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Pyrrolizidine alkaloids enhance alcohol-induced hepatocytotoxicity *in vitro* in normal human hepatocytes

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Abstract. – OBJECTIVE: Herbal remedies containing pyrrilidozine alkaloids (PA)s can induce liver damage, including hepato-sinusoidal obstruction syndrome (HSOS) or veno-occlusive liver disease (VOD). Some individuals misusing alcohol consume also teas and/or herbal remedies containing PA. The interaction or additive toxicity of alcohol to PA toxicity needs to be addressed. The objectives of this study are 1) to review the scientific literature on the PA-induced liver toxicity; 2) identify possible mechanism(s) involved in PA-induced hepatocytotoxicity in the presence or absence of ethanol (EtOH) *in vitro* in normal human hepatocytes (NHH) in primary culture. To respond to the first objective, we systematically search all the literature engines (PubMed, Google Scholar) for liver induced damage due to PAs and summarize the results in an introductory systematic review.

ORIGINAL ARTICLE EXPERIMENTAL DESIGN AND METHODS: Cells were exposed to one dose of 100 mmol/L EtOH for 24 hrs and to 2 doses of 100 mmol/L EtOH for consecutive 24 hrs periods, in the presence or absence of PAs (10 mg/mL), or the caspase-3 inhibitor IDN-1965 (50 μmol/L). Cells were analyzed for apoptosis by light microscopy, immuno-histochemistry, measuring cytokeratin-18 fragmentation, and transmission electron microscopy (TEM) (6000 cells/treatment). Cytotoxicity was determined using succinate dehydrogenase (SDH) activity, an enzyme specific to the mitochondria.

RESULTS: In NHH cells, a 100 mmol/L dose of Et-OH resulted in 22±2.5 apoptosis ($p<0.001$ *vs.* control). Two consecutive doses of 100 mmol/L Et-OH for 24 hrs each caused 36±3.0% apoptosis ($p<0.001$ *vs.* control and $p<0.05$ *vs.* one dose Et-OH). Pre-treatment with 50 μmol/L caspase inhibitor significantly reduced Et-OH-induced apoptosis [12±1.5% in 100 mmol/L ($p<0.05$) and 20±4.0% in 2×100 mmol/L ($p<0.001$)]. In addition, pre-treatment with 50 μmol caspase inhibitor in cells treated with PA + EtOH reduced apoptosis significantly (*vs.* non-exposed to caspase-inhibitor): Δ -22±3.0 % ($p<0.05$). HPC significantly decreased apoptosis compared to conditions lacking this supplementation in cells treated with EtOH-exposed cells present ballooning, Mallory bodies, changes in mitochondrial cristae and apoptosis by TEM. Pre-treatment with 50 μmol caspase inhibitor significantly reduced 100 mmol/L EtOH-induced (one dose) in NHH by 14±0.5% ($p<0.05$) compared to cells not exposed to the caspase-inhibitor. In cells treated concomitantly with PA and EtOH 100 mM Mallory-bodies and apo-necrotic cells have been observed. Pre-treatment with 50 μmol caspase inhibitor reduced the mitochondrial damage. A significant depletion in glutathione (GSH) was observed in Et-OH treated cells after 1 and 2 treatments ($p<0.001$ *vs.* control). Treatment with Et-OH enhanced *PA*-induced GSH-depletion and resulted in a significant increase in *PA*-induced cytotoxicity ($p<0.001$ *vs.* Et-untreated cells). Exposure to EtOH increased the cell culture media levels of the pro-inflammatory cytokine TNF. PA + EtOH-treated cells increased TNF-α levels in media compared to EtOH alone [86±8 *vs.* 53±5 pg/mL in cells exposed to 100 mmol/L EtOH ($p<0.05$) and 218±14 *vs.* 179±8 pg/mL in cells exposed to 2×100 mmol/L EtOH ($p<0.05$)].

CONCLUSIONS: PA up-regulates EtOH-induced hepatocytotoxicity by inducing the inflammatory cytokines and enhancing the apoptotic effects of ethanol. There is a need for monitoring herbal medicine in order to optimize traditional medicine use and maximize the clinical benefits. Additionally, there is necessary to communicate to physicians the possible negative results of herbal remedies use. Also, the interactions between herbal remedies and drugs of misuse should be communicated to consumers.

Key Words:
Alcohol-induced liver damage, Apoptosis, Caspase, CYP 2E1, Glutathione, Herbal-induced hepatotoxicity, Normal human hepatocytes, Mitochondria, Pyrrilidozine alkaloids, Reactive oxygen species, Transmission electron microscopy, Veno-occlusive disease of the liver.

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Abbreviations

ADP = adenosine diphosphate; ALD = alcoholic liver disease; ALF = acute liver failure; α-MEM = minimum essential medium; ANOVA = one-way analysis of variance; ATP = adenosine triphosphate; ccCK = caspased cleved cytokeratine; CYP = cytochrome P-450; DNA, deoxyribonucleic acid; DTNB = 5,5-dithiobis-2-nitrobenzoic acid; EDTA = ethylene diamine tetra-acetic acid (disodium salt, dihydrate); EtOH = ethanol; GC = gas chromatography; GSH = glutathione; GSSG = oxidized glutathione, glutathione disulfide; HILI = herbal-induced liver injury; HPLC = high-performance liquid chromatography; IDN = caspase 3-inhibitor IDUN-Pharma; IFN-γ = interferon gamma; IL = interleukin; LPS = lipopolysaccharide; MDA = malondialdehyde; MDB = Mallory-Denk bodies; MS = mass spectroscopy; NADPH = reduced nicotinamide adenine dinucleotide phosphate; NHH = normal human hepatocytes; PA = pyrrolizidine alkaloid; PBS = phosphate buffered saline; RANTES = regulated upon activation, normal T-cell expressed and secreted; CCL5 = "CC" chemokine; ROS = reactive oxygen species; SD = standard deviation; SDH = succinate dehydrogenase; SSA = 5-sulfo-salicyclic acid; TEM = transmission electron microscopy; TLC = thin layer chromatography; TNF-α = tumor necrosis factor alpha; UPLC = ultra-performance liquid chromatography; UHPLC-MS = Ultra-high performance liquid chromatography-triple quadrupole-mass spectrometry; VOD=HSOS=BCS = Veno-occlusive liver disease=hepato-sinusoidal obstruction syndrome- Budd-Chiari syndrome.

Introduction

Herbal Remedies Containing Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids (PAs) are toxic to animals and humans. These alkaloids are found in more than 6,000 plants within the Asteraceae, Boraginaceae, Compositae, and Fabaceae families[1-3]. Plants are reported to have PAs such as senecionine, retrorsine, riddeliine, integerrimine, neosenkirkine, and florosenine[4]. The PAs contain a double bond in the ring nucleus, an esterified hydroxyl group, and a branched carbon in at least one of the ester side chains[5]. The hepatotoxicity was reported after ingestion of the herbs belonging to following families of plants Boraginaceae (*Heliotropium, Trichodesma, Symphytum* [Comfrey]), Compositae (*Senecio* [Bush Teas], *Eupatorium*), Leguminosae [(*Crotalaria*), Germander (*Teucrium chamaedrys*), Greater Celandine (*Chelidonium majus*)], and Scrophulariaceae (*Castilleja*)[6-13].

Traditional Herbal Medicine-Induced Human Toxicity

Herbal medications and natural products are often mistakenly suggested to have minimal toxicity while being rich in health benefits. However, high amounts of herbal medicine can damage individuals. Herbal medicines contain a complex mixture of chemical compounds, both beneficial and toxic[14-16]. Alissa[17] shows the beneficial effects of natural products, while Brazier et al[18] and Shi et al[19] also show the possible interactions of drugs used by health-care professionals and herbal medicine. Many natural products interact with other herbs, drugs of use and misuse, environmental pollution[20,21]. Ernst[22] reported the incidence of heavy metal contamination, which 64% of samples collected in India contained significant amounts of lead (64% mercury, 41% arsenic and 9% cadmium). In 1999, Zimmerman[16] alerted the clinicians on the need of identification of the possible liver damage due to these remedies. Drug-induced or herbal-induced-liver injury, including VOD, relies on both chronological and clinical criteria[15].

However, although analytical methods have confirmed that numerous herbal teas contain PAs, there are still licensed and registered commercial products that are available in the market for consumers to purchase[23]. Many herbal teas marketed for infants pregnant women, or lactating mothers contain traces of PA that may be harmful to the exposed child[24]. Analytical chemistry techniques such as high-performance liquid chromatography (HPLC), LC-ion trap mass spectrometry (LC-MS), gas chromatography (GC), mass spectroscopy (MS), ultra-high performance liquid chromatography-triple quadru-pole-mass spectrometry (UHPLC-MS)-and thin layer chromatography (TLC), have been conducted in order to provide quantitative data regarding the amount of PAs[25-33]. Xia et al[34] are quantifying PAs and pyrrole-protein adducts (DHP-protein) and the resulting 7,9-di-C2H5O-DHP by HPLC-ES-MS/MS multiple reaction analysis.

Human Hepatocytotoxicity from Pyrrolizidine Alkaloids

Pyrrolizidine alkaloid poisoning is due by the ingestion of grain contaminated with PAs[35,36] or by consumption of herbal teas[37-41]. Another source of exposure to PA is from honey contaminated with PAs[42-45]. Milk could also contain traces of PAs if the cows eat PAs-contaminated supply[46]. A case of a newborn infant with hepatic VOD was reported. The mother was drinking herbal tea containing PAs and the child via breast milk became intoxicated PAs[47]. Additionally, Rasenack et al[48] described VOD in a fetus caused by PAs origi-

nated in mother's food. Moreover, the traditional medicine would be applied by farmers to their livestock. *Senecio oxyriifolius DC* is given to animals with swelling and *Senecio tamoides DC* is administered to animals with anthrax[49,50]. The meat from these animals can produce hepatotoxicity to humans[51]. However, there are interspecies differences in phytochemical profile leading to hepatotoxicity[52-54]. Livestock poisoning, primarily liver damage, caused by consumption of plants containing 1,2-dehydro-pyrrolizidine ester alkaloids and the corresponding N-oxides has economic impact, particularly in Australia, South Africa, South America and the United States [55,56]. The relationship between the exposure and the hepatic toxicity is not always clear, since patients may be taking multiple preparations, making very hard the identification of a single offending agent. In addition, the individuals may have concomitant liver diseases, such as alcohol-induced liver damage or hepatitis B or C viral infections. A specific test for humans, lymphocyte toxicity assay, may detect idiosyncratic adverse reaction due either to medication or natural products. This test confirms a possible diagnosis of drug-induced, herbal-induced or animal-venom-induced liver injury[57-60]. In 1978, Datta et al[61] described 6 cases of VOD after medicinal *Heliotropium eichwaldii* ingestion. The herb, containing the PA heliotrine, was identified in three of the patients. Two patients presented with fulminant hepatic failure while the other four patients had decompensated cirrhosis. Chauvin's team[62] described Heliotrope food poisoning, in Tadjikistan leading to herbal-induced liver injury (HILI). Ascites occurs in 96% of patients, hepatomegaly in 85% and elevated liver enzymes in 92%[63]. Numerous case reports in human and veterinary medicine indicate that PAs at high doses induce hepatic VOD. Willmot et al[64] clinically described the symptoms of hepatic VOD by ascites, hyperbilirubinemia, hepatomegaly, and abdominal pain. Others also reported the same symptoms[65-67]. Kakar et al[68], described hepatic VOD in Western Afghanistan after exposure to flour contaminated with PAs. Both acute and chronic PA-induced toxicity have been reported in humans as being dose and frequency of the exposure dependent[68]. Conradie et al[65] described two pairs of toddler twins that ingested PA-natural medicine to be diagnosed with VOD due to HILI. In one family, both siblings survived, albeit with hepatic damage. In the other family, one twin died within 24 h and the second one-month after admission with a diagnosis of VOD. In both cases, the presence of the toxic PA, retrorsine, was determined. Neuman et al[69] reported a 71-year-old Caucasian woman, originally from South Africa that presented to the emergency room being cachectic with a distended abdomen. The liver enzymes were six times upper limit of normal. The liver biopsy presented central areas with marked hepatocellular inflammation and atrophy, as well as centro-lobular necrosis consistent with VOD of the liver. The history revealed that the patient had been using multiple herbal remedies. Upon the discontinuation of the herbal remedies the patient improved clinically and all liver functions returned to normal. A diagnostic serum marker was validated to identify hepatotoxicity caused by PAs. We used patient's lymphocytes to diagnose PA-induced VOD[69]. The personalized analysis may be used to improve herbal product safety. A cohort study provides evidence that traditional herbal medicine composed of herbs from the Asteraceae, Fabaceae, and Lamiaceae families have associated risk for liver fibrosis regardless of one's HIV status[70]. A problem arises for HIV patients when novel antiretroviral therapy is neglected for traditional African herbal medicine. The problem is double since the antiretroviral therapy needs to be taken regularly, and the traditional remedies and life style (alcohol misuse) may interact with the antiretroviral leading to liver damage[71-73]. Furthermore, in the Msambweni community of Kenya, *Senecio syringitolius* is used as an antimalarial remedy[74]. Medicinal, homeopathic or natural remedies containing PA-rich herbs also induced serious liver VOD, in many parts of the world[75,76]. Therefore it is a need to facilitate and to support rigorous research and education on medicinal therapies and natural health products based on non-invasive biomarkers and personalized medicine

PA Metabolism/Mechanism of Action

PAs undergo three main metabolic pathways. PAs that are hydrolyzed to a carboxylic acid or N-oxidized to a N-oxide metabolite are non-toxic and soluble in water thus excreted *via* urine[77]. PAs undergo biotransformation by CYP3A its reactive metabolites[78-80]. CYP3A oxidize the PAs, followed by the dehydrogenation of the necine ring. The phenomenon produces a dehydro-pyrrolizidine compound, a toxic pyrrolic ester that acts as an electrophile. Thus, CYP3A inducers could increase the susceptibility of PA-induced toxicity, CYP3A inhibitors could prevent toxic outcomes since inhibitors yield less dehydro-PAs[81]. The excess of pyrrolizidine N-oxide metabolites metabolites can be further transformed into toxic

epoxides and necine bases[81]. Glutathione is the central antioxidant, reacting with most of the reactive oxygen species (ROS), except superoxide anions. *In vivo* and *in vitro* studies have shown that PA-induced VOD have been linked to the depletion of glutathione in hepatocytes and sinusoidal endothelial cells, indicative to PA-induced oxidative stress[82-84]. Cattles that have been intoxicated presented liver injury due to *Senecio* spp. showed higher activity of copper-zinc superoxide dismutase as indicative of lipid peroxidation. He et al[85], show that senecionine and other PAs are conjugated by glucuronic acid in humans and animals. *In vivo* and *in vitro* studies suggest that oxidative stress and apoptosis of hepatocytes are responsible for liver injury[86]. Several models show concentration-dependent PA-induced depletion of glutathione indicative to oxidative stress by PAs, or reduced expression of p53 have conducted an *in vitro* study and demonstrated that toxic PAs not only induce apoptosis, but also clump tubulin cytoskeleton leading to necrosis[87-94].

Inflammatory response is part of PA-induced VOD. Cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and endothelin-I (ET-1) are secreted by monocytes in response to PAs[95]. Bile acid homeostasis is also compromised. Xiong et al[96] has investigated PAs-induced toxicity by studying the change in metabolomics and genomic profiles of the hepatocytes. Patients exposed to PA toxicity showed an elevated activity of alanine and aspartate aminotransferase. The same liver enzymes pattern showed by the intoxicated patients was observed in an *in vivo* alcohol model of PA-hepatotoxicity[97]. Previously, Neuman[98] showed the importance of pro-inflammatory cytokines in alcohol-induced hepatotoxicity. Therefore we hypothesize that a combination of PA and alcohol consumption will contribute to an elevation of the inflammation.

Previous Research

HepG2 cells have shown senecionine-induced dose- and time-dependent cytotoxicity assessed by MTT[93,94]. Other used bromodeoxyuridine incorporation assay, neutral red uptake assay, resazurin assay, and lactate dehydrogenase release assay[99]. Moreover, insect cell line and injection bioassay also show agreed conclusion that PAs are cytotoxic in a dose-dependent manner[100]. L-02 cells also show dose-dependent and time-dependent that senecionine and other PAs such as adonifoline, senecionine, monocrotaline, and isoline deplete cellular glutathione level and increase the level of oxidized glutathione resulting a decreased ratio of glutathione to oxidized glutathione. N-acetyl-cysteine, the precursor to glutathione, and antioxidant compounds, lowered the susceptibility of PA-induced hepatocytotoxicity[93] and glutathione synthesis inhibitor increased the susceptibility of PA-induced hepatocytotoxicity[93,94]. Primary mice hepatocytes have shown that senecionine and other PAs induce apoptotic DNA laddering, caspase-3 activation, and decreased level of Bcl-xL, an anti-apoptotic protein[93] thus concluding that PAs share a common hepatotoxic signaling pathway that involves the degradation of Bcl-xL protein and activation of the intrinsic apoptotic pathway, mediated by the mitochondria[101,102]. Our previous research[88] regarding *Senecio*-induced toxicity showed that aqueous extract of *Senecio* induced cytotoxicity in a dose-dependent and time-dependent manner determined by ELISA and terminal dUTP nick-end labeling in cells. Furthermore, glutathione depletion was observed when treated with *Senecio* extract and N-acetyl-cysteine was shown to potentially reduce cytotoxicity induced by *Senecio*. Lastly, caspase-3 and caspase-9 inhibitors were demonstrated to prevent apoptosis associated with aqueous *Senecio* extract[84]. Many signals during apoptosis induction aim at mitochondria and cause hypergeneration and release of superoxide anions after the opening of the permeability transition pore and the disruption of the mitochondrial membrane potential[84]. The reduction of cellular glutathione levels can sometimes be the cause, sometimes the consequence of ROS-mediated apoptosis. Glutathione serves two major functions during the regulation of apoptosis. It balances against ROS created by multiple signaling pathways, enzymatic reactions or mitochondria and it inhibits sphingomyelinase, the key enzyme for the generation of ceramide, a second messenger that is intrinsically interwoven with the generation of ROS and with activation of execution-caspases[84]. In the present research, we used normal human hepatocytes (NHH) to continue our research in understanding the mechanism behind PA-induced hepatotoxicity. In particular, we observed apoptosis and the effect of inflammatory response by measuring cytokine secretion that may arise from exposure to aqueous extract in the presence of the plant-induced hepatotoxicity. We opted to expose the cells to ethanol because the model of ethanol-induced hepatocytotoxicity is well characterized by us. In addition, the role of key cyto-

kines in this model has been previously studied in our laboratory[103,104]. Ethanol exposure resulted in elevated levels of TNF-α leading to liver cell apoptosis. Therefore, we hypothesize that there will be changes in inflammatory biomarkers and increased level of apoptosis when aqueous plant extract is added to ethanol-treated NHH. The properties investigated in the present research will provide supporting evidence regarding the PA-induced hepatocytotoxicity in the presence of alcohol.

Materials

Herbal Remedy Preparation

The herbal remedy was provided by the parents of one of the patients and consisted of a mixture of dried plant material (Figure 1)[65]. The dried mixture was ground to a fine powder. For experiments, 1 g of the powdered material was extracted by suspension in 10 mL boiling (distilled) water and infusing for 15 min. The suspension was centrifuged and the supernatant filtered through Whatman No. 1 filter paper and then filter-sterilized using 0.22 um filter (Waters Corporation, Milford, MA, USA.). PAs from the powdered plant material was extracted twice with 0.05 mol/L sulfuric acid through a glass column with alkalinized celite. The aqueous phase was then extracted with dichloromethane and evaporated to dryness. PA extraction method was performed accordingly to the methods by van Wyk et al[105]. Detection of PAs in this preparation was performed using a gas chromatography-mass spectrometry (GSMS) method of Holstege et al[106].

Figure 1. The row material containing PAs.

GC/MS analysis revealed the presence of retrorsine. A mass spectrum from the peak at 22.26 min indicates characteristic ions of retrorsine.

Cell Culture

Normal human hepatocytes (NHH) were obtained from partial liver transplantation donors, using a collagenase perfusion. These cells are not contaminated with non-parenchymal cells and have a stable phenotype. NHH are in primary culture. They are free of viruses or bacterial contamination. Cells were shown to retain morphological features of hepatocytes by light and electron microscopy. The functionality of the NHH parenchymal cells was proven by demonstrating glucose-6-phosphatase activity, transferrin, and albumin secretion, as well as small but sustained inducibility of 7-ethoxycoumarin O-diethylase activity (CYP2B1) and p-nitroso-dimethyl-aminine dimethylase activity (CYP2E1)[65]. We measured p450s activities in the form of ethoxy resorufin O-dealkylase (EROD), benzo[a]pyrene-hydroxylase, aryl hydrocarbon hydroxylase (BROD), and 1-ethoxy-coumarin demethylase activities, which are functional markers for CYP1A1, CYP1A2, CYP2B1. EtOH exposure showed an IC_{50} of 33.56±0.72 μM for NHH. Cells were seeded in flasks (1×10^6 cells/mL). The cell counts were monitored using a Coulter counter (Coulter Electronics Inc., Hialeah, FL, USA). Cells in long-term cultures were grown in α-MEM supplemented with 10% v/v heat inactivated fetal bovine serum (FBS). At the beginning of the experiment, when cells reached 70% confluence, the growth medium was removed from the culture flasks. The cultures were washed twice with phosphate buffered saline (PBS) and fresh serum-free medium was used as base for all the treatments. Cells were maintained in a humidified atmosphere of 95% O_2-5% CO_2 at 37°C. The pH of the media was maintained at 7.4.

Materials Used for Cultured Cells

Bovine serum albumin (BSA), L-buthionine-(S,R)-sulfoximine (BSO), *N*-acetylcysteine, MTT (formazan 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), GSH reductase**,** GSH standard, NADPH (reduced nicotinamide adenine dinucleotide), 5-sulfosalicyclic acid (SSA), EDTA (ethylene diamine-tetra-acetic acid), and DTNB (5,5-dithiobis-2-nitrobenzoic acid) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Minimal essential medium (α-MEM) was obtained from Gibco (Burlington, Ontario, Cana-

da). Trypsin was purchased from Difco (Detroit, MI, USA) and was prepared as a 1% solution. The kit for protein determination was obtained from Bio-Rad Laboratories (Richmond, CA, USA). PBS (phosphate buffered saline without Ca^{2+} or Mg^{2+}, pH 7.4) was used to wash cells and to remove medium. All plastic ware for cell cultures was obtained from Falcon (Becton Dickinson, Oxnard, CA, USA). All of the remaining reagents were of analytical grade, obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental Design

The cells have been seeded in the 96-well round bottom and were exposed to alpha medium (control), one dose of 100 mmol/L EtOH for 24 hrs and to 2 doses of 100 mmol/L EtOH for consecutive 24 hrs periods, in the presence or absence of PAs (10 mg/mL) or the caspase-3 inhibitor IDN-1965 (50 μmol/L). Cells were incubated at 37°C (95% O_2-5% CO_2). Cytotoxicity was determined using succinate dehydrogenase (SDH) activity, an enzyme specific to the mitochondria. The cells were analyzed for viability as previously described[65]. Also, cells were analyzed for apoptosis by light microscopy and transmission electron microscopy (TEM) and by measuring cytokeratin-18 fragmentation (cleavage). The cells underwent lysis and were exposed to the detector antibody for 1 h at room temperature. After that, the cells were incubated for another 30 min with SA-HRP (horseradish peroxidase) conjugate. The reaction was stopped and the absorbance was read (dual lengths 450/595 nm). The intensity of the color was proportional to the number of nucleosomes in the sample. For each treatment, six wells per plate in five different plates were quantitated. The results were reported as percent apoptosis *vs.* control, with non-treated cells taken as 0% apoptosis. The standard curve comprised of six replicates from each of two plates. Taking two standard deviations above the mean of zero, we defined that the assay was able to distinguish 0.7% sensitivity. The GSH assay is based on the principle that GSH can be measured by an enzymatic recycling procedure in which it is sequentially oxidized by Dinitro-5-thiobenzoic acid and reduced by NADPH in the presence of glutathione reductase. The rate of formation of Dinitro-5-thiobenzoic acid can be measured using a spectrophotometer and GSH levels quantitated by reference to a standard curve as described by us previously [93,94]..

Cytokines and Chemokines

The media was collected for cytokine determination. All the cytokines were evaluated using enzyme-linked immunosorbent-assay – ELISA, as follows: IL-1b, VEGF (PeproTech Asia, Rehovot, Israel), IL-6, TNF-α (eBioScience, Frederick, MI, USA). The assays showed 96% sensitivity and 92% specificity. The tests were performed according to manufacturer specifications.

Microscopy Morphology Analysis

Cells in long-term cultures were grown in α-MEM supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS). At the beginning of the experiment, when cells reached 70% confluence, the growth medium was removed from the culture flasks. The cultures were washed twice with phosphate buffered saline (PBS) and fresh serum-free medium was used as the base for all of the treatments. The cells were prepared for light microscopy (LM) and transmission electron microscopy (TEM) studies using a standard procedure as outlined below[88]. Six flasks of cells were used for each group: α-MEM only, plant extract. After the period of 24 h incubation, the media was removed and cells were washed twice with PBS. Five mL of 1% trypsin was added to each flask for 2 min. Cells were washed again with PBS and then re-suspended in plain media. Pellets were immediately fixed in 2.5% v/v glutaraldehyde for a minimum of 24 h. Blocks of cells were separated, post-fixed in 1% v/v osmium tetra-oxide, dehydrated with a graded series of acetone concentrations and embedded in Araldite resin. Sections (1 micron thick) were viewed by light microscopy. For light microscopy studies an Olympus microscope equipped with Leco 2005 Image Processing and Analysis System (Leco Instr., Toronto, Ontario, Canada) software were used. Cells were considered apoptotic if the classic features of pyknotic nuclei, cytoplasmic condensation and nuclear chromatin fragmentation could be observed. Representative blocks were selected, subjected to ultra-thin sectioning and stained with uranyl acetate and lead citrate for transmission electron microscopy. Electron micrographs were taken with a transmission electron microscope JEOL 1200 E x II (JOEL Institute Inc., Peabody, MA, USA). Ultrastructural findings were examined in five different grids per flask in each experiment. On each grid, 200-400 cells were examined. An average of 9000 (300 cells/grid x number of grids/flask x 6 flasks/treatment) cells were analyzed for each treatment. We used standard criteria for the morphological identification of cellular structures. When cells were

assessed by electron microscopy, cell shrinkage, electron dark cytoplasm, and apoptotic bodies were considered criteria for classical apoptosis[104]. Only intact hepatocytes with nuclei were assessed both for light microscopy and transmission electron microscopy. The system used for light microscopy morphometry was a modulator high-performance image processing and analysis system, extended with a high-resolution camera. For each block, 5 slides were studied and the 60 hepatocytes per slide were measured. The morphological dimensions (particle sizing) were implemented by a combination of hardware and software to ensure an optimized performance of Microsoft^{R} Visual Basic^{TM} .

Statistical Analysis

All data are expressed as a means ± standard deviation (SD). Statistical significance, defined as *p*-value less than 0.05, between control and treated cells were analyzed using Student's *t*-test. Statistical analysis was conducted using SPSS v.22.0.0.0 (SPSS Inc. Chicago, IL, USA) and graphs were constructed using GraphPad Prism v.6.0c (San Diego, CA, USA).

Results

Apoptosis, Glutathione

In NHH cells, a 100 mmol/L dose of EtOH resulted in 22±2.5 apoptosis ($p<0.001$ *vs.* control) (Figure 2i). Two consecutive doses of 100 mmol/L EtOH for 24 hrs each caused 36±3.0% apoptosis ($p<0.001$ *vs.* control and $p<0.05$ *vs.* one dose). Pre-treatment with 50 µmol/L caspase inhibitor significantly reduced EtOH-induced apoptosis [12±1.5% in 100 mmol/L ($p<0.05$) and 20±4.0% in 2×100 mmol/L ($p<0.001$)]. PAs significantly enhanced apoptosis [12±1.5% in 100 mmol/L ($p<0.05$) and 44±4.0% in 2×100 mmol/L ($p<0.001$)]. In addition, pre-treatment with 50 µmol caspase inhibitor in cells treated with PA + EtOH reduced apoptosis significantly (*vs.* non-exposed to caspase-inhibitor): Δ -22±3.0 % ($p<0.05$). Pre-treatment with 50 µmol caspase inhibitor significantly reduced 100 mmol/L EtOH-induced (one dose) in NHH by 14±0.5% ($p<0.05$) compared to cells not exposed to the caspase-inhibitor. In cells treated concomitantly with PA and EtOH 100 mM Mallory-bodies and apo-necrotic cells have been observed. Pre-treatment with 50 µmol caspase inhibitor reduced the mitochondrial damage. In addition the pre-treatment significantly reduced 100 mmol/L EtOH-toxicity 14±0.5% ($p<0.05$) compared to cells not exposed to the caspase-inhibitor. The basal apoptosis level of NHH without any treatment was calculated at 3.5%. Treatment of ethanol increased the level of apoptosis for both single dose of 100 mM and two consecutive doses of 100 mM to 22% and 36%, respectively ($p<0.005$). PA exposure augmented level of apoptosis regardless of ethanol treatment, in as such that PA increased apoptosis in control cells to 20%, cells treated with single dose of 100mM ethanol to 32% and two doses of 100 mM ethanol to 45% ($p<0.001$). Cells treated with single dose of 100 mM ethanol and IDN presented 10% apoptosis, while cells treated with two doses of 100 mM ethanol in the presence of IDN presented 14% apoptosis. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) showed a significant depletion in Et-OH treated cells after 1 and 2 treatments ($p<0.001$ *vs.* control) (Table I). Treatment with ethanol enhanced *PA*-induced GSH-depletion and resulted in a significant increase in *PA*-induced cytotoxicity ($p<0.001$ *vs.* EtOH-untreated cells).

TNF-α

The basal level of TNF-a secreted by normal human hepatocytes without any treatment was 13 pg/mL (Figure 2ii). Treatment of ethanol increased the concentration of TNF-α for both single dose of 100 mM (to 53 pg/mL) and two consecutive doses of 100 mM (to 179 pg/mL), respectively ($p<0.01$). PA exposure exacerbated the level of TNF-a production regardless of ethanol treatment, in as such that PA increased TNF-a concentration in control cells to 16 pg/mL, cells treated with single dose of 100 mM ethanol to 156 pg/mL and two doses of 100 mM ethanol to 256 pg/mL ($p<0.05$). IDN showed no statistically sig-

Table I. Glutathione levels in tissue culture.

Treatment	GSH (mg/mL) protein)	SD	GSH (nmol/mg (% control)
Control-a MEM	17.1	0.9	100
EtOH-100 mM	15.0	1.1	87
PA 10.00 (mg/mL)	15.0	0.7	87
PA 10.00 (mg/mL) +EtOH 100 mM	10.0	1.5	58
2 x PA 10 (mg/mL)	10.5	1.7	61
2x EtOH 100 mM	7.5	2.5	44
2 x PA (mg/mL) + 2x EtOH 100 mM	3.5	1.9	20

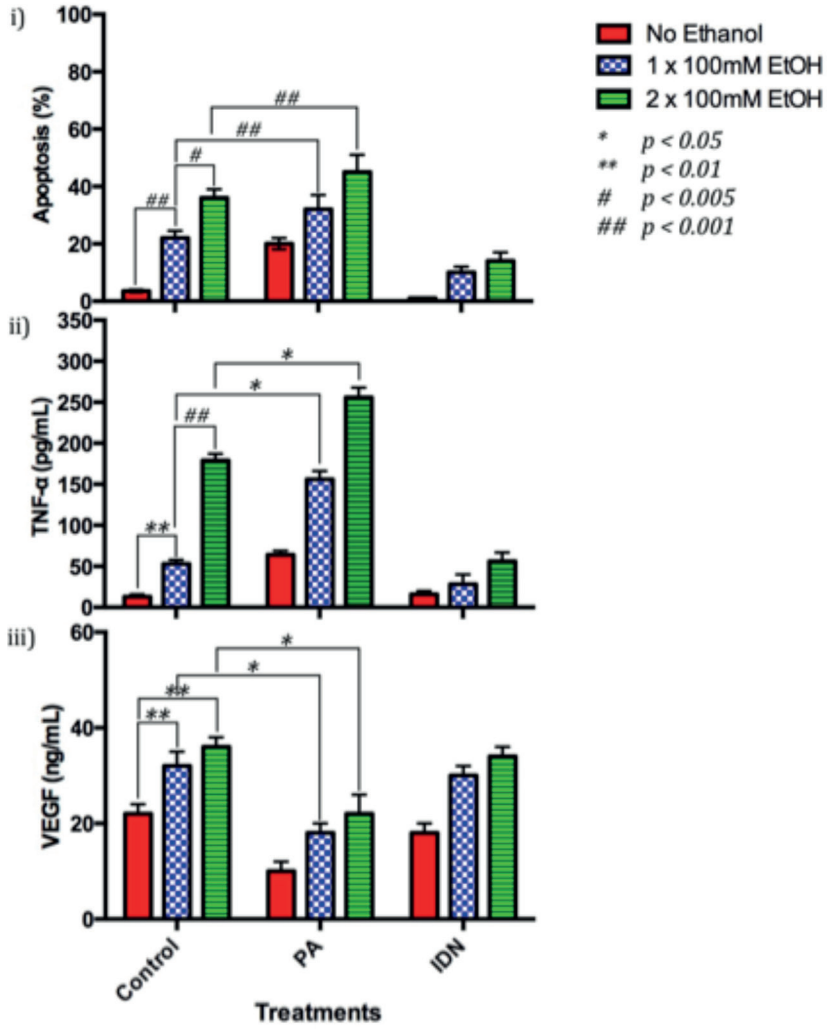

Figure 2. Normal human hepatocytes were pre-treated with one dose of 100 mM EtOH, two doses of 100 mM EtOH, or a-MEM (*control*). Cells were exposed to one dose of EtOH for 24 hrs, whereas other cells have been exposed two consecutives doses for 24 hrs each. Cells were then exposed to PA, caspase inhibitor-IDUN, or α-MEM (*control*). The cells have been collected for microscopy. The media was collected for cytokine determination. All the cytokines measurements and apoptosis were evaluated using ELISA. ***(i)*** Apoptosis measurments. Cells that are exposed to one dose of ethanol showed more apoptosis (p<0.001). Two consecutive doses of ethanol showed more apoptosis than one dose (p<0.005). Cells that were exposed to PA showed greater degree of apoptosis regardless of EtOH exposure (p<0.001). ***(ii)*** TNFα concentration. Cells that are exposed to one dose of ethanol showed greater level of TNFα (p<0.01). Two consecutive doses of ethanol showed greater level of TNFα than one dose (p<0.001). Cells that were exposed to PA showed greater level of TNFα regardless of EtOH exposure (p<0.05). ***(iii)*** VEGF concentration Cells that are exposed to one dose of ethanol showed greater level of VEGF (p<0.01). Two consecutive doses of ethanol showed greater level of VEGF than one dose (p<0.01). Cells that are exposed to PA showed a significant decreased level of VEGF, regardless of EtOH exposure (p<0.05).

nificant change when it is added to control cells. However, the cells exposed to ethanol in the presence of IDN showed a decrease in TNF-a production in as such that cells treated with single dose of 100 mM ethanol decreased to 28 pg/mL and cells treated with double dose of 100 mM ethanol decreased to 56 pg/mL. The results from the IDN treatment indicate that a toxicological insult that induces apoptosis is required for an inflammatory response.

VEGF

The basal level of VEGF secreted by normal human hepatocytes without any treatment was 22 pg/mL (Figure 2iii). Treatment of ethanol increased the concentration of VEGF for both single dose of 100 mM and two doses of 100 mM to 32 pg/mL and 36 pg/mL, respectively (p<0.01). PA exposure decreased the level of VEGF production regardless of ethanol treatment, in as such that PA decreased VEGF concentration in control

cells to 10 pg/mL, cells treated with single dose of 100 mM ethanol to 18 pg/mL and two doses of 22 pg/mL mM ethanol to 256 pg/mL ($p<0.05$). IDN showed no statistically significant change when it is added to cells regardless of their ethanol exposure, about the non-treated cells. The results from the IDN treatment indicate that down-regulation of VEGF is independent from PA-induced apoptosis. Interleukins 1 and 6 did not present significant differences between the different treatments. Figure 2 presents the significant results.

Microscopy

The immunohistochemistry image is provided in Figure 3. Human hepatocytes treated with solely ethanol exhibit large lipid droplets pushing aside the liver cell nucleus and altering its cellular morphology. When human hepatocytes are treated with both ethanol and exposed to PAs, they lack normal cellular morphology to a greater extent. In addition to the large lipid droplets, inflammation is readily apparent when the cells have been exposed to the toxic Et-OH and PA. During the process of controlled cell death by apoptosis, the intact cytokeratin 18, situated in cytoplasm is cleaved (Asp 396 neo-epitope). Caspased cleved cytokeratine 18 (ccCK18) indicate only apoptosis not necrosis.

Figure 4 presents cells in which the measurements of lipid froplets were performed via the morphometric measurements. Figure 5 shows a transmission electron microscopy picture of NHH treated with PA and EtOH. The cells are linked by tight junctions. The hepatocytes are not homogenous presenting destoreted mitochondria without cristae and unregulated nuclei. An apoptotic cell with chromatin condensation in the nucleus can be seen. The apoptotic cell is shrunk, detached from the other cells. However, the membrane of the hepatocyte preserved its integrity.

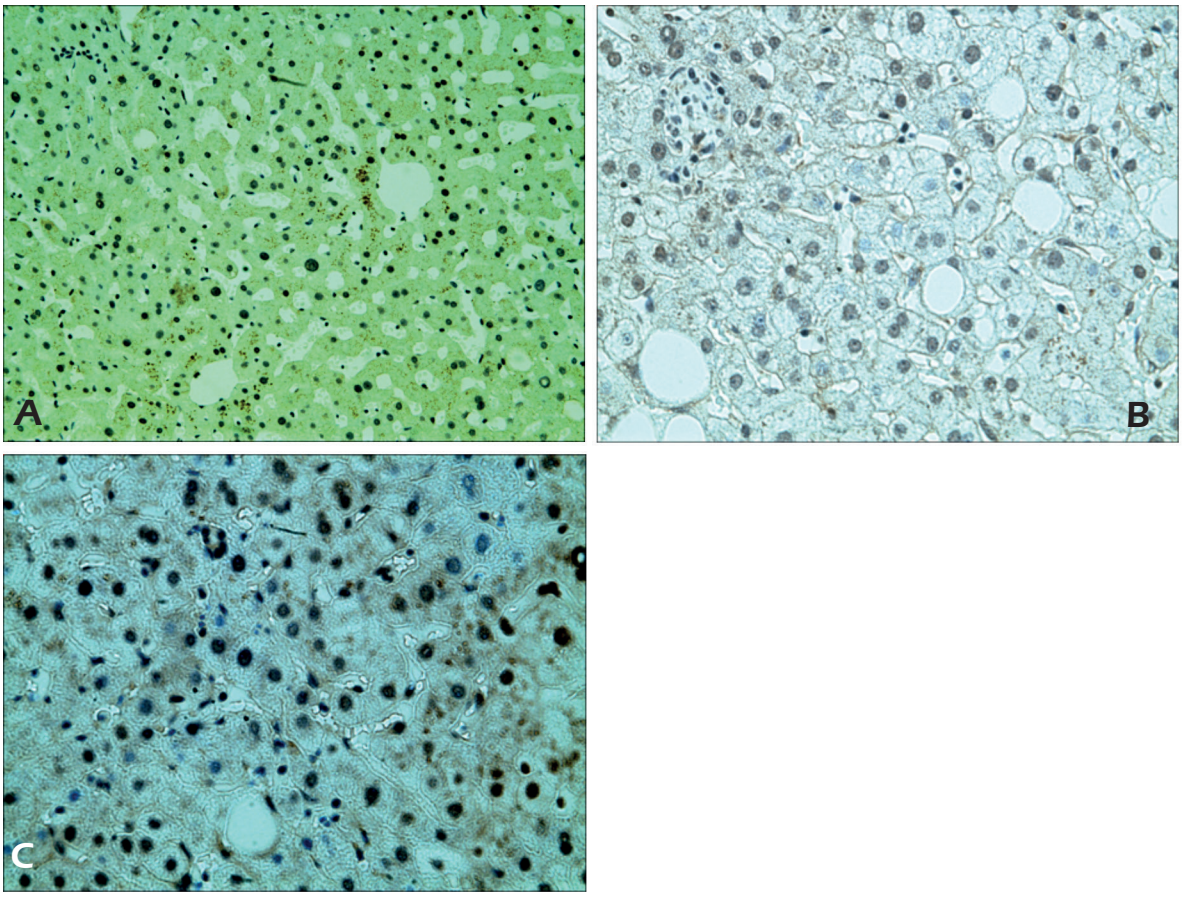

Figure 3. Immunohistochemistry (M30-cytokeratine-8) of NHH treated with ***(A)*** one dose of ethanol. Large lipid droplets occupy some cells, few apoptotic nuclei. ×20. ***(B)*** Normal human hepatocytes exposed to two consecutive dosage of 100 mM ethanol presenting very large lipid droplets; cells with foamy cytoplasm. Some apoptotic bodies can be observed. ×40. ***(C)*** Cells treated with 2 doses of EtOH in the presence of Pas. Cytoplasm is foamy, with large lipid droplets, most of the cells present picnotic nuclei, some are apoptotic and apoptotic bodies can be seen. ×40.

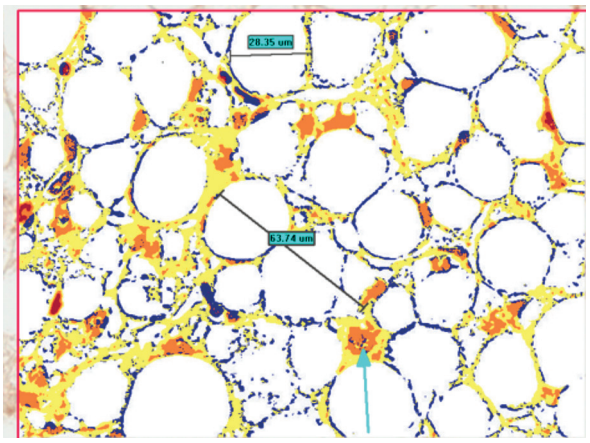

Figure 4. NHH treated with 2 × 100 mM Et0H presenting large lipid droplets. The morphometric measurments of the diameter of the lipid droplets is shown by black lines. Also the blue arrow is pointing apoptotic bodies. × 100.

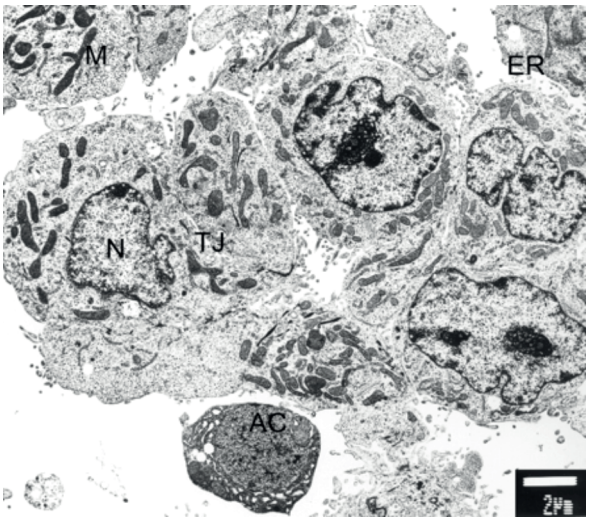

Figure 5. TEM of NHH treated with 2 × EtOH 100 mM in the presence of 2 × PA (10 mg/mL). The cells are irregulate, however there are linked by tight junctions (TJ). Nucleus (N) is not homogenous both in form and in content. Elongated mitochondria (M) with no cristae can be observed. Endoplasmic reticulum (ER) is shown in one of the hepatocytes. An apoptotic cell (AC) with highly chromatic nucleus is presented. In the AC cytoplasm there are numerous lipid droplets showing lipotoxicity.

Discussion

Apoptosis and TNF-α

PA-induced apoptosis in the current study is closely proportional to apoptosis. This suggests that TNF-a, is associated with the apoptosis activity induced by PA. In murine models, enhanced apoptosis of hepatocytes induced by TNF-a is associated with inflammation, fibrosis, and increased risk for hepatocellular carcinoma[107]. TNF-a induced apoptosis in normal cell, which would result in poor perfusion to the liver and lead to VOD and exacerbate liver damage.

Alcoholic hepatitis is an extensively studied liver disease that involves pro-inflammatory cytokines such as TNF-α[98]. Although TNF-a contributes to the elevated level of apoptosis, oxidative stress may play an equal or even greater role in regards to the ability for PA to induce apoptosis. Our previous research has demonstrated that PAs deplete cellular glutathione level due to oxidative stress from its reactive metabolite, while antioxidants or N-acetyl-cystine, the precursor to glutathione, can alleviate cell death[103]. Similarly, ethanol induced a concentration-dependent reduction of glutathione level in hepatocytes. PA-exposure to cells that have been treated with ethanol would deplete cellular and mitochondrial glutathione pool at a great extent thus contributing to greater cytotoxicity. Furthermore, there is evidence that shows ethanol suppress glutathione synthesis, thus disrupting the ability for hepatocytes to re-synthesize endogenous antioxidants for cellular protection from further oxidative stress[108]. It is expected that the oxidative stress contributed by both PA and ethanol would synergistically exacerbate hepatocytotoxicity.

VEGF

This is the first experiment to report that PA decreases expression of VEGF in cells as well as release of VEGF in the cell media. Serum VEGF level is a diagnostic biomarker that may play a role in the prognosis of VOD. Similarly, in animal models, acute exposure of PA increases VEGF level from hepatic endothelial cells. Nyska et al[109] proposes that the increase of VEGF level is due to the hypoxic environment occluded by enlarged hepatocytes, whereas Moye et al[110] proposes that VEGF level is increased to compensate the PA-induced apoptosis of endothelial cell. Despite the discrepancy, our *in vitro* model uses normal human hepatocytes at normoxic conditions, whereas the findings by Nyska et al[109] are under hypoxic conditions. The p65 subunit of NF-κB (NF-κB65) expression was reduced in mice treated with *Senecio brasiliensis* with inflammation induced by carrageenan[111]. Thus the lowered VEGF expression can be explained by the down-regulation of the NF-κBp65, which in return down-regulates HIF-1a and its downstream pro-angiogenic genes

such as VEGF. The mechanism remains unclear but from our findings and previous research, we suggest that PAs have the ability to down-regulate NF-κB and its downstream genes such as VEGF and this may be a contributing factor to the pathogenesis of veno-occlusive disease. As the disease progresses, hypoxic state due to the abnormal deficit of VEGF and hepatomegaly induces gene expression of HIF-1α increasing VEGF. Nevertheless, further investigation is required to understand the molecular mechanism responsible for VEGF down-regulation by PAs and potential cross-talking pathways. Indicine N-oxide is a PA found in *Heliotropium indicium* and has been produced semi-synthetically for phase I and II clinical trials in patients with advanced solid tumors and leukemia[112-114]. Attempts are being made to develop an indicine N-oxide analog that can be used as an anti-cancer agent, while being less toxic to the patient. Miser et al[113,114] carried out a phase II clinical trial in children with relapsed acute leukemia and although indicine N-oxide showed some anti-leukemic activity, it was associated with severe and irreversible hepatotoxicity. Although PA appears to be a promising compound for anti-cancer therapy due to its cytotoxic and angiogenesis inhibiting property, due to its severe hepatotoxicity, clinical application is limited[112-114].

Cytokines-Extracellular Matrix-VOD

Metallo-peptidase-9 and c-Jun N-terminal kinase activity are believed to be involved in the pathogenesis of PA-VOD. Nakamura's team treating rats with a non-specific tyrosine kinase inhibitor such as VEGF-receptor 2 (Sorafenib) they reduce the severity of PA-VOD[115]. Similarly, regorafenib, a multikinase inhibitor, was shown to reduce the severity of PA-induced VOD in rats alongside with decreased activity of metalloproteinase-9[116]. Furthermore, sesamol has also shown to attenuate PA-induced VOD such that treated rats show less inflammatory cell recruited to the liver, down-regulation of matrix metallo-proteinase-9, and up-regulation of tissue inhibitor of matrix metallo-proteinase-1[117]. In humans, VOD, previously called Budd-Chiari syndrome (BCS), is resulting from obstruction of the hepatic venous outflow tract that typically presents with abdominal pain, jaundice, and ascites without liver failure. However, BCS may also evolve to acute liver failure (ALF). The Acute Liver Failure Study Group (ALFSG) described the clinical features and outcomes of 20 ALF due to BCS In-hospital mortality were approximately 60%. Vascular causes of fulminant hepatic failure include hepatic vein thrombosis, veno-occlusive disease, and ischemic hepatitis. BCS mandates prompt diagnosis and management for successful outcomes[118].

Conclusions

PAs-containing species induce apoptosis in normal human hepatocytes. In addition, cells are susceptible to a greater degree of liver damage, when they are exposed to both PAs and alcohol. Therefore, individuals with preexisting liver injury or simultaneously misusing alcohol or a xenobiotic that induce liver damage may be more susceptible to PA-induced hepatotoxicity. Our research suggests that inflammation may play a role in the pathogenesis of PA-induced hepatotoxicity, as indicated by TNFα. However, this requires further investigation in its relations to the clinical symptoms found in patients with PA-induced VOD as well as other cross-talking pathways such as the NF-κB or other cytokines. Our present research suggests that PA is capable of down-regulating VEGF, which can be further investigated as an angiogenesis inhibitor for cancer therapy. It is important to note that, although cytotoxicity and down-regulation of VEGF seems to characterize PA as a promising compound for anti-cancer medicine, its clinical application is limited due to its potency to induce liver damage. If PA were to be used as medicine, it must be monitored for its hepatotoxic effect, while retaining its cytotoxicity and ability to decrease levels of VEGF. The findings of our research open the way for better understanding of ROS-and cytokines-dependent signaling pathways involved in the processes of PA-induced hepatocytotoxicity, on one side and natural antitumor mechanisms on the other. The knowledge of these mechanisms may enable therapeutic interference in the future. PAs-induced liver toxicity is a concern that demonstrates the lack of pharmacovigilance regarding traditional medicine. Therefore, a greater degree of safety regulation is required to assess the toxicological profiles of traditional medicine that are available to the public. Positive properties of complementary and traditional medicine may include improvement of disease-specific outcomes. Drug-herb interactions leading to hepat otoxicity negatively impacts the patient and health care professionals. We conclude that personalized medicine and assessing individual risk to herbal-induced liver injury should be equally important for naturopaths

and patients since alternatives for the herbals involved in this toxicity may be needed for future treatment.

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Conflict of interest

The authors declare no conflicts of interest.

References

1) Smith LW, Culvenor CCJ. Plant sources of hepatotoxic pyrrolizidine alkaloids. J Nat Prod 1981; 44: 129-152.
2) Hartmann T. Chemical ecology of pyrrolizidine alkaloids. Planta 1999; 207: 483-495.
3) Lindigkeit R, Biller A, Buch M, Schiebel HM, Boppre M, Hartmann T. The two faces of pyrrolizidine alkaloids: the role of the tertiary amine and its N-oxide in chemical defense of insects with acquired plant alkaloids. Eur J Biochem 1997; 245: 626-636.
4) Grue MR, Liddell JR. Pyrrolizidine alkaloids from Senecio chrysocoma. Phytochem 1993; 33: 1517-1519.
5) Culvenor CC, Edgar JA, Jago MV, Outteridge A, Peterson JE, Smith LW. Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. Chem Biol Interact 1976; 12: 299-324.
6) Benninger J, Schneider HT, Schuppan D, Kirchner T, Hahn EG. Acute hepatitis induced by greater celandine (Chelidonium majus). Gastroenterology 1999; 117: 1234-1237.
7) Crijns AP, de Smet PA, van den Heuvel M, Schot BW, Haagsma EB. Acute hepatitis after use of a herbal preparation with greater celandine (Chelidonium majus). Ned Tijdschr Geneeskd 2002; 146: 124-128.
8) Rifai K, Flemming P, Manns MP, Trautwein C. Severe drug hepatitis caused by Chelidonium. Internist (Berl) 2006; 47: 749-751.
9) Schneider J, Tsegaye Y, Tensae M, Selassie S, Haile T, Bane A, Ali A, Mesfin G, Seboxa T. Veno-occlusive liver disease: a case report. Ethiop Med J 2012; 50 Suppl 2: 47-51.
10) Stickel F, Seitz HK. The efficacy and safety of comfrey. Public Health Nutr 2000; 3: 501-508.
11) Teschke R, Glass X, Schulze J, Eickhoff A. Suspected greater celandine hepatotoxicity: liver-specific causality evaluation of published case reports from Europe. Eur J Gastroenterol Hepatol 2012; 24: 270-280.
12) Teschke R, Glass X, Schulze J. Herbal hepatotoxicity by Greater Celandine (Chelidonium majus): causality assessment of 22 spontaneous reports. Regul Toxicol Pharmacol 2011; 61: 282-291.
13) Teschke R, Wolff A, Frenzel C, Schulze J. Review article: Herbal hepatotoxicity--an update on traditional Chinese medicine preparations. Aliment Pharmacol Ther 2014; 40: 32-50.
14) Zimmerman HJ, Lewis JH. Chemical- and toxin-induced hepatotoxicity. Gastroenterol Clin North Am 1995; 24: 1027-1245.
15) Pantano F, Tittarelli R, Mannocchi G, Zaami S, Ricci S, Giorgetti R, Terranova D, Busardò FP, Marinelli E. Hepatotoxicity Induced by "the 3Ks": Kava, Kratom and Khat. Int J Mol Sci 2016; 17: 580.
16) Zimmerman HJ. Drug-induced liver disease. In: Hepatotoxicity. The adverse effects of drugs and other chemicals on the liver, 1st ed, Appleton-Century-Crofts, New York, 1999.
17) Alissa EM. Medicinal herbs and therapeutic drugs interactions. Ther Drug Monit; 2014; 36: 413-422.
18) Brazier NC, Levine MA. Drug-herb interaction among commonly used conventional medicines: a compendium for health care professionals. Am J Ther 2003; 10: 163-169.
19) Shi S, Klotz U. Drug interactions with herbal medicines. Clin Pharmacokinet 2012; 51: 77-104.
20) Robinson O, Want E, Coen M, Kennedy R, van den Bosch C, Gebrehawaria Y, Kudo H, Sadiq F, Goldin RD, Hauser ML, Fenwick A, Toledano MB, Thursz MR. Hirmi Valley liver disease: a disease associated with exposure to pyrrolizidine alkaloids and DDT. J Hepatol 2014; 60: 96-102.
21) Neuman MG, Cohen L, Opris M, Nanau RM, Hyunjin J. Hepatotoxicity of pyrrolizidine alkaloids. J Pharm Pharm Sci 2015; 18: 825-843.
22) Ernst E. Heavy metals in traditional Indian remedies. Eur J Clin Pharmacol 2002; 57: 891-896.
23) Schulz M, Meins J, Diemert S, Zagermann-Muncke P, Goebel R, Schrenk D, Schubert-Zsilavecz M, Abdel-Tawab M. Detection of pyrrolizidine alkaloids in German licensed herbal medicinal teas. Phytomed 2015; 23: 647-656.
24) Madge I, Cramer L, Rahaus I, Jerz G, Winterhalter P, Beuerle T. Pyrrolizidine alkaloids in herbal teas for infants, pregnant or lactating women. Food Chem 2015; 187: 491-498.
25) Martinello M, Cristofoli C, Gallina A, Mutinelli F. Easy and rapid method for the quantitative determination of pyrrolizidine alkaloids in honey by ultra performance liquid chromatography-mass spectrometry: an evaluation in commercial honey. Food Control 2014; 37: 146-152.
26) Bolechova M, Casavsky J, Pospichalova M, Kosubova P. UPLC-MS/MS method for determination of selected pyrrolizidine alkaloids in feed. Food Chem 2015; 170: 265-270.

27) Crews C, Driffield M, Berthiller F, Krska R. Loss of pyrrolizidine alkaloids on decomposition of ragwort (Senecio jacobaea) as measured by LC-TOF-MS. J Afric Food Chem 2009; 57: 3669-3673.

28) Crews C, Berthiller F, Krska R. Update on analytical methods for toxic pyrrolizidine alkaloids. Anal Bioanal Chem 2010; 396: 327-338.

29) Dubecke A, Beckh G, Lullmann C. Pyrrolizidine alkaloids in honey and bee pollen. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28: 348-358.

30) Griffin CT, O'Mahony J, Danaher M, Furey A. Liquid chromatography tandem mass spectrometry detection of targeted pyrrolizidine alkaloids in honeys purchased within Ireland. Food Anal Methods 2015; 8: 18-31.

31) Griffin CT, Danaher M, Elliott CT, Kennedy DG, Furey A. Detection of pyrrolizidine alkaloids in commercial honey using chromatography-ion trap mass spectrometry. Food Chem 2013; 136: 1577-1583.

32) Oplatowska M, Elliott CT, Huet AC, McCarthy M, Mulder PP, von Holst C, Delahaut P, Van Egmond HP, Campbell K. Development and validation of rapid multiplex ELISA for pyrrolizidine alkaloids and their N-oxides in honey and feed. Anal Bioanal Chem 2014; 406: 757-770.

33) Ruan J, Gao H, Li N, Xue J, Chen J, Ke C, Ye Y, Fu PP, Zheng J, Wang J, Lin G. Blood pyrrole-protein adducts--a biomarker of pyrrolizidine alkaloid-induced liver injury in humans. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2015; 33: 404-421.

34) Xia Q, Zhao Y, Lin G, Beland FA, Cai L, Fu PP. Pyrrolizidine alkaloid-protein adducts: potential non-invasive biomarkers of pyrrolizidine alkaloid-induced liver toxicity and exposure. Chem Res Toxicol 2016; 15; 1282-1292.

35) Mayer F, Luthy J. Heliotrope poisoning in Tadjikistan. Lancet 1993; 342: 246-247.

36) Selzer G, Parker RGF. Senecio poisoning exhibiting as Chiari's syndrome. A report on twelve cases. Am J Pathol 1951; 27: 165-185.

37) Culvenor CCJ, Clarke M, Edgar JA, Frahn JL, Jago MV, Peterson JE, Smith LW. Structure and toxicity of the alkaloids of Russian comfrey (Symphytum x uplandicum Nyman), a medicinal herb and item of human diet. Experientia 1980; 36: 377-379.

38) Huxtable RJ. Herbal teas and toxins: novel aspects of pyrrolizidine poisoning in the United States. Perspect Biol Med 1990; 24: 1-14.

39) McGee JOD, Patrick RS, Wood CB, Blumgart LH. A case of veno-occlusive disease of the liver in Britain associated with herbal tea consumption. J Clin Path 1976; 29: 788-794.

40) Steenkamp V, Stewart MJ, Zuckerman M. Clinical and analytical aspects of pyrrolizidine poisoning caused by South African traditional medicine. Ther Drug Monit 2000; 22: 302-306.

41) Deinzer ML, Thomson PA, Burgett DM, Isaacson D. Pyrrolizidine alkaloids: their occurrence in honey from tansy ragword (Senecio jacobaea L.). Science 1977; 195: 494-499.

42) Edgar JA, Roeder E, Molyneux RJ. Honey from plants containing pyrrolizidine alkaloids: a potential threat to health. J Agric Food Chem 2002; 50: 2719-2730.

43) Edgar JA, Colegate SM, Boppre M, Molyneux RJ. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28: 308-324.

44) Kempf M, Wittig M, Reinhard A, von der Ohe K, Blacquière T, Raezke KP, Schreier P, Beuerle T. Pyrrolizidine alkaloids in honey: comparison of analytical methods. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28: 332-347

45) Kempf M, Wittig M, Schönfeld K, Cramer L, Schreier P, Beuerle T. Pyrrolizidine alkaloids in food: downstream contamination in the food chain caused by honey and pollen. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28: 325-331.

46) Hoogenboom LAP, Mulder PPJ, Zeilmaker MJ, van den Top HJ, Remmelink GJ, Brandon EFA, Klinjnstra M, Meijer GAL, Schothorst R, Van Egmond HP. Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28: 359-372.

47) Roulet M, Laurini R, Rivier L, Calame A. Hepatic veno-occlusive disease in newborn infant of a woman drinking herbal tea. J Pediatr 1988; 112: 443-436.

48) Rasenack R, Muller C, Kleinschmidt M, Rasenack J, Wiedenfeld H. Veno-occlusive disease in a fetus caused by pyrrolizidine alkaloids of food origin. Fetal Diagn Ther 2003; 18: 223-225.

49) McGaw LJ, Eloff JN. Ethno-veterinary use of Southern African plants and scientific evaluation of their medicinal properties. J. Ethnopharmacol 2008; 119: 559-574.

50) Jacobs J, Sing S. Ecology and management of tansy ragword (Senecio jacobaea L.). United States Department of Agriculture Natural Resources Conservation Service Invasive Species Technical Note 2009, 1-13.

51) Rosemann GM, Botha CJ, Eloff JN. Distinguishing between toxic and non-toxic pyrrolizine alkaloids and quantification by liquid chromatography-mass spectrometry. Phytochem Lett 2014; 8: 126-131.

52) Castells E, Mulder PPJ, Perez-Trujillo M. Diversity of pyrrolizidine alkaloids in native and invasive Senecio pterophorus (Asteraceae): implications for toxicity. Phytochem 2014; 108: 137-146.

53) Conforti F., Loizzo MR, Statti GA, Houghton PJ, Menichini F. Biological properties of different extracts of two Senecio species. Int J Food Sci Nutr 2006; 57: 1-8.

54) Lin G, Wang JY, Li N, Li M, Gao H, Ji Y, Zhang F, Wang H, Zhou Y, Ye Y, Xu HX, Zheng J. Hepatic sinusoidal obstruction syndrome associated with consumption of Gynura segetum. J Hepatol 2011; 54: 666-673.

55) Stegelmeier B. Pyrrolizidine alkaloid-containing toxic plants (Senecio, Crotalaria, Cynoglossum, Amsinckia, Heliotropium, and Echium spp). Vet Clin North Am Food Anim Pract 2011; 27: 419-428.

56) Molyneux RJ, Gardner DL, Colegate SM, Edgar JA. Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28: 293-307.

57) Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clin Biochem 2000; 33: 517-524.

58) Neuman MG, Shear NH, Jacobson-Brown PM, Katz GG, Neilson HK, Malkiewicz IM, Cameron RG, Abbott F. CYP2E1-mediated modulation of valproic acid-induced hepatocytotoxicity. Clin Biochem 2001; 34: 211-218.

59) Krivoy N, Struminger L, Bendersky R, Aviv I, Neuman MG, Pollack S. Rifampin-induced thrombocytopenia and hemolysis: diagnosis by a novel in-vitro lymphocyte toxicity assay. The Israel Med Association J 2001; 3: 536-537.

60) Neuman MG, Ishay J, Waron M, Scapa E, Eshchar J. Hepatotoxic effects of repeated administration of the Oriental hornet (Vespa orientalis) venom. J Clin Lab Anal 1990; 4: 453 456.

61) Datta DV, Khuroo MS, Mattocks AR, Aikat BK, Chhuttani PN. Herbal medicines and veno-occlusive disease in India. Postgrad Med J 1978; 54: 511-515.

62 Chauvin P, Dillon JC, Moren A. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993. Sante 1994; 4: 263-268.

63) Grobler AC, Koen M, Bouwers F. Planttoksiene en lewersiektes by kinders in Pretoria en omgewing. Tijdschr Kindergeneeskd 1997; 65: 99-104.

64) Willmot FC, Robertson GW. Senecio disease, or cirrhosis of the liver due to Senecio poisoning. Lancet 1920; 196: 848-849.

65) Conradie J, Stewart MJ, Steenkamp V. GC/MS identification of toxic pyrrolizidine alkaloids in traditional remedies given to two sets of twins. Ann Clin Biochem 2005; 42: 141-144.

66) Richardson P, Guinan E. The pathology, diagnosis, and treatment of hepatic veno-occlusive disease: current status and novel approaches. Br J Haematol 1999; 107: 485-493.

67) Wadleigh M, Ho V, Momtaz P, Richardson P. Hepatic veno-occlusive disease: pathogenesis, diagnosis and treatment. Curr Opin Hematol 2003; 10: 451-462.

68) Kakar F, Akbarian Z, Leslie T, Mustafa ML, Watson J, van Egmond HP, Omar MF, Mofleh J. An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. J Toxicol 2010; 2010: 313280.

69) Neuman MG, Winkler RE. Veno-occlusive disease of the liver induced by herbal medicine. Rom J Hepatology 2008; 2: 39-51.

70) Auerbach BJ, Reynolds SJ, Lamorde M, Merry C, Kukunda-Byobona C, Ocama P, Semeere AS, Ndyanabo A, Boaz I, Kiggundu V, Nalugoda F, Gray RH, Wawer MJ, Thomas DL, Kirk GD, Quinn TC, Stabinski L; Rakai Health Sciences Program. Traditional herbal medicine use associated with liver fibrosis in rural Rakai, Uganda. PLoS One 2012; 7: e41737.

71) Neuman MG, Steenkamp V. Toxicity profile of pyrrolizidine alkaloid-containing medicinal plants: emphasis on Senecio species. Indian J Biological Sc, Intern J Biomedical Pharm Sc f IJBPS-11-13-2009; 3: 13, 26-30, 104-108.

72) Neuman MG, Schneider M, Nanau RM, Parry C. Hepatic, gastrointestinal and pancreatic adverse reactions of HAART: role of alcohol and HIV medication. Int J Hepatology 2012; 2012: 760706.

73) Schneider M, Chersich M, Neuman MG, Parry C. Alcohol consumption and HIV/AIDS: the neglected interface. Addiction 2012; 107: 1369-1371.

74) Nguta JM, Mbaria JM, Gakuya DW, Gathumbi PK, Kiama SG. Antimalarial herbal remedies of Msambweni, Kenya. J Ethnopharmacol 2010; 128: 424-432.

75) Bach N, Thung SN, Schaffner F. Comfrey herb tea-induced hepatic veno-occlusive disease. Am J Med 1989; 87: 97-99.

76) Sperl W, Stuppner H, Gassner I, Jdumaier W, Dietze O, Vogel W. Reversible hepatic veno-occlusive disease in an infant after consumption of pyrrolizidine-containing herbal tea. Eur J Pediatr 1995; 165: 112-116.

77) McLean E. The toxic actions of pyrrolizidine (Senecio) alkaloids. Pharmacol Rev 1970; 22: 429-483.

78) Chou MW, Fu, PP. Formation of DHP-derived DNA adducts in vivo from dietary supplements and Chinese herbal plant extracts containing carcinogenic pyrrolizidine alkaloids. Toxicol Ind Health 2006; 22: 321-327.

79) Dai J, Zhang F, Zheng J. Retrorsine, but not monocrotaline, is a mechanism-based inactivator of P450 3A4. Chem Biol Interact 2010; 183: 49-56.

80) Tu M, Li L, Li H, Ma Z, Chen Z, Sun S, Xu S, Zhou H, Zeng S, Jiang H. Involvement of organic cation transporter 1 and CYP3A4 in retrorsine-induced toxicity. Toxicology 2014; 322: 34-42.

81) Yang YC, Yan J, Churchwell M, Beger R, Chan PC, Doerge DR, Fu PP, Chou MW. Development of a 32P-postlabeling/HPLC method for detection of dehydroretronecine-derived DNA adducts in vivo and in vitro. Chem Res Toxicol 2001; 14: 91-100.

82) Chen Y, Ji L, Wang H, Wang Z. Intracellular glutathione plays important roles in pyrrolizidine alkaloids-induced growth inhibition on hepatocytes. Environ Toxicol Pharmacol 2009; 28: 357-362.

83) Chen Y, Ji L, Xiong A, Yang L, Wang Z. Involvement of intracellular glutathione in regulating isoline-induced cytotoxicity in human normal liver L-02 cells. Toxicol Ind Health 2013; 29: 567-575.

84) Katz GG, Shear NH, Malkiewicz IM, Valentino K, Neuman MG. Signaling for ethanol-induced apoptosis and repair in vitro. Clin Biochem 2001; 34: 218-235.

85) He YQ, Yang L, Liu HX, Zhang JW, Liu Y, Fong A, Xiong AZ, Lu YL, Yang L, Wang CH, Wang ZT. Glucuronidation, a new metabolic pathway for pyrrolizidine alkaloids. Chem Res Toxicol 2010; 23: 491-599.

86) Chojkier M. Hepatic sinusoidal-obstruction syndrome: toxicity of pyrrolizidine alkaloids. J Hepatol 2003; 39: 437-446.

87) Ji LL, Zhang M, Sheng YC, Wang ZT. Pyrrolizidine alkaloid clivorine induces apoptosis in human normal liver L-02 cells and reduces the expression of p53 protein. Toxicology in Vitro 2005; 19: 41-46.

88) Neuman MG, Jia AY, Steenkamp V. Senecio latifolius induces in vitro hepatocytotoxicity in a human cell line. Can J Physiol Pharmacol 2007; 85: 1063-1075.

89) Zuckerman M, Steenkamp V, Steward MJ. Hepatic veno-occlusive disease as a result of a traditional remedy: confirmation of toxic pyrrolizidine alkaloids as the cause, using an in vitro technique. J Clin Pathol 2002; 55: 676-679.

90) Yeong ML, Swinburn B, Kennedy M, Nicholson G. Hepatic veno-occlusive disease associated with comfrey ingestion. J Gastro Hepatol 1990; 5: 211-214.

91) Steenkamp V, Stewart MJ, van der Merwe S, Zuckerman M, Crowther NJ. The effect of Senecio latifolius a plant used as a South African traditional medicine, on a human hepatoma cell line. J Ethnopharmacology 2001; 78: 51-58.

92) Bondan C, Soares JC, Cecium M, Lopes ST, Graca DL, da Rocha RX. Oxidative stress in erythrocytes of cattle intoxicated with Senecio sp. Vet Clin Pathol 2005; 34: 354-357.

93) Popat A, Shear NH, Stewart M, Thomson S, Malkiewicz I, Neuman MG. The hepatotoxicity of Callilepis laureola, a South African herbal medicine, in HepG2 cells in vitro. Clin Biochem 2001; 34: 219-227.

94) Popat A, Shear NH, Malkiewicz I, Thomson S, Neuman MG. Mechanism of Impila (Callilepis laureola)-induced cytotoxicity in Hep G2 cells. Clin Biochem 2002; 35: 57-64.

95) Coppell JA, Brown SA, Perry DJ. Veno-occlusive disease: cytokines, genetics, and haemostasis. Blood Rev 2003; 17: 63-70.

96) Xiong A, Yang F, Fang L, Yang L, He Y, Wan YJ, Xu Y, Qi M, Wang X, Yu K, Tsim WK, Wang Z. Metabolomic and genomic evidence for compromised bile acid homeostasis by senecionine, a hepatotoxic pyrrolizidine alkaloid. Chem Res Toxicol 2014; 27: 775-786.

97) Xiong A, Fang L, Yang X, Yang F, Qi M, Kang H, Yang L, Tsim K W-K, Wang Z. An application of target profiling analyses in the hepatotoxicity assessment of herbal98-medicines: comparative characteristic fingerprint and bile acid profiling of Senecio vulgaris L. and Senecio scandens Buch. Ham Anal Bioanal Chem 2014; 406: 7715-7727.

98) Neuman MG. Mechanisms of alcoholic liver disease: cytokines. Clin Biochem 2001; 34: 163-166.

99) Li YH, Kan WLT, Li N, Lin G. Assessment of pyrrolizidine alkaloid-induced toxicity in an in vitro screening model. J Ethnopharmacol 2013; 150: 560-567.

100) Nuringyas TR, Verpoorte R, Klinkhamer PGL. Toxicity of pyrrolizidine alkaloids to Spodoptera exigua using insect cell lines and injection bioassays. J Chem Ecol 2014; 40: 609-616.

101) Ji L, Chen Y, Liu T, Wang Z. Involvement of Bcl-xL degradation and mitochondrial-mediated apoptotic pathway in pyrrolizidine alkaloids-induced apoptosis in hepatocytes. Toxicol Appl Pharmacol 2008; 231: 393-400.

102) Ji L, Liu T, Chen Y, Wang Z. Protective mechanisms of N-acetyl-cysteine against pyrrolizidine alkaloid clivorine-induced hepatotoxicity. J Cell Biochem 2009; 108: 424-432.

103) Neuman MG, Cameron RG, Shear NH, Bellentani S, Tiribelli C. Effect of tauroursodeoxycholic and ursodeoxycholic acid on ethanol-induced cell injuries in human Hep G2 cell line. Gastroenterology 1995; 109: 555-563.

104) Neuman MG, Shear NH, Bellentani S, Tiribelli C. Role of cytokines in ethanol-induced cytotoxicity in vitro in Hep G2 cells. Gastroenterology 1998; 115: 157-166.

105) van Wyk BE, Verdoorn GH, Schutte AL. Distribution and taxonomic significance of major alkaloids in the genus Podalyria. Biochem Systematic Ecol 1992; 20: 163-172

106) Holstege DM, Seiber JN, Galey FD. Rapid multi-residue screen for alkaloids in plant material and biological samples. J Agric Food Chem 1995; 43: 691-699.

107) Takehara T, Tatsumi T, Suzuki T, Rucker III E B, Hennighausen L, Jinushi M, Miyagi T, Kanazawa Y, Hayahi N. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gasteroenterology 2004; 127: 1189-1197.

108) Speisky H, Macdonald A, Giles G, Orrego H, Israel Y. Increased loss and decreased synthesis of hepatic glutathione after ethanol administration. Biochem J 1985; 225: 565-572.

109) Nyska A, Roomaw CR, Foley JF, Maronpot RR, Malarkey DE, Cummings CA, Shyamal P, Moyer CF, Allen DG, Travlos G, Chan PC. The hepatic endothelial carcinogen riddelliine induces endothelial apoptosis, mitosis, S phase, and p53 and hepatocytic vascular endothelial growth factor expression after short-term exposure. Toxicol App Pharmacol 2002; 184: 153-164.

110) Moyer C, Allen D, Basabe A, Maronpot RR, Nyska A. Analysis of vascular endothelial growth factor (VEGF) and a receptor subtype (KDR/flk-1) in the liver of rats exposed to riddelliine: a potential role in the development of hemangiosarcoma. Exp Toxic Pathol 2004; 55: 455-465.

111) de Souza RR, Bretanha LC, Dalmarco EM, Pizzolatti MG, Frode TS. Modulatory effect of Senecio brasiliensis (Spreng) Less. In a murine model of inflammation induced by carrageenan into the pleural cavity. J Ethnopharmacol 2015; 168: 373-379.

112) Niwa H, Ogawa T, Yamada K. An efficient enantioselective synthesis of (+)-indicine N-oxide, an antitumor pyrrolizidine alkaloid. Tetrahedron Letters 1989; 30: 4985-4986.

113) Miser JS, Smithson WA, Krivit W, Hughes CH, Davis D, Krailo MD, Hammond GD. Phase II trial of ind-

icine N-oxide in relapsed pediatric solid tumors. Invest New Drugs 1991; 9: 339-342.

114) Miser JS, Smithson WA, Krivit W, Hughes CH, Davis D, Krailo MD, Hammond GD. Phase II trial of indicine N-oxide in relapsed acute leukemia of childhood. Am J Clin Oncol 1992; 15: 135-140.

115) Nakamura K, Hatano E, Narita M, Miyagawa-Hayashino A, Koyama Y, Nagata H, Iwaisako K, Taura K, Uemoto S. Sorafenib attenatues monocrotalien-induced sinusoidal obstruction syndrome in rats through suppression of JNK and MMP-9. J Hepatology 2012; 57: 1037-1043.

116) Okuno M, Hatano E, Nakamura K, Miyagawa-Hayashino A, Kasai Y, Nishio T, Seo S, Taura K, Uemoto S. Regorafenib suppresses sinusoidal obstruction syndrome in rats. J Surg Res 2015; 193: 693-703.

117) Periasamy S, Hsu DZ, Chen SY, Yang SS, Chandrasekaran VR, Liu MY. Therapeutic sesamol attenatues monocrotaline-induced sinusoidal obstruction syndrome in rats by inhibiting matrix metalloproteinase-9. Cell Biochem Biophys 2011; 61: 327-336.

118) Parekh J, Matei VM, Canas-Coto A, Friedman D, Lee WM; Acute Liver Failure Study Group. Budd-Chiari syndrome causing acute liver failure: a multicenter case series. Liver Transpl 2017; 23: 135-142.