Pyrrolizidine alkaloids enhance alcohol-induced hepatocytotoxicity \textit{in vitro} in normal human hepatocytes

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\textbf{Abstract.} – \textbf{OBJECTIVE:} Herbal remedies containing pyrrolidine alkaloids (PAs) 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 hepato-cytotoxicity in the presence or absence of ethanol (EtOH) \textit{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.

\textbf{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.

\textbf{RESULTS:} In NHH cells, a 100 mmol/L dose of EtOH resulted in 22±3.5 apoptosis (p<0.01 vs. control). Two consecutive doses of 100 mmol/L EtOH for 24 hrs each caused 36±3.0% apoptosis (p<0.01 vs. control) and apoptosis (p<0.05 vs. one dose EtOH). 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)]. 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 EtOH treated cells after 1 and 2 treatments (p<0.001 vs. control). Treatment with EtOH 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)].

\textbf{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.

\textbf{Key Words:} Alcohol-induced liver damage, Apoptosis, Caspase, CYP 2E1, Glutathione, Herbal-induced hepatotoxicity, Normal human hepatocytes, Mitochondria, Pyrrolizidine 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 cleaved cytokeratine; CYP = cytochrome P-450; DNA, deoxyribo nucleic acid; DTPNB = 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; HIL = herbal-induced liver injury; HPLC = high-performance liquid chromatography; IDN = caspase 3-inhibitor IDUN-Pharma; IFN-γ = interferon gamma; IL = interleukin; LPS = lipopolysaccharide; MDA = malondiadehyde; 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 expressin; 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 = hepato-sinusoidal obstruction syndrome; VOD/HOS = BCS = Ve- no-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 families3-5. Plants are reported to have PAs such as senecionine, retrorsine, ridelidine, integerrime, neosenkirkine, and floresinenc6. 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 chains5. The hepatotoxicity was reported after ingestion of the herbs belonging to following families of plants Boraginaceae (Heliotropium, Trichodesma, Synphytum [Comfrey]), Compositae (Senecio [Bush Teas], Eupatorium), Leguminoises ([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 toxic14-16. Alissa7 shows the beneficial effects of natural products, while Brazier et al18 and Shi et al19 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 pollution20,21. Ernst22 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, Zimmerman16 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 criteria3.

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 purchase23. Many herbal teas marketed for infants pregnant women, or lactating mothers contain traces of PA that may be harmful to the exposed child24. 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 quadrupole-mass spectrometry (UHPLC-MS)-and thin layer chromatography (TLC), have been conducted in order to provide quantitative data regarding the amount of PAs25-33. Xia et al34 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 PAs25,26 or by consumption of herbal teas37-41. Another source of exposure to PA is from honey contaminated with PAs25,26. Milk could also contain traces of PAs if the cows eat PAs-contaminated supply42-45. Milk could also contain traces of PAs if the cows eat PAs-contaminated supply46. 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 PAs47. Additionally, Rasenack et al48 described VOD in a fetus caused by PAs origin-
Hepatocytotoxicity of pyrrolidizine alkaloids in vitro

described hepatic VOD by ascites, hyperbilirubinemia, hepatomegaly, and abdominal pain. Others also reported the same symptoms. Kakar et al 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. Conradie et al described two pairs of toddler twins that ingested PA-rich 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 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 centrolobular 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. 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. 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. Furthermore, in the Msambweni community of Kenya, Senecio syringiotolius is used as an antimalarial remedy. Medicinal, homeopathic or natural remedies containing PA-rich herbs also induced serious liver VOD, in many parts of the world. 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. PAs undergo biotransformation by CYP3A its reactive metabolites. 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. The excess of pyrrolizidine N-oxide metabolites metabolites can be further transformed into toxic
epoxides and necine bases\textsuperscript{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\textsuperscript{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\textsuperscript{86}, show that senecione 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\textsuperscript{85}. 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\textsuperscript{87-94}. 

Inflammatory response is part of PA-induced VOD. Cytokines such as tumor necrosis factor alpha (TNF-\(\alpha\)), interleukin-1 beta (IL-1\(\beta\)), and endothelin-I (ET-1) are secreted by monocytes in response to PAs\textsuperscript{86}. Bile acid homeostasis is also compromised. Xiong et al\textsuperscript{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\textsuperscript{97}. Previously, Neuman\textsuperscript{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\textsuperscript{93,94}. Other used bromodeoxyuridine incorporation assay, neutral red uptake assay, resazurin assay, and lactate dehydrogenase release assay\textsuperscript{99}. Moreover, insect cell line and infection bioassay also show agreed conclusion that PAs are cytotoxic in a dose-dependent manner\textsuperscript{100}. L-02 cells also show dose-dependent and time-dependent that senecione and other PAs such as adonifoline, senecione, monocrotaline, and isolate deplete cellular glutathione level and increase the level of oxidized glutathione resulting in 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\textsuperscript{93} and glutathione synthesis inhibitor increased the susceptibility of PA-induced hepatocytotoxicity\textsuperscript{93,94}. Primary mice hepatocytes have shown that senecione and other PAs induce apoptotic DNA laddering, caspase-3 activation, and decreased level of Bcl-xL, an anti-apoptotic protein\textsuperscript{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\textsuperscript{101,102}. Our previous research\textsuperscript{93} 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\textsuperscript{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\textsuperscript{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\textsuperscript{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-
Hepatocytotoxicity of pyrrolidizine alkaloids in vitro

Kines in this model has been previously studied in our laboratory. 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). 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. Detection of PAs in this preparation was performed using a gas chromatography-mass spectrometry (GSMS) method of Holstege et al.

**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-diethylation activity (CYP2B1) and p-nitroso-dimethyl-aminine dimethylase activity (CYP2E1). 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 IC50 of 33.56±0.72 µM for NHH. Cells were seeded in flasks (1 x 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% O2-5% CO2 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-di-phenyl-tetrazolium bromide), GSH reductase, GSH standard, NADPH (reduced nicotinamide adenine dimucleotide), 5-sulfosalicylic 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-
Tryptsin 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-1β, 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\(^{48}\). 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

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58
assessed by electron microscopy, cell shrinkage, electron dark cytoplasm, and apoptotic bodies were considered criteria for classical apoptosis\textsuperscript{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\textsuperscript{R} Visual Basic\textsuperscript{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 (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 \((p<0.001 \text{ vs. control})\) (Figure 2i). Two consecutive doses of 100 mmol/L EtOH for 24 hrs each caused 36±3.0\% apoptosis \((p<0.001 \text{ vs. control and } p<0.05 \text{ vs. one dose})\). Pre-treatment with 50 µmol/L caspase inhibitor significantly reduced EtOH-induced apoptosis \([12±1.5\% \text{ in 100 mmol/L } (p<0.05) \text{ and } 20±4.0\% \text{ in } 2\times100 \text{ mmol/L } (p<0.001)]\). PAs significantly enhanced apoptosis \([12±1.5\% \text{ in 100 mmol/L } (p<0.05) \text{ and } 44±4.0\% \text{ in } 2\times100 \text{ mmol/L } (p<0.001)]\). In addition, pre-treatment with 50 µmol caspase inhibitor in cells treated with PA + EtOH reduced apoptosis significantly \((\Delta -22±3.0\% \text{ } (p<0.05))\). Pre-treatment with 50 µmol caspase inhibitor significantly reduced 100 mmol/L EtOH-induced apoptosis \((\text{one dose})\) 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 \((p<0.001 \text{ vs. control})\) (Table I).

Table I. Glutathione levels in tissue culture.

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

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-
significant change when it is added to control cells. However, the cells exposed to ethanol in the presence of IDN showed a decrease in TNF-α production in 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...
Hepatocytotoxicity of pyrrolizidine alkaloids in vitro

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 cytokeratin 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 destoroted 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.
Discussion

**Apoptosis and TNF-α.**

PA-induced apoptosis in the current study is closely proportional to apoptosis. This suggests that TNF-α, is associated with the apoptosis activity induced by PA. In murine models, enhanced apoptosis of hepatocytes induced by TNF-α is associated with inflammation, fibrosis, and increased risk for hepatocellular carcinoma. TNF-α 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. Although TNF-α 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-cysteine, the precursor to glutathione, can alleviate cell death. 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 resynthesize endogenous antioxidants for cellular protection from further oxidative stress. 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 proposes that the increase of VEGF level is due to the hypoxic environment occluded by enlarged hepatocytes, whereas Moye et al 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 are under hypoxic conditions. The p65 subunit of NF-κB (NF-κBp65) expression was reduced in mice treated with Senecio brasiliensis with inflammation induced by carrageenan. Thus the lowered VEGF expression can be explained by the down-regulation of the NF-κBp65, which in return down-regulates HIF-1α 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{13}. Similarly, regorafenib, a multikinase inhibitor, was shown to reduce the severity of PA-induced VOD in rats alongside with decreased activity of metalloproteinase-9\textsuperscript{16}. 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 metalloproteinase-9, and up-regulation of tissue inhibitor of matrix metalloproteinase-1\textsuperscript{17}. 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\textsuperscript{18}.

**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 hepatotoxicity, 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 hepatotoxicity 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.

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