

Full Length Research Paper

Hepatotoxicity of aqueous extract and fractionated methanol extract of *Phytolacca americana* by isolated rat liver perfusion system

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Phytolacca americana is a perennial plant native to North America and other parts of the world. It is well known for several medicinal properties despite being considered to have digestive toxicity (especially hepatotoxicity). Our objective is to examine whether extract of aerial parts of *P. americana* could produce biochemical changes by Isolated Rat Liver Perfusion (IRLP) system, which is ideal for studying biochemical alterations of chemicals with minimum neural-hormonal effects. In this study, the liver was perfused with Kerbs-Henseleit buffer, containing different concentration of aqueous extract of the aerial parts of *P. americana* (10, 20, 40, 50, 100 mg/kg) and CHCl₃, ethanol and methanol fraction (10, 20, 40 mg/kg) were added to the buffer and perfused for 2 h. During the perfusion, many factors including amino-transferase activities were assessed as indicator of liver viability. Consequently, sections of liver tissue were examined for any histo-pathological changes. The results showed that histo-pathological changes in liver tissues were related in a dose-dependent manner to methanol extract of aerial parts *Phytolacca americana* concentrations. Doses of 50, 100 mg/kg caused significant ($P < 0.05$) histo-pathological changes.

Keywords: *Phytolacca americana*, Liver perfusion, Kerbs-Henseleit buffer, histo-pathological changes, amino-transferase activity.

INTRODUCTION

Phytolacca americana, Pokeweed is a medical herb from Phytolaccaceae, shrubby perennial plant growing up to 10 feet in height native to eastern North America and other parts of the world. One hundred and fifty species of *Phytolacca* are available in western and northwestern of Iran, mainly in the coastal and forest areas; however, it is rarely and in most cases, accidentally used in Iran (Zargari, 1981; Mirhaydar, 1994). The leaves are alternate with coarse texture with moderate porosity and can reach nine inches in length. Leaves are medium green and with an unpleasant odor and mostly the boiled leaves, are

used in a popular salad (called grandmother salad), in the American diet.

The flowers have 5 regular parts with upright stamens and are up to 0.2 inches wide. They have white petal-like sepals without true petals. A shiny dark purple berry held in racemous clusters on pink pedicles with a pink peduncle. Pedicles are without berries have a distinctive rounded five part calyx. It also has tan cortex, white pulp, moderate number of rootlets. Transversely cut root slices show concentric rings, and has no nitrogen fixation ability (Santillo, 1993; Murray and Pizzorno, 1999).

Known constituents are alkaloids (betanidine, betanine, phytolaccine, prebetanine), triterpene saponins (phytolaccoside A, B, C, D, E, F, G or esculentoside E, Phytolaccagenin, jaligonic acid, esculentic acid, 3-oxo-30-carbomethoxy-23-norolean-12-en-28-oic acid, esculentic acid,

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phytolaccagenic acid, oleanolic acid), triperine alcohols (alpha spinasterol, alpha spinasteryl-beta-D-glucoside, 6 palmityl-delta 7-stigmasterol-delta-D-glucoside, 6 palmytityl-alph-spinasteryl-6-D-glucoside), and others (phytolaccatoxin, canthomicrol, astragaline, protein PAP-R, pokeweed nitrogen glycoprotein's (pa¹-pa⁵), pokeweed antiviral protein (PAP), caryophylline, histamine, GABA, tannin, starch) (Newall et al., 1996; Takahashi et al., 2001; Tyler, 1987).

P. americana or poke root (common name) has been most commonly used for its laxative properties. The dried root has found application in relieving pain, reducing inflammation, treating rheumatism and arthritis along with various skin diseases (Woo and Kang, 1976), Modern researchers are investigating the plant to determine if it possesses any anti-viral, anti-cancer, antifungal, or immune stimulant properties (Goldestein et al., 1973), despite being considered to have digestive toxicity especially hepato-toxicity (Harkness et al., 2003; Stein, 1975; Heinrich et al., 2004).

In this study, isolated rat liver perfusion was employed to evaluate *P. americana* hepato-toxicity and its correlation to biochemical changes.

MATERIALS AND METHODS

Animals

Male albino Wistar rats (6 to 8 weeks), weighing 200 - 250 g were used for all experiments. They were housed individually in standard rat cages in a room on a 12- h light- dark cycle at 22°C (22 ± 1°C) and 50 ± 5% with relative humidity, including food and water *ad libitum*. The animals were adapted to the condition for 7 days prior to the beginning of the experiments (Woo and Shin, 1976). The experiments were performed during the day time (08:00 - 16:00 h). Each animal was used once only. A research proposal was prepared according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethical Committee (IAEC) of Mazandaran University of Medical Sciences approved the proposal.

Plant

Aerial parts (flowered browse) of *P. americana* were collected from Mazandaran (a Northern state in Iran) in April 2007 and identified and confirmed by Department of Pharmacognosy. A voucher specimen (No.0506-16) has been deposited in Tehran School of Pharmacy Herbarium. Aerial parts were dried at room temperature and powdered before extraction. One hundred grams of the powdered sample was extracted at room temperature by percolation with methanol/water (80:20, 400 ml × 3 times). The resulting extract was concentrated over a rotary vacuum evaporator until a solid extract sample was obtained (Samsamshariat et al., 1981). The extract was prepared in phosphate buffer (pH = 7.4) for pharmacological studies.

Experimental design

Rats were divided into five treatment group and control group. Each group contained four male rats and their livers were perfused by a single dose of 10, 20, 40, 50 and 100 mg/kg of aqueous extract of

aerial parts of *P. americana* with CHCl₃, ethanol and methanol fraction (10, 20, 40 mg/kg) respectively (total = 16 groups). Control livers were perfused with the perfusion buffer. Following the preliminary study, the dose of 100 mg/kg was chosen for the remaining of the study in order to evaluate the hepato-toxicity of *P. americana* (Dehpour et al., 1999).

Buffer

Perfusion fluid was made of Kerbs-henseleit buffer. The perfusion medium consisted of 118.9 mM Na Cl, 4.76 mM KCl, 1.19 mM KH₂PO₄, 2.55 mM CaCl₂ and 24.8 mM NaHCO₃, at 37°C. Glucose (1%, w/v) is usually added (Jeong et al., 2004; Wolkoff, 1987). The perfusion medium was gassed continuously with carbogen (95% O₂, 5% CO₂) (Figure 1).

Perfusion conditions and parameters of liver viability

Temperature, perfusion pressure, flow rate and perfusion fluid pH were closely monitored during the perfusion, particularly, during the first 30 min of equilibration (Wolkoff, 1987). These parameters were initially checked every 10 to 15 min and the experiment did not begin until they had reached constant and acceptable values. The temperature in the perfusion system was also set and maintained at 37°C. Perfusion pressure was not raised above 10 - 15 cm of water with a flow rate of approximately 2 ml/min/g liver weight, to provide adequate oxygenation. The perfusion fluid pH was always set between 7.2 and 7.4 by adjusting the CO₂ gases. As soon as perfusion began, the liver developed an even, light-brown color, was soft and kept moistened. Serum amino-transferases activities (ALT and AST) serve as indicators of liver viability during perfusion which was determined in samples of perfusion medium.

Biochemical determinations

The activities of aspartate aminotransferase (AST) activity and alanine aminotransferase (ALT) activity in the perfusion fluid were assayed using a commercial Kit of Zist himie (Tehran, Iran).

Histological studies

The liver was completely excised and freed of any extraneous tissue. Multiple samples were then taken from each liver (mean 3 mm) and placed in 10% neutral buffered formalin. The liver was cut into small pieces, sections prepared and stained by Eosin-Hematoxylin and examined blind for histo-pathological changes.

Surgery

The rats were anesthetized with ether. Heparin (500 unit; I.P.) was used to prevent blood clotting prior to anesthesia (Cheung et al., 1996). An incision was made along the length of the abdomen to expose the liver. Sutures were placed loosely around the common bile duct, which then was annulated with PE-10 tubing and secured. Sutures were then placed loosely around the inferior vena cava, above and below the renal veins. The distal suture around the vena cava was tightened and an 18 g polyethylene catheter was inserted and placed above the renal vein. The diaphragm was incised and the inferior vena cava ligated suprahepatically. Following attachment of the perfusion tubing to the cannulate, the liver was perfused in situ through the portal vein (Cheung et al., 1996).

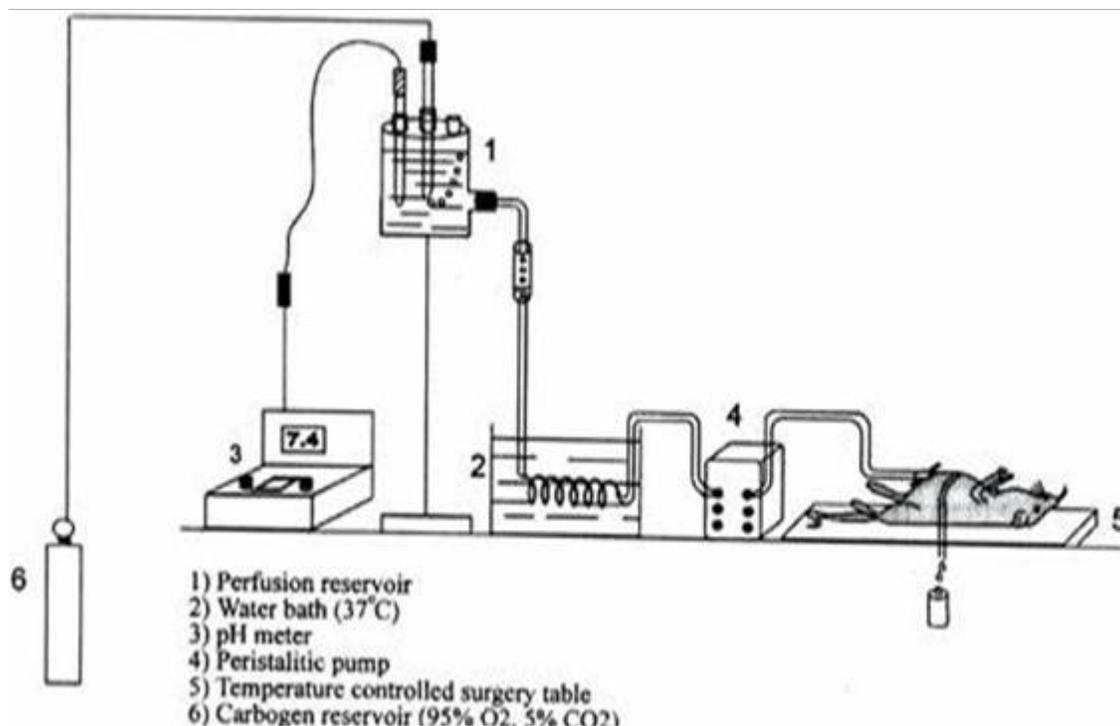


Figure 1. Schematic diagram of a rat liver perfusion system.

Analyses of the data

Statistical analysis was performed using SPSS for Windows (Ver.10, SPSS, Inc., Chicago, USA). All values were analyzed by one-way analysis of variance (ANOVA) and expressed as mean \pm standard error in the mean of 4 rats (S.E.M). Student-Newman-Keuls test were used to evaluate the significance of the obtained results. $P < 0.05$ was considered to be significant (Bermeyer and Bernt, 1980).

RESULTS

Activity of serum amino-transferases enzymes changes

The results of present study showed that the aqueous extract of aerial parts (flowered browse) of *P. americana* significantly increased the activity of amino-transferase enzymes in a dose-dependent manner ($P < 0.01$) at 60th min, in comparison with control at single dose of 10, 20, 40, 50 and 100 mg/kg (Figure 2). Furthermore, the activity of enzymatic methanol extract fractionates increased compared with the control group (Figures 3A and B).

Light microscope observation

Histo-pathological studies using a light microscope showed significant hepatocellular damage including necrosis and infiltration, due to aqueous extract of aerial parts (flowered browse) of *P. Americana* (Figure 4b) when compared

to control group (Figure 4a). In addition, other histo-pathological parameters including the number of Kupffer and mononuclear cells, edematous cells and cell degeneration changed significantly with aqueous extract and CHCl₃, ethanol and methanol fractions of aerial parts (flowered browse) of *P. americana* respectively (Table 1).

DISCUSSION

The liver has been identified as the most important tissue target for *P. americana* in rats (Jeong et al., 1997). The pain relief composition is prepared from roots of the Phytolacca family, particularly the species of Phytolacca Americana (Potter and Clarke, 1900). On the other hand the anti-cancer effects, appear to work primarily based upon anti-tumor and anti-inflammatory properties, along with immune stimulant functions (Larson, 2007), Phytolaccatoxin and related triterpene saponins, believed to be the primary toxic constituents, are present within berry juice and other parts. Other toxic constituents have also been identified including the alkaloid phytolaccine (in small amounts), the alkaloid phytolaccotoxin, as well as a glycoprotein and histamines (Armstrong and Yo, 2007; Winston, 2004; Rossini et al., 1976). Our data showed that administration of aqueous extract and methanol extract fractionates of aerial parts (flowered browse) of *P. Americana* causes edema which can be assessed by histo-pathological examination (Table 1).

Our findings are in agreement with the fact that an

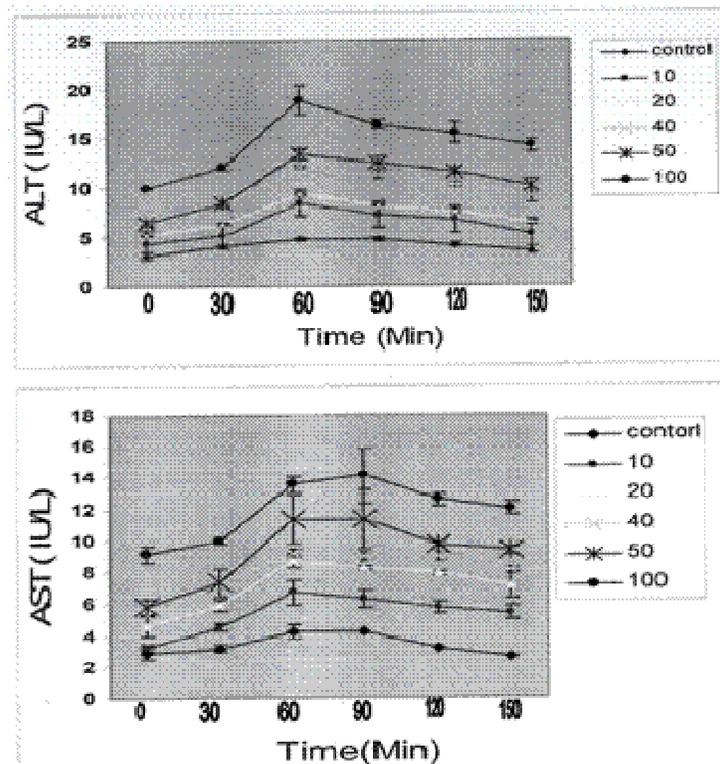


Figure 2. Activity of ALT (alanine transferase) and AST (asparate transferase) enzymes aqueous extract of aerial parts of *Phytolacca Americana* at differences times. Values are presented as mean \pm SEM (N = 4), ***P < 0.001 with respect to control (ANOVA followed by Newman-Keuls multiple comparisons test).

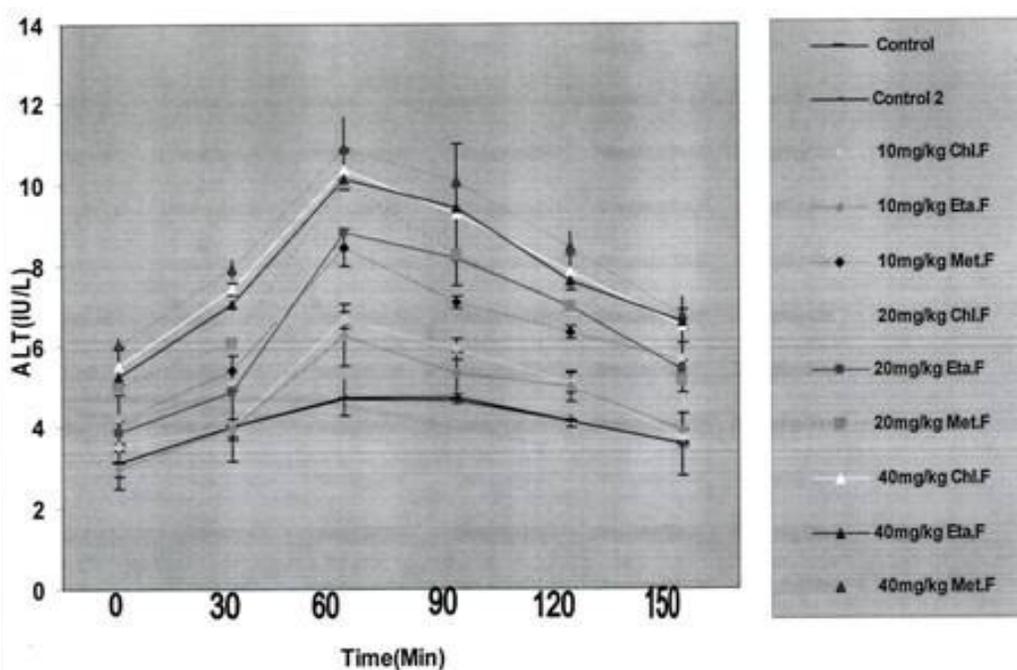


Figure 3A. Activity of ALT (alanine transferaes) enzyme fractionated methanol extract of aerial parts of *Phytolacca Americana* at differences times. Values are presented as mean \pm SEM (N = 4), ***P < 0.001 with respect to control, (ANOVA followed by Newman-Keuls multiple comparisons test).

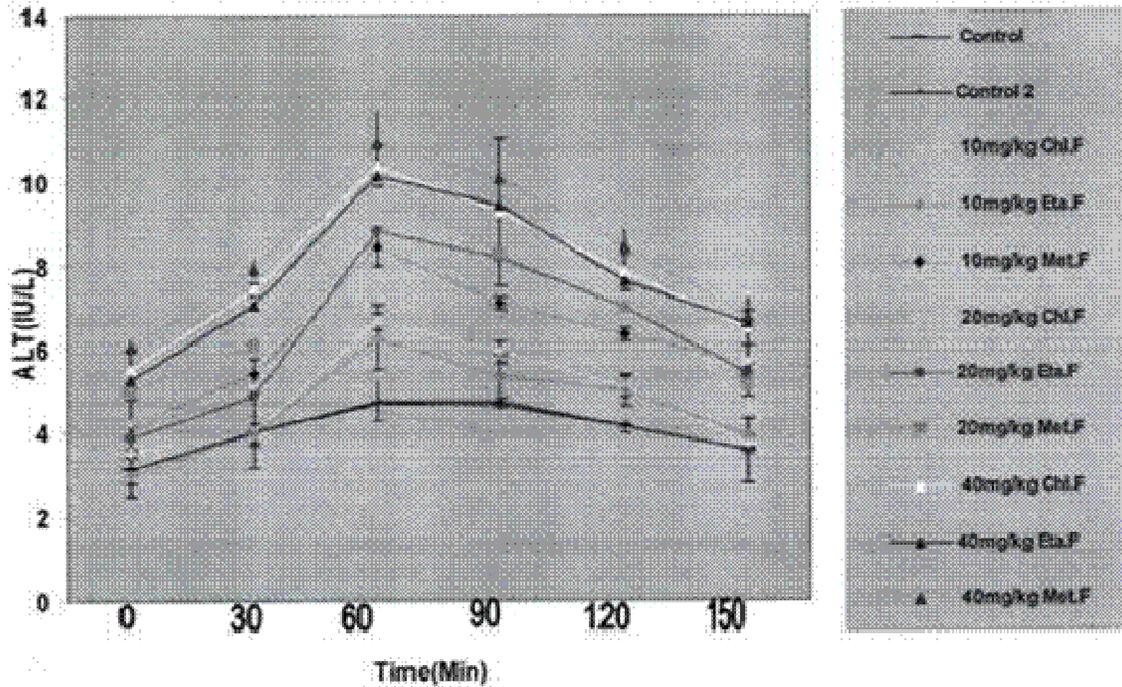


Figure 3B. Activity of AST (aspartate transferase) enzyme fractionated methanol extract of aerial parts of *Phytolacca Americana* at differences times. Values are presented as mean \pm SEM (N = 4), ***P < 0.001 with respect to control (ANOVA followed by Newman-Keuls multiple comparisons test).

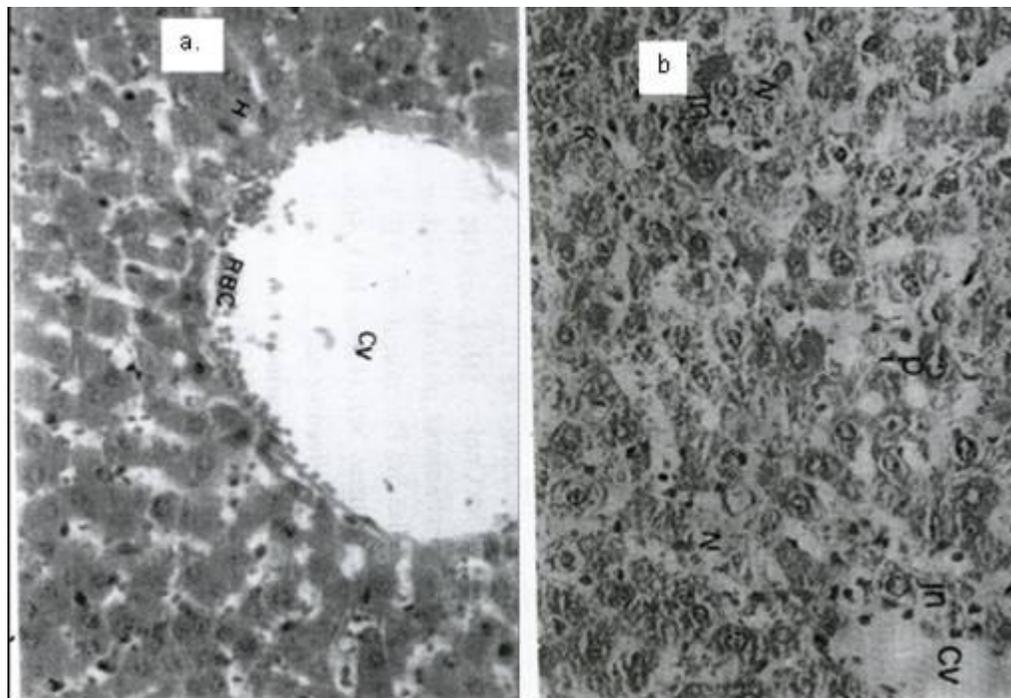


Figure 4. Photomicrograph of lobules from control group and *Phytolacca americana* - treated liver. Control showed red blood cells (RBC), hepatocytes (H) and central veins (CV). Staining shows that cytoplasm was acidophilic and surround by a bright basophilic nucleus (a). *Phytolacca americana* perfused liver (50 mg/kg) showed limited changes in lobules of liver and hepatocellular necrosis (N), with infiltration (IN) of mononuclear cells and accumulation of necrotic Kupffer cells (K), with pyknotic (P) nuclei (b).

Table 1. Histo-pathological effects of aqueous extract and CHCl₃, ethanol and methanol fractionated extract of aerial parts of *Phytolacca americana*.

Histopathological parameters	Control	<i>Phytolacca Americana</i> (mg/kg)					CHCl ₃ fraction (mg/kg)			Ethanol fraction (mg/kg)			Methanol fraction (mg/kg)		
		10	20	40	50	100	10	20	40	10	20	40	10	20	40
Kupffer cells	+	+	+	++*	+++**	+++**	+	+	++*	+	+	++*	+	++*	+++**
Edematous cells	+	+	+	+	++*	+++**	+	+	++*	+	+	++*	+	++*	+++**
Mononuclear cells	+	-	+	+	+++*	+++**	+	+	+	+	+	++*	+	++*	+++**
Degeneration	-	+	+	+	++*	+++**	-	+	+	-	+	++*	+	++*	+++**
Necrosis	-	-	-	+	++*	+++**	-	+	++**	-	+	++*	+	++*	+++**

- = No effect, + = minor effect, ++ = medium effect, +++ = major effect.

*P < 0.05, **P < 0.01, significantly different from control using Fisher exact test. Data are means of three replicates

oncotic agent can cause increase in pressure (Chen and Cosgriff, 2000). In addition, isolated organs have a time-dependent tendency to absorb water, as with a relatively protein-free medium water which gradually escapes from the vascular space and therefore interstitial edema develops (Wolkoff, 1987). Histo-pathological examination revealed significant hemolysis as assessed by the hemolytic index (Figure 4b).

This can be due to altered calcium homeostasis concomitant with a significant increase in cytosolic calcium, which has been previously reported for *P. americana* in liver (Jeong et al., 2004). Moreover, the disturbances of intracellular calcium homeostasis have been shown to be associated with a variety of toxicological and pathological processes (Cheung et al., 1996). Accumulation of *P. americana* in the liver as the target organ has been shown to cause degeneration (Goldestein et al., 1973). In a similar manner the results of this study also showed liver degeneration (Table 1). This in fact could be a result of *P. americana* recep-tor binding, which is sufficient to affect different cells. In this study, significant necrosis was also observed in the liver at *P. americana* doses of 50 and 100 mg/kg. *P. Americana* induces formation of reactive oxygen species and an oxidative stress, resulting in lipid peroxidation (Karami

et al., 2001). This may explain the observed necrosis (Figure 4b). We have also observed cell death followed by cell proliferation with hyperplasia nodules in Kupffer cell by *P. americana*. Therefore, the results of our study, in agreement with others (Goldestein et al., 1973; Heinrich et al., 2004), demonstrate that liver perfusion is a suitable model in order to study the hepatotoxicity of chemicals (such as *P. americana*). More studies, however, are needed to further elucidate the exact mechanism by which *P. americana* induces hepatotoxicity.

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