TOXICOLOGICAL AND HISTOPATHOLOGICAL EFFECTS OF *Plumbago indica* ROOT EXTRACT IN HAMSTERS

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Abstract

***The Plumbago indica* roots have been used as traditional medicines against many maladies; however, the information of toxicity has been needed when consumed in the high volume or for a long period. The objective of this study was to investigate the toxicity of crude extract from *P. indica* root (cPI) in hamsters by mortality, behavior changes, histochemical and histopathological changes. Golden Syrian hamsters were randomly divided into 5 groups and administered with cPI at 0, 100, 400, 1,000, and 3,000 mg/kg orally for 24 h and every day for 7 days for studying acute and subacute toxicity assessments, respectively. Neither mortality nor behavior changes of animals were found during acute and subacute toxicity tests. Weights of liver and kidney in cPI treated at 3,000 mg/kg were significantly increased after oral administration at 24 h and 7 days. The biochemical parameters revealed insignificant differences in the liver function tests (ALT and AST) and kidney function tests (creatinine and BUN). The morphological hepatic lobules in the subacute assessment were better than acute assessment. Although glomeruli swelling and angiectasis in the kidney were found in cPI treated group at 3,000 mg/kg for both the 24 h and 7 days, the renal tubular compartments had become similar to the control groups. The LD_{50} of cPI is classified as category 5 (2,000-5,000 mg/kg). These results suggest that the precaution of users should be concern harmful to hepatotoxic property and renal function in early acute and subacute periods at the high dose of cPI extract.**

Keywords: ***Plumbago indica*, toxicity, histopathology, biochemical test**

Introduction

Plumbago indica Linn. (Syn. *Plumbago rosea*) belongs to the Plumbaginaceae family, *Plumbago* genus, which consists of 25 species globally. It is a shrubby perennial plant common in tropical South East Asia and Central Asia, which grows in open grassland areas, in India, Malaysia, and Thailand. The ethanol root extract of *P. indica* in the Thai traditional medicine had been used to cure the several gastrointestinal problems including diarrhea, dyspepsia, and anti-flatulence and also used to

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treat skin infections and parasitic infections (Atjanasuppat *et al*., 2009; Ratanavalachai *et al*., 2015; Minsakorn *et al*., 2019). In addition, *P. indica* root extract has been recorded to have antibacterial activity (Paiva *et al*., 2003; Kaewbumrung and Panichayupakaranant, 2014; Ketpanyapong and Itharat; 2016), antimalarial activity (Thiengsusuk *et al*., 2013; Sumsakul *et al*., 2015), and this extract combined with other herbs have been used as an ingredient for cancer treatments in Asian traditional medicine (Makchuchit *et al.*, 2017). Although the roots of this plant have been reported as an antifertility activity, it caused the abortion and induced anti-estrogenic with the prolonged estrous cycle in female Wistar rats (Savadi and Alagawadi, 2009; Padhye *et al*., 2010; Sandeep *et al*., 2011). The roots of *P. indica* extract have been reported to be a powerful poison. In rodents, it had dose-related toxic side effects, including diarrhea, skin rashes, and hepatic and reproductive toxicity (Padhye *et al*., 2010). The major bioactive compound exhibited in the *P. indica* root extract is known to be plumbagin that is able to isolate from the root, leaf and stem bark of some plant families including *Plumbaginaceae*, *Ebenceae*, *Dioncophyllaceae*, *Ancestrocladaceae* and *Droseraceae* (Solomon *et al.*, 1993). However, potential toxicities in Thai traditional medicine remedies are often ignored due to a lack of scientific researches regarding effective-dosage range, safeness, and toxic doses. Therefore, scientific information about the toxicity, side effects and precaution of traditional herbal have been currently focused.

Preliminary screening studies in our laboratory using the ethanolic root extract of *P. indica* has shown an against parasitic motility and killed the immature stage of liver fluke (unpublished data). Before undertaking a detailed study of the antiparasitic effect of this plant in the animal model, it is important to evaluate the toxic effects of the extract, which is essential where a safe dose has to be selected in the animal model. In general, rats and mice are used as rodent models for toxicity studies and inexpensive cost. But they are unfit with some studies such as parasitic infections with *Opisthorchis viverrini* or *Fasciola gigantica*. Only the hamster is susceptible to these parasites. The lethal dose and hepatotoxicity studies of the *P. indica* root extract in mice and rats were reported already (Solomon *et al.*, 1993; Sukkasem *et al*., 2016). Hence, we filled in the gap of scientific study which focused on investigating potential harmful effects, safety profile, as well as histopathological changes induced by the crude root extract of *P. indica* (cPI) on hamsters.

Materials and Methods

Chemicals

Standard plumbagin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Darmstadt, Germany), and methanol (HPLC grade) was purchased from Merck Millipore (Germany). Harris's hematoxylin and Eosin Y 1% aqueous solution were purchased from Bio Optica (Milan, Italy). Xylene and Permount® were bought from Fisher Scientific (Loughborough, UK).

Collection and Preparation of Crude Extract of *P. indica* root

Roots of *P. indica* were collected and purchased from Chiang Mai Province, Thailand. The characteristics of *P. indica* trees, roots and flowers were identified and authenticated by Dr. Santi Wattana, Institute of Biology, Scientific Botany of Suranaree University of Technology (SUT voucher; N. Saowakon 01, SUT Botanical Garden, Suranaree University of Technology, Nakhon Ratchasima, Thailand). The roots of *P. indica* were air-dried, cut into small pieces, blended with an electric blender, and then sieved through a 250 µm mesh to obtain a fine powder. The crude extract of *P. indica* root (cPI) was prepared by soaking 100 g of *P. indica* root powder in 1,000 ml of 95% ethanol at room temperature for 7 days within an incubator shaker at 250 rpm (Innova 42/42R, New Brunswick Scientific, France). The macerate was filtered and concentrated with a rotary evaporator (Rotavapor R-210/215, BUCHI, Switzerland) under reduced pressure. Then, the extracts were lyophilized into powder by a lyophilizer (Freezone, LABCONCO Co, MO) at -40°C for 3 days. The yield of cPI extract powder was 9.20 %, which was stored in a refrigerator at -20°C until use. The cPI power was weighed and dissolved in 0.1% dimethyl sulfoxide (DMSO, Sigma, Darmstadt, Germany) to obtain the required concentrations.

Evaluation of Plumbagin in cPI by using High-Performance Liquid Chromatography (HPLC) Technique

The plumbagin content of many parts of *P. indica* especially root (Unnikrishnan *et al*., 2008). The cPI was evaluated for the amount of plumbagin using high-performance liquid chromatography (HPLC) which was modified from Nayak *et al.* (2015). Briefly, the C18 reversedphase column (4×250 mm, particle size 4 um, Agilent Technology Co, CA) coupled with an Agilent 1100 series HPLC System (Agilent, Satan Clara, CA) were calibrated

by eluting with water–methanol with gradient elution as follows: 0 min, 2:98; 10 min, 50:50; 30 min, 100:0; and 40 min, 100:0. The methanol solution was used as a blank solution before evaluated the cPI with a flow rate 0.5 ml/min. The experiment was performed triplicate. The standard plumbagin solution and cPI were freshly dissolved in methanol (HPLC grade, Merck, Germany), mixed for 5 min and filtered through a 0.45 μm membrane filter before use. The stock plumbagin solution and cPI solution were prepared at 100 μg/ml and then were injected in triplicates at 10 μl in each time. Plumbagin, cPI and combine plumbagin with cPI were detected by chromatogram at a wavelength of 254 nm. The chromatographic analysis was performed base on the retention time, peak area and the UV spectra of the reference standard of plumbagin.

Animal Preparation

Six-week-old male and female Golden Syrian hamsters used in this experiment were purchased from Laboratory Animal, Faculty of Medicine, Khon Kaen University, Thailand. Hamsters (80-100 g) were acclimatized for 7 days prior to the experiments. They were housed in stainless steel cages with free access to normal diet and water *ad libitum*. The room temperature was maintained at 25±2°C, with relative humidity 45-50% and a 12 h daylight/dark cycle. All experimental procedures in this study were approved and modified following the recommendations of the Committee of Animal Care, Suranaree University of Technology, Thailand (SUT approach; 4/2558).

Toxicity Study

The toxicological study was divided into two parts, acute and subacute treatments based on OECD (OECD/OCDE, 2001) guidelines and Solomon *et al.* (1993). After acclimatization, sixty hamsters were randomly divided into 5 groups with 12 animals per group with 3 females and 3 males per group, and the experiments were repeated twice. For the acute toxicity test, the hamsters had fasted for 12 h overnight, and then administered with chemicals as follows: Group 1 was received 0.1% DMSO in distilled water as a control group by oral gavage in both sexes, while animals in groups 2-5 were orally administered with a single dose of cPI (dissolved in 0.1% DMSO in distilled water) at 100, 400, 1,000, and 3,000 mg/kg. The hamster behaviors were continuously observed at 1 h, 6 h, 24 h, and daily, respectively. According to OECD 423 guidelines (OECD/OCDE, 2001), the toxicity signs and symptoms were examined, including mortality, salivation, lethargy, diarrhea, chill or tremor, and convulsion. We were also attentive to changes in body weight, respiratory, circulatory, posture, and locomotion (central nervous system), skin and fur, eyes and mucous membranes evaluation, as well as alterations in the behavioral patterns. After sacrifice, blood samples were collected for biochemical evaluations indicating the renal and liver functions, including levels of blood urea nitrogen (BUN), creatinine (Cr), alanine transaminase (ALT) and aspartate transaminase (AST), respectively. The internal organs (heart, lung, liver, kidney, spleen, ovary, uterus, testis and epididymis) were collected, weighed and then fixed in 10% neutral-buffered formalin fixative solution for histopathological examination.

In the subacute toxicity test, sixty hamsters were divided into 5 groups as in the acute toxicity test (3 females and 3 males in each group for twice); four groups were receiving different cPI dosages (100, 400, 1,000, and 3,000 mg/kg) and the control group was receiving 0.1% DMSO dissolved in distilled water. The dosages were decided according to previous studies by Solomon *et al.* (1993). The animals were given daily oral administration with gavage for further 7 days, the animals were observed for the same physical and behavioral changes recommended by the OECD 423. At the end of the experiment, the animals were fasted overnight, anesthetized with an overdose of pentobarbital and then sacrificed by cardiac puncture. The blood and visceral tissue samples were collected and performed for histopathological and biochemical analysis.

Histopathological Examination

The conventional histopathological examination was performed using paraffin-embedded stained with hematoxylin and eosin (H&E). Tissue samples were washed twice in 70% ethanol with 10 min/wash to remove the fixative solution. Then, they were dehydrated in serial ethanol solutions with increasing concentrations up to 100%, cleared with xylene, infiltrated and embedded in paraffin blocks. After that, paraffin blocks were sectioned at 5 μm in thickness and stained with H&E staining, followed by dehydration, clearing, and mounting with permount media. Photographs of five sections of each tissue sample were taken by a light microscope (80i, Nikon, Japan) at 400x magnification.

Biochemical Analysis

After the cardiac puncture, the blood samples were allowed to clot in tubes without anticoagulant and then the sera were separated by centrifugation at 12,000 rpm for 15 min by using Hettich U320R centrifuge machine. Subsequently, the sera were evaluated by using semi-automatic analyzer (Ortho Clinical Diagnosis: VITROS 5600, Miami, USA)

and commercially available test kits (Ortho Clinical Diagnosis, New Jersey and Erba Diagnostics Manheim, Miami, USA) for the levels of aspartate aminotransferase (AST: Vitros Chemistry Products Kit 165 5281, Miami, USA), alanine aminotransferase (ALT: Kit 843 3815, Miami, USA), creatinine (CRE: Erba kit BLT00020, Ohio, USA), and blood urea nitrogen (BUN: Erba kit BLT00061, Ohio, USA) at Clinical Laboratory of Suranaree University of Technology Hospital. The serum of control group was used as the baseline of the liver enzyme function.

Statistical Analysis

Results of all experiments were reported as means±SD and processed by using SPSS13.0 software. The data were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's-b test. The differences between groups were considered statistically significant for $p<0.05$.

Results

Analysis of Plumbagin in the Ethanolic *P. indica* Root Crude Extract by Using HPLC Technique

Identification and quantification of plumbagin in the ethanol *P. indica* root extracts were performed on the basis of the retention time (RT) and peak area of plumbagin authentic standards. The medthod validation parameters showed in Table 1. The limit of detection (LOD) and limit of quantification (LOQ) of the method were 0.021 and 0.062 µg/ml, respectively. The method showed a good linear relationship between the concentration of plumbagin in the range of 0.1 to 5 µg/ml and the HPLC peak area at 254 nm with the linear regression equation of Y = 75.582X-6.2693 (r^2 = 0.9991). The percentage relative standard deviation (%RSD) of the peak area and retention time of plumbagin at each concentration of the standard solution were 14-22% and 1.1-1.2%, respectively. Accuracy of the method was evaluated as %recovery of the plumbagin added. The accuracy was 99.71±0.23%.

Table 1. Validation parameters of the analytical method for quantification of plumbagin in *P. indica* crude extract

Parameters	
Sensitivity	
LOD (µg/ml)	0.021
LOQ (µg/ml)	0.062
Specificity	
at λ 254 nm	
Linerity (concentration 0.1-5 µg/ml)	
Linear regression equation	Y=75.582X – 6.2693
Coefficient of determination (r^2)	0.9991
Precision (%RSD) (concentration 0.1-5 µg/ml)	
Peak area	14-22
Retention time	1.1-1.2
Accuracy (%recovery)	99.71±0.23

The result showed specificity for plumbagin, there was peak interference in the early retention time of plumbagin standards whereas the chromatograms of the blanks or methanol (Figure 1(a)) had no interference. The retention time of plumbagin in the *P. indica* crude extract correlated to those of the plumbagin standards, in which the retention times of the standards plumbagin in methanol were 5.313 and 5.309 min at 1 ppm and 5 ppm, respectively (Figures 1(b) and 1(c)). The retention time of ethanolic *P. indica* crude extract at 50 ppm was 5.309 min. The root extract of *P. indica* contained plumbagin about 3.28±0.28 ppm (y = ax+b; r^2 = 0.9986) (Figure 1(d)) which was similar to the plumbagin standard, but its peak showed less than the standard. There was peak interference in early retention time as the plumbagin standard. Therefore, we applied the standard plumbagin at 1 ppm with the *P. indica* crude extract at 12.5 ppm, had 1.32±0.41 ppm of plumbagin.

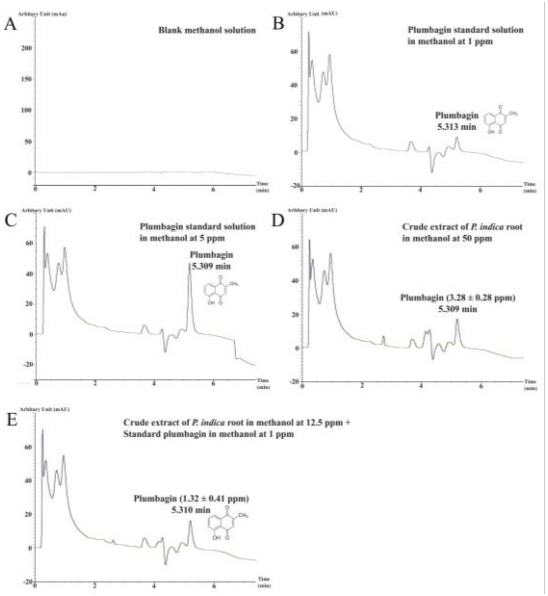

Figure 1. The specificity validation for the HPLC analytical method for quantification of plumbagin standard in methanol and methanolic *P. indica* root crude extract. (A) Blank methanol solution. (B) Plumbagin standard solutions at 1 ppm and (C) 5 ppm. (D) The crude extract of *P. indica* root at 50 ppm. (E) Combination mixture between plumbagin at 1 ppm + crude extract of *P. indica* root at 12.5 ppm

Toxicity Study

After 24 h oral administration, the acute toxicity test showed that a single dose of all groups treated with cPI (100-3,000 mg/kg) caused no adverse effect on hamster behavior up to 7 days after administration. Neither morbidity nor mortality in all treated groups was seen during acute and subacute assessments by following OECD guidelines (OECD/OCDE, 2001). Although the mean value of body weights of animals in cPI 3,000 mg/kg after 24 h (or a day) was higher than both the control group and other cPI-treated groups, there were no significant differences in body weight between treated and control groups (Table 2). The internal organ weights of cPI-treated groups were not significantly different from the control group except for the liver and kidney weights of the groups treated with cPI at 3,000 mg/kg which were significantly higher than others as shown in Table 2. The liver index value calculated from liver weight per body weight indicated liver enlargement from pathological changes. The value of liver index in treated with cPI 3,000 mg/kg also was statistically different when compared to the control and other cPI treatment groups. The group treated with cPI 3,000 mg/kg showed significantly heavier kidneys, in fact, an increase of 1.2 folds over the control group was observed. In kidney weight among animals receiving cPI treatment at 3,000 mg/kg displayed significant differences with the control and other cPI treatment groups. During the acute period, the weights of heart and lung slightly increased in cPI treated groups, but they were not significantly different from the control group. Reproductive organ weights of females and males in Table 2 indicated that no adverse effect on reproductive organ weights in both sexes due to show no significant difference in organ weight.

In the subacute toxicity test, there was no evidence of pathological change and no mortalities among treatment groups or the control group. The body weights increased slightly in both the control group and treated groups but there was not significantly different (Table 2). The internal organ weights of cPI-treated groups at 3,000 mg/kg were significantly higher for the liver, heart, and lung when compared to the control and other groups. The liver index value of the group treated with cPI at 3,000 mg/kg was also significantly higher compared to the other groups. Moreover, the female reproductive organs weights of the group treated with cPI at 3,000 mg/kg were significantly higher than other cPI treatment groups, whereas the male reproductive organ weights showed no significant difference when compared with control and treated groups.

Table 2. Body weight and internal organs weight of hamsters after treatment with cPI at 24 h and 7 days.

		Control		cPI treatment (mg/kg bw)							
				100		400		1,000		3,000	
		1D	7D	1D	7D	1D	7D	1D	7D	1D	7D
Body weight	**Before**	100.00±9.12	96.25±18.87	87.50±15.00	86.25±13.77	88.75±10.31	85.00±10.00	93.75±11.09	97.00±8.12	87.50±6.45	105.00±14.72
	After	96.25±18.87	100.00±7.07	86.25±13.77	97.50±2.89	85.00±10.00	88.75±16.52	97.00±8.12	97.50±5.00	105.00±14.72	111.25±10.31
Liver		2.79±0.15	3.39±0.15	3.39±0.42	3.45±0.37	3.27±0.62	3.26±0.71	3.26±0.25	3.43±0.26	5.44±0.85*#	5.25±1.35*#
Liver index		0.0035±0.0002	0.0039±0.0003	0.0042±0.0004	0.0044±0.0006	0.0041±0.0004	0.0051±0.0008*	0.0038±0.0002	0.0045±0.0002	0.0050±0.0006*#	0.0058±0.0006*#
Kidney		0.88±0.10	1.01±0.08	0.94±0.09	1.02±0.09	0.94±0.09	0.98±0.17	0.91±0.03	1.02±0.07	1.14±0.05*#	1.19±0.11
Heart		0.35±0.04	0.39±0.04	0.37±0.05	0.39±0.01	0.37±0.04	0.42±0.04	0.37±0.04	0.42±0.03	0.44±0.04	0.49±0.04*#
Lung		0.65±0.04	0.66±0.05	0.60±0.10	0.67±0.06	0.68±0.15	0.68±0.09	0.67±0.04	0.67±0.06	0.72±0.09	1.05±0.41*#
Spleen		0.15±0.02	0.16±0.02	0.18±0.03	0.15±0.01	0.17±0.05	0.19±0.06	0.16±0.02	0.19±0.01	0.21±0.04	0.20±0.06
Uterus and Ovary		0.38±0.01	0.24±0.02	0.42±0.13	0.29±0.03	0.55±0.08	0.27±0.10	0.21±0.02	0.13±0.01	0.28±0.02	0.39±0.07#
Testis		4.08±0.06	4.36±0.01	3.47±0.27	5.33±0.20	4.23±0.52	5.08±0.59	4.48±0.30	5.42±0.69	3.68±1.11	4.09±0.05

Data are expressed as mean ± SD (n=6) versus control using One way ANOVA followed by Tukey's-b test. The values were considered to be significantly difference at $p<0.05$, *; different from the control group, #; compare within treated groups.

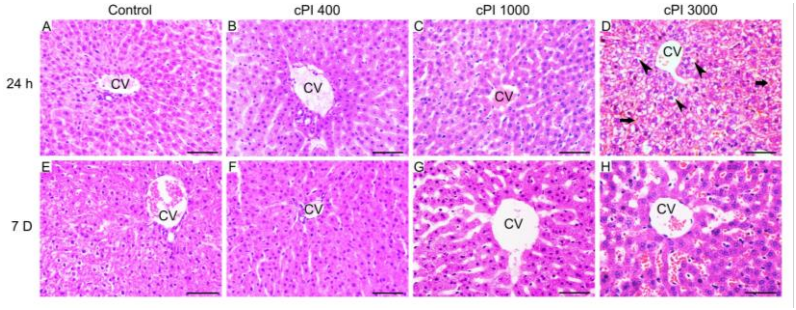

Figure 2. Histopathological photomicrographs of liver section from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of liver section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). No pathological changes present in the hepatic lobule except the highest cPI oral administration (3,000 mg/kg). Size bars for comparison are 10 µm H&E (x400). CV = Central vein, arrow head = Cytoplasmic vacuole, arrow = Red blood cell

Histopathological Changes

No gross morphological changes were found in tissue samples from cPI treatment groups when compared to the control group. Histopathological changes of internal organs were assessed in the acute and subacute assessments as shown in Figure 2. In the control group, the hepatic lobule was polyhedral in shape and lying by hepatocytes in a radial pattern. The center of the hepatic lobule was a central vein. Histopathological changes in hepatic lobule were found in the only group that was treated with cPI 3,000 mg/kg. We found the acute necrosis pattern in the centrilobular area after 24 h treatment. The swelling of nucleus and cytoplasm of hepatocytes were found at the center of lobule and red blood cell congestion and cellular hypertrophy and vacuolization occurred (Figure 2 (d)). Although the changes were observed spreading throughout the hepatic area, the overall histological changes on day 7 (Figure 2(h)) had been improved when compared to what appeared at 24 h. Almost the morphology of hepatic lobules exhibited similar features to those in the vehicle control group, while the red blood congestion had been still observed. These hepatic histopathological findings showed that the cPI extract induced hepatotoxicity in the dose-dependent pattern especially in the early acute stage.

The histology of the kidney was presented in Figure 3. After 24 h oral administrations of cPI at 100 to 400 mg/kg, no histological changes in the kidney of the cPI-treated groups compared to the control. Histopathological changes were found in both the 1,000 and 3,000 mg/kg cPI treatment groups. Epithelial linings in proximal tubules were swollen that caused the absence of luminal space, and angiectasis or red cell congestion in the glomerulus were observed (Figure 3). Moreover, the red blood cell congestion in the renal interstitial space was observed in the acute stage. Surprising, renal histology observed after 7 days in the cPI treatment group at 3,000 mg/kg, appeared better than at 24 h as the renal tubular epithelial cells recovered and look like as control group with open luminal space. However, the angiectasis in the glomerulus and interstitial space had still presented in some renal cortical areas. While the heart and

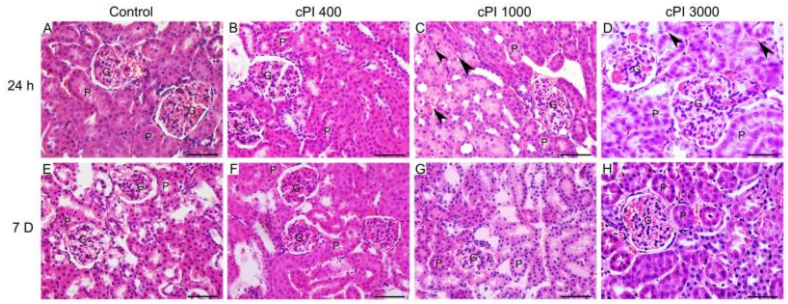

Figure 3. Histopathological photomicrographs of kidney section from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of kidney section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). The proximal tubules present swelling and red blood cell congestion in the interstitial space. Size bars for comparison are 10 µm H&E (x400). G = Glomerulus, P = Proximal convoluted tubule, arrow head = Red blood cell

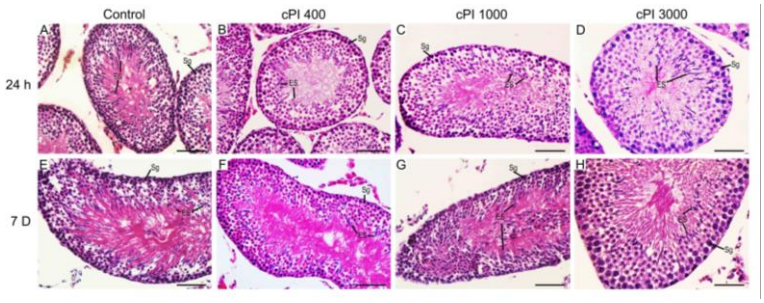

Figure 4. Histopathological photomicrographs of seminiferous tubule section from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of seminiferous tubule section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). No pathological changes present in all cPI treatment groups. Size bars for comparison are 10 µm H&E (x400). Sg = Spermatogonium, ES = Elongate spermatid

lung weights were significantly greater in comparison to the control group, no definite histopathological changes were observed in these organs, except for some of lung samples where red blood cell accumulations in alveolar ducts and sacs were observed (data not shown).

The testes of all cPI-treated groups exhibited no histological changes in either acute or subacute treatments (Figure 4). As well, no histopathological changes were observed in the ovaries or the epithelial lining of the endometrium in all cPI-treated groups (Figure 5).

Biochemical Blood Analysis

The biochemical blood analysis of liver and kidney function tests in male and female are shown in Table 3. The AST, ALT, and AST/ALT ratio levels of cPI treatment groups in 24 h showed higher than the control, and there was no significant difference between control and other cPI treatment groups. The renal function test presented by BUN, creatinine and its ratio, the cPI increased both plasma BUN and creatinine levels in a dose-dependent manner, there was no significant difference when compared with the control and within other cPI treatment groups. In the subacute assessment, cPI reduced both the ALT and AST levels in a dose-dependent pattern, then liver ratio also had decreased as well. There were no statistical differences between the levels of BUN, creatinine and the ratio of BUN/creatinine in kidney function tests between the treated and control groups.

Discussion

In this study, the amount of plumbagin in the cPI extract was 6.56±0.56 % dry weight which higher than the previous studies of the chloroform extract of PI root using TLC of 0.17% (Ariyanathan *et al*., 2010) and the methanolic extract of PI root using HPLC of 0.18% (Sukkasem *et al*., 2016). The solvent and method of extraction were different between previous studies (Rajasekaran *et al*., 2011; Sukkasem *et al*., 2016) (Rajasekaran chose Soxhlet extraction using ethanol at 68ºC for 72 h and Sukkasem method performed with Soxhlet extraction using methanol at it boiling temperature for 3h) and our work (maceration in ethanol at room temperature for a week with shaking and make concentration by rotator evaporation) might involve on the yield of the extracts. Therefore, the results are possibly different. In addition, the extended duration of extraction and prolonged heating time of the crude extract could make chemical degradation, the bioactive compound of the extract was lost even though using in the same method (Paiva *et al*., 2004). We concerned about the temperature, made plumbagin loss. But we missed the stability of plumbagin in cPI in this work. It was a limitation of our experiment.

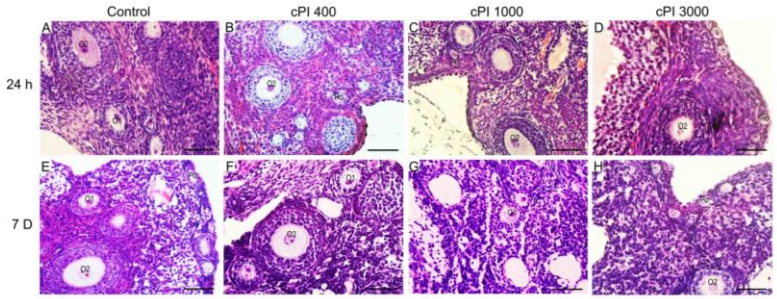

Figure 5. Histopathological photomicrographs of an ovary section from the control (A) and treated-groups (B-D) in 24 h (acute stage). Histopathological photomicrographs of an ovary section from the control (E) and treated-groups (F-H) in 7 days (subacute stage). No pathological changes present in all cPI treatment groups. Size bars for comparison are 10 µm H&E (x400). Po = Primordial ovum, O1 = Primary ovum, O2 = Secondary ovum

Table 3. Biochemical parameters of hamsters after treatment with cPI at 24 h and 7 days

	Control		cPI treatment (mg/kg bw)							
			100		400		1000		3000	
	24 h	7 days	24 h	7 days	24 h	7 days	24 h	7 days	24 h	7 days
AST (U/L)	105.25±13.12	139.50±12.35	112.25±13.33	99.75±16.58	86.50±18.16	152.75±11.93	103.50±17.74	118.00±13.76	112.00±16.75	102.50±18.15
ALT (U/L)	62.20±12.68	71.75±17.02	90.50±13.81	53.25±14.73	69.00±18.92	68.75±15.65	64.00±5.89	62.00±10.74	69.00±14.91	61.00±9.49
AST/ALT	0.99±0.32	1.85±0.43	1.38±0.88	1.50±0.50	1.49±0.57	1.24±0.25	1.37±0.48	1.68±0.28	0.98±0.17	1.63±0.79
BUN (mg/dl)	0.50±0.22	0.53±0.01	0.52±0.25	0.58±0.20	0.58±0.25	0.62±0.27	0.69±0.15	0.62±0.09	0.59±0.14	0.59±0.12
Cr (mg/dl)	4.81±0.79	4.10±0.14	4.54±0.83	5.40±0.31	6.31±0.60	6.21±0.44	6.23±0.83	5.73±0.70	5.32±0.98	5.94±0.38
BUN/Cr	0.10±0.03	0.13±0.01	0.11±0.04	0.11±0.01	0.09±0.02	0.09±0.02	0.11±0.03	0.11±0.01	0.11±0.01	0.10±0.02

Data are expressed as mean ± SD (n=6) versus control using One way ANOVA followed by Tukey's-b test. The values were considered to be significantly difference at $p<0.05$, *; different from the control group, #; compare within treated groups. AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, BUN=Blood urea nitrogen, Cr=Creatinine.

Knowledge of traditional medicinal plant toxic side effects is limited and thus their applications are potentially dangerous. In order to examine the safety of *P. indica* root extract, we are the first reporter who performed the toxicological and histological assessments of oral administration of cPI in Golden Syrian hamsters. by general gross appearance, histomorphological changes and liver and kidney function tests. In the acute toxicity test of our study, hamsters given oral administration of cPI at 100, 400, 1,000, and 3,000 mg/kg were still alive at the end of the experiments and no behavior changes were observed from 24 h and 7 days. Regarding differences OECD 423 guidelines, if all animals survived after their scheduled termination, the extract is deemed to have low toxicity and should be classified in category 5 (less toxic), with the LD_{50} estimated between 2,000-5,000 mg/kg. Although the pharmacokinetic information on the metabolism, distribution, and excretion of plumbagin in humans have not been available yet, there is some information on its oral bioavailability of the rat model. The LD_{50} of the methanolic extract of *P. indica* root in mice with the oral administration was reported at 1,250 mg/kg and 1,148.15 mg/kg in rats, respectively (Solomon *et al*., 1993). They also reported the mortality of mice after oral administration of crude root extract of *P. indica* at the concentration 1,000 mg/kg was 20%. We compared the general information of rats and hamsters did not differ in the weight in the mature stage. Therefore, we followed the LD_{50} of rats. In ideal, the dosages of PI extract of 100, 400, and 1,000 mg/kg did not make hamster dead, whereas cPI higher than 1,150 mg/kg gonna induce the mortality of hamsters similar to rat and we tried to find out the maximum concentration of cPI that killed hamster 50%. A large amount of extract (more than 2,000 mg/kg) can not make animal dead, it implies that is a low toxic (OECD/OCDE., 2011). Our findings showed that there was no mortality in acute and subacute assessments in the highest of cPI. It did not mean that the cPI had a low poison, we confirmed this hypothesis by evaluation of internal organ weight and histopathological changes of hamsters. The increasing of organ weights are used to predict the pathological changes that occurred in those organs. After 24 h, the significantly increased weights of liver and kidneys were observed in the animal treated with cPI at 3,000 mg/kg. It seems to be the harm in the acute observation. We concerned the powerful poison of cPI in prolonged use, especially in females. Because it was used as an expelled hematinic substance in females (Savadi and Alagawadi, 2009; Padhye *et al*., 2010; Sandeep *et al*., 2011). Therefore, we observed the side effect of the subacute toxicity test. The weights of liver, heart, and lung of animals treated with 3,000 mg/kg exhibited significant greater than the control and other cPI treatment groups whereas the kidney weights of all cPI treatment groups were no significant difference with the control group. The findings reflected that the toxic side effect of cPI directly acted on the liver and kidney depended on the dose-depended response.

Examinations for histopathological changes also confirmed the results from organ weights. The histopathological changes of liver and kidney showed severe damage at 24 h (acute stage) and morphological changes at day 7 had been better than 24 h. A main function of the liver is biotransformation and detoxification of exogenous chemical compounds; therefore, the most common of hepatic injury by drug or chemical substance is inflammation such as acute hepatitis with or without cholecystitis. The morphological changes consisted of the portal and parenchymal cell swelling and hepatocellular changes such as swelling, hypertrophy or necrosis (Ramachandran *et al*., 2009). In this study, the concentrations of cPI at 100, 400, and 1,000 mg/kg did not show the histomorphological changes in the liver while the highest concentration (3,000 mg/kg) of cPI caused the severe hepatic injury. This work correlated with the previous study, the hepatic histopathology caused by the PI extract was indicated from the dose of 200 to 1,000 mg/kg/day (Sukkasem *et al*., 2016).

The morphological changes of cell injury were found by nuclear shrinkage caused the chromatin clumping, nuclear fragmentation, and nucleus fading with or without intracellular degenerations. After cell injury, cellular permeability is lost and allows the water influx in the cytoplasm, indicating hepatic damage, which is yet lethal but can induce liver necrosis if an animal has continuously exposed to causes. In our study, hepatic necrosis and angiectasis in the hepatic lobules in the cPI treatment may have been the cause of the liver weight increase. Moreover, the hepatic sinusoid may lose the vascular permeability, therefore white blood cells and red blood cells are able to migrate through the interstitial space, leading to red blood cell congestion, white blood cell infiltration, extravasation of blood, necrosis, and fibrosis in the organ (Silva-Cunha *et al.*, 2009). When the liver is overloaded, chemical toxin accumulates in the bloodstream that is able to induce acute renal injury. Thus, the glomeruli and proximal tubular swelling and angiectasis in glomerulus were found in the renal cortex leading to damage of renal tissue as indicated by “acute glomerulonephritis” (Silva-Cunha *et al.*, 2009, Tain *et al.*, 2015). In the present study, the renal tissue damages were found within 24 h after oral administration and renal tubular

injury on day 7 showed better than 24 h. It is possible that the kidney should be excreted more toxin in the urine. Padhye *et al.* (2010) reported that after 24 h oral administration in animal, plumbagin reaches the maximum concentration in plasma within 1-2.5 h. The half-life of plumbagin is approximately 1.3-15 h, and the main portion of the drug metabolites is excreted in urine (51%) and in feces (49%) (Padhye *et al.*, 2010).

Normally, the hepato-cellular toxicity is evaluated by measuring levels of enzymatic markers (AST and ALT levels) for identifying liver damage (Calil Brondani *et al.*, 2017). AST is not specific to the liver because it is produced by skeletal muscles and erythrocyte, whereas ALT is produced in the hepatocyte. The increased levels of ALT and AST in the plasma imply that the liver injury occurs. In the present study, the levels of AST and ALT did not significant differences among treatment groups and the control in the acute toxicity test. Levels of AST and ALT in the cPI treatment groups showed higher than the control group in 24 h, whereas those levels less than control in day 7. It implied that cPI induced the hepatic injury in the acute oral administration, these results correlated with the histomorphological changes and previous studies (Solomon *et al*.,1993; Sukkasem *et al*., 2016). Cellular adaptations can occur after cell injury if the cell is able to reverse or recover as normal shape (Rajasekaran *et al*., 2011), claimed the hepatoprotective effect of PI extract in Wistar albino rats induced hepatotoxicity using paracetamol. Perhaps the animals recovered as the histopathological observation in the subacute stage showed no negative effects. The liver function remained within the physiological limits which implied the reversible of hepatic the adaptation. Other studies reported similar findings regarding the hepatoprotective effect of plants (Calil Brondani *et al.*, 2017; Lima *et al.*, 2017). Urea and creatinine are markers for kidney damages. There was no evidence of cPI inducing nephrotoxicity since the values of BUN and creatinine displayed greater than control, but they were no significant difference. These results were similar to the previous study, the value was still within the physiological limits (Lima *et al.*, 2017). This is the first report to compare the liver enzymatic function and kidney function tests relate with pathological changes in early acute and subacute whereas the previous studies reported the liver function test in 14 days and 30 days in mice and rats, respectively (Solomon *et al*.,1993; Sukkasem *et al*., 2016). The feeding of cPI caused the liver enzymatic elevation and increasing of kidney function, made to be the liver injury. From these findings, we suggested that scientists should be aware of the doses per safety and side effect of *P. indica* when using this plant in higher doses in the animal model in a future study.

Conclusions

This present study indicates that the ethanolic root extract of *P. indica* showed no toxic effects when administrated orally in doses less than 1,000 mg/kg. These present results confirm the potent cytotoxic properties of the *Plumbago indica* root extract. we suggest that the user should be concern about the toxicity effect in liver and kidney at the high dosage of *P. indica* root extract.

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References

Ariyanathan, S., Saraswathy, A., Rajamanickam, G.V. (2010). Quality control standards for the roots of three Plumbago species. Indian J Pharm Sci.,72:86-91.

Atjanasuppat, K., Wongkham, W., Meepowpan, P., Kittakoop, P., Sobhon, P., Bartlett, A., and Whitfield, J. P. (2009). *In vitro* screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. J. Ethnopharmacol., 123(3):475-482.

Calil Brondani, J., Reginato, F.Z., da Silva Brum, E., de Souza Vencato, M., Lima Lhamas, C., Viana, C., da Rochac, M.I.U.M., Bauermann, L.F., and Manfron, M.P. (2017). Evaluation of acute and subacute toxicity of hydroethanolic extract of *Dolichandra unguis- cati* L. leaves in rats. J. Ethnopharmacol., 202:147-153.

Kaewbumrung, S. and Panichayupakaranant, P. (2014). Antibacterial activity of plumbagin derivative- rich *Plumbago indica* root extracts and chemical stability. Nat Prod Res., 28(11):835-837.

Ketpanyapong, W. and Itharat, A. (2016). Antibacterial Activity of Thai Medicinal Plant Extracts Against Microorganism Isolated from Post-Weaning Diarrhea in Piglets. J Med Assoc Thai., 99 (4): S203-S210.

Lima, N.V., Arakaki, D.G., Saldanha Tschinkel, P.F., Silva, A.F. Avellaneda Guimarães, R.C., Hiane, P.A., and Ferreira Júnior, M.A. (2017). First comprehensive study on total determination of nutritional elements in the fruit of the *Campomanesia adamantium* (Cambess.): Brazilian cerrado plant. Int. Arch. Med., p. 9.

Makchuchit, S., Rattarom, R., and Itharat, A. (2017). The anti-allergic and anti-inflammatory effects of Benjakul extract (a Thai traditional medicine), its constituent plants and its some pure constituents using *in vitro* experiments. Biomed. Pharmacother., 89:1,018-1,026.

Minsakorn, S., Nuplod, K., Puttarak, P., Chawengkirttikul, R., Panyarachun, B., Ngamniyom, A., Charoenkul, T., Jaisa-

Aad, M., Panyarachun, P., Anuracpreeda, P. (2019). The Anthelmintic Effects of Medicinal Plant Extracts Against Paramphistome Parasites, *Carmyerius spatiosus*. Acta Parasitol., 64(3):566-574.

Nayak, P., Sharma, M., Behera, S., Thirunavoukkarasu, M., and Chand, P. (2015). High-Performance liquid chromatographic quantification of plumbagin from transformed rhizoclones of *Plumbago zeylanica* L.: Inter-Clonal variation in biomass growth and plumbagin production. Appl Biochem Biotechnol., 175:1,745-1,770.

Organisation for Economic Co- operation and Development [OECD/OCDE], (France). (2001). OECD Guideline for testing of chemical; acute oral toxicity- acute toxic class method. 423.

Padhye, S., Dandawate, P., Yusufi, M., Ahmad, A., and Sarkar, F.H. (2010). Perspectives on medicinal properties of plumbagin and its analogs. Med. Res. Rev., 32(6):1,131-1,158.

Paiva, S.R., Figueiredo, M.R., Tânia Verônica Aragão, T.V., Kaplan, M.A.C. (2003). Antimicrobial activity *in vitro* of plumbagin isolated from *Plumbago* species. Mem. Inst. Oswaldo. Cruz., 98(7):959-961.

Paiva, S.R., Lima, L.A., Figueiredo, M.R., Kaplan, M.A. (2004). Plumbagin quantification in roots of Plumbago scandens L. obtained by different extraction techniques. An Acad Bras Cienc.,76:499-504.

Rajasekaran, A., Periasamy, M. (2011). Protective effect of ethanolic root extract of *Plumbago indica* L. on paracetamol induced hepatotoxicity in rats. Afr J Pharm Pharmacol., 5:2,330-2,334.

Ramachandran, R. abd Kakar, S. (2009). Histological patterns in drug-induced liver disease. J Clin Pathol., 62:481-492.

Ratanavalachai, T., Thitiorul, S., Tanuchit, S., Itharat, A., Sakpakdeejaroen, I. (2015). In vitro enhancement of doxorubicin genotoxic activities and interference with cell cycle delay by Plumbago indica root ethanolic extract in human lymphocytes. J Med Assoc Thai.; 98:S38-S44.

Sandeep, G., Dheeraj. A., Sharma, N.K., Jhade, D., Bharti, A. (2011). Effect of plumbagin free alcohol extract of *Plumbago zeylanica* Linn. root on reproductive system of female Wistar rats. Asian Pac J Trop Med., 4(12):978-984.

Savadi, R.V., Alagawadi, K.R. (2009). Antifertility activity of ethanolic extracts of *Plumbago indica* and *Aerva lanata* on albino rats. Int J Green Pharmacy., 3 (3): 230-233.

Silva-Cunha, A., Fialho, S.L., Naud, M.C., and Behar-Cohen, F. (2009). Poly-ε-caprolactone intravitreous devices: An *in vivo* study. J. Physiol. Pharmacol., 50(5):2312-2318.

Solomon, F.E., Sharada, A.C., and Devi, P.U. (1993). Toxic effects of crude root extract of *Plumbago rosea* (Rakta chitraka) on mice and rats. J. Ethnopharmacol., 38(1):79-84.

Sumsakul, W., Mahavorasirikul, W., and Na- Bangchang, K. (2015). Inhibitory activities of Thai medicinal plants with promising activities against malaria and cholangiocarcinoma on human cytochrome P450. Phytother. Res., 29(12):1926-1933.

Sukkasem, N., Chatuphonprasert, W., Tatiya-Aphiradee, N., Jarukamjorn, K. (2016). Imbalance of the antioxidative system by plumbagin and *Plumbago indica* L. extract induces hepatotoxicity in mice. J Intercult Ethnopharmacol., 5(2):137-145.

Tain, Y.L., Hsu, C.N., Chan, J.Y.H., and Huang, L.T. (2015). Renal transcriptome analysis of programmed hypertension induced by maternal nutritional insults. Int. J. Mol. Sci., 16(8):17826-17837.

Thiengsusuk, A., Chaijaroenkul, W., and Na- Bangchang, K. (2013). Antimalarial activities of medicinal plants and herbal formulations used in Thai traditional medicine. Parasitol. Res., 112(4):1475-1481.

Unnikrishnan, K.P., Sudhakar Raja, S., and Balachandran, I. (2008). A reverse phase HPLC-UV and HPTLC methods for determination of plumbagin in Plumbago indica and Plumbago zeylanica. Indian J. Pharm. Sci. 70(6):844-847.