J Biochem Tech (2012) 4(1):541-548
ISSN: 0974-2328

Toxicological, biochemical and histopathological evaluation of Tridham, a siddha medicine in Wistar albino rats

Ravindran Jaganathan, Vijaya Ravinayagam, Sachdanandam Panchanadham, Shanthi Palanivelu*

Received: 22 September 2012 / Received in revised form: 06 June 2013, Accepted: 06 June 2013, Published online: 30 June 2013

Abstract

Tridham (TD), a polyherbal formulation is assessed for its acute (72 hr) and subacute toxicity (28 days) and also its significance on histological, hematological and biochemical variations in albino wistar rats. Body weight and general behavior of animal was observed throughout the experimental period and at the end of the study period organ weight, haematological and biochemical parameters of blood and urine, as well as kidney and liver histology were evaluated. Results of the studies performed indicated no toxic clinical symptoms or histopathological lesions in both acute and subacute toxicity, which clearly shows that TD extract has high margin of safety.

Keywords: Toxicity assessment, Polyherbal formulation, Tridham, *Terminalia chebula, Eleocarpus ganitrus, Prosopis cineraria.*

Introduction

Plants have broader uses than as just food and genetic reservoirs. Medicinal plants have been used for centuries to treat wide variety of ailments (Vaidya and Devasagayam, 2007). The presence of secondary metabolites in plants has been associated in most of their therapeutic activities (Ogunleye and Ibiyoye, 2003). Herbal medicines are now considered a part of Complementary and Alternative medicine (CAM) and are gaining popularity due to their potent antioxidant activity, minimal side effects and economic viability (Auddy et al. 2003). Active principles from natural sources have contributed significantly to the development of new drugs from medicinal plants (Cox and Balick, 1994).

Ravindran Jaganathan, Vijaya Ravinayagam, Shanthi Palanivelu*

Department of Pathology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani campus, Chennai, Tamilnadu, India, 600113

* Email: pshanthi9@yahoo.co.in

Sachdanandam Panchanadham

Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani campus, Chennai, Tamilnadu, India, 600113

Among the different type of herbal medicine, Siddha medicine is a form of traditional medicine, used for nearly 10,000 years by Indian people. This ancient Siddha System of Medicine has its own unique nature in respect of physiology, pathology, pharmacology, and therapeutics (Shanmugavelan and Sundararajan, 1992).

Tridham (TD) is a combination of three plant ingredients including seed coats of *Terminalia chebula*, fruits of *Eleocarpus ganitrus* and leaves of *Prosopis cineraria* in equal proportions. It is used by traditional practitioners practicing Siddha medicine and indigenous systems. TD has been proved for its potential anticancer and antioxidant properties against Aflatoxin B_1 induced rat hepatocellular carcinoma (Vijaya et al. 2011).

The medicinal properties of *Terminalia chebula*, a constituent of TD, has been acknowledged from olden times and were detailed by Charaka in his text "Charaka Samhita" (Gandhi and Nair, 2005) and it also called as "King of Medicines". It has been used widely in ayurveda, unani and homeopathy (Chattopadhyay and Bhattacharyya, 2007; Mahesh et al. 2009). Extensive studies reported on T. chebula for its wide spectrum of biological properties such as anti-bacterial (Kim et al. 2006), anti- fungal (Bonjar, 2004), anti-diabetic (Sabu and Kuttan, 2002) antioxidant (Cheng et al. 2003; Lee et al. 2005), anti-cancer (Saleem et al. 2002), hepatoprotective (Tasduq et al. 2006; Lee et al. 2007) and anti-mutagenic (Grover and Bala, 1992). *E. ganitrus*, which belongs to the Elaeocarpaceae family, is used in ayurveda for treating various diseases such as mental illness, epilepsy, asthma, hypertension, anti-aging, arthritis, hysteria, cough and hepatic diseases (Gaurav et al. 2010). The E. ganitrus fruit contains several phytoconstituents such as alkaloids, steroids, triterpenoids tannins, flavonoids, carbohydrates and cardiac glycosides (Farnsworth, 1966). Singh et al. 2000 reported a significant amount of phytocomponents such as isoelaeocarpicine, elaeocapine isoelaeocarpine and quercetin, gallic, ellagic acids and rudrakine. Three new ellagic acid derivatives of eleocarpaceae, 4-Omethylellagic acid 3-0-a-rhamnoside, 4-O-methylellagic acid 30-(300-O-acetyl)-a-rhamnoside and 4-O-methylellagic acid 30-(400-Oacetyl)- a-rhamnoside in addition to the known ellagic acid derivative, 4-O-methylellagic acid 30-(200,300-di-O-acetyl)-a-rhamnoside are used in multi targeted therapy of cancer (Heber, 2008) and a significant antioxidant competence due to its rich content of tannins and flavonoids (Sathish kumar et al. 2008). *Prosopis* species has been widely used

to cure basic ailments in the traditional system of medicine (Kirtikar and Basu, 1984). *P. cineraria* is considered to have a number of medicinal effects such as anti-helminthic, anti-cancer, antibacterial, antifungal, antiviral, anti-diabetic and many other pharmacological properties (Chopra et al. 1956; Sharma et al. 2010).

Qualitative chemical exposition studies on TD, carried out in our laboratory, showed the presence of various phytochemicals such as flavonoids, tannins, phenols, steroids, carbohydrates, saponins, terpenoids and proteins - the presence of several phytoconsituents was also identified by spectroscopy analysis such as FT-IR. A well-known polyhydroxyphenolic compound 3,4,5-trihydroxybenzoic acid (Gallic acid) that can be found in various natural products has been isolated by us through column chromatography and characterized by a series of experiments, involving NMR, IR, MS and single crystal X-ray crystallography (Vijaya et al. 2012).

With the resurgence in the use of herbal medicines recently, there is an increased awareness of the need for data on the safety and potential toxicity of medical plants. Animal toxicity studies are mandatory to establish the adverse effects of the drug. Despite the use of TD in antioxidant, anticancer and active principle studies, no study has been published in the scientific literature about its toxicological and histopathological profile. The present study scrutinizes the acute and subacute toxicity profiles, including histopathological evaluation, of TD in Wistar albino rats.

Materials and Methods

Drug and Chemicals

Drug (TD) ingredients were freshly collected and prepared according to the formula used by some Siddha practitioners to treat hepatocellular carcinoma. All other chemicals used were of highest purity and analytical grade.

TD preparation

TD is an amalgamation of *T. chebula* seed coats, dry seeds of *E. ganitrus* and *P. cineraria* leaves. The three plants were collected and submitted for botanical authentication to the Department of Centre for Advance Study (CAS) in Botany, University of Madras, Guindy Campus, Chennai, India. The authenticated herbarium numbers was, CASBH-16 for *T. chebula*, CASBH-17 for *E. ganitrus*, and CASBH-18 for *P. cineraria*.
The ingredients were washed, air dried in shade and then finely ground. The components were then mixed in equal proportions on weight basis to get TD mixture. The extract of TD was prepared in 3:1(v/w) ratio by adding 30 ml of water to 10 grams of combined TD and mixed well. The mixture was mixed by using a shaker for 12 hours. The mixture was subsequently filtered using filter paper and the clear filtrate (aqueous extract) was collected in a beaker. The filtrate was then lyophilized under vacuum pressure to yield a powder. The lyophilized extract was stored in airtight containers in a dry dark place.

Animals and diet

Healthy Albino Wistar strain rats, of either were used in this study. The animals were obtained from Central Animal House Facility, Taramani Campus, University of Madras, Taramani, Chennai – 600113, India. The animals were housed in polypropylene cages under a controlled environment with 12 ± 1h light/dark cycles. The temperature was maintained between 27 and 37º C and was fed with standard pellet diet (Gold Mohor rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water ad libitum. The study has got the approval from the Institutional Animal Ethical Committee (IAEC) and conducted according to ethical guidelines of Committee for the Purpose of Control and Super- vision of Experiments on Animals (CPCSEA). The IAEC numbers for the present study are 06/005/07 and 06/012/08.

Experimental design

Acute toxicity study

The acute toxicity of TD was evaluated in rats using the up and down procedure of OECD guidelines (OECD 2000). In accordance with the limit test, single dose of TD (2000 mg/kg b. wt) was orally administrated. Healthy Wistar albino rats weighing 150 ± 20 g were divided into 7 groups (Groups I-VII) of 6 each animals each. The drug was administered orally through gastric intubation in aqueous phase at doses of 50, 100, 250, 500, 1000 and 2000 mg/kg b.wt. The control group received 0.5 ml of the vehicle alone. The animals were observed continuously for 72 h for any signs of behavioral changes and mortality.

Subacute toxicity study

Wistar albino rats of (150-180 g) were divided into 5 groups (Groups I-V) of 6 animals each and were housed under the same conditions as described above. The drug was administered for 28 days at doses of 50, 100, 200 and 500 mg/kg b.wt, respectively. The control animals (Group I) received 0.5 ml of the vehicle alone. Toxic manifestations and mortality were monitored daily till the end of the study.

Body weight changes

Initially, the body weights of the individual animals were recorded on the 1st day of the study before the administration of drug. Thereafter, at the end of the 2nd, 3rd, and 4th weeks of the experimental period, animal weights were recorded and compared to that of control animals.

Organ weight analysis

On completion of 30th day, both the control and drug treated animals were deprived of food overnight and sacrificed by cervical decapitation. Vital organs like liver, kidney, lung, spleen and heart from each animal were separated after thorough perfusion of the organs with neutral saline, and pressed with the help of tissue paper to remove any moisture. The isolated organs were observed for morphological changes such as the presence of any kind of lesions, etc., and the individual organs from each animal were weighed. The results were compared to that of the control animals.

Hematological indices

At the end of treatment period, blood samples were collected by retro orbital puncture of all control and experimental rats and submitted to biochemical and hematological tests. Hemoglobin (Hb) content in blood was estimated using the method of Drabkin and Austin (1932). Red blood cell (RBC) and White blood cells (WBC) count were determined by the method of Chesbrough and Mc Arthur, 1972.

For the hepatic function, alanine aminotransferases (ALT), and aspartate aminotransferases (AST) , alkaline phosphotase (ALP) were determined, while for the renal function, serum urea (Natelson et al. 1951), uric acid (Caraway, 1963), creatinine (Owen et al. 1954) were evaluated. Blood glucose was assessed for carbohydrate

metabolism analysis by the method of Sasaki and Matsui (1974) and protein was also estimated.

Urine analysis

For urine analysis, 24 h urine samples were collected by placing the animals in the metabolic cage with free access to tap water without administration of feed. The urine was free from faecal contamination and was collected using a 50 ml beaker maintained at 0ºC in an ice-bath. Toluene is used as a preservative while collecting the sample. The sediments present in the urine were removed by centrifugation and the collected urine was used for biochemical estimations.

Histopathological studies

Histopathological studies were carried out in liver and kidney tissues of control and experimental rats. Tissues were fixed in 10% buffered formalin, routinely processed and embedded in paraffin wax. Consecutive sections were cut at a thickness of 4µm, stained with hematoxylin and eosin (H&E) (Culling, 1974). The pathological observations of all tissues were performed on gross and microscopic bases. A minimum of three fields of each tissue slide was morphologically evaluated.

Statistical analysis

The values were expressed as mean ± SD. The results were computed statistically (SPSS software package, version 7.5 for windows) using one-way analysis of variance (ANOVA). Post hoc testing was performed for inter-group comparison using Student-Newman-Kuel multiple comparison test. Values of $p < 0.05$ were considered significant.

Results and Discussion

Acute toxicity

In the acute toxicity study, body weight, food and water consumption were found to be unaffected by the treatment of TD. No mortality or significant changes in general behavior was observed up to the maximum dose level of 2000 mg/kg b.wt.of the drug administered orally. This is the single highest dose recommended by OECD guidelines-423 (Ecobichon, 1997). Mortality and general behavioral changes are often the first signs of toxicity observed during acute toxicity studies (Carol, 1995). Thus our results indicate that TD does not cause any acute toxicity.

Subacute toxicity

In order to evaluate the adverse effect of repeated daily exposure of drug, subacute toxicity study was carried out. Subacute studies are immensely valuable in assessing the safety profile of phytomedicines (Aniagu et al. 2005). To determine dose related toxic effects, doses of 50, 100, 200 and 500 mg/kg b.wt, of TD extract were administered for the experimental duration of 28 days. TD at the specified doses did not produce any significant changes in animals, as evidenced by the absence of toxic symptoms with no changes in water/food ingestion and general behaviors.

Effect of TD on Body and organ weight changes

Body weights of animals in all the groups were observed throughout the duration of study and presented in Fig. 1. Administration of TD was found to change the body weights of all the rats but this was not statistically significant. The observed weight gain in the TD treated

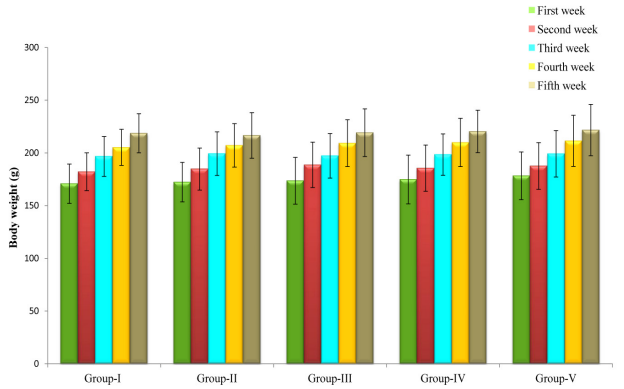

Figure 1. Effect of TD on body weight in subacute toxicity studies. Values are expressed as mean ± SD for 6 rats. Comparisons were made between Group I with Group II, III, IV and V.

animals shows that the administrated herbal combinational drug does not have any untoward action that affect the growth of the animals. A change in body weight is an uncomplicated and sensitive index to study the detrimental effects of drugs and chemicals (Teo et al. 2002). In general toxic nature of the drug leads to abnormalities in body weight (Etuk and Muhammad, 2010). No significant change in the body weight was noticed among the control (Group I) and TD treated groups (Group II to Group V).

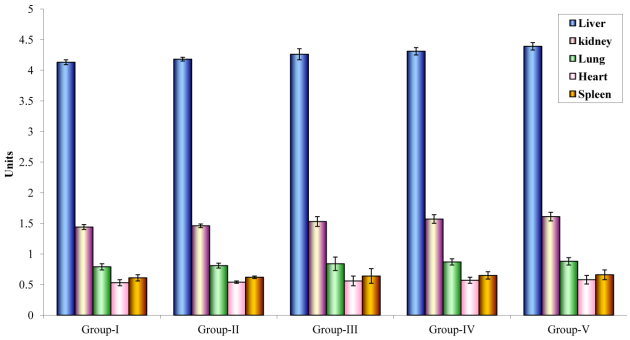

Figure 2. Effect of TD on organ weight in subacute toxicity studies. Values are expressed as mean ± SD for 6 rats. Comparisons were made between Group I with Group II, III, IV and V. Units: Liver and Kidney: grams (g), Heart, Lung and Spleen: milligrams (mg).

The retainment of the body weight in drug treated animals is an important finding that shows the nontoxic nature of the drug TD. A decline in the body weight and its associated organ weights are significant indices for toxicity after drug exposure (Thanabhorn et al. 2006).

A morphological change gives a clear evidence of serious changes leading to organ dysfunction due to the drug (Andrew et al. 2003). The results of the observation in vital organs such as liver, kidney, heart, lung, and spleen indicated that there were no signs of any inflammation or toxicity in both control as well as in the TD treated animals. The organ weight changes of control and drug treated rats are presented in Fig. 2. The statistical results suggest that there are no significant changes in the organs weight among the control (Group I) and TD treated groups (Group II to Group V). Notable changes in the organ weight and in biochemical parameters are indicators of the toxicity of the drug (Vaghasiya et al. 2011). The adverse metabolic effect of toxic substances is reflected in the structure and function of vital organs such as heart, liver, kidney, lung and spleen (Dybing et al. 2002). The TD treated groups revealed no significant differences in body weight or weight of

organs such as heart, lung, liver, kidney and spleen. The present evaluation clearly shows that TD does not produce any toxicological effects on the body and organ weights.

Effect of TD on Hematological parameters

Hematological parameters such as Hb, WBC and RBC were evaluated to study the alterations in the hematological system due to toxicity of drug. The toxic effects of TD on the hematological parameters are depicted in Table 1. The parameters (Hb and RBC) in all treated groups remain within the normal limits. A significant increase in the WBC level is observed with the dose of 500mg/kg b.wt.

Table 1. Effect of TD on hematological parameters in blood for subacute toxicity*

Parameters	GROUP I (Control)	GROUP II (50mg/kg b.wt.)	GROUP III (100mg/kg b.wt.)	GROUP IV (200mg/kg b.wt.)	GROUP V (500mg/kg b.wt.)
Hb (g/dl)	13.87±1.66	14.15±1.71	14.68±1.75	14.96±2.04	15.04±2.12
RBCx (10^6/mm^3)	8.41±0.91	8.63±0.96	8.13±0.89	8.37±0.96	8.55±0.88
WBC x (10^3/mm^3)	7.98±0.11	6.98±0.72	7.12±0.91	7.56±0.83	7.84±0.93*

*Values are expressed as mean ± SD for 6 rats. Comparisons were made between Group I with Group II, III, IV and V. The symbol * represents the statistical significance at $p < 0.05$. Hb- hemoglobin, RBC- red blood cells, WBC- white blood cells

Evaluation of hematological parameters is an important and sensitive index, considered vital in toxicity studies during extrapolations of experimental data to clinical study (Olson et al. 2000). The consumption of toxic plants will cause an alteration in the hematological profile (Sani et al. 2009). Drugs associated with toxic effect are reported to cause haemolytic anaemia and organ dysfunction eventually producing a significant change in some biomarkers in the blood profile (Echobicon, 1992). In the present study, hemoglobin and RBC levels in all treated groups were showed no significant changes and remained within the normal limits as compared with control rats. Total WBC count showed an increase in the TD treated animals, but this was not statistically significant. Such an increase in WBC level directly indicates the strengthening of the organism defense (Chang-Gue et al. 2003; Stanley et al. 2005). This elevation in WBC count suggests that TD contains an immune potentiating effect. Non-significant level of Hb, RBC, and WBC was observed, which may indicate the nontoxic beneficial effect of TD on the hematological profile.

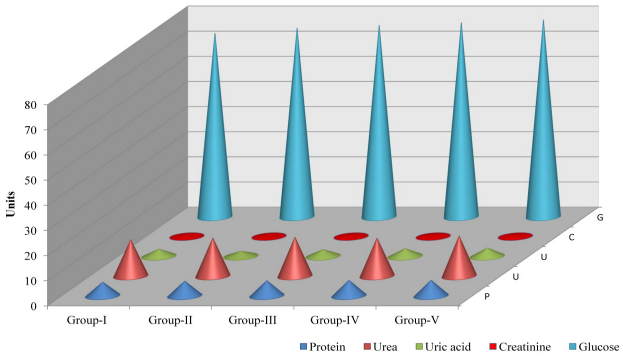

Figure 3. Levels of protein, urea, uric acid and glucose in blood in subacute toxicity study of TD. Values are expressed as mean + SD for 6 rats. Comparisons were made between Group I with Group II, III, IV and V. Units: Protein – g/dl, Urea, Uric acid, Creatinine, and Glucose – mg/dl.

The effect of TD on blood parameters such as protein, urea, uric acid, creatinine and blood glucose were presented in Fig. 3. These biochemical variables in both control and treated groups remained within normal levels without any significant difference. Urea, uric acid and creatinine are end products of protein metabolism and must be removed continually to ensure protein metabolism in the cells (Guyton, 1981). Renal toxicity of the administered drug can be evaluated by studying changes in renal function.

An increased plasma urea, uric acid and creatinine level with simultaneous decrease in urine urea, uric acid and creatinine level shows the diminution of glomerular filtration rate and acute renal failure (Renugadevi et al. 2009). Luyckx et al. (2002) reported that renal dysfunction is associated with adverse reactions of herbal remedies. In the present investigation, a non significant alteration was observed in plasma urea, uric acid and creatinine levels and as well as in urine urea, uric acid and creatinine levels. Therefore it can be inferred that the herbal combination drug TD did not exert any toxic effect on the kidneys. *Terminalia chebula* is the one of the components of TD, which has a nephron-protective effect on rats (Rao and Nammi, 2006). *E. ganitrus* also possesses renal protective effect (Gaurav et al. 2010). The presence of these components may contribute to nephroprotective effect of TD. Protein synthesis is an important function of the normal liver cell. Thus, protein levels seem to be a true index and a reliable indicator of liver function in drug overdose (Hutchinson et al. 1980; Martin and Friedmen, 1998). In the present study protein levels of plasma and urine shows no significant changes in TD treated animals as compared to control animals. It indicates the nontoxic nature of TD on protein.

Effect of TD on Biochemical and urine analysis

Hepatic function has been examined by the evaluation of the marker enzymes. Table 2 represents the marker enzymes (ALT, AST and ALP) in serum, liver and kidney. Transaminases (ALT, AST) and ALP are good indices of liver and kidney damage, respectively (Martin et al. 1981). No significant toxic changes were observed in the levels of ALT, AST and ALP in serum, liver and kidney of treated groups when compared with the control rats. The biological role of transaminases (ALT and AST) and ALP is concerned with the inter conversion of highly important metabolite. The enzymes serve as an index of liver cell injury (Hanley et al. 2005). In the present investigation, the levels of transaminases ALT, AST and ALP in serum, liver and kidney of TD treated and control animals were evaluated. In general, an amendment in ALT, AST and ALP levels may reveal a change on cellular permeability or cellular damage (Kashaw et al. 2011). However, no such deleterious changes were found in the levels of liver marker enzymes such as ALT, AST and ALP in serum, liver and kidney of TD treated groups when compared with the control rats. The current results clearly indicated that treatment with TD did not induce any harmful biochemical effects on the animals Table 3 depicts the urinary levels of protein, urea, uric acid and creatinine. The renal function test shows that all the parameters were found to vary, but this variation was not statistically significant when compared with that of the control rats.

Table 2: Effect of TD on marker enzymes in serum, liver and kidney for subacute toxicity*

PARAMETERS	GROUP I (Control)	GROUP II (50mg/kg b.wt.)	GROUP III (100mg/kg b.wt.)	GROUP IV (200mg/kg b.wt.)	GROUP V (500mg/kg b.wt.)
			Serum		
Serum AST	82.85±9.41	84.62±10.11	85.67±9.72	87.14±9.12	88.33±10.18
Serum ALT	18.35±9.61	19.73±2.36	21.56±3.91	22.83±2.95	23.14±2.98
Serum ALP	233.13±25.15	235.75±24.51	236.74±29.54	237.94±24.4	238.43±26.24
			Liver		
AST	19.67±2.38	20.19±2.64	20.66±3.14	21.18±2.73	21.93±3.02
ALT	15.23±1.65	15.31±1.94	15.73±2.12	16.03±2.17	16.64±2.33
ALP	3.85±0.42	3.86±0.41	3.87±0.43	3.88±0.46	3.89±0.48
			Kidney		
AST	14.23±2.12	14.76±1.71	15.26±3.16	16.18±2.18	16.94±2.63
ALT	12.24±2.12	12.46±1.81	13.19±1.92	13.78±2.31	14.13±1.95
ALP	2.78±0.32	2.81±0.39	2.84±0.29	2.93±0.31	2.97±0.37

*Values are expressed as mean ± SD for 6 rats. Comparisons were made between Group I with Group II, III, IV and V. Units: AST and ALT - µmoles of pyruvate liberated/min/mg protein, ALP - U/L. AST- aspartate transaminase, ALT- alanine transaminase, ALP- alkaline phosphatase

Table 3: Effect of TD on biochemical parameters in urine for subacute toxicity*

PARAMETERS	GROUP I (Control)	GROUP II (50 mg/kg b.wt.)	GROUP III (100 mg/kg b.wt.)	GROUP IV (200 mg/kg b.wt.)	GROUP V (500 mg/kg b.wt.)
Protein (mg/24 hr urine)	11.43±2.15	11.48±1.94	11.59±1.78	11.54±1.42	11.65±1.77
Urea (mg/24 hr urine)	11.13±1.22	11.87±1.58	12.14±1.62	12.58±1.91	12.72±2.16
Uricacid (mg/24 hr urine)	3.67±0.44	3.73±0.48	3.86±0.43	3.89±0.42	3.97±0.47
Creatinine (mg/24hr urine)	7.21±0.79	7.48±0.82	7.89±1.02	8.11±1.29	8.29±1.16

*Values are expressed as mean ± SD for 6 rats. Comparisons were made between Group I with Group II, III, IV and V.

Effect of TD on Histopathology

Histological evaluation was carried out to characterize the biological response factors. Figs. 4 and 5 show the histopathological examination of liver and kidney respectively. The assessment of histopathology of liver and kidney showed normal architecture implied no detrimental changes and morphological alterations in control and TD treated animals. As well, gross and pathological examinations of all other internal organs revealed no pathological abnormality (data not shown). Gross morphology of all organs and histopathology of liver and kidney in all animals (Group-I to Group-V) showed normal appearance. This indicates that the Siddha preparation, TD, did not exert any toxic effect on the animals. In fact, one of the ingredients of TD, *T. chebula* has been reported to exert a hepatoprotective effect, including reduction of hepatocyte swelling and neutrophilic infiltration and prevention of necrosis in rat liver (Lee et al. 2005).

Conclusions

In conclusion, the results of the acute toxicity study reveal that TD did not lead to mortality and anomaly in general behavior at the maximum dose of 2000 mg/kg b.wt. Subacute toxicity study discloses that TD may be considered as a safe drug, as it did not cause any fatality and it did not show any notable morphological, hematological, biochemical or adverse histopathological changes in rats. Based on this scientific appraisal, it can be concluded that the extracts of the TD have a high margin of safety as it did not induce any toxicological effects.

Acknowledgments

One of the authors, Mr. J. RAVINDRAN, acknowledges the Indian Council of Medical Research (ICMR), New Delhi, India for the financial assistance given in the form of Senior Research Fellowship (No.3/2/2/189/2009/NCD-III).

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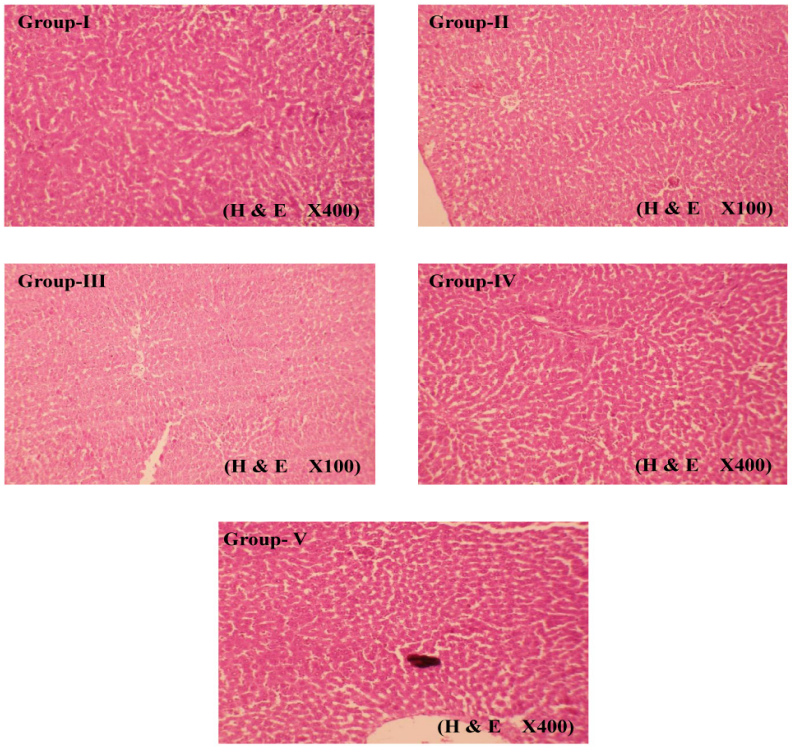

Figure 4. Histopathological evaluation (H & E) of hepatic tissue in control and experimental animals of TD subacute toxicity study. Group-I: Control animals, Group-II: TD treated (50 mg/kg b.wt.), Group-III: TD treated (100 mg/kg b.wt.), Group-IV: TD treated (200 mg/kg b.wt.), Group-V: TD treated (500 mg/kg b.wt.).

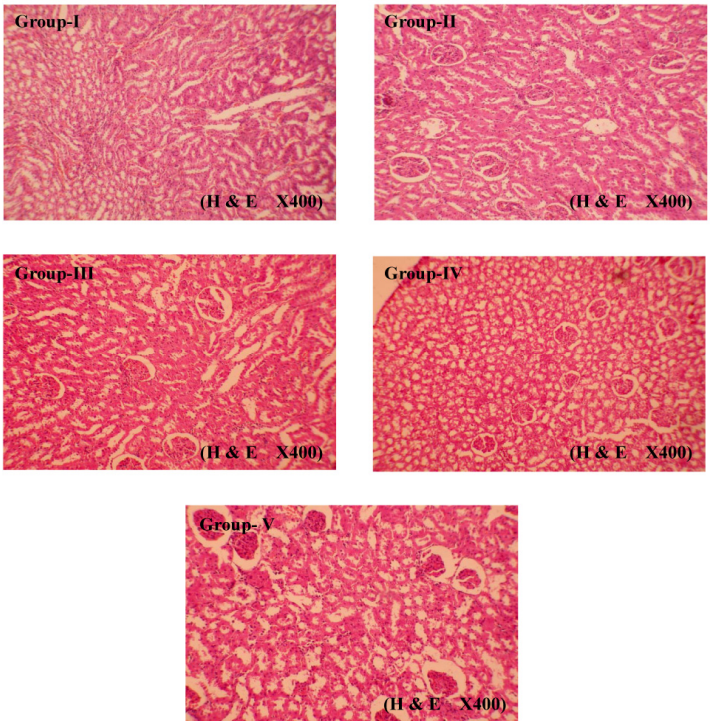

Figure 5. Histopathological (H & E) evaluation of Kidney tissue in control and experimental animals of TD subacute toxicity study. Group-I: Control animals, Group-II: TD treated (50 mg/kg b.wt.), Group-III: TD treated (100 mg/kg b.wt.), Group-IV: TD treated (200 mg/kg b.wt.), Group-V: TD treated (500 mg/kg b.wt.).

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