ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 8, Issue 2, 2015

ISSN - 0974-2441

Research Article

SAFETY EVALUATION OF PETROLEUM ETHER, ACETONE AND HYDROALCOHOLIC EXTRACTS OF *HOLOPTELEA INTEGRIFOLIA* (ROXB.) PLANCH. BARK BY ORAL ADMINISTRATION IN WISTAR RATS

KUMAR D[1], PATIL PA[2], HARSHA V HEGDE[1*], ROY S[1], KHOLKUTE SD[1]

[1]Regional Medical Research Centre (ICMR), Belgaum, Karnataka, India - 590010. [2]Department of Pharmacology, USM-KLE, Belgaum, Karnataka, India 590010. Email: harshavh@rediffmail.com

Received: 20 October 2014, Revised and Accepted: 24 December 2014

ABSTRACT

Objective: There are no reports on safety information of *Holoptelea integrifolia* (Roxb.) Planch. bark extracts by oral administration for long duration. This study is planned to add information on the safety margin of *H. integrifolia*.

Methods: Acute oral toxicity study was carried out in the consideration of OECD guideline 425. Animals were observed for mortality, morbidity, behavior and toxic symptoms for 14 consecutive days. Sub-acute toxicity was planned by employing the OECD guideline 407. Dose 50, 100 and 200 mg/kg of the extracts were administered orally, daily for 28 consecutive days. Animals were observed for toxicity sign, morbidity and mortality. Body weight, relative organ weight, biochemical, hematological parameters and histopathology of vital organs like liver and kidney were evaluated.

Results: In the acute oral toxicity study, all the extracts were found to be safe at 1000 mg/kg. However, out of three extracts, petroleum ether extract of the plant showed mild damage to liver and kidney in sub-acute toxicity studies.

Conclusion: Hydroalcoholic extract of *H. integrifolia* is found to be safe, compared to the acetone and petroleum ether extracts for the long-term use.

Keywords: *Holoptelea integrifolia*, Bark, Acute toxicity, Chronic toxicity

INTRODUCTION

Most of the population in developing countries still depends on alternative systems of medicine. In India, apart from qualified Ayurvedic practitioners, many unqualified traditional healers dispense a variety of herbal preparations for common ailments. World Health Organization recommends that, the plants used for the treatment of disease need to be scientifically investigated for their toxicity [1]. Toxicity profile of the plant helps to understand the severity of toxicity for that particular herbal drug corresponding to particular dose and duration. *Holoptelea integrifolia* (Roxb.) Planch. is routinely used by the local traditional practitioners in the rural area of Northern Karnataka of India for the cure of various diseases viz.; bronchitis, obesity [2], inflammation, gastritis, dyspepsia, vomiting, piles, wound healing, leprosy, diabetes, diarrhea, rheumatism and skin disease [3]. *H. integrifolia* is from the family Ulmaceae. It is a large sized tree, found throughout India, tropical and subtropical parts of Asia and Africa [4]. The aqueous leaf extract of *H. integrifolia* is reported to possess anti-inflammatory activity [5]. Major chemicals reported from *H. integrifolia a*re 1,4-napthalenedione, Holoptelene A, Holoptelene B, Friedelin, Epifriedelinol, 2 aminenapthaquinone, Hexacosanol, Octacosanol, β-amyrin and β-sitosterol [6]. Traditional Ayurvedic uses of *H. integrifolia* are disorder of the spleen, fever, jaundice, urinary disorder, laxative [3] and also used as an adjuvant for various therapies. *H. integrifolia* is scientifically studied for its anti-inflammatory activity [7], anti-diarrheal effect in castor oil induced diarrhea [8], adaptogenic effect of ethanolic extracts [9], anti-diabetic effect [10], anti-oxidant, wound healing property [11] and for anti-helminthic activity [12], The plant has also been screened for anti-bacterial activity against *Staphylococcus aureus, Basillus subtilis, Escherichia coli, Pseudomonas aeurginosa* and found effective [13]. *H. integrifolia* parts are being frequently prescribed by the traditional Ayurvedic practitioners for various ailments as a single plant or in combination with other plant drugs. To the best of our knowledge, there are no reports on oral administration of *H. integrifolia* extracts for its sub-acute exposure. Therefore, there is a need to develop safety profile of *H. integrifolia* for its oral administration in sub-acute exposure based on hematological, biochemical and vital organ parameters. This study planned to explore the lethal dose range and effect on sub-acute administration at hematology, biochemistry and vital organs to develop safety profile, which will guide for the better understanding of the least toxic dose and duration for administration.

MATERIALS AND METHODS

Experimental animals

The study was conducted in healthy, Wistar rats of male sex weighing between 150 g and 200 g and Swiss mice weighing 20 to25 g. Animals were purchased from Shree Venkteshwara Traders, Bangalore, India. All animals were acclimatized for 7 days in the laboratory with natural light and dark cycle. Animals were fed with standard rodent pellet, tap water ad libitum. Details of the experimental procedures were reviewed and approved by Institutional Animal Ethics Committee, constituted as per CPCSEA guidelines.

Drugs and chemicals

Specific kits for serum aspartate aminotransferase, alanine transaminase, alkaline phosphatase, creatinine and urea estimation were procured from local supplier, manufactured by Erba Diagnostic Mannheim GmbH Labs, Germany. Hematoxylin and eosin Y were procured from Fisher Scientific, Hampton, NH.

Plant material

H. integrifolia (Roxb.) Planch. (RMRC-525) bark was collected from the forest areas of Gokak (Belgaum). Plant specimens were authenticated by qualified taxonomist and voucher specimen is deposited at the herbaria of Regional Medical Research Centre (ICMR), Belgaum, Karnataka, India.

Extraction and yield
The coarse powder of the shade-dried bark was subjected for successive continuous soxhlet extraction with petroleum ether, acetone and 70% ethanol. Solvents were evaporated using a rotary evaporator to prepare solvent free extracts.

Acute toxicity studies
Acute oral toxicity test of *H. integrifolia* bark extracts were conducted in Swiss mice for the limit test. The main test of up and down procedure was as per acute oral toxicity guideline 425 of Organization for Economic Co-operation and Development (OECD). Petroleum ether, acetone and hydroalcoholic extracts of *H. integrifolia* were dissolved in dimethyl sulfoxide (DMSO) and administered orally in animals. Overnight fasted with water ad libitum animals were orally administered with the dose of 2000 mg/kg and continuously observed up to 6 hrs for morbidity, mortality, other toxic signs and subsequently for 14 days. If animal showed mortality, then switched to main test to find out LD50 range. The dose for the main test was selected from default progression factor on the basis of mortality, morbidity, onset duration and severity of toxic sign. If the animal survives, then another two animals were administered with the same dose sequentially at 48 hrs intervals with the same observation as in the first animal. The high dose at which animal died and low dose at which survived was considered as LD50 range [14].

Sub-acute toxicity studies
Sub-acute toxicity studies were conducted in Wistar rats as per the OECD 407 guideline. Rats were distributed in 10 groups with 6 animals in each group. Group I received DMSO as vehicle and all the extracts were administered in the dose of 50, 100 and 200 mg/kg. Group II-IV received petroleum ether extract of *H. integrifolia* (HIPE), whereas Group V-VII received acetone extract (HIAC) and Group VIII-X received hydroalcoholic extract (HIHY). All the extracts and vehicle were administered orally every day for 28 consecutive days. Body weights of all animals were measured on day one before starting the study and on every 7 days interval till 28th day. Next day, overnight fasted with water ad libitum animals were anesthetized by halothane to collect the blood from retro-orbital plexus in to the tubes, with or without ethylenediaminetetraacetic acid (EDTA) for hematological or biochemical assessment and subsequently animals were sacrificed to collect the organs [15].

Biochemical profile
Clotted blood tubes were subjected for centrifugation at 5000 revolutions per minute for 10 minutes to separate the serum. Sera were used to estimate various enzymes viz. serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), alkaline phosphatase (ALP), creatinine, urea and protein as per the manufacturer instructions (Erba Mannheim Germany).

Hematological profile
EDTA mixed blood samples were used for red blood cells count, hemoglobin (Hb), mean corpuscular Hb (MCH), MCH concentration (MCHC), mean corpuscular volume, total white blood cell count, differential white blood cell count (neutrophils, lymphocytes, monocytes, basophils and eosinophils) and platelet count. Hematological parameters were estimated using fully automated hematological analyzer (Accurex, complete blood count 360).

Relative body weight
After blood collection, animals were sacrificed to collect the vital organs like liver, kidney, spleen, heart and lungs. The weights of the organs were measured and relative organ weight was calculated.

$$\text{Relative organ body weigh ratio} = \frac{\text{Weight of organ}}{\text{Weight of animal}} \times 100$$

Histopathology assessment
Five millimeter thick sections of liver and kidney were prepared using paraffin wax embedded tissue. They were stained with hematoxylin and eosin after processing and mounted in DPX mounting medium.

Statistical analysis
Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test and P≤0.05 was considered as significant. Results were expressed as mean ± standard deviation.

RESULTS

Plant extract yield
The percentage yield of extracts were 0.89%, 2.26%, 7.41% in petroleum ether, acetone and hydro-alcoholic solvents, respectively.

Acute toxicity
All three extracts found toxic at limit dose 2000 mg/kg and caused mortality. In the main test, hydroalcoholic, acetone and petroleum ether extracts found safe at 1000 mg/kg and toxic at 1750 mg/kg.

Sub-acute toxicity
All extracts treated groups (50, 100 and 200 mg/kg dose at every 24 hrs interval oral administration for a period of 28 days) did not produce any signs of toxicity, mortality and morbidity.

Relative organ weight ratio
Relative organ weight ratio of kidneys, spleen and lungs did not alter significantly in any extract treated groups. However, relative weight ratio of heart were found significantly (P<0.01) changed in *H. Integrifolia* acetone and petroleum ether extracts treated group at 200 mg/kg. Whereas, liver weight ratio was found significantly (P<0.01) altered in 200 mg/kg of acetone extract, 100 and 200 mg/kg of petroleum ether extract treated groups (Table 1).

Biochemical results
Biochemical profiles of serum indicated that, the elevation in urea was not significant in any treated groups. However, creatinine found significantly (P<0.01) elevated in acetone and petroleum ether extracts at 200 mg/kg. SGPT found significantly (P<0.05) elevated only in 200 mg/kg of acetone extract treated group. ALP was found increased significantly (P<0.001 and P<0.01) at 200 mg/kg of acetone and petroleum ether extracts treated groups. SGOT found significantly

Table 1: Relative body organ weight ratio of *H. integrifolia* extracts treated groups

Organ	Treatments									
	Vehicle	HIAC 50 mg/kg	HIAC 100 mg/kg	HIAC 200 mg/kg	HIHY 50 mg/kg	HIHY 100 mg/kg	HIHY 200 mg/kg	HIPE 50 mg/kg	HIPE 100 mg/kg	HIPE 200 mg/kg
Liver	2.36±0.14	2.74±0.03	2.68±0.11	3.45±0.33**	2.78±0.10	2.95±0.29	2.95±0.33	2.96±0.10	3.26±0.12**	3.38±0.09**
Kidneys	0.62±0.03	0.75±0.03	0.72±0.04	0.78±0.07	0.73±0.04	0.73±0.08	0.68±0.03	0.77±0.04	0.72±0.03	0.73±0.03
Spleen	0.37±0.01	0.45±0.01	0.43±0.04	0.46±0.02	0.43±0.01	0.39±0.05	0.45±0.01	0.42±0.01	0.43±0.02	0.46±0.05
Lungs	0.62±0.01	0.71±0.02	0.64±0.01	0.64±0.05	0.71±0.03	0.61±0.02	0.66±0.08	0.66±0.06	0.69±0.12	0.61±0.03
Heart	0.29±0.01	0.35±0.01	0.36±0.01	0.40±0.03**	0.35±0.01	0.36±0.02	0.37±0.03	0.34±0.02	0.38±0.02	0.40±0.01**

(N=6) *P≤0.05, **P≤0.01, HIAC: *H. integrifolia* acetone extract, HIHY: *H. integrifolia* hydroalcoholic extract, HIPE: *H. integrifolia* petroleum ether extract, *H. integrifolia: Holoptelea integrifolia*

Table 2: Biochemical parameters of *H. integrifolia* various extracts treated groups

	Treatments									
	Vehicle	**HIAC 50 mg/kg**	**HIAC 100 mg/kg**	**HIAC 200 mg/kg**	**HIHY 50 mg/kg**	**HIHY 100 mg/kg**	**HIHY 200 mg/kg**	**HIPE 50 mg/kg**	**HIPE 100 mg/kg**	**HIPE 200 mg/kg**
ALP	49.23±12.40	50.02±17.08	54.13±3.18	68.35±7.97**	49.82±1.84	50.53±3.37	51.54±0.95	51.21±3.96	55.78±4.31	68.50±8.15**
SGPT	69.11±12.09	73.07±5.08	76.19±3.39	92.75±3.58*	70.32±2.80	73.07±16.22	80.73±12.07	74.46±3.47	89.57±7.95	110.20±13.63**
SGOT	99.94±17.79	102.04±1.03	117.67±5.45	123.76±6.89*	99.17±7.45	111.98±7.76	126.11±12.78**	115.16±8.62	126.11±10.16**	156.76±35.93**
Urea	128.35±70.32	127.25±1.53	133.64±1.39	139.68±3.44	118.51±5.97	133.07±4.91	137.03±9.22	129.96±2.12	133.71±5.83	146.21±3.42
Creatinine	1.17±0.18	1.22±0.06	1.34±0.03	1.54±0.22**	1.14±0.09	1.21±0.03	1.27±0.08	1.22±0.06	1.39±0.02	1.62±0.14**
Bilirubin	29.88±0.71	32.62±0.22	33.35±3.94	33.96±2.55*	31.74±2.37	32.57±0.19	33.81±1.63*	31.74±4.45	32.63±0.12	34.34±1.99*

(N=6) *P≤0.05, **P≤0.01, HIAC: *H. integrifolia* acetone extract, HIHY: *H. integrifolia* hydroalcoholic extract, HIPE: *H. integrifolia* petroleum ether extract, *H. integrifolia: Holoptelea integrifolia*, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic-pyruvic transaminase, ALP: Alkaline phosphatase

Table 3: Hematological parameters of *H. integrifolia* various extracts treated groups

	Treatments									
	Vehicle	**HIAC 50 mg/kg**	**HIAC 100 mg/kg**	**HIAC 200 mg/kg**	**HIHY 50 mg/kg**	**HIHY 100 mg/kg**	**HIHY 200 mg/kg**	**HIPE 50 mg/kg**	**HIPE 100 mg/kg**	**HIPE 200 mg/kg**
RBC (10^2/l)	8.092±0.40	8.08±0.13	8.00±0.35	6.71±0.68**	8.01±0.09	7.80±0.59	6.79±0.82**	8.02±0.05	8.07±0.24	7.72±0.37
WBC (10^9/l)	16.12±10.18	13.50±43.81	13.06±51.63	12.40±16.56*	12.55±11.29*	11.70±12.99***	11.83±11.25**	14.98±67.05	14.53±30.79	13.46±10.93
Hb (g/dl)	15.51±0.86	15.18±0.20	15.43±0.16	14.06±0.60***	15.26±0.27	15.2±0.35	15.15±0.10	15.3±0.10	14.75±0.40	14.61±0.33*
Platelet (10^9/l)	693.60±972.22	809.83±146.34**	828.83±388.29**	845.16±874.30**	724.83±499.66	740.50±201.07	845.83±178.26**	755.66±399.03	824.83±134.22**	960.66±545.00**
MCH (pq)	18.46±1.42	18.91±0.61	19.00±0.53	19.36±1.59	19.13±0.39	19.4±2.33	19.93±0.31	18.26±0.53	18.58±0.24	19.2±0.96
MCHC (g/dl)	31.78±1.02	32.5±0.34	33.11±0.90*	33.3±0.86*	32.63±0.13	33.25±0.31*	33.76±1.21**	32.61±0.19	32.75±0.61	33.08±0.63
MCV (fl)	55.41±3.49	57±2.53	58.13±2.07	58.91±1.93	57.33±2.85	59.33±2.29	65.66±5.45**	57.2±2.42	58.36±1.45	61.96±5.36**

(N=6) *p≤0.05, **P≤0.01, ***P≤0.001, HIAC: *Holoptelea integrifolia* acetone extract, HIHY: *Holoptelea integrifolia* hydroalcoholic extract, HIPE: *H. integrifolia* petroleum ether extract, RBC: Red blood count, WBC: White blood count, Hb: Hemoglobin, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, *H. integrifolia: Holoptelea integrifolia*

(P<0.05 and P<0.01) elevated in acetone and hydroalcoholic extracts at 200 mg/kg; petroleum ether extract (P<0.01) at 100 and 200 mg/kg treated groups (Table 2).

Hematological results

All three extracts of *H. integrifolia* did not produce any alteration in neutrophils, lymphocytes, eosinophils, basophils, monocytes and MCH, and hence not incorporated in the results table. Significant (P<0.001) decrease in hemoglobin was observed in 200 mg/kg acetone extract treated group and mean corpuscular volume was found significantly (P<0.05) elevated in 200 mg/kg of hydroalcoholic and petroleum ether extracts treated groups. However, red blood cells found decreased significantly (P<0.01) in acetone and hydroalcoholic extracts at 200 mg/kg treated groups. White blood cells found significantly (P<0.05) decreased in acetone extract 200 mg/kg and hydroalcoholic extract 50 mg/kg treated groups. Hydroalcoholic extract 100 and 200 mg/kg showed significant (P<0.001 and P<0.01) changes in white blood cell count respectively. MCHC significantly (P<0.05) elevated in 100 mg/kg of hydroalcoholic and acetone extracts and also in 200 mg/kg of acetone extract treated groups (P<0.01). Platelet count was observed significantly (p<0.01) increased in all doses of acetone extract, 200 mg/kg of

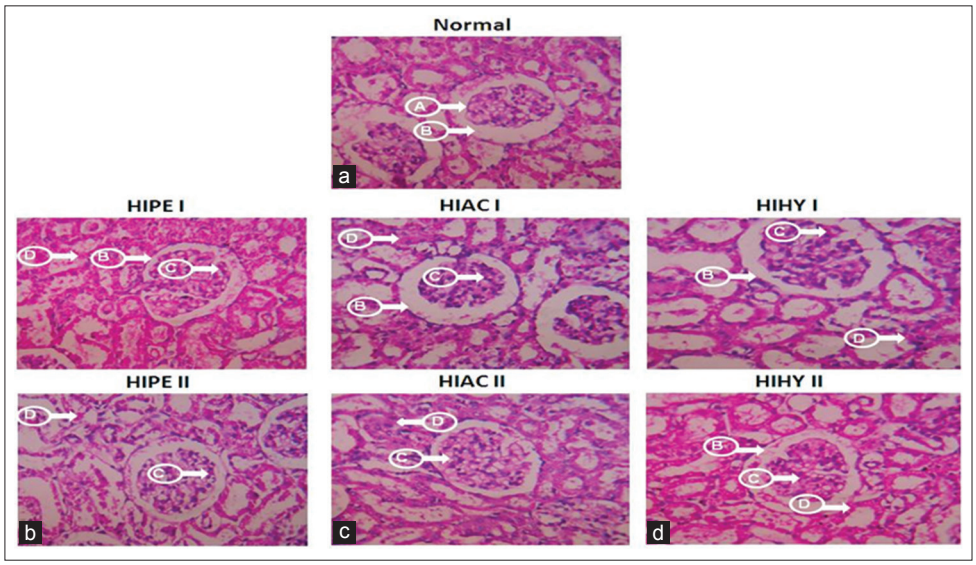

Fig. 1: Histopathology images of the kidney of various treated groups, A: Glomerular, B: Bowmen space, C: Glomerular congestion, D: Tubular congestion, HIPE I: *Holoptelea integrifolia* petroleum ether extract 100 mg/kg, HIPE II: *Holoptelea integrifolia* petroleum ether extract 200 mg/kg, HIHY I: *Holoptelea integrifolia* hydroalcoholic extract, 100 mg/kg, HIHY II: *Holoptelea integrifolia* hydroalcoholic extract 200 mg/kg, HIAC I: *Holoptelea integrifolia* acetone extract 100 mg/kg, HIAC II: *Holoptelea integrifolia* acetone extract 200 mg/kg

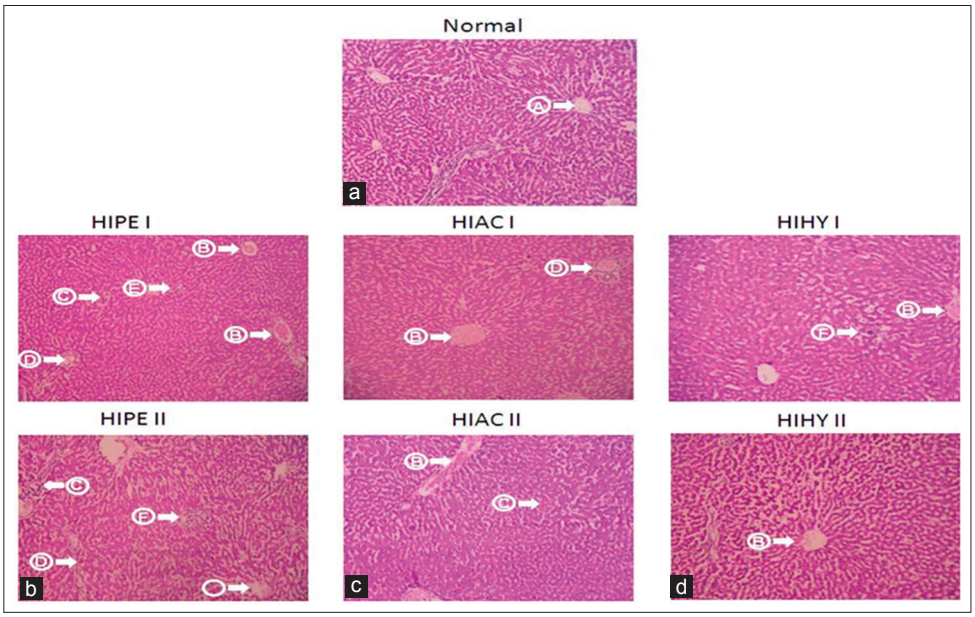

Fig. 2: Histopathology images of the liver of various treated groups, A: Central vein, B: Central vein congestion, C: Inflammatory cells, D: Portal triaditis, E: Sinus congestion, F: Spotty necrosis, HIPE I: *Holoptelea integrifolia* petroleum ether extract 100 mg/kg, HIPE II: *Holoptelea integrifolia* petroleum ether extract 200 mg/kg, HIHY I: *Holoptelea integrifolia* hydroalcoholic extract 100 mg/kg, HIHY II: *Holoptelea integrifolia* hydroalcoholic extract 200 mg/kg, HIAC I: *Holoptelea integrifolia* acetone extract 100 mg/kg, HIAC II: *Holoptelea integrifolia* acetone extract 200 mg/kg

hydroalcoholic extract and 100, 200 mg/kg of petroleum ether extract treated groups (Table 3).

Histopathology

Histopathology image of the liver confirmed the mild damage in petroleum ether and acetone extract treated groups. Mild tubular congestion and glomerular congestion were observed in kidneys of acetone extract treated group (Fig. 1). Inflammation, central vein congestion, portal triaditis and spotty necrosis were observed in liver of acetone and petroleum ether extract treated groups (Fig. 2).

DISCUSSION

Toxicity evaluation is also the major concern like efficacy of any plant based drug, because all plants on the earth are not safe for consumption. Most of the preparations of Ayurveda, Siddha and traditional practitioners are based on herbs, which they use to cure various diseases. *H. integrifolia* is one such plant, which is routinely used for various ailments like bronchitis, obesity, inflammation, gastritis, dyspepsia, vomiting, piles, wound healing, leprosy, diabetes, diarrhea, rheumatism and skin disease. World Health Organization suggested that, the plants used for the treatments of disease are needed to be scientifically investigated for their safety [1]. Therefore, this study was planned to probe into the safety profile of *H. integrifolia* extracts on acute and sub-chronic administration. The results of the acute toxicity study indicated that *H. integrifolia* showed mortality at 2000 mg/kg in all extracts treated groups and found safe at 1000 mg/kg. All three extracts of *H. integrifolia* neither show significant change in body weight nor any signs of toxicity at 50, 100 and 200 mg/kg over a period of twenty eight days of treatment. Results of the biochemical studies reflect mild liver and kidney toxicity, whereas hematology profile indicated the platelet increase which might lead to coagulopathy. Based on the resulted data, it can be concluded that hydroalcoholic extract of *H. integrifolia* is safer, compared to acetone and petroleum ether extracts on daily dose for 28 consecutive days.

ACKNOWLEDGEMENTS

First author (Dushyant Kumar) is thankful to Indian Council of Medical Research (ICMR) for providing Senior Research Fellowship to carry out this work. Authors are also thankful to Dr. Anand Ojha, Dr. Ashutosh Shinde, Mr. Jalalappa and Mr. Mohit Adhikari for their help and support.

REFERENCES

1. WHO (World Health Organization), 2008. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems. Geneva.
2. Rajbhandari M, Wegner U, Julich M, Schopke T, Mentel R. Screening of Nepalese medicinal plants for antiviral activity. J Ethnopharmacol 2001; 74: 251-255.
3. Kumar D, Kumar K, Gupta J, Bishnoi N, Kumar S. A mini review on chemistry and biology of *Holoptelea integrifolia* Roxb. Planch (Ulmaceae). Asian Pac J Trop Biomed 2012; 1200-1205.
4. Anonymous, Quality standards of Indian medicinal plants, Indian Council of Medical Research, New Delhi. 2004; 5: 246-255.
5. Sharma S, Lakshmi KS, Patidar A, Chaudhary A, Dhaker S. Studies on anti-inflammatory effect of aqueous extract of leaves of *Holoptelea integrifolea* Planch. in rats. Ind J Pharmacol 2009; 41(2): 87-88.
6. Sandhar HK, Kaur M, Tiwari P, Salhan M, Sharma P, Prasher S, Kumar B. Chemistry and Medicinal properties of *Holoptelea integrifolia*. Int J Drug Dev Res 2011; 3(1): 06-11.
7. Kalpana, Upadhyay A. Anti -inflammatory evaluation of ethanolic extract of leaves of *Holoptelea integrifolia*, Planch. Annal Biol Res 2010; 1(2): 185-195.
8. Sharma S, Lakshmi KS, Rajesh T. Evaluation of antidiarrhoeal potentials of ethanolic extract of leaves of *Holoptelea integrifolia* in mice model. Int J Pharm Tech Res 2009; 1(3): 832-836.
9. Kumar B, Puri S, Debnath J, Tiwari P, Salhan M, Kaur M, Mittal A. Comparative pharmacological evaluation of adaptogenic activity of *Holoptelea integrifolia* and Withania somnifera. Int J Drug Dev Res 2011, 3(1): 84-98.
10. Sharma S, Khatri P, Pandey A, Jakhetia V, Chaturvedi L, Dwivedi N. Anti-diabetic screening of leaves extract of *Holoptelea integrifolia* Roxb. Int J Pharma Res Dev 2010; 2(10): 66-71.
11. Reddy BS, Reddy RK, Naidu VGM, Madhusudan K, Agwane SB, Sistla R. Evaluation of antimicrobial, antioxidant and wound healing potentials of *Holoptelea integrifolia*. J Ethnopharmacol 2008; 115: 249-256.
12. Durga N, Paarakh PM. Evaluation of anti-helminthic activity of stem bark of *Holoptelea integrifolia* (Roxb.) Planch. Int J Res Ayur Pharm 2010; 1 (2); 637-641.
13. Paarakh PM and Durga N. Antibacterial activity of different extracts of stem bark of *Holoptelea integrifolia*. Int Res J Pharm 2011; 2(1): 111-113.
14. OECD (2008), Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.
15. OECD (2008), Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.