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Toxicity Studies of *Trema orientalis* in Rats with Respect to Certain Haematological and Biochemical Parameters

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

An experimental study was carried out to assess the toxicological aspects of methanolic extract of leaves and stems of *Trema orientalis* (METO) in rats. Repeated dose 28-day oral toxicity study of METO was performed in male Wistar albino rats as per the OECD guidelines 407. In total, 24 rats were assigned in four groups of six in each group. Group I served as control, while Group II, III and IV rats were orally gavaged with METO daily at dose levels of 250, 500 and 1000 mg/kg respectively for 28 days. The experimental rats were apparently healthy throughout the experimental period with respect to feed intake and body weight gain. Haematological parameters such as total erythrocytes count (TEC), total leukocyte count (TLC), haemoglobin (Hb) and packed cell volume (PCV) were assessed from blood on day 14 and day 28 in the repeated dose oral toxicity study. Also, certain biochemical parameters like AST, ALT, ALP, BUN and creatinine from serum were estimated. TLC was reduced significantly ($p < 0.05$) in group IV of METO treated rats. The significant ($p < 0.05$) increase in creatinine values of group IV on 28th day, associated with occurrence of various phytochemicals. Other haematological and biochemical parameters showed no significant changes in METO treated groups. The study concluded that the doses of *T. orientalis* resulted in pulmonary, hepatic and renal damage in experimental rats and might be toxic to the domestic animals also, which need to be investigated in future.

Keywords: T. orientalis; toxicity study; Wistar albino rats; haematological and biochemical.

1. INTRODUCTION

"Trema orientalis (L) Blume is a small to medium sized tree belonging to Cannabaceae family. The toxic principle of *T. orientalis* is trematoxin, a glycoside which contains steroidal saponins. Additional constituents in the plant include tannins, flavonoids, phytosterols, triterpenes and various xanthone components, which are responsible for the pathophysiologic qualities of the plant" [1]. Its medicinal properties have been utilized to treat respiratory, inflammatory and helminthic diseases [2]. The separation and identification of biologically active substances and molecules from the medicinal plants have contributed in discovery of new medicinal products and also identification of toxic principles [3,4-6]. "Suspected toxicity conditions in goats after ingestion of *T. orientalis* leaves have been noticed by the veterinarians across Karnataka state particularly in and around Shivamogga and Dharwad district. As per the information received regarding the incidences of the plant toxicity, the ailing goats had exhibited abnormal clinical signs such as incoordination, apathy, tenesmus, paddling movements and coma before death on fifth day of ingestion of the plant. Thus, in addition to the numerous traditional claims of the medicinal properties of the aerial parts of the plant and the incidences of suspected toxicity in goats necessitated the studies on the toxicological aspects and pharmacological properties of *T. orientalis* in animals" [7]. The main objective of the study is to conduct the safety evaluation of the extract of *T. orientalis* in rats with reference to haematology and certain biochemical parameters.

2. MATERIALS AND METHODS

This experimentation was performed on rats in small animal facility of Veterinary College Shivamogga. The fresh aerial parts of *T. orientalis* plant were gathered from different regions of Soraba taluk, Shivamogga district, Karnataka in the months of April and May 2022. Dr. Rajeshwari, N. Professor, Department of Botany and Seed Technology, Sahyadri Science College, Shivamogga, verified the plant's taxonomic identification. The plant material was further processed to obtain methanolic extract of *Trema orientalis*. The botanical characteristics of the plant is shown in Fig. 1.

An electronic scale was used to weigh 1kg of fine powder, which was then soaked in 5 litres of methanol (99% SDFCL) at 1:5 ratio in glass containers which were kept closed at room temperature [8].

After one week, the contents were filtered and concentrated in vacuum using a Rotary Evaporator. Then concentrated and dried completely in the incubator at 40 °C for one day. Finally obtained product is methanolic extract of aerial parts of *Trema orientalis* (METO).

1.a. Whole plant

1.b. Branches

1.c. Flowers

1.d. Fruit

1.e. Seed

1.f. Leaves

Fig. 1. Identification and authentication of *Trema orientalis* plant

For haematological and biochemical analysis of blood and serum test kits containing reagent 1 (R_1) and reagent 2 (R_2) (Alpha Technologies) for each parameter such as aspartate amino transferase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine were commercially procured for the biochemical examination of the serum. The test kit containing R_1 and R_2 for the parameter alkaline phosphatase (ALP) was commercially purchased from Erba Diagnostics FZ, Dubai for the serum biochemical analysis.

2.1 Toxicity Studies of Methanolic Extract of Aerial Parts of *T. orientalis*

"The repeated dose sub-acute toxicity study was conducted for 28 days as per broader outlines of OECD 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents)" [9].

2.1.1 Experimental animals

"Four to five weeks old Wistar albino male rats weighing around 150±10 g were procured from Adita biosys private limited, Tumakuru (Reg No: -1868/PO/RcBt/S/16/CPCSEA. All the experimental rats were housed in polypropylene cages and acclimatized to laboratory conditions of 12 h light/dark cycle, 22 ± 3^0C housing temperature, relative humidity of 50-60 % and ventilation of 12-15 air cycles per hour for one week before the experiment. The rats were provided with standard pellet feed and *ad libitum* water throughout the experiment" [7]. The guidelines prescribed by CCSEA were followed during the course of experimental study, with duly approval from the Institutional Animal Ethics Committee of Veterinary College, Shivamogga. Prior to the commencement of the experimental study, the experimental rats were acclimatized to the laboratory environment for seven days.

2.1.2 Selection and preparation of doses

Three doses *viz:* 250, 500 and 1000 mg/kg were chosen as low, medium and high doses, respectively in accordance with OECD 407 [9] based on earlier pharmacological research on methanolic extract of aerial parts of *T. orientalis* (METO) plant leaves conducted by Hemalatha et al. [10,11,12]. Previous studies revealed that the METO had little toxic effects on liver and kidneys. The LD_{50} was above 2000 mg/kg**.** The rats were administered with the METO for a period of 28 days by oral gavaging technique as a single dose separately for each rat every day at the scheduled time in early morning, before providing feed and water. The experimental design for repeated dose 28-day oral toxicity study of METO in rats is presented in (Table 1).

2.1.3 Observation of animals

Throughout the 28-day trial period, the general clinical observations were conducted once a day while considering the time frame for the possible effects on health following administration of METO. The rats were observed twice a day, once in the morning and again in the evening, for overall health conditions, mortality and morbidity. Changes in skin, fur, eyes, mucous membranes, secretions, excretions and autonomic activity were noted. Changes in gait, posture and responsiveness to handling, as well as the existence of clonic or tonic movements and stereotyped behaviours were also documented. The clinical indications like hypoactivity, ataxia, paraphimosis, emaciation, haematuria, hypothermia, dyspnea and cyanosis were thought to be connected to overall morbidity.

Table 1. Experimental design for repeated dose 28-day oral toxicity study of METO in rats

Sl. No	Group	No. of rats	Treatment	Dosing
1	Group I	6	Control	Administered (*per os*) with single dose of normal saline (1.5 ml)
2	Group II	6	Low dose	Administered (*per os*) daily with single low dose (250 mg/kg) of *T. orientalis* extract.
3	Group III	6	Medium dose	Administered (*per os*) daily with single medium dose (500 mg/kg) of *T. orientalis extract*.
4	Group IV	6	High dose	Administered (*per os*) daily with single high dose (1000 mg/kg) of *T. orientalis* extract.

2.1.4 Haematology

Blood was subjected to assessment of haematological parameters, viz. TEC, TLC, Hb and PCV by using automatic veterinary haematology analyser (Exigo®, Sweden), on day 14 and day 28 in the repeated dose oral toxicity study. On day 14, blood was collected (less than 1% of b.w) through retro-orbital plexus in EDTA coated vacutainers, under mild sedation with ketamine and xylazine (75 mg+10 mg/kg i.p) through micro haematocrit capillary tubes. On day 28, the terminal blood sample was also collected similarly.

2.1.5 Serum biochemistry

The biochemical parameters *viz:* Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Blood urea nitrogen (BUN) and Creatinine (CRT) were estimated on day 14 and 28 by the biochemical analyser (HY-SAC Vet Version: A/6 Semi-auto Chemistry Analyser, Hycel® Handelsges Austria). The serum biochemistry profile was determined under standard laboratory procedure. On day 14, blood was collected (less than 1% of b.w) through retro-orbital plexus under mild sedation with ketamine and xylazine (75 mg+10 mg/kg i.p) through micro haematocrit capillary tubes in vacutainers. Similarly, the terminal blood sample was also collected on day 28. The values of AST, ALT and ALP were estimated as per the IFCC method guidelines. The parameters of BUN and creatinine were estimated based on the GLDH and Jaffe's methods, respectively. Data of both the haematological and biochemical parameters were expressed as mean ± SEM. Differences were considered significant at *p <0.05 when compared test groups v/s control group by One-way Analysis of Variance (ANOVA) with Dunnet's multiple comparisons test using Graph Pad Prism 7.2.0 (435) software.

3. RESULTS AND DISCUSSION

The general observations of experimental rats are depicted in (Table 2).

The objective of the current study was to assess the toxicological characteristics of the methanolic extract of aerial parts of *T. orientalis* plant with respect to haematological and biochemical parameters. "Wistar albino rats were used for safety evaluation of METO by repeated dose 28-day oral toxicity study" [7]. Repeated dose 28-day oral toxicity study of methanolic extract of *T. orientalis* aerial parts was performed in male Wistar albino rats following broader outlines of OECD 407 [9]. "The experimental rats were divided into four groups (n=6 per group), group I served as control, group II, II and IV rats were administered with METO at doses of 250, 500 and 1000 mg/kg respectively for 28 days" [7]. The experimental rats were apparently healthy throughout the experiment period without the observation of any abnormal clinical signs or notable changes with respect to bodyweights and feed intake. The maximum tolerable dose of METO in rats was investigated to be more than 2000 mg/kg.

Table 2. General observations of experimental groups (n=6) of rats in repeated dose 28-day oral toxicity study of METO

Group	Group I	Group II	Group III	Group IV
Treatment and dose	**Normal saline**	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
No. of rats	6	6	6	6
Feeding	N*	N*	N*	R**
Fur condition	NAD	NAD	NAD	NAD
Mucous membrane	NAD	NAD	NAD	NAD
Eye colour	NAD	NAD	NAD	NAD
Activity	NAD	NAD	NAD	NAD
Convulsion	NOB	NOB	NOB	NOB
Locomotion	NAD	NAD	NAD	NAD
CNS depression	NOB	NOB	NOB	NOB
Faecal consistency	Pellet	Pellet	Pellet	Pellet
Mortality observed	0	0	0	0
Mortality (%)	0	0	0	0

**N: Normal; **R: Reduced; NOB: Not Observed; NAD: No Abnormality Detected*

By using the particular solvents, the extraction procedures could separate the medicinally useful components of plant species from the inert components. The type of solvent employed in the extraction method is a key factor in the successful evaluation of biologically active chemicals from plant parts [13]. Methanol is used as the solvent to obtain biologically active compounds like anthocyanins, flavones, lactones, phenones, polyphenols, saponins, terpenoids, tannins and xanthoxyllines [14]. The individual qualities of the bioactive components have been evaluated to determine the solvent system for the extraction process. It is possible to extract the bioactive ingredient from natural compounds using a variety of solvent systems [15].

There was increase in the haematological parameters viz: TEC, Hb, PCV and serum biochemical parameters (AST, ALT, BUN, ALP and creatinine) in all the experimental groups on day 28. TLC was reduced significantly ($p < 0.05$) in group IV of METO treated rats. The significant ($p < 0.05$) increase in creatinine values from that of control group in the present toxicity study on 28th day, could be associated with the occurrence of various phytochemicals (saponins, tannins, flavonoids, phenolic compounds, glycosides, phytosterols and triterpenoids) in the extract. Even, there was higher creatinine and ALT values noted in group IV, suggesting the possible damages to kidney and liver. The findings of TLC, creatinine, PCV and ALT values could be correlated with the studies of Hemalatha et al. [10,11]. The results of haematological parameters are shown in (Table 3) and Figs. 2 to 5.

On day 14, the haematological parameters, in each experimental group were within the normal range. The findings of the current study suggested that METO administration up to 14 days did not significantly affect the haematological parameters when administered in lower doses, inferring relative safety of METO for the hematopoietic system. Evaluation of the haematological parameters in the current study showed that, with the exception of a decrease in TLC and increase in PCV in group IV on day 28 of the repeated dose oral toxicity study the (Mean ± S.E.M) values of Hb, TEC, neutrophils, lymphocytes, monocytes and eosinophils were within normal range and no significant variation was observed.

On day 14, biochemical parameters such as AST, ALT, ALP, BUN and creatinine of all the test group rats were within the normal range. On day 28, there was marginal increase in the values of AST, ALT and BUN in the groups III and IV. Similarly, AST and ALT values where higher in a group III and IV on day 28 as compared to the other two study groups. There was higher creatinine level and significant difference in group IV with respect to other groups. The extent of increase in ALP was higher in group IV compared to the other two test groups. The extent of increase in BUN was higher in group IV and group III compared to group II. It was noticed that the renal damage was less severe than hepatic damage on day 28. The results of biochemical parameters are shown in (Table 4) and Fig. 6 to 9.

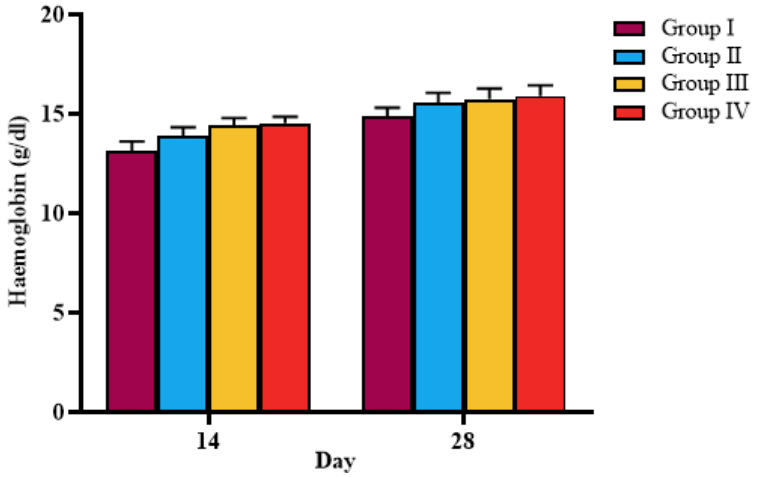

Fig. 2. Haemoglobin (g/dl) Mean ± S.E.M values on day 14 and 28

Table 3. Haematolgical parameters in experimental groups (n=6) on day 14 and 28 of the study (Mean ± S.E.M values)

Haematological parameters	Days	Groups			
		I	II	III	IV
Hb (g/dl)	14	13.13 ± 0.48	13.87 ± 0.45	14.41 ± 0.38	14.53 ± 0.33
	28	14.87 ± 0.44	15.53 ± 0.53	15.74 ± 0.54	15.91 ± 0.53
TEC (10^6/µL)	14	6.94 ± 0.33	7.63 ± 0.27	7.44 ± 0.39	7.35 ± 0.40
	28	7.52 ± 0.22	8.27 ± 0.30	8.11 ± 0.37	8.01 ± 0.51
TLC (10^3/µL)	14	8.53 ± 0.39	5.11 ± 0.21	4.92 ± 0.23	4.86 ± 0.23*
	28	7.83 ± 0.29	4.89 ± 0.16	4.76 ± 0.36	4.63 ± 0.30*
PCV (%)	14	45.75 ± 0.96	52.25 ± 1.12	53.12 ± 1.31	52.75 ± 1.27*
	28	48.25 ± 1.04	54.35 ± 1.42	54.85 ± 1.58	54.40 ± 1.39*

Note: *Data were analysed by one-way ANOVA followed by Dunnett's multiple comparisons test and compared with control group. *p < 0.05*

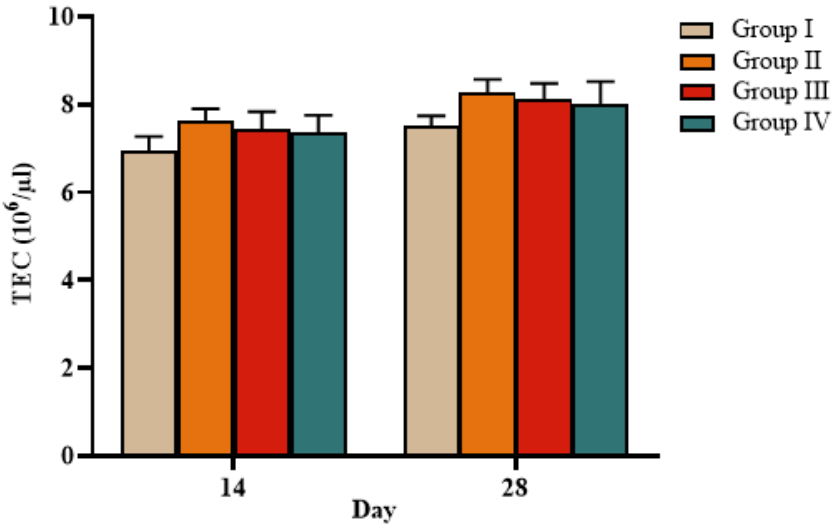

Fig. 3. Total Erythrocyte Count (10^6/µL) Mean ± S.E.M values on day 14 and 28

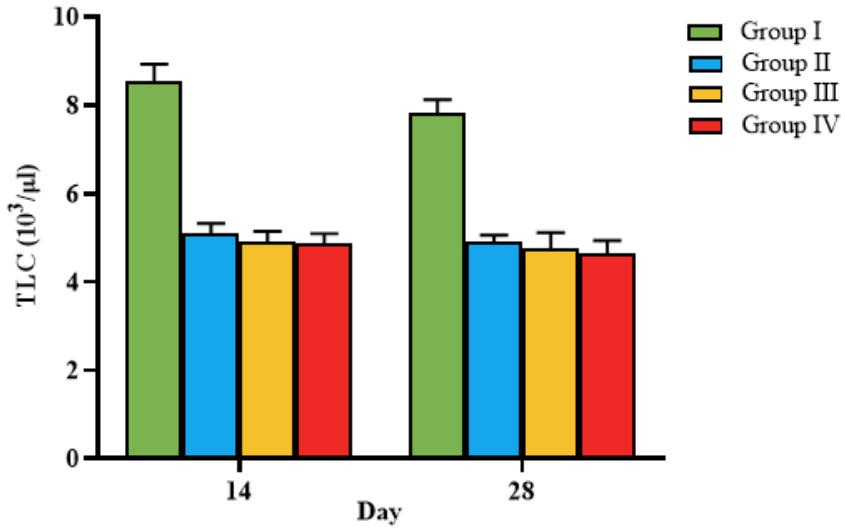

Fig. 4. Total Leukocyte Count (10^3/µL) Mean ± S.E.M values on day 14 and 28

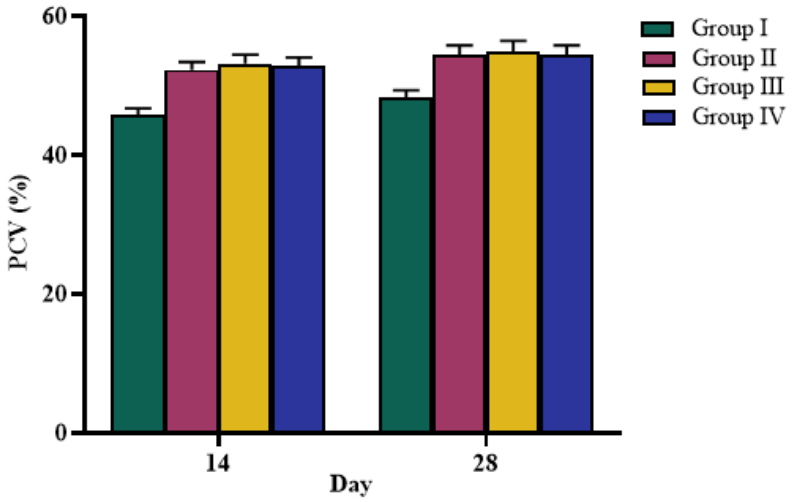

Fig. 5. Packed Cell Volume (%) Mean ± S.E.M values on day 14 and 28

Table 4. Biochemical parameters in experimental groups (n=6) on day 14 and 28 of the study (Mean ± S.E.M values)

Biochemical parameter	Day	Group			
		I	II	III	IV
AST (IU/L)	14	179.23 ± 8.64	193.34 ± 11.89	218.06 ± 9.46	229.86 ± 10.33
	28	272.54 ± 7.63	288.84 ± 8.91	302.65 ± 9.66	312.72 ± 7.89
ALT (IU/L)	14	36.75 ± 2.51	43.26 ± 2.93	50.37 ± 1.63	56.25 ± 2.44
	28	43.16 ± 1.91	54.63 ± 3.30	62.77 ± 2.19	72.38 ± 1.30
ALP (IU/L)	14	175.26 ± 8.83	191.33 ± 15.12	198.16 ± 17.85	208.77 ± 11.02
	28	218.63 ± 9.04	227.37 ± 8.07	241.18 ± 17.80	252.33 ± 9.04
BUN (mg/dl)	14	12.65 ± 1.18	14.35 ± 0.58	16.15 ± 0.48	18.13 ± 0.64
	28	15.18 ± 0.66	17.96 ± 0.31	19.71 ± 0.53	21.68 ± 0.82
Creatinine (mg/dl)	14	0.52 ± 0.03	0.67 ± 0.04	0.85 ± 0.07	1.13 ± 0.09*
	28	0.69 ± 0.04	0.89 ± 0.09	1.05 ± 0.14	1.28 ± 0.15*

Note: *Data were analysed by one-way ANOVA followed by Dunnett's multiple comparisons test and compared with control group. *p < 0.05*

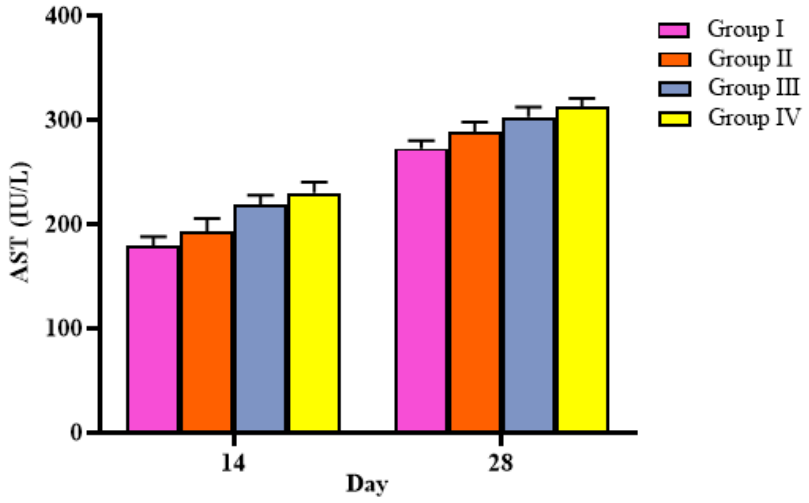

Fig. 6. Aspartate transaminase (IU/L) Mean ± S.E.M values on day 14 and 28

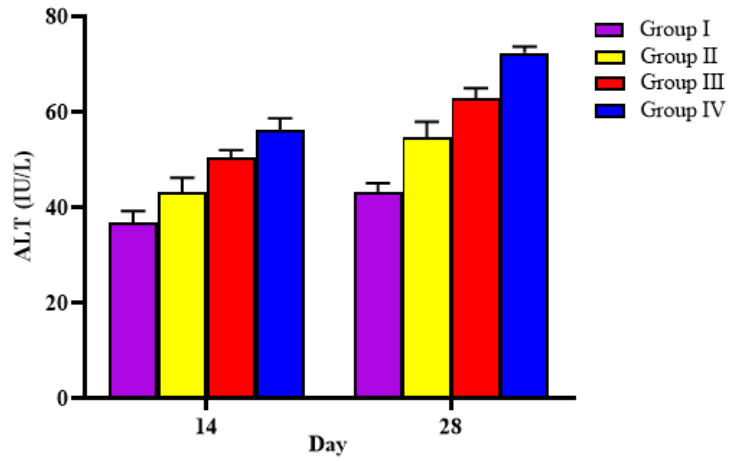

Fig. 7. Alanine transaminase (IU/L) Mean ± S.E.M values on day 14 and 28

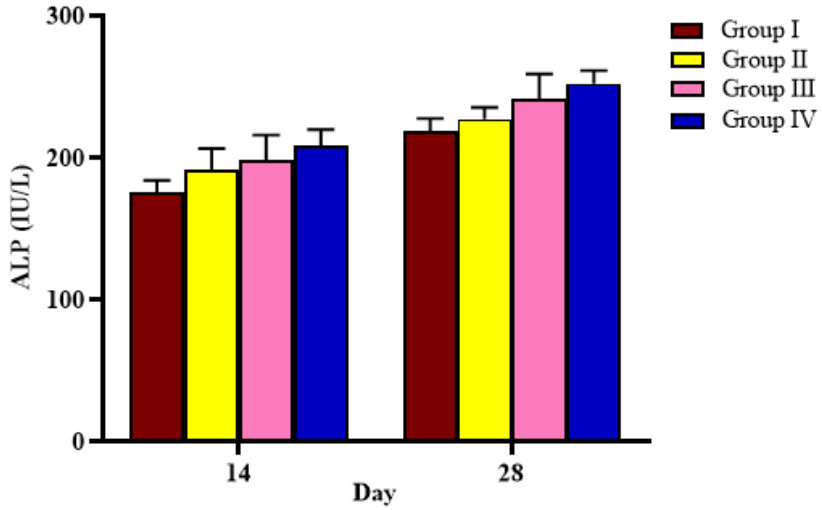

Fig. 8. Alkaline phosphatase (IU/L) Mean ± S.E.M values on day 14 and 28

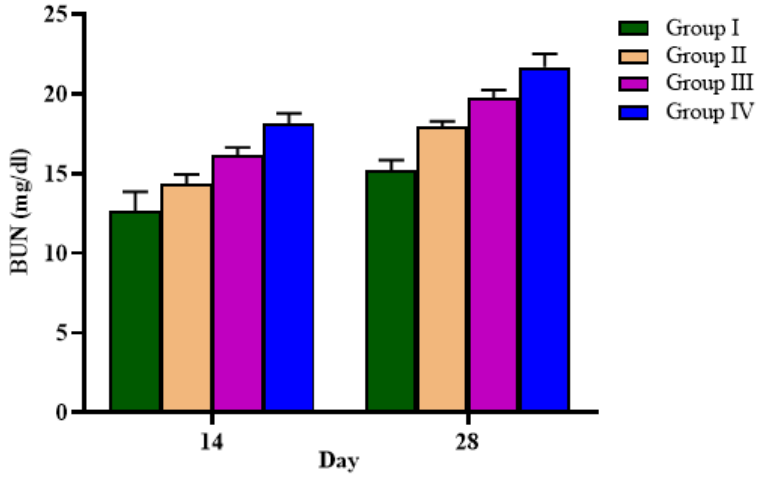

Fig. 9. Blood urea nitrogen (mg/dl) Mean ± S.E.M values on day 14 and 28

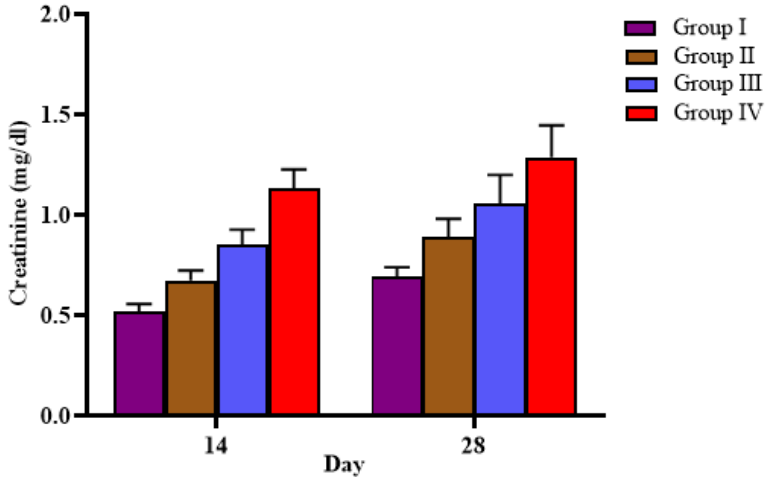

Fig. 10. Creatinine (mg/dl) Mean ± S.E.M values on day 14 and 28

The findings of the present study are in accordance with [5 & 10], which showed *T. orientalis* would result in significant hepatic damage on chronic administration. The values of creatinine were significantly ($p < 0.05$) altered in group III and IV of the study up to 28 days indicating the moderate renal damage as compared to the significant hepatic damage, which could be attributed to the glycosidic toxic principle, trematoxin present in *T. orientalis* plant [12]. The hepatic damage with respect to METO in the present study was well reflected with the increased in levels of ALT and ALP enzymes on day 28 in Wistar albino rats.

4. CONCLUSION

Correlating with the phytoconstituents of *T. orientalis* and the previous studies, Trematoxin, the toxic glycoside found in the seeds could be the contributing factor that would have major role in the pulmonary, hepatic and renal damage caused by continuous administration in rats. The rise in the creatinine values in group IV rats could be associated with the occurrence of saponins, tannins, flavonoids, phenolic compounds, glycosides, phytosterols and triterpenoids in the plant extract. In animal husbandry conditions a number of variables, including concentration of phytoconstituent, ambient circumstances, management and nutrition may exacerbate toxicity in domestic animals. The present study concluded that *T. orientalis could* be significantly hepatotoxic and pneumotoxic, thus might be the reason for the suspected toxicity conditions reported in goats after ingestion of *T. orientalis* leaves under the husbandry conditions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ETHICAL APPROVAL

The experimental protocol was started following the approval of Institutional Animal Ethics Committee of Veterinary College Shivamogga as per CCSEA guidelines vide: No. VCS/IAEC/SA-71/2022-23 and dated: 06.08.2022.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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