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Chronic toxicity studies of *Tuttha bhasma* ($CuSO_4.5H_2O$) in Wistar Albino Rats

Research Article

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Abstract

Tuttha Bhasma (TB) is an important metal based drug used in Ayurveda. It has widespread therapeutic uses in the treatment of metabolic disorders. Despite of its therapeutic utility there is a lack of data related to its safety. Hence the present study was undertaken to evaluate chronic toxicity of TB in Wistar albino rats. Chronic toxicity study was conducted as per AYUSH guidelines 170 and the parameters such as ponderal changes; biochemical, hematological and histopathological examinations were recorded. Based on the overall toxicity profile significant changes in the serum SGPT was noted in therapeutic and higher dose levels. We could see the serum cholesterol, SGOT, SGPT and albumin levels were significantly elevated at lower dose compared to the control group. Histopathology of kidney showed cell infiltration and sections from liver showed mild to moderate fatty changes at higher dose levels. Histopathology of jejunum revealed there was mild to moderate epithelial erosion and shortening of epithelial layer. However overall toxicity study parameters showed the test drug TB is well tolerated and no dose dependent toxicity symptoms were observed. Based on the findings we could conclude that *Tuttha bhasma* is much safer to use at therapeutic dose level. However, at higher dose levels there are chances of organ toxicity.

Key Words: Sub-acute toxicity, *Tuttha bhasma*, Atherosclerosis, Histopathology, Cell infiltration, Anemia.

Introduction

Indian system of medicine comprises of wide range of therapeutics, derived from minerals, metal and herbal based drugs. The herbo-mineral drugs being considered as most potent drugs with long shelf life. However, in the recent years the safety issue being one of the most concerned over human uses (1). Numbers of references are available extolling the therapeutic efficacy of herbo-mineral drugs used in both the present and earlier day literature stretching for a period of around thousand years. These preparations have received utmost attention of the leading luminaries of Ayurveda though out the ages.

Tuttha bhasma (TB) is a drug classified under '*MaharasaVarga'* in the texts of Ayurveda (2). Chemically it is known as copper sulphate with chemical formula CuSO4.5H2O. It is commonly known as blue stone or blue vitriol. It is reported that TB has potent antimicrobial activity (3). It has been reported that the repeated administration of TB could induce vascular endothelial growth factor (VEGF) expression in the wound (4). The classical references acclaim its efficacy in the various ailments such as obesity, atherosclerosis, cardiovascular diseases, chronic skin diseases, eye disorders, pain, asthma and hemorrhoids (5). *Tuttha bhasma* is mentioned as pungent (*Katu*), alkali (*Kshara*), astringent (*Kashaya*), light to digest (*Laghu*), emetic (*Vamaka*), scraping (*Lekhana*), piercing (*Bhedana*) and hot (*Usna*) in potency, thus it has potentials to treat many diseases (5).

Many literatures exists about the methods of preparing metal or mineral based drugs and tests to be carried out to determine the quality of the obtained product. Considering that they are in use since millennium and reports about their toxic effects being few it can be assumed that the earlier *Acharyas* had mastered the technique of detoxifying these potentially dangerous substances (6). Besides its widespread therapeutic utility we need to establish the scientific data pertaining to its toxicity profile and hence the study was carried out to evaluate its chronic toxicity profile in Wistar albino rats.

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Methodology

Preparation of *Tuttha bhasma*

Tuttha bhasma was prepared according to the classical reference at SDM Pharmacy, Udupi. All the ingredients for preparing *Tuttha bhasma* were authenticated and prepared according to *Rasaratna Samuchchaya* following Ayurveda formulary of India at SDM Ayurveda Pharmacy, Udupi (7). The processing of *Tuttha bhasma* (Copper sulphate) involved S*hodhana* (Purification) and *Marana* (Incineration with specific herbs). As part of *Shodhana* (Purification) *Tuttha* was

ground well with the juice of *Nimbu* (*Citrus medica*) in a stone edge runner in the prescribed format. As part of *Marana* process (Incineration procedure) it is added with S*huddha Gandhaka* (Purified Sulphur) and *Suhuddha Tankana* (Purified Borax), ground with *Nimbu swarasa* (*Citrus medica*) and subjected to *Laghu Puta* (following specific temperature pattern). Then, it is collected; finely finished product was obtained, which was used for the pharmacological investigations.

Animal dose fixation

According to the classical texts the human dose of *Tuttha bhasma* was 125mg per day (7). The rat dose was calculated according to Paget and Barnes table 1964 by extrapolating human therapeutic dose in rat dose (11.25 mg/kg) (8).

Experimental animal

The chronic toxicity was conducted in Wistar albino rats of both sexes weighing 120g ± 20 body weight. The animals were maintained at standard lab conditions at animal house. Animals were housed in polypropylene cage having a dimension of 525 x 330 x 230 mm (5 rats each) with paddy husk bedding material. The experimentation was carried out according to CPSCA guidelines after obtaining the approval of IAEC (No: SDMCAU/ IAEC/ PH-02/ 2016-17).

Experimental design

The chronic toxicity study was carried out according to AYUSH guidelines 170, for testing Ayurveda, Unani, Siddha and other traditional medicine of India (9, 10). Rats were grouped into 4 different categories each with 10 male and 10 female. Group I rats were considered as control group and rats were orally administered with the 0.5% carboxyl methyl cellulose solution. Group II, III and IV rats were treated with the test drug (*Tuttha bhasma*) at different dose levels. The chronic toxicity of TB was assessed after single daily administration of TB at therapeutic dose 11.25 mg/kg (TED), 56.25 mg/kg (5 times of TED) and 5.625 mg/kg (half the TED), for a period of 90 consecutive days. TB was given as solution in 0.5% carboxyl methyl cellulose. The chronic toxicity was evaluated by assessing the body weight change at weekly time intervals and organs weight at the end of experimental period. The biochemical assessment like SGOT, SGPT, serum alkaline phosphatase activity, serum total protein, serum creatinine, blood urea and blood glucose and haematological changes such as haemoglobin, WBC, RBC, PCV, MCV, MCH, RDWCV, RDWSD, MCHC and platelet count (11, 12) were done based on standard procedure (GB Tricell 60-3 Part Differential Double chamber- Haematology analyser, Genuine Biosystem). On 90th day rats were sacrificed. The weights of the organs such as heart, brain, liver, kidney, lungs, trachea, testis, uterus, seminal vesicles, ovaries, spleen and stomach were carried out. The histopathological examinations of the important organs were carried using standard protocol (13).

Statistical analysis:

The data obtained were expressed in mean ± SEM and statistically analysed using one way ANOVA followed by Dunnet's multiple comparison post hoc tests using Graph Pad prism version 6.01 (Graph pad Software, Inc., USA) and a '*p*' < 0.05 were considered statistically significant.

Results

Effect of TB on percentage body weight change

Tuttha bhasma administred at half the therapeutic dose caused significant increase in the percentage body weight measured at 1st , 8th and 12th weeks of experimental period compared to control group rats (*p<0.05, ** p<0.01). Repeated administration of TB at therapeutic and five times of therapeutic dose caused significant increase in the percentage change in the body weight measured during 1st, 4th, 8th and 12th weeks of experimental period as compared to control rats (*p<0.05, **p<0.01) (Table 1).

Effect of TB on organs weight

Repeated administration of TB at three different dose levels showed no significant changes in the weights of brain, spleen, trachea, ventral prostrate and uterus as compared to control group. TB administred at half the therapeutic dose caused significant increase in the stomach, lungs and seminal vesicle weight in male rats, whereas ovary weight was significantly reduced in female rats as compared to control group. TB administered at therapeutic dose caused significant increase in the weights of liver, kidney, lungs and stomach in female rats, whereas in significant decrease in the lungs weight in female rats as compared to control group. TB administered at five times of therapeutic dose caused significant increase in the weights of lungs, stomach and seminal vesicle in male rats and significant decrease in the jejunum weight in female rats as compared to control group (Table-2).

Effect of TB on haematological parameters

TB administered at half the therapeutic dose significantly increased total WBC count and RDWSD, whereas levels of MCV and MCHC were significantly decrease in female rats as compared to control group. TB administered at therapeutic dose caused significant decrease in the blood MCV, MCH and RDWSD and significantly increased in MCHC levels in male rats. Female rats showed a significant increase in the MCH and significant decrease in the MCV level as compared to control group. TB administered at five times of therapeutic dose caused significant decrease in haemoglobin, PCV and MCHC level in female rats as compared to control group (Table -3).

Effect of TB on biochemical parameters

TB administered with half the therapeutic dose significantly increased serum SGPT, albumin, cholesterol and blood glucose level in male rats, whereas serum total protein, albumin and creatinine were significantly decreased in female rats compared to control group. *Tuttha bhasma* at therapeutic dose significantly

decreased serum SGOT, ALP, total protein and total bilirubin in male rats, whereas female rats showed significant decrease in the serum SGPT, ALP, creatinine and significant increase in the serum albumin level as compared to control group. TB administred at five times of therapeutic dose caused significant increase in serum SGPT and TG levels in male rats, whereas significant increase in the serum urea level was observed in female rats as compared to control group (Table-4).

Table 1: Effect of TB on percentage changes in the body weight.

Group	1st week		4th week		8th week		12th week	
	Male	Female	Male	Female	Male	Female	Male	Female
Control	7.28 ± 1.56	7.15 ± 1.24	42.56± 3.16	20.54±3.13	44.54 ±4.59	26.7±4.33	57.37±2.92	44.99±6.55
TB TEDx½	20.68 ±1.45 **	10.51 ± 3.20	51.49 ±4.4	39.75 4±3.49	93.83±6.24**	71.17±6.37**	141.78±5.76**	91.0±9.10*
TB TED	21.97 ±1.98 **	15.65 ±1.41*	71.84± 3.22 **	49.53±4.35 *	137.95± 6.63**	88.32±6.84**	82.70±4.87**	110.89±6.28**
TB TED x 5	36.84 ±3.18**	28.05±1.22**	116.54±9.14**	79.14±8.31**	207.49±15.65**	132.05±12.18**	274.93±15.49**	157.51±16.87**

Data expressed in mean± SEM, p value less than 0.05 and 0.01 considered as statistically significant compared to control. TB- *Tuttha bhasma.*

Tuttha bhasma administred at half the therapeutic dose caused significant increase in the percentage body weight measured at 1st , 8th and 12th weeks of experimental period compared to control group rats (*p<0.05, ** p<0.01). Repeated administration of TB at therapeutic and five times of therapeutic dose caused significant increase in the percentage change in the body weight measured during 1st, 4th, 8th and 12th weeks of experimental period as compared to control rats (*p<0.05, **p<0.01).

Table 2: Effect of repeated administration of three different dose levels of TB on organs weight.

Organs weight (g)	Groups							
	Control		TB TED x ½		TB TED		TB TEDx5	
	Male	Female	Male	Female	Male	Female	Male	Female
liver	9.88± 0.58	8.49±0.58	10.55±0.89	9.80±0.54	8.51±0.55	10.88±0.54**	11.95±0.99	9.85±0.55
Brain	1.45±0.15	1.9±0.09	1.94±0.10	1.69±0.04	1.48±0.01	1.39±0.05	1.95±0.15	1.59±0.05
heart	0.95±0.01	0.99±0.05	0.99±0.04	0.91±0.04	1.10±0.04	0.95±0.04	0.90±0.15	0.94±0.04
Kidney	1.98±0.09	1.45±0.05	1.95±0.08	1.01±0.09	2.45±0.09	2.40±0.05**	2.04±0.14	1.80±0.10
Spleen	1.04±0.08	0.85±0.05	1.08±0.19	0.94±0.09	0.89±0.07	0.58±0.04	1.58±0.18	0.91±0.01
Lungs	2.55±0.59	1.85±0.18	3.08±0.08*	1.04±0.15	1.95±0.18**	3.58±0.05**	3.05±0.19*	2.89±0.04
Trachea	0.50± 0.00	0.44±0.04	0.44±0.09	0.48±0.05	0.48±0.04	0.40±0.04	0.59±0.05	0.55±0.05
Jejunum	0.85±0.08	0.85±0.08	0.98±0.08	0.89±0.08	0.80±0.04	0.58±0.08**	0.85±0.09	0.69±0.04*
Stomach	1.88±0.05	1.49±0.02	4.18±0.09**	1.44±0.09	1.59±0.005	3.55±0.04**	2.98±0.14*	1.88±0.05
Testis	3.99±0.08		4.15±0.19		3.89±0.14		3.99±0.11	
Ventral Prostate	0.81±0.09		0.85±0.09		0.89±0.08		0.59±0.05	
Seminal vesicle	0.84±0.04		1.54±0.04 **		0.88±0.05		1.08±0.008*	
Uterus		0.94±0.10		0.96±0.18		0.89±0.05		1.06±0.09
Ovary		0.90±0.05		0.48±0.003*		0.51±0.04**		0.58±0.10

Data expressed in mean± SEM, *p<0.05, **p<0.01 in comparison to control group.

Repeated administration of TB at three different dose levels showed no significant changes in the organs weight such as brain, spleen, trachea, ventral prostrate and uterus as compared to control group. TB administred at half the therapeutic dose caused significant increase in the stomach, lungs and seminal vesicle weight in male rats, whereas ovary weight was significantly reduced in female rats as compared to control group. TB administered at therapeutic dose caused significant increase in the weights of liver, kidney, lungs and stomach in female rats, whereas in significant decrease in the lungs weight in female rats as compared to control group. TB administered at five times of therapeutic dose caused significant increase in the weights of lungs, stomach and seminal vesicle in male rats and significant decrease in the jejunum weight in female rats as compared to control group.

Table 3: Effect of three different dose levels of TB on haematological parameters in albino rats.

Parameters	Groups							
	Control		TD (TED x ½)		TD(TED)		TD(TED x 5)	
	Male	Female	Male	Female	Male	Female	Male	Female
Total WBC count /mm³	10025±1680.2	9145.71±688.64	13400±1100.8	14192±1521.4*	12371.42±1104.9	9225±613.30	11150±767.68	8943.33±1208.1
RBC count 10^6/µL	8.45±0.27	7.94±0.25	8.65±0.19	7.97±0.21	8.20±0.32*	7.21±0.02	7.98±0.28	7.65±0.14
Haemoglobin g/dL	15.56±0.11	14.98±0.21	14.48±0.41	15.96±0.11	16.84±0.33	15.56±0.21	15.28±0.27	13.16±0.21**
PCV (%)	45.11±0.45	44.87±0.92	45.3±1.42	42.16±0.30	46.21±0.84	42.56±0.42	41.91±0.62	41.11±0.58**
MCV (fl/cell)	54.2±0.40	54.90±0.34	52.81±0.47	53.18±0.89	51.56±0.37**	54.5±0.21*	56.14±0.99	56.14±0.76
MCH (pg/cell)	18.02±0.22	21.01±0.2	18.56±0.26	17.86±0.13**	17.2±0.15*	21.15±0.3**	18.38±0.13	20.4±0.23
MCHC (g/dL)	34.92±0.2	35.22±0.22	35.48±0.1	33.86±0.11**	35.92±0.3*	35.98±0.21	35.38±0.2	34.23±0.01*
Platelet count (10^3/ml)	7.02±0.47	7.01±0.58	7.67±0.59	7.50±0.31	7.51±0.16	7.18±0.28	7.81±0.40	6.76±0.23
RDWCV (%)	14.67±0.3	13.34±0.5	14.45±0.3	15.1±0.11*	14.21±0.2	14.3±0.12	14.34±0.4	14.018±0.12
RDWSD (%)	25.18±0.4	25.87±0.4	27.46±0.5	28.12±1.05	24.89±0.5**	23.52±0.15	27.91±0.4	26.15±0.13

Data: Mean± SEM, *p<0.05, **p<0.01 in comparison to control group.

TB administered at half the therapeutic dose significantly increased total WBC count and RDWSD, whereas levels of MCV and MCHC were significantly decrease in female rats as compared to control group. TB administered at therapeutic dose caused significant decrease in the blood MCV, MCH and RDWSD and significantly increased in MCHC levels in male rats. Female rats showed a significant increase in the MCH and significant decrease in the MCV level as compared to control group. TB administered at five times of therapeutic dose caused significant decrease in haemoglobin, PCV and MCHC level in female rats as compared to control group.

Table 4: Effect of different dose levels of TBbiochemical parameters in albino rats.

Parameters	Groups							
	Control		TB (TED x ½)		TB (TED)		TB (TEDx5)	
	Male	Female	Male	Female	Male	Female	Male	Female
SGOT IU/L	137.18±6.22	119.7±14.0	124.60±7.54	158.2±14.14	86.71±12.39**	85.3±5.56	134.16±10.11	138±8.04
SGPT IU/L	65.71±1.79	62.85±4.31	93.8±9.42**	72.2±4.93	52.42±5.94	33.5±6.91*	80.5±7.34*	70.33±9.36
ALP IU/L	356.14±59.70	500.7±7.62	609.2±72.03	290.4±82.28	220.14±12.35*	344±51.07*	612.83±53.86	576.6±115
Total protein g/dL	8.14±0.21	7.25± 1.08	6.96±0.22	5.34±0.79*	4.15±0.45**	8.61±0.19	7.13±0.12	8.01±0.18
Albumin g/dL	3.41±0.16	3.67±0.08	5.10±0.17*	3.08±020*	4.02±0.138	5.12±0.18*	3.85±0.23	4.89±0.25
Total bilirubin mg/dL	0.26±0.12	0.28±0.46	0.24±01	0.40±0.12	0.09±0.10**	0.13±0.23	0.22±0.11	0.21±0.44
Direct bilirubin mg/dL	0.09±0.02	0.07±0.08	0.05±0.00	0.06±0.01	0.06±0.01	0.08±0.01	0.05±0.00	0.05±0.01
Cholesterol mg/dL	48.14±2.35	54±2.18	62.10±4.33**	68±9.49	51.28±2.60	90.4±4.28**	39.90±4.14	46.26±4.28
Triglycerides mg/dL	90.85±9.44	132.42±15.50	71.20±9.35	97±18.55	69.27±4.12	120.37±18.71	52.93±11.62*	77.26±8.60
Blood glucose mg/dL	115.85± 7.30	118.85±5.63	171.31±13.71**	118.8±6.72	125.29±9.15	123.15±8.56	145.33±2.85	125.13±6.30
Urea mg/dL	38.81±1.20	35.48±2.33	36.2±3.82	38±5.15	30.15±2.13	38±5.15	28.93±0.97	45.23±3.57**
Creatinine mg/dL	0.44±0.01	0.75±0.18	0.50±0.22	0.20±0.22**	0.39±0.08	0.42±0.13**	0.33±0.13	0.55±0.13

Data related to biochemical parameters were expressed in mean± SEM, p value less than 0.05 and 0.01 were considered as statistically significant as compared to control group. TB- *Tuttha bhasma*

TB administered with half the therapeutic dose significantly increased serum SGPT, albumin, cholesterol and blood glucose level in male rats, whereas serum total protein, albumin and creatinine were significantly decreased in female rats compared to control group. *Tuttha bhasma* at therapeutic dose significantly decreased serum SGOT, ALP, total protein and total bilirubin in male rats, whereas female rats showed significant decrease in the serum SGPT, ALP, creatinine and significant increase in the serum albumin level as compared to control group. TB administred at five times of therapeutic dose caused significant increase in serum SGPT and TG levels in male rats, whereas significant increase in the serum urea level was observed in female rats as compared to control group.

Histopathology

The histopathological examination of the organs such as heart, brain, uterus, ovary, seminal vesicles, lymph node, trachea, colon, and adrenal glands didn't show any pathological changes. The notable pathological changes were observed in rats administered with *Tuttha bhasma* administered at five times of therapeutic dose. The histopathology observation revealed mild fatty changes in liver sections from two rats and mild cellular infiltration in section from one rat. There was mild haemorrhage in kidney sections from one rat and all other found to be having normal cytoarchitecture (Figure- 1, 2). Histopathology examination of spleen tissues revealed an increase in white pulp proportion in two female and three male rats (Figure-3). TB administred at five times of therapeutic dose caused mild to moderate erosion of epithelial layer, shortening of epithelial layer of jejunum tissues (Figure-4).

Figure - 1: Photomicrograph of Liver

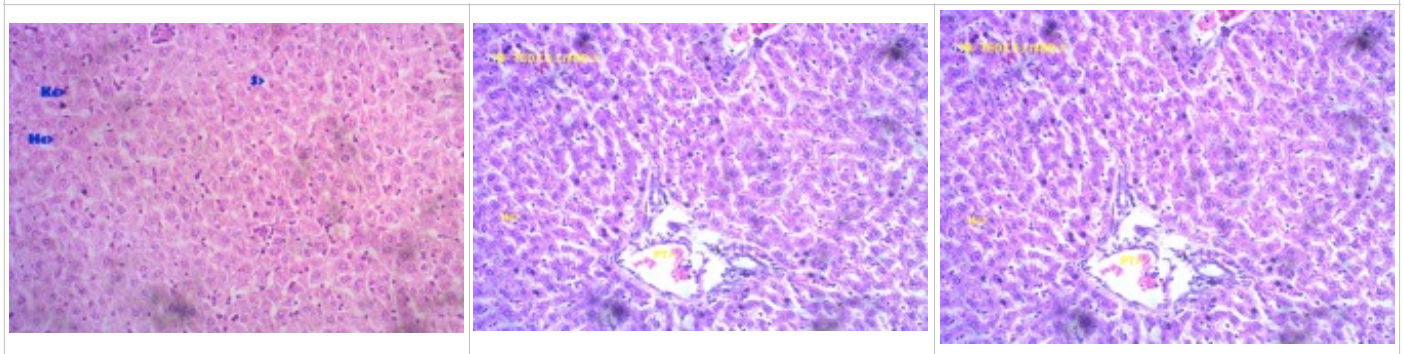

Fig 1a	Fig 1b	Fig 1c

Photomicrographs of liver tissues taken at ×200 magnification. (1a) Normal cytoarchitecture (Control group), (1b) mild fatty changes and mild cellular infiltration (TB TEDx5 female rats), (1c) (TB TEDx5 male rat).

Figure– 2: Photomicrograph of Kidney

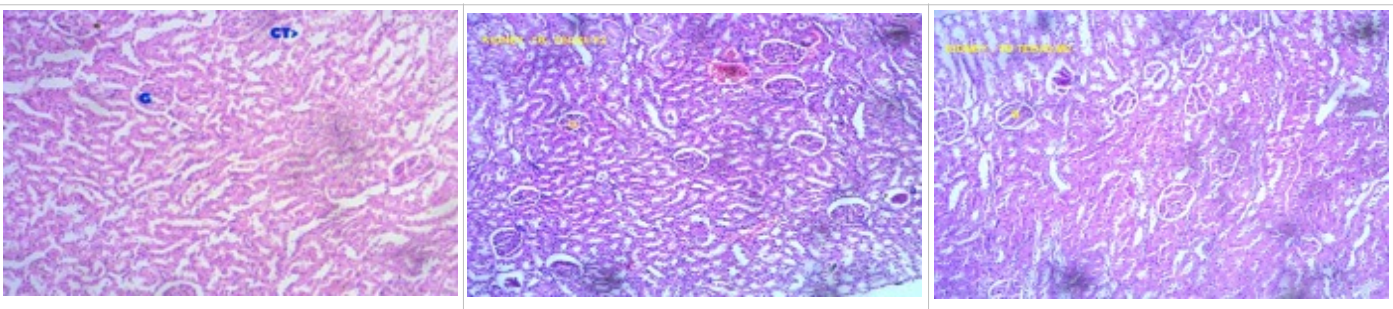

Fig-2a	Fig-2b	Fig-2c

Photomicrographs of kidney tissues taken at ×200 magnification. (2a) Normal cytoarchitecture (Control group), (2b) cell infiltration and degenerative changes (TB TEDx5 female rats), (2c) Normal cytoarchitecture (TB TEDx5 male rat).

Figure – 3: Photomicrograph of Spleen

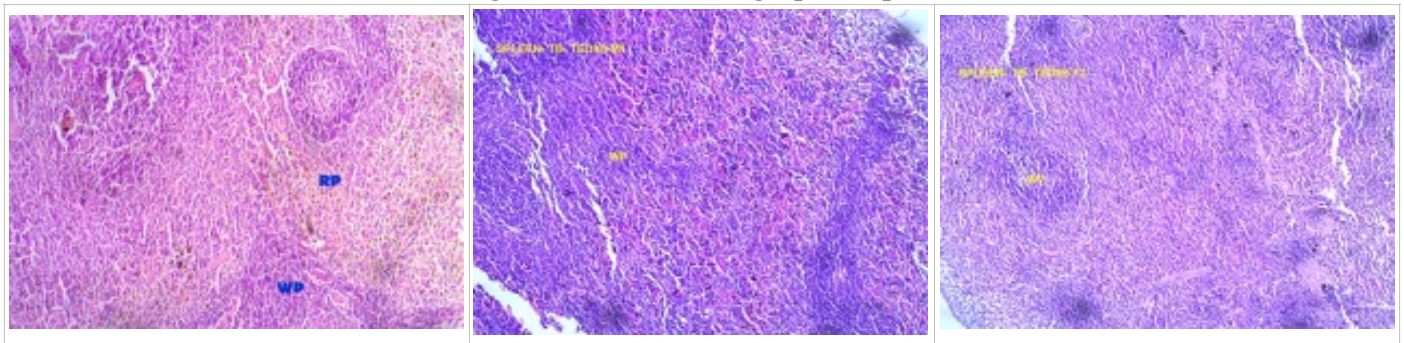

Fig-3a	Fig-3b	Fig-3c

Photomicrographs of Spleen tissues taken at ×200 magnification. (3a) Normal cytoarchitecture (Control group), (3b and 3c) There was increase in white pulp proportion of spleen was observed (TB, TEDX5 male and female

Figure – 4: Photomicrograph of Jejunum

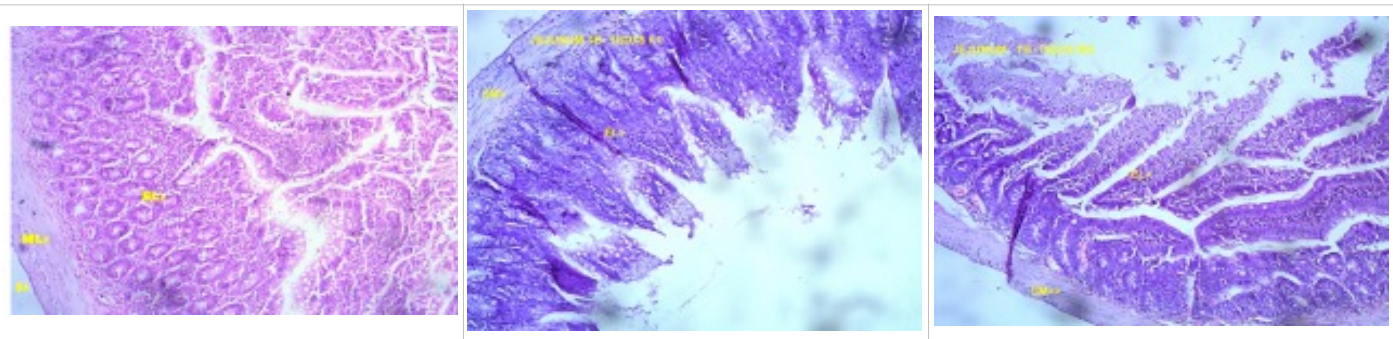

Fig-4a	Fig-4b	Fig-4c

Photomicrographs of Jejunum tissues taken at ×200 magnification. (4a) Normal cytoarchitecture (Control group), (4b and 4c) There was mild epithelial erosion, shortening of epithelial layer was observed (TB, TEDX5 male and female rats).

Discussion

The repeated dose toxicity study of TB provides information regarding possible health hazards in human health. Thus in the present study we have administered test drug at three different dose levels for 90 consecutive days and parameters such as body weight changes, organs weight, haematological, biochemical and histological change of the important organs were examined to provide information regarding its safety in clinical use. The present study might provide information on the major toxic effects; indicate target organs and possibility of accumulation. Chronic toxicity studies would be helpful in predicting the potential to cause damage to the vital organs like brain, kidney, liver and reproductive organs. In the present study we could observe a time dependant increase in the percentage body weight change in three different dose levels of TB as compared to the control group. Generally the body weight gain change can be considered as an index of degenerative effect. The present study TB administered at higher dose for longer duration (12 week) has showed significant increase in the percentage body weight and hence we could say no potential to cause any serious or significant degenerative changes or loss of tissue. Thus the time dependant increase in the percentage body weight gain provides evidence to rejuvenator nature of the drug.

The data related to haematological parameters were almost in the normal range as compared to control group. However there was significant decrease in the Hb, PCV and MCHC level especially in female rats at five times and half of therapeutic dose compared to control group. TB at therapeutic dose had shown increased RBC and RDWSD level and significant reduction in the parameters such as MCV, MCHC and MCH level. This indicated the long term treatment with TB might have potential to cause fatigue and anaemic changes (14).

Total fourteen biochemical parameters were measured in the rat's serum samples. Important changes observed were, TB at therapeutic dose significantly increased cholesterol and albumin level, whereas SGOT, SGPT, ALP and total bilirubin level was significantly decreased. TB administered at five times of therapeutic dose significantly increased serum SGPT level whereas triglyceride level was significantly decreased. TB at half the therapeutic dose a significantly increased serum sugar, cholesterol, SGPT and albumin level in male rats whereas significantly decreased serum creatinine, total protein and albumin level in female rats. The observed changes do not indicate any serious pathological implications. Analysis of the data shows that the urea, albumin, globulin and creatinine values of male and female rats were lower. Based on this it can be inferred that it does not indicate renal insufficiency, may be indicative of increased formation of creatinine because of the increased body weight gain. Overall changes in serum total protein and albumin levels were comparable with that of control rats and found moderate increase in the serum level. Moderate decrease in serum bilirubin level was observed at TED dose but not with other two dose level- indicating self-limiting nature. Electrolyte level remained normal- thus overall analysis of the biochemical parameters do not indicate any serious functional impairment in the important organs. Organ weight analysis show increase or decrease but the tendency was inconsistent and not dose dependent. Histopathological examination of jejunum showed only mild epithelial erosion, liver tissues with mild fatty changes and cell infiltration in kidney tissues in some rats. The histopathology of major organs such as brain, heart, reproductive organs didn't reveal any marked degenerative changes.

Conclusion

Overall assessment regarding TB is safer at three different dose levels. Its careful administration is not likely to cause any serious toxic outcomes at the therapeutic dose level; however repeated administration of *Tuttha bhasma* at higher dose level for longer duration might cause organ toxicity.

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The present study was funded by RGUHS, Bangalore, Govt. of Karnataka to Muralidhar R (RGU: ADV.RES: Proposal-AY-259:2015-16) and infrastructure and laboratory facilities were provided by SDM research centre, Udupi, Karnataka.

Conflicts of interest

There are no conflicts of interest with any of the researchers involved in this project.

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