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Acute and subchronic toxicity study of *Tamra Bhasma* (incinerated copper) prepared with and without *Amritikarana*

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ABSTRACT

Background: *Tamra Bhasma* (TB) is one among herbo-metallic preparations extensively used in routine ayurvedic practice. In the present era, *Bhasma* preparations used in ayurvedic system of medicines are always under stern observations for containing heavy metals which may raise the question of safety aspect.
Objective: In the present study, TB prepared with and without *Amritikarana* was subjected to toxicity study to ascertain the role of *Amritikarana* on safety profile of TB in rats.
Materials and methods: Both the samples of TB were administered to rats for 28 consecutive days at the doses of 5.5, 27.5, and 55 mg/kg. The effects of both drugs were assessed on ponderal changes, hematological, serum biochemical, and histopathology of various organs.
Results: Results showed that both the samples of TB did not produce any sign and symptoms of toxicity at therapeutic dose level (5.5 mg/kg) and therapeutic equivalent dose (TED) × 5 (27.5 mg/kg) while at higher dose of TED × 10 (55 mg/kg) TB has mild toxicity in liver, kidney, heart, and thymus on repeated administration for 28 days in rats. The sample without *Amritikarana* has more magnitude of toxicity than the sample with *Amritikarana*.
Conclusion: From the present study, it is concluded that TB with *Amritikarana* was found to be relatively safer than TB without *Amritikarana* at different dose levels in rats and hence suggest for safely use in humans at therapeutic dose level. It proves the role of *Amritikarana* in the preparation of TB.

1. Introduction

Ayurveda advocates therapeutic uses of mineral and metallic preparations in many diseases since century in clinical practice. Knowing the possibilities of toxic effects, seers emphasized on following set of exclusive pharmaceutical procedures such as *Shodhana* (purification and/or detoxification), *Marana* (incineration and/or calcination), and *Amritikarana* that converts the metals and minerals into *Bhasma* (calcined powders) [1,2]. *Bhasmas* are unique preparations which are safely being practiced in Ayurveda without any noticeable side effects can be considered as a testimony to their safety, but no objective verifiable data exist to support many such claims. Preclinical studies of ayurvedic drug provide scientific basis for their traditional use and to prove that they are safe and efficacious [3].

Tamra Bhasma (TB) (incinerated copper) is one among such ayurvedic herbo-metallic preparation used in the treatment of *Udara* (ascites), *Pandu* (anemia), *Svasa* (asthma), and *Amlapitta* (hyperacidity) [4]. Though wide therapeutic utility of TB has been mentioned in classics, it is reported as poison as or more than that if not processed or purified properly as per classical methods [5]. To indicate its toxic potential, *Ashtamahadoshas* (eight major ill effects) have been quoted in classics [6]. Previous studies reported safety of TB in animal models [7,8]. Role of *Shodhana* in safety of TB was also reported in animals [9]. Though number of studies have been carried out in direction of safety of *Bhasmas*, concerns are always being raised on ayurvedic formulations for the presence of heavy metals [10,11]. *Amritikarana* is exclusively mentioned for TB and *Abhrak Bhasma* which is said to eliminate all the blemishes from the end

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product. There is a need to provide scientific basis to establish the impact of this procedure. Hence, the present study is aimed to evaluate the acute and subchronic toxicity studies of TB prepared with and without *Amritikarana* in rats.

2. Materials and methods

2.1. Drugs and chemicals

Two samples of TB with and without *Amritikarana* were prepared by following standard guidelines as prescribed in ayurvedic classics. Copper scraps with 99.89% pure copper was procured from local industrial area, Jamnagar, India. It was subjected to general and specific purification procedure followed by incineration after mixed with purified sulfur, *Kajjali* (black sulfide of mercury), and juice of *Citrus jambhiri* Lush. In *Amritikarana*, it was mixed with half part purified sulfur and juice of *C. jambhiri* Lush, kept in the corm of *Amorphophallus campanulatus* Linn. It was subjected to heat treatment and labeled as *Tamra Bhasma* with *Amritikarana* (TBA) [12]. Another sample was subjected up to *Marana* and labeled as TB without *Amritikarana* [13]. All chemicals used in the study were of analytical grade.

2.2. Animals

Wistar strain albino rats of either sex, weighing 200 ± 20 g, were used as per the guidelines of the Institutional Animal Ethics Committee (IAEC). The animals were obtained from the animal house attached to the pharmacology laboratory, IPGT and RA, Jamnagar. The animals were maintained under ideal husbandry conditions in terms of standard conditions of temperature (23 ± 2 °C), relative humidity (50–60%) and exposed to 12 h light and dark cycles. All animals were exposed to the same environmental conditions and were maintained on standard diet and drinking water *ad libitum*. The experimental protocol was approved by the IAEC/14/2013/16 as per guideline of committee for the purpose of control and supervision of experiments on animals in India.

2.3. Dose fixation

As per classical guideline, the therapeutic clinical dose of TB is 30 mg twice a day (60 mg/day) [14]. The suitable dose for rats was calculated by referring to table of Paget and Branes [15] and was found to be 5.5 mg/kg body weight of rat (considered as TED). The test drug was administered orally (licking) along with honey as adjuvant with the help of oral cannula.

2.4. Acute toxicity study

Young, healthy, nulliparous, and nonpregnant Wistar strain albino female rats were selected and acclimatized for 7 days before the experiment. Both test drugs along with adjuvant were orally administered at limit dose of 2000 mg/kg to overnight fasted female rats by following Organization for Economic Cooperation and Development (OECD) 425 guideline [16]. The rats were observed closely for behavioral changes, signs and symptoms of toxicity, and mortality, if any continuously for the first 6 h and thereafter periodically up to 14 days.

2.5. Subchronic toxicity

Animals were divided into seven groups, each comprising three male and three females. Rats were randomized into six groups, each consisting of six rats comprising three male and three female. Group I was kept as control group, received vehicle as honey (2 ml/kg, orally). Group II to IV were administered with test drug TB without *Amritikarana* along with adjuvant at TED (5.5 mg/kg, orally), TED × 5 (27.5 mg/kg, orally), and TED × 10 (55.0 mg/kg, orally), respectively. Group V to VII were administered with test drug, TBA along with adjuvant at TED (5.5 mg/kg, orally), TED × 5 (27.5 mg/kg, orally), and TED × 10 (55.0 mg/kg, orally), respectively. The suspensions of test drugs were administered orally once a day for 28 consecutive days [17].

Initial body weight of all animals was recorded. General behavioral pattern was observed once a week by exposing each animal to open arena. On the 29th day, animals were weighed again and anesthetized with diethyl ether. Supraorbital plexus was punctured under light anesthesia and blood was collected by capillary in two different types of tubes, one containing anticoagulant fluid for hematological parameters and another plain tube for serum biochemical investigations. Then, the rats were sacrificed and the abdomen was opened through midline incision to record the autopsy changes followed by dissecting out the important organs.

Hematological analysis was performed by using an automatic hematological analyzer (Swelab, Sweden). Total red blood cell (TRBC), hemoglobin (Hb), hematocrit, mean corpuscular volume, mean corpuscular Hb, mean corpuscular Hb concentration, white blood cell (WBC), neutrophils, lymphocyte percentage, eosinophils percentage, monocyte percentage, packed cell volume (PCV), and platelet count were measured from the blood samples.

Serum biochemical parameters were carried out by using fully automated biochemical random access analyzer (BS-200, Lilac Medicare Pvt. Ltd., Mumbai). The studied parameters were blood glucose [18], urea [19], creatinine [20], total cholesterol [21], high-density lipoprotein (HDL)-cholesterol [22], triglyceride [23], very-low-density lipoprotein (VLDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, total protein [24], albumin, globulin [25], alkaline phosphatase [26], serum glutamic oxaloacetic transaminase (SGOT) [27], serum glutamic pyruvic transaminase (SGPT) [28], uric acid [29], direct bilirubin [28], total bilirubin [30], and serum calcium [31].

All the important internal organs were carefully dissected namely, liver, kidney, heart, lungs, trachea, intestine spleen, thymus, lymph node, testis, seminal vesicle, prostate, uterus, and ovary. After noting signs of gross lesion and ponderal changes of major organs, all were transferred to 10% phosphate buffered formalin solution for fixation and later on subjected to dehydrating, wax embedding, sectioning, and staining with hematoxylin and eosin for histological evaluation. The slides were viewed under trinocular research Carl-Zeiss's microscope at various magnifications to note down the changes in the microscopic features of the tissues.

2.6. Statistical analysis

The data are expressed as mean ± standard error of mean for six rats per experimental group. One-way analysis of variance was used to compare the mean values of quantitative variables among the groups followed by Holm–Sidak multiple *t*-test for unpaired data by using Sigmastat software (version 3.5, Systat Software Inc.) to determine significant difference between groups at $P < 0.05$.

3. Results

3.1. Acute toxicity study

The results of acute toxicity showed that both the samples of TB along with adjuvant did not affect any behavioral changes and other parameters during entire experimental period of 14 days.

Both TB and TBA did not show any signs and symptoms of toxicity and mortality when given orally at a dose of 2000 mg/kg.

3.2. Subchronic toxicity study

Behavioral changes were not observed in treated groups during the course of subchronic toxicity study in comparison to control group. No mortality was observed in both samples of TB at TED × 10, TED × 5, and TED dose levels during the experimental period.

Weight gain was observed in normal control rats during subchronic toxicity study. Effects of TB and TBA on change in body weight showed that weight gain was observed in all treated groups, but percentage change in body weight pattern in treated groups did not differ significantly from the changes observed in control groups [Table 1]. Subchronic administration of TB at different dose levels lead to nonsignificant effect on change in relative weight of nine organs except significant increase was observed in relative weight of heart in TBA at TED level [Table 2]. TBA at all dose levels produced nonsignificant increase in relative weight of thymus, spleen, and testis in a uniform manner while other changes were not distinct in comparison to control group.

Effect of samples of TB on hematological parameters [Table 2] revealed that out of the twelve parameters studied, nonsignificant increase in WBC count and neutrophil in TB at TED × 10 dose level while significant increase in monocyte count in TB at TED × 5 dose level in comparison to control group. Significant increase in Hb, PCV, and TRBC were observed in TB and TBA treated groups at TED and TED × 5 dose levels [Table 3]. Both samples of TB at all dose levels produced nonsignificant increase in platelet count in comparison to control group.

Among the 18 serum biochemical parameters, significant decrease in blood sugar was found in TB at TED dose level and TBA treated drug at TED × 5 and TED × 10 levels, while significant increase in cholesterol was found in TB and TBA treated groups at TED and TED × 5 dose levels, while higher dose of both drugs produced nonsignificant increase in serum cholesterol level. Significant increase in serum triglyceride, LDL, and VLDL level was found in TB at TED × 10 treated groups while nonsignificant increase in triglyceride and VLDL level in all TBA treated groups in comparison to control group. HDL cholesterol was significantly increased in TB treated group at TED dose level and TBA treated group at TED and TED × 5 treated groups while both at higher dose levels produced nonsignificant increase in comparison to control group. Significant increase in albumin was observed at all dose levels of TB and higher dose level of TBA. The changes observed in remaining biochemical parameters were statistically nonsignificant in comparison to control group [Table 4].

Microscopic examination of all the organs obtained from control group exhibited normal cytoarchitecture. TB with and without *Amritikarana* at TED × 10 dose level did not affect the cytoarchitecture of the spleen, lung, stomach, lymph node, trachea, adrenal, prostate, seminal vesicle, testis, ovary, and uterus. Administration of TB at TED × 10 causes mild fatty changes in the heart but TBA at TED × 10 showed normal cytoarchitecture [Fig. 1], while both drugs at higher dose level showed micro fatty changes in liver [Fig. 2]. Mild cell infiltration and edema in the kidney in TB at TED × 10, while mild fatty changes in TBA at TED × 10 dose level [Fig. 3] were observed and both drugs at higher dose produced mild decrease in cellularity in cytoarchitecture of thymus [Fig. 4]. However, all these changes were not observed in both the drugs treated groups at TED × 5 and TED dose levels in rats that suggest that drug is devoid of drastic toxicity.

4. Discussion

Metals and minerals are extensively used in ayurvedic formulations since Vedic period, which became an important part of ayurvedic therapeutics. To make them for therapeutic use, they have to pass through a set of classical pharmaceutical processes known as *Shodhana*, *Marana*, etc. The possible impurities that remain in *Bhasma* after *Marana* process are advocated to be removed by another specialized procedure known as *Amritikarana*. [32] In classics, it is mentioned that *Amritikarana* increases therapeutic efficacy of *Bhasma* [33]. The word, *Amritikarana* itself explains the importance of this procedure, i.e., the process by which *Amrititva* is imparted to the matter. This procedure is recommended for all the *Bhasmas* but claimed to be essential mainly in *Abhraka* and TB. It should be done after confirmation of *Bhasma Siddha Lakshanas*. In this process, quality of *Bhasma* increases along with change in regular color and appearance.

Acute toxicity was carried out to record immediate adverse signs and symptoms after the administration of single dose of drug at dose levels that are several folds higher than the therapeutic equivalent dose (2000 mg/kg). OECD 425 guideline for oral acute toxicity study were employed using a female sex to reduce variability and as a means of minimizing the number of animals used. This is because there is little difference in sensitivity in LD_{50} test between the sexes; however, in those cases where differences were observed, females were generally slightly more sensitive [34]. In this study, TB *s* at the dose level of 2000 mg/kg, orally did not produce any observable toxic effects during the entire duration of acute toxicity study and all female rats survived 14 days of observation suggest that LD_{50} value may be higher than 2000 mg/kg by oral route. As per UN classification, any substance which has oral LD_{50} of more than 2000 mg/kg is considered as low hazard potential and categorized as UN 6.1 PG III [35]. Thus, as per the above criterion, both TB with or without *Amritikarana* can be categorized as substances with low health hazard potential (Class 4 of GHS and UN 6.1 PG III).

On subchronic administration of TB with or without *Amritikarana* showed significant increase in Hb, red blood cell (RBC) and PCV but not found to be in dose-dependent manner. In the present study, increased Hb content is due to the increase observed in the production of erythrocytes and PCV in these treated groups.

Table 1
Effect of *Tamra Bhasma* on body weight of albino rats.

Groups	0 day	1st week	2nd week	3rd week	4th week
Control	210.66 ± 11.02	233.33 ± 12.29***	239.33 ± 11.73***	262.00 ± 9.94*	241.66 ± 6.58**
TB TED	194.33 ± 9.58	210.33 ± 10.07	213.33 ± 9.24*	213.33 ± 9.78	212.66 ± 10.97
TB TED × 5	187.66 ± 10.99	205.66 ± 14.88*	208.66 ± 13.38*	205.00 ± 12.31	210.00 ± 17.88*
TB TED × 10	206.00 ± 9.71	216.33 ± 8.57	222.33 ± 12.81	228.83 ± 12.51*	232.33 ± 16.37*
TBA TED	182.33 ± 8.00	193.33 ± 7.14**	206.66 ± 7.13**	221.33 ± 11.12*	206.66 ± 12.561
TBA TED × 5	186.66 ± 9.54	195.33 ± 8.43**	205.00 ± 8.46***	224.00 ± 5.61**	213.33 ± 4.21
TBA TED × 10	195.33 ± 9.95	212.33 ± 9.38	216.33 ± 9.45***	222.33 ± 10.39**	221.66 ± 13.76*

Data presented as mean ± SEM, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (paired *t*-test). SEM = Standard error of mean, TED = Therapeutic equivalent dose, TB = *Tamra Bhasma without Amritikarana*, TBA = *Tamra Bhasma* with *Amritikarana*.

Table 2
Effect of *Tamra Bhasma* samples on relative weight of organs.

Relative weight	Control group	*Tamra Bhasma* without *Amritikarana*			*Tamra Bhasma* with *Amritikarana*		
		TED	TED × 5	TED × 10	TED	TED × 5	TED × 10
Heart (mg/100 g)	284.26 ± 10.97	290.55 ± 9.30	307.29 ± 12.75	276.12 ± 15.86	332.00 ± 12.77*	310.26 ± 13.37	302.70 ± 14.89
Liver (g/100 g)	3.06 ± 0.17	2.97 ± 0.12	3.22 ± 0.13	3.10 ± 0.06	2.96 ± 0.21	3.45 ± 0.13	3.24 ± 0.13
Spleen (mg/100 g)	201.01 ± 10.72	200.78 ± 13.26	212.09 ± 12.96	211.56 ± 12.84	235.79 ± 13.78	235.77 ± 16.61	215.35 ± 12.04
Kidney (mg/100 g)	691.18 ± 17.31	684.25 ± 25.74	751.01 ± 23.38	660.20 ± 28.13	722.47 ± 31.24	734.34 ± 10.94	712.74 ± 27.10
Thymus (mg/100 g)	134.60 ± 27.92	151.61 ± 10.16	168.69 ± 11.68	144.85 ± 12.49	183.18 ± 5.33	176.65 ± 11.15	148.78 ± 12.03
Testis (mg/100 g)	921.62 ± 61.24	1074.91 ± 43.72	1103.66 ± 43.55	857.87 ± 17.64	1071.21 ± 72.44	1113.97 ± 50.34	1082.62 ± 33.57
Prostate (mg/100 g)	158.30 ± 19.87	99.90 ± 11.99	151.89 ± 12.64	96.55 ± 18.57	149.30 ± 20.84	174.13 ± 24.95	117.99 ± 9.22
Uterus (mg/100 g)	252.46 ± 65.42	275.70 ± 58.59	227.10 ± 42.78	230.11 ± 3.16	383.30 ± 105.87	194.96 ± 37.15	228.63 ± 67.33

*$P < 0.05$ when compared to control group. TED = Therapeutic equivalent dose.

Table 3
Effect of *Tamra Bhasma* on hematological parameters.

Parameters	Control group	*Tamra Bhasma* without *Amritikarana*			*Tamra Bhasma* with *Amritikarana*		
		TED	TED × 5	TED × 10	TED	TED × 5	TED × 10
TWBC (10^3/mL)	8016.67 ± 526.89	8333.33 ± 463.08	8616.67 ± 633.20	9750.00 ± 1050.95	7333.33 ± 615.18	9250.00 ± 867.85	7916.67 ± 207.23
Neutrophil (%)	22.33 ± 2.42	20.33 ± 2.23	18.33 ± 2.89	26.60 ± 5.16	20.00 ± 4.30	22.00 ± 2.57	24.83 ± 2.24
Lymphocyte (%)	73.17 ± 2.50	75.33 ± 2.12	77.00 ± 2.82	68.40 ± 5.71	76.00 ± 4.62	74.00 ± 2.57	70.33 ± 2.15
Eosinophil (%)	2.67 ± 0.33	2.67 ± 0.33	2.17 ± 0.17	2.80 ± 0.58	2.17 ± 0.31	2.17 ± 0.17	2.83 ± 0.31
Monocyte (%)	1.83 ± 0.17	1.67 ± 0.21	2.50 ± 0.22*	2.20 ± 0.20	1.83 ± 0.31	1.83 ± 0.17	2.00 ± 0.26
Hb (g/dL)	14.37 ± 0.27	15.83 ± 0.27**,@	15.18 ± 0.35*,@	14.84 ± 0.30	15.92 ± 0.20***,@	15.80 ± 0.36*,@	14.80 ± 0.27
PCV (%)	44.70 ± 1.20	49.40 ± 1.28*,@	47.57 ± 1.51	46.14 ± 0.84	49.13 ± 0.55**,@	48.30 ± 0.56*,@	45.02 ± 0.53
TRBC (10^6/mL)	7.89 ± 0.25	8.90 ± 0.29*,@	8.54 ± 0.35	8.33 ± 0.17	8.72 ± 0.13*	8.62 ± 0.16*	8.00 ± 0.11
MCV (fL)	56.70 ± 0.64	55.60 ± 0.67	55.82 ± 0.70	55.40 ± 0.22	56.38 ± 0.40	56.13 ± 0.81	56.32 ± 0.82
MCH (pg/red cell)	18.25 ± 0.33	17.85 ± 0.40	17.87 ± 0.35	17.80 ± 0.37	18.27 ± 0.076	18.07 ± 0.41	18.50 ± 0.42
MCHC (g/dL)	32.18 ± 0.29	32.10 ± 0.41	31.97 ± 0.29	32.18 ± 0.65	32.40 ± 0.24	32.20 ± 0.36	32.90 ± 0.30
Platelets (10^3/mL)	997.00 ± 143.59	1127.33 ± 23.24	1177.67 ± 88.36	1150.40 ± 101.33	1213.67 ± 66.57	1086.17 ± 34.76	1122.33 ± 61.56

Data presented as mean ± SEM, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control group (unpaired *t*-test), @$P < 0.05$ compared to control group (ANOVA followed by Holm–Sidak test). TED = Therapeutic equivalent dose, SEM = Standard error of mean, TWBC = Total white blood cell, Hb = Hemoglobin, PCV = Packed cell volume, TRBC = Total red blood cell, MCV = Mean corpuscular volume, MCH = Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration.

Table 4
Effect of *Tamra Bhasma* on biochemical parameters.

Parameters	NC	TB TED	TB TED × 5	TB TED × 10	TBA TED	TBA TED × 5	TBA TED × 10
FBS (mg/dL)	114.33 ± 6.09	86.00 ± 4.38*,@	102.33 ± 2.46	100.00 ± 6.05	100.33 ± 6.10	97.50 ± 3.66*	94.17 ± 2.60*,@
Serum cholesterol (mg/dL)	47.33 ± 2.59	69.00 ± 6.27*,@	59.17 ± 4.30*	56.67 ± 5.80	63.17 ± 3.03*	67.00 ± 6.51*,@	55.33 ± 3.40
Triglycerides (mg/dL)	57.33 ± 4.36	56.67 ± 3.40	57.00 ± 8.41	71.67 ± 4.28*	81.83 ± 11.78	74.83 ± 7.04	76.83 ± 8.50
HDL (mg/dL)	27.83 ± 3.28	41.17 ± 2.27*	35.00 ± 2.73	36.17 ± 3.49	45.00 ± 2.57*,@	47.00 ± 3.97*,@	34.50 ± 1.98
LDL (mg/dL)	8.03 ± 1.59	16.50 ± 4.67	12.77 ± 2.11	8.20 ± 1.88	8.33 ± 1.73	7.83 ± 2.71	5.48 ± 1.29
VLDL (mg/dL)	11.47 ± 0.87	11.33 ± 0.68	11.40 ± 1.68	14.37 ± 0.87*	14.28 ± 2.35	14.97 ± 1.41	15.37 ± 1.70
SGPT (IU/L)	56.17 ± 2.50	63.67 ± 5.83	65.83 ± 6.47	65.83 ± 9.22	82.17 ± 28.11	70.67 ± 6.64	60.50 ± 3.53
SGOT (IU/L)	166.00 ± 13.47	171.33 ± 11.04	176.50 ± 13.40	172.00 ± 6.74	162.83 ± 9.99	173.17 ± 13.26	179.67 ± 18.66
ALP (IU/L)	205.67 ± 17.22	170.17 ± 15.49	222.67 ± 26.10	267.83 ± 51.75	206.50 ± 58.21	173.83 ± 36.55	174.33 ± 20.40
Total bilirubin (mg/dL)	0.52 ± 0.07	0.47 ± 0.03	0.57 ± 0.08	1.37 ± 0.83	0.35 ± 0.06	0.37 ± 0.03	0.62 ± 0.08
Direct bilirubin (mg/dL)	0.13 ± 0.02	0.17 ± 0.02	0.13 ± 0.02	0.47 ± 0.32	0.10 ± 0.00	0.10 ± 0.00	0.13 ± 0.02
Blood urea (mg/dL)	62.67 ± 6.06	64.17 ± 4.83	64.50 ± 2.31	59.33 ± 3.22	70.67 ± 4.97	71.67 ± 4.59	61.33 ± 5.10
Creatinin (mg/dL)	0.55 ± 0.034	0.62 ± 0.040	0.57 ± 0.042	0.52 ± 0.017	0.53 ± 0.042	0.55 ± 0.034	0.52 ± 0.040
Protein (g/dL)	6.93 ± 0.20	7.25 ± 0.13	7.02 ± 0.08	6.87 ± 0.14	7.20 ± 0.33	7.18 ± 0.25	7.17 ± 0.25
Albumin (g/dL)	3.38 ± 0.21	3.90 ± 0.08@	3.83 ± 0.08@	3.95 ± 0.13*,@	3.63 ± 0.08	3.77 ± 0.05	3.83 ± 0.09@
Globulin (g/dL)	3.55 ± 0.37	3.35 ± 0.14	3.18 ± 0.14	2.91 ± 0.21	3.57 ± 0.39	3.42 ± 0.25	3.33 ± 0.18
Uric acid (mg/dL)	1.00 ± 0.19	0.87 ± 0.99	0.82 ± 0.09	1.20 ± 0.37	0.97 ± 0.22	0.90 ± 0.19	0.85 ± 0.15
Serum calcium (mg/dL)	8.85 ± 0.15	8.50 ± 0.17	8.78 ± 0.20	9.55 ± 0.33	9.10 ± 0.07	9.35 ± 0.14*	9.18 ± 0.38

Data presented as mean ± SEM, *$P < 0.05$ compared to control group (unpaired *t*-test), @$P < 0.05$ compared to control group (ANOVA followed by Holm–Sidak test). SEM = Standard error of mean, NC = Normal control, FBS = Fasting blood sugar, HDL = High-density lipoprotein, LDL = Low-density lipoprotein, VLDL = Very-low-density lipoprotein, SGPT = Serum glutamic pyruvic transaminase, SGOT = Serum glutamic oxaloacetic transaminase, ALP = Alkaline phosphatase, TED = Therapeutic equivalent dose, TB = *Tamra Bhasma without Amritikarana*, TBA = *Tamra Bhasma* with *Amritikarana*.

Significant increase in monocyte percentage was observed at middle dose level of TB. The fact that the observed effect was not dose-dependent and is not remarkably deviant from the normal range of values indicates that they do not indicate any serious toxicity potential. Previous study also revealed significant increase in total RBC count, PCV, and platelet count at different dose level of TB on administration for 45 days in albino rats [9]. Copper is essential for the synthesis of Hb and also supports absorption of iron from intestine, which may responsible for increase in RBC related parameters in rats [36].

Significant decrease in fasting blood sugar at TED level of TB and higher dose level of TBA suggest role of copper in glucose metabolism. Copper plays important role in carbohydrate metabolism by stimulating insulin binding, hexose transport, and lipogenesis

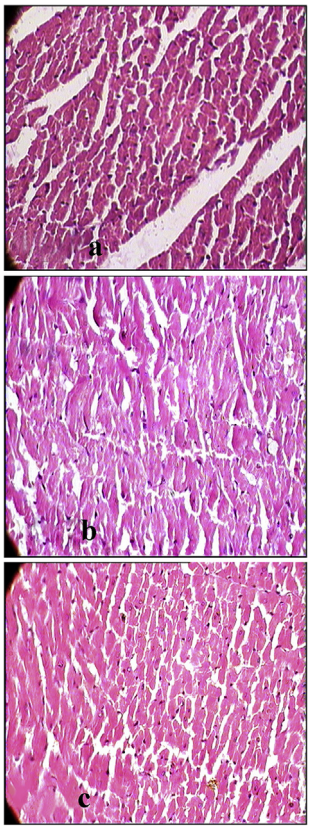

Fig. 1. Photomicrographs of sections of the heart taken at ×400 (a) normal cytoarchitecture (control group) (b) fatty changes (*Tamra Bhasma* therapeutic equivalent dose, ×10) (c) normal cytoarchitecture (*Tamra Bhasma* with *Amritikarana* therapeutic equivalent dose, ×10).

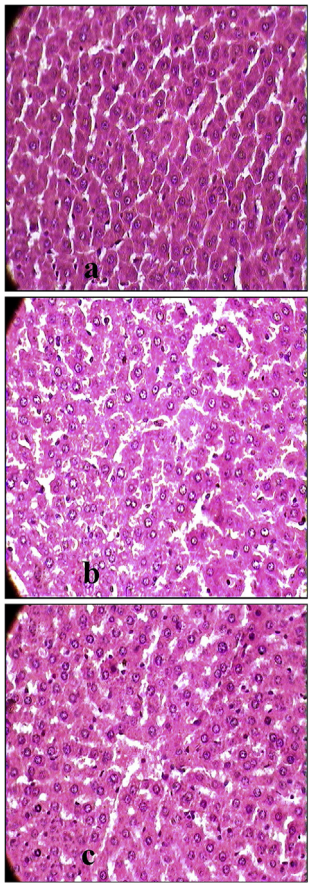

Fig. 2. Photomicrographs of sections of the liver taken at ×400 (a) normal cytoarchitecture (control group) (b) micro fatty changes (*Tamra Bhasma* therapeutic equivalent dose, ×10) (c) micro fatty changes (*Tamra Bhasma* with *Amritikarana* therapeutic equivalent dose, ×10).

in vivo and *in vitro* [37]. This may be the reason behind decreased blood sugar level. However, the observed values are still within normal range [38].

Significant increase in cholesterol level was found in TB and TBA treated groups at TED and TED × 5 dose levels while higher dose of both drugs produced nonsignificant increase in serum cholesterol level. Significant increase in serum triglyceride, LDL, and VLDL level was found in TB at TED × 10 treated groups while nonsignificant increase in triglyceride and VLDL level in all TBA treated groups in comparison to control group. This suggests that the test drugs may have effect on cholesterol turnover in the body; however, parameters were not affected to significant extent at higher dose level in TBA treated group. Though tendency toward increasing of lipid profile is seen in TBA treated group, it may not be indicative of any drastic influence on lipid metabolism, but TB treated rats prone to produce hyperlipidemia and related disorders.

HDL-cholesterol was significantly increased in TB treated group at TED dose level and TBA treated group at TED and TED × 5 treated groups while both at higher dose levels produced nonsignificant increase in comparison to control group. This activity seen at therapeutic dose levels in TB and TBA can have good therapeutic application because elevation of HDL-cholesterol level will be quite useful in patients with hypercholesterolemia conditions. However, same was not observed at higher dose levels in both the treated group.

Parameters related to normal liver functioning such as SGPT, SGOT, alkaline phosphatase, and bilirubin were not altered to significant extent at all dose levels in both treated groups in comparison to control group. The values observed were still within normal range [38]. Significant increase in albumin was observed at all dose levels of TB and higher dose level of TBA. Albumin is a most abundant protein in human blood plasma and synthesized in liver as preproalbumin. However, the values of total protein, globulin, uric acid, calcium, urea, and creatinine in treated groups and control group are still within normal range that suggests that both drugs are devoid of any drastic toxic effect in rats at therapeutic dose level.

The results of histopathological studies revealed that mild changes were observed in four visceral organs, i.e., thymus, heart, liver, and kidney at higher dose level. TBA showed less magnitude of adverse changes compared to TB without *Amritikarana*. However, these changes were not observed in both the drug treated groups at lower dose levels in rats, which suggest that the drug is devoid of drastic toxicity. Earlier studies with TB also reported safety of the sample at TED level [9]. Considering this, it can be said that the *Bhasma* is safe at TED levels, and mild changes can manifest at higher dose levels.

Overall, effect of both drugs, TB and TBA at different dose levels compared with control group. Further, there is no significant difference between TB and TBA treated groups in any of the observed parameters. TB and TBA produced almost same magnitude of effect except TB produced changes on alkaline phosphatase, triglyceride, LDL-cholesterol, and total bilirubin. TBA produced more effect on HDL-cholesterol which may have good therapeutic application and will be quite useful in patients with hypercholesterolemia conditions. In histopathological observations, TBA

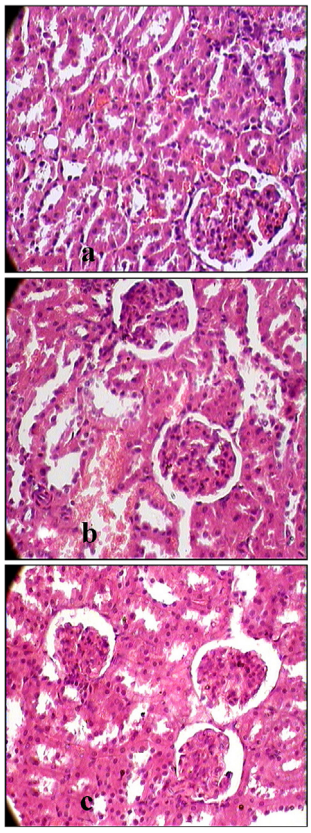

Fig. 3. Photomicrographs of sections of the kidney taken at ×400 (a) normal cytoarchitecture (control group) (b) mild cell infiltration and edematous changes (*Tamra Bhasma* therapeutic equivalent dose, ×10) (c) mild fatty changes (*Tamra Bhasma* with *Amritikarana* therapeutic equivalent dose, ×10).

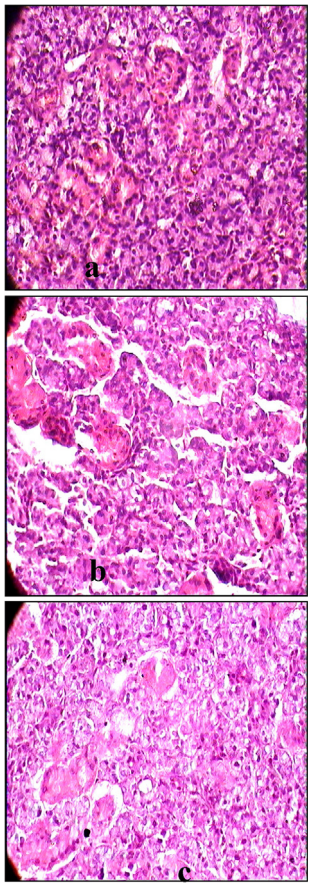

Fig. 4. Photomicrographs of sections of the thymus taken at ×400 (a) normal cytoarchitecture (control group) (b) mild decrease in cellularity (*Tamra Bhasma* Therapeutic equivalent dose, ×10) (c) mild decrease in cellularity (*Tamra Bhasma* with *Amritikarana* therapeutic equivalent dose, ×10).

showed less magnitude of adverse changes compared to TB. TB produced fatty changes in heart, which was absent in TBA treated group. Considering all these, it suggests that, though TB is not much toxic at therapeutic dose level has potential to produced adverse effects when administered for longer duration at higher dose levels. Hence, *Amritikarana* is must for internal administration of TB.

5. Conclusion

From the present study, it is concluded that TBA and TB at the dose level of 2000 mg/kg, orally did not produce any observable toxic effects and mortality in acute toxicity study which suggest that LD_{50} value may be higher than 2000 mg/kg by oral route and can be categorized as substances with low health hazard potential. The results of subchronic toxicity concluded that TB can be relatively safe at therapeutic dose levels in rats. However, TB at TED × 10 dose level, equivalent of which are not likely to be ever employed in clinical conditions, has prone to produce toxic changes in liver, kidney, and heart and also impact on immune-related organs such as thymus as revealed by ponderal, hematological, biochemical, and histopathological parameters in rats. At higher dose level, a sample without *Amritikarana* has more magnitude of toxicity than the sample with *Amritikarana*. This study demonstrates the rational of *Amritikarana* in the safety of TB. Hence, it can be inferred that *Amritikarana* is must for internal administration of TB in clinical practices.

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Conflicts of interest

None declared.

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