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Subacute Oral Toxicity Study of New Formulation of *Bacopa monnieri* in Rats

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Abstract

Brahmi (*Bacopa Monnieri:* BM) is widely used for its memory enhancing properties. The problem with commercially available BM formulations is that, its dose and duration of therapy is high, which indicates; that the permeability of its active components (bacosides) into the brain is meagre and its high doses cause gastric irritation. Hence we hypothesized and prepared a new formulation of BM extract (BME) which can have improved bioavailability so its dose can be low, frequency and duration of therapy is less, to have better tolerability and efficacy. We found that the new lipophylic formulation (20% phosphatidylcholine) of BM had better penetration into the brain due to the increased lipid solubility and had better pharmacokinetic profile than previously marketed formulations. We evaluated subacute toxicity of new formulation of BME and there was no morbidity & mortality, effects on body weight, food consumption, organ weight, urine analysis, hematology, histopathology & serum biochemical parameters. The biochemical parameters like Glucose, Blood Urea, Calcium, SGOT, and SGPT, Creatinine, Cholesterol, Total Bilirubin, Direct Bilirubin and Total Protein doses not shown any significant changes as compared to standard and control group of animals. We can conclude that new formulation of bacopa with 20% phosphotydylcholine is better and safe for oral administration.

Keywords: Bacopa, Phosphatidylcholine, Subacute Toxicity Study

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1. Introduction

Bacopa, also known as *Bacopa monnieri (BM)*, *Herpestis monniera*, water hyssop, and Brahmi, has been used in the Ayurveda for centuries. It has been used as a brain tonic to enhance memory, learning, and concentration.[1] Recent research has focused primarily on BM's cognitive enhancing effects, specifically memory and learning. It is also used in Alzheimer's disease (AD) and dementia.[2] The available psychotherapeutic agents do not subserve the therapeutic demand of mental health problems hence herbal remedies are used with therapeutic hope in such patients.[3]

The problem with commercially available BM formulations is that these have higher recommended dose for the disease like Alzheimer's and dementia & longer duration of therapy which indicates that the permeability of bacosides into the brain is meagre. Furthermore, its high doses and longer duration of therapy causes gastric irritation.[4]

Hence we hypothesized and plan to prepare a new formulation of BM extract (BME) which can have improved bioavailability and more ability to cross BBB so its dose can be low, frequency and duration of therapy less, to have better tolerability and efficacy.

The toxicological study is mandatory after the development of a new drug or formulation as per the guidelines specified by "Organisation for Economic Co-operation and Development" (OECD). In the assessment and evaluation of the toxic characteristics of a chemical, the determination of oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained by acute testing. This study provides information on the possible health hazards likely to arise

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from repeated exposure over a relatively limited period of time. The method comprises of basic repeated dose toxicity study that may be used for chemicals on which a 90 day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary to a long term study. The duration of exposure should normally be 28 days although a 14day study may be appropriate in certain circumstances.[5]

2. Objectives

To assess the subchronic toxicity of marketed and new formulation of BM administered orally to rats for 28 days. This will provide a rational basis for toxicological risk assessment in humans.

3. Material and Methods

Selection of doses: The dose of test substances for 28 day toxicity study was selected on the basis of assay of new formulation.

4. Preparation of Samples

The test substances were suspended in 0.5% carboxy methyl cellulose (CMC). Concentration of both the samples was 10mg/ml.

5. Animal care and handling

The experiment was carried out on adult Wistar albino rats, weighing between 140-180gm, of either sex. The animals were acclimatized for five days prior to experiment in standard laboratory conditions in cross ventilated animal house of the Institute at temperature 22±3°C, relative humidity 45–55%, light and dark cycles of 12:12 hours, fed standard pallet diet and given water *ad libitum*. The toxicological study was approved by the institutional animal ethics committee.[5]

6. Procedure

Healthy young adult rats were randomly assigned to either control or treatment groups. The test compounds were administered by gavage. The rats were divided into three groups comprising of 10 animals, five male and five female, in each group. At the end of study, animals were euthanized by chloroform, and blood collected through retro-orbital puncture, then animals were killed by cervical dislocation for the study of the organs.

The animal groups constituted were:

Group I: Control, received vehicle 1ml/100gm

Group II: Received standard formulation (30.83mg/kg p.o.) once daily for 28 days

Group III: Received test formulation (45.628mg/kg p.o.) once daily for 28 days

6. Collection of Urine

The rats were kept in metabolic cages and urine samples were collected. The animals had free access to drinking water during the urine collection period. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4^0C.[6]

7. Blood collection

At the end of study, blood was collected by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min. at room temperature for serum analysis. Serum was separated by centrifugation at 3000 rpm at 4˚C for 15 min. and utilized for the estimation of bio-chemical parameters.[7]

Animals were killed and liver, brain and kidney were excised and fixed in normal saline solution. The fixed organs were cut to approximately 1mm thick slices, and after tissue processing, paraffin sections of 5µm thickness were prepared and then stained with haematoxylin and eosin (H&E).[8]

8. Parameters

Morbidity/ Mortality: The rats were observed once daily for morbidity/ mortality during the entire observation period.

Body weight: Body weight of each rat was recorded just prior to the administration of test formulations on day 0and subsequently weekly thereafter.

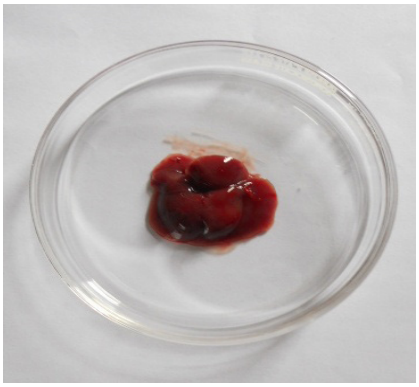
Haematology: At the end of the test period the hematocrit, haemoglobin concentration, erythrocyte count, leucocyte count, platelet count and differential leucocyte count were done.

Clinical Biochemistry:[9-11]

- Serum analysis: The clinical biochemical parameters in serum evaluated at the end of the test period were blood glucose, total cholesterol, total protein, albumin, SGOT, SGPT, total bilirubin, creatinine and urea.
- Urine analysis: The urine analysis parameters evaluated at the end of the test period included urine volume, appearance, pH, specific gravity, creatinine, protein
- and glucose.

9. Organ weight and histopathology

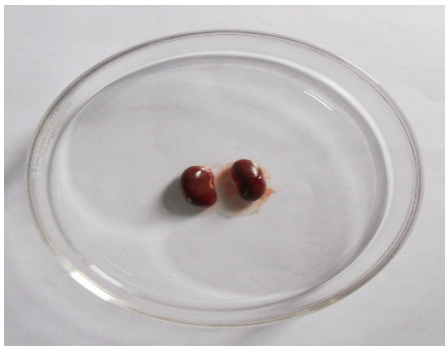
At the end of the study the rats were killed and various organs were excised, their wet weight measured and then organs were fixed in normal saline solution for histological examination.



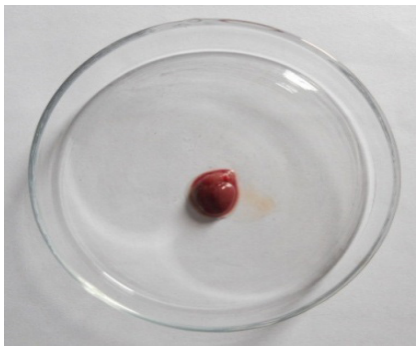
Isolated liver



Isolated spleen



Isolated kidney



Isolated heart



Isolated brain



All organs in formalin solution

Figure 1. Isolated organs.

10. Statistical Analysis

All the values are expressed as mean±standard error of mean (SEM) and analyzed for ANOVA and posthoc Tukey-Kramer Multiple Comparisons Test by employing statistical software, GraphPad InStat 3.1 Mean differences were considered significant if the p value was less than 0.05.

11. Results

On the analysis of various parameters of 28 days subacute toxicological study in rat as per the OECD guidelines we found that.

Table 1. Mortality data

Groups	No. of rats treated	(%) Mortality (upto 28 days)	
		Male	Female
I	5 Males + 5 Females	0	0
II	5 Males + 5 Females	0	0
III	5 Males + 5 Females	0	0

The data of the table shows that no mortality occurred in any of the animal groups.

The data shows that no significant change was found in mean body weight of animals on the given days of observation.

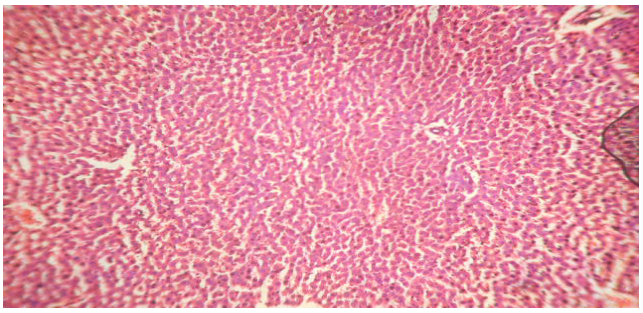
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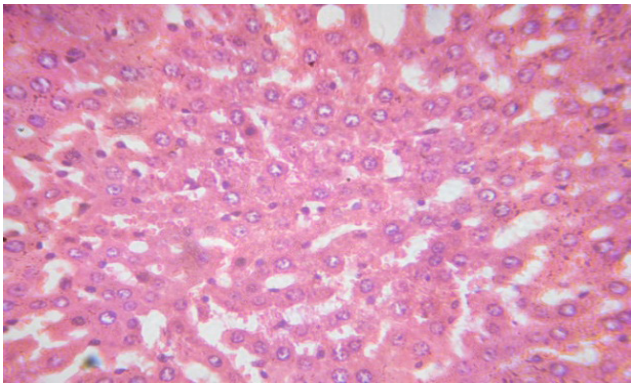
There were no treatment related changes in biochemical parameters.

Urine volume and pH of test groups increased with interventional drug administration.

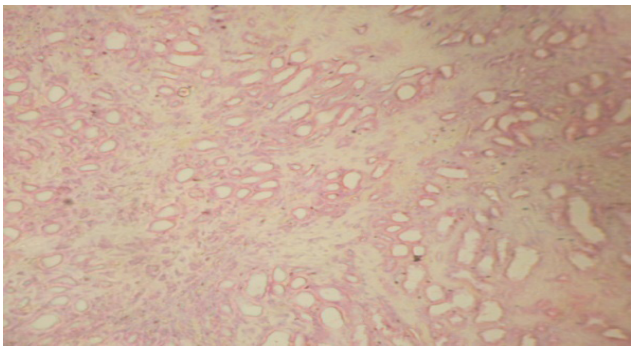
No significant change was found in the weight of the various organs in both treatment groups as compared to the control.



(A). Histopathological Slide (Group I, Liver).

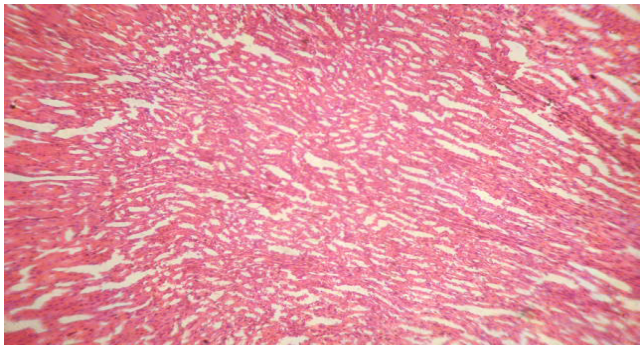


(B). Histopathological Slide (Group I, Kidney).

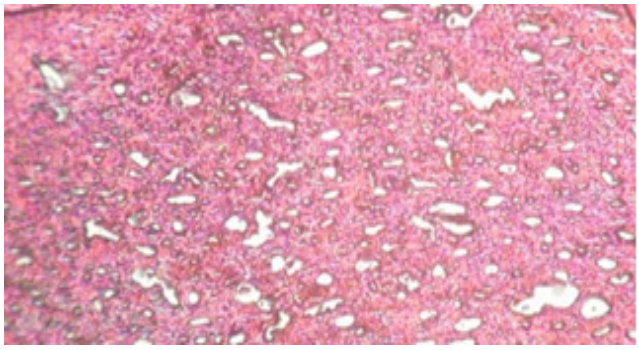


(C). Histopathological Slide (Group I, Brain).

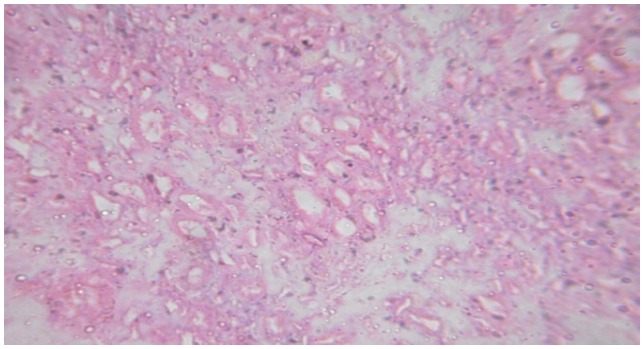
Figure 2. Histopathology of Group I: Control Group.



(A). Histopathological Slide (Group II, Liver).

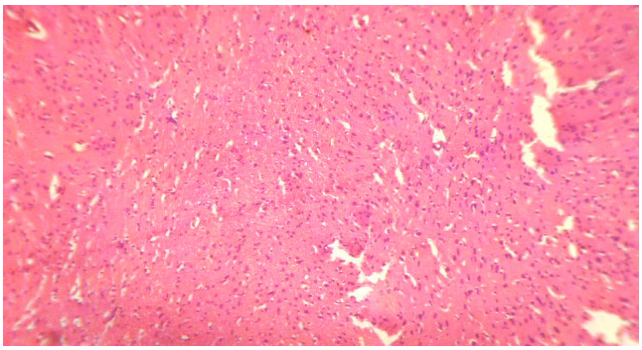


(B). Histopathological Slide (Group II, Kidney).

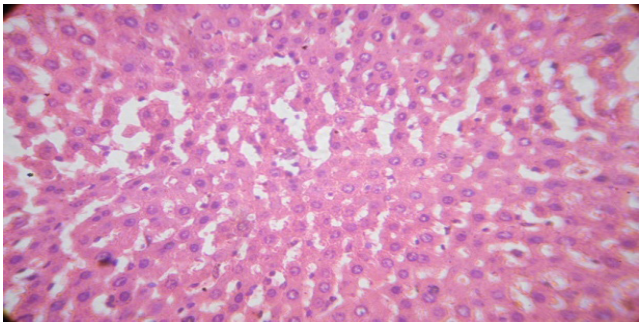


(C). Histopathological Slide (Group II, Brain).

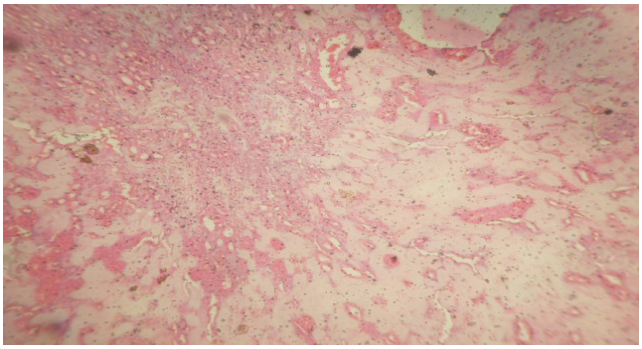
Figure 3. Group II: Standard Group.



(A). Histopathological Slide (Group III, Liver).



(B). Histopathological Slide (Group III, Kidney).



(C). Histopathological Slide (Group III, Brain).

Figure 4. Group III: Test Group.

Table 2. Mean Body Weight

Body Weight (gm)	Groups					
	I		II		III	
	Male	Female	Male	Female	Male	Female
Day 0	145.6±5.34	140.8±5.57	165.32±3.43	155.0±5.09	176.8±3.79	172.44±4.56
Day 7	145.0±5.76	140.0±6.05	164.5±3.79	153.6±4.49	174.0±4.54	170.4±4.9
Day 14	142.6±4.78	142.8±4.44	161.52±4.56	152.0±5.34	173.11±5.87	168.04±4.21
Day 21	143.0±4.74	142.5±4.52	160.4±4.5	150.7±5.41	172.0±5.74	167.0±5.63
Day 28	143.45±5.23	143.2±5.34	160.56±5.78	150.6±4.98	170.76±5.41	165.5±5.62

All values are mean ± SEM, n = 5

Table 3. Haematological data

Haematological parameters	Groups					
	I		II		III	
	Male	Female	Male	Female	Male	Female
Hb (g/dl)	14.9±1.21	14.3±0.45	14.7±1.34	14.4±0.3	14.4±1.23	14.6±0.49
RBC (millions/mm^3)	7.2±1.43	6.9±1.78	7.4±1.22	6.7±1.71	7.1±1.09	6.5±1.07
WBC (thousands/mm^3)	5.5±1.87	4.4±1.65	5.4±1.23	4.6±1.07	5.4±0.98	4.66±1.37
Platelet (x 10^3/µl)	425±5.57	395.5±7.54	423±8.93	395.7±7.23	426±6.65	395.3±9.53
Hematocrit (%)	44.2±3.07	42.4±3.83	41.5±3.45	40.7±3.97	41.4±2.97	39.3±2.76

All values are mean ± SEM, n = 5

Table 4. Differential leucocyte count

Differential (%)	Groups					
	I		II		III	
	Male	Female	Male	Female	Male	Female
Neutrophils	20.6±1.34	22±1.54	20.3±1.16	24±1.15	20.5±1.24	21.0±0.87
Basophils	0	0	0	0	0	0
Eosinophils	1.8±0.14	1±0.09	1.6±0.1	1.2±0.13	1.5±0.19	1.0±0.18
Lymphocytes	75.4±2.39	75.0±3.73	75.7±3.27	72.5±2.76	75.9±2.55	75.8±2.89
Monocytes	2.2±0.17	2.0±0.07	2.4±0.18	2.3±0.17	2.1±0.11	2.2±0.22

All values are mean ± SEM, n = 5

Table 5. Biochemical data

Biochemical parameters	Groups					
	I		II		III	
	Male	Female	Male	Female	Male	Female
Blood Glucose (mg/dl)	90.8±2.56	100.3±3.39	91.7±2.54	102.4±3.65	90.2±2.22	90.87±2.0
T-Cholesterol (mg/dl)	112.34±4.39	111.6±4.87	112.4±4.76	111.9±4.56	111.8±5.69	112.2±4.87
Total protein (g/dl)	4.5±0.19	4.3±0.12	4.2±0.15	4.6±0.11	4.1±0.15	4.3±0.15
Albumin (g/dl)	2.9±0.12	2.7±0.10	2.3±0.19	2.6±0.15	3.0±0.12	2.8±0.13
Total Bilirubin (mg/dl)	0.3±0.07	0.5±0.02	0.4±0.03	0.3±0.03	0.2±0.01	0.3±0.06
SGOT (IU/l)	55.7±2.19	52.0±2.76	54.37±2.87	55.98±2.73	53.0±2.39	52.0±3.59
SGPT (IU/l)	22.7±2.33	22.7±2.09	20.87±2.76	23.8±2.54	24.76±2.03	22.0±2.77
Creatinine (mg/dl)	0.4±0.03	0.2±0.01	0.5±0.05	0.3±0.07	0.6±0.05	0.4±0.02
Urea (mg/dl)	21±2.87	23.3±2.9	23.5±2.0	22.7±2.18	21.4±2.14	22.8±2.76

All values are mean ± SEM, n = 5

Table 6. Urine analysis

Urinary parameters (24-hr urine)	Groups					
	I		II		III	
	Male	Female	Male	Female	Male	Female
Appearance	Light yellow	Light yellow	Yellow	Yellow	Yellow	Yellow
pH	7.6±0.32	7.8±0.68	8.2±0.88	8.5±0.48	8.3±0.57	8.4±0.64
Urine volume (ml)	5.34±0.22	5.87±0.38	6.38±0.72	6.87±0.48	7.17±0.43	7.5±0.12
Specific gravity	1.013±0.00	1.016±0.006	1.015±0.00	1.018±0.00	1.015±0.00	1.019±0.00
Albumin (mg/24hr)	56.6±2.39	59.9±2.98	60.43±2.87	61.3±2.09	60.98±2.8	62.0±2.22
Glucose	Absent	Absent	Absent	Absent	Absent	Absent

All values are mean ± SEM, n = 5

Table 7. Organ Weights

Organ Weights (gm)	Groups					
	I		II		III	
	Male	Female	Male	Female	Male	Female
Liver	5.67±0.76	5.25±0.45	5.97±0.31	5.88±0.64	6.2±0.59	6.6±0.54
Kidney	0.8±0.06	0.87±0.08	1.06±0.054	1.05±0.05	1.5±0.08	1.6±0.06
Brain	1.1±0.34	1.2±0.33	1.4±0.45	1.5±0.14	1.6±0.39	1.6±0.25
Spleen	0.67±0.05	0.71±0.02	0.74±0.05	0.75±0.03	0.77±0.04	0.78±0.04
Heart	0.42±0.04	0.38±0.03	0.64±0.02	0.5±0.05	0.65±0.04	0.68±0.0.3

All values are mean ± SEM, n = 5

When histological findings of various organs of test groups were compared to normal, no changes were found in the cellular part.

12. Discussion

The trend of using herbal formulations is increasing globally, the reason being better acceptability and belief that all natural products are safe. But there is evidence that using some Ayurvedic medicine involves potentially serious risks, including toxicity. Despite the widespread use, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies.[12]

Our research shows that, the new lipophylic formulation of BM based on 20% phosphatidylcholine has shown better penetration into the brain due to the increased lipid solubility and has better pharmacokinetic profile then previously marketed formulations. Due to high penetration of bacosides and their metabolites in the brain, the dose, frequency and duration of therapy found to be decreased; in present study after performing subacute toxicological study of new formulation we did not find any morbidity and mortality in rats after 28 days of the drug administration. Our study is also supported by Allen et al 2007 and they found that the drug is well tolerated up to the dose 500 mg/kg on 14 days repeated dose oral toxicity and A subchronic oral toxicity study for 90 days in rats at the dose levels of 85, 210 and 500 mg/kg did not reveal any evidence of morbidity, mortality and toxicity. [13]

There was no significant change in mean body weight from start to end of the study [Table 2] the changes in body weight have been used as an indicator of adverse effects of drugs /chemicals. The results suggest that with the administered oral doses the new formulation of BME was non-toxic in rats.[14]

In 28 days the hematological study reveals anomalies in body metabolic processes and the blood profile delivers vital information on the response of the body to injury, deficiency and/or stress. No significant difference in blood parameters were observed between treatment groups treated with new formulation of BME, standard and control group. Mostly all the values remained within the normal limits [Table 3, 4] for rats and did not suggest toxic effects in subacute toxicity study. The determination of such parameters is important in the study of safety of the product with therapeutic intentions. Analysis of parameter related to the blood is relevant to risk evaluation as the hematological system has a higher predictive value for toxicity in humans (91%) with assays in rodents and non-rodents.[15]

Blood glucose is indicator of diabetes mellitus. High range of cholesterol indicates the risk of stroke being higher.[16]

There were no adverse effects on usual markers of liver and kidney toxicity (plasma levels of liver enzymes, ALT and AST, bilirubin, urea and creatinine).Hence it may be concluded that the new formulation of BME did not induce any significant damage to these organs when compared to the standard and control group of animals.

Increase of transaminases (SGOT, SGPT) which are indicators of liver damage, the AST is also found in a large no of tissues, such as heart, lung, skeletal muscle and kidney, whereas ALT is primarily limited to liver. Thus the latter is considered a highly sensitive indicator of hepatotoxicity. Since the new formulation of BME did not alter SGOT and SGPT, it can be concluded that BME does not have any hepatotoxicity.[15]

Raised urea and non-protein nitrogen level in blood have been observed with impaired renal function or in acute renal failure. Since the new BME formulation dose did not show any significant change it indicates that this formulation does not show any renal toxicity [Table 5].

In urine analysis, urine volume and pH of test groups was increased, indicating the excretion of sample through kidney and presence of basic compound in urine. These changes may not indicate the toxicologically sign of kidneys [Table 6].

No significant change was found in the weight of the various organs in sample treated groups when compared to normal control [Table 7]. None of the rats showed any macroscopic lesions in test sample treated groups.

The histological study of various organs [Figure 2-4] of test group when compared to standard and control, no changes were seen in cellular part. This corroborated the results of biochemical analysis. Hence we can say that the oral administration of new formulation of BME, after 28 days subacute toxicological study, was well tolerated by the treated rats.

13. Conclusion

The haematological, biochemical, urinalysis and histological results indicating that the test samples of

BME were safe when administered orally for 28 days in rats. Hence it can be concluded that the new formulation of BME which has more penetration into brain with better pharmacokinetic profile, is safe for oral administration. It will help the patients suffering from disease like AD and dementia.

14. References

1. Mukherjee DG, Dey, CD. Clinical trial on Brahmi. I. J Exp Med Sci. 1996; 10:5–11.
2. Holcomb LA, Dhanasekaran M, Hitt AR, Young KA, Riggs M, Manyam BV. Bacopa monniera extract reduces amyloid levels in PSAPP mice. J Alzheimers Dis. 2006; 9(3):243–51.
3. Husain GM, Mishra D, Singh PN, Rao CV, Kumar V. Ethnopharmacological review of native traditional medicinal plants or brain disorders. Pharmacognocy Review. 2007; 1:20–8.
4. Morgan A, Stevens J. Does Bacopa monnieri improve memory performance in older persons? Results of a randomized, placebo-controlled, double-blind trial. J Altern Complement Med. 2010; 16(7):753–9.
5. OECD Guidelines for the Testing of Chemicals (No. 407, Section 4: Health Effects) Repeated Dose 28-Day Oral Toxicity in Rodents (Adopted on 12 May 1981 and Updated on 27 July 1995). [cited in 2015]. Available from: http://www.oecd.org/chemicalsafety/testing/37477972.pdf
6. Sridevi K, Ravishankar K, Kiranmayi GVN. Evaluation of diuretic and antiurolithiatic activities of ethanolic leaf extract of basellaalba. Int J Pharm. 2014; 4(1):145–9.
7. Dash DK, Yeligar VC, Nayak SS, Ghosh T, Rajalingam D, Sengupta P et al. Evaluation of hepatoprotective and antioxidant activity of Ichnocarpus frutescens (Linn.) R.Br. on paracetamol-induced hepatotoxicity in rats. Trop J Pharm Res. 2007; 6(3):755–65.
8. Chapter 1. [cited in 2015]. Available from: http://www.rajswasthya.nic.in/RHSDP%20Training%20Modules/Lab.%20Tech/Histo/Introduction.pdf
9. Chitra B, Ramaswamy RS, Suba V. Toxicity Evaluation of Purna Cantirotaya Centuram, a Siddha Medicine in Wistar Rats. Int sch res notices. 2015; 1–10.
10. Complete Urinalysis. [cited in 2015]. Available from: http://www.highlands.edu/academics/divisions/scipe/biology/labs/cartersville/2122/urinalysis.htm
11. Cho SH, Yoon Y, Yang Y. The Evaluation of the Body Weight Lowering Effects of Herbal Extract THI on Exercising Healthy Overweight Humans: A Randomized Double-Blind, Placebo-Controlled Trial. Evid Based Complementary Altern Med. 2013; 1–8.
12. Pattanik N, Singh AV, Panday RS, Singh BS, Kumar M, Dixit SK et al. Toxicological and free radical scavenging property of tamrabhasma. Indian J ClinBiochem. 2003; 18(2):181–9.
13. Allan JJ, Damodaran A, Deshmukh NS, Goudar KS, Amit A. Safety evaluation of a standardized phytochemical composition extracted from Bacopa monnieri in Sprague-Dawley rats. Food Chem Toxicol. 2007; 45(10):1928–37.
14. Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V. A 90 day oral gavage toxicity study of d-methylphenidate and d, l-methylphenidate in Sprague–Dawley rats. Toxicology. 2002; 179:183–96.
15. Feldman DL, Mogelesky TC, Sharif R, Sawyer WK, Jeune M, Hu CW et al. The in vitro and ex vivo antioxidant properties and hypolipidemic activity of CGP 2881. Atherosclerosis. 1999; 144(2):343–55.
16. Cholesterol Abnormalities and Diabetes. [cited in 2015]. Available from: http://www.heart.org/HEARTORG/Conditions/Diabetes/WhyDiabetesMatters/Cholesterol-Abnormalities-Diabetes_UCM_313868_Article.jsp