EVALUATION OF TOXICITY AND ANTIASTHMATIC POTENTIAL OF AN AYURVEDIC FORMULATION ON EXPERIMENTAL ANIMALS

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ABSTRACT

The aim of this study was to explore acute and chronic toxicity as well as antiasthmatic potential of the ayurvedic formulation Nayopayam Kashayam on experimental animals. The present study was targeted for the study of its toxicity profile along with its antiasthmatic activity. The acute toxicity study was carried out using OECD 425 CPCSEA guideline in albino Wistar rats. Oral acute toxicity study was performed at 2000mg/kg orally, which was considered as limit dose. The chronic toxicity study was carried out with administration of Nayopayam Kashayam at three therapeutic equivalent doses i.e. TED (45mg/kg, orally), TEDx5 (225 mg/kg, orally) and TEDx10 (450 mg/kg, orally) for 90 days. Further, antiasthmatic study was carried out using histamine-induced bronchospasm in guinea pig model. The results of acute toxicity studies showed that drug did not create any signs and symptoms of toxicity and no mortality was shown to an oral dose of 2000 mg/kg in rats. The results of chronic toxicity study showed that the drug even at level as high as dose of TEDx10 had no significant effect at all on hematological and body weight parameters, however mild to moderate unfavorable changes in kidney and liver were indicated. The experiential changes were not seen at the lower dose levels. The drug also showed a marked decrease in hiccups of asthma during antiasthmatic study. Hence, it is suggested that the Nayopayam Kashayam, prepared as per the traditional method, is secured/safe for utilization and treatment of asthma at the therapeutic dose level.

Keywords: Nayopayam Kashayam, toxicity, Asthma

ABBREVIATIONS

TED: Therapeutic effective dose; **IAEC**: Institutional Animal Ethics Committee; **mg/d** Milligram/day; **OECD**: Organization for Economic Co-operation and Development; **RBC**: Red blood cells; **Hb:**Haemoglobin; **PCV**: Packed Cell Volume; **MCV**: Mean Corpuscular Volume; **MCH**: Mean Corpuscular Haemoglobin; **MCHC**: Mean Corpuscular Haemoglobin Concentration; **WBC**: White Blood Cells; **%N**: Percentage Neutrophill; **%L**: Percentage Lymphocyte; **PC**: Platelet Count; **HDL**: High Density Lipoprotein; **VLDL**: Very Low Density Lipoprotein; **LDL**: Low Density Lipoprotein; **SGOT**: Serum Glutamic Oxaloacetic Transaminase; **SGPT**: Serum Glutamate–Pyruvate Transaminase.

INTRODUCTION

Ayurveda is an ancient Indian traditional system of medicine. The medicinal treatment offered by it shares a great historical background. It has enhanced the living conditions of various communities of the world[1]. The Indian traditional practioners have developed these processes to detoxify chemical transformation and to modify the therapeutic potential of the combinatorial formulations. Extensive use of these formulations since decades without any unwanted effect is the best proof of their therapeutic efficacy and safety[2].

The main problem with these formulations is the non-existence of preclinical or clinical data to support such claims. Preclinical or clinical data of Ayurvedic formulations provides scientific basis for their traditional use and to prove that they are safe and efficacious[3]. Nayopayam Kashayam is a herbal decoction and it contains *Sida cordifolia* (Bala), *Cuminum cyminum* (Jiraka) and *Zingiber officinale* (Viswam), as described in Sahasrayoga. It is a very good bronchodilator and carminative. It is indicated for respiratory diseases like hiccups (Hikka), bronchial asthma (Tamakassasa), gas trouble and cardiac diseases[4].

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Since reports of toxicity evaluation of this classical preparation were not available during extensive literature review, it was thought worthwhile to undertake detailed toxicity and antiasthmatic assessment in albino rats.

MATERIALS AND METHODS

Nayoayam Kashayam formulation was procured from an ayurvedic shop in Raipur, C.G., India.

Experimental animals

Wistar female albino rats of either sex weighing 200±20 g bodyweight were used for the study. The animals were maintained under ideal husbandry conditions in terms of standard conditions of temperature (23±2°C) and relative humidity (50 to 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 Institutional Animal Ethical Committee (CIP/IAEC/2015-2016/065) and is registered (Regd. No.1321/PO/Re Bi/S/10/CPCSEA) as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, India.

Dose selection

The recommended human dose of Nayopayam Kashayam is 45 mL/day. The dose was converted for albino Wistar rat (200-250 g) using conversion table. The suitable dose for the rat was found to be 0.73 mL, calculated by referring to the table of Paget and Barnes[5] and this was considered as therapeutic equivalent dose (TED).

Acute toxicity studies

Young, healthy, non-pregnant Wistar-strain albino female rats were selected and acclimatized for seven days before the experiment. The Nayopayam Kashayam was orally administered at a limit dose of 2000 mg/kg to overnight fasted female rats in sequential manner as per OECD 425 guidelines[6,7]. The rats were observed closely for behavioral changes, signs and symptoms of toxicity and mortality continuously for the first six h; and thereafter, periodically up to 14 d. The body weight of each rat was noted on the last day and the rats were sacrificed. The abdomen was opened through mid-line incision to record the autopsy changes, followed by dissecting the important organs for histopathological changes.

Chronic toxicity studies

The chronic toxicity study was carried out by standard guideline with modification as per experimental need[8]. Rats were randomized into six groups of six rats in each with three males and three females. Group I was kept as control group, received vehicle as honey solution in distilled water (5 mL/kg, orally). Groups II to IV were administered with test drug Nayopayam Kashayam along with adjuvant at TED (45 mg/kg, orally), TED×5 (225 mg/kg, orally) and TED×10 (450 mg/kg, orally), respectively. The suspensions of test drug were administered orally once-a-day for 90 consecutive days in the main study. Additional six animals were kept in satellite control group (V) and in the recovery TED×10 treated groups (VI) for observation after the treatment period, and thereafter weekly during the study period. At the end of experimental periods, blood was withdrawn by the retro-orbital puncture under light-ether anesthesia using capillary tube for estimation of serum biochemical and hematological parameters. The body weight of each rat was noted on the last day and the rats were sacrificed. The abdomen was opened through mid-line incision to record the autopsy changes, followed by dissecting out the important organs.

Hematological analysis was performed using an automatic hematological analyzer (Swelab, Sweden). The parameters were total red blood cell (RBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC), neutrophils percentage (%N), lymphocyte percentage (%L), eosinophil percentage, monocyte percentage and platelet count (PC).

Serum bio-chemical parameters were determined using fully automated biochemical random access analyser (BS-200, Lilac Medicare Pvt. Ltd., Mumbai). The parameters were blood glucose[8], urea[9], creatinine[10], total cholesterol[11], HDL-cholesterol[12], triglyceride[13], VLDLcholesterol[14], LDL-cholesterol[15], total protein[16], albumin, globulin[17], alkaline Phosphatase[18], SGOT[19], SGPT[20], uric acid[21], direct bilirubin[22], total bilirubin[23] and serum calcium[24].

The important internal organs, namely, liver and kidney, were carefully dissected. After noting for any signs of gross lesion and ponderable 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 haematoxylin and eosin (H and E) for histological evaluation by light microscopy. 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 for reversibility or persistence of any toxic effects. The duration of

post-treatment period was fixed as 30 d (total of 120 d, including 90 d treatment period and 30 d recovery period). All the animals were dosed with constant dose volume of 5 mL/kg, orally. The rats were observed daily, carefully for any overt and apparent signs and symptoms of toxicity. The bodyweight change of an individual rats was noted initially.

Histamine induced bronchospasm in guinea pigs

Experimental conditions

Male guinea pigs weighing 300-400g were used for antiasthmatic study. The animals were maintained under normal laboratory conditions and kept in standard polypropylene cages at room temperature and 50 to 60% relative humidity and provided with standard diet and water ad *libitum*. All animals were kept in experimental room. The room was well- ventilated (> 10 air changes per hour) with 100 % fresh air. A 12-h light/dark photoperiod was maintained[25].

Dosing and administration of test compound

Guinea pigs were kept in a closed histamine chamber and exposed to an aerosol of 0.1 % w/V histamine dihydrochloride through a nebulizer. The time required for the onset of preconvulsive dyspnoea (PCD) was recorded from the time of aerosol exposure to the onset of dyspnoea leading to the appearance of convulsions. As soon as PCD commenced, animals were immediately withdrawn from the inhalation box and placed in fresh air to recover. Guinea pigs were divided into different groups, with six animals in each group. Test group was subcutaneously treated with Nayopayam Kashayam and salbutamol at a dose 2 mg/kg body weight orally was administered to the standard group. All the groups were given a single dose treatment for seven days. On the seventh day, last dose was administered and the time for the onset of PCT was recorded on 0 day. The increases in PCD onset time by the animals was calculated by the following formula[25].

Percent increase in PCD time = [After treatment - Before Treatment / After Treatment x 100]

Statistical analysis

The data is expressed as mean±standard error of mean for six rats per experimental group. One-way analysis of variance (ANOVA) was used to compare the mean values of quantitative variables among the groups, followed by Dunnet's multiple t-test for unpaired data to determine significant difference between groups at $P<0.05$.

RESULT AND DISCUSSION

Acute toxicity study of test drug was carried out to record immediate adverse signs and symptoms of drug in female rats at dose levels that are several folds higher than the therapeutic equivalent dose. Administration of Nayopayam Kashayam did not affect any behavioral changes and other parameters observed during the acute toxicity test in female rats. No signs and symptoms of toxicity and mortality were observed up to oral dose of 2000 mg/kg of test drug in rats. Further, drug did not affect the cytoarchitecture of major organs like kidney and liver, which suggests that LD50 value may be higher than 2000 mg/kg by oral route. As per UN classification, any substance, which has oral LD50 of more than 2000 mg/kg, is considered as low hazard potential and categorized as UN 6.1 PG III[26]. Thus, as per the above criterion, Nayopayam Kashayam can be categorized as substance with low health hazard potential (Class 4 of GHS and UN 6.1 PG III). There were no behavioral changes observed in Nayopayam Kashayam treated groups during the course of chronic toxicity study. No symptoms of toxicity and mortality were observed in treated groups at TED×10, TED×5 and TED dose levels and TED×10 in the recovery study.

Normal body weight gain was observed in control rats during main study (90 d) as well as recovery study (120 d). An increase in body weight was found in Nayopayam Kashayam treated groups at all dose levels. Changes in body weight are an important factor to monitor the health of an animal. Loss of body weight is usually the first sign indicating the onset of an adverse effect. The dose, at which body weight loss is by 10% or more, is considered to be a toxic dose, irrespective of whether or not it is accompanied by any other changes[27,28]. The percentage change in body

Table I: Relative weight of organs of rats recorded during chronic toxicity study

Relative weight	Control	TED	TEDx5	TEDx10	Recovery TEDx10
Liver (g/100g)	7.06±0.03	6.65±0.09*	6.66±0.02	7.16±0.22	8.26±0.24
Kidney (mg/100g)	1.33±0.02	1.30±0.01	1.31±0.02	1.35±0.01*	1.32±0.54

*The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05, compared with control group.*

Table II: Hematological parameters in rats recorded during chronic toxicity study

Hematological parameter	Control group	TED	TEDx5	TEDx10	Recovery TEDx10
Bleeding time	11.43±0.43	11.88±0.88	11.45±0.45*	11.03±0.01	11.74± 0.24
Clotting Time	55.32± 0.72	53.21 ±0.49*	52.73 ±0.36	56.73± 0.34	55.32±0.34
WBC count(10^3/mL)	10161±380.11	4235.75±163.01	7133.5±92.12*	4211.5±90.19	4212.6±155.02
Lymphocyte count(%)	72.05±1.46	73.625±0.4*8	64.125±0.63	77.75±2.22	78.25±0.64
Monocyte count(%)	1.22±0.07	1.14±0.14	1.18±0.17	1.21±0.19	1.20±0.16
Hemoglobin(g/dl)	12.075±0.05	12.28±0.26	13.275±0.28*	12.26±0.25	13.024±0.32
Neutrophil Count(%)	20.04±0.04	20.04±0.04	30.003±0.5*	15.75±0.23	18.32±0.52
Eosinophi Count(%)	6.19±0.21	5.29±0.42*	4.42±0.46	4.45±0.32	5.64±0.28
R.B.C. Count(106/mL)	6.525±0.07	6.7025±0.0*6	6.855±0.05	6.64±0.09	6.90±0.34
Platelet Count(10^3/mL)	7.83±0.12	7.627±0.09	7.20±0.05*	7.62±0.12	7.74±0.52
Mean Platelet value	6.22±0.17	6.11±0.11	6.62±0.22	6.35±0.13*	6.86±0.64
Packed cell volume(%)	32.23±0.12	34.25±0.73	37.3±0.22	33.4±0.22	34.52±0.25
Mean corpuscular volume (fl)	48.39±0.09	51.41±0.24*	53.31±0.29	47.23±4.23	52.54±0.64
Meancorpuscular hemoglobin (pg/red cells)	18.12±0.03	19.59±0.006	18.88±0.09*	18.26±0.02	18.92.0.82
Red cell distribution width(µm)	12.28±0.15	14.37±0.23	14.1±0.19	13.3±0.21*	14.53±0.51

*The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05, compared with control group*

weight pattern in test drug treated groups did not differ significantly from the changes observed in the control groups, which suggests the absence of serious toxic effect of Nayopayam Kashayam during chronic administration in rats. In liver (Table I), Nayopayam Kashayam treated group showed no significant decrease in relative weight of liver in TED treated group, TEDx5 and TEDx10 in comparison to the normal control whereas in case of kidney TED and TEDx5 showed no significant decrease in weight but TEDx10 showed significant increase in weight. Normally, decrease in the weight of an organ is indicative of loss of tissue mass in that organ, exception being the secretary organs in which the decrease in weight sometimes is seen along with the increased activity.

In the present study, there were no remarkable changes observed in the relative weight of the organs at higher doses of test drugs. Hence, it may be understood that the drugs do not tend to produce any serious toxic effect on the relative weight of the important internal organs in chronic toxicity studies.

Analysis of the effects of Nayopayam Kashayam on hematological parameters (Table II) revealed non-significant increase in bleeding time, clotting time and WBC count at each of the dose level studied in the main study in comparison to the control group. The same was the case with lymphocyte and monocyte counts. Hemoglobin count showed significant increase in TEDX10 and recovery group which may be due to increase in formation of the same. The drug showed significant increase in neutrophils in TEDX10 and decrease in recovery group. The increase in neutrophils is a good indication for immune responses which might be responsible for its asthmatic potential. RBC count was normal in the entire group along with PC, MPV, PCV, MCV, MCH and RCDW. However all the values were within the normal range[28]. This clearly indicates that the test drug did not affect the cellular and non-cellular elements of the blood to a significant extent.

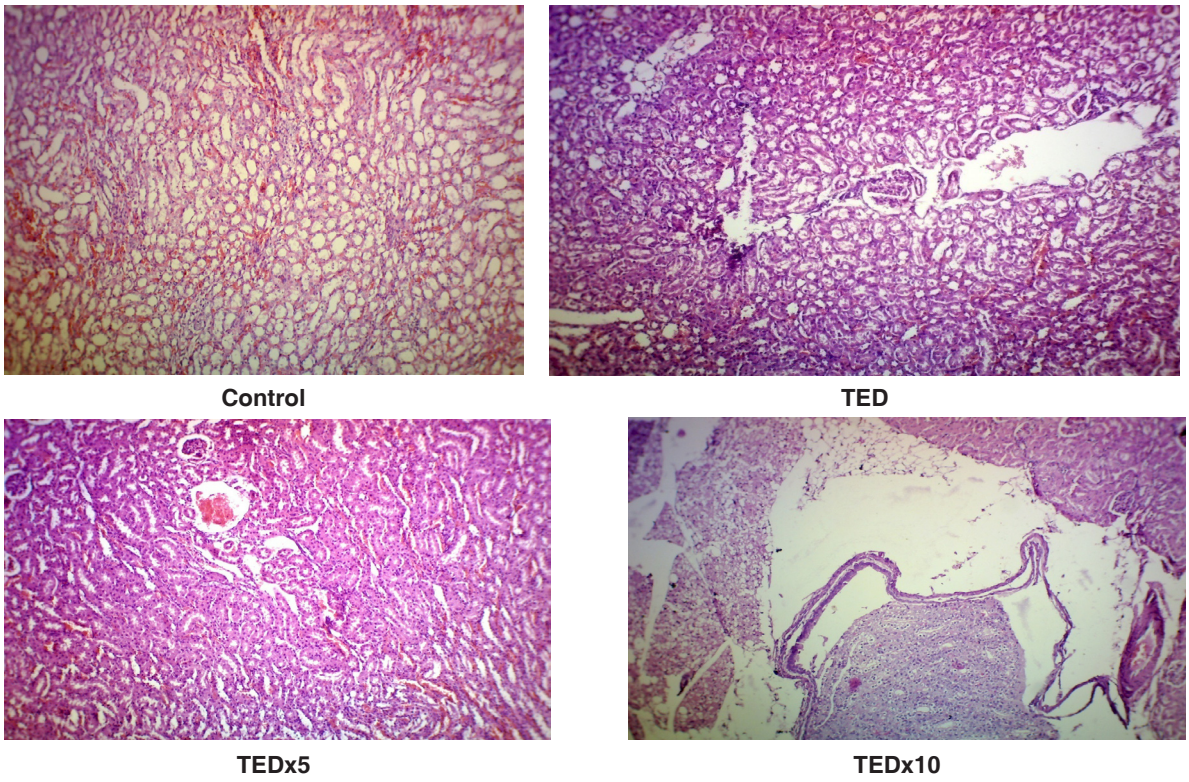

Fig. 1: Histopathology of kidney tissues (x400 magnification) (A) Normal cytoarchitecture (control group), (B) edema and fatty changes (TED×5), (C) oedema, pigment deposition and fatty changes (TED×10)

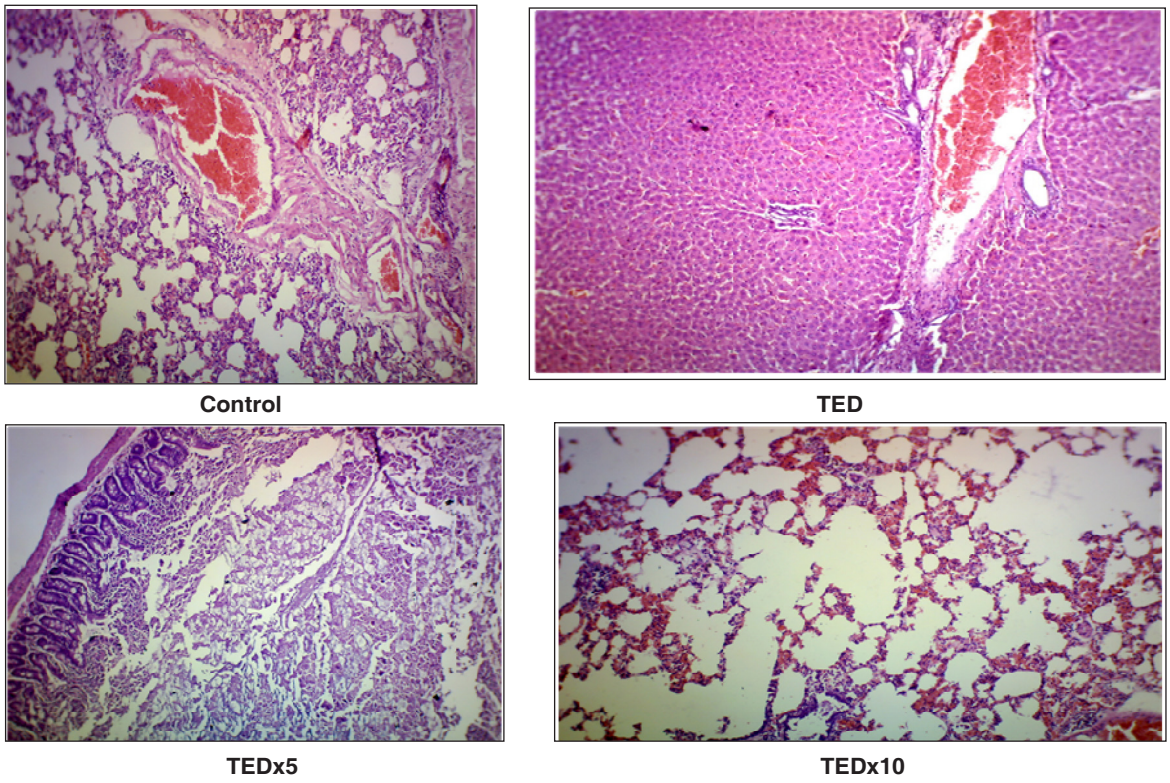

Fig. 2: Histopathology of liver tissues (x400 magnification) (A) Normal cytoarchitecture (control group), (B) pigment deposition, mild necrosis and fatty changes (TED×5), (C) pigment deposition, mild necrosis and fatty changes (TED×10)

Table III: Biochemical parameters in rats recorded during chronic toxicity study

Biochemical Parameter	Control group	TED	TEDx5	TEDx10	Recovery TEDx10
Serum Creatinine (mg/dl)	1.08±0.08	1.4±0.27*	1.6±0.16	0.77±16	1.5±0.34
Total Serum Protein (g/dl)	6.53±0.24	6.85±0.11	6.79±0.16*	6.66±0.31	6.62±0.54
Serum globulin(g/dl)	6.66±0.46	6.01±0.41	5.35±0.17*	6.78±0.30	6.82±0.78
SGPT(IU/L)	27.33±0.56	70.16±0.15	63.77±2.93	18.26±0.73	30.02±0.51
SGOT (IU/L)	164±1.24	31.8±0.81	176.14±1.58*	13.47±0.39	32.58±0.31
Serum alkaline phosphatase (IU/L)	70.43±0.40	52.9±0.62*	14.16±0.29	64.12±0.49	50.81±0.54
Serum cholesterol(mmol/L)	71.08±0.10	45.09±0.09	68.02±0.48	69.55±0.19*	65.04±0.04
Serum triglyceride(mmol/L)	177.30±1.36	60.59±0.40	98.85±0.16*	30.51±0.41	169.54±0.35
Serum HDL Cholesterol (mg/dl)	14.29±0.22	9.0±0.05	13.33±0.28	13.82±0.11*	12.85±0.08
Serum LDL(mg/dl)	21.25±0.17	70.83±31.32*	27.105±15.96	49.74±0.02	20.86±0.45
TGL/HDL Ratio	35.23±0.31	19.29±0.19	12.15±12.15*	6.09±6.11	22.54±0.56
Blood Urea (mg/dl)	1.51±0.45	12.35±0.47	18.11±0.53*	6.02±0.45	2.45±0.05
Serum Uric acid (mg/dl)	1.57±1.51	6.63±6.60	7.30±7.33*	2.17±2.18	2.45±0.42
Blood glucose(mg/dl)	73.25±0.37	50.19±0.16	56.03±0.03	64.07±0.09*	71.48±0.52

*The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05, compared with control group.*

Table IV: Effects of Nayopayam Kashayam on histamine-induced bronchospasm in guinea pigs

Group	Preconvulsion Time (sec)		
	Before Treatment	After Treatment	% Increase in time of PCT
Test	27.93± 0.47	55.98± 0.85	50. ± 0.64
Standard	25.19 ±0.92	65.94 ±1.04	61.79±0.46

*The results are expressed as mean±SEM, where n=6. SEM: Standard error of mean. *P<0.05, compared with control group*

All the other hematological parameters were more or less near to normal, which indicated that there is no major change after administration of the test drug. Analysis of the effects of Nayopayam Kashayam on biochemical parameters (Table III) revealed a significant increase in creatinine level, no significant increase in total serum protein, significant decrease in serum globulin except in TEDx 10, no significant decrease in serum globulin, significant increase in SGPT and significant decrease in SGOT except in TEDx5, significant decrease in alkaline phosphatase and significant increase in blood sugar level. After discontinuation of test drug in the recovery group, the observed changes in glucose level, creatinine and triglyceride were almost same, as seen in the recovery control group (Table IV).

The histopathological studies of the organs showed that Nayopayam Kashayam along with adjuvant at highest dose level exhibited mild to moderate changes in kidney and liver in comparison to the control group. Nayopayam Kashayam produced mild pigment deposition, fatty changes in epithelium and oedematous changes in kidney tubule (Fig. 1). Nayopayam Kashayam TED×10 and TED×5 treated groups exhibited pigment deposition, mild necrosis and fatty changes in the liver in comparison the control group (Fig. 2).

In the case of histamine induced bronchospasm in guinea pig, there was percentage increase in preconvulsion time after treatment with the test drug; however it was less than standard salbutamol solution. This clearly indicates that the test drug might be effective in asthma.

CONCLUSION

The results obtained during the study clearly depicts that the ayurvedic formulation Nayopayam Kashayam showed no sign of toxicity and is safe when properly manufactured and consumed as per directed instruction. The ingredients used in the formation of formulation have various medicinal properties such as hepatoprotective, antioxidant etc and, as a whole Nayopayam Kashayam is a good remedy for the treatment of asthma.

REFERENCES

1. Pandey R., Tiwari R. K. and Shukla S. S.: Omics: A newer technique in herbal drug standardization and quantification, **J. Young. Pharm.**, 2016, 8(2), 76-81.
2. Shukla S. S., Tiwari R. K., Sharma A. and Pandey R. K.:A review on mechanism and plants used for diabetic nephropathy: A curse of diabetes, Mintage , **J. Phar. Med. Sci**., 2019, 8(4), 1-13.
3. Chauhan A., Semwal D. K., Mishra S. P. andSemwal R.B.: Ayurvedic research and methodology: Present status and future strategies, **Ayu**, 2015, 36(4), 364–369.
4. Shah S.: Ayurveda: The Conventional Indian Medicine System and its Global practice, **IJIST**, 2019, 4(1), 13-33.
5. Paget G.E. and Barnes J.M. Toxicity tests, Evaluation of drug activities: Pharmacometrics, New York: Academic Press, 1964, 205-210.
6. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl425-508.pdf.
7. https://www.oecd.org/chemicalsafety/testing/Revision-OECD-TG408- repeated-dose-90-day-oral-toxicity-study-in-rodents.pdf.
8. http://ayush.gov.in/sites/default/files/File779%20%20%204.pdf.
9. Pennock C.A., Sellers D.J. and Longdon K.J.: A comparison auto analyzer method for the estimation of glucose in blood, **Clin. Chim. Acta**, 1973, 48, 193-201.
10. Talke H. and Schubert G.E.: Enzymatic urea determination in the blood and serum in Warburg optical test, **Klin. Wochenschr.,** 1965, 43, 174-175.
11. Slot C.: Plasma creatinine determination: A new and specific Jaffe reaction method, **Scand. J. Clin. Lab. Invest.**, 1965, 17(4), 381-387.
12. Roeschlau P., Bernt E. and Gruber W.A.: Enzymatic determination of total cholesterol in serum, **J. Clin. Chem.,** 1974, 12 226-229.
13. Dominiczak M., Mc Namara J., Nauk M., Wiebe D. and Warnick G.: Measurement of high-density-lipoprotein cholesterol, Handbook of lipoprotein testing, 2nd ed. Washington DC, AACC Press, 2000,819.
14. Fossati P. and Prencipe L.: Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. **Clin. Chem.**, 1982, 28(10), 2077-2080.
15. Tietz N.W.: Text book of Clinical Chemistry, Philadelphia (PA), WB Saunders, 1986,579.
16. Doumas B.T., Arends R.L. and Pinto P.: In standard methods of clinical chemistry, Chicago. Academic Press, 1972, 7, 175-7189.
17. Wilkinson J.H., Boutwell J.H. and Winsten S.: Evaluation of a new system for kinetic measurement of serum alkaline Phosphatase, **Clin. Chem.**, 1969, 15(6), 487-95.
18. Tietz N.W.: Clinical guide to laboratory tests, 3rd edition, Philadelphia (PA). WB Saunders, 1995, 76.
19. Burtis C.A. and Ashwood E.A.: Textbook of Clinical Chemistry, 3rd edition, Philadelphia (PA), WB Saunders 1999, 652- 1136.
20. Kabasakalian P., Kalliney S.and Wescott A.: Determination of uric acid in serum, with use of uricase and tribromophenol-aminoantipyrinechromogen, **Clin.Chem.**, 1973, 19(5), 522-524.
21. Pearlman P.C. and Lee R.T.: Detection and measurement of total bilirubin in serum with use of surfactants as solubilizing agents, **Clin.Chem.**, 1974,20(4), 447-453.
22. Biggs H.G. andMoorehead W.R.: 2-Amino-2-methyl-1-propanol as the alkalizing agent in an improved continuous-flow cresolphthaleincomplexone procedure for calcium in serum. **Clin.Chem.**, 1974, 20(11), 1458-1460.
23. Sharma A., Tiwari R. K., Sharma V., Pandey R. K. and Shukla S.S.: Toxicological Studies of Chaturmukha Rasa,an Ayurvedic Formulation, **Indian J. Pharm. Edu. Res.**, 2019, 53 (4), 688-694.
24. Pandey R.K., Shukla S.S., Tiwari R.K. and Chauhan N.S.:Peganamharmala Indian traditional plant: a scientific update, Spatula DD, 2018, 6 (4) 103-112.
25. Masakazu I. and Peter J. B.:Histamine H3 receptors modulate antigen-induced bronchoconstriction in guinea pigs, **J. Allergy Clin. Immunol.,** 1990,86(4), 491-495.
26. Gad S.G.: Animal Models in Toxicology, Boca Raton, CRC press, 2007, 147-217
27. Sathya T., Murthy B. andVardhini N.:Genotoxicity evaluation of certain bhasmas using micronucleus and Comet assays, **Int. J. Alt. Med,** 2009, 7, 1-5.
28. Kumar A., Nair A.G.C., Reddy A.V.R. andGarg A.N.: Availability of essential elements in bhasmas: analysis of Ayurvedic metallic preparations by INAA, **J. Radioanal. Nucl. Chem.**, 2006,270(1) 173-180.