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Original Research Article

Acute and Sub-Acute Toxicity Studies of Dhatupaushtik Churna

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ABSTRACT

Ayurvedic formulations have been utilised in healthcare for thousands of years and have made significant contributions to human health protection and improvement. Although its treatments have been used for millennia and are known to be non-toxic or considered non-toxic, there is a lack of verifiable data to support the numerous claims. The current investigation sheds light on the acute and subacute oral toxicity of Dhatupaushtik Churna aqueous extract in Swiss mice and Wistar rats. Acute toxicity assessment was studied using male Swiss mice weighing 20-25 g, and the subacute toxicity testing was done using male Wistar rats weighing 150-200 g. The acute toxicity study was carried out according to OECD (Organisation for Economic Co-operation and Development) guidelines 423. There were no deaths of mice at an oral dose of 2000 mg/kg in the acute toxicity studies. The extract-treated rats (200 and 400 mg/kg, p.o.) showed no significant differences compared to the control group in the subacute toxicity investigation. The liver, kidney, pancreas, and testis weights remained unchanged, while haematological and biochemical markers remained stable. The findings revealed that Dhatupaushtik Churna had no discernible effect on the parameters evaluated at the doses used in the study. However, further investigation is required to confirm its safety.

Keywords: Dhatupaushtik churna, Aqueous extract, Swiss mice, Wistar rats, Acute toxicity study, Subacute toxicity study.

Introduction

For the development of novel medications, a toxicological examination is deemed critical. According to the U.S. Food and Drug Administration (F.D.A.), screening novel compounds for toxicity and pharmacological activity in animals is vital. Herbs and herbal products are used in many developing countries to meet their healthcare demands. Plants are utilised to make a range of allopathic/western medications used to treat various diseases in both developing and developed countries. Ayurvedic medicine is still practiced in India, where over 85 percent of the population employs basic plant extracts/formulations to treat various ailments. However, folkloric usage of herbs for treating diseases without attention to their harmful effects may lead to future health issues.[1] The use of plants or plant components is fast growing because they have fewer or no side effects than synthetic medications.[2] According to World Health Organization (WHO) data, more than 80% of the world's population, primarily from developing countries, rely on herbal medicines for their healthcare needs. Natural medicines as therapy are becoming increasingly popular because people believe that using herbal medications is safe for a long time, even though certain herbal or mineral-based treatments have harmful effects. As a result, the demand for toxicological studies of traditional medicines commonly used by the general public without safety indications is increasing. According to traditional Ayurvedic medicine, the formulations are made up of a single plant, a mixture of plants / components, and plants and minerals.[3] As a result, the possibility of drug-drug interactions which might potentially decrease the patient's therapeutic output still exist. In some polyherbal formulations, there has been no evidence of drug-drug interactions.[4] Ayurveda is gradually gaining popularity and ensuring humanity's well-being. For the simple fact that it is effective and has fewer side effects, an increasing number of people are turning to Ayurveda to live a disease-free life. Ayurvedic remedies such as Dhatupaushtik churna have a long history of use, till researchers are now extolling their effectiveness. Due to its therapeutic qualities, Dhatupaushtik Churna is utilised in Ayurveda and Unani healthcare systems to treat sexual and other nerve disorders.[5] Dhatupaushtik Churna aids in the management of the body's vitality and vigour concerns. It contains potent compounds that help to boost stamina and libido. It helps males with a variety of sexual issues.[6] Dhatupaushtik Churna is a herbal medication that treats impotence, erectile dysfunction and premature ejaculation. The formulation (3 to 6 g) is taken twice a day, after lunch and dinner. This can be mixed with cow milk to make it more digestible. The exact dose of Dhatupaushtik Churna depends on the age, strength, digestive power of the patient, the nature of the illness, the state of the viscera and humours, and the properties of individual drugs.[7] Gokhru (*Tribulus terrestris* L.; Family: Zygophyllaceae), Ashwagandha (*Withania somnifera* L.; family: Solanaceae), and Safed musli (*Chlorophytum borivilianum* L.; Family: Asparagaceae) are three essential ingredients that are blended in equal proportions (Table 1) to make Dhatupaushtik Churna. The toxicity study for Dhatupaushtik Churna is not reported to date. Thus, the study examined the acute and sub-acute (15-day) toxicity of Dhatupaushtik Churna administered via oral gavage to Swiss mice and Wistar rats.

Table 1: Composition of Dhatupaushtik Churna

Common Name	Scientific Name	Part used	Quantity
Gokhru	*Tribulus terrestris* L.	Seed	1 part
Ashwagandha	*Withania somnifera* L.	Root	1 part
Safed musli	*Chlorophytum borivilianum* L.	Root	1 part

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Materials and Methods

Plant materials

The three ingredients of Dhatupaushtik Churna were purchased in January 2020 from Herbal House, Kolkata, West Bengal, India. They were authenticated by Dr. Suchandra Samanta Mondal, Assistant Professor, Department of Botany, Krishnanagar B. Ed. College, Krishnanagar, Nadia, West Bengal, India (Reference no Cert/01-03/20).

Preparation of Dhatupaushtik Churna

The Dhatupaushtik Churna was made according to the Ayurvedic Formulary of India's standard procedure.[8] To make Dhatupaushtik Churna, all components were shade-dried and pulverised individually, then sieved through a #80 sieve before being combined in equal amounts.

Extraction of Dhatupaushtik Churna

The powdered drug (500 g) was extracted by maceration with 2.5 L distilled water for 48 h. The aqueous extract was filtered through a Whatman filter paper and evaporated to dryness over a regulated hot water bath maintained at 60-70ºC to obtain a yield of 8.12% (w/w). The dried extract was kept in the refrigerator at 4°C until ready for use. The crude extract was resuspended in distilled water regularly during the administration of experimental animals.

Experimental animals

Adult healthy male Swiss mice weighing 20-25 g and male Wistar rats weighing between 150-200 g were used to evaluate acute and sub-acute toxicity studies. They were kept in separate polypropylene cages having stainless steel grills at the top. The animals were acclimatised for a week to laboratory conditions in the animal house. They were maintained at 22 ± 2ºC temperature, 65% humidity and 12/12 hours light/dark cycle.[9] They were kept with free access to standard diet and water *ad libitum.*[10] The Institutional Animal Ethical Committee of Bengal School of Technology, Sugandha, Hooghly, India, approved all experimental protocols (Proposal no: 1726/CPCSEA/IAEC/2021-007).

Acute toxicity study

The studies were conducted over Swiss mice as per Organization for Economic Cooperation and Development (OECD) guidelines 423, with a few minor modifications.[9] Adult healthy male Swiss mice were used. The animals were randomly selected and divided into three groups, each containing six male mice kept in their cages for one week before dosing to allow acclimatisation at laboratory conditions. All mice in the three groups were fasted overnight before extract administration. After the fasting period, all animals were weighed, and their dosages were calculated based on their body weight. The extract was dissolved in distilled water, and the test was carried out according to methods of Sumanta Mondal *et al*.[11] and Shivhare *et al*.[12]

Aqueous extract of Dhatupaushtik churna (AEDC) was administered orally at 1000 mg/kg and 2000 mg/kg, respectively for groups II and III as per body weight of mice. Group I was used as control group that received only distilled water (3 mL/kg, p.o.). After administration of extract and solvent as control, the animals were kept under close observation continuously for first hour followed by observation in every five minutes up to four hours and thereafter once in a day for 14 days. The animals were monitored for mortality, behavioral, neurological, and other toxic signals. The animals were weighed daily and finally on 15th day, last weights were measured, and average weights were calculated. One-fifth and one-tenth of maximum tolerated doses[13] of Dhatupaushtik churna aqueous extract used in acute toxicity (2000 mg/kg, p.o.) was determined for subacute toxicity study (200 and 400 mg/kg, p.o.).

Subacute toxicity study

The subacute toxicity of Dhatupaushtik churna aqueous extract was performed following the method of Halim *et al*.[14] and Mondal *et al*.[11] Healthy adult male Wistar rats were randomly assigned into three groups each containing six male rats. Group I received only distilled water (3 mL/kg, p.o.) via oral route for 14 days. Group II and III received AEDC at 200 and 400 mg/kg, p.o., once daily for 14 days. All 18 rats were observed every day for any kind of physiological and behavioral modifications. The body weight for each rat along with their food and water intake were monitored on daily basis. On 15th day, the animals were sacrificed and blood samples were collected for determination of hematological parameters[15] such as percentage of hemoglobin (Hb%), red blood cell count (R.B.C.), white blood cell count (W.B.C.), differential count (D.C.) which includes neutrophils (N), lymphocytes (L), basophils (B) and biochemical parameters[16] such as blood urea, aspartate transaminase (A.S.T.), alanine transaminase (A.L.T.), serum creatinine and alkaline phosphatase (A.L.P.) were measured using commercially available kits., supplied by Span Diagnostics Ltd., Surat, India. Histopathological studies were performed on organ samples of liver, pancreas and kidney. After euthanasia, the main organs were surgically removed and treated in a 10% buffered formalin solution (pH 7.4). The tissue samples were dehydrated in a graded sequence of ethanol (70–99.9%), rinsed in toluene, and then wrapped in paraffin after fixing. The sample was then stained with hematoxylin and eosin after thin tissue slices of 5 µm were churn out on a rotating microtome. Microscopically, the slices were examined for pathological evaluations, and photomicrographs were recorded.[17-19]

Statistical analysis

The findings were expressed as mean ± standard error of mean (S.E.M.).[20] One-way of analysis of variance (ANOVA) was applied for determination of significant difference. Dunnet's t-test was used to analyse the inter group significance. For all studies, the degree of significance for acceptation was taken as $p<0.05$.

Results and Discussion

Acute toxicity

In the evaluation of specific hazardous effects of a pharmacological product on the species, organ, and dosage, preclinical toxicity tests on numerous biological systems are utilised. In preclinical toxicity research, rats are frequently utilised as predictors of access to toxicity in humans, saving time, money, and effort.[21] When Dhatupaushtik Churna aqueous extract was administered orally to mice at dosages of 1000 and 2000 mg/kg, p.o., which are several times greater than the therapeutic equivalent dose, unpleasant signs and symptoms were not reported at a single dose of the drug in an acute toxicity study. Because the test dose of Dhatupaushtik Churna did not induce any death in the test animals over the 14-day observation period, it's LD_{50} (Lethal Dose 50%) is predicted to be larger than 2000 mg/kg, p.o.[22] Furthermore, the animals showed no indication of behavioral alterations. As a result, Churna can be deemed relatively non-toxic, according to earlier published literature.[23, 24]

Subacute toxicity

In the sub-acute toxicity study, Dhatupaushtik Churna aqueous extract at doses of 200 and 400 mg/kg, p.o. did not produce any significant changes for increment of body weight (Figure 1), which proves that the extract does not have any adverse effects on body weight.[25,26] The weights alteration of liver, kidney, pancreas and testis (Figure 2) was found almost negligible in the experimental groups as compared with control group which suggest that the extract was non-toxic to vital organs. The hematological and biochemical parameters are shown in Tables 2, 3 and Figure 3. It was observed that there were no significant changes in several liver function parameters (Table 2) such as total bilirubin, conjugated bilirubin, total protein, albumin, total cholesterol, triglyceride, A.L.T., A.S.T. and A.L.P. when compared with control group. A hike in above mentioned parameters reflects hepatocyte damage.[27] Blood urea and serum creatinine levels remain normal (Figure 3) which showed that extract do not interfere with renal function and renal integrity.[28] No significant changes were observed in several hematological parameters (Table 3) such as hemoglobin (Hb), total count (T.C.) of R.B.C. and W.B.C., differential count (D.C.) of WBC and platelet count as compared with control

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group that confirms non-toxic nature of extract and does not affect circulating red cells, hematopoiesis or leucopoiesis.

The effects of the histopathological analysis of the liver, kidney, pancreas, and testis are presented in Figure 4. The histopathology examination results revealed no significant deterioration in the separate organs. The retention of normal lobular architecture was found in numerous segments of the liver. Hepatocytes are arranged in single cell cords spreading from the central vein and appear normal. At the dose levels of the extracts examined, no non-specific lobular hepatitis was observed. There was no evidence of biliary stasis, granuloma, dysplasia, or malignancy, and various sections examined from a renal biopsy confirmed that the glomeruli, tubules, interstitium, and blood vessels were all normal in size and shape. Acute tubular necrosis or glomerular alterations were not present, and several sections of the pancreas indicated normal architecture. Histopathological lesions, granuloma, dysplasia, or malignancies were not found. Sections of the reproductive organ i.e., testis for male rats showed normal pathology for both the control and extract treated groups.

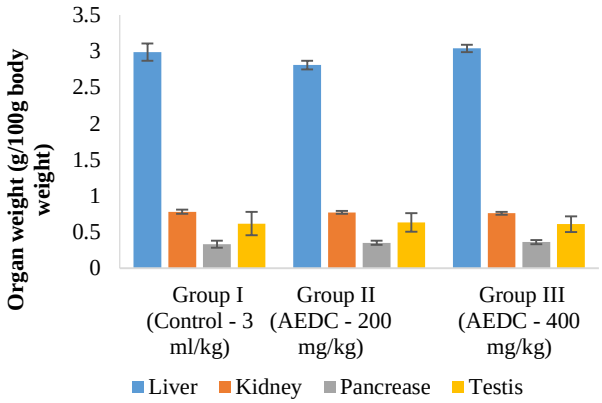

Figure 2: Effects of 14 days oral administration of Aqueous extract of Dhatupaushtik churna (AEDC) on organ weights in rats. Values are expressed as mean ± S.E.M from six observations.

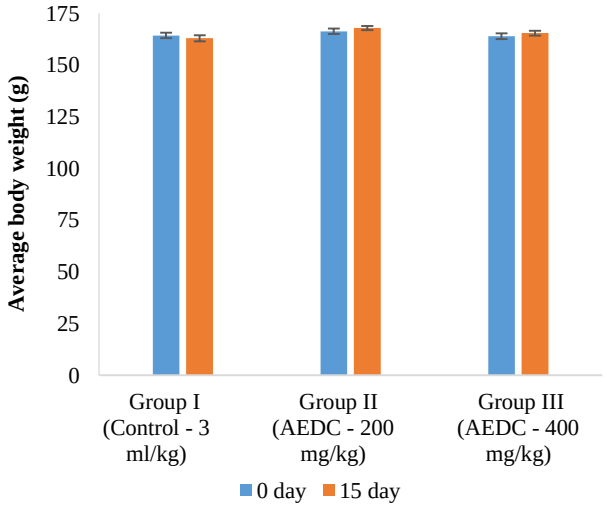

Figure 1: Effects of Rats body weight in 14 days treatment with aqueous extract of Dhatupaushtik churna (AEDC). Values are expressed as mean ± S.E.M from six observations.

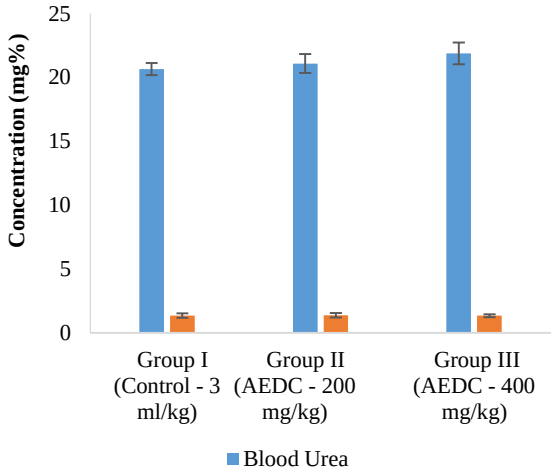

Figure 3: Effect of Dhatupaushtik churna aqueous extract on kidney function in rats. Values are expressed as mean ± S.E.M from six observations.

Table 2: Effects of Dhatupoustic churna aqueous extract on biochemical parameters for liver function tests in rats

Parameter	Control (3 mL/kg, p.o.)	Dhatupaushtik churna aqueous extract (200 mg/kg, p.o.)	Dhatupaushtik churna aqueous extract (400 mg/kg, p.o.)
Total bilirubin (µmol/l)	13.11 ± 1.09	13.79 ± 1.11	13.98 ± 1.14
Conjugated bilirubin (µmol/l)	3.46 ± 0.91	3.88 ± 0.07	3.89 ± 0.07
Total protein (g/l)	69.73 ± 5.34	71.16 ± 5.39	72.37 ± 7.56
Albumin (g/l)	37.45 ± 2.51	36.12 ± 2.34	37.82 ± 2.27
Total cholesterol (mg/dl)	62.49 ± 5.31	63.21 ± 6.35	64.05 ± 6.71
Triglyceride (mg/dl)	82.47 ± 6.02	81.92 ± 6.27	83.58 ± 6.59
ALT (IU/l)	24.87 ± 3.12	24.63 ± 3.18	25.09 ± 3.01
AST (IU/l)	9.96 ± 1.59	10.73 ± 1.67	11.51 ± 1.73
ALP (IU/l)	112.76 ± 6.07	114.29 ± 7.51	115.06 ± 8.42

Values are expressed as mean ± S.E.M from six observations. A.L.T.: Alanine transaminase also called Serum Glutamic Pyruvate Transaminase (SGPT); A.S.P.: Aspartate transaminase also called Serum Glutamic Oxaloacetate Transaminase (SGOT); A.L.P.: Alkaline Phosphate.

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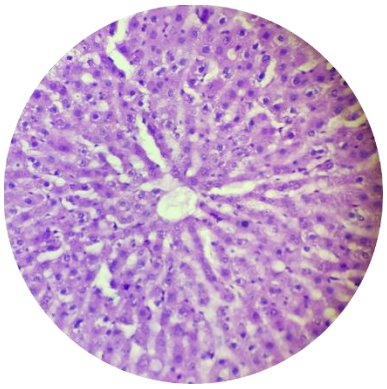
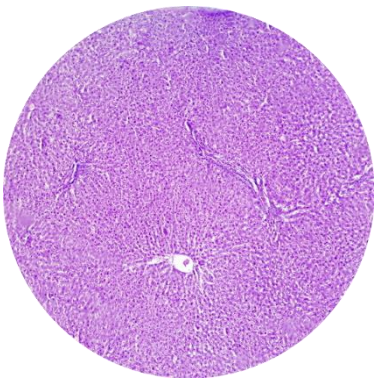
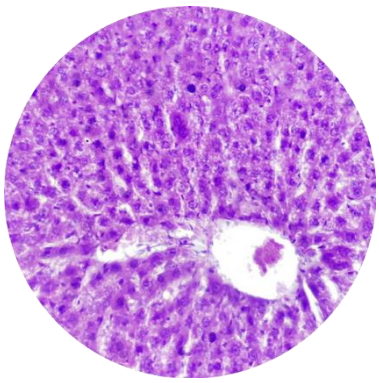
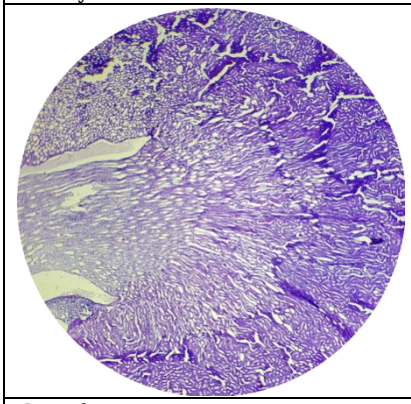
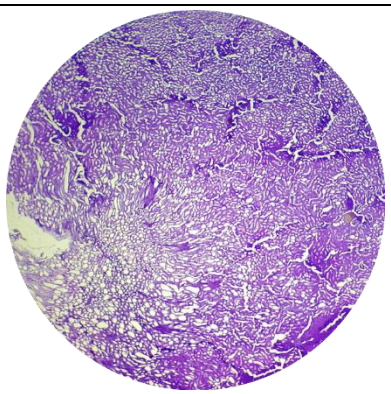
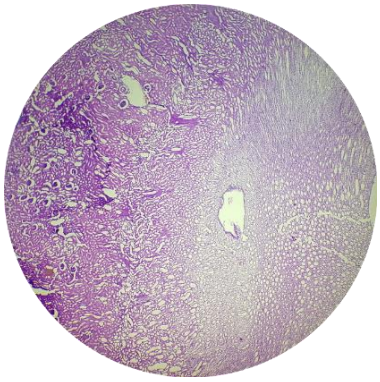
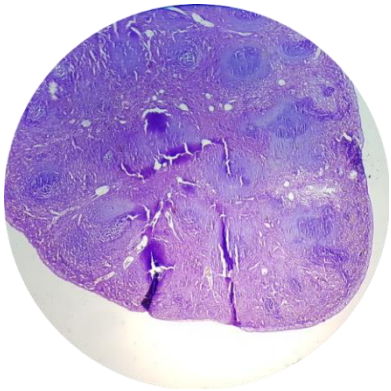
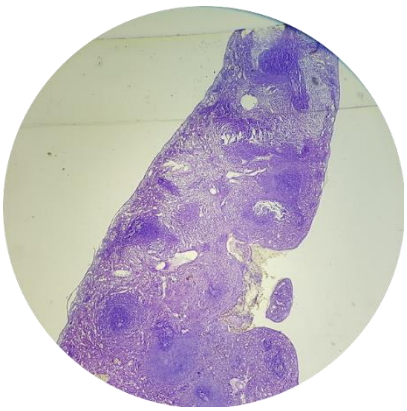
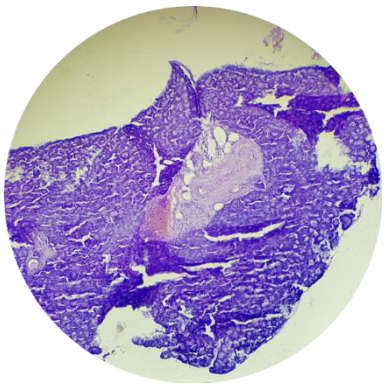
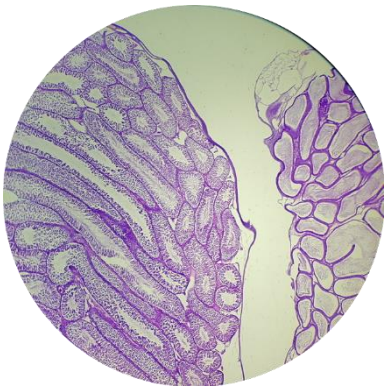
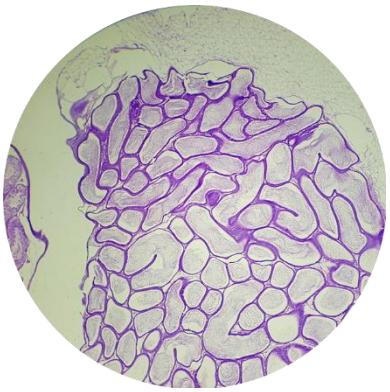
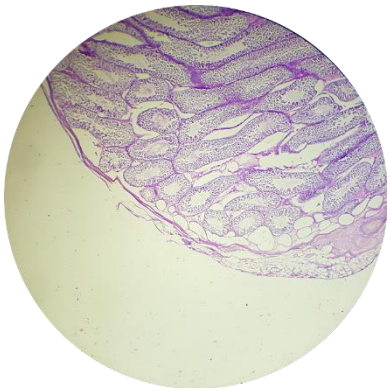
Liver		
		
Control	AEDC (200 mg/kg, b.wt., p.o.)	AEDC (400 mg/kg, b.wt., p.o.)
Kidney		
		
Control	AEDC (200 mg/kg, b.wt., p.o.)	AEDC (400 mg/kg, b.wt., p.o.)
Pancreas		
		
Control	AEDC (200 mg/kg, b.wt., p.o.)	AEDC (400 mg/kg, b.wt., p.o.)
Testis		
		
Control	AEDC (200 mg/kg, b.wt., p.o.)	AEDC (400 mg/kg, b.wt., p.o.)

Figure 4: Histopathological study of vital organs from Wistar male rats.
Photomicrographs of histological findings of different organs from male rats treated with normal control vehicle and AEDC at doses of 200 and 400 mg/kg, body weight, p.o., for a period of 15 days (hematoxylin-eosin staining, 40 × magnification, magnified parts in testis = 100 × magnification)

Table 3: Effect of Dhatupaushtik churna aqueous extract on some hematological parameters

Parameter	Control (3 mL/kg, p.o.)	Dhatupaushtik churna aqueous extract (200 mg/kg, p.o.)	(400 mg/kg, p.o.)
Hb (%)	12.92 ± 0.47	13.69 ± 0.76	14.25 ± 0.81
RBC (X 10^{12}/l)	8.87 ± 0.72	8.78 ± 0.64	9.56 ± 0.78
WBC (X 10^{9}/l)	13.14 ± 0.61	13.25 ± 0.65	14.07 ± 0.69
Platelet (X 10^{9}/l)	536.76 ± 37.69	567.32 ± 38.58	563.95 ± 38.86
Neutrophils (%)	43.81 ± 3.12	44.35 ± 3.97	45.03 ± 4.05
Lymphocytes (%)	55.82 ± 5.63	55.17 ± 6.28	55.89 ± 6.31
Eosinophils (%)	1.45 ± 0.15	1.52 ± 0.16	1.26 ± 0.14
Monocytes (%)	1.07 ± 0.11	1.02 ± 0.09	0.99 ± 0.11
Basophils (%)	00 ± 00	00 ± 00	00 ± 00

Results are expressed as mean ± S.E.M from six observations. H.B.: Haemoglobin; R.B.C.: Red Blood Cells; W.B.C.: White Blood Cells.

Conclusion

Based on the findings, it can be concluded that Dhatupaushtik churna aqueous extract administered for 15 days at doses of 200 and 400 mg/kg, p.o., has relatively minor toxic potential. Dhatupaushtik churna was shown to be safe to use and can be used widely as a traditional medication, according to the findings. However, further study is required to investigate and confirm its safety and effectiveness in humans.

Conflict of Interest

The Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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References

1. Ghosh D, Mondal S, Ramakrishna K. Acute and sub-acute (30-day) toxicity studies of *Aegialitis rotundifolia* Roxb., leaves extract in Wistar rats: safety assessment of a rare mangrove traditionally utilised as pain antidote. Clin Phytosci. 2019; 5(1):1-16.
2. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 2014; 4(177):1-10.
3. Cock IE. The safe usage of herbal medicines: counterindications, crossreactivity and toxicity. Phcog Commun. 2015; 5(1):2-38.
4. Che CT, Wang ZJ, Chow MSS, Lam CWK. Herb-Herb Combination for Therapeutic Enhancement and Advancement: Theory, Practice and Future Perspectives. Molecules. 2013; 18(5):5125-5141.
5. Anonymous. Ministry of Health and Family Welfare. Department of Ayush: Government of India. Volume- I. Ghaziabad, India: Pharmacopoeia Commission for Indian Medicine and Homoeopathy; 1999; 19-20p.
6. Chaddha V, Nayak S, Solanki SS. Preliminary Studies and Evaluation of Dhatupaushtik Churna: A Traditional Ayurvedic Formulation. Int J Med Pharm Sci. 2011; 1(1):11-27.
7. Ministry of Health and Family Welfare. Department of Ayush: Government of India. Volume 4. Ghaziabad, India: Pharmacopoeia Commission for Indian Medicine and Homoeopathy; 2004; 122-123p.
8. Kansabanik S, Mukhi S, Das C, Das D, Bose A. Establishment of Quality Control Parameters of Nagarmotha Churna. Res J Pharm Tech. 2019; 12(12):5967-5971.
9. Diener W, Mischke U, Kayser D, Schlede E. The Biometric Evaluation of the OECD Modified Version of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 1995; 69(10):729-734.
10. Anonymous. National Research Council, Guide for the Care and Use of Laboratory Animals. (8th ed.). National Academy Press. Wash, DC, U.S.A. 2011; 246p.
11. Mondal S, Panigrahi N, Sancheti P, Tirkey R, Mondal P, Almas S, Kola V. Evaluation of toxicological, diuretic, and laxative properties of ethanol extract from *Macrothelypteris torresiana* (Gaudich) aerial parts with In-silico docking studies of polyphenolic compounds on carbonic anhydrase II: An enzyme target for diuretic activity. Pharmacogn Res. 2018; 10(4):408-416.
12. Shivhare Y, Dangi S, Sonil P, Singh P, Baghel SS. Acute toxicity study of Aqueous extract of *Coccinia indica* (Roots). Asian J Res Pharm Sci. 2011; 1(1):23-25.
13. Walum E. Acute oral toxicity. Environ Health Perspect. 1998; 106(Suppl 2):497-503.
14. Halim SZ, Abdullah NR, Afzan A, Rashid BAA, Jantan I, Ismail Z. Acute toxicity study of *Carica papaya* Leaf extract in *Sprague Dawley* Rats. J Med Plant Res. 2011; 5(20):1867-1872.
15. John MB. Laboratory Medicine Hematology. (4th ed.). St. Louis; CV. Mosby Co. 1972; 1198p.
16. Pieme CA, Penlap VN, Nkegoum B, Taziebou CL, Tekwu EM, Etoa FX, Ngongang J. Evaluation of acute and subacute toxicities of aqueous ethanolic extract of leaves of *Senna alata* (L.) Roxb (Ceasalpiniaceae). Afr J Biotechnol. 2006; 5(3):283-289.
17. Ghai CL. A Text Book of Practical Physiology. India; Jaypee Brothers: 1995; 119p.
18. Mondal S, Ghosh D, Sagar N, Ganapaty S. Evaluation of Antioxidant, Toxicological and wound healing Properties of *Hibiscus rosa-sinensis* L. (Malvaceae) ethanolic leaves extract on different Experimental animal models. Indian J Pharm Educ Res. 2016; 50(4):620-637.

19. Ghosh D, Mondal S, Ramakrishna K. Acute and sub-acute (30-day) toxicity studies of *Aegialitis rotundifolia* Roxb., leaves extract in Wistar rats: safety assessment of a rare mangrove traditionally utilised as pain antidote. Clin Phytosci. 2019; 5(1):1-16.
20. Baghel SS, Dangi S, Soni P, Singh P, Shivhare Y. Acute Toxicity Study of Aqueous Extract of *Coccinia indica* (Roots). Asian J Res Pharm Sci. 2011; 1(1):23-25.
21. Kramer JA, O'Neill E, Phillips ME, Bruce D, Smith T, Albright MM, Bellum S, Gopinathan S, Heydorn WE, Liu X, Nouraldeen A, Payne BJ, Read R, Vogel P, Yu X-Q, Wilson A.G.E. Early toxicology signal generation in the mouse. Toxicol Pathol. 2010; 38(3):452-471.
22. Dixon WJ. The Up-and-Down Method for Small Samples. J Am Stat Assoc. 1965; 60(312):967-978.
23. Kennedy G, Ferenz RL, Burgess BA. Estimation of acute oral toxicity in rats by determination of the approximate lethal dose rather than the LD_{50}. J Appl Toxicol. 1986; 6(3):145-148.
24. Ateba SB, Simo RV, Mbanya JC, Krenn L, Njamen D. Safety profile and gender specific differences of a aqueous extract of *Eriosemala urentii* (Leguminosae) in acute and subchronic (28 days) oral toxicity studies in Wistar rats. Food Chem Toxicol. 2014; 65(3):27-32.
25. Winder CV, Lembke LA, Stephens MD. Comparative bioassay of drugs in adjuvant induced arthritis in rats, flufenamic acid, mefenemic acid and phenylbutazone. Arthritis Rheumatol. 1969; 12(5):472-482.
26. Teo S, Stirling D, Thomas S, Hobermann A, Kiorpes A, Khetani V. A 90-Days Oral Gavage Toxicity Study of D-Methyl Penidate and DL Methyl Penidate in *Sprague-Dawley* rats. Toxicol. 2002; 179(3):183-196.
27. Aniagu SO, Nwinyi FC, Ajoku DD, Dzanma SL, Lamaniel K. Toxicity Studies in rats fed nature cure bitters. Afr J Biotechnol. 2005; 4(1):72-78.
28. Kaneko JJ. Clinical Biochemistry of Domestic Animals. San Diego; Academic Press. 1989. 496-537 p.