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Sowmya M
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Kavya S
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Kaveri KR
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

Shashank AS
Department of Life Sciences, CHRIST (Deemed to be University), Bangalore, Karnataka, India

Corresponding Author:
Sowmya M
Department of Studies and Research in Zoology, Tumkur University, Tumakuru, Karnataka, India

A critical study on the acute oral toxicity of polyherbal formulation of *Rosmarinus officinalis* L. AND *Withania somnifera* L. in wistar Albino Rats

Sowmya M, Kavya S, Kaveri KR and Shashank AS

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Abstract
The present work was planned to evaluate minimum dose level and pathomorphological alterations in Wistar albino rats after administration of polyherbal formulation (*Rosmarinus officinalis*) and Ashwagandha (*Withania somnifera*) in 3:1 at 2 g/kg body weight accordance with the OECD guidelines 423. All the animals that were orally exposed to polyherbal formulation extracts were sacrificed after the Fourteen days trial. On gross examination, no specific lesions were observed in any of the organs, whereas microscopic examination revealed no changes. As there was zero mortality even after 14 days of exposure, it was concluded that the LD_{50} value was more than 2 g/kg body weight, and the above polyherbal formulation was considered non-toxic. The present study analyzed the minimum dose level of polyherbal formulation extract *Rosmarinus officinalis* and *Withania somnifera* and observed slight weight gain in all the experimental rats. Still, this study could not monitor any mortality or pathomorphological changes, so it can be concluded that the extracts of *Rosmarinus officinalis* and *Withania somnifera* are non-toxic or safe at 2 g/kg body weight. However, further studies with more experimental animals are warranted to ascertain polyherbal formulation's toxicity and safety level and use this to prepare new therapeutic agents in Ayurvedic medicine in the coming days.

Keywords: *Rosmarinus officinalis*, *Withania somnifera*, LD_{50}, polyherbal formulation

1. Introduction
Ayurvedic science is India's oldest holistic (whole-body) healing system. The belief of Ayurveda is to protect human beings from unnecessary suffering by improving overall health and longevity. The Sarangdhar Samhita, a work of Ayurvedic literature, introduced the idea of polyherbal as a means of enhancing therapeutic efficacy; the therapeutic effect resulting from the combination of two herbs in a particular ratio would be much better as compared to a single herbal plant [1]. In developing countries, many of the local population depend on traditional medicines due to their easy availability, traditional background, knowledge of these plants, believed that they are harmless [2]. Various synthetic medicines are known to relieve symptoms by acting on a particular molecular target. Herbal medicines provide various therapeutic benefits and are effective in the treatment of chronic illnesses, including cancer, diabetes, and many other disorders, as well as in helping patients restore their health [3]. Even if they are shown to be effective in various clinical and pharmacological evaluations, there is a need to ensure their safety to reap their full benefits [4].

Herbal medication therapy is regarded as a standard procedure used as an alternative to traditional medicine for treating many human problems since ancient times. *Rosmarinus officinalis* L., popularly known as rosemary, is an aromatic plant used for culinary and medicinal purposes [5]. Rosemary has been described as a wonder drug in literature, mainly for its antioxidant and anti-inflammatory properties, which are attributed to the presence of a rich source of active metabolites such as flavonoids, carnosol, rosmanol, carnosic acid, rosmarinic acid, and other phenolic compounds [6]. Many pharmacological studies have reported anti-tumor, anti-inflammatory, antioxidant, anticancer, antibacterial, analgesic, and hepatoprotective activities of secondary metabolites and extracts of *Rosmarinus officinalis* L. [7].

Withania somnifera, Indian ginseng, is a popular Indian plant with high medicinal value for its pharmacological and nutraceutical effects. It is a perennial shrub known as Ashwagandha in Sanskrit and belongs to the family Solanaceae. Over 300 formulations, including the root of this plant, were utilized in conventional medicine systems like Ayurveda, Unani, and Siddha to treat a wide range of physiological conditions [8].

It was widely used to treat many therapeutic disorders in humans, alone or in conjunction with other plants. It has a broad range of pharmacological properties for application in biological approaches, including antibacterial, anti-inflammatory, anticancer, anti-stress, antioxidant, neurodegenerative, cardioprotective immunomodulatory, hemopoietic, and rejuvenating properties.

The protection, diagnosis, and treatment of many diseases involve using traditional and alternative medicine. Despite the widespread use of *Rosmarinus officinalis* in conventional medicine, no comprehensive toxicity studies on animals have been reported. Many preclinical studies in rabbits and rodents have shown the proapoptotic activity of *Withania somnifera* extracts against carcinoma cells. Comprehensive toxicological studies on acceptable model animals are required to determine a safe dose of herbal formulation for human use. The current study, "Acute oral toxicity study of polyherbal formulation (*Rosmarinus officinalis* and *Withania somnifera* in 3:1)" in Wistar rats, was performed by following OECD Guideline 423 in the Liveon bio labs, Tumkur, which is mainly intended to investigate the acute (limited dose) toxicity of these two herbal extracts of Ashwagandha root and rosemary leaf in Wister rats to determine the minimum safety threshold for herbal extracts.

2. Materials and Methods

2.1 Collection of plant material

The plant material of *R. officinalis* (Rosemary leaves) and *W. somnifera* (Ashwagandha root) were collected from the local farms and market of Tumkuru district situated extends from 13.34°N 77.1°E coordinates of Karnataka, India. The leaves and root materials are extensively washed with distilled water to remove soil and dust, and then dried under shade for 5-7days. The dried material is milled into powder and stored in airtight desiccators for polyherbal formulation.

2.2 Extraction and preparation of Polyherbal formulation

The dried leaf powder of *Rosmarinus officinalis* of about was extracted with 95% v/v ethanol using a Soxhlet apparatus after being first defatted with petroleum ether (at 60–80 °C). The extract was then subjected to evaporation to remove the solvent, which was then accurately weighed, and its color and composition were recorded. The Ashwagandha root powder was used; 1500mg powder extract of rosemary leaves and the 500 mg powder of Ashwagandha root were combined with 10 ml of distilled water to create the polyherbal formulation in a 3:1 ratio, and that is used in further study [7, 20].

2.3 Experimental animal and maintenance

The experimental investigations were performed on healthy female Wister Albino rats (*Rattus norvegicus*) that were nulliparous and non-pregnant. The rats aged 8 to 12 weeks with an average weight of about 160g to 180g were selected. The animal handling and experimentation were carried out following standard protocols recommended by the "Association for Assessment and Accreditation of Laboratory Animal Care" (AAALAC) and registered with "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA) guidelines, Forests and Climate change, Ministry of Environment, Government of India. Additionally, Liveon Biolabs Private Limited guarantees that animal experiments are carried out following the advice of the rules for laboratory animal facilities published in the Indian Gazette in 2018.

3. Experimental design and procedures

3.1 Acute oral toxicity study

The research was conducted in compliance with OECD Guideline No. 423, "Acute Oral Toxicity Study (Acute Toxic Class Method)," which was adopted on December 17, 2001. Three Wistar rats were used in each phase. After a 16-hour fast, the rats were administered a single dose of about 2 g/kg body weight through gavage with an 18G intubation cannula in phases I. Unlike phase II, which consists of three stages, the dose for treating female Wister rats was the same, 2 g/kg body weight.

4. Results

The current study was undertaken to study the acute oral toxicity of polyherbal formulation in female Wister albino rats. Various observations were made during the survey to calculate the rats' minimum lethal level (LD_{50}).

The extraction of *Rosmarinus officinalis* leaf powder with 95% ethanol yielded 16% on a dry matter basis, to which the root powder of *Withania somnifera* was mixed at a 3:1 ratio in 10ml distilled water to obtain a polyherbal formulation.

First, one set of three rats (n=3) received the maximal oral dose, or 2 g/kg body weight, and was monitored for 14 days. The animals survived and did not exhibit any acute poisoning symptoms or indicators. To examine six animals, the same dose was given to the second set of rats (n=3). In this investigation, all six animals lived and were healthy for the two weeks of monitoring without exhibiting any toxicity-related signs or symptoms. According to the above finding, it is safe to utilize both the plant extracts in animals up to an oral dose of 2 g/kg because the acute oral LD50 of the polyherbal formulation in rats was more than 2 g/kg.

The body weight of each animal was noted at the time of receipt, on the first day before the administration of the test item, on days 8 and 15, and during the observation period. Any of the treated animal's body weight did not alter due to the treatment; instead, it increased on days 8 and 15 relative to day one body weight. Table 1 and Graphs 1 and 2 display the values for the individual animal body weights.

Table 1: Individual animal body weight (g)

Step	Dose (g/kg body weight)	Sex	Animal No.	Volume Administered (ml)	Time of Dosing	Body Weight (gram) Days			Body Weight Gain (%)	
						Day 1	Day 8	Day 15	Day 1-8	Day1-15
I	2	F	1	1.7	10:20 to 10:25	177.3	185.2	201.01	4.46	13.37
			2	1.8		177.76	187.6	197.81	5.54	11.28
			3	1.9		187.31	200.14	214.12	6.85	14.31
			Mean			179.06	195.38	180.79	190.98	204.31
			SD			7.37	9.06	5.65	8.02	8.64
II	2	F	4	1.8	10:18 to 10:23	175.12	186.51	210.1	6.50	19.97
			5	1.8		178.14	184.1	214.3	3.35	20.30
			6	1.9		184.18	194.1	212.4	5.39	15.32
			Mean			183.62	200.61	179.15	188.24	212.27
			SD			4.02	3.31	4.61	5.22	2.10

Key: F: Female, SD -Standard Deviation

Graph 1: The Graph showing Body weight in Grams V/S Experimental period in days.

Graph 2: The Graph showing Body weight in Grams V/S Experimental period in days

Daily observations of animals were made during the exposure period. After dosing, animals were individually examined for toxic symptoms and death at least once within the first 30 minutes, one hour, two hours, and four hours (10 minutes at each point), and every day after that for a total of 15 days. Changes in the circulatory, autonomic, respiratory, and behavior patterns like tremors, convulsions, salivation, diarrhea, and lethargy, were all observed. Throughout the experiment, animals were checked twice daily for mortality and illness. No clinical toxicity was seen in any of the treated animals in step 1 (2g/kg body weight) on day one at 30 minutes, the first hour, the second hour, the third hour, and the fourth hour after treatment, or throughout the 15-day investigation. In step 2 (2g/kg body weight), none of the treated animals showed any clinical symptoms of toxicity on day one at 30 minutes, the first hour, the second hour, the third hour, or the fourth hour after treatment, or for the duration of the 15-day investigation. Table 2 outlines the clinical symptoms in more depth.

Table 2: Individual animal clinical signs

Step	Dose (g/kg body weight)	Animal No.	Sex	Days																		
				1						2	3	4	5	6	7	8	9	10	11	12	13	14	15
				30 Min	1hr	2hr	3hr	4hr	*														
I	2	1	F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		3		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
II	2	4		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		5		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		6		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Key: 1= Normal; * Observation at end of the day; F = Female; hr = Hour; min = Minutes.

All of the animals were slaughtered after the 14-day observation period was up to do a thorough post-mortem examination. Gross pathological analysis on the day of necropsy (Day 15) revealed no exterior or interior macroscopically abnormalities. Table 3 displayed the specifics of each gross pathological finding.

Table 3: Individual animal gross pathological findings

Step	Dose (g/kg Body weight)	Animal No.	Sex	Gross Pathological Findings	
				External	Internal
I	2	1	F	NAD	NAD
		2		NAD	NAD
		3		NAD	NAD
II	2	4		NAD	NAD
		5		NAD	NAD
		6		NAD	NAD

Key: NAD: No Abnormalities Detected; F: Female.

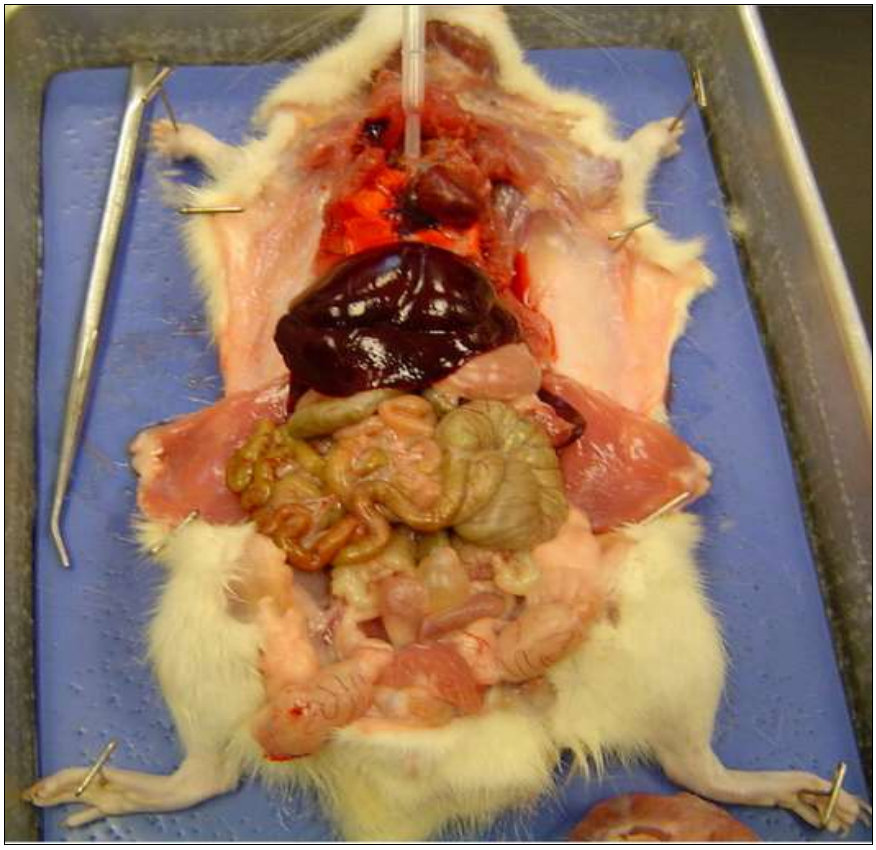

Fig 1: Postmortem examination of treated animal

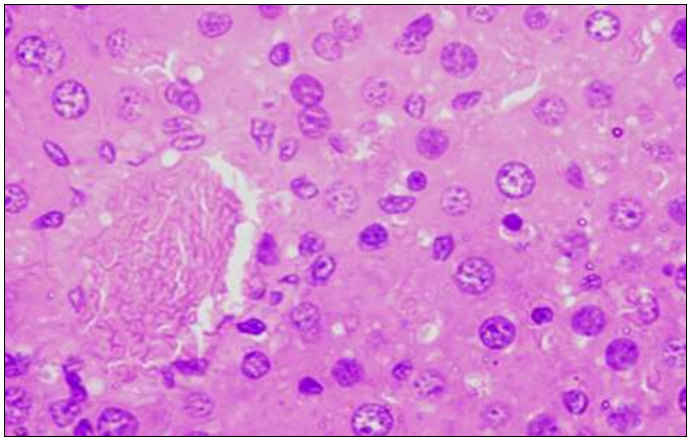

Fig 2: Liver Showing normal centrilobular area and Hepatocytes. (H&E, 400X

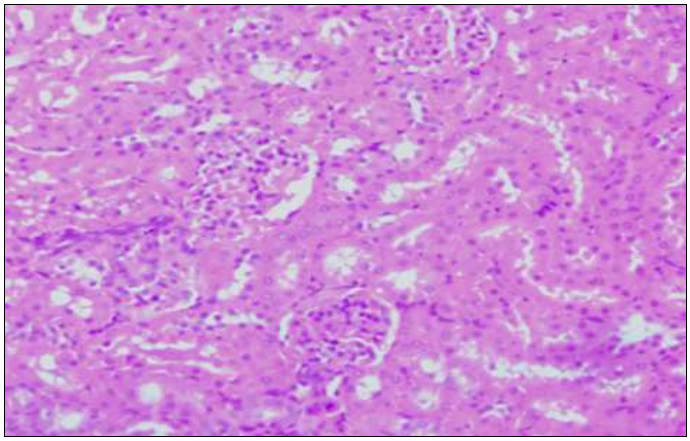

Fig 3: Kidney. Showing normal glomeruli and tubules. (H&E, 100X)

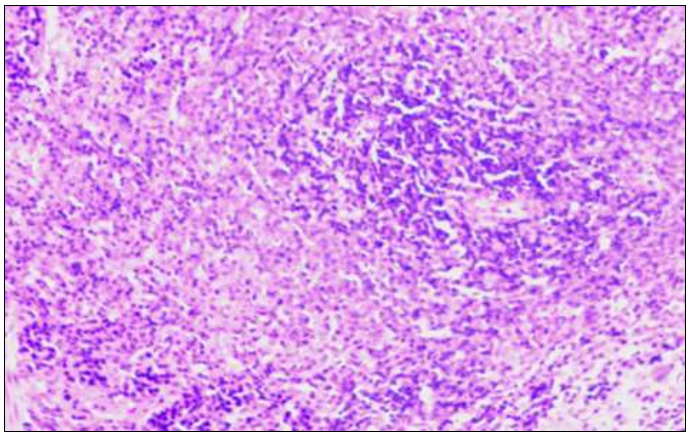

Fig 4: Spleen; Showing normal red and white pulp. (H&E, 100X)

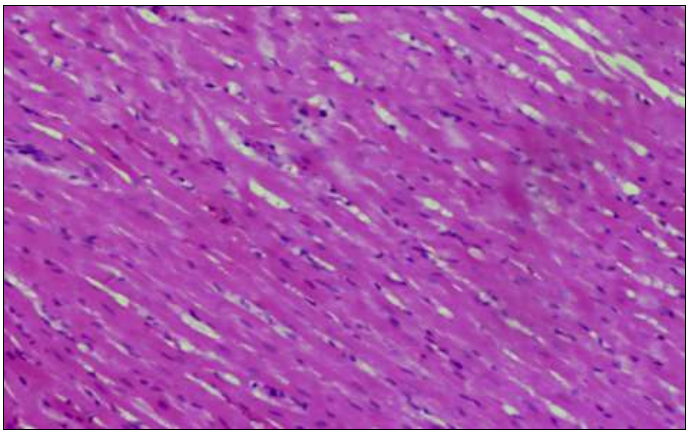

Fig 5: Heart. Showing normal myocardial muscle fibres. (H&E, 100X)

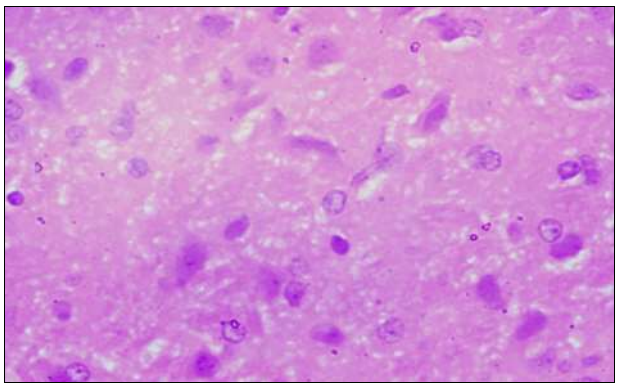

Fig 6: Brain. Showing normal neuronal cells and Cerebral Parenchyma. (H&E, 400X

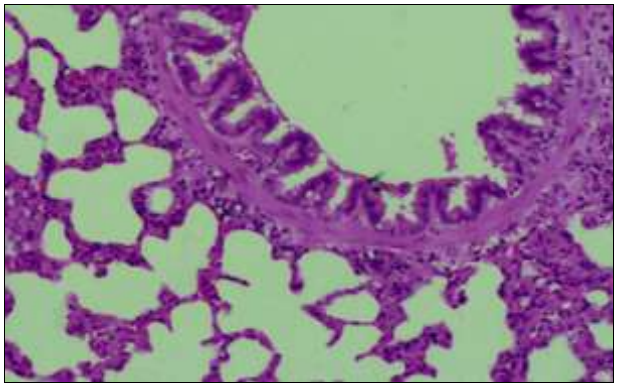

Fig 7: Lungs. Showing normal alveolar Structure and Bronchi. (H&E, 100X

Fig 1, 2, 3, 4, 5, 6 and 7: Photomicrograph of liver, Kidney, Spleen, Heart, Brain, Lungs section from rats treated with 2 g/kg body weight/day

5. Discussion

Medicinal herbs are the basics for the treatment of many diseases. Many plants are known for their medicinal properties since they have been used extravagantly in curing diseases both in animals and humans because of various bioactive compounds comprising primary and secondary metabolites. These can cause beneficial and detrimental effects on the animal's health. Unaware of harmful effects, the administration of surplus plants produces toxicity. Toxins are abundant in most plants as these toxins act as self-defence mechanisms and deter many herbivores from feeding. Different plant species have a wide variety of compounds that affects normal physiological homeostasis leading to death.

Research on lesser-known medicinal plants continues to yield valuable ideas for developing novel medicines [9]. The toxicological database of these substances also becomes increasingly specialized as more pharmacological and clinical data on therapeutic plants becomes available. Because so many people utilize herbal medicines for self-medication, it is essential to determine their effectiveness and safety [10]. Despite the paucity of information on the pharmacology and toxicology of the most widely used herbal treatments [11]. To confirm the safety of herbal remedies, toxicological studies must be conducted, just like with their synthetic counterparts [12]. Therefore, efforts to clarify herbal medications' health advantages and disadvantages need to be stepped up.

In the absence of additional information, single-dose experiments are carried out to determine the extent of toxicity. If herbal remedies contained biologically active components, information on acute toxicity would typically be necessary [13]. The adopted recommendations for acute toxicity testing generally recommend postmortem toxicology of treated animals [14]. Each animal's gross pathological findings are considered prospective sources of knowledge on the target organ or system and the selected test agent's toxicity level [15]. There is little knowledge of the harmful effects of medicinal plants *Withania somnifera* and *Rosmarinus officinalis*. These facts informed the design of the current investigation, which examined the fatal dose and pathomorphological changes to explore the acute oral toxic effects of polyherbal formulation in rats.

An acute oral toxicity study is essential to identify the dose that could prove lethal to animals. In the present study, no mortality or signs of toxicity were observed immediately after dosing through the 14 days observation period, which indicated that the LD_{50} of the polyherbal formulation is greater than 2g/kg body weight. This follows the findings of [16, 17]. They reported that administration of *Rosmarinus officinalis* and *Withania somnifera* at the rate of 2g/kg body weight showed no mortality or toxicological changes thought the 14 days in rats. In this study, we found that the body weight of all the animals increased significantly on days 8 and 15, which is consistent with the findings of [15], who found that all the female rats treated with the polyherbal veterinary drug for 14 days saw a slight increase in body weight. Still, they could not detect any death rates or significant toxico-pathological changes [18] reported that the polyherbal formulation was non-toxic despite noticing a slight increase in the body weight of all the rats used in their study.

Throughout the current study, there were no clinical indications of harm in any of the treated animals on Day one at 30 minutes, an hour, two hours, three hours, and four hours after treatment, as well as throughout the experimental period of four days. Clinical signs of toxicity include changes in skin, fur, eyes, and mucous membranes, as well as respiratory, circulatory, autonomic, and nervous systems, and somatomotor activity and behavior pattern tremors, convulsions, and salivation. Similar finding and noted the absence of clinical symptoms following a single oral dosage of 2 mg/kg polyherbal formulation. After a single oral dosage of 2 mg/kg Body weight of *Rosmarinus officinalis*, [16, 18] also found no unusual symptoms or behavioral alterations. Similar study found no acute mortality at 1100 mg/kg; however, there was a rapid increase in the death rate with an additional 100 mg increment in the dose. The LD_{50} of the extract was discovered to be 1260 mg/kg body weight, and no animals survived after receiving an injection of 1500 mg/kg body weight. *Withania somnifera* was a safe polyherbal formulation component with an LD_{50} value larger than 5000mg/kg body weight [19].

At a dose level of 2 mg/kg body weight, we could not detect appreciable pathological alterations in any of the rats treated in the current investigation. Similar findings were reported by [19], who noted no perceptible pathological alterations in any of the rats given 2 mg/kg of *Rosmarinus officinalis*. However, when tested the acute oral toxicity of polyherbal veterinary medicines over 14 days, they found no evidence of death, toxicity changes, or significant pathological alterations up to the dose level of 5000mg/kg body weight [15].

However, further studies with more experimental animals are warranted to ascertain polyherbal formulation's toxicity and safety level and use this to prepare new therapeutic agents in Ayurveda medicine in the coming days.

6. Conclusions

The present study analyzed the minimum dose level of polyherbal formulation extract *Rosmarinus officinalis* and *Withania somnifera* and observed slight weight gain in all the experimental rats. Still, it could not monitor any mortality or pathomorphological changes, so it can be concluded that the extracts of *Rosmarinus officinalis* and *Withania somnifera* are non-toxic or safe at 2g/kg body weight. However, further studies with more experimental animals are warranted to ascertain the toxicity and safety level of polyherbal formulation and use this to prepare new therapeutic agents in ayurvedic medicine in the future days; by the end of the trial, all three animals were still alive. Gross necropsy and clinical signs and symptoms did not reveal any significant findings. *Rosmarinus officinalis* and *Withania somnifera* were combined in a polyherbal formulation with an LD50 of more than 2 mg/kg (Category 5 according to OECD guideline 423 for acute Toxicity Studies), making it virtually harmless.

7. Acknowledgments

Liveon Biolabs Private Limited Tumakuru is gratefully acknowledged for providing experimental animals and accessing this work to happen in their lab.

8. Conflict of interest

The authors declare no conflicts of interest.

9. Ethics statement

Liveon Biolabs Private Limited guarantees that animal experiments are carried out following the advice of the rules for laboratory animal facilities published in the Indian Gazette in 2018.

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