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Comparative Assessment of Acute Toxicity of *Sanjivani Vati* Prepared With Two Different Species of Aconite Through Fish Embryo Toxicity Test

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ABSTRACT

The presented study deals with evaluating the Acute toxicity of *Sanjivani Vati* **(**SV), an ayurvedic medicament prepared with two different species of Aconite *viz.* naturally sourced sample and marketed sample employing Fish Embryo Toxicity (FET) using OECD guideline 236.All the raw botanicals were authenticated before the preparation of the drug. Two groups of *Sanjivani Vati* namely G-1 and G-2 were prepared from *Aconitum napellus* L., and *Aconitum balfourii* Holmes ex Stapf. respectively. Their aqueous extraction was done using a soxhlet apparatus**.** They were used as test drugs further. The study was conducted at CSIR-IHBT, Himachal Pradesh. No indications of toxicity were detected in the initial limit test, OECD 236 for both the groups of SV. The median lethal concentration of G-1 (naturally sourced sample) i.e. 535.53 µg/mL was found less than the median lethal concentration of G-2 (marketed sample) i.e. 652.81 µg/mL. The findings suggest that both the tested species of Aconite could be utilized for drug preparation following thorough processing as per the classical guidelines. Nonetheless, caution is advised regarding dosage administration. Preferably, the drug sourced from natural origins should be utilized owing to variations in potency.

Keywords: Aconite; Acute toxicity; FET; OECD 236; *Sanjivani Vati***;** Zebrafish embryo

NOMENCLATURE

SV	: Sanjivani vati
FET	: Fish embryo toxicity
OECD	: Organization for economic cooperation and development
API	: Ayurvedic pharmacopoeia of india
LC_{50}	: Median lethal concentration
EC_{50}	: Median effective concentration

1. INTRODUCTION

The evaluation of the toxicity profile of traditional medicines, despite their long-standing history of safe usage, is imperative to uphold public health standards and ensure continued trust in these therapeutic practices. While traditional medicines, such as Ayurveda, have been relied upon for generations and are often perceived as safe due to their natural origins, modern scientific scrutiny offers a deeper understanding of potential risks and benefits. Comprehensive toxicity evaluation provides valuable insights into the safety margins and potential adverse effects of traditional medicines, enabling informed decision-making by healthcare professionals and consumers alike. Thus, the evaluation of toxicity for Ayurvedic medicaments is necessary for globalization to ensure compliance with international trade regulations, protect consumer health, harmonize safety standards, facilitate market access, address health concerns, and promote cultural sensitivity and acceptance of traditional medicine systems.

Sanjivani Vati (SV) is an extensively used multi component Ayurvedic medicine, outlined in the Ayurvedic Formulary of India[1]. It is widely recommended by Ayurvedic practitioners for addressing a range of conditions such as fever, indigestion, dysentery, Gastroenteritis, etc. It comprises ten ingredients of herbal origin including a couple of Schedule E-1 drugs viz. *Vatsanabha*(*Aconitum ferox* Wall. Ex Ser.) and *Bhallataka*(*Semecarpus anacardium* Linn.). Furthermore, Cow's urine holds significant importance as one of the primary constituents, utilized for *Bhavana* (impregnation)[2]. Despite its established History of Safe Use (HoSU), SV lacks a comprehensive toxicity study to ascertain its safety more precisely. Additionally, concerns arise regarding one of its ingredients i.e. *Vatsanabha*, historically recognized as one of the most poisonous plants. It is being sold as a mixture of various species in Indian markets. Previous research has highlighted significant qualitative and quantitative variations in the total alkaloid content among different species of *Vatsanabha*[3]. However, the potential impact of utilizing different species on the safety profile of various formulations remains unexplored.

The adoption of the European policy REACH i.e. Registration, Evaluation, Authorisation, and Restriction

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of Chemicals[4] underscores a clear protocol to actively encourage the development of alternative methodologies as per the 3Rs principle, which emphasizes "Replacement, Reduction, and Refinement[5]." This directive prioritizes the utilization of data generated through validated alternative methods whenever feasible. Consequently, there is a growing impetus to replace conventional animal-based approaches for assessing acute toxicity and teratogenicity with innovative approaches such as adverse outcome pathway-based biomarker experiments[6] and in vitro testing[7]. Additionally, there is a push for the adoption of more 3Rs-compatible in vivo models[8,9]. Notably, alternative in vitro studies and in vivo assays, including those utilizing fish embryos, offer the potential for high-throughput testing[10], aligning with the overarching goal of promoting ethical and efficient methods for chemical safety assessment. The Zebrafish embryo acute toxicity test is one such method utilised for evaluating acute toxicity, employing embryonic and larval models. This approach is deemed appropriate due to the resemblances observed between the human and zebrafish genomes, as well as similarities in the physiology of their, nervous, digestive, and vascular systems. Therefore, the current experiment deal with the evaluation of Acute toxicity of SV prepared with two different species of *Vatsanabha* employing Fish Embryo toxicity (FET) in the Zebrafish model using OECD guideline 236.

2. MATERIAL AND METHODS

2.1 Procurement of Raw materials

All requisite raw botanicals including a sample of *Vatsanabha* were purchased from a local vendor, Jaipur, in dried form. Another sample of *Vatsanabha* was collected from Chicham Khas, Himachal Pradesh (Elevation - 4300m, Lat. 32.339601^{0}, Long. 77.997743^{0}). Fresh *Gomutra* (Cow's urine) was collected from nearby cowsheds in the early morning.

2.2 Authentication of Raw material

The procured samples of individual crude drugs were authenticated by the Raw Materials Herbarium and Museum, Delhi, under National Institute of Science Communication and Policy Research (CSIR). The authentication numbers for its constituents issued by the institute are as follows: *Vidanga* (4081-82-2), *Nagara* (4081-82-8), *Krishna* (4081-82-9), *Pathya* (4081-82-6), *Amala*(4081-82-5), *Bibhitaka*(4081-82-4),*Vacha* (4081-82-3), *Guduchi* (4081-82-7), *Bhallataka* (4081-82-1). Apart, the fresh sample of *Vatsanabha* was identified and authenticated as *Aconitum napellus* L. (BSI/BGIR/1/TECH./2021/027/80) by Botanical Survey of India, Botanical Garden of Indian Republic, Noida. The marketed sample of *Vatsanabha* was identified and authenticated as *Aconitum balfourii* Holmes ex Stapf (RRDR/AIIA/129) at Taxonomy and Herbarium laboratory, Regional Raw Drug Repository (RRDR), All India Institute of Ayurveda, New Delhi. The voucher specimens and herbarium have been preserved in the respective Institutes. Furthermore, the confirmation of species *Vatsanabha* was also done by DNA fingerprinting(BLAST RID: *Aconitum napellus* L.-1RWPUC26013 and *Aconitum balfourii* Holmes ex Stapf – 130MZKGG013).

2.3 Preparation of *SanjivaniVati*

Two groups of *Sanjivani Vati* namely G-1 and G-2 were prepared in the Pharmaceutical Laboratory, *Rasashastra* and *Bhaishajya Kalpana* Department, All India Institute of Ayurveda as per the official reference[11]. G-1 and G-2 can be stated as *Sanjivani Vati* prepared from *Aconitum napellus* L. and *Aconitum balfourii* Holmes ex Stapf respectively. The formulation composition is depicted in Table 1.All the relevant physicochemical parameters for both of the test drugs were found within permissible limit as per the Ayurvedic Pharmacopoeia of India (API Part I, Volume III).The analytical study was also conducted at All India Institute of Ayurveda, New Delhi.

Table 1. Table 1: Formulation Composition of *Sanjivani Vati*

S. No.	Ingredient	Latin name	Part used	Ratio
1.	Vidanga	*Embeliaribes* Burn.	Dried Fruit	1
2.	Nagara	*Zingiber officinale* Rosc.	Dried Rhizome	1
3.	Krishna	*Piper longum*Linn.	Dried Fruit	1
4.	Pathya	*Terminalia chebula* Retz.	Dried Pericarp	1
5.	Amala	Embelicaofficinalis Gaertn.	Dried Pericarp	1
6.	Bibhitaka	*Termenalia bellirica* Roxb.	Dried Pericarp	1
7.	Vacha	*Acoruscalamus* Linn.	Dried Rhizome	1
8.	Guduchi	*Tinospora cordifolia* Miers ex Hook. & Thoms.	Dried Stem	1
9.	Shuddha Bhallataka	Processed *Semecarpusanacardium* Linn.	Dried Fruit	1
10.	Shuddha Visha	Processed *Aconitum napellus* L. (G-1) /*Aconitum balfourii* Holmes ex Stapf (G-2)	Dried Root tuber	1
11.	Gomutra	Cow urine		Q.S. for Bhavana

2.4 Preparation of Test Samples

The extraction for both G-1 and G-2 was carried out in the soxhlet apparatus using water as a solvent. The resultant filtrates were evaporated to dryness in an oven at 40 ^{0}C. The extracts thus obtained were used as test samples for the Zebrafish Embryo Acute Toxicity Test.

2.5 Test Organism and Ethical Approval

The test subjects employed for the study were the embryo of *Danio rerio* i.e. short fin Zebrafish, wild-type. The test was performed at the Zebrafish Research Facility, Pharmacology and Toxicology Laboratory, Institute of Himalayan Bio-resources and Technology under the Council of Scientific and Industrial Research (CSIR-IHBT). Approval for the study was granted by the Institutional Animal Ethics Committee on August 18, 2023, under CPCSEA(1381/GO/ReBiBt/S/10/CPCSEA).

The experimental procedure involved dispersing the test sample in conditioned water(Temperature: 25-27 °C, Conductivity: 400-600µS, pH: 7.0-7.5, Oxygen saturation: 95-98 %)maintained by the fish system i.e. Zebtec, Tecniplast, Italy to attain various concentrations as mentioned below. The sample after dispersion in the water was sonicated for 5 minutes, and the solution of the final test concentration was changed every 8 h during incubation to avoid precipitation. Initially, the limit test was conducted at 100 mg/L following OECD TG 236 guidelines. In this test, newly fertilized eggs (20 for each observation) were exposed to 100 mg/L of the test drugs for 96 hours at 26 ± 1 °C in a BOD incubator, maintaining conventional water conditions as prescribed in OECD 236; S. No. 12. An additional group of 20 embryos were exposed to solvent, that served as a solvent control. Four observations were made on each tested embryo at interval of 24-hour to assess toxicity, including(A) coagulation of fertilised embryos; (B) Lack of somite formation; (C) Lack of detachment of tail-bud from the yolk sac; and (D) absence of a heartbeat.

Subsequently, the trial was conducted at various concentrations of the test sample (200-1000 µg/mL) to determine LC_{50}. Before commencing the test, the test chambers were conditioned with the experimental solutions for 24 hours. Then, 20 embryos were incubated at each concentration in conventional inert chambers as per OECD 236; S. No. 1 for 96 hours. Throughout incubation, consistent changes were made using a semi-static renewal procedure, ensuring embryos remained covered with a small amount of prior test solutions to prevent dehydration. Efforts were made to reduce stress while handling and observing them. Embryos were examined under a microscope, and images were captured every 24 hours until 96 hours for signs of toxicity. Notably, the developmental phase never surpassed the threshold for unprotected phases of development outlined by existing animal welfare laws by the European Union[12,13].Mortality of embryos/larvae was recorded at all tested concentrations throughout the 96-hour incubation period. This procedure was employed for both the test drugs.

3. RESULTS

No indications of toxicity were found in embryos in the limit test for both tested samples. On incubating at a concentration of 100 mg/L G-1 and G-2, all the embryos exhibited normal growth without any mortality. The developmental stages during a limit test for G-1 and G-2 have been shown in Fig. 1 and 2 respectively. Also, solvent control group exhibited complete survival of the embryos without any adverse effect.

In brief, by the 24-hour mark (Fig. 1-A & Fig. 2-A), the basic organization of the embryo is evident, with identifiable anatomical features like somites, otoliths, notochord, eye anlage, and heart anlage. Also, the tail curling of the embryo can be seen. By 48 hpf(Fig. 1-B & Fig. 2-B), the development of important sensory organs namely the eye and ear have been observed. The caudal fin was also formed. Also, different partitions of the brain and spine over the notochord are distinguishable. By 72 hpf(Fig. 1-C & Fig 2-C), the whole anatomy got predominantly developed. Also, the embryos were poised for hatching. The fins have undergone further development. By 96 hpf(Fig. 1-D & Fig 2-D), the volume of yolk sac resorption has significantly decreased along with the development of the swim bladder. Also, the intestinal tract is completely formed. All the observations at each stage were found to correspond with the normal development of zebrafish embryos[14].

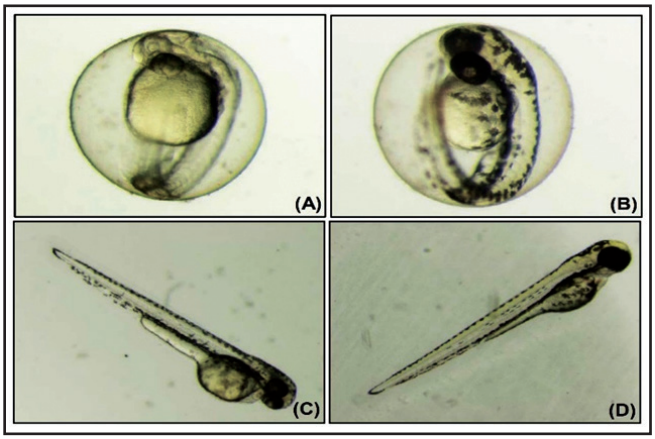

Figure 1. Development of embryos in limit test (100 mg/L of G1) @ (A) 24 hours, (B) 48 hours, (C) 72 hours, and (D) 96 hours.

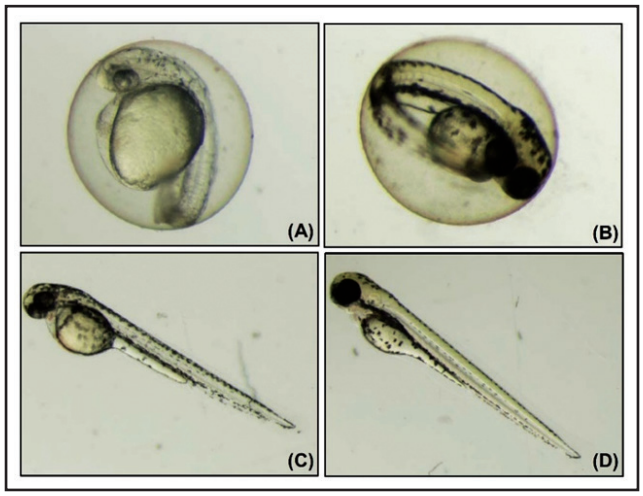

Figure 2. Development of embryos in limit test (100 mg/L of G2) @ (A) 24 hours, (B) 485 hours, (C) 72 Hours, and (D) 96 hours.

However, mortality and abnormalities were observed at higher tested concentrations of test samples, based on which the LC_{50} was calculated. The toxicity observed at various tested concentrations of G-1 and G-2 during different observational endpoints have been depicted in Fig. 3 (A to E) and Fig. 4 (A to E) respectively. A representative image from each tested concentration is shown in the figure at a time point where maximum toxicity was observed. At a concentration of 200 µg/mL, blood congestion within the pericardial region was observed, resulting in the absence of heartbeat at 96h. At 400 µg/mL, reduced or lack of somite formation was seen at 48 h. Also, impaired development was evident at 48h with a concentration of 600 µg/mL. Further, on increasing the concentration to 800 µg/mL, defects were noticed in the early stages of development with a short tail or no tail noted at 24 h. Likewise, a lack of tail detachment was found with 1000 µg/mL at 24 h. Both the experimental groups exhibited comparable morphological alterations at different concentrations.

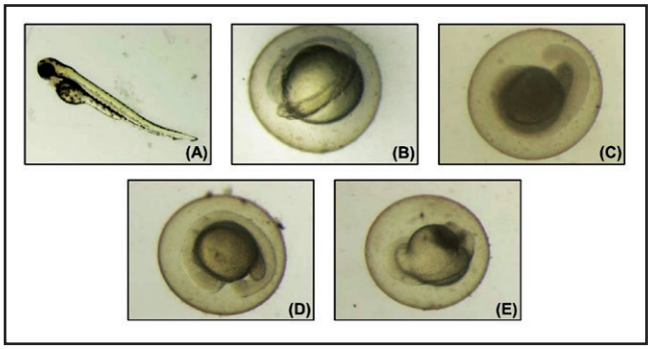

Figure 3. Toxicity observed at various tested concentrations of G1 during different observational endpoints. A representative image from each tested concentration is shown in the figure at a time-point where maximum toxicity was observed (A: 200 µg/mL at 96 h; B: 400 µg/mL at 48 h; C: 600 µg/mL at 48 h; D: 800 µg/mL at 24 h and; E: 1000 µg/mL at 24 h).

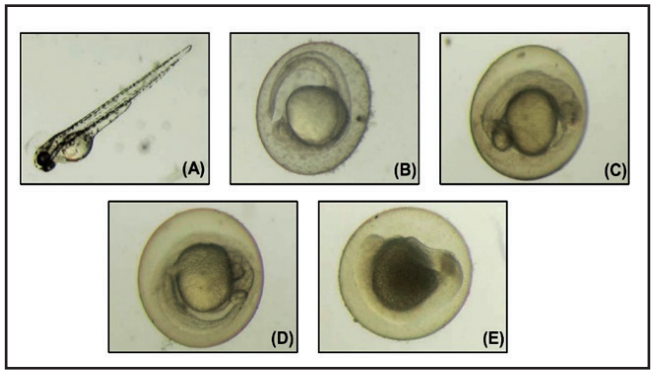

Figure 4. Toxicity observed at various tested concentrations of G2 during different observational endpoints. A representative image from each tested concentration is shown in the figure at a time-point where maximum toxicity was observed (A: 200 µg/mL at 96 h; B: 400 µg/mL at 48 h; C: 600 µg/mL at 48 h; D: 800 µg/mL at 24 h and; E: 1000 µg/mL at 24 h).

3.1 Data Analysis

The data for mortality was utilized to compute LC_{50} employing the Probit analysis[15] with minor modifications. The data for mortality at each tested concentration was transformed into corresponding probit values using a probit table[16]. To determine the probit, the percentage of larvae dead within the 96-hour incubation period for both 0 % and 100 % were adjusted as follows: 0 % dead as 100 (0.25/n) and 100 % dead as 100 (n-0.25/n). Here, 'n' represents the total number of larvae[17].The probit values were then plotted on the Y-axis graphically against log concentrations on the X-axis. The concentration corresponding to 50 % mortality on the logarithmic scale was noted. The antilogarithm of this value yielded the LC_{50} measurement. This analysis has been represented in Fig. 5 and Fig. 6 for tested samples. The calculated LC_{50} value of G1 was found to be 535.53 µg/mL i.e. antilog 2.72878 of corresponding Probit 5. For G-2, the LC_{50} value was found to be 652.81 µg/mL i.e. antilog 2.81479 of corresponding Probit 5.

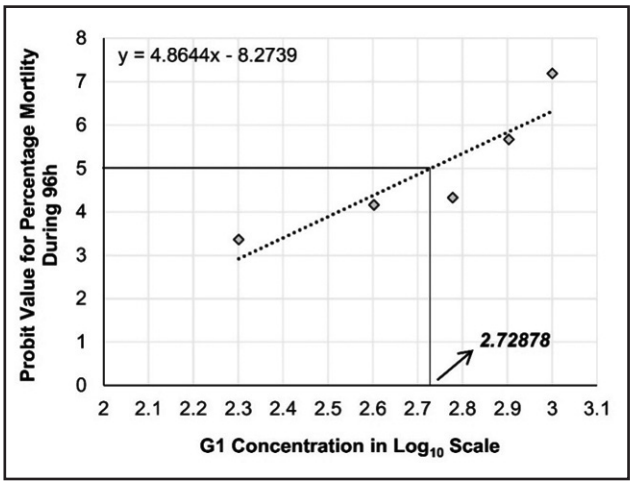

Figure 5: Probit analysis for G-1.

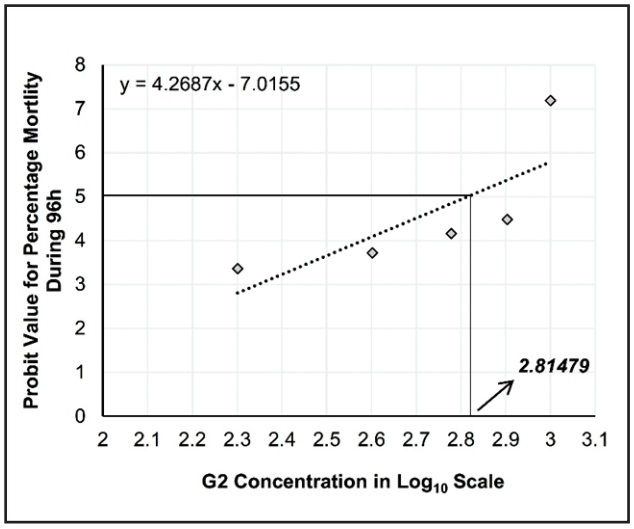

Figure 6. Probit analysis for G-2.

4. DISCUSSION

The Fish Embryo Acute Toxicity (FET) test employing Zebrafish embryos, as outlined in OECD test guideline (TG) 236, serves as an alternative to conventional acute fish toxicity testing like the OECD Acute Fish Toxicity Test (TG 203). To ensure comparable sensitivity to the acute fish test, the original FET test was streamlined to focus solely on four morphological core endpoints as mentioned above. These endpoints were chosen due to their direct or indirect correlation with mortality, making them practical for screening by proficient technical personnel and involving relatively simple morphological alterations[14].This FET test has gained wide recognition in scientific circles and even proved to have a greater sensitivity and closer correlation to humans compared to other models[18,19].Thus, this model has been employed in the current study to assess the acute toxicity of a frequently used medicament i.e. *Sanjivani Vati* prepared with two distinct species of Aconite.

On reviewing the outcomes, it can be inferred that both the test drugs were entirely safe in the initial limit test, suggesting their suitability for drug formulation. However, these findings necessitate validation through alternative and diverse models. Furthermore, the signs of toxicity were noted with increasing concentrations. At the highest concentration of 1000 µg/mL, the toxicity was evident in the early stages of development while defects in later stages of development were manifested in lower concentrations of the drug. Thus, both the test drugs exhibited concentration-dependent mortality. All these alterations were similar at different time points in both groups, indicating a similar nature among different species within the same genus. Looking over the toxicity signs closely, the cardiotoxic potential of the drug could be understood. The possible reason behind it may be its crucial ingredient i.e. *Vatsanabha* which consists of Aconitine. Aconitine is classified as a diester-diterpene alkaloid, well-known for its arrhythmogenic effects. A study on zebrafish embryos reported that aconitine-induced cardiac dysfunction and apoptosis were related to Ca^{2+} signaling pathway[20].When discussing additional indicators of toxicity, the failure of tail detachment is observed during the initial stage of development and can either serve as an indicator of general delayed development The reasons behind the failure of the tail to detach are still unclear, and it is essential to focus not only on its incidence and severity but also on the possibility of its recovery[14].These indications of toxicity could also be linked to Piperlongumine, an alkaloid present in another component of the test drug i.e. long pepper which has been observed to stimulate a rise in the number of inter segmental vessels and impede early-stage development in *Daniorerio* embryos. Additionally, it has been noted to impact heart formation and heart rate[21]. Apart, the absence or lack of somite formation indicates severe lethality. Somites play a crucial role in the formation of various structures such as the vertebrae, ribs, skeletal muscles, and skin, emphasizing their significance in the subsequent development of the embryo[22].

In addition, the LC_{50} value for both groups was determined as it provides a quantitative measure of the toxicity of a substance, indicating the concentration at which it is lethal to 50 % of the test population within a specified exposure period[23]. Moreover, the LC_{50} values frequently serve as the cornerstone for numerous advanced assays in higher tiers, including behavioral assessments and more targeted and mechanistic evaluations of toxicity using adult animals[14].On comparing the values, it was observed that the median lethal concentration of G-1 was found less than the median lethal concentration of G-2. It indicates the variation in potency between a naturally sourced drug sample and one procured from the market. Also, aconitine was identified and quantified in both the Aconitum species through High Performance Thin Layer Chromatography (Mobile Phase: Toluene: Ethyl acetate: Diethyl amine-70:20:10 v/v/v) as: *Aconitum napellus* L.-440.32 ug/100 mg and *Aconitum balfourii* Holmes ex Stapf.-16.90ug/100mg.Moreover, these results could be further substantiated through the calculation of median effective concentration i.e. EC_{50}.

5. CONCLUSION

No indications of toxicity were detected in the initial limit test, OECD 236 for *Sanjivani Vati* prepared from *Aconitum napellus* L., and *Aconitum balfourii* Holmes ex Stapf. This suggests that both the tested species of Aconite could be utilized for drug preparation following thorough processing as per the classical guidelines. Nonetheless, caution is advised regarding their dosage administration. Moreover, additional studies may also be required to elucidate the findings.

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Prof. (Vd.) Pradeep Kumar Prajapati is the Vice-Chancellor of Dr. Sarvepalli Radhakrishnan Rajasthan Ayurved University, Jodhpur. He holds expertise in pharmaceutical processing of traditional and contemporary Ayurvedic dosage forms, her bomineral formulations, establishing the safety profile of metallo-mineral formulation, and pharmaco epidemiological studies.
He contributed to the conception and design of the work and in the supervision of the study.