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Toxicological Evaluation of Polyherbal Insty Granules: An Evidence of Preclinical Safety

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Abstract: Insty, a polyherbal formulation, is widely used to treat upper respiratory ailments, especially cold and cough. However, no toxicological evaluation of the formulation has been conducted to date at the preclinical level as per regulatory guidelines. With this in mind, this study aimed to assess its preclinical toxicity (acute, repeated dose, reproductive, and genotoxicity) along with the phytochemical estimation of important constituents. Briefly, acute toxicity in mice was evaluated by oral administration of a limited dose of 1 or 5 g/kg. Mice were observed for signs of toxicity (mortality or morbidity) for 2 weeks. In case of repeated dose toxicity, the rats were orally gavaged with Insty (1 g/kg) consecutively for 28 days, followed by hematology, hepatic, and renal function tests. Furthermore, a two-generation (F0-F2) reproductive toxicity study was performed in rats at the clinically used

dose of Insty i.e. 0.24 g/kg. This includes the calculation of viability, reproductive health indices, and histopathology of the testis and ovary. The granules were also subjected to genotoxic evaluation using the micronucleus test after 2 weeks of administration (1 and 5 g/kg). High-performance liquid chromatography (HPLC) was used to measure important phytochemicals, such as salicin, glycyrrhizin, and vasicine. Our data showed no signs of mortality or morbidity in the acute toxicity tests. The hematological, hepatic, and renal parameters also did not reveal any significant differences compared to the control. In the case of reproductive toxicity assessment, the viability and reproductive health indices were comparable between the treated and control groups over the two generations. Similarly, histopathology of the testis and ovary also revealed an intact architecture. Genotoxic evaluation also showed comparable erythrocyte scoring (micronucleus count and ratios) between the treated and control groups. HPLC measurements revealed undetectable salicin, while trace levels of glycyrrhizin (4.99 mg) and vasicine (5.75 mg) were found per sachet. In conclusion, the present study is the first to extensively investigate and advocate for the safer nature of the widely used Insty, suggesting its continued consumption in humans.

Keywords: acute toxicity; repeated dose toxicity; reproductive toxicity; genotoxicity

复方益气颗粒的毒理学评价：临床前安全性的证据

摘要：因斯蒂是一种多种草药配方，广泛用于治疗上呼吸道疾病，尤其是感冒和咳嗽。然而，按照监管指南，迄今为止尚未在临床前水平对该配方进行毒理学评估。考虑到这一点，本研究旨在评估其临床前毒性（急性、重复剂量、生殖和遗传毒性）以及重要成分的植物化学评估。简而言之，通过口服 1 或 5 g/kg 的有限剂量来评估小鼠的急性毒性。观察小鼠 2 周是否有毒性迹象（死亡或发病）。在重复剂量毒性的情况下，给大鼠连续 28 天口服管饲因斯蒂（1
g/kg），然后进行血液学、肝功能和肾功能测试。此外，在临床使用的因斯蒂剂量（即 0.24 g/kg）下对大鼠进行了两代（F0-
F2）生殖毒性研究。这包括计算睾丸和卵巢的活力、生殖健康指数和组织病理学。在给药 2 周（1 和 5 g/kg）后，还使用微核试验对颗粒进行了遗传毒性评估。高效液相色谱法 (HPLC) 用于测量重要的植物化学物质，例如水杨苷、甘草甜素和vasicine。我们的数据显示急性毒性测试中没有死亡或发病的迹象。与对照组相比，血液学、肝脏和肾脏参数也没有显示出任何显着差异。在生殖毒性评估的情况下，两代治疗组和对照组之间的活力和生殖健康指数相当。同样，睾丸和卵巢的组织病理学也显示出完整的结构。遗传毒性评估还显示，治疗组和对照组的红细胞评分（微核计数和比率）相当。HPLC测量显示未检测到水杨苷，而每袋中发现微量甘草甜素（4.99毫克）和甘草酸（5.75毫克）。总之，本研究是首次广泛调查和倡导广泛使用的因斯蒂的更安全性质，表明其在人类中继续食用。

关键词：急性毒性; 重复剂量毒性; 生殖毒性; 遗传毒性

1. Introduction

Humans are provided with a wealth of plants, which have been used for medicinal purposes since antiquity. Additionally, they are the most important source of modern-day allopathic medicines, as most active pharmaceuticals are isolated from natural sources [1]. The use of medicinal plants for healing purposes is a common practice worldwide, and supersedes the consumption of modern-day medications. An estimated 80% of the global population relies on the ancient wisdom of combating diseases and illnesses. This practice is also extremely popular in Pakistan because of its affordability, availability, and belief in safety [2]. An estimated fifty thousand practitioners of herbal medicines are working in the country, with two-thirds of them working in rural areas [3]. Additionally, the country is an enriched habitat for medicinal plants, which has led to ever-increasing exports. On the one hand, it is brining revenue worth billions of dollars, but if not regulated, it may lead to species extinction from local habitats [4].

During the last century, the development and sale of herbal medicines have not been appropriately regulated around the globe, and they are mostly available over the counter without the need for prescription. Conversely, the present century is significantly changing the dynamics of this healing system, as the use of this traditional knowledge is becoming increasingly regulated with the emerging phytopharmaceutical platform. These specialized pharmaceuticals pick the ancient wisdom of healing through medicinal plants and align them with modern day requirements, thereby leading to the development

of botanicals, the newer terminology for plant-based medicinal products [5]. In some countries, phytopharmaceuticals are classified as separate classes of drugs [6]. Presumably, with the involvement of regulation and industry, there are discussions regarding the safety of these products, leading to the development of guidelines for their development. There has been an increase in the demand for the efficacy and toxicity evaluation of these products, similar to allopathic medicines, along with chemical characterization, which most of them lack. The long history of its use no longer supports the notion of safety. In this regard, regulatory bodies have been developing and updating guidelines for the industry, which is indeed a challenge due to its multi-constituent nature and fluctuating chemical diversity [7-8].

Insty, developed in 1997, is a commonly used polyherbal formulation for respiratory issues such as sore throat, cough, flu, nasal congestion, fever, cold, headache, and body ache. The product is based on the traditional wisdom of eight medicinal herbs.

Table 1 Composition of poly-herbal Insty granules

S. No.	Composition of Insty (Medicinal Plants)	Quantity per sachet used for extract preparation (mg)	Percentage of each herb (%)
1.	*Salix alba*	275	35.7
2.	*Glycyrrhiza glabra*	205	26.6
3.	*Adhatoda vasica*	115	14.9
4.	*Thea sinensis*	50	6.49
5.	*Viola odorata*	40	5.19
6.	*Valeriana officinalis*	40	5.19
7.	*Foeniculum vulgare*	30	3.89
8.	*Eucalyptus globulus*	15	1.95
	Total	770	99.91

Source: Developed by the authors

Insty herbal granules are commonly used owing to their unique blend of herbs, making it a very effective formulation. Despite their long history of use in humans, they still lack proper toxicological evaluation at both the pre-clinical and clinical levels, as per regulatory guidelines. Therefore, the present study was designed to assess the safety of Insty in laboratory animals. The outcome of the study will help practitioners make more informed decisions with confidence in product safety.

2. Materials and Methods

2.1. Animals

Non-magnetic resonance imaging (NMRI) for mice (25–30 g) and Wistar rats (200 g) of either sex were obtained from the Animal Resource Facility of Herbion Pak. Pvt. Ltd. They were housed under standard environmental conditions (25 ± 1 °C and 12 h dark/light cycle. Food and water were provided *ad libitum*. All experiments were performed according to the ethical guidelines for the care and use of laboratory animals (UK 1986).

2.2. Chemicals

The following chemicals were used in the study: glycyrrhizic acid, D-salicin, glacial acetic acid, ketamine and formaldehyde were obtained from Sigma Aldrich (USA). Vascine was provided by PhytoLab (Germany), whereas orthophosphoric acid was provided by Merck (Germany). Acetonitrile and methanol were purchased from Honeywell (USA). Insty herbal granules (Batch No. 2320 078) were provided by the R&D section of Herbion, Pakistan.

2.3. Acute Toxicity

Mice were divided into control and treatment groups (n= 5/sex). The control group was treated with distilled water (10 ml/kg), whereas the treated group received Insty Herbal Granules orally at the limit dose of 1 and 5 g/kg and was observed for any signs of toxicity for 14 days as per the guidelines provided by the Organization of Economic Co-operation & Development [9].

2.4. Repeated Dose Toxicity

Rats (n=5) were divided into two groups: vehicle control and treatment groups (Insty granules, 1 g/kg). Rats were orally administered the respective treatments for 28 days (as per OECD guideline 407), followed by collection of blood for hematology (complete blood picture) and assessment of hepatic [liver function test, alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, and total proteins] and renal (creatinine and urea) function indicators. These assessments were performed in respective hematology and chemical analyzers at the diagnostic facility Dr. Panjwani Center for Molecular Medicine & Drug Research (PCMD), International Center for Chemical & Biological Science (ICCBS), University of Karachi (UoK), Karachi, Pakistan.

2.5. Reproductive Toxicity

The effect of Insty on the reproductive health of the rats was assessed according to the guidelines

mentioned in the Red Book (FDA, 2000). Animals (F_0, 10 per sex per dose) were treated with total daily clinical dose (0.24 g/kg) of Insty for 10 weeks prior to mating and during mating. The administration of the product was continued in female rats after mating until lactation in F_1 litters. F_1 litters (one male and one female pup from each mating pair) were treated until adulthood and bred to obtain F_2 litters. Similarly, the control groups received distilled water. The following were noted as indicators of reproductive health.

2.5.1. Reproductive Health Indices & Viability Index

The following indices were calculated for both generations, F_1 and F_2:

Female fertility: Percent of mating that results in pregnancies

Gestation: Pregnancy that results in at least one live offspring

Live-born: Number of pups born alive/total number of pups born

Weaning: Survive of pups till day 21

Percentage by sex: Gender of pups, i.e., male or female from total births

Viability index: Survival from birth (day zero) – day 4, day 4 – day 7, day 7 – day 14 and day 14 – day 21.

2.5.2. Histopathology

The histopathology of male and female reproductive organs, that is, ovaries and testes, was performed after continuing the treatment for more than 1 month in male rats, whereas the treatment of female rats was initiated with male rats but was continued until weaning of newborns, that is, post-lactation in female animals. Organs were harvested at the animal house facility of Herbion Naturals and samples were submitted to the Histopathology Lab at Dow University of Health Sciences, Karachi, Pakistan.

2.6. Genotoxicity

Wistar rats (n=5) were divided into two groups: control (drinking water, 5 ml/kg) and treatment (Insty, 1 or 5 g/kg). After 14 days of oral gavage with the respective treatments, the rats were euthanized to obtain the femur. Bone marrow was harvested using 3 ml of Hank's buffered salt solution (HBSS, pH 7.4). The marrow pellet was re-suspended in HBSS (200 ml), and the smear was air-dried, fixed (100% methanol), and stored for 24 h. The slides were stained with Giemsa (5% in phosphate buffer), rinsed, air-dried, and mounted with DPX. Erythrocyte scoring was performed using a compound microscope (Motic BA210; USA).

The genotoxicity assessment (erythrocyte scoring) process comprised the manual scoring of two slides from each animal in the control and treatment groups. We focused on three different types of erythrocytes: normochromatic erythrocytes (NCEs), polychromatic erythrocytes (PCEs), and micro-nucleated polychromatic erythrocytes (MNPCEs). A total of 200 erythrocytes were observed to determine the ratio of PCE/NCE, while MNCPE occurrence was evaluated after analysis of 2000 PCEs, as reported previously (10).

2.7. Phytochemistry

The quantitative estimation of important phytochemicals, that is, glycyrrhizin, salicin, and vasicine, was performed as follows:

2.7.1. Standard & Sample Preparation

The working standards of ammonium glycyrrhizinate (100µg/ml) and salicin (500µg/ml) were prepared in 0.8% solution of ammonia and 50% methanol, respectively. Vasicine HCl was prepared at a concentration of 200µg/ml in the mobile phase. Fresh working standards were prepared daily, sonicated, and filtered through a 0.45µm syringe filter before injection.

Insty granules (equivalent to the content of one sachet) were ground first and diluted with ammonia (0.8%) and 50% methanol (50%), sonicated, and filtered (0.45µm syringe filter) before HPLC analysis of glycyrrhizin and salicin, respectively. Vasicine was extracted by transferring the content of one sachet in a dividing funnel with water and concentrated ammonia, followed by extraction with chloroform (3x). Residues obtained after evaporation of chloroform were dissolved in the mobile phase (25 ml), filtered, and subjected to quantitative determination of vasicine.

2.7.2. Chromatographic Conditions

Chromatography was performed using an HPLC (Shimadzu Prominence LC2030, Japan) equipped with a built-in quaternary pump, column compartment, autosampler, and PDA detector. Glycyrrhizin, salicin, and vasicine were individually analyzed with slight modifications to previously published chromatographic conditions [11-12]. Glycyrrhizin was quantified using a Phenomenex Luna C-18 column (250 × 4.6 mm×5µ), mobile phase (water: glacial acetic acid: acetonitrile at a ratio of 54:6:40 v/v) at 25°C, a flow rate of 1.0 ml/min, and lambda (254 nm). Salicin was analyzed using a Phenomenex Luna C18 column (250 × 4.6 mm×5µ) with a trifluoroacetic acid (0.01%): acetonitrile (92:8 v/v) mobile phase at 30°C, flow rate of 1.0 mL/min, and detection at 270 nm. In case of vasicine, estimation was performed using a Bondapak TM C18 column (300 × 3.9 mm×10µ), while mobile phase consisted of 0.1 M phosphate buffer with, acetonitrile and glacial acetic acid (85:15:1 v/v) at room temperature along with flow rate of 0.7 ml/min and detection at 300 nm.

2.8. Statistical Analysis

Data are presented as mean ± SEM (n=5). Statistical analysis was performed by one-way ANOVA followed by post-hoc analysis (least significant difference) using SPSS software (v26.0, Chicago, USA). The minimum level of significance was set at $p<0.05$.

3. Results

3.1. Acute Toxicity

No mortality was observed in mice receiving 1 or 5 g/kg of herbal Insty granules. The skin, fur, eyes, mucus membrane, behavior, bodily movement, heart, and respiration rates were normal. No signs of tremors, convulsions, salivation, diarrhea, lethargy, sleepiness, or coma were observed compared to control rats.

3.2. Repeated Dose Toxicity

None of the CBC parameters (Hemoglobin, RBC count, HCV/PCV, MCH, MCHC, WBC count, and platelet count) were found to be significantly altered in the treatment group compared to the control.

The graph depicts the mean ± SEM (n=5) of the hematological parameters following repeated doses (1 g/kg) of Insty granules (Figure 1). None of the parameters showed significant alterations compared with the control group.

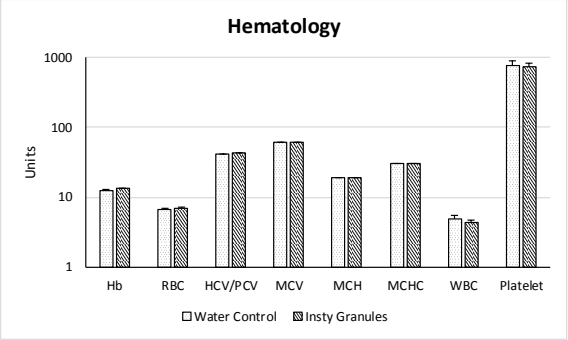

Figure 1. Effect of Insty granules on the hematology of rats *(Source: Developed by the authors*)

In the case of hepatic and renal function indicators, no significant difference was noted between the test and control groups.

Table 2. Effect of Insty granules on hepatic and renal function of rats

Treatment Group	Hepatic Function					Renal Function	
	ALT	ALP	Total Bilirubin	Direct Bilirubin	Protein	Urea	Creatinine
Insty (1g/kg)	66±16	180±44	0.4±0.07	0.1±0	6±0.2	37±1.5	0.8±0.1
Control	61±5	209±18	0.3±0.1	0.1±0	5.4±0.2	35±1.4	0.7±0.1

Source: Developed by the authors

3.3. Reproductive Toxicity

The results obtained in various indicators of reproductive toxicity are as follows:

3.3.1. Reproductive Health Indices & Viability Index

Our data showed that all other reproductive health indices (fertility, gestation, live birth, and weaning) along F_1-F_2 generations between the treated and control groups were comparable (Table 3).

The viability of pups on days 4, 14, and 21 was also comparable between the treatment and control groups (Table 4).

Table 3. Effect of Insty treatment on reproductive health indices of rats

	F_1 Generation		F_2 Generation	
Parameter	Treatment (%)	Control (%)	Treatment (%)	Control (%)
Female Fertility	70	80	42	43
Gestation	100	100	100	100
Live Born	100	100	100	100
Weaning	87	80	82	83
Male Percentage	49	51	55	43
Female Percentage	51	49	45	57

Source: Developed by the authors

Table 4. Effect of Insty treatment on viability index of rats

	F_1 Generation Viability Index (%)		F_2 Generation Viability Index (%)	
Days	Treatment	Control	Treatment	Control
4	100	100	100	100
7	95	88	100	100
14	95	88	100	100
21	87	80	82	83

Source: Developed by the authors

3.3.2. Histopathology

In both F0 and G1 generations, histopathological evaluation of male (testis) and female (ovary) reproductive organs did not reveal any signs of inflammation or structural alterations between the treated and control groups. In both groups, microscopic examination of the ovaries revealed an intact architecture. The ovarian capsule and horns were intact, and no pathology was observed. Follicles and corpus luteum were observed in the ovaries of both groups of animals (Figure 2). In the testes, the sections showed testicular tissue with intact seminiferous tubules. The basement membrane remains intact. The spermatogenesis was normal. The interstitium contained Leydig cells. No evidence of atypical cells or inflammation in any other pathology was observed.

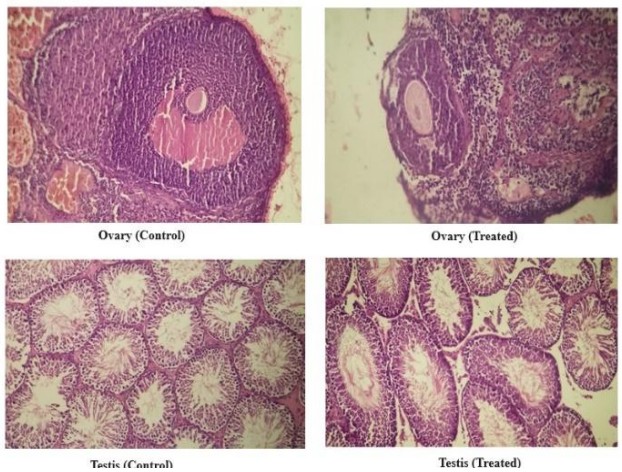

Figure 2. Microscopic images of ovary and testis of rats (*Source: Developed by the authors*)

The images depict the microscopic view of the ovaries and testes of rats that underwent a two-generation reproductive toxicity study. The images of the treated and control groups were similar and did not reveal any signs of pathology.

3.4. Genotoxicity

Similar to the control, the incidence of MNPCEs in Insty (1 g/kg) was 3 per 2000 PCEs. A slight increase to seven MNPCEs was noted in the Insty (5 g/kg) group. Furthermore, the PCE/NCE ratio was comparable among all groups (Table 5).

Table 5. Effect of Insty treatment on the erythrocyte scoring in genotoxicity test

Groups	MNPCE (Count)	PCE/NCE (%)
Insty (1g/kg)	3	46
Insty (5g/kg)	7	51
Control	3	53

Source: Developed by the authors

3.5. Phytochemistry

The HPLC analysis revealed the absence of salicin in the sample, while glycyrrhizin and vasicine were found to be 4.99 mg and 5.75 mg per sachet, respectively.

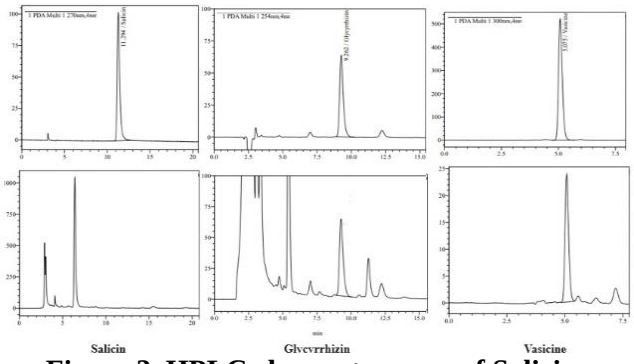

Figure 3. HPLC chromatograms of Salicin, Glycyrrhizin and Vasicine (*Source: Developed by the authors*)

Figure 3 depicts HPLC chromatograms of the three important phytochemicals present in the herbs used in the production of herbal granules. In the case of salicin, the chromatogram on the top showed the peak of standard salicin at 11.294 min, while no peak was found for a similar retention time in the Insty sample. However, against the standard peaks of glycyrrhizin (9.262 minutes) and vasicine (5.075 minutes), the peaks on the similar retention times were also observed in Insty samples, which were quantified to be 4.99 mg and 5.75 mg per sachet, respectively.

4. Discussion

Humans are provided with a wealth of medicinal plants, which are used by both traditional healers and modern phytopharmaceuticals. In the case of industry, there has been a continuous increase in regulations by healthcare authorities. A long history of its use is no longer accepted as proof of safety. Therefore, the present study was designed for the toxicological evaluation of polyherbal Insty granules at the pre-clinical level.

To identify the safe dose range, the granules were tested for mortality and morbidity using doses of 1 and 5 g/kg. Notably, no mortality or obvious signs of morbidity were observed for two weeks in the treated mice. This outcome, despite using extremely high doses, advocates the safe nature of the granules. To further assess any internal systemic manifestations, a repeated dose toxicity study was performed at 1 g/kg, followed by evaluation of hematology, liver, and kidney function [13]. Although a limited dose was used in this study continuously for 28 days, our data showed that all hematological parameters were significantly unaltered compared to the control (Figure 2). Hepatic and liver function indicators were similar to those of the control. This further supports the safety of instant granules.

Two generation (F0-F2) toxicity studies are commonly used assess the effect of test substances on reproductive health [14-15]. Our data shows that none reproductive health indices (female fertility, gestation, live-born, weaning and gender percentage) was affected by the clinically used dose (0.24 g/kg) of granules till two generations. Additionally, the viability index until day 21 was comparable to that of the control rats. Furthermore, histological evaluation of both testes and ovaries did not reveal any signs of pathology or structural alterations in the treated group (Figure 2). Taken together, these results suggest that Insty granules have no toxicological effects on the reproductive health of rats until two generations.

The mammalian micronucleus test is routinely used to evaluate the genotoxic potential of a test substance [16]. At a limit dose of 1 g/kg, the micronucleus count and PCE/NCE ratio were similar to those of the control group (Table 6). Similar to the control, the incidence of MNPCEs in Insty (1 g/kg) was 3 per 2000 PCEs. A slight increase to seven MNPCEs was noted in the Insty (5 g/kg) group. Furthermore, the PCE/NCE ratio was comparable among all groups (Table 5).

Table 5. Effect of Insty treatment on the erythrocyte scoring in genotoxicity test

Groups	MNPCE (Count)	PCE/NCE (%)
Insty (1g/kg)	3	46
Insty (5g/kg)	7	51
Control	3	53

Source: Developed by the authors

It was only at the highest dose limit dose i.e. 5 g/kg), and an increase in the micronucleus count was observed. This indicates that the insty granules has a huge safe dose range from the genotoxicity point of view, but still at some point, it may cause it.

Natural products contain a wide range of constituents, which underlie their efficacy and toxicity. However, some of these are considered as biomarkers. Insty composition suggested that out of eight plants, *Salix alba*, *Glycyrrhiza glabra*, and *Adhatoda vasica* accounted for 77%. Hence, one biomarker from each of the three plants, that is, salicin (*Salix alba*), glycyrrhizin (*Glycyrrhiza glabra*), and vascine (*Adhatoda vasica*), was selected and quantified in the granules using HPLC. Salicin is contraindicated by the European Medicines Agency (EMA) during pregnancy owing to its reproductive toxicity [17]. Notably, we did not find any traces of salicin in polyherbal Insty granules which probably underlies the lack of any toxicological effects on reproductive health in the present study. The absence of salicin in Insty is consistent with the EMA recommendations, which is attributed to improved extraction. A literature search revealed that glycyrrhizin was relatively safe. The acceptable daily intake limit of glycrrhizin and vaccine was reported to be 0.05 – 0.229 mg/kg/day [18], which is far higher than its daily intake through Insty granules i.e. 4.99 mg per sachet. With reference to vasicine, to the best of our knowledge, we could not find any daily intake limits in the literature. In general, it has been reported to be safe [19], and our data revealed 5.75 mg of vascine per sachet. Hence, our phytochemical findings support the non-toxic nature of Insty granules observed in the present study.

5. Conclusion

In conclusion, polyherbal Insty granules did not show any signs of toxicity (acute, systemic, reproductive, and genotoxicity) at the preclinical level. To the best of our knowledge, this is the first detailed toxicological evaluation of this formulation to date. One of the major limitations of this study is that it was conducted in a single species, despite recommendations by regulatory bodies to do so in at least two species before drawing conclusions. However, this study advocates for its safer nature and implies that its use should be continued in humans. Future studies should be conducted in humans to confirm their safe consumption.

Declarations

Author Contributions

Conceptualization, S.B.; methodology, M.M.U. and A.H.A.; formal analysis, F.G. and M.M.U.; data curation, F.G., M.M.U. and A.H.A.; writing—original draft preparation, all authors contributed equally; writing—review and editing, S.B.; supervision, S.B.; project administration, S.B. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data presented in this study are available in this article.

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Funding information is not available.

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Institutional Review Board Statement

The animal study protocol was approved by the Ethics Committee of Herbion Pak. Pvt. Ltd.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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