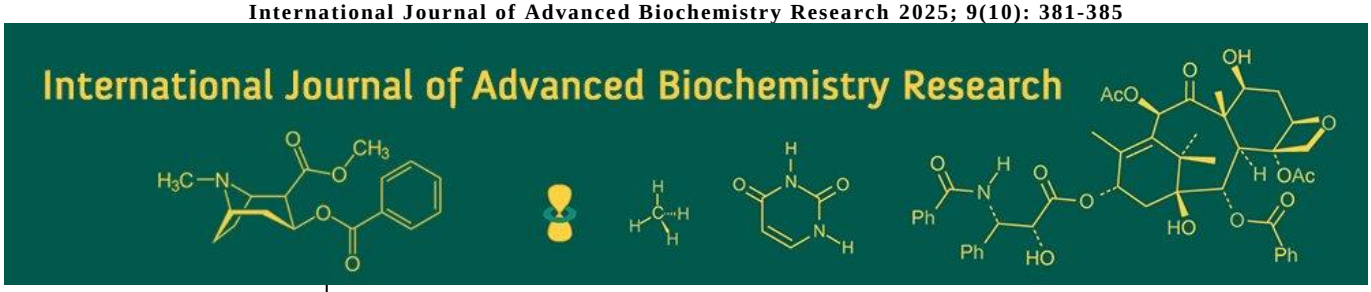

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Subacute toxicological evaluation of *Catharanthus pusillus* Methanolic extract in wistar rats

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Abstract
The present study evaluated the subacute toxicity of alcoholic extracts of *Catharanthus pusillus* (Murray) G. Don (CPE) in Wistar rats in accordance with OECD Test Guideline 407. Forty healthy rats (20/sex) were divided into four groups (I-IV; n = 10/group). Group I served as control (distilled water), while Groups II-IV received CPE orally at 750, 1500, and 3000 mg/kg body weight, respectively, for 28 consecutive days. All animals survived until terminal sacrifice, and no treatment-related effects were observed on body weight, hematology, or clinical chemistry, except for a minimal increase in platelet counts in females of Groups II and IV. Absolute and relative spleen weights were increased in treated males, while relative liver weight was elevated in Group IV males. Histopathology revealed hepatocellular necrosis in two male animals of Group IV and splenic lymphoid hyperplasia in male animals of Groups II, III and IV, whereas no other test article-related microscopic changes were noted. Immunohistochemistry demonstrated increased CYP2E1 expression following CPE administration, with no effect on CYP3A1. Based on these findings, a No Observed Adverse Effect Level (NOAEL) could not be established.

Keywords: *Catharanthus pusillus*, immunohistochemistry, CYP3A1, wistar rats

Introduction
India is globally renowned for its rich heritage of traditional medicine and remarkable biodiversity of medicinal plants. Approximately 85% of traditional medicines worldwide are plant-derived, and nearly 7,500 species are used in India by various ethnic communities (Farnsworth *et al.*, 1985) [9]. As the world's largest producer of medicinal herbs, India is often referred to as the "Botanical Garden of the World" (Ahmedulla & Nayar, 1999) [1]. Medicinal plants contain diverse bioactive compounds—including alkaloids, glycosides, terpenes, phenolics, flavonoids, tannins, essential oils, and toxalbumins—that can exert both therapeutic and toxicological effects depending on dose, chemical composition, and mode of use (Satish, 2002; Groten, 2013) [19, 11]. Plant poisoning remains a significant cause of economic loss in Indian livestock production due to mortality, reduced productivity, abortions, and associated veterinary costs (Khandare, 2015) [15]. *Catharanthus pusillus* (CP), a perennial herb of the family Apocynaceae, is notable for both its medicinal and toxicological significance (Navitha *et al.*, 2012) [18]. The plant is characterized by white latex, a carrot-shaped taproot, quadrangular reddish stems, and oblong leaves (Figure 1). Traditionally, CP is used for its antimicrobial, antineoplastic (Subbaiyan *et al.*, 2013) [20], antioxidant (Thingujam *et al.*, 2015) [22], anthelmintic, and wound-healing properties (Gajalakshmi *et al.*, 2013) [10]. Its roots, leaves, and latex are also employed in the treatment of skin and liver disorders, leprosy, ulcers, tumors, rheumatism, asthma, and cardiac ailments (El-Sayed & Cordell, 1981; Chandran & Saj, 2015) [6, 7, 5]. These effects are largely attributed to its alkaloid content, which also contributes to its potential toxicity.
Field observations indicate that CP toxicity is common in livestock in parts of Gujarat, particularly the Virampur region of Banaskantha district, where it is locally known as "Marchadi." Outbreaks in cattle and buffaloes are reported annually, with high morbidity and production loss, and in severe cases, progression to coma and death. In the absence of a specific antidote, only symptomatic treatment is available. Despite frequent incidents, scientific data on the toxicodynamics, target organ effects, and LD_{50} of CP in animals are

scarce. Therefore, the present 28-day oral toxicity study in Wistar rats was undertaken to Evaluate clinical and behavioural alterations following CP extract administration, assess hematological and biochemical changes, and Investigate pathomorphological alterations in major organs.

Materials and Methods

The experimental protocol (Protocol No. VETCOLL/IAEC/2021/17/PROTOCOL-12) was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science and Animal Husbandry, Kamdhenu University, Sardarkrushinagar, Gujarat, India. All experimental procedures were conducted in accordance with the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), New Delhi.

Experimental Animals and Management

A total of 40 healthy Wistar rats (20 males and 20 females) were procured from the Animal Facility of Cadila Pharmaceuticals Ltd., Dholka, Gujarat, India. Animals were acclimatized prior to experimentation and maintained under standard laboratory conditions (12 h light-dark cycle, temperature 22±2 °C, relative humidity 50±10%) with free access to pelleted feed and water ad libitum. All management and treatment procedures adhered to CCSEA guidelines.

Collection and Authentication of Plant Material

Catharanthus pusillus (Murray) G. Don (CP), locally known as "Marchadi" is commonly found around Virampur village, Banaskantha district, Gujarat—particularly during September to October, when incidences of bovine toxicity are highest. The plant was collected from a farm reporting toxicity in buffaloes and subsequently identified and authenticated by the Department of Botany, Hemchandracharya North Gujarat University, Palanpur.

Preparation of Alcoholic Plant Extract

Collected plants were washed, shade-dried, powdered, and sieved to obtain a fine powder. The powdered material was packed in a thimble and extracted using methanol in a Soxhlet apparatus, with three successive extractions over 8 hours at the boiling point of methanol (56 °C), following the method described by Harborne (1964) [12]. The extract was concentrated to dryness and stored in airtight sample bottles at 4 °C until use.

Experimental Design

The 40 rats were randomly assigned to four groups (I-IV), each consisting of five males and five females: Group I (Control): Received distilled water orally for 28 days. Group II: Received CP extract (CPE) at 750 mg/kg body weight orally for 28 days. Group III: Received CPE at 1500 mg/kg body weight orally for 28 days. Group IV: Received CPE at 3000 mg/kg body weight orally for 28 days. All animals were observed daily for clinical signs and behavioural alterations throughout the experimental period.

Clinical Observations and Body Weight Monitoring

All animals were observed twice daily for clinical signs of morbidity and mortality throughout the study. Body weights were recorded on Day 1 and at weekly intervals thereafter. On Day 29, surviving rats were fasted overnight, euthanized, and terminal body weights were recorded prior to necropsy.

Organ Collection and Relative Organ Weights

Following euthanasia, a complete necropsy was performed. Organs including the liver, kidneys, lungs, heart, brain, spleen, adrenals, thymus, ovaries, epididymides, and testes were collected. All organs were fixed in 10% neutral buffered formalin, except testes and epididymides, which were initially preserved in modified Davidson's fluid for 48 h and then transferred to formalin. The liver, kidneys, adrenals, thymus, spleen, brain, heart, epididymis, and testes were trimmed and weighed immediately to determine absolute and relative organ weights.

Hematology and Serum Biochemistry

On Day 29, blood samples were collected from the retro-orbital plexus of all surviving rats. Samples were placed in EDTA tubes for hematological analysis and clot activator tubes for biochemical analysis. Hematological parameters—including TEC, Hb, HCT, MCV, MCH, MCHC, platelet count, WBC, TLC, and DLC—were analyzed using a fully automated hematology analyzer (Exigo™ EOS, Boule Diagnostics AB, Sweden). Giemsa-stained smears were prepared within 3 h of collection to assess platelet and erythrocyte morphology and basophil count. Biochemical parameters— including ALT, AST, ALP, total protein, albumin, urea, creatinine, triglycerides, phosphorus, calcium, magnesium, cholesterol, glucose, iron, uric acid, and GGT—were measured using an automated clinical chemistry analyzer (Randox Monaco, Randox Laboratories Ltd., UK).

Histopathology

Fixed tissues were trimmed, labeled, and washed for 2 h under running tap water. Dehydration was performed in graded isopropyl alcohol (30-100%), cleared in xylene, and paraffin-embedded using an automated tissue processor (Leica TP1020) and embedding station (Leica EG1160). Sections (4-5 µm) were cut with a rotary microtome (Leica RM2255) and mounted on poly-L-lysine-coated slides. Slides were deparaffinized, rehydrated, and stained with Harris' hematoxylin, differentiated in acid alcohol, blued in ammonia water, and counterstained with eosin. After dehydration and clearing in xylene, sections were mounted with DPX. Staining was performed using an automated stainer (Gemini AS, Thermo Scientific) following standard protocols (Luna, 1968; Suvarna *et al.*, 2012) [16, 21].

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver tissues from male rats were sectioned at 4 µm on poly-L-lysine-coated slides. Sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval in EDTA buffer (pH 8.5). Endogenous peroxidase activity was blocked with 3% H_2O_2, followed by incubation with primary antibodies against CYP3A1 (1:500) and CYP2E1 (1:200). After washing with Tris-buffered saline, slides were incubated with HRP-conjugated secondary reagent (Dako REAL™ EnVision™) for 30 min at room temperature, and color was developed using DAB chromogen. Sections were counterstained with Gill's hematoxylin, dehydrated, cleared, and mounted for microscopic evaluation.

Statistical Analysis
Data on body weights, hematological and biochemical parameters, and organ weights were analyzed using two-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical significance was set at $p<0.05$.

Results and Discussion
Clinical Observations and Body Weight
No abnormal clinical signs were observed in Groups I and II throughout the 28-day study. One male in Group III and two males and one female in Group IV showed intermittent diarrhea from Day 9 onwards. One Group IV female exhibited temporary circling movements but recovered spontaneously. Similar clinical signs, including tremors and restlessness, have been reported in rats following administration of Catharanthus roseus extracts at high doses (Vutukuri *et al.*, 2017; Kevin *et al.*, 2012) [24, 14]. No mortality was recorded in any group, aligning with previous findings in rats treated with Catharanthus extracts (Ukoha *et al.*, 2017; Ajuru *et al.*, 2019) [23, 2]. Body weight remained unaffected at all dose levels (up to 3000 mg/kg), which agrees with earlier reports in rats exposed to Catharanthus extracts (Kevin *et al.*, 2012) [14].

Hematology and Biochemistry
No significant changes in hematological parameters were observed in males. In females, a significant increase in platelet count was noted in Groups II and IV, while other parameters were comparable to controls. Terpenoid indole alkaloids in Catharanthus species (e.g., vindoline, catharanthine, yohimbine) may have vincristine-like activity, contributing to thrombocytosis (Mackin *et al.*, 1995; Allen *et al.*, 2021; Zárate *et al.*, 2001) [17, 3, 26]. Biochemical parameters remained within normal limits in all groups, indicating no functional impairment of major organs, similar to findings in Catharanthus toxicity in sheep (Aydogan *et al.*, 2015) [4].

Organ Weights
No significant changes in absolute or relative organ weights were observed in females. In males, an increase in spleen (Groups II-IV) and relative liver weight (Group IV) was noted. These changes may reflect lymphoid hyperplasia and mild hepatic alterations. Sex-related physiological differences may explain the male-specific effects, as reported previously (Jothy *et al.*, 2011; Yuet *et al.*, 2013) [13, 25].

Gross and Histopathology
No gross lesions were observed in any group. Microscopically, male rats in Group IV showed minimal hepatocellular necrosis (Figure 2), vacuolation, and neutrophilic inflammation localized to periportal and midzonal regions (Figure 3). Lower dose groups showed no hepatic lesions. Splenic lymphoid hyperplasia (Figure 4) was observed in all Group IV males and in some Group II and III males, characterized by expansion of the periarteriolar lymphoid sheath (Figure 5) and prominent germinal centers. Other organs, including brain, kidneys, lungs, heart, adrenals, thymus, reproductive organs, GI tract, salivary glands, and lymph nodes, were unremarkable. Similar hepatic changes have been reported with Catharanthus roseus extracts in rats (Ajuru *et al.*, 2019; Elshama *et al.*, 2014) [2, 8]. The observed lymphoid hyperplasia may reflect immunostimulation due to bisindole alkaloids in *Catharanthus pusillus* (El-Sayed & Cordell, 1981) [6, 7].

Immunohistochemistry
Because liver lesions occurred only in males, CYP expression was evaluated in this group. CYP2E1 showed strong pericentral cytoplasmic immunoreactivity in controls and increased midzonal and periportal staining in Groups II-IV, suggesting enzyme induction (Figure 6). CYP3A1 expression remained comparable among all groups. These findings suggest that CPE may induce or activate CYP2E1, contributing to its metabolism. Similar CYP modulation has been reported with vindoline in rats (Zhang *et al.*, 2022) [27].

Fig 1: *Catharanthus pusillus* (Murray) G Don. Plant

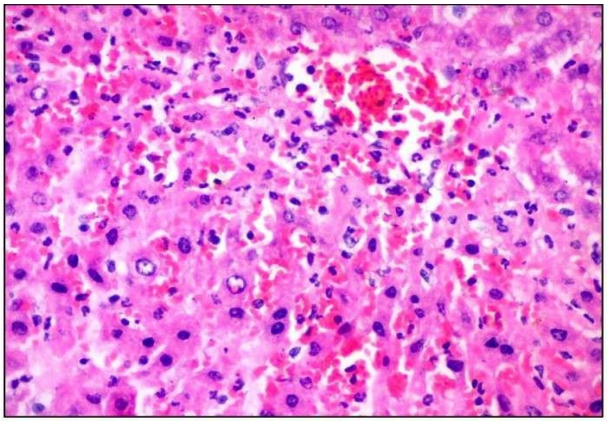

Fig 2: Group IV (Male): Liver showing minimal hepatocellular necrosis with infiltration of neutrophils H&E, 400X.

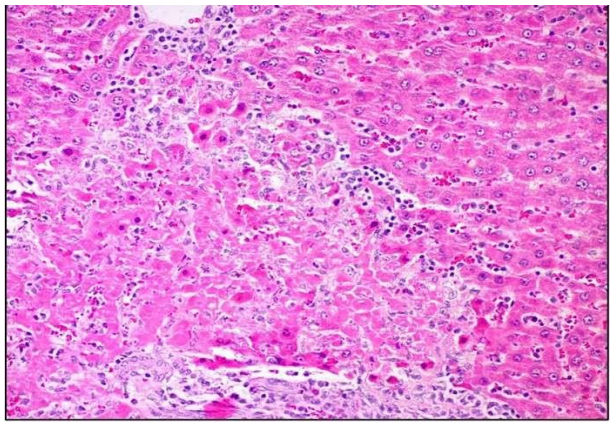

Fig 3: Group IV (Male): Liver showing focal extensive area of hepatocellular necrosis involving periportal and midzonal hepatocytes H&E, 400X.

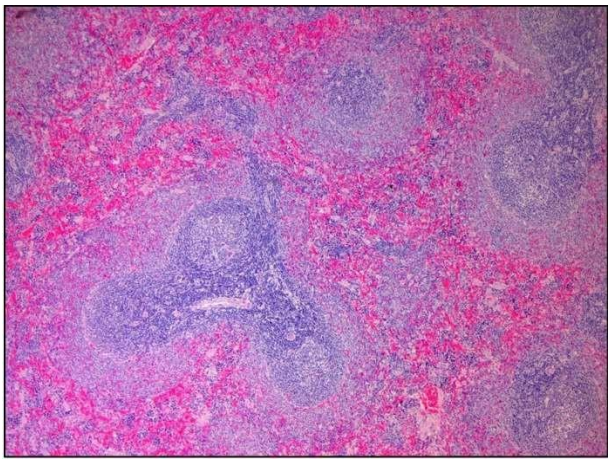

Fig 4: Group IV(Male): Spleen showing expansion of PALS area prominent secondary follicle andmarginal zone H&E, 50X.

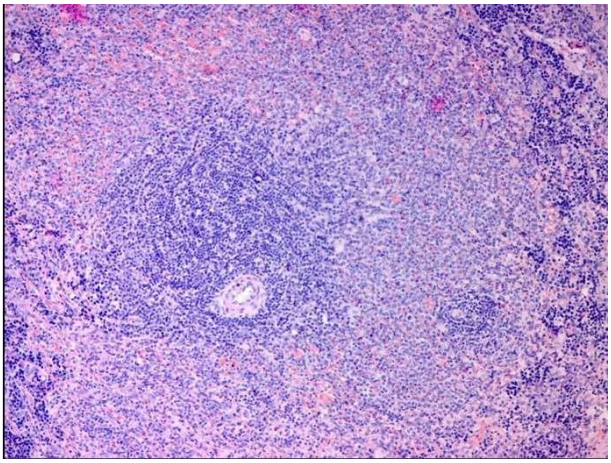

Fig 5: Group III (Male): Spleen showing prominent PALS, secondary follicle and marginal zone H&E, 100X

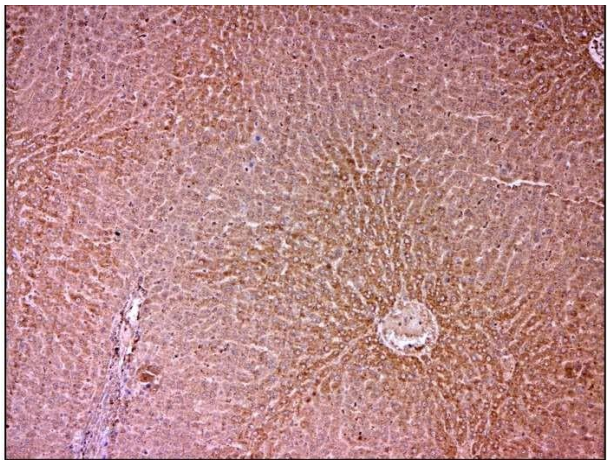

Fig 6: Group IV (Male): Liver showing strong immunoexpression of Cytochrome P450 CYP2E1 in pericentral while midzonal & periportal hepatocytes shows moderate immunoexpression Immunoperoxidase staining, Metal enhanced DAB chromogen, Gills hematoxylin, 100X.

Conclusion

This study demonstrated that 28-day oral administration of *Catharanthus pusillus* methanolic extract (CPE) up to 3000 mg/kg body weight did not produce significant changes in body weight, hematological, or biochemical parameters. However, dose-related increases in spleen and liver weights were observed, accompanied by minimal hepatocellular necrosis and splenic lymphoid hyperplasia. Immunohistochemical analysis revealed induction of CYP2E1 expression, indicating metabolic activation, while CYP3A1 remained unaffected. These findings suggest that CPE induces subacute toxic effects at higher doses. Therefore, a No Observed Adverse Effect Level (NOAEL) could not be established under the conditions of this study.

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