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Original Article

Preclinical Safety Assessment of a Polyherbal Formulation: Acute, Subchronic, and Mutagenic Evaluation

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ABSTRACT

Introduction: Before market approval, novel herbal medicines and bioactive compounds require rigorous genotoxicity and safety evaluations. A polyherbal formulation derived from *Dasiphora fruticosa*, *Cynara scolymus*, and *Rosa acicularis* has previously demonstrated antioxidant and nephroprotective properties. However, comprehensive toxicological and mutagenicity evaluations are needed to support its clinical development. In this study, we aimed to evaluate the acute and subchronic toxicities and mutagenic potential of this polyherbal formulation. **Methods:** Acute oral toxicity was assessed in C57BL/6 mice using a two-phase protocol based on Lorke's method. Subchronic toxicity was evaluated in Wistar rats following OECD guideline 407, with daily oral administration of the polyherbal formulation at doses of 500 and 1,000 mg/kg for 28 days. Mutagenicity was assessed using the Muta-Chromoplate (Ames test) kit according to OECD guideline 471. **Results:** Acute toxicity evaluation determined that the median lethal dose (LD_{50}) of the polyherbal formulation exceeds 5,000 mg/kg, categorising it as practically nontoxic. The subchronic toxicity assessment revealed that doses of 500 and 1,000 mg/kg had no significant effects on body and organ weight, haematological and biochemical parameters, and histopathological features compared with the controls. Furthermore, the Ames test confirmed that the polyherbal formulation had no mutagenic activity. **Conclusions:** The polyherbal formulation exhibited no acute toxicity at doses up to 5,000 mg/kg, and no adverse effects were observed in a 28-day subchronic toxicity study. Furthermore, its favourable safety profile was further confirmed by its lack of mutagenic potential. Collectively, these findings provide a robust foundation for continued preclinical and clinical development of the polyherbal formulation.

KEYWORDS: acute toxicity; mutagenicity; phytochemicals; polyherbal formulation; safety evaluation; subchronic toxicity

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INTRODUCTION

Genotoxicity refers to the capacity of a substance to damage the genetic material in somatic cells, potentially leading to carcinogenesis. Mutations that occur in germ cells can result in heritable genetic alterations. Common mutation types, such as duplications, insertions, deletions, and point mutations, are implicated in the pathogenesis of hereditary diseases and various cancers[1]. Various *in vitro* (cell-based) and *in vivo* (animal-based) assays are employed to determine the genotoxic potential of chemical substances. Among these, the Ames test is widely regarded as the gold standard *in vitro* assay for genotoxicity assessment[2].

The global burden of cancer continues to increase. In 2015, cancer accounted for 9 million deaths, and the World Health Organization estimates that this figure will increase to 11.4 million by 2030[3]. Numerous chemical substances and various physical factors, including UV light and γ-radiation, have been linked to the development of human cancer, since the formation of DNA damage (also referred to as DNA adducts or lesions) caused by these agents constitutes a critical initial step in carcinogenesis[4]. Consequently, inhibiting mutagenesis has emerged as a promising strategy for cancer prevention and therapeutic intervention[5].

Drug development typically progresses through four stages: discovery, preclinical research, clinical trials, and post-marketing surveillance. This process spans approximately 10–15 years before market approval is achieved. Given that adverse drug reactions (ADRs) remain a significant challenge in clinical practice, affecting approximately 6.5% of hospitalised patients, preclinical studies are particularly crucial for confirming the safety and non-toxicity of candidate compounds[6,7]. Mortality related to ADRs has been reported in 0.1% of internal medicine inpatients and 0.01% of surgical patients[8]. Notably, several drug-associated risks remain unidentified during pre-market clinical trials and may only become apparent years after commercialisation. For instance, in the United States, 10% of drugs approved between 1975 and 1999 were subsequently associated with serious adverse effects[9]. Similarly, in Canada, 40% of drugs withdrawn from the market between 1963 and 2004 were removed within 3 years of approval owing to safety concerns[10,11]. These findings underscore the critical need for rigorous preclinical safety evaluations.

Mongolia's rich biodiversity and long-standing tradition of herbal medicine use offer a strong foundation for developing plant-based therapeutics. Plants containing bioactive compounds, such as flavonoids, cynarin, alkaloids, glycosides, quercetin, rutin, luteolin, and vitamin C, are typically employed for their well-documented anti-inflammatory, antioxidant, and antibacterial properties[12-14]. These

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plants have traditionally been used in the treatment of liver and gallbladder diseases, kidney disorders, hypertension, anemia, diarrhea, dysentery, gastrointestinal ailments, fever, cough, and edema affecting the limbs and the entire body[15-17]. Based on this rationale, *Dasiphora fruticosa* L., *Cynara scolymus* L. (artichoke), and *Rosa acicularis* L. were selected to formulate a polyherbal formulation. The phytochemical composition, antioxidant capacity, and nephroprotective effects of this formulation have been previously characterised[18-20]. However, comprehensive toxicological and mutagenicity evaluations are needed to support its clinical development. Accordingly, this study aimed to further evaluate the toxicological profile of the polyherbal formulation.

MATERIALS AND METHODS

Preparation of the polyherbal formulation

The flowers of *Dasiphora fruticosa* L., aerial parts of *Cynara scolymus* L. (artichoke), and fruits of *Rosa acicularis* L. were sliced into 3–5 mm fragments and extracted using distilled water at a 1:5 (w/v) ratio to obtain aqueous extracts. Separately, the flowers of *Dasiphora fruticosa* L. and aerial parts of *Cynara scolymus* were extracted with 70% ethanol at the same ratio. The ethanol was removed under reduced pressure using a rotary vacuum evaporator to concentrate the extracts. The concentrated ethanolic and aqueous extracts were lyophilised using a freeze dryer (BK-FD10 Series; Biobase, China) to yield dry powders for subsequent experiments.

The polyherbal formulation consists of three components: the flowers of *Dasiphora fruticosa* L., aerial parts of *Cynara scolymus* L., and fruits of *Rosa acicularis* L. with a ratio of 1:2:2.

Experimental animals

C57/BL6 mice were obtained from Experimental animal center, Institute of biomedical science, MNUMS. Wistar rats were obtained Drug Research Institute of Monos Group LLC. The animals were housed in the vivarium of the Department of Pharmacology, Mongolian National University of Medical Science, under standard laboratory conditions with ad libitum access to food and water.

Ethical statement

The study protocol was approved by the Ethical Review Committee of the Mongolian National University of Medical Science (approval no. 2024/3-04) and ethical conclusions were issued by protocol number 25-26/01-01. The study was conducted according to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

Acute toxicity study

The acute toxicity (LD_{50}) of the polyherbal formulation was evaluated in C57BL/6 mice (body weight: 24–27 g; age: 6–8 weeks) using the method described by Lorke (1983),[21] conducted in two phases.

In Phase I, healthy male mice were randomly assigned to the control and experimental groups. The control group received distilled water (10 mL/kg), whereas the experimental groups (n=3 per group) received the polyherbal formulation via gavage at doses of 500, 1,500, and 2,500 mg/kg. The physical condition of the mice and signs of toxicity were monitored for 2 h, and mortality was recorded at 24, 48, and 72 h. In Phase II, based on the Phase I results, three additional groups (n=3 per group) received the polyherbal formulation at higher doses of 3,000, 4,000, and 5,000 mg/kg.

Subchronic toxicity study

Subchronic toxicity was assessed according to OECD guidelines 407[22]. Wistar rats were randomly divided into three groups: an untreated healthy control group (n=6), which received distilled water daily; a group that received the polyherbal formulation at 500 mg/kg (n=6); and a group that received the polyherbal formulation at 1,000 mg/kg (n=6) via gavage. All treatments were administered for 28 consecutive days.

Body weight was recorded, after which blood samples were collected for haematological evaluation to determine the safety profile of the polyherbal formulation. The analyses focused on key haematological indices, including red blood cell count (RBC), haemoglobin (HGB), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width–standard deviation (RDW-SD), red cell distribution width–coefficient of variation (RDW-CV), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), platelet-to-large cell ratio (PLCR), plateletcrit (PCT), and white blood cell count (WBC). All measurements were conducted using a Sysmex PocH-100i haematology auto-analyser (Sysmex Corporation, Japan).

Biochemical parameters, including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), urea, and creatinine, were measured using an automated biochemical analyzer (ICUBIO iChem-520, Shenzhen, China)[23], and histopathological analyses were performed as previously described[24].

In vitro Ames test for mutagenicity

The mutagenic potential of the polyherbal formulation was evaluated using the Muta-Chromoplate™ kit (Environmental Bio-detection Products Inc.), based on the validated bacterial reverse mutation assay (Ames test, OECD 471)[1]. This assay detects mutagens in environmental samples, food, cosmetics, and biological fluids.[25] The assay was performed using *Salmonella typhimurium* (TA100) under sterile conditions, following the manufacturer's instructions as well as the protocol described by Hubbard et al[26]. The polyherbal formulation was tested at concentrations of 5.0, 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/mL in triplicate in a 24-well plate, with each well containing nutrient broth, S9 metabolic activation components, and standard mutagens. Wells that turned purple were classified as negative, whereas yellow-coloured wells were classified as positive. Sodium azide (SA) was used as a positive control for TA100, and 2-aminoanthracene (2-AA) was used for all strains with S9 activation. The same solvent system used for the tested samples was used as the negative control.

Statistical analysis

Data are presented as the mean ± SD with a 95% confidence interval. Statistical analyses were performed using IBM SPSS Statistics for Windows version 26. Group comparisons were conducted using ANOVA followed by Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$.

RESULTS

Acute toxicity study

All mice were monitored for 24 and 72 h post-treatment. The control group received 10 mL/kg distilled water orally, whereas the treatment groups were administered a single oral dose of the polyherbal formulation at 500, 1,500, 2,500, 3,000, 4,000, and 5,000 mg/kg via gavage. No signs of toxicity or mortality were observed at any of the tested doses (500–5,000 mg/kg). These findings indicate that the oral LD_{50} of the polyherbal formulation exceeds 5,000 mg/kg, classifying it as practically non-toxic according to OECD toxicity guidelines (Table 1).

Subchronic toxicity study

The body weights of the rats were measured on Days 1, 7, 14, 21, and 28 following polyherbal formulation administration and compared with the baseline values and those of the control group.

Throughout the study, the rats were closely monitored for signs of toxicity and behavioral alterations, including changes in locomotor activity, respiration, urination, defecation, diarrhea, tremors, convulsions, and mortality. All observations were systematically recorded. Oral administration of polyherbal formulation for 28 days did not produce any signs of toxicity or behavioural abnormalities, and all rats survived throughout the study duration. However, a significant increase in body weight was observed in groups receiving 500 and 1,000 mg/kg of the polyherbal formulation compared with the control group (Figure 1). The body weights of both control rats and those treated with polyherbal formulation increased significantly from Day 1 to Days 7, 14, 21, and 28 ($p < 0.05$).

Macroscopic examination of the liver, spleen, heart, lungs, and kidneys revealed no significant differences between the control group and groups that received 500 or 1,000 mg/kg of the polyherbal formulation. Furthermore, polyherbal formulation administration for 28 days did not induce significant differences in the absolute or relative weights of the liver, spleen, heart, lungs, or kidneys between the experimental and control groups (Figure 2). Taken together, these findings indicate that prolonged polyherbal formulation administration did not markedly impact organ mass.

Haematological analysis of rats treated with 500 and 1,000 mg/kg of the polyherbal formulation revealed no statistically significant differences in key blood parameters, including red and white blood cell counts, platelet counts, haemoglobin levels, and haematocrit, compared with the control group (Table 2).

Similarly, biochemical assessments revealed no significant differences in liver (AST, ALT, and ALP) or renal function markers (creatinine and urea) between the experimental and control groups after 28 days (Table 3).

Table 1. Acute toxicity of the polyherbal formulation following oral administration

Phase	Group	Dose (mg/kg)	Mortality (at 24 h)	Mortality (at 48 h)	Mortality (at 72 h)
	I	500	0	0	0
I	II	1,500	0	0	0
	III	2,500	0	0	0
	IV	3,000	0	0	0
II	V	4,000	0	0	0
	VI	5,000	0	0	0

number of animals per group (n) =3.

Table 2. Effects of the polyherbal formulation on haematological parameters in rats during the subchronic toxicity study

Parameter	Groups		
	Control	500 mg/kg	1,000 mg/kg
WBC (10^3/μL)	8.83 ± 1.36	6.53 ± 1.50	8.67 ± 3.04
RBC (10^6/μL)	6.51 ± 0.88	6.43 ± 0.67	7.43 ± 0.46
HGB (g/dL)	12.02 ± 1.73	12.27 ± 1.05	13.28 ± 0.90
HCT (%)	41.07 ± 0.95	41.00 ± 1.24	43.35 ± 2.62
MCV (fL)	60.77 ± 9.50	60.37 ± 3.96	58.42 ± 3.08
MCH (pg)	19.25 ± 1.97	18.90 ± 0.76	17.88 ± 0.82
MCHC (g/dL)	31.88 ± 2.35	31.45 ± 1.14	30.65 ± 1.16
RDW-SD (fL)	30.62 ± 1.95	31.97 ± 4.38	32.90 ± 7.18
RDW-CV (%)	12.72 ± 1.78	12.93 ± 1.81	14.22 ± 3.59
PDW (%)	8.25 ± 0.43	8.30 ± 0.32	8.03 ± 0.32
MPV (fL)	7.45 ± 0.68	7.25 ± 0.23	7.02 ± 0.21
PLCR (%)	6.47 ± 1.28	7.12 ± 1.06	5.93 ± 1.27
PCT (%)	0.85 ± 0.13	0.70 ± 0.14	0.75 ± 0.10
PLT (10^3/μL)	1142.67 ± 59.67	1045.00 ± 96.48	1062.83 ± 83.10

Table 3. Effects of the polyherbal formulation on biochemical parameters in rats during the subchronic toxicity study.

Parameter	Groups		
	Control	500 mg/kg	1,000 mg/kg
Alanine transaminase (U/L)	61.78 ± 10.08	65.52 ± 6.82	72.22 ± 3.29
Aspartate transaminase (U/L)	117.27 ± 9.77	124.55 ± 9.25	128.18 ± 7.99
Alkaline phosphatase (U/L)	339.42 ± 47.80	324.60 ± 75.23	387.98 ± 53.38
Urea (mmol/L)	7.17 ± 1.35	7.89 ± 0.53	8.47 ± 0.80
Creatinine (μmol/L)	48.70 ± 2.79	44.71 ± 3.81	45.24 ± 2.01

Table 4. Mutagenicity evaluation of the polyherbal formulation using the Ames test.

Condition	Salmonella typhimurium (TA100)	
	(S9 -)	(S9 +)
Sterile control	0 ± 0	0 ± 0
Negative control (-)	2 ± 1*	2.3 ± 0.57*
Positive control (+)	21 ± 1	21.3 ± 0.57
5 mg/mL	0 ± 0*	14.6 ± 0.57*
2.5 mg/mL	0 ± 0*	10.6 ± 2.08*
1.25 mg/mL	6 ± 2*	11 ± 3*
0.625 mg/mL	5 ± 1*	9 ± 1*
0.312 mg/mL	6.3 ± 1.52*	8 ± 1*
0.156 mg/mL	1.6 ± 0.57*	7 ± 1*

*The values for the positive controls significantly differed ($p < 0.05$) from those of the other groups.

Histopathological study

Microscopic examination of liver sections from both untreated control rats and those administered 500 or 1,000 mg/kg of the polyherbal formulation revealed no evidence of necrosis, fatty degeneration, inflammation around the portal areas, or fibrosis. Kidney tissue analysis revealed normal glomerular structure without focal or diffuse necrosis in the proximal or distal tubules. Moreover, histological assessment of the cardiac muscle revealed normal cardiomyocyte morphology, with no pathological changes observed in either the control or treatment groups.

In vitro Ames test for mutagenicity

The mutagenic potential of the polyherbal formulation was assessed using aqueous extracts of its herbal constituents (*Dasiphora fruticosa*, *Cynara scolymus* (artichoke), and *Rosa acicularis*) at concentrations of 5.00, 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/mL. The assay was performed *in vitro* using a Muta-Chromoplate kit (Ames test), with and without S9 metabolic activation. The mean values were calculated and compared across treatments.

In the absence of S9 activation, no bacterial colony growth was observed at 5.00 and 2.50 mg/mL, likely as a result of the antibacterial properties of the extract, necessitating further dilution for colony visualisation. However, at lower concentrations (1.25–0.15625 mg/mL), bacterial colonies were clearly observed. Conversely, in the presence of S9 mix, colony formation was evident across all tested concentrations.

The positive controls (SA for the non-activated system and 2-AA for the S9-activated system) produced significantly higher numbers of revertant colonies compared to the polyherbal formulation treated samples ($p < 0.05$), thus validating the assay results. Collectively, these results indicate that the aqueous extract of the polyherbal formulation does not exhibit mutagenic activity under the tested conditions (Table 4).

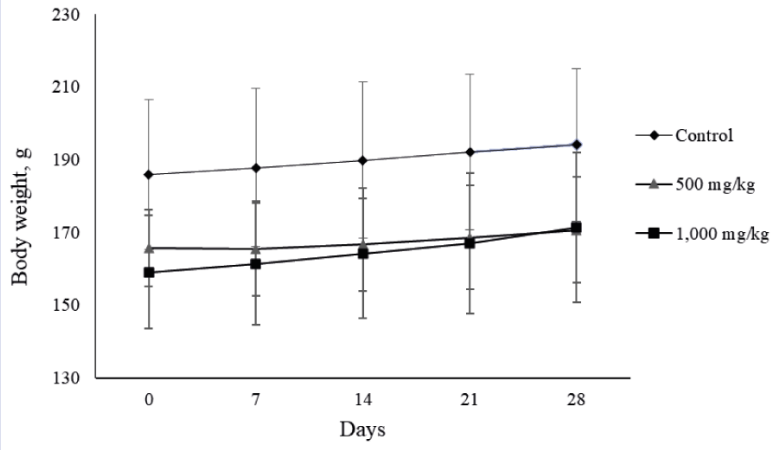

Figure 1. Body weight measurements of rats administered the polyherbal formulation. *Data is presented as the mean ± SEM.

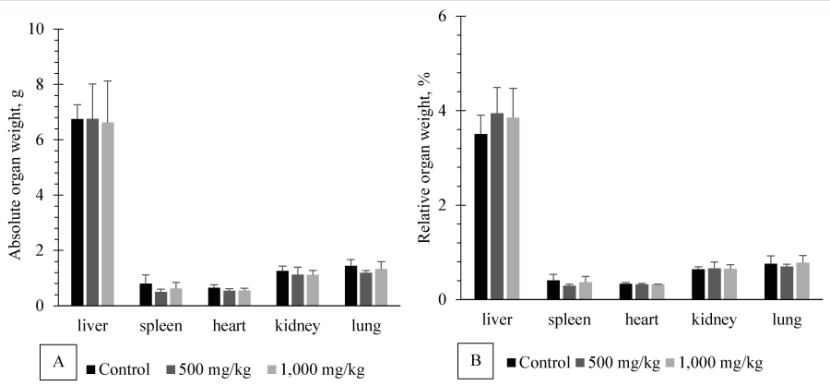

Figure 2. Absolute (A) and relative (B) organ weights of rats treated with the polyherbal formulation for 28 days.

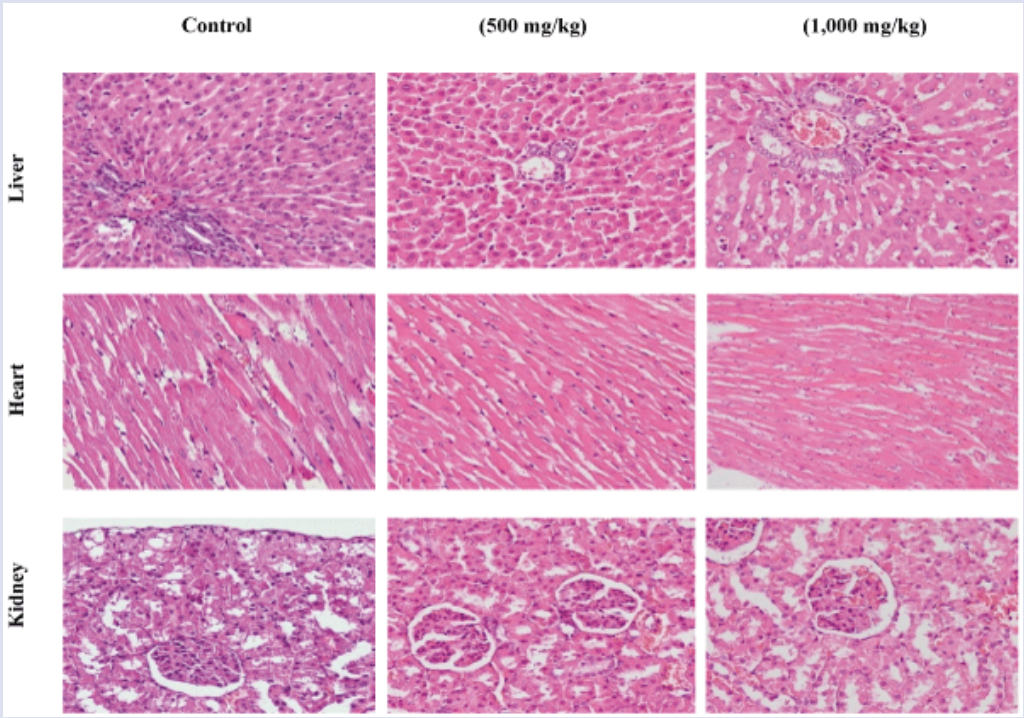

Figure 3. Histological structures of the liver, heart, and kidneys in experimental rats. H&E staining, ×40 magnification

DISCUSSION

Safety assessments are a critical step in developing herbal therapeutics. This study evaluated the acute and subchronic toxicities and mutagenic potential of a polyherbal formulation in accordance with internationally recognised preclinical standards. The combined *in vivo* and *in vitro* results confirmed the safety of the formulation, supporting its advancement to clinical trials[27].

Acute toxicity evaluation revealed that the LD_{50} of the polyherbal formulation exceeds 5,000 mg/kg, classifying it as practically non-toxic according to the OECD guidelines. This finding aligns with previous research that has indicated that many herbal formulations are generally well-tolerated even at high doses[28-30]. For instance, Dram et al. (2020) reported no toxicity in mice administered *Potentilla anserina* L. extract at a maximum tolerated dose of 345.6 g/kg[31]. Similarly, Tian et al. (2025) found that *Potentilla freyniana* Bornm rhizome extract did not exhibit acute toxicity in rats at doses up to 4,000 mg/kg, with an LD_{50} of 8,510 mg/kg[32]. Moreover, research conducted in Mongolia further corroborates these findings, with LD_{50} values exceeding 5,000 mg/kg reported for Antidiabet-3 (containing *Cynara scolymus*, *Dasiphora fruticosa*, and *Tribulus terrestris*),[33] *Cynara scolymus* aqueous extract (>5 g/kg),[20] and *Dasiphora fruticosa* L. extract (4 g/kg)[34].

Subchronic toxicity studies further support the safety of *Cynara scolymus* and its related botanical formulations. Bemidinezhad et al. (2023) reported no adverse effects after 12 weeks of treatment with an artichoke leaf formulation at doses of 600 and 1,200 mg/kg[35]. Zhang et al. (2012) observed no toxicity from *Rosa laevigata* flavonoids administered at 500–2,000 mg/kg over a 90-day period,[36] and similarly, Olfat et al. (2020) observed no physiological or histological changes in rats administered 5 g/kg of artichoke extract for 4 weeks[37].

In this present study, the 28-day subchronic toxicity evaluation revealed no significant changes in body weight, organ weight, haematological or biochemical parameters, or histopathological features. Vital organs, including the liver, kidneys, and heart, remained structurally and functionally intact, confirming the safety of the formulation at the organ level.

The polyherbal formulation consists of *Dasiphora fruticosa*, *Cynara scolymus*, and *Rosa acicularis*, which are all abundant in polyphenolic compounds, such as quercetin, rutin, luteolin, and various flavonoid derivatives recognised for their antioxidant properties[38,39]. The absence of biochemical or histopathological abnormalities observed in this study can likely be attributed to these constituents[40].

Although some studies have reported context-dependent genotoxicity, which is typically dose- or condition-specific, Regiane et al. (2012)[41] noted genotoxic effects in HepG2 cells exposed to artichoke leaf extract. However, low-dose pretreatment attenuated hydrogen peroxide-induced DNA damage, suggesting potential antigenotoxic effects[41]. Moreover, Goryacha et al. (2022) further demonstrated that *Dasiphora fruticosa* L. possesses mechanisms that prevent the accumulation of harmful substances, supporting its antigenotoxic and antimutagenic properties[42].

Under in vitro conditions, the genoprotective activity of cynarin was evaluated. Cynarin demonstrated no genotoxic effects, as evidenced by the absence of numerical and structural chromosomal abnormalities, the lack of sister chromatid exchanges, the absence of micronucleus formation, and negative comet assay results[43]. Moreover, evaluation of the genotoxic effects of flavonoids using the SMART (Somatic Mutation and Recombination Test) assay revealed a statistically significant reduction in spot mutations compared with groups exposed solely to the damaging agent[44]. Likewise, vitamin C administered at a dose of 500 mg/kg markedly reduced $FeSO_4$ (200 mg Fe/kg)-induced chromosomal aberrations (CAs) and DNA damage[45]. The absence of mutagenic activity in the Phytonephro-SAN preparation aligns with these findings and indicates that the cynarin, vitamin C, and flavonoid constituents of the formulation do not exhibit genotoxic properties.

The Ames test remains a benchmark assay that is used to assess mutagenicity. In the present study, the polyherbal formulation inhibited bacterial growth at higher concentrations in the absence of S9 metabolic activation, indicating its potential antibacterial activity. At lower concentrations and in the presence of S9 activation, colony formation was observed that was not significantly different from that in the negative control ($p < 0.05$), confirming the absence of mutagenic activity. Collectively, these findings provide robust evidence for the genetic safety of the polyherbal formulation[46]. Overall, this study represents the first comprehensive safety evaluation of this specific polyherbal formulation and establishes baseline data on its acute, subchronic, and mutagenic profiles. Given the potential for synergistic interactions between phytoconstituents in polyherbal formulations, integrated toxicological assessments are essential to guide clinical development.

CONCLUSION

The polyherbal formulation exhibited a favourable safety profile, with an LD_{50} exceeding 5,000 mg/kg, classifying it as practically nontoxic according to the OECD guidelines. The subchronic administration of the formulation at doses of 500 and 1,000 mg/kg over 28 consecutive days produced no mortality or significant alterations in physiological, biochemical, or histological parameters. Furthermore, the formulation exhibited no mutagenic activity in the Ames assay. Taken together, these findings support the continued development of this polyherbal formulation as a safe and promising candidate for clinical evaluation and further studies will be conducted to assess its stability and technological properties.

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