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Original Article

Safety Evaluation of a Standardized *Dalbergia sissoo* Leaf Extract (DSLE)

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ABSTRACT

Background: *Dalbergia sissoo*, also known as Indian Rosewood, is a plant known for its medicinal value with the leaves used traditionally for its effect on joint inflammation and joint pain. Recently, *Dalbergia sissoo* leaves and its derived novel compound Caviunin glycoside (CAFG) was shown to alleviate symptoms and signs of osteoarthritis in a rat model. **Objectives:** The objective of the study was to investigate potential adverse effects, if any, of a standardized *Dalbergia sissoo* leaf extract (DSLE) in rats following sub-chronic administration. **Materials and Methods:** DSLE was standardized to a composition of not less than 2% w/w CAFG and not less than 6% w/w total flavonoids. In the sub-chronic study, Wistar rats (10/sex/group) were administered via gavage 0 (control), 310, 620, 1240 mg/kg body weight/day of DSLE for 90 days. Two additional groups received 0 and 1240mg/kg /day of the extract for 90 days, followed by a 28-day reversal phase. **Results:** The study revealed that administration of the DSLE did not result in any toxicologically significant treatment related changes in clinical observation, ophthalmic examination, body weight, body weight gain, feed consumption, clinical pathology and organ weight. The haematology and serum chemistry parameters compared to the control group were also within the normal laboratory limits. Terminal necroscopy did not indicate any treatment related gross or histopathological findings. The results of genotoxicity studies as evaluated by gene mutations in Salmonella typhimurium, and *in vitro* chromosome aberration assay did not reveal any genotoxicity of the extract. **Conclusion:** Based on the results of this study, the no observed-adverse-effect level (NOAEL) for this standardized *Dalbergia sissoo* leaf extract was determined as 1240 mg/kg body weight/day, the highest dose tested.

Key words: *Dalbergia sissoo*, Dietary ingredient, Leaf extract, Safety, Toxicity, *Sissoo*, Bone health.

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INTRODUCTION

Dalbergia sissoo (*D. sissoo*), commonly known by the names Indian rosewood and Sheesham, belongs to the legume family (Fabaceae). It is a perennial tree found in tropical to subtropical climates in natural and planted forests in the Indian subcontinent.[1] In addition to its use as timber for fine furniture, the tree has many reputed medicinal properties and has been used culturally for various ailments.[2] Various parts of *D. sissoo* are traditionally used in providing health benefits. For example, its bark and wood are known to exhibit anti-inflammatory, anti-nociceptive, antioxidant and anti-helminthic activities. Its leaves and leaf extracts have been shown to possess anti-inflammatory and anti-diabetic properties as well.[1]

An earlier study demonstrated that the ethanolic extract of *D. sissoo* leaves possesses anti-inflammatory activity, wherein inhibition of oedema induced in rats was observed.[3] Evaluation of the constituents of *D. sissoo* leaf extract for osteogenic activity revealed that genstein, biochanin A, pratensein, biochanin 7-O-glucoside and caviunin 7-O-[β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside] (CAFG) resulted in significant osteogenic activity.[4] The isolated novel isoflavonoid CAFG was present in a higher percentage in the *D. sissoo* leaf extract. Furthermore, experiments in osteopenic ovariectomized mice indicated the osteogenic potential of CAFG as an alternative for anabolic therapy for the condition of osteoporosis.[5]

Recently, a preclinical study was conducted with a standardized *D. sissoo* leaf extract and its derived novel compound CAFG, with an aim to treat the clinical symptoms of osteoarthritis and understand the mechanism.[6] The authors concluded from the study that the *D. sissoo* leaf extract alleviated symptoms and signs of osteoarthritis in the rat model. Although further investigations are required to understand the detailed mechanism, they concluded that the CAFG in IL-1β induced

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chondrocytes by its anti-inflammatory activity inhibits cartilage degradation and subchondral bone loss.[6]

A clinical study of *D. sissoo* leaf extract conducted in post-menopausal women to evaluate its anti-osteoporotic efficacy reported that *D. sissoo* leaf extract was well tolerated by all participant at given dose of 600mg/day orally for a period of one year.[7]

For centuries, natural products, such as medicinal plants, have been the basis for the treatment of various ailments. However, in screening natural products for pharmacological activity, assessment and evaluation of the safety of long-term use or consumption of medicinal plants is important. In order to establish a comprehensive safety profile of the standardized *D. sissoo* leaf extract (DSLE) appropriate toxicological evaluation was carried out. The objective of the study was to investigate adverse effects, if any, of a standardized DSLE preparation in genotoxicity studies as evaluated by the Ames test, and *in vitro* chromosome aberration assay, and in a repeat-dose sub-chronic toxicity study in rats. The effects of DSLE preparation were investigated in a dose-response manner.

MATERIALS AND METHODS

Preparation of extract

The *D. sissoo* leaves used in the experiment were procured from Anand (Gujarat, India). Plant material identified, and a voucher specimen (PHPL/HB/029.A) was deposited at the Anand Agricultural University (Gujarat, India) for identification. The shade dried and pulverized leaves (1000 g) were extracted thrice with 4L of ethanol and water (9:1) at 50 ± 5°C for 3 hr by the reflux method. The solvent mixture was filtered using Whatman paper and concentrated under reduced pressure. The obtained dried DSLE was further triturated in ethanol at room temperature, and the alcohol-soluble part was concentrated and dried to yield 51 g. The physical and chemical specifications of the product have been fully developed (Table 1). The DSLE was further used for the purification of compounds (1–5) and identification.[4]

Test substance

The DSLE was provided by Pharmanza Herbal Pvt. Ltd., Gujarat, India. The extract is a brown color powder and was standardized to contain not less than 2.0% caviunin 7-O-[β-D-apiofuranosyl-(1→6)-β-Dglucopyranoside (CAFG) and not less than 6% w/w total flavonoids (Biochanin-7-O-Glucoside, Genstein, Pratensein and Biochanin A) as analysed by high-performance liquid chromatography (HPLC) analysis. Appropriate amounts of the test substance were dissolved by slowly adding the pure water (Milli Q) and crushing the solid extract by a standardized procedure to ensure homogeneity.

High-performance liquid chromatography profiling of DSLE

High pressure liquid chromatography system (Shimadzu Corporation Ltd, Kyoto, Japan) was used, connected to a SIL-30AC auto sampler, LC-30AD quaternary CTO-20AC column oven and SPD-20A UV detector. The data was analyzed by laboratory solution software. It was equipped with a RP C_{18} Shim-pack GIST column (4.6 X 250 mm, 5 µm particle size). Glacial acetic acid (0.2% v/v) in water and Acetonitrile (HPLC GR,Rankem) was used as the mobile phase. Solvent flow rate was set to be 1.0mL/min and a gradient elution profile was used for the identification and separation of the main components of the DSLE. The chromatograms were obtained by eluting the samples at 259nm for quantification and identification (Figure 1) of total flavonoids as Caviunin 7-O-B-D-glucopyranoside, Biochanin-7-O-Glucoside, Genstein, Pratensein and Biochanin A.

Table 1: **Physical, chemical and microbiological specifications of *Dalbergia sissoo* leaf extract (DSLE).**

Parameter	Specifications	Assay method
	Physical parameters	
Appearance	Powder	Visual
Color	Light to Dark Greenish Brown	Visual
Taste	Bitter	Physical
	Chemical parameters	
Loss on Drying	NMT 6.00%	USP <731>
Total DS Flavonoids	NLT 6.00%	In-house
Caviunin 7-O-[β-D-apiofuranosyl-(1→6)-β-Dglucopyranoside (CAFG)	NLT 2.00%	In-house
	Heavy metals	
Lead	< 5 ppm	USP <233>, ICP-MS
Arsenic	< 3 ppm	USP <233>, ICP-MS
Cadmium	< 1 ppm	USP <233>, ICP-MS
Mercury	< 2 ppm	USP <233>, ICP-MS
	Microbiological parameters	
Total Plate Count	< 10000 cfu/g	USP <2021>
Yeast and Mold	< 1000 cfu/g	USP <2021>
Coliforms	< 30 mpn/g	FDA-BAM Chapter 4
Escherichia coli	Absent/10g	USP <2022>
Salmonella sp.	Absent/10g	USP <2022>
Staphylococcus aureus	Absent/10g	USP <2022>

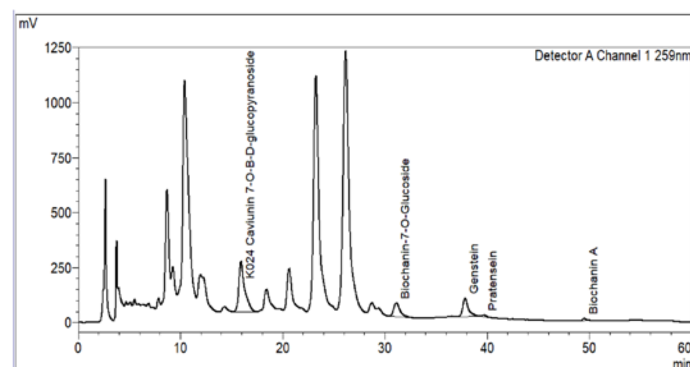

Figure 1: HPLC Chromatogram of Standardized DSLE.

Genotoxicity Studies

Ames test

This test was carried out to investigate the potential of DSLE to induce gene mutations (Ames test) and was performed at GLP certified laboratory in compliance with The Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP). *Salmonella typhimurium* strains TA98, TA100, TA102,

TA1535, TA1537 were used, and the plate incorporation method in the presence or absence of a S9 metabolic activation system was applied. Based on the solubility and precipitation test results eight different concentrations were selected for a pre-experiment. Based on the pre-experiment results, the test item that were tested were viz., 0.002, 0.002, 1.582, and 5 mg/plate for the main study, both in the presence of metabolic activation (+S9) and in the absence of metabolic activation (-S9). Chemicals used as positive control for assays without or with metabolic activation included sodium azide, methyl methane sulfonate, and 2-aminoanthracene. S9 fraction (Aroclor 1254-induced; Analab, USA) with cofactor was used to mimic the metabolic activation system.

The plate incorporation method was employed, and the following components were added sequentially: A: phosphate buffer; B: each testing concentration of DSLE, negative or positive control solution; C: overnight culture of the *Salmonella typhimurium* strains (containing approximately 1-2 × 10^9 cells/ml); D: molten top agar with histidine/biotin. The contents were mixed and poured onto the surface of minimal glucose agar plates. When the top agar was solidified, the plates were inverted and placed in an incubator for 48 to 72 hr at 37 ± 1°C. Following which the colonies were counted. All experiments were carried out in triplicate.

A test item was considered a mutagen, if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA100 and TA 102) or thrice (TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed. A dose dependent increase was considered biologically relevant if the threshold exceeded at more than one concentration. An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative control and vehicle control such an increase is not considered biologically relevant.

In vitro chromosomal aberration assay

The potential of DSLE to induce structural and numerical chromosome aberrations was evaluated in human lymphocytes. The test was performed following GLP guidelines and in accordance with the OECD guideline for testing of chemicals #473, *In vitro* Mammalian Chromosome Aberration Test (1997), in the presence and absence of metabolic activation system. Three different test concentrations based on the solubility of DSLE (5 mg/ml maximum concentration), was tested unless limited by solubility, precipitation and effect on media pH of the test item. The treatment of cultures with the test substance was conducted in a minimum of two independent phases. For Phase – I 1% and for Phase – II 2% of the S9 mixture was used with the metabolic activation system. Proliferating cells were used and the cell culture was prepared in RPMI-1640 medium 48-50 hr prior to exposure. Negative and vehicle control (solvent), and positive control were maintained, both in the presence and absence of metabolic activation system. The medium of the proliferating blood culture was removed by centrifugation at 1000-1500 rpm for 10 min and cells suspended in plain medium mixed with S9 mix and in complete media mixed with phosphate buffer for the treatment in the presence and absence of metabolic activation system respectively. For Phase II, cultures were harvested at the end of incubation of 22-25 h after treatment.

Harvesting and processing cells

Before 3-5 hr of harvesting, 240 μL of colcemid (10 μg/ml) was added to each of the culture tube/flask, and incubated at 37± 2°C. The cultures were harvested by centrifugation at 1000-1500 rpm for 10 min, 22-25 hr after beginning of treatment. The supernatant was discarded and the cells were re-suspended in approximately 7-8 ml of freshly prepared, warm (37± 2°C) hypotonic solution of potassium chloride (0.075 M KCl). The cell suspension was allowed to stand for 30 min then centrifuged and supernatant removed. Freshly prepared chilled (5-6 mL) Carnoy's fixative (3:1 methanol: acetic acid solution) was added and allowed to stand for 5 min. This process was repeated twice and after final centrifugation, the supernatant was removed completely, and the cell pellet was re-suspended in 0.3 to 5mL of Carnoy's fixative. Slides were prepared by dropping the cell suspension onto a clean microscope slide and stained with 5% fresh Giemsa stain. All slides, including those of positive and negative controls, were independently coded before microscopic analysis.

Analysis of metaphase cells

A minimum of 1000 cells were counted in different fields of the slide per culture and the number of metaphases were recorded for mitotic index (MI) calculation. At least 300 cells were equally divided among the two cultures and a well spread metaphase plates per concentration were scored for cytogenetic analysis using a 100x oil immersion objective. Chromosomal and chromatid breaks, acentric fragments, deletions, exchanges, polyploidy (including end reduplication) and disintegrations were recorded as structural chromosome aberrations. Gaps were recorded as well, but they were not included in the calculation of the aberration rates. Only metaphases with 46 ± 2 centromere regions were included in the analysis. Also, to describe a cytotoxic effect, the mitotic index (% cells in mitosis) was determined.

Subchronic study

Study design

The subchronic study was performed according to a well-designed protocol based on Organization for Economic Co-operation and Development (OECD) guidelines for testing chemicals.[8] The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Protocol No. APC/2016-IAEC/1619).

Animals

For the subchronic study, Wistar strain rats were used and were procured from National Centre for Laboratory Animal Sciences (CPCSEA Registration No: 154/99/CPCSEA) National Institute of Nutrition, (ICMR), Hyderabad. The study was performed at the Anand Pharmacy College, Anand, Gujrat, India. A total of 120 male and female rats 5-6 weeks old, were selected after physical and behavioural examination for the subchronic study. Selected females were nulliparous and non-pregnant. The animals were maintained according to standard guidelines. The animals were housed in groups of five in standard polypropylene cages with stainless steel top grill under controlled conditions in a room ventilated with fresh air, with 15 to 20 air changes per hour. Clean paddy husk was used as a bedding material. The room temperature was maintained at 22±3°C with relative humidity between 30 and 70% and a 12 hr light/dark cycle. The animals were allowed to acclimatize for a minimum of five days before the initiation of experiments. A standard rat pellet diet (purchased from Pranav Argo Pvt. Ltd, India) was provided

ad libitum throughout the study period. Drinking water was provided *ad libitum* in polypropylene bottles with stainless steel sipper tubes.

Treatment

In the subchronic study, Wistar strain rats (10/sex/group) were randomly divided into six groups. At randomization, the animals were approximately 5-6 weeks old and their body weight was within ±25% of the overall mean of each sex (weight range for male 80 -110 g and females 70 - 100 g). Rats (10/sex/group) were treated orally (gavage) with DSLE at dose levels of 0 (Group I- control), 310 (Group II- low dose), 620 (Group III- mid dose), and 1240 (Group IV- high dose) mg/kg body weight/day (dosing volume 10 mL/kg) for 90 days. Two additional groups of animals for the recovery study received 0 (Group V) and 1240 (Group VI) mg/kg/day of DSLE for 90 days, followed by no additional treatment for 28 days. The use levels of DSLE in the 90 days and recovery study were based on a human equivalent dose of 5 mg/kg/day of CAFG, a pharmacological study in ovariectomized mice that led to enhanced bone formation (Kushwaha *et al*., 2014). In the course of the subchronic study, all animals were provided *ad libitum* feed, until the day prior to the scheduled euthanasia. At the completion of the 90-day treatment period, all animals in Group I to IV were euthanized. In the recovery group, after completion of the treatment period of 90 days, animals were kept under post-treatment observations for 28 days and then euthanized.

Parameters Investigated

Clinical Observation, Body Weight and Feed Consumption

All animals were observed twice daily for morbidity and mortality. Clinical examinations included any abnormal physical and behavioral changes. The observations included changes in skin, fur, eyes, mucus membrane, and autonomic activity like lacrimation, piloerection, pupil size and unusual breathing pattern. Changes in gaits, posture, response to handling, presence of clonic or tonic movements, stereotypic activities like excessive grooming, repetitive circling, etc, were observed. The time of onset, intensity and duration of such symptoms, if any, were recorded. Ocular examinations were conducted on all animals prior to the initiation of experiments and during the day prior to euthanasia. Individual animal body weight for treatment and recovery groups were recorded at least weekly, beginning on the day before the initiation of treatment. Mean body weight and mean body weight changes were calculated for the corresponding intervals. Final body weight was recorded one day prior to the scheduled necropsy. The amount of feed and water consumed by each cage of animals was recorded weekly. Feed intake was calculated as g/animal/day for the corresponding bodyweight intervals. The water intake was calculated as ml/animal/day.

Clinical Pathology

After the end of the experimental protocol, animals were anesthetized by intraperitoneal (i.p) administration of ketamine (50mg/kg) and xylazine (10mg/kg) mixture and blood was collected in vial that were pre-filled with the EDTA and Citrate for the analysis of hematological and biochemical parameters from each animal by retro-orbital plexus method. Hematological analysis of the blood samples was performed using an automatic hematology analyzer (Nihon kohden, celltecα, Japan). The parameters which were evaluated included: hemoglobin (Hb); red blood cells (RBC) count; mean corpuscular volume (MCV); pack cell volume (PCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); prothrombin time(PT); activated partial prothrombin time (APTT); platelets (PLT); white blood cell (WBC) count; eosinophil's, lymphocytes and monocytes count. Biochemical parameters were performed by using an automated analyzer (Turbochem 100, USA).

Macroscopic and Microscopic Examinations

A complete necropsy was performed on all animals. Animals were euthanized under carbon dioxide inhalation anesthesia. The necropsies included but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. Many tissues and organs were collected and were placed in 10% neutral-buffered formalin. At the scheduled necropsies, the selected organs were weighed from all animals, and for thorough histopathological examinations, tissues were processed into paraffin blocks, sectioned at nominal 5 μm, mounted on glass microscope slides and stained with hematoxylin and eosin. The pathologist who performed the histological assessments of the slides was blinded to the treatment.

Statistical Analysis

Results were expressed as the mean ± S.E.M. Statistical analysis was performed using version 6.0 of the Graph Pad Prism statistical program. One-way ANOVA was used, followed by Dunnett's Test for parametric multiple comparisons between the vehicle control and the treatment groups. Differences were considered significant when the p value was less than 0.05 ($p < 0.05$).

RESULTS

Genotoxicity studies

Bacterial reverse mutation test

The DSLE was assessed for its potential to induce gene mutations according to the plate incorporation test (Trial -I) and the pre-incubation test (Trial- II) using Ames tester strains (TA98, TA100, and TA102, TA1535 and TA1537) along with the different positive controls for different strains. The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the negative, vehicle and positive controls were tested in triplicates. Based on the solubility and precipitation test results eight different concentrations (0.002, 0.005, 0.016, 0.050, 0.158, 0.501, 1.582 and 5 mg/plate were selected for pre-experiment. Based on the pre-experiment results, DSLE was tested with the following concentrations: 0.50, 0.158, 0.501, 1.582 and 5mg/plate for the main study, both in the presence and absence of metabolic activation.

No substantial increase in revertant colony numbers of any of the tester strains were observed following treatment with DSLE at any dose level in both the confirmatory trials, in the presence or absence of metabolic activation. There was no evidence of a trend with high mutation rates with an increase in concentrations. Also, the spontaneous reversion rates in the negative, vehicle and positive controls were within the range of historical data. The reference mutagens showed a distinct increase in induced revertant colonies in all the tester strains both in the presence as well as in the absence of metabolic activation without indicating cytotoxicity. Therefore, it was concluded that the DSLE tested was not mutagenic under the experimental conditions.

In vitro chromosomal aberration assay

Chromosomal aberration *in vitro* assay was performed to assess the potential of DSLE induced structural/numerical chromosomal aberrations in two phases. The induction of cytogenetic damage in human lymphocytes was assessed with and without metabolic activation. Mitotic index was calculated for different concentrations of DSLE at 4, 2, 1, 0.5, 0.25 mg/ml and the average mitotic index was 63.75, 53.82, 50.71, 52.23, 50.86 and 39.44 percent respectively. Since the concentration of 1 mg/ml DSLE indicated a mitotic index of more than 50%, a concentration of 1, 0.5 and 0.25 were selected for cytogenetic analysis. The percentage of chromosomal aberration in the negative control without

metabolic activation was 27%, while with metabolic activation was 36%, respectively. In the positive treatment group with metabolic activation, the chromosomal aberration was 60% and 62% without activation. At the concentration of 1, 0.5 and 0.25 mg/ml DSLE the corresponding percent chromosomal aberration were 25 and 21%; 24% and 31%; and 28 and 26% with and without metabolic activation, respectively. Since the ratio of percent chromosomal aberration between the positive and negative controls were more than 2; while that of the DSLE treated sample and negative control were less than 2, it was concluded that DSLE does not have the potential to induce structural/numerical chromosomal aberrations in both the phases under the experimental conditions of the study.

Subchronic study

Survival, Clinical Observation and Body Weight

All animals survived until the scheduled necropsy in both the 90-day study group and the recovery group. Physical and behavioural examinations did not reveal any treatment-related adverse effects. Compared to control Group, no treatment-related biologically significant effects of DSLE were noted on body weight or body weight gain at dose levels up to 1240 mg/kg/day (Figure 2A, 2B). During this period, the activity of the animals was found to be normal. In the recovery group, no significant changes in body weight gain were noted during the course of the study. These results suggest that administration of DSLE at levels up to 1240 mg/kg/day to rats for 90 days had no adverse effects on clinical observations and body weight. Similarly, in the recovery group also no adverse effects of DSLE were noted.

Feed and Water Consumption

The overall feed consumption of animals receiving DSLE was generally similar to that of the controls (data not shown). Similarly, the feed consumption in the recovery group was similar to the respective control. The results of feed consumption show that in spite of lower body weight, females consumed a similar amount of the feed compared to males. There were no treatment-related significant adverse effects of the DSLE on feed consumption. The quantity of water consumed during the course of study by DSLE treated animals was comparable to the respective controls in 90 day and recovery study groups (data not shown). These results show that administration of the DSLE at levels up to 1240 mg/kg/day to rats does not affect feed and water consumption.

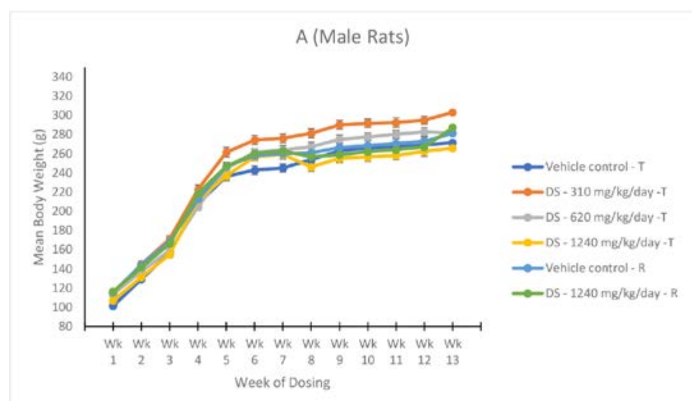

Figure 2A: Effect of DSLE on Body Weights in Male Rats.
Mean body weights for male rats during a 90-day oral (gavage) toxicity study and 28 day recovery study with DSLE. The values are presented as means ± standard error. * Represent values significantly different from control at $p<0.05$.

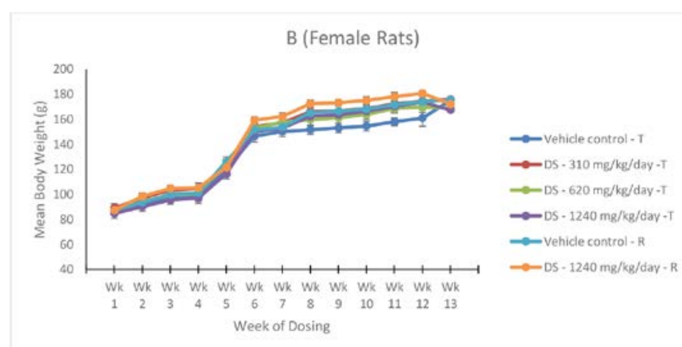

Figure 2B: Effect of DSLE on Body Weights in Female rats.
Mean body weights for female rats during a 90-day oral (gavage) toxicity study and 28 day recovery study with DSLE. The values are presented as means ± standard error. * Represent values significantly different from control at $p<0.05$.

Clinical Pathology

Hematology

There were no treatment-related adverse effects of DSLE on hematology parameters in male and female rats (Table 2). However, some statistically significant differences were noted when the control and treatment groups were compared. In male rats, a statistically significant increase in lymphocytes, MCV, MCH, MCHC were observed in groups treated with 1240 mg/kg DSLE, while a statistically significant decrease was noted in WBC, platelets, Hb, RBC and PCV. Also noted was a decrease in Hb, RBC, Platelets, PCV and an increase in WBC and eosinophils in the recovery group at the top dose of 1240 mg/kg. Increase in WBC and lymphocytes were observed at 310 and 620 mg/kg dose of DSLE compared with the vehicle control group while, a statistically significant decline was observed in platelets at the 620 mg/kg dose. All the differences which were observed where however within the laboratory range. Essentially, the other hematology parameters did not show statistically significant differences as compared with the vehicle control group. (Table 2).

In the female rats, significant decline in monocytes were noted in the reversal groups and decreased eosinophils were also observed in the reversal high dose group. While, significant increase was observed at the 1240 mg/kg dose, in the reversal group with regard to Hb, RBC, Lymphocytes, platelets, PCV and PT. Also, a significant increase was noticed in PT at the 310 and 620 mg/kg dose DSLE. Again however, these changes where within the historical laboratory range and hence were considered as incidental and not treatment related.

Biochemical

The activities of serum enzymes like AST, ALT and ALP, and other important biochemical constituents like Glucose, Creatinine, Urea, Triglyceride, Cholesterol, and total Bilirubin, GGT which are indicative of metabolic and pathological abnormalities, exhibited no significant change in experimental animals as compared to vehicle control animals. Biochemical analysis presented a statistically significant decrease in creatinine level at 310 and 620 mg/kg body weight doses of DSLE, while a significant increase in creatinine levels were observed at 1240 mg/kg in the reversal group. In addition, total bilirubin significantly increased at 310 mg/ kg and reversed at the 1240 mg/kg dose but was within the laboratory limit/range (Table 3). Rest of the parameters were demonstrated to have no statistically significant difference between the vehicle control group and animals treated with DSLE. While in female rats, a high dose (1240mg/kg) presented a significant increase in cholesterol level but the range was within the limit. Rest all the

Table 2: Effect of *Dalbergia sissoo* leaf extract (DSLE) on hematological parameters inmale and female rats.

Hematological values of male and female Rats orally treated with *Dalbergia sissoo* leaf extract (DSLE)								
Parameters	Units	Sex	90-day dosing period				Reversal group	
			Vehicle Control	DSLE (mg/kg/day)			Vehicle Control	DSLE (mg/kg/ day)
			0	310	620	1240	0	1240
Hb	g/dl	M	13.51 ± 0.23	12.77± 0.39	12.83 ±0.17	11.33±0.26****	12.54±0.24	11.77±0.37***
		F	10.20 ± 0.26	9.86± 0.51	11.02 ±0.19	10.74±0.33	11.37 ±0.55	12.37±0.39***
RBC	10^6/cm	M	7.87 ±0.17	7.53 ±0.20	7.86 ±0.14	6.10±0.15****	7.16±0.25	6.86±0.19**
		F	5.26±0.11	5.08 ±0.26	5.86 ±0.11	5.59±0.16	5.99± 0.34	6.33± 0.21**
WBC	10^3/cm	M	7.03 ± 0.24	9.18 ± 0.66*	9.05 ± 0.47*	4.80 ± 0.40*	8.00 ± 0.73	9.26 ± 0.52*
		F	6.96 ± 0.59	6.33 ± 0.61	7.50 ± 0.53	7.36 ± 0.51	8.70 ± 0.91	5.80 ± 0.32
Lymphocytes	%	M	61.75 ±3.02	70.30± 1.46*	70.40± 1.66*	78.29±1.52****	69.14±2.53	67.30±2.59
		F	70.33 ±1.38	74.44± 2.67	65.89± 1.65	68.20±1.65	71.17 ±2.54	78.78 ±3.35*
Monocytes	%	M	2.87 ±0.22	3.90± 0.52	2.00± 0.25	3.28±0.35	4.44±0.58	4.40±0.63
		F	5.55 ±0.24	5.44± 0.37	5.66± 0.33	5.80±0.13	1.83 ±0.16****	1.33± 0.16****
Eosinophil	%	M	1.75± 0.16	2.40± 0.16	2.20± 0.20	2.14±0.34	3.00±0.32*	3.00±0.39*
		F	3.77± 0.14	3.55± 0.24	3.55± 0.24	3.20±0.24	3.00 ±0.00	2.22± 0.32***
Platelets	10^3/cm	M	781.70 ± 27.89	690.20 ± 32.64	647.80 ± 19.60**	550.00 ± 28.21****	69.37 ± 37.29	677.90 ± 26.02*
		F	429.44 ± 20.51	367.22 ± 51.06	467.00 ± 12.59	468.50 ± 25.33	521.67 ± 35.95	582.56 ± 26.41**
PCV	%	M	38.44 ±0.78	36.39± 1.00	36.01 ±0.36	31.11±0.75****	34.91±0.89**	33.79±0.89***
		F	27.07±0.59	25.91± 1.27	29.61±0.47	28.33±0.87	30.33 ±1.65	33.20±1.16***
MCV	fL	M	48.85 ±0.25	48.33 ±0.25	48.07 ±0.24	51.00±0.32**	50.16±0.66	49.29±0.61
		F	51.40±0.41	51.06±0.42	50.52±0.39	50.67±0.32	50.62 ±0.41	52.38± 0.40
MCH	pg	M	17.04 ±0.17	16.95± 0.12	17.18 ± 0.12	18.59±0.20***	17.94±0.23	17.18±0.40
		F	19.36±0.20	19.31± 0.17	18.79± 0.10	19.21±0.10	19.02 ±0.29	19.54± 0.12
MCHC	%	M	34.90 ±0.30	35.09 ±0.15	35.07 ±0.17	36.43±0.24**	35.83±0.31	34.80±0.42
		F	37.67 ±0.24	37.81±0.24	37.20 ±0.21	37.92±0.09	37.55± 0.34	37.32 ±0.28
PT	s	M	48.13 ±8.81	37.10± 4.28	36.10± 1.74	37.88±3.80	28.38±0.90*	36.40±2.29
		F	23.90 ±2.81	38.50± 3.27*	40.10± 2.73**	27.20±3.78	35.10 ±3.77	37.60 ±4.18*
APTT	s	M	38.78± 4.98	28.90 ±2.20	38.78 ±1.90	38.80±4.99	39.88±3.94	41.33±3.99
		F	46.25± 5.90	46.67 ±2.40	50.80 ±2.35	31.36±7.36	53.80 ±2.78	51.20± 2.93

Values are mean ± SEM for 10 rats in each group

RBC = red blood cells; WBC = white blood cells; PCV = packed cell volume; MCV = mean cell volume; MCH = mean cell hemoglobin; MCHC = mean corpuscular hemoglobin concentration; CT = clotting time; APTT = activated partial prothrombin time fL= femtolitre

*Statistically significantly different from vehicle controls ($p < 0.05$).**Statistically significantly different from vehicle controls ($p < 0.02$).***Statistically significantly different from vehicle controls ($p < 0.01$).****Statistically significantly different from vehicle controls ($p < 0.001$).

parameters were demonstrated as not significantly different between vehicle and DSLE treated animals. (Table 3) In addition, at high dose levels, these parameters were not altered and hence not considered as treatment related.

Organ Weight

No treatment related changes in organ weight (liver, Thymus, R-kidney, R-testes, Heart, Lung and Ovary) were noted in male and female rats following administration of DSLE. No statistically significant differences were observed in the weight of organs of DSLE treated groups compared to the control group except the heart weight of a male rat at low dose (310mg/kg DSLE) exhibited a significant difference compared to control Group. (Table 4).

Macroscopic and Microscopic Examinations

Macroscopic examination

There were no treatment-related macroscopic findings at the scheduled necropsy following administration of DSLE to rats. All macroscopic

Table 3: Effect of *Dalbergia sissoo* leaf extract (DSLE) on serum chemistry parameters in male and female rats.

Clinical biochemistry values of male and female rats orally treated with *Dalbergia sissoo* leaf extract (DSLE)								
Parameters	Units	Sex	90-day dosing period				Reversal group	
			Vehicle Control	DSLE (mg/kg/day)			Vehicle Control	DSLE (mg/kg/day)
			0	310	620	1240	0	1240
Creatinine	mg/dL	M	0.52 ±0.01	0.42 ±0.04*	0.40 ±0.01**	0.50 ±0.02	0.55 ±0.02	0.65 ±0.01**
		F	0.56 ±0.05	0.66 ±0.01	0.68 ±0.03	0.58±0.01	0.59± 0.01	0.54± 0.006
Urea	mg/dL	M	37.37 ±1.40	41.46± 2.36	40.43 ±1.88	40.61± 0.78	35.37 ±1.38	37.52± 1.52
		F	60.60 ±5.72	52.23± 9.24	62.47 ±0.36	66.88± 3.20	67.99± 2.09	67.58± 2.20
Glucose	mg/dL	M	95.14 ±2.61	82.85 ±4.60	94.22±6.59	86.77 ±3.89	96.24 ±2.12	96.87± 2.57
		F	106.4 ±2.47	108.4 ±5.66	114.8±3.54	127.4 ±5.51	116.4 ±2.57	106. 8± 3.45
Cholesterol	mg/dL	M	37.88 ±3.94	39.12± 1.17	41.27 ± 1.50	39.96 ±1.34	45.57 ±2.28	44.16 ±2.82
		F	63.23 ±2.85	72.30± 3.11	69.17 ± 3.04	76.03 ±4.49*	65.81 ±2.51	72.80 ±1.59
Triglycerides	mg/dL	M	38.11 ±5.35	52.40 ±7.69	48.93 ±2.31	55.05 ±5.20	37.99 ±3.45	52.29± 5.10
		F	73.22± 4.16	70.22 ±7.00	82.62 ±5.93	88.10± 5.96	65.22± 6.91	87.45 ±3.04
ALP	IU/L	M	112.00 ±10.34	132.06± 10.18	148.01± 13.81	124.03 ±7.39	122.3 ±08.21	128.1 ±10.79
		F	93.95± 11.52	118.1±04.20	119.9± 14.98	103.1 ±6.67	100.05± 5.56	100.8 ±6.12
ALT	U/L	M	46.10 ±2.65	44.78± 3.86	47.54 ±3.02	49.52± 2.83	52.10 ±2.65	45.66 ±2.23
		F	40.46 ±1.80	37.79 ±2.46	34.88 ±1.04	42.73± 1.31	36.30 ±1.28	40.17 ±3.02
AST	U/L	M	98.56 ±7.82	116.10± 6.01	87.89 ±8.08	87.18± 4.16	84.78 ±4.17	84.10± 5.08
		F	73.30 ±2.86	74.62±5.85	79.62 ±4.47	84.80 ±4.24	79.90± 2.82	81.80 ±3.83
Total bilirubin	mg/dL	M	0.02 ±0.002	0.08 ±0.03**	0.05± 0.009	0.050 ±0.006	0.04± 0.01	0.07 ±0.005**
		F	0.06 ±0.009	0.06 ±0.01	0.03 ±0.003	0.13 ±0.05	0.12 ±0.03	0.08 ±0.02
GGT	U/L	M	2.02 ±0.96	1.70± 0.26	1.04 ±0.29	3.60± 0.95	0.50 ±0.04	3.60± 1.32
		F	0.05 ±0.02	0.08 ±0.04	0.33±0.14	0.27 ±0.15	0.09 ±0.07	0.04 ±0.01

Values are mean ± SEM for 10 rats in each group

ALP=alkaline phosphatase; ALT=alanine aminotransferase; AST=aspartate aminotransferase; GGT=gamma-glutamyl transferase

*Statistically significantly different from vehicle controls ($p < 0.05$) **Statistically significantly different from vehicle controls ($p < 0.02$)

Table 4: Effect of *Dalbergia sissoo* leaf extract (DSLE) on organ weight in male and female rats.

Organ weight of Male and Female Rats orally treated with *Dalbergia sissoo* leaf extract (DSLE)								
Organ	Units	Sex	90-day dosing period				Reversal group	
			Vehicle Control	DSLE (mg/kg/day)			Vehicle Control	DSLE (mg/kg/day)
			0	310	620	1240	0	1240
Liver	g	M	9.54 ± 0.47	9.980± 0.46	9.55 ±0.26	9.27 ±0.23	10.57±0.15	10.18 ±0.14
		F	9.07 ± 0.14	8.32± 0.34	7.32 ±0.32	8.52±0.13	8.62±0.12	8.63± 0.13
Thymus	g	M	0.24 ±0.01	0.27 ±0.008	0.24 ±0.01	0.27 ±0.01	0.24±0.004	0.24±0.01
		F	0.20 ±0.01	0.20 ±0.02	0.17 ±0.02	0.16±0.02	0.20± 0.02	0.21 ±0.02
R-kidney	g	M	1.10 ±0.04	1.24± 0.07	1.07 ±0.05	2.01± 0.99	0.97±0.01	1.09± 0.03
		F	0.77 ±0.07	0.85± 0.08	0.82 ±0.03	0.86±0.03	0.88 ±0.02	0.88 ±0.02
R-Testicle	g	M	1.48 ±0.03	1.59 ±0.06	1.54 ±0.09	1.44 ±0.03	1.27±0.02	1.43± 0.07
		F	0.12 ±0.01	0.10 ±0.004	0.11 ±0.01	0.12±0.01	0.11 ±0.004	0.10 ±0.004
Heart	g	M	0.98 ±0.02	1.38± 0.05****	1.11 ± 0.05	0.94 ±0.03	0.89±0.02	1.05±0.04
		F	0.86 ±0.05	0.85± 0.03	0.73 ± 0.04	0.83±0.03	0.80± 0.01	0.88 ±0.005
Lungs	g	M	1.37 ±0.06	1.66 ±0.16	1.47 ±0.08	1.60 ±0.06	1.71±0.02	1.58±0.09
		F	1.32 ±0.07	1.26 ±0.14	1.16 ±0.09	1.36±0.04	1.35 ± 0.03	1.39 ± 0.03

Values are mean ± SEM for 10 rats in each group

*Statistically significantly different from vehicle controls ($p < 0.05$) ****Statistically significantly different from vehicle controls ($p < 0.001$)

changes noted were considered to be spontaneous and/or incidental in nature and unrelated to the treatment. There were no treatment-related histopathological findings. Similarly, in the recovery group no treatment related macroscopic or microscopic changes were noted. All findings observed were consistent with normal background lesions in clinically normal rats of the age and strain used in this study and were considered spontaneous and/or incidental in nature and unrelated to the treatment. These results suggest that administration of DSLE at dose levels up to 1240 mg/kg/day to rats for 90 days had no adverse macroscopic or microscopic effects

Microscopic examination

Histopathological examinations were performed for male rats: brain, lung, heart, liver, spleen, kidney and testis to assess whether or not organs or tissues had been damaged. In few DSLE treated samples of the lung showed thickening of alveolar septa and peri bronchial lymphoid aggression but that was not statistically significant. Also, in the liver some samples presented mild fatty changes while heart and kidney presented mild congestion although they were not toxicologically significant. Overall Histopathological examination did not reveal any major specific microscopic lesions (Figure 3).

In female rats, brain, lung, heart, spleen, kidney and ovary were evaluated for the histopathological examination to assess whether or not organ or tissue had been damaged. All the DSLE treated samples did not indicate any pathological changes except in the lung of high dose (reversal group) animal that presented inflammation and hemorrhages. However, this was observed only in one animal, the rest all presented no pathological changes in the lung as well (Figure 4).

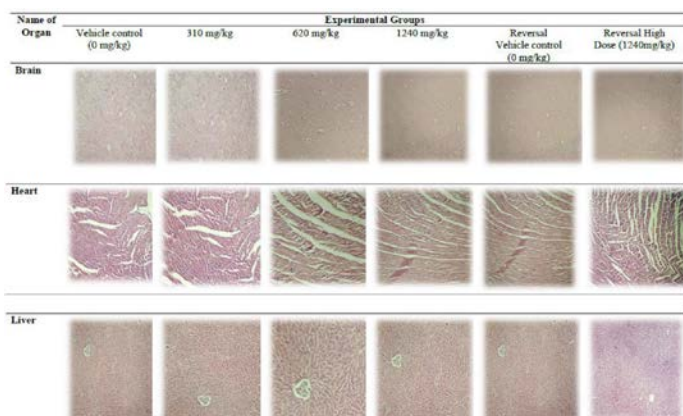

Figure 3: Histopathology of male organs.

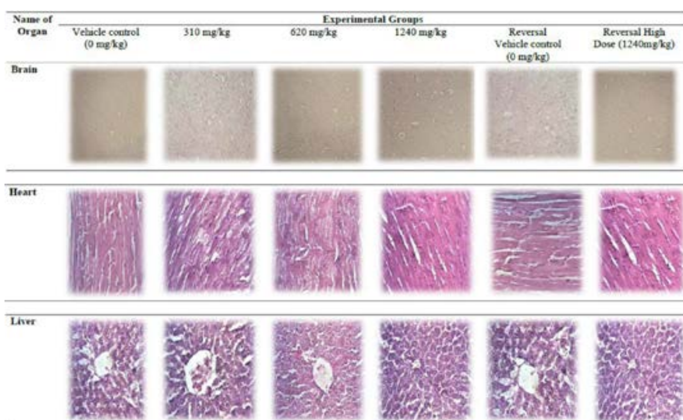

Figure 4: Histopathology of female organs.

DISCUSSION

The initial step in screening natural products for their pharmacological activity is the assessment and evaluation of the characteristics of a natural product extract, fraction, or compound. Regardless of the beneficial pharmacological effects of the natural product, detailed knowledge about chronic toxicology is of prime importance. In recent years, the safety of long-term use or consumption of natural products is becoming very vital as the traditional herbal medicines have surpassed ethnic boundaries and reached worldwide.

Previous studies have shown that various parts of the plant of *D. sissoo* have exerted antimicrobial, neural, cardiac, antioxidant, antiparasitic, anti-diabetic, anti-inflammatory, analgesic, osteogenic, dermatological, gastrointestinal, effects to name a few.[9] An acute toxicity study showed that the ethanol bark extract of *Dalbergia sissoo* was non-toxic up to 3000 mg/kg body weight in Swiss albino mice.[10] Another acute toxicity study that was carried out on Wistar rats where alcoholic bark extracts at a dose of 50, 100, 300, 1000, and 3000 mg/kg body weight were administered after overnight fasting. Acute toxicity studies did not reveal any toxic symptoms or death in any of the animals up to the dose level of 3000 mg/kg body weight.[11] In another study, with 90% ethanolic extract of *D. sissoo leaves*, was also safe up to 3000 mg/kg, administered orally in rats.[3] In a study to test the efficacy of DSLE in ovariectomized rats, a model for post-menopausal osteopenia was used to evaluate the skeletal effects of an extract made from the leaves and pods of Dalbergia. The authors concluded from the study that the oral doses of DSLE in the preclinical setting are effective in preventing estrogen deficiency-induced bone loss.[12]

Several safety and efficacy studies on the use of different parts of the *D. sissoo* plant with potential therapeutic use have been investigated in experimental studies, as well as in human clinical trials.[7,13] These studies do not raise any new safety concerns.[10,11,14] Choosing the appropriate tests and dosing regimens that will demonstrate an adequate margin of exposure is a critical step in establishing human safety. Since no toxic effects were found during the acute toxicity or efficacy studies (5mg/kg/day CAFG in ovariectomized mice led to enhanced bone formation), appropriate the study where the human equivalent dose was determined and study was designed to assess the subchronic toxicity of DSLE up to 90 days in rats. To prepare the comprehensive toxicology data of *D. sissoo,* the current study was undertaken to evaluate and focus on the genotoxicity and subchronic toxicity of DSLE in an animal model. The toxic effects of DSE evaluated at the doses of 310 mg/kg, 620 mg/kg and 1240 mg/kg in rats for 90 days with two reversal dose group (vehicle control and 1240mg/kg DSE group). These reversal groups were observed for 28 days after completion of 90 days of treatment schedule for development of any post treatment toxicity.

In the 90-day toxicity study and 28 days of reversal study, all the rats appeared healthy and gained weight normally. No changes were observed in the habit of food consumption in both male and female rats. This suggests that the DSLE did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. Essentially DSLE did not adversely affect the nutritional benefits, such as weight gain and stability of appetite. There were no indications of any test substance-related neuro-behavioural toxicity symptoms throughout the study. There were no changes in organ weights of male and female rats of control and treatment groups except increase in heart weight of a male rat in low dose (310 mg/kg) group. Though the heart weight was increased, it was within the normal range without any histo-pathological

abnormalities. Haematological and biochemical analysis suggested that there are no significant changes in majority of parameters in control and the treatment groups. Though some sporadic variations in haematological parameters were observed in male and female rats of 90 days groups and reversal groups, the changes observed were not toxicologically relevant as the increase was low in magnitude and within the historical control range (normal physiological range).

The activities of serum enzymes and other important biochemical parameters which are indicative of metabolic and pathological abnormalities exhibited no significant change in experimental animals as compared to vehicle control animals. However, stray variations were observed but again were within the expected historical laboratory range. Individual gross morphology and histopathology of male and female rats exhibited no significant abnormality in 90 days in the treatment groups and reversal groups except in the lung of high dose (reversal group) in a female rat that presented inflammation and hemorrhages. As this change was observed in only one animal, it was considered incidental and not treatment-related.

Ames and chromosomal aberration tests were the two genotoxicity studies conducted with DSLE. Ames test was performed to investigate the potential of DSLE to induce gene mutations using the *Salmonella typhimurium* strains. No substantial increase in revertant colony numbers in *Salmonella typhimurium* strains were observed with DSLE treatment. Also, DSLE did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Similarly, *in vitro* chromosomal aberration assay also revealed that DSLE was non-toxic. Chromosomal aberration assay was performed with three different test concentrations of DSLE. It was found that the ratio between positive to negative control was more than 2, while the treated sample to negative control was less than 2. So, it was concluded that DSLE did not induce chromosomal aberration in the cytogenetic analysis.

In summary, the results of the present subchronic toxicity study suggest that oral administration of the DSLE at levels up to 1240 mg/kg bw/day does not cause adverse effects in male and female rats. Based on the results of the study, the no-observed effect level (NOAEL) of the DSLE was found to be 1240 mg/kg bw/day, the highest dose tested. The findings from present investigations also suggest that DSLE is unlikely to cause any genotoxic effects. In light of these findings, it can be concluded that DSLE was not toxic in all the doses studied herein and did not produce any signs or evident symptoms of subchronic oral toxicity. These results establish the safety of DSLE and offer reassurance to explore its therapeutic value to alleviate signs and symptoms of osteoarthritis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DSLE: *Dalbergia sissoo* Leaf Extract.

Author contribution

Tejal Gandhi: Conceptualization, Methodology, Supervision, Resources, Project administration and Review; **Hital Shah:** Methodology, Investigation, Validation, Formal analysis, Writing, Review and Editing; **Ritu Trivedi:** Investigation, Validation, Formal analysis, Writing, Review and Editing; **Amol Deshmukh:** Supervision, Project administration, Writing and Editing; **Lal Hingorani:** Conceptualization, Methodology, Resources, Supervision.

SUMMARY

- 90 days sub chronic toxicity study on standardised *Dalbergia sissoo* leaf extract in rats.
- *Dalbergia sissoo* leaf extract didn't produce any adverse effects at dose up to 1240mg/kg/day in rats.
- Standardised *Dalbergia sissoo* leaf extract in Ames test and Chromosomal Aberration assay didn't exhibit genotoxic effects.

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