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Evaluation of Clastogenic Potential of Ethanolic Extract of Leaf of *Couroupita guianensis* using Micronucleus Test in Mice

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Abstract

Medicinal plants are being used since ancient times in different tribes in India for numerous medicinal purposes. The essential facets of plant-based medicines, such as safety, quality, and efficiency, must be evaluated. *Couroupita guianensis* is a medicinally important plant, being used conventionally for curing varieties of medical ailments. From the safety point of view, limited information exists for the *C. guianensis* plant. Considering the facts mentioned above, this study evaluates the genotoxic potential of ethanolic leaf extract of *C. guianensis*, through an *in vivo* micronucleus test , which was conducted on mice (Strain: Hsd: ICR (CD1)). The main study consisted of three groups (5 male mice per group): vehicle control (corn oil), a treatment group (2000 mg/kg body weight), and positive control (Mitomycin-C, 1.0 mg/kg body weight). The methodology followed was the standard *in vivo* procedure as per the internationally approved OECD TG 474 guidelines with a two-day dosing schedule separated by 24 hours. Dose-volume used was 10 mL/kg body weight. Mice in each group exhibited normal behavior and were found clinically normal after dosing. Toxicity to bone marrow, as well as any statistically significant rise in the percent micronucleated polychromatic erythrocytes (% MNPCE), was not detected in mice that were treated with *C. guianensis* at 2000 mg/kg body weight when compared with the data of concurrent vehicle control. Statistically, a significant rise in the % MNPCE was obtained in mice treated with Mitomycin-C (1.0 mg/kg body weight), which confirmed the test system's suitability, the procedures, and efficiency of conditions employed in the test. Our study concluded that the plant is not showing any genotoxicity and can be used as a medicine to cure various reported ailments.

Keywords: *Couroupita guianensis*, Ethanolic Leaf Extract, Genotoxicity, Medicinal Plant, Micronucleus Test

1. Introduction

A wide variety of naturally available pharmacologically important active molecules are used to treat numerous diseases[1,2]. Amongst them, herbal products are suggested for various health-related needs. Even the base of our modern aged drugs is plant-based[3]. Different parts of plants or their extracts are being used for consumption or external body applications for ages. Nowadays, more than forty thousand plant species have been implemented by pharmaceutical industries for the treatment of a variety of therapeutic conditions[4].

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One such medicinally important plant is *Couroupita guianensis*, commonly known as cannonball tree (Nagalingam). Nagalingam tree has gained universal attention because of its vast beneficial features, which include analgesic, anti-inflammatory, antibiotic, and antiarthritic properties. It has also been utilised to treat tumorous diseases, fertility, ulcers, stress, immunomodulatory and wound healing activities[5–8]. Almost all parts of the tree are reported to be used for treating various diseased conditions. Flowers of the plant have been documented to treat scorpion bites, colds, intestinal gas formation, and stomachache. Even the infusions made from the blend of *C. guianensis* flowers and leaves were reported to cure inflammatory conditions[9]. Also, it has been used in treating skin diseases, flu, stomach ache, toothache, and pathogenic infections[10]. Its antimicrobial, antifungal, antiseptic, antidepressant, antimalaria, anticancer, and antihelmintic activities have been scientifically proven[11–14].

The allopathic potential of *C. guianensis* has proved by the researcher by showing the plant growth inhibitory effect, which could help to study weed management[15]. Results of preliminary studies also showed promising antitubercular agents[6]. The methanolic extract of the leaf of *C. guianensis* has potent antioxidant, scavenging activity, a free radical activity that suggests the therapeutic potentials of *C. guianensis*[7]. In general, plant extracts are in use for different medicinal purposes, such as *Gentiana* genus demonstrated pharmacological properties such as protection from hepatic, cardiovascular, gastrointestinal disorders, and immunomodulatory effects[8].

Despite these many uses, pharmacological benefits, and increasing demands of herbal medicines, the safety concerns of the plants are not yet thoroughly evaluated. It is a complete misbelief that herbal drugs are non-toxic upon consumption. The cytological or genetic toxicity of plants seems only after regular consumption for a longer time[16]. Plants are also reported to be initiating factors for DNA damages, cell mutations, and natural toxic elements[17]. Consequently, the safety and toxicity of medicinally important plants should be well-thought-out on a serious note, and else the illiteracy may result in dangerous to lethal consequences[18]. Hadi *et al.*[19] conducted genotoxicity of few pyrrolizidine alkaloids in HepG2 and Huh6, human hepatoma cell lines using micronucleus frequency evaluation. *Dioscorea* Rhizome Water Extract (DRWE) was evaluated using the genotoxic battery of tests as per OECD TGs to check its toxicity profile[20]. Considering the pharmacological properties of *Rhus verniciflua* Stokes (RVS), including anticancer activities, safety evaluation was conducted by Ames, micronucleus and comet assay[21]. Studies were conducted with mice lymphocytes to evaluate the protection provided by *Origanum vulgare* L. against the cyclophosphamide-induced genotoxicity[22]. A review published on medicinal plants of Himalayan regions revealed antimutagenic effects against EMS-induced genotoxicity in mice by micronucleus test and chromosomal aberration test. The reduction profiles in the MN frequency were observed due to the activity of methanolic extract of *Melissa officinalis* at different test concentrations[23].

Therefore, despite the employment of the plant as traditional medicine, no scientific evidence is presently telling its toxicity profile[18]. Limited information on the cytotoxicity, genotoxicity, and mutagenic potential of the *C. guianensis* is available in the literature. Thus, the increasing trend of utilising herbal medicines with limited safety data has enhanced the scope for screening of its safety characteristics. Therefore, evaluation of *in vivo* genotoxicity using globally accepted OECD TG (OECD Test No. 474) considered as a suitable model for herbals by different researchers[20–23].

In summary, there are several studies describing the traditional and pharmaceutical uses of *C. guianensis*[6–8,15,19,24]. Gothai *et al.* have described the safety of *C. guianensis* based on data of *in vitro* studies[18]. Gothai *et al.* also reported the absence of significant cytotoxicity nor genotoxicity in the *in vitro* studies[18]. The author also opined to conduct further safety evaluation using *in vivo* animal studies to further evaluate the *in vivo* relevance of the *in vitro* studies[18]. Therefore, many research works were conducted on the pharmacological properties of *C. guianensis.* A very few toxicology studies were carried out as per the globally accepted regulatory guidelines. Furthermore, the critical data and evidence of safety evaluation before its regulatory approval in properly conducted studies are lacking, as per the current requirements of genotoxicity and mutagenicity studies. Accordingly, the objective of this research was to assess the genotoxic potential of ethanolic extract of leaf extract of *C. guianensis* using *in vivo* micronucleus test[25]. As per the EFSA Scientific Committee recommendations, the Micronucleus test[25] in mice was conducted to detects clastogenic response to further support the *in vivo* relevance of the *in vitro* genotoxicity test results[26,27].

2. Material and Methods

2.1 Materials

The chemicals used in the study were procured from Sigma Aldrich, Merck, HiMedia and Qualigens as described in Table 1. Instruments and equipment used in the study are described in Table 2.

Table 1. Solvents and chemicals

Solvents and Chemicals	Source	Lot Numbers
Methanol	Qualigens	6472941020
Foetal Bovine Serum	HiMedia	0000410605
Corn oil	Sigma	MKCQ3257
Immersion oil	HiMedia	0000385251
NaH2PO4	Merck	DL6D663605
Na2HPO4	Sigma	BCBR4509
Disinfectant	Dettol 2.5%; Reckitt Benckiser	GH773
DPX mountant	Qualigens	6383220720
Giemsa solution	Hi Media	0000400553

Table 2. Instruments and equipment used in the study

Name of the Instrument/Equipment	Details
Balances	Electronic Weighing Scale – Smart Axis Electronics (Capable of measuring 5 g to 3 kg)
	Ohaus, (Capable of measuring 10 mg to 210 g)
Metal Cannula	CW12 ILA (Size: 18G x 15 cm)
Syringe	BD 1 mL disposable syringe
Needles	26½ G (0.45 mm x 13 mm) and 24 x 1" G (0.6 mm x 25 mm), B.D. Precision Glide
Vacuum Desiccator	Tarsons (CO_2 Chamber)
Centrifuge	Thermoscientific
Binocular Microscopes	Eclipse Ci-L; Eclipse 80i; E600 and Eclipse Ni-U (Fluorescence), Nikon
Tattoo Machine	AIMS™
Refrigerator	LG Electronics Inc.
Bench Top Autoclave	Kumar, India
Deep-Freezer (-20^{0}C)	Vestfrost Solutions
Deep Freezer (-80^{0}C)	SANYO Electronics Co., Ltd.
Micropipettes	Eppendorf AG
Millipore Water Purification	Merck Millipore water purification system
Vortex Mixer	Remi Elecktrotechnik Ltd.
RO System	Hi-tech Water Technology Pvt., Ltd., and Kent RO water
Microprobe Thermometer	Physitemp Instruments Inc.
Ultrasonic Cleaning Bath	Spectralab
pH meter	Thermoscientific

2.2 Collection of Plant Materials and Preparation of Leaf Extract of *C. guianensis*

Leaves of *C. guianensis* plant were collected at the Jai Research Foundation garden, Valvada, Gujarat, India. The collection site falls within the Gujarat plains of the humid tropical climate. Plant identification and authentication were made by Bhagvati Herbal and Healthcare Private Limited, Plot No.785/4, 40 Shed Area GIDC, Vapi, Valsad GJ 396195. The voucher specimens (JRF/Genotox/18-19/01) for authentic Leaves of *Couroupita guianensis* were deposited at the Mutagenicity department, Jai Research Foundation, Valvada 396105, Gujarat.

Ethanolic extract of *C. guianensis* leaves was prepared using a Soxhlet apparatus. The leaves of *C. guianensis* were collected and air-dried in the shed. Around 25 g of dried leaves plant material was crushed, using a pestle and mortar, to provide a greater surface area. A volume of 250 ml of ethanol was used for extraction. The process was run for a total of 4 hours till colourless extract was seen in the siphon. The solvent was then filtered using Whatman filter paper, and the rotary vacuum evaporator was used to dry the filtrate. Dried powdered material was collected and stored in a refrigerator for further toxicological evaluation.

2.3 Animals

The test was undertaken in accordance with the 'Guidelines for Laboratory Animals Facility' published by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The project proposal for the experimentation was authorised by the Institutional Animal Ethics Committee (IAEC), Jai Research Foundation (Animal Ethics Approval Number: JRF/IAEC/2020/317). JRF is also accredited with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) that encourages that the animals used for science should be handled humanely. Hsd: ICR (CD1) mice (*Mus musculus*) procured from the Animal breeding facility, JRF, were acclimatised into the experimental procedure room for a period of five days. The room in which the animals were acclimatised was having controlled temperature as well as light. The light was with a 12-12 h light-dark cycle. At the same time, the temperature and relative humidity were maintained to 25 ± 2 ^{0}C and 61 ± 5%, respectively. The feed used for the mice was commercially pelleted feed and water *ad libitum*. The animals were 9-10 weeks old and weighed 32–42 g on the day I of dosing (main study). The average body weight of the animals used for the study was within the limit of ± 20% to that of the mean body weight for both sexes at the time of initiation of dosing.

In-house developed software was employed to randomise the study animals into 3 groups using the censored randomisation method. During acclimatisation, animals were marked using a non-toxic marker pen. After randomisation, the individual animal number was tattooed on the tail of mice using a tattoo machine. In order to make the identification appropriate, the cages used to accommodate mice were attached with labels showing specific study number, test item code, group number and sex, dose, type of study, cage number and animal number.

2.4 Dose Range-finding Study

A dose range-finding study was performed using mice to assess the cytotoxicity of ethanolic extract of *C. guianensis* to bone marrow (suppression of polychromatic to total erythrocytes ratio). Each group of animals (consisting of a male and female), which were treated at different dose concentrations (viz., 500, 1000, and 2000 mg/kg body weight) orally through gavages for two consecutive days. The body temperatures of all the individual animals were measured before dosing, 2 and 5 hours after each dosing, and before sacrifice using a microprobe thermometer[28–30].

2.5 Dosing

Based on the range-finding test, the limit dose of 2000 mg/kg of body weight for male animals was selected. Animals were treated using two treatments separated by 24 hours with a dose volume of 10 mL/kg body weight. For the animals of all the groups, G I: Vehicle control, G II: Treatment Group, and G III: Positive control, the volume taken was 10 mL/kg body weight. A quantity of 2000 mg of plant extract was suspended in 10 ml of corn oil to get a 200 mg/mL dose. The dose formulation of ethanolic extract of *C. guianensis* leaves was administered orally to mice using a metal cannula attached to a 1 mL disposable syringe. Animals in group I only received corn oil orally on both days. Positive control group animals were administered with a single injection of Mitomycin-C intraperitoneally at the dose level of 1.0 mg/kg body weight on day 2 of dosing (Table 3). All animals were weighed before dosing each day and before sacrifice. The clinical signs of toxicity were recorded before dosing, post-dosing, and before sacrifice.

2.6 Slide Preparation

Within 18 - 24 h following the last treatment, mice in group I and group II were sacrificed by CO_2 asphyxiation[31]. The positive control group (group III) was sacrificed 24 hours after the last treatment by CO_2 asphyxiation[32]. From the sacrificed animals, femur bones were collected, and the epicondyle ends were separated. The bone marrow cells were ejected out in centrifuge tubes using a 1 mL syringe and a 24–gauge needle by flushing and aspirating approximately

3 mL foetal bovine serum. The aspirated bone marrow substance was mixed using the syringe to make a single-cell suspension. This bone marrow content was centrifuged at 1500 rpm for 10 minutes. After discarding the supernatant, the cell pellet with 0.2–0.3 mL of the medium was mixed properly using a Pasteur pipette, and the suspension was smeared on a clean slide and allowed to air dry. Appropriately marked, two replicates of slides were prepared for each animal. Slides were exposed to methanol in order to get the cells fixed on the slide surface, and it was air-dried for 15–20 minutes. 5% Giemsa in phosphate buffer was used to stain the slides for 25 minutes[33]. Distilled water was used to rinse the slides to remove the excess amount of stain before air drying and mounting. To conduct an unbiased scoring process, the slide identification was covered with unique code numbers.

Table 3. Details of positive control

Name	Mitomycin-C
Lot Number	SLBX4310
CAS Number	50-07-7
Appearance	Grey-blue powder
Route of Administration	Intraperitoneal
Dose	1 mg/kg body weight
Manufactured by	Sigma
Storage	2-8^{0}C
Date of Expiry	August 2022

2.7 Scoring of Bone Marrow Micronucleus

One out of two slides of each animal were used to evaluate erythrocytes with micronucleus. Whereas the other slide was kept in reserve to be used for scoring when required. The slides were observed for the possible formation of micronucleus in polychromatic (PCEs) as well as Normochromatic Erythrocytes (NCEs) under a light microscope (Nikon Eclipse E600, Eclipse Ni-U Fluorescence, Nikon Eclipse 80i, and Nikon Ci-L). At least 4000 PCEs were screened per mouse to evaluate the presence of micronucleated immature erythrocytes. A minimum of 500 NCEs was scored to determine the P/E ratio. The masked labels were removed, and all the slides were decoded after scoring.

2.8 Calculation and Statistical Analysis

The P/E ratios were calculated using the following equation:

$$\frac{P}{E} = \frac{PCE}{PCE + NCE}$$

where, PCE = Polychromatic Erythrocytes and NCE = Normochromatic Erythrocytes

The percent Micronucleated Polychromatic Erythrocytes (%MNPCE) were also calculated using the following equation:

$$\%\ MNPCE = \frac{MNPCE \times 100}{Total\ PCE}$$

The data of percent micronucleated polychromatic erythrocytes (% MNPCE), body weight, and P/E ratio for both the sexes were subjected to statistical analysis. Homogeneity of variance was analyzed by Bartlett's test before Analysis of Variance (ANOVA) and Dunnett's t-test. Student's t-test[34] was employed to define the level of significant difference between the control and the treated groups, wherever data did not meet the homogeneity of variance.

3. Results

3.1 Dose Range Finding Study

Normal behavior was observed in all mice in control and groups treated at 500, 1000, and 2000 mg/kg body weight, post-treatment and pre-sacrifice. Mortality was not noticed in any mice treated at 500, 1000, and 2000 mg/kg body weight. Animals that survived after two days of dosing were sacrificed, and bone marrow content was collected to evaluate the toxicity to bone marrow (suppression of polychromatic to total erythrocytes ratio). Significant increase in body temperature, i.e., > 1^{0}C or significant decrease in body temperature, i.e.,> 3^{0}C, was not observed in both male and female animals up to 5 h after day 1 and day 2 of dosing, treated at 500, 1000, and 2000 mg/kg body weight and in the control animals. The results of mean body temperature in mice of both sexes can be seen in Tables 4 and 5. Therefore, body temperature was not recorded during the main study experiment. From the results of the range-finding study, the limit dose of 2000 mg/kg body weight for only male animals was selected using the two-day dosing schedule with the vehicle and positive control groups.

Table 4. Summary of mean body temperature - dose range finding study in male (Number of animals = 2 animals/sex/group)

Group	Dose of *C. guianensis* leaf extract, mg/kg of body weight	Body Temperature, ^{0}C						
		Before dosing (Day 1)	After dosing (Day 1)		Before dosing (Day 2)	After dosing (Day 2)		Before sacrifice
			2 h	5 h		2 h	5 h	
G I	Vehicle control	37.2 ± 0.4	37.7 ± 0.1	37.3 ± 0.6	37.4 ± 0.6	36.9 ± 0.0	37.3 ± 0.6	37.5 ± 0.4
G II	500	37.4 ± 0.6	37.2 ± 0.4	37.3 ± 0.6	37.4 ± 0.6	36.9 ± 0.1	36.9 ± 0.0	37.4 ± 0.3
G III	1000	37.0 ± 0.2	37.3 ± 0.5	37.4 ± 0.6	37.4 ± 0.8	37.2 ± 0.4	37.6 ± 0.3	37.1 ± 0.2
G IV	2000	37.2 ± 0.1	36.9 ± 0.0	37.3 ± 0.7	36.9 ± 0.0	37.5 ± 0.5	37.1 ± 0.2	37.4 ± 0.5

Table 5. Summary of mean body temperature - dose range finding study in female (number of animals = 2 animals/sex/group)

Group	Dose of *C. guianensis* leaf extract, mg/kg of body weight	Body Temperature, ^{0}C						
		Before dosing (Day 1)	After dosing (Day 1)		Before dosing (Day 2)	After dosing (Day 2)		Before sacrifice
			2 h	5 h		2 h	5 h	
G I	Vehicle control	37.3 ± 0.7	37.2 ± 0.4	37.7 ± 0.1	37.1 ± 0.2	37.7 ± 0.1	37.5 ± 0.4	37 ± 0.1
G II	500	37.1 ± 0.4	37.3 ± 0.5	37.2 ± 0.4	37.3 ± 0.6	37.7 ± 0.4	37.6 ± 0.5	36.9 ± 0.0
G III	1000	36.9 ± 0.0	37.5 ± 0.5	37.4 ± 0.4	37.5 ± 0.5	37.3 ± 0.6	37.3 ± 0.6	37.1 ± 0.1
G IV	2000	37.2 ± 0.4	37.9 ± 0.1	37.2 ± 0.4	37.3 ± 0.4	37.4 ± 0.2	37.3 ± 0.6	36.9 ± 0.0

Note: Corn oil was used in vehicle control group.

3.2 Main Study

Normal behavior was observed in all mice in the controls and groups treated at 2000 mg/kg body weight. No mortality was detected in any of the male mice from the vehicle control, treatment, and positive control groups. Any statistically significant change in the body weight was not seen in any animal from positive control or treatment groups when compared with the concurrent vehicle control group, which can be observed from Table 6.

Table 6. Summary of mean body weight: male (number of animals = 5 males/group)

Group	Dose, mg/kg of body weight	Body weight, grams (Mean ± SD)		
		Day 1	Day 2	Before sacrifice
G I	Vehicle control	37.8 ± 3.70	37.6 ± 2.70	38.0 ± 2.74
G II	*C. guianensis* leaf extract: 2000	38.6 ± 2.30	38.0 ± 1.22	38.0 ± 1.58
G III	Mitomycin-C: 1.0	-	37.4 ± 3.78	37.6 ± 4.93

Key: SD = Standard Deviation, - = Not Applicable
Note: Body weight of positive control animals was not recorded on day one since positive control animals were not treated on day one.

3.3 Micronucleated Polychromatic Erythrocytes (MNPCE)

Table 7 shows a summary of MNPCE in Bone Marrow Cells.

No toxicity to bone marrow [decreases in polychromatic to total erythrocytes ratio (P/E)] was noticed in male animals

Table 7. Summary of micronucleated polychromatic erythrocytes in bone marrow cells (number of animals = 5 males/group)

Group	Dose, mg/kg of body weight	Total PCE	MNPCE			P/E Ratio Mean ± SD
			Total	Mean ± SD	%MNPCE Mean ± SD	
G I	Vehicle control	22540	6	1.200 ± 0.447	0.024 ± 0.009	0.497 ± 0.002
G II	*C. guianensis* leaf extract: 2000	22524	6	1.200 ± 0.447	0.024 ± 0.009	0.496 ± 0.007
G III	Mitomycin-C: 1.0	22547	257	51.400 ↑↑ ± 4.722	1.142 ↑↑ ± 0.108	0.494± 0.006

Key: PCE = Polychromatic Erythrocytes, MNPCE = Micronucleated Polychromatic Erythrocytes, P/E = Polychromatic Erythrocytes corresponding to Normochromatic Erythrocytes/ Total Erythrocyte,.↑↑ = Significantly higher than the control at 1% level (p≤0.01)

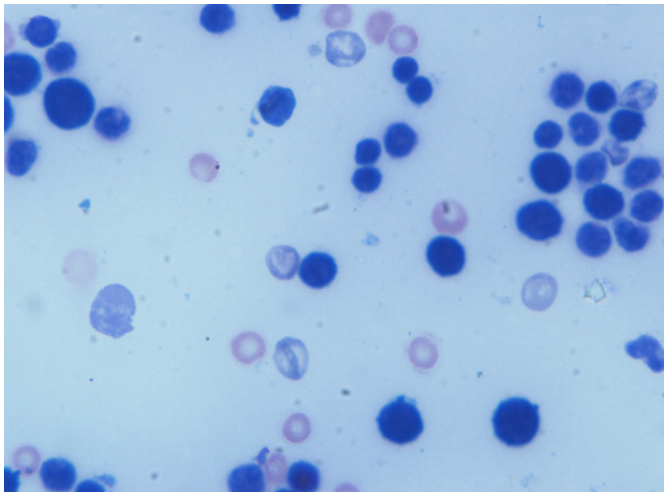


Photo 1. Bone Marrow Slide without Micronuclei (Vehicle Control Group)

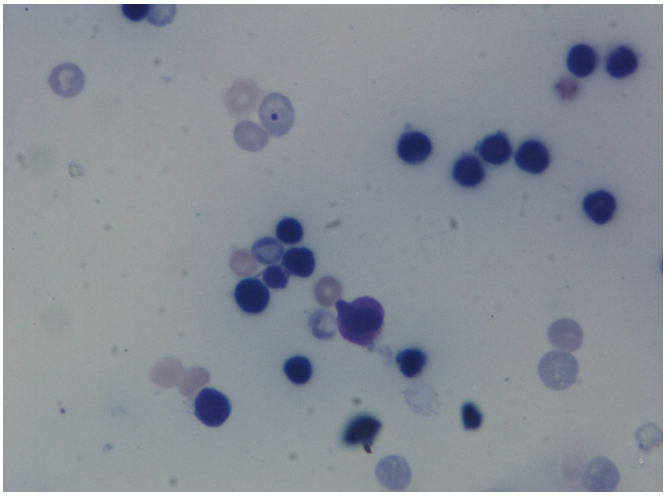


Photo 3. Bone Marrow Slide with Micronuclei (Positive Control Group).

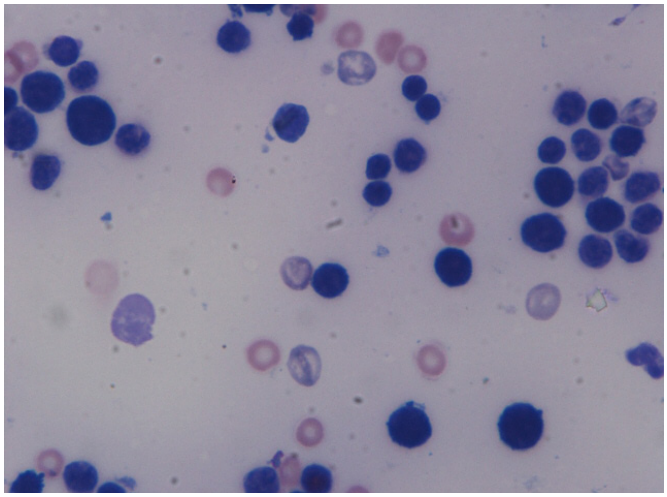


Photo 2. Bone Marrow Slide without Micronuclei (Treatment Control Group).

of positive control groups when compared to the concurrent vehicle control group. Statistically, no significant decrease in polychromatic to total erythrocytes ratio (P/E) was obtained in male mice treated at 2000 mg/kg body weight when compared to the concurrent vehicle control.

Mean P/E ratios were 0.497 and 0.496 for male animals treated at the dose level of 0.0 (vehicle control group) and 2000 mg/kg body weight, respectively. Percent reduction of 0.20% was observed in male animals treated at 2000 mg/kg body weight. The Mean P/E ratio observed in animals treated with Mitomycin-C (1.0 mg/kg body weight) was 0.494. Mean %MNPCE values were 0.024 and 0.024 for male animals treated at 0.0 (vehicle control group), and 2000 mg/kg body weight, respectively. The Mean % MNPCE observed in animals treated with Mitomycin-C (1.0 mg/kg body

weight) was 1.142. No significant increase in % MNPCE was detected in any mouse of treatment groups when compared to the concurrent vehicle control. Statistically, a significant rise observed in %MNPCE in animals treated with Mitomycin-C (1.0 mg/kg body weight) demonstrated the suitability of the test system, procedures employed, and efficiency of the test conditions employed in the test.

4. Discussions

Herbal products are widely used since ancient times as a remedy for the diversity of medical ailments. *C. guianensis* is one such plant with stated antibacterial, antimycobacterial, antimicrobial, antioxidant, antitumor, antiulcer, antinociceptive, anthelmintic, antifertility, and antifungal activities[5–8]. Its chemical constituent, indirubin is reported to have antibacterial, antifungal activity and is actively used for the treatment of chronic myelocytic leukemia. Isatin, a chemical constituent of *C. guianensis* flower extract, has been used as a prophylactic agent, prevents free radical-induced cancer, acts as a chemotherapeutic agent to kill cancer cells, and has antioxidant and anticancer activities against human promyelocytic leukemia 60 cells. The plant extract of *C. guianensis* is equally effective as paracetamol for its analgesic activity and indomethacin for its anti-inflammatory activity[35]. Similarly, *C. guianensis* flower extract was found to have a protective effect against chloramphenicol-induced toxicity in bone marrow as well as hematological changes like aplastic anaemia, leucocytosis, and thrombocytosis[36]. Even scientists have suggested the use of *C. guianensis* plant extract as an effective and environmentally sustainable bio-insecticide for the control of whiteflies, *Bemisia tabaci* [37].

C. guianensis has shown good therapeutic potential, such as plant growth inhibitory effect, which will help in agriculture for weed management[15]. It also showed promising properties which will act as an antitubercular agent[6]. Other studies conducted on different medicinal plants also showed the similar types of medicinal properties such as *Gentiana genus* provides protection from hepatic, cardiovascular, gastrointestinal disorders as well as immunomodulatory effects[8]. *Origanum vulgare* L. demonstrated protective effects to neutralise the adverse effects of cyclophosphamide against the damages of normal cells in patients in chemotherapy[22].

Similar to useful medicinal properties showed by other plant extracts[8,22,23], *C. guianensis* has been reported to be used for a wide variety of traditional usage[6,7], but the determination of method of engagement, separation, purification and pharmacokinetics studies of active compounds are yet not done completely. Besides, people are becoming more anxious concerning the safe use of plant extracts, and hence more emphasis has been kept on investigating mutagenicity, toxicity, etc., of traditionally utilized natural products[38]. The efficacy and safety aspects of the plants should be determined using various *in vitro*, *in vivo*, or *ex vivo* assays to make the plant a good medicinal resource in the upcoming future[5].

Limited studies are conducted to evaluate the toxicological profile of *C. guianensis*. *In vitro* and *in vivo* cytotoxicity potential of *C. guianensis* using Vero cell line and brine shrimp naupliim (or nauplii), respectively, and genotoxicity potential on DNA of Vero cell lines were determined by Sivapragasam *et al.,* to identify its harmful effects. However, no cytotoxicity or genotoxicity was found[18]. Although few studies indicate that the plant is non-toxic at higher doses, further testing needs to be carried out to decide its safety with respect to the genotoxicity endpoint. Genotoxicity assessment of plant extracts is a good measure to examine possible collateral effects and establish safe utilisation of plants. Some researchers reported that the use of available human hepatoma cell lines HepG2 and Huh6 for genotoxicity testing endpoints were found to be suitable for the assessment of Pyrrolizidine Alkaloids (PAs) induced genotoxicity and results also confirmed previously demonstrated potency rankings using micronucleus assay[19].

For evaluation of genotoxic potential, *in vivo* micronucleus test in mice following the globally accepted OECD TG (OECD Test No. 474) was found a suitable model as per the research published by different researchers[20–23,25,39–44].

Hence, with the main objective of the determination of the genotoxic potential of ethanolic extract of *C. guianensis* plant leaves, we have performed *in vivo* micronucleus test. Induction of micronucleus under *in vivo* conditions is a very important genotoxicity test endorsed by the regulatory authorities worldwide for the determination of product safety[25,45]. Increased frequency of MNPCEs in treated mice indicates the induction of chromosome damage. A decrease in the P/E ratio caused due to direct cytotoxicity, while micronuclei formation and heavy DNA damages lead to cell death or apoptosis[46]. In the present study, neither increase in the %MNPCEs nor a decrease in P/E has been observed between the treated and control population. This indicates that the ethanolic extract of *C. guianensis* leaves is not genotoxic even at the dose of 2000 mg/kg. Results of dose range-finding study as well the main study revealed the absence of any bone marrow toxicity and these results were in line with earlier published data in which acute toxicity study revealed zero mortality and normal behavior in mice upon oral administration of methanolic flower extract and ethanolic leaf extract up to 2000 mg/kg and 3000 mg/kg

doses, respectively[47,48]. Mechanistically micronucleus test provides details on the mechanism involved in damage to the chromosome termed as clastogenicity as well aneugenicity. Observed results showed that the extract of *Couroupita guianensis* did not demonstrate any adverse effects on the target organ therefore, no clinical symptoms or effect on thermoregulation was observed[30,29]. No effect on body temperature was observed therefore extract of *C. guianensis* did not reveal any impact on erythropoiesis leading to lagging of whole chromosome, i.e. aneugenic response[30,29]. Therefore, the results revealed that extract of *Couroupita guianensis* did not show genotoxic response and our finding are in line with the findings of Han et al. (2019)[49]. *In vitro* studies conducted using *C. guianensis* flower extract also demonstrated absence of any direct DNA damage in comet assay[18]. The absence of genotoxicity observed in the present research is supported by earlier research work in which pharmacologically significant effects such as antioxidant, scavenging activity and free radical activity activities were found in Leaf extracts of *C. guianensis* which might be showing protecting effect leading to the absence of any genotoxic effect[7]. Similarly, *Origanum vulgare* L. demonstrated protective effects to neutralise the adverse effects of cyclophosphamide against the damages of normal cells in patients in chemotherapy[22]. Some research work also demonstrated the antimutagenic effects against EMS-induced genotoxicity in mice by micronucleus test and chromosomal aberration test due to the activity of methanolic extract of *Melissa officinalis*[23]. *C. guianensis* has been used in ethnomedicine for the treatment of various ailments. The results observed in this research are in line with the earlier research work and provide further proof of safety[50,51].

Additionally, studies conducted with glycolic extract of *Ziziphus joazeirobark* by MNT test in mice bone marrow did not show genotoxic effects[40]. Results of studies provided experimental evidence about the absence of genotoxicity due to Myelophil, an ethanol extract mixture of *Astragali radix* and *Salviae radix*, therefore considered safe for genotoxicity endpoint[41]. Author demonstrated the absence of mutagenic and clastogenic response of herbal preparation *Euphorbia triaculeata* extract in the *in vivo* micronucleus and chromosome aberration test[42]. Genotoxicity testing conducted with GHX02, which is a herbal mixture containing Gwaruin (*Trichosanthes kirilowii*), Haengin (*Prunus armeniaca*), Hwangryeon (*Coptis japonica*) and Hwangkeum (*Scutellaria baicalensis*) found safe in all the three battery of genotoxicity assays[43]. Results of genotoxicity evaluation of CinDura® (GMCT) using Ames test and the micronucleus test in mouse revealed it is neither mutagenic nor clastogenic and found to be safe[44]. Studies on extracts of *Combretum leprosum* Mart. (*Combretaceae*) showed that that the ethanolic extracts of leaves and stem bark found to safe, and it did not have cytotoxic, genotoxic or mutagenic potential[39]. Results of the battery of test conducted for genotoxicity evaluation of *Dioscorea* Rhizome water extract (DRWE) showed that DRWE was neither showed mutagenic (Ames test) nor clastogenic (*in vitro* chromosome aberrations and *in vivo* MNT test) response[20]. Pharmacologically important plant extract of *Rhus verniciflua* Stokes (RVS) did not reveal either mutagenic (Ames test) or clastogenic response (*in vitro* chromosome aberration test) or DNA damaging response (Comet assay) and further concluded to be safe for genotoxicity endpoints[21]. Therefore, results of genotoxicity testing of *C. guianensis,* were in line with the earlier published results of similar type of studies conducted with different medicinally important plants[20–23,39–44].

5. Conclusion

The present study reported the non-genotoxic nature of ethanolic extract of *C. guianensis* leaves using the i*n vivo* micronucleus test in the mice model. Various safety aspects of *C. guianensis* plant remains to be carried out despite lots of medicinal properties and no such toxicity studies have been reported for the plant yet. Therefore, we have tried to justify that the plant can be a potential source of the medicinally important molecule without any clastogenic response, i.e., genotoxicity. Our study concluded that the plant is not showing any genotoxicity and can be used as a medicine to cure various reported ailments.

6. Conflict of Interest

The authors declare that they have no conflicts of interest.

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