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Evaluation of Mutagenic Potential of Ethanolic Extract of Leaves of *Couroupita guianensis* using *Salmonella typhimurium* Tester Strains

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Abstract

India has a golden resource and well-recorded traditionally practised incomparable awareness of herbal medicine. Not all herbals are considered safe as minimal data is available concerning the safety of *Couroupita guianensis* to cause mutations. Considering the enormous therapeutic potential and the absence of specific safety data, we planned this research to provide data on the mutagenic potential of *Couroupita guianensis*. Therefore, the present study was conducted with ethanolic extract of leaves of *Couroupita guianensis* using *Salmonella typhimurium* tester strains TA1537, TA1535, TA98, TA100, and TA102 at different concentrations (viz., 156.25, 312.5, 625, 1250, 2500, and 5000 µg/plate) along with the negative control and respective positive controls. The methodology followed was the standard plate incorporation method as per the internationally approved OECD TG 471 guidelines. The study was performed with and without S9 mix using the plate incorporation method. Results were evaluated after meeting the acceptance criteria for the study. The extracts did not show mutagenicity when compared to the negative control (spontaneous mutation) and respective positive controls. There was no concentration-response increase of revertant colonies in treatments from any strain. This is the first of its kind of research having been conducted following all the internationally set guidelines and evidenced the safety of the plant material. Therefore, it is concluded that ethanolic extract of leaves of *Couroupita guianensis* did not show mutagenic potential in bacterial reverse mutation test with and without S9 mix.

Keywords: Reverse Mutation, Bacteria Strains, *Couroupita guianensis*

1. Introduction

India is having a good history of using herbal plants as medicines for various purposes. Though these different practical uses of various medicinally important plants are reported and used inherently from ancient times by different tribes throughout India, scientific evidence of most plants is not reported. Herbal medicines are recommended for different health care needs, even the basic knowledge for modern medicine. People are utilising different plant parts or their extracts for consumption for external body applications since ancient times. Pharmaceutical industries have implemented greater than forty thousand plant species for making their medicinal products treating numerous therapeutic conditions[1]. Despite traditional uses and increasing demand for herbal

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medicine, one should consider that with pharmacological benefits, plants can also be caught up as an initiating factor in DNA damage, cell mutations, and natural toxic elements[2]. It is a complete misconception that the consumption of herbal drugs is non-toxic. The cytotoxicity and genotoxicity of plants become apparent only after regular consumption for a longer period of time[3]. Thus, the safety and toxicity of medicinally important plants should be considered on a serious note[4]. Traditionally utilise natural products should be investigated properly for their mutagenicity, toxicity, etc[5].

Couroupita guianensis Aubl. attracted worldwide attention because of its immense pharmacological properties[6]. Almost all parts of the tree have been used traditionally for treating various ailments. Even the infusions made from the blend of *C. guianensis* flowers and leaves were reported for anti-inflammatory properties[7], skin diseases, flu, stomach-ache, toothache, and pathogenic infections[8]. Scientific studies have proven its antimicrobial, antifungal, antiseptic, antidepressant, antimalaria, anticancer, antihelmintic activities[9–12] and insecticidal activities for safer environment[13,14]. *C. guianensis* has been employed in traditional medicine but limited scientific evidence is there signifying its toxicity profile[4].

Otabe *et al.*[15] studied genotoxicity of aspartame was using Ames pre-incubation assay with *Salmonella typhimurium* tester strains TA1537, TA1535, TA100, and TA98; as well as Escherichia coli tester strain WP2 uvrA; and *in vivo* micronucleus using Crlj:CD1(ICR) mice. To evaluate the genotoxicity, Han *et al.*[16] performed tests on *Opuntia ficus-indica* var. saboten (OFIS), which included the chromosomal aberration test, the mammalian micronucleus test as well as Ames test. Studies were conducted as per OECD and GLP-compliant battery for toxicological evaluation in order to evaluate the safety of an extremely pure *Germanium sesquioxide*[17]. Genotoxicity of herbal formulation CinDura® (GMCT) was conducted using Ames and micronucleus test[18]. Therefore, Ames test is considered and the most commonly used test for assessment of the mutagenic capability of medicinal plants[19]. The assay was also performed to assess the antioxidant potential of ethanolic extract of leaves extract of *C. spinosa* against the genotoxicity caused by KBrO3 using micronucleus test[20]. A review published by Wang *et al.*[21] reported genotoxicity evaluation of *Dendrobium* species, *D. speciosum*. Mainly leaf extract using *in vitro* models exhibited genotoxic effects in an *in vitro* studies. Overall, very limited data is available for the genotoxic potential of *C. guianensis* plant extract[22,23], which necessitates further research to demonstrate its toxicological safety as per recent methodologies. Considering all the published data, it is revealed very limited data is generated with recent regulatory guidelines to consider *C. guianensis* safety for the mutagenicity and genotoxicity endpoints, which is a piece of key evidence to demonstrate the safety of *C. guianensis* before getting approval to commercialise it as a safe ayurvedic drug. Therefore, the current study was performed with the objective to assess the mutagenic potential of ethanolic leaves extract of *C. guianensis* on *Salmonella typhimurium* tester strains using the recent and globally accepted testing requirements[24].

2. Material and Methods

2.1 Chemicals

The chemicals used in the study were procured from Sigma Aldrich, Merck Qauligens, Gibco, SRL and HiMedia as described in Table 1.

2.2 Plant Collection

Leaves of *C. guianensis* were collected from the Jai Research Foundation, Valvada, Gujarat, India. Plant identification and authentication were made by Bhagvati Herbal and Healthcare Private Limited, Valsad, Gujarat 396195. The voucher specimens (JRF/Genotox/18-19/01) of *C. guianensis* were deposited at the Department of Toxicology (Mutagenicity), Jai Research Foundation, Gujarat.

2.3 Extraction

The collected leaves were washed with distilled water, dried under shaded conditions at room temperature, and leaves were crushed to make a fine powder using an electric blender. A volume of 50 gm of powder was used for extraction in a Soxhlet apparatus. At 250 ml of ethanol was used for extraction, and the process continued up to 4 hrs. Further, whatman filter paper was used in order to filter the extract, and it was subjected to a rotary vacuum evaporator to concentrate the extract.

2.4 Tester Strains

The test was intended to evaluate the mutagenic capability of ethanolic leaves extract of *C. guianensis* employing histidine-deficient strains of *Salmonella typhimurium*, TA1537, TA1535, TA98, TA100, and TA102 in the presence and absence of the metabolic activation system (5% v/v S9 mix)[24]. Positive controls utilised for different strains with and without S9 mix are listed in Table 2.

Table 1. Positive controls used for each tester strain in presence and absence of metabolic activation system

Sr. No.	Chemical Name	Catalogue Number	Make
1	Magnesium Sulphate	13005	Qualigens
2	Citric Acid	C7129	Merck
3	Potassium Phosphate Dibasic	15665	Qualigens
4	Sodium Ammonium Phosphate	59506	Sigma Aldrich
5	Dextrose Anhydrous	6178091001730	Merck
6	Sodium Dihydrogen Phosphate	1.936240521	Merck
7	Disodium Hydrogen Phosphate	S5136-500G	Sigma Aldrich
8	D-Glucose-6-Phosphate	G7250	Sigma Aldrich
9	β - NADP	N3886	Sigma Aldrich
10	Magnesium Chloride	69396	SRL
11	Potassium Chloride	P5404	Sigma Aldrich
12	L- Histidine	H8125	Sigma Aldrich
13	D- Biotin	B4501	Sigma Aldrich
14	L- Tryptophan	T0254	Sigma Aldrich
15	Bacto Agar	210410	BD
16	Sodium Chloride	55886	Sigma Aldrich
17	Ampicillin	CM5645	Himedia
18	Tetracycline	CM5219	Himedia
19	Oxoid Nutrient Broth	CM0067	Oxoid
20	Crystal Violet	GRM114	Himedia
21	Dimethyl Sulfoxide	Q43486	Qualigens
22	Nutrient Agar	M1269-500G	Himedia
23	Sabouraud Chloramphenicol Agar	M1067	Himedia
24	2- Aminoanthracene	A38800	Sigma Aldrich
25	9 - Aminoacridine Hydrochloride Hydrate	A38401	Sigma Aldrich
26	2- Nitrofluorene	N16754	Sigma Aldrich
27	Sodium Azide	S2002	Sigma Aldrich
28	Mitomycin - C	M4287	Sigma Aldrich
29	4- Nitroqunoline - N - Oxide	N4181	Sigma Aldrich

Table 2. Positive controls used for each tester strain in presence and absence of metabolic activation system

Tester Organism	In the absence of Metabolic activation system			In the presence of Metabolic activation system		
	Positive control	Solvent	Conc., µg/plate	Positive control	Solvent	Conc., µg/plate
TA1537	9-Aminoacridine hydrochloride hydrate	Dimethyl sufoxide	75.0	2-Aminoanthracene	Dimethyl sufoxide	10.0
TA1535	Sodium azide	Distilled water	0.5			10.0
TA98	2- Nitrofluorene	Dimethyl sufoxide	7.5			5.0
TA100	Sodium azide	Distilled water	5.0			5.0
TA102	Mitomycin-C	Distilled water	0.5			10.0

2.5 Mutagenicity Experiment

The highest exposure concentration selected was 5000 µg/plate. The extract was selected based upon the precipitation test. The treatment was performed by the plate incorporation technique in triplicate for each concentration of *C. guianesis*, negative and positive controls. A volume of 100 µL of tester strains suspensions (1-2 x10^9 cells/mL), 100 µL of extract was used to achieve test concentrations between156.25 and 5000 µg/plate, while positive controls and negative controls were added to respective tubes. A 0.5 mL volume of 5% v/v S9 mixture or 0.2 M phosphate buffer having a pH of 7.4 were added individually in 2.0 ml of melted top agar. The mixture in each individual tube was mixed properly before pouring immediately onto a minimal glucose agar (bottom agar) plate.

Once the agar poured on the plate got solidified, the plates were kept on an incubator in inverted condition at 37 ±1^{0}C for 48 hrs. Revertant colonies developed following the incubation were enumerated using an automated colony counter. The obtained results were compared with that of the respective number of spontaneous revertant colonies developed in the control plates (solvent/vehicle control plates). Mutagenic index (MI) was calculated using the below-mentioned formula:

$$MI = \frac{Number\ of\ revertant\ colonies\ in\ test}{Number\ of\ revertant\ colonies\ in\ negative\ control}$$

A sample with MI greater than 2 is considered mutagenic[17,18].

3. Results

Bacterial reverse mutation test is used to detect revert mutations by growing treated strains in the medium lacking specific amino acid, for which the strains used were auxotrophic. Here strains used were histidine auxotrophs, counting of revertant strains were made on minimal glucose agar plate without histidine.

Precipitation was not observed at the tested concentration up to 5000 µg/plate. Hence, 5000 µg/plate was selected as the top concentration to be tested for mutagenicity test in the presence (5% v/v S9 mix) and absence of the metabolic activation. All the tester strains used for the assay were tested for cell viability before the actual assay, represented in Table 3.

Also, before the assay, to meet the criteria for a valid assay, tester strain integrity, presence of a cell wall mutation, requirement of the biotin except for TA102 (which is biotin-dependent), sensitivity to UV exposure except for TA102 (which is a wild type), histidine dependence, presence of pKM101 plasmid, i.e., ampicillin resistance, presence of pAQ1 plasmid, i.e., tetracycline resistance was determined and results of which are represented in Table 4.

Sterility check of all test components (top agar, S9 mix, ethanol, test items, MGA plate, ONB solution, and 0.2 M Sodium Phosphate buffer) was performed along with a mutagenicity test. There was no contamination observed in any plate used for the study (Table 5).

Table 3. Cell Viability Test for Tester Strains

Tester strain used	OD at 660 nm	Viable number of cells (x10^9 CFU/mL)
TA1537	0.486	1.87
TA1535	0.589	2.24
TA98	0.560	2.13
TA100	0.520	1.99
TA102	0.507	1.95
Control Blank	0.00	0.00

Key: OD = Optical density, CFU = Colony forming unit

Table 4. Genotype Confirmation Test for Tester Strains

Name of the Test		*Salmonella typhimurium* Tester Strains				
		TA1537	TA1535	TA98	TA100	TA102
Histidine Dependence		NG	NG	NG	NG	NG
Biotin Dependence		NG	NG	NG	NG	G
Histidine and Biotin Dependence		G	G	G	G	G
***rfa* Mutation**		ZI	ZI	ZI	ZI	ZI
DNA repair (*uvrB*)		NG*	NG*	NG*	NG*	G*
R - factor Resistance	**Ampicillin Resistance**	NG	NG	G	G	G
	Tetracycline Resistance	NG	NG	NG	NG	G

Key: NG = No growth, G = Growth, ZI = Zone of inhibition, * = On irradiated side Results: Strains have retained their genetic characteristics.

Table 5. Sterility Test for the Operating System (Mutagenicity Test)

Sterility checks for	Number of colonies
Top agar	NIL
S9 mix	NIL#
Solvent (Ethanol)	NIL
T.I. Stock solution*	NIL
MGA Plate (Blank)	NIL
ONB Solution	NIL
Sodium Phosphate Buffer	NIL

* = Lowest concentration of Test Item stock solution, # = 5% v/v S9 mix, ONB = Oxoid Nutrient Broth, MGA = Minimal glucose agar, T.I. = Test item, NIL = No colonies

As described in the regulatory guidelines, the actual Mutagenicity test was performed in the triplicate on each strain at different concentrations of ethanolic leaf extract ranging from 156.25 to 5000 µg/plate, respective positive controls; and negative control. Tables 6 and 7 represent the average number of His+ revertant colonies in the negative controls treatment plates, and the positive controls, in the absence and presence of the S9. Figure 1 shows dose-response curves with and without the S9 mix.

Table 6. Mean Count of His+ Revertant Colonies in Negative Control, Positive Controls and Treatment Plates in the Absence of Metabolic Activation

[*C. guianesis* leaf extract], µg/plate	His+ Revertant Colonies/Plate (Absence of Metabolic Activation), Mean ± SD				
	TA1537	TA1535	TA98	TA100	TA102
NC (Ethanol)	8.67±1.53	20.00±2.00	18.33±3.51	135.33±7.02	228.33±6.03
156.25	8.00±2.65	20.00±1.00	18.00±2.65	131.67±6.66	228.33±2.31
312.5	8.67±3.06	20.33±2.08	17.67±0.58	133.33±8.62	227.33±6.81
625	7.67±3.06	19.67±3.79	18.33±6.43	134.00±10.82	226.33±6.51
1250	8.33±2.52	19.67±3.21	20.00±3.00	132.67±5.69	226.33±6.43
2500	8.00±3.46	19.00±4.36	17.33±4.04	134.67±8.14	224.00±7.00
5000	7.33±1.53	19.67±7.57	17.33±2.52	134.67±9.02	225.00±4.00
PC	297.00±29.51	427.00±25.94	607.33±71.36	995.33±68.72	1175.00±31.05
2Aa	-	-	-	136.67±10.60	-

Table 7. Mean Count of His+ Revertant Colonies in Negative Control, Positive Control and Treatment Plates in the Presence of Metabolic Activation

[*C. guianesis* leaf extract], µg/plate	His+ Revertant Colonies/Plate (Presence of Metabolic Activation at 5% v/v S9 mix), Mean ± SD				
	TA1537	TA1535	TA98	TA100	TA102
NC (Ethanol)	10.67±1.53	22.33±3.06	25.33±4.16	136.00±8.89	238.67±3.51
156.25	10.00±2.00	23.00±2.65	23.00±4.00	132.00±6.24	234.00±6.24
312.5	10.33±2.31	22.33±3.51	25.33±1.53	133.33±6.03	236.67±4.16
625	10.33±1.53	21.67±1.53	22.67±2.08	134.33±6.51	237.67±3.06
1250	11.00±1.00	21.00±2.00	25.67±2.52	133.67±7.09	235.67±3.51
2500	10.00±3.61	22.00±7.21	23.67±1.53	134.00±5.00	237.33±2.52
5000	9.67±1.53	21.33±2.08	22.67±5.69	135.33±9.61	237.33±4.04
PC-2A	309.33±42.16	464.00±69.76	721.33±67.26	1035.00±58.95	1198.00±53.45

Key: NC = Negative control, PC = Positive control [TA98 = 2-Nitrofluorene (7.5 µg/plate), TA100 = Sodium azide (5 µg/plate), TA102 = Mitomycin-C (0.5 µg/plate)], 2Aa = 2-Aminoanthracene (10 µg/plate for TA102 and 5 µg/plate for TA98 and TA100), - = Not applicable.

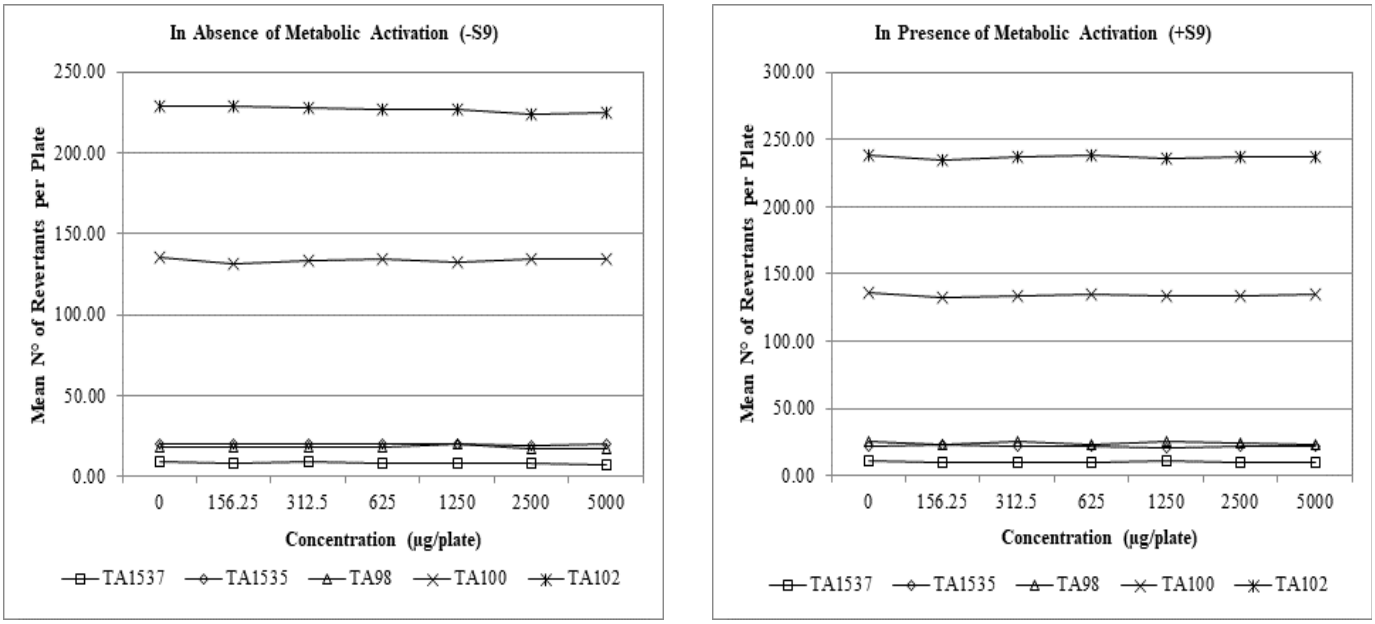


Figure 1. Dose-response curves.

The results of negative control treatments for all strains used were within the normal historical limits. Positive controls (+S9 and -S9) displayed a clear-cut rise in the number of revertants when equated to the parallel control. This rise proved the effectiveness of the test system and the appropriateness of the processes completed in the study. Any rise in the mean number of colonies was not observed in tester strain TA100 treated with 2-aminoanthracene without S9, but a clear increase was observed with S9. This rise exhibited the proficiency of the S9 fraction utilized in this study. Normal growth was detected up to the assessed strength of 5000 µg/plate with and without S9 mix in all tester strains.

Results revealed no positive mutagenic effect in all the tester strains at the tested concentration between 156.25 and 5000 µg/plate in the absence and presence (5% v/v S9 mix) of the metabolic activation, when compared with the concurrent negative control. Statistical evaluation did not reveal any considerable impact, i.e., the numbers of revertant mutants in extract tested groups were equal to the negative control. From the result, it can be reflected that there was no statistical difference in the number of revertant mutants for any strain used in negative control and treatment groups. Thus, the Mutagenic indices calculated were ≈ 1, which depicts that the plant extract is not mutagenic. Therefore, the results suggest that the plant extract was not mutagenic to any tester strain, neither with (5% v/v S9 mix) nor without metabolic activation.

4. Discussion

It is impervious to determine the safety of herbals before we consider them for drug activities[4]. People are becoming more and more concerned about the safe use of plant extracts, and hence it has been emphasized that traditionally utilised natural products should be investigated properly for their mutagenicity, toxicity, etc[5]. One of the key safety parameters in evaluating any drug candidate is the evaluation of genotoxicity which is an essential requirement of an early safety evaluation before considering it as a drug candidate[4]. Research has proved that genotoxic agents possess nucleophilic properties that cause harmful consequences to DNA which leads to mutation and, in several cases, turns to cancers[25–28]. For detection of DNA damage caused by any test materials or herbals, testing for three different endpoint is necessary to meet the international regulatory requirements and very first requirement is gene mutation testing in prokaryotes[29], secondly *in vitro* mammalian cell tests to check clastogenicity and aneugenicty and lastly *in vivo* relevance by using micronucleus test[30,31]. Bacterial reverse mutation assay is one is the most sensitive test with a sensitivity of more than 85% when compared with existing data. Therefore, it is one of the most preferred test amongst the scientific community for the evaluation of the mechanism of actions of molecules for mutagenicity. It is also one of the first tests needed for the evaluation of safety with respect to genotoxic effects, and without the Ames test, genotoxicity test data almost not accepted by different regulatory agencies over the globe.

C. guianensis is one of the most important plants having numerous pharmacological effects. Very limited data is available on its toxicity testing[32–34]. Results of the *in vitro* studies with Vero cell line[4] and *in vivo* studies with mice at 2000 mg/kg[35] showed the absence of any toxicity due to crude extract. Results of studies also confirmed that *C. guianensis* flower extract did not exert a genotoxic effect in Vero cells[4]. Studies using ethanolic extract of flowers of *C. guianensis* confirmed the absence of toxicity due to that crude ethanol extract or its fractions. In contrast, its isolate isatin from *C. guianensis* flower revealed cytotoxicity against HL60 cells[36]. As the concentration might be higher in individual isolates, the isolated compounds might show more toxicity than the plant whole crude compounds. As the mutagenicity data is lacking in currently available data, we decided to evaluate the leaf extract of *C. guianensis* using bacterial reverse mutation test.

In the present study, ethanolic extracts of leaves of *Couroupita guianensis* did not show mutagenicity towards tester strains TA1537, TA1535, TA98, TA100, and TA102. These results coincide with Edziri *et al.*[37] findings that methanol extract of *Peganum harmala* did not show any mutagenicity. Mechanistically bacterial reverse mutation test provides details on the mechanism involved in causing particular reverse mutations. Observed results showed that the extract of *Couroupita guianensis* did not demonstrate any mutagenic effects in the target of histidine operon-containing strains of *S. typhimurium*. This further demonstrates the absence of the base-pair substitution mutations as well as frameshift mutations. Therefore, the results demonstrated that the extract of *Couroupita guianensis* had no mutagenic effects and our findings are in line with the findings of Han *et al.* (2019)[16]. Studies conducted using *C. guianensis* flower extract does not show any direct DNA damage in *in vitro* comet assay. *C. guianensis* has been used in ethnomedicine for the treatment of various ailments, so observed results in the current research were in line with the earlier research work and these results further provide proof of safety[38,39].

Research conducted with several plant-based extracts established the usefulness of Ames test for the evaluation of mutagenicity *in vitro*. Methanolic seeds extracts of *Abrus precatorius* did not show mutagenicity against TA100 up to 0.5 mg/L concentration[40]. Extract from *Astragali radix* and *Salviae miltiorrhizae Radix* did not exhibit any mutagenicity against the tested bacterial strains TA1537, TA1535, TA100, TA98, and WP2uvrA in presence as well as in the absence of S9 up to 5000 µg/plate. Further, the extract did show any toxicity in the chromosomal and micronucleus test[41]. *Zataria multiflora* extracts did not show mutagenicity against *S. typhimurium* (TA98, TA100), which also act as antimutagenicity agents[42]. Eight different plant aqueous extracts were studied for their mutagenicity effect on TA98 and TA100. The extract did not shows mutagenicity against the tested bacteria by Ames test and also did not show toxic effects (survivability of the cells)[43]. Therefore observed results in our present study are in line with several studies conducted using botanical extracts[40–43].

Additionally, studies conducted with aspartame, a sugar substitute, did not show potential for mutagenic or genotoxic activity[15]. Extract of the *Opuntia ficus-indica* were evaluated, and the results did not show any mutagenicity effects towards *S. typhimurium* or *E. coli* with or without S9 activation[16]. Any evidence of mutagenicity was not revealed while *Germanium sesquioxide* (Organic Germanium) was assessed toxicologically in a bacterial reverse mutation test and no clastogenic response in an *in vitro* mammalian chromosomal aberration test[17]. Results of the ames test and the micronucleus test in mouse bone marrow revealed that CinDura® (GMCT) did not have either mutagenic or clastogenic potential[18]. Ethanolic leaves extract of *C. spinosa* against the genotoxicity revealed a protective by abolishing the oxidative DNA damage by decreasing the frequency of micronuclei[20]. *Dendrobium* species. *D. speciosum* leaves exhibited genotoxic effects in an *in vitro* study[21].

In the present study, extracts of the *C. guanensis* did not reveal any mutagenicity by the number of colonies increased with the increase in concentration compared to control. Our results confirmed by Moosavi *et al.*[40], who reported that the EPA and GenPharmTox guidelines say that the test chemical, tested with the help of a bacterial reverse mutation test, is only concluded to be mutagenic if the mutant frequency (induction factor) is 2.0 or above. But here, in this study, it is well proven that the ethanolic extract of *C. guianensis* leaves was not mutagenic even at the top dose of 5000 µg/plate without and with S9. Being a traditionally utilized medicinal plant for various purposes, so many aspects of the safety of *C. guianensis* plant are remained to be studied. No such genotoxicity studies have been reported for the plant yet with internationally accepted regulatory guidelines. So, we have tried to make it firm that the plant can become a potential source of the medicinal molecule without any side effects of toxicity or mutagenicity. Our study concludes that the plant is not showing any toxicity and can be used as a medicine to cure various reported ailments. As per EFSA recommendations, data on *in vitro studies, including Ames* and *in vitro* micronucleus assay, is required. Further *in vivo* testing is recommended to confirm the negative results of *in vitro*

tests[44]. In genetic toxicity testing battery, one *in vivo* study result supports the relevance of *in vitro* test results[45].

5. Conclusion

Ethanolic extract of *C. guianensis* leaves was not mutagenic up to 5000 µg/plate concentrations. In conclusion, we can use this plant as a potential source of medicinal purpose without any side effects such as toxicity or mutagenicity.

6. Conflict of Interest

The authors declare that they have no conflicts of interest.

7. Acknowledgement

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8. References

1. Idu M, Onyibe HI, Timothy O, Erhabor JO. Ethnomedicinal flora of Otuo people of Edo State, Nigeria. Asian J Plant Sci. 2008; 7(1):8212. https://doi.org/10.3923/ajps.2008.8.12
2. Plewa MJ, Wagner ED. Activation of promutagens by green plants. Annu Rev Genet. 1993; 27:93–113. https://doi.org/10.1146/annurev.ge.27.120193.000521. PMid:8122914
3. Mattana CM, Cangiano MA, Alcaráz LE, *et al.* Evaluation of cytotoxicity and genotoxicity of acacia aroma leaf extracts. Sci World J. 2014; 2014. https://doi.org/10.1155/2014/380850. PMid:25530999. PMCid: PMC4228826
4. Gothai S, Vijayarathna S, Chen Y, *et al. In vitro* and *in vivo*-scientific evaluation on cytotoxicity and genotoxicity of traditional medicinal plant *Couroupita guianensis* aubl. flower. Pharmacologyonline. 2019; 2:24–38.
5. de Queiroz FM, de Oliveira Matias KW, da Cunha MMF, Schwarz A. Evaluation of (anti)genotoxic activities of *Phyllanthus niruri* L. in rat bone marrow using the micronucleus test. Brazilian J Pharm Sci. 2013; 49(1):137–48. https://doi.org/10.1590/S1984-82502013000100015
6. Sheba LA, Anuradha V. An updated review on *Couroupita guianensis* Aubl: A sacred plant of India with myriad medicinal properties. J Herbmed Pharmacol. 2020; 9(1):1–11. https://doi.org/10.15171/jhp.2020.01
7. Elumalai A, Eswaraiah M, Didala A. Investigations on anti-oxidant, anti-arthritic and anti-platelet studies in *Couroupita guianensis* Aubl leaves by in-vitro methods. Pharma Sci Monit. 2012;3(3):2262-2269.
8. Umachigi SP, Jayaveera KN, Kumar CKA, Kumar GS. Antimicrobial, wound healing and antioxidant potential of *Couroupita guianensis* in rats. Pharmacologyonline. 2007; 3:269–81.
9. Khan MR, Kihara M, Omoloso AD. Antibiotic activity of *Couroupita guianensis*. J Herbs, Spices Med Plants. 2003; 10(3):95–108. https://doi.org/10.1300/J044v10n03_10
10. Kavitha R, Kamalakannan P, Deepa T, Elamathi R, Sridhar S, Suresh JK. *In vitro* antimicrobial activity and phytochemical analysis of Indian medicinal plant *Couroupita guianensis* aubl. J Chem Pharm Res. 2011; 3(6):115–21.
11. Sivakumar T, Shankar T, Geetha G. Efficacy of *Couroupita guianensis* against selected human pathogens. Adv Biol Res (Rennes). 2012; 6(2):59–63.
12. Prabhu V, Ravi S. Quantification of quercetin and stigmasterol of *Couroupita guianensis* aubl by HPTLC method and in-vitro cytototoxic activity by MTT assay of the methanol extract against HeLa, NIH 3T3 and HepG2 cancer cell lines. Int J Pharm Pharm Sci. 2012; 4(4):126–30.
13. Baskar K, Ignacimuthu S, Jayakumar M. Toxic effects of *Couroupita guianensis* against Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae). Neotrop Entomol. 2015; 44(1):84–91. https://doi.org/10.1007/s13744-014-0260-7. PMid:26013016
14. Maheswaran R, Baskar K, Ignacimuthu S, Packiam SM, Rajapandiyan K. Bioactivity of *Couroupita guianensis* Aubl. against filarial and dengue vectors and non-target fish. South African J Bot. 2019; 125:46–53. https://doi.org/10.1016/j.sajb.2019.06.020
15. Otabe A, Ohta F, Takumi A, Lynch B. Mutagenicity and genotoxicity studies of aspartame. Regul Toxicol Pharmacol. 2019; 103:345–51. https://doi.org/10.1016/j.yrtph.2018.01.023. PMid:29408486
16. Han EH, Lim MK, Lee SH, Rahman MM, Lim YH. An oral toxicity test in rats and a genotoxicity study of extracts from the stems of *Opuntia ficus-indica* var. saboten. BMC Complement Altern Med. 2019; 19(1). https://doi.org/10.1186/s12906-019-2442-7. PMid:30691445. PMCid:PMC6350306

17. Reddeman RA, Glávits R, Endres JR, *et al.* A toxicological evaluation of *Germanium sesquioxide* (Organic Germanium). J Toxicol. 2020; 2020. https://doi.org/10.1155/2020/6275625. PMid:32322266. PMCid:PMC7160733

18. Dodda S, Alluri VK, Golakoti T, Sengupta K. Acute, subacute, and genotoxicity assessments of a proprietary blend of *Garcinia mangostana* fruit rind and *Cinnamomum tamala* leaf extracts (CinDura®). J Toxicol. 2020; 2020. https://doi.org/10.1155/2020/1435891. PMid:32802054. PMCid:PMC7414347

19. Dantas FG da S, Castilho PF de, Almeida-Apolonio AA de, Araújo RP de, Oliveira KMP de. Mutagenic potential of medicinal plants evaluated by the Ames Salmonella/microsome assay: A systematic review. Mutat Res - Rev Mutat Res. 2020;786. https://doi.org/10.1016/j.mrrev.2020.108338. PMid:33339578

20. Al-Anazi KM, Al-Mareed AA, Farah MA, Ali MA, Hailan WAQ, Al-Hemaid FM. Protective effect of *Capparis spinosa* Extract against potassium bromate induced oxidative stress and genotoxicity in mice. Evidence-based Complement Altern Med. 2021;2021. https://doi.org/10.1155/2021/8875238. PMid:33531925. PMCid:PMC7837764

21. Wang YH. Traditional uses, chemical constituents, pharmacological activities, and toxicological effects of *Dendrobium* leaves: A review. J Ethnopharmacol. 2021;270. https://doi.org/10.1016/j.jep.2021.113851. PMid:33485987

22. Gupta VH, Gunjal MA, Wankhede SS, Deshmukh VS. Neuropharmacological evaluation of the methanolic extract of *Couroupita guianensis* Aubl. Flower in Mice. Int J Pharm Pharmacol Res. 2012; 1(5):242–6.

23. Elumalai A, Bargavi K, Krishna S, And Eswaraiah MC. Evaluation of anti-oxidant and hepatoprotective activity of *Couroupita guianensis* Leaves. J Cell Tissue Res. 2013; 13(2):3745–8.

24. Organ Econ Co-operation Dev. OECD Test No. 471: Bacterial reverse mutation test[Internet]. 1997. https://doi.org/10.1787/9789264071247-en

25. De Flora S, Izzotti A, Randerath K, *et al.* DNA adducts and chronic degenerative diseases. Pathogenetic relevance and implications in preventive medicine. Mutat Res - Rev Genet Toxicol. 1996;366(3). https://doi.org/10.1016/S0165-1110(96)00043-7

26. Andreassi MG, Botto N, Colombo MG, Biagini A, Clerico A. Genetic instability and atherosclerosis: Can somatic mutations account for the development of cardiovascular diseases? Environ Mol Mutagen. 2000;35(4). https://doi.org/10.1002/1098-2280(2000)35:4<265::AID-EM1>3.0.CO;2-M

27. Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. 2003; 523–4. https://doi.org/10.1016/S0027-5107(02)00317-2

28. Ross CA, Margolis RL. Neurogenetics: Insights into degenerative diseases and approaches to schizophrenia. Clin Neurosci Res. 2005;5(1 SPEC. ISS.). https://doi.org/10.1016/j.cnr.2005.07.001

29. Test No. 471: Bacterial Reverse Mutation Test. OECD; 2020. https://doi.org/10.1787/9789264071247-en

30. Sánchez F, Radaeva TV, Nikonenko BV, *et al.* Multigenic control of disease severity after virulent Mycobacterium tuberculosis infection in mice. Infect Immun. 2003;71(1). https://doi.org/10.1128/IAI.71.1.126-131.2003. PMid:12496157. PMCid:PMC143141

31. Food. International Conference on Harmonisation. Guidance on S2(R1) genotoxicity testing and data interpretation for pharmaceuticals intended for human use; availability. Notice [Internet].2012.

32. Johara FT, Aovi FI. Pharmacological screening of *in vitro* activity of methanol extract of nagalinga flower. Pharmacologyonline. 2020;2.

33. George SA, Bhadran S, Sudhakar M, Harini BP. Comprehensive *in vitro* evaluation of pharmacological activities of selected mass spectrometry profiling of *Flacourtia jangomas* flower extract. Asian J Pharm Clin Res. 2017; 10(5):237–44. https://doi.org/10.22159/ajpcr.2017.v10i5.17419

34. Begum K, Shammi M, Hasan N, Asaduzzaman M, Appiah KS, Fujii Y. Potential allelopathic candidates for land use and possible sustainable weed management in South Asian ecosystem. Sustain. 2019;11(9). https://doi.org/10.3390/su11092649

35. Gupta VH, Wankhede SS, Gunjal MA, Juvekar AR. Antidepressant like effect of *Couroupita guianensis* Aubl. flowers in animal model of depression. Int J Toxicol Pharmacol Res. 2012; 4(2):12–16.

36. Pinheiro MMG, Fernandes SBO, Fingolo CE, Boylan F, Fernandes PD. Anti-inflammatory activity of ethanol extract and fractions from *Couroupita guianensis* Aublet leaves. J Ethnopharmacol. 2013; 146(1):324–30. https://doi.org/10.1016/j.jep.2012.12.053. PMid:23313951

37. Edziri H, Mastouri M, Mahjoub A, *et al.* Toxic and mutagenic properties of extracts from Tunisian traditional medicinal plants investigated by the neutral red uptake, VITOTOX and alkaline comet assays. South African J Bot. 2011; 77(3):703–10. https://doi.org/10.1016/j.sajb.2011.03.007

38. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Investig Suppl. 1968; 97:77–89.

39. Pradhan D, Panda PK, Tripathy G. Evaluation of the immunomodulatory activity of the methanolic extract of *Couroupita guianensis* aubl. Flowers in rats. Nat Prod Radiance. 2009; 8(1):37–42.

40. Moosavi M, Jalali A, Siahpoosh A, Farajzadeh-Shikh A, Kianipur F. Assessing mutagenicity of methanolic exteract of *Abrus precatorius* seeds using ames bioassay. J Med Sci. 2013; 13(2):118–23. https://doi.org/10.3923/jms.2013.118.123

41. Lee JS, Cho JH, Lee DS, Son CG. Genotoxicity evaluation of an ethanol extract mixture of *Astragali radix* and *Salviae miltiorrhizae Radix*. Evidence-based Complement Altern Med. 2018; 2018. https://doi.org/10.1155/2018/5684805. PMid:30402128. PMCid: PMC6198562

42. Sharififar F, Dehghan-Noudeh G, Moshafi M, *et al.* Antimutagenic activity of major fractions of *Zataria multiflora* Boiss by Ames method. Asian J Pharm. 2015; 9(3):195–9. https://doi.org/10.4103/0973-8398.160316

43. Abdul-Hafeez EY, Karamova NS, Ilinskaya ON. Evaluation of mutagenic and antimutagenic potential of stem bark aqueous extracts of eight trees by the bacterial reverse mutation assay. Ecol Genet. 2018; 16(3):55–61. https://doi.org/10.17816/ecogen16355-61

44. EFSA. Guidance for submission for food additive evaluations. EFSA J. 2012;10(7). https://doi.org/10.2903/j.efsa.2012.2760

45. Hardy A, Benford D, Halldorsson T, *et al.* Clarification of some aspects related to genotoxicity assessment. EFSA J. 2017; 15(12):5113. https://doi.org/10.2903/j.efsa.2017.5113. PMid:32625393