J. Pharm. Res. 2018;17(3):17–26

Print ISSN: 0973-7200
Online ISSN: 2454-8405

Journal of Pharmaceutical Research

ORIGINAL ARTICLE

Antigenotoxicity of Semecarpus Anacardium: In Vitro and In Vivo

Mohamed Sauf Anam K S[1], S V Rajendra[1,*], Raman Dang[2]

[1] *Department of Pharmacology, Krupanidhi College of Pharmacy, Carmelaram, Varthur Hobli, Bengaluru, Karnataka, India*
[2] *Principal, Krupanidhi College of Pharmacy, Carmelaram, Varthur Hobli, Bengaluru, Karnataka, India*

ARTICLE INFO

Article history:
Received 25.07.2018
Accepted 12.09.2018
Published 22.09.2018

* *Corresponding author.*
S V Rajendra

https://doi.org/10.18579/jopcr/v17.3.mohamed

ABSTRACT

Objectives: Genotoxicity is a word in genetics defined as the destructive effect of a cell's genetic material (DNA or RNA) on its integrity. Currently, most of the therapeutics prescribed for cancer treatment show genotoxicity owing to their natural properties (for example, Nitrosoureas, Cisplatin, 5-Fluorouracil, Ionizing radiation). The present study on the antigenotoxic potential of Semecarpus anacardium leaf extract in vitro and in vivo opens a greater window for the safety of anticancer drugs.

Methods: The study included the AMES test, micronucleus assay, chromosomal aberration test, Allium cepa assay, and in vitro antioxidant activity assay to elucidate the activity of Semecarpus anacardium leaf extract. Cyclophosphamide, a potent alkylating agent was used as a positive control to produce genotoxicity and Semecarpus anacardium leaf extract was assumed to protect it. The doses of Semecarpus anacardium leaf extract used were 50,100, 200 μg/mL for the Allium cepa assay and 50, 100, and 200 mg/kg for the micronuclei assay and chromosomal aberration test. Mutant strains of Salmonella typhimurium were used for the AMES test. Swiss albino mice were used for in vivo studies and divided into five groups of six animals each. Onion roots were used in the Allium cepa assay.

Findings: The aqueous extract of Semecarpus anacardium leaf showed antigenotoxic activity against CP-induced genotoxicity in mammalian bone marrow erythrocyte micronucleus assay (MNA), chromosomal aberration (CA), and Allium cepa assays in a dose-dependent manner. Similar to the AMES test, it was unable to produce any genotoxic effects. It showed maximum antigenotoxic activity when administered at a dose of 200 mg/kg in the micronucleus assay and chromosomal aberration test and 200 μg/mL in the Allium cepa assay. The leaf extract also had good antioxidant activity, with an IC50 value of 37.239 μg/mL for DPPH scavenging activity and an IC50 value of 34.191 μg/mL in superoxide scavenging activity.

Novelty: Semecarpus anacardium leaf extract possesses antigenotoxic and antioxidant activities and can be used as a combination therapy with potent and novel anticancer molecules.

Keywords: Semecarpus anacardium; In Vitro; In Vivo; AMES test

INTRODUCTION

Novel physical and chemical agents obtained from medicinal plants have a positive impact on human health; however, these agents may also have mutagenic and cytotoxic effects and can cause serious health problems[1]. Plant components mediate their effects by interacting with biological targets to provide a therapeutic effect and are one of the major sources for providing pure chemical agents to modern (allopathic) medicine[2,3].

Semecarpus anacardium (Anacardiaceae) is a deciduous tree distributed in the sub-Himalayan tract and hotter parts of India[4]. Commonly known as "Bhelwa", it is a deciduous tree that is up to ten meters tall. Different parts of this plant have been traditionally used to treat rheumatism, asthma, neuralgia, anthelmintic infections, cancer, and psoriasis[5]. Most studies have been performed on the nut and fruit parts of Semecarpus. anacardium L. (Bhallatak, nut shell) as the fruit was found to display hypocholesterolemic properties and prevent cholesterol-induced atheroma in rabbits with hypercholesterolaemia[6]. In vitro acetyl cholinesterase activity (AChE) of methanolic extracts of stem bark of Semecarpus. anacardium have been previously investigated[7]. The ethyl acetate extract showed in vivo anti-inflammatory activity against carrageenan-induced rat paw oedema[8].

KRUPANIDHI COLLEGE OF PHARMACY

The use of plants as prebiotic and complementary drugs for the treatment of diseases is increasing daily[9]. The World Health Organization (WHO) reported that 80% of the population in developing countries is dependent on folk medicine for their health and well-being[10]. The naturally occurring synergistic effects of many plant compounds make them more effective in treating diseases[11]. Despite their broad therapeutic potential and general approval, herbal remedies are still limited owing to the absence of a standard dosage regimen and comprehensive toxicity information[12]. Thus, screening therapeutic plants for their safety, quality, and potency is crucial. The Ames mutagenicity assay (Ames test), widely recognized as the most reliable in vitro test for genotoxicity determination, is the preferred method for this purpose. The present study was undertaken to evaluate the presence of phytochemicals, antioxidant potential, and antimutagenic activity in Semecarpus anacardium.

METHODOLOGY

Preparation of Semecarpus anacardium leaf extract and dose selection

Plant material was collected and authenticated by the GKVK Agricultural Institute, Hebbal, Bangalore. The leaves were then shade-dried for 14 days. On the day of extraction, leaves weighing 200 g were dried in a hot air oven and subsequently converted into a fine powder. The material was added to a round-bottom flask which was then filled with water. A reflux condenser was attached to its end and a reflux condensation process was initiated which was continued for five hours. After filtration through a muslin cloth, the material was collected in a beaker. It was concentrated in a china dish by evaporation, and thus, leaf extract was prepared by repeating the same process for five batches.

Phytochemical screening

The water extract of Semecarpus anacardium (WESA) was subjected to qualitative analysis to investigate the presence of various phytochemical constituents, such as polyphenols, alkaloids, glycosides, saponins, tannins, and flavonoids[13].

Tests for alkaloids

- **Dragendroff's test:** 1 mL of Dragendroff's reagent (potassium bismuth iodide solution) was added to 1 mL of the extract, formation of an orange-red precipitate indicated the presence of alkaloids.
- **Mayer's test:** 1 mL of Mayer's reagent (potassium mercuric iodide solution) was added to 1 mL of the extract and the formation of cream coloured precipitate confirmed the presence of alkaloids.
- **Hager's test:** 3 mL of Hager's reagent (saturated aqueous solution of picric acid) was added to 1 mL of the extract, and the presence of alkaloids was confirmed by the formation of a yellow precipitate.

Tests for flavonoids

- **Shinoda test:** The plant extract was treated with 5 mL of 95% ethanol, and a few drops of HCL and magnesium turnings (0.5 g) were added. The change in the colour of the solution to pink indicates the presence of flavonoids.
- **Ferric chloride test:** The plant extract was treated with ferric chloride solution; the appearance of a blackish-blue colour solution indicates the presence of flavonoids.

Tests for tannins

- **Lead acetate solution:** Lead acetate solution was treated with the plant extract; the yellow precipitate indicates the formation of tannins.
- **Ferric chloride solution:** The plant extract was treated with 5% $FeCl_3$ solution. The blue colour indicates the presence of tannins.

Test for proteins

- **Biuret test:** 3 mL of extract was added to 4% sodium hydroxide, and a few drops of 1% $CuSO_4$ solution were added. A pink or violet colour indicates the presence of a protein.
- **Millon's test:** 5 mL of Millon's reagent was added to 3 mL of extract, which led to white precipitates, indicating the presence of proteins.

Test for saponins (Froth Test)

To a small quantity of plant extract, 20 mL of distilled water was added and shaken in a graduated cylinder for 15 min lengthwise. Formation of foam indicated the presence of saponins.

Test for steroids, tri-steroids and cardiac glycosides

- **Salkowski's test:** The extract in chloroform was treated with a few drops of H_2SO_4, after which the moisture was shaken and allowed to stand for a few minutes. The formation of a red colour in the chloroform layer indicated the presence of steroids.
- **Liebermann- Burchard test:** The extract in chloroform was treated with 1 mL of acetic anhydride and a few drops of H_2SO_4. The reddish-violet colour formation at the junction indicates the presence of steroids.
- **Keller killani's test:** The extract was treated with glacial acetic acid and few drops of ferric chloride. To this mixture, 2 mL of a conc. H_2SO_4 was then added to the sides of the test tubes. A reddish-brown colour at the junction of the two layers and bluish-green colour in the upper layer indicate the presence of cardiac glycosides.

Test for Carbohydrates

- **Molisch test:** The extract was treated with α-naphthol in 95% ethanol. Add few drops of Conc. H_2SO_4 was then added along the sides of the test tubes. The formation of a violet ring at the junction indicated the presence of carbohydrates.
- **Fehling's test:** The plant extract was treated with equal volumes of 1 mL of Fehling A and 1 mL of Fehling B solution and boiled for one minute. The mixture was boiled for 5-10 minutes in a water bath. A reddish-brown colour was obtained due to the formation of cuprous oxide which indicated the presence of carbohydrates.
- **Barfoed's test:** The extract was treated with copper acetate in water and glacial acetic acid and heated in a water bath. The formation of a red precipitate indicates the presence of carbohydrates.

Antioxidant activity of Semecarpus anacardium

DPPH Radical scavenging activity [14]

The antioxidant activity of WETP was measured using the 2,2-diphenyl-2-picrylhydrazyl (DPPH) method. One millilitre of DPPH (0.01mM) was added to 3 mL of METP at various concentrations. The reaction mixture was then incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm against a blank. The free radical-scavenging activity of the plant extract was determined by comparison with that of the methanol control. A blank was prepared without the addition of the extract. Ascorbic acid at various concentrations (10, 20, 30, 40, and 50 μg/ml) was used as the standard. A lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. DPPH radical scavenging ability was calculated using the following equation:

$$DPPH\ Scavenged\ \% = \frac{A(Control) - A(Test)}{A(Control)} X\ 100$$

Super oxide radical- scavenging activity [15]

Measurement of superoxide anion scavenging activity of different extracts was performed based on the method described by Nishimiki [16] with slight modifications. About 1 mL of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solution of S. anacardium in water were mixed. The reaction was initiated by adding 100 μL phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples. The decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The % of inhibition was calculated by using following equation; % Inhibition= [(Absorbance control – Absorbance sample)/Absorbance control] x 100

In vitro genotoxicity assay

AMES test

The potential mutagenic effects of the plant extracts were investigated using a plate incorporation procedure [17]. The assay was performed using the Salmonella typhimurium strains TA1535 and TA1538. Approximately 100 μl of the bacterial stock was incubated in 20 mL of Oxoid Nutrient broth for 16 h at 37°C on an orbital shaker. The overnight culture (0.1 mL) was added to 2 mL of top agar (containing traces of biotin and histidine) together with a 0.1 mL test solution (the plant extract, solvent control, or positive control) and 0.5 mL phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured onto the surface of the agar plate and incubated for 48 h at 37°C. Following the incubation, the number of revertant colonies (mutants) was counted. All cultures were prepared in triplicate (except for the solvent control, where five replicates were performed) for each assay. This assay was repeated twice. The positive control (reference standard) used was sodium azide at a concentration of 5 μg/plate for the TA 1535 strain, and the positive control (Reference standard) used was nitrofluorine at a concentration of 5 μg/plate for the TA 1538 strain. Determination of mutagenic and antimutagenic potential of Semecarpus anacardium occurs via two different mechanisms [base pair and frame shift mutation] in Salmonella typhimurium strains.

Experimental groups

- Group 1: TA 1535 Control
- Group 2: TA 1535 Standard (Sodium azide 5 μg/plate)
- Group 3: TA 1535 Test (Semecarpus anacardium 10 mg/plate)
- Group 4: TA 1535 Standard (Sodium azide 5μg/plate) + Test (Semecarpus anacardium 10 mg/plate)
- Group 5: TA 1538 Control
- Group 6: TA 1538 Standard (Nitro fluorine 5 μg/plate)Group 7: TA 1538 Test (Semecarpus anacardium 10 mg/plate)
- Group 8: TA 1538 Standard (Nitro fluorine 5μg/plate) + Test (Semecarpus anacardium 10 mg/plate).

Allium cepa assay

The Allium cepa assay is an efficient test for chemical screening and in situ monitoring of the genotoxicity of environmental contaminants. The test organism is usually a common onion of the Stuttgarter variety, which is mainly having the size 15-22 mm and weight of 2-4 g. Allium cepa chromosomal aberration test was carried out according to the methods described by Ragunathan et al. and Ping et al. [16,18]. Cell proliferation [mitotic induction or depression] and chromosomal abnormalities in the meristematic root

cells of Allium cepa have been evaluated.

Experimental groups

- Group 1: Control
- Group 2: Cyclophosphamide Treated (40 μg/mL)
- Group 3: Cyclophosphamide (40 μg/mL) + 50 μg/mL of aqueous extract of SA
- Group 4: Cyclophosphamide (40 μg/mL) + 100 μg/mL of aqueous extract of SA
- Group 5: Cyclophosphamide (40 μg/mL) + 200 μg/ mL of aqueous extract of SA

Onions, as specified above, were kept for 4-5 days in different beakers filled with water for proper root growth. They were exposed to different concentrations (50,100,200 μg/mL) of Semecarpus anacardium in Petri dishes, and cyclophosphamide (40μg/mL) was used as the positive control. For detection of protective effect of Semecarpus anacardium, a few onions were exposed to cyclophosphamide in presence of the same concentration range of Semecarpus anacardium mentioned above at the same time. After 24 h (at least one mitotic cycle), chemically exposed roots of the different onions were collected in glass vials containing a fixative solution of methanol and glacial acetic acid (3:1) and stored overnight at 4°C.

The next day, roots were placed in a clear Petri dish containing a mixture of 2% aceto orcein and 1N HCl in a 5:1 ratio. Next, the Petri dish was heated gently for 2-5 sec in a yellow flame because it emits less heat and was kept for 1-1.5 h. Subsequently, a small drop of 45% acetic acid was placed on a clear microscopic glass slide, and the root tip (approximately 1-2 mm) was placed on the glass slide with acetic acid. It was then kept for a few seconds to wash off excess stain. A cover slip was also placed above it in such a way that no air bubbles were formed, and the root tips were squashed slightly with a gentle tap of the thumb or matchstick. Finally, the open interface of the coverslip and slide was sealed with paraffin or DPX so that the cells would not dry. Five slides were prepared and analysed for each treatment group to determine the mitotic index and various chromosomal aberrations.

In vivo genotoxicity assay

Six-week-old Swiss albino mice of either sex weighing 25–30 g were procured from Adita Biosys Private Limited, Tumkur. They were housed and acclimatized in a well-ventilated animal house at the Krupanidhi College of Pharmacy, Bangalore, India. Laboratory conditions were maintained for 10 days prior to the experiment at a controlled temperature (25 $\pm$ 4°C) and relative humidity (50-60) % with a 12h light and dark cycle with food and water ad libitum as per the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The Institutional Ethical Committee approved the experimental protocol (number 2016/PCOL/07/KCP/IAEC.41. Dose selection was performed according to previously published data on the toxicity of Semecarpus anacardium [19].

Induction of genotoxicity and evaluation of antigenotoxic activity by micronucleus test

The antigenotoxic activity of Semecarpus anacardium against cyclophosphamide-induced genotoxicity in mice bone marrow erythrocytes was evaluated.

Experimental groups

- Group 1: Control
- Group 2: Cyclophosphamide Treated (40 mg/kg i.p)
- Group 3: CP (40mg/kg i.p) + 50 mg/kg of aqueous extract of SA orally
- Group 4: CP (40mg/kg i.p) + 100 mg/kg of aqueous extract of SA orally
- Group 5: CP (40mg/kg i.p) +200 mg/kg of aqueous extract of SA orally

Micronucleus test procedure

Micronucleus assay was performed according to OECD guidelines (TG-474) [20]. Thirty animals were assigned to this assay, with six animals per group. One group of animals (Group 1) was used as a control and was administered distilled water. The second group (Group 2) was treated with cyclophosphamide (40 mg/kg body weight) i.p. for two consecutive days, and the last three groups (Group 3, Group 4, and Group 5) were given Semecarpus anacardium 50 mg/kg, 100 mg/kg, and 200 mg/kg body weight orally along with cyclophosphamide. Semecarpus anacardium leaf extract was dissolved in water and administered in divided doses before and 4 h after cyclophosphamide injection. After 48 h of treatment, the animals were sacrificed, and the femur bone was collected. The proximal ends of the bone were opened and the bone marrow was flushed into a centrifuge tube with chilled phosphate-buffered saline (pH-7.4).

The bone marrow suspension was centrifuged at 3000 rpm for 10 min. The supernatant was discarded, and the pellets were re-suspended with 0.2 mL of the same phosphate buffer saline. A drop of the cell suspension was placed on a clear slide and smeared. The smear was air-dried and fixed with methanol for 10 minutes. After fixation, slides were stained with Giemsa solution (1x) for 15 min and covered with dibutyl phthalate Polystyrene Xylene (DPX) to make the slides permanent. 1000 polychromatic erythrocytes (PCEs) were observed at 1000x magnification with oil immersion for each treatment group.

Chromosomal aberration test procedure [21–23]

Chromosomal aberration test was performed according to previously published methods [24]. The same grouping of animals (Group 1 to Group 5) and treatments were maintained in this method, as mentioned in section 2.5.1, with six animals per group. Colchicine (metaphase arresting agent) was administered to animals at 4 mg/kg, i. p., 3 h

before sacrifice. After that, the animals were sacrificed, and both femur bones were dissected, followed by opening of the proximal end of the bones with a small incision. The bone marrow was flushed with 1.5 mL of 2.2% freshly prepared chilled sodium citrate solution in a centrifuge tube and centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatant was removed and 1.5 mL of 0.075 M potassium chloride solution was added to the centrifuge tubes, and the cells were re-suspended and incubated for 30 min at 37°C. After incubation, the cell suspensions were centrifuged, and the supernatant was replaced with freshly prepared cold fixative methanol and glacial acetic acid (3:1) and kept for 10 min. The suspensions were then centrifuged for 10 min at the same rpm (3000 rpm). Afterwards, the supernatants were discarded by leaving one or two drops of fixative with cell pellets. Cells were smeared by dropping the cell-fixative suspension in clear glass slides and air drying for 10 min.

Air-dried slides were then stained with 1x Giemsa stain for 15 min and washed gently in water to remove excess stain. Finally, the slides were dried and mounted with DPX. At least five slides were made for each treatment group, and 100 well-separated metaphase chromosomes were analyzed for any type of aberration.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Dunnett's comparison test, and all results are presented as mean ± standard error of the mean (SEM). GraphPad Prism software version 5 was used for the statistical analysis.

RESULTS

Extraction

The percentage yield was found to be 3.24%.

Phytochemical screening

Preliminary phytochemical screening revealed the presence of various phytoconstituents in the extract, such as alkaloids, flavonoids, tannins, proteins, saponins, steroids, tri-steroids, cardiac glycosides, and carbohydrates.

In vitro antioxidant activity

WEKP at graded concentrations was tested for antioxidant activity using two different in vitro models. It was observed that the WEKP scavenged free radicals in a concentration-dependent manner in the models studied. WEKP shows antioxidant activity comparatively less than that of the standard drug ascorbic acid.

DPPH radical scavenging

The half-maximal inhibitory concentration, also known as the IC50 value, is denoted in μg/mL, and it is said that the lower the IC50 value, the greater the free radical scavenging by the antioxidant compound. The IC50 value of ascorbic acid was 7.283 μg/mL, and the IC50 value of leaf extract of Semecarpus anacardium leaf extract was 37.239 μg/mL. The DPPH-scavenging activity of the leaf extract of Semecarpus anacardium and the reference standard ascorbic acid are presented in Table 1.

Table 1: DPPH scavenging activity at different concentrations of leaf extract of Semecarpus anacardium and Ascorbic acid

Sr. No	Concentration of Ascorbic acids (μg/mL)	Scavenging effect of Ascorbic acid (%)	Concentration of Semecarpus anacardium (μg/ mL)	Scavenging effect of Semecarpus anacardium (%)
1	3	38.82±2.82	25	35.53 ± 2.04
2	6	46.41±1.30	50	42.60 ± 1.61
3	9	53.31±2.83	100	64.63 ± 1.05
4	12	61.62±1.21	200	68.29 ± 2.42
5	15	74.38±1.98	400	71.95 ± 1.17
6	18	83.11±1.02	800	79.26 ± 2.13

Super oxide radical- scavenging activity

The half-maximal inhibitory concentration, also known as the IC50 value, is denoted in μg/mL, and it is said that the lower the IC50 value, the greater the free radical scavenging by the antioxidant compound. The IC50 value of ascorbic acid was 11.432 μg/mL and IC50 Value of Semecarpus anacardium leaf extract was 34.191 μg/mL (Table 2).

Table 2: Superoxide scavenging activity at different (Standard)

Sr. No.	Concentration of Ascorbic acid (μg/mL)	Scavenging effect of Ascorbic acid (%)	Concentration of Semecarpus anacardium (μg/ mL)	Scavenging effect of Semecarpus anacardium (%)
1	3	18.82±2.22	25	37.83 ± 2.44
2	6	27.62±1.36	50	48.96 ± 1.03
3	9	39.41±2.01	100	56.63 ± 1.47
4	12	48.58±1.51	200	63.19 ± 2.04
5	15	69.58±2.07	400	68.95 ± 2.17
6	18	78.14±1.21	800	70.26 ± 1.08

In vitro genotoxicity assay

AMES test

The AMES test was carried out to evaluate the genotoxic and antigenotoxic potential of Semecarpus anacardium leaf extract. The assay was performed without metabolic activation which was carried out by addition of the S9 mix, and the number of reverted colonies was counted. Based on these results, it can be concluded that Semecarpus anacardium leaf extract at a dose of 10 mg/plate is non-mutagenic. It was also noted that in the presence of

mutagens, the number of revertant (histidine +) colonies of Salmonella was not reduced, suggesting that the Semecarpus anacardium leaf extract does not have any antimutagenic properties. The TA 1535 strain was used for checking the base pair mutation, and the TA 1538 strain was used for observing frameshift mutations. The results are presented in Table 3 and Figure 1.

Table 3: Results for AMES test for different treatment groups

Groups	Treatment	Number of colonies
1	TA 1535 Control	29 ± 2.673
2	TA 1535 Standard	787 ± 15.668
3	TA 1535 Test	27 ± 4.532
4	TA 1535 Test + Standard	721 ± 14. 247
5	TA 1538 Control	24 ± 3.853
6	TA 1538 Standard	592 ± 21.744
7	TA 1538 Test	21 ± 4.724
8	TA 1538 Test + Standard	523 ± 25.125

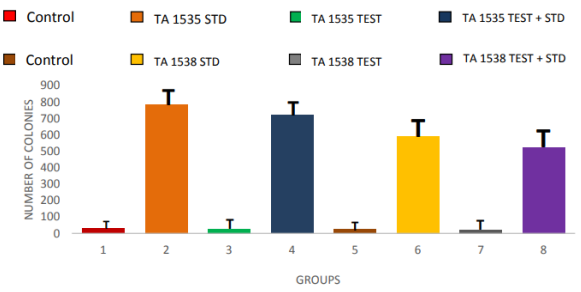

Fig. 1: Graphical representation of AMES test for different groups

Allium cepa assay

Using this assay, the mitotic depressant and genotoxic/antigenotoxic activity of Semecarpus anacardium leaf extract were evaluated. The mitotic index (M.I) was calculated using the following formula: Mitotic index (M. I) = [(number of dividing cells/total number of cells) × 100].

The leaf extract of Semecarpus anacardium considerably reduced the number of dividing cells in a dose-dependent manner. There was a significant reduction in the mitotic index from 2.6 ± 0.830 to 2.2 ± 0.372 when the dose of leaf extract was increased from 50 μg/mL to 200 μg/mL showing that it has mitotic depressant activity in a dose dependent manner (Table 4 and Figure 2).

Allium cepa cells were subjected to chromosomal analysis to study the effect of Semecarpus anacardium leaf extract on cyclophosphamide-induced aberrations. A significant reduction in the chromosomal aberration percentage was visible as they were reduced from 1.3 ± 3.56 to 0.9 ± 2.53, 0.5 ± 2.67 and 0.3 ± 2.15 in Group 3, Group 4, and Group 5 in a dose depended manner (Table 5 and Figure 3).

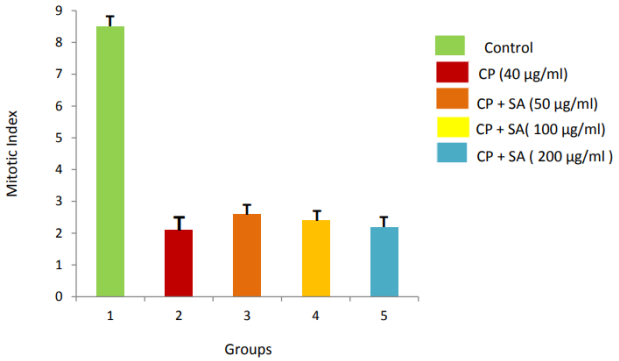

Fig. 2: Graphical representation of mitotic index

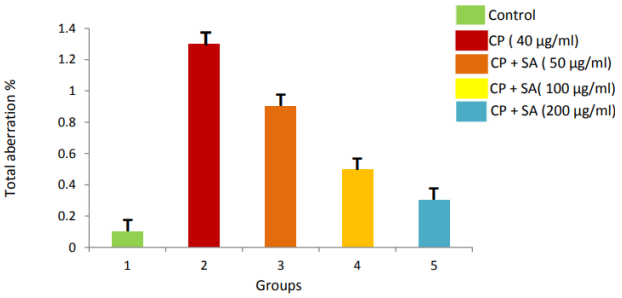

Fig. 3: Graphical representation

In vivo genotoxicity assay

Micronucleus Assay

Micronucleus assay was performed and the number of micro nucleated polychromatic erythrocytes (MNPCEs) were counted for the determination of antigenotoxic (protective) effect of Semecarpus anacardium leaf extract on mice bone marrow erythrocytes. The leaf extract significantly reduced the number of MNPCEs ($P < 0.05$) in a dose-dependent manner when administered simultaneously with cyclophosphamide. The MNPCE count in the positive control cyclophosphamide (40 mg/kg) group was 29 ± 1.623. However, when 50, 100, and 200 mg/kg Semecarpus anacardium leaf extract was administered along with cyclophosphamide in Gr-3, 4, and 5, a significant reduction in count to 16.66 ± 1.441, 9.33 ± 1.247, and 5 ± 0.816, respectively, was observed in a dose-dependent manner. The maximum percentage reduction in the micronucleus polychromatic erythrocyte cell count was observed in Group 5 (82.75%) (Table 6 and Figure 4).

Chromosomal aberration (CA) test

The same treatment plan was followed in the micronucleus assay. Metaphase arrested cells of about hundred in

Table 4: Mitotic index in different treatment groups

Groups	Treatment	Total cells	Dividing cells				Non dividing cells	Mitotic index (MI)
			P	M	A	T	INT	
1	Distilled Water	5000	13 5	12 4	76	90	4575	8.5 ± 1.432
2	CP 40 μg/mL	5000	30	28	21	26	4895	2.1 ± 1.814
3	CP + WESA 50 μg/mL	5000	44	32	25	29	4870	2.6 ± 0.830
4	CP + WESA 100 μg/mL	5000	43	33	20	24	4880	2.4 ± 1.471
5	CP + WESA 200 μg/mL	5000	36	30	19	25	4890	2.2 ± 0.372

Table 5: Chromosomal aberrations in Allium cepa Assay

Groups	Treatment	Chromosomal aberrations	Chromosomal aberration %
1	Control	5	0.1 ± 2.42
2	Cyclophosphamide treated (40 mg/kg i.p)	65	1.3 ± 3.56
3	Cyclophosphamide (40 mg/kg i.p) + 50 mg/kg of Aqueous extract of Semecarpus anacardium orally	45	0.9 ± 2.53
4	Cyclophosphamide (40 mg/kg i.p) + 100 mg/kg of Aqueous extract of Semecarpus anacardium orally	25	0.5 ± 2.67
5	Cyclophosphamide (40 mg/kg i.p) + 200 mg/kg of Aqueous extract of Semecarpus anacardium. orally	15	0.3 ± 2.15

Table 6: Micronuclei frequencyin different treatment groups frequency in different treatment groups

Groups	Treatment	MNPCE's	% Reduction
1	Control	1.33 ± 0.272	-
2	Cyclophosphamide treated (40 mg/kg i.p)	29 ± 1.623a	-
3	Cyclophosphamide (40 mg/kg i.p) + 50 mg/kg of Aqueous extract of Semecarpus anacardium orally	16.66 ± 1.441b	42.55
4	Cyclophosphamide (40 mg/kg i.p) + 100 mg/kg of Aqueous extract of Semecarpus anacardium orally	9.33 ± 1.24 c	67.82
5	Cyclophosphamide (40 mg/kg i.p) + 200 mg/kg of Aqueous extract of Semecarpus anacardium. orally	5 ± 0.816d	82.75

All values are represented as the mean ± SEM, n = 5. Statistics: a P< 0.05 when compared to normal control and b, c & d P< 0.05 when compared with the standard group and analyzed with One-way ANOVA followed by Dunnett's comparison test.

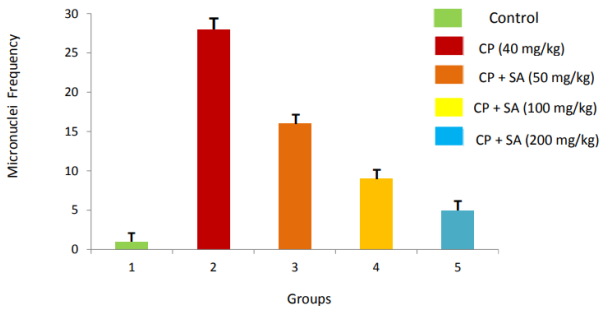

Fig. 4: Graphical representation of Micronucleus assay

number were observed in each group, for chromosomal abnormalities and the observations were made. The aqueous extract of Semecarpus anacardium reduced the number of chromosomal abnormalities induced by cyclophosphamide in mouse bone marrow cells. The total aberration % was calculated using the following formula:

Total aberration % = (Total no of aberrations / 500) × 100

The total aberration % was found to be 25.69 ± 0.32% in cyclophosphamide-treated group 2, which was reduced significantly to 16.33 ± 1.57, 9.23 ± 1.64%, and 6.12 ± 1.09 in groups 3, 4, and 5, respectively, in a dose-dependent manner when the leaf extract was administered along with cyclophosphamide (Table 7 and Figure 5).

Table 7: Chromosomal Aberration in different treatment groups

Groups	Treatment	Total aberration %	% Protection
1	Control	2.33 ± 0.48	-
2	Cyclophosphamide treated (40 mg/kg i.p)	25.69 ± 0.32 a	-
3	Cyclophosphamide (40 mg/kg i.p) + 50 mg/kg of Aqueous extract of Semecarpus anacardium orally	16.33 ± 1.57b	40
4	Cyclophosphamide (40 mg/kg i.p) + 100 mg/kg of Aqueous extract of Semecarpus anacardium orally	9.23 ± 1.64 c	64
5	Cyclophosphamide (40 mg/kg i.p) + 200 mg/kg of Aqueous extract of Semecarpus anacardium. orally	6.12 ± 1.09 d	76

All values are represented as the mean ± SEM, n = 5. Statistics: a P< 0.05 when compared to normal control and b, c & d at P< 0.05 when compared with the standard group and analyzed with One-way ANOVA followed by Dunnett's comparison test.

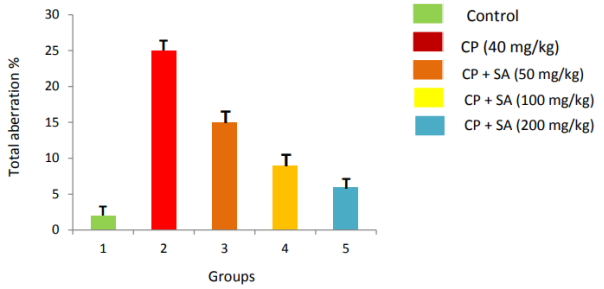

Fig. 5: Graphical representation of total aberration percentage

DISCUSSION

The antigenotoxic activity of Semecarpus anacardium was evaluated using AMES test, micronucleus assay, chromosomal aberration test and Allium cepa assay. In vitro antioxidant activity studies were also carried out to elucidate the mechanism by which Semecarpus anacardium displays antigenotoxic action.

In the Salmonella (AMES) test, sodium azide and nitro fluorine (5 μg/plate) were able to cause gene mutations in mutant strains of Salmonella typhimurium TA 1535 and TA 1538, respectively, which was confirmed by the colony count of standard groups. The number of colonies in the Semecarpus anacardium leaf extract (10 mg/plate)-treated groups was very low (control group) for both strains. From this study, it is clear that the leaf extract did not possess any genotoxic activity. When the leaf extract was administered along with mutagens, such as sodium azide and terfluorene, there was no significant decrease in the number of revertant colonies. It was also found that the leaf extract was unable to resist sodium azide-induced base pair mutations in Salmonella TA 1535, and nitro fluorine induced a frameshift mutation in strain TA 1538. Hence, it acts as both a non-mutagenic and non-antimutagenic[25].

From the micronucleus assay, it was clear that cyclophosphamide administered at a dose of 40 mg/kg for two consecutive days caused a significant (P<0.05) increase in micronuclei frequency in mice bone marrow erythrocytes[25]. In cyclophosphamide-treated animals, there were significant changes in NCE and PCE counts. However, when Semecarpus anacardium at doses of 50, 100, and 200 mg/kg was administered along with cyclophosphamide, it was found that SA showed potential antigenotoxic activity and was effective in reducing the MNPCE count in a significant manner. Semecarpus anacardium significantly reduced (P<0.05) the count of micronucleated polychromatic erythrocytes generated in the mice bone marrow erythrocytes in Groups 3, 4, and 5. From the results obtained, it is clear that Semecarpus anacardium acts against cyclophosphamide-induced genotoxicity in Swiss albino mice. Micronuclei are well-known biomarkers of DNA strand breaks[26]. It was concluded from the study that the leaf extract of Semecarpus anacardium has antioxidant activity and helps reduce cyclophosphamide-induced oxidative stress. It also alters membrane permeability and exhibits antigenotoxic activity[27]. The number of micronuclei was found to be higher after cyclophosphamide treatment and significantly decreased after leaf extract treatment which was administered along with cyclophosphamide at the same concentration. Hence, Semecarpus anacardium possesses antigenotoxic activity.

Chromosomal analysis was performed to provide further evidence of antigenotoxic activity. From the chromosomal aberration test, it was found that Semecarpus anacardium leaf extract acted against cyclophosphamide-induced chromosomal abnormalities. Based on the theory of formation of micronuclei and CA, it can be concluded that DNA damage in the S phase causes formation of CA in the M phase, and the unrepaired CA is responsible for the formation of micronuclei. This means it can be hypothetically concluded that Semecarpus anacardium leaf extract modulates the M-G0 cell cycle check point by which it repairs the CA and inhibit the transfer of damaged genomic components into daughter cells[28].

From further investigation using Allium cepa assay, we can conclude that Semecarpus anacardium leaf extract has mitotic depressant activity in onion root meristematic cells.

This means that it causes a significant decrease in the mitotic index in a dose-dependent manner when compared to the control groups. Cyclophosphamide was given at 40 μg/mL and it caused maximum number of chromosomal aberrations. However, when Semecarpus anacardium leaf extract was administered along with CP, a significant reduction in chromosomal abnormalities was observed in a dose-dependent manner. These aberrations are mainly due to spindle poisoning, spindle inhibition, and inhibition of tubulin synthesis via many pathways [28].

Furthermore, the DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of Semecarpus anacardium leaf extract was evaluated, and the results showed that the antioxidant activity was increased by increasing the concentration of Semecarpus anacardium leaf extract. The antioxidant activities of Semecarpus anacardium leaf extract and ascorbic acid were investigated by analyzing their superoxide radical-scavenging activity. Semecarpus anacardium leaf extract showed significant antioxidant activity as compared to the standard ascorbic acid.

The leaf extract of Semecarpus anacardium is not genotoxic but has antigenotoxic activity, as it can reduce the number of micronuclei, chromosomal abnormalities, and spindle formation in different test models in a dose-dependent manner. This is because of the presence of antioxidant compounds, such as flavonoids and polyphenols, which are found in Semecarpus anacardium leaves.

CONCLUSION

This study demonstrated that the leaf extract of Semecarpus anacardium possesses potent antigenotoxic activity due to the presence of antioxidant compounds. It also possessed mitotic depressant activity in the Allium cepa assay. Further estimation of the different molecular markers of various pathways associated with genotoxicity and cytotoxicity will prove the exact mechanism of action of the leaf extract as an antigenotoxic compound.

REFERENCES

1. Aremu AO, Cheesman L, Finnie JF, Van Staden J. Mondia whitei (Apocynaceae): A review of its biological activities, conservation strategies and economic potential. *South African Journal of Botany*. 2011;77(4):960–971. Available from: https://dx.doi.org/10.1016/j.sajb.2011.06.010.
2. Aggarwal BB, Ichikawa H, Garodia P, Weerasinghe P, Sethi G, Bhatt ID, et al. From traditional Ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. *Expert Opinion on Therapeutic Targets*. 2006;10(1):87–118. Available from: https://dx.doi.org/10.1517/14728222.10.1.87.
3. Salazar-Gómez A, Ontiveros-Rodríguez JC, Pablo-Pérez SS, Vargas-Díaz ME, Garduño-Siciliano L. The potential role of sesquiterpene lactones isolated from medicinal plants in the treatment of the metabolic syndrome – A review. *South African Journal of Botany*. 2020;135:240–251. Available from: https://dx.doi.org/10.1016/j.sajb.2020.08.020.
4. Kirtikar KR, Basu BD. Indian Medicinal Plants;vol. 3. 2nd ed. Singh BSMP, editor. 1975.
5. Chadha YR. Raw Materials Series. New Delhi. Publications & Information Directorate, CSIR. 1989. Available from: https://niscpr.res.in/knowledgeproducts/wealthofindia.
6. Sharma A, Mathur R, Dixit VP. Hypocholesterolemic activity of nut shell extract of Semecarpus anacardium (Bhilawa) in cholesterol fed rabbits. *Indian journal of experimental biology*. 1995;33(6):444–452. Available from: https://europepmc.org/article/med/7590951.
7. Vinutha B, Prashanth D, Salma K, Sreeja SL, Pratiti D, Padmaja R, et al. Screening of selected Indian medicinal plants for acetylcholinesterase inhibitory activity. *Journal of Ethnopharmacology*. 2007;109(2):359–363. Available from: https://dx.doi.org/10.1016/j.jep.2006.06.014.
8. Selvam C, Jachak SM, Bhutani KK. Cyclooxygenase inhibitory flavonoids from the stem bark of Semecarpus anacardium Linn. *Phytotherapy Research*. 2004;18(7):582–584. Available from: https://dx.doi.org/10.1002/ptr.1492.
9. Akkol EK, Karpuz B, Sobarzo-Sánchez E, Khan H. A phytopharmacological overview of medicinal plants used for prophylactic and treatment of colitis. *Food and Chemical Toxicology*. 2020;144:111628–111628. Available from: https://dx.doi.org/10.1016/j.fct.2020.111628.
10. Grujičić D, Marković A, Vukajlović JT, Stanković M, Jakovljević MR, Ćirić A, et al. Genotoxic and cytotoxic properties of two medical plants (Teucrium arduini L.and Teucrium flavum L.) in relation to their polyphenolic contents. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2020;852:503168–503168. Available from: https://dx.doi.org/10.1016/j.mrgentox.2020.503168.
11. Khan I, Ahmad B, Azam S, Hassan F, Aziz A, Rehman N, et al. Pharmacological activities of Justicia adhatoda. *Pakistan Journal of Pharmaceutical Sciences*. 2018;31(2). Available from: https://pubmed.ncbi.nlm.nih.gov/29618423/.
12. Raina P, Chandrasekaran CV, Deepak M, Agarwal A, Ruchika KG. Evaluation of subacute toxicity of methanolic/aqueous preparation of aerial parts of O. sanctum in Wistar rats: Clinical, haematological, biochemical and histopathological studies. *Journal of Ethnopharmacology*. 2015;175:509–517. Available from: https://dx.doi.org/10.1016/j.jep.2015.10.015.
13. Godard T, Fessard V, Huet S, Mourot A, Deslandes E, Pottier D, et al. Comparative in vitro and in vivo assessment of genotoxic effects of etoposide and chlorothalonil by the comet assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 1999;444(1):103–116. Available from: https://dx.doi.org/10.1016/s1383-5718(99)00100-x.
14. Arulpriya P, Lalitha P, Hemalatha S. Antimicrobial testing of the extracts of Samanea saman (Jacq.) Merr. *Der Pharma Chemica*. 2010;2(6):73–83. Available from: https://www.derpharmachemica.com/pharma-chemica/antimicrobial-testing-of-the-extracts-of-samanea-saman-jacqmerr.pdf.
15. Vasavi M, Vedicherala B, Vattam KK, Ahuja YR, Hasan Q. Assessment of Genetic Damage in Inflammatory, Precancerous, and Cancerous Pathologies of the Esophagus Using the Comet Assay. *Genetic Testing and Molecular Biomarkers*. 2010;14(4):477–482. Available from: https://dx.doi.org/10.1089/gtmb.2010.0006.
16. Evans HJ. Cytological Methods for Detecting Chemical Mutagens. In: and others, editor. Chemical Mutagens: Principles and Methods for Their Detection;vol. 4. Bonston. Springer. 1976;p. 1–29. Available from: https://link.springer.com/chapter/10.1007/978-1-4684-0892-8_1.
17. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*. 1983;113:173–215. Available from: https://dx.doi.org/10.1016/0165-1161(83)90010-9.
18. Newcomb TG, Loeb LA. Oxidative DNA Damage and Mutagenesis. In: Nickoloff JA, Hoekstra MF, editors. DNA Damage and Repair. Humana Press. 1998;p. 65–84. Available from: https://link.springer.com/book/10.1007/978-1-59259-455-9.
19. Patwardhan B, Saraf MN, David SB. Toxicity of Semecarpus anacardium extract. *Ancient science of life*. 1988;8:106–115. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3331355/.
20. S2B Genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals. 1998. Available from: https://www.fda.gov/media/

71971/download.
21. Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. *Proceedings of the National Academy of Sciences*. 1973;70(8):2281–2285. Available from: https://dx.doi.org/10.1073/pnas.70.8.2281.
22. Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 2000;455:29–60. Available from: https://dx.doi.org/10.1016/s0027-5107(00)00064-6.
23. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*. 2011;26(1):125–132. Available from: https://dx.doi.org/10.1093/mutage/geq052.
24. Obe G, Pfeiffer P, Savage JRK, Johannes C, Goedecke W, Jeppesen P, et al. Chromosomal aberrations: formation, identification and distribution. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 2002;504:17–36. Available from: https://dx.doi.org/10.1016/s0027-5107(02)00076-3.
25. Fotakis G, Timbrell JA. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*. 2006;160(2):171–177. Available from: https://dx.doi.org/10.1016/j.toxlet.2005.07.001.
26. Morita T, Nagaki T, Fukuda I, Okumura K. Clastogenicity of low pH to various cultured mammalian cells. Mutation Research/Fundamental and Molecular Mechanisms of. *Mutagenesis*. 1992;268(2):297–305. Available from: https://pubmed.ncbi.nlm.nih.gov/1379335/.
27. Scott D, Galloway SM, Marshall RR, Ishidate M, Brusick D, Ashby J, et al. Genotoxicity under extreme culture conditions. *Mutation Research/Reviews in Genetic Toxicology*. 1991;257(2):147–205. Available from: https://dx.doi.org/10.1016/0165-1110(91)90024-p.
28. Sivasankari P, Kaur N, Reddy S, Vivekanandam KS, S. Micronucleus index: An early diagnosis in oral carcinoma. *Journal of the Anatomical Society of India*. 2008;57:8–13. Available from: https://www.researchgate.net/publication/281612865_Micronucleus_Index_An_Early_Diagnosis_In_Oral_Carcinoma.