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RESEARCH ARTICLE

Antigenotoxicity of Tuberous Rhizomes of *Kaempferia rotunda* Linn. in Cyclophosphamide Induced Genotoxicity: *In vitro* and *In vivo* Study

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Abstract

Objectives: To investigate the potential of a methanolic extract of *Kaempferia rotunda* Linn. rhizomes to mitigate the genotoxicity caused by cyclophosphamide. **Methods**: In this study, swiss albino mice of either sex were given a low dose of 200 mg/kg and a high dosage of 400 mg/kg of Kaempferia rotunda methanolic extract, followed by cyclophosphamide as a challenge, to test for antigenotoxic action. *In vivo* chromosomal aberration and micronuclei assays were conducted. Hydroxyl scavenging activity was conducted *in vitro*. Mention the statistical tool used. **Findings**: A significant decrease in PCE/NCE ratio was seen at doses of 200 mg/kg (1.763±0.445) and 400 mg/kg (1.241±0.871) of *Kaempferia rotunda* Linn. A significant reduction (18±2.54) in the chromosomal aberrations was found at 400 mg/kg of *Kaempferia rotunda* Linn. DPPH free radical scavenging activity of *K. rotunda* methanolic extract, demonstrated an increased IC50 value of 24.09μg/ml, in comparison to standard Ascorbic acid with IC50 of 8.17 μg/ml. The methanolic extract of *K. rotunda* exhibited a significant hydroxyl radical scavenging activity with an IC_{50} value of 10.22μg/ml when compared to standard Gallic acid with IC_{50} of 10.22μg/ml. **Novelty**: New medicines are being developed for the treatment of complicated diseases but these medicines are themselves associated with a number of side effects that range from minor to severe intensity. The medicines from nature on the other hand appear to be more effective than the synthetic counterpart. Our investigation confirms the genoprotective action of *Kaempferia rotunda* Linn.

Keywords: Genotoxicity; Chromosomal Aberration; Methanolic extract; Free radicals; Kaempferia rotunda linn

1 Introduction

Testing for potential genotoxic properties is vital to handle safety hazards or to provide a risk evaluation for a novel chemical or product. *Kaempferia rotunda* Linn. has several medical applications in both allopathic as well as ayurvedic medicine. The rhizomes of K. galanga are utilized as an antipyretic, expectorant, anabolic, diuretic, anti-tussive,

and carminative, as well as for the curing of asthma, rheumatism, gastrointestinal ailments, wounds, epilepsy, and skin disorders [1]. Extracts and purified compounds from select *Kaempferia* species are used for the treatment of knee osteoarthritis and the inhibition of a breast cancer resistance protein (BCRP), anti-inflammatory, anti-acne, anticholinesterase, anti-obesity-induced dermatopathy, wound healing, anti-drug resistant strains of *Mycobacterium tuberculosis*, neuroprotective, anti-nociceptive, human immunodeficiency virus type-1 (HIV-1) inhibitory activity, *in vitro* anti-allergenic, and larvicidal activity against *Aedes aegypti* [1].

It is well-known that oxidative stress due to the overproduction of reactive oxidative species (ROS) contributes significantly to the onset and progression of diabetes, cancer, cardiovascular, and neurological disorders. Bioactive chemicals found in medicinal plants have the ability to prevent or repair ROS-induced damage, which is known to cause cancer in its early stages [2]. Consequently, it's critical to prioritize studies on plants with antigenotoxic as well as free-radical scavenging characteristics. This study aimed to evaluate the antigenotoxicity of *Kaempferia rotunda* Linn. *in vitro* and *in vivo*.

2 Methodology

2.1 Preparation of *Kaempferia rotunda* rhizomes extract and dose selection

The plants were collected from Bermiok (Martam) Sikkim, India, identified and authenticated at Central Ayurveda Research Institute, Bengaluru with the authentication letter number- RRCBI-427. The rhizomes of *Kaempferia rotunda* were washed with distilled water and allowed to air dry at the ambient temperature (25°C) for five days. The methanolic extract of *Kaempferia rotunda* was done by soxchelt extraction. Dose selection was done according to the previous literature available [3].

2.2 Experimental animals

Swiss albino mice, six weeks old of both sexes weighing 30-35g were obtained from Krupanidhi College of Pharmacy, Bengaluru, India. This study was approved by the Institutional Ethical Committee with approval No. KCP/IAEC/PCOL/106/2022. They were housed and acclimatized in a well-ventilated animal house. In accordance with the guidelines of the Committee for the Control and Supervision on Experiments on Animals (CCSEA), laboratory conditions were maintained for 10 days prior to the experiment, with food and water available at all times, a 12-hour light and dark cycle, and controlled temperature (24±4°C and relative humidity of 50–60%) [Table 1].

2.3 Experimental methods: *In vivo* models

2.3.1 Micronucleus assay

OECD criteria (TG-474) were followed in performing the micronucleus test. 24 animals were assigned for this assay containing 6 animals per group. Distilled water was administered to one group of animals (Group 1), which was treated as Normal. Groups 3 and 4 received the extract of *Kaempferia rotunda* (200 and 400mg/kg), which was dissolved in water and administered orally along with cyclophosphamide. Group 2 received treatment with cyclophosphamide (40mg/kg body weight) i.p. for two consecutive days.

After 48h of treatment animals were sacrificed and femur bone was collected. Bone marrow cells were removed from the bone by opening its proximal ends and flushing them with cooled phosphate buffer saline (pH-7.4) in a centrifuge tube. Following a 10-minute centrifugation at 3000 rpm for the bone marrow suspension, the pellets were reconstituted using 0.2 ml of the same phosphate buffer saline. On a transparent slide, a drop of suspension of cells was added, creating a smear. The smear was air dried and fixed with methanol for 10min after fixation slides were stained with Giemsa solution (1x) for 15min. Micronucleus assay was performed according to the above-mentioned procedure.1000 polychromatic erythrocytes (PCEs) were observed in 100x magnification with oil immersion for each treatment group.

2.3.2 Bone marrow Chromosomal Aberration (CA test)

Colchicine (metaphase arresting agent) was given to animals at about 4mg/kg i.p 3 hours before sacrifice. After that, animals were sacrificed and both femur bones were dissected which was followed by the opening of the proximal end of the bones with a small incision [4]. After that, 1.5 ml of cold sodium citrate solution (2.2 %) was used to remove the bone marrow into a centrifuge tube, and centrifuged for 10 minutes at 3000 rpm. Following centrifugation, the supernatant was discarded, and 1.5 ml of a potassium chloride solution with a concentration of 0.075 M was added to the centrifuge tubes. The cells were then reconstituted and incubated for 30 minutes at 37°C. Following incubation, cell suspensions were centrifuged, and the supernatant was replaced with a newly made cold fixative, a 3:1 ratio of glacial acetic acid to methanol, which was then

maintained for ten minutes. Subsequently, the suspensions were centrifuged at the same speed (3000 rpm) for 10 minutes. Following that, supernatants were disposed of by placing a drop or two of fixative beside the cell pellets. Cells were smear-made by placing the cell-fixative solution onto transparent glass slides and letting them air dry for ten minutes. After allowing the slides to air dry, 1x Giemsa stain was applied, and the excess stain was carefully removed by gently washing them in water and later the slides were dried. At least five slides were made for each treatment group and 100 well-separated metaphase chromosomes were analyzed for aberrations[5].

2.4 Experimental methods: *In vitro* models

2.4.1 In vitro Allium cepa assay

Cell proliferation and chromosomal abnormalities in meristematic root cells of *Allium cepa* have to be evaluated in the *Allium cepa* assay for proper root growth. They were exposed to different concentrations of *Kaempferia rotunda* Linn. (200,400μg/ml) of in petri-dish and cyclophosphamide (40μg/ml) was considered a positive control. For the detection of the protective effect of *Kaempferia rotunda* Linn., a few samples of onions were simultaneously exposed in the same concentration range of *Kaempferia rotunda* Linn. mentioned above at the same time. The roots of several onions were collected in glass vials containing a fixative solution of methanol and glacial acetic acid (3:1) after 24 hours (at least one mitotic cycle) of chemical exposure. The vials were then refrigerated overnight at 40°C. The following day, roots were transferred onto a petri dish that was filled with a 5:1 combination of 1N HCl and 2% acetoorcein. The petri dish was then held for one-to-one and a half hours and heated gradually for two to five seconds over a yellow flame, as it emits less heat. Next, the root tip (about 1-2 mm) was placed on a transparent microscopic glass slide that had a little drop of 45% acetic acid on it. To determine the mitotic index and other chromosomal abnormalities, five slides were prepared and examined for every treatment group[6].

2.4.2 2,2-diphenylpicrylhydrazyl (DPPH) radical-scavenging activity

The *Kaempferia rotunda* Linn extract at 10, 20, 40, 60, and 80μg/ml concentration and ascorbic acid (standard) was tested for DPPH radical scavenging activity. A sonicator was employed to disintegrate the material evenly. 6 ml of 0.004% DPPH solution was pipetted into each test tube after the required concentrations were achieved. The test tubes were left in the dark for 30 minutes while the ambient temperature was measured. Simultaneously, DPPH was administered to the test tubes labelled as blanks, which contained only ethanol. Each test tube's absorbance was measured at 517 nm using a UV spectrophotometer after 30 minutes[7]. The percentage of inhibition was obtained using the following formula:

% inhibition = [(Blank absorbance- Sample absorbance)/ Blank absorbance] X 100

Ac= absorbance of control

As = absorbance of sample

2.4.3 Hydroxyl radical scavenging assay

The test was carried out with 2.8 mM H_2O_2, 40 mM $FeCl_3$, 100 mM EDTA, and various dilutions of the chemicals in a 2.5 mM phosphate buffer (PBS) solution with a pH of 7.4 were included in this 1.0ml reaction volume. Addition of 0.1 mM ascorbic acid and 90 minutes of incubation at 37°C initiate the reaction at the beginning. Following incubation, it was heated for eight minutes at 100°C and then 1mL of 2.5% TCA (trichloroacetic acid) and 1mL of TBA (thiobarbituric acid) (0.7% in 0.05 N KOH) was added[8]. After cooling down, the mixture was measured at 532 nm to determine the pink colour that was produced. Gallic acid was used as the standard. The following formula is used to calculate the percentage inhibition of hydroxyl radicals (% scavenged):

% Scavenged = [AC-AS)/AC] $\times$100

Where AC = absorbance of the control,

AS = absorbance in the presence of the sample of extracts or standards.

2.4.4 Statistical analysis

Graph pad prism software version 10.0.3 was used for the statistical analysis. The experimental data obtained were represented as Mean$\pm$SEM and Multiple comparisons were performed by one-way ANOVA followed by Dunnet's post hoc comparison test with $p<0.05$ as the limit of statistical significance.

3 Results and Discussion

3.1 *In vivo* methods

3.1.1 Micronuclei frequency in different treatment groups

The antigenotoxic activity of *Kaempferia rotunda* was investigated by performing a micronuclei assay, bone marrow chromosomal aberration test as well as antioxidant activity. In the micronucleus assay, it was evident that cyclophosphamide significantly reduced micronuclei frequency and PCE percentage in mouse bone marrow erythrocytes. Furthermore, extract of *Kaempferia rotunda* Linn. significantly decreased MNPCE count. Also, we have noted the reduction in the number of micro-nucleated polychromatic erythrocytes at 200 and 400 mg/kg of *Kaempferia rotunda* extract. When *K. rotunda* 200 and 400mg/kg were given along with cyclophosphamide, it showed a significant decrease in PCE/NCE ratio in group 3 and group 4 (1.763±0.445 and 1.241±0.871). The maximum percentage reduction in the micronuclei polychromatic erythrocyte cell count was found in group 4 [Table 1, Figure 1]. *Kaempheria* plant extracts and isolated compounds demonstrate numerous and promising biological and pharmaceutical activities, rhizome ethanolic extracts of *K. galanga* and the purified component ethyl trans p-methoxycinnamate (105) demonstrate moderate cytotoxic activity against human cholangiocarcinoma (CL-6) cells with IC_{50} of 64.2 and 49.4 μg mL^{-1}, respectively. Significant cholangiocarcinoma (CCA) efficacy as indicated by suppressing tumour growth and lung metastasis in CL6-xenografed mice.[(1)]. Acetone, petroleum ether, chloroform, and MeOH extracts of *K. galanga* rhizomes show moderate cytotoxicity in a brine shrimp lethality bioassay compared with vincristine sulfate as the reference compound. Moreover, a methanolic extract of *K. galanga* rhizomes induces Ehrlich ascites carcinoma (EAC) cell death in a dose-dependent manner[(1)].

Table 1. Micronuclei frequency in different treatment groups

Groups	**Treatment**	**MNPCE'S** (mean ± SEM)	**PCE/NCE ratio** (mean ±SEM)
1	Normal control (vehicle)	8.815±1.216	2.087±0.728
2	Treatment control (Cyclophosphamide 40mg/kg)	28.40±2.708##	2.143±0.444#
3	Low dose (Cyclophosphamide+ K. rotunda 200mg/kg)	14.79±1.560**	1.763±0.445**
4	High dose (Cyclophosphamide+ K. rotunda 400mg/kg)	10.98±2.134*	1.241±0.871*

All values were represented as MEAN±SEM where n=4, by employing one-way ANOVA and Dunnett's test where #P< 0.05, ##P< 0.01, ### p < 0.001normal control vs treatment control (Cyclophosphamide) *P < 0.05, **P< 0.01, *** p < 0.001 respectively were used to denote significance levels when comparing all low-dose and high-dose treated groups vs treatment control.

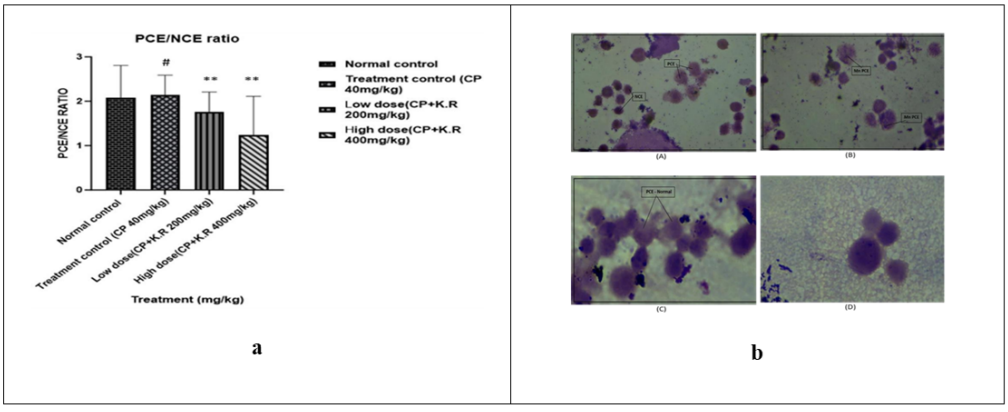

Fig 1. a: Graphical representation of PCE/NCE ratio; b: PCE and NCE in different treated groups. A-PCE and NCE in low dose treated group, B- Micro nucleated PCE in treatment control group, C- PCE cells in normal control group and D-NCE in highdose treated group

3.1.2 Bone marrow Chromosomal Aberration test

The methanolic extract of *Kaempferia rotunda* was able to reduce the number of chromosomal abnormalities which were induced by cyclophosphamide in mouse bone marrow cells. The maximum reduction (10.61±0.950) was observed in chromosomal aberrations in the cell count found at 400 mg/kg [Table 2]. Chromosome aberrations were found to decrease after methanolic extract treatment with cyclophosphamide, indicating antigenotoxic activity of *K. rotunda* against cyclophosphamide-induced abnormalities, suggesting potential antigenotoxic properties.

Table 2. Bone marrow CA in different treatment groups

Groups	Treatment	% Total aberrations
1	Normal control (vehicle)	7.97±0.928
2	Treatment Control (Cyclophosphamide 40mg/kg)	19.23±1.25#
3	Low dose (Cyclophosphamide+ K. rotunda 200mg/kg)	14.22±0.955*
4	High dose (Cyclophosphamide+ K. rotunda 400mg/kg)	10.61±0.950**

All values were represented as MEAN±SEM where n=4, by employing one-way ANOVA and Dunnett's test where #P< 0.05, ##P< 0.01, ### p < 0.001normal control vs treatment control (Cyclophosphamide) *P < 0.05, **P< 0.01, *** p < 0.001 respectively were used to denote significance levels when comparing all low-dose and high-dose treated groups vs treatment control.

3.2 *In vitro* methods

3.2.1 Allium cepa assay

The methanolic extract of *K. rotunda* considerably reduced the number of dividing cells in a dose-dependent manner. There was an increase in the mitotic index (6.48±0.45 and 7.94±0.12) when the dose of rhizome extract was increased from 200μg/ml to 400μg/ml showing that it has mitotic activity [Table 3].

Table 3. Mitotic index in different treatment groups

Groups	Treatment	Total cells	Dividing cells				Non-dividing cells (INT)	Mitotic index
			P	M	A	T		
1	Normal control (vehicle)	5000	128	112	69	61	4630	3.65±0.53
2	Treatment control (Cyclophosphamide 40μg/ml)	5000	30	28	25	21	4902	4.89±0.34#
3	Low dose CP+ K. rotunda 200μg/ml	5000	55	46	28	30	4888	6.48±0.45*
4	High dose CP + K. rotunda 400μg/ml	5000	51	32	26	27	4853	7.94±0.12**

All values were represented as MEAN±SEM where n=4, by employing one-way ANOVA and Dunnett's test where #P< 0.05, ##P< 0.01, ### p < 0.001normal control vs treatment control (Cyclophosphamide) *P < 0.05, **P< 0.01, *** p < 0.001 respectively were used to denote significance levels when comparing all low-dose and high-dose treated groups vs treatment control.

Allium cepa cells were then subjected to chromosomal analysis in order to study the effect of methanolic extract of *K. rotunda* against cyclophosphamide-induced aberrations (21±3.61 and 18±2.54). A significant reduction in the chromosomal aberration (21±3.61 and 18±2.54) were visible as they were reduced at 200 and 400 μg/ml [Table 4 and Figure 2]. Cyclophosphamide, given at 40μg/ml, caused the most chromosomal aberrations. However, when *K. rotunda* was combined with cyclophosphamide, there was a significant reduction in chromosomal abnormalities.

Table 4. Percentage of Chromosomal aberrations

Groups	Treatment	Chromosomal aberrations	% Chromosomal aberrations
1.	Normal control (vehicle)	8	8±2.46
2.	Treatment control (Cyclophosphamide40μg/ml)	52	52±3.39###
3.	Low dose (CP+ K. rotunda 200μg/ml)	21	21±3.61**
4.	High dose (CP + K. rotunda 400μg/ml)	18	18±2.54*

All values were represented as MEAN±SEM where n=4, by employing one-way ANOVA and Dunnett's test where #P< 0.05, ##P< 0.01, ### p < 0.001normal control vs treatment control (Cyclophosphamide) *P < 0.05, **P< 0.01, *** p < 0.001 respectively were used to denote significance levels when comparing all low-dose and high-dose treated groups vs treatment control.

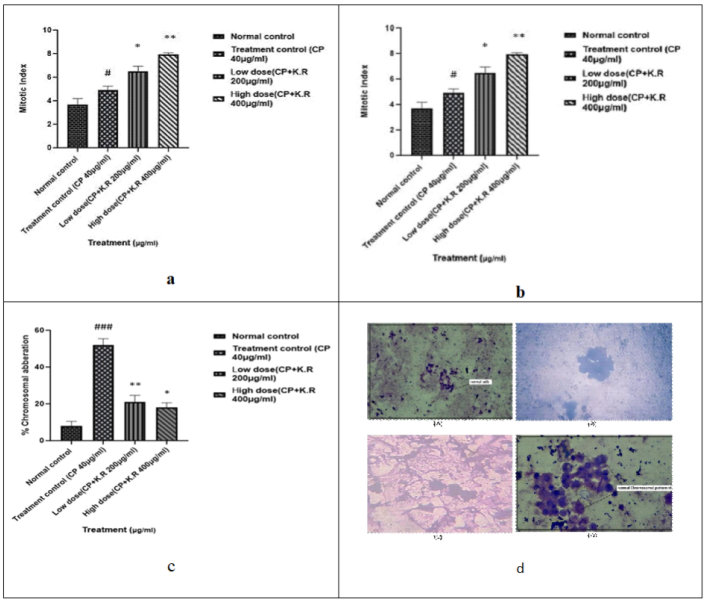

Fig 2. a: Graphical representation of Chromosomal Aberration; b: Mitotic Index assay; c: Chromosomal aberration; d: Bone marrow chromosomal aberration, A-Normal cells, B- Chromosomal aberration in the chromosomes of cells of low dose treated group, C-Polyploidy in treatment control group, D-Chromosomal aberration in the chromosomes of cells of high dose treated group

3.2.2 DPPH radical scavenging activity

DPPH free radical scavenging activity of *K. rotunda* methanolic extract demonstrated an increased IC_{50} value in comparison to standard Ascorbic acid. The plant exhibits free radical scavenging activity, according to the DPPH scavenging assay [Table 5 and Figure 3]. DPPH free radical scavenging activity was shown by the methanolic extract of *K. rotunda* rhizomes, with an IC50 value of 24.09μg/ml, substantially greater in comparison to standard ascorbic acid with an IC_{50} value of 8.17μg/ml. This indicates the plant's antioxidant properties.

Table 5. DPPH radical scavenging assay

Conc (μg/ml)	Scavenging effect of standard ascorbic acid (%)	Scavenging effect of rhizomes of *K. rotunda* (%)
10	17.34	48.75
20	25.26	49.00
40	47.82	61.50
60	73.23	74.75
80	85.56	75.50

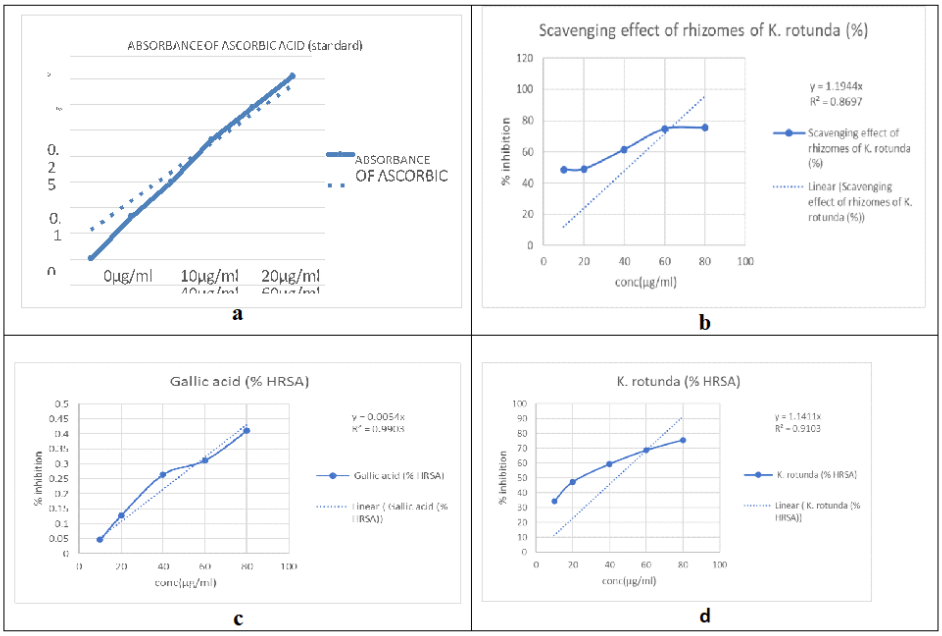

Fig 3. a:DPPH free radical scavenging activity of Ascorbic acid; b: DPPH free radical scavenging activity of methanolic extract of *K. rotunda* ; c: Hydroxy radical scavenging activity of Gallic acid; d: Hydroxy radical scavenging activity of methanolic extract of *K. rotunda*

3.2.3 Hydroxyl radical scavenging activity

The methanolic extract of *K. rotunda* exhibited a significant dose-dependent hydroxyl radical scavenging activity with an increased IC50 value when compared to standard Gallic acid [Table 6 and Figure 3]. From the Hydroxyl radical scavenging activity, it can be said that the plant has the hydroxyl radical scavenging activity. In comparison to standard gallic acid, which has an IC50 value of 10.22μg/ml, the methanolic extract of *K. rotunda* rhizomes exhibited hydroxyl radical scavenging action with an IC50 value of 21.74μg/ml. It may be concluded from the Hydroxyl radical scavenging assay that the plant possesses free radical scavenging properties.

Table 6. Hydroxyl radical scavenging assay

Concentration (μg/ml)	Gallic acid (% HRSA)	*K. rotunda* (% HRSA)
10	13.45	34.25
20	22.76	47.21
40	48.12	59.20
60	67.34	68.50
80	89.54	75.13

The methanolic extracts of the rhizomes of *K. angustifolia* reported strong antioxidant activity against DPPH expressed with 615.92 mg Trolox equivalent (TE)/g of extract. In an azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay, MeOH extracts showed good antioxidant properties with a value of 38.87 mg TE/g. However, n-hexane extract exhibited significant antioxidant activity with 901.76 mg TE/g in a cupric-reducing antioxidant capacity assay, while EtOAc extracts exhibited significant reduction ability against ferric-reducing antioxidant power (FRAP) with a value of 342.23 mg TE/g. Also, kaempfolienol showed potent free radical scavenging activity in a DPPH assay, as well as, 2′-hydroxy-4,4′,6′-trimethoxychalcone in ABTS, CUPRAC, and FRAP assays. A methanol extract of rhizomes of *K. galanga* exhibited concentration-dependent antioxidant activity in DPPH, ABTS, and nitric oxide (NO) radical scavenging assays. Moreover, the essential oil extracts of conventionally propagated and *in vitro* propagated *K. galanga* had significant DPPH radical scavenging activity. As well, the ethanol extract of *K. rotunda* exhibited antioxidant activity in a DPPH assay with IC_{50} (67.95 μg/mL)[(1)].

Study findings revealed that *K. rotunda* rhizome methanolic extract demonstrated antigenotoxic activity, reduced chromosomal aberrations, and the number of micronuclei. Based on a preliminary phytochemical study, the extracts contained alkaloids, tannins, saponins, steroids, terpene, flavonoids, phenolics, proteins, and carbohydrates. These findings suggest that the presence of flavonoids and polyphenols may be the reason for the observed antigenotoxicity.

Numerous therapeutic plant extracts have generally been reported to be either anti-mutagenic or anti-genotoxic[9,10]. This is due to the fact that crude extracts are made up of a variety of phytochemicals that may operate additively, antagonistically, or synergistically. Additionally, the rhizomes of *Kaempferia rotunda* may be employed for therapeutic reasons; however, the therapeutic dose would need to be evaluated.

4 Conclusion

From the study result, it is evident that the methanolic extract of rhizomes of *Kaempferia rotunda* Linn possesses potent antigenotoxic activity. The highest antigenotoxic activity was observed at 400 mg/kg of *Kaempferia rotunda* Linn extract. Modern drugs often cause side effects that can be challenging for patients. Herbal medicine, when used appropriately, may have fewer and milder side effects, contributing to improved quality of life during treatment. Further estimation of different molecular markers of various pathways associated with genotoxicity and cytotoxicity will prove the exact mechanism of action of *Kaempferia rotunda* Linn. as an antigenotoxic compound.

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