**Antimutagenic and antigenotoxic activity of *Curcuma caesia* Roxb. against Cyclophosphamide.**


## ABSTRACT:

The most effective procedure for preventing human cancer and other free radicals generated diseases is the use of antimutagens and anticarcinogens obtained from natural products i.e from medicinal plants, in everyday life. Medicinal plants are endowed with components such as bioactive compounds which can act as a means to block or reverse the carcinogenesis done by various mutations at early stages. Moreover, the medicinal plants can easily be metabolised, inexpensive, effective and early applicable without any side effects to control cancer and many other diseases. The treatment of cancer and other dreadful diseases with the synthetic drugs cause various side effects which can again lead to sequential secondary mutations and ultimately occurrence of various types of cancer. Therefore many medicinal, edible as well as herbal plants because of their less side effects have been tested worldwide for the antimutagenic and antigenotoxic activities and have been proved to inhibit the mutagenic and carcinogenic activities of some chemical mutagens. There are also reviews that have been proved that plants having potential antioxidant activities have antimutagenic activity. Rhizome of *Curcuma caesia* Roxb. has been used for many medicinal purposes like in treating leucoderma, asthma,tumors,piles, bronchitis,cancer etc. It also known for its antifungal, antidepressant, anti-inflammatory, antiulcer, hepaprotective activity. Rhizomes of *C.caesia* Roxb. are also suggested to be used in dysentery, diarrhoea and cough. Therefore the current investigation was to investigate the antimutagenic and antigenotoxic activity of *Curcuma caesia* Roxb. against cyclophosphamide. In the present study, different extracts of the rhizome of *Curcuma caesia* Roxb. were tested initially for the presence of various phytochemicals through preliminary phytochemical screening. In the four different extracts such as ethyl acetate extract (EaECC), ethanolic extract (EECC), methanolic

extract (MECC) as well as aqueous extract (AECC) of the rhizome of *Curcuma caesia* Roxb., many phytochemicals such as alkaloids, carbohydrates, flavanoids, phenolics, reducing sugars, terpenes, steroids, starch, saponins and tannins etc were detected. Total phenolic contents were also examined in the four extracts and it was ranged as MECC= 52.11mg GAE/g d.wt, EECC=68.64 mg GAE/g d.wt, EaECC=38.0 mg GAE/g d.wt, AECC= 4.82 mg GAE/g d.wt of the extract. The highest concentration of phenols was measured in ethanolic extract followed by methanolic extract, ethyl acetate and aqueous extracts. The reducing power of *Curcuma caesia* Roxb. rhizome extracts was also tested and it was found to be dose dependent in all the extracts. The maximum absorbance was observed with EECC at 1000µg/mL and was more or near to the standard compound ascorbic acid at 200µg/mL. The reducing ability of the extracts follows the order: EECC> MECC> EaECC > AECC. All the four extracts such as EaECC, EECC, MECC and AECC were tested for their antioxidant activities through DPPH, $ABTS^{+}$ and superoxide anion radical scavenging assays. The extracts showed scavenging activity towards DPPH, $ABTS^{+}$ and superoxide anions. In DPPH assay, the ethanol fraction was found to be the most active free radical scavenger exhibited (86.914% inhibition against DPPH at a concentration of 800 µg/ml) compared to ascorbic acid (94.717%) (200 µg/ml). Likewise the crude methanolic, ethyl acetate and aqueous extract showed scavenging activity with a percent inhibition of 83.104%, 70.44% and 69.19% at their highest concentration of 800 µg/ml against DPPH. The IC50 value ranges in the order of 418µg/ml (EECC)>441.90 µg/ml (MECC)>561 µg/ml (EaECC)> 591µg/ml (AECC). The inhibitory effect of all extracts against $ABTS^{+}$ was compared with standard compound Gallic acid. The highest inhibitory effect against $ABTS^{+}$ was found to be exhibited by EaECC against $ABTS^{+}$ with the percentage inhibition of 41.15% at its lowest concentration (5µg/ml) and 77.93% at its highest concentration (100 µg/ml) which lies in between 70.57% and 92.78% of Gallic acid standard at the concentrations of 60µg/ml and 80µg/ml. The IC50 value of EaECC

is 14.44 µg/ml as compared to that of Gallic acid (4.37µg/ml). The highest inhibitory effect of other extracts EECC, MECC and AECC was shown at their highest concentration (100 µg/ml) with the percentage inhibition of 63.76 %, 64.78% and 54.73% against $ABTS^+$ with the IC50 value of 43.63µg/ml, 51.994 µg/ml and 76.992 µg/ml respectively. All the extracts showed dose dependent inhibition against $ABTS^+$ free radical in the order of EaECC> EECC> MECC> AECC. In superoxide anion scavenging ability of different extracts of *C.caesia* Roxb. it was found that among the different extracts, ethyl acetate (EaECC) fraction of rhizomes was the most effective with an inhibitory effect of 77.88% at the concentration of 20µg/ml, whereas EECC, MECC and AECC exerted the inhibition percentage of 75.26%, 71.83% and 60.7% respectively at the same concentration (20µg/ml). EaECC exhibits 89.77% at the concentration of 200µg/ml while EECC, MECC and AECC exerted the percent inhibition of 88.36%, 84.03% against $O_2^-$ anion radical respectively at the concentration of 200µg/ml. Gallic acid standard showed maximum $O_2^-$ anion radical scavenging activity with the percentage inhibition of 94.05% at its highest concentration of 200µg/ml. Based on the promising antioxidant activity, ethyl acetate, ethanolic, methanolic and aqueous extracts were evaluated for their antimutagenic activity employing Ames test using two salmonella strains TA98 and TA 100 in the presence of the metabolic activator S9 against the indirect acting mutagen cyclophosphamide (CP). All the extracts were found to inhibit the mutagen cyclophosphamide in dose dependent manner. The percentage inhibition of EaECC against cyclophosphamide starts from 78.26% to 93.07% in TA98 and it was found to be significantly different ($p< 0.01$) as compared to positive control and in TA100 the percentage inhibition of EaECC against the mutagen cyclophosphamide starts from 59.99% to 72.55% and was statistically significant at $p<0.02$ as compared to the positive control group.. At all the doses of EECC, the antimutagenic response was significant ($p<0.01$) against both the strains TA98 and TA100 with the percent antimutagenicity from 77.99 to 90.95% for TA98 followed by TA100

with percent antimutagenicity starting from 57.77% to72.55%. Similar trend was followed for methanoilc extract of *Curcuma caesia* Roxb. At all doses of MECC, the antimutagenic response was significant at ($p<0.01$) with the percent mutagenicity decrease from 54.66% to 76.41% in case of TA98 followed by TA100 with the percent mutagenicity decrease from 49.65% to 60.80% in MECC. At the dose of 50µg of AECC antimutagenic response was insignificant with percent inhibition of 29.30% but at 500 µg, the antimutagenic response was significant ($p<0.05$) with percent inhibition of 37.51% and at the dose of 5000 µg it was significant ($p<0.01$) (57.57% inhibition) in case of TA98 and in case of TA100 in AECC the significant level shown was ($p<0.01$) for all concentrations with the percent mutagenicity decrease from 29.30% to 57.57%. In the acute toxicity studies, treatment of animals with different extracts of *Curcuma caesia* Roxb. EaECC, EECC and MECC did not show any sign of toxicity during the period of investigation. No mortality was observed even at the highest dose of 2000mg/ kg. b. wt.of the animals. Animals from both the control as well *C.caesia* extracts treated groups showed normal patterns of awareness, somato motor activity, touch response and sound response. There was no sign of change in behavior, skin and fur color, no salivation, diarrhea, lethargy, tremors, sleep as well as coma. Antigenotoxic activity of the extracts EaECC, EECC and AECC was tested through micronucleus assay. The damaging effect done by cyclophosphamide (CP) on DNA (micronuclei formation) was observed to be more in positive control groups at the dose of 50mg/kg.b.wt of CP. Percentage of micronuclei formed in PC group were high (15.36%) as compared to negative control (NC) groups (0.46%) significantly ($p<0.002$). However, pre-treatment of each extract at different concentrations (100, 250, 500mg/kg.b.wt of mice) reduces the DNA damage. The percentage inhibition of DNA damage: by EaECC was found to be 46% ($p<0.01$), 57.03% ($p<0.01$) and 71.87($p<0.001$); by EECC: was found to be 43.8%, 54.42% and 69% significantly ($p<0.01$ and $p<0.05$) at the concentrations of 100, 250, 500mg/kg.b.wt of mice as compared to the positive control

groups. The pre treatment of different concentrations of MECC followed by CP also reduces the micronuclei formation significantly ($p<0.005$, $p<0.01$, $p<0.001$) as compared to the positive control groups. The reduction percentage of micronuclei by MECC was 41.77%, 48.43% and 68.75% at different concentrations (100, 250, 500mg/kg.b.wt respectively). There was no sign of genotoxicity in the treatment with each extract only, since the values of micronuclei formation were almost near to normal groups and they are not significantly different from the negative control groups. The increased levels of SGOT and SGPT in the serum resulting from the leakage from liver due to the treatment of cyclophosphamide was found to be more in the positive control groups significantly at $p<0.01$ in SGOT analysis and $p<0.0001$ in case of SGPT analysis as compare to negative control groups. The present study shows that the levels of SGOT (444U/ml) and SGPT (101.33U/ml) in positive control groups were statistically higher as compared to negative control groups (43U/ml in SGOT and 6U/ml in SGPT) indicating the toxicity to the liver. But the pre-treatment of each extract at different concentrations (100, 250 and 500mg/kg body wt) reduces the leakage of SGOT and SGPT from the liver which was indicated by the reduction of their levels signifying the protective effect of each extract. Biochemical analysis such as lipid peroxidation assay, GSH assay, GR assay and protein content were also performed in both the liver and kidney of mice. In positive control groups in liver, the lipid peroxidation form was more i.e., 0.550 nmole of TBARS/ g tissue ($p<0.005$) as compared to negative control group (0.120 nmole TBARS/g tissue) which was significantly different and the level of GSH in the positive control groups was found to be reduced, 0.007 nmole of GSH/g tissue significantly ($p<0.0001$) as compared to negative control groups with the value of 0.182 nmole of GSH/g tissue. Besides the lipid peroxidation and decreasing level of GSH caused by CP in the positive control in the liver, the amount of GR and protein were also found to be reduced in the liver significantly as compared to negative control groups. The level of GR in the liver of

CP treated group was found to be 0.048 µmoles of NADPH oxidized/min/g tissue while the negative control group was found to be 1.920 µmoles of NADPH oxidized/min/g tissue. It was found to be significantly different ($p<0.0001$). The levels of protein oxidized in the CP treated group were also higher as compare to the negative control group. The total protein thiol content in the negative control group was found to be 22.58 mg/g wt of tissue as compared to the positive control group with the value of -13.75 mg/g wt (decrease level is because of protein oxidation) of tissue which is significantly different as compared to the normal group ($p< 0.05$). But the pretreatment of all the extracts studied showed the inhibition of lipid peroxidation as well as increases the level of GSH, GR and protein content in the liver in the order of EaECC> EECC> MECC. Like the liver, the reactive metabolites of CP also affect the lipid membrane of kidney leading to lipid peroxidation and decrease the level of GSH, GR and protein content. Peroxidation level of kidney in the positive control group was found to be 0.936 nmole of TBARS/ g tissue, which is significantly high ($p<0.02$) as compared to the negative control group with the value of 0.236 nmole of TBARS/ g tissue. Likewise, the value of GSH in the positive control group was found to be 0.011 nmole of GSH/g tissue which is significantly reduced ($p<0.05$) from the negative control group 0.082 nmole of GSH/g tissue. Cyclophosphamide toxicity in the kidney also reduces the enzymatic antioxidant level of GR and the total protein concentration in the kidney. The level of GR in the positive control group of mice was found to be 0.851 µmoles of NADPH oxidized/min/g tissue as compared to negative control group of mice with the value of 2.148 µmoles of NADPH oxidized/min/g tissue which is significantly different ($p<0.005$). The total concentration of protein was also found to be reduced in the positive control group with the value of -7.33 µg from 20.75 µg in negative control group which is significantly different($p<0.001$). However the pretreatment of all extracts studied protects the changes brought about by cyclophosphamide in kidney. Histopathological analysis of liver reveals that the liver of the

negative control group of animals showed regular cellular architechture with distinct hepatic cells, sinusoidal spaces and clear central vein. On the other hand, section of liver from the mice treated with CP alone (grp II) showed inflammation in the central vein and decrease in sinusoidal spaces. The pretreatment of all the extracts EaECC, EECC and MECC at different concentrations 100mg, 250mg and 500mg (grp III, IV and V) before the administration of CP (50mg/kg.b.wt) revealed a better presentation of normal architechture of liver. Each extract at the dose of 500mg/kg.b.wt helps in generation of normal architecture of the liver as compare to other lower dose groups. The treatment of mice with highest concentration 500mg/kg.b.wt of each extract only showed normal liver architecture. Likewise in the kidney also, the reactive metabolites of CP change the renal architecture which was again regenerated by the treatment of different concentrations of each extracts. The antiproliferation activity of EaECC, EECC and MECC was tested using human breast cancer cell lines MDAMB231 and lung cancer cell lines Calu6 through MTT assay. It was found that EaECC shows antiproliferating activity against breast cancer cell lines MDAMB 231 and lung cancer cell lines Calu6 with percentage inhibition of 79.48% and 69.75% respectively at the highest concentration of 80 µg/ml. The other extracts EECC and MECC also showed similar pattern of cancer cell antiproliferation activity while the control shows 100% proliferation. The IC50 values of each extract EaECC, EECC and MECC against the cancer cell line MDAMB231 are 35.89µg/ml, 82.12 µg/ml and 89.33 µg/ml respectively. The antiproliferating activitity of EaECC, EECC and MECC was also tested against lung cancer cell lines Calu6. Ethyl acetate extract demonstrates dose dependent antiproliferative activity against cancer cell lines Calu6 with the percentage inhibition of 32.37% at the lowest concentration, 5µg/ml and 69.75% at the highest concentration, 80µg/ml with the IC 50 value of 38.405µg/ml. EaECC showed strong cytotoxic activity while EECC and MECC showed moderate cytotoxic activity in cancer cell lines Calu6. EECC showed percentage inhibition of 33.18% at its

lowest concentration, 5µg/ml and 48.87% at its highest concentration (80µg/ml) with the IC 50 value of 93.24 µg/ml. MECC showed moderate antiproliferating activity with the percentage inhibition of 31.81% at its lowest concentration (5µg/ml) and 50% at its highest concentration with the IC 50 value of 83.84 µg/ml. MECC showed more strong cytotoxic activity than EECC against Calu6 based on its low IC 50 value. In the GCMS analysis, 29 compounds were found to present in the ethyl acetate extract and 44 compounds in the ethanolic extract of *Curcuma caesia* Roxb. All the compounds identified were match with the data in NIST library. Majority of the compounds were identified as terpenes and sesquiterpenes. The qualitative UV-Visible spectrum profile of ethyl acetate extract of the rhizome of *Curcuma caesia* Roxb was selected at wavelength from 260 to 400nm due to sharpness of the peaks in between this region. The profile showed the peaks at 300nm, 320 and 380nm with the absorption of 1.196, 0.863 and 0.371 respectively and the UV-Visible profile of ethanolic extract of *Curcuma caesia* Roxb was selected at the wavelength of 200 to 400nm and the spectrum profile showed the peaks at 200, 300, 320, 340 and 360nm with the absorption of 0.748, 1.067, 0.737, 0.494 and 0.294 respectively. The relative precise positions and intensities of maxima give valuable information on the nature of the flavanoids. May be because of the presence of various compounds in the rhizome of *Curcuma caesia* Roxb, the bioactivities shown by the plant is so effective and the present analysis of the results suggested that the extracts and different fractions of *Curcuma caesia* Roxb.at different concentrations exhibited protective activities through various assays. Therefore the results of the present study may help in the development of novel medicines for using in treatment of cancer and other diseases.

**Keywords:** Phytochemical screening, total phenol content, reducing power assay, antioxidant assay, antimutagenicity assay, acute toxicity studies, antigenotoxicity study, biochemical assays, histopathological

analysis, cytotoxicity assays, GC-MS and UV-visible spectral analysis.