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Genoprotective effects of gallic acid against cisplatin induced genotoxicity in bone marrow cells of mice

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Chemotherapeutic drugs are used for the treatment of cancer. However, the use of these drugs is limited due to their side effects on normal cells. One of the measures to detect chemoprotection of plant extracts is to evaluate their anticlastogenic effects. In this study, we report the anticlastogenic effect of gallic acid (GA) against cisplatin (Csp), a chemotherapeutic drug, in Swiss albino mice. Three different doses of GA (100, 200 and 400 mg per kg bw) were administered orally to the experimental animals in 0.2 mL quantity for 5 days at 24 hour intervals. Cisplatin, the positive control agent (10 mg per kg bw), was administered intraperitoneally (i.p.) in 0.1 mL quantity. Overall, the results showed that the pretreatment of GA caused a remarkable decrease in Csp induced micronucleus frequency and DNA damage in bone marrow cells of mice. The results suggest that GA showed potent antigenotoxic effects against Csp induced damage in mice bone marrow cells.

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Introduction

Humans are continuously exposed to exogenous or endogenous types of mutagenic/carcinogenic agents through various sources, which ultimately cause DNA damage.[1] The exposure of living organisms including humans to toxicants may result from various events such as intentional ingestion, occupational exposure and environmental exposure as well as accidental and suicidal poisoning. The mutagenic or carcinogenic effects of genotoxic agents directly or indirectly cause genetic damage and ultimately result in the transformation of normal cells to cancer cells. Cisplatin is one of the most potent chemotherapeutic drugs which is being used effectively against various solid tumors including testicular, ovarian, breast, lung, bladder, head, neck, *etc.*[2] The cytotoxicity of cisplatin is due to its ability to form DNA adducts which include DNA–protein cross-links, DNA monoadducts and inter/intra cross-links and there is a strong association between the formation of DNA adducts and DNA strand breaks.[3] Induced DNA damages activate various signaling pathways which repair the damage or promote cell death. Thus, the signaling pathways that regulate apoptosis have a significant impact on deciding cellular responsiveness to cisplatin.[4] The micronucleus induced by cisplatin eventually results in cell death, genomic instability or cancer development.[5] Cisplatin exposure increases the frequency of mutant erythrocytes which in turn causes damage to the hematopoietic cells and ultimately increases the incidence of secondary leukemia.[6] Cisplatin is frequently used in combination therapies with one or additional drugs with positive outcomes. The hope is that the drugs will work together, producing synergistic or at least additive effects in killing cancer cells, while producing no additional side effects on normal cells.[7,8]

Micronuclei (MN) are small acentric chromatin fragments, formed whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter cells, which lag behind during the anaphase of the cell division and appear in the following interphase as the micronucleus. The MN assay was developed as a short-term screening test. Boller and Schmid[9] used the term micronucleus and used it for the genotoxic analysis for the first time and Heddle[10] applied this technique using bone marrow erythrocytes.[11] Among the currently available test systems, the micronucleus assay is the most widely applied method due to its simplicity, reliability, sensitivity and proven suitability for the broad-spectrum determination of DNA damage at the chromosome level.[12]

The micronucleus assay has been considered as a primary assay to determine the genotoxic potential of chemicals. The alkaline comet assay complements the MN test. The alkaline comet assay has been widely accepted as a simple, sensitive and rapid tool for assessing DNA strand breaks, alkali labile sites and repair in individual eukaryotic and prokaryotic cells and it has increasingly found applications in diverse fields

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ranging from genetic toxicology to human epidemiology. It has gained wide acceptance as a valuable tool in fundamental genotoxicity testing, human biomonitoring, DNA damage and repair studies.[13–16] The comet assay is also used to assess the level of DNA damage in neonatal sepsis.[17] This assay has been widely accepted in studying DNA damage and repair in healthy individuals in clinical studies and in monitoring risk of DNA damage/lifestyle. A combination of comet and micronucleus assays can be used as an important tool to explore the genotoxic potential of benzene in rats.[18]

The biological effects of chemotherapeutic drugs result from direct targeted damage to nuclear DNA. In humans, the continuous damage in genetic materials of somatic and germinal cells can accelerate the risk of cancer. In the search of agents to prevent the risk of chemotherapeutic drugs, it has been shown that phytochemicals can play a pivotal role. Plant phytochemicals have the potential to prevent DNA damage. Phytochemicals with antigenotoxic and cytotoxic activities have great importance for the development of new chemotherapeutic drugs or to be used as an adjuvant to enhance the potential of chemotherapeutic drugs.[19]

Gallic acid (GA) (3,4,5-trihydroxybenzoic acid) is a polyhydroxy-phenolic compound and is present in the form of free acids, esters, catechin derivatives and hydrolysable tannins; also, it is one of the major bioactive phenolic compounds present in plants.[20,21] Gallic acid is well known for its antioxidant properties as a pure compound or as a bioactive complex of plants.[22–24] Along with its antioxidant properties, there are other reports on the *in vivo* and *in vitro* immunomodulatory activity of gallic acid as an active component of a plant.[25–28] Gallic acid possesses significant anti-inflammatory properties and prevents the expression of inflammatory chemicals including cytokines and histamines and can be used to treat inflammatory allergic diseases.[29]

Information available on GA and its medicinal uses has initiated us to investigate its anticlastogenic/antigenotoxic effects against the cisplatin induced clastogenic effect on bone marrow cells of Swiss albino mice. For this study, the most sensitive and reliable tests such as micronucleus and comet assays have been employed.

Materials and methods

Experimental animals

Swiss albino mice belonging to *Mus musculus* species, bred and maintained in the institutional animal house, were used for the experiment. They were housed in polypropylene shoe box-type cages, bedded with rice husk and kept in an air-conditioned room at 23 °C (±2 °C) and with a relative humidity of 50 ± 5%;they were fed with a pelleted diet (Amruth Feeds, India) and water *ad libitum*. A 12 : 12, light : dark cycle was followed. 8–10 weeks old animals with an average body weight of 25 ± 2 g were used for the experiments. For this study, a total of 10 different groups were maintained (control, solvent control, 3 groups of GA, 1 group of Csp, 1 group of solvent+Csp, 3 groups of different doses of GA+Csp). Based on the sample size, five animals (3 females + 2 males) per group were maintained separately. Care and experimental procedures were carried out as per the Guidelines of the Committee for the purpose of control and supervision of experiments on animals (CPCSEA), India. All groups of animals were kept under absolute hygienic conditions as per the recommended procedures by fulfilling the necessary ethical standards. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Mangalore University prior to the initiation of experiments (IAEC) (MU/AZ/99/2013-14/IAEC dt:2.04.2013).

Chemicals

Gallic acid and cisplatin were procured from Sigma Aldrich and other chemicals were purchased from Merck, SRL and Himedia, India.

Dose and treatment schedule

Nair and Nair[23] have reported the LD_{50} value of gallic acid in rats as 5000 mg per kg bw. In view of that, three different doses of gallic acid (100, 200 and 400 mg per kg bw) were selected for our study. Experimental animals were orally administered with 0.2 mL quantity of different doses of gallic acid prepared in 0.5% carboxymethyl cellulose (CMC) for 5 consecutive days. Csp (10 mg per kg bw) dissolved in 0.9% saline was administered i.p. in 0.1 mL quantity on the 5th day, one hour after the last treatment of gallic acid and was used as a positive control agent. After 24 hours of the treatment, bone marrow cells were extracted and processed for the micronucleus and comet assays.

Bone marrow micronucleus (BMN) assay

Bone marrow MN preparations were made by employing the modified method of Schmid[30,31] and by following the standard guidelines of OECD.[32] Here, 3% bovine serum albumin (BSA) prepared in phosphate buffered saline (PBS; pH 7.2) was used as a suspending medium. The bone marrow suspension was centrifuged at 1500 rpm and the pellet was resuspended in a required quantity of BSA mixed thoroughly and a drop of suspension was smeared on clean slides and air dried. The dried slides were fixed in methanol and stained with buffered (pH 6.8) May-Grunwald-Giemsa. According to the OECD guidelines (1997),[32] 2000 polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) in the corresponding field were screened from each animal to score the MN and to determine the PCE/NCE ratio.

Comet assay

The alkaline comet assay was performed according to the method of Singh *et al.*[33] From each animal, 100 cells were analysed using a fluorescence microscope (Olympus BX51 microscope), with a magnification of 100×. These comets were classified and analysed by visual scoring as five categories or levels of damage according to the percentage of DNA in the tail, as follows: 0: no damage 1: low damage, 2: moderate

damage, 3: high damage and 4: severe damage. Scores depict the extent of damage that is tail formation as observed after staining.[34]

Statistical analysis

The statistical significance of the results was tested by employing one-way ANOVA and Dunnett's *post hoc* tests using Graph Pad Prism 5 (Graph Pad Software, Inc., CA, USA). GA treated groups were compared with a negative control 0.5% CMC treated group. GA combined treatment groups were compared with Csp treated groups. Differences with a *P*-value of 0.05 or lower were considered to be statistically significant. The significance of the obtained results was designated as $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

Results

Effect of gallic acid on cisplatin induced MN in mice bone marrow cells

Cisplatin significantly increased the frequency of MN in PCE and total MN in bone marrow cells of mice compared to the negative control group (Fig. 1). Additionally, reduction in the normal PCE/NCE ratio was also observed. Also, gallic acid did not induce significant MN at any of the doses tested compared to the CMC solvent control. In the combined treatment groups, GA significantly reduced the Csp induced MN frequency at all the three doses tested. Further addition of GA improved the PCE/NCE ratio to the normal level. A dose dependent percentage inhibition of MN was observed along with the positive agent cisplatin. All the three doses of GA showed positive response as a protective agent against Csp. Among the three doses tested, the lowest dose 100 mg per kg bw was found to be more effective than the other two doses. The results are presented in Table 1 and Fig. 2.

Table 1 Frequency of MN in bone marrow cells of mice treated with gallic acid and cisplatin

Treatment (mg kg^{-1})	MNPCE (%)	Total MN (%)	PCE/NCE
0.9% Saline	0.16 ± 0.018	0.09 ± 0.013	1.10 ± 0.024
0.5% CMC	0.10 ± 0.013	0.06 ± 0.012	1.10 ± 0.020
GA100	0.02 ± 0.010	0.01 ± 0.000	1.17 ± 0.004
GA200	0.01 ± 0.010	0.01 ± 0.000	1.20 ± 0.006
GA400	0.01 ± 0.012	0.01 ± 0.000	1.19 ± 0.002
Csp10	3.70 ± 0.111^{a}	2.47 ± 0.059^{a}	0.57 ± 0.013^{a}
CMC + Csp10	3.60 ± 0.051	2.35 ± 0.024	0.59 ± 0.001
GA100 + Csp10	1.50 ± 0.037^{b}	0.79 ± 0.027^{b}	0.85 ± 0.010^{b}
GA200 + Csp10	1.84 ± 0.032^{b}	1.00 ± 0.010^{b}	0.83 ± 0.000^{b}
GA400 + Csp10	2.50 ± 0.064^{b}	1.23 ± 0.012^{b}	0.78 ± 0.008^{b}

Values are expressed in mean ± SE, (n = 5). 2000 PCE scored from each animal. One-way ANOVA followed by Dunnett's *post hoc* test. $^{a}P < 0.001$ when compared to saline. $^{b}P < 0.001$ when compared to cisplatin.

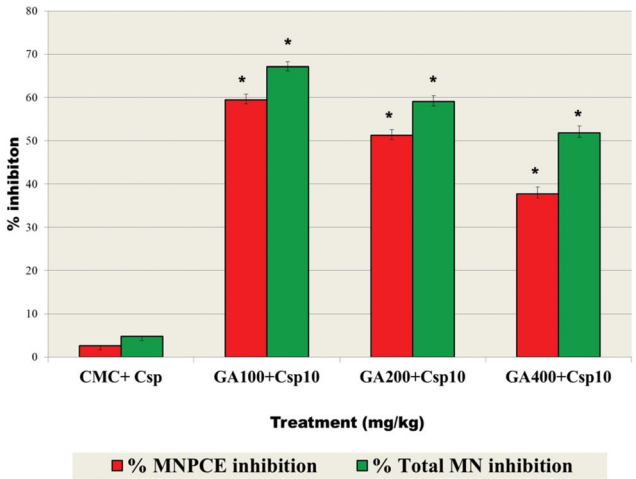

Fig. 2 Effect of gallic acid on MNPCE and Total MN induced by cisplatin; values are expressed in mean ± SE, (n = 5), one way ANOVA followed by Dunnett's *post hoc* test, *$P < 0.001$ compared to cisplatin.

Effect of gallic acid on cisplatin induced DNA damage in mice bone marrow cells

Cisplatin administration significantly increased the number of comets grading from 1 to 4 compared to the negative control group (Table 2). The ability of Csp to cause DNA strand breaks in bone marrow cells of mice shows its genotoxic potential. Gallic acid did not induce significant bone marrow cell DNA damage compared to the CMC solvent control. GA pretreatment significantly reduced the genotoxic effects of

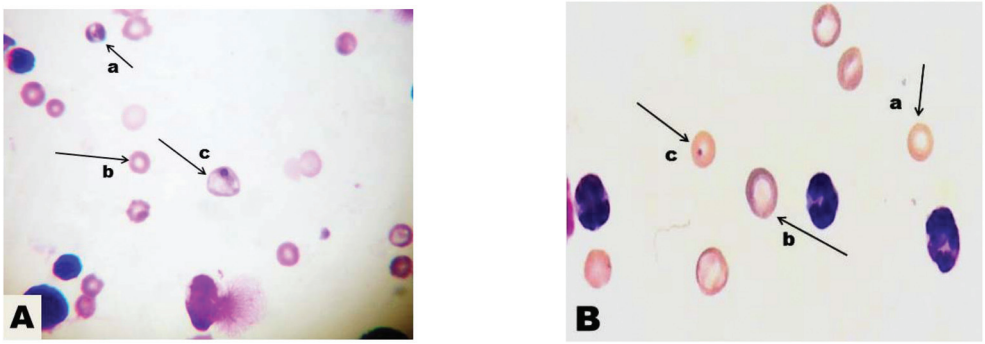

Fig. 1 Micronucleus induced by cisplatin in bone marrow cells of mice. A: (a) Polychromatic erythrocytes; (b) normochromatic erythrocytes; (c) polychromatic erythrocytes with the micronucleus. B: (a) Normochromatic erythrocytes; (b) polychromatic erythrocytes; (c) normochromatic erythrocytes with the micronucleus.

Table 2 Effect of gallic acid on cisplatin induced DNA damage in mice bone marrow cells

	DNA damage					
Treatment (mg kg^{-1})	0	1	2	3	4	% of comet
0.9% saline	95.4	3.20	1.40	—	—	4.00 ± 0.219
0.5% CMC	96.4	2.40	1.20	—	—	3.20 ± 0.334
GA100	98.4	1.60	—	—	—	1.60 ± 0.219
GA200	98.0	2.00	—	—	—	2.20 ± 0.179
GA400	97.4	2.60	—	—	—	2.60 ± 0.219
Csp10	41.6	15.0	18.0	19.4	6.00	58.4 ± 0.456^a
CMC + Csp10	44.4	19.0	16.0	15.0	5.20	55.6 ± 1.117
GA100 + Csp10	86.8	9.60	1.80	1.80	—	13.2 ± 0.657^b
GA200 + Csp10	7.40	13.8	9.60	4.00	—	27.6 ± 0.456^b
GA400 + Csp10	64.6	18.4	10.2	6.60	—	35.4 ± 0.456^b

Values are expressed in mean ± SE, (n = 5). 100 cells scored from each animal. One-way ANOVA followed by Dunnett's *post hoc* test. $^aP < 0.001$ when compared to saline. $^bP < 0.001$ when compared to cisplatin.

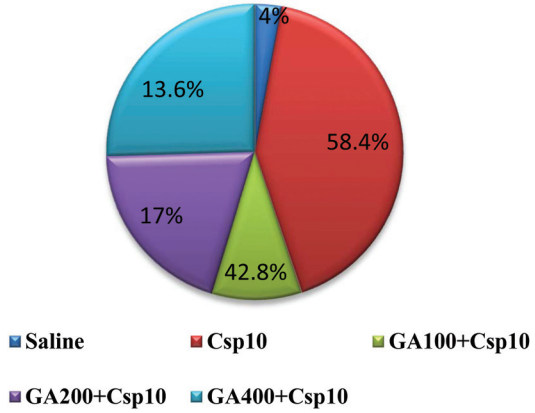

Fig. 4 Effect of gallic acid on cisplatin induced DNA strand breaks in mice bone marrow cells.

Csp on bone marrow cells at all the three doses tested (Table 2).

Cisplatin significantly increased the migration of DNA from the head of the comet to the tail and thereby increased the total percentage of DNA strand breaks in bone marrow cells (Fig. 3). The total percentage of comets induced by Csp was significantly very high when compared to the negative control group (Fig. 4). The presence of different types of comets demonstrates the extent of bone marrow cell DNA strand breaks induced by Csp in mice. The increase in the percentage of type-3 DNA damage showed the presence of fragmented DNA in the tail of the comet which indicates the high levels of damage caused by cisplatin. Gallic acid showed promising genoprotective effects against Csp induced DNA damage. The reduction in the amount of fragmented DNA was observed at all three doses of gallic acid, also the effect was significant when compared to the Csp treated group. However, the maximum effect was observed at a GA dose of 100 mg per kg bw.

Discussion

The objective of the current study is to investigate the possible protective effects of gallic acid against cisplatin induced clastogenic/genotoxic effects on bone marrow cells of Swiss albino mice. In our study, gallic acid significantly reduced the cisplatin induced damage in bone marrow cells. These results were confirmed by bone marrow MN and comet assays. Gallic acid (100, 200 and 400 mg kg^{-1}) did not induce significant MN at any of the doses tested compared to the CMC control. GA in combination with cisplatin showed significant reduction in the MN frequencies in bone marrow cells at all the doses tested. Even though all three doses of gallic acid showed protection to the somatic cells, the maximum anticlastogenic effect was observed at a GA dose of 100 mg per kg bw.

The anticlastogenic mechanism of gallic acid can be explained based on the earlier studies conducted by some investigators. The potent genotoxic effect of cisplatin is due to the generation of oxygen/nitrogen free radicals during chemo-

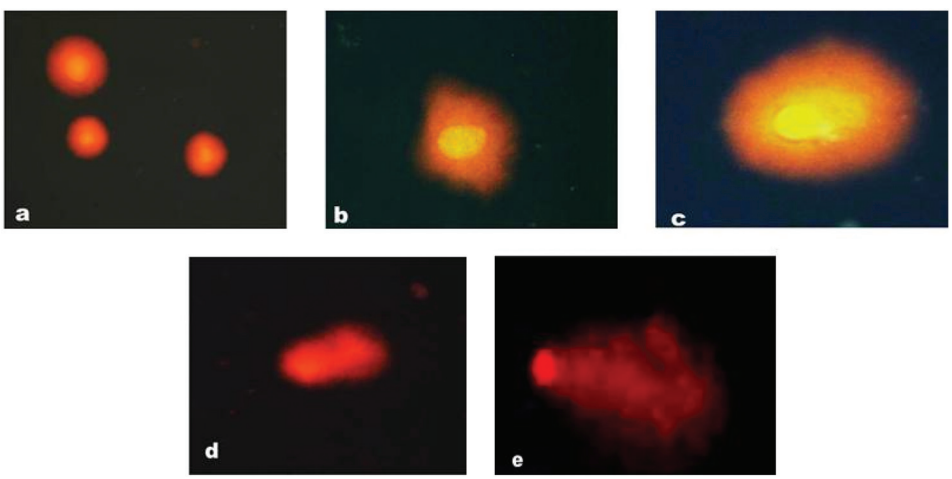

Fig. 3 Cisplatin induced bone marrow cell DNA strand breaks detected using the comet assay. (a) Normal cell; (b) little DNA damage; (c) moderate DNA damage; (d) extensive DNA damage; (e) completely damaged DNA.

therapy which causes DNA damage. Micronuclei can be mainly initiated by chromosomal segregation errors or damaged DNA or due to the malfunction of mitotic spindles caused by cisplatin.[35] Ascorbic acid with its antioxidant and free radical scavenging properties ameliorated the cisplatin induced mutagenesis and oxidative damage in mice.[36] Adejuwon *et al.*[37] evaluated the anticlastogenic effects of the *Launaea taraxacifolia* leaf extract against cisplatin induced micronuclei in bone marrow cells of mice. In another study, epigallocatechin gallate, a polyphenolic compound which is a potent antioxidant, obtained from green tea offered protection against MN induced by Csp in bone marrow cells of Swiss albino mice.[38] In view of these reports, the antioxidant and free radical scavenging capacity of gallic acid may be attributed to its anticlastogenic effects.

The antigenotoxic effect of GA on bone marrow cells of mice was further assessed using the alkaline comet assay. The alkaline comet assay is an appropriate method for the evaluation of antigenotoxic effects of GA because it detects the initial DNA lesions associated with the cisplatin generated free radicals and reactive species. The alkaline comet assay is widely used to measure the extent of DNA damage using the comet parameters such as the tail length and the percentage of DNA in the tail. Based on the tail length and the percentage of DNA in the tail, the severity of damage is categorized into no damage, mild, moderate and extensive damage. In our study, the frequency of comets and the extent of bone marrow cell DNA damage was found to be significantly increased in cisplatin treated animals, compared to the negative control group. These findings from our study are related to the work done by El-Bialy and Rageh,[39] wherein authors have correlated the number of comets formed to the extent of DNA damage caused. The ability of Csp to induce DNA damage in bone marrow cells of mice showed its genotoxic potential. The genotoxic effects of cisplatin are interrelated to free radical generation by Csp. Cisplatin is the platinum based drug and hence, its cytotoxicity is mainly mediated by the covalent linking of two strands of DNA, thus it prevents their separation and blocks transcription, segregation and replication.[40] The cisplatin induced DNA adducts and cross-links are poorly repaired by DNA repairing enzymes.[41] Accordingly, the possible mechanism of the cisplatin induced DNA strand break may be due to the accumulation of free radicals. The free radicals were generated which may influence the permeability of the mitochondrial membrane permeability transition pore. Thus, the opening of the mitochondrial transition pore permits the release of cytochrome C from the mitochondria to the cytosol and activates the mitochondria dependent pathway leading to apoptosis.[42] Cisplatin has also been shown to induce DNA damage in erythrocytes of Wistar albino rats. It induces DNA damage by promoting the generation of free radicals which attack and destroy erythrocytes.[37] Free radical mediated DNA damage in mice bone marrow cells has been reported by Choudhary *et al.*[38] In our study, cisplatin showed significant genotoxic effects by increasing the micronucleus and the DNA strand break frequency in bone marrow cells of mice. However, the coadministration of GA exhibited significant dose-dependent antimutagenic activity against cisplatin as assessed by MN and comet assays. Even though all the three doses of GA significantly ameliorated cisplatin induced chromosomal and DNA damage in mice bone marrow cells, the maximum anticlastogenic effect of GA was observed at a dose of 100 mg kg^{-1}.

Plants and their components have been studied for their protective effects against cisplatin induced DNA damage. Sayed[43] reported the ameliorative effects of *Echinacea* extract against Csp induced chromosomal aberrations, MN and comets in bone marrow cells of mice. Serpeloni *et al.*[44] reported the protective effects of a dietary pigment chlorophyll b against cisplatin induced chromosomal breakage and MN in bone marrow and peripheral blood cells of mice. Santos *et al.*[45] studied the ameliorative effects of a carotenoid bixin, isolated from annatto seeds against the detrimental effects of cisplatin in rat pheochromocytoma cell lines, in which, bixin modulated the genotoxic effects of Csp by reducing the frequency of MN and DNA breaks. Bilberry, an effective antioxidant, deteriorated the cisplatin induced oxidative stress and DNA damage in rat peripheral blood cells.[42] Antioxidant properties may be the reason for the protective effects of these plants.

From the above findings it is evident that the possible protection of gallic acid against the clastogenic effect induced by Csp may be due to the maintenance of the balance between the free radicals and enhanced antioxidant enzyme activities. The literature available on anticlastogenic effects of GA is scant. Nair and Nair[23] reported the anticlastogenic potential of GA against gamma radiation induced damage in peripheral erythrocytes and bone marrow cells of mice. According to them, radiation induced oxidative stress had increased the frequency of chromosomal damage and MN in erythrocytes and bone marrow cells of rats. Based on their observation, they suggested that the probable mechanism involved in the protective effect of GA against radiation is through scavenging the free radicals and/or induction of DNA repair enzymes. However, plants containing gallic acid as their bioactive components have been studied for their anticlastogenic potential. Ghosh *et al.*[46] evaluated the anticlastogenic potential of *Hibiscus sabdariffa* extract against arsenic induced DNA damage in male Swiss albino mice by using the comet assay. The biochemical profile of this plant suggested that the presence of the polyphenol component GA may have contributed to its protective effect. Lee *et al.*[47] have investigated the *in vitro* and *in vivo* antioxidative effects of *Paeonia lactiflora* plant active components including GA and methyl gallate against hydrogen peroxide and potassium bromate induced oxidative damage, in which, the GA and methyl gallate have significantly ameliorated the hydrogen peroxide induced oxidative DNA damage in mammalian fibroblast cell lines. They have also shown that the oral administration of GA significantly reduced the frequency of the micronucleus in peripheral erythrocytes, indicating the inhibition of potassium bromate induced toxicity in mice through the antioxidant mechanism. However, the free radical scavenging ability of GA was further confirmed by the

in vitro free radical scavenging assay, in which GA at a concentration of 0.401 μg mL^{-1} exhibited 50% scavenging effects on 2,2-diphenyl-L-picrylhydrazyl (DPPH) radicals. Since the gallic acid is a phenolic compound with high antioxidant potential, it has not shown a significant frequency of MN and DNA strand breaks at any of the doses tested. However, a further increase in the concentration of gallic acid may induce the clastogenic effect due to its prooxidant activity. Among the three doses of gallic acid (100, 200 and 400 mg per kg bw) in combination with cisplatin, the lower dose of GA of 100 mg per kg bw was found to be more effective than higher doses. It can be noticed that the maximum effect was observed at lower doses and may be due to the prooxidant activity of gallic acid at higher doses. GA is naturally found in fruits, vegetables and other plant parts. They may have many favourable biological effects due to their antioxidant and free radical scavenging abilities. However, at higher doses, they act as prooxidants. Tourino *et al.*[48] reported the antioxidant/prooxidant effects of bioactive polyphenolics. According to them, the most effective antioxidants are also the most cytotoxic and effective anti-proliferative agents, which may be due to the dual antioxidant/prooxidant effect of polyphenols.

Conclusion

In conclusion, our results confirmed the protective effects of gallic acid against cisplatin induced genotoxicity in bone marrow cells of Swiss albino mice. The present findings indicate that the antioxidant property of gallic acid may play a pivotal role in preventing the chromosomal and DNA strand break frequencies induced by cisplatin. The protection exerted by gallic acid against oxidative stress induced DNA damage can be attributed to its free radical scavenging activity.

Conflicts of interest

There are no conflicts of interest to declare.

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