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AMELIORATIVE POTENTIAL OF CROCUS SATIVUS WITH SPECIAL REFERENCE TO GENOTOXICITY AND CYTOTOXICITY IN TUMOR BEARING MICE

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ABSTRACT: Cancer is considered as one of the most dreadful diseases of the 20th century and is a major concern. Chemotherapy is the most common modality used for cancer treatment, but is unable to achieve satisfactory effects because of its severe side effects and dose-limiting toxicity. Ample evidence shows that herbal medicine has evolved as a solution for cancer chemoprevention and new drug development. *Crocus sativus* is reported to be useful in treating various human disorders. It's main constituents have memory improving, anti-oxidant, anti-depressant and anti-tumor properties. The present study was therefore, carried out to unveil the protective potential of saffron in terms of the chromosome damage in tumor bearing mice and cytotoxicity using mice skin cells and human peripheral blood lymphocytes. Chromosomal aberration assay was carried out to unveil the genotoxicity of various saffron extracts. MTT-cytotoxicity assay was carried out to explore the cytotoxicity of the saffron extracts using human peripheral blood lymphocytes and mice skin cells. As compared to standard drug 35mg/kg, 65mg/kg, 165mg/kg and 200mg/kg dosage of saffron stigma were found to produce statistically lower mean aberrant metaphases ($p<0.05$). Besides, 65mg/kg, 165mg/kg and 200mg/kg dosage of saffron bulb were found to produce statistically lower mean aberrant metaphases ($p<0.05$). MTT cytotoxicity assay of mice skin cells revealed maximum cytoprotection by 20% methanol stigma and minimum cytotoprotection by 100% saffron bulb extract. MTT cytotoxicity assay of lymphocytes revealed minimum cytoprotection by 100% saffron bulb and maximum cytoprotection by 100% methanol stigma.

KEYWORDS: *Crocus sativus*, genotoxicity, cytotoxicity, MTT assay

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1. INTRODUCTION

Cancer is considered as one of the most dreadful diseases claiming millions of lives each year. If detected early, surgical intervention may be applicable as an efficacious therapeutic measure. However, for better outcomes, many patients still need additional treatments such as chemotherapy and radiotherapy. Chemotherapy is the most widely used modality but is unable to achieve satisfactory effects because of its severe side effects and dose-limiting toxicity. Even newly designated drug therapies with specific tumor targets are reported with many undesirable adverse effects[1,2]. To date, no ideal approach has been found to obtain satisfactory effects against cancer.

An appropriate strategy for cancer prevention or treatment could be a combined approach, including the application of synthetic or natural agents to inhibit cancer development [2, 3]. Growing evidence shows that plants, such as vegetables, spices and herbs, have evolved as a solution for cancer chemoprevention and new drug development [4-6]. Compared to traditional cancer therapies, natural remedies have advantages, including little or no toxicity and low cost [7-9]. Herbal medications have already been used as an alternative treatment in cancer patients [10,2]. On an epidemiological basis, long-term consumption of certain botanicals, such as Asian ginseng, has been associated with reduced cancer incidence [11]. The anticancer effects of ginseng have also been shown in experimental studies [12,13]. Research that explores new botanical candidates with potential anticancer effects is imperative, and it supplies new data for developing safer and efficacious anticancer therapies [14,15]. Saffron, cultivated in various countries like Spain, Iran, Itlay, Switzerland and India [16] is reputed to be useful in treating various human disorders and is used in worldwide as a folk medicine [17]. The main constituents especially crocins which are coloured components present in Saffron have memory improving properties [18], anti-oxidant [19], anti-depressant [20], anti-tumor [21]. Traditionally Saffron has been considered as antispasmodic, aphrodisiac, galactogogue, expectorant and sedative. It has also been used in folk remedy against small pox, scarlet fever, colds, Insomnia, asthma, tumor and other diseases of kidney, liver, spleen and the brain [22]. Saffron extract is also useful for neuro disorders accompanying memory impairment, treatment of mild to moderate depression, reduces stress induced anorexia [23]. Preventive effect on tracheal responses and serum level of inflammatory mediators, improves liver function and have chemopreventive effect against liver cancer has also been reported [24]. Keeping in view the aforementioned properties of *Crocus sativus,* the present study was carried out to unveil the protective potential of saffron in terms of the chromosome damage in tumor bearing mice and cytotoxicity using mice skin cells and human peripheral blood lymphocytes.

2. MATERIAL AND METHODS

2.1 Collection and authentication of plant

Crocus sativus plant was collected from District Pulwama of Jammu & Kashmir. The plant was

identified by a Senior Taxonomist at University of Kashmir on the basis of its organoleptic and microscopic examination. Plant Herbarium was submitted at University of Kashmir with Voucher Numbers. 2458, 2459-A, 2459-B, 2459-C. Saffron stigma was collected and shade dried before extraction.

2.2 Extraction of Saffron

Crystallization method was used to extract the saffron stigma. For saffron bulb Soxhlet apparatus extraction method was used. 80% ethanol was chosen as the best extraction solvent. Saffron stigma (12gm) was suspended in 25ml ethanol at 0°C and shaken by vortex for 2 minutes after centrifugation for 10 minutes at 4000rpm the supernatant was separated. Twenty five millilitres of 80% ethanol were added to sediment and extraction was repeated again. The total volume of solvent consumption in extraction process was 200ml. The resulting solution was kept in a thick walled glass container for one week at -5°C in darkness. The container was sealed during that period. The obtained crystals were separated from the solution and washed with acetone to remove remaining water.

2.3 Experimental Animals

Adult *Swiss albino* mice aged 6-8 weeks with an average weight of 30gms were obtained from animal house of Jawaharlal Nehru Cancer Hospital & Research, Bhopal. The animals were housed in standard polypropylene cages and maintained in air conditioned animal house (20-25^{0}C relative humidity 70-75%) in a 12-hour light-dark cycle. The animals were fed on a standard laboratory diet and water *ad-libitum*. The studies were done with the prior permission and approval of CPCSEA and norms of IAEC Ref.No.500/01/a/CPCSEA/2001. Mice were grouped into 14 groups of 4 animals each. Six groups were given oral dosage of 10, 35, 65, 100, 165 and 200mg/kg body weight saffron stigma extract and six were given oral dosage of similar concentrations of saffron bulb extract twice daily. Control group was fed with double distilled water and one group was given oral dosage of standard drug(Doxorubicin) and used as drug control.

2.4 Chromosomal aberration assay

Chromosomal aberration assay was carried out as per the method of Preston et al, 1987 with slight modifications [25]. An intraperitoneal injection of colchicine (0.025%) was given to all mice two hours prior to euthanasia. Mice were dissected by cervical dislocation after 2 hours of colchicine injection. Animals were dissected and femoral bones were exposed and excised. Bone marrow was aspirated by flushing with normal saline in the centrifuge tube. The suspension flushed in the tube properly to get good cell suspension. This suspension was centrifuged for 10 minutes at 1000rpm. Supernatant was discarded & pellet was treated with pre-warmed (37^{0}C) 0.57M KCl on cyclomixer. Above suspension was left in water bath (37oC) for 20min. The suspension was centrifuged &

supernatant was discarded. Pellet was treated with freshly prepared Carnoy's fixative on cyclomixer & centrifuged. The supernatant was discarded. Above step of treatment with Carnoy's fixative was repeated 5 times to get debris free white pellet. Carnoy's fixative (quantity sufficient) was added to the pellet to get a good cell suspension. The slides of the above suspension were prepared by using Air Drop Method. The slides were stained with Giemsa Stain for 5 minutes and observed under microscope under 100X magnification. Number of cells having aberrations were scored (total 50 metaphases were analysed per animal).

2.5 MTT-cytotoxicity assay

Cytotoxic effect of all the extracts was determined as per Razzaq et al., 2011[26]. 96-well culture plates were seeded at 10,000 cells per well and incubated in CO_2 incubator (Heracell, Heraceus Germany) for 24-hours. Experiment was carried out by adding 50µl of prepared concentrations of all the extracts and stock solutions of all the extracts 1mg/1ml) diluted to 20, 40, 60, 80, 100µg/ml) in triplicate into appropriate wells and reincubated for 72-hours at the same condition. Media alone was taken as Blank and media plus cells were treated as control. MTT solution was prepared at 5mg/ml in PBS and was filtered through a 0.2µm filterate. 20µl MTT solution was added to each well and mixed by tapping gently on the side of the tray and incubated at 37^0C for 4-hours. 100µl of old media containing MTT was then gently replaced by 100µl DMSO into each well and to dissolve the formazane by pipetting several times. The absorbance was measured on ELISA plate reader (STAFFA Diagnostic Pvt. Ltd. Chennai-India), at a test wavelength of 492nm and a reference wavelength of 630nm. Concentration of each extracts was analysed in 5-wells and experiment was done in triplicate. The percentage growth inhibition was assessed taking percentage cell growth into consideration. The treatment showing highest percentage growth was considered to have lowest percentage growth inhibition.

2.6 Statistical analysis

Statistical analysis was carried out using Origin 8.0 Software. Student's t test was performed on the means of aberrant metaphases and the percentage growth of cells in the *in-vitro* cytotoxicity analysis of saffron dosages.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Chromosomal aberration assay

Saffron stigma extract

The mean aberrant metaphases in tumor bearing mice of different treatment groups and controls revealed maximum aberrant metaphases (18.28±1.52) in standard drug group (Figure 1). In the normal control group the mean aberrant metaphases was the minimum (Figure 2). Different

treatment groups of saffron stigma revealed different number of mean aberrant metaphases. As compared to standard drug 35mg/kg, 65mg/kg, 165mg/kg and 200mg/kg dosage of saffron stigma were found to produce statistically lower mean aberrant metaphases ($p<0.05$)(Figure 3). However, the mean aberrant metaphases in all dosage of saffron stigma extract were found to be statistically higher as compared to normal control group. The difference between the mean aberrant metaphases of 10mg/kg dosage of saffron stigma and drug control groups was found to be statistically non-significant ($p >0.05$) (Table 1).

Table 1: Showing number of different chromosomal abnormalities in various treatment groups of Saffron stigma and the control groups.

Groups	Aberrant Metaphases (Mean± SEM)
Normal Control	2.12±0.23
Tumor Control	7.28±1.34
Standard Drug	18.28±1.52
Saffron Stigma 10mg/kg	16.85±1.37
Saffron Stigma 35mg/kg	13.57±1.30*
Saffron Stigma 65mg/kg	10.85±0.59*
Saffron Stigma 100mg/kg	9.15±1.43*
Saffron Stigma 165mg/kg	8.28±2.82*
Saffron Stigma 200mg/kg	5.57±2.53*

*****Statistically significant genoprotection as compared to standard drug.

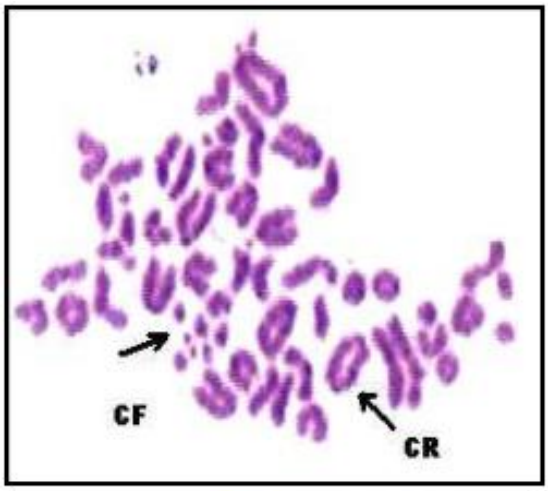

Figure 1: Metaphase showing ring chromosomes and chromosome fragments in the standard drug treatment group.

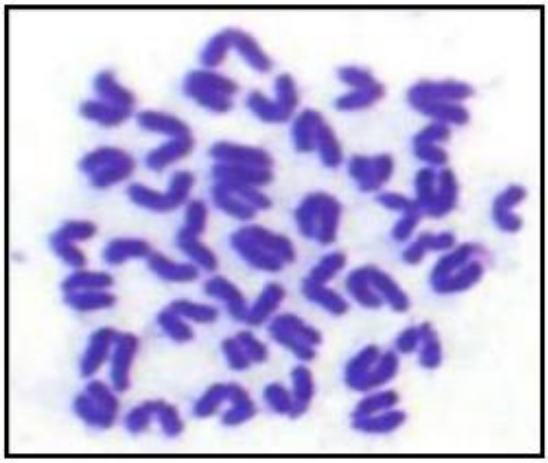

Figure 2: Metaphase showing normal chromosomes in the control group.

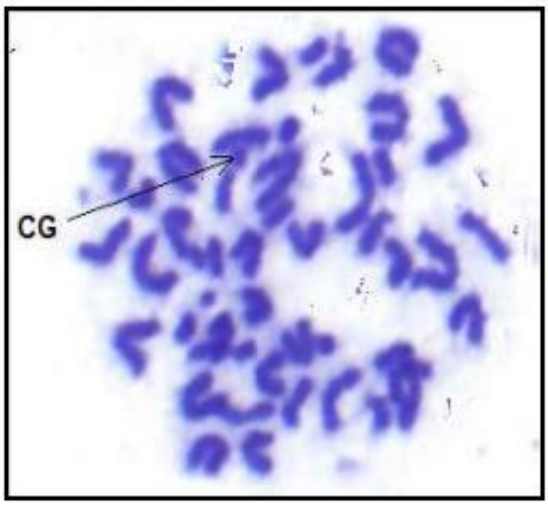

Figure 3: Metaphase showing chromosome gap in the saffron stigma treatment group.

Saffron bulb extract

The mean aberrant metaphases in tumor bearing mice of different treatment groups of saffron bulb and controls revealed maximum aberrant metaphases (18.28±1.52) in standard drug group. Similar number of mean aberrant metaphases (18.0±2.13) was recorded in the 10mg/kg dosage of saffron bulb group. As compared to standard drug 65mg/kg, 165mg/kg and 200mg/kg dosage of saffron bulb were found to produce statistically lower mean aberrant metaphases ($p<0.05$)(Figure 4). However, the mean aberrant metaphases in all dosage of saffron bulb extract were found to be statistically higher as compared to normal control group. The difference between the mean aberrant

metaphases of 35mg/kg dosage group and drug control group was found to be statistically non significant ($p > 0.05$)(Table 2).

Table 2: Showing number of different chromosomal abnormalities in various treatment groups of Saffron bulb and the control groups.

Groups	Aberrant Metaphases (Mean± SEM)
Normal Control	2.12±0.23
Tumor Control	7.28±1.34
Standard Drug	18.28±1.52
Saffron bulb 10mg/kg	18.0±2.13
Saffron bulb 35mg/kg	16.52±1.32
Saffron bulb 65mg/kg	14.15±1.23*
Saffron bulb 100mg/kg	13.25±1.43*
Saffron bulb 165mg/kg	11.20±1.52*
Saffron bulb 200mg/kg	11.23±1.54*

*****Statistically significant genoprotection as compared to standard drug.

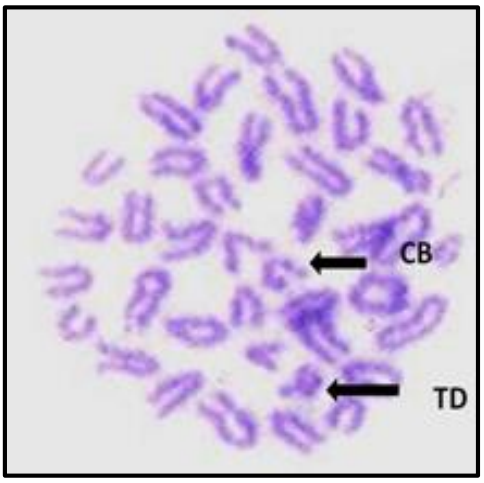

Figure 4: Metaphase showing chromatid break and terminal deletion in the saffron bulb treatment group.

3.1.2 Cytoprotection study using mice skin cells

For cytoprotection study, MTT assay of on mice skin cells was carried out that revealed maximum cytotoxicity by 100% saffron bulb and minimum cytotoxicity was shown by 20% methanol stigma. In ethanolic stigma extract group, 100% ethanol stigma showed the maximum cell damage, while as 40% ethanol stigma concentration showed the maximum protection. All the concentrations of ethanol stigma extract were found to exhibit better cytoprotection as compared to cyclophosphamide. Besides all the concentrations (20%, 40%, 60%, 80% and 100%) of ethanol stigma extract were found to exhibit decreased cytotoxicity as compared to carboplatin. In methanolic stigma extract group, 100% methanol stigma showed the maximum cytotoxicity, while as 20% methanol stigma showed best protective effect. However, all the concentrations of methanolic stigma extract were found to exhibit better protective effect as compared to cyclophosphamide as well as carboplatin. In saffron bulb extract group, 100% saffron bulb dosage provided the minimum cytoprotection, while as 60% dosage offered the maximum protective effect. As compared to carboplatin, all the dosages of saffron bulb exhibited better cytoprotection. Besides, all the concentrations of saffron bulb extract were found to produce better cytoprotection as compared to cyclophosphamide. Comparing 20% concentration of methanol stigma, ethanol stigma and saffron bulb, 20% saffron bulb produced the minimum cytoprotection, while as 20% methanol stigma produced maximum cytoprotection. Comparing 40% concentration of methanol stigma, ethanol stigma and saffron bulb, 40% saffron bulb produced the minimum cytoprotection, while as 40% methanol stigma produced maximum cytoprotection. Comparing 60% concentration of methanol stigma, ethanol stigma and saffron bulb, 60% saffron bulb produced the minimum cytoprotection, while as 60% methanol stigma produced maximum cytoprotection. Comparing 80% concentration of methanol stigma, ethanol stigma and saffron bulb, 80% ethanol stigma produced the minimum cytoprotection, while as 80% methanol stigma produced maximum cytoprotection. Comparing 100% concentration of methanol stigma, ethanol stigma and saffron bulb, 100% ethanol stigma produced the minimum cytoprotection, while as 100% methanol stigma exhibited maximum cytoprotection (Table 3).

3.1.3 Cytoprotection using peripheral blood lymphocytes

The results of MTT assay of lymphocytes revealed minimum cytoprotection by 100% saffron bulb and maximum cytoprotection by 100% methanol stigma. In ethanolic stigma extract, 40% ethanol showed the minimum cytoprotection, while as 100% ethanol stigma concentration showed the maximum cytoprotection.

Table 3: **Showing mean absorbance and percentage growth in various treatment groups of saffron in mice skin cells.**

Groups	Absorbance (Mean± SEM)	Percentage Growth
Cell Only	1.335±0.0590	
DDW	2.13±0.1500	168.83
Carboplatin	0.861±0.1349	64.49
Cyclophosphamide	0.343±0.0116	25.69
20% EtOH Stigma	1.416±0.2720	116.06
40% EtOH Stigma	1.586±0.0829	118.8*
60% EtOH Stigma	1.439±0.2257	107.79
80% EtOH Stigma	1.483±0.0096	100.59
100% EtOH Stigma	1.218±0.221	91.23
20% MeOH Stigma	2.368±0.1836	177.37#*
40% MeOH Stigma	1.992±0.1968	149.21
60% MeOH Stigma	2.022±0.0827	151.46
80% MeOH Stigma	1.826±1.1600	136.77
100% MeOH Stigma	1.680±0.0311	125.84
20% Saffron Bulb	1.523±0.1485	110.89
40% Saffron Bulb	1.442±0.0326	105.03
60% Saffron Bulb	1.4106±0.0362	106.43*
80% Saffron Bulb	1.246±0.0318	104.33
100% Saffron Bulb	1.180±0.0185	100.38

*Maximum cytoprotection within the respective group.

#Best cytoprotection *in-vitro* in mice skin cells.

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All the concentrations of ethanol stigma extract were found to exhibit better cytoprotection as compared to cyclophosphamide. Besides all the concentrations (20%, 40%, 60%, 80% and100%) of ethanol stigma extract were found to exhibit better cytoprotection as compared to carboplatin. In methanolic stigma extract group, 20% methanol stigma showed the minimum cytoprotection, while as 100% methanol stigma showed maximum cytoprotection. However, all the concentrations of methanolic stigma extract were found to exhibit better cytoprotection as compared to cyclophosphamide as well as carboplatin. In saffron bulb extract group, 40% saffron bulb dosage provided minimum cytoprotection, while as 80% dosage showed the maximum cytoprotection. All the concentrations of saffron bulb extract were found to exhibit better cytoprotection as compared to cyclophosphamide. Comparing 20% concentration of methanol stigma, ethanol stigma and saffron bulb, 20% saffron bulb exhibited the minimum cytoprotection, while as 20% methanol stigma produced the maximum cytoprotection. Comparing 40% concentration of methanol stigma, ethanol stigma and saffron bulb, 40% saffron bulb produced the minimum cytoprotection, while as 40% methanol stigma produced maximum cytoprotection. Comparing 60% concentration of methanol stigma, ethanol stigma and saffron bulb, 60% saffron bulb exhibited the minimum cytoprotection, while as 60% methanol stigma produced maximum cytoprotection. Comparing 80% concentration of methanol stigma, ethanol stigma and saffron bulb, 80% saffron bulb produced the minimum cytoprotection, while as 80% methanol stigma produced the maximum cytoprotection. Comparing 100% concentration of methanol stigma, ethanol stigma and saffron bulb, 100% saffron bulb showed the minimum cytoprotection, while as 100% methanol stigma produced the maximum cytoprotection.

Table 4: **Showing mean absorbance and percentage growth in various treatment groups of saffron in lymphocytes.**

Groups	Absorbance (Mean± SEM)	Percentage Growth
Cell Only	0.999±0.1191	
DDW	1.105±0.097	110.65
Carboplatin	1.046±0.0724	104.7
Cyclophosphamide	0.228±0.0054	22.82
20% EtOH Stigma	1.324±0.2975	132.53
40% EtOH stigma	1.155±0.2446	115.61
60% EtOH Stigma	1.651±0.1701	165.26
80% EtOH stigma	1.495±0.2390	149.64
100% EtOH Stigma	1.845±0.1653	184.6*

20% MeOH Stigma	1.415±0.1872	141.64
40% MeOH Stigma	1.86±0.1333	186.18
60% MeOH Stigma	1.76±0.1395	176.17
80% MeOH Stigma	2.038±0.0898	204.02
100% MeOH Stigma	2.206±0.059	220.82#*
20% Saffron Bulb	1.210±0.0330	123.97
40% Saffron Bulb	1.018±0.0388	102.85
60% Saffron Bulb	1.18±0.0589	118.08
80% Saffron Bulb	1.328±0.0528	132.89*
100% Saffron Bulb	1.123±0.0376	112.37

*Maximum cytoprotective effect within the respective group.
Maximum cytoprotection in lymphocytes in-vitro.

3.2 Discussion

3.2.1 Chromosomal aberration assay

The chromosomal aberration assay was carried out to analyse the genomic protection of saffron extracts. The mean aberrant metaphases in tumor bearing mice of different treatment groups and controls revealed maximum aberrant metaphases (18.28±1.52) in standard drug group. Different treatment groups of saffron stigma revealed different number of mean aberrant metaphases. As compared to standard drug 35mg/kg, 65mg/kg, 165mg/kg and 200mg/kg dosage of saffron stigma were found to produce statistically lower mean aberrant metaphases ($p<0.05$). As compared to standard drug 65mg/kg, 165mg/kg and 200mg/kg dosage of saffron bulb were also found to produce statistically lower mean aberrant metaphases ($p<0.05$). These treatments could significantly reduce the number of chromosomal aberrations in mice, thereby offering considerable genomic protection as compared to the standard drug. Our results are in concordance with that of Premkumar *et al* (2001), who observed that pretreatment with the aqueous extract of saffron (composed mainly by carotenoids) in experiments with *Swiss albino* mice significantly inhibits the genotoxicity of cisplatin, cyclophosphamide, mitomycin, and urethane [27]. In a study on cytogenetic behaviour of crocin on cultured lymphocytes from leukemic patients, after crocin affection a statistically significant decrease of the sister chromatid exchanges frequency of lymphocytes was observed. The results further indicated that crocin mainly reduced significantly the DNA damages with being cytoprotective [28]**.** Literature suggests that saffron rich in carotenoids might exert its chemopreventive effects by the modulation of lipid peroxidation, antioxidants, and detoxification systems [29]**.**

3.2.2 Cytoprotection study using mice skin cells

For cytoprotection study, MTT assay of on mice skin cells was carried out that revealed maximum cytotoxicity by 100% saffron bulb and minimum cytotoxicity was shown by 100% methanol stigma. All the concentrations of ethanol stigma extract were found to exhibit better cytoprotection as compared to cyclophosphamide as well as carboplatin. Besides, all the concentrations of methanolic stigma extract were found to exhibit better protective effect as compared to cyclophosphamide as well as carboplatin. Similar results were obtained in the saffron bulb extract treatment groups. It was observed that saffron extract at higher dosages offers considerable cellular protection in normal mice skin cells. So far no study has been carried out to investigate the cytoprotective potential of saffron in mice skin cells.

3.2.3 Cytoprotection using peripheral blood lymphocytes

MTT cytotoxicity of peripheral blood lymphocytes was carried out to unveil the *in-vitro* cytoprotection of the extracts. The results revealed maximum cytotoxicity by 100% saffron bulb extract, however, saffron stigma extract at higher dosage was found to produce minimum toxicity and therefore offering considerable cytoprotection. The results further suggest that all the dosages of saffron stigma extract produce less cytotoxicity as compared to standard chemotherapy drug, cyclophosphamide. Methanol stigma extract was found to be the best in terms of cytoprotection in peripheral blood lymphocytes. Although, little data is available about the *in-vitro* toxicity of saffron extracts on peripheral blood lymphocytes and mice skin cells, our results validate the safe usage of saffron extract. Besides, ample aforementioned evidence suggests tissue protection by saffron and its constituents [30,31]

4. CONCLUSION

The results of the present study validate the protective potential of *Crocus sativus*. The plant offers significant genomic protection and cytoprotection. The study further concludes that the plant can be explored as an adjuvant to chemotherapy which is very much essential considering the existing chemotherapeutic drugs which have a lot of side effects. Further research is required to validate the mode of action of the plant and its active constituents in preventing the chromosome damage and cellular toxicity.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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