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Research article

Comparative antigenotoxic effects of aqueous leaf extracts of different cultivars of *Chrysanthemum morifolium* R. against genotoxicity induced by mercuric chloride using *Allium cepa* L. root chromosomal aberration assay

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Key words: Chromosomal aberrations, *Allium cepa*, *Chrysanthemum morifolium*, antigenotoxicity.

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Abstract

Plant bioassays are simple, inexpensive, accurate and direct methods to test the effect of a substance on the living systems and are considered important in the development of new drugs. One such bioassay is *Allium cepa* root chromosomal aberration assay. The present investigation was carried out to test the antigenotoxic potential of aqueous leaf extracts of different cultivars of *Chrysanthemum morifolium* against the genotoxicity induced by mercuric chloride. Simultaneous treatment of onion root tips with mercuric chloride and different concentrations of aqueous leaf extracts of different cultivars of chrysanthemum resulted in dose dependent decrease in frequency of chromosomal aberrations as compared to those induced by treatment with mercuric chloride alone. Maximum genoprotective potential was shown by Yellow coin cultivar, while minimum was shown by Cameo cultivar, against mercuric chloride induced genotoxicity.

Introduction

Our environment is full of toxic chemicals which occur either naturally or are the result of anthropogenic activities. They are continuously discharged into the air, water and soil resources, from where they make their way into the living systems. Once they enter a biological system it becomes very difficult to eliminate them. Of various toxic chemicals present in the environment, heavy metals pose a great threat to living systems [1-5]. Among various heavy metals, mercury is a major environmental contaminant which is used in the manufacture of thermometers, barometers, electrolytes, pesticides, paints, coating of mirrors, dental fillings etc [6]. High Solubility of mercury in water and the easiness with which it can shift to gaseous phase are the most important features of this heavy metal [7]. These properties explain the ability and effectiveness of mercury to move in different ecosystems and remain there for longer durations, and later on get deposited in soil and water bodies [8]. When mercury is in the form of water soluble salts, like mercuric chloride, it can prove to be highly poisonous. Studies regarding toxicity of mercury are mostly from humans and animals [9-10]. Mercury is known to induce a number of health problems in the living beings [11-13]. It has been demonstrated that in low doses, Hg can induce chromosomal aberrations including c-mitosis and spindle alternations [14-15].

Use of plants as medicines pre-dates written human history. The estimated number of plant species on earth is about 2,50,000. It is estimated that 35,000 to 70,000 species have, at one time or another, been used in some cultures for medicinal purposes. Medicinal plants and herbal medicines account for a significant percentage of the pharmaceutical market. About 122 different compounds have been identified in modern medicines which have been derived from traditional plant sources [16]. WHO is fully aware of the importance of herbal medicines for the health of the population in today's world. Herbal plants which can be eaten raw promote youthfulness and improve health status. Herbal medicines have been recognized as safe and readily available resources and their use is being encouraged [17]. Many plant extracts have been shown to possess anti-cancer, anti-bacterial, anti-oxidative and anti-proliferative properties [18-21]. Different bioassays have been developed to evaluate the genotoxic potential of certain chemicals and at the same time antigenotoxic effects of certain plant extracts. *Allium cepa* root chromosomal aberration assay (AlRCAA) is one such assay which has been widely used for the assessment of genotoxicity and antigenotoxicity of various plant extracts [22-26].

Chrysanthemum, (family Asteraceae) is an important cut flower plant, which also has medicinal properties. Flower extracts of this plant are used in the cure of various ailments like fever, cough, asthma, cold, influenza, skin

irritation, hypertension and angina. The plant has also been known to show antigenotoxic potential in many studies [27-30]. Although, large data is available on the bioactivities of the flowers of this plant species, we have little information about the bioactivities of the leaves. So, the present study was planned to examine the antigenotoxic activities of aqueous leaf extracts of ten cultivars of *Chrysanthemum morifolium* (Basanti, Bravo, Cameo, Flirt, Garden Beauty, Jaya, Sadbhawna, Winter Queen, Yellow Charm and Yellow Coin) against the genotoxicity induced by mercuric chloride in AlRCAA.

Experimental

Materials and methods

Preparation of extracts

Ten cultivars of *Chrysanthemum morifolium* (Basanti, Bravo, Cameo, Flirt, Garden Beauty, Jaya, Sadbhawna, Winter Queen, Yellow Charm and Yellow Coin) were chosen for the present study. Certified and disease free plants were procured from PAU, Ludhiana, Punjab. Fresh leaves of all cultivars were taken separately, washed thoroughly and kept in shade until completely dry. The dried leaves were powdered in a grinder. 25 g leaf powder was suspended in 250 ml of water and kept in incubator shaker at 120 rpm and 50^{0}C for 16 h. The suspension was then filtered and filtrate was considered as 100% chrysanthemum leaf extract. The filtered extracts were then stored at -20^{0}C till further use. 100% leaf extract was considered as the stock solution. Different concentrations (25%, 50%, 75%) of the extract were prepared from the stock solution.

Treatment with mercuric chloride

Mercuric chloride was purchased from Thomas Baker Chemicals, Mumbai, India while glacial acetic acid, orcein stain and other chemicals were purchased from Qualigens, Mumbai, India. 1000 ppm stock solution of mercury was prepared by dissolving 135 mg of mercuric chloride in 100 ml of tap water. Afterwards different concentrations of mercury (1, 0.75, 0.5, 0.2, 0.1 and 0.05 ppm) were prepared by serial dilutions of the stock solution.

Healthy bulbs of *Allium cepa* L. were grown in tap water in coupling jars. When the length of the roots reached about 1.0 cm they were transferred to different concentrations of the mercuric chloride. The length of roots for each concentration was noted after 5 days. From different concentrations of mercuric chloride, EC_{50} value was found to be 0.75 ppm, so this concentration of mercuric chloride was used as positive control for the cytological investigations (Table 1, Figure 1).

Antigenotoxic effect of chrysanthemum

Antigenotoxic effects of different concentrations of aqueous leaf extracts of *Chrysanthemum* were analyzed on mercuric chloride (0.75 ppm) induced chromosomal aberrations in AlRCAA. Onion roots were grown in water in coupling jars till they were 0.5-1.0 cm long. The bulbs were then shifted to other coupling jars having 0.75 ppm of mercuric chloride with different concentrations of the aqueous leaf extracts of each of ten cultivars of *Chrysanthemum* for 3 h. Roots tips were then thoroughly washed. Roots grown only in tap water served as negative control where as the root tips treated with 0.75 ppm of mercuric chloride concentration served as positive control.

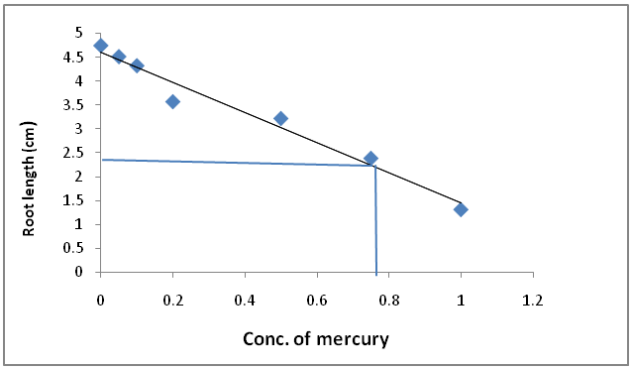

Figure 1. Effect of different concentrations of mercury on roots of *Allium cepa*.

Table 1. Effect of different concentrations of mercury on length of *Allium cepa* roots.

Conc. of mercury (ppm)	Root Length in cm (Mean ± SE)
NC	4.74 ± 0.050
0.05	4.51 ± 0.033
0.10	4.32 ± 0.176
0.20	3.57 ± 0.265
0.5	3.22 ± 0.144
0.75	2.41 ± 0.333
1.0	1.32 ± 0.088

The roots were then washed with water. They were plucked with the help of forceps and fixed in Farmer's fluid (glacial acetic acid: ethanol:: 1:3) for 24 hours.

Fixed root tips were first hydrolyzed in 1N HCl in a watch glass with intermittent heating up to 60^{0}C for 1 min. The roots were then transferred to a watch glass containing staining solution (1N HCl: aceto-orcein:: 1:9). The roots tips were then again heated intermittently up to 60^{0}C in a watch glass for 10-15 minutes and kept aside for 30-40 minutes. The extreme tips of the roots were cut with a sharp scalpel and then squashed in a drop of 45% acetic acid. Photomicrographs were taken with the help of Olympus binocular microscope.

Aceto-orcein stain was prepared by mixing 2g of orcein dye in 100 ml of 45% boiling glacial acetic acid. The

mixture was brought to room temperature, filtered and then stored in brown bottles.

Scoring

The slides of squashed root tips were observed and scored under binocular microscope to study different chromosomal aberrations. 300 dividing cells were scored for each concentration.

Statistical analysis

A linear relationship between different concentrations of the aqueous leaf extracts of each cultivar of *C. morifolium* and percent inhibition of chromosomal aberrations was obtained by the regression and correlation analysis (using excel). Regression coefficient for relation between concentration of the extract and percent inhibition in chromosomal aberrations for each extract was calculated.

Results and discussion

Results

The results showing effect of mercuric chloride on onion root length are presented in Table 1. Increasing concentration of mercury showed decrease in length of roots, when compared with negative control. The EC_{50} value for mercury was found to be 0.75 ppm which caused nearly 50% inhibition of root length as compared to control. This concentration was used as positive control for induction of chromosomal aberrations in *Allium cepa* root tip cells. Treatment of onion root tips with 0.75 ppm mercury caused induction of different kinds of chromosomal aberrations. C-mitosis, delayed anaphase, vagrants, laggards, stickiness, chromosomal bridges, ring chromosomes and chromosomal breaks constituted the spectrum of chromosomal aberrations in the root tip cells.

A dose dependent decrease in the induction of chromosomal aberrations induced by mercury in the root tip cells of *Allium cepa* was observed following simultaneous treatment with different concentrations of aqueous leaf extracts of different cultivars of chrysanthemum and 0.75 ppm mercury (Table 2). The antigenotoxic potential of different cultivars varied slightly from one another. Bravo and Yellow Coin cultivars showed maximum antigenotoxic potential (77.42%), followed by Basanti, Yellow Charm and Garden Beauty (73.55%), Flirt (72.90%), Winter Queen (71.61%), Jaya (70.96%) and Sadbhawna (67.09%). Minimum anti genotoxicity was displayed by Cameo (64.51%).

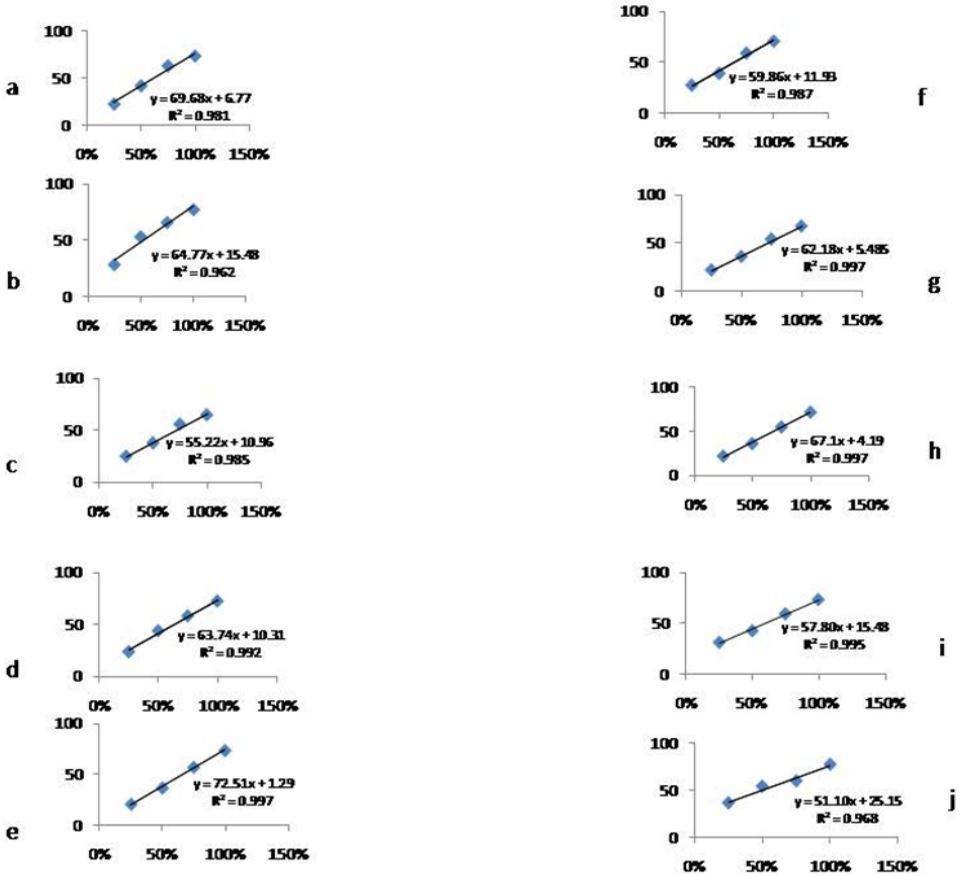

Figure 2. Relationship between simultaneous treatment with different concentration of aqueous leaf extracts of different cultivars* of *Chrysanthemum morifolium* and percent inhibition of chromosomal aberrations in AlRCCA. *a. Basanti, b. Bravo, c. Cameo, d. Flirt, e. Garden Beauty, f. Jaya, g. Sadbhawna, h. Winter Queen, i. Yellow Charm, j. Yellow Coin.

Table 2. Effect of mercury (0.75 ppm) and simultaneous treatment of *Allium cepa* root tips with different concentrations of aqueous leaf extracts of different cultivars of *C. morifolium* and mercury (0.75 ppm) on induction of chromosomal aberrations in *Allium cepa* root tip cells.

		Number of aberrant cells/300dividing cells										
Name of Cultivar	Conc of ext. (%)	Cm	Vg	Lg	Da	St	Cb	Rc	Ck	Total	PA	PI
NC	-	3	2	1	2	4	-	-	1	13	4.33	-
PC	-	29	16	14	22	20	29	2	36	168	56.0	-
BS	25	25	15	11	18	16	17	-	31	133	44.33	22.58
	50	16	12	8	13	11	16	-	27	103	34.33	41.93
	75	10	9	5	7	8	13	-	18	70	23.33	63.22
	100	9	8	3	5	7	8	-	14	54	18.0	73.55
BR	25	24	12	11	17	15	21	-	25	125	41.67	27.74
	50	18	9	9	7	9	17	-	17	86	28.67	52.90
	75	14	7	6	5	7	13	-	14	66	22.0	65.8
	100	9	5	5	3	5	11	-	10	48	16.0	77.42
CM	25	25	12	10	16	14	21	-	32	130	43.33	24.51
	50	20	11	13	10	9	21	-	26	110	36.67	37.42
	75	16	9	8	8	9	17	-	15	82	27.33	55.48
	100	13	7	7	6	7	15	-	13	68	22.67	64.51
FL	25	26	10	14	14	17	18	-	31	130	43.34	24.51
	50	17	9	12	11	13	19	-	18	99	33.0	44.51
	75	15	8	7	9	9	16	-	13	77	25.67	58.71
	100	12	6	5	8	6	7	-	11	55	18.33	72.90
GB	25	30	9	12	20	16	21	-	29	137	45.67	20.0
	50	25	11	8	14	11	20	-	23	112	37.33	36.13
	75	18	7	8	8	13	9	1	16	80	26.67	56.77
	100	9	7	7	5	7	8	-	11	54	18.0	73.55
JY	25	22	13	12	14	15	20	-	29	125	41.67	27.74
	50	19	10	9	11	10	22	-	26	107	35.67	39.35
	75	14	5	8	10	10	19	-	10	76	25.33	59.35
	100	13	5	3	8	6	13	-	10	58	19.33	70.96
SB	25	17	10	12	21	22	24	-	29	135	45.0	21.29
	50	23	9	9	16	17	18	-	21	113	37.67	35.48
	75	16	5	7	15	14	11	-	17	85	28.33	53.55
	100	9	7	6	11	9	10	-	12	64	21.33	67.09
WQ	25	25	15	9	20	13	27	-	25	134	44.67	21.93
	50	18	10	11	15	17	21	1	19	112	37.33	36.13
	75	16	10	8	17	4	16	-	12	83	27.67	54.84
	100	12	6	4	6	6	12	-	11	57	19.0	71.61
YCh	25	27	10	12	17	16	23	-	15	120	40.0	30.96
	50	20	13	12	20	9	10	1	16	102	34.0	42.58
	75	22	8	7	16	10	3	-	10	76	25.33	59.35
	100	13	7	5	6	10	4	-	9	54	18.0	73.54
YC	25	21	9	7	19	17	21	-	17	111	37.0	36.77
	50	15	8	10	10	12	13	-	16	84	28.0	54.19
	75	17	7	8	12	7	15	-	9	75	25.0	60.0
	100	11	4	5	8	7	5	-	8	48	16.0	77.42

NC- Negative control, PC- Positive control (mercury, 0.75 ppm), Cm- C-mitosis, Vg- Vagrant, Lg- Laggard/s, Da- Delayed anaphase/s, St- Stickiness, Cb- Chromosomal bridge/s, Rc- Ring Chromosome/s, Ck- Chromosomal break/s, PA- Percent aberrations, PI- Percent inhibition (a-b/a-c) X 100, where a= number of aberrant cells induced by positive control, b= number of cells induced by extract and mercuric chloride, c= number of aberrant cells induced by negative control), BS-Basanti, BR- Bravo, CM- Cameo, FL- Flirt, GB- Garden Beauty, JY- Jaya, SB- Sadbhawna, WQ- Winter Queen, YCh- Yellow Charm, YC- Yellow Coin.

The regression analysis revealed positive correlation between percent inhibition of chromosomal aberrations and the concentration of the extract (Figure 2).

Discussion

Natural products, in particular which originate from plants are extremely important as therapeutic agents. Our dietary food contains substances which may have anti

mutagenic/anticarcinogenic/antigenotoxic effects that can prevent or reverse some of the effects of mutagens/carcinogens/genotoxins [31]. Therefore, it is important to assess both; the genotoxic properties of the chemicals which are present in the environment as well as the properties of the natural products to protect the genetic material from the damage caused by use of these substances.

In the present study, genoprotective potential of aqueous leaf extracts of ten different cultivars of *Chrysanthemum morifolium* was studied using *Allium cepa* root chromosomal aberration assay. The *Allium cepa* test, first introduced by Levan (1938), is a widely used test system as it is an excellent model for *in vivo* studies, where roots grow in direct contact with the substance of interest causing possible damage to the DNA by that substance or the protection offered to the genetic material by use of another substance can be studied [32]. This test is very suitable for the genotoxicity/antigenotoxicity studies because of certain advantages- (i) Root growth is very sensitive to the presence of pollutants in the environment (ii) Mitotic phases are very clear in onion (iii) Stable karyotype (iv) Clear and quick response to the presence of genotoxins (v) Small number and large size of the chromosomes [33]. Abnormalities in chromosomes such as change in the structure of chromosomes or loss/gain of chromosomes are associated with enhanced risk of carcinogenesis and in the progression of neoplastic transformation [34].

In this study, the genotoxicity of mercury was first assessed using *Allium cepa* assay using its different concentrations. The most important macroscopic parameter is root length [35] so EC_{50} (half effective concentration, at which inhibition in onion root length was half the maximum length) was calculated using this parameter, which was 0.75 ppm. Simultaneous treatment of onion root tips with 0.75 ppm of mercury and aqueous leaf extracts (25%, 50%, 75% and 100%) of ten different cultivars of *Chrysanthemum morifolium* resulted in dose dependent decrease in frequency of chromosomal aberrations. The protective function of chrysanthemum extract in the simultaneous treatment can be attributed to the presence of flavonoids in the leaf extracts which function as antimutagenic agents [36]. Flavonoids have been utilized in folk as well in the modern medicines around the world. These antimutagenic substances act directly on the substances that induce DNA by mutations inactivating them chemically or enzymatically or by sequestering reactive molecules [37].

As the results suggest, protection by the extracts is higher at higher concentrations, which clearly mean that the bioactive ingredients which are responsible for the antigenotoxicity are present in greater amounts in the higher concentrations. Earlier studies in phytochemical analysis of the plants have revealed the presence of various substances which include flavonoids, sesquiterpenoids, triterpenoids and quinic acid caffeates [38-42]. All these compounds possibly act synergistically. Flavonoids, in particular have been extensively reported in chrysanthemum [36, 43-44] and have shown many pharmacological properties including antiinflammatory, immunoregulatory, antimutagenic, antitumor and hepatoprotective effects [45-46].

Conclusion

To conclude, the present study indicates the genoprotective potential of aqueous leaf extracts of different cultivars of *Chrysanthemum morifolium*. Although flowers of this plant species are already in use in the form of chrysanthemum tea in many parts of world since many decades, use of leaves have an additional advantage as they are available for most part of the year. This study is the first of its kind to bring out the genoprotective potential of the leaves of *Chrysanthemum morifolium* which can be further explored in other systems before one can recommend their usage in any form.

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