Cytogenetic Estimation of Broccoli Extract on Dexamethasone Treated Mice

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Abstract
The current study was intended to evaluate the active compounds found in hydro-alcoholic extract of *Brassica oleracea var. italica*(broccoli), also to estimates the percentage of chromosomal aberrations (CAs) ,mitotic index (MI) and micronuclei (MN) formation in mouse bone marrow cells .The mice were divided into, negative group received (0.2 ml of Distilled Water)intraperitoneally ,positive group I was given single dose of dexamethasone (0.4 mg/kg for 24 h) and positive group II (acute toxicity assay) administrated with (0.2 ml) of high level dose (800 mg/kg) of extract for 5 days. Interaction groups treated with different doses of extract (50,100,200,400 and 600 mg/kg) for 5 days in addition to a single (0.4 mg/kg) of dexamethasone per each dose of extract**.** High level dose (800mg/kg) of broccoli extract had show null genotoxicity for bone marrow cells of mouse. Dexamethasone showed significant effects (P< 0.05) in reducing mitotic index and increased chromosomal aberrations and micronuclei in mouse bone marrow cells (*in vivo*). Broccoli extract significantly increased MI and reduced (CA ,MN) (P< 0.05) especially at does (400 mg/kg).
Keywords- mitotic index, Micronuclei formation ,Chromosomal aberrations , Dexamethasone, *Brassica oleracea var. italica* (broccoli), hydro-alcoholic extract.

I. Introduction

A large number of studies confirm that reduction of chronic degenerative diseases risks such cancers and others belong to great and regular consumption of fruits and vegetables (1). The World Health Organization (WHO) has documented the importance of herbal medicine suggesting that it is a great number of natural products submitted to as chemo preventive agents that have antioxidant, anti-carcinogenic and anti-mutagenic activity (2). Broccoli is a latent healthy vegetables which has antioxidant activity because of its high rate of phenolic content, it also encompasses mixture of antioxidant compounds like carotenoids, ascorbic acid and alpha tocopherol which famous to forbid onset of chronic disease (3). Synthetic pharmaceutical drugs with glucocorticoids GCs had similar effects, are used in cases, ranging from skin diseases to brain tumors, dexamethasone and its derivatives are almost pure GCs *(4)*. Dexamethasone is administered to treat many inflammatory and autoimmune diseases, like rheumatoid arthritis and bronchospasm .Idiopathic thrombocytopenic purpura (immune thrombocytopenia), a decrease in numbers of platelets due to an immune problem, responds to dexamethasone administration (*5)*.

II. Materials and Methods

A. Doses and concentrations of the Broccoli extract

Six doses of the extract were used (50,100, 200,400, 600 and 800 mg/kg). To prepare the essential doses and concentrations, the extract was thawed in D.W and filtrated by filter paper (5).

B. Dose and concentration of the dexamethasone

Dexamethasone (8mg/2ml) ampoule which was produced by (MEDOCHIE LTD ,Cyprus), it was earned from Al Karama Teaching Hospital as packed in glass ampoule of a capacity 2 ml**.** For mouse injection, a dose of 0.4 mg/kg was tested , so concentration has been found causes genotoxicity in mouse bone marrow (*6*). To prepare the required dose and concentration, which equivalent to (0.01 mg/mouse), with distilled water the drug solution was diluted.

C. Laboratory animals

Swiss male mice (80) of BALB / C were earned from National Center for Drug Control and research / Ministry of Health / Baghdad. Mice age at the start of experiments was 8-12 weeks and their mass was 25±2 gram. They were separated into groups, each group was put in a separate plastic cage (details and information of these groups are described below). The animals were preserved at a temperature of 23 – 25°C, they had free excess to water and food (standard pellets).

D. Administration of laboratory animals

1) Control groups

Three categories of controls were used for this experiment and treated as follow:
Group I: Negative control (10 mice) treated with (0.2 ml) D.W.
Group II: positive group I (10 mice) treated with dexamethasone (0.01 mg).

was injected for 24 hr, and then the mice were killed, bone marrow samples were gained and cytogenetic analyses were done.

Group III: positive group II (10 mice) treated with (0.2 ml) of extract (800 mg/kg).

2) **The interaction studies**

The interaction between broccoli extract and dexamethasone was carried out to estimate the role of the extract in reduction the cytogenetic effects of this drug in male mice. The animals (50 mice) were separated to five groups (10 mice in every groups) and every group treated (0.2 ml) (IP) with one of these five doses of the extract (50,100,200,400 and 600 mg/kg) for 5 successive days (one dose / one day). Single dose of dexamethasone 0.4 mg/kg (0.25 ml) injected after 2 hr from final dose of extract, and then the mice were murdered after 24hr from dexamethasone treatment, bone marrow samples were submitted for cytogenetic analysis.

III. CYTOGENETIC EXPERIMENTS

A. Chromosome preparation from somatic cells of the mouse bone marrow

The procedure was completed according to Allen *et al.* (1977)(7). The mice were injected (I.P.) with (0.25 ml) of colchicine with concentration of (125µg/ml) 2 hours before murdering the animals. The femur bone was injected with five ml of PBS was carefully. The test tubes were put in the centrifuge apparatus at speed of 2000 rpm for 10 min. Five ml of pre warmed (37C°) potassium chloride (KCL) was added to tubes as a hypotonic solution (0.075 M). Then the tubes were incubated in water bath at (37 C º) for 20 minute with continuous shaking from time to time. The incubated tubes were centrifuged for 10 min at 2000 rpm. The fixative solution was added (drop by drop) with shaking continuously, the volume was completed to 5 ml. The tubes were put at (4 C º) for 30 min in order to fix the cells. The fixed tubes were centrifuged for 10 min at 2000 rpm. The fixation step was repeated three times to make cells suspended finally in 2 ml of the fixative solution. By a micro pipette (4-5) drops from cell suspension were dropped vertically on slide .Later, the slides were kept in remote place to dry overnight. With Giemsa stain the slides were stained for 15 min, and then washed with D.W. Five slides for each animal were ready for cytogenetic analysis.

IV. CYTOGENETIC ANALYSIS

A. ***Mitotic index (MI) assay***

The slides were examined below high power (40 x) of light microscope, and 1000 of mitotic and non mitotic cells were counted. The percentage rate was calculated for the mitotic cells according to the following equation(8):-

$$\text{“Mitotic index } (\%) = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total Count}} \right) \text{x } 100$$

B. ***Chromosomal aberration (CA) assay***

The ready prepared slides were examined beneath the oil immersion lens for 100X metaphase stage cells per each slide , and the cells ought to be at the metaphase stage of the mitotic division where the chromosomal aberrations were obvious and the percentage of these aberrations be estimated(7).

C. Micronucleus MN assay

The experiment was been done dependent on method of (Schmid, 1975)(9) .The animal is sacrificed by, and the femur bone was obtained . The bone was gapped with 1ml of human plasma (heat inactivated) to accumulate the cellular content inside the test tube. The test tube was centrifuged (1000 rpm) for five minutes. The cellular precipitate is gently mixed, and a thin smear is form on a clean slide. The slide is left for (24 hr.) at a standard room temperature to dry. The slides are fixed by absolute methanol for (5 min.), then stained by Giemsa stain for (15 min), then washed by D.W and left to dry. Five slides for every animal are prepared for micronucleus test. The slides are examined under the oil immersion lens, and at least 1000 polychromatic erythrocytes (PCE) are examined for the appearance of micronucleus. The micronucleus index is got using the following equation (10):

$$\text{Micronucleus Index} = \left(\frac{\text{Numberof Micronuclei}}{\text{TotalCountof PCE}} \right) \text{x } 100 \text{ .}$$

V. DETECTION OF ACTIVE COMPOUNDS IN BROCCOLI

(A) Determination of total phenol content

Adding in test tubes 1ml of gallic acid concentrations (20,40,60,80 and 100 mg\ml). Add 5ml of distilled water for each tube. Add 0.5 ml of Folin Ciocaleu reagent. Then add Sodium Carbonate 1.5 ml (20%) for 5 min. Then, volume completed to 10 ml. On 750 nm ,absorbance was measured via spectrophotometer apparatus.

Sample preparation with concentration 1mg\ml. Use gallic acid as a standard.

(B) Determination of total Flavonoids

Adding in test tubes 1ml of qurcetin concentrations (25,50,100,200,300,400 and 500 mg\ml). Add 4ml of distilled water for each tube. Add 0.3 ml of Sodium Nitrate (5%) for 5 min. then add 0.3 ml of Aluminum Chloride (10%) for 6 min. final addition was 2 ml of Sodium Hydroxide 2M then, volume completed to 10 ml. On 510 nm ,absorbance was measured via spectrophotometer apparatus**.** Sample preparation with concentration 1mg\ml. Use qurcetin as a standard.

VI. STATISTICAL ANALYSIS

The gained data were statistically analyzed by using a 2*2contengency table (X2)(11). The difference is considered significant when the probability rate less than $p<0.05$.

VII. Results and discussion

i. Cytogenetic analysis of animal groups

The results of metaphase test are presented in table (I). There are significant differences when we compare between negative control and positive control (I and II) and this differences were due to the toxic effect of dexamethasone alone by reducing the mitotic index (MI) and broccoli extract alone. And also there is a significant different when we compare the interaction groups of broccoli (600, 400, 200, 100 and 50) with negative control. All these results were statistically significant ($p<0.05$).

Table I: Percentages of mitotic index in bone marrow of mice for negative control, positive control groups and treatment groups.

Dose	No. of animals	No. of examined cells	NO. of dividing cells	**%
Negative control	5	25000	1417	5.668
Dexamethasone (0.4 mg\kg)	5	25000	1030	*a 4.12
broccoli extract (800mg\kg)	5	25000	1437	*a5.748
Dexamethasone and Broccoli (600 mg\kg)	5	25000	725	* b 2.9
Dexamethasone and Broccoli(400 mg\kg)	5	25000	1310	*b 5.25
Dexamethasone and Broccoli (200 mg\kg)	5	25000	1230	*b4.92
Dexamethasone and Broccoli (100 mg\kg)	5	25000	935	*b3.74
Dexamethasone and Broccoli (50 mg\kg)	5	25000	675	*b2.7

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p<0.05$).

Table II: Percentages of different types of chromosomal aberrations (CA) in bone marrow of mice for negative control, positive control groups and treatment groups.

Groups	No. of animal	NO. of examined cell	Chromosomal Aberration													
			Acentric Fragment		Gap		Break		Fragment		Ring		Polyploidy		Total	
			NO.	%	NO.	%	NO.	%	NO	%	NO.	%	NO	%	NO.	%
Negative control	5	500	140	28	13	2.6	28	5.6	8	1.6	1	0.2	0	0	190	38
Dexamethasone (0.4 mg\kg)	5	500	198	39.6	16	3.2	79	15.8	32	6.4	5	1	1	0.2	330	*a 67.2
broccoli extract (800mg\kg)	5	500	95	19	6	1.2	3	0.6	6	1.2	2	0.4	4	0.8	116	*a 23.2
Dexamethasone and Broccoli (600 mg\kg)	5	500	125	25	25	5	20	4	45	9	50	10	20	4	285	*b 57
Dexamethasone and Broccoli(400 mg\kg)	5	500	22	110	10	2	20	4	5	1	10	2	3	0.6	158	*b31.6
Dexamethasone and Broccoli (200 mg\kg)	5	500	115	23	26	5.2	15	3	10	2	35	7	0	0	201	*b40.2
Dexamethasone and Broccoli (100 mg\kg)	5	500	165	33	40	8	20	4	15	3	65	13	0	0	305	*b61
Dexamethasone and Broccoli (50 mg\kg)	5	500	150	30	35	7	50	10	30	6	50	10	0	0	315	*b63

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p<0.05$)

Table (II) shows the results of CAs types for control groups and the series Interaction groups. The interaction treatment was carried out between single dose (0.4mg/kg) of dexamethasone and multiple dose of broccoli (600, 400, 200, 100 and 50 mg\kg). The results had a great difference when compared with the negative

control group , positive control I, and with positive group II.

The results of this experiments showed the ability of broccoli extract to reduce the effect of the drug in the mouse bone marrow on CAs. Also the plant extract have an ability to reduce the effect of median drug doses.

Table III: Percentages of micronuclei (MN) in bone marrow of mice for negative control, positive control groups and treatment groups .

groups	No. of animal	No. of examined cells	micronuclei	%
control	5	25000	711	2.824
Dexamethasone(positive control I)	5	25000	1610	[a*]6.44
Broccoli 800mg\kg(positive control II)	5	25000	525	[a*]2.1
Dexamethasone and Broccoli (600 mg\kg)	5	25000	1425	[*b]5.7
Dexamethasone and Broccoli (400 mg\kg)	5	25000	1050	[*b] 4.2
Dexamethasone and Broccoli (200 mg\kg)	5	25000	1354	[*b]5.416
Dexamethasone and Broccoli (100 mg\kg)	5	25000	1525	[*b] 6.1
Dexamethasone and Broccoli (50 mg\kg)	5	25000	1550	[*b]6.2

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p<0.05$)

The natural incidence of MN for negative controls was 2.824%. dexamethasone increased the micronucleus to 6.44% and broccoli extract decreased to 2.1% respectively. This gradient of values is consider significant ($p<0.05$) with negative control. Table (III) showed the high percentage of MN among Interaction groups of broccoli (50mg\kg)(6.2%), which gave significant differences when compared with negative control, positive control I and positive control II.

Dexamethasone is pure GCs (4). GCs may be attributed to changes in the genesis, programmed cell death (apoptosis) of osteoblasts and metabolic activity(12). thus supporting their use of chemotherapeutic agents for lymphomas, leukemias and myeloma (13). Also they influence the expression of osteoblastic proteins that control both cell-cell and cell-matrix exchanges(14). On other hand, extracts of broccoli sprouts (have either indol-3-carbinol or sulforaphane as the primary enzyme inducer) were extremely effective in reducing the frequency, multiplicity, and rate of growth of mammary tumors(15). Corticosteroid drug have genotoxic and mutagenic effects such as sister chromatid exchanges (SCEs) in bone marrow cells moreover chromosomal aberrations (CAs) in mouse spermatocytes(16). Because that genotoxicity, the MN value is directly proportional with CAs, CAs patterns shown in figure 1 While figure 2 shows MN formation on mice bone marrow cells.

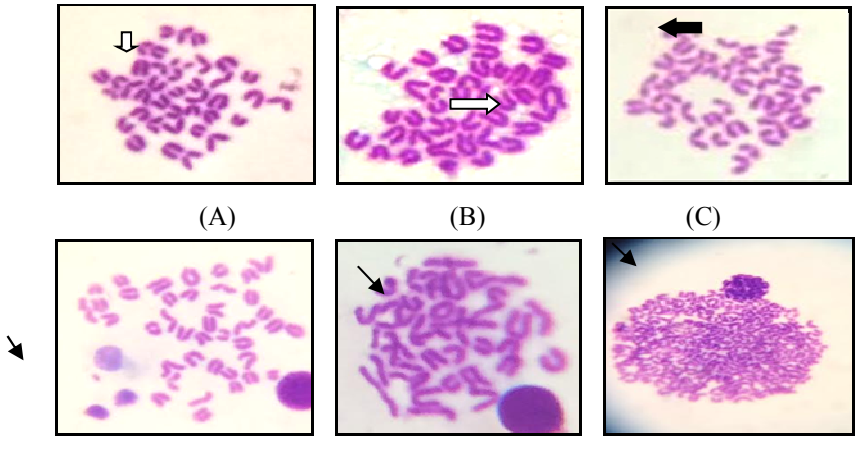

Figure 1: Chromosomal aberrations in mice bone marrow at (100x). A: acentric fragment B: Gap C: break D: fragment E: Ring F: polyploidy.

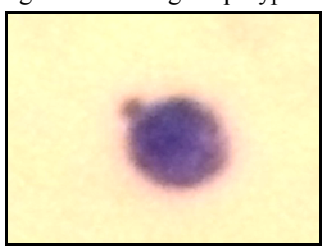

Figure 2: MN formation at (100x).

ii. Chemical Analysis of broccoli extract

1.Detection of total phenol content and Flavonoids content

Results obtained by S.P showed that broccoli extract had high concentrations of phenolic compounds that as explained in table (IV). Absorbance is measured at a 750nm wavelength, then the sample prepared with concentration (1 mg\ml) . Finally , the measurement of phenols depended on gallic acid units (mg\ml) as a standard. while, the total flavonoids content done by concentration (1 mg\ml), then the absorbance measured with (510nm) wavelength via S.P apparatus as shown in table (IV)too. Qurcetin was used as a standard (1 ml) with selection of concentrations(25, 50, 100,200,300,400 and 500). The measurement of Flavonoids depended on qurcetin units(mg\ml) as a standard.

Table(IV): The sequence of total phenolic and flavonoid compounds found in the sample of broccoli extract using spectrophotometer assay.

No.	Test	Conc. Mg\ml	ABS. nm	Duplicate
1	Total phenols	70	0.186	1
		67.30	0.179	2
		68.65	0.1825	Rang
2	Total Flavonoids	140	0.026	1
		123.8	0.023	2
		131.9	0.0245	Rang

Numerous groups of phytochemicals in specific vegetables have been identified for their potential reduction of cancer risk. For instance, broccoli sprouts are a rich source of polyphenols, which have antimutagenic and antioxidative activities(17). Flavonoids are the biggest group of phenolic compounds and contain basic skeleton consists of three rings (C6-C3-C6). They are classified into six main classes according to their exchange pattern in the B- and C-rings, which are flavan-3-ols, flavones, anthocyanins, flavonols, isoflavones and flavanones (18).

VIII. Conclusions

The study was concluded that broccoli is safe and non-toxic plant, which did not show significant biological effects on mouse in tested dose. Dexamethasone has been observed to be a genotoxic in mice, which demonstrated the ability to decrease in mitotic index, increase chromosomal aberrations and micronuclei induction. The intermediate dose (400 mg/kg) Broccoli's extract could be considered as an optimum and suitable dose, in terms of dexamethasone toxic effect reduction in mice. This effect may belong to the natural compounds of broccoli's, such as flavonoids and phenols, which can abolish the detrimental effects of dexamethasone drug following extract treatment.

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