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Original Article

Micronucleus formation assay and phagocytic index in mice administered water leaf extracts of *Camellia sinensis*

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Summary

Background: Green tea *Camellia sinensis* is non fermented tea. The tea is an infusion of flavorful leaves that has been consumed for centuries as a beverage and is valued for its medicinal properties. Over the last few decades green tea has been subjected to many scientific and medical studies to determine the extent of its long-purported health benefits, with some evidence suggesting regular green tea drinkers may have lower chances of heart disease and developing certain types of cancer. Green tea has also been claimed useful for weight loss management.

Methods: Three doses of the hot water extract were used (19.2, 38.4 and 57.6 mg/kg) to investigate micronucleus formation and phagocytic index. Mice were divided into two groups. Group one; control and group two; mice were administered with three doses of the green tea extract orally as a single dose (0.1 ml) per day for 7 days. Then the mice were sacrificed in day 8 for immunological and cytogenetic assessments.

Results: Results revealed that the first and third doses of green tea hot water extract were significantly effective in enhancing the values of phagocytic index and reducing micronucleus formation.

Conclusions: Hot water may be a good solvent to extract active component from *Camellia sinensis* especially catechin, polyphenols, polysaccharides, flavonoids and vitamins. The high dose of hot water extracts of *Camellia sinensis* showed an excellent enhancing effects on the function of immune system of mice, moreover, reduction in micronucleus formation was recorded at such dose.

Key words: Green tea, Cytogenetic, Immunological effect

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Introduction:

Green tea is a type of tea made solely from the leaves of *Camellia sinensis*, that has undergone minimal oxidation during processing. Many varieties of green tea have been created in countries where it is grown, these varieties can differ substantially due to variable growing conditions, processing and harvesting time(1).The phytochemical screening of tea revealed the presence of alkaloids, saponins, tannins, catechin and polyphenols. The cardinal antioxidative ingredient in the green tea extract is green tea catechins which comprise four major epicatechin derivatives; namely, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) (2). Other components include three kinds of flavonoids, known as kaempferol, quercetin, and myricetin. A remarkably higher content of myricetin which may have some bioactivity against pathogen is detected in tea and its extracts than in many other plants (3). Antimutagenic activity of green tea was studied *in vivo* which include metaphase chromosome analysis, micronucleus analysis and sister chromatid exchange assays (4).

The micronucleus test is a method devised primarily for screening of mutagenic chemicals for chromosome breaking effect. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome lagging at anaphase or from a centric chromosomal fragments (5). Micronucleated polychromatic erthrocytes (PCE) was particularly useful index of an *in vivo* bone marrow cytogenetic damage, and such finding formed the basis to develop a simple *in vivo* assay based on an identification of micronuclei in PCE of mouse bone marrow. Since then, many researchers have employed this assay for the assessment of mutagenic effects induced by different mutagens (6)

It had been becoming clear that there was a relationship between antioxidants in green tea and immune functions. The protection against lipid peroxidation attributed to dietary antioxidants. Antioxidants prevent the loss of membrane fluidity which in turn determines much of the protective functions of immune cells (7). There was increasing evidence that green-tea polyphenols had anti-inflammatory effects, possibly mediated by their antioxidant properties, for instance, epigallocatechin gallate inhibits okadaic acid which induced tumor necrosis factor (TNF) production and gene expression in BALB/3T3 cells (8). Green-tea polyphenols also inhibit nitric oxide (NO) production in peritoneal exudates (macrophage) cells, inhibit lipopolysaccharide (LPS)-induced NO production and iNOS gene expression in isolated peritoneal macrophages by decreasing TNF-B activation, block TNF- gene expression by inhibiting NF-B

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activation, and reduce inflammatory responses (9).

Materials and methods:

Preparation of Plant Extracts

The air-dried leaves of green tea (*Camellia sinensis*) were collected from locally market. The leaves were grinded into powdery form using a sterile electric grinder.

Twenty five grams of the leaves powder were extracted for 10 min in 500 ml of distilled water using the soxhlet apparatus and the source of heating was hot water bath (70°C). The leaf extract solution was then evaporated at 45°C using a rotary evaporator, and the resultant crude extract was frozen at -20°C until use to prepare the required doses (10). Three doses of the hot water extract were prepared (19.2, 38.4 and 57.6 mg/kg). These doses were correspondent to 10, 20 and 30%, respectively of the LD_{50} dose in mice (192mg/kg) of the green tea (11).

Sixteen albino male mice were divided into two groups. **Group I**: Mice treated with normal saline (negative controls) (4 mice) and **Group II**: Mice were administered with three doses of the water extract (19.2, 38.4 and 57.6 mg/kg) (12 mice). The tested materials were administered as a single dose (0.1 ml) per day for 7 days. Then the mice were sacrificed in day 8 for laboratory assessments.

The evaluation of phagocytosis was carried out according to Metcalf (12) with some modifications on phagocytes obtained from the peritoneum of mice. The animal was anaesthetized with chloroform, and then injected intraperitoneially with 3 ml of normal warm saline (37°C). After that, the abdominal region was massaged for 3 minutes. The animal was dissected, and the peritoneal cells were collected with a pasture pipette and transferred to a clean test tube. The tube was centrifuged (2000 rpm/minutes) for 5 minutes. The cells were suspended in 1 ml of normal saline, counted and their number was adjusted to 10^6 cell /ml. Also, the cell viability was assessed using trypan blue stain.

To carry out phagocytosis, 0.2 ml of cell suspension, 0.1 ml of heat-killed yeast suspension and 0.1 ml of human plasma AB were mixed in a test tube and incubated in a shaking water bath (37°C).After 30 and 60 minute incubations, smears were made and the slides were air-dried, and then stained with Giemsa stain for 15 minutes. The slides were examined under oil immersion lens (100X), and at least 100 yeast-phagocytic and non-phagocytic cells were randomly counted. The phagocytic activity was expressed as a phagocytic index, which was calculated using the following equation:

Phagocytic Index (%) =

$$\left(\frac{\text{Number of Phagocytic Cells}}{\text{Total Count}}\right) \times 100$$

Micronucleus formation assay was carried out according to Schmid (13). The mouse was sacrificed by cervical dislocation, and then dissected to obtain the femur bone. After cutting both ends of the bone, it was grapped from the middle with a forceps in a vertical position over the edge of a test tube, and then the cellular content was collected in test tube with (2 ml) of a heat inactivated (56°C for 30 minutes) human AB plasma. The test tube was centrifuged (1000 rpm) for 10 minutes, and the supernatant was discarded. The cellular deposit was gently mixed, and a thin smear was made on a clean slide, and air-dried at room temperature. The smear was fixed with absolute methanol for 5 minutes, and then air-dried at room temperature, stained with Giemsa stain for 15 minutes, and rinsed with distilled water. The slides were examined under oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus formation. The micronucleus index was obtained using the following equation:

Micronucleus Index (micronucleus/cell) =

$$\left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}}\right) \times 100$$

Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using the computer programme SPSS version 7.5.The difference was considered significant when the probability value was equal or less than 0.05.

Results:

In this study three doses (19.2, 38.4 and 57.6 mg/kg) of *Camellia sinensis* (green tea) extracts was evaluated for their immunological and cytogenetic effects on albino male mice.

Phagocytosis after 30 and 60 minutes of incubation

Mice treated with green tea extract showed a significant increased in phagocytosis after 30 and 60 minutes of incubation {(77±2), (50±3) and (64±1 %)} and {(83±1), (56±3) and (74±2) % respectively }as compared with control {(3±24) % and (44±2) % respectively} table (1) and (2).

Table (1) : Phagocytic index of peritoneal cells (mean ± standard error) in male mice treated with hot watery extracts of Green tea and control group after 30 minutes incubation.

Groups	Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm .blood)	Statistical Evaluation
Control group	0.00	34±2	A
Group 1	19.2	77±2	B
Group 2	38.4	50±3	C
Group 3	57.2	64±1	D

Different letters in the same column:
significant difference ($P \leq 0.05$) between means.

Table (2): Phagocytic index of peritoneal cells (mean ± standard error) in male mice treated with hot water extracts of Green tea and control group after 60 minutes incubation.

Groups	Dose (mg/kg)	Mean ± Standard Error (cells/ cu.mm .blood)	Statistical Evaluation
Control group	0.00	44±2	A
Group 1	19.2	83±1	B
Group 2	38.4	56±3	C
Group 3	57.6	74±2	D

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

Figure (1): Phagocytosis of Candida albicans by Macrophage.

The micronucleus formation was assessed in polychromatic erythrocytes of bone marrow. The first and third doses showed a significant decrease in micronucleus frequency (0.004±0.0006) and (0.002±0.0002) micronucleus/cell, respectively as compared with control group (0.007±0.0004 micronucleus/cell.). The second dose caused a mild decrease in micronucleus frequency (0.006±0.0085 vs. 0.007±0.0004 micronucleus/cell) table (3).

Table (3): Micronucleus formation in bone marrow cells (mean ± standard error) in male mice treated with hot water extracts of Green tea and control group.

Groups	Dose (mg/kg)	Mean ± Standard Error (cells/cu.mm .blood)	Statistical Evaluation
Control group	0.00	0.007±0.0004	A
Group 1	19.2	0.004±0.0006	B
Group 2	38.4	0.006±0.0085	AB
Group 3	57.6	0.002±0.0002	C

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

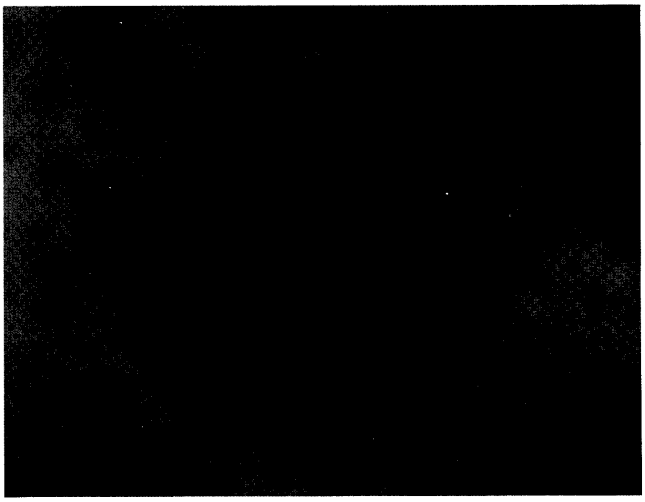

Figure (2): Micronucleus formation in mice treated with green tea extract

Discussion:

Results demonstrated that a treatment with green tea extracts showed a significant increase in phagocytic index. Recently, it had been demonstrated that catechin-polysaccharide complex of tea extracts had a very important immunomodulating activity. Phagocytic cells recognize the molecular features of catechin-polysaccharide complex in green tea extract through their receptors and may be associated with the immunostimulating activity of the catechin-polysaccharide complex (3). A study reported in the Proceedings of the National Academy of Sciences indicated that an amino acid called L-theanine found in black, green and oolong tea can boost the body's defense against disease (14). Researchers at Harvard Medical School gave 11 test subjects, five to six cups of tea equal to 230 mg of L-theanine, for four weeks. Results showed a three- to four-fold increase in interferon-gamma production and white blood cells from the same groups of test subjects and they found that tea contains chemicals known as alkylamine antigens, as an intact molecule and its precursor form is L-theanine which is found in some bacteria, tumour cells, parasites and fungi (15). Results of genetic evaluations showed that a treatment with green tea extracts was associated with a significant reduction in micronucleus formation. This result agreed with Edwin (16) who showed that the tea extracts decreased the micronuclei (MN) induced by cyclophosphamide and regular intake of tea may improve the antioxidant status *in vivo* and thereby reduce the risk of cancer and coronary heart disease. Tea catechins and polyphenols are effective scavengers of reactive oxygen species *in vitro*, especially of superoxide, and hydrogen peroxide which play a key role in tissue damage (17) , (18). The catechins may also act indirectly as antioxidants, through their effects on the transcription factors and enzyme activities (19). EGCG can act as an antioxidant by trapping peroxyl radicals. It inhibits lipid peroxidation and is capable to protect erythrocyte membrane-bound ATPases against oxidative stress (20).The anticarcinogenic effect of EGCG includes many other effects reviewed by Park (21), Bode (22) and Sarcar (23). Apart

from the antioxidant activity, mentioned it is the modulation of cell signaling cascades through stimulation of a negative cell cycle regulators as p53, p57 and other proapoptotic proteins which plays a decisive role. The study also found that the antimutagenic activity of green tea polyphenols (GTP) implies that it may affect carcinogen metabolism, DNA adduct structure, the interaction of ultimate carcinogen and the scavenging of free radicals.

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