Antimutagenicity and Anticancer Effects of *Citrus Medica* Fruit Juice

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Abstract- Currently cancer is considered as one of the main factors of mortality globally. Many chemicals in our environment can cause genetic mutations and are potentially responsible for millions of cancer-related deaths. Nowadays the scientists are looking for food materials which can potenthially prevent the cancer occurrence. The purpose of this research is to examine antimutagenicity and anticancer effect of *Citrus Medica* fruit juice.In present study human astrocytoma cancer cells were cultured in DMEM (Gibco),supplemented with 10% fetal calf serum,peniciline-streptomycin,L-glutamine and incubated at 37 ºC for 2 days.In addition cancer cell line were treated by half-ripe and ripe *Citrus Medica* fruit juice and cellular vital capacity was determined by MTT. The *Citrus Medica* fruit juice was subsequenthy evaluated in terms of antimutagenicity and anticancer properties by a standard reverse mutation assay (Ames Test). This was performed with histidine auxotroph strain *of Salmonella typhimurium* (TA100) .Thus, it requires histidine from a foreign supply to ensure its growth.The aforementioned strain gives rise to reverted colonies when expose to carcinogen substance (Sodium Azide). During MTT, human astrocytoma cell line revealed to have a meaningful cell death when compared with controls (P<0.01). In Ames Test the fruit juice prevented the reverted mutations and the hindrance percent of half-ripe *Citrus Medica* was 71.7% and ripe *Citrus Medica* was 34.4% in antimutagenicity test and this value in anticancer test was 83.3% and 50% in half-ripe *Citrus Medica* and ripe *Citrus Medica* respectively.This is the first study that have revealed antimutagenicity and anticancer effect of *Citrus Medica* fruit juice and the effects were higher in half-ripe *Citrus Medica* in comparison to the riprned one.

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Key words: Antimutagenicity, anticancer, *citrus limon*, human astrocytoma cancer cells, Ames Test

Introduction

Nowadays cancer is one of the mortality factors in the world which takes place in result of different causes such as mutagenesis and carcinogen chemicals in the environment. Environmental agents which serve as mutagens are cancer factors. According to the statistics almost more than 75% of cancers have an environmental origin (1,2). Genetic damages and changes in DNA sequences and genes mutations and other changes in chromosomal structure play an important role in cancer (3).

Most of mutagenic and carcinogen agents display their destructive effects through free radicals including reactive oxygen's species (ROS). So that antioxidants are able to reduce ROS. ROS have a role in etiology of diseases such as cancer, cardiocellular, nerves problems and senescence. So daily consumption of antioxidants enhances immunity of the body against free radicals production and serves as anticancer agent (4,6). Some of the fruits and vegetables are considered as the main anticancer foods, because of their abundant antioxidants such as phenols, vitamin C, vitamin E, beta-carotene and lipotene (7).

Citrus is the most interesting one among these fruits (8,9). Ames test is one of the most current test to assay anticancer and antimutagenesis effects using bacteria with special mutants (10,11) and the material is used on cancerous cells in vitro. This research has been tried to consider anticancer effects of half-ripe and ripe *Citrus medica* fruit juice on cancerous cells and also through Ames test.

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Patients and Methods

In this research, the method of vital capacity test (MTT) has been used in order to consider cytotoxicity of *Citrus medica* fruit juice on cancerous cell lines (in vitro) and results have been calculated in terms of stimulation index and assessed by t-test.

Ames test has been used as a current method to assess anticancer and antimutagenesis effect of fruit juice on mutant bacteria, *Salmonella typhimurium*, and results have been assessed by one way-ANOVA followed by the Duncan post hoc test on the basis of bacterial colonies in selected conditions.

Culture of human astrocytoma cancer cell line (line 1321)

Cells have been cultured in DMEM medium contained 10-20% fetal calf serum (FBS) and in incubator contained 5% CO_2 at 37 ºC. After growth and reproduction of the cells, in order to take examinations and cells incubation with target materials, adherent cells separated from flask bottom by trypsin 0.25% and after counting, almost 5000 cells transferred to flasks. In any cases, 3 flasks have been considered for each test with 3 repeats for all tests. All examinations have been accomplished after 18 hours incubation in flasks of cell culture plates (full adherence of cells to plates), because some of the above cells were adherent, and in order to evaluate they should be at normal condition of growth.

MTT staining

In this technique, color effect of MTT on cells has been used in which alive cells, contained purple crystals as a result of color reduction by mitochondrial dehydrogenase of alive cells, would be countered and alive cells percentage would be determined by the following formula:

Viability= (alive cells number/ whole cells cultured) ×100

After 18 hours in order to full adherence of cells to the plate, different concentrations of the fruit juice (0 as control, 25,50,100,500 µl/ml) have been added to cells and plates were incubated for 48 hours at 37 ℃ and 5% CO_2.

MTT staining is on the basis of MTT (dimethylthiazol diphenyl tetrazolium bromide) reduction into an insoluble blue- purple product (Formazan) by mitochondrial reductase in alive cells. MTT solution contains 50 mg MTT powder in 10 ml PBS (0.15 M) which has been diluted by 10 times with PBS to get 0.5 mg/ml solution of MTT, Then the solution was autoclaved. After 48 hours incubation of cancerous cells with different concentrations of the fruit juice, the plates incubated at 37 ℃ with 5% CO_2 then stained with MTT mg/ml 0.5 and after 3-5 hours incubation at 37 ℃, the supernatant liquid was removed and replaced by 200 µl isopropanol (Merck, Germany) which was added to the relevant wells. The relevant plate was shaked for 10-15 min on shaker. Then after, the relevant plate was read by a micro titer plate reader (ELISA-reader, Organon-Teknika, Netherland) on 570 nanometer. Toxicity level was calculated by the following formula:

$$cytotoxicity\% = \frac{1-mean\ \ absorbance\ \ of\ \ toxicant \times 100}{Mean\ \ absorbance\ \ of\ \ negative\ \ control}$$

Viability% = 100 - Cytotoxicity %

To diminish test error level, MTT strain was added to some wells without cells and along with other wells, absorbance level was read and ultimately subtracted from whole the absorbance.

Ames test

Salmonella typhimurium TA100 used for Ames test. The mutant strain ,in need of histidine, directly receipt from professor Ames. Fresh bacterial culture should be used for test and incubation time of bacterial culture in nutrient broth should not be more than 16 hours. Appropriate bacterial concentration was considered1-2×10^9 cells/ ml.

After consideration of cytotoxicity effect of fruit juice on cancer cells, according to Ames, fruit juice was added to test tube containing 0.5 ml of the overnight fresh bacterial culture, 0.5ml of histidine and biotin solution (0.5 mM histidin/0.5N biotin), 10 ml top agar (50 gr/lit Agar + 50 gr/lit NaCl), sodium azide as a carcinogene (1.5 µgr/ml Sadium azide) and then content of this tube distributed on the surface of minimum medium of glucose agar (%40 glucose), after 3 second shaking incubation was performed at 37℃ for 48 hours. Each treatment was repeated 3 times. In the test after 48 h incubation at 37℃, reversed colonies were counted in control and test plates and after angular conversion , results were compared by analysis variance. Most materials in their original form are inactive in terms of carcinogenic effects and most materials to become metabolically are active to display mutagenesis properties. So it is necessary to add a microsomal sterile fruit juice to mammalian tissue like rat. After 10 h starvation, livers of 10 male rats were separated. Starvation stimulates and enhances liver enzymes secretion. Livers homogenized in 0.15M potassium chlorideand centrifuged for 10 mins in 9000 rpm in at 4℃. Supernatant (S9 mixture) was removed and mixed with necessary cofactors including

moved and mixed with necessary cofactors including NADP and G-6p(glucose 6 phosphate) and then 0.5ml of the solution was added to Top agar in order to consider anticancer effect.

Also after the counting colonies in anticancer-antimutagenesis test, prevention percentage or antioxidant activity has been calculated as follows (12):

$$prevention \quad percent = (1-\frac{T}{M})\times 100$$

T is reversed colonies in each Petri dish under carcinogen and fruit juice and M is reversed colonies in petri dishes related to positive control (mutagen).

Results

Vital capacity test:

The results of MTT test on cancerous cells under various concentrations of fruit juice, has been shown in figure 1, the cancerous cells lost their vital capacity and there was a significant difference between half-ripe and ripe fruit juice effect on growth depression of cancerous cells and preventive effect of half-ripe fruit juice was more than ripe fruit juice ($P<0.01$).

Results of anticancer and antimutagenesis effect of the fruit juice:

The results of colony counting in Ames test under 25 µl/ml of the fruit juice (with regard to the results of vital capacity test) showed that there was a significant difference between half-ripe and ripe fruit juice antimutagenesis effect on colony growth with controls (distilled water and sodium azide) ($P < 0.01$) .Also, there was a significant difference between half-ripe and ripe fruit juice effect in which reversing effect of half-ripe fruit juice was more than ripe fruit juice. According to figure 2, half-ripe and ripe fruit juice have a strong antimutagenesis effect.

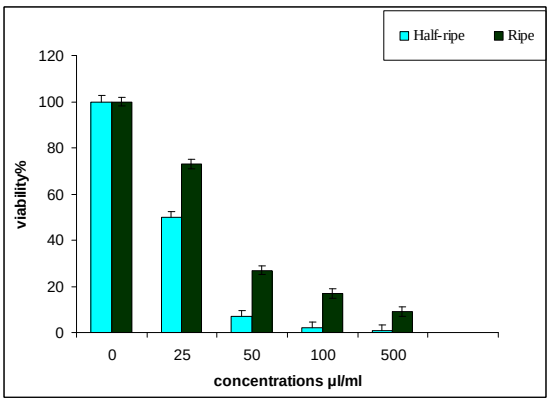

Figure1. Results of MTT test on cancerous cells under various concentrations of fruit juice

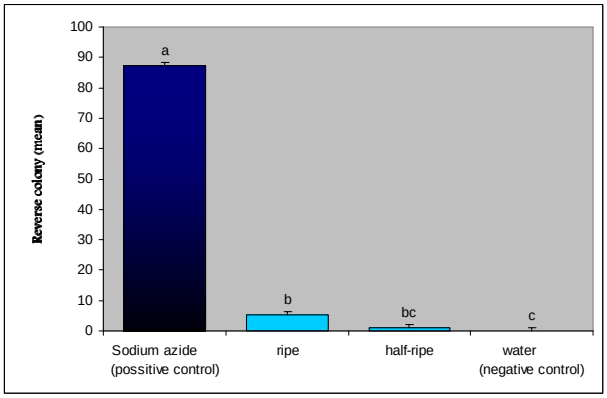

Figure 2. Results of colony counting in Ames test under 25 µl/ml of the fruit juice in mutagenesis test

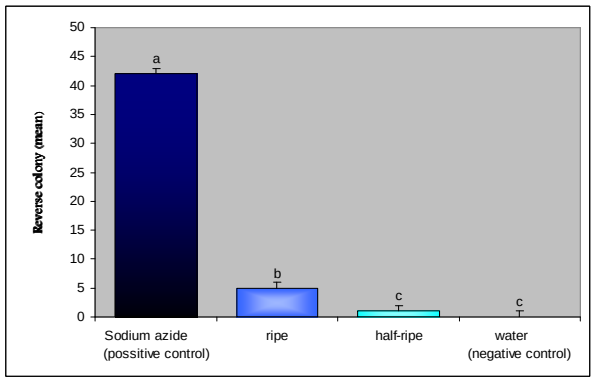

Figure 3. Results of colony counting in Ames test under 25 µl/ml of the fruit juice in carcinogenesis test

To consider anticancer effect, Ames test was repeated after adding S9 in order to metabolic activation of fruit juice.

Half-ripe and ripe fruit juice displayed a significant difference at anticancer effect on colonial growth rather than control (distilled water and sodium aside) ($P < 0.01$) also, reversing effect of half-ripe fruit juice was more than ripe fruit juice. According to figure 3, half-ripe and ripe fruit juice have a strong anticancer effect.

Discussion

Since usual methods on cancer treatment (surgery, chemical treatment, radiotherapy) have an effect on natural dividing cells, in addition to tumor cell, and kill or arrest their cell division (13). In recent years, herbals found widespread use in prevention and treatment of cancer which in this procedure, tumor cells are controlled while natural cells remain intact (14). The effect of diverse antioxidant foods on cancer and cardiovascu-

lar disease has been proved and it has been revealed that these materials cause to enhance long life by 60% (15). During laboratory researches on poly metoxilated flavonoides including tungertin, it has been revealed that these materials have antioxidant and anticancer effects and preservative effect on neurons (16). Nijveldt (2001) showed limonins effects (flavonoids) on cell cycle which caused to changes in cell division and/or cell death (apoptosis)(17). Li et al. (2005) has bean revealed that nobilin (flavonoid of citrus peels) has anticancer, antivirus and antiinflammation activity (18). So far, anticancer and antimutagenesis effect of half-ripe and ripe fruit juice have not been reported, in the present study vital capacity test and Ames test were used to consider its anticancer effect with special emphasizes on application of *salmonella typhimurium* to identify antimutagenesis and anticancer level of chemicals. In this research, half-ripe and ripe fruit juice displayed anticancer and antimutagenesis effect which half-ripe fruit juice was more effective than ripe fruit juice. According to the Ames theory which presented in 1982, in case the number of colonies on positive control medium (contained carcinogen) is two times more than test sample, the substance will be considered as an antimutagenesis and anticancer. According to the Ames theory, when prevention percent ranges between 25-40%, mutagenesis effect in this test sample is assumed medium and when prevention percent is more than 40, mutagenesis effect of the test sample is strong and in case prevention percent is less than 25, mutagenesis effect is negative which the case is true to consider anticancer effect by adding S9 for metabolic activation (11,10) This was found in the fruit juice. In vitro study on fruit juice effect on cancerous cell culture revealed that the fruit juice severely repressed division of cancerous cells which in this study the effect of half-ripe *Citrus medica* fruit juice was more than ripe one.

This case is represented by considering prevention or antioxidant percent of fruit juice that all kinds of fruit juice have preservative effects. As revealed by antimutagenesis test, *Citrus medica* fruit juice solely has the ability to express antimutagenesis effect. In this research, we have examined this fruit juice with rat liver extract (S9). Reason of adding S9 to the fruit juice is that some of anti cancer substances remain inactive and can not attach to DNA till enter into an being with electrophilic enzymes. Bacteria lack this system , so liver extract S9 is applied as active system of cytochrome P-450/P-448 for activation of the materials (19). Also the result showes, fruit juice with rat liver extract displays anticancer activity .

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