

## Antimutagenicity effect of *Citrus nobilis*

Maliheh Entezari\*, Seyed Javad Hosseini

Islamic Azad University, Tehran Medical Branch, Tehran, Iran

\* Corresponding author: email address: [mentezari@iautmu.ac.ir](mailto:mentezari@iautmu.ac.ir) (M.Entezari)

### 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 potentially prevent the cancer occurrence. The purpose of this research is to examine antimutagenicity and anticancer effect of *Citrus nobilis*. The *Citrus nobilis* was subsequently evaluated in terms of antimutagenicity 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 exposed to carcinogen substance (Sodium Azide). In Ames Test the *Citrus nobilis* prevented the reverted mutations and the hindrance percent of *Citrus nobilis* was 72.46%. This is the first study that has revealed antimutagenicity effect of *Citrus nobilis*.

**Keywords:** Antimutagenicity; *Citrus nobilis*; 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 antimutagenesis effect using bacteria with special mutants. This research has been tried to consider antimutagenesis effect of *Citrus nobilis* through Ames test. The Ames test uses mutant strains of *Salmonella typhimurium* that cannot grow in the absence of the amino acid histidine because a mutation has occurred in a gene that encodes one of the nine enzymes used in the pathway of histidine synthesis that prevents translation of a functional enzyme, and thus the conversion of the catabolic intermediate to histidine cannot be completed. Therefore, the Ames mutants are auxotrophic and are called *histidine-dependent* or *his<sup>-</sup>* (pronounced his<sup>-</sup> minus) mutants because they can only grow if histidine is supplied in the growth medium. So far, anticancer and antimutagenesis effect of *Citrus nobilis* has not been reported, in the present study Ames test was used to consider its anticancer effect with special emphasizes on application of *salmonella typhimurium* to identify antimutagenesis and anticancer level of chemicals [10,11].

## MATERIALS AND METHODS

Ames test has been used as a current method to assess antimutagenesis effect of *Citrus nobilis* on mutant bacteria, *Salmonella typhimurium*. *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 considered  $1-2 \times 10^9$  cells / ml. According to Ames, *Citrus nobilis* was added to test tube containing 0.5ml of the overnight fresh bacterial culture, 0.5ml of histidine and biotin solution (0.5mM histidine / 0.5mM biotin), 10 ml top agar (50 gr/lit Agar + 50 gr/lit NaCl), sodium azide as a carcinogene (1.5 µgr/ml Sodium 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°C for 48 hours. Each treatment was repeated 3 times. In the test after 48 h incubation at 37°C, 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 chloride and centrifuged for 10 mins in 9000 rpm in at 4°C. Supernatant (S9 mixture) was removed 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.

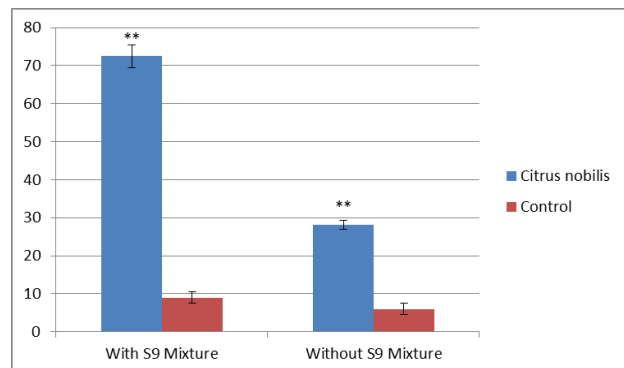
Also after the counting colonies in anticancer-antimutagenesis test, prevention percentage or antioxidant activity has been calculated as follows [12]:

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

T is reversed colonies in each Petri dish under carcinogen and *Citrus nobilis* and M shows reversed colonies in petri dishes related to positive control (mutagen).

## RESULTS

The results of colony counting in Ames test showed that there was a significant difference between *Citrus nobilis* antimutagenesis effect on colony growth with control (sodium azide) ( $P < 0.01$ ).



**Figure 1.** Results of prevention percentage in Ames test of the *Citrus nobilis* in mutagenesis test

To consider anticancer effect, Ames test was repeated after adding S9 in order to metabolic activation of *Citrus nobilis*.

## 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]. Our environment is full of potential carcinogens such as UV light, industrial pollutants, pesticides, food additives, and natural products such as tobacco. Some of these carcinogens are genotoxic, i.e. they can cause cancers because they can change the nucleic acid sequence of DNA *directly*, and they are also called mutagens (chemicals that cause mutations).

Obviously, it is important to have a rapid and inexpensive assay for testing chemicals we suspect are carcinogenic. 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 cardiovascular 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 been revealed that nobilin (flavonoid of citrus peels) has anticancer, antiviral and antiinflammation activity [18]. The Ames test was developed by Bruce Ames, Professor of Biochemistry at UC-Berkeley, as a fast and sensitive assay of the ability of a chemical compound or mixture to induce mutations in DNA [19].

A mutation is any change in a DNA sequence from the original sequence of nucleic acids, and mutations happen all the time in cells. Sometimes it is because a mutagen comes from the outside of the cell and in some manner creates changes in the DNA. Often the mutations are just errors that occur during DNA replication when cells divide. In fact, there is an average of nearly one mutation (error) in DNA every time one cell divides. The cells have mechanisms to repair the mutated DNA, and they usually do, but if a mistake is overlooked, the change in the DNA is carried on in future replications in the cell. This scenario represents one way that a "spontaneous" mutation can occur, because there was no obvious cause on which to blame the mutation [20,21].

To determine the number of revertants following exposure to a mutagen, and differentiate between the mutant strain (*his*<sup>-</sup> auxotrophs) and the new mutants we may generate (*his*<sup>+</sup> revertants), the Ames test uses a chemically defined medium, in which the amounts of every ingredient are known. If a *his*<sup>-</sup> culture were placed on a chemically defined minimal agar lacking histidine, only those cells that have mutated to *his*<sup>+</sup> (revertants) would grow and form colonies. In theory, the number of colonies that revert and grow is proportional to the mutagenicity of the test chemical. [21]

The chemically defined medium used for the Ames test actually has a trace (growth limiting) amount of histidine. Trace amounts of histidine in the medium are necessary because some mutagenic agents react preferentially with actively replicating DNA. When *his*<sup>-</sup> strains are plated on this medium, they grow until they run out of histidine (only 2-3 cell divisions lasting about one hour), and the result is a faint, nearly invisible lawn of growth within the overlay.

Conversely, revertant bacteria should form large colonies because their growth is not limited because they can produce their own histidine. Each large colony represents one revertant bacterium and its offspring. By definition in the Ames test, a mutagen is any chemical agent that results in more than twice the number of mutants as occur spontaneously, and thus is potentially carcinogenic for humans.

Because the assay does not use a live animal model, it is inexpensive, easy, and fast. 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 *Citrus nobilis*.

As revealed by antimutagenesis test, *Citrus nobilis* has the ability to express antimutagenesis effect. In this research, we have examined this *Citrus nobilis* with rat liver extract (S9). Reason of adding S9 to the *Citrus nobilis* is that some of anticancer 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[22]. The result showed, *Citrus nobilis* with rat liver extract displays anticancer activity.

**CONCLUSION**

Totally, according to the obtained results and comparison with other studies, it may be

**REFERENCES**

1. Moller P, Wallin J, Kundsén I. Oxidative stress associated psychological stress and life-style factor. *Chem. Bio Interact.* 1996;102:1-36.
2. Namiki M. Antioxidants/antimutagens in foods. *Critical Rev. Food Sci. Nutr.* 1990; 29:273-300.
3. Mccord J.M. Free radicals and pro-oxidants in health and nutrition. *Food Technol.* 1994;48:106-110.
4. Elrkson PM, Thompson HS. Antioxidant: What role do they play in physical activity and health? *Mutagenesis.* 2001;H;Abstract.
5. Hirota F. Green tea: Cancer preventive beverage and /or drug. *Elsivier science Irland LTD.* 2002;188:9-13.
6. Ching Chen S, Chang K. Mutagenicity and antimutagenicity studies of Tannic acid and related compounds. *Food and chemical Toxicology.* 2000;38:1-5.
7. Ghasemian A, Mehrabian S, Majd A. Peel Extracts of two Iranian cultivars of Pomegranate (*punica granatum*) have antioxidant and antimutagenic activities. *Pakistan Journal Biological Sci.* 2006; 7:1402-1405.
8. Jacob R, Hasegawa Sh, Manners G. The potential of Citrus Limnoids as anticancer agents. *perishables Handling Quarterly.* 2000;102:6-8.
9. Mazaki M, Ishii T, Uyeta M. Mutagenicity of hydrolysates of citrus fruit juice. *Mutat Res.* 1982;101:283-91.
10. Ames BN. Methods for detecting carcinogens and mutagens with the Salmonella mammalian microsome mutagenicity test. *utat Res.* 1976;31:347-349.
11. Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc, Natl. Acad. Sci.* 1973;70:2281-2285.
12. Ong T, Wong WZ, Stewart JD, Brockman HE. Chlorophyllin: A potent antimutagen against environmental and dietary complex mixture. *Mutation Res.* 1986;173:111-115.

recommended that *Citrus nobilis* has good antimutagenesis effects.

13. Chabner BA, Friedman MA. Progress against rare and not so-rare cancer. *New Engl J Med.* 1992;236:564-68.
14. Franks LM, Teich NM. Introduction to the cellular and molecular biology of cancer. New York: Oxford university press; 1997.
15. Sunj J, Chu YF, Wu X. Antioxidant and antiproliferative activities of common fruits. *J Agric Food Chem.* 2002;25:7449-54.
16. Bennett JP, Gompert S, Wollenweber E. Inhibitory effects of natural flavonoids on secretion from mast cells and neutrophils. *Arzneimittelforschung.* 1981;31:433-7.
17. Nijveldt RJ, Nood EV, Boelens PG. Flavonoids: A review of probable mechanisms of action and potential applications. *Am J Clin Nut.* 2001;74:418-25.
18. Li S, Yu H, Ho CT. Nobiletin: efficient and large quantity isolation from orange *Citrus nobilis*. *Pub Med Nj.* 2005;8901-8520.
19. Hashemi M, Nouri Long M, Entezari M, Nafisi S, Nowroozii H. Anti-mutagenic and pro-apoptotic effects of apigenin on human chronic lymphocytic leukemia cells. *Acta Med Iran.* 2010; 48:283-8.
20. Entezari M, Majd A, Falahian F, Mehrabian S, Hashemi M, Ardeshiry Lajim A. Antimutagenicity and anticancer effects of *Citrus medica* fruit Juice. *Acta Med Iran.* 2009;47: 373-7.
21. Entezari M, Hashemi M, Ashki M, Ebrahimian S, Bayat M, Azizi Saraji AR, et al. Studying the effect *Echinophora platyloba* extract on bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans*, *Aspergillus flavus* and *Aspergillus niger*) invitro. *World Journal of Medical Sciences* 2009; 4: 89-92.
22. Hakura A, Shimada H, Nakajima M, Sui H, Kitamoto S, Suzuki S, Satoh T. Salmonella /human S9 mutagenicity test: a collaborative study with 58 compounds. *Mutagenesis.* 2005;20:217-22.