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Research in Pharmacy 2(2) : 39-45, 2012 ISSN : 2231-539X
www.researchinpharmacy.com

Regular Article

Inhibition the mutagenicity of vitamin a by using *Olea europaea* extract

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This study aims to investigate the predisposition of the extraction of the extract of olive leaves (*Olea europaea* of the family Oleaceae, or the olive family) to frustrate the excretion of the inherent toxic caused by vitamin A (henceforth VA). After being sure that the extraction is inane of any negative effects on the living creature, the following tests are adopted, the mitotic index test of the medulla ossium of the bones, and the chromosomal aberration test. The results showed that toxic dose of vitamin A have cytogenetic effect. It cusses increased in bone marrow cell proliferation and increase in chromosome malformation, the olea leaf extract showed anti-mutagenic activity against the cytogenetic effect of vitamin A it decrees chromosome malformation and kept the Mitotic index in normal level.

Key words: Chromosome aberration, cytogenetic effect, antimutagenic effect.

The Vitamin A (VA) is one of the necessary micronutrient in all stages of life (Zile, 1998). This micronutrient was unknown until 1913 when the scientists discovered that it can stop the night weak sight (Mclaren & Frigg, 2001). In the year 1932, it was discovered that (β-Carotene) is the basic substance of VA, then it was termed as the pro-vitamin. It is composed of two molecules of the vitamin which joined by their vertices (A-A). And when we eat (β-Carotene) the VA is produced through the interaction with the enzymes found in the intestine. These enzymes break the (β-Carotene) into two molecules of VA absorbed in the fat.

The VA is carried by the blood as a complex composition joined to the fats or proteins and it is called cailomicron. This composition can not easily pass through the defecation system; therefore, 90% of it is stored in the liver. Then it is transferred from the liver to the target tissue as Retinol or Retinoic Acid (RA) by a protein joined to the Retinol. (Wessells & Hopson, 1988).

The active form of the VA, or Retinoic Acid (RA), is connected by the Retinoid Receptors. It carries the organizing sign of growth. Each deficiency or over dose of VA during the fetus growth will lead to the congenital malformation (Zile, 1998). The gastrointestinal digestion process and then the oxidation process of the VA in the tissues of the body produce several products, two of which are called Trans RA & 9 cis all. They work as subsidiary hormones in a number of the known functions of the VA inside the body and they might interfere with the congenital malformation in a specified mechanism (Kochhar, et al 1993).

VA is available in the animal products only, such as liver which is very rich in VA since three ounces contain about 30000 I.U. of the VA (Gibney, 2000). It is also available in following, the kidneys, the yolk of the egg, and the fat extracted milk. The (β-Carotene) is found in the yellow fruits such as carrot, sweet potatoes, gourd, yellow melon, and in the vegetables with dark leaves such as cabbage and spinach (McLaren & Frigg, 2001). The VA is sensitive for the ionic radiation whereas retinol and (β-Carotene) are constant during most cooking processes and they are resistant for the acids and bases. As for the food stuff processes, such as pasteurization or taking out water, they usually cause minute loss of the VA.

The acute intoxication occurs to infants when they are given one dose of 25000 I.U. per the body weight and the high doses of 2000000 taken by the adults cause the symptoms of the acute intoxication. There are several cases of the acute intoxication noticed in the North Pole which result from taking over doses of the VA. The over dose of the VA causes the danger on the newly born kids if it is taken by pregnant women.

The Signs of the intoxication by VA, these can take the following syndromes; the occurrence of blurred vision, bone pain, diarrhea, dryness of the skin and mucous tissues, fatigue, headache, loss of hair, liver damage and liver enlargement, nausea, skin rashes, small cracks and scales on the lips and at the corners of the mouth, and hepatic fibrosis and cirrhosis.

The treatment of the intoxication by VA is to stop taking any supplements containing VA or supplied with it, table 1 shows the daily doses of VA.

Table 1: The Typical Daily Doses of VA.

Category	Dose
Infants (less than one year)	10250 I.U. per day
Children 1-3 years old	10333 I.U. per day
Children 4-6 years old	10667 I.U. per day
Children 7-10 years old	20333 I.U. per day
Individuals 11 years and more	30333 I.U. per day
pregnant and wet-nurses (1st to 6th month)	4000 I.U. per day

Cytogenetic assays

- ***The Mitotic Index***

A body cell divides into two structural cells. Each one contains the same number of chromosomes of the mater cell. This process is complete by two steps: the first is the division of the nucleus into two. This process is called the nuclear division. The second is the cytoplasm division. Generally, the division process is considered the centre of two important biological processes, i.e. the processes of growth and reproduction in living systems. It can be also termed the life cycle of the cell which takes different spans of time according to the living species and the tissue and the environmental circumstances.

The coefficient of the mitotic index can be calculated as an indicator since it can be counted as a ratio of the number of the cells which are in different cytodieresis to the total number of the cells (divided or undivided) which are usually (1000) in number. (King et. al. 1982)

- ***Chromosomal Aberration Test***

This test is one of the tests used in the study of the cellular genetic effects in the bone marrow of the mammals so as to discover the mutagens (Kliesch et al. 1982). Chromosomal aberration occurs automatically if there is any factor which can affect either the natural integration of the chromosomes or the mechanism of their movement during the cellular divisions, or both. Therefore, many materials of environment polluting and the

cellular toxic lesions control, which have he ability to be mutagens, have also the ability to urge chromosomal aberration (Malhi, 1978). These changes are of two basic types: the first occurs as a result of defect in the chromatid and is called the chromosome break. The other kind of changes occurs in one of the chromatids and it is called the chromatic break. The physical factors, such as radiation, cause chromosomal changes regardless of the division phase of the cell during which it is exposed to. The effect of the chemical agents or factors depends on the division phase of the cell. Here, the effect is confined to the S-phase, or the phase of building DNA. The chemical factors can be studied in the following tropical phase (Lucas, 1993). This test was used to discover the effects of chemical and physical materials either directly or indirectly.

Materials and Methods

The Design of the Experiment

White albino rats 14±2 weeks with body weight 300±50 g, animals are divided into three main groups. The first one is the negative control group which is given distilled water only. The second group was divided into two positive subgroups, the first one is the positive control group which is given VA with 30000 I.U. \ kg., and the second positive control group which is given the extraction of the leaves of olea with the dose 500 ml/kg.

The third group in which the animals are given the toxic dose of VA of 30000 I.U.\kg and they are also given the therapeutic dose of the leaves extract of olea 500 ml/kg.

Solutions

1) Phosphate Buffer Saline (PBS). The following materials should be prepared in advance (0.8 gm of NaCl, 0.2 gm of PCl, 1.15 gm of Na_2HPO_4 and 0.2 gm of KH_2PO_4. These materials are dissolved in 500 ml. of distilled water, then the volume is completed to 1000 ml. After adjusting the acidity of the solution to pH= 7.2. then it is sterilized. (121^{c}C, 1 bar for 20 minutes), the solution is divided into 10 equal volumes and kept in the fridge at (4^{c}C). (Cambell, *et al*. 1964)

2) The Colchicine Solution. The Colchicine Solution is prepared immediately before using it. This is carried out by dissolving two tablets (1 gram each) of the drug in 1 ml. of solution No.1, then each animal is injected with 0.25 ml. in the intrapretonial membrane (Allen *et al*., 1977).

3) Hypotonic Solution. The solution is prepared by dissolving 5.75 gm. of KCl in 500 ml. of distilled water, then the volume is completed to 1000 ml. It is sterilized and kept in the fridge (4^{c}C) after dividing it into ten equal volumes (Allen, *et al*. 1977).

4) Fixative Solution. It is prepared instantaneously (or 30 minutes before using it) by mixing absolute methanol with glacial acetic acid (CH_3OH). The ratio is 1:3 volume. The solution kept in the fridge at (4^{c}C) (Allen, *et al*. 1977).

5) Giemsa Stain Solutions.

- **a.** Sodium Bicarbonate is prepared by dissolving 7.5 g. of sodium bicarbonate ($NaHCO_3$) in 50 ml distilled water, then the volume is completed to 100 ml. (Allen, *et. al*. 1977).
- **b.** Stock Solution of Giemsa Stain. 3.8 g. of Giemsa stain solution powder is dissolved in 25 ml. of glycerin solution. The solution is put in water bath (60^{c}C) for two hours with continuously shaking it. The solution is left for 30 minutes at 37^{c}C, then 75 ml. of absolute methanol is gradually added and simultaneously mixed. The solution is filtered and kept in a dim bottle as stock solution of Giemsa stain.

Before the staining procedure with Giemsa stain, the following materials are mixed, 1 ml. of stock solution of Giemsa stain 1.15 ml. of absolute methanol, 0.5 ml. of sodium bicarbonate, and 40 ml. of distilled water (Allen, *et al*. 1977).

Preparation of the plant extract

The plant extract is prepared according to the method of Sato *et al*. (1990) with some modification. Specific weight of the ground dried leaves mixed with the dispert solution (20 methanol: 80 distilled water volumes) at 1g/3ml. The mixture is uniformed by electric blender for 30 minutes in room temperature. The solution is filtered by using gauze fabric for getting transudated solution which is concentrated by using rotary evaporator. Then it dried in oven at 50^{c}C for 24 hours dispert is kept in a dry place until it is used.

Mitotic Index

The Mitotic Index of the bone marrow is studied according to Allen *et al*. (1977), where the animal is injected with 0.25 ml. of Colchisen (solution No. 2) in the pseudo membrane before 3 hours of dissection. The following procedure is adopted in preparing the bone marrow cells suspension.

1- The animal is gourde by chloroform, and it is fixed on the panel of anatomy.
2- A long cut is made from its hypocelom to the uppermost of the chest for revealing different organs and getting the intended ones.
3- The bone of the right meros is distracted by cutting the ligaments and the extrusion of the muscles from it. After the meros is cut, the bone marrow is taken from it by pumping 10 ml. of (solution No. 1) from one of its ends and the bone marrow is discharged into a test tube from the other end.
4- A Pasteur injector is used to rapture the interrelated linkage of the cells. This can carried out by quickly pulling out the solution several times until all the agglomerations of the cells disappeared.
5- The tube containing the bone marrow solution is put in the centrifuge with (2000 rpm\min) for 10 minutes. The clear limpid material is neglected.
6- The cells are washed once again with the (PBS) solution (10 ml.) and the previous step is repeated.
7- 10 ml. of warm low tension (solution No. 3) is gradually added to the residue of the cells with continuous shaking and the tube is kept in watery bath (37^{c}C) for 30 minutes with shaking the tube each five minutes. Then it put in the centrifuge with (2000 rpm\min) for 10 minutes and the clear limpid material is neglected.
8- Before carrying out the last step, the fixative solution has been prepared (solution No. 4) and it has been kept in the fridge at (4^{c}C). The bone marrow cells are fixed by adding 10 of the fixative solution as drops poured into the test tube with continuous shaking to prevent clustering of the cells. It is centrifuged with (2000 rpm\min for 10 minutes and then it is put in the fridge at (4^{c}C) for 30 minutes. The clear and limpid material is neglected. This step is repeated once again and the cells are suspended by the cold fixative solution (2 ml.).
9- 3-4 drops of the bone marrow cells suspension are poured on clean glass plate from a suitable height (3 feet).
10- The plate is left to dry up, then it is stained with concentrated stain solution (solution No. 11) for 10 minutes, then it is washed with distilled water and left to dry up.

To calculate the modules of bone marrow cells, 1000 cells are classified into divided cells and not divided ones by using the magnifying power of the oil lens, then the coefficient of division is obtained according to the following ratio

Mitotic Index (%) = The number of the dividing cells X 100 / The total number

Results and Discussion

The study of the coefficient of cells of the bone marrow of rats

Table no. 2 explains the coefficient of cells of rats bone marrow. These animals are treated with 30000 IU of VA and 500 ml/kg of the body weight of the extracted methanol of olive leaves as a single dose. The animals treated with VA highly raised the value of the coefficient of the division in comparison with the negative control group. The animals treated with the plant extraction show little raise in the value of coefficient of the division if compared with the negative control group. The treatment the of animals with VA and the extraction of the olive leaves plant results in lowering the value of the coefficient of the division towards the value of the negative control group. This means that the extraction of the olive leaves has the ability to reduce the toxic effect of VA which has led to the high augmentation of the ratio of the blood cells.

Table 2. Bone marrow cell proliferation value.

MI	Treatment
5.16±0.42	Negative control
8.8±0.46	Olea leaf extract only
20±2.88 *	VA only
5.4±0.23	Extract + VA

M±Se * significant ($p< 0.05$)

The study of the chromosomal aberration

Figure (1) shows percentage of chromosomal aberration and Table no. 3 shows different type of chromosomal aberration in the bone marrow cells of the rats figure (2) which are treated with 30000 IU dose of VA and 500 ml/kg of the body weight of the extraction of the olive leaves in single dose. Treatment by VA causes increase in chromosome aberration in all types, while the treatment by VA with extract causes decrease in aberration of chromosome compare with negative control.

Table 3. Types of chromosome aberration in bone marrow cell.

Treatment	Chromatid Break	Chromosome Break	Aneuploidy	Polyploidy
Negative control	1.70±0.10	0.93±0.13	0.80±0.23	0.40±
Olea leaf extract only	4.00±1.5	4.00±1.15*	2.00±1.00	0
VA only	9.33±2.02*	10.00±1.15*	4.00±0.57*	2.00±0.57*
VA + extract	5.00±1.15	5.00±1.15*	2.00±0.57	1.00±0.57

M±Se * significant ($p< 0.05$)

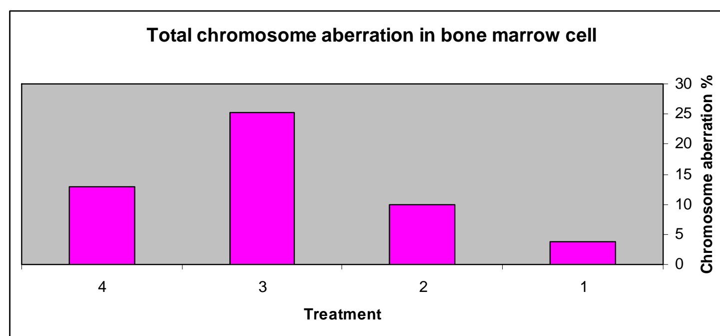

Figure 1. Total chromosome aberration in bone marrow cells. (1) Negative control. (2) Treated by olea extract only. (3) Treated by VA only. (4) Treated by VA+ olea extract

The results of the study are in full agreement with the results of the studies on ability of the phenol materials found in the extraction of the olive leaves to reduce the toxic effect of some mutagenic and carcinogenic materials since they are antioxidation materials. Benavente Gaccia, has proved that the phenol materials in the olive leaves reduce the mutagenic effect of X-ray if compared with some other materials. Fig 1(2) shows the effect of VA on chromosomes of rats, the aneuploidy, polyploidy, chromatid break, and chromosome break are markedly visible.

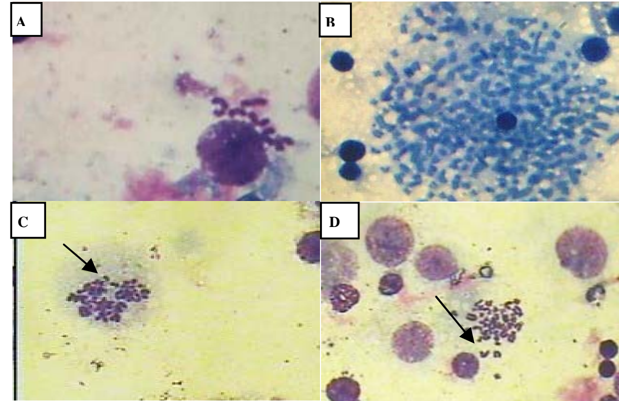

Figure 1. Chromosome aberration in bone marrow cell in rats treated by VA. (A) aneuploidy (B) polyploidy (C) chromatid break (D) chromosome break.

Acknowledgement

I would like to take this opportunity to thank those who had been involved in completing this research paper. This includes colleagues, the Librarians, the Laboratory Assistant and others at the Babylon University, who had provided their best efforts in assisting me to obtain the necessary information to complete this research. I am very grateful to have their share of thoughts and knowledge on this particular field.

Above all, I would like to thank Allah for His will, the strength, and the knowledge he has provided me.

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