**Research Article**

# Effect of the Total Lycium barbarum Carotene on Normal Human Blood Lymphocytes

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Received: 27.10.19, Revised: 10.12.19, Accepted: 30.12.19

## ABSTRACT

The pioneering and recognition of novel medications, that increase the ability of the immune system tasks has become a very crucial target for the Immune-pharmacology researches as well as for the Onco-therapist. Our scientific work demonstrated the favorable effects of Iraqi wild type Lycium barbarum active components as an immunomodulating agent. The immune stimulating effect of the extracted total carotene notified by the proliferation of the normal blood lymphocytes, which estimated by the MTT assay and the alterations of IL-10 and TNF-α levels that evaluated by ELISA technique. The elevations of those two cytokines have been demonstrated after 2 and 4 hours exposure of the extract at concentrations (125 and 250μg/ml). The results of the present study indicated that Lycium barbarum Carotenoids can behave as an immunostimulant, acted to promote IL-10 production and increase up to four folds TNF-α level especially after 2 hours exposure and at a lower concentration (125μg/ml).

**Keywords:** Lycium barbarum carotene, Immunomodulation, IL-10, TNF-α Elisa assay, MTT assay.

## INTRODUCTION

Studies that included to understand the effects of several natural compounds and how they act to protect mutagenic and carcinogenic compounds. One of these natural compounds is carotene. Reducing cancer incidence through a carotenes rich diets had been concluded in many studies, also their benefits in different chronic diseases. The the potent antioxidant activity for carotenoids is an important factor for all these activities (1). Beta-carotene may enhance immune cell function that played an important role immune in cancer prevention (2).

Among the traditional medicine plant that are well-known in Chinese is Lycium barbarum. This plant contained very important active constituents, with many pharmacological importance, as anti-aging agent (3), immune boosting (4) beside the anti-cancer effects (5). Little (if not) researches that included the Iraqi wild Lycium barbarum and their biological activities which in the present study was found that Iraqi wild Lycium fruit was rich with the carotenes compound estimated as β –carotene, and the plant was considered as a good source for(Total carotene)which might give the plant focusing for being cultivated in well and improved conditions(6). The aims of this study is to investigate the cytotoxic activity of the extracted carotene on cell culture of normal human blood lymphocytes through the MTT assay, then to determine by ELISA technique the level for two important interleukins; the IL-10& TNF-α.

## MATERIALS & METHODS

Plant Collection:

A quantity of ripe Lycium barbarum fruits were collected From the ground of University of Baghdad which is located at Al-Jadriya district in the center of Baghdad, and classified at collage of science in the herbarium of the Biology Department, Baghdad University/Iraq.

Extraction and Determination of the L.barbarum Total Carotene Content in the Fruit

Firstly; a quantity of one gram dried powder from

L.barbarum fruits had been homogenized with 3 ml distilled water with aid of Ceramic mortar. Secondly a liquate of 2 mL absolute ethanol was added to powdered fruit in order to denaturized all proteins that may present in the plant fruits. Thirdly; the yielded mixture was transferred to separate funnel, and about 10 mL n-hexane was added with gentle shaking. Two layers were separated; the organic hexane layer which might contain the extracted carotenes, while the unwanted aqueous layer should be discarded. Qualitative and Quantitative determination of total carotenes content in the fruit were carried out as employed in reference (6).

Carotene Immunomodulation Effects(in vitro)

The extracted L. barbarum total carotene had been employer for immune modulation determination through; lymphocytes proliferation test (MTT test), and the Interleukins (IL-10 &TNF-α) levels determination.

Lymphocyte Culturing and Viable Counting (7,8): -

-From eight healthy volunteers (with age between 25 to 35 years and no medications have been taken for at least ten days), 5ml peripheral vein blood were placed in separated glass vacuumed tubes containing anticoagulant EDTA with continuous shaking.

-All blood samples were diluted with PBS (pH=7.2) in a ratio of 1:1.

-The diluted blood samples were layered each onto 3ml lymphocyte separation fluid (lymphprep; s.g.:1.077g/l), then all tubes were centrifuged for half an hour at 2000 rpm.

- With the aid of sterile Pasteur pipette, the separated lymphocyte cells were collected and transferred to new 10ml vacuumed tubes, to be washed with 5ml of RPMI 1640, and centrifuged for about10 minutes at 2000 rpm. Finally the cells were again suspended in complete growth medium that contained 10% fetal calf serum, and incubated at 370C overnight(380.

Measurement of Lymphocytes proliferation by MTT Assay (9):-

To each well of the 96-microtiter plate, 100μl of the isolated lymphocytes (104cell/well) was seeded and incubated for 2 hours.

An extracted carotene stock solution (1000μg/ml) was prepared to get the following concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.9) μg/ml. All prepared liquids were sterilized with 0.22 μm Millipore filter. The seeded lymphocytes in plate culture were treated with 100 μl of extracted carotene in triplicate from each concentration. A positive control of 0.1% PHA (phytohemagglutinin) was employed, and untreated lymphocyte wells as negative control. A bout 50 μl of MTT dye (2mg/ml) was given to all wells after incubating the plate 20 hours at 37C0. The plate was centrifuged for 5mimutes at 1500rpm at the end of 4 hours from the dye incubation period to remove the supernatant by fine needle. Finally 100μl DMSO was added to all wells to dissolved the fromazan crystals that formed through metabolism of MTT day in the living mitochondrial lymphocytes. The result color was read 620 nm immediately by ELISA reader. With the equation, the present viable lymphocytes were calculated and then evaluated statistically:

[Absorbance of the test /Absorbance of negative control]X 100.

Effect of the Extracted Carotene on Cytokines Level (10)

- To determine the effect of extracted carotene at three concentration(500, 250, and 125 )μg/ml on two cytokines(IL-10 and TNF-α) secreted by normal lymphocytes, 1ml of suspended cells (1X106cell/well), were treated with 1 ml of each sterile concentration from the extracted carotene for 2 and 4 hours interval each time in separated plate (39). The untreated cells were considered as negative control. Then both plates were centrifuged for 20 minutes at 2000 rpm and the supernatants were separated in two tubes to be kept at -20C0 for ELISA kit estimation.

The Cytokine IL-10 Level and The cytokine TNF-α Level

According to "US Biological" instructions for TNF-α and IL10 procedure documents of the United State Biological Catalog Number (T9160-01), the levels of both cytokines in the collected supernatants for extracted carotene treated lymphocytes at different concentrations were calculated through plotting the absorbance of each sample at 450nm against their concentrations. Through the straight line equation obtained from the standard curve for TNF- α and IL-10, both cytokines level in all samples were

calculated and evaluated statistically(40-44).

## STATISTICAL ANALYSIS

To estimate the relations of different factors, Statistical Analysis System- SAS (2004) was applied in the present study. Both the minimum significant difference (LSD) test and the Duncan test at the comparative between means were also employed in this study.

## RESULTS

i-Total Carotene Content in Lycium barbarum Fruits : The result showed that the fruit contained total carotenes 0.33 mg/g dry weight of fruit(6).

ii-Lymphocyte Proliferation determination by MTT assay:

Figure (1) shows that eight concentrations from extracted carotene affect normal human Lymphocyte by MTT assay/24 hours reading at 620 nm with negative and positive control.

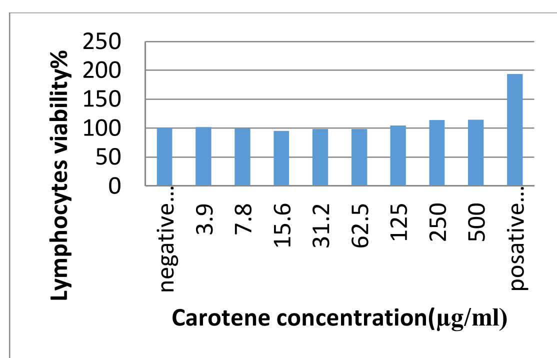



**Fig.1: MTT results at 620 nm for different concentrations of L.barbarum extracted carotene with (0.1% PHA) as positive control on normal human lymphocytes.**

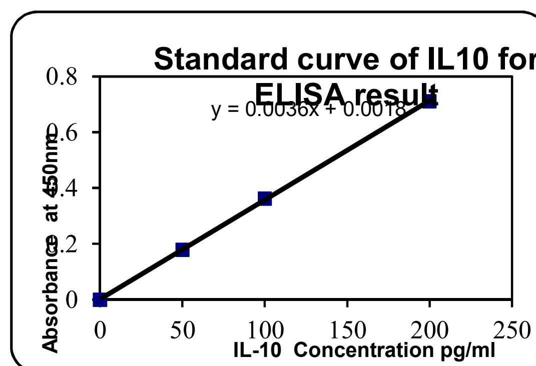
As shown in figure (1) the extracted carotene acted as immune stimulant by enhancing lymphocyte proliferation especially at concentration 125µg/ml and above. The results indicated the mechanism by which extracted carotenes boosting immunity and human health through its antioxidants effects, and minimize the toxic effects of reactive oxygen species (ROS) which seems to be a predictable factor for many diseases like cancer, cardiovascular, neurodegenerative diseases and aging and carotene regulate immune function and all these illnesses (1).

In an animal study by Wu and co, 2006 on L.barbarum explained that the plant anti-cancer activity was through immune enhancements by elevating numbers of CD4+and CD8+ T cells to alleviate the immunosuppression and intensify the anti-cancer ability of the immune system (12). Other studies, found that Lycium barbarum able to activate T cells, and the percentage of cells in G0/G1 phase was risen (13,14)

iii-Effect of Extracted Carotene from L.barbarum on Cytokine:

A standard curve for both interleukins was plotted separately Figure ( 2, A&B)

-A




-B-

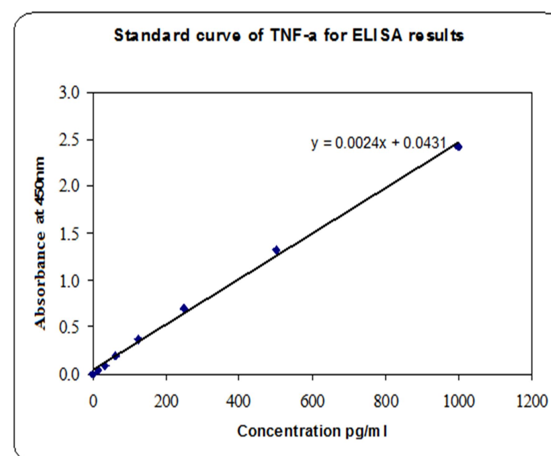



**Fig.2: Standard curve of IL-10 (A) and TNF-α (B) analyzed by ELISA, R2=0.98 and 0.93 respectively**

The effect of the extracted carotene from L. barbarum on cytokine level after 2 and 4 hours exposure was summarized in Table(1).

**Table 1: Impact Of Different Carotene Concentrations And Exposure Time (2 , 4 Hr.) On Lymphocytes IL-10 &TNF Concentration**

| Carotene Concentration (µg/ml) | IL-10 (pg/ml) | | LSD Value | TNF (pg/ml) | | LSD Value |
|---|---|---|---|---|---|---|
| | 2 hr. | 4 hr. | | 2 hr. | 4 hr. | |
| 500 | 89.33 ±7.68d | 122.53 ±7.63c | 30.08 * | 600.00 ±9.96b | 444.40 ±15.82d | 51.91 * |
| 250 | 157.67 ±7.33c | 228.10 ±7.70a | 29.53 * | 629.31 ±9.89b | 801.00 ±17.50a | 55.69 * |
| 125 | 225.33 ±8.95a | 164.33 ±25.56bc | 75.19 NS | 966.3± 50.11a | 513.67 ±9.990c | 141.83 * |
| Control | 200.00 ± 0.0b | 220.00 ± 0.00ab | 0.00 | 676.0 ± 0.0b | 6500 ± 0.00b | 0.00 |
| LSD Value | 22.65 * | 45.27 * | ---- | 84.83 * | 41.72 * | ---- |

* (P<0.05).

Means having different small letters at the same column are significant different.

The impact of different concentrations of carotene on interleukin level produced by treated lymphocytes after 2 and 4 hours, showed different manner. Carotene increased significantly IL-10 level on ($p \leq 0.05$) at the concentrations 125 µg/ml to reach the level of (225.33 pg/ml) after 2 hours exposure and (228.1 pg/ml) after 4 hours of carotene exposure at 250 µg/ml concentration, in comparison with control. The same was for TNF-α level; a significant increase was at 125 µg/ml after 2 hours of carotene exposure and at 250 µg/ml after 4 hours(966.3 pg/ml and 801 pg/ml) respectively as compared with control.

Usually IL2, Tumor Necrosis Factor and Interferon Gamma synthesized by collection of cells called T-helper 1(CD4+).

T-helper 1 main function are cytotoxicity and regional inflammatory activities, so it's responsible for eliminating the foreign biological invaders like viruses, bacteria and parasites.

While, T-helper 2 cells have a major role in the Antibody based immunity for example they can be activated and proliferated to produce immunoglobulins that acts against free-living microorganisms. So to obtain ordinary immunological response, the body needs to make a balance between those two kinds of biological immunological tools.

Many Immunological tests have been implicated in many researches that concern carotenoids and their story with the immune system (15). Seifter et al. (16) found an obvious stimulatory property of β-carotene on the development of the thymus gland and a great escalation in the calculations of thymic small lymphocytes. Lymphocyte blastogenesis in rats (17), pigs (18), and cattle (19) has been seen as a stimulatory biological activity of β-carotene.

Studies showed that the Oral administration of β-carotene to human adults lifts the number of T-helper and T inducer cells (20,21). The lymphocytes numbers that carry NK, IL-2 and transferrin receptors have been risen fundamentally in the peripheral blood mononuclear cells (PBMCs) of humans that given β-carotene orally as a supplement (22,23), Also it promotes the NK cytotoxicity (24)

At the same time, long period of supplementation with β-carotene to old age but not middle-aged men elevated natural killer cell activity (25). In vitro, β-carotene stimulated hamster macrophages to generate TNFα (26). ROS Activation by TNFα will elevates the breakdown of Inhibitory-κB from NFκB, and then the entering this transcriptional factor into the nucleus, that will releases the cytokines, chemokines, cell adhesion molecules, and acute phase proteins; also it will permit the activity of the apoptotic antagonist effect. Alternatively, intracellular ROS may directly rise NFκB (26). Besides cell-mediated and humoral immune responses, β-carotene has been shown to arrange nonspecific cellular host defense (27).

Tjoelker et al. (28) showed that the alimentary β-carotene that given to dairy cows through the stressful drying off period could activate neutrophils opsinization and bacterial eliminating ability. Bendich and Shapiro were the first to study the specific role of carotenoids on the immunological response (17). They presented that rats supplied with canthaxanthin, a carotenoid with no provitamin A action, had an increased mitogen-induced lymphocyte proliferation; dietary β-carotene illustrated similar picture. Later researches explain the same immune-supporting action of carotenoids without provitamin A action, particularly lutein, lycopene, astaxanthin and

canthaxanthin. Canthaxanthin boosted the expression of activation markers for T-helper and NK cells in human PBMC in vitro (29). Jyonouchi et al. (30) presented that lutein and astaxanthin heightened the ex vivo immunoglobulin response of mouse splenocytes to T-cell antigens.

Schwartz et al. (31) illustrated elevated cytochrome oxidase and peroxidase activities in macrophages incubated with canthaxanthin, β-carotene, and α-carotene in contrast to the incubation with 13-cis retinoic acids. All of these alterations signalize increased respiratory outbursts by the macrophages when they are presented to carotenoids.

Interleukin-10 and TNF-α are two significant cytokines in anti-tumor immune response. Interleukin-10 (IL-10) is a pleiotropic cytokine that has a remarkable impact on the control of the immunological response (32). This biological molecule can strongly suppress macrophages activity, inhibiting the production of pro-inflammatory cytokines [e.g., tumor necrosis factor α (TNF-α) and IL-6] and impairing antigen presentation/T cell activation, by restraining expression of major histocompatibility complex class II, B7-1, and B7-2 (33).The anti-inflammatory leverage of IL-10 can be strengthening by reinforcing the production of soluble(s) TNF receptors (R) and IL-1R antagonist. In the opposite to its actions on macrophages, IL-10 encourages the propagation of mast cells, B and T cells, and consolidates T cell responses to IL-2 (34).

The main interest of IL-10 studies has been to know how IL-10 intercede deactivation of cytokine production, But this still argumentative; precisely the capability of IL-10 to prevent lipopolysaccharide (LPS)-induced gene expression which mediated transcriptionally via the block of the NF-κB pathway. Though, more proof also proposes that IL-10 can work by means of post-transcriptional process by destabilizing mRNA, in the matter of TNF-α and the chemokine KC. This influence needs the AU-rich elements in the 3′ untranslated region.

Moreover, these researches showed the impacts of IL-10 are indirect and that IL-10 is prompting a gene that its product is in charge of intermediate the destabilization of mRNA (35). Investigations that applied microarray analysis to characterize IL-10-inducible genes in the existence and loss of the potent pro-inflammatory stimulus LPS, These researches have clarified 19 inducible genes for IL-10, Three of these genes, IL-1ra, SOCS3, and CD163, have formerly been observed as being organized by IL-10; yet, the other 16 exemplify novel IL-10-inducible genes primarily noticed in a study by Kaur et al., (2006)(36). The results of our study indicated that Lycium Carotenoids are pigments accountable for the red, orange and yellow coloration of plants and animals. They may be profitable in two ways; they have a powerful antioxidant activity, and they work as an immunostimulants, acted to elevate TNF-α level and enhance IL-10 production precisely at lower concentration (125μg/ml) after 2 hours of exposure about four folds of their normal TNF-α level.

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