RESEARCH PAPER | Biotechnology | Volume : 5 | Issue : 12 | December 2015 | ISSN - 2249-555X

Effect of TheTotal Lycium barbarum Carotene on Normal Human Blood Lymphocytes

KEYWORDS: Lycium barbarum carotene , Immunomodulation, IL-10, TNF-α Elisa assay, MTT assay

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ABSTRACT The discovery and identification of a new drugs, which can potentiate the immune function has become an important goal of researches in immune-pharmacology and oncotherapy. This study demonstrates the favorable effects of Iraqi wild type Lycium barbarum active components as immunomodulating agent. The immune stimulating effect of the extracted total carotene showed normal blood lymphocytes proliferation estimated by MTT assay and alteration in IL-10 and TNF-α level by ELISA technique caused both cytokines level elevation after 2 and 4 hours exposure at concentrations .The result of the present study indicated that Lycium Carotenoids as pigments responsible for the red, orange and yellow coloration of plants, can behave as an immunostimulants, acted to promote IL-10 production and increase TNF-α level specially at lower concentration(125µg/ml) after 2 hours of exposure about four folds of their normal TNF-α level.

Introduction

Studies are in progress to understand how natural compounds may provide protection against toxic, mutagenic and carcinogenic activities of chemical compounds. One of these natural compounds are carotene. Many studies have shown an association between diets rich in carotenioods and a reduced incidence of many forms of cancer, and also several other chronic diseases it has been suggested that the antioxidant properties of these compounds are a causative factor(1). Since the immune system plays a major role in cancer prevention, it has been suggested that beta-carotene may enhance immune cell function(2).

Lycium barbarum, a well-known in Chinese traditional medicine and foodstuff, contained different active components which have many proposed pharmacological and biological effects, including anti-aging activity (3), immune modulation (4) and anti-cancer activity (5). All studies and researches on *Lycium barbarum* biological active components were done on the Chinese grown plant, while there is little(if not) researches about the Iraqi wild type plant. The color components of the fruit are a group of caroteniods, a major component of *Lycium* which make up 0.03-0.5% of the dried fruit in cultivate Chinese type, while in the Iraqi wild *Lycium* fruit, the total caroteniods compound estimated as β –carotene is about 0.031%of the dried fruits(6). In spite of the lower contents of *Lycium* active constituents in wild Iraqi plant than the cultivated Chinese fruit, the plant was considered a good source for both constituents(polysaccharides and carotene)which might give the plant important focusing to improve the cultivated conditions of this plant(6). The aim of the present study was to investigate the cytotoxic activity of the extracted carotene towards normal human blood lymphocytes cell (by MTT assay) ,and then to determine the cytokines level in lymphocytes cultured cells represented by (IL-10& TNF-α) as a mediators of immune regulation by ELISA technique at cellular level.

Material and Method

i-Plant Collection:

Ripe orange small fruits from *Lycium barbarum* trees grown as a wild plant were collected from Al-Jadriya district at University of Baghdad, and classified by the herbarium of the Biology Department, collage of Science at Baghdad University/Iraq.

ii-Plant Extraction and Determination of the Total Carotene Content in the Fruit were mentioned in a published paper(6)

iii- Immunomodulation Determination(*in vitro*)

To determine *in vitro* immune effects for total carotene extracted from *Lycium barbarum* ; lymphocytes culturing and viable counting was employed in each step; lymphocytes proliferation(MTT Assay), cytokines level(IL-10 &TNF-α) were estimated.

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

-From eight healthy volunteers five milliliters of venous blood were taken from each, their ages in the range of (25-35) years old, never taken medication at least 10 days ago.
-Each five milliliters was transferred into vacuumed tubes containing 0.2% EDTA as anticoagulant with continuous gentle shaking.
-The human peripheral blood was diluted with PBS(pH=7.2) in 1:1 ratio.
-About 5 milliliters of the diluted cell suspension was layered onto three milliters of Ficoll separation fluid(lymphprep; specific gravity=1.077g/l, placed into vacuumed tube (10 ml capacity).
-The tubes were centrifuged at 2000 rpm for 30 minutes.
-The lymphocyte cells were collected with sterile Pasteur pipette, transferred into 10ml vacuumed tubes, suspended with 5ml RPMI 1640, and centrifuged for 10minutes at 2000 rpm. The step was repeated twice.
-The isolated lymphocyte cells were collected again and suspended in RPMI-1640 medium supplemented with 10% fetal calf serum, containing100 units/ml penicillin, and 100µg/ml streptomycin, then transferred into appropriate tissue culture flask and incubated for 18 hours at 37^{0}C in 5% CO_2 incubator.

2-Measurement of Lymphocytes proliferation by MTT Assay (9) :-Aliquot of 100µl of the suspended cells was seeded in each of the 96 well microtiterplate, (10^4cell/well). The plate was incubated at least for 2 hours in a CO_2incubator.

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-Serial concentrations from the extracted carotene were prepared from the stock solution (1000µg/ml)to get (500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.9) µg/ml, then sterilized with 0.22 µm Millipore filter.
-Then 100 µl from each concentration of the extract was added in triplicate to each well of the lymphocytes seeding plate .Control positive was employed as 10 µl of 0.1% PHA solution (phytohemagglutinin), while negative control represented by untreated lymphocyte cells suspended in medium.
-The plate was incubated in a CO_2 incubator for 24 hours at $37C^0$.
-Finally, 50 µl of MTT dye (2mg/ml) was added to all wells, then incubate for further 4 hours. The plate was centrifuged for 5mimutes at 1500rpm at the end of the dye incubation period.
-Then the medium was removed gently by fine gauge needle after centrifugation.
-The MTT-formazan crystals which formed only by live cells were dissolved with100µl DMSO added to all wells.
-Absorbance at 620 nm was recorded immediately by ELISA reader.
-Viable cell Lymphocytes as a percentage was calculated as followed:
[Absorbance of the test /Absorbance of negative control] X 100.
-A comparison between the results of carotene extracts at different concentrations were statistically calculated to choose the most effective dosages of each extract that may cause lymphocytes proliferation that to be used in further experiment as immunostimulants.

3-Determination of the Cytokine Level by ELISA Technique(10)

-Measuring 1000µl of lymphocyte suspended cells were seeded in each of the 24 well tissue culture plate($1X10^6$cell/well), two plates were needed, one plate incubated for 2 hours intervals and the other incubated for 4 hours.
-Both plates were incubated at least for 2 hours in a CO_2incubator before treatments.
- Three concentrations from extracted carotenes were chosen, these are: (500, 250,and 125)µg/ml as they showed the potent proliferative effect for the normal lymphocytes. They were prepared from carotene stock solution, then all solutions were sterilized with 0.22 µm Millipore filter.
-To each plate one milliliter/well was added from each carotene concentration in triplicate. Negative control represented by untreated cells suspended in growth medium was included.
- One plate was incubated for 2 hours, the other incubated four 4 hours in CO_2 incubator at $37C^0$.
-At the end of each interval times all wells were aspirated and transferred in separated vacuum tubes and centrifuged for 20 minutes at 2000 rpm.
-The supernatants of each tube were separated and kept at $-20C^0$ to be estimated by ELISA kit.

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

The work was done following the instruction of USBiological TNF-α and IL10 kit protocol/Biochemical &Biological Reagents ,United State Biological. Catalog No(T9160-01). The supernatants of treated lymphocytes at different concentrations of extracted carotene (previous steps) were applied in this test. At the end of experiment a standard curve for each cytokine different concentrations was plotted with their absorbance at 450nm, then all test reading were calculated according to straight line equation obtained from the standard curve and both TNF- α and IL-10 level of the treated lymphocytes in the supernatant were obtained and evaluated statistically.

Statistical Analysis(11):

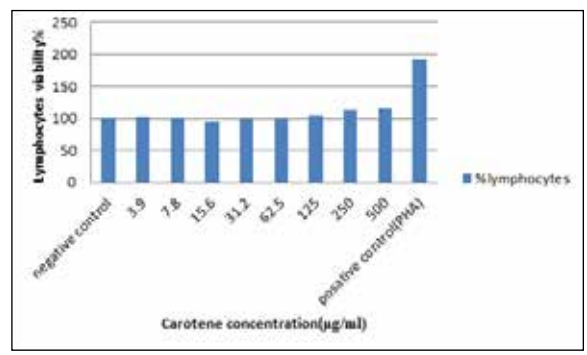
The Statistical Analysis System- SAS (2004) was applied for all results to show effect of difference concentration and other factors in studied parameters. The least significant difference (LSD) test and Duncan test at the comparative between means in this study

The Results

i-Total Carotene Content in *Lycium barbarum* **Fruits were mentioned in a published paper(6)**

ii-Lymphocyte Proliferation determination by MTT assay:

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



Figure(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 act as immune stimulant by enhancing lymphocyte proliferation specially at concentration 125µg/ml and above. Several human trials, using dietary beta-carotene supplementation with a wide range of intakes, have been undertaken to address this hypothesis. The general conclusion of these studies is that this compound can enhance cell-mediated immune responses, particularly in the elderly. Certain caroteniods, acting as antioxidants, can potentially reduce the toxic effects of reactive oxygen species (ROS). These ROS, and therefore caroteniods, have been implicated in the etiology of diseases such as cancer, cardiovascular and neurodegenerative diseases and aging(1). Recent studies on the role of caroteniods in gene regulation, apoptosis and angiogenesis have advanced our knowledge on the possible mechanism by which caroteniods regulate immune function and cancer.

Various *in vitro* studies about the mechanism of the plant cytotoxicity were differ from one cell culture to another depend on whether whole plant extract was used or any of the plant component. In fact, many nutritive and non-nutritive phytochemicals with diversified pharmacological properties have shown promising responses for the prevention and treatment of various cancers, including different types of cancer. Most of the animal studies done on *L.barbarum* explained the anti-cancer effects of the plant were through immune enhancements and prevent the development of complications or even tendency to carcinogenesis by increasing numbers of $CD4^+$and $CD8^+$ T cells to relieve the immunosuppression and enhance the anti-tumor function of the immune system(12). T lymphocytes play a central role in adaptive immunity. Chen *et al* (2008),

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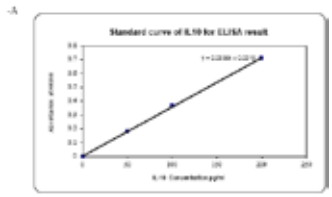
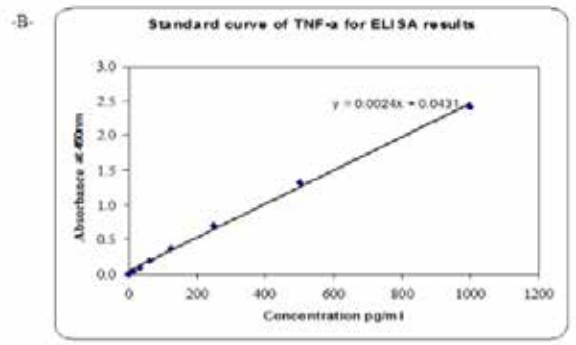
found that *Lycium barbarum* able to activate T cells, at the same time, the percentage of cells in G0/G1 phase was increased (13), thus because T cells spontaneously arrest in G0 and may remain quiescent for long period of time until exposed to specific antigen or mitogens that initiates a cascade of biochemical events leading the resting T cells to enter the cell cycle then proliferating and differentiating(14).For this reason the plant active components had been used as immune stimulant or immune adjuvant.

***iii*-Effect of Extracted Carotene from *L.barbarum* on Cytokine Levels (IL-10 and TNF-α)**

In order to trace cytokines (IL-10 and TNF-α) level in the supernatant of the treated lymphocytes with carotene at different concentrations and exposure time (2 and 4 hours) as well as control culture and standard solutions, ELISA was used and standard curve for both interleukins was plotted separately Figure(2)





Figure(2) Standard curve of IL-10 (A) and TNF-α (B) analyzed by ELISA, R^2=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)Effect 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	75.19NS	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 effect of different concentrations of carotene on cytokine level produced by treated lymphocytes after 2 and 4 hours, has 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.

Cytokines are soluble molecules that mediate cell-to-cell interactions. Cytokines commonly measured include IL-2, TNFα and IFN-γ produced by the CD4+Th1 cell subset, and IL-4, IL-5, IL-6 and IL-10 produced by the Th2 subset. The Th1 cells mediate cytotoxic and local inflammatory reactions, and therefore play important roles in combating intracellular pathogens including viruses, bacteria and parasites. The Th2 cells are more effective in humoral immunity, i.e., they stimulate B cells to proliferate and produce antibodies against free-living microorganisms. Therefore, a normal immune response will require a balance between the Th1 and Th2 subsets.

Studies on the role of caroteniods on immune response have generally used several key immune function assays(15). Seifter et al. (16) reported a marked stimulatory action of β-carotene on the growth of the thymus gland and a large increase in the number of thymic small lymphocytes. The stimulatory activity of β-carotene on lymphocyte blastogenesis has similarly been demonstrated in rats (17), pigs (18), and cattle (19). Increased numbers of Th and T inducer lymphocytes have been reported in human adults given oral β-carotene supplementation (20,21). The number of lymphoid cells with surface markers for NK cells and for IL-2 and transferrin receptors also was increased substantially in peripheral blood mononuclear cells (PBMC) from individuals supplemented with β-carotene (22,23). Enhanced NK cell cytotoxicity was observed in human subjects given oral β-carotene (24). Similarly, long-term β-carotene supplementation to elderly but not middle-age men increased NK cell activity (25). In vitro, β-carotene induced hamster macrophages to produce TNFα (26). Activation of TNFα by ROS increases the dissociation of IκB from NFκB, and the subsequent translocation of this transcription factor to the nucleus, resulting in the production of cytokines, chemokines, cell adhesion molecules, and acute phase proteins; this activation also produces an anti-apoptotic effect. Alternatively, intracellular ROS may directly increase NFκB(26). Besides cell-mediated and humoral immune responses, β-carotene has been shown to regulate nonspecific cellular host defense(27). Tjoelker et al. (28) reported that dietary β-carotene stimulated phagocytic and bacterial killing ability of neutrophils from dairy cows during the stressful drying off period. A specific role of caroteniods on immune response was first reported by Bendich and Shapiro (17). They showed that rats fed canthaxanthin, a carotenoid with no

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provitamin A activity, had a heightened mitogen-induced lymphocyte proliferation; dietary β-carotene showed similar action. Subsequent studies have similarly reported the immunoenhancing action of carotenoids without provitamin A activity, notably lutein, lycopene, astaxanthin and canthaxanthin. Canthaxanthin enhanced the expression of activation markers for Th and NK cells in human PBMC in vitro (29). Jyonouchi et al. (30) reported that lutein and astaxanthin increased the ex vivo antibody response of mouse splenocytes to T-cell antigens. Schwartz et al. (31) reported increased cytochrome oxidase and peroxidase activities in macrophages incubated with canthaxanthin, β-carotene, and α-carotene compared with incubation with 13-*cis* retinoic acid. All of these changes indicate increased respiratory bursts by the macrophages when they are exposed to carotenoids. .

Interleukin-10 and tumor necrosis factor- α are two important cytokines in antitumor immunity. Interleukin-10 (IL-10) is a pleiotrophic cytokine that has an important role in regulating the immune response (32). This cytokine potently inactivates macrophages, inhibiting the expression of proinflammatory cytokines [e.g., tumor necrosis factor α (TNF-α) and IL-6] and disabling antigen presentation/T cell activation, by inhibiting expression of major histocompatibility complex class II, B7-1, and B7-2 (33).

The anti-inflammatory activity of IL-10 is augmented by enhancing the release of soluble(s) TNF receptors (R) and IL-1R antagonist. In contrast to its activities on macrophages, IL-10 induces the proliferation of mast cells, B and T cells, and enhances T cell responses to IL-2(34). A major focus of IL-10 research has been to identify the mechanism by which IL-10 mediates suppression of cytokine synthesis. This remains a controversial field; specifically, the ability of IL-10 to inhibit lipopolysaccharide (LPS)-induced gene expression has been shown to be transcriptionally mediated via the inhibition of the nuclear factor-κB pathway. However, further evidence also suggests that IL-10 can act through a post-transcriptional mechanism via destabilizing mRNA, in the case of TNF-α and the chemokine KC. This effect requires the AU-rich elements in the 3′ untranslated region . Furthermore, these reports suggest that the effects of IL-10 are indirect and that IL-10 is inducing a gene whose product is responsible for mediating the destabilization of mRNA (35).

In one study used microarray analysis to identify IL-10-inducible genes in the presence and absence of the powerful pro-inflammatory stimulus LPS. These studies have identified 19 inducible genes for IL-10. Three of these genes, IL-1ra, SOCS3, and CD163, have previously been shown as being regulated by IL-10; however, the other 16 represent novel IL-10-inducible genes first identified in a study by Kaur *et al.,* (2006)(36). The result of the present study indicated that *Lycium* Carotenoids are pigments responsible for the red, orange and yellow coloration of plants and animals. They may be beneficial in two ways; they have a powerful antioxidant activity, and they can behave as an immunostimulants, acted to increase TNF-α level and promote IL-10 production specially at lower concentration(125µg/ml) after 2 hours of exposure about four folds of their normal TNF-α level.

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