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Molecular Immuno-response Effects for Iraqi *Lycium barbarm* Carotenes upon Normal Human Lymphocytes Culture

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Abstract

Objectives: The study aimed to investigate the effects of Iraqi *Lycium barbarm* carotene on normal human blood lymphocytes at molecular level.

Methods: The normal human blood lymphocytes culture was exposed to Carotene extracted from Iraqi *Lycium barbarm* to estimate the interleukins expression in treated lymphocytes represented by IL-10 and TNF-α as a moderator of immune response at a molecular scale, and their CD markers changes expression (CD3, CD4 & CD8) by flow-cytometer apparatus.

Results: Total carotenes content was 0.33 mg/g powdered dried fruit. Potent carotene effect was appeared at concentration of 500 µg/ml for treated lymphocytes with increasing CD3 level to get upper level after 2 hours interval, while after 4 hours exposure both CD4 and CD8 levels increased dramatically for lymphocytes treated with 125 µg/ml, and 250 µg/ml *Lycium* carotene. An alteration in cytokines gene expression for IL-10 production in response to carotene treatments at (125, 250, 500) µg/ml for both intervals, with suppress in tumor necrosis factor (TNF-α) gene expression in relation to β-actin the internal control and the PHA mitogenic agent.

Conclusion: The study concluded that the carotenes extracted from *Lycium barbarm* fruits seemed to initiate the immune system toward Th2 cell activation rather than Th1, besides the extract may potentiate IL-10 production.

Key words: *Lycium barbarm*, carotenoids, interleukin-10, TNF-α, CD4, CD8

Introduction

Lycium barbarm (*L. barbarm*) belongs to Solanaceae family is one of the most notable Chinese medicinal herbal species and has a famous tradition usage as a food and medicinal plant in various Asian countries, especially in Chinese traditional medicine, known with different names; wolfberry, boxthorn, Chinese wolfberry, and gugija in Korea and kuko in Japan.[1] Leaves and Fruits of *L. barbarm* are exceedingly applied as herbal and medicinal tea in China, Southeast Asia, Europe, and North America. This plant is naturally grown in warm regions of Iraq, known locally as 'Awsaj'. The institute of the Chinese medicinal monographs recorded the plant to be the "nourishing liver and kidney, enhancing eyesight, enriching blood, invigorating sex, reducing rheumatism" and so on.[2] Its main functions were reported as immunity improvement,[3] Antioxidant,[4] Anti-microbial,[5] Anti-cancer,[6] improving hemopoiesis,[7] Anti-aging, and sexual tonic.[8] Studies are in progress to understand how these compounds may or may not provide protection against toxic, mutagenic and carcinogenic activities of chemical compounds. The beautiful orang of lycium fruit indicated the high plant content of "zeaxanthin" as one of predominant carotenoids group in nature, also called physalien or physalin.[9] Therefore, this fruit is considered as good food source in zeaxanthin content.[10]

The present study represented a novel work that high lightened the biological activity for the wild Iraqi *Lycium barbarm* intended as a great worth for researcher knowledge specially the immunomodulatory effects Therefore, current research including this plant was aimed to estimate the effects of the extracted carotene towards normal human blood lymphocytes (CD3, CD4 & CD8) by using flow-cytometer, and to investigate IL-10 and TNF-α gene expression in the cultured lymphocytes using RT-PCR.

Materials and Methods

Plant Selection and Collection

The plant was collected at end of September as the small ripe wild *Lycium barbarm* orange fruits had been naturally found in Al-Jadriya district of Baghdad University to be classified by Biology Department herbarium at the college of science.

The Extraction of the Total Carotenes from the Fruits[11]

Using porcelain mortar, the dried *L. barbarm* fruits powder in a quantity of 1 g was homogenized well with 3 ml distilled water, then 2 ml absolute ethanol was added to be mixed well with aid of vortex and transferred to separator funnel contained 10 ml *n*-hexane and gentle mixing. Two layers were separated, the organic hexane layer which contained the extracted carotene and the aqueous layer. The aqueous was extracted many times with *n*-hexane. The final volume of the combined *n*-hexane layers was measured representing the final volume of the total carotenes extracted from one gm *L. barbarm* fruit which was evaporated and weighted as total carotene.

Immunomodulation Determination (*in vitro*)

Lymphocyte Culturing

From 8 healthy volunteers with an age ranged between (25–35), the venous blood (5 ml) was collected and transferred into separated vacuumed tube. All volunteers appeared healthy, and they never took any kind of medication for at least 10

days. A General protocol for lymphocytes separation was preceded in the current study.[12]

Cell Viable Counting for the Isolated Lymphocytes

According to general protocol included in reference for viable cells counting, a freshly Trypan blue 1% PBS dye was prepared.[12] Viable cells appeared transparent in contrast to the dead cells that could be easily distinguished under light microscope within seconds as violet color.[13] Calculation for viable lymphocyte cells was done before any of the following immune tests.[14]

Treating the Isolated Lymphocytes with the Extracted Lycium Total Carotenes

Lymphocytes suspended cells (1 ml) in concentration of 10^6 cell/well were seeded, in two tissue culture plate of the 24 wells to be incubated in a CO_2 incubator for at least for 2 hours before treatments. Three concentrations from the extracted carotenes (125, 250, and 500 µg/ml) were used for two intervals for carotene exposure time; one plate incubated for 2 hours and the other for 4 hours had been used.[15] At the end of each exposure period, both plates were centrifuged to separate the supernatant from the pellets.

Detection of CD4 and CD8 Markers by Flow Cytometry

The CD4 and CD8 estimation protocol (EXBIO/NadSafinou) were applied using 10 µl FITC (for CD4 marker), or PE (for CD8 marker)–conjugated monoclonal anti-human CD marker provided in the kit. The isolated pellets from each well were separately treated and subjected to flow cytometry apparatus for analysis.[16]

Extraction and Purification of Total RNA

Using Geneaid total RNA Mini kit, in aseptic condition and all equipment, glassware, hood-benches, gloves, and even electrophoresis tank were thoroughly cleaned and washed with RNase-free water, treated with 0.1% diethylpyrocarbonate solution (DEPC), then left 10minutes in a vacuumed-hood, to insure RNase-free condition and to remove all residual traces of DEPC solution. Total RNA was measured by using a spectrophotometer at 260 nm wavelengths and the purity of RNA was measured by determining the ratio of A_{260}/A_{280} nm. All samples were at ratios of above 1.75. The purified RNA is ready for RT-PCR and other tests. The work was done according to Geneaid kit protocol.

Electrophoresis

Gel electrophoresis was done according to as follows:[17]

- Agarose gel (0.8–1)g dissolved in100 ml of TBE 1X buffer (Tris HCl: Boric acid: EDTA in D.D. water) was prepared in a quantity enough for pouring in appropriate tray.
- Mixing 8 µl extracted Total RNA with 2 µl loading dye (Bromophenol blue and glycerin).
- After about two hours running the apparatus was stopped, and the gel was removed from the tank and to ensure the presence of RNA's bands, the gel was visualized by staining with ethidium bromide that incorporated in RNA strands (ribosomal RNA considered double-stranded molecule due to extensive secondary structure). Pictures were taken under UV camera system.

Detection of Interleukin -10 (IL-10) and Tumor Necrosis Factor-α (TNF- α) mRNA Expression by RT-PCR

Total RNA which extracted from treated lymphocytes with the extracted carotene in above step for all samples, were reverse transcript to complementary DNA (cDNA) according to (Accu Power Rocket Script RT Pre-Mix) which is ready lyophilized master-mix containing all components for first-strand cDNA synthesis (in 96 wells of 20 µl total volume) from purified Poly (A) or total RNA templates.[18] Table 1 showed primers used and their sequences and the predicted product size.

- All samples (the extracted total RNA for lymphocytes treated with three concentrations and with two exposure time) designated as in Table 2.
- The recommended amounts of each extracted RNA sample and the specific primers that should be added for RT-PCR Pre-Mix tubes were shown in the Table 3.
- The reaction was performed under the condition shown in Table 4, using Thermal cycle PCR Apparatus (Labnet international Multigene/USA).

Electrophoresis for the RT-PCR Product

At the end of the reaction a gel electrophoreses was applied with (1.5%) Agarose gel and Ladder DNA of 100 bp, internal control β-actin, PHA also –ve control and distilled water

Table 1. **Human cytokine primers (IL-10 and TNF- α) and their sequences and predicted product size(Bp = Base pair; F = forward sequence; R = reverse sequence)**

Cytokine	The sequences	Product
IL-10	F: 5'TACGGCGCTGTCATCGATTT3' R: 5'AAGGTTTCTCAAGGGGCTGG3'	273 bp
TNF-α	F: 5'TCTCGAACCCCGAGTGACCAA3' R: 5'ACCGCACCTCGACTCCTAT3'	123 bp
β-actin (internal control)	R: 5'AACCCCAAGGCCAACCGCGAGA AGATGACC3' R: 5'GGTGATGACCTGGCCGTCAGG-CAGCTCGTA3'	416 bp

Table 2. **Symbols of all samples used in RT-PCR system**

Sample treatment	Concentration (µg/ml)	Interval	Symbol
Total carotenes	500	2 hours	C1/2 h
Total carotenes	250	2 hours	C2/2 h
Total carotenes	125	2 hours	C3/2 h
Total carotenes	500	4 hours	C1/4 h
Total carotenes	250	4 hours	C2/4 h
Total carotenes	125	4 hours	C3/4 h
(β-actin) internal control			+ve
PHA	0.1%		PHA
Lymphocytes culture only (–ve control)			–ve

Table 3. **The recommended quantities of components for RT- PCR kit**

Component	Amount/sample
Total RNA of sample	10 pmole-5 µg (3 µl)
Primer	20 pmole each (2 µl each)
ddH_2O with DEPIC	Up to20 µl

Table 4. **Conditions for PCR Program**

Step	Temperature	Time	Cycle
cDNA synthesis	60°C	30 minutes	1
Pre-Denaturation	95°C	5 minutes	1
Denaturation	94°C	30 seconds	40
Annealing	55°C	1 minutes	–
Extension	72°C	1 minutes	–
Final extension	72°C	5 minutes	1

RNase free. The same procedure mentioned in the previous step was applied and the results for both cytokine PCR products were visualized under UV system.

Statistical Analysis

The Statistical Analysis System-SAS (2018)was used to present the effect of different concentrations and factors in the studied parameters.[19] To compare between the means of the variations in this study, the least significant difference (LSD) test and Duncan test were used.

Results

The Total Carotenes Content in the Fruits

The final volume of total carotene extracted from 1 g dried powdered fruit with *n*-hexane was 165 ml which analyzed qualitatively and quantitatively, and the concentration of total carotene extracted from the dried fruits of *L. barbarm* was 0.33 mg/g and all qualitative and quantitative results was authorized in previous study.[20]

Effect of *L. barbarum* Total Carotenes on CD4, CD8 and CD3 Markers

To determine the level of CD markers as normal lymphocytes treated with different *Lycium* carotenes concentrations, a flow-cytometer was used to pick the change in CD markers percentage. Table 5 showed CD markers percentage expressed by lymphocytes after Carotene treatment for two exposure times (2 and 4) hours.

As shown in Table 5, the most potent effect was shown for the lymphocytes treated with extracted carotene at concentration of 500 µg/ml by increasing CD3 percentage to upper level after 2 hours treatment, while CD4 and CD8 levels increased dramatically when normal lymphocytes treated with 125 µg/ml, and 250 µg/ml *Lycium* carotene for 4 hours exposure.

Table 5. **% CD Markers of lymphocyte treated with different concentrations (125, 250, 500) µg/ml of extracted carotene for two exposure times (2 and 4) hours**

Concentration (µg/ml) carotene	CD3	CD4	CD8	CD3	CD4	CD8
	For 2 hours exposure*			For 4 hours exposure*		
500	38.1%	0.0%	0.1%	0.0%	0.6%	0.0%
250	36.3%	0.0%	0.0%	0.0%	0.6%	0.3%
125	0.0%	0.0%	0.0%	0.1%	0.3%	0.4%
Control	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

*CD marker level less than 0.1% represented by 0.0% as the kit sensitivity declared.

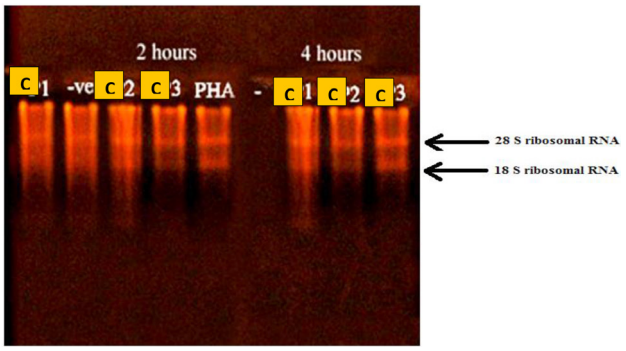

Fig. 1 **Agarose gel electrophoresis for 8 µl extracted total RNA of treated lymphocytes with extracted carotene mixed with 2 µl Bromophenol Blue loading dye. Bands were fractionated by electrophoresis on 1% Agarose gel in TBE 1X buffer pH = 8 (5 Volts/cm, current of 60 Ampere for 1 hour) visualized under UV light after staining with Ethidium Bromide. Lane C1, C2, C3: Lymphocytes treated with (500, 250, 125) µg/ml *Lycium* carotene respectively, Lane PHA: Lymphocytes treated with PHA, Lane (–ve): Lymphocytes without treatment, and Lane (–): Distilled water only. Treatment was made for (2 and 4) hours.**

Effect of Extracted Carotenes from *L. barbarm* on Gene Expression for Cytokines

A. Total RNA in Lymphocytes Treated with L. barbarm Carotenes

Gel electrophoreses indicated the presence of clear two bands which mimic that related to genes for 28 S and 18 S ribosomal RNA as shown in Figure 1.

B. The Expression of (IL-10) and (TNF- α) genes in Lymphocytes treated with Extracted Carotene

In order to detect the expression of specific gene (IL-10 and / or TNF-α) as well as alteration of gene expression which may be directed the immune defense in response to treating Lymphocytes with *Lycium barbarm* carotene at different concentrations toward humoral or cellular immune defense, Reverse transcriptase polymerase chain reaction (RT-PCR) technique was applied in this study. The results shown in Figure 2 indicated that alteration of gene expression of normal lymphocytes toward IL-10 production in response to *Lycium barbarm* extracted carotene treatments at concentrations (125, 250, 500) µg/ml were obvious for all treatments and for both

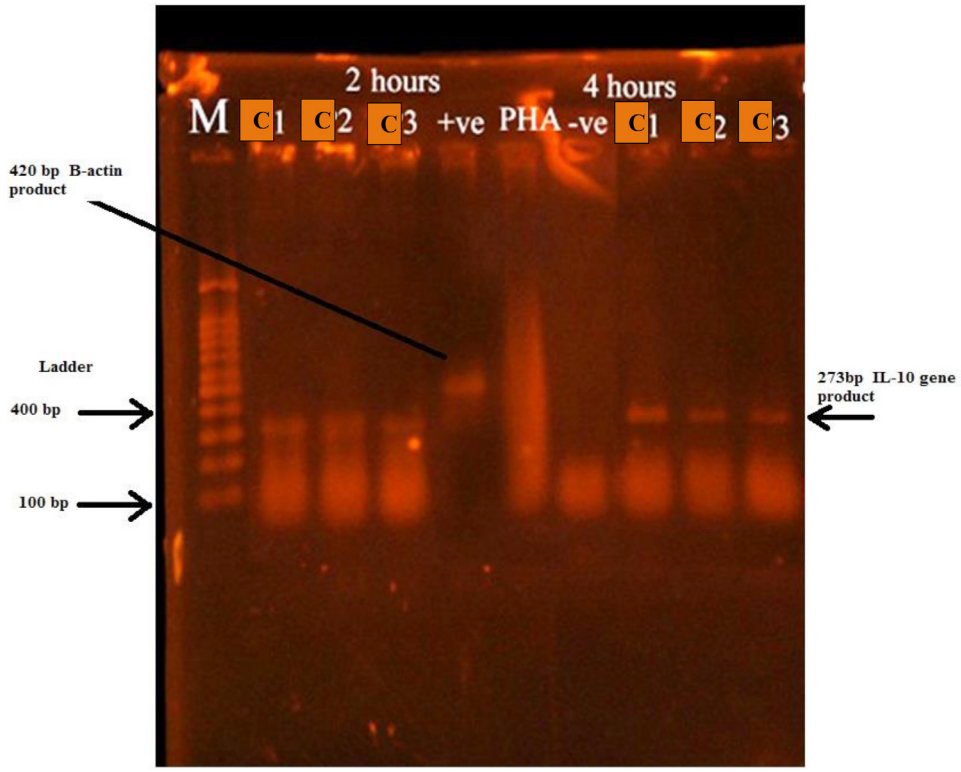

Fig. 2 **Agarose gel electrophoresis for amplified mRNA for IL-10 gene expression of the lymphocytes treated with different *L. barbarum* carotenes concentrations. Bands were fractionated by electrophoresis on 1.5% Agarose gel (5 volts/cm, current 60 ampere, 2 hours, TEB 1X buffer) analyzed under UV light after staining with ethidium bromide. Lane M: 100 bp ladder, lane +ve: β-actin internal control, lanes C1, C2, C3: lymphocytes treated with 500, 250 and 125 µg/ml extracted carotenes respectively, lane –ve: negative control, lane PHA: lymphocytes treated with PHA. Treatment for (2 and 4) hours intervals.**

interval times 2 and 4 hours in corresponding to negative control (untreated cells) and the internal control (β-actin gene that gave product of 420 bp). The PHA, which is a mitogenic for T lymphocytes which binds to N-acetyl galactosamine glycoproteins expressed on the surface of T cells then activates the cell proliferation has not any effect on IL-10 expression.[21]

With respect to gene expression for tumor necrosis factor (TNF-α), human peripheral blood lymphocyte cells exposed to *Lycium* total carotenes, do not show any TNF-α expression at all concentrations (125, 250, 500) µg/ml and for (2 and 4) hours intervals, as well as the negative control, in relation to (β-actin) the internal control and the PHA mitogenic agent as shown in Figure 3.

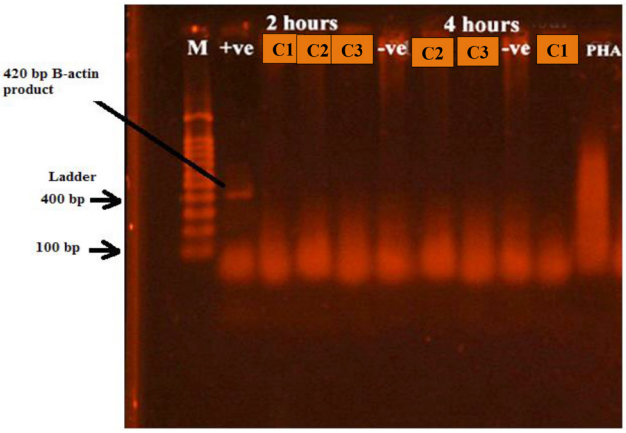

Fig. 3 **Agarose gel electrophoresis for amplified mRNA of TNF-α from treated lymphocytes with different *L. barbarm* extracted carotene concentrations. Bands were fractionated by electrophoresis on 1.5% Agarose gel (5 volts/cm, current 60 ampere, 2 hours, TEB 1X buffer) analyzed under UV light after staining with ethidium bromide. Lane M: 100 bp ladder, lane +ve: β-actin internal control, lanes C1, C2, C3: lymphocytes treated with 500, 250 and 125 µg/ml extracted carotene respectively, lane –ve: negative control, lane PHA: lymphocytes treated with PHA. Treatment for (2 and 4) hours intervals.**

Discussion

In a study by reported that; β–carotene administration may cause enhancement of immunoglobulin levels and CD4+ cells count.[22] Other study by concluded that; The CD4-CD8 ratio were raised after the administration of β-carotene in 9 month, whereas throughout the study the natural killer cells, virgin T cells, memory T cells, and cytotoxic T cells remained unaltered, suggesting that it takes a long time to induce alterations of lymphocyte surface markers.[23]

Carotenoids can stimulate B- and T-lymphocytes proliferation and increase the activity of macrophages and cytotoxic T-cells, for production of cytokines.[24] Recently, the focusing on carotenoid-rich vegetables and fruits for decreasing the risk of many diseases has been attributed to the major carotenoids, such as β-carotene, lutein, zeaxanthin, and lycopene which the wild Iraqi *L. barbarm* fruit rich in. In a study confirmed that administration of carotenoid compounds such as Vit. A decreased the side effects of the conventional anticancer drug Methotrexate (MTX); that considered as protective in chemotherapy.[25] In one study which acts to estimate carotene

production from ex plants in tissue culture technique to successfully yield a plant product rich with carotenoids compound as there is a great demand to natural carotene source in medical field.[26]

The possible mechanisms for these compounds' activity may contribute to that; β-carotene can block the nuclear translocation of the NF-κB p65 subunit which had an inhibitory effect on phosphorylation and degradation of the NF-κB inhibitor. This may explain the antioxidant activities for this bioactive molecule in quenching free radicals, and reducing the damage caused by (ROS) reactive oxidant species that causes lipid peroxidation. Furthermore, carotenoids can play important roles in immune-regulation and immune-stimulation in vertebrates.[24]

Conclusion

Since cytokines play a prominent role in the development of immune response, in the current study the extracted carotenes seemed to initiate the immune system toward Th2 cell activation rather than Th1, besides the extract may potentiate IL-10 production. Results including IL-10 and TNF-α level with or without carotenes for each cytokine expression detection, give an indication that *L. barbarm* directed body defense toward Th2 rather than Th1 immune response since cytokines of the treated lymphocytes with this active immune stimulant component have been increased in level toward IL-10 (the pro-inflammatory cytokine inhibitor) with remarkable elevation of $CD8^+$ after 4 hours of exposure.

The safety, metabolism, and molecular biological properties of carotenoids should be elucidated through further studies before they are used to prevent carcinogenesis.

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Conflict of Interest

None.

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