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The Immunomodulation Effect of Staphyloxanthin Produced by Staphylococcus Aureus

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Abstract--*The results was indicated from a total of(70) isolate of staphylococcus (22.8%) isolated from 306 sample of different clinical sources that is primary diagnosis as S. aureus depending on cultural, morphological and biochemical test were collected from patients suffering from different disease infection such as wound ,UTI ,blood , eye , vagina , tonsil , nasal , burn infections and Bacteremia who had certain clinical. And the burn, wound and Nasal infection was the most accessible site for S. aureus (30.8 ,30.8,29.8)% respectively .the results was indicated the skim milk agar was the best medium of staphyloxanthin production , therefore the optimum condition of pigment production at 37°C for 72 hr .the extraction of staphyloxanthin was recorded highly significant by ethyl acetate and ethanol method . the column chromatography method used to purification of staphyloxanthin extract. as well as the results was recorded the antimodultory effect of staphyloxanthin (crude and partied in vivo and increasing in level of cytokines that induced by extracts effect). Conclusion: Staphyloxanthin acts as anti-oxidant ,anti-inflammatory ,as well as the carotenoid molecules could play an important role in regulation of the immune response. therefore staphyloxanthin represented as antimodulater effect during infection .*

Key words*--staphyloxanthin , antimodulatar,, purification ,cytokines*

I. INTRODUCTION

Staphylococcus aureus was a Gram positive, non-motile and non-spore-forming bacterium. *S. aureus* was being an opportunistic pathogen capable of causing a diverse spectrum of acute and chronic infections (1). The golden pigment staphyloxanthin of *S. aureus* colonies distinguished it from other *Staphylococci* and related Gram-positive cocci. Staphyloxanthin also acted as a virulence factor. It has an antioxidant action that helps the microbe evade death by reactive oxygen species produced by the host immune system (2). Therefore the membrane-bound carotenoid which was played a role in the environmental fitness of *S. aureus* ,Carotenoids may also stabilized the *S. aureus* membrane during infection and pathogenesis (3). by (Marshall and Rodwell)(4) was determined their chemical structures ,which are triterpenoid carotenoids, possessing a C_{30} chain, it's chemical formula(C51H78O8). It was not a very stable character. Pigmentation is being usually apparent after 18 to 24 h of growth at 370C but was more pronounced when cultures were held at room temperature for 24 to 48hr. Staphyloxanthin was a typical secondary metabolite. It was not necessary for the growth and reproduction of *S. aureus* but might serve a role in survival in infected hosts and in combating the immunesystem(5).

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II. MATERIAL AND METHODS

Bacterial characterization

From 306 sample of different clinical sources (70) isolate (22.8%) primary diagnosed as *S. aureus* depending on cultural morphological and biochemical test. These characteristics include; colonial morphology, size of colony, color and the effect on the media such as blood hemolysis, pigments appear on Milk agar and ability to ferment mannitol. Bacterial isolates were examined and identified by microscopic, biochemical test and Vitek2 system characteristics (6). The confirmed identification was down by Vitec system, Results were expressed as defined by the manufacturer as 96% to 100%, excellent identification; 93% to 95%, very good identification; 89% to 92%, good identification; 85% to 88%, acceptable identification; below 85%, no identification (7).

Detecting the ability of *S. aureus* isolates to produce staphyloxanthin

A volume of 100 µl of the inoculum from each isolate (70 isolate) was streaked on Milk agar medium, incubated at 37°C for two days and then incubated at 20°C for two days. Appearance of growth with pigment (orange, yellow) indicates a positive result (8).

Quantitative Assay of production pigment

Bacterial cell were harvested from skim agar and placed in sterile plane tube containing 3ml of sterilized normal saline, then vortex and centrifugation at 5000rpm for 10min , the supernatant was transferred to new curate tube and the absorption of solution was measured at 450nm using spectrophotometer and determined the isolate with highly concentration of staphyloxanrhin (4). .

Extraction and purification of staphyloxanthin pigment

Extracting by ethanol and ethyl acetate according to (3)

1-The pigment of *Staphylococcus aureus* (STX) was extracted from isolate S.25 by ethanol and ethyl acetate, methanol, chloroform.

After extraction of staphyloxanthin from S.25 isolate by ethanol and ethyl acetate methods, the absorption of the pigment was calculated at 450nm and the amount of pigment was calculated by using the following equation (9)

$$\text{Total carotenoid unite / cell} = \frac{V(A-0.0051)}{0.175W}$$

Where as:-

A : Is the absorbance value of the diluted staphyloxanthin extraction at 450nm.

V: Is the final volume of the extract staphyloxanthin .

W(g): Is the weight of the dried powder of staphyloxanthin.

0.175: Is the extraction coefficient of carotenoids

Purification by Thin-layer chromatography (TLC) according o (f Wieland, *et al.*, 1994)(5) -

The TLC was spotted with two drop of crude extract of staphyloxanthin (final concentration 2mg /ml) in the prepared line near one end of aluminum oxide plate, then the plate was put in the jar contain solvent

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(benzene –methanol-acetic acid 87:11:2(vol/vol/vol) which act as mobile phase Most carotenoid are readily detected as colorless spotted and ,the Rf calculated according to the following equation

$$RF = \frac{\text{Distance of sample movement}}{\text{Distance of spot solvent movement}}$$

6--Purification of staphykoxanth by column chromatography (according of 3)

After extraction of staphyloxanthin by ethanol and ethyl acetate from S.25 isolate, the pigment was purified by using column chromatography. The crude extract was dissolved in ethyl acetate and subjected to silica gel column chromatography ((1.5 × 80)cm. The colored fractions were eluted with ethyl acetate and ethanol; , and the individual fractions were evaporated to dryness. The staphyloxanthin peak fractions were pooled to assay staphyloxanthin activity and pigment concentration.

In vivo staphyloxanrhin extract as immunomodulatory against pathogenic bacteria

Infection of mice

Mice were challenged intraperitoneally with 0.5 ml of *pseudomonas aeruginosa* equal to (1.5×10^8) CFU/ml McFarland turbidity tube.

Experimental design

18 animals were divided into 3 groups , two of them (treatment and protective) were sub divided into two groups ,each group consist of 3 mice , third group represented as control which consist of 3 mice as following

Un treated control (3 mice): mice injected with normal saline after infection for three days.

Treatment groups 6 mice (3 mice for crude and 3 mice of purified): three mice were injected with crude extract , three mice were injected with purified extract of stphyloxanthin (2mg / 2ml) after infection for 3 days.

Protective groups which composed of 6 mice(3 mice for crude and 3 mice of purified) 3 injected with crude and 3 injected with purified extract of staphyloxanthin (2mg /2 ml) for three days before and after infection.

After 6, 10 days post-infection and treatment, two ml of blood was collected from eye. The blood was dispensed in a plain tube to collect serum after clotting, blood was centrifuged at 2000 rpm for 15 minutes at room temperature, and then serum was separated.

Assay Procedure for IL-10, and TNF-α

Instruction of manufacture was followed, and they are summarized in the following steps:

1. The plate was washed three times by, added 200 µl of Washing Solution to each well then aspirated the wells to remove liquid and Solution every well. Then invert plate to remove remaining solution and blot on paper towel.
2. 100 µl of standard or sample was added to each well in duplicate. Then covered with the Plate Sealer which provided and left at room temperature for two hours.

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3. The plate was washed four times after Aspirate the wells.
4. 100µl of the diluted detection antibody (0 ug/ml) was added per well then covered with the Plate Sealer provided. Left at room temperature for two hours.
5. The, plate was aspirated and washed four times.
6. 100 µl of the diluted Color Development Enzyme (1:20 dilute) was added every well. Then coved Red with the Plate Sealer provided. Then incubated half hour at room temperature (or 37°C for 30 minutes).
7. Plate was aspirated and washed four times.
8. 100, µl of color, development solution was added to each well. Then left at room temperature for a proper color, development. (1-11 minutes)
9. Then added 100 µl of the stop solution to each well
10. To read the plate, A microliter plate reader was used at 470 nm wavelength.

III. RESULTS AND DISCUSSION

Isolation and Identification of staphylococcus aureus

From a total 70 isolate of *staphylococcus* (table1) isolated from 306 different clinical sources (primary diagnosing as *S. aureus* depending on biochemical ,morphological ,and cultural characteristics were collected from patients suffering from different disease Infection such as wound ,UTI ,blood , eye , vagina , tonsil , nasal , burn infections and Bacteremia who had certain clinical.. These samples were collected from many hospitals in Baghdad. .All colonies from primary culture were purified by subculture on blood agar and then re-inoculated on Mannitol Salt Agar at 37°C for 24 hr these isolates were marked by the letter S.(10,11).

Table 1. Percentage of *Staph aureus* with difference Source infection

No.	Type of sample	Total	No of sample	Percentage (%)
1	Blood	28	7	25.00
2	Burn	42	13	30.95
3	Eyes swab	16	1	6.25
4	Ears swab	19	2	10.52
5	Urine	32	7	21.88
6	Nasal swan (medical staph)	35	8	22.86
7	Nasal swab (patients)	40	12	30.00
8	Tonsils swab (patients)	38	6	15.79
9	Vaginal swab	14	1	7.14
10	Wound swab	42	13	30.95
Total		306	70	22.87
Chi-Square		----	----	8.945 **
** (P<0.01).				

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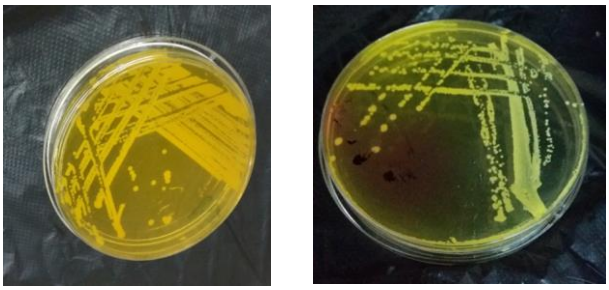
Cultural characteristics for *S. aureus* isolates appeared when isolated bacteria grown on its selective media, the colony morphology of isolates on mannitol salt agar and blood agar, these isolates were characterized by raised, smooth, glistering ,translucent with varied pigmentation production, on blood agar some isolates showed B- hemolysis and some others showed a- hemolysis and all isolates grew on mannitol salt agar and fermented mannitol , in figure (1) was showed only *S. aureus* formed a large golden colonies surrounded by wide yellow zones due to fermenting the mannitol and producing acid which turned the color of the medium from pink to yellow and growth on this medium can be improved in the presence of 7.5% NaCl (. 12,13,14)

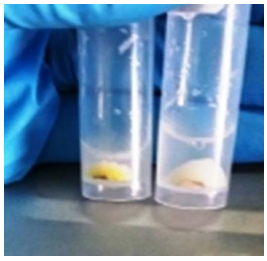


S.25 **S.68**

Figure 1 S. aureus on mannitol salt agar

4-Detection the ability of Isolates to produce Staphyloxanthin

In figure(2- A,B,C) showed the ability of *Staphylococcus* isolates for staphyloxanthin production that identified by culturing of *Staphylococcus (*70) isolates on skim milk agar media at 37°C for two days, then inculcated at 28°C for two ,after harvesting and centrifugation the bacterial growth and calculate the concentration of staphyloxanthin pigment at wave length 450 nm in which high peak of pure pigment absorbance (4;8). .



Figure(2- A) detection the ability of isolates of S. aureus to produce staphyloxanthin

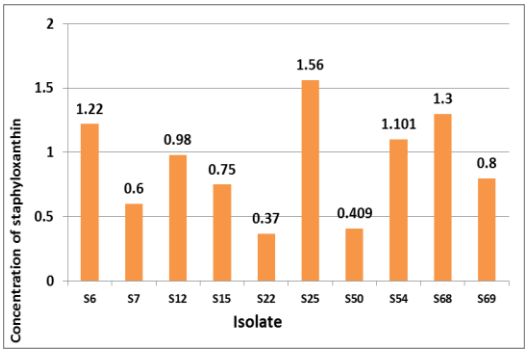


Figure (2-B) showed the productively of staphyloxanthin with different isolate at($P<0.05$)

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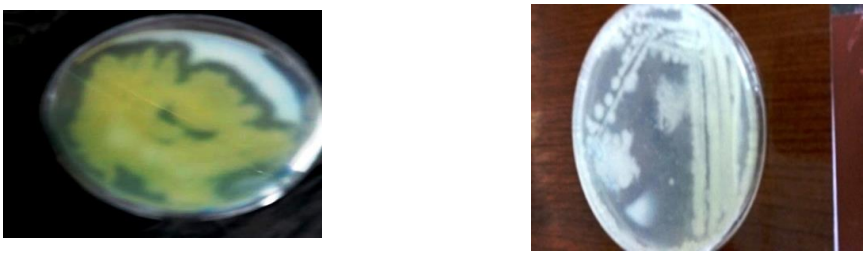

A B

figure 2 -C showed production of staphyloxanthin on skim milk agar ,(A) S.25 isolate producing of staphyloxanthin ,(B) S.3 isolate non producing staphyloxanthin .

Extraction of staphyloxanthin pigment

Staphyloxantn was exstracted by using different solvent chloroform (15) ethyle acetate and ethanol (3) and methanol(4; 5) in table (2) and figure (3) showed the signficant differences in methods of extraction of pigment ,as well as the highely concentaration of staphyloxanthin was obtained from extraction by ethyle acetate and ethanol ,with absorbity of staphyloxanthin that was recorded (0.683 at wave length (450nm) and table (2) , figure (3) was showed the signficant difference($P<0.05$) in exteaction of pgment and the cancentration of pigment was deremined at 450nm, the high peak of pure pigment (5;16).

Table 2 Extraction of staphyloxanthin by different methods

Extraction methods	**Concentration of ABS**
Chloroform	0.226 ± 0.002 b
Methanol	0.345 ± 0.009 b
Ethyl acetate and ethanol	0.683 ± 0.006 a
LSD value	0.208 *
* ($P<0.05$). Means having with the different letters in same column differed significantly.	

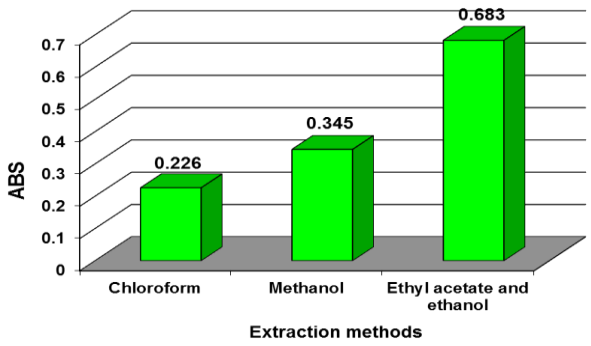

Figure 3 showed exstraction of staphyloxanthin by using different solvents

In others study carotenoids were soluble in apolar solvents, including edible fats and oils,. Basically carotenoids were lip soluble; they are usually extracted from the plant sources with organic solvents (chloroform, hexane, acetone, petroleum ether, etc.) The majority methods of extraction of carotenoids from plant sources make use of organic solvents such as hexane, ethanol, acetone, methanol, , and petroleum ether

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,therfoure It has been observed that the stability of carotenoid extracts obtained with hexane/acetone or hexane/ethanol was higher than that of extracts obtained with other organic solvents, such as chloroform, methanol (17) .Also polar solvents (ethanol and acetone) enhanced the solubilisation of the polar lutein. This was probably related to the relative solubility of lutein in ethanol and acetone which was 15–40 folds higher than the respective one in hexane (18). After extraction the of staphyloxanthin from isolates S.25 by ethanol and ethyle acetate the amount of crude extract of the pigment was calculated quantitivily (154 .94) unite /cell at absorbinaity 450nm. (8). .

purfication of staphyloxanthin .

Thin layar chromotogrophy (TLC)

the thin layar chromotogrophy (TLC) method was used to purfity of staphyloxanthin . TLC was ageneral method used to purfiy of secondary metabolitis producte(19). the figure (4) showed the RF value(0.38) which was simillar to(Marshal and Willmoth , 1981; Wieland etal., 1994)(4;5) with molcular weight 819.17 dalton . After the experiment, the spots are visualized often this can be done simply by projecting ultraviolet light onto the sheet .

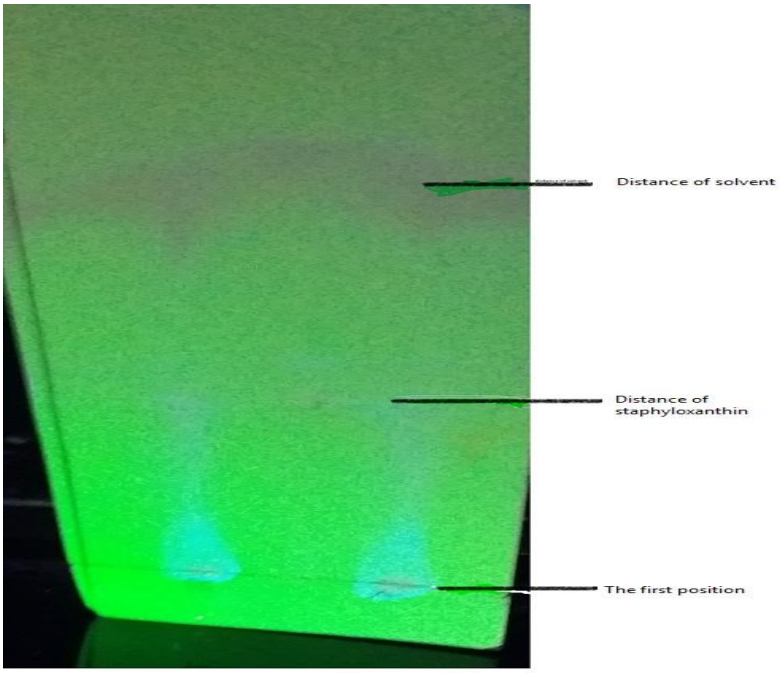

Figure 4 purification of staphyloxanthin from S.25 by using thin layer choreography (molecular weight of staphyloxanthan is 819.17 Dalton , mobile phase ,benzene-methanol- acetic acid 87:11:2 (vol/vol/vol).

Purification of staphyloxanthin by Colum chromatography

Purification of staphyloxanthin was carried out by a Colum chromatography and used using silica gel after soulbilzation the silica gel in ethanol and pooled in column with then developing the column with a solvent , the pigment fractions were pooled and passed through silica gel column ,the fractionation yielded (one peak) one fraction of peaks as absorbance reading at wave length (450nm) and other fraction (tubes 1-

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13)containing the purified extract of staphyloxanthin (3ml) that was demonstrated in (Alexandra Pelz‡ *et al*., 2005)(3).Column chromatography step increased the purity of the isolated pigments, while the spectral quality of the main constituent remains comparable (20).

the result was showed in table(3) and figure(5) the difference significant in concentration of staphyloxanthin in tubes containing the purified extract and the extract in tube (7) was recorded (0.998) highly concentration of ,then the purified fraction evaporated in rotary evaporator and the amount of pigment of purified extract was calculated quantitivity (17.02114 unite /cell) for purified extract and at absorbency 450 nm according to equal was demined by (9). .

Table 4 showed purification of crude extract in Colum chromatography

1	0.0 ± 0.00 c
2	0.0 ± 0.00 c
3	0.0 ± 0.00 c
4	0.0 ± 0.00 c
5	0.208 ± 0.005 bc
6	0.581 ± 0.018 b
7	0.998 ± 0.041 a
8	0.970 ± 0.033 a
9	0.668 ± 0.026 ab
10	0.439 ± 0.012 b
11	0.344 ± 0.007 bc
12	0.028 ± 0.001 c
13	0.00 ± 0.00 c
LSD value	0.361 *
* ($P<0.05$). Means having with the different letters in same column differed significantly.	

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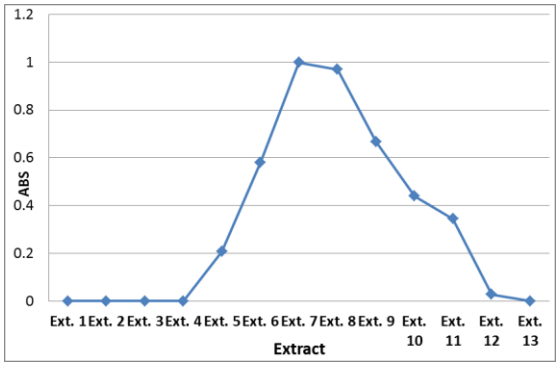

Figure 5 showed purification of layer of purified extract

In vivo staphyloxanrhin extract as immunomodulatory against pathogenic bacteria

In table (4) ,(5) The immunomodulatory of crude and purified extract of staphyloxanthin was showed that three groups were significant differences at (P<0.05) in elevated levels of cytokines (IL1-0,TNF-a) in both treatment and protective groups when contrast with control group that was non treated with staphyloxanthin extract . The differences in levels of cytokines may be due to inducing of immune response by staphyloxanthin and this elevated in the cytokines may be lead to decreasing in the number of the pathogenic bacteria. .

Cytokines are soluble molecules that mediate cell-to-cell interactions. Cytokines such as IL-2, TNFα and IFN-γ that 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, 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 (21). .

Table 4 Effect of staphyloxanthin extract on cytokines level (TNF-α) in mice

The Group	**Mean ± SE of concentration of TNF-α (pg/ml)**		**LSD value**
Group 1: treatment : Infected with *Pseudomonas* and treatment with staphyloxanthin pigment (1 mg)	**Purified**	**crude**	16.039 *
	123.23 ± 7.35	25.03 ± 1.69	
Group 2: Protective: treatment with staphyloxanthin extract before and post infection with *P. aeruginosa*	99.81 ± 5.02	48.56 ± 2.76	12.763 *
Group 3: Control : infected with *Pseudomonas* and treatment with normal saline	23.75 ± 2.50	23.75 ± 2.50	----
LSD value	19.406 *	8.215 *	----

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* (P<0.05).

Table 5 Effect of staphyloxanthin extract on cytokines level (IL-10) in mice

The Group	**Mean ± SE of concentration of TNF-α (pg/ml)**		**LSD value**
Group 1: Infected with *Pseudomonas* and treatment with staphyloxanthin pigment (1 mg)	**Purified** 36.50 ± 2.18	**crude** 26.67 ± 1.45	5.286 *
Group 2: Protective treatment with staphyloxanthin extract before and post infection with *P. aeruginosa*	31.07 ± 1.94	48.56 ± 2.57	6.091 *
Group 3: Control infection with *Pseudomonas* and treatment with normal slain	16.54 ± 1.06	16.54 ± 1.06	----
LSD value	7.335 *	8.183 *	----
* (P<0.05).			

Boon P. and Jean S.,2004. (22) was reporting the action role of β-carotene on the growth of the thymus gland and a large increase in the number lymphocytes that demonstrated in rats and cattle and oral β-carotene supplementation in human audits was causing an increase the numbers of Th and T inducer lymphocytes. *in vitro,* β-carotene was induced hamster macrophages to produce TNF-α when ROS was activated TNF-α lead to increase 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 . ROS were important in primary immune response ,this activation by ROS also produced an anti-apoptotic effect(23). As the results of previous studies have shown that carotenoids could be directly stimulated the immune response by inducing lymphocyte proliferation, immunoglobulin and cytokine production, and gene regulation. And the results was showed that the link between carotenoids and the immune system was mediated by oxidative stress.(24).

Carotenoids were might alleviated the negative effects of large amounts of free radicals that produced by some immune cells (such as macrophages and heterophils in order to kill pathogens (25-26).

In other studies by 27;28(Møller,et al., 2002; ; Chew B.P.1& Park J.S. 2014) were showed carotenoid molecules could play an important role in both the activation and regulation of the immune system by enhance antibody production and activation of the plasma complement cascade (a set of proteolytic enzymes) that promote inflammation, as well as the agglutination and marking of invasive microbes for destruction by lysis or phagocytosis Additionally, immune responses were increasing oxidative stress, the antioxidant activity of carotenoids had been implicated in ameliorating damage was caused by free radicals, such as nitric oxide (NO),that produced by immune cells when responding to pathogenic threats , Carotenoids could play a key role

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in stimulate attacks on foreign cells after the skin has been breached by activating an unidentified feature of the acquired immune system.

Diet-derived carotenoids can play an important role in physiological functions because of their antioxidant and immunostimulant properties, Dietary astaxanthin was a xanthophyll carotenoid, that founded in various microorganisms and marine animals and had a prominent role to stimulate lymphocyte proliferation, to increase natural killer cell cytotoxicity and the delayed-type hypersensitivity response, and increase the number of total T and B cells in the peripheral blood.(29;30). .

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