Production and application of Spirulina platensis rich in fatty acids, and vitamins

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Abstract: *Spirulina platensis* is a microscopic blue-green alga in the shape of a spiral coil, living both in sea and fresh water .It is widely used as health food due to its protein content, vitamins and active substances for immune system. Polyunsaturated fatty acids amount to 46.548 %(w/w) of total lipids.Among the essential fatty acids detected in El Khadra lake water body in Waadi El Natroun micro- alga, cholesterol decreasing γ-linolenic acid with 0.986%(w/w) .Vitamin A amounts to 120.13 µg/100g, vitamin C amounts to 540.34 µg/100g and vitamin D amounts to 105.6 µg/100g were found . Vivo studies revealed *Spirulina* effectiveness on Triglycerides(TG) ,Total cholesterol(TC),High density lipoprotein-chloestero(HDL-ch), body weight ,serum calcium., serum iron, and serum ferritin after treatment of the experimental rabbits for 30 days.

[Aly, M.S, Amber.S. Gad. El Sayed M. Kamel. **Production and application of Spirulina platensis rich in fatty acids, and vitamins**. Journal of American Science 2011;7(2):36-45]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: *Spirulina platensis*, γ-linolenic acid, vitamins, hypercholesterolemia , serum calcium, prothrombin. , serum iron, serum ferritin.

1. Introduction:

Spirulina is a natural health food as a blue-green algae.It contains beneficial nutrients that are readily digested and absorbed by the body, so none of its nutritional benefits are lost. It is excellent in combating imbalances arising from lifestyle habits and it is effective in overcoming and preventing various disorders arising from a poorly balanced diet, including insufficient intake of vegetables as it supplies several of the vitamins that all living beings need to carry on metabolic processes or prevent some serious diseases ;Cingi *et. al* .(2008): This cyanobacterium is important for its content of polyunsaturated fatty acids as it is frequently rich in gamma-linolenic acid (GLA), and also provides alpha-linolenic acid (ALA), linoleic acid (LA), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA(;Babadzhanov *et. al.*(2004), beta-carotene and other antioxidants pigments;Ciferri (1983) ; Gemma *et. al.* (2002); anti-virals sulphated polysaccharides; Noaman *et. al.* (2004), antimicrobials sterols. *Spirulina* contains a number of vitamins: B1, B2, B3 B6 ,B9, vitamin C, vitamin D, vitamin E; Babadzhanov, *et. al*. (2004) and B12, Watanabe *et. al.* (2002).

*Spirulina i*s accepted as functional foods, which are defined as products derived from natural sources, whose consumption is likely to benefit human health and enhance positive medical properties These foods are used as a supplement/ingredient or as a complete food to enhance the performance and state of the human body, or improve a specific bodily function. It is being widely studied for its possible antioxidant, antibacterial, and antiparasitic properties, and for several medical conditions such as allergies**;** Mao *et. al*. (20**0**5**)** ulcers, anemia, heavy-metal poisoning, Barmejo-Bescós *et. a*l. (2008**)**, and radiation poisoning, Misbahuddin *et. al.(*2006); Raj *et. al*. **(**2008). *Spirulina* or its extracts can prevent or inhibit cancer in humans and animals and has other medical effects;Wang *et. al*. (2005)[=]

Functional foods are used mainly as products to nourish the human body after physical exertion or as a preventive measure against ailments. *Spirulina* contains unusually high levels of gamma-linolenic acid,(GLA),an essential polyunsaturated fatty acid; Otleş and Pire .(2001); Babadzhanov, *et. al.*(2004). The aim of this study is to evaluate fatty acids and vitamins contents of *S. platensis* isolated from Waadi El Natroun and studying its effect on cholesterol TG,TC ,HDL levels , body weight , and serum calcium ,serum iron and serum ferritin in the blood of experimental animals.

2. Materials and Methods:

Micro-organisms:

Spirulina platensis used in this study were obtained from El-Khadra lake at Wadi El Natroun, Egypt, characterized by extreme conditions of pH 10.5 and salt concentration of 0.55 M,,(Aly ,2000).

Maintenance stock media:

Zarrouk's synthetic medium was used for maintaining and batch culture preparation of *S.platensis*(SP) as described according to Ali and Amber (2010).

Harvesting the biomass:

After seven days incubation , the algal biomass were harvested with the 40 µm mesh size cloth **,** filtered and washed with distilled water to remove the salts from the algal surface then, the washed slurry was divided into tow sets ,first for determination of vitamins and fatty acids which stored at -20°C while the second set was dried at 50°C for I h and milled ;Ali and Amber (2010). The dried samples were milled and stored in plastic container to be used in feeding experimental animals.

Extraction and Identification of vitamins; Qian and Sheng (1998): Ten grams of SP tissue fresh weight were homogenized with methanol for extraction of water soluble vitamins while acetone–chloroform (30:70 v/v) was used for extraction of fat soluble vitamins The mixtures were shaken on a vortex mixer for 5 min, centrifuged at 4000 rpm for 5 min and filtered through a Millipore filter (45 ìm). The filtrates were evaporated under nitrogen and the residues were re-dissolved in 1ml water for water soluble vitamins and in 1 ml butanol for fat soluble vitamins were quantified by HPLC Vitamin C was analyzed by AOAC(1995), α-tocoferol (vitamin E) by HPLC;Manz and Vuilleumier(1988), β- carotene by spectrophotometric method, AOAC(1995) .

Lipids were obtained from lyophilized biomass sample according to Folch *et. al.* (1957) lipids were extracted with chloroform/ methanl (2+1 v/v) purified in methanol/ water (2:1 v/v), containing 9 g Na Cl to remove sugar, salts, protein and concentrated in a rotary evaporator residual solvents were evaporated. Lipids were gravimetrically estimated.

Extraction and Identification of fatty acids (FA): (Isik *et. al. 2006;* Diarman *et. al. 2009).*

FA were analysed by IUPAC (1982) method with Thermoquest Trace GC. FID detector (250 ºC) and SP-2330 Fused silica capillary column 30 m.-0.25 mm ID-0.20 µm (film thickness)of cyanopropyl were also used. Air was adjusted 350 ml/min. 35 ml/min H_2 and 30 ml/min He were used. The range, carrier ratio, split flow and split ratio were 1, 0.5 ml/ min, 75 ml/min and 1/150, respectively. Oven temperature was 120 ºC (up to 220 ºC with the adding of 5ºC. The sample injection was 0.5 µL. The FA was identified by comparing them in their retention time with standards obtained from Sigma.

Vivo studies.

Healthy adult(1-1.5 kg)t white Newzeland rabbits were kindly provided by NRC,Egypt , housed and maintained under a constant temperature of 30±1°C.i,e. animals were acclimatized to laboratory conditions before the experiment with a week .Rabbits were given food and water *ad libitum* along the period of the treatment. . Animals were randomly divided into two groups (n=10 per group) and treated for a period of a months as follows: 1) Group 1 (SP-treated group): animals were fed on a standard diet as 100 mg *Spirulina* /k weight) (100mg powder dissolved in 10 ml sterilized water) by a gavage daily for one month and given water for 30 days ; 2) Group 2 (untreated control group): animals were fed on a standard diet until the termination of the experiment; Body weight of the animal was recorded every 10days.

Biochemical analysis

After 15 days fasting following the end of the experimental time ,the animals were cut in ears , blood samples were collected from the marginal vein of the ear every 10 days for one month in clean dry test tube . The samples were kept for 30 min at room temp to clot then centrifuged at 3000 rpm for 10 min. Clear serum was divided into aliquots and was used for the biochemical determinations.

Aliquot of serum samples were kept at -20°C until used for determinations.

Total cholesterol (TC) was evaluated according to Richmond (1973).

Triglcerides(TG)) were evaluated according to Trinder (1969).

High density lipoprotein cholesterol (HDL-CH) was evaluated according to Lopez-Virella *et. al.* (1977).

Calcium serum was evaluated according to Gindler and King (1977).

Ferritin serum was evaluated according to White *et. al*. (1986).

Iron serum. was evaluated according to Dreux (1977).

Atherogenic index (AI)=TC-HDL-ch/HDl-ch.AI when increased than 2 lead to atherosclerosis.

Statistical analysis:

Data are expressed in mean ± SE of three replicates, results were considered significant when p<001.

3. Results and Discussion

Fatty acids content in the local strain of *S.platensis:*

A concentrated source of nutrients such as *Spirulina* is loaded with fats, starches and calories. *Spirulina* contains 7 % lipid, and most of that is in the form of essential fatty acids that promote cholesterol normalization. The essential fatty acids sometimes called vitamin F, include linoleic, linolenic and arachidonic acid. They are used by the body to manufacture prostaglandins, the hormonal regulators of blood pressure and capillary resilience ;Tomaselli *et. al.* (1988) **.**Otleş and Pire (2001) determined the composition of *Spirulina platensis* fatty acid composition. The essential fatty acids are involved in respiration in all the cells, and are especially important to oxygen transport. They affect the health of the hair, skin and nails, and help break up cholesterol in the blood stream. Isik *et. al.*(2006) found that chemical composition of *Spirulina* related to the environmental cultivation conditions, salinity, light intensity, pH which determines the solubility of CO_2 and minerals in the medium. Data presented in table 1 indicated that PA amounted to 33.452%(w/w). Quoc and Dubacq (1997) ; Romano *et. al*. (2000); Mühling *et. al. (*2000*)* and Tomaselli *et. al.* (1988) studied the temperature influence on *S. platensis* M2 and determined the fatty acids contents of *S. platensis* at the different temperatures, They observed that the lessening temperature led to the decrease of the C16:0 content. Similarly, Tomaselli *et. al.* (1988) and Romano *et. al.* (2000)reported the increase in C16:1 level by the low temperatures 26 ºC. On the contrary, Quoc and Dubacq (1997) reported the increase in the level of C18:2 with the lowering of the temperature. It was shown that cyanobacteria responded to a decrease in ambient growth temperature by de-saturating the fatty acids of membrane lipids to compensate for the decrease in membrane fluidity at low temperatures ;Tomaselli *et. al.* (1988). In addition, the proportion of de-saturated fatty acids increases by the decrease in temperature . Oliveira *et. al*. (1999) reported the increase of the C18:3 with lowering temperature .The percentage of C18:2 decreased at the 26 ºC. In agreement with these studies, table 1 indicated the level of C16:0 was found to be 33.452*%*(w/w), palmitolleic fatty acid (16:1) 1.262, (w/w)% of total lipids which is used to fight weight gain., while C18:2.yield was 5.523% (w/w). Table 1, showed also,the content of C18:3: was small 0.986% (w/w)..;oleic acid (C18:1) yields 37.988%(w/w).; linoleic acid (C18:2) represents 5.523 %(w/w)., LA content was similar to a previous study 10-37% (w/w).**;**Diraman *et. al*.(2009).

GLA (γ-linolenic) acid (C18:3) that is associated with pharmaceuticals and nutraceuticals amount to 0.986%(w/w) of lipids .However, *S. platensis* is a very rich source in γ-linolenic acid. It has been found that contents and composition of fatty acids are temperature dependent in *S.platensis*; an increase in temp reduce the composition of fatty acids in membrane lipids, Colla *et. al.* (2008). GLA yield depends on dark and light cycles ,indoor or outdoor cultivation , harvest time,age of the culture, This low yield in fatty acids in Wadi El Natroun *S.platensis* may be due to the fact stated by ;Diraman *et. al.* (2009) who confirmed that, the mechanism of fatty acids composition are not fully understood ,variation in percentage concentrations may be due to temp.or sodium nitrate concentration in the synthetic media used .Table 1 showed that PA and OA were the most abundant.

Caprylic acid (C8:0) yield is 1.578 (w/w) % known with its strong anti-fungal properties, Capric acid (C10:0) yield is 6.154% w/w)used in making artificial fruit flavors. Lauric acid (C12:0) helps in curing skin infections and dandruff,; myristic acid (C14:0) represents 2.761%, palmitic acid (C16:0) yield is 33.4(w/w) %,; stearic acid (C18:0)represents 5.08 (w/w) % , and arcachedic acid (C20:0) yield is 2.761%. γ-linolenic acid (GLA and),linoleic fatty acid (LA) are poly and mono- unsaturated fatty acids. Table2 indicated the amount of γ-linolenic acid was only 0.986% (w/w)). Total saturated fatty acids amount to 53.452.% (w/w). GLA in particular has a role in lowering the decrease blood cholesterol level; Ishikwa *et. al.* (1989) used it in treatment of hypercholesterolemia.

Table (1). (%) Fatty acid in *S. platensis* biomass.

Fatty acid	Concentration%(w/w)	Fatty acid	Concentration% (w/w)
Short chain (CA)C8:0	1.578	Unkown	0.789
(CA) C10:0	6.154	(SA)C18:0	5.089
Long chain (lA)C12:0	1.657	(OA)C18:1	37.988
(MA)C14:0	2.761	(LA)C18:2	5.523
(PA)C16:0	33.452	(GLA)C18:3	0.986
(POL)C16:1	1.262	Very long chain (AA) (C20:0)	2.761

Table 2. Concentration % of γ-linolenic poly unsaturated fatty acid in *S.platensis* biomass

GLA% (w/w)	UFA% w/w)	L○○A% (w/w)	OA% (w/w)	SF% w/w)
0.986	46.548	5.523	37.9	53.452

Total fatty avidsTFA,linoleic fatty acid(LA), oleic fatty acid(OA)- γ-linolenic fatty acids(GLA)-Unsaturated fatty a cids(USf).

Vitamins content in the local strain of *S. platensis* :

S. platensis biomass could be obtained under the optimum laboratory cultivation conditions for the quantification of β-carotene, vitamin A and α-tocopherol . *Spirulina* contains a number of vitamins: B1 (thiamine), B6 (pyridoxine), B9 (folic acid), vitamin C, vitamin D, vitamin E and B12. (cobalamin), , biotin, pantothenic acid, beta carotene (source of vitamin A, Elizabeth and and Lillian (1968) ; Watanabe *et. al.* (2002); García-Martínez *et. al.*((2007), *Spirulina* is rich in vitamin B12 Vitamin B12 (cobalamin), ascorbic acid, (vitamin C) ,tocopherols(vitamin E) ,phylloquinone (vitamin K 1) and menaquinones (vitamin K 2), vitamins:A (B-carotene); B1(thiamine), B2 (riboflavin), B3 (niacin), were also detected in *Spirulina* extract in a satisfactory amounts; García-Martínez *et. al.*((2007).

***S.platensis* and vitamin A:**.Table (3) indicated that *Spirulina* contains 120.13 µg / 100g wet weight. Vitamin A helps form and maintains healthy teeth, skeletal and soft tissue, mucous membranes, and skin. It is also known as retinol because it produces the pigments in the retina of the eye; Duester(2008**)**.

***S.platensis* and vitamin C (ascorbic acid) :**Table (3) indicated that *Spirulina* contains 540.34µg / 100g wet weight .Ascorbic acid is required for healing of wounds, the production of digestive enzymes and connective tissue, brain and nerve function, formation of teeth and bones, glandular activity. Also vitamin C aids in the absorption of iron and protection of cells, B complex vitamins, vitamin E and vitamin A from oxidation . *Spirulina* helps in curing all immune system problems as well; Quereshi *et. al.* (1994).

***S.platensis* and vitamin E:** Table (3) indicated that *Spirulina* contains Tocopherol or vitamin E (105.6µg/100gwet weight).This nutrient protects heart and vascular health, promotes oxygenation of cells; Zingg ,and Azzi(2004), and retards aging. Vitamin E deficiency i*n* humans results in ataxia (poor muscle coordination with shaky movements), decreased sensation to vibration, lack of reflexes, and paralysis of eye muscles. One particularly severe symptom of vitamin E deficiency is the inability to walk.

Table (3). Vitamins in *S. platensis* biomass.

Vitamin	Concentration (µg/100g) wet biomass
(C)Ascorbic	540.34
B-carotein(A)	120.13
A- tocopherol (E)	105.6

In vivo studies

Effect of *S.platensis* on experimental animals body weight:

Table 4 shows the change recorded in rabbits that had been fed *Spirulina* diet for 4 weeks ,as the body weight of each animal along the experimental period (one month) is given. Kumar *et. al.* (2010) stated that *S.platensis* has diverse biological effect due to high content of highly digestible protein, vitamins, beta-carotene ,phycocyanin and other pigment. Table 4 shows increasing percentage of change in the animals weight with time in all the groups under treatment as compared to control groups, this comes in agreement with Yin, *et.al*. (2008). As the body weight of each animal was recorded every 10 days, the recorded increase in the 3rd period in the experimental animals is 2.10 ±.30 (Kg) refers to the "free-feeding" weight of an animal, "The rabbit's *ad libitum* weight was about 300 grams**.**

Table (4). Mean weight of rabbits over the test period ±SD

Weight elevation (%)	Test (Kg)	Control (Kg)	Period(10 days)
7.462	**1.40±0.11**	**1.34±0.05**	**1**
6.211	**±0.2011.66**	**1.61±0.10**	**2**
5.376	**2.10±0.30**	**1.86±0.12**	**3**

Effect of *S.platensis* on the cholesterol titres:

Early interest in *S.platensis* focused mainly on its potential as a source of protein and vitamins but recently more attention has been made to study its therapeutic use and number of reports suggested its beneficial effect in acute allergic rhinitis, anti-cardiotoxic ,anti-hepatotoxic, anti-nephrotoxic; Kumar *et. al.* (2010). Table 5 indicated that after 30 days feeding with *Spirulina* , TG reached 48.23±2.11 mg % compared to the control, TC 45.61±3.21% mg % reached **a** decrease to 18.27±0.28 mg % compared to the control 25.24±0.42 mg % , HDL-Ch recorded 17.91±1.50 mg % increase as compared to the control 12.23±1.15 mg % under the same laboratory conditions, as total blood cholesterol below 200 mg/dL indicating the relatively low risk of coronary heart disease , even with a low risk this comes in agreement with , Edlin *et. al*. (2009).

Serum levels of total glycerides and free glycerol are important indices of lipid metabolism and cardiovascular disease risk. Khan *et. al.*(2005). Triglycerides(TG) are a type of fat in the bloodstream and fat tissue. Too much of this type of fat, as tabl;e 5 indicated this value reached 48.23±2.11 mg % can contribute to the hardening and narrowing of arteries. This puts risk of having a heart attack or strok**;** John *et. al*.(2008) .Diseases such as diabetes, obesity, kidney failure or alcoholism can cause high triglycerides. Often, high triglycerides occur along with high levels of cholesterol, which is an important component for the

manufacture of fat-soluble vitamins including vitamin A, vitamin D, vitamin E, and vitamin K. HDL particles are able to remove cholesterol from atheroma within arteries and transport it back to the liver for excretion or re-utilization, which is the main reason why the cholesterol carried within HDL particles, termed HDL-C, is sometimes called "good cholesterol". higher levels of HDL-C recorded 17.91±1.50 mg % as data presented in table 5 seem to indicate fewer problems with cardiovascular diseases, while low HDL-cholesterol levels (less than 40 mg/dl or about 1 mmol/L) have indicated rates for heart disease .

There are several types of cholesterol, each made up of lipoproteins and fats. Each type of lipoprotein contains a mixture of cholesterol, protein and a type of fat (triglyceride), but in varying amounts. Nayaka *et. al*.(1988). Of the lipoprotein types, VLDL contains the highest amount of triglyceride. Because it contains a high level of triglyceride, having a high VLDL level means the increased risk of coronary artery disease, which can lead to a heart attack or stroke. A normal VLDL cholesterol level is between 5 and 40 milligrams per deciliter. Since higher levels of LDL particles promote health problems and cardiovascular disease, they are often called the bad cholesterol particles, (as opposed to HDL particles, which are frequently referred to as good cholesterol or healthy cholesterol particles). VLDL-cholesterol is a minor lipid component of very low-density lipoprotein (VLDL) particles of VLDL particle; Ren *et. al.* (2010). A study involving geriatric patients determined that *Spirulina* helped to significantly reduce the LDL-to-HDL ratio after four months of supplementation; Park *et. al.* (2008). Treatment with over a six week period, exhibited significant changes in cholesterol and blood pressure as it lowered total cholesterol, increase HDL cholesterol, lower triglycerides; and lower systolic ; Torres-Duran *et. al.* (2007).

Table 5 showed that AI 0.71±0.13 for the control and 0.27±0.11 for the treatment .i.e. not the least evidence for atherosclerosis. Arteriosclerosis is hardening of the arteries. This condition not only thickens the wall of arteries, but also causes stiffness and a loss of elasticity. Over time, the arteries become harder and harder as they are slowly damaged by high blood pressure. Atherosclerosis is the most common type of arteriosclerosis, or hardening of the arteries, and caused by plaque building up in the vessel. Over time the plaque causes thickening of the walls of the artery, when AI increased than 2 lead to atherosclerosis.

Table (5).Effect of *S. platensis* tablets(100mg/Kg) in lipid profile of the rabbits ±SE.

Period	group	TGmg%	TC mg%	HDL-ch mg%	Al
1	control	48.93±5.84	20.63±3.06	14.43±2.45	0.726±0.124
	test	58.8±3.60	26.5±2.82	11.22±1.42	1.74±0.266**
2	control	46.8±5.96	14.93±1.15	9.22±0.99	0.93±0.223
	test	49.58±3.83	19.48±0.43	18.08±1.74**	0.37±0.23*
3	control	45.61±3.21	25.24±0.42	12.23±1.15	0.71±0.13
	test	48.23±2.11	18.27±0.28	17.91±1.50	0.27±0.11*

*p<0.1 corresponding to control

**p<0/001 corresponding to control.

**p<0/001 corresponding to control

Effect of *S.platensis* on serum calcium level:

Serum calcium is a test that measures how much calcium is in blood. The presence of free calcium was a necessary condition of co- agulation as certain salts of citrate and oxalates electrolytes have low solubility product, so it can be used as anti-co-agulation. However, in combination with prothrombin, calcium acts not as free element but as a complex that could interfere in blood clotting; Levelock and Porterfield (1952). Fig .1 shows the increase in the serum calcium level of the treated than the control sample in the three periods, which is prominent in the 2nd period, as it amounts to 11.3mg/dl compared to 5.43mg/dl of the control test. Also, the parameter is still high in the 3rdperiod as it measures 9.6 mg/dl compared to 7.1 mg/dl of the control. This decrease may be due to the use of this calcium by the body. the body uses vitamin D to help transport calcium to the bones When blood calcium levels drop too low, the vital mineral is "borrowed" from the bones. It is returned to the bones from calcium supplied through the diet. . Calcium is the most abundant mineral in the body; the bones and teeth accounting for about 99% of the total body stores.

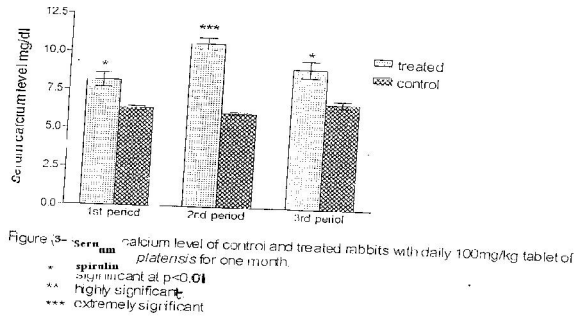

Figure (3- $Serum_{calcium}$ level of control and treated rabbits with daily 100mg/kg tablet of *spirulin platensis* for one month.
* significant at p<0.05
** highly significant
*** extremely significant

Fig. (1) S. platensis effect on serum calcium.

Effect of *S.platensis* on serum iron and serum ferritin:

Free iron is toxic to cells as it acts as a catalyst in the formation of free radicals from reactive oxygen species via the Fenton Reaction. Orino et. *al.*(2001). Hence body uses an elaborate set of protective mechanisms to bind iron in various tissue compartments. Within cells, iron is stored in a protein complex as ferritin. The amount of ferritin in blood (serum ferritin level) is directly related to the amount of iron stored in the body, i.e. under steady state conditions, the serum ferritin level correlates with total body iron stores; thus, the serum ferritin is the most convenient laboratory test to estimate iron stores as it serves to store iron in a non-toxic form, to deposit it in a safe form, and to transport it to areas where it is required, Seckback (1982).The serum ferritin concentration is a clinical parameter measured widely for the differential diagnosis of anemia. Ferritin is a ubiquitous intracellular protein that stores iron and releases it in a controlled fashion. In humans, it acts as a buffer against iron deficiency and iron overload Seckback (1982). The function and structure of the expressed ferritin protein varies in different cell types. Fig (2,3) illustrates the effect of 100 mg *Spirulina*/ kg daily administration to the rabbits . Control and treated samples showed a significant decrease in serum ferritin in the third period due to the use of its iron in hemoglobin formation for example as high ferritin is correlated to iron in excess. Serum iron increase significantly in the 3rd period as it reached 5.5mg/dl compared to 2mg/dl of the control. Ferritin decreased in the 3rd period 12 mg /dl because of iron consumption in hemoglobin but it still in a higher concentration than control. 9.4 mg/dl.

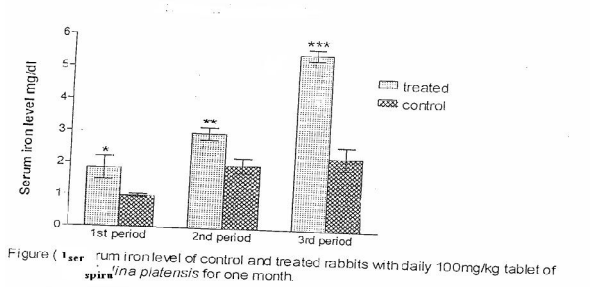

Figure (I_{ser} rum iron level of control and treated rabbits with daily 100mg/kg tablet of $spirul$*ina platensis* for one month.

Fig. (2) S. platensis effect on serum iron.

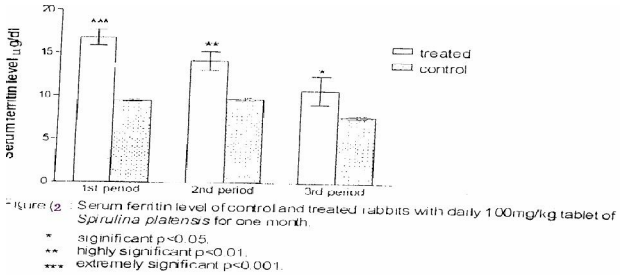

Figure (2 : Serum ferritin level of control and treated rabbits with daily 100mg/kg tablet of *Spirulina platensis* for one month.
* significant p<0.05.
** highly significant p<0.01.
*** extremely significant p<0.001.

Fig. (3) S. platensis effect on serum ferritin.

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12/2/2010