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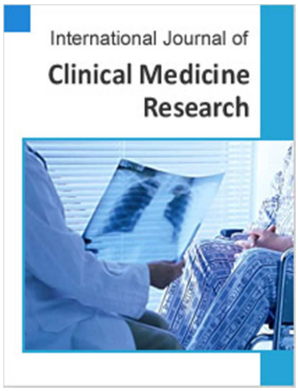

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Clinical Chemistry and Microbiology Studies in Egyptian Thalassemic Children Infected with Hepatitis C Virus

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Abstract
The present study was conducted to evaluate the efficiency of natural *Spirulina* preparation in the treatment thalassemic children infected with hepatitis C virus (HCV). Twenty five (25) Egyptian thalassemic children infected with HCV (12 male, and 13 female children), aged from 3 to 8 years were subjected in this study. Volunteers were supplied with dried *Spirulina* (250 mg /kg /day) by oral uptake with their usual thalassemic drugs. Throughout 6 months treatment, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), albumin, total bilirubin, and quantitative (real time) PCR for HCV were assessed. The main parameters of healing for these complicated cases were the promising appearance of 3 cases with –ve (0.0 IU/ml) HCV count in patient serum by real time PCR quantification; and lowering of ALT, and AST less than 40 U/L in 13 female cases, and 12 male cases, respectively after 6 months of treatment with *Spirulina*.

1. Introduction

Thalassaemia is an inherited autosomal recessive blood disease that originated in the Mediterranean region. In thalassemia, the genetic defect which could be either mutation or deletion, results in reduced rate of synthesis or no synthesis of one of the globin chains that make up hemoglobin. This can cause the formation of abnormal hemoglobin molecules, thus causing anemia [1]. The two major forms of the disease, α and β are prevalent in discrete geographical clusters around the world.

Many complications were record as non desired symptoms all over the world, accompanying thalassemia cases [2].

Hepatitis C virus (HCV) is a small (50 nm) in size, enveloped, single-stranded, positive sense RNA virus. It is the only known member of the *hepacivirus* genus in the family *Flaviviridae*. There are six major genotypes of HCV, which are indicated numerically (e.g., genotype 1, genotype 2, etc.). Based on the NS5 gene there are three major and eleven minor genotypes. A seventh genotype — 7a — has also been described

[3]. Hepatitis C is an infectious disease caused by HCV that primarily affects the liver. In some cases, those with cirrhosis will go on to develop liver failure or other complications, including liver cancer or life-threatening esophageal varices and gastric varices an estimated 180 million people worldwide are infected with hepatitis C [4]. Treatment is generally recommended for patients with proven HCV infection and persistently abnormal liver function tests. Current treatment is a combination of 22 pegylated interferon-alpha- 2a or 22 pegylated interferon-alpha- 2b (brand names Pegasys or PEG-Intron) and the antiviral drug ribavirin for a period of 24 or 48 weeks [5]. Several alternative therapies are claimed by their proponents to be helpful for hepatitis C, or are being researched to see if they can be effective treatments. Among them are milk thistle, ginseng, colloidal silver, licorice root (or its extract glycyrrhizin), lactoferrin, 1TJ-108 (a mixture of herbs used in Japanese Kampo medicine), schisandra, and oxymatrine (an extract from the sophora root) [6].

Spirulina comes from two different species of cyanobacteria: *Arthrospira platensis*, and *Arthrospira maxima*. *Spirulina* contains an unusually high amount of protein, between 55% and 77% by dry weight, depending upon the source. It is a complete protein, containing all essential amino acids, though with reduced amounts of methionine, cysteine, and lysine when compared to the proteins of meat, eggs, and milk. It is, however, superior to typical plant protein, such as that from legumes [7]. *Spirulina* is also 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). *Spirulina* contains vitamin B1 (thiamine), B2 (riboflavin), B3 (nicotinamide;'), B6 (pyridoxine), B9 (folic acid), vitamin C, vitamin D, and vitamin E. *Spirulina* is a rich source of potassium, and also contains calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium, and zinc [8]. The proteins, lipids, and carbohydrates in *Spirulina* are digested, absorbed, and metabolized by the humans upon oral consumption. *Spirulina* can be consumed at a dose of 3–20 g/day without manifestation of any adverse effects. Studies on the acute, subchronic, and chronic toxicity and mutagenicity of *Spirulina* have revealed no specific body or organ toxicity or genotoxicity [9].

Clinical application of *Spirulina* has suggested that the constituents, which make up these specialized blue-green algae, may provide for certain antiretroviral activities. Calcium *Spirulina*, a sulfated polysaccharide and component of *Spirulina platensis*, has received the greatest attention. Studies suggest that it may offer protection against different membranes viruses. Various *in vitro* studies of *Spirulina platensis* indicated that calcium *Spirulina* inhibits the replication of harmful viruses, including; human cytomegalovirus, HIV-1, measles, mumps, and influenza A [10]. This polysaccharide may prove equally effective at minimizing the rate of replication of the herpes simplex virus (HSV-1), while also inhibiting its penetration into certain host cells [11].

2. Materials and Methods

2.1. Application on Human Volunteers

This study was done according to guidelines of Egyptian ministry of Health and Population decree 95/year 2005 for medical research, good clinical practice, Declaration of Helsinki and World Health Organization Guidelines [12]. Spirulina was approved by the FDA (Food Drug Administration) by the issuance of a GRAS (generally recognized as safe) certificate. The FDA has stated that Spirulina can be legally marketed as a food or food supplement without risk to human health [13].

Follow up of 25 (12male, and 13 female) thalassemic children infected with HCV, aged from 3 to 8 years was performed, the laboratory tests were performed at start before uptaking *Spirulina* and after 6 months from uptaking Spirulina. Volunteers were supplied with dried Spirulina (250 mg /kg/day) by oral uptake.

2.2. Blood Sampling and Serum Preparation

Blood samples were taken from patients and healthy children at the Hematology & Oncology unit, Tanta University Hospital**.** All samples were directed for the following tests:

2.2.1. Liver Function Tests

I. Determination of Serum Alanine Aminotransferase (SGPT-ALT)

Method:

Kinetic method for the determination of ALT activity is according to the recommendation of the Expert panel of the IFCC (International Federation of Clinical Chemistry), without Pyridoxal phosphate activation [14].

Reaction principle:

(1). 2- Oxoglutarate + L- glutamate $\xleftarrow{GPT}$ L-gluatmate + pyruvate

(2). Pyruvate+ NADH+H^+ $\xleftarrow{LDH}$ L-lactate + NAD^+

Table 1. *Procedures of Determination of serum Alanine Aminotransferase.*

Pipette into cuvettes	37°C
Sample	100 µl
Working reagent	1000 µl
Mix and then read the absorbance after 1 minute and at the same time start the stopwatch and then read the absorbance again exactly after 30, 60 and 120 seconds.	

Calculation:

The rate of absorbance changes through 30, 60 and 120 seconds were calculated at 340 nm of wave length according to this equation:

$$\text{At 340 nm, Equation} = \Delta A_S \times 1745$$

II. Determination of Serum Aspartate Aminotransferase (SGOT-AST)

Method:

It was revealed that the kinetic method for the determination of AST activity was determined according to the recommendations of the Expert Panel of the IFCC (International Federation of Clinical Chemistry), without pyridoxal phosphate activation [15].

Reaction Principle:

(1). 2-Oxoglutarate + L-aspartate $\xrightleftharpoons{GOT}$ L-glutamate + oxaloacetate

(2). Oxaloacetate + NADH + H^+ $\xrightleftharpoons{MDH}$ L-malate + NAD^+

Table 2. *Reagents Used in Determination of Serum Aspartate Aminotransferase.*

Buffer	Buffer / Enzyme reagent	
	TRIS buffer (ph 7.8)	100 m mol/l
	L-aspartate	300 m mol/l
	LDH	≥ 0.9 k U/l
	MDH	≥ 0.6 k U/l
Substrate	Substrate	
	2-oxoglutarate	60 m mol/l
	NADH	0.9 m mol/l

The entire contents of substrate bottle were poured into bottle contained buffer. The working reagents stable for 4 weeks at 2-8°C.

Procedure:

(1) Pipette into cuvettes at 37°C 100 µL Sample and 1000 µL of working reagent.

(2) Mix and then read the absorbance after 1 minute and at the same time start the stopwatch. Read the absorbance again exactly after 30, 60 and 120 seconds.

Calculation:

The rate of AS changes through 30, 60 and 120 seconds were calculated at 340 nm of wave length according to this equation:

$$\text{At 340 nm, Equation} = \Delta A_S \times 1745$$

III. Determination of Serum Albumin

Method:

Bromocresol green forms with albumin in citrate buffer a colored complex. The absorbance of this complex is proportional to the albumin concentration in the sample [16].

Contents:

(A) Working reagent:

(1)	Citrate buffer (pH 4.2)	30mmol/l
(2)	Bromocresol green	260 µmol/l

(B) Standard:

(1)	Albumin	4 g/dl
(2)	Sodium azide	0.095%

Procedure:

Ten ml from standard, collected serum samples were added to 1 ml from working reagent and the mixture was left at room temperature for 5 min. The absorbance of standard and serum sample was read at 578 nm against reagent blank [17].

Calculation:

Albumin concentrations of sample were estimated by using the following equation:

$$C = 4 \times \Delta A_{sample} / \Delta A_{STD} \text{ [g/dl]}$$

IV. Determination of Serum Total Bilirubin

Principles of reaction*:*

Most chemical methods for bilirubin determination are based on the reaction between diazotized sulfanilic acid and bilirubin in the presence or absence of an organic solvent to distinguish free from conjugated bilirubin on a differential solubility basis. In aqueous solution only conjugated or direct bilirubin will react. To measure total bilirubin, it is necessary to add an accelerator or solvent [18].

Reagents:

(1) Reagent 1:	Sulfanilic acid	31 mmol/I
	HCI	0.2 N
(2) Reagent 2:	Sodium nitrite	28 mmol/I
(3) Reagent 3:	Caffeine	0.28 mol/l
	Sodium benzoate	0.55 mol/
(4) Reagent 4:	Tartrate	0.99 mol/I
	NaOH	2.0 N

Wave length adjustment of F- T- 2 Spectrophotometer (Italy):

Total Bilirubin 578 nm (560-600 nm)

Method:

0.2 ml from reagent 1 poured in two different tubes; one for sample and one for blank). For specimen, one drop from reagent 2 (100 ml) was added, 1 ml from reagent 3 was added for test and samples under examination from serum and 0.2 ml was added to test and blank.

The later mixtures were incubated at 20-25°C for 10 minutes. One ml from reagent 4 was added to test and standard.

The mixture incubated for 5 min at 20-25°C. The absorbances for samples or specimen against blank were reading at 578 nm. Total bilirubin was calculated by following equation:

$$\text{Total bilirubin} = A_s \times 10.8$$

Table 3. *Procedures of Determination of Serum Total Bilirubin.*

	Specimen blank	Specimen
R_1	0.2 ml	0.2 ml
R_2		1 drop
R_3	1.0 ml	1.0 ml
Specimen	0.2 ml	0.2 ml
Mix, and incubate for 10 minutes at 20 - 25°C. Add		
R_4	1.0 ml	1.0 ml
Mix, and incubate for 5 minutes at 20-25°C. Read absorbance of specimen against specimen blank at 580 nm. The color intensity is stable for 30 min.		

2.2.2. Renal Function Tests

I. Determination of Serum Creatinine Level

Principle of reaction:

Creatinine in the sample reacts with picrate in alkaline medium forming a colored complex. The complex formation rate is measured in a short period to avoid interference [19].

Composition:

(1) Reagent A: Sodium Hydroxide 0.4 mol, detergent.

(2) Reagent B: Picric acid 25 m mol.

(3) Reagent C: Glucose/urea/creatinine standard: glucose 100 mg/dL, urea 50 mg/dL, creatinine 2 mg/dL, aqueous primary standard.

Procedure:

(1) Bring the working reagent and photometer to 37°C.

(2) Pipette into a cuvette: 1 ml working reagent A, 1 ml reagent B, and 0.1 ml standard or sample.

(3) Mix and insert into photometer, start stopwatch.

(4) Record the absorbance at 500 nm after 30 sec. (A_1), and 90 sec. (A_2).

Calculation:

The creatinine concentration in the sample is calculated using the following general formula:

$$[(A_2\text{-}A_1)_{\text{sample}} / (A_2\text{-}A_1)_{\text{standard}}] \times C_{\text{standard}} \times \text{Sample dil.} = C_{\text{sample}}$$

II. Determination of Serum Urea

Principle:

Urea in the sample is hydrolyzed enzymatically into ammonia (NH_4^+) and CO_2. Ammonia ions formed react with salicylate and hypochlorite (NaClO), in presence of the catalyst nitroprusside, to form a green indophenol [20].

(1) $\text{Urea} + H_2O \xrightarrow{\text{Urease}} NH_4^+ + CO_2$

(2) $NH_4^+ + \text{Salicylate} + \text{NaClO} \xrightarrow{\text{Nitroprusside}} \text{Indophenol}$

The intensity of the color formed is proportional to the urea concentration in the sample.

Table 4. *Reagents Used in Determination Serum Urea.*

R1 Standard	Urea aqueous primary standard 50 mg/dL	
R2 enzymes	Urease	30000U/L
	Phosphate pH 6.7	50 m mol
R3 Buffer	EDTA	2 m mol
	Na salicylate	400 m mol
	Na nitroprusside	10 m mol
R4 NaClO	NaClO	140 m mol
	NaOH	150 m mol

Table 5. *Procedures of Determination of Serum Urea.*

	Blank	Standard	Specimen
R3	1 ml	1 ml	1 ml
R2	1 drop	1 drop	1 drop
R1	-	10 µL	-
Specimen	-	-	10 µL
Mx, incubate for 3 min. at 37°C, or for 5 min. at 20-25°C, then add:			
R4	200 µL	200 µL	200 µL
Mix, incubate at 37°C for 5 min., or at 20-25°C for 10 min. Measure absorbance of specimen ($A_{specimen}$), and standard ($A_{standard}$) at 578 nm against blank within 60 min.			

Calculation:

$$(A_{\text{specimen}}/A_{\text{standard}}) \times 50 = \text{urea in the sample (mg/dL)}$$

(3) Quantitative (real time) PCR for HCV The quantification of RNA-based HCV in patients' samples was performed according to the following protocol:

(a) RNA Purification and Reverse Transcriptase (RT-PCR) Experiment

Total RNAs were isolated from sample serum in liquid lipase dependent medium, using Trizol (Gibcol) according to the manufacturer's protocol. First strand cDNA was synthesized using Advantage RT-for-PCR Kit (Clontech, alo Alto, CA, USA). The first strand cDNA was used as the template in PCR experiment to the viral nucleic acid using the primer (HCV-F4-5'-GAC ACT CCA CCA TTA TCA CT-3').

(b) Real Time PCR Amplification Conditions

The cDNA of the samples were subjected to semi-quantitive PCR using the primer that mentioned above. The Real time PCR reaction consist of 12.5 µl of 2x Quantitech SYBR® Green RT Mix (Fermentase.com), 1µl of 25 pm/µl primer, 1 µl of the cDNA (50 ng), 9.25 µl of RNase free water for a total of 25 µl. Samples were spun before loading in the Rotor's wells. The real time PCR program was as follows: initial denaturation at 95°C for 10 min.; 40 cycles of 95°C for 15 sec.; annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene- 6000-system (Qiagen, USA). The 18S rRNA gene was used as housekeeping gene (reference gene) in this test. Threshold cycle (CT) values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred, for each amplification plot. Comparative quantification analysis was done using Rotor-Gene-6000 Series Software according to [21].

3. Results

3.1. Liver Function Tests

3.1.1. Determination of Serum Alanine Aminotransferase (SGPT-ALT)

The results in Table 6 showed that, quantitative ALT at start was ranging from 16 to 338 but after 6 months was ranging from 10 to 200. The results also revealed that, there was significant decrease in ALT after 6 months compared to at start (P value 0.05).

Table 6. *Evaluation of Serum Alanine Aminotransferase.*

Groups	ALT (U/L)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	16	-	338	94.3	±	83.84	2.03	0.05*
After 6 months	10	-	200	56.9	±	44.85		

P value is statistically highly significant at< 0.05 level

3.1.2. Determination of Serum Aspartate Aminotransferase (SGOT-AST)

The results in Table 7 showed that, AST at start was ranging from 23 to 287 but after 6 months was ranging from 11 to 165.

The results also revealed that, there was significant decrease in AST after 6 months compared to at start (P value 0.01).

Table 7. *Evaluation of Serum Aspartate Aminotransferase.*

Groups	AST (U/L)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	23	-	287	89.88	±	67.66	2.6	0.01*
After 6 months	11	-	165	53.6	±	37.89		

P value is statistically highly significant at< 0.05 level.

3.1.3. Determination of Serum Albumin

The results in Table 8 showed that, Albumin at start was ranging from 3.3 to 4.8 but after 6 months was ranging from 3.7 to 4.6. The results also revealed that, there was significant increase in albumin after 6 months compared to at start (P value< 0.01).

Table 8. *Evaluation of Serum Albumin.*

Groups	Albumin						T-Test	
	Range			Mean	±	SD	T	P-value
AT Start	3.3	-	4.8	3.848	±	0.340	4.59	<.001*
After 6 months	3.7	-	4.6	4.188	±	0.224		

P value is statistically highly significant at< 0.05 level

3.1.4. Determination of Total Bilirubin

The results in Table 9 showed that, Total bilirubin at start was ranging from 0.3 to 5 but after 6 months was ranging from 0.2 to 8. The results also revealed that, there were no significant differences in total bilirubin between at start and after 6 months (P value 0.35).

Table 9. *Evaluation of Serum total bilirubin.*

Groups	Total bilirubin						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	0.3	-	5	1.76	±	1.55	0.94	0.35
After 6 months	0.2	-	8	1.41	±	1.55		

P value is statistically highly significant at < 0.05 level

3.2. Renal Function Tests

3.2.1. Determination of Serum Creatinine Level

The results in Table 10 showed that, Creatinine at start was ranging from 0.2 to 0.9 but after 6 months was ranging from 0.2 to 0.7. The results also revealed that, there was significant decrease in Creatinine after 6 months compared to at start (P value 0.012).

Table 10. *Evaluation of Serum Creatinine.*

Groups	Creatinine (mg/dl)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	0.2	-	0.9	0.548	±	0.150	2.73	0.012*
After 6 months	0.2	-	0.7	0.456	±	0.189		

P value is statistically highly significant at< 0.05 level

3.2.2. Determination of Serum Urea

The results in Table 11 showed that, Urea at start was ranging from 15 to 126 but after 6 months was ranging from 11 to 28. The results also revealed that, there was significant decrease in Urea after 6 months compared to at start (P value 0.050).

Table 11. *Evaluation of Serum Urea.*

Groups	Urea (mg/dl)						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	15	-	126	26.2	±	21.777	2.07	.050*
After 6 months	11	-	28	17.04	±	5.334		

P value is statistically highly significant at< 0.05 level

3.3. Quantitative (Real Time) PCR for HCV

The results in Table 12 showed that, quantitative real time PCR for Hepatitis C virus at start was ranging from 350 to 4920000 but after 6 months was ranging from 0 to 120700. The results also revealed that, there was no significant difference in quantitative real time PCR for Hepatitis C virus between at start and after 6 months (P value 0.16).

Table 12. *Evaluation of Quantitative Real Time PCR for Hepatitis C virus.*

Groups	Quantitative real time PCR for Hepatitis C virus						T-Test	
	Range			Mean	±	SD	t	P-value
AT Start	350	-	4920000	352300	±	1029283	1.42	0.16
After 6 months	0	-	120700	57900	±	55194.62		

P value is statistically highly significant at< 0.05 level.

4. Discussion

At first, alanine amino transferase (ALT) was determined as a liver function indicator, which was significantly decreased from 94.3 ± 83.84 to 56.9 ± 44.85 after 6 months of the treatment. These records were confirmed by the grouping of individual case reports, as only 5 cases (out of total 25 cases) possessed ALT level less than 40 U/L at the start of treatment, while number of cases was significantly increased to 8 cases of ALT level less than 40 U/L at the end at treatment.

Another liver function indicator was the recording of aspartate amino transferase (AST), which was significantly decreased to from 89.88 ± 67.66 to 53.6 ± 37.89 after 6 months of the treatment. This was confirmed by recording only 2 cases possessed AST level less than 40 U/L at the start of treatment, while the number of cases was significantly increased to 12 cases (equal to 48% of total cases) of AST level less than 40 U/L at the end at treatment.

In the present study serum albumin was determined as a liver function indicator, which was significantly increased from 3.848 ± 0.340 to 4.188 ± 0.224 after 6 months of the treatment. Also serum total bilirubin was determined as a liver function indicator, which was decreased from 1.76 ± 1.55 to 1.41 ± 1.55 after 6 months of the treatment.

These findings were found to be in agreement with the study that recoded long-term outcomes of hepatitis patients with improved ALT levels to 40 U/L in 23 cases throughout the course of treatment with different algal food additives. [22]. Another study recorded the same ALT level enhancement during the management of HCV infection in 34 cases in Italy [23].

These records were confirmed in other studies that detected the improvement of AST levels in more than 28 cases of resistant HCV cases, treated with different natural products, especially from algal sources [24]. Another study confirmed the improvement of AST levels during the treatment of HCV with *Spirulina* extracts in India [25].

In the present study, serum creatinine was determined as a kidney function indicator, which was significantly decreased from 0.548 ± 0.150 to 0.456± 0.189 after 6 months of the treatment. Also serum urea was determined as a kidney function indicator, which was significantly decreased from 26.2 ± 21.777 to 17.04 ± 5.334 after 6 months of the treatment

Other studies can agree with the records in the present study, as study recorded an observable improvement in kidney function parameters during the treatment of thalassemic cases with natural products [26].

A main parameter for healing of HCV infection of treated children was the quantification of HCV in patient serum by real time PCR. In the present study serum quantitative real time PCR for Hepatitis C virus was determined, which was significantly decreased from 352300 ± 1029283 to 57900 ± 55194.62 after 6 months of the treatment.

These records were confirmed by recording a very high HCV count (> 1000000 IU/ml) in 2 cases at the start of treatment that was opposite to 3 cases of normal HCV count (0.0 IU/ml) at the end of treatment as a highly promising result for HCV treatment in the present study.

These findings were in agreement with other studies; as study recorded a high inhibition ratio of HCV by a plant-derived flavonoid treatment [27]. Also, ketoamide protease inhibitors decreased the count of HCV in treated patients in 16 cases out of 20 cases to less than 10000 IU/ml after 3 months of treatment [28].

5. Conclusion

The thrust of this study to measure liver function tests, kidney function tests and quantitative real time PCR for hepatitis C virus in thalassemic Children Infected with Hepatitis C Virus for evaluation the effect of Spirulina on blood chemistry and hepatitis c virus. This study proved that there were improvements in the results of liver function tests, kidney function tests and quantitative real time PCR for hepatitis C virus after 6 months from uptaking Spirulina.

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