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Protective Effect of *Nigella sativa* Oil against Methotrexate Induced Hepatotoxicity in Children with Acute Lymphoblastic Leukemia

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Abstract

Background: Acute Lymphoblastic Leukemia (ALL) is the most common childhood malignancies representing about one third of all pediatric cancers. Adding methotrexate to leukemia treatment protocols has been associated with an increased survival rate in children with ALL. The efficacy of this agent is often limited by its toxicity which can be reduced if supplemented with anti-oxidants. *Nigella sativa* has antioxidant property through different mechanisms.

Objective: The aim of this work was to study the role of *Nigella sativa* oil in the protection against hepatotoxicity induced by methotrexate therapy in children with ALL and the impact on the treatment outcome.

Patients and methods: The present study was conducted in the period between July 2010 and July 2013 on 40 children with newly diagnosed ALL including 28 males and 12 females, with mean age value of 9.17 ± 3.81 years and they were divided into 20 patients of ALL under methotrexate therapy included in ALL treatment protocol, delayed leukovorin rescue (10 mg/m^2 orally or IV every 6 hours for five doses beginning 48 hours after start of methotrexate infusion and *Nigella sativa* oil in form of soft gelatin capsule 450 mg in dose of 80 mg/kg/day on three divided doses for one week after each methotrexate dose (Group II) and 20 patients of ALL under methotrexate therapy included in ALL treatment protocol, delayed leukovorin rescue (10 mg/m^2 orally or IV every 6 hours for five doses beginning 48 hours after start of methotrexate infusion and placebo for one week after each methotrexate dose (Group III). This study also included 20 healthy children as a control group (11 males and 9 females) with their mean age value of 9.1+ 2.9 (Group I). All patients included in the study were subjected to the following investigations: Complete blood picture, bone marrow aspiration, cytochemistry, immunophenotyping and liver function testes.

Results: There were no significant difference in serum bilirubin, alanine aminotransferase, aspartate amino-transferase, alkaline phosphase levels and prothrombin time between group II and group III but there was significant difference between group II and group III compared to controls. There was no significant difference in total protein, albumin, globulin levels, and albumin globulin ratio between studied groups. There were non-significant increase in total, direct and indirect serum bilirubin, serum ALT, AST, and alkaline phosphatase levels and prothrombin time in group II after methotrexate and *Nigella sativa* oil therapy but there was significant increase in group III after treatment with methotrexate and placebo with significant difference between group II and III after therapy. There were significant differences in overall and disease free survival between group II and group III.

Conclusion: Oral administration of *Nigella sativa* oil in leukemic children can prevent MTX hepatotoxicity and improved survival in patients with ALL.

Recommendations: *Nigella sativa* oil is recommended adjuvant drug as hepatoprotective agent in patients with ALL who received methotrexate therapy.

Keywords: Acute lymphoblastic leukemia; Hepatotoxicity; Methotrexate; Thymoquinone; *Nigella sativa* oil

Introduction

ALL is the most common childhood malignancies, representing nearly one third of all pediatric cancers [1]. Among children with ALL, 75%-85% survive free of leukemia recurrence at least 5 years from diagnosis with current treatment that incorporate systemic combination chemotherapy with or without cranial radiation [2].

Methotrexate (MTX) is a key drug in the curative regimen of children with ALL [3]. Hepatotoxicity is a common complication of long term treatment with MTX and is defined as an injury of the liver associated with impaired liver function caused by exposure to the drug [4]. Methotrexate hepatotoxicity causes discontinuation of chemotherapy and may affects the overall prognosis and outcome of the disease [5]. In children with cancer, dose reduction or withdrawal of therapy in case of abnormal liver function tests might be more harmful than continuing treatment [6].

The underlying mechanism of MTX hepatotoxicity remains unclear. However, it was reported that MTX causes oxidative stress in liver tissue. MTX is metabolized and stored in hepatocytes in polyglutamated form. The presence of higher levels of polyglutamates causes a longer intracellular presence of the drug which was suggested as a mechanism for hepatotoxicity [7]. There is a debate about the concurrent use of antioxidants with chemotherapy [5] *Nigella sativa* is the black cumin herb belongs to Ranunculaceae family of flowering plants and genus of about 14 species [8] Thymoquinone (TQ) is the main active constituent of volatile oil of the black seed [9].

The oil of *N. sativa* and TQ are known to possess strong antioxidant

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activities. TQ has been shown to inhibit non-enzymatic peroxidation in ox brain phospholipid liposomes also it has extremely high superoxide anion radical-scavenging abilities as effective as Superoxide Dismutase (SOD) [10].

Nigella sativa might protect the liver against the ischemia reperfusion injury. An excessive production of oxygen free radicals has been reported in ischemic re-perfused liver, leading to tissue damage [11].

Also hepatotoxicity caused by acetaminophen as shown by significant increases in alanine aminotransferase (ALT), total nitrate/nitrite, and lipid peroxide and decreased glutathione was prevented by 5 days of 2 mg/kg/day of oral TQ in mice [12].

Aim of the Work

The aim of this work was to study the role of the *Nigella sativa* oil (NSO) in the protection against hepatotoxicity induced by methotrexate therapy in children with acute lymphoblastic leukemia and the impact on the treatment outcome.

Study Design

After research ethical committee approval and informed written parental consent from all participants in this research, this study was carried out on 40 patients of newly diagnosed ALL (28 males and 12 females). They were attendants to Oncology Unit, Pediatric Department, Tanta University Hospital in the period between July 2010 and July 2013. Their ages ranged from 4-13 years with mean value of 9.17 ± 3.81 years and they were divided into 20 patients of ALL under methotrexate therapy included in ALL treatment protocol, delayed leukovorin rescue (10 mg/m^2 orally or IV every 6 hours for five doses beginning 48 hours after start of methotrexate infusion and *Nigella sativa* oil in form of soft gelatin capsule 450 mg (Baraka; Pharco Pharmaceuticals) in dose of 80 mg/kg/day on divided doses for one week after giving methotrexate therapy (Group II) and 20 patients of ALL under methotrexate therapy included in ALL treatment protocol, delayed leukovorin rescue (10 mg/m^2 orally or IV every 6 hours for five doses beginning 48 hours after start of methotrexate infusion and placebo for one week after giving methotrexate therapy (Group III). This study also included 20 healthy children as a control group (11 males and 9 females) with their age ranged from 6-15 years and mean age value of 9.1 ± 2.9 (Group I). Diagnosis and classification of ALL were made according to French–American–British (FAB) criteria and immunophenotype analyses. The immunophenotyping was pre-B (CD19, CD22, CD10-), common ALL (CD19, CD22, CD10-), and T-ALL (CD3, CD5, CD7). The clinical data from all patients were obtained, including age at diagnosis, sex, presence of fever, pallor, purpura, bone ache, hepatomegaly, splenomegaly and lymphadenopathy. All patients were subjected to follow up for two year to evaluate their prognosis.

Protocol of Treatment

Induction (6 weeks)

Vincristine 1.5 mg/kg/m^2/week IV (days 0, 7, 14, 21, 28, 35), Doxorubicin 25 mg/m^2/ week IV infusion (days 0, 7, 14, 21, 28, 35), L-Asparginase 6000 µ/m^2 SC on alternate days for 10 doses, and Prednisone 40 mg/m^2/day for 6weeks orally. On day 21, bone marrow aspiration was done. In non-responding cases, we add Etopsoide 100 mg/m^2/dose IV (days 22, 25, 29), Cyclophosphamide 750 mg/ m^2/dose IV infusion (days 22, 25, 29), Aracytin 100/m^2/dose IV (days 22, 25, 29), and high dose methotrexate 5 g/m^2 over 4 hours on day 28 [13].

Consolidation (9 weeks)

IV methotrexate 1 gm/m^2/dose over 24 hour infusion on days 0, 21, 42 and 63, Mercaptopurine 60 mg/m^2 orally daily on days 0-13 and 28-41, Vincristine 1.5 mg/m^2 IV on days 14, 21, 42 and 49, PEG Asparaginase 2,500 units/m^2 IM on days 14 and 22, Cyclophosphamide 750 mg/m^2/dose IV infusion on days 0 and 28, Aracytin 100/m^2/dose IV on days 1-4, 8-11, 29-32 and 36-39 and age-adjusted intrathecal methotrexate on days 1,8,15 and 22 [14,15].

Interim maintenance (6 weeks)

Vincristine 1.5 mg/m^2 per day IV on days 0, 10, 20, 30, 40, IV methotrexate starting dose of 100 mg/m^2/dose over 10-15 minutes on day 0 thereafter escalate by 50 mg/m^2/dose on days 10, 20, 30 and 40, PEG Asparaginase 2,500 units/m^2 IM on days 1 and 21 and age-adjusted IT Methotrexate on days 0 and 30 [15].

Delayed –intensification (6 weeks)

Oral dexamethasone (10 mg/m^2/day on days 1-7 and 14-21, IV vincristine 1.5 mg/m^2 on days 0, 7 and 14, IM or IV pegylated L-asparaginase 2500 u/m^2 on day 4, doxorubicin 25 mg/m^2 IV push on days 0, 7 and 14, IV cyclophosphamide 1 gm/m^2 over 30 minutes on day 28, oral 6-thioguanine 60 mg/m^2 on days 28 to 41, cytarabine 75 mg/m^2 on days 29-32 and 36-39 and age-adjusted intrathecal methotrexate on day 28 [16].

Maintenance (30 months)

Weekly IV Methotrexate 20 mg/m^2 in B cell and 30 mg/m^2 in T cell ALL, Prednisone 120 mg/m^2/day for 5 days every 3 weeks, Vincristine 2 mg/m^2 IV every 3 weeks, 6-mercaptopurine 50 mg/m^2/day orally for 14 days every 3 weeks and age-adjusted IT Ara-C and methotrexate every 18 weeks [17].

Inclusion criteria

Children with newly diagnosed ALL who were treated with MTX based protocol.

Exclusion criteria

1. Patients with ALL who were positive for hepatitis A, B, C.
2. ALL patients who received medications, other than chemotherapy, that may affects the liver functions.

For all patients the following Laboratory investigations were done including:

Complete Blood Count (CBC).

One ml venous blood was collected in EDTA tubes from each patient for CBC [18].

Bone marrow examination with morphological, cytochemistry and immunophenotypic classification:

One ml BM for BM morphologic, cytochemistry and immunophenotyping [18].

Liver function tests to assess methotrexate hepatotoxicity

3.8 ml venous blood was collected from each patient, and delivered into two tubes. 1.8 ml blood into tube containing 0.4 ml sodium citrate for prothrombin time and activity and 2 ml into a plain tube for assessment of other liver function tests including total, direct and indirect serum bilirubin, total serum protein and its fractions albumin

and globulin, albumin globulin ratio (A/G ratio), serum alkaline phosphatase, serum Alanine Aminotransferase (ALT) and serum Aspartate Aminotransferase (AST) [19-25].

Statistics

Data were collected and analyzed using Statistical Package for Social Science (SPSS) (version 12). All Data were expressed as in terms of mean values ± SD. Comparisons of parameters among groups were made using paired t test. Two-group comparisons were performed nonparametrically using Mann-Whitney U test. All statistical tests were two tailed, and $P<0.05$ was considered statistically significant.

Results

- The most common presenting clinical manifestations in studied patients were pallor, purpura and fever followed by hepatomegaly, splenomegaly and lymphadenopathy with no significant difference between group II and III (Table1).
- There was no significant difference in immunophenotyping between group II and group III (Table 2).
- There were no significant differences in total serum bilirubin, ALT, AST, alkaline phosphase levels and prothrombin time between group II and group III at time of diagnosis but there was significant difference between group II and group III compared to control (Table 3).
- There were no significant differences in total protein, albumin, globulin levels, and albumin globulin ratio between studied groups at time of diagnosis.
- There were non-significant increase in total, direct and indirect serum bilirubin, serum ALT, AST, and alkaline phosphatase levels and prothrombin time in group II after methotrexate and *Nigella sativa* oil therapy but there was significant increase in group III after treatment with methotrexate and placebo with significant difference between group II and III after therapy (Table 4).
- There was no significant difference in total serum protein, serum albumin, globulin levels and A/G ratio in group II and group III before and after methotrexate therapy with no significant difference between group II and III after therapy (Table 4).
- There was significant difference in prognosis regarding complete remission, relapse, death, disease free survival and Overall survival between group II and group III (Figures 2 and 3, Table 5).

Clinical manifestations	Group II	(20 patients)	Group III	(20 patients)	X2	P
	N	%	N	%		
Fever	15	75	16	80	0.140	0.704
Pallor	16	80	15	75	0.140	0.704
Purpura	16	80	15	75	0.140	0.704
Bone ache	6	30	5	25	0.130	0.723
Hepatomegaly	13	65	12	60	0.110	0.743
Splenomegaly	12	60	12	60	0.000	1.00
Lymphadenopathy	10	50	9	45	0.100	0.751

Table 1: Clinical manifestations of the studied patients.

Immunophynotyping	Group II (20 patients)		Group III (20 patients)		T	P
	N	%	N	%		
Early pre-B	6	30	5	25	0.668	0.745
Pre-B	11	55	12	60	0.968	0.558
T-cell	3	15	3	15	0.000	1.00

Table 2: Immunophenotyping of the studied patients.

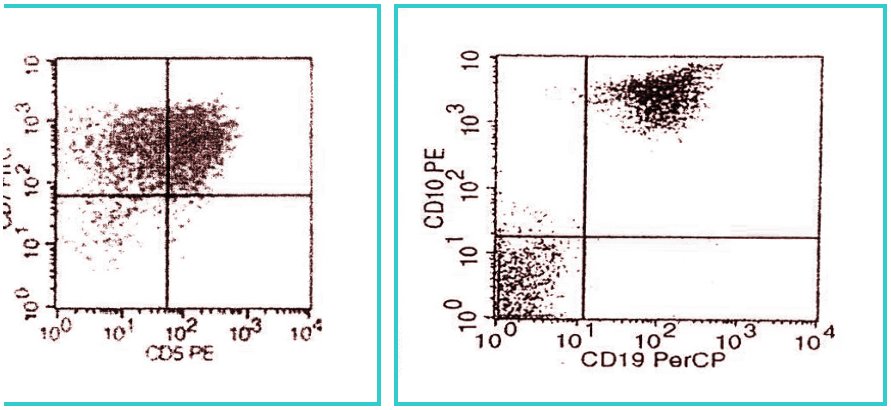

Figure 1: Immunophenotyping of some patients included in this study, to the left, a case T-cell ALL with CD 7 and CD 5 positive and to the right, a case of B-cell ALL with CD19 positive and CD 10 negative.

Parameters		Group I (No= 20)	Group II (No= 20)	Group III (No= 20)
Total serum bilirubin (mg/dl)	Mean ± SD	0.70 ± 0.09	0.82 ± 0.22	0.80 ± 0.12
	P - Value	0.034 ‡	0.635 ★	0.031♠
Serum ALT (u/l)	Mean ± SD	17.85 ± 4.06	57.69 ± 17.63	60.10 ± 20.36
	P - Value	0.001‡	0.068 ★	0.001♠
Serum AST(u/l)	Mean ± SD	25.75 ± 7.97	74.4 ± 28.06	59.65 ± 20.36
	P - Value	0.001‡	0.89 ★	0.002♠
Alkaline phosphatase (u/l)	Mean ± SD	189 ± 85.41	221.09 ± 59.63	231.8 ± 87.24
	P - Value	0.042‡	0.635 ★	0.039♠
Total serum protein (gm/dl)	Mean ± SD	6.99 ± 0.70	6.74 ± 1.13	7.32 ± 0.64
	P - Value	0.448‡	0.524 ★	0.335♠
Serum albumin (gm/dl)	Mean ± SD	4.10 ± 0.34	3.83 ± 0.52	3.93 ± 0.37
	P - Value	0.589‡	0.447 ★	0.665♠
Serum globulin (gm/dl)	Mean ± SD	2.91 ± 0.459	2.89 ± 0.71	3.17 ± 0.63
	P - Value	0.102‡	0.269 ★	0.223♠
Albumin globulin ratio	Mean ± SD	1.40 ± 0.34	1.40 ± 0.32	1.20 ± 0.21
	P - Value	1.00‡	0.086 ★	0.096♠
Prothrombin time (seconds)	Mean ± SD	13.99 ± 0.81	14.68 ± 1.05	14.37 ± 0.48
	P - Value	0.034‡	0.886 ★	0.049♠

Significant *p-value*<0.05, non-significant *p-value*>0.05
‡ Between Group I & Group II,
♠ Between Group I & Group III, ★Between Group II & Group III

Table 3: Comparison between patients and control group regarding liver function tests at time of diagnosis.

Parameters	Group II (No= 20)	Group III (No= 20)	t. test	P. value
Total serum bilirubin (mg/dl) Pre-treatment Post-treatment t. test P. value	 0.8 ± 0.12 0.83 ± 0.14 1.2 0.23	 0.82 ± 0.29 2.21 ± 0.83 7.38 0.000*	 0.47 7.4	 0.64 0.000*
Direct bilirubin (mg/dl) Pre-treatment Post-treatment t. test P. value	 0.22 ± 0.04 0.23 ± 0.04 1.00 0.33	 0.23 ± 0.05 1.08 ± 0.51 7.38 0.000*	 -1.17 -7.35	 0.25 0.000*
Indirect bilirubin (mg/dl) Pre-treatment Post-treatment t. test P. value	 0.56 ± 0.17 0.58 ± 0.19 0.92 0.36	 0.58 ± 0.27 1.13 ± 0.35 6.00 0.000*	 0.39 6.5	 0.69 0.000*
Serum ALT (u/l) P re-treatment Post-treatment t. test P. value	 55.85 ± 9.48 57.1 ± 6.53 0.44 0.66	 54.75 ± 8.27 103.6 ± 24.39 8.78 0.000*	 1.57 8.8	 0.13 0.000*
Serum AST(u/l) Pre-treatment Post-treatment t. test P. value	 56.9 ± 9.21 59.9 ± 5.09 1.8 0.78	 58.15 ± 6.42 99.85 ± 17.43 10.45 0.000*	 0.77 9.5	 0.44 0.000*
Alkaline phosphatase (u/l) Pre-treatment Post-treatment t. test P. value	 213.5 1 ± 5.74 220.85 ± 25.03 1.27 0.21	 215.15 ± 27.81 482.8 ± 29.47 8.00 0.000*	 0.29 7.9	 0.77 0.000*
Prothrombin time (seconds) Pre-treatment Post-treatment t. test P. value	 14.77 ± 1.12 14.92 ± 0.97 1.83 0.83	 15.12 ± 1.33 16.18 ± 1.14 3.24 0.04*	 1.58 4.05	 0.13 0.01*
Total serum protein (gm/dl) Pre-treatment Post-treatment t. test P. value	 6.74 ± 1.13 7.13 ± 0.79 0.652 0.428	 7.32 ± 0.64 7.24 ± 0.71 0.358 0.210	 0.558 0.668	 0.524 0.550

Serum albumin (gm/dl) Pre-treatment Post-treatment t. test P. value	3.83 ± 0.52 3.95 ± 0.38 0.963 0.811	3.93 ± 0.37 3.80 ± 0.31 0.612 0.258	0.887 0.753	0.589 0.158
Serum globulin (gm/dl) Pre-treatment Post-treatment t. test p. value	2.89 ± 0.61 3.05 ± 0.64 0.899 0.335	3.38 ± 0.53 3.41 ± 0.65 0.774 0.621	2.114 0.841	0.125 0.637
Albumin globulin ratio Pre-treatment Post-treatment t. test P. value	1.40 ± 0.32 1.37 ± 0.38 0.214 0.330	1.20 ± 0.21 1.12 ± 0.23 0.635 0.447	1.587 0.244	0.086 0.336

**Significant when p-value<0.05, non-significant when p-value>0.05. ®Liver function is the main value of peaked level determined on successive days after giving each methotrexate dose. Persistent elevation of liver enzymes more than 2 folds for one month warrants a reduction of or discontinuation of methotrexate therapy* [25]

Table 4: Mean values of liver function tests before and after MTX therapy® in group II and III during follow up period.

Prognosis		Group		Total
		GII	GIII	
Complete remission	N	13	10	23
	%	65	50	57.5
Relapse	N	5	7	12
	%	25	35	30
Died	N	2	3	5
	%	10	15	12.5
Total	N	20	20	40
	%	100	100	100
Chi-Square	X^2	3.552		
	P-value	0.029*		

**significant.*

Table 5: Prognosis of patients after follow up for two years.

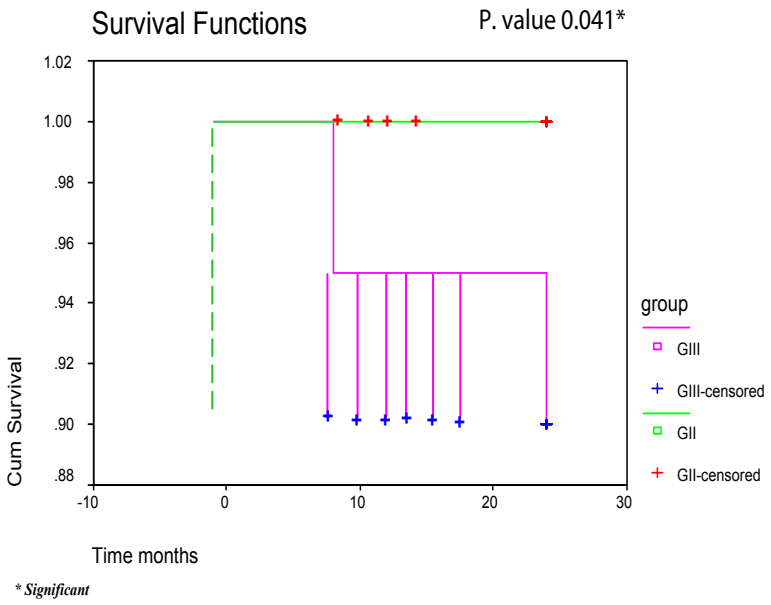

Figure 2: Disease free survival in group II and group III.

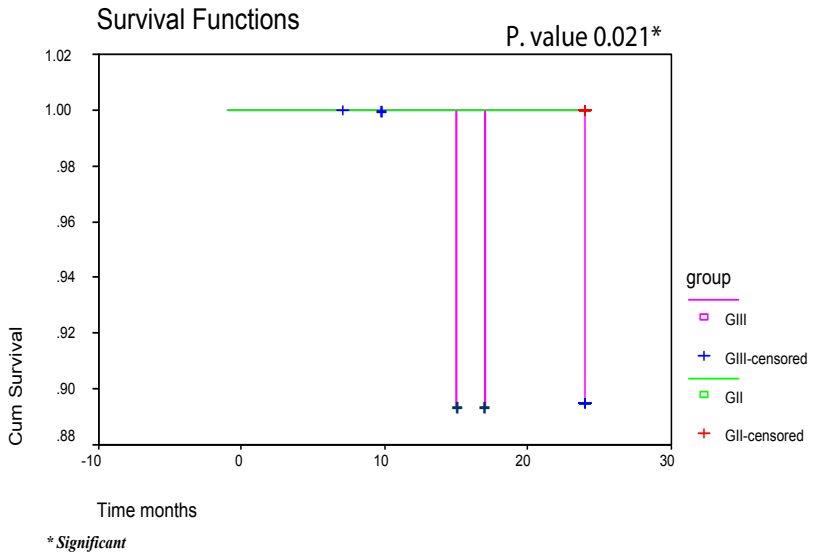

Figure 3: Overall survival in group II and group III.

Discussion

MTX is a folic acid antagonist that can cause unpredictable serious side effects [26]. Daily combination therapy of folic acid with MTX has been widely suggested as it reduces hepatic toxicity of MTX however; it is still controversial whether the use of folic acid reduces therapeutic efficiency of MTX or not [27]. Therefore, new antioxidant agents have been put on experimental trials against systemic oxidative damage caused by MTX [28].

The oil of *N. sativa* and TQ are known to possess strong antioxidant activities. TQ has been shown to inhibit non-enzymatic peroxidation in ox brain phospholipid liposomes also it has extremely high superoxide anion radical-scavenging abilities as effective as Superoxide Dismutase (SOD) [10]. The present study aimed to evaluate the protective effect of *Nigella sativa* oil on liver of children with ALL during treatment with MTX in oncology unit, Pediatric department, Tanta university hospital to helps these patients to complete the course of treatment without interruption and improve the prognosis and outcome of the disease.

In this study, the most common presenting clinical manifestations in studied patients were pallor, purpura and fever followed by hepatomegaly, splenomegaly and lymphadenopathy with no significant difference between group II and III. This is consistent with Biswas et al., [29] and Karimi et al., [30] who found the same results. Fever in ALL patients may be caused by pyrogenic cytokines released from leukemic cells, including interleukin-1, tumor necrosis factor and interleukin-6, but in about 30% of patients, fever is caused by infection [31].

In this study there was significant lower hemoglobin level and platelet count and significant higher white blood cell count in group II and group III compared to control with no significant difference between group II and III. This is consistent with Silverman and Sallan 2003 who found anemia and thrombocytopenia in the majority of cases with ALL at diagnosis (32) and Biswas et al., 2009, who found leucocytosis in 88% of their studied patients (29).

There was a significant higher serum bilirubin, ALT, AST and alkaline phosphatase levels and prothrombin time in group II and group III compared with control with no significant difference between group II and group III. After therapy there were non-significant increase in total, direct and indirect serum bilirubin, serum ALT, AST, and alkaline phosphatase levels and prothrombin time in group II but there was significant increase in group III with significant difference between group II and III after therapy.

These results were in agreement with Dogar et al., who investigate the efficacy of *Nigella sativa* seeds in ALL and found that no significant increase in ALT, AST, total serum bilirubin and alkaline phosphatase, in group received *Nigella sativa* oil in comparison with group not received [33] Ilham and Firas, [34], who investigate the effect of maintenance therapy for childhood ALL on the liver and found that seven of 30 children with ALL receiving daily oral 6–mercaptopurine and weekly methotrexate developed both hepatocellular destruction and intrahepatic cholestasis with abnormally elevated levels of serum aminotransferases enzymes, alkaline phosphatase and total serum bilirubin that mainly indicate biliary tract disorder [34] and El-Gharieb et al., who studied the hepatoprotective effect of NSO and vitamin E on liver of liver of workers exposed to organophosphorus insecticide, there was a significant reduction in serum hepatic transaminases in group received NSO [35] .

There was no significant difference in the total serum protein, albumin, globulin levels, and A/G ratio between group II and group III compared to control, also there is no significant difference between group II and group III after MTX therapy. This is in agreement Ilham and Firas [34] who found the same results. This could be explained this by the ability of the liver to increase protein, albumin biosynthesis during diseases associated with protein loss, or in presence of liver cell damage or injury induced by cytotoxic drugs, until the parenchymal damage or loss is severe [36].

Conclusion

Our study showed that oral administration of *Nigella sativa* oil in leukemic children prevented the side effects of MTX treatment on the liver in patients with ALL which lead to improvement in OAS and DFS.

Recommendations

Nigella sativa oil and its active constituent thymoquinone is recommended adjuvant drug as hepatoprotective agent in patients with ALL who received MTX therapy.

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