

Study of Iraqi Spinach Leaves (Phytochemical and Protective Effects Against methotrexate-Induced hepatotoxicity in rats)

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Abstract

Spinach, Spinacia oleracea L is a popular vegetable belonging to the family *Chenopodiaceae*. This study was concerned with extraction of compounds in Iraqi spinach leaves, preliminary phytochemical evaluation, identification of two biological important flavonols, quercetin and kaempferol in spinach leaves and evaluation of the protective effect of aqueous spinach extract on methotrexate (MTX) induced hepatotoxicity in rats. The percentage yield of extraction procedure, identification of spinach by chemical tests and identification of flavonols by thin layer chromatography (TLC) and High performance liquid chromatography (HPLC) were fully described in this study. The results indicate that the percentage of quercetin in spinach leaves is more than the percentage of kaempferol in the same plant. The rats were divided into three groups as control, MTX group following a single dose of MTX (20 mg/kg, i.p) saline was administered for 5 days and the MTX+aqueous spinach extract group were rats received 200mg/kg orally of aqueous spinach extract 7days before and 5 days after MTX treatment. MTX administration increased the MDA and decreased GSH, ALP while these changes were reversed in aqueous spinach extract treated group. Histological changes observed in MTX treated group was improved by aqueous spinach extract treatment. The protective effect of aqueous spinach extract against MTX- induced hepatotoxicity could be attributed to the combined effects of its constituents.

Key words: Spinach, Kaempferol , Quercetin , Methotrexate, Oxidative stress.

دراسة عن أوراق السبانخ العراقي (الكيمياء النباتية وتأثيراتها الواقية ضد تسمم الكبد الناتج عن الميثوتريكسيت في الجرذان)

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الخلاصة

السبانخ *Spinacia oleracea L* هو عبارة عن خضار شعبية تنتمي إلى عائلة *Chenopodiaceae* هذه الدراسة تهتم باستخلاص المركبات الفينولية من أوراق السبانخ العراقي، وتقييم الكيمياء النباتية الأولية، وتحديد الفلافون الهامتين بايولوجيا، كايامفيرول و كويرسيتين في أوراق السبانخ وتقويم التأثير الوقائي لمستخلص السبانخ المائي ضد تسمم الكبد الذي يسببه الميثوتريكسيت (MTX) في الجرذان . تم وصف النسبة المئوية للمستخلص واجريت الاختبارات الكيمائية للسبانخ بشكل كامل وتحديد الفلافون بواسطة الطبقة الرقيقة للفصل اللوني و الفصل اللوني السائل ذات الكفاءة العاليه في هذه الدراسة. تشير النتائج الى ان نسبة كويرسيتين في أوراق السبانخ هو أكثر من نسبة كايامفيرول في نفس النبات. تم تقسيم الجرذان إلى ثلاث مجموعات على النحو التالي السيطره ,مجموعة MTX بعد جرعه واحده من MTX (٢٠ ملغم / كغم في البريتون) تم إعطاء المحلول الملحي لمدة ٥ أيام في مجموعه MTX + مستخلص السبانخ المائي تلقت الجرذان (٢٠٠ ملغم / كغم) مستخلص السبانخ المائي عن طريق الفم ٧ ايام قبل و٥ ايام بعد اعطاء MTX. سبب اعطاء MTX زياده في مستوى MDA وانخفاض في GSH و ALP في حين انعكست هذه التغييرات في المجموعه التي تلقت مستخلص السبانخ المائي. تم تحسين معاملة تغيير النسيجية التي لوحظت في مجموعة MTX باعطاء مستخلص السبانخ المائي ويمكن أن يعزى التأثير الوقائي لمستخلص السبانخ المائي ضد تسمم الكبد التي يسببها MTX الى التأثير المشترك لمكونات اوراق السبانخ .

الكلمات المفتاحية : السبانخ ، كايامفيرول, كويرسيتين, ميثوتريكسيت, الاجهاد التاكسدي.

Introduction

Spinach is a leafy green vegetable that came originally from south western Asia and is now grown in most parts of the world. Scientifically it is known as *Spinacia oleracea L*, belonging to the family *Chenopodiaceae* (Figure1), locally known as ispanakh. ⁽¹⁾ Spinach is packed

with vitamins, minerals and a number of anti-oxidants components like polyphenols, flavonoids and carotinoids which are shown to possess anti-inflammatory effects, anti-mutagenic potential, anti-neoplastic effects, as well as chemo-preventive activities. ^(2,3)

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Figure 1: *Spinacia oleracea*

At least 13 different flavonoid compounds had been discovered by researchers in Spinach leaves⁽⁴⁾ among these flavonoids, quercetin and kaempferol are the most important and widely spread flavonol class (containing the typical C₆-C₃-C₆ structure)⁽⁵⁾ these flavonols provide the body with anti-oxidant protection, so they have significant influence against kidney damage, toxic liver damage, neurovascular complications and diabetic complications^(6, 7). Methotrexate (MTX), a folic acid antagonist is widely used as a cytotoxic chemotherapeutic agent; however its associated with hepatotoxicity which is considered to be a major clinical side-effect.⁽⁸⁾ The toxicity of MTX in the liver seems to relate to the generation of reactive oxygen species (ROS)^(9,10). The present study deals with the investigation of

phytochemicals found in the plant by ethanolic and aqueous spinach leaves extract and the possible protective effects of aqueous spinach extract against hepatic damage caused by methotrexate (MTX) in rats.

Materials and Methods

Plant materials

Fresh leaves of Spinach were purchased from vegetable markets of Baghdad; the plant was identified by the department of pharmacognacy, college of pharmacy/ university of Baghdad; and authenticated by the Herbarium of Baghdad University. 100 gram of fresh spinach leaves collected and dried under shade, powdered and divided into two parts: the first part, 50 grams of powdered leaves were packed in a thimble of soxhlet extractor. 100 ml of petroleum ether (40-60 °C) was used in soxhlet for 3 hours to get rid of lipids and fats to give fraction 1 (F1). The defatted powder after drying over-night were extracted with 100 ml of 96% ethanol for 72 hours by soxhlet extractor to give fraction two (F2) as shown in (Figure 2)⁽¹¹⁾. In the second part, 50 grams of powdered plant material was extracted with 100 ml of distilled water by cold maceration method, this step was repeated for three times to give fraction three (F3) as shown in (Figure 2)⁽¹²⁾. After that, the extracts (F1, F2 and F3) were filtrated and then concentrated under reduced pressure at a temperature not exceeding 40 °C to give greenish colored residues.

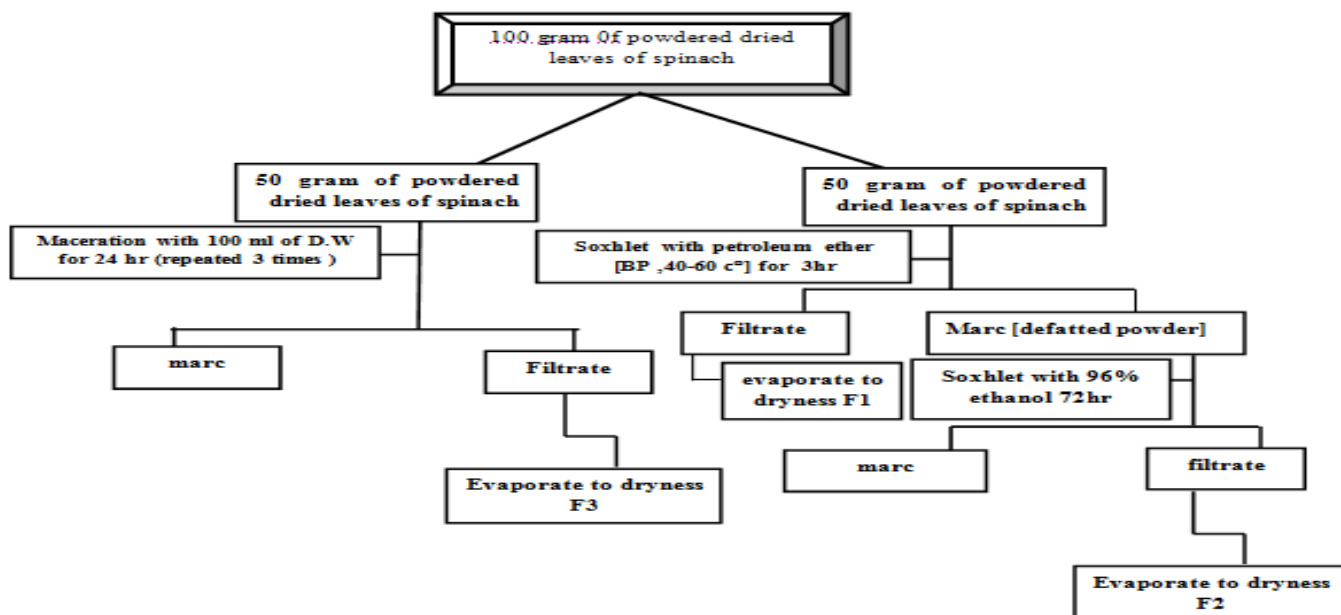


Figure 2: Schematic procedure for first and second parts of spinach leaves extraction^(11,12)

Phytochemical evaluation

Phytochemical evaluation of spinach leaves were done by making general tests for fixed oils, glycosides, alkaloids, phenolic compound, tannins and flavonoids.

1. Test for fixed oils ⁽¹³⁾

Small quantities of various extracts (F1, F2 and F3) were separately pressed between two filter papers; the appearance of permanent spot oil on the paper indicates the presence of fixed oils.

2. Test for glycosides ⁽¹⁴⁾**a - Baljet's test:**

1ml from each extracts (F1, F2 and F3) + 1ml of picric acid make it alkaline with sodium hydroxide → orange color.

b- Keller-Kiallian's test:

1ml from extracts (F1, F2 and F3) + 2ml of glacial acetic acid + 1 drop of 0.1% ferric chloride solution → then add 1ml of H₂SO₄ (drop by drop) → the junction between two liquid layers indicates the presence of the sugar part of glycosides.

c- Brontrager's test:

1ml of F1 and F2 extract + 1ml of diluted ammonia solution → pink color. While F3 was treated with chloroform and then chloroform layer was separated. To this equal amount of dilute ammonia solution added → pink color, showing the presence of glycosides.

3. Test for saponins ⁽¹⁵⁾

Each extract (F1, F2 and F3) was diluted with 10 ml of distilled water and it was agitated in test tube for 15 seconds and standing for 15 minutes, the formation of not less than 1cm layer of foam shows the presence of saponins.

4. Test for alkaloids ⁽¹⁶⁾

A small portion of extracts (F1, F2 and F3) were tested with various reagents for the presence of alkaloids.

Mayer's reagent → cream precipitation.

Dargendroff's reagent → orange brown precipitation.

Wagner's reagent → reddish brown precipitation.

5. Test for phenolic compounds and tannins ⁽¹⁴⁾

The tests for phenolic compounds and tannins were carried out with following reagents:

a. 5% ferric chloride solution → deep green or deep blue.

b. 10% lead acetate solution → white precipitation.

c. 1% potassium dichromate solution → orange precipitate.

6. Test for flavonoids ⁽¹¹⁾

a. With aqueous sodium hydroxide solution

Anthrocyanins → blue to violet color.

Flavones and flavonol → yellow color.

Flavonones → yellow to orange color .

b. With concentrated sulphuric acid

Anthrocyanins → yellow orange color.

Flavones and flavonol → yellow to orange color.

Flavonones → orange to crimson color.

Identification of quercetin and kaempferol as example of flavonoids

Spinach is loaded with flavonoids which act as antioxidants; identification of these flavonoids was performed by:

1. Identification of flavonoids (quercetin and kaempferol) by TLC: - ⁽⁶⁾

Using ready made aluminum plates of silica gel GF254, ultraviolet light detector at 254 nm wave length as detection method, standard flavonoids in comparison with three different solvent systems S1, S2 and S3.

Standard flavonoids are:

quercetin (Fluka-Austria) and kaempferol (Sigma-Aldrich, USA).

Different developing solvent systems were:-

S1 = Chloroform: Acetone: Formic acid (75:16.5: 8.5)

S2 = Chloroform: Methanol (90:10)

S3 = Toluene: Chloroform: Acetone (40:25:35).

2- Identification of flavonoids in HPLC

Further identification to the flavonoids in plant extracts (F2 and F3) were performed by HPLC. For qualitative estimation comparison of retention time obtained at identical chromatographic conditions of plant extract with authentic standards was done.

Table 1: HPLC conditions ⁽⁶⁾

Sample	Mobile phase	Column	Flow rate	Detection
Quercetin	Acetonitrile: methanol : glacial acetic acid (70:30: 0.1)	C ₁₈ 5 mm x 150 mm	0.5 ml / min	UV. Detector at λ 306 nm
Kaempferol	methanol : water (7.5 : 92.5)	C ₁₈ ODS	1.5 ml / min	UV. detector at λ 308 nm

For quantitative estimation the following equation was used to calculate the percentage of the compounds in the plant: - ⁽¹⁷⁾

Percentage of compound in the plant

$$= \left\{ \left(\frac{\text{AUC of plant sample}}{\text{AUC of the standard}} \right) \right\} * \text{Conc. St} * \text{DF} * 100 / (\text{weight of the plant in the extraction})$$

The weight of the plant used in the extraction was 50 gm

AUC = Area under the curve.

DF = Dilution factor.

Conc. St. = Concentration of the standard used in HPLC.

Pharmacological studies

Preparation of aqueous spinach extract

The roots of spinach were removed by sharp knife then the remaining portion was thoroughly washed several times with tap water and finally with distilled water and the water was removed by keeping on drying cabinet for 2 hours at room temperature. These were kept on a rectangular netted plastic basket for 2 hours to remove washing water. Then, the leaves and soft stalks were separated and chopped into small pieces and kept under an electric fan at room temperature then these were dried fully. The dried spinach was grinded with an electric grinder and then the grinded mass was sieved with 2mm wire sieve to get fine powder. Fifteen gram of spinach powder was weighed and thoroughly mixed with 1000 ml deionized distilled water and kept for 24 hours at room temperature. After that the solution was filtered and used.⁽¹⁸⁾

Experimental protocol

Eighteen white Albino rats of both sexes, weighing 200-250g were used in this study; the rats were obtained from and maintained in the animal house of the College of Pharmacy, University of Baghdad under conditions of controlled temperature. The animals were fed commercial pellets. The rats were divided into three groups:

Group 1 - normal control rats.

Group 2- MTX (20 mg/kg i.p) following a single dose of MTX saline will be administered for 5 days⁽¹⁹⁾.

Group 3 – MTX(20 mg/kg i.p) + spinach extract 200 mg/kg body weight orally in drinking water daily 7 days prior to and 5 days after MTX administration.

Biochemical estimations

Determination of the serum concentrations of the liver enzymes AST, ALT, ALP and Bilirubin were measured in serum samples obtained from all groups of rats. Liver tissue homogenate was prepared by standard procedure⁽²⁰⁾ and the contents of malondialdehyde

(MDA)⁽²¹⁾ and reduced glutathione (GSH)⁽²²⁾ were analyzed in liver tissue homogenate.

Microscopic evaluation

For the light microscopic investigations, tissue specimens from the liver were fixed with 10% formaldehyde and processed routinely for embedding in paraffin. Tissue sections of 5 µm were stained with hematoxylin and eosin (H and E)⁽²³⁾.

Statistical analysis

Results were expressed as mean ± SD (standard deviation). Using Student's t-test, the level of statistical significance was set at P<0.05.

Results

The dried leaves of *Spinacia oleraceae* L. was divided into two parts, the first one defatted with petroleum ether and extracted with 96% ethanol by soxhlet apparatus while the second one extracted with distilled water by cold maceration method. The percentage yield of plant extracts was as following:

Petroleum ether → 1.2%

Ethanol → 5.5%

Aqueous → 6.25%

Preliminary phytochemical evaluation

The various extract of dried leaves were subjected for phytochemical screening which shows the presence of different compounds in plant extracts as shown in (Table 2).

Table 2 : Preliminary phytochemical evaluation of dried leaves of *Spinacia oleraceae*.

Active constituents	Tests	F1	F2	F3
Fixed oil	Spot test	+	-	-
Glycosides	Baljet's test	-	+	+
	Keller kiallan's test	-	+	+
	Brontrager's test	-	+	+
Saponins	Foam test	-	+	+
Alkaloids	Mayer's test	-	-	-
	Dragendroff's test	-	-	-
	Wagner's test	-	-	-
Phenolic compounds and tannins	FeCl ₃ test	-	+	+
	Lead acetate test	-	+	+
	Potassium dichromate test	-	+	+
Flavonoids	Dil. NaOH solution	-	+	+
	Conc. H ₂ SO ₄	-	+	+

Where + =present ; - =absent

Identification of flavonoids by TLC

TLC of the extracts (F1, F2 and F3) obtained from dried leaves of Iraqi spinach

confirms the presence of quercetin and kaempferol in F2 and F3 only but not in F1 when comparison was made with standards, as represented in (Table 3) and (Figures 3, 4 and 5).

Table 3 : R_f value of plant extracts (F2 and F3), standard quercetin and kaempferol.

	S1	S2	S3
Solvent system	0.5	0.42	0.72
R_f value of quercetin in F2	0.49	0.41	0.71
R_f value of quercetin in F3	0.48	0.4	0.7
R_fvalue of kaempferol standard	0.7	0.6	0.82
R_f value of kaempferol F2	0.72	0.61	0.84
R_f value of kaempferol F3	0.71	0.59	0.83

S1=Chloroform: Acetone: Formic acid (75:16.5:8.5)
 S2 = Chloroform: Methanol (90:10)
 S3 = Toluene: Chloroform: Acetone (40:25:35).

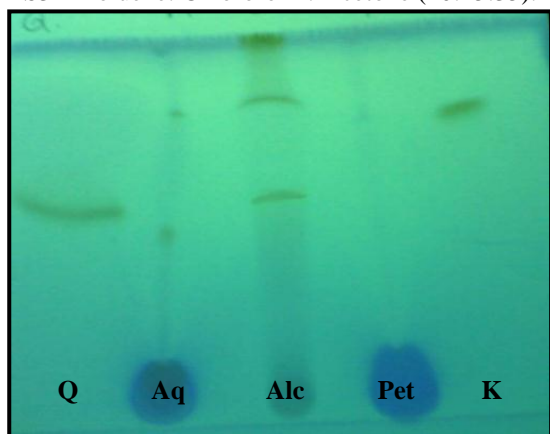


Figure 3 : TLC plate of spinach leaves extracts and standard, using S1 mobile phase

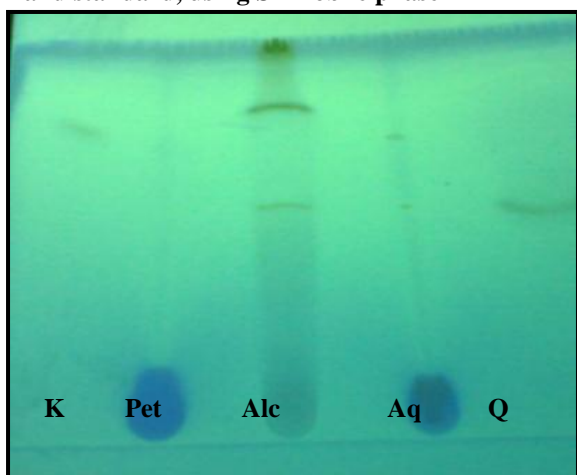


Figure 4: TLC plate of spinach leaves extracts and standard, using S2 mobile phase

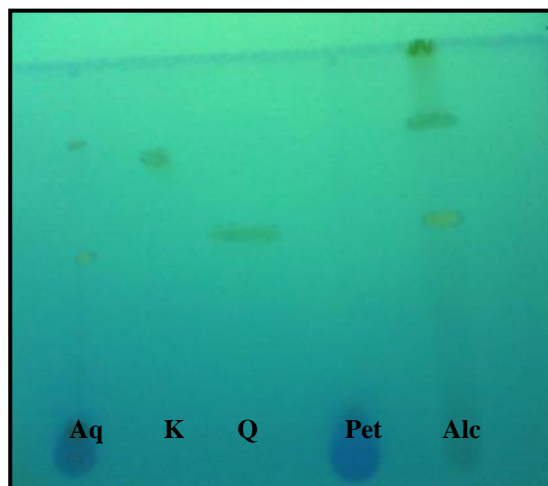


Figure 5: TLC plate of spinach leaves extracts and standard, using S3 mobile phase

Where Q=quercetin standard, K= kaempferol standard, Pet=petroleum ether extract, Alc=alcoholic extract and Aq=aqueous extract.

Identification of flavonoids by HPLC

Further identification to the quercetin and kaempferol in plant extracts (F2 and F3) were performed by HPLC in which the retention time of both standards (quercetine and kaempferol) and the plant extracts (F2 and F3) were identical as represented in the figures below:

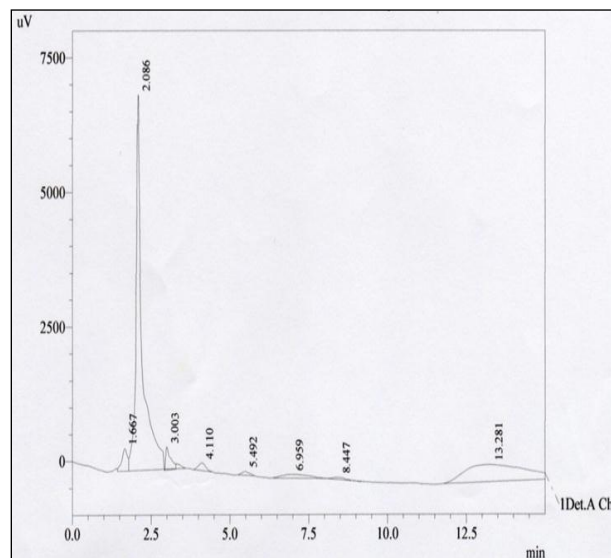


Figure 6: HPLC analysis of kaempferol standard

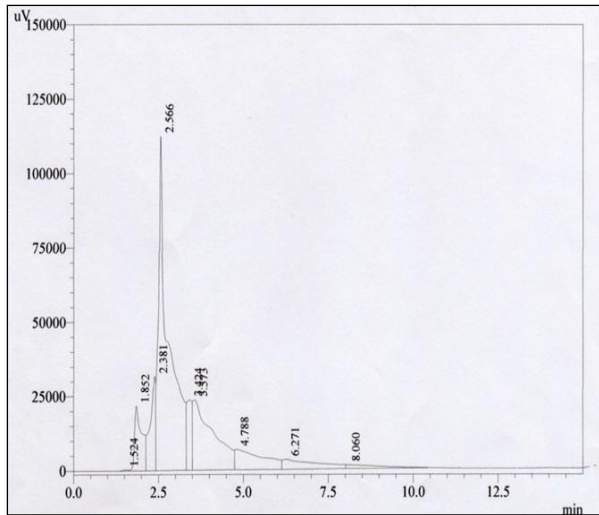


Figure 7: HPLC analysis of aqueous extract of Iraqi spinach leaves

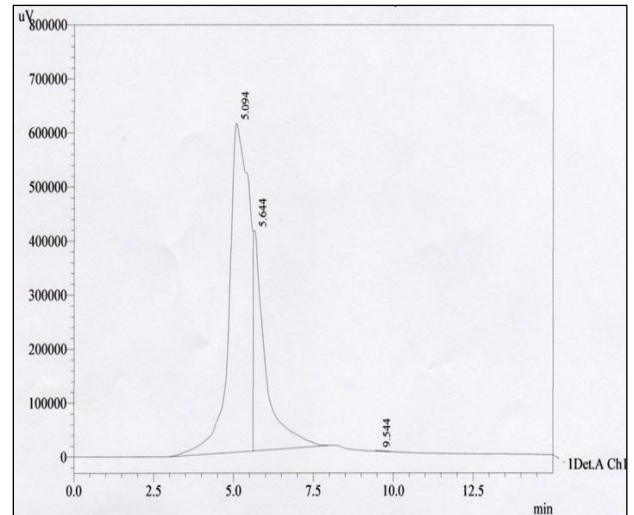


Figure 10 : HPLC analysis of alcoholic extract of Iraqi spinach leaves

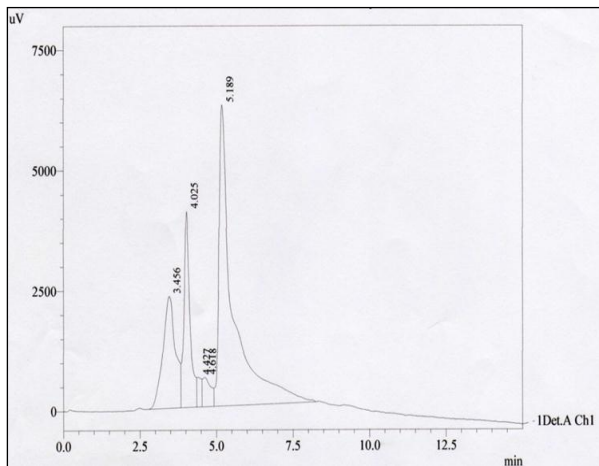


Figure 8: HPLC analysis of alcoholic extract of Iraqi spinach leaves

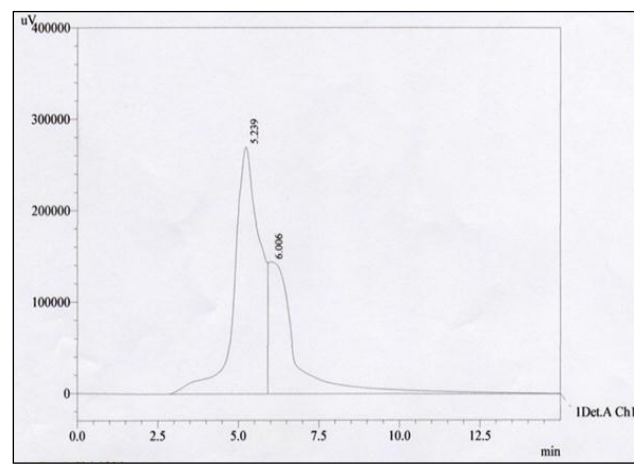


Figure 11: HPLC analysis of aqueous extract of Iraqi spinach leaves

For quantitative estimation, the percentage of quercetin in both F2 and F3 were higher than the percentage of kaempferol in the same extract, as shown in table (4).

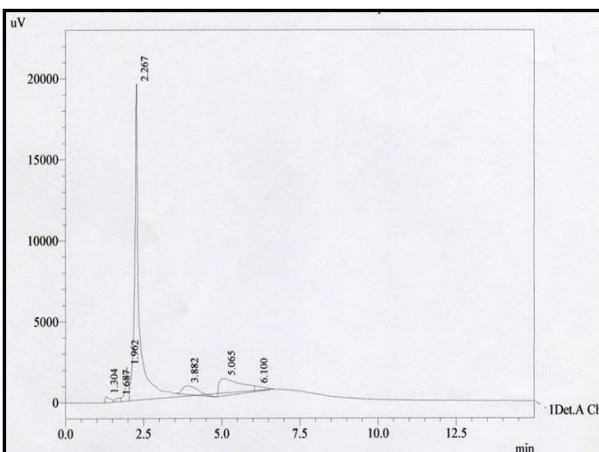


Figure 9 : HPLC analysis of quercetin standard

Table 4: data showing extractive values (percentage w/w) of quercetin and kaempferol in Iraqi spinach leaves

Extraction solvent	quercetin	kampferol
Ethanolic extract	0.268 %	0.12 %
Aqueous extract	0.149 %	0.096 %

Biochemical parameters

As shown in table 5, the level of MDA, a major degradation product of lipid peroxidation, was found to be significantly higher and level of GSH was significantly decreased in the MTX-

treated group when compared with control group ($p < 0.05$).Methotrexate cause no significant change in AST, ALT and bilirubin level ($p > 0.05$) and decrease in ALP level compared to control group ($p < 0.05$).While Rats treated with an oral concentrations (200 mg / kg) of Spinach extract 7 days prior to and 5 days after single i.p of methotrexate (20 mg/kg) resulted in significant decrease in MDA and increase GSH and ALP ($p < 0.05$) and produce no significant change in bilirubin, ALT and AST compared to methotrexate group ($p > 0.05$).In the livers of rats from the

control-treated rats, hepatocytes showed a normal histological appearance (Figure12). In the MTX-treated rats, showed rare scattered dysplastic hepatocytic changes, periportal inflammatory cell infiltration and sinusoidal dilatation were observed (Figure 13 and 14) , while in the MTX+ aqueous spinach extract treated rats affected to a significantly less degree than those in the group given MTX alone showed minimal periportal lymphocytic infiltration and lesser sinusoidal dilatation(Figure 15).

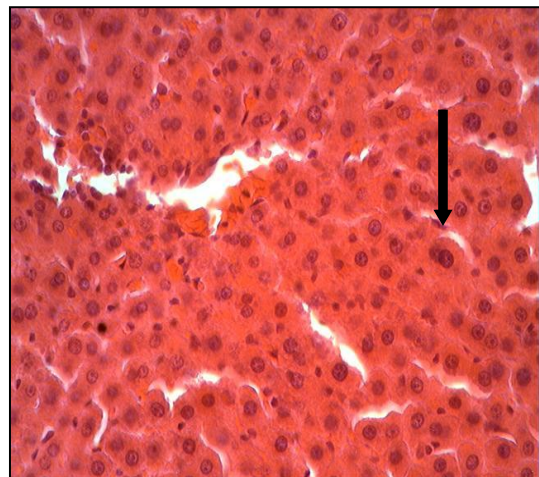
Table 5: Effect of methotrexate and aqueous spinach extract on liver biochemical parameters, MDA and GSH of rats

	Control	MTX	MTX+ spinach
AST(U/L)	105.7 ± 19.05	121.67 ±17.45	118.67 ± 40.1
ALT(U/L)	64.65 ± 24.32	45.83 ± 6.31	73.5 ± 23.25 **
Alp (U/L)	519 ± 93.92	185.33 ± 68.85 *	339.5 ±179.82 **
Bilirubin (U.Mol/L)	0.54 ± 0.11	0.61 ± 0.02	0.61 ± 0.12
MDA (nmol/gtissue)	1522.22 ± 277.08	4386.96 ±508.72 *	3069.01 ± 244.7 **
GSH (µmol/g tissue)	38.87 ± 4.28	29.21 ±1.93 *	35 ± 2.23 **

Data are presented as mean ± SD.
 n= number of animals.
 *P<0.05 with respect to control group.
 **P<0.05 with respect to methotrexate group.



Figure 12: Normal histological appearance of liver in control group (H and E X 200)



group (H&E x 400)

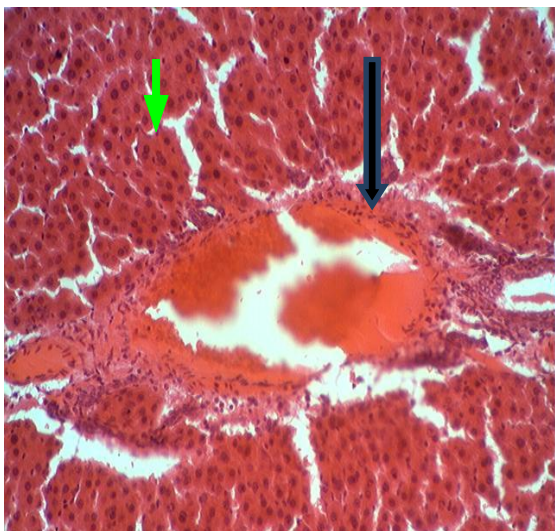


Figure 14: Periportal inflammatory cell infiltration (→) and sinusoidal dilatation (→) in methotrexate treated group (H&E x 200)

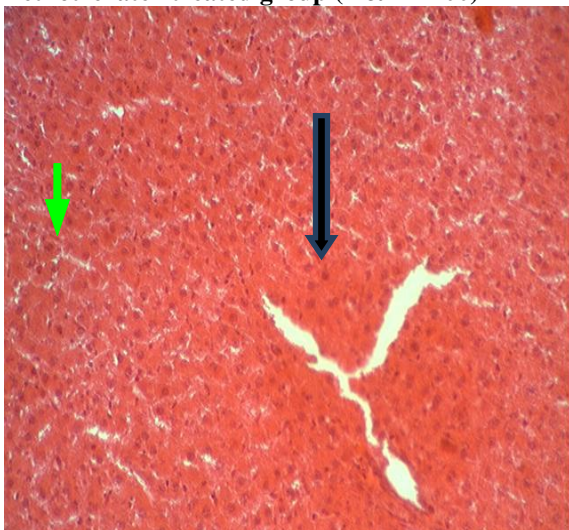


Figure 15: minimal periportal lymphocytic infiltration and sinusoidal dilatation (→) in MTX + aqueous spinach extract treated group (H&E x 200)

Discussion

Preliminary phytochemical studies showed the presence of flavonoids, glycosides, tannins, saponins and polyphenolic compounds on both ethanolic and aqueous extracts. Identification of quercetin and kaempferol as examples to flavonoids by TLC and HPLC indicates the presence of these flavonoids and give the results that the percentage of quercetin in Iraqi spinach leaves is more than the percentage of kaempferol in the same plant. The study examined the role of oxidative stress in an attempt to suggest a possible mechanism of MTX-induced hepatotoxicity in a rodent model. The results of the studies indicate that MTX causes oxidative tissue damage by increasing lipid peroxidation in the liver tissue and decreasing

the level of GSH. These results were consistent with other studied. ^(24, 25) The increased MDA levels as observed in our study can be attributed to the lipid peroxidation can be induced by MTX itself or as a result of a possible increase in ROS induced by MTX. ⁽²⁶⁾ The ROS thus formed may lead to cellular damage by peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown. ^(27, 25) It has been shown that treatment with MTX leads to a reduction in the effectiveness of antioxidant defense systems and that cellular levels of glutathione are reduced by MTX. ⁽²⁶⁾ Although statistically no significant differences in AST was observed, ALT and bilirubin level in methotrexate treated group and this result are consistent with other studies. ^(25, 28) this may be due to administration of methotrexate for short duration or may need high dose to produce change in the level of these enzymes but the level of ALP decreased. This reduction could be explained by the noticeable damage that affected the endothelial cells lining the blood sinusoids and blood vessels in the portal tracts, which are considered to be the main sites at which the ALP acts. ⁽²⁹⁾ Previous studies have shown that many antioxidants, including N-acetylcysteine ⁽³⁰⁾ and grape seed extract ⁽³¹⁾ have protective effects in the MTX injury of the liver in rat. At microscopic level, a single dose of MTX resulted in significant liver injury. The mechanism by which MTX causes hepatotoxicity due to its binding to the enzyme dihydrofolic reductase, thus preventing conversion of folic acid to its active form, folinic acid. This in turn blocks the synthesis of nucleic acids, certain amino acids and indirectly proteins which lead to damage of organelles and plasma membranes of hepatic parenchymal cells interfering with their function ⁽³²⁾. Aqueous spinach extract supplementation reduced MTX-induced oxidative liver damage these data are in accordance with the findings of a recent study which demonstrated that spinach leaves contain natural antioxidant system which provide free radical scavenger properties and hepatic protection against CCL₄ ⁽³³⁾ and radiation ⁽³⁴⁾ induced by oxidative stress.

Conclusion

Phytochemical investigation of the aqueous and ethanolic extracts of leaves of *Spinacia oleracea* showed the presence of flavanoids, glycosides, saponins, tannins, phenolic compounds and treatment of rats with aqueous spinach extract before and after MTX application provided significant protection from the hepatotoxicity of MTX could be attributed to the combined effects of its constituents as the leaves are rich in flavonoids and *p*-coumaric acid.

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