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, Kaempherol , Quercetin, Methotrexate, Oxidative stress.

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. Spinach is packed with vitamins, minerals and a number of antioxidants 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.

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At least 13 different flavonoid compounds had been discovered by researchers in Spinach leaves among these flavonoids, quercetin and kaempferol are the most important and widely spread flavonol class (containing the typical C₆-C₃-C₆ structure). These flavonols provide the body with antioxidant protection, so they have significant influence against kidney damage, toxic liver damage, neurovascular complications and diabetic complications. 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. The toxicity of MTX in the liver seems to relate to the generation of reactive oxygen species (ROS). 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. 100gram 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). 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). 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.
**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**
   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**
   - **Baljet’s test:**
     1 ml from each extracts (F1, F2 and F3) + 1 ml of picric acid make it alkaline with sodium hydroxide → orange color.
   - **Keller-Killiani’s test:**
     1 ml from extracts (F1,F2 and F3)+ 2 ml of glacial acetic acid+ 1 drop of 0.1% ferric chloride solution → then add 1 ml of H₂SO₄ (drop by drop) → the junction between two liquid layers indicates the presence of the sugar part of glycosides.
   - **Bronteiger’s test:**
     1 ml of F1 and F2 extract+1 ml 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**
   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 1 cm layer of foam shows the presence of saponins.

4. **Test for alkaloids**
   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**
   The tests for phenolic compounds and tannins were carried out with following reagents:
   - 5% ferric chloride solution → deep green or deep blue.
   - 10% lead acetate solution → white precipitation.
   - 1% potassium dichromate solution → orange precipitate.

6. **Test for flavonoids**
   - With aqueous sodium hydroxide solution → Anthocyanins → blue to violet color.
   - Flavonoids and flavonols → yellow color.
   - Flavonones → yellow to orange color.
   - With concentrated sulphuric acid:
     - Anthocyanins → yellow to orange color.
     - Flavonoids and flavonols → 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:**
   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)

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mobile phase</th>
<th>Column</th>
<th>Flow rate</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>Acetonitrile: methanol :</td>
<td>C$_{18}$</td>
<td>0.5 ml / min</td>
<td>UV. Detector at 2306 nm</td>
</tr>
<tr>
<td></td>
<td>glacial acetic acid (70:30:0.1)</td>
<td>5 mm x 150 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>methanol : water (7.5 : 92.5 )</td>
<td>C$_{18}$</td>
<td>1.5 ml / min</td>
<td>UV. Detector at 2308 nm</td>
</tr>
<tr>
<td></td>
<td>ODS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

\[
\frac{(\text{AUC of plant sample})}{(\text{AUC of the standard})} \times \text{Conc. St} \times \text{DF} \times 100)/\text{(weight of the plant in the extraction)}
\]

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

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 oleracea 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>
<thead>
<tr>
<th>Active constituents</th>
<th>Tests</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed oil</td>
<td>Spot test</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Baljet’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Keller kiallan’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brontrager’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Foam test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Mayer’s test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dragendorff’s test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wagner’s test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenolic compounds and tannins</td>
<td>FeCl3 test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Potassium dichromate test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Dil. NaOH solution</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Conc. H2SO4</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>0.5</td>
<td>0.42</td>
<td>0.72</td>
</tr>
<tr>
<td>$R_f$ value of quercetin in F2</td>
<td>0.49</td>
<td>0.41</td>
<td>0.71</td>
</tr>
<tr>
<td>$R_f$ value of quercetin in F3</td>
<td>0.48</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>$R_f$ value of kaempferol standard</td>
<td>0.7</td>
<td>0.6</td>
<td>0.82</td>
</tr>
<tr>
<td>$R_f$ value of kaempferol F2</td>
<td>0.72</td>
<td>0.61</td>
<td>0.84</td>
</tr>
<tr>
<td>$R_f$ value of kaempferol F3</td>
<td>0.71</td>
<td>0.59</td>
<td>0.83</td>
</tr>
</tbody>
</table>

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 bellow:

**Figure 6**: HPLC analysis of kaempferol standard
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).

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

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>quercetin</th>
<th>kaempferol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanollic extract</td>
<td>0.268 %</td>
<td>0.12 %</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>0.149 %</td>
<td>0.096 %</td>
</tr>
</tbody>
</table>

**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

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

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)](image1.png)

![Figure 13: Rare scattered dysplastic hepatocytic changes in methotrexate treated group (H&E x 400)](image2.png)
The study examined the role of MTX in 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 studies. 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. The ROS thus formed may lead to cellular damage by peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown. 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. 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. 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. Previous studies have shown that many antioxidants, including N-acetylcysteine and grape seed extract 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 dihydrofolate 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. 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 CCL-4 and radiation induced by oxidative stress.

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 studies. 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. The ROS thus formed may lead to cellular damage by peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown. 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. 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. 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. Previous studies have shown that many antioxidants, including N-acetylcysteine and grape seed extract 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 dihydrofolate 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. 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 CCL-4 and radiation induced by oxidative stress.

Conclusion

Phytochemical investigation of the aqueous and ethanolic extracts of leaves of Spinacia oleracea showed the presence of flavonoids, 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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