Astaxanthin affects oxidative stress biomarkers in methotrexate-induced hepatotoxicity

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Abstract---Methotrexate is a chemotherapeutic and immunosuppressive drug used in the treatment of cancer, psoriasis, rheumatoid arthritis and several other disorders. Its use predisposes to hepatotoxicity which is a serious side effect. The current study aimed to assess the oxidative-stress-causing potential of methotrexate to liver cells and evaluate the hepatoprotective activity of the potent antioxidant astaxanthin, by affecting the oxidative stress biomarkers GSH (glutathione), SOD (superoxide dismutase), CAT (catalase) and MDA (malondialdehyde). A model of methotrexate-induced liver toxicity was employed on male rats. Thirty-six rats were divided into six groups; a negative control group, methotrexate induction group given (20 mg/kg) I.P. on day 13, three groups pretreated with oral astaxanthin in ascending doses (50, 75 and 100 mg/kg) for 14 days before methotrexate, and a conventional therapy group pretreated with silymarin (200mg/kg). The use of methotrexate significantly decreased liver tissue GSH, SOD and CAT while significantly increasing MDA, compared to the negative control. On the other side, astaxanthin used in all three doses significantly normalized these biomarkers. This study revealed that since astaxanthin significantly increased GSH, SOD and CAT while decreasing MDA that minimizes oxidative stress, it could be used as pretreatment in patients treated with methotrexate to decrease its hepatotoxicity.
**Keywords**--astaxanthin, methotrexate, hepatotoxicity, oxidative stress.

**Introduction**

Drug Induced liver injury (DILI) is a major clinical issue in diagnosis and treatment of patients. It can frequently lead to drug development failure and drug withdrawal which is financially burdening for the pharmaceutical industry (Garcia-Cortes et al. 2020). The liver is subject to great toxicity of xenobiotics due to its detoxifying function (Said, Al-Khashali, and Al-Khateeb 2011). Methotrexate (MTX) is an effective medicine used in the treatment of autoimmune disorders like rheumatoid arthritis (RA), idiopathic arthritis, vasculitis, Crohn’s disease and dermatological diseases including psoriasis and refractory atopic dermatitis (Devarbhavi et al. 2021). Its mechanism of action is mainly by inhibiting the enzyme 5-aminomidazole-4-carboxamide ribonucleotide transformylase (ATIC). This enzyme catalyzes the last step in biosynthesis of purine (Cronstein and Aune 2020; J. Kim et al. 2018). Patients undergoing MTX-therapy are at risk of developing hepatic injury and MTX was used in this experiment to induce hepatotoxicity.

One of the pathological characteristics of DILI is excessive oxidative stress (Inam Sameh et al. 2022). In hepatocytes, MTX increases reactive oxygen species (ROS) production leading to lipid peroxidation and increase in products like MDA (Ekińci-Akdemir et al. 2018). Additionally, it downregulates intracellular antioxidants such as SOD, CAT and GSH. Lipid peroxidation and hepatocyte membrane damage and destruction of liver cells by increased oxidative stress releases excessive amounts of liver enzymes like aspartate aminotransferase (AST) and alanine transaminase (ALT) released into the bloodstream which can be measured as indicators of liver injury (Kareem, Abdullah, and Waheed 2021). Additionally, oxidative stress induces the creation of pro-inflammatory cytokines, which worsen the disease in cells (Chauhan et al. 2020). Astaxanthin (ASX), is formally named 3,3'-dihydroxy-ß,ß-carotene-4,4'-dione (Ambati et al. 2014). It is a red carotenoid pigment found in various microorganisms and marine animals, such as shrimp, crab, and salmon. ASX outperforms beta-carotene and vitamin E in addition to coenzyme Q10 in antioxidant capacity. It is produced by chemical synthesis from carotene or by biological extraction from the sources mentioned previously. Synthetic astaxanthin is less safe and less stable than the natural form, and is thus not allowed in the human diet under FDA regulations. Astaxanthin produced from a microalga called Haematococcus pluvialis, gives the highest yield for human consumption (Gao, Zhu, and Xu 2021; Pan, Wang, and Gu 2018; Pereira et al. 2021).

**Methodology**

This study started after approval by the Animal Ethics Committee in the Pharmacology and Toxicology Department College of Pharmacy/ Mustansiriyah University. Starting with pretreatment of the rats with astaxanthin, followed by induction with methotrexate (as described in the study design), scarifying of the
animals, and ending with the evaluation of the oxidative stress biomarkers GSH, SOD, CAT and MDA in samples.

**Animals and study design**

Thirty-six male Wistar rats weighing between 200- and 250 grams were used to conduct this study. It was performed according to the regulations of the Animals Ethics Committee in the College of Pharmacy, Mustansiriyah University. The rats were purchased from the Iraqi Center for Cancer Research, and housed in big comfortable cages there. For 10 days, the rats were allowed to acclimate in a controlled environment, including temperature (22°C), humidity (40-50%) and a light schedule of 12 hours’ light-dark cycles. They were allowed food and water *ad libitum* (Luay, Mustafa, and Jaber 2021). Thereafter, the rats were divided randomly into six groups of six rats each. The groups were described as follows (figure 1):

- **Group 1 (n=6):** Negative control, rats received the vehicle of ASX suspension (distilled water and Tween 20) orally (P.O.) for 14 days and a single injection of N.S. intraperitoneally (I.P.) on day 13 only.
- **Group 2 (n=6):** Positive or induction control group. It received the vehicle of ASX suspension P.O. for 14 days, in addition to a single intraperitoneal administration of methotrexate (20 mg/kg) I.P. on day 13 of the experiment.
- **Group 3 (n=6):** Treatment group of oral astaxanthin suspension at a dose of 50 mg/kg daily for 14 days, in addition to a single intraperitoneal administration of methotrexate (20 mg/kg) I.P. on day 13 of the experiment.
- **Group 4 (n=6):** Treatment group of oral astaxanthin at a dose of 75 mg/kg daily for 14 days, in addition to a single intraperitoneal administration of methotrexate (20 mg/kg) on day 13 of the experiment.
- **Group 5 (n=6):** Treatment group of oral astaxanthin suspension at a dose of 100 mg/kg daily for 14 days, in addition to a single intraperitoneal administration of methotrexate (20 mg/kg) on day 13 of the experiment.
- **Group 6 (n=6):** Conventional treatment group of oral silymarin (from the medicinal plant *Silybum marianum* (Mshemish et al. 2011)) suspension at a dose of 200 mg/kg daily for 14 days, in addition to a single intraperitoneal administration of methotrexate (20 mg/kg) on day 13 of the experiment.

Figure 1. Study design
On the 15th day of the experiment, the rats were fixed in flat position, after which they were deeply anaesthetized (loss of corneal and toe pad reflexes) by I.P injection of ketamine (80 mg/kg) and xylazine (10mg/kg). The animals were afterwards decapitated and liver samples were obtained for analysis. It was homogenized according to kits manufacturers’ instructions and stored at -40 °C for biochemical assays.

**Determination of serum superoxide dismutase levels**

The levels of SOD in blood serum were measured employing the quantitative sandwich ELISA technique. Serum samples and standards were pipetted into wells pre-coated with SOD-specific antibodies. This was followed by washing to remove any unbound substances and addition of a SOD-specific biotin-conjugated antibody to the wells. The washing was repeated. Avidin-conjugated HRP was then added to the wells, followed by another wash. A substrate solution was then added to the wells after a wash to remove any unbound avidin-enzyme reagent. A yellowish color in proportion to the amount of SOD developed formed. The color was halted by addition of a stopping solution and its intensity measured spectrophotometrically at 450 nm. The concentrations of SOD were expressed in (ng/mL).

**Determination of serum catalase levels**

By employing the quantitative sandwich enzyme immunoassay technique, CAT levels were measured. Standards and samples were pipetted into the wells of a microplate pre-coated with an antibody specific for CAT. All CAT present was bound by the immobilized antibody. A biotin-conjugated antibody specific for CAT was added to the wells. Following a wash, avidin conjugated HRP was added to the wells. The wells were washed again to remove any unbound avidin-enzyme reagent followed by addition of a substrate solution to the wells. The amount of CAT bound in the initial stage determined the color. The color development was stopped by a stopping solution and the intensity of the color was measured using a microplate reader set to 450 nm to give the activities of catalase in (IU/L).

**Determination of serum malondialdehyde (MDA) levels**

The total activity of MDA which is the end product of lipid peroxidation of polyunsaturated fatty acids could be measured by sandwich ELISA. Standards and rat serum samples were pipetted into the wells of a microplate pre-coated with an antibody specific for MDA. All MDA present was bound by the immobilized antibody. A biotin-conjugated antibody specific for MDA was added to the wells. Following a wash, avidin conjugated HRP was added to the wells. The wells were washed again to remove any unbound avidin-enzyme reagent followed by addition of a substrate solution to the wells. The amount of MDA bound in the initial stage determined the color. The color development was stopped by a stopping solution and the intensity of the color was measured using a microplate reader set to 450 nm to give the concentrations of MDA in (nmol/mL).
**Determination of GSH in liver tissues**

By employing the “Double Antibody Sandwich” technique, the principle was based on the properties of the target analyte (GSH) with more than two potential epitopes that could be detected concurrently by both the pre-coated capture antibody (anti-Rat GSH monoclonal antibody) and the detection antibody (a biotinylated polyclonal antibody). The steps were as follows: The antibodies were pre-coated to the plate. By washing, all antibodies and impurities that did not stick to the plate, were removed. Irrelevant proteins were used to block the remaining spots on the plate. Then, the prepared tissue samples containing the GSH was added, which resulted in the GSH immobilization by the GSH-specific capture antibodies, leading to the formation of an antigen-antibody combination. All unbound particles and impurities were then removed by washing the wells. The wells were subsequently filled with a biotin-labeled antibody that was specific for GSH, resulting in an antibody-antigen-antibody complex. This was followed by washing of the plate to remove unbound antibodies and impurities. Then, HRP and avidin were added to the wells and bound with the biotin-labeled antibodies. The amount of reporter enzyme in the sample was then positively linked with the amount of GSH. After that, the wells were washed again to remove any remaining contaminants. Finally, HRP reaction substrates were introduced, and sample concentrations in (μg/mL) could be computed based on the coloring changes at 450 nm wavelength.

**Results**

The effect of astaxanthin pretreatment on oxidative stress biomarkers (SOD, CAT, MDA and GSH) levels in MTX-induced hepatotoxicity

The following table shows the effect of astaxanthin on serum oxidative stress biomarkers (SOD, CAT and MDA) in methotrexate-induced-hepatotoxicity in male rats’ model.

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (ng/mL)</th>
<th>CAT (IU/L)</th>
<th>MDA (nmol/mL)</th>
<th>GSH (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>136.48±2.08a</td>
<td>12.21±1.64a</td>
<td>1.50±0.31c</td>
<td>22.99±2.65a</td>
</tr>
<tr>
<td>Induction</td>
<td>78.18±4.20d</td>
<td>7.23±1.16b</td>
<td>5.03±0.19a</td>
<td>8.10±1.14b</td>
</tr>
<tr>
<td>ASX(50mg/kg)</td>
<td>89.70±7.32cd</td>
<td>10.73±1.63a</td>
<td>2.60±0.44b</td>
<td>17.82±5.08ab</td>
</tr>
<tr>
<td>ASX(75mg/kg)</td>
<td>91.63±2.35cd</td>
<td>10.27±0.99ab</td>
<td>1.53±0.18c</td>
<td>24.01±3.15a</td>
</tr>
<tr>
<td>ASX(100mg/kg)</td>
<td>119.78±5.28b</td>
<td>10.83±2.03a</td>
<td>1.03±0.16c</td>
<td>25.94±3.95a</td>
</tr>
<tr>
<td>Silymarin(200mg/kg)</td>
<td>102.21±3.42c</td>
<td>11.08±1.35a</td>
<td>1.18±0.15c</td>
<td>17.94±3.42ab</td>
</tr>
<tr>
<td>LSD</td>
<td>15.72</td>
<td>3.00</td>
<td>0.76</td>
<td>9.97</td>
</tr>
</tbody>
</table>

The results were expressed as mean ±SD. Results with unidentical superscripts (a, b, c) are significantly different (p<0.05). Results with two different letters indicate no significant difference; (ab): no significant difference with (a) nor (b), (bc): no significant difference with (b) nor (c). LSD: Least significant difference.
Serum SOD mean concentration significantly declined \([p<0.05]\) in the induction group (78.18±4.20 ng/mL) in comparison to the negative control group (136.48±2.08 ng/mL). When treated with ASX at the doses 50 mg/kg and 75 mg/kg, the serum mean concentration of SOD significantly increased \([p<0.05]\) to (89.70±7.32 ng/mL) and (91.63±2.35 ng/mL) compared to the induction group. The difference between these two results and the result of the control group were non-significant \([p>0.05]\). Moreover, there was a significant rise in serum SOD mean concentration \([p<0.05]\) in the ASX-treated group with the dose 100 mg/kg (119.78±5.28 ng/mL) as well as the silymarin treated group (102.21±3.42 ng/mL) when compared to the induction group. These two treated groups demonstrated significant differences \([p<0.05]\) with the negative control group; figure (2).

![Figure 2. The effect of ASX on serum SOD levels](image)

The results were expressed as mean±SD. Results with unidentical small letters (a, b, c, d) are significantly different \((p<0.05)\). Results with two different letters indicate no significant difference; (ab): no significant difference with (a&b). Serum mean catalase activity significantly decreased \([p<0.05]\) in the induction group (7.23±1.16 IU/L) in comparison to the negative control group (12.21±1.64 IU/L). When treated with ASX at the dose 50 mg/kg, the serum mean catalase activity significantly increased \([p<0.05]\) to (10.73±1.63 IU/L) compared to the induction group. On the other side, when comparing this ASX group to the control group, a non-significant difference was demonstrated \([p>0.5]\). The serum catalase activity of the group treated with 75 mg/kg ASX was (10.27±0.99), which were non-significant from the induction group \([p>0.05]\). Moreover, there was a significant rise in serum catalase activity \([p<0.05]\) in the ASX-treated group with the dose 100 mg/kg (10.83±2.03 IU/L) as well as the silymarin treated group (11.08±1.35 IU/L) when compared to the induction group. The groups treated with all three doses of ASX and silymarin showed no significant difference \([p>0.05]\) to the negative control group; figure (3).
The results were expressed as Mean±SD. Results with unidentical small letters (a, b) are significantly different (p<0.05). Results with two different letters indicates no significant difference; (ab): no significant difference with (a&b). Serum MDA mean concentration significantly increased [p<0.05] in the induction group (5.03±0.19 nmol/mL) in comparison to the negative control group (1.50±0.31 nmol/mL). When treated with ASX at the dose 50 mg/kg, the serum mean concentration of MDA significantly decreased [p<0.05] to (2.60±0.44 nmol/mL) compared to the induction group. Moreover, there was a significant decline in serum MDA mean concentration [p<0.05] in the ASX-treated group with the doses 75 mg/kg (1.53±0.18 nmol/mL) and 100 mg/kg (1.03±0.16 nmol/mL) as well as the silymarin treated group (1.18±0.15 nmol/mL) when compared to the induction group. The groups treated with the two higher doses of ASX and silymarin demonstrated no significant difference [p>0.05] to the negative control group; figure (4).
The results were expressed as mean±SD. Results with unidentical small letters (a, b, c) are significantly different (p<0.05). Tissue mean GSH concentration significantly decreased [p<0.05] in the induction group (8.10±1.14 μg/mL) in comparison to the negative control group (22.99±2.65 μg/mL). When treated with ASX at the dose 50 mg/kg, the tissue mean GSH concentration non-significantly increased [p>0.05] to (17.82±5.08 μg/mL) compared to the induction group. Moreover, there was a significant rise in tissue GSH concentration [p<0.05] in the ASX-treated groups with the doses 75 mg/kg (24.01±3.15 μg/mL) and 100 mg/kg (25.94±3.95 μg/mL) when compared to the induction group. These ASX treated groups showed significant difference [p<0.05] to the negative control group. Both the group treated with the smallest dose of ASX and with silymarin (17.94±3.42 μg/mL) showed no significant difference to the induction group [p>0.05]; figure (5).

Figure 5. The effect of ASX on tissue GSH levels

The results were expressed as mean±SD. Results with unidentical small letters (a, b) are significantly different (p<0.05). Results with two different letters indicate no significant difference; (ab): no significant difference with (a&b).

Discussion

Oxidative stress interrelates with inflammation contributing to cardiovascular diseases. Astaxanthin decreases oxidative stress and potentiates the activity of antioxidant enzymes. Owing to its unique chemical structure, it aligns into the cell membranes decreasing lipid peroxidation and maintaining membrane structure (Pereira et al. 2021). Excess formation of reactive oxygen species (ROS) is partly responsible for the methotrexate induced liver injury. Fortunately, liver cells have both enzymatic and non-enzymatic defenses against ROS to maintain the redox balance (C. H. Kim, Kim, and Nam 2021). Superoxide dismutases (SODs) are a family of enzymes that act as the first line of defense against ROS in the enzymatic defense system. They catalyze the dismutation of superoxide anions (O$_2$.−) which is the reduction of O$_2$.− to O$_2$ and hydrogen peroxide (H$_2$O$_2$) (Yan and Spaulding 2020). In this study, administration of MTX significantly decreased the serum level of SOD, as compared to the negative control group. Pretreatment with (100 mg/kg) ASX significantly increased the SOD level.
In a study on the effects of apigenin in methotrexate induced hepatotoxic rats, MTX administration significantly depleted the level of SOD, which is in agreement with the current study (Goudarzi et al. 2021). Additionally, mice given MTX to examine the alleviating effects of *Moringa oleifera* leaf extract to hepatotoxicity, also suffered a significant decrease in SOD level (Soliman et al. 2020). In liver injured mice induced by acetaminophen, ASX pretreatment alleviated the decline in SOD induced by acetaminophen thus decreased oxidative stress (Zhang et al. 2017). Rats exposed to ambient air showed a significant decline in SOD level and a rise in oxidative stress. Treatment with ASX significantly improved the level of SOD and mitigated the oxidative stress caused by ambient air exposure (Johnson et al. 2021). The hydrogen peroxide produced with the help of SOD is further metabolized by the antioxidant enzymes catalase or glutathione peroxidase. Catalase enzymatically converts this highly reactive species to water and oxygen in a two-step reaction (Nandi et al. 2019).

Administration of MTX, in this study, significantly decreased the level of catalase in the serum of the rats in the induction group compared to the negative control group. This is in accordance with the results obtained in a study by F. Nur Ekińci-Akdemi et al where the level of CAT significantly decreased by a single IP injection of (20mg/kg) MTX (Ekińci-Akdemir et al. 2018). Additionally, CAT level significantly decreased by MTX administration in a study performed to explore the hepatorenal protective effects of the plant *Glycyrrhiza glabra* in rats (Chauhan et al. 2020). Pretreatment with ASX in all its three doses in addition to the conventional treatment silymarin significantly increased the level of CAT in serum normalizing its value. This in line with a study involving the toxic insecticide thiacloprid. While thiacloprid decreased CAT level, ASX treatment ameliorated the toxic effect by increasing CAT activity (Abou-Zeid et al. 2021). In diabetic rats, ASX significantly increased CAT level when compared to the negative control group (Toprak and Dedeoğlu 2022).

Malondialdehyde (MDA) is a biomarker of lipid peroxidation as it is one of the end products of peroxidation of poly unsaturated fatty acid arachidonic acid (PUFA AA) found in biological cell membranes (Tsikas 2017). MDA is highly reactive and is considered to be a toxic second messenger that spreads and amplifies oxidative damage (Mas-Bargues et al. 2021). Methotrexate toxicity provokes increased lipid peroxidation as demonstrated by increased level of MDA. This is shown in the current experiment where the serum level of MDA in the MTX induction group significantly rose in comparison to the negative control group. In agreement with that, rats induced with (20 mg/kg) MTX showed a significant accumulation of MDA in a study involving the effects of *Quillaja saponaria* bark saponin on hepatotoxicity (Abdel-Reheim et al. 2022). Another study about spirulina demonstrated increased level of MDA in the group given MTX (Khafaga and El-Sayed 2018).

Fortunately, astaxanthin pretreatment significantly decreased in a dose-dependent manner the level of serum MDA when compared to the induction group in the present study. This is in agreement with the animal study by L. Cui et al demonstrating the effects of ASX inhibiting the oxidative stress of esophageal cancer induced by N-nitrosomethylbenzylamine by means of decreasing serum MDA level (Cui et al. 2019). Moreover, in a model of sepsis in rats, ASX reduced
oxidative stress through decreasing MDA serum level (Zhou et al. 2015). Glutathione is a reducing tripeptide particularly concentrated in liver tissues (Lu 2020). It has many functions, including shielding proteins from oxidative stress by reversibly glutathionylating active thiols. Through the mechanism of redox cycling, which involves frequent reduction-oxidation (SH to SS) events at active site cysteine residues, GSH also keeps a number of antioxidant enzymes in their reduced state (Sreekumar, Ferrington, and Kannan 2021). Since GSH is a nucleophile, it reacts with electrophilic xenobiotics. They become consequently more soluble and can be excreted easily. Thus large amounts of GSH are required especially in DILI (Vairetti et al. 2021).

In the present experiment, MTX administration significantly lowered tissue GSH in the induction group when compared to the negative control group. An animal study of Petroselinum crispum hepatoprotective effects in methotrexate-induced liver injury by B. Ertas et al showed comparable results by MTX administration (Ertas et al. 2021). Additionally, MTX injection in a study in mice evaluating the hepatoprotective activity of vitamin C, significantly decreased the tissue level of GSH (Mohammed and Al-Gareeb 2021). Astaxanthin pretreatment significantly increased dose-dependently tissue level of GSH in comparison to the negative control group. This was in agreement with the study by X. Lin in mice exposed to aircraft noise. Pretreatment with ASX alleviated the cognitive deficits produced in these mice by increasing GSH thus decreasing the oxidative injury to the heart, gut and hippocampus (Lin et al. 2022). Liver oxidative stress in Wistar rats given formaldehyde for 14 days was likewise alleviated by treatment with ASX through increment of liver tissue GSH ( . et al. 2019).

Conclusion

This study concluded that pretreatment of ASX can alleviate methotrexate-induced hepatotoxicity by downregulation of oxidative stress in addition to lipid peroxidation through increasing liver tissue GSH, serum SOD and serum CAT, while increasing serum MDA levels.

References


