

## Astaxanthin effect on apoptotic biomarkers in methotrexate-induced liver injury

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### Abstract:

Methotrexate is used in the treatment of cancer, psoriasis, rheumatoid arthritis and several other disorders. It has a hepatotoxic potential side effect. Patients who have no access to alternative medications face a serious

challenge as a result. The current study aimed to assess the apoptotic potential of methotrexate on liver cells and evaluate the hepatoprotective activity of the potent antioxidant astaxanthin, by downregulation of apoptotic biomarkers caspase 9 and caspase 3.

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) on day 13, three groups pretreated with 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 increased liver tissue caspase 9 and caspase 3 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 decreased caspase 9 and caspase 3 that are involved in the apoptotic pathway, it could be used as pretreatment in patients treated with methotrexate to alleviate its hepatotoxicity.

**Key words:** Methotrexate, hepatotoxicity, astaxanthin, caspase 9 and caspase 3.

### تأثير الاستازانثين على موت الخلايا المبرمج في سمية الكبد المستحثة بالميثوتريكسيت

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### الخلاصة:

يستخدم الميثوتريكسيت في علاج السرطان والصدفية والتهاب المفاصل الروماتويدي والعديد من الاضطرابات الأخرى. بسبب تأثيره السام على الكبد، فإن استخدامه مقيد بشدة. نتيجة لذلك، يواجه المرضى الذين لا يستطيعون الوصول إلى الأدوية البديلة تحديات خطيرة. تهدف الدراسة الحالية إلى تقييم إمكانية موت الخلايا المبرمج للميثوتريكسيت في خلايا الكبد وتقييم النشاط الوقائي للكبد لمضاد الأكسدة القوي؛ الاستازانثين، عن طريق تقليل الكاسباس 9 والكاسباس 3. تم استخدام نموذج لسمية الكبد التي يسببها الميثوتريكسيت في ذكور الجرذان. تم تقسيم ستة وثلاثين جرذاً إلى ست مجموعات. مجموعة تحكم سلبية، مجموعة حث سمية الكبد بالميثوتريكسيت (20 مجم / كجم) في اليوم 13، ثلاث مجموعات تمت معالجتها مسبقاً باستخدام استازانثين بجرعات تصاعديّة (50 و 75 و 100 مجم / كجم) لمدة 14 يوماً قبل الميثوتريكسيت، ومجموعة العلاج التقليدية المعالجة مسبقاً باستخدام سيليمارين (200 مجم / كجم).. أدى استخدام الميثوتريكسيت إلى زيادة كبيرة في أنسجة الكبد بالكاسباس 9 وكاسباس 3 مقارنة بمجموعة التحكم السلبي. على الجانب الآخر، أدى استخدام الاستازانثين في الجرعات الثلاث إلى تحسين هذه المؤشرات الحيوية بشكل ملحوظ.

كشفت هذه الدراسة أنه نظرًا لأن أستازانتين قلل بشكل كبير من كاسبيس 9 وكاسباس 3 المتضمن في مسار موت الخلايا المبرمج ، فيمكن استخدامه كعلاج مسبق في المرضى الذين يعالجون بالميثوتريكسيت للتخفيف من سمية الكبد.

**الكلمات المفتاحية:** ميثوتريكسين، أستازانتين، تسمم الكبد، كاسبيس 9، كاسبيس 3.

## Introduction

Drug Induced liver injury (DILI) is a major clinical problem pertaining to the diagnosis and treatment. It can frequently lead to drug development failure and drug withdrawal(1). Drug withdrawal is financially burdening for the pharmaceutical industry(2). The liver is subject to great toxicity of xenobiotics due to its detoxifying function(3).

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(4). It acts mainly by inhibiting the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC). This enzyme catalyzes the last step in biosynthesis of the de novo purine biosynthesis pathway(5,6).

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 hepatocyte apoptosis. In hepatocytes, MTX upregulates caspase 9 and caspase 3 thus increases the apoptosis cascade resulting in cell death(7). Additionally, MTX alters the homeostasis of pro-apoptotic marker Bcl-2 (B-cell lymphoma 2) and the anti-apoptotic marker bax (Bcl2-Associated X Protein) in hepatocytes(8). MTX induces liver steatosis via disturbances in the mitochondrial respiratory chain and impairment in fatty acid oxidation. The mitochondrial membrane potential ( $\Delta\Psi_m$ ) can be altered by excessive production of ROS leading to release of cytochrome c from mitochondria to cytosol(9). This cytochrome c activates the initiator caspase, caspase 9 which is normally

found in phosphorylated inhibited form. Caspase 9 initiates apoptosis by activating the downstream executioner caspases 3 and 7(10). Destruction of liver cells by increased apoptosis releases excessive amounts of liver enzymes like AST (aspartate aminotransferase) and ALT (alanine aminotransferase) into the bloodstream which can be measured as indicators of liver injury(11). Also, depletion of GSH (glutathione) and increment of MDA (malondialdehyde) is observed in liver tissue(12).

Astaxanthin(ASX), is chemically named 3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione(13). 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 forbidden to use in the human diet under FDA regulations. Astaxanthin produced from a microalga called *Haematococcus pluvialis*, gives the highest yield for human consumption(14–16).

## 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 apoptotic marker's caspase 9 and caspase 3 in rat liver tissue.

## 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*(17). 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* (18)) 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.

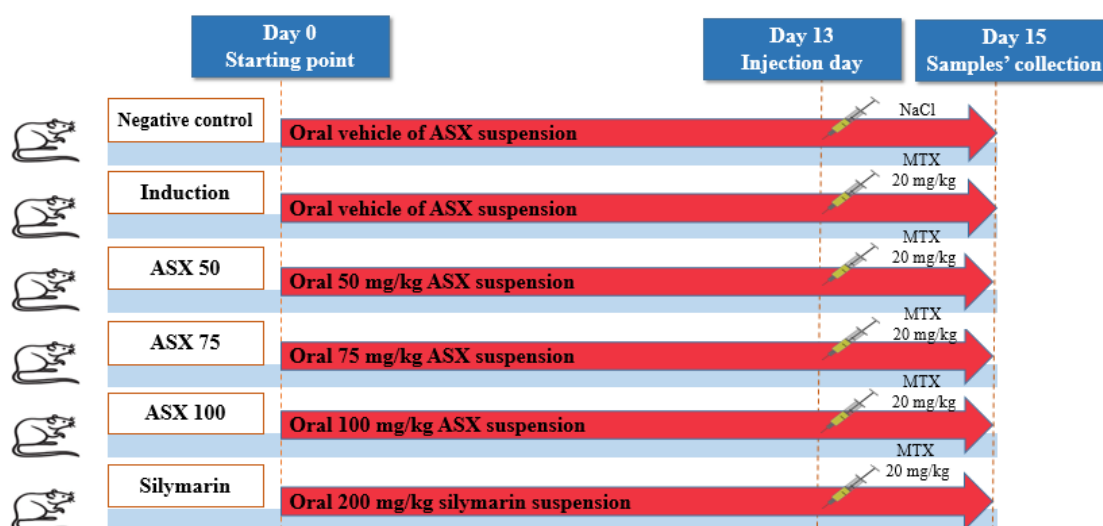


Figure (1): Study design.

On the 15<sup>th</sup> 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 Caspase 9 in liver tissue by ELISA technique**

A monoclonal antibody specific for caspase-9 had been pre-coated on the microtiter plate. Standards and homogenized tissue samples were added to the microtiter plate wells. Caspase-9 bound to the antibody pre-coated wells. A standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for caspase-9 was added to each well to “sandwich” the immobilized caspase-9 on the plate. The procedure was employed according to manufacturers’ instructions (kit catalogue number: MBS264306, company: Mybiosource). After microtiter plate incubation, the wells were thoroughly washed to remove all unbound components. Then, in each well, substrate solutions were added. Over a short incubation period, the enzyme (HRP) and substrate were allowed to react. The hue of the wells containing only caspase-9 and enzyme-conjugated antibody changed. The color shift was detected spectrophotometrically by the Humareader at a wavelength of 450 nm after the enzyme-substrate reaction was stopped by adding a sulphuric acid solution.

### **Determination of Caspase 3 in liver tissue by ELISA technique**

By the same technique and principle explained previously, caspase 3 was detected. The pre-coated antibody was an anti-Human Caspase-3 monoclonal antibody, while the detection antibody was a biotinylated polyclonal antibody.

According to manufacturers’ instructions (catalogue number: MBS018987, company: Mybiosource), the steps were as follows:

The plate had been pre-coated with antibodies. All antibodies that did not adhere to the plate were washed away. Irrelevant proteins were used to block the remaining antibodies on the plate. The sample containing the target analyte (caspase 3) was then added, resulting in immobilization of the target analyte by analyte-specific capture antibodies, resulting in the development of an antigen-antibody combination. All unbound particles and potential impurities were then removed by washing the wells. The wells were subsequently filled with a biotin-labeled antibody that was specific for the target analyte, resulting in an antibody-antigen-antibody combination. After that, the plate was washed to eliminate any unbound antibodies. Then, horseradish peroxidase and avidin was added to the wells and bound with the biotin-labeled antibodies. The amount of reporter enzyme in the sample was positively linked with the amount of target analyte. Thereafter, the wells were washed again. Finally, HRP reaction substrates were added. The enzyme-substrate reaction was stopped by addition of sulphuric acid solution and the ample concentrations were estimated based on color changes using the Humareader apparatus.

## **Results**

### **The effect of astaxanthin pretreatment on tissue apoptotic biomarkers (caspase 9 and caspase 3) levels in MTX-induced hepatotoxicity**

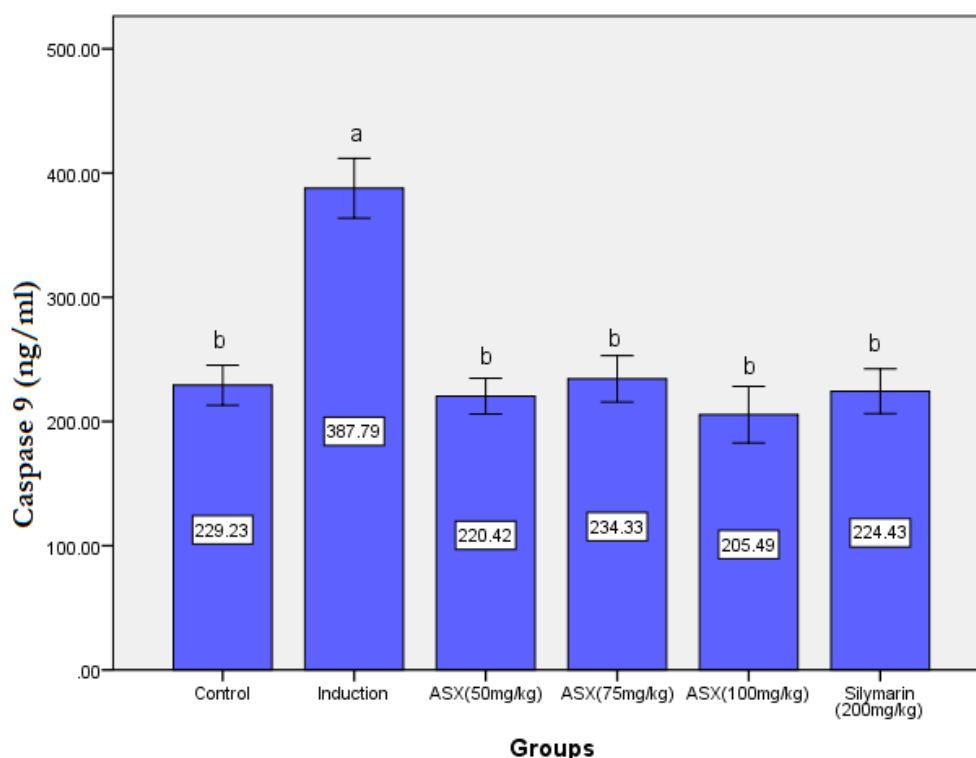
The following table shows the effect of astaxanthin on tissue caspase 9 and 3 level in methotrexate-induced-hepatotoxicity in male rats’ model.

**Table (1): The effect of ASX on apoptotic biomarkers in methotrexate-induced-hepatotoxicity in male rats' model.**

Group	Caspase 9 (ng/ml)	Caspase 3 (ng/ml)
Negative Control	229.23±16.12 <sup>b</sup>	11.46±1.61 <sup>b</sup>
Induction	387.79±24.14 <sup>a</sup>	34.20±3.01 <sup>a</sup>
ASX (50mg/kg)	220.42±14.43 <sup>b</sup>	11.38±1.66 <sup>b</sup>
ASX (75mg/kg)	234.33±18.66 <sup>b</sup>	11.46±1.61 <sup>b</sup>
ASX (100mg/kg)	205.49±22.78 <sup>b</sup>	10.17±1.20 <sup>b</sup>
Silymarin(200mg/kg)	224.43±18.01 <sup>b</sup>	10.80±0.73 <sup>b</sup>
LSD	55.84	4.84

The results were expressed as Mean ±SD. Results with unidentical superscripts (a, b) are significantly different ( $P<0.05$ ). LSD: least significant difference.

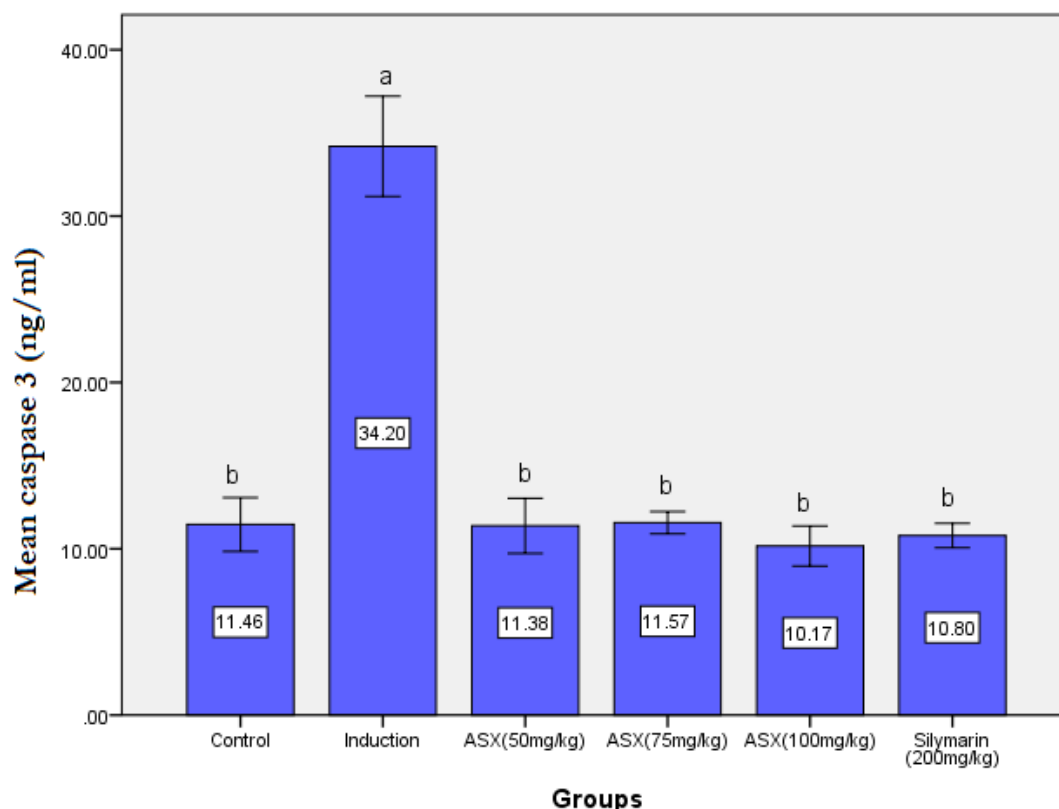
Tissue caspase 9 mean concentration significantly increased [ $p<0.05$ ] in the induction group (387.79±24.14) ng/ml in comparison to the negative control group (229.23±16.12) ng/ml. When treated with ASX at the dose 50mg/kg, the tissue means concentration of caspase 9 significantly decreased [ $p<0.05$ ] to (220.42±14.43) ng/ml compared to the induction group. Moreover, there was a significant decline in tissue caspase 9 mean concentration [ $p<0.05$ ] in the ASX-treated group with the dose 75mg/kg (234.33±18.66) ng/ml and 100mg/kg (205.49±22.78) ng/ml as well as the silymarin treated group (224.43±18.01) ng/ml when compared to the induction group. The groups treated with all three doses of ASX and silymarin demonstrated no significant difference [ $p>0.05$ ] to the negative control group; figure (2).



**Figure (2): The effect of ASX on tissue caspase 9 levels. The results were expressed as Mean±standard deviation. Results with unidentical small letters (a, b) are significantly different ( $P<0.05$ ).**

Tissue caspase 3 mean concentration significantly increased [ $p < 0.05$ ] in the induction group ( $34.20 \pm 3.01$ ) ng/ml in comparison to the negative control group ( $11.46 \pm 1.61$ ) ng/ml. When treated with ASX at the dose 50mg/kg, the tissue means concentration of caspase 3 significantly decreased [ $p < 0.05$ ] to ( $11.38 \pm 1.66$ ) ng/ml compared to the induction group. Moreover, there was a significant decline

in tissue caspase 3 mean concentration [ $p < 0.05$ ] in the ASX-treated group with the dose 75mg/kg ( $11.46 \pm 1.61$ ) ng/ml and 100mg/kg ( $10.17 \pm 1.20$ ) ng/ml as well as the silymarin treated group ( $10.80 \pm 0.73$ ) ng/ml when compared to the induction group. The groups treated with all three doses of ASX and silymarin demonstrated no significant difference [ $p > 0.05$ ] to the negative control group; figure (3).



**Figure (3): The effect of ASX on tissue caspase 3 levels. The results were expressed as Mean $\pm$ standard deviation. Results with unidentical small letters (a, b) are significantly different ( $P < 0.05$ ).**

## Discussion

Methotrexate is widely used in clinical practice. However, upregulation of tissue apoptosis and the compromised antioxidant defense system among other things are major reasons for the side effects of MTX(19). Alleviating medical side effects via natural products with strong antioxidative and anti-apoptotic abilities has become a research hotspot(20). To

conduct this study, astaxanthin was used to prevent liver injury caused by the treatment with methotrexate. Examination of the apoptotic pathway was employed. In this study, the group treated with methotrexate showed a significant increase in the level of both caspases (9 and 3) in tissues compared to the negatively control group. This is in agreement with N. Pinar *et al* who observed significant increases in



Bax and caspase-3 activity concomitant with decreased Bcl-2 activity the MTX group of rats (21). In a study by N. Mahmoud *et al* methotrexate induced apoptosis as observed by increased Bax and caspase 3 while declined Bcl 2(8). In rats treated with 20 mg/kg methotrexate, the activities of caspase 9 and caspase 3 were significantly higher in the MTX treated group compared to the negative control group, which is in agreement with the current study(22).

When pretreating with ASX in all three doses, the caspase 9 and caspase 3 level measured by ELISA were significantly decreased when compared to the induction group. This antiapoptotic potency of ASX was also explored by Li S. *et al* in mice subject to ischemia reperfusion injury where the protein level of caspase-9 and caspase-3 significantly decreased in the ASX treated group(23). S. Zhu *et al* found that astaxanthin decreased caspase 3 expression levels in LX-2 hepatic stellate cells (24). In ischemia reperfusion injury in rat ovaries, administration of ASX significantly decreased Caspase 3, IL-1  $\beta$ , and IL-6 expressions, and could be used as treatment for that injury(25).

## Conclusion

This study concluded that pretreatment of ASX can alleviate methotrexate-induced hepatotoxicity by downregulation of caspases 9 and 3 within the apoptotic pathway in liver tissues.

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