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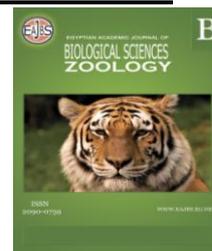


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The Effect of *Bombyx mori* Larvae Extract in Reducing the Toxicity of Methotrexate in the Fetus of Female Albino Rats

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ABSTRACT

Methotrexate (MTX) is a chemotherapeutic agent and immune system suppressant. It is used to treat cancer, autoimmune diseases, and ectopic pregnancy. Silkworm (*Bombyx mori*) larvae can be used as a bioreactor for the production of low-cost vaccines against different infectious diseases due to their huge value as a health food, especially for cardiac and diabetic patients.

In the present study, about 200 fetuses from female rats were examined. We divide pregnant female rats into five groups; group 1 used as negative control received distilled water, group 2 used as positive control and received buffer of *Bombyx mori* larvae extract, group 3 was treated with MTX at the 12th day of gestation (at organogenesis phase), group 4 was treated with *Bombyx mori* larvae extract at 7th, 9th, 11th, 13th and 15th days of gestation (during the organogenesis period), & group 5 was injected by MTX the 12th day of gestation as well as *bombyx mori* larvae extract at 7th, 9th, 11th, 13th and 15th days of gestation. Animals of all groups will be sacrificed on the 20th day of gestation periods. Then all fetus rats were removed for examination. Livers of all fetus rats were removed for histological and biochemical examination. The morphological examination of the fetuses showed that MTX causes growth retardation represented by a decrease in fetal body weight, body length and tail length. In addition, MTX induced an elevation in the examined liver oxidative stress biomarkers plus myeloperoxidase activity and a decrease in reduced glutathione content and catalase activity. Histopathological studies of fetal liver tissues showed congestion of central vein in MTX group and ballooning degeneration of hepatocytes, perivascular inflammatory cells infiltration and strong deposition of collagen fibers. Noteworthy, *Bombyx mori* larvae induced marked improvement in injuries associated with MTX administration.

INTRODUCTION

Chemotherapies are a type of anti-cancer drug treatment. They work by killing cancer cells by moving throughout your body and are called a systemic treatment. It is also given to cancers with an elevated risk of micrometastatic disease (Perry, 2008).

Methotrexate was made in 1947 and initially came into medical use to treat cancer, as it was less toxic than the then-current treatments (Sneider, 2005). It is used to treat cancer and autoimmune diseases such as breast cancer, leukemia, lung cancer, lymphoma, osteosarcoma, gestational choriocarcinoma, chorioadenoma destruens, and hydatidiform mole (Weinblatt, 2013). In addition, it's used to treat some types of autoimmune diseases such as psoriasis, rheumatoid arthritis, and Crohn's disease. On the other hand, as with other chemotherapeutic agents, MTX exerts prominent oxidant effects in the liver (Vardi *et al.*, 2010).

Bombyx mori larvae (*B.mori.L*) is native to Asia, spins a cocoon of fiber that is the source of commercial silk. It feeds on mulberry tree leaves with a cheap life cycle and with no ethical issues involved, so we chose it for this investigation (Hamamoto *et al.*, 2008). Natural silk has fibroin and sericin which are used in human body tissues such as skin, bone, nerve. Also, it has anti-cancer, anti-tyrosinase, anti-coagulant, anti-oxidant, anti-bacterial, and anti-diabetic properties (Khosropanah *et al.*, 2021).

Larvae are a source of numerous chemical constituents, such as adipokinetic hormone, insulin-like growth factor-II, chymotrypsin inhibitors, b-N-acetyl glucosaminidase, DOPA, quinone amine conversion factor and sex pheromone bombykol (Marumoto *et al.*, 1992, Ishibashi *et al.*, 1992, Shinohara *et al.*, 1993, Aso *et al.*, 1995 and Nagamatsu *et al.*, 1995).

Silkworm larvae create Interleukin-3 (IL-3) which is biologically identical to IL-3 produced from mammalian cells, so, it is used as a bioreactor for the production of low-cost vaccines against different infectious diseases (Datta, 1994).

Also, diabetic and cardiac patients use processed larvae in diets due to their low cholesterol content (De, 1991).

MATERIALS AND METHODS

The experiment was performed on a white albino rat as its gestation period is short (about 21 days) and has a large number of litters. Also, they characterized by their genetic stability and a very low rate of spontaneous malformation (Hai, 1989, Tuchmann, 1966 and Banerjee, 1973). We determine rat copulation and numbers of corpora lutea easily by the vaginal smear examination method (Edwards, 1968). The present experimental study complies with the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Females of 11-13 weeks old were selected for the present study and vaginal smears were prepared every morning. Then the vaginal smear was examined under the light microscope, for 5 days to select the female with regular estrus (Snell, 1956). Two females with regular estrus cycles were selected in the pro-estrus stage and caged together with one male overnight under controlled environmental conditions of temperature, humidity and light. Every morning, the vaginal smear is used to determine the first day of gestation by the presence of sperms in the smear (McClain & Becker, 1975).

Experimental Design:

Mating occurred under the normal conditions, about 40 pregnant female rats were divided into five groups (n= 6- 8, each) as follow:

- Group 1: Normal control group and was received distilled water.
- Group 2: Buffer control group and was received buffer of *Bombyx mori* larvae extract (aqueous phosphate buffer at pH= 7 composed of Na₂HPO₄, KH₂PO₄, NaCl, KCl).
- Group3: Methotrexate (MTX) (14mg/Kg, once orally) was administered at the 12th of gestation (at organogenesis phase) (Coleshowers *et al.*, 2010).

-Group4: *Bombyx mori* larvae (*B.mori.L*) extract (45 mg/kg, intramuscular) was given at 7th, 9th, 11th, 13th and 15th days of gestation (during the organogenesis period) (Cho *et al.*, 1998).

-Group5: Methotrexate (MTX) (14mg/Kg, once orally at the 12th day) and *Bombyx mori* larvae extract (45 mg/kg, intramuscular at 7th, 9th, 11th, 13th and 15th days of gestation.

-Animals of all groups will be sacrificed at the end of gestation periods at the 20th day of gestation. Fetuses of all pregnant rats will be removed at 10% neutral formalin buffer for at least one week to be used in our study.

Preparation of *Bombyx mori* (*B.mori.L*) Liposome:

Bombyx mori larvae in the fifth age were collected, homogenized, filtered to obtain the homogenate. Then, the homogenate is lyophilized and obtained a crude dry extract of the larvae. Every 2 grams of larvae give 100 mg powder. Liposomal preparation occurred, with slight modification, by mixing of 50 mg of dry extract, 150 mg cholesterol (Shaanxi Sangherb Bio-Tec, china), 500 mg phosphatidyl choline (Shaanxi M.R Natural Product Co., Ltd., China) in 100 ml chloroform until the mixture is completely dissolved. The mixture then was put in a rotary evaporator (lab first scientific, China) of 40 rpm. After complete evaporation, the aqueous phosphate buffer pH 7 composed of Na₂HPO₄, KH₂PO₄, NaCl, KCl was added to obtain a total volume of 10 milliliters (Nounou *et al.*, 2005).

1-Signs of Toxicity:

Growth retardation: the following parameters were recorded after the fixation at 10% neutral formalin buffer for at least one week to be used in our study:

- a) Fetuses body weight
- b) Body length
- c) Tail length

2-Biochemical Parameters:

a-Determination of Hepatic Inflammatory Marker; Myeloperoxidase (MPO) Activity:

MPO activity was done using a kinetic colorimetric method described by Bradley *et al.* (Bradley *et al.*, 1982).

b-Determination of Hepatic Oxidative Stress Biomarkers; Contents of Malondialdehyde (MDA) and Reduced Glutathione (GSH), and Catalase (CAT) Activity, As Well As Nitrite/Nitrate Index (NOx):

Liver contents of MDA and GSH, as well as CAT were performed according to reagent kits (Biodiagnostic Company, Giza, Egypt). Additionally, vanadium trichloride was used to reduce nitrate to nitrite according to (Miranda *et al.*, 2001) in nitric oxide assay. The method of nitrite estimation is based on Griess reaction that was performed using the kit provided by Biodiagnostic.

3-Histological Examinations; Stain With H&E And Masson's Trichrome:

At the 20th day of gestation, one lobe of half numbers of the liver of fetus rats of all groups was fixed in 10% neutral formalin buffer for at least one week followed by washing with tap water. Then samples were processed using a graded ethanol series and embedded in paraffin. Paraffin sections were cut into 6µm-thick slices and stained with hematoxylin and eosin for light microscopic examination. Additionally, other liver sections were stained with Masson's trichrome stain.

4-Statistical Analysis:

Results were expressed as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer Multiple Comparison Test. Probability values of less than 0.05 were considered statistically significant. The graphs were drawn using a prism computer program (GraphPad software

Inc. V5, San Diego, CA) (Armitage & Berry, 1987).

RESULTS

A-Growth Retardation:

The morphological examination of the fetuses showed that MTX causes growth retardation represented by a decrease in fetal body weight, body length and tail length (Figs. 1, 2&3)

1-The Bodyweight of Fetuses on the 20th Day of Gestation:

The present results showed that the bodyweight of the embryo decreased significantly in MTX treated group by 13% as compared to the control saline group, while it increased significantly in *B.mori. L*-treated group by 19% as compared to MTX treated group.

2-The Total Body Length of Fetuses at 20thday of Gestation:

The total body length decreased significantly in the case of the group which received *B.mori.L*+MTX by 18% as compared to the control saline group, and by 17% compared to the buffer group.

3-The Tail Length of Fetuses at the 20th Day of Gestation:

Fetal tail length decreased significantly in the group which received MTX by 29% as compared to the control saline group, and by 26% as compared to the buffer group. Whereas, the tail length increased significantly in the group that received *B.mori.L* and the group that received *B.mori.L*+MTX by 18% and 31% respectively as compared to the group which received MTX only.

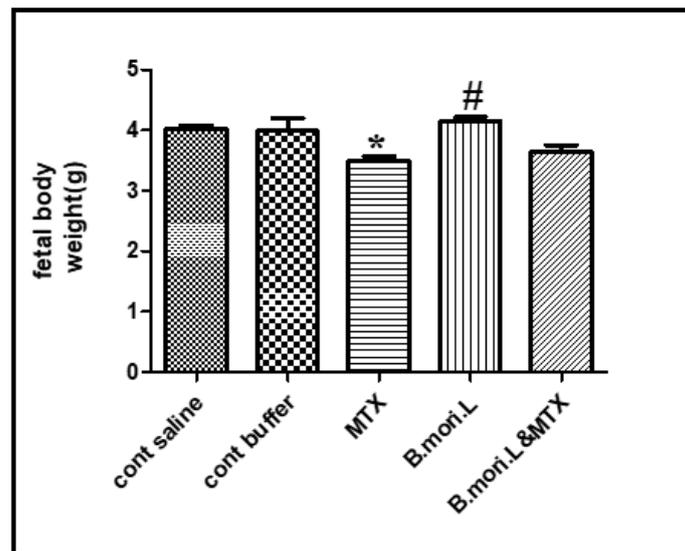


Fig. 1: Effect of MTX or/and *B.mori.L* on body weight of fetuses maternally received MTX or/and *B.mori.L*.

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05

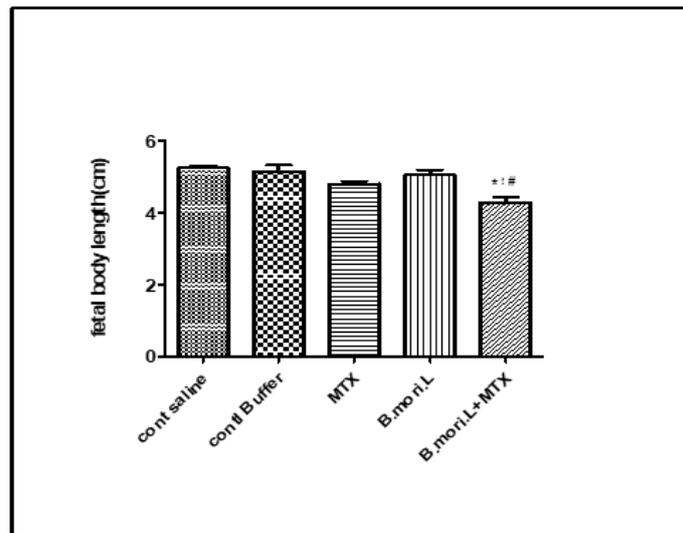


Fig. 2: Effects of MTX or/and *B.mori.L* on body length of fetuses maternally received the treatment

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L*= *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation.

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05.

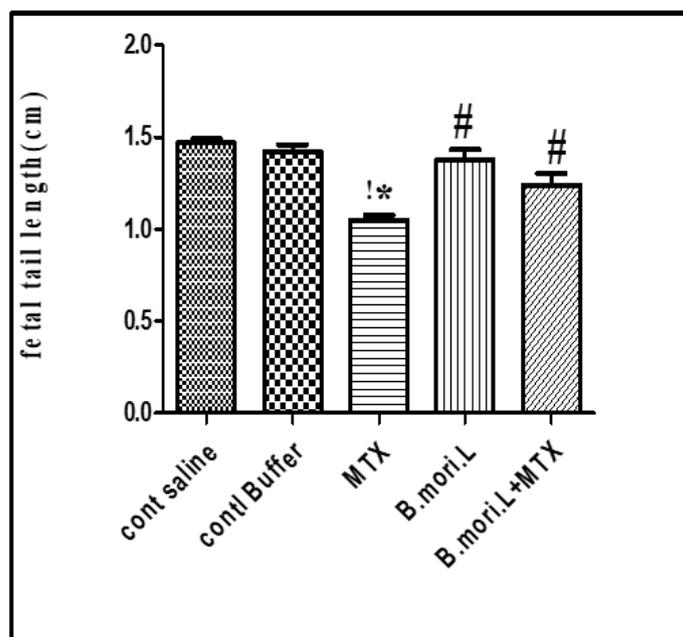


Fig. 3: Effects of MTX or/and *B.mori.L* on the tail length of fetuses maternally received the treatment

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L*= *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation.

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05

Morphological Malformations:

- Control Fetuses:

The body of the fetus on the 20th day of gestation appeared straight on both back and neck regions and the head was also noticeably straight dorsally, thus appearing smaller in size in relation to the body length.

In the head region, the eyes were closed with upper and lower eyelids, and the external auditory meatus was completely invisible, being covered with the fully developed ear pinnae. The fore and hind limbs are formed of well-developed bones and their extremities had well-developed digits with well-demarcated phalanges and claws.

The abdominal region of those fetuses acquired a cylindrical shape ending with the tail. The skin is obviously thickened.

-Effects of MTX or/and *B.mori.L* on External Anomalies at 20th Day of Gestation:

The anomalies of fetuses are represented in (Figs. 4,5,6&7). Examination of fetuses maternally received saline showed hematoma and paralysis rate by 16% and anomalies in the tail by 5%. Mothers received buffer, fetuses showed hematoma rate by 6%, paralysis by 11% and anomalies in the tail by 3%. While mothers were treated with MTX, their fetuses marked hematoma by 44% plus paralysis by 31%, odema by 9%, contraction by 13% and bending in the tail by 38%. Fetuses maternally treated with *B.mori.L* extract showed hematoma by 25%, paralysis by 13% and anomalies in the tail by 2%. On the other hand *B.mori.L*+MTX, this group showed hematoma and paralysis by (19%).

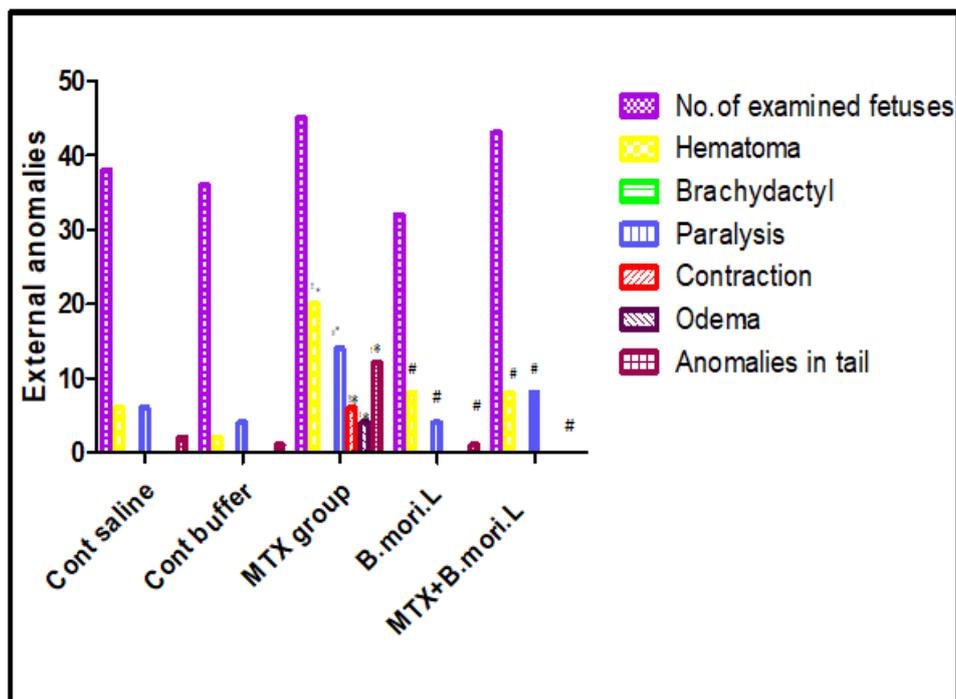


Fig. 4: Effects of MTX or/and *B.mori.L* on external anomalies in the fetuses at 20th day of gestation:

Histogram showing the incidence of external anomalies where (Cont. saline) = control treated with saline. (Cont buffer) = control treated with buffer. MTX = treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation. *B.mori.L* +MTX = bombyx mori larva extract and methotrexate.

* Significantly different from normal control saline group at $p < 0.05$.

! significantly different from normal control buffer group at $p < 0.05$.

significantly different from MTX group at $p < 0.05$.

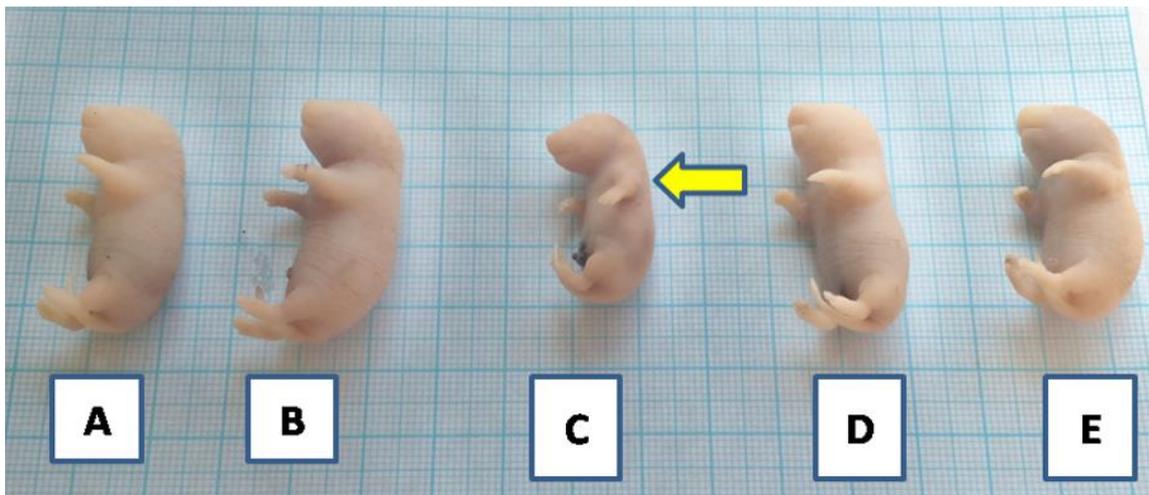


Fig. 5: photomicrograph Showed fetal morphological structures of 20th days of gestation. , where, (A) Fetus of Cont. saline=control treated with saline showed normal morphological structure, (B) Fetus of Cont buffer= control treated with buffer showed normal morphological structure. (C) Fetus of MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation, where yellow arrow indicates to fore limb paralysis. (D) Fetus of *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation showed normal morphological structure. (E) Fetus of *B.mori.L*+MTX = *bombyx mori* larva extract and methotrexate.

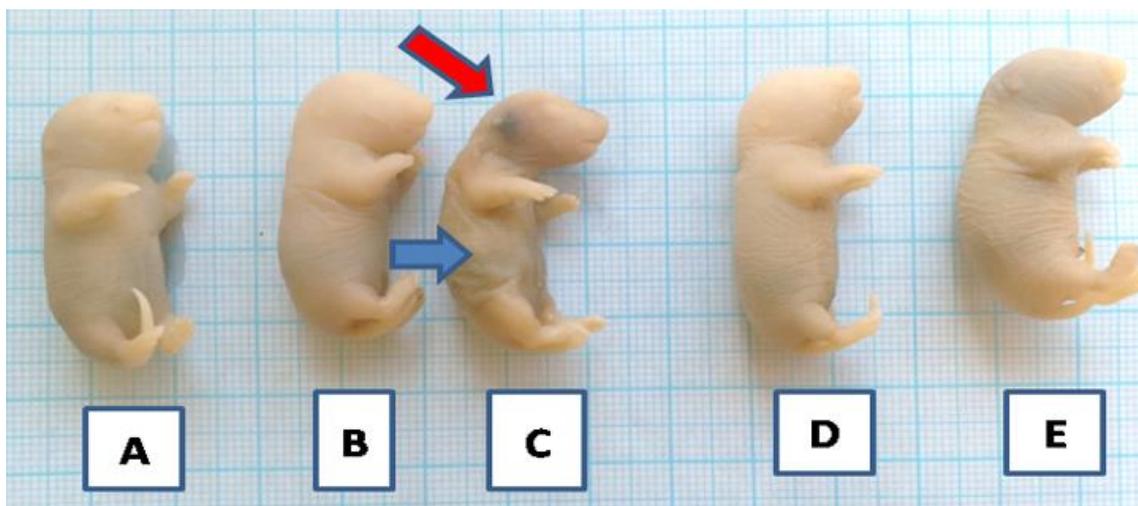


Fig. 6: photomicrograph Showed fetal morphological structures of 20th days of gestation. , where, (A) Fetus of Cont. saline=control treated with saline showed normal morphological structure, (B) Fetus of Cont buffer= control treated with buffer showed normal morphological structure. (C) Fetus of MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation, where red arrow indicate to heamatoma and blue arrow refers to contraction in the skin. (D) Fetus of *B.mori.L*= *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation showed normal morphological structure. (E) Fetus of *B.mori.L*+MTX = *bombyx mori* larva extract and methotrexate at 7th, 9th, 11th, 12th, 13th, 14th, 15th, 16th days of gestation showed normal morphological structure.

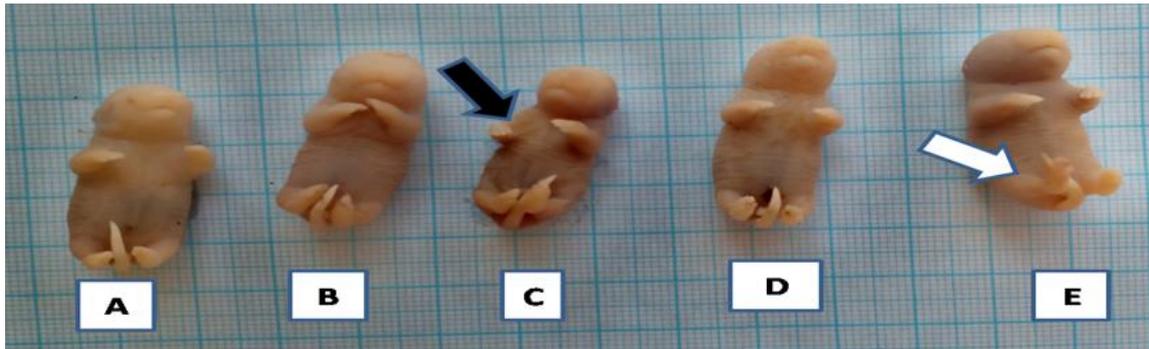


Fig. 7: photomicrograph Showed fetal morphological structures of 20th days of gestation. , where, (A) Fetus of Cont. saline=control treated with saline showed normal morphological structure, (B) Fetus of Cont buffer= control treated with buffer showed normal morphological structure. (C) Fetus of MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation, where black arrow refers to paralysis in fore limb. (D) Fetus of *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation showed normal morphological structure. (E)

Fetus of *B.mori.L* +MTX = *bombyx mori* larva extract and methotrexate at 7th, 9th, 11th, 12th, 13th, 14th, 15th, 16th days of gestation the white arrow showed bending in tail.

Skeletal Anomalies of Fetuses Maternally Treated with Saline or Buffer Extract (Fig. 8 A&B):

The fetuses of the group maternally received saline, group (A), and group (B) received buffer extract showing normal and complete skeletal system formation.

-Skeletal Anomalies of Fetuses Maternally Treated with MTX, *B.mori.L*, MTX+*B.mori.L* (Fig. 8 C, D &E):

The fetuses of group (C), figure (8) maternally treated with MTX showed a lack of ossification of skull and metatarsal bones (line segment), concave in the sacral and caudal vertebrae. The fetuses of group (D), figure (8), treated with *B.mori.L* at 7th, 9th, 11th, 13th, 15th days of gestation had normal and complete skeletal system formation. In addition, the fetuses of group (E), figure (8), treated with *B.mori.L* +MTX showed ossification of the skull with mild bending in the caudal vertebrae only, as well as mild ossification for the metatarsal bones were found.

Histopathological Studies on The Liver:

All groups of fetuses were stained with hematoxylin and esion stain (H&E) method and Masson's trichrome method.

1-Hematoxylin and Eosin Stain (H&E) Method:

The fetal liver maternally received saline and buffer showed the normal histological structure of hepatic parenchyma. While in MTX treated group which appears as ballooning degeneration of hepatocytes (small arrow) and perivascular inflammatory cells infiltration (long arrow). Noteworthy, both groups of *B.mori.L* extract, either alone or co-administered with MTX, showed slight activation of Kupffer cells figure (9).

2-Masson's Trichrome Method:

The fetal liver of groups maternally received saline and control buffer showed normal weak histochemical reaction for collagen fibers in the normal control group and buffer control group. Whereas, in MTX treated group showed a strong positive histochemical reaction for collagen fibers. On the other hand, both groups of *B.mori.L* extract, either alone or co-administered with MTX, showed weak histochemical reaction for collagen fibers figure (10).



Fig. 8: Photographs of skeleton of fetuses on the 20th day of gestation maternally received: (A) saline and (B) Buffer, showing normal and complete skeletal system formation. (C) MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation showed, lack of ossification of skull (arrow) and metatarsal bones(line segment), concave in the sacral and caudal vertebrae (head arrow). (D) *B.mori.L*= *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation showed normal and complete skeletal system formation was occurred. (E) *B.mori.L*+MTX= *bombyx mori* larva extract and methotrexate showed ossification of skull with mild bending in the caudal vertebrae only (red arrow). Also, mild of ossification for the metatarsal bones were found.

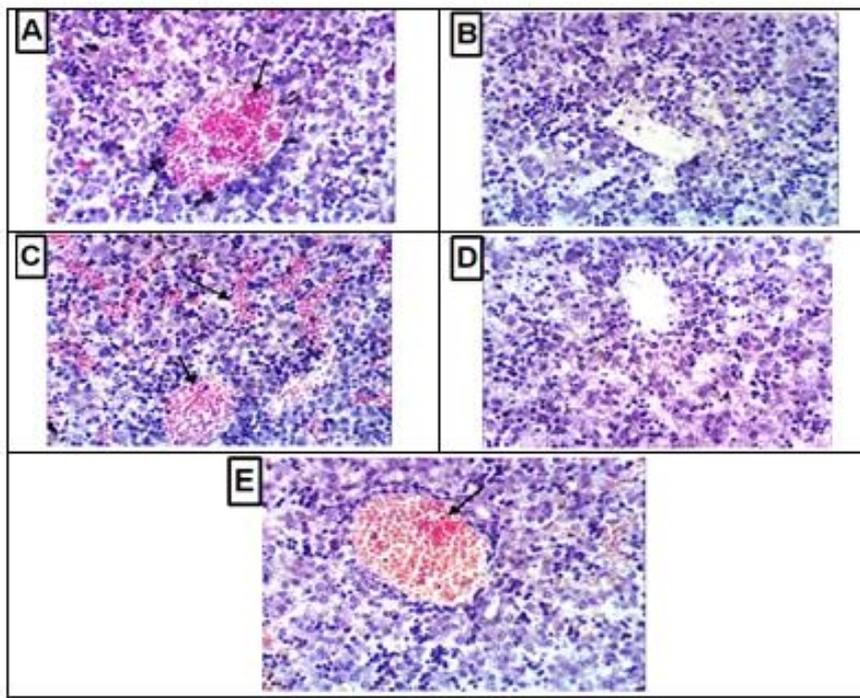


Fig.9: photomicrograph showed histological section of liver of fetuses rats stained by (H&E) method, where. (A) Cont. saline=control treated with saline, (B) Cont buffer= control treated with buffer. (C) MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. (D) *B.mori.L*= *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation. (E) *B.mori.L*+MTX= *bombyx mori* larva extract and methotrexate

All liver sections of control saline & *B.mori.L*+MTX treated group (A, E) showed congestion of central vein (H & E X 400). Liver sections of MTX treated group (C) showed congestion of central vein and hepatic sinusoids (H & E X 400).

Liver sections of control buffer group & *B.mori.L* group (B,D) showed no histopathological changes (H & E X 400).

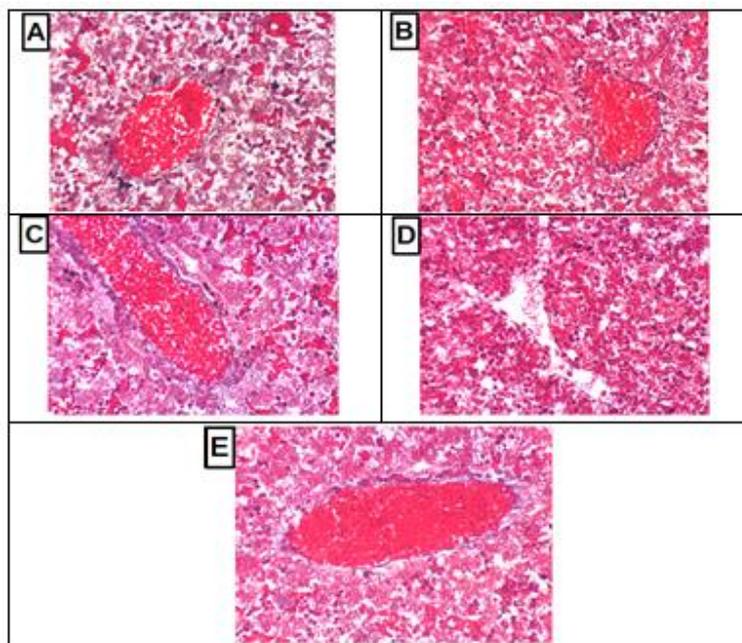


Fig. 10: photomicrograph showed histological sections of liver of fetuses rats stained by Masson's Trichrome method, where. (A) Cont. saline=control treated with saline, (B) Cont buffer= control treated with buffer. (C) MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. (D) B.mori.L= bombyx mori larva extract at 7th, 9th, 11th, 13th, 15th days of gestation. (E) B.mori.L+MTX = bombyx mori larva extract and methotrexate.

All liver sections of control saline & control buffer groups (A, B) showed normal weak histochemical reaction for collagen fibers in the portal triad (Masson's Trichrome stain X 400).

Liver section of MTX-treated group (C) showed strong positive histochemical reaction for collagen fibers (Masson's Trichrome stain X 400).

Liver sections of *B.mori.L* & *B.mori.L*+MTX-treated groups (D, E) showed weak positive histochemical reaction for collagen fibers (Masson's Trichrome stain X 400).

Liver section (D, E) showed Kupffer cells activation (arrow) (H & E X 400).

Biochemical Studies in Liver:

1. Effects of MTX or/and *B.mori.L* on Myeloperoxidase (MPO) Activity in Liver of Fetuses:

Administration of MTX caused elevation in MPO activity significantly by 220% and 250% respectively as compared to the control saline and control buffer groups; respectively. On the other hand *B.mori.L* group decreased MPO activity significantly by 71% and 70% respectively as compared MTX group in *B.mori.L* group and *B.mori.L*+MTX group; respectively (Fig. 11).

2.Effects of MTX or/and *B.mori.L* on Reduced Glutathione (GSH) Content in Liver of Fetuses:

This study revealed that GSH content was obviously declined in MTX group by 35% and 33% respectively as compared to control saline and control buffer groups; respectively; In contrast, *B.mori.L* group increased GSH content significantly by 19%, 22% and 83% respectively as compared to control saline, control buffer and MTX groups; respectively. Also, GSH content increase significantly in *B.mori.L*+MTX group as compared to control saline, control buffer and MTX groups by 15%,18% and75% ;respectively (Fig.12).

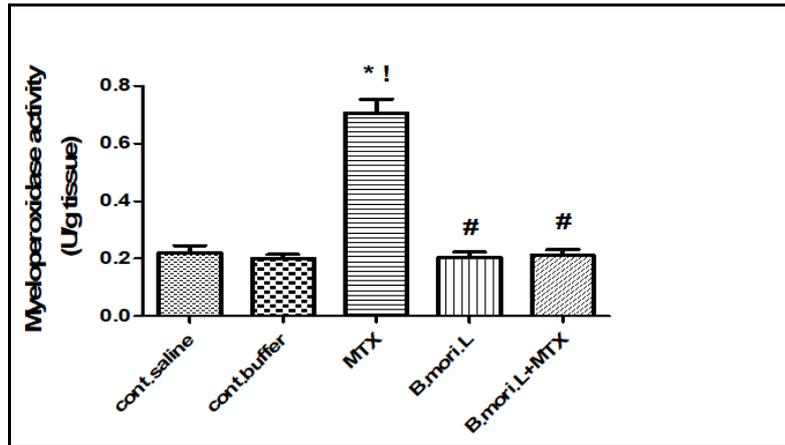


Fig. 11: Effects of MTX or/and *B.mori.L* on MPO activity in liver of fetuses.

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation.

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05

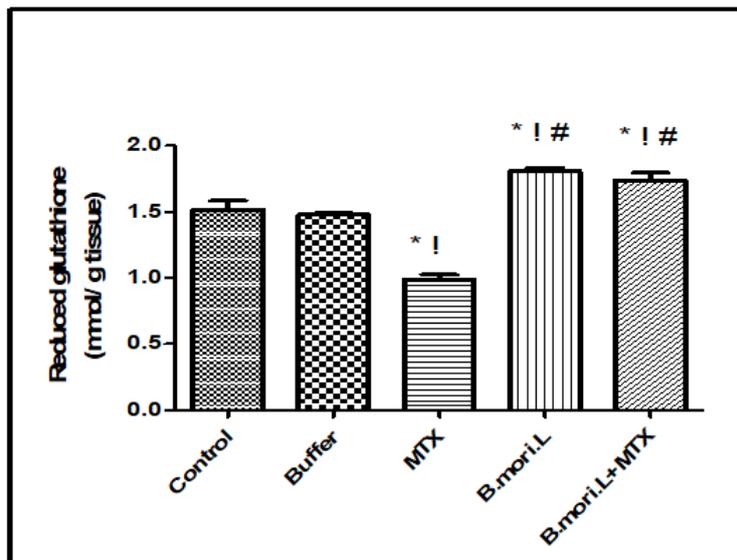


Fig. 12: Effects of MTX or/and *B.mori.L* on liver activity of GSH in fetuses.

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation. *B.mori.L* +MTX = *bombyx mori* larva extract and methotrexate.

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05

3.Effects of MTX or/and *B.mori.L* on Catalase Activity in Liver of Fetuses:

The existing study disclosed that catalase activity was clearly decreased significantly in MTX group by 40% and 44% respectively as compared to normal control and buffer control groups; respectively. In addition, there was a significant increase in catalases activity in *B.mori.L* extract by 24% and 106% respectively as compared to control saline and MTX groups; respectively. In addition, catalase activity increase

significantly in *B.mori.L*+MTX group by 82% as compared to MTX group (Fig. 13).

4.Effects of MTX or/and *B.mori.L* on Nitrite/Nitrate (NO_x) Content in Liver of Fetuses:

In the present study, NO_x content was apparently increased in MTX and control buffer groups by 59% and 20% as compared to the control saline group; respectively.

In addition, increase in NO_x content in MTX group by 33% as compared to the buffer group. In contrast, *bombyx mori* larvae extract decreased NO_x contents in *B.mori.L* and *B.mori.L*+MTX groups significantly by 31% and 29% respectively as compared to MTX group; respectively (Fig. 14).

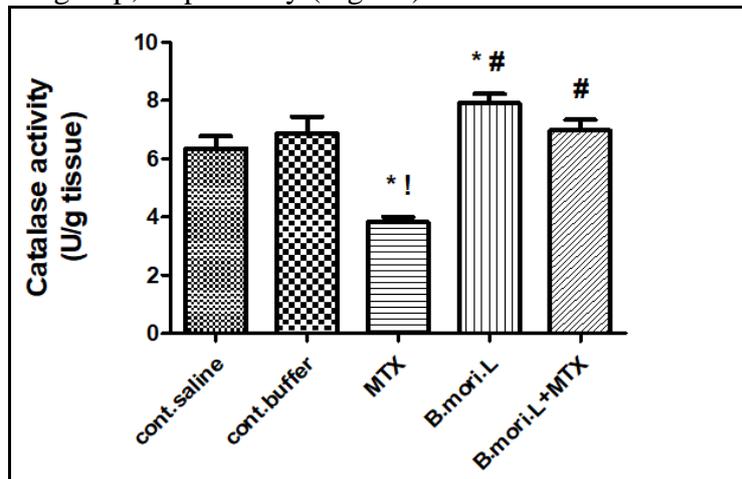


Fig. 13: Effects of MTX or/and *B.mori.L* on catalase activity in liver of fetuses.

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation.

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05

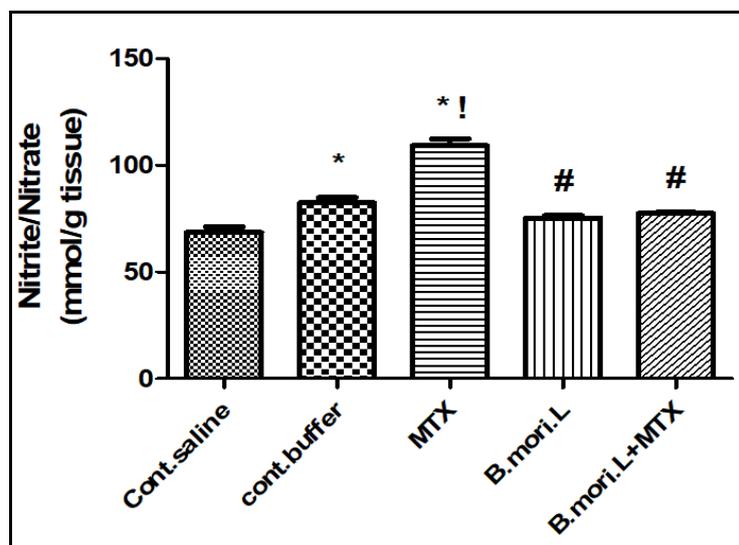


Fig. 14: Effects of MTX or/and *B.mori.L* on NO_x content in liver of fetuses.

Each value indicates the mean±SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer= control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L* = *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation.

* Significantly different from normal control saline group at p<0.05

! significantly different from normal control buffer group at p<0.05

significantly different from MTX group at p<0.05

5. Effects of MTX or/and *B.mori.L* Malondialdehyde (MDA) Content in Liver of Fetuses:

In the present study MDA contents were markedly elevated in MTX, *B.mori.L* and *B.mori.L*+MTX groups by 68%, 28% and 24%; respectively; as compared to the control saline group. In addition, MDA content increase significantly in MTX group by 50% as compared to the control buffer group. In contrast, MDA content decreased significant in *B.mori.L* and *B.mori.L*+MTX groups by 24% and 26% as compared to MTX group; respectively (Fig. 15).

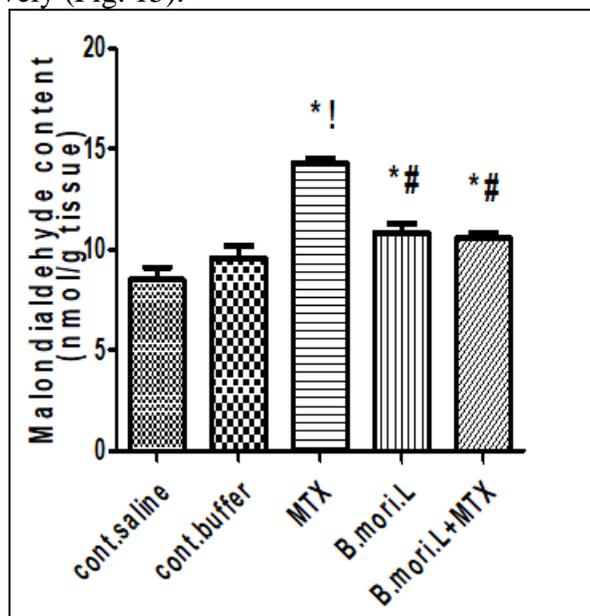


Fig. 15: Effects of MTX or/and *B.mori.L* on MDA content in liver of fetuses.

Each value indicates the mean \pm SEM of 6 animals statistical analysis was carried out by one way ANOVA followed by Tukey Multiple Comparison Test. Cont. saline=control treated with saline. Cont buffer=control treated with buffer. MTX=treated with methotrexate (14mg/Kg) at 12th, 14th and 16th days of gestation. *B.mori.L*= *bombyx mori* larva extract at 7th, 9th, 11th, 13th, 15th days of gestation. *B.mori.L* +MTX = *bombyx mori* larva extract and methotrexate.

* Significantly different from normal control saline group at $p < 0.05$

! significantly different from normal control buffer group at $p < 0.05$

significantly different from MTX group at $p < 0.05$

DISCUSSION

Our investigation is the first one to study the advanced effect of *Bombyx mori* larvae (*B.mori.L*) extract in reducing the toxicity of methotrexate (MTX) on albino rats' fetuses.

Regarding the fetal body length, in the case of MTX our results showed a significant reduction in the tail length and this is accepted with (Nguyen *et al.*, 2002) For the skeletal system, some skeletal anomalies were present in fetuses maternally treated with MTX as, lack of ossification of bones skull and metatarsal bones and concave in the vertebral column (sacral and caudal vertebrae). According to (Addar & M. H, 2004) a patient who was taking oral methotrexate for psoriasis gave birth to a fetus with craniofacial, skeletal, cardiovascular and gastrointestinal anomalies. In this case there was only a short, low-dose exposure to MTX in the first trimester (Usta *et al.*, 2007). Another case report cited a human woman who was given MTX at 5 weeks gestation for suspected ectopic pregnancy. In actuality, she had an intrauterine pregnancy, which survived and resulted in multiple skeletal anomalies and ambiguous genitalia (Xue-ping *et al.*, 1991)

A similar case was described in which MTX was given to a woman at 5 weeks gestation for suspected ectopic. Later in the pregnancy, an 8-week twin intrauterine pregnancy was diagnosed and spontaneously reduced to a singleton. The surviving singleton was born with a birth weight less than the fifth percentile and with hypertelorism, facial nerve palsy, scoliosis and cardiovascular abnormalities, among others (Singh *et al.*, 2002). On the other hand *B.mori.L* decoctions are used for the treatment of facial palsy and pain, primary trigeminal neuralgia, vocal nodules and vocal polyps (Sen, 1991).

In this study, the livers of fetuses in MTX group revealed some histopathological changes which appear as ballooning degeneration of hepatocytes and perivascular inflammatory cells infiltration demonstrated using H&E stain while livers in *B.mori.L*+MTX treated group showed improvement in histopathological alterations. The nuclear changes may indicate a decrease in cellular activity (De-zi, 1991). Liver in *B.mori.L*+MTX treated group showed Kupffer cells activation, liver injury and the release of inflammatory mediators primarily from Kupffer cells, neutrophils and monocytes are recruited to the liver and subsequently amplify the inflammation response by secreting more inflammatory mediators (Cetinkaya *et al.*, 2006). Kupffer cells are the resident macrophages in the liver (Liaskou *et al.*, 2012). In response to injury, Kupffer cells release inflammatory signals including cytokines, chemokines, growth factors, and reactive oxygen species (Mandal *et al.*, 2011). Depending in part on the type of mediators released, this response may result in progression or attenuation of liver damage. In addition, these extracellular signals may participate in the regulation of hepatobiliary transporters (Fukui *et al.*, 1991).

Also, the current study revealed a strong deposition of collagen fibers in livers that were treated with MTX. According to (Campion *et al.*, 2009), MTX-induced proliferation of myofibroblasts, in an injured liver, progressed to the deposition of collagen. On the other hand, *B. mori. L*+MTX group showed a weak reaction of collagen fibers. This result signifies the antifibrotic action of *B.mori.L*. The decrease of glycogen in the hepatocytes as detected with Periodic acid–Schiff is a staining method used to detect polysaccharides such as glycogen, and many substances such as glycoproteins, glycolipids and mucins in tissues, the reaction may give an idea about the disturbance in the process of glycogenolysis in these cells. The enzymes involved in this process may be affected by MTX (Ohbayashi *et al.*, 2010). This observation is confirmed by (Kremer *et al.*, 1995) who reported that there was a decrease in glycogen particles in hepatocyte treated by MTX. Here, we found an increase in collagenous fibers around the central vein, portal tract and between hepatocytes was clearly observed in MTX group as demonstrated by using Masson's trichrome stain. This observation confirmed the studies which had been reported concerning the hepatic fibrosis (Kim & Seo, 2001)

In fact, MTX in this work induced oxidative stress revealed by a significant reduction in liver GSH but it induced significant elevations in the content of MDA and NO_x, as well as MPO activity as compared to control animals. These achievements were in agreement with previous reports (Hemeida *et al.*, 2008) and these may be due to that MTX can inhibit some antioxidant enzymes which sequentially may cause lipid peroxidation to enlarge due to diminution in the activities of protective antioxidant enzymes such as catalase (Jahovic *et al.*, 2003). Moreover, MTX inhibits cytosolic NADP-dependent dehydrogenase and NADP malic enzyme and leads to a decrease in intracellular NADPH levels (Jahovic *et al.*, 2003). In fact, NADPH is crucial for glutathione reductase enzyme that maintains the levels of GSH, which is an important cytosolic antioxidant substance (Valderrama *et al.*, 2007). Meanwhile, the reduction in GSH content may be due to the reduction of glutathione reductase activity (Coleshowers

et al., 2010).

Really, we found that *B. mori*. *L* administration caused elevation in GSH content and a reduction in the contents of MDA and NO_x, along with MPO activity. Moreover, larvae improved the activity of protective antioxidant enzymes, catalase. According to (Hu *et al.*, 2018), the effects of larvae may be due to that larva possess potential radical scavenging properties. These are in agreement with the previous findings (Zhao *et al.*, 2014).

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