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# Evaluation of the influences of sesame oil and flaxseed oil on mice exposed to Methotrexate via isozyme electrophoresis and Semi-Quantitative RT-PCR

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**Abstract**

**The genetic alterations due to the Methotrexate (MTX) influences in numerous biological systems including mammals are still unclear. In the present study, some Isozyme systems (Esterase, Malate dehydrogenase, Lactate dehydrogenase and Superoxide dismutase) and Semi-quantitative RT-PCR techniques were used for evaluating the influences of sesame oil and flaxseed oil on mice exposed to Methotrexate. The density and the numbers of some isozyme bands in some mice body organs were alerted under the experimental conditions. The highest relative Sod expression value was induced by sesame oil. Both Isozyme and Semi-quantitative RT-PCR techniques were informative in exploring gene expression in certain mice body organs. The developed molecular and biochemical markers could be informative in understanding the physiological consequences of gene expression analysis under certain experimental conditions. Further investigations are required to explore the true influences of sesame and flaxseed oil against the toxicity induced by various toxic factors at molecular and/or biochemical levels.**

**Keywords: Sesame, Flaxseed, Gene expression, Methotrexate**

## INTRODUCTION

Infectious Methotrexate (MTX) is generally used to treat malignancies and autoimmune disorders. The molecular genetic alterations due to methotrexate (MTX) influence in numerous biological systems, including mammals, are still unclear. Methotrexate causes hepatotoxicity due to its effect on the intestinal mucosa. So, the intestinal barrier functions could be disrupted, permitting bacteria to translocate to the liver and cause hepatotoxicity [1, 2, 3]. The detection of the effects of methotrexate was carried out via various laboratory techniques, including histological, biochemical, cytogenetic, and molecular approaches. The MTX side effects on patients, including skin pain, intestinal mucous membrane damage, eye burning, fever, folate metabolism inhibition, intestinal toxicity, and allergic reactions, were discussed in many investigations [4, 5, 6]. Flaxseed plant seed (*Linium usitatissimum*) oil is widely used as an edible oil in many countries. Various uses and nutritional values of this oil have been discussed in many investigations [1] [7][8]. In addition, flaxseed oil was considered a renal protective factor in rat males due to its antioxidant effects (prevention of oxidative stress).


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Concerning sesame oil, the antioxidant and anti-inflammatory properties and the effectiveness of this oil in reducing atherosclerosis and the risk of cardiovascular disease were confirmed [9]. The rats fed sesame seed extracts detected a significant decrease in plasma triglycerides, cholesterol, and very low-density lipoprotein. Also, the levels of free cholesterol and cholesterol ester could be altered by decreasing acyl-CoA cholesterol acyltransferase activity due to sesame oil effects [10]. We can conclude that sesame oil supplementation has anti-inflammatory, antioxidant, and anti-atherogenic effects [1][9][11]. The sensitivity of semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR) in quantitatively assessing the expressions of various genes was confirmed [12–15]. Also, this technique is specific and informative for investigating the differential regulation of certain genes [16].

This investigation aimed to assess the influences of sesame and flaxseed oil on male mice exposed to Methotrexate via isozyme electrophoresis and Semi-Quantitative RT-PCR techniques.

## MATERIALS AND METHODS

Mice male individuals (25 ± 5g) were housed for 7 days under standard conditions (25˚C and a 12/12 h light/dark cycle). The study was conducted following the ethical guidelines of Animal Care at KAU, KSA.
The sesame oil and Flaxseed oil were purchased from Sigma–Aldrich Corp., St. Louis, MO, USA.
The mice individuals (n=30) were divided into 6 groups (five individuals per group (From G1 to G6) as follows:
Group 1(control group = C): mice individuals (n=5) were intraperitoneally injected (IP) with NaCl solution (0.9%).
Group 2 (MTX group = T1): mice individuals (n=5) were IP by a single dose of MTX (20 mg $kg^{-1}$ body weight).
Groups 3 (MTX +Sesame oil = T2): mice individuals (n=5) were administered oral gavage daily with sesame oil (600 mg/kg) before the MTX dose.
Group 4 (MTX + Flaxseed oil = T3); mice individuals (n=5) were administered oral gavage daily with Flaxseed oil (2 g/kg body weight) before the MTX dose.
Group 5 (Sesame oil = T4): mice individuals (n=5) were administered oral gavage daily with sesame oil (600 mg/kg body weight).
Group 6 (Flaxseed oil = T5) mice individuals were administered oral gavage daily with Flaxseed oil (2 g/kg body weight).
On day 40, the blood samples were withdrawn from each mice individual. Also, the mice individuals were killed, and the liver, kidney and spleen organs were excised, weighed, frozen [4] and kept at -80°C.

**Isozyme extraction**
Isozyme samples were extracted from three mice body organs (liver, kidney and spleen). Also, the serum samples were prepared for separation via polyacrylamide gel electrophoresis as described by Mansour *et al.* [4].

**Isozyme separation**
Esterase (a-naphthylacetate), Malate dehydrogenase (Mdh), Lactate dehydrogenase (Ldh) and Superoxide dismutase (SOD) isozyme systems were applied to differentiate biochemical differences among the control and treated samples.
Electrophoretic conditions, gel preparation, staining and distaining were carried out according to Mansour *et al*. [4], Pasteur *et al*. [17], and Elsebaie and saad [18**]**.

**Semi-quantitative RT-PCR**
The RNA samples were extracted from the liver tissue (100 mg) using TRIzol as Rio et al. described [19] . The samples were quantitatively measured as described by Romero *et al.* [20].
A semi-quantitative RT-PCR method was applied to evaluate Sod gene expression under the experimental conditions. According to Meadus [21] (2003), the cDNA synthesis was carried out.
As a control, B -actin gene fragments were amplified .The B Actine primer pairs were as follows:B-Actin_F: GGCACCACACCTTCTACAATG and B-ctin_R: GGGGTGTTGAAGGTCTCAAAC. The PCR reaction and condition were carried out according to Wei *et al.* [22].
Regarding the SOD gene fragments, the primer pairs were Sod_F: TTTTTGCGCGGTCCTTTCCTG and Sod_R: GGTTCACCGCTTGCCTTCTGCT. The PCR reaction and condition were carried out according to Singh *et al*. [23].

**Data analysis**
Concerning the B-Actin and SOD gene fragments, the bands were analyzed using the Gel Analyzer 19.1 software (WWW.gelanalyzer.com).

## RESULTS

**Isozyme polymorphism**
The electrophoretic patterns of the isozymes (Figures 1,3,5 and 7) in the estimated samples (serum, liver, kidney and spleen) shown notable bands. The total number of calculated bands and isozyme resolutions were evaluated (Table 1). The averages of the band frequencies (ABF) were calculated and presented in Table (2).

**Esterase**

The highest ABF value (0.98) was detected in the serum esterase pattern (14 bands). On the other hand, the lowest ABF (0.8) value was detected in the spleen esterase pattern (Figure 1). Only one serum esterase band (relative front equal 0.05) was absent in the control sample.

Regarding the liver esterases, out of 20 detected bands, four bands (at relative fronts 0.425, 0.45, 0.8, and 0.988) were absent from the C sample (control). Only one esterase band (relative act equal to 0.1) was absent in three samples (T3, T4, and T5). A total of 20 esterase bands were identified in the kidney tissue (Figure 1) (relative fronts ranged from 0.05 to 0.738). One band was a control-specific esterase band (relative front = 0.188). On the other hand, one band at the relative front (0.588) was absent in the control sample. Also, two bands (at relative fronts 0.688 and 0.713) were absent from four samples (C, T1, T2, and T3). The results showed that the band at the relative front of 0.738 was absent in the T5 (flaxseed oil) sample. Concerning the spleen tissue, the characteristic bands ranged from 8 to 9. Two bands (at relative fronts of 0.3 and 0.525) were absent from the control sample (C). Also, band number 6 (Rf = 0.438) was a control-specific band. The relative fronts and distribution of the band frequencies of each evaluated esterase isozyme in each evaluated tissue are presented in Figure 2.

**Table (1)**: The resolutions of isozymes in the serum, liver, kidney and spleen of mice

| **Tissue** | | **Serum** | | **Liver** | | **Kidney** | | **Spleen** | |
|---|---|---|---|---|---|---|---|---|---|
| **System** | **En** | **Res.** | **NDB** | **Res.** | **NDB** | **Res.** | **NDB** | **Res.** | **NDB** |
| Est | 3.1.1.1 | +3 | 14 | +3 | 20 | +3 | 20 | +2 | 10 |
| Mdh | 1.1.1.37 | +2 | 15 | +2 | 9 | +2 | 3 | +2 | 1 |
| Ldh | 1.1.1.27 | +3 | 10 | +3 | 3 | +3 | 5 | +3 | 6 |
| Sod | 1.15.1.1 | +3 | 8 | +2 | 3 | +2 | 3 | +2 | 4 |

Res. = resolution, Est = Esterase, Sod= Superoxide dismutase, Mdh= Malate dehydrogenase, Ldh= Lactate dehydrogenase, En= Enzyme number, +3= strong, +2=Moderate reaction and NBD= Number of detected bands.

**Table (2)**: Number of detected isozyme bands in each treatment for each tissue of mice

| | | **C** | **T1** | **T2** | **T3** | **T4** | **T5** | **ABF** | **SD** |
|---|---|---|---|---|---|---|---|---|---|
| Serum | Est. | 13 | 14 | 14 | 14 | 14 | 14 | 0.98 | 0.04 |
| | Mdh | 7 | 6 | 6 | 7 | 7 | 9 | 0.46 | 0.34 |
| | Ldh | 9 | 9 | 9 | 9 | 9 | 9 | 0.75 | 0.26 |
| | Sod | 7 | 5 | 6 | 6 | 6 | 6 | 0.67 | 0.37 |
| Liver | Est. | 16 | 20 | 20 | 19 | 19 | 19 | 0.94 | 0.12 |
| | Mdh | 8 | 8 | 8 | 8 | 8 | 8 | 0.89 | 0.28 |
| | Ldh | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 0 |
| | Sod | 2 | 3 | 3 | 3 | 2 | 2 | 0.83 | 0.29 |
| Kidney | Est. | 17 | 17 | 16 | 16 | 18 | 17 | 0.84 | 0.29 |
| | Mdh | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 0 |
| | Ldh | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 0 |
| | Sod | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 |
| Spleen | Est. | 8 | 9 | 7 | 8 | 8 | 8 | 0.8 | 0.34 |
| | Mdh | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| | Ldh | 6 | 6 | 6 | 4 | 6 | 6 | 0.94 | 0.09 |
| | Sod | 2 | 2 | 1 | 1 | 1 | 3 | 0.42 | 0.4 |

Est.= Esterase, Sod= Superoxide dismutase, Mdh= Malate dehydrogenase, Ldh= Lactate dehydrogenase, C= control, T1 = MTX group , T2 = MTX +Sesame oil, T3 = MTX + Flaxseed oil, T4 = Sesame oil, T5 = Flaxseed oil and ABF = average of band frequencies SD= Standard deviation.

**Malate dehydrogenase**

15 serum Malate dehydrogenase bands (Figure 3) were identified (the relative fronts ranged from 0.068 to 0.919). Only one serum Mdh band (relative front equal 0.117) was (C) sample specific band. Regarding the liver Malate dehydrogenase (9 bands), the relative fronts ranged from 0.133 to 0.881). The control sample (C) has a specific marker (band number 4) at the relative front equal 0.376. No variations were identified in both Kidney (3 detected bands) and spleen (one detected band) Mdh banding patterns (Figure 3). The Relative fronts and distribution of the band frequencies of each evaluated Mdh isozyme in each estimated tissue are presented in Figure (4).

**Lactate dehydrogenase**

Lactate dehydrogenase banding patterns are presented in Figure (5). A total of 10 serum Lactate dehydrogenase bands were detected according to the Rf for each band. Only one serum Lactate dehydrogenase band (Relative front equal 0.13) was absent from the (C) control sample. A total of 3 liver Ldh bands were characterized. No polymorphism was observed in liver and Kidney (5 common Ldh bands) Ldh banding patterns. A total of two Ldh bands were absent from the sample T3 at the relative fronts 0.528 and 0.624 (Figure 5). The Relative fronts and distribution of the band frequencies of each evaluated Ldh isozymes in each investigated tissue was explored in Figure (6).

**Superoxide dismutase**

A total of 8 serum Superoxide dismutase bands were recognized (Figure 7). Some serum Superoxide dismutase bands were specific for the control (C) sample (Rf:0.154 and 0.383). Regarding the liver Superoxide dismutase banding pattern (Figure 7), the band (Rf = 0.43) is detected in only three samples T1, T2 and T3. No variations were detected in the Kidney Sod pattern (Figure 7). Concerning the spleen banding (4 bands) pattern, only one band (Rf = 0.94) was specific for the sample T5. The band at Rf equal 0.711 was detected in both C and T5. The Relative fronts and distribution of the band frequencies of each evaluated Sod isozyme in each estimated tissue were presented in Figure (8).

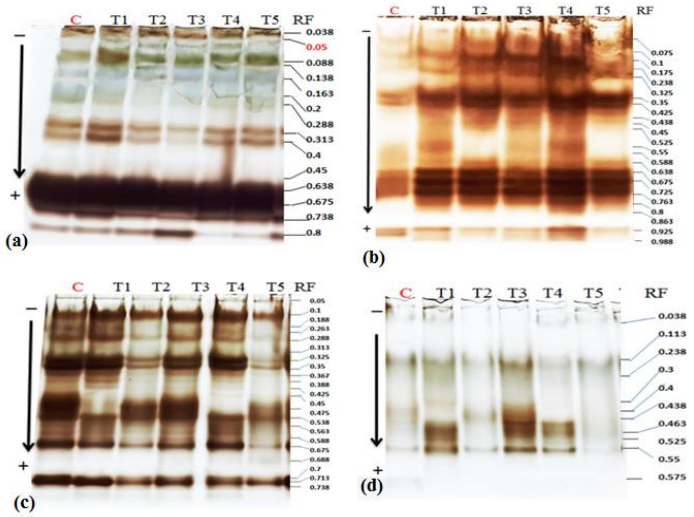


**Fig.1:** The Esterase banding patterns observed in different samples from serum (a), liver (b), kidney (c), and spleen (d) tissues. Serum (a), Liver (b), Kidney (c) and Spleen (d) tissues. C= Control, T1= MTX, T2= MTX + Sesame oil, T3 = MTX + Flaxseed oil, T4= Sesame oil and T5 = Flaxseed oil.

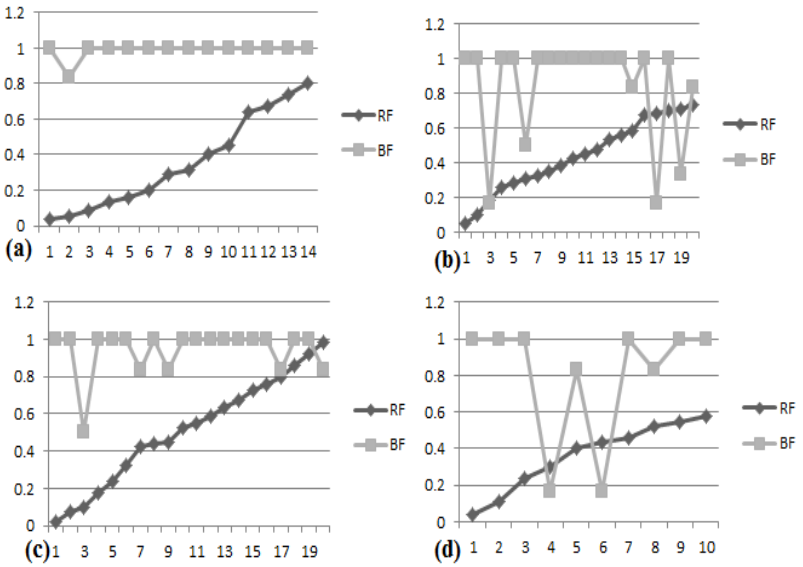


**Fig.2:** Distribution of band frequencies (BF) and Relative fronts (RF) for each evaluated esterase isozyme. Serum (a), Liver (b), Kidney (c) and Spleen (d) tissues.

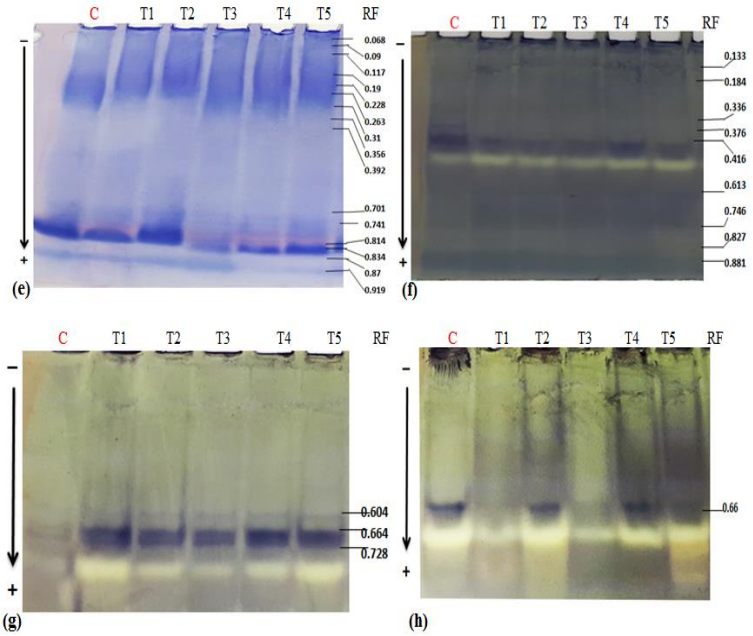


**Fig.3:** The Malate dehydrogenase banding patterns from different samples from Serum (e), Liver (f), Kidney (g) and Spleen (h) tissues. C= Control, T1= MTX, T2= MTX + Sesame oil, T3 = MTX + Flaxseed oil, T4= Sesame oil and T5 = Flaxseed oil.

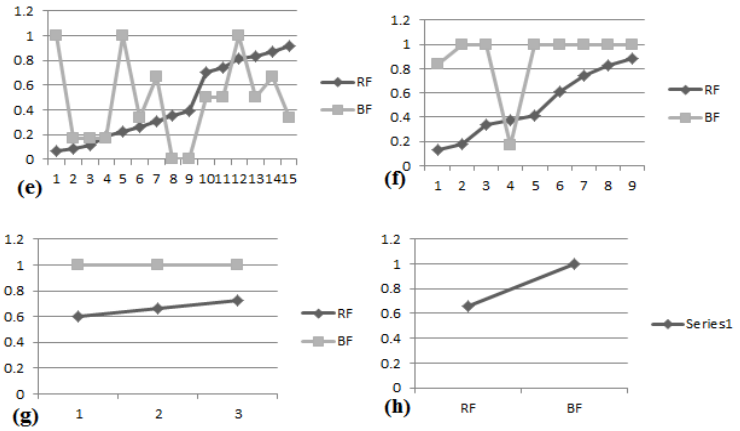


**Fig.4:** Distribution of Band frequencies (BF) and Relative fronts (RF) for each evaluated Malate dehydrogenase isozymes. Serum (e), Liver (f), Kidney (g) and Spleen (h) tissues.

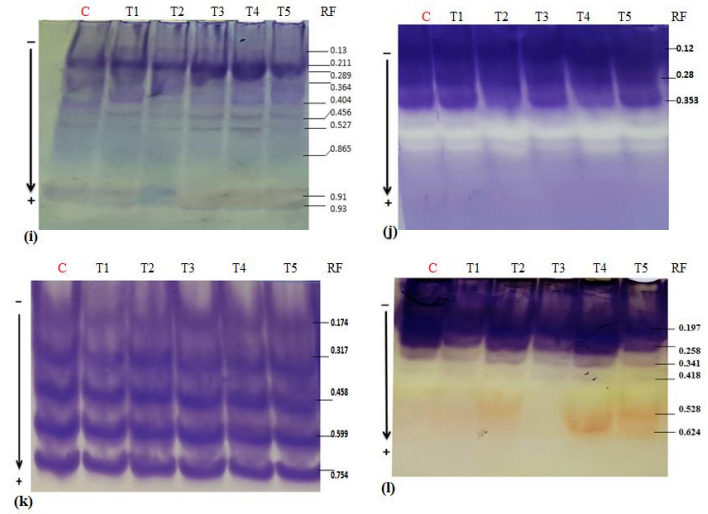


**Fig.5:** The Lactate dehydrogenase banding patterns from different samples from Serum (i), Liver (j), Kidney (k) and Spleen (l) tissues. C= Control, T1= MTX, T2= MTX + Sesame oil, T3 = MTX + Flaxseed oil, T4= Sesame oil and T5 = Flaxseed oil.

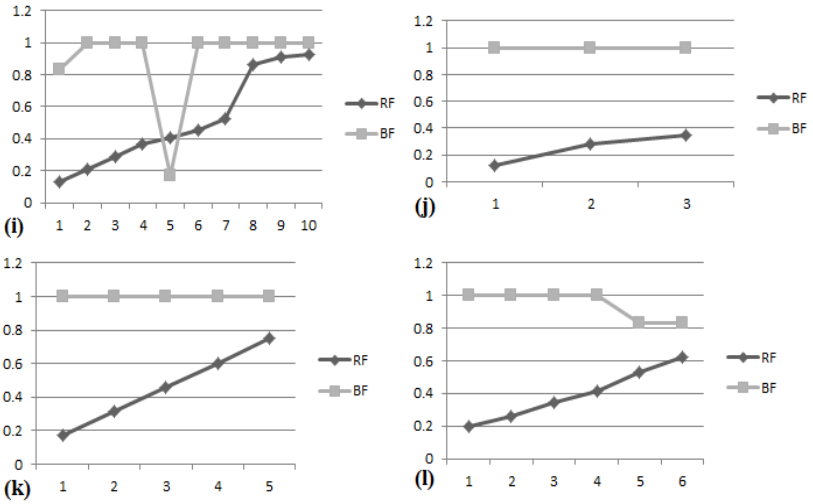


**Fig.6:** Distribution of Band frequencies (BF) and Relative fronts (RF) for each evaluated Lactate dehydrogenase isozymes. Serum (i), Liver (j), Kidney (k) and Spleen (l) tissues.

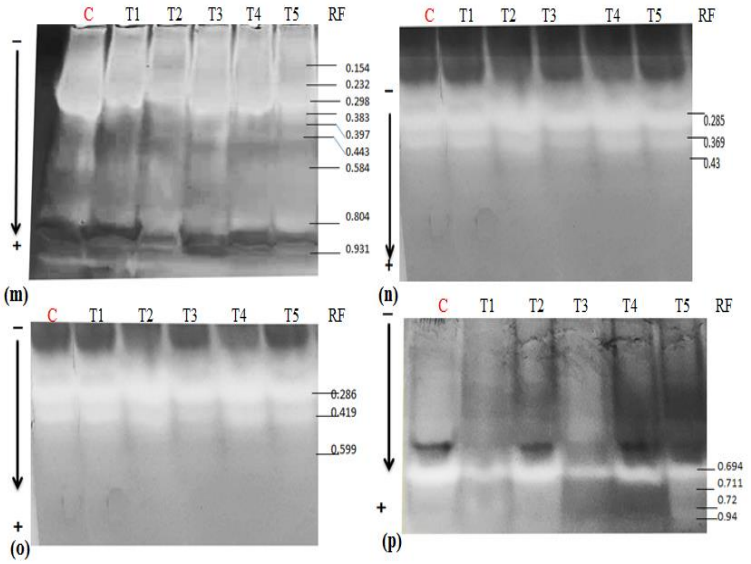


**Fig.7:** The Superoxide dismutase banding patterns from different samples. Serum (m), Liver (n), Kidney (o) and Spleen (p) tissues. C= Control, T1= MTX, T2= MTX + Sesame oil, T3 = MTX + Flaxseed oil, T4= Sesame oil and T5 = Flaxseed oil.

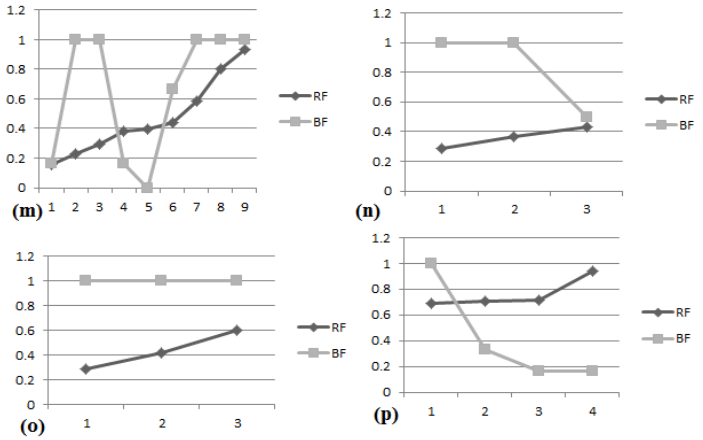


**Fig.8:** Distribution of Band frequencies (BF) and Relative fronts (RF) for each evaluated Superoxide dismutase isozymes. Serum (m), Liver (n), Kidney (o) and Spleen (p) tissues.

**Analysis of liver SOD gene expression**

A semiquantitative RT-PCR was applied to measure the Sod gene expression in the liver tissues to verify between the experimental and control individuals under the experimental conditions. Analysis of gene expression in the liver tissue exhibited that the expression levels of Sod were affected by the treatment types. The T4 (Sesame oil) calculated the highest relative expression value. On the other hand, the lowest value was detected in the T1 treatment (MTX). The results exhibited that the SOD expression levels under the treatment conditions were upregulated in the T2 (MTX+S), T3(MTX+F), T4(Sesame oil) and T5 (Flaxseed oil) compared with the control and MTX (T1) samples. The relative Sod expression under the T2 (MTX+ Sesame oil) is lower than T4 (Sesame oil) conditions. Also, the relative Sod expression under the T3 (MTX+ Flaxseed oil) is lower than the Sod expression under T5 (Flaxseed oil) conditions (Figure 9).

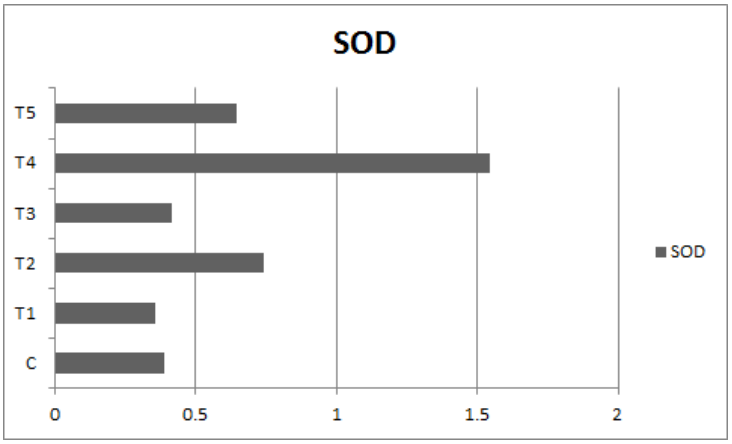


Fig. 9: Average relative gene expressions of liver SOD under different experimental conditions. The experimental conditions are as follows: C = Control samples, T1 = (MTX), T2 = (MTX+ sesame oil), T3 = (MTX+ flaxseed oil), T4 = (Sesame oil), and T5 = (Flaxseed oil).

## DISCUSSION

In the present study, some isozyme systems (Esterase, Malate dehydrogenase, Lactate dehydrogenase, and Superoxide dismutase) and semi-quantitative RT-PCR techniques were used to evaluate the influences of sesame oil and flaxseed oil on mice exposed to methotrexate. The density and numbers of some isozymes in some mice's body organs were detected under the experimental conditions. The influences of the MTX are related to concentration and exposure time. Al-Maruf et al. [24] confirmed that the rat liver exposed to the MTX (300 M) enhanced the mitochondrial injury. They found that MTX-induced cytotoxicity caused by ROS (reactive oxygen species) formation and GSH oxidation leads to oxidative stress in rat hepatocytes. The dose-dependent mode affected many enzymes, such as LDH, ALT, ALP, and AST enzyme activities and mRNA levels, under certain experimental conditions [25]. The cells were affected when exposed to a higher concentration of reactive oxygen species. Some isozymes could be informative markers for differential gene expression in numerous body organs in various organisms, including mice [4], fish [26], and snails [18]. The present study showed that some mouse body organ samples were affected by treatment types under experimental conditions. On the other hand, the total numbers of some isozyme bands (kidney Mdh, spleen Mdh, liver Ldh, and kidney Ldh) were not changed. These observations were confirmed in some other studies in various organisms [18, 26, 27], including mice [4]. In the present study, the samples were quantitatively evaluated to identify the usefulness of the RNA extraction method from the liver cells and the percent value of the mRNA to cDNA as recommended by Romero et al. [20]. The results showed that the expression levels of Sod were affected by the treatment types. The highest relative expression value was calculated in T4 (sesame oil). On the other hand, the lowest value was detected in the T1 treatment (MTX). Comparatively with the control, the downregulation of Sod expression in the T1 sample (MTX) may be due to enhancement of the reactive oxygen species [24, 28, 29]. The positive effects of the sesame and/or flaxseed oils were reflected by the UP-regulation of the liver Sod gene expression in T2 (MTX+ sesame) and T3 (MTX+ flaxseed). The antioxidant (prevention of oxidative stress), anti-inflammatory properties, and effectiveness of sesame oil in reducing atherosclerosis and the risk of cardiovascular disease were confirmed by Hsu and Parthasarathy [9]. Flaxseed oil was considered a renal protective factor in rat males [1] due to its antioxidant effects (prevention of oxidative stress). The results indicated the sensitivity of this technique in reflecting the Sod gene expression under the experimental conditions. The sensitivity of this technique to quantitatively assess the expressions of various genes was confirmed [12–16]. Also, this technique was successfully applied for detecting Sod, TGF-1, and DUSP1 expressions in normal and keratoconic cultured corneal stromal fibroblasts [30].

## CONCLUSION

Exposure to MTX can impact the density and quantity of specific isozyme bands in certain organs of mice. Both isozyme electrophoresis and semi-quantitative RT-PCR techniques proved valuable in investigating gene expression across different organs of mice under the experimental conditions. The developed molecular and biochemical markers could be informative in

understanding the physiological consequences of gene expression analysis. Further investigations are required to explore the true influences of sesame and flaxseed oil against the toxicity induced by various toxic factors at molecular and/or biochemical levels.

**Conflict of Interest**

The Authors declared no conflict of interest.

**Author contributions**

Bakhsh, Noor, Saad Yasser Mohamed and Rabah, Samar planned the work, explained the results, made the write and illustrations. Saeed, Mahmoud Ahmad statistically analyzed the data.

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