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ORIGINAL ARTICLES

Subchronic Feeding Study of Fenitrothion Residues in Maize and The Protective Action of Purslane (*Portulaca oleracea* L)] Extract on Rats

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ABSTRACT

After spraying of maize plant, during cultivation with ^{14}C-fenitrothion organophosphorus insecticide, the obtained seeds contain about 1.6 µg insecticide/g seeds. The effect of subchronic feeding ,for 45 days ,of these harvested seeds on rats and the role of natural purslane herb extract in protection against toxicity damage induced were studied. Analytical evaluations were performed by detecting erythrocyte and plasma cholinesterase activities, liver and kidney functions and lipid profile. The assessment of feeding rats with treated seeds after 45 days led to an inhibition in cholinesterase enzyme activity (ChE) over 40% in plasma and erythrocyte. The obtained results showed a significant elevation in the activity of liver enzyme 64% for alanine aminotransferase(ALT), 58% for aspartate aminotransferase(AST) and 35% for alkaline phosphatase(ALP), whereas a moderate decrease in levels of albumin and total protein was observed. A moderate increase in blood urea nitrogen and creatinine concentration was also observed. The detection levels of cholesterol and triglycerides showed a small increase (15%). On feeding rats with obtained maize seeds mixed with purslane herb (*Portulaca oleracea* L)] extract led to an increase in cholinesterase enzyme activity, albumin and total protein as well as a decrease in liver, kidney parameters and lipid profile. These data suggest that purslane herb extract have a beneficial effect on reducing the toxicological effects induced by fenitrothion insecticide residues and the protective individual for oxidative stress diseases.

***Key words*:** Fenitrothion, insecticide residues, toxicity protective effect, purslane.

Introduction

Humans are potentially exposed to pesticides either directly, as workers in green-houses and in agriculture, or indirectly, via food consumption. The toxicity of organophosphates has been studied extensively in short and long terms in animal tests over the past several decades (El-Shenawy, 2010)

Fenitrothion (O, O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate, is widely used for controlling a wide range of insects and pests. It is marketed under the different trade names Sumithion, Novathion and Metathion. It is a broad-spectrum organophosphorus insecticide, non-systemic and non-persistent (Briggs, 1992), and moderately toxic to mammals and has found extensive use as a residual spray in homes for malaria control and public health applications (Uygun, *et al*, 2005). It is employed in agriculture to control insects on rice, fruits, cereals, soybeans, coffee, wheat, barley, and sesame seeds (Saber, *et al*, 2005). It is also used as a grain protectant and it finds use in horticulture, forestry, domestic and public health applications (Tomlin, 1997).

Several reports showed that fenitrothion had a significant increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and serum level of total protein, creatinine and urea compared with of the control level of rat (Kadry,*et al,* 2001, Farghaly and El-Maghraby, 2008).

Maize (*Zea mays L*.) is an economic and popular plant in Egypt, which is grown in more than one season. It is a high source of carbohydrate and good quality oil for human food, livestock feed and raw material for industrial processing (Ramessar, *et al*, 2008). Recently, corn oil production increased markedly and is refined into high quality oil for the food industry such as sweetener and starch (Feng, *et al*, 2002).

Herbs are powerhouses of nutrition and if it is used wisely and regularly, it can replace costly pills and supplements, and even some drugs. Portulaca oleracea (Portulacaceae family) is listed in the World Health Organization as one of the most used medicinal plants and it has been given the term 'Global Panacea (Samy,*et al,* 2005). Although purslane is considered as a weed in Egypt and in the United States because of its growth patterns, it can be eaten as a leaf vegetable, with a slightly sour and salty taste in Europe, Asia and Mexico (Cros, 2007). Purslane is a rich source of omega-3 fatty acids (alpha-linolenic acid in particular), which are beneficial in congenital heart disease (CHD) and certain cancers. It has a beneficial effect on cholesterol and triglyceride levels, in heart disease, and in strengthening the immune system (Low, and Rodd, 1994). It also

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contains vitamins mainly vitamin A, vitamin C, and some vitamin B and carotenoids, as well as dietary minerals, such as magnesium, calcium, potassium and iron (Simopoulos, *et al*, 1992) . The plant possesses marked antioxidant activity (Shyu, *et al*, 2009). In Traditional Chinese Medicine, it is used to treat infections or bleeding of the genito-urinary tract as well as dysentery. The fresh herb may also be applied topically to relieve sores and insect or snake bites on the skin (Bensky, *et al*, 2004). Purslane has been reported to possess antifungal effects, with marked activity against the genus trichophyton (Ki-Bong, *et al*, 2000).

This work was performed to investigate the effect of subchronic feeding of rat with fenitrothion residues on maize seeds and to study the protective action of purslane extract on rats by assessing RBCs and plasma cholinesterase activities, liver and kidney functions and lipid profile.

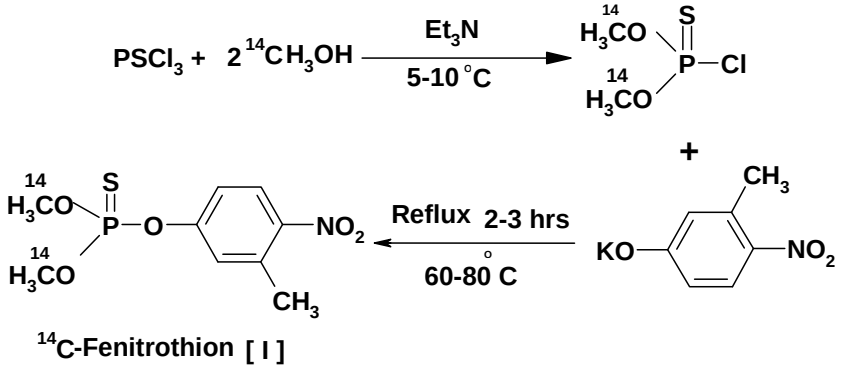
Materials and Method

Chemicals:

Synthesis of ^{14}C-fenitrothion [I]:

^{14}C-Fenitrothion-labelled at carbon atom of methyl groups-was synthesized by a single vesseled reaction in our laboratory (Scheme.1)according to Farghaly,*et al* (2007). This method involved the condensation of 0.007 mole of ^{14}C-methanol in toluene to 0.003 mole of thiophosphorylchloride in presence of triethylamine 0.006 mole . The mixture was stirred at 5-10˚C for 30 minutes and was filtered to remove the separated triethylamine hydrochloride and then the filtrate was evaporated under vacuum. The obtained O, O-dimethylthiophosphoryl chloride in toluene was allowed to react with 0.003 mole of potassium salt of 3-methyl-4-nitrophenol while stirring for 30 minutes at room temperature.This mixture was refluxed for 2-3 hours at 60-80 ˚C, cooled followed by addition of cold water and the organic layer was separated, dried over anhydrous sodium sulphate. The solvent was evaporated under reduced pressure to give yellow-brown liquid (b.p 140-145˚C, 0.1 mm) yield 70-75 % (Scheme 1). The product was purified by preparative thin layer chromatography (TLC) (Silica gel 60 F_{254} pre-coated plates 20×20 cm layer thickness 0.5 mm, Merck, Germany). The prepared ^{14}C-fenitrothion had a specific activity 2.96 MBq/mg and a radiometric purity 98 %.

Pure non-labelled fenitrothion and its main degradation products were synthesized in our laboratory for comparison purposes (Fig 1).



Scheme 1: Synthesis of ^{14}C-Fenitrothion

Synthesis of fenitrooxon (II):

Oxidation of fenitrothion by mixing pure fenitrothion solution (1 mM) in acetonitrile with 10 molar excess of bromine solution (10 mM) in acetonitrile followed by vortexing the mixture for a few seconds. The completion of the reaction was checked by silica gel TLC by using suitable mobile phases Hexane: Benzene (1:2 v/v). Isolation of the oxidised product fenitrooxon was achieved by preparative TLC (silica gel) using the same mobile phases as used in analytical TLC gave colorless liquid (b.p 146.5-148˚C, 0.7 mm) yield 85-90%, purity over 90 % (Young, *et al* , 2000).

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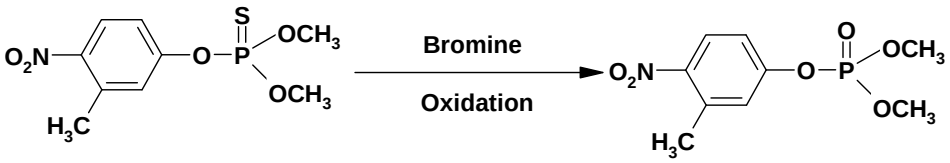
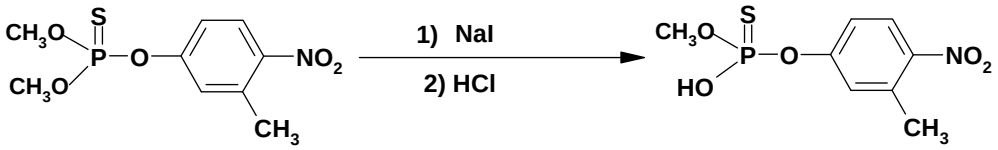


Fenitrothion **Fenitrooxon [II]**

Synthesis of desmethylfenitrothion (III):

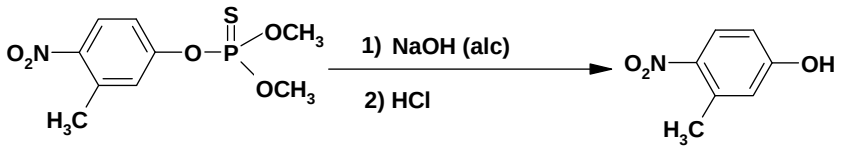
The sodium salt of desmethylfenitrothion was obtained by reacting equimolar amounts of fenitrothion (0.005mole) and sodium iodide (0.005mole) in acetone (25 ml) at room temperature for 6 hours. The solvent was evaporated to give crystals of sodium salt desmethylfenitrothion, recrystillized from acetone-ethanol (m.p 67-71˚C). Free desmethylfenitrothion was liberated by treatment with a few drops of 1N HCl yield 80%, purity 75 % (Akhtar, and Foster, 1977).



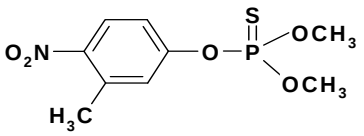
Fenitrothion **Desmethylfenitrothion [III]**

Synthesis of 3-methyl-4-nitrophenol (IV):

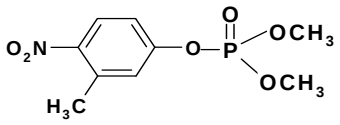
One gram of pure fenitrothion was dissolved in 5% alcoholic potassium or sodium hydroxide (10mL) and heated on water bath for 30 minutes. The solvent was evaporated, few drops of water were added and extracted with diethyl ether, followed by acidification of phenol salt with 1N HCl, the solution was left over night and the pure phenol was obtained by crystallization from water (m.p. 125 -128 ˚C) yield 90 % (Akintonwa, and Hutson, 1967).



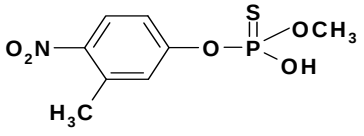
Fenitrothion **3-Methyl- 4-Nitrophenol [IV]**



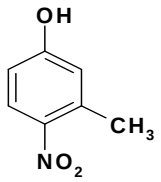
Fenitrothion [I]



Fenitrooxon [II]



Desmethylfenitrothion [III]



3-methyl-4-nitrophenol [IV]

Fig. 1: Fenitrothion and its main degradation products

Seeds:

Pesticide-free Zea-mays seeds (Var. Giza.1 hybrid) were obtained from Agricultural Research Centre (Cairo). The seeds were cleaned from any dockage and impurities before cultivation.

Field Experiment:

Sound whole seeds of maize were cultivated under normal field conditions in controlled, isolated field area. Irrigation, fertilization and soil management were conducted as practiced in the field according to the guide lines of the Egyptian Ministry of Agriculture (2007).[25] Shortly at blooming stage, leaves of plants were treated twice, 23 days apart, with ^{14}C-fenitrothion at dose of 6 mg/plant which equivalent 0.8 µCi. At maturity seeds were collected, dried and crushed for feeding and determination of their radioactivity to know the exact radioactive fenitrothion residues in the obtained maize seeds. In parallel experiments, only the non-labelled fenitrothion insecticide was used for preparing maize seeds for feeding rats.

Purslane Extract:

Purslane (*Portulaca oleracea* L) was purchased from local market and the parts of the plant suitable for consumption were used, which consisted of the soft upper stems of the plant and leaves. The purslane plant was extracted by stirring with methanol at room temperature for one week, the methanolic extract evaporated under vacuum and freeze-drying in Mycotoxin Central Laboratory, National Research Centre for future use.

Thin Layer Chromatographic (TLC) Analysis:

Residues were characterized by TLC on silica gel plates (20×20 cm; 0.25 mm thickness) with fluorescent indicator (Kiesel gel 60 F_{254}**,** Merck, Germany) using suitable systems for fenitrothion. Authentic samples were run alongside as references for identification and the plates were seen under UV-light at 254 nm and spots were made visible by spraying the plates with vanillin/sulphuric acid (2.5 %; v/v), (80 ml of ethanolic solution of vanillin were added slowly while stirring to 400 ml of ice cold concentrated sulphuric acid and then kept in a dark bottle),the plates were sprayed with the reagent, followed by heating at 110 ˚C for 5 minutes.

High Performance Liquid Chromatography Analysis (HPLC):

Analysis by HPLC was afforded on a Helwet Packard (1100 U.S.A) equipped with gradient system pumping, solvent programmer, the stainless steel column (125×4 mm I.D) was packed with 5 µm ODS-Hypersil, variable-wavelength UV detector operated at 254 nm. The mobile phase consisted of a mixture of acetonitrile: water (1:1 by volume).

Radiometric Measurements:

The obtained maize seeds were crushed and assayed for their radioactivity by combusting a definite weight (100 mg) in Harvey Biological Oxidizer (Model OX-600) followed by Liquid Scintillation Counting (LSC) (Packard Model RI-CARB 2300 TR) in vials using a dioxane-based scintillation cocktail. The internal standard (^{14}C n-hexadecane 0.2 µCi/ml=74 KBq/ml) technique was used for quench correction. The scintillator can be prepared by adding 100 g naphthalene, 10 g 2,5-diphenyloxazole (PPO) and 0.25 g 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl)-benzol (POPOP) to one liter of dioxane (L'Annunziata, 1979).

Toxicological Studies:

In order to study the toxicological potential of fenitrothion residues in maize, a subchronic feeding study on rat for 45 days was carried out. White male albino rats weighting 80-100 g were obtained from the Animal House in National Research Centre, Dokki, Cairo. The rats were housed in stainless steel cages with a maximum of four rats per cage provided with a free supply of feed and water. These were randomly divided into four groups as follow:

Group I: Control rats fed with normal diet free from any pesticides or plant extract.
Group II: Rats fed with normal diet containing plant extract [purslane (*Portulaca oleracea* L)] (0.01g extract/rat/day).
Group III: Rats fed with total maize seeds which treated with fenitrothion (10g maize/rat/day).

Group IV: Rats fed with total maize seeds treated with fenitrothion mixed with purslane extract (0.01g extract/10g maize/rat/day).

Maize- purslane diet was prepared by adding 1g purslane extract to 1kg crushed maize seeds and shaked manually for at least 30 minutes to ensure good distribution. At the end of the experimental period, animals were sacrificed by cervical decapitation. Blood was collected from arbitral plexus and divided into two parts; the first part was collected in a dry test tube, left at room temperature to clot and then centrifuged at 3000 rpm for 10 minutes to separate the serum that was used for the assay of biochemical parameters. The other part was collected in heparinized tubes for the assay of cholinesterase activity in both plasma and red blood cells (RBCs). Plasma and RBCs cholinesterase activities were determined according to Ellman method (1961), as modified by Gorun *et al*, (1978). Serum ALT and AST activities were measured by the dinitophenylhydrazene (DNPH) method according to

Reitman and Frankel (1957) Alkaline phosphatase activity in serum was estimated by the method of King (1965); albumin according to Doumas, *et al*, (1971), total protein according to Gornall, *et al*, (1949), cholesterol according to Richmond, (1973) and triglyceride according to Fassati and Prencipe, (1982). The kidney damage was evaluated by measuring the blood urea nitrogen according to Patton, and Crouch, (1977) and creatinine level according to Harry and Abraham, (1968) All blood parameters were carried out by using kits from Biodiagonostic Company in Egypt.

Statistical Analysis:

All data in the text were presented as means ± standard deviation (means ± S.D.). Statistical analysis was carried out using Student's *t*-test.

Results:

Table 1 showed the chromatographic analyses by TLC and HPLC which exhibit R_f and R_t of fenitrothion and its main degradation products in the obtained maize seeds after cultivation.

Table 1: R_f and R_t of fenitrothion and its main degradation products.

Compound	R_f values			R_t values*
	Sys. 1	Sys. 2	Sys. 3	
Fenitrothion [I]	0.90	0.75	0.90	9.32
Fenitrooxon [II]	0.82	0.44	0.74	2.47
Desmethyl fenitrothion [III]	0.72	0.35	0.78	0.96
3-methyl-4-nitrophenol [IV]	0.80	0.38	0.76	2.24

System 1= Toluene: Ethyl acetate: Acetic acid (5:7:1)
System 2= Toluene: Acetic acid (7:1)
System 3= Acetonitrile: Water: Ammonia (85:14:1)
(*)UV detector at 254nm, mobile phase: acetonitrile: water (1:1).

Vanilin/sulfuric acid reagent:

Fenitrothion (I) and fenitrooxon (II) gave pink,

desmethylfenitrothion (III) gave yellow while phenol (IV) gave blue.

As demonstrated in Table 2, rats (group 3) fed with fenitrothion residues in maize seeds had a significant inhibition in plasma and RBCs cholinesterase, with percent change 59.3 and 56.9% from control respectively. Feeding rats with maize seeds mixed with purslane extract (group 4) significantly countered the toxic effect of fenitrothion and resulted in values of plasma and RBCs cholinesterase nearly from control groups.

Table 2: Effect of methanolic extract of purslane on cholinesterase activities of rats fed with fenitrothion residues in maize seeds for 45 days.

Group	Plasma-Cholinesterase level (μ mole/mL)		RBC-Cholinesterase level (μ mole/mL)	
	Means [a] ± SD	%	Means [a] ± SD	%
Control	1.77 0.09	-----	3.67 0.13	-----
Control + E	1.80 0.09	-----	3.68 0.12	-----
Fenitrothion	1.05 0.07 ***	40.7	2.09 0.11***	43.1
Fenitrothion + E	1.66 0.03	6.2	3.36 0.12	8.4

a: Results are expressed as mean ± SD for four samples
E = Methanolic extract of purslane
*** Significance at P< 0.001

As shown in Table 3 rats (Group 3) developed liver damage as indicated by the increased serum levels of liver marker enzymes ALT (64%), AST (58%) and ALP(35.7) as well as decreased in albumin (21%) and total protein contents (15%) with respect to control group (group 1).

Feeding rats with maize seeds mixed with purslane extract (Group 4) showed significantly attenuated in the liver markers levels resulted in values small increases about fourth of the total increase in ALT and AST (group 3).

Table 3: Effect of methanolic extract of purslane on liver function of rats fed with fenitrothion residues in maize seeds for 45 days.

Group	ALT (U/L)		AST (U/L)		Alk. Phosphatase (U/L)		Albumin (g/dL)		Total protein (g/dL)	
	Means [a] ± SD	%	Means [a] ± SD	%	Means [a] ± SD	%	Means [a] ± SD	%	Means [a] ± SD	%
Control	36.75 3.86	----	86.0 5.16	----	220.0 9.93	----	3.42 0.18	----	7.05 0.18	----
Control + E	35.0 3.90	----	85.0 4.54	----	219.0 8.14	----	3.35 0.17	----	6.98 0.19	----
Fenitrothion	60.25 4.27***	63.9	136.0 5.89***	58.1	298.5 10.75*	35.7	2.70 0.11***	21	5.99 0.15***	15
Fenitrothion + E	42.5 4.40	15.6	98.5 7.7	14.5	221.6 8.4	0.7	3.09 0.10	9.6	6.89 0.10	2.3

a: Results are expressed as mean ± SD for four samples
E = Methanolic extract of purslane
* Significance at P< 0.05
*** Significance at P< 0.001

Table 4 shows a marked elevated level of serum kidney function indices, creatinine (27.8%), and urea (39%) in group 3 in relation to control animals (group 1).

Similarly fenitrothion treated rats received purslane (group 4) greatly prevented the deviation in the kidney function indices fourth of total activity in urea and half in case of creatinine if compared with group 3.

Table 4: Effect of methanolic extract of purslane on kidney function of rats fed with fenitrothion residues in maize seeds for 45 days.

Group	Urea (mg %)		Creatinine (mg %)	
	Means [a] ± SD	%	Means [a] ± SD	%
Control	49.8 4.2	-----	0.90 0.04	-----
Control + E	45.5 2.8	-----	0.94 0.07	-----
Fenitrothion	69.25 5.6***	39	1.15 0.07***	27.8
Fenitrothion + E	54.65 2.1	9.7	1.02 0.05	13.3

a: Results are expressed as mean ± SD for four samples
E = Methanolic extract of purslane
*** Significance at P< 0.001

There was a significant increase in both cholesterol (15.4%) and triglycerides (16.2%) levels in treated rats (group 3) if compared with control group (Table 5). However it was observed that the group 4 received fenitrothion residues along with purslane extract had normal cholesterol and triglycerides levels comparable to that of control group.

Table 5: Effect of methanolic extract of purslane on lipid profile of rats fed with fenitrothion residues in maize seeds for 45 days.

Group	Cholesterol (mg %)		Triglycerides (mg %)	
	Means [a] ± SD	%	Means [a] ± SD	%
Control	104.62 5.12	-----	85.63 4.61	-----
Control + E	95.75 5.56	-----	78.9 2.74	-----
Fenitrothion	120.75 5.62*	15.4	99.55 2.23**	16.2
Fenitrothion + E	95.00 6.98	-----	84.75 3.40	-----

a: Results are expressed as mean ± SD for four samples
E = Methanolic extract of purslane
* Significance at P< 0.05; ** Significance at P< 0.01

It is worthy to mention that, no effects are observed on feeding rats group 2 during toxicity experiment if compared with control group.

Desiccation:

The widespread use of pesticides in public health and agricultural programs has caused severe environmental pollution and potential health hazards including acute and chronic cases of human poisoning (Kamath, and Rajini, 2007).

Organophosphorus and pyrethroid group of pesticides are the most commonly used in agriculture today. Both are efficiently absorbed and rapidly redistributed to various organs as part of their disposal mechanism. Risk assessment for environmental toxicants especially pesticides is currently undergoing a refocusing to induce chronic health effects from long-term, low level-exposure (Ritz, and Yu, 2000).

On the other hand, there is no doubt that the use of alternative and traditional medicine system is increasing throw the world, and as a consequence many traditional medicines have came under renewed security and are being investigated for efficacy and safety.

The present work aimed to evaluate the effect of purslane extract on induced chronic toxicity of fenitrothion residues in maize seeds during feeding rats.

The inhibition of acetylcholinesterase (AChE) has been a focal point given that most organophosphorus pesticides cause this effect (Gomes,*et al*, 1997) The present study revealed that fenitrothion intoxication induced the classical inhibitory effect of organophosphorus (Table 2), these findings runs parallel with that previously recorded (Okahashi, *et al*,2006, and Hassan, 2005) ,An observable significant improvement in the activity of acetylcholinesterase (AChE) enzymes was recorded in purslane extract supplemented groups (Table 2). Liver is the most sensitive organ to biochemical changes therefore its functions are greatly affected by pollutants resulting in an increase in serum enzymes levels (Hassan, 2005) Concerning the toxic effect of fenitrothion on the liver parameters ALT, AST, ALP, total protein and albumin. The results revealed elevation in the activity of each of ALT, AST, and ALP enzymes and decrease in the level of each of total protein and albumin in serum of treated animals (group 3), these findings coincide with previous studies that showed a significant increase in liver enzymes in rats and humans exposed to fenitrothion and chlorpyrifos (Elhalwagy,2008, Patil, *et al* ,2003 Alsahhaf, 2006 and Al-Jahdali *et al* , 2007)The obtained results showed that the treatment of fenitrothion intoxicated rats with purslane extract (group 4) decreased serum activities of ALT ; AST and ALP, as well as increase of total protein and albumin level because it contain omega-3, omega-6 and phenolic compounds as antioxidants (Ivo,*et al*,2009).

In the present study the extract of purslane show improvement in biochemical parameters as a result of hepatotoxin challenge, indicating amelioration of the functional status of the liver. The protective effect due to mixing with purslane extract strongly indicated the possibility of the extract being able to prevent and/or mitigate any leakages of marker enzymes into circulation condition the hepatocytes to accelerate regeneration of parenchymal cells and preserves the integrity of the plasma membranes and hence restores these enzymes levels (Al-Howiriny,*et al*,2004).

Kidneys are responsible for the elimination of metabolic wastes and control of the amount and composition of the body fluids. Nephrotoxicity is toxicity to the kidneys. It can result in systemic toxicity causing low ability to excrete body wastes, inability to maintain body fluid and electrolyte balance and decreased synthesis of essential hormones (e.g., erythropoietin) (Finn,1977). The results obtained provided the evidence of functional renal damages induced by fenitrothion exposure of rat's .A significant increase in creatinine and urea levels was recorded in the treated animals in the end of experiment. These data are in line with those obtained by several authors in the study of toxicity of fenitrothion on experimental animals (Kadry,*et al,*2001 and Farghaly *et al*, 2007) The treatment of fenitrothion intoxicated rats with purslane extract made a decreasing in serum creatinine, and serum urea level due to its ability to treatment nephrotoxicity. Recently, it is worthy to mention that these results are in accordance with those of other authors (Nitha, and Janardhanan; 2008 and Hozayen,*et al* ; 2001)

The importance of plasma levels of cholesterol and triglycerides in the pathogenesis of atherosclerosis has been noted by a number of studies. The obtained data revealed elevation in cholesterol and triglycerides levels in serum of animals treated with fenitrothion residues. These findings are in agreement with recent reports by other authors (Alsahhaf, 2006 and Afshar, *et al* 2008)

As shown in the present work, administration of purslane extract resulted in a significant reduction of serum cholesterol and triglycerides

The obtained data suggest that, the purslane herb extract have a beneficial effect, by reducing the toxicological effects induced by fenitrothion insecticide residues in liver and kidney functions, lipid profile, cholinesterase activity, albumin and total protein and therefore, the protective individual for oxidative stress diseases.

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