JJBS

Volume 12, Number 4,September 2019
ISSN 1995-6673
Pages 385 - 393

Jordan Journal of Biological Sciences

Protective Effects of the Aqueous Extract of Black Mulberry Leaves, *Morus nigra*, on Chlorpyrifos Toxicity in Male Albino Rats

AlaaEddeen M. Seufi[1,2*], Hoda A Mohammed[3], Farouzina I Moussa[4], Ahmed A Seif El-Din[5], Muhammad El-Saadani[6], Tarek H. Taha[7] and Elsayed E. Hafez[3]

[1]Department of Basic Sciences, Deanship of Common First Year, Jouf University, KSA; [2]Department of Entomology, Faculty of Science, Cairo University, Egypt; [3]Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications; [4]Zoology Department, Faculty of Science; [5]Pharmacognosy Department, Faculty of Pharmacy; [6]Biochemistry Department, Faculty of Science, Alexandria University, Alexandria; [7]Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research And Technological Applications, New Borg El Arab, 21934, Alexandria, Egypt.

Received October 10, 2018; Revised November 6, 2018; Accepted November 20, 2018

Abstract

The present study investigates the protective efficacy of the aqueous extract of *Morus nigra* leaves in rats exposed to toxicity by chlorpyrifos (CPF). The hematological indices (RBCs, WBCs, and platelets' counts, Hb level, hematocrit percent, MCH, MCV and MCHC), oxidant/ antioxidant status (Malondialdehide (MDA), Total Antioxidant Capacity (TAC)), and the biochemical profile (AChE and LDH enzymes, Blood Glucose (BG), Cholesterol (Chol), Triglycerides (TG) and creatinine) are all investigated in this study. In addition, differential display results will be analyzed to draw the genetic relationship between the experimental groups. The results reveal a significant reduction in WBCs, RBCs counts, Hb, hematocrit percentage, TAC, AChE activity, BG, TG, and Chol. A significant rise in platelets' count, MDA and LDH activities were also observed. Insignificant difference in MCV, MCH, MCHC and creatinine levels in the CPF-treated rats was recoded compared to the negative control. The administration of CPF-BMB combination led to a recovery to the normal state of health regarding most of the CPF-influenced parameters. The differential display Polymerase Chain Reaction (DD-PCR) results reinstated hematological and biochemical results by grouping CPF-BMB combinations with negative control in the same lineage. Conclusively, the findings of this study suggest that some useful parameters are prognostic biomarkers of organophosphorus intoxication, and can throw light on the potential of the aqueous extract of *M. nigra* leaves as a natural auspicious and secure detoxifying agent. Finally, further studies on *M. nigra* will reveal many other bioactive substances with therapeutic, pharmacological, and nutritional values.

Keywords: Chlorpyrifos, Black mulberry leaves, Insecticides toxicity, Antioxidant activity, Acetylcholinesterase

1. Introduction

Pesticides are unique contaminants because they are intentionally released into the environment to elicit toxicity in certain pest species. Unfortunately, the lack of selectivity often leads to problems in humans and other non-target species. Organophosphorus (OP) pesticides are the major chemical class of insecticides used in the world today. Chlorpyrifos (CPF) belongs to the OP class of pesticides, serving as an insecticide and acaricides. CPF is widely used, because of its greater stability, persistence, and less toxicity than other OP pesticides (Richardson *et al.*, 1993; Mileson *et al.*, 1998). CPF is the active component in a wide array of pesticide formulations. According to the U.S. Environmental Protection Agency (EPA), CPF has been extensively studied due to its potential neurotoxicity (Richardson, 1995; Pope, 1999). Although CPF is no longer registered for residential use in the U.S. (U.S. EPA, 2012), there is still a high potential for human exposure, since it is widely utilized in agricultural applications (Lee *et al.*, 2007). CPF has been associated with asthma, and reproductive, developmental and acute toxicity. As for its acute effects, the EPA classifies CPF as class II: moderately toxic. Recent research indicates that children exposed to CPF while in the womb have an increased risk of delays in mental and motor development at age three. An increased occurrence of pervasive developmental disorders such as attention deficit hyperactivity disorder (ADHD) was reported, too. CPF induces toxicity through inhibition of acetyl cholinesterase (AChE) in different tissues including liver, kidney, and spleen (Sultatos *et al.*, 1985; Sultatos, 1987; Mileson *et al.*, 1998; Slotkin *et al.*, 2006). With extensive AChE inhibition, the neurotransmitter acetylcholine (ACh) accumulates in the synapses of the central and peripheral nervous systems, which in turn leads to overstimulation of postsynaptic cholinergic receptors and signs of cholinergic

* Corresponding author e-mail: alaaseufi@yahoo.com, alaaseufi@ju.edu.sa.

neurotoxicity (Silver, 1974; Ecobichon, 1996; Savolainen, 2001). CPF exposure causes inhibition of antioxidant enzyme activities and increases in the levels of hydrogen peroxide (H_2O_2) in rat brains and liver (Gultekin *et al.*, 2001; Verma and Srivastava, 2001and 2003). Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent producing free radicals or reactive oxygen species (ROS). In turn, they start chain reactions which lead to cell damage. Antioxidants terminate these chain reactions by several mechanisms such as removing free radical intermediates, and inhibiting other oxidation reactions (Sies and Masumoto, 1997). Many antioxidants elicit protective effects on OP-induced acute poisoning in animal models (Gultekin *et al.*, 2001; Altuntas *et al.*, 2002). Medicinal plants are abundant sources of antioxidants. These plants demonstrated an ability to combat ROS-induced oxidative damage. Fruits and leaves of berries including black mulberry, raspberry, and strawberry contain a high content of antioxidants (Matsumoto *et al.*, 2004). Black mulberry, in particular, has exhibited the highest capacity to inhibit O_2, H_2O_2, and OH radicals among forty-one fruits and vegetables (Wang and Jiao, 2000). Many articles have been published on the bioactivity of *M. nigra*, and its protective and curative capacity against many toxigenic substances (Shukla *et al.*, 2014; Akhlaq *et al.*, 2016; de Freitas *et al.*, 2016; Sánchez-Salcedo *et al.*, 2017; Zhang *et al.*, 2018).

Accordingly, the main objective of this study is to investigate the efficacy of *M. nigra* leaves' aqueous extract to reduce CPF-induced hematological and biochemical alterations in adult male albino rats. Genetic relationships between the experimental groups will be investigated, too.

2. Materials and Methods

2.1. Plant Materials

Leaves of the black mulberry, *Morus nigra*, were collected from ten farms at Behera governorate, Kafr El Dawar Center, Egypt. The collected leaves were labeled and transferred to the laboratory, washed with tap water, air dried, and stored until further investigations.

2.2. Preparation of the Plant Extract

Fifty grams of oven-dried leaves (50°C) of *M. nigra* were soaked in 1 liter of boiling water. The dried leaves were steeped in boiling water for two-three hours. The extract was filtered using 0.1 mm Whatman's filter paper. The filtrate was transferred into 50-ml flasks and evaporated in the oven at 60°C for three days. The extract was dissolved in phosphate-buffered saline (PBS) [8 g NaCl + 1.15 g KH_2PO_4+ 0.2 g KCl dissolved in 1L of distilled water, and the pH was justified to 7.4]. The extract was stored at -20°C until processed.

2.3. Animal Groups

Fifty male albino rats (*Rattus norvegicus*), (twelve to fourteen-week-old), weighing (180± 20 g) were originally obtained from the Institute of Graduate Studies and Research Farm (Alexandria University, Alex., Egypt). The rats were acclimatized for at least one week prior to experimentation. They were kept in plastic cages at room temperature (22-25°C) and a photoperiod of 12L: 12D. Crowding was avoided, and the animals were provided with a sufficient amount of balanced diet. An animal was judged healthy on the basis of general activity, feeding behavior, and absence of overt disease symptoms. The rats were randomly assigned to one of the following five groups: Control: the control group received corn oil; the CPF-treated group: received oral LD_{50} (13.5 mg/ kg body weight (b.wt)) of CPF ethyl in corn oil every alternate day (Goel *et al.*, 2005 ; 2006); CPF-BMB1 group: received LD_{50} of CPF ethyl+ aqueous extract of 0.15 g/ kg b.wt black mulberry leaves; CPF-BMB2 group: received LD_{50} of CPF ethyl+ aqueous extract of 0.25 g/ kg body b.wt mulberry leaves; and CPF-BMB3 group: received LD_{50} of CPF ethyl+ aqueous extract of 0.4 g/ kg b.wt black mulberry leaves. Doses of the black mulberry leaves' extract were derived from the curve of total phenolic content as previously described (Shukla *et al.*, 2014). Solutions were freshly prepared every forty-eight hours, and were given to the rats using stomach gavage. During the experimental period, the animals were examined for any observable signs and abnormalities, and even death. They were also weighed weekly during the test period before feeding. Blood samples were collected from the anterior jugular vein of rats. The blood samples were passed for hematological and biochemical analysis. The experimental protocols were approved by the Ethical Committee of Alexandria University.

2.4. Hematological Parameters

Packed hemoglobin (Hb), total erythrocyte count (RBCs), hematocrit percent, platelets' count, and leukocyte count (WBCs) were evaluated. Mean corpuscular volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were calculated, too. All calculations were carried out by a built-in computer software.

2.5. Biochemical Parameters

Malondialdehyde (MDA) concentration, total antioxidant capacity (TAC), acetyl cholinesterase activity (AChE), lactate dehydrogenase activity (LDH), blood glucose (BG), triglycerides (TG), total cholesterol (Chol), and creatinine levels were investigated. The animals were fastened before blood-sampling whenever needed. All chemicals were purchased from Sigma-Aldrich and all kits were purchased from BioRad, USA. Experimental procedures were carried out according to the manufacturer's protocols.

2.6. Molecular Examinations

2.6.1. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the blood samples using the TriAzol reagent procedure (BioFlux, Germany). Then, RNA pellet was resuspended in DEPC-treated water. The RNA quantity and quality were determined by using spectrophotometer and gel-electrophoresis. The isolated RNA was converted into cDNA using the cDNA synthesis system kit (Fermentas, Germany) in the presence of oligo-dT primer. Each 25 µL of the reaction mixture contained 2.5 µL (5x) buffer with $MgCl_2$, 2.5 µL (2.5 mM) dNTPs, 1 µL (10 pmol) primer, 2.5 µL RNA (2mg/ml) and 0.5 unit Reverse Transcriptase Enzyme (MVLR, Fermentas). The thermal cycler equipment was programmed at 42 °C for one hour, 72 °C for ten minutes and the PCR product was stored at 4°C until use.

2.6.2. Differential Display Polymerase Chain Reaction (DD-PCR)

cDNA was used as a template for DD-PCR reaction. Reaction conditions were performed according to Hafez *et al.* (2013). Sequences and annealing temperature of each primer were summarized in Table 1. The number and length of the amplified fragments were analyzed according to Liang and Pardee (1992).

Table 1. Names, sequences and annealing temperatures of five arbitrary primers employed in this study.

Primer name	Primer sequence `5 to `3	Annealing Temp.
NAR47	CGG CAG CGC C	45 °C
NAR48	CCT TTC CCT C	47 °C
NAR50	ACG GAG TTG GAG GTC	53 °C
NAR51	TGC GCC GAA TTA TGC GG	53 °C
NAR52	GTA AAA CGA CGG CAA	48 °C

2.7. Statistical Analyses

Experiments were repeated thrice, and the data were collected. The test for homogeneity of variances was carried out. One-way analysis of variance (ANOVA) and multiple comparison tests were applied as needed. The effects of the leaves' extract of black mulberry on biochemical, hematological parameters were analyzed. The significance level was justified to 95 % confidence interval (P< 0.05). Statistical analyses of data were performed using SPSS software (Ver. 20.0).

3. Results

3.1. Hematological Parameters

The effects of different treatments on RBCs, Hb and hematocrit percentage were examined. The results presented in Table 2 reveal that the CPF-group showed significant (P< 0.05) decreases of RBCs (3.0± 0.1 million/ mm^3), Hb (6.8± 0.3 g/ dL) and hematocrit percentage (32.0± 1.2 %) compared to the negative controls (5.2± 9.5 million/ mm^3, 14.4±0.3 g/dL and 45.8±1.1%, respectively). The results showed that the administration of CPF-BMB combinations abolished the reduction of RBCs, Hb, and hematocrit percentages (Table 2).

Table 2 demonstrates that the CPF-treated rats showed a significant increase (P<0.05) in platelets' count (272333± 12148) compared to the negative controls (257333± 13413). It is also evident that the simultaneous administration of CPF in the BMB1, BMB2 and BMB3 groups exhibited significant decrease (P<0.05) in platelets' count (205000± 5240, 203333± 8432 and 206666± 9189, respectively) compared to both positive and negative control groups.

In addition, the results in Table 2 clearly show that the CPF-administration resulted in a significant decrease (P< 0.05) of the WBCs' count (202.3 ± 31) compared with the negative control (206.8 ± 6). However, significant protective effects (P< 0.05) were observed in the case of treatment with CPF-BMB1 (207.8 ± 7), CPF-BMB2 (207.5 ± 8) and CPF-BMB3 (206.2 ± 8) compared to the positive control (Table 2).

The hematological parameters MCV, MCH and MCHC of both of the control and treated rats were shown in Figure 1. Statistical analysis of data revealed that no significant changes (P> 0.05) were observed in MCV, MCH and MCHC of the treated rats (Figure 1) when compared to both negative control (untreated rats) and positive control (CPF-treated rats).

Table 2. Effect of different treatments on some hematological indices in white albino rats. Control: -ve control group received corn oil, CPF: CPF-treated group received oral LD50 (13.5 mg/ kg body weight) of CPF ethyl in corn oil every alternate day, CPF-BMB1: received LD50 of CPF ethyl+ aqueous extract of 0.15 g/ kg body weight black mulberry leaves, CPF-BMB2: received LD50 of CPF ethyl+ aqueous extract of 0.25 g/ kg body weight black mulberry leaves and CPF-BMB3: received LD50 of CPF ethyl+ aqueous extract of 0.4 g/ kg body weight black mulberry leaves.

	Parameter				
	Platelet / µL	WBCs (cell/ µL)	RBCs	Haemoglobin	Haematocrit (%)
Control	257333 ±13413	206.8 ±6	5.2± 9.5	14.4± 0.3	45.8±1.1
CPF	272333 ±12148	202.3 ±31	3.0± 0.1	6.8± 0.3	32.0±1.2
CPF-BMB1	205000 ±5240	207.8 ±7	4.8± 8.3	12.6± 0.2	39.1±2.1
CPF-BMB2	203333 ±8432	207.5 ±8	5.1± 6.1	14.1± 0.3	42.3±3.3
CPF-BMB3	206666 ±9189	206.2 ±8	5.2± 7.4	14.5± 0.7	46.1±2.2

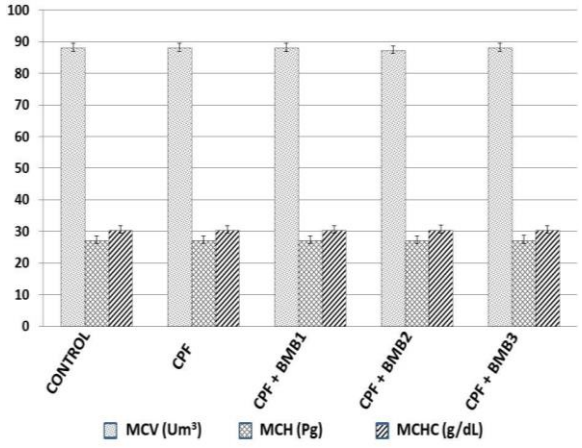

Figure 1. Effect of different treatments on mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) in white albino rats. Control: -ve control group received corn oil, CPF: CPF-treated group received oral LD_{50} (13.5 mg/ kg body weight) of CPF ethyl in corn oil every alternate day, CPF-BMB1: received LD_{50} of CPF ethyl+ aqueous extract of 0.15 g/ kg body weight black mulberry leaves, CPF-BMB2: received LD_{50} of CPF ethyl+ aqueous extract of 0.25 g/ kg body weight black mulberry leaves and CPF-BMB3: received LD_{50} of CPF ethyl+ aqueous extract of 0.4 g/ kg body weight black mulberry leaves.

3.2. Biochemical Parameters

Figure 2 demonstrates a variation in serum MDA levels in all of the tested groups. It is obvious that the concentrations of serum MDA were significantly increased (P< 0.05) with the CPF-treatment (10.00± 2.34 nmol/mL) compared to the negative control group (7.06± 2.26 nmol/mL). Serum MDA concentrations displayed a significant decrease (P< 0.05) in the cases of CPF-BMB1

(11.1± 3.148 nmol/mL), CPF-BMB2 (10. 370± 2.118 nmol/mL), and CPF-BMB3 (6.4 ±8.597 nmol/mL) when compared to the CPF-group (12.9± 2.989 nmol/mL).

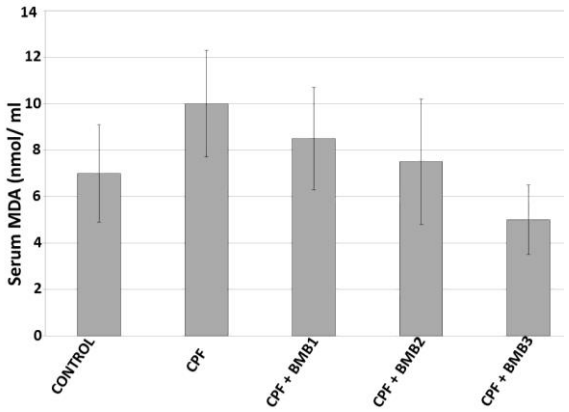

Figure 2. Effect of different treatments on mean concentration serum malondialdehyde (MDA) in white albino rats. Control: -ve control group received corn oil, CPF: CPF-treated group received oral LD_{50} of CPF ethyl in corn oil every alternate day, CPF-BMB1: received LD_{50} of CPF ethyl+ aqueous extract of 0.15 g/ kg body weight black mulberry leaves, CPF-BMB2: received LD_{50} of CPF ethyl+ aqueous extract of 0.25 g/ kg body weight black mulberry leaves and CPF-BMB3: received LD_{50} of CPF ethyl+ aqueous extract of 0.4 g/ kg body weight black mulberry leaves.

Figure 3 presents TAC of serum in all of the tested groups. CPF-administration significantly (P< 0.05) decreased serum's TAC (1.1033 ± 0.1023) mM/L compared with the negative control rats (1.1410 ± 0.3706) mM/L. However, the administration of CPF-BMB1, CPF-BMB2 and CPF-BMB3 significantly antagonized the effects of CPF (P< 0.05), resulting in a significant increase of TAC (1.2273 ± 0.2486, 1.1473 ± 0.1359 and 1.2130 ± 0.2033, respectively) mM/L.

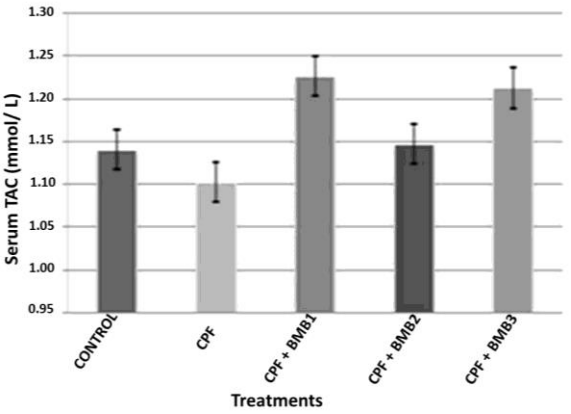

Figure 3. Effect of different treatments on total antioxidant capacity (TAC) in white albino rats' serum. Control: -ve control group received corn oil, CPF: CPF-treated group received oral LD_{50} of CPF ethyl in corn oil every alternate day, CPF-BMB1: received LD_{50} of CPF ethyl+ aqueous extract of 0.15 g/ kg body weight black mulberry leaves, CPF-BMB2: received LD_{50} of CPF ethyl+ aqueous extract of 0.25 g/ kg body weight black mulberry leaves and CPF-BMB3**:** received LD_{50} of CPF ethyl+ aqueous extract of 0.4 g/ kg body weight black mulberry leaves.

Figure 4 shows that CPF-supplementation decreased the AChE activity (25794.7± 1505.4) compared with the negative control group (26525.2± 423.9). The administration of CPF-BMB combination led to a significant increase (P< 0.05). However, the CPF-treated rats showed a significant increase (P< 0.05) in the LDH activity compared to the negative control group (4469.7± 789.0 and 3235.3± 591.8 U/L, respectively), whilst the administration of CPF-BMB combination resulted in a dose-dependent gradual decrease of LDH activity compared to the positive control group (Figure 4). The decreases were significant (P< 0.05) in the cases of CPF-BMB2 and CPF-BMB3 when compared to the positive control group.

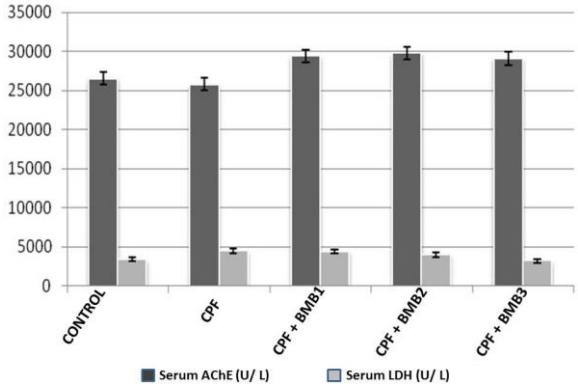

Figure 4. Effect of different treatments on serum acetylcholinesterase (AChE) and lactate dehydrogenase (LDH) activity in white albino rats. Control: -ve control group received corn oil, CPF: CPF-treated group received oral LD_{50} of CPF ethyl in corn oil every alternate day, CPF-BMB1: received LD_{50} of CPF ethyl+ aqueous extract of 0.15 g/ kg body weight black mulberry leaves, CPF-BMB2: received LD_{50} of CPF ethyl+ aqueous extract of 0.25 g/ kg body weight black mulberry leaves and CPF-BMB3: received LD_{50} of CPF ethyl+ aqueous extract of 0.4 g/ kg body weight black mulberry leaves.

Figure 5 shows that CPF-treatment resulted in a significant decrease (P<0.05) in the blood glucose level (25.5± 4.2) mg/dl compared with the negative control group (94.5± 12.8) mg/dl. Furthermore, the administration of CPF-BMB combinations (73.8 ± 11.9, 63.1 ± 15.9 and 75.9 ± 13.4 mg/dl) produced a significant increase (P<0.05) in blood glucose levels compared to the positive control group (Figure 5).

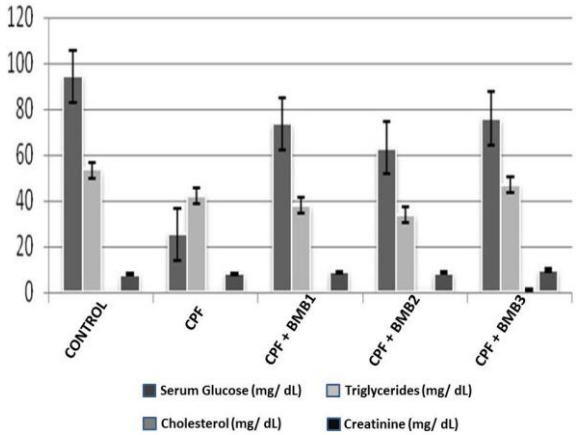

Figure 5. Effect of different treatments on blood glucose (BG), triglycerides (TG), cholesterol (Chol), and creatinine concentrations in the serum in white albino rats. Control: -ve control group received corn oil, CPF: CPF-treated group received oral LD_{50} of CPF ethyl in corn oil every alternate day, CPF-BMB1: received LD_{50} of CPF ethyl+ aqueous extract of 0.15 g/ kg body weight black mulberry leaves, CPF-BMB2: received LD_{50} of CPF ethyl+ aqueous extract of 0.25 g/ kg body weight black mulberry leaves and CPF-BMB3: received LD_{50} of CPF ethyl+ aqueous extract of 0.4 g/ kg body weight black mulberry leaves.

In addition, Figure 5 indicates that the CPF-treatment produced a significant decrease ($P< 0.05$) of serum triglycerides (42.4 ± 6.3) mg/dl compared with negative control group (53.5± 7.6) mg/dl. Treatment with CPF-BMB3 exhibited an insignificant increase ($P> 0.05$) of serum triglycerides (47.0 ± 8.5) mg/dl when compared to both control groups. Both of the CPF-BMB1 and CPF-BMB2 groups presented a significant decrease ($P< 0.05$) of serum triglycerides when compared to the negative control. However, this decrease was significant ($P< 0.05$) in the case of the CPF-BMB2 group only when compared to the positive control group (Figure 5).

Moreover, the results presented in Figure 5 show that the CPF-treated rats showed an insignificant decrease ($P> 0.05$) in serum cholesterol (0.7062± 0.1515) mg/dL compared to the negative control group (1.0035± 0.7865) mg/dL. However, the administration of CPF-BMB combination antagonized the effects of the CPF treatment. The increase of cholesterol concentration was significant ($P< 0.05$) in the cases of CPF-BMB2 and CPF-BMB3 groups (1.1450± 0.1838 and 1.1548± 0.1309 mg/dL, respectively) compared to the positive control group (Figure 5).

The increase of serum creatinine level was insignificant ($P> 0.05$) in the case of the CPF-treated rats (8.05± 1.4) mg/dL when compared with the negative control group (7.78 ± 0.2) mg/dL. No significant difference in the serum creatinine level (P> 0.05) was observed when rats were treated with any of the three combinations CPF-BMB1, CPF-BMB2 or CPF-BMB3 (8.78 ± 0.9, 8.58 ± 0.5 or 9.78 ± 1.1 mg/dL, respectively) in comparison to the positive control group (Figure 5).

3.3. Differential Display and DD-PCR Analysis

Figure 6 presents results of differentially-displayed DNA bands of the control and treated samples using five primers. Collectively, they reflected the molecular pattern of up and down expressed genes in this experiment. The total numbers of bands resolved in 2 % agarose gel for both of the control and treated samples were 5, 4, 8, 10 and 5 bands in the case of Nar47, Nar48, Nar49, Nar50, and Nar51 primers, respectively (molecular size≈ 700 to 80 bp). The average number of bands per sample was 6.4. However, the average number of bands was 5.2, 5 and 3.2 bands in the cases of negative control, positive control and CPF-BMB treatments, respectively. Out of thirty-two bands, five monomorphic (15.6 %) and twenty-seven (84.4 %) polymorphic bands were recorded. Out of the five monomorphic bands, four bands were reported in the negative control, and one band in the CPF-BMB treatment (Figure 6). Some common bands in both of the controls and the treated samples were recorded. Few treatment-induced bands were observed (genes were turned on). On the other hand, some bands were observed in controls and disappeared in the treated groups (genes were turned off). Many bands were densely illuminated reflecting the overexpression of these genes. One treatment-specific band was noticed in the case of CPF-BMB3 with Nar51 primer. On the whole, these results revealed that many upregulated (turned on or overexpressed) and down-regulated genes (turned off) were observed in different treatments using the selected primers (Figure 6).

A dendrogram was constructed using the results of differential display. The maximum band divergence was exhibited in the lineage II (four phylogenetic groups). However, the lineage I appeared as a separate phylogenetic group (positive control). Negative control was clustered with CPF-BMB1, CPF-BMB2 and CPF-BMB3 in lineage II (Figure 7). It divides into two groups (Group I and Group II). Group I clustered the CPF-BMB combination at the three concentrations in three sister clades. Whilst Group II counts negative control in a monophyletic sister clade. The general topology of the tree illustrates that the CPF-BMB1 combination is closer to the negative control group (Figure 7).

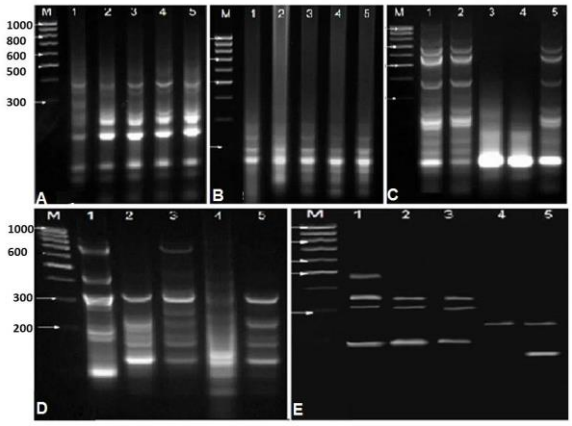

Figure 6. Effect of different treatments on differential display banding pattern (DD-PCR) in white albino rats' serum using five selected primers (A: Nar47, B: Nar48, C: Nar49, D: Nar50 and E: Nar51). M: 100 bp DNA ladder, lanes 1-5: -ve control group, CPF-treated group, CPF-BMB1 group, CPF-BMB2 group and CPF-BMB3 group, respectively.

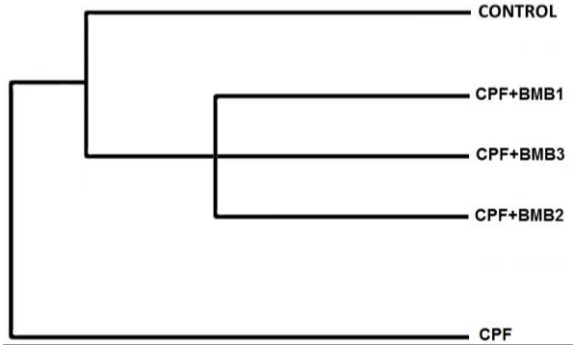

Figure 7. A dendrogram developed on the ground of similarity index of differentially displayed sera in white albino rats using five selected primers. Control: -ve control group, CPF: CPF-treated group, CPF-BMB1, CPF-BMB2 and CPF-BMB3 are the groups treated with combinations of chlorpyrifos and black mulberry leaves' extract.

4. Discussion

The extensive application of pesticides is usually accompanied by serious problems of pollution and health hazards. It is well-known that many pesticides could produce toxic and adverse effects on experimental animals through their mode of action or by production of free radicals (Khan, 2006). The present study is aimed at investigating the efficacy of BMB leaves' water extract as a potential protectant against organophosphate toxicity using CPF insecticide. To achieve this goal, the

hematological profile (RBCs, WBCs, and platelets' counts, Hb, hematocrit percentage, MCH, MCV and MCHC), oxidant/ antioxidant stress (MDA and TAC), and the biochemical profile (glucose, cholesterol, triglycerides, creatinine, AChE and LDH enzymes) were investigated. Moreover, differential display results were analyzed to draw the genetic relation between the experimental groups.

Collectively the results of this study point out that the application of CPF insecticide led to significant adverse effects ($P< 0.05$) on most of the tested hematological indices compared to the negative control group. Agreeable results were presented by Ambali *et al.* (2007 and 2011) who reported a reduced RBC count, Hb, and hematocrit percentage in the Wistar rats. Reduced Hb of dogs was acquired after 8, 24, and 36 hours of exposure to organophosphate (OP) insecticides (Ola-Davies *et al.*, 2018). Similar results were produced by Hundekari *et al.* (2013) who discovered a reduced Hb in 150 clinically-diagnosed OP-poisoning cases of patients admitted to Patil Medical College Hospital. In this study, the reduced WBC count (Leucopenia: abnormal reduction in WBCs) is in consistence with previous results (Hundekari *et al.*, 2013; Ola-Davies *et al.*, 2018). This leucopenia could be assigned to lymphopenia (subtype of leucopenia: abnormal reduction in lymphocyte' count in blood). It has been formerly denounced as a result of CPF-exposure (Goel *et al.*, 2006; Ambali *et al.*, 2010). On the other hand, Raghu *et al.* (2014) described a case of Leucocytosis (above the normal WBC count in blood) due to OP-poisoning. The excessive platelets' count (thrombocytosis) due to CPF-administration may be an indication of the increased risk of blood clots. Del Pardo-Lu (2007) adduced abnormal blood indices (RBCs, WBCs, platelets' counts, Hb, hematocrit and MCV) of cutflower farmers vulnerable to pesticides. The reduced RBC count of the CPF-group attracts attention to possible oxidative stress. It leads to lipid peroxidation in RBCs' membranes, auto-oxidation of hemoglobin, and limited repair processes. All these processes lead to decrease the lifespan of RBCs (Rice-Evans and Baysal, 1987; Halliwell and Chirico, 1993). In the present study, changes were statistically insignificant ($P> 0.05$) in the cases of MCV, MCH, and MCHC indices. Controversial results were conveyed in the study of Ola-Davies *et al.* (2018) who recorded a significant increase in the MCV and MCH indices, but an insignificant change of the MCHC index. However, the application of CPF-BMB combinations repealed or nullified the adverse effects of OP toxicity when compared to the positive control group ($P< 0.05$). This protective or antitoxic effect could be attributed to the higher content of bioactive compounds including many antioxidants (Zhang *et al.*, 2018). BMB leaves' components may play a vital role to enhance the defense system against oxidative stress induced by OP-poisoning (Luczaj *et al.*, 2004; Ostrowska *et al.*, 2004; Dobrzynska *et al.*, 2005).

To divulge the mechanisms behind the former results, the oxidant/ antioxidant status and biochemical profile were examined. The significant increase in the MDA concentration ($P< 0.05$) due to the CPF-application is an indicator of increased lipid peroxidation. Meanwhile, the significant inhibition of TAC ($P< 0.05$) is a result of the increased oxidative stress which led to the inhibition of the antioxidant enzymes. Similar results were exhibited in OP-poisoned Wistar rats (Kopjar *et al.*, 2018), OP-poisoned patients at the Zagazig Hospital, Egypt (Abbas *et al.*, 2016), OP-poisoned patients at Patil Medical College Hospital, India (Hundekari *et al.*, 2013), and in OP-poisoned rats (Ojha *et al.*, 2013). In the meantime, the increased oxidative stress by CPF-application was revoked by the application of CPF-BMB combinations compared to the positive control group ($P< 0.05$). Emphasized nutritional value and antioxidant activity of BMB' leaves have been presented (Iqbal *et al.*, 2012). In addition, they determined total phenolics (16.21–24.37 mg gallic acid equivalent/g), total flavonoids (26.41–31.28 mg rutin equivalent/g), and ascorbic acid (0.97–1.49 mg/g). The leaf extract was tested for TAC using three methods: the DPPH method (TAC= 1.89–2.12 mM Trolox equivalent/g), the ABTS method (TAC= 6.12–9.89 mM Trolox equivalent/g) and a ferric-ion method (0.56–0.97 mM Trolox equivalent/g) of dried leaves (Iqbal *et al.*, 2012).

The significant decrease of AChE as a result of OP-poisoning in the present study is harmonious with the results introduced by many previous investigations: OP-poisoned Wistar rats (Kopjar *et al.*, 2018), OP-poisoned patients at Zagazig Hospital, Egypt (Abbas *et al.*, 2016), OP-poisoned Wistar rats (Chanez *et al.*, 2015), OP-poisoned seventeen-year-old patient (Cavari *et al.*, 2013), OP-poisoned patients at Patil Medical College Hospital, India (Hundekari *et al.*, 2013), OP-poisoned common crap (Banaee *et al.*, 2013), OP-poisoned patients at Bijapur Hospital, India (Hundekari *et al.*, 2012), OP-poisoned rats (Ojha *et al.*, 2013), and OP-poisoned cutflower farmers (Del Pardo-Lu, 2007). AChE functions to regulate cholinergic neurotransmission by catalyzing breakdown of the neurotransmitter, acetylcholine. AChE is the main target of inhibition by OP compounds. They were considered a class of irreversible inhibitors of AChE (NPIC, 2012). Repeatedly, the effects of CPF application were overruled by the CPF-BMB administration. This may be attributed to the presence of many reversible AChE inhibitors in the BMB' leaf extract (de Freitas *et al.*, 2016), due to the atropine-sensitive prokinetic effects of the extract (Akhlaq *et al.*, 2016), resulting from the cholinomimetic substances which trigger the enzyme activity, or from the unblocking mechanism of the enzyme.

The significant increase of the LDH activity by CPF-poisoning indicates a severe cellular damage. This result coincides with past results of OP-poisoned patients admitted to Government Villupuram Medical College and Hospital, India (Sangeetha *et al.*, 2017), OP-poisoned patients admitted to emergency department of tertiary care Hospital, India (Swaminathan and Roopa, 2017), OP-poisoned fish, *Catla catla* (Abhijith *et al.*, 2016), OP-poisoned patients admitted to the emergency department of SCB Medical College Hospital, India (Panda *et al.*, 2014), and OP-poisoned common crap (Banaee *et al.*, 2013). Cellular damage is antagonized by the well-investigated anti-oxidant activity of BMB (Iqbal *et al.*, 2012; Zou *et al.*, 2012; Sánchez-Salcedo *et al.*, 2017; Zhang *et al.*, 2018), resulting in a dose-dependent significant correction of the LDH activity.

Levels of blood glucose, triglycerides and cholesterol were significantly lowered by the application of CPF insecticide. Many glycemic changes including hypoglycemia to hyperglycemia have been reported to be associated with OP-poisoning (Meller *et al.*, 1981; Shobha

and Prakash, 2000; Rahimi and Abdollahi, 2007; Banaee *et al.*, 2013; Malekirad *et al.*, 2013; Chanez *et al.*, 2015). These glycemic changes may be attributed to cholinergic changes associated with OP-poisoning and/ or to pancreatic dysfunction. In addition, significant decreases in triglycerides and cholesterol levels due to the OP-poisoning were observed. The decrease of blood glucose (BG), triglycerides (TG) and cholesterol (Chol) levels may be attributed to higher energetic needs to overcome OP-poisoning and/ or to counteract the oxidative stress induced by OP-poisoning. It may be a reflection of the lipid and glucose metabolism (liver dysfunction), too. Han *et al.* (2016) reported increased TG and Chol levels in OP-poisoned patients admitted to the Tertiary University Hospital, Korea. Chanez *et al.* (2015) observed a rise in the TG and Chol of OP-poisoned Wistar rats. Banaee *et al.* (2013) noticed increased TG and Chol levels in OP-poisoned common carp. Malekirad *et al.* (2013) presented increased Chol and BG levels and decreased TG levels in OP-poisoned farmers. Hundekari *et al.* (2012) reported a reduced Chol level in OP-poisoned patients at Bijapur Hospital, India. The change was insignificant in the case of creatinine index. Similarly, Ola-Davies *et al.* (2018) showed an insignificant change in dogs' creatinine after 8, 24, and 36 hours of OP-exposure. On the other hand, Cavari *et al.* (2013) and Raghu *et al.* (2014) described high creatinine levels in two OP- poisoned case studies. After proper treatment, the renal functions were corrected. Furthermore, an abnormal creatinine level was reported in OP-poisoned cutflower farmers (Del Pardo-Lu, 2007). Statistical analysis of DD-PCR results manifested the close genetic relation between negative control and CPF-BMB combinations by gathering them in the same phylogenetic group.

In conclusion, this piece of work stresses the detrimental effects of CPF insecticide and the protective capacity of the BMB extract. It was found that CPF results in adverse reactions that increase oxidative stress, cell damage, metabolic and enzymatic disorders. Most of these adverse reactions could be overruled by the confirmed antioxidant activity and by other suggested metabolic antitoxifying mechanisms. In addition, this work reveals that most of the investigated parameters could be considered good prognostic biomarkers.

Acknowledgments

The authors would like to thank Prof. Dr. Fatma H. Galal for her valuable criticism which helped improve the style of this manuscript.

Conflict of interest

The authors declare that there is no conflict of interest

References

Abass M, Arafa M, Elfakharany Y and Atteia H. 2016. Role of zinc deficiency in the susceptibility to delayed polyneuropathy in organophosphorus intoxicated patients in zagazig university hospitals, Zagazig, Egypt. *Egypt J Forensic Sci Appli Toxicol* **16(2):** 17-33.

Abhijith B, Ramesh M and Poopal R. 2016. Responses of metabolic and antioxidant enzymatic activities in gill, liver and plasma of *Catla catla* during methyl parathion exposure. *J Basic Appl Zool* **77:** 31-40.

Akhlaq A, Mehmood M, Rehman A, Ashraf Z, Syed S, Bawany S, Gilani A, Ilyas M and Siddiqui B. 2016. The prokinetic, laxative, and antidiarrheal effects of *Morus nigra*: Possible muscarinic, Ca (2+) channel blocking, and antimuscarinic mechanisms. *Phytother Res* **30(8):** 1362-1376.

Altuntas I, Delibas N and Sutcu, R. 2002. The effects of organophosphate insecticide methidathion on lipid peroxidation and antioxidant enzymes in rat erythrocytes: Role of vitamins E and C. *Hum Exp Toxicol* **21:** 681-685.

Ambali S, Abubakar A, Shittu M, Yaqub L, Anafi S and Abdullahi A. 2010. Chlorpyrifos-induced alteration of hematological parameters in Wistar Rats: Ameliorative effect of Zinc. *Res J Env Toxicol* **4(2):** 55-66.

Ambali S, Akanbi D, Igbokwe N, Shittu M, Kawu M and Ayo J. 2007. Evaluation of subchronic chlorpyrifos poisoning on hematological and serum biochemical changes in mice and protective effect of vitamin C. *J Toxicol Sci* **32(2):** 111-120.

Ambali S, Akanbi D, Oladipo O, Yaqub L and Kawu M. 2011. Subchronic chlorpyrifos-induced clinical, hematological and biochemical changes in Swiss albino mice: Protective effect of vitamin E. *Int J Biol Med Res* **2(2):** 497-503.

Banaee M, Haghi B and Ibrahim A. 2013. Sub-lethal toxicity of chlorpyrifos on common carp, *Cyprinus carpio* (Linnaeus, 1758): biochemical response. *Int J Aqu Biol* **1(6):** 281-288.

Cavari Y, Landau D, Sofer S, Leibson Tand Lazar I. 2013. Organophosphate poisoning-induced acute renal failure. *Pediatr Emer Care* **29:** 646-647.

Chanez L, Chahira R, Sofiane L, Samantha T, Larbi D, Daniel M, Philippe S and Giulia G. 2015. Environmental effect of parathion methyl on biochemical changes and detoxification capacity. *Int J Sci Eng Res* **6(5):** 361-367.

de Freitas M, Fontes P, Souza P, Fagg C, Guerra E, Nobrega Y, *et al.* 2016. Extracts of *Morus nigra* L. leaves standardized in chlorogenic acid, rutin and isoquercitrin: tyrosinase inhibition and cytotoxicity. *PLoS ONE* **11(9):** e0163130.

Del Prado-Lu J. 2007. Pesticide exposure, risk factors and health problems among cutflower farmers: a cross sectional study. *J Occup Med Toxicol* **2:9**, doi:10.1186/1745-6673-2-9.

Dobrzynska I, Szachowicz-Petelska B, Ostrowska J, Skrzydlewska E and Figaszewski Z. 2005. Protective effect of green tea on erythrocyte membrane of different age rats intoxicated with ethanol. *Chem Biol Interact* **156:** 41–53.

Ecobichon D. 1996. Toxic effects of pesticides, In: Klassen CD,(Ed) **Casarett and Doull's Toxicology**, (5th Ed.), McGraw-Hill, New York, pp. 643–698.

Goel A, Dani V and Dhawan D. 2006. Role of zinc in mitigating the toxic effects of chlorpyrifos on hematological alteration and electron microscopic observations in rat blood. *Bio Metals* **19(5):** 483-492.

Goel A, Dani V and Dhawan D. 2005. Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. *Chem Biol Interact* **156:** 131-140.

Gultekin F, Delibas N, Yasar S and Killinc I. 2001. *In vivo* changes in antioxidant systems and protective role of melatonin and a combination of vitamin C and vitamin E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in rats. *Arch Toxicol* **75:** 88-96.

Hafez E, Hashem M, Balbaa M, El-Saadani M and Ahmed S. 2013. Induction of new defensin genes in tomato plants via pathogens-biocontrol agent interaction. *J Plant Pathol Microb* **4:** 167.

Halliwell B and Chireco S. 1993. Lipid peroxidation: It's mechanism, measurements and significances. *Am Clin Nur* **57:** 715-725.

Han D, Kim D and Park Y. 2016. Blood lipid profile as a prognostic factor in patients with organophosphate poisoning. *Korean J Emer Med* **27(1):** 61-68.

Hundekari I, Suryakar A and Rathi D. 2013. Acute organophosphorus pesticide poisoning in North Karnataka, India: oxidative damage, haemoglobin level and total leukocyte. *Afr Health Sci* **13(1):** 129-136.

Hundekari I, Surykar A, Dongre N and Rathi D. 2012. Acute poisoning with organophosphorus pesticide: patients admitted to a hospital in bijapur, karnataka. *J Krishna Inst Med Sci Univ* **1 (1):** 38-47.

Iqbal S, Younas U, Sirajuddin, Chan K, Sarfraz R and Kamal Uddin M. 2012. Proximate composition and antioxidant potential of leaves from three varieties of mulberry (*Morus sp.*): A comparative study. *Int J Mol Sci* **13:** 6651-6664.

Khan M. 2006. Protective effect of black tea extract on the levels of lipid peroxidation and antioxidant enzymes in liver of mice with pesticide induced liver injury. *Cell Biol Funct* **24:** 332-372.

Kopjar N, Žunec S, Mendaš G, Micek V, Kašuba V, Mikolić A, Lovaković B, Milić M, Pavičić I, Čermak A, Pizent A, Vrdoljak A and Želježić D. 2018. Evaluation of chlorpyrifos toxicity through a 28-day study: Cholinesterase activity, oxidative stress responses, parent compound/ metabolite levels, and primary DNA damage in blood and brain tissue of adult male Wistar rats. *Chemico-Biologic Interact* **279:** 51–63.

Lee W, Alavania M, Hoppin J, Rusiecki J, Kamel F, Blair A and Sandler D. 2007. Mortality among pesticide applicators exposed to chlorpyrifos in the agricultural health study. *Environ Health Perspect* **115(4):** 528-534.

Liang P and Pardee A. 1992. Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. *Science* **157:** 967-971.

Luczaj W, Waszkiewicz E, Skrzydlewska E and Roszkowska-Jakimiec W. 2004. Green tea protection against age-dependent ethanol-induced oxidative stress. *J Toxicol Environ Health A* **67:** 595-606.

Malekirad A, Faghih M, Mirabdollahi M, Kiani M, Fathi A and Abdollahi M. 2013. Neurocognitive, mental health, and glucose disorders in farmers exposed to organophosphorus pesticides. *Arch Ind Hyg Toxicol* **64:**1-8.

Matsumoto M, Hara H, Chiji H and Kasai T. 2004. Gastroprotective effect of red pigments in black chokeberry fruit (*Aronia melanocarpa* Elliot) on acute gastric hemorrhagic lesions in rats. *J Agric Food Chem* **52(8):** 2226-2229.

Meller D, Fraser I and Kryger M. 1981. Hyperglycemia in anticholinesterase poisoning. *Can Med Assoc J* **124:** 745-748.

Mileson B, Chambers J, Chen W, Dettbarn W, Erich M, Eldefrawi A, Gaylor D, Hamernik K, Hodgson E, Karczmar A, Padilla S, Pope C, Richardson R, Saunders D, Sheets L, Sultatos L and Wallace K. 1998. Common mechanism of toxicity: A case study of organophosphorus pesticides. *Toxicol Sci* **41:** 8–20.

NPIC. 2012. Annual report of national pesticide information center. *Retrieved 27/ 6/ 2018*.

Ojha A, Yaduvanshi S, Pant S, Lomash V and Srivastava N. 2013. Evaluation of DNA damage and cytotoxicity induced by three commonly used organophosphate pesticides individually and in mixture, in rat tissues. *Environ Toxicol* **28(10):** 543-52.

Ola-Davies O, Azeez O, Oyagbemi A and Abatan M. 2018. Acute coumaphos organophosphate exposure in the domestic dogs: Its implication on haematology and liver functions. *Int J Vet Sci Med* **6:** 103-112.

Ostrowska J, Luczaj W, Kasacka I, Rozanski A and Skrzydlewska E. 2004. Green tea protects against ethanol-induced lipid peroxidation in rat organs. *Alcohol* **32:** 25-32.

Panda S, Nanda R, Mangaraj M, Mishra P and Rao E. 2014. Laboratory abnormalities in patients with organophosphorus poisoning. *Ind Med Gaz* **148(1):** 6-12.

Pope C. 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J Toxicol Environ Health, Part B* **2(2):** 161-181.

Raghu K, Kumar S, Haneef M and Kumar R. 2014. Acute Kidney Injury in Organophosphorous Poisoning, How Frequently You See? *Sch J Med Case Rep* **2(11):** 752-754.

Rahimi R and Abdollahi M. 2007. A review on the mechanisms involved in hyperglycemia induced by organophosphorus pesticides. *Pestic Biochem Phys* **88:** 115-121.

Rice-Evans C and Baysal E. 1987. Iron mediated oxidative stress in erythrocytes. *Biochem* **244:** 191-196.

Richardson R. 1995. Assessment of the neurotoxic potential of chlorpyrifos relative to other organophosphorus compounds, a critical review of the literature. *J Toxicol Environ Health* **44(2):** 135-165.

Richardson R, Moore T, Karjyali U and Randall J. 1993. Chlorpyrifos: assessment of potential for delayed neurotoxicity by repeated dosing in adult hens with monitoring of brain acetylcholinesterase, brain and lymphocyte neurotoxic esterase and plasma butyrylcholinesterase activity. *Fund Appl Toxicol* **2:** 89-96.

Sánchez-Salcedo E, Amorós A, Hernández F and Martínez J. 2017. Physicochemical properties of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves, a new food supplement. *J Food Nut Res* **5(4):** 253-261.

Sangeetha K, Mohandoss R, Ajay V, Deepavarshini P and Kumar G. 2017. Clinical utility of creatine kinase and lactate dehydrogenase as severity markers of organophosphorus compound poisoning. *Eur J Pharm Med Res* **4(1):** 597-601.

Savolainen K. 2001. Understanding the toxic actions of organophosphates. Agents 2. *In*: Krieger R., (Ed). **Handbook of Pesticide Toxicology**, Academic Press, New York, pp. 1013–1036.

Shobha T and Prakash O. 2013. Glycosuria in organophosphate and carbamate poisoning. *J Assoc Physicians India* **48:**1197-1199.

Shukla RK, Painuly D, Shukla A, Singh J, Porval A and Vats S. 2014. *In vitro* biological activity and total phenolic content of *Morus nigra* seeds. *J Chem Pharm Res*., **6(11):** 200-210.

Sies H and Masumoto H. 1997. Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. *Adv Pharmacol* **38:** 229-246.

Silver A. 1974. The Biology of Cholinesterases. American Elsevier Pub. Co., *Frontiers in Biology* **36:** 596.

Slotkin T, Levin E and Seidler F. 2006. Comparative developmental neurotoxicity of organophosphate insecticides: Effects on brain development are separable from systemic toxicity. *Environ Health Perspect* **114:** 746-751.

Sultatos L. 1987. The role of the liver in mediating the acute toxicity of the pesticide methyl parathion in the mouse. *Drug Metab Dispos* **15:** 613-617.

Sultatos L, Minor L and Murphy S. 1985. Metabolic activation of phosphorothioate pesticides: role of the liver. *J Pharmacol Exp Ther* **232:** 624-628.

Swaminathan K and Roopa T. 2017. A Study on biochemical parameters in patients with organophosphorus poisoning. *IOSR J Dent Med Sci* **16(10):** 16-19.

U.S. EPA. 2012. US Environmental Protection Agency FIFRA SAP Meeting on Chlorpyrifos Health Effects, April 10-13, **ID:** EPA-HQ-OPP-2012-0040.

Verma R and Srivastava N. 2001. Chlorpyrifos-induced alteration in levels of thiobarbitunc acid reactive substances and glutathione in rat brain. *Ind J Exp Biol* **39:** 174-177.

Verma R and Srivastava N. 2003. Effect of chlorpyrifos on thiobarbituric acid reactive substances, scavenging enzymes and glutathione in rat tissues. *Ind J Biochem Biophys* **40:** 423-428.

Wang S and Jiao H. 2000. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen. *J Agric Food Chem* **48(11):** 5677-5684.

Zhang H, Ma Z, Luo X and Li X. 2018. Effects of mulberry fruit (*Morus alba* L.) consumption on health outcomes: A mini-review. *Antioxidants* **7: 69**, doi:10.3390/antiox7050069.

Zou Y, Liao S, Shen W, Liu F, Tang C, Chen C-Y and Sun Y. 2012. Phenolics and antioxidant activity of mulberry leaves depend on cultivar and harvest month in southern china. *Int J Mol Sci* **13:** 16544-16553.