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Evaluation of sub-chronic chlorpyrifos poisoning on immunological and biochemical changes in rats and protective effect of eugenol

Ibrahim A. Elelaimy, Hany M. Ibrahim, Faten R. Abdel Ghaffar and Yahia S. Alawthan

Ibrahim A. Elelaimy, Hany M. Ibrahim, Faten R. Abdel Ghaffar
Department of Zoology,
Faculty of Science, Minufiya University, Shebin El-Kom, Egypt.

Yahia S. Alawthan
Department of Zoology,
Faculty of Science,
Ibb University, Yemen.

For Correspondence
Ibrahim A. Elelaimy
Department of Zoology,
Faculty of Science, Minufiya University, Shebin El-kom, Minufiya, Egypt.
Tel: +02-048-2362923
Fax: +02-048-223568

ABSTRACT

Chlorpyrifos (CPF) is one of the most widely used organophosphorus (OP) insecticides until 2000 when the United States Environmental Protection Agency restricted some of its domestic uses due to its toxicity. Despite this, CPF remains one of the most widely used OP insecticides. Eugenol is a flavoring agent used in cosmetic and food products. Furthermore, eugenol acts as a pro-oxidant and as an anti-oxidant, under certain circumstances. Therefore, the present study aims to evaluate the possible immuno-toxicogical consequences produced by CPF on different immunological aspects and to assess the protective role of eugenol in attenuating the CPF-induced immunotoxicity. The changes in humoral and cell mediated-immunity were evaluated by measuring the level of immunoglobulin (IgG), lymphocyte viability, neutrophil phagocytic function assay, total white blood cells count (WBC) and relative differential white blood cells count. On the biochemical level, estimation of nitric oxide (NO) level and catalase activity was also undertaken. The treatment with CPF showed an inhibitory effect on the level of lymphocyte viability, neutrophil phagocytic index, total white blood cells count, relative lymphocyte count, IgG concentration and catalase activity. On the other hand, a high level of NO was detected upon animal treatment with CPF. Interestingly, eugenol pre- and post-treatment to CPF-treated group improved the lymphocyte viability, total white blood cells count, relative lymphocyte count, catalase activity and the NO level. Moreover, eugenol pre- and post-treatment recovered phagocytic activity of neutrophils and restored IgG level. In conclusion, eugenol has protective and curative roles in attenuating the CPF-induced immunotoxicity.

Keywords: chlorpyrifos, eugenol, lymphocyte, phagocytosis, IgG.

INTRODUCTION

Chlorpyrifos (CPF) is one of the most widely used organophosphate (OP) insecticides until 2000 when the United States Environmental Protection Agency restricted some of its domestic uses due to its toxicity. Despite this, CPF remains one of the most widely used OP insecticides. Anemia and alteration in other hematological parameters have been recorded following repeated CPF exposure (Goel *et al.,* 2006b; Ambali *et al.,* 2010a).

Although the mechanism of acute CPF toxicity involves acetylcholinesterase (AChE) inhibition, other mechanisms unrelated to AChE inhibition, including the induction of oxidative stress, have been implicated (Gultekin et al., 2001; Ambali et al., 2007; Ambali et al., 2010a,b). As a lipophilic molecule, CPF easily passes through the cells into the cytoplasm (Uzun et al., 2010). Once inside the cell, CPF induces damage to the cellular molecules (Ncibi et al., 2008).

OP pesticides were reported as immunotoxicants in the early 1970s by Ercegovich (1973) and Street and Sharma (1975). In addition, Street and Sharma (1975) observed thymic atrophy and reductions in splenic germinal centers in rabbits following exposure to 1.5 mg/kg/day of malathion. A number of organophosphate pesticides including malathion, methylparathion and trichlorphon have displayed immunotoxicity in laboratory studies (Galloway and Handy, 2003). Casale et al. (1983) noticed suppression of primary humoral immune responses to a T-cell-dependent antigen in rodents treated orally with cholinergic doses of parathion (16 mg/kg), malathion (720 mg/kg), or dimethyl dichlorovinyl phosphate (DDVP; 120 mg/kg). the authors found that suppression was absent at noncholinergic doses, suggesting that stress may have played a role. Furthermore, Casale et al. (1989) compared several organophosphate compounds for their ability to inhibit human serum complement-mediated lysis of sheep red blood cells (SRBCs).

The effects of CPF on selected functions of immune system in male Fisher 344 rats, CPF (5.0 mg/kg twice weekly for 28 day) was found to impaire T-lymphocyte blastogenesis induced by concanavalin and phytohemagglutinin (Blakley et al., 1999). Also humoral immunity (anti-SRBCs), a T-lymphocyte macrophage-dependent response, was also reduced (Blakley et al., 1999). On the other hand, CPF increased the relative percentage expression of $CD5^+$ and $CD8^+$ (Blakley et al., 1999). In this study, it has been selected an OP insecticide, CPF, which is presently being most widely used for a variety of agricultural and public health applications throughout the world (Richardson, 1995). A resultant consequence of extensive use of CPF is that more human population and other nontarget organisms will become exposed to it and would thus increase the frequency of toxic sequelae attributed to this compound (Sultatos, 1994). In order to interpret such effects and the mechanistic understanding of its toxicity, there is an essential need to study the status of the immune response to CPF intoxication.

Eugenol is the main component of oil of cloves that originate from the *Syzygium* species (Kollmannsberger and Nitz, 1994) and is also present in the essential oils of many other plants, including cinnamon, basil and nutmeg (Rompelberg et al., 1996a,b). It is a flavoring agent used in cosmetic and food products (Opdyke, 1975) and It is widely used in dentistry as a cement material with zinc oxide or as a sedative agent (Markowitz et al., 1992). Eugenol molecules, like other antioxidant compounds, acts as a pro-oxidant, (A substance that can produce oxygen byproducts of metabolism that can cause damage to cells), and as an anti-oxidant, (A substance that protects cells from the damage caused by free radicals), (Atsumi et al., 2000; Ogata et al., 2000; Fujisawa et al., 2002) under certain circumstances. In a study to evaluate the effect of eugenol on antioxidant status of rat intestine, Vidhya and Devaraj, (1999) suggested that eugenol is nontoxic, protective and induces glutathionine S-transferase (GST) and thereby it may facilitate the removal of toxic substances from the intestine. Actually, there are no reports of the eugenol protective effects on CPF induced immunotoxicity in rats. Therefore, the aim of the present study was extended to assess the protective and curative role of the eugenol in attenuating the CPF-induced toxicity.

MATERIALS & METHODS

Insecticide used

Chlorpyrifos

0,0-diethyl 0-(3,5,6-trichloro-2-pyridyl) phosphoro-thioate was obtained commercially at a concentration of 48% from Help Pesticides and Chemical Company, Free zone, New Damietta, Egypt. The desired concentrations were prepared freshly when needed by diluting the pesticide with corn oil. The oral LD50 of chlorpyrifos was 135 - 163 mg/kg in Wister rats (Worthing and Walker, 1987).

Tested material

Eugenol

2-Methoxy-4-[2-propenyl] phenol (C_{10} H_{12} O_2) was purchased from Sigma, USA.

Experimental animals

Five to six weeks old, healthy male albino Wister rats with average weight of 100 – 120 g were obtained from the Egyptian Organization for Serology and Vaccination, Ministry of Health, Cairo, Egypt. All animals were kept under controlled laboratory conditions in the animal room, Zoology Department, Faculty of Science, Minufiya University. Animals were housed in standard plastic rodent cages with enough space for their activity. Standard rodent food and clean water were supplied *ad libitum.* The animals were acclimatized to laboratory condition for at least one week before the initiation of the experiments. The animals were used after approval of Institutional Animal Ethical Committee.

Experimental protocol

Exp. 1: Protective action of eugenol

The experimental animals were divided into four groups as follows:

G (1): (10 rats) served as control group and orally received only vehicle corn oil. 5 rats received the vehicle for one week and 5 rats received the vehicle for two weeks.

G (2): (10 rats) were orally administered with 100mg/kg/day eugenol. 5 rats received the eugenol for one week and 5 rats received the eugenol for two weeks.

G (3): (10 rats) were orally administered 13.5mg/kg/day CPF. 5 rats received the CPF for one week and 5 rats received the CPF for two weeks.

G (4): (10 rats) received eugenol (100mg/kg/day) orally, 1 hour before the CPF treatment. 5 rats received the eugenol followed by CPF for one week and 5 rats received the eugenol followed by CPF for two weeks.

All treatments were continued daily and the animals were sacrificed at the end of the 1st or 2nd week of the treatments.

Exp. 2: Curative effect of eugenol

The experimental animals were divided into two main groups:

G (1): (10 rats) served as control group and received only vehicle, corn oil.

G (2): (20 rats) received orally the CPF only (25mg/kg/twice weekly) for four weeks then, subdivided into two subgroups as follows:

SG (a): (10 rats) received the vehicle alone orally 5 rats received the vehicle daily for one more week and 5 rats received the vehicle for two weeks.

SG (b): (10 rats) received eugenol (100mg/kg/day) orally 5 rats received for one week and 5 rats received for two weeks.

The animals were sacrificed at the end of the 5th or 6th week of the treatment.

Blood and tissue sampling

At the end of the designed period, animals were anesthetized with halothane, dissected immediately, and blood was collected from the hepatic portal vein of the rats. Each blood sample was divided into two tubes, one of them was mixed with heparin and the other was permitted to clot. The tubes were centrifuged in a cooling centrifuge at 3000 rpm for 15 min to separate blood serum. The separated serum was sampled into clean tubes and kept in a deep-freezer at -20 ^{o}C.

Liver was homogenized in ice-cold 100 mM phosphate buffer (pH 7.4) using a Potter–Elvehjem homogenizer fitted with a Teflon Plunger. The homogenates were centrifuged at 14,000 ×g for 20 min and the resulting supernatant was kept in a deep-freezer at -20 ^{o}C till further analyses were performed.

Immunological parameter

Total leukocytes count (TLC), relative differential white blood cells count; was done manually according to method described by Dacie and Lewis, (1991).

Lymphocyte viability assay

Lymphocytes were isolated from the heparinized blood sample according to the method of Boyum et al. (1968). Briefly, lymphocytes were isolated from blood by density gradient centrifugation on Histopaque-1077. The mononuclear cells from the buffy coat were carefully pipetted out with sterilized Pasteur pipette and pelleted at 400 ×g for 10 min. The cell pellet was suspended in 1 ml of growth medium (RPMI-1640) containing 10% fetal bovine serum (FBS) and recommended concentration of antibiotic solutions. The viability of lymphocytes was estimated by trypan blue exclusion method (Leffell, 1990).

Phagocytic activity of the neutrophils

Briefly, neutrophils were isolated from the heparinized blood sample according to the method of Markert et al. (1984) 0.1 ml of aliquot of cell having density of 10×10^6 cells/ml was mixed with 0.1 ml RPMI-1640 containing 20% fetal calf serum (FCS) and 100×10^6 cells/ml heated treated yeast cells. The mixture was incubated for 60 min at 37°C with occasional shaking. The tubes were then immersed in ice cold water to stop the reaction. The phagocytic index was estimated by checking the phagocytic cells under ordinary light microscope according to the method of (Timothy et al., 1997).

IgG concentration

IgG levels in the blood serum of animals were measured by using radial immunodiffusion (RID) plates (The binding site Co. UK) according to the manufacturer's recommendations.

Biochemical parameters

Catalase activity

Protein content was determined in liver homogenate for the assessment of catalase enzyme according to the method of Lowry et al. (1951). Catalase activity as antioxidant enzyme was determined in the liver homogenate according to the method of Aebi, (1974).

Nitric oxide (NO) content

NO content of the liver homogenate was determined as nitrite and nitrate by spectrophotometer according to previously described method (Miranda et al., 2001).

Statistical analysis

For statistical analysis the SPSS computer program was used. The statistical analysis was carried out by one-way ANOVA setting the probability level to $P<0.05$, *post hoc* analysis of group differences was performed by LSD test. The treated groups were compared both with each other and with untreated control groups.

RESULTS

Pre-treatment (protective) effect of eugenol

Total leukocytes count

As shown in Table (1a), CPF significantly reduced (P<0.001) the total leukocytes count at the 2nd week of intoxication compared to control group. Furthermore, eugenol pre-treatment to CPF-intoxicated animals resulted in a significant elevation (P<0.001) in the total leukocytes count when compared to that of CPF treated group alone. The data also showed that the treatment of rats with eugenol alone for the same period had no significant change in the total leukocytes count when compared to control group.

Differential leukocytes count

CPF treatment for two weeks caused a significant decrease (P<0.05) in the relative lymphocytes count and a significant increase in relative granulocyte counts. The relative

monocytes count remains without any significant change. Fortunately, eugenol pre-treatment to CPF intoxicated animals improved the over all changes and restored back the differential counts to the normal (Table 1b).

Table. (1a): Total leukocytes count in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Groups	TLC ($10^3/mm^3$)			
	1st week		2nd week	
	Mean	**± SD**	**Mean**	**± SD**
Control	13.9 [a]	± 0.67	14.0 [a]	± 0.69
Eugenol	14.2 [a]	± 0.79	14.2 [a]	± 0.79
Chlorpyrifos (CPF)	13.6 [a]	± 0.66	10.5 [b] ***	± 0.94
Eugenol + CPF	13.6 [a]	± 0.76	13.0 [ac] ***	± 0.82

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Table. (1b): Differential leukocytes count in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Parameter	Groups	Treatment Period			
		1st week		2nd week	
		Mean	**±SD**	**Mean**	**±SD**
LYMPHOCYTES COUNT (%)	Control	52.8 [a]	± 3.96	53.0 [a]	± 3.16
	Eugenol	54.6 [a]	± 4.04	52.8 [a]	± 3.70
	Chlorpyrifos CPF)	51.6 [a]	± 2.96	46.4 [b***]	± 4.03
	Eugenol+ CPF	53.8 [a]	± 2.86	51.6 [ac*]	± 3.50
MONOCYTES COUNT (%)	Control	8.2 [a]	± 0.83	8.8 [a]	± 0.84
	Eugenol	8.8 [a]	± 0.83	9.4 [a]	± 0.14
	Chlorpyrifos (CPF)	3 9.0 [a]	± 0.71	3 9.6 [a]	± 1.14
	Eugenol+ CPF	3 8.8 [a]	± 0.84	3 9.4 [a]	± 1.14
GRANULOCYTES COUNT (%)	Control	39.0 [a]	± 3.39	38.2 [a]	± 2.77
	Eugenol	36.6 [a]	± 3.57	37.8 [b]	± 2.77
	Chlorpyrifos (CPF)	39.4 [a]	± 2.30	44.0 [b**]	± 3.46
	Eugenol+ CPF	37.4 [a]	± 3.29	39.0 [ac*]	± 2.54

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001) Means in the same columns assigned with the same letter show insignificant differences between these values.

Lymphocytes viability

As shown in Table (1c), CPF significantly reduced (*P*<0.001) the viability of lymphocytes at the 2nd week of intoxication (78.6±4.9%) compared to that of the control group (92.2±4.7%).

In contrary, the viability of rat lymphocytes was significantly increased (*P*<0.05) upon pre-treatment with eugenol to CPF intoxicated animals (85.8±5.3%) when compared to that of CPF treated group (78.6±4.9%). The data also showed that treatment of rats with eugenol alone for the same period had no significant change in the lymphocyte viability when compared to the control group.

Table. (1c): Lymphocytes viability in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Groups	LYMPHOCYTE VIABILITY (%)			
	1st week		2nd week	
	Mean	**± SD**	**Mean**	**± SD**
Control	91.2 [a]	± 3.1	92.2 [a]	± 4.7
Eugenol	90.0 [a]	± 3.5	90.8 [a]	± 3.7
Chlorpyrifos (CPF)	88.6 [a]	± 3.4	78.6 [b] ***	± 4.9
Eugenol + CPF	90.4 [a]	± 3.4	85.8 [c] *	± 5.3

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Phagocytic index

Table (1d), illustrates the effect of CPF and eugenol on the phagocytic function of neutrophils, expressed as phagocytic index. A significant decrease (P<0.01) was observed in rats upon the treatment with CPF at the 2nd week of intoxication (64.2±2.8%) when compared to control animals (77±2.9%). On the other hand, eugenol pre-treatment to CPF-treated group significantly increased phagocytic index (74.4±5.3%) when compared to that of CPF treated group.

Table. (1d): Phagocytic index in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Groups	PHAGOCYTIC INDEX (%)			
	1st week		2nd week	
	Mean	**± SD**	**Mean**	**± SD**
Control	76.2 [a]	± 3.3	77.0 [a]	± 2.9
Eugenol	76.2 [a]	± 6.2	79.2 [a]	± 3.7
Chlorpyrifos (CPF)	74.6 [a]	± 4.9	64.2 [b] **	± 2.8
Eugenol + CPF	75.4 [a]	± 3.9	74.4 [ac] **	± 5.3

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Immunoglobulin G concentration (IgG)

The level of immunoglobulin G (IgG) in serum of male rats was shown in Table (1e). The results indicated that CPF alone caused a significant decrease (*P*<0.05) in the level of IgG at the 2nd week of treatment (8368±1192 mg/L) when compared to control animals (10252±555 mg/L). On the other hand, pre-treatment with eugenol for two weeks improved (*P*<0.05) the IgG level (10188±509 mg/L) when compared to the CPF group.

Table. (1e): Serum immunoglobulin (IgG) Levels in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Groups	IgG Conc. (mg/L)			
	1st week		2nd week	
	Mean	**± SD**	**Mean**	**± SD**
Control	10412 [a]	± 731	10252 [a]	± 555
Eugenol	11100 [a]	± 632	10632 [a]	± 501
Chlorpyrifos (CPF)	10800 [a]	± 877	8368 [b] *	± 1192
Eugenol + CPF	10632 [ab]	± 755	10188 [ac] *	± 509

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Catalase activity (CAT)

Catalase activity exhibited highly significant inhibition (P<0.001) at 1st and 2nd weeks of CPF treatment when compared to that of the control animals. As shown in Table (1f), levels of CAT at 1st and 2nd week of CPF treatment were 35.14±1.92 and 21.34±3.41, compared to that of control group (42.72±1.80 and 44.53±2.00) respectively. There was no significant difference between the eugenol treated animals and the control group. Eugenol pre-treatment to the CPF-intoxicated animals resulted in a significant elevation (P<0.001) in the catalase activity (39.51 ±1.75, 35.21 ±2.42) when compared to the CPF group.

Table. (1f): Liver catalase (CAT) activities in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Groups	CATALASE ACTIVITY (10^{-3} U/ mg protein / Sec.)			
	1st week		2nd week	
	Mean	± SD	Mean	± SD
Control	42.72 [a]	± 1.80	44.53 [a]	± 2.00
Eugenol	43.82 [a]	± 3.51	43.06 [a]	± 3.72
Chlorpyrifos (CPF)	35.14 [b] ***	± 1.92	21.34 [b] ***	± 3.41
Eugenol + CPF	39.51 [c] **	± 1.75	35.21 [c] ***	± 2.42

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Nitric oxide levels

Liver nitric oxide levels are shown in Table (1g), the results demonstrate that CPF significantly increased (P<0.001) NO levels in livers male rats after two weeks compared to control group.

The mean level of NO at 2nd week of CPF treatment was 4.40±0.21 compared of that of control group (3.62±0.36). On the other hand, after two weeks eugenol pre-treatment to the CPF-intoxicated animals normalized the nitric oxide content (3.44±0.32) compared of that of control group.

Table. (1g): Liver nitric oxide (NO) levels in chlorpyrifos intoxicated male rats pre-treated with eugenol.

Groups	NO LEVELS (mmoles/g tissue)			
	1st week		2nd week	
	Mean	± SD	Mean	± SD
Control	3.39 [a]	± 0.34	3.62 [a]	± 0.36
Eugenol	3.42 [a]	± 0.27	3.61 [a]	± 0.35
Chlorpyrifos (CPF)	3.81 [b]	± 0.34	4.40 [b] ***	± 0.21
Eugenol + CPF	3.41 [ac]	± 0.21	3.44 [ac] ***	± 0.32

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Post-treatment (Curative) effect of eugenol

Total leukocytes count

As shown in Table (2a), four weeks of CPF treatment significantly reduced (P<0.001) the total leukocytes count when compared to control group. Furthermore, eugenol post-treatment to CPF intoxicated animal resulted in a normalization of the total leukocytes count when compared to the CPF treated group alone.

Table. (2a): Total leukocytes count following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Groups	TLC (10^3/mm^3)			
	5th week		6th week	
	Mean	± SD	Mean	± SD
Control	13.4 [a]	± 0.98	13.7 [a]	± 0.72
Chlorpyrifos (CPF)	9.6 [b] ***	± 0.81	11.5 [b] ***	± 0.76
CPF + Eugenol	12.0 [c] **	± 0.97	13.0 [ac] **	± 0.81

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD).
Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Differential leucocytes count

CPF intoxication of animals caused a significant decrease (P<0.05) in the relative lymphocytes and a significant increase in relative granulocyte counts, without any significant change on the relative monocytes count. Eugenol post-treatment to CPF intoxicated animals showed a remarkable improvement of the over all changes (Table 2b).

Table. (2b): Relative differential leukocytes count following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Parameter	Groups	Treatment Period			
		5th week		6th week	
		Mean	±SD	Mean	±SD
LYMPHOCYTES COUNT (%)	Control	52.2 [a]	± 3.49	51.0 [a]	± 3.16
	Chlorpyrifos (CPF)	43.0 [b**]	± 2.83	46.4 [a]	± 4.04
	CPF+ Eugenol	48.2 [ac*]	± 4.03	51.6 [ac*]	± 3.51
MONOCYTES COUNT (%)	Control	8.6 [a]	± 0.90	8.6 [a]	± 0.89
	Chlorpyrifos (CPF)	9.4 [a]	± 0.55	9.6 [a]	± 1.14
	CPF+ Eugenol	3 8.8 [a]	± 0.83	3 9.4 [a]	± 1.14
GRANULOCYTES COUNT (%)	Control	39.2 [a]	± 2.77	40.4 [a]	± 2.07
	Chlorpyrifos (CPF)	47.6 [b**]	± 2.79	44.0 [a]	± 3.46
	CPF+ Eugenol	43.0 [ac*]	± 4.53	39.0 [ac*]	± 2.54

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD).
Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001) Means in the same columns assigned with the same letter show insignificant differences between these values.

Lymphocyte viability

CPF treatment significantly reduced (P<0.001) the viability of lymphocytes compared to control group (Table 2c). Moreover, eugenol post-treatment to CPF intoxicated animals significantly increased (P<0.01) the viability of rat lymphocytes when compared to the CPF treated group.

Table. (2c): Lymphocytes viability following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Groups	LYMPHOCYTE VIABILITY (%)			
	5th week		6th week	
	Mean	± SD	Mean	± SD
Control	93.40 [a]	± 4.22	93.00 [a]	± 4.00
Chlorpyrifos (CPF)	74.20 [b] ***	± 3.70	82.80 [b] **	± 5.89
CPF + Eugenol	82.60 [c] **	± 6.47	91.20 [ac] **	± 3.49

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. [b] Significantly different from controls. [c] Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Phagocytic index

As shown in Table (2d), At 5th and 6th week, CPF treatment significantly reduced (P<0.01) the phagocytic index of rat neutrophil (56.80±4.15% and 63.40±3.36%) when compared to control group (76.20±3.42% and 76.20±3.78%) respectively, whereas eugenol post-treatment to CPF intoxicated animals significantly increased (P<0.01) the phagocytic index (62.80 ±3.35%, 71.80 ±2.77%) respectively, when compared to the CPF treated group.

Table. (2d): Phagocytic index following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Groups	PHAGOCYTIC INDEX (%)			
	5th week		6th week	
	Mean	± SD	Mean	± SD
Control	76.20 a	± 3.42	76.20 a	± 3.78
Chlorpyrifos (CPF)	56.80 b **	± 4.15	63.40 b **	± 3.36
CPF + Eugenol	62.80 c **	± 3.35	71.80 ac **	± 2.77

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. b Significantly different from controls. c Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Immunoglobulin G concentration (IgG)

As shown in Table (2e), At the 6th week of CPF treatment the serum IgG concentration was significantly reduced (8692±790 mg/L) when compared to control group (10420±766 mg/L), while eugenol post-treatment to CPF intoxicated animals significantly increased (P<0.05) and restored the IgG level (10128 ±1048 mg/L) when compared to that of CPF treated group. Similar results were obtained at the 5th week of treatment.

Table. (2e): Serum immunoglobulin (IgG) concentration following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Groups	IgG Conc. (mg/L)			
	5th week		6th week	
	Mean	± SD	Mean	± SD
Control	10260 a	± 0665	10420 a	± 0766
Chlorpyrifos (CPF)	07628 b **	± 1102	08692 b *	± 0790
CPF + Eugenol	08720 b	± 1449	10128 ac *	± 1048

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. b Significantly different from controls. c Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Catalase activity (CAT)

CPF treatment for four weeks caused a significant inhibition (*P*< 0.001) in the liver catalase activity when compared to that of the control animals.

As shown in Table (2f), the levels of catalase at 5th and 6th week of CPF treatment were 25.31±4.20 and 29.25±2.99 respectively, compared of that of control group (42.85±1.72 and 42.51±2.00). At 5th and 6th week, eugenol post-treatment to the CPF-intoxicated animals resulted in a significant elevation (*P*<0.001) in the liver catalase activity (32.92±3.99, 38.81±2.71), respectively, when compared to that of CPF treated group.

Table. (2f): Liver catalase (CAT) activities following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Groups	CATALASE ACTIVITY (10^{-3} U/ mg protein / Sec.)			
	5th week		6th week	
	Mean	± SD	Mean	± SD
Control	42.85 a	± 1.72	42.51 a	± 2.00
Chlorpyrifos (CPF)	25.31 b ***	± 4.20	29.25 b ***	± 2.99
CPF + Eugenol	32.92 c ***	± 3.99	38.81 ac ***	± 2.71

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. b Significantly different from controls. c Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

Nitric oxide levels (NO)

As shown in Table (2g), CPF treatment for four weeks significantly increased (*P*<0.01) the nitric oxide content in rat liver when compared to control group. The levels of NO at 5th and 6th week of CPF treatment were 4.62±0.24 and 4.44±0.32, compared of that of control group (3.36±0.29 and 3.42±0.42) respectively. Eugenol post-treatment to CPF intoxicated animals normalized the NO levels (4.01±0.23, 3.76±0.33) when compared to CPF-treated animals.

Table. (2g): Liver nitric oxide (NO) levels following eugenol post-treatment to male rats intoxicated with chlorpyrifos.

Groups	NO LEVELS (mmoles/g tissue)			
	5th week		6th week	
	Mean	± SD	Mean	± SD
Control	3.36 a	± 0.29	3.42 a	± 0.42
Chlorpyrifos (CPF)	4.62 b **	± 0.24	4.44 b **	± 0.32
CPF + Eugenol	4.01 c **	± 0.23	3.76 ac **	± 0.33

Number of animals/group = 5, Data are expressed as: mean ± standard deviation (SD). Means assigned with the same letter show insignificant differences between these values. b Significantly different from controls. c Significantly different from CPF-treated animals. Significant * (P<0.05), High significant ** (P<0.01), and Very high significant *** (P<0.001)

DISCUSSION

The present study investigated the immunotoxic effect of CPF commonly used in agricultural purposes. Furthermore, it was extended to investigate the ability of eugenol to modulate the immunotoxic effect of this pesticide.

The results of the present study demonstrated a significant decrease in the total leukocytes and relative lymphocytes counts and a significant increase in relative granulocytes count in rats treated with CPF when compared to that of the control group. However no significant change was demonstrated upon the treatment of eugenol.

These findings are consistent with the results of Goel et al. (2006a) who reported that CPF treatment decreased the total leukocytes and lymphocytes count and caused an elevation in the neutrophils count. Neutrophils are the first line of defense against infectious agents, tissue injury, parasites and inflammatory or foreign materials and exert their activity by eliminating foreign material by phagocytosis (Kobayashi et al. 2003). So, the observed decrease in leukocyte counts following intoxication with CPF could be attributed either to the slower rate of production of leukocytes or due to their inhibited release into the blood circulation (Goel et al., 2006a). A similar decrease in leukocyte counts was observed in rodents which were intoxicated with

another organophosphate, monochrotophos for a long term study (Janardhan and Sisodia 1990). A significant decrease in the total bone marrow cells count was indicated to be a plausible rationale for the observed depression in the total and differential counts of leukocytes of the rats exposed to chronic doses of primiphos-methyl (Rajini et al. 1987). Also, in a study to evaluate haemato-biochemical and immunopathophysiological changes following feeding of broiler chicks with 20 ppm fenvalerate (synthetic pyrethroid), 2 ppm monocrotophos (organophosphate) and 2 ppm endosulfan (chlorinated hydrocarbon), Garg et al. (2004), found that total leukocytes and T-lymphocytes count was lower in all treated groups as compared to control group. Recent studies revealed that, leukopenia following CPF treatment, apparently due to lymphopenia, neutropenia, and monocytopenia in the CPF treated animals (Ambali et al., 2007; Ambali et al., 2011).

The present results also, demonstrated that eugenol pre or post-treatment to CPF intoxicated rats normalized the otherwise altered levels of white blood cell counts when compared to CPF treated alone. These finding are consistent with the results of Goel et al. (2006a) who showed that zinc coadministration to CPF treated animals raised the otherwise decreased total leukocytes count. Also, zinc treatment to the CPF poisoned animals significantly improved the overall lymphocyte and neutrophil counts (Goel et al., 2006a).

The present results also, demonstrated a significant reduction in lymphocytes viability in CPF-intoxicated rats compared to those of the control group. Co-exposure of CPF with eugenol restored the cell viability. Many reports have identified two potential cellular targets for CPF, cell signaling cascades from one side and the expression and function of gene transcription factors from the other side (Song et al., 1997; Crumpton et al., 2000a,b). Reactive oxygen interacts with receptors, second messengers and transcription factors, altering gene expression and influencing cell growth and survival (Palmer and Paulson, 1997). Crumpton et al. (2000a,b) showed that when PC12 cell suspensions were treated acutely with CPF for 10 min, ROS generation was increased in a concentration- dependent manner. The present results are in consistent with previous studies especially that reported by Gultekin et al. (2006) who noticed that CPF decreased the viability of HepG2 cells in a dose dependent manner and the pre-incubation with melatonin prior to CPF application caused an increase in cell viability. Also, Raha et al. (2005) demonstrated that 10mM H_2O_2 produced a significant reduction in viability of cultured human microvascular endothelial cells and the H_2O_2-induced alterations were completely prevented by pre-incubating the cells with 10 μg/ml green tea polyphenol for 1hour. Moreover, Ou et al. (2006) illustrated that eugneol inhibited the reactive oxygen species (ROS) generation, intracellular calcium accumulation, and the subsequent mitochondrial membrane potential collapse, cytochrome c release and caspase-3 activation induced by oxLDL. The cytotoxicity and apoptotic features induced by a cytotoxic concentration (200 microg/ml) of oxLDL were also attenuated by eugenol (Ou et al., 2006). Recent study demonstrated that there was a significant increase in the level of radical generation, NADPH oxidase and myeloperoxidase activity, lipid, protein, DNA damage and oxidized glutathione level following treatment with nicotine, which were significantly reduced by eugenol and N-acetylcysteine supplementation (Kar Mahapatra et al., 2009). Our findings together with the previously mentioned ones suggest the potential use and benefit of eugenol as a modulator of CPF-induced cellular damage and it may be used as an immunomodulatory drug against CPF toxicity.

Phagocytic index showed a significant reduction in CPF-intoxicated rats compared to those of the control group. Co-exposure of CPF with eugenol recovered the CPF-inhibitory effect on the phagocytic function of neutrophils. In this respect few reports have been done to evaluate the effects of CPF on phagocytosis therefore, the current data come in agreement with the others especially those recorded by Queiroz et al. (1999) who reported that there was a considerable reduction in the ability of neutrophils from exposed workers to carbamate and OP pesticides to kill *Candida albicans*. In the pervious report the authors concluded that exposure to carbamates and OP insecticides may lead to changes in neutrophils function even in workers presenting no impairment in the cholinesterase (ChE) activity (Queiroz et al. 1999). Also, Wysocki et al. (1987) showed a significant decrease in the neutrophils activity in workers exposed to OP pesticides as demonstrated using nitroblue- tetrazolium test (NBT) and this reduction was in linear correlation with a reduced ChE activity.
Similar obtained results were reported by Harford et al. (2005) who demonstrated that endosulfan and CPF caused a significant reduction in the phagocytic function of head kidney cells from four native Australian fish, but the results of endosulfan was more significant than those observed by CPF. Also, Siwicki et al. (1990) reported that trichlorphon an OP insecticide decreased phagocytic ability of neutrophils and in phagocytic index in carp (*Cyprinus carpio*) at high dose. Moreover, Chang et al. (2006) supported those observed results and reported that phagocytic activity and clearance efficiency to *L. garvieae* significantly decreased when prawns were exposed to 0.2 and 0.4 mg L^{-1} trichlorfon for 48 h.
The improvement in the phagocytic action in animals pre or post-treated with eugenol can explained by the data of Kar Mahapatra et al. (2011) who demonstrated that both phagocytosis indexes and chemotactic indexes of murine macrophages were significantly reduced upon the nicotine treatment, which were significantly improved in a time-dependent increase when cells were treated with eugenol and N-acetylcysteine.

Moreover, the present data revealed that CPF significantly decreased the serum immunoglobulin G (IgG) concentration; however, the co-adminstration of CPF with eugenol significantly restored the IgG level. These results were consistent with some previous reports, Blakley et al. (1999) reported that humoral immunity (anti-sheep red blood cell), a T-lymphocyte macrophage dependent response, was reduced in rats when CPF administered by oral gavage twice weekly for 28 days at a dose of 5.0 mg/kg. Also, Aly and El-Gendy, (2000) found that a single oral dose of dimethoate (16 mg/kg) significantly decreased serum total immunoglobulins (Ig) and IgM, while IgG was non-significantly

deceased. Furthermore, the results of the same study also revealed that dimethoate caused a significant decrease in the number of plaque forming cell (PFC) in a time dependent manner.
Banerjee et al. (1998) showed that sub-chronic doses of malathion exposure caused an attenuation in antigen-induced antibody response, suppression of PFC and induced differential degrees of humoral and cell-mediated immune suppression in male albino mice, rats and rabbits. The organophosphate-induced immunosuppression may result from direct action of acetylcholine upon the immune system or it may be secondary to the toxic chemical stress associated with cholinergic poisoning (Casale et al., 1983). Kowalczyk-Bronisz et al. (1992) found that the pesticide chlorfenwinfos exerts immunotropic effect in mice and after high doses the strong suppressive effect in PFC and E-rosettes was observed. The significant increase in IgG antibodies following eugenol pre or post-treatment in CPF-intoxicated animals may explained by Hilton et al. (1996) who showed that exposure to eugenol, as respiratory allergens, was associated with a statistically significant increase in serum IgE concentrations when initial application concentrations of 2.5% were used. Moreover, the percentage of B cells ($B220^+$ or IgG/IgM^+ cells) was increased with isoeugenol, and eugenol as tested allergens using the local lymph node assay (Gerberick et al., 2002).

The present results also, showed that the treatment with CPF significantly caused an inhibition in the liver CAT and increased the liver NO level, while eugenol pre or post-treatment to CPF-intoxicated animals improved their activities. These results are coincident with those obtained by Goel et al. (2005) who reported that CPF intoxication (13.5mg/kg/day) caused a significant inhibition in the level of CAT activity in the liver of male Wistar rats. Gultekin et al. (2000) showed that, CPF-ethyl caused an in vitro increase in lipid peroxidation in human erythrocytes. Also, OP insecticides such as phosphomidon, trichlorfon and dichlorvos have been reported to induce an oxidative stress as shown by enhanced malondialdehyde (MDA, a marker of lipid peroxidation) production (Naqvi and Hasan, 1992; Yamano and Morita, 1992).

Oxygen free radicals and hydroperoxides, collectively termed reactive oxygen species (ROS), are produced by the univalent reduction of dioxygen to superoxide anion (O_2-), which in turn disproportionate to H_2O_2 and O_2 either spontaneously or through a reaction catalyzed by superoxide dismutase (SOD) (McCord and Fridovich, 1969). Endogenous H_2O_2 may be converted by CAT to H_2O (Kehrer, 1993) or otherwise it may generate the highly reactive free hydroxyl radical (OH−) by the Fenton reaction (McCord and Dary, 1978; Rigo et al., 1977), which is widely believed to be mainly responsible for oxidative damage (Halliwell and Gutteridge, 1984; Halliwell and Gutteridge, 1990). The low levels of CAT following the CPF treatment could possibly be contributed to the consumption of this enzyme in converting the H_2O_2 to H_2O (Goel et al., 2005). It has been also shown that CAT activity was inhibited by free radicals, such as singlet oxygen and superoxide and peroxyl radicals (Kono and Fridovich, 1982; Escobar et al., 1996). Therefore, CAT may be inhibited by both CPF itself and increased ROS induced by CPF (Gultekin et al., 2006).

The present results also demonstrated that the treatment with CPF significantly increased the liver nitric oxide level and the co-administration of CPF with eugenol significantly inhibits the rise in the level of nitric oxide. These results were in consistence with the previous finding of Bouchaud et al. (1994) who demonstrated that the soman administration at the LD_{50} leads to an increased activity of NO synthase in the cerebral endothelial cells from the 6th hour after poisoning. Crittenden et al. (1998) explained that the methyl parathion increased nitrite production by macrophages in mice treated with 1, 3, or 6 mg/kg/day. Gupta et al., (2001) reported that the carbamate and OP pesticides caused a significant increase in nitric oxide level of rat brain which related to an increase in citrulline levels while the pretreatment of an antioxidant significantly prevented the increase in the level of citrulline. Furthermore, Zhou et al. (2002) showed that the patients with acute OP pesticide poisoning showed a significant increase in plasma nitric oxide level with a significant decrease in the activities of SOD, CAT and AChE in erythrocytes.

Co-administration of eugenol with CPF to rats resulted in a marked improvement of the liver CAT and NO activities when compared to the group which received CPF alone. One of the possible explanations for the observed recovery of various enzyme activities involved in the detoxification following eugenol treatment could be related to its hepatoprotective influence by acting as antioxidants (Nagababu et al., 1995). Antioxidant effects can be classified into (1) direct action scavenging reactive oxygen species, and (2) the inhibition of the formation of reactive oxygen species (Halliwell, 1995). Eugenol was reported to exert protective role against many genotoxins and carcinogens (Francis et al., 1989; Rompelberg et al., 1993; Rompelberg et al., 1996a,b; Abraham, 2001). Biochemical studies in mice and rats have demonstrated its role as an effective inducer of detoxifying phase II enzymes (Rompelberg et al., 1993; Thompson et al., 1990). Furthermore, eugenol was reported to inhibit lipid peroxidation by acting as a chin-breaking antioxidant (Nagababu and Lakshmaiah, 1992; Nagababu and Lakshmaiah, 1994; Kumaravelu et al., 1995; Nagababu et al., 1995). Moreover, Tiku et al. (2004) revealed that eugenol exerted significant protection against oxidative stress and has a radioprotective potential. In addition, Vidhya and Devaraj, (1999) suggested that eugenol is nontoxic, protective and induces GST and thereby it may facilitate the removal of toxic substances from the rat intestine.

Our finding is also, in agreement with Li et al. (2006) who reported that eugenol and isoeugenol inhibited lipopolysaccharide (LPS)-dependent production of NO, which was due to the inhibition of protein synthesis of (iNOS). Furthermore, Nangle et al. (2006) found that eugenol treatment improved the level of nitric oxide production in streptozotocin-induced diabetic rats.

Therefore, it is quite possible to conclude that eugenol, being an antioxidant, might have scavenged the generated free radicals and in turn provided a protection against CPF-induced oxidative stress. In conclusion, CPF exhibited an immuotoxic

effect in male albino rats, and CPF immunotoxicity could be protected and cured by the use of eugenol in the experimental animals.

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DISCLOSURE

The authors declare that there is no conflict of interest.

REFERENCES

Abraham S.K. Anti-genotoxicity of trans–anethole and eugenol in mice. Food. Chem. Toxicol. 2001; 39: 493-498.

Aebi H. Catalase. In: Methods of Enzymatic Analysis. Bergemeyer, H. V. (ed.), Public Academic Press, New York, 1974; 673-684.

Aly N.M and El-Gendy K.S. Effect of dimethoate on the immune system of female mice. J. Environ. Sci. Health, B. 2000; 35(1): 77-86.

Ambali S., Akanbi D., Igbokwe N., Shittu M., Kawu M., Ayo J. Evaluation of subchronic chlorpyrifos poisoning on hematological and serum biochemical changes in mice and protective effect of vitamin C. J Toxicol Sci. 2007; 32(2):111-20.

Ambali S.F., Abubakar A.T., Shittu M., Yaqub L.S., Anafi S.B. and Abdullahi A. Chlorpyrifos-induced alteration of hematological parameters in Wistar rats: Ameliorative effect of zinc. Research Journal of Environmental Toxicology 2010a; 4(2): 55-66.

Ambali S.F., Abubakar A.T., Shittu M., Yaqub L.S., Kobo P.I. and Giwa A. Ameliorative Effect of Zinc on Chlorpyrifos-Induced Erythrocyte Fragility in Wistar Rats. New York Science Journal, 2010b; 3(5): 117-122.

Ambali S.F., Ayo J.O., Esievo K.A., Ojo S.A. Hemotoxicity induced by chronic chlorpyrifos exposure in wistar rats: mitigating effect of vitamin C. Vet Med Int. 2011; 2011:945439.

Atsumi T., Fujisawa S., Satoh K., Sakagami H., Iwakura I., Ueha T., Sugita Y., Yokoe I. Cytotoxicity and radical intensity of eugenol, isoeugenol or related dimers. Anticancer Res. 2000; 20(4): 2519–2524.

Banerjee B.D., Pasha S.T., Hussain Q.Z., Koner B.C., and Ray A. A comparative evaluation of immunotoxicity of malathion after subchronic exposure in experimental animals. Ind. J. Exp. Biol. 1998, 36(3): 273-282 .

Blakley B.R., Yole M.J., Brousseau P., Boermans H., and Fournier M. Effect of chlorpyrifos on immune function in rats. Vet. Hum. Toxicol. 1999, 41(3): 140-144.

Bouchaud C., Chollat-Namy A., Duserre S., Delamanche I.S. The role of nitric oxide in the neuropathology in soman intoxication. Brain. Res. 1994; 660(2): 249-254.

Boyum A. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 1968, 21: Supp, 97:7.

Casale G.P., Bavari S., and Connolly J.J. Inhibition of human serum complement activity by diisopropylfluorophosphate and selected anticholinesterase insecticides. Fund. Appl. Toxicol. 1989; 12: 460-468.

Casale G.P., Cohen S.D., and DiCapua R.A. The effects of organophosphate-induced cholinergic stimulation on the antibody response to sheep erythrocytes in inbred mice. Toxicol. Appl. Pharmacol. 1983; 68: 198-205 ().

Chang C.C., Lee P.P., Liu C.H., Cheng W. Trichlorfon, an organophosphorus insecticide, depresses the immune responses and resistance to *Lactococcus garvieae* of the giant freshwater prawn *Macrobrachium rosenbergii*. Fish Shellfish Immunol. 2006, 20 (4): 574-585.

Crittenden P.L., Carr R., Pruett S.B. Immunotoxicological assessment of methyl parathion in female B6C3F1 mice. J. Toxicol. Environ. Health. 1998; 54(1):1-20.

Crumpton T.L., Seidler F.J., and Slotkin T.A. Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos?. Brain Res. 2000a, 121:189–195.

Crumpton T.L., Seidler F.J., and Slotkin T.A. Developmental neurotoxicity of chlorpyrifos *in vivo* and *in vitro*: effects on nuclear transcription factors involved in cell replication and differentiation. Brain Res. 2000b, 857: 87–98.

Dacie J.V. and Lewis S.M. Practical Haematology. Churchill Livingstone UK, 7th Ed (1991).

Ercegovich C.D. Relationships of pesticides to immune responses. Fed. Proc. 1973, 32: 2010-2016.

Escobar J.A., Rubio M.A., Lissi E.A. SOD and catalase inactivation by singlet oxygen and peroxyl radicals. Free Rad. Biol. Med. 1996; 20: 285-290.

Francis A.R., Shetty T.K. and Bhattacharya R.K. Modification of the mutagenicity of aflatoxin B1 and N- methyl- N-nitrosoguanidine by certain phenolic compounds. Cancer Lett. 1989; 45: 177-182.

Fujisawa S., Atsumi T., Kadoma Y. Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Toxicology. 2002; 177: 39–54.

Galloway T., and Handy R. Immunotoxicity of organo-phosphorous pesticides. Ecotoxicology. 2003, 12(1-4): 345-363.

Garg U.K., Pal A.K., Jha G.J., and Jadhao S.B. Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides in broiler chicks. Int Immunopharmacol. 2004, 4(13):1709-1722.

Gerberick G.F., Cruse L.W., Ryan C.A., Hulette B.C., Chaney J.G., Skinner R.A., Dearman R.J., Kimber I. Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. Toxicol Sci. 2002; 68(2):420-8.

Goel A., Dani V., and Dhawan D.K. Chlorpyrifos-induced alterations in the activities of carbohydrate metabolizing enzymes in rat liver: the role of zinc Toxicol. Lett. 2006a, 163(3): 235–241.

Goel A., Dani V., Dhawan D.K. Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. Chemico-Biological Interactions 2005; 156: 131–140.

Goel A., Dani V., Dhawan D.K. Role of zinc in mitigating the toxic effects of chlorpyrifos on hematological alterations and electron microscopic observations in rat blood. Biometals. 2006b; 19(5):483-92.

Gultekin F., Ozturk M. and Akdogan M. The effect of organophosphate insecticide chlorpyrifos-ethyl on lipid peroxidation and antioxidant defense system in human fetus- an in vitro study. Ind. J. Exp. Biol. 2000; 30: 352-354.

Gultekin F., Delibas N., Yasar S., Kilinc I. *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. 2001; 75(2):88-96.

Gultekin F., Patat S., Akca H., Akdogan M., and Altuntas I. Melatonin can suppress the cytotoxic effects of chlorpyrifos on human hepG2 cell lines. Hum. Exp. Toxicol. 2006, 25(2): 47-55.

Gupta R.C., Milatovic D., Dettbarn W.D. Nitric oxide modulates high-energy phosphates in brain regions of rats intoxicated with diisopropylphospho -rofluoridate or carbofuran: prevention by N-tert-butyl-alpha-phenylnitrone or vitamin E. Arch Toxicol. 2001; 75(6): 346-356.

Halliwell B. Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol. 1995; 49 (10): 1341-8.

Halliwell B. and Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 1984; 219: 1–14.

Halliwell B. and Gutteridge J.M. Role of free radicals and catalytic metal ions in human disease: an overview. Meth. Enzymol. 1990; 186: 1–85.

Harford A.J., O'Halloran K., and Wright P.F. The effects of *in vitro* pesticide exposures on the phagocytic function of four native

Australian freshwater fish. Aquat. Toxicol. 2005, 75(4): 330-342.

Hilton I., Dearman R.J., Fielding I., Basketter D.A, Kimber I. Evaluation of the sensitizing potential of eugenol and isoeugenol in mice and guinea pigs. .J Appl Toxicol. 1996; 16(5):459-64.

Janardhan A., and Sisodia P. Monocrotophos: short-term toxicity in rats. *Bull.* Environ. Contam. Toxicol. 1990; 44: 230–239.

Kar Mahapatra S., Bhattacharjee S., Chakraborty S.P, Majumdar S., Roy S. Alteration of immune functions and Th1/Th2 cytokine balance in nicotine-induced murine macrophages: immunomodulatory role of eugenol and N-acetylcysteine. Int Immunopharmacol. 2011, 11(4):485-95.

Kar Mahapatra S., Chakraborty S.P., Majumdar S., Bag B.G., Roy S. Eugenol protects nicotine-induced superoxide mediated oxidative damage in murine peritoneal macrophages in vitro. Eur J Pharmacol. 2009, 623(1-3):132-40.

Kehrer J.P. Free radicals as mediator of tissue injury and disease. Crit. Rev. Toxicol. 1993; 23: 21–48.

Kobayashi S.D., Voyich J.M., DeLeo F.R. Regulation of the neutrophilmediated inflammatory response to infection. Microbes Infect. 2003, 5: 1337–1344.

Kollmannsberger H., and Nitz S. About the flavoring composition of high-Drude-Extract: 3 cloves (*Syzygium aromaticum*). Chim. Mikrobiol. Technol. Lebensmitt. 1994; 16: 112–123.

Kono Y., and Fridovich I. Superoxide radical inhibits catalase. J. Biol. Chem. 1982; 257: 5751-5754.

Kowalczyk-Bronisz S., Gieldanowski J., Bubak B., and Kotz J. Studies on effect of pesticide Chlorfenwinfos on mouse immune system. Arch. Immunol. Ther. Exp., (Warsz). 1992; (5-6): 283-289.

Kumaravelu P., Dakshinamoorthy D.P., Subramaniam P., Devaraj H. and Devaraj N.S. Effect of eugenol on drug-metabolizing enzymes of carbon tetrachloride-intoxicated rat liver. Biochem. Pharm. 1995; 49: 1703-1707.

Leffell M.S. Assessment of purity and viability. In: Falk JA, Goeken NE. eds. The ASHI laboratory manual.2nd edition. Lenexa. ASHI. (1990) 38.

Li W., Tsubouchi R., Qiao S., Haneda M., Murakami K., Yoshino M. Inhibitory action of eugenol compounds on the production of nitric oxide in RAW264.7 macrophages. Biomed. Res. 2006; 27(2): 69-74.

Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with Folin's phenol reagent. J. Biol. Chem. 1951; 193: 265–275.

Markert M., Andrews P.C., and Babior B.M. Measurement of O_2^- production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. Meth. Enzymol. 1984, 105: 358-65.

Markowitz K., Moynihan M., Liu M., Kim S. Biologic properties of eugenol and zinc oxide-eugenol. A clinically oriented review. Oral Surg Oral Med Oral Pathol. 1992; 73(6):729-37.

McCord J.M. and Fridovich I. Superoxide dismutase, an enzymatic function of erythrocupricin (haemocuprein). J. Biol. Chem. 1969; 224: 6049–6055.

McCord J.M. and Dary Jr, E.D. Superoxide dependent production of hydroxyl radical catalyzed by iron–EDTA complex. FEBS Lett. 1978; 86: 139–142.

Miranda K.M., Espey M.G., Wink D.A. A rapid, simple spectrophotometric method for simultaneous detection of Nitrate and nitrite. Nitric oxide: Biology and Chemistry 2001; 5 (1): 62-71.

Nagababu E. and Lakshmaiah N. Inhibitory effect of eugenol on non-enzymatic lipid peroxidation in rat liver mitochondria. Biochem. Pharm. 1992; 43: 2393-2400.

Nagababu E., and Lakshmaiah N. Inhibition of microsomal lipid peroxidation and monooxygenase activities by eugenol. Free Radical. Res. 1994; 20: 253-266.

Nagababu E., Ssikeran B., Lakshmaiah N. The protective effects of eugenol on carbon tetrachloride induced hepatotoxicity in rats. Free Radical. Res. 1995; 23: 617-627.

Nangle M.R., Gibson T.M., Cotter M.A., Cameron N.E. Effects of eugenol on nerve and vascular dysfunction in streptozotocin-diabetic rats. Planta. Med. 2006; 72(6): 494-500.

Naqvi S.M., Hasan M. Acetylhomocysteine thiolactone protection against phosphamidon-induced alteration of regional superoxide dismutase activity in central nervous system and its correlation with altered lipid peroxidation. Ind. J. Exp. Biol. 1992; 30: 850-852.

Ncibi S., Ben Othman M., Akacha A., Krifi M.N., Zourgui L. *Opuntia ficus indica* extract protects against chlorpyrifos-induced damage on mice liver. Food Chem Toxicol. 2008; 46(2):797-802.

Ogata M., Hoshi M., Urano S., Endo T. Antioxidant activity of eugenol and related monomeric and dimeric compounds. Chemical and Pharmaceutical Bulletin 2000; 48: 1467–1469.

Opdyke D.L.J. Monographs on fragrance and raw materials: eugenol. Food and Cosmetic Toxicology 1975; 13: 545–547.

Ou H.C., Chou F.P., Lin T.M., Yang C.H., Sheu W.H. Protective effects of eugenol against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells. Food Chem Toxicol. 2006, 44(9):1485-95.

Palmer H.J., and Paulson K.E. Reactive oxygen species and antioxidants in signal transduction and gene expression. Nutr. Rev. 1997, 55 (10): 353-61.

Queiroz M.L., Fernandes M.D., and Valadares M.C. Neutrophil function in workers exposed to organophosphate and carbamate insecticides. Int. J. Immunopharmacol. 1999, 21(4): 263-270.

Raha D.K., Hanb D.W., Hyun Sook Baeka H.S., Hyond S.H., and Parkb J.C. Prevention of reactive oxygen species-induced oxidative stress in human microvascular endothelial cells by green tea polyphenol. Toxicol. Lett. 2005*;* 155: 269– 275.

Rajini P.S., Viswanatha S., and Krishnakumari M.K. Effect of pirimiphos-methyl, an organophosphorus insecticide on hematological parameters in albino rats. Indian J Exp Biol.1987; 25: 190–193.

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

Rigo A., Stevenato R., Finazzi A.A, Rotilio G. An attempt to evaluate the rate of Haber Weiss reaction by OH radical scavengers. FEBS Lett. 1977; 80: 130–132.

Rompelberg C.J.M, Verhage H. and Van Bladern P.J. Effects of naturally occurring alkenylbenzenes eugenol and trans-anethole on drug metabolizing enzymes in rat liver. Food and Chemical Toxicology 1993; 31: 637-645.

Rompelberg C.J.M, steenwinkel M.J., Van Austen J.G., Van Delft J.H., Baan R.A. and Verhagen H. Effects of eugenol on the mutagenicity of benzo(a)pyrene and the formation of benzo(a)pyrene-DNA adducts in the lamda-lac Z-transgenic mouse. Mut. Res. 1996a; 369: 87-96.

Rompelberg C.J.M., Evertz S.G.C.J., Bruijntjes-Rozier G.C.D.M., van den Heuvel, P.D., Verhagen H. Effects of eugenol on the genotoxicity of established mutagens in the liver. Food and Chemical Toxicology 1996b; 34: 33-42.

Siwicki A.K., Cossarini-Dunier M., Studnicka M., Demael A. *In vivo* effect of the organophosphorus insecticide trichlorphon on immune response of carp (*Cyprinus carpio*). II. Effect of high doses of trichlorphon on nonspecific immune response. Ecotoxicol. Environ. Saf. 1990, 19 (1): 99-105.

Song X., Seidler F.J., Saleh J.L., Zhang J., Padilla S., and Slotkin T.A. Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade. Toxicol. Appl .Pharmacol. 1997, 145(1):158-174.

Street J.C. and Sharma R.P. Alteration of induced cellular and humoral immune responses by pesticides and chemicals of environmental concern: quantitative studies of immunosuppression by DDT, Aroclor 1254, carbaryl, carbofuran, and methylparathion. Toxicol. Appl. Pharmacol. 1975, 32: 587-602.

Sultatos L.G. Mammalian toxicology of organophosphorus pesticides, J. Toxicol. Environ. Health 1994, 43: 271–289.

Thompson D., Constantin-Teodosiu D., Egestad B., Mickos H and Moldeus P. Formation of glutathione conjugates during oxidation of eugenol by microsomal fraction of rat liver and lung. Biochem. Pharm. 1990, 39: 1587-1595.

Tiku A.B., Abraham S.K. and Kale R.K. Eugenol as in vivo radioprotective agent. J. Radiat. Res. 2004, 45:435-440.

Timothy B.H., David A.M., Pamela A.F and George T.R. A Cytotoxicity Evaluation of Antimicrobial and Non-Microbial Wound Cleansers. Derma Sciences 1997, 5(1): 1-6.

Uzun F.G., Demir F., Kalender S., Bas H., Kalender Y. Protective effect of catechin and quercetin on chlorpyrifos-induced lung toxicity in male rats. Food Chem Toxicol. 2010; 48(6):1714-20.

Vidhya N., and Devaraj S.N. Antioxidant effect of eugenol in rat intestine. Indian J Exp Biol. 1999; 37(12):1192-1195.

Worthing CR and Walker SB, eds (1987) The Pesticide Manual: A World Compendium, 8th ed. The British Crop Protection Council, Thornton Heath, UK.

Wysocki J., Kalina Z., and Owczarzy I. Effect of organophosphoric pesticides on the behaviour of NBT-dye reduction and E rosette formation tests in human blood. Int. Arch. Occup. Environ. Health 1987, 59(1): 63-71.

Yamano T., Morita S. Hepatotoxicity of trichlorfon and dichlorvos in isolated rat hepatocytes. Toxicology 1992; 76: 69-77.

Zhou J.F., Xu G.B., Fang W.J. Relationship between acute organophosphorus pesticide poisoning and damages induced by free radicals. Biomed Environ Sci. 2002; 15(2): 177-186.