

THE PROTECTIVE ROLE OF PROPOLIS AGAINST CHLORPYRIFOS INDUCED OXIDATIVE STRESS AND DNA DAMAGE IN THE LIVER OF MALE ALBINO RATS

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Abstract

Chlorpyrifos (CPF) is a conventional chlorinated organophosphate insecticide, it used widely to control a variety of pests in agriculture and animal farm. Acute and chronic exposure to CPF can elicit several adverse effects including oxidative stress and DNA damage. Twenty-eight rats were divided randomly into four groups of seven rats each. The first group was served as control and received distilled water. The second one received aqueous extract of propolis (100 mg/kg). The third group was administered 2.5 mg/kg of chlorpyrifos (1/30 LD₅₀). The fourth group was received propolis two hours before CPF administration. All animals were orally intubated five days/week for four weeks. Results showed that oral administration of CPF led to a significant increase in liver enzymes alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) as well as malondialdehyde (MDA) level beside the percentage of DNA damage in liver tissue were increased in contrast with control group. Decrease in serum cholinesterase (ChE) activity as well as liver reduced glutathione (GSH) level, glutathione-S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) activities were observed. Co-administration of propolis to CPF-treated group improved all biochemical parameters towards the control value although this treatment couldn't normalize it. The data provide evidence for co-administration of propolis to CPF-treated animals ameliorate the oxidative stress and DNA damage revealed the antioxidant property of propolis.

Key words: chlorpyrifos, oxidative stress, DNA damage, propolis, albino rats, liver

INTRODUCTION

Agricultural Pesticides are used widely in the agricultural production, fruits and vegetables even though they can have adverse health effects on consumers (Keikotlhaile *et al.*, 2010). Organophosphorous (OP) pesticides are the major chemical class of insecticides used in the world today (Bardin *et al.*, 1994). Chlorpyrifos, (O, O-diethyl-o-(3, 5, 6-trichloro-2-pyridinyl) phosphothioate), is a conventional chlorinated organophosphate insecticide, and widely used to control a variety of pests in agriculture and animal farm. It is a well-known acetyl cholinesterase inhibitor which leads to

accumulation of acetylcholine and results in excessive stimulation of postsynaptic receptors and consequent signs of toxicity (Mehta *et al.*, 2009). Chlorpyrifos has been known to generate oxidative stress by inhibiting enzymatic antioxidant defense (Verma & Srivastava, 2003), and also induces DNA damage (Mehta *et al.*, 2009). Liver is the main site for all metabolic processes and detoxification of toxic compounds. Thus, it is likely to be affected also by CPF (Joshi *et al.*, 2007).

Propolis (bee glue) is the generic name for the resinous substances collected by honey bees from various plant sources. Propolis contains more than 300 components, including phenolic aldehyde, quinines, coumarins, steroids, amino acids and inorganic compounds (Kanbur *et al.*, 2009). It has gained popularity and used extensively in healthy drinks and foods to improve well-being and prevent diseases, also it possesses several biological and antioxidant properties (Marquele *et al.*, 2005). Thus, the present study was conducted to evaluate the efficacy of propolis aqueous extract against the oxidative stress and DNA damage in liver tissue resulting from chlorpyrifos administration.

MATERIALS AND METHODS

Chemicals used

Chlorpyrifos "CPF" (48% EC) was obtained from Department of Pesticides Analysis, Central Agricultural Pesticides Lab., Dokki, Egypt. 2-thiobarbituric acid (TBA), 1-chloro 2,4-dinitrobenzoic acid (CDNB), reduced glutathione, nicotinamide adenine dinucleotide phosphate (NADPH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), pyrogallol, trichloro acetic acid, sodium azide and Tris-HCl were purchased from Sigma-Aldrich. All other chemicals and solvents analytical grade purchased from Merck.

Experimental protocol

Animals

A total of 48 adult male albino rats (*Rattus norvegicus*) weighting 180 ± 10 grams and 12-14 weeks age were obtained from the breeding colony of the Mammalian and Aquatic Toxicology Department, Central Agricultural Pesticides Lab, Giza, Egypt. The animals were randomly housed in well ventilated wire-plastic cages and wood shaving as bedding materials at a temperature of $25 \pm 2^\circ\text{C}$ and humidity of $55 \pm 5\%$ and normal light/dark cycle. The animals were maintained on the standard laboratory feed and water *ad libitum*.

Acute oral toxicity study

The acute oral median lethal dose (LD₅₀) was undertaken according to the Environmental Protection Agency (EPA) guideline (EPA, 1996). A twenty rats were divided into four groups (each five animals) and the mortality was recorded through the

first 6 hours up to 14 days post intubation. The value of LD₅₀ was calculated using special tables given by Weil (1952).

Sub-acute study

Twenty eight animals were segregated into 4 groups of 7 rats each. The first group was served as control and received distilled water. The second one was received aqueous extract of propolis 100 mg/ Kg (Faried *et al.*, 2015). The third group was administered 2.5 mg/kg of chlorpyrifos (1/30 LD₅₀). The fourth group was received propolis 2hr before chlorpyrifos administration. All animals were orally intubated five days/week for four weeks.

Sample collection

At the end of the experiment, the animals were starved overnight and the blood samples were collected from retro-orbital venous plexus under diethyl ether anaesthesia. Finally rats were sacrificed, liver of each animal were quickly removed and washed in normal saline solution. The obtained sera and the liver were stored at -20°C until used for biochemical assays.

Biochemical estimations

Serum ALAT and ASAT activities were estimated according to the method of Young (1990) using the commercial kits of MDSS GmbH, (Germany) according to manufacture instruction. Plasma ChE activity was determined according to the method of Ellman *et al.* (1961) by using acetyl thiocholin iodide as substrate and activity expressed as $\mu\text{m}/\text{min}/\text{ml}$.

Lipid peroxidation and antioxidant enzymes

Tissue preparation

The liver was homogenized individually in 1.17% KCl on an ice bath using Teflon homogenizer. The whole homogenate was used to measure MDA and GSH. The homogenate was centrifuged at 10,000 xg for 20 min. and the supernatant was used for measurements of GPx, GST, GR activities and protein content.

Lipid peroxidation

Content of MDA in liver tissue homogenate, indicator for lipid peroxidation, was assayed in the form of TBA-reactive species (TBARS) according to the method of Wills (1966), and the level of MDA was expressed as nmol /g tissue.

Estimation of GSH concentration

Reduced glutathione was determined by the method of Ellman (1959). The liver homogenate (0.5 ml) was precipitated with 2 ml of 5% TCA, then centrifuged at 3200 xg for 20 min. one ml of supernatant was added to 0.5 ml of Ellman's reagent and 3.0

ml of phosphate buffer (0.2M PH 8.0). The absorbance was read at 412 nm and the content of GSH was expressed as mg/g tissue.

Determination of GPx activity

GPx activity was carried out according to the method Necheles *et al.* (1969) which based on the measurement of residual reduced glutathione remaining by Ellman's reagent (Ellman, 1959) in the presence of cumene hydroperoxide as a secondary substrate. The specific activity of enzyme expressed as $\mu\text{M}/\text{min}/\text{mg}$ protein. The protein content in tissue fraction was done based on the method of Bradford (1976).

Determination of GST activity

The GST enzyme activity was assay by the method of Habig *et al.* (1974). The reaction medium contains 0.95 ml of phosphate buffer PH 6.5 (0.1 M), 20 μl of 1.0 mM GSH, 20 μl homogenate and 10 μl of 1.0 mM CDNB. The absorbance was measured at 340 nm and the specific activity was expressed in $\mu\text{m}/\text{min}/\text{mg}$ protein.

Determination of GR activity

The activity of GR was determined using the method of Coldberg & Spooner (1983). The enzyme activity was measured spectrophotometrically by following decrease in absorbance of NADPH at 340 nm in a sodium phosphate buffer (50 mM, pH 7.6) containing 1 mM EDTA, 0.17 mM NADPH, and 3.3 mM oxidized glutathione. The specific activity was expressed as $\mu\text{m}/\text{min}/\text{mg}$ protein.

Single Cell Gel Electrophoresis

The comet assay was performed under alkaline conditions by procedure of Singh *et al.* (1988). A freshly prepared suspension of liver was mixed with 0.7% low melting point agarose and casted to precoated slides with 1% normal melting agarose. The cells were then lysed for 1 hour at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. The slides were then placed in the electrophoretic buffer consisting of 300 mM NaOH, 1 mM EDTA, pH > 13 for 40 min. Electrophoresis was conducted for 30 min at electric field strength 0.56 V/cm (300 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5 and stained with ethidium bromide before examination under a fluorescence microscope (Lx 400, Labomed, USA) with 590 nm as a barrier filter. Analysis was performed on coded slides by one reader to avoid variability by comet 5 image analysis software (Liverpool, UK) linked to a CCD camera.

RESULTS AND DISCUSSION

As seen in (Table 1), no significant differences were observed in the serum ALAT and ASAT activities in the group that was administered propolis alone. On the other hand, a significant increase in ALAT and ASAT activities in CPF group and propolis administered group in association with chlorpyrifos when compared with control group.

The treatment with propolis alone did not induce any significant alteration in serum ChE activity compared with control group. Treatment rats with CPF alone and propolis administered group in combination with chlorpyrifos reduced ChE activity compared with control group.

The administered propolis group did not display any significant difference in liver MDA level in comparison with the control group. On the other hand, a significant elevation in the liver MDA level was observed in both groups that administered CPF alone and that was administered propolis in association with CPF.

The present study revealed that rats treated with propolis extract alone resulted in a significant increase in reduced glutathione level, GPx and GR activity, and no significant change in GST activity was observed. However, chlorpyrifos treated group produced a significant decrease in GSH level, GPx, GST and GR activities. Co-administration of propolis extract to chlorpyrifos treated group improved the level of GSH as such as GPx, GST and GR activities, but the data still below the normal values as compared to the control group (table 1).

The CPF-treated group revealed a significant increase in tail DNA, damage index, tail length and tail moment, and the less effect was observed in co-administration propolis group compared with control one (table 2 and figure 1).

Table 1. The protective role of propolis on chlorpyrifos induced alteration in liver enzymes, cholinesterase and antioxidant enzymes.

Groups	ALAT U/L	ASAT U/L	CHE µm/min/ ml	MDA nmol/ g tissue	GSH mg/ g tissue	GPX µm/min/ mg protein	GST µm/min/ mg protein	GR µm/min/ mg protein
Control	34.85 ± 0.40	77.85 ± 1.56	209.92 ± 8.50	340.64 ± 10.98	25.94 ± 0.33	348.88 ± 4.59	204.96 ± 5.87	178.02 ± 1.49
Propolis	35.00 ± 0.30 ^{c,d}	79.28 ± 0.42 ^{c,d}	195.46 ± 9.66 ^c	338.63 ± 13.40 ^{c,d}	29.60 ± 0.34 ^{a,c,d}	363.38 ± 1.79 ^{a,c,d}	209.19 ± 5.06 ^{c,d}	181.58 ± 0.74 ^{a,c,d}
chlorpyrifos	44.42 ± 0.57 ^{a,b,d}	99.14 ± 0.85 ^{a,b,d}	145.42 ± 7.13 ^{a,b,d}	756.38 ± 30.26 ^{a,b,d}	18.75 ± 0.48 ^{a,b,d}	279.22 ± 3.67 ^{a,b,d}	136.90 ± 3.55 ^{a,b,d}	139.98 ± 0.81 ^{a,b,d}
Propolis+chlorpyrifos	38.28 ± 0.42 ^{a,b,c}	90.42 ± 0.64 ^{a,b,c}	183.14 ± 6.84 ^{a,c}	522.87 ± 16.39 ^{a,b,c}	21.40 ± 0.46 ^{a,b,c}	332.08 ± 3.18 ^{a,b,c}	166.09 ± 2.98 ^{a,b,c}	161.25 ± 0.94 ^{a,b,c}

The data presented as mean ± SE, (n=7); (a) significantly different at p<0.05 in compared to the control group; (b) compared to propolis; (c) compared to chlorpyrifos and (d) compared to propolis plus chlorpyrifos.

Table 2. The protective role of propolis on chlorpyrifos induced alteration in DNA.

Groups	Tail DNA	Damage Index	Tail Length	Tail Moment
Control	3.00 ±0.70	3.80 ±0.83	1.84 ±0.13	5.81 ±0.69
Propolis	2.80 ±0.83 ^{c,d}	3.4 ±1.14 ^{c,d}	1.74 ±0.07 ^{c,d}	5.01 ±0.18 ^{c,d}
chlorpyrifos	33.80 ±3.27 ^{a,b,d}	72.8 ±8.31 ^{a,b,d}	7.45 ±0.64 ^{a,b,d}	23.45 ±1.61 ^{a,b,d}
Propolis+chlorpyrifos	18.40 ±2.07 ^{a,b,c}	30.80 ±5.31 ^{a,b,c}	4.05 ±0.18 ^{a,b,c}	13.52 ±0.51 ^{a,b,c}

The data presented as mean ± SE, (n=7); (a) significantly different at p<0.05 in compared to the control group; (b) compared to propolis; (c) compared to chlorpyrifos and (d) compared to propolis plus chlorpyrifos.

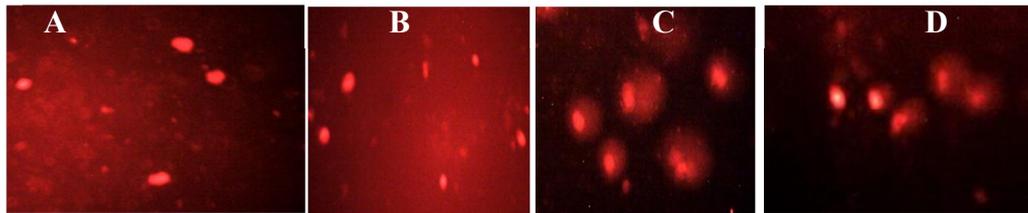


Fig. 1. Microscopic scoring of DNA damage in liver tissue. (A) control group, (B) propolis group, (C) chlorpyrifos group, and (D) propolis plus chlorpyrifos group.

The liver plays an important role in the detoxification of xenobiotics, the disturbance of liver homeostasis under oxidative imbalance is sufficient to alter the normal physiological functioning of any organism (Joshi *et al.*, 2007). The organophosphate insecticides induced an increase in ALAT and ASAT activities in both groups that administered CPF only and in association with propolis and this fact is a conventional indicator of liver injury. When the liver cell membrane is damaged, varieties of enzymes normally located on the cytosol are released into the blood stream, elevation in ALAT and ASAT indicates the utilization of amino acids for the oxidation or for glucogenesis and is used to determine liver damage (Rao, 2006).

The results revealed that either CPF alone or combination with propolis induced a significant inhibition in the activity of ChE compared to the control rats. CPF after ingestion, is subjected to microsomal oxidation in the liver, resulting in the formation of CPF-oxone, which is a potent inhibitor of AChE in the peripheral tissues and CNS (Mehta *et al.*, 2009).

Pesticides are known to produce oxidative stress, results of the present study showed that exposure to CPF caused increase in lipid peroxidation, which has been used

as measure of xenobiotics-induced oxidative stress, which was originally defined as the disequilibrium between pro-oxidants and antioxidants in biological system (Kelly *et al.*, 1998). Which leads to damage of cellular macromolecules or cell death by free radicals. The increase in MDA levels may be due to a possible increase in free radicals caused by CPF (Gultekin *et al.*, 2001). Rats that were administered propolis in association with CPF in compared to the CPF alone, showed decrease in liver MDA level, may be due to scavenging of free radicals that cause lipid peroxidation or may be due to inhibition of xanthine oxidase which is known to cause free radical to be generated by propolis (Harris *et al.*, 2000).

GSH acts as reducing agent and a vital substance in detoxification, also provides antioxidant protection in the aqueous phase of cellular system (Rana *et al.*, 2002) GSH also acts as substrate and co-substrate in many essential enzymatic reactions such as GPx and GST.

Chlorpyrifos treated group produced a significant decrease in GSH level, GST, GPx and GR activities. The data are consistent with Gupta *et al.* (2010) who found that chlorpyrifos lead to oxidative damage to various tissues or cells. CPF is known to cause depletion of reduced glutathione and decrease in the ratio of GSH/GSSG in rat tissues which is maintained intracellularly through the action of GR and NADPH dependent reaction, and causes inhibition of antioxidant enzyme activities in liver rats (Verma & Srivastava, 2003).

Depletion of cellular GSH below a critical level prevents the conjugation of xenobiotics to GSH and enables them to freely combine covalently with DNA, RNA or cell proteins resulting in cellular damage (Meister & Anderson, 1993). Reduction of GSH by xenobiotics below a certain level allow the enhancement of lipid peroxidation and induction of ROS, which could also cause formation of DNA single strand breaks (Banu *et al.*, 2001), as it clear in the present study in which CPF causes DNA damage in liver tissues, as evidenced by increase in DNA migration which was detected by comet assay.

Animals that were administered propolis in association with chlorpyrifos were alleviated the negative effects of CPF-treated group on GSH level, GPx, GST and GR activities. This may be due to the phenolic compounds existing in propolis extract which responsible for the antioxidant activity (Kanbur *et al.*, 2009).

CONCLUSION

The present study clearly shows that CPF exposure generates oxidative stress in rat hepatic tissue which is an additional toxic effect other than DNA damage. Which was also corroborated by the protection provided by aqueous extract of propolis.

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الدور الوقائي لصمغ النحل ضد التأثير التأكسدي والخلل الناجم في الحامض النووي من مبيد الكلوربيريفوس في كبد ذكور الجرزان البيضاء

خيري عبدالمنعم ابراهيم و سعاد أحمد ابراهيم

قسم سمية المبيدات للثدييات والأحياء المائية- المعمل المركزي للمبيدات- مركز البحوث الزراعية - الدقي

يعتبر مبيد الكلوربيريفوس من المركبات الفوسفورية العضوية التقليدية وعلى الرغم من انه يستخدم على نطاق واسع ضد مجموعة متنوعة من الآفات الزراعية الا ان التعرض الحاد والمزمن لهذا المبيد يؤدي الى العديد من الآثار الضارة بما في ذلك الأوكسدة الفائقة وتلف الحمض النووي وذلك يهدف هذا العمل الى ايجاد مادة طبيعية تقي من مثل هذه الاضرار. تم استخدام ثمانية وعشرين من ذكور الجرزان البيضاء موزعين عشوائياً إلى أربع مجموعات. المجموعة الأولى كانت بمثابة المجموعة الضابطة وجرعت بالماء المقطر والثانية جرعت بـ ١٠٠ ملليجرام/كجم من المستخلص المائي لصمغ النحل. اما المجموعة الثالثة جرعت بـ ٢,٥ ملليجرام/كجم من مبيد الكلوربيريفوس (٣٠/١ من الجرعة نصف المميتة) والرابعة فقد جرعت بنفس الجرعة من المستخلص المائي لصمغ النحل قبل تجريع المبيد بساعتين. وقد تم تجريع جميع الحيوانات عن طريق الفم خمسة أيام في الاسبوع لمدة اربعة اسابيع. وظهرت النتائج الى ان التعرض لمبيد الكلوربيريفوس ادى الى زيادة ذات دلالة احصائية في انزيمات الكبد و في المقابل فقد وجد انخفاض معنوي في نشاط انزيم الكولين استراز في المصل. اما في انسجه الكبد فقد زاد مستوي مادة المالوندايالديهايد ونسبة الحامض النووي المجزئ وفي المقابل فقد انخفض مستوى مادة الجلوتاثيون وكذلك النشاط الانزيمي لكلا من الجلوتاثيون-اس-ترانسفيريز و انزيم الجلوتاثيون ريدكتيز عند المقارنة بالمجموعة الضابطة. اوضحت النتائج ايضا ان المستخلص المائي لصمغ النحل قد ادى الى تحسن ذو دلالة احصائية في الخلل الناجم عن التعرض لمبيد الكلوربيريفوس لذلك من الممكن ان يستخدم كمادة وقائية لجميع المشتغلين في مجال المبيدات.

الكلمات المفتاحية: مبيد الكلوربيريفوس- التأثير التأكسدي- الخلل الناجم من الحامض النووي- صمغ النحل- الجرزان البيضاء- الكبد.