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Propolis relieves the cardiotoxicity of chlorpyrifos in diabetic rats via alleviations of paraoxonase-1 and xanthine oxidase genes expression

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ABSTRACT

Pesticides cardiotoxicity in case of diabetic-induced cardiac complications is unidentified. The probable amelioration role of propolis is gauged against the cardiotoxic effects of chlorpyrifos in the diabetic rats through paraoxonase-1 (PON1) and xanthine oxidase (XO) genes dysregulation. Fifty-six male rats were distributed (n = 7) into eight groups. The first one saved as control whereas the 2nd, 3rd, and 4th were kept for propolis aqueous extract (100 mg/kg), diabetes (60 mg/kg streptozotocin) and chlorpyrifos (2.5 mg/kg), respectively. The 5th was diabetes/chlorpyrifos combination, while 6th, 7th, and 8th were intubated with propolis for four weeks after diabetic induction, chlorpyrifos intoxication, and their combination, respectively. The plasma glucose, lipid profiles, cardiac enzymes and interleukin-6 (IL-6) significantly elevated, while insulin decreased in the diabetic and combination groups. Although the cardiac acetylcholinesterase, total thiols, and PON1 significantly reduced after diabetic and/or chlorpyrifos gavage, the protein carbonyl, superoxide dismutase, catalase, and XO significantly elevated. The mRNA genes expression of PON1 and XO have also confirmed the enzymatic activities. Interestingly, propolis significantly restored the hyperglycemia, hypoinsulinemia, hyperlipidemia, IL-6 elevations, and antioxidant defense system disorder. These records revealed that the immunomodulatory, anti-diabetic and antioxidant tasks are fine pointers for the cardiovascular defender of propolis especially during diabetes and/or pesticides exposure.

1. Introduction

Diabetes mellitus (DM) is one of the mutual health problems as the variety of diabetic patients worldwide is rising very fast and the major cause of demise in diabetes is the cardiovascular disease (Westermann et al., 2009). Myocardial dysfunction and structural variations are the common distinctive features of cardiomyopathy in diabetic (Yu et al., 2012), which has signals of primary-onset diastolic, late-onset systolic dysfunctions and associated with DM (Huynh et al., 2010).

The cardiac complication of diabetes is characterized by microvascular and macrovascular snags of chronic hyperglycemia. The microvascular hitches affect minor blood vessels and comprise the neuropathy, nephropathy, and retinopathy, whereas the macrovascular complications encompass the cardiovascular, cerebrovascular and peripheral artery diseases (Faria and Persaud, 2017) which arise from a number of hyperglycemia-induced mechanisms as oxidative damage (Atta et al., 2018). A previous study revealed that extreme generation of reactive oxygen species (ROS) is possibly involved in the pathophysiology of diabetic cardiomyopathy (DCM) as the heart is predominantly prone to oxidative injury (Umbarkar et al., 2015). Oxidative destruction is intensified by the disturbance in the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and xanthine oxidase (Kumar et al., 2013).

Xanthine oxidase (XO) acts as a vital natural root of ROS and the influence of this enzyme in countless pathological progressions including diabetes has been well recognized (Higgins et al., 2011). Conferring to preceding study, circulating and tissue XO are amplified in experimental diabetes which is donated to superoxide creation (Matsumoto et al., 2003). Paraoxonase-1 (PON1), a calcium-dependent esterase, is an alternative antioxidant enzyme faithfully allied with high-density lipoproteins ((Sentí et al., 2003) and its activity is abnormally diminished in diabetes mellitus with cardiovascular impediments (Durrington et al., 2001). PON1 also catalyzes an extensive range of substrates hydrolysis including organophosphates as chlorpyrifos (Draganov and La Du, 2004).

Chlorpyrifos, (O, O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate), is a straight organophosphorus insecticide that extensively controls a multiplicity of pests in agriculture and animal homestead

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(Testai et al., 2010). It is a famous of the acetylcholinesterase (AChE) inhibitor which leads to acetylcholine buildup, consequences in an excessive postsynaptic receptors stimulation and subsequent signs of toxicity (Mehta et al., 2009). Creation of reactive oxygen species encourages antioxidant system disturbance and oxidative damage are also implicated in the mechanisms of chlorpyrifos toxicity (Uchendu et al., 2012).

Natural products are appreciated the basis of bioactive composites and have been used for therapeutic resolves in all over the world, and about 80% of medications used in fast developing countries are by-products of phytoextracts (Halim and Misra, 2011). Propolis, a gummy natural product, comprises > 300 biochemical components (Marquele et al., 2005). It has expanded popularity and used broadly in healthy drinks and foods to rally well-being and avoid diseases as inflammation, heart diseases, diabetes and owns numerous biological possessions as anticancer, antibiotic, antifungal, and antioxidant activities (Kowalski and Makarewicz, 2017).

The antioxidant possessions of propolis and its active constituents have also been proposed to be accountable for its ability to moderate the markers of cardiovascular disease (Kocot et al., 2018). In this issue, Fuliang et al. (2005) proposed that propolis could modulate the metabolism of blood glucose and lipid, leading to reduce the outputs of lipid peroxidation and hunt the free radicals in the diabetic rats and reduced the doxorubicin produced-cardiomyopathy (Chopra et al., 1995). Further, the active components of propolis as caffeic acid phenethyl ester have prevented the chronic hypertensive (Salmas et al., 2017), and reduced xenobiotics-induced the cytotoxic injuries including pesticides (Abdel-Daim and Abdellatief, 2018).

In the meantime, there were no records in the literature for the estimation of the pesticides adversative effects in case of diabetes. So, the current study was originally directed to evaluate the impacts of chlorpyrifos on the cardiac PON1 and XO genes expression when it has administered to non-diabetic and/or diabetic rats. The subsequent one is to define if propolis can alleviate the probable shifts in the regulation of the genes triggered by the designated pesticide and/or diabetic which may have interpretations in human management with these accidental influences.

2. Materials and methods

2.1. Chemical used

Streptozotocin (STZ) obtained from MP Chemical Company (France) and chlorpyrifos (48% of the technical grade in emulsifiable concentrate) gained from Pesticides Analysis Department, Central Agricultural Pesticides Laboratory, Dokki, Egypt. Wholly other chemicals and substrates procured from Sigma Chemicals Company (USA).

2.2. Propolis preparation

The crude propolis obtained from honey bee colonies of Beekeeping Research Section, Plant Protection Research Institute, Agriculture Research Centre, Dokki, Giza, Egypt. Aqueous extract of propolis was equipped conferring to the scheme of Nagai et al. (2003) by cutting the crude into small pieces, grounded and finally extracted with five volumes in double distilled water then shaking at 30 °C for three days. The extract was filtered and the supernatant lyophilized (Labconco Lyophilizer, USA).

2.3. Experimental protocol

2.3.1. Animals husbandry

A 56 of outbred male albino rats (*Rattus norvegicus*) aged 12–14 weeks (180 ± 10 g) gained from the breeding colony of the Mammalian Toxicology Department, Central Agricultural Pesticides Laboratory, Giza, Egypt. The animals haphazardly housed in suitable plastic cages (3–4 rats/cage) with stainless steel wire lids and wood shavings as bedding material. The rats kept under typical laboratory environments (22 ± 2 °C, 60–70% relative humidity and a 12-h light/dark cycle), fed a usual diet of marketable pellets and received water ad libitum. All animal procedures directed in agreement with the recommendation criteria of the experimental animal care and the current protocol was accepted (CU/I/F/66/17) by the Cairo University Institutional Animal Care and Use Committee.

2.3.2. Induction of diabetes

Diabetic was convinced by a single (60 mg/kg) intraperitoneal injection of STZ (Al-hariri, 2012). Subsequently, the animals had free admittance to food and given a 5% glucose solution in their drinking water overnight to counter the hypoglycemic shock (Bhandari et al., 2005). The rats were considered diabetic for supplementary research only if they had hyperglycemia (glucose levels ≥300 mg/dl) at 72 h after STZ injection. The diabetic state of animals watched for its steadiness for seven sequential days after diabetic initiation.

2.3.3. Study design

Animals were arbitrarily distributed into eight groups ($n = 7$) as the following: the negative control group (G1) orally received 0.5 ml of distilled water, (G2) received 100 mg/kg of propolis aqueous extract (Hemieda et al., 2015), (G3) was a positive control for diabetic, (G4) administered 2.5 mg/kg of chlorpyrifos and (G5) received the same dose of chlorpyrifos after diabetic induction (combination). The aqueous extract of propolis intubated to (G6), (G7) and (G8) after diabetic induction, during chlorpyrifos intoxication, and their combination, respectively. The rates have orally received the propolis 2 h before chlorpyrifos administration for a five days/week through four weeks. The dose of chlorpyrifos chosen relied on plentiful records from our laboratory gained consuming the acute oral median lethal dose study.

2.3.4. Samples

By the finale of the study course, the animals lightly anesthetized and the blood samples poised in sanitized and heparinized tubes from retro-orbital plexus for serum and plasma separation, correspondingly. Finally, rats sacrificed and the heart from each animal rapidly detached, washed with ice-cold normal saline and the tissue was blotted on a filter paper for excess buffer removal. The gotten sera, plasma, and cardiac tissue were frozen until used for biochemical assays.

2.4. Biochemical assay

Plasma glucose, serum total cholesterol, serum triglycerides (TG), serum high-density lipoprotein (HDL-cholesterol) estimated agreeing to construction protocol of colorimetric kits (MDSS, Hannover, GmbH), while the low-density lipoprotein (LDL-cholesterol) calculated according to Friedewald et al. (1972). Plasma lactate dehydrogenase (LDH) and total creatine kinase (CK) measured by Salucea company kits (Netherlands). Plasma insulin and interleukin-6 (IL-6) determined by rats Enzyme-linked Immunosorbent Assay kits (Immunoconceptin, Sacramento, USA).

2.5. Cardiac tissue analysis

Cardiac tissues homogenized in precooled 50 mM potassium phosphate buffer pH 7.5 containing 1.0 mM ethylenediamine tetra-acetic (EDTA), and the supernatants frozen for further use. The content of the total protein measured by Bradford (1976) method with bovine serum albumin as standard. Total thiol determined by the method based on the progress of a yellow color when 0.01 M 5,5-dithiobis (2-nitrobenzoic acid) added to 0.2 M Tris-HCl buffer pH 8.2 and sample (Sedlak and Lindsay, 1968). Protein carbonyl (PC) testified according to Reznick and Packer (1994) scheme while SOD and CAT activities measured by the procedures of Marklund and Marklund (1974) and

Aebi (1984), respectively. The enzymatic activity of PON1 done in 50 mM glycine buffer pH 10 containing 1.0 mM paraoxon, and 1.0 mM calcium chloride (Hernández et al., 2004), while XO activity determined by Bergmeyer et al. (1974) protocol. The activity of AChE tested in a reaction mixture comprising 38 mM Tris HCl pH 8.5, 1.0 mM dithio-bis-2 nitrobenzoic acid, and 1.0 mM acetylthiocholine iodide (Ellman et al., 1961).

2.6. Estimation of PON1 and XO genes expression

The cardiac RNA extracted according to instruction rules of QIAamp RNeasy Mini kit (Qiagen, GmbH). The real-time polymerase chain reaction (*RT*-PCR) kit with a single stage (Qiagen, GmbH) was used. The RNA template (3 µl) exploited in a 25 µl reaction comprising 12.5 µl of the 2× SYBR Green PCR Master Mix, 0.25 µl of Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer (Metabion, GmbH), and 8.25 µl of RNase-free water. The primer sequences of PON1 were 5′-TGAGAGCTTCTATGCCACAAATG-3′ (sense), and 5′-CCATGACAGG CCCAAGTACA-3′ (antisense). Those for XO were 5′-GACTCACTTCAA CCAGAAGC-3′ (sense), and 5′-CTGGTTCAGAAAAGGAAGTG-3′ (antisense). Expression normalized to the β-actin gene as an internal housekeeping control and its primers were 5′-TCCTCCTGAGCGCAAG TACTCT-3′ (sense), and 5′-GCTCAGTAACAGTCCGCCTAGAA-3′ (antisense), and each set of assessments involved a negative control.

The reverse transcription has started the reaction at 50 °C for 30 min and the primary denaturation did at 95 °C for 5 min. The amplification monitored by 40 cycles of the following sequential steps: 15 s at 94 °C (denaturation), 30 s at 60 °C (annealing) for PON1 (Hafez et al., 2014), 56 °C for XO (Kumar et al., 2013), 60 °C for β-actin (Banni et al., 2010), and 30 s at 72 °C (extension). The dissociation stage added after the amplification one to verify the specificity of the PCR products and calculable analysis achieved by the measurement of the threshold cycle (Ct) values during the exponential phase of amplification. The MX3005P software (Agilent Technologies, GmbH) was used for Ct values measurement and the variations of mRNA genes expression calculated by "ΔΔCt" method (Yuan et al., 2006).

2.7. Statistical analysis

Data of continuous variables articulated as mean ± standard error (M ± SE) of seven animals and standard computer program (SPSS for Windows, release 25.0, IBM SPSS Inc., USA) used for data entrance and analysis. The variances between groups evaluated using one-way ANOVA followed by Turkey's honestly significant difference (HSD) test and a $P < .05$ set as statistically significant differences. The correlation was also assessed by 2-tailed Pearson correlation coefficient to study the relationships between the quantitative variables. Joint action analysis was done by Mansour et al. (2017) intention.

3. Results

3.1. Diabetes characterization

Hyperglycemia noticeable in the diabetic and combination groups as the plasma glucose significantly raised and obviously decreased in the insulin as compared with control ($P < .001$). While propolis supplementation was significantly reduced the glucose and increased the insulin levels when these groups matched with their parallel without propolis administration ($P < .05$), it didn't extend to the normal range. Although chlorpyrifos group scored a significant decrease ($P < .01$) in insulin level when compared with control and propolis co-treatment restored this effect, there was a non-significant increase in glucose level. There were noteworthy changes also in insulin and glucose levels when the combination group compared with chlorpyrifos alone ($P < .001$), but the non-significant variance documented when it compared with diabetic one (Table 1).

3.2. Lipid profiles

Cholesterol, triglycerides, and LDL remarkably increased ($P < .01$) in diabetic groups as compared with control one. The HDL scored the same pattern with the omission in diabetic alone which noted the non-significant change. Chlorpyrifos group also displayed a significant increase ($P < .05$) in cholesterol and LDL levels as compared with control. The co-treatment with propolis significantly decreased ($P < .05$) the lipid profiles in all groups when compared with the same sets without propolis supplementation. The combination group scored also significant variances in triglycerides, HDL and LDL levels paralleled with chlorpyrifos alone ($P < .001$), and with diabetic one among the cholesterol and HDL (Table 1).

3.3. Cardiac enzymes

Plasma LDH and total CK significantly elevated in diabetic and combination groups as compared with control ($P < .01$). There were also significant differences in LDH when combined group compared either with diabetic or chlorpyrifos alone ($P < .05$). While the same effect recorded in diabetic/propolis and diabetic/chlorpyrifos/propolis groups, the LDH activity noticeably ($P < .005$) decreased in these groups when matched with the same one without propolis administration (Table 1).

3.4. IL-6

Plasma IL-6 significantly ($P < .05$) elevated in the diabetic and combination groups as compared with control. While the propolis was significantly ($P < .005$) decreased the IL-6 levels in diabetic/propolis and diabetic/chlorpyrifos/propolis groups as compared with their parallel without propolis administration, these rises still quiet as compared with control (Table 1).

3.5. Oxidative stress in the cardiac tissue

As seen in the Table 2, the enzymatic activity of the cardiac SOD significantly elevated ($P < .001$) in all experimental groups as compared with normal one with the exclusion of the propolis group. The same influence recorded in CAT activity with the exception the groups of propolis and propolis/chlorpyrifos, where they scored the non-significant difference as compared with control. However, administration of propolis to diabetic, chlorpyrifos and their combination groups significantly recovered the rise of SOD and CAT activities to the nearly normal one (Table 2).

While the enzymatic activity of PON1 in the cardiac tissue significantly increased after oral administration of propolis alone ($P < .001$), the rest groups scored significant reductions as compared with control. In contrast, the specific activity of the cardiac XO noted a significant reduction in the propolis group alone, but other groups documented significant elevations as compared with control. Although propolis was significantly restored the reductions of PON1 after complementation to diabetic, chlorpyrifos and their combination, it didn't standardize the enzyme activity. Whereas propolis succeeded to regulate the activity of XO after diabetes and chlorpyrifos intoxication, it didn't normalize their combination (Table 2).

There were significant reductions in the cardiac AChE activities ($P < .005$) in all experimental groups with excluding propolis and propolis/diabetic groups as compared with control. Supplementation with propolis was significantly restored the enzyme activity to the nearly normal level. The same pattern observed in total thiol levels with the exception in the groups of propolis alone and when co-treated with chlorpyrifos where they scored non-significant variations as compared with control. In contrast, the protein carbonyl levels significantly elevated ($P < .005$) in the groups of diabetic alone and combination as compared with control and supplementation with propolis was repaired

Table 1
Efficiency roles of propolis on diabetic and/or chlorpyrifos induced amendment in plasma glucose, lipids profiles, cardiac enzymes, insulin, and interleukin-6.

	G1	G2	G3	G4	G5	G6	G7	G8
Glucose mg/dl	121.25 ± 3.96	110.42 ± 3.69^{c,e,f,h}	277.48 ± 6.67^{a,b,d,f,g,h}	129.32 ± 3.75^{c,e}	284.97 ± 7.15^{a,b,d,f,g,h}	146.54 ± 9.02^{a,b,c,e,g}	106.02 ± 7.62^{c,e,f,h}	150.52 ± 4.88^{a,b,c,e,g}
Cholesterol mg/dl	111.41 ± 4.06	100.36 ± 3.50^{c,d,e,f,g,h}	178.15 ± 4.61^{a,b,d,e,f,h}	127.89 ± 4.31^{a,b,c}	205.08 ± 4.20^{a,b,c,f,g,h}	118.30 ± 4.72^{b,c,e,h}	117.04 ± 3.06^{b,e,h}	142.57 ± 3.90^{a,b,c,e,f,g}
Triglycerides mg/dl	60.36 ± 1.44	60.59 ± 1.18^{c,e,f,h}	81.80 ± 3.40^{a,b,d,g}	67.59 ± 1.66^{c,e}	90.55 ± 6.03^{a,b,d,f,g,h}	72.51 ± 2.14^{a,b,e,g}	60.84 ± 2.50^{c,e,f,h}	77.91 ± 2.83^{a,b,e,g}
HDL mg/dl	36.62 ± 1.21	38.45 ± 1.29	33.62 ± 1.28^{e,h}	35.10 ± 1.28^{e,h}	68.87 ± 5.37^{a,c,d,f,g,h}	36.79 ± 1.33^{e,h}	37.71 ± 1.55^{e,h}	47.01 ± 1.60^{a,c,d,e,f,g}
LDL mg/dl	54.10 ± 2.86	43.27 ± 4.32^{c,d,e,f,h}	124.20 ± 2.66^{a,b,d,f,g,h}	73.14 ± 4.24^{a,b,c,e}	119.38 ± 5.1^{a,b,d,f,g,h}	66.13 ± 4.23^{b,c,e}	58.09 ± 4.09^{c,e,h}	80.08 ± 3.65^{a,b,c,e,g}
CK total U/L	147.34 ± 5.62	135.38 ± 6.77	828.73 ± 39.5^{a,d,e,f,h}	164.68 ± 8.62^{c,e}	1209.07 ± 98.9^{a,c,d,f,g,h}	228.85 ± 14.32^{c,e}	145.05 ± 7.60^{e}	258.50 ± 12.96^{c,e}
LDH U/L	230.43 ± 11.15	205.67 ± 9.68^{c,e,f,h}	689.12 ± 19.1^{a,b,d,e,f,g}	244.51 ± 10.10^{c,e,h}	1111.82 ± 31.2^{a,b,c,d,f,h}	310.32 ± 12.1^{a,b,c,e,h}	237.77 ± 10.83^{c,h}	657.74 ± 32.9^{a,b,d,e,f,g}
Insulin µg/L	3.92 ± 0.05	3.81 ± 0.05^{c,d,e,f,h}	1.92 ± 0.04^{a,b,d,f,g,h}	3.30 ± 0.11^{a,b,c,e,h}	1.86 ± 0.06^{a,b,d,f,g,h}	3.19 ± 0.06^{a,b,c,e,g}	3.70 ± 0.09^{c,e,f,h}	2.63 ± 0.08^{a,b,c,d,e,g}
Interleukin-6 Pg/ml	29.2 ± 0.35	27.71 ± 0.50^{c,e,f,h}	50.45 ± 2.43^{a,b,d,f,g}	33.22 ± 0.23^{c,e,h}	55.24 ± 2.68^{a,b,d,f,g,h}	38.96 ± 0.48^{a,b,c,e,g}	29.62 ± 1.04^{c,e,f,h}	42.39 ± 1.16^{a,b,d,e,g}

Each value represents the mean ± SE. (a) significant compared to G1 (control), (b) significant compared to G2 (propolis), (c) significant compared to G3 (diabetic), (d) significant compared to G4 (chlorpyrifos), (e) significant compared to G5 (diabetic/chlorpyrifos), (f) significant compared to G6 (diabetic/proplis), (g) significant compared to G7 (proplis/chlorpyrifos), (h) significant compared to G8 (diabetic/chlorpyrifos/proplis).

these dissimilarities (Table 2).

3.6. Correlations analysis

The 2-tailed Pearson correlations among different investigated biochemical parameters revealed that there were significant negative correlations ($P < .01$) between the cardiac PON1 and plasma glucose, total cholesterol, TG, HDL, LDL, CK, LDH and IL-6 with −0.629, −0.715, −0.574, −0.275, −0.748, −0.588, −0.598 and − 0.655 correlation coefficients (r), respectively. In contrast, the plasma insulin level scored a significant positive correlation with 0.715 correlation coefficient. Although there were significant negative correlations ($P < .01$) between the cardiac PON1 and PC, SOD, CAT and XO ($r = -0.548$, −0.760, −0.725 and − 0.805, respectively), AChE and total thiol scored significant positive correlations ($r = 0.800$ and 0.754, respectively).

Anti-clockwise, there were significant positive correlations ($P < .01$) between the cardiac XO and plasma glucose, total cholesterol, TG, HDL, LDL, CK, LDH and IL-6 ($r = 0.724$, 0.834, 0.705, 0.639, 0.760, 0.739, 0.820 and 0.793, respectively) beside that the plasma insulin recorded a significant negative correlation ($r = -0.787$). While there were significant positive correlations ($P < .01$) between the cardiac XO and PC, SOD and CAT ($r = 0.760$, 0.854 and 0.873, respectively), AChE and total thiol scored significant negative correlations ($r = -0.815$ and − 0.750, respectively).

3.7. Joint action analysis

The joint action analysis revealed that the diabetic/chlorpyrifos group has different types of interaction among the quantifiable biochemical markers. Generally, an additive interaction effect detected in plasma glucose, cholesterol, triglyceride, and IL-6 as well as the cardiac SOD and XO. The potentiation interaction recorded in plasma CK, LDH, and HDL beside the cardiac PC and CAT. The plasma insulin and LDL scored an antagonistic effect and the pattern observed in the cardiac AChE, total thiol, and PON1 (Table 3).

3.8. Cardiac PON1 and XO mRNA genes expression

The mRNA genes expression of PON1 and XO have confirmed the enzymes specific activities where there was significant up-regulation ($P < .01$) in PON1 by 1.7 fold and down-regulation in XO by 0.32 fold after propolis administration as compared with control. There was significant down-regulation ($P < .01$) in the mRNA of PON1 after

Table 2
Efficiency roles of propolis on diabetic and/or chlorpyrifos induced amendment in the cardiac AChE, total thiol, PC, SOD, CAT, PON1, and XO.

	G1	G2	G3	G4	G5	G6	G7	G8
AChE µM/min/mg protein	152.77 ± 4.32	147.08 ± 6.00^{c,d,e,g,h}	99.61 ± 4.70^{a,b,d,e,f,g}	60.93 ± 3.21^{a,b,c,g,h}	49.44 ± 2.20^{a,b,c,f,g,h}	136.19 ± 4.05^{c,d,e,h}	122.19 ± 6.84^{a,b,c,d,e,h}	98.27 ± 4.54^{a,b,d,e,f,g}
Total Thiols µM/mg protein	100.94 ± 4.72	109.05 ± 2.46^{c,d,e,f,g,h}	76.67 ± 2.73^{a,b,g}	82.82 ± 1.12^{a,b}	71.96 ± 2.84^{a,b,f,g}	86.34 ± 3.85^{a,b,e}	90.59 ± 3.70^{b,c,e}	81.47 ± 1.76a,b
PC µM/mg protein	122.25 ± 3.60	121.53 ± 2.96^{c,e}	154.35 ± 6.31^{a,b,e,g}	137.00 ± 4.37^{e}	190.35 ± 5.5^{a,b,c,d,f,g,h}	136.10 ± 4.60^{e}	125.47 ± 12.07^{c,e}	144.20 ± 2.61^{e}
SOD U/mg protein	44.48 ± 2.25	38.66 ± 1.55^{c,d,e,f,g,h}	96.82 ± 3.17^{a,b,d,e,f,h}	68.37 ± 2.25^{a,b,c,e,h}	125.51 ± 3.80^{a,b,c,d,f,g,h}	68.86 ± 3.23^{a,b,c,e,h}	58.69 ± 1.98^{a,b,e,h}	85.65 ± 3.4^{a,b,c,d,e,f,g}
CAT µM/min/mg protein	56.57 ± 2.24	49.57 ± 2.47^{c,d,e,f,g,h}	111.32 ± 7.21^{a,b,d,e,f,g}	80.27 ± 2.29^{a,b,c,e}	146.80 ± 4.64^{a,b,c,d,f,g,h}	80.49 ± 3.13^{a,b,c,e}	65.25 ± 1.61^{b,c,e,h}	92.22 ± 3.20^{a,b,e,g}
PON1 µM/min/mg protein	257.88 ± 14.71	323.70 ± 15.6^{a,c,d,e,f,g,h}	116.68 ± 5.17^{a,b,f,g,h}	117.21 ± 3.55^{a,b,f,g,h}	102.78 ± 3.14^{a,b,f,g,h}	200.33 ± 3.95^{a,b,c,d,e}	188.67 ± 5.41^{a,b,c,d,e}	183.00 ± 5.98^{a,b,c,d,e}
XO µM/min/mg protein	194.47 ± 15.21	106.81 ± 6.03^{a,c,d,e,f,g,h}	385.57 ± 24.7^{a,b,e,f,g}	392.44 ± 22.7^{a,b,e,f,g}	583.07 ± 24.3^{a,b,c,d,f,g,h}	249.40 ± 17.4^{b,c,d,e,h}	230.79 ± 17.6^{b,c,d,e,h}	367.41 ± 24.5^{a,b,e,f,g}

Each value represents the mean ± SE. (a) significant compared to G1 (control), (b) significant compared to G2 (propolis), (c) significant compared to G3 (diabetic), (d) significant compared to G4 (chlorpyrifos), (e) significant compared to G5 (diabetic/chlorpyrifos), (f) significant compared to G6 (diabetic/proplis), (g) significant compared to G7 (proplis/chlorpyrifos), (h) significant compared to G8 (diabetic/chlorpyrifos/proplis).

Table 3
Joint action analysis on the different studied parameters in diabetic and chlorpyrifos insecticide.

Plasma				Cardiac			
Parameters	Effect	I.I	Joint action	Parameters	Effect	I.I	Joint action
Glucose	Increase	0.99	Additive	AchE	Decrease	1.25	Antagonism
Cholesterol	Increase	1.03	Additive	Total Thiols	Decrease	1.08	Antagonism
Triglycerides	Increase	1.01	Additive	PC	Increase	1.07	Potentiation
HDL	Increase	1.53	Potentiation	SOD	Increase	1.02	Additive
LDL	Increase	0.87	Antagonism	CAT	Increase	1.06	Potentiation
CK total	Increase	1.36	Potentiation	PON1	Decrease	1.52	Antagonism
LDH	Increase	1.43	Potentiation	XO	Increase	0.99	Additive
Insulin	Decrease	1.1	Antagonism				
Interleukin-6	Increase	1.00	Additive				

Interaction Index (I·I) = 1 ± 0.05 for additive effect. In cases of positive effect on the baseline values (increase) > 1 for potentiation, or < 1 for antagonism. In cases of negative effect on the baseline values, ≤ 1 for potentiation, or > 1 for antagonism.

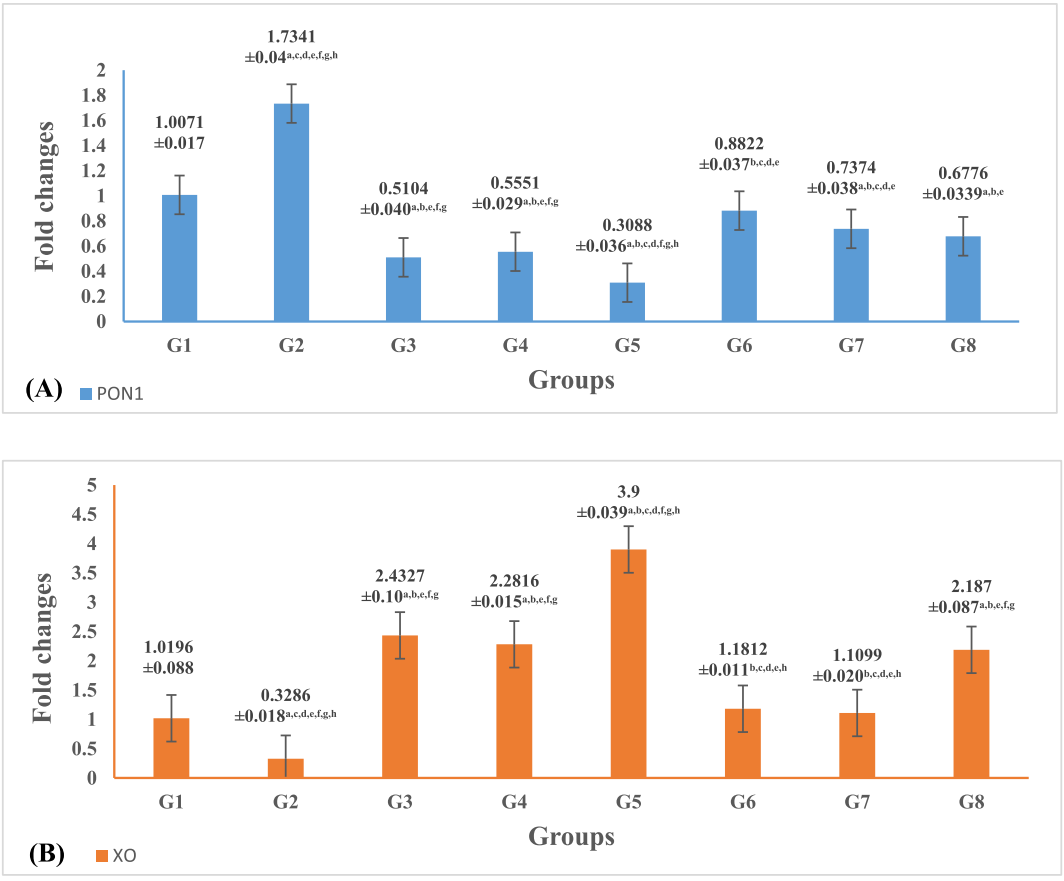

Fig. 1. Fold changes of PON1 (A) and XO (B) mRNA gene expressions. (a) significant compared to G1 (control), (b) significant compared to G2 (propolis), (c) significant compared to G3 (diabetic), (d) significant compared to G4 (chlorpyrifos), (e) significant compared to G5 (diabetic/chlorpyrifos), (f) significant compared to G6 (diabetic/proplis), (g) significant compared to G7 (proplis/chlorpyrifos), (h) significant compared to G8 (diabetic/chlorpyrifos/proplis).

diabetic, chlorpyrifos intoxication and their combination by 0.51, 0.55 and 0.308 folds, respectively as compared with control one. However, the mRNA of XO up-regulated in the same groups by 2.43, 2.28 and 3.9 folds, respectively as compared with control ($P < .01$). While the mRNA levels of PON1 and XO didn't return to the normal values after the combination group co-treated with propolis, this supplementation standardized those enzymes in case of diabetic alone. Supplementation of propolis with the chlorpyrifos intoxication also normalized the mRNA of XO gene expression and this group still has a significant down-regulation in PON1 as compared with control (Fig. 1).

4. Discussion

To our knowledge, the current study is considered the primary one for the evaluation of the anti-diabetic and/or antioxidant characteristics of propolis aqueous extract against the cardiotoxic effects of chlorpyrifos in case of diabetic-induced cardiac complications. Firstly, we have verified the representative signs of diabetes as hyperglycemia, hypoinsulinemia, combined hyperlipidemia associated with IL-6 elevation especially in the combined group and some of these effects still existent in diabetic alone and chlorpyrifos intoxicated one. These influences may result chiefly from the adverse metabolic impacts and inflammatory reactions after pancreatic beta-cells destructions by STZ and/or the cholinergic disturbance mechanism of chlorpyrifos.

In fact, STZ is selectively arriving pancreatic beta-cells via the glucose transporter and the progress of hyperglycemia in rats is principally due to direct pancreatic beta-cells necrosis (Furman, 2015) as STZ is a forceful agent for DNA methylation and nitric oxide donor. Further, it produced DNA strand disruptions via nuclear synthetase release which leads to obstruction of proinsulin synthesis and finally diabetes implementation (Sudhakara et al., 2012).

Sequentially, lipoprotein lipase (LPL) is obstructed due to hyperglycemia and hypoinsulinemia, as insulin is a forceful activator for LPL, in adipose tissue resulted in triglycerides accumulation. Insulin also has a direct optimistic impact on the LPL gene in promoting LPL synthesis, whereas hypercholesterolemia in diabetic rats results from enlarged intestinal absorption and cholesterol synthesis (Hussien and Shoman, 2015). Correspondingly, due to lack of insulin, triglycerides are broken down to give glycerol and fatty acids in the liver β-oxidation to synthesize acetyl CoA which leads to a rise in cholesterol biosynthesis consequences to total cholesterol and LDL-cholesterol elevations.

On the other hand, accumulations of acetylcholine as a response to chlorpyrifos toxicity is considered a promising explanation for the insulin decline in plasma (Peris-Sampedro et al., 2015). It is well acknowledged that acetylcholine prompts the release of the catecholamines in the adrenal medulla which could trigger transitory hyperglycemia and insulin resistance by diminishing insulin-stimulated translocation of glucose transporters to the plasma membrane (Ziegler et al., 2012) which may be followed in the present study.

In the existing study, the effect of chlorpyrifos in lipid profiles noted as hypercholesterolemia and the intrinsic mechanisms of this effect haven't been recognized yet (Peris-Sampedro et al., 2015). However, the rise in total cholesterol level may be attributed to the effects of chlorpyrifos on the penetrability of the liver cell membrane and/or the impasse of the liver bile ducts, which reduces or stops cholesterol excretion into the duodenum (Ogutcu et al., 2008; Elsharkawy et al., 2013). Also, chlorpyrifos disturbs the crucial enzymes linked to lipid metabolism as monoacylglycerol lipase and fatty acid amide hydrolase (Quistad et al., 2006) which leads to hypercholesterolemia and LDL elevations.

Actually, chemical interactions represent a deviation from simple additivity and the potentiation is observed when the effect of a chemical is enhanced by other whereas the interference between two different compounds leads to antagonism (Hernández et al., 2013). The HDL potentiation reaction may be resulted mainly from the cholesteryl ester transfer protein decay by STZ. Cholesteryl ester transfer protein smooths the transmission of cholesteryl esters from HDL to apoB100-containing lipoproteins in interchange for triglyceride (J. Niesor et al., 2012). The deficiency in the transfer associated with diabetic chiefs to a substantial rise in HDL cholesterol levels (Aslan et al., 2013) which fails to reduce atherosclerosis. So, there were many restrictions in the anti-atherogenic role of HDL levitation (Malara et al., 2016) as realized in the combination group.

Our results suggested that, as chlorpyrifos and STZ individually didn't alter the HDL and induced LDL elevations, the antagonistic effect recorded in the mixture with LDL to decline HDL potentiation response. On the other hand, STZ could prevent the biosynthesis of proinsulin plus chlorpyrifos induced insulin resistance and didn't succeed to hyperglycemia initiation may be via inducing the antagonistic action in plasma insulin and additive effect in glucose. Also, cholesterol has scored an additive effect which may consequence from hypercholesterolemia induced by both STZ and chlorpyrifos.

Consistently, we noted that the plasma IL-6 considerably increased in diabetic rats and the combined group which has verified an additive effect. This effect may be produced mainly as diabetic complications and/or immune system activation.

Really, the progressive of beta-cell failure by STZ has been considered the main basis of the cell mass condense. There is evidence that inflammatory mediators may not only represent markers for metabolic aberrations but may also contribute energetically to beta-cell death. Additionally, apoptotic cells by themselves can provoke the initiation of the innate immune system and the conditions of diabetes can rise the creation of proinflammatory cytokines including IL-6 (Alexandraki et al., 2006). However, an additive effect was scored in the combined group as chlorpyrifos induced inflammatory responses via IL-6 (Mense et al., 2006), the selected dose didn't succeed for the orientation of this effect individually.

Our results revealed that hyperglycemia and hyperlipidemia associated with significant elevations in plasma cardiac enzymes (LDH and total CK) in the diabetic group and the potentiation effect scored in the combined one. These influences may be attributed to the pathogenesis of cardiac dysfunction through diabetes and/or cardiotoxic effects of chlorpyrifos.

Recently, the pathogenesis of cardiac disturbances in diabetes is occasioned from the trouble in intracellular Ca^{2+} signaling during the contractile cycle lead to a decline upstroke phase of the Ca^{2+} transient due to the drop in the discharge of Ca^{2+} from the sarcoplasmic reticulum (Teshima et al., 2000). In addition, the diastolic decay of the Ca^{2+} transient is diminished due to condensing the activity of the sarco-endoplasmic reticulum Ca^{2+}-ATPase pump (Ganguly et al., 1983) which may induce diabetic cardiomyopathy. On the other hand, chlorpyrifos cardiotoxic effects may occur via direct myocardial endothelial damage and the destructions of myocardial cells which lead to the release of the cardiac enzymes into the bloodstream (Wakf et al., 2018). As the designated dose of chlorpyrifos induced a non-significant elevation in cardiac enzymes, a potentiation response verified in the combined group.

Indeed, the oxidative stress is the main cause of the diabetes pathogenesis and its complications which notable by myocardial cellular structures and functional amendments. Oxidative injury is associated with histopathological alterations in the heart tissue which can be attenuated by declines in the antioxidant ability of the cardiac cells after production of ROS that leads to marked myolysis, degeneration, vacuolation of myocardial fibers and finally to failure (Atta et al., 2018). Oxidative damage is involved also in the cardiotoxic effect of chlorpyrifos which associated with many histopathological alterations as congested thickened arteries, edema, disorganization, vacuolization, and degeneration in myocardial fibers with separation of myofibrils (Wakf et al., 2018).

Subsequently, the steady status of cardiomyopathy may be up via oxidative stress scenario which has observed as significant reductions in the cardiac total thiols and PON1 beside the elevations in enzymatic activities of SOD, CAT and XO in diabetic and/or chlorpyrifos groups. The dual analysis revealed that the diabetic/chlorpyrifos group scored additive effects in SOD and XO, whereas the potentiation interactions recorded in PC and CAT lastly, total thiol and PON1 planned antagonistic effects.

Our results proposed that the diabetic-induced IL-6 elevation may modulate PON1 mRNA and protein levels. In this respect, insulin resistance during diabetic cardiomyopathy increased the mitochondrial ROS production by debility of the anti-atherosclerosis enzymes (Giacco and Brownlee, 2010). Frequently, the creations of ROS induced-oxidative stress and raises in the cytokines as IL-6 resulted in the development of an inflammatory response leading to negative regulation of the PON1 gene. However, there are three possible mechanisms involved in chlorpyrifos induced negative transcriptional regulation: (1) modulation of the PON1 promoter during the biotransformation; (2) alteration of some transcription factors by cytokines; and (3) through a direct regulation of the small heterodimer partner gene which exerts repressive activity on transcription factor present in the PON1 promoter and posttranscriptional regulation (Medina-Díaz et al., 2017). As chlorpyrifos didn't induce a significant elevation in IL-6, it could down-regulated PON1 via mechanism totally varied from diabetic and induced antagonistic action in a combined group.

Vitally, our results showed that XO scored a negative correlation with PON1 and the mechanism by which the diabetic and/or

chlorpyrifos induced this up-regulation is still unclear. However, XO mRNA gene expression may be over-expressed as this enzyme has been occupied in free radical creation due to xanthine oxidoreductase raise during diabetes and/or chlorpyrifos intoxication. Actually, the hypoxanthine is oxidatively catalyzed via XO to xanthine and uric acid which finally generated O_2^- and H_2O_2 (McCord et al., 1985) as an intracellular origin of ROS that participated in the oxidative burst which induced the diabetic cardiomyopathy (Kumar et al., 2013). XO also facilitates the pesticides-induced oxidative stress through inducible nitric oxide synthase self-mechanism, which might be planned by inflammatory cytokines (Singh et al., 2015). As both diabetic and chlorpyrifos possibly could utilize XO in free radical production, an additive effect observed in a concoction.

We also anticipated that oxidative damage in cardiac tissue didn't ensue by ROS generation via XO up-regulation alone, but PC elevation involved also as a positive correlation scored between them and a negative one with PON1. Protein carbonyls are the best common indicator of protein oxidation and created when reducing sugars react with lysine remains of proteins with the subsequent formation of advanced glycation end products as central bases of ROS (Nowotny et al., 2015). So, the raises of cardiac PC levels may be resulted from impairment of the protein structure by side chains oxidation of some amino acid residues due to diabetic and/or chlorpyrifos intoxication. Chlorpyrifos also induced potentiation reaction when combined with diabetes as itself scored non-significant elevation.

The elevations in the cardiac SOD and CAT activities due to diabetes and/or chlorpyrifos may be reflected a compensatory mechanism to evoke oxidative stress as they scored a positive correlation with XO and PC while negative one with PON1. SOD is the primary line of defense against the oxy-radical harmful effects in the cell and CAT is the key antioxidant enzyme in the straight removal of ROS. Superoxide ions are removed by SOD in mitochondria which catalyzed by dismutation (Kinnula and Crapo, 2004) and the subsequent H_2O_2 is eliminated by CAT, however, the post-translational regulation is responsible for amplified SOD and CAT after oxidative stress (Hassani et al., 2018). As both diabetes and chlorpyrifos may affect directly on dismutation, an additive effect scored in their combination. However, the CAT activity depends on the quantity of H_2O_2 induced by SOD and diabetes scored higher effect on CAT, chlorpyrifos may potentiate the rise response.

The decline in the cardiac total thiol content in diabetes and/or chlorpyrifos groups may be attributed to the utilization of total thiol through ROS generation as it scored negative correlations with PC, XO, SOD and CAT, however, it documented a positive correlation with PON1. The decline in the thiol levels noted as a part of their defense role contrary to free radicals induced by chlorpyrifos (Wakf et al., 2018) which leads to glutathione depletion as it has a compensatory mechanism of the antioxidants to combat the oxidative stress. As STZ-induced diabetic has affected on total thiols via suppression of thiol antioxidative genes (Liang and Pietrusz, 2007) which completely diverse from chlorpyrifos, antagonistic action documented in a combined group.

The inhibition of the cardiac AChE in diabetic rats may consequence from oxidative stress as it recorded a positive correlation with PON1 and total thiols, while, the negative correlation logged with XO, CAT, SOD, and PC. Diabetes is associated with cholinergic alterations where insulin resistance produces lipid peroxidation and decrease membrane fluidity which leads to a decline in the activity of AChE and acetylcholine level (Ghareeb and Hussen, 2008). Meanwhile, the direct bind and/or interaction of chlorpyrifos with cardiac muscarinic receptors could inhibit the AChE activity (Howard and Pope, 2002), a combined effect reached to an antagonism.

Noticeably, propolis aqueous extract significantly alleviated the hyperglycemia, hypoinsulinemia, hyperlipidemia, IL-6 elevation and oxidant/antioxidant turbulences to nearly the normal levels in diabetic and/or chlorpyrifos groups. The amelioration efficiency may result from antidiabetic, antioxidant and immunomodulatory effects of propolis.

The antidiabetic characteristic of propolis aqueous extract resulted from its components like vitamins, polyphenols, and amino acids which may hinder the IL-1β generation besides nitric oxide synthase and finally prevent the β-cells destruction (Matsushige et al., 1996). Propolis also modulates glucose levels via inhibition of α-glucosidase, essential for carbohydrate digestion, that would be expected to improve insulin sensitivity.

The hypolipidemic efficiency of propolis aqueous extract may be attributed to flavonoids comprising compounds which hinder the triglyceride and cholesterol hepatic synthesis via inhibitions of the 3-hydroxy-3 methyl-glutaryl-CoA reductase and acyl-cholesterol-O-Acyl transferase (Bok et al., 1999). Propolis also obstructs the lipids uptake in the digestive tract, eliminates LDL from the blood, increases the activity of degradation enzymes, and finally increases the HDL by refining the liver ATP-binding cassette transporters (Nader et al., 2010).

The anti-inflammatory action of propolis may outcome from the prevention of arachidonic acid release from the cell membrane by chrysin and caffeic acid phenethyl ester (Lee et al., 2004) which leads to the suppression of pro-inflammatory accomplishments of cyclooxygenase and inducible nitric oxide synthase. Taken together, hypoglycemic, hypolipidemic, and immunomodulatory actions of propolis are good indicators for its potential as a cardiovascular protector which leads to the cardiac enzymes modulation (LDH and total CK) in blood circulation.

Our results suggested that the primary mechanisms for antioxidant activity of propolis are basically due to free radical predator via PON1 mRNA up-regulation and obstruction of ROS creation by XO mRNA down-regulation. According to the literature, propolis biophenols contents may inhibit the enzymatic systems involved in ROS initiation reactions as XO (Harris et al., 2000). Caffeic acid phenethyl ester, an active element of propolis, excites the transcription issue of the antioxidant enzymes and triggers the antioxidant stress comeback (Mapesa et al., 2011) which may clarify the elevations of PON1 and PC reduction. Gastric acid secretion encouraged by caffeic acid phenethyl ester also stimulated the acetylcholine agonist receptors (Borrelli et al., 2005) may explain the stimulatory effect of propolis on AChE activity. Pinocembrin, propolis abundant flavonoid, can diminish intracellular ROS indirectly by increasing intracellular glutathione (Jin et al., 2015) leading to the neutralization of total thiol content and it may contribute to the major significant alleviations in antioxidant activity of CAT and SOD.

5. Conclusions

Our results provide a significant potentiation action of chlorpyrifos on diabetes mellitus induced cardiomyopathy via cumulative the vulnerability of cardiac tissue to oxidative injury. However, co-treatment with propolis alleviated such effects perhaps by the improvement of PON1 and XO mRNA genes regulation which resulted in the enrichment of the cellular enzymatic and/or non-enzymatic antioxidants. According to our finding, the dietary propolis may recover the risk of cardiovascular diseases which resulted from the prognosis of diabetes complications and/or pesticides exposure, however, more research could be conduct to focus in this topic.

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