Cardiovascular Toxic Effects of Chlorpyrifos: A Possible Protective Role for Pomegranate Extracts

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Introduction

At present, there are more than 65,000 chemicals used as insecticides released daily into the environment [1]. Many of insecticides including organophosphorus compounds (OP) have toxic effects on human and other organisms [2]. Chlorpyrifos [O,O-diethyl 0-3,5,6-trichloro-2-pyridylphosphoro-thioate (CPF)] is one among a broad spectrum OP insecticides widely used for agricultural pest control, sterilization and industrial applications [3]. CPF like other organophosphates exerts its toxic effects mainly by inhibiting the action of acetylcholinesterase enzyme activity [4]. It has also demonstrated that CPF toxicity is closely related to increased oxidative stress with accumulation of lipid peroxides in different organs [5], causing several health hazards [6].

Cardiovascular system (CVS) affection is one of the most frequent sequelae of exposure to OP poisoning. CVS manifestations of OP poisoning include sinus tachycardia, sinus bradycardia, hypertension, and impaired heart rate [7]. Heart tissue has reported to produce high levels of free radicals in response to CPF poisoning which make it particularly sensitive to oxidative stress and peroxidative damage [8]. This effect represents a serious risk for progression of cardiovascular system changes, including myocardial necrosis and increased serum levels of cardiac marker enzymes; creatinine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) [9]. ECG alterations have also been reported following CPF poisoning, as presented by prolonged corrected QTc interval, ST segment elevation, low-amplitude T waves, and prolonged PR interval [10]. Previous studies also described derangements in the blood pressure regulation, thermoregulation, and other autonomic nervous system functions in CPF-insecticide poisoning [11].

Punica granatum L. (family Punicaceae), commonly called pomegranate, is described as nature's power fruit. Pomegranate is a...
plant used in folkloric medicine to induce beneficial effects on human health and disease prevention [12]. It is particularly native to the Mediterranean region and has widely cultivated by the ancient Egyptians over 3000 years ago [13]. Pomegranate is generally used as an astringent, cardiotonic and digestive agent. Also, it is highly effective in chronic diarrhea, dyspepsia, colitis, dysentery, piles and uterine disorders [14]. Pomegranate is a good source of polyphenolic components such as ellagic acid and ellagitannin [15] which act as primary anti-inflammatory, anti-carcinogenic, platelets aggregation inhibiting and antioxidant [16]. Moreover, pomegranate extracts were found to provide protection against free radicals-induced lipid peroxidation and DNA damage [17].

Given its wide spread health benefits, the present study aimed to investigate protective effects of either pomegranate juice (PJ) or pomegranate peel methanol extract (PPME) against CPF-induced cardiotoxicity and whether these effects are mediated through normalizing ECG alterations and cardiac oxidative status.

Materials and Methods

Chemicals

Chlorpyrifos (CPF) was obtained from Misr for Agricultural Development Company, Cairo, Egypt under trade name Dursban. CPF was orally administered at a dose level equivalent to 1/20 of LD50 (6.75 mg/kg) in distilled water for 60 successive days. This selected dose was based on previous studies in which 1/20 LD50 of CPF induced biochemical alterations in rats without morbidity [18]. Stock solution was prepared by bringing CPF to room temperature then taking a certain amount from CPF and dilute it in distilled water (0.25 ml of CPF was dissolved in 250 ml distilled water) and the working solution was freshly prepared daily for dosing [19].

Preparation of pomegranate extracts

Pomegranate juice (PJ) and pomegranate peel methanol extract (PPME) were prepared as described by Abdel Monem et al. [12]. The fresh ripened pomegranate fruits were purchased from a local market at Mansoura City. Ten kg of pomegranate were washed and manually peeled, without separating the seeds. Juice was obtained using an electrical blender, filtered with a buchner funnel and immediately diluted with distilled water to volume of 1:3 and stored at -20°C until used [20]. Pomegranate peels were manually separated, air dried at room temperature and powdered by an electric mill. The powder (25 g) was extracted by mixing with 100 ml methanol at 30°C for 1 h using a magnetic stirrer. The extract was filtered to remove the peel particles. The residue was re-extracted with the same solvent. The extracts were pooled and concentrated under vacuum at 40°C [12,21].

Experimental design

Thirty six adult male albino rats weighing 170-180 g were used in the present study. The rats were obtained from the Urology and Nephrology center, Mansoura University, Mansoura, Egypt. The rats were maintained under normal laboratory conditions of aeration and temperature (25 ± 2°C). They were provided with water and normal laboratory diet ad libitum. Care and use of experimental animals were carried out as guided by the Animal Care Committee of Mansoura University, Mansoura, Egypt. All experimental protocols and procedures were approved by, institutional review board (IRB)-Mansoura Faculty of Medicine (code # phd/17.9.101).

After one week of acclimation, rats were randomly divided into six equal groups (six animals/each) as follows: Group 1 received normal laboratory diet without any treatment and served as control; Group 2 received PJ orally at dose (3 ml/kg b.wt) [12]; Group 3 received PPME orally at dose (200 mg/kg b.wt) [22]; Group 4 received CPF orally at dose (6.75 mg/kg) [18]; Group 5 and Group 6 were given CPF and then administered with PPME and PJ, respectively at doses exactly similar to the above mentioned groups. Treatment in all groups was given orally by gastric gavage and continued for 60 successive days.

ECG, heart rate and blood pressure

Following the last treatment, ECG, heart rate and blood pressure were recorded under light ether anesthesia, using Biopac student lab system (software BSL 3.7.5), data acquisition unit MP45, biopac electrode lead set X2, and disposable vinyl electrodes (3 electrodes per rat) [23], at physiology department, Faculty of Medicine, Mansoura University, Egypt.

Collection of blood samples and harvesting heart

At the end of the experimental period, overnight fasted rats were sacrificed and blood samples were collected to obtain sera by centrifugation at 855 × g for 10 min. Sera were kept at -200°C for later biochemical analysis. Immediately after collecting blood, animals were dissected. Heart from each rat was removed, cleaned with saline solution (0.9%), then divided longitudinally into two parts, the right part was homogenized in distilled water (1/10 weight/volume), centrifuged for 10 min at 855 × g and the resulting supernatant was transferred into eppendorf tubes and preserved at -80°C until used for various biochemical assays. Samples from the left part were frozen at -80°C until used for Flow cytometry analysis, while the other samples were fixed in 10% formalin for histopathological examination.

Assay of cardiac enzymes (CK-MB, LDH) and troponin 1 (cTn1)

Cardiac enzymes (CK-MB, LDH) and cTn1 were measured using commercially available kits according to the manufacturer's instructions. Kits for CK-MB were purchased from Spectrum Company, Egypt, while kits for LDH were purchased from Elitech Company, Franc, and Kits for cTn1 were obtained from Siemens Company, USA.

Estimation of oxidative stress and antioxidant markers (MDA, SOD, GSH)

Malondialdhyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) were assessed by colorimetric kits obtained by colorimetric kits obtained by Bio-Diagnostic Company, Giza, Egypt according to the manufacturer's instructions.

Assessment of cell cycle phases% and apoptotic markers (Bax, Caspase-3, p53, Bcl-2)

Flow cytometric analysis of cardiac Bax, caspase-3, P53, Bcl-2 and cell cycle phases (G0/G1, S-and G2/M %) were determined according to the method of Tribukait et al. [24], using FACS caliber flow cytometer (Becton Dickinson, Sunnyvale, CA, USA), equipped with a compact air cooled low power 15 m watt argon laser beam (488 nm). Average evaluated nuclei per specimen are 20,000 (120 nuclei/s).
Dean and Jett computer program for mathematical analysis was used to obtain the DNA histograms [25].

**Histopathological examination**

After fixation, the heart tissue was embedded in paraffin, then sectioned and stained with hematoxylin and eosin (H&E) [26].

**Statistical analysis**

Data were expressed as Mean ± SE. Comparison for parametric data was carried out by analysis of variance (ANOVA) followed by Tukey’s post hoc analysis for intergroup multiple comparisons. Results were considered significant at p<0.05.

**Results**

**ECG, heart rate and blood pressure**

Treatment of normal rats with PPME or PJ did not produce significant changes in all ECG variables, heart rate (HR) and blood pressure [systolic and diastolic (SBP & DBP)] compared to control group. Exposure to CPF caused marked ECG alterations presented as, significant decrease in RR interval, elevation in ST and prolongation in corrected QTc interval, with increase in the HR and BP. Administration of CPF with either PPME or PJ tended to restore the above described changes where significant increase in RR interval and significant reduction in the elevated ST segment, corrected QTc interval, HR and BP were noted (Table 1 and Figures 1A-1C).

<table>
<thead>
<tr>
<th>Group</th>
<th>DBP (mmHg)</th>
<th>SBP (mmHg)</th>
<th>HR (bpm)</th>
<th>QTc (msec)</th>
<th>ST (mv)</th>
<th>RR (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88.33 ± 2.01</td>
<td>124.00 ± 1.44</td>
<td>220.86 ± 1.72</td>
<td>0.17 ± 0.00</td>
<td>5.50 ± 1.00</td>
<td>0.27 ± 0.00</td>
</tr>
<tr>
<td>PPME</td>
<td>88.67 ± 1.08</td>
<td>124.17 ± 1.62</td>
<td>220.33 ± 4.37</td>
<td>0.17 ± 0.00</td>
<td>5.70 ± 1.00</td>
<td>0.27 ± 0.00</td>
</tr>
<tr>
<td>PJ</td>
<td>88.67 ± 3.07</td>
<td>124.67 ± 2.91</td>
<td>220.00 ± 6.01</td>
<td>0.17 ± 0.01</td>
<td>5.50 ± 1.00</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>CPF</td>
<td>128.00 ± 3.69a</td>
<td>171.00 ± 2.85a</td>
<td>417.52 ± 3.53a</td>
<td>0.27 ± 0.01a</td>
<td>14.20 ± 5.00a</td>
<td>0.14 ± 0.002a</td>
</tr>
<tr>
<td>CPF+PPME</td>
<td>104.50 ± 3.18ab</td>
<td>143.33 ± 3.85ab</td>
<td>286.46 ± 4.10ab</td>
<td>0.20 ± 0.01ab</td>
<td>7.80 ± 1.00ab</td>
<td>0.22 ± 0.001ab</td>
</tr>
<tr>
<td>CPF+PJ</td>
<td>113.67 ± 5.38ab</td>
<td>145.83 ± 4.17ab</td>
<td>339.48 ± 10.76abc</td>
<td>0.21 ± 0.01abc</td>
<td>9.80 ± 0.04abc</td>
<td>0.19 ± 0.01abc</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE (n=6). a: significant (p<0.05) when compared with control group, b: significant (p<0.05) when compared with CPF group and c: significant (p<0.05) when compared with CPF+PPME group.

**Table 1:** Blood pressure (DBP-SBP), heart rate and ECG (QTc-ST-RR) in different studied groups.

**Cardiac enzymes (CK-MB, LDH) and troponin 1 (cTn1)**

Non-significant changes in the activities of CK-MB, LDH and the level of cTn1 were observed following administration of PJ or PPME to normal rats, however, exposure to CPF showed significant increase in serum activities of CK-MB, LDH and cTn1 level. Subsequent administration of either PPME or PJ to CPF exposed rats was found to reduce the above mentioned changes near to normal levels (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>CK-MB(U/l)</th>
<th>LDH(U/l)</th>
<th>cTn1(ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.38 ± 1.21</td>
<td>235.55 ± 27.01</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>PPME</td>
<td>86.64 ± 5.70</td>
<td>201.33 ± 5.04</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>PJ</td>
<td>85.35 ± 1.40</td>
<td>208.14 ± 25.00</td>
<td>0.30 ± 0.00</td>
</tr>
<tr>
<td>CPF</td>
<td>174.60 ± 10.68a</td>
<td>971.11 ± 82.46a</td>
<td>0.88 ± 0.46a</td>
</tr>
<tr>
<td>CPF+PPME</td>
<td>125.05 ± 14.91b</td>
<td>522.77 ± 83.43ab</td>
<td>0.24 ± 0.16ab</td>
</tr>
<tr>
<td>CPF+PJ</td>
<td>137.90 ± 1.70abc</td>
<td>559.91 ± 21.79ab</td>
<td>0.31 ± 0.04ab</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE & (n=6). a: significant (p<0.05) when compared with control group and b: significant (p<0.05) when compared with CPF group.

**Table 2:** Serum creatine kinase-MB (CK-MB), lactic dehydrogenase (LDH) and C troponin 1 (cTn1) level in different studied groups.

**Cardiac MDA, GSH and SOD activity**

CPF-exposed rats exhibited significant increase in myocardial MDA, in parallel with significant decreases in the level of GSH and SOD activity, whereas co-administration of CPF with either PPME or PJ showed a reverse pattern, where significant reduction in myocardial MDA with significant increase in both GSH and SOD were recorded compared to CPF group. On the other hand, administration of PPME or PJ to normal rats showed no significant changes in all tested parameters (Table 3).

<table>
<thead>
<tr>
<th>Group</th>
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<th>cTn1(ng/l)</th>
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<td>113.67 ± 5.38ab</td>
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<td>339.48 ± 10.76abc</td>
</tr>
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</table>

Values are represented as mean ± SE (n=6). a: significant (p<0.05) when compared with control group, b: significant (p<0.05) when compared with CPF group and c: significant (p<0.05) when compared withCPF+PPME group.

**Table 3:** Cardiac MDA, GSH and SOD activity in different studied groups.

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Table 3: Cardiac malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) in different studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/g)</th>
<th>GSH (mg/gm)</th>
<th>SOD (U/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.39 ± 5.61</td>
<td>19.35 ± 0.50</td>
<td>836.01 ± 87.53</td>
</tr>
<tr>
<td>PPME</td>
<td>59.96 ± 2.66</td>
<td>19.54 ± 1.71</td>
<td>842.26 ± 48.59</td>
</tr>
<tr>
<td>PJ</td>
<td>59.91 ± 2.64</td>
<td>19.54 ± 1.30</td>
<td>842.68 ± 52.15</td>
</tr>
<tr>
<td>CPF</td>
<td>162.30 ± 11.85a</td>
<td>5.99 ± 0.67a</td>
<td>479.62 ± 41.23a</td>
</tr>
<tr>
<td>CPF+PPME</td>
<td>82.27 ± 4.95b</td>
<td>15.04 ± 1.60b</td>
<td>756.96 ± 57.86b</td>
</tr>
<tr>
<td>CPF+PJ</td>
<td>81.64 ± 6.83b</td>
<td>14.87 ± 1.49b</td>
<td>562.26 ± 43.78ab</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE & (n=6). a: significant (p<0.05) when compared with control group and b: significant (p<0.05) when compared with CPF group.

Table 4: Apoptotic proteins (Bax, caspase 3, P53, Bcl-2) and G0/G1, S-phase and G2/M% phases in different studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>G2M %</th>
<th>S-phase%</th>
<th>G0/G1 %</th>
<th>BCL-2 %</th>
<th>P53 %</th>
<th>Caspase3%</th>
<th>Bax %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.67 ± 0.22</td>
<td>7.51 ± 0.35</td>
<td>24.59 ± 2.67</td>
<td>52.22 ± 0.79</td>
<td>58.08 ± 0.17</td>
<td>44.48 ± 0.46</td>
<td>31.98 ± 0.30</td>
</tr>
<tr>
<td>PPME</td>
<td>3.26 ± 0.21</td>
<td>6.96 ± 1.25</td>
<td>24.52 ± 1.53</td>
<td>51.24 ± 0.26</td>
<td>57.46 ± 0.39</td>
<td>44.27 ± 0.19</td>
<td>31.44 ± 0.15</td>
</tr>
<tr>
<td>PJ</td>
<td>3.24 ± 0.16</td>
<td>6.64 ± 0.15</td>
<td>24.49 ± 2.62</td>
<td>51.06 ± 0.15</td>
<td>56.77 ± 0.95</td>
<td>45.46 ± 0.11</td>
<td>31.15 ± 0.25</td>
</tr>
<tr>
<td>CPF</td>
<td>0.79 ± 0.08a</td>
<td>3.73 ± 0.48a</td>
<td>56.19 ± 5.13a</td>
<td>23.67 ± 0.64a</td>
<td>76.55 ± 1.12a</td>
<td>62.32 ± 0.59a</td>
<td>50.19 ± 0.88a</td>
</tr>
<tr>
<td>CPF+PPME</td>
<td>2.80 ± 0.56ab</td>
<td>5.12 ± 0.22ab</td>
<td>35.37 ± 2.90ab</td>
<td>45.56 ± 0.15ab</td>
<td>61.77 ± 0.25ab</td>
<td>47.46 ± 1.06ab</td>
<td>36.95 ± 0.44ab</td>
</tr>
<tr>
<td>CPF+PJ</td>
<td>2.66 ± 0.29ab</td>
<td>4.65 ± 0.32ab</td>
<td>36.20 ± 3.17ab</td>
<td>44.32 ± 0.34ab</td>
<td>62.01 ± 1.17ab</td>
<td>49.68 ± 0.19ab</td>
<td>38.38 ± 0.49ab</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SE & (n=6). a: significant (p<0.05) when compared with control group and b: significant (p<0.05) when compared with CPF group.

Discussion

Cardiotoxic effects of organophosphorus (OP) compounds are the main cause of death in case of severe poisoning because ventricular fibrillation may suddenly develop after exposure leading to cardiac death (Figure 2A) [27]. In the present study, it was demonstrated that exposure of rats to CPF for 60 days caused increased HR and BP with ECG changes characterized by significant decrease in RR intervals with prolonged QTc interval and elevated ST segment. Such results were in agreement with previous study demonstrating short RR interval and prolonged QTc interval in rats exposed to OP poisoning [28]. ECG changes could be due increase in the cellular Na⁺ and decrease in K⁺ contents [29] and impairment of mitochondrial metabolism [30] which affect RR wave amplitude [28]. Such abnormalities coupled with delay in impulse conduction through AV bundle are known to cause cardiotoxicity [28]. In the present study, pretreatment with either...
PPME or PJ in CPF-exposed rats caused significant improvement in ECG pattern (increased RR interval, and decreased ST and QTc interval) with lowering of BP and HR. The protective effects of PPME and PJ on cardiac electrical activities have been demonstrated in previous studies. In a rat model with doxirubicin-induced myocardial injury Fard et al. [31] reported that administration of whole fruit extract of pomegranate restored QTc interval up to normal level. Also, in isoproterenol-induced myocardial injury, Khatib et al. [32] found that pretreatment with PJ markedly attenuated the significant elevation in ST-segment, prolonged QTc interval and increased HR, suggesting that PJ stabilizes the cell membrane potential under this condition (Figures 2B-2E).

According to previous studies, CPF cardiotoxic effect may occur via direct myocardial endothelial damage and destruction of myocardial cells. As a result, cardiac enzymes CK-MB and LDH are released into the blood stream and serve as biomarkers for certain types of heart diseases, including myocardial, myocarditis and heart failure [33]. In the current study, CPF poisoning caused significant increase in serum cardiac enzymes (CK-MB and LDH), as well as myocardium specific cTn1 which is widely used as a predictor for heart damage. These results are in accordance with previous studies showing significant elevation in cardiac biomarkers (CK-MB, LDH and cTn1) in animals exposed to the OP compound dichlorvos (Figure 2F) [9]. Recently, Ahmed et al. [34] demonstrated that treatment with different pomegranate extracts normalized the cardiac enzymes (LDH and CK-MB) and improved the cardiac morphology in rats treated with dimethylnbenz anthracene (DMBA) and carbon tetrachloride (CCl4). In agreement, the present study demonstrated that pretreatment with either PPME or PJ to CPF exposed rats caused significant decrease in serum levels of cardiac enzymes (CK-MB and LDH) and myocardium specific cTn1, indicating strong protective effect of these extracts, probably through maintaining membrane integrity and/or permeability thereby preventing leakage of these cardiac biomarkers into the blood [35].

Accompanying the aforementioned data, more frequent manifestations of increased oxidative stress is thought to be involved in cardiotoxicity associated with CPF poisoning [9]. Likewise, Casas et al. [36] reported that diazinon OP insecticide causes excessive generation of mitochondrial ROS and induction of oxidative stress. As support, current data demonstrated increased oxidative stress, evidenced by significant elevation of MDA with reduction in endogenous antioxidants (GSH and SOD), suggesting imbalanced redox state in the myocardial cells following CPF exposure. This goes widely with the presently observed histopathological changes characterized by cytoplasmic vacuolization, edema in the connective tissue, disorganization and degeneration in cardiac muscle fibers of CPF-exposed rats, which in all may result from an increase of ROS generation in the heart tissue [8]. However, pretreatment of PPME or PJ caused significant improvement in the myocardial oxidative status, with normalized features of myocardial suggesting antioxidant effects of both PPME and PJ. Park et al. [37] demonstrated that pomegranate extracts are a well-known source of polyphenolic compounds, including tannins and flavonoids that show powerful antioxidant activities in various experimental models [16].

For more interpretation, CPF toxicity may involve mechanisms other than oxidative stress. One such mechanism is induction of apoptosis which is among the major forms of cell death [38]. CPF-induced apoptosis is characterized by loss of mitochondrial potential, appearance of nuclear fragmentation and down regulation of Bcl-2 [11]. Other studies reported induction of apoptosis in various organs through activating caspase pathways [6]. In the present study, prolonged exposure to CPF caused significant increase in the apoptotic proteins, Bax, caspase-3 and p53, as well as G0/G1 phase%, but decrease in the antiapoptotic protein Bcl-2, S- and G2/M phases%, indicating cell cycle arrest and induction of apoptosis in myocardial cells following CPF exposure. Induction of apoptosis might be due to peroxidation of the mitochondrial membrane by ROS, leading to loss of cell integrity, increase in membrane permeability, and DNA damage that contribute to cell death [39]. However there is now strong evidence that antioxidant supplements having the ability to inhibit DNA fragmentation and apoptosis [40]. Similar findings were demonstrated in the current study, where administration of CPF with either PPME or PJ, as a rich source of antioxidants caused significant reduction in G0/G1 phase %, Bax, caspase3 and p53 with significant increase in Bcl-2, S-and G2/M phases%, suggesting effective antiapoptotic properties of PPME and PJ, in association with potent activity for neutralizing CPF-induced ROS generation (Figures 2F and 2G).
Conclusion

The present data, provided evidence for an association between CPF exposure and cardiac toxicity, as evidenced by alterations of ECG pattern (ST, RR, QTc), elevated BP and augmented HR. Such association is mediated through disruption of cardiac oxidative status, induction of apoptosis, leakage of cardiac marker enzymes and cTnI, which in all are considered as diagnostic markers for cardiac dysfunction. Administration of either PPME or PJ tended to protect against CPF-induced cardiotoxicity, probably through its antioxidant, antiapoptotic and membrane stabilizing properties. Thus, regular consumption of pomegranate extracts can be considered as powerful cardioprotective agents.

Finally we recommend a proper strategies should be applied to restrict the use of insecticides especially OP for their hazards and health risks and also many research should be done to discover another protective natural substances to offend the insecticides health risks.

References


