Research Paper

Chlorpyrifos-induced oxidative stress and tissue damage in the liver, kidney, brain and fetus in pregnant rats: The protective role of the butanolic extract of *Paronychia argentea* L.

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ABSTRACT

Objective: Toxicity of pesticides is thought to be due to reactive oxygen species (ROS). Due to their antioxidant property, polyphenols in plant extracts may afford protection from pesticide toxicity. In the present study, we evaluated the protective effect of a butanolic extract of *Paronychia argentea* L. against toxicity caused by the organophosphorus pesticide, chloropyriphos ethyl (CE).

Materials and Methods: Pregnant albino Wistar rats were used. Pesticide and plant extract were administered daily by oral gavage from the 6th to the 15th day of gestation. Plasma and tissue malondialdehyde (MDA), blood reduced glutathione (GSH) and erythrocyte superoxide dismutase (SOD) activities were estimated. MDA levels were estimated in plasma and different organs (liver, kidney, brain, placenta and in the fetuses and their livers) as an indicator of lipid peroxydation (LPO).

Results: The data showed a significant increase in plasma and tissue LPO levels in animals treated with the pesticide while the effect was attenuated by the plant extract (CE-ex). Also, CE caused a significant decrease in antioxidant enzyme activity and this effect was partially reversed in groups treated with the plant extract. The pesticide induced embryotoxicity and resulted in resorption, fetal death and a reduced implant number.

Conclusion: It can be concluded that CE can lead to an increase in LPO production in adult and fetal tissues, while treatment with the plant extract leads to protection against CE toxicity. The decrease in LPO levels and the increase in GSH and SOD enzyme activities after treatment with the plant extract revealed its antioxidant property.

KEY WORDS: Chlorpyrifos, flavonoids, lipid peroxidation, oxidative stress, *Paronychia argentea* L., pregnant rat

Introduction

Pesticide chemicals can induce oxidative stress by generating free radicals and altering antioxidant levels of the free radical scavenging enzyme activity.[1] Chlorpyrifos (CE) is still widely used in Algeria as a pesticide for crops and farm animals. It induces toxicity through inhibition of acetyl cholinesterase (AChE) but also involves multiple mechanisms besides the inhibition of AChE.[2] Exposure to endosulfan and chlorpyrifos can differentially modify endogenous antioxidants like SOD, GPX and GSH, which can lead to the development of oxidative stress in some tissues.[3] Chlorpyrifos intoxication causes a significant decrease in the reduced glutathione (GSH), catalase (CAT) and glutathione-*S*-transferase (GST) activities.[4]

Plant extracts with antioxidant activity are traditionally used to strengthen the natural immune defences. Many studies have focused on the antioxidant effect of flavonoids,[5] resulting in their identification as potential antioxidants and anticancer agents.[6]

Several antioxidant dietary compound classes have been suggested to have health benefits. Evidence shows consumption of these products leads to a decrease in various pro-inflammatory and / or oxidative stress biomarkers.[7]

Paronychia argentea Lamb, which is widely distributed in East of Algeria, is used in folk medicine for the treatment of several disorders. Its aerial parts are used for the treatment of respiratory infections, abdominal pain and as an antistress

agent. No published studies are available till date describing its pharmacological or antioxidant effects.

As this plant is already being widely used as an herbal tea in Algeria, it is important to elucidate the role of its extract in protection from the oxidative stress caused by CE.

Materials and Methods

1. Chemicals and plant extract

Chlorpyrifos ethyl (CE) [*O*, *O*-diethyl-*O*-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate], which is being widely used as an insecticide in Algeria, was used in this study. Fresh mature *Paronychia argentea* L. plants were collected from Mila, East of Algeria in May, 2003 and identified by Prof. Dr. Khalfallah N., S. N. V. Dept.; Constantine University, Algeria. Powdered, air-dried, aerial parts (1 kg) of *Paronychia argentea* were macerated thrice at room temperature with methanol-water (MeOH-H_2O, (7:3 v/v) for 48 h. After filtration and concentration under reduced pressure, the residue was dissolved in water and re-extracted successively with chloroform ($CHCl_3$), ethyl acetate (EtOAc) and finally with *n*-butanol (*n*-BuOH). The organic layers were dried over anhydrous Na_2SO_4 (sodium sulfate) Then the solvents were removed under reduced pressure, leading to three extracts, $CHCl_3$ (6 g), EtOAc (8 g) and *n*-BuOH (36 g) extracts. In this study, we used the *n*-butanolic extract.

2. Experimental animals and treatments

Female albino Wistar rats, each weighing 175-195 g, were obtained from the Pasteur Institute, Algiers and randomized into eight groups of ten rats per group. A set of five rats was housed in a plastic cage in a temperature- and light-controlled room and the animals had free access to food and tap water. All animals were fed a commercial diet during the experiment. Daily doses of the pesticide and plant extract were administered by gavage from the 6th to the 15th day of gestation. The eight groups of rats fell into two categories. The first one served as a control and consisted of four groups out of which one was untreated and received only corn oil and the other three groups received 50, 100 or 200 mg/kg doses of the plant extract. The second category was also made up of four groups: one group was treated with CE (20 mg/kg) whereas the rats in the other three groups received the same dose of CE along with 50, 100 and 200 mg/kg doses of the plant extract. The plant extract was administered orally by gavage 15 min before oral administration of CE (20 mg/kg per day, once a day in corn oil) also by gavage. All dosing was started at the same time in the morning (0800 h) to avoid the effects of biological rhythm changes.

3. Methods of analysis

On day 19, animals were dissected under ether anesthesia and a blood sample was collected and used for the estimation of plasma malondialdhyde (MDA) as an indicator of lipid peroxidation (LPO) levels. An aliquot of heparinized blood was used for estimating GSH content. In addition, red blood cells were saved for estimation of SOD activity. After perfusion of the animal with ice-cold 0.9% NaCl through the jugular vein, the liver, kidney and brain were removed, weighed and homogenized in ice-cold potassium chloride (1.15% KCl) to give a 20% (w/v) tissue suspension. After centrifugation, the homogenate was used for the estimation of LPO levels.

Fetuses were removed, weighed and examined for any external congenital malformation. They were then assigned to two groups: the first group was homogenized in ice-cold 1.15% KCl to give 10% (w/v) tissue suspension. As mentioned earlier, the homogenate was separated by centrifugation and used for the determination of LPO levels in the fetus. The fetuses in the other group were dissected and their livers and placenta removed. These organs were treated and used as described before for both the estimation of placental and fetal liver LPO levels.

Lipid peroxidation assay

The formation of thiobarbituric acid-reactive substances (TBARs) in plasma and organs of adults (liver, kidney and brain) and of fetuses (placenta and liver) was monitored as an index of lipid peroxidation according to a previously described colorimetric method.[9] Briefly, 0.5 ml of plasma or 10% homogenate was taken in a 10 ml centrifuge tube to which 3 ml of 1% phosphoric acid and 1 ml of 0.6% aqueous thiobarbituric acid (TBA) were added. The mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml of *n*-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation and its absorbance was measured at 532 nm. Positive controls of MDA were also taken to determine the amounts of MDA formed in the organ / tissue homogenates, expressed as nanomoles of MDA per ml of plasma or per gram of wet tissue.

Glutathione level content

Determination of reduced glutathione level (GSH) in blood was carried out according to the previously described colorimetric method.[10] This method depends on the reaction of reduced glutathione with 5, 5-dithiobis, 2-(nitrobenzoic acid) that can be measured colorimetrically. The yellow color developed was measured at 412 nm against a blank reagent. GSH level was expressed as mg/dl blood.

SOD activity

Rats were fasted overnight and blood was withdrawn into EDTA. Samples were immediately centrifuged at 1500 *g* for 15 min. After separation of plasma, the packed erythrocytes were washed three times with a cold isotonic saline solution (0.9% NaCl) and centrifuged for 10 min at 1500 *g*. The washed red blood cells were lysed with ice-cold redistilled water. The suspension was centrifuged twice to obtain erythrocyte membranes and hemolysate. Hemoglobin was removed from the erythrocyte lysate by precipitating with ice-cold chloroform and ethanol. The remaining solution was shaken, centrifuged and the clear top layer was used for the determination of SOD enzyme activity.

Measurement of erythrocyte SOD activity was based on the SOD-mediated inhibition of the reaction of xanthine with xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolilum chloride to form a red formazan dye.[11] The SOD activity is expressed as IU/ml.

4. Statistical analysis

Statistical analysis was carried out using the SPSS for Windows Version 8.0 program. Data for each group of animals was subjected to analysis of variance (ANOVA). Values are given as mean ± standard deviation (SD). The statistical evaluation

of the results was carried out using two-tailed, paired student's *t*-tests. Significance was set at $P < 0.05$. A student's *t*-test was used to compare the difference between treated and control groups. Statistical significances of differences were calculated with one-way analyses of variance followed by Student-Newman-Keul's multiple-range test.

Results

The effect of CE and plant extract on the fetus.

Fetus weight

Rats treated with CE showed a significant decrease in fetal weights ($P < 0.001$) as compared to control [Figure 1A]. However, the plant extract protected the fetuses from this effect and caused a significant increase ($P < 0.001$) in fetal weight as compared to CE-treated rats as seen in groups CE-ex 200 and CE-ex 100.

Fetus and implant number

A significant decrease ($P < 0.001$) in fetus number was noted in the CE-treated group as compared to the control. In contrast, treatment with plant extract caused a significant increase ($P < 0.001$) in fetus number in groups CE-ex 200 and CE-ex 100 when compared to CE-treated rats [Figure 1B]. These results indicate that gestational exposure to this pesticide inhibited fetal growth and that a dose of 100 mg/kg of the plant extract had a protective effect against CE. Significant ($P < 0.001$) reduction in the number of total implants was found in animals treated with CE and there were high rates of pre- and postimplantation losses in pregnant rats.

The effects of different treatments on plasma, organ and fetal LPO levels

Plasma LPO levels

The present study showed that CE caused a significant increase in TBAR levels. It was clearly demonstrated that plasma LPO levels increased significantly ($P < 0.001$) after treatment with CE. MDA levels were significantly higher in the pesticide-treated group as compared to the control. The two higher doses of the plant extract caused a significant decrease ($P < 0.001$) in plasma LPO (MDA) levels compared to control and CE-treated rats [Figure 2A]. Plant extract treatment along with CE caused a significant decrease ($P < 0.001$) in plasma LPO levels compared to CE-treated rats. A dose of 100 mg/kg of the extract restored the plasma LPO levels to that of the control. These results demonstrate that this extract can protect an organism from oxidative damage induced by CE.

Tissue LPO levels

Tissue MDA levels have been used as an indicator of LPO *in vivo*. As shown in Figure 2B, MDA levels in tissues of CE-treated rats were higher than those of control rats. These results support the concept that CE toxicity increases the susceptibility of tissues of the liver, kidney and brain to CE-mediated free radical damage. The data shown in Figure 2B demonstrates that CE treatment causes a significant increase ($P < 0.001$) in liver, kidney and brain LPO levels, which was countered by the plant extract which restored the LPO levels to control values.

Furthermore, as seen earlier, the two higher doses of the plant extract (100 and 200 mg/kg) when given in combination with CE, caused a significant decrease ($P < 0.001$) in tissue LPO levels compared to CE-treated rats while the lowest dose (50 mg/kg) of the extract did nothave much effect. Thus, this

Figure 1: The effect of CE and plant extract on the fetus weight A) and fetus growth B) in pregnant Wistar albino rats. Each value represented mean ± SD, n = 10. a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$; ns: non significant, compared to control or CE-treated rats. A: difference between control and all treatments. B: difference between CE and plant extract treatments

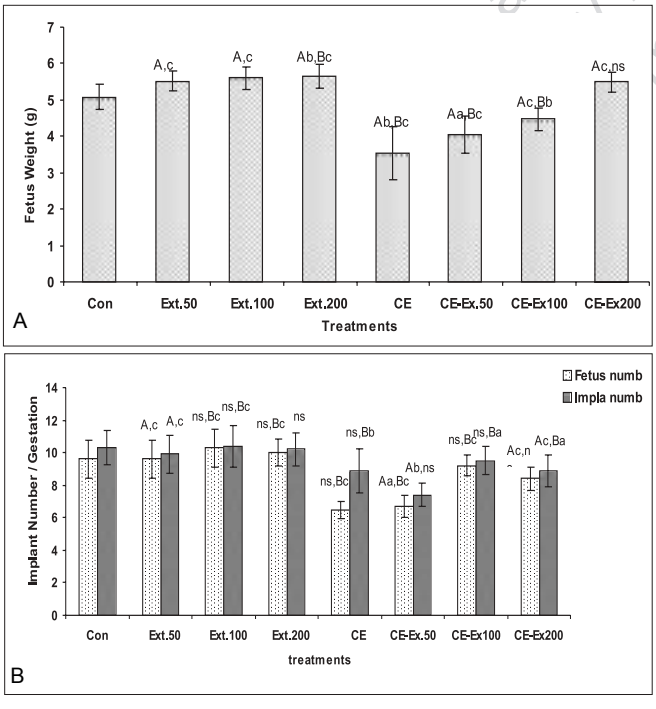
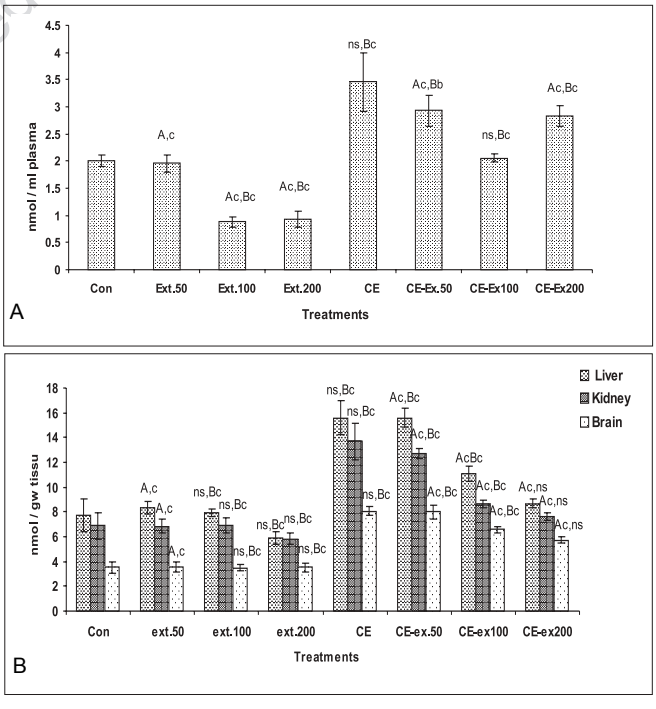


Figure 2: The effect of CE and plant extract on plasma A) and liver kidney and brain tissues B) LPO levels in pregnant Wistar albino rats. Each value represented mean ± SD, n = 10 a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$; ns: non significantnt, compared to control or CE-treated rats. A: difference between control and all treatments. B: difference between CE and plant extract treatments



study shows that the extract of *Paronychia argentea* L. protected cells and their subcellular structures from oxidative damage by decreasing MDA levels elevated due to exposure to pesticides or possibly other oxidative insults.

Placenta, fetal and fetal liver LPO levels

Data shown in Figure 1 suggests that CE treatment affects fetal growth and results in decreased fetal weights. Figure 3 shows a significant increase ($P < 0.001$) in the entire fetal as well as in fetal liver and placenta LPO levels in animals treated with CE. As seen earlier, the higher two doses of the plant extract were found to significantly ($P < 0.001$) decreasefetal placental LPO levels compared to control rats even when administered alone. However, the combination treatment of CE and the three doses of plant extract caused a significant decrease ($P < 0.001$) in fetal as well as fetal liver and placental LPO levels compared to that of CE-treated rats.

The effect of CE and plant extract on GSH content and SOD enzyme activity

Blood GSH

As shown in Figure 4A, CE treatment caused a significant decrease ($P < 0.001$) in GSH values. The two higher doses of the plant extract when given with CE, were found to restore GSH levels in the blood closer to control values. *SOD activity*

The results in Figure 4B showed a significant decrease ($P < 0.001$) in SOD activity in CE-treated animals when compared to control. Consistent with other results in this study, the two higher doses of the plant extract when given with CE, were found to restore SOD activity in the erythrocytes closer to control values.

Discussion

The results of the present study showed that CE treatment caused a significant decrease in fetal weights ($P < 0.001$) when compared to those of control. The effect of this pesticide may be explained by the fact that the fetus is not protected from the toxicity caused by the exposure of the mother to an oral dose of pesticide. The butanolic extract of *P. argentea* L. protected the fetuses from this effect and caused a significant increase ($P < 0.01$; $P < 0.001$) in fetal weights compared to that CE-treated rats. This could be explained by the observation that the plant extract treatment caused an increase in food consumption by the animals. Rats treated with CE showed a significant decrease in fetus number and an increase in embryonic mortality reaching almost 20%. These results are in agreement with other studies[12] using the same pesticide. In addition, another report describes a similar harmful impact on the development of preimplantation embryos *in vitro* and *in vivo* by another herbicide, paraquat.[13]

Literature reports claim that the central mechanism of pesticide action is via changes in the cellular oxidative status. Conclusive evidence demonstrates that supplementation with black tea extract protects against the free radical-mediated oxidative stress in hepatocytes of animals suffering from pesticide-induced live injury.[19] Endosulfan (13 mg/kg/day), an organochlorine insecticide, caused degeneration in mouse kidney due to oxidative stress.[15] Treatment with the insecticide, rotenone, increased xanthine oxidase enzyme activity and lipid peroxidation in liver tissue.[17] The pyrethroid insecticide, cypermethrin, significantly ($P < 0.05$) induced free radical production in plasma, liver, brain and testes.[18] Isoflavone was found to confer notable protection against cypermethrin-induced oxidative stress in rabbit plasma, liver, brain and testes.[18] Levels of MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, have been considered as an important indicator of lipid peroxidation.[14] In this study, we have shown that CE treatment increase MDA levels in plasma, liver, kidney and brain of the female rats as well as in the entire fetus and fetal liver and placenta. Plant extract treatment was found to significantly ($P < 0.001$) decrease LPO levels in all of these tissues in both the female rats and the fetuses. This

Figure 3: The effect of CE and plant extract on fetus, placenta and fetus' Liver LPO levels in pregnant Wistar albino rats. Each value represented mean ± SD, n = 10 a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$; ns: non significantnt, compared to control or CE-treated rats. A: difference between control and all treatments. B: difference between CE and plant extract treatments

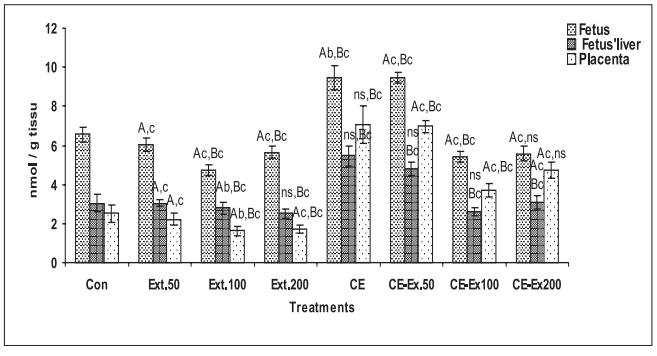
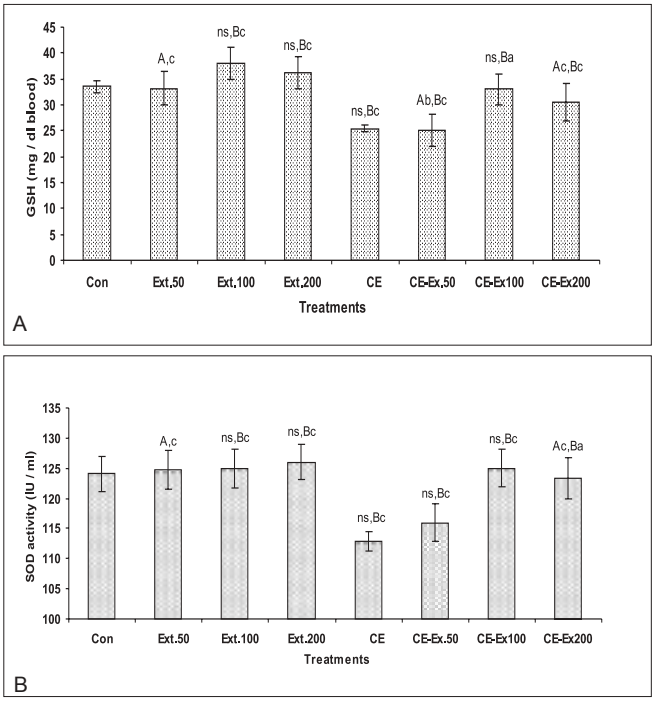


Figure 4: The effect of CE and plant extract on GSH content (A) and SOD enzyme activity (B) in pregnant Wistar albino rats. Each value represented mean ± SD, n = 10. a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$; ns: non significant, compared to control or CE- treated rats. A: difference between control and all treatments. B: difference between CE and plant extract treatments



is consistent with other observations regarding other plant extracts which were also found to be potent inhibitors of lipid peroxidation.[6] Thus, it appears that the orally administered butanolic extract of *Paronychia argentea* protects against CE-induced toxicity possibly through the inhibition of increased LPO in addition to inhibition of triglyceride accumulation, plasma membrane destruction and neutrophil infiltration in the liver tissue.

Unpublished data

Glutathione is the cell's natural antioxidant, which destroys free radicals formed in cells. Significant dose-dependent depletion of GSH levels and perturbations in antioxidant enzyme levels further confirmed the potential of the insecticide, fenvalerate to induce oxidative stress in hepatic tissue.[21] Subchronic exposure to the insecticide, dimethoate (6 and 30 mg/kg) resulted in a decrease in glutathione levels in both liver and brain tissues of male Wistar rats.[22] It was concluded that the oxidative stress due to dimethoate may be ascribed to the induction of Cytochrome P450, inhibition of AChE and disturbance in the activities of GSH and GST enzymes causing lipid peroxidation and histological changes in the liver and the brain.[22] In this present study, we observed a similar decrease in GSH levels in the pregnant female rats treated with CE. Co-administration of the plant extract with CE was found to restore the GSH levels to those found in control rats.Besides the decrease in GSH and increase in MDA (LPO) levels, we found that CE-treated rats also showed a significant decrease in SOD activity. This decrease in SOD activity could be due to the increased production of ROS as evident from the increased LPO levels due to CE treatment. However, a few studies have indicated that superoxide radicals can also inhibit catalase (CAT) activity and the increased H_2O_2 levels resulting from CAT inhibition could finally inhibit SOD activity. Thus, increased formation of MDA could be due to both increase in pesticide-induced ROS formation and SOD inhibition.[23] In contrast, plant extract treatment was found to significantly increase SOD enzyme activity in the erythrocytes of the pregnant rats, which could explain the decrease in LPO levels.[24] Increase in SOD activity should also accelerate the removal of the ROS. However, it is not clear how this plant extract increases SOD activity. There may be two explanations for this effect: plant extract may directly affect enzyme transcription or decrease superoxide production.

Conclusion

In conclusion, the present study suggests that the butanolic extract of *Paronychia argentea* L. can prevent or slow down the oxidative damage induced by CE in rats. The effects of CE on LPO and GSH levels and on SOD activity were reversed by treatment with plant extract. Further studies to identify the active compounds in the butanolic extract of *Paronychia argentea* L. and determine their structure and mechanism of action are in progress. In addition, further work is required to clarify how this plant extract increases antioxidant enzyme activity (directly by gene transcription or indirectly through superoxide production).

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