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FULL PAPER

The effect of crocetin and rutin on acetylcholinesterase and butyrylcholinesterase enzymes reactivation in acute poisoning by diazinon in mice

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Acute poisoning by Organophosphate (OP) pesticides is an essential clinical problem in rural Asia. Crocetin (CRO) and Rutin (RU) are the nucleophile compounds with no toxicity effects. In the present study, the feasibility of crocetin and rutin administration as therapeutic agents for OP poisoning was studied and compared with 2-(pralidoxime) PAM administration in mice. CRO and RU at doses of (50, 100, 200 mg/kg) were administered intraperitoneal (IP) 15 minutes after a single intraperitoneal injection of Diazinon (DIZ) (LD50=366 mg/kg). Atropine (ATR; 20 mg/kg, IP) and pralidoxime (2-PAM; 30 mg/kg, ip) were used alone or together as standard therapy or control in different (12) groups. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured after 3 and 24 hours as markers of OP toxicity. Significant increase of AChE and BChE activity was observed by the CRO and RU at all doses as compared with the DIZ group. CRO at the dose of 100 mg/kg and RU at the dose of 200 mg/kg significantly increased the AChE activity and CRO and RU at the dose of 200 mg/kg significantly increased the BChE enzyme activity in comparison to DIZ+PAM and DIZ+ATR after 3 hours. It is concluded that CRO and RUT are more effective than 2-PAM to reactivation and also prevention of re-inhibition of the reactivated enzyme after 3 and 24 hours. High nucleophilic properties of CRO and RU can be considered as the mechanism proposed for AChE reactivation.

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KEYWORDS

Organophosphate; acetylcholinesterase; butyrylcholinesterase; crocetin; rutin; diazinon; enzymes reactivation.

Introduction

Organophosphate (OP) can be used in pesticide industries and the chemical weapons in the form of aerosols or dust. In addition to the human skin or the human respiratory system, the mucous membranes quickly absorb the OP. The organophosphate mechanism of toxicity is to inhibit the Acetylcholinesterase (AChE), leading to the formation of a permanent covalent bond to the enzyme [1]. Moreover, it inhibits Butyrylcholinesterase.

Organophosphates interfere in the nervous system, specifically at its cholinergic junctions and specific synapses in the CNS central nervous system [2]. Suppressing the AChE leads to stopping the degradation of acetylcholine, which is the neurohumoral mediator at the cholinergic junctions. Consequently, the patient experiences excessive stimulation and depression induced

by the accumulation of acetylcholine. To treat the patient, the airway should be controlled for adequate oxygenation[3]. The patient must be intubated when he faces respiratory distress due to laryngospasm, bronchospasm, bronchorrhea, or seizures. The necessity of intubation can be removed in the case of immediate treatment using the atropine. The patient's heart should be instantly monitored and pulse oximetry must be done. The electrocardiogram (ECG) should be accomplished [4]. Using intravenous magnesium sulfate was recommended for organophosphate toxicity [5].

Since OP can considerably increase the pulmonarysecretions, it can lead to respiratory failure. In this case, Atropine can be used to dry pulmonary secretions and reduce the excessive airway secretions [6]. Pralidoxime Nucleophilic is recommended as an early treatment for OP poisoned persons. It binds to the OP molecule and stimulates the phosphorylated AChE [7]. Pralidoxime is an effective antidote that is recommended for treatment, which can reverse muscle paralysis. Pralidoxime is recommended for the first 48 h of OP poisoning, but it is not effective after 48 hours due to the aging phenomena [8]. Diazinon (O,O-diethyl-O-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate) is an organophosphate pesticide, which has been applied in the agricultural industry and living house to control the insect population for several years. There is limited evidence for diazinon carcinogenicity [9]. However, it has different adverse effects on non-target species including humans. But, the underlying nephrotoxic effect and mechanism have not been fully understood [10]. Thousands of people die every year due to pesticide poisoning worldwide [11]. Domestic use of the particular type of the organophosphate insecticides such as diazinon has been restricted in recent years after publishing the findings of neurotoxicity and unpredicted environmental persistence [12].

Crocetin (CRO) is a natural substance, which is the component of saffron extract used as a herbal medicine found in old Chinese documents [13]. It is a carotenoid (8,8′-diapo-8,8′-carotenoic acid) with a symmetrical chemical structure that contains diterpenic and seven double bonds and four methyl groups [14]. The positive effect of crocetin on diabetic neuropathy, hyperglycemia, antimicrobial, anti-apoptotic, anti-inflammatory, anti-oxidation, free radical scavenging, and its great potential to inhibit neuronal disease have been confirmed [15, 16]. During the last decade, numerous studies have been performed on the beneficial effect of saffron extracts that work as natural antioxidants to reduce free radicals [17]. The antioxidant effect of the rutin (quercetin-3-rhamnosyl glucoside), which is known as vitamin p, has been approved by different experiments and studies [18, 19], and it is recognized for intense DPPH radical scavenging activity. Pesticide poisoning is growing in developing countries, including Iran. This study aims to protect Acetylcholinesterase and Butyrylcholinesterase enzymes from the damage of poisoning, and its reactivating using crocetin and rutin. Crocetin and rutin are the two potential agents that have the capability of improving the enzyme's activity. The current study revealed the treatment effect of crocetin and rutin on poisoning in mice models.

Materials and methods

Crocetin (CRO) 91%, rutin 94%, and diazinon 95 % were purchased from Shahre Daru Co (Tehran, Iran), Razak Pharmaceutical Co (Tehran, Iran), and Sigma-Aldrich (China).

Experimental design

Male wistar mice (20-25 g) were used for the current study that were provided from the Laboratory Animals Research Center belonging to the Faculty of Pharmaceutical at

Mazandaran University of Medical Sciences. The mice were kept under a controlled environment with temperature 24 ± 1 C°, and 55 ± 5% relative humidity. Their house light was controlled to simulate a regular 12-hour day-night cycle with full access to nutrition and water [20]. All the experiments were performed under the ethics committee's protocols of the Mazandaran University of Medical Sciences. Totally 13 groups of animals were selected, including six mice for each group. One of the groups only received normal saline, taken as the control group. The second group received only diazinon that was solved in normal saline with 25 mg/mL concentration, and injected via IP. The third, fourth, and fifth groups received diazinon solved in normal saline and the different doses of crocetin (50,100 and 200 mg/kg). The sixth, seventh, and eighth groups received diazinon, which solved in normal saline and a different dose of rutin (50, 100, and 200 mg/kg). The ninth group received diazinon solved in normal saline and atropine (10 mg/kg). The tenth group received diazinon solved in normal saline and pralidoxime (30 mg/kg). The eleventh group received diazinon solved in normal saline and atropine (10 mg/kg), and pralidoxime (30 mg/kg). The twelfth group received diazinon solved in normal saline and atropine (10 mg/kg), pralidoxime (30 mg/kg), and crocetin. The thirteenth Group received diazinon solved in normal saline and Atropine (10 mg/kg), pralidoxime (30 mg/kg), and rutin. After 3 and 24 hours injection, mice were anesthetized with chloroform and killed, respectively, and 1 mL blood was taken from the tail and heart. It was used to measure the acetylcholinesterase enzyme and butyrylcholinesterase esterase to analyze the treatment effect.

We have used the GraphPad Prism 6 software to analyze the data to report the result in the form of mean ± SD. The one-way analysis of variance test was used to determine the statistical significance when set at $P < 0.001$. The LD50 (Lethal Dose of toxin that reduced the number of live animals to 50%) assay was performed on the cytotoxic effects of diazinon on mice [21]. The mice were housed in the animal laboratory center. Different concentrations of diazinon (300,350,400 mg/kg) were injected into every three groups of mice. On reducing the number of live animals to 50%, the LD50 was calculated. The LD50 was calculated by means of the control group. Figure 1 represents the calculated results of the viability versus dose with a diagram scale. As a result, the value of LD50 was equal to 366 mg/kg [22].

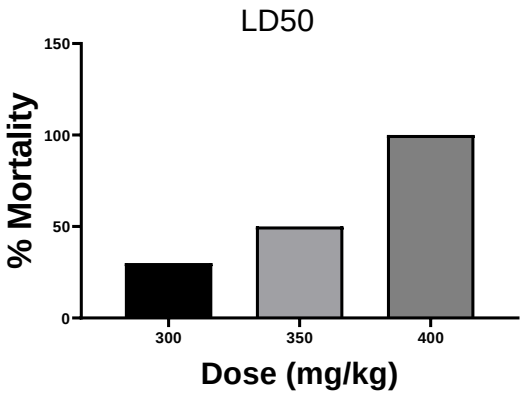


FIGURE 1 Calculated results of the viability versus dosage with a diagram scale. As a result, the value of LD50 was equal to 366 mg/kg

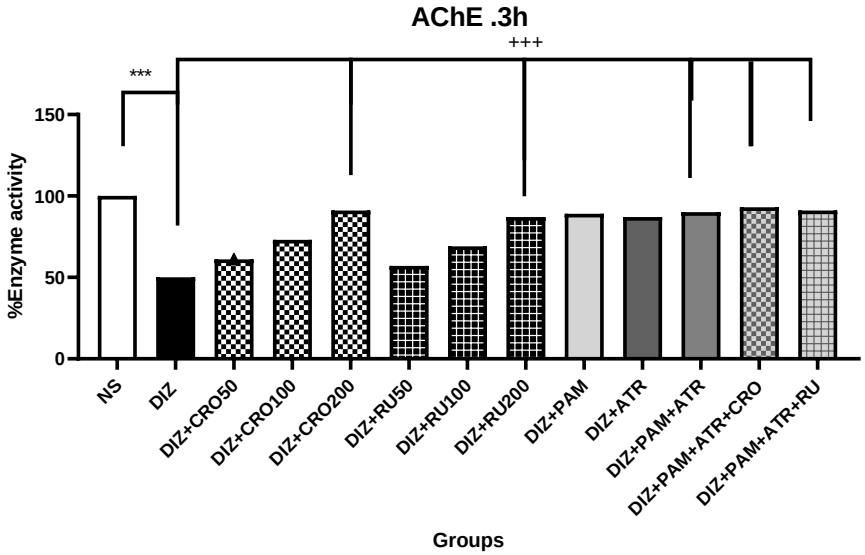
Results and discussion

The effect of crocetin and rutin on Cholinesterase enzyme reactivation in acute poisoning by Diazinon in mice showed significant ($P < 0.001$) variation between the different groups. Acetylcholinesterase enzyme activity dramatically ($P < 0.001$) increased in the ATR and PAM groups as a conventional treatment compared to the DIZ group. Using the CRO as a new treatment showed the acceptable result when acetylcholinesterase enzyme activity was ($P < 0.001$) augmented in the DIZ+CRO group at different concentrations comparing to the DIZ group (Firure 1). The best concentration of CRO was 200 mg/mL with 40% augmentation in the

acetylcholinesterase enzyme activity, which was more effective than ATR and PAM. Rutin also was used to recover the effect of the poison on acetylcholinesterase enzyme activity. The results reveal that RTU had the same trend as CRO when by increasing the dosage the level of acetylcholinesterase increased. 200 mg/l of rutin can reach the acetylcholinesterase activity to 87%, which is slightly smaller than the CRO group. The combination of the new agents RU and CRO with conventional treatment agents ATR and PAM e.g., DIZ+ATR+PAM+CRO group and DIZ+ATR+PAM+CRO, do not show a dramatic difference with pure agent groups.

The experiment was repeated after 24 hours (Figure 2(b)) and no significant change was observed on the level of acetylcholinesterase enzyme activity

(a)



(b)

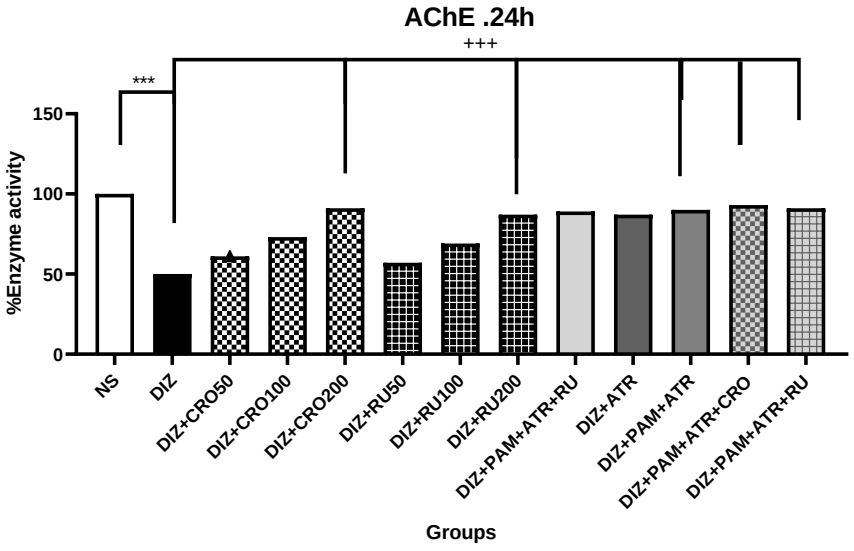
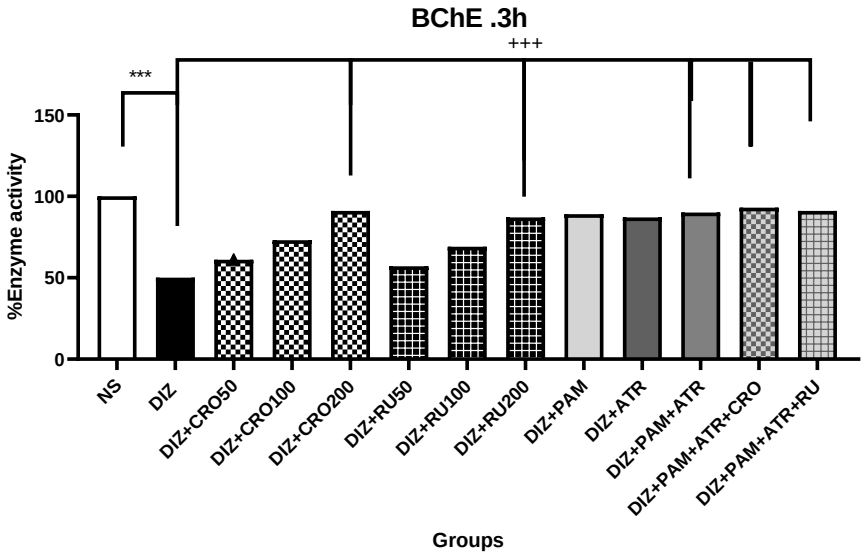


FIGURE 2 Effects of crocetin and rutin on acetylcholinesterase activity in RBC after (a) 3 hours, (b) 24 hours. The groups are NS (mice that received intraperitoneal injection normal salinee), DIZ (The mice group reciving intraperitoneal injection of diazinon), DIZ+CRO 50,100 and 200 (The mice group reciving intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of crocetin of 50, 100 and 200 mg/mL), DIZ+RU 50,100 and 200 (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of rutin with 50, 100 and 200 mg/mL), DIZ+PAM (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL), DIZ+ATR (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal

injection of atropine 10 mg/mL), DIZ+ATR+PAM (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL and Atropine 10 mg/kg), DIZ+ATR+PAM+CRO (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL , atropine 10 mg/kg and 200 mg/kg CRO), DIZ+ATR+PAM+RU (The mice group reciving intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL , atropine 10 mg/kg and 200 mg/kg RU). The statistics denoted as mean ± SD, n=6. **P < 0.01; ***P < 0.001 compared with diazinon group. NS denotes Normal Saline; DIZ, Diazinon; CRO, Crocetin; RU, Rutin; PAM, Pralidoxim; ATR, Atropine; SD, standard deviation.

We have also analyzed the effect of the same agents on the butyrylcholinesterase enzyme activity. The results show that all the different groups' agents have approximately the same effect on butyrylcholinesterase enzyme activity as acetylcholinesterase. The level of butyrylcholinesterase activity was considerably (P < 0.001) increased in the ATR and 2-PAM groups compared to the DIZ group as a routine treatment. CRO and RU are also more effective in 200 mg/mL dosage. When their power to recover butyrylcholinesterase enzyme activity is comparable with the conventional treatment, e.g., ART and 2-PAM. No meaningful difference was observed on the effect of the combined groups such as the DIZ+ATR+PAM+CRO group and DIZ+ATR+PAM+RU group. The experiment was repeated after 24 hours (figure 3(b)), and no significant change was observed on the level of acetylcholinesterase enzyme activity. As a result, the treatment was working well. The results of the present study showed that the acetylcholinesterase enzyme and butyrylcholinesterase enzyme activity were inhibited due to the poisoning with diazinon. The present study revealed that crocetin and rutin could be as effective as a conventional treatment of the poisoning, e.g., atropine and pralidoxime, or even work better of them and recover the enzyme activity. Numerous studies were conducted about the treatment of pesticide or insecticide poisoning. In this study, the role of crocetin and rutin in reactivating the enzyme was evaluated in male mice. Atropine and pralidoxime were used as a reference treatment of the poisoning. Experimental and clinical research has revealed that various enzymes, e.g., acetylcholinesterase and butyrylcholinesterase, are influenced by diazinon poisoning. This study showed a significant increase in the enzymes for the group that received crocetin and rutin and a combination of them after the diazinon injection comparing to the group that only received diazinon. According, crocetin and rutin could recover the AChE and BChE enzymes in RBC and plasma. Consequently, crocetin and rutin have a protective effect on poisoning treatment with diazinon even better than the main therapy of this poisoning (atropine and Paralidoxim).

(a)



(b)

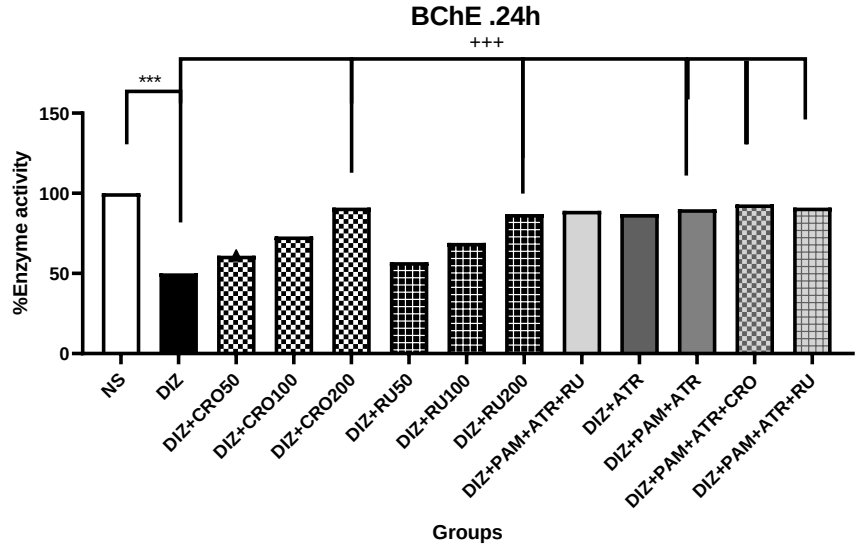


FIGURE 3 Effects of crocetin and rutin on butyrylcholinesterase activity in RBC after (a) 3 hours, (b) 24 hours. The groups are NS (mice that received intraperitoneal injection normal salinee), DIZ (The mice group reciving intraperitoneal injection of diazinon), DIZ+CRO 50,100 and 200 (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of crocetin of 50, 100 and 200 mg/mL), DIZ+RU 50,100 and 200 (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of rutin with 50, 100 and 200 mg/mL), DIZ+PAM (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL), DIZ+ATR (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of atropine 10 mg/mL), DIZ+ATR+PAM (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL and Atropine 10 mg/kg), DIZ+ATR+PAM+CRO (mice that received intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL , atropine 10 mg/kg and 200 mg/kg CRO), DIZ+ATR+PAM+RU The mice group reciving intraperitoneal injection of diazinon and 30 minutes after poisoning received intraperitoneal injection of pralidoxim 30 mg/mL , atropine 10 mg/kg and 200 mg/kg RU). The statistics denoted as mean ± SD, n=6. **P < 0.01; ***P < 0.001 compared with diazinon group. NS denotes Normal Saline; DIZ, Diazinon; CRO, Crocetin; RU, Rutin; PAM, Pralidoxim; ATR, Atropine; SD, standard deviation.

Shadnia *et al.* [23] studied the protective effects of two different agents on diazinon-induced oxidative stress in rats reported that acetylcholinesterase activity was reduced as an organophosphate toxicity marker. Moreover, the plasma butyrylcholinesterase (BChE) could be another marker together with Ache for organophosphate poisoning [24].

Saffron extracts were recognized as a source of innovative inhibitor of Acetylcholinesterase when it was introduced by Geromichalos *et al.* [25] as moderate AChE inhibitory activity in an in Vitro study.

Rutin and crocetin's antioxidant and anti-inflammatory properties were reported in the literature [26-30]. Hariri *et al.* [31] used the safranal and crocin to treat and reduced the effect of diazinon poisoning on genotoxicity and hematological indicators in rats and reported a significant growth in micronucleus indices with diazinon. The results showed that crocin, safranal, and vitamin E decreased diazinon hematological toxicity, but they did not stop the genotoxicity produced by diazinon.

Besides, crocetin can attach to the negative charge spots and hydrophobic parts of the AChE and growth cholinergic activity [32]. The crocetin existance has been monitored in the rat brain after intragastric usage of GJ-4, which is a crocin fraction with particular components that is the main reason for hindering the AChE in the brain. It can improve the mice's learning and restore cognitive decline [33].

Kartick *et al.* [34] showed that the rutin can has a protective effect on lipid peroxides and antioxidants in myocardial infarction produced by isoproterenol. They reported that rutin holds antioxidant activity in experimental myocardial infarction caused by isoproterenol consumption. Besides, the antioxidative properties of the rutin on acetylcholinesterase and butyrylcholinesterase activities were proved by Ademosun *et al.* [35]. In another study, Anesti *et al.* [36] reported that rutin has a noteworthy anxiolytic effect and an anticholinesterase activity in a certain part of mice brain areas. Anesi *et al.* [36] showed that the rutin could dramatically reduce the AChE in the all parts of rat bran. On the other hand, Yan *et al.* [37] described that forsythiaside might be more effective than rutin in quenching the intrinsic fluorescence of AChE to treat Alzheimer's disease. Adefegha *et al.* [38] emphasize the higher inhibitory effects of rutin on Fe2+- and lipid peroxidation produced by sodium nitroprusside. They also informed that rutin and quercetin significantly hold down the malondialdehyde (MDA) production, but a combination of rutin and quercetin has better performance. The present study shows that rutin and crocetin can be used as an effective agents to increase the AChE and BChE activity levels reduced by diazinon poisoning. But, the combination of these two agents or combination of them with ATR and 2-PAM don't show a significatnt difference in the enzyme level in comparison with each of them.

Conclusion

Administration of crocetin and rutin with a dosage of 200 mg/kg can recover the enzyme activity to slightly less than the normal value. It seems that crocetin and rutin have a protective effect on the reactivation of enzyme acetylcholinesterase and butyrylcholinesterase activity that inhibited by diazinon. Besides, using the CRO and RUT as a treatment is more effective than 2-PAM after 24 hours due to the aging phenomena that affect 2-PAM output. Finally, the combination of the mentioned agents did not show any prevalence over the single administration of them to recover the enzyme activity.

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