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THE PROTECTIVE EFFECT OF CROCIN ON PANCREATIC DISORDERS RESULTING FROM NICOTINE PRESCRIPTION IN MALE BALB/C MICE

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ABSTRACT

Purpose: Healthy ESBL carrier patients are major challenge in control of infections produced by members of *Entrobacteriaceae*. The aims of the present study were to investigate the isolation of TEM, SHV and CTX type ESβLs producing *E. coli*, *Edwardsiella*, and *Erwinia spp*. from feaces of carriers.
Methods: Two hundred fresh stool samples collected from non-hospitalized and hospitalized patients were cultured on MacConkey agar supplemented with 2 mg/L cefotaxime. After 24 hr. incubation at 37°C the *E. coli*, *Erwinia and Edwardsiella spp* were identified by routine biochemical tests. Combined tests were carried out to select ESBLs producing bacteria and susceptibility of isolates was determined by disc diffusion method. Multiplex-PCR was used to identify TEM, SHV and CTX type ESβLs producing isolates.
Results: Of the 34.5% bacteria resistant to cefotaxime, 81.63% and 55% ESBL producing organisms were recovered from inpatients and outpatients respectively. *E. coli* was the predominant ESBL-producing organism; One *Erwinia and* three *Edwardsiella* producing ESBL were detected. Overall, carbapenems including imipenem and meropenem and amikacin were the antibiotics most active against the ESBL-producing organisms. The overall prevalence of these ESBL genes was 73.92%, including: the *bla*TEM and *bla*SHV genes alone in 27.45% and 5.88% respectively; *bla* CTX-M were not distinguished alone in any of the isolates
Conclusion: More than one ESBL was produced by most isolates carried by patients and Carbapenems, imipenem and meropenem continue to show good *in vitro* activity against the isolates. Patients can act as a source of ESBL producing bacteria in hospitals.

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Introduction

Nicotine is a natural alkaloid. It is composed of carbon, hydrogen and nitrogen, which the ratio of carbon, hydrogen and nitrogen is C10, H14, and N2 (1). Nicotine is extracted from dried leaves of Nicotiana tabacum and millions of people around the world are exposed to it through smoking (2-3). Nicotine is a compound affecting physiological structures of the body, for example, it has different stimulating effects on the secretion of the hormone insulin and glucagon (4-5). Nicotine is an alkaloid found in nicotina tabacum and it is one member of Solanaceae plant family. It was extracted for the first time from nicotina tabacum in 1828 by two German chemists known as Posselt and Reiman (6). Although there are various toxic and harmful substances in tobacco smoke, nicotine has been considered as the most important substance of cigarette smoke, since it causes addiction by affecting brain cells in addition to damage to the function of various parts of the body especially the blood circulatory system (7). Nicotine has various effects on single-cell and multicellular organisms. Nicotine readily passes through the blood –brain obstacle. This sudden burst of nicotine is caused by stimulating the adrenal glands leading to release of epinephrine. In addition, there is sudden release of glucose and increased breathing, heart rate and vasoconstriction and increased alertness (8). Nicotine in pancreatic beta cells causes the secretion of insulin through direct impact on nicotine receptors of acetylcholine (9). Consumption of alcoholic beverages and cigarettes is one of the serious problems that endanger the health of human society. According to the assessment of the World Health Organization (WHO), nicotine caused 12% of deaths worldwide in 2000 (10). Nicotine in the form of in vivo and in vitro induces oxidative stress (11). Unwanted side effects of some chemical drugs make it justified to pay attention to the possible effects of medical plants on the function of different parts of the body. Due to their availability, minimal side effects, and reasonable price, medicinal plants have always been considered as an alternative to synthetic drugs. On the other hand, most of the plants leave very few side effects in patients (12). In this regard, Saffron has a special place in nutrition of the people. Evidence suggests that saffron due to its numerous antioxidant compounds such as crocin has been considered in the treatment of certain diseases (13). Saffron with scientific name of Crocus sativus belongs to the family of lily, and it is an herbaceous, perennials, and without stem plant (14). Saffron is a small perennial plant from the lily family with a height of 30-10 cm and it is bulbous plant that its bulb is almost spherical and covered with a thin brown membranes. The used part of this plant the style and stigma three branches which is called Saffron is well known that odor is aromatic and slightly bitter taste. (15). Glycoside crocin is composed of carotenoid known as crocetin saffron and sugars which are responsible for color of saffron (16). Saffron, crocin, and crocetin have removing effect on free radicals and antioxidants (17). Sativus causes weakened acquiring and strengthening of the conditioned place preference expression caused by morphine and it alone causes conditioned place preference in the small male and female mice. For this reason, it has been suggested that saffron may be useful for the treatment of psychological dependence to opioids in humans (18). Saffron in small male and female mice has a decreasing effect on the acquiring and expressing the behavioral sensitivity to morphine and it removes the effect of morphine in inducing increased movement activity. For this reason, it has been suggested that its use might be useful during the drug withdrawal or even later it (19). The pancreas is a gland that is relatively stiff and crispy at the white and grayish color that it secrets the digestive juices and insulin (20). Mature pancreatic in terms of morphology and function has two endocrine and exocrine duct parts. Endocrine part includes islets of Langerhans that are cellular accumulation derived from endoderm and they are between exocrine parts sporadically. Islets of Langerhans have four types of cells that synthesize the peptide hormones: insulin (cell β), glucagon cells (cells α), somatostatin (cell γ) and pancreatic polypeptide cells (21). Insulin is a hormone with two polypeptide chains and weighed about 5,800 Daltons, secreted from Islets of Langerhans beta cells in the pancreas (22). NO is a free radical and one of the 10 small molecules in nature involved in many biological processes (23) and it has diverse role such as vasodilators, inhibition of platelet aggregation, dilatation of the bronchi and regulate air routes, regulation of renal function and relaxation of the stomach. It has also anti-tumor and anti-microbial activity, so that stimulation of macrophages by various microorganisms such as bacteria, viruses, parasites and fungi leads to production of large amounts of NO that has toxic effects (toxicity) and eventually kills the pathogenic microorganisms (24-25). Given that millions of people around the world are exposed to nicotine through smoking or inhaling pesticides and at the same time they are exposed to all kinds of stressors, investigating the intervention effects different stresses with nicotine on the function of various body organs is necessary. According to increasing number of consumers of nicotine, it is necessary to study its effects on the function of pancreatic secretion and serum level of insulin. Accordingly, this study was conducted to evaluate the protective effect of crocin on pancreatic disorders induced by nicotine in small mice.

Method

In this study, 48 male mice (balb / c) with a weight range of 30-27 g were purchased from Razi Institute of Iran and they were used in the study. For one week before the start of the experiment, they were kept in animal house under laboratory conditions and at temperatures 20 ± 2, 12 hours in darkness and 12 hours in lighting conditions. To keep the mice, standard cages in medical school that there are 6 mice in each cage were used. In this study, the harmful dose of nicotine was 2.5 mg/kg and multiple doses of crocin were injected intraperitoneally for 28 days. Mice were divided in two control and experimental groups. The experimental group was divided into eight groups, including control group: received distilled water, the experimental group 1 received 2.5 mg / kg nicotine, experimental group 2: received 12.5 mg / kg crocin + 2.5 mg / kg of nicotine, the experimental group 3: received 25 mg/kg crocin + 2.5 mg / kg nicotine, the experimental group 4:

received 50 mg / kg crocin + 2.5 mg / kg nicotine, the experimental group 5, 6 and 7: each of them received 12.5, 25, and 50 mg / kg crocin. For group saline considered as a control group, injection of saline solution 0.9% constructed by Razi serum-producing Company was used. For the experimental group, crocin was weighted based on the mentioned concentration and normal injectable saline 0.9 percent was used to dissolve crocin and obtain the doses studied. To dissolve nicotine, normal saline solution 0.9 % was used. After fixing pancreas, the preparation process of tissues was performed by Automatic Tissue processors device and based on the common method of histology (paraffin method) that involves passing through the samples from the ascending alcohols (100-100-96 -90-80-70-60%) then passing through Xylene for transparency and removing the opacity created in the process of dewatering and finally crossing the paraffin to impregnate and to fill the vacuum created in the fat pores created in the stage of passing through Xylene. At this stage, Merck paraffin with a melting temperature of 57 ° C was used. After finishing the impregnating stage by Lockhart molds, molding was carried out. Then, serial cuts were prepared from each from the considered mold. Among all the sections, 5 samples were selected for coloring, respectively. Accordingly, 10 lam of pancreas of each mouse was prepared. To prepare the desired color, 5 g hematoxylin powder was dissolved in 50 cc alcohol. Then, 100 g potassium alum powder was dissolved at low heat in distilled water. Then, hematoxylin and dissolved solution of potassium alum were combined with each other. Then, they were heated to the point that they reached to boiling point while shaking them. Arriving reaching to boiling point, solution was immediately removed from heat and 2.5 g Sodium Iodate powder was added to it. Finally, we placed the solution immediately in a container of water. Then, 20 cc of glacial acetic acid was added to it, and filter paper was used to make it smooth. Then, we kept the solution within the dark glass. To prepare the eosin color, 1.5 g eosin powder was dissolved in 20 ml of distilled water and then 120 ml of 96% ethanol was added to it. Then, to prepare a working (600 ml eosin color), 450 ml of ethanol 80% was added to 150 ml of stock color solution. Finally, 4.5 ml glacial acetic acid was added to it. Twenty-four hours after the last injection, the animals were anesthetized with chloroform and bloodletting was performed of their heart using 5 cc syringes. Blood samples were incubated for 15 min at 37 ° to be clotted. Then, the plotted blood was centrifuged for 15 min at 3000 rpm to isolate its serum. Isolated serum was kept at -20 ° C until measuring the hormone insulin, amylase and nitric oxide. Then, pancreas of animals was removed and stored in 10% formalin solution.Serum insulin levels were measured using a kit (Monobind) by ELISA device. In order to measure amylase level, after de-freezing the serum samples placed at 20 ° C, Roche kit and using Autoanalyzer device (automated analyzer Architect 16200, Abbott, IL, United States), amylase levels in blood serum of animals were measured. NO was measured by Griess reaction using microplate method. To study pancreatic tissue, pancreas was fixed in 10% formalin solution. After 72 hours, the formalin was rinsed by water. Dewatering was performed with ascending alcohols and molding in paraffin and cuts by 5 micrometers in diameter were prepared from tissue by microtome. The prepared cuts were colored using hematoxylin and eosin method to be examined by light microscopy. Pancreatic samples of each tissue, a tissue mold was prepared and serial cut was prepared. Then, 10 cuts were colored from each sample and they were studied separately by two people. Control samples were considered as natural and changes caused by nicotine and crocin were evaluated. After the preparation of the Lam of each group, 5 fields in 100x magnification of microscope were randomly selected. After counting the number of islets, its average was obtained. To measure the mean diameter of islets, 5 islets were selected of each lam. Then, large and small diameter of each islet was determined in micrometer and by placement it in the following formula, the mean diameter of each islet was obtained.

$$MD=\sqrt{L \times S \times \text{magnification}}$$

(MD: mean diameter, S: small diameter of islet, L: large diameter of islet, and magnification: lens magnification)

For statistical analysis, SPSS software (version 16) was used and quantitative data were compared using one-way ANOVA and Tukey test and $P<0.05$ was considered significant.

Findings

The results of the investigation of pancreatic weight and the weight of the mice among the studied groups show significant decrease in pancreatic weight and the weight of the mice between group receiving nicotine and the saline group ($p< 0.05$). In addition, increasing the weight of the mice and pancreatic weight in the groups receiving crocin in all doses and crocine plus nicotine (25 and 50 mg/kg) was observed in comparison with the nicotine group ($p< 0.05$) (Chart 1 and 2).

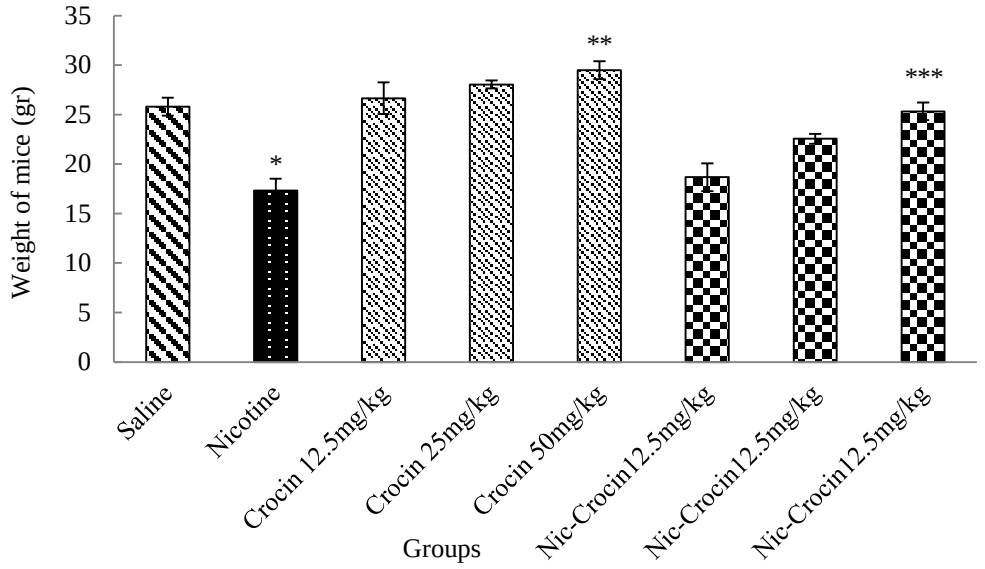

Chart 1. Comparison of changes in weight of mice among the groups studied. *Significant decrease in nicotine group compared to saline (control) group ($p<0.05$). ** Significant increase in all crocin groups compared to nicotine group ($p<0.05$). *** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

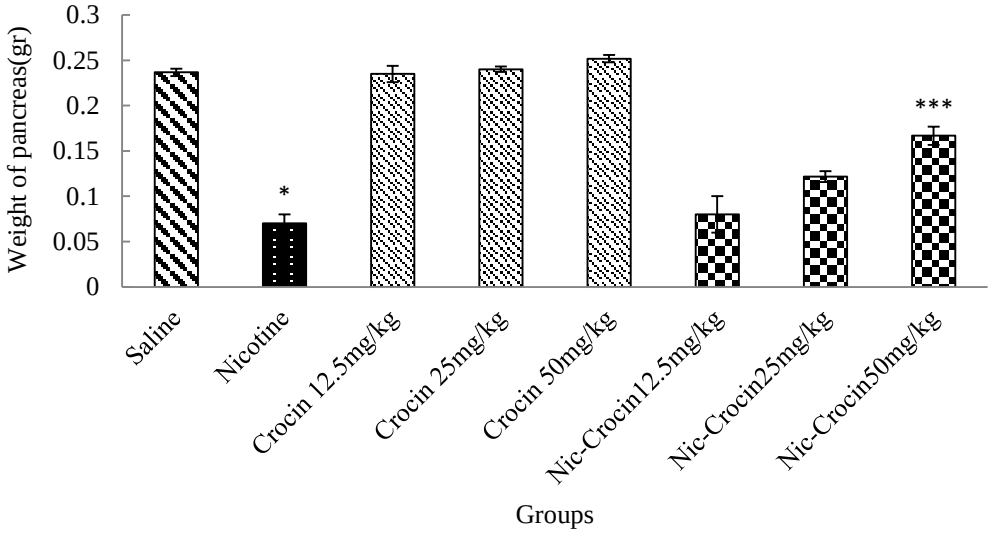

Chart 2. Comparison of changes in weight of pancreas among the groups studied. *Significant decrease in nicotine group compared to saline (control) group ($p<0.05$). ** Significant increase in all crocin groups compared to nicotine group ($p<0.05$). *** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

The results of measuring levels of nitric oxide in blood serum showed a significant increase among the group receiving nicotine in comparison with the group receiving saline ($p< 0.05$). In the all groups receiving of crocine and crocine plus nicotine (25 and 50 mg/kg), there is significant reduction compared with nicotine group ($p< 0.05$)(Chart 3).

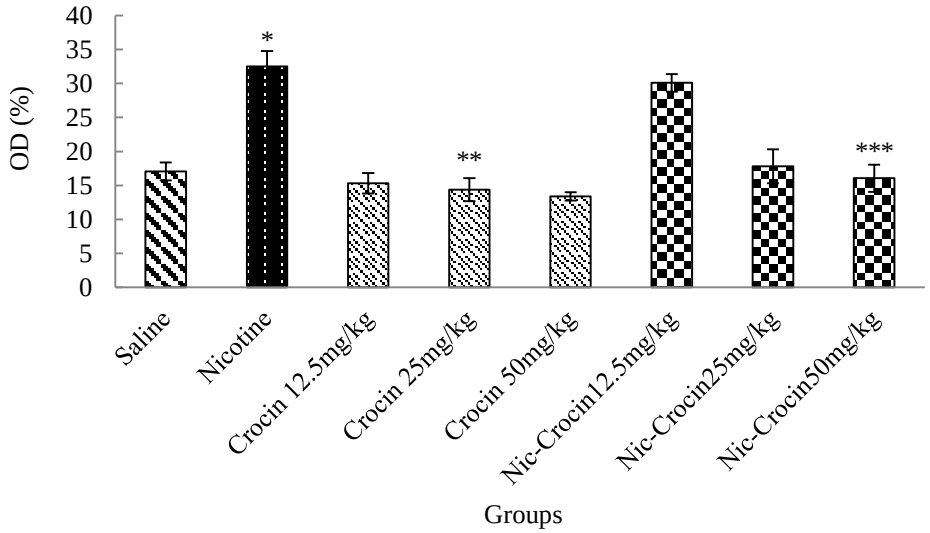

Chart 3. The effect of different concentrations of nicotine plus crocin on the levels of nitric oxide in the study groups. *Significant increase in nicotine group compared to saline (control) group ($p<0.05$). ** Significant decrease in all crocin groups compared to nicotine group ($p<0.05$). *** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

The results of the investigating the diameter and the number of islets in treatment groups showed a significant reduction between group received nicotine compared with the saline(control) group in diameter and the number of islets ($p< 0.05$). Statistical analysis did not show a significant difference in the diameter size of the islets among groups receiving different doses of crocine and crocin plus nicotine compared with the group receiving nicotine alone ($p< 0.05$) (Chart 4). In addition, significant increase was observed between groups receiving crocine in all doses and nicotine plus crocine (25 and 50 mg/kg) in the number of islets in comparison with group receiving nicotine ($p< 0.05$)(Chart 5).

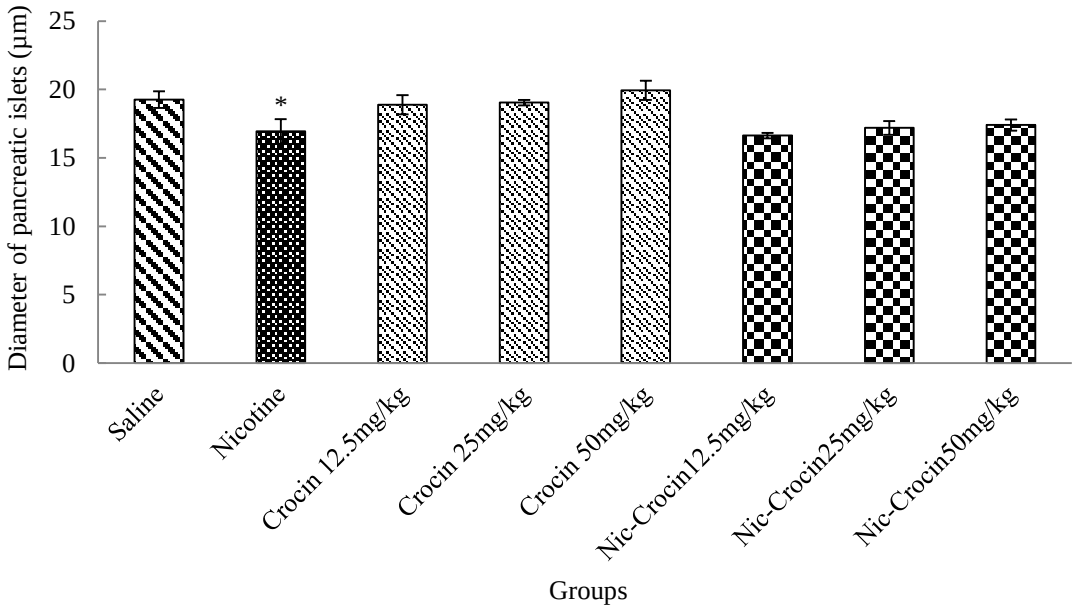

Chart 4.The effect of different concentrations of crocin-nicotine on the diameter of the islets of Langerhans in the study groups. *Significant decrease in nicotine group compared to saline (control) group ($p<0.05$).

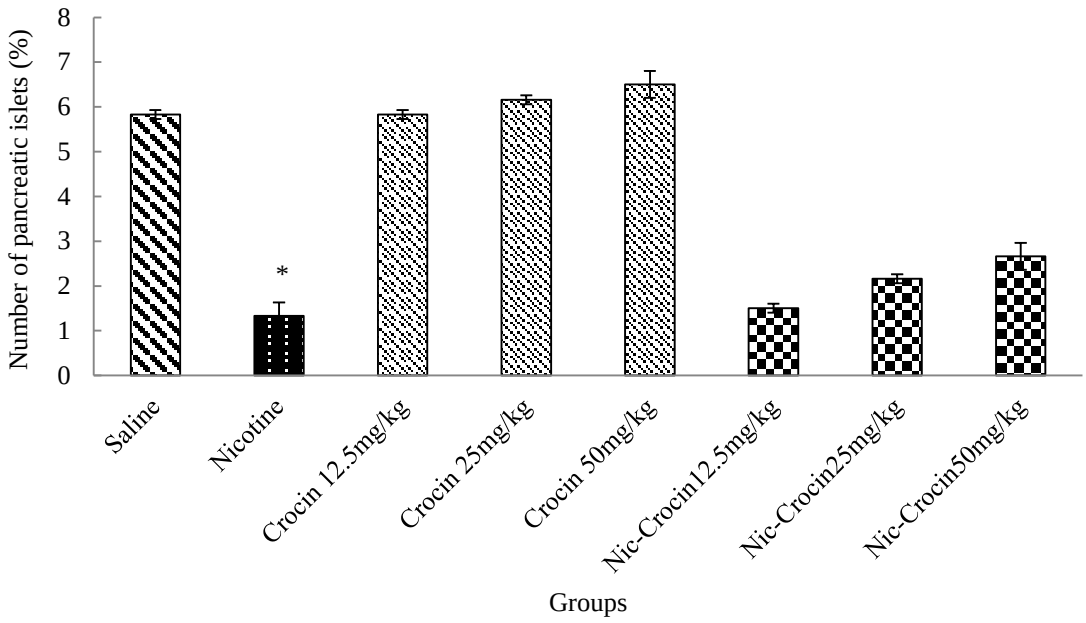

Chart 5. The effect of different concentrations of nicotine-crocin on the number of islets of Langerhans in the study groups. *Significant decrease in nicotine group compared to saline (control) group ($p<0.05$). ** Significant increase in all crocin groups compared to nicotine group ($p<0.05$). *** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

The results of investigating the level of insulin hormone in blood serum and the glucose levels in the experimental groups showed a significant increase in nicotine group compared with the control (saline) group($p< 0.05$). Group receiving nicotine + crocin with dose of 25 and 50 mg/kg showed significant increase in serum level of insulin compared to the group receiving nicotine alone ($p< 0.05$) (Chart 6). In addition, in the groups receiving crocine with all doses and crocin plus nicotine (25 and 50 mg/kg), statistical analysis showed a significant reduction in serum level of glucose compared to group receiving nicotine ($p< 0.05$) (Chart 7).

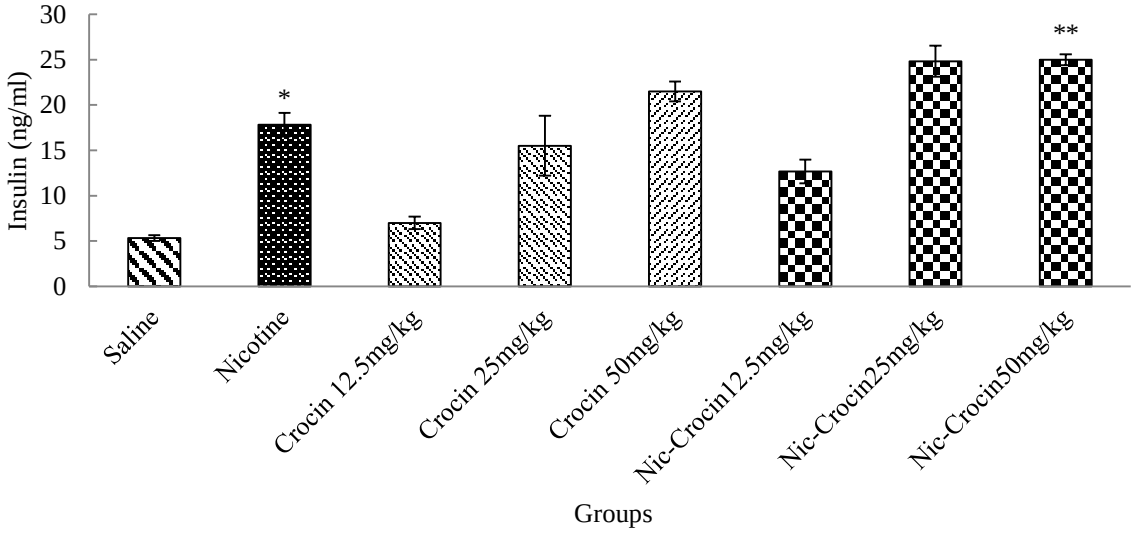

Chart 6. The effect of different concentrations of nicotine-crocin on insulin levels in the study group. *Significant increase in nicotine group compared to saline (control) group ($p<0.05$). ** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

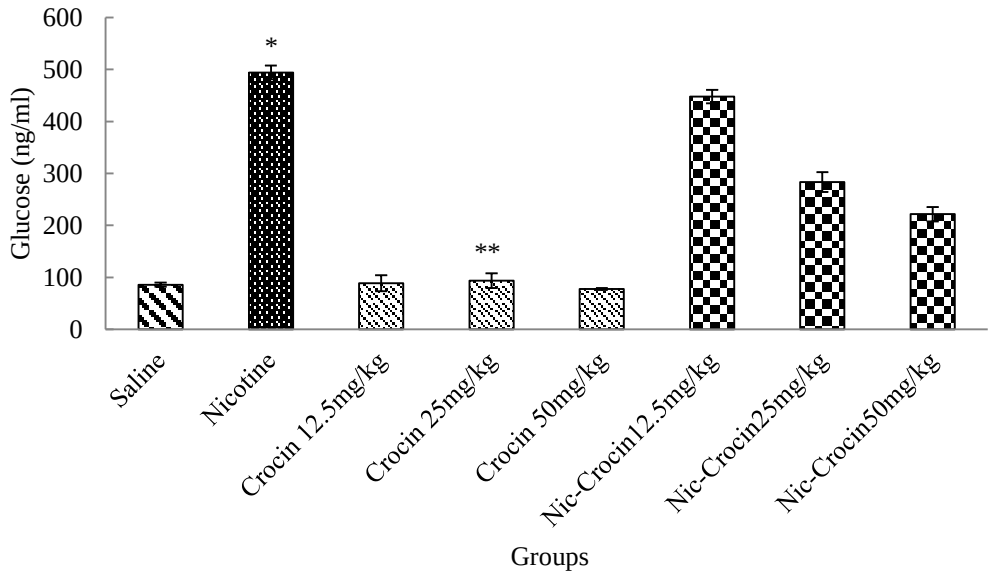

Chart 7. The effect of different concentrations of nicotine-crocin on glucose levels in the study group. *Significant increase in nicotine group compared to saline (control) group ($p<0.05$). ** Significant decrease in all crocin groups compared to nicotine group ($p<0.05$). *** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

The results of the statistical analysis of the level of amylase in blood serum in experimental groups showed significant increase between nicotine group and the control (saline) group($p< 0.05$). In addition, in groups receiving crocine in all doses and crocin-nicotine with doses of 25 and 50 mg/kg, a significant decrease in the amylase level was observed compared with the group receiving nicotine alone ($p< 0.05$) (Chart 8).

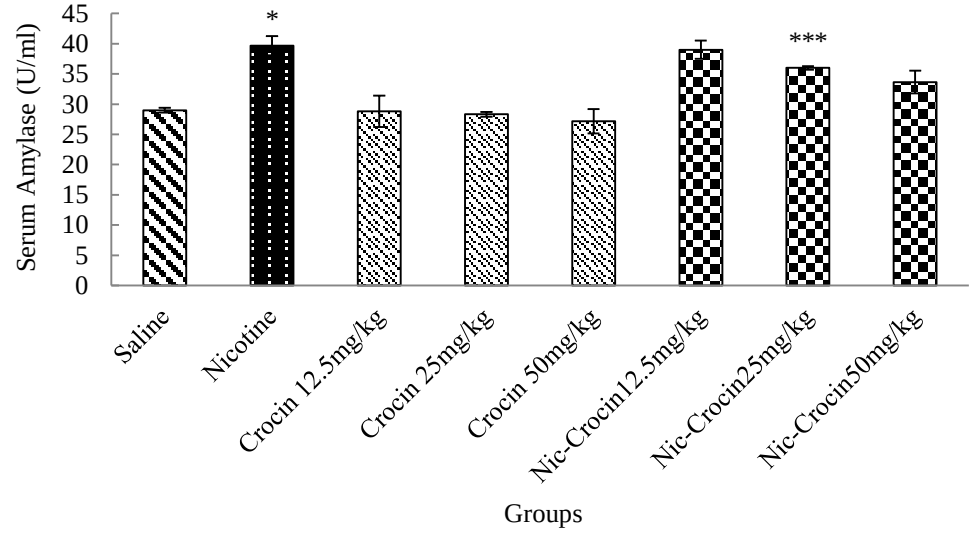

Chart 8. The effect of different concentrations of nicotine-crocin on serum amylase levels in study groups. *Significant decrease in nicotine group compared to saline (control) group ($p<0.05$). ** Significant increase in all crocin groups compared to nicotine group ($p<0.05$). *** Significant increase in crocine plus nicotine (25 and 50 mg/kg) groups compared to nicotine group ($p<0.05$).

Histological images of pancreas in group treated with nicotine and crocin have been in Figures 1 and 2.

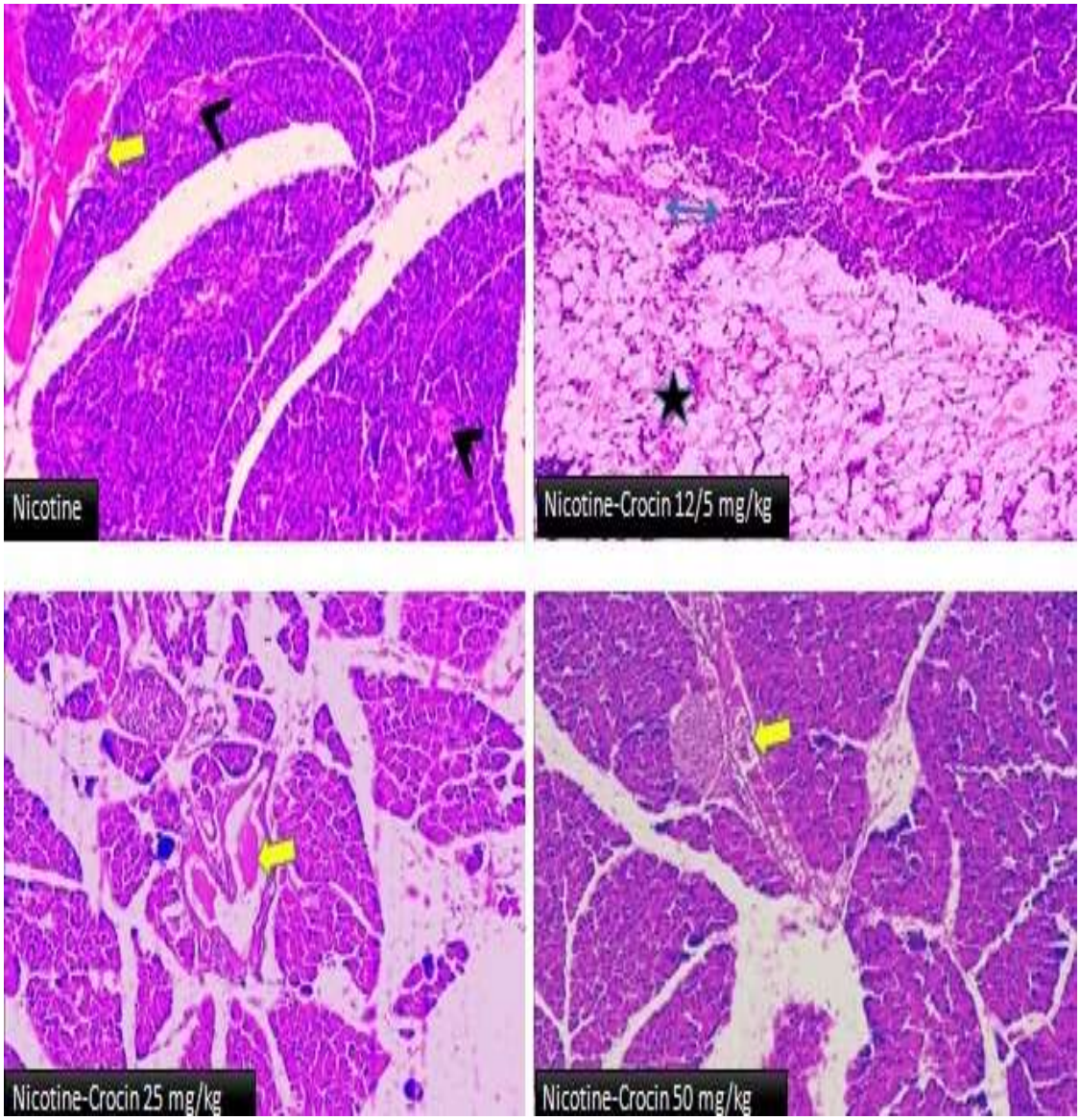

Figure 1. The experimental groups receiving nicotine-crocin with doses of 12.5, 25 and 50 mg/kg, creating fat tissue (arrow)in tissues of the pancreas and reduction in small of islet (arrow tip), blooding in pancreas tissue (large arrow) and infiltration of lymphocytes (double arrow) (magnification 100 ×) (coloring H & E)

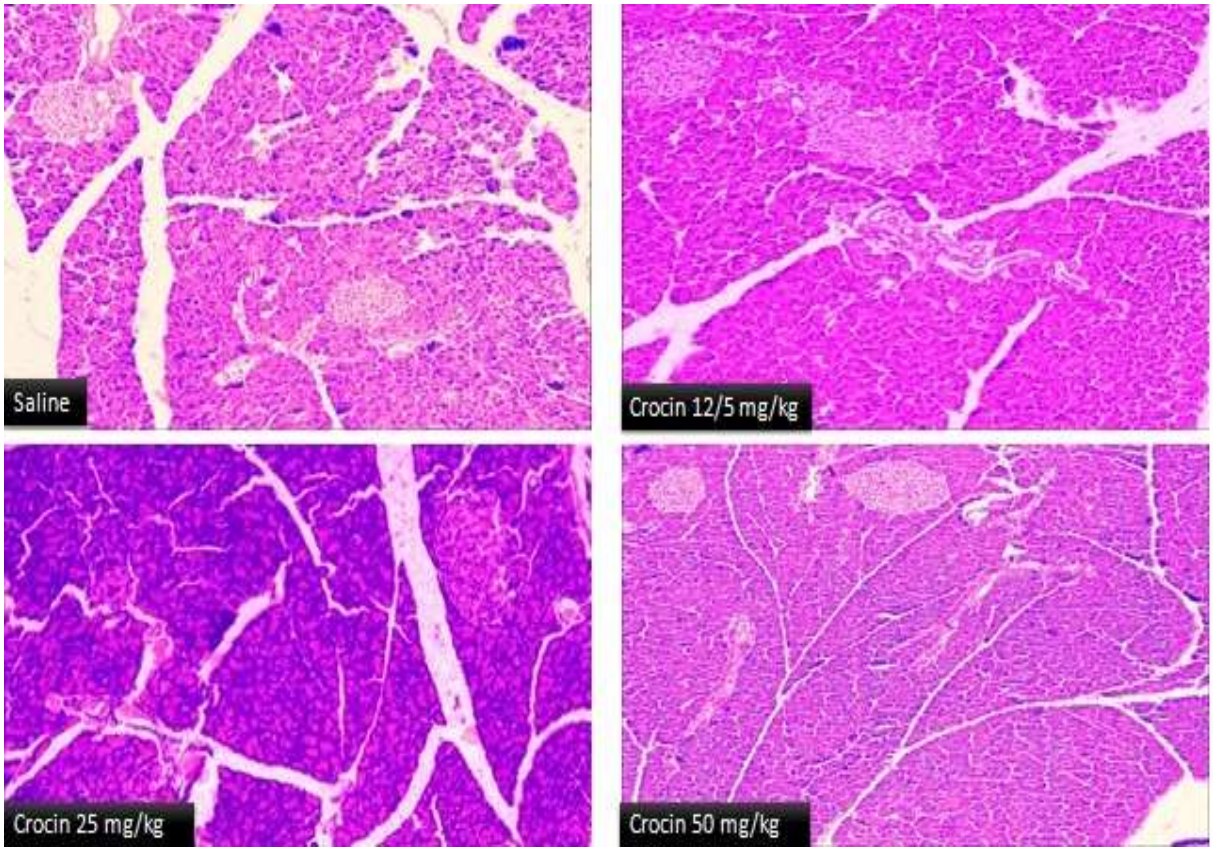

Figure 1. The experimental groups receiving crocin with doses of 12.5, 25 and 50 mg/kg, Islets of Langerhans with specified range and healthy veins are seen in groups (magnification 100 ×) (H & E coloring)

Discussion

In order to investigate and determine precisely the effects of cigarette smoke, it is necessary that different compounds of it especially nicotine that is the main composition in tobacco to be studied separately. The second reason that emphasizes on the importance of the studying toxic effects of the nicotine is that this substance is used as a drug to quit the habit of smoking and also the treatment of some diseases, such as Alzheimer's, Parkinson, and ulcerative colitis (26). Balakrishnan et al study showed that prescription of nicotine reduces body weight that is consistent with our results (27). In addition, Bruin et al study showed that nicotine induced beta cells in rodent model as well as an increase in apoptosis and damage to the islet cells (28). Nicotine may potentially induce noradrenaline and subsequently narrowing of the arteries and reduced blood flow to the organs. Nicotine also causes reduction in angiogenesis gastric mucosa through the inhibition of nitric oxide production and it stops the cell production process (29). Animal studies suggest that antioxidants are capable to remove free radicals (30). Antioxidants can cause damage to nitric oxide system (proteins enzymes, substrates and cofactors) and therefore reduce the production of nitric oxide. Hydroxyl radicals produced by nitric oxide and superoxide cause intervention in the pathogenesis process (31). Nicotine in high doses cause apoptosis in pancreatic islets, and also through oxidative stress caused by nicotine activates the mitochondrial pathway of apoptosis. The study conducted by Somm et al shows that the apoptosis effects caused by nicotine are not limited to pancreatic β- cells, but also they have impact on α cells and reduce glucagon gene expression. In prenatal mice that were exposed to nicotine, overexpressing of adiponectin caused antagonistic effects in sensitivity to insulin in fat tissue (32). Bruin et al study reveals that exposure to nicotine reduces the number of beta cells and defects in glucose intolerance that these results are consistent with the results of the present study. Nicotine reduces the density of the beta cells in rodents model and apoptosis of beta cells and non- proliferation of pancreases islets of Langerhans (33). The results of the study conducted by Hazman et al showed that crocin significantly reduces the expression of TNF-α and IL-6 in serum and also decreases the level of the expression of TNF-α and IFN-γ in pancreatic tissues of diabetic mice. In addition, due to its anti-inflammatory and antioxidant property, it prevents damage to the pancreatic beta cells (34). The results of the study conducted by Bruin et al show that nicotine exposure reduces the density of the beta cells and an increase in the rate of apoptosis through mitochondrial pathway in beta cells, which these results are consistent with the results of our study (35). Studies show that exposure of pancreas with nicotine leads to activation of protein BCL2 and increased apoptosis in pancreatic beta cells. Additionally, as result of BCL2 protein activation by nicotine, it causes mitochondria destruction and damage to pancreatic tissue (36). The results of the study conducted by Tamaddonfard et al show that crocin significantly enhances the secretion of insulin in diabetic mice with STZ (37). In this study, in the group

receiving the nicotine, changes were observed in pancreatic tissue in the form of vascular congestion and bleeding in the pancreas artery, and reduced size in islets of Langerhans. Damage to pancreatic tissue due to nicotine can be caused by the leakage of intracellular enzymes to maintain the integrity and stability of cell membrane or regeneration of damaged pancreatic cells (38). As a result of crocin prescription along with nicotine, with only mild degenerative changes were observed and no necrosis was found, showing the protective effects of crocin against nicotine toxicity. In addition, the results of the study conducted by Onyesom et al have shown in smokers in addition to dysfunction in parotid and salivary gland, the amount of alpha amylase both in serum and saliva of smokers increased significantly compared to non-smokers significantly, which it is consistent with the results of the present study (39). Based on the results of the present investigation, it can be said that the prescription of crocin as a powerful antioxidant on animals with healthy pancreas and in groups receiving nicotine, especially in high doses, can be effective in the function of pancreas. In addition, the results of this study showed that the possible antioxidant properties of crocin are effective on the amount of the insulin and blood glucose levels in healthy groups and also in groups treated with nicotine. They have also protective effects against free radicals and they are effective in improving the function and structure of pancreas. It is recommended that further studies to be conducted for studying the molecular analysis of the effects of nicotine and crocin on the pancreas in terms of gene expression. In addition, it is recommended that apoptotic effects of nicotine to be examined on Islets of Langerhans, especially beta cells. Finally, according to the findings of this study, it seems that taking crocin in certain dose to be effective in improving the function of pancreas, while more studies are recommended to identify the mechanism of action of this substance.

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