

# Naringenin and Vanillin Mitigate Cadmium-Induced Pancreatic Injury in Rats *via* Inhibition of JNK and p38 MAPK Pathways

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## ABSTRACT

**Background:** Cadmium can induce pancreatic injury via oxidative stress, inflammation and apoptosis. Naringenin (NGN) and vanillin (VLN) exert antioxidant, anti-inflammatory, and anti-apoptotic effects.

**Objective:** The likely ameliorative effects of NGN, VLN and their combination were studied in rats exposed to cadmium-induced pancreatic injury. **Materials and Methods:** Rats received NGN (50 mg/kg/day, p.o.), VLN (100 mg/kg/day, p.o.), or NGN + VLN for 7 days and one injection of CdCl<sub>2</sub> (2 mg/kg, i.p.) on the 6<sup>th</sup> day. **Results:** Cadmium significantly lowered serum amylase and insulin levels. Cadmium also caused significant increments of malondialdehyde, tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , nuclear factor- $\kappa$ B p65, Bax/Bcl-2 ratio and phosphorylated c-Jun N-terminal kinase (p-JNK) and p38 mitogen-activated protein kinases (MAPKs) and significant decrements of reduced glutathione and catalase in the pancreas of rats received CdCl<sub>2</sub>. Additionally, CdCl<sub>2</sub> caused marked histopathological necrosis and significantly increased caspase-3 expression in pancreatic tissue. The cadmium-induced biochemical, histopathological and immunohistochemical changes were significantly ameliorated by NGN, VLN and NGN + VLN. However, NGN + VLN caused more significant ameliorative effects than did NGN and VLN alone. **Conclusion:** NGN, VLN and NGN + VLN afforded significant protection of pancreas in rats exposed to cadmium insult through modulation of JNK and p38 MAPK pathways and inhibition of oxidative stress, inflammation and apoptosis.

**Key words:** Naringenin, Vanillin, CdCl<sub>2</sub>, JNK/MAPK, p38/MAPK, Pancreas.

## INTRODUCTION

Cadmium, the divalent heavy metal cation, is a prevalent environmental pollutant of major cosmopolitan interest. Batteries, electroplating, alloys, paint pigments, plastics and fertilizers industries are the main sources of occupational exposure to cadmium. Consumption of contaminated water, smoking tobacco and combustion of fossil fuel are the major sources of non-occupational cadmium exposure.<sup>1,2</sup> Exposure to minute amounts of cadmium can lead to toxicity because the acceptable blood cadmium level in humans is very low ranging from 0.03 to 0.12  $\mu$ g/dl. In addition, cadmium accumulates readily in the human body due to lack of effective elimination mechanisms and the elimination half-life of cadmium from the body may reach up to 45 years.<sup>3,4</sup> It was also reported by the WHO that allowable cadmium quantities are 3  $\mu$ g/l in drinking water, 5 ng/m<sup>3</sup> in air and the endurable cadmium intake is 7  $\mu$ g/kg/week.<sup>5</sup> On the other hand, it was recommended by the ATSDR that the minimum risk level of human cadmium exposure by chronic oral ingestion is 0.1  $\mu$ g/kg/day.<sup>6</sup> The WHO had also indicated 5  $\mu$ g cadmium/g creatinine in urine as a biological threshold for occupational cadmium exposure. On the contrary, it was evidenced that cadmium adversely affects health at exposure limits below the threshold level.<sup>7</sup>

Cadmium toxicity has a negative impact on all body organs, including the pancreas. Previous studies

showed that cadmium exposure lead to pancreatic  $\beta$ -cell injury, impairment of insulin secretion, development of diabetes mellitus and suppression of exocrine pancreatic functions.<sup>8,9</sup> It is also considered as a risk factor for pancreatic cancer in humans.<sup>10</sup> It was revealed that cadmium-induced oxidative stress up-regulated c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs) signaling pathways in pancreas, which resulted in enhancement of inflammatory responses and induction of apoptosis.<sup>11</sup>

Naringenin (NGN) is a flavonoid compound found mainly in grapefruit and vanillin (VLN) is a phenolic aldehyde obtained mainly from vanilla bean. Both NGN and VLN have antioxidant, anti-inflammatory, anti-apoptotic and anti-carcinogenic activities.<sup>12,13</sup> It was reported in the literature that NGN and VLN significantly protected against streptozotocin-induced pancreatic  $\beta$ -cell injury and diabetes mellitus.<sup>14-16</sup> In addition, NGN mitigated acute pancreatitis induced by caerulein and L-arginine in mice.<sup>17</sup> The current work was done to investigate the possible protective effects of NGN, VLN and NGN + VLN in a rat model of cadmium-induced pancreatic injury and to elucidate the feasible mechanisms mediating these protective effects.

## MATERIALS AND METHODS

### Chemicals

NGN, VLN and CdCl<sub>2</sub> were purchased from Sigma-Aldrich, USA. VLN and CdCl<sub>2</sub> were

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dissolved in physiological saline and NGN was prepared in 0.5% carboxymethylcellulose (CMC) solution. The selected doses and routes of administration of NGN, VLN and CdCl<sub>2</sub> were reported in the literature.<sup>18-20</sup>

## Animals

Male Sprague-Dawley rats (220-250 g weight) were purchased from the National Research Center, Giza, Egypt. Rats were housed at 24°C, 45% humidity, 12 h/12 hr dark-light cycle and left to acclimatize for 7 days prior to the experiments. They were supplied with commercial chow and tap water *ad libitum*. The study was approved by the Research Ethics Committee, Faculty of Medicine, Minia University (approval number: 145:12019). The international guidelines of use and care of laboratory animals were followed.

## Experimental strategy

The rats were randomly divided into 5 equal groups ( $n = 6$ , each) as follows:

- Group 1 (control) received a single i.p. injection of physiological saline (vehicle of CdCl<sub>2</sub>).
- Group 2 received physiological saline, p.o., for 7 days and a single injection of CdCl<sub>2</sub> (2 mg/kg, i.p.) on the 6<sup>th</sup> day.
- Group 3 received NGN (50 mg/kg/day, p.o.) for 7 days and a single i.p. injection of CdCl<sub>2</sub> (2 mg/kg) on the 6<sup>th</sup> day.
- Group 4 received VLN (100 mg/kg/day, p.o.) for 7 days and a single i.p. injection of CdCl<sub>2</sub> (2 mg/kg) on the 6<sup>th</sup> day.
- Group 5 received a combination of NGN (50 mg/kg/day, p.o.) and VLN (100 mg/kg/day, p.o.) for 7 days and a single i.p. injection of CdCl<sub>2</sub> (2 mg/kg) on the 6<sup>th</sup> day.

## Sampling and biochemical analyses

Euthanasia of the rats by i.p. urethane (1 g/kg) was done at the end of experiments. Blood samples were aspirated through a left ventricular pierce and a colorimetric kit was used to assess serum amylase (Biodiagnostic, Egypt) and an ELISA kit was used to estimate serum insulin (Elabscience Biotechnology Inc., USA). Dissection of the pancreas and homogenization in cold PBS (pH 7.4, 0.05 M) were done. Pancreatic homogenates were centrifuged at 5000 rpm at 4°C for 10 min. Malondialdehyde (MDA), reduced glutathione (GSH) and catalase (CAT) activity were measured in pancreatic homogenates by colorimetric kits (Biodiagnostic, Egypt). Moreover, ELISA kits were used to assess IL-1 $\beta$ , TNF- $\alpha$ , phosphorylated c-Jun N-terminal kinase (p-JNK) (R and D Systems, USA), p38 MAPK (MyBioSource, USA), Bax (Biovision, Inc., USA) and Bcl-2 (Novus Biologicals, USA) in pancreatic homogenates.

Additionally, a portion of the pancreatic homogenate was re-centrifuged at 15000 rpm for 30 min at 4°C and the resultant pellet (nuclear fraction) was used to measure nuclear factor- $\kappa$ B p65 (NF- $\kappa$ B p65) by an ELISA kit (Novus Biologicals, USA).

## Histopathological analysis

Pancreatic tissue was fixed in 10% formalin solution, dehydrated in alcohol and embedded in paraffin. Sections at 5  $\mu$ m were cut and stained with hematoxylin and eosin (H and E). An expert who was unaware of slide identity examined the slides under light microscope. The morphological injuries of pancreas were evaluated through tissue degeneration, inflammatory cell infiltration, hemorrhage and perivascular inflammation. A score ranging from 0 to 4 for each parameter was used and high scores indicate severe damages.<sup>21</sup>

## Immunohistochemical analysis

Paraffin blocks were cut into 4  $\mu$ m-thick sections and mounted on positively charged glass slides. Sections were deparaffinised, gradually rehydrated and treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min to block endogenous peroxidase activity. Antigen retrieval was done by boiling the slides in 10 mM citrate buffer (pH 6.0) for 10 min and then cooled at room temperature for 20 min. Sections were incubated with rabbit polyclonal antibody for rat caspase-3 (Thermo Scientific, USA, 1:500) for 30 min. After washing with phosphate buffer solution (PBS), the slides were incubated with biotinylated secondary antibody for 10 min, streptavidin peroxidase complex for 5 min and finally with DAB, as chromogen for 3 min. The slides were counterstained by hematoxylin for 1 min. Dehydration with ascending grades of ethyl alcohol was done, followed by clearance, mounting and finally covered with cover slips. Negative controls were performed, in which the sections were stained by the same technique but using PBS instead of the primary antibody.<sup>22</sup>

The light microscope was used to show caspase 3 immunostaining. Then, the slides were inspected by an image analyzer computer system using software Leica Qwin 500 (Leica Microsystems Imaging Solutions Ltd, UK). The immunopositive cells were calculated in 10 non-overlapping fields of the tissue sections of each rat in all groups.

## Statistics

The results are expressed as mean  $\pm$  S.E.M. Data analysis was done by one-way ANOVA test followed by Tukey test for *post hoc* comparisons using GraphPad Prism Software Program (version 6.01). The significance level was at  $p < 0.05$ .

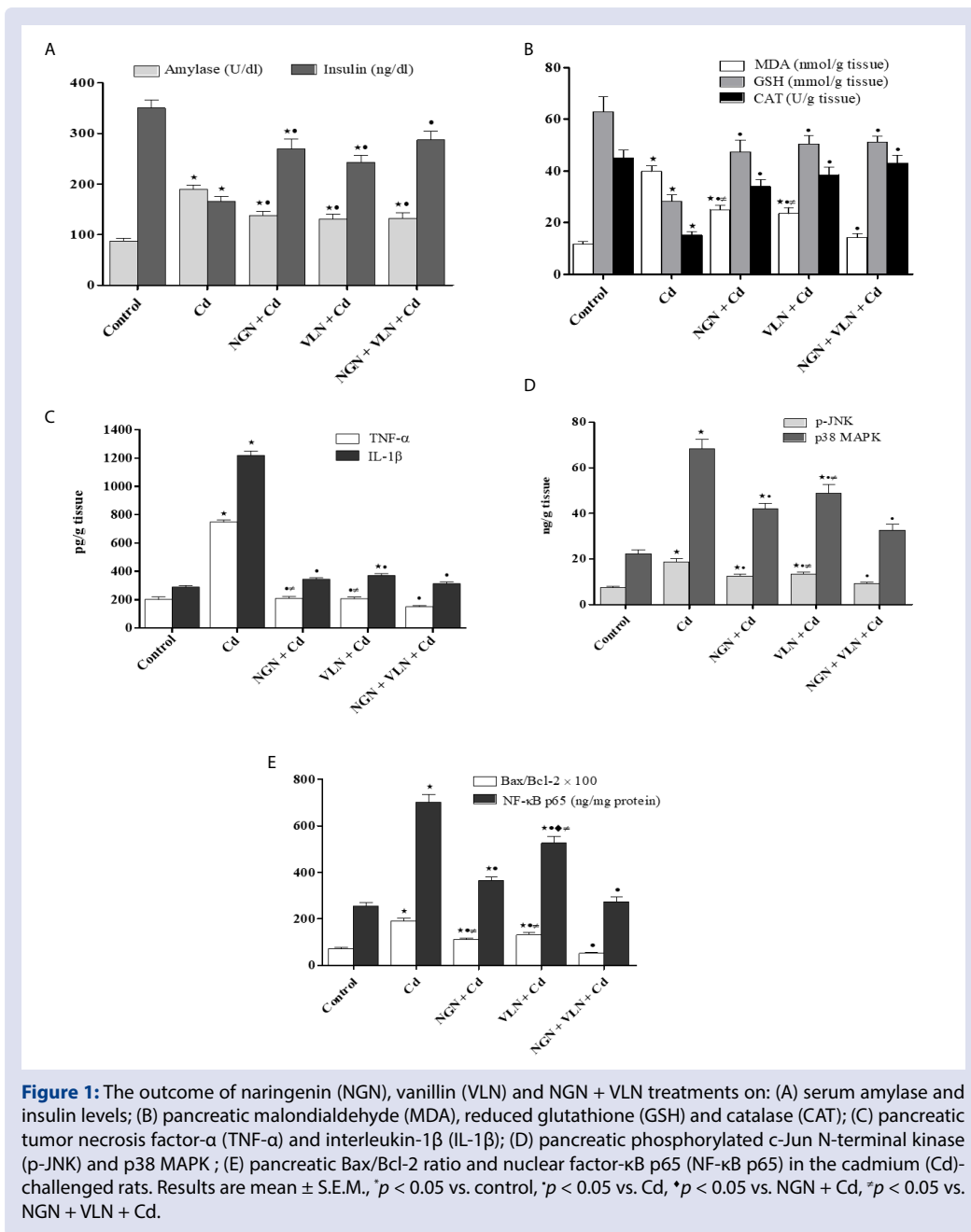
## RESULTS

### Biochemical observations

Administration of one dose of CdCl<sub>2</sub> (2 mg/kg, i.p.) resulted in a significant increment of serum amylase ( $p < 0.05$ ) and a significant decrement of serum insulin ( $p < 0.05$ ), as compared with the corresponding control values (Figure 1A). Treatment with NGN, VLN and NGN + VLN resulted in significant decreases of serum amylase ( $p < 0.05$ ) and significant increases of serum insulin ( $p < 0.05$ ) in the cadmium-challenged rats (Figure 1A). In addition, cadmium insult caused significant elevations of MDA, TNF- $\alpha$ , IL-1 $\beta$ , p-JNK, p38 MAPK, Bax/Bcl-2 ratio and NF- $\kappa$ B p65 ( $p < 0.05$ ) and significant decreases of GSH and CAT ( $p < 0.05$ ) in the rat pancreas (Figures 1B-1E). Contrarily, significant reductions of MDA, TNF- $\alpha$ , IL-1 $\beta$ , p-JNK, p38 MAPK, Bax/Bcl-2 ratio and NF- $\kappa$ B p65 ( $p < 0.05$ ) and significant increases of GSH and CAT ( $p < 0.05$ ) were observed in the pancreas of cadmium-challenged rats treated with NGN, VLN and NGN + VLN (Figures 1B-1E). The improvements detected with NGN + VLN treatment were more significant than treatment with NGN or VLN alone.

### Histopathological observations

Administration of CdCl<sub>2</sub> resulted in marked degeneration of pancreatic tissue, vascular dilatation and congestion, dilatation and desquamation of the epithelial lining of pancreatic ducts and inflammatory cell infiltration. In addition, islets of Langerhans showed necrotic areas and cells with vacuolated cytoplasm (Figure 2). Treatment with NGN and VLN attenuated the cadmium-induced pancreatic injury; however, NGN + VLN preserved the normal histological picture of the pancreas and maintained the integrity of islets of Langerhans (Figure 2). Additionally, the cadmium-induced increase of pancreatic injury score was significantly reduced ( $p < 0.05$ ) by NGN, VLN and NGN + VLN. Again, the decrement of pancreatic injury score was more significant with NGN + VLN than did with NGN or VLN alone (Figure 2).



**Figure 1:** The outcome of naringenin (NGN), vanillin (VLN) and NGN + VLN treatments on: (A) serum amylase and insulin levels; (B) pancreatic malondialdehyde (MDA), reduced glutathione (GSH) and catalase (CAT); (C) pancreatic tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β); (D) pancreatic phosphorylated c-Jun N-terminal kinase (p-JNK) and p38 MAPK; (E) pancreatic Bax/Bcl-2 ratio and nuclear factor-κB p65 (NF-κB p65) in the cadmium (Cd)-challenged rats. Results are mean ± S.E.M., \**p* < 0.05 vs. control, †*p* < 0.05 vs. Cd, \**p* < 0.05 vs. NGN + Cd, †*p* < 0.05 vs. NGN + VLN + Cd.

### Immunohistochemical observations

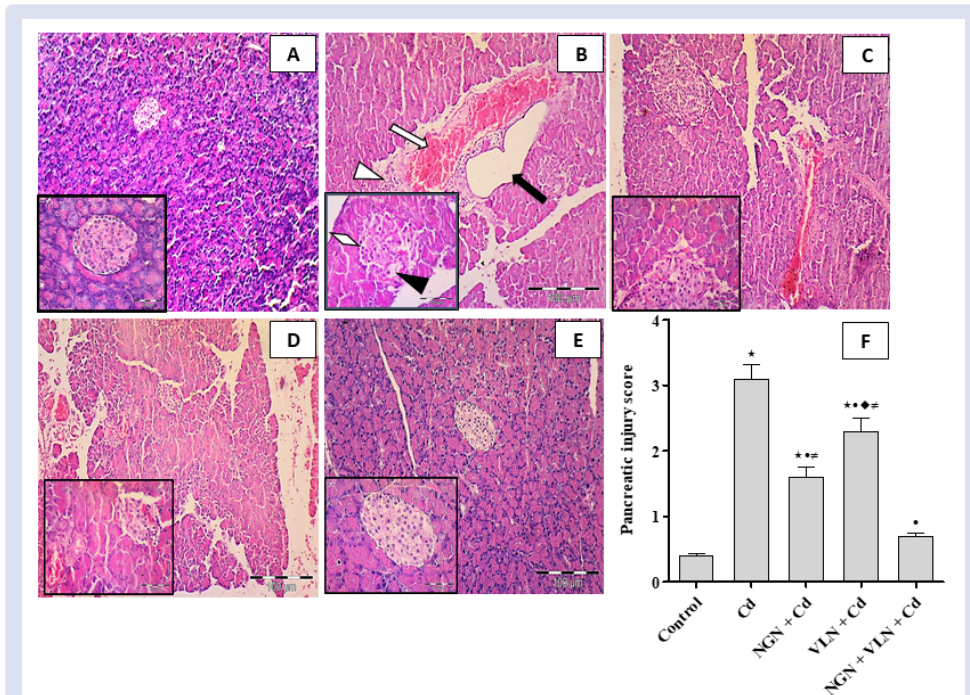
The pancreas of rats received CdCl<sub>2</sub> showed a significant rise of caspase-3 immunoreactivity (*p* < 0.05) in comparison with the control rats (Figure 3). Contrarily, treatment with NGN, VLN and NGN + VLN significantly reduced pancreatic caspase-3 expression (*p* < 0.05) in cadmium-challenged rats. Additionally, NGN + VLN caused a more significant reduction of pancreatic caspase-3 expression (*p* < 0.05) than did NGN or VLN alone (Figure 3).

### DISCUSSION

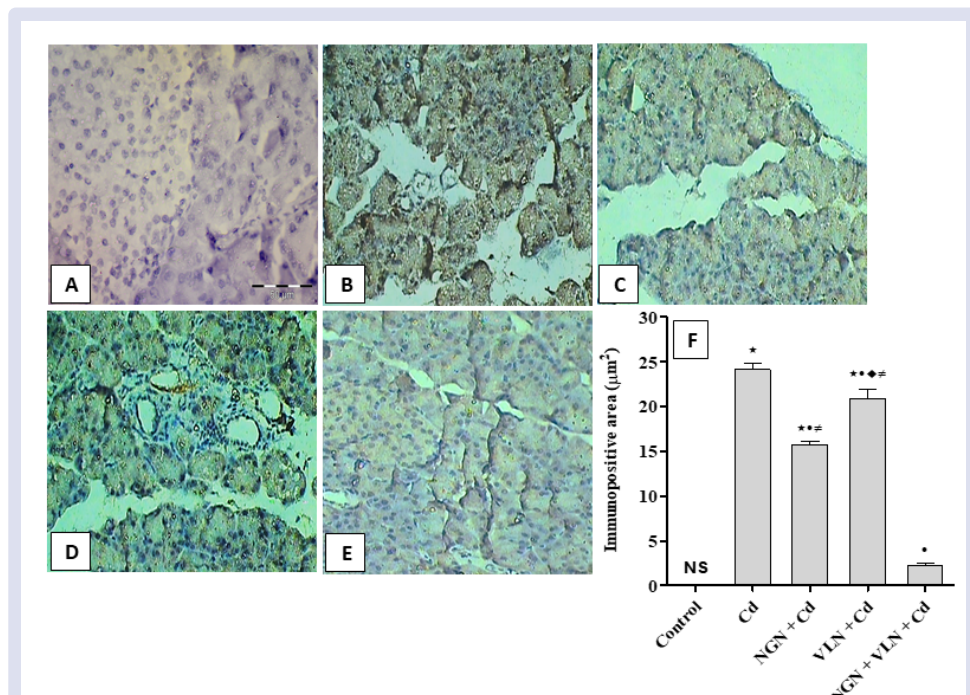
Prior studies denoted that cadmium exposure caused pancreatic tissue injury and disruption of endocrine and exocrine pancreatic functions. It was reported that cadmium intoxication intensified the generation of reactive oxygen species (ROS), depleted the endogenous antioxidant defenses, enhanced lipid peroxidation of cellular biomembranes and increased production of MDA, the end result of lipid peroxidation.<sup>8-10</sup>

Additionally, upregulation of NF-κB-dependent inflammatory cascades with increased inflammatory cytokine production were involved in cadmium-induced tissue injury.<sup>23</sup> This is in consistence with the current investigation, which revealed that cadmium insult increased MDA, decreased GSH and CAT and raised NF-κB p65, TNF-α and IL-1β in the pancreatic tissue of rats.

Moreover, it was demonstrated that cadmium exposure induced cell death via activation of the mitochondrial apoptotic pathway. Similar to the present results, prior studies showed that cadmium exposure increased the production of Bax, the pro-apoptotic protein and decreased the production of Bcl-2, the anti-apoptotic protein. Disruption of the balance between Bax and Bcl-2 enhances the mitochondrial membrane permeability with the resultant release of mitochondrial cytochrome C into the cytoplasm. Subsequently, activation of caspase family of proteases takes place and activated caspase-3 eventually executes apoptotic cell death.<sup>24,25</sup>



**Figure 2:** H and E (100×) of rat pancreas and islets of Langerhans (inserted figures, 400×) from: (A) control group displaying normal histology of pancreas, and normal islet of Langerhans; (B) cadmium (Cd) group showing dilatation and desquamation of epithelial lining of pancreatic ducts (black arrow), vascular dilatation and congestion (white arrow), inflammatory cell infiltration (white head) and islets of Langerhans showing empty areas (black head) and cells with vacuolated cytoplasm (white diamond); (C and D) naringenin (NGN) + Cd and vanillin (VLN) + Cd, respectively, showing improvements in the histological architecture the pancreas and reductions in pancreatic injury; (E) NGN + VLN + Cd group showing marked improvement and preservation of the normal histology of pancreatic tissue; (F) pancreatic injury score. Results are mean ± S.E.M., \* $p < 0.05$  vs. control, \* $p < 0.05$  vs. Cd, \* $p < 0.05$  vs. NGN + Cd, \* $p < 0.05$  vs. NGN + VLN + Cd.



**Figure 3:** Caspase-3 immunohistochemistry of rat pancreas (400×) from: (A) control group showing negative staining (NS); (B) cadmium (Cd) group showing a significant increment of caspase-3 immunostaining in brown color; (C, D and E) naringenin (NGN) + Cd, vanillin (VLN) + Cd and NGN + VLN + Cd, respectively, showing significant decreases of caspase-3 positivity; (F) immunopositive area (µm<sup>2</sup>). Results are mean ± S.E.M., \* $p < 0.05$  vs. control, \* $p < 0.05$  vs. Cd, \* $p < 0.05$  vs. NGN + Cd, \* $p < 0.05$  vs. NGN + VLN + Cd.

Both NGN (4',5,7-trihydroxy flavanone), and VLN (4-hydroxy-3-methoxybenzaldehyde) are natural compounds with prominent antioxidant, anti-inflammatory and anti-apoptotic effects. It was reported in the literature that NGN and VLN significantly protected against oxidative stress and inflammatory injuries of various organs, including the pancreas. Both agents act as free radical scavengers, reduce generation of ROS, maintain endogenous antioxidant capacity, prevent lipid peroxidation of biomembranes and decrease the production of inflammatory cytokines.<sup>12,13</sup> This is consistent with the present investigation which revealed that treatment with NGN, VLN and NGN + VLN caused significant decrements of MDA, NF- $\kappa$ B p65, TNF- $\alpha$  and IL-1 $\beta$  and significant preservation of GSH and CAT in the pancreas of cadmium-challenged rats. The current work also demonstrated that NGN, VLN and NGN + VLN significantly decreased Bax/Bcl-2 ratio and caspase-3 activation in pancreas of rats challenged with cadmium and thus offered protection against cell apoptosis. This is in agreement with previous investigations, which showed comparable results with NGN and VLN treatments.<sup>26,27</sup>

The MAPKs, particularly JNK and p38 MAPKs, is a family of protease enzymes involved in the regulation of cell proliferation, differentiation and apoptosis. Upon activation via phosphorylation, MAPKs phosphorylate other protein kinases and transcription factors, including many inflammatory and apoptotic biomarkers.<sup>28</sup> Oxidative stress with increased ROS generation is a well-known inducer of MAPKs. It was reported that cadmium-mediated oxidative stress induced phosphorylation and activation of JNK and p38 MAPKs and subsequently enhanced the downstream inflammatory and apoptotic cascades.<sup>23,29</sup> In addition, previous studies showed that activation of MAPKs increased the release of NF- $\kappa$ B p65 unit in the cytoplasm, which upon nuclear translocation promote the transcription of inflammatory cytokines and augment inflammatory responses.<sup>23,30</sup> It was also revealed that cadmium can induce the mitochondrial apoptotic pathway via up-regulation of JNK and p38 MAPKs resulting in increased Bax/Bcl-2 ratio, and caspase-3 activity.<sup>25,31</sup> This is similar to the current work, which showed that cadmium exposure increased p-JNK and p38 MAPKs, NF- $\kappa$ B p65, Bax/Bcl-2 ratio and caspase-3 and in rat pancreas. Besides, previous investigations, similar to the present one, showed that NGN and VLN provided their anti-inflammatory and anti-apoptotic effects via down-regulation of JNK and p38 MAPKs signaling pathways with subsequent reduction of inflammatory cytokines, Bax/Bcl-2 ratio and caspase-3.<sup>32-35</sup>

In addition, NGN, VLN and NGN + VLN significantly reduced serum amylase and increased serum insulin in rats received cadmium indicating that exocrine and endocrine pancreatic functions were preserved. Besides, similar cadmium-induced histopathological pancreatic tissue injury were observed in previous studies.<sup>36,37</sup> These histopathological changes were significantly ameliorated by NGN, VLN and NGN + VLN.

## CONCLUSION

NGN, VLN and NGN + VLN significantly protected against pancreatic injury and dysfunction induced by cadmium in rats. The protective effects of these agents were mediated via down-regulation of JNK and p38 MAPKs signaling pathways and inhibition of oxidative stress, inflammation and apoptosis. The protective effect afforded by NGN + VLN was more significant than NGN or VLN alone.

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## CONFLICTS OF INTEREST

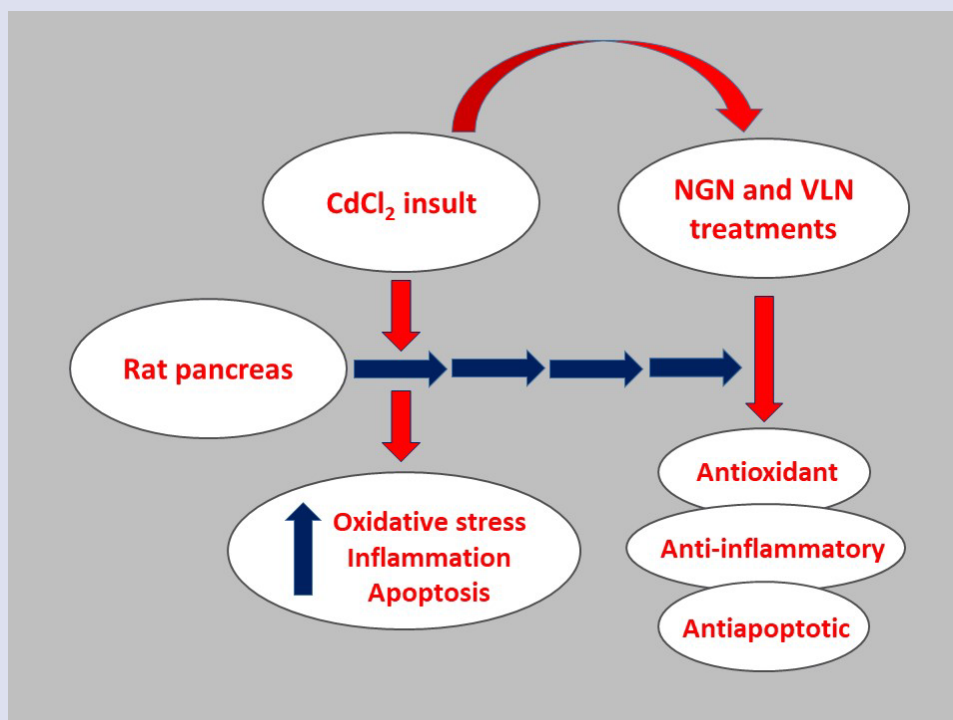
All the authors declare that there are no conflicts of interest.

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## GRAPHICAL ABSTRACT



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