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Original article

Diosmetin alleviates nonylphenol-induced liver damage by improving biochemical, inflammatory, apoptotic and histological profile in rats

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ABSTRACT

Nonylphenol (NP) is an environmental pollutant that is recognized for its hazardous effects on humans and animals. NP has potential to induce oxidative stress that leads to hepatic toxicity. Diosmetin (DIOS) is a naturally occurring bioflavonoid that possesses several biological properties. The current study was designed to ascertain the curative effects of DIOS against NP prompted hepatotoxicity in rats. 32 male albino rats were randomly categorized in 4 groups i.e., control (0.1 % DMSO), NP (50 mg/kg), NP + DIOS (50 mg/kg + 100 mg/kg) and DIOS (100 mg/kg) group. Our results revealed that NP instigated substantial reduction in the antioxidant enzymes activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GSR), glutathione peroxidase (GPx), glutathione S-transferases (GST) and glutathione (GSH). NP administration raised the levels of reactive oxygen species (ROS) as well as the levels of malondialdehyde (MDA). Treatment with DIOS significantly ($p < 0.05$) recovered activities of antioxidant enzymes, ROS, and TBARS levels. Furthermore, DIOS treatment ameliorated the NP-induced increased level of inflammatory markers i.e., nuclear factor kappa B (NF-κB), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα) and cyclooxygenase-2 (COX-2) activity. In addition, DIOS co-treatment also recovered the NP-provoked escalated levels of pro-apoptotic proteins (Bax, caspase-3 and caspase-9) and substantially reduced level of anti-apoptotic protein (Bcl-2) to normal levels. Besides, DIOS treatment recovered the potential histopathological damages in liver tissues. Therefore, DIOS might be an effective therapeutic agent for alleviating NP-induced hepatic toxicity due to its antioxidant, anti-inflammatory and anti-apoptotic potential.

1. Introduction

Due to the growing population, urbanization and uncontrolled industrialization, the environmental pollution has become a serious issue in recent years (Liu et al., 2020). Nonylphenol (NP) is one of the most commonly encountered persistent organic pollutants (POPs) present in the environment (Guo et al., 2021). It is extensively utilized in the formulation of oil additives for lubrication, plastic items, cleansing agents, dyes, surfactants, cosmetic items, and resin (Jubendradass et al., 2012). Domestic and industrial waste water discharge, agricultural and urban runoff, can introduce NP into aquatic environments (Sengul and Cevdet, 2017). In many countries, its presence is reported in soil, river water, sediments, terrestrial and aquatic biota (Soares et al., 2008).

Oral ingestion, inhalation, and skin contact are the most common ways for humans to be affected by NP (Chokwe et al., 2017). Its exposure can damage brain (Aydogan et al., 2008), pancreas (Li et al., 2017), testis (Ijaz et al., 2021) and liver (Kazemi et al., 2016). The liver carries out hundreds of indispensable functions to maintain health and homeostasis (Sun and Karin, 2008). NP administration prompts oxidative stress (OS) in rat's liver (Ke et al., 2021). OS is related to the production of reactive oxygen species (ROS) such as, hydroxyl radicals, hydrogen peroxide and superoxide (McMillian et al., 2004) that cause an imbalance

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between anti-oxidants and pro-oxidants that leads to hepatotoxicity. Previous researches showed that NP exposure can cause disruption in hepatic parenchyma (Kazemi et al., 2016), ballooning (Kourouma et al., 2015), congestion, vacuolar degeneration and steatosis in the liver (Shirdel et al., 2020).

Flavonoids are known for their pharmacological and biological properties (Ruiz-Cruz et al., 2017). Flavonoids have been reported as an essential component in many nutraceutical, pharmaceutical, medicinal, and cosmetic items because they exhibit wide range of health-promoting properties (Panche et al., 2016). Diosmetin (DIOS), a bioflavonoid known as 3, 5, 7-trihydroxy-4-methoxy flavone, is abundantly present in spermin, citrus fruits, legume leaves and certain medicinal herbs, including *Rosa agrestis*, *Origanumvulgare*, *Lespedeza davurica*, *Chrysanthemum morifolium* and *Robinia pseudoacacia* (Patel et al., 2013). DIOS is reported to exhibit anti-inflammatory (Lee et al., 2020), antibacterial (Huang et al., 1992), antioxidant (Liao et al., 2014), anti-mutagenic (Wang et al., 2014) and anti-apoptotic (Yang et al., 2017) activities. Considering the therapeutic potential of DIOS, the current research was carried out to investigate the protective potential of DIOS against NP induced hepatotoxicity in rats.

2. Materials and methods

2.1. Animals

Thirty-two male albino rats were procured from the Animal House of the University of Agriculture, Faisalabad. Animals were kept at a standard temperature of 25 ± 1 °C under 12-hour light/dark cycles in an animal house. Department Ethical board of the University of Agriculture, Faisalabad, approved all the protocols.

2.2. Chemicals used

Nonylphenol; CAS no: 84852–15-3; Purity: 99 % and diosmetin; CAS no: 520–34-3; Purity: ≥98 % were purchased from Sigma Ald (Germany).

2.3. Experimental design

Thirty-two rats were segregated in 4 groups (n = 8/group) and housed in separate steel mesh cages. The following doses were administered to them: control group (0.1 % DMSO); NP administered group (50 mg/kg b.wt of NP dissolved in DMSO); NP + DIOS administered group (50 mg/kg b.wt of NP and 100 mg/kg b.wt of DIOS was provided by oral gavage) and DIOS administered group (100 mg/kg b.wt of DIOS was given orally). After 30-days of treatment, all rats were anesthetized by ketamine and sacrificed. The samples of blood were taken from heart using heparinized syringes and centrifuged at 3000 rpm for almost 15 min for plasma separation, which was then stored at −20 °C for further analysis. Hepatic samples of all rats were separated and stored at −80 °C for biochemical analysis.

2.4. Biochemical analysis

Chance and Maehly (1955) procedure with slight modifications was used to assess the catalase (CAT) activity. Kakkar et al. (1984) methodology was used to evaluate the superoxide dismutase (SOD) activity. Carlberg and Mannervik (1975) approach was used to assess glutathione reductase (GSR) activity. The spectrophotometric methodology described by the Jollow et al. (1974) was used to evaluate the glutathione peroxidase (GPx) activity in liver tissue homogenate. The ROS concentration was estimated in homogenate by the process of Hayashi et al. (2007). The level of malondialdehyde (MDA) in liver tissues was determined using the procedure developed by Ohkawa et al. (1978). To take recordings, a spectrophotometer (UV–Visible/NIR-UH5700) was used.

2.5. Estimation of inflammatory markers

ELISA kits were used to estimate the inflammatory markers i.e. NF-κB, IL-1β, IL-6, TNFα levels and COX-2 activity in hepatic homogenate according to the guidelines of the manufacturer (Bosterbio, China). The recordings were taken with an ELISA plate reader (Mark™ microplate absorbance reader1681130).

2.6. Estimation of apoptotic markers

The pro-apoptotic and anti-apoptotic markers i.e. Bax, Bcl-2, caspase-3 and caspase-9 were analyzed from hepatic homogenate by using Cusabio ELISA kits (Cusabio Technology Llc, Houston, TX, USA). ELISA plate reader (Mark™ microplate absorbance reader1681130) was used to take recordings.

2.7. Histopathological analysis

Histopathological examination was performed to assess NP-induced damages in hepatic tissues. Initially, liver samples were rinsed gently in 0.9 % chilled saline, fixed in a 10 % formalin solution for 24 h, dehydrated in ascending grades (80 %, 90 %, and 100 %) of alcohol and embedded in paraffin wax. Thin segments of paraffin embedded tissues were partitioned though a microtome stained with Hematoxylin and Eosin stains and finally examined under the light microscope (Nikon, 187,842, Japan) at 40X, and images were taken by using a camera (Canon, EOS 250D).

2.8. Statistical analysis:

Data was presented as Mean ± SEM. One-way ANOVA followed by the tukey test was used for comparative evaluations of various groups by applying GraphPad prism 5 software. The level of significance was set as $p < 0.05$.

3. Results

3.1. Ameliorative effect of DIOS on antioxidant markers

The effect of DIOS on NP-induced decreased activities of antioxidant enzymes are illustrated in Table 1. Furthermore, NP administration resulted in substantial ($p < 0.05$) reduction in CAT, SOD, GSH, GPx, GST and GSR activities compared to the control. Treatment with DIOS along with NP remarkably ($p < 0.05$) increased the activities of antioxidant enzymes in liver when compared to NP intoxicated group. No significant difference was observed in the activities of antioxidant enzymes between DIOS administered group and control group.

3.2. Ameliorative effect of DIOS on oxidative stress markers

The effect of DIOS and NP on oxidative stress parameters i.e., MDA and ROS levels, are presented in Table 2. The level of MDA, was increased considerably ($p < 0.05$) in NP group as compared to the control group (Table 2). ROS also showed a similar trend, with a significant ($p < 0.05$) increase in NP administered group compared to the control group. In comparison to NP-treated group, co-treatment with DIOS markedly ($p < 0.05$) decreased MDA and ROS levels in the liver homogenate. Besides, no significant difference in ROS and MDA levels was seen between DIOS-treated group compared to the control (Table 2).

Table 1
Effect of NP and DIOS on antioxidant markers in rat's liver.

Groups	CAT (U/mg protein)	GPx (U/mg protein)	SOD (U/mg protein)	GSR (nM NADPH oxidized/min/mg tissue)	GST (nM/min/mg protein)	GSH (μM/g tissue)
Control	9.55 ± 0.71[a]	12.8 ± 0.59[a]	5.35 ± 0.18[a]	3.51 ± 0.14[ab]	26.02 ± 0.88[a]	15.87 ± 0.58[a]
NP	4.35 ± 0.20[b]	7.03 ± 0.19[b]	3.04 ± 0.11[b]	1.20 ± 0.14[c]	11.66 ± 0.66[c]	7.56 ± 0.79[c]
NP + DIOS	7.62 ± 0.26[a]	11.24 ± 0.85[a]	4.68 ± 0.25[a]	2.80 ± 0.14[b]	21.6 ± 0.64[b]	11.24 ± 0.38[b]
DIOS	9.59 ± 0.81[a]	12.93 ± 0.93[a]	5.42 ± 0.17[a]	3.57 ± 0.23[a]	26.22 ± 0.94[a]	15.79 ± 0.81[a]

Means of Control, NP, NP + DIOS and DIOS groups that do not have similar letter are substantially different.

Table 2
Effect of NP and DIOS on oxidative stress markers in rat's liver.

Groups	ROS (U/mg tissue)	MDA (nmol/mL)
Control	1.26 ± 0.06[c]	0.63 ± 0.06[c]
NP	5.66 ± 0.12[a]	1.91 ± 0.11[a]
NP + DIOS	1.84 ± 0.15[b]	1.02 ± 0.08[b]
DIOS	1.25 ± 0.06[c]	0.63 ± 0.05[c]

Means of Control, NP, NP + DIOS and DIOS groups that do not have similar letter are substantially different.

3.3. Ameliorative effect of DIOS on inflammatory markers

The effect of DIOS on NP induced increased levels of inflammatory hepatic markers such as NF-κB, TNFα, IL-6, IL-1β and COX-2 activity are demonstrated in Table 3. NP treatment resulted in a substantial ($p < 0.05$) escalation in inflammatory markers as compared with control. In NP + DIOS group a considerable ($p < 0.05$) decrease in these inflammatory markers was seen in comparison with the NP treated group. Whereas, in DIOS only treated group the mean values of inflammatory markers were similar to control group.

3.4. Ameliorative effect of DIOS on apoptotic markers

The effects of DIOS and NP on apoptotic and anti-apoptotic markers in liver are shown in Table 4. NP-intoxicated group had significantly ($p < 0.05$) higher level of the apoptotic markers (Bax caspase-3, and caspase-9) than control group, while level of anti-apoptotic marker, Bcl-2, was significantly ($p < 0.05$) reduced. DIOS co-treatment with NP, considerably ($p < 0.05$) reduced the levels of Bax, caspase-3 and caspase-9, whilst Bcl-2 level was significantly ($p < 0.05$) raised, compared to the NP group. In DIOS only treated group the mean values of apoptotic markers were similar to control group.

3.5. Effect of NP and DIOS on liver histopathology

To analyze the NP-induced liver damage histopathology was performed. The histopathological analysis indicated that NP treatment induced disruption in the liver parenchyma, inflammatory cells infiltration, vacuolization, central vein disruption, nucleus aggregation and necrosis as compared to control group. But, the above damages were significantly reduced in the rats co-treated with DIOS and NP. In the DIOS only treated group the histological patterns were almost similar to control group.

4. Discussion

The present study examined the hepatoprotective effect of DIOS against NP-prompted toxicity in rats. The current research revealed that NP exposure reduced the activities of antioxidant enzymes including GSR, GPx, CAT, GST, SOD and GSH, while elevated the concentration of ROS and MDA level in liver. Generally, in pathogenesis of various liver ailments, OS and high lipid peroxidation (LPO) play a significant part (Zhao et al., 2010). Antioxidant enzymes act as a first line of defense against OS by minimizing the ROS generation and protecting the biological molecules (DNA, proteins and lipids) (Ighodaro and Akinloye, 2018). CAT and SOD are two most vital antioxidant enzymes present in body for scavenging free radicals (Xia et al., 2015). SOD catalyzes the formation of H_2O_2 from highly reactive superoxide anion·H_2O_2 produced inside cells is then converted into H_2O and O_2 by CAT, which inhibits H_2O_2 from being converted to a more active species like hydroxyl radical (Yang et al., 2017). Diminution in CAT and SOD activities indicates the failure of liver to remove hydrogen peroxide generation which may be due to antioxidant enzymes inactivation via excessive ROS formation prompted by NP (Kaplowitz et al., 1985). Oxidation of unsaturated fatty acids in cell membranes results in ROS production (Mansour et al., 2006). However, Treatment with DIOS substantially restored the activities of antioxidant

Table 3
Effect of NP and DIOS on inflammatory markers in rat's liver.

Groups	NF-κB (n/g tissue)	TNFα (ng/g tissue)	IL-1β (ng/g tissue)	IL-6 (ng/g tissue)	COX-2 (ng/g tissue)
Control	14.5 ± 0.65[a]	6.63 ± 0.51[a]	22.93 ± 1.03[a]	6.49 ± 0.39[a]	24.55 ± 0.87[a]
NP	61.03 ± 4.32[b]	17.40 ± 0.81[b]	81.91 ± 186[b]	25.83 ± 1.19[b]	78.76 ± 3.47[b]
AUC + NP	23.90 ± 1.13[a]	10.77 ± 0.60[a]	33.17 ± 0.92[c]	13.60 ± 0.33[c]	34.88 ± 1.14[c]
AUC	14.04 ± 0.56[a]	8.64 ± 0.45[a]	22.50 ± 1.54[a]	6.43 ± 0.38[a]	24.18 ± 1.11[a]

Means of Control, NP, NP + DIOS and DIOS groups that do not have similar letter are substantially different.

Table 4
Effect of NP and DIOS on apoptotic markers in rat's liver.

Groups	Bax (pg/mL)	Bcl-2 (ng/mL)	Caspase-3 (pg/mL)	Caspase-9 (pg/mL)
Control	2.50 ± 0.26[a]	17.74 ± 0.67[a]	1.45 ± 0.163[a]	3.24 ± 0.13[a]
NP	8.9 ± 0.56[b]	5.86 ± 0.19[b]	13.83 ± 0.53[b]	21.34 ± 1.38[b]
NP + DIOS	2.99 ± 0.17[c]	14.81 ± 0.67[c]	3.16 ± 0.23[c]	5.56 ± 0.22[c]
DIOS	2.44 ± 0.18[a]	17.78 ± 0.74[a]	1.38 ± 0.17[a]	3.21 ± 0.14[a]

Means of Control, NP, NP + DIOS and DIOS groups that do not have similar letter are substantially different.

enzymes and reduced the levels of ROS and MDA that may be attributed to antioxidant potential of DIOS.

NP exposure can cause liver impairment due to inflammation. The results of current study also demonstrated that the NP triggered the activation of NF-κB, along with inflammatory cytokines including TNFα, IL-1β, IL-6 and COX-2 levels. TNFα and IL-1β are two important pro-inflammatory cytokines that are activated by inflammation and OS (Dong et al., 2014). COX-2 activity was increased in the liver tissues of NP-treated rats in this study, indicating liver injury in NP-treated rats. Our results are in line with the study conducted by (Subbaramaiah and Dannenberg, 2003) that NP elevated the activity of COX-2 and many cytokines levels implicated in inflammation, such as IL-6, IL-1β and TNFα, along with NF-κB activation. However, co-treatment with DIOS inhibited NF-κB production and increased the levels of IL-1β, IL-6, TNFα and COX-2. The anti-inflammatory properties of DIOS may be responsible for the decreased levels of inflammatory markers. These
findings confirmed the anti-inflammatory function of DIOS in the liver tissues.

An excessive amount of ROS may trigger apoptosis and oxidative stress. Apoptosis plays a significant role in hepatotoxicity (Zhang et al., 2003). Our results revealed that NP exposure, increased the level of Bax, caspase-3 and caspase-9 and reduced the levels of Bcl-2. The present research demonstrated that NP exposure led to OS, leading to caspase-3 activation, resulting in apoptosis. Apoptosis is induced by various signals that change mitochondrial membrane permeability, which leads to the release of mitochondrial intermembrane proteins, such as cytochrome *c* (Budihardjo et al., 1999). Cytochrome *C*, a pro-apoptotic factor released from outer mitochondrial membrane to cytoplasm, forms a complex with Apaf-1 and procaspase-9, resulting in activation of caspase-9. Activated caspase-9 activates the caspase-3 leading to apoptosis (Arya et al., 2011). Bax functions as a pro-apoptotic protein. While Bcl-2 functions as an anti-apoptotic protein (Klanova et al., 2022). Our results indicated that DIOS treatment augmented the level of the anti-apoptotic protein Bcl-2 while down-turning the level of apoptotic markers Bax, caspase-3 and caspase-9. These finding suggested that DIOS has an anti-apoptotic effect against NP, which could be attributable to regulation of these apoptotic markers.

The histopathological examination revealed that NP administration caused architectural alteration in liver tissues. Furthermore, NP administration disturbs the balance between antioxidant enzymes and ROS which results in OS induction in the liver of rats (McMillian et al., 2004). NP also increases lipid peroxidation in liver tissues which leads to morphological defects. NP exposure resulted in disruption in liver parenchyma, swelling of supporting and connective tissues, necrosis, nucleus aggregation, inflammatory cells infiltration and vacuolization (Jubendradass et al., 2012). However, the above damages were mitigated in the rats co-treated with DIOS. These restorative effects exhibited by DIOS might be due to antioxidant, anti-inflammatory and anti-apoptotic properties.

5. Conclusion

In conclusion, our investigation demonstrated that DIOS exhibits strong protective efficacy against OS that is a key part of NP induced hepatic damage. The levels of inflammatory markers, apoptotic markers, activities of endogenous antioxidant enzymes and histological anomalies were successfully recovered with DIOS treatment. The therapeutic potential of DIOS may be attributed to its hepatoprotective, antioxidant, anti-apoptotic and anti-inflammatory activities. The results of current study signify that diosmetin may be considered as a promising therapeutic agent for the treatment of various liver disorders that are linked to oxidative stress.

Declarations

Ethics approval: All the experimental protocols for animal handling and treatment were reviewed and monitored by ethical committee of University of Agriculture, Faisalabad, in line with the European Union of animal care and experimentation (CEE Council 86/ 609) approved protocol.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All the data is contained in the manuscript.

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Author contribution

RA, NE, and MUI designed the study, conceived the study, and analyzed the results. RA and MUI conceived an initial part of the study, performed the experiment, histology and helped in compiling the results. SR, AA, RA, HA, NWA, and TA helped in writing the results. MUI, SR, RA, NE, TA, HA, and AA made a substantial contribution in the interpretation of data and revising the manuscript for intellectual content. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jksus.2022.102392.

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