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Original article

Tangeretin ameliorates bisphenol induced hepatocyte injury by inhibiting inflammation and oxidative stress

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ABSTRACT

Bisphenol A (BPA) is an industrial toxicant that can potentially damage the liver. Tangeretin (TGN) is a natural flavonoid that displays various pharmacological activities. This experiment was carried out to evaluate the protective effects of TGN against BPA-induced hepatic impairment in the male albino rat. Twenty-four male albino rats were equally divided into four different groups: control, BPA (100 mg/kg), BPA + TGN (100 mg/kg + 50 mg/kg) and TGN (50 mg/kg). BPA exposure significantly decreased the activities of catalase (CAT), superoxidase dismutase (SOD), peroxidase (POD), glutathione reductase (GSR), glutathione S-transferase (GST), and glutathione (GSH) content while substantially increasing the thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H_2O_2) levels. A substantial increase in the levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) was also observed in BPA treated rats. Moreover, BPA significantly increased the inflammatory markers, including tumor necrosis factor-α (TNF-α), nuclear factor kappa-B (NF-κB), Interleukin-6 (IL-6), Interleukin-1β (IL-1β)levels, cyclooxygenase-2 (COX-2) activity, and histopathological damages. However, co-treatment with TGN efficiently minimized the BPA-induced biochemical, inflammatory, and histopathological impairments in rat liver. The present study shows that TNG has significant potential to avert BPA-induced liver damage to its antioxidant and anti-inflammatory properties.

1. Introduction

Bisphenol A (BPA) is one of the conspicuous environmental contaminants commonly used to manufacture polycarbonates, epoxy resins, food containers (food wrapping and can glazing), and several plastic products. The extensive use of these products has led to high content BPA in several biological fluids, including amniotic fluid, neonatal blood, and human breast milk (Vandenberg et al., 2012). The primary route of BPA exposure is through ingestion (Ribeiro et al., 2017), but inhalation and transdermal absorption are possible secondary routes of exposure, particularly in individuals who work in companies that deal with BPA-based products (Biedermann et al., 2010). BPA can be released from the walls of the food containers, water bottles, and dental sealants and enters into the atmosphere, water, and food items (Bjornsdotter et al., 2017). Studies have demonstrated that BPA exposure induces detrimental effects on humans and animals (Biedermann et al., 2010). The United States Environmental Protection Agency (EPA) has ranked the BPA a third most hazardous environmental toxicant (Nahar et al., 2012).

Acute BPA exposure and its toxic ability increase concerns about its effects on various body organs (Lee et al., 2014). BPA shows short-term, subchronic, and acute toxicity (Eweda et al., 2020). Several studies have reported the effects of BPA on the body weight, kidneys, and liver at various doses (Tyl, 2008). Numerous investigations have reported that BPA exposure instigates hepatic damage and induces oxidative stress (Thoene et al., 2017; Eid

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et al., 2015). It has been reported that the liver is considered a major site of or BPA absorption and metabolism (Kim et al., 2016). Recent studies have reported that elevated concentration of BPA in urine has a close association with serum markers of abnormal hepatic activities (Lang et al., 2008). In vivo study indicated that BPA can induce liver injuries through reactive oxygen species (ROS) production, which consequently damages the liver (Hassan et al., 2012).

Phytochemicals are getting attention due to their better efficacies and fewer side effects (Omar et al., 2016). Flavonoids are dietetic compounds that are present in an extensive amount in fruits and vegetables. They exhibit significant pharmacological properties (Bakar et al., 2019; Ijaz et al., 2020). Tangeretin (TGN) is a bioactive flavonoid extracted from the peel of citrus fruits, which possesses significant antioxidant, anti-cancer, and anti-inflammatory activities (Ashrafizadeh et al., 2020). Furthermore, due to its pharmacological properties, TGN can be used to reduce ROS production and boost the efficiency of antioxidant enzymes (Chen et al., 2007). So, the current study was planned to assess the protective effects of orally administrated TGN against BPA-induced hepatotoxicity in rats by determining hepatic serum markers, antioxidants enzymes, lipid peroxidation, Inflammatory markers, and histological damages.

2. Material and methods

2.1. Chemicals

Bisphenol A and Tangeretin were purchased from Sigma-Aldrich, Germany.

2.2. Animals

Twenty-four albino male rats (180–220 g) were used for this research. Rats were kept in steel cages in the animal and care center of the University of Agriculture, Faisalabad (UAF). Rats were divided into different groups and kept in separate cages sustained under controlled conditions at 26 ± 2℃, maintained 12 h day/night, and provided a standard diet and water during the whole experiment. Rats were handled in compliance with the European Union of animals care and experimentation (CEE Council 86/609) guidelines.

2.3. Experimental protocol

Rats were divided equally into four groups (n = 6/group) and treated as follows; Group 1st was considered to control and provided a regular diet and water. Group 2 was administered with BPA (100 mg/kg) orally, the 3rd group was co-administrated with BPA (100 mg/kg) and TGN (50 mg/kg) orally, and the 4th group was treated with TGN (50 mg/kg) orally. The experiment was set for 30 days. After the trial, the rats were treated with anesthesia, decapitated, and blood samples were assembled in sterile tubes. The liver was separated and cleaned with normal saline. After dissection, the liver was cut into two pieces; one was packed in zipper bags and stored at −80 °C for biochemical analysis. The other was preserved in a 10% neutral formalin buffer solution for histopathological examination.

2.4. Estimation of antioxidant enzymes

The procedure of Chance and Maehly (1955) was used to measure the activity of CAT and POD. SOD activity was measured by following the process of Nishikimi et al. (1972). GST was calculated by following the procedure of Couri and Abdel-Rahman (1979). The activity of GSR was estimated by the process of Carlberg and Mannervik (1975). The concentration of GSH was measured from tissue homogenates by following the method of Sedlak and Lindsay (1968). H_2O_2level was assessed by following the process of Pick and Keisari (1981). The level of TBARS was measured through the method of Ohkawa et al. (1979).

2.5. Analysis of hepatic serum markers

Evaluation of ALT, AST, and ALP levels was determined by specific kits Purchased from abcam (USA).

2.6. Inflammatory markers assessment

Commercially available kits were used to assess the inflammatory markers of the hepatic tissues. NF-κB, TNF-α, IL-1β, IL-6 levels, and COX-2 activity were determined with a rat ELISA kit (Shanghai-YL-Biotech. Co. Ltd., China). Analyses were completed by following the manufacturer's instructions through ELISA Plate-Reader (BioTek, Winooski-VT, USA).

2.7. Histopathological analysis

Liver tissue was washed in 0.9% cold saline and fixed in a 10% formalin buffer solution for the histopathological assessment for 24 h. After its fixation, samples were dehydrated using different ethyl alcohol grades (70, 80, 90, 100%), cleared in xylene, and then embedded in paraffin wax. A microtome machine was used to cut4-5 μm thick sections (Leica RM 2155, England). The hematoxylin-eosin stain was used to stain these sections. A light microscope (Nikon, 187842, Japan) was used for the histopathological examinations of these sections. Leica LB microscope-connected camera was used to capture the photographs of tissues.

2.8. Statistical analyses

Data are shown as means ± SEM. The normality of data was first tested using Levene's test. A one-way analysis made comparisons of the differences of variance (ANOVA) followed by Fisher's LSD for multiple comparisons or nonparametric Kruskal-Wallis as appropriate. Statistical significance was taken at$P < 0.05$. All analyses were performed using Minitab software.

3. Results

3.1. Effect of BPA and TGN on biochemical markers

The activities of antioxidants enzymes, including CAT, SOD, POD, GSR, GST, and GSH content, were significantly ($p < 0.05$) decreased in BPA administered rats compared to the control group. Furthermore, When the rats were administrated with TGN and BPA, these antioxidant enzymes' activities were substantially ($p < 0.05$) elevated when compared to the BPA intoxicated rats. TGN alone treatment showed regular activity of antioxidant enzymes (Table 1).

3.2. Effect of BPA and TGN on TBARS and H_2O_2 levels

Levels of TBARS and H_2O_2 were significantly ($p < 0.05$) increased in BPA intoxicated rats compared to the control group. The supplementation of TGN and BPA significantly lowered the levels of TBARS and H_2O_2 compared to BPA-exposed groups. TGN alone treatment maintained the normal levels of TBARS and H_2O_2 (Table 2).

Table 1
The effect of TGN on CAT, SOD, POD, GSR GST, and GSH activities against BPA induced hepatotoxicity in rats.

Groups	**CAT** (U/mg protein)	**POD** (U/mg protein)	**SOD** (U/mg protein)	**GSR** (nM NADPH oxidized/min/mg tissue)	**GST** (nM/min/mg protein)	**GSH** (μM/g tissue)
Control	7.90 ± 0.30[a]	6.41 ± 0.12[a]	5.45 ± 0.18[a]	3.40 ± 0.16[a]	21.06 ± 1.20[a]	15.32 ± 0.45[a]
BPA (100 mg/kg)	4.18 ± 0.22[b]	2.94 ± 0.12[b]	2.42 ± 0.15[b]	1.82 ± 0.09[b]	11.19 ± 0.61[b]	8.02 ± 0.29[b]
BPA (100 mg/kg) **+ TGN** (50 mg/kg)	7.13 ± 0.22[a]	5.78 ± 0.20[c]	5.18 ± 0.13[a]	2.75 ± 0.23[c]	18.22 ± 0.54[c]	14.35 ± 0.18[c]
TGN (50 mg/kg)	7.84 ± 0.36[a]	6.40 ± 0.16[a]	5.52 ± 0.19[a]	3.32 ± 0.24[a]	21.53 ± 0.84[a]	15.70 ± 0.50[a]

Values in the same column that do not share a superscript are significantly different.

Table 2
The effect of TGN on TBARS and H_2O_2 levels in rat liver after BPA exposure.

Groups	**TBARS** (nm TBARS/min/mg tissue)	**H_2O_2** (nM/min/mg protein)
Control	13.87 ± 0.34[a]	1.36 ± 0.10[a]
BPA (100 mg/kg)	25.35 ± 1.06[b]	4.15 ± 0.11[b]
BPA (100 mg/kg) **+ TGN** (50 mg/kg)	17.95 ± 1.30[c]	2.30 ± 0.15[c]
TGN (50 mg/kg)	14.43 ± 0.56[a]	1.48 ± 0.12[a]

Values in the same column that do not share a superscript are significantly different.

3.3. Effect of BPA and TGN on hepatic serum markers

Results of hepatic serum markers assessment indicated that the BPA exposure led to severe liver injuries that were evicted by a significant ($p < 0.05$) rise in the levels of ALT, ASP, and ALP when compared to the control. However, levels of these hepatic serum markers were substantially ($p < 0.05$) reduced in the co-treated rats in comparison to the BPA-administered group (Table 3).

3.4. Effect of BPA and TGN on inflammatory markers

BPA administration significantly ($p < 0.05$) elevated the inflammatory parameters; NF-κB, TNF-α, IL-6, IL-1βlevels, and COX-2 activities in comparison to the control group. While TGN administration significantly ($p < 0.05$) decreased the levels of these inflammatory parameters in the cotreated rats compared to the BPA treated group. No increase in inflammatory markers was noted in the TGN alone administered group (Table 4).

3.5. Effect of BPA and TGN on histopathology

Histopathological findings of control, BPA intoxicated, BPA + TGN, and TGN supplemented groups are illustrated in Fig. 1. The Control group indicated the regular architecture of hepatocytes, central vein, and sinusoidal spaces (A). The liver of the BPA-treated group revealed the severe deformation of cellular structure with noticeable blockage of central and portal vein, more dilated sinusoid, and disorganized hepatic cells (B). Our results represented that co-administration with TGN resulted in the recovery of morphological parameters of hepatic tissues. A significant decrease in the intensity of inflammation, blood vessel congestion, necrotic cells, and sinusoidal dilation was noticed in BPA + TGN treated rats (C). Any structural abnormalities such as inflammatory cell infiltration, sinusoidal dilation, and necrosis were not observed in the TGN administrated group (D).

Table 3
TGN on serum markers of the liver (ALT, ALP, and AST) in rats against BPA administration.

Groups	**ALT** (U/L)	**AST** (U/L)	**ALP** (U/L)
Control	43.24 ± 1.80[a]	58.73 ± 2.83[a]	75.31 ± 2.35[a]
BPA (100 mg/kg)	132.6 ± 7.35[b]	221.7 ± 5.90[b]	136.6 ± 5.13[b]
BPA (100 mg/kg) **+ TGN** (50 mg/kg)	75.03 ± 3.28[c]	82.66 ± 3.63[c]	94.61 ± 2.89[c]
TGN (50 mg/kg)	50.52 ± 3.51[ac]	66.77 ± 5.19[ac]	76.48 ± 3.79[a]

Values in the same column that do not share a superscript are significantly different.

4. Discussion

Bisphenol A is an environmental toxicant that is being released from the lining of food cans, water bottles, polycarbonate plastics, and dental sealants (Bjornsdotter et al., 2017). Due to its widespread presence in the environment, animals are often exposed to BPA in their daily life. It has been revealed that BPA can cause oxidative stress and produce various disorders in the brain and other organs of the body (Miyagawa et al., 2007). Several studies have reported that BPA exposure instigates hepatic damage and induces oxidative stress (Kim et al., 2016). The natural plant-derived flavonoid TGN has various pharmacological activities. Therefore, this research was planned to focus on assessing the toxic potential of BPA and investigating the curative effects of the TGN against BPA-induced damage in the liver.

In this experiment, a remarkable decline in antioxidant enzyme activities of CAT, SOD, POD, GSR, GST, and GSH content and substantial elevation in TBARS and H_2O_2 levels was observed. Several studies have elucidated that the BPA produces free radicals (Kim et al., 2016), which usually leads to disturbance in the defensive system of antioxidant enzymes (Wang et al., 2019). These antioxidant enzymes provide cellular protection against ROS-induced oxidative stress (Uzunhisarcikli et al., 2016; Latif et al., 2020). The imbalance of free radical oxygen induces oxidative stress, disrupting the equilibrium state of antioxidants and oxidants, inclining towards oxidation (Liu et al., 2021). Catalase (CAT) is a hemeprotein that catalyzes the H_2O_2 into oxygen and water and protects cells from oxidative impairment of hydroxyl radical and H_2O_2 (Safhi et al., 2016). SOD plays a defensive role against free radicals and eliminates them by converting them into less toxic radicals (Stinghen et al., 2014). GSR converts the glutathione disulfide into GSH. GSH protects mammalian cells against oxidative stress by minimizing levels of H_2O_2 as well as other peroxides (Deponte, 2013). GST is a multifunctional enzyme involved in detoxification processes due to the catalytic conjugation of glutathione present in it (Allocati et al., 2018). Previous researchers also reported that BPA accumulation reduced the activates of antioxidant enzymes (Meli et al., 2020). Due to BPA exposure, the level of TBARS increased, which displayed sufficient lipid peroxidation due to a decrease in the antioxidant mechanism (Ali et al., 2018). Apart from the endogenous-antioxidant enzymes, antioxidants can also be obtained from natural resources (plants) to overcome OS (Nahid et al., 2017). Therefore, TGN remarkably suppressed oxidative stress and scavenged free radicals by raising the activities of CAT, SOD, POD, GSR, GST, and GSH along with a

Table 4
TGN on inflammatory parameters (NF-κB, TNF-α, IL-1β, IL-6 levels, and COX-2 activities) in the liver of BPA-administered rats.

Groups	NF-κB (ng/g tissue)	TNF-α (ng/g tissue)	IL-1β (ng/g tissue)	IL-6 (ng/g tissue)	COX-2 (ng/g tissue)
Control	15.7 ± 0.27[a]	7.29 ± 0.26[a]	25.2 ± 0.32[a]	6.01 ± 0.20[a]	26.5 ± 1.25[a]
BPA (100 mg/kg)	68.7 ± 1.69[b]	15.9 ± 0.44[b]	84.3 ± 1.88[b]	19.2 ± 0.44[b]	76.9 ± 3.67[b]
BPA (100 mg/kg) **+ TGN** (50 mg/kg)	28.1 ± 0.79[c]	8.29 ± 0.27[c]	36.0 ± 1.53[c]	7.59 ± 0.29[c]	39.9 ± 1.33[c]
TGN (50 mg/kg)	15.2 ± 0.27[a]	6.85 ± 0.23[a]	23.9 ± 0.59[a]	5.92 ± 0.20[a]	25.9 ± 1.26[a]

Values in the same column that do not share a superscript are significantly different.

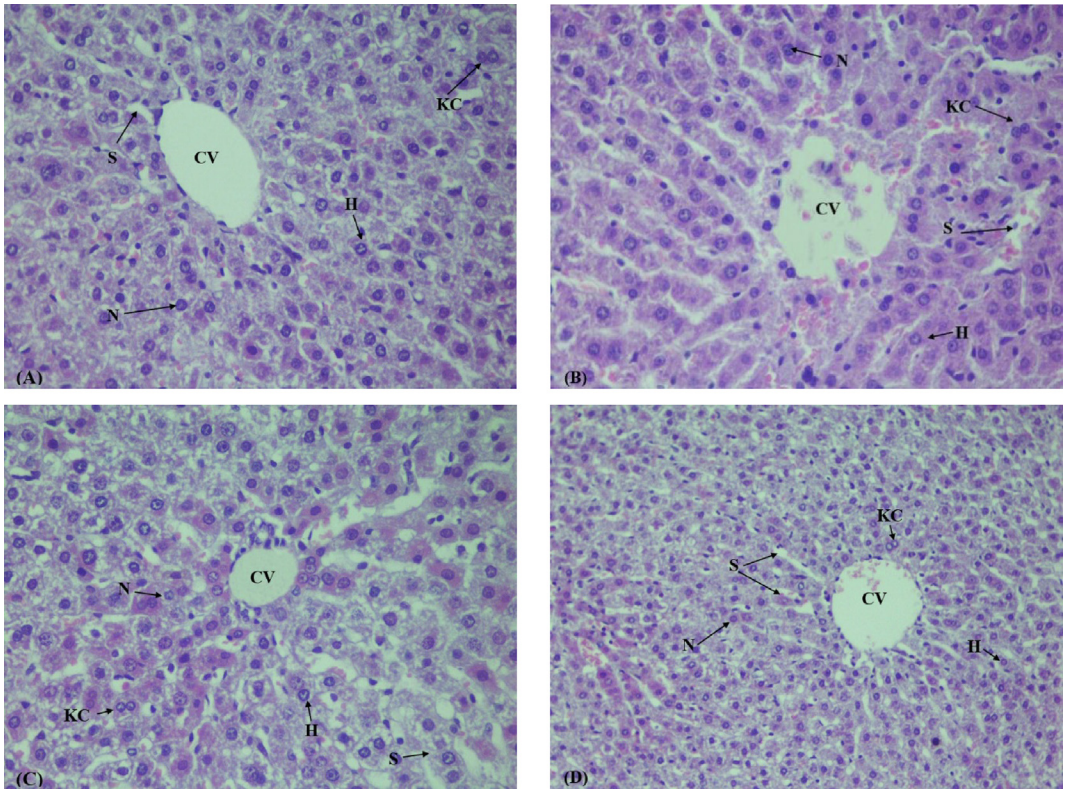

Fig. 1. Light microscopy of liver tissues was obtained from different groups (H&E 40X). (A): Control (normal histoarchitecture). (B): BPA (100 mg/kg.); Tissues showing extensive and marked necrosis. (C): BPA (100 mg/kg) + TGN (50 mg/kg); decrease in necrosis throughout the liver tissues and recovery of damaged tissues. (D): Treatment with TGN (50 mg/kg); normal histoarchitecture almost as in control group. CV; Central venule. H; Hepatocytes. KC; Kupffer cells. S; Sinusoids. N; Nucleus.

reduction in TBARS and H_2O_2, probably due to its antioxidant potential.

It has been well documented that liver tissue damage can be evaluated through ALT, AST, and ALP evaluation (Vagvala and O'Connor, 2018). Our experimental data revealed that BPA exposure remarkably increased the serum markers of liver function. It has been previously reported that elevated serum markers indicate tissue damage in the liver (Vagvala and O'Connor, 2018). The deleterious effects of BPA might be linked to its reactive intermediate metabolites formed during its metabolism in the body. Previous investigations show that overproduction of ROS damages the structural integrity of liver cells that may result in increased hepatic serum markers (Vagvala and O'Connor, 2018). However, co-treatment with TGN prevented the BPA-induced hepatotoxicity which has been displayed by the reduced level of these serum enzymes; AST, ALT, and ALP, which indicates the hepatoprotective nature of TGN.

Inflammation plays a significant role in the association of BPA with hepatic damage. In this analysis, BPA treatment elevated the levels of NF-κB, TNF-α, IL-6, IL-1β, and COX-2 activities. NF-κB activation plays a vital role in the manifestation of pro-inflammatory cytokines like NF-κB, TNF-α, IL-6, IL-1β, and COX-2 that are linked with acute inflammatory responses and other ROS related disorders (Kandemir et al., 2018). NF-kB activation leads to increased secretion of TNF-α, IL-1β, IL-6 through gene upregulation, contributing to acute liver injury and progression (Sun and Karin, 2008). COX-2 is an inductive form of COX and an additional critical inflammation marker, which plays an essential biological role in inflammation (Gandhi et al., 2017). In this analysis, the activity of COX-2 was elevated in hepatic tissues of BPA-treated groups, which indicates hepatotoxicity in BPA-treated rats. TGN treated rats showed decreased levels of NF-κB, IL-1β, TNF-α, IL-6, and COX-2 activities. This normalization may be credited to the anti-inflammatory potential of TGN. These results solidify the anti-inflammatory role of TGN on liver tissues.

Microscopic evaluation of hepatic tissues approved the BPA-induced histopathological damages. The histopathological observation revealed that BPA caused substantial injuries in the morphology of hepatic tissue, induced inflammation, sinusoid dilation, and necrosis in the rat's liver. The investigation indicated that BPA intoxication caused the peroxidation of membrane lipids in the hepatic tissues. Our findings are consistent with the previous study of (Eweda et al., 2020), who reported BPA-induced hepatic and cardiac tissue injuries in rats. However, BPA-induced abnor-

malities were cured by co-administration with TGN. The TGN has curative potential and possesses antioxidant properties, which may decrease the impairment in liver tissues, which is also consistent with the normalization of hepatic serum markers, followed by TGN treatment.

5. Conclusion

In conclusion, the outcomes of our investigation demonstrated that TGN presented excellent ameliorative potential against BPA-induced oxidative stress, which is one of the essential mediators of BPA-induced liver damage. TGN treatment significantly restored the activities of antioxidant enzymes, levels of liver serum markers, regulated the inflammatory markers and histological architecture. This hepatoprotective property of TGN is attributed to its antioxidant and anti-inflammatory potential. Finally, it can be stated that TGN might hold some prospects at the clinical level in future studies for the restoration of hepatic dysfunctions in animals and humans, followed by BPA exposure. The limitation of the study is that there was a limited sample size and duration of the study. This limitation can be addressed in subsequent studies.

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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