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RESEARCH ARTICLE Indian Journal of Animal Research

Hepatoprotective Mechanism of Apigenin *via* Suppression of Oxidative Inflammatory Signaling and Apoptosis against Hepatotoxicity Induced by CCl_4 in Rats

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ABSTRACT

Background: Carbon tetrachloride (CCl_4) is a critical hepatotoxicant causing liver injury and fibrosis via hepatic production of reactive oxygen species (ROS). Apigenin (APG) is a natural bioactive compound and flavonoid antioxidant. We, therefore, evaluated whether APG could mitigate CCl_4-mediated hepatotoxicity.

Methods: Rats were randomly divided and administered APG and/or CCl_4 in Control group, CCl_4 group, APG + CCl_4 groups (APG: 10 and 20 mg/kg bw) and APG groups (APG: 10 and 20 mg/kg bw) 2 times per week for 7 consecutive weeks.

Result: Rats exposed to CCl_4 demonstrated marked increases in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and monoamine oxidase (MAO) activities and decreased hepatic malondialdehyde (MDA) level compared to control. The hepatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) decreased appreciably. The CCl_4 intoxication caused significant increases in inflammatory cytokines (IL-6 and TNF-α) and apoptosis markers, while the anti-inflammatory cytokines (IL-4 and IL-10) decreased with evident histopathological lesions compared to control. APG-dose-dependently-prevented these hepatic alterations.

Key words: Apigenin, Carbon tetrachloride, Hepatotoxicity.

INTRODUCTION

Environmental toxicants are ubiquitous and have been implicated in the pathophysiology of chronic diseases and organ damage (Famurewa *et al*., 2022). Carbon tetrachloride is among these environmental pollutants that inadvertently enter the human body and trigger pathologies. It is an exogenous industrial solvent with a strong affinity for the liver and it has been recognized as a hepatotoxicant (Zhou *et al*., 2020). In spite of its hepatotoxic and nephrotoxic stress in humans and experimental animals, it is still being used in dry cleaning, fumigation of grains, insecticide and filling fire extinguishers (Que *et al*., 2022). The hepatic metabolism of CCl_4 via the action of cytochrome P450-dependent monooxygenases results in the production of its hepatic metabolites, trichloromethyl (CCl_3) and trichloromethyl peroxyl ($^{\cdot}OOCCl_3$) reactive oxygen species (ROS) El-(Hadary and Hassanien, 2016). The metabolites are potent free radicals that trigger subsequent reactive oxygen species (ROS) generation. Hepatic diseases, including hepatic cirrhosis and fibrosis have been associated with ROS effect (El-Hadary and Hassanien, 2016). The prevailing mechanism of CCl_4-induced hepatotoxic damage is consistent with the potential of its oxidative metabolites to cause hepatic antioxidant impairment leading to oxidative stress, and pro-inflammatory activation Yang *et al*. (2018). Oxidative stress is capable of initiating membrane lipid peroxidation and, finally cell death (Zhou *et al*., 2020). The trichloromethyl radical can react with sulfhydryl groups of glutathione enzymes and other protein thiols to cause deficit in glutathione metabolism and consequently reduces cell activities of superoxide dismutase and catalase (Almatroodi

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et al., 2020). Existing literature has implicated the involvement of oxidative pro-inflammation and apoptosis in CCl_4-induced hepatic pathologies Yue *et al*. (2020).

A robust body of literature reports that natural antioxidants are efficacious in preventing oxidative stress-related liver pathologies (Almatroodi *et al*., 2020). Apigenin (4,5,7-trihydroxyflavone) is a natural polyphenolic flavone widely found in fruits, herbs and vegetables (Shankar *et al*., 2017). Systematic investigations on the health benefits of APG have shown its several pharmacological effects, including antioxidant, anti-inflammatory, antidiabetic, anti-Alzheimer's disease, anticancer, antiviral and antihypertensive (DeRango-Adem and Blay, 2021 Ahmad *et al*. (2019) and Wu *et al*., 2021). Therefore, the study herein was designed to explore APG's possible hepatoprotective effect and mechanism against CCl_4-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride (Cat. No. 56-23-5) was purchased from Loba Chemie (India), olive oil (Cat. No. EL 40-105) was purchased from AGROVIM (Greece) and apigenin (Cat. No. 520-36-5) was purchased from Matrix Scientific (Columbia, SC, USA). Reagent kits for liver function markers (ALT: E-BC-K235-S and AST: E-BC-K236-M). The kits for SOD (SOD: Cat. No. SD 2521), CAT (CAT: Cat.No. CA 2517) and GPx (GPx: Cat. No. GP 2524) activities and MDA (MDA: Cat. No. MD 2529) level were obtained from BioDiagnostics, Giza, Egypt. The MAO kit (EMAO-100) was purchased from BioAssay Systems, CA, USA. The kits for cytokines were procured from OriGene Technologies Inc., Rockville, MD and MyBioSource, Inc., San Diego, USA, while ELISA kits for apoptosis markers were obtained from PEVIVA, USA.

Experimental animals

Thirty male rats (weighing 200-220 g) were used which were obtained from the Faculty of Science at King Faisal University, Kingdom of Saudi Arabia. The experimental design used in this study was approved by the Department of Chemistry Research and Ethics Committee, College of Science, King Faisal University, Kingdom of Saudi Arabia, with reference number KFU-REC/2020-09-02. The rats were housed in a laboratory animal room under standard management conditions of a temperature of 20-25°C and the exposure time to light per day was 12 hours.

Experimental design

Following 14 days of acclimatization, rats were randomly divided into six groups (n=5/group).

Group I (Control): Rats received an intraperitoneal (i.p.) injection of olive oil (3 ml/kg b.w.).

Group II (CCl_4): Rats received CCl_4 (30% in olive oil) (3 ml/kg b.w., i.p) [20].

Group III (APG + CCl_4): Rats received APG (10 mg/kg, orally) + CCl_4 (3 ml/kg b.w., i.p).

Group IV (APG + CCl_4): Rats received APG (20 mg/kg, orally) + CCl_4 (3 ml/kg bw, ip).

Group V (APG): Rats received APG (10 mg/kg b.w, orally).

Group VI (APG): Rats received APG (20 mg/kg b.w, orally).

The treatment was performed twice per week for seven consecutive weeks. The doses of CCl_4 and APG doses were chosen according to Liu *et al*. (2018) and Anusha *et al*. (2017), respectively.

Blood samples and liver tissues were collected at the end of the experimental period from all experimental animals. The serum was separated and liver samples were taken for the biochemical analysis and histological examination.

Biochemical analysis

Determination of liver function indices

Liver enzymes, including AST and ALT, were quantitatively estimated in serum using commercial kits, following the manufacturer's instructions. Monoamine oxidase (MAO) was analyzed using a commercial kit.

Determination of oxidative stress markers

Liver antioxidant CAT, SOD, GPx and TBARS as MDA levels were measured using standard assay kits, following the procedures of the kits' manufacturer.

Determination of inflammatory markers

The inflammatory cytokines interleukin-6 (IL-6), interleukin-4 (IL-4) and interleukin-10 (IL-10) levels were measured in serum using rat ELISA kits.The tumor necrosis factor-α (TNF-α) was analyzed using ELISA kit. The analyses were done according to the manufacturers' instructions.

Apoptotic markers

According to the protocols described in the ELISA kits.

Histopathological analysis

Liver samples from each group were fixed in 4% formaldehyde for 24 h, dehydrated in ascending ethanol series, embedded in paraffin, sectioning (4 µm thick) and stained with hematoxylin and eosin dye (H and E) and examined under light microscope. The histopathological alterations in tissue sections were scored and an average value was determined as follows: normal histostructure (0), mild (1), moderate (2) and severe (3) following extensive alterations according to their histopathological findings (Bancroft and Gamble 2002).

Statistical analysis

The SPSS software was used for data analysis and results presented as mean ± SEM. The one-way analysis of variance (ANOVA) was used to determine statistical differences among groups, followed by a post hoc "LSD test: the least significant difference." A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effect of APG on liver function indices

ALT and AST are enzymes mainly confined within the hepatocytes, although they are also found in the heart, kidney, blood cells and pancreas (Aja *et al*. 2020) while monoamine oxidase is a mitochondrial enzyme with high

activity in the brain, GIT and hepatic tissue (Jaka *et al*. (2021). CCl_4 significantly increased the serum activities of ALT, AST and MAO compared to normal control. However, the administration of APG prevented these toxic effects in a dose-dependent mannerreduction (Table 1). Similar result was obtained by Liu *et al*. (2018) and Almatroodi *et al*. (2020). Elevations in activities of ALT and AST is due to cellular leakage and loss of functional integrity of hepatic cell membrane, whereas elevated ALP activity is a marker of hepatic-cholestatic damage while increased hepatic MAO activity reveals mitochondrial injury (Abou Seif, 2016). Furthermore, hepatic necrosis which was observed in our histopathological analysis has been implicated as a contributory factor to ALT and AST release into the blood circulation. Contrarily, concomitant administration of APG (10 and 20 mg/kg body weight) to rats inhibited CCl_4-mediated hepatic damage dose-dependently in this study. This result agree with that study of Yue *et al*. (2020).

Effect of APG on oxidative stress markers

It is known that SOD, CAT and GPx are cellular antioxidant enzymes that scavenge ROS and thus promote antioxidant mechanism. CCl_4 sigmificantly reduced the activities of SOD, CAT and GPx and increased MDA level.

However, the administration of APG (at 10 and 20 mg/kg body weight) significantly increased the activities of these enzymes and decreased MDA level compared to CCl_4 group. APG exerted significant dose-dependent reduction on MDA alone (Table 2). These findings can be confirm by previous reports (Ubhenin *et al*., 2016). The significant depression in the hepatic activities of SOD, CAT and GPx implies the overwhelming oxidative imbalance exerted by the CCl_4 metabolites leading to antioxidant imbalance and/or oxidative stress in the rat liver exposed to CCl_4.

The chief mechanism underlying CCl_4 hepatotoxicity is oxidative stress arising from the hepatic metabolites of CCl_4 (trichloromethyl and trichloromethyl peroxyl radicals). These hepatic metabolites, are ROS generators and consumers of antioxidant balance (El-Hadary and Hassanien, 2016). Intriguingly, trichloromethyl can react with sulfhydryl groups present in glutathione, GPx and protein thiols to form an oxidative complex, exerting deleterious effects on SOD and CAT (El-Hadary and Hassanien, 2016).

Interestingly, the APG administration scavenged the ROS and enhanced hepatic antioxidant homeostasis. This was evident through prominently elevated hepatic activities of SOD, CAT and GPx and decreased level of MDA compared to CCl_4 group, in consonance with earlier studies (Raskovic *et al*., 2017 and Ubhenin *et al*., 2016). Consequently, appreciably indicating ability of APG to inhibit oxidative stress.

It was noteworthy to observe that the two doses of APG failed to demonstrate dose-dependent increases in SOD, CAT and GPx activities but only in MDA level (Table 2). A robust body of literature indicates APG antioxidant property (DeRango-Adem and Blay, 2021). It is a natural polyphenolic flavonoid flavone with ROS-scavenging activity and other pharmacological properties (Salehi *et al*. 2014). By implication, therefore, APG demonstrates a hepatoprotective effect against CCl_4 oxidative stress *via* its antioxidant property. The free hydroxyl groups present on the A/B rings of APG are responsible for the antioxidant effects of this flavone (Singh *et al*. 2014).

Effect of APG on inflammatory markers in CCl_4-intoxicated rats

In CCl_4 group, the levels of IL-6 and TNF-α significantly increased while the levels of anti-inflammatory markers, IL-4 and IL-10 is significantly reduced compared to normal control. This result indicates that CCl_4 provokes pro-inflammation and depresses anti-inflammation (Yang *et al*., 2018). The oxidative stress observed herein might have enhanced the induction of cytokine expression. Oxidative stress status may trigger the nuclear translocation of nuclear factor-kappa B (NF-κB) to stimulate the expression of cytokine proteins Edeogu *et al*. (2020), which has been reported to occur during CCl_4 hepatotoxicity (Tsai *et al*. 2017).

However, APG administration prominently abrogated the effect of CCl_4 on these cytokines. Interestingly, the two

Table 1: Effect of APG on liver function indices (U/L) in CCl_4-intoxicated rats.

	ALT	AST	MAO
Control	37.8±1.1	55.6±1.1	21.6±0.8
CCl_4	86.8±1.8^a	245.6±1.5^a	59.8±0.9^a
APG 10 + CCl_4	62.4±0.5^b	195±10.1^b	39.6±1.3^b
APG 20 + CCl_4	46.8±1.2^{bc}	134.6±1.4^{bc}	27.8±0.9^{bc}
APG 10	37.1±0.5	56.6±0.9	21.2±1.1
APG 20	37.2±0.9	54.6±1.3	21.4±0.7

Data were displayed as mean±SEM (n = 5 rats/group). CCl_4: Carbon tetrachloride; APG: Apigenin (where 10 and 20 refer to the dose in mg/kg bw); ^{a}p<0.05: Significant when compared to control group in the same column. ^{b}p<0.05: Significant when compared to CCl_4 group in the same column. ^{c}p<0.05: Significant when compared to APG 10 + CCl_4 group in the same column.

Table 2: Effect of APG on liver oxidative stress markers in CCl_4-intoxicated rats.

	CAT (U/mg protein)	SOD (mU/mg protein)	GP_X (mU/mg protein)	MDA (nmol/gm tissue)
Control	34.6±1.5	55.4±1.7	55.6±1.1	5.0±0.2
CCl_4	17.6±1.3^a	19.8±0.9^a	25.6±1.1^a	25.4±1.2^a
APG 10+CCl_4	27.4±1.0^b	31.8±1.4^b	35.4±1.2^b	18.4±1.2^b
APG 20+CCl_4	31.4±0.6^b	36.2±1.2^b	43.8±1.8^b	13.4±0.9^{bc}
APG 10	35.5±1.3	54.2±1.5	55.4±1.2	5.6±0.1
APG 20	33.4±1.3	53.6±0.8	54.8±1.4	5.4±0.1

Data are displayed as mean±SEM (n = 5 rats/group). CCl_4: Carbon tetrachloride; APG: Apigenin (where 10 and 20 refer to the dose in mg/kg bw); ^{a}p<0.05: Significant when compared to control group in the same column. ^{b}p<0.05: Significant when compared to CCl_4 group in the same column. ^{c}p<0.05: Significant when compared to APG 10 + CCl_4 group in the same column.

Table 3: Effect of APG on inflammatory markers (pg/ml) in CCl_4 intoxicated rats.

	Pro-inflammatory		Anti-inflammatory	
	IL-6	TNF-α	IL-4	IL-10
Control	37.2±1.2	51.4±1.7	38.4±1.4	52.4±1.2
CCl_4	364.6±1.3^a	392±1.0^a	26.4±1.1^a	24.4±1.2^a
APG 10 + CCl_4	222.4±5.2^b	220.8±5.2^b	30.2±2.6^b	35.7±0.9^b
APG 20 + CCl_4	134.4±1.1^{bc}	115±1.5^{bc}	34.6±0.9^b	43.4±1.2^b
APG 10	36.6±0.9	52.2±1.9	37.2±0.6	51.4±1.0
APG 20	36±1.5	51.2±1.2	39.8±0.9	52.2±1.3

Data were displayed as mean ± SEM (n = 5 rats/group). CCl_4: Carbon tetrachloride; APG: Apigenin (where 10 and 20 refer to the dose in mg/kg bw); ^{a}p<0.05: Significant when compared to control group in the same column. ^{b}p<0.05: Significant when compared to CCl_4 group in the same column. ^{c}p<0.05: Significant when compared to APG 10 + CCl_4 group in the same column.

doses of APG expressed a dose-dependent effect on IL-6 and TNF-α only (Table 3). Accumulating number of studies demonstrate that APG can suppresses inflammatory cascades (Salehi *et al*.,2019).

Effect of APG on apoptosis markers in CCl_4-intoxicated rats

Serum levels of M30 and M65 are indicator of apoptosis of cells undergoing necrosis and cell death de Haas *et al*. (2008). The release of TNF-α promotes cell apoptosis leading to hepatocyte cell death (Li *et al*., 2020). The CCl_4 significantly increased the level of M65 and M30 in comparison to the normal control (Fig 1). The induction of apoptosis by CCl_4 in this study corroborate the findings of previous studies (Li *et al*., 2020).

On the contrary, the APG doses significantly reduced the levels of M65 and M30 compared to CCl_4 group (Fig 1).

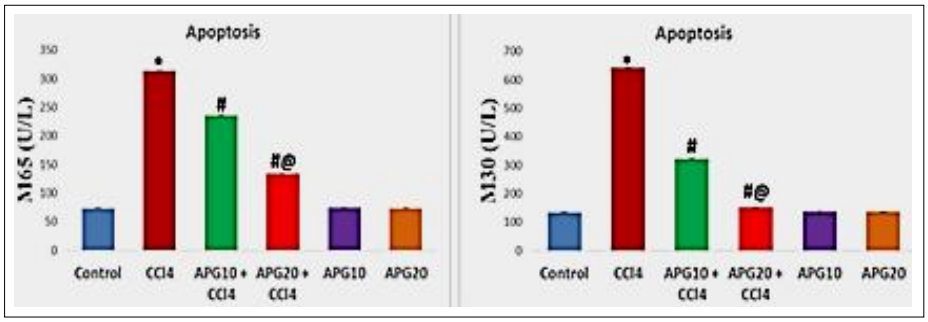

Fig 1: Effect of APG on apoptosis markers in CCl_4-intoxicated rats. CCl_4: carbon tetrachloride; APG: apigenin (where 10 and 20 refer to the dose in mg/kg bw); *p < 0.05: significant when compared to control group. $^{\#}$p < 0.05: significant when compared to CCl_4 group. $^{@}$p < 0.05: significant when compared to APG 10 + CCl_4 group.

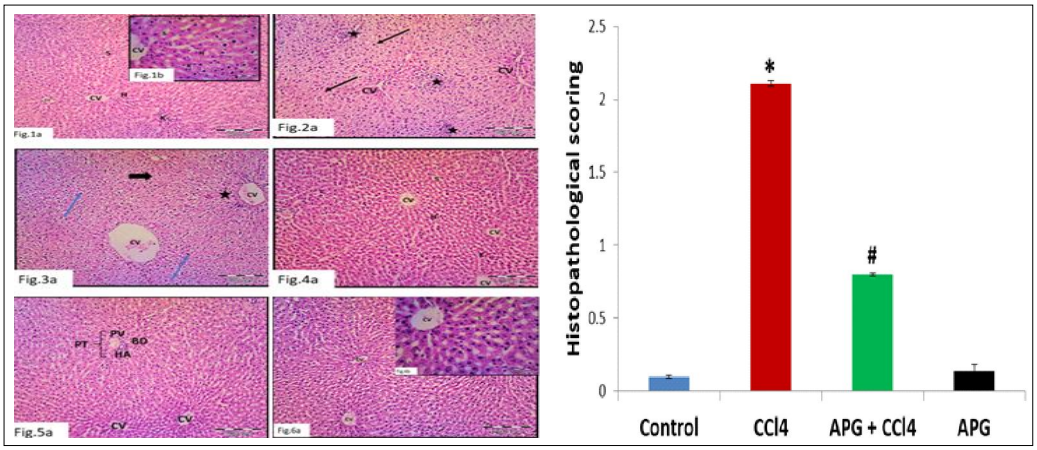

Fig 2: Photomicrograph representation of the effect of APG on liver histology of CCl_4-exposed rats (H and E stain). Control group (1a); CCl_4 group (2a), APG + CCl_4 group (3a and 4a) and APG group (5a and 6a). Control showed normal hepatocytes (H), blood sinusoids (S) and central vein (CV). The liver from CCl_4 group showed infiltration of inflammatory cells (star), severe hepatic necrosis (arrow) and cytoplasmic degeneration. The APG + CCl_4 groups revealed ameliorated structures showing mildly congested central vein (CV), cytoplasmic degeneration (thick arrow), Kupffer cells (K) and Kupffer cellular infiltration (star). APG group showed normal structures consistent with normal hepatocytes (H), Kupffer cells (K), central vein (CV) and blood sinusoid (S). Values are expressed as mean ± SEM (n=5). *Significant when compared to control (p < 0.05); #significant when compared to CCl_4 group.

Interestingly, the two doses of APG revealed a dose-dependent effect on M65 and M30, respectively.

Also, there was dose-dependent antiapoptotic effects of APG as mentioned in previous studies (Mohamed *et al*., 2020 and Zhong *et al*., 2017).

Histopathological analysis

Fig 2 showed that, the control group (1a), the liver architecture appears normal with hepatocytes, blood sinusoids and central vein. On the contrary, the liver histological analysis from CCl_4 group revealed inflammatory cells infiltration (star), cytoplasmic vacuolation and degeneration and severe hepatic necrosis (arrow) (2a). The oxidative milieu created by CCl_4 may be the cause of these histopathologic lesions. The observed infiltration of inflammatory cells in our histopathological analysis could also account for the increased IL-6 and TNF-α levels in this study (Yeh *et al*. 2013).

The administration of APG in the APG + CCl_4 groups ameliorated the CCl_4-induced alterations to mild lesions (3a and 4a). The APG only did not alter the liver structures (5a and 6a).

CONCLUSION

The present study demonstrated the hepatotoxic effect of CCl_4 and emphasized that APG possesses a mechanistic hepatoprotective effect against CCl_4 induced hepatotoxicity via abrogation of oxidative stress, pro-inflammation and apoptosis. Chiefly, these beneficial effects can be attributed to antioxidant and anti-inflammatory activities of APG .

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Conflict of interest

The authors declare no conflict of interests.

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