Original article

Epigallocatechin gallate ameliorates tetrahydrochloride-induced liver toxicity in rats via inhibition of TGFβ / p-ERK/p-Smad1/2 signaling, antioxidant, anti-inflammatory activity

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ABSTRACT

Chronic liver disease is a worldwide health problem. Carbon tetra hydrochloride is an environmental toxin which is regarded as highly toxic and a potential human carcinogen. It can cause liver damage through the generation of metabolites and production of free radicals. Green tea contains catechins such as Epigallocatechin gallate which has been found to reduce the inflammation, oxidative stress, and fibrosis in experimental animal models. Hence, it represents a good source to prevent or ameliorate several chronic diseases. Silymarin is extracted from milk thistle seeds and has been found to be an effective agent to reduce the oxidative stress and free radical production and thereby exert protective effects in chronic liver conditions. The present study was planned to keep in view the above-mentioned facts. We included thirty rats in our study and divided them into five groups, each having six rats and the study continued for 8 weeks. Group I received normal saline; Group 2 received i.p. CCl4 injections; Group 3 received CCl4 i.p. injection and Epigallocatechin gallate (EGCG) oral gavage, Group 4 received CCl4 i.p. injection and silymarin by oral gavage; and Group 5 received CCl4 i.p. injection and combined EGCG + silymarin by oral gavage. The study found that in group 2, CCl4 induced significant elevation of ALT and MDA and reduced GSH thereby signifying increased oxidative stress. CCl4 also significantly increased inflammatory (TNFα, NFκB, IL1β, and TGFβ) as well as fibrotic markers (p-ERK and p-Smad1/2 protein expression). EGCG and silymarin significantly reversed the previously mentioned parameters either alone or in combination; however, the effect was more pronounced in case of EGCG. We conclude that EGCG and silymarin possess liver protective effects through their antioxidant, anti-inflammatory, and antifibrotic action.

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1. Introduction

Chronic liver injury is the foremost etiological factor and a harbinger of liver fibrosis and cirrhosis, leading to morbidity and mortality (Setiawan et al., 2016). The causative liver injury may result from hepatitis, metabolic liver disease, biliary obstruction, and non-alcoholic fatty liver disease (Sánchez-Valle et al., 2012).

The fibrotic changes occur due to increased production and deposition of extracellular matrix (ECM) rich in collagen, which develops into fibrous tissues if not managed properly. Distortion of the liver architecture by the deposition of fibrous tissue leads to cirrhosis, the most predisposing factor for hepatocellular carcinoma (HCC), which in turn may precipitate liver cell failure (Tacke and Trautwein 2015).

The fibrotic activity of the hepatic stellate cells (HSCs) is promoted mainly by the cytokines and reactive oxygen species (ROS).(Cheng et al., 2019). Fibrosis could be stimulated via another

important signaling pathway: transforming growth factor-beta 1 (TGF-β1)/Smad3 via stimulation of ECM-rich collagen I and III production (Duan et al., 2014). Mitigation of TGF-β1/Smad3 signaling may represent an interesting way to attenuate inflammatory and oxidative stress and decrease fibrotic activities.

Natural products have been found to be of great value in many research studies for the treatment and prevention of many induced diseases (Mostafa-Hedeab et al., 2015, Helal et al., 2017),(Mahmoud et al., 2017). Green tea is one of the natural products used for thousands of years that contains many bioactive ingredients rich in flavonoids as epigallocatechin (EGC), epigallocatechin-3-O-gallate (EGCG), and epicatechin-3-O-gallate (ECG), among which EGCG is the most powerful and active compound (Khokhar and Magnusdottir 2002, Wang et al., 2018). Long-term consumption of green tea catechins may have beneficial effects in obesity, type II diabetes and coronary artery disease (Chacko et al., 2010). Several reports demonstrated the beneficial effects of catechins' and their effectiveness against tumor cell growth and neurodegenerative diseases (Higdon and Frei 2003).

In a *meta*-analysis done by (Yin et al., 2015), they observed that green tea consumption was associated with significant protective effects on liver diseases which include a decreased risk hepatic steatosis, hepatitis, cirrhosis, and hepatocellular carcinoma (Yin et al., 2015). In an experimental study on non-obese type 2 diabetic rats, low dose EGCG supplementation reduces the risk of liver injuries as was evident by decreased gene expression for the proinflammatory cytokines and fibrosis-related matrix metalloproteinases (Mochizuki et al., 2019). Green tea polyphenols including EGCG reduce the severity of liver injury by decreasing the expression of iNOS and nitrotyrosine leading to lowered concentrations of inflammatory mediators (Chen et al., 2004). Few researchers have studied the effects of green tea on liver injuries with conflicting results (Cho et al., 2021) (Wang et al., 2018). Green tea products were systematically reviewed by the US Pharmacopeia Dietary Supplement Information Expert Committee and they observed 34 reports of liver damage which included acute hepatitis and fulminant liver failure (Sarma et al., 2008).

Silymarin has long been used as a hepato-protective agent in many parts of the world (Down et al., 1974). In a study done by Tsai et al, silymarin restored the CCl4-induced liver damage and reversed the liver fibrosis which was evident by decreased levels of liver enzymes and reversal of altered expressions of α-smooth muscle actin respectively (Tsai et al., 2008). In another experimental study, silymarin caused upregulation of hepatic anti-oxidative genes like catalase, glutathione peroxidase, and super oxide dismutase genes and reversed the adverse effects of oxidative stress in broiler chickens model (Baradaran et al., 2019). Silymarin is composed of a complex mixture of flavonolignans and flavonoid compounds including silybin, silychristin, isosilybin, silydianin, and taxifolin. All the above mentioned compounds have free radical scavenging properties (Reina and Martínez 2016).

The present study was designed in view of conflicting reports regarding the effects of green tea extracts on the liver. We aim to investigate the actions of EGCG and silymarin against CCl4-induced liver hepatotoxicity by evaluating their anti-inflammatory, antioxidant, and antifibrotic activities.

2. Animal & methods

Thirty Male Wistar rats were incorporated in the current research, weighing 180–200 g, obtained from the Animal House, Medical College, Cairo University (Egypt). Animals were taken care of under normal conditions with temperatures of 23 ± 2 °C with a 12:12-h light:dark cycle. They were allowed for diet and ad libitum. The study protocol was approved by the bioethics committee, Cairo University (Egypt). All animals were kept for two weeks for adaptation, then were classified into five groups, each of which consisted of six animals.

Group 1: received olive oil (1 ml/kg) twice per week by intraperitoneal (i.p.) injection plus a daily oral dose of 1% carboxymethyl cellulose (CMC) for eight weeks and served as a normal control group.

Group 2: was given twice weekly dose of 1 ml of CCl$_4$:olive oil in a ratio of (1:1 v/v) (Hardjo et al., 2009) and 1% CMC was given orally as a single daily dose for a period of eight weeks and served as an untreated group.

Group 3: received 1 ml CCl$_4$ twice weekly and 300 mg/kg EGCG (Lukitasari et al., 2020) in 1% CMC daily and served as an EGCG-treated group.

Group 4: received 1 ml CCl$_4$ twice weekly and (20 mg/kg) silymarin daily (Wang et al., 2018) for eight weeks and served as a silymarin-treated group.

Group 5: received 1 ml CCl$_4$ twice weekly and 300 mg/kg EGCG solvated in 1% CMC daily plus 20 mg/kg silymarin daily oral dose and served as a combined-treated group.

The treatment continued for eight weeks. The CCl$_4$ was given by i.p. injection while silymarin and EGCG were given via oral route by oral gavage.

At the end of experimental period of eight weeks, blood samples were collected and stored for serum separation. Under general anesthesia, all the rats were sacrificed; the livers were removed and washed. Each liver was divided into two halves; one half fixed in 10% formalin for pathological examination and the other half was stored at −80 °C and utilized for molecular study.

2.1. Assay of liver function and proinflammatory markers

The blood samples were manipulated to separate the serum. Serum samples were used to estimate Alanine aminotransferase (ALT), reduced glutathione (GSH), and malondialdehyde (MDA). These activities were analyzed using commercially ready-to-use kits; ALT was measured (Spinreact, Girona, Spain).

2.2. Real-time PCR

Lysis of all the liver samples was done followed by extraction of total RNA and purification by Gene JET Kit (Thermo Fisher Scientific Inc., Germany, #K0732). A 48 well plate StepOne real-time PCR system (Applied Biosystems, Foster City, USA) was used for doing the PCR. Analysis was carried out using Applied Biosystems software version 2. Reverse transcription followed by quantitative PCR amplification with Bioline, a median life sciences Company, U.K. (SensiFAST^{TM}SYBR®Hi-ROX) One-step Kit (catalog number PI-50217 V) was carried out for twenty nanograms of purified RNA obtained from each sample. cDNA synthesis was carried out for 20 min. at 45°C and subsequently reverse transcriptase enzyme inactivation was done for 10 min at 95°C. Furthermore, PCR amplification was done for 40 cycles at 95°C for 10 sec, at 58°C for 30 sec and at 72°C for 1 min. ΔCt method was used to normalize the changes in the expression of each target gene relative to the mean cycle threshold (C.T.) values of the housekeeping gene GAPDH. Table 1. shows all the examined genes with their sequence of primers.

2.3. Western blot

Tissues lysis was carried out in RIPA buffer followed by separation through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The tissue lysates obtained were mixed with lamellae buffer and boiled for 5 min. This was followed by protein separation through SDS-PAGE and subsequent transfer to Immobilon membrane (Millipore). The antibodies used were

Table 1
Sequence of Primers for the Studied Genes.

Gene	Sequence	Gene bank accession number
TNFα	Forward primer 5′- ACTTTGGAGTGATCGGCCCC −3′	NM_000594.4
	Reverse primer 5′- TGGGCTACAGGCTTGTCACT −3′	
NFκβ 1	Forward primer 5′- GCTTAGGAGGGAGAGCCCAC −3′	NM_003998.4
	Reverse primer 5′- GGCAGTGCCATCTGTGGTTG −3′	
TGF	Forward primer 5′- GCCGTGGAGGGGAAATTGAG −3′	NM_000660.7
	Reverse primer 5′- ACCTCGGCGGCCGGT −3′	
IL1β	Forward primer 5′- CCTGAGCTCGCCAGTGAAATG-3′	NM_000576.3
	Reverse primer 5′- ATGGCCACAACAACTGACGC-3′	
GAPDH	Forward primer 5′- TCCTGTTCGACAGTCAGCCG −3′	NM_002046.7
	Reverse primer 5′- CCCCATGGTGTCTGAGCGAT −3′	

anti-Phospho-ERK antibody (Boster Biological Technology, Pleasanton CA, USA, Catalog # P00030) and Smad1/2 antibody (Santa Cruz Biotechnology, Inc. Catalog # sc-7960). Incubation was done in 5% nonfat dry milk, Tris-HCL, 0.1% Tween 20 for 1 hr. This was followed by addition of primary antibodies to the membrane carrying samples and subsequent incubation at 4℃ overnight. A 2-hour incubation was carried out at room temperature for secondary antibodies. The samples are washed twice in n $1 \times$ TBS-T. Quantification of the target proteins was done by the densitometric analysis of the immunoblots against the control sample by β-actin protein normalization using Image analysis software on the ChemiDoc MP imaging system (version 3) produced by Bio-Rad (Hercules, CA).

2.4. Histopathological examination

Fixation of the liver samples was done in 10% formalin for a period of 48 h. This was followed by dehydration of samples achieved by passing through through ascending grades of alcohol. This is followed by removing excess alcohol through clearing by using xylene. The process of embedding was accomplished using paraffin wax. Rotary microtome was used for cutting the sections of 5 μm and then stained with hematoxylin and eosin (H&E). Light microscopic examination of at least three slides were examined from each rat liver specimen.

2.5. Statistical analysis

The Statistical Package of Social Science (SPSS) (version 26) was used to generate results. The normality of the data was tested using the Shapiro-Wilk test. As the data were normally distributed, they were presented as mean and standard error of mean (SEM). For comparison, an ANOVA test was used to compare groups. Post hoc multiple comparisons were made using the Least Significance difference (LSD) test. A p-value of ≤ 0.05 was considered significant.

3. Results

3.1. EGCG improves liver functions in treated rats

We estimate the serum level of alanine transferase (ALT) to evaluate liver function. CCL4-induced hepatotoxicity group

showed significantly increased ALT levels contrary to the control group. The silymarin-treated and EGCG-treated groups depicted significantly decreased levels of ALT when compared to the untreated group. However, the combined group depicted a much more reduction in the ALT levels as compared to the administration of silymarin or EGCG alone (Table 2).

3.2. EGCG treatment improves the antioxidant activities

CCL4-induced hepatotoxicity group depicted oxidative stress as shown by significantly increased Malondialdehyde (MDA) and significantly decreased reduced glutathione (GSH) in contrast to the control group.

EGCG or silymarin administration significantly decreased MDA and increased GSH levels in comparison to the CCl4 group. Co-administration of Silymarin and EGCG significantly restored the antioxidant capacity (Table 3).

3.3. EGCG attenuates the liver inflammatory gene expression via suppression of TNF-α-induced NF-κB axis activation

In the CCL4-induced hepatotoxicity group, inflammatory genes expression (TNFα, NFκB, IL1β, and TGFβ) levels exhibit a significant increase in comparison to the control group.

Significant downregulation of TGFβ TNFα, NFκB, IL1β, and TGFβ gene expression was observed in group 3 and group 4 treated with ECGC or silymarin, respectively.

Combined administration of Silymarin and EGCG in group 5 significantly downregulates the TNFα, NFκB, IL1β an TGFβ gene expressions compared to either of them alone (Fig. 1).

3.4. ECGC downregulates p-ERK and p-SMAD1/2 protein expression

To evaluate the possible anti-fibrinogenic protective effect of EGCG, we estimate the protein expression levels of the phosphorylated ERK (p-ERK) and SMAD1/2 (p-SMAD1/2) through the western blot technique.

CCL4-induced hepatotoxicity group depicted significantly increased protein expression in comparison to the control group.

EGCG treated group or silymarin treated group depicted significantly decreased p-ERK and p-SMAD1/2 protein expression in contrast to the non-treated group.

The combined silymarin + EGCG group depicted significantly attenuated protein expression of p-ERK and p-SMAD1/2 compared to either of them alone or non-treated groups, respectively (Fig. 2).

3.5. Histopathology examination

The liver slides from the control group depicted normal hepatic architecture on histopathological examination (Fig. 3).

Sections of the liver from the CCL4-induced hepatotoxicity rat group depicted hydropic and fatty changes in the hepatocytes as well as hepatocyte necrosis (Fig. 4a). Portal tracts, as well as hep-

Table 2
ALT (U/ml) among studied groups.

Group 1 (normal group)	30.67 ± 2.14
Group 2 (CCL4 group)	105 ± 6.59 *
Group 3 (EGCG treated group)	36.17 ± 1.08 ¥
Group 4 (Silymarin treated group)	53.5 ± 2.63 ¥ €
Group 5 (Silymarin + EGCG treated group)	24 ± 1.81 ¥ € £

ANOVA test followed by LSD as a Post hoc test.
* sig. Compared to the group I
¥ sig. Compared to group 2.
€ sig. Compared to group 3
£ sig. compared to group 4.

Table 3
Antioxidant activities among studied groups.

	MDA (nmol/mL)	GSH (U/mg)
Group 1 (normal group)	0.283 ± 0.026	444.17 ± 21.29
Group 2 (CCL4 group)	2.435 ± 0.036 *	136.5 ± 5.03 *
Group 3 (EGCG treated group)	0.867 ± 0.082 ¥	360.5 ± 7.89 ¥
Group 4 (Silymarin treated group)	1 0.553 ± 0.067 ¥ ∈	299.5 ± 7.37 ¥ ∈
Group 5 (Silymarin + EGCG treated group)	0.382 ± 0.043 ¥ ∈ £	463.5 ± 29.45 ¥ ∈ £

ANOVA test followed by LSD as a Post hoc test.
* sig. Compared to the group I.
¥ sig. Compared to group 2.
∈ sig. Compared to group 3.
£ sig. compared to group 4.

atic parenchyma, showed infiltration by the mononuclear cells and vascular congestion. In addition, fibrosis of the portal tracts and the hepatic parenchyma with the formation of bridging fibrosis was also observed (Fig. 4b).

EGCG treated group liver sections show the presence of normal hepatocyte cords of cells, along with hepatocytes showing fatty and hydropic changes. Mild mononuclear inflammatory cell infiltrates were also seen (Fig. 5).

Sections of the liver from the silymarin treated group depicted the many normal cords of hepatocyte cords and hepatocytes with hydropic and fatty changes. Mild to moderate mononuclear inflammatory cell infiltrate was also observed (Fig. 6).

Sections of the liver from the combined silymarin + EGCG group show nearly normal architecture of liver parenchyma. However, mild mononuclear inflammatory cell infiltrates and vascular congestion is observed (Fig. 7).

4. Discussion

Green tea is an active enriched compound that has antioxidant activities. Here we tested its ability to ameliorate the CCL4-induced liver fibrosis. We also evaluated the antioxidant role of silymarin which has been attributed to its rich flavonoid content.

Oxidative stress is of considerable importance in the genesis of several diseases, including liver injury, as approved in many previous reports (Mahmoud et al., 2017) (Shahataa et al., 2016) (Mohamed et al., 2021). Excessive reactive oxygen species (ROS) production induces hepatocyte injury through membrane lipid peroxidation (Sun et al., 2018). The CCl4 is metabolized within liver cells with formation of highly reactive trichloromethyl and peroxyl radicals, mainly due to cytochrome P450. It then binds covalently to the cellular macromolecules initiating hepatocyte cellular peroxidation (Weber et al., 2003). CCL4, in addition, stimulates the production of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) which in turn mediates the production of ROS in the liver (Cheng et al., 2019). Oxidative stress induces damage to liver via induction of pro-inflammatory genes resulting in tissue injury and ROS liberation (Reyes-Gordillo et al., 2017).
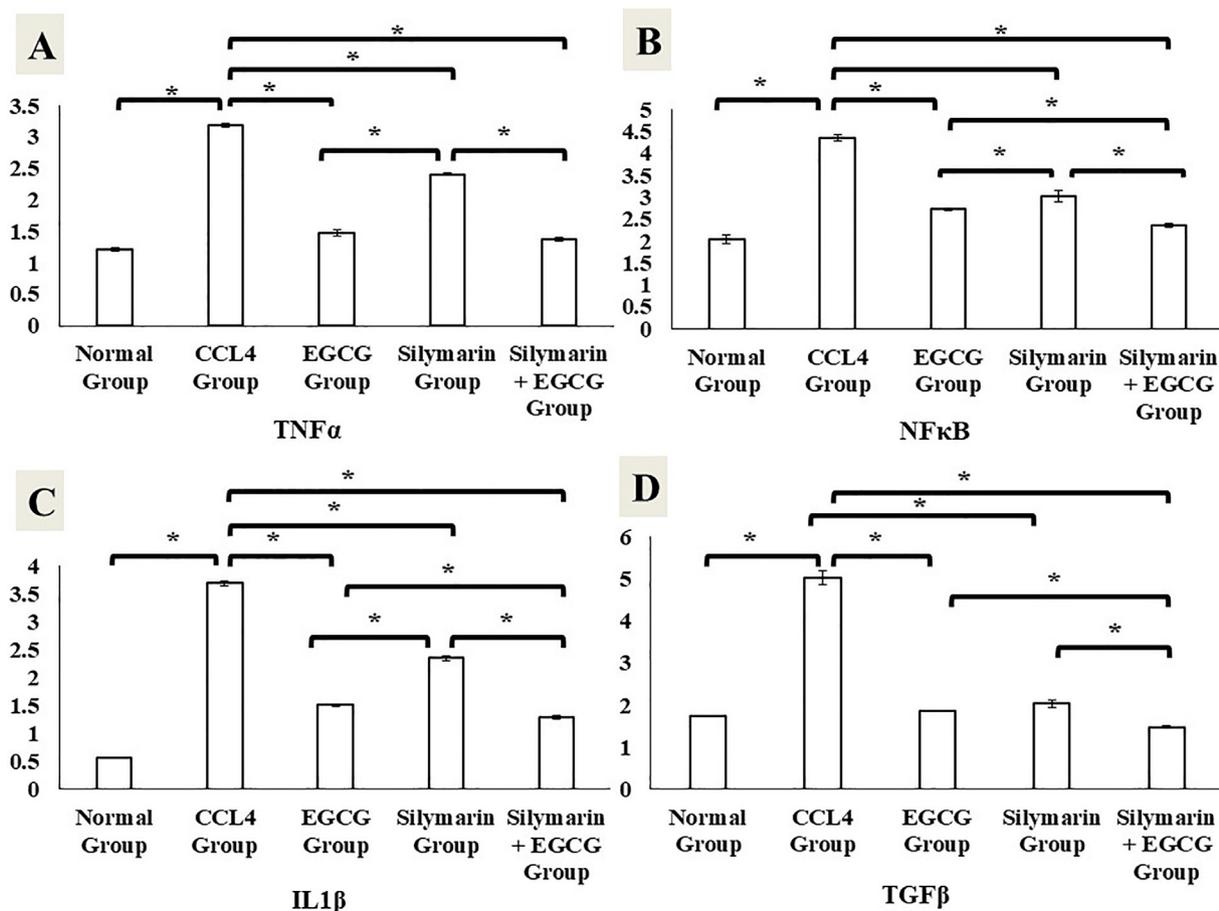

Fig. 1. Liver inflammatory genes expression parameters among studied groups (Gene expression relative to GAPDH (RT-PCR). * Significant differences.- ANOVA test followed by LSD as a Post hoc test.
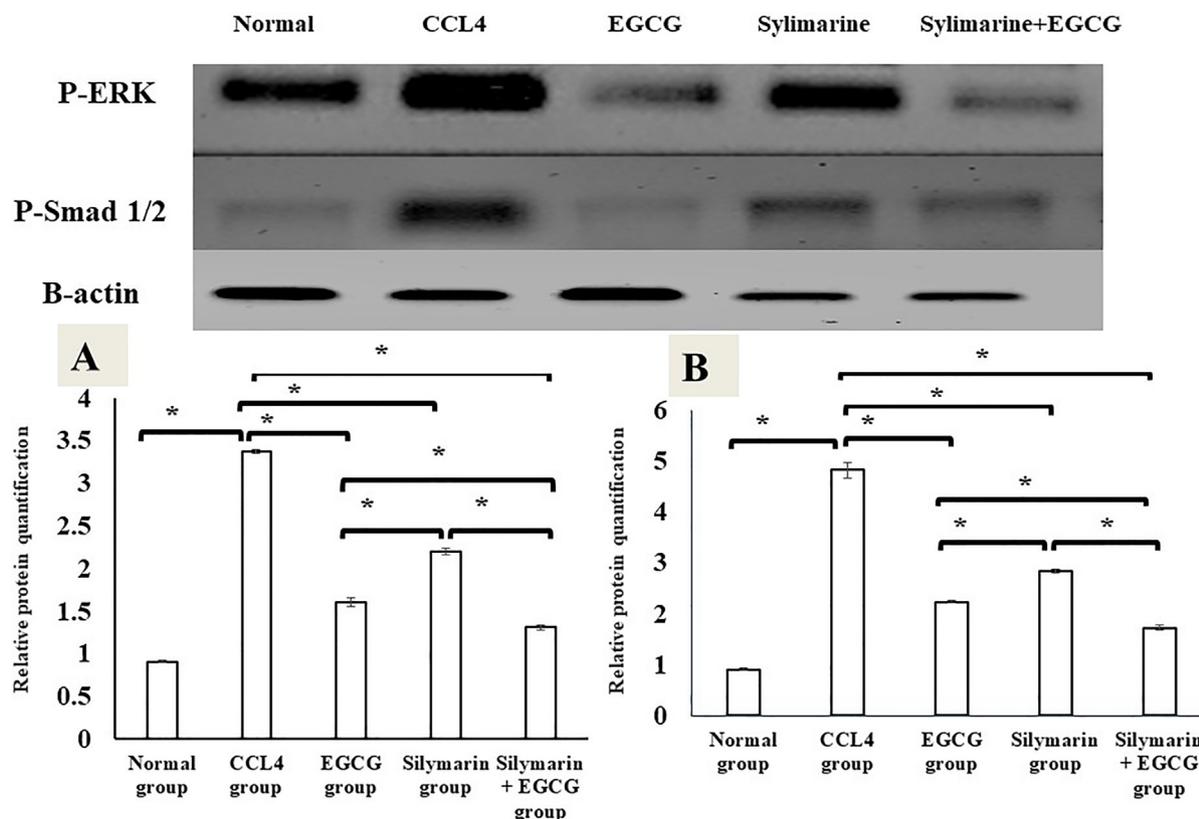
Fig. 2. p-Smad1/2 (A) and p-ERK (B) among studied groupsp-SMAD1/2 and p-ERK was quantified using the western blot technique. * Significant differences.- ANOVA test followed by LSD as a Post hoc test.
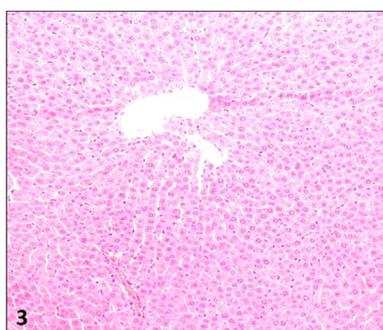

Fig. 3. Representative histological sections of the liver (200 × magnification, H & E stain) from the control group depicting the normal architecture of the liver parenchyma.

Fig. 4a. Representative histological sections of the liver (200 × magnification, H & E stain) from group 2 depicting hydropic changes in the hepatocytes and necrosis of the hepatocytes, especially in the centrilobular areas.

In our study, the liver antioxidant capacity evaluation showed a significantly elevated level of MDA and significantly reduced GSH in the liver of the CCl4-intoxicated rats.

CCl4-induced liver inflammation and subsequent fibrosis are indicated by up-regulation of the major fibrogenic cytokine TGF-β (Thomes et al., 2016).CCl4-derived radicals increased hepatic cellular lipid peroxidation and increased the generation of ROS, which result in significant activation of the NF-κB transcription factor. Once activated, the NF-κB enhances several inflammatory mediators, which include TNF-α, and IL-1 β (Sun et al., 2013). We observed significant elevations of NF-κB transcription factor and other cytokines.

In summary, CCL4 group significantly increased ALT levels, increased MDA and decreased GSH levels when compared to the untreated group. We also observed upregulation of proinflammatory gene expression in the liver tissues. Such deleterious findings
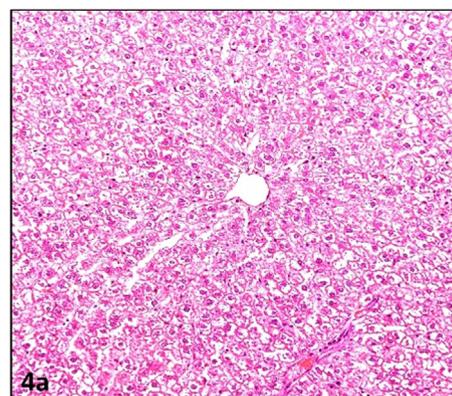
due to CCl4 effects on the liver tissue have been observed by other researchers like Dutta et al 2018, Peng et al 2019, and Ullah et al 2020 (Dutta et al., 2018, Peng et al., 2019, Ullah et al., 2020).

CCL4-induced hepatotoxic changes in the liver include reversible and irreversible changes in the hepatocytes such as hydropic changes, fatty changes, and necrosis of the hepatocytes. CCL4-induced vascular congestion and infiltration by the mononuclear cells. Such changes were observed by Ullah et al., Dutta et al., and Peng et al. in their studies (Dutta et al., 2018, Peng et al., 2019, Ullah et al., 2020). In addition, fibrosis of the portal tracts and the hepatic parenchyma with the formation of bridging fibrosis was also observed. Similar findings were observed by other researchers such as Dutta et al. and Son et al. (Son et al., 2007, Dutta et al., 2018).
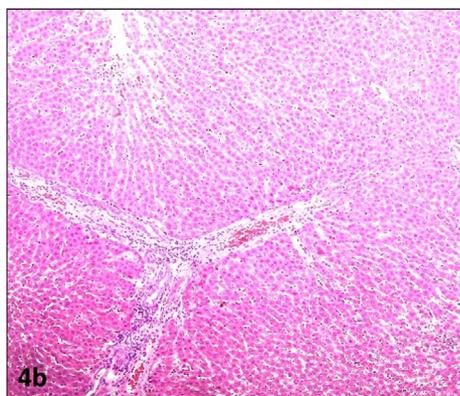
Fig. 4b. Representative histological liver sections (100 × magnification, H & E stain) from group 2 depict bridging fibrosis of the portal tract and infiltration by the mononuclear cells and vascular congestion.
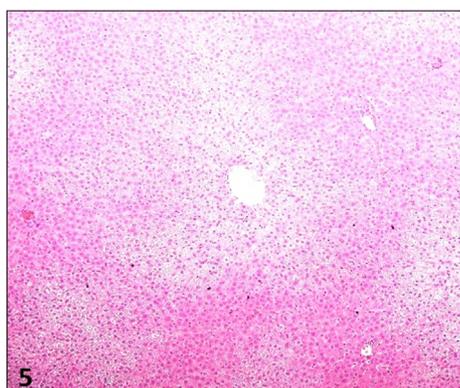

Fig. 5. Representative histological sections of the liver (100 × magnification, H & E stain) from group 3 shows normal hepatocyte cords of cells, hepatocytes with fatty changes and hydropic changes, as well as the presence of mild mononuclear inflammation.
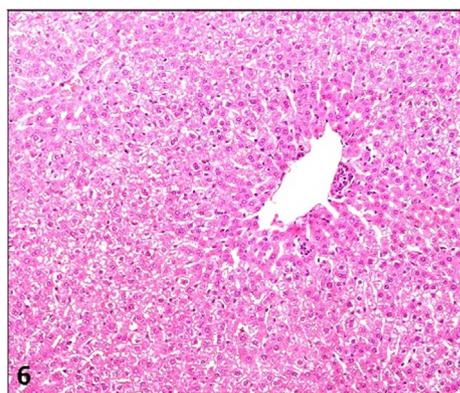

Fig. 6. Representative histological sections of the liver (200 × magnification, H & E stain) from group 4 shows hepatocytes with hydropic and fatty changes with many normal hepatocytes as well as the presence of mild to moderate mononuclear inflammatory cell infiltrate.

We observed that all the altered parameters induced by CCL4-induced liver inflammation were significantly ameliorated by administering EGCG, Silymarin, or its combinations. Together with our free radical scavenging evaluation, such data suggest that catechins principally modified the oxidative stress and its associated abnormalities. A significant inhibition of proinflammatory cytokines (TNF-α, TGF-β) was observed and there was reduction in the products of peroxidative damage (MDA). In all treated groups, gene
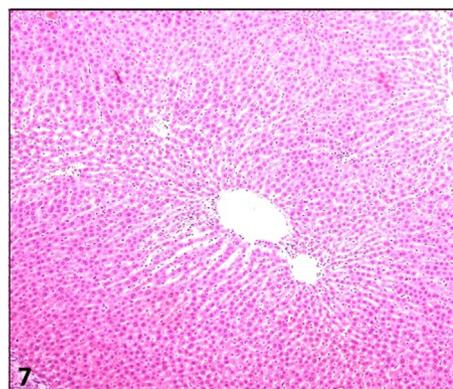

Fig. 7. Representative histological sections of the liver (100 × magnification, H & E stain) from group 5 shows a nearly normal architecture of liver parenchyma. Mild mononuclear inflammatory cell infiltrates and vascular congestion is observed.

expression of the TNFα, NFκB, IL1β, and TGFβ was markedly downregulated.

In contrast to our results was study of Lambert et al.., (Lambert et al., 2010), reported that EGCG can cause hepatotoxicity, increased lipid peroxidation, and inflammatory markers.

The discrepancy between his and our results stemmed from the dose employed; Lambert et al. used larger EGCG doses (1500 mg/kg) and (750 mg/kg), whereas our dose was 300 mg/kg.

Kucera et al., (Kucera et al., 2015) found that in an isolated hepatocyte cell line a greater EGCG dose causes hepatic cellular injury and degradation of liver function, as well as increased generation of reactive oxygen species. These parameters were reversed when a low EGCG dose was used (Kucera et al., 2015). The difference between this result and ours could be related to the fact that they used a different model in which they used a hepatocyte cell line, whereas our experiment was conducted in a rat model.

IL-17 triggers the Kupffer cells to discharge cytokines like IL1β, TNF-α, and TGF-β (Meng et al., 2012). TNF-α results in stimulation of stellate cells and subsequent synthesis of the ECM (Connolly et al., 2009) and inhibits apoptosis of activated HSCs via upregulation of NF-κB and others (Saile et al., 2001). The reduction of TNF-α significantly attenuated liver inflammatory changes, fibrosis, and necrosis, this was approved by Koca et al., in a non-alcoholic steatohepatitis rat model using specific TNF-α antibodies (Koca et al., 2008).

In the current work, EGCG alone or in combination reversed the upregulation of TNF-α gene expression induced by CCl4. Our results run with Sakata et al., who demonstrated the beneficial effect of green tea in improving liver function in a double-blinded controlled trial included Non-alcoholic fatty liver disease (NAFLD) (Sakata et al., 2013).

Leukocytes activation trigger synthesis of cytokines like TNF-α and IL-1. The nuclear factor-κB (NF-κB) signaling pathway activates type II epithelial cells (Pober and Sessa 2007). NF-κB is present in an inactive form attached to NF-κB inhibitor κB(IκB). However, when stimulated by cytokines like TNF-α, it becomes activated through the canonical pathway resulting in N.F.- κB p50/p65 heterodimer activation (Hayden and Ghosh 2014). The NF-κB p50/p65 heterodimer stimulates gene expression of many cytokines as interleukin 6, monocyte chemoattractant protein-1, IL-8, and others (Pober and Sessa 2007). These elements have a vital role in endothelial dysfunction (Zhang 2008). The inhibition of NF-kB will protect against these dysfunctions. A previous report of our lab demonstrated the beneficial effect of inflammatory and oxidant pathway attenuation in the protection of the liver against cardiac and hepatic damage in type-II diabetic rats (Mohamed et al., 2021).

In the present study, significant inhibition of NF-kB by EGCG was observed. This inhibition was followed by a significant attenuation of TNFα, IL1β, and TGFβ. The ability of EGCG to inhibit NF-kB is previously reported by Sing et al. (Singh et al., 2011), indicating EGCG as a good therapeutic agent for treating various diseases (Fang et al., 2019). The anti-inflammatory property of EGCG is related to the plentiful and powerful catechins in green tea. Its anti-inflammatory activity was proved in vitro (Singh et al., 2002), through reduction of TNF and NF-κB (Rasheed et al., 2009). It was found that EGCG inhibits the release of various cytokines, and TGF-β in Osteoarthritis fibroblast-like synoviocytes and monocytes stimulated with calcium crystals (Oliviero et al., 2013).

In our study, the protective effects of silymarin and EGCG were observed histologically with a decrease in the fatty change, hydropic change, necrosis, inflammation, and fibrotic changes. The protective effects of green tea extract which is rich in polyphenols has been reported by Cui et al in an experimental model of liver injury in mice induced by CCl4 (Cui et al., 2014). Protective effects of silymarin and poncirin were observed in CCL4 induced liver injury by Ullah et al. (Ullah et al., 2020).

TGF-β1, through its binding to its membrane receptor, stimulates Smad signaling resulting in increased extracellular matrix (ECM) components (Duan et al., 2014). The role of TGF-β1 suppression in attenuating liver injury or fibrosis has been demonstrated earlier (Lang et al., 2011). The previous report showed that CCl4/diethylnitrosamine in a hepatocarcinogenesis rat model is associated with activation of TGF-β1/Smad3 signaling stimulation (Mahmoud et al., 2017).

Previous reports have demonstrated the Smad2/3 signaling pathway's role in fibrotic changes in various organs (Zeisberg et al., 2007) (Kim et al., 2006). TGF-β receptor type I kinase induces phosphorylation of Smad2/3 resulting in the deposition of type I and III collagen that initiate the process of liver fibrosis. This notion was supported by a report that showed that TGF-β1/Smad2/3 signaling markedly attenuated collagen production (Sun et al., 2017). The TGF-β induces not only Smad2/3 phosphorylation but also induces Smad1 phosphorylation indicating the importance of Smad1 phosphorylation in TGF-β normal transcription functions (Ramachandran et al., 2018). The important role of Smad1 in collagen formation in response to TGF-β is proved. At the same time, Smad2/3 is inhibited by the Smad1 action (Dufton et al., 2017); the profibrotic activity of Smad1 takes place even without the presence of Smad3 activation (Pannu et al., 2007).

We observed an upregulation of TGF-β1 gene expression as well Smad1/2 phosphorylation in the livers of CCL4 group rats. Treatment with either EGCG, silymarin, or its combination attenuates expression of TGF-β1 gene in the livers of CCl4-intoxicated rats. Our results are supported by reports of a study done by Wang et al. (Wang et al., 2018), which showed the fibrotic action of Smad1/2 phosphorylation upregulation in CCl4-induced rats.

ERK is one of the earliest Smad1 nuclear transcription inhibitors (Kretzschmar et al., 1997). In the Smad1 linker domain, ERK phosphorylates the specific residues resulting in reversing BMP/Smad-stimulated transcription. In our study, TGF-β and phosphorylated Smad1 and Smad2 protein expression was significantly attenuated in the livers of the EGCG-treated rats. This proves our hypothesis that the EGCG's protective role is mediated via stimulation of the ERK and Smad1/2 phosphorylation interaction.

5. Conclusions

EGCG and silymarin alone or in combination ameliorated the CCl4-driven liver damage by improving the liver function and reducing the oxidant activity, causing a reduction in MDA and an increase in GSH. Furthermore, expression of TNFα, NFκB, IL1β, and TGFβ genes was significantly reduced. Downregulation of p-ERK and p-Smad1/2 protein expression was noted.

We conclude that EGCG and silymarin offer liver protection through their antioxidant, anti-inflammatory, and antifibrotic actions. The protective effects of EGCG may be related to its phenolic content.

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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