International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 05-01-2022, Accepted: 20-01-2022, Published: 05-02-2022
Volume 7, Issue 2, 2022, Page No. 197-203

Catechin isolated from *Withania coagulans* modulates ethanol-induced inflammatory and apoptosis pathways in zebrafish liver

Y Swarnalatha[1*], S Sivakkumar[2], Juliet[3]
[1] Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India
[2] Department of Gunapadam, National Institute of Siddha, Tambaram Sanatorium, Chennai, Tamil Nadu, India
[3] Siddha Central Research Institute, Chennai, Tamil Nadu, India

Abstract
Among life-threatening ailments, ethanol-induced liver is a major cause of deaths worldwide. The disease progression is a result of the increased permeability of intestine leading to inflammation and hepatotoxicity. Hence, in order to explore the possible cures or interventions to curtail disease progression, the present study evaluated the hepatoprotective and antioxidant activities of catechin (flavonoid) isolated from *Withania coagulans* in zebrafish. Catechin was isolated and purified using column chromatography, and molecular weight was determined through LCMS. Findings showed 0.5% of catechin was effective in hepatoprotective, hepatic antioxidant enzymes uplifting and minimizing inflammatory genes. This is due to membrane integrity and downregulating apoptotic genes. Normal anatomical features of hepatic tissue in the cathechin-treated group confirmed these observations. A high content of catechin in *W. Coagulans* helps improve the rate of recovery from ethanol-induced hepatotoxicity.

Keywords: *Withania coagulans*, hepatotoxicity, hepatoprotective, inflammatory genes, apoptotic genes, hepatic antioxidant enzymes

Introduction
Liver is the one of the largest organs in human body and plays a crucial role in fundamental metabolic functions such as homeostasis, lipids, and proteins and carbohydrates metabolisms. It performs detoxification functions. In alcoholic liver disease, hepatic steatosis is the first and most common consequence of alcohol insult, and, in most of the heavy intake cases, disease develops up to 90-95% (World Health Organization 2014) [1] and as much as 5-20% of alcohol consumers eventually develop severe liver disease that leads to mortality in all most all of those cases. Damage to liver cell causes inflammation that leads to cirrhosis (O'Shea RS *et al* 2010) [2].
Continuous consumption of alcohol can lead to both fibrosis and cirrhosis or at the very least can lead to any one of the two. Fibronectin is a glycoprotein that contributes a major potion to extracellular matrix. Some past studies showed that the deposition of fibronectin increases the risk of fibrosis in the perivenular area of liver. Steatohepatitis occurs because of the relocation of excessive triglycerides to hepatocytes. Ethanol exposure in liver originates from various oxidation mechanisms such as oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress, with all of these later leading to alteration of lipogenic and lipolytic pathways and immunological responses. Furthermore, oxidation mechanisms are also influenced by antioxidant defense systems in cells, of which these are the two main groups: enzymatic and non-enzymatic antioxidants. Multiple antioxidants (SOD, CAT, and GSH) are crucial in the enzyme protection network inside the cell and regulate detoxification, dismutation, and decomposition (Xinxin Huang *et al* 2019). Out of these antioxidant enzymes CAT and GSH are involved in the detoxification process of ROS and act as scavengers that remove superoxide radicals.
Hepatic marker enzymes are a diverse and significant group of biomarkers that can be used to assess liver toxicity; these are ALT (Alanine aminotranferase), ALP (Alkaline phosphatase), and AST (Aspartate aminotransferase) that get released into blood due to liver damage (Qureshi SA *et al* 2019) [4]. The present study's findings help to understand the pathogenesis of hepatic steatosis (Aseervatham Anusha Amali *et al*) and liver injury induced by alcohol and the protective role of Catechin (flavonoid) isolated from *Withania coagulans*. The plant withania coagulans, a medicinal plant, has been proven to be an effective cure for various disease conditions and that it is hypolipedemic and hypoglycemic has also been proven (Rumana Ahmad *et al* 2017) [6]. Hence, based on the findings from studies that focused on antioxidant enzyme activities, hepatic marker enzyme, inflammatory and apoptotic gene expression, histological studies using H&E staining were performed in adult zebrafish.

Methodology
Preparation of plant extract
Flowers of *Withania coagulans* (paneer phool) was collected from siddha central research institute, Chennai. Flowers were soaked in ethanol for five days, and crude ethanol extract was prepared. This crude extract was

used for isolating active compounds using column chromatography (25 to 50 cm); a piece of glass wool was kept at the bottom of the column and packed with silica gel grade of 60-120 mesh size. The stationary phase of column was mixed with methanol to pack properly. *Withania coagulans* crude extract was mixed with a small quantity of silica powder and methanol to prepare slurry that is used as stationary phase. This mixture was loaded onto the column and eluted using a solvent comprising methanol and ethyl acetate (40:60) which works best in isolating flavonoids. As many as 70 fractions were collected and checked for the presence flavonoids. And out of these 70 fractions, 5 fractions showed the presence of flavonoids, that is, in elutes 12 to 17. These fractions were then subjected to LC-MS in order to obtain the molecular weight of active metabolites.

Maintenance of experimental animals

Zebrafish (n=90), wild type were separated into three basic groups and in each group 30 fishes were maintained. The first group was control and named Group 1, and the fish in this group obviously weren't treated; the second group, Group 2, was ethanol-treated (0.05v/v), and Group 3 was treated with the combination of ethanol (0.05v/v) and Catechin (0.5%/L). Ethanol was poured into the water and was replaced every two days along with water. Fish were fed twice a day with fish food for the whole duration of experiment (21 days). At the end of the experimental period, fish were collected, dissected, and their liver was separated and stored for further use.

Assay of enzymatic antioxidants

The enzymatic antioxidant levels were assayed from liver tissue homogenate. Enzymatic antioxidants like reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were also quantified following the standard protocols for all the three experimental groups.

Liver marker enzymes

Liver homogenate was used to assess the liver marker enzymes. Liver marker enzymes like alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) were estimated from tissue homogenate following the methodology reported in King J and king J.

Histopathology of liver samples

A histopathological examination was performed on the liver tissue in all the three experimental groups; liver samples collected from 5 fishes in each group were pooled and were then fixed in 4% paraformaldehyde. The samples were then cryosectioned after embedding them in agarose and were later stained using hematoxylin and eosin.

Gene expression assessment

A Q-PCR analysis was performed using TRIZOL reagent based on manufacturer's protocol, and cDNA was synthesized and quantified using Nanodrop at 260 nm. PCR amplification was run, and gene expression essay was conducted using the formula $2^{-\Delta\Delta ct.}$ Forward and reverse primers were designed for IL-1B, IL-6, IL-10, TNF-α, Caspase3 and 9, Bcl2, and Bax and *Gapdh* was used to normalize the levels (Table 1). Statistical analyses

Table 1: Primer details of apoptotic and inflammatory genes.

S.no	Gene Name	Primer Details	Amplicon length
1	*Caspase3*	F-5'AACATTCTCAGTCTCAGGTT3'	124
		R-5'AAGAACAGAGGCAAGTGAA3'	
2	*Caspase9*	F-5'GCGTCTGATGAACTTGATGC3'	150
		R-5'GTTTTCCTCCAGCACACGAT3'	
3	*Bcl2*	F-5'GATGGCGTCCCAGGTAGATA3'	144
		R-5'AACGGGTGGAACACAGAGTC3'	
4	*BAX*	F-5'ACAGGGATGCTGAAGTGACC3'	112
		R-5'TTTCCATCCAGCTCATCTCC3'	
5	*IL-6*	F-5'TCTGCTACACTGGCTACA3'	104
		R-5'ACATCCTGAACTTCGTCTC3'	
6	*IL-10*	F-5'GAGACCATTCTGCCAACA3'	100
		R-5'TATCCCGCTTGAGTTCCT3'	
7	*IL-1Beta*	F-5'GCTGGAGATCCAAACGGATA3'	137
		R-5'GCTGGAGATCCAAACGGATA3'	
8	*TNF-α*	F-5'CTTCCTCAGACCACGGAAAA3'	101
		R-5'AACCCATTTCAGCGATTGTC3'	
9	*Gapdh*	F-5'AACGGATTCGGTCGCATTGG3'	132
		R-5'GGTGGAGTCGTACTGGAACA3'	

The results obtained were analyzed to derive the mean ± SEM values, and furthermore, a two-way analysis of variance using the Duncan multiple range test was also carried out. Results of experimental groups showed a *p* value of less than 0.001. The *p* values arrived at were highly significant in the case of Group 3 = ***$p<0.001$; **$p<0.01$; *$p<0.05$.

Results

We are confident that the effects of ethanol and the protective role of isolated flavonoid-Catechin were well observed in the present study. Now we discuss our findings in specific areas as given below.

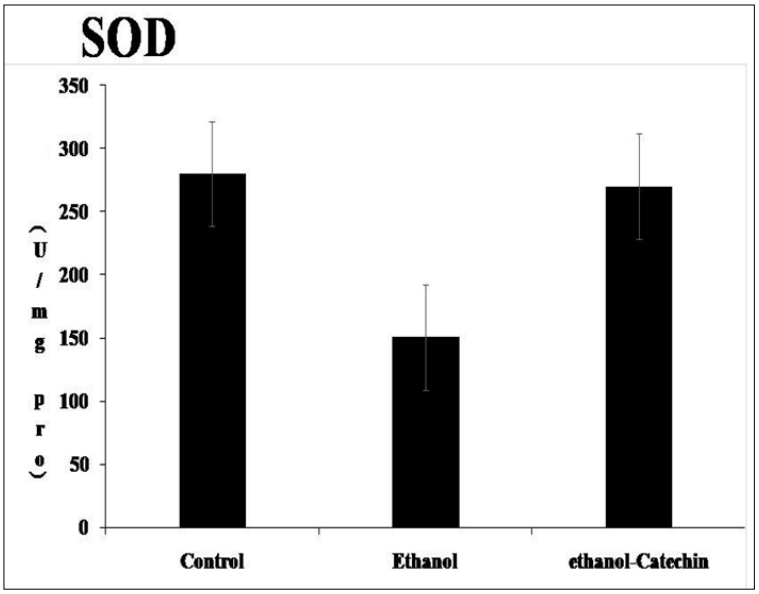
Toxicity studies

Toxicity effect of isolated catechin was studied to fix the drug dosage levels. Catechin was fed to four groups in different concentrations—100 mg, 250 mg, 500 mg, and 1000 mg. We found that till 1000 mg there was no toxicity effect of catechin on zebra fish. Hence, in the present study we chose to use the 500 mg as the optimum concentration level based on the survival rate and morphological changes such as body length and tail length, heart development, and facial edema.

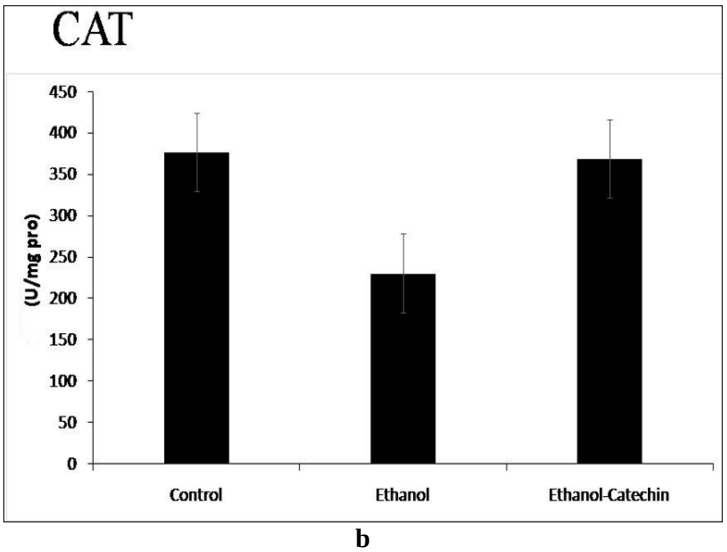
A survival rate of 10% was reduced in group treated with 1000 mg catechin and a death rate of only 2% was observed in the treated group<AQ: Please provide the concentration level here; is it 500 mg?>. Thus, in the present study, a dosage 500 mg concentration was fixed as the optimum concentration level.

Antioxidant activity assay

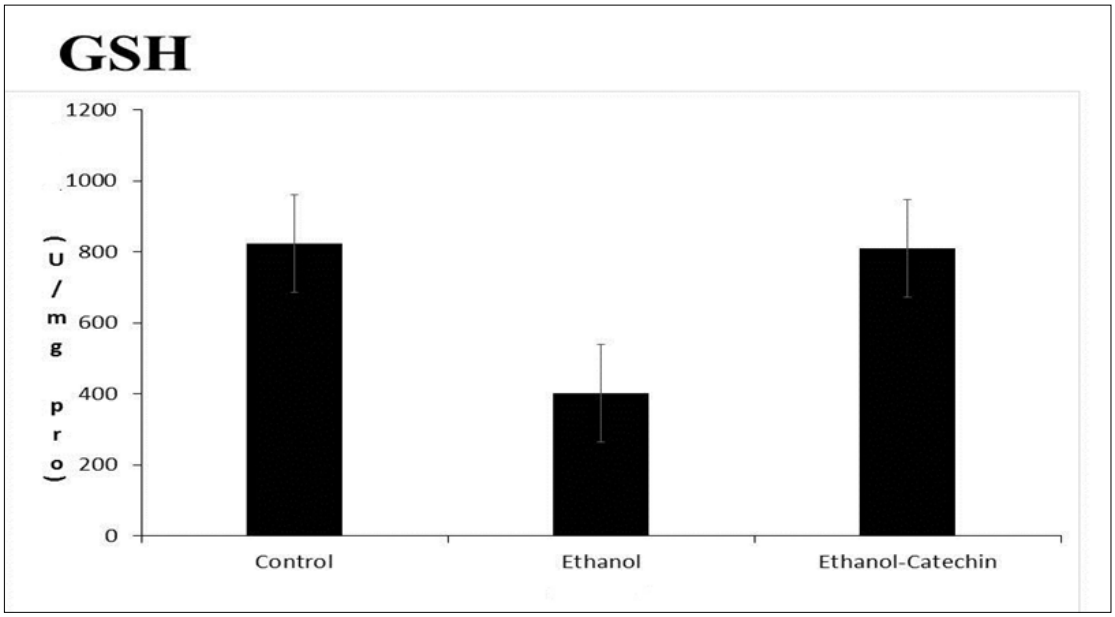
Enzymatic antioxidants like SOD, CAT, and GSH were used as first-line defense to counter the oxidative damage in liver. Fig.1 a-c shows the enzymatic antioxidant levels in the liver tissue for all the three experimental groups. A significant increase in the antioxidant levels were observed in the catechin-treated group than in the ethanol-treated group.



a



b



c

Fig 1abc: Antioxidant enzyme levels.

Hepatic marker enzymes assay

Hepatic marker enzymes are the key enzymes used in diagnosing the liver function. The liver marker enzymes used are AST, ALT, ALP, and LDH. Significantly higher levels of liver marker enzymes in liver tissues indicate the level of hepatocellular damage in group 2. Fig. 2 illustrates the different levels of liver marker enzymes. There was a significant decrease in the liver marker enzymes in the ethanol–catechin treated group compared to the ethanol-treated group.

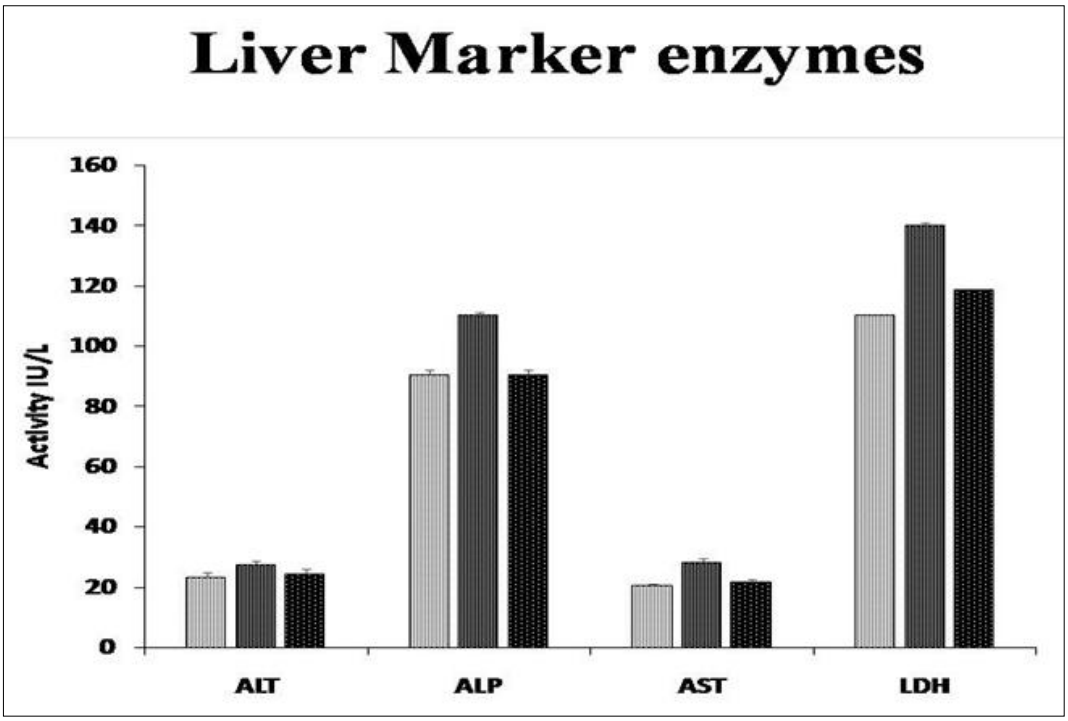


Fig 2: Liver marker enzymes

Histopathological observations

A histological study was carried out using hematoxylin and eosin staining of the liver after 21 days of treatment with catechin and ethanol. In the control group, liver cells showed normal representation, but in the ethanol-treated group hepatic cells showed necrosis with large and small vacuoles of fatty bodies accumulation with disbanding of cytoplasm, where vacuoles pushed the nucleus to one side and left a space for vacuoles to occupy (Fig. 3). This indicates the inflammatory state of the liver when compared to the normal liver of the control group. Whereas in the catechin-treated group, liver cells showed regular architecture, and there was no noticeable inflammation or vacuolization.

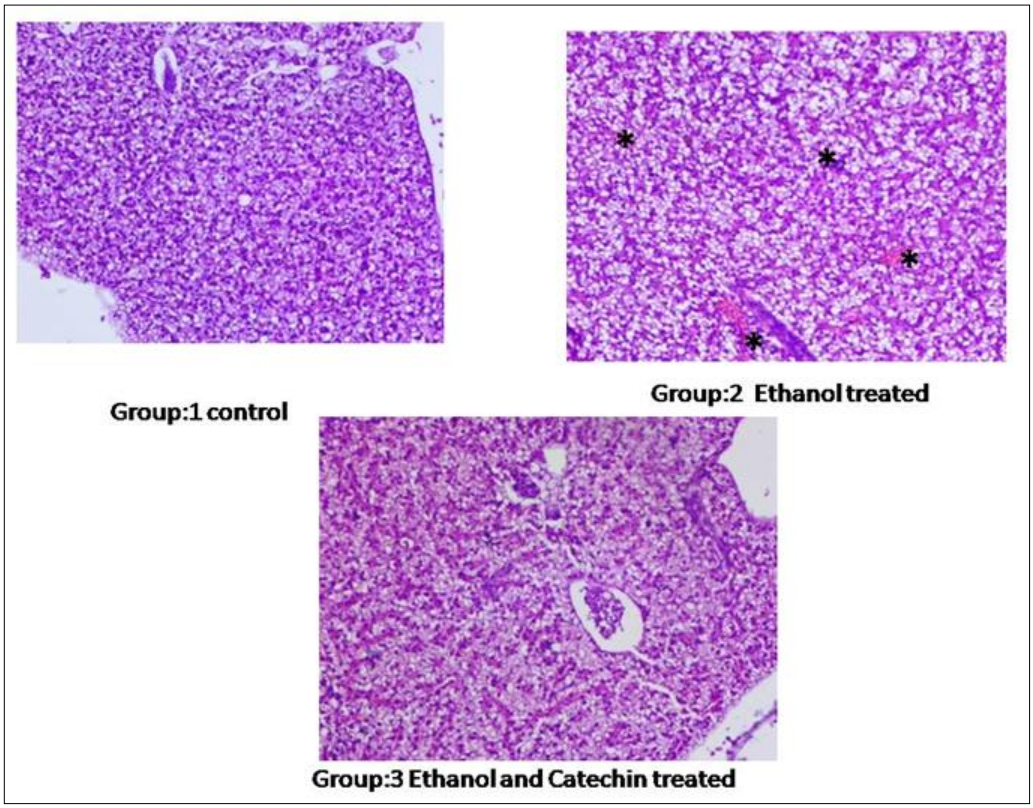


Fig 3: H&E staining of Liver cells.

Gene expression indicating inflammation and apoptosis

The expression levels of Interleukin-1b (IL-1b), Interleukin-6, Interleukin- 10, and TNF- α (IL-1B, IL-6, IL-10, and TNF-α; Fig.4) were significantly increased in the alcohol-induced group compared to the control group. However, in catechin-treated group, inflammatory factors' expression levels were normal. We observed that the inflammatory reactions caused by ethanol were normal when treated with catechin. The transcription levels of caspase 3, caspase 9, and Bcl2/Bax were significantly upregulated in the ethanol-treated fish group. However, in the catechin-treated group the apoptotic gene expression was found to be normal.

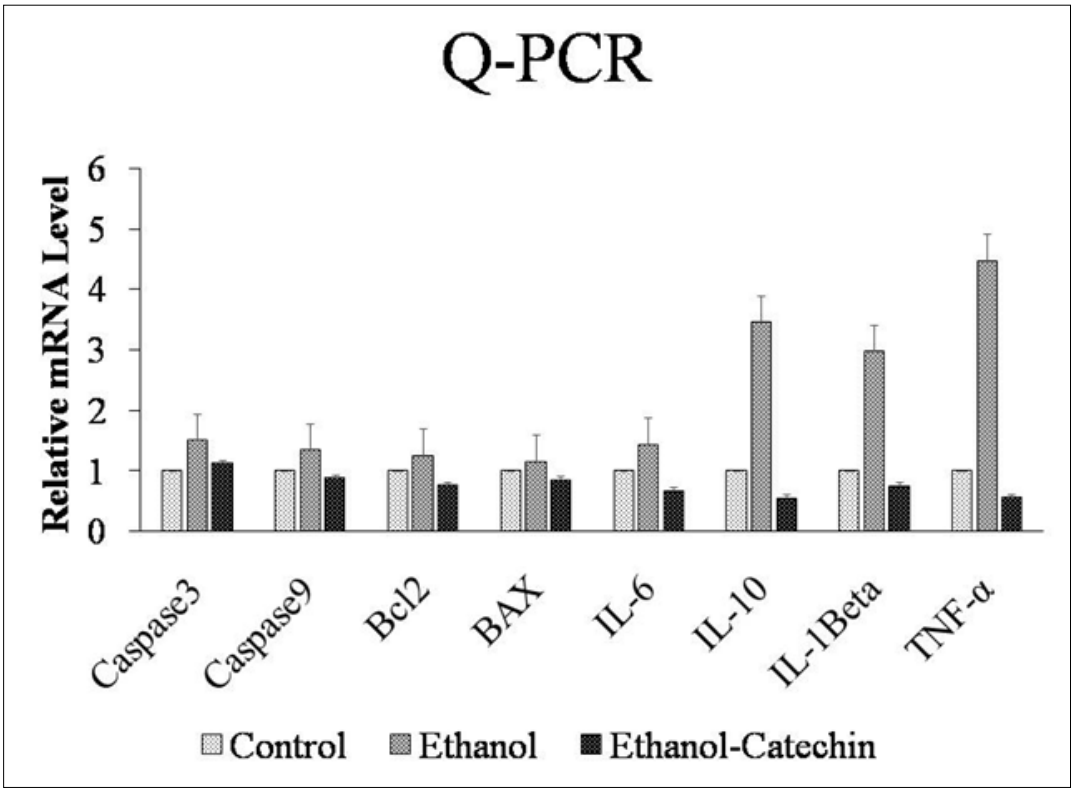


Fig 4: Apoptotic and inflammatory gene expression levels.

Discussion

In the present study, ethanol-induced hepatic metabolic de-arrangements were considered factors that play a major role in the development of hepatic steatosis. We came to this conclusion by studying the findings of biochemical analysis and the molecular and histological changes. Inflammation has been observed to directly result in steatohepatitis. This is because the first line defense mechanism was insufficient to protect the enzymes against oxidative stress.

In the present study, CAT, SOD, and GSH activities of the toxic group were reduced and highly influenced to detoxify ROS produced during the detoxification process. However, in the catechin-treated group, levels of first-line defense enzyme were equal to that of the control group, including inflammatory mediators. This was understood to be clearly a result of the significant levels of decrease in inflammation, apoptotic mediators, and hepatic cell structure normalization.

The ROS generated can also affect normal liver metabolism, as it induces an increase in the levels of hepatic marker enzymes. ALT, AST, ALP, and LDH are important intracellular hepatic marker enzymes that diagnose the quality of liver function. Inflammatory liver injury alters the levels of hepatic marker enzymes as well as the membrane permeability and leakage of marker enzymes. However, in the catechin-treated group, there was a significant decrease in the levels of the hepatic marker enzymes. This is due to the elevated levels of the first-line defense system. Hepatocellular membrane's structural integrity was restored due to the catechin activity, and the hepatic marker enzymes and LDH were observed to be at normal levels in this group. Catechin protects hepatocytes from oxidative damage so that fibrogenesis is reduced by further suppression of inflammation and enhancing significantly the level of xenobiotic detoxification by antioxidant enzymes. Ethanol-induced hepatocelluar toxicity was correlated with histological changes observed for all groups. In this case, we found that hepatocyte structure was destroyed by ethanol through lipid globule accumulation. This is due to the inflammatory (IL-1B, IL-6, IL-10, and TNF-α; Hongtan Wu *et al* 2018) [9] intermediates' infiltration in the central vein, which created the fat vacuoles leading to hepatocyte degeneration and necrosis. Among apoptotic proteins, Bax is the most important pro-apoptotic protein that forms a dimer with Bcl2 to promote apoptosis, and our findings showed that apoptotic factors caspase 3, caspase 9, and Bcl2/Bax were significantly upregulated (except Bax, which was downregulated) in ethanol-treated group. However, in Group 3, the apoptotic gene expression was found to be remarkably low (Bax levels were increased), and this was normalized and positively correlated with antioxidant enzyme levels and the role of catechin as a protective agent. Inflammatory factors and apoptotic factors may be the reason for hepatotoxicity in zebrafish liver in ethanol-treated group. With the treatment of catechin (flavonoid; Chen *et al* 2012, Gao C *et al* 2014, Tian *et al* 2014) [11], liver structure was normalized. We correlated the inflammation-related genes with first-line defense enzymes (Ana Claudia Reis Schneider *et al* 2017), which are well-correlated to prove our findings. Results show ethanol

a. Induced membrane permeability and aggregation of the ROS (Ardiansya H 2007) [14];
b. Significantly increased gene transcription (IL-1B, IL-6, IL-10, and TNF-α); and
c. Subsequently affected the expression of caspase 3, caspase 9, and Bcl2/Bax.

Overall, our study findings show that catechin can be a good solution to treat ethanol-induced hepatotoxicity (Venkatakrishnan K *et al* 2018) [15].

Conclusion

The present study showed ethanol induced inflammation in hepatic tissue and enhanced hepatotoxicity. Ethanol significantly reduced ROS content in the liver in the inflammatory state. Hepatocytes expressed enhanced levels of inflammatory genes and reduced levels of first-line-defense enzyme expressions. Therefore, the ROS produced may influence the intracellular enzymes and lead to a rise in the levels of hepatic marker enzymes. This finding may be significantly relevant to considering natural antioxidants to treat ethanol-induced hepatotoxicty.

Conflict of interest

The authors declare there is no conflict of interest

Funding statement

There is no funding

References

1. World Health Organization. Global status report on alcohol and health. WHO Library Cataloguing-in-Publication Data, 2014. Available from: http://www.worldgastroenterology.org/UserFiles/file/guidelines/probiotics-and-prebiotics-english-2017.pdf.
2. O'Shea RS, Dasarathy S, McCullough AJ. Alcoholic liver disease. Hepatol,2010:51(1):307-328. Available from: doi: 10.1002/hep.23258
3. Xinxin Huang, Yuanyuan Li, Tantan Wang, Hui Liu, Jiaqi Shi and Xuesheng Zhang. Evaluation of the Oxidative Stress Status in Zebrafish (Danio rerio) Liver Induced by Three Typical Organic UV Filters (BP-4, PABA and PBSA) Int. J. Environ. Res. Public Health,2020:17:651.

4. Qureshi SA, Jahan M, Lateef T, Ahmed D, Rais S, Azmi MB. Presence of gallic acid and rutin improve the hepatoprotective strength of Withania coagulans. *Pak J Pharm Sci*,2019:32(Supplementary)):301–308.
5. Aseervatham Anusha Amali, Ravikumar Deepa Rekha, Cliff Ji-Fan Lin, Wei-Lun Wang, Hong-Yi Gong, Gour-Mour Her & Jen-Leih Wu, Thioacetamide induced liver damage in zebrafish embryo as a disease model for steatohepatitis, Journal of Biomedical Science,2006:13:225–232.
6. Rumana Ahmad N. Srivastava Mohsin AliKhan Evaluation of apoptotic activity of *Withania coagulans* methanolic extract against human breast cancer and Vero celllines Journal of Ayurveda and Integrative Medicine, July–September,2017:8(3):177–183.
7. King J. The phosphohydrolases—acid and alkaline phosphatases. In: Practical Clinical Enzymology. London: D. Van Nostrand Co Ltd, 1965a, 191-208.
8. King J. The dehydrogenase (or) oxidoreductase? Lactate dehydrogenase. In: Practical Clinical Enzymology. London: D. Van Nostrand Co Ltd, 1965b, 83-93.
9. Hongtan Wu, Haiyue Pang, Yupei Chen, Lisen Huang, Huaxin Liu, Yongbiao Zheng, *et al*. Anti-Inflammatory Effect of a Polyphenol-Enriched Fraction from Acalypha wilkesiana on Lipopolysaccharide Stimulated RAW 264.7 Macrophages and Acetaminophen Induced Liver Injury in Mice, Oxidative Medicine and Cellular Longevity, Article ID 7858094, 2018.
10. Chen J. *et al*. Studies of the protective effect and antioxidant mechanism of blueberry anthocyanins in a $CC1_4$-induced liver injury model in mice. Food Agr. Immunol,2012:3:352–362.
11. Gao CY, Tian CR, Zhou R, Zhang RG, Lu YH. Phenolic composition, DNA damage protective activity and hepatoprotective effect of free phenolic extract from *Sphallerocarpus gracilis* seeds. Int. Immunopharmacol,2014:20:238–247.
12. Tian LM. *et al*. Chemical composition and hepatoprotective effects of polyphenol rich extract from *Houttuynia cordata* Tea. J. Agric. Food Chem,2012:60:4641–4648.
13. Ana Claudia Reis Schneider, Cleandra Gregório, Carolina Uribe-Cruz, Ranieli Guizzo, Tais Malysz, Maria Cristina Faccioni-Heuser, Larisse Longo, Themis Reverbel da Silveira, Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver, World J Hepatol,2017:9(8):418–426.
14. Ardiansyah, Indrayani Natural Antioxidants Dietary and Lipid Oxidation Analysis in Zebrafish (*Brachydanio rerio*) Tissue HAYATI Journal of Biosciences, September 2007, 87–92
15. Venkatakrishnan K, Chiu HF, Cheng JC, *et al*. Comparative studies on the hypolipidemic, antioxidant and hepatoprotective activities of catechin-enriched green and oolong tea in a double-blind clinical trial. Food Funct,2018:9(2):1205-1213.