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Carvacrol inhibits cytochrome P450 and protects against binge alcohol-induced liver toxicity

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ABSTRACT

Alcoholism is a serious addiction that can lead to various health complications such as liver fibrosis, steatosis, and cirrhosis. Carvacrol is present in many plant-based essential oils and used as a preservative in the food industry. In this study, we have investigated the hepatoprotective role of carvacrol against ethanol-induced liver toxicity in mice. To determine the effect of carvacrol on liver injury parameters, 5 doses of 50% ethanol (10 mL/kg body weight) were orally administered every 12 h for inducing the hepatotoxicity in experimental mice. Interestingly, carvacrol pre-treatment (50 and 100 mg/kg) reversed the ethanol-induced effects on liver function, antioxidant markers, matrix metalloproteinases activities, and histological changes. Moreover, carvacrol binds to the active pocket of cytochrome P450 (Cyt P450) and inhibits its expression. Thus, our finding suggests carvacrol can be used as an adjuvant for the amelioration of alcohol-induced hepatotoxicity.

1. Introduction

Drinking alcohol is part of social customs and etiquette (Sudhinaraset et al., 2016). Alcohol has been traditionally used for medicinal, religious, as well as social purposes (Egea et al., 2016). However, drinking alcohol causes inflammation in various organs such as liver, kidney, brain, and within the reproductive system (Barve et al., 2017). After absorption in the gastrointestinal tract, alcohol is directed to various organs with the majority sent to the liver, further inducing metabolic and functional changes (Chowdhury and Gupta, 2006). The liver is vital to the detoxification process for the removal of toxic and other waste material present in the body (Allen et al., 2011), and it also helps maintain metabolic homeostasis. An imbalance in metabolic reactions leads to the expression of various hepatic metabolism genes and to steatosis, which mediates accumulation of liver fat (Rasineni and Casey, 2012). Furthermore, steatotic liver can lead to chronic steatosis, cirrhosis, and, finally, to hepatocarcinoma (Veteläinen et al., 2007). It is important to the pathophysiological and molecular aspects in liver hepatic steatosis. Various signaling pathways play crucial roles in defining the inflammation induced by alcohol intoxication.

The family of mitogen-activated protein kinase (MAPK) is associated in the majority of signaling pathways (Lawan and Bennett, 2017). The MAPK family comprises extracellular signal regulated kinases 1 and 2, p38, and the c-Jun NH_2 terminal kinase. Various cellular stressors and external stimuli trigger the activation of the MAPK pathway which leads to lipid deposition in hepatocytes (Roseet al., 2010). Previous research revealed that hepatic steatosis was reduced in mice expressing JNK2 but not JNK1 (Singh et al., 2009; Czaja, 2010). The MAPK p38 protein is involved in the fatty acid β-oxidation pathway that is crucial for lipid metabolism in hepatocytes (Flach et al., 2011). Furthermore, oxidative stress also plays a crucial role in alcohol-induced inflammation as well as liver steatosis (Mandrekar and Szabo, 2009). The acute and chronic alcohol-induced inflammations have been found to have high oxidative bursts that enhances the oxidation of DNA, proteins, and lipids (Wang et al., 2012). Although numerous researches have conducted research on alcohol-induced inflammation previously, more detailed molecular studies about this pathophysiological condition are needed. Identifying a phytochemical that has anti-

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inflammatory properties with little or no side effects is one such area requiring further investigation (Owolabi et al., 2018).

The treatment of hepatic illnesses has an extended history, and this is a pioneering field of study by employing traditional therapeutic plants and their various phytochemicals against hepatic toxicity. All the medicinal plants, as well as the ingested food material, have established diverse effects on living systems. Although there have been miscellaneous studies directed towards the assessment of hepatoprotective activity of phytochemicals, most of investigations have been directed towards their antipyretic, antibacterial, anticancer and antiviral activities. Overall, liver-protective medicinal plants possess a diverse range of phytochemicals, such as phenols, monoterpenes, flavonoids, etc. (Madrigal et al., 2014). Moreover, different phytochemicals are used to reduce acute or chronic inflammatory injuries. In this study, we have chosen to evaluate the anti-inflammatory effect of carvacrol on alcohol-induced inflammation in mice. Carvacrol (2-methyl-5-(1-methylethyl)-phenol) is a monoterpenoid with various biological properties, including strong antibacterial, anticancer, and antioxidant properties (Baser, 2008; Khan et al, 2017, 2018b; Khan et al., 2018a). In support to this, carvacrol is known to have anti-inflammatory potential against thioacetamide-induced liver injury in rats (Hussein et al., 2017), and attenuates N-nitrosodiethylamine-induced liver injury in rats (Rajan et al., 2015). Recently, Mohebbati et al. (2018) also reported the hepatoprotective effect of carvacrol in a rat experimental model.

In this study, we investigated whether carvacrol has hepatoprotective activity against alcohol-induced liver inflammation in mice. We evaluated the decreased levels of biochemical parameters which were elevated by the alcohol intoxication. Moreover, the elevated matrix metalloproteinases (MMP)-2, and −9 levels were also found to be normalized after carvacrol treatment. We also demonstrated that carvacrol has ability to reduced cytochrome P450 (Cyt P450) in a dose-dependent manner, which was enhanced by alcohol intoxication, thus carvacrol protecting the alcohol-induced liver inflammation.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in this study were of superior analytical grade. Carvacrol, dimethyl sulfoxide (DMSO), eosin, hematoxylin, and tetramethylbenzidine (TMB) were obtained from Sigma commercial source, St. Louis, USA.

2.2. Experimental animals

Adult male ICR mice (6 weeks, 24–26 g) were housed under standard conditions of animal maintenance at 24 ± 1 °C with 55% relative humidity, and an alternate 12 h light/dark cycle. Animals were fed on water ad-libitum and standard pellet diet. Animals described as fasted had only free access to water but were deprived from food for 24 h. Animal experiments were approved by the animal ethical committee of Daegu University, Republic of Korea and were performed in accordance with national guidelines and regulations. Procedures involving animals and their care conformed to institutional guidelines that comply with national and international laws and policies.

2.3. Experimental design

The mice were randomly divided into 5 groups with 8 mice each. Group-I mice, termed as vehicle control (control) were orally fed on water. Group-II mice (ethanol control, ethanol) were, on day 6, intragastrically administered with 5 doses of 50% ethanol (10 ml/kg) at 12 h intervals between each dose. Group-III mice (ethanol + carvacrol [50 mg/kg], (CA-50) were orally administered 50 mg/kg carvacrol for 3 consecutive days; on day 4, the mice were administered 5 doses of 50% ethanol (10 ml/kg) at 12 h intervals between each dose. Group-IV mice (alcohol + carvacrol [100 mg/kg], CA-100) were orally administered 50 mg/kg carvacrol for 3 consecutive days; on day 4, the mice were administered 5 doses of 50% ethanol (10 ml/kg) at 12 h intervals between each dose. A dose of 100 mg/kg of silymarin was orally administered to the mice of group-V [alcohol + silymarin (SL)] for 3 consecutive days and, followed by intragastrically administration of 5 doses of 50% ethanol (10 ml/kg) at 12 h intervals between each dose, starting on day 6. The selection of the administration of carvacrol and ethanol doses was made based on the pilot study. A previously reported method of Koneru et al., (2016) was adopted for inducing alcohol-mediated acute liver injury in animals.

2.4. Determination of the levels of liver injury biomarkers

The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), alkaline phosphatase (ALP), total protein (TP), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) were estimated using commercially available diagnostic kits (BioVision, Inc., Milpitas, CA).

2.5. Determination of MPO, NO, SOD, and GSH

The levels of myeloperoxidase (MPO), nitric oxide (NO), super oxide dismutase (SOD), and reduced glutathione (GSH) were determined using commercially available kits purchased from Sigma-Aldrich, USA.

2.6. Gelatin zymography

A previously described method reported by Ganguly et al. (2005) was adopted for the analysis of gelatin zymography. In brief, RIPA buffer was used for the preparation of tissue homogenate. SDS-PAGE of the tissue homogenate was performed with gel containing gelatin (1 mg/mL). Next, the gel was subsequently washed with Triton X-100 (2.5%) and maintained in the developing buffer overnight at 37 °C. Finally, the gel was stained with Coomassie brilliant blue for 20 min and, after de-staining, a picture of the gelatin bands were taken.

2.7. In situ zymography

To estimate the localization of gelatin in tissue, we performed *in situ* gelatin zymography as described by Hadler-Olsen et al. (2010) with some modifications. DQ gelatin zymography is a sensitive technique used to visualize gelatin in tissue. The highly fluorescent green color observed and measured after quenching informs on gelatinase enzyme activity. Tissue sections were embedded in paraffin, further sectioned (5 mm thick) on a cryostat microtome, followed by incubation at 60 °C overnight followed by incubation of the sections with DQ gelatin (40 mg/mL) for 2–3 h at 37 °C under CO_2 condition. Finally, the tissue sections were washed with PBS, and visualized with a TS 100, NIKON fluorescent microscope (Japan).

2.8. Histopathological examination

For histopathological analysis, tissue sections were fixed in 10% formalin PBS buffer followed by alcohol-mediated dehydration. Further, tissues were sectioned, and tissue staining was performed using hematoxylin and eosin (H & E) stains.

2.9. Immunohistochemistry

Tissue liver sections (10 μm) were de-paraffinized followed by 10 min incubation in 3% H_2O_2 in order to reduce the activity of endogenous peroxidase. In brief, after blocking for 20 min, tissues were incubated overnight with the primary polyclonal rabbit antibody raised against Cyt P450 (1:100, Bioworld) at 4 °C. Next, the sections were washed with PBS and then incubated with secondary goat anti-rabbit

antibody at 37 °C for 30 min. Visualization of binding sites of the antibody was performed by incubating the sample with DAB-substrate for 10 min at room temperature.

2.10. Immunoblotting

Homogenized tissues and/or mitochondria were subjected to sonication using an ice-cold RIPA lysis buffer containing a cocktail of protease inhibitor followed by centrifugation of the lysates (10,000 *g*; 4 °C). Then, the supernatant was collected and the contents of protein in the supernatant were determined employing the bi-cinchoninic acid (BCA) method. Equal amount of protein (100 μg) was loaded on polyacrylamide gel (10%), and bands were separated by SDS-PAGE followed by transferring to PVDF membrane. Blocking of membrane was performed at room temperature for 1 h in a TBST buffer solution of 5% bovine serum albumin (BSA) followed by membrane incubation at 4 °C overnight with the primary antibody in the same TBST-BSA buffer solution. Detection of specific proteins was performed using the primary antibody at an appropriate dilution ratio.

2.11. In silico analysis

For *in silico* analysis, different modules of Schrodinger software (Schrödinger, LLC, New York, 2017) were used to execute molecular docking and to analyze the interaction between carvacrol and hemoprotein Cyt P450.

2.11.1. Retrieval and preparation of protein and ligand structures

Three-dimensional structure of Cyt P450 was obtained from the RCBSPDB database (https://www.rcsb.org/). Prior to docking obtained protein was optimized for docking by the Protein Preparation Wizard (Schrödinger Release, 2017–4: Schrödinger Suite, 2017–4 Protein Preparation Wizard; Epik; Impact; Prime, 2017).

2.11.2. Prediction of binding site and molecular docking

For the prediction of active site, Schrodinger application Sitemap was used in the Cyt P450 hemoprotein (Schrödinger Release, 2017–4: Sitemap, 2017). Moreover, for validating binding energy, we followed module of Schrodinger, Glide Ligand Docking (Schrödinger Release, 2017–4: Glide; 2017). For entire receptor structure, we selected grid for Glide Ligand docking. Binding affinity was also calculated with Schrodinger application Prime-MMGBSA (Schrödinger Release, 2017–4: Prime, 2017). The calculation of binding affinity was done with the following equation:

ΔG(bind) = E_complex (minimized) − [E_ligand (minimized) + E_receptor (minimized)]where E_complex is the minimized total energy in complex, E_ligand is the minimized energy of the ligand and E_receptor is the minimized energy for the receptor.

2.12. Statistical analysis

Mean SD values were statistically analyzed using a Student's t-test, while value of $p < 0.05$ was regarded as significantly different. Significance of the statistical analysis was confirmed by Student's t-test at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. Results

3.1. Effect of carvacrol on histopathological changes

First, we determined the liver-to-body weight ratio, which was found to be suppressed in the carvacrol-treated group, in contrast to the ethanol-intoxicated group, at 100 mg/kg carvacrol (Fig. 1A) ($p*** < 0.001$) and similarly in the silymarin group ($p*** < 0.001$) compared to the ethanol. For the histopathological examination, we performed H & E staining (Fig. 1B). In an alcohol-treated group, we found hemorrhagic lesions and distorted morphology as compared to the control. Moreover, micro-steatosis was observed in the alcohol-treated group. However, in the carvacrol-treated group, the damage caused by alcohol decreased in a dose-dependent manner. In addition, periodic acid-Schiff (PAS) staining revealed glycogen accumulation in the ethanol-intoxicated and carvacrol-treated groups. The glycogen content in serum decreased in a dose-dependent manner as depicted in Fig. 1C. Furthermore, we determined that the triglyceride (TG) content was significantly lower in the carvacrol-treated group (100 mg/kg) in contrast to ethanol-intoxicated group ($p*** < 0.001$) and similarly in the silymarin group ($p*** < 0.001$) compared to the ethanol (Fig. 1D).

3.2. Carvacrol affects liver injury parameters

The AST, ALT, ALP, TP, TNF-α, and IL-1β (Fig. 2A–F) levels were significantly higher in the alcohol-treated group as compared to the control (untreated) group. However, the alcohol and pretreated-carvacrol (50–100 mg/kg) groups showed lower levels of the above-mentioned parameters ($p*** < 0.001$). Moreover, the silymarin-pretreated group (50 mg/kg) showed significantly lower levels of the above-mentioned parameters.

3.3. Carvacrol binds to Cyt P450 and inhibits its expression

Cyt P450 plays a crucial role in alcohol-induced liver inflammation. It is involved in the detoxification of many metabolites; however, it also causes adverse reactions. Thus, we evaluated the levels of Cyt P450 in the groups treated with ethanol and carvacrol. Immunohistochemistry analysis revealed that the Cyt P450 expression decreased in a dose-dependent manner in contrast to the ethanol-intoxicated group (Fig. 3A and B). These results were further validated by immunoblot analysis, which also showed the dose-dependent inhibition of Cyt P450 in the carvacrol-treated group similar to that of the silymarin group ($p*** < 0.001$) compared with ethanol treated group (Fig. 3C and D). *In vitro* results also supported these findings, revealing a high docking score and strong binding affinity for the docking interaction between Cyt P450 hemeprotein and carvacrol. Eighteen carvacrol docking poses exhibited the lowest binding energy (−6.87 kcal/mol) and depicted a crucial interaction with the active pocket of Cyt P450 (Fig. 4A, B, and C). Furthermore, we estimated a binding capability of −26.03 KD, and a two-dimensional diagram indicated a hydrogen bond formation (Fig. 4D and E) between asparagine 228 and glutamine 332 with the minimum distance between the residues ranging from 2.07 Å to 2.15 Å. The additional *in silico* properties are provided in Table 1 and Table 2.

3.4. Carvacrol affects the level of liver antioxidant enzymes

The major culprit of alcohol-induced inflammation is oxidative stress. As shown in Fig. 5, an imbalance in antioxidant enzymes was observed after alcohol treatment in the mice. The elevation of MPO and NO levels due to alcohol (Fig. 5A and B) was reversed by treatment with carvacrol ($p*** < 0.001$). The reduction in the antioxidant enzyme levels was reversed in the carvacrol-treated group ($p*** < 0.001$) (Fig. 5C and D). Moreover, the levels of antioxidant enzymes such as catalase, GR, SOD1, and Nrf-2 (Fig. 5E and F) that were reduced after treatment with alcohol were improved with carvacrol treatment. These results indicate that carvacrol attenuates oxidative stress.

3.5. Effect of carvacrol on matrix metalloproteinase (MMP) content

The release of MMPs after alcohol intoxication is indicative of damage caused by the substance to liver hepatocytes. *In situ* gelatin zymography was used to evaluate the MMPs secreted after alcohol intoxication (Fig. 6A) and depicted a decrease in the levels of MP-2 and -9 (Fig. 6B). These results were further validated by immunoblot analysis (Fig. 6C). MMP secretion from damaged hepatocytes was higher in the

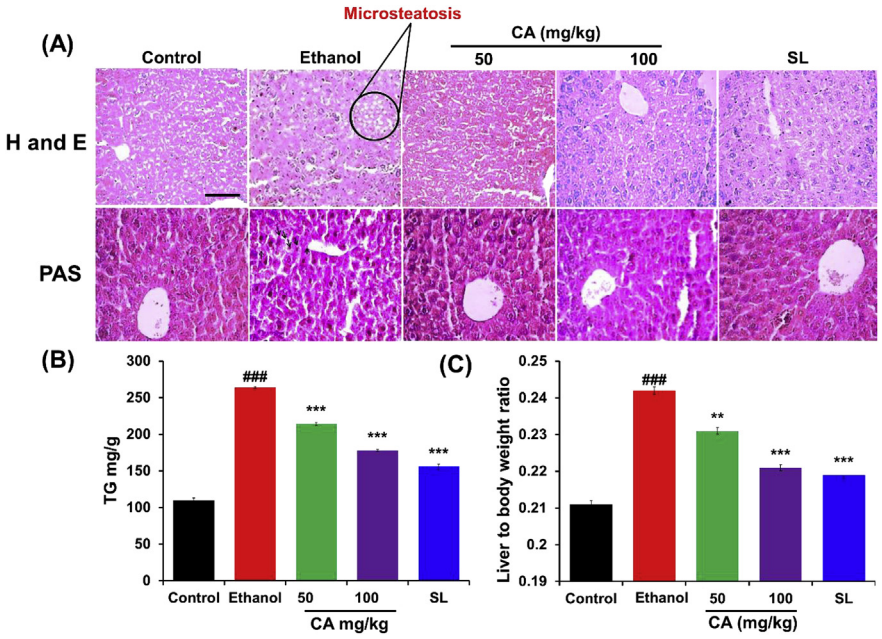

Fig. 1. A) H & E staining and light microscopic examination of liver tissue from carvacrol- or alcohol-treated mice. B) PAS staining for hepatocellular glycogen storage (glycogen in red). C) The graph represents the %TG content. D) Liver-to-body weight ratio. The data are represented as the mean ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

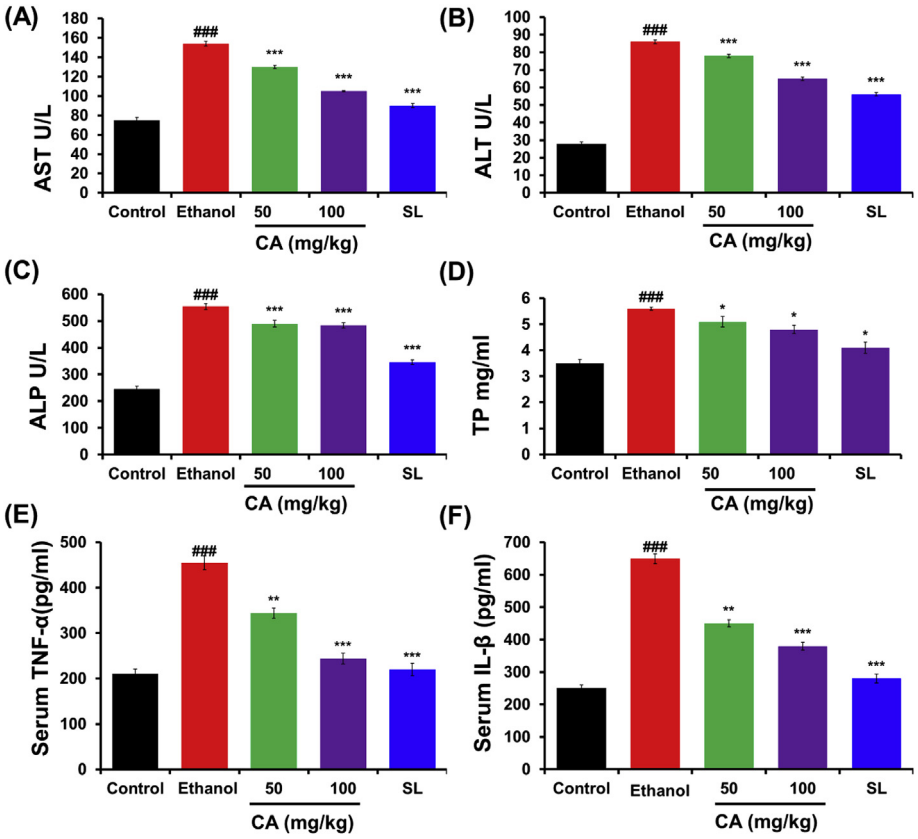

Fig. 2. Effect of carvacrol on alcohol-induced liver injury to biochemical markers (A) AST, (B) ALT, (C) ALP, (D) TP, (E) Serum-TNF and (F) Serum-IL-1β. Bar diagrams are depicted as the means ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol.

ethanol-intoxicated group; however, the MMP levels had decreased in a dose-dependent manner in the carvacrol-treated group ($p < 0.05$).

3.6. Effect of carvacrol on MAPK pathway and inflammatory markers

Inflammatory markers were found to be elevated in the alcohol-treated group, including p-JNK, p-ERK, p-P38, p-AKT, iNOS, TNF-α, eNOS, nNOS, and NFκB 65 (Fig. 7A). However, after treatment with carvacrol, the levels of inflammatory markers were reduced in a dose-dependent manner as compared to the positive control (silymarin) as shown in the image J graph (Fig. 7B). Moreover, the results of densitometry analysis support the finding of the dose-dependent decrease of inflammatory markers by carvacrol treatment. Therefore, carvacrol reduces the alcohol-induced inflammatory marker levels.

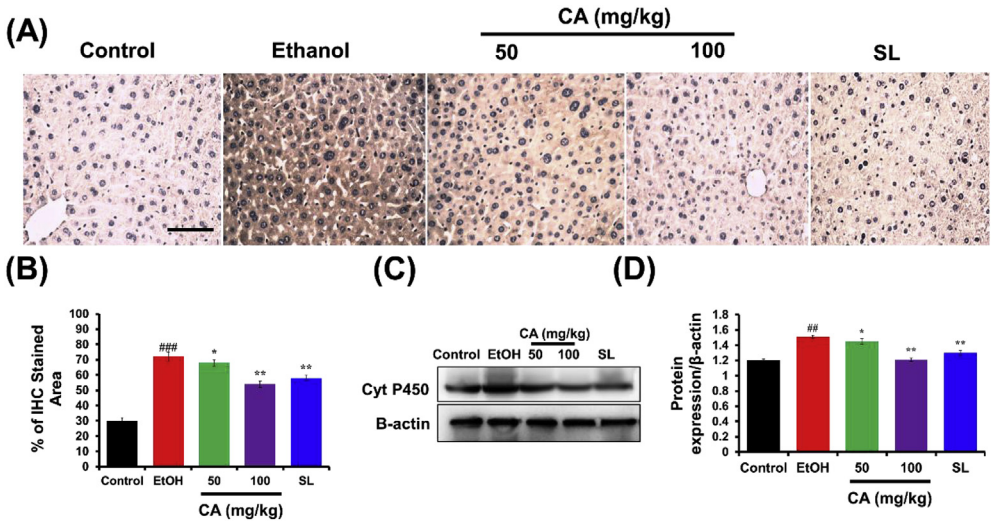

Fig. 3. A) Effect of carvacrol on Cyt P450 levels as determined by immunohistochemistry in alcohol-induced liver tissue (scale bar = 0.1 mm). B) Image J analysis of Cyt P450 positive area. C) The dose-dependent reduction in Cyt P450 protein levels and its D) image J analysis. Bar diagrams are depicted as the means ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol.

3.7. Carvacrol reverses autophagy dysregulation caused by ethanol intoxication

Autophagy plays an important role in maintaining homeostasis. In this study, after ethanol intoxication, ATG5, Beclin-1, and LC-3 II levels were found to be lowered in comparison to that of the control ($^{###}p < 0.001$). However, after treatment with carvacrol (50 and 100 mg/kg), the proteins were found to be at normal levels ($^{***}p < 0.001$). Thus, the data indicates that autophagy is restored after treatment with carvacrol (Fig. 8A and B).

4. Discussion

Health benefits are achieved from a diet rich in various phytochemicals (Slavin and Lloyd, 2012). The thyme essential oil is rich in thymol and carvacrol with some monoterpenes (Chouhan et al., 2017). A daily diet that includes the proper amount of carvacrol reduces the risk of various diseases. Carvacrol has various biological activities such as antimicrobial, antitumor, anti-inflammatory, angiogenic, anti-parasitic, and insecticidal etc. (Baser, 2008). In our previous research, we showed that carvacrol has potent antibacterial and anticancer properties (Khan et al., 2017, 2018a; 2018b). In this study, we evaluated the hepatoprotective potential of carvacrol against binge alcohol-induced liver damage.

Table 1
Binding energy of Carvacrol and the degree of conformational change after binding wit Cyt P450.

Proteins	Ligand	Best Pose[a]	Binding energy (KJ/mol)	G-score (Kcal/mol)[b]	Binding Affinity (kD)
CytP450	Carvacrol	18	−28.74	−6.87	−26.03

[a] Best binding position.
[b] ΔG Score for calculating binding energy.

Acute alcohol drinking causes liver inflammation. In our study, the liver steatosis caused by alcohol was abrogated by carvacrol treatment (50 and 100 mg/kg). Kim et al. (2013) have reported similar results on carvacrol protecting hepatic steatosis in mice. Furthermore, treatment with carvacrol resulted in negative hepatic PAS staining and the elimination of glycogen content in comparison to an alcohol-treated group (Fig. 1). Similar results were also observed by Uyanoglu et al. (2008)

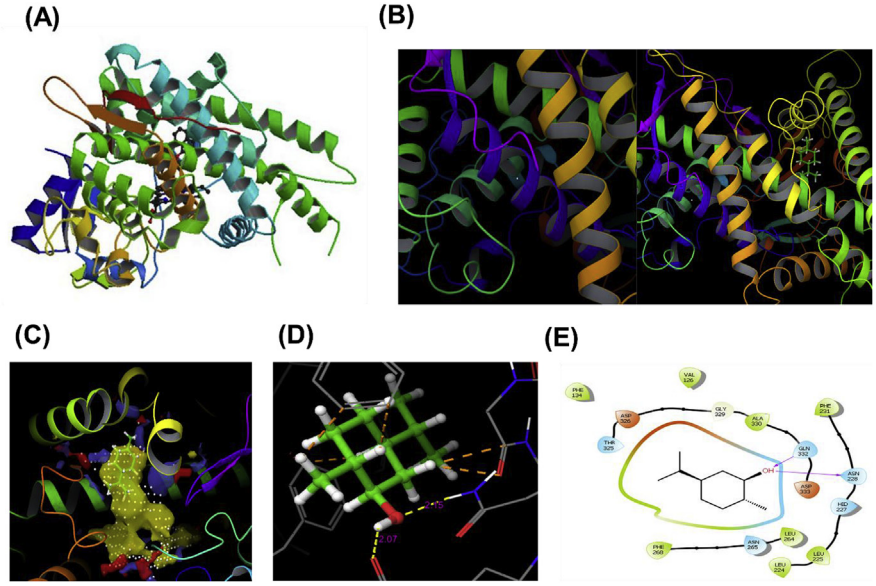

Fig. 4. *In silico* analysis of carvacrol with Cyt P450. (A) The molecular docking of Cyt P450, (B) comparative view, (C) active site binding, (D) H-bond interaction, and (E) 2-D interaction diagram.

Table 2
Descriptive properties of selected target protein for molecular docking.

Protein name	Uni-Prot ID	PDB ID	Chain[a]	Length[b]	Gene[c]	Classification[d]
Cyt P450	Q64483	3PM0	A	507	*Cyp1b1*	Oxidoreductase

[a] Total number of subunits present in the protein.
[b] Total length of all the subunits in the protein.
[c] Gen encoding for the protein.
[d] Family of protein.

and Baser (2008) with reduction in glycogen content as well as liver fibrosis in rats. Alcohol consumption disrupts the cellular environment which directly leads to elevation in the serum levels of markers such as AST, ALT, ALP, TP, TNF-α, and IL-1β (Fig. 2). Elevated serum parameters also indicate necrosis and mitochondrial damage. In our previous investigation we observed that carvacrol reduces the alcohol-induced elevation of AST, ALT, TP, TNF-α, IL-1β as well as triglyceride levels (Kim et al., 2013). Kim et al. (2013) results proved that high fat diet-induced hepatic steatosis was protected by carvacrol treatment in mice. The cellular enzyme possesses high levels of Cyt P450 as well as alcohol dehydrogenase enzymes. The formation of reactive oxygen species plays a critical role during ethanol metabolism, which also results in lipid peroxidation. This cytotoxic mechanism leads to cellular toxicity and ultimately cell death. The level of Cyt P450 was found to be reduced in a dose-dependent manner as validated by immunocytochemistry as well as immunoblotting results (Fig. 3). In our current investigation we found that Cyt P450 levels were reduced due to the binding of carvacrol to its active site, as supported by *in silico* analysis (Fig. 4). The activation of Cyt P450 by ethanol toxicity triggers oxidative stress and further hepatic cellular as well as mitochondrial injury. The binding of carvacrol to the active site of the Cyt P450 reduces the downstream pathway. The Cyt P450 enzyme is very well known for its detoxification of xenobiotics. Carvacrol reduces the levels of Cyt P450 that were elevated due to alcohol toxicity, and the enzyme inhibition by carvacrol blocks the alcohol-induced hepatotoxicity. Similarly, the role of carvacrol in regulation of Cyt P450 enzyme in rats was well elaborated by Aristatile et al. (2014). The activated oxidative burst, such as MPO, NO, SOD, and GSH, was reduced by carvacrol as observed by the elevated levels of antioxidants, such as catalase, GR, SOD1, and Nrf-2, which help reduce oxidative stress (Fig. 5). These antioxidant enzymes play a critical role in detoxification and in the scavenging of ROS as well as protection of the cellular environment. These results are in accordance with the antioxidant properties previously reported (Bakır et al., 2016; Kılıç et al., 2016). Bakır et al. (2016) and Kılıç et al. (2016) shown that carvacrol has ability to regulate the oxidative stress induced by inflammation inducing agents in rats.

Alcohol consumption creates oxidative stress, subsequently leading to the degradation of the extracellular matrix (ECM) and the elevation of MMPs, and resulting in hepatic steatosis (Beier and Arteel, 2012; Bataller and Brenner, 2005). According to the results of previous reports, carvacrol reduces MMP levels that are elevated due to inflammation (Subramaniyan et al., 2014). Subramaniyan et al. (2014) explored the mechanism of decreased mast cell number as well as MMP's expression level in rats. In the present investigation, we found the enhancement of MMPs by alcohol which was also reduced due to

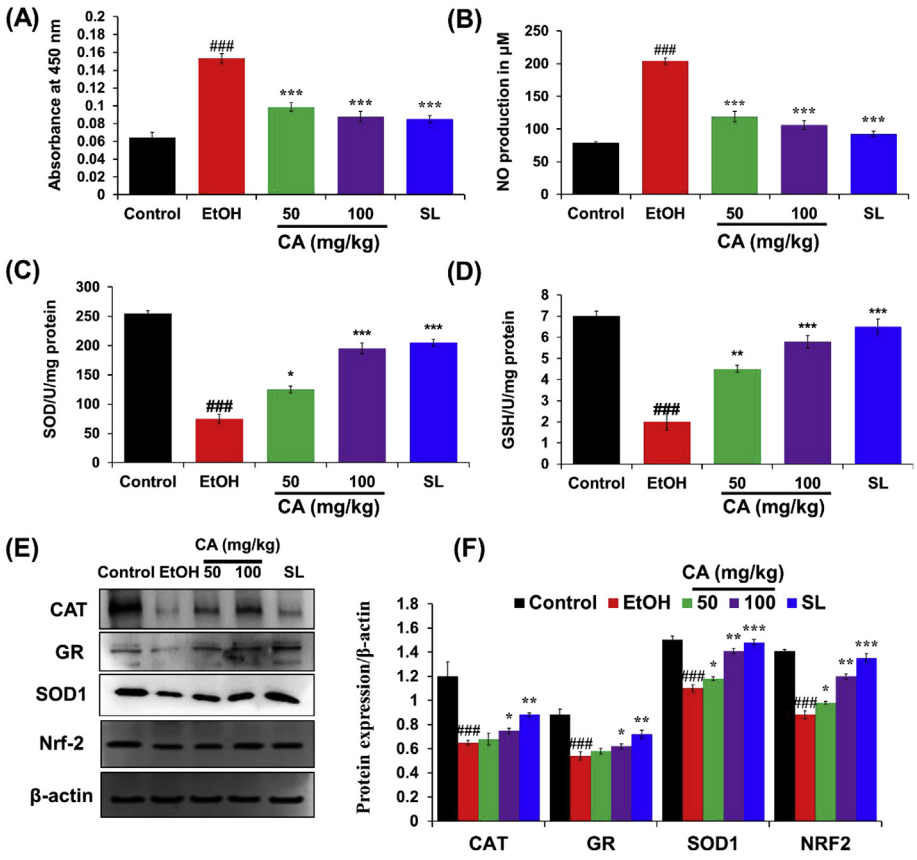

Fig. 5. Effect of carvacrol on alcohol-induced oxidative stress in liver tissue. Represented in this figure are the (A) Myeloperoxidase assay, (B) Nitric oxide levels, (C) SOD levels, (D) GSH levels and (E) Western blot analysis of the oxidative stress markers as well as (F) Image J analysis. Bar diagrams are depicted as the means ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol.

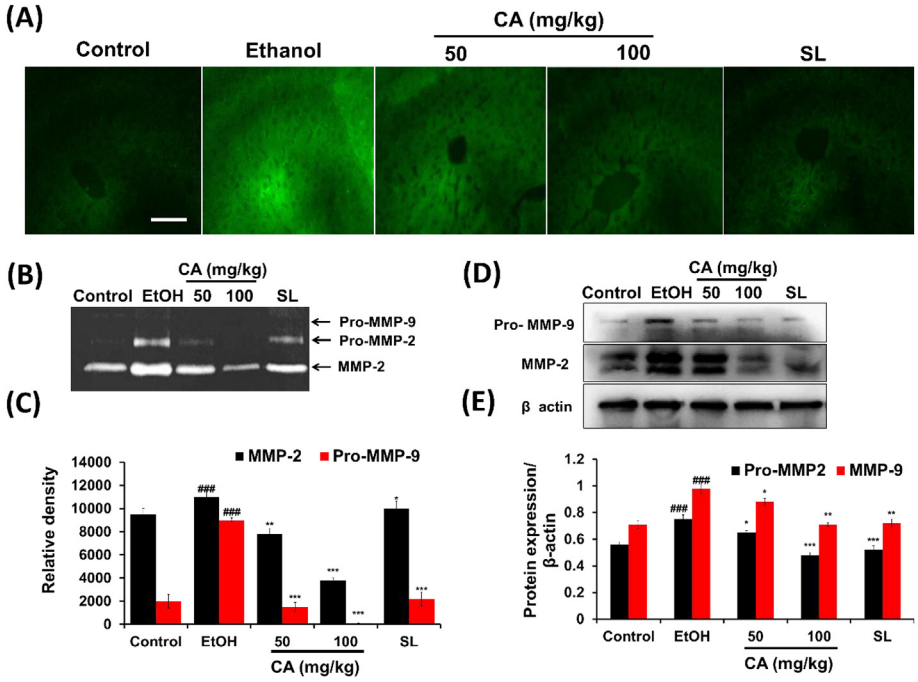

Fig. 6. Effect of carvacrol on alcohol-induced MMPs in liver tissue. Represented in this figure are A) DQ gelatin of liver tissue sections (scale bar = 0.1 mm), B) Gelatin zymography, and C) Western blot analysis of MMP markers. Bar diagrams are depicted as the means ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol.

carvacrol treatment. MMPs play a crucial role in the remodeling of hepatic tissue; carvacrol blocks the release of the MMPs, ultimately helping to restore the hepatic tissue structure, which is similar to the hepatoprotective activity of curcumin (Lee et al., 2013). Furthermore, we also confirmed the preventive role of carvacrol against alcohol-induced upregulation of MMP-9 by both gelatin and *in situ* zymography (Fig. 6). It has been previously reported that the elevated expression of MMP-9 is directly linked to the activation of inflammatory markers such as within the MAPK pathway (Hu et al., 2018).

MAPKs are key players in controlling cell growth via proliferation, differentiation, and the cell death mechanism (Hommes et al., 2003). The MAPK family consists of various cytoplasmic proteins that are activated upon phosphorylation at key serine or threonine residues. The protein phosphorylation activates downstream nuclear proteins such as NF-κB which secretes inflammatory cytokines. Currently, the MAPK pathway is a new target of therapeutic drugs (Hommes et al., 2003; Vahdati Hassani et al., 2017; Ali et al., 2018), and the development of nutraceuticals such as carvacrol could be a promising avenue for future therapy. The results of previous investigation and the current study showed that carvacrol normalized the otherwise elevated MAPK levels resulting from alcohol treatment. Our data shows that carvacrol reduces the elevated MAPK levels in a dose-dependent manner, and that it also reduces cytokine levels (Fig. 7A).

Alcohol intoxication is responsible for the dramatic elevation of inflammatory cytokines (i.e., TNF-α, IL-1β, etc.) (O'Halloran et al., 2016). Moreover, other reports have also suggested that TNF-α and oxidative stress were drastically increased in chronic alcohol-induced liver diseases (Beier et al., 2011; Bharrhan et al., 2011). In our study, we found that the levels of MAPK downstream targets such as NF-κB, iNOS, eNOS, and the pro-inflammatory markers elevated in the ethanol-intoxicated group but were at lower levels in the carvacrol-treated group. This indicates that carvacrol has the ability to reduce alcohol-induced inflammatory markers and cytokines (Fig. 7B).

Several lines of evidence support the hypothesis that alcohol

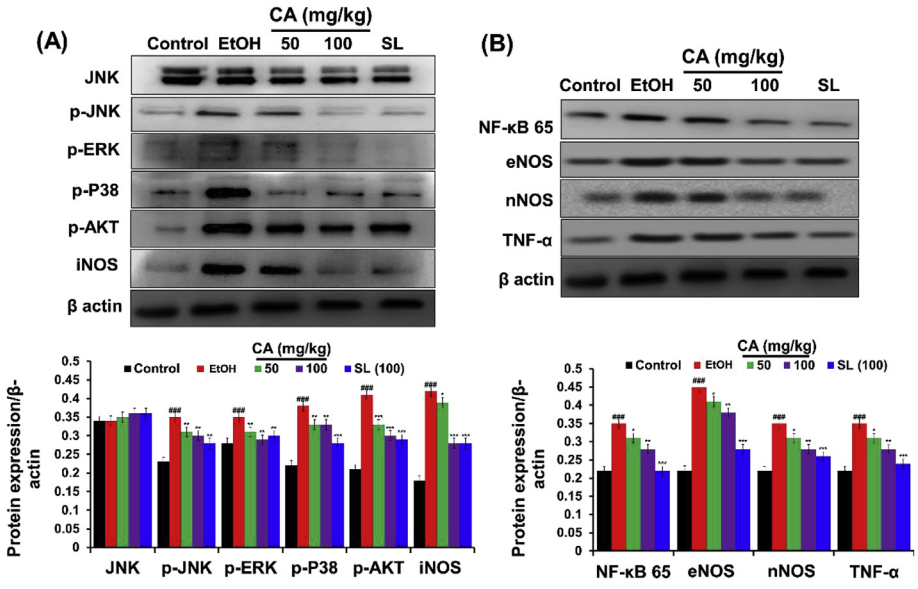

Fig. 7. Effect of carvacrol on MAPK kinase pathway and other inflammatory proteins. Represented in this figure are (A) the MAPK kinase pathway proteins (JNK, p-JNK, p-ERK,p-P38, p-AKT) of liver tissue sections and (B) other inflammatory protein levels (NF-κB 65, eNOS, nNOS, Inos, TNF-α) after treatment with carvacrol. Bar diagrams are depicted as the means ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol.

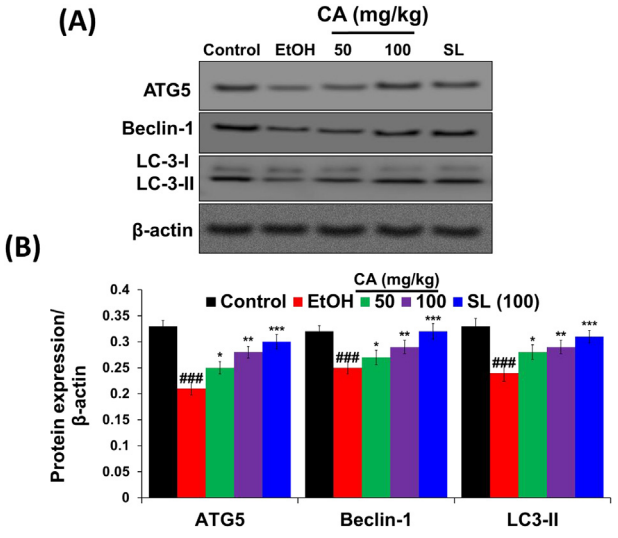

Fig. 8. Effect of carvacrol on alcohol-induced autophagy in liver tissue. Bar diagrams are depicted as the means ± SD of three independent experiments. $^{###}P < 0.001$ vs Control; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs ethanol.

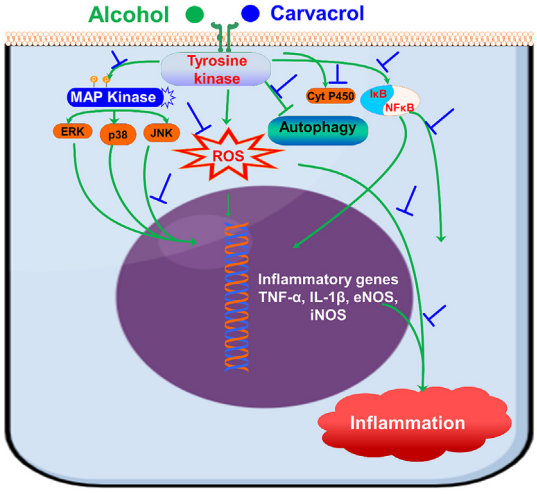

Fig. 9. Schematic representation of the mechanism of the carvacrol ameliorates alcohol induced liver toxicity. Alcohol intoxication in hepatocytes causes activation of MAPK markers such as ERK, p38 and JNK which further translates the signal to synthesize inflammatory proteins. Moreover, autophagy plays crucial role in maintaining the homeostasis of the cell, alcohol intoxication instigates abrogates autophagy. Most importantly, Cyt P450 enzymes plays crucial role to eradicate xenobiotics, found to be enhance after alcohol intoxication, and after carvacrol treatment, it was reduced dose dependently. Overall, carvacrol ameliorates inflammation stimuli intoxicated by alcohol.

consumption inhibits autophagy (Ding et al., 2010; Rautou et al., 2010), which is a cell survival mechanism. Previous reports suggest that chronic alcohol intoxication reduces the autophagic vacuole formation. Protein aggregates also known as Mallory-Denk bodies are found in alcoholic patients. The elevated levels of Mallory-Denk bodies prove that autophagy is suppressed in patients with alcohol-induced liver disease. The actual mechanism behind the suppression of autophagy is still unclear (Rautou et al., 2010). Previous reports have shown that ethanol intoxication reduces AMPK protein levels and inhibits autophagy by mTOR pathway. Ethanol is also responsible for the obstruction of microtubules and microfilaments; because these structures are crucial for autophagosome formation, their inhibition may explain the suppression of autophagy (Rautou et al., 2010). We observed the suppression of autophagy in liver tissue due to alcohol treatment. However, the autophagic process was re-established in a dose-dependent manner in the carvacrol-treated group. This proves that carvacrol has the ability to restore autophagy (Fig. 8) (Kang et al., 2019; Chu et al., 2019).

Alcohol intoxication in hepatocytes causes activation of MAPK pathway which further translates the signals to synthesize inflammatory proteins. Autophagy plays a vital role in maintaining the homeostasis of the cell, alcohol intoxication initiates abolishment of autophagy. Most importantly, Cyt P450 enzyme plays a crucial role to eradicate xenobiotics, which is found to be enhanced after alcohol intoxication, and after carvacrol treatment, it was reduced dose-dependently. Overall, in this study we found that carvacrol ameliorates inflammation stimuli intoxicated by alcohol as explained in Fig. 9.

5. Conclusion

Thus, carvacrol ameliorates alcohol-induced histological changes, restores liver-to-body weight ratio and antioxidants levels, and inhibits the elevation of Cyt P450 and MAPK protein levels. Furthermore, carvacrol reduces NF-κB expression as well as downstream markers such as iNOS, eNOS etc., and it stabilizes MMP activity in liver tissue. Moreover, it restores the autophagy process. Therefore, further investigation including future clinical trials is required to determine whether a carvacrol-enriched diet can be used as adjuvant therapy for treating alcohol-induced diseases.

Conflicts of interest

All authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110582.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.110582

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