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Hepato-protective Effect of Clove Bud Polyphenols (*Syzygium aromaticum L.*) (Clovinol®) by Modulating Alcohol Induced Oxidative Stress and Inflammation

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Abstract

The pathogenesis of alcoholic liver diseases progresses from fatty liver to hepatic inflammation and necrosis to fibrosis. Clove buds (*Syzygium aromaticum L.*) are one of the richest sources of dietary polyphenols with many traditional medicinal uses. Hence, the present work attempted to evaluate the hepatoprotective activity of a standardized polyphenol-rich extract of clove buds (*Clovinol*). The experiment was conducted on Wistar rats designated into three groups. First group was vehicle control and hepatotoxicity was induced to the second group by the administration of ethanol at the fixed dose of 12.5 g/kg body wt., orally for 30 days. *Clovinol* (100 mg/kg body wt.) was co-administered with ethanol in the third group. The liver toxicity was assessed by the estimation of liver marker enzymes in addition to which, assays of antioxidant enzymes, inflammatory markers, and liver histopathology studies were also conducted. Ethanol treatment significantly increased ($p<0.05$) liver function markers (SGOT and SGPT) and reduced ($p<0.05$) the antioxidant enzymes (SOD, CAT, and GPx) and GSH. It also increased the WBC count, inflammatory markers (nitrite, CRP, COX-2, IL-6 and TNF-α) and lipid peroxidation, significantly ($p<0.05$). Supplementation of *Clovinol* showed significant ($p<0.05$) reversal of all these biochemical and molecular variables indicating the efficacy of *Clovinol* in the downregulation of alcohol-induced oxidative stress and inflammatory changes, bringing about a significant reduction in the associated liver pathology. To summarize, we found that *Clovinol* could be a potential functional ingredient for liver health.

Keywords: Clove buds, hepatotoxicity, *Clovinol*, antioxidants, inflammation

1. Introduction

Alcoholic liver diseases (ALD) pose serious health concern and is responsible for approximately 4% of all deaths annually and 5% of all disabilities worldwide (Singh et al., 2016). Despite the tremendous scientific advances made in hepatology and related areas, liver problems are still on the rise (Mujeeb et al., 2011). Yet, there are no specific strategies for its management or treatment. Most of the treatment strategies are accompanied by serious side effects and other health implications (McDowell Torres et al., 2010). Hence, developing management systems, especially based on safe natural agents has been generating considerable interest, of late.

Alcohol is metabolized mostly in the liver. Its by-products, such as acetaldehyde were reported to be more toxic than alcohol itself (Gao and Bataller, 2011). Chronic alcohol consumption damages liver, advancing through distinct pathological features such as alcoholic fatty liver which may further progress through alcoholic steatohepatitis, fibrosis and finally to cirrhosis (Cohen and Nagy, 2011). Excess of free radicals generated during alcohol metabolism can affect the mechanism of the antioxidant defense systems. Chronic consumption of alcohol can induce the activation of the Kupffer cells which enhances reactive oxygen species (ROS) generation and increases the oxidative stress. Activated Kupffer cells also release gut-derived endotoxins-lipopolysaccharides (LPS) to produce pro-inflammatory cytokines like tumour necrosis factor-α (TNF-α) and

interleukin-6 (IL-6) leading to hepatotoxicity (Wheeler, 2003). Besides these, it stimulates NF-κB, a transcriptional factor which triggers the induction of inflammatory genes (Zima and Kalousova, 2005) and leads to matrix degradation (Tilg et al., 2011). The cirrhosis of the liver is also characterized by over-expression of extracellular matrix proteins from hepatic stellate cells (HSC). HSCs ordinarily store vitamin A and upon activation triggers liver fibrosis and further to cirrhosis (Hernández-Gea et al., 2012).

Spices are popular food flavouring agents known for their distinct flavour profiles and utility in traditional medicine (Kaefer and Milner, 2008). The dried flower buds of clove (*Syzygium aromaticum L.*), an evergreen tropical plant belonging to Myrtaceae family, have many pharmacological effects including the antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, analgesic, antispasmodic, antinociceptive, anticarminative and antiseptic properties (Cortés-Rojas et al., 2014; Zheng et al., 1992). It is a rich source of eugenol rich volatile oil (12-16% (v/w), phenolic compounds like hydrolysable tannins, phenolic acids, and flavonoids (Chaieb et al., 2007). Besides, earlier reports have suggested clove polyphenols to have *in vivo* antioxidant, anti-inflammatory and gastroprotective activities (Issac et al., 2015; Johannah et al., 2015). Since oxidative stress and associated inflammatory changes are crucial in the development of alcoholic liver diseases, the present study investigated the effect of clove bud polyphenols (hereinafter referred to as '*Clovinol*') on alcohol-induced liver toxicity in the chronic alcohol-treated rat model.

2. Materials and Methods

2.1 Materials

All the chemicals used were of analytical grade from Merck, Bangalore, India. ELISA kits and antibodies were purchased from Sigma-Aldrich, Bangalore, India. RT-PCR kit was purchased from Eppendorf India Ltd, Chennai, India. Liver function markers were analysed using respective kits provided by M/s Agappe Diagnostics Pvt Ltd, Bangalore, India. Plasma CRP (C-reactive proteins) level was measured using the kit manufactured and supplied by M/s Diasys Diagnostics, Germany.

2.2 Preparation and Characterization of Clovinol

Clovinol has been characterized in detail in an earlier study (Johannah et al., 2015) and was prepared by hydro-ethanolic extraction followed by purification and spray drying as reported earlier (Issac et al., 2015). Matured and dried Indonesian clove buds were used in the preparation of *Clovinol* with a polyphenol content of 41.2% (w/w) as gallic acid equivalent. Nutritional and microbial analysis were also carried out to ensure the quality. Tandem mass spectrometric analysis indicated the presence of various polyphenols including flavonoids, phenolic acids, hydrolysable tannins, and their glycosides. Absence of methyl eugenol was confirmed by GC/M/MS analysis.

2.3 Animals

Selected rats having an average body weight of 150 ± 10 g were acclimatized for a period of 14 days in ventilated cages and housed in an air-conditioned room at 24 ± 2 ℃ and relative humidity of 60 ± 5% with a 12 h light and dark cycle. All animal experiments were carried out in compliance to the approved ethical norms by the Institutional Animal Ethics Committee (IAEC) recognized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (registration no: CAF/361/2015). Animals were kept on a balanced pellet diet (M/s Amrut Laboratory Animal Feeds, Maharashtra, India) and water *ad libitum*.

2.4 Experimental Design for Hepatotoxicity Study

Twenty four animals were randomly divided into three groups each containing eight animals per group, as follows.

Group I : Normal control rats (N)

Group II : Ethanol treated rats (12.5 g/kg body weight of 90% (v/v) (ET)

Group III : ET + *Clovinol* treated (100 mg/kg body weight).

Ethanol, *Clovinol* and distilled water (vehicle) were administrated by intragastric oral gavage on a daily basis at the same time after being deprived of food for 10 h beforehand. The dose of *Clovinol* was fixed to 100 mg/kg body weight based on earlier reports which showed this dose to be the minimum effective dose for consumption (Johannah et al, 2015). After 30 days of study period, overnight fasted rats were sacrificed by euthanasia. Blood was collected by direct heart puncture into EDTA coated and non-EDTA vials for analysing haematological and biochemical parameters. White blood cell (WBC) count was determined using a haematology analyser. Serum glutamic-pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT) and alkaline

phosphatase (ALP) were measured by standard kits (Agappe Diagnostic kits, Pvt. Ltd, India, product No. 11408005, 11401001). Serum was separated from the clotted blood sample by centrifuging at 5000 rpm for 10 min at 4 ℃ and was stored at −20 ℃ for analyses. Liver was dissected out for biochemical and molecular studies and proteins were estimated (Lowry et al., 1951). The samples of liver tissues from each group were taken and washed in PBS buffer and kept in 10% formalin for histopathological examinations. The liver tissues for biochemical analysis were weighed and homogenized in either 0.25 M sucrose or 0.15 M Phosphate buffer depending upon the assay it is subjected to. The homogenate was then centrifuged and the supernatant was collected for the assays.

2.5 Measurement of Endogenous Antioxidants and Lipid Peroxidation in Tissues

The supernatant obtained by centrifugation of the homogenate of the liver tissue was employed for the analysis of various antioxidant enzymes. Catalase (CAT) activity was determined by measuring the rate of decomposition of hydrogen peroxide at 240 nm and expressed in terms of units per mg protein (Aebi, 1984). The superoxide dismutase (SOD) activity in the tissues homogenized in sucrose was estimated by the nitro blue tetrazolium (NBT) reduction method of (Kakkar et al., 1984). Glutathione peroxidase (GPx) activity in the tissues homogenized in sucrose was performed by the method of Lawrence and Burk based on the oxidation of glutathione in the presence of H_2O_2 (Lawrence and Burk, 1976). The non-protein thiol, glutathione (GSH) activity was measured based on the reaction with 5,5-dithiobis-2-nitrobenzoicacid (DTNB) reagent (Benke and Murphy, 1974). Thiobarbituric acid reactive substances **(**TBARS) were estimated (Ohkawa et al., 1979) and expressed as mmol of malondialdehyde produced.

2.6 Estimation of Plasma Nitric Oxide (NO) Levels

Plasma NO was measured as its breakdown product of nitrite (Grisham et al, 1996). NO rapidly converts into nitrite and nitrate in the presence of H_2O, Total production of NO therefore may be determined by measuring the stable NO metabolite nitrite (NO_2^-). Equal volume of tissue supernatant and Griess reagent (1% sulphanilamide and 0.1% N-[naphthyl] ethylenediamine dihydrochloride 1:1) was mixed and absorbance was measured at 550 nm.

2.7 Cyclooxygenase (COX) Activity

COX activity was assayed by the method of Shimizu et al (1981). Lysed monocytes isolated from control, standard and test groups were incubated with Tris-HCl buffer (pH 8.0), 5 mM glutathione and 5 mM hemoglobin for 1 min at 25^{o}C. The reaction was initiated by the addition of 200 μM arachidonic acid and terminated after 20 min incubation at 37^{o}C by the addition of 10% trichloroacetic acid (TCA) in 1N HCl. Following centrifugal separation and addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 530 nm.

2.8 RT-PCR Analysis for IL-6 and TNF- α

The gene level expression of TNF-α and IL-6 mRNA were measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from liver tissues using RNA isolation mini kit (Sigma-Aldrich, Bangalore, India) as per the manufacturer's instructions. Primer sequences for rat IL-6, TNF-α and GADPH were as en below.

Gene	Forward primer	Reverse primer
IL-6	5'CCACTGCCTTCCCTACTTCA3'	5'TGGTCCTTAGCCACTCCTTC3'
TNF-α	5'GTCGTAAACCACCAAGC3'	5'GACTCCAAAGTAGACCTGCCC3'
GAPDH	5'CCTGCTTCACCACCTTCTTG3'	5'ATCCCATCACCATCTTCCAG3'

GAPDH (glyceraldehyde 3-phosphate dehydrogenase) primers were used as an internal control for RNA loading. RT-PCR was performed in an Eppendorf thermocycler. Reverse transcription and DNA amplification was done separately with two step RT-PCR kit. RNA template, dNTPs, oligo (dT) and reverse transcriptase enzyme were subjected to reverse transcription and the DNA was amplified with relevant primers. The denaturation step was at 94 ℃ for 4 min; 35 cycles at 94 ℃ for 30 s, primer annealing for 30 s, 72 ℃ for 1 min; and then a final extension for 3min at 72 ℃. The PCR products subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide, visualized under a UV-transilluminator and the relative intensities of bands of interest were measured on a GelDoc 2000 scanner (Bio-Rad, CA, USA) with scan analysis software.

2.9 Histopathological Analysis of Liver Tissue

The entire liver was rapidly dissected out and ultrathin sections (5μm) were fixed by immersing in 10% formalin solution at room temperature. For histological examinations, paraffin-embedded tissue sections were de-waxed

with xylene and stained with hematoxylin-eosin (H&E). The tissue samples were then examined and photographed under an optical microscope of 100X magnification (Olympus-Magnus trinocular microscope, Noida, India) for observation of structural abnormality.

2.10 Statistical Analysis

The results were analyzed using a statistical program SPSS/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was employed for comparison test of significant differences among groups. Pair fed comparisons between the groups was made by Duncan's multiple range tests and $p < 0.05$ was considered as significant.

3. Results

3.1 Preparation and Characterization of Clovinol

Matured and dried Indonesian clove buds with an average polyphenol content of 11.5% GAE and 6.6% volatile oil content were sampled from a 1000 kg lot and used as the plant material for the preparation of *Clovinol*. A process of hydro-ethanolic extraction followed by purification, evaporation at controlled temperature (<50◦C) and spray drying was developed for the preparation of *Clovinol* as a polyphenol-rich water soluble extract powder. The free flowing powder of *Clovinol* showed mild taste and aroma characteristic of clove with a polyphenol content of 31.2% (w/w) as gallic acid equivalent. Upon nutritional analysis, *Clovinol* was found to contain 46% carbohydrate, 4% protein, 5.3% fat, 1.5% ash and 0.5% dietary fibre. Moisture content was 2.1% with an average density of 0.37 g/mL bulk density; with less than 300 cfu/g total aerobic plate count and 20 cfu/g total yeast and mold. No traces of Escherichia coli or Salmonella were detected, indicating its adherence to standard microbial specification for food ingredients. Tandem mass spectrometric analysis of *Clovinol* indicated the presence of various polyphenols including flavonoids, phenolic acids, hydrolysable tannins, and their glycosides [18, 20]. Gallic acid, ellagic acid, chlorogenic acid, quercetin, luteolin, eugenol, and eugenol acetate were identified and confirmed in *Clovinol* by comparing the LC/MS/MS details either with reference compounds or with literature data. Thus, the major polyphenols in *Clovinol* were already shown to be widely present in the plant kingdom and possess varying health beneficial pharmacological effects. Further, GC/M/MS analysis confirmed the absence of methyleugenol in *Clovinol*.

3.2 Effect of Clovinol on Total WBC Counts

Total WBC count was increased significantly ($p< 0.05$) in ethanol treated rats as compared to the normal control group. Upon *Clovinol* supplementation for 30 days, WBC count was decreased significantly ($p < 0.05$) as shown in Table 1.

3.3 Effect of Clovinol on Liver Function Markers

Serum levels of specific liver enzymes were measured to determine the liver function of each rat. The liver damage induced by ethanol significantly elevated the serum level of specific liver enzymes SGOT, SGPT and ALP as compared to the other groups. *Clovinol* supplementation for 30 days significantly ($p< 0.05$) lowered the levels of these marker enzymes. The data indicated that *Clovinol* counterbalanced the alcohol induced liver toxicity in an effective manner. The results were shown in Fig. 1.

3.4 Effect of Clovinol on Inflammatory Markers

The concentration of CRP was increased significantly ($p < 0.05$) in ethanol treated rats. But, 30 days of *Clovinol* supplementation could significantly decrease the CRP enhancement ($p< 0.05$) as shown in Table 1.

Ethanol treatment enhanced in COX activity significantly ($p < 0.05$) whereas supplementation of *Clovinol* lowered the same significantly ($p < 0.05$), as compared to ethanol treated rats (Table 1). Similarly, on ethanol exposure, nitrite levels increased significantly ($p < 0.05$), but decreased significantly ($p < 0.05$) when *Clovinol* was supplemented along with ethanol treatment (Table 1).

3.5 Effect of Clovinol on Molecular Markers and Gene Expression

The mRNA expressions of molecular markers like IL-6 and TNF-α were upregulated on ethanol exposure and was downregulated significantly ($p <0.05$) when subjected to *Clovinol* in addition to ethanol (Figure 2). But, supplementation of *Clovinol* was found to be efficient to suppress the upregulation of IL-6 andTNF-α.

3.6 Effect of Clovinol on Antioxidant Enzymes in Liver Tissue

The activity of the antioxidant enzymes (SOD, CAT and GPx) and GSH were analyzed in liver. Among ethanol treated rats, SOD, CAT, GPx and GSH were lower than those in the normal rats, indicating severe cellular damage upon ethanol treatment. Upon *Clovinol* administration along with ethanol, significant ($p<0.05$) enhancement in the

levels of these antioxidant enzymes and GSH were observed, indicating the survival of hepatocytes (Fig. 4). The extent of lipid peroxidation was judged by measuring the TBARS values in liver tissues. The results indicated a significant increase ($p< 0.05$) in ethanol treated rats. When *Clovinol* was supplemented for 30 days, a significant ($p< 0.05$) reduction in TBARS values were observed (Table 1).

Table 1. Effect of *Clovinol* on inflammatory markers

Groups	WBC (cellsx10^3/ml)	CRP (mg/ml)	NO (µmol/mg)	Total COX (ODshift/ mgprotein)	TBARS (nmol/g)
Normal	3.4 ±0.02	52±3.32	9.6 ±0.11	3.9 ±0.14	2.8 ±0.02
ET	8.0 ±0.03[a]	76 ±6.64 [a]	16.1 ±0.13[a]	7.4 ±0.21[a]	7.6 ±0.04 [a]
Clovinol	4.7 ±0.01 [a,b]	60 ±3.44 [a,b]	11.2 ±0.09 [a,b]	4.8 ±0.17 [a,b]	3.7 ±0.03 [a,b]

3.7 Liver Histopathology

Among the normal control rats normal liver morphology was observed. There was no evidence of inflammation/necrosis/hemorrhage or cholestasis. In ethanol treated rats (ET) irregular, swollen hepatocytes with mild fatty change containing fat droplets as well as fatty degeneration were noticed. Some of the hepatocytes show degenerative changes (DH) and binucleation. In *Clovinol* treated rats, hepatocytes had more of a normal morphology but were multinucleated indicating cellular regeneration. No evidence of inflammation/ necrosis/ hemorrhage or cholestasis was observed among Clovinol treated rats (Fig. 5). These indicated that the liver of Clovinol treated rats were protected against ethanol induced damage.

4. Discussion

There are no FDA approved regimens for treating alcoholism, and most of the synthetic drugs produce adverse side effects. Hence, we investigated the possibilities of food-derived, natural alternatives for protection against chronic alcohol consumption, a major reason for chronic liver diseases, globally. Clove buds, rich in polyphenols such as flavonoids, hydrolysable tannins of ellagic acid and gallic acid (Issac et al., 2015) is one of the best antioxidant spice, often referred to as the 'Champion of spices' (Perez-Jimenez, 2010; Milind, 2011; Shan et al., 2005). Besides, there have been reports on hepatoprotective effects of clove essential oil and clove extracts (Hadary and Ramadan Hassanien, 2015; Al-Okbi et al., 2014; Ali et al., 2014). The present study investigated the efficacy of *Clovinol*, the polyphenols of clove buds, as a hepatoprotective agent by assessing its effects on liver function markers as well as inflammatory and oxidative stress markers in the rat model for ethanol toxicity.

Elevation in ALP, SGOT and SGPT was reported to be gradual in the first stage of liver inflammation and increased further on liver failure, indicating cellular damage, obstructive damage (cholestasis or blockage of bile flow) and loss of functional integrity of hepatic cell membrane (Friedman, 2003). Consistent with these findings, we found that the hepatic toxicity marker enzymes- ALP, SGOT and SGPT- were significantly increased on ethanol treatment. However, *Clovinol* supplementation was found to cause significant suppression of these activities, indicating its inhibitory effect on hepatotoxicity. Though clove essential oil has already been reported to effectively lower liver toxicity markers in CCl_4 induced hepatotoxicity (Hadary and Ramadan Hassanien, 2015; Al-Okbi et al., 2014), no reports on the hepatoprotective activity of clove polyphenols are reported so far. The present study demonstrates the hepatoprotective action of *Clovinol* for the first time.

The pathogenesis of liver damage leading to necrosis, cholestasis, fibrosis and cirrhosis has been closely associated with a significant elevation in WBC counts (Bataller and Brenner, 2005). Elevated WBC count was also observed in the animals subjected to excessive alcohol (Gao, et al., 2017). Likewise, we also found ethanol treatment to induce significant elevation in WBC, whereas *Clovinol* supplementation was found to restore it back to the normal conditions. Earlier reports suggest that the inflammation, cellular leakage and tissue damage upon ethanol treatment might be primarily evident in the enhanced levels of CRP, an acute phase plasma protein synthesized within the hepatocytes (Black et al., 2004). The transcriptional control of cytokine IL-6 was reported to cause CRP elevation under conditions of inflammation and was associated with the continuous loss of hepatocytes and necrosis in the liver parenchyma (Black et al., 2004). The production of TNF-α is one of the earliest events in many types of liver injury, which trigger the production of other cytokines and recruits inflammatory cells, kills hepatocytes, and initiates a healing response that includes fibrogenesis (Neuman et al., 2015). In progressive liver diseases, the counterbalancing of pro-inflammatory and anti-inflammatory cytokines might tilt in favor of the pro-inflammatory axis, thereby compromising the efficacy of the anti-inflammatory cytokines in controlling inflammation and fibrosis. Pro-inflammatory cytokines like TNF-α and IL-6 are pivotal in the development and progression of alcohol-related liver dysfunction and is associated with elevated serum

levels of the interleukins such as- IL-1, and IL-8 (Tilg, 2009). Similarly, ethanol-treated rats showed significant enhancement in CRP, TNF-α and IL-6 levels indicating induction of inflammatory response, chronicity of which may assist the disease progression. But, administration of *Clovinol* significantly reduced the CRP level and suppressing TNF-α and IL-6 expressions indicating its protective effect against ethanol-induced inflammatory response.

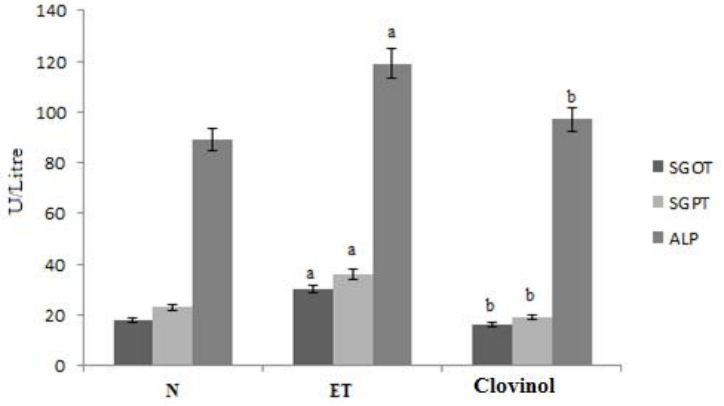

Figure 1. Effect of *Clovinol* on liver toxicity markers

The values are expressed as mean ± SEM of eight rats in each group. U: SGOT- µmol of oxaloacetate liberated/min/mg protein. U: SGPT- µmol of pyruvate formed /min/mg protein. U: ALP- amount of enzyme to decompose 1 µmole of P-NPP/minute at 25 °C. 'a' - Statistical difference with control group at $P \leq 0.05$. 'b' - Statistical difference with ethanol treated rats at $P \leq 0.05$.

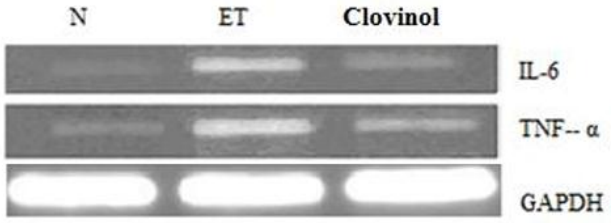

Figure 2. mRNA level of IL-6 and TNF- α.

mRNA expression studies were carried out in triplicates band intensities were plotted against GAPDH standards. The values are expressed as Mean ± SEM for 3 different experiments.

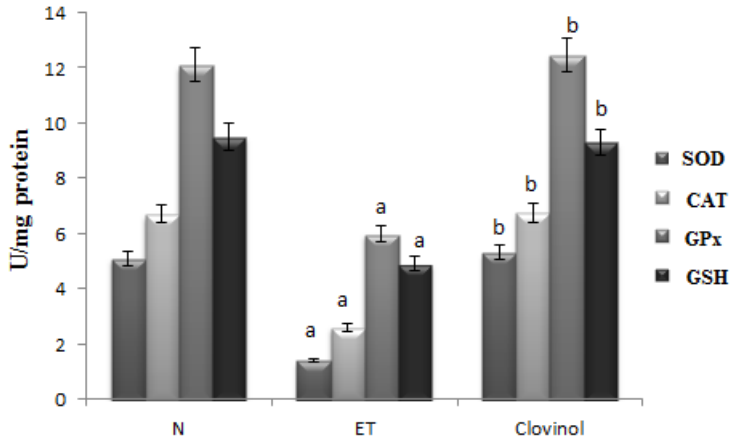

Figure 3. Effect of *Clovinol* on the level of SOD, CAT, GPx and GSH

The values are expressed as mean ± SEM of with eight rats in each group. a - Statistical difference with control group at $P \leq 0.05$. b - Statistical difference with ethanol treated rats at $P \leq 0.05$. SOD: U- enzyme

concentration required to inhibit chromogen production by 50% in 1 min. Catalase: U-μmolH_2O_2 decomposed/min. GPx: U-μmol NADPH oxidized / min. GSH: U-mmol/100g.

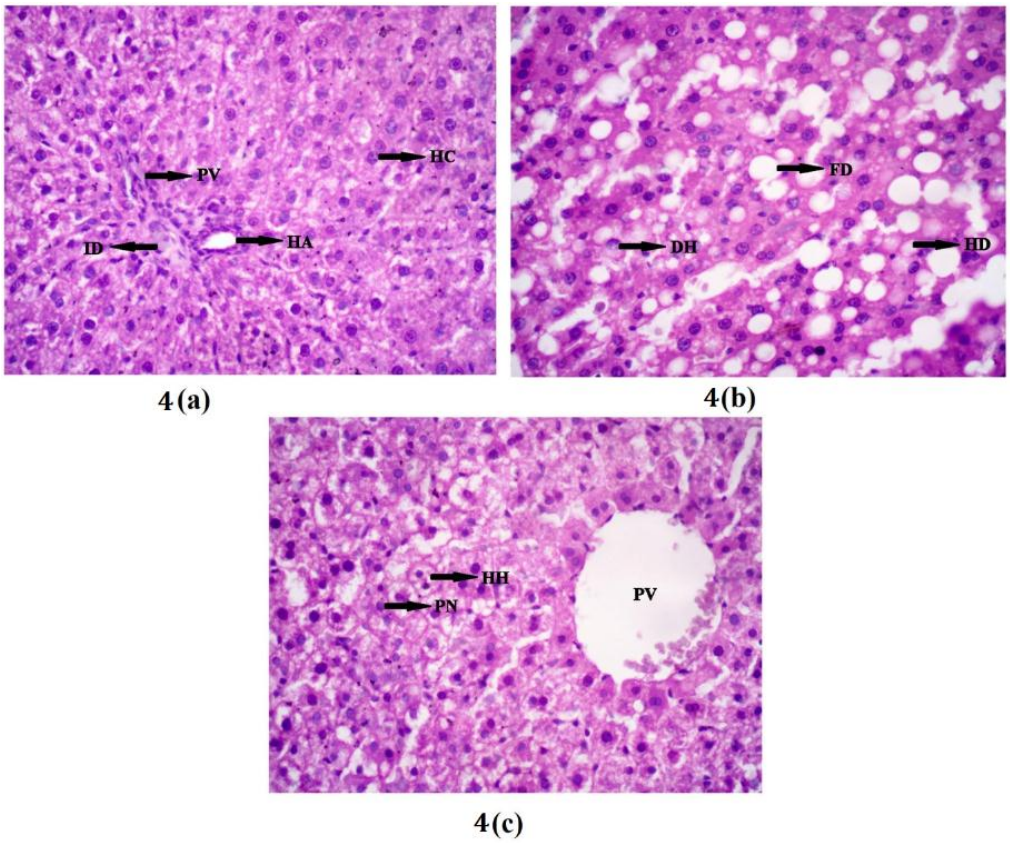

Figure 4. Liver Histopathology of experimental rats (H&E stain 100X)

(a) Normal liver contains well-arranged hepatocytes, portal vein, well circulating hepatic artery and Interlobular Duct. (b) In ET rats, hepatocytes were irregular and mild fatty change were noted (hepatocytes are swollen and contain fat droplets of varying size). Fatty degeneration was also noted. Some of the hepatocytes are degenerated (DH) and shows binucleation. (c). In *Clovinol* treated rats, hepatocytes were regular. Some of the hepatocytes were large and contain two or more nuclei with prominent nucleoli. Though a mild Kupffer cell proliferation was noted, there was no evidence of inflammation/necrosis/haemorrhage or Cholestasis.

Alcohol-induced oxidative stress is linked to the metabolism of ethanol involving both microsomal and mitochondrial systems. SOD is an endogenous oxidoreductase which catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich, 1989). Superoxides ($O_2^{\bullet-}$), capable of generating hydroxy radicals (OH•), are toxic to cells and hence play an important role in alcohol-induced liver damage (Das and Vasudevan, 2007). CAT is yet another enzyme capable of decomposing toxic H_2O_2 produced by the free radicals to non-toxic water and oxygen (L ü et al., 2010). Similarly, GPx is an antioxidant enzyme involved in the detoxification of H_2O_2 with the help of the endogenous antioxidant GSH (L ü et al., 2010). In this study, we found decrease in the activities of SOD, CAT and glutathione metabolizing GPx with concurrent depletion of GSH on ethanol treatment, indicating the increased oxidative stress which has been implied in the pathogenesis of alcohol-related diseases. Eugenol rich fraction of clove has earlier been reported to mitigate thioacetamide induced liver damage (Ali et al., 2014). Interestingly, *Clovinol* too could combat the oxidative stress as was evident in significant enhancement of the activities of these enzymes with significant replenishment of the endogenous antioxidant GSH.

Though nitric oxide ($NO^{\bullet}$) production is a protective mechanism at lower concentrations, it might cause tissue damage at higher levels (Hon et al., 2002). Stable metabolites of NO (nitrites, nitrates and peroxynitrites) were found to be increased in alcoholics, in which potent oxidants like peroxynitrite mediate most of the toxicity among alcohol abuse (Radi et al., 2002). Enhanced NO• biosynthesis *via.* iNOS may also contribute to platelet aggregation, endothelial dysfunctions, and even motor disturbances (Wang et al., 1995). NO• can also diffuse

freely into the mitochondria and reacts rapidly with intra-mitochondrial superoxide (Radi et al., 2002). Our results are in agreement with these findings and show an increase in NO levels with upregulation of iNOS. But *Clovinol* could effectively reduce the NO level and downregulate iNOS expression suggesting its efficacy in reversing nitrogen free radicals induced oxidative stress.

During *Clovinol* supplementation, a marked reduction in lipid peroxidation gives evident from the decrease in TBARS values, probably due to better scavenging of free radicals. The involvement of free radicals in the peroxidation of membrane phospholipids and formation of lipid peroxide or hydrogen peroxide has been reported in various stages of liver pathogenesis (Bianchi, 1997). Significantly high TBARS values in animals treated with ethanol to induce hepatocyte damage with generation of cytokines were evident in the present study also.

The above discussed results have been found to be supported by histopathological analysis. In ethanol treated rats, swollen irregular hepatocytes containing fat droplets and degenerative hepatocytes were found. In *Clovinol* treated rats, hepatocytes were regular, though multinucleated hepatocytes indicating regeneration were present along with mild Kupffer cell proliferation and absence of inflammation/ necrosis/ hemorrhage or cholestasis. All these confirmed that *Clovinol* could improve the liver health even in the cases of chronic alcohol exposure.

In conclusion, we found that *Clovinol* could effectively confer hepatoprotection against ethanol induced toxicity to liver. Alcohol metabolism generates reducing equivalents, induces CYP2E1, generating ROS. Excess ROS would increase the oxidative stress, damaging cells. As a consequence of increased oxidative stress, lipid peroxidation increases and GSH is depleted (Wu and Cederbaum, 2003). *Clovinol* was found to reduce all these changes. *Clovinol* is polyphenol-enriched and the antioxidant potential of the phenolics are attributable to its reducing potential, free radical scavenging and decomposition of peroxides (Bettaieb et al., 2010). *Clovinol* was also found to suppress iNOS induction downregulating TNF-α and interfering with NF-κB activation (Rodrigues et al., 2009). This in turn, downregulated IL-6 and hence ameliorating nitroso-oxidative stress and inflammatory response evoked by ethanol. Hence, *Clovinol*, the taste and odour minimised water soluble powder, might form an effective therapeutic strategy and/or functional ingredient against ethanol induce damage to liver. The dosage in rats when extrapolated to humans, as regulated by US FDA guidelines (2005) was found to be 13 mg/kg body weight per day which is of clinical significance.

Conflict of Interest

Authors disclose the conflict of interest. *Clovinol*® is the patent pending and registered formulation of M/s Akay Flavours & Aromatics Pvt Ltd, Cochin, India.

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