

Eugenol-rich Fraction of *Syzygium aromaticum* (Clove) Reverses Biochemical and Histopathological Changes in Liver Cirrhosis and Inhibits Hepatic Cell Proliferation

ORIGINAL
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Background: Dried flower bud of *Syzygium aromaticum* (clove) is rich in eugenol, an antioxidant and antiinflammatory compound that can protect liver against injury. Clove, besides eugenol, also contains other pharmacologically active phytochemicals such as β -sitosterol and ascorbic acid. This study reports the effect of eugenol-rich fraction (ERF) of clove on liver cirrhosis induced by thioacetamide.

Methods: Cirrhosis of the liver, which predisposes to hepatocellular carcinoma, was induced by administering thioacetamide (0.03%) in drinking water for 16 weeks. Cirrhotic animals were divided into two groups; the treated group was administered ERF for 9 weeks, one week after discontinuation of thioacetamide, while the other group received normal saline for a similar duration of time.

Results: The treatment with ERF, as determined by histopathology and through a battery of biochemical markers of hepatic injury, oxidative stress and drug metabolizing enzymes, significantly ameliorated the signs of liver cirrhosis. It lowered the elevated levels of alkaline phosphatase, γ -glutamyl transferase and other biochemical changes in liver cirrhosis. Histopathology of the liver corroborated the effect of ERF with biochemical findings. ERF treatment further inhibited cell proliferation, as demonstrated by reduced [³H]-thymidine uptake.

Conclusions: Data provide evidence supporting the protective action of ERF on liver cirrhosis. The study assumes significance because cirrhosis predisposes the liver to cancer, which is characterized by abnormal cell proliferation. ERF in this study is reported to inhibit hepatic cell proliferation and at the same time decrease oxidative stress, which might be the mechanism of protection against liver cirrhosis. (J Cancer Prev 2014;19:288-300)

Key Words: Liver, Fibrosis, Cirrhosis, Clove, Cell proliferation

INTRODUCTION

Eugenol, a methoxyphenol component of clove (*Syzygium aromaticum*, Family *Myrtaceae*), has been reported for a number of pharmacological effects, including the antioxidant, anti-inflammatory, analgesic, anesthetic, antipyretic, antiplatelet, antianaphylactic, antidepressant, anticonvulsant, antihyperglycemic, antibacterial, antifungal and antiviral effects. The uses and benefits of eugenol isolated from various sources including *Syzygium aromaticum* and *Ocimum sanctum* are many.¹ Eugenol and its derivatives in clove are potent antioxidants,² which could be due to their ability to form complexes with reduced metals;

eugenol and structurally related compounds have been reported to inhibit iron-mediated lipid peroxidation and autooxidation of Fe²⁺ ion. Recently the effect of eugenol on hepatic glucose production and AMP-activated kinase signaling in hepatocyte and C57BL/6J mice³ suggested eugenol or eugenol-containing fractions as promising therapeutic agent. In the study, eugenol effectively ameliorated hyperglycemia through inhibition of hepatic gluconeogenesis by modulating calcium calmodulin dependent kinase kinase-AMP activated kinase-CREB binding protein signaling pathway. In the liver, eugenol has been investigated for its antioxidant, antiinflammatory and DNA protective properties.⁴

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The dried flower bud of *Syzygium aromaticum* is used in food for aroma, and in medicine for its carminative, antispasmodic,⁵ anticarcinogenic⁶ and other properties. It is known to inhibit platelet aggregation and alter arachidonic acid metabolism in human platelet,⁷ besides showing the antiviral activity against herpes simplex,⁸ and antioxidant action in aflatoxicosis.⁹ The essential oil extracted from clove is used as topical application to relieve pain and promote healing.¹⁰ Clove is a rich source of free eugenol, eugenol acetate, caryophyllene, sesquiterpene ester, phenylpropanoid and β -caryophyllene,^{11,12} besides tannins and triterpenoids.^{13,14} It is used topically as analgesic in dental clinic and is antinociceptive, which might be due to α_2 -adrenergic and opioidergic receptors, but not serotonergic receptor.¹⁵ Eugenol or 4-allyl-2-methoxyphenol (molecular weight: 164.20), the major constituent in clove, is an allyl chain-substituted guaiacol (2-methoxyphenol) which can also lower uric acid and is indicated in the treatment of rheumatoid arthritis.¹⁶ It can induce apoptosis via the caspase-dependent pathway in human osteosarcoma cell.¹⁷ Eugenol is also known for its dose-dependent suppressive and enhancing effects on the immune response.¹⁸ Clove oil and eugenol microemulsions have been found beneficial in fatty liver and dyslipidemia.¹⁹ The formulation in microemulsion provides a delivery system for oral administration of eugenol in homogeneous, water-based and thermodynamically stable dosage form. In CCl_4 -induced hepatotoxicity in rats, eugenol protects liver injury when given concurrently or soon after the CCl_4 treatment.²⁰ A high dose of eugenol in its pure form, however, augments injury.

The cirrhosis of the liver is characterized by over-expression of extracellular matrix proteins (EMP) from hepatic stellate cells (HSC), which ordinarily store vitamin A and upon activation lead to liver fibrosis²¹; the fibrosis of the liver progresses to cirrhosis, which predisposes liver to cancer. Plant-derived molecules such as silybin have been found to inhibit the progression of liver fibrosis and hence cirrhosis and cancer by various mechanisms. Silybin, for example, reduces the pro-fibrogenic potential of HSC in the liver by inhibiting the phosphorylation of I κ B,²² the small protein that inhibits the activation of NF- κ B.²³ Recently, chebulagic acid and chebulinic acid, two hydrolysable tannins of tropical almond (*Terminalia chebula*) fruits, have been reported to exhibit cytotoxic and antiproliferative effects on HSC-T6 cells at a concentration as low as 20 μmol .²⁴ These tannins considerably decreased the synthesis of collagen, procollagen I ($\alpha 1$) and III, and suppressed the activation of plasminogen activator inhibitor-1, consequently facilitating the resolution of fibrosis through Smad pathway. Other bioactive principles in

clove including eugenol²⁵ and sanguinarine, a benzophenanthridine alkaloid,²⁶ might ameliorate the inflammatory response, fibrosis and cirrhosis. This study reports the curative and antiproliferative effect of eugenol-rich fraction (ERF) of clove in rats treated with a liver cirrhosis protocol and attributes the inhibitory effect to the decrease in production of free radicals.

MATERIALS AND METHODS

Clove was procured from the local market. ERF was prepared by crushing the clove in an iron mortar and extracting the fraction in Soxhlet at 60-70°C for 6 hours continuously in 50% ethanol. The extracted material was evaporated to dryness under reduced pressure at 40-50°C and suspended in methanol (2 mg/mL) for chemical fingerprinting. The fraction was stored in an autoclaved airtight container in refrigerator. Various chemicals and reagents used in this study were of highest purity grade purchased from standard commercial sources in India.

1. Characterization of the fraction by high-performance thin-layer chromatography and thin-layer chromatography autographic assay

The fraction (2 mg/mL methanol) was filtered through a 0.45 micron membrane filter and analyzed using a CAMAG high-performance thin-layer chromatography (HPTLC) system equipped with an automatic thin-layer chromatography (TLC) sampler Linomet 5, TLC scanner 3 and integrated software WinCats version 3 (CAMAG, Muttenz, Switzerland). For chromatographic separation, the sample was applied onto a pre-coated silica gel HPTLC plate of 0.20 mm thickness with an automatic TLC sampler (Linomet 5) under N_2 gas flow. The sample was applied 10 mm above the bottom of the plate and 10 mm from the side; the space between the two spots was 8 mm. Linear ascending development was carried out for 30 minutes at room temperature in CAMAG twin trough chamber (10 × 10 cm) pre-saturated with mobile phase consisting of toluene, ethyl acetate and formic acid in a 60 : 40 : 8 ratio for eugenol; hexane : ethyl acetate : acetic acid (75 : 25 : 1) for β -sitosterol; and acetonitrile : acetone : water : acetic acid (80 : 5 : 15 : 2) for ascorbic acid. The length of the chromatogram run was 8 cm. For the development, the plate was dried in an oven at 60°C for 5 minutes and the post-chromatographic derivatization (of eugenol and β -sitosterol) was done in 5% anisaldehyde, followed by heating at 110°C for 2 minutes. The plate was scanned in absorption mode at 210-500 nm. Standards (eugenol, β -sitosterol and ascorbic acid) were prepared in methanol and used to

identify respective components in the fraction.

The presence of antioxidants in the fraction was demonstrated by TLC autography.²⁷ Briefly, ERF (5 μ L in methanol) was spotted on silica gel plate (10 \times 10 cm) and run on TLC plate in a mixture (solvent) of hexane, ethyl acetate and glacial acetic acid (75 : 25 : 0.5) for 20 minutes. The plate was dried and sprayed with 0.2% 2,2-diphenyl-2-picrylhydrazyl (DPPH) in methanol and examined. Yellow spot against purple background indicated the presence of antioxidants in the extract. The radical scavenging activity of ERF against stable DPPH was also determined spectrophotometrically. In the presence of antioxidant, DPPH is reduced and changes color from deep violet to light or yellow, which can be measured at 517 nm. Free radical scavenging activity of ERF was determined according to the method of Yamaguchi et al.²⁸ in a reaction mixture consisting of 0.1 mL ERF (5 mg/mL methanol), 0.3 mL methanol and 0.4 mL DPPH (0.3 mM in methanol). The mixture was vigorously shaken and incubated in dark at room temperature for 10 minutes. The optical density (OD) was determined at 517 nm. Blank was consisted of 0.4 mL DPPH and 0.4 mL methanol. Pyrogallol was used as positive control. The scavenging activity of DPPH was calculated as follows: scavenging activity (%) = [(OD of the blank - OD of the sample)/OD of blank] \times 100.

2. Animals and treatment protocol

Inbred female rats (Wistar strain) weighing 140-180 g were used throughout the study. Animals were housed in polypropylene cages at $21 \pm 2^\circ\text{C}$, > 40% humidity and a 12-hour light-dark cycle, and provided with pellet diet and water *ad libitum*. Animals were randomized (N = 5) and acclimatized for a week before starting the treatment protocol. The study was approved by the Institutional Animal Ethics Committee (No. 121), duly constituted by the Committee for the Purpose of Control and Supervision of Experiments on Animals.

The experimental animals were divided into two groups, Gp I and II. Gp I was administered normal saline for 16 weeks and Gp

II was given thioacetamide (TAA) (0.03% in drinking water) for a similar duration to induce cirrhosis. After the completion of treatment with TAA, animals were left untreated for one week to ensure elimination of TAA metabolites that might interfere with the drug, and thereafter, Gp I was subdivided into two subgroups, IA and IB. Subgroup IA, the normal control group, was continued with normal saline, while subgroup IB was administered ERF for 9 weeks. Gp II was also divided into two subgroups: IIA and IIB. While no treatment was given to IIA, IIB (cirrhotic animals) was administered ERF for 9 weeks, as shown in Figure 1. The treatment protocol was terminated by sacrificing the animals from both treatment and control groups at the same time point. The dose of ERF to be administered orally was prepared in 1% gum acacia in distilled water. ERF was administered at a dose of 80 mg/100 g body weight for the specified duration of time. The dose was based on preliminary studies done in our laboratory on the eugenol-rich fraction, and not pure eugenol, which might be toxic at high doses. Administration of ERF at a dose specified in this study did not show any sign of toxicity on vital organs in any animal. Animals were fasted 18 hours before starting the treatment. Chemicals and consumables used in this study were purchased from standard commercial sources.

3. Gross morphology and histopathology of the liver

Rats, sacrificed by cervical dislocation, were dissected to collect the liver. Gross morphological examination of the liver obtained from the treated and control rats was done and documented. For histology, a small piece of the left lobe of liver was removed and fixed in 10% buffered formalin, embedded in paraffin and cut into thin films. Sections were processed and stained in hematoxylin-eosin according to the standard protocol. Histological grade of fibrosis was determined using the Metavir scoring system. A pathologist, who was not aware of the treatment groups, examined the slides.

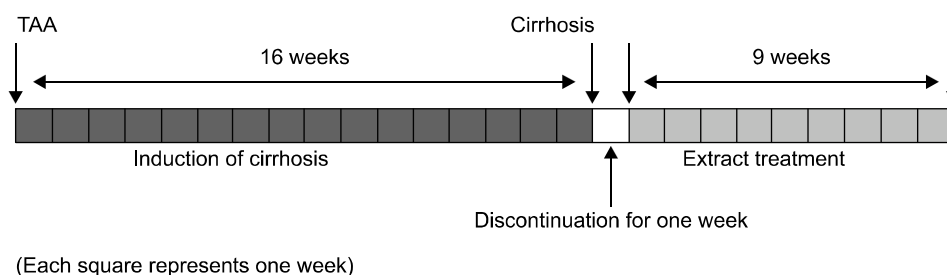


Figure 1. Treatment plan. Cirrhosis was induced in rat by administering thioacetamide (TAA) in drinking water (0.03%) for 16 weeks, followed by the treatment with eugenol-rich fraction (ERF) for 9 weeks. ERF was administered 1 week after the TAA treatment was discontinued to ensure elimination of metabolites of TAA. Dose and mode of treatment have been described in MATERIALS AND METHODS.

4. Biochemical analyses

Method for the preparation of sample for biochemical analysis has been described in one of our earlier publication.²⁹ Serum alkaline phosphatase (ALP) and hepatic γ -glutamyl transferase (GGT) were determined according to spectrophotometric methods described by Kind and King³⁰ and Orłowski and Meister,³¹ respectively, using diagnostic kits. Succinate dehydrogenase (SDH) was determined by the method of Kun and Abood.³² Briefly, in 5 mL calibrated centrifuge tube, 0.1 M phosphate buffer (0.5 mL, pH 7.4) was mixed with 0.2 M sodium succinate (0.5 mL) and 10% homogenate (0.2 mL). Final volume was made up to 1 mL with distilled water and 1.0 mL of freshly prepared triphenyl-tetrazolium chloride (0.1%) was mixed with the mixture and incubated at 38°C for 20 minutes. After incubation, acetone (7 mL) was added to the mixture and vigorously shaken. Precipitate was centrifuged and clear supernatant was drawn off for measuring absorbance at 420 nm. The enzyme activity was expressed as μg tetrazolium reduced/mg tissue.

Catalase was measured by the method described by Claiborne.³³ The assay mixture consisted of 1.99 mL phosphate buffer (0.05 M, pH 7.0), 1.0 mL of H_2O_2 (0.0019 M) and 10 μL phenazine methosulfate (10% w/v) in a total volume of 3.0 mL in quartz cuvette. Decrease in absorbance due to disappearance of H_2O_2 was recorded at every 30 seconds up to 3 minutes at 230 nm. The activity was calculated using extinction coefficient and expressed as nmole H_2O_2 consumed/min/mg protein. Glutathione peroxidase, glutathione reductase (GR) and glutathione S-transferase (GST) were also measured according to published protocols.³⁴⁻³⁶ The GR and glutathione peroxidase activity was calculated using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ and expressed as nmole NADPH oxidized/min/mg protein. GST was expressed as nmole 1-chloro-2,4-dinitrobenzene conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. Xanthine oxidase (XO) was determined according to the method of Stripe and Della Corte³⁷ and enzyme activity was expressed as μmole uric acid produced/mg protein.

Lipid peroxidation (LPO) was measured from the tissue homogenate by the method described by Bernheim et al.³⁸ This method measures the concentration of malondialdehyde. Trichloroacetic acid was used in this method to eliminate interference caused by the malondialdehyde precursors. Briefly, the reaction mixture containing phosphate buffer (1.8 mL, 0.1 M, pH 7.4) and tissue homogenate (0.2 mL, 10%, w/v) was incubated at 37°C in a shaker water bath for 1 hour. The reaction was terminated by trichloroacetic acid (1.0 mL, 10%) followed by

thiobarbituric acid (1.0 mL, 0.67%) and kept in boiling water bath for 20 minutes. The reaction mixture was cooled in ice and centrifuged at $2,500 \times g$ for 10 minutes. The absorbance of the supernatant was taken at 432 nm against reagent blank. LPO was expressed as nmole of malondialdehyde formed/mg protein at 37°C using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. Hepatic glutathione (GSH) was measured by the method of Jollow and coworkers³⁹ by precipitating the homogenate (1.0 mL) with sulphosalicylic acid (4%, 1.0 mL). The sample was kept for 1 hour at 4°C followed by centrifugation at $1,200 \times g$ for 15 minutes. Absorbance of the assay mixture, which consisted of supernatant (0.1 mL), phosphate buffer (2.7 mL, 0.01 M, pH 7.4) and freshly prepared (0.2 mL) 5,5'-dithiobis-2-nitrobenzene (4 mg/mL in 0.1 M phosphate buffer, pH 7.4) in a total volume of 3.0 mL was determined at 412 nm. Concentration of glutathione was expressed as $\mu\text{mole/g}$ liver.

5. Cell proliferation assay

The proliferation of hepatocytes was measured by [³H]-thymidine uptake method. Briefly, the treatment group was administered ERF orally for three consecutive days; TAA was given 45 minutes after administering the last dose of ERF on day 3. In the positive control (TAA alone treated group), TAA was administered as a single intraperitoneal injection (400 mg/kg body weight) in saline. After 16 hours of TAA administration, animals were injected with [³H]-thymidine (20 mCi/0.2 mL saline/100 g body weight, intraperitoneal injection) and sacrificed exactly after 2 hours by cervical dislocation. The normal control rats were administered with saline. In each group, liver was excised quickly, washed in ice-cold saline (0.9% NaCl) and processed for the estimation of hepatic DNA.

The DNA was isolated and incorporation of [³H]-thymidine into DNA was determined by the method of Smart et al.⁴⁰ Briefly, rats given [³H]-thymidine (20 $\mu\text{Ci}/200 \mu\text{L}/100 \text{ g}$ body weight, intraperitoneal injection) 16 hours after TAA administration, were sacrificed two hours after [³H]-thymidine injection. About 2 g of liver was taken to make 20% homogenate in 10 mL cold water. An equal volume of ice-cold trichloroacetic acid (10%) was added to the homogenate and the mixture was centrifuged at 5,000 rpm for 10 minutes. Supernatant was discarded and the precipitate was dissolved in 5 mL ice-cold trichloroacetic acid (5%) and spun at 5,000 rpm for 10 minutes. Supernatant was discarded again and the pellet was dissolved in 5 mL of 10% ice-cold perchloric acid (PCA). The mixture was kept for 18 hours at 4°C and centrifuged after 18 hours at 5,000 rpm for 10 minutes. The pellet was collected and mixed with 5 mL ice-cold PCA (5%) and

centrifuged at 5,000 rpm for 10 minutes at 4°C. The precipitate was incubated with 5 mL warm PCA (10%) in boiling water bath for 30 minutes. Sample was centrifuged again and the supernatant was filtered through a Whatman-50 filter paper to get clear solution, which was used for the estimation of DNA and counting radioactivity. For counting radioactivity, solution (200 µL) was added to the scintillation vial containing 5 mL scintillation fluid and counted in the scintillation counter (LKB-Wallace1410; Pharmacia Biotech, Espoo, Finland). The amount of DNA in the filtrate was estimated by diphenylamine method.⁴¹ Amount of [³H]-thymidine incorporated into DNA was expressed as disintegration/min (DPM)/µg DNA. The scintillation fluid was prepared by dissolving naphthalene (52 g), 2,5-diphenyloxazole (3.25 g) and 1,4-bis (5-phenyl-2-oxazole) benzene (0.065 g) in a mixture containing 1,4-dioxane (250 mL), toluene (aldehyde free, 250 mL) and methanol (150 mL). Scintillation

fluid was prepared immediately before use and kept in a dark bottle.

6. Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was done using one-way ANOVA (Dunnett's test). *P* < 0.05 was taken as the level of significance.

RESULTS

1. Liver weight-body weight ratio and gross morphology and histopathology of the liver

TAA in drinking water (0.03%) for 16 weeks induced liver cirrhosis in rat, which was evident by the gross morphological examination of the liver, its histology and a battery of biochemical tests. Rats treated with TAA for 16 weeks showed

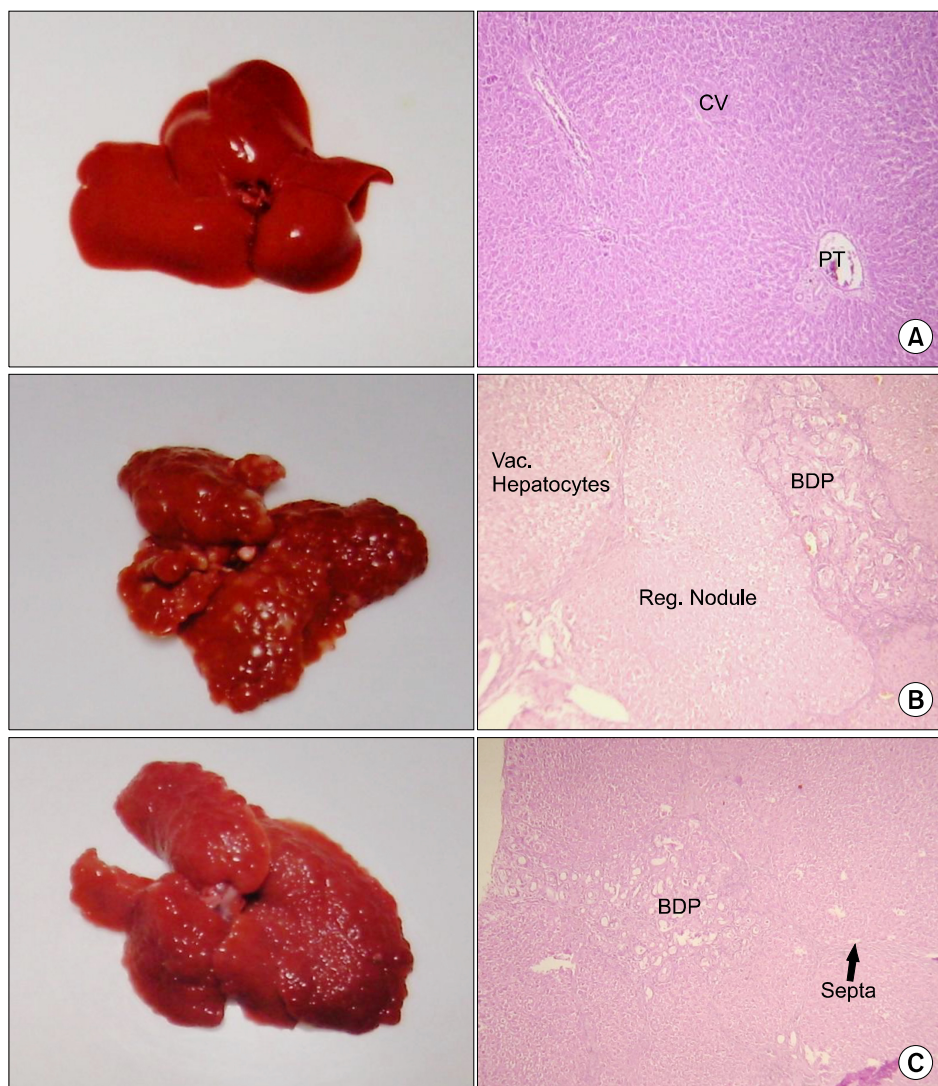


Figure 2. Gross morphology and histopathology of the liver in (A) control, (B) cirrhotic, and (C) ERF-treated rats. PT, portal triad; CV, central vein; BDP, bile duct proliferation.

signs of liver cirrhosis, which are shown in Figure 2. Representative histopathological section of normal liver showed a normal lobular architecture with central vein and radiating hepatic cords (Fig. 2A). The section of the liver from cirrhotic rats showed proliferating bile ducts in the septal area and a large nodule of regenerating hepatocytes (Fig. 2B). Vacuolization of hepatocytes was evident in the upper left corner of the tissue section obtained from cirrhotic animals. In this slide, septa were comparatively thick (Fig. 2B). Hepatic parenchyma in ERF treated rats was more like normal and showed a focus of bile duct proliferation (Fig. 2C). The liver of TAA treated rats shrunk considerably and liver weight-body weight ratio increased when compared with control rats (Fig. 3A). Tests reported an increase in activity of serum ALP and hepatic GGT (Fig. 3B and 3C), the two hepatic biochemical markers in liver cirrhosis. ERF reversed these changes. Treatment with ERF caused a significant decrease in liver weight-body weight ratio, decreased the activity of ALP and GGT, and caused a considerable improvement in hepatic tissue architecture when compared with the cirrhosis group. Nodules, which are a prominent feature of liver cirrhosis, reduced considerably in ERF treated rats.

2. Oxidative stress markers and enzymes involved in drug metabolism

LPO is a prominent feature in rats with cirrhosis (Fig. 4A). The increase in LPO was accompanied by a concomitant decrease in glutathione, which indicate oxidative stress, an important factor for the development of liver fibrosis and cirrhosis. ERF significantly decreased elevated LPO in the whole liver homogenate and increased glutathione (Fig. 4A). The activity of GR, enzyme involved in glutathione metabolism, was found to be decreased in liver cirrhosis (Fig. 4B). Glutathione peroxidase (Fig. 4C) and catalase (Fig. 4D), the two enzymes involved in peroxide metabolism, were also decreased in this group, as was the SDH (Fig. 4E). ERF significantly increased the activity level of these enzymes. In cirrhotic rats, there was an increase in activity of XO and GST, the two enzymes involved in drug metabolism (Fig. 4F and 4G). Both of these enzymes were significantly low in ERF treated rats when compared with cirrhotic animals.

3. [³H]-thymidine uptake

The uptake of radiolabeled thymidine (³H]-thymidine) by hepatic DNA was 20,000 dpm/ μ g DNA in normal control group,

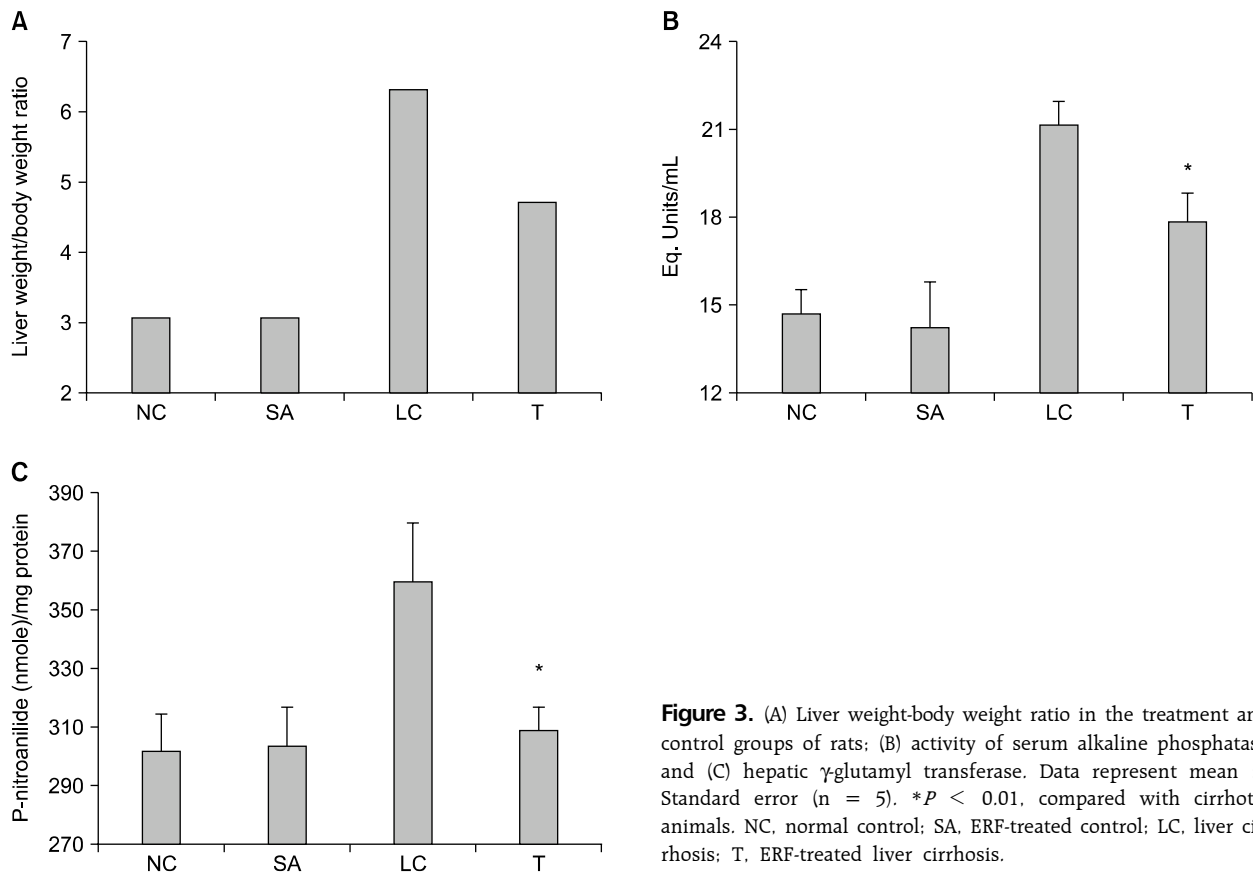


Figure 3. (A) Liver weight-body weight ratio in the treatment and control groups of rats; (B) activity of serum alkaline phosphatase and (C) hepatic γ -glutamyl transferase. Data represent mean \pm Standard error (n = 5). * $P < 0.01$, compared with cirrhotic animals. NC, normal control; SA, ERF-treated control; LC, liver cirrhosis; T, ERF-treated liver cirrhosis.

which increased to 30,000 dpm in rats treated with TAA. ERF significantly reduced the increased uptake of thymidine (Fig. 5). In this group (T, Fig. 5), thymidine uptake was much less than the value reported in control.

4. High-performance thin-layer chromatography finger print and thin-layer chromatography autography of eugenol-rich fraction

The chemical fingerprint of ERF revealed high concentration of eugenol. Out of nine peaks observed when TLC plate was scanned at 210-500 nm, eugenol peak occupied the highest area (Fig. 6A). The other components in ERF, which included β -sitosterol and ascorbic acid, are not shown as they are commonly present in many plant species. ERF was further demonstrated for antioxidant activity with the help of TLC autographic assay. The presence of yellow spot against a purple background confirmed the presence of antioxidant in the fraction (Fig. 6B). Quantification of data showed 90% free radical scavenging activity when compared with pyrogallol, a reference antioxidant used as control in autographic assay (Fig. 6C).

DISCUSSION

Cirrhosis of the liver is end stage of liver fibrosis which if persist may lead to liver cancer.⁴² It is characterized by the deposition of EMP in the tissue in excess. In rat, cirrhosis induced by TAA produces many changes own to cirrhosis in human.⁴³ In this study, liver cirrhosis was induced in rat by TAA, and confirmed by gross morphology and histopathology of the liver and a number of biochemical parameters. The body weight of animals suffering from cirrhosis reduced considerably, which might be attributed to alterations in nutrient absorption and metabolic utilization due to hepatic stress; the portal hypertension and impaired bile acid metabolism are known to affect nutrient utilization,⁴⁴ and hence lower the body weight. Activity of ALP and GGT was increased in liver cirrhosis, which is in agreement with data reported earlier.^{45,46} ERF protected the liver against these changes. Previous studies on liver have shown the effect of eugenol, the major constituent in ERF, to protect against injury inflicted by TAA (300 mg/kg body weight, intraperitoneal injection, for the last 2 days at 24-hour interval) at a dose of 10.7 mg/kg body weight/day, orally, for 15 days. In this study, animals were sacrificed on 16th day.⁴ Eugenol is also

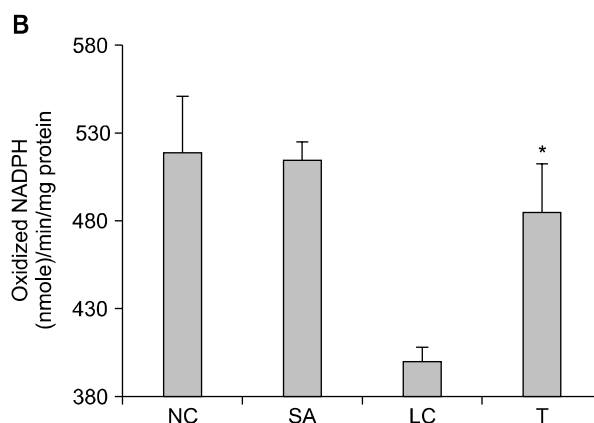
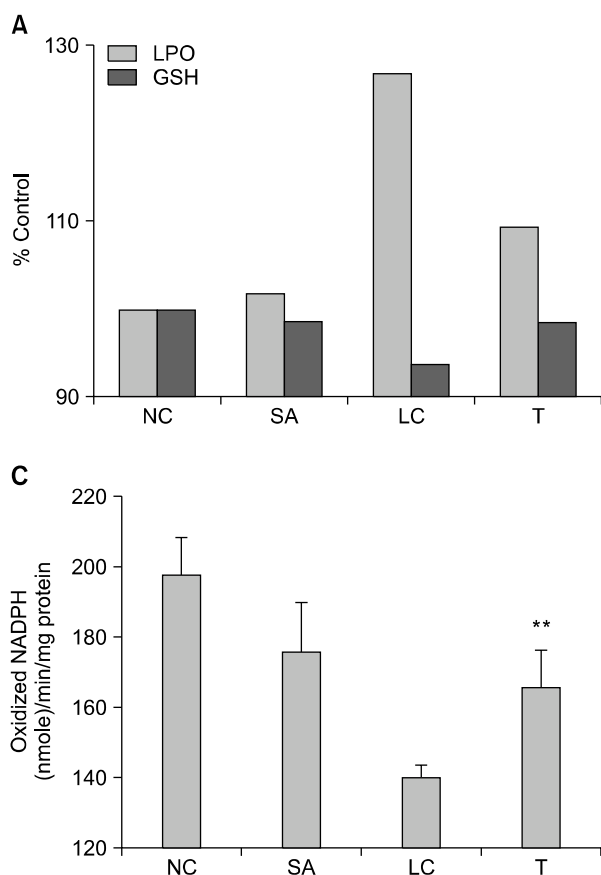


Figure 4. (A) Hepatic lipid peroxidation (LPO) and glutathione (GSH); values are shown as percent of control. Activity of (B) hepatic glutathione reductase, (C) glutathione peroxidase, (D) catalase, (E) succinate dehydrogenase, (F) xanthine oxidase, and (G) glutathione S-transferase. Data represent mean \pm SE ($n = 5$). * $P < 0.01$, ** $P < 0.03$, when compared with cirrhotic animals. NC, normal control; SA, ERF-treated control; LC, liver cirrhosis; T, ERF-treated liver cirrhosis.

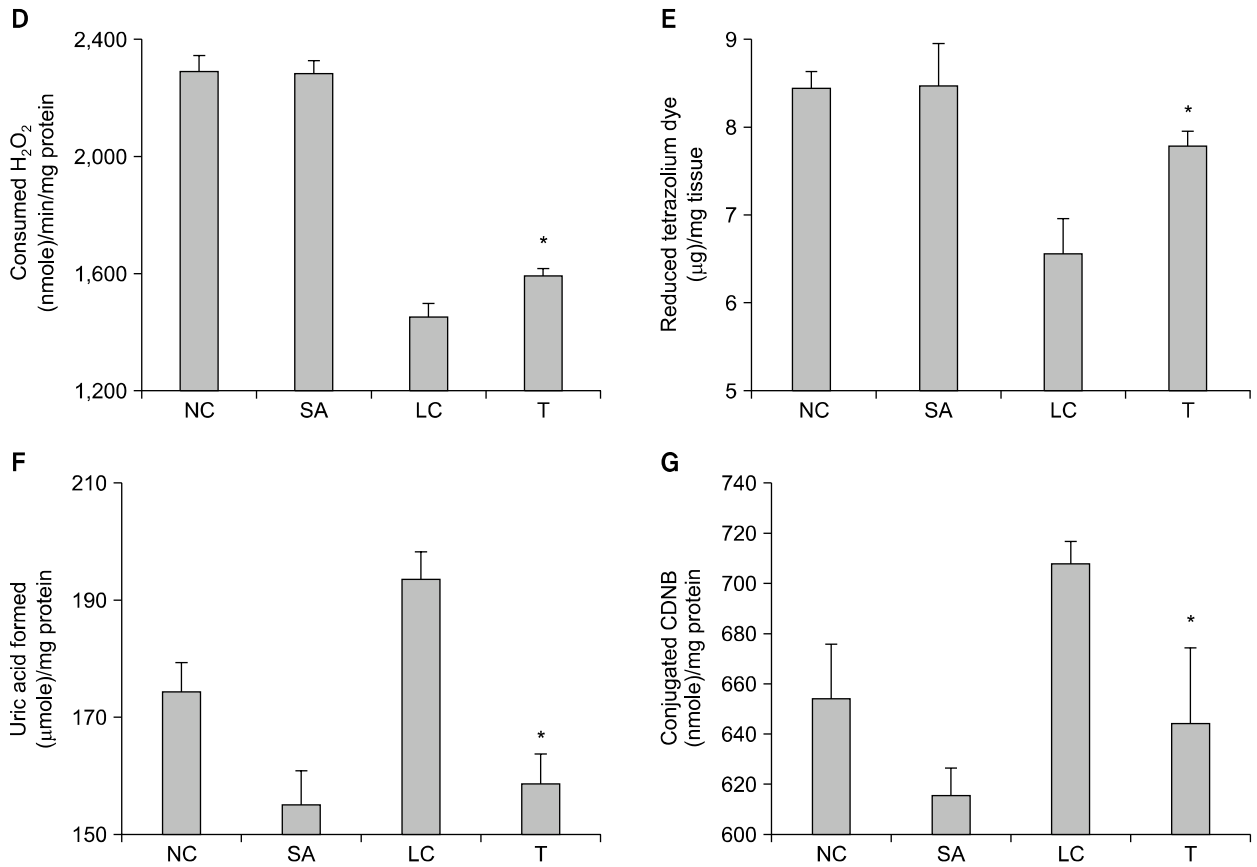


Figure 4. Continued.

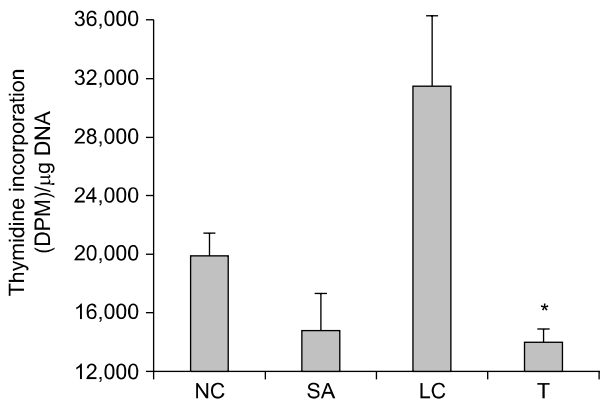


Figure 5. Effect of eugenol-rich fraction (ERF) on [³H]-thymidine uptake by hepatic cell in rats. Data represent mean ± Standard error (n = 5). *P < 0.01, when compared with cirrhotic animals. DPM, disintegration per minute; NC, normal control; SA, ERF-treated control; LC, liver cirrhosis; T, ERF-treated liver cirrhosis.

reported to protect CCl₄-induced liver injury at a low dose (5-25 mg/kg), particularly when given concurrently or soon after rather than much before CCl₄²⁰ at high dose and in pure form, eugenol is reported to augment tissue damage.⁴⁷

A number of phytochemicals in plants of medicinal

importance have been reported to protect the liver against injury by various mechanisms. In one of the mechanisms involving oxidative stress,⁴⁸⁻⁵⁰ the reactive oxygen species either extract hydrogen from unsaturated membrane lipids and initiate LPO or react with the sulfhydryl groups, triggering a chain of peroxidation reactions leading to cell injury and the development of fibrosis and cirrhosis. Eugenol and its derivatives have been reported to protect against oxidative injury and tissue damage,^{2,4,20} which might be a mechanism of action of ERF in liver cirrhosis. Eugenol is also reported to induce apoptosis (via caspase-dependent pathway) in human osteosarcoma cell,¹⁷ which could be important as in liver cirrhosis, which is characterized by the activation of HSC,²¹ apoptosis of activated HSC might be a mechanism to protect the liver against cirrhosis; activated HSCs produce EMP and cause fibrosis. Active ingredients of plant origin, such as silybin, have been reported to reduce the pro-fibrogenic potential of HSC in the liver by inhibiting phosphorylation of IκB,²² the small protein that inhibits the activation of NF-κB,²³ a signaling molecule involved in apoptosis. In a recent study,⁵¹ eugenol is reported to trigger apoptosis in breast cancer cells through E2F1/survivin down-

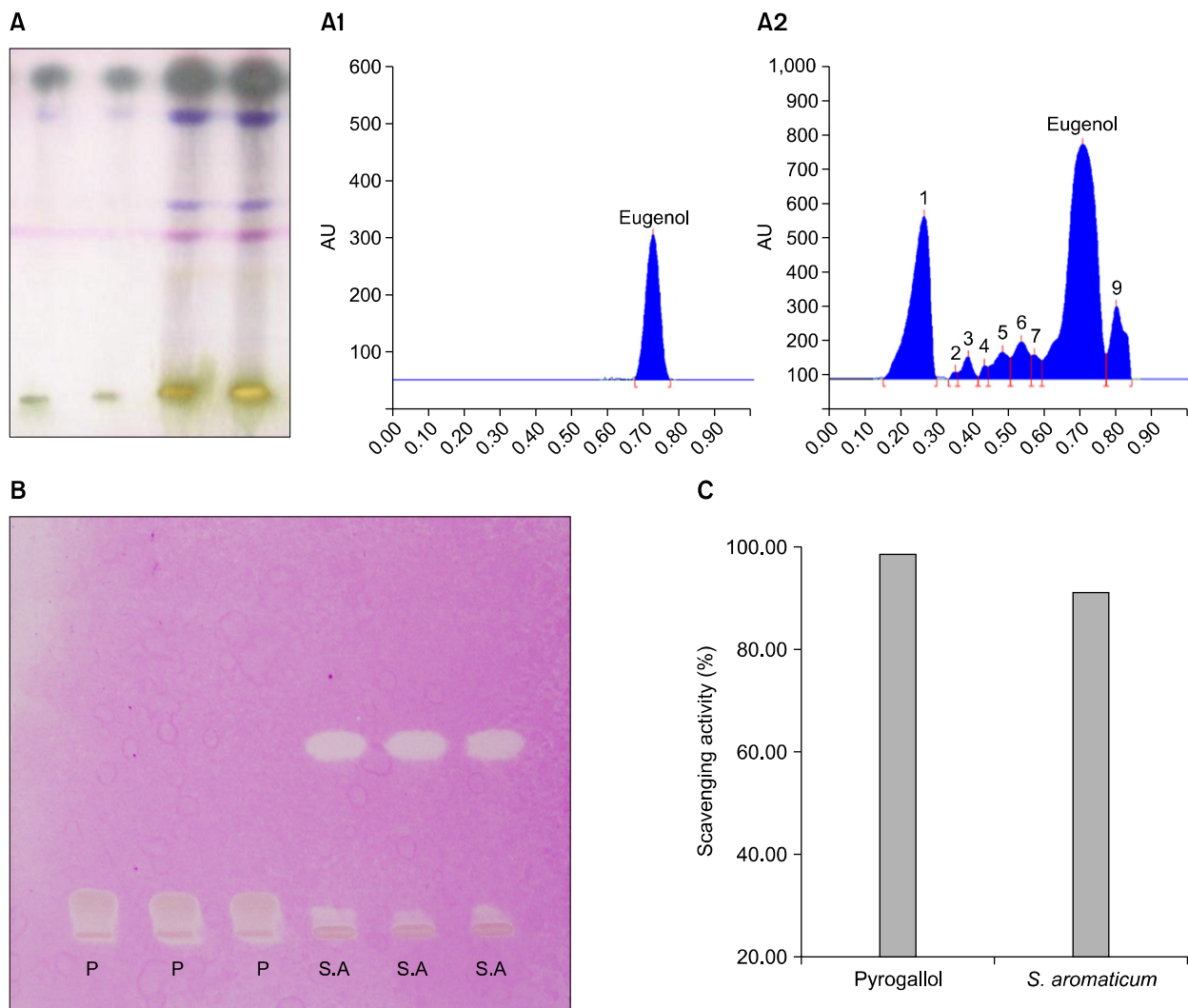


Figure 6. (A) Photographic images of high-performance thin-layer chromatography (HPTLC) plates showing the fingerprint of eugenol in eugenol-rich fraction (ERF). Eugenol (λ_{\max} : 228 nm, R_f : 0.77 ± 0.04) and its respective peak in standard (A1) and fraction (A2) are shown; (B) thin-layer chromatography (TLC) autographic image; and (C) free radical scavenging activity of ERF, compared with pyrogallol. P, pyrogallol; S.A, *Syzygium aromaticum*

regulation. A probe into the mechanistic aspects suggested that eugenol precludes carcinogenesis in mouse by preventing oxidative stress and inflammation and by inducing apoptosis. Eugenol markedly inhibited proteins involved in tumor promotion and inflammation: ornithine decarboxylase, inducible nitric oxide synthase and cyclooxygenase-2, and the levels of proinflammatory cytokines (interleukin-6, tumor necrosis factor [TNF]- α , and dinoprostone), as well as NF- κ B, which regulates expression of these genes.⁵² The activation of HSC is hallmark of liver fibrosis/cirrhosis, and ERF might act by triggering apoptosis of HSC, thus resolving cirrhosis. Inhibition of TAA-induced hepatocyte proliferation by eugenol supports this hypothesis. Recently, active ingredients in tropical almond fruit, chebulagic

acid and chebulinic acid, have been reported to considerably decrease the synthesis of collagen, procollagen I (α 1) and III, and suppress the activation of plasminogen activator inhibitor-1, thus, facilitating the resolution of fibrosis through Smad pathway in HSC-T6 cells at concentration as low as 20 μ mol.²⁴ This study demonstrates a considerable increase in cell proliferation by TAA, which was inhibited by ERF.

Eugenol has been found to attenuate oxidative injury. In a study in Sprague-Dawley rats, eugenol inhibited gentamicin-induced oxidative injury to (renal) tissue and restored normal function at a dose of 100 mg/kg administered 4 days before and 6 days concurrently with gentamicin (80 mg/kg body weight).⁵³ LPO represents a degradative process in the tissue arising from the

production and propagation of free radicals, primarily involving membrane polyunsaturated fatty acids and the production of end products such as malondialdehyde and 4-hydroxynonenal.⁵⁴ Increase in LPO is hallmark of injury caused by free radicals. In acute doxorubicin cardiac toxicity in rats (induced by a single intraperitoneal injection of doxorubicin, 20 mg/kg), eugenol treatment started 2 days before doxorubicin administration and continued for 5 consecutive days significantly decreased LPO with a concomitant reduction in elevated serum creatine kinase and lactate dehydrogenase, restoring the electrocardiographic disturbances from doxorubicin administration.⁵⁵ This study demonstrated a decrease in LPO in ERF treated rats, indicating a common antioxidant mechanism in tissue repair in various tissues, including the liver. In rats with cirrhosis, progressive increase in LPO and a concomitant decrease in GSH have been reported.^{56,57} This study found ERF to cause a significant reduction in peroxidation of lipids, a prominent feature of TAA-induced cirrhosis, with a concomitant increase in GSH and enzymes involved in GSH metabolism, such as GR, which is decreased in liver cirrhosis, was increased after ERF treatment. Eugenol has been earlier shown to inhibit LPO in CCl₄-induced liver injury when given thrice orally, prior to (-1 hour), along with (0 hour), and after (+3 hours) intraperitoneal administration of CCl₄ (0.4 mL/kg). At a dose of 5 or 25 mg/kg, given orally for 3 consecutive days, eugenol did not alter the level of aspartate aminotransferases and microsomal enzymes, cytochrome P450 (CYP) reductase, glucose-6-phosphatase, and xenobiotic-metabolizing enzymes; since eugenol is metabolized and cleared rapidly from the body. The protective effect against CCl₄ toxicity was more prominent when eugenol was given concurrently or soon after, rather than much before CCl₄ treatment. In our study on liver cirrhosis, ERF treatment was given 9 weeks post injury, and a significant improvement in various antioxidant enzymes and enzymes involved in drug metabolism was observed (Fig. 4). ERF might inhibit non-enzymatic peroxidation⁵⁰ due to the presence of antioxidants and the ability to quench free radical and significantly protect against the degradation of CYP during LPO, suggesting its potential use as a therapeutic agent. Typically, a chemical therapeutic agent either inhibits CYP or induces phase II detoxifying enzymes. Eugenol is reported to suppress CYP1A induction by 7,12-dimethylbenz[a]anthracene through decreased aryl hydrocarbon receptor activation and subsequent DNA binding, and also increase the expression and activity of NAD(P)H: quinone oxidoreductase, a major detoxifying enzyme for 7,12-dimethylbenz[a]anthracene, through nuclear factor-erythroid 2 related factor 2 binding to antioxidant response element in

quinone oxidoreductase gene,⁵⁸ suggesting protective effect against genotoxicity, presumably through suppression of 7,12-dimethylbenz[a]anthracene activation and induction of its detoxification.

Glutathione peroxidase and catalase are important enzymes involved in peroxide metabolism. ERF increased the activity of these enzymes. The activity of SDH, a mitochondrial enzyme linked to adenosine triphosphate synthesis,⁵⁹ also decreased in cirrhotic rats, but increased after ERF treatment. Decrease in SDH might be due to deficiency in one or more electron transport chain components in cells of cirrhotic rats. Increase in SDH can be attributed to the antioxidant activity of a molecule.⁶⁰ Eugenol has been demonstrated, both in vitro and in vivo, to have antioxidant property.⁵⁰ This study provides evidence supporting the free radical scavenging activity of ERF, as demonstrated by the DPPH method. Quantification of data showed 90% free radical scavenging activity of ERF, when compared with pyrogallol, an antioxidant used as reference (Fig. 6). Recently, aqueous and ethanolic fractions of clove have been found in macrophages to be immunomodulatory.⁶¹ In vitro evaluation of aqueous and ethanolic constituents of clove extract imparted potential anti- and pro-oxidant effects in cells, depending on the concentration, and on activation state of macrophages themselves at the time of exposure to the extract; although it did not show a distinct cytotoxic activity. In lipopolysaccharide-stimulated cells, the release of TNF- α was significantly affected by the extracts (ethanolic extract was suppressive at all doses tested, while the aqueous extract was so up to 1 μ g/mL and then became stimulatory); in contrast, nearly every dose of either extract appeared to stimulate interleukin-6 release from lipopolysaccharide-treated cells.⁶¹ In an in situ microcirculation assay, eugenol at a dose of 250 mg/kg significantly decreased the number of leukocytes that rolled, adhered and migrated to perivascular tissue, highlighting its role in diminishing excessive leukocyte migration in inflammation.⁶² The histology of the tissue from ERF treated rats indicate diminished leukocyte migration in treated rats.

Eugenol is reported as a dual inhibitor of platelet-activating factor and arachidonic acid metabolism.⁶³ Rats receiving 10 mg eugenol/kg/day for 15 days and subjected to an ischemia (induced for 45 minutes) followed by reperfusion (for 6 hours) showed improvement in both liver function and structure, and inhibition of ischemia/reperfusion-induced increase in TNF- α , as well as hepatic NF- κ B p65 and caspase-3 expression. The treatment also inhibited the degree of loss in GSH and of rise in malondialdehyde in the liver, which is in agreement with the results of

this study (Fig. 4A). However, eugenol at a dose of 100 mg/kg body weight, augmented the damage, displaying significant increases in oxidant, inflammatory and apoptotic proteins in comparison with the levels seen in ischemia/reperfusion-only rats. In our study, ERF, which contained other antioxidants such as the ascorbic acid and β -sitosterol, besides eugenol, did not show any sign of toxicity.

A considerable volume of literature suggests an increase in activity of drug metabolizing enzymes other than CYPs in tissue injury. We found a significant increase in XO and also GST in liver cirrhosis. XO and GST decreased significantly in ERF treated rats. XO is a phase 1 and GST is a phase 2 drug metabolizing enzyme. XO belongs to an enzyme family called the molybdenum iron-sulfur flavin hydroxylase. These proteins generate free radicals while catalyzing the reaction and have been reported to contribute to liver injury.²⁹ The generation of reactive oxygen species in excess of the capacity of cell to counteract its effect can initiate HSC proliferation.⁶⁴ ERF caused a decrease in XO, which suggests possible role of molybdenum iron-sulfur proteins in liver cirrhosis. ERF also decrease GST, the phase 2 drug-metabolizing enzyme also involved in prostaglandin metabolism. Inhibition of GST could be important because of its role in inflammation, and hence liver fibrosis. Eugenol in ERF,²⁵ and possibly sanguinarine, a benzophenanthridine alkaloid,²⁶ might be what could ameliorate the inflammatory response, inhibiting the development of fibrosis and cirrhosis in liver.

ERF, in this study, is suggested to inhibit the progression of liver fibrosis and development of cirrhosis by ameliorating oxidative stress and inhibiting inflammation, as demonstrated by leukocyte migration. ERF counteracted the effect of oxidative stress and, at the same time, reduced the generation of reactive oxygen species by XO. It inhibited LPO at initiation or propagation stage or at both, and might therefore interfere with the chain reactions by trapping active oxygen. Eugenol in ERF may be metabolized to dimer (dieugenol), which is known to inhibit LPO at the level of propagation of free radical chain reaction. The protective effect of eugenol can be compared with α -tocopherol against oxidative stresses. NF- κ B might be an important signaling molecule involved in protection of liver against fibrosis/cirrhosis by eugenol. The bis-eugenol, but not eugenol, has been reported to inhibit the degradation of I κ B α and inhibit expression of inflammatory cytokines at both gene and protein levels.²⁵

Overall, results indicate that ERF suppressed oxidative stress and inflammation and inhibited HSC proliferation, leading to a concomitant reduction in EMP deposition and resolution of liver

cirrhosis. The anti-proliferative and molecular mechanism of eugenol-induced apoptosis has been studied in cancer,⁶⁵ which is also characterized by increased cell proliferation. The study has clinical implication as the animal model of liver cirrhosis in this study mimics alcohol-induced liver cirrhosis in human liver.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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