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Hepatoprotective Effect of *Syzygium aromaticum* Extract on Acute Liver Injury Induced by Thioacetamide

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ABSTRACT
Dried flower bud of *S. aromaticum* contains eugenol, gallic acid, ellagic acid, β-sitosterol, ascorbic acid, and other phytoconstituents. This study reports that a 50 % ethanolic extract of *S. aromaticum* can attenuate liver necrosis in animal model. Liver injury was induced by single intraperitoneal injection of thioacetamide (400 mg/kg b. w.). Oral administration of the extract (800 mg/kg b. w.) for three consecutive days could significantly ameliorate the changes associated with hepatic injury, particularly the levels serum biochemical markers of liver injury and oxidative stress. The protective action may be attributed to eugenol, which has been shown to be present in the test extract. The study has implication in human as rat model of hepatic injury produced by thioacetamide mimics the changes observed in alcohol induced liver injury in human.

Keywords: *S. aromaticum*, eugenol, hepatic enzymes, oxidative stress, HPLC.

INTRODUCTION

Viruses, chemicals, drugs and a number of substances including alcohol have been reported to inflict necrosis of liver, which is a complex process, characterized by simultaneous activation of multiple deregulated pathways that culminate in the loss of cell membrane integrity and thus, the leakage of cellular constituents. [1] Presence of pharmacologically active constituents in human diet such as eugenol (Fig. 1) in *Syzygium* spp. (family, *Myrtaceae*) may be beneficial for liver. Dried flower buds of clove (*Syzygium aromaticum*) are rich source of eugenol, which is reported for antibacterial [2], and anti-inflammatory activity. [3] Traditionally clove has been used as diuretic, odontalgic, stomachic, and condiment with carminative and stimulant activity. [4] Clove is safe to use, and the LD_{50} of one of its major active principle, eugenol, in mice and rats is 3,000 and 2,680 mg/kg, respectively. [5] We propose clove as a dietary constituent that can protect liver against injury.

MATERIALS AND METHODS

Animals

Female wistar rats weighing 160-180 g were housed in large spacious polypropylene cages, and kept in an environmentally controlled room (temperature: 21 ± 2°C, humidity: >40%) with a 12 h light-dark cycle. They were provided pellet diet and water *ad libitum*. The study was approved by the Animal Ethics Committee under proposal no. 232.

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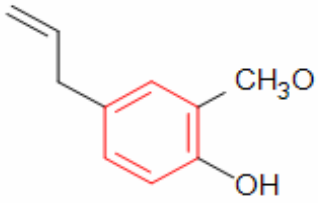

Fig. 1: Chemical structure of eugenol

Material and preparation of the dose

Dried flower buds of *S. aromaticum* were purchased from commercial fruit market in Delhi. The sample was identified, and a specimen voucher number (BRL/A-2/03) was assigned to it. Dried cloves were crushed in an iron mortar, and subjected to extraction in a Soxhlet apparatus at 60-70°C for 6 hours continuously in 50 % ethanol. The extracted material was evaporated to dryness under reduced pressure, and suspended in distilled water in 1 % gum acacia as suspending agent. The suspended extract was stored in sterilized airtight vials in refrigerator till further use. All chemicals used in this study were of the highest purity commercially available in India.

Induction of liver necrosis

Liver necrosis was induced by thioacetamide (400 mg/kg b. w.). [6] Briefly, 24 female rats (Wistar strain) were randomly divided into four groups, each having six animals. Group I was administered normal saline. Group II and IV were given the *S. aromaticum* extract (800 mg/kg b. w./day, orally) for

three consecutive days. On third day of the experiment, Groups III and IV were injected thioacetamide (400 mg/kg body weight; freshly dissolved in 0.9 % NaCl), 45 minutes after administering the last dose. The animals were anaesthetized and the blood from each animal was collected. Subsequently, the animals were euthanized by cervical dislocation after 18 h. Liver was excised quickly, washed in ice-cold saline (0.9 % NaCl), and processed for subcellular fractionation and biochemical and histopathological analysis.

Biochemical analysis

After completion of the experiment, for the analyses of serum biochemical markers of hepatic injury, the blood collected from the abdominal aorta was processed to obtain the serum as per the standard procedure. Serum aminotransferases were determined by Reitman and Frankel [7], alkaline phosphatase by Kind and King [8], and Ɣglutamyl transferase by Orlowaski & Meister. [9] Various oxidative stress related parameters such as lipid peroxidation [10], reduced glutathione [11], glutathione reductase [12], glutathione peroxidase [13], catalase [14], and xanthine oxidase [15] were estimated by described procedures.

Histopathological Analysis

Rat livers were quickly removed after autopsy and fixed in 10 % formalin. The sections were cut and then stained by hematoxylin and eosin. These were observed under microscope.

Characterization of eugenol in extract by HPLC

The HPLC analysis of eugenol standard as well as extract was carried out on Shimadzu (Japan) high-performance liquid chromatography, equipped with a C18 column (300 mm×3.9 mm, alphabond) obtained from Supelco. The isocratic mobile phase was methanol-water (95:5, v/v). The mobile phase was filtered through 0.45µm millipore filters before use. The injection volume was 20 µl and the flow rate was 1.2 ml/min. Eugenol was detected at 254 nm in the standard as well as extract. Retention time for the elution of eugenol was 2.430 min, and the system was operated at room temperature (25°C).

Statistical analysis

All data obtained during the investigation were expressed as mean±S.E.M (n=5). For determining the statistical significance, one-way analysis of variance and Dunnett's test were employed. *p* values less than 0.05 were considered significant. [16]

RESULTS AND DISCUSSION

Considering the variety of factors that can damage hepatic tissue, protection of liver injury by dietary constituents is of utmost significance. Clove is often used in diet, and one of its major ingredients, eugenol, has been reported for beneficial pharmacological actions. In this study, we found the effect of clove extract supplementation on serum biochemical markers of liver necrosis in rat model (Table 1). When compared to the liver necrosis group, clove extract could significantly attenuate the changes in the level of aminotransferases, alkaline phosphatase and γ-glutamyltranspeptidase. Elevation of the plasma levels of cytoplasmic and mitochondrial enzymes is a sensitive indicator of liver necrosis, and has been correlated with an increase in the activity of these enzymes. [17-18] Decreased levels of these enzymes indicate stabilization of plasma membrane and protection of hepatocytes against damage caused by thioacetamide. Liver histopathology (Fig. 2) corroborated with the biochemical findings (Table 1). While inflammatory cell infiltration in the central vein area with dilatation of sinusoids and thrombosed central vein at were reported in necrosis group, clove extract treated rats showed well maintained hepatic parenchyma and mild inflammatory cell filtration.

The efficacy of any hepatoprotective drug is essentially dependent on its capability to either reduce the harmful effects of a hepatotoxin or to maintain the normal physiological mechanisms that are perturbed by the hepatotoxin. As can be seen in Table 2, liver necrosis group exhibited changes suggesting oxidative stress such as increased lipid peroxidation and decreased glutathione (Table 2). Biomarkers of oxidative stress measured in this study (lipid peroxidation, and glutathione) increased in liver necrosis, and the levels attenuated in treated rats. GSH constitutes the first line of defense against free radicals and is a critical determinant of the tissue susceptibility to oxidative damage. In this study, clove extract increased the level of glutathione, which might be due to the antioxidant potential of eugenol in it. This is further supported by the ability of the extract to inhibit lipid peroxidation. Further, a significant decrease was observed in glutathione reductase in necrosis, and an increase in rats treated with the extract. Glutathione reductase is involved in the recycling of GSH at the expense of NADPH.

Xanthine oxidase is a source of reactive oxygen species. We observed an increase in the level of this enzyme, which might contribute to the generation of reactive oxygen species.

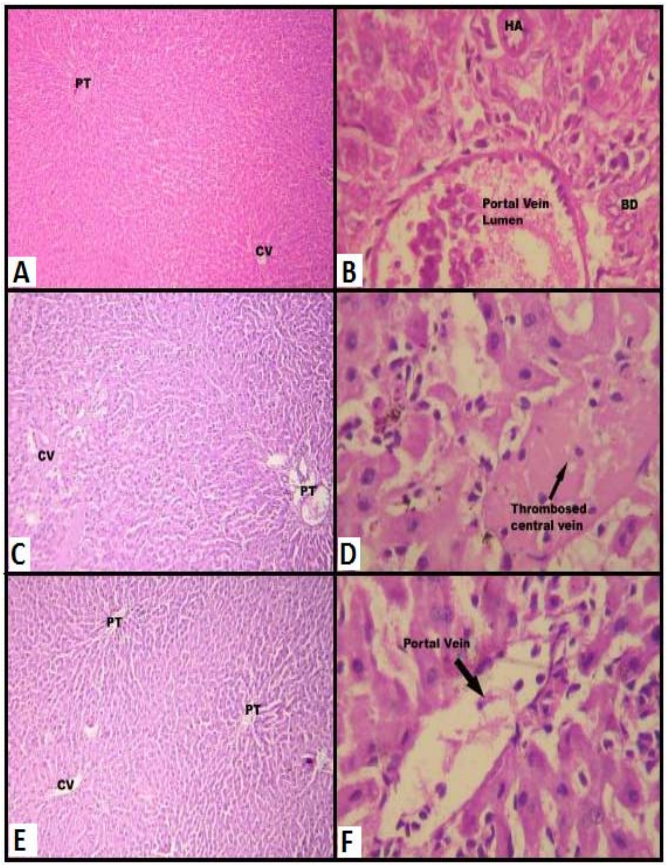

Fig. 2: Histopathological photographs of the liver from the control and treated (S. aromaticum extract) group of rats. (A) Normal control, (B) Section of the liver from extract alone treated rats, (C) Section of the liver from necrosis group, (D) Treated with the clove extract followed by treatment with the hepatotoxin. HA: Hepatic artery, BD: Bile duct. (A: 100x, B: 400x)

Table 3: Effect of S. aromaticum **on antioxidant enzymes of hepatic origin**

Groups	GR	GPx	Catalase
I	752.45±34.24	857.06±72.62	4858.68±375.72
II	745.33±5.20	848.37±25.87	4727.47±188.60
III	500.19±42.79	561.04±19.92	2966.73±276.71
IV	693.37±31.28*	773.72±24.20*	4472.69±342.40*

Data represents mean ± S.E.M (n=5). *p<0.05; statistically significant when compared to group III. GR: glutathione reductase (nmole NADPH oxidized/min/mg protein), GPx: glutathione peroxidase (nmole NADPH oxidized/min/mg protein), and CAT: catalase (nmole H_2O_2

Fig. 3: HPLC chromatogram of eugenol (254 nm). (A) Standard, (B) S. aromaticum extract

Table 1: Effect of the test drug on serum markers of liver necrosis

Groups	ALT	AST	ALP	GGT
I	70.82±0.26	137.87±2.00	17.02±0.24	335.52±4.46
II	70.69±0.43	134.69±1.83	17.03±1.43	344.25±1.68
III	89.57±2.70	156.32±2.32	28.31±0.87	599.95±30.84
IV	79.61±0.31*	144.37±3.03*	20.71±1.26*	400.65±13.80*

Data represent mean ± S.E.M (n=5). *p <0.05; statistically significant when compared to group III. ALT: alanine aminotransferase (Units/ml), AST: aspartate aminotransferase (Units/ml), ALP: alkaline phosphatase (Equivalent Units/ml), and GGT: Jglutamyl transpeptidase (nmole of p-nitroanilide/mg protein). Group I: Normal Control, Group II: Rats treated with *S. aromaticum* extract alone, Group III: Liver necrosis, Group IV: Rats treated with extract of *S. aromaticum* followed by the induction of necrosis.

Table 2: Effect of S. aromaticum **extract on biomarkers of oxidative stress in rat model of liver necrosis**

Groups	LPO	GSH	XO
I	274.59±14.50	1493.16±38.29	93.78±2.16
II	266.83±5.56	1485.07±27.99	87.80±5.23
III	899.97±35.03	1252.39±15.00	133.41±10.37
IV	510.29±46.42*	1424.56±11.01*	101.45±5.78*

Data represents mean ± S.E.M (n=5). *p<0.05; statistically significant when compare to group III. LPO: lipid peroxidation (nmole MDA formed/mg protein), GSH: reduced glutathione (µmole of GSH/gm tissue), and XO: xanthine oxidase (µmole of uric acid formed/mg protein). Group I: Normal Control, Group II: Rats treated with *S. aromaticum* extract alone, Group III: Liver necrosis, Group IV: Rats treated with extract of *S. aromaticum* followed by the induction of necrosis.