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THE PROTECTIVE EFFECTS OF EUGENOL ON CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN RATS

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Our earlier studies *in vitro* have shown that eugenol inhibits liver microsomal monooxygenase activities and carbon tetrachloride ($CC1_4$)-induced lipid peroxidation (Free Rad. Res. 20, 253–266, 1994). The objective of the present investigation was to study the *in vivo* protective effect of eugenol against $CC1_4$ toxicity. Eugenol (5 or 25 mg/kg body wt) given orally for 3 consecutive days did not alter the levels of serum glutamic oxalacetic transaminase (SGOT), microsomal enzymes such as cytochrome P_{450} reductase, glucose-6-phosphatase (G-6-Pase) xenobiotic-metabolizing enzymes (aminopyrine-N-demethylase, N-nitrosodimethylamine-demethylase and ethoxyresorufin-O-deethylase) and liver histology. Doses of eugenol (5 or 25 mg/kg) administered intragastrically to each rat on three consecutive days i.e. 48 hr, 24 hr and 30 min before a single oral dose of CCl_4 (2.5 ml/kg body wt) prevented the rise in SGOT level without appreciable improvement in morphological changes in liver. Eugenol pretreatment also did not influence the decrease in microsomal cytochrome P_{450} content, G-6-Pase and xenobiotic-metabolizing enzymes brought about by CCl_4. Since eugenol is metabolized and cleared rapidly from the body, the dose schedule was modified in another experiment. Eugenol (0.2, 1.0, 5.0 or 25 mg/kg) when given thrice orally i.e. prior to (−1 hr) along with (0 hr) and after (+ 3 hr) the i.p. administration of CCl_4 (0.4 ml/kg) prevented significantly the rise in SGOT activity as well as liver necrosis. The protective effect was more evident at 1 mg and 5 mg eugenol doses. However, the decrease in microsomal G-6-Pase activity by CCl_4 treatment was not prevented by eugenol suggesting that the damage to endoplasmic reticulum is not protected. The protective effect of eugenol against CCl_4 induced hepatotoxicity is more evident when it is given concurrently or soon after rather than much before CCl_4 treatment.

KEY WORDS: Eugenol, Antioxidant, Carbon tetrachloride, Hepatotoxicity, Monooxygenase. Free Radicals.

INTRODUCTION

The toxicity of several chemicals is due to their conversion to radicals, which in turn may generate reactive oxygen species (ROS) during metabolism.[1–3] Carbon tetrachloride (CCl_4) has been extensively studied as a model of xenobiotic-induced lipid peroxidation and hepatotoxicity. Bioactivation of CCl_4 by hepatic microsomal cytochrome P450 results in generation of $\dot{C}Cl_3$ radicals which covalently bind to cellular constituents or react with O_2 and initiate lipid peroxidation leading to cell death.[4–6] It

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is becoming increasingly evident that natural and synthetic antioxidants play an important role in protecting tissues against oxidative damage caused by free radicals.[7–11] However, in recent times, natural antioxidants are receiving greater attention in view of the reports about toxic effects of synthetic ones.[12–14]

Eugenol is the principal constituent (70–90%) of clove essential oil and is also present in many essential oils of plants especially in basil and cinnamon.[15] It is used mainly as a fragrance and flavouring agent. It is also used as an analgesic in dental materials and non-prescription drug products, as an insect repellent and as an intermediate in chemical synthesis.[16,17] In traditional medicine, the essential oil of cloves has been used in the treatment of flatulent colic, chronic diarrhoea and other gastrointestinal disorders.[18] Cloves, basil and cinnamon are components of many Ayurvedic (Indigenous Indian system) medicinal preparations.[19] The Joint Food and Agricultural Organisation (FAO)/World Health Organisation (WHO) Expert Committee on Food Additives established a conditional acceptable daily intake of 0–2.5 mg eugenol/kg. body weight for humans.[20]

Previous studies have shown that α-tocopherol,[21] ellagic acid,[22] curcumin[23] and silymarin[24] protect against CCl_4 induced hepatic necrosis. Recently, we reported that eugenol inhibits nonenzymatic lipid peroxidation in liver mitochondria[25] and microsomal lipid peroxidation induced by Fe^{+3}-ADP-NADPH, CCl_4-NADPH and cumene hydroperoxide system *in vitro*.[26] More recent studies have shown that eugenol effectively quenches trichloromethyl peroxyl radicals ($CCl_3O\dot{O}$) with a second order rate constant of $5.8 \times 10^6 M^{-1}S^{-1}$.[27] The objective of the present study is to find out the possible protective effect of eugenol *in vivo* against CCl_4 induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals

Eugenol, L-aspartate, L-oxoglutarate, maleic acid, glutathione, sodium isocitrate, aminopyrine, ethoxyresorufin, resorufin, glucose-6-phosphate, cytochrome c, N-nitrosodimethylamine, NADP, NADPH, NADH, malic dehydrogenase (EC 1.1.1.37), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and isocitrate dehydrogenase (EC 1.1.1.42) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Animals and treatment

Two experiments were conducted. In the first experiment, inbred male Wistar/NIN rats weighing 200–225 g were used. Rats were fasted for 16 to 18 hr prior to administration of CCl_4 and food was given 3 hr after dosing. Eugenol doses (5 mg or 25 mg/kg body wt) were administered by gavage as a suspension in 5% soluble starch adjusting vehicle volume to 5 ml/kg body weight. Each dose of eugenol was administered to a rat thrice on three consecutive days i.e. 48 hr, 24 hr and 30 min before dosing with CCl_4 (2.5 ml/kg) in liquid paraffin (1:1 v/v) also by gavage as a single dose. The untreated rats received respective vehicles. Dosing schedule is shown in Fig. 1A. The control as well as treated animals were sacrificed 24 hr after CCl_4 dosing. Blood was collected and livers were thoroughly perfused with ice cold saline. The livers were excised and homogenised in 4 volumes of 1.15% KCl solution. Liver microsomes were prepared from the homogenates as described by Omura and Sato.[28] The microsomes were

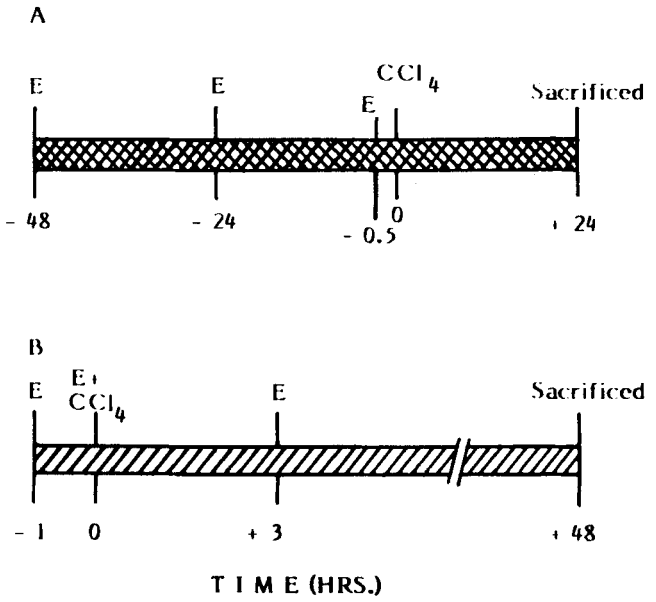


FIGURE 1 Dosing schedule for giving CCl_4 and eugenol (E) to rats. (A) Rats were given 5 or 25 mg eugenol per kg body weight intragastrically at indicated time points. With 5 mg doses at 3 time points, total amounted to 15 mg/kg and with 25 mg doses likewise the total amount was 75 mg/kg. Single dose of CCl_4 (2.5 ml/kg) was given intragastrically as indicated in the figure. (B) Rats were given doses of 0.2 mg, 1 mg, 5 mg and 25 mg eugenol per kg body weight intragastrically. Each dose was given thrice at the time points indicated such that the total dose was 3 times the individual doses mentioned i.e 0.6 mg, 3 mg, 15 mg and 75 mg of eugenol/kg. A single dose of CCl_4 (0.4 ml/kg) was given i.p. as indicated in the figure.

suspended in isotonic KCl (1.15%), stored at −70°C and used within 7 days. Protein content was determined by the method of Lowry *et al.*[29] Cytochrome P-450 content in microsomes was determined as described by Omura and Sato.[28] Cytochrome P-450 reductase activity was determined by the cytochrome c reduction method.[30] Aminopyrine-N-demethylase, N-nitrosodimethylamine (NDMA) demethylase and ethoxyresorufin-O-deethylase were determined according to Mazel;[31] Kawanishi[32] and Burke *et al.*,[33] respectively. Glucose-6-phosphatase (G-6-Pase) activity was determined by the method of Swanson.[34] Serum glutamic oxalacetic transaminase (SGOT) was determined according to Karmen.[35] The effect of eugenol on SGOT activity *per se* was tested by adding eugenol directly to serum before starting the assay. Eugenol did not influence the original activity of SGOT.

For the second experiment male Wistar/NIN rats weighing 200–250 g were used. Rats were given different doses (0.2, 1, 5 and 25 mg/kg) of eugenol suspended in 5% soluble starch by gavage adjusting administered volume to 2.5 ml/kg body wt. Each dose was given thrice at different time intervals which included prior to (− 1 hr) along with (0 hr) and after (+ 3 hr) the administration of CCl_4. Thus rats getting 0.2, 1.0, 5.0 or 25 mg/kg doses received a total of 0.6, 3, 15 or 75 mg/kg respectively taking the sum of three administrations into consideration. Carbon tetrachloride (0.4 ml/kg) in peanut oil was given i.p. adjusting the administered volume to 2.5 ml/kg body wt. Any possible interference between eugenol and CCl_4 at the level of intestinal absorption is avoided

by i.p. administration of CCl_4. Dosing schedule is shown in Fig 1b. Blood was collected after 24 hr from the eye (Orbital Sinus) and rats were sacrificed after 48 hr following CCl_4 dose. Blood and livers were collected and liver microsomes were prepared as described in the first experiment. Serum GOT and microsomal G-6-Pase were determined as described in the first experiment.

Histopathological examination

Small slices from the middle lobe of liver were fixed in 10% neutral buffered formalin solutions. Tissues were processed and 6 μm paraffin sections were stained with hematoxylin-eosin. Hepatocellular necrosis was graded as follows. No necrosis = 0.0, (Figure 4.1), necrosis around centrilobular vein = 1.0, (Figure. 4.2) necrosis or fatty changes involving 1/3 of the lobule = 2.0 (Figure 4.3), necrosis of more than 1/3 of lobule = 3.0 (Figure 4.4).

Statistics

Data were statistically analysed using student's 't' test taking $P < 0.05$ as significant. Values are given as Means ± SE.

RESULTS

Effect of treatment with eugenol alone on serum GOT, microsomal enzymes and liver histology

Rats treated with eugenol for 3 consecutive days did not show any change in the activities of GOT (Figure 2), cytochrome P-450 reductase, monooxygenases (Aminopyrine-n-demethylase, NDMA-demethylase and ethoxyresorufin-O-deethylase), glucose-6-phosphatase and cytochrome P-450 content (Table 1,2). Eugenol treatment also did not alter the normal hepatic architecture (Table 2).

Effect of eugenol on CCl_4 induced liver damage

The intragastric administration of CCl_4 (Experiment 1) resulted in a marked increase in the activities of serum GOT. Pretreatment of rats with eugenol 48 hr, 24 hr and 30 min before administration of CCl_4 resulted in significant decrease in serum GOT activities (Figure 2). Histopathological examination of livers after 24 hr of CCl_4 administration showed centrilobular necrosis. The necrosis score in animals pretreated with lower and higher doses of eugenol was similar and not significantly different in CCl_4 treated group (Figure 4) (Table 2). As expected, CCl_4 administration produced a marked decrease in cytochrome P-450 content, monooxygenase activities and G-6-pase without affecting cytochrome P-450 reductase. Eugenol treatment did not influence the alteration in cytochrome P-450 content or enzyme activities brought about by CCl_4 administration (Table 1 and 2).

Intraperitoneal administration of CCl_4 (0.4 ml/kg) (Experiment 2) also resulted in a marked increase in the activity of serum GOT (Figure 3). Administration of eugenol in three divided doses which include prior to (−1 hr) along with (0 hr) and after (+ 3 hr) CCl_4 administration significantly decreased CCl_4 induced serum GOT activities in a dose dependent manner upto 5 mg/kg. However, at higher doses (25 mg/kg) the trend was reversed and serum GOT activities tended to remain high (Figure 3). By 48 hr after

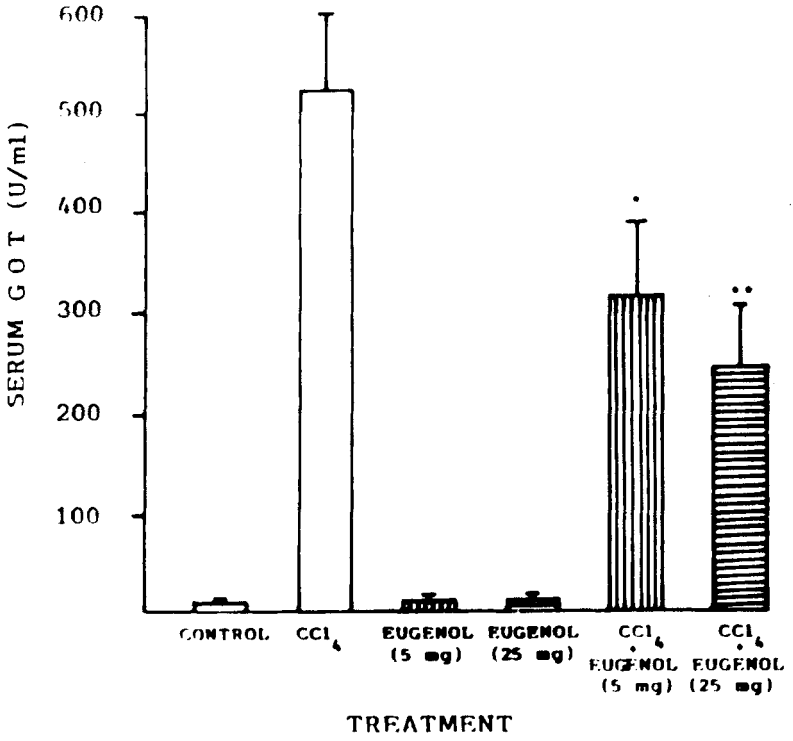


FIGURE 2 Effect of CCl_4 administration on SGOT activity of rats pretreated with eugenol: Eugenol and CCl_4 were administered to rats as mentioned in figure 1a. Serum was collected after 24 hr of CCl_4 dose and assayed for GOT activity. Data are presented as means ± SE. Each group contained 6 animals. An asterisk indicated a significant difference between CCl_4 and eugenol + CCl_4 treatments. *P<0.05; **P>0.01.

TABLE 1
Effect of eugenol-pretreatment on hepatic monooxygenase activities of CCl_4-treated rats (experiment 1)

Treatment	Monooxygenase (nmol/mg protein/min.)		
	Aminopyrine-N-demethylase	NDMA Demethylase	Ethoxyresorufin-O-deethylase
Control	5.72 ± 0.23	1.35 ± 0.18	0.230 ± 0.050
CCl_4 (2.5 ml/kg)	0.20 ± 0.02*	0.12 ± 0.02*	0.010 ± 0.003*
Eugenol (5 mg/kg)	4.90 ± 0.35	1.54 ± 0.04	0.203 ± 0.038
Eugenol (25 mg/kg)	5.34 ± 0.52	1.28 ± 0.06	0.210 ± 0.060
Eugenol (5 mg/kg) + CCl_4 (2.5 ml/kg)	0.21 ± 0.06*	0.18 ± 0.03*	0.007 ± 0.002*
Eugenol (25 mg/kg) + CCl_4 (2.5 ml/kg)	0.22 ± 0.05*	0.09 ± 0.02*	0.006 ± 0.002*

Rats were given eugenol and CCl_4 as mentioned in Fig. 1A.
Results are mean ± SE for 6 rats
*Significantly different from control * P <0.01.

TABLE 2
Effect of pretreatment with eugenol on microsomal changes and hepatic necrosis induced by CCl_4 in rats (*Experiment 1*)

Treatment	Cyt. P450[(a)]	Cyt. P450[(b)] reductase	G-6-Pase[(c)]	Hepatic necrosis score
Control	0.86 ± 0.08	47 ± 6	9.2 ± 0.66	0
CCl_4 (2.5 ml/kg)	0.33 ± 0.04*	42 ± 4	1.5 ± 0.15*	2.7 ± 0.210
Eugenol (5 mg/kg)	0.79 ± 0.06	46 ± 5	8.6 ± 0.57	0
Eugenol (25 mg/kg)	0.80 ± 0.03	50 ± 8	8.3 ± 0.35	0
Eugenol (5 mg/kg) + CCl_4 (2.5 ml/kg)	0.31 ± 0.04*	40 ± 4	1.75 ± 0.26*	2.5 ± 0.224
Eugenol (25 mg/kg) + CCl_4 (2.5 ml/kg)	0.27 ± 0.04*	38 ± 5	1.91 ± 0.14*	2.5 ± 0.341

Rats were given CCl_4 and eugenol as mentioned in Fig. 1A
(a) nmol/mg protein; (b) nmol Cyt. c reduced/mg protein/min; (c) μmol Pi released/hr/mg protein.
Results are mean ± SE for 6 rats.
*Significantly different from control *P <0.01.

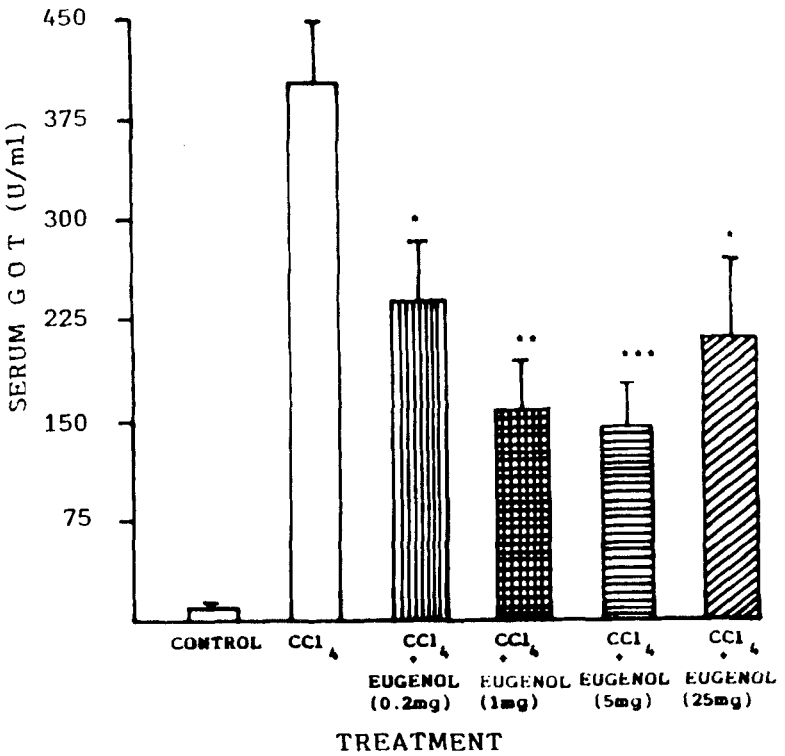


FIGURE 3 Effect of CCl_4 administration on SGOT activity of rats treated with eugenol: Eugenol and CCl_4 were administered to rats as mentioned in Fig. 1b. Serum was collected after 24 hr of CCl_4 dosing and assayed for GOT activity. Data are presented as mean ± SE of 6 rats in each group. An asterisk indicated a significant difference between CCl_4 and eugenol + CCl_4 treatment. *P<0.05, **P>0.01, ***P<0.001.

TABLE 3
Effect of eugenol treatment on microsomal P450, G-6-Pase and hepatic necrosis in rats administered CCl_4 (Experiment 2)

Treatment	Cyt-P450[a]	G-6-Pase[b]	Hepatic[c] necrosis score
Control	0.74 ± 0.10	10.5 ± 0.57	0.0
CCl_4 (0.4 ml/kg)	0.38 ± 0.04*	2.6 ± 0.41*	2.0 ± 0.0
Eugenol (0.2 mg/kg) + CCl_4 (0.4 ml/kg)	0.36 ± 0.03*	2.8 ± 0.24*	2.0 ± 0.0
Eugenol (1.0 mg/kg) + CCl_4 (0.4 ml/kg)	0.41 ± 0.04*	2.4 ± 0.11*	1.5 ± 0.28*
Eugenol (5.0 mg/kg) + CCl_4 (0.4 ml/kg)	0.34 ± 0.028*	2.5 ± 0.22*	1.25 ± 0.25*
Eugenol (25.0 mg/kg) + CCl_4 (0.4 ml/kg)	0.45 ± 0.036*	2.2 ± 0.10*	2.0 ± 0.0

Rats were given CCl_4 and eugenol as mentioned in Fig. 1B
(a) nmol/mg protein
(b) μmol Pi released/hr/mg protein.
* (a) (b) Significantly different from controls $P<0.01$
* (c) Significantly different from CCl_4 treatment $P<0.01$
Results are mean ± SE for 4–6 rats.

CCl_4 treatment serum GOT activities almost reverted to near basal level in all groups (data not shown). Histopathological examination of livers revealed the highest centrilobular necrosis score in CCl_4 treated rats. Treatment with doses of 1 mg and 5 mg eugenol per kg body weight gave significant protection against CCl_4-induced liver damage (Table 3) (Figure 4). However eugenol at lower (0.2 mg/kg) or higher doses (25 mg/kg) did not afford similar protection against necrotic changes (Table 3). Eugenol treatment did not protect cytochrome P-450 degradation and CCl_4 induced deactivation of G-6-pase (Table 3).

DISCUSSION

The results of the present study show some protective effects of eugenol *in vivo* against CCl_4 induced hepatotoxicity. In contrast to *in vitro* results[26] eugenol treatment for 3 days did not inhibit monooxygenase activities. Pretreatment of rats with eugenol prior to administration of CCl_4 prevented the CCl_4 induced rise in SGOT but not the morphological changes in the liver. The lack of protection may be due to rapid elimination of eugenol from the body.[36,37] The presence of the compound at the site of injury during free radical attack may be necessary to offer protection. Thus, when eugenol was administered just before, along with and soon after CCl_4 administration, eugenol could protect against cellular damage as well as GOT release. However, eugenol did not protect against damage to endoplasmic reticulum by CCl_4 as judged by levels of cytochrome P-450, monooxygenase and G-6-pase activities. This could be due to insufficient amounts of eugenol retained in endoplasmic reticulum (E.R.) because eugenol itself is a substrate for microsomal cytochrome P-450.[38] To compete with CCl_4 bioactivation and to intercept primary radical accumulation larger amounts of eugenol might be needed.

The exact mechanism of necrosis following E.R. damage is not known. However, it

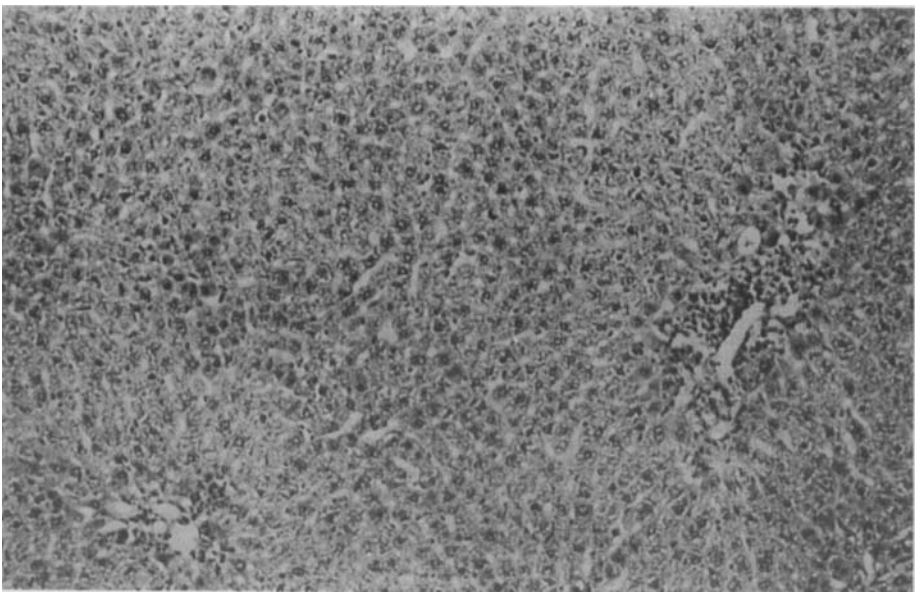
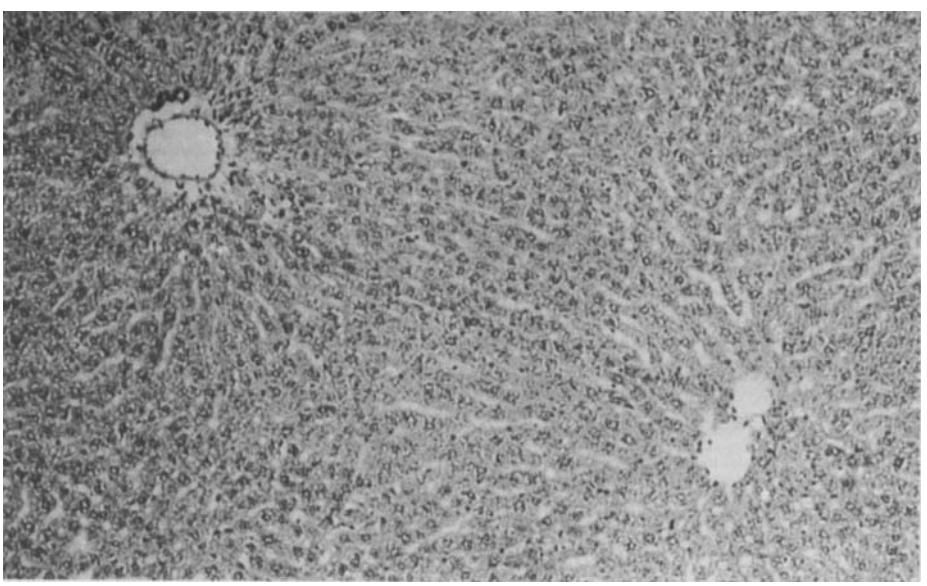


FIGURE 4 Examples of liver necrosis of rats treated with eugenol and CCl_4.
4.1 No necrosis, necrosis score (NS) = 0



4.2 Necrosis around centribolular vein, Necrosis score (NS) = 1.

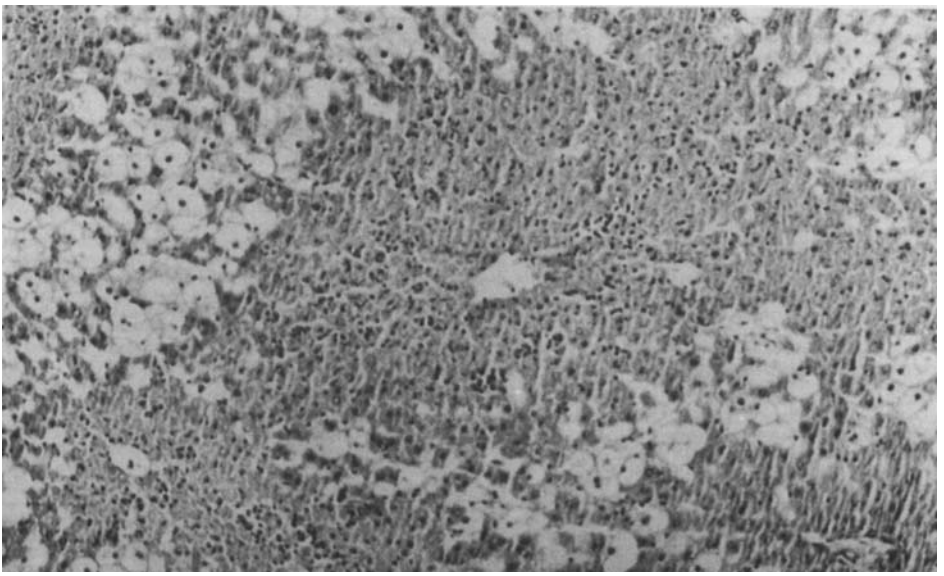
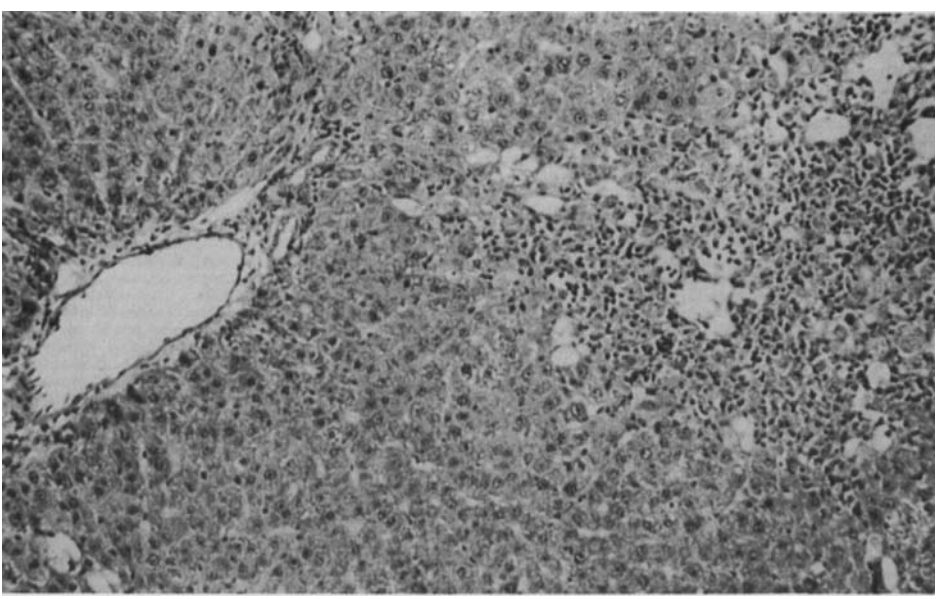


FIGURE 4 *continued*
4.3 Necrosis or fatty changes involving 1/3 of the lobule, (NS) = 2.



4.4 Necrosis more than 1/3 of lobule, (N.S) = 3.

is believed that lipid derived secondary radicals formed in E.R. attack mitochondrial, lysosomal and plasma membranes leading to cell necrosis.[39,40,41] Eugenol could be incorporated into these membranes due to its hyrophobic nature and offer protection against the toxic radicals. The efficacy of eugenol was found to be within the permitted levels by FAO/WHO expert committee on Food Additives (maximum permitted intake is 2.5 mg/kg wt).

In summary, not only does eugenol inhibits CCl_4-NADPH mediated peroxidation in microsomes *in vitro* but it also offers some protection *in vivo* against CCl_4 induced hepatotoxicity. The protective effect of eugenol is evident when it is given concurrently or soon after rather than much before CCl_4 treatment.

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