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Toxicological Studies on the Essential Oil of *Eugenia caryophyllata* Buds

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Abstract – The essential oil (EC-oil) obtained from the buds of *Eugenia caryophyllata* (Myrtaceae) was examined for its free radical-scavenging activity, cytotoxicity, and *in vivo* toxicity. To find the xenobiotic properties of EC-oil, serum thiobarbituric acid reactive substances (TBARS) level and hepatic drug-metabolizing enzyme activities were measured. It was found that EC-oil displayed xenobiotic properties like bromobenzene. The cytotoxicities of eugenol and of the EC-oil were greatly attenuated by the sulfhydryl-containing *N*-acetyl-L-cysteine (NAC), suggesting that eugenol was susceptible to nucleophilic sulfhydryl. In addition, eugenol also showed potent free radical-scavenging activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Moreover, methyleugenol considerably exhibited less cytotoxicity and less potent free radical-scavenging activity than eugenol, and the cell viability of the methyleugenol was more increased with NAC treatment than the eugenol. These results indicate that the phenolic OH in eugenol may play a crucial role in both cytotoxicity and free radical-scavenging activity. The fashion on oxidative stress and hepatic drug-metabolizing enzyme activities of eugenol resembled those of bromobenznene.

Keywords – *Eugenia caryophyllata*, Myrtaceae, essential oil, eugenol, glutathione, xenobiotics

Introduction

The essential oil (EC-oil) of the buds of *Eugenia caryophyllata* (*Syzigium aromaticum*, Myrtaceae) is used as a local anesthetic and analgesic and for sterilizing purposes (Harborne and Baxter, 1993). It is known that this material contains eugenol, acetyleugenol, chavicol, acetyl salicylate, and α-, β-humulenes (Zheng *et al.*, 1992). EC-oil is well known for its therapeutic benefits. The essential oil of *E. caryophyllata* is reported to have anti-fungal, anticonvulsant (Harborne and Baxter, 1993), antimutagenic (Miyazawa and Hisama, 2001), and anticarcinogenic activities (Zheng *et al.*, 1992). In addition, eugenol has been reported to participate in photochemical reactions (Mihara and Shibamoto, 1982) and to possess antioxidant activity (Ogata *et al.*, 2000), insecticidal activity (Park *et al.*, 2000) and photo-cytotoxicity (Atsumi *et al.*, 2001). Eugenol, was also reported to have mosquito-repellent activity (Chogo and Crank, 1981) and to act as an anthelmintic (Asha *et al.*, 2001). And the buds of Eugenia caryophyllata have been traditionally used as a herbal drug to treat dyspepsia, acute/chronic gastritis and diarrhea (Han, 2001).

Since EC-oil produced lipid peroxidation activity in our toxicological assay in rats, the oil was analyzed, phytochemically isolated and further more examined for its biological properties. Based on reported biological properties and chemical composition of EC-oil, I attempted to isolate the main component eugenol and to prepare methyleugenol and also tested the *in vitro* cytotoxicity and free radical-scavenging activity of eugenol and its *in vivo* toxicity. And I focused on aspects of allyl group of eugenol capable of showing electrophilicity, and upon its phenolic OH, for antioxidant ability and further investigated hepatic drug-metabolizing enzyme activities, i.e., cytochrome P450, xanthine oxidase and aldehyde oxidase in comparison with thiobarbituric acid reactive substances (TBARS) values in the rat, which allow one to distinguish xenobiotics from other biologically active substances.

Experimental

Plant material and steam distillation – Commercially available plant material (buds) was purchased for the essential oil extraction. The plant was identified as *Eugenia caryophyllata* by Dr. Sang-Cheol Lim (Department of Botanical Resources, Sangji University, Wonju, Korea). The buds (2.0 kg) were extracted by steam distillation for 4 h. The distillate was extracted with diethyl ether, dehydrated with anhydrous sodium sulfate and solvent removed by evaporation using a rotatory evaporator at

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40 °C. The residual oil (21 g) obtained was used for chemical analysis, further purification and biological assays.

Animals – Experimental animals were purchased from the Korean Experimental Animal Co. and were adapted to constant conditions (temperature: 20 ± 2 °C, humidity: 40-60%, light/dark cycle: 12 hr) for more than two weeks. Sprague-Dawley male rats weighing 100-120 g were used for the experiments. Animals were fasted 24 hours before the experiments, but offered *ad lib*. Animals were sacrificed at fixed time (10:00 A.M.-12:00 A.M.) to account for diurnal enzyme activity variations.

Sample treatment – Sampels were dissolved in 10% Tween 80 and diluted 10% with saline for the animal experiment. Rats in the untreted group were offered with vehicle only. Samples were orally administered at 5 and 10 mg/kg of eugenol and at 480 mg/kg of bromobenzene for 7 d for the animal experiment. Twenty-four hour after the last administration, the blood was collected after anesthesia with CO_2 gas.

Isolation of enzyme sources – After being anesthetized with CO_2, the animals were killed by decapitation. Blood was collected from the aorta and from the liver. Liver tissues were homogenized in ice-cool in four-volumes of 0.1 M sodium phosphate buffer (pH 7.4). This suspension was then centrifuged cold at 600 × g, 10 min. The supernatant was again centrifuged (10,000 × g, 20 min), the pellet was resuspended with an equivalent volume of 0.1 M sodium phosphate buffer (pH 7.4) and recentrifuged (105,000 × g, 30 min). The resultant mitochondrial fraction was used as catalase source and the supernatant obtained from centrifugation of the last one (105,000 × g, 60 min) was used as an enzyme source of cytosolic fraction to measure the activities of xanthine oxidase and aldehyde oxidase. The resultant precipitate was centrifuged (105,000 × g, 60 min) in 0.1 M sodium phosphate buffer (pH 7.4) and the microsomal fraction obtained was used as an enzyme sources to measure the activities of cytochrome P450, aminopyrine *N*-demethylase, and aniline hydroxylase.

Measurement of TBARS – Animals were sacrificed by decapitating the blood from the abdominal aorta under slightly anesthesia with CO_2. The liver was exhaustively perfused with ice-cold normal saline through the portal vein until it was uniformly pale and then weighed. The TBARS in the liver was measured as a marker of lipid peroxidation using the method of Ohkawa *et al.* (1979) Briefly, an aliquot (0.4 ml) of 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of 8.1% SDS, 1.5 ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was heated at 95 °C for 1 h, cooled, and extracted with 5.0 ml of *n*-butanol : pyridine (15 : 1). Absorbance of the *n*-butanol : pyridine layer was measured at 532 nm was to determine TBARS.

Hepatic microsomal enzyme activities (cytochrome P450, aminopyrine *N*-demethylase cytochrome P450, aniline hydroxylase) – Cytochrome P450 activity was determined according to the method of Omura and Sato (1964), aminopyrine N-demethylase activity by the method of Nash (1953). The enzyme activity of aniline hydroxylase, was measured by the method of Bidlack and Lowry (1982).

Hepatic non-microsomal enzyme activities (xanthine oxidase and aldehyde oxidase) – Xanthine oxidase activity was determined using the method of Stripe and Della (1969). In brief, a mixture of 3.0 ml of 0.1 M potassium phosphate buffer (pH 7.5) and 0.1 ml of 60 μM sodium xanthine (substrate) was reacted at 37 °C. After the reaction, the protein in the mixture was removed by adding 20% trichloroacetic acid. The absorbance of the supernatant obtained was measured at 292 nm. The activity of xanthine oxidase was calculated using a standard calibration curve, and the unit of enzyme activity was expressed as nmoles of uric acid produced of 1 mg protein per minute. The enzyme activity of aldehyde oxidase was determined as described by Rajagopalan *et al.* (1968) 0.1 M potassium phosphate buffer (pH 7.5), *N*-methylnicotinamide (substrate) and enzyme solution were reacted, and the absorbance of the 2-pyridone produced was measured at 300 nm. Enzyme activity was calculated using a standard calibration curve, and expressed as nmoles of the 2-pyridone produced of 1 mg of protein per minute.

Glutathione *S*-transferase activity – Glutathione S-transferase activity was determined according to the method reported by Habig *et al.* (1974). Briefly, 75 μl of 40 mM of reduced glutathione was added to 0.1 M potassium phosphate buffer (pH 6.5) in the test tube, and then 100 μl of the enzyme solution was added. In the blank, the reaction was terminated by 20% trichloroacetic acid. In each of the test- and blank solutions, the mixture was reacted at 25 °C for 5 min and then reacted with 25 μl of 2,4-dinitrochlorobenzene (substrate). The reaction was quenched with 20% trichloroacetic acid and centrifuged. Absorbance was measured at 340 nm after centrifuging in every case. Glutathione S-transferase activity (nmole) was calculated using a molar absorption coefficient (9.6 mM^{-1} cm^{-1}).

GC-MS analysis – Column {DB-1 (length 30 meters, i.d. 0.25 mm, film thickness 0.25 μm, J&W Scientific, USA)}; Column temperature program {init. temp. 50 °C (3 min), the increased (at 8 °C/min) to 250 °C (10 min)}; solvent cut (3 min); temperatures {injector (250 °C), transfer

line (250 °C), ion source (150 °C), manifold (70 °C)}, detector {Electron Impact-Quadrupole 1 (EI energy 70 eV); carrier gas {He (99.99%), flow rate (1.5 ml/min)}.

Isolation of eugenol – Extracted oil was subjected to silica gel column chromatography (Art No. 7734, Merck, Germany, 3 × 21 cm, 60 g) with the eluent of *n*-hexane-ethyl acetate (10 : 1) as eluent. The eluate was collected in 30 fractions of 10 ml, and checked by spraying vanillin-sulfuric acid reagent. Fractions showing similar TLC patterns were combined and dried under reduced pressure. Fractions collected with retention volumes between 160- and 280 ml were dried *in vacuo* to yield a colorless oil (compound **1**, R_f 0.34, 2.2 g). This compound was identified by physicochemical data: Compound **1**-Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) δ : see literature (Kwon *et al.*, 2001), EI-MS (70 eV) *m/z* (rel. int. %): 164.1 (100), 149.1 (30), 103.1 (19).

Methylation – Eugenol (0.8 g) was dissolved in 150 ml of acetone and 10 ml of dimethylsulfate (DMSO) containing K_2CO_3 (2.3 g) was added. This reaction mixture was then heated under reflux for 8 h. After cooling, the mixture was poured into 150 ml of H_2O and extracted with ether in a separation funnel. The diethylether fraction obtained was dehydrated with anhydrous sodium sulfate and filtered, dried and passed through silica gel column, as described above. Fractions showing the major component by TLC were combined and dried *in vacuo* to yield a colorless oil (methyleugenol).

Methyleugenol – Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.35 (2H, d, J = 6.7 Hz, H-1'), 3.88, 3.87 (2 × OCH_3), 5.09 (1H, dm, J = 16.7 Hz, H-3'$_{trans}$), 5.11 (1H, dm, J = 10.0 Hz, H-3'$_{cis}$), 5.98 (2H, ddt, J = 16.7, 10.1, 6.7 Hz), 6.75 (1H, dd, J = 8.0, 1.8 Hz, H-6), 6.75 (1H, d, 1.8 Hz), 6.82 (1H, d, J = 8.0 Hz, H-5); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 40.5 (C-1'), 56.5, 56.6 (OCH_3), 112.1 (C-5), 112.7 (C-3'), 116.2 (C-2), 121.9 (C-6), 133.4 (C-2'), 138.3 (C-1), 148.2 (C-4), 149.7 (C-3).

MTT Assay – The *in vitro* tests with 3LL cells (a human lung carcinoma cell line) were carried out according to a method described previously (Denizot, 1996). Cells (1×10^4) were seeded in each well of a 96-well microtiter plate containing 100 μl of RPMI medium supplemented with 10% FBS/well and incubated overnight. The test samples, EC-oil, eugenol and methyleugenol were dissolved in dimethylsulfoxide (DMSO) and were added after serial dilution (the final DMSO concentrations in all assays did not exceed 0.01%). Twenty-four hours after seeding, 100 μl new media or test samples were added, and the plates were incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue which formed in the cells was dissolved by adding 50 μl DMSO. Optical density was measured at 540 nm.

DPPH Radical-Scavenging Effect – The scavenging effect was determined by measuring the intensity of quenching DPPH radical as described by Xiong *et al.* (1996) and expressed as the percent scavenging (%) of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical *vs.* that of the control. The IC_{50} values were calculated from regression plots of the average percent reduction of DPPH radical from three separate tests versus the concentration of the tested compound.

Statistics – The data are presented as means ± S.D. Statistical significance was determined using Duncan's multiple range test.

Results and Discussion

Biological properties of EC-oil as xenobiotics – A substantial quantity of essential oil (EC-oil) was obtained from the buds of *E. caryophyllata*. In order to find whether induces oxidative or antioxidative effects *in vivo*, we examined the effects on lipid peroxidation and on hepatic drug-metabolizing enzyme activities in rats treated with EC oil for 7 days. Rats administered with EC-oil showed higher serum TBARS levels than normal rats, suggesting that EC-oil causes lipid peroxidation due to oxidative stress. Moreover, EC-oil reduced glutathione content and glutathione S-transferase activity (GST), indicating that it consumes glutathione for detoxification processes (Table 1). Both aminopyrine demethylase and aniline hydroxylase, which belong to the hepatic microsomal cytochrome P450 enzymes, were activated by EC-oil. A variety of xenobiotics activate microsomal cytochrome P450 enzymes, which initially form epoxides from mutagens with strong electrophilicity, carcinogens such as benzopyrenes and aflatoxines provides examples, and these epoxides may cause further lipid peroxidation, which is closely associated with the formation of reactive oxygen species (ROS) (Schaffner, 1975; Singh and Rao, 1993). The hepatic microsomal enxymes, aldehyde oxidase and xanthine oxidase, are both capable of generating hydroxyl radical, and were considerably increased in the serum. Hepatic epoxide hydrolase was significantly reduced in rats treated the EC-oil, suggesting that aromatic compounds in EC-oil might undergo epoxidatiopn in the liver (Halliwell, 1978; Denecke and Fanburg, 1980). The involvement of the aromatic ring in epoxidation process might be presumed examples provided by other aromatic ring (s) in xenobiotics. Overall,

Table 1. Effect of the *Eugenia caryophyllata* essential oil (EC-oil) on hepatic drug-metabolizing enzymes

group	normal	EC-oil		bromobenzene
		5 mg/kg	10 mg/kg	480 mg/kg
MDA[2]	$23.9 \pm 0.37^{1),e}$	40.6 ± 0.43^{b}	50.2 ± 0.30^{a}	46.9 ± 0.53^{a}
GST[3]	208.6 ± 11.43^{a}	123.3 ± 10.97^{c}	117.6 ± 9.42^{c}	177.4 ± 19.1^{b}
Glut.[4]	2.17 ± 0.21^{a}	1.14 ± 0.12^{d}	$1.44 \pm 0.09^{c,d}$	$1.89 \pm 0.20^{a,b}$
epox. hyd.[5]	12.2 ± 0.24^{a}	7.32 ± 0.19^{e}	7.43 ± 0.18^{e}	4.30 ± 0.22^{d}
amin. dem.[6]	2.84 ± 0.13^{d}	3.87 ± 0.12^{b}	4.12 ± 0.11^{b}	6.43 ± 0.63^{a}
anil. hydroxyl.[7]	0.60 ± 0.09^{d}	$1.09 \pm 0.10^{a,b}$	1.14 ± 0.07^{a}	$0.90 \pm 0.21^{b,c}$
ald. oxid.[8]	1.36 ± 0.09^{c}	3.16 ± 0.14^{b}	3.18 ± 0.17^{b}	4.17 ± 0.33^{a}
xanth. oxid.[9]	2.33 ± 0.16^{e}	4.89 ± 0.18^{c}	5.54 ± 0.23^{b}	5.96 ± 0.27^{a}

Rats were intraperitoneally injected daily for seven days and animals were decapitated 24 h after the last injection. [1]Values are means ± S.D. for six experiments. [a-d]Values with the same superscript letter are not significantly different in each row ($p < 0.05$); Unit: [2]nmol/g of tissue, [3]1,2-dinitro-4-nitrobenzene nmol/mg protein/min, [4]μmol/g of tissue, [5]nmol/g of tissue, [6]HCHO nmol/mg protein/min, [7]p-aminophenol nmol/mg protein/min, [8]2-pyridone nmol/mg protein/min, [9]uric acid nmol/mg protein/min.

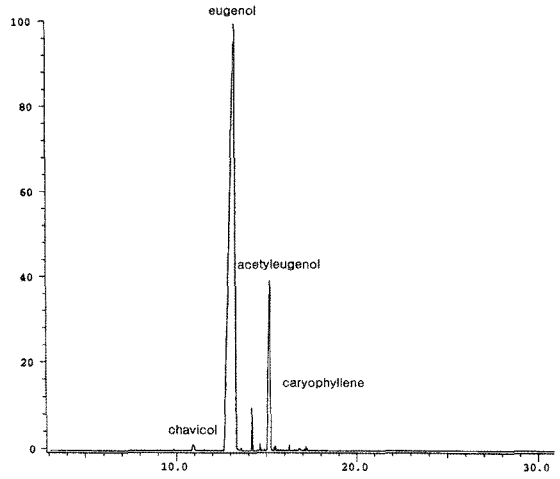

Fig. 1. Gas chromatogram of the essential oil (EC-oil) of *E. caryophyllata.*

an examination of the *in vivo* data led to the finding that the effects of samples on hepatic drug-metabolyzing enzymes are a key common feature of xenobiotics, as for bromobenzene used for a positive control.

Antioxidative and cytotoxic properties of eugenol – To confirm the composition of the EC-oil, it was analyzed using GC-MS, which indicated the most abundant eugenol in the oil and the trace components, acetyleugenol and caryophyllene (Fig. 1). Eugenol was purified from EC-oil by silica gel column chromatograpgy, and was indentified by NMR spectroscopic data. In general, phenolic OH of natural aromatics shows free radical-scavenging effect while exomethylene in allyl group produces electrophilic properties in biology. The orientation of *p*-OH of eugenol to the allyl group may change the biological properties of free radical-scavenging effect due to phenolic OH or of cytotoxicity due to the electrophilic allyl group. Therefore, eugenol was methylated to produce methyleugenol and the structure was indentified by NMR spectroscopic method.

Table 2. IC_{50} values of eugenol, methyleugenol and EC-oil in the DPPH assay

compound	IC_{50} (μg/ml)
eugenol	7.7 ± 0.2
methyleugenol	92.4 ± 2.4
EC-oil	10.2 ± 2.1

The values represent the mean of three independent experiments.

Eugenol was found to have a more potent free radical-scavenging effect (IC_{50} 7.7 μg/ml) than methyleugenol (IC_{50} 92.4 μg/ml) or EC-oil (IC_{50} 10.2 μg/ml) in the DPPH assay (Table 2). These results indicate that the ionizable phenolic OH of eugenol in EC-oil may be responsible for the ROS scavenging effect. EC-oil containing abundant eugenol also exhibited potent antioxidative ability. The IC_{50}s of EC-oil was calculated by using the results of MTT testing (43.5 μg/ml against HL-60 cell, 126.4 μg/ml against HepG-2 cell). Eugenol had IC_{50} values of 39.2 μg/ml, and 183.6 μg/ml against HepG-2, whereas methyleugenol had higher IC_{50} values (76.3 μg/ml against HL-60 cell, and 222.2 μg/ml against HepG-2 cell). It is notable that methylation of eugenol decreases its cytotoxicity in the cells (Fig. 2). We presumed that the *p*-OH with respect to the allyl substituent may contribute to the enhanced electrophilicity of exomethylene and that the effect of *p*-

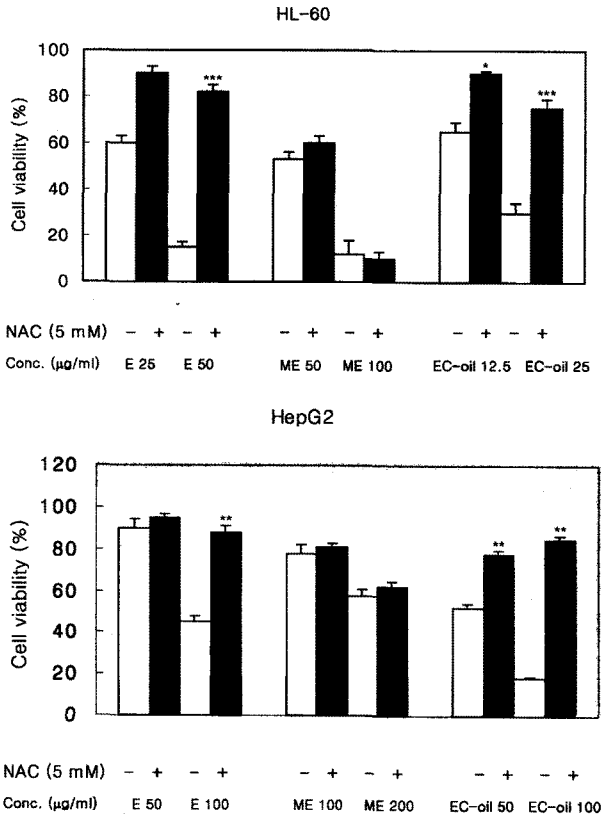

Fig. 2. Cytotoxic effects of *N*-acetyl-L-cysteine (NAC, 5 mM) on eugenol (E), methyleugenol (ME) or the essential oil of *E. caryophyllata* (EC-oil) on HL-60 (above) and HepG2 (below) cell lines. Each column represents the mean ± S.D. of at least three independent experiments performed in triplicates. Asterisk (*) indicates means that are significantly different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) from the control.

OCH_3 is weaker than that of *p*-OH. Furthermore, the addition of *N*-acetyl-L-cysteine (NAC) significantly decreased the cytotoxic effects of eugenol and EC-oil on HL-60 cells, but less potently decreased their effects on HepG2 cells. Although the cytotoxicities of eugenol were considerably prevented by NAC, those of methyleugeol were not significantly inhibited. These results suggest that eugenol has a more powerful electrophilic allyl group possibly enhanced by the *p*-positioned OH than methyleugenol. Based on the above experimental results of MTT- and DPPH assays, the phenolic OH in eugenol may play a crucial role in both cytotoxicity and free radical-scavenging activity.

Certain phenolic compounds can be converted into quinone forms, though this is dependent on substituent positions, e.g. 1,2- or 1,4-disubstituents. As shown in Fig. 3 eugenol could be transformed into an oxidized species with a highly conjugated form (semiquinone), which might represent the actual cytotoxic form. Moreover, species *a*

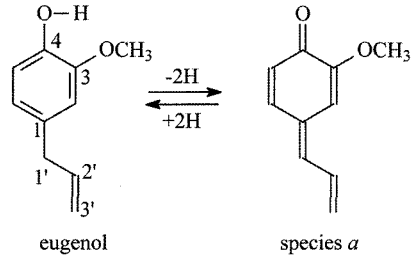

Fig. 3. Proposed eugenol redox changes.

may be more susceptible to NAC-conjugation than eugenol itself. This proposal is supported by the reported photodynamic behavior of eugenol (Atsumi *et al.*, 2001). In fact, this redox change in the case of eugenol may be related to the biological properties, i.e., cytotoxic- or its free radical-scavenging ability.

Xenobiotic properties of eugenol – Measurement of *in vitro* antioxidative or cytotoxicity is not enough for the elucidation of xenobiotic properties of eugenol. As shown in Fig. 4, treatment of rats with eugenol increased serum TBARS values at 5 or 10 mg/kg dose, suggesting that eugenol produces oxidative stress in rats. Since we have reported that cinnamaldehyde with potent cytotoxicity activity lowered serum TBARS value (Choi *et al.*, 2001), the present results suggest that eugenol has a xenobiotic property. Bromobenzene, which is often used for the induction of lipid peroxidation, is one of typical xenobiotics (Lee *et al.*, 2002). As shown in Fig. 4, treatment with bromobenzne increased serum TBARS value, aminopyrine *N*-demethylase-, aniline hydroxylase-, aldehyde oxidase-, xanthine oxidase-, and xanthine oxidase activities of rat liver while it led to the decrease in hepatic glutathione *S*-transferase and epoxide hydrolase activities and glutathione content. This pharmacologic fashion represents the xenobiotic properties. And it was found that the pharmacologic fashion of eugenol resembled the xenobiotic fashion of bromobenzene as shown in Fig. 4. Although acetaminophen is being clinically used for an analgesic medicinal drug, it is also known as a xenobiotic causing lipid peroxidation in the body and therefore used for the induction of oxidative stress in animal experiments (Choi *et al.*, 2002). Therefore, it is noted that a clinical agent, EC-oil, should be more carefully used based on its xenobiotic properties.

In conclusion, eugenol is the major component of the EC-oil with xenobiotic properties. It was also concluded that *p*-positioned OH in eugenol against allyl group contributes the enhancement of its electrophilic properties.

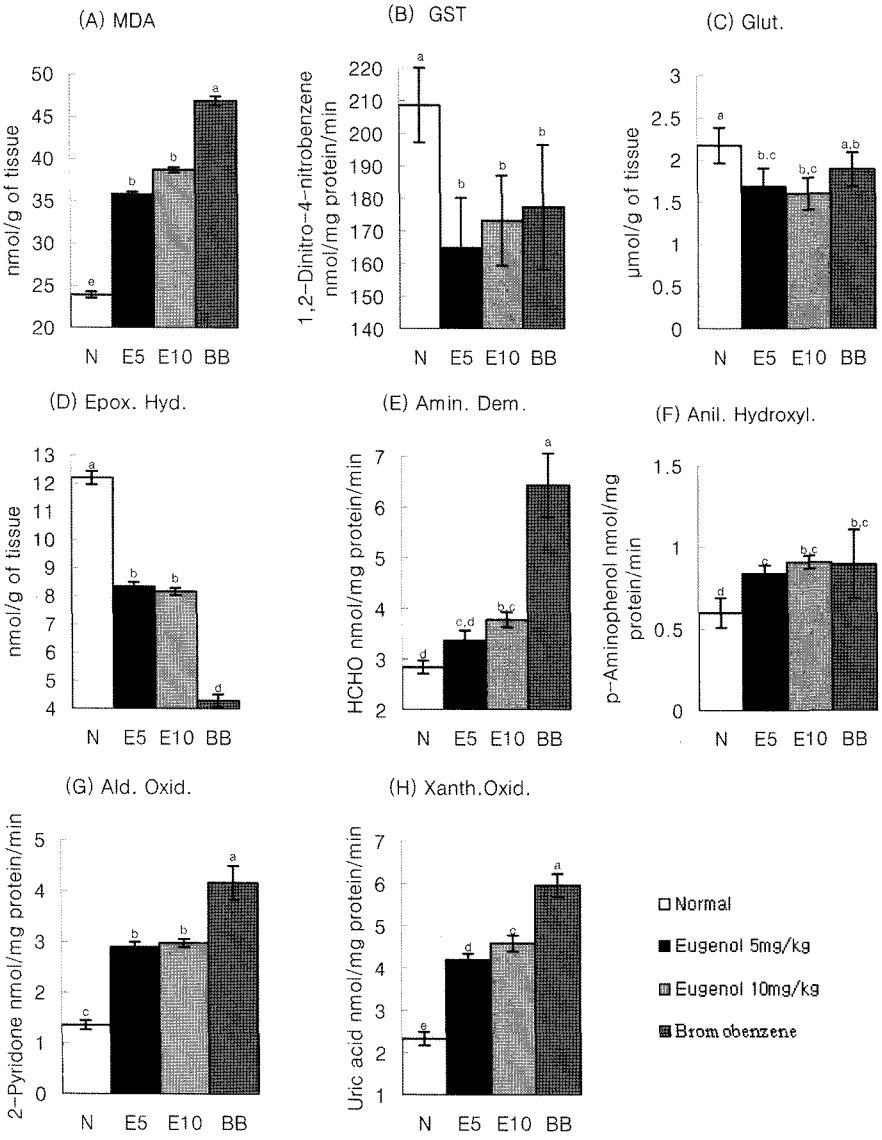

Fig. 4. Effect of eugenol on lipid peroxidation and hepatic drug-metabolizing enzymes. Bromobenzene (BB) was orally administered (480 mg/kg). Values on the graphic bar with the same superscript letter are not significantly different in each graph ($p < 0.05$).

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