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Inhibitory Effects of the Essential Oils on Acetaminophen-Induced Lipid Peroxidation in the Rat

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Abstract – Inhibitory effects of the essential oils obtained from ten herbs were tested on acetaminophen-induced lipid peroxidation in the rat. The oil of *Artemisia princeps* var. *orientalis* buds (AP-oil) showed the most significant hepatic malondialdehyde value which was comparable to those of ascorbic acid and methionine. This was warranted by the protective effect on hepatic glutathione depletion. Overview of the data on the activities of hepatic microsomal enzymes, aminopyrine *N*-demethylase and aniline hydroxylase led to the notice that the suppressed activities of those enzymes are mainly responsible for the anti-lipid peroxidation. The interpretation of GC-MS data on the AP-oil revealed the ingredient of cineol, thujone, carvone, borneol, camphor and terpineol.

Keywords – Essential oil, acetaminophen, lipid peroxidation, *Artemisia princeps* var. *orientalis*

Introduction

Free radicals producible from superoxide anion radical are the unambiguous reason for the lipid peroxidation. One of the reactive oxygen species, superoxide anion radical, could be generated mostly from the reaction of enzyme with cofactors of flavoprotein and hemoprotein which include cyclooxygenase, NADPH oxidase, xanthine oxidase, lipoxigenase, cytochrome P-450 and etc (Schaffner, 1975; Singh *et al.*, 1993; Morgan, 1997). Immunologically enhanced state in animals results in the high activities of these enzymes (Halliwell *et al.*, 1978; Deneke *et al.*, 1980; Freeman *et al.*, 1986). Free radicals cause lipid peroxidation by targeting the unsaturated fatty acid moiety of cell membrane to generate mainly malondialdehyde (MDA) (Ji *et al.*, 1991; Radi *et al.*, 1991). Therefore, the observation of hepatic MDA can be an indicative for the aging, which is a non-specific concept in the disease of adult. A number of natural products that is active for the many types of disease have been so far revealed to be effective for the lipid peroxidation. Essential oils obtainable from plants are composed of volatile and mostly fragrant components. We tested the inhibitory effects on the acetaminophen-induced lipid peroxidation in the rat. Successively, the enzyme activities of aniline hydroxylase, aminipyrine *N*-demethylase and glutathione content were also measured. Plant materials were mostly chosen to monitor the ingredients of the essential oil as much as possible.

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Materials and Methods

Plant material and steam distillation – The used plant materials were as follows: the rhizomes of *Zingiber officinale*, the buds of *Eugenia caryophyllata*, the stem barks of *Cinnamomum cassia*, the root barks of *Paeonia moutan*, the rhizomes of *Curcuma longa* and the herbs of *Chrysanthemum sibiricum*. All the commercially available ones were dried plant materals and they were identified by G. T. Kim (Division of Applied Plant Sciences, Sangji University, Korea). The bulb of *Allium sativum* was also purchased from the market in Wonju, Korea, and it was in the fresh state. The aerial part of *Allium victorialis* var. *platyphyllum* was available from National Alpine Agricultural Experiment Station, Pyongchang, Korea, and it was also in the fresh state. Others, the herbs of *Agastache rugosa*, the buds of *Artemisia princeps* var. *orientalis* were collected in Mt Taegi, Gangwon province, Korea, and the former one was collected on July, 2001 and the latter was on October, 2001. The fresh plant material was preserved at 5°C prior to the extraction. Especially, the bulbs of *A. sativum* and the aerial part of *A. victorialis* var. *platyphyllum* were macerated for the enzyme activation 1 h before the extraction. Other plant

materials were used in the finely cut state. These plant materials (0.50-2.0 kg) were extracted by steam distillation for 6 h. The distilled liquid was extracted with diethyl ether and dehydrated with anhydrous sodium sulfate followed by evaporation at 40°C. The residual oil was weighed and the yield was shown as follows: *Curcuma longa* (0.15%), *Artemisia princeps* var. *orientalis* (0.12%), *Paeonia moutan* (1.6%), *Eugenia caryophyllata* (1.26%), *Chrysanthemum sibiricum* (0.15%), *Agastache rugosa* (0.32%), *Allium sativum* (0.09%), *Zingiber officinale* (0.15%), *Cinnamomum cassia* (1.13%), *Allium victorialis* var. *platyphyllum* (0.04%). The obtained essential oils were used for the assays. Voucher specimens are deposited in the herbarium of Life Science and Natural Resources, Sangji University, Wonju, Korea.

Animal and treatment – Sprague-Dawley male rats were purchased from Korean Experimental Animal Co. and were adapted in constant conditions (temperature: 20±2°C, humidity: 40-60%, light/dark cycle: 12 hr) for more than two weeks. For the animal experiments, Sprague-Dawley male rats weighing 150±10 g were used. The essential oils were pretreated with 50 mg/kg (i.p.) once a day for 7 days before acetaminophen injection. Phenobarbital (75 mg/kg, i.p.) was treated for 3 days and acetaminophen (800 mg/kg) suspended by 1% tween 80 was intraperitoneally administered at the final day according to the method of Poulsen *et al.* (1985). The animals were fasted 24 hours prior to the experiments. Considering the variation of enzyme activity during a day, the animals were sacrificed at a set time (10:00 A.M.-12:00 A.M.).

Isolation of enzyme sources – After being anesthetized with CO_2 gas, the blood was decapitated from the abdomen aorta. The liver was washed by fluxing in saline and the liver tissue was homogenized in a four-fold quantity of ice-cooled 0.1M sodium phosphate buffer (pH 7.4). This suspension was centrifuged in the cool state (600×g, 10 min). The supernatant was again subjected to centrifugation (10,000×g, 20 min) and the precipitate was taken. This was subsequently suspended with an equivalent volume of 0.1 M sodium phosphate buffer (pH 7.4) and further subjected to centrifugation (105,000×g, 30 min). The supernatant obtained from centrifugation of the final one (105,000×g, 60 min) was discarded. The resulting precipitate was centrifuged (105,000×g, 60 min) in 0.1 M sodium phosphate buffer (pH 7.4) and the obtained microsomal fraction was used for the enzyme sources to measure the cytochrome P_{450}, aminopyrine *N*-demethylase, and aniline hydroxylase activities.

Material and reagents – NADH and NADPH were obtained from Sigma Chemical Co. and malondialdehyde and pyridine from Aldrich Chemical Co.. Reduced glutathione and oxidized glutathione were obtained from Fluka Chemical Co. and thiobarbituric acid from Katayama Chemical Co. respectively.

Measurement of malondialdehyde – The thiobarbituric acid (TBA) reactive substance in the liver was measured as a marker of lipid peroxidation by the method reported by Ohkawa *et al.* (1979) (4). An aliquot (0.4 ml) of the 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of an 8.1% SDS, 1.5 ml of a 20% acetate buffer (pH 3.5) and 1.5 ml of a 0.8% TBA solution. The mixture was heated at 95°C for 1 h. After cooling, 5.0 ml of n-butanol: pyridine (15:1) was added for extraction, and the absorbance of the *n*-butanol:pyridine layer at 532 nm was measured to identify the TBA reactive substance.

Measurement of aminopyrine *N*-demethylase activity – The aminopyrine N-demethylase activity was determined according to the Nashs method (1953). Briefly, the solution (2 ml) containing 2 mM aminopyrine, 0.5 mM NADPH, 10 mM $MgCl_2$, 150 mM KCl and 1 mM semicarbazide in 0.1 M Na^+/K^+ phosphate buffer (pH 7.5) was reacted for 15 min. The reaction was subsequently quenched by adding 15% $ZnSO_4$ and saturated $Ba(OH)_2$. This reactant was centrifuged and the supernatant was colored with Nash reagent. Finally, the activity of aminopyrine *N*-demethylase was calculated from the absorbance at the 415 nm wavelength.

Measurement of aniline hydroxylase activity – The aniline hydroxylase activity was determined according to the method reported by Bidlack and Lowery (1982). Briefly, 1 mM aniline, 0.5 mM NADPH and the enzyme sources were added to the solution containing 50 mM Tris. HCl buffer (pH 7.4), 10 mM $MgCl_2$ and 150 mM KCl to a final volume adjusted to 2.0 ml. This solution was reacted at 37°C for 20 min and the reaction was terminated by adding 20% trichloroacetic acid. The reaction mixture was then centrifuged, and 10% Na_2CO_3 and 0.2N NaOH/2% phenol were added to the obtained supernatant. This solution was reacted at 37°C for 30 min and the absorbance was measured at 640 nm. The enzyme activity was calculated from a standard calibration curve. The unit of enzyme activity was expressed as the amount (nmole) of *p*-aminophenol produced per minute in 1 mg of protein.

Measurement of glutathione content – Cysteine was quantitatively measured by the modified protocol of Gaitonde *et al.*s method (1967). The glutathione content was determined by subtracting cysteine-SH from nonprotein bound-SH. 10 % Trichloroacetic acid was added to the homogenate and followed by centrifugation and then 0.5 ml acetic acid and 0.5 ml ninhydrin reagent were added in the supernatant. This was heated for 10 min and cooled.

Immediately after the addition of 3 ml ethanol in that solution, the absorbance was measured.

GC-MS Analysis – Column [DB-1 {100% dimethyl polysiloxane; 0.25 mm i.d.×30 m length, J&W Scientific, (USA)}], Column temp. program {init. temp. 260°C (splitless), temp. increase velocity (16°C/min), final temp. 260°C (20 min)}; detector {Electron Impact-Quadrupole 1 (EI energy 70 eV); carrier gas {He (99.99%), flow rate (1.5 ml/min)}; transfer line temp. 260°C; Gas chromatogram was measured using Varian 3400 (USA) and electron impact-Mass spectra (EI-MS, ionization voltage 70 eV) were taken on a Finnigan Mat TSQ-700.

Cineol: Rt 6.71 min, MS (70 eV) m/z (%): 154.1 (100), 139.1 (68), 125.1 (15), 111.1 (71), 108.1 (93), 81.1 (94); Thujone: Rt 8.23 min, MS (70 eV) m/z (%): 150.1 (20), 135.1 (8), 122.1 (25), 107.1 (100), 91.1 (47), 79.1 (19); Carvone: Rt 8.51 min, MS (70 eV) m/z (%); Camphor: Rt 9.08, 152.2 (49), 137.1 (6), 109.1 (29), 95.1 (100), 81.1 (61); Terpineol: Rt 9.88 154.1 (31), 136.1 (20), 111.1 (72), 93.1 (55), 86.1 (30), 71.1 (100)

Statistics – The data is reported as a mean±S.E.. Statistical significance was analyzed by the Students t-test.

Results and Discussion

Excessive administration of acetaminophen, an analgesic/antipyretic drug, has been reported to cause lethal damage of necrosis on liver- and kidney tissues (Jollow *et al.*, 1974; Prescott *et al.*, 1983). Oxidative process of acetaminophen by hepatic cytochrome P-450 forms the metabolic intermediate which lead hepatic necrosis by inducing at least 50% depletion of glutathione. Although in the common dose acetaminophen could be metabolized to the formation of sulfate- and glucuronic acid-conjugates, the excessive dose has a main pathway of glutathione conjugation and therefore causes the glutathione depletion (Miners *et al.*, 1984; Lin *et al.*, 1986).

The anti-lipid peroxidative effects of ten essential oils in the rat, which are represented as a marker of hepatic MDA content, were shown in Fig. 1. The MDA content in the control was around three-fold higher than that of the normal. Methionine and ascorbic acid used for the positive control considerably suppressed the MDA of the control. All the essential oils suppressed the MDA but the values are higher than those of methionine or ascorbic acid-treated rats and the normal rats. No essential oil showed the higher value than that of the control. We have reported that the essential oil of *E. caryophyllata* could increase the MDA value of the normal rat (Park *et al.*, 2001). In addition, we have also reported the inhibitory

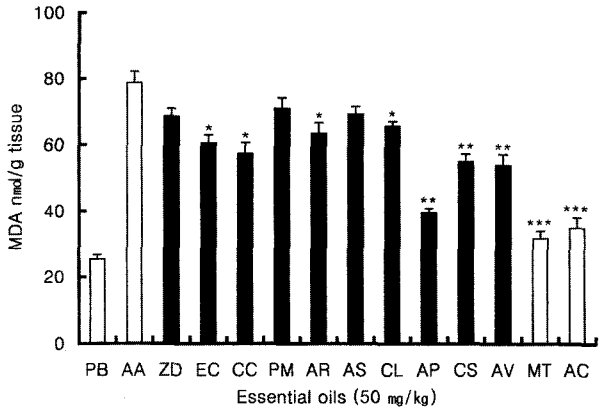

Fig. 1. Protective effects of the essential oils on the lipid peroxide induced by acetaminophen in the rat.
AA (acetaminophen), MT (methionine), AC (ascorbic acid); Other abbreviations represent the essential oils: ZD (*Zingiber officinale*), EC (*Eugenia caryophyllata*), CC (*Cinnamomum cassia*), PM (*Paeonia moutan*), AR (*Agastache rugosa*), AS (*Allium sativum*), CL (*Curcuma longa*), AP (*Artemisia princeps* var. *orientalis*), CS (*Chrysanthemum sibiricum*), AV (*Allium victorialis* var. *platyphyllum*); Values are mean±S.E. for 3 experiments (5 animals for each experiment). Abbreviations: PB (phenobarbital). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with the acetaminophen treatment.

effect of the essential oil of *C. cassia* on the hepatic MDA of the normal rat (Choi *et al.*, 2001). Based on our previous and the present result, it was suggested that many essential oil could decrease the MDA only in the induced lipid peroxidation as in acetaminophen treatment. The bud oil of *Artemisia princeps* var *orientalis* showed the most significant effect among the tested oils. It was also notable that the oil of *Allium victorialis* var. *platyphyllum* herbs showed the more potent effect than that of *Allium sativum* bulbs because both oils contain disulfide derivatives (Lee *et al.*, 2001; Hikino *et al.*, 1986). Although many beneficial bioactivities have been reported (Chalier *et al.*, 1998; Munday *et al.*, 1999), the present anti-lipid peroxidative effect of the essential oil of *Allium satvum* is not potent.

Hepatic cytochrome P-450, one of hepatic microsomal enzymes, can be classified into aniline hydroxylase and aminopyrine *N*-demethylase, which are responsible for the Phase I reaction. The enzymes generate superoxide anion radical during the action, so that their activities could be a motive power for the lipid peroxidation. In Fig. 2 and 3, aniline hydroxilase and aminopyrine *N*-demethylase activities in the rat induced by acetaminophen administration were shown. The treatment with acetaminophen alone caused considerably higher activities of aniline hydroxylase and aminopyrine *N*-demethylase than any other treatment. Ascorbic acid showed highly significant activity whereas methionine showed no activity. This indicated that methionine shows the anti-lipid peroxidative effect through

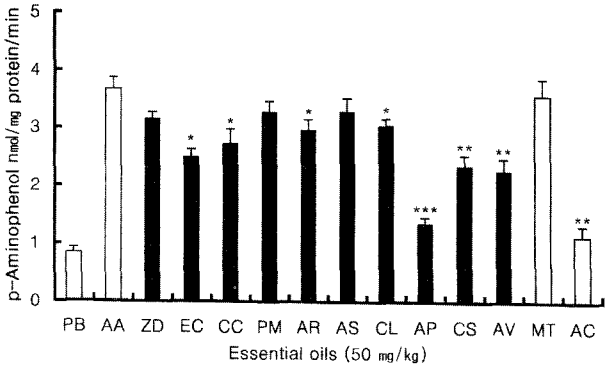

Fig. 2. Protective effects of the essential oils on the hepatic glutathione depletion induced by acetaminophen in the rat.
AA (acetaminophen), MT (methionine), AC (ascorbic acid); Other abbreviations represent the essential oils: ZD (*Zingiber officinale*), EC (*Eugenia caryophyllata*), CC (*Cinnamomum cassia*), PM (*Paeonia moutan*), AR (*Agastache rugosa*), AS (*Allium sativum*), CL (*Curcuma longa*), AP (*Artemisia princeps* var. *orientalis*), CS (*Chrysanthemum sibiricum*), AV (*Allium victorialis* var. *platyphyllum*); Values are mean±S.E. for 3 experiments (5 animals for each experiment). Abbreviations: PB (phenobarbital). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with the acetaminophen treatment.

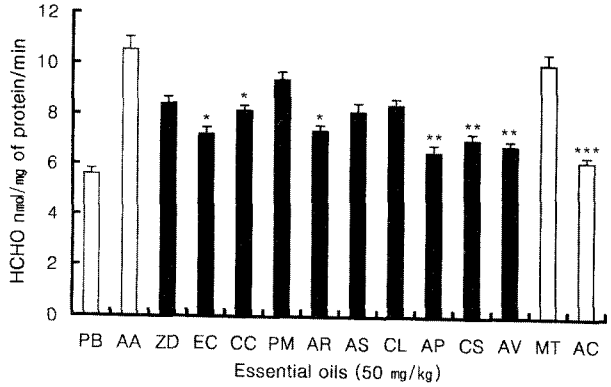

Fig. 3. Protective effects of the essential oils on aniline hydroxylase activity induced by acetaminophen.
AA (acetaminophen), MT (methionine), AC (ascorbic acid); Other abbreviations represent the essential oils: ZD (*Zingiber officinale*), EC (*Eugenia caryophyllata*), CC (*Cinnamomum cassia*), PM (*Paeonia moutan*), AR (*Agastache rugosa*), AS (*Allium sativum*), CL (*Curcuma longa*), AP (*Artemisia princeps* var. *orientalis*), CS (*Chrysanthemum sibiricum*), AV (*Allium victorialis* var. *platyphyllum*); Values are mean±S.E. for 3 experiments (5 animals for each experiment). Abbreviations: PB (phenobarbital). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with the acetaminophen treatment.

the different pathway than cytochrome P-450 inhibition. Since the trend in inhibitory effect on cytochrome P-450 activity was comparable with the effect on MDA content, the inhibition of essential oils on this enzyme are responsible for the anti-lipid peroxidation in the present assay system. Acetaminophen administration exhibited much lower content of glutathione than no treatment (Fig. 4). All the pretreatment prohibited the reduction of glutathione but their activities

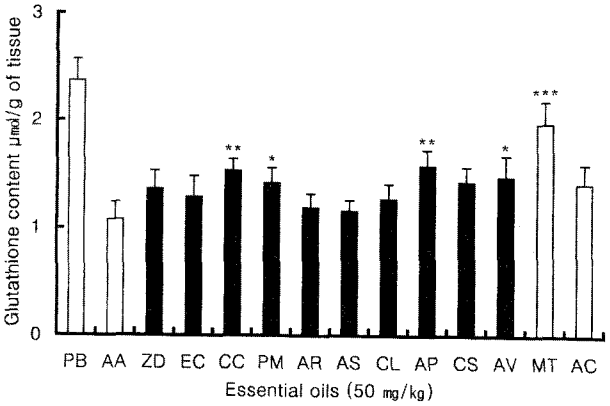

Fig. 4. Protective effects of the essential oils on hepatic glutathione content induced by acetaminophen in the rat.
AA (acetaminophen), MT (methionine), AC (ascorbic acid); Other abbreviations represent the essential oils: ZD (*Zingiber officinale*), EC (*Eugenia caryophyllata*), CC (*Cinnamomum cassia*), PM (*Paeonia moutan*), AR (*Agastache rugosa*), AS (*Allium sativum*), CL (*Curcuma longa*), AP (*Artemisia princeps* var. *orientalis*), CS (*Chrysanthemum sibiricum*), AV (*Allium victorialis* var. *platyphyllum*); Values are mean±S.E. for 3 experiments (5 animals for each experiment). Abbreviations: PB (phenobarbital). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with the acetaminophen treatment.

were not potent except that of methionine. The increase of glutathione in methionine-treated rat could explain the anti-lipid peroxidative effect of methionine.

Through the investigation on anti-lipid peroxidative effect of the essential oils, the oil of *Artemisia princeps* var. *orientalis* buds was found to be the most significant anti-lipid peroxidative agent among the tested oils. GC-MS analysis was undertaken to find the composition of the oil. The interpretation of the respective MS data on the peak of AP-oil chromatogram revealed the ingredient of cineol, thujone, carvone, borneol, camphor and terpineol.

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