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THE EFFECTS OF *URTICA DIOICA* L. LEAF EXTRACT ON ANILINE 4-HYDROXYLASE IN MICE

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Abstract: The effects of hydroalcoholic (80 % ethanol-20 % water) extract of *Urtica dioica* L. on microsomal aniline 4-hydroxylase (A4H) were investigated in the liver of Swiss albino mice (8-10 weeks old) treated with two doses (50 and 100 mg/kg body weight, given orally for 14 days). The activities of A4H showed a significant increase in the liver at both dose levels of extract treatment. The hydroalcoholic extract of *Urtica dioica* induced the activities of A4H that had been increased by treatment of metal ions (Mg^{2+} and Ca^{2+}) and the mixture of cofactors (NADH and NADPH). At saturated concentration of cofactor, microsomal A4H exhibited significantly even higher activities in the presence of the mixture of cofactors than NADPH and NADH. Mg^{2+} and Ca^{2+} ions acted as stimulants *in vitro*. The present results suggest that the hydroalcoholic extract of *Urtica dioica* may have modalatory effect on aniline hydroxylase at least in part and enhance the activity of A4H adding metals ions and cofactors.

Keywords: Urtica *dioica* L., aniline 4-hydroxylase, cofactor, metal ions

Xenobiotic detoxification is controlled mainly by the liver . Phase I enzyme reactions of many organisms are catalyzed by mixed function oxidase (MFO) systems via several oxidation, reduction and hydrolysis reactions (1). Since some of the main organs are susceptible for developing tumors, this led to the idea of examining the inducibility of phase I enzymes. Aniline 4-hydroxylase (A4H) has been known as a member of mixed-function oxidases belonging to P450 2E1 gene family and it catalyzes transformation of toxic aniline to a non-toxic derivative, *p*-aminophenol (2). Aniline is converted to *p*-aminophenol by cytochrome P450-dependent aniline hydroxylase. *p*-Aminophenol is an acute nephrotoxin of the pars recta of the promixal tubule (3). It was also reported after *in vivo* investigations that cytochrome P450 and a cytchrome c reductase are required for reconstitution of microsomal A4H activity in rat (4). *In vivo* studies with liver microsomes have shown that A4H activity was dependent upon pH, incubation period, temperature, tissue studied, nutritional state and pretreatment of organism (5).

Urtica dioica L. (*Urticaceae*) or stinging nettle traditionally employed as a folklore remedy for wide spectrum of ailments is nowadays incorporated into a number of herbal medicinal preparations. Leaves of this plant have been reported to exhibit hypotensive effect (6), anti-inflammatory effect (7), to use in the therapy of prostatic hyperplasia (8), diuretic activity (9) and immunomodulatory activity (10). The data of the studies clearly suggested that *Urtica dioica* L. contained constituents altering the activities of major drug metabolizing enzyme whose route of activity is mainly via cytochrome P450 systems (11, 12).

We found that *Urtica dioica* L. had modulatory effects on phase I enzyme systems expected for A4H activity (11). According to article survey, there is no information about potential effect of *Urtica dioica* L. on modulatory effects of A4H activity, requirements of cofactors (NADH, NADPH and NADH-NADPH) and metal ions (Ca^{2+} and Mg^{2+}) in *Swiss albino* mice. A4H induce microsomal phase I enzyme systems, thus it is important to find an activator for A4H. We thought that A4H activated by *Urtica dioica* L. could decline the effects of toxic aniline, increase *p*-aminophenol in microsomal xenobiotic detoxification systems and regulate the requirement of cofactor and metal ions for A4H, effective in these assays.

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MATERIALS AND METHODS

Chemicals

NADH, NADPH and trichloroacetic acid (TCA) were obtained from Merck A. G. Darmstadt, Germany. Bovine serum albumin (BSA), cholic acid, folin and Ciocalteu's phenol reagent, aniline, butylated hydroxyanisole (BHA), *p*-aminophenol and HEPES were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were obtained form local firms and were of the highest purity grade.

Preparation of plant

Urticaceae plants were collected from the Samsun-Turkey in April (2007) during the early

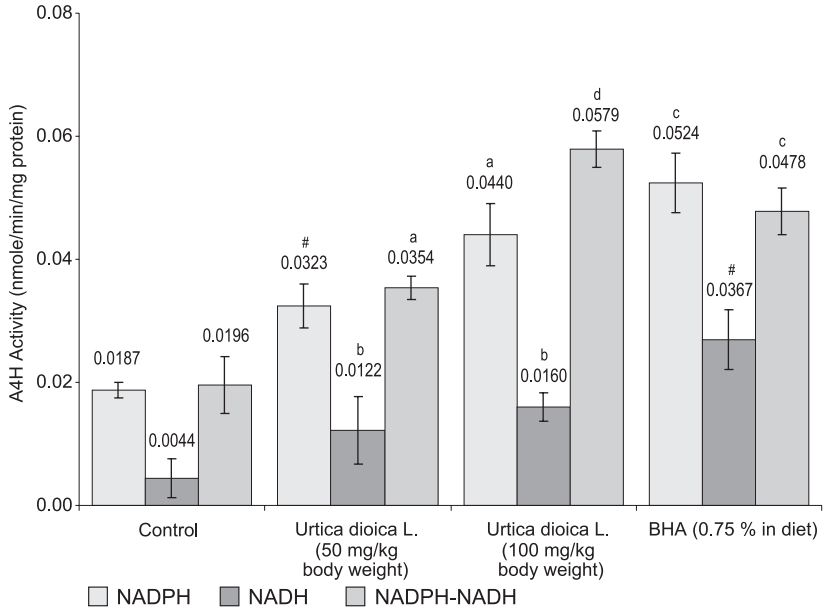

Figure 1. Modulatory effect of two different doses of *Urtica dioica* L. (*Urticaceae*) leaf extract and BHA hepatic aniline 4-hydroxylase (A4H) cofactor requirement in mice.
The statistical analysis according to control values: [a] $p < 0.01$; [b] $p < 0.05$; [c] $p < 0.001$); [d] $p < 0.005$; [#]not significant

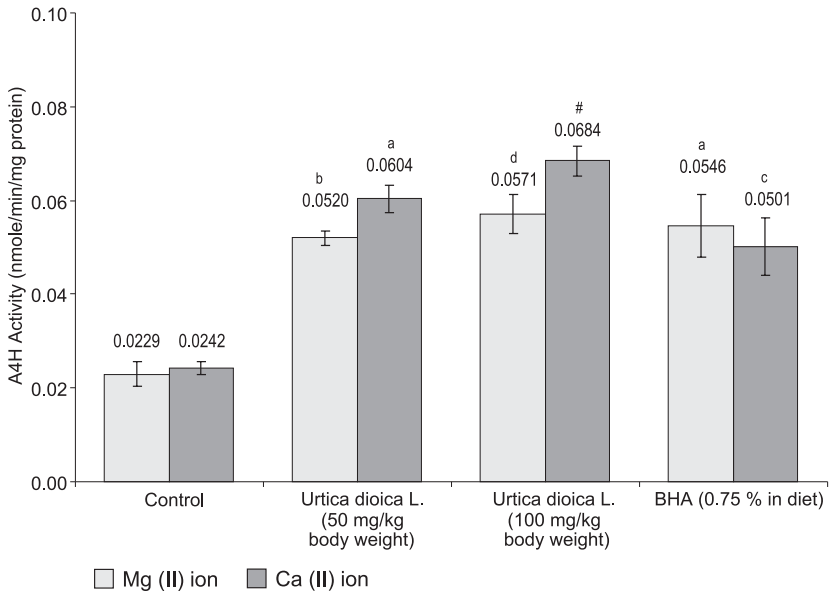

Figure 2. Modulatory effect of two different doses of *Urtica dioica* L. (*Urticaceae*) leaf extract and BHA on the dependence of microsomal aniline 4-hydroxylase (A4H) activity on Ca^{2+} and Mg^{2+} in mice. The statistical analysis according to control values: [a] $p < 0.01$; [b] $p < 0.05$; [c] $p < 0.001$); [d] $p < 0.005$; [#]not significant

Table 1. Cofactor requirement for A4H activity in the effect of control, two different doses of *Urtica dioica* L. (*Urticaceae*) leaf extract and BHA

Cofactor	Final conc (mM)	Control		50 mg/kg body weight.		100 mg/kg body weight		BHA (0.75 % in diet)	
		Activity (nmole/min/ mg protein)	Activity (%)	Activity (nmole/min/ mg protein)	Activity (%)	Activity (nmole/min/ mg protein)	Activity (%)	Activity (nmole/min/ mg protein)	Activity (%)
Control	-	-	0	-	0	-	0	-	0
NADPH	0.50	0.0187 ± 0.0012	**100.0**	0.0323 ±0.0035	**100.0**	0.0440 ±0.0051	**100.0**	0.0524 ±0.0049	**100.0**
NADH	0.50	0.0044 ± 0.0032	**23.5**	0.0122 ±0.0054	**37.7**	0.0160 ±0.0024	**36.3**	0.0269 ±0.0048	**51.3**
NADH-NADPH	0.25-0.25	0.0196 ± 0.0047	**104.8**	0.0354 ±0.0019	**109.5**	0.0579 ±0.0029	**131.5**	0.0478 ±0.0037	**91.2**
NADPH-generating system		0.0185 ± 0.0024	**98.9**	0.0315 ±0.0037	**97.5**	0.0432 ±0.0029	**98.2**	0.0492 ±0.0051	**93.7**
Glucose -6-phosphate $MgCl_2$	2.50 2.50								
Potassium phosphate buffer (pH 7.4) $NADP^+$	14.20 0.25								
Glucose-6-phosphate dehydrogenase	0.25 units								

hours of the day in the 19 Mayýs University Campus. They were then dried shade and sliced. Afterwards, it was soxhletted using a mixture of ethanol (80%) and doubly distilled water (20%) tree times. The mixture was lyophilized.

Animals and treatments

Swiss albino male mice (8-9 weeks old; 28-32 g) were divided into four groups of five mice per group. Group I (n = 5) was fed a normal diet and given doubly-distilled water through oral gavage, daily for 14 days. The first group was used as a control. The second group (n = 5) was fed a normal diet and treated with 50 mg/kg b. w. *U. dioica* extract through oral gavage, dissolved in 0.100 mL of double-distilled water, daily for 14 days. The third group (n = 5 was fed a normal diet and treated with 100 mg/kg b. w. of *U. dioica* extract through oral gavage, dissolved in 0.100 mL of double-distilled water. The fourth group (n = 5) was designated as a positive control group. The animals were orally on diet containing 0.75 % BHA daily for 14 days. The animals were sacrificed 24 h after the last treatment.

Preparation of subcellular fractions in the liver

Mice were starved overnight and sacrificed by cervical dislocation. The entire liver was perfused with ice-cold 0.9 % NaCl in a short time to eliminate any possible effects due to diurnal variation and rinsed in cold 0.15 M Tris-KCl buffer (pH 7.4). Liver homogenates were prepared in ice-cold 0.15 M Tris-KCl buffer (pH 7.4) and centrifuged at 10,000 rpm for 20 min at + 4°C. The microsomal fraction (pellet) was precipitated by centrifuging the supernatant at 100 000 × g for 60 min at + 4°C and stored in liquid nitrogren until assay of A4H (13).

Determination of protein

Microsomal protein was determined by the method of Lowry using bovine serum albumin (BSA) as a standard at 660 nm (14). A standard curve of 0 to 100 µg/mL BSA was also constructed and was used for calculation of protein amounts in microsomes.

Assay of aniline 4-hydroxylase (A4H)

Aniline hydroxylase activity was determined by measuring the amount of *p*-aminophenol formed from aniline hydrochloride as described in (15). The incubation mixture contained HEPES buffer (0.2 mM, pH 7.4, 0.5 mL), aniline (10 mM, 0,1 mL) cofactors (10 mM NADPH or 10 mM NADH or 5.0 mM NADPH-5.0 mM NADH, or NADPH generating system, 0,05 mL) and microsomal enzyme (2 mg microsomal protein, 0.25 mL) in a final volume of 1.0 mL. The reactions were started by addition of cofactor and detected at 630 nm. NADPH generating system was prepared as previously described (16). NADPH generating system consisted of 1.3 mM NADP, 3.3 mM $MgCl_2$, 3.3 mM glucose 6-phosphate, and 0.4 U/mL of glucose 6-phosphate dehydrogenase (Table 1). For metal ions, aqueous solutions of Ca^{2+} and Mg^{2+} were used during metal ion incubation experiments (15, 17). The incubation mixture contained HEPES buffer, aniline, microsomal enzyme, metal ions (0,05 mL, 1.0 mM Ca^{2+} or 1.0 mM Mg^{2+}) in a final volume of 1.0 mL.

Statistical analysis of data

The results are presented as the mean ± S.D. Statistical analysis was applied using ANOVA following Dunnett's *t*-test. Values were evaluated indicating a significant differences referring to control groups. The SPSS 10.0 (Statistical Program for Social Sciences) was used for the statistical analysis.

RESULTS AND DISCUSSION

In this study, application of the hydroalcoholic extract of *Urticaceae* to mice showed a marked effect on A4H. The administrations of *Urticaceae* can effectively adjust the activity of this enzyme that is vital for enzymatic and nonenzymatic activation pathways. Two different doses of *Urticaceae* leaf extract (50 and 100 mg/kg of extract/body weight of mice/day for 14 days) and BHA had discernible effects on the animals. BHA was used as a positive control to prove a chemoprotectant in assorted animal models of specific carcinogenicity and detoxification (17, 18).

Cytochrome P450 consists of a family of cytochrome P450 hemoproteins, NADPH-cytochrome P450 reductase, and NADH-cytochrome b_5 reductase which are cytochrome P450-dependent monooxygenases or mixed-function oxidases. Their primary tasks are detoxifications of both endogenous and exogenous compounds (5). In these detoxification reactions, in microsomal system involving the CYP450, electron flows from NADPH or NADH through a flavoprotein CYP450 to different isomorphic factors of CYP450. The main function of phase I metabolism is to prepare a compound for phase II metabolism. Phase II metabolism leads to true detoxification of drugs and xenobiotics which results in water soluble products which can be easily excreted (19). The findings of this study were based on the microsomal A4H activity from normal mice liver using aniline as the substrate. The results of this investigation are in generel agreement with the earlier reports which indicate reduced ability of mice liver to metabolize aniline when compared to other species (20). It was illustrated in sheep and rabbit liver microsomes that NADPH was necessitated for A4H activity (21). At saturating concentrations of cofactor, the mice liver microsomes exhibited much higher activities in the presence of NADPH and NADPH-NADH than that of NADH which support the precondition of NADPH for A4H activity (17). According to our previous investigation, *Urtica dioica* L. has biological significance in the activation of A4H both in phase I and II metabolism (17). The active component of this plant may regulate A4H in mixed function oxidase systems, which are responsible for removal of xenobiotics and catalyzing transformation of toxic aniline to a non-toxic derivative, *p*-aminophenol. The specific activities of A4H in microsomes of the liver were compared with their control groups. NADH and/or NADPH at saturating concentration (0.5 mM) were used to determine the cofactor requirements for A4H (Table 1, Figure 1). It was seen that A4H activity was at the maximum in the presence of NADPH, however, in the presence of NADH, it was marginally active (77% less in control group, 63% in group II and 64% in group III). The activity was higher in control group by 4.8%, in group II by 9.5% and in group III by 31.5% when these two cofactors were used together. Additionally, the activity reached in control group 98.9% in group II 97.5% and in group III 98.2% with the presence of an NADPH-generating system. Also BHA regulated cofactor requirement and supported previous investigation (11, 17). In the liver microsomal system, A4H activities increased dramatically and to similar extents in mice receiving the BHA diet. BHA feeding caused a marked elevation of aniline hydroxylase activity in mice.

Although earlier observations have shown inhibitory effects of several divalent metal ions, the effects of Mg^{2+} and Ca^{2+} on A4H activity were stimulatory at 5.0 mM concentration (21-24). The maximum activity shown by 5.0 mM Mg^{2+} and 5.0 mM Ca^{2+} was in line with the earlier research (17). Accumulation of toxic aniline in tissues may be prevented by detoxification pathways, since the exposure of mice to such divalent cations leads to an increase in A4H activity by stimulation. The *Urticaceae* extracts can activate the transformation of aniline to *p*-aminophenol because of more positive effect of Mg^{2+} and Ca^{2+} ions. This may be an evidence for relationship between metal ions and the formation of *p*-aminophenol. Figure 2 shows the effect of Mg^{2+} and Ca^{+2} on the specific activity of A4H assayed in microsomal system of the liver. For *in vitro* treatment of Mg^{2+} the activity was strengthened at the lower dose of treatment 2.27 times and at a higher dose of treatment 2.49 times. BHA was found to be quite effective in A4H activity (2.38 times). For *in vitro* treatment of Ca^{2+}, the activity of A4H exhibited a significant increase by 2.49 times in group II, 2.82 times in group III, and 2.07 times in group IV.

To conclude, application of *Urtica dioica* L. may have an important effect on A4H activity, cofactor requirement and the effects of metal ions. The significant differences in the levels of A4H activity and the effects of cofactors (NADPH, NADH, NADH-NADPH) and metal ions (Mg^{2+} and Ca^{2+}) might be taken as reliable markers for evaluating and supporting drug metabolization potential (phase I reactions) of this plant. The increase in A4H activity gives rise to elevated action of metabolite which can be strongly activated by biotransformation enzyme system. This system can also shift the xenobiotic metabolism equilibrium towards detoxification or might reinforce its importance in cytoprotection and chemoprevention.

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