Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: https://www.tandfonline.com/loi/iphb20

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To cite this article: Serdar Karakurt, Aslı Semiz, Gurbet Celik, Ayşe Mine Gencler-Ozkan, Alaattin Sen & Orhan Adali (2013) *Epilobium hirsutum* alters xenobiotic metabolizing CYP1A1, CYP2E1, NQO1 and GPx activities, mRNA and protein levels in rats, Pharmaceutical Biology, 51:5, 650-658, DOI: 10.3109/13880209.2012.762404

To link to this article: https://doi.org/10.3109/13880209.2012.762404

Published online: 26 Mar 2013.

Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2013; 51(5): 650–658
 DOI: 10.3109/13880209.2012.762404

informa
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RESEARCH ARTICLE

Epilobium hirsutum alters xenobiotic metabolizing CYP1A1, CYP2E1, NQO1 and GPx activities, mRNA and protein levels in rats

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Abstract

Context: Natural products have attracted increasing interests due to their use in flavoring, nutrition, cosmetics, pharmacy and medicine. *Epilobium hirsutum* L. (Onagraceae) is known for its analgesic, antimicrobial, and antiproliferative activity. CYP1A1 and CYP2E1, xenobiotic metabolizing enzymes, serve as a metabolic activation route yielding reactive metabolites that are eliminated by the action of NQO1 and glutathione peroxidase (GPx) enzymes.
Objective: This study investigated *in vivo* effects of *Epilobium hirsutum* (*EH*) on CYP2E1, CYP1A1, NQO1 and GPx activities, protein and mRNA expressions in liver.
Materials and methods: Male Wistar Albino rats were injected with *EH* at a dose of 37.5 mg/kg i.p. daily for 9 d. CYP2E1, CYP1A1, NQO1 and GPx activities, protein and mRNA levels were determined by enzyme assays, Western blotting and qPCR, respectively.
Results: CYP1A1 associated ethoxyresorufin-*O*-deethylase activity of control and *EH*-treated animals were found as 6.54 ± 1.21 and 4.48 ± 1.67 nmol/min/mg, respectively. CYP2E1 associated aniline 4-hydroxylase of control and *EH* group were 0.537 ± 0.011 and 0.109 ± 0.01 nmol/min/mg, respectively. However, *EH* treatment increased the GPx and NQO1 activities from 0.069 ± 0.015 to 0.107 ± 0.026 nmol/min/mg and from 163.34 ± 92 to 588.3 ± 14 nmol/min/mg, respectively. Furthermore, protein and mRNA expression analysis revealed that CYP1A1 and CYP2E1 levels were decreased while those of NQO1 and GPx increased after *EH* treatment.
Discussion and conclusion: Our current data suggest that the metabolism of xenobiotics, including drugs, may be altered due to changes in the expression and activity of these proteins by *EH*.

Keywords

Cytochrome P450, drug metabolism, glutathione peroxidase, liver enzymes, medicinal plant, NQO1, onagraceae

History

Received 20 July 2012
Revised 30 November 2012
Accepted 18 December 2012
Published online 26 March 2013

Introduction

Naturally produced plant origin chemicals have gained crucial importance over the last decades because of their diverse biological effects in cancer, aging, cardiovascular diseases and their antioxidant activity. Herbs are used in many ways, including flavoring, nutrition, beverages, dyeing, cosmetics and medicine. It is an undeniable fact that plants have been used as treatment for human health from time immemorial. *Epilobium hirsutum* L. (Onagraceae), the largest genus of the family, is widely distributed all over the world and consists of approximately 200 species. It is found in moist wastelands of the Mediterranean region, Europe, Asia and Africa. The species *Epilobium hirsutum* (*EH*) possesses polyphenolics including steroids, tannins and flavonoids (Barakat et al., 1997; Ivancheva et al., 1992). HPLC analysis of the plant *EH* carried out by Barakat et al. (1997) showed the presence of gallic, protocatechuic, ellagic, valoneic dilactone, *p*-coumaric acids, methyl gallate, *p*-methoxy gallic acid methyl ester, kaempferol, quercetin and myricetin. Total phenolic content of *EH* was reported to be 4.03 ± 0.12 mg of gallic acid equivalents (GAE)/100 g of dry weight (Wojdyło et al., 2007). The body of the plant has been reported as an anti-inflammatory and edema preventer (Hiermann, et al., 1986). In Anatolia, the leaves and roots of *EH* are used as an antifebrile drug and for the treatment of constipation and prostate cancer (Everest & Ozturk, 2005; Tita et al., 2001). Moreover, *EH* has also been found to prevent benign prostate tumors (Vitalone et al., 2001, 2003). Aqueous extracts of the plant have been shown to be effective as edema and tumor deterrents in rats and reported to have an antimicrobial effect, especially on *Pseudomanas pyocyanea*, *Candida albicans* and *Staphylacoccus aureus* (Battinelli et al., 2001).

The microsomal cytochrome P450 (CYP) monooxygenase system has a key role in toxicity and carcinogenesis by bioactivating a variety of xenobiotics such as drugs, food additives, industrial solvents and pollutants to highly reactive and mutagenic metabolites. Although, the main function of these CYP enzymes is detoxification of these compounds, many CYP isoforms catalyze the metabolic activation of

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procarcinogens to their ultimate carcinogenic forms (Guengerich & Shimada, 1991). CYP1A1, one of the members of P450s, metabolizes a variety of polycyclic aromatic hydrocarbons (PAHs) to mutagenic metabolites. PAHs such as benzo[*a*]pyrene (BaP), the major substrate of CYP1A1 enzymes, play a major role in lung cancer development (Hecht, 1999). Although BaP is not directly mutagenic, its metabolism via CYP1A1 enzymes resulted in the formation of a highly reactive BaP-7,8-diol-9,10-epoxide that forms covalent DNA adducts, especially with deoxyguanosine. The microsomal P450 enzyme CYP2E1 is characterized by the oxidation of ethanol. Alcohol, benzene and pyridine inducible CYP2E1 metabolizes many low-molecular weight drugs, toxicants and carcinogens, such as acetaminophen, nitrosamines, phenol, benzene, 4-nitrophenol, carbon tetrachloride, chloroform, pyrazole and vinyl chloride (Arinc et al., 2000, 2007; Gonzalez, 2005). The increase in the activities of CYP1A1 and CYP2E1 has been shown to potentiate the activation of carcinogenic metabolites. It has also been shown that CYP1A1 and CYP2E1 are associated with the formation of lung and breast cancers (Cleary et al., 2010; Nakachi et al., 1993).

The hazardous effects of CYP enzymes can be eliminated by the action of Phase II and antioxidant enzymes such as NADPH quinone oxidoreductase 1 (NQO1) and glutathione peroxidase (GPx). NQO1 is an obligate two-electron reductase and can protect against natural and exogenous quinones reduced to hydroquinones in a single two-electron step. Moreover, two electron reduction by NQO1 leads to the formation of antioxidant compounds such as vitamin E quinone, coenzyme Q10 and their complement hydroquinones (Beyer et al., 1996; Siegel et al., 1997). Additionally, NQO1 has been shown to stabilize tumor suppressor protein p53, (Anwar et al., 2003). Therefore, NQO1 can be categorized as an antioxidant and cancer-preventive enzyme. The enzyme GPx, a selenocysteine-containing protein, has been found in both the cytosol and the mitochondria. GPx has a central role in controlling the amount of reactive oxygen species (ROS) and therefore buffering oxidative stress. This antioxidant enzyme has a variety of functions in mammals as it reduces fatty acid hydroperoxides, H_2O_2, phospholipid hydroperoxides and cholesterol hydroperoxides (Imai et al., 1998). GPx knockout mice were found extremely susceptible to oxidative stressors such as paraquat (de Haan et al., 1998). Furthermore, its absence has been found to be associated with cardiovascular diseases and stroke incidences (Freedman et al., 1996). Therefore, inhibition of the CYP1A1 and CYP2E1 enzymes and activation of GPx and NQO1 might be accepted as a better cancer chemoprevention strategy.

Materials and methods

Chemicals

Aniline was obtained from Fisher Scientific Company, Chemical Manufacturing Division (Fair Lawn, NJ). $NADP^+$ and NADPH were obtained from Applichem GmbH (Darmstadt, Germany). Formaldehyde was obtained from Fluka A.G. (Buchs, Switzerland). Bicinchoninic acid, D-glucose-6-phosphate monosodium salt, D-glucose-6-phosphate dehydrogenase, NDMA, Tris, HEPES and ethoxyresorufin were purchased from Sigma-Aldrich (St. Louis, MO). The CYP1A1, CYP2E1, GPx and NQO1 antibodies were purchased from Santa Cruz (Santa Cruz, CA) and Abcam (Cambridge, UK). Primers were made by Bio Basic Inc (ON, Canada). All other chemicals and solvents were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

Plant material and extract preparation

Flowering aerial parts of *EH* were collected from Golyaka, Duzce, Turkey, at an altitude of 563 m, in July 2009. The plant was identified and a voucher specimen (No: AEF 25812) was deposited in the Herbarium of Faculty of Pharmacy at Ankara. Air-dried and powdered plant material was subjected to active maceration in ddH_2O by using a Heidolph mechanic shaker at 300 rpm at room temperature for 8 h. The extract obtained was filtered from filter paper and dried in a freeze-dryer (Christ Gamma 2-16 LSC, Osterode am Harz, Germany) and weighed. The total concentration of phenolic compounds in the *EH* extracts was determined using a series of gallic acid standards as described by the method of Singleton & Rossi (1965) and the results were given as mg GAE per 100 g of extract. The extract was gassed with nitrogen to prevent oxygen interaction, and stored at −20 °C for injection to rats.

Animals and treatments

Male Wistar Albino rats (12 weeks old) weighing 200–250 g were used. They were housed at the University Animal House in standard conditions and fed with a standard diet with water *ad libitum*. All experimental procedures in animals such as the administration of substances by intraperitoneally (i.p.), collection of blood and tissue, etc., were performed to the national standards under appropriate regimes with veterinary services and licensed projects. Rats were randomly assorted into the following two groups: Group I (control I, 10 rats) rats were treated with water i.p. daily for 9 d; Group II (*EH*, 30 rats) rats were treated with *EH* water extract 37.5 mg/kg, i.p. daily for nine consecutive days. At the end of the experimental period and following 16 h of fasting, the animals were sacrificed. Blood samples were taken from the aorta to determine the serum enzymes. The livers were isolated and rinsed with cold physiological saline and stored at −80 °C until analyzed.

Preparation of tissue subcellular fractions

The tissues were homogenized in 4 parts homogenization solution (1.15% KCl containing 3 mM EDTA, 0.5 mM APMSF, 0.3 mM ε-aminocaproic acid, 0.15 mM butylated hydroxytoluene, 0.025% Triton X-100) using a tissue homogenizer with a teflon pestle at 4 °C. The subcellular fractions of rat tissues were prepared by a standard differential centrifugation procedure as described previously (Sen & Kirikbakan, 2004).

The amounts of proteins in various fractions were measured with BCA (bicinchoninic acid) as described by Smith et al. (1985) using crystalline bovine serum albumin as a standard.

Measurement of enzyme activities

7-Ethoxyresorufin-*O*-deethylase (EROD) activity was determined by the method of Burke & Mayer (1974) as optimized

by Arinc & Sen (1994). Formation of resorufin from 7-ethoxyresorufin was measured by spectrofluorometric method. Typical reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.8 containing 0.2 M NaCl, 1.2 mg BSA, 1.5 μM 7-ethoxyresorufin, 100 μg microsomal protein, 0.5 mM NADPH in a final volume of 2 ml in a fluorometer cuvvette. The reaction was initiated by the addition of substrate and followed for 2 min in spectrofluorometer (Varian Cary Eclipse, Mulgrave, Australia) at 535 nm (excitation) and 585 nm (emission).

Aniline 4-hydroxylase activity was measured by the quantification of *p*-aminophenol as modified according to the method of Imai et al. (1966). The reaction mixture contained 10 mM aniline, 1.5 mg microsomal protein, 100 mM HEPES buffer, pH 7.6 and NADPH generating system consisting of 0.5 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose 6-phosphate, 0.5 units of glucose 6-phosphate dehydrogenase, 14.6 mM HEPES buffer, pH 7.8 in a final volume of 0.5 ml. The reaction was carried out for 25 min at 37 °C.

NDMA *N*-demethylase activity of rat liver microsomes was determined by the method of Gorsky & Hollenberg (1989), and formaldehyde formed was measured by the method of Nash (1953) as modified by Cochin & Axelrod (1959). The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.7, 2.5 mM NDMA, 0.5 mM NADPH-generating system consisting of 0.5 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose 6-phosphate, 0.5 units of glucose 6-phosphate dehydrogenase, 14.6 mM HEPES buffer, pH 7.8 and 1.5 mg liver microsomal protein in a final volume of 1.0 ml. The reaction was terminated by the addition of 1.0 ml 0.75 N perchloric acid solution after a 20 min incubation period at 25 °C.

GPx activity was determined according to the method of Paglia & Valentine (1967) with the modification of Sadi & Guray (2009). The reaction mixture contained 85 mM Tris-HCl, pH 8.0, 2 mM GSH, 0.24 units of glutathione reductase, 4 mg of cytosolic protein and 0.066 mM NADPH. One unit activity was described as the amount of NADPH consumed in 1 min by 1 mg protein containing cytosolic fraction.

Rat liver NQO1 enzyme activity was determined according to the method of Ernster (1967) as modified by Karakurt & Adali (2011). Standard enzyme assay mixtures contained 25 mM potassium phosphate buffer, pH 7.8, 4 mg of cytosolic fraction, 0.7 mg BSA, 0.2 mM NADPH and 40 μM DCPIP in a final volume of 1 ml. DCPIP reduction reaction was followed continuously at 600 nm for 2 min by Schimadzu UV-160 A UV visible spectrophotometer (Kyoto, Japan). The enzyme activity was calculated by using an extinction coefficient of $0.021\ \mu M^{-1}\,cm^{-1}$.

Lactate dehydrogenase activity was determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. Enzyme assay mixture contained 0.2 M Tris-HCl, pH 7.5, 6.6 mM NADH and 30 mM sodium pyruvate. One unit causes the oxidation of one micromole of NADH per minute at 25 °C, under the above specified conditions.

Enzyme activities were measured in triplicates ($n = 3$) from at least two individual experiments ($n \geq 2$).

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 4% stacking gel and 8.5% (CYP isoforms) and 12% (NQO1 and GPx) separating gels in a discontinuous buffer system as described by Laemmli (1970). Rat liver microsomal and cytosolic proteins were analyzed by Western blot, essentially as described by Towbin et al. (1979). Proteins were transferred from the gel to a nitrocellulose membrane by using the trans-blot electrophoretic transfer cell (Bio-Rad, Richmond, CA) containing Tris-glycine buffer, pH 8.3 and methanol. The transfer was carried out at 0–4 °C for 90 min at 90 V (400 mA). Primary antibodies in TBS containing 5% non-fat dried milk and nitrocellulose sheet were incubated at room temperature for 2 h with shaking. After three washes with TBST, the blot was further incubated with HRP conjugated secondary antibodies for 1 h and then washed three times with TBST. In order to detect antibody-bound immunoreactive proteins, nitrocellulose sheets were incubated with Pierce ECL Western Blotting Substrate solution. The gels were photographed using a computer-based gel imaging instrument and analyzed using the Image J software (Bethesda, MA).

RNA isolation, cDNA synthesis and quantitative real time PCR

Total RNA was isolated from 70 mg of liver tissue described by Sen et al. (2001). The isolated RNA was quantified by measuring the absorbance at 260 nm and its purity assessed by the 260/280 nm ratio, and the integrity was checked using 1% agarose gel. Reverse transcription of RNA to cDNA was carried out using Moloney-Murine Leukemia Virus Reverse Transcriptase and Oligo (dT) 18 Primer (Fermentas, Hanover, MD). Reverse transcription reaction mixture (20 μL) contained 2 μg total RNA, 4 μL of buffer, 1 μL of oligo dT (18), 2 μL of 10 mM dNTP and 1 μl of ribolock. The mixture was incubated at 70 °C for 5 min, and then 1 μL of Moloney-Murine Leukemia Virus Reverse Transcriptase was added. The resulting mixture was initially incubated at 42 °C for 60 min, and then heating the mixture to 70 °C for 10 min inactivated the reverse transcriptase. cDNA obtained was stored at −80 °C until further use.

The effect of *EH* extract on mRNA expression in rat liver was studied by quantitative Real Time PCR (qRT-PCR) using Corbett Rotor Gene 6000 (Corbett Life Science, Concorde, NSW, Australia). The final reaction mixture (25 μl) contained 500 ng cDNA, 0.5 μM reverse and forward primers and 1X Maxima® SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD) and RNAase free ddH_2O. In order to detect DNA contamination NTC (no template control) was used during the reaction. As an internal standard, ''housekeeping'' gene GAPDH was used. DNA was amplified in a reaction mixture containing the primers as given in Table 1. The qRT-PCR program consisted of the following temperature profile: initial denaturation at 95 °C for 10 min, 40 cycles of melting at 95 °C for 20 s, annealing temperature varied depending on the gene as shown in Table 1, for 30 s, and extension at 72 °C for 30 s. The primers were designed using Primer 3 program (Cambridge, MA) and entered into the NCBI Blast to confirm the specificity for *Rattus norvegicus*. Each set of primers was

Table 1. Analysis of mRNA expression by qRT-PCR. Table showing the name of gene, primer pairs used for amplification, annealing temperature and size of the PCR product.

Gene	NCBI reference sequence	Sequence (5′->3′)	Annealing temp. (°C)	Size of PCR product (base pair)
CYP2E1	NM_031543.1	F → GTCTGAGGCTCATGAGTTTG R → TCTGGAAACTCATGGCTG	55	628
CYP1A1	NM_012540.2	F → GGAACTCGTTTGGATCACC R → ATGCCAATGTCCAGCTCTC	58	393
GPx	NM_183403.2	F → ACCGATCCCAAGCTCATCA R → TCTCAAAGTTCCAGGACACATCTG	54	64
NQO1	NM_017000.3	F → TCCGCCCCCAACTTCTG R → TCTGCGTGGGCCAATACA	56	63
GAPDH	NM_017008.3	F → TGATGACATCAAGAAGGTGGTGAAG R → TCCTTGGAGGCCATGTGGGCCAT	60	240

Table 2. The effect of *EH* on the activities of rat liver CYP2E1 and CYP1A1 associated enzymes; aniline 4-hydroxylase, NDMA *N*-demethylase and EROD, antioxidant enzyme; GPx and phase II enzyme; NQO1.

	Aniline 4-hydroxylase (nmol/min/mg)	NDMA *N*-demethylase (nmol/min/mg)	EROD (pmol/min/mg)	GPx (nmol/min/mg)	NQO1 (nmol/min/mg)	Lactate dehydrogenase (unit/mg)
Control[a]	0.537 ± 0.011	0.192 ± 0.011	6.54 ± 1.21	0.069 ± 0.015	163.34 ± 92	619.5 ± 80.2
EHT[a]	0.109 ± 0.01**	0.159 ± 0.012*	4.48 ± 1.67**	0.107 ± 0.026**	588.3 ± 138**	687.6 ± 91.6
Change	80%↓	32%↓	69%↓	1.55 fold↑	3.6 fold↑	1.01 fold↑

[a]Male Wistar rats were divided into two groups. Control group ($n = 10$) was not treated and EHT group ($n = 30$) was intraperitoneally administered *EH* extract (37.5 mg/kg). Enzyme activities were determined as described in the ''Materials and Methods'' section. Values are mean ± SD for triplicate determinations. EHT; *EH* treated.
Significantly different from control by two-tailed Student's *t*-test, *$p < 0.05$, ** $p < 0.0001$.

carefully chosen to avoid primer dimerization and to ensure specificity of amplification. Specific annealing temperatures were verified by the use of a gradient thermal cycler.

Statistical analysis

Statistical analyses were performed by Student's *t*-test using GraphPad Prism 5.0 statistical software package for Windows (La Jolla, CA). All results were expressed as means with their standard deviation (SD). $p < 0.05$ was taken as the minimum level of significance.

Results

Effects of EH on rat liver CYP2E1, CYP1A1, GPx and NQO1 enzyme activities

The effects of i.p. administered *EH* extract on rat liver CYP2E1, CYP1A1, GPx and NQO1 enzyme activities are summarized in Table 2. The cytotoxic effect of *EH* was also studied by measuring LDH. Spectrophotometric analysis of LDH showed that *EH* does not cause any cytotoxicity in the rat liver (1.01-fold). Studies of *EH* treatment in rat liver CYP2E1 associated activities showed that *EH* has a greater inhibitory effect on aniline 4-hydroxylase enzyme activity (80% decrease) $p < 0.0001$, than on NDMA *N*-demethylase activity (32% decrease) $p < 0.05$. EROD associated with CYP1A1, an executive isoform for the formation of mutagenic metabolites, was also measured by spectrofluorometer using 7-ethoxyresorufin as a substrate. Nine days of 37.5 mg/kg *EH* injection of rats significantly inhibited liver EROD activity from 6.54 ± 1.21 pmol/min/mg to 4.48 ± 1.67 pmol/min/mg ($p < 0.0001$). In addition to CYP enzyme activities, the activities of the antioxidant enzymes GPx and NQO1 were also measured after i.p dose of *EH* (Table 2). *EH* treatment of rats increased liver GPx and NQO1 activities by 1.55- ($p < 0.0001$) and 3.6-fold ($p < 0.0001$), respectively, compared to control group.

Effects of EH on rat liver CYP2E1, CYP1A1, GPx and NQO1 protein expressions

Effects of *EH* on rat liver CYP2E1, CYP1A1, GPx and NQO1 protein levels were analyzed by Western blotting (Figures 1–4A). Our results showed that CYP2E1 and CYP1A1 protein levels significantly decreased, 63% ($p < 0.0001$) and 54% ($p < 0.0001$), respectively, in *EH*-treated rats compared to control animals (Figures 1B and 2B). Beside CYP protein expression, protein levels of the antioxidant enzymes GPx and NQO1 were also determined. Treatment of rats with *EH* caused an induction on the protein expression of GPx and NQO1 as 1.93- and 2.97-fold, respectively (Figures 3B and 4B). Moreover, cross analyses of enzyme activity versus protein expression showed that there is a correlation between protein expression levels and corresponding enzyme activities in *EH* treated and control groups as shown in Figures 1–4C.

Effects of EH on rat liver CYP2E1, CYP1A1, GPx and NQO1 mRNA expression

The effects of *EH* on CYP2E1, CYP1A1, GPx and NQO1 mRNA expression in rat liver were analyzed using qRT-PCR and results are shown in Figure 5. After 9 d i.p. treatment of

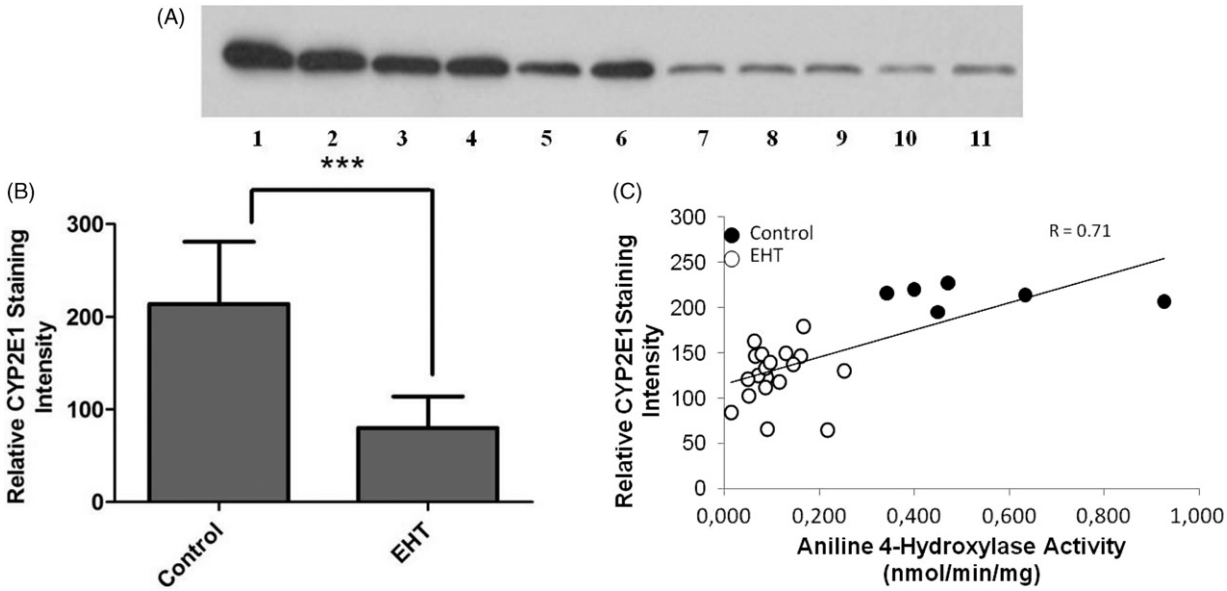

Figure 1. Effects of *EH* treatment on CYP2E1 protein level of rat liver. (A) Representative immunoblot of liver microsomal CYP2E1 protein in experimental control (lanes 1–6) and EHT (lanes 7–11) groups. (B) Comparison of CYP2E1 protein expression of the control ($n = 10$) and EHT treated ($n = 30$) groups. Experiments were repeated at least three times ($n \geq 3$). ***Significantly different from respective control value ($p < 0.0001$). (C) Correlation between liver microsomal aniline 4-hydroxylase and relative CYP2E1 protein expression in rat. The correlation coefficient ($r = 0.71$) was calculated by the least squares linear regression method. The solid line represents the line of best fit.

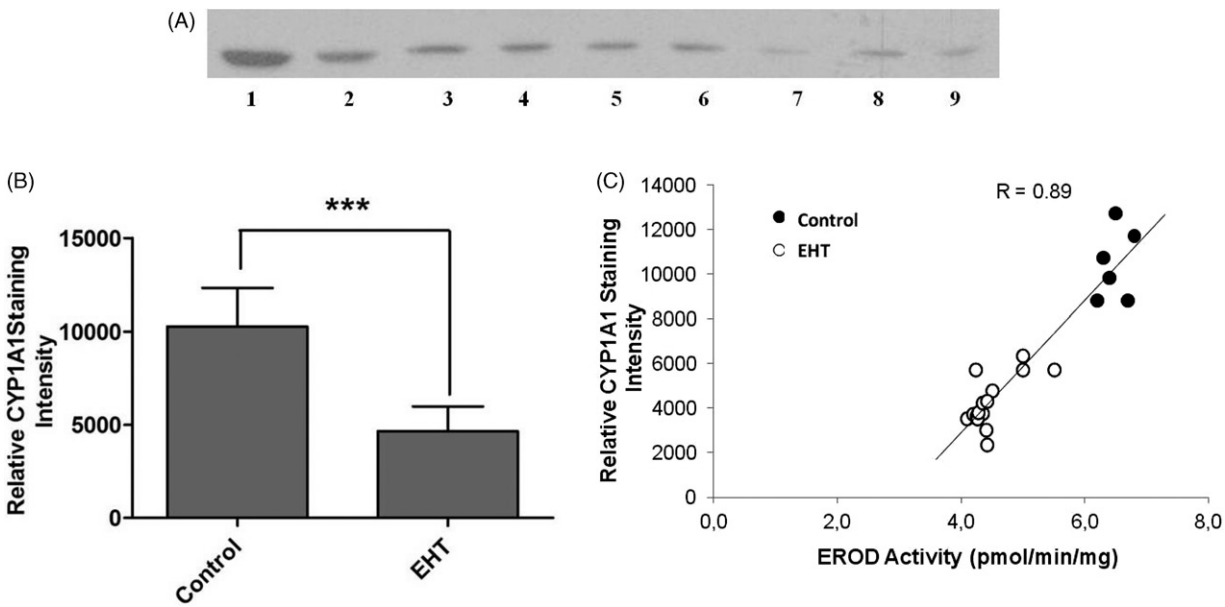

Figure 2. Effects of *EH* treatment on CYP1A1 protein level of rat liver. (A) Representative immunoblot of liver microsomal CYP1A1 protein in experimental control (lanes 1–2) and EHT (lanes 3–9) groups. (B) Comparison of CYP1A1 protein expression of the control ($n = 10$) and EHT treated ($n = 30$) groups. Experiments were repeated at least three times ($n \geq 3$). ***Significantly different from respective control value ($p < 0.0001$). (C) Correlation between liver microsomal EROD activity and relative CYP1A1 protein expression in rat. The correlation coefficient ($r = 0.89$) was calculated by the least squares linear regression method. The solid line represents the line of best fit.

the rats with *EH*, mRNA expression of CYP2E1 and CYP1A1 was decreased 1.48- ($p < 0.0001$) and 6.7-fold ($p < 0.0001$), respectively. In contrast, *EH* treatment increased GPx and NQO1 mRNA expression as 3.5- and 4.97-fold, respectively, compared to the control group ($p < 0.0001$).

Discussion

Increasing popularity towards traditional medicine such as herbal remedies or dietary supplements leads investigators to examine the actions of phytochemicals on xenobiotic metabolism and their antioxidant, antitumor, anticarcinogenic and antimutagenic effects. *EH* has a worldwide distribution and is being used as a folk medicine in Asia, America and Europe. It has been reported that *EH* extract exhibited the highest antioxidant activity and highest antioxidant concentration among 32 plants extracts (Wojdyło et al., 2007). The amount of total phenolic compounds found in the *EH* extract used in the present study was 13.96 mg of GAE/100 g of dry weight.

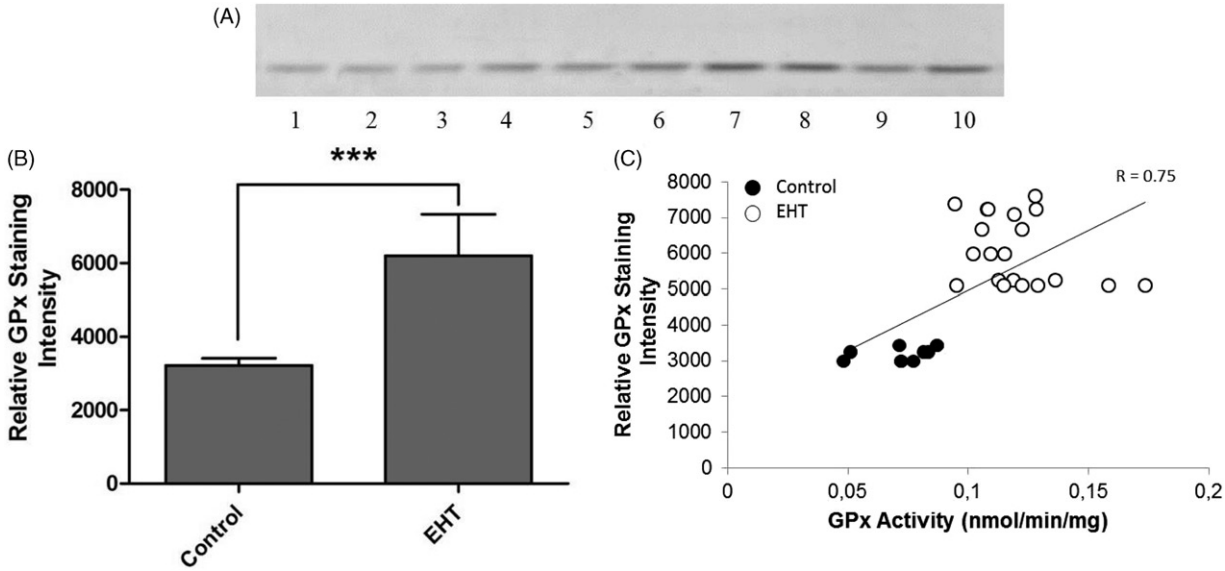

Figure 3. Effects of *EH* treatment on GPx protein level of rat liver. (A) Representative immunoblot of liver microsomal GPx protein in experimental control (lanes 1–3) and EHT (lanes 4–10) groups. (B) Comparison of GPx protein expression of the control ($n = 10$) and EHT treated ($n = 30$) groups. Experiments were repeated at least three times ($n \geq 3$). ***Significantly different from respective control value ($p < 0.0001$). (C) Correlation between liver cytosolic GPx activity and relative GPx protein expression in rat. The correlation coefficient ($r = 0.75$) was calculated by the least squares linear regression method. The solid line represents the line of best fit.

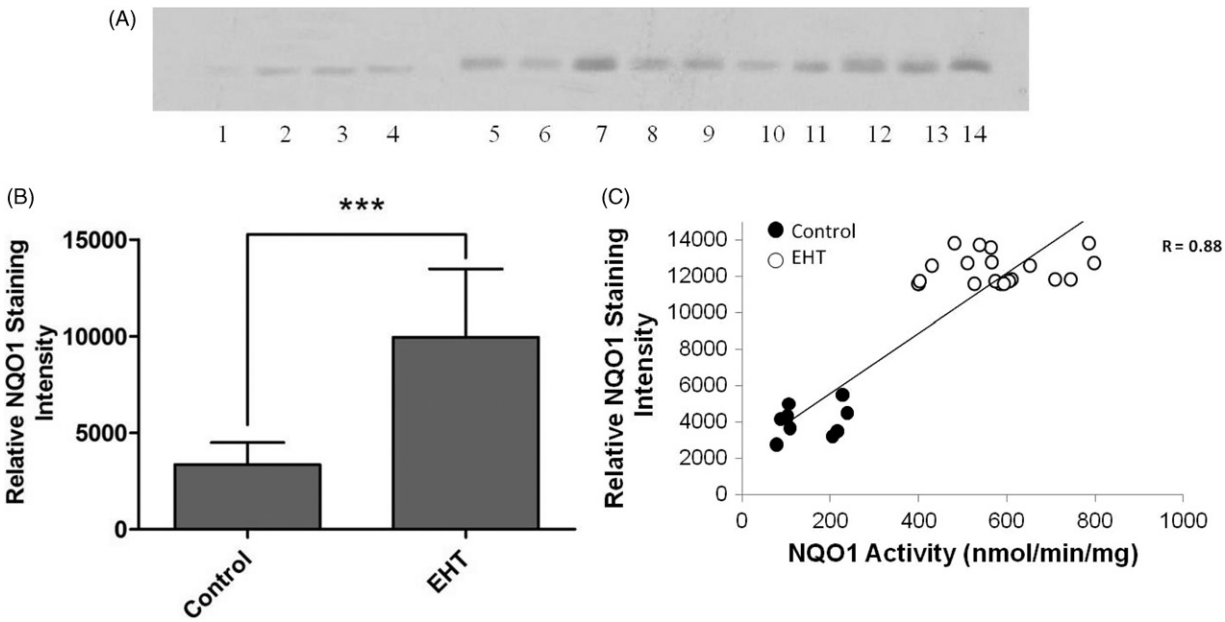

Figure 4. Effects of *EH* treatment on NQO1 protein level of rat liver. (A) Representative immunoblots of liver microsomal NQO1 protein in experimental control (lanes 1–4) and EHT (lanes 5–14) groups. (B) Comparison of NQO1 protein expression of the control ($n = 10$) and EHT treated ($n = 30$) groups. Experiments were repeated at least three times ($n \geq 3$). ***Significantly different from respective control value ($p < 0.0001$). (C) Correlation between liver cytosolic NQO1 activity and relative NQO1 protein expression in rat. The correlation coefficient ($r = 0.88$) was calculated by the least squares linear regression method. The solid line represents the line of best fit.

Hence, we have undertaken this study to elucidate its effects on several phase I and phase II enzymes.

The data presented in this study indicate that the intraperitoneal administration of water extract of *EH* to rats caused remarkable changes in xenobiotic metabolizing phase I, phase II and antioxidant enzymes in the rat liver. Numerous studies have shown that rodent and human CYP1A1, CYP2E1, NQO1 and GPx enzymes catalyze similar reactions. Protein sequencing analyses of human and rat CYP1A1 and CYP2E1 showed that both proteins sharing more than 80% of amino acid similarity. EROD and aniline 4-hydroxylation were also accepted as a probe reaction in rats (Kravchenko et al., 2012; Martin et al., 2003). Therefore, in the present study, the rat is used as a model organism for screening these enzymes.

Our results have shown that *EH* decreased the EROD activity associated with CYP1A1 ($p < 0.0001$) and NDMA *N*-demethylase ($p < 0.05$) and aniline 4-hydroxylase ($p < 0.0001$) activities of CYP2E1 while increasing NQO1

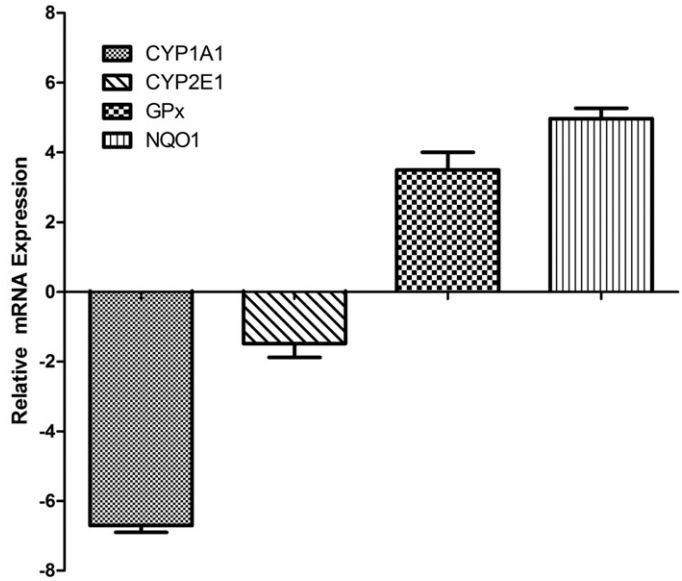

Figure 5. Effects of *EH* treatment on rat liver CYP2E1, CYP1A1, GPx and NQO1 mRNA expression. Alterations in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments ($n \geq 3$) and expressed as relative mean ± SD. Effects of *EH* on mRNA levels of the tested genes were normalized to housekeeping GAPDH mRNA. Fold of inhibition was calculated by the following formula: $2^{-\Delta\Delta Ct}$, where ΔΔCt: ΔCt (treated) – ΔCt(control); ΔCt (treated): ΔCt (CYPs) – ΔCt(GAPDH); ΔCt(control): ΔCt (CYPs) – ΔCt(GAPDH).

($p < 0.0001$) and GPx ($p < 0.0001$) enzyme activities. It is well known that CYP1A1 is the main member of cytochrome P4501A family, which could be induced by PAHs, and involved in the metabolic activation of PAHs and heterocyclic amines (McManus et al., 1990). Metabolism of PAHs by CYP1A1 produces reactive products that are irreversibly bound to protein and DNA leading to toxic and carcinogenic events (Miller & Miller, 1966). The risk assessment for many diseases, especially cancer, might decrease with the inhibition of CYP1A1 because of its role in the formation of such reactive products.

Similarly, CYP2E1 has a wide range of substrates which are largely exogenous such as industrial solvents, protoxins and procarcinogens (Gonzalez, 2005). Furthermore, such drugs used by humans as acetaminophen, chlorzoxazone and methoxyflurane have been also shown to be metabolized by CYP2E1. Hence, inhibition of CYP2E1 may prevent the conversion of such procarcinogens to their carcinogenic forms such as nitrosamine, acrylamide, phenol and benzene. Therefore, the inhibitory mechanisms on CYP1A1 and CYP2E1 seen in the present study with *EH* extract might be regarded as a protective effect because of the probable suppression of the tumor formation induced by PAHs, drugs and other carcinogens.

As a result of various enzyme catalyzed reactions including CYPs, the oxygen-centered free radicals also known as ROS are produced. In the case of any imbalance between production of ROS and cellular antioxidant capacity or when there is a reduction in this capacity, oxidative stress may occur. One of the crucial enzymes in the defense system against ROS as well as neoplasia is NQO1 (Karakurt & Adali, 2011). hNQO and rNQO1 have a high degree of sequence similarity as 86% and structural similarity (Faig et al., 2000). NQO1 has also a function in the antioxidant and cancer preventing processes (Landi et al., 1997). Moreover, NQO1 induction might reduce the formation of the semiquinone radicals, which are the products of quinones metabolized by CYP enzymes not NQO1. Furthermore, higher NQO1 activity was shown to reduce oxidative DNA damage and prevent estrogen-induced breast cancer via decreasing the levels of 17β-estradiol (E2)-quinones capable of interacting with DNA by converting E2-quinones to E2-catechols (Singh et al., 2012). Therefore, the induction of NQO1 by *EH* might help to eliminate toxic metabolites and ROS from cells and is important in early defense against carcinogenesis.

It has been long known that the first line of defense against ROS is the GPx. GPx is the enzyme reducing H_2O_2 and organic ROOH to water and alcohols (ROH). Increased levels of GPx activity may enhance the resistance against ROS. GPx1-overexpressing mice were found to be more resistant to paraquat-induced lethality than GPx1 knockout mice (Cheng et al., 1998). Apart from this, increased levels of GPx is also protective against cardiovascular diseases, since ROS such as superoxide radicals or hydrogen peroxides cause changes in the vascular tone and structure (Battin & Brumaghim, 2009). As a result, combining induction of GPx together with NQO1 by the *EH* treatment could be a reasonable significant enhancement against the toxicity of various agents.

In addition to allosteric control mechanisms of enzymes, expression of CYP enzymes as well as phase II and antioxidant enzymes are known to be controlled at the transcriptional and translational levels. Recent studies showed that mRNA levels of CYP genes were also affected by expression of micro RNAs (Choi et al., 2012; Mohri et al., 2010). Therefore, to elucidate the means of control mechanism of those liver enzymes at the transcriptional and translational levels, Western blot and qRT-PCR analysis were performed, in addition to activity studies. Immunoblot analysis showed that protein levels of those enzymes have been altered via *EH* administration to rats. CYP1A1 and CYP2E1 protein levels significantly decreased ($p < 0.0001$) while GPx and NQO1 protein levels increased 1.93- and 2.97-fold, respectively. Furthermore, *EH* plant extract has remarkable effects on mRNA expression levels of CYP1A1, CYP2E1, GPx and NQO1 enzymes. CYP1A1 and CYP2E1 mRNA levels were decreased 6.7- and 1.48-fold, respectively, while those of GPx and NQO1 were increased 3.5- and 4.97-fold, respectively, upon injection of plant extract to rats.

Although, understanding the mechanism of the regulation of those genes by *EH* is beyond the scope of the current study, our results provide further evidence demonstrating that the effect of *EH* is at the gene expression level.

Conclusions

The presented evidence points out that *EH* alters the activity and expression of enzymes involved in xenobiotics activation-detoxification pathways. Therefore, modulatory effect of *EH* on these enzymes suggests an inherent chemopreventive action against a group of chemicals including carcinogens and drugs. However, necessary precautions such as medical advice should be taken regarding the usage of this plant in replacement treatments since it reveals possible interactions with drugs and dietary foods.

Declaration of interest

The authors report no declarations of interest.

This work is supported by Scientific and Technological Research Council of Turkey (TUBITAK), Project No: 109R012, Turkey.

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