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EFFECTS OF FOLK MEDICINAL PLANT *EPILOBIUM HIRSUTUM L.* AND ITS INGREDIENT ELLAGIC ACID ON RAT LIVER BILE ACID SYNTHESIZING CYPS IN RATS

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Abstract

The phytochemical compounds are used mostly in medicine as alternative medicine. Excessive cholesterol is removed mainly through conversion to bile acids in mammals, cytochromes P450 initiate all quantitatively significant pathways of cholesterol metabolism and bile acid biosynthesis.

This study was aimed to investigate the possible potency of medicinal plant *Epilobium hirsutum L.* (EHT) extract and its ingredient ellagic acid (EAT) on rat liver cholesterol and bile acid metabolizing enzymes. In addition to molecular studies, bioactive compound of EHT extracts was identified using Liquid Chromatograph-Mass Spectrometry (LC-MS) technique. The water extracts of EHT and EAT were injected intraperitoneally as 37.5 mg/kg and 20 mg/kg for 9 days, respectively. Then, in vivo effects of the EHT and EAT extract on rat liver cholesterol and bile acid metabolizing CYPs were analyzed by determining protein and mRNA expression levels using western blotting and qRT-PCR techniques, respectively. In addition, serum cholesterol level of animals was determined. EHT caused 0.6, 0.8 and 0.6 fold decrease in protein expression of CYP7A1, CYP8B1 and CYP7B1, respectively, whereas 1.3 fold increase in protein expression of CYP27A1 was observed. EHT caused 2.1, 1.4 and 4.1 fold decrease in mRNA expression of CYP7A1, CYP27A1, CYP7B1, respectively. However, mRNA expression of CYP8B1 was decreased 2.3 fold by EHT treatment. EAT led to 0.7, 0.8 and 0.8 fold decrease in protein expression of CYP27A1, CYP8B1, CYP7B1, respectively, but one fold increase of protein expression of CYP7A1. EAT caused 2.6, 1.7, 1.8 and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1, respectively.

EHT-treated and EAT-treated animals also showed a significant reduction in total cholesterol level compared to control animals. Based on doses used that applied in this study, EHT and EAT is quite safe and can be used for drug development without any toxicity.

Keywords: *Epilobium hirsutum L., ellagic acid, bile acid metabolizing enzymes, cholesterol*

Introduction

Phytochemicals known as non-nutritive active compounds are found naturally in plants, and more than a thousand of them are known. They are not fundamental to human life, but recent research showed that they have preventive and curative properties. They act as mimicking hormones and antioxidants and stimulating enzymes; they can also destroy bacteria, enhance the immune system and detoxify carcinogens by activating CYP and phase II enzymes. While some phytochemicals work with other nutrients such as vitamins, some others work alone.

CYPs play a vital role in biological and clinical processes because they possess numerous substrates both endogenous compounds including steroids and fatty acids, and exogenous compounds including drugs, environmental chemicals and pollutants [1-12] In mammals, excessive cholesterol is removed mainly through conversion to bile acids, and cytochromes P450s (P450s or CYPs) initiate all quantitatively important pathways of cholesterol metabolism and bile acid biosynthesis [Figure 1, 13]. The bile acid pathway is essential in maintaining normal cholesterol levels. There are two pathways namely the classical pathway and the acidic pathway involved in bile acid synthesis catalyzed by CYP7A1 and CYP27A1, respectively. The first and rate-limiting enzyme of the neutral (classic) pathway is cholesterol 7α-hydroxylase (CYP7A1). Microsomal cholesterol 7α-hydroxylase is a hepatic enzyme; therefore, this pathway takes place solely in the liver. The alternative pathway is initiated by mitochondrial CYP27A1 (sterol 27-hydroxylase). Unlike CYP7A1; CYP27A1 is a multi-functional mitochondrial enzyme, expressed in many tissues such as lung, kidney, liver, prostate, brain, small intestine, arterial endothelium, skin, fibroblast and macrophages. In contradistinction to the neutral pathway, the acidic pathway may start in extra-hepatic tissues. Sterol 12 α-hydroxylase, microsomal cytochrome P450 (CYP8B1), is essential for the production of cholic acid and balance of ratio between cholic acid and chenodeoxycholic acid [14-16]. It is a liver specific enzyme and has broad substrate specificity. CYP7B1, oxysterol 7α-hydroxylase, is present in many tissues including brain, lung, liver, prostate and kidney. Hydroxylation reaction catalyzed by CYP7B1 can occur either in carbon 7- or carbon 6- area. CYP7B1 participates in 7-alpha-hydroxylation of 25- and 27-hydroxycholesterol in an alternative pathway of bile acid synthesis [9, 17-19].

Epilobium hirsutum L.(EHT) is also commonly called as great-hairy willow herb, codlins and cream, European fireweed, apple-pie and cherry-pie. *Epilobium hirsutum L.*, great willow-herb, grows in Eurasia, North Africa, Europe, Southern Australia and the United States. *Epilobium hirsutum L.* was used traditionally to treat gastrointestinal and prostate diseases, menstrual disorder, stomach ulceration, gastritis and sleeping disorders [20-23]. *Epilobium hirsutum L.* also has anti-bacterial, anti-microbial, anti-exudative, anti-nociceptive, anti-septic and anti-phlogistic effects [23-27]. There are many medical studies to investigate of these biological impacts of *Epilobium hirsutum.* Wojdylo *et al.* [28] studied 32 Polish herbs which have rich phenolic content including *Epilobium hirsutum*. They suggested some plants have rich phenolic content like *Epilobium hirsutum* could be used a natural antioxidant source. Furthermore, to evaluate the anti-tumor action of *Epilobium hirsutum L.* (Onagraceae) and *Maclura aurantiaca* (Moraceae), Wojdylo *et al.* [28] treated mice with these two plants extract. *Epilobium* treated mice showed prolonged the lifetime and the highest antioxidant activity. Moreover, because phenolic compound can be used as free radical scavengers, Ebrahimzadeh *et al.* [29] detected a linear relationship between phenolic content and chelation activity. In that research, *Epilobium hirsutum* and *Mentha arvensis* showed a good chelator activity [29].

Karakut *et all* suggested *EHT* may alter expressions of protein and mRNA and activity of drug metabolizing enzymes such as CYP1A1 and CYP2E1 [27]. Then, Celik *et al* researched effect of *EHT* on drug metabolizing CYP2B, CYP2C, CYP2D and CYP3A[12].Their result supports using *EHT* as herbal remedy inhibits drug clearance due to decreasing expression and activity of these CYPs.

Ellagic acid (EAT) is therapeutic agent for many diseases and it is found in pomegranate, strawberries, cloudberry, blueberry, blackberry, grapes, raspberries, cranberries, pecans, walnuts and black currants [30]. Ellagic acid in foodstuff is found either generally conjugated with a glycoside moiety

or part of ellagitannins [31]. For the first time, Tell [32] proposed that ellagic acid has a potent activity of anti-carcinogenic and anti-mutagenic by making DNA-adduct, which ellagic acid masks binding side of DNA in order to protect engagement of carcinogen or mutagen agents. Furthermore, it has been showed that pomegranate exhibits anti-oxidant activity owing to its phytochemical content [33]. Yu *et al* [33] indicated that pomegranate extract has good effects in atherosclerotic animals due to presence of ellagic acid [33-34]. Pomegrate juice includes not only ellagic acid both also pro-estrogenic and estrogenic compounds that they have effects on spread and growth of prostate tumors. So, it is important to create a treatment establish on one compound such as ellagic acid for hormone dependent disorder [35]. Also, phenolic compounds including ellagic acid can help cell to show anti-oxidant defense by inhibiting the expression of CYP and NADPH oxidase [36]. Larrosa *et al.* [37] researched apoptotic effect of ellagic acid in colon cancer. It has been found that ellagic acid induces apoptosis in colon cancer cells. According to results taken from studies of human bladder cancer, ellagic acid has effects on subcellular signaling by inducing p53 and p21, and decreasing COX-2 cycling D1 and nuclear factor kappa (NF_KB) [38]. Moreover, in another investigation, to study anti-inflammatory and anti-nociceptive activity of ellagic acid, it was been suggested ellagic acid has a beneficial effect on inflammatory pathway by inhibiting COX (cyclooxygenase), Interleukin (IL) 1-Beta, mutagen activated protein kinases and tumor necrosis factor alpha induced activation of activator protein 1, by inducing iNOS (nitric oxide synthetase), COX2 [39-40].

Methods

Preparation of Extraction of Epilobium hirsutum L.

Epilobium hirsutum L. was collected from Gölyaka, Düzce, Turkey, 563 m. altitude in June. Air-dried and powdered 50 g plant samples for each taxon were subjected to maceration in sterile ddH2O by using Heidolph mechanic shaker at 300 rpm/minute at RT (room temperature) for 8 hours. Plant extracts obtained were filtered through Whatman filter paper, dried in a freeze dryer (Christ Gamma 2-16 LSC), and weighed. Then, the extracts of plants were gassed with nitrogen to prevent oxygen interaction, and held in at -20°C until use for the assays.

Phenolic Profile of Plant

Identification and quantification of the bioactive compounds of *EHT* was carried out by Liquid Chromatography Mass Spectrometry (LC-MS) (AGILENT 6460 Triple Quadrupole System (ESI+Agilent Jet Stream) coupled with AGILENT 1200 Series HPLC"). For analysis of bioactive compounds of *EHT* extract; ellagic acid, gallic acid, caffeic acid, *p*-coumaric acid and quercetin dehydrate were selected as standards based on the literature.

Determination of Total Phenolic Content (TPC)

Total phenolic content was determined by the Folin-Ciocalteu method [41]. *EHT* extract was diluted as 0,2 mg/ml and 0,6 mg/ml. 20 µl of sample was mixed with 100 µL of 1:4 diluted % Folin-Ciocalteu's phenol reagent and mixed by pipetting. 80 µL of 10% sodium carbonate solution was added in the 96 well plate and shaken vigorously. Then, the mixture incubated at room temperature for 30 minutes in the dark. The absorbance was measured at 750 nm using a Multiskan™ GO microplate reader (Thermo Scientific, USA). The calibration curve was drawn using range of concentrations between 25-200 µg/ml gallic acids. The results were expressed in mg gallic acid equivalents (GAE)/g fresh matter. The experiments were carried out triplicates and repeated three times.

Determination of Total Flavonoid Content (TFC)

Determination of total flavonoid content was performed by colorimetric method of [42] in which sodium nitrite reacts with aluminum chloride and flavonoids and generates the red color. *EHT* extracts were diluted as 1 mg/ml and 1,5 mg/ml. 20 µL of each extract was added into 96 well plate containing 80 µL of distilled water and then 6 µL of 5% sodium nitrite ($NaNO_2$) was added to the wells. Five minutes later, 6 µL of 10 % aluminium chloride ($AlCl_3$) solution was added. After waiting 6 minutes, 40 µL of 1 M sodium hydroxide (NaOH) was added and the total volume was completed up to 200 µL with distilled water and incubated at room temperature for 30 min. The absorbance of the mixture was measured at 510 nm using a Multiskan™ GO microplate reader (Thermo Scientific, USA). Total flavonoid content was

calculated based on the calibration curve prepared by using catechin solutions at concentrations from 50 to 300 µg/ml. The experiments were carried out triplicates and repeated three times.

Animal Studies

12 weeks old healthy male Wistar Albino rats (*Rattus norvegicus*) which weighing 200-250 g were purchased. The water extract of *EHT* and its ingredient (ellagic acid=EAT) were intraperitoneally (i.p.) injected to the rats. For the experiment, animals were divided into two groups. Rats in control group never made any application, but they were kept under the same conditions with other groups during the experiment. The water extract of *EHT* and EAT were injected intraperitoneally as 37.5 mg/kg and 20 mg/kg for 9 days. At the end of the treatment protocol given above and after 16 h of fasting, the rats were euthanized with decapitation. Blood was collected by heart puncture, and the livers were isolated and stored at -80 ºC till preparation of microsomes and mitochondrial fractions.

Protein Expression Analysis by Western Blotting Technique

Microsomal and S1.5 fractions of the rat livers were prepared based on Schenkman and Cinti method (1978) [43] as optimized by Şen and Kırıkbakan (2004) [44]. The protein concentrations of microsomes and S1.5 were determined according to the method of Lowry *et al.* (1951) [45] using BSA (Crystalline bovine serum albumin) as the standard. In the SDS-PAGE method, proteins were separated in 4% stacking gel and 8.5% separating gel through a discontinuous buffer system. Protein samples were diluted with 4X sample dilution buffer in a ratio 1:3 (1 part buffer and three parts sample) to obtain 2 mg/ml of protein. The proteins were transferred to nitrocellulose membrane. The membrane was incubated with blocking solution (5% nonfat dry milk in TBST) for one night. The blot was incubated with primary antibody for 2 hours. Before incubation with secondary antibody, the blot was washed with TBST 3 times to remove excessive and nonbinding antibody. After 1-hour incubation with secondary antibody, the blot was washed again with TBST three times for 5 minutes each. The end, the blot was incubated with substrate solution (alkaline phosphate substrate solution). Finally, the image was photographed by a gel imaging instrument which based on a computer, (Infinity 3000, Vilber Lourmat, Marne-la-Vallee Cedex1, France) through Infinity-Capt Version 12.9 software. The density of protein bands was quantified using Image J visualization software (Scion Image Version Beta 4.0.2).

mRNA Expression Analysis by Quantitative Real Time PCR (qRT-PCR)

The impact of *EHT* and its ingredient EAT on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 gene expressions was analyzed by quantitative Real Time PCR (qRT-PCR) using Corbett Rotor Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137). Quantitative Real-Time PCR was carried out by using Light Cycler FastStart DNA Master^{Plus} SYBR Green I (Roche Applied Science, Basel, Switzerland). The assay was performed manufacturer's protocols. 5 µl PCR-grade water and a sample in which reverse transcriptase was omitted during reverse transcription, were included in every PCR-run as negative controls to confirm that there is no genomic DNA contamination in the cDNA samples. After addition of 3 µl of cDNA, 2 µl gene-specific primer for CYPs and 12.5 µl Fast Start Universal SYBR Green Master (ROX), reaction volume was completed to 25 µl with RNase-free distilled water. All samples were run in triplicates, no template control (NTC) was used to determine contamination. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), a housekeeping gene, was used as an internal standard. The primers were designed using NCBI, and the blasts were confirmed the based on *Rottus norvegius.* Primer sequences, annealing temperatures and product sizes for gene expression analysis by real-time PCR were given in Table 1.

Determination of Total Cholesterol

Rat serum total cholesterol levels were determined by enzymatic and spectrophotometric techniques. Cholesterol is found in two forms in the blood as free form or esterified form. In this method, enzymes (cholesterol esterase and cholesterol oxidase) are very specific to determine the forms of cholesterol. Cholesterol esterase converted the cholesterol esters into the cholesterol. Next, the cholesterol was oxidized by cholesterol oxidase to produce ketone cholest-4-en-3-one and hydrogen peroxide. After the reaction between horseradish

peroxidase and hydrogen peroxide, a colored compound obtained gave an absorbance at 540-570 nm.

Statistical analysis

Statistical analysis was performed by student t-test using GraphPad Prism 6.0 statistical software package for Windows. Results were expressed as means with their Standard derivation (SD) and $p<0.05$ were chosen as a level of significance.

Results

LC-MS Analysis of Epilobium hirsutum L.

Recently, Liquid chromatography-Mass Spectrometry (LC-MS) has attained popularity owing to its specificity and sensitivity. This has made it an essential tool for analyzing the pharmacological profiles of herbs. *EHT* extract was analyzed and quantified for the presence of 5 phenolic compounds (Table 2), including ellagic acid, gallic acid, caffeic acid, *p*-coumaric acid, and quercetin dehydrate. LC-MS chromatogram of the *EHT* extract and 4 phenolic compounds are presented in Fig. 2, while the chromatogram of ellagic acid is shown in Fig. 3 since it was analyzed using different column and solvent system. According to quantitative LC-MS analysis, gallic acid (11.906 ppm) was the major compound among the standard phenolics used in this study in *EHT p*-coumaric acid amount was 1.0642 ppm, while quercetin dehydrate was less than 0.625 ppm, and the ellagic acid value was calculated less than 0.31 ppm.

Total Phenolic and Flovanoid Content of Epilobium hirsutum L.

The total phenolic and flavonoid content of *EHT* was determined as defined in Materials and Methods. The data represented in Table 2 reveals that the total phenolic content of *EHT* was 225 ±2.123 mg GAE/g dried extract and the total flavonoid content of *EHT* was 19 mg CE/ g dried extract.

Effects of EHT and EAT on Rat Liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 Protein Expression

The impact of *EHT* and its major ingredients EAT on rat liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) protein expression were determined via western blot, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37kDa) as an internal control. Fig.4 shows the results of Western blot analysis of the CYP proteins and GAPDH, with co-immunostaining to compare the relative expressions. The intensity of immunoreactive protein bands of *EHT*, EAT and control were evaluated with Image J. Relative expressions of *EHT* with reference to housekeeping GAPDH protein are illustrated in Fig. 4. The protein expressions of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 in *EHT*-treated rats were significantly different from control rats. *EHT* caused a 0.6, 0.8, and 0.6 fold decrease in protein expression of CYP7A1, CYP8B1, and CYP7B1 respectively, and a 1.3 fold increase in CYP27A1 (***p<0,0001) (Fig. 4).

The protein expression levels of CYP27A1 and CYP8B1 in ellagic acid-treated rats were significantly different from control rats. The densitometric analysis of the liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 displayed in Fig.5 shows that EAT caused a 0.7, 0.8 and 0.8 fold decrease in protein expression of CYP27A1, CYP8B1, and CYP7B1 respectively, but a 1 fold increase of CYP7A1.

Effects of EHT and EAT on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA Expression

Changes in mRNA expression of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 due to the treatment of *EHT* and EAT were measured by quantitative real-time PCR (qRT-PCR). qPCR results of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA levels are presented in Fig. 6. GAPDH was used to normalize the results. Livak method [46] was applied to determine the mRNA expression changes of CYPs. *EHT* treatment led to significant changes in mRNA expression of CYP7A1, CYP8B1, and CYP7B1. *EHT* promoted a 2.1, 1.4 and 4.1 fold decrease in mRNA expression of CYP7A1, CYP27A1, and CYP7B1 respectively, with respect to controls and normalized to GAPDH expression as an internal reference and a 2.3 fold increase in mRNA expression of CYP8B1.

qRT-PCR data for the effects of EAT on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 expression is presented in Fig. 7. The relative mRNA expression of CYP7A1, CY8B1, and CYP7B1 in ellagic acid-treated rats was increased significantly compared to control animals. EAT treatment induced a 1.8, 1.3, 2.3, and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 respectively, with

respect to controls and normalized with GAPDH expression as an internal reference.

Blood Serum Total Cholesterol Levels in Control and Treated Rats

Rat serum total cholesterol levels were determined by enzymatic and spectrophotometric techniques as described in the Materials and Methods section. Serum samples obtained from *EHT* and its major ingredients EAT treated rats were determined, and results were given in Table 2. For calculation of total cholesterol concentration of samples, a standard curve obtained by different concentrations of cholesterol was used. *EHT* (46.25±2.306) and EAT (46±3.572)-treated animals showed a significant decrease in total cholesterol level compared to control animals (70.5±1.905) (Fig. 8).

Discussion

Until now, two-thirds of the plants identified have medicinal importance, and they have been used as a medicine for human health and a supplement to the maintenance of a healthy lifestyle for centuries. Many species of plants have been documented to have medicinal properties and beneficial impacts on health, especially as antioxidant activity, anti-carcinogenic potential, anti-microbial, anti-inflammatory, anti-mutagenic effects and digestive stimulation action [47].

CYPs (Cytochromes P450 enzymes) play biological and clinical vital role because they possess numerous substrates both endogenous compounds including steroids and fatty acids, and exogenous compounds including drugs, environmental chemicals and pollutants [48-49, 3-10]. This is the first study to demonstrate the *in vivo* effects of medicinal plant *EHT* and its major ingredients EAT on rat liver bile acid metabolizing CYPs (CYP7A1, CYP27A1, CYP8B1 and CYP7B1). The water extracts of *EHT* was injected i.p. as 37.5 mg/kg for nine days, respectively. *In vivo* effects of *EHT* and its major ingredients ellagic acid on rat liver bile acid metabolizing CYPs were analyzed by determining protein and mRNA expression levels using western blotting and qRT-PCR techniques, respectively.

In this study, total cholesterol levels were determined by enzymatic and spectrophotometric technique. When we compared the total cholesterol level of *EHT* (46.25±2.306) treated animals to control (70.5±1.905) in this study, they showed a significant decrease in total cholesterol levels. Protein and mRNA expressions of bile acid metabolizing enzymes were analyzed in *EHT*-treated samples. According to results, *EHT* caused 0.6, 0.8 and 0.6 fold decrease in protein expression of CYP7A1, CYP8B1, CYP7B1, respectively, on the contrary, 1.3 fold increase in protein expression of CYP27A1. *EHT* caused 2.1, 1.4 and 4.1 fold decrease in mRNA expression of CYP7A1, CYP27A1, CYP7B1, respectively, with respect to controls and normalized to GAPDH expression as an internal reference, however, 2.3 fold increase in mRNA expression of CYP8B1. Fig. 9 indicates that the decrease of CYP27A1 mRNA expression does not interpret with a proper decrease of CYP27A1 protein levels. Moreover, the increase of CYP8B1 mRNA expression does not correlate with a fitting increase of CYP8B1 protein expression.

According to results, EAT caused 0.7, 0.8 and 0.8 decrease in protein expression of CYP27A1, CYP8B1, CYP7B1, respectively, but one fold increase of protein expression of CYP7A1. EAT caused 2.6, 1.7, 1.8 and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 respectively, with respect to controls and normalized with GAPDH expression as an internal reference. There is only a correlation between relative mRNA and protein expressions of ellagic acid-treated CYP7A1 enzyme as shown Fig. 9.

In appears that when mRNA and protein expression of CYPs were compared, there was no significant correlation. The expressions of CYPs gene is regulated transcriptionally, post-transcriptionally, translationally and post-translationally. These mechanisms are important for steady-state protein concentration. There are some reasons for these poor correlation or non-correlation. Translation and protein degradation are as important as mRNA transcription and stability of the final product of protein. Transcription is first and major target for gene regulation. But transcriptional regulation is not enough for describe protein abundance. One of the explanation is micro RNA (miRNAs), 21 nucleotide long non-coding RNA, mediates gene expressions. Generally, miRNAs repress the protein synthesis by inhibiting translation and/or via facilitating

deadenylation and following degradation of mRNA targets [50].

There is a correlation relative to protein and mRNA expression of CYP7A1 and CYP7B1 in *EHT*-treated samples. The first and rate-limiting enzyme in the neutral pathway is cholesterol 7α-hydroxylase (CYP7A1). Bile acids can regulate cholesterol homeostasis via bile acid synthesis pathway. Bile acid synthesis is regulated by many factors, including bile acids, nutrients, diets, and hormones, in particular through regulating transcription of CYP7A1 gene. Besides, many liver-specific transcription factors, commonly nuclear receptors have an important role in cholesterol and bile acid metabolism [51-54].

A mentioned above, regulating transcription of CYP7A1 gene is very important bile acid synthesis. LXR and FXR play a crucial role coordinately way on bile acid synthesis, transport and absorption in liver and intestine, and cholesterol metabolism in the liver and peripheral tissues. When cholesterol increases in liver, oxysterols activate LXRα, which stimulates conversion of cholesterol to bile acids by inducing CYP7A1 transcription. Increased bile acid synthesis and pool stimulate FXR, which inhibits CYP7A1 to decrease bile acid synthesis but stimulates BSEP (bile salt export pump) expression to excrete bile acids into bile. Thus, bile acid- regulated genes and nuclear receptors are promising target for drug development for decreasing the serum cholesterol and triacylglycerol levels, and liver and cardiovascular diseases [51-52].

According to our protein and mRNA expression results, *EHT* repressed the CYP7A1 transcription while EAT induced it. Decreased cholesterol level leads to repression of CYP7A1 enzyme production. *EHT*-treated animals showed a significant reduction in total cholesterol levels. *EHT*-treated animals also showed a decrease in protein and mRNA expression of CYP7A1 enzyme, since decreased cholesterol suppresses the CYP7A1 enzyme transcription. Our data indicates that *EHT* may affect FXR and BSEP, which resulted in activation of FXR and BSEP transcriptional activity and thus, inhibition of CYP7A1 expression. To create the bile acids that are needed by the body *EHT* activates the alternative pathway inducing CYP27A1, due to activation of BSEP. However, EAT induces production of CYP7A1 which is the rate limiting enzyme of classic pathway.

In conclusion, the synthesis of bile acids from cholesterol by CYP-catalyzed reactions can be altered due to the changes in CYP protein and mRNA expressions which may be resulted by the treatment of animals with *EHT* and EAT. *EHT* and EAT-treated animals showed a significant decrease in total cholesterol levels. Also, *EHT*-treated animals showed a decrease in protein and mRNA expression of CYP7A1 enzyme, since decreased cholesterol suppresses the CYP7A1 enzyme transcription. To create the bile acids that are needed by the body *EHT* activates the alternative pathway inducing CYP27A1. However, EAT induces production of CYP7A1 which is the rate limiting enzyme of classic pathway. Therefore, bases on doses and time point that applied in this study, the medicinal plant *EHT* and its polyphenolic compound, EAT, may have regulatory effects on bile acid metabolizing enzymes. In conclusion, the synthesis of bile acids from cholesterol by CYP-catalyzed reactions may be altered due to the changes in CYP protein and mRNA expressions which may be resulted by the treatment of animals with *EHT* and EAT*; EHT* and EAT is quite safe and can be used for drug development without any toxicity.

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Table 1. Primer sequences, annealing temperatures and product sizes for gene expression analysis.

Gene	Sequence(5'→ 3')	Annealing Temperature (°C)	Product Size (bp)
CYP7A1	F→ TGCCTTCTGTTACCGAGTGATGTT	60	518
	R→ACCGGCAGGTCATTCAGTTGCACT		
CYP27A1	F→ ATG TGG CCA ATC TTC TCT ACC	55	163
	R→ GGG AAG GAA AGT GAC ATA GAC		
CYP8B1	F→ CAGGTTGGAAGCCGAGACAT	60	158
	R→ TGCCCACTGGCCATCTTTAG		
CYP7B1	F→ CCTTCTCTTTGCGGTCACCT	60	121
	R→ TTTCAGGGCCATGCCAAGAT		
GAPDH	F→ GATGACATCAAGAAGGTGGTGAAG	60	240
	R→ TCCTTGGAGGCCATGTGGGCCAT		

Table 1. Quantitative LC-MS analysis of selected bioactive compounds from *Epilobium hirsutum L.*; and total phenolic and flavonoid content of the plant.

Bioactive compound	*Epilobium hirsutum L.*
Gallic acid	11,906 ± 0,1810
Cafeic acid	≤ 0,31 ppm
Quercetin dhydrate	≤ 0,625ppm
p-coumaric acid	1,0642±0,0027
Ellagic acid	≤ 0,31 ppm
Total phenolic content	225±2, 123 (mgGAE/g dried extract±SD)
Total flavonoid content	19,2593±0,8225 (mg CE/g dried extract±SD)

Figure 1. Synthesis and metabolism of bile acids (Klaassen & Cui 2015)

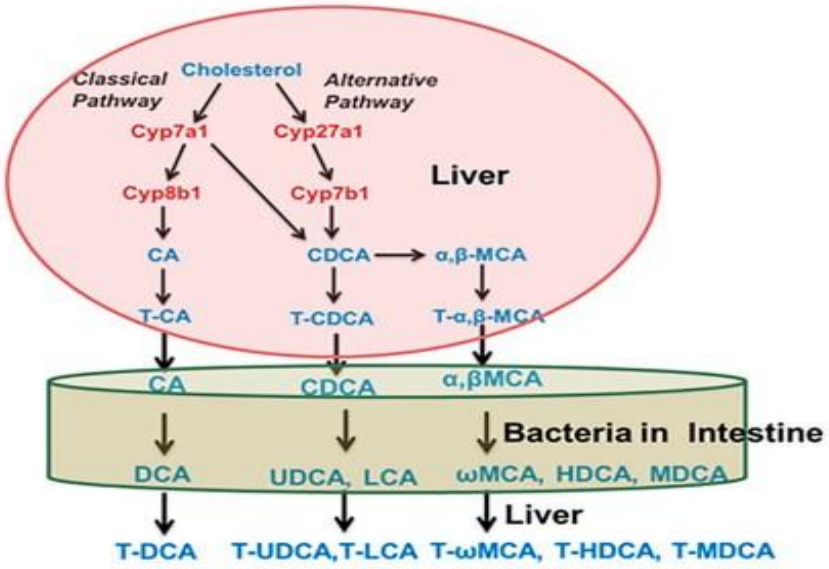

Figure 2. LC-MS chromatogram of *EHT* extract. Bright green line shows mixture of standards (total 5 ppm). Dark green line shows *EHT* extract (10 mg/ml).

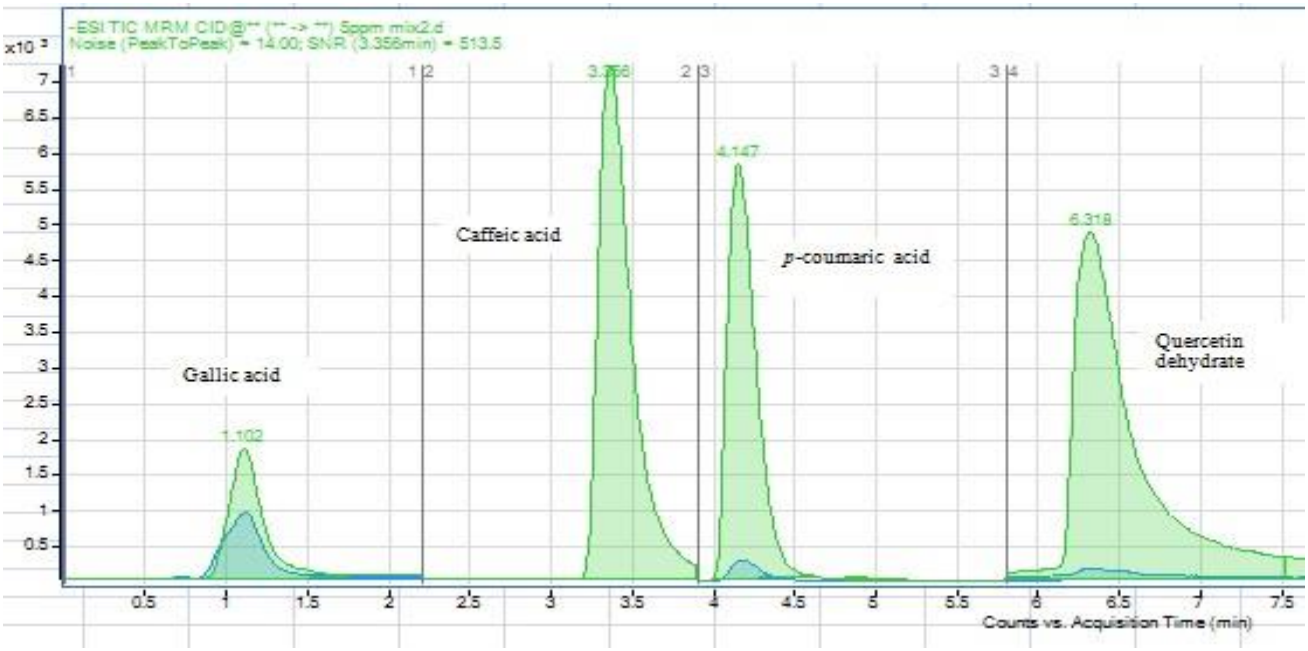

Figure 3. LC-MS chromatogram of *EHT* extract. Grey line shows 2.5 ppm EAT. Blue line shows *EHT* extract (10 mg/ml).

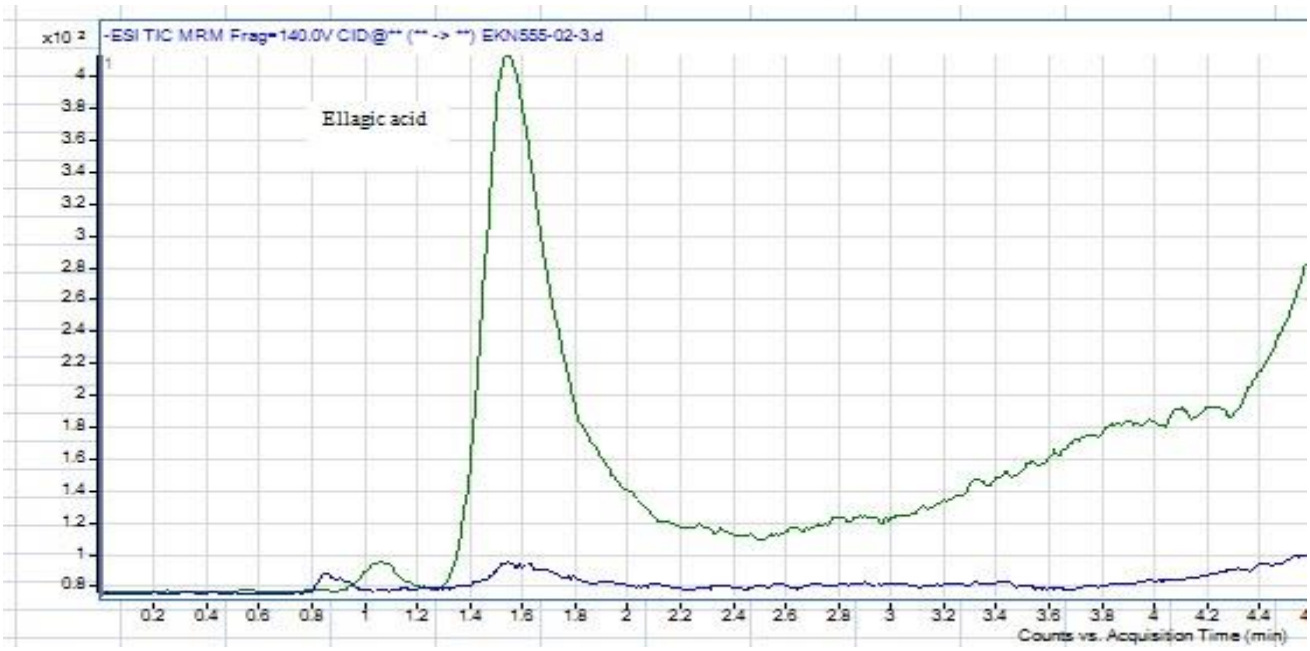

Figure 4 (A) Representative immunoblot of liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) proteins in experimental control and EHT. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) was used as an internal standard for each membrane. (B) Comparison of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 protein expression of the control (n=21) and EHT treated (n=16) groups. Band quantification was expressed as the mean ±SD of the relative intensity with respect to that of GAPDH, used as the internal control. Asterisks denote the level of significance: ***p<0, 0001.

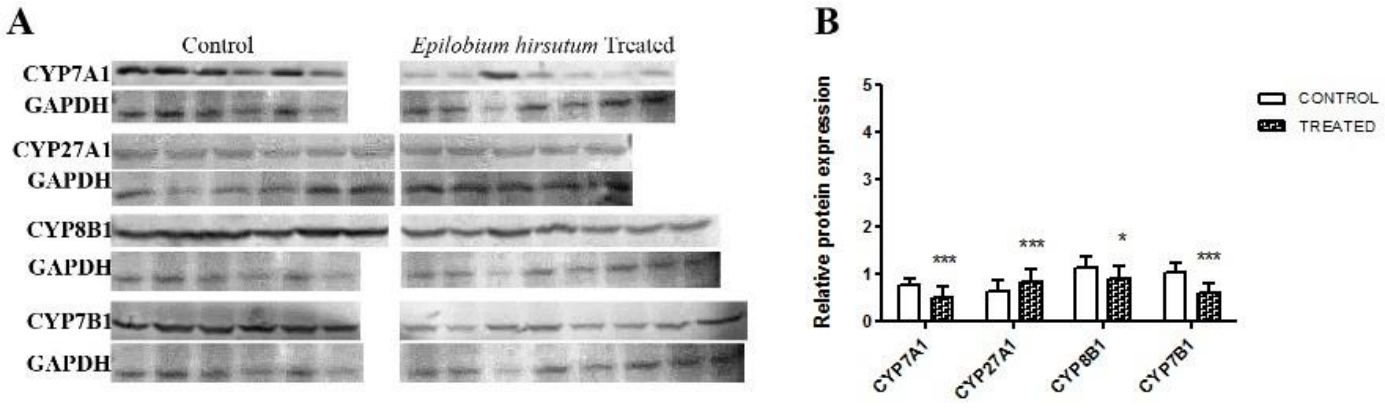

Figure 5. (A) Representative immunoblot of liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) proteins in experimental control and EAT. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) was used as an internal standard for each membrane. (B) Comparison of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 protein expression of the control (n=6) and EAT treated (n=6) groups. Band quantification was expressed as the mean ±SD of the relative intensity with respect to that of GAPDH, used as the internal control. *Significantly different from the respective control value $p<0.005$.

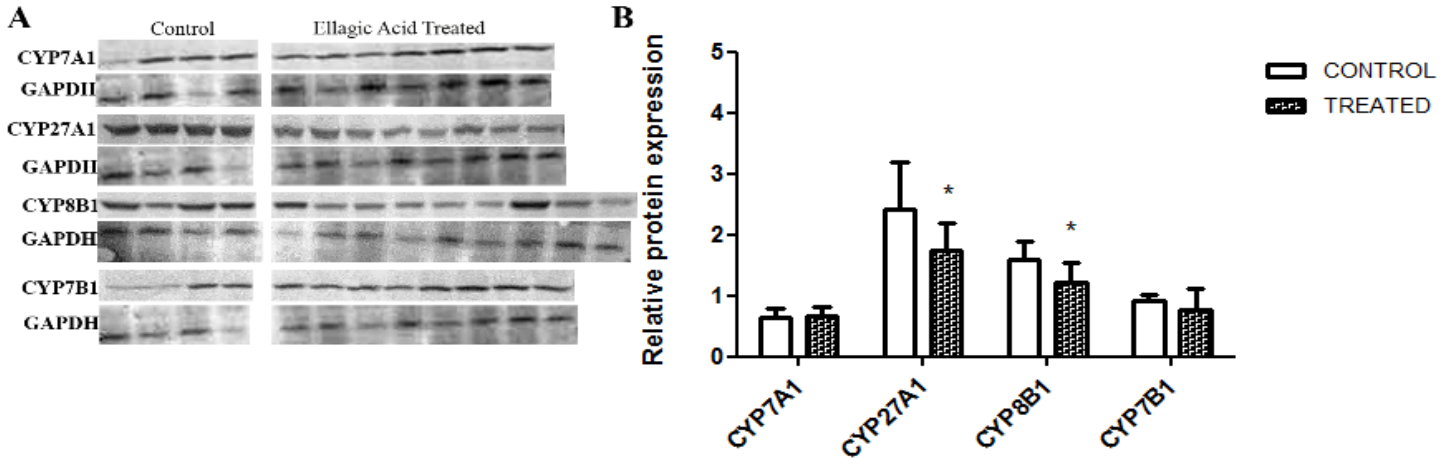

Figure 6. Comparison of in vivo effects of EHT on rat liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA expression. The quantities are expressed as mean ±SD of the relative expression.

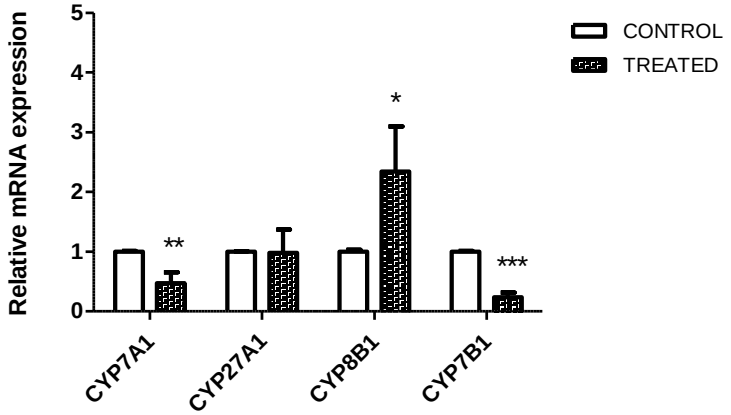

Figure 7: Comparison of *in vivo* effects of EAT on rat liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA expressions. The quantities were expressed as mean ±SD of the relative expression.

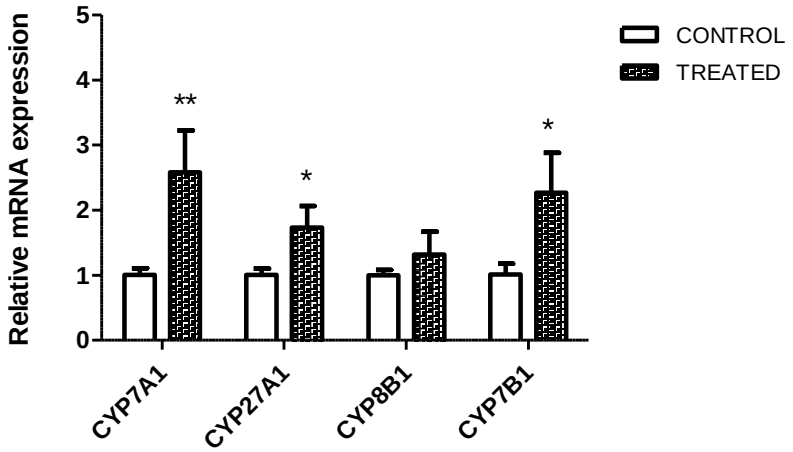

Figure 8. Comparison of blood serum total cholesterol levels between. EHT, EAT treated, and control animals. *Significantly different from the respective control value p<0.05.

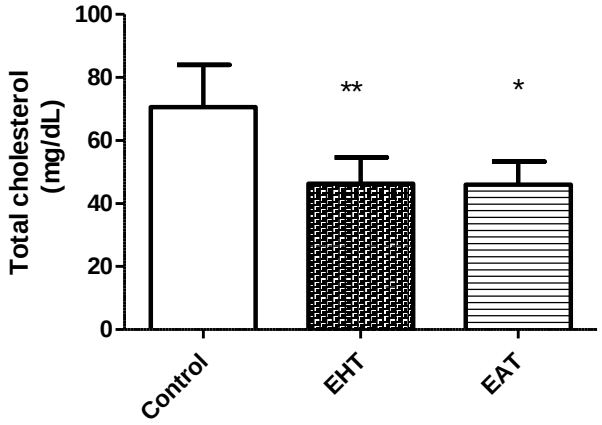

Figure 9. Effects of *EHT* and EAT on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions.I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.