

Assessment of hepatoprotective activity of *Achillea biebersteinii* ethanol extract on carbon tetrachloride-induced liver damage in rats

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Summary

Achillea biebersteinii Afan. (Asteraceae) is claimed to be effective for treatment of jaundice by public in eastern Turkey. In the present study, hepatoprotective effect of *A. biebersteinii* ethanol extract was evaluated against carbon tetrachloride (CCl₄)-induced subacute hepatotoxicity in rats. For the activity assessment on CCl₄-induced hepatic injury the following biochemical parameters were evaluated: Plasma and hepatic tissue malondialdehyde formation, and liver tissue glutathione, catalase levels, -as well as plasma enzyme levels (aspartate transferase, alanine transferase and alkaline phosphatase). Increasing doses of ethanol extract reduced plasma ALP levels. On the contrary, all doses of the extract increased plasma AST and ALT levels. Ethanol extract did not show any significant hepatoprotective activity at 250 and 500 mg/kg doses, whereas a weak activity was observed at 750 mg/kg. On the other hand, at all the tested doses, ethanol extract caused improvement in antioxidant defense potential (liver glutathione, catalase levels). Moreover, total phenolic content of *A. biebersteinii* was found to be 6.21 ±0.004 mg gallic acid equivalent/g extract.

Key Words: *Achillea biebersteinii*, Lipid peroxidation, Serum enzymes, Glutathione, Catalase

Received:19.11.2012

Revised:11.12.2012

Accepted:13.12.2012

Achillea biebersteinii Etanol Ekstresinin Sıçanlarda Karbontetraklorür-nedenli Karaciğer Hasarı Üzerindeki Hepatoprotektif Etkisinin Değerlendirilmesi

Özet

Achillea biebersteinii Afan. (Asteraceae) bitkisi, Türkiye'nin doğu bölgelerinde halk tarafından sarılık tedavisinde etkili olduğu belirtilmektedir. Bu çalışmada, *A. biebersteinii* etanol ekstresinin hepatoprotektif aktivitesi, sıçanlarda oluşturulan karbon tetraklorür (CCl₄)-nedenli subakut hepatotoksosite modelinde değerlendirilmiştir. CCl₄-nedenli karaciğer hasarını değerlendirmek için plazma ve karaciğer malondialdehit oluşumu, karaciğer dokusundaki glutatyon, katalaz seviyeleri ve plazma enzim seviyeleri (aspartat transferaz, alanin transferaz ve alkalin fosfataz) incelenmiştir. Etanol ekstresi artan dozlarda plazma ALP seviyelerini düşürmüştür. Aksine, etanol ekstresi denenen tüm dozlarda plazma AST ve ALT seviyelerini arttırmıştır. Etanol ekstresi 250 ve 500 mg/kg dozlarda herhangi bir anlamlı hepatoprotektif aktivite göstermezken 750 mg/kg dozda zayıf bir aktivite görülmüştür. Diğer yandan, etanol ekstresi test edilen tüm dozlarda, antioksidan savunma potansiyelinde (Karaciğer glutatyon, katalaz seviyeleri) düzelmeye neden olmuştur. Bundan başka, *A. biebersteinii* total fenol içeriği 6.21 ±0.004 mg gallik asit ekivalent/g ekstre olarak bulunmuştur.

Anahtar Kelimeler: *Achillea biebersteinii*, Lipit peroksidasyon, Serum enzimleri, Glutatyon, Katalaz

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INTRODUCTION

The liver is responsible for many important functions, including detoxification, protein synthesis, within the body, and the loss of those functions can cause significant damages. Therefore, treatment of liver diseases is extremely important. Among liver diseases, viral hepatitis can be treated with interferon therapy or with antiviral drugs such as lamivudine, ribavirin (1). In other words, treatment options are very limited. For this reason, discovery of new drugs is important in the treatment of liver diseases.

Numerous medicinal plants used in folk medicine against various diseases with the ethnobotanical field surveys conducted in Anatolia have been reported. Many scientific studies were designed on the plant species used as folk remedy. The research results are usually in good agreement with the traditional uses of the tested plants. Therefore, discovery of new drug molecules from plants based on the evaluation of folk medicines is a very important research subject.

Achillea genus belongs to Asteraceae family, and is widely distributed in Turkey. The plant is known as “yılan çiçeği, civan perçemi, sarı çiçek, çetüğe, püjan, yavşan, kılıç otu, sarılık otu, ayvadene, tatarcı otu, kedi tırnağı, tavuk kıcı”. In the German Commission E Monographs, European, German, USA, French, and British Pharmacopoeias, dried aerial parts with its flowers of *A. millefolium* L., Yarrow, are stated to possess therapeutic activities against a wide range of ailments, including fever, cold and infectious diseases including influenza, gastrointestinal disorders, prevention of inflammation of the gastric mucosa and intestinal colic, hypertension, amenorrhea, sinusitis, cystitis, wounds, diarrhea, liver and biliary disorders. In Turkish folk medicine, the flowers and leaves of *A. biebersteinii* Afan. also have been used to treat cold, urinary infection, abdominal pain, stomachache, furuncle, menstruation problems, hemorrhoid and dizziness (2-7). Additionally, the plant is used to treat jaundice in Kars Province (8). In previous phytochemical studies, the phytochemical constituents of *A. biebersteinii* aerial parts have been reported by several research groups to include essential oil, sesquiterpene lactones, flavonoids,

alkaloids, tannins, polyacetylenes, triterpenes, sterols (including β -sitosterol), coumarins, and phenolic acids (9-12).

The possible hepatoprotective activity of *A. biebersteinii* aerial parts has not been reported so far. Therefore, in this study, we aimed to evaluate the hepatoprotective effect of *A. biebersteinii* aerial parts, using the CCl_4 -induced subacute liver injury model in rats. Liver damage was evaluated by using biochemical parameters (plasma and liver tissue MDA [malondialdehyde], transaminase enzyme levels in plasma [AST-aspartate transaminase, ALT-alanine transferase, ALP-alkaline phosphatase] and liver GSH [glutathione] and CAT [catalase] levels). Moreover, total phenol content of *A. biebersteinii* was determined using Folin-Ciocalteu reagent.

MATERIAL AND METHODS

Plant material

Achillea biebersteinii Afan. was collected from Palandöken Mountain, Erzurum, Turkey in August 2009 and identified by Professor Dr. Mecit Vural of Department of Biology, Faculty of Science and Art, Gazi University, Ankara, Turkey. A voucher of the plant is stored in the herbarium of Gazi University, Faculty of Pharmacy (GUE 2930). Aerial parts with flowers were dried under shade and powdered coarsely before extraction.

Preparation of plant extract

The dried and coarsely powdered aerial parts of *A. biebersteinii* (50 g) were macerated with 70% ethanol by continuous stirring at room temperature, and then evaporated to dryness under reduced pressure [EtOH extract, yield 24.8%].

Determination of total phenols

Ethanol extract of the plant (100 μL) was mixed with 0.2 mL Folin-Ciocalteu reagent 2 mL of H_2O , and 1mL of 15% Na_2CO_3 , and the mixture was measured at 765 nm after 2 h at room temperature. The mean of the three readings was used and the total phenolic content was expressed in milligram of gallic acid equivalents/g extract. A calibration curve with different concentrations of gallic acid was created as

$$y = 0.3195x - 0.0223 \quad (r^2 = 0.9819) \quad (13).$$

Pharmacological procedures

Test animals

Female Wistar-albino rats (150-200 g) purchased from the Animal House of Gazi University (Ankara, Turkey) were used in the experiments. The animals left for 2 days for acclimatization to animal room conditions were maintained on standard pellet diet and water *ad libitum*. The food was withdrawn on the day before the experiment, but free access of water was allowed. A minimum of six animals was used in each group. Institutional Animal Ethical Committee of the Gazi University approved (G.U.ET-11.057) the experimental protocol used in the present study.

Preparation and administration of test samples

The animals were divided into 5 groups, each consisting of 6 rats. The control group was given 0.5% CMC (carboxymethylcellulose) suspension by a gastric gavage (per os). Extract groups were orally treated with EtOH extract (in 250, 500 and 750 mg/kg bw. doses) (bw., body weight) and 50% CCl₄ in liquid paraffin (2.5 ml/kg bw., per os) 60 min after the administration of each extract. The control group (untreated) and carbon tetrachloride group (positive control) received 0.5% CMC suspension and 50% CCl₄ in liquid paraffin (2.5 ml/kg bw., per os) 60 min after the administration of vehicle (14).

Experimental procedure

60 min after the administration of the last dose on 5th day, except the control group rats, each of the carbon tetrachloride (CCl₄) group and test group of animals was challenged with 50% CCl₄ in liquid paraffin (2.5 ml/kg bw., per os) to induce hepatic injury. Twenty-four hours after the hepatotoxin administration, blood samples were withdrawn by cardiac puncture and then rats were anesthetized with ketamine/xylazine. Blood samples collected in heparinized tubes were centrifuged at 3000 \times g (4 °C) for 10 min to obtain plasma. Plasma samples were used to determine the lipid peroxide levels as well as to test AST, ALT and ALP activities. On the other hand, liver of each rat was promptly removed and a part was used to determine the tissue levels of MDA, CAT and GSH.

Aspartate transferase (AST), alanine transferase (ALT) and alkaline phosphatase (ALP) in plasma IBL Turkey reagents were also used in measurement of AST, ALT and ALP levels in plasma samples. All enzyme levels were expressed as U/L.

Determination of plasma lipid peroxidation level

The methodology described by Kurtel et al. (1992) was used (15). Briefly, 1mL of plasma sample as combined with 2.0 mL of trichloroacetic acid (TCA; 15% w/v) -thiobarbituric acid (TBA; 0.375%), -0.25 N HCl and mixed thoroughly and centrifuged at 10.000 \times g for 10 min. The supernatant was mixed with 20 μ L of butyl hydroxy toluene (BHT; 0.02% in 95% EtOH, w/v) to prevent further oxidation and heated for 15 min in a boiling water bath. After cooling under running water, the flocculent precipitate was removed by centrifugation at 10.000 \times g for 5 min. Absorbance of the sample was measured at 532 nm against blank that contained all the reagents except plasma.

Determination of lipid peroxidation in liver tissue

The method of Ohkawa et al. (1979) as modified by Jamall and Smith (1985) was used to determine the lipid peroxidation in tissue samples (16, 17). Rats were anesthetized with ketamine/ xylazine. The liver of each rat was immediately excised and chilled in ice-cold 0.9% NaCl and then perfused via the portal vein with ice-cold 0.9% NaCl. Afterwashing with 0.9% NaCl, 1.0 g of wet tissue was weighted exactly and homogenized in 9 mL of 0.25 M sucrose using a Teflon homogenizer to obtain a 10% suspension. The cytosolic fraction was obtained by a two-step centrifugation first at 1000 \times g for 10 min, then at 2000 \times g for 30 min at 4°C. A volume of the homogenate (0.2 mL) was transferred to a vial and was mixed with 0.2 mL of 8.1% (w/v) sodium dodecyl sulphate solution, 1.5 mL of a 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 mL of 0.8% (w/v) solution of TBA, and the final volume was adjusted to 4.0 mL with distilled water. Each vial was tightly capped and heated in boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test samples and 10%

TCA were transferred into a centrifuge tube and centrifuged at $1000 \times g$ for 10 min. The absorbance of the supernatant fraction was measured at 532 nm. Control experiment was processed using the same experimental procedure, except for the TBA solution, replaced with distilled water. Due to the peroxidative effect of CCl_4 on tissues; the livers of CCl_4 -treated rats were used as positive controls. 1,1,3,3-Tetraethoxypropan was used as standard for calibration of the curve.

Nonprotein sulfhydryl groups (cellular GSH) in liver tissue

Two hundred milligrams of liver was homogenized in 8.0 mL of 0.02 M EDTA in an ice bath. The homogenates were kept in the ice bath until used. Aliquots of 5.0 mL of the homogenates were mixed in 15.0 mL test tubes with 4.0 mL distilled water and 1.0 mL of 50% TCA. The tubes were centrifuged for 15 min at approximately $3000 \times g$ about 2.0 mL of supernatant was mixed with 4.0 mL of 0.4 M Tris buffer, pH 8.9, 0.1 mL Ellman's reagent [5,5'-dithiobis(2-nitro-benzoic acid)] (DTNB) and the sample was shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results were expressed as $\mu\text{mol} / \text{g}$ tissue (18).

Determination of catalase activity

Liver catalase activity was assayed according to the method developed by Aebi 1984 (19). Briefly, 500 mg wet weigh tissue was homogenized by 1% Triton X-100 in 50 mM phosphate buffer. Homogenate was centrifuged at $2500 \times g$ for 10 minutes. Upper phase was removed and kept at -80°C , until the analysis. The catalase activity was determined based on the rate of decrease in absorbance at 240 nm which is caused by hydrolyzation of H_2O_2 by the catalase originating from the sample.

Statistical analysis

The data obtained were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls posthoc tests for the significant interrelation between the various groups using InStat computer software. $P < 0.05$ was considered to be statistically significant from the control.

RESULTS AND DISCUSSION

Since ancient times, plants have played important roles throughout the world in treating diseases. Therefore, folk medicines are also important sources for drug development researches. For this purpose, in this study, hepatoprotective activity of *A. biebersteinii* used for the treatment of jaundice as folk medicine in Kars province was evaluated. CCl_4 -induced liver damage model is frequently used for evaluation of hepatoprotective effects of plant extracts. The changes associated with CCl_4 -induced hepatic injury are similar to that of acute viral hepatitis (20). This xenobiotic is converted to a highly reactive trichloromethyl radical ($CCl_3\cdot$) in the body to which attacks membrane phospholipid stimulating lipid peroxidation (21). A reduced glutathione dependent mechanism can protect the liver microsomal membrane against CCl_4 -induced lipid peroxidation. Moreover, antioxidant enzymes (SOD, catalase and GPx) protect against oxidative tissue damage (22). CAT converts hydrogen peroxide into form water and oxygen. This way, toxic hydrogen peroxide is detoxified. The reduction in the activity of CAT enzyme may result in a number of harmful effects due to the accumulation of hydrogen peroxide (23).

Administration of CCl_4 to rats for a 5 days period caused significant ($p < 0.001$) rise in AST (274.8%), ALT (160.1%), and ALP (187.7%) levels in plasma, when compared with normal controls. Increasing doses of the extract reduced plasma ALP levels. On the contrary, all doses of the extract increased plasma AST (0.4-21.4%) and ALT (20.2-24.4%) levels in a dose dependent manner (Table 1).

Plasma MDA levels were slightly higher in the CCl_4 -treated group. Administration of ethanol extract at a dose of 750 mg/kg decreased plasma MDA level compared to those in CMC group. Results indicated that liver lipid peroxidation level (43.0%) increased significantly in CCl_4 -treated group as compared to those in the control group. Dose-dependent decreases in tissue MDA levels were observed after the administration of all the tested doses of ethanol extract (Table 2).

Table 1. Effect of *A. biebersteinii* ethanol extract on plasma transaminase and alkaline phosphatase enzymes levels against CCl₄-induced liver damage

Materials	AST (U/L ±SEM)	% Change	ALT (U/L ±SEM)	% Change	ALP (U/L ±SEM)	% Change
CMC	8.2 ±0.6		52.6 ±3.4		74.2 ±7.8	
CCl ₄ ^a	30.9 ±5.2***	+274.8	136.8 ±7.1***	+160.1	213.4 ±11.0***	+187.7
250 mg/kg ^b	37.5 ±3.8	+21.4	170.2 ±6.5**	+24.4	207.1 ±12.3	-3.0
500 mg/kg ^b	32.0 ±3.6	+3.8	166.2 ±4.8**	+21.5	201.9 ±10.3	-5.3
750 mg/kg ^b	30.9 ±2.5	+0.4	164.3 ±6.7**	+20.2	176.6 ±9.6	-17.2

[Results were expressed as mean ±SEM].

^a Compared to vehicle control (0.5% CMC).

^b Compared to carbon tetrachloride (CCl₄) as hepatotoxin.

^c (+) represents percentage of increase and (-) represents decrease in each value when compared to either vehicle or CCl₄. **p <0.01 significant from control or CCl₄. ***p <0.001 significant from control or CCl₄.

As seen in Table 3, the level of CAT in the liver (76.5%) decreased in CCl₄-treated group. The administration of ethanol extract significantly increased CAT activity compared to those in the CCl₄-treated group. Administration of ethanol extract increased the activities of CAT in extract group rats to prevent the accumulation of excessive free radicals and protected the liver from CCl₄ intoxication.

A small reduction in the GSH level (2%) was observed in the liver tissues of CCl₄ group rats. The GSH contents of liver tissues were augmented by the administration of *A. biebersteinii* ethanol extract at all the tested doses (0.9-10.1%) (Table 3).

Total phenolic content of *A. biebersteinii* was found to be 6.21 ±0.004 mg gallic acid equivalent/g extract.

Consequently, for all tested doses, the extract caused impairment in antioxidant defense potential (liver glutathione, catalase levels). But, *A. biebersteinii* ethanol extract was observed to be ineffective in normalizing CCl₄-induced increase of plasma transaminase levels.

These findings revealed that, *A. biebersteinii* ethanol extract was not adequate to produce significant hepatoprotective and antioxidant effects at 250 and 500 mg/kg doses. But, among the tested doses,

Table 2. Effect of *A. biebersteinii* ethanol extract on plasma and liver MDA levels against CCl₄-induced liver damage

Materials	Plasma MDA level (nmol/ml ±SEM)	% Change	Liver homogenate MDA level (nmol/g wet weight ±SEM)	% Change
CMC	6.25 ±0.02		98.4 ±3.3	
CCl ₄ ^a	6.28 ±0.02	+0.5	140.7 ±5.4***	+43.0
250 mg/kg ^b	6.32 ±0.04	+0.6	133.6 ±3.8	-5.1
500 mg/kg ^b	6.27 ±0.04	-0.2	131.1 ±5.4	-6.8
750 mg/kg ^b	6.24 ±0.05	-0.6	129.6 ±1.9	-7.9

[Results were expressed as mean ±SEM].

^a Compared to vehicle control (0.5% CMC).

^b Compared to carbon tetrachloride (CCl₄) as hepatotoxin.

^c (+) represents percentage of increase and (-) represents decrease in each value when compared to either vehicle or CCl₄. ***p <0.001 significant from control or CCl₄.

Table 3. Effect of *A. biebersteinii* ethanol extract on liver GSH and CAT levels against CCl₄-induced liver damage

Materials	Tissue GSH (µmol/g ±SEM)	% Change	Tissue CAT level (k/g protein ±SEM)	% Change
CMC	113.0 ±3.5		53.1 ±3.7	
CCl ₄ ^a	110.7 ±8.3	-2.0	12.5 ±0.9***	-76.5
250 mg/kg ^b	98.9 ±6.9	+0.9	26.6 ±1.6***	+113.1
500 mg/kg ^b	120.9 ±9.1	+9.2	21.2 ±1.3*	+69.6
750 mg/kg ^b	121.9 ±5.1	+10.1	18.9 ±2.0*	+51.3

[Results were expressed as mean ±SEM].

^a Compared to vehicle control (0.5% CMC).

^b Compared to carbon tetrachloride (CCl₄) as hepatotoxin.

^c (+) represents percentage of increase and (-) represents decrease in each value when compared to either vehicle or CCl₄. *p <0.05 significant from control or CCl₄. ***p <0.001 significant from control or CCl₄.

maximum hepatoprotective activity was observed at 750 mg/kg dose.

In conclusion, we did not find significant scientific evidence to sufficiently support the declared strong hepatoprotective effect of *A. biebersteinii* ethanol extract at the tested doses on liver damage. On the other hand, the data obtained in the present study showed that *A. biebersteinii* ethanol extract possessed healing activity on liver CAT, GSH and plasma ALP parameters. Further studies will be done for evaluation of hepatoprotective activity of *A. biebersteinii* aqueous extract.

ACKNOWLEDGEMENT

This study was financially supported by The Research Fund of Gazi University (EF 02/2011-45).

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