Protective Effects of *Rheum Turkestanicum* Janischagainst Diethylnitrosamine-Induced Hepatocellular Carcinoma in Rats

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Research Article

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Abstract

Aim of the study Hepatocellular carcinoma (HCC) is common cancer that causes many deaths worldwide. Recent studies have reported anti-cancer effects of *R. turkestanicum* against various cell lines including leukemia cervical tumor, and breast cancer. In this study, we aimed to identify the effect of *R. turkestanicum* against diethylnitrosamine (DEN)-induced HCC.

Methods Wistar rats were divided into four groups of control, DEN, DEN + 100 mg/kg or 400 mg/kg of hydroethanolic extract of the plant roots.

Results After four months, the animals in the DEN group showed HCC foci in the liver, an increase of hepatic lipid peroxidation, attenuation of hepatic antioxidant capacity, an increase of blood liver enzymes (ALT, AST, and ALP), bilirubin, albumin, creatinine, glucose, and reduction of the body weight. The plant extract could decrease the levels of liver enzymes, total bilirubin, direct bilirubin, albumin, urea, and creatinine in the blood. Also, the extract attenuated oxidative stress and improved pathological changes in the liver. Quantitative real-time PCR revealed a decrease in gene expression of Wnt/β-catenin and Akt and an increase in PTEN as the tumor suppressor gene.

Conclusion The extract of *R. turkestanicum* reduced DEN-induced liver changes through inhibiting oxidative stress and attenuating the expression of Wnt/β-catenin and Akt and elevating PTEN expression.

Introduction

Hepatocellular carcinoma (HCC) is common cancer that causes many deaths worldwide. Different risk factors play role in the development of HCC such as fungal toxins (aflatoxins), food additives, alcohol, hepatitis viral infection (HBV and HCV), air and water pollutants, and toxic industrial chemicals [1]. Diethylnitrosamine (DEN), a hepatocarcinogen compound [2], is found in cheddar cheese, tobacco products, fried meals, agricultural chemicals, cosmetics, and pharmaceutical agents [3]. The DEN-induced HCC is an experimental model which extensively used to induce liver cancer for research purposes [4]. Various studies have reported that oxidative stress and reactive oxygen species (ROS) have key roles in the carcinogenesis of DEN [5]. The exposure of animals to DEN leads to infiltration of macrophages and neutrophils in liver tissue and a large number of cytokines are released from immune cells. The high production of ROS may damage some biomolecules such as DNA, proteins, and lipids in hepatocytes [6].

Various signaling pathways have been suggested to involve in the initiation and promotion of HCC. The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway, Wnt/β-catenin pathway, Ras/mitogen-activated protein kinase (MAPK) signaling, the ubiquitin/proteasome degradation system, and receptor tyrosine kinase pathways are some of the well-known pathways involved in HCC [7]. Compounds that target these signaling pathways might be candidates for developing therapeutics for HCC.

Rheum turkestanicum Janisch belongs to the Polygonaceae family and grows widely in the central regions of Asia. In the traditional medicine, the root of this plant has been used for the treatment liver problems such as jaundice [8, 9]. Some of the main active compounds found in *R. turkestanicum* include rhein, emodin, chrysophanol, physcion, glcogallin, epicatechin, and quercetin [9]. *R. turkestanicum* also contains active compounds in the treatment of cancer [10]. *In vitro* studies have reported that *R. turkestanicum* can reduce the viability of carcinoma cells [11]. However, studies on the anticancer effect of *R. turkestanicum* are limited to *in vitro* works and no *in vivo* study has been done so far. Therefore, the present study was designed to evaluate the effects of *R. turkestanicum* on DEN-induced HCC in the rat.

Materials And Methods

Chemicals

DEN was purchased from Tinab Shimi Khavaremianeh Co., Mashhad, Iran. 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB), *n*-butanol, ethylenediaminetetraacetic acid disodium (Na_2EDTA), 2-thiobarbituric acid (TBA), Tris, phosphoric acid (1%), hydrochloric acid (HCl), trichloroacetic acid (TCA), and methanol were bought from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) was obtained from Sigma (St. Louis, USA).

Preparation of extract

The *R. turkestanicum* was collected from Razavi Khorassan Province, NW Dargaz, Zarrin-Kuh Protected Area (37°38'13"N, 58°55'09"E, height =727 m) in June 2019. Mr. Joharchi defined the voucher specimen number (21377) for the plant and kept it in Ferdowsi University of Mashhad Herbarium. The extract was prepared by the maceration technique. The roots were cleaned, dried in shadow, powdered by a mill, and then macerated in ethanol (70%) for 72 h. Then, the extract was filtered through a metal mesh (106 µm pore size), centrifuged (300 g, 5 min), and dried in an

oven at 40 ºC. The amount of obtained extract was 55%. It was stored at -20 ºC until further use.

Evaluation of phenolic compounds

A sample of 20 µl of the plant extract (10 mg/ml) was added to 100 µl of Folin-Ciocalteu reagent and 300 µl of sodium carbonate solution (1 mol/L). Then, the volume was adjusted to 2 mL with deionized water. After 2 h, the optical density of the solution was determined at 765 nm by a spectrometer. A standard curve was generated for gallic acid (0, 50, 100, 150, 250, and 500 mg/L) and the concentration of phenolic compounds was expressed as milligram of equivalent gallic acid [12].

Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS was performed in an AB SCIEX QTRAP (Shimadzu) liquid chromatography coupled with a triple quadrupole Mass Spectrometer. Liquid chromatography separation was performed on a Supelco C18 (15 mm×2.1 mm×3 µm) column. The MS analysis was performed in the negative mode of ionization

to screen as many ions as possible and to ensure that the greatest number of metabolites extracted from the *R. turkestanicum* sample was detected. The eluent flow rate was set at 0.5 ml/min. The mobile phase gradient was programmed as follows: starting with 90% of 0.1% aqueous formic acid, isocratic conditions were maintained for 5 min, and then a 15-min linear gradient to 35% methanol with 0.1% formic acid was applied. From 20 to 35 min the acidified methanol was increased to 100%, followed by 5 min of 100% acidified methanol, and 5 min at the start conditions to re-equilibrate the column. The mass spectra were acquired in a range of 200 to 1000 within the scan time of 45 minutes. Mass feature extraction of the acquired LC-MS data and maximum detection of peaks were acquired using MZmine analysis software package, version 2.3.

Animals

Forty adult male Wistar rats of about 180-200 g were bought from Animal House, Faculty of Medicine, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. The animals were kept in an appropriate light and temperature and had no special limitation for food and water. All protocols carried out according the national laws and in accordance with the National Institutes of Health guidelines for the use and care of laboratory animals. The research proposal and its methods were confirmed by the Ethics Committee of MUMS (ethical code number: IR. MUMS. MEDICAL. REC. 1398.370).

Experimental Protocol

Following one week acclimatization period, the animals were randomly divided into four groups of ten rats as below:

Group 1: The normal control group received saline intraperitoneally (i.p.) for the first month and drinking water for a further three months.

Group 2: The HCC group to which DEN was weekly administrated at a dose of 75 mg/kg, i.p. for one month followed by oral administration (100 mg/kg/day) for a further three months.

Groups 3 and 4: The extract was given at doses of 100 and 400 mg/kg in drinking water from day 1 of DEN administration for 4 months.

The weight of animals was measured every two weeks. After four months, the rats were fasted overnight, then anesthetized by ketamine (50 mg/kg)-xylazine (10 mg/kg). The blood was collected from cardiac puncture, and the serum was separated to evaluate the levels of glucose, alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin, direct bilirubin, albumin, urea, and creatinine. The liver was separated and washed with normal saline. The KCl buffer (1.5%, pH 7.4) was used to homogenize liver tissue for the determination of malondialdehyde (MDA) level, superoxide dismutase (SOD) activity, and thiol content.

Determination malondialdehyde level

The amount of MDA, as the final product of lipid peroxidation [13], was determined using TBA reagent. A sample of 0.5 ml of liver homogenate was mixed with 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) and then warmed in a water bath for 45 min. After cooling the mixture, *n*-butanol (4 ml) was added, vortexed (1 min), and centrifuged at 20,000 rpm for 20 min. The absorbance of this complex was read at 532 nm [14].

The measurement of thiol groups

The level of total SH groups in the liver was measured using the DTNB. The reaction of SH groups with DTNB produces a yellow-colored complex with an absorbance peak at 412 nm. A sample of 0.5 ml of the tissue homogenate was mixed with 1 ml of Tris-EDTA (pH: 8.6) and absorbance was measured at 412 nm against Tris-EDTA buffer alone (A1). After adding DTNB, the mixture was put at room temperature for 15 min and the absorbance was read again (A2). DTNB was applied as blank (B). The below formula was used to estimate the amount of thiol [12].

Thiol concentration (mM) = (A2-A1-B) × 1.07/(0.05 × 13.6).

Evaluation of SOD activity

The SOD activity in the liver tissue was evaluated by a colorimetric method as described previously [15]. Briefly, the tissue homogenized was mixed with MTT and pyrogallol solution and then incubated for 5 min at room temperature. Finally, DMSO was added and the absorbance was recorded at 570 nm. The SOD activity was expressed as units per milligram protein.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The RNA extraction kit (Pars Tous, Iran) was applied to separate RNA from the liver. The amount and purity of extracted RNAs were determined by NanoDrop ND-1000 (Thermo Fischer Scientific, USA). Reverse transcription was carried out using an ExcelRT™ Reverse Transcription Kit II (SMOBIO, Taiwan). Quantitative real-time polymerase chain reaction (qRT-PCR) was done via Light Cycler 96 Real-Time PCR System (Roche, Germany) with RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). The primer sequences are listed in Table 1. The expression level of different genes was calculated via the $2^{-\Delta\Delta Ct}$ method.

Histopathological study

For each rat, liver specimens were removed and fixed in 10% formalin. Then, they were embedded in paraffin wax, cut into sections with ~5 μm thickness, and stained with hematoxylin and eosin dye. Each tissue sample was examined for the presence of dysplastic nodules and the number and size of HCC foci.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test for multiple comparisons. The change of body weight was carried out by repeated-measures two-way ANOVA during various times of research. Data were expressed as mean ± standard error of the mean (SEM). The *p*-value less than 0.05 was considered to be statistically significant.

Results

Phytochemical evaluation of *R. turkestanicum* extract

The content of total phenolic compounds in *R. turkestanicum* was found to be 189 mg gallic acid equivalent per gram of the extract. The mass spectra of *R. turkestanicum* extract are shown in Figure 1. Comparing the spectra with literature data on other Rheum species showed at least 22 compounds in the extract (Table 2). These compounds include anthraquinones (e.g. emodin, emodin glycosides, physione, rhein, and rhein derivatives), fatty acids (palmitic acid), and flavonoids (e.g., epicatechin and quercetin).

Effect of *R. turkestanicum* on body weight and survival

Before induction of HCC, the body weight was not statistically different between the experimental groups. As shown in Figure 2, DEN decreased body weight significantly from the 8^{th} week. The repeated measures ANOVA confirmed that the body weight altered over time ($p < 0.05$) and that there was a difference in the weight between experimental groups ($p < 0.05$). Administration of *R. turkestanicum* at the dose of 400 mg/kg increased body weight in HCC rats. However, the change of weight by the extract was not significant except in the last week ($p<0.05$).

The mortality rate was observed as 0% (0/10), 50% (5/10), 40% (4/10) and 20% (2/10) in control, DEN, DEN + 100 mg/kg of the extract and DEN + 400 mg/kg of the extract, respectively.

Effect of *R. turkestanicum* on the serum biochemical factors

As shown in Figure 3a-c, DEN increased the serum level of AST, ALT, and ALP activity ($p < 0.001$). Administration of the plant extract significantly decreased AST (400 mg/kg, $p < 0.001$), ALT (400 mg/kg, $p < 0.01$) and ALP (100 mg/kg, $p < 0.05$; 400 mg/kg, $p < 0.001$) compared to the untreated DEN group.

DEN also increased the levels of direct bilirubin, total bilirubin, and creatinine compared to control rats ($p < 0.001$) (Fig. 4a-f). On the other hand, the serum levels of albumin ($p < 0.01$) and glucose ($p < 0.001$) significantly decreased by DEN. DEN-induced HCC displayed no significant alteration in the levels of urea. Administration of the extract restored the effects of DEN on direct bilirubin (400 mg/kg, $p < 0.001$), total bilirubin (100 mg/kg, $p < 0.05$; 400 mg/kg, $p < 0.001$), albumin (400 mg/kg, $p < 0.05$), and glucose (400 mg/kg, $p < 0.01$), and creatinine (400 mg/kg, $p < 0.01$).

Effect of *R. turkestanicum* on the liver oxidative stress

As shown in Figure 5a-c, DEN significantly ($p < 0.001$) increased the level of MDA which is an index of lipid peroxidation. Also, the content of thiol compounds and the activity of SOD in the liver of the DEN group were lower than those in normal control rats ($p < 0.001$). *R. turkestanicum* decreased DEN-induced oxidative stress through reducing the lipid peroxidation (100 mg/kg, $p < 0.01$; 400 mg/kg, $p < 0.001$) and elevating both the thiol content (400 mg/kg, $p < 0.05$) and SOD activity (400 mg/kg $p < 0.01$).

Effect of *R. turkestanicum* on the expression of genes involved in Wnt/β-catenin and PI3K/Akt pathways

There was no significant difference in the expression of PI3K mRNA between the experimental groups (Fig. 6a-e). However, the expression of Akt, Wnt4, and β-catenin genes significantly increased in HCC rats compared to the normal control group ($p < 0.05$). Administration of the plant extract at the dose of 400 mg/kg significantly decreased the expression of Akt, Wnt4, and β-catenin compared to the HCC group ($p < 0.05$). The expression of PTEN was significantly suppressed in the HCC group when compared to the control group ($p < 0.01$). The extract could increase the expression of PTEN at the dose of 400 mg/kg ($p < 0.05$).

Evaluation of hepatic histopathology

Macroscopic and microscopic images of the livers isolated from different study groups are illustrated in Figure 7. The macroscopic observations showed that the livers of the control group have normal appearance while cirrhosis, necrosis, and macroscopic nodules were observed in the DEN-induced HCC group. The macroscopic alterations in groups that received extract were lower in comparison with the DEN group but could not reverse to normal status.

Microscopic evaluation revealed normal hepatocytes and liver architecture in the normal control group. The liver of rats that received DEN displayed abnormal architecture including cirrhosis and dysplastic nodules progressing to HCC with extensive pleomorphism and hyperchromatic nuclei. As shown in Table 3, the HCC foci were observed in both DEN control and DEN-treated groups. The size of HCC foci in treatment groups was lower than the DEN control group but this difference was not statistically significant. In groups that received the plant extract, both of the doses significantly decreased the largest HCC nodule in comparison with the DEN group (100 mg/kg, $p < 0.05$; 400 mg/kg $p < 0.001$).

Discussion

The liver has an important role in the metabolism of various compounds such as drugs, carcinogens, and chemicals [28]. DEN as a chemical carcinogen of N-nitroso compounds activates cytochrome P-450 enzymes in hepatocytes and leads to HCC in experimental animal models [29]. Also, carcinogenesis by DEN is strongly associated with the excessive production of free radicals and decreasing the anti-oxidant enzymes which trigger time-dependent necrosis through accumulating oxidized thiol proteins [30, 31]. In recent years, studies have reported beneficial effects of natural compounds in the prevention and management of cancers including HCC [32–34]. It has been shown the extract has anti-oxidant activity and anti-cancer effects against different cancer cell lines [11]. In the present study, the anticancer effect

of *R. turkestanicum* root extract was investigated in a rat model of HCC. Results showed that co-treatment with 400 mg/kg of the extract improves oxidant/antioxidant status and function of the liver, which were accompanied by increased body weight and survival.

As expected DEN reduced SOD activity and thiol content and induced lipid peroxidation in the liver. This hepatic oxidative stress was associated with increased levels of liver enzymes (AST, ALT, and ALP) and bilirubin and decreased concentrations of albumin and glucose in the blood. The *R. turkestanicum* extract could restore these parameters, which was in agreement with antioxidant and hepatoprotective effects of this plant reported by other studies [9, 12, 35]. Also, there are reports that protective effects of *R. turkestanicum* against organ toxicity induced by doxorubicin [36], gentamicin [37], $HgCl_2$ [38], and hexachlorobutadiene [37] is mediated through its antioxidant activity.

Inhibition of cancer progression and increasing survival are the main goals of any anticancer therapy. In the present study, *R. turkestanicum* extract reduced the mortality rate in the rat model of HCC. The percent of death was 50% in the rats who received DEN alone compared to 20% in those who were treated with the extract (400 mg/kg). We assumed that this decrease in animal death to be due to the improvement of liver pathology. However, although *R. turkestanicum* extract could reduce the weight of the liver and the size of the largest HCC nodule, the other pathological characteristics of livers from treated rats were comparable with those of non-treated animals. Therefore, the effect of *R. turkestanicum* on mortality is not only because of cytotoxic effect against HCC cells but most probably due to improving liver function (as judged by decreased bilirubin and liver enzymes and increased albumin in serum).

The beneficial effects of *R. turkestanicum* on the HCC may be related to its bioactive ingredients including chrysophanol [39], emodin [40], aloe-emodin [41], and quercetin [42]. The LC-MS analysis of *R. turkestanicum* extract showed the presence of a high amount of quercetin and emodin which belong to flavonoids and anthraquinones, respectively. Previous studies have reported anti-cancer activity of emodin [40], quercetin [42], and chrysophanol [39] against HepG2 liver cancer cells. Also, we found that *R. turkestanicum* extract has a high level of phenolic compounds (189 mg/g extract). The role of natural phenolic compounds in cancer prevention has been well established [43].

Studies have shown that the expression of some genes involved in signaling pathways is modified in patients with liver cancer. Two common pathways related to HCC pathogenesis include PI3K/Akt/mTOR and Wnt/β-catenin pathways [44, 45]. The PI3K/Akt/mTOR pathway plays role in the control of survival, cell growth, and apoptosis [46]. The PTEN is a tumor suppressor gene that inhibits this pathway. Mutation of this gene prevents apoptosis and enhances cell proliferation and migration, which may lead to the development of human cancer such as HCC [47]. Similarly, dysregulation of the expression of genes involved in β-catenin-dependent Wnt signaling alters liver metabolism and may lead to inappropriate transcription of some oncogenic target genes [48, 49]. In the present study, change in the expression of PI3K mRNA was not statistically significant following HCC induction. However, DEN significantly increased the expression of Akt, Wnt, and β-catenin and significantly decreased the expression of PTEN. These findings are in agreement with our previous work that DEN changed the

expression of PTEN, Wnt, and β-catenin while had no significant effect on the PI3K gene [34]. Treatment with *R. turkestanicum* extract (400 mg/kg) could restore the PTEN expression and downregulated the expression of Wnt4 and β-catenin genes. It has been shown that some bioactive ingredients of *R. turkestanicum* can modulate the PI3K/Akt/mTOR pathway. For example, quercetin prevented hepatic fibrosis [50] and increased the sensitivity of breast cancer to doxorubicin via PTEN/Akt pathway [51, 52]. Also, emodin increased the sensitivity of HCC cells to sorafenib via Akt signaling [53]. Also, in line with the results of the present work, previous studies have reported that some nature-derived compounds can modulate the Wnt/*β*-catenin signaling in cancer cells and the liver of HCC animals [54–56]. One of the limitations of the present study is that we did not investigate the effect of *R. turkestanicum* on protein expression of PI3K, Akt, PTEN, Wnt4, and β-catenin. The activity of the PI3K/Akt/PTEN pathway is also determined by their phosphorylation and further studies are needed to clearly explain molecular mechanisms of the effects of *R. turkestanicum* on HCC.

Considering the body surface area of humans and rats, the dose of 400 mg/kg of *R. turkestanicum* extract in the rat is approximately equal to 60 mg/kg in humans (i.e. 4 g for a 70-kg person) [57]. In our previous study, we evaluated acute and sub-acute toxicity of *R. turkestanicum* root following oral administration. After 4 weeks, the extract at the dose of 400 mg/kg induced no mortality or significant changes in body weight, hematological parameters, serum biochemical factors (related to the kidney and liver functions), and histopathology of the heart, liver, kidney, and brain. Therefore, it seems that short-term use of the extract does not produce significant toxicity (in humans up to a dose of 60 mg/kg). However, toxicological data about chronic use of the extract are not available and should be assessed by future studies.

Conclusion

This research suggests that oral administration of *R. turkestanicum* has hepatoprotective effects through inhibiting oxidative stress and inhibiting Wnt4/β-catenin and Akt pathways, and therefore increases survival of HCC rats. The present research might serve as the basis for future clinical studies on improving the function of the liver in patients with cirrhosis/HCC and increasing their survival.

Abbreviations

alanine transaminase (ALT), alkaline phosphatase (ALP), analysis of variance (ANOVA), aspartate aminotransferase (AST), diethylnitrosamine (DEN), 5,5’-dithio-bis-[2-nitrobenzoic acid] (DTNB), ethylenediaminetetraacetic acid disodium salt (Na2EDTA), hepatocellular carcinoma (HCC), hydrochloric acid (HCl), Liquid chromatography-mass spectrometry (LC-MS), malondialdehyde (MDA), mammalian target of rapamycin (mTOR), potassium chloride (KCl), phosphoinositide 3-kinase (PI3K), phosphatase and tensin homolog (PTEN), quantitative real-time polymerase chain reaction (qRT PCR), reactive oxygen species (ROS), standard error of the mean (SEM), superoxide dismutase (SOD), trichloroacetic acid (TCA), thiobarbituric acid (TBA)

Declarations

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Authors' contributions

GA and RA conducted the conception and design of the project, RA performed the experiments and statistical analysis; HA participated in the experiments and prepared a draft of the manuscript. MF and SM performed RT-PCR and the data analysis. HA, KA, HS, AM participated in the experiments. HM and IM performed LC-Ms analysis. JA was involved in the histopathological evaluation, GA and RA provided the final revision of the manuscript. All authors contributed to manuscript preparation and approved the submitted version.

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Availability of data and materials

The authors confirm that the data supporting the findings of the present study are available within the article [and/or] its supplementary materials.

Declarations

Ethics approval and consent to participate

The animal experimental protocol was approved by the national laws and in accordance with the National Institutes of Health guidelines for the use and care of laboratory animals. The research proposal and the experimental protocol were confirmed by the Ethics Committee of MUMS with ethical code number: IR. MUMS. MEDICAL. REC. 1398.370.

Consent for publication

All authors have seen and approved the manuscript being submitted.

Competing interests

The authors declare no competing interests.

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Tables

Table 1. Primer sequences of Akt, PI3K, PTEN, Wnt4, β-Catenin, and GAPDH.

Gene name	Forward (5'->3')	Reverse (5'->3')	Product length (bp)
AKT	CCATTGTGAAGGAGGGCTGG	AGTTGTTGAGTGGGGACTCG	150
PI3K	TCCTGATCTTCCTCGTGCTG	AAAGCCATTTTCCCGGACAC	150
PTEN	GGGAAAGGACGGACTGGTG	GCGCCTCTGACTGGGAATAG	141
Wnt4	TGGCCTTTGCAGTGACAAGA	CCCTTGCTTCTCTCTCGGAC	175
B-Catenin	TACTAGAGGTCGTGAGCCTT	TGGTATAGACAGACGCCCCT	159
GAPDH	CAGGGCTGCCTTCTCTTGTG	ACCAGCATCACCCCATTTGA	217

Table 2. Peak assignment of metabolites in the hydro-ethanol extract of *R. turkestanicum* using LC-MS in the negative mode.

Peak No.	Compound	RT (min)	[M-1] (*m/z*)	Ref.
1	6-Methyl-Rhein	20.4	297.72	[16]
2	6-Methyl-Rhein-diacetate	28.8	381.18	[17]
3	Emodin	18.9	269.52	[16, 18]
4	Emodin-8-O-glucopyranoside	30.8	431.22	[19, 20]
5	Kaempferol	16.1	285.3	[21]
6	Revandchinone 1	9.6	520.86	[16]
7	Revandchinone 2	2.9	673.44	[16]
8	Palmitic acid	24.7	255.72	[22]
9	Chrysophanol	14.4	253.2	[16, 18]
10	Epicatechin	44.9	289.26	[16]
11	Desoxyrhaponticin	26.5	403.2	[23]
12	Glucogallin	27.0	331.26	[24]
13	Danthron	19.6	239.58	[25]
15	Physcion	39.2	283.62	[16, 18]
16	Piceatannol	18.1	243.48	
17	Gallocatechol	44.9	305.28	[26]
18	9-octadecenoic acid	25.2	281.28	[22]
19	Quercetin	11.6	301.32	[22]
20	Rhein	39.1	283.68	[16, 18]
21	Rheochrysin	29.4	445.58	[26]
22	Rhododendrin	38.4	327.54	[27]

Table 3. A summary of pathological characteristics of the liver sections in DEN-induced HCC rats. The *R. turkestanicum* extract (RE) was administrated at doses of 100 mg/kg (RE100) and 400 mg/kg (RE400)

for 16 weeks (n = 10). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to DEN group.

Groups	Number of HCC foci	Size of HCC foci (mm)	Largest HCC nodule (mm)	Liver weight (g)
Control	0	0	0	10.83 ± 0.65**
DEN	4.3±0.37	8.109 ± 0.85	13.67±1.4	16.20 ± 1.412
DEN+RE100	3.3±0.42	7.600 ± 0.82	9.667 ±0.33*	14.56 ± 0.94
DEN+RE400	3±0.33	5.900 ± 0.71	5±0.5***	11.90 ± 0.66*

Figures

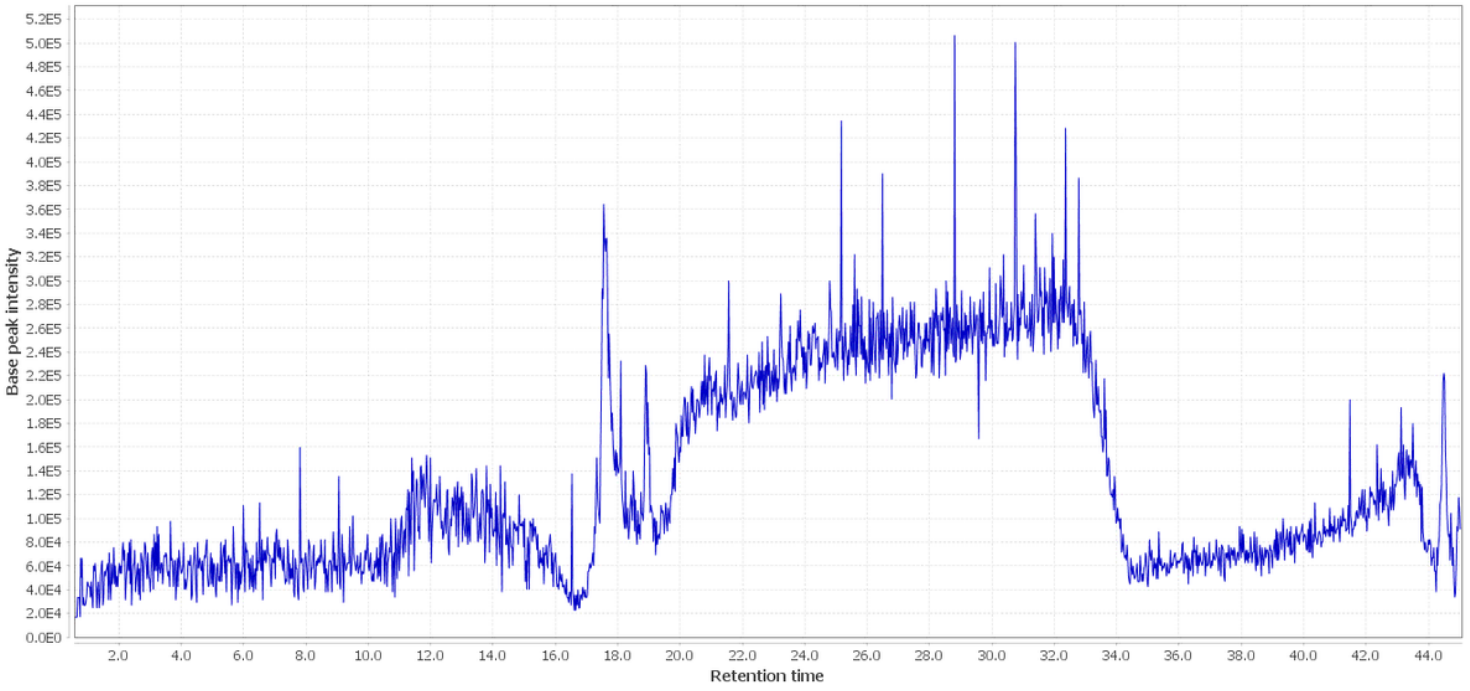

Figure 1

The LC-MS analysis of a hydroethanolic extract of R. turkestanicum root.

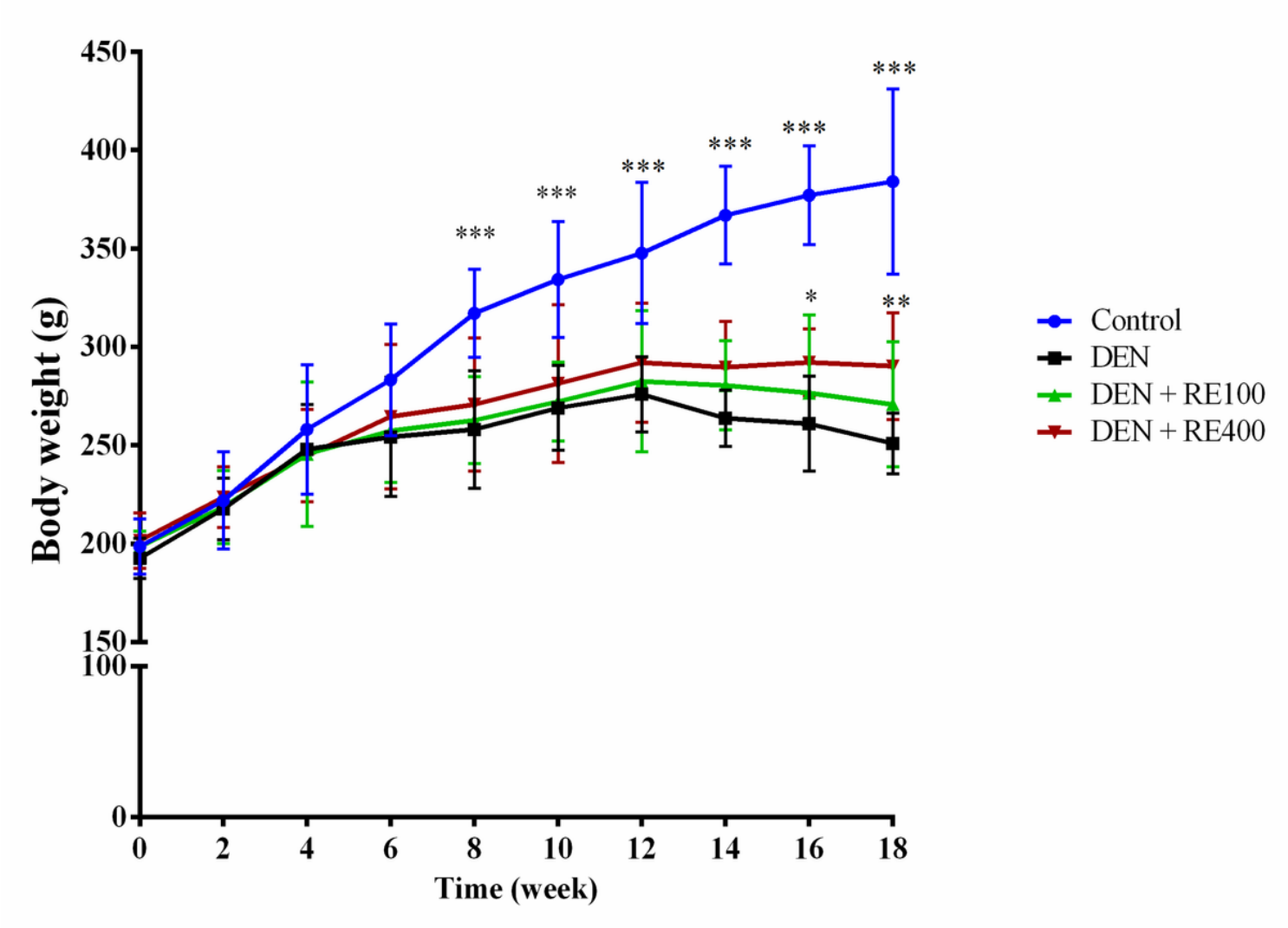

Figure 2

Effect of R. turkestanicum extract (RE) on body weight in HCC rats. The extract was administrated at doses of 100 mg/kg (RE100) and 400 mg/kg (RE400) for 16 weeks in HCC rats (n = 10). The body weight was measured every two weeks. Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001 compared to DEN group

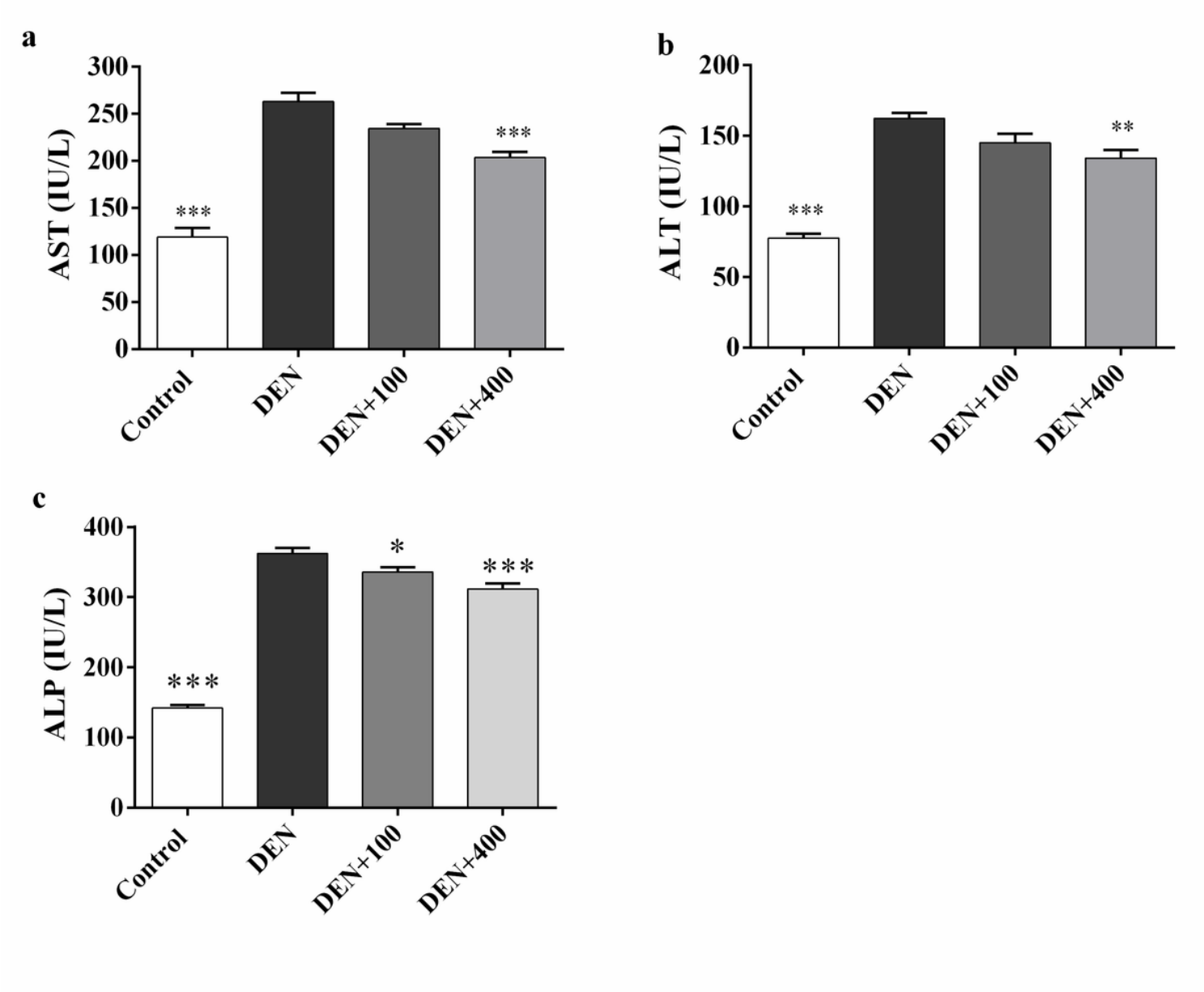

Figure 3

Effect of R. turkestanicum extract (RE) on liver enzymes. The extract was administrated at doses of 100 mg/kg (RE100) and 400 mg/kg (RE400) for 16 weeks (n = 10). The level of liver enzymes was measured in serum after 16 weeks. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to DEN group

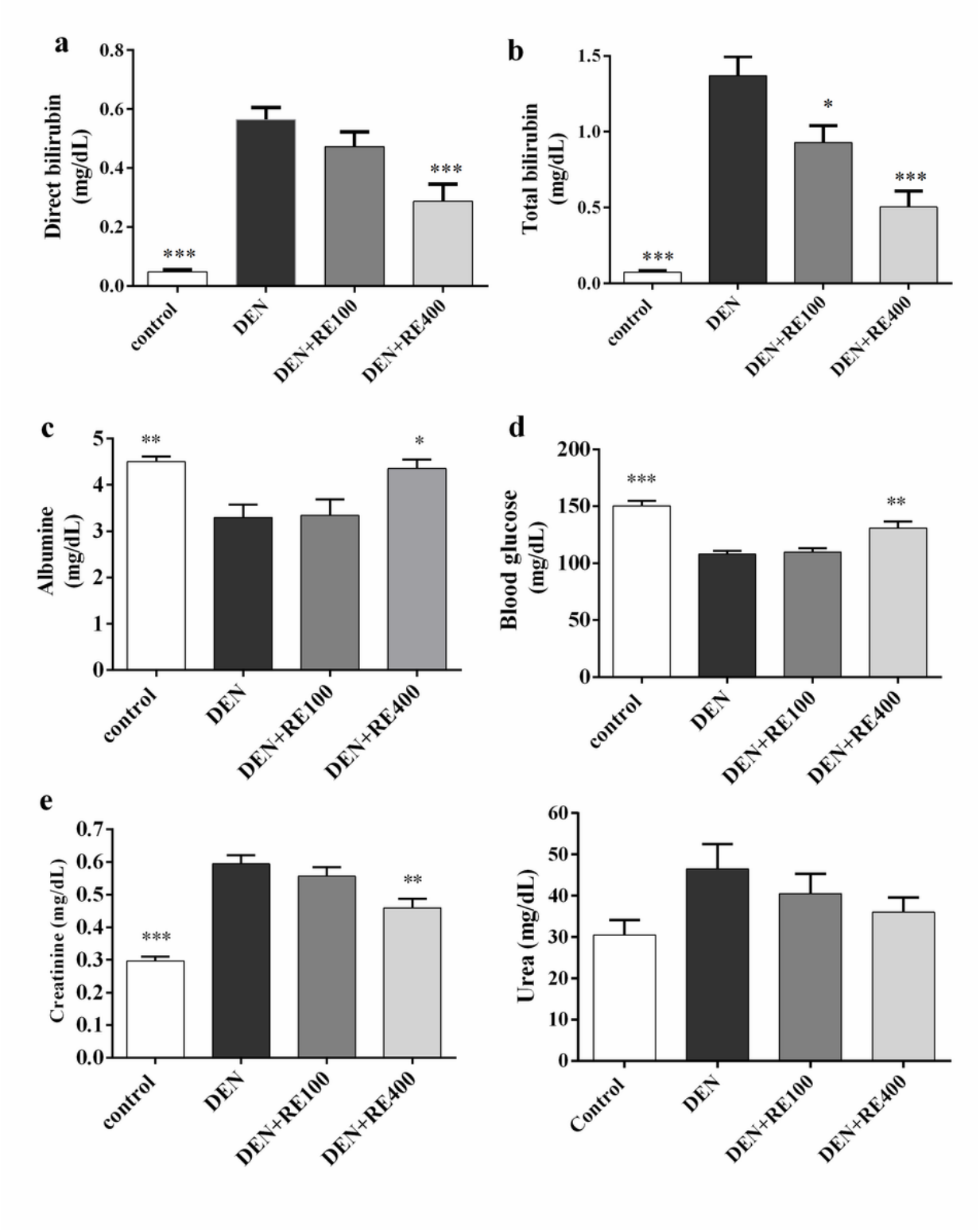

Figure 4

Effect of R. turkestanicum extract (RE) on the serum biochemical factors. The extract was administrated at doses of 100 (RE100) and 400 mg/kg (RE400) for 16 weeks (n = 10). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to DEN group

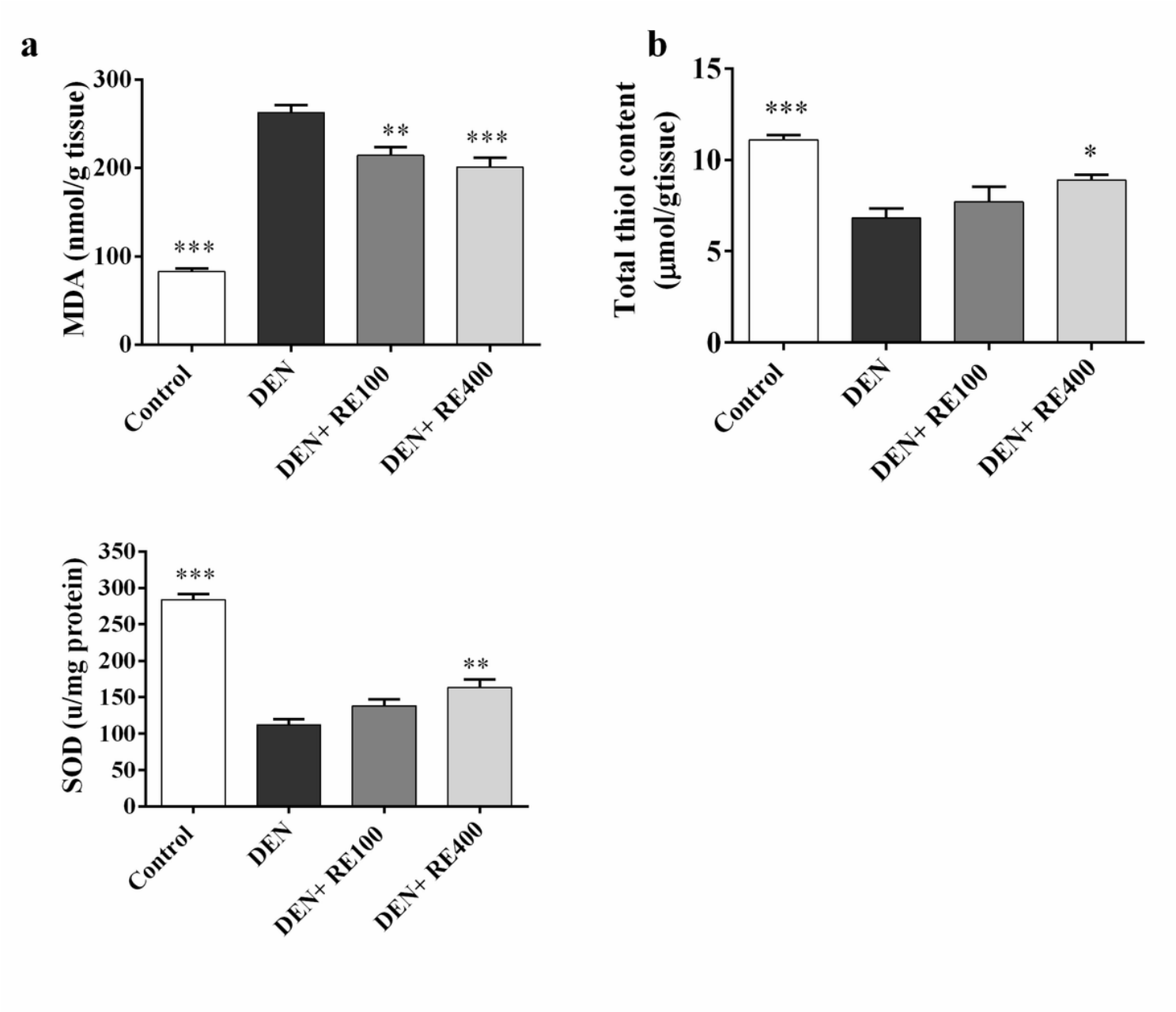

Figure 5

Effect of R. turkestanicum extract (RE) on the level of MDA (a), thiol groups (b), and SOD activity (c) in the liver tissue of DEN-induced HCC rats. The extract was administrated at doses of 100 (RE100) and 400 mg/kg (RE400) for 16 weeks (n = 10). Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to DEN group

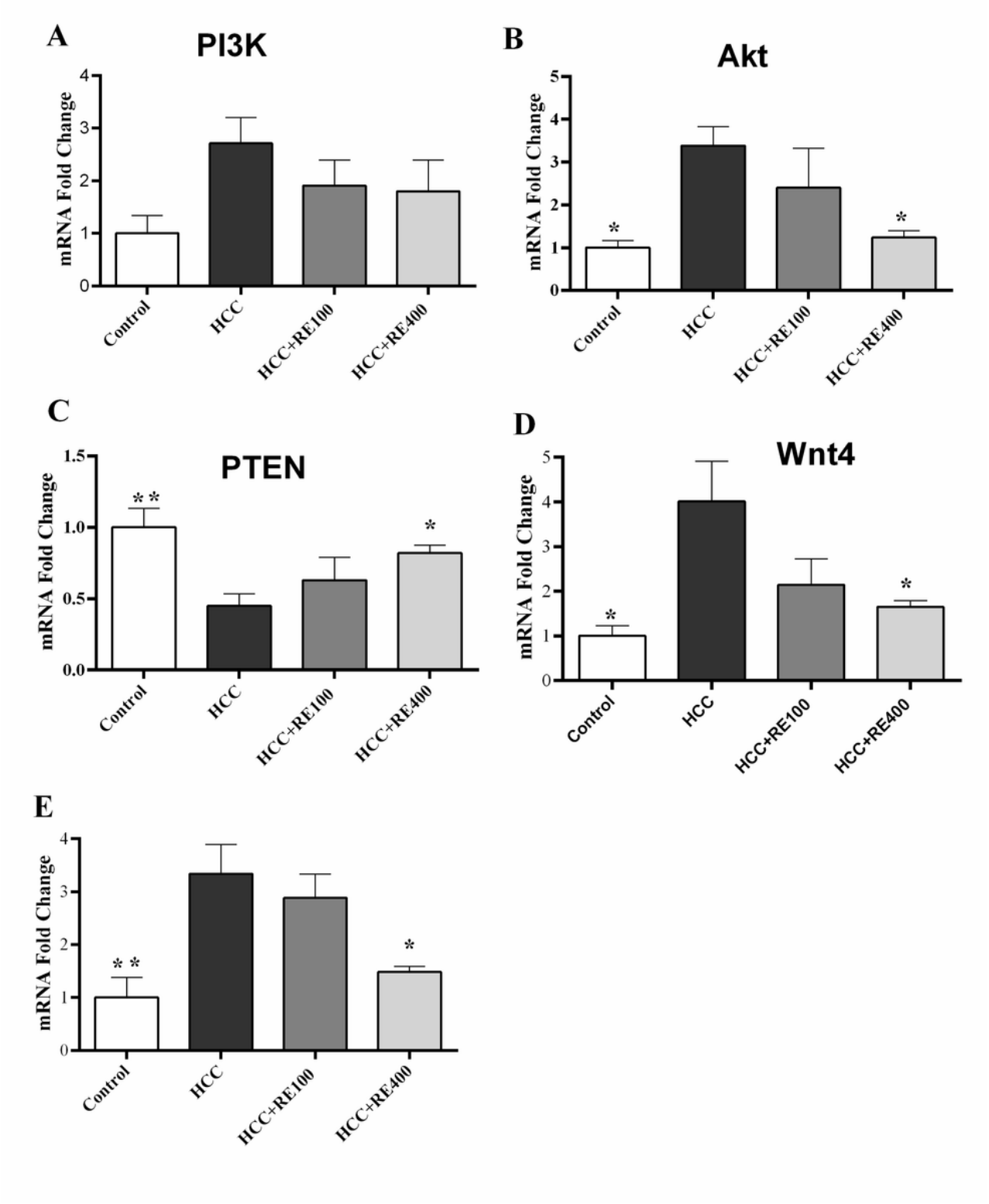

Figure 6

Effect of R. turkestanicum extract (RE) on the expression of PI3K (a), Akt (b), PTEN (c), Wnt4 (d), and β-catenin (e) in the liver of DEN-induced HCC rats. The extract was administrated at doses of 100 (RE100) and 400 mg/kg (RE400) for 16 weeks (n = 10). Data are expressed as mean ± SEM. *p<0.05, **p<0.01 compared to DEN group

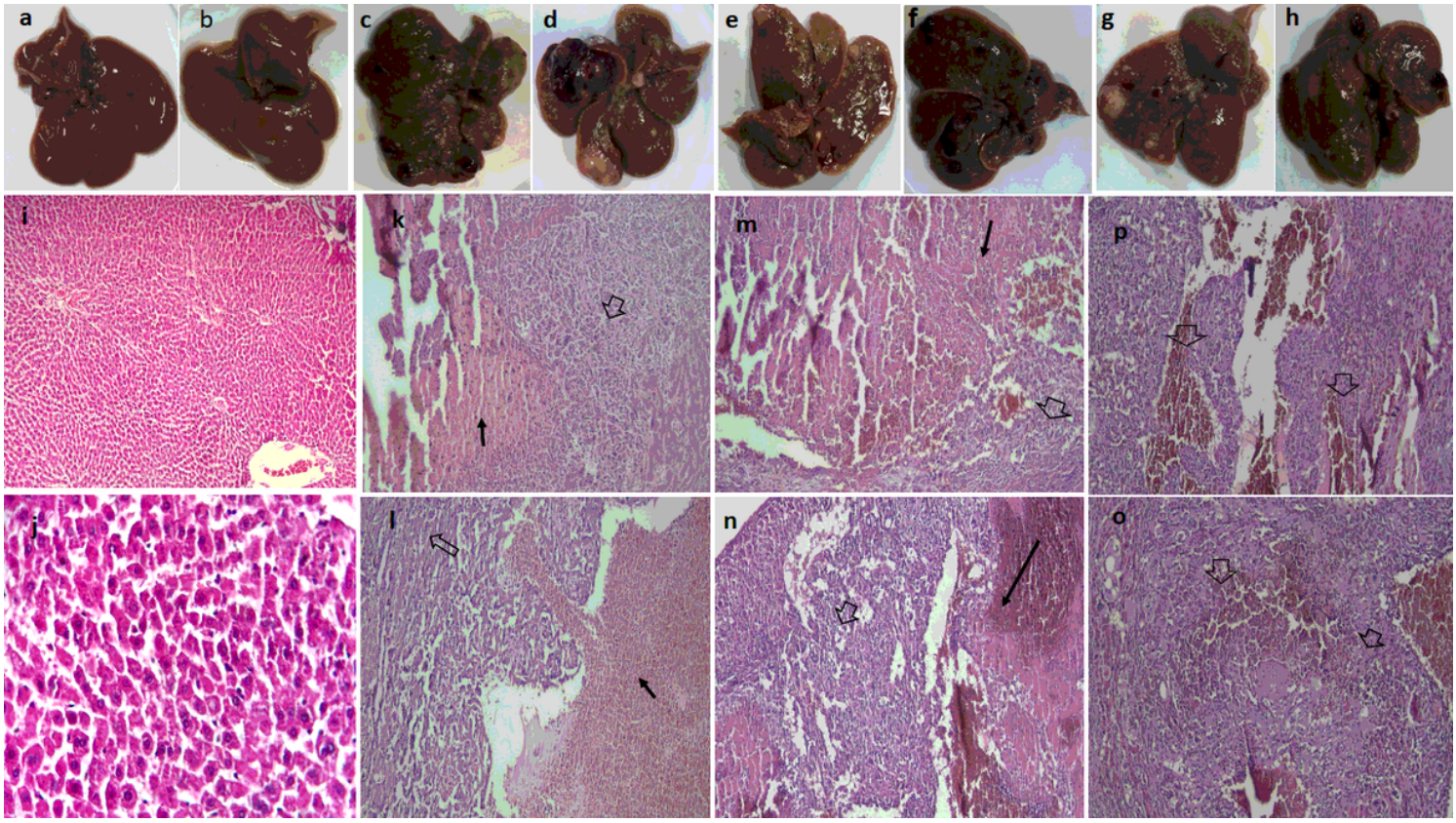

Figure 7

The effects of DEN and R. turkestanicum on the macroscopic and microscopic appearance of the liver. a and b (control) shows normal appearance of the liver; c and d displaying of cirrhosis and macroscopic nodules in DEN-induced HCC; E-H macroscopic changes in groups treated with the extract; i and j (control) showing the normal microscopic architecture of the liver; k and l (HCC) displaying nuclear enlargement, hyperchromasia, and remarkable pleomorphism; m, n, p, and q (100 and 400 mg/kg of the extract) show pleomorphism. The thin and thick arrows show necrosis and tumor sites, respectively. Magnification:100x

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