Research article

Phytochemical identification, acute and subchronic oral toxicity assessments of hydroalcoholic extract of *Acroptilon repens* in BALB/c mice: A toxicological and mechanistic study

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ABSTRACT

Acroptilon repens (L.) DC, commonly known as Rhaponticum repens, is a popular traditional phytomedicine. The current study was conducted to evaluate the acute and subchronic toxicity of the hydroalcoholic extract of this herb with regard to its terpenoid contents in a BALB/c mice model and to investigate the toxicity of this medicinal herb. Identification of extract components of the plant was done using gas chromatography (GC)-mass spectrometry. In order to establish the acute toxicity model, a single dose of 2000 mg/kg of the extract was given orally to male mice and in the subchronic toxicity study, the extract was consecutively administered at doses 250, 500, and 1000 mg/kg for 28 days. After 28 and 42 days, signs of toxicity and mortality were observed. Organ weight changes and the toxicity-associated parameters such as biochemical indicators, oxidative stress indices, mitochondrial parameters, apoptosis-associated gene expression levels, and pro-inflammatory cytokines were evaluated along with the histopathological examination. GC analysis showed that the terpenoids are the major components of the extract. The LD50 value (2 g/kg) was obtained in the acute toxicity assay; the subchronic administration caused a significant elevation in the serum biomarkers as well as in the levels of lipid peroxidation, protein carbonyl, and ROS. Besides, significant reductions in the superoxide dismutase and catalase activities were observed. This toxic effect was further confirmed by histological studies, cytokine assay, and gene expression assays. Following the treatment discontinuation, the abnormalities in the values of biochemical parameters and histopathological changes returned to normal. These findings demonstrate that the subchronic administration of the hydroalcoholic extract of *A. repens* can reversibly cause toxicity by inducing oxidative stress and mitochondrial dysfunction.

1. Introduction

Acroptilon repens is a perennial herbaceous plant belonging to the family Asteraceae [1]. This plant is one of the most invasive weed species that are more competitive than other weedy species in occupying areas [2]. The competitiveness of this plant has been related to its ability to release harmful chemicals with considerable allelopathic activities [3]. Allelopathy is the negative effect of a plant in inhibiting the growth of other plants [4]. *Acroptilon repens* or Rhaponticum repens is widespread in numerous countries, particularly in Mongolia, Iran, Turkey, Armenia, the United States, and Canada [5]. *A. repens* has been used in traditional medicine as an emetic, antiepileptic and anti-malaria around the world

[6]. Recent studies on the *A. repens* have shown it to have pharmacological properties such as antimicrobial [2], antipyretic, lipid-lowering and antidiabetic effects [7]. Previous study also reported that the extract of *A. repens* have strong cytotoxic activities against tumor cell lines P-388 [8] and HL-60 [9]. In few regions, it has been reported that this species causes poisoning and illness in the horse. Especially, following the prolonged ingestion that can eventuate in chewing disease or equine nigropallidal encephalomalacia (ENE) [10]. Previous studies has shown that several sesquiterpene lactones and flavonoids have isolated from *A. repens*, which have significant toxicities [11]. It has been reported that this plant contains two toxic compounds of benzoflavone and acropeltin lactone sesquiterpene [12].

Repin, a kind of sesquiterpene lactones, is the most abundant active ingredient in the extract of *A. repens* and is the most toxic agent among several sesquiterpene lactones which have been known to exert neurotoxic and cytotoxic effects [13]. The main functional groups in repin are characterized by the presence of an a-methylenebutyrolactone moiety and epoxides [14]. Repin, as an extremely reactive electrophile, readily conjugates with endogenous cellular nucleophiles, including macromolecular targets such as proteins, DNA, glutathione, and lipids [15]. Repin has been shown to reduce cell viability and glutathione levels in PC12 cells and cultured neonatal mouse astrocytes as well as to induce oxidative stress in the mouse brain. Repin accelerated the rate of Reactive oxygen species (ROS) generation in PC12 cells (derived from a pheochromocytoma of the rat adrenal medulla) than can explain its mechanism in the pathogenesis of ENE [16]. Moreover, neurotoxic effects on primary cultures of fetal rat brain cells following the exposure to sesquiterpenoids have been reported [17]. Several studies demonstrate that the pathogenesis of many neurodegenerative diseases such as ENE may involve ROS production and consequential mitochondrial dysfunction and energy failure [18]. There is sufficient evidence on the indication of mitochondrial dysfunction and ROS generation during the establishment of these diseases [19]. Despite limited evidence on the mechanism of repin-induced toxicity, there were not any comprehensive experimental and mechanistic studies on the toxicity of *A. repens* with regard to various components existing within its extract. Therefore, the current study attempts to assess the various aspects of the oral acute and subchronic toxicity of *A. repens* and its key component, the repin, on the possible target organs in mice. Furthermore, a wide range of indicators such as biochemical parameters, oxidative stress indices, proinflammatory cytokines, mitochondrial functions, and apoptotic factors as well as the histological status were evaluated to better comprehend various aspects and the mechanics underlying the toxicity of *A. repens* in a murine model.

2. Materials and methods

2.1. Plant material and extraction

Whole parts of the plant were collected from Damghan, Semnan, Iran, during flowering season in July 2020, and a voucher specimen was deposited at the Herbarium of Research Institute of Mazandaran Pharmaceutical Sciences Branch, Sari, Iran.

Shade-dried powdered material weighing 700 g was extracted by the percolation method using methanol. The filtrate obtained through Whatman filter paper was concentrated using a rotary evaporator at 45 °C temperature. The solvent was completely removed to obtain the sticky crude extracts because of the existing wax in it. Then, to remove the wax, methanol was added to sticky crude extracts, and the solution was stored in a -15 °C freezer for 48 h in which the waxes finally accumulated on the top of the solution and were discarded. Finally, powdered material was obtained and used for toxicological tests in the present study.

2.2. Gas chromatography and mass spectrometry (GC–MS)

GC–MS was performed using Hewlett-Packard 5890 GC with an HP5970 MSD system equipped with an HP-5MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm) and interfaced with a Varian ion trap detector. The oven temperature was 45–280 °C at a rate of 5 °C/min; the injection temperature was 280 °C; transfer line temperature was 280 °C; the carrier gas was helium with a linear velocity of 36.3 cm/s; the split ratio was 10:1; the ionization energy, 70 eV; the scan time, 1 s; and the mass range, 40–320 amu.

The percentage of different components of the extract was calculated automatically from the peak area without any correction. Retention indices (RI) of compounds were determined relative to the retention times of a series of n-alkanes with linear interpolation. The components of the extract were identified by comparing their mass spectra with those

from a Wiley GC–MS library or with authentic compounds and then confirmed by comparing the measured retention indices, either with those of authentic compounds or with other literature data.

2.3. Animals

Male BALB/c mice (25–35 g) were used for the study. The animals were housed in standard cages under controlled conditions (25 ± 2 °C and relative humidity of 60 %) and with free access to tap water and a standard pellet diet. All animal experiments were performed according to the ethical principle of the animal handling protocol approved by the local Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (IR.MAZUMS.REC.1397.1763).

2.4. Experimental design

2.4.1. Acute toxicity study

The oral acute toxicity study of hydroalcoholic extract of *A. repens* was performed following the Organisation of Economic Co-operation and Development (OECD) guideline 420 to examine adverse effects of the extract on different vital organs (liver, kidney, heart, and brain) and the biochemical parameters (alanine aminotransferase, alkaline phosphate, creatinine, and blood urea nitrogen (BUN). All the mice were fasted overnight from food prior to dosing with the extract but were allowed free access to water [20].

The initial dose of 300 mg/kg was chosen because no data on *in vivo* and *in vitro* toxicity study was available for the extract. We didn't find any symptoms of toxicity in the preliminary study with a mouse using a dose of 300 mg/kg, so the dose was increased to 2,000 mg/kg. Further preliminary tests showed that no toxicity symptoms were developed at a dosage of 2,000 mg/kg. Moreover, the main test was continued with a dose of 2,000 mg/kg on 4 additional mice. Each animal was monitored for any signs of toxicity and mortality immediately after dosing, at 4 h and at 24 h of intervals, and twice daily for 14 days. Then, the substance will be ranked and categorized according to the Global Harmonized System (GHS) for chemicals with acute toxicity [20].

After overnight fasting, on day 15, the mice were anesthetized using ketamine/xylazine (50 mg/kg and 10 mg/kg, respectively). The mice liver, kidney, heart, and brain were removed, cleaned in normal saline solution, and weighed. The relative organ weight of each animal was calculated by organ weight/body weight [21].

2.4.2. Subchronic toxicity study

A repeat-dose oral toxicity study was conducted in accordance with the OECD guideline 407 [22] for 28 days on the vital organs (liver, kidney, heart, and brain) to evaluate adverse effects and possible toxicity mechanisms of extract. The animals were randomly stratified into five groups of nine animals: The control animals orally received normal saline and the other three test groups received the plant extract at low dose (250 mg/kg), middle dose (500 mg/kg), and high dose (1000 mg/K), respectively. For the satellite group, in order to assess reversibility effects, the extract at the high dose of 1000 mg/kg was given once daily to additional group of mice for 28 days, and kept for another 14 days without treatment. The extract was dissolved in saline solution and administered by gavage once daily for 28 consecutive days. At the end of the experimental period, all animals were anesthetized with ketamine/xylazine (50 and 10 mg/kg, respectively; i.p). Their vital organs (liver, kidney, heart, and brain) were collected, cleaned with saline, and weighed. Relative organ weight was calculated as described earlier.

2.5. Sample collection

2.5.1. Biochemical analysis of blood serum

Animal blood samples were collected in heparinized tubes by cardiac puncture and then centrifuged (300×g, 10 min) to obtain serum. The biochemical parameters including liver enzymes alanine transaminase

(ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP), lipid profiles, Creatinine (Cr), BUN, and glucose levels were measured using standard kits (Pars Azmoon, Tehran, Iran) and an automated hematology analyzer (KX-21N™).

2.5.2. Preparation of liver and brain mitochondria in the subchronic study

Liver and brain tissues were instantly removed by a fine scissor. The tissues were washed and minced in cold mannitol solution (0.225 M D-mannitol, 75 mM sucrose, and 0.2 mM EDTA). The minced liver and brain were gently homogenized in a glass homogenizer, then centrifuged at $1000 \times g$ for 10 min at 4 °C. After removing nuclei and intact cells, the supernatant was centrifuged ($7000 \times g$, 15 min, 4 °C) to precipitate the heavy mitochondrial fraction (packed lower layer). The supernatant was removed and the heavy mitochondrial fraction was resuspended in mannitol solution and recentrifuged twice at $7000 \times g$ for 15 min at 4 °C. Sedimentations of the heavy mitochondrial were suspended in a buffer containing 0.05 M Tris–HCl buffer (pH 7.4), 20 mM KCl, 0.25 M sucrose, 1 mM Na_2HPO_4, and 2 mM MgCl2 at 4 °C before analyses.

2.5.3. Protein concentration

Protein concentrations in the samples were determined using the Bradford method [23]. Then, mitochondrial suspensions were added to a 96-well plate and mixed with Bradford reagent and after 5 min of incubation at room temperature in the dark, optical densities of samples were measured at 595 nm.

2.6. Oxidative stress indices assays in the subchronic study

2.6.1. Measurement of lipid peroxidation in isolated mitochondria

Malondialdehyde (MDA) determination as an index of lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARSs) method. Briefly, 100 μl of the mitochondrial suspension was added to a reaction mixture containing 20% trichloroacetic acid and 0.1 M thiobarbituric acid (TBA), 0.24 N HCl, and then heated for 30 min at 90 °C to complete the reaction. After centrifugation ($10000 \times g$, 5 min), the absorbances of the supernatants were measured at 532 nm using a microplate reader (BioTek, USA).

2.6.2. Measurement of carbonyl proteins in isolated mitochondria

Carbonyl groups as indicators of protein oxidation were examined through reaction with 2, 4-dinitrophenylhydrazine (DNPH) reagent in a colorimetric assay. Isolated liver and brain mitochondria were incubated in the presence of 10 mM 2,4-Dinitrophenylhydrazine (DNPH) in 2 M hydrochloric acid (HCl) at room temperature for 60 min and 20% trichloroacetic acid was added to the mixture. After centrifugation ($1500 \times g$, 5 min), the supernatant was removed and the sediment was washed with ethanol-ethyl acetate (1:1 v/v) to dissolve it in a buffer consisted up of guanidine hydrochloride (6 M) and potassium phosphate (20 mM). The absorbances were measured at 370 nm.

2.6.3. Reactive oxygen species (ROS) in isolated mitochondria

Determination of the mitochondrial ROS was performed using the fluorescent probe DCFH-DA. Briefly, isolated mitochondria were resuspended into a buffer containing 125 mM sucrose, 10 mM HEPES, 65 mM KCl, 20 μM Ca^{2+}, and 5 mM sodium succinate (pH = 7.2). DCFH-DA was added (final concentration, 10 mM) to mitochondria and incubated at 37 °C for 30 min. The fluorescence intensities of samples were examined using a fluorescent microplate reader with an excitation at 485 nm and emission at 525 nm.

2.6.4. Antioxidant enzymes assay

The activity of catalase (CAT) and superoxide dismutase (SOD) in isolated liver and brain mitochondria was measured using CAT and SOD assay kit (ZellBio GmbH, Germany) according to the manufacturer's instruction.

2.7. Mitochondrial function assays in the subchronic study

2.7.1. MTT assay

The quantitative colorimetric method for determination of mitochondrial succinate dehydrogenases activity by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was performed in isolated mice liver and brain mitochondria. The mitochondrial suspension was incubated with 0.4% of MTT at 37 °C for 30 min. Formazan crystals were solubilized in dimethyl sulfoxide (DMSO) and the optical densities were measured at 570 nm using a microplate reader (BioTek, USA).

2.7.2. Mitochondrial membrane potential (MMP) assay

Rhodamine 123, a cationic fluorescent dye, is commonly used to monitor MMP changes. The mitochondrial fractions were incubated with a reaction mixture containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 20 μM Ca^{2+} (pH 7.2) at 37 °C for 30 min. The reaction was initiated by adding 10 μM of rhodamine 123 and the fluorescence intensities were measured with excitation and emission wavelengths of 507 nm and 527 nm, respectively.

2.7.3. The mitochondrial permeability transition pore (MPTP) assay

MPTP is a transmembrane protein in the inner membrane of the mitochondria that could be stimulated by mitochondrial matrix Ca^{2+} accumulation. The opening of MPTPs was validated by adding 250 μM Ca^{2+} to the isolated mitochondria and observation of mitochondrial swelling. The isolated mitochondria were suspended in a swelling buffer consisted up of 125 mM sucrose, 65 mM KCl, 10 mM HEPES, and 1 mM $MgCl_2$

2.8. Quantitative real-time PCR analysis in the subchronic study

Total RNA was extracted from liver tissues using an RNA purification kit according to the manufacturer's instructions. RNA concentration and purity were measured using the spectrophotometer with ratios of absorbance at 280/260 nm. First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA by reverse transcription kit according to the manufacturer's guidelines (Thermo Fisher, USA). Quantitative real-time PCR used a Rotor-Gene Q cycler with an SYBR green kit (TaKaRa, Japan) and specific forward and reverse primers. PCR mixture (25 μl) included 1 μl of each primer (10 pmol/μl), 2 μl of cDNA, 12.5 μl of $2\times$ SYBR Green PCR Master Mix, and 8.5 μl of RNase-free water. The mRNA relative abundance was determined according to the delta-delta Ct ($\Delta\Delta$Ct) method and the expressions of the studied genes were compared to the expression of Glyceraldehyde-3-phosphate

Table 1. Primer sequences for apoptosis-related genes.

Gene name	Description	Primer sequences	Product size (bp)
GAPDH	F	5′-TGACGTGCCGCCTGGAGAAAC-3′	210
	R	5′-CCGGCATCGAAGGTGGAAGAG-3′	
Bcl-2	F	5′-TTGACGCTCTCCACACACATG-3′	150
	R	5′-GGTGGAGGAACTCTTCAG GGA-3′	
p53	F	5′-CTACTAAGGTCGTGAGACGCTGCC-3′	120
	R	5′-TCAGCATACAGGTTTCCTTCCACC-3′	
Bax	F	5′-TATGGGACACCAGGAGGGTA-3′	166
	R	5′-CGTAGACCTTGCGGATAAAGC-3′	
Caspase-3	F	5′-CTGAAGGCTCCTGGTTTA-3′	104
	R	5′-CTCGATCTTGCGGTCCCTC-3′	

F: indicates forward primer; R: indicates reverse primer; P53: Tumor protein P53; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; Caspase-3: cysteine-aspartic proteases-3; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

dehydrogenase (GAPDH). Table 1 shows the primer sequences used for real-time PCR analyses.

2.9. Pro-inflammatory cytokines assay in the subchronic study

The levels of TNF-a, IL-1b, and IL-6 in supernatants of the liver and brain tissues were determined following the tissue dissociation using mouse ELISA kits (eBioscience, USA) according to the manufacturer's instructions.

2.10. Histopathological examination in the subchronic study

The liver and brain tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and cut in 5 μm thickness for hematoxylin-eosin (H&E) staining. The prepared slides were observed for histopathological alterations by an expert pathologist using a light microscope.

2.11. Statistical analysis

All data were analyzed with GraphPad software and expressed as mean ± SD. Comparisons among multiple groups were done using one-way ANOVA and Tukey's *posthoc* comparison. Differences were considered statistically significant when *P*-value was below 0.05.

3. Results

3.1. Percentages of constituents isolated from the extract of A. repens

Table 2 exhibits the constituents of the extract of *A. repens* with their percentages. Twenty-one components (representing 97.57% of the extract) were identified. Some sesquiterpenes compounds found extract including Caryophyllene oxide (25.67 %), Cholest-5-en-3-ol (3. beta.) (19.81%), 1-Tetradecanol (10.05 %), n-Hexadecanoic acid (5.87 %), Z-3-Hexadecen-7-yne (4.24 %), Spiro[4.4]nonan-2-one (3.62 %),

Alloaromadendrene oxide (3.39 %), and (+) spathulenol (3.39%) were also determined (Figure 1).

3.2. Acute toxicity evaluation

There were no signs of toxicity and mortality in the acute toxicity studies within 72 h–14 days after extract administration. The lethal dose, 50% (LD50) of this extract was estimated to be above 2000 mg/kg. The data indicated no statistically significant differences between control and extract-treated mice in the amount of food and water consumed.

Furthermore, the results showed that an increase in body weight of the treatment group was not significantly different compared to the control group. This study represented that administration of hydro-alcoholic extract of *A. repens* at a dose of 2000 mg/kg had no effect on body weight in mice. Also, significant changes in weights and relative weight of organs did not observe in each mice at a dose of 2000 mg/kg for 14 days (Table 3).

3.3. Subchronic toxicity evaluation

The results demonstrated that daily oral administration of hydro-alcoholic extract of *A. repens* (250, 500, and 1000 mg/kg) for 28 days did not cause any mortality in mice. The amount of food and water intake and changes in body weight on male mice indicated no significant differences between the treatment group and the control group during the treatment period.

Moreover, there was no significant difference observed between the treatment and the control group in all mice organ weight and relative organ, including the heart, liver, kidneys, and brain at each dose level, although an increase in the organ weight and the relative organ was observed. The satellite group showed no significant changes in the amount of food and water and body weight (Table 3).

3.4. Serum biochemical indices analysis

The effects of hydroalcoholic extract of *A. repens* on the biochemical parameters in the acute and subchronic studies are presented in Table 4. In an acute study, the results showed that the extract administration made no statistically significant changes on the biochemical parameters compared to the control, while the subchronic study showed significant changes in the biochemical parameters.

In a subchronic study, Animals were treated with the extract at 250, 500, and 1000 mg/kg doses for 28 days, and several biochemical factors including ALT, AST, ALP, HDL, LDL, TG, Cr, BUN, and glucose were measured. As represented in Table 3, serum ALT, AST, and ALP levels showed a significant increase in 500 and 1000 mg/kg extract received groups compared to the control group (P < 0.01 and P < 0.001, respectively). Besides, 500 and 1000 mg/kg extracts resulted in a significant decrease in the levels of LDL and TG along with a remarkable increase in HDL level (P < 0.05 and P < 0.01, respectively), as shown in Table 4.

As shown in the satellite group, extract at a dose of 1000 mg/kg caused an increase in ALT, AST, and ALP levels. However, this effect was not significant and returned to normal after treatment stopped. According to these results, the effect of hydroalcoholic the extract of *A. repens* on biochemical parameters was reversible (Table 4).

3.5. Histopathological analysis of organs

Histopathological changes in the tissues of mice treated with *A. repens* extract in the subchronic study were evaluated by H&E staining. Histopathology evaluation was observed at the end of the experiment over 28 days and 42 days. Liver, kidney, heart and brain of mice were observed. The evaluation of histopathology of kidney, heart and brain in the control, treatment and even satellite groups showed normal tissue structure at all doses of extract (Figure 2a).
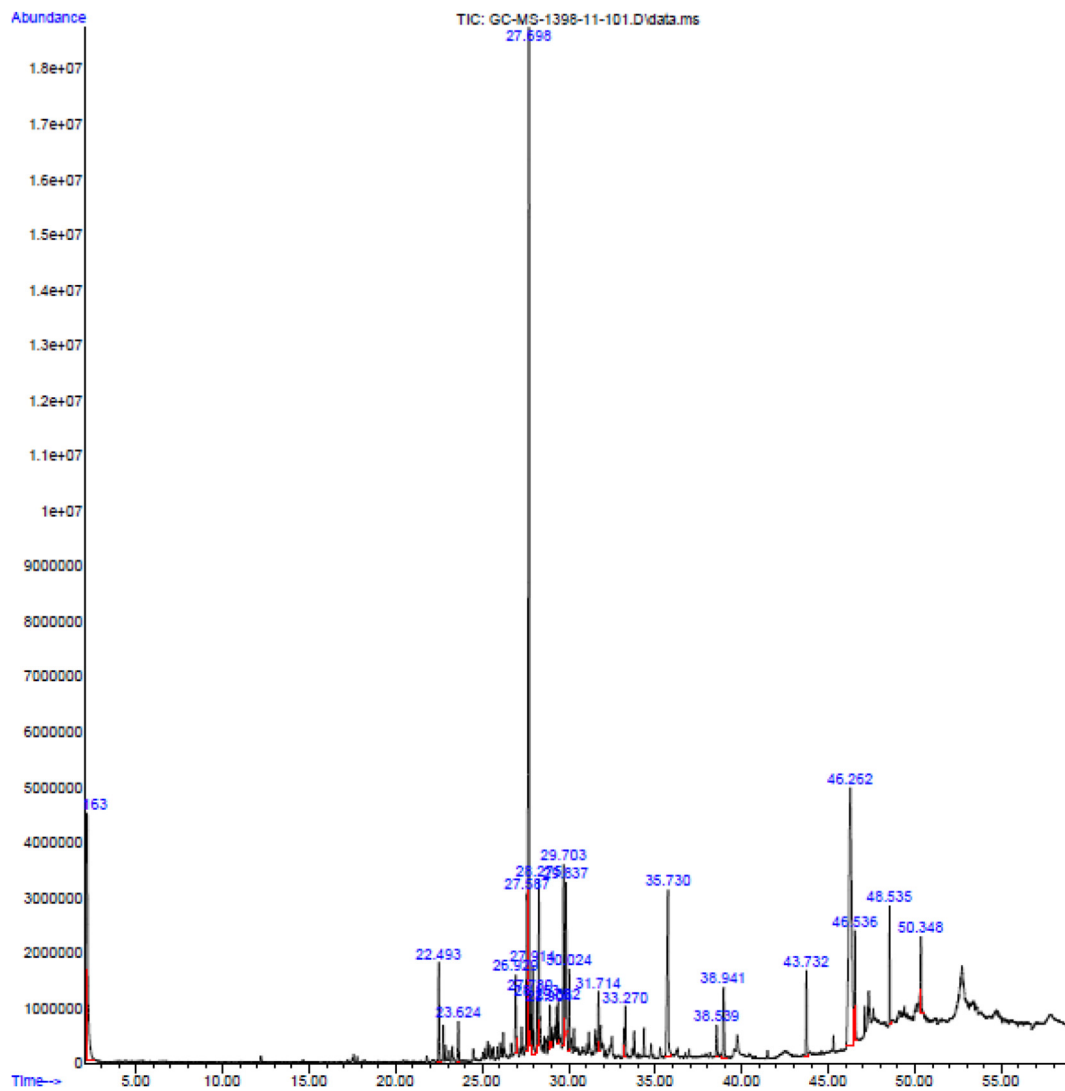
Table 2. Percentage composition of the extract of whole parts of Acroptilon *repens* L.

Compound	RT[a]	RI[b]	%
1-Tetradecanol	2.16	1365	10.05
Alloaromadendrene oxide(1)	22.49	1216	3.39
(-)-Caryophyllene oxide	23.62	1213	1.76
(+) spathulenol	27.58	1450	3.18
Caryophyllene oxide	27.69	1450	25.67
Spiro[4.5]decan-7-one1,8-dimethyl-8,9-epoxy-4-isopropyl	27.82	1450	1.02
salvial-4(14)-en-1-one	27.91	1450	2.50
Cyclohexane,1,5-diethenyl	28.15	3319	1.09
Spiro[4.4]nonan-2-one	28.27	3387	3.62
caryophylla-4 dien-5α-ol	28.90	1450	0.97
caryophylla-3dien-5.β-ol	29.38	1450	0.86
Z-3-Hexadecen-7-yne	29.70	1454	4.24
4-Oxo-α-ylangene	29.83	1419	3.02
Aristol-9-en-3-ol	30.02	1450	1.74
2-propen-1-ol	31.71	8706	1.16
2-Pentadecanone	33.27	2113	0.89
n-Hexadecanoic acid	35.73	1954	5.87
Heneicosane	38.53	2457	0.82
Phytol	38.94	2456	2.08
Eicosane	43.73	2289	1.79
Cholest-5-en-3-ol (3.beta.)	46.26	3244	19.81
Pentacosane	46.53	3005	2.04

[a] Retention time.
[b] Retention indices on DB-5.

Figure 1. Gas chromatographic profile of Acroptilon *repens*.

Table 3. Organ weights, relative organ weights, Bodyweight, body weight gain of mice treated orally with hydroalcoholic extract of *A. repens* for 14 and 28 days.

Parameters	Acute toxicity (mg/kg BW)		Subchronic toxicity (mg/kg BW)				Satellite
	Control	2000	Control	250	500	1000	
Initial weight (g)	28.142 ± 0.144	29.158 ± 1.809	25.121 ± 0.818	25.166 ± 2.446	25.132 ± 0.556	26.444 ± 3.288	25.113 ± 1.316
Final weight (g)	30.426 ± 0.712	33.743 ± 2.092	32.761 ± 1.554	32.001 ± 3.809	32.761 ± 0.405	34.832 ± 3.118	33.412 ± 2.12
BWG (g)	2.284 ± 0.614	4.585 ± 1.81	7.64 ± 1.086	6.835 ± 2.912	7.629 ± 0.58	8.388 ± 3.12	8.299 ± 2.04
Food intake(g)	24.295 ± 1.703	25.007 ± 1.554	46.088 ± 3.149	43.750 ± 4.263	45.332 ± 3.50	45.448 ± 3.167	44.842 ± 2.29
Water intake(ml)	33.117 ± 3.019	36.193 ± 3.66	62.341 ± 2.416	61.513 ± 2.322	61.166 ± 1.429	63.148 ± 2.114	62.514 ± 3.237
Organ weights							
Liver (g)	1.446 ± 0.04	1.523 ± 0.001	1.386 ± 0.027	1.418 ± 0.04	1.287 ± 0.03	1.644 ± 0.008	1.545 ± 0.023
Liver (%)	5.138 ± 0.0127	4.51 ± 0.089	4.230 ± 1.41	4.431 ± 0.141	3.928 ± 0.18	4.719 ± 0.089	4.624 ± 1.084
Kidney (g)	0.193 ± 0.003	0.202 ± 0.0019	0.243 ± 0.001	0.238 ± 0.0034	0.250 ± 0.017	0.261 ± 0.019	0.249 ± 0.012
Kidney (%)	0.634 ± 0.036	0.598 ± 0.13	0.741 ± 0.056	0.743 ± 0.05	0.763 ± 0.091	0.749 ± 0.027	0.736 ± 0.056
Heart (g)	0.146 ± 0.011	0.145 ± 0.009	0.187 ± 0.023	0.190 ± 0.07	0.188 ± 0.01	0.202 ± 0.01	0.194 ± 0.04
Heart (%)	0.479 ± 0.0046	0.476 ± 0.066	0.570 ± 0.014	0.593 ± 0.063	0.587 ± 0.075	0.579 ± 0.055	0.58 ± 0.032
Brain (g)	0.224 ± 0.048	0.219 ± 0.005	0.228 ± 0.048	0.230 ± 0.018	0.228 ± 0.02	0.237 ± 0.064	0.232 ± 0.085
Brain (%)	0.739 ± 0.183	0.649 ± 0.227	0.695 ± 0.057	0.718 ± 0.194	0.695 ± 0.144	0.668 ± 0.340	0.694 ± 0.248

Though *A. repens* treatment could not induce remarkable alterations in kidney, heart and brain histology, liver tissues of *A. repens* extract-treated animals showed some alterations including some degrees of damage in the liver structure, multifocal necrosis, apoptosis, hemorrhage, and edema in tissue histology compared to in the control group (Figure 2b and Table 5), While in the satellite group was not seen the

Table 4. The changes of serum biochemical parameters after intragastric administration of *A. repens* extract for consecutive 14 and 28 days in mice.

Parameters	Acute toxicity treatment (mg/kg BW)		Subchronic toxicity treatment (mg/kg BW)				Satellite
	Control	2000	Control	250	500	1000	
ALP(U/L)	139.14 ± 12.63	147.63 ± 5.16	138.67 ± 10.56	143.01 ± 8.32	201.18 ± 17.14[b]	393 ± 37.51[c]	148.02 ± 17.24
ALT(U/L)	50.34 ± 4.33	54.4 ± 2.19	51 ± 6.01	54 ± 10.67	108.63 ± 20.29[b]	223.34 ± 21.71[c]	73.42 ± 8.35
AST(U/L)	68.25 ± 14.12	73.14 ± 10.72	66.34 ± 17.09	73.12 ± 7.69	180 ± 28.23[b]	327.38 ± 9.45[c]	82.16 ± 13.22
HDL	16.13 ± 12.2	16.77 ± 4.10	15.31 ± 4.163	23.55 ± 8.01	28.19 ± 16	71 ± 8.71[b]	17 ± 6.29
LDL	120.32 ± 14.12	121.42 ± 8.36	120.67 ± 21.54	108.32 ± 11.82	63.1 ± 24.19[a]	29.33 ± 8.62[b]	116.02 ± 4.73
TG	16.36 ± 4.22	19.74 ± 3.35	15.54 ± 9.53	18.01 ± 15.66	31 ± 18.39[a]	57.4 ± 13.31[b]	18.4 ± 6.29
FBS	118 ± 3.14	121 ± 2.35	119 ± 2.64	118 ± 10.12	121 ± 8	116 ± 26.9	106.45 ± 14.7
Cr (mg/dL)	0.30 ± 0.04	0.29 ± 0.17	0.29 ± 0.12	0.32 ± 0.16	0.32 ± 0.13	0.31 ± 0.01	0.30 ± 0.04
BUN (mg/dL)	19.37 ± 1.94	20.55 ± 0.72	20.48 ± 1.34	21.56 ± 2.18	21.33 ± 1.21	22.34 ± 1.55	22.62 ± 2.19

Data are expressed as Means ± SD of nine animals in each group. Pluses indicate significant differences.
[a] Compared to the control group, $p < 0.05$.
[b] Compared to control group, $p < 0.01$.
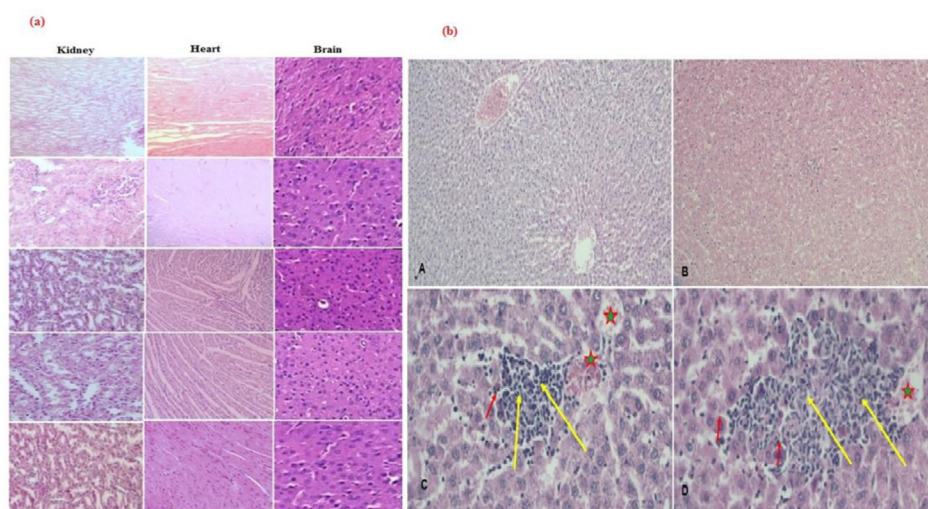[c] Compared to control group, $p < 0.001$.

Figure 2. Histopathological finding of the liver, kidney, heart and brain stained with hematoxylin and eosin, (H & E) in sub-chronic study. in normal control, *A. repens* extract (250, 500 and 1000 mg/kg/day) and satellite groups without any tissue damage (with magnification *100) in kidney, heart and brain tissues (a). Histopathological appearance of liver tissues in (A with magnification *100) normal control without any tissue damage; (B with magnification *100) liver section from *A. repens* extract (250 mg/kg/day) showing normal liver structure; (C and D with magnification *400) treatment with doses of 500 and 1000 mg/kg/day of *A. repens* extract showing multifocal hepatic necrosis, apoptosis, hemorrhage and edema. Necrosis showed by large arrow, apoptosis showed by small arrow and hemorrhage showed by stars (b).

Table 5. Grading of the Histopathological changes in the mice liver after exposure with orally hydroalcoholic extract of *A. repens* for 28 days.

Histopathological findings	Control	Extract (250 mg/kg)	Extract (500 mg/kg)	Extract (1000 mg/kg)
Multifocal necrosis	0	0	+++	+++
Apoptotic body	0	0	+	++
Hemorrhage	0	0	++	+
Edema	0	0	+	++

hepatic lesions including edema, hemorrhage and apoptotic bodies in mice treated with 1000 mg/kg these result suggestion that *A. repens* extract had a reversible effect on microscopic examination.

In the continuation of the study in order to explore the subchronic toxicity mechanism of the *A. repens* extract, liver and brain tissues were selected and the parameters of oxidative stress, mitochondrial function, inflammatory biomarkers, and cell death in tissues were evaluated.

3.6. Effects of A. repens on oxidative stress indices of liver and brain

Oxidative damage is a possible mechanism for extract-induced hepatotoxicity and neurotoxicity. Hence, the content of oxide products resulting from oxidative damage was assessed in liver and brain

mitochondria. Assessment of lipid peroxidation and formation of protein carbonyl showed that uptake of both 500 and 1000 mg/kg extract significantly increased the content of Malondialdehyde (MDA) (33% and 66.9%, respectively) and protein carbonyl (164.51% and 306.25%, respectively) in mitochondria isolated from liver compared to control group (Figures 3 and 4; P < 0.05, P < 0.01 and P < 0.001, respectively).
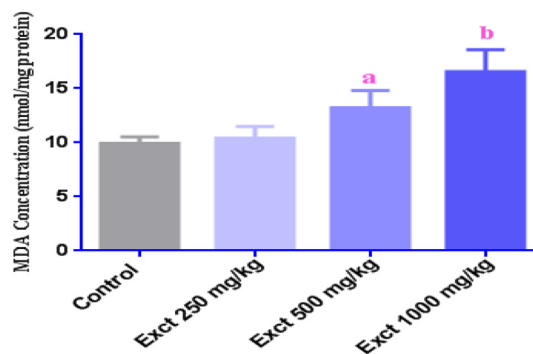

Figure 3. Effect of Acroptilon *repens* extracts on the levels of MDA in mice liver mitochondria. Data are expressed as Means ± SD of nine animals in each group. [a] ($p < 0.05$), [b] (p < 0.01), significantly different as compared to the control group.
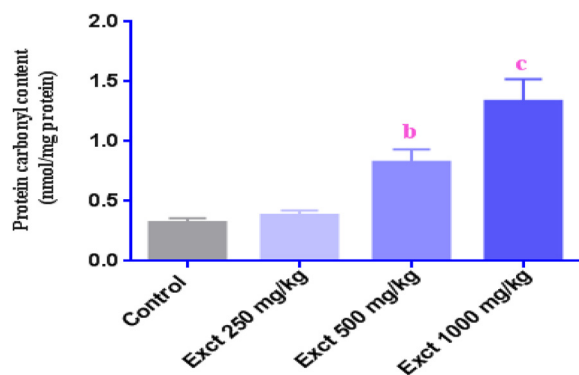
Figure 4. Effect of Acroptilon *repens* extract on the levels of protein carbonyl formation in mice liver mitochondria. Data are expressed as Means ± SD of nine animals in each group. [b] ($p < 0.01$), [c] ($p < 0.001$), significantly different as compared to control group.

Figure 6. Effect of Acroptilon *repens* extract on the levels of protein carbonyl formation in mice brain mitochondria. Data are expressed as Means ± SD of nine animals in each group. [b] ($p < 0.01$) significantly different as compared to control group.

Administration of 1000 mg/kg extract caused a significant increase (30.27%) in the mitochondrial TBARS and protein carbonyl content (133.33%) in the brain compared to the control group (Figures 5 and 6; P < 0.05 and P < 0.01) in the subchronic toxicity.

The data showed a significant increase in ROS levels in mice treated with 500 and 1000 mg/kg extract (P < 0.01 and P < 0.001, respectively). Moreover, 500 and 1000 mg/kg doses caused a significant accumulation of ROS in brain mitochondria (P < 0.05 and P < 0.01, respectively; Figure 7a,b). Oral subchronic administration of extract caused a notable decrease in the activities of antioxidant superoxide dismutase (SOD) and catalase (CAT) enzymes in liver and brain tissues (Table 6).

3.7. Effects of A. repens extract on mitochondrial succinate dehydrogenase activity

The activity of succinate dehydrogenase (SDH) enzyme in liver and brain mitochondria was assessed by MTT assay. Our data revealed that the subchronic extract treatments in 250, 500, and 1000 mg/kg doses resulted in a significant decrease in SDH enzyme activity in the liver (P < 0.05, P < 0.01, and P < 0.001, respectively; Figure 8a). Succinate dehydrogenase activities of brain tissues were significantly reduced in mice treated with 500 and 1000 mg/kg of extract compared to the control group (Figure 8b; P < 0.05 and P < 0.01, respectively).

3.8. Effect of A. repens extract on mitochondrial membrane potential

As shown in Figure 9a,b, a decrease in mitochondrial membrane potential was documented in the case of subchronic extract administration (P < 0.01 and P < 0.001). This decrease was more evident in mice
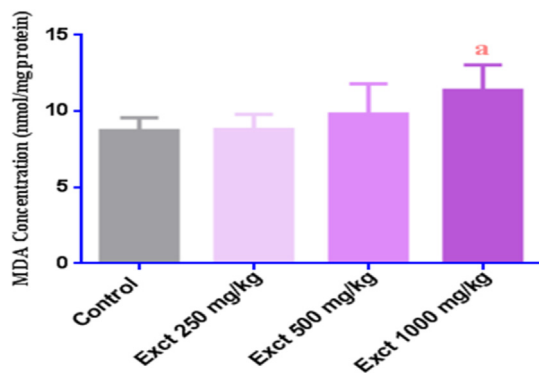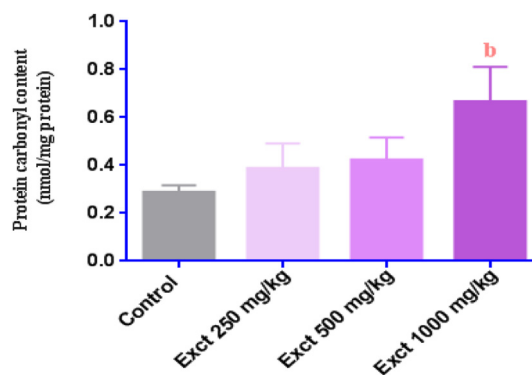

Figure 5. Effect of Acroptilon *repens* extracts on the levels of MDA in mice brain mitochondria. Data are expressed as Means ± SD of nine animals in each group. [a] ($p < 0.05$) is significantly different as compared to the control group.

treated with 500 and 1000 mg/kg doses (21% and 33%, respectively). Also, 1000 mg/kg caused a significant (P < 0.01) decrease in the mitochondrial membrane potential to 23% in mice brains when compared with the control group.

3.9. Effect of A. repens extract on mitochondrial permeability transition (MPTP)

The permeability of the inner mitochondrial membrane was monitored by the swelling test. There were remarkable changes in the MPTP of *A. repens* extract-treated groups in the liver and brain (Figure 10a,b). The mitochondrial swelling was more obvious in liver mitochondria in the subchronic toxicity.

3.10. A. repens extract-mediated apoptotic effects in hepatic tissue

Effects of the studied extract on the hepatocyte cell death were evaluated by examining the apoptosis-related gene expressions using quantitative real-time polymerase chain reaction (qRT-PCR). The mRNA expression of liver apoptosis proteins and anti-apoptotic proteins was detected among the groups in the subchronic toxicity.

The gene expression of P53, a key regulator of apoptosis, shows a significant increase in hepatic tissue of mice treated with extract at 500 mg/kg and 1000 mg/kg dosages (P < 0.05 and P < 0.001, respectively). Treatment groups of hydroalcoholic extract significantly decreased mRNA expression levels of B-cell lymphoma 2 (Bcl-2) at 250 and 500 mg/kg doses (P < 0.05 and P < 0.001, respectively).

There was a significant difference between 1000 mg/kg extract groups and the control group in the expression of Caspase-3 and Bax genes (Figure 11). The gene expressions of Caspase 3 and Bax were significantly increased in 1000 mg/kg extract groups compared with the control group (P < 0.001). However, no significant differences of Caspase 3 and Bcl-2 Associated X-protein (BAX) expression were found between 250 and 500 mg/kg extract-treated groups and the control group (Figure 11). There were no significant differences between the groups in the expression patterns of pro-apoptotic and anti-apoptotic genes in the brain tissues of the treated mice.

3.11. Effect of A. repens extract on cytokines in liver and brain

Pro-inflammatory cytokines are involved in the initiation and progression of inflammatory cascade during tissue damage events. Figure 12 shows that the levels of liver TNF-α were significantly increased in the mice receiving 1000 mg/kg extract (P < 0.01) in the subchronic study. There was also a significant increase in the levels of IL-1β and IL-6 in liver tissues of mice treated with 500 and 1000 mg/kg doses of extract (P <
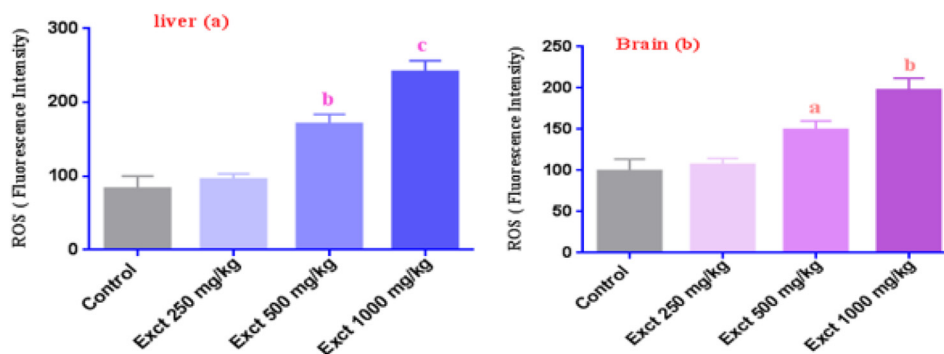
Figure 7. Effect of Acroptilon *repens* extract on the levels of reactive oxygen species (ROS) formation in (a) liver and (b) brain mitochondria. Data are expressed as Means ± SD of nine animals in each group. [b] ($p < 0.01$), [c] ($p < 0.001$), significantly different as compared to control group.

Table 6. Effect of *A. repens* extract on the levels of antioxidant enzyme in the subchronic study.

Groups	Liver		Brain	
	CAT (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)
Control	9.87 ± 0.35	66.87 ± 5.93	9.64 ± 0.58	68.32 ± 4.26
Exct 250 mg/kg	9.22 ± 0.61	58.67 ± 3.43[a]	9.51 ± 1.14	64.25 ± 5.19
Exct 500 mg/kg	8.38 ± 0.22[a]	51.38 ± 6.30[b]	8.60 ± 0.36	58.86 ± 2.14[b]
Exct 1000 mg/kg	6.91 ± 1.57[c]	45.41 ± 7.34[c]	8.14 ± 1.28[a]	53.55 ± 3.81[c]

Data are expressed as Means ± SD of nine animals in each group.
[a] Compared to control group, $p < 0.05$.
[b] Compared to control group, $p < 0.01$.
[c] Compared to control group, $p < 0.001$.

0.05 and P < 0.01, respectively). However, no significant changes were observed in levels of pro-inflammatory cytokine in brain tissue.

4. Discussion

Toxicological evaluations of medicinal plants are necessary to examine the possible toxicities of such plants and to assure the safety of the candidate herbs. Though it is extensively used in the traditional treatment of various ailments including diabetes, there is little experimental data on the safety of *A. repens* extract. The current study was carried out to explore the acute and subchronic toxicities of *A. repens*

and its terpene compounds in an animal model. To the best of our knowledge, this is the first study illustrating the mechanisms underlying the toxicity of bioactive compounds of hydroalcoholic extract of *A. repens*. Few studies have identified the chemical components of the extract of *A. repens* from other regions [24, 25]. In the current study, sesquiterpenes were found to be major compounds and the most common component was caryophyllene oxide (Table 2). Several studies reported that the major constituent identified in the essential oil of *A. repens* were mainly sesquiterpenes [2, 26]. These compounds, as a subcategory of the terpenes, constitute a large group of secondary metabolites that are widely distributed in a variety of plants [25]. Previous studies reported that some sesquiterpenes isolate from *A. repens* have neurotoxicity in rodents and primary cultures of fetal rat brain cells [17, 27]. The Sesquiterpene Lactones family has been introduced as the most probable cause of toxicity induced by *A. repens* [15, 28]. Whereas these components are categorized as volatile substances, the composition of the studied extract was evaluated by GC-MS with a special focus on the volatile fraction of the extract of *A. repens*. The volatile fraction of the *A. repens* extract comprises a considerable list of active components that the hepatotoxic properties of this extract are also attributable to them. Thus, we focused on the volatile components as evaluated by the GC-MS analysis.

In the acute toxicity study, the median lethal dose (LD50) of the extract was >2000 mg/kg (non-toxic) following single oral administration in male mice. Hydroalcoholic extract of *A. repens* revealed no mortality during 14 days of the experiment. While the results of the subchronic study showed that liver and brain injuries were the primary toxic effects following the administration of subchronic doses of *A. repens*. Moreover, the findings showed that the subchronic treatment with hydroalcoholic extract causes a significant increase in oxidative
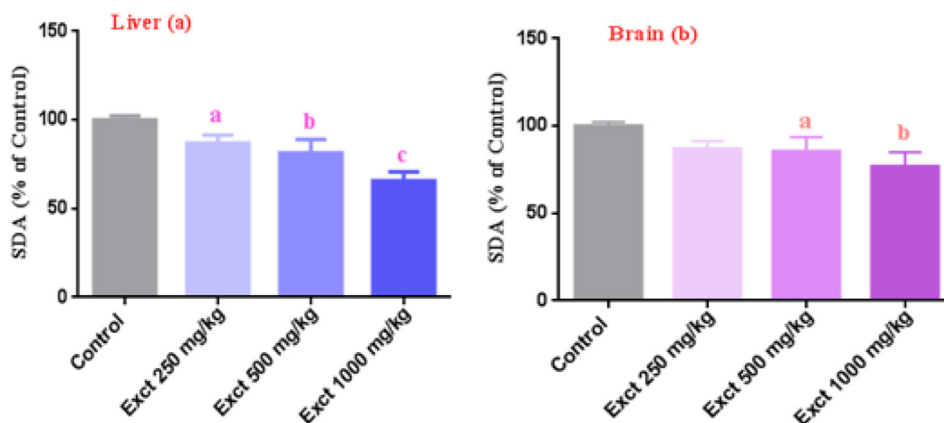

Figure 8. Effect of Acroptilon *repens* extract on mitochondrial succinate dehydrogenase activity (SDA) in mice (a) liver and (b) brain as evaluated by MTT test. Data are expressed as Means ± SD of nine animals in each group. [a] ($p < 0.05$), [b] ($p < 0.01$), [c] ($p < 0.001$) significantly different as compared to control group.
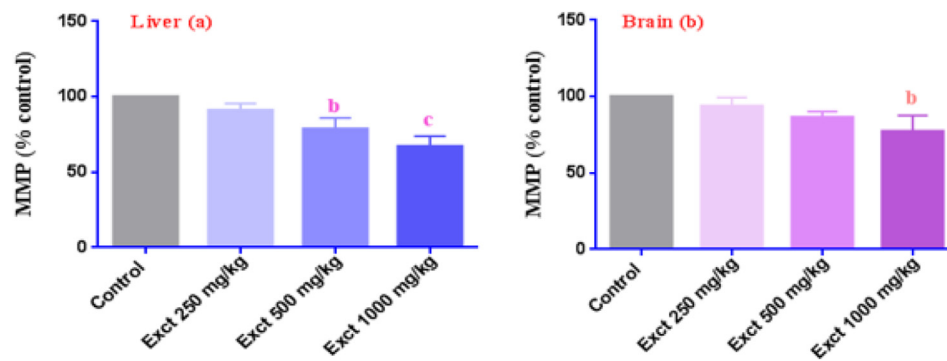
Figure 9. Effect of Acroptilon *repens* extract on mitochondrial membrane potential (MMP) in mice (a) liver and (b). Data are expressed as Means ± SD of nine animals in each group. [b] ($p < 0.01$), [c] ($p < 0.001$) significantly different as compared to control group.
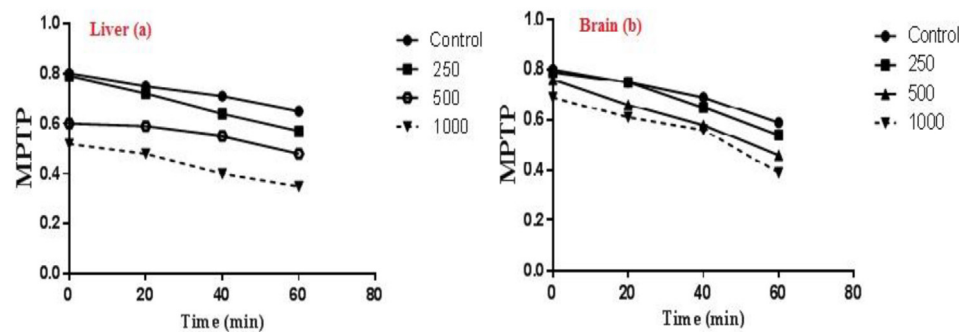

Figure 10. Effect of Acroptilon *repens* extract on (a) liver and (b) mitochondrial permeability transition (MPTP). *A. repens* caused mitochondrial swelling.

stress, impairment in mitochondrial function, inflammation, and apoptosis in mice liver.

In general, hepatocellular damage is directly reflected in the increase of serum hepatic enzymes, such as ALP, AST, and ALT, and their pathological changes. A recent study showed that the mice treated with 1000 mg/kg of *A. repens's* extract for 28 days had elevated levels of liver function enzymes, obvious hepatocellular necrosis, and multifocal infiltration of mononuclear cells [29]. Based on the results, the hydroalcoholic extract exhibited a noticeable increase in serum levels of ALT, AST, and ALP after subchronic treatment with extract at the dose of 500 and 1000 mg/kg. The present study findings indicated that there was organ target damage. This result was confirmed by the histopathology result that showed organs damaged in response to high dose treatment. The satellite group showed that all the toxic symptoms, biochemical and pathological disorder response was reversible when the treatment stopped. The previous study reported that the duration and intensity of exposure to toxic substances also can affect the form and toxicity of a particular material [30].

Mitochondrial dysfunction can cause oxidative stress which results in a vicious cycle of mitochondrial ROS and organelle dysregulation, ultimately inducing apoptosis [31]. Therefore, we hypothesized that extract-induced tissue damages and cell death linked with its metabolite or cellular oxidative status in the subchronic study. ROS has been shown to induce apoptotic cell death through depletion of endogenous antioxidants and over-production of lipid peroxidation [32].

Malondialdehyde (MDA), a lipid peroxidation product, is widely used as a marker of oxidative stress. Prolonged oxidative stress can cause changes in the activity of antioxidant enzymes (SOD and CAT)

that are commonly used to prevent or reduce tissue damage caused by oxidative stress. SOD metabolizes free radicals and dismutates superoxide anions to H_2O_2 and protects cells from lipid peroxidation. Studies have demonstrated that oxidative damages are caused by the over-production of the lipid peroxidation molecules, products of protein oxidation, and decrease in the activities of antioxidant enzymes [33]. Zhan et al. reported that a new sesquiterpenoid compound from *A. repens's* extract showed a potent cytotoxic activities on the tissue culture through hypoxic mechanism [8]. Also, acute hypoxia has been shown to increase the production of ROS in the cell by changing the activity of cytochrome chains, which are responsible for the oxidative phosphorylation of mitochondria [34]. Our findings showed that lipid peroxidation, protein carbonyl, and ROS contents significantly increased but CAT and SOD levels decreased in the liver and brain of mice treated with extract (500 and 1000 mg/kg) for 28 days. Due to hypoxic potentials of *A. repens's* extract [8], the results presented in this study further verify that extract administration-induced oxidative stress could occur along with an increase in the oxidation products and decreased in the activities of antioxidant enzymes.

Impairment of mitochondrial function is closely associated with many anti- and pro-apoptotic proteins in the terpene extract-induced toxicity. Apoptotic proteins can affect mitochondrial function in different ways [35]. These proteins may cause loss of the mitochondrial membrane potential ($\Delta\Psi$) and the swelling of the matrix of mitochondria through the formation of membrane pores or an increase in the permeability of the mitochondrial membrane, finally causing apoptotic effectors to leak out [36, 37].
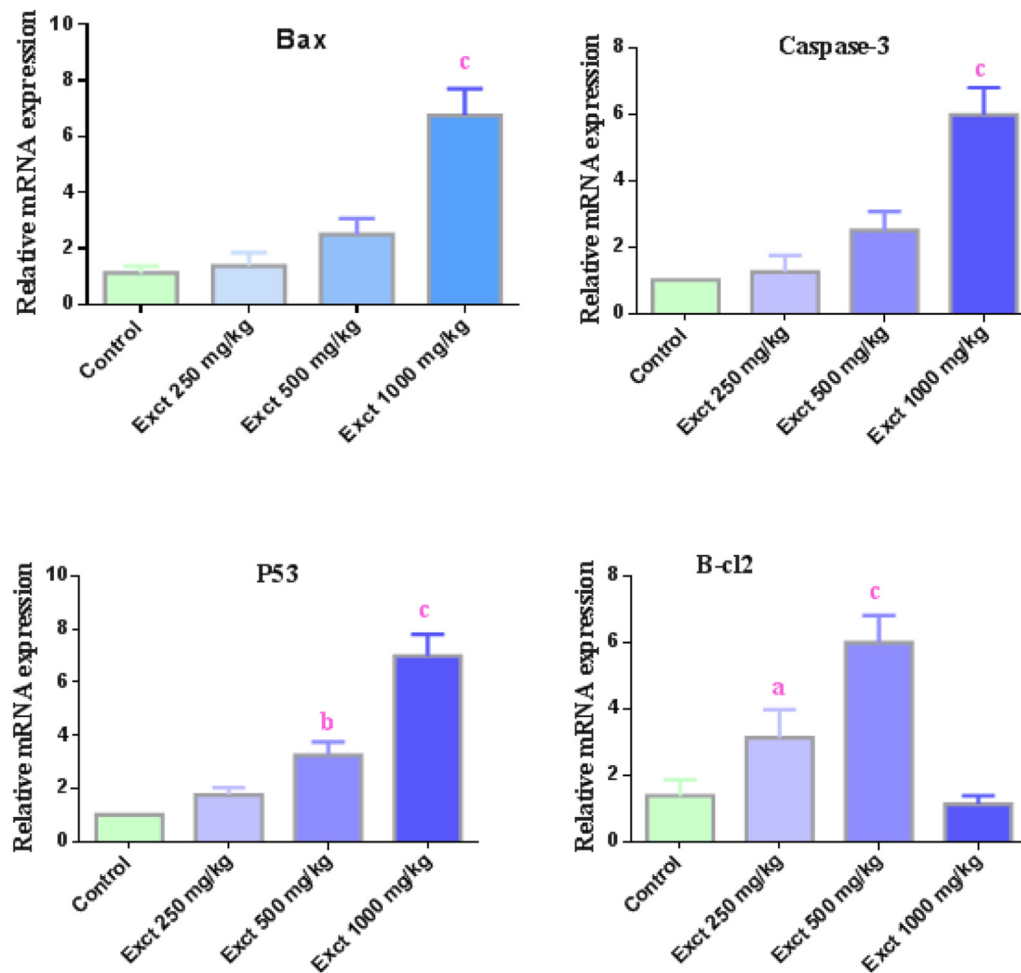
Figure 11. Effects of Acroptilon *repens* extract on mRNA expression in liver tissue of mice. Expressions of apoptosis-related genes of liver (Bax, Bcl-2, Caspase3, P53). Data are expressed as Means $\pm$ SD of nine animals in each group. [a] ($p < 0.05$), [b] ($p < 0.01$), [c] ($p < 0.001$) significantly different as compared to control group.

As shown in the result (Figures 8, 9, and 10 a and b), most doses of 500 and 1000 mg/kg of hydroalcoholic extract in the subchronic study cause mitochondrial damages in mice livers and brains, suggesting that hydroalcoholic extract induces apoptosis [38]. Several studies confirm that mitochondrial dysfunction conveys the apoptosis process [39, 40]. The mitochondrial pathways of apoptosis known as the intrinsic pathway are regulated by the Bcl-2 family of signaling modulators, including anti-apoptotic proteins Bcl-XL and Bcl-w and proapoptotic Bax and Bak proteins [41]. The primary function of Bcl-2 protein, an important factor in the inhibition of apoptosis, is to preserve mitochondrial integrity and prevent cytochrome-c efflux through restraining pro-apoptotic BAX/BAK [42]. On the other hand, the main function of Bax is to accelerate apoptosis, together with Bcl-2, which plays a crucial role in the regulation of apoptosis [43]. Bax is a downstream effector of p53-induced apoptosis and is transcriptionally regulated by p53. The p53 protein has been associated with the regulation of Bcl-2 signaling via direct interaction with Bax [44], Bak [45], Bcl-2, and Bcl-xL [46]. However, one of the central executioners of apoptosis is caspase-3 (cysteine protease), which is known to be a core modulator of apoptosis in mammals [47]. The Bcl-2 protein has been shown to act downstream of caspase-3 and to cause in an anti-apoptotic manner [48]. As shown in Figure 11, the expression of Bcl-2 and Bax proteins were significantly down-regulated,

while the expression of caspase-3 was up-regulated in the mice treated with *A. repens* extract (1000 mg/kg) for 28 days. These data suggest that the hydroalcoholic extract might induce apoptosis in the damaged hepatic and neuronal cells via a p53-dependent pathway, ultimately leading to caspase-3 activation in the subchronic study.

Overproduction of ROS/RNS in the process of oxidative metabolism triggers the inflammatory response resulting in the synthesis and secretion of proinflammatory cytokines. There is a close correlation between inflammatory cytokines and oxidative stress [49]. TNF-α, IL-6, and IL-1β are the key cytokines in the development and progression of oxidative stress as they are associated with mitochondrial ROS production that occurs under the influence of the NF-κB signaling pathway. The NF-κB transcription factors translocate into the nucleus and initiate the transcription of the inflammation-related genes such as Inducible nitric oxide synthase (iNOS) and cyclooxygenase type-2 (COX2), which are involved in inflammatory and immune responses [50]. These Pro-inflammatory cytokines can induce fibrosis, apoptosis, and acute phase responses that cause organ dysfunction and tissue damage [51]. Since the levels of these pro-inflammatory cytokines were significantly higher in mice receiving hydroalcoholic extract for 28 days, the compounds found in the extract might induce acute and/or subchronic inflammatory responses in various tissues.
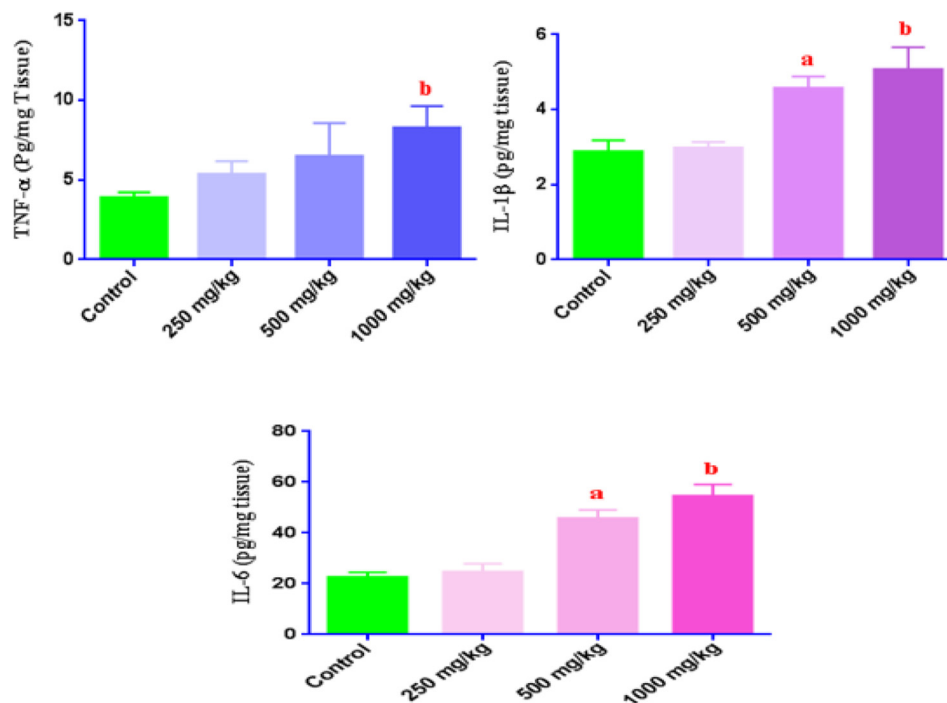
Figure 12. Effect of Acroptilon *repens* extracts on TNF-α, IL-1β, IL-6 production in liver tissue of mice. Data are expressed as Means ± SD of nine animals in each group. [a] ($p < 0.05$), [b] ($p < 0.01$) significantly different as compared to the control group.

5. Conclusions

Acute (2000 mg/kg) toxicological studies of *A. repens* hydroalcoholic extract did not cause any mortality nor toxicity of the main organs in mice. The extract did not induce a toxic effect at the low dose (250 mg/kg) in long term treatment. The toxic effect started to appear at the middle dose (500 mg/kg) and high dose (1000 mg/kg) but the effect was reversible, as shown in the satellite group. However, treatment for 28 days with over 500 mg/kg hydroalcoholic extract caused oxidative stress and apoptosis via reduction of non-enzymatic antioxidant, antioxidant enzymes, and the expression of anti-apoptotic factors along with an increased expression of apoptotic proteins. The information presented in this study will help to clarify the mechanisms underlying the sesquiterpenoids components-induced toxicity in mice in the subchronic study.

Declarations

Author contribution statement

Ayat Dashti: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Shokrzadeh: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Karami and Emran Habibi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

The data that has been used is confidential.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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