Study of the antioxidant effects of *Eremostachys laciniata* rhizome extracts in isolated rat hepatocytes

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Abstract

Eremostachys laciniata, having rich flavonoid content, is expected to have a considerable antioxidant effect. In this study We used ACMS (Accelerated cytotoxic or protective mechanism screening technique) to evaluate the possible antioxidant effect of *E. laciniata* rhizome against oxidative cell damages induced by different types of oxidative stress such as iron-8-hydroxyquinolin (IQ) complex and copper in freshly isolated liver cells. The extracts were prepared with n-hexane, dichloromethane and methanol. Hepatocytes were isolated from male Sprague-Dawley rats by a two-step collagenase perfusion. Cell viability was measured by trypan blue exclusion method. DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was used to evaluate the antioxidant activity. ROS formation was measured by using DCFDA (2, 7-dichlorofluorescin diacetate) probe, mitochondrial membrane potential (MMP) was assessed by rhodamine 123 fluorescence and lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS) assay. The MET extract was demonstrated to possess a significant radical scavenging activity (RC50%=0.212). Unlike MET extract, the n-hexane and dichloromethane extracts showed toxic effects in cell suspensions. The MET extract significantly decreased cell death and ROS formation induced by IQ complex and copper and demonstrated protective effects against copper-induced mitochondrial membrane potential collapse and lipid peroxidation. The protection induced by MET extract can be attributed to antioxidant characteristics of the phenylethanoids content.

Keywords: Eremostachys Laciniata, Oxidative stress, Hepatocyte, Iron-quinolin, Copper.

1. Introduction

Oxygen (O_2) is a vital element for most organisms, however it could turn into toxic compounds causing oxidative damage to important biological systems. This threat can be reduced by antioxidant agents. Although the human body's antioxidant systems function effectively, oxidative damages accumulate at biological systems over the time. This may accelerate aging and age-related degenerative diseases (1). Nowadays, the role of Reactive Oxygen Species (ROS) is well-established in inflammatory and age-related degenerative diseases such as cancer, diabetes, alzheimer, parkinsonism, cirrhosis, cataracts, cardiovascular disease and arthritis (2, 3). Existence of antioxidants such as carotenoids, flavonoids, cinnamic acid, benzoic acid, folic acid, ascorbic acid, tocopherols and phenolic compounds accumulated mostly in plants has encouraged researchers around the world to evaluate antioxidants with herbal origin (4). Lamiaceae family is one of these

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unique plants with effective antioxidant composi-tions. *E. laciniata* from Lamiaceae family is a pe-rennial plant with bushy roots and pale purple or white flowers. It is one of the 15 endemic species in Iran which is grown in eastern and western Asia (5). The extracts of roots and flowers of this plant have traditionally been used to treat headaches, allergies, asthma, coughs, colds and liver disease (6, 7). Studies have shown the anti-bacterial (6), anti-inflammatory (8), pain relieving (9) and free radical-scavenging effects of this herb (10). In Ira-nian traditional medicine, the roots of some spe-cies belonging to the genus Eremostachys has been used as an analgesic and anti-inflammatory agent and applied topically for the treatment of bruises, and localized pain and swelling (7, 11). Previous Phytochemical study on *E. laciniata* established the presence of mono- and sesqui-tepenes, iridoid glycosides, phenylethanoids and phytosterols (12, 13). Considering medicinal use of *Eremostachys* species and the various pathophysiologic role of oxidative stress in different diseases, it is estimat-ed that the most beneficial effects of this species are related to anti-oxidant components. Thereby, in this study, the protective effects of MET, n-hex-ane and dichloromethane extracts of *E. laciniata* rhizomes on the oxidative damages induced by the different systems of iron- 8-hydroxyquinoline (IQ) complex and copper (II) chloride ($CuCl_2$) in the isolated rat hepatocytes were evaluated.

2. Materials and methods
2.1. Materials

N-hexane, methanol, dichloromethane, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2',7'-di-chlorofluorescin diacetate (DCFH-DA), ethylene glycol tetra acetic acid (EGTA), N-(2-hydroxy-ethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), Bovine serum albumin (BSA), vitamin C, heparin sodium salt (grade 1-A), trypan blue and all buffer salts were purchased from Merck (Germany). Rhodamine 123 of Fluka (Italy) and collagenase A (from *Clostridium histolyticum*) of Roche diagnostics (Indianapolis, USA) companies were used in this study.

2.2. Plant material

The rhizomes of E*remostachys laciniata* (L) Bunge were collected during September–Octo-ber 2005 from Ajabshir country in East Azarbaijan province in Iran (37° 36′ 46.7″ N latitude, 46° 11′ 15.6″ E longitude and altitude 1900 m above sea level). A voucher specimen (TUM-ADE 0204) has been retained in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Science, and in the herbarium of the Plant and Soil Science Department, University of Aberdeen, Scotland (ABD).

2.3. Extraction and isolation of compounds

The dried and ground rhizomes of *E. la-*

Table 1. Molecular formula and weights of the 15 compounds isolated from dried and ground rhizomes *Eremostachys laciniata* (100 g).

Compound name	Molecular formula	Weight (mg)
9-epi-Phlomiol (1)	$C_{17}H_{26}O_{13}$	16.2
9-epi-pulchelloside II (2)	$C_{17}H_{26}O_{12}$	8.3
6-β-Hydroxy-7-epi-loganin (3)	$C_{17}H_{26}O_{11}$	10.0
Lamalbide (4)	$C_{17}H_{26}O_{12}$	98.9
Sesamoside (5)	$C_{17}H_{24}O_{12}$	78.9
6'-O-β-D-Glucopyranosyl-sesamoside (6)	$C_{23}H_{34}O_{17}$	1.8
Shanzhiside methyl ester (7)	$C_{17}H_{26}O_{11}$	13.9
5, 9-epi-Phlomiol (8)	$C_{17}H_{26}O_{13}$	9.5
Phloyoside II (9)	$C_{17}H_{25}O_{12}C_{1}$	5.1
5,9-epi-Penstemoside (10)	$C_{17}H_{26}O_{11}$	2.0
6,9-epi-8-O-Acetyl-shanzhiside metyl ester (11)	$C_{19}H_{27}O_{12}$	6.5
Forsythoside B (12)	$C_{34}H_{44}O_{19}$	15.0
Verbascoside (13)	$C_{29}H_{36}O_{15}$	7.9
Stigmasterol (14)	$C_{29}H_{48}O$	2.3
β-Sistosterol (15)	$C_{29}H_{50}O$	2.8

ciniata (100 g) were successively Soxhlet extracted, using n-hexane, dichloromethane and methanol (1.1L each). All these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C to yield 1.52 g, 0.92 g and 14.72 g of dried n-hexane, DCM and MeOH extracts, respectively. The relative physical characteristics, retention times and weights of the 15 compounds (1-11: iridoid glycosides, 12 and 13: phenylethanoids, 14 and 15: phytosterols) obtained by Delazar *et al*. are outlined in Table 1. The chemical structures of these compounds were elucidated by spectroscopic means (12) .

2.4. Animals

Sprague-Dawley male rats (220–250 g), were provided by animal research laboratory of Tabriz University of medical sciences. Animals transferred to the research section and acclimatized over a period of one week before experiments. The research section was maintained at a temperature of 23 °C, relative humidity of 36 ± 5 and a light/dark cycle of 12/12 hours with adequate ventilation and food and water accessibility. The maintenance, care and treatments of the rats were complied with National Institute of Health guideline for the humane use of laboratory rats and the guideline of Tabriz University of Medical Sciences.

2.5. Isolation and incubation of hepatocytes

The animals were anesthetized by intraperitoneal (i.p.) injection of phenobarbital and the portal vein cannulation was performed for buffer circulation. Hepatocytes were isolated using a two-step collagenase perfusion technique with slight modifications on the method by Moldéus *et al*. (14, 15). After isolation, the cells were suspended at a density of 10^6 cells mL^{-1} in Krebs-Henseleit buffer (pH=7.4) supplemented with 12.5 mM HEPES. The mixture was incubated under an atmosphere of 95% O_2 and 5% CO_2 in continuous rotating round bottomed 50 mL^{-1} flask at 37 °C. Each flask contained 10 mL^{-1} of hepatocyte suspension which was pre-incubated for 30 min before the addition of other chemicals.

2.5. Cell viability

The viability of isolated hepatocytes was evaluated by trypan blue (0.2% w/v) exclusion method and the percentage of cells excluding the dye was determined. Cell viability was determined immediately after isolation and at scheduled time intervals during 180 min incubation period. Approximately 85% to 90% of hepatocytes were viable at the time of isolation (16).

2.6. Evaluation of antioxidant activity of E. laciniata extracts by DPPH assay

This assay is a quick, easy and repeatable test to assess the ability of different compounds in radical scavenging. In this test, 2, 2-Diphenyl-1-picrylhydrazyl is used as the oxidant agent. The oxidant solution of DPPH in concentration of 0.08 mg/mL was prepared in solvents of methanol and chloroform. MET solution (5 mL) was added to 5 mL of each concentration of MET extract. Similarly, chloroform solution (5 mL) was added to 5 mL of each concentration of n-hexane and also dichloromethane extract. After 30 minutes, the absorbance was measured at a wave length of 517 nm by a spectrophotometer. RC% for each concentration was calculated. Using RC% versus concentration curve, the RC_{50} was determined and compared to the standard anti-oxidant of quercetin (17).

2.7. Evaluation of E. laciniata extract on cytotoxic damages induced by IQ complex and CuCl₂

After the initial incubation (30 min without adding any chemicals), different concentration of extracts (250, 500 and 1000 µg/ml) were immediately added to the other compounds according to the protocols in table 2. After 60, 120 and 180 minutes of adding the last agent, 100 µL from each flask were stained by 100 µL of trypan-blue 0.1% w/v and the percentage of cell death for each flask was counted by hemacytometer and the survival rate was calculated. This experiment was repeated three times and the mean values±SD are expressed as the final results.

2.8. Determination of intracellular reactive oxygen species (ROS)

Production of ROS was monitored by the fluorescence emission of DCFDA (18). After the initial incubation (30 min without adding any chemicals), different concentration of extracts (250, 500 and 1000 µg/ml) were immediately added to the other compounds according to the protocols in table 2 with the difference that the $CuCl_2$ concentration was 25 µM in this assay. To determine the rate of extract inhibitory effect on

ROS generation, DCFDA (solubilized in DMSO) was added to the hepatocyte incubation. This compound diffuses easily through the cell membrane and is de-esterified enzymatically by intracellular esterase to non-fluorescent compound of dichlorofluorescin (DCF) (19). Following by oxidation, the fluorescent compound of DCF is produced and the fluorescence intensity was measured using fluorescence spectrofluorometer at λ EM=530 and λ EX=485 nm. Clearly, the fluorescence intensity and the rate of oxidation are dependent to cellular ROS level.

2.9. Mitochondrial membrane potential assay (Δψmit)

To evaluation of the protective antioxidant effect of extract on mitochondrial membrane potential in isolated rat hepatocytes, the changes in Δψmit were assayed using uptake and retention of rhodamine123 dye in cells (20-22). Isolated cells were incubated with different concentration of MET extract (250, 500 and 1000 μg/mL) and 50 μM of $CuCl_2$ as an agent to induce oxidative stress. After 10 min of incubation with original media containing 1 μM Rhodamine123, the cells were centrifuged, and the capacity of mitochondria to uptake the dye in the supernatant was measured with spectrofluorimeter at the excitation wave length of 501 nm and the emission wave length of 520 nm. The amount of rhodamine accumulation in the supernatant was inversely proportional to the membrane potential of the cells (23).

2.10. Effect of E. laciniata extract on CuCl2 induced lipid peroxidation

Hepatocyte lipid peroxidation was determined by TBARS assay. After the initial incubation (30 min without adding any chemicals), different concentration of extracts (250, 500 and 1000 μg/ml) were immediately added to the other compounds according to the protocols in table 2, with the only exception of $CuCl_2$ concentration being 25μM in this assay. After 60, 120 and 180 minutes of adding the last agent, 1 mL aliquot of hepatocyte suspension was treated with 250 μL TCA (70%) and after 5 min incubation in ice-cold temperature, was centrifuged in 3000 rpm for 5min. The supernatant was boiled with TBA (0.8%) for 20 min and finally the absorbance was determined at 530 nm via UV spectrophotometer (24).

2.11. Statistical analysis

Data are expressed as mean±SD. Statistical comparisons were carried out using a one-way analysis of variance (ANOVA) followed by the Tukey test (post-hoc). Values with $P<0.05$ are considered significant.
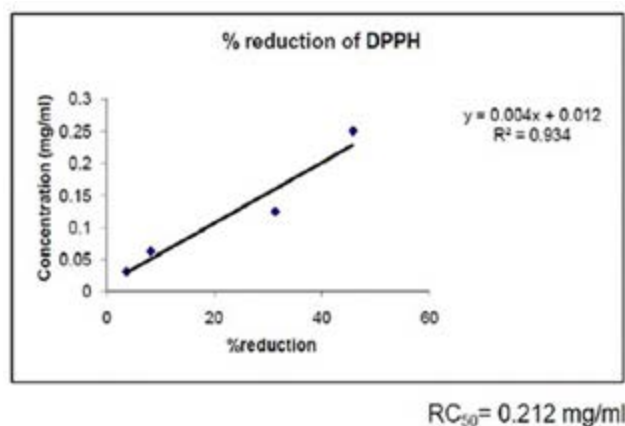
3. Results
3.1. DPPH assay

The result of DPPH test showed that in comparison to quercetin (RC_{50}=0.0035 mg mL^{-1}), MET extract of E. laciniata with RC_{50}=0.212 mg/mL can be proposed as a moderate anti-oxidant agent (figure 1). For n-hexane and dichloromethane extracts, significant anti-oxidant activity was

Table 2. Evaluation of the extract effect on cytotoxic damage induced by IQ and $CuCl_2$ complex.

Evaluation of the antioxidant effect of *Eremostachys Laciniata* MET extract on oxidative damage induced by IQ complex

Flask number	1	2	3	4	5	6	7	8
compounds	Control	EXT	Fe 3 μM+HQ 25 μM (Complex1)	Fe 6 μM+HQ 50 μM (Complex2)	Fe 12 μM+HQ 100 μM (Complex3)	(Complex1)+EXT 500 μg/mL	(Complex2)+EXT 500 μg/mL	(Complex3)+ EXT
added to cell suspension		500 μg/mL						500 μg/mL

Evaluation of the antioxidant effect of *Eremostachys Laciniata* extracts on oxidative damage induced by $CuCl_2$ complex

Flask number	1	2	3	4	5	6
compounds	Control	EXT	Cucl2	Cucl2 50 μM	Cucl2 50 μM	Cucl2 250 μM
added to cell suspension		1000 μg/mL	50 μM	+ EXT 250 μg/mL	+ EXT 500 μg/mL	+ EXT 1000 μg/mL

Figure 1. Anti-oxidant effect of MET extract of *E. Laciniata*. Concentration (mg/ml) vs % reduction for extract by DPPH free radical scavenging assay method is represented.

not demonstrated.

3.2. The effect of E. laciniata extract on cytotoxic damages induced by IQ complex and CuCl$_2$

According to the table 3, trivalent iron in complex with hydroxy quinoline can penetrate into the cell and cause toxicity in comparison to control group (P<0.001). Furthermore, the extract prevented the toxic effect of IQ complex and the cell death was reduced significantly (P<0.001) as compared to their control group (complex 1, 2 and 3).

In addition, the protective effects of extract against the CuCl$_2$ toxicity were demonstrated in comparison to the control group (P<0.001). Also, extract showed reduction of cell death in groups containing of CuCl$_2$ in comparison with CuCl$_2$ group without extract (P<0.001).

3.3. Effects of E. laciniata extract on intracellular reactive oxygen species formation

Copper induced significant level of ROS formation in comparison to the control (figure 2). Inversely, in the group treated with MET extract, the ROS formation level was significantly lower. Also in all groups receiving different concentrations of MET extract and copper as the toxic substance, the rate of ROS formation was significantly lower in comparison to the group treated with copper without extract. The intensity of fluorescence was dependent on extract concentration and time. Unlike the MET extract, both the DCM and n-HEX induced a significant increase in ROS level compared to the control group.

3.4. Effects of E. laciniata extract on mitochondrial membrane potential (Δψm)

Figure 3 demonstrates the mitochondrial membrane potential (Δψm) in test groups and
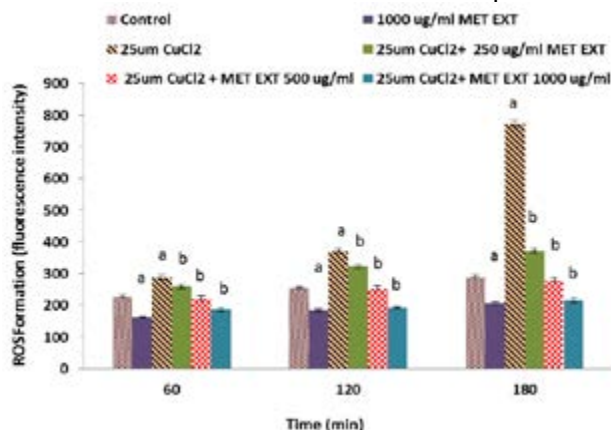
Figure 2. ROS formation rate in different concentrations of MET extract of *E. laciniata*. Hepatocytes were incubated with copper and extracts (250, 500 and 1000 μg/ml) and ROS formation was measured at different time intervals. ROS formation was expressed as fluorescent intensity units. Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey test. a significant difference (P<0.05) in comparison with control group; b significant difference (P<0.05) in comparison with CuCl$_2$ (25 μm) group.
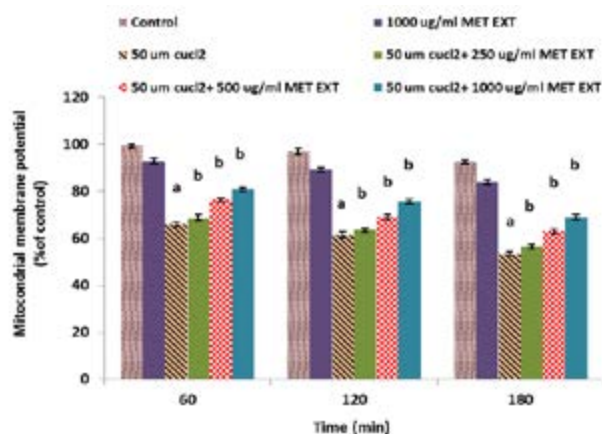
Figure 3. Effect of MET extract of *E. laciniata* on mitochondrial membrane potential. Hepatocytes were incubated with CuCl$_2$ and extracts (250, 500 and 1000 μg/ml) and mitochondrial membrane potential changes were evaluated at different time intervals. Mitochondrial membrane potential was expressed as fluorescent intensity units. Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey test. a significant difference (P<0.05) in comparison with control group; b significant difference (P<0.05) in comparison with CuCl$_2$ (25μm) group.

control group, where the 100% of rhodamine 123 trapping occurred in the intact mitochondria. The results revealed that CuCl$_2$ caused a rapid time-dependent decline of mitochondrial membrane potential as an apparent marker of mitochondrial dysfunction. Meanwhile, mitochondrial membrane potential collapse was prevented by treatment of hepatocytes with MET extract in comparison to CuCl$_2$ group. Furthermore this test was also performed for DCM and n-HEX extracts, but there was significant increase in mitochondrial membrane potential collapse rate in comparison to the control group.

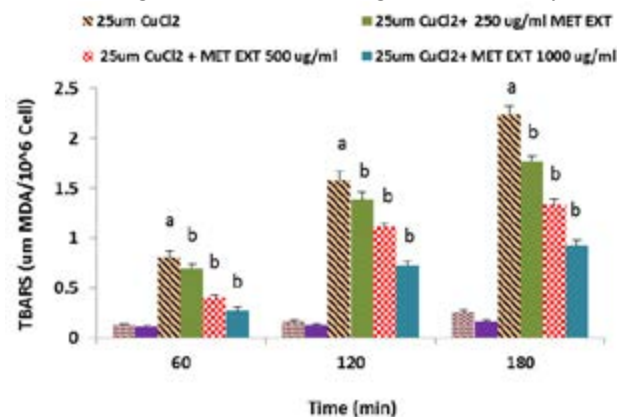### 3.5. Effects of E. laciniata extract on lipid peroxidation

Hepatocytes of control group revealed no significant lipid peroxidation during the incuba-

tion period. Meanwhile a remarkable amount of TBARS was formed in CuCl$_2$-treated rat hepatocytes (P<0.05) as compared to the control group. Furthermore, administration of different concentrations of MET extract considerably (P<0.05) prevented lipid peroxidation in comparison to the CuCl$_2$-treated hepatocytes (figure 4).

In groups treated with DCM and n-HEX extracts, the amount of TBARS were significantly higher than CuCl$_2$-treated rat hepatocytes (P<0.05), which indicated the lipid peroxidation induced by DCM and n-HEX extracts.

4. Discussion

In this study, the protective effects of MET, n-hexane and dichloromethane extracts of E. laciniata was evaluated on the oxidative damages induced by the different systems of iron-

Figure 4. Effect of MET extract of *E. laciniata* on CuCl$_2$-induced lipid peroxidation. Hepatocytes were incubated with CuCl$_2$ and extracts (250, 500 and 1000 μg/ml) and rate of lipid peroxidation was determined by measuring color absorbance at 530 nm. Values are expressed as mean±SD of three separate experiments (n=3)

8-hydrxy-quinoline (IQ) and copper (II) chloride (CuCl$_2$) complex in the isolated rat hepatocytes.

Isolated hepatocytes are widely acclaimed as a model for assessing the cell viability and cell metabolism and eventually toxicological studies. Using this model and ACMS technique (ACMS: Accelerated Cytotoxicity Mechanism Screening) various systems for the evaluation of protective and antioxidant properties of extract were designed (25). The toxicity of iron and copper used in this study is dependent on their ability in catalyzing and production of free radicals. In another word, the feature that made iron and copper as common catalyst in oxidation reactions is that they can easily accept and donate electrons and act as free radicals and damaging cellular macromolecules (26). In addition to catalytic role, iron can act as a reagent and directly react with oxygen and be involved in ROS production through perferryl intermediates (27). Further, it is demonstrated that 8-hydroxyquinoline increases the capacity of iron in lipid peroxidation (28) and is used as a lipophilic chelator. Our results confirmed previous findings related to high toxicity of iron-quinoline complex (28).

According to figure 1, the results of DPPH assay showed that the MET extract of *E.laciniata* (RC$_{50}$=0.212 mg/mL), have a moderate antioxidant capacity compared to the quercetin (RC$_{50}$=0.0035 mg/mL). The antioxidant activity was not observed with n-hexane and dichloromethane extracts. Protective effects of MET extract of *E. laciniata* is probably related to the presence of compounds such as flavonoids, iridoids and phenylethanoid glycosides. Lack of protective effect in DCM and n-hexane extract and their dose-dependent cytotoxicity can be attributed to labdane diterpenes and aldehyde content of these extracts (13).

In evaluation of the antioxidant effect of the extracts on oxidative damage induced by IQ and CuCl$_2$ complex, in all groups of containing the extract and different concentrations of toxic agents, significant reduction in cell death were recorded as compared to their control group (table 2). The exact mechanism of the extract in confronting toxicity of iron-quinoline complex and copper is unknown. Nevertheless, a possible explanation for this protective effect could be the ability of extract to prevent the breakdown of DNA bands and depletion of cellular ATP due to poly ADP polymerase activation in repair process of DNA.

Furthermore, inhibition of DNA binding of iron-quinoline and copper complex result in less induction of specific site breakdown in phosphodiester skeleton of DNA. This effect of extract can be attributed to inhibitory effect on oxidative degradation of deoxy ribose base pairs (29). It is also probable that extract could interdict in hydroxyl radical formation as a major ROS involved in oxidative damage.

In addition to determination of the intracellular reactive oxygen species test, extract significantly lowered the rate of ROS formation. Copper is able to penetrate into the cells and cause oxidation reactions and reactive oxygen species formation resulting in a sharp increase in conversion of non-fluorescent form of dichloro fluorescein di-acetate into fluorescent form. In turn these processes results in amplification of absorption at various test time which indicates the percentage of hepatocyte death. Different mechanisms may be involved in the protection, in which the antioxidant role of extract especially phenylethanoids of Verbascoside and Forsythoside B may have an effective role in trapping free radicals and other intermediates of oxidation can be the main mechanisms.

Membrane damages of iron and copper complexes result in impaired calcium homeostasis and endonucleases activation which eventually cause DNA damage in an apoptosis-like pathway. Confronting damage of toxic substances can be supposed a presumable protective role of extract (28). Furthermore, mechanisms such as ferrous chelating, prevention of iron reaction with oxygen and peroxide, maintaining iron in oxide form (unable to reduce oxygen) or even negation of radical intermediates formation and finally debarment of enzyme deactivation due to oxidative damage could be involved in the protective role of *E. laciniata* extract (30). In addition, the role of extract composition on enzymatic antioxidant system and also the other aspects of cell-protection are not ignorable.

Clear evidence of a prominent role of MPT (mitochondrial permeability transition) in copper toxicity has been demonstrated. In MPT phenomenon, permeability transition result in non-specific conductance of solutes less than 200 Da into the cell. Finally this process caused mitochondrial depolarization, inhibition in coupling of oxidative phosphorylation, ATP depletion and os-

Haleh Vaez *et al.*

motic expansion of colloid with large range in the matrix section of the inner membrane. Coupling inhibition and ATP depletion result in necrotic death and lesser amount of mitochondrial expansion. Following release of cytochrome C and other pro-apoptic factors eventually cause apoptosis. Inhibition of oxidative damage which is a major factor in MPT, could be the mechanism of extract protection. Other protective mechanisms could consist items such as boosting cellular detoxification processes and antioxidant system and also reaction with reactive oxygen intermediates which in general could prevent MPT attacks and subsequent collapse of MMP.

The collapse rate observed for DCM and n-HEX extracts were significantly higher in comparison to control group. Probably compounds such as the labdane diterpenes caused the reduction of mitochondrial membrane potential in iso-

lated rat hepatocytes. For a detailed understanding of these mechanisms, further experiments on the isolated compounds of extract are necessary.

The MET extract in all used doses could prevent the oxidative damage caused by copper and the decrease in lipid peroxidation, was statistically significant. The mechanisms involved in this protection could be due to factors such as strengthening the processes of cell detoxicifying and enzymatic and non-enzymatic antioxidant systems and reaction with reactive intermediates, which all could prevent the chainlike oxidative degradation of fatty acids and consequently malondialdehyde production .

5. Conclusion

The result of this study showed that the protective effects of MET extract against oxidative damage induced by iron-quinoline and $CuCl_2$

Table 3. Result of the evaluation of antioxidant effect of the extracts on oxidative damages induced by IQ and $CuCl_2$ complex.

Groups	Cytotoxicity mean±SD (% trypan blue uptake)		
	60 min	120 min	180 min
Control 1	16±0.58	18.67±0.67	23.33±0.88
MET EXT 500 µg/mL	14±0.58	16.67±0.33	21.67±0.88
Fe^{3+} 3 µM + HQ 25 µM (Complex 1)	30.33±0.88 a	36±1.15 a	41±1.15 a
Fe^{3+} 6 µM + HQ 50 µM (Complex 2)	35±0.58 a	39.33±0.67 a	42.67±0.88 a
Fe^{3+} 12 µM + HQ 100 µM (Complex 3)	39.67±1.53 a	42.33±1.55 a	46.33±1.53 a
Complex 1 + MET EXT 500 µg/mL	22.67±1.2 b	28.33±0.88 b'	33±0.58 b'
Complex 2 + MET EXT 500 µg/mL	29.33±1.2 c	34.33±1.2 c'	37.67±0.88 c'
Complex 3 + MET EXT 500 µg/mL	34.67±0.88 d	37.67±0.88 d'	41.67±0.88 d'
Control 2	16±0.58	18.67±0.67	23.3±0.88
MET EXT 1000 µg/mL	15±0.58	18.33±0.33	23.67±0.88
$CuCl_2$ 50 µM	65.33±1.45 e	77.67±0.88 e	87.67±1.45 e
$CuCl_2$ 50 µM + MET EXT 250 µg/mL	55±1.15 f'	72.67±0.88 f''	82.67±0.67
$CuCl_2$ 50 µM + MET EXT 500 µg/mL	47.33±1.2 f	71.33±0.88 f'	80.67±1.45 f'
$CuCl_2$ 50 µM +MET EXT 1000 µg/mL	37±1.85 f	51±1.52 f	69.33±1.45 f

Isolated rat hepatocytes (106 cells mL-1) were incubated at 37 °C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH 7.4). The results shown represent the Mean±SD for three independent experiments.

a Significantly different from control 1 group (P<0.001); b Significantly different from Complex 1 group (P<0.001); b' Significantly different from Complex 1 group (P<0.01);

c Significantly different from Complex 2 group (P<0.001); c' Significantly different from Complex 2 group (P<0.05); d Significantly different from Complex 3 group (P<0.001); d' Significantly different from Complex 3 group (P<0.05).

e Significantly different from $CuCl_2$ 50 µM group (P<0.001); f Significantly different from $CuCl_2$ 50 µM group (P<0.001); f' Significantly different from $CuCl_2$ 50 µM group (P<0.01); f'' Significantly different from $CuCl_2$ 50 µM group (P<0.05).

systems reduced the oxidative stress of these cytotoxic agents. In addition, our findings were in consistent with previous findings which confirmed the role of reactive oxygen species, as the main factor in the cytotoxicity of these damaging elements. Furthermore, the toxic effects of n-hexane and DCM extracts of the plant were demonstrated. The results of this study could be applied in various areas of pharmaceutical and clinical services and disease prevention, and the rhizome extract of *E. laciniata* could be used in pharmaceutical formulations and dietary supplements. Also in the presence of effective and adequate studies on animal models, perhaps the plant can be used to treat human chronic liver disorders. The effects of each

composition of extract especially phenylethanoids with more antioxidant activity is suggested to evaluated in a separate experiments to determine the best part in oxidative diseases.

Conflict of interest
None declared.

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