Hepatoprotective activity of White Horehound (Marrubium vulgare) extract against Cyclophosphamide toxicity in male rats

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Hepatoprotective activity of White Horehound (Marrubium vulgare) extract against Cyclophosphamide toxicity in male rats

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Abstract

The hepatoprotective activity of *Marrubium vulgare* against cyclophosphamide toxicity in Wistar rats was evaluated. Adult male rats were divided into 4 groups of six each: a control group, a group injected with cyclophosphamide (150 mg/kg) for 3 days, a group orally given a *Marrubium vulgare* aqueous extract (500 mg of dry leaves /kg/day) for 30 days then treated with cyclophosphamide, and a group receiving only *Marrubium vulgare* for 30 days. After 33 days of treatment, activities of alanine amino transferase (ALAT), aspartate amino transferase (ASAT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were determined in serum. Moreover, lipid peroxidation level and superoxide dismutase (SOD) activities, catalase (CAT) and glutathione peroxidase (GPx) were measured in liver. Alterations of these hepatic biomarkers and increased lipid peroxidation confirmed cyclophosphamide-induced liver toxicity. Cyclophosphamide also decreased the enzymatic defense system against oxidative stress. However, when this drug was administered in rats given *Marrubium vulgare* extract, all the biological parameters underwent much less alteration. Administration of *Marrubium vulgare* extract was found to be beneficial by attenuating cyclophosphamide-induced liver damage. The protective effect of the plant is mainly attributed to its antioxidant properties and the existence of phenolic acids and flavonoids, as highlighted by HPLC-based analysis.

Keywords: Cyclophosphamide,*Marrubium vulgare*,Oxidative stress,Liver
Introduction

Cyclophosphamide (CP) is an antineoplastic agent from the oxazophorines group. It has been proven to be effective against some autoimmune diseases and a variety of cancer types such as lymphomas, neuroblastomas, breast cancer, ovary adenocarcinoma, myeloma and chronic lymphocytic leukemia (Baumann and Preiss 2001). During clinical application, it is important to prevent or reduce CP-induced DNA damage (Tripathi and Jena 2008). Notwithstanding the fact that it acts as an efficient chemotherapeutic mediator, CP intake is associated with several significant adverse effects such as mutagenicity, carcinogenicity, immunosuppression, cardiac toxicity, and lung and kidney toxicity leading to the formation of reactive oxygen species as well as lipid peroxidation (Stankiewicz et al. 2002). Previous studies have shown that therapeutic doses of CP could be toxic to liver (Snover et al. 1989; Sulkowska et al. 2002). CP is metabolized via the hepatic P450 enzymatic system resulting in the formation of 4-hydroxycyclophosphamide and its tautomer aldocyclophosphamide which induce an oxidative stress by producing hydroxyl radicals and altering the antioxidant status of a cell (Selvakumar et al. 2005). Other findings suggest that CP depresses antioxidant defense in liver while its metabolism generates superoxide anions and hydrogen peroxide (H$_2$O$_2$) (Singh et al. 2014). Additionally, Tripathi et al. (2010) point out that CP, administered to mice at a dose of 100 and 200 mg/kg, i.p., induces DNA damage in both bone marrow cells and peripheral blood lymphocyte cells. Injection of CP into rats also results in a repression of GPx as well as SOD activities and, on the other hand, an increase in TBARS content in blood. The oxidative stress emerging from excessive generation of reactive oxygen species as well as flawed antioxidant defense system may account for many side effects of CP treatment.

Several experimental studies have sought to prevent troublesome side effects caused by drugs. Treatment materials used for this purpose include herbal medicine as an ancient discipline relying on herbal remedies or plant extracts.
M. vulgare L. (Lamiaceae) naturally grows as a native plant in Northern and Southern USA and is also found in the Mediterranean area (Tunisia, Alegria, etc) as well as Western Asia as far as India. This plant is used in various traditional medical applications and is characterized by anti-diabetic, anti-oxidant and antibacterial properties (Boudjelal et al. 2012; Ghedadba et al. 2014; Mubashir et al. 2008). It is also claimed to induce gastroprotective (De Oliveira et al. 2011) and anti-inflammatory (Kanyonga et al. 2011) activities. Phytochemically, M. vulgare is characterized by the presence of a variety of compounds such as polyphenols, tannins, flavonoids, diterpenes and saponins (Bouterfas et al. 2013; El Bardai et al. 2003; Kurbatova et al. 2003). The extract of M. vulgare has been proven to have hepatoprotective effects in rats (Akther et al. 2013; Elberry et al. 2010). The antioxidant properties of M. vulgare extract have been confirmed by its ability to trap reactive oxygen species (ROS), inhibit lipid peroxidation and enhance the efficiency of endogenous antioxidant systems (Akther et al. 2013).

This work aimed to investigate the hepatoprotective effect of M. vulgare on CP-induced toxic damage to rats. In the first part of this study, some biochemical parameters (i.e. alanine aminotransferase (ALAT), lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT) and alkalinephosphatase (ALP) activities) were measured in blood serum. In addition, lipid peroxidation level and antioxidant enzyme (SOD, GPx, and CAT) activities were determined in liver. In the second part, a phytochemical study of the M. vulgare extract was carried out to identify some antioxidant substances.

**Materials and methods**

**Chemicals**

Cyclophosphamide (Endoxan) was obtained as a powder from the pharmacy of the Regional Hospital of Gafsa, Tunisia, to be dissolved in saline for injection. The CP dose used in the present study was derived from previous reports (Senthilkumar et al. 2005) and determined on the basis of some preliminary experiments.
Preparation of the M. vulgare extract

Samples of *M. vulgare* were collected in November 2013 from the Gafsa area located in south-west Tunisia. They were identified by a botanist at Faculty of Sciences of Gafsa, Tunisia. The plant leaves were washed, dried at a room temperature in the dark then ground. 1g of the obtained dry powder was added to 100 ml of boiling distilled water. After reaching room temperature (ca. 15 min), the prepared infusion was filtered in order to carry out biological tests on the experimental animals.

Experimental design

Rats were divided into 4 groups of 6 each: (1) a control group (C); (2) a group of rats fed with CP by intraperitoneal injection (150 mg/kg) for 3 successive days, following the protocol of Senthilkumar et al. (2005) (CP); (3) a group of rats pre-treated with *M. vulgare* extract at a dose corresponding to 500 mg of dry leaves powder/kg body weight per day given by oral gavage for 30 successive days (Elberry et al. 2010), then the rats were injected with CP (CPM); and (4) a group of rats receiving *M. vulgare* extract for only 30 successive days (M).

After 33 days, all the animals were quickly sacrificed via decapitation in order to minimize the treatment stress. Blood serum was obtained by centrifugation (1500 × g, 15 min, 4°C) and the livers were removed, cleaned of fat and weighed. All samples were stored at −80°C until use. All the experimental procedures were conducted in compliance with Guidelines on the Use of Living Animals in Scientific Investigations.

Biochemical assays

Based on Yagi's (1976) method, lipid peroxidation level was measured by the quantity of thiobarbituric acid reactive substances (TBARS). The test involved combination of 125 µl of supernatant (S1) and 175 µl of 20% trichloroacetic acid containing 1% butyl-hydroxytoluene and centrifuged (1000 × g, 10 min, 4°C). Then, 200 µl of supernatant (S2) was mixed with 40 µl of HCl (0.6 M) and 160 µl of thiobarbituric acid (0.72 mM). The mixture was heated at
80°C for 10 min and the absorbance was measured at 530 nm. The total TBARS amount was calculated using an extinction coefficient of 156 mM−1·cm−1 and expressed in nmoles/mg protein.

Catalase (CAT) activity was measured by the method described by Aebi (1984). The reaction mixture (1 ml) consisted of 100 mM phosphate buffer at a pH of 7, 100 mM H$_2$O$_2$ and 20 µl (about 1 - 1.5 mg of protein) of liver homogenate. In order to track the decomposition of H$_2$O$_2$ at 25°C, the decrease in absorbance was measured at 240 nm for 1 min. Enzyme activity was determined by means of an extinction coefficient of 0.043 mM−1·cm−1 and expressed in µM H$_2$O$_2$ destroyed min/mg protein.

The total superoxide-dismutase (SOD) activity was determined by measuring its capacity to exert inhibitory effect on the photoreduction of nitrobluetetrazolium (NBT) (Sun et al. 1988). One unit of SOD represents inhibition of NBT photoreduction by 50%, and the activity was expressed as units/mg protein, at 25°C.

Glutathione-peroxidase (GPx) activity was assayed using the method described by Flohe and Gunzler (1984) at 25°C and expressed as µmoles of GSH oxidized/min/g protein.

Protein content in tissue extracts was determined using Lowry et al.'s method (1951) and thus bovine serum albumine as a standard. The serum activities of alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) were identified using kit methods (Spinreact).

**Assay of Free Radical-Scavenging Activity on DPPH**

The Assay of Free Radical-Scavenging Activity of *M. vulgare* was measured using the stable radical DPPH according to Grzegorczyk et al.'s (2007) protocol. An aqueous solution of *M. vulgare* (1 ml) at various concentrations (50 - 400 µg/ml) was added to 1 ml of a 0.1
methanolic solution of DPPH and kept at 27°C for 30 min. This was followed by reading the absorbance at 517 nm.

DPPH radical-scavenging activity (RSA), expressed as a percentage, was calculated using the following formula:

\[
\text{RSA\%} = \frac{\text{ADPPH} - (\text{Asample} - \text{Acontrol})}{\text{ADPPH}} \times 100
\]

**Ferric reducing antioxidant power (FRAP) of Marrubium vulgare extract**

The FRAP of *M. vulgare* extract was determined in accordance with the protocol described in Chu et al. (2000). 2.5 ml of Potassium phosphate buffer (0.1 M, pH 6.6) as well as 2.5 ml of 1% (w/v) potassium ferricyanide were combined with 1.0 ml of *M. vulgare* extract solution at various concentrations (50-500µg/ml). The reaction was incubated at 50°C for 20 min, then 2.5 ml of 10% (w/v) trichloroacetic acid was added. After that, water (2.5 ml) and 0.5 ml of 0.1% (w/v) FeCl3 was added to 2.5 ml of the reaction mixture and incubated at 28°C for 30 min to facilitate color change. The absorbance was measured at 700 nm as a function of *M. vulgare* extract concentration (µg/ml) and compared with ascorbic acid (AA) used as a standards.

**Extraction of *M. vulgare* Phenolic Acids and Flavonoids**

The dried powder of *M. vulgare* (1g) was mixed with 10 ml of extraction solution (methanol 80%), agitated for 10 min, and then centrifuged at 12,000 × g for 5 min. A 0.5 ml aliquot of supernatant was added to 0.5 ml of acetone and agitated. After that, the homogenate was centrifuged (12,000 × g for 5min). A Speed Vac device was used to dry the homogenate to be used for HPLC analysis of phenolic acids and flavonoids (Hfaiedh et al. 2013).
**HPLC Analysis Conditions**

Examination by liquid chromatography was carried out using a Varian Prostar HPLC system equipped with a ProStar 230 ternary pump, a manual injector and a ProStar 330 diode array detector. HPLC separation of the active compounds was conveyed on a 5µm particle C-18 reversed-phase column (Varian, 150 × 4.6 mm). The mobile phase was composed of solvent A: water and acetic acid (98:2 v/v), and solvent B: water, acetonitrile and acetic acid (58:40:2 v/v). The gradient elution used was: 0-80% B for 55 min, 80-100% B for 15 min and 100-0% B for 5 min. The flow rate amounted to 0.9 ml/min with an injection volume of 20 µl. Compound identification was carried out at 280 nm for phenolic acids and at 360 nm for flavonoids on the basis of a comparison between the retention time as well as mass spectra of the peaks in the injected extracts and those of HPLC standard compounds.

**Results**

**Effects of treatments on plasma biochemical parameters**

Treatment with CP resulted in a significant increase in ASAT, ALAT, LDH and ALP activities compared with the control (Table 1). In the case of CP-treated rats receiving *M. vulgare*, all the aforementioned biomarkers reverted to almost normal values.

**Oxidative damage**

CP significantly increased the hepatic TBARS concentration compared to controls (Fig.1). Administration of *M. vulgare* extract led to a reduction in the TBARS levels.

**Antioxidant activities**

The exposure of rats to CP induced significant adverse effects on the liver redox status; SOD, CAT and GPx markedly decreased compared to controls (fig.1). However, when the animals received *M. vulgare* extract, the antioxidant enzymatic defense system was significantly corrected.
DPPH radical scavenging activity

As shown in Fig. 2, free radical scavenging activities of *M. vulgare* extract were measured by DPPH assay. It is clear from Fig. 2 that the anti-DPPH activity increased with increasing *M. vulgare* concentration until a maximum concentration (400 µg/ml) was reached.

Based on the curve presented in Fig. 2, effective concentration (EC50) of *M. vulgare* extract, which gave 50% inhibition of the DPPH radical, was found to be 180 ± 2.54 µg/ml. This indicated that the anti-DPPH activity was relatively lower than that of ascorbic acid 110 ± 4.12 µg/ml.

FRAP test

As can be clearly seen in Fig. 3, *M. vulgare* extract was capable of reducing Fe³⁺ to Fe²⁺ at different concentration ranges. The reducing power of this plant increased with increasing concentrations and was able to serve as an electron donor. The reducing power of *M. vulgare* extract at 500 µg/ml was found to be 0.37 ± 0.021 and thus significantly lower than that of ascorbic acid, which was used as positive control (1.02 ± 0.04), at the same concentration.

HPLC analysis of marrubium extracts phenolic acids and flavonoids

The HPLC-based analysis of *M. vulgare* extract indicated the presence of phenolic acids and flavonoids. As shown in Fig. 4, this plant contained 32 unknown compounds and six known phenolic acids: Gallic, Catechic, Caffeic, Epicatechic, Vanillic and Coummarin acids, with a retention time of 7.179 min, 15.144 min, 17.285 min, 18.387 min, 21.131 min and 27.096 min, respectively. The HPLC elution profile of flavonoids displayed in Fig. 5 revealed 36 compounds absorbed at 360 nm including three known flavonoids; Rutin, Quercetin and Kaempferol, with retention time of 20.717 min, 29.883 min and 33.019 min, respectively.

Thus, *M. vulgare* proved to be rich in antioxidant compounds (i.e. phenolic acids and flavonoids) which enabled it to have a protective effect on toxicity.
Discussion

CP is an alkylating agent widely used in chemotherapy to slow or prevent cell proliferation. CP is converted by the liver oxidase system mixed function into two metabolites: phosphoramide mustard and acrolein. Phosphoramide mustard is responsible for therapeutic activity, and metabolite acrolein induces inactivation of microsomal enzymes resulting in increased reactive oxygen species (ROS) generation as well as lipid peroxidation (Kehrer and Biswal 2000). In our study, CP injection at a dose of 150 mg/kg caused oxidative stress exhibited by an increase in lipid peroxidation level as well as weakening of the antioxidative status in the hepatic tissue of rats. The obtained results are in agreement with those reported in other works suggesting that CP administration induces a significant increase in TABRS level in liver (Lata et al. 2014; Manda and Bhatia 2003). An increase in TBARS level may alter the cellular membrane structure and then block cellular metabolism. These factors may explain the negative effects of CP on hepatocytes. CP-induced hepatotoxicity can also be explained by mitochondrial membrane potential alteration caused by acrolein in hepatocytes (Lindley et al. 2002; Luo et al. 2005; Pass et al. 2005). The same metabolite induces necrosis and apoptosis in hepatic tissue as reported by Cuce et al. 2015. In fact, in liver, hepatocytes are essentially affected by ROS and oxidative stress, which could result in apoptosis and necrosis and is an explanatory factor that could account for hepatotoxicity. Indeed, the release of cytochrome c from damaged mitochondria during oxidative stress induced caspase cascade. During cellular apoptosis, chromatin condensation as well as DNA fragmentation occur without damaging the plasma membrane. An alteration in signalling pathways regulates apoptosis and thus prevents its occurrence (Kang and Reynolds 2009). There are various factors involved in the regulation of apoptosis such as Bel-2. In fact, Bel-2-family proteins are critical regulators of apoptosis, and their primary site of action is on the outer mitochondrial membrane (Lindsay et al. 2011). Once liver cells were damaged by oxidative stress,
mitochondrial membranes were impaired, which resulted in almost complete inhibition of the antiapoptotic effect of Bcl-2 described by Schwartz and Waxman (2001).

CP administration was found to induce disturbances of the antioxidant system indicated by changes observed in enzymatic antioxidant parameters. CP injection reduced superoxide dismutase, CAT and glutathione peroxidase activities in liver. It’s known that the activities of SOD and GPx behave in two different ways face to oxidative stress; an overexpression of the enzyme in a first stage and then their inhibition if the stress is permanent. It was noted that CP metabolized by cytochrome P450 induced an excessive generation of free radicals (Olayinka et al. 2015), which may explain the severe alteration of antioxidant enzyme status in the liver. In fact, CP metabolism produced highly reactive electrophiles accompanied by attenuation of GSH in CP-treated group that can be associated with the electrophilic burden on the cells and the formation of acrolein. The results found in this study are similar to those of Haque et al. (2001); Rajasekaran et al. (2002); Selvakumar et al. (2005); Shanmugarajan et al. (2008) who point out that SOD, CAT and GPx play a critical role in the fight against free radicals and their elimination which explains the decrease in the activity of the three enzymes after the CP administration that induced hepatotoxicity. However, in the case of rats pre-treated with M. vulgare, the perturbation was significantly attenuated, which was indicated by reduction in lipid peroxidation level and enhancement of antioxidant states (SOD, GPx and CAT) when compared with those of normal rats, as suggested by Akther et al. (2013), Haque et al. (2003) as well as Zarei and Shivanandappa (2013). This beneficial effect can be explained by the modulation of the cellular GSH pool which was found to improve the antioxidant and free radical-scavenging activity. It is also suggested that the components of M. vulgare appeared to have protective effect against CP-generated aggressive oxidants.

The protective effect of M. vulgare can be attributed to the presence of phenolic acids and flavonoids as revealed by the HPLC analysis. In fact, phenolic acids and flavonoids were able
to release a hydrogen proton from their hydroxyl group, trap free radicals and prevent CP-induced liver damage.

Based on the HPLC analysis of *M. vulgare* extract, phenolic acids (acid Gallic, Catechin, Caffeic, Epicatechin, Vanillic and Coummarin) and flavonoids (Rutin, Quercetin and Kampferol) were identified. Similar findings have been reported by Boudjelal et al. (2012) and Pukalskas et al. (2012), emphasizing that *M. vulgare* is an influential factor to counteract the oxidative stress-induced liver damage.

Another indicator of CP-induced hepatic toxicity was perturbation of biochemical parameters (ASAT, ALAT, LDH and ALP) which are markers of hepatic damage. In the present study, damaged of hepatocytes can been associated to the increase activities of ASAT, ALAT, LDH and ALP in serum after injection by CP for 3 successive days which is in agreement with previous reports indicating that these biochemical markers increases during membrane hepatocytes damage (Alabbassi 2010; Habibi et al. 2014; Shokrzadeh et al. 2006). Based on the experiments performed in this study, *M. vulgare* extract was found to be beneficial in reducing the pathological shifts of all the biochemical parameters studied. The reduction of these parameters is mainly due to the presence of antioxidant properties capable of scavenging free radicals and protecting cellular structures against cytotoxic effects as shown by the DPPH, Ferric reducing power (FRAP). Results reported by several researchers (e.g. Ghedadba et al. 2014; Kadri et al. 2011) are in agreement with the findings obtained in the present study which emphasizes the alleviating effect of *M. vulgare* on liver damage (Elberry et al. 2010; Mubashir et al. 2009).

**Conclusion**

In this study, it was found that aqueous extract of *M. vulgare* possesses protective activity against CP-induced hepatotoxicity and oxidative stress. This efficiency is mainly attributed to antioxidant properties as well as the presence of phenolic acids and flavonoids in this plant.
Acknowledgments

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Conflict of interest:

The authors declare that there is no conflict of interest.

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**Table 1.** Blood ALAT (UI/L), ASAT (UI/L), ALP (UI/L) and LDH (UI/L) levels after 33 days of treatment in: controls (C), Cyclophosphamide-treated rats (CP), *M. vulgare* treated rats (M) and Cyclophosphamide treated rats with *M. vulgare* (CPM). Values are the mean of 6 measurements ± SD. ** *p*≤0.01, as compared to control group (C). ++ *p*≤0.01, as compared to treated group (CP).

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<td>ALP</td>
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<td>ASAT</td>
<td>22 ± 2.82</td>
<td>45.5 ± 6.65**</td>
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<td>ALAT</td>
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<td>22.25 ± 2.75**</td>
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<td>LDH</td>
<td>692 ± 38.38</td>
<td>1312.5 ± 32.63**</td>
<td>846.25 ± 58.56++</td>
<td>723.33 ± 67.71++</td>
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Captions of figures:

Figure 1. TBARS (nmol/mg protein) levels and activities of SOD (I.U./mg protein), GPX(I.U./mg protein) and CAT(I.U./mg protein) in liver after 33 days of treatment in controls (C), Cyclophosphamide-treated rats (CP), Cyclophosphamide-treated rats with *Marrubium vulgare* extract (CPM) and *M. vulgare* treated rats (M). Values are the mean of 6 measurements ± SD. ** $p \leq 0.01$ compared with control group (C). ++ $p \leq 0.01$ compared with treated group (CP).

Figure 2. Scavenging activity of *M. vulgare* extract (at final concentration ranging from 100 to 400 µg/ml) on the DPPH radical. Each value is repeated in 3 separate assays ± SD. RSA: Radical scavenging activity.

Figure 3. Reducing power of *M. vulgare* and the synthetic antioxidant ascorbic acid, at different concentrations.

Figure 4. HPLC profile of phenolic acids ($\lambda = 280$ nm) from *M. vulgare* aqueous extract. Peaks (1) Gallic Acid, (2) Catechic Acid, (3) Caffeic Acid, (4) Epicatechic Acid, (5) Vanillic Acid, (6) Coummarin Acid and 32 unknown compounds. The HPLC analyses were performed using a varian Prostar HPLC equipped with a reverse phase C-18 column (Varian, 150 mm × 4.6 mm, particle size 5 µm). The mobile phase consisted of water: acetic acid (98:2 v/v) (A) and water: acetonitrile: acetic acid (58:40:2 v/v) (B). The elution gradient used was: 0-80% B for 55 min, 80-100% B for 15 min and 100-0% B for 5 min. The flow rate was 0.9 ml/min and the injection volume was 20 µl.

Figure 5. HPLC profile of flavonoids ($\lambda = 360$ nm) from *M. vulgare* aqueous extract. Peaks (1) Rutin, (2) Quercetin, (3) Kaempferol and 33 unknown compounds. The HPLC analyses were performed using a varian Prostar HPLC equipped with a reverse phase C-18 column (Varian, 150 mm × 4.6 mm, particle size 5 µm). The mobile phase consisted of water: acetic acid (98:2 v/v) (A) and water: acetonitrile: acetic acid (58:40:2 v/v) (B). The elution gradient
used was: 0-80% B for 55 min, 80-100% B for 15 min and 100-0% B for 5 min. The flow rate was 0.9 ml/min and the injection volume was 20 µl.