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Effects of Salvia officinalis and Thymus vulgaris on oxidant-induced DNA damage and antioxidant status in HepG2 cells

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a b s t r a c t

Salvia officinalis (SO) and *Thymus vulgaris* (TV) are medicinal plants well known for their curative powers. However, the molecular mechanisms responsible for these abilities of sage and thyme have not been fully understood yet. In this study we investigated the composition and the quantitative estimation of plant extracts, the protective effects of plant extracts against hydrogen peroxide- and 2,3-dimethoxy-1,4-naph-thoquinone-induced DNA damage, and levels of enzymatic and non-enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, glutathione) in human HepG2 cells. To measure antioxidative activity of plant extracts we used three assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The results showed that the oxidant-induced DNA lesions were significantly reduced in cells pre-treated with the plant extracts studied. The observed DNA-protective activity could be explained by both elevation of GPx activity in cells pre-treated with SO and TV and antioxidant activity of SO and TV.

Keywords:Salvia officinalis extract, Thymus vulgaris extract, Antioxidant activity, Human hepatoma HepG2 cells, Comet assay, Antioxidant enzymes

1. Introduction

Oxygen is essential to many living organisms. It provides energy for biomass-to-fuel conversion necessary for the biological processes. However, the metabolism of oxygen generates 'free radicals' – reactive oxygen species (ROS) which represent a class of highly reactive molecules. They are derived from oxygen and generated by metabolic processes in human beings and by external factors such as pollution, radiation or some dietary habits. When overproduced during different conditions such as excessive exercise, hypoxia and/or antioxidant system failure, ROS destroy macromolecules such as DNA, proteins and lipids and are detrimental to cells (Alho & Leinonen, 1999). The oxidative damage of biological molecules is an important event in the development of various human disorders that result from oxidative stress overwhelming the biological defence system. There is increasing evidence to suggest that many degenerative diseases, such as brain dysfunction, cancer, heart diseases, and weakened immune system, could be the results of cellular damage caused by free radicals. Antioxidants present in the human diet may play an important role in disease prevention. The dietary intake of antioxidants or compounds that ameliorate or enhance the biological antioxidant mechanisms can prevent and in some cases help in the treatment of oxidative-related disorders including cancer (Halliwell & Gutteridge, 1999).

Salvia officinalis L. (SO) and *Thymus vulgaris* L. (TV) (Lamiacae) are common aromatic medicinal plants native to Mediterranean countries, widely used in medicine and cooking (Tsai, Lin, Lin, & Yang, 2011). Application of plant extracts prepared from SO and TV has a long tradition in human society, as these extracts manifest remarkable biological effects (antibacterial, fungistatic, virustatic, antioxidant, analgesic) and have preventive and therapeutic activity against many diseases (e.g., bronchial asthma, inflammatory affection, hepatotoxicity, atherosclerosis, ischaemic heart disease, cataracts, cancer, insufficient sperm mobility) (Slameňová, Horváthová, Šramková, & Lábaj, 2007).

The antioxidant properties of SO reside mainly in its phenolic nature (Baricevic & Bartol, 2000) and are described in several studies/reviews (Lamaison, Petitjean-Freytet, & Carnat, 1991; Shan, Cai, Sun, & Corke, 2005; Zheng & Wang, 2001). Lima, Andrade, Seabra, Fernandes-Ferreira, and Pereira-Wilson (2005) determined significant increase of the liver antioxidant enzyme glutathione-S-transferase (GST) activity in rats and mice of sage drinking groups. Similar effects were observed also in in *vitro* conditions, as in rat hepatocytes (isolated from the livers of sage drinking rats) a

significant increase of glutathione (GSH) level and GST activity was found. Additionally, the treatment of rats with water extracts of sage for 5 weeks protected rat hepatocytes against azathioprine toxicity (Amin & Hamza, 2005).

Youdim and Deans (1999) measured changes in antioxidant enzyme activity of different organs during the lifetime of rats. They found out that dietary supplementation of *T. vulgaris* reduced the unfavourable age-related decline in activities of superoxide dismutase in liver and heart of old rats. These results highlighted the benefit of TV as a dietary antioxidant. Alcohol extract of TV leaves manifested hepatoprotective effects in rats and antibacterial action (Oyewole, Owoseni, & Faboro, 2010). Investigation of effects of thyme essential oil compounds on human lymphocytes showed that a short-term treatment of cells with low concentrations of TV oil components protected their DNA against several genotoxins [(2-amino-3-methylimidazo[4,5-f]-quinoline (IQ), mitomycin C (MMC) and hydrogen peroxide (H_2O_2)] (Aydin, Başaran, & Başaran, 2005).

Despite all the above mentioned beneficial effects of SO and TV at the level of multicellular living organisms, little is known about the antioxidant effects of SO and TV extracts at the cellular level. The use of mammalian cell systems in *vitro* is very useful in investigating the ability of natural compounds to reduce or eliminate genotoxic effects of oxidative carcinogens. These systems have a spectrum of protective mechanisms that shield important cellular biomacromolecules against toxic effects of oxidants at their disposal and simulate innate conditions in living organisms.

In this study we analysed the chemical composition and measured the concentrations of the main phenolic components of SO and TV extracts and evaluated their potential protective effects against hydrogen peroxide (H_2O_2)- and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ)-induced oxidative DNA damage in HepG2 cells. Furthermore we investigated the activities of enzymatic and non-enzymatic antioxidants (namely superoxide dismutase – SOD, glutathione peroxidase – GPx, and intracellular glutathione – iGSH) in HepG2 cells treated with SO and TV extracts. To determine antioxidant activity of SO and TV extracts we used 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays.

2. Materials and methods

2.1. Chemicals

S. officinalis L. (SO) and *T. vulgaris* L. (TV) extracts were prepared and provided by Calendula Inc. (Nová Ľubovňa, Slovakia). Production of dry extracts from leaves of SO and haulm of TV were carried out in an enamel boiler using 40% ethanol as the extraction reagent. After centrifugation of extracted plant materials the concentration of liquid extracts were adjusted by condensation in a vacuum evaporator and then dried in a spray oven. Dry matter of both extracts was 96 ± 1%. The plant extracts were kept dry and in the dark, and diluted in medium to reach the final concentrations (0.01–100 mg/ml) just before use.

HPLC-grade methano-formic acid (98–100%) was purchased from Merck (Germany). Water was purified using a QPLUS185 system from Millipore (Billerica, MA, USA). The following reagents and compounds were used: 2,2′-azinobis(ethyl-2,3-dihydrobenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) from Sigma–Aldrich (St. Louis, MO, USA) and sodium persulfate ($Na_2S_2O_8$) from Merck (Darmstadt, Germany). The following standards were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (+)-catechin hydrate, rosmarinic acid, syringic acid, p-coumaric acid and luteolin from Sigma–Aldrich; caffeic acid, protocatechuic acid and hesperidin from Fluka (Buchs, Switzerland); homoorientin and isovitexin from LGC Standards (London, UK).

Hydrogen peroxide (H_2O_2; Sigma–Aldrich) was stored at 4 °C and diluted in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free) to a final concentration of 300 μM immediately before treatment of HepG2 cells. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ; Sigma–Aldrich) was kept at a concentration of 50 mM at −20 °C. HepG2 cells (1×10^6) were treated with 50 μM DMNQ in petri dishes (Ø = 40 mm) for 30 min at 37 °C in a CO_2 incubator. From each sample 250 μl (2.5×10^5 of HepG2 cells) were taken, added to 750 μl of PBS in Eppendorf tubes and centrifuged (1000 rpm, 5 min). After centrifugation, the supernatants were removed from each sample and low-melting-point agarose (LMP; 300 μl) was added to the cell residues. The cells were mixed and spread (2×10^4) on a base layer of 1% normal-melting-point (NMP) agarose placed on microscopic slides and covered with cover slips.

Formamidopyrimidine-DNA-glycosylase (Fpg; Biolabs, Biotech Slovakia) was stored at −20 °C and diluted in a buffer containing 40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA; Sigma–Aldrich), pH 8, prior to use. The dilution used was 0.2 U/slide.

RANSOD kit was purchased from Randox Laboratories Ltd. (Crumlin, UK) and glutathione reductase, glutathione, NADPH from Sigma–Aldrich. Media and chemicals used for cell cultivation were purchased from Gibco BRL (Paisley, UK). All other chemicals were of analytical grade from commercial suppliers.

2.2. Chromatographic determination of bioactive compounds of SO and TV by high-performance liquid chromatography (HPLC) with diode-array detection (DAD) and mass spectrometry (MS)

Phenolic compounds in sage and thyme extracts (25 mg of dry extract for 1 ml of water) were analysed by Agilent Technologies 1200 Series HPLC–DAD–MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with Zorbax XDB C8 (150 × 4.6 mm, 3.5 μm) column. The phytochemical resolution was carried out using a mobile phase composed of water (solvent A) and methanol (solvent B), both acidified with 0.1% (v/v) formic acid, at a flow rate of 1 ml/min; the injection volume of all samples was 4 μl. Elution was conducted with a linear gradient program: 0–30 min: 15–50% B; 30–35 min: 50–100% B. MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 120 V; drying gas temperature, 350 °C; gas flow (N_2), 12 l/min; nebuliser pressure, 35 psi g. The instrument was operated in positive and negative ion mode, scanning from *m/z* 100 to 1000. Individual compounds were identified by comparing their retention times and spectra with those for standards or on the basis of available literature spectral data. For quantitative determination of analytes, the calibration curves were generated by the integration of the areas of absorption peaks determined during chromatographic analysis of serial dilutions of available standards.

2.3. Cell culture

Malignant cell line HepG2 (human hepatocellular carcinoma cells) was obtained from Prof. A.R. Collins (University of Oslo, Oslo, Norway). The cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum and antibiotics (penicillin 200 U/ml, streptomycin 100 μg/ml, kanamycin 100 μg/ml) on plastic petri dishes (Ø = 10 cm) at 37 °C in humidified atmosphere of 5% CO_2.

2.4. Cytotoxicity of SO and TV

HepG2 cells were seeded into the series of 96 well plates at a density of 2×10^4/well and cultured in complete RPMI 1640

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medium. Exponentially growing cells were then pre-incubated in the presence of SO (0.01–100 mg/ml) and TV (0.01–100 mg/ml) or without extracts (control) for 24 h and used for testing of cytotoxicity by the MTT assay. MTT test is a colorimetric method for measuring the activity of mitochondrial enzymes that reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphentltetrazolium bromide), a yellow tetrazole, to purple formazan. This reduction takes place only when reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells. In our experiments, we incubated the properly treated HepG2 cells in 100 μl of complete RPMI medium + 50 μl of 1 mg/ml MTT solution for 4 h. For each sample at least four wells were used. Then, MTT solution was removed, 100 μl of dimethyl sulfoxide (DMSO; Sigma–Aldrich) were added to each well and plates were placed on an orbital shaker for 30 min to completely dissolve the formazan crystals. Absorbance at 540 nm was measured using an xMark™ microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and background absorbance at 690 nm was subtracted.

2.5. Single cell gel electrophoresis (SCGE, the comet assay)

The procedure of Singh, McCoy, Tice, and Schneider (1988) was used with minor modifications suggested by Slameňová et al. (1997). In brief, in the conventional comet assay used in combination with H_2O_2-treatment there were 2×10^4 aliquots of HepG2 cells embedded in 0.75% LMP agarose. This cell suspension was spread on a base layer of 1% NMP agarose in PBS on microscopic slides and covered with cover slips. After solidification of the gels, the cover slips were removed and treatment of cells with 300 μM H_2O_2 (5 min on ice in the dark) was carried out. The samples treated with DMNQ were processed as described in Section 2.1. The slides containing HepG2 cells treated with H_2O_2 were then placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10 and 1% Triton X-100, at 4 °C) for 1 h to remove cellular proteins. Slides were transferred to an electrophoresis box and immersed in an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13). After 40 min unwinding time, a voltage of 25 V (0.3 A) was applied for 30 min at 4 °C.

In the modified comet assay (Collins, Duthie, & Dobson, 1993) used for detection of oxidative DNA lesions induced by DMNQ, the gels were washed after lysis twice for 10 min in endonuclease buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, pH 8) and incubated with Fpg enzyme for 30 min at 37 °C. The control slides were incubated with endonuclease buffer containing BSA.

The following steps of unwinding and electrophoresis were identical both in the conventional and modified procedure, i.e., the slides were neutralised with 3 × 5 min washes with Tris–HCl (0.4 M, pH 7.4), and stained with ethidium bromide (EtBr, 5 μg/ml; Sigma–Aldrich). EtBr-stained nucleoides were examined with a Zeiss Imager.Z2 fluorescence microscope using computerised image analysis (Metafer 3.6, Meta Systems GmbH, Altlussheim, Germany). The percentage of DNA in the tail (% of tail DNA) was used as a parameter for measurement of DNA damage (DNA strand breaks). One hundred comets were scored per each sample in one electrophoresis run.

2.6. Reduced glutathione content

Intracellular glutathione (iGSH) was measured by flow cytometry (CANTO II; Becton Dickinson, Franklin Lakes, NJ, USA) using monochlorobimane (MCB; Sigma–Aldrich) staining for iGSH. 1–2×10^6 control HepG2 cells and the same amount of cells treated with SO or TV extracts were stained with 40 μM MCB at room temperature for 20 min. Cells were chilled by addition of ice-cold PBS at 0 °C, to stop the enzyme-dependent staining reaction. HepG2 cells were then washed, maintained at 4 °C and 5 μl of propidium iodide (PI; 1 mg/ml) were added. PI fluorescence was used to exclude the dead cells from the analysis. Fluorescence of MCB–GSH conjugate, representing iGSH content, was detected using 405 nm excitation laser and 450/50 emission bandpass filter (Violet 1-A). Viable cells were analysed by FCS Express 4.0 (De Novo Software, Los Angeles, CA, USA).

2.7. Antioxidant enzyme activity assays

HepG2 cells were treated with SO (2, 1 and 0.5 mg/ml) or TV (1, 0.5 and 0.1 mg/ml) for 24 h. For determination of superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GPx, EC 1.11.1.9) activities, we used 3×10^4 treated and untreated (control) HepG2 cells, which were solved 1:1 in 0.1% Triton X-100. For determination of SOD we used 1.5×10^4 of HepG2 cells and the RANSOD kit. The method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The activity is measured by the degree of reaction inhibition. For GPx determination according to the method of Paglia and Valentine (1967), we used 3×10^4 HepG2 cells and cumene hydroperoxide as a substrate.

2.8. Antioxidant activity

The antioxidant potential was evaluated using the following in *vitro* methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays.

The antioxidant activity of SO and TV was measured in terms of DPPH free radical scavenging ability with slight modifications (Tagashira & Ohtake, 1998). Quercetin was used as a reference/positive compound. A methanol and water solutions of SO and TV (50 μl) at different concentrations was placed in a cuvette and 1 ml of 23.7 μg/ml methanol solution of DPPH radical was added followed by incubation for 30 min. The decrease in absorbance at 517 nm was determined with a Specol 221 spectrophotometer. All determinations were performed in triplicate. The percentage inhibition of DPPH radical by the samples was calculated according to the following formula:

$$\text{DPPH scavenging activity } (\%) = [(AC(0) - AA(t))/AC(0)] \times 100$$

where AC(0) is the absorbance of the control (without antioxidant) at $t = 0$ min and AA(t) is the absorbance of the sample (with antioxidant) at $t = 30$ min. A dose–response curve was plotted for determining the SC_{50} values. SC_{50} was defined as the concentration sufficient to obtain 50% of maximum scavenging capacity.

Total antioxidant activity (TAA) was investigated using FRAP assay, based upon reduction of Fe^{3+}–TPTZ complex under acidic conditions. Increase in absorbance of blue-coloured ferrous form (Fe^{2+}–TPTZ complex) is measured at 593 nm. FRAP reagent was freshly prepared by mixing 25 ml of acetate buffer (300 mM, pH 3.6), 2.5 ml of TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.5 ml of $FeCl_3$ (20 mM) in water solution. One hundred microlitres of each extract dissolved in appropriate solvent were added to 4.5 ml of FRAP reagent, stirred and incubated for 30 min; absorbance was measured at 593 nm using FRAP working solution as blank. All determinations were performed in triplicate. The results were calculated on a basis of a standard curve obtained using quercetin and expressed as the relative quercetin equivalents (Kukic et al., 2008).

For on-line profiling of antioxidants, the HPLC–DAD system (Agilent Technologies) was connected to a Pinnacle PCX Derivatisation Instrument (Pickering Laboratories, Inc., Mountain View, CA, USA) and UV–vis detector (MWD; Agilent Technologies). The

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conditions of chromatographic separation of bioactive compounds present in *Salvia* and *Thymus* infusions were identical with those described above. The post-column derivatisation procedure was done according to Kusznierewicz, Piasek, Bartoszek, and Namiesnik (2011). In all experiments, a 0.5 ml (PTFE, 0.25 mm, 10 m) reaction coil heated to 130 °C was used. The derivatisation reagent was prepared as follows: ABTS was dissolved in aqueous sodium persulfate (2.45 mM) to obtain a concentration of 7 mM. The mixture was stored in the dark at room temperature for 12 h and then diluted with methanol to a concentration of 30% (v/v). ABTS solution was fed into the system at a flow rate of 0.2 ml/min. Chromatograms after derivatisation with ABTS were registered at 734 nm. The equation of the standard line (Trolox concentration = *f*(peak area)) determined for ABTS derivatisation reagent was used to calculate TE values from the peak areas of analytes obtained for plant extracts following derivatisation.

2.9. Statistical analysis

The results represent a mean from 3 to 5 experiments ± standard deviation (SD). For the H_2O_2-treatment three parallel slides and for the DMNQ-treatment, six parallel slides (three for incubation with Fpg enzyme and three for incubation with endonuclease buffer) were made in one experiment. The differences between defined groups were tested for statistical significance using Student's *t*-test ($^{*,+}p < 0.05$; $^{**,++}p < 0.01$; $^{***,+++}p < 0.001$). For the purpose of comparing dose–response effects linear regression analysis was used.

3. Results

3.1. Chemical composition of plant extracts

UV-chromatograms of sage and thyme extracts recorded at 270 nm are shown in Fig. 1. The phenolic acids and flavonoids were separated within 25 min. Table 1 compiles the data for the main detected peaks. All listed peaks showed higher intensity when negatively ionised than when positive ionisation mode was used. The identification of peaks was confirmed by comparison with standard compounds or with published data. On the basis of chromatographic and spectrometric data of available standards, seven compounds were identified and a further 15 structures were deduced according to UV and MS spectra comparison with literature data. Among these 22 compounds, there were 11 phenolic acids and 11 flavonoids – mostly flavone glycosides. The mass spectra collected during analysis reveal fragmentation patterns characterised by a major molecular ion peak that in the case of positive ionisation occurs often as sodium adduct [M+Na] (Table 1). In the case of almost all glycosides, the loss of sugar moiety was observed. The quantitative estimation of phenolic compounds shown in Table 1 was calculated as concentration in dry mass of extracts (mg/g). Since reference compounds were not available for most of the detected phenolics, luteolin glycosides were quantitated as homoorientin (at 350 nm), apigenin glycosides as isovitexin (at 330 nm), caffeic acid derivatives as rosmarinic acid (at 330 nm), and flavanone glycosides as hesperidin (at 285 nm).

3.2. Effects of plant extracts on HepG2 cell viability

Cytotoxic effects of 24-h treatment of different concentrations of SO (0–100 mg/ml) and TV (0–100 mg/ml) extracts were evaluated in HepG2 cells by the MTT assay. The results are summarised in Fig. 2. IC_{50} values (median inhibitory concentrations that cause approximately 50% cell death) were 5.7 mg/ml for SO and 4.3 mg/ml for TV extracts, respectively. Further studies assessing genotoxic and DNA-protective effects of plant extracts were assessed at $IC_{\sim 10-40}$.

3.3. DNA-protective effects of plant extracts against DNA strand breaks induced by H_2O_2

The level of DNA single strand breaks induced in HepG2 cells by H_2O_2 was determined by SCGE. As a suitable concentration of H_2O_2 300 μM was chosen for our further experiments (data not shown). The percentage of tail DNA corresponding to the level of DNA strand breaks at this H_2O_2 concentration was ~50% and it was regarded as a positive control. The reduction of H_2O_2-induced DNA damage by 24-h pre-treatment of HepG2 cell with SO (2, 1 and 0.5 mg/ml) or TV (1, 0.5 or 0.1 mg/ml) is shown in Fig. 3A. DNA damage induced by 300 μM H_2O_2 was significantly decreased by SO at concentrations of 2 and 1 mg/ml and by TV at concentrations of 1 and 0.5 mg/ml. The level of DNA strand breaks induced in HepG2 cells by SO and TV extracts alone did not differ significantly from the level of DNA strand breaks in untreated control cells (data not shown).

3.4. DNA-protective effects of plant extracts against DNA strand breaks and Fpg-sensitive sites induced by DMNQ

Fig. 3B shows DNA-damaging effects of DMNQ on HepG2 cells. Preliminary experiments showed a high sensitivity of HepG2 cells against the genotoxic effects of DMNQ (data not shown). For experiments in which DNA-protective effects of SO and TV were investigated we chose 30-min treatment of cells with 50 μM DMNQ. The level of both oxidative DNA lesions (Fpg-sensitive sites) and DNA frank breaks was determined by the modified comet assay in three groups of cells. These represented: (i) control untreated cells, (ii) cells treated with DMNQ (50 μM for 30 min), and (iii) cells pretreated with SO (2, 1 and 0.5 mg/ml) or TV (1, 0.5 and 0.1 mg/ml) and then exposed to DMNQ. Results show that SO and TV significantly reduced the levels of both types of DMNQ-induced DNA lesions at all concentrations tested.

3.5. Effects of plant extracts on antioxidant status of HepG2 cells

HepG2 cells were treated with SO (1–4 mg/ml) or TV (0.1–1 mg/ml) or without extracts (control) for 24 h; iGSH content, superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) activity were determined. Treatment of HepG2 cells with TV at 0.1 mg/ml had no effect, whereas higher TV concentrations (1 and 0.5 mg/ml) induced significant increase of GPx activity compared to the control cells. Treatment of cells with SO (4, 2 and 1 mg/ml) also increased GPx activity significantly (Fig. 4A). On the other hand, SOD activity determined in cells treated with TV or SO extracts was significantly lower than SOD activity of control HepG2 cells (Fig. 4B). As for the iGSH content, we did not find any significant differences in iGSH content in untreated control HepG2 cells and cells treated with SO or TV extracts (data not shown).

3.6. Antioxidant activity of plant extracts

Table 2 presents the radical-scavenging activity of plant compounds evaluated by DPPH and ABTS assays. SO and TV extracts displayed antioxidant activities lower than that measured for the quercetin positive control. The ability of an antioxidant to quench free radicals by hydrogen donation was exploited for the on-line HPLC-coupled profiling of antioxidants in SO and TV extracts. This approach enabled the characterisation of antioxidant phytochemicals in these extracts, as well as determination of their individual antioxidant activities. The examples of chromatograms for extracts prepared from sage and thyme obtained during on-line antioxidant

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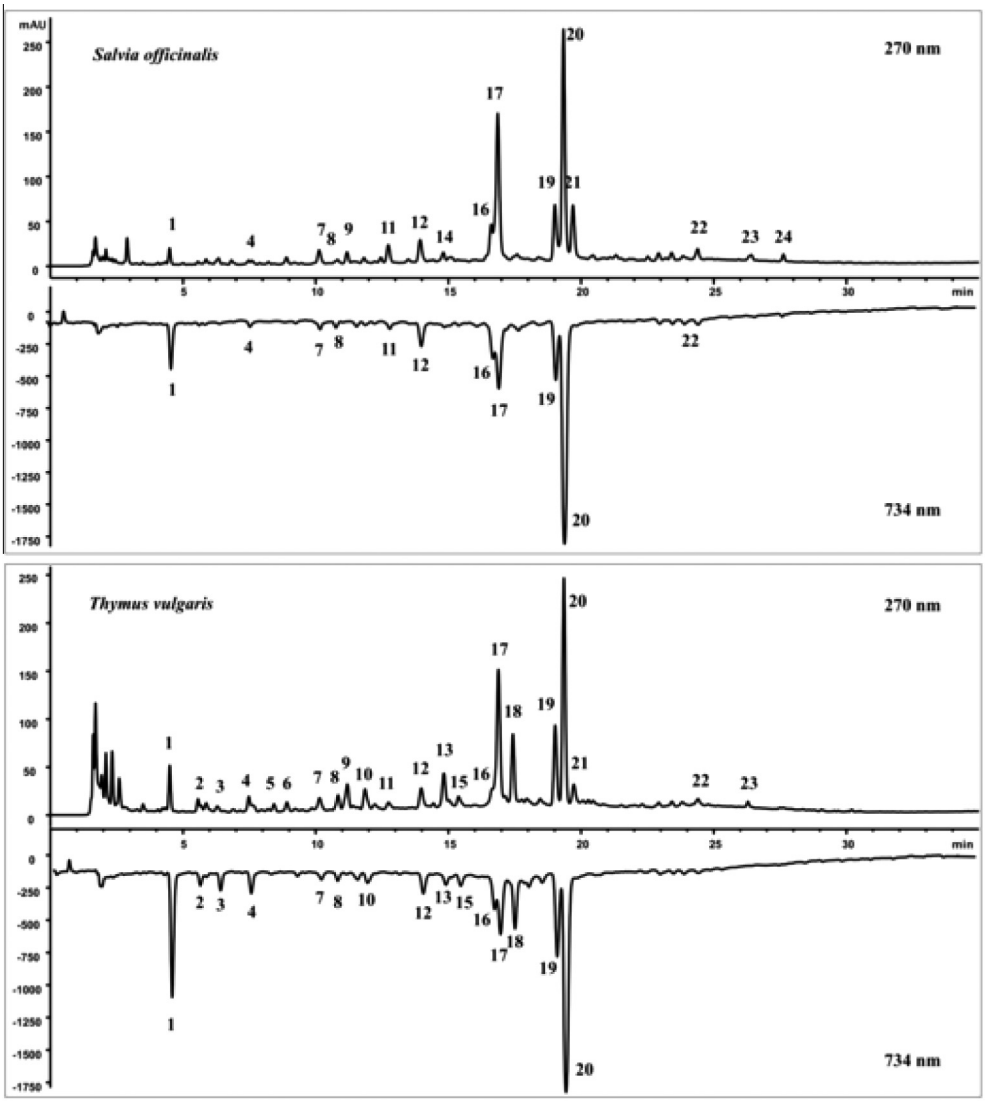

Fig. 1. The sample HPLC chromatograms of phenolic compounds (270 nm) obtained for infusions prepared from *S. officinalis* and *T. vulgaris* dried extracts along with the profiles of antioxidants detected on-line with ABTS (743 nm). Compound names corresponding to each peak are given in Table 1.

profiling with ABTS as derivatisation reagent are presented in Fig. 1. The post-column detection of the reduction of the ABTS radical in relation to the content of antioxidants detected is reflected by the negative chromatogram at 734 nm. As can be seen, rosmarinic acid (peak No. 20) and other caffeic acid derivatives (peakss Nos. 1, 18 and 19) are the major analytes responsible for antioxidant activity of samples studied. The contribution of luteolin glycosides (peak Nos. 16 and 17) is markedly smaller. Additionally the antioxidant activity of separated compounds was calculated as Trolox equivalents (TE [mg/g]), and these values are presented in Table 1. The sum of Trolox equivalents of individual antioxidants shows 25% higher activity of TV extract ($\sum$ 87 mg/g) than SO extract ($\sum$ 66 mg/g). The same relation was observed in FRAP assay where reducing power of TV extract was also 25% higher than SO extract: 474 vs. 354 mg of quercetin equivalents per gram of extract, respectively.

4. Discussion

S. officinalis and *T. vulgaris*, observed in our study, are very important representatives of medicinal plants. The leaves of *S. officinalis* (SO), well known for their antioxidant properties, are used in the food processing industry, but they are applicable also in many areas of human health (Eidi, Eidi, & Bahar, 2006). The genus *Thymus* comprises 300–400 species, several of which are utilised in folk medicine. *T. vulgaris* (TV) is the most important medicinal type of *Thymus*; it has been recommended to cure dry coughs, bronchitis, indigestion and gastritis (Hajimehdipoor, Shekarchi, Khanavi, Adib, & Amri, 2010).

Cytotoxicity of plant extracts studied was determined with help of MTT assay. Our results showed that 24-h long treatment of cells with SO and TV extracts affected cell viability in a dose-dependent manner; IC_{50} values equalled 5.7 mg/ml for SO and 4.5 mg/ml for TV extracts, respectively. Aherne, Kerry and O'Brien (2007) determined the viability of Caco-2 cells following 24-h long treatment with various concentrations of sage and reported IC_{50} values at much higher concentrations of sage extract. The discrepancy between our and their results could be connected with the fact that the cytotoxicity of sage and thyme extracts is dependent on different factors, including geographical location of the plant, parts of plants used for extracts preparation, kind of extraction protocol (solvent, temperature, time, etc.). It is very interesting that even the drying methods of plants used for preparation of extracts could

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Table 1
The qualitative and quantitative data on phenolic compounds present in *S. officinalis* and *T. vulgaris* obtained during chromatographic analyses.

Class	Peak No.	Compound	t_R (min)	Mass data		*S. officinalis*		*T. vulgaris*	
				MS(+)	MS(−)	(mg/g)[a]	TE[b] (mg/g)	(mg/g)[a]	TE[b] (mg/g)
HCA	1	Danshensu	4.6	221 [M+Na]	197 [M−H], 395 [2M−H]	0.32	3.84	0.90	10.21
HBA	2	Protocatechuic acid	5.7	155 [M+H]	153 [M−H]	0.01	0.42	0.15	2.02
Fol	3	(+)Catechin	6.4	291 [M+H]	289 [M−H]	nd	nd	1.38	2.11
HCA	4	Salvianolic acid I isomer	7.6	561 [M+Na]	583 [M+HCOO]	0.20	1.17	0.69	2.52
HCA	5	Caffeoyl hexoside	8.6	181 [M+H−Hex], 365 [M+Na]	179 [M−H−Hex], 341 [M−H]	nd	nd	0.22	0.91
HCA	6	Coumaroyl hexoside	9.1	165 [M+H−Hex], 349 [M+Na]	163 [M−H−Hex], 325 [M−H]	nd	nd	0.30	0.80
HCA	7	Caffeic acid	10.3	181 [M+H]	179 [M−H]	0.68	1.72	0.59	1.43
HBA	8	Syringic acid	11.1	221 [M+Na]	197 [M−H]	0.29	0.72	0.77	1.34
Fon	9	Diglucosyl-apigenin	11.4	595 [M+H]	593 [M−H]	0.66	nd	2.22	0.59
Fva	10	Eriodictyol-glucoside	12.1	289 [M+H−Hex], 473 [M+Na]	449 [M−H]	0.23	1.08	0.31	2.15
Fon	11	Luteolin-diglucoronide	13.0	639 [M+H]	637 [M−H]	1.16	0.79	0.70	0.56
Fva	12	Hesperetin-glucoside	14.3	303 [M+H−Gluc], 465 [M+H]	463 [M−H]	0.36	3.73	0.32	1.97
Fva	13	Eriodictyol-glucoronide	15.1	289 [M+H−Glur], 465 [M+H]	463 [M−H]	nd	nd	0.60	2.80
HCA	14	Methylmelitric acid A	15.2	575 [M+Na]	551 [M−H]	0.45	2.04	nd	nd
Fon	15	Luteolin-glucoside	15.7	287 [M+H−Gluc], 449 [M+H]	447 [M−H]	nd	nd	1.65	2.09
Fon	16	Luteolin-hexoside	16.9	287 [M+H−Hex], 449 [M+H]	447 [M−H]	2.71	5.58	2.04	4.31
Fon	17	Luteolin-glucoronide	17.2	287 [M+H−Glur], 463 [M+H]	461 [M−H]	1.16	8.96	1.16	7.92
HCA	18	Unknown Mw = 522	17.6	545 [M+Na]	521 [M−H]	nd	nd	4.78	6.90
HCA	19	Salvanolic acid K isomer	19.4	579 [M+Na]	555 [M−H]	2.71	6.72	3.10	8.68
HCA	20	Rosmarinic acid	19.7	383 [M+Na]	359 [M−H]	16.33	28.5	14.72	26.61
Fon	21	Apigenin-glucoronide	20.1	271 [M+H−Glur], 447 [M+H]	445 [M−H]	4.32	nd	1.80	nd
Fon	22	Luteolin	24.7	287 [M+H]	285 [M−H]	0.47	0.91	0.28	0.61

[a] Contents of compounds in mg/g of dry weight of extract.
[b] Antioxidant activity of compound determined by HPLC coupled post-column derivatisation with ABTS, expressed as Trolox equivalents (TE) in mg/g of dry weight of extract. Each value is a mean of three replicates and standard deviations do not exceed 15%. Abbreviations: t_R, retention time; HCA, hydroxycinnamic acids and derivatives; HBA, hydroxybenzoic acids and derivatives; Fon, flavones; Fol, flavanols; Fva, flavanones; MS(+), positive ionisation mode; MS(−), negative ionisation mode; nd, not detected; Gluc, glucoside; Glur, glucoronide; Hex, hexose.

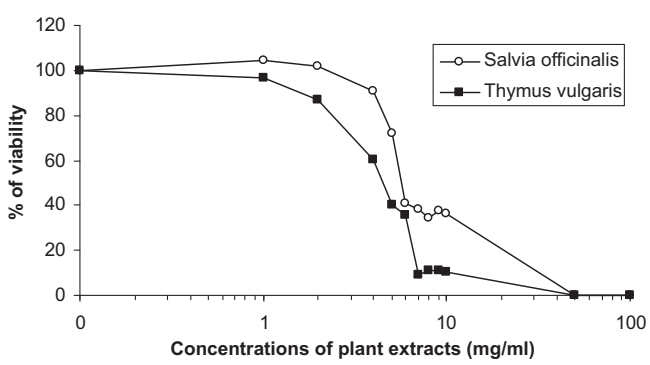

Fig. 2. Cytotoxicity/viability of HepG2 cells treated with *S. officinalis* or *T. vulgaris* extracts (0–100 mg/ml) for 24 h.

be responsible for the content of phenolics and flavonoids as well as antioxidant activity of extracts (Hamrouni-Sellami et al., 2013).

It is well-known that oxidative stress occurs when free radical production exceeds the antioxidant capacity of a cell. Most reactive oxygen species (ROS), such as hydroxyl radicals (OH), hydrogen peroxide and superoxide anions, can damage crucial cellular compounds such as lipids, carbohydrates, proteins and DNA (Halliwell & Gutteridge, 1999). Superoxide anions are thought to be involved in inflammatory reactions, since they are produced by phagocytic cells which produce hydroxyl radicals and singlet oxygen. In this study, we used two strong oxidants, hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) for induction of DNA damage. H_2O_2 induces predominantly DNA breaks via the formation of $^{\cdot}$OH radicals by the Haber–Weiss reaction, which is catalysed by ferric ion. Like all reactive oxygen species, it is generated from nearly all sources of the oxidative cycle and has the ability to diffuse in and out of cells and tissues. Among ROS,$^{\cdot}$OH has an extremely high reactivity with DNA, lipids and proteins, leading to cellular injury (Barbouti, Doulias, Nousis, Tenopoulou, & Galaris, 2002). The second oxidant used, DMNQ, belongs to the quinones. Their toxicity is induced by two principal mechanisms; they (i) react covalently with thiols (reduced glutathione-GSH) or the cysteine residues of proteins, in order to form arylation products that eventually cause cellular damage (Tapper, Sheedy, Hammermeister, & Schmieder, 2000); or (ii) induce oxidative stress via redox cycling. DMNQ has no arylating moiety in its structure and several studies have established that its toxicity is mediated via redox cycling. Quinones undergo one-electron reduction to yield semiquinone radicals, which are then reoxidised in cooperation with molecular oxygen to form superoxide anions. These are converted into ROS (hydrogen peroxide, hydroxyl radical, singlet oxygen) through various pathways within cells (Gant, Rao, Mason, & Cohen, 1988).

Exposure of HepG2 cells to 300 μM H_2O_2 or 50 μM DMNQ resulted in significant single strand DNA breaks formation. The plant extracts themselves did not induce DNA damage at the concentration ranges studied. The highest protective effects of SO and TV against H_2O_2- or DMNQ-induced DNA damage were observed in HepG2 cells at 2 mg/ml (SO) and 0.5 mg/ml (TV). Our results are in accordance with findings of Aherne, Kerry and O'Brien (2007), who described the DNA-protective activity of *S. officinalis* against H_2O_2-induced DNA damage in Caco-2 cells.

Antioxidant effectiveness of plant extracts in *vitro* is probably caused by their ability to act as reducing agents and free radical scavengers or as quenchers of singlet oxygen formation. Some authors ascertained the fact that phenolic compounds were able to chelate metal ions. Melidou, Riganakos, and Galaris (2005) found that intracellular binding of iron is responsible for the protection offered by flavonoids against H_2O_2-induced DNA damage. On the other hand complexation of plant extracts with metal ions results in a significant reversal from antioxidant to pro-oxidant properties for the resulting complexes (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003).

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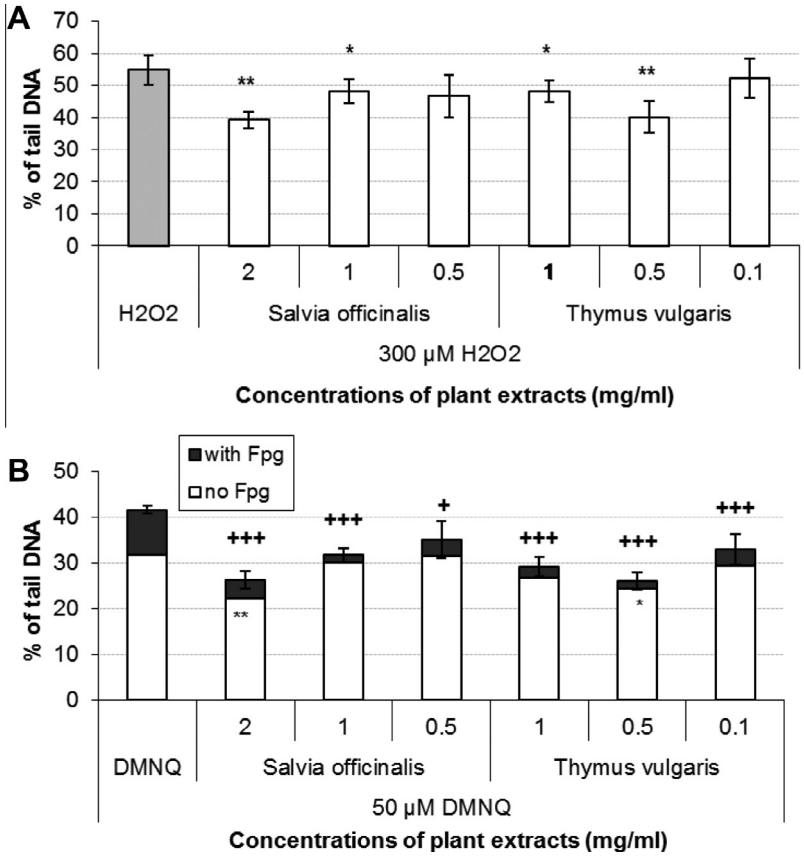

Fig. 3. The levels of DNA damage in HepG2 cells pre-treated with *S. officinalis* or *T. vulgaris* extracts for 24 h and then exposed either to 300 µM H_2O_2 (A) or 50 µM DMNQ (B). White or grey bars or portions of bars indicate frank DNA breaks and alkali-labile sites and black portions of bars symbolise additional Fpg-sensitive sites. Data represent means ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ indicate significant differences compared to H_2O_2- or DMNQ-induced frank DNA breaks and alkali-labile sites and $^{+}p < 0.05$; $^{+++}p < 0.001$ indicate significant differences compared to DMNQ-induced Fpg-sensitive sites, respectively.

The genoprotective effects of SO and TV extracts could be caused, at least in part, by their free radical-scavenging efficiency and reducing power, as a result of their phenolic and/or non-phenolic constituents. Antiradical effects of SO and TV have already been evaluated by the most common radical-scavenging assays using 1,1-diphenyl-2-picrylhydrazil (DPPH) radical (Lamaison et al., 1991) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical (Shan et al., 2005), as well as on the basis of their oxygen radical absorbance capacity (ORAC) (Zheng & Wang, 2001). SO and TV manifested antioxidant capacity also in the system of HepG2 cells treated with tert-butyl hydroperoxide (Lima et al., 2007). Utilising DPPH, FRAP and ABTS assays we verified reducing power and radical-scavenging activities of SO and TV extracts used in our experiments. We found out that the sage and thyme extracts that we used manifested significant antioxidant activity.

The destruction of reactive oxygen intermediates and free radicals involves the activities of SOD, catalase, GPx, and iGSH. The antioxidant defence enzymes, namely SOD and catalase, protect aerobic cells against O_2 toxicity and lipid peroxidation. Glutathione peroxidase (GPx) is localised in the cytoplasm and mitochondria. It catalyses the degradation of various peroxides, by oxidation of glutathione and formation of its conjugates. GPx has more affinity to H_2O_2 than to catalase. In the present study both plant extracts significantly increased GPx, while SOD was found to have a decreasing trend. Similar results were obtained in our preliminary experiments with plant extracts from *Hypericum perforatum* and *Lavandula angustifolia*, in which no effects of natural compounds on SOD activity and elevation of GPx activity were found (unpublished data). Youdim and Deans (1999), and Kuresh, Youdim, and Stanley (1999) found a decline of SOD activity during the lifetime of rats but the activity of GPx increased significantly. The observed age-related changes were suppressed in rats whose diet was supplemented with thyme oil. The authors suggested that cellular antioxidants were under homeostatic control and that dietary antioxidant reduced endogenous antioxidant synthesis; thereby they nullified the expected beneficial effect of the supplement. Changes in the level of one particular antioxidant can be compensated by a change in a different antioxidant with no benefit to the overall antioxidant system. The exact mechanism by which SO and TV affect various antioxidant parameters is not clear yet. It is possible that the antioxidant properties of SO and TV are utilised by the cells, thus sparing the intracellular antioxidant systems, such as SOD and GPx. It is also possible that SO and TV influence other cellular systems, suggesting that more detailed examination of further antioxidant and cellular parameters is required.

Analysis of the chemical composition of the plant extracts used in our experiments showed a similar phytochemical profile of both extracts. Rosmarinic acid was the main constituent of both *S. officinalis* and *T. vulgaris* extracts, representing 16.3% and 14.7%, respectively. Comparison of the two extracts with respect to cytotoxicity, DNA-protection and antioxidant capacity did not show any significant differences. The contents of polyphenolic compounds did not differ a lot (about 32 and 38 mg/g dw, respectively). The antioxidant activity of SO extract is a little lower than that of TV (66 and 87 TE mg/g dw, respectively) and apparently results from lower polyphenol content. We assume the similar effects in the biological experiments are due to the similar profile and concentration of antioxidants. Kontogianni et al. (2013) who analysed *Rosmarinus officinalis* (RO) and *S. officinalis* (SO) extracts by HPLC

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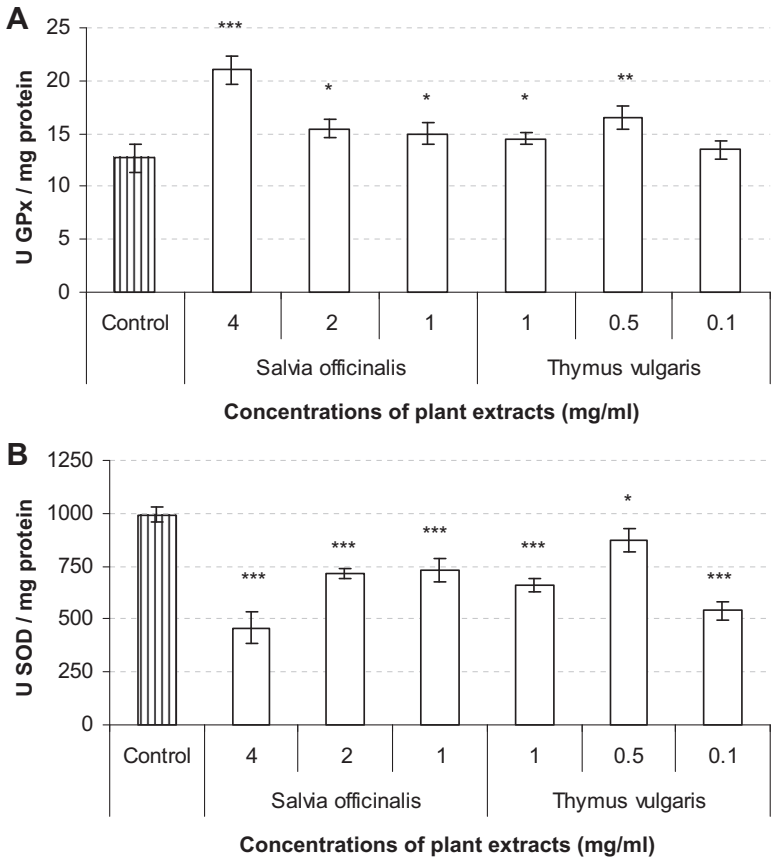

Fig. 4. The activity of enzymes glutathione peroxidase (GPx: A) and superoxide dismutase (SOD: B) in HepG2 cells treated with *S. officinalis* or *T. vulgaris* extracts for 24 h. Data represent means ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ indicate significant differences compared to the control.

Table 2
DPPH and ABTS scavenging activities of *S. officinalis* and *T. vulgaris* extracts. Quercetin was used as a positive control.

Compounds	DPPH[a,b]		ABTS			
	SC_{50}[a] (mg/l)	r^2	SC_{50}[b] (mg/l)	r^2	SC_{50}[a] (mg/l)	r^2
S. officinalis	69.09	0.9738	50.79	0.9947	19.89	0.996
T. vulgaris	44.69	0.9876	49.57	0.9942	13.84	0.989
Quercetin	2.79	0.998	–	–	1.167	0.9987

Data represent the means of three independent experiments.
[a] Solvent/dissolving agent MeOH.
[b] Solvent/dissolving agent H_2O.

and NMR, found higher antioxidant and cytotoxic effects in RO extract in comparison with SO extract. They proved that the higher antioxidant activity of RO extract is caused by the higher content of phenolic abietane diterpenoids, such as carnosic acid and its derivatives, carnosol and rosmanol isomers, while the cytotoxic activity of RO could be attributed to carnosic acid and the triterpenoids betulinic acid and ursolic acid.

Prevention is a strategy more effective than treatment of chronic diseases. Regular use of herbs in meals may be beneficial for consumers. The diet in which herbs are added generously provides a variety of bioactive phytochemicals. These could help in supporting good health by protecting tissues against ROS-induced damage and preventing the onset of chronic diseases. The findings of our study suggest that the consumption of sage and thyme in a proper amount is certainly beneficial; however, our toxicity data suggest that the consumption of high doses of these plants could be harmful for human beings. Based on the results of this paper it would be interesting to investigate whether SO and TV extracts could protect experimental animals against diseases caused by oxidative stress.

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