TITLE: Evaluation of toxic/protective effects of the essential oil of *Salvia officinalis* on freshly isolated rat hepatocytes

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RUNNING TITLE: Hepatotoxicity of Sage Essential Oil

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KEYWORDS: *Salvia officinalis*; sage; essential oil; toxic effects; antioxidant effects; hepatoprotective effects; hepatocytes.

ABREVIATIONS: DMSO – dimethyl sulfoxide; EO – essential oil; GC – gas chromatography; GC-MS – GC coupled to mass spectrometry; GSH – reduced glutathione; GSSG – oxidised glutathione; GSx – total glutathione; LDH – Lactate dehydrogenase; TBARS – thiobarbituric acid reactive substances; *t*-BHP – *tert*-butyl hydroperoxide.

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1 ABSTRACT: For this study the essential oil (EO) of sage (*Salvia officinalis* L.) was
2 isolated from air-dried vegetative aerial parts of the plants by hydrodistillation and
3 analysed by GC and GC-MS. A total yield of 12.07 mg of EO per g of plant dry mass
4 was obtained and more than 50 compounds identified. The major compounds were *cis*-
5 thujone (17.4 %), α-humulene (13.3 %), 1,8-cineole (12.7 %), *E*-caryophyllene (8.5 %)
6 and borneol (8.3 %). The EO fraction of sage tea was also isolated by partition with
7 pentane and the respective components identified. The toxic and antioxidant protective
8 effects of *S. officinalis* EO were evaluated on freshly isolated rat hepatocytes. Cell
9 viability (LDH leakage), lipid peroxidation and glutathione status were measured in
10 experiments undertaken with cells (suspensions of 1×10^6 cells per millilitre) exposed to
11 EO alone (toxicity of the EO; *t*-BHP as positive control); and with cells exposed to EO
12 and an oxidative compound (*t*-BHP) together (in EO protection evaluation; quercetin as
13 positive control) for 30 min. The results show that the EO is not toxic when present at
14 concentrations below 200 nl/ml; it was only at 2000 nl EO/ml that a significant LDH
15 leakage and GSH decrease were observed indicating cell damage. In the range of
16 concentrations tested, the EO did not show protective effects against *t*-BHP-induced
17 toxicity.
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26 INTRODUCTION

27 Sage (*Salvia officinalis* L.) enjoys the reputation of being a panacea because of
28 its wide range of medicinal effects: it has been used as an antihydrotic, spasmolytic,
29 antiseptic and anti-inflammatory and in the treatment of mental and nervous conditions
30 (Baricevic and Bartol, 2000). Sage is also used traditionally in food preparation.

31 Recently several authors reported the antioxidant properties of sage and some of
32 its constituents, mainly phenolic compounds such as carnosic, rosmarinic, caffeic and
33 salvianolic acids as well as other phenolic structure-based compounds (Cuvelier et al.,
34 1994; Hohmann et al., 1999; Lu and Foo, 2001; Wang et al., 1998; Zupko et al., 2001).
35 Sage is, therefore, one of the favourite candidate species as a source of natural
36 antioxidants in health care products. The essentaial oils (EOs) of some other plants have
37 also been shown to have antioxidant and hepatoprotective activities (Cuppett and Hall,
38 1998; Teissedre and Waterhouse, 2000), although the potentially hepatoprotective
39 effects of sage EO have, to our knowledge, not been investigated. However, potentially
40 toxic effects of sage EO especially to the liver – the main detoxifying organ – may also
41 exist and place limitations on the use of sage.

42 Sage EO is a complex mixture of tens of volatile compounds including
43 monoterpenes, sesquiterpenes and diterpenes. Importantly, the EO contributes the
44 unique flavour of sage and justifies the use of sage as a food condiment. Although some
45 data already exist relating to the toxicity or antioxidant properties of some individual
46 compounds (such as thujone and eugenol, respectively) (Cuppett and Hall, 1998; Millet
47 *et al.*, 1981), to assess the effects of the complex mixture of EO as it reaches the
48 consumer remains the best way to predict and prevent possible deleterious effects of its
49 use.

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50 The present study aims to evaluate the toxic versus antioxidant protective
51 activities of the essential oil from *S. officinalis* on freshly isolated rat hepatocytes. As
52 specific objectives the experiments had in view (a) to test whether the use of the *S.*
53 *officinalis* EO of known composition has any adverse effects on the liver, and (b) test
54 for any antioxidant effects of sage EO on liver cells challenged with an oxidizing agent
55 (*tert*-butyl hydroperoxide – *t*-BHP) and compare it to the effects of the reference
56 antioxidant quercetin.
57
58 MATERIALS AND METHODOS
59 **Chemicals**. All reagents were of analytical grade. The following reagents were
60 obtained from Sigma (St. Louis, MO, USA): collagenase (grade IV), *tert*-butyl
61 hydroperoxide (*t*-BHP), bovine serum albumin, HEPES (N-[2-
62 Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]), reduced glutathione (GSH),
63 oxidised glutathione (GSSG), glutathione reductase (EC 1.6.4.2.), 5,5'-dithio-bis-(2-
64 nitrobenzoic acid) (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced
65 form (β-NADPH), β-nicotinamide adenine dinucleotide reduced form (β-NADH),
66 quercetin and 2-thiobarbituric acid (TBA).
67 **Plant material and isolation of the essential oil.** The essential oil used in this
68 study was obtained by hydrodistillation of 226 g of air-dried vegetative aerial parts of *S.*
69 *officinalis* plants cultivated in an experimental farm located in Arouca, Portugal, and
70 collected on April, 2001. The absolute quantification of each compound was tentatively
71 achieved by GC using 5α-cholestane as an internal standard. For this purpose,
72 subsamples of 10 g from the same plant material were submitted to hydrodistillation in
73 a Clevenger type apparatus over 1 h, using volumes of 1.0 mL of n-hexane, containing
74 5α-cholestane (1mg/mL), for retention of the hydrodistillate components.

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75 **Preparation of sage tea and isolation of the essential oil constituents.**
76 Considering that sage is traditionally used as a tea, an infusion of sage was prepared by
77 pouring 150 ml of boiling water onto 2 g of the same dried plant material and allowing
78 to steep for 5 min. The essential oil constituents of the tea were extracted, at room
79 temperature, with 5 mL of n-pentane containing 5α-cholestane (1mg/mL).
80 **Analysis conditions of essential oils and procedures.** The samples of the
81 essential oils and essential oil constituents from tea containing the internal standard 5α-
82 cholestane were analyzed by GC and GC-MS. GC analyses were performed using a
83 Perkin-Elmer Autosystem gas chromatograph equipped with a fused silica DB5 column
84 (30 m long × 0.25 mm i.d., 0.25 μm film thickness composed by 5 % phenyl
85 methylpolysiloxane, J & W Scientific). Injections were performed in a split/spliless
86 injector with the splitter opened at the 1:13 split ratio under a column head pressure of
87 12.5 psi and H_2 as carrier gas at a flow rate of 1.49 ml/min. Oven temperature was
88 programmed from 60 to 285 ºC at 3 ºC min^{-1}. Injector and detector temperatures were
89 300 and 320 ºC, respectively. The same column and conditions were used in the
90 analysis performed with the GC-MS with the exception of the carrier gas which was
91 helium instead of H_2. The GC-MS consisted of a Perkin-Elmer 8500 gas chromatograph
92 equipped with a fused silica DB5 column, the same as that for GC, connected to a
93 Finnigan MAT ion trap detector (ITD; software version 4.1) operating in EI mode at 70
94 eV. Identification of the chromatogram peaks as well as quantification of the
95 compounds was performed following the methodology previously described (Santos-
96 Gomes and Fernandes-Ferreira, 2001).
97 **Animals.** Adult male Wistar rats, weighing 200-250 g, were used as hepatocyte
98 donors. The rats were kept in polyethylene cages lined with wood shavings, with wire
99 mesh top and acclimated to ambient temperature (20 ± 2 ºC) and humidity and natural

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100 light/dark cycle. The animals had free access to standard rat chow and tap drinking
101 water, and were kept in our facilities for at least 2 weeks prior to use.

102 **Hepatocyte isolation and incubation.** Hepatocyte isolation was performed
103 between 10.00 am and 11.00 am by collagenase perfusion of the liver as previously
104 described (Moldeus et al., 1978) with some modifications. In brief, the liver was
105 perfused *in situ* via the portal vein with a calcium-free Hank's solution with 0.67 %
106 albumin and 12.5 mM HEPES (pH 7.4) at 37 ºC at a flow rate of 10 ml/min, then with a
107 Hank's solution with 12.5 mM HEPES containing 0.025 % collagenase and 0.44 %
108 (w/v) $CaCl_2$ at the same flow rate and temperature for 10 min. The cells were dispersed
109 in Ca^{2+}-free Hank's solution with 0.67% albumin and 12.5 mM HEPES and washed by
110 low speed centrifugation (50 × g) for 2 min. The cells were then ressuspended in Krebs-
111 Henseleit solution with 12.5 mM HEPES and washed two more times. The hepatocytes
112 viability was > 80% as estimated by trypan blue exclusion.

113 Incubations were performed at 37ºC in suspensions of 10^6 viable cells per
114 milliliter in Krebs-Henseleit solution with 12.5 mM HEPES (pH 7.4) gassed with
115 carbogen (95 % O_2 and 5 % CO_2). The hepatocytes were always preincubated for 60
116 min at 37ºC before the beginning of the experiments. Both toxic and protective effects
117 of *S. officinalis* EO were tested on hepatocyte suspensions. In toxicity experiments the
118 cells were incubated with 10 μl/ml of EO dissolved in DMSO at final concentrations of
119 0, 2, 20, 200 and 2000 nl/ml cell suspension for 30 min. *tert*-Butyl hydroperoxide (*t*-
120 BHP) 0.75 mM was used as a positive control for cell damage. In hepatoprotective
121 experiments 10 μl/ml of EO dissolved in DMSO at final concentrations of 0, 2, 20, 200
122 and 2000 nl/ml were added to the cell suspensions 5 min before the addition of *t*-BHP
123 0.75 mM and allowed to incubate simultaneously for 30 minutes. The reference
124 antioxidant quercetin was used as positive control in the protective experiments. The

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125 cells were incubated with 10 μl/ml of quercetin dissolved in DMSO at final
126 concentrations of 0, 1.6, 12.5, 50 and 200 μM for 5 min before addition of *t*-BHP 0.75
127 mM for 30 min. At the end of experiments, aliquots of cell suspensions were taken for
128 measurement of lactate dehydrogenase (LDH) leakage, malondialdehyde, GSH and
129 GSSG contents.
130 **Analytical procedures.** Cell death was estimated by quantification of LDH
131 activity in the incubation medium versus total LDH activity in the samples (the results
132 are shown as percentages of control cell viability). The enzyme activity was measured at
133 room temperature by quantification of NADH by continuous spectrophotometry (at 340
134 nm) on a plate reader (Spectra Max 340pc, Molecular Devices).
135 The extent of lipid peroxidation was determined indirectly by the
136 malondialdehyde formation after the breakdown of polyunsaturated fatty acids,
137 measured by the thiobarbituric acid reactive substances (TBARS) assay at 535 nm as
138 described previously (Fernandes et al., 1995).
139 The glutathione content of cell suspensions was determined by the DTNB-GSSG
140 reductase recycling assay as described in Anderson (1985), with some modifications.
141 Briefly, 200 μl of cell suspension was added to 200 μl of 10 % (w/v) 5-sulfosalicylic
142 acid for protein precipitation and centrifuged 2 min at 12000 rpm. Supernatant aliquots
143 were taken out for measurement of total glutathione (GSx) following the DTNB
144 oxidation at 415 nm and compared with a standard curve. The final concentrations of
145 the assay reagents were 0.6 mM DTNB, 0.21 mM NADPH and 2 U/ml glutathione
146 reductase. For the GSSG determination, 100 μl of supernatant was derivatized with 2 μl
147 of 2- vinylpyridine and 10 μl of 50 % (v/v) ethanolamide and mixed continuously for 60
148 min. GSSG was then measured as described above for total glutathione. The GSH
149 content was calculated by subtracting GSSG content from the total glutathione content.

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150 **Statistical analysis.** Data are expressed as means ± SEM (n=5) (cells obtained
151 from 5 different rats). Significant differences between control and *t*-BHP treated cells
152 were determined by Student's *t*-test. Significant differences within EO series (EO toxic
153 and protective evaluation) were determined by one-way ANOVA followed by Dunnett's
154 test. Differences were considered significant when $P \leq 0.05$.
155
156 RESULTS
157 **Essential oil and essential oil constituents of tea from *S. officinalis* leaves.** The yield
158 of the essential oil obtained by hydrodistillation of air-dried sage aerial parts was 13.3
159 μl per gram of biomass dry weight, corresponding to 1.2% (w/w). The composition of
160 the *S. officinalis* EO used in the experiments here reported is shown in Table 1, where
161 compounds are listed according to their Kovats retention indexes. The EO includes
162 around 60 compounds most of which were identified (Santos-Gomes and Fernandes-
163 Ferreira, 2001). Around 88% of the compounds can be distributed into three terpene
164 groups: oxygen-containing monoterpenes (47.7%), sesquiterpene hydrocarbons
165 (24.5%), and monoterpene hydrocarbons (15.9%). The major compounds were *cis*-
166 thujone (17.4 %), α-humulene (13.3 %), 1,8-cineole (12.7 %), *E*-caryophyllene (8.5 %)
167 and borneol (8.3 %).
168 In the preparation of the tea only 2.99 % of total essential oil content was
169 extracted from the biomass and found in the water giving a final concentration of 4.81
170 μg of EO per ml of tea (Table 1). However, the presence of the individual essential oil
171 constituents in tea was not proportional to their specific amount in the oil extracted by
172 hydrodistillation. From the approximate 60 compounds detected in the essential oil of *S.*
173 *officinalis* only 25 were found as essential oil constituents of the respective tea (Table
174 1). Oxygen-containing monoterpenes corresponded to 86% of the tea essential oil

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175 constituents. The sum of *cis*-thujone and camphor concentrations (1.68 and 0.51 μg.ml^{-1}, respectively) corresponded to almost 45% of all the constituents (Table 1). With
177 respect to the composition of EO present in tea it is not surprising that oxygen-
178 containing monoterpenes, such as *cis*-thujone and camphor, are proportionately more
179 abundant in tea than other EO constituents in view of the higher solubility in water of
180 this group of compounds relatively to the other ones.

181 Surprisingly, the values of the specific contents of the alkanes *n*-undecane, *n*-
182 hexacosane and *n*-octacosane determined in the tea were higher than the corresponding
183 values determined in the essential oil (Table 1) obtained by hydrodistillation. The
184 respective loss during hydrodistillation is a possibility that can not be ruled out.

185 **EO toxicity experiments.** EO toxicity was tested in hepatocyte suspensions
186 exposed to the EO (0-2000 nl/ml) and compared to that of *t*-BHP (0.75 mM). Cell
187 viability measured by LDH leakage of rat hepatocytes (fig. 1) did not change
188 significantly after 30 min incubation with 0 – 200 nl of EO per ml of cell suspension.
189 However, 2000 nl/ml (0.2 % (v/v)) caused a significant decrease in cell viability. Lipid
190 peroxidation (Table 2) did not change after the incubations with different concentrations
191 of EO. Total glutathione (GSx) (Table 2) was significantly lowered by incubation with
192 the highest oil concentration tested (2000 nl/ml) but not by any of the other
193 concentrations. This decrease in GSx was accompanied by a decrease in GSH (fig. 2),
194 where incubations with 200 nl EO/ml also produced a significant decrease in GSH when
195 compared to the control. There were no significant changes in GSSG content (Table 2)
196 produced by incubation of the cells with the EO.

197 The incubations of hepatocyte suspensions with the positive control 0.75 mM *t*-
198 BHP for 30 min resulted in significant decreases in cell viability (fig. 1), GSH (fig. 2) as

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199 well as GSx content and an increase in lipid peroxidation (Table 2). We also observed a
200 perceptible increase of GSSG content (P = 0.0556) with this toxicant.
201 **EO protection experiments.** Hepatoprotection of the EO was tested in
202 hepatocyte suspensions exposed simultaneously to the EO (0-2000 nl/ml) and *t*-BHP
203 (0.75 mM) and the protective effects of the oil compared to those of quercetin.
204 The addition of 0.75 mM *t*-BHP to hepatocytes resulted in significant cell death
205 after 30 min was evident from LDH leakage (fig 3). EO in the concentrations of 2-2000
206 nl/ml did not reduce cell mortality. Quercetin, on the other hand, significantly reduced
207 LDH leakage at 200 μM. Lipid peroxidation induced by t-BHP (Table 4) was also
208 significantly reduced by co-incubation with quercetin whereas the EO had no protective
209 effects at any of the concentrations tested (Table 3). Addition of *t*-BHP significantly
210 reduced cell GSH content but no protective effects on the glutathione balance could be
211 detected for quercetin or the EO (fig 4). Co-incubation of t-BHP with 2000 nl/ml EO
212 seems to further decrease the GSH levels in the cells.
213
214 DISCUSSION
215 The liver is very active in metabolizing foreign compounds. The
216 biotransformation of xenobiotics by the liver involves chemical modifications that
217 globally increase their water solubility thereby facilitating their elimination from the
218 organism in bile and urine. These reactions may, however, result in the formation of
219 compounds of greater toxicity. Although the large biotransforming ability of the liver
220 allows efficient elimination of the most toxic compounds, it also makes hepatocytes a
221 vulnerable target for toxic effects. Damage to the cell's constituents such as lipid
222 peroxidation may occur. Reduced GSH plays an important role in hepatocytes defence
223 (Kedderis, 1996; Reed, 1990). The most common pathway of glutathione depletion in

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224 xenobiotic toxicity is excessive consumption of GSH without recovery (Castell et al.,
225 1997). Severe GSH depletion leaves cells more vulnerable to oxidative damage which
226 causes progressive deterioration of macromolecules, cell structure and may lead to cell
227 death. GSH level could therefore determine the cytotoxicity of a xenobiotic and its
228 ultimate effects on cell survival.

229 Our data shows that sage EO was not toxic to rat hepatocyte suspensions when
230 present in concentrations below 200 nl/ml for 30 min; it was only at 2000 nl of EO per
231 ml of hepatocyte suspension that a significant LDH leakage and GSH decrease occurred
232 indicating cell damage. *cis*-Thujone, the major constituent of sage EO (17.4%, see
233 Table1), has been shown to be neurotoxic and implicated in the effects of absinth
234 consumption (Hold *et al.*, 2000; Hold *et al.*, 2001). Results by Bonkovsky and co-
235 workers (Bonkovsky *et al.*, 1992) and our own preliminary data (not shown) indicates
236 that *cis*-thujone in concentrations below 1 mM (960 nl EO/ml) does not decrease
237 hepatocyte viability and seem therefore not to be toxic to the liver. Judging by thujone
238 toxicity alone we would not expected toxicity to the liver of EO concentrations below
239 200 nl/ml. However, a decrease in hepatocyte GSH was apparent at this EO
240 concentration indicating some degree of toxicity. This emphasises the need for the
241 evaluation of the toxicity of the actual EO – a mixture of compounds – since the toxicity
242 of the mixture can not in all cases be inferred from the toxicity of its individual
243 compounds.

244 The toxicity induced by *t*-BHP is well-known and is well characterized
245 (Fernandes et al., 1995; Joyeux et al., 1990; Tseng et al., 1997) and our results are in
246 agreement with these published reports. Our data also show that when compared with *t*-
247 BHP-induced toxicity, which is the result of GSx and GSH depletion, GSSG increase
248 and lipid peroxidation, cell death induced by the EO at 2000 nl/ml on liver cells does

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249 not seem to be mediated by lipid peroxidation and GSH oxidation with concomitant
250 GSSG increase. The EO toxicity may be due to the presence of reactive compounds
251 and/or reactive metabolites generated by metabolism of EO. The depleted glutathione
252 was possibly recruited to act as a nucleophilic scavenger of some compounds and/or
253 their metabolites with electrophilic properties. The cellular death indicated by LDH
254 leakage could also be due to a solvent effect of the essential oil, whereby the
255 hydrophobic compounds exert a direct effect on cellular membranes disturbing the
256 physico-chemical properties of the bilayer culminating in the disruption of cellular
257 volume and cell death (Berry et al., 1991; Kedderis, 1996).
258 The hepatoprotective effects of the EO were tested by co-incubation with the
259 toxicant *t*-BHP and compared with those of the reference antioxidant quercetin.
260 Quercetin shows concentration-dependent protective effects against *t*-BHP-induced
261 toxicity in almost all parameters measured. Quercetin was preferred to other known
262 antioxidants as a positive control because of its plant origin, DMSO solubility and for
263 having a well characterised protective effect against *t*-BHP-induced toxicity (Joyeux et
264 al., 1990). In contrast, the presence of sage EO did not reduce hepatocyte death due to *t*-
265 BHP toxicity. The co-incubation with the EO resulted in a tendency for a further
266 decrease in GSH and increased lipid peroxidation. In spite of the reputation of some
267 plant EOs as antioxidants and hepatoprotectants in animal models (Teissedre and
268 Waterhouse, 2000 and references therein), our results do not show antioxidant
269 protective effects of *S. officinalis* EO against *t*-BHP-induced liver toxicity.
270 The EO obtained by hydrodistillation used in this study was somewhat lower
271 than that obtained from fresh leaves of the same sage cultivar (Santos-Gomes and
272 Fernandes-Ferreira, 2001). The composition of sage EO extracts varies and reflects
273 extraction conditions as well as time of harvest and the plant's growth conditions such

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274 as climate, soil composition and light intensity (Santos-Gomes and Fernandes-Ferreira,
275 2001). In April, the composition of the essential oil from *S. officinalis* plants grown in
276 Portugal (Santos-Gomes and Fernandes-Ferreira, 2001) and in Israel (Putievsky et al.,
277 1986) are characterised by the lowest levels of *cis*-thujone and camphor. The levels of
278 these two compounds in the EO used (Table 1), were slightly lower than the
279 corresponding minimum values from the profile defined by the standard ISO 9909
280 (1999) for the essential oil of *S. officinalis* - *cis*-thujone (18-43%), camphor (4.5-24.5%)
281 (Bruneton, 1999). It is therefore important that detailed information is available on the
282 material being tested or used in the food industry.

283 In summary, our results indicate that the hepatoprotective properties attributed to
284 *S. officinalis* seem not to be due to the essential oil fraction. At doses higher than 200
285 nl/ml acting directly on the liver the EO may cause toxicity. The neurotoxicity of
286 thujones and camphor, major compounds *of S. officinalis* EO, as well as hepatotoxicity
287 of sage EO reported herein, justify concerns with the consumption of high doses of sage
288 products.

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294

295 ACKNOWLEDGMENT
296 CFL is supported by the Foundation for Science and Technology, Portugal, grant
297 SFRH/BD/6942/2001. This work was supported by FCT research grant
298 POCTI/AGR/43482/2001.

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Table 1. Composition of the essential oil and essential oil constituents of tea prepared from vegetative aerial parts of *S. officinalis* L. plants collected in April, before anthesis.

Compound		Essential oil from *Salvia officinalis*		Essential oil constituents from *Salvia officinalis* tea		
	RI[a]	%	µg/g dry wt	%	µg/g dry wt	µg/ml of tea
1-Butyl acetate	813	*tr*[b]	2.1	-	-	-
cis-2-Methyl-3-methylene-hep-5-ene	847	0.7	68.1	-	-	-
trans-2-Methyl-3-methylene-hep-5-ene	857	0.1	7.6	2.1	5.2	0.069
Tricyclene	920	0.1	11.3	-	-	-
α-Thujene	924	0.1	8.9	-	-	-
α-Pinene	930	4.1	399.1	*tr*	0.3	0.005
Camphene	946	3.1	298.4	*tr*	0.4	0.006
Sabinene	970	0.1	6.3	-	-	-
β-Pinene	974	4.5	437.0	*tr*	0.5	0.007
Myrcene	988	0.5	48.0	-	-	-
n-Decane	1000	*tr*	1.3	-	-	-
α-Phellandrene	1004	0.1	5.5	-	-	-
α-Terpinene	1016	0.2	18.9	-	-	-
p-Cymene	1022	0.1	13.7	-	-	-
Limonene	1024	1.5	142.4	-	-	-
1,8-Cineole	1033	12.7	1703.9	18.1	66.3	0.884
Z-β-Ocimene	1034	0.3	26.5	-	-	-
E-β-Ocimene	1044	0.4	39.2	-	-	-
γ-Terpinene	1055	0.1	4.4	-	-	-
cis-Linalool oxide	1072	0.1	6.7	-	-	-
Terpinolene	1086	0.2	15.7	-	-	-
n-Undecane	1100	*tr*	0.8	1.1	2.7	0.036
Linalool	1101	*tr*	*tr*	-	-	-
cis-Thujone [= (-)-thujone]	1103	17.4	2491.4	34.0	125.9	1.678
trans-Thujone [=(+)-thujone	1114	3.9	518.5	5.3	20.1	0.268
α-Campholenal	1125	0.1	13.5	-	-	-
Not identified	1129	0.2	29.9	*tr*	0.9	0.013
Camphor	1143	3.3	445.4	10.6	38.4	0.512
cis-Pinocamphone [= (*cis-3-*)-Pinanone]	1160	0.7	97.8	1.1	4.3	0.057
Borneol	1165	8.3	1109.5	13.8	52.3	0.697
Pinocamphone isomer (T)	1172	0.2	30.3	1.1	2.0	0.027
4-Terpineol	1176	0.2	25.6	*tr*	1.2	0.016
α-Terpineol	1189	0.3	32.7	-	-	-
Not identified	1201	0.3	43.0	2.1	9.2	0.123
Bornyl acetate	1283	0.9	127.9	*tr*	1.3	0.017
cis-Sabinyl acetate	1290	0.1	9.8	-	-	-
Not identified	1322	0.3	47.2	-	-	-
Not identified	1328	0.1	11.3	-	-	-
δ-Elemene	1334	0.1	8.8	-	-	-
trans-Carvyl acetate	1337	*tr*	0.5	-	-	-
cis-Carvyl acetate	1362	0.1	18.5	-	-	-
Neryl acetate	1364	0.3	38.8	-	-	-
β-Bourbonene + Geranyl acetate	1383+1384	0.1	10.8	-	-	-
E-Caryophyllene	1416	8.5	836.9	*tr*	1.0	0.014
Aromadendrene or α-Guaiene (?)	1436	0.8	82.2	-	-	-
α-Humulene	1450	13.3	1305.7	1.1	1.6	0.022
allo-Aromadendrene	1458	0.1	13.2	-	-	-
Germacrene D isomer #3	1473	0.5	46.5	-	-	-
Germacrene D	1477	0.1	4.6	-	-	-
α-Selinene	1491	0.5	49.4	1.1	2.0	0.026
δ-Cadinene	1520	0.6	62.9	-	-	-
Caryophyllene oxide	1580	0.5	65.9	1.1	3.2	0.043
Viridiflorol	1595	6.2	834.0	*tr*	1.4	0.019
Widdrol (?)	1606	0.7	96.4	-	-	-
Z-α-*trans*-Bergamatol acetate	1801	0.1	8.9	-	-	-
Not identified	1903	*tr*	4.1	1.1	2.4	0.032
Manool	2063	2.4	248.7	1.1	1.8	0.024
n-Hexacosane	2600	*tr*	1.1	3.2	9.9	0.132
n-Octacosane	2800	*tr*	2.6	2.1	6.5	0.087
Grouped components						
Monoterpene hydrocarbons		15.9	1553.8	2.1	6.5	0.086
Oxygen-containing monoterpenes		47.7	6565.5	86.2	320.6	4.274
Monoterpenyl esters		1.8	243.7	0.0	1.3	0.017
Sesquiterpene hydrocarbons		24.5	2410.2	2.1	4.6	0.062
Oxygen-containing sesquiterpenes		7.4	996.4	1.1	4.6	0.062
Sesquiterpenyl esters		*tr*	8.9	-	-	-
Oxygen-containing diterpenes		2.4	248.7	1.1	1.8	0.024
Others		0.3	38.3	7.4	21.5	0.286
Total		100.0	12065.4	100.0	360.8	4.810

[a] Kovats retention index relatively to *n*-alkanes in a DB-5 column. [b] *tr*, trace amounts (< 0.05)

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Table 2. Effect of 30 min exposure of hepatocyte suspensions to *S. officinalis* essential oil on GSSG and GSx content and on lipid peroxidation expressed as TBARS formation (means ± SEM). n=5; # P≤0.05 between control and *t*-BHP treated cells; * P≤0.05 when compared with control cells.

Parameter	Sample					
	***t*-BHP**	**EO (nl/ml)**				
	(0.75 mM)	0 (contr)	2	20	200	2000
TBARS (nmol/million cells)	1.99 ± 0.16 #	0.94 ± 0.12	0.89 ± 0.11	0.87 ± 0.09	0.79 ± 0.14	0.89 ± 0.12
GSx content (nmol/million cells)	35.2 ± 1.7 #	45.1 ± 3.5	43.9 ± 4.2	43.4 ± 3.2	39.7 ± 4.1	28.4 ± 3.3 *
GSSG content (nmol/million cells)	13.1 ± 1.6	9.2 ± 0.9	9.5 ± 0.8	9.8 ± 0.9	10.1 ± 1.7	10.2 ± 2.0

Table 3. Anti-hepatotoxic effect of *S. officinalis* essential oil on hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min, on GSSG and GSx content and on lipid peroxidation expressed as TBARS formation (means ± SEM). n=5; # P≤0.05 between control and cells treated only with *t*-BHP.

Parameter	Sample					
	(-) control	***t*-BHP (0.75 mM) + EO (nl/ml) series**				
		0	2	20	200	2000
TBARS (nmol/million cells)	0.92 ± 0.09	2.08 ± 0.13 #	2.14 ± 0.23	2.20 ± 0.20	2.22 ± 0.30	2.88 ± 0.59
GSx content (nmol/million cells)	44.6 ± 4.9	35.9 ± 2.2	31.1 ± 3.0	33.4 ± 2.6	29.4 ± 2.6	22.5 ± 3.3
GSSG content (nmol/million cells)	10.5 ± 1.1	13.5 ± 1.9	12.7 ± 2.1	12.6 ± 1.8	11.7 ± 1.8	10.6 ± 1.8

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Table 4. Anti-hepatotoxic effect of quercetin (positive control) on hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min, on GSSG and GSx content and on lipid peroxidation expressed as TBARS formation (means ± SEM). n=5; # $P \leq 0.05$ between control and cells treated only with *t*-BHP; * $P \leq 0.05$ when compared with cells treated only with *t*-BHP.

Parameter	Sample					
	(-) control	*t*-BHP (0.75 mM) + quercetin (μM) series				
		0	1.6	12.5	50	200
TBARS (nmol/million cells)	0.92 ± 0.09	2.08 ± 0.13 #	2.09 ± 0.24	1.35 ± 0.15*	0.72 ± 0.04*	0.79 ± 0.10*
GSx content (nmol/million cells)	44.6 ± 4.9	35.9 ± 2.2	37.7 ± 1.9	37.0 ± 1.8	35.2 ± 1.0	31.9 ± 3.2
GSSG content (nmol/million cells)	10.5 ± 1.1	13.5 ± 1.9	13.8 ± 1.9	12.7 ± 2.1	11.2 ± 1.8	8.7 ± 1.2

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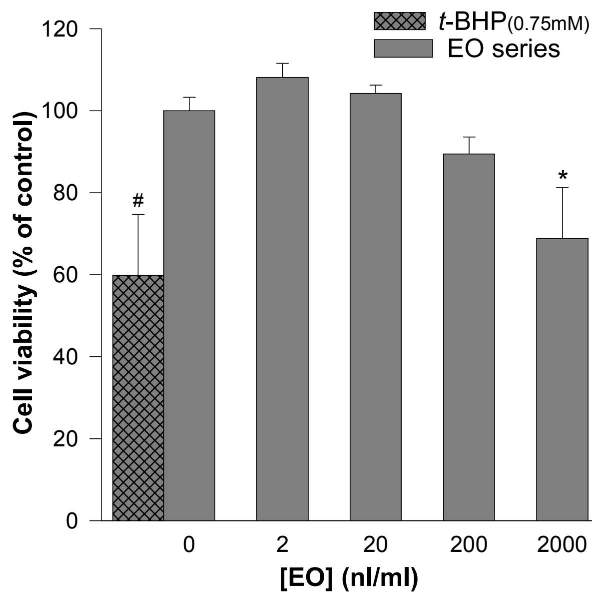

Figure 1. Effect of essential oil of *S. officinalis* on cell viability (measured by % of LDH leakage) in hepatocyte suspensions after 30 min of exposure (means ± SEM). n=5; # P≤0.05 between control and *t*-BHP treated cells; * P≤0.05 when compared with control cells.

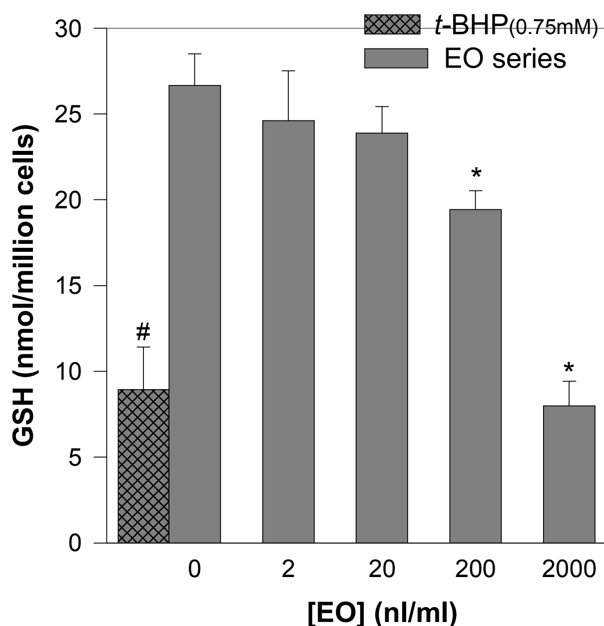

Figure 2. Effect of essential oil of *S. officinalis* on hepatocyte suspensions GSH content after 30 min of exposure (means ± SEM). n=5; # P≤0.05 between control and *t*-BHP treated cells; * P≤0.05 when compared with control cells.

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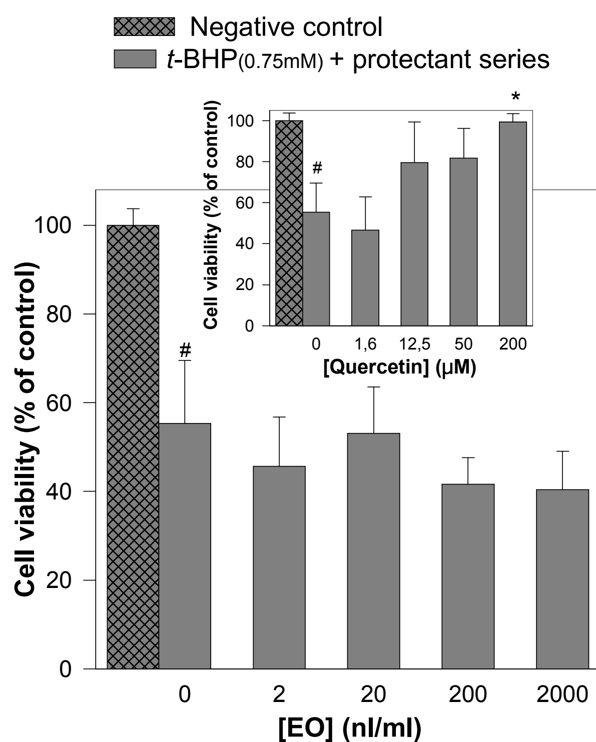

Figure 3. Effect of essential oil of *S. officinalis* and quercetin (insert) on cell viability (measured by % of LDH leakage) of hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min (means ± SEM). n=5; # P≤0.05 between control and cells treated only with *t*-BHP; * P≤0.05 when compared with cells treated only with *t*-BHP.

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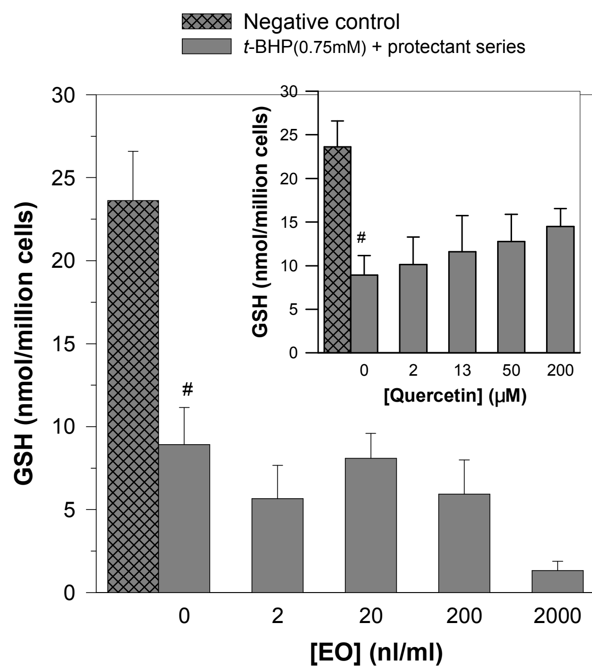

Figure 4. Effect of essential oil of *S. officinalis* and quercetin (insert) on GSH content of hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min (means ± SEM). n=5; # $P \leq 0.05$ between control and cells treated only with *t*-BHP.

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