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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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.

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.

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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 $\times$ 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 $\pm$ 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 ($50 \times g$) 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 $P \leq 0.05$.

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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$^-$

176   ^{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.

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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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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	RI[a]	Essential oil from *Salvia officinalis*			Essential oil constituents from *Salvia officinalis* tea		
		%	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)

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	*t*-BHP (0.75 mM)	Sample				
		EO (nl/ml)				
		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	(-) control	Sample				
		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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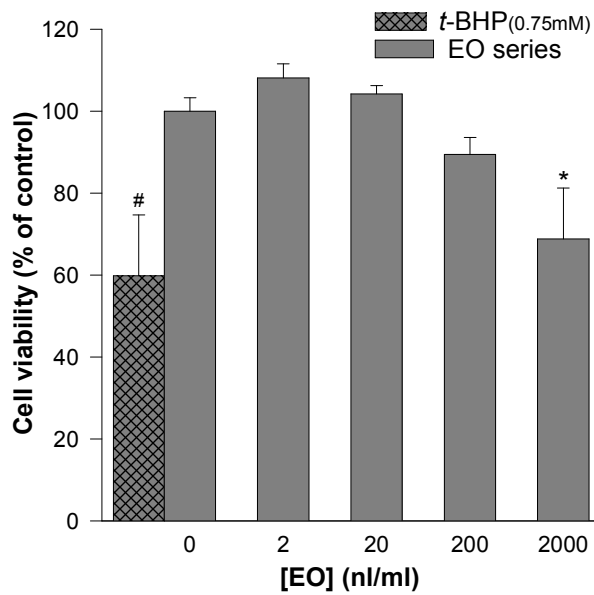
Figure 1. Effect of essential oil of *S. officinalis* on cell viability (measured by % of LDH leakage) in hepatocyte suspensions after 30 min of exposure (means ± SEM). n=5; [#] P≤0.05 between control and *t*-BHP treated cells; * P≤0.05 when compared with control cells.
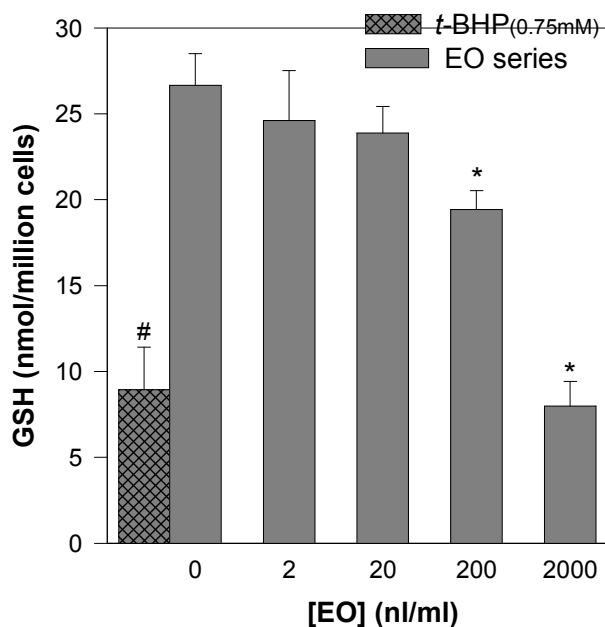
Figure 2. Effect of essential oil of *S. officinalis* on hepatocyte suspensions GSH content after 30 min of exposure (means ± SEM). n=5; [#] P≤0.05 between control and *t*-BHP treated cells; * P≤0.05 when compared with control cells.

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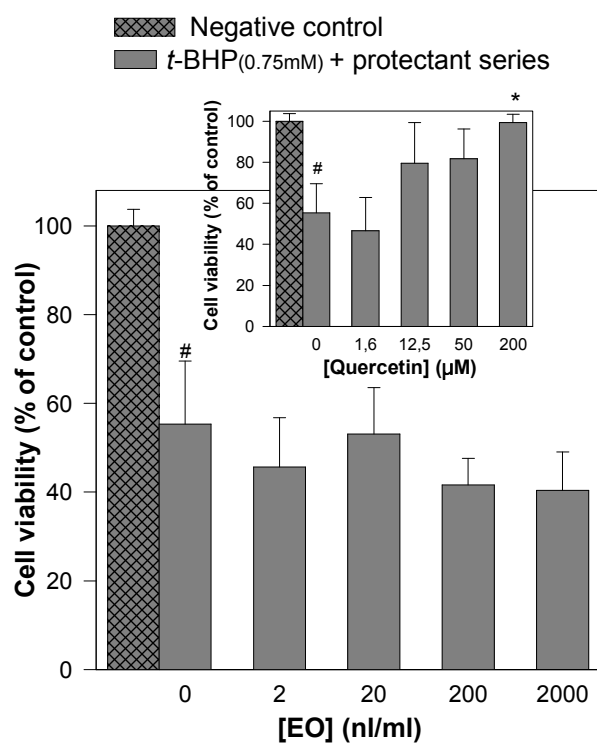
Figure 3. Effect of essential oil of *S. officinalis* and quercetin (insert) on cell viability (measured by % of LDH leakage) of hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min (means ± SEM). n=5; [#] P≤0.05 between control and cells treated only with *t*-BHP; * P≤0.05 when compared with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

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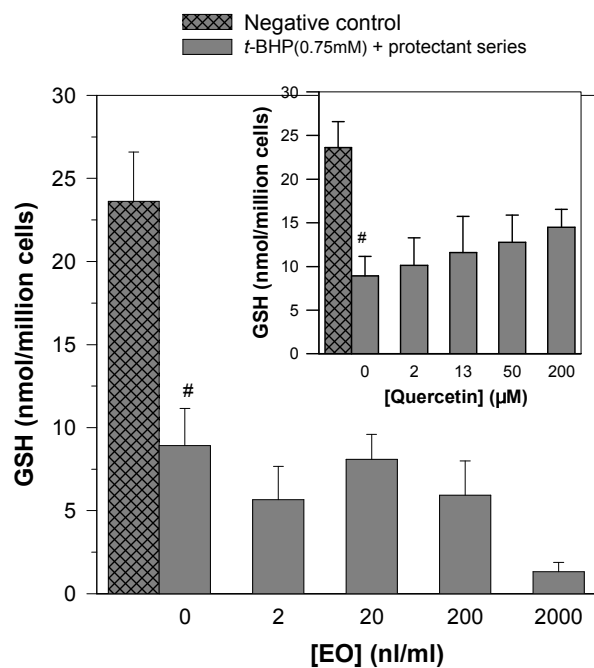
Figure 4. Effect of essential oil of *S. officinalis* and quercetin (insert) on GSH content of hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min (means ± SEM). n=5; [#] P≤0.05 between control and cells treated only with *t*-BHP.