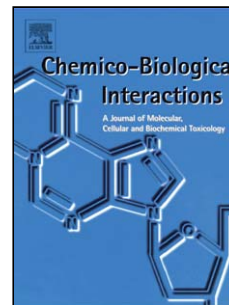


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Water and methanolic extracts of *Salvia officinalis* protect HepG2 cells from *t*-BHP induced oxidative damage

Authors: Cristovao F. Lima^a, Patricia C.R. Valentao^b, Paula B. Andrade^b, Rosa M. Seabra^b, Manuel Fernandes-Ferreira^a, Cristina Pereira-Wilson^{a*}

Addresses: ^a Department/Centre of Biology, School of Sciences, University of Minho, 4710-057 Braga, Portugal. ^b REQUIMTE, Pharmacognosy Laboratory, Faculty of Pharmacy, University of Porto, 4050-047 Porto, Portugal.

* **Corresponding author:** Cristina Pereira-Wilson; telephone +351 253604318; fax +351 253678980; e-mail cpereira@bio.uminho.pt

1 **Abstract**

2 Common sage (*Salvia officinalis* L., *Lamiaceae*) is an aromatic and medicinal
3 plant well known for its antioxidant properties. Some in vivo studies have shown the
4 biological antioxidant effects of sage. However, the intracellular antioxidant
5 mechanisms of action are still poorly understood. In this study, we evaluated the
6 cytoprotective effects of two sage extracts (a water and a methanolic extract) against
7 *tert*-butyl hydroperoxide (*t*-BHP)-induced toxicity in HepG2 cells. The most abundant
8 phenolic compounds present in the extracts were rosmarinic acid and luteolin-7-
9 glucoside. Both extracts, when co-incubated with the toxicant, protected significantly
10 HepG2 cells against cell death. The methanolic extract, with a higher content of
11 phenolic compounds than the water extract, conferred better protection in this in vitro
12 model of oxidative stress with liver cells. Both extracts, tested in a concentration that
13 protects 80% against cell death (IC₈₀), significantly prevented *t*-BHP-induced lipid
14 peroxidation and GSH depletion, but not DNA damage assessed by the comet assay.
15 The ability of sage extracts to reduce *t*-BHP-induced GSH depletion by 62% was
16 probably the most relevant contributor to the observed cytoprotection. A good
17 correlation between the above cellular effects of sage and the effects of their main
18 phenolic compounds was found. When incubated alone for 5 hours, sage extracts
19 induced an increase in basal GSH levels of HepG2 cells, which indicates an
20 improvement of the antioxidant potential of the cells. Compounds present in sage
21 extracts other than phenolics may also contribute to this latter effect. Based in these
22 results, it would be of interest to investigate whether sage has protective effects in
23 suitable in vivo models of liver diseases, where it is known that oxidative stress is
24 involved.

25

26 **Keywords:** *Salvia officinalis* L. / Phenolic Compounds / Antioxidant Effects / HepG2
27 cells / *tert*-Butyl Hydroperoxide.

28

29

30 **1. Introduction**

31 Reactive oxygen species (ROS) and other free radicals are produced during the
32 normal cell metabolism and they are a necessary and normal process that provides
33 important physiological functions [1,2]. The production of ROS and other free radicals
34 is normally compensated by an elaborate endogenous antioxidant system. However, due
35 to many environmental, lifestyle and pathological factors, an excess of radicals can be
36 accumulated in cells resulting in oxidative stress. Because of their high reactivity,
37 accumulation of radicals above cells' defenses may affect cellular functionality and
38 integrity by damaging critical molecules, such as the DNA, proteins, carbohydrates and
39 lipids, which ultimately can cause cell death. In fact, oxidative stress has been
40 recognized to be involved in the etiology of several diseases, including liver diseases
41 [3,4]. The liver, because of its high metabolic activity and its anatomical positioning to
42 receive blood from the gastrointestinal tract, is vulnerable to toxicity from a variety of
43 drugs and environmental contaminants. Consequently, mechanisms of cytoprotection
44 relevant to the liver are of particular interest. Natural antioxidants have been proposed
45 and utilized as therapeutic agents to counteract liver damage [3,4].

46 *Salvia officinalis* L. (*Lamiaceae*) is an aromatic and medicinal plant of
47 Mediterranean origin well known for its antioxidant properties, mainly due to its
48 composition in phenolic compounds [5]. Sage extracts revealed strong antioxidant
49 activity in several assays: by increasing the stability of food oils [6-10], in an assay
50 based on the disappearance of methyl linoleate in a lipophilic solvent under strong

51 oxidizing conditions [11,12], by the ability to scavenge DPPH[•] [13] and ABTS^{•+} free
52 radicals [14] as well as by having oxygen radical absorbance capacity (ORAC assay)
53 [15]. In addition, the reported superoxide and hydroxyl radicals scavenging activities
54 using the electron spin resonance technique [16] and the protective effects against
55 enzyme-dependent and enzyme-independent lipid peroxidation [17,18] of sage extracts
56 also showed its antioxidant potential. More recently, results from in vivo studies suggest
57 a biological antioxidant effect of sage. The drinking of a sage infusion (tea) for 14 days
58 was reported to improve liver antioxidant status in mice and rats [19]. Also, the
59 treatment of rats with a water extract of sage for 5 weeks was shown to protect against
60 the hepatotoxicity of azathioprine [20]. However, little is known about the active
61 compounds and cellular mechanisms action. Only in a small experiment using
62 fibroblasts, performed by Masaki et al. (1995), sage antioxidant effects were related
63 with cytoprotective effects. In their study, a sage extract protected significantly against
64 cell death induced by a superoxide-generating system [16]. Very recently, a hydro
65 alcoholic extract of sage was reported to possess neuroprotective effects against
66 amyloid β (A β)-induced toxicity in PC12 cells, and the effect was attributed, at least in
67 part, to rosmarinic acid [21].

68 In this study we propose to evaluate the potential antioxidant/cytoprotective
69 effects of two sage extracts (a water and a methanolic crude extracts) against *tert*-butyl
70 hydroperoxide (*t*-BHP)-induced oxidative damages in HepG2 cells. This hepatoma cell
71 line is considered a good tool to study the toxic/cytoprotective and genotoxic/
72 antigenotoxic effects of compounds to liver cells [22]. Furthermore, this model of in
73 vitro hepatotoxicity (*t*-BHP and HepG2 cells) was recently used to evaluate the
74 cytoprotective effects of individual phenolic compounds, which included the two most
75 representative ones of the above sage extracts – rosmarinic acid and luteolin-7-

76 glucoside [22]. Here, the concentration of sage extracts that protected 50% (IC₅₀)
77 against *t*-BHP-induced cell death were determined in order to establish their
78 cytoprotective potential. Subsequently, IC₈₀ values, a concentration that effectively
79 protects against cell death, were used to evaluate the effects of each extract on three
80 markers of oxidative damage: lipid peroxidation, intracellular glutathione levels and
81 DNA damage. The importance of modulation of these parameters by sage extracts in the
82 protection against *t*-BHP-induced cell death is discussed. Throughout the experiment
83 quercetin was used as a positive control.

84

85 **2. Materials and methods**

86 *2.1. Chemicals*

87 Minimum Essential Medium Eagle (MEM), *tert*-butyl hydroperoxide, quercetin
88 and Bradford reagent were purchased from Sigma (St. Louis, MO, USA). Fetal Bovine
89 Serum (FBS) was obtained from Biochrom KG (Germany). All others reagents were of
90 analytical grade.

91

92 *2.2. Plant material, preparation of sage extracts and analysis of their phenolic* 93 *composition*

94 *Salvia officinalis* L. plants were cultivated in an experimental farm located in
95 Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were
96 lyophilised and kept at -20° C. Voucher specimen is kept in an active bank under the
97 responsibility of the DRAEDM (Direcção Regional de Agricultura de Entre Douro e
98 Minho) from the Portuguese Ministry of Agricultural.

99 The dried and powdered aerial plant material (4 g) was extracted with 2 × 100
100 ml of 90% methanol in water at room temperature, using an ultrasonic bath (15 min).

101 The filtered extract (SOME) was evaporated to dryness under reduced pressure at 40°C
102 and a yield of 26.2% (w/w) was obtained.

103 Considering that sage is traditionally consumed as a tea, an infusion of sage
104 (SOI) was also prepared following a previous methodology [19]. In brief, 300 ml of
105 ultrapure Milli Q boiling water were poured over 4 g of lyophilised aerial plant material
106 and allowed to steep for 5 min. The filtered extract was lyophilised to dryness and a
107 yield of 25.8% (w/w) was obtained.

108 Phenolic compounds present on SOME and SOI extracts were identified and
109 quantified by HPLC/DAD as described in Santos-Gomes et al. (2002) [23] and Lima et
110 al. (2005) [19] for each extract, respectively.

111

112 *2.3. Antiradical activity*

113 The free radical scavenging (antiradical) activity of sage extracts was studied
114 against two radicals: the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) and
115 the superoxide radical.

116 For DPPH scavenging activity, after addition of different concentrations of
117 extract to DPPH (90µM), the percentage of remaining DPPH was determined at
118 different times from the absorbance at 515 nm using a plate reader spectrophotometer.
119 At steady state, the percentage of remaining DPPH was plotted against the
120 concentration of the extract and the amount of antioxidant necessary to decrease by 50%
121 the initial DPPH concentration (IC₅₀) calculated. We also present the parameter
122 antiradical efficiency (AE) [24] using the estimated T_{IC50} – time needed to reach the
123 steady state at the corresponding IC₅₀ concentration, where $AE = 1/(IC_{50} \times T_{IC50})$.

124 The superoxide radical scavenging activity was determined using the phenazine
125 methosulphate-NADH nonenzymatic assay as previously described [25].

126

127 *2.4. Cell culture*

128 HepG2 cells (hepatocellular carcinoma cell line) were obtained from the
129 American Type Culture Collection (ATCC) and maintained in culture in 75 cm²
130 polystyrene flasks (Falcon) with MEM containing 10% FBS, 1% antibiotic-antimycotic
131 solution, 1 mM sodium pyruvate, 10 mM HEPES and 1.5 g/l sodium bicarbonate under
132 an atmosphere of 5% CO₂ at 37°C.

133

134 *2.5. Experimental outline*135 *2.5.1. Assay for protection against t-BHP-induced toxicity in HepG2 cells*

136 In order to determine the concentration of sage extract/querletin that protects the
137 cells 50% from the oxidative damage (IC₅₀), cells were incubated with 2 mM of *t*-BHP
138 for 5 h to induce significant cell death as previously described [22]. HepG2 cells were
139 plated in 24-multiwell culture plates at 2.5×10⁵ cells per well. The prevention of LDH
140 leakage (cell death) was measured in co-incubations with sage extract/querletin
141 dissolved in DMSO (1% v/v final concentration, controls with DMSO only) at several
142 concentrations. The IC₅₀ and the Hill slope – slope from the plotted sage
143 extract/querletin's concentrations (in logarithm) versus cell death protection relative to
144 the control (2 mM *t*-BHP, 5 h) – were calculated graphically using a computer program
145 (GraphPad Prism, version 4.00, GraphPad Software Inc.). Based on the dose-response
146 curves of protection against cell death by sage extract/querletin, the IC₈₀ concentrations
147 were estimated and used in the following experiments to evaluate the protective
148 potential of the compounds on several cellular parameters as previously described [22].

149 Briefly:

150

151 2.5.2. *Evaluation of the effects of sage extract/queracetin at the IC₈₀ concentration*
152 *against t-BHP-induced lipid peroxidation and GSH depletion in HepG2 cells.*

153 In order to evaluate the potential protective effect of sage extract/queracetin at
154 IC₈₀ concentration against *t*-BHP-induced lipid peroxidation and GSH depletion, cells
155 were incubated with 2 mM *t*-BHP for 5 h. HepG2 cells were plated in 6-multiwell
156 culture plates at 7.5×10^5 cells per well. Forty hours after plating, the medium was
157 discarded and fresh medium containing 2 mM *t*-BHP and/or the IC₈₀ concentration of
158 sage extract/queracetin was added. Both sage extracts and queracetin did not change
159 significantly the pH of the culture medium at their IC₈₀ concentration. Five hours later,
160 cell culture medium and cell scrapings were harvested and kept at -80°C for following
161 quantification of lipid peroxidation and glutathione levels.

162

163 2.5.3. *Evaluation of the effects of sage extract/queracetin at the IC₈₀ concentration*
164 *against t-BHP-induced DNA damage in HepG2 cells*

165 In order to evaluate the potential protective effect of sage extract/queracetin at
166 IC₈₀ concentration against *t*-BHP-induced DNA damage, cells were incubated with 200
167 μM *t*-BHP for 1 h. HepG2 cells were plated in 6-multiwell culture plates at 5×10^5 cells
168 per well. Sixteen hours after plating, the medium was discarded and fresh medium
169 containing 200 μM *t*-BHP and/or the IC₈₀ concentration of sage extract/queracetin was
170 added to the cells. After 1 h incubation, cells were rinsed with warm PBS and then
171 incubated for 5 min with 0.125% (w/v) trypsin in PBS. The cells were then harvested in
172 PBS to be used in the alkaline version of the comet assay for evaluation of DNA
173 damage.

174

175 2.6. *Biochemical analysis*

176 2.6.1. LDH

177 To assess the extend of cell death caused by *t*-BHP, the determination of lactate
178 dehydrogenase leakage to the culture medium was used as indicator of plasma
179 membrane integrity of HepG2 cells. LDH activity was measured spectrophometrically
180 at 30°C as previously described [19].

181

182 2.6.2. Lipid peroxidation

183 The extent of lipid peroxidation was estimated by the levels of malondialdehyde
184 measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm
185 following a methodology previously described [26] with some modifications [19]. The
186 results are expressed as nmol/mg of protein using a molar extinction coefficient of
187 $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

188

189 2.6.3. Glutathione content

190 The glutathione levels of HepG2 cells were determined by the DTNB-GSSG
191 reductase recycling assay as previously described [27], with some modifications [28].
192 The results are expressed as nmol GSH/mg of protein.

193

194 2.6.4. Protein

195 Protein content was measured with a Bradford Reagent purchased from Sigma
196 using bovine serum albumin as a standard.

197

198 2.7. Comet assay

199 The alkaline version of the single cell gel electrophoresis (comet) assay was
200 performed based in previous descriptions [29-31] with slight modifications [22]. The

201 comet images were analysed using the semiquantitative method of visual scoring [32].
202 Each cell was classified in five classes according to the intensity of fluorescence in the
203 comet tail, attributing a value of 0, 1, 2, 3 or 4 from undamaged to maximal damage. In
204 this way, the total score for 100 images can range from 0 (all undamaged) to 400 (all
205 maximally damaged), the overall DNA damage of the cell population expressed in
206 arbitrary units.

207

208 2.8. Statistical analysis

209 Data are expressed as means \pm SEM. Statistical significances were determined
210 using a one-way ANOVA followed by the Student-Newman-Keuls post-hoc test. *P*
211 values ≤ 0.05 were considered statistically significant.

212

213 3. Results

214 3.1. Phenolic composition of sage extracts and their antiradical activity

215 A methanolic (SOME) and a water (SOI) extract were prepared from aerial parts
216 of *Salvia officinalis* and analysed for phenolic compounds by HPLC/DAD (Table 1).
217 Eight phenolic compounds were identified, 5 phenolic acids and 3 flavonoids, SOME
218 having the highest content. SOME's main phenolic compound was rosmarinic acid
219 whereas SOI's were rosmarinic acid and luteolin-7-glucoside.

220 The antiradical activity of both extracts was then evaluated against DPPH and
221 superoxide radicals (Table 2). SOME, with higher content in phenolic compounds, had
222 higher antiradical activity against DPPH presenting a lower IC_{50} and a higher antiradical
223 efficiency than SOI extract. The activity of both extracts was smaller than the positive
224 control quercetin. Regarding the scavenging of superoxide radical, SOI extract showed
225 a higher antiradical activity than SOME.

226

227 *3.2. Potential cytoprotective effects of sage extracts*

228 The potential cytoprotective effects of both sage extracts against the cell death
229 induced by *t*-BHP were evaluated in HepG2 cells (Table 3, Fig. 1). *t*-BHP 2 mM for 5
230 hours was previously shown to induce oxidative damage to HepG2 cells causing about
231 40-50% of cell death [22]. As shown in Fig. 1, both extracts protected against cell death
232 in a dose-dependent manner. SOME had, however, higher cytoprotective activity (lower
233 IC₅₀) than SOI (Table 3). The Hill slope was also higher in SOME than SOI (Table 3),
234 which indicates a narrower concentration (in logarithm) range from 0 to 100% of
235 cytoprotective activity of SOME (Fig 1).

236

237 *3.3. Effects of sage extracts on lipid peroxidation, glutathione levels and DNA damage*

238 To study the effects of sage extracts against lipid peroxidation, GSH depletion
239 and DNA damage induced by *t*-BHP, concentrations that effectively protect against cell
240 death (IC₈₀) were used. IC₈₀ concentrations were used to determine if the same level of
241 cytoprotection for each extract correlate with similar effects on the above mentioned
242 parameters. IC₈₀ concentration for each extract (Table 3) was estimated based on the
243 curves presented in Fig. 1 and, as can be seen in Fig. 2, *t*-BHP-induced cell death was
244 prevented by around 80% by both sage extracts as well as quercetin. No significant cell
245 death was observed in incubations of HepG2 cells with sage extracts or quercetin alone
246 (Fig. 2).

247 As shown in Fig. 3, *t*-BHP-induced lipid peroxidation was significantly
248 decreased by around 25% by both extracts. Quercetin also significantly protected
249 against lipid peroxidation by 30%. None of the extracts, when incubated alone with
250 HepG2 cells, induced significant lipid peroxidation.

251 *t*-BHP-induced GSH (reduced glutathione) depletion was also significantly
252 inhibited by both extracts by around 62% while quercetin inhibited GSH depletion by
253 only 40% (Fig. 4). The increase in GSSG levels induced by *t*-BHP was slightly
254 decreased by both sage extracts and quercetin, although the effect was not statistically
255 significant (data not shown). When the cells were incubated with the extracts alone, a
256 significant increase in the basal GSH levels (Fig. 4) was observed for SOME (15%). On
257 the other hand, quercetin induced a decrease in the basal levels of GSH.

258 The incubation of HepG2 cells for 1 h with 200 μ M of *t*-BHP induced
259 significant DNA damages without cell death [22], conditions that can be used to assess
260 effects of compounds or extracts against DNA damage by the comet assay. As shown in
261 Fig. 5, contrarily to what happened with quercetin, both sage extracts did not protect
262 HepG2 cells against DNA damage induced by *t*-BHP. None of the tested extracts
263 induced DNA damages at IC₈₀ concentration when incubated alone with HepG2 cells.

264

265 **4. Discussion and conclusions**

266 Since oxidative stress has been recognized to be involved in the etiology of
267 several liver diseases [3,4] and because the liver is very susceptible to toxic effects,
268 natural antioxidants and plant extracts have been proposed as therapeutic agents to
269 counteract liver damage. *Salvia officinalis* is well known for its antioxidant activity,
270 mainly based on results from several subcellular and noncellular in vitro studies [5].
271 Previous work in our laboratory has shown the ability of sage tea drinking to improve
272 liver antioxidant status in mice and rats [19]. That was, however, not enough to protect
273 against CCl₄-induced hepatotoxicity in mice and, instead, a herb-toxicant interaction
274 was observed [33]. On the other hand, in an in vivo experiment, Amin and Hamza
275 (2005) have shown that the treatment of rats with a water extract of sage for 5 weeks

276 protected against the hepatotoxicity of azathioprine [20]. However, despite all these
277 effects, little is known about the active compounds and mechanisms of antioxidant
278 protection of sage extracts at cellular level.

279 Here, the potential antioxidant and cytoprotective effects of sage crude extracts,
280 a methanolic (SOME) and a water extract (SOI), were tested against *t*-BHP-induced
281 toxicity in HepG2 cells. Both sage extracts, in co-incubations with the toxicant, showed
282 protective effects against *t*-BHP-induced cell death. SOME revealed higher
283 cytoprotective activity than SOI, as shown by the lower IC₅₀ obtained for this extract
284 against *t*-BHP-induced cell death compared to that of SOI extract. This biological
285 activity is in agreement with the literature where sage's antioxidant activity has been
286 attributed to its phenolic compounds, more abundant in the methanolic extract.

287 In this model of cytoprotection, because effects were tested in co-incubations
288 with the toxicant, the antioxidant protection may reflect mainly direct actions on *t*-BHP
289 toxicity [22]. These direct effects would include, besides the antiradical scavenging or
290 hydrogen-donating activity measured in this study, the compounds' ability to chelate
291 metal ions [34]. Since ROS [35], *t*-BHP radicals [36,37] and intracellular iron ions [38]
292 are involved in the toxicity of *t*-BHP, direct effects on these parameters would tend to
293 reduce the level of damage. Antiradical activity of sage is well known from previous
294 studies [11,13-16] and was also shown here against DPPH and superoxide radicals.
295 Considering the composition of the extracts in phenolic compounds, they most likely
296 also possess the ability to chelate metal ions [34].

297 Irrespective of their antiradical and metal chelating ability of extracts, they will
298 act as intracellular antioxidants if only the compounds permeate cell membranes. Our
299 previous results underscored the importance of the compound's lipophilicity, in addition
300 to its antioxidant potential, for biological activity [22]. Incubation of HepG2 cells with

301 *t*-BHP induced significant lipid peroxidation, GSH depletion and DNA damage. At
302 IC₈₀, both sage extracts significantly prevented lipid peroxidation and GSH depletion,
303 but failed to prevent DNA damage. In general, there seems to be a good correlation
304 between the many biological effects of sage extracts and those of their main phenolic
305 constituents, rosmarinic acid and luteolin-7-glucoside. These compounds have
306 previously shown in this experimental model to possess cytoprotective activities (IC₅₀'s
307 of 69 μM and 78 μM, respectively) [22]. Although both these compounds have lower
308 lipophilicity than quercetin, they too were able to protect against *t*-BHP-induced
309 toxicity in HepG2 cells (albeit with a 3 times higher IC₅₀ than quercetin). In our
310 previous study, rosmarinic acid and luteolin-7-glucoside also protected significantly
311 against *t*-BHP-induced lipid peroxidation and intracellular GSH depletion, as was the
312 case here for the sage extracts. They seem, therefore, to permeate cell membrane, at
313 least in some extent, and in the case of luteolin-7-glucoside, the removal of the
314 glucoside moiety would probably increase bioavailability.

315 The fact that sage extracts did not prevent DNA damage may be explained by
316 the low lipophilicity of the compounds present. In our previous study, the main phenolic
317 compounds present in this sage extracts, rosmarinic acid and luteolin-7-glucoside,
318 showed poor ability to prevent DNA damage induced by *t*-BHP [22]. In that study, the
319 lipophilicity of phenolic compounds appeared to be of even greater importance for DNA
320 protection than for cytoprotective effects. Only antioxidant compounds with
321 hydrophobicities similar to quercetin were able to protect against DNA damage induced
322 by *t*-BHP in HepG2 cells.

323 Based on previous studies, lipid peroxidation and DNA damage seem not to be
324 as relevant for the *t*-BHP-induced cell death as GSH depletion [22,35]. GSH depletion
325 has been suggested as primary mechanism of *t*-BHP-induced toxicity in liver cells

326 [35,39,40]. GSH plays an important role in hepatocyte defence against ROS, free
327 radicals and electrophilic metabolites [41,42]. A severe GSH depletion leaves cells
328 more vulnerable to oxidative damage and is normally associated with calcium
329 homeostasis disruption, which ultimately causes cell death [42]. The prevention of *t*-
330 BHP-induced GSH depletion in about 40% has previously been suggested as a major
331 contribution to cytoprotective effects in a same experimental model [22]. Thus, the 62%
332 protection against GSH depletion was probably the most relevant effect of the extracts
333 used in this study. In agreement with this in vitro data, Amin and Hamza (2005) [20]
334 showed the ability of sage to protect in vivo against the hepatotoxicity of azathioprine, a
335 drug that acts by depleting GSH levels.

336 Although rosmarinic acid and luteolin-7-glucoside present in the extracts may
337 contribute to the observed prevention of GSH depletion induced by *t*-BHP, they cannot
338 be the sole explanation for the effects of sage extracts on the GSH levels. In the same
339 experimental model, both phenolic compounds were shown to have some pro-oxidant
340 effects decreasing slightly GSH levels when incubated alone with HepG2 cells for 5
341 hours [22], an effect similar to what was observed in this study with quercetin – the
342 positive control. For some phenolic compounds and, in particular, quercetin, the
343 formation of quinone metabolites are thought to mediate the formation of conjugates
344 with GSH, decreasing its basal levels [43,44]. Contrarily to the effects of incubations
345 with the individual phenolic compounds present in the extracts, when sage extracts were
346 incubated alone with HepG2 cells for 5 hours, a slight increase in GSH levels was
347 observed, which was significant for the SOME extract. Compounds other than phenolics
348 present in the extracts appear, therefore, to be important for this effect of sage extracts
349 in HepG2 cells. Since the increase in GSH levels was accompanied by an increase in the
350 total glutathione levels (and not to a reduction in GSSG levels), sage extracts seem to

351 have an ability to increase the *de novo* synthesis of glutathione. In a previous study,
352 after a stress-induced GSH depletion, SOI given in vivo to rats restored GSH levels of
353 subsequent hepatocyte cultures to a higher value than controls [19], which also
354 suggested an increase in the *de novo* glutathione synthesis.

355 In conclusion, this study showed clearly the antioxidant effects at cellular level
356 of sage, namely preventing cell death, lipid peroxidation and GSH depletion induced by
357 *t*-BHP in HepG2 cells. The protection of cell viability conferred by sage extracts
358 seemed to be due mainly to their ability to prevent GSH depletion (by about 60%). This
359 work also showed a good correlation of the above cellular effects of sage with the
360 effects of their main phenolic compounds, rosmarinic acid and luteolin-7-glucoside.
361 Nevertheless, unknown compounds other than phenolics also seem to contribute to the
362 antioxidant effects of sage on basal GSH levels. In fact, this work showed for the first
363 time the ability of sage (mainly the methanolic extract) to increase basal GSH levels,
364 probably by the induction of glutathione synthesis, an effect that may be relevant in the
365 face of oxidative stress. Based on these results, it would be of interest to investigate
366 whether sage has protective effects in suitable in vivo models of liver diseases, where it
367 is known that oxidative stress is involved.

368

369

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Results (tables)

Table 1 – Composition ($\mu\text{g}/\text{mg}$ extract) in phenolic compounds of *S. officinalis* methanolic extract (SOME) and *S. officinalis* infusion (SOI).

Compound	SOME	SOI
Phenolic acids		
Rosmarinic acid	132.2	52.0
Caffeic acid	tr	0.8
Ferulic acid	tr	0.5
3-Caffeoylquinic acid	tr	tr
5-Caffeoylquinic acid	tr	tr
Flavonoids		
Luteolin-7-glucoside	1.2	19.7
4',5,7,8-Tetrahydroxyflavone	0.1	0.9
Apigenin-7-glucoside	tr	0.4

tr – trace amounts

Table 2 – Antiradical activity of the sage extracts and quercetin against DPPH and superoxide radical.

Extract/compound	DPPH ^a		Superoxide radical ^b
	IC ₅₀ (μg/ml)	AE (×10 ⁻³) ^c	IC ₅₀ (μg/ml)
SOME	13.5 ± 0.5	6.12	162 ± 39
SOI	14.9 ± 0.3	5.14	14.4 ± 1.4
Quercetin	3.43 ± 0.07	13.2	10.6 ± 1.0

^a Values represent mean ± SD of 5 replicates.

^b Values represent mean ± SD of 3 independent experiments with 3 replicates each.

^c AE – antiradical efficiency: $AE = 1/(IC_{50} \times T_{IC50})$, where T_{IC50} is the time needed to reach the steady state at the corresponding IC₅₀ concentration.

Table 3 – Potential cytoprotective effects^a of the sage extracts against *t*-BHP-induced toxicity in HepG2 cells.

Extract/Compound	IC ₅₀ (μg/ml)	Hillslope	IC ₈₀ (μg/ml)
SOME	7.6 ± 0.5	1.89 ± 0.23	16
SOI	101.4 ± 11.3	1.02 ± 0.13	~250
Quercetin	6.5 ± 0.5	1.95 ± 0.28	13

^a Tested in co-incubations with 2 mM of *t*-BHP (5 h) in HepG2 cells. IC₅₀ and the Hillslope were taken from the plotted dose-response curve (Fig. 1). IC₈₀ concentration was estimated from the same dose-response curve. Values are mean ± SEM of at least 4 independent experiments.

Results (figures)

Fig. 1 – Dose-response effect of the sage extracts against *t*-BHP-induced toxicity in HepG2 cells. After incubating HepG2 cells with 2 mM of *t*-BHP and sage extracts/querceetin for 5 h, protection against cell death (as measured by LDH leakage) versus sage extract/querceetin concentrations (in logarithm) were plotted in order to take the IC₅₀ and Hillslope of each compound (Table 3). Values are mean ± SEM of at least 4 independent experiments.

Fig. 2 – Effects of sage extracts at IC₈₀ concentration against *t*-BHP-induced cell death. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with sage extract/querceetin at IC₈₀ concentration and cell viability measured by LDH leakage. Values are mean ± SEM, n = 5. *** P≤0.001 when compared with the negative control. #### P≤0.001 when compared with the *t*-BHP control.

Fig. 3 – Effects of sage extracts at IC₈₀ concentration against *t*-BHP-induced lipid peroxidation in HepG2 cells. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with sage extract/querceetin at IC₈₀ concentration and lipid peroxidation measured by TBARS assay. Values are mean ± SEM, n = 5 (100% = 2.25 nmol/mg). *** P≤0.001 when compared with the negative control. ## P≤0.01 and #### P≤0.001 when compared with the *t*-BHP control.

Fig. 4 – Effects of sage extracts at IC₈₀ concentration against *t*-BHP-induced decrease in GSH levels in HepG2 cells. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with sage extract/querceetin at IC₈₀ concentration and GSH levels determined by the DTNB-GSSG reductase recycling assay. Values are mean ± SEM, n = 5 (100% = 72.4

nmol/mg). * $P \leq 0.05$ and *** $P \leq 0.001$ when compared with the negative control. ##
 $P \leq 0.01$ and ### $P \leq 0.001$ when compared with the *t*-BHP control.

Fig. 5 – Effects of sage extracts at IC_{80} concentration against *t*-BHP-induced DNA damage in HepG2 cells. HepG2 cells were incubated with *t*-BHP 200 μ M (1 h) and/or with sage extract/quercetin at IC_{80} concentration and DNA damage evaluated by the comet assay. DNA damage was assessed by the semiquantitative method of visual scoring. Values are mean \pm SEM, n = 4 (100% = 187.1 arbitrary units). *** $P \leq 0.001$ when compared with the negative control. ### $P \leq 0.001$ when compared with the *t*-BHP control.

