Drinking of *Salvia officinalis* tea increases CCl_4-induced hepatotoxicity in mice

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Abbreviations: CCl_4 – carbon tetrachloride; CYP – cytochrome P450; CYPR – NADPH-cytochrome P450 reductase; EROD – ethoxyresorufin-*O*-dealkylation; GSH – glutathione (reduced form); GST – glutathione-s-transferase; GPox – glutathione peroxidase; GR – glutathione reductase; H&E – hematoxylin and eosin; PNP-H – paranitrophenol hydroxylation ; PROD – pentoxyresorufin-*O*-dealkylation; *t*-BHP – *tert*-butyl hydroperoxide

Abstract

In a previous study, the drinking of a *Salvia officinalis* tea (prepared as an infusion) for 14 days improved liver antioxidant status in mice and rats where, among other factors, an enhancement of glutathione-S-transferase (GST) activity was observed. Taking in consideration these effects, in the present study the potential protective effects of sage tea drinking against a situation of hepatotoxicity due to free radical formation, such as that caused by carbon tetrachloride (CCl_4), were evaluated in mice of both genders. Contrary to what was expected, sage tea drinking significantly increased the CCl_4-induced liver injury, as seen by increased plasma transaminase levels and histology liver damage. In accordance with the previous study, sage tea drinking enhanced significantly GST activity. Additionally, glutathione peroxidase was also significantly increased by sage tea drinking. Since CCl_4 toxicity results from its bioactivation mainly by cytochrome P450 (CYP) 2E1, the expression level of this protein was measured by Western Blot. An increase in CYP 2E1 protein was observed which may explain, at least in part, the potentiation of CCl_4-induced hepatotoxicity conferred by sage tea drinking. The CCl_4-induced hepatotoxicity was higher in females than males. In conclusion, our results indicate that, although sage tea did not have toxic effects of its own, herb-drug interactions are possible and may affect the efficacy and safety of concurrent medical therapy with drugs that are metabolized by phase I enzymes.

1. Introduction

Chronic liver diseases are common worldwide and are characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (Loguercio and Federico, 2003; Vitaglione et al., 2004). There are increasing evidences that free radicals and reactive oxygen species play a crucial role in the various steps that initiate and regulate the progression of liver diseases independently of the agent in its origin (Loguercio and Federico, 2003; Vitaglione et al., 2004). By virtue of its unique vascular and metabolic features, the liver is exposed to absorbed drugs and xenobiotics in concentrated form. Detoxification reactions (phase I and phase II) metabolize xenobiotics aiming to increase substrate hydrophilicity for excretion. Drug-metabolizing enzymes detoxify many xenobiotics but bioactivate or increase the toxicity of others (Jaeschke et al., 2002). In case of bioactivation, the liver is the first organ exposed to the damaging effects of the newly formed toxic substance. Therefore, protective mechanisms relevant to the liver are of particular interest.

Because free radicals and reactive oxygen species play a central role in liver diseases pathology and progression, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Vitaglione et al., 2004). Additionally, recent studies have suggested that natural antioxidants in complex mixtures ingested with the diet are more efficacious than pure compounds in preventing oxidative stress-related pathologies due to particular interactions and synergisms (Vitaglione et al., 2004). Natural antioxidants may act as protectors of several pathologies not only as conventional hydrogen-donating compounds (antiradical activity) but, more importantly, may exert modulatory effects in cells through actions in antioxidant, drug-metabolizing and repairing enzymes as well as working as signaling molecules in important cascades for cell survival (Ferguson et al., 2004; Williams et al., 2004).

Salvia officinalis L. (common sage) is a medicinal plant well known for its reputation of being a panacea and for its strong antioxidant properties attributed to its constitution in phenolic compounds (rosmarinic acid being the most representative) (Cuvelier et al., 1994; Baricevic and Bartol, 2000). In an in vivo study using rats, treatment with a sage water extract for 5 weeks protected against the hepatotoxicity of azathioprine, a drug that acts by reducing GSH levels, revealing the antioxidant properties of this extract (Amin and Hamza, 2005). Drinking of sage tea (prepared as an infusion) for 14 days also improved liver antioxidant status in mice and rats. It significantly increased the activity of a phase II detoxifying enzyme, glutathione-S-transferase (GST), and protected against lipid peroxidation and GSH depletion induced by an oxidant insult (*tert*-butyl hydroperoxide) in rat hepatocytes in primary culture (Lima et al., 2005). In view of these observations we hypothesised that sage tea would have protective effects in an *in vivo* situation of free radical-mediated hepatotoxicity, such as that caused by the well known hepatotoxin carbon tetrachloride (CCl_4). Therefore, in the present study, we evaluate the potential hepatoprotective effects of sage tea drinking for 14 days against a subsequent acute toxic dose of CCl_4 in mice.

In the liver, CCl_4 metabolism begins with the formation of the trichloromethyl radical ($CCl_3^{\bullet}$) through the action of cytochrome P450 (CYP) enzymes, phase I drug-metabolizing or detoxifying enzymes. This radical can also react with oxygen to form its highly reactive derivative trichloromethylperoxy radical ($CCl_3OO^{\bullet}$). Both radicals initiate chain reactions of direct and indirect bond formation with cellular molecules (nucleic acids, proteins, lipids and carbohydrates) impairing crucial cellular processes that may ultimately culminate in extensive cell damage and death (Weber et al., 2003). The bioactivation of CCl_4 is mainly executed by the CYP 2E1 isozyme, but at higher

concentrations CYP 2B1, CYP 2B2 and CYP 3A (only in humans) are capable of attacking this haloalkane (Weber et al., 2003).

Because the bioactivation of the drug needs to occur in this model of hepatotoxicity, effects on the activity of CYP enzymes and in particular the expression of CYP 2E1 should be considered when studying effects on CCl_4 toxicity. It is well known today that the inhibition of CYP 2E1 decreases CCl_4 hepatotoxicity. On the other hand, the induction of this cytochrome increases the drug's hepatotoxicity (Weber et al., 2003). Since pharmaceutical drugs may also be metabolized by CYP enzymes, drug-drug interactions are possible and have been recognized between herbal medicines and conventional drugs, which may affect the safety of phytomedicine users (Ioannides, 2002; Izzo, 2005; Hu et al., 2005).

Finally, gender is another factor that should be studied. Because CYP enzyme activities are known to be gender dependent (Kato and Yamazoe, 1992; Clewell et al., 2002; Meibohm et al., 2002), the extension of cell damage caused by toxicants that are metabolized by phase I enzymes may be significantly different in males and females. We therefore evaluated the gender effect on the potential protection against CCl_4-induced hepatotoxicity conferred by sage tea drinking in mice.

2. Materials and methods

2.1. Chemicals

Glutathione reductase (EC 1.6.4.2.), glucose-6-phosphate dehydrogenase (EC 1.1.1.49.), aprotinine, *tert*-butyl hydroperoxide (*t*-BHP), 7-ethoxyresorufin, 7-pentoxyresorufin and Bradford reagent were purchased from Sigma (St. Louis, MO, USA). The rabbit polyclonal antibody against CYP 2E1 protein was purchased from StressGen (Victoria, Canada). All other reagents were of analytical grade.

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96 *2.2. Plant material, preparation of sage tea and composition in phenolic and volatile*
97 *compounds*

98 *Salvia officinalis* L. plants were cultivated in an experimental farm located in
99 Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were
100 lyophilized and kept at -20 ºC. Considering that sage is traditionally used as a tea, an
101 infusion of sage was routinely prepared as in a previous study by pouring 150 ml of
102 boiling water onto 2 g of the dried plant material and allowing to steep for 5 min (Lima
103 et al., 2005). This preparation produced a 3.5 ± 0.1 mg of dry weight extract per ml of
104 infusion, with rosmarinic acid (362 μg/ml of infusion) and luteolin-7-glucoside (115.3
105 μg/ml of infusion) as a major phenolic compounds and 1,8-cineole, *cis*-thujone, *trans*-
106 thujone, camphor and borneol as major volatile compounds (4.8 μg/ml of infusion)
107 (Lima et al., 2005).

108

109 *2.3. Animals*

110 Twenty male and twenty female Balb/c mice, 6-8 weeks (male: 20.3 ± 2.4;
111 female: 17.6 ± 1.9), were purchased from Charles River Laboratories (Spain) and
112 acclimated to our laboratory animal facilities for at least one week before the start of the
113 experiments. During this period, the animals were maintained on a natural light/dark
114 cycle at 20 ± 2 ºC and given food and tap water *ad libitum*. The animals used in this
115 experiment were kept and handled in accordance to our University regulations that
116 follows the *Guidelines for the Humane Use and Care of Laboratory Animals*.

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118 *2.4. CCl_4-induced hepatotoxicity in mice*

Twenty male Balb/c mice were randomly divided into two groups (five per cage), given food *ad libitum* and either drinking water (tap) or sage tea *ad libitum* for 14 days (beverage was renewed daily). Twenty four hours before the end of the experiment, half the animals of each drinking group received an ip injection of CCl_4 in order to observe the hepatic injury effects (Chung et al., 2005). CCl_4 was administered ip at 20 μl/kg in olive oil (8 ml/kg) to induce liver injury as previously described (Chen et al., 2004), and controls received vehicle only. At the end of the experiment, animals were sacrificed by cervical dislocation and plasma collected for measurement of transaminase activities (ALT-alanine aminotransferase and AST-aspartate aminotransferase). The livers were also collected, frozen in liquid nitrogen and kept at -80 ºC for later analysis and measurement of several liver parameters.

The same experimental outline was used for the twenty female Balb/c mice.

2.5. Biochemical analysis

Histological examinations

A fresh piece of the liver from each mouse, previously trimmed to approximately 2 mm thickness, was rapidly immersed in Bouin's solutionand kept for 24 h at 4 ºC. Fixed tissues were then processed routinely for embedding in paraffin, sectioned (5 μm), deparaffinized and rehydrated using standard techniques. The extent of CCl_4-induced liver damage was evaluated based on morphological changes in liver sections stained with hematoxylin and eosin (H&E) using standard techniques. Histological damage was expressed using the following score system: 0 - absent; + - few; + + - mild; + + + - moderate; + + + + - severe; and, + + + + + - extremely severe.

Liver homogenates and microsome isolation

For measurement of the activities of GST, glutathione peroxidase (GPox), glutathione reductase (GR) and NADPH-cytochrome P450 reductase (CYPR) in mice liver, a piece of tissue was homogenized individually in a phosphate/glycerol buffer pH 7.4 (Na_2HPO_4 20 mM; β-mercaptoethanol 5 mM; EDTA 0.5 mM; BSA 0.2% (w/v); aprotinine 10µg/ml and glycerol 50% (v/v)) and centrifuged at 10,000 × *g* at 4 ºC for 10 min and the supernatant collected.

For measurement of the activities of cytochromes P450 and analysis of the expression level of CYP 2E1 protein, liver microsomes were isolated by differential centrifugation as described elsewhere (Barbier *et al.*, 2000). In brief, a piece of the liver was homogenized in homogenization buffer (80 mM K_2HPO_4, 80 mM KH_2PO_4 (pH 7.4), 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM phenylmethanesulfonyl fluoride) and centrifuged at 12,000 × *g* at 4 ºC for 20 min. The supernatant was collected and centrifuged at 105,000 × *g* at 4 ºC for 1 h. Microsomal pellets were resuspended in homogenization buffer, rapidly frozen in liquid nitrogen and stored at -80 ºC.

Enzyme activities

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), GST and GR activities were measured spectrophotometrically as previously described (Lima et al., 2005). GPox activity was also measured as previously described by Lima et al. (2006).

The CYPR activity was determined indirectly by measuring its NADPH-cytochrome *c* reductase activity as previously described (Phillips and Langdon, 1962) with the modifications introduced by Plaa and Hewitt (1982) and the results expressed as nmol cytochrome c reduced per minute per mg of protein (mU/mg).

Microsomal ethoxyresorufin-*O*-dealkylation (EROD) and pentoxyresorufin-*O*-dealkylation (PROD) were determined according to Burke *et al*. (1985) with some modifications (Pearce *et al.*, 1996). Briefly, liver microsomes (0.2 mg) were incubated at 37 ºC in 1 ml (final volume) incubation mixture containing 100 mM KH_2PO_4 (pH 7.4), 7.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM NADP – 5 mM glucose-6-phosphate/0.5 U/ml glucose-6-phosphate dehydrogenase and either 7-ethoxyresorufin (5 μM) or 7-pentoxyresorufin (10 μM) in the EROD or PROD activities, respectively. Reactions were started by addition of the NADPH-generating system and were stopped after 5 min by addition of 2 ml of ice-cold acetone. After centrifugation, the amount of resorufin was determined fluorometrically with a Perkin Elmer LS50 spectrophotometer (Perkin-Elmer Ltd., Buckinghamshire, UK). The activity was expressed as pmol resorufin formed/min/mg microsomal proteins using a standard curve of resorufin.

Paranitrophenol hydroxylation (PNP-H) in liver microsomes was assessed according to the methodology previously described by Allis and Robinson (1994), following specrophotometrically at 480 nm the formation 4-nitrocatechol. Briefly, 0.2 mg of microsomal proteins were pre-incubated for 5 min at 37 ºC with 1 mM *p*-nitrophenol and 100 mM Hepes (pH 6.8). Five minutes after adding the NADPH-generating system, the formation of 4-nitrocatechol was followed at 480 nm at 37 ºC on a plate reader spectrophotometer and the results expressed as pmol 4-nitrocatechol formed/min/mg microsomal proteins using the extinction coefficient of 3.567 $mM^{-1}.cm^{-1}$.

Glutathione content

The glutathione content of mice livers was determined by the DTNB-GSSG reductase recycling assay as previously described (Lima *et al*., 2004). The results are expressed as nmol GSH/mg of liver.

Protein

Protein content of liver homogenates was determined with Bradford Reagent using bovine serum albumin as a standard. Protein content of liver microsomes was determined by the Lowry method (Lowry *et al.*, 1951).

2.6. CYP 2E1 expression analysis

The expression of CYP 2E1 protein was analyzed by Western Blot. Electrophoretic separation of microsomal proteins (15 μg) was performed in 12% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) using the mini-PROTEAN 3 electrophoresis cell (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to the method of Laemmli (1970). The separated proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Buckinghamshire, UK) using the method of Towbin and collaborators (1979). The PVDF membranes were blocked with 5% nonfat dry milk overnight at 4°C and the immunoblots exposed to rabbit polyclonal antibody against CYP 2E1 protein. Immunodetection was performed using horseradish peroxidase–labeled donkey anti-rabbit IgG antibody (Amersham Biosciences, Buckinghamshire, UK) and developed with ECL reagents (Amersham Biosciences) according to manufacturer's instructions. The amount of protein was quantified by densitometry analysis on the SigmaScan Pro 5 program (SPSS Inc., San Rafael, CA, USA) and expressed as percentage of the protein level present in control situation.

2.7. Statistical Analysis

Data are expressed as means ± SEM (n=5). Statistical significances (P values < 0.05) were evaluated by two-way ANOVA based on gender and treatment group (water

drinking + saline ip; water drinking + CCl_4 ip; sage tea drinking + saline ip; sage tea drinking + CCl_4 ip) followed by the Student-Newman-Keuls post hoc test. ALT and AST data were natural logarithm transformed prior to statistical analysis in order to stabilize the variance.

3. Results

The effect of drinking of sage tea for 14 days (instead of water) on the hepatotoxicity of CCl_4 was evaluated in mice of both genders challenged with a single dose of CCl_4 (20 μl/kg, ip). Plasma transaminase activities were measured 24 h after CCl_4 administration as markers of liver injury (Fig. 1). Elevated ALT and AST activities were observed due to CCl_4 administration, which is always higher in females compared with males. Both males and females that had been drinking sage tea were significantly more sensitive to the hepatotoxic effects of CCl_4 than their control counterparts, as indicated by increased plasma transaminase activities.

<Insert figure 1 here>

CCl_4 is a hepatotoxicant known to produce a characteristic centrilobular pattern of degeneration and necrosis (Weber et al., 2003). Histological examination of H&E-stained liver sections was conducted 24 h after CCl_4 administration to confirm the pattern of hepatotoxicity and compare the extent of liver injury between control and sage tea drinking animals (Table 1). Morphological findings were consistent with plasma transaminase observations. The CCl_4 induced histopathological changes in the liver with significant degeneration and necrosis of hepatocytes in the centrilobular region and with perivenular inflammatory infiltrates. These CCl_4-induced histopathological changes were significantly potentiated in the sage tea drinking group of mice with about 50-60% of total area presenting signs of degeneration, necrotic

<Insert table 1 here>

241 regions and higher leukocyte infiltration. Also histologically, the liver damage induced
242 by the CCl_4 in mice appear to be higher in females than in males.
243 CCl_4 is a hepatotoxic chemical that requires metabolic activation by phase I
244 drug-metabolizing enzymes and therefore it was important to monitor the effects of sage
245 tea drinking on the activity of some CYP enzymes. For that, EROD, PROD and PNP-H
246 were measured in liver microsomal fractions (Table 2). Comparing the groups where
247 CCl_4 was not administered, although not statistically significant, sage tea drinking
248 increased slightly, between 8% and 13%, the activity of CYP 1A and CYP 2E1 in both
249 genders. The activities of CYP 2B and CYP 2E1 in females was lower and higher,
250 respectively, when compared with males. Twenty four hours after administration, CCl_4
251 hepatotoxicity was also reflected in the decrease observed for the activities of the CYP's
252 measured as well as in the majority of the others enzyme activities (Table 3).
253 Comparing drinking groups, the decrease in these enzyme activities after CCl_4
254 administration was also consistent with the higher toxicity in sage tea groups, since it
255 was in general significantly higher in sage tea than water drinking mice.
256 The CYPR is an essential enzyme for microssomal P450-mediated
257 monooxygenase activity, which by interaction with the different CYP's transfers the
258 essential electron from NADPH (Backes and Kelley, 2003; Henderson et al., 2003).
259 Therefore, its activity was measured (Table 2), and was found to be significantly higher
260 in female mice, which indirectly may contributed to higher toxicity of CCl_4 in females.
261 Sage tea drinking induced 21% the activity of this cytochrome, but only in female mice.
262 The bioactivation of CCl_4 is mainly executed by CYP 2E1 (Weber et al., 2003).
263 It is also known that modulatory effects on the expression of CYP 2E1 affects the CCl_4-
264 induced hepatotoxicity (Weber et al., 2003). Therefore, in addition to the measurement
265 of some CYP enzyme activities which included the CYP 2E1, the expression of this

<Insert table 2 here>

<Insert table 3 here>

cytochrome was evaluated by Western Blot (Fig. 2). Sage tea drinking for 14 days increased significantly the amount of CYP 2E1 protein in females (24%) abut it only slightly increased in males (8%). In sage tea drinking mice, the decrease on CYP 2E1 protein induced by CCl_4 was most severe in females.

<Insert figure 2 here>

After bioactivation, CCl_4-induced hepatotoxicity is mediated by primary and secondary bond formation of reactive species to critical cellular molecules such as DNA, lipids, proteins or carbohydrates (Weber et al., 2003). Thus, detoxifying enzymes (such as GST) and antioxidant enzymes (such as the pair GPox/GR) are important against the cell stress situation caused by CCl_4. To monitor effects at this level, three glutathione-related enzymes were measured (Table 3) and gender differences were observed in all of them. The activity of GST in males was significantly increased by sage tea drinking, as previously observed in other study (Lima et al., 2005). GPox activity was also increased by sage tea drinking but significantly only in females. Hepatic GSH is an important intracellular antioxidant that can scavenge free radicals and could be important in the defense against radical-mediated hepatotoxicity. Alterations in GSH and oxidized glutathione (GSSG) levels are therefore an important indicator of oxidative stress. Comparing the groups where CCl_4 was not administered, there was no effect of sage tea drinking on GSH and GSSG levels in male and female mice (Table 3). Twenty four hours after CCl_4 administration, GSH levels decreased significantly only in females from the sage tea drinking group. GSSG levels increased significantly after CCl_4 administration in both genders but only in the sage tea drinking groups (Table 3). This increase was significantly higher in females than males. As a result, glutathione data also suggest higher cell damage induced by CCl_4 in the sage tea drinking groups in females.

290 Finally, soluble protein measured after 10,000 × *g* centrifugation (Table 3)
291 corroborates the previous results. Comparing the groups where CCl_4 was not
292 administered, the higher soluble protein found in the sage tea drinking groups suggests
293 induction of protein expression. The decrease in soluble protein, with concomitant
294 precipitation of damaged proteins, found after the haloalkane administration suggests
295 higher toxicity of CCl_4 in the sage tea drinking groups and in females.
296
297 **4. Discussion**
298 In a previous study, sage tea drinking significantly increased (rat and mouse)
299 liver GST activity and protected against GSH depletion and lipid peroxidation induced
300 by an oxidant agent (Lima et al., 2005). Considering these beneficial effects on liver
301 antioxidant status the present study was carried out in order to evaluate whether sage tea
302 drinking would reduce the extent of hepatic injury induced by CCl_4 in male and female
303 mice. In a recently published work, GST was implicated as an important defence
304 mechanism during the early stages (1–6 h) of the CCl_4-induced liver injury (Dwivedi et
305 al., 2006). GST is a phase II enzyme that plays a key role in cellular detoxification of
306 xenobiotics, electrophiles and reactive oxygen species through their conjugation to GSH
307 (Mates, 2000). Besides an essential substrate to GST and GPox, GSH is also an
308 important intracellular antioxidant (hydrogen-donating compound) that spontaneously
309 neutralizes several electrophiles and reactive oxygen species (Lu, 1999). After
310 bioactivation of CCl_4, in addition to dangerous free radical formation and subsequent
311 reactive oxygen species formation, a sequence of chain reactions can be initiated that
312 leads to lipid peroxidation (Weber et al., 2003). Since sage tea drinking has also been
313 shown to decrease lipid peroxidation induced by *tert*-butyl hydroxide in rat hepatocyte
314 primary cultures (Lima et al, 2005), this also suggested here possible beneficial effects

315 against CCl_4. However, contrary to our hypothesis, sage tea drinking increased
316 significantly the CCl_4-induced hepatotoxicity in mice.

317 CCl_4 becomes toxic upon activation mainly through CYP 2E1, and an induction
318 or an over-expression of this cytochrome correlates with higher CCl_4 toxicity (Weber et
319 al., 2003; Chan et al., 2005). Sage tea drinking for 14 days increased the expression
320 level of CYP 2E1. In agreement with this, the activity of this cytochrome was also
321 slightly increased by sage tea drinking. This could provide an explanation for the higher
322 CCl_4 toxicity in tea drinking mice. CYP 2E1 protein is localized predominantly in the
323 central zone of the liver lobule (Forkert et al., 1991), which explains the typical
324 centrilobular region of hepatocyte injury observed after CCl_4 administration. This
325 pattern of centrilobular toxicity was more extensive in sage tea versus water drinking
326 mice. After CCl_4 bioactivation, the resulting $CCl_3^{\bullet}$ radical binds covalently to CYP 2E1,
327 either to the active site of the enzyme or to the heme group, thereby causing suicide
328 inactivation (Weber et al., 2003). After drug administration to sage tea drinking mice,
329 CYP 2E1 levels, originally higher, decreased to significant lower levels. A decrease in
330 CYP 2E1 expression and activity after CCl_4 exposure seem to reflect inactivation of the
331 protein, which is consistent with the increased CCl_4 hepatotoxicity in this drinking
332 group. However, to confirm increased CCl_4 bioactivation through CYP 2E1 in sage tea
333 drinking mice than the water drinking cohorts, measurement of covalent binding of
334 $^{14}CCl_4$-derived radiolabel to liver tissue would have to be done. The simultaneous
335 increases in GST and GPox activities by sage tea drinking, and possibly other
336 detoxifying and antioxidant enzymes, seem to have been incapable of neutralizing
337 increased CCl_4 toxicity. Also, the previously observed beneficial effect of sage tea
338 against lipid peroxidation (Lima et al., 2005) seemed to be insufficient to block CCl_4-
339 induced damage. The increased levels of CYP 2E1 protein and activity induced by sage

340 tea drinking may, thus, at least in part, provide an explanation for the obtained results –
341 an herb-toxicant interaction between sage tea and CCl_4 that potentiated the haloalkane's
342 toxicity.

343 Herb-drug interactions have been described for a variety of plants used as
344 phytomedicines, many of them by case reports of interactions between herbs and
345 pharmaceutical drugs (Izzo, 2005; Hu et al., 2005). CYP isozymes are particularly
346 vulnerable to modulation by the diverse active constituents of herbs (Zhou et al., 2003).
347 This important phase I drug-metabolizing enzyme system is responsible for the
348 metabolism of a variety of xenobiotics and some important endogenous substances such
349 as steroids and prostaglandins (Anzenbacher and Anzenbacherova, 2001; Tamasi et al.,
350 2003). Although CYP-mediated reactions are primarily detoxification processes, certain
351 substrates are metabolically activated resulting in the generation of reactive
352 intermediates with increased toxicity and mutagenicity (Jaeschke et al., 2002; Tamasi et
353 al., 2003). Many pharmaceutical drugs are also metabolized by these phase I enzymes
354 and modulation of CYPs by herbs may either exacerbate the undesirable effects (by
355 increasing toxicity) or antagonize the actions (by increasing clearance) of concurrent
356 medical therapy (Stedman, 2002). In addition, severe hepatic injury may be caused by
357 chemicals or natural toxins metabolically activated by drug-metabolizing enzymes as a
358 result of occupational, household or environmental exposure, emphasizing the need for
359 understanding mechanisms of action of herbal extracts. Thus, although interspecies
360 differences in xenobiotic metabolism are well documented (Caldwell, 1992), the drug-
361 toxicant interaction between sage tea and CCl_4 reported here highlight possible herb-
362 drug interactions between this extract and drugs metabolized by the liver. However, as
363 far as we know, there were no reports of drug-drug interactions between sage tea and
364 pharmaceutical drugs or environmental contaminants. In this particular study, where a

herb-drug interaction was observed, sage tea replaced almost 100% the water that the animal consumed, since food is provided as dry pellets. Therefore, by taking 1 or 2 cups of sage tea, a person never reaches the dose of sage extract ingested by mice in this study. So, it seems that the moderate, traditional drinking of sage tea by people most likely does not result in adverse interactions with other drugs. It should, however, be kept in mind that, if a phytomedicine with a higher dose of sage is taken over an extended period of time, an opportunity for enzyme induction could occur and undesirable interactions take place. Additionally, interindividual differences in drug metabolism, for example due to genetic polymorphism of CYP genes (Tamasi et al., 2003; Wu and Cederbaum, 2005), could increase the susceptibility of different populations or individuals for herb-drug interactions.

Many of these drug-metabolizing enzymes and also antioxidant enzymes are known to be gender dependent (Chaubey et al., 1994; Clewell et al., 2002; Sverko et al., 2004), which may ultimately differentially affect the toxicity of drugs between male and female individuals of the same specie (Kato and Yamazoe, 1992; Meibohm et al., 2002; Chanas et al., 2003). The hepatotoxicity of CCl_4 to females was higher than to males in both drinking groups. Looking to all measured parameters, several gender differences were observed which can explain the higher toxicity to female mice. In terms of drug bioactivation, although the activity of CYP 2E1 was lower in females, the expression of CYP 2E1, the activity of CYP 2B family and the activity of CYPR were higher in females which seems to indicate an increased ability to metabolise CCl_4 in females. In terms of cell defences against drug-induced injury, although GPox activity was higher in females, GST activity is significantly higher in males. At least during the initial stage of CCl_4-induced hepatotoxicity, GST is more likely to confer protection, since CCl_4 toxicity is mediated by strong free radicals.

These CYP modulatory as well as antioxidant effects of plant extracts have often been attributed to phenolic and monoterpenic compounds (Elegbede et al., 1993; Banerjee et al., 1995; Birt et al., 2001; Ren et al., 2003; Ferguson et al., 2004). Flavonoids are a diverse group of polyphenols that are produced by several plants (Havsteen, 2002). In relation to phase I and phase II drug-metabolizing enzymes, flavonoids have been reported to possess several modulatory effects, either inducing or decreasing the expression of these enzymes and also either as potent inhibitors or stimulators of enzyme activities, depending on structure, concentration, and assay conditions (Zhou et al., 2003; Ferguson et al., 2004). Rosmarinic acid is the predominat phenolic compound in sage tea (Lima et al., 2005). The oral administration of rosmarinic acid in rats was previously shown not to induce phase I and phase II enzymes (Debersac et al., 2001), and, therefore, was possibly not the responsible for the effects observed in our study. Luteolin-7-glucoside, the major flavonoid present in sage tea, and also monoterpenes present in the essential oil fraction, could, on the other hand, be good candidates. However, pre-treatment of rats with luteolin-7-glucoside was recently found to protect significantly against CCl_4-induced toxicity, and its effects attributed to the compound's antioxidant properties acting as scavenger of reactive oxygen species (Zheng et al., 2004). Most likely, the sage tea effects observed here were a result of interactions and synergisms among the different compounds and metabolites present, which makes it difficult to attribute them to any particular compound or family of compounds.

In conclusion, the present work showed that sage tea drinking for 14 days significantly potentiated CCl_4-induced hepatic injury in mice, to a higher degree in females, as a result, at least in part, of an induction of CYP 2E1. In addition, although sage tea did not have toxic effects of its own and in fact seemed to improve the

antioxidant status of the liver, the observed herb-toxicant interaction may affect the efficacy and safety of concurrent medical therapy with drugs that are metabolized by phase I enzymes.

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Results (tables)

Table 1 – Effect of sage tea drinking for 14 days on CCl_4-induced hepatotoxicity as observed by liver histological examinations.

Microscopic observation	Drinking group	Male		Female	
		without CCl_4	with CCl_4	without CCl_4	with CCl_4
Hepatocyte degeneration (balooning)	water	0	+ +	0	+ +
	sage tea	0	+ + + +	0	+ + + + +
Hepatocyte necrosis	water	0	+	0	+ +
	sage tea	0	+ + +	0	+ + + +
Infiltration of leukocytes (inflammation)	water	+	+ + +	+	+ + +
	sage tea	+	+ + + +	+	+ + + +

0 - absent; + - few; + + - mild; + + + - moderate; + + + + - severe; + + + + + - extremely severe.

Table 2 – Effects of sage tea drinking (for 14 days) and CCl_4 on CYP activities in mice liver.

Enzyme	Drinking group	Male		Female	
		without CCl_4	with CCl_4	without CCl_4	with CCl_4
CYP 1A (pmol/min/mg)	water	52.8 ± 3.1 [a]	39.3 ± 3.4 [b]	46.9 ± 4.8 [a]	33.1 ± 2.2 [b]
	sage tea	58.8 ± 2.4 [a]	27.5 ± 1.4 [c]	53.0 ± 3.1 [a]	19.5 ± 3.2 [c]
CYP 2B (pmol/min/mg)	water	8.6 ± 0.7 [a]	7.7 ± 0.6 [a]	15.4 ± 2.9 [a] *	9.5 ± 0.9 [b]
	sage tea	9.3 ± 0.8 [a]	5.1 ± 0.5 [a]	14.3 ± 1.3 [a] *	6.7 ± 1.6 [b]
CYP 2E1 (pmol/min/mg)	water	0.63 ± 0.05 [a]	0.28 ± 0.04 [b]	0.51 ± 0.06 [a] *	0.26 ± 0.03 [b]
	sage tea	0.68 ± 0.02 [a]	0.16 ± 0.02 [c]	0.57 ± 0.04 [a] *	0.08 ± 0.03 [c]
CYPR (mU/mg)	water	15.2 ± 0.7 [a]	13.4 ± 0.3 [b]	19.1 ± 0.5 [a] *	17.8 ± 0.7 [a] *
	sage tea	15.8 ± 0.6 [a]	10.2 ± 0.5 [c]	23.2 ± 0.5 [b] *	12.2 ± 0.6 [c] *

Values are means ± SEM, n=5. Drinking groups of the same gender with the same letter notation are not significantly different from each other ($P > 0.05$). * $P < 0.05$, significantly different when compared with the same treatment group from males.

CYP 1A1/2, CYP 2B1/2, CYP 2E1, and CYPR represents EROD, PROD, PNP-H and CYP reductase activities, respectively.

Table 3 – Effects of sage tea drinking (for 14 days) and CCl_4 on glutathione-related enzymes, glutathione levels and soluble protein in mice livers.

Parameter	Drinking group	Male		Female	
		without CCl_4	with CCl_4	without CCl_4	with CCl_4
GST (mU/mg)	water	305 ± 15 [a]	307 ± 12 [a]	128 ± 5 [a] *	115 ± 6 [a] *
	sage tea	369 ± 30 [b]	237 ± 23 [c]	144 ± 4 [a] *	76 ± 8 [b] *
GPox (mU/mg)	water	432 ± 20 [a]	456 ± 14 [a]	779 ± 12 [a] *	772 ± 12 [a] *
	sage tea	493 ± 30 [a]	570 ± 25 [b]	888 ± 19 [b] *	694 ± 25 [c] *
GR (mU/mg)	water	24.1 ± 0.9 [a]	24.0 ± 0.3 [a]	20.1 ± 0.6 [a,b] *	21.1 ± 0.2 [a] *
	sage tea	25.2 ± 1.0 [a]	20.9 ± 1.1 [b]	22.4 ± 0.4 [a] *	18.3 ± 0.5 [b] *
GSH (nmol/mg liver)	water	7.61 ± 0.24 [a]	7.48 ± 0.22 [a]	7.46 ± 0.33 [a]	8.36 ± 0.16 [a]
	sage tea	6.53 ± 0.34 [a]	8.18 ± 0.56 [a]	6.71 ± 0.14 [a]	4.53 ± 1.09 [b] *
GSSG (nmol/mg liver)	water	0.26 ± 0.02 [a,b]	0.23 ± 0.02 [a,b]	0.19 ± 0.02 [a]	0.27 ± 0.06 [a]
	sage tea	0.19 ± 0.02 [b]	0.31 ± 0.03 [a]	0.22 ± 0.03 [a]	1.35 ± 0.20 [b] *
Protein (mg protein/g liver)[1]	water	195.7 ± 4.3 [a]	200.4 ± 2.8 [a]	194.1 ± 3.6 [a]	180.1 ± 1.2 [b] *
	sage tea	215.8 ± 4.3 [b]	171.7 ± 4.2 [c]	214.3 ± 2.9 [c]	154.8 ± 4.9 [d] *

[1] Liver soluble proteins measured in the supernatant after a centrifugation of 10,000 × *g* for 10 min at 4 ºC by the Bradford reagent using bovine serum albumin as a standard.

Values are means ± SEM, n=5. Drinking groups of the same gender with the same letter notation are not significantly different from each other ($P > 0.05$). * $P < 0.05$, significantly different when compared with the same treatment group from males.

GST: glutathione-s-transferase; GPox: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione (reduced form); GSSG: glutathione: oxidized form.

Results (figures)

Fig. 1 – Effects of sage tea drinking for 14 days on CCl_4-induced increase in plasma transaminase activities. (A) ALT: alanine aminotransferase; (B) AST: aspartate aminotransferase. Values are means ± SEM, n=5. For statistical evaluation, these data were natural logarithm transformed in order to stabilize the variance. Drinking groups of the same gender with the same letter notation are not significantly different from each other (P > 0.05). * *P*< 0.05, significantly different when compared with the same treatment group from males.

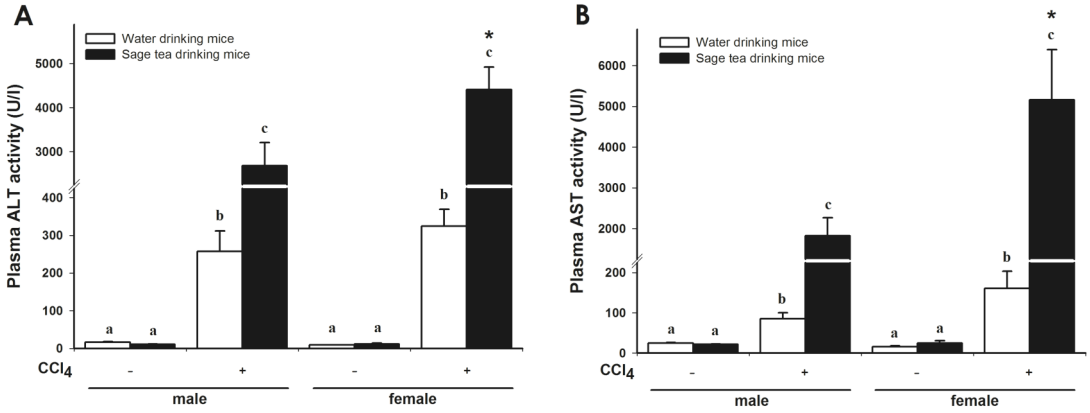

Fig. 2 – Effects of sage tea drinking (for 14 days) and CCl_4 on expression of CYP 2E1 in the liver of male and female mice. Each gel lane was loaded with fifteen µg of microsome proteins for the Western blotting analysis. (A) Results obtained from five mice of each group. Mean ± SEM. Groups of the same gender with the same letter notation are not significantly different from each other (P > 0.05). * *P*< 0.05, significantly different when compared with the same treatment group from males. (B) Representative images of the imunodetection of CYP 2E1 by Western Blot from 2 animals for each group.