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Effects of *Salvia officinalis* in the liver: Relevance of glutathione levels

Abstract

Salvia officinalis L. (*Lamiaceae*, common sage) is an aromatic plant that grows in Portugal well known for its medical properties. Antioxidant and antidiabetic effects are among the medicinal properties attributed to this plant. However, both still lack of biological experimental confirmation. Since sage compounds, after gastrointestinal absorption, first pass through the liver, giving an opportunity to accumulate to considerable concentrations, the antioxidant and antidiabetic studies reported here focused on the liver. In addition, the liver is a biotransforming organ, susceptible to toxic effects of xenobiotics and affected by several liver diseases where oxidative stress is known to be involved. Therefore, these studies allowed us to observe, besides the benefits of sage consumption to the liver, the possible toxic effects of sage and interactions with other drugs. Three different extracts of sage were studied: the essential oil (EO), a methanolic extract and a water extract (prepared as a tea).

In chapter 2, sage EO was tested in freshly isolated rat hepatocytes. No direct antioxidant effects were observed in liver cells. On the contrary, at higher concentrations (more than 2 µl/ml), sage EO induced significant cell death, which was accompanied by GSH (glutathione – reduced form) depletion but not by lipid peroxidation. The GSH depletion induced by sage EO was probably an important mechanism that explains EO toxic effects.

Then, after an *in vivo* experiment with mice, where sage tea drinking (for 14 days) improved the liver antioxidant status (chapter 3), the direct effects of sage were studied on HepG2 cells (a human hepatoma cell line) in chapter 4. Sage extracts protected against cell death induced by *tert*-butyl hydroperoxide – a model of oxidative stress – revealing their direct antioxidant effects at cellular level. Sage was able to act at a cell critical parameter, the GSH levels, preventing GSH depletion. The cytoprotective effect of sage was found to be dependent on its composition in phenolic compounds and their antiradical activity.

Also in chapter 4, not only did sage extracts prevent GSH depletion in a situation of oxidative stress, they also increased basal GSH levels in HepG2 cells. This fact indicated a capacity of sage extracts to improve basal cell antioxidant defences. The increase in basal GSH levels may possibly have happened by induction of *de novo* glutathione synthesis, which was corroborated by a different set of experiments using rat hepatocytes in culture (chapter 3). In that study, sage tea given *in vivo* restored hepatocyte GSH levels after collagenase isolation to higher levels when compared with controls. Sage tea was, in addition, able to induce significantly the activities of GST (chapter 3), GPox and NADPH cytochrome P450 reductase

and also, slightly, some CYP enzymes (chapter 5) in mice liver. These indirect antioxidant effects may leave liver cells better prepared to face oxidative stress and toxicants.

However, contrarily to what was expected, sage tea drinking did not protect carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice (chapter 5). Instead, sage tea potentiated the toxicity of CCl_4 in mice liver of both genders. This herb-toxicant interaction may be explained, at least in part, by the significant induction of CYP 2E1 protein by sage tea. Since the dose of sage tea used in this study was much higher than what is usually taken by humans, sage tea-drug interactions are not likely to happen in humans. Nevertheless, these results draw attention to possible herb-drug interactions between sage and drugs metabolised by the liver.

Regarding the antidiabetic effects (chapter 6), sage tea was found to lower fasting plasma glucose in mice indicating effects on gluconeogenesis. This was confirmed in the experiments using normal rat hepatocytes in culture, where sage tea drinking induced a decrease in gluconeogenesis in response to glucagon. In addition, sage tea drinking increased glucose uptake capacity and, sage EO further increased hepatocyte sensitivity to insulin and inhibited gluconeogenesis. Overall, these effects resemble those obtained with the pharmaceutical drug metformin used in the treatment and prevention of type 2 *diabetes mellitus*. In primary cultures of hepatocytes isolated from streptozotocin-induced diabetic rats none of these activities, however, was observed. Nevertheless, the metformin-like effect observed here suggests a possible type 2 diabetes preventive potential of sage tea, mainly in people at risk of developing it.

In conclusion, the antioxidant effects of sage, at cellular and liver levels, were demonstrated by these results as well as its hypoglycaemic effects. In order to apply sage products as a therapeutical tool for diabetes and liver diseases more research has, however, to be done. Nevertheless, sage products may now be considered as a functional food or a food supplements that could have a beneficial impact in low cost prevention strategies of diabetes and liver diseases. In addition, the treatment and/or the prevention of other kind of diseases where oxidative stress is known to be involved, such as cancer and neurodegenerative diseases, may also benefit from the regular consumption of sage. Care should be taken, however, not to use high doses of sage products, over extended periods of time, in combination with conventional pharmaceutical drugs, since undesired herb-drugs interaction may take place.

Efeitos de *Salvia officinalis* no fígado: Relevância dos níveis de glutationa

Resumo

Salvia officinalis L (*Lamiaceae*, salva comum) é uma planta aromática que cresce espontaneamente em Portugal, bastante conhecida pelas suas propriedades medicinais. Duas das principais propriedades medicinais atribuídas a esta planta são como antioxidante e antidiabética. No entanto, ainda não existem suficientes evidências experimentais que demonstrem estes efeitos e em particular os mecanismos inerentes em sistemas biológicos. No presente trabalho, os estudos dos efeitos antioxidantes e antidiabéticos foram realizados no fígado e em hepatócitos. Uma vez que os compostos da salva passam primeiro pelo fígado após a absorção gastrointestinal, estes podem atingir concentrações consideráveis neste órgão possibilitando a indução de efeitos biológicos. Além disso, o fígado tem uma grande capacidade de biotransformação, é bastante susceptível a efeitos tóxicos de xenobióticos e é também afectado por bastantes doenças onde se reconhece o envolvimento de stresse oxidativo. Deste modo, para além dos prováveis benefícios do consumo de salva para o fígado, estes estudos permitiram-nos observar possíveis efeitos tóxicos desta planta e interacções com outros compostos. Três tipos de extractos foram estudados: o óleo essencial (OE), um extracto metanólico e a extracto aquoso de salva preparado sob a forma de um chá.

Numa primeira série de experiências (capítulo 2), usando ensaios com suspensões de hepatócitos de rato, observou-se que o OE de salva não teve efeitos antioxidantes. Pelo contrário, a altas concentrações (maiores que 2 μl/ml), o óleo de salva induziu significativamente morte nas células, a qual foi acompanhada por uma depleção nos níveis de GSH (forma reduzida da glutationa) mas não por uma indução da peroxidação lipídica. A diminuição dos níveis de GSH foi provavelmente um dos mecanismos responsáveis pelos efeitos tóxicos do OE.

Em seguida, após uma experiência *in vivo* com ratinhos onde se verificou que o chá de salva melhorava a resposta antioxidante do fígado (capítulo 3), no capítulo 4 foram estudados os efeitos directos de salva em células HepG2 (linha celular de hepatoma humano). A salva protegeu contra a morte celular induzida pelo *tert*-butil hidroperóxido – um modelo de stresse oxidativo – revelando o seu potencial antioxidante directo em células. A sua acção nos níveis de GSH foi muito importante, prevenindo significativamente a sua depleção. O efeito citoprotector dos extractos da salva foi dependente da composição dos extractos em compostos fenólicos bem como da sua actividade antiradicalar.

Também no capítulo 4, verificou-se que os mesmos extractos eram capazes de elevar os níveis basais de GSH em células HepG2, indicando uma capacidade em melhorar as defesas

antioxidantes. O aumento de GSH poderá ter acontecido através da indução da síntese *de novo* da glutationa, o que é também corroborado por uma experiência anterior com hepatócitos de rato em cultura (capítulo 3). Nessa experiência, o chá de salva dado *in vivo* fez recuperar os níveis de GSH dos hepatócitos, após o isolamento com colagenase, para valores mais altos do que na situação controlo. Adicionalmente, o chá de salva induziu significativamente as actividades das enzimas GST (capítulo 3), GPox e NADPH citocromo P450 redutase bem como, em alguma extensão, algumas enzimas citocromo P450 em fígado de ratinho (capítulo 5). Estes efeitos antioxidantes indirectos parecem deixar o fígado mais bem preparado para combater uma situação de stresse oxidativo e o efeito tóxico de xenobióticos.

No entanto, contrariamente ao que era esperado, o tratamento prévio com chá de salva não protegeu a hepatotoxicidade induzida pelo tetracloreto de carbono (CCl_4) em ratinhos (capítulo 5). Em vez disso, ocorreu uma potenciação da toxicidade do CCl_4. Esta interacção pode ser explicada, pelo menos em parte, pelo aumento significativo induzido pelo chá da proteína CYP 2E1. Estes resultados chamam a atenção para possíveis interacções entre a salva e fármacos ou outros xenobióticos metabolizados pelo fígado. No entanto, isso provavelmente não acontecerá, a menos que uma elevada quantidade de salva for consumida por um longo período de tempo.

Em relação aos estudos das actividades antidiabéticas da salva, descritos no capítulo 6, verificou-se que o chá desta planta reduziu a glucose plasmática em jejum em ratinhos, indicando efeitos ao nível da gluconeogénese. Isto foi confirmado com experiências utilizando hepatócitos em cultura isolados de ratos, nos quais o chá de salva reduziu a indução da gluconeogénese pelo glucagon. Além disso, verificou-se que o chá de salva aumentou o consumo de glucose pelas células e que o OE aumentou a sensibilidade das células à insulina e inibiu a gluconeogénese. No geral, estes efeitos assemelham-se aos obtidos com o fármaco metformina, usado no tratamento e prevenção da diabetes. No entanto, em hepatócitos isolados de ratos diabéticos induzidos pela estreptozotocina, estes efeitos não foram observados. Os resultados sugerem contudo, que a salva poderá ser utilizada na prevenção da diabetes tipo 2, principalmente em pessoas em risco de a desenvolver.

Em conclusão, os resultados aqui apresentados confirmam os efeitos antioxidantes e hipoglicémicos de salva em sistemas biológicos, embora o seu uso terapêutico contra doenças do fígado e a diabetes necessite de mais investigação. No entanto, neste momento a salva pode ser encarada como um suplemento alimentar que poderá ter efeitos benéficos na prevenção, a baixo custo, da diabetes tipo 2 e de doenças do fígado. Para além disso, a prevenção de outro tipo de doenças também poderá ser considerada, principalmente aquelas onde é conhecido o envolvimento de stresse oxidativo, tal como o cancro e doenças neurodegenerativas. Por último, torna-se relevante relembrar que o consumo de salva, por períodos prolongados de tempo, em conjunto com certos fármacos poderá levar a efeitos indesejados devido a interacções planta-fármaco.

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Abbreviations

ARE	Antioxidant Response Element
BHT	Butylated Hydroxytoluene
BHA	Butylated Hydroxyanisole
CAT	Catalase
CCl_4	Carbon Tetrachloride
CYP	Cytochrome P450
EO	Essential Oil
GPox	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione – reduced form
GSSG	Glutathione – oxidised form
GST	Glutathione *S*-Transferase
H_2O_2	Hydrogen Peroxide
IGT	Impaired Glucose Tolerance
$^{\cdot}NO$	Nitric Oxide
$O_2^{\cdot -}$	Superoxide Anion
$^{\cdot}OH$	Hydroxyl Radical
OH	Hydroxyl Group
RNS	Reactive Nitrogen Species
$RO^{\cdot}$	Alkoxyl Radical
$ROO^{\cdot}$	Peroxyl Radical
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
t-BHP or *t*-BOOH	*tert*-Butyl Hydroperoxide

Preface

In Portugal, agriculture is still an important source of income for many families. With the European common market, many agricultural products from other European countries appear in Portugal at much lower price. Consequently, Portuguese agriculture is facing a severe crisis and alternative added value crops are urgently needed.

Some studies are ongoing in the Department of Biology in the University of Minho with aromatic and medicinal plants that grow spontaneously or are cultivated in Portugal aiming to help identify new and advantageous agricultural products for the Portuguese producers. Aromatic plants have for long been used as sources of fragrances to the cosmetic and perfume industries as well as source of flavours for food and beverage industries. In addition, medicinal plants are known to possess bioactive compounds that are also used in the pharmaceutical industries. Therefore, the production of selected added value bioactive compounds ("molecular farming") may constitute an advantageous alternative to Portuguese agriculture.

A way to add value to a plant product is to find new pharmaceutical applications for it. The classification of a given plant as medicinal has been based in its empirical use, since ancient times, in the treatment of several disorders. Confirming attributed medicinal properties and researching new ones is necessary to increase the pharmaceutical interest of a particular medicinal plant. Therefore, this research project arose from the necessity to find and study new bioactivities and bioactive compounds as a part of an integrated program of research and exploitation of aromatic and medicinal plants to be implemented in Portugal. In particular, this work aimed to increase our knowledge about two recognised effects of *Salvia officinalis* – the antioxidant and antidiabetic effects.

Furthermore, we are witnessing a growing interest in the use of natural products over synthetic ones by consumers, and, therefore, also by the food, pharmaceutical, cosmetic and other industries in order to embrace the emerging market interests. As a result of the increasing interest of phytopharmaceutic products and the need to ensure the safety of consumers, there is an effort of the European Union to create a simple and applicable legislation for this type of products. It was also our concern to clarify possible toxic effects of *S. officinalis* and interactions with pharmaceutical drugs, which may condition the application of products of this plant.

Finally, as in a pharmaceutical research, the use of cells and animals were an inevitable way to reach the proposed objectives. The 3 R's policy (Replacement, Reduction and Refinement) were, however, always taken in consideration. Whenever possible, the use of animals were avoided and replaced by cellular or chemical assays. The experiments using laboratory animals followed the *Guidelines for the Humane Use and Care of Animals in Experimentation* [Van Zutphen LFM, Baumans V & Beynen AC (2001) *Principles of Laboratory Animal Science*, revised edition. Elsevier Science BV, Amsterdam].

Chapter 1

General Introduction

1.1. Chapter overview

Since immemorial times, Man has been using plants not only for their nourishment but also for the therapy of diverse of their ailments. Mainly through the method of trial and error, several herbs were chosen to heal diverse disorders and this knowledge brought to the present from generation to generation. Nowadays, many medicinal plants have been researched and some of their attributed empiric therapeutic properties proved to be true.

Salvia officinalis (common sage) is one of the most known and used aromatic and medicinal plant, to which several ethnopharmaceutical properties are attributed. Many of them are thought to be mediated by many bioactive compounds present in this plant, such as phenolic compounds and terpenes. As an aromatic plant, sage produces essential oil where a variety of monoterpenes and sesquiterpenes can be found. As medicinal plant, sage produces strong antioxidant compounds, such as rosmarinic acid (main compound) and other phenolic-based compounds, which are thought to be the key for several therapeutic properties attributed to sage.

Since oxidative stress and free radicals have been implicated in the origin and progression of several age-related chronic diseases, plant antioxidants have been proposed as therapeutic agents to counteract them. However, besides their antioxidant effects, such type of plant compounds are known to be beneficial against several disorders also by their modulation of several enzyme activities, protein expression and important cell signalling cascades.

The liver is an important organ in the metabolic homeostasis of the body. However, due to its metabolic features and localisation, it is very vulnerable to toxic effects of xenobiotics, which can induce several steps of liver damages – from inflammatory to fibrotic processes. Antioxidants have also been proposed as therapeutic agents to counteract liver diseases, since reactive species are known to play a crucial role in liver diseases induction and progression. Additionally, because plant compounds are xenobiotics, they can induce toxicity to the liver, which highlight the importance of performing studies with liver cells. Moreover, possible enzyme and protein induction conferred by these products could provide an opportunity to mechanisms of interaction with other important drugs.

From the mechanisms behind liver cells injury, glutathione takes a vital place. The liver is the human organ with highest concentrations of glutathione. More than in other tissues, the levels of this intracellular antioxidant are very important in the protection against oxidative stress originated in the liver cells. Also, a drastic decrease of the reduced form of this tripeptide is known to be the central step in a cascade of events that culminate in extensive cell damage and death. Moreover, besides the detoxifying function of cellular glutathione, the recognition that this thiol can modulate signal transduction processes has recently been increasing.

The liver is also very important in the regulation of the plasma levels of glucose. In the diabetes disease, which is characterised by a hyperglycaemic situation originated by a deregulation of insulin function and/or secretion, liver cells did not do their work properly. *Diabetes mellitus* attained epidemic proportions in many countries and the available medication is far from resolving the problem, which makes it as a major public health concern. The liver is one of the potential targets for the treatment of diabetes and plants are currently again being used in the search for new possible drugs for its management. Diabetes is also associated with several complications where oxidative stress is known to be implicated, which make plant antioxidants interesting compounds to be researched against this chronic disease.

1.2. *Salvia officinalis*

Salvia officinalis L. (common sage, garden sage or Dalmatian sage) is a medicinal and aromatic plant of the *Lamiaceae* (= *Labiatae*) family, native to Mediterranean countries (mentioned in the Pharmacopeia of Portugal), which today is cultivated all over the world. Sage is a small, evergreen perennial plant with short woody stems that branch extensively, with blue and in some cases violet flowers (Fig. 1).

S. officinalis is a popular herb commonly used as a culinary spice for flavouring and seasoning that has also been used for centuries in folk medicine for the treatment of a variety of ailments. The botanical name of sage is a clear reference to the important curative properties of the plant: the genus name *Salvia* comes from the Latin *salvāre* meaning "to save" or "to heal" and *officinalis* means medicinal (Dweck, 2000; Miura *et al.*, 2002). Its ancient use as a medicinal plant can be shown by several old reports. For example, as cited by Dweck (2000), in an Anglo-Saxon manuscript we can read "why should man die when he has sage?"

Fig. 1. *Salvia officinalis* L. (*Lamiaceae*) plant.

Because of the wide range of traditional medicinal effects, sage enjoys the reputation of being a panacea: it has been used as anti-inflammatory and against perspiration and fever (antihydrotic properties); as spasmolytic, antiseptic/ bactericidal and astringent; in skin and hair care; against rheumatism and sexual debility; for menstrual and menopausal problems; to treat nervous and mental conditions; among others (Lu & Foo, 1999; Dweck, 2000; Wang *et al.*, 2000).

Sage extracts, mostly essential oil (EO), are also widely used in the food, drug, beverage, cosmetic and fragrance industries. In the past decades, sage has been submitted to extensive studies due to its antioxidant properties. Because of the side-effects of synthetic antioxidants, such as butylated hydroxytoluene (BHT) used as food preservative, on cancer risk, the interest on antioxidants of natural sources has increased considerably in recent years (Deans & Simpson, 2000). Sage has become an important source of antioxidants used in food industry, which had additional wider implications for the dietary intake of natural antioxidants (Deans & Simpson, 2000). Due to the increasing scientific evidence of the health benefits of natural antioxidants, *S. officinalis* turned into a good candidate as a source of those compounds for the cosmetic and pharmaceutical industries.

1.2.1. Bioactive compounds

It is sage's high production of secondary metabolites that gives it its medicinal and aromatic properties and makes it a rich source of bioactive compounds.

S. officinalis is the specie of the genus *Salvia* with the highest EO production (Giannouli & Kintzios, 2000). EO is a mixture of volatile compounds where there is a predominance of terpenes of lower molecular weight, mainly monoterpenes and sesquiterpenes, which for being volatile make part of the aroma and fragrance of aromatic plants (Cuppett & Hall, 1998). Plant EOs are currently used in food flavouring, in aromatherapy, in health care products, in perfumes and as preservatives against food spoilage (Giannouli & Kintzios, 2000). In a direct relationship with plant biological effects, EOs have been proven to possess antimicrobial and antifungal activities and also insecticide properties (Deans & Waterman, 1993).

Due to the low boiling points of the EO compounds, they can be recovered from the plant tissues by hydrodistillation. The composition of sage EO is very complex and could contain more than 50 compounds, being α-thujone (Fig. 2), α-humulene, 1,8-cineole (Fig.2), *E*-caryophyllene, camphor and borneol the major constituents (Perry *et al.*, 1999b; Giannouli & Kintzios, 2000; Santos-Gomes & Fernandes-Ferreira, 2001). The EO composition varies due to many factors such as plant organ, season, culture conditions and culture site (Perry *et al.*, 1999b; Santos-Gomes & Fernandes-Ferreira, 2001). For example, the major compound, α-thujone (Fig.2), can vary from 18% to more than 50% of the EO of the plant (Perry *et al.*, 1999b; Giannouli & Kintzios, 2000; Santos-Gomes & Fernandes-Ferreira, 2001). Sage EO has shown some ability to act as food antioxidants/preservatives (preventing rancidity in

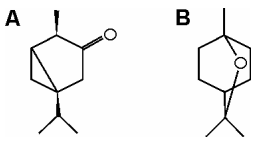


Fig. 2. Chemical structures of two of the most representative compounds present in *Salvia officinalis* essential oil: the oxygen-containing monoterpenes α-thujone (**A**) and 1,8-cineole (**B**).

food) by inhibiting lipid peroxidation (Cuppett & Hall, 1998). The use of sage EO should, however, be cautious because of its known neurotoxic and convulsant effects, mainly due to the high contents in α-thujone and camphor (Millet *et al.*, 1981; Baricevic & Bartol, 2000; Hold *et al.*, 2000).

Sage also contains important bioactive terpenoids such as the triterpenoids ursolic acid (Fig. 3) and oleanolic acid (Ulubelen, 2000; Topcu, 2006). These highly lipophilic compounds, although not showing antioxidant activity, may turn in a near future promising in combating inflammatory and cancer diseases (Baricevic & Bartol, 2000). Both compounds have been shown to have hepatoprotective, anti-inflammatory, antihyperlipidemic and antitumorigenic properties (Liu, 1995; Baricevic & Bartol, 2000).

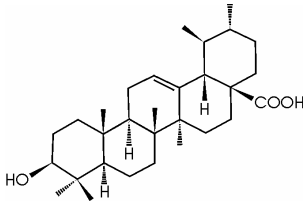


Fig. 3. Chemical structure of ursolic acid, a pentacyclic triterpenic acid.

In the 1950s, as a result of the studies performed by Chipault and collaborators, plants (spices) were shown to possess antioxidant capacity. Sage was one of the spices studied by Chipault and showed to be a good source of antioxidants useful as food preservatives (Cuppett & Hall, 1998). These studies were then extended to other plants, but with the use of synthetic antioxidants (*e.g.* BHT and butylated hydroxyanisole - BHA), the natural compounds were for some years forgotten. In the 1990s, the safety of the synthetic antioxidants was, however, been questioned, since it was shown that long term exposures to these compounds in the diet could produce tumours in animals (Deans & Simpson, 2000). Although the actual effects of BHT and BHA on human cancer risk are unknown, these concerns have lead to an increased interest in plant antioxidants as food preservatives because of their higher safety and acceptability (Deans & Simpson, 2000). As a result, sage becomes one of the most interesting plants of research due to its high content in antioxidant compounds.

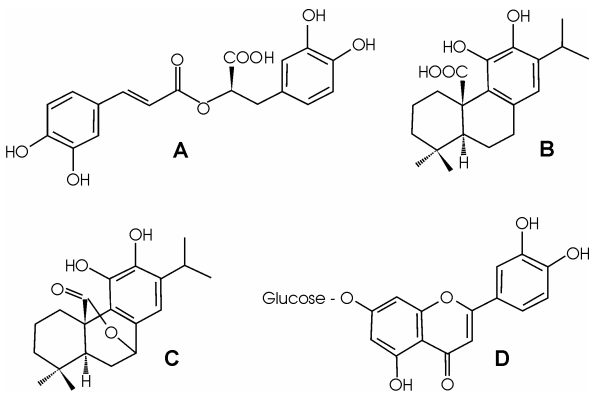


Fig. 4. Chemical structures of rosmarinic acid (**A**), carnosic acid (**B**), carnosol (**C**) and luteolin-7-glucoside (**D**).

The phenolic rosmarinic acid (Fig. 4) is the compound that more contributes to the antioxidant activity of sage (Lamaison *et al.*, 1991; Wang *et al.*, 1998; Deans & Simpson, 2000). Besides this phenolic acid, phenolic diterpenes such as carnosic acid and carnosol (Fig. 4) were found in sage and reported to possess strong antioxidant activities (Cuvelier *et al.*, 1994). Additionally, many other phenolic compounds were

found in *S. officinalis* that could contribute to its antioxidant properties, such as sagecoumarin and salvianolic acids (caffeic acid derivatives) as well as the flavonoid luteolin-7-glucoside (Fig. 4) and other phenolic glycosides (Lu & Foo, 1999; Lu *et al.*, 1999; Wang *et al.*, 1999; Lu & Foo, 2000; 2001; 2002). Apart from the use in the food processing industry, these phenolic compounds are currently appointed as useful in the area of human health (Pearson *et al.*, 1997) since they are thought to be the key for the several therapeutic properties attributed to medicinal plants.

1.2.2. Biological and pharmacological activities

Besides their traditional uses, many biological and pharmacological activities have been scientifically researched and attributed to *S. officinalis*.

Sage EO is still employed in flavouring condiments, cured meats, liqueurs and bitters (Baricevic & Bartol, 2000). Besides its usage as a flavouring and antioxidant agent, sage EO exhibit antibacterial and antifungal activities (Baricevic & Bartol, 2000; Araujo *et al.*, 2003; Rota *et al.*, 2004; Pereira *et al.*, 2004) as well as antimutagenic and insect repellent effects (Simic *et al.*, 1998; Baricevic & Bartol, 2000; Vujosevic & Blagojevic, 2004). Insecticidal activity was also reported for the methanolic extract (Pavela, 2004) as well as antimutagenic activity for water extract of sage (Samejima *et al.*, 1995).

Both the *n*-hexane and the chloroform extracts of leaves of *S. officinalis* were reported to possess strong anti-inflammatory activities and its activity related with its content in ursolic acid (Baricevic & Bartol, 2000; Baricevic *et al.*, 2001). Polysaccharides extracted from sage showed recently immunomodulatory activity using the comitogenic thymocyte test (Capek *et al.*, 2003; Ebringerovda *et al.*, 2003; Capek & Hribalova, 2004). Sage phenolics were also reported to possess immunomodulatory and antileishmanial activities (Radtke *et al.*, 2003). In addition, sage extracts obtained with polar solvents showed to possess hypoglycaemic and anti-hyperlipidemic effects in mice (Alarcon-Aguilar *et al.*, 2002; Ninomiya *et al.*, 2004), and spasmolytic and hypotensive actions in other animal models (Todorov *et al.*, 1984).

Some studies using human patients showed that sage had antiviral activity against herpes infections (Saller *et al.*, 2001), was effective in the treatment of menopausal symptoms (de Leo *et al.*, 1998) and was efficacious in the treatment of acute pharyngitis (Hubbert *et al.*, 2006).

The antioxidant properties of sage were extensively studied using *in vitro* studies. Sage alcoholic extracts revealed strong antioxidant activity by increasing the stability of food oils (Cuppett & Hall, 1998; Miura *et al.*, 2002; Zainuddin *et al.*, 2002; Ozcan, 2003; Jaswir *et al.*, 2005) contributing to its application in food industry. The antioxidant activity of sage extracts were also shown using other methods, such as by the accelerated autoxidation of methyl

linoleate (Cuvelier *et al.*, 1994; Cuvelier *et al.*, 1996), by the ability to scavenge $DPPH^{\cdot}$ (Lamaison *et al.*, 1991) and $ABTS^{\cdot}$ free radicals (Shan *et al.*, 2005) as well as by the oxygen radical absorbance capacity (ORAC assay) (Zheng & Wang, 2001). With the increasing interest of application of plant antioxidant compounds in the human health over the past 15 years, information relating sage antioxidant activity with pharmacological activity begun to appear in the literature. Sage extracts were reported to possess superoxide and hydroxyl radicals scavenging activities using the electron spin resonance (ESR) spin-trapping technique (Masaki *et al.*, 1995; Madsen *et al.*, 1997). Additionally, sage also showed the capacity to inhibit lipid peroxidation of liposomes in enzyme-dependent and enzyme-independent lipid peroxidation assays (Masaki *et al.*, 1995; Hohmann *et al.*, 1999; Zupko *et al.*, 2001). Since the composition in lipids of biological tissues is complex, it is very difficult to assess the health effects of antioxidants using an experimental model that uses a single lipid type and oxidation catalysts (Decker, 1997). To better relate with human health, the use of cell systems is preferable in the evaluation of the bioactivity of an antioxidant. However, only few studies with sage extracts are available. Until 2002, only Masaki and its collaborators (1995) performed a small experiment using fibroblasts and related sage antioxidant effects with cytoprotective effects. In their study, sage extract significantly prevent cell death induced by a superoxide-generating system (Masaki *et al.*, 1995).

The references in old European books to the memory-improving activities of *S. officinalis* (Perry *et al.*, 1999a) recently led researchers to perform studies aiming the ability of sage extracts to attenuate the cognitive decline seen in dementia. In 1996, Perry and collaborators reported the anticholinesterase activity of sage EO in human brain tissue (post-mortem) that could in part explain the reported memory-enhancement properties (Perry *et al.*, 1999a; Perry *et al.*, 2000). In addition, based on a study with a close specie of *S. officinalis*, *Melissa officinalis*, modulation of mood and cognitive performance has been suggested for sage extracts (Akhondzadeh *et al.*, 2003; Kennedy *et al.*, 2006). Recently, using a rat model, the ethanolic extract of sage was shown to potentiate memory retention and to interact with the muscarinic and nicotinic cholinergic systems that are involved in the memory retention process (Eidi *et al.*, 2006). Moreover, a double-blind, placebo-controlled, and crossover study using 30 healthy participants confirm the improvement of mood and on cognitive performance by *S. officinalis* (Kennedy *et al.*, 2006). The same study also reported a dose-dependent *in vitro* inhibition of acetylcholinesterase and, to a greater extent, butyrylcholinesterase by an ethanol extract of sage. Some of the previous studies suggested *S. officinalis* as a possible potentially novel natural treatment for Alzheimer's disease. In fact, Akhondzadeh *et al.* (2003) performed a randomized double blind placebo-controlled trial using patients with mild to moderate Alzheimer's disease. They showed a significantly better outcome of cognitive functions for sage than placebo patients group indicating the efficacy of this plant in the management of mild to

moderate Alzheimer's disease (Akhondzadeh *et al.*, 2003). In addition, a hydro alcoholic extract of sage was also recently reported to have neuroprotective effects against amyloid β (Aβ)-induced toxicity in PC12 cells, which corroborate the potential use in the treatment of Alzheimer's disease (Iuvone *et al.*, 2006). This activity was attributed, at least in part, to rosmarinic acid.

1.3. Oxidative stress

The appearance of oxygen as a diatomic molecule (O_2) in the Earth's atmosphere over 2500 millions years ago resulted in the emergence of a new environment. During a long evolutionary process, new organisms utilising O_2 for production of energy by the use of O_2-dependent electron transport chains arose in this environment – the aerobic organisms. Due to the toxicity of O_2, these organisms only survived because they developed in parallel efficient defences to protect them against it (Fig. 5) (Halliwell & Gutteridge, 1999).

In normal physiological conditions, the production of free radicals and other reactive species (derived from the use of O_2) is approximately balanced by the antioxidant defence system (Halliwell, 1996; Halliwell & Gutteridge, 1999). The balance is, however, not perfect because oxidative damage occurs to DNA, proteins, lipids, and small molecules in living systems also under normal oxygen conditions (Halliwell & Gutteridge, 1999; Willcox *et al.*, 2004). Normally, damaged molecules are repaired or replaced. However, in certain circumstances a significant imbalance between reactive species and antioxidant defence system can occur, a situation referred as oxidative stress, which can result in extensive damage with accumulation of oxidised molecules (Halliwell, 1996; Halliwell & Gutteridge, 1999; Willcox *et al.*, 2004). This disturbance between the pro-oxidant and antioxidant state in favour to the former can result from a diminishment of antioxidants (for example due to mutations or malnutrition) and/or from an increased production of reactive species (Halliwell, 1996; Halliwell & Gutteridge, 1999). The latter can, for example, result from the exposure to environmental oxidant conditions (Fig. 5), such as ultraviolet radiation, hyperoxia, drugs and toxins (for instance present in polluted air and cigarette smoke); and also by increased production by cellular systems under pathological conditions (such as in chronic inflammatory diseases and ischemia) (Halliwell, 1996; Dalton *et al.*, 1999; Willcox *et al.*, 2004).

At higher levels of oxidative stress, severe injuries to cell molecules, such as DNA, proteins and lipids, may happen, which may result in extensive cell death (see section 1.6.3). Nevertheless, cells often tolerate mild oxidative stress by upregulating the synthesis of antioxidant defence mechanisms through changes in gene expression (Halliwell, 1996; Halliwell

& Gutteridge, 1999; Willcox *et al.*, 2004). Therefore, despite their potential to cause damage, reactive species have been shown in recent years to be involved in modulation of signal transduction cascades and redirection of gene expression under physiological and pathological conditions (Dalton *et al.*, 1999; Finkel & Holbrook, 2000; de Magalhaes & Church, 2006).

1.3.1. Reactive species

Reactive species are produced in cells as a result of normal metabolism as well as due to extracellular agents (Fig. 5) and have a pro-oxidant action if its levels rise above the

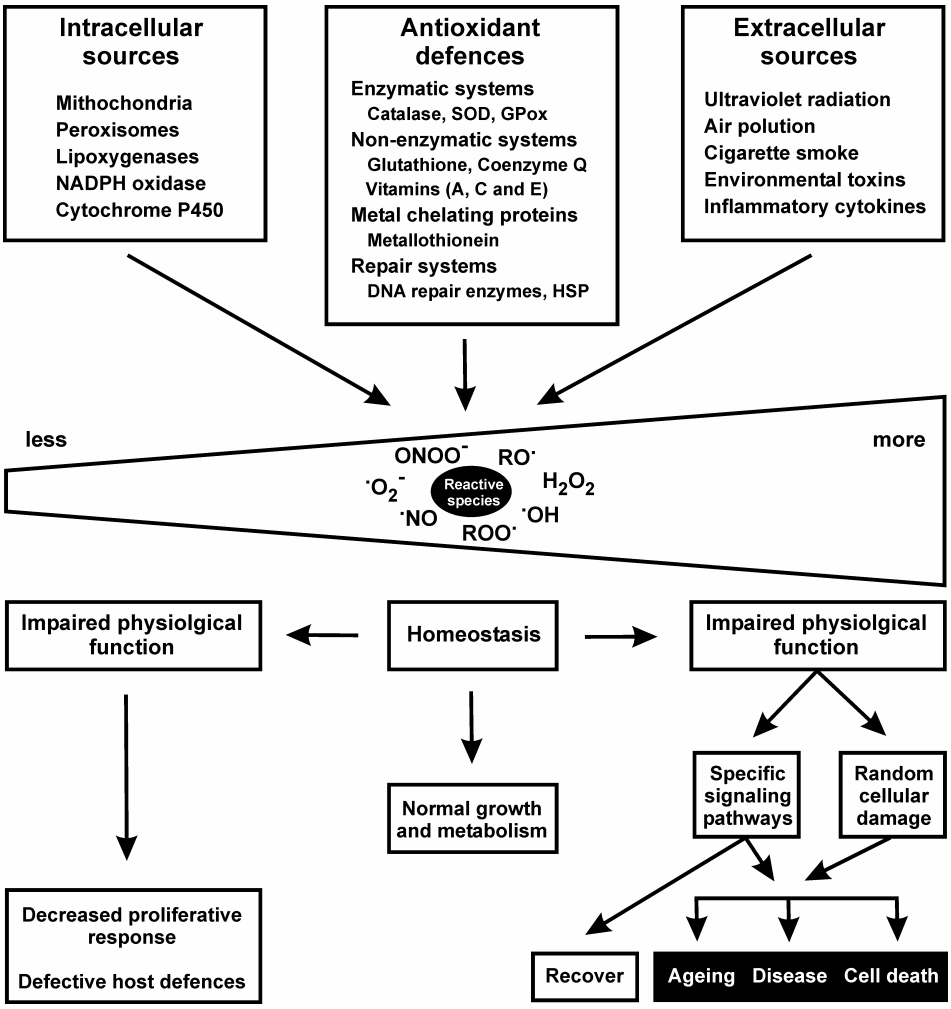


Fig. 5. Sources and cellular responses to reactive species. Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, extracellular agents can trigger oxidants production. A sophisticated antioxidant defence system counteracts and regulates overall reactive species levels to maintain physiological homeostasis. Lowering oxidants below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defence. Similarly, increased oxidants may also be detrimental and lead to cell death, disease or to acceleration in ageing and age-related diseases. The oxidative stress caused by increased reactive species is thought to result from random damage to proteins, lipids and DNA. In addition to these effects, a rise in oxidants may also constitute a stress signal that activates specific redox-sensitive signalling pathways. Once activated, these diverse signalling pathways may have either damaging or potentially protective functions. SOD - superoxide dismutase, GPox - glutathione peroxidase, HSP - heat shock proteins. [Adapted from Finkel & Holbrook (2000)]

homeostatic set point. The main reactive species produced are (but not limited to) reactive oxygen species (ROS) and reactive nitrogen species (RNS). They include free radicals and non-radical species. A free radical can be defined as any specie capable of independent existence that contains one or more unpaired electrons. The presence of unpaired electrons makes free radicals highly reactive because they require another electron to complete the last orbital and become stable (Halliwell & Gutteridge, 1999; Willcox *et al.*, 2004).

ROS encompass a number of chemically reactive molecules derived from oxygen which include both free radicals, such as superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$), and non-radicals such as hydrogen peroxide (H_2O_2). Some non-radicals are also considered as reactive species because they can act as oxidising agents and/or can be easily converted into radicals (Evans & Halliwell, 2001). Some ROS are extremely reactive, such as $^{\cdot}OH$, while some are less reactive (poor oxidant), such as $O_2^{\cdot -}$ and H_2O_2 (Nordberg & Arner, 2001). Moreover, some of these species, such as $O_2^{\cdot -}$ and $^{\cdot}OH$, are extremely unstable whereas others, like H_2O_2, are freely diffusible and relatively long-lived (Finkel & Holbrook, 2000).

The superoxide anion, created from molecular oxygen by the addition of an electron, lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it is produced (Nordberg & Arner, 2001). The most important source of $O_2^{\cdot -}$ in eukaryotic cells is the leakage of electrons from mitochondrial electron transport chain (Fig. 6). It can also be formed by autoxidation reactions and by certain enzymes, such as xanthine oxidase, lipoxygenase and cyclooxygenase (Nordberg & Arner, 2001; Halliwell, 2001). Superoxide anion cannot directly attack DNA, lipids and many proteins. However, it can decrease the activity of certain enzymes including some antioxidant defence enzymes (such as catalase and glutathione peroxidase), NADH dehydrogenase and ribonucleotide reductase among others (Willcox *et al.*, 2004). At elevated levels, $O_2^{\cdot -}$ can also mobilise small amounts of iron from the iron-storage protein ferritin and attack the active sites of some enzymes containing iron-sulphur clusters, such as aconitase, causing their inactivation. This last process is also accompanied by iron release (Halliwell, 2001). Aside from direct damage, $O_2^{\cdot -}$ can be more cytotoxic by generating other reactive species, such as H_2O_2, by the addition of one more electron (Fig. 6).

Hydrogen peroxide, apart of its production from the dismutation of superoxide by superoxide dismutase (SOD):

$$2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

can also be produced directly by several other enzymes, such as glycollate oxidase and xanthine oxidase (Halliwell, 2001). Hydrogen peroxide is poorly reactive with most biomolecules and appears unable to directly oxidise DNA, lipids and proteins, except for a few proteins which have hyper-reactive thiol groups or methionine residues, such as glyceraldehyde-3-phosphate dehydrogenase (Halliwell & Gutteridge, 1999; Halliwell, 2001). Although H_2O_2 is not a free

radical, it is highly important much because of its ability to penetrate biological membranes (Fig. 6) and to form much more damaging species such as ˙OH (Nordberg & Arner, 2001; Halliwell, 2001; Willcox *et al.*, 2004).

The ˙OH can attack and damage all biomolecules (carbohydrates, lipids, proteins and DNA) but only at the site where it is produced due to its high reactivity (short half-life) and very low diffusion distance (Yu, 1994; Halliwell & Gutteridge, 1999; Nordberg & Arner, 2001; Halliwell, 2001). The radical is formed from H_2O_2 in a reaction catalysed by metal ions (Fe^{2+} or Cu^{+}) known as the Fenton reaction:

$$H_2O_2 + Cu^{+}/Fe^{2+} \rightarrow {}^{\cdot}OH + OH^{-} + Cu^{2+}/Fe^{3+} \qquad \text{(Reaction 1)}$$

Superoxide plays an important role as reductant agent in connection with Reaction 1 by recycling the metal ions:

$$O_2^{\cdot -} + Cu^{2+}/Fe^{3+} \rightarrow O_2 + Cu^{+}/Fe^{2+} \qquad \text{(Reaction 2)}$$

Ascorbic acid can also act as a reductant agent. Although ascorbate normally acts as a powerful scavenger of reactive species, it can become pro-oxidant in the presence of iron (or copper)

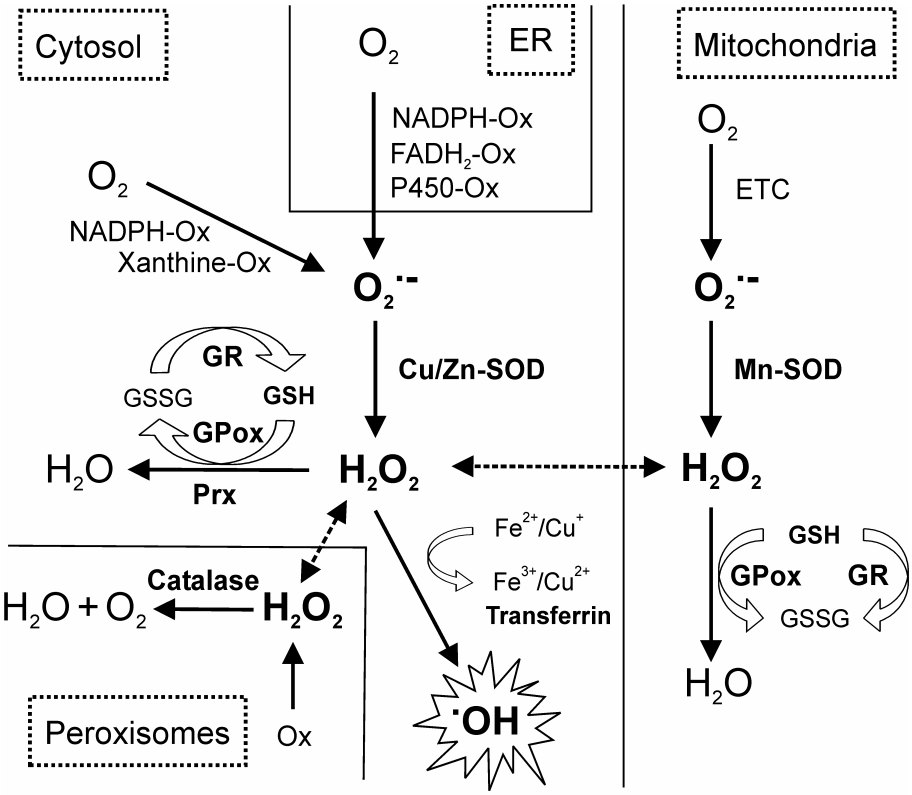


Fig. 6. Simplified nonstoichiometric scheme of generation of reactive oxygen species in cells and the action of some intracellular antioxidants. Superoxide is produced in significant amounts intracellularly, mainly both in the cytosol and in mitochondria (due to escape of electrons from the respiratory chain). Two molecules of superoxide rapidly dismutate, either spontaneously or via superoxide dismutases to dioxygen and hydrogen peroxide, the latter permitting flux of ROS between different cellular compartments. Hydrogen peroxide can be enzymatically metabolised to dioxygen and water by a number of different enzyme systems or converted to the hydroxyl radical, which is extremely reactive, via a chemical reaction catalysed by transition metals. ER - endoplasmic reticulum, ETC - electron transport chains, Ox - oxidases, GSH - reduced glutathione, GSSG - oxidised glutathione, GR - glutathione reductase, GPox - glutathione peroxidase, SOD - superoxide dismutase, Prx - peroxiredoxins. Reactive oxygen species and endogenous antioxidants are bolded. [Adapted from Nordberg & Arner (2001) and Willcox *et al.* (2004)]

ions, damaging biomolecules by promoting the formation of $^{\cdot}OH$ (Halliwell, 2001). The sum of Reactions 1 and 2 is the Haber-Weiss reaction (Reaction 3), transition metals thus playing an important role in the formation of hydroxyl radicals (Nordberg & Arner, 2001).

$$H_2O_2 + O_2^{\cdot -} \rightarrow {}^{\cdot}OH + OH^- + O_2 \qquad \text{(Reaction 3)}$$

Thus, although iron and copper are required by the human body for its normal function, these transition metals are dangerous due to their ability to transfer single electrons (they can be considered as radicals; Halliwell & Gutteridge, 1999; Halliwell, 2001). For example, iron can oscillate between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states making it a powerful catalyst of free radical reactions. Fortunately, iron and copper ions, in chemical forms that can work as catalysts, are in short supply *in vivo*. The body contains a system of transport and of storage proteins that ensure that as much iron and copper as possible is safely sequestered in noncatalytic forms (Halliwell, 1996; Gutteridge & Halliwell, 2000). However, oxidative stress and cell injury can induce the liberation of metal ions (and, therefore be in a catalytic form) for free radical reactions from ferritin and heme proteins (Gutteridge & Halliwell, 2000). As mentioned above, $O_2^{\cdot -}$ have an important role in these reactions. Besides its importance in the formation of $^{\cdot}OH$, transition metal ions can also be deleterious catalysing the formation of reactive species by autoxidation reactions and participating in the formation of alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals (Halliwell & Gutteridge, 1999; Halliwell, 2001).

Nitric oxide ($^{\cdot}NO$) is included in the RNS and in several aspects is similar to $O_2^{\cdot -}$. Despite its unpaired electron, it does not readily react with most biomolecules. It plays important physiological functions, such as vasodilatation and neurotransmission functions (Halliwell & Gutteridge, 1999; Halliwell, 2001). Nitric oxide also works as free radical scavenger generating mainly less reactive molecules (Nordberg & Arner, 2001). However, in excess, $^{\cdot}NO$ can inhibit cytochrome oxidase in mitochondria, leading to increased leakage of electrons and more $O_2^{\cdot -}$ formation (Halliwell, 2001). Then, $^{\cdot}NO$ can react with $O_2^{\cdot -}$ to give peroxynitrite ($ONOO^-$), which is highly cytotoxic (Halliwell & Gutteridge, 1999; Nordberg & Arner, 2001; Halliwell, 2001). Peroxynitrite is also responsible for the formation of other dangerous reactive species. Thus, generation of $ONOO^-$ *in vivo* can lead to oxidation and nitration of lipids, DNA and amino acid residues on proteins (Halliwell, 2001).

Besides the formation of ROS and RNS, other reactive species can also be formed by the metabolism of drugs and toxins. Carbon tetrachloride (CCl_4), for example, is metabolised forming high reactive free radicals such as $^{\cdot}CCl_3$ and $CCl_3OO^{\cdot}$ (Halliwell & Gutteridge, 1999; Weber *et al.*, 2003). Another example is the organic hydroperoxide – *tert*-butyl hydroperoxide (*t*-BHP or *t*-BOOH) which can be metabolised to form peroxyl (*t*-$BHOO^{\cdot}$) and alkoxyl (*t*-$BHO^{\cdot}$) radicals (Davies, 1989; VanderZee *et al.*, 1996).

1.3.2. Cellular antioxidant defences

Due to the continuous formation of reactive species *in vivo*, aerobic organisms only survived because they develop antioxidant defences. Antioxidant is a term widely used but rarely defined. For example, for a chemist an antioxidant is a molecule that has the ability to scavenge free radicals. In the food industry, the term is implicitly restricted to chain-breaking inhibitors of lipid peroxidation and consequent rancidity in food materials, such as α-tocopherol. Indeed, food scientists often simply connect antioxidants to inhibitors of lipid peroxidation (Halliwell & Gutteridge, 1999). However, reactive species generated *in vivo* damage many targets other than lipids, including proteins and DNA. Therefore, a broader definition of an antioxidant has been given by Halliwell and Gutteridge in 1989 to include living organisms as '*any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate*' (Halliwell & Gutteridge, 1999). The antioxidants can either directly or indirectly protect cells against the adverse effects of reactive species (Mates, 2000). Some antioxidants are strategically compartmentalised in subcellular organelles within the cell to provide maximum protection (Fig. 6).

The cellular antioxidants may defend against reactive species by (Fig. 7):

(1) inhibiting their generation (**preventive antioxidants**);

(2) removing or degrading them to less harmful products (**removal antioxidants**);

(3) and, presenting themselves for oxidation (**scavenging antioxidants**).

Additionally, antioxidants may protect target biomolecules against oxidative damage by other mechanisms (*e.g.* heat shock proteins, which are also repair proteins). Also, ROS/RNS (in moderate levels) and certain cellular signal molecules, such as cytokines, can enhance the cellular antioxidant defences (for example, by upregulating gene expression) (Halliwell *et al.*, 1995; Halliwell & Gutteridge, 1999). Since under normal conditions oxidative damage to DNA, proteins, lipids and other molecules has been demonstrated in living organisms, scientists have realised that the cellular antioxidant defences may be incomplete. Hence, the **repair antioxidants** (Fig. 7) have been added to the antioxidant defences (Halliwell & Gutteridge, 1999). Critical structures in the cell are protected not only by the ability to maintain reactive species in low levels, but also by other mechanisms, such as the repair or elimination of damaged molecules (Cui *et al.*, 2004). Therefore, in brief, the antioxidant defence system can include any substance that neutralises the potential damaging effects of reactive species.

As a first line of defence, the preventive antioxidants suppress radical formation by binding to, and sequestering, oxidation promoters and transition metal ions (Fig. 7), such as iron and copper, which contain unpaired electrons and strongly accelerate free radical formation (Cui *et al.*, 2004). Examples are transferrin and lactoferrin (which bind ferric ions), caeruloplasmin

(which binds Cu, catalyzes the oxidation of ferrous ions to ferric due to its ferroxidase activity, and increases the binding of iron to transferrin), haptoglobins (which bind hemoglobin), hemopexin (which binds heme), and albumin (which binds copper and heme).

Next, removal antioxidants catalytically degrade specific reactive species (Fig. 7) converting them into less harmful products (Halliwell & Gutteridge, 1999). Examples are the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPox). SOD converts $O_2^{\cdot -}$ to H_2O_2,

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

which is not a free radical by itself, but is a precursor of the highly reactive hydroxyl radical (Fig. 6). Mammalian cells have in the mitochondria a SOD enzyme containing manganese at the active site (Mn-SOD). A SOD with copper and zinc at the active site (Cu/Zn-SOD) is also present largely in the cytosol (Halliwell, 1996). There is also an extracellular mammalian SOD (EC-SOD) (Mates, 2000). Detoxification of hydrogen peroxide (Fig. 6) is carried out by (i) CAT, which decomposes H_2O_2 to water and oxygen,

$$2H_2O_2 \rightarrow H_2O + O_2$$

and is an exclusive peroxisomal enzyme in most tissues (Halliwell *et al.*, 1995); or by (ii) GPox,

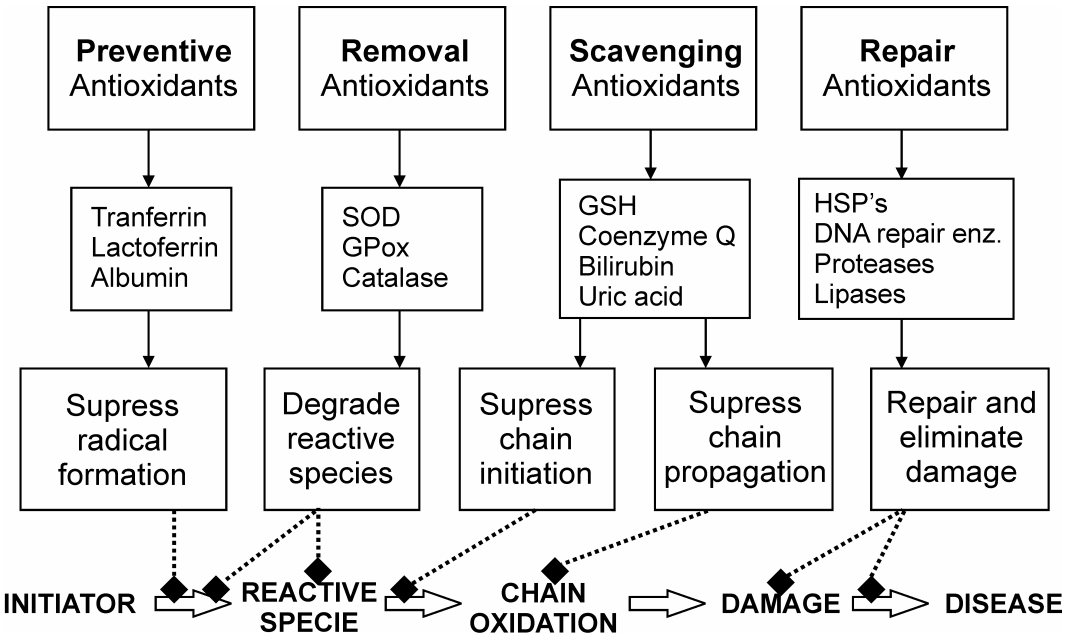


Fig. 7. Cellular antioxidants defences against reactive species. The preventive antioxidants suppress radical formation whereas the removal antioxidants catalytically degrade specific reactive species converting them into less harmful products. Then, oxidative chain reactions initiated by reactive species that escaped from the above antioxidant defences are terminated by the scavenging or chain-breaking antioxidants. They act by presenting themselves for oxidation at an early stage in the oxidation chain reactions, inhibiting its initiation and preventing chain propagation. The repair antioxidants act as the last line of defence by repairing damage or eliminating damaged molecules. SOD - superoxide dismutase, GPox - glutathione peroxidase, GSH - reduced glutathione, HSP - heat shock protein. [Adapted from Willcox *et al.* (2004)]

which reduces H_2O_2 to water in the presence of glutathione in reduced form:

$$2\ GSH + H_2O_2 \rightarrow 2H_2O + GSSG$$

GPox catalyse also the reduction of others hydroperoxides (ROOH) that may appear in the cell. The GPox is assisted by glutathione reductase (GR) to recycle oxidised glutathione (GSSG):

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Other H_2O_2-removing systems exist, such as peroxidases that use the dithiol-containing protein thioredoxin as a substrate (peroxiredoxins) (Nordberg & Arner, 2001; Evans & Halliwell, 2001). The enzymes GR, GPox and peroxiredoxin are present in several cellular compartments, including mitochondria (Halliwell, 2001). The antioxidant enzymes work to remove most superoxides and peroxides before they react with metal ions to form $^{\cdot}OH$. Since they suppress the generation of a more reactive free radical, they are also referred for some authors as preventive antioxidants (Willcox *et al.*, 2004).

Then, oxidative chain reactions initiated by reactive species that escaped the above antioxidant defences may be terminated by low molecular mass scavenging antioxidants (Fig. 7). The scavenging or chain-breaking antioxidants act by presenting themselves for oxidation at an early stage in the oxidation chain reactions, inhibiting its initiation and preventing chain propagation (Cui *et al.*, 2004). Lipid soluble and water soluble scavengers act in cellular environments that are either hydrophobic or hydrophilic, respectively. The major endogenous lipophilic scavengers are coenzyme Q and bilirubin, while glutathione and uric acid function in the aqueous environment. Glutathione is an important cell antioxidant that, besides a cofactor for GPox, is a potent nucleophilic scavenger of free radicals via conjugation reactions, either chemically or enzymatically (catalysed by glutathione *S*-transferase - GST) (Reed, 1990; Yu, 1994). Diet plays a vital role in the production of scavenging antioxidants by providing essential nutrient antioxidants such as the lipophilic vitamin E (α-tocopherol) and β-carotene as well as the hydrophilic vitamin c (ascorbic acid) (Halliwell & Gutteridge, 1999; Willcox *et al.*, 2004). Also important for the antioxidant defences are essential minerals obtained from diet, since many of them are vital cofactors for enzymatic antioxidants (*e.g.* selenium), as well as plant phenolic compounds, such as flavonoids (see section 1.4.1).

The repair (antioxidant) system acts as the last line of defence by repairing damage or eliminating damaged molecules (Fig. 7). These include lipases, proteases, heat shock proteins, DNA repair enzymes, and transferases (Willcox *et al.*, 2004). For example, DNA repair enzymes act by excising and replacing damaged bases in the DNA whereas heat shock proteins repair or refold slightly damaged proteins and increase the turnover of severe damaged proteins (Halliwell & Gutteridge, 1999; Evans & Halliwell, 2001).

1.3.3. Oxidative stress and disease

Because of their high reactivity, accumulation of radicals above cells' defences may affect cellular functionality and integrity by damaging critical molecules, such as the DNA, proteins, carbohydrates and lipids, which ultimately can cause cell death. In fact, oxidative stress has been recognised to be involved in the aetiology of several age-related and chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Tiwari, 2004; Cui *et al.*, 2004; Ceriello & Motz, 2004; Klaunig & Kamendulis, 2004; Ballinger, 2005; Gibson & Huang, 2005; Rolo & Palmeira, 2006). Even aging and age-related loss of physiological fitness have been attributed to oxidative stress (Finkel & Holbrook, 2000; Cadenas & Davies, 2000). In some human diseases, oxidative stress is, however, a secondary phenomenon, not the primary cause of the disease, which does not mean that oxidative stress is in this cases unimportant (Halliwell, 1996).

1.4. Plant compounds as antioxidants

Foods and nutrients play a vital role in normal functioning of the body. They are helpful in maintaining the health of the individual and in reducing the risk of various diseases. From the acceptance of this fact, worldwide evolved the concept of "functional food", *i.e.*, any altered food or ingredient that could give a beneficial effect beyond that provided by the nutrients that it normally contains (Ferrari, 2004). On the other hand, a non altered food (or part of it) with health or medical benefits is defined by the American Dietetic Association (as cited by Ferrari, 2004) as "nutraceutical", which include products such as vitamins, minerals, fruits, plant and animal extracts. These nutraceuticals are believed to be useful in the prevention and treatment of several diseases (Ferrari, 2004; Ramaa *et al.*, 2006). Based in epidemiologic studies, animal research, clinical trials and nutritional biochemistry studies, these dietary supplements are currently recognised as beneficial in the coronary heart disease, cancer, osteoporosis and other chronic and degenerative diseases, such as diabetes, Parkinson's and Alzheimer's diseases (Mandel *et al.*, 2005). This gave impetus to investigate the mechanisms of action of nutraceuticals and related bioactive compounds.

The health benefits of nutraceuticals of plant origin, phytochemicals, are currently the most studied. Based on epidemiological studies, diets rich in fruits and vegetables and other plant foods (including tea and wine) are associated with a decreased risk of premature death and mortality from chronic age-related diseases, such as cardiovascular diseases and some types of cancer (Stanner *et al.*, 2004; Ferrari, 2004; Neuhouser, 2004; Liu, 2004). Because oxidative

stress plays a central role in aging and chronic and age-related diseases, plant antioxidants, such as vitamin C, vitamin E, carotenoids (*e.g.* β-carotene, lycopene and lutein) and phenolic compounds (*e.g.* flavonoids), have been proposed as components responsible for the health promotion of those plant foods (Willcox *et al.*, 2004; Stanner *et al.*, 2004). The hypothesis that plant antioxidants are responsible for the health promotion effects is supported by some experiments with animals, cultured human cell lines and chemical *in vitro* studies (Scalbert *et al.*, 2005; Halliwell *et al.*, 2005). For example, experimental studies on animals or cultured cells support a role for flavonoids and other phenolic compounds in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes and osteoporosis (Jiang & Dusting, 2003; Neuhouser, 2004; Tsuda *et al.*, 2004; Scalbert *et al.*, 2005; Nichenametla *et al.*, 2006). However, it is very difficult to predict from these results the effects of the intake of phenolic compounds on disease prevention in humans. One of the reasons is that these studies have often been conducted at doses or concentrations far beyond those observed in humans (Scalbert *et al.*, 2005). Maximal plasma concentrations achieved are low, usually not more than 1 μM, in part because of their poor gastrointestinal absorption and rapid metabolism by human tissues and colonic bacteria. Many of the products of metabolism, such as methylated and glucuronidated forms, have decreased direct antioxidant activity (Halliwell *et al.*, 2005). Due to the low concentrations reached *in vivo*, phytochemicals and other nutraceuticals have been suggested to exert beneficial actions on cells not through their direct antioxidant effects but rather through their modulation of proteins (and enzymes), gene expression and cell signalling cascades (Williams *et al.*, 2004; Mandel *et al.*, 2005). Moreover, by these mechanisms, the bioactive form of a plant antioxidant *in vivo* is not necessarily the natural phytochemical, but conjugates and metabolites arising up on absorption and first pass in the liver (Spencer *et al.*, 2004; Williams *et al.*, 2004).

Some human clinical trials using plant antioxidants have led to controversial results regarding protective action against some chronic age-associated diseases (Stanner *et al.*, 2004; Scalbert *et al.*, 2005). These studies usually involved the administration of single antioxidant nutrients given at relatively high doses. Results of trials investigating the effect of a balanced combination of antioxidants at levels achievable by diet are still awaited (Stanner *et al.*, 2004). In fact, as proposed by Liu (2004), it is the additive and synergistic effect of phytochemicals in fruits and vegetables that may be responsible for the *in vivo* antioxidant effects. This would explain why no single antioxidant can replace the combination of natural phytochemicals in fruits and vegetables to achieve the health benefits (Liu, 2004).

Successful application of plant antioxidants as drugs require a much greater understanding of their pharmacological properties, including their rates of absorption, tissue distribution, metabolism and the microenvironment in which they must act (Finkel & Holbrook, 2000).

1.4.1. Phenolic compounds and antioxidant activity

Previous studies have shown that many dietary phenolic compounds derived from plants are more effective antioxidants *in vitro* than vitamins E or C or the carotenoids, and thus might contribute significantly to the protective effects *in vivo* (Rice-Evans *et al.*, 1997).

Phenolics compounds are ubiquitous in foods of plant origin (*e.g.* fruits, vegetables, cereals, nuts, wines, whiskies, beer, teas and cocoa), although their concentrations can vary by many orders of magnitude and are influenced by several factors, including species, variety, light, degree of ripeness, processing and storage. Plants produce over 8000 phenolic compounds via the shikimate, mevalonate and phenylpropanoid pathways. They are most frequently found in nature as conjugates, for example with monosaccharides and disaccharides (glycosides), and as such tend to be water soluble. Some of these products of secondary metabolism are essential to the plant's physiology, being involved in diverse functions such as pigmentation, pollination, protection against UV-B irradiation, and pathogen and predator resistance (Duthie & Crozier, 2000).

Fruits like apple, grape, pear, cherry, and various berries contain up to 200–300 mg phenolic compounds per 100 g fresh weight. Typically, a glass of red wine or a cup of tea or coffee contains about 100 mg phenolic compounds. The total dietary intake is about 1 g/d. It is much higher than that of all other known dietary antioxidants, about 10 times higher than that of vitamin C and 100 times higher than those of vitamin E and carotenoids (Scalbert *et al.*, 2005).

1.4.1.1. Chemistry of phenolic compounds

The term phenolic compound embraces a wide range of plant substances that possess one or more aromatic rings with one or more hydroxyl groups (hydroxybenzenes; phenols) and generally are categorised as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Croft, 1998; Ferguson, 2001; Jiang & Dusting, 2003; Liu, 2004). The nomenclature of natural phenolic compounds is sometimes confusing. The term polyphenol is widely used and refer to phenolic compounds containing two or more phenol groups. However, currently, the terms polyphenols or polyphenolics are used to refer to phenolic compounds regardless of the number of phenol groups in the molecules (Jiang & Dusting, 2003).

1.4.1.1.1. *Phenolic acids*

Phenolic acids seem to be universally distributed in plants. They are usually divided in two main groups (Fig. 8): benzoic acids, containing seven carbon atoms (C6–C1), and cinnamic acids, comprising nine carbon atoms (C6–C3). These compounds exist

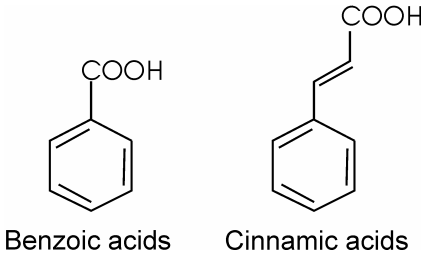


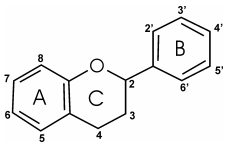
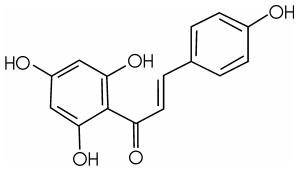
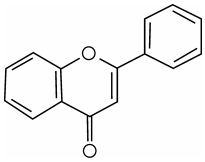
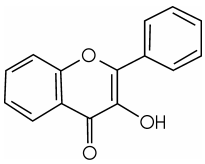
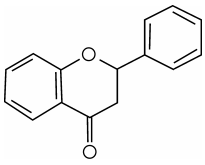
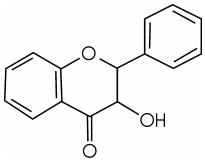
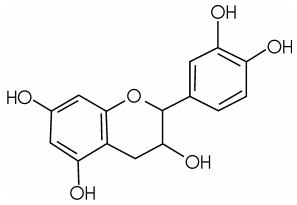
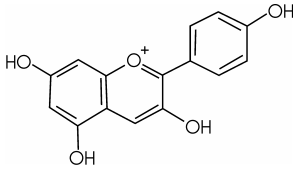
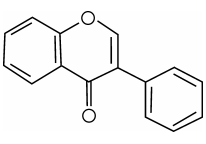
Fig. 8. Generic structure of phenolic acids.

predominantly in the hydroxylated form, therefore being generally named hydroxybenzoic and hydroxycinnamic acids, respectively (Liu, 2004; Nichenametla *et al.*, 2006; Fresco *et al.*, 2006). Phenolic acids derived from hydroxybenzoic acid include gallic acid, vanillic acid, procatechuic acid and syringic acid. Phenolic acids derived from hydroxycinnamic acid include *p*-coumaric acid, caffeic acid, and ferulic acid (Liu, 2004; Nichenametla *et al.*, 2006). Natural phenolic acids, occurs both in the free or conjugated forms (Fresco *et al.*, 2006). For example, chlorogenic acid is the ester of caffeic acid and quinic acid (Liu, 2004). Due to their structural similarity, several polyphenols are considered as acid analogs such as rosmarinic acid (Fresco *et al.*, 2006). Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid commonly found in the family *Lamiaceae* (Petersen & Simmonds, 2003).

1.4.1.1.2. *Flavonoids*

Flavonoids are ubiquitous in most plants and the largest class of polyphenols with more than 4000 distinct compounds identified. They are C15 compounds composed with a common phenylbenzopyrone structure (C6-C3-C6), consisting of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring, the C ring (Table 1) (Ross & Kasum, 2002; Ren *et al.*, 2003; Liu, 2004). The A ring are biosynthetically derived from acetate and has a characteristic hydroxylation pattern at the 5 and 7 position. The B ring is synthesised from the shikimate and is usually 4'-, 3'4'- (cathecol B ring), or 3'4'5'-hydroxylated (Croft, 1998; Ross & Kasum, 2002). Differences in the generic structure of the heterocycle C ring are used to classify them as chalcones, flavonols, flavones, flavanols, flavanones, flavanonols, anthocyanidins, and isoflavones (Table 1). Individual differences within each group result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation of these groups (Rice-Evans *et al.*, 1996). Flavones and flavonols have a double bond between 2 and 3 position. Flavonols are different from flavones in that they have a hydroxyl group in the 3 position (Table 1). Flavanones, flavanonols and flavanols are characterised by the presence of a saturated carbon bond between position 2 and 3 (Table 1). Flavanones have a keto (oxo) group at position 4, flavanols have frequently a hydroxyl (OH) group at position 3 whereas flavanonols have both a keto group at position 4 and an OH group at position 3 (Nichenametla *et al.*, 2006). Flavanols are represented by catechins, which are phenolic compounds mainly present in *Camellia sinensis*. The isoflavonones are derived by cyclisation of the chalcones, such that the B ring is located at the 3 position. Because they are structurally similar to estrogens, having estrogen-like biological activities, isoflavones are frequently referred to as phytoestrogens (Jiang & Dusting, 2003; Galati & O'Brien, 2004). Anthocyanidins are highly coloured pigments that give the red and blue colours in some fruits and vegetables.

Table 1. The chemical structures and major food sources of the 8 major flavonoid subgroups. [Adapted from Birt *et al.* (2001) and Moon *et al.* (2006)]

Structure		Examples	OH-substitution	Major food sources
Basic structure				
Chalcones		Chalcone		Hops, beer
Flavones		Chrysin Apigenin Luteolin	5,7 5,7,4’ 5,7,3’,4’	Parsley, thyme, celery, sweet red pepper, honey, propolis
Flavonols		Galangin Kaempferol Fisetin Quercetin Myricetin	5,7 5,7,4’ 7,3’,4’ 5,7,3’,4’ 5,7,3’,4’,5’	Onions, cherries, apples, broccoli, kale, tomato, berries, tea, redwine, tartary buckwheat
Flavanones		Naringenin Eriodictyol	5,7,4’ 5,7,3’,4’	Oranges, grapefruit, prunes
Flavanonols		Taxifolin	5,7,3’,4’	Limon, aurantium
Flavanols (flavan-3-ols)		Catechin Epicatechin Epigallocatechin	(+)-OH* (−)-OH* 5’	Tea, apples, cocoa, red wine
Anthocyanidins		Pelargonidin Cyanidin Delphinidin	 3’ 3’,5’	Cherries, grapes, blueberries
Isoflavones		Daidzein Genistein	7,4’ 5,7,4’	Soya beans, legumes

* Position of the OH group at position 3 relative to the phenolic group at position 2. Therefore, the prefix “epi” is given to a *cis* configuration between 3-OH group and 3-phenolic group.

1.4.1.2. Antioxidant activity

The antioxidant potential of phenolics was one of the earliest activities proposed for these compounds, since they were found to stabilise foods by retardation of rancidity and extension of shelf-life (Bors *et al.*, 1997). These compounds are, currently, recognised as beneficial for health limiting damages imposed by oxidative stress due to their antioxidant effects. They can either act directly, *i.e.* helping regulating overall reactive species levels to maintain physiological homeostasis by scavenging them and/or preventing their formation, or indirectly by stimulating endogenous antioxidant defence systems (Scalbert *et al.*, 2005).

Firstly, flavonoids are considered antioxidants because they are able to scavenge electrons of free radicals (Rice-Evans *et al.*, 1996). Phenolic compounds easily donate a hydrogen atom from an aromatic OH group to a free radical, because they are able to stabilise an unpaired electron through its delocalisation (Duthie & Crozier, 2000). Although numerous data exist for phenolic compounds, the correlation between antioxidant activity and chemical structure is far from clear. Different methods of assessment, varying substrate systems, and differences in concentrations of active antioxidants all have contributed to the confounding of the issue (Rice-Evans *et al.*, 1996). Nevertheless, the main structural features of flavonoids required for efficient radical scavenging could be summarised as follows (Rice-Evans *et al.*, 1996; Croft, 1998):

(1) an *ortho*-dihydroxy (catechol) structure in the B ring, for electron delocalisation;
(2) the 2,3 double bond in combination with a 4-keto function in the C ring provides electron delocalisation from the B ring;
(3) the 3- and 5-OH groups with 4-oxo function in A and C rings are required for maximum radical scavenging potential, since it provides hydrogen bonding to the keto group.

The addition of other hydroxyl groups can also increase, in some cases, the compound's scavenging activity, as for example the *meta* 5,7-dihydroxy in the A ring of flavonoids (Rice-Evans *et al.*, 1997). Phenolic acids may also be good radical scavengers, particularly those possessing the catechol-type structure, such as caffeic and rosmarinic acids (Croft, 1998). The free radical scavenging activity of phenolic compounds is important for their direct antioxidant activity by breaking the free radical chain reactions, inhibiting its initiation and preventing chain propagation (Rice-Evans *et al.*, 1996; Croft, 1998). In some conditions, these compounds can, however, be pro-oxidant if the resulting phenolic radical is not stable enough and results itself in chain-propagating radical. Further oxidation by reaction with another radical can, nevertheless, inactivate the phenolic radical (Croft, 1998).

Another pathway of direct antioxidant action of phenolic compounds, particularly in oxidation systems using transition metal ions such as copper or iron, is chelation of the metal ions (Rice-Evans *et al.*, 1997; Croft, 1998). Chelation of catalytic metal ions may prevent their

involvement in Fenton-type reactions, which can generate highly reactive hydroxyl radicals (Croft, 1998). For metal chelation, the two points of attachment of transition metal ions to flavonoid molecule are the 3',4' dihydroxy groups in the B ring and the 4-keto in combination with either the 5-hydroxy or 3-hydroxy groups in the A and C ring (Rice-Evans *et al.*, 1996; Rice-Evans *et al.*, 1997; Mira *et al.*, 2002; Williams *et al.*, 2004). The ability of phenolic compounds to react with metal ions may, in certain conditions, also render them pro-oxidant. For example, some flavonoids can, presumably, reduce Cu^{2+} to Cu^{+} and hence allow the formation of initiating radicals (Croft, 1998).

However, their classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation for cellular antioxidant effects of phenolic compounds, mainly by three reasons (Williams *et al.*, 2004; Scalbert *et al.*, 2005):

- phenolic compounds are extensively metabolised in the body, decreasing their direct antioxidant activity;

- antioxidant properties of polyphenols largely depend on their chemical and physico-chemical environment, which varies according to tissues and physiological conditions;

- concentration of flavonoids and their metabolites accumulated *in vivo* (*e.g.* in plasma or in organs) are lower (high nanomolar, low micromolar) than those recorded for small molecule antioxidant nutrients such as ascorbic acid and α-tocopherol. Consequently, phenolic compounds are unlikely to express beneficial action *in vivo* through outcompeting antioxidants such as ascorbate, which are present at higher concentrations (high micromolar).

It should, therefore, not be a surprise that no correlation between the antioxidant potency of various phenolic compounds measured *in vitro* and their biological activity determined *in vivo* or at the cellular level has ever been published (Scalbert *et al.*, 2005).

Phenolic compounds most likely exert their antioxidant effects *in vivo* by stimulating endogenous antioxidant defence systems. Certain polyphenols can induce phase II enzymes such as GST that will enhance the excretion of oxidising species, or induce antioxidant enzymes such as metallothionein (a metal-binding protein with antioxidant property) (Ferguson, 2001; Ross & Kasum, 2002). Polyphenols may also inhibit cytochromes P450 (CYPs) or enzymes such as cyclooxygenase or lipoxygenase that have oxidant activities (Ferguson, 2001; Moon *et al.*, 2006). Moreover, flavonoids may enhance antioxidant enzymes such as GPox (Ferguson, 2001).

1.4.1.3. Other biological effects

Besides their antioxidant effects, phenolic compounds exhibit a wide variety of other biological effects that may be beneficial to cells and organism, including antiviral, antibacterial, anti-inflammatory, anticarcinogenic and estrogenic-like effects among others (Rice-Evans *et al.*, 1996). Flavonoids and other phenolic compounds are complex molecules and multiple cellular

biological activities have been reported that could account for their biological effects, such as telomerase inhibition, modulation of signal transduction pathways, inhibition of cyclooxygenases and lipoxygenases, decrease of xanthine oxidase and sulfotransferase activities, interaction with sirtuins and cellular drug transport systems, compete with glucose for transmembrane transport, interfere with cyclin-dependent regulation of the cell cycle, and affect platelet function (Halliwell *et al.*, 2005). For example, focusing only on anticarcinogenic effects, many mechanisms of action have been identified for flavonoids and other polyphenols (Birt *et al.*, 2001; Ren *et al.*, 2003; Ferguson *et al.*, 2004). They include carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation, reversal of multidrug resistance, induction of detoxification enzymes, regulation of the host immune system, and maintenance of genomic stability. Not only one but rather some combination of these mechanisms is expected to result in both cancer chemoprevention and chemotherapy (Birt *et al.*, 2001; Ren *et al.*, 2003).

There is an emerging view that flavonoids, and their metabolites, act *in vivo* most likely as modulators of protein and lipid kinase signalling cascades. Many of the above activities may be accomplished by interactions of flavonoids with specific proteins central to intracellular signalling cascades. Inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular function by altering the phosphorylation state of target molecules and/or by modulating gene expression (Williams *et al.*, 2004). Again, focusing on the anticarcinogenic effects, modulation of several signal transduction pathways have been related to the chemopreventive activities of these compounds. These effects are believed to occur by the regulation of signalling pathways such as nuclear factor-*k*B (NF-*k*B), activator protein-1 (AP-1) or mitogen-activated protein kinases (MAPK). By modulating cell signalling pathways, polyphenols activate cell death signals and induce apoptosis in precancerous or malignant cells resulting in the inhibition of cancer development or progression (Fresco *et al.*, 2006). The induction of detoxifying enzymes (increase expression level) by polyphenols are mediated by the antioxidant response element (ARE)/electrophile response element (EpRE), which is located in the promoter region of related genes (Ferguson, 2001; Chen & Kong, 2004; Fresco *et al.*, 2006).

1.5. Liver

The liver is a large organ making up about 2 per cent of human body weight (Hinton & Grasso, 1995). The liver is strategically positioned in the circulation to perform its task of maintaining the body's metabolic homeostasis. It is the first organ that by the portal vein comes

in contact with the venous blood after its exposure to the stomach and intestines. Therefore, the liver is the first organ exposed to absorbed nutrients, metals, drugs, environmental toxicants and metabolic by-products of bacteria present in the gastrointestinal tract, which need to be processed before entering the systematic circulation (Moslen, 1996; Haussinger, 1996).

Among the many functions of the liver are (Hinton & Grasso, 1995; Moslen, 1996; Haussinger, 1996):

- Bile acid synthesis, bile formation and biliary excretion – exocrine function;
- Synthesis of most plasma proteins (except immunoglobulins) and very low density lipoproteins (VLDL) – endocrine function;
- Recycling of plasma proteins and old red blood cells;
- Maintenance of glucose, lipid, amino acid, ammonia and bicarbonate homeostasis of the body;
- Processing of absorbed nutrients as well as endo- and xenobiotics – biotransformation and detoxification.

1.5.1. Liver cell types and tissue structural organisation

The hepatocytes, the parenchymal cells of the liver, comprise about 90% of the cellular volume of the liver and exhibit a clearly defined polarity. The polarity of the hepatocyte is maintained by three different plasma membrane domains that can be recognised both morphologically and functionally. The highly specialised canalicular (or apical) membrane is rich in microvilli and comprises 10-15% of the membrane surface area of the cell; the smooth lateral membrane accounts for 15% of the surface area, and the sinusoidal (or basolateral) membrane, also rich in microvilli, comprises at least 70% of the cell surface (Diaz, 2000). Hepatocytes are assembled into sheets, each a single cell thick, which bifurcate and fuse to give a most complex network. Through this network run the liver capillaries, termed sinusoids (Fig. 9A). Between cells within the sheet run small branching channels called bile canaliculus (Hinton & Grasso, 1995).

Four main types of non-parenchymal cells are also present (Fig. 9A), *i.e.* endothelial cells and Kupffer cells (resident macrophages) lining the sinusoids, fat-storing Ito cells, and pit cells (liver associated lymphocytes) (Hinton & Grasso, 1995; Puviani *et al.*, 1998). Endothelial cells, unlike the endothelial of normal capillaries, do not form a continuous barrier but are penetrated by fenestrations which allow a free exchange of proteins between the blood within the sinusoids and the "Space of Disse", which lies between the endothelial cell and the hepatocyte (Hinton & Grasso, 1995).

In terms of cell number, hepatocytes represent about 65% of the liver. The discrepancy between cell number and volume is due to the fact that hepatocytes are much larger than the

other liver cells. Cell volume of hepatocytes depends, however, on the nutritional status. After 48 hours of starvation, it may decrease as much as 45% (Puviani *et al.*, 1998).

The liver is organised into operational units (Fig. 9B): the lobule (classic manner) and the acinus. The hexagonal lobule is divided into three regions: the centrilobular (near the hepatic vein), midzonal and periportal regions. Nowadays, the acinus is, however, preferred as a concept of a functional hepatic unit (Plaa, 1991; Moslen, 1996). The base of the acinus is formed by the terminal branches of the portal vein and hepatic artery that extend out the portal tracts (Moslen, 1996). The acinus has three zones that, fortunately, coincide with the three regions of the lobule (Fig. 9B): zone 1 is closest to the entry of blood and receives the highest concentration of oxygen and nutrients; zone 3 is adjacent the terminal hepatic vein; and, zone 2 is intermediate. The organisation of these operational units leads to a gradient of oxygen and nutrients. For example, cells in zone 1 tend to have more mitochondria and higher respiration rates than cells in zone 3 (Kedderis, 1996). The zonation of metabolic functions has important implications for chemically induced toxicity due in part to the differential expression of enzymes and the concentration gradients of nutrients, oxygen, cofactors and toxicant across the acinus (Kedderis, 1996).

1.5.2. Liver as a biotransforming organ

The liver is the highest metabolic organ. As such, many molecules are catabolised in the liver by diverse enzymes. Many lipophilic endo- (formed within the body) and xenobiotics (foreign molecules to the body, either of natural origin or man-made), potentially toxic, may be

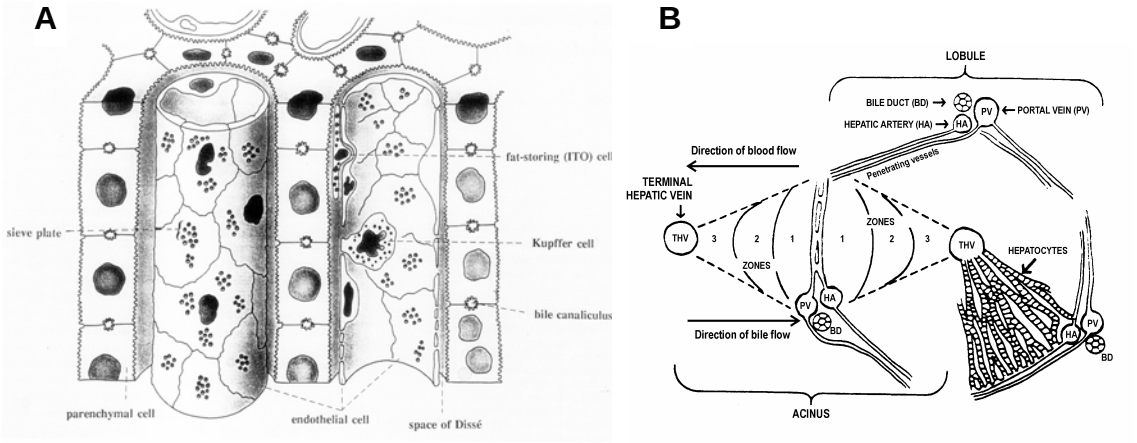


Fig. 9. (**A**) A three-dimensional representation of the organisation of parenchymal (hepatocytes) and non-parenchymal liver cells. Endothelial and Kuppffer cells line the sinusoids, thereby separating the sinusoidal space from the perisinusoidal space of Disse. The two spaces communicate via endothelial fenestrations that allow access of blood plasma (but not erythrocytes) to parenchymal cells. [From Haussinger (1996)]; (**B**) Schematic representation of liver operational units: the classic lobule and the acinus. The acinus extends from the terminal portal venule along the sinusoids to the draining terminal hepatic venule and is formed by tubes (20-30 cells long) of parenchymal cells. [Adapted from Moslen (1996)]

easily absorbed and systematically distributed, which makes them difficult to eliminate from the body in their original form. The process in which endo- and xenobiotics are changed from hydrophobic to hydrophilic molecules to facilitate elimination from the body is called biotransformation (Sipes & Gandolfi, 1991; Parkinson, 1996).

The highest quantity and diversity of enzymes involved in biotransformation occurs in the liver. The reactions metabolised by these drug-metabolising enzymes are generally divided into two groups, called phase I and phase II (Fig. 10). Phase I reactions involve chemical modification of the molecule by hydrolysis, reduction and oxidation. These reactions expose or introduce a functional group (-OH, -NH_2, -SH or -COOH) and usually result in only small increase in water solubility. Phase II reactions involve biosynthetic reactions where the xenobiotic or a phase I-derived metabolite is covalently linked to an endogenous molecule, producing a conjugate (Fig. 10). These biotransformations reactions include glucuronidation, sulfatation, acetylation, methylation, conjugation with glutathione and amino acids (such as glycine, taurine and glutamic acid). Most phase II reactions result in a large increase in xenobiotic hydrophobicity, and greatly promote the excretion of foreign chemicals in bile and urine (Sipes & Gandolfi, 1991; Hinton & Grasso, 1995; Parkinson, 1996).

The most important group of enzymes involved in phase I reactions are the cytochromes P450-dependent mixed-function oxygenase (MFO) system in the membranes of endoplasmatic reticulum. This system is actually a coupled enzyme system composed by two enzymes: NADPH-cytochrome P450 reductases and a heme-containing enzyme, commonly referred to as cytochrome P450 enzymes. The system is termed mixed-function oxygenase because electrons are taken from NADPH to reduce oxygen and oxidise or hydroxylate substrates. Cytochrome P450s are a large group of enzymes classified into families and subfamilies according to their amino acid sequences and substrate specificity, which catalyse a wide variety of reactions. The endoplasmatic reticulum also contains a second NADPH-dependent mixed-function amine oxidase system, the flavin-containing monooxygenases (FMO) (Sipes & Gandolfi, 1991; Kedderis, 1996).

Among the different phase II enzymes, which are mostly present in cytosol, glutathione *S*-transferase represents an important biotransforming enzyme to the liver. The GST catalyses

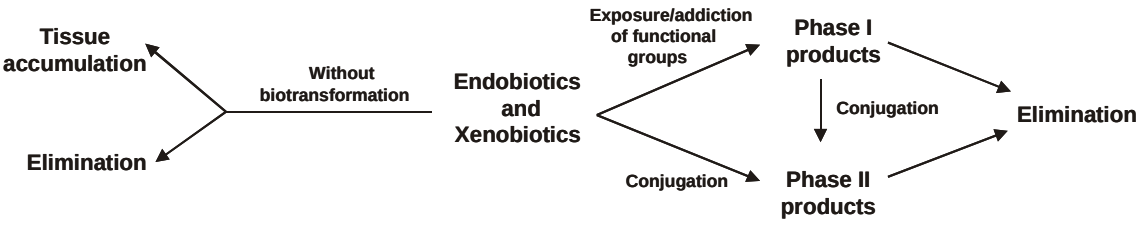


Fig. 10. Schematic integration of phase I and phase II biotransformation reactions. [Adapted from Sipes & Gandolfi (1991)]

the reaction of the nucleophilic sulfhydril of glutathione (GS^-) with compounds containing electophilic carbon (mostly) atoms. Substrates for GST share three common features: they are hydrophobic, they contain an electrophilic atom, and they react nonenzymatically with glutathione at some measurable rate (Sipes & Gandolfi, 1991; Parkinson, 1996).

1.5.2.1. Biotransformation

In general, drug metabolism can be considered as protective or a detoxification process in that it converts lipophilic compounds (which accumulation can overwhelm and injury cells) and active xenobiotics into water soluble and easily excreted inactive metabolites. However, biotransformation is not strictly related to detoxification. Phase I enzymes, and to lesser extent some phase II enzymes, can increase the toxicity of a xenobiotic and produce highly reactive metabolites from a inert molecule which becomes, therefore, toxic. The formation of products with enhanced activity and toxicity is often termed bioactivation and frequently leads to mutagenicity and carcinogenicity (Sipes & Gandolfi, 1991; Hinton & Grasso, 1995; Kedderis, 1996). Additionally, because electron transfer to molecular oxygen occurs during the phase I reaction sequence, ROS can occasionally dissociate from the cytochrome prior to oxidation of the substrate, leading to oxidative stress and cellular damage (Haussinger, 1996).

Reactive intermediates from bioactivation can interact with nucleophilic sites on tissues constituents, such as the sulfhydryl group of glutathione and cystein, or the amino or hydroxyl groups present in DNA, RNA and proteins. This covalent interaction with tissue macromolecules is thought to be the key factor in the toxic effects produced by xenobiotics. For many chemicals, these reactive intermediates can be detoxified without adverse cellular effects, provided that there is a balance between rates of formation and rates of detoxification. When this balance is disturbed, either by enhanced production of reactive intermediates or diminished capacity for their detoxification, formation of reactive metabolites can be associated with cellular injury (Sipes & Gandolfi, 1991).

1.5.2.2. Drug-drug interactions

Phase I enzymes and in particular CYPs are particularly vulnerable to induction (increased expression levels) and inhibition (enzyme activity) by a variety of xenobiotics, including constituents of herbs (Parkinson, 1996; Zhou *et al.*, 2003). This can lead to adverse drug-drug interactions.

Induction of CYPs would be expected to increase the activation of procarcinogens to DNA-reactive metabolites, leading to increase tumor formation, as well as other toxicants such as CCl_4 and other haloalkanes (Parkinson, 1996). Therefore, hepatic injury induced by chemicals or natural toxins metabolically activated by drug-metabolising enzymes may occur

from occupational, household or environmental exposure. Inhibition of CYPs can also be deleterious by decreasing the clearance of toxicants in blood.

Additionally, the pharmacokinetic behaviour of pharmaceutical drugs can be severely affected due to modulation of CYPs by xenobiotics. In case of pharmaceutical drugs that need biotransformation to exert their effects, an inhibiton of the CYP needed for the desired reaction may compromise the therapeutical goal of a drug therapy. On the contrary, induction of CYP can increase rapidly the clearance of pharmaceutical drugs compromising its action (Parkinson, 1996).

Gender and interindividual variations of CYP expression levels (Meibohm *et al.*, 2002; Tamasi *et al.*, 2003) could increase the susceptibility of different populations or individuals to herb-drug interaction effects.

1.5.3. Liver diseases and oxidative stress

Liver injury is a common pathology, both acute and chronic, quite often characterised in its chronic evolution by a progressive process from steatosis to hepatocellular carcinoma, through chronic hepatitis, fibrosis and cirrhosis (Loguercio & Federico, 2003; Vitaglione *et al.*, 2004). These processes involve apoptosis, necrosis, inflammation, immune responses, ischemia, altered gene expression and regeneration in hepatocyte, Kupffer and endothelial cells (Loguercio & Federico, 2003).

The knowledge about the molecular events that induce liver cell damage (see section 1.6) and disease progression is continuously improving. There is increasing evidences that ROS and RNS (and, therefore, oxidative stress) play a crucial role in the various steps that initiate and regulate the progression of liver diseases, such as alcoholic liver disease, nonalcoholic steatohepatitis and hepatitis type C independently from the type of etiologic agents (Loguercio & Federico, 2003; Vitaglione *et al.*, 2004; Tanikawa & Torimura, 2006). Oxidative stress is also involved in liver damage induced by alcohol abuse, viral infection, alteration of lipid and carbohydrate metabolism and xenobiotics (Loguercio & Federico, 2003; Vitaglione *et al.*, 2004).

Because oxidative stress plays a central role in liver diseases, the use of antioxidants (both natural and synthetic) have been proposed as therapeutic agents, as well as drug coadjuvants, to counteract liver damage (Loguercio & Federico, 2003; Vitaglione *et al.*, 2004).

1.6. Biochemical and molecular mechanisms of cell injury and death

A wide range of chemicals, environmental pollutants, dietary constituents, pharmaceutical agents and natural products can cause hepatotoxicity in humans. The liver is particularly susceptible to toxic agents mainly for two reasons (Jaeschke *et al.*, 2002): (1) after gastrointestinal absorption, most xenobiotics that enter the blood flow come directly to the liver ("first pass") by the portal vein in a concentrated form before being distributed to other organs; and (2) the liver is the primary site for biotransformation, which exposes the liver to metabolites, some of which, as a result of bioactivation, may be more toxic than the original chemical. The hepatotoxic effects can then be reflected, at the organ level, in diverse liver lesions, such as the ones mentioned above (section 1.5.3, hepatitis, steatosis, fibrosis, cirrhosis, etc.) (Pessayre *et al.*, 1999).

At cellular level (here, mainly hepatocyte), the different biochemical and molecular mechanisms of cell injury inflicted by chemicals, can, according to Kedderis (1996), be organised in four general categories: direct effects of toxicants, covalent binding of reactive metabolites, induction of oxidative stress and disruption of calcium homeostasis, as detailed below. It must be stressed that many of the following mechanisms can be induced simultaneously by one toxicant. Sometimes different mechanisms can operate at different doses of the toxicant. Therefore, there is not one single cause for cell damage and death (Kedderis, 1996).

1.6.1. Direct effects of toxicants

The simplest general way involved in cell damage is a direct effect of the toxicant upon critical cellular systems. Many organic solvents are capable of causing damage to cells by their direct solvent or surfactant effects on the lipid membranes (Berry *et al.*, 1991; Kedderis, 1996). These effects will ultimately disrupt cellular volume homeostasis and lead to cell death. Certain toxicants bind to actin and disrupt cell cytoskeleton, resulting also in increase plasma membrane permeability (Kedderis, 1996). Various chemicals (including metal ions) bind to mithocondrial membranes and enzymes, disrupting energy metabolism and cellular respiration, while others act as direct inhibitors and uncouplers of mithochondrial electron transport. As a result, membrane potential can be lost increasing, therefore, plasma membrane permeability, disrupting calcium homeostasis and, ultimately, causing cell death (Kedderis, 1996; Castell *et al.*, 1997).

1.6.2. Covalent binding of reactive metabolites

Many hepatotoxins are metabolically activated to high reactive metabolites (generally electron-deficient species termed electrophiles) that can covalently bind, *i.e.* irreversible binding, to cellular macromolecules such as proteins and DNA (Kedderis, 1996; Castell *et al.*, 1997). Because of their ability to participate in many different biotranformation reactions, CYP isozymes are usually involved in reactions leading to covalent binding. Electrophiles, intermediate radicals and conjugates can react with nucleophilic functional groups of proteins to form stable bonds (Castell *et al.*, 1997). This results in protein and enzyme inactivation which can affect and impair critical cellular functions (Kedderis, 1996). Highly reactive electrophiles can also, by covalent binding, form DNA adducts which may result in somatic mutations. This genotoxic action may in certain circumstances result in a tumour (Kahl, 1999).

1.6.3. Induction of oxidative stress

Another way by which hepatotoxins can induce liver cell damage and death is the generation of oxidative stress either by increasing reactive substances, such as ROS, and/or by decreasing cellular antioxidant defences (Kedderis, 1996; Castell *et al.*, 1997). Some intermediate ROS formed by the toxicant or even reactive metabolites can initiate lipid peroxidation that in turn, by a cascade of reactions, form more reactive substances. Oxidative stress, in addition to covalent binding, also leads to a decrease in cellular glutathione levels, which has high implications to cell survival (Kedderis, 1996; Castell *et al.*, 1997; Kahl, 1999). Cellular glutathione can decrease for several reasons (Castell *et al.*, 1997): (1) oxidation to GSSG; (2) conjugation with metabolites or endogenous proteins; and (3) reduced *de novo* synthesis. Lipid peroxidation of mitochondria and depletion of glutathione levels may alter calcium homeostasis which induce several cellular processes that ends in cell death (Kedderis, 1996; Castell *et al.*, 1997; Pessayre *et al.*, 1999).

1.6.3.1. Lipid peroxidation

A important type of molecular lesion induced by free radicals is lipid peroxidation of polyunsaturated lipids (those that contain two or more carbon-carbon double bonds) of cells, particularly present in membranes (Berry *et al.*, 1991; Halliwell & Gutteridge, 1999). The first step in this process (Initiation, Fig. 11) is the formation of lipid radicals (Halliwell & Gutteridge, 1999). A free radical ($R^{\cdot}$) with enough reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$) of a polyunsaturated lipid (LH) leads to a lipid radical ($L^{\cdot}$). The carbon radical ($-{}^{\cdot}CH-$) is usually stabilised by a molecular rearrangement to form a conjugated

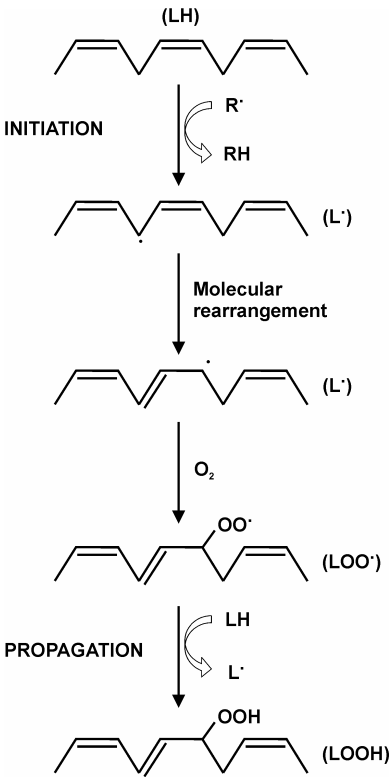


Fig. 11. Representation of the initiation and propagation reactions of lipid peroxidation. [Adapted from Halliwell & Gutteridge (1999) and Spiteller (2001)]

diene. This radical quickly adds molecular oxygen to form a peroxyl radical ($LOO^{\cdot}$). The peroxyl radical may react with another lipid molecule to form a hydroperoxide (LOOH) and a new lipid radical (Halliwell & Gutteridge, 1999; Pessayre *et al.*, 1999; Spiteller, 2001). Lipid peroxidation may thus extend from one lipid to the other or along the same lipid chain (Propagation, Fig. 11). Eventually, during lipid peroxidation, unsaturated lipids are oxidised and cut into small fragments, such as alkanes, malondialdehyde and alkenals (Pessayre *et al.*, 1999). By the Fenton reaction, metal ions may react with lipid peroxides in a similar way to their reaction with H_2O_2, producing the alkoxyl radical ($LO^{\cdot}$) (Halliwell & Gutteridge, 1999).

The first consequence of this process is a profound alteration in the physico-chemical properties of cell membranes (cytosolic, mitochondrial, etc.) and the loss of membrane enzyme activities (Castell *et al.*, 1997; Halliwell & Gutteridge, 1999). Lipid radicals, as well as most lipid degradation products, such as malondialdehyde and alkenals, are chemically very reactive and, if not detoxified or removed from the cell by antioxidant mechanisms, may cause additional cellular damage such as enzyme inactivation, protein and DNA damage (Castell *et al.*, 1997).

1.6.3.2. Glutathione levels

Glutathione (γ-glutamylcysteinylglycine) is a tripeptide composed by glutamate, cysteine and glycine, which exists in thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms (Kaplowitz *et al.*, 1985; Lu, 1999). Glutathione is a fairly ubiquitous in aerobic organisms, found in most plants and animals, being the major low-molecular-mass thiol compound that generally exists in millimolar (1-12 mM) concentrations (Kaplowitz *et al.*, 1985; Sies, 1999; Dringen *et al.*, 2000). Within the cell, glutathione exists mainly (>98%) as GSH, but some is also present as GSSG, thioether, mercaptide or other thioester forms (GSH conjugates) (Hammond *et al.*, 2001). Glutathione is synthesised in every cell of the body, but the liver is quantitatively the major site of synthesis (Hammond *et al.*, 2001). Therefore, the liver is one of the organs with the highest content of GSH (Kaplowitz *et al.*, 1985; Lu, 1999), hepatocytes containing about 10% of total body pool of GSH (Loguercio & Federico, 2003).

Glutathione has important functions as antioxidant (is a strong nucleophile), in detoxifications reactions and as a transport and storage form of cysteine (Lu, 1999; Dringen *et al.*, 2000). Because GSH is the major intracellular nonprotein thiol, it maintains the cellular reducing environment in cells keeping sulfhydryl groups of cytosolic proteins in the reduced form, which is essential for its optimal activity (Dringen *et al.*, 2000; Hammond *et al.*, 2001). Also, there is increasing evidence that glutathione has a role in control of many cellular processes such as signal transduction, gene expression and apoptosis (Sies, 1999; Hammond *et al.*, 2001).

Regarding its role in cellular antioxidant defences, GSH reacts directly with radicals in nonenzymatic reactions and is the electron donor in the reduction of peroxides catalysed by GPox. GSH is regenerated from GSSG within cells in a reaction catalysed by GR, transferring reduction equivalents from NADPH. Glutathione can also inactivate reactive metabolites by conjugation via enzymatic mechanisms, reactions catalysed by GST (Reed, 1990; Castell *et al.*, 1997; Griffith, 1999). Thus, glutathione depletion can impair the cellular antioxidant defences, resulting in oxidative stress, which may lead to cell injury and death (Reed, 1990; Kedderis, 1996; Castell *et al.*, 1997).

Glutathione depletion, from excessive consumption of GSH without adequate recovery (when needs exceed the capacity of the liver to increase its synthesis), together with the direct covalent binding to protein thiols and/or the direct oxidation of protein thiols, tend to decrease protein thiols, which also has severe toxicological implications (Castell *et al.*, 1997; Pessayre *et al.*, 1999):

(1) A first consequence is the formation of disulphur bonds between different molecules of actin, forming inactive actin aggregates. This destroys the microfilamentous network of cytoskeleton allowing the formation of fragile plasma membrane blebs.

(2) A second consequence of the oxidation of protein thiols is to decrease the activity of plasma membrane calcium translocases, whose role is constantly to extrude calcium from the hepatocytes. It results, therefore, in an increase of cellular calcium which has several toxicological consequences (see below, section 1.6.4.).

(3) A last consequence of the oxidation of protein tiols is to permeabilise the mitochondrial inner membrane, decreasing membrane potential and the ability to form ATP.

Because the enzyme catalase is not present in mitochondria, they are totally dependent on GPox, and hence GSH, to detoxify hydroperoxides (Castell *et al.*, 1997). Therefore, this and the latter consequence of oxidation of protein thiols, make mitochondria particularly susceptible to oxidative stress and xenobiotics.

1.6.4. Disruption of calcium homeostasis

Calcium regulates a wide variety of crucial physiological functions in the cell. Moreover, intracellular calcium is also involved in many pathological and toxicological processes where it accumulates in dying cells (Kedderis, 1996; Castell *et al.*, 1997; Halliwell & Gutteridge, 1999). The cellular cytosolic free Ca^{2+} concentration is very low (approximately 0.1 μM) compared with the concentration of Ca^{2+} in extracelular fluids (1-2 mM). This gradient is maintained by an ATP-dependent Ca^{2+} extrusion translocases. Inside the cell, Ca^{2+} concentration is also controlled by active sequestration into intracellular stores, such as mitochondria and endoplasmatic reticulum, as well as by Ca^{2+}-binding proteins (*e.g.* calmodulin) (Kedderis, 1996; Castell *et al.*, 1997; Halliwell & Gutteridge, 1999). This equilibrium or calcium homeostasis can be altered in the course of toxic phenomena, such as by inhibitors of the several cellular calcium translocases. However, most toxicants act indirectly by modifying membrane properties, reducing the energy available, inducing lipid peroxidation of mitochondria and generalised oxidative stress with consequent depletion of GSH (Castell *et al.*, 1997).

There are several toxicological consequences of the disruption of cellular calcium homeostasis that can lead to cell death (Kedderis, 1996; Castell *et al.*, 1997; Halliwell & Gutteridge, 1999; Pessayre *et al.*, 1999):

(1) Activation of Ca^{2+}-dependent proteases (calpains) that cleave actin-binding proteins, eliminating the plasma membrane 'anchorage' of the cytoskeleton and, consequently, allowing membrane blebbing.

(2) Activation of Ca^{2+}-dependent and calmodulin-dependent phospholipases, leading to a stimulation of arachidonate metabolism and increased plasma membrane permeability.

(3) Activation of Ca^{2+}-dependent transglutaminases, which form protein aggregates causing irreparable damage and functionality of those proteins.

(4) Activation of Ca^{2+}-dependent endonucleases, which cut DNA between nucleosomes resulting in extensive DNA fragmentation.

1.6.5. Cell death

The end result of these various cellular and molecular mechanisms may be the death of the cell, either by necrosis (pathological and accidental – murder) or apoptosis (physiological and programmed - suicide). Among other factors, the type of cell death can depend on the concentration of the toxic agent. Moderate concentrations can apparently induce apoptosis, while higher concentrations cause cell necrosis (Castell *et al.*, 1997; Halliwell & Gutteridge, 1999).

Necrosis is caused by a rapid collapse of the cell's internal homeostasis characterised by cell swelling, membrane lysis and release of cellular contents (Castell *et al.*, 1997; Halliwell & Gutteridge, 1999). This is usually a consequence of the profound loss of mitochondrial function and resultant ATP depletion, leading to a loss of ion homeostasis and volume regulation, with increased influx of Ca^{2+}. The latter activates a number of nonspecific hydrolases, like proteases, nucleases and phospholipases (Kaplowitz, 2002). Necrosis is accompanied by an inflammatory response in the surrounding tissue areas.

Apoptosis involves cell shrinkage, chromatin condensation and nuclear disassembly as well as fragmentation of the cell into apoptotic bodies with intact plasma membranes. These are rapidly phagocytosed by neighbouring cells or macrophages (Castell *et al.*, 1997; Halliwell & Gutteridge, 1999; Kaplowitz, 2002). Apoptosis is under precise genetic control, in which RNA and protein synthesis as well as energy (ATP) is required, and the degradation of the cell occurs in a much more orderly fashion than in necrosis (Kedderis, 1996; Castell *et al.*, 1997). At the molecular level, apoptosis represents a collection of intricate pathways with more than 100 different proteins actively participating in the apoptotic phenomena, such as signal transduction proteins and execution proteins, which results in the dismantling of the cell and inactivation of repair (Gosslau & Chen, 2004). The process involves the participation of specialised proteolytic enzymes – caspases – which contain cysteine residues at the active sites and cleave at the aspartate residues. Thus, specific proteins are cleaved, leading to both activation (execution proteins) and inactivation (repair proteins) of specific targets (Kaplowitz, 2002). There are two major pathways that initiate apoptosis: an extrinsic (receptor mediated) and intrinsic (mitochondrial mediated), which are cross-linked. In addition, mitogenic and stress responsive pathways are involved in the regulation of apoptotic signalling (Gosslau & Chen, 2004).

1.7. Diabetes

Diabetes mellitus (the Latin word *mellitus* meaning 'honey-sweet', refers to the taste of diabetic urine) is a chronic metabolic disorder characterised by elevated blood glucose (hyperglycaemia) and urinary glucose excretion (Halliwell & Gutteridge, 1999). The most common forms of *diabetes mellitus* are type 1 and type 2 diabetes, which result from defects in insulin secretion and/or insulin action (Costacou & Mayer-Davis, 2003). Insulin-dependent *diabetes mellitus* (IDDM, or type 1), which accounts for about 10% of all cases of diabetes, generally occurs in young patients and is characterised by the marked inability of the pancreas to secrete insulin because of autoimmune destruction of the beta cells. Noninsulin-dependent *diabetes mellitus* (NIDDM, or type 2), the more prevalent form of diabetes, is characterised by a

combination of peripheral insulin resistance and/or a β-cell insulin-secretory defect that varies in severity (Poitout & Robertson, 1996; Mandel *et al.*, 2005; Rolo & Palmeira, 2006). It typically occurs in individuals older than 40 years who have a family history of diabetes, but it is noteworthy, that the manifestation of type 2 diabetes is frequent now in younger ages (Jermendy, 2005; Mandel *et al.*, 2005).

Chronic elevation of blood glucose is the fundamental cause of diabetic complications, affecting multiple organs in both type 1 and type 2 diabetes. Diabetes is associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke and limb amputation. Diabetes is also associated with microvascular complications in the retina, glomerulus and vasa nervorum which can lead to the failure of these tissues/organs (Brownlee, 2001).

The prevalence of type 2 diabetes has been increasing alarmingly worldwide. It has been estimated that from 1995 to the year 2025, the prevalence of diabetes will increase by 42% among adults living in the developed world and by 170% among adults in developing countries (Costacou & Mayer-Davis, 2003). An estimated 300 million people age 20 and older will have diabetes by the year 2025, and 366 million by the year 2030 (Costacou & Mayer-Davis, 2003; Jermendy, 2005; Rolo & Palmeira, 2006).

1.7.1. Diabetes and the liver

The liver plays a major role in glucose homeostasis maintaining normal concentrations of blood glucose within a very narrow range by its ability to store glucose as glycogen and to produce glucose from glycogen breakdown or gluconeogenic precursors (Roden & Bernroider, 2003; Klover & Mooney, 2004; Rolo & Palmeira, 2006). In healthy individuals, hepatocytes can respond, under hormonal control, to either feeding or fasting conditions by storing or producing glucose as necessary (Klover & Mooney, 2004). In the fasting state, the effects of glucagon avoid hypoglycemia by stimulating gluconeogenesis and glycogenolysis. These pathways are related with the hepatic glucose release to systemic circulation to fuel the function of organs and tissues (Roden & Bernroider, 2003; Klover & Mooney, 2004; Rolo & Palmeira, 2006). Postprandially, insulin prevents hyperglycaemia, in part, by suppressing hepatic gluconeogenesis and glycogenolysis and facilitating hepatic glycogen synthesis, in addition to its increasing glucose absorption in muscle and adipose tissue (Roden & Bernroider, 2003; Klover & Mooney, 2004).

In type 2 diabetes, dysregulation of insulin action (insulin resistance) will have profound effects on hepatic glucose homeostatic pathways and circulating glucose levels. In the hepatocyte, glycogenolysis and gluconeogenesis are less efficiently suppressed in response to

insulin despite adequate circulating glucose levels (Roden & Bernroider, 2003; Klover & Mooney, 2004). This leads to inappropriate glucose release by the liver and results in hyperglycaemia which can occur both after meals and during fasting. It should be noted that insulin resistance also affects the skeletal muscle which is responsible for much of insulin-dependent glucose uptake and utilisation and contributes very significantly to the hyperglycaemia of this disorder (Klover & Mooney, 2004). In summary, impairments in the insulin signal transduction pathway appear to be critical lesions contributing to insulin resistance and type 2 diabetes, where glucose is overproduced by the hepatocyte and is ineffectively metabolised by other organs (Klover & Mooney, 2004).

Moreover, it was recently found that the hepatic glutathione is involved in the synthesis of a putative hormone referred to as hepatic insulin-sensitising substance (HISS) (Guarino *et al.*, 2003). Therefore, this recent finding supports the idea that glutathione, an important intracellular antioxidant, may also be essential for insulin action.

Therefore, the liver could be seen as a potential target for the treatment of diabetes and, because the available medication is far from resolving this disease and its complications, plants are currently being used in the search for new possible drugs for its management.

1.7.2. Diabetes and oxidative stress

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of *diabetes mellitus* (Maritim *et al.*, 2003; Willcox *et al.*, 2004). Looking only to studies on the most prevalent type, type 2, the association between oxidative stress and diabetes is mainly of two types: diabetic patients relative to matched controls possess increased production of ROS (and other free radicals) and/or possess impaired antioxidant defences , such as plasma glutathione levels (West, 2000; Maritim *et al.*, 2003; Bruce *et al.*, 2003; Willcox *et al.*, 2004).

However, although a great amount of data shows a link between oxidative stress and diabetes, whether oxidative stress is important to the development and progression of diabetes and its complications is still under debate. For some authors, oxidative stress does not have a primary role in the pathogenesis of diabetes and diabetic complications. They argue that oxidative stress does not occur at an early stage in diabetes, preceding the appearance of complications. Oxidative stress is viewed as a merely common consequence of the tissue damage, reflecting the presence of complications (Baynes & Thorpe, 1999). In this regard, as Baynes and Thorpe (1999) said, “the treatment of diabetes with antioxidant therapy is like applying water to a burning house, certainly helpful in limiting the conflagration, but also a little late in the process”.

In spite of the sceptics, it is increasingly accepted that oxidative stress, generated as a result of hyperglycaemia, has an important role in causing the secondary complications of diabetes, such as atherosclerosis, nephropathy, retinopathy and neuropathy (West, 2000). Four main mechanisms, by which the diverse microvascular and macrovascular pathologies may result from hyperglycaemia, have been implicated: increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux (Brownlee, 2001; Maritim *et al.*, 2003; Rolo & Palmeira, 2006). Until recently, there was no unifying hypothesis linking these four mechanisms. This issue may now be resolved, since each of the four different pathogenic mechanisms could reflect a single hyperglycaemia-induced process: overproduction of superoxide by the mitochondrial electron transport chain (Brownlee, 2001; Rolo & Palmeira, 2006). Mitochondria are the principal source of ROS in cells as a result from imperfectly coupled electron transport. Normally, only 0.1% of total oxygen consumption leaks from the respiratory chain to generate ROS, which can be dealt with the endogenous antioxidant system. Hyperglycaemia induces an increase in electron flux through the mitochondrial electron transport chain, which, consequently, generates higher concentrations of free radical anion superoxide (Brownlee, 2001; Rolo & Palmeira, 2006). This overproduction of $O_2^{\cdot -}$ may not be sustained by the antioxidant system inducing, therefore, a situation of oxidative stress and damage. Being ROS a central player in the appearance of various secondary complications in diabetes, inhibition of ROS production and/or enhancement of ROS scavenging might be a beneficial therapy (Brownlee, 2001; Rolo & Palmeira, 2006). In this context, medicinal plants rich in antioxidants compounds may become, therefore, an interesting tool for the treatment of diabetes complications.

Although there is a genetic component, oxidative stress may also, for some authors, be an important factor in the development and progression of type 2 diabetes, at least in some groups at risk – for example the obese people. According to this hypothesis, oxidative stress can be seen as a pathogenic mechanism linking insulin resistance (the first step of the development of type 2 diabetes), impaired glucose tolerance (IGT) and overt diabetes (West, 2000; Ceriello & Motz, 2004). In predisposed subjects, the combination of excess caloric intake and reduced physical activity induces a state of insulin resistance, during which blood glucose is maintained near normal levels by compensatory hyperinsulinemia. When β-cells are no longer able to compensate for insulin resistance, by adequately increasing insulin production, IGT appears. This is characterised by excessive postprandial hyperglycemia, with fasting glucose being in normal range. Persistence of imbalance between caloric intake and expenditure eventually leads to overt diabetes, characterised by high glycaemia in any condition whether fasting or postprandial (Ceriello & Motz, 2004).

When caloric intake exceeds the energy expenditure, an excess of mitochondrial ROS is also generated increasing the activity of the citric acid cycle. As a compensatory mechanism against ROS, cells reduce glucose transporter type-4 (GLUT4) translocation to the plasma membrane, resulting in resistance to insulin-stimulated glucose uptake in muscle and adipose tissues (Ceriello & Motz, 2004). Because β-cells are notably not dependent on insulin for glucose uptake, which take here place via facilitative diffusion, these cells are even more susceptible to overfeeding. In addition, β-cells are particularly sensitive to ROS because they have low levels of antioxidant defences. Therefore, is not surprising that oxidative stress can markedly impair insulin secretion of β-cell during increased glucose metabolism (Ceriello & Motz, 2004). The increase of insulin resistance and/or the decrease in compensatory insulin secretory response can lead to the appearance of IGT. Repeated exposure to hyperglycemia may lead to β-cell dysfunction that may become irreversible over time. This in association with the dysregulation of insulin action in hepatocytes and peripheral tissues leads to overt diabetes (Ceriello & Motz, 2004).

Concluding, in accordance with this last hypothesis and the overall oxidative stress involved in complications of diabetes, the antioxidant therapy, also based in natural compounds, can be seen as a good possible development to oppose the increasing epidemic of diabetes, a real emergency in our future.

The lower antioxidant defences of diabetic patients make them, in addition, more vulnerable to other related diseases where oxidative stress is involved, such as cardiovascular diseases.

1.7.3. Diabetes as a preventable disease

The prevalence of type 2 diabetes is increasing worldwide due to a combination of different factors: increasing lifespan, sedentary lifestyle, excessive intake of high energy foods, increasing prevalence of overweight/obese people (Lai, 2002). Taking into consideration the increasing prevalence of type 2 diabetes and the high costs involved in its treatment, the primary prevention of this disease arises as an important issue (Lai, 2002; Costacou & Mayer-Davis, 2003; Jermendy, 2005).

Before the establishment of the disease, those individuals more at risk of developing type 2 diabetes present the first signs of abnormal glucose metabolism such as IGT and/or impaired fasting glucose (Simpson *et al.*, 2003). This provides an asymptomatic period at the beginning of the progression of diabetes, where preventive interventions can be applied. There is now substantial evidence that type 2 diabetes could be considered as a preventable disease through changes in lifestyle that include, among others, dietary factors (Costacou & Mayer-

Davis, 2003; Simpson *et al.*, 2003; Jermendy, 2005). However, the difficulty in maintaining lifestyle changes over the long term justifies the need for pharmacotherapeutic support, and recent studies have shown beneficial effects of metformin and acarbose in the retardation of the progression from IGT to type 2 diabetes (Simpson *et al.*, 2003). However, any pharmacological intervention in an asymptomatic population raises ethical considerations in addition to practical and economic issues. Dietary supplements with plasma glucose lowering properties could provide a culturally acceptable and economically viable alternative to pharmaceutical interventions at this stage. Plant extracts or natural compounds can easily be used as functional foods or food supplements and could have a beneficial impact in low cost prevention strategies of diabetes. In fact, some epidemiological evidences and recent studies of hypoglycaemic effects of plant extracts and polyphenols provide a promising future to the use of plant products in the prevention of diabetes (Costacou & Mayer-Davis, 2003; Scalbert *et al.*, 2005; Mandel *et al.*, 2005).

1.8. References

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Chapter 2

Sage Essential Oil: is it Antioxidant Also to the Liver?

2.1. Chapter overview

Sage essential oil is widely used in the food, drug, beverage, cosmetic and fragrance industries. Previous experiments performed by Farag *et al.* (1989)[1] showed a relatively weak antioxidant activity of sage EO. The authors measured the antioxidant activity of sage EO in a linoleic acid emulsion system. This antioxidant activity was then related to the ability of EO to inhibit rancidity in food materials, *i.e.* to act as food preservatives[1]. But, how can this antioxidant activity of sage EO be also observed in cells? To answer this question, the effect of EO was tested *in vitro* using freshly isolated rat hepatocytes. The use of hepatocytes, which are highly metabolic cells, is valuable also to verify potential toxic effects of sage EO. Sage EO has already some limitations in its use due to its known neurotoxic and convulsant effects, mainly related to the content in α-thujone and camphor[2]. However, the toxic as well as protective/antioxidant effects of sage EO to the liver was, to our knowledge, never been investigated.

When hepatocytes were exposed to *t*-BHP, sage EO was not able to protect cells from damage and, therefore, did not show antioxidant effects in this type of cell assays. However, although sage EO did not possess direct antioxidant effects in hepatocytes, there is still the possibility that these low molecular weight compounds have the ability to modulate certain proteins or cell signalling processes, such as the ones related to the antioxidant or detoxifying systems of cells (see chapter 3).

At reasonable concentrations, as the ones when EO is used as food supplement, sage EO did not show toxic effects to rat hepatocytes. However, at the concentration of 2 µl EO/ml significant hepatocyte cell death was observed. Interestingly, the toxic effect of the mixture of tens of compounds, present in sage EO, was not mediated by lipid peroxidation. This is in agreement with the effects of sage EO in inhibiting rancidity in food products. A significant decrease in GSH content was observed by sage EO. Because there was no significant increase in GSSG levels, depletion in GSH was most likely via conjugation reactions with some constituents (possibly terpenes and/or their metabolites) with electrophilic properties. The significant GSH depletion induced by sage EO provides an explanation for the toxic effects observed for higher concentrations in liver cells. Taking into account the hydrophobic properties of the EO, its direct solvent effect on cellular membranes can not be ruled out as a contributing factor for cell death.

Our results did not indicate any antioxidant effects of *S. officinalis* EO on rat

[1] Cuppett SL & Hall CA, 1998. Antioxidant activity of the *Labiatae*. In: *Advances in Food and Nutrition Research*, Academic Press, London, pp. 245-271.

[2] Millet Y, Jouglard J, Steinmetz MD, Tognetti P, Joanny P & Arditti J, 1981. Toxicity of some essential plant oils. Clinical and experimental study. *Clinical Toxicology* **18**, 1485-1498.

hepatocytes, probably because of its poor concentration in monoterpenes with a hydroxyl group on an aromatic ring (*e.g.* eugenol and borneol) or with an ethylidene group (*e.g.* linalool)[1].

In conclusion, at doses higher than 200 nl/ml, acting directly on the liver, the EO may cause toxicity. The neurotoxicity of thujones and camphor, which are the major compounds of *S. officinalis* EO, as well as the hepatotoxicity of sage EO reported herein, justify some concern with the consumption of high doses of sage products containing EO.

2.2. Methods

The following experimental protocols (see Appendix) were used in this chapter:

- P1: Biological Models – Rat Hepatocyte Isolation and Use
- P4: Cell Viability Assays – LDH Leakage
- P6: Cell viability Assays – Trypan Blue Staining
- P7: Metabolite Measurements – TBARS (Lipid Peroxidation)
- P8: Metabolite Measurements – Glutathione Levels
- P10: Metabolite Measurements – Total Protein

2.3. Publication

This chapter comprises the following publication:

Lima CF, Carvalho F, Fernandes E, Bastos M, Santos-Gomes P, Fernandes-Ferreira M & Pereira-Wilson C, 2004. Evaluation of toxic/protective effects of the essential oil of *Salvia officinalis* on freshly isolated rat hepatocytes. *Toxicology in Vitro* **18(4)**, 457-465.

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

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Abstract

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

Keywords: *Salvia officinalis*; Sage; Essential oil; Toxic effects; Antioxidant effects; Hepatoprotective effects; Hepatocytes.

1. Introduction

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

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

Sage EO is a complex mixture of tens of volatile compounds including monoterpenes, sesquiterpenes and diterpenes. Importantly, the EO contributes the unique

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

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flavour of sage and justifies the use of sage as a food condiment. Although some data already exist relating to the toxicity or antioxidant properties of some individual compounds (such as thujone and eugenol, respectively) (Cuppett and Hall, 1998; Millet et al., 1981), to assess the effects of the complex mixture of EO as it reaches the consumer remains the best way to predict and prevent possible deleterious effects of its use.

The present study aims to evaluate the toxic versus antioxidant protective activities of the EO from *S. officinalis* on freshly isolated rat hepatocytes. As specific objectives the experiments had in view (a) to test whether the use of the *S. officinalis* EO of known composition has any adverse effects on the liver, and (b) test for any antioxidant effects of sage EO on liver cells challenged with an oxidizing agent (*tert*-butyl hydroperoxide – *t*-BHP) and compare it to the effects of the reference antioxidant quercetin.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. The following reagents were obtained from Sigma (St. Louis, MO, USA): collagenase (grade IV), *tert*-butyl hydroperoxide (*t*-BHP), bovine serum albumin, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]), reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (EC 1.6.4.2.), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), β-nicotinamide adenine dinucleotide reduced form (β-NADH), quercetin and 2-thiobarbituric acid (TBA).

2.2. Plant material and isolation of the essential oil

The EO used in this study was obtained by hydrodistillation of 226 g of air-dried vegetative aerial parts of *S. officinalis* plants cultivated in an experimental farm located in Arouca, Portugal, and collected on April, 2001. The absolute quantification of each compound was tentatively achieved by GC using 5α-cholestane as an internal standard. For this purpose, sub-samples of 10 g from the same plant material were sub-mitted to hydrodistillation in a Clevenger type apparatus over 1 h, using volumes of 1.0 ml of *n*-hexane, contain-ning 5α-cholestane (1mg/ml), for retention of the hydrodistillate components.

2.3. Preparation of sage tea and isolation of the essential oil constituents

Considering that sage is traditionally used as a tea, an infusion of sage was prepared by pouring 150 ml of boiling water onto 2 g of the same dried plant material and allowing to steep for 5 min. The EO constituents of the tea were extracted, at room temperature, with 5 ml of *n*-pentane containing 5α-cholestane (1mg/ml).

2.4. Analysis conditions of essential oils and procedures

The samples of the EOs and EO constituents from tea containing the internal standard 5α-cholestane were analyzed by GC and GC-MS. GC analyses were performed using a Perkin-Elmer Autosystem gas chromatograph equipped with a fused silica DB5 column (30 m long × 0.25 mm i.d., 0.25 μm film thickness composed by 5% phenyl methylpolysiloxane, J & W Scientific). Injections were performed in a split/spliless injector with the splitter opened at the 1:13 split ratio under a column head pressure of 12.5 psi and H_2 as carrier gas at a flow rate of 1.49 ml/min. Oven temperature was programmed from 60 to 285 ºC at 3 ºC min^{-1}. Injector and detector temperatures were 300 and 320ºC, respectively. The same column and conditions were used in the analysis performed with the GC-MS with the exception of the carrier gas which was helium instead of H_2. The GC-MS consisted of a Perkin-Elmer 8500 gas chromatograph equipped with a fused silica DB5 column, the same as that for GC, connected to a Finnigan MAT ion trap detector (ITD; software version 4.1) operating in EI mode at 70 eV. Identification of the chromatogram peaks as well as quantification of the compounds was performed following the methodology previously described (Santos-Gomes and Fernandes-Ferreira, 2001).

2.5. Animals

Adult male Wistar rats, weighing 200-250 g, were used as hepatocyte donors. The rats were kept in polyethylene cages lined with wood shavings, with wire mesh top and acclimated to ambient temperature (20 ± 2 ºC) and humidity and natural light/dark cycle. The animals had free access to standard rat chow and tap drinking water, and were kept in our facilities for at least 2 weeks prior to use.

2.6. Hepatocyte isolation and incubation

Hepatocyte isolation was performed between 10.00 am and 11.00 am by collagenase perfusion of the liver as previously described (Moldeus et al., 1978) with some modifications. In brief, the liver was perfused in situ via the portal vein with a calcium-free Hank's solution with 0.67% albumin and 12.5 mM HEPES (pH 7.4) at 37 ºC at a flow rate of 10 ml/min, then with a Hank's solution with 12.5 mM HEPES containing 0.025 % collagenase and 0.44% (w/v) $CaCl_2$ at the same flow rate and temperature for 10 min. The cells were dispersed in Ca^{2+}-free Hank's solution with 0.67% albumin and 12.5 mM

HEPES and washed by low speed centrifugation (50×*g*) for 2 min. The cells were then ressuspended in Krebs-Henseleit solution with 12.5 mM HEPES and washed two more times. The hepatocytes viability was >80% as estimated by trypan blue exclusion.

Incubations were performed at 37 ºC in suspensions of 10^6 viable cells per milliliter in Krebs-Henseleit solution with 12.5 mM HEPES (pH 7.4) gassed with carbogen (95% O_2 and 5% CO_2). The hepatocytes were always pre-incubated for 60 min at 37 ºC before the beginning of the experiments. Both toxic and protective effects of *S. officinalis* EO were tested on hepatocyte suspensions. In toxicity experiments the cells were incubated with 10 µl/ml of EO dissolved in DMSO at final concentrations of 0, 2, 20, 200 and 2000 nl/ml cell suspension for 30 min. *tert*-Butyl hydroperoxide (*t*-BHP) 0.75 mM was used as a positive control for cell damage. In hepatoprotective experiments 10 µl/ml of EO dissolved in DMSO at final concentrations of 0, 2, 20, 200 and 2000 nl/ml were added to the cell suspensions 5 min before the addition of *t*-BHP 0.75 mM and allowed to incubate simultaneously for 30 minutes. The reference antioxidant quercetin was used as positive control in the protective experiments. The cells were incubated with 10 µl/ml of quercetin dissolved in DMSO at final concentrations of 0, 1.6, 12.5, 50 and 200 µM for 5 min before addition of *t*-BHP 0.75 mM for 30 min. At the end of experiments, aliquots of cell suspensions were taken for measurement of lactate dehydrogenase (LDH) leakage, malondialdehyde, GSH and GSSG contents.

2.7. Analytical procedures

Cell death was estimated by quantification of LDH activity in the incubation medium versus total LDH activity in the samples (the results are shown as percentages of control cell viability). The enzyme activity was measured at room temperature by quantification of NADH by continuous spectrophotometry (at 340 nm) on a plate reader (Spectra Max 340pc, Molecular Devices).

The extent of lipid peroxidation was determined indirectly by the malondialdehyde formation after the breakdown of polyunsaturated fatty acids, measured by the thiobarbituric acid reactive substances (TBARS) assay at 535 nm as described previously (Fernandes et al., 1995).

The glutathione content of cell suspensions was determined by the DTNB-GSSG reductase recycling assay as described in Anderson (1985), with some modifications. Briefly, 200 µl of cell suspension was added to 200 µl of 10% (w/v) 5-sulfosalicylic acid for protein precipitation and centrifuged 2 min at 12,000 rpm. Supernatant aliquots were taken out for measurement of total glutathione (GSx) following the DTNB oxidation at 415 nm and compared with a standard curve. The final concentrations of the assay reagents were 0.6 mM DTNB, 0.21 mM NADPH and 2 U/ml glutathione reductase. For the GSSG determination, 100 µl of supernatant was derivatized with 2 µl of 2-vinylpyridine and 10 µl of 50% (v/v) ethanolamide and mixed continuously for 60 min. GSSG was then measured as described above for total glutathione. The GSH content was calculated by subtracting GSSG content from the total glutathione content.

2.8. Statistical analysis

Data are expressed as means ± SEM (n = 5) (cells obtained from 5 different rats). Significant differences between control and *t*-BHP treated cells were determined by Student's *t*-test. Significant differences within EO series (EO toxic and protective evaluation) were determined by one-way ANOVA followed by Dunnett's test. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Essential oil and essential oil constituents of tea from S. officinalis leaves

The yield of the EO obtained by hydrodistillation of air-dried sage aerial parts was 13.3 µl per gram of biomass dry weight, corresponding to 1.2% (w/w). The composition of the *S. officinalis* EO used in the experiments here reported is shown in Table 1, where compounds are listed according to their Kovats retention indexes. The EO includes around 60 compounds most of which were identified (Santos-Gomes and Fernandes-Ferreira, 2001). Around 88% of the compounds can be distributed into three terpene groups: oxygen-containing monoterpenes (47.7%), sesquiterpene hydrocarbons (24.5%), and monoterpene hydrocarbons (15.9%). The major compounds were *cis*-thujone (17.4%), α-humulene (13.3%), 1,8-cineole (12.7%), *E*-caryophyllene (8.5%) and borneol (8.3%).

In the preparation of the tea only 2.99% of total EO content was extracted from the biomass and found in the water giving a final concentration of 4.81 µg of EO per ml of tea (Table 1). However, the presence of the individual essential oil constituents in tea was not proportional to their specific amount in the oil extracted by hydrodistillation. From the approximate 60 compounds detected in the EO of *S. officinalis* only 25 were found as EO constituents of the respective tea (Table 1). Oxygen-containing monoterpenes corresponded to 86% of the tea EO constituents. The sum of *cis*-thujone and camphor concentrations (1.68 and 0.51 $\mu g.ml^{-1}$, respectively) corresponded to almost 45% of all the constituents (Table 1). With respect to the composition

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

Compound	RI[a]	EO from *S. officinalis*		EO constituents from *S. 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 □-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

Table 1 (*continued*)

Compound	RI[a]	EO from *S. officinalis*		EO constituents from *S. officinalis* tea		
		%	μg/g dry wt	%	μg/g dry wt	μg/ml of tea
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).

of EO present in tea it is not surprising that oxygen-containing monoterpenes, such as *cis*-thujone and camphor, are proportionately more abundant in tea than other EO constituents in view of the higher solubility in water of this group of compounds relatively to the other ones.

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

3.2. EO toxicity experiments

EO toxicity was tested in hepatocyte suspensions exposed to the EO (0-2000 nl/ml) and compared to that of *t*-BHP (0.75 mM). Cell viability measured by LDH leakage of rat hepatocytes (Fig. 1) did not change significantly after 30 min incubation with 0 – 200 nl of EO per ml of cell suspension. However, 2000 nl/ml (0.2% (v/v)) caused a significant decrease in cell viability. Lipid peroxidation (Table 2) did not change after the incubations with different concentrations of EO. Total glutathione (GSx) (Table 2) was significantly lowered by incubation with the highest oil concentration tested (2000 nl/ml) but not by any of the other concentrations.

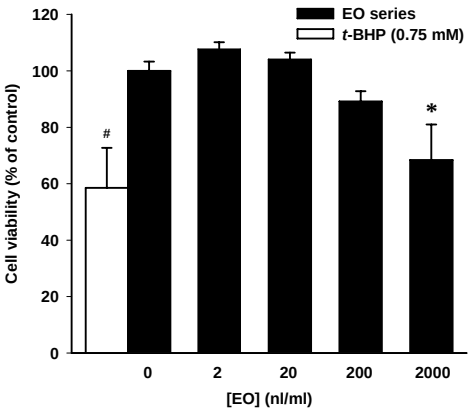

Fig. 1. Effect of EO of *S. officinalis* on cell viability (measured by % of LDH leakage) in hepatocyte suspensions after 30 min of exposure (means ± SEM). n=5; # $P \leq 0.05$ between control and *t*-BHP treated cells; * $P \leq 0.05$ when compared with control cells.

This decrease in GSx was accompanied by a decrease in GSH (Fig. 2), where incubations with 200 nl EO/ml also produced a significant decrease in GSH when compared to the control. There were no significant changes in GSSG content (Table 2) produced by incubation of the cells with the EO.

Table 2
Effect of 30 min exposure of hepatocyte suspensions to *S. officinalis* EO on GSSG and GSx content and on lipid peroxidation expressed as TBARS formation (means ± SEM)

Parameter	Sample					
	t-BHP (0.75 mM)	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

n = 5.

$P \leq 0.05$ between control and *t*-BHP treated cells.

* $P \leq 0.05$ when compared with control cells.

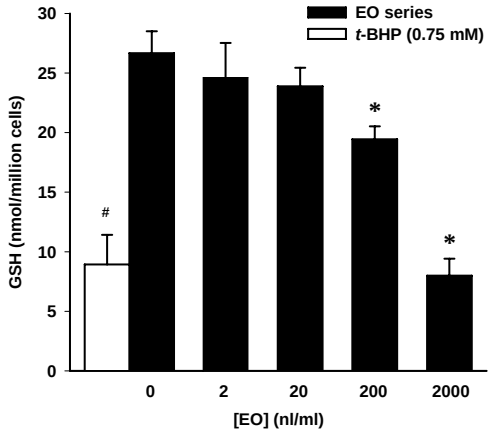

Fig. 2. Effect of EO of *S. officinalis* on hepatocyte suspensions GSH content after 30 min of exposure (means ± SEM). n=5; # $P \leq 0.05$ between control and *t*-BHP treated cells; * $P \leq 0.05$ when compared with control cells.

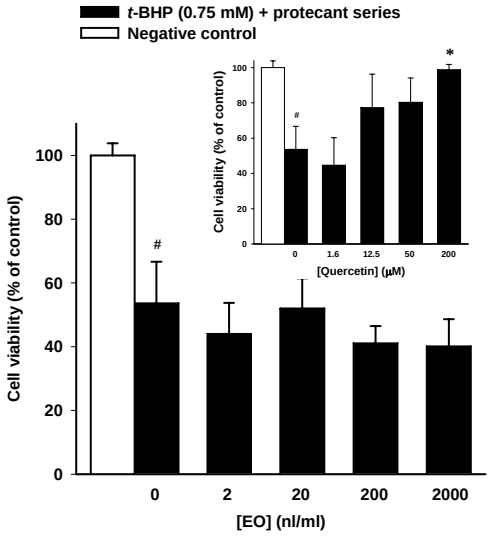

Fig. 3. Effect of EO of *S. officinalis* and quercetin (inset) on cell viability (measured by % of LDH leakage) of hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min (means ± SEM). n=5; # $P \leq 0.05$ between control and cells treated only with *t*-BHP; * $P \leq 0.05$ when compared with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

The incubations of hepatocyte suspensions with the positive control 0.75 mM *t*-BHP for 30 min resulted in significant decreases in cell viability (Fig. 1), GSH (Fig. 2) as well as GSx content and an increase in lipid peroxidation (Table 2). We also observed a perceptible increase of GSSG content (P = 0.0556) with this toxicant.

3.3. EO protection experiments

Hepatoprotection of the EO was tested in hepatocyte suspensions exposed simultaneously to the EO (0-2000 nl/ml) and *t*-BHP (0.75 mM) and the protective effects of the oil compared to those of quercetin.

The addition of 0.75 mM *t*-BHP to hepatocytes resulted in significant cell death after 30 min was evident from LDH leakage (Fig 3). EO in the concentrations of 2-2000 nl/ml did not reduce cell mortality. Quercetin, on

Table 3
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)

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

n = 5.
# $P \leq 0.05$ between control and cells treated only with *t*-BHP.

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)

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

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.

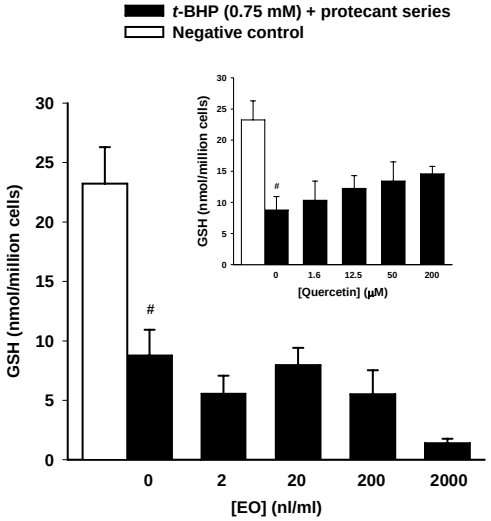

Fig. 4. Effect of EO of *S. officinalis* and quercetin (inset) on GSH content of hepatocyte suspensions damaged by *t*-BHP (0.75 mM) for 30 min (means ± SEM). n=5; # $P \leq 0.05$ between control and cells treated only with *t*-BHP.

the other hand, significantly reduced LDH leakage at 200 μM. Lipid peroxidation induced by *t*-BHP (Table 4) was also significantly reduced by co-incubation with quercetin whereas the EO had no protective effects at any of the concentrations tested (Table 3). Addition of *t*-BHP significantly reduced cell GSH content but no protective effects on the glutathione balance could be detected for quercetin or the EO (Fig 4). Co-incubation of *t*-BHP with 2000 nl/ml EO seems to further decrease the GSH levels in the cells.

4. Discussion

The liver is very active in metabolizing foreign compounds. The biotransformation of xenobiotics by the liver involves chemical modifications that globally increase their water solubility thereby facilitating their elimination from the organism in bile and urine. These reactions may, however, result in the formation of compounds of greater toxicity. Although the large biotransforming ability of the liver allows efficient elimination of the most toxic compounds, it also makes hepatocytes a vulnerable target for toxic effects. Damage to the cell's constituents such as lipid peroxidation may occur. GSH plays an important role in hepatocytes defence (Kedderis, 1996; Reed, 1990). The most common pathway of glutathione depletion in xenobiotic toxicity is excessive consumption of GSH without recovery (Castell et al., 1997). Severe GSH depletion leaves cells more vulnerable to oxidative damage which causes progressive deterioration of macromolecules, cell structure and may lead to cell death. GSH level could therefore determine the cytotoxicity of a xenobiotic and its ultimate effects on cell survival.

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

The toxicity induced by *t*-BHP is well-known and is well characterized (Fernandes et al., 1995; Joyeux et al., 1990; Tseng et al., 1997) and our results are in agreement with these published reports. Our data also show that when compared with *t*-BHP-induced toxicity, which is the result of GSx and GSH depletion, GSSG increase and lipid peroxidation, cell death induced by the EO at 2000 nl/ml on liver cells does not seem to be mediated by lipid peroxidation and GSH oxidation with concomitant GSSG increase. The EO toxicity may be due to the presence of reactive compounds and/or reactive metabolites generated by metabolism of EO. The depleted glutathione was possibly recruited to act as a nucleophilic scavenger of some compounds and/or their metabolites with electrophilic properties. The cellular death indicated by LDH leakage could also be due to a solvent effect of the EO, whereby the hydrophobic compounds exert a direct effect on cellular membranes disturbing the physico-chemical properties of the bilayer culminating in the disruption of cellular volume and cell death (Berry et al., 1991; Kedderis, 1996).

The hepatoprotective effects of the EO were tested by co-incubation with the toxicant *t*-BHP and compared with those of the reference antioxidant quercetin. Quercetin shows concentration-dependent protective effects against *t*-BHP-induced toxicity in almost all parameters measured. Quercetin was preferred to other known antioxidants as a positive control because of its plant origin, DMSO solubility and for having a well characterised protective effect against *t*-BHP-induced

toxicity (Joyeux et al., 1990). In contrast, the presence of sage EO did not reduce hepatocyte death due to *t*-BHP toxicity. The co-incubation with the EO resulted in a tendency for a further decrease in GSH and increased lipid peroxidation. In spite of the reputation of some plant EOs as antioxidants and hepatoprotectants in animal models (Teissedre and Waterhouse, 2000 and references therein), our results do not show antioxidant protective effects of *S. officinalis* EO against *t*-BHP-induced liver toxicity.

The EO obtained by hydrodistillation used in this study was somewhat lower than that obtained from fresh leaves of the same sage cultivar (Santos-Gomes and Fernandes-Ferreira, 2001). The composition of sage EO extracts varies and reflects extraction conditions as well as time of harvest and the plant's growth conditions such as climate, soil composition and light intensity (Santos-Gomes and Fernandes-Ferreira, 2001). In April, the composition of the EO from *S. officinalis* plants grown in Portugal (Santos-Gomes and Fernandes-Ferreira, 2001) and in Israel (Putievsky et al., 1986) are characterrised by the lowest levels of *cis*-thujone and camphor. The levels of these two compounds in the EO used (Table 1), were slightly lower than the corresponding minimum values from the profile defined by the standard ISO 9909 (1999) for the EO of *S. officinalis* - *cis*-thujone (18-43%), camphor (4.5-24.5%) (Bruneton, 1999). It is therefore important that detailed information is available on the material being tested or used in the food industry.

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

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Reprinted from *Toxicology In Vitro*, 18, Lima CF, Carvalho F, Fernandes E, Bastos M, Santos-Gomes P, Fernandes-Ferreira M, Pereira-Wilson C, Evaluation of toxic/protective effects of the essential oil of *Salvia officinalis* on freshly isolated rat hepatocytes, 457-465, Copyright (2004), with permission from Elsevier.

Chapter 3

Sage Tea: Does it Really Have Antioxidant Effects on the Liver?

3.1. Chapter overview

The antioxidant activity of essential oils and extracts obtained from aromatic and medicinal plants by extraction with organic solvents has been extensively studied with several *in vitro* techniques. However, little is known about the antioxidant effects of polar extracts of these plants prepared as infusions or decoctions. Triantaphyllou *et al.* (2001) studied different water extracts from herbs of the *Lamiaceae* family, which included *Salvia fruticosa* but not *Salvia officinalis*. They concluded that these extracts were rich in polyphenols and had a remarkable capacity in retarding lipid oxidation[1]. Matsingou *et al.* (2003) also tested a *S. fruticosa* water extract and they concluded that this herb possesses interesting antioxidant effects, mainly due to polyphenolic and nonpolyphenolic (such as carnosic acid) substances present in the extract[2].

Salvia officinalis is extensively used as a medicinal plant and several bioactivities for different types of extracts were already experimentally shown, including antioxidant activities. However, the most common form of consumption of this plant, the sage tea (prepared as an infusion), has received little attention. A question arises: does sage tea consumption really confer antioxidant protection *in vivo* or is this an utopia? To test whether sage tea has antioxidant potential *in vivo*, we studied the effects of sage tea drinking in mice and rats. Since the liver is the organ first passed by sage tea constituents and its metabolites after the gastrointestinal absorption, we focused the studies on liver cells. Moreover, the liver is a highly metabolic organ, which underscores the importance to perform the studies in these cells, since they are a good tool to look for potential toxic effects of chemicals. In an attempt to identify tea constituents associated with the observed bioactivities, phenolic and volatile compounds present in the sage tea were analysed.

Since sage tea was given to the animals replacing all the water they consumed, in the first set of experiments performed with mice, the potential toxic effects of sage tea drinking to the liver were evaluated. As shown by the plasma transaminase levels, the replacement of water with sage tea for 14 days did not induce liver toxicity. On the other hand, a significant increase in about 10% of liver GST and GR activities was observed in mice of sage drinking groups, which indicate already some antioxidant capacity of sage tea *in vivo*.

Then, in the second set of experiments, the potential antioxidant effects of sage tea were also tested in rats in experiments that comprised *in vivo* and *in vitro* studies. After 14 days of

[1] Triantaphyllou K, Blekas G & Boskou D, 2001. Antioxidative properties of water extracts obtained from herbs of the species *Lamiaceae*. *International Journal of Food Sciences and Nutrition* **52**, 313-317.

[2] Matsingou TC, Petrakis N, Kapsokefalou M & Salifoglou A, 2003. Antioxidant activity of organic extracts from aqueous infusions of sage. *Journal of Agricultural and Food Chemistry* **51,** 6696-6701.

sage tea drinking, rat hepatocytes were isolated for the establishment of primary cultures. Immediately after hepatocyte isolation, GST activity was significantly increased (24%) in hepatocytes isolated from sage tea drinking rats when compared with controls. After 4 hours in culture, GSH content of the hepatocytes increased. The increase in GSH levels was significantly higher in hepatocytes isolated from sage tea drinking rats. Also, GST activity remained itself significantly higher in hepatocytes from sage tea than from water drinking rats. These results showed an improvement of the antioxidant status of rat hepatocytes from sage tea drinking animals. Finally, when the hepatocyte cultures were exposed to an oxidant agent, *t*-BHP, although cell death was not prevented, some protection against *t*-BHP-induced lipid peroxidation and GSH depletion was conferred by sage tea drinking.

Therefore, this study showed that *S. officinalis* tea positively affects the antioxidant status of the liver and may have hepatoprotective potential that justifies further studies. The improvement of the antioxidant response by sage tea could possibly be beneficial in many liver diseases, where it is known that oxidative stress plays an important role.

The improvement of GST and GR activities and GSH levels afforded by sage tea may tentatively be attributed to the flavonoids and certain compounds present in the EO fraction. It is known that these compounds can induce phase II detoxifying enzymes such as GST. On the other hand, the higher GSH levels obtained in hepatocytes isolated from sage tea drinking rats, after 4 hours in culture, can be the result of an enhancement of *de novo* glutathione synthesis. For example, an up-regulation of both phase II enzymes and the key enzyme involved in *de novo* glutathione synthesis, by polyphenols, has already been observed[1]. So, although not studied here, there is the possibility that, also in this case, the interaction of some compounds present in sage tea with, for example, the antioxidant response element (ARE) could result in a higher GST activity and increased GSH recovery.

3.2. Methods

The following experimental protocols (see Appendix) were used in this chapter:

- Biological Models – Mice were housed as described in P3 and used to see effects of sage tea drinking in the liver.
- P1: Biological Models – Rat Hepatocyte Isolation and Use
- P4: Cell Viability Assays – LDH Leakage

[1] Mulcahy RT, Wartman MA, Bailey HH & Gipp JJ, 1997. Constitutive and *beta*-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *The Journal of Biological Chemistry* **272**, 7445-7454.

- P6: Cell Viability Assays – Trypan Blue Staining
- P7: Metabolite Measurements – TBARS (Lipid Peroxidation)
- P8: Metabolite Measurements – Glutathione Levels
- P10: Metabolite Measurements – Total Protein
- P11: Enzyme Activities – GR
- P13: Enzyme Activities – GST
- P16: Enzyme Activities – ALT and AST

3.3. Publication

This chapter comprises the following publication:

Lima CF, Andrade PB, Seabra RM, Fernandes-Ferreira M & Pereira-Wilson C, 2005. The drinking of a *Salvia officinalis* infusion improves liver antioxidant status in mice and rats. *Journal of Ethnopharmacology* **97(2)**, 383-389.

The drinking of a *Salvia officinalis* infusion improves liver antioxidant status in mice and rats

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Abstract

In this study, we evaluate the biosafety and bioactivity (antioxidant potential) of a traditional water infusion (tea) of common sage (*Salvia officinalis* L.) in vivo in mice and rats by quantification of plasma transaminase activities and liver glutathione-*S*-transferase (GST) and glutathione reductase (GR) enzyme activities. The replacement of water by sage tea for 14 days in the diet of rodents did not affect the body weight and food consumption and did not induce liver toxicity. On the other hand, a significant increase of liver GST activity was observed in rats (24%) and mice (10%) of sage drinking groups. The antioxidant potential of sage tea drinking was also studied in vitro in a model using rat hepatocytes in primary culture. The replacement of drinking water with sage tea in the rats used as hepatocyte donors resulted in an improvement of the antioxidant status of rat hepatocytes in primary culture, namely a significant increase in GSH content and GST activity after 4 h of culture. When these hepatocyte cultures were exposed to 0.75 or 1mM of *tert*-butyl hydroperoxide (*t*-BHP) for 1 h, some protection against lipid peroxidation and GSH depletion was conferred by sage tea drinking. However, the cell death induced by *t*-BHP as shown by lactate dehydrogenase (LDH) leakage was not different from that observed in cultures from control animals. This study indicates that the compounds present in this sage preparation contain interesting bioactivities, which improve the liver antioxidant potential.

Keywords: *Salvia officinalis* L. infusion; Glutathione status; Antioxidant effects; Rat hepatocytes; Mice; *tert*-Butyl hydroperoxide

1. Introduction

The oxidative damage of biological molecules is an important event in the development of a variety of human disorders that result from overwhelming the biological defense system against oxidative stress, drugs and carcinogens. The intake in the human diet of antioxidant compounds, or compounds that ameliorate or enhance the biological antioxidant mechanisms, can prevent and in some cases help in the treatment of some oxidative-related disorders and carcinogenic events (Havsteen, 2002).

Natural plant products have been used empirically for this purpose since ancient times and a tendency is emerging today for their increased use. *Salvia officinalis* L. (Lamiaceae) is a common aromatic and medicinal plant native from mediterranean countries that is in widespread use.

Experimental evidence already exists for a variety of bioactivities for different types of extracts of *Salvia officinalis* such as antioxidant, anti-inflammatory, hypoglycaemic and anti-mutagenic activities (Cuvelier et al., 1994; Wang et al., 1998; Hohmann et al., 1999; Baricevic and Bartol, 2000; Baricevic et al., 2001; Zupko et al., 2001; Alarcon-Aguilar et al., 2002). However, the properties of sage infusion (hereafter, referred to as tea), the most common form of consumption of this plant, have received little attention.

Many bioactivities have been researched and detected in tea and in infusions (or water extracts) of other plants. Among them, the phenolic content of different plants have been shown to have antioxidant activities and the capacity

to modulate xenobiotic metabolizing enzymes involved in drug and carcinogen activation and detoxification (Ferguson, 2001; Triantaphyllou et al., 2001). Several studies showed that black and green tea (*Camellia sinensis*) enhance phase II enzymes (Khan et al., 1992; Yu et al., 1997; Bu-Abbas et al., 1998). A water-soluble extract of rosemary also induced both phase I and phase II enzymes (Debersac et al., 2001a,b). However, the use of natural products may also result in toxic effects, which underscore the need to understand the biological effects of natural compounds. Toxic effects to the liver, the main xenobiotic metabolizing organ, are particularly relevant.

In the present study, we evaluate the biosafety and bioactivities of sage tea in vivo with mice and rats and in vitro using rat hepatocytes in primary culture. Toxic effects to the liver of sage tea drinking are tested in vivo on mice monitoring the plasma transaminase activities. The liver glutathione content and glutathione reductase (GR) and glutathione-*S*-transferase (GST) activities in the mouse livers and freshly isolated rat hepatocytes were also evaluated. In addition, primary cultures of hepatocytes isolated from sage tea and water drinking rats were challenged with the oxidant *tert*-butyl hydroperoxide (t-BHP) and the antioxidant protection conferred by sage tea drinking evaluated.

2. Materials and methods

2.1. Chemicals

Collagenase (grade IV), *tert*-butyl hydroperoxide, glutathione reductase (EC 1.6.4.2.), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), William's Medium E (WME) and Bradford reagent were purchased from Sigma (St. Louis, MO, USA). *L*-Lactate dehydrogenase (EC 1.1.1.27) and *L*-malate dehydrogenase (EC 1.1.1.37) were purchased from Roche (Germany). All others reagents were of analytical grade.

2.2. Plant material, preparation of sage tea and analysis of its phenolic and volatile compounds

Salvia officinalis L. plants were cultivated in an experimental farm located in Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were lyophilized and kept a -20ºC. Considering that sage is traditionally used as a tea, an infusion of sage was routinely prepared by pouring 150 ml of boiling water onto 2 g of the dried plant material and allowing to steep for 5 min. This produced an infusion of 3.5 ± 0.1 mg (mean ± S.E.M., n = 6) of extract dry weight per ml of infusion (0.35%, w/v) and a yield of 26.3% (w/w) in terms of initial crude plant material dry weight.

Phenolic compounds were analysed by HPLC/DAD. Freeze-dried (Labconco Freeze Dry System) extract (0.01 g) was redissolved in 1ml of ultrapure Milli Q water and aliquots of 20 μl were injected in an HPLC/DAD system. Separation and identification of phenolic compounds by HPLC/DAD were performed as previously described (Santos-Gomes et al., 2002). The volatile constituents of the tea (150 ml) were extracted, at room temperature, with 5 ml of *n*-pentane containing 5α-cholestane (1 mg/ml). The volatile compounds were then identified by GC and GC-MS as previously described (Lima et al., 2004).

2.3. Animals

Female Balb/c mice (8–10 weeks) and male Wistar rats (150–200 g) were purchased from Charles River Laboratories (Spain) and acclimated to our laboratory animal facilities for at least 1 week before the start of the experiments. During this period, the animals were maintained on a natural light/dark cycle at 20 ± 2ºC and given food and tap water ad libitum. The animals used in the two experiments were kept and handled in accordance to our University regulations. In experiment 1, mice were used to evaluate in vivo the liver toxicity of sage tea drinking for 14 days and changes in the liver glutathione levels as well as in the activities of glutathione-related enzymes. In experiment 2, rats from two different drinking groups (water and sage tea) were used for hepatocyte isolation for establishment of primary cultures. The primary cultures of hepatocytes isolated from sage tea and water drinking rats were challenged with the oxidant *tert*-butyl hydroperoxide and the antioxidant protection conferred by sage tea drinking evaluated.

2.4. Experiment 1

Ten female Balb/c mice were randomly divided into two groups, given food ad libitum and either water (tap) or sage tea ad libitum for 14 days (beverage was renewed daily). On day 15, the animals were sacrificed by cervical dislocation and blood samples collected for measurement of plasma transaminase activities (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)). The livers were also collected, frozen in liquid nitrogen and kept at -80ºC for later analysis of glutathione content and activities of glutathione reductase and glutathione-*S*-transferase.

2.5. Experiment 2

Eight male Wistar rats were randomly divided into two groups and given food ad libitum with either water (tap) or sage tea ad libitum for 14 days (beverage was renewed daily). On day 15, hepatocytes were isolated and used to establish primary cultures.

Hepatocyte isolation was performed between 10:00 a.m. and 11:00 a.m. by collagenase perfusion as previously described by Moldeus et al. (1978) with some modifications (Lima et al., 2004). Cell viability was >85% as estimated by the trypan blue exclusion test. Aliquots of the cell suspensions were kept a -80ºC for measurement of GR and GST activities and quantification of glutathione levels at the start

of the in vitro experiments, i.e., time zero of primary cultures. Then, cells were suspended in William's medium E supplemented with 10% fetal bovine serum (FBS), 10^{-9} M insulin and 10^{-9} M dexamethasone and seeded onto six-well culture plates at a density of 1×10^6 cells/well. The culture plates were incubated at 37ºC in a humidified incubator gassed with 5% CO_2/95% air.

Three hours after plating, the culture medium was replaced with WME supplemented with 10% FBS and *t*-BHP 0, 0.75 or 1mM for 1 h to induce cytotoxicity (Rush et al., 1985). To assess the protection conferred by sage tea drinking culture medium and cells were collected and the activities of lactate dehydrogenase (LDH), GR and GST determined. The levels of malondialdehyde (MDA) and glutathione were also measured.

2.6. Biochemical analysis

2.6.1. Enzyme activities

2.6.1.1. ALT and AST. The alanine aminotransferase and aspartate aminotransferase activities were measured spectrophotometrically in plasma of mice following NADH oxidation (at 30ºC) at 340 nm on a plate reader (Spectra Max 340pc, Molecular Devices). For ALT activity, the reaction mixture consisted of 200 mM *L*-alanine, 25 µM pyridoxalphosphate, 0.12 mM NADH, 12 units/ml *L*-lactate dehydrogenase and 10.5 mM alpha-ketoglutarate in 50 mM imidazole (pH 7.4). For AST activity, the reaction mixture consisted of 40 mM aspartate, 25 µM pyridoxalphosphate, 0.12 mM NADH, 8 units/ml *L*-malate dehydrogenase and 7 mM alpha-ketoglutarate in 50 mM imidazole (pH 7.4). The activities are expressed as µmol of substrate oxidized per minute per liter of plasma (U/L).

2.6.1.2. GR and GST. For measurement of mice liver glutathione reductase and glutathione-*S*-transferase activities, the livers were homogenised 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 10 µg/ml and glycerol 50%, v/v) and centrifuged at 10,000 × *g* for 10 min at 4ºC and the supernatant collected. In the case of the cells collected after exposure to *t*-BHP (primary cultures of hepatocytes) as well as the time zero hepatocyte aliquots, the samples were homogenised by sonication in phosphate/glycerol buffer pH 7.4, centrifuged at 10,000 × *g* for 10 min at 4ºC and the supernatant collected.

The GR activity was measured spectrophotometrically at 340 nm following NADPH oxidation at 30ºC. The reaction mixture consisted of 3 mM GSSG, 2.5 mM EDTA and 0.12 mM NADPH in 50mM Hepes (pH 7.4). The activity is expressed as nmol of NADPH oxidized per minute per milligram protein (mU/mg).

The GST activity was measured spectrophotometrically at 340 nm following the formation of GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) at 30ºC. The reaction mixture consisted of 1mM GSH and 1mM CDNB (dissolved in ethanol) in 50 mM Hepes (pH 7.4). The activity was calculated using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ and expressed as nmol of conjugate per minute per milligram protein (mU/mg).

2.6.1.3. LDH. To assess the extent of cell death caused by *t*-BHP, the determination of lactate dehydrogenase activity in the culture medium was used as indicator of plasma membrane integrity of hepatocytes. The enzyme activity was measured at 30ºC by quantification of NADH (0.28 mM) consumption by continuous spectrophotometry (at 340 nm) on a plate reader using pyruvate (0.32 mM) as substrate in 50 mM phosphate buffer (pH 7.4). The results are expressed as µmol of substrate oxidized per minute per milligram protein (U/mg).

2.6.2. Lipid peroxidation

The extent of hepatocyte lipid peroxidation was estimated by the levels of malondialdehyde. The thiobarbituric acid reactive substances (TBARS) assay at 535 nm was used as described previously (Fernandes et al., 1995) but with some modifications for cultured hepatocytes. Briefly, 360 µl of culture medium was precipitated with 60 µl of 50% trichloroacetic acid. After centrifugation, 300 µl of the supernatant were added to an equal volume of 1% thiobarbituric acid and the mixture was heated for 10 min in a boiling water bath, allowed to cool and the absorbance measured at 535 nm. The results are expressed as nmol MDA/mg of protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

2.6.3. Glutathione content

The glutathione content of mice livers, time zero hepatocyte aliquots and 4 h cultured rat hepatocytes were determined by the DTNB-GSSG reductase recycling assay as previously described (Anderson, 1985), with some modifications (Lima et al., 2004). The results are expressed as nmol GSH per milligram of protein.

2.6.4. Protein

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

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. The comparison between the means of treatment (sage tea) and control group was performed using Student's *t*-test. For primary cultures of hepatocytes a two-way ANOVA followed by the Bonferroni post-hoc test were employed to compare the in vivo treatments (water versus sage tea) and in vitro treatments (*t*-BHP concentrations). *P*-values ≤ 0.05 were considered statistically significant.

Table 1
Phenolic and volatile compounds of sage tea

Component	%	μg/ml
Water	99.65	
Phenolic acids		
Rosmarinic acid	0.04	362.0
Flavonoids		163.7
Luteolin 7-glucoside	0.01	115.3
Others luteolin glycosides (3)	< 0.01	48.5
Volatile components	<< 0.01	4.8
1,8-Cineole		0.9
cis-Thujone [= (-)-thujone]		1.7
trans-Thujone [=(+)-thujone]		0.3
Camphor		0.5
Borneol		0.7
Others (20)		0.7
Unknown	0.30	2972.0

3. Results

3.1. Phenolic and volatile compounds in sage tea

The infusion is composed of the phenolic compounds rosmarinic acid and four luteolin glycosides - luteolin-7-glucoside being the most representative flavone (Table 1), which constitutes 0.05% of total wet weight. In this sage infusion, we also identified 25 volatile compounds with 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor and borneol being the most representative (85% of total volatile fraction). The most representative volatile compounds and their quantification are presented in Table 1.

3.2. Experiment 1

Water replacement with sage tea for 14 days did not affect food consumption and body weight in mice during the experiment (data not shown). However, drinking was slightly different between the two groups - water drinking group: 11.0 ± 0.4 ml/day/100 g; sage tea drinking group: 10.0 ± 0.5 ml/day/100 g of body weight. Plasma ALT and AST activities (Table 2) were not different between water and sage drinking animals. Also the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in the mice livers were not different between the two groups (Table 2).

Table 2
Effect of sage tea on plasma transaminase activities, liver glutathione levels and liver glutathione-related enzyme activities after 14 days of treatment in mice

Parameter	In vivo beverage	
	Water	Sage tea
ALT (U/L)	36 ± 6	30 ± 6
AST (U/L)	90 ± 11	89 ± 11
GR (mU/mg)	13.4 ± 0.1	14.7 ± 0.4 *
GST (mU/mg)	107 ± 3	119 ± 2 *
GSH (nmol/mg)	46.1 ± 0.9	47.4 ± 1.9
GSSG (nmol GSH equiv/mg)	2.1 ± 0.1	2.0 ± 0.2

Values are means ± S.E.M., n = 5.
* $P \leq 0.05$ when compared with the respective control.

The activities of glutathione-related enzymes, GR and GST, were significantly higher (10%) in livers of sage tea drinking mice (Table 2).

3.3. Experiment 2

The replacement of drinking water with the sage tea did not affect food and drink consumption as well as body weight of rats (data not shown).

Immediately after collagenase isolation glutathione levels of rat hepatocytes were similar in the two groups (Table 3), water and sage tea drinking, and smaller than

Table 3
Effect of sage tea consumption (in vivo for 14 days) on *t*-BHP-induced toxicity in primary culture of rat hepatocytes and on liver glutathione levels and liver glutathione-related enzymes activities of rat hepatocytes after collagenase isolation

Parameter	In vivo beverage	Rat hepatocytes (after isolation)	Primary cultures of rat hepatocytes - *t*-BHP (mM)		
			0	0.75	1
LDHextr (U/mg)	Water	-	0.06 ± 0.01	0.40 ± 0.03**	0.72 ± 0.09***
	Sage tea	-	0.09 ± 0.03	0.40 ± 0.07*	0.78 ± 0.14***
TBARS (nmol/mg)	Water	-	0.10 ± 0.06	1.89 ± 0.09***	3.38 ± 0.45***
	Sage tea	-	0.03 ± 0.02	1.30 ± 0.27*	2.62 ± 0.45***
GSH (nmol/mg)	Water	21.9 ± 1.3	38.1 ± 2.7	25.0 ± 0.6***	12.5 ± 1.2***
	Sage tea	20.4 ± 3.1	51.4 ± 3.6$^{\#\#}$	36.3 ± 1.4***$^{\#\#}$	23.3 ± 2.1***$^{\#\#}$
GSSG (nmol GSH equiv/mg)	Water	tr	0.9 ± 0.5	7.9 ± 0.5**	8.4 ± 1.2***
	Sage tea	tr	0.7 ± 0.2	9.3 ± 2.3***	9.7 ± 1.2***
GR (mU/mg)	Water	21.4 ± 1.6	22.0 ± 0.9	19.9 ± 2.2	20.4 ± 2.5
	Sage tea	21.5 ± 1.2	25.5 ± 2.9	24.0 ± 1.7	19.9 ± 0.3
GST (mU/mg)	Water	209 ± 4	168 ± 9	162 ± 12	135 ± 8
	Sage tea	260 ± 18$^{\#}$	210 ± 9$^{\#}$	184 ± 7	153 ± 4*

Values are mean ± S.E.M., n = 4 (except rat hepatocytes after isolation, n = 3). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ when compared with the respective control. $^{\#}$ $P \leq 0.05$, $^{\#\#}$ $P \leq 0.01$ and $^{\#\#\#}$ $P \leq 0.001$ between the water and sage tea in the same situation, tr: trace amounts.

those of mice. GST activity was significantly enhanced in isolated rat hepatocytes from sage tea drinking animals (Table 3) with an increase of 1.24-fold relative to the water drinking group. No differences were observed in GR activity.

There was a marked increase in GSH values from time zero hepatocyte aliquots to 4 h cultured hepatocytes (Table 3), both from water and sage tea drinking animals. However, comparing the values (*t*-BHP 0 mM) measured in the primary cultures, a significantly higher GSH content (1.35-fold) was observed (Table 3) after 4 h of culture (3 h of pre-incubation plus 1 h with 0mM of *t*-BHP) in hepatocytes of sage drinking animals. After 4 h in culture, the GST activity decreased somewhat but remained higher (1.25-fold) in the cells from sage drinking animals. The GR activity was also somewhat increased in the hepatocytes of sage drinking rats although not significantly.

Incubation of rat hepatocyte primary cultures with *t*-BHP at 0.75 or 1 mM for 1 h resulted in significant cell damage as shown by a strong increase in LDH activity in the culture medium, higher cellular lipid peroxidation and GSSG levels, as well as a significant decrease in GSH levels (Table 3). *t*-BHP did not affect GR activity and only at the concentration of 1 mM was the GST activity significantly reduced (Table 3) when compared with the respective controls.

The extent of *t*-BHP-induced lipid peroxidation was lower in cells of sage tea drinking animals. This effect was only marginally non-significant ($P = 0.051$). The GSH levels of hepatocytes challenged with *t*-BHP remained significantly higher in the cultures of sage tea drinking rats (Table 3). Following exposure to *t*-BHP the reduction of GSH in hepatocytes of the sage tea drinking group was not as dramatic as the one observed in hepatocytes from water drinking animals (Fig. 1) being significantly different at 1 mM of *t*-BHP. However, when exposed to 0.75 or 1mM of *t*-BHP no protective effect of sage tea drinking was observed in LDH leakage nor in GSSG content (Table 3).

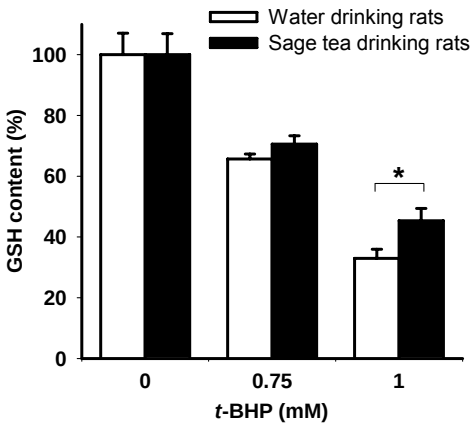

Fig. 1. Effect of sage tea consumption (in vivo for 14 days) on *t*-BHP-induced decrease in GSH content of primary hepatocyte cultures, presented as percentage from control. Absolute values presented in Table 3. Values are mean ± S.E.M., n = 4. * $P \leq 0.05$, significantly different with Student's *t*-test.

4. Discussion

The present study shows that sage tea drinking had no toxicity to the liver and no adverse effects on growth parameters neither in mice nor in rats. It also shows that sage tea drinking positively affected the antioxidant status of the liver, mainly the GST and GR activities of the mice livers and GST activity in rats.

The positive effects of sage tea drinking were also present in cultured hepatocytes. Immediately after isolation GST activity was higher in cells from sage tea drinking rats. At this point GSH levels were not different from those of control cells. After 4 h in culture, GSH content increased in both groups. However, this increase was dramatically higher in cells isolated from sage tea drinking animals indicating better recovery of this group of cells from the oxidative stress imposed by collagenase isolation.

Also following treatment with *t*-BHP, GSH content and GST activity remained significantly higher in the cells from tea drinking animals. This higher antioxidant status was probably the cause of the smaller extent of lipid peroxidation induced by *t*-BHP to these cells compared to those of water drinking animals. However, in spite of this, cell death, as indicated by LDH leakage, was not prevented in the cells of sage tea drinking animals. Although not done in this study, cell recovery after the removal of the toxic might have been higher in cells of sage tea drinking animals.

An enhancement of GST activity and other phase II enzymes due to treatment with water extracts of plants, namely *Camellia sinensis* and *Rosmarinus officinalis* has been reported (Bu-Abbas et al., 1998; Debersac et al., 2001b), and related to cancer chemoprevention (Saha and Das, 2003). In accordance with this, we also found an enhancement of GST activity in the livers of both mice and rats due to sage tea drinking for 14 days. The observed increase in liver GST activity after tea drinking was smaller in comparison with other studies, for example with the water-soluble extract of rosemary (Debersac et al., 2001a,b) and *Camellia sinensis* (Bu-Abbas et al., 1998). Apart from the differences in extract composition, this may be due to the fact that our water extract was much more diluted, only about 0.35% (w/v), than that used in the above mentioned studies.

According to the work done by Debersac et al. (2001b), where individual compounds were administered orally to rats, rosmarinic acid (also the most abundant phenolic compound present in this sage tea) could not be responsible for the observed increase in GST activity. This effect could be due to the luteolin glycosides, since induction of GST activity has been reported as the result of dietary ingestion of certain antioxidant flavonoids (Siess et al., 1996; Birt et al., 2001; Ross and Kasum, 2002; Ren et al., 2003). There is also a possibility that components of the essential oil fraction present in sage tea could contribute to the increase in the GST activity, since monoterpenes (including camphor) have been reported

to induce phase II enzymes such as GST and UGT (Elegbede et al., 1993; Banerjee et al., 1995). Unidentified compounds present in this water extract belonging to other classes of compounds, such as aminoacids, organic acids, sugars and other polar compounds could also contribute to the observed effects. It should also be kept in mind that due to the complexity of the mixture that plant extracts are, a synergistic interaction between the compounds could be the ultimate cause for the observed effects.

A higher content of glutathione as well as increased activity of GST and GR were present in the cells from sage tea drinking animals indicating a better recovery from collagenase treatment. Glutathione is the major cellular nucleophile and provides an efficient detoxification pathway for a variety of electrophilic reactive metabolites (Reed, 1990; Kedderis, 1996; Lu, 1999). The higher activity of GR could contribute to the maintenance of glutathione in the reduced form when challenged with *t*-BHP. In addition, an enhancement of de novo glutathione synthesis by the hepatocytes of sage drinking animals induced by a possible bioactive compound present in the sage water extract can not be ruled out. Some studies suggest that the enhancement of phase II enzymes by antioxidants, such as polyphenols present in plant water extracts, is achieved by upregulating the corresponding genes by interaction with antioxidant response elements (AREs) that trancriptionally regulate these genes (Ferguson, 2001). It has also been shown that the γ-glutamylcysteine synthetase (γ-GCS), a key enzyme in de novo glutathione synthesis, is also transcriptionally regulated by AREs (Griffith, 1999; Lu, 1999; Myhrstad et al., 2002), and it is known that several treatments that induce expression of phase II detoxifying enzymes also result in elevated γ-GCS activity as well as increased intracellular GSH levels (Mulcahy et al., 1997). So, although not studied, there is the possibility that also in this case, the interaction of some compounds present in the water extract of sage with AREs in vivo, would result in a higher GST and γ-GCS activities and explain the significant increased GSH recovery after 4 h in culture of hepatocytes of sage tea drinking rats.

Concluding, this study shows that the *Salvia officinalis* water extract obtained and consumed as the plant's herbal tea positively affects the antioxidant status of the liver and may have hepatoprotective potential that justify further studies. Because failure to cope with oxidative stress is a common factor in the aetiology of many diseases sage's effects on the improvement of the antioxidant response could provide an explanation for the wide ranging medicinal properties attributed to sage.

Acknowledgments

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Reprinted from *Journal of Etnhopharmacology*, 97, Lima CF, Andrade PB, Seabra RM, Fernandes-Ferreira M, Pereira-Wilson C, The drinking of a *Salvia officinalis* infusion improves liver antioxidant status in mice and rats, 383-389, Copyright (2005), with permission from Elsevier.

Chapter 4

Sage: Antioxidant Effects in Liver Cells

4.1. Chapter overview

The antioxidant activity of sage extracts obtained by organic solvents is well known, mainly based in several noncellular and subcellular *in vitro* techniques. However, little is known about their antioxidant effects at cellular level. Some previous experiments are, even so, indicative of a good cellular antioxidant activity for sage organic extracts. For example, an aqueous methanolic extract of *Salvia officinalis* conferred protective effects against enzyme-dependent and enzyme-independent lipid peroxidation[1]. Until recently, only a small experiment using fibroblasts, performed by Masaki and its collaborators (1995), showed some cellular antioxidant effects of a sage hydro alcoholic extract[2]. Moreover, it was also now reported that a hydro alcoholic extract of sage possess neuroprotective effects against amyloid beta-induced toxicity to PC12 cells, and the effect attributed, at least in part, to the rosmarinic acid[3]. Apart from these studies with organic extracts, we previously also tested the potential biological activity of the sage essential oil fraction (chapter 2) and of a sage aqueous extract, prepared as a tea (chapter 3). Sage tea showed some potential antioxidant effects in liver cells, whereas sage essential oil did not.

Therefore, in the following studies, the potential antioxidant and cytoprotective effects of a sage water (as an infusion) extract (SOI) and also a sage methanolic extract (SOME) were tested against oxidative damages induced by *t*-BHP to the hepatocyte cell line, HepG2. As stated before, the search for hepatoprotective activities is of particular interest since the liver is vulnerable to toxic effects from a variety of drugs and environmental contaminants, because of its high metabolic activity and anatomical positioning receiving blood from the gastrointestinal tract. Moreover, also due to the liver's anatomical positioning, sage tea constituents and possible metabolites will accumulate in relative high concentrations in the liver, after the gastrointestinal absorption, which emphasise also the importance of these studies in liver cells. Since the antioxidant activity of sage has been attributed to the phenolic compounds, they were quantified in the sage extracts used (SOI and SOME). In addition, the potential hepatoprotective effect of their main phenolic compounds and other structure-related phenolic compounds were studied.

The hepatoprotective potential of both sage extracts and phenolic compounds were

[1] Hohmann J, Zupko I, Redei D, Csanyi M, Falkay G, Mathe I & Janicsak G, 1999. Protective effects of the aerial parts of *Salvia officinalis*, *Melissa Officinalis* and *Lavandula angustifolia* and their constituents against enzyme-dependent and enzyme-independent lipid peroxidation. *Planta Medica* **65**, 576-578.

[2] Masaki H, Sakaki S, Atsumi T & Sakurai H, 1995. Active-oxygen scavenging activity of plant extracts. *Biological & Pharmaceutical Bulletin* **18**, 162-166.

[3] Iuvone T, De Filippis D, Esposito G, D'Amico A & Izzo AA, 2006. The spice sage and its active ingredient rosmarinic acid protect PC12 cells from amyloid-beta peptide-induced neurotoxicity. *Journal of Pharmacology and Experimental Therapeutics* **317**, 1143-1149.

tested against *t*-BHP-induced toxicity in HepG2 cells. All the extracts and phenolic compounds studied, when co-incubated with the toxicant, protected significantly HepG2 cells against cell death. The methanolic extract, with a higher content of phenolic compounds, conferred higher protection better than the water extract. The results obtained with the phenolic compounds underscored the importance of the compound's lipophilicity in addition to its antioxidant potential for their biological activity.

In an attempt to explain the observed cytoprotective activities, the effects of sage extracts and phenolic compounds on three markers of cellular oxidative stress (lipid peroxidation, glutathione levels and DNA damage) were evaluated. Both sage extracts significantly prevented lipid peroxidation and GSH depletion, but failed to prevent DNA damage induced by *t*-BHP. The main phenolic compounds present in the extracts, rosmarinic acid and luteolin-7-glucoside, also significantly prevented lipid peroxidation and GSH depletion, but had poor ability to prevent DNA damage. This indicates, therefore, a good correlation between the biological effects of sage extracts and those of their main phenolic compounds. Only the flavonoid aglycones tested, luteolin and quercetin, significantly decreased DNA damage. Therefore, the lipophilicity of the natural antioxidants appeared to be of even higher importance for DNA protection than for cell survival. It seems that, based on other reports and in our own observations, the potential of sage extracts and phenolic compounds to protect against GSH depletion, at least in about 40%, was probably the most relevant effect for the observed hepatoprotection.

Interestingly, sage extracts, when incubated alone with HepG2 cells for 5 hours, induced an increase in GSH levels, which indicates an improvement of the cells' antioxidant potential. However, the above effect can not be attributed solely to the phenolic compounds, which did not induce the same effect, emphasising the importance of constituents other than phenolic compounds present in sage extracts. In fact, rosmarinic acid and luteolin-7-glucoside, as well as quercetin and luteolin, induced a slight decrease in GSH levels, when incubated alone with HepG2 cells for 5 hours. Since the increase in GSH levels, induced by sage extracts, was accompanied by an increase in total glutathione levels, sage extracts seem to have an ability to increase *de novo* glutathione synthesis. This is in accordance with previous observations (see chapter 3), where the ability of a same water extract (sage tea) to increase the *de novo* glutathione synthesis was suggested. In that study, after a stress-induced GSH depletion, sage tea given *in vivo* restored GSH levels, measured *in vitro* in rat hepatocytes, to a higher value than in controls.

In conclusion, this study has shown cellular antioxidant effects of sage extracts, which could be attributed, at least in part, to their phenolic compounds. Other unknown compounds present in the extracts seem, in addition, to induce positive effects on basal GSH levels, which can be of interest against a situation of oxidative stress induced in liver cells. Therefore, sage

extracts can be considered as a possibility in the prevention and/or in the treatment of liver diseases where it is known that oxidative stress is involved.

4.2. Methods

The following experimental protocols (see Appendix) were used in this chapter:

- P2: Biological Models – Culture of HepG2 Cells and Use
- P4: Cell viability Assays – LDH Leakage
- P5: Cell viability Assays – MTT Assay
- P7: Metabolite Measurements – TBARS (Lipid Peroxidation)
- P8: Metabolite Measurements – Glutathione Levels
- P10: Metabolite Measurements – Total Protein
- P11: Enzyme Activities – Glutathione Reductase
- P12: Enzyme Activities – Glutathione Peroxidase
- P13: Enzyme Activities – Glutathione S-Transferase
- P17: Others – Comet Assay
- P19: Others – Antiradical Activity: DPPH and Superoxide Radical

4.3. Manuscript

This chapter comprises the following manuscript:

Lima CF, Valentao PCR, Andrade PB, Seabra RM, Fernandes-Ferreira M & Pereira-Wilson C, 2006. Water and methanolic extracts of *Salvia officinalis* protect HepG2 cells from *t*-BHP induced oxidative damage. Submitted to *Journal of Ethnopharmacology*.

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

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Abstract

Common sage (*Salvia officinalis* L., *Lamiaceae*) is an aromatic and medicinal plant well known for its antioxidant properties. In this study, we propose to evaluate the antioxidant effects of sage extracts at cellular level. For that, the antioxidant/hepatoprotective potential of two sage extracts (a water and a methanolic extract) against *tert*-butyl hydroperoxide (*t*-BHP)-induced toxicity to HepG2 cells was assessed. The most abundant phenolic compounds present in the extracts were rosmarinic acid and luteolin-7-glucoside. Both extracts, when co-incubated with the toxicant, protected significantly HepG2 cells against cell death. The methanolic extract, with a higher content of phenolic compounds than the water extract, conferred better protection, to an extent similar to the positive control, quercetin. Both extracts, tested in a concentration that protects 80% against cell death (IC_{80}), significantly prevented *t*-BHP-induced lipid peroxidation and GSH depletion, but not DNA damage. The ability of sage extracts to protect 62% from GSH depletion was probably the most relevant contributor for the observed hepatoprotection. When incubated alone for 5 hours, sage extracts induced an increase in GSH levels in HepG2 cells, which indicates an improvement of the antioxidant potential of the cells. In conclusion, this study demonstrates the antioxidant/hepatoprotective potential of sage extracts which suggest that may be used in the prevention or treatment of liver diseases where it is known that oxidative stress is involved.

Keywords: Salvia officinalis L; Phenolic compounds; Antioxidant effects; HepG2 cells; *tert*-Butyl hydroperoxide

1. Introduction

Reactive oxygen species (ROS) and other free radicals are produced during the normal cell metabolism and they are a necessary and normal process that provides important physiological functions (Cui *et al.*, 2004; Willcox *et al.*, 2004). The production of ROS and

other free radicals is normally compensated by an elaborate endogenous antioxidant system. However, due to many environmental, lifestyle and pathological factors, an excess of radicals can be accumulated in cells resulting in oxidative stress. Because of their high reactivity, accumulation of radicals above cells' defences may affect cellular functionality and integrity by damaging critical molecules, such as the DNA, proteins, carbohydrates and lipids, which ultimately can cause cell death. In fact, oxidative stress has been recognised to be involved in the aetiology of several diseases, including liver diseases (Loguercio and Federico, 2003; Vitaglione *et al.*, 2004). The liver, because of its high metabolic activity and its anatomical positioning to receive blood from the gastrointestinal tract, is vulnerable to toxicity from a variety of drugs and environmental contaminants. Consequently, mechanisms of cytoprotection relevant to the liver are of particular interest. Natural antioxidants have been proposed and utilised as therapeutic agents to counteract liver damage (Loguercio and Federico, 2003; Vitaglione *et al.*, 2004).

Salvia officinalis L. (*Lamiaceae*) is an aromatic and medicinal plant of Mediterranean origin well known for its antioxidant properties, mainly due to its composition in phenolic compounds (Baricevic and Bartol, 2000). Sage extracts revealed strong antioxidant activity in several assays: by increasing the stability of food oils (Cuppett and Hall, 1998; Miura *et al.*, 2002; Zainuddin *et al.*, 2002; Ozcan, 2003; Jaswir *et al.*, 2005), in an assay based on the disappearance of methyl linoleate in a lipophilic solvent under strong oxidising conditions (Cuvelier *et al.*, 1994; Cuvelier *et al.*, 1996), by the ability to scavenge $DPPH^{\cdot}$ (Lamaison *et al.*, 1991) and $ABTS^{\cdot}$ free radicals (Shan *et al.*, 2005) as well as by having oxygen radical absorbance capacity (ORAC assay) (Zheng and Wang, 2001). In addition, the reported superoxide and hydroxyl radicals scavenging activities using the electron spin resonance technique (Masaki *et al.*, 1995) and the protective effects against enzyme-dependent and enzyme-independent lipid peroxidation (Hohmann *et al.*, 1999; Zupko *et al.*, 2001) of sage extracts also showed its antioxidant potential. Recently, the drinking of a sage infusion (tea) for 14 days was reported to improve liver antioxidant status in mice and rats (Lima *et al.*, 2005). Additionally, the treatment of rats with a water extract of sage for 5 weeks was shown to protect against the hepatotoxicity of azathioprine (Amin and Hamza, 2005). However, despite all these effects, little is known about the antioxidant effects of sage extracts at the cellular level. Only in a small experiment using fibroblasts, performed by Masaki *et al.* (1995), sage antioxidant effects were related with cytoprotective effects. In their study, a sage extract protect significantly against cell death induced by a superoxide-generating system (Masaki *et al.*, 1995). Very recently, a hydro alcoholic extract of sage was reported to possess neuroprotective effects against amyloid β (Aβ)-induced toxicity in PC12 cells, and the effect attributed, at least in part, to the rosmarinic acid (Iuvone *et al.*, 2006).

In this study we proposed to evaluate the potential antioxidant/hepatoprotective effects of two sage extracts (a water and a methanolic crude extracts) against *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damages in HepG2 cells. This model of hepatotoxicity was recently used to evaluate the *in vitro* hepatoprotective effects of individual phenolic compounds, which included the two most representative ones of the above sage extracts – rosmarinic acid and luteolin-7-glucoside (Lima *et al.*, 2006a). Here, the concentration of sage extracts that protected 50% (IC_{50}) against *t*-BHP-induced cell death were determined in order to establish their hepatoprotective potential. Subsequently, IC_{80} values, a concentration that effectively protects against cell death, were used to evaluate the effects of each extract on three markers of oxidative damage: lipid peroxidation, intracellular glutathione levels and DNA damage. The importance of modulation of these parameters by sage extracts in the protection against *t*-BHP-induced cell death is discussed. Throughout the experiment quercetin was used as a positive control.

2. Materials and methods

2.1. Chemicals

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

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

Salvia officinalis L. plants were cultivated in an experimental farm located in Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were lyophilised and kept at -20º C.

The dried and powdered aerial plant material (4 g) was extracted with 2 × 100 ml of 90% methanol in water at room temperature, using an ultrasonic bath (15 min). The filtered extract (SOME) was evaporated to dryness under reduced pressure at 40ºC and a yield of 26.2% (w/w) was obtained.

Considering that sage is traditionally consumed as a tea, an infusion of sage (SOI) was also prepared following a previous methodology (Lima *et al.*, 2005). In brief, 300 ml of ultrapure Milli Q boiling water were poured over 4 g of dried aerial plant material and allowed to steep for 5 min. The filtered extract was lyophilised to dryness and a yield of 25.8% (w/w) was obtained.

Phenolic compounds present on SOME and SOI extracts were identified and quantified by HPLC/DAD as described in Santos Gomes *et al.* (2002) and Lima *et al.* (2005) for each extract, respectively.

2.3. Antiradical activity

The free radical scavenging (antiradical) activity of sage extracts was studied against two radicals: the stable free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and the superoxide radical.

For DPPH scavenging activity, after addition of different concentrations of extract to DPPH (90μM), the percentage of remaining DPPH was determined at different times from the absorbance at 515 nm using a plate reader spectrophotometer. As suggested by Sanchez-Moreno *et al.* (1998), the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration (IC_{50}) was expressed in terms of initial concentration of DPPH. We also present the parameter antiradical efficiency (AE) (Sanchez-Moreno *et al.*, 1998) using the estimated T_{IC50} – time needed to reach the steady state at the corresponding IC_{50} concentration, where $AE = 1/(IC_{50} \times T_{IC50})$.

The superoxide radical scavenging activity was determined using the phenazine methosulphate-NADH non-enzymatic assay as previously described (Valentao *et al.*, 2001).

2.4. Cell culture

HepG2 cells (hepatocellular carcinoma cell line) were obtained from the American Type Culture Collection (ATCC) and maintained in culture in 75 cm^2 polystyrene flasks (Falcon) with MEM containing 10% FBS, 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37ºC.

2.5. Experimental outline

2.5.1. Assay for protection against t-*BHP-induced toxicity in HepG2 cells*

In order to determine the concentration of sage extract/quercetin that protects the cells 50% from the oxidative damage (IC_{50}), cells were incubated with 2 mM of *t*-BHP for 5 h to induce significant cell death as previously described (Lima *et al.*, 2006a). HepG2 cells were plated in 24-multiwell culture plates at 2.5×10^5 cells per well. The prevention of LDH leakage (cell death) was measured in co-incubations with sage extract/quercetin dissolved in DMSO (1% v/v final concentration, controls with DMSO only) at several concentrations. The IC_{50} and the Hillslope – slope from the plotted sage extract/quercetin's concentrations (in logarithm) versus cell death protection relative to the control (2 mM *t*-BHP, 5 h) – were calculated graphically using a computer program (GraphPad Prism, version 4.00, GraphPad Software Inc.). Based on the dose-response curves of protection against cell death by sage extract/quercetin, the IC_{80} concentrations were estimated and used in the following experiments to evaluate the protective potential of the compounds on several cellular parameters as previously described (Lima *et al.*, 2006a). Briefly:

2.5.2. Evaluation of the effects of sage extract/quercetin at the IC_{80} concentration against t-*BHP-induced lipid peroxidation and GSH depletion in HepG2 cells.*

In order to evaluate the potential protective effect of sage extract/quercetin at IC_{80} concentration against *t*-BHP-induced lipid peroxidation and GSH depletion, cells were incubated with 2 mM *t*-BHP for 5 h. HepG2 cells were plated in 6-multiwell culture plates at 7.5×10^5 cells per well. Forty hours after plating, the medium was discarded and fresh medium containing 2 mM *t*-BHP and/or the IC_{80} concentration of sage extract/quercetin was added. Five hours later, cell culture medium and cell scrapings were harvested and kept at -80ºC for following quantification of lipid peroxidation and glutathione levels.

2.5.3. Evaluation of the effects of sage extract/quercetin at the IC_{80} concentration against t-*BHP-induced DNA damage in HepG2 cells*

In order to evaluate the potential protective effect of sage extract/quercetin at IC_{80} concentration against *t*-BHP-induced DNA damage, cells were incubated with 200 μM *t*-BHP for 1 h. HepG2 cells were plated in 6-multiwell culture plates at 5×10^5 cells per well. Sixteen hours after plating, the medium was discarded and fresh medium containing 200 μM *t*-BHP and/or the IC_{80} concentration of sage extract/quercetin was added to the cells. After 1 h incubation, cells were rinsed with warm PBS and then incubated for 5 min with 0.125% (w/v) trypsin in PBS. The cells were then harvested in PBS to be used in the alkaline version of the comet assay for evaluation of DNA damage.

2.6. Biochemical analysis

2.6.1. LDH

To assess the extend of cell death caused by *t*-BHP, the determination of lactate dehydrogenase leakage to the culture determination of lactate dehydrogenase leakage to the culture medium was used as indicator of plasma membrane integrity of HepG2 cells. LDH activity was measured spectrophometrically at 30ºC as previously described (Lima *et al.*, 2005).

2.6.2. Lipid peroxidation

The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm following a methodology previously described (Fernandes *et al.*, 1995) with some modifications (Lima *et al.*, 2005). The results are expressed as nmol/mg of protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

2.6.3. Glutathione content

The glutathione levels of HepG2 cells were determined by the DTNB-GSSG reductase recycling assay as previously described (Anderson, 1985), with some modifications (Lima *et al.*, 2004). The results are expressed as nmol GSH/mg of protein.

2.6.4. Protein

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

2.7. Comet assay

The alkaline version of the single cell gel electrophoresis (comet) assay was performed based in previous descriptions (Klaude *et al.*, 1996; Uhl *et al.*, 1999; Uhl *et al.*, 2000) with slight modifications (Lima *et al.*, 2006a). The comet images were analysed using the semiquantitative method of visual scoring (Duthie and Dobson, 1999). Each cell was classified in five classes according to the intensity of fluorescence in the comet tail, attributing a value of 0, 1, 2, 3 or 4 from undamaged to maximal damage. In this way, the total score for 100 images can range from 0 (all undamaged) to 400 (all maximally damaged), the overall DNA damage of the cell population expressed in arbitrary units.

2.8. Statistical analysis

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

3. Results

3.1. Phenolic composition of sage extracts and their anti-radical activity

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

The antiradical activity of both extracts was then evaluated against DPPH and superoxide radicals (Table 2). SOME, with higher content in phenolic compounds, had higher antiradical activity against DPPH presenting a lower IC_{50} and a higher AE than SOI extract. The activity of both extracts was smaller than the positive control quercetin. Regarding the activity against superoxide radical, SOI extract had a comparable antiradical activity to that of quercetin, higher than that of SOME.

3.2. Potential hepatoprotective effects of sage extracts

The potential hepatoprotective effects of both sage extracts against the cell death induced by *t*-BHP were evaluated in HepG2 cells (Table 3, Fig. 1). *t*-BHP 2 mM for 5 hours

Table 1. Composition (μg/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		Superoxide radical[c]
	IC_{50} (g/kg DPPH)[a]	AE[b] ($\times 10^{-3}$)	IC_{50} (μg/ml)
SOME	381 ± 14	0.22	162 ± 39
SOI	419 ± 9	0.18	14.4 ± 1.4
Quercetin	96.6 ± 1.9	0.47	10.6 ± 1.0

[a] Values represent mean ± SD of 5 replicates.

[b] AE - Antiradical efficiency

[c] Values represent mean ± SD of 3 independent experiments with 3 replicates each.

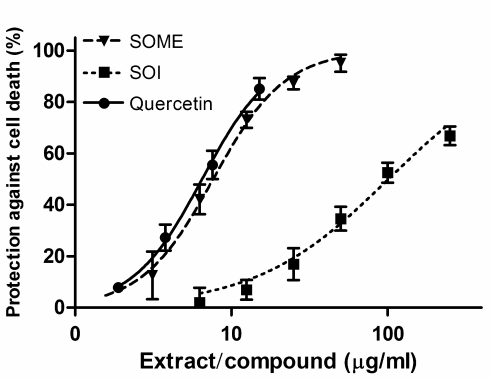

Fig. 1. Dose-response effect of the sage extracts against *t*-BHP-induced toxicity in HepG2 cells. After incubating HepG2 cells with 2 mM of *t*-BHP and sage extracts/quercetin for 5 h, protection against cell death (as measured by LDH leakage) versus sage extract/quercetin concentrations (in logarithm) were plotted in order to take the IC_{50} and Hillslope of each compound (Table 3). Values are mean ± SEM of at least 4 independent experiments.

was previously shown to induce oxidative damage to HepG2 cells causing about 40-50% of cell death (Lima *et al.*, 2006a). As shown in Fig. 1, both extracts protected against cell death in a dose-dependent manner. SOME had, however, higher hepatoprotective activity (lower IC_{50}) than SOI (Table 3), and in the same range of that of quercetin. The Hillslope was also higher in SOME than SOI (Table 3), which indicates a narrower concentration (in logarithm) range from 0 to 100% of hepatoprotective activity of SOME (Fig 1).

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

To study the effects of sage extracts against lipid peroxidation, GSH depletion and DNA damage induced by *t*-BHP, concentrations that effectively protect against cell death (IC_{80}) were used. IC_{80} concentrations were used to determine if the same level of cytoprotection for each extract correlate with similar effects on the above mentioned parameters. IC_{80} concentration for each extract (Table 3) was estimated based on the curves presented in Fig. 1.

As shown in Fig. 2, *t*-BHP-induced lipid peroxidation was significantly decreased by around 25% by both extracts. Quercetin also significantly protected against lipid peroxidation by 30%. None of the extracts, when incubated alone with HepG2 cells, induced significant lipid peroxidation.

t-BHP-induced GSH (reduced glutathione) depletion was also signify-cantly inhibited by both extracts by around 62% while quercetin inhibited GSH depletion by only 40% (Fig. 3). The increase in GSSG levels induced by *t*-BHP was slightly decreased by both sage

Table 3. Potential hepatoprotective 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. IC_{50} and the Hillslope were taken form the plotted dose-response curve (Fig. 1). IC_{80} concentration was estimated from the same dose-response curve. Values are mean ± SEM of at least 4 independent experiments.

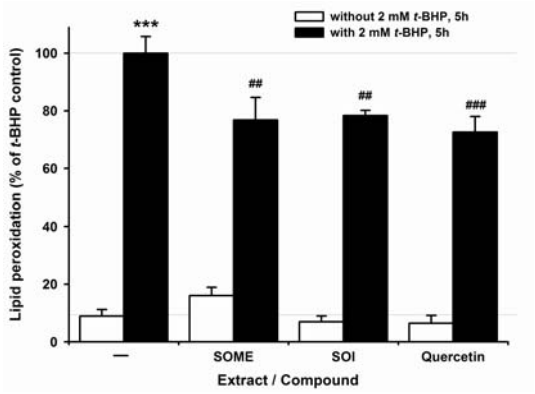

Fig. 2. Effect of sage extracts at IC_{80} concentration against *t*-BHP-induced lipid peroxidation in HepG2 cells. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with sage extract/quercetin at IC_{80} concentration and lipid peroxidation measured by TBARS assay. Values are mean ± SEM, $n = 5$ (100% = 2.25 nmol/mg). *** $P \leq 0.001$ when compared with the negative control. ## $P \leq 0.01$ and ### $P \leq 0.001$ when compared with the *t*-BHP control.

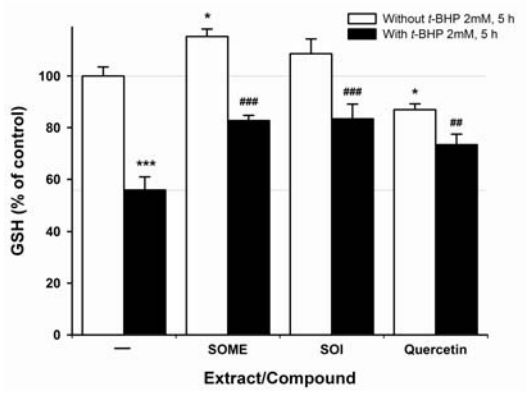

Fig. 3. Effect of sage extracts at IC_{80} concentration against *t*-BHP-induced decrease in GSH levels in HepG2 cells. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with sage extract/quercetin at IC_{80} concentration and GSH levels determined by the DTNB-GSSG reductase recycling assay. 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.

extracts and quercetin, although the effect was not statistically significant (data not shown). When the cells were incubated with the extracts alone, a significant increase in the basal GSH levels (Fig. 3) was observed for SOME (15%). On the other hand, quercetin induced a decrease in the basal levels of GSH.

The incubation of HepG2 cells for 1 h with 200 μM of *t*-BHP induced significant DNA damages without cell death (Lima *et al.*, 2006a), conditions that can be used to assess effects of compounds or extracts against DNA damage by the comet assay. As shown in Fig. 4, contrarily to what happened with quercetin, both sage extracts did not protect HepG2 cells against DNA damage induced by *t*-BHP. None of the tested extracts induced DNA damages at IC_{80} concentration when incubated alone with HepG2 cells.

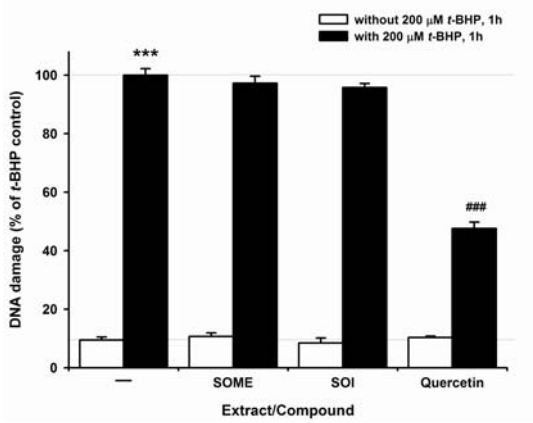

Fig. 4. Effect of sage extracts at IC_{80} concentration against *t*-BHP-induced DNA damage in HepG2 cells. HepG2 cells were incubated with *t*-BHP 200 μM (1 h) and/or with sage extract/quercetin at IC_{80} concentration and DNA damage evaluated by the comet assay. DNA damage was assessed by the semiquantitative method of visual scoring. Values are mean ± 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.

4. Discussion and conclusions

Since oxidative stress has been recognised to be involved in the aetiology of several liver diseases (Loguercio and Federico, 2003; Vitaglione *et al.*, 2004) and because the liver is very susceptible to toxic effects, natural antioxidants and plant extracts have been proposed as therapeutic agents to counteract liver damage. *Salvia officinalis* is well known for its antioxidant activity, mainly based in several subcellular and noncellular *in vitro* studies (Baricevic and Bartol, 2000). Recently, sage tea drinking was reported to improve liver antioxidant status in mice and rats (Lima *et al.*, 2005). That was, however, not enough to protect against CCl_4-induced hepatotoxicity in mice and, instead, a herb-toxicant interaction was observed (Lima et al, 2006b). In another experiment, the treatment of rats with a water extract of sage for 5 weeks was, on the other hand, shown to protect against the hepatotoxicity of azathioprine (Amin and Hamza, 2005). However, despite all these effects, little is known about the antioxidant and protective effects of sage extracts at cellular level.

Here, the potential antioxidant and hepatoprotective effects of sage crude extracts were tested against *t*-BHP-induced toxicity in HepG2 cells. Both a methanolic (SOME) and a water extract (SOI) of sage, in co-incubations with the toxicant, showed protective effects against *t*-BHP-induced cell death. SOME extract revealed higher hepatoprotective activity than SOI. This is in agreement with the literature where sage's antioxidant activity has been attributed to its phenolic compounds, more abundant in the methanolic extract. This was shown by the lower IC_{50} obtained for this extract against *t*-BHP-induced cell death compared to that of SOI extract.
In this model of cytoprotection, because effects were tested in co-incubations with the toxicant, the antioxidant protection may reflect mainly direct actions on *t*-BHP toxicity (Lima et al, 2006a). These direct effects would include, besides the antiradical scavenging or hydrogen-donating activity measured in this study, the compounds' ability to chelate metal ions (Rice-Evans *et al.*, 1996). Since ROS (Martin *et al.*, 2001), *t*-BHP radicals (Davies, 1989; VanderZee *et al.*, 1996) and intracellular iron ions (Hix *et al.*, 2000) are involved in the toxicity of *t*-BHP, direct effects at both these levels would tend to reduce the extension of damage. Antiradical activity of sage was shown here against DPPH and superoxide radicals and also by previous studies (Lamaison *et al.*, 1991; Cuvelier *et al.*, 1994; Masaki *et al.*, 1995; Zheng and Wang, 2001; Shan *et al.*, 2005). Considering the composition of the extracts in phenolic compounds, they most likely also possess the ability to chelate metal ions (Rice-Evans *et al.*, 1996).

Apart from the antiradical and metal chelation ability of the extracts, they will only act as intracellular antioxidants if the compounds permeate cell membranes. Previous results underscored the importance of the compound's lipophilicity in addition to its antioxidant potential for biological activity (Lima *et al.*, 2006a). The two main phenolic compounds present in these extracts, rosmarinic acid and luteolin-7-glucoside, were previously tested in this

experimental model and shown to possess similar hepatoprotective activities (IC_{50}'s of 69 μM and 78 μM, respectively; Lima *et al.*, 2006a).

In an attempt to explain the observed cytoprotective effects of the sage extracts, we looked at their effects at IC_{80} concentration on three markers of cellular oxidative stress: lipid peroxidation, glutathione levels and DNA damage. Incubation of HepG2 cells with *t*-BHP induced significant lipid peroxidation, GSH depletion and DNA damage. At IC_{80}, both sage extracts significantly prevented lipid peroxidation and GSH depletion, but failed to prevent DNA damage. In a previous study, both rosmarinic acid and luteolin-7-glucoside also significantly prevented lipid peroxidation and GSH depletion. Both these compounds had poor ability to prevent DNA damage induced by *t*-BHP (Lima *et al.*, 2006a). There seems to be, therefore, a good correlation between the biological effects of sage extracts and those of their main phenolic compounds.

Based on previous studies, lipid peroxidation and DNA damage seem not to be as relevant for the *t*-BHP-induced cell death as GSH depletion (Martin *et al.*, 2001; Lima *et al.*, 2006a). GSH depletion has been suggested as the primary mechanisms of *t*-BHP-induced toxicity in liver cells (Jewell *et al.*, 1986; Buc-Calderon *et al.*, 1991; Martin *et al.*, 2001). GSH plays an important role in hepatocyte defence against ROS, free radicals and electrophilic metabolites (Kedderis, 1996; Castell *et al.*, 1997). A severe GSH depletion leaves cells more vulnerable to oxidative damage and is normally associated with calcium homeostasis disruption, which ultimately causes cell death (Castell *et al.*, 1997). The prevention of *t*-BHP-induced GSH depletion in at least 40% has previously been suggested as a major contribution to cytoprotective effects in the experimental model used here (Lima *et al.*, 2006a). Thus, the 62% protection against GSH depletion was probably the most relevant effect of the extracts used in this study.

Although rosmarinic acid and luteolin-7-glucoside present in the extracts may contribute to the observed prevention of GSH depletion induced by *t*-BHP, they cannot be the sole explanation for the effects of sage extracts on the GSH levels. In the same experimental model, both phenolic compounds showed to decrease slightly GSH levels when incubated alone with HepG2 cells for 5 hours (Lima *et al.*, 2006a), an effect similar to what was observed in this study with quercetin – the positive control. On the contrary, when sage extracts were incubated alone with HepG2 cells for 5 hours, a slight increase in GSH levels was observed, which was significant for the SOME extract. Compounds other than phenolics present in the extracts appear, therefore, to be important for this effect of sage extracts in HepG2 cells. Since the increase in GSH levels was accompanied by an increase in the total glutathione levels (and not to a reduction in GSSG levels), sage extracts seem to have an ability to increase the *de novo* synthesis of glutathione. A previous study also showed that, after a stress-induced GSH depletion, SOI given *in vivo* to rats restored GSH levels of subsequent hepatocyte cultures to a

higher value than controls (Lima *et al.*, 2005). In that study, the ability of SOI to increase the *de novo* glutathione synthesis was also suggested. In agreement with this, Amin and Hamza (2005) showed the ability of sage to protect *in vivo* the hepatotoxicity of a drug that acts by depleting GSH levels.

In conclusion, this study showed clearly the antioxidant effects at cellular level of sage, namely preventing cell death, lipid peroxidation and GSH depletion induced by *t*-BHP. These extracts seem also to have positive effects on GSH levels that can be relevant in the face of a situation of oxidative stress. The observed hepatoprotective effects suggest the use of sage in the treatment for liver diseases where it is known that oxidative stress is involved. The phenolic compounds present in the sage extracts are probably contributors to the observed hepatoprotection but other unknown compounds may also be important, mainly in the induction of glutathione synthesis.

Acknowledgments

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4.4. Publication

This chapter comprises also the following publication:

Lima CF, Fernandes-Ferreira M & Pereira-Wilson C, 2006. Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life Sciences* **79(21)**, 2056-2068.

Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels

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Abstract

In the present work, the potential hepatoprotective effects of five phenolic compounds against oxidative damages induced by tert-butyl hydroperoxide (*t*-BHP) were evaluated in HepG2 cells in order to relate in vitro antioxidant activity with cytoprotective effects. *t*-BHP induced considerable cell damage in HepG2 cells as shown by significant LDH leakage, increased lipid peroxidation, DNA damage as well as decreased levels of reduced glutathione (GSH). All tested phenolic compounds significantly decreased cell death induced by *t*-BHP (when in co-incubation). If the effects of quercetin are given the reference value 1, the compounds rank in the following order according to inhibition of cell death: luteolin (4.0) > quercetin (1.0) > rosmarinic acid (0.34) > luteolin-7-glucoside (0.30) > caffeic acid (0.21). The results underscore the importance of the compound's lipophilicity in addition to its antioxidant potential for its biological activity. All tested phenolic compounds were found to significantly decrease lipid peroxidation and prevent GSH depletion induced by *t*-BHP, but only luteolin and quercetin significantly decreased DNA damage. Therefore, the lipophilicity of the natural antioxidants tested appeared to be of even greater importance for DNA protection than for cell survival. The protective potential against cell death was probably achieved mainly by preventing intracellular GSH depletion. The phenolic compounds studied here showed protective potential against oxidative damage induced in HepG2 cells. This could be beneficial against liver diseases where it is known that oxidative stress plays a crucial role.

Keywords: Phenolic compounds; Liver; Oxidative stress; HepG2 cells; *tert*-butyl hydroperoxide; Antioxidants

Introduction

An overall increase in cellular levels of reactive oxygen species (ROS) above the cells' defenses results in oxidative stress that can ultimately cause cell death. Oxidative stress has been recognized to be involved in the etiology of several age-related and chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Tiwari, 2004; Cui et al., 2004; Ceriello and Motz, 2004; Klaunig and Kamendulis, 2004; Willcox et al., 2004; Ballinger, 2005; Gibson and Huang, 2005). In particular with respect to liver diseases such as hepatocellular carcinoma, viral and alcoholic hepatitis and non-alcoholic steatosis, it is known that ROS and reactive nitrogen species play a crucial role in disease induction and progression (Adachi and Ishii, 2002; Loguercio and Federico, 2003; Vitaglione et al., 2004). The liver is particularly susceptible to toxicants since the portal vein brings blood to this organ after intestinal absorption. The absorbed drugs and xenobiotics in a concentrated form can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic processes (Jaeschke et al., 2002).

Because oxidative stress plays a central role in liver diseases pathology, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Vitaglione et al., 2004). This same idea has also been suggested for other oxidative stress-based chronic diseases (Tiwari, 2004; Willcox et al., 2004). In fact, several epidemiological studies have shown that diets rich in fruit and vegetables and other plant foods (including tea and wine) are associated with a decreased risk of premature death and mortality from chronic diseases, such as cardiovascular diseases and some types of cancer (Stanner et al., 2004; Scalbert et al., 2005). Phenolic compounds (PhC), and in particular polyphenols, are believed to be, at least in part, responsible for such effects. Results from some human clinical trials support the role of these

compounds in the prevention of some chronic diseases (Ren et al., 2003; Spencer et al., 2004; Tiwari, 2004; Willcox et al., 2004; Scalbert et al., 2005).

Today much is known about the chemistry and antioxidant potential of PhC as a result of in vitro chemical and sub-cellular studies (Rice-Evans et al., 1997; Croft, 1998). However, besides their strong free radical scavenging activity, PhC can also act as antioxidants by chelating metal ions, preventing radical formation, and indirectly by modulating enzyme activities and altering the expression levels of important proteins, such as antioxidant and detoxifying enzymes (Ferguson, 2001; Ross and Kasum, 2002; Ferguson et al., 2004). Few studies, however, address the biological effects of PhC, and the ones performed using cellular and in vivo models indicate a poor correlation between the antioxidant potency of PhC measured in vitro and the compound's biological activity. The biological effect of PhC and their in vivo circulating metabolites will ultimately depend on their cellular uptake and/or the extent to which they associate with cell membranes (Spencer et al., 2004).

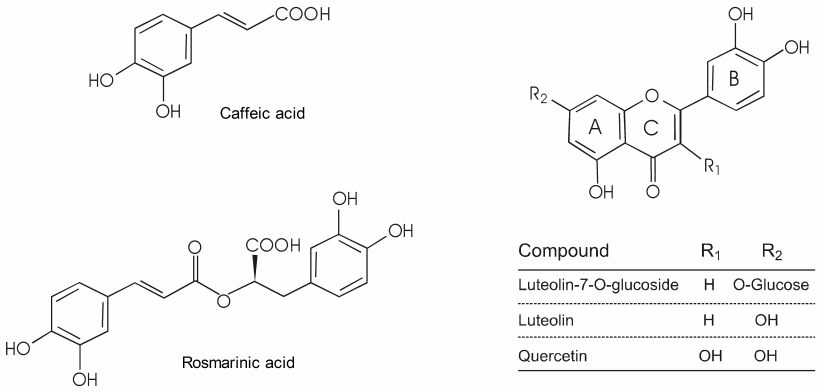
HepG2 cells, a human hepatoma cell line, are considered a good model to study in vitro xenobiotic metabolism and toxicity to the liver, since they retain many of the specialized functions which characterize normal human hepatocytes (Knasmuller et al., 1998). In particular, HepG2 cells retain the activity of many phase I, phase II and antioxidant enzymes ensuring that they constitute a good tool to study cytoprotective, genotoxic and antigenotoxic effects of compounds (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004). Recently, studies of cytoprotection by natural antioxidants in HepG2 cells have increasingly been using *tert*-butyl hydroperoxide (*t*-BHP), an organic hydroperoxide, as the toxic agent (Thabrew et al., 1997; Kinjo et al., 2003; Mersch-Sundermann et al., 2004; Lee et al., 2005a,b; Alia et al., 2006). *t*-BHP can bemetabolized in the hepatocyte by glutathione peroxidase, generating oxidized glutathione (GSSG) (Sies and Summer, 1975; Rush et al., 1985). GSSG is converted back to reduced glutathione (GSH) at the expense of NADPH by glutathione reductase (GR). Depletion of GSH and NADPH oxidation are associated with altered calcium homeostasis, leading to loss of cell viability (Bellomo et al., 1982; Martin et al., 2001). Alternatively, *t*-BHP can be converted into its peroxyl and alkoxyl free radicals by cytochrome P450 enzymes and by free iron-dependent reactions. These free radicals can subsequently initiate lipid peroxidation, form covalent bonds with cellular molecules (such as DNA and proteins) and further decrease GSH levels. The latter effect, in addition to altering calcium homeostasis, affects mitochondrial membrane potential, eventually causing cell death (Rush et al., 1985; Nicotera et al., 1988; Masaki et al., 1989; Davies, 1989; Buc-Calderon et al., 1991; Kass et al., 1992; VanderZee et al., 1996; Hix et al., 2000).

In this study we evaluate the hepatoprotective effects of PhC against *t*-BHP-induced oxidative damage inHepG2 cells, in order to relate in vitro antioxidant activity with cytoprotective effects. Two phenolic acids, caffeic acid and rosmarinic acid (an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid), and three flavonoids, luteolin (flavone), luteolin-7-glucoside (flavone glycoside) and quercetin (flavonol), were used (Fig. 1). Firstly, the concentrations of PhC that protected by 50% (IC_{50}) against *t*-BHP-induced cell death were determined. Based on the IC_{50} values for each compound, biological activity was related to both antiradical efficiency and hydrophobicity. Subsequently, IC_{80} values, a concentration that effectively protects 80% of the cells against *t*-BHP-induced cell death, were used to evaluate the effects of each compound on several markers of oxidative damage, such as intracellular glutathione, lipid peroxidation, glutathione-related enzyme such as glutathione-*S*-transferase (GST), GR and glutathione peroxidase (GPox), as well as on DNA damage. The relative importance of effects of PhC on these parameters to protection against *t*-BHP-induced cell death is discussed.

Materials and methods

Chemicals

Minimum Essential Medium Eagle (MEM), *tert*-butyl hydroperoxide, quercetin, rosmarinic acid, caffeic acid and Bradford reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biochrom KG (Germany). Luteolin and luteolin-7-O-glucoside were purchase from Extrasynthese (Genay, France). All other reagents were of analytical grade.



Compound	R_1	R_2
Luteolin-7-O-glucoside	H	O-Glucose
Luteolin	H	OH
Quercetin	OH	OH

Fig. 1. Chemical structures of the phenolic compounds used in this study.

Cell culture

HepG2 cells (hepatocellular carcinoma cell line), obtained from the American Type Culture Collection (ATCC), were maintained in culture in 75 cm^2 polystyrene flasks (Falcon) with MEM containing 10% FBS, 1% antibiotic–antimycotic solution, 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate under an atmosphere of 5% CO2 at 37°C.

Assay for t-BHP cytotoxicity and protection by phenolic compounds

HepG2 cells were plated in 24-multiwell culture plates at 2.5×10^5 cells per well. To study *t*-BHP cytotoxicity, 40 h after plating, the medium was discarded and fresh medium containing *t*-BHP at various concentrations was added. At different time points, cellular viability was determined by the MTT assay (Mosmann, 1983) and by lactate dehydrogenase (LDH) leakage assay (Lima et al., 2005). In order to determine the concentration of PhC that protects 50% of the cells from damage induced by the toxicant (IC_{50}), cells were incubated with 2 mM of *t*-BHP for 5 h to induce significant cell death. The prevention of LDH leakage (cell death) was measured in co-incubations with PhC dissolved in DMSO (1% v/v final concentration, controls with DMSO only) at several concentrations. The IC_{50} and the Hill slope — the slope of the PhC concentrations (in logarithm) plotted versus cell death protection relative to the control (2 mM *t*-BHP, 5 h) — were calculated graphically using a computer program (GraphPad Prism, version 4.00, GraphPad Software Inc.). Based on the dose–response curves of cell death protection by PhC against the *t*-BHP-induced oxidative damage in HepG2 cells, the IC_{80} concentrations were estimated and used in the following experiments to evaluate the protective potential of the compounds on several cellular parameters.

Evaluation of the effects of t-BHP and PhC at the IC_{80} concentration on lipid peroxidation, glutathione levels and glutathione-related enzyme activities in HepG2 cells

HepG2 cells were plated in 6-multiwell culture plates at 7.5×10^5 cells per well. 40 h after plating, the medium was discarded and fresh medium containing 2 mM *t*-BHP and/or the IC_{80} concentration of each PhC was added. 5 h later, cell culture medium and cell scrapings were harvested and kept at −80°C for following quantification of several parameters. Cell scrapings were harvested in lysis buffer (25 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 1 mM EGTA, 100 μM PMSF, pH 7.5) after rinsing the cells with PBS (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Evaluation of the effects of t-BHP and PhC at the IC_{80} concentration on DNA damage in HepG2 cells

HepG2 cells were plated in 6-multiwell culture plates at 5×10^5 cells per well. To study *t*-BHP-induced DNA damage, 16 h after plating, the medium was discarded and fresh medium containing *t*-BHP at various concentrations was added. After 1 h of incubation, cells were rinsed in warm PBS and then incubated for 5 min with 0.125% (w/v) trypsin in PBS. The cells were then harvested in PBS to be used in the alkaline version of the comet assay for evaluation of DNA damage. To study the protective potential of PhC at IC_{80} concentration on *t*-BHP-induced DNA damage, cells were incubated with 200 μM *t*-BHP for 1 h to induce significant DNA damage. For that, 16 h after plating, the medium was discarded and fresh medium containing 200 μM *t*-BHP and/or the IC_{80} concentration of each PhC was added to the cells. After 1 h incubation, cells were treated as above to carry out the comet assay.

Comet assay

The single cell gel electrophoresis (comet) assay was performed based on previous descriptions (Klaude et al., 1996; Uhl et al., 1999, 2000) with slight modifications. Briefly, 40,000 cells in PBS were centrifuged (80 ×*g*, 2 min), the pellet was mixed with 100 μl of low melting agarose 0.5% (w/v) in PBS, at 37 °C and spread on agarose coated slides. The agarose was allowed to set at 4°C for 10 min, and then the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with NaOH, triton X-100 1% v/v added fresh) at 4°C for 2 h. After being rinsed with distilled water, the slides were immersed in a horizontal electrophoresis tank with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) at 4°C and exposed for 40 min to allow alkaline unwinding. Afterwards, electrophoresis was carried out under alkaline conditions for 20 min, 300 mA, at 0.8 V/cm in a cold room (4°C). Finally, the slides were neutralized by washing three times for 5 min each with 0.4 M Tris, pH 7.5, at 4°C, fixed with methanol and kept at 4°C until evaluation. For the analysis of the comet images, the DNA was stained with ethidium bromide and scored under a fluorescent microscope using a computer assisted image analysis system and/or a visual scoring method avoiding analyzing cells at the edges of the gel. The computer image analyses were done using a public domain image-analysis program — NIH image (Helma and Uhl, 2000), and the results expressed in terms of tail length, tail moment and % DNA in tail of 50 cells in 4 independent experiments. In the semiquantitative method of visual scoring, the comet images were classified in five classes according to the intensity of fluorescence in the comet tail, attributing a value of 0, 1, 2, 3 or 4 from undamaged to maximal damage. In this way, the total score for 100 images can range from 0 (all undamaged) to 400 (all maximally damaged) giving the overall DNA damage of the cell population expressed in arbitrary units (Duthie and Dobson, 1999; Duthie, 2003).

Biochemical analyses

Lipid peroxidation

The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm following a methodology previously described (Lima et al., 2005). The results are expressed as nmol/mg of protein using a molar extinction coefficient of $1.56\times10^5\ M^{-1}cm^{-1}$.

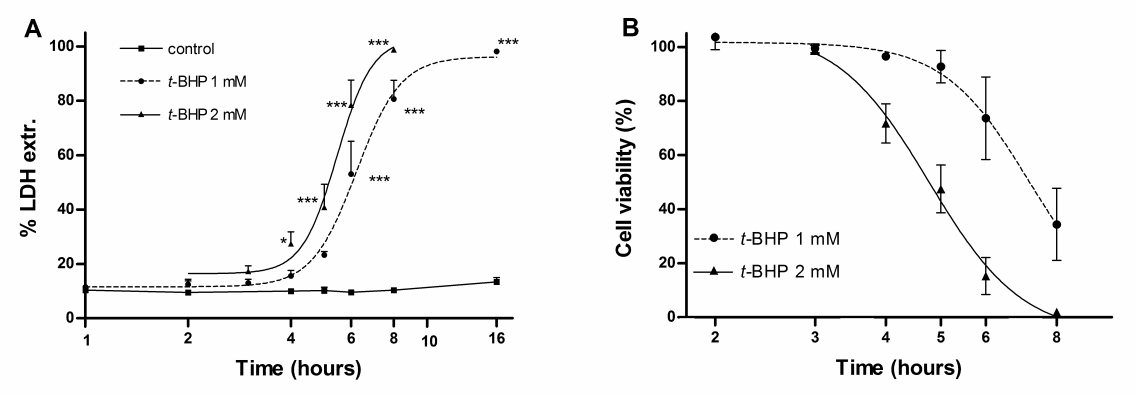


Fig. 2. *t*-BHP-induced toxicity in HepG2 cells. HepG2 cells were incubated with *t*-BHP 1 mM and 2 mM for different time periods and cell viability measured by LDH leakage (% of LDH in the extracelular medium) (A) and MTT assay (B). Time scale was logarithmized in order to obtain sigmoidal response curves. Values represent the mean ± SEM, $n = 4$. In A: $*P \leq 0.05$ and $***P \leq 0.001$ when compared to the same time point in the control situation.

Glutathione levels

The glutathione levels from the cell cultures were determined by the DTNB–GSSG reductase recycling assay as previously described (Anderson, 1985),with some modifications (Lima et al., 2004). The results are expressed as nmol GSH/mg of protein.

Glutathione-related enzyme activities

For measurement of the glutathione-related enzyme activities, the cell scraping homogenates were centrifuged at 10,000 ×*g* for 10 min at 4°C and the supernatant collected.

GST and GR activities were measured spectrophotometrically at 30 °C as previously described (Lima et al., 2005) and the results expressed in nmol/min/mg protein (mU/mg).

The selenium-dependent and -independent GPox activity was assayed as previously described (Martin-Aragon et al., 2001) with some modifications. Briefly, GPox activity was measured at 30°C following NADPH oxidation at 340 nm on a plate reader spectrophotometer (SpectraMax 340pc, Molecular Devices, Sunnyvale, CA, USA) in the presence of 1 mM GSH, 0.18 mM NADPH, 1 mM EDTA, 0.5 U/ml GR and 0.7 mM *t*-BHP in 50 mM imidazole (pH 7.4). The activity was expressed as nmol of substrate oxidized per minute per mg of protein (mU/mg).

Protein

Protein content was measured with the Bradford Reagent purchased from Sigma using bovine serumalbumin as a standard.

Antiradical activity

The free radical scavenging (antiradical) activity of PhC was studied against two radicals: the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the superoxide radical.

For DPPH scavenging activity, after the addition of different concentrations of PhC to DPPH (90 μM), the percentage of the remaining DPPH was determined at different times from the absorbance at 515 nm using a plate reader spectrophotometer. As suggested by Sanchez-Moreno et al. (1998), the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration (IC_{50})was expressed in terms of initial concentration of DPPH to make the results easier comparable with other published results. However, we put the results of the PhC in terms of moles instead of grams to better relate the results with the chemical structures (Fig. 1) of the PhC studied. We also calculated the parameter antiradical efficiency (AE) (Sanchez-Moreno et al., 1998) using the estimated T_{IC50} — the time needed to reach the steady state at the corresponding IC_{50} concentration, where $AE = 1/(IC_{50} \times T_{IC50})$. Finally, a new parameter is also shown — the Hill slope, the graphically calculated slope from the plotted PhC concentration (in logarithm) versus the remaining DPPH concentration (GraphPad Prism). The higher this value, the narrower the concentration range from 0 to 100% of antiradical activity. This graph was also used to calculate the IC_{50} of each compound.

The superoxide radical scavenging activity was determined using the phenazine methosulphate-NADH nonenzymatic assay as previously described (Valentao et al., 2001). As for DPPH

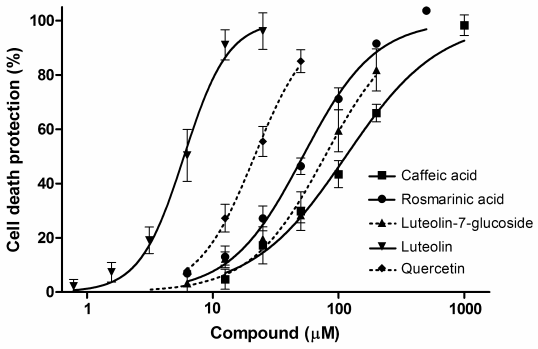


Fig. 3. Dose–response effect of the tested PhC against *t*-BHP-induced toxicity in HepG2 cells. After incubating HepG2 cells with 2 mM of *t*-BHP and individual PhC for 5 h, protection against cell death (as measured by LDH leakage) versus PhC concentration (in logarithm) were plotted in order to take the IC_{50} and Hill slope of each compound (Table 1). Values are the mean ± SEM of at least 4 independent experiments.

Table 1
Potential hepatoprotective effects of the tested PhC against *t*-BHP-induced toxicity in HepG2 cells

Compound	IC_{50} (μM)	Hill slope	IC_{80} (μM)
Caffeic acid	114.1 ± 11.5	1.17 ± 0.16	370
Rosmarinic acid	69.2 ± 5.3	1.48 ± 0.16	180
Luteolin-7-*O*-glucoside	78.0 ± 7.6	1.47 ± 0.22	200
Luteolin	5.9 ± 0.5	2.46 ± 0.44	11
Quercetin	23.5 ± 1.4	2.12 ± 0.27	45

Hepatoprotective effects of PhC were tested in co-incubations with 2 mM of *t*-BHP (5 h) in HepG2 cells. IC_{50} and the Hill slope were taken from the plotted dose–response curve (Fig. 3). IC_{80} concentration was estimated from the same dose–response curve. Values are the mean ± SEM of at least 4 independent experiments.

assay, we also show the Hill slope from the graphics used to calculate the IC_{50} (GraphPad Prism).

Measurement of the partition coefficients

The degree of hydrophobicity of the PhC was examined by measuring the partition coefficients taken in logarithm using an *n*-octanol/HEPES system (K_{ow}) as previously described (Areias et al., 2001), at ambient temperature (~ 25°C).

Statistical analysis

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

Results

t-BHP cytotoxicity

The cytotoxicity of t-BHP to liver cells has been extensively studied although its mechanisms of action have not been totally established (Sies and Summer, 1975; Cadenas and Sies, 1982; Bellomo et al., 1982; Rush et al., 1985; Jewell et al., 1986; Nicotera et al., 1988; Masaki et al., 1989; Davies, 1989; Buc-Calderon et al., 1991; Kass et al., 1992; Hix et al., 2000; Martin et al., 2001). Recently, HepG2 cells have been used to study the hepatotoxicity of *t*-BHP (Kim et al., 1998, 2000; Piret et al., 2002, 2004; Alia et al., 2005), and this model suggested to evaluate the protective properties of natural compounds and plant extracts against oxidative damages (Thabrew et al., 1997; Kinjo et al., 2003; Lee et al., 2005a,b; Alia et al., 2006). However, because the cell's response to *t*-BHP depends on culture conditions, we first studied HepG2 cells' response to *t*-

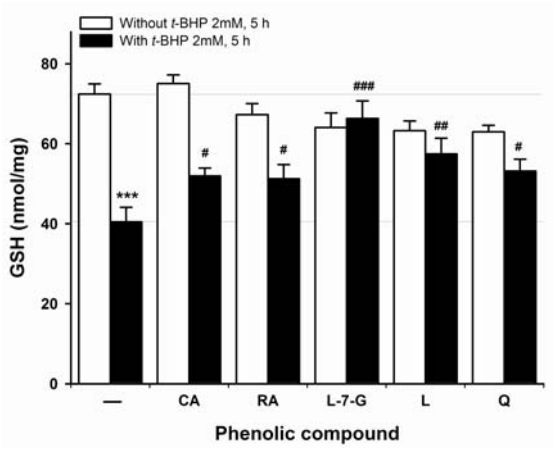


Fig. 4. Effects of *t*-BHP and PhC at the IC_{80} concentration on reduced glutathione levels in HepG2 cells. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with individual PhC (CA - caffeic acid; RA - rosmarinic acid; L-7-G - luteolin-7-glucoside; L - luteolin; Q - quercetin) at IC_{80} concentration and GSH levels measured. Values are the mean ± SEM, $n = 5$. $^{***}P \leq 0.001$ when compared with the negative control. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ and $^{\#\#\#}P \leq 0.001$ when compared with the *t*-BHP control.

Table 2
Effects of *t*-BHP nad PHC at IC_{80} concentration on lipid peroxidation and oxidized glutathione levels in HepG2 cells

Phenolic compound	*t*-BHP 2 mM, ~ 5 h	Parameter	
		TBARS (nmol/mg)	GSSG (nmol GSH equiv/mg)
–	-	0.20 ± 0.05	1.2 ± 0.3
	+	2.25 ± 0.13***	5.0 ± 0.4***
Caffeic acid	-	0.19 ± 0.10	1.2 ± 0.4
	+	1.54 ± 0.10###	4.2 ± 0.1
Rosmarinic acid	-	0.15 ± 0.05	1.2 ± 0.3
	+	1.71 ± 0.08###	3.5 ± 0.3#
Luteolin-7-glucoside	-	0.15 ± 0.02	1.3 ± 0.2
	+	1.74 ± 0.08###	3.8 ± 0.5
Luteolin	-	0.20 ± 0.07	1.6 ± 0.4
	+	1.66 ± 0.12###	4.4 ± 0.4
Quercetin	-	0.15 ± 0.06	1.5 ± 0.3
	+	1.64 ± 0.12###	4.6 ± 0.2

HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with individual PhC at IC_{80} concentration and lipid peroxidation (as estimated by TBARS assay) and GSSG levels measured. Values are the mean ± SEM, $n = 5$ (TBARS), $n = 4$ (GSSG). $^{***}P \leq 0.001$ when compared with the negative control. $^{\#}P \leq 0.05$ and $^{\#\#\#}P \leq 0.001$ when compared with the *t*-BHP control.

Table 3
Effects of *t*-BHP nad PHC at IC_{80} concentration on glutathione-related enzyme activities in HepG2 cells

Phenolic compound	*t*-BHP 2 mM, ~ 5 h	Enzyme activity (mU/mg)		
		GST	GR	GPox
–	-	24.7 ± 1.0	25.9 ± 0.8	18.2 ± 0.5
	+	23.5 ± 0.5	21.8 ± 0.9*	6.3 ± 0.6***
Caffeic acid	-	24.4 ± 0.8	25.6 ± 1.3	17.8 ± 0.3
	+	23.1 ± 0.9	23.2 ± 0.8	7.5 ± 0.8
Rosmarinic acid	-	23.1 ± 0.4	23.6 ± 0.4	16.1 ± 0.5
	+	24.5 ± 0.3	21.3 ± 0.9	5.5 ± 0.5
Luteolin-7-glucoside	-	20.6 ± 0.6**	23.6 ± 0.3	16.0 ± 0.5
	+	23.7 ± 0.6	22.7 ± 0.8	5.7 ± 0.5
Luteolin	-	22.6 ± 0.6	24.6 ± 0.3	16.1 ± 0.7
	+	26.1 ± 0.8	23.2 ± 0.8	5.6 ± 0.7
Quercetin	-	24.0 ± 1.0	25.9 ± 1.0	17.2 ± 0.4
	+	22.6 ± 0.8	22.9 ± 0.6	8.6 ± 1.1#

HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with individual PhC at IC_{80} concentration and the activities of GST, GR and Gpox measured. Values are the mean ± SEM, $n = 5$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ when compared with the negative control. $^{\#}P \leq 0.05$ and when compared with the *t*-BHP control.

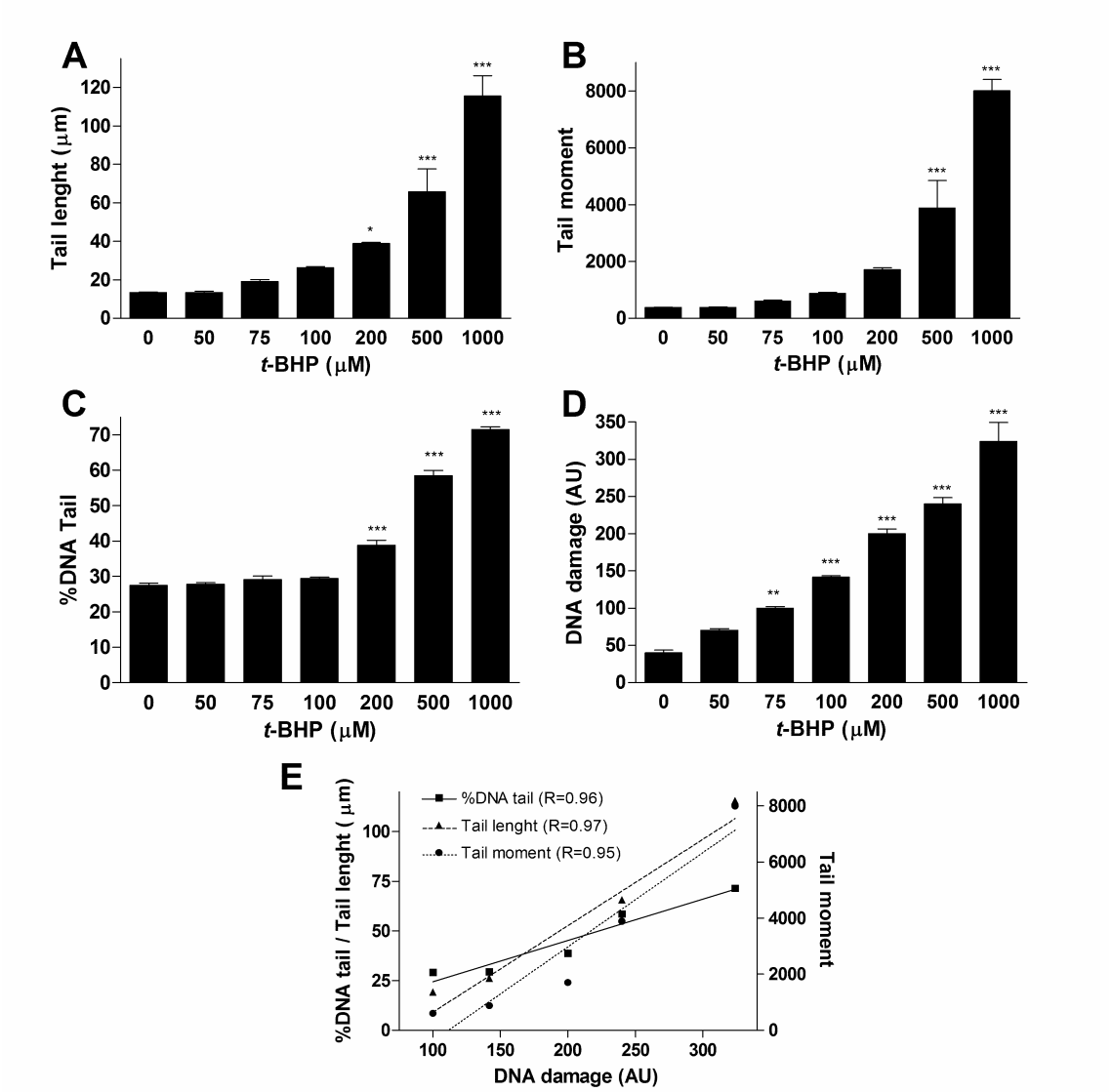


Fig. 5. *t*-BHP-induced DNA damage in HepG2 cells. HepG2 cells were incubated with different concentrations *t*-BHP for 1 h and DNA damage assessed by the comet assay. Comet images were examined by computer assisted image analysis system (A — tail length; B — tail moment; C — % DNA in the tail) and by a semiquantitative method of visual scoring (D). The correlation coefficients between the semiquantitative method and the computer assisted parameters are given in graph E. Values are the mean ± SEM, $n = 4$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared with the control.

BHP dose (1 mM and 2 mM) and incubation time (1–16 h) by measuring LDH leakage and by the MTT assay (Fig. 2). All our experiments with HepG2 cells were done with a culture medium containing 10% (v/v) FBS. LDH leakage and MTT assay gave similar results for effects on cell viability in response to *t*-BHP at both studied concentrations (Fig. 2). In the subsequent studies, cell incubations were performed with 2 mM of *t*-BHP for 5 h to induce 40–50% of cell death (Fig. 2) and used to evaluate the hepatoprotective potential of PhC against this oxidant insult.

Potential hepatoprotective effects of the PhC against t-BHP-induced toxicity in HepG2 cells

The potential hepatoprotective effects of the five PhC against the *t*-BHP-induced toxicity (2 mM, 5 h) was evaluated by determining the protection of cell viability, as measured by LDH leakage (Fig. 3) in HepG2 cells. From the graphically computed values (Fig. 3), IC_{50} and Hill slope values for each compound were obtained (Table 1). As shown in Table 1, of the tested compounds, luteolin had the highest protective activity against *t*-BHP-induced toxicity. The glycosylation of the hydroxyl group at position 7, present in luteolin-7-glucoside, significantly decreased both IC_{50} and Hill slope (Table 1). Quercetin, the flavonol of luteolin, in addition to a higher IC_{50} also had a lower Hill slope, indicating a lower hepatoprotective potential when compared to the flavone. Rosmarinic and caffeic acids had lower protective potentials against the oxidant insult to HepG2 cells when compared to the flavonoids — higher IC_{50} values and lower Hill slopes (Table 1). Comparing the phenolic acids (Table 1), the polyphenol rosmarinic acid had higher hepatoprotective potential than caffeic acid, which correlates well with the presence of one more ortho dihydroxy phenolic structure (Fig. 1).

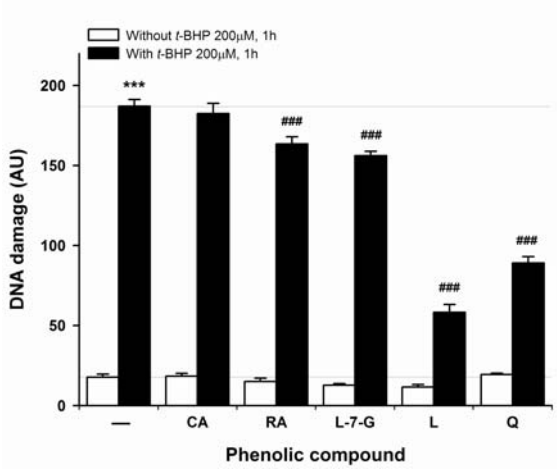


Fig. 6. Effects of *t*-BHP and PhC at IC_{80} concentration on DNA damage in HepG2 cells. HepG2 cells were incubated with *t*-BHP 200 μM (1 h) and/or with individual PhC at IC_{80} concentration and DNA damage evaluated by the comet assay. DNA damage was assessed by the semiquantitative method of visual scoring. Values are the mean ± SEM, $n = 4$. $^{***}P \leq 0.001$ when compared with the negative control. $^{###}P \leq 0.001$ when compared with the *t*-BHP control.

Based on the dose-response curves of protection from cell death, the PhC IC_{80} concentrations were extrapolated (Table 1) and used to evaluate the effects of each compound against *t*-BHP-induced oxidative injuries in HepG2 cells in terms of lipid peroxidation, glutathione levels, glutathione-related enzyme activities and DNA damage. The level of protection of cell viability obtained for each compound was correlated with the effect on each of the several parameters outlined above.

Effects of the t-BHP and PhC at the IC_{80} concentration on lipid peroxidation, glutathione levels and glutathione-related enzyme activities in HepG2 cells

The incubation of HepG2 cells with 2 mM *t*-BHP for 5 h decreased cell viability by 40–50% (Fig. 2), along with a significant increase in lipid peroxidation and GSSG levels (Table 2), as well as a decrease in GSH levels (Fig. 4). The toxicant also significantly decreased the GR and GPox activities and had no significant effect on GST activity (Table 3).

All the PhC tested at IC_{80} concentration decreased significantly the *t*-BHP-induced increase in lipid peroxidation (Table 2), caffeic acid being the most powerful with a 35% reduction and the weakest being luteolin-7-glucoside with a 25% reduction. None of the PhC significantly changed lipid peroxidation and GSSG levels in cells incubated alone (without *t*-BHP) for 5 h. As shown in Table 2, all the compounds reduced the *t*-BHP-induced increases in GSSG levels, but the effect was significant only for rosmarinic acid.

The decrease in the GSH levels induced by *t*-BHP was significantly attenuated by all of the PhC (Fig. 4). Luteolin-7-glucoside showed the best protective effect (81%) against the *t*-BHP-induced decrease in GSH levels, followed by luteolin (53%), quercetin (40%), caffeic acid (36%) and rosmarinic acid (34%). When HepG2 cells were incubated alone with the PhC for 5 h, rosmarinic acid and the three tested flavonoids slightly decreased basal GSH levels, although not significantly (Fig. 4). When this effect is taken into consideration, luteolin-7-glucoside almost completely prevented the decrease of GSH induced by the toxicant.

When incubated alone with HepG2 cells, luteolin-7-glucoside decreased significantly the GST activity by 17% (Table 3). As observed in Table 3, the *t*-BHP-induced decreases in GR and GPox activities were only slightly attenuated by the PhC, and only quercetin showed a significant protective effect (19%) on GPox activity.

t-BHP-induced DNA damage in HepG2 cells

The extent of DNA damage produced by 1-hour incubations with increasing concentrations of *t*-BHP were determined by the comet assay and the images analyzed both by computer assisted program and visual scoring. This model of *t*-BHP-induced DNA damage in HepG2 cells has been used by other authors (Woods et al., 1999, 2001). As stated previously, due to the effects of culture conditions a dose–response to *t*-BHP on

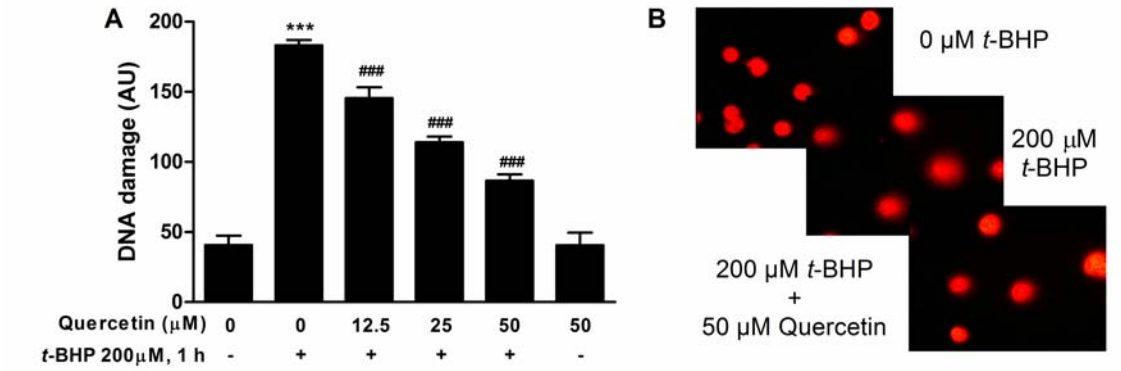


Fig. 7. Dose-dependent protection of *t*-BHP-induced DNA damage in HepG2 cells by quercetin (A). HepG2 cells were incubated with *t*-BHP 200 μM (1 h) and/or with quercetin at different concentrations and DNA damage evaluated by the comet assay. DNA damage was assessed by the semiquantitative method of visual scoring. Values are the mean ± SEM, $n = 4$. $^{***}P \leq 0.001$ when compared with the negative control. $^{###}P \leq 0.001$ when compared with the *t*-BHP control. B—Representative pictures of the comet assay results.

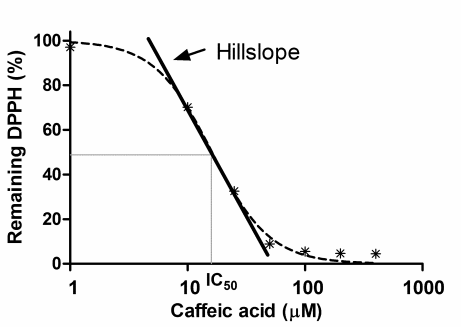


Fig. 8. Dose-dependent DPPH scavenging activity of caffeic acid. Different concentrations of caffeic acid were added to the ethanolic solution of DPPH and the discoloration measured spectrophotometrically at 515 nm. At the time point where all tested concentrations had reached the steady state (9 min), the percentages of the remaining DPPH were plotted against the corresponding caffeic acid concentrations (in logarithm). From this graph, the IC_{50} and Hill slope were taken (Table 4). Values represent the mean ± SD of 5 replicates.

DNA damage was studied. As shown in Fig. 5, *t*-BHP concentrations of 200 μM and higher result in significant DNA damage as visualized by the comet assay. The semiquantitative method of visual scoring used has been extensively validated by comparison with computerized image analysis systems and correlates well with more quantitative measures, such as % DNA in the tail and tail moment (Duthie, 2003). Our results also showed good correlations between the semiquantitative method and the parameters given by computer analysis system (Fig. 5E). To evaluate the effect of the PhC at IC_{80} concentration on *t*-BHP-induced DNA damage, HepG2 cells were co-incubated for 1 h with the different PhC plus 200 μM *t*-BHP, and the DNA damage was assessed using the alkaline version of the comet assay (results scored using the semiquantitative method). Incubation conditions of 1 h with 200 μM *t*-BHP were chosen to test the protective effects of PhC because intermediate damage to the DNA was produced (~ 200 AU).

Effects of PhC at the IC_{80} concentration on t-BHP-induced DNA damage in HepG2 cells

Of the PhC tested, quercetin and luteolin conferred the best protection against *t*-BHP-induced DNA damage (Fig. 6). Even if the IC_{80} concentration for luteolin is 4 times lower than that for quercetin (Table 1), luteolin gave better protection than the flavonol (76% and 58%, respectively) (Fig. 6). Both quercetin (Fig. 7) and luteolin (data not shown) showed a concentration-dependent DNA protection. As shown in Fig. 7B, the protective effect of quercetin was visually clear in the comet assay images. Rosmarinic acid (14%) and luteolin-7-glucoside (18%) also protected significantly from DNA damages, although to a much lower extent. At IC_{80} concentration, caffeic acid did not show protection of the DNA. None of the PhC tested induced DNA damage when incubated alone for 1 h at IC_{80} concentration (Fig. 6).

Antiradical activity

The antiradical activity of the PhC used in this study was evaluated by the DPPH and superoxide radical scavenging assays. Fig. 8 shows graphically the results from the DPPH scavenging assay of caffeic acid as an example, which was used to calculate the IC_{50} and the Hill slope for the compound. Rosmarinic acid had the best IC_{50} values both against DPPH and superoxide radicals (Tables 4 and 5). The IC_{50} values in both antiradical activity assays for caffeic acid were, as expected, significantly higher than those for rosmarinic acid, but both compounds showed similar Hill slopes. Quercetin presented lower IC_{50} values than the other flavonoids against both radicals (Tables 4 and 5). On the other hand, quercetin had the lowest AE (Table 4). Comparing luteolin with its glucoside, the aglycone had a slightly lower IC_{50} and a higher Hill slope against both radicals. In the case of the DPPH scavenging activity, the higher AE value with a similar IC_{50} means that for luteolin-7-glucoside the time needed for it to reach the steady state at the corresponding IC_{50} concentration was shorter than for luteolin.

The antiradical activity of some of these PhC has been extensively studied by many authors (Sanchez-Moreno et al., 1998; Moridani et al., 2003; Butkovic et al., 2004; Parejo et al., 2004; Kosar et al., 2004), and our results are, in general, in agreement with theirs.

Partition coefficients

The degree of hydrophobicity of the PhC was examined by measuring the partition coefficients using an n-octanol/HEPES

Table 4
DPPH scavenging activity of the tested PhC

Compound	IC_{50} (mmol/mol DPPH)	Hill slope	AE ($\times 10^{-3}$)
Caffeic acid	179.6 ± 4.1	2.03 ± 0.06	0.81
Rosmarinic acid	102.6 ± 2.2	2.07 ± 0.09	0.53
Luteolin-7-*O*-glucoside	277.3 ± 14.9	1.48 ± 0.06	1.21
Luteolin	263.9 ± 11.0	1.66 ± 0.03	0.70
Quercetin	126.0 ± 2.4	1.66 ± 0.05	0.36

Different concentrations of each PhC were added to the ethanolic solution of DPPH and the discoloration measured spectrophotometrically at 515 nm. From the results expressed as the percentage of the remaining DPPH obtained for each PhC concentration (Fig. 8), the IC_{50} and Hill slope were taken. From the results, the AE was also calculated for each PhC. Values represent the mean ± SD of 5 replicates.

Table 5
Superoxide radical scavenging activity of the tested PhC

Compound	IC_{50} (μM)	Hill slope
Caffeic acid	99.1 ± 5.3	1.02 ± 0.06
Rosmarinic acid	21.0 ± 0.9	0.95 ± 0.04
Luteolin-7-*O*-glucoside	50.4 ± 2.4	0.93 ± 0.05
Luteolin	45.3 ± 3.0	1.70 ± 0.19
Quercetin	35.1 ± 3.3	1.69 ± 0.25

Using the phenazine methosulphate-NADH nonenzymatic assay, superoxide radicals were produced continuously and measured spectrophotometrically at 560 nm. In co-incubations with individual PhC at several concentrations, the scavenging of superoxide radical was measured and from the plotted results the IC_{50} and the Hill slope were taken.Values represent the mean ± SD of 3 independent experiments with 3 replicates each.

Table 6
Experimental partition coefficients values obtained for each tested PhC

Phenolic compound	K_{ow}
Caffeic acid	-0.89 ± 0.10
Rosmarinic acid	-0.44 ± 0.13
Luteolin-7-glucoside	1.22 ± 0.01
Luteolin	2.68 ± 0.05
Quercetin	2.60 ± 0.09

Partition coefficient values in logarithm (K_{ow}) were measured in an *n*-octanol/ HEPES (20 mM, pH 7.4) system. Values are the mean ± SD of 3 independent experiments.

system. Flavonoids are much more hydrophobic than phenolic acids (Table 6). As expected, the glycosylation of the hydroxyl group at position 7 of luteolin decreased considerably the degree of hydrophobicity of this compound. Luteolin had a slightly higher K_{ow} than that of quercetin (Table 6). The experimentally determined hydrophobicity of these two flavonoids has often been referred in the literature, but the results are controversial. Some authors describe luteolin as more hydrophobic than quercetin (Brown et al., 1998; Areias et al., 2001; Murata et al., 2004) whereas others hold the opposite to be true (Moridani et al., 2003). The computer program that can be accessed at http://www.esc.syrres.com, the KowWin (LogKow) software, gives a lower degree of hydrophobicity for quercetin than for luteolin, 1.48 and 2.36, respectively. This program uses fragmental analysis of the compound's structure for the prediction and the computed values show usually a high correlation with quoted experimental values ($r^2 = 0.95$).

Discussion

The present work demonstrates that all the tested PhC possess protective effects against t-BHP-induced cell death in HepG2 cells. Conferred protection decreased in the following order: luteolin > quercetin > rosmarinic acid > luteolin-7-glucoside > caffeic acid as shown by IC_{50} values. Considering the compounds' hydrophobicity (luteolin > quercetin > luteolin-7-glucoside > rosmarinic acid > caffeic acid) and the antiradical activity evaluated both for DPPH (rosmarinic acid > quercetin > caffeic acid > luteolin > luteolin-7-glucoside) and superoxide radical (rosmarinic acid > quercetin > luteolin > luteolin-7-glucoside > caffeic acid) scavenging activities, the results show that the hepatoprotective potential of these PhC correlates primarily with their degree of hydrophobicity and only secondarily with their antiradical capacity. In fact, Rice-Evans et al. (1996) and Spencer et al. (2004) suggested that the antioxidant biological activity of PhC will depend more heavily on the extent to which they associate, interact and permeate cell membranes than on its antiradical activity alone. In agreement with this, it was only for compounds with comparable hydrophobicities, such as the two tested phenolic acids, that a direct correlation between biological activity and antiradical activity was obtained.

The importance of the compound's lipophilicity in addition to the antiradical capacity is corroborated by comparisons between structurally related compounds. When luteolin is glycosylated at position 7 in the A ring to become luteolin-7-glucoside, the compound's hydrophobicity decreases dramatically. As a result, although the antiradical activity of luteolin-7-glucoside was only slightly affected (5% to 11%), its biological activity decreased dramatically (about 13 times lower) when compared with that for luteolin. The results observed for quercetin and luteolin also implicate hydrophobicity as an important factor for this cytoprotective antioxidant effect of compounds. The absence of the hydroxyl group at position 3 (C ring) decreases the antiradical (hydrogen-donating) activity of luteolin while increasing its hydrophobicity relative to quercetin. In agreement with the previously stated, in co-incubations with *t*-BHP, luteolin showed the best protection with an IC_{50} four times lower than that for quercetin. Also, in certain types of non-cellular lipophilic oxidation systems, luteolin showed higher antioxidant effects than those of quercetin (Brown et al., 1998; Filipe et al., 2001; Hirano et al., 2001).

The importance of the compounds' hydrophobicity is also shown by comparing the results between rosmarinic acid and luteolin-7-glucoside. Although rosmarinic acid had higher antiradical scavenging activity, because the degree of hydrophobicity of luteolin-7-glucoside was higher than rosmarinic acid, both compounds showed similar biological effect (similar IC_{50} values).

Because our model of cytoprotection tests the PhC in co-incubations with the toxicant, their antioxidant effects may reflect mainly their direct actions on mediators of *t*-BHP toxicity. These direct effects include, besides the antiradical scavenging or hydrogen-donating activity measured in this study, the compounds' ability to chelate metal ions (Rice-Evans et al., 1996). Iron chelation could indeed be important for the protection against *t*-BHP toxicity, which is known to be mediated by intracellular iron ions (Hix et al., 2000). PhC may also indirectly act as antioxidants in cells by modulating the activity of antioxidant, detoxifying and repairing enzymes as well as enzymes involved in the bioactivation of xenobiotics (Ferguson, 2001; Ross and Kasum, 2002; Ferguson et al., 2004). In the present study, where short term simultaneous incubations were used, PhC protection through increased activity of glutathione-related enzymes seems not to be relevant. In fact, the activity of GST, an important phase II detoxifying enzyme (Ferguson, 2001; Ferguson et al., 2004), was decreased rather than increased in controls exposed to luteolin-7-glucoside, the only compound that had a significant effect on glutathione-related enzymes. Longer term pre-incubations would provide the opportunity for induction of proteins and enzymes, such as antioxidant enzymes, by interaction with antioxidant response elements (Ferguson et al., 2004).

t-BHP-induced cell death was accompanied by increased lipid peroxidation and GSSG levels, and DNA damage as well as decreased GSH levels and glutathione-related enzyme activity. The increase in GSSG levels was not in the same range as the decrease in GSH levels. This indicates that *t*-BHP reduced GSH levels mainly through formation of GSH conjugates rather than oxidation to GSSG. These effects are in accordance with previous studies in liver cells (Sies and Summer, 1975; Bellomo et al., 1982; Rush et al., 1985; Jewell et al., 1986; Nicotera et al.,

1988; Masaki et al., 1989; Buc-Calderon et al., 1991; Kass et al., 1992; Thabrew et al., 1997; Martin et al., 2001; Kinjo et al., 2003; Alia et al., 2005, 2006; Lee et al., 2005a,b). However, particularly in HepG2 cells, *t*-BHP exposure conditions are different among different studies published so far (Thabrew et al., 1997; Kim et al., 1998, 2000; Piret et al., 2002, 2004; Kinjo et al., 2003; Alia et al., 2005, 2006; Lee et al., 2005a,b). Previous reports indeed alert to the fact that different origins of HepG2 clones, culture medium composition and cultivation time (age of cells) may affect the experimental outcome through differences in sensitivity towards drugs (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004). It therefore becomes imperative to characterize the cells' response to the toxicant as well as the experimental conditions used for the detection of protective effects of test compounds.

In an attempt to explain the observed cytoprotective effects of the tested PhC, we looked at their effects at IC_{80} concentration on several markers of cellular oxidative stress, such as lipid peroxidation, glutathione levels and DNA damage.

t-BHP-induced lipid peroxidation in HepG2 cells was attenuated by all tested PhC at IC_{80} concentrations to a similar extent, of about 30% (25% to 35%). A good correlation seems to exist between hepatoprotective effects and the prevention of lipid peroxidation. The ability of PhC to chelate metal ions and/or to act as chain breaking antioxidants by scavenging (as hydrogen donors) lipid alkoxyl and peroxyl radicals (Rice-Evans et al., 1996; Brown et al., 1998) could provide an explanation for the observed reduction in lipid peroxidation. Nevertheless, the extent of this reduction was relatively small, only about 30%. This indicates that it is most likely not only through reduction of lipid peroxidation that PhC protect HepG2 cells against death. In agreement with this, previous reports indicated that *t*-BHP-induced toxicity was not mediated by lipid peroxidation (Rush et al., 1985; Jewell et al., 1986; Buc-Calderon et al., 1991; Martin et al., 2001). Moreover, our own observations (data not shown) and a previous work (Rush et al., 1985) reported that incubations of liver cells with the oxidant pair ascorbate/iron induced massive cell lipid peroxidation without significantly affecting cell viability. Preservation of cell viability seems therefore to depend also on effects at other levels.

All tested PhC also significantly attenuate the decrease of GSH levels induced by *t*-BHP at their IC_{80} concentrations. GSH plays an important role in hepatocyte defence against ROS, free radicals and electrophilic metabolites (Kedderis, 1996; Castell et al., 1997). A severe GSH depletion leaves cells more vulnerable to oxidative damage by radicals and increases protein thiolation or oxidation of SH groups that may lead to alterations in cellular calcium homeostasis (Castell et al., 1997). A sustained increase in cytosolic calcium levels results in activation of enzymes (phospholipases, non-lysomal proteases, endonucleases) and cytoskeletal damage which ultimately causes cell death (Castell et al., 1997). The decrease of GSH levels has indeed been suggested as one of the primary mechanisms of *t*-BHP-induced toxicity in liver cells (Jewell et al., 1986; Buc-Calderon et al., 1991; Martin et al., 2001) that is generally followed by an increase in the intracellular levels of calcium (Bellomo et al., 1982; Nicotera et al., 1988; Buc-Calderon et al., 1991; Kass et al., 1992). Thus, the potential of PhC to maintain GSH at reasonably high levels could be of great importance against *t*-BHP-induced toxicity. Therefore, the ability of the tested PhC in preventing against *t*-BHP-induced GSH depletion by about 40% was probably a major contribution to their cytoprotective effects. In the case of luteolin-7-glucoside, there was a higher protection (~ 80%) of GSH levels that did not reflect higher cytoprotection (all compounds were tested at their IC_{80} concentration). This may have been due to the observed inhibitory effect of luteolin-7-glucoside on GST having a sparing effect on GSH. Because protection by PhC against increases of GSSG levels induced by *t*-BHP was weak, it seems that PhC protect against the decrease of GSH levels mainly by preventing the formation of GSH conjugates rather than oxidation to GSSG.

In spite of this general protection of GSH, when incubated alone, PhC decreased GSH levels by 5%in the case of rosmarinic acid and between 10% and 14% for the tested flavonoids. Although not statistically significant, this effect seems to indicate some pro-oxidant activity of these compounds. Previous studies also found a decrease in GSH induced by flavonoids (Duthie et al., 1997; Galati et al., 2002). For flavonoids with a 3′,4′-dihydroxyl group on the B ring (catechol B ring), as is the case here, the decrease of GSH levels was found to be through formation of GSH conjugates instead of oxidation to GSSG (Galati et al., 2002).

Incubations of HepG2 cells with *t*-BHP induced DNA damage in a concentration-dependent manner, as visualized by the comet assay. Exposure to 200 μM *t*-BHP induced significant DNA damage without inducing cell mortality (data not shown). This seems to indicate that *t*-BHP-induced DNA damage was not implicated in the cell death induced by this organic peroxide in HepG2 cells. In fact, caffeic acid, at IC_{80} concentration, significantly decreased *t*-BHP-induced cell death without protecting DNA from damage. Also, previous reports showed a dissociation between the oxidative DNA damage induced by *t*-BHP from the killing of hepatocytes (Coleman et al., 1989; Latour et al., 1995). Latour and collaborators (1995) ruled out both the formation of oxidized DNA bases and the activation of a calcium-dependent endonuclease as mechanisms by which *t*-BHP induces DNA single strand breaks. They showed that *t*-BHP causes DNA single strand breaks most likely by covalent binding of free radicals to DNA by mechanisms dependent on iron ions (Latour et al., 1995). Iron-dependent reactions have been proposed as the key factor to the DNA damage induced by *t*-BHP since it can be prevented by iron chelators but not by free radical scavengers, such as butylated hydroxytoluene and trolox (Coleman et al., 1989; Latour et al., 1995; Guidarelli et al., 1997; Sestili et al., 1998, 2002). Recently, another study using a different model showed the importance of iron chelation on DNA protection over free radical scavenger activity (Melidou et al., 2005). In our study, where the compounds were tested at their IC_{80} concentration (concentration that protected 80% against cell death), only luteolin and quercetin conferred a very clear protection against DNA damage. An ortho dihydroxy phenolic structure is one of the requirements for PhC ability to chelate transition metal ions such as copper and iron (Rice-Evans et al., 1996; Williams

et al., 2004). All the compounds used in this study possess this element, but only luteolin and quercetin conferred noticeable protection against DNA damage. It seems therefore, that even more than in the case of preserved cell viability, the degree of hydrophobicity of the compound is an important factor for protecting from DNA damage, since this could explain the higher effects obtained for luteolin and quercetin. Also in accordance with this are the results obtained from the comparison between quercetin and luteolin themselves. Metal ion chelation ability of flavonoids appears to be not only dependent on the presence of the catechol B ring but also on an oxo group at position 4 in C ring in combination with a hydroxyl group either at position 5 or 3 (Mira et al., 2002; Williams et al., 2004). Therefore, quercetin probably has higher metal ion chelation ability than luteolin, which lacks the OH group at position 3. In fact, previous results showed higher capacity of quercetin to chelate iron and copper than luteolin (Mira et al., 2002). Our results show that luteolin, although at a concentration 4 times lower, protected DNA against damage better than quercetin, which emphasizes the importance of the compounds' lipophilicity. Also others have already drawn attention to the fact that the biological effects of a compound would be a direct function of its lipophilicity, which is expected to increase the cellular uptake of these agents, as well as their subcellular localization in lipid compartments (Sestili et al., 2002; Spencer et al., 2004). Studies using other models and/or different cell types showed that luteolin had higher potential to decrease DNA damage than quercetin (Noroozi et al., 1998; Romanova et al., 2001; Horvathova et al., 2004, 2005), or the opposite-quercetin having higher ability to reduce DNA damage than luteolin (Horvathova et al., 2003; Melidou et al., 2005). As well, higher cytoprotective effects of luteolin over quercetin were found by some authors (Kaneko and Baba, 1999; Sasaki et al., 2003), although others reported the opposite (Ishige et al., 2001). It seems, therefore, that the protective potential of luteolin and quercetin is cell type specific and/or dependent on the agent used to induce DNA damage. Nevertheless, although DNA damage induced by *t*-BHP in HepG2 cells seems not to be a crucial event for cell death, this experimental model can be of use to extensively study the protective potential of PhC against DNA damage. It would be, for example, a good model for structure–activity relationships between several classes of flavonoids.

In conclusion, the PhC studied here showed protective effects against oxidative damages induced in HepG2 cells that could be of use against liver diseases where it is known that oxidative stress plays a crucial role. Moreover, their protective potential seems to be dependent on the compound's lipophilicity in conjunction with its antioxidant activity. Their effects on protection against *t*-BHP-induced GSH depletion seem to be an important factor for preserving cell viability.

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4.5. Supplementary data

Because previous reports have stressed the antioxidant potential of water extracts of a related specie of *S. officinalis*, *Salvia fruticosa*[1,2], we present here the results of an additional study on the antioxidant potential of an infusion of this specie for comparison with *S. officinalis* (see section 4.3).

Salvia fruticosa plants were cultivated in an experimental farm located in São Pedro de Merelim (Braga), Portugal, and were collected in September, 2003. The leaves and the smaller branches of the plants were lyophilised and kept at -20ºC.

Salvia infusions (teas) were prepared in the same day following a previous methodology (see section 3.3), where 300 ml of ultrapure Milli Q boiling water was poured over 4g of lyophilised-plant material and allowed to steep for 5 min. The filtered extract was then lyophilised to dryness and the following yield in terms of initial dry weight plant material was obtained:

- *Salvia officinalis*: 25.8% (w/w);
- *Salvia fruticosa*: 20.4% (w/w).

Phenolic compounds were then analysed by HPLC/DAD as previously described (see section 3.3) and the results presented in the following table:

Phenolic Compound	*Salvia officinalis* (µg/mg extract)	*Salvia fruticosa* (µg/mg extract)
Phenolic acids		
Rosmarinic acid	52.00	71.49
Caffeic acid	0.82	0.13
Ferulic acid	0.52	0.03
3-Caffeoylquinic acid	tr	tr
5-Caffeoylquinic acid	tr	tr
Flavonoids		
Luteolin-7-glucoside	19.74	tr
6-Hydroxyluteolin-7-glucoside	-	22.66
4´,5,7,8-Tetrahydroxyflavone	0.90	28.64
Apigenin-7-glucoside	0.43	0.59

[1] Triantaphyllou K, Blekas G & Boskou D, 2001. Antioxidative properties of water extracts obtained from herbs of the species *Lamiaceae*. *International Journal of Food Sciences and Nutrition* **52**, 313-317.

[2] Matsingou TC, Petrakis N, Kapsokefalou M & Salifoglou A, 2003. Antioxidant activity of organic extracts from aqueous infusions of sage. *Journal of Agricultural and Food Chemistry* **51,** 6696-6701.

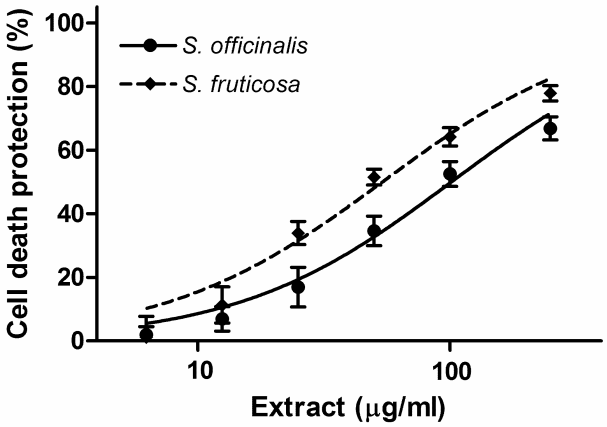
Although the yield of *S. fruticosa* infusion was lower than that of *S. officinalis*, the extract obtained had a higher content in phenolic compounds, and specially a higher content in flavonoids, that common sage.

The antiradical activity was, then, measured for both extracts against two radicals: DPPH and superoxide anion (see experimental protocol 19 - P19 - in the appendix section). The results are presented in the following table:

Infusion	DPPH		Superoxide radical
	IC_{50} (g/kg DPPH)	AE ($\times 10^{-3}$)	IC_{50} (μg/ml)
Salvia officinalis	419 ± 9	0.18	14.4 ± 1.4
Salvia fruticosa	350 ± 14	0.19	9.7 ± 0.9

As expected from the composition of the extracts in phenolic compounds, *S. fruticosa* had higher antiradical activity than *S. officinalis*, as shown by the lower IC_{50}'s and the high antiradical efficiency (AE) in the case of DPPH.

Considering these results, a high cellular antioxidant effects was expected for *S. fruticosa*. To test that, this extract was used to study the potential hepatoprotective effect of *S. fruticosa* against *t*-BHP-induced toxicity in HepG2 cells. Using the same methodology described in the section 4.3. and 4.4., the following graph was obtained for both plants:



As expected, the above graph shows that *S. fruticosa* infusion has higher hepatoprotective activity than *S. officinalis*. The same can be observed in the following table, where the IC_{50} of *S. fruticosa* is lower than of that of common sage:

Infusion	IC_{50} (μg/ml)	Hillslope
Salvia officinalis	101.4 ± 11.3	1.02 ± 0.13
Salvia fruticosa	54.0 ± 4.1	1.01 ± 0.09

Interestingly, the Hillslopes of both extracts were similar.

In conclusion, based on these results, *S. fruticosa* seems to be an interesting medicinal plant to be research in terms of antioxidant effects, with a higher activity than *S. officinalis*. The presence in the *S. fruticosa* infusion of a flavonoid aglycone, 4´,5,7,8-tetrahydroxyflavone, in much higher concentration than in *S. officinalis*, could help explain the much higher cellular antioxidant effects due to its high hydrophobicity than luteolin-7-glucoside.

Chapter 5

Sage Tea: is it Hepatoprotective *in Vivo*?

5.1. Chapter overview

Chronic liver diseases are common worldwide and may range from steatosis to hepatocellular carcinoma. Increasing evidence suggests the involvement of free radicals and reactive oxygen species in the various steps that initiate and regulate the progression of liver diseases. Therefore, protective mechanisms relevant to the liver are of particular interest and, because oxidative stress is involved in that diseases, dietary antioxidants have been proposed, as therapeutic agents, to counteract liver damages.

Salvia officinalis is a medicinal plant that can be viewed as a possible agent for prevention and/or treatment of liver diseases. According to our previous studies, this can be, in fact, a possibility. Sage tea, the most common form of consumption of this plant, was shown to improve liver antioxidant status in mice and rats (chapter 3). Besides an enhancement of a phase II detoxifying enzyme (GST), sage tea induced a better recovery of rat hepatocytes, in culture, from the stress imposed by the collagenase isolation, namely with a significant increase in GSH content after 4 hours of culture. Moreover, protection against lipid peroxidation and GSH depletion induced by an oxidant insult in cultured rat hepatocytes showed that antioxidant protection was conferred to hepatocytes by sage tea drinking. In addition, sage tea was also tested *in vitro* against an oxidant insult using HepG2 cells (chapter 4). Sage tea has shown hepatoprotective effects and the ability to increase the basal glutathione levels, which also emphasise the antioxidant properties of sage at cellular level. Recently, Amin and Hamza (2005) reported the *in vivo* hepatoprotective effect of a water extract of sage[1]. The treatment of rats with that extract, for 5 weeks, was shown to protect against the hepatotoxicity induced by azathioprine, a drug that acts by decreasing GSH levels, emphasising the antioxidant properties of this extract.

In view of all these results, the *in vivo* hepatoprotective effect of sage tea was hypothesised against toxicity induced through free radical formation, such as that caused by the well known hepatotoxin, carbon tetrachloride. Therefore, the potential hepatoprotective effect of sage tea drinking for 14 days against a subsequent acute toxic dose insult with 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 histologic liver damage. In accordance with the previous study (chapter 3), 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 CYP 2E1, the expression level of this protein was evaluated by Western Blot. An

[1] Amin A & Hamza AA, 2005. Hepatoprotective effects of *Hibiscu*s, *Rosmarinus* and *Salvia* on azathioprine-induced toxicity in rats. *Life Sciences* **77**, 266-278.

increase in CYP 2E1 protein was observed in sage tea drinking mice, which may explain, at least in part, the potentiation of CCl_4-induced hepatotoxicity, conferred by sage tea. The modulatory effects of sage tea drinking expressed by increased GST and GPox activities, and possibly other detoxifying and antioxidant enzymes, seem to have been unable of neutralising the injuries inflicted to the cells. The CCl_4-induced hepatotoxicity was shown to be higher in females than in males, which may be explained by gender differences observed in some parameters measured, such as CYP 2E1, GST and GPox.

The drug-toxicant interaction between sage tea and CCl_4 observed here, highlight possible herb-drug interactions between this extract and drugs metabolised by the liver. However, because there were no reports of herb-drug interactions between sage tea and pharmaceutical drugs, and because in this experiment sage tea replaced in almost 100% the water consumed by the animals, it seems unlikely that the normal consumption of sage tea by humans would result in interactions between sage tea and other drugs. We should, however, keep in mind that, if a sage product is taken over an extended period of time, an opportunity for enzyme induction could occur and mechanisms of interaction take place.

In conclusion, these results indicate that, although sage tea did not have toxic effects of its own and, in fact, seemed to improve the antioxidant status of the liver, herb-drug interactions are possible that may affect the efficacy and safety of concurrent medical therapy with drugs that are metabolised by phase I enzymes. In addition, based on our previous studies and, also, in the study performed by Amin and Hamza (2005), the hepatoprotective effect of sage tea *in vivo*, using different toxicity models, must not be ignored.

5.2. Methods

The following experimental protocols (see Appendix) were used in this chapter:

- P3: Biological Models – Mice as Experimental Model
- P8: Metabolite Measurements – Glutathione Levels
- P10: Metabolite Measurements – Total Protein
- P11: Enzyme Activities – Glutathione Reductase
- P12: Enzyme Activities – Glutathione Peroxidase
- P13: Enzyme Activities – Glutathione S-Transferase
- P14: Enzyme Activities – Cythocrome P450s
- P15: Enzyme Activities – NADPH-Cythocrome P450 Reductase
- P16: Enzyme Activities – ALT and AST
- P18: Others – Western Blot of CYP 2E1

5.3. Publication

This chapter comprises the following publication:

Lima CF, Fernandes-Ferreira M & Pereira-Wilson C, 2006. Drinking of *Salvia officinalis* tea increases CCl_4-induced hepatotoxicity in mice. *Food and Chemical Toxicology*, ***in press*** (DOI: 10.1016/j.fct.2006.09.009).

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

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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.

Keywords: *Salvia officinalis* L. infusion; Mice; CCl_4-induced hepatotoxicity; Herb-drug interaction; Gender differences

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 (Loguer-cio 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.,

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

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 representtative; 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 hypothesized 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 ($^{\cdot}CCl_3$) 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 ($CCl_3OO^{\cdot}$). 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 exten-sive 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.

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

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

2.3. Animals

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

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 -80ºC 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

2.5.1. Histological examinations

A fresh piece of the liver from each mouse, previously trimmed to approximately 2 mm thickness, was rapidly immersed in Bouin's solution and kept for 24 h at 4ºC. 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.

2.5.2. 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 10 µg/ml and glycerol 50% (v/v)) and centrifuged at 10,000*g* at 4ºC for 10 min and the super-natant 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 4ºC for 20 min. The super-natant was collected and centrifuged at 105,000*g* at 4ºC for 1 h. Microsomal pellets were resuspended in homogenization buffer, rapidly frozen in liquid nitrogen and stored at -80 ºC.

2.5.3. Enzyme activities

Alanine aminotransferase (ALT), aspartate aminotrans-ferase (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 37ºC 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 a resorufin formed/min/mg microsomal proteins using a standard curve of resorufin.

para-Nitrophenol 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 $mM^{-1}.cm^{-1}$.

2.5.4. 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.

2.5.5. 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 et al. (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

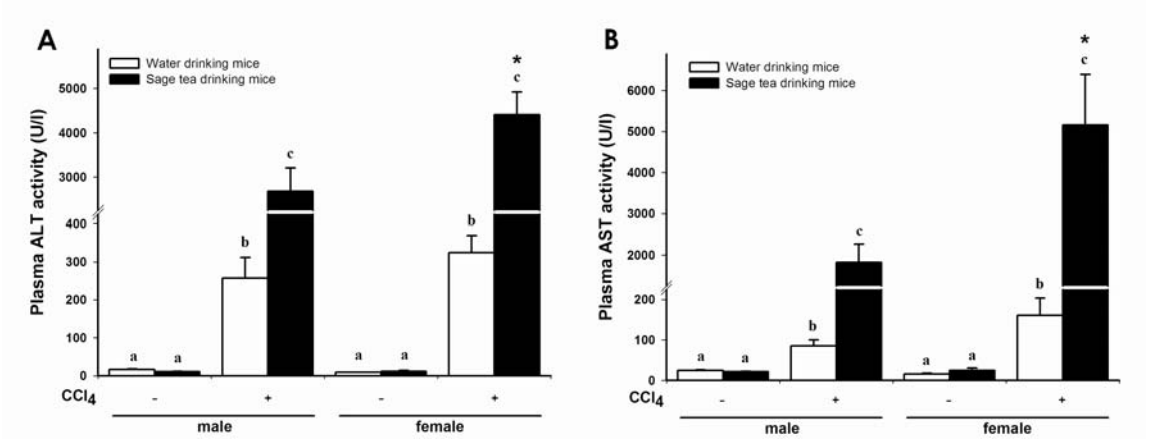

Fig. 1. Effects of sage tea drinking for 14 days on CCl_4-induced increase in plasma transaminase activities. (A) ALT: alanine aminotransferase; (B) AST: aspartate aminotransferase. Values are means ± SEM, $n = 5$. For statistical evaluation, these data were natural logarithm transformed in order to 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.

to the hepatotoxic effects of CCl_4 than their control counterparts, as indicated by increased plasma transaminase activities.

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 a 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 regions and higher leukocyte infiltration. Also histologically, the liver damage induced by the CCl_4 in mice appeared to be higher in females than in males.

CCl_4 is a hepatotoxic chemical that requires metabolic activation by phase I drug-metabolizing enzymes and therefore it was important to monitor the effects of sage tea drinking on the activity of some CYP enzymes. For that, EROD, PROD and PNP-H were measured in liver microsomal fractions (Table 2). Comparing the groups where CCl_4 was not adminis-tered, although not statistically significant, sage tea drinking increased slightly, between 8% and 13%, the activity of CYP 1A and CYP 2E1 in both genders. The activities of CYP 2B and CYP 2E1 in females was lower and higher, respectively, when compared with males. Twenty four hours after administration, CCl_4 hepatotoxicity was also reflected in the decrease observed for the activities of the CYP's measured as well as in the majority of the others enzyme activities (Table 3). Comparing drinking groups, the decrease in these enzyme activities after CCl_4 administration was also consistent with the higher toxicity in sage tea groups, since it was in general significantly higher in sage tea than water drinking mice.

The CYPR is an essential enzyme for microssomal P450-mediated monooxygenase activity, which by interaction with the different CYP's transfers the essential electron from NADPH (Backes and Kelley, 2003; Henderson et al., 2003). Therefore, its activity was measured (Table 2), and was found to be significantly higher in female mice, which indirectly may contributed to higher toxicity of CCl_4 in females. Sage tea drinking induced 21% the activity of this cytochrome, but only in female mice.

The bioactivation of CCl_4 is mainly executed by CYP 2E1 (Weber et al., 2003). It is also known that modulatory effects on the expression of CYP 2E1 affects the CCl_4-induced hepatotoxicity (Weber et al., 2003). Therefore, in

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)[a]	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] *

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

addition to the measurement of some CYP enzyme activities which included the CYP 2E1, the expression of this cytochrome was evalu-ated by Western Blot (Fig. 2). Sage tea drinking for 14 days increased significantly the amount of CYP 2E1 protein in females (24%) but 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.

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 dif-ferences 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 signi-ficantly 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.

Finally, soluble protein measured after 10,000*g* centrifugation (Table 3) corroborates the previous results. Comparing the groups where CCl_4 was not administered, the

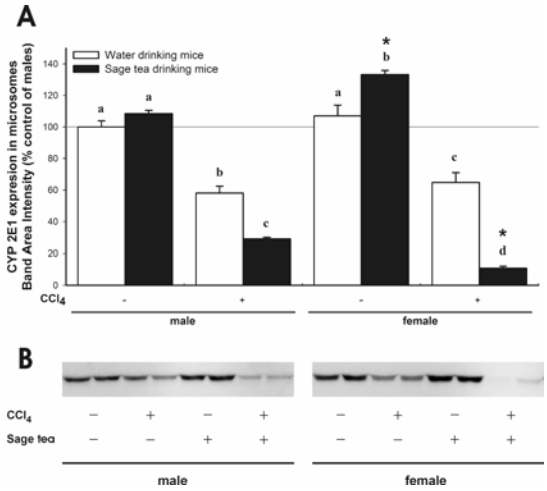

Fig. 2. Effects of sage tea drinking (for 14 days) and CCl_4 on expression of CYP 2E1 in the liver of male and female mice. Each gel lane was loaded with 15 µg of microsome proteins for the Western blotting analysis. (A) Results obtained from five mice of each group. Mean ± SEM. Groups of the same gender 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.

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higher soluble protein found in the sage tea drinking groups suggests induc-tion of protein expression. The decrease in soluble protein, with concomitant precipitation of damaged proteins, found after the haloalkane administration suggests higher toxicity of CCl_4 in the sage tea drinking groups and in females.

4. Discussion

In a previous study, sage tea drinking significantly increased (rat and mouse) liver GST activity and protected against GSH depletion and lipid peroxidation induced by an oxidant agent (Lima et al., 2005). Considering these beneficial effects on liver antioxidant status, the present study was carried out in order to evaluate whether sage tea drinking would reduce the extent of hepatic injury induced by CCl_4 in male and female mice. In a recently published work, GST was implicated as an important defence mechanism during the early stages (1–6 h) of the CCl_4-induced liver injury (Dwivedi et al., 2006). GST is a phase II enzyme that plays a key role in cellular detoxification of xenobiotics, electrophiles and reactive oxygen species through their conjugation to GSH (Mates, 2000). Besides an essential substrate to GST and GPox, GSH is also an important intracellular antioxidant (hydrogen-donating compound) that spontaneously neutralizes several electrophiles and reactive oxygen species (Lu, 1999). After bioactivation of CCl_4, in addition to dangerous free radical formation and subsequent reactive oxygen species formation, a sequence of chain reactions can be initiated that leads to lipid peroxidation (Weber et al., 2003). Since sage tea drinking has also been shown to decrease lipid peroxidation induced by *tert*-butyl hydroxide in rat hepatocyte primary cultures (Lima et al, 2005), this also suggested here possible beneficial effects against CCl_4. However, contrary to our hypothesis, sage tea drinking increased significantly the CCl_4-induced hepatotoxicity in mice.

CCl_4 becomes toxic upon activation mainly through CYP 2E1, and an induction or an over-expression of this cytochrome correlates with higher CCl_4 toxicity (Weber et al., 2003; Chan et al., 2005). Sage tea drinking for 14 days increased the expression level of CYP 2E1. In agreement with this, the activity of this cytochrome was also slightly increased by sage tea drinking. This could provide an explanation for the higher CCl_4 toxicity in tea drinking mice. CYP 2E1 protein is localized predominantly in the central zone of the liver lobule (Forkert et al., 1991), which explains the typical centrilobular region of hepa-tocyte injury observed after CCl_4 administration. This pattern of centrilobular toxicity was more extensive in sage tea versus water drinking mice. After CCl_4 bioactivation, the resulting $^{\cdot}CCl_3$ radical binds covalently to CYP 2E1, either to the active site of the enzyme or to the heme group, thereby causing suicide inactivation (Weber et al., 2003). After drug administration to sage tea drinking mice, CYP 2E1 levels, originally higher, decreased to significant lower levels. A decrease in CYP 2E1 expression and activity after CCl_4 exposure seem to reflect inactivation of the protein, which is consistent with the increased CCl_4 hepatotoxicity in this drinking group. However, to confirm increased CCl_4 bioactivation through CYP 2E1 in sage tea drinking mice than the water drinking cohorts, measurement of covalent binding of $^{14}CCl_4$-derived radiolabel to liver tissue would have to be done. The simultaneous increases in GST and GPox activities by sage tea drinking, and possibly other detoxifying and antioxidant enzymes, seem to have been incapable of neutralizing increased CCl_4 toxicity. Also, the previously observed beneficial effect of sage tea against lipid peroxidation (Lima et al., 2005) seemed to be insufficient to block CCl_4-induced damage. The increased levels of CYP 2E1 protein and activity induced by sage tea drinking may, thus, at least in part, provide an explanation for the obtained results – an herb-toxicant interaction between sage tea and CCl_4 that potentiated the haloalkane's toxicity.

Herb-drug interactions have been described for a variety of plants used as phytomedicines, many of them by case reports of interactions between herbs and pharmaceutical drugs (Izzo, 2005; Hu et al., 2005). CYP isozymes are particularly vulnerable to modulation by the diverse active constituents of herbs (Zhou et al., 2003). This important phase I drug-metabolizing enzyme system is responsible for the metabolism of a variety of xenobiotics and some important endogenous substances such as steroids and prostaglandins (Anzenbacher and Anzenbache-rova, 2001; Tamasi et al., 2003). Although CYP-mediated reactions are primarily detoxification processes, certain substrates are metabolically activated resulting in the generation of

reactive intermediates with increased toxicity and mutagenicity (Jaeschke et al., 2002; Tamasi et al., 2003). Many pharmaceutical drugs are also metabolized by these phase I enzymes and modulation of CYPs by herbs may either exacerbate the undesirable effects (by increasing toxicity) or antagonize the actions (by increasing clearance) of concurrent medical therapy (Stedman, 2002). In addition, severe hepatic injury may be caused by chemicals or natural toxins metabolically activated by drug-metabolizing enzymes as a result of occupational, household or environmental exposure, emphasizing the need for understanding mechanisms of action of herbal extracts. Thus, although interspecies differences in xenobiotic metabolism are well documented (Caldwell, 1992), the drug-toxicant interaction between sage tea and CCl_4 reported here highlight possible herb-drug interactions between this extract and drugs metabolized by the liver. However, as far as we know, there were no reports of drug-drug interactions between sage tea and 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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Chapter 6

Metformin-Like Effect of Sage Tea: is it Useful in Diabetes Prevention?

6.1. Chapter overview

Besides the important function as biotransforming organ, the liver also plays a major role in glucose homeostasis. It maintains normal concentrations of blood glucose within a narrow range by its ability to store glucose as glycogen and to produce glucose through glycogen breakdown or from gluconeogenic precursors. In type 2 diabetes, deregulation of insulin action (insulin resistance) has marked effects on hepatic glucose homeostatic pathways resulting in high circulating glucose levels. Therefore, the liver could be seen as a potential target for the treatment of diabetes and, because the available medication is far from being 100% efficient, plants are again being screened in the search for new potential agents that can help manage the disease.

Salvia officinalis is one of the plants used for the traditional treatment of *diabetes mellitus*[1] and some of its extracts have shown to have hypoglycaemic effects in normal and diabetic animals. Alarcon-Aguilar *et al.* (2002) showed that a water-ethanolic extract from *S. officinalis*, injected intraperitoneally, had hypoglycaemic effects in fasted normoglycaemic mice and in fasted mildly alloxan-induced diabetic mice[2]. In addition, Eidi *et al.* (2005) showed, recently, that a sage methanolic extract, given intraperitoneally, reduced significantly serum glucose in fasted streptozotocin-induced diabetic rats without changes in insulin levels[3]. Sage EO has also been referred to possess hypoglycaemic activity[4].

Considering the reputation of *S. officinalis* in the regulation of glucose levels, the hypoglycaemic properties of a sage tea was evaluated using normal mice and rats. Replacing water with sage tea for 14 days lowered fasting plasma glucose in normal mice but had no effect on glucose clearance in response to an intraperitoneal glucose tolerance test. This indicated effects at level of the liver, namely on gluconeogenesis. Therefore, in a following set of experiments, hepatocyte primary cultures of sage tea drinking rats showed, after stimulation (incubation with high concentrations of glucose), high glucose uptake capacity and decreased gluconeogenesis in response to glucagon. Sage EO, added *in vitro* to the cultures, further increased hepatocyte sensitivity to insulin and inhibited gluconeogenesis. Overall, these effects resemble those of the pharmaceutical drug metformin, a known inhibitor of gluconeogenesis

[1] Swanston-Flatt SK, Day C, Flatt PR, Gould BJ & Bailey CJ, 1989. Glycaemic effects of traditional European plant treatments for diabetes. Studies in normal and streptozotocin diabetic mice. *Diabetes Research* **10**, 69-73.

[2] Alarcon-Aguilar FJ, Roman-Ramos R, Flores-Saenz JL & Aguirre-Garcia F, 2002. Investigation on the hypoglycaemic effects of extracts of four Mexican medicinal plants in normal and alloxan-diabetic mice. *Phytotherapy Research* **16**, 383-386.

[3] Eidi M, Eidi A & Zamanizadeh H, 2005. Effect of *Salvia officinalis* L. leaves on serum glucose and insulin in healthy and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology* **100**, 310-313.

[4] Baricevic D & Bartol T, 2000. The biological/pharmacological activity of the *Salvia* genus. **In:** Kintzios SE (Ed.), *SAGE - The Genus Salvia*, Harwood Academic Publishers, Amsterdam, pp. 143-184.

used in the treatment and prevention of type 2 *diabetes mellitus*. The results also show an important role of the essential oil fraction for the hypoglycaemic effects of sage. In primary cultures of rat hepatocytes isolated from streptozotocin-induced diabetic rats, none of these activities induced by sage tea drinking were, however, observed.

The metformin-like effect of sage tea indicates, nevertheless, a possible type 2 diabetes preventive potential, mainly in people at risk of developing it as is the case of those who present impaired glucose tolerance. In fact, there is now substantial evidence that type 2 diabetes could be considered as a preventable disease through changes in lifestyle that include, among others, dietary factors. Therefore, sage products can easily be utilised as a functional food or food supplements that could have a beneficial impact in low cost prevention strategies of diabetes.

Besides the potential use of sage in diabetes prevention, further studies with other animal models of type 2 diabetes could provide additional information on the therapeutical effects of sage tea and/or sage EO.

Nevertheless, the regular consumption of sage tea can, in addition to help preventing type 2 diabetes through its metformin-like effect, be a source of antioxidants with their wide range of health benefits. In particular on the liver, the previous studies (chapter 3, 4 and 5) showed the ability of sage in potentiating the antioxidant defences. In turn, the beneficial effects of sage tea on the liver glutathione levels may, apart from the antioxidant and detoxifying function, also be important to glucose homeostasis. As suggested by Guarino *et al.* (2003), liver glutathione may indirectly improve the liver mediated insulin response *in vivo*[1]. Since oxidative stress is involved in diabetic complications and possibly in the onset of diabetes (see section 1.7.2), the antioxidant effects of sage may also help, indirectly, in the prevention and treatment of diabetes. Therefore, although little is known about the sage effects in this field, the study presented here opened a range of research opportunities that should be explored. For example, it is known that the erythrocyte glutathione content of patients with type 2 diabetes is significantly decreased, lowering their antioxidant potential[2]. Also in diabetic rat models, GSH content is decreased in several organs including the liver when compared with healthy controls[3]. Therefore, the beneficial effects of sage tea on GSH levels could also contribute to increase the antioxidant potential of patients with type 2 diabetes, whose defences are normally decreased,

[1] Guarino MP, Afonso RA, Raimundo N, Raposo JF & Macedo MP, 2003. Hepatic glutathione and nitric oxide are critical for hepatic insulin-sensitizing substance action. *American Journal of Physiology - Gastrointestinal Liver Physiology* **284**, G588-G594.

[2] Bravi MC, Armiento A, Laurenti O, Cassone-Faldetta M, de Luca O, Moretti A & de Mattia G, 2006. Insulin decreases intracellular oxidative stress in patients with type 2 *diabetes mellitus*. *Metabolism Clinical and Experimental* **55**, 691-695.

[3] Kamalakkannan N & Prince PSM. 2006. Rutin improves the antioxidant status in streptozotocin-induced diabetic rat tissues. *Molecular and Cellular Biochemistry*, ***in press*** (DOI: 10.1007/s11010-006-9244-1).

helping them to face an oxidative stress condition.

6.2. Methods

The following experimental protocols (see Appendix) were used in this chapter:

- P1: Biological Models – Rat Hepatocyte Isolation and Use
- P3: Biological Models – Mice as Experimental Model
- P4: Cell Viability Assays – LDH Leakage
- P9: Metabolite Measurements – Glucose
- P10: Metabolite Measurements – Total Protein
- P20: Others – Coating with Collagen

6.3. Publication

This chapter comprises the following publication:

Lima CF, Azevedo MF, Araujo R, Fernandes-Ferreira M & Pereira-Wilson C, 2006. Metformin-like effect of *Salvia officinalis* (common sage): is it useful in diabetes prevention? *British Journal of Nutrition* **96(2)**, 326-333.

Metformin-like effect of *Salvia officinalis* (common sage): is it useful in diabetes prevention?

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Common sage (*Salvia officinalis* L.) is among the plants that are claimed to be beneficial to diabetic patients, and previous studies have suggested that some of its extracts have hypoglycaemic effects in normal and diabetic animals. In the present study, we aimed to verify the antidiabetic effects of an infusion (tea) of common sage, which is the most common form of this plant consumed. Replacing water with sage tea for 14 d lowered the fasting plasma glucose level in normal mice but had no effect on glucose clearance in response to an intraperitoneal glucose tolerance test. This indicated effects on gluconeogenesis at the level of the liver. Primary cultures of hepatocytes from healthy, sage-tea-drinking rats showed, after stimulation, a high glucose uptake capacity and decreased gluconeogenesis in response to glucagon. Essential oil from sage further increased hepatocyte sensitivity to insulin and inhibited gluconeogenesis. Overall, these effects resemble those of the pharmaceutical drug metformin, a known inhibitor of gluconeogenesis used in the treatment and prevention of type 2 diabetes mellitus. In primary cultures of rat hepatocytes isolated from streptozotocin (STZ)-induced diabetic rats, none of these activities was observed. The present results seem to indicate that sage tea does not possess antidiabetic effects at this level. However, its effects on fasting glucose levels in normal animals and its metformin-like effects on rat hepatocytes suggest that sage may be useful as a food supplement in the prevention of type 2 diabetes mellitus by lowering the plasma glucose of individuals at risk.

***Salvia officinalis* L.: Diabetes: Metformin: Rat hepatocyte: Hypoglycaemic effects**

Diabetes mellitus is a disease characterised by increased plasma glucose levels that is the result of an insufficient production of (type 1 diabetes) and/or decreased tissue response to (type 2 diabetes) the pancreatic hormone insulin. Type 1 diabetes involves an autoimmune disease in which the insulin-secreting β-cells of the pancreas are destroyed by the individual's immune system. In type 2 diabetes, insulin resistance in the peripheral tissues strains insulin secretion, which leads to subsequent failure of the β-cells of the pancreas (Klover & Mooney, 2004). Type 2 diabetes accounts for the majority (85–90%) of cases and is likely to become even more prevalent over the coming decades because of the increasing rates of childhood and adult obesity and the tendency for developing countries to embrace Western lifestyles (Williams & Pickup, 2004). Nowadays, diabetes mellitus is a major public health concern that has in many countries attained epidemic proportions.

Glucose is an essential nutrient for the human body, and mechanisms of glucose homeostasis aim to maintain the blood glucose level within a narrow range, about 5–7 mmol/l (Klover & Mooney, 2004; Williams & Pickup, 2004). In healthy individuals, blood glucose concentrations are maintained by the balance between glucose entry into the circulation from intestinal absorption and glucose uptake into peripheral tissues such as muscle and adipose tissue. Circulating levels of insulin increase after meals, stimulating GLUT-4-mediated glucose uptake by the peripheral tissues, thereby preventing hyperglycaemia. During periods when there is no intestinal glucose absorption, blood levels do not decrease drastically because the liver releases glucose into the circulation in response to the counter-regulatory pancreatic hormone glucagon, which stimulates both glycogen breakdown (glycogenolysis) and gluconeogenesis (i.e. the formation of new glucose from substrates, for example glycerol, lactate and amino acids such as alanine).

The anti-hyperglycaemic effects of insulin include the suppression of glucose output from the liver, inhibiting both glycogenolysis and gluconeogenesis. In healthy individuals, relatively low concentrations of insulin are needed to suppress hepatic glucose output (Roden & Bernroider, 2003; Williams & Pickup, 2004). In type 2 diabetes, however, not only does hyperglycaemia exist postprandially, where it reveals the inability of insulin to increase peripheral glucose uptake, but also elevated blood glucose levels persist even during fasting owing to increased gluconeogenesis in the liver (Roden & Bernroider, 2003; Klover & Mooney, 2004).

Before the disease has become established, those individuals more at risk of developing type 2 diabetes show the first signs of abnormal glucose metabolism, such as impaired glucose tolerance and/or impaired fasting glucose (Simpson *et al.* 2003). This provides an asymptomatic period at the beginning of the progression of type 2 diabetes, during which preventive interventions can be applied. Previous studies have shown that

Abbreviations: EO, essential oil; FBS, fetal bovine serum; IpGTT, intraperitoneal glucose tolerance test; STZ, streptozotocin.

changes in dietary habits and sedentary behaviour can reduce the progression from impaired glucose tolerance to type 2 diabetes by 50–60% (Chiasson *et al.* 2002; Simpson *et al.* 2003). However, the difficulty of maintaining lifestyle changes in the long term justifies the need for pharmacotherapeutic support, and recent studies have shown beneficial effects of metformin and acarbose during the progression from impaired glucose tolerance to type 2 diabetes (Simpson *et al.* 2003). Metformin mainly inhibits gluconeogenesis, whereas acarbose reduces intestinal glucose absorption. But any pharmacological intervention in an asymptomatic population raises ethical considerations in addition to practical and economic issues.

Dietary supplements with glucose-lowering properties could provide a culturally acceptable and economically viable alternative to pharmaceutical interventions at this stage. However, in spite of growing interest in the effects of herbs and food supplements on glucose control in diabetes, information remains insufficient (Day, 1998; Yeh *et al.* 2003). Plants have for centuries been used in folk medicine, and their beneficial effects have been well described. *Salvia officinalis* L. (common sage) is among those which are reputed to possess antidiabetic properties (Baricevic & Bartol, 2000).

Recently, Alarcon-Aguilar *et al.* (2002) showed that a water ethanolic extract from *S. officinalis* injected intraperitoneally had hypoglycaemic effects in fasted normoglycaemic mice and in fasted mildly alloxan-induced diabetic mice. In addition, Eidi *et al.* (2005) showed that a sage methanolic extract given intraperitoneally significantly reduced serum glucose level in fasted streptozotocin (STZ)-induced diabetic rats without changes in insulin level. Sage has a high essential oil (EO) content (Giannouli & Kintzios, 2000). The EO has also been tested and proved to be hypoglycaemically active in normal and in alloxan-induced diabetic rats (Baricevic & Bartol, 2000) but not in STZ-induced diabetic rats (Eidi *et al.* 2005).

With the present study, we aimed to evaluate the hypoglycaemic properties of a sage infusion (hereafter referred to as sage tea), the most common form of human sage consumption, and to shed some light on possible mechanisms of action. In mice and rats treated for 14 d with sage tea, we evaluated *in vivo* the response to an intraperitoneal glucose tolerance test (ipGTT), and, in primary cultures of hepatocytes isolated from normal and STZ-induced diabetic rats, the effect on responses to glucose, insulin and glucagon. The *in vitro* effects of sage EO were also investigated.

Materials and methods

Chemicals

Collagenase (grade IV), William's medium E, Dulbecco's modified Eagle's medium, dexamethasone, insulin, glucagon, 1,1-dimethylbiguanide hydrochloride (metformin), STZ and Bradford reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). Glucofix for glucose measurements was acquired from A. Menarini Diagnostics (Firenze, Italy). All others reagents were of analytical grade.

Plant material, preparation of sage tea, isolation of essential oil and analysis of its constituents

Salvia officinalis L. plants were grown in an experimental farm located in Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were lyophilised and kept at -20ºC. The sage tea was routinely prepared as in a previous study by pouring 150 ml boiling water onto 2 g dried plant material and allowing it to steep for 5 min (Lima *et al.* 2005). This preparation produced a 3·5(SEM 0·1) mg extract dry weight per ml infusion, with rosmarinic acid (362 μg/ml infusion) and luteolin 7-glucoside (115·3 μg/ml infusion) as major phenolic compounds, and 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor and borneol as the major volatile components (4·8 μg/ml infusion; Lima *et al.* 2005). The EO was obtained by hydrodistillation, and the compounds were identified by GC and GC–MS in a previous work (Lima *et al.* 2004). The EO included approximately sixty compounds, the most abundant being *cis*-thujone (17·4 %), *alpha*-humulene (13·3 %), 1,8-cineole (12·7 %), *E*-caryophyllene (8·5 %) and borneol (8·3 %; Lima *et al.* 2004).

Animals

Female Balb/c mice (8–10 weeks) and male Wistar rats (150–200 g) were purchased from Charles River Laboratories (Barcelona, Spain) and acclimated to our laboratory animal facilities for at least 1 week before the start of the experiments. During this period, the animals were maintained on a natural light–dark cycle at 20(SD 2) ºC and given food and tap water *ad libitum*. The animals used in the experiments were kept and handled in accordance with our university regulations, which follow the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985).

To study the effects of sage-tea-drinking, sage tea was given to mice and rats *ad libitum* for 14 d as a replacement for their water, as previously performed (Lima *et al.* 2005). The volumes consumed were not significantly different between water and sage tea in both normal mice and rats (Lima *et al.* 2005). Diabetes was induced in rats by the intraperitoneal injection of a freshly prepared STZ solution (50 mg/kg in 0·1 M-citrate buffer, pH 4·5). Experiments with diabetic rats were carried out 1 week after STZ injection. During this period, diabetes was well established, with polydipsia, polyuria and non-fasting blood glucose levels of over 250 mg/dl. The animals were used in four different experiments.

Experiment 1

This experiment aimed to evaluate the hypoglycaemic potential of the sage tea in normal mice, an ipGTT being performed on animals from two different groups (water and sage-tea-drinking). Twenty female Balb/c mice were randomly divided into two groups, and given food and either tap water or sage tea *ad libitum* for 14 d (the beverage being renewed daily). On day 15, an ipGTT (intraperitoneal injection of 300 g glucose/l in physiological saline in a dose of 5·83 ml/kg mouse) was performed in mice that had been fasted for 3 h (half of the animals from each group being a control group, with an intraperitoneal injection of saline alone). Blood samples were collected 45 min after the intraperitoneal injection, and plasma was used for glucose measurements.

Experiment 2

In this experiment, primary cultures of rat hepatocytes from overnight fasted normal animals, in a medium with low

concentrations of glucose and a gluconeogenic substrate (lactate), were used to evaluate the modulation by sage tea of hepatocyte glucose production. Eight male Wistar rats were randomly divided into two groups and given food *ad libitum* with either tap water or sage tea *ad libitum* for 14 d (the beverage being renewed daily). Hepatocyte isolation was performed between 10.00 and 11.00 hours by collagenase perfusion, as previously described by Moldeus *et al.* (1978) with some modifications (Lima *et al.* 2004), from overnight-fasted normal animals. Cell viability was over 85%, as estimated by a trypan blue exclusion test.

Cells were suspended in Dulbecco's modified Eagle's medium (containing 5·6 mM-glucose) supplemented with 10 mM-lactate, fetal bovine serum (FBS; 100 ml/l), 10^{-9} M-insulin and 10^{-9} M-dexamethasone, and were seeded onto six-well culture plates at a density of 1×10^6 cells/well. Cells were incubated at 37ºC in a humidified incubator gassed with 50 ml/l CO_2/air. After plating (to allow for cell attachment), culture medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10 mM-lactate, 100 ml FBS/l and none, one or more of the following compounds: 10^{-7} M-glucagon, 10^{-3} M-metformin and/or sage EO (4 nl/ml). After 24 h incubation, the medium was recovered for glucose quantification. Metformin was used as positive control.

Experiment 3

In this experiment, primary cultures of normal rat hepatocytes in media with high (11 and 22 mM) concentrations of glucose (to mimic postprandial and diabetic conditions) were used to evaluate the effect of the sage tea on the glucose consumption capacity of the cells. Eight male Wistar rats were randomly divided into two groups and given food *ad libitum* with either tap water or sage tea *ad libitum* for 14 d (the beverage being renewed daily).

Hepatocytes were isolated from normal animals as described above, and cells were suspended in William's medium E (containing 11 mM-glucose) supplemented with 100 ml FBS/l, 10^{-9} M-insulin and 10^{-9} M-dexamethasone, and seeded onto six-well culture plates at a density of 1×10^6 cells/well. Cells were incubated at 37ºC in a humidified incubator gassed with 50 ml/l CO_2/air. After plating, culture medium was replaced with William's medium E supplemented with 100 ml FBS/l and none, one or more of the following compounds: glucose (to a final concentration of 22 mM), 10^{-7} M-insulin and/or 4 nl sage EO/ml. After 24 h incubation, the media were recovered for glucose quantification.

Experiment 4

In this experiment, primary cultures of hepatocytes from STZ-induced diabetic rats were used in media with low and high concentrations of glucose (both containing the gluconeogenic substrate lactate) to evaluate effects of sage-tea-drinking on cell glucose production. Eight STZ-induced diabetic rats (male Wistar) were randomly divided into two groups and given food *ad libitum* with either tap water or sage tea *ad libitum* for 14 d (the beverage being renewed daily). Because the diabetic rats were polydipsic, sage-tea-drinking animals were pair-fed with the non-diabetic animals given diluted sage tea, in order to ensure a similar intake of tea dry weight to that of the rats in experiments 2 and 3.

Hepatocyte isolation from diabetic animals were performed as above, and cells were suspended in Dulbecco's modified Eagle's medium containing either 5·6 mM or 22 mM-glucose, supplemented with 10 mM-lactate, 100 ml FBS/l, 10^{-9} M-insulin and 10^{-9} M-dexamethasone, and seeded onto six-well culture plates at a density of 1×10^6 cells/well. The culture plates were incubated at 37ºC in a humidified incubator gassed with 50 ml/l CO_2/air. After plating, culture medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10 mM-lactate, 100 ml FBS/l and none, one or more of the following compounds: glucose (to a final concentration of 22 mM), 10^{-7} M-insulin, 10^{-7} M-glucagon, 10^{-3} M-metformin and/or 4 nl sage EO/ml. After 24 h incubation, the medium was recovered for glucose quantification. Metformin was used as a positive control.

Plating periods of 24 h were used in cell cultures from normally fed animals for cell attachment. In an attempt to preserve the altered physiological conditions, introduced by both the fasting and the STZ-induced diabetes, cells were plated for 3 h before exposure to the different test conditions. In the fasted condition, the results of plating for 24 h are also presented for comparison.

In all experiments with rat hepatocytes, lactate dehydrogenase activity was measured in the media to ensure no toxicity of the treatment on the cell layer.

Biochemical analysis

The concentrations of glucose in the mouse plasma and culture media were measured using a colorimetric enzymatic method (Glucofix) following the manufacturer's specifications.

The lactate dehydrogenase activity of the culture media was used as an indicator of hepatocyte plasma membrane integrity. The activity of the enzyme was measured at 30ºC by quantifying NADH consumption by continuous spectrophotometry on a plate reader (Spectra Max 340pc; Molecular Devices, Sunnyvale, CA, USA; Lima *et al.* 2005).

Protein content was measured with the Bradford reagent purchased from Sigma-Aldrich using bovine serum albumin as a standard.

Statistical analysis

Data are expressed as means with standard errors of the means (SEM). Two-way ANOVA followed by the Student–Newman–Keuls *post hoc* test (SigmaStat, version 2.03; SPSS Inc., San Rafael, CA, USA) was employed in experiment 1 to compare the effects of the *in vivo* beverage (water *v.* sage tea) and the ipGTT (intraperitoneal saline *v.* intraperitoneal glucose). In experiments 2, 3 and 4 (in which two replicates were used for each experimental condition), the same statistical test was employed to compare the effects of the *in vivo* beverage (water *v.* sage tea) and the *in vitro* treatments (in this case, when a significant effect was obtained, a paired student's *t* test was employed to find the differences between each treatment). *P* values ≤ 0·05 were considered statistically significant.

Results

Experiment 1

A period of 14 d of sage-tea-drinking significantly lowered fasting (3 h and 45 min) plasma glucose concentration from 8·8 mM to 6·8 mM ($P \leq 0{\cdot}01$) in normal mice (Table 1). In response to an ipGTT, a significant increase in plasma glucose was observed at 45 min in both groups (Table 1), although no differences were observed between the water and sage-tea-drinking groups.

Experiment 2

When hepatocytes from overnight fasted rats were plated with 5·6 mM-glucose- and 10 mM-lactate-containing medium, there was a release of glucose (mainly due to gluconeogenesis) into the medium. Hepatocyte glucose production increased in response to glucagon (Fig. 1(A)) in cells from water-drinking animals but was not statistically significant in cells from sage-tea-drinking animals (Fig. 1(A,B)). In general, hepatocyte glucose production (Fig. 1(A)) was lower in cells isolated from sage-tea-drinking rats than in those of water-drinking controls ($P \leq 0{\cdot}05$), and the difference became significant in the glucagon + EO groups (Fig. 1(A)). When incubated with sage EO, a significant decrease in hepatocyte glucose production was observed in both drinking groups (Fig. 1(A)). In co-incubations with glucagon, sage EO significantly decreased glucose production in response to glucagon (Fig. 1(A)).

Metformin (a known inhibitor of gluconeogenesis) significantly lowered hepatocyte glucose production capacity, even when co-incubated with glucagon, in cells from both water- and tea-drinking animals (Fig. 1(B)).

Experiment 3

Hepatocyte glucose consumption measured after 24 h incubation was higher in 22 mM-glucose medium (Fig. 2(B)) than in 11 mM-glucose medium (Fig. 2(A)), and increased in response to insulin. Glucose consumption was significantly higher ($P \leq 0{\cdot}01$) in cells isolated from tea dinking rats under all circumstances tested.

When the cells were incubated with sage EO, no significant differences in hepatocyte glucose consumption were obtained, although consumption was higher in cells isolated from the sage-tea-drinking animals. In co-incubations with insulin, sage EO significantly potentiated the hormone's effects on glucose consumption (Fig. 2(A,B)).

Experiment 4

In contrast with the situation for cells from healthy animals, when hepatocytes from STZ-induced diabetic rats were plated with medium containing 22 mM-glucose (and 10 mM-lactate), glucose production (and not consumption) was observed. There was no stimulation of glucose consumption by insulin (Fig. 3). Sage-tea-drinking did not modify this situation. In addition, EO did not inhibit hepatocyte glucose production. Only metformin was able to reduce the glucose production of hepatocytes isolated from diabetic water and sage-tea-drinking rats (Fig. 3).

When hepatocytes from STZ-induced diabetic rats were plated with 5·6 mM-glucose (and 10 mM-lactate) containing medium, glucose production was similar in cells isolated from both water and sage-tea-drinking rats (Fig. 4). Glucagon did not further stimulate glucose production (Fig. 4). As above, no effect was observed for EO. Once again, metformin significantly reduced (by about 60%) hepatocyte glucose production in cells from both water and sage-tea-drinking rats (Fig. 4).

All the treatments in the primary cultures did not induce lactate dehydrogenase release to the medium, an indicator that there was no cell toxicity in any of the *in vitro* treatments.

Table 1. Plasma glucose concentration in mice in response to an intraperitoneal glucose tolerance test (ipGTT; 45 min) performed on mice fasted for 3 h previously treated with or without sage tea for 14 d

(Values are means and standard errors of the mean, *n* 5)

		Plasma glucose (mM)	
Group	*In vivo* beverage	Mean	SEM
Control	Water	8·8	0·6
	Sage tea	6·8 **	0·4
ipGTT	Water	10·5 *	0·3
	Sage tea	10·4 †††	0·4

Mean values were significantly different compared with the water + control group *$P \leq 0{\cdot}05$, **$P \leq 0{\cdot}01$.

Mean values were significantly different when compared with the water + control group: ††† $P \leq 0{\cdot}001$.

Discussion

The present work shows that sage-tea-drinking significantly reduced fasting plasma glucose level in mice. This suggested an inhibition of gluconeogenesis and/or glycogenolysis in the liver. In agreement with this, rat overall hepatocyte glucose production was lower in cells isolated from sage-tea-drinking animals than controls. Furthermore, stimulation with glucagon did not increase gluconeogenesis significantly in cells from sage-tea-drinking animals. Sage EO, although not as effective as metformin, produced a significant decrease in hepatocyte gluconeogenesis. In addition, the response of the hepatocytes to insulin was significantly increased by sage EO. These data suggests a metformin-like effect for sage tea and in particular for the EO fraction of *Salvia officinalis* L. These effects were, however, not observed in hepatocytes isolated from STZ diabetic animals, in which only metformin, a drug used in the treatment and prevention of type 2 diabetes, was effective in reducing glucose production. The effects of metformin were not modified by sage-tea-drinking, which seems to imply that sage tea, although not effective in diabetics, would not interfere negatively with metformin therapy.

Although using a different extract and experimental methodology, the hypoglycaemic effects of sage have previously been reported by others (Alarcon-Aguilar *et al*. 2002; Eidi *et al*. 2005). Alarcon-Aguilar *et al*. (2002) showed that, 4 h after an intraperitoneal injection of a sage water ethanolic extract, blood glucose decreased significantly in fasted normal mice and in fasted mildly alloxan-diabetic mice, but not in fasted severely alloxan-diabetic mice. Although the authors stated that insulin might have mediated the hypoglycaemic effect of

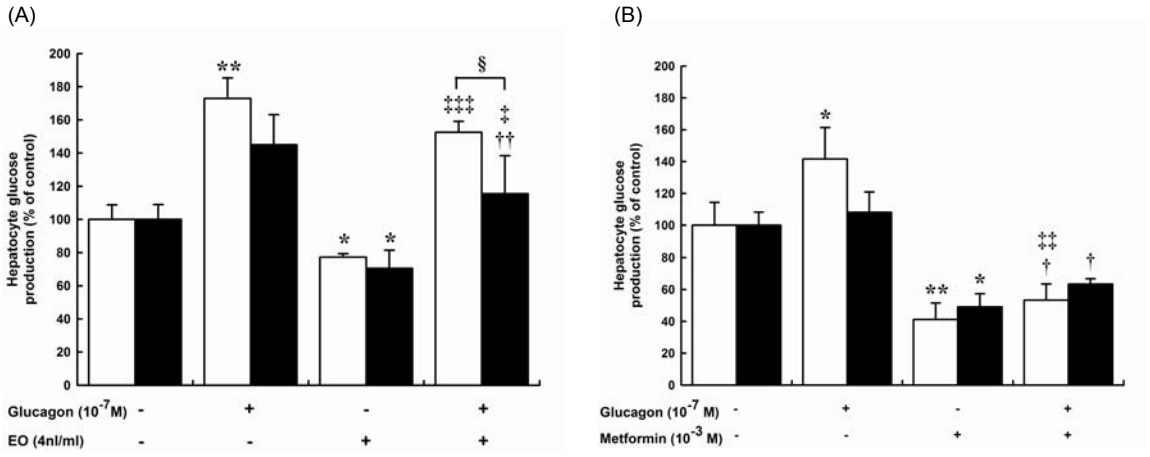

Fig. 1. Hepatocyte glucose production (24 h) by primary cultures of rat hepatocytes (isolated from overnight-fasted animals; □) and the effects of previous *in vivo* treatment with sage tea (for 14 d, ■) on hepatocyte responses to glucagon (10^{-7} M), essential oil (EO; 4 nl/ml) and metformin (10^{-3} M). The initial glucose concentration of the medium was 5·6 mM, and experiments were performed 24 h (A) or 3 h (B) after plating. Values were means with standard errors of the means shown by vertical bars (*n* 4). (A) Water-drinking rats: 100% = 6·4 (SEM 0·6) μmol glucose/mg protein; sage-tea-drinking rats: 100% = 6·2 (SEM 0·6) μmol glucose/mg protein. (B) Water-drinking rats: 100% = 8·2 (SEM 1·2) μmol glucose/mg protein; sage-tea-drinking rats: 100% = 9·5 (SEM 0·8) μmol glucose/mg protein. Mean values were significantly different compared with the respective control group: **P* ≤ 0·05, ***P* ≤ 0·01. Mean values were significantly different compared with the respective glucagon group: †*P* ≤ 0·05, ††*P* ≤ 0·01. Mean values were significantly different compared with the respective EO group (A) or metformin group (B): ‡*P* ≤ 0·05, ‡‡*P* ≤ 0·01, ‡‡‡*P* ≤ 0·001. Mean value was significantly different between the water and sage tea groups: §*P* ≤ 0·05.

the extract, once the animals were tested in the fasted condition, it seems likely that an inhibition of gluconeogenesis was the cause of the effects observed in their study, as indeed suggested by the present results. Additionally, Eidi *et al*. (2005) showed that, 3 h after an intraperitoneal injection of a sage methanolic extract, blood glucose decreased significantly in fasted STZ-diabetic rats but not in fasted normal rats. This effect was not accompanied by an increased release of insulin (Eidi *et al*. 2005).

In human subjects, the abnormal glucose metabolism observed in both prediabetic states and in overt type 2 diabetes results in part from a deregulation of glucose production by the liver, which is mainly caused by an unrestrained glucagon stimulation of gluconeogenesis. In these individuals, therefore,

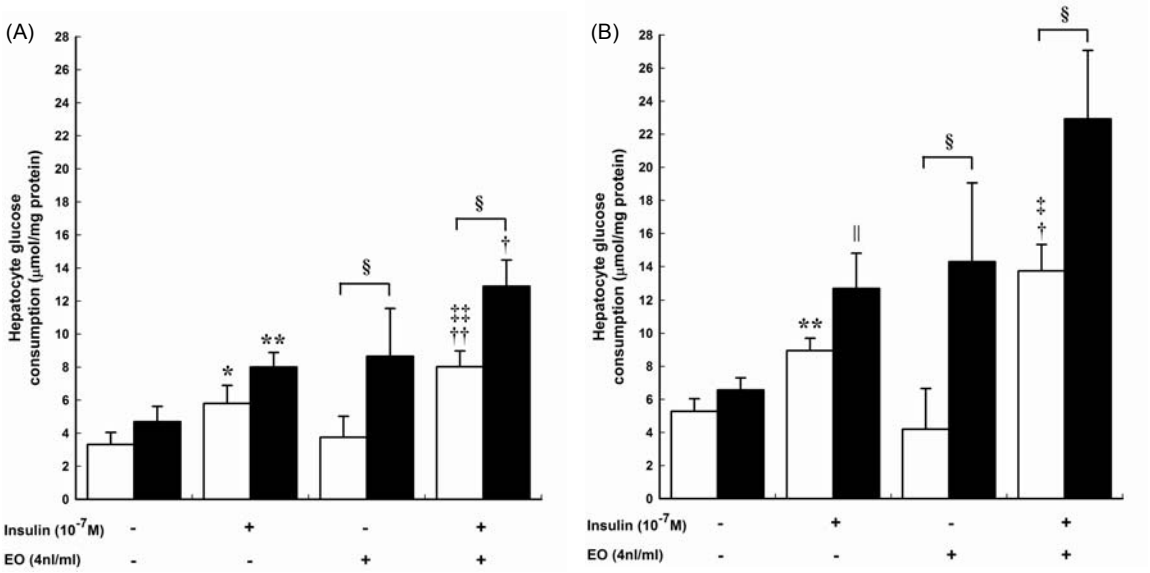

Fig. 2. Glucose consumption (24 h) by rat hepatocytes in primary cultures (□), and effects of previous *in vivo* treatment with sage tea (for 14 d; ■) on hepatocyte responses to insulin (10^{-7} M) and essential oil (EO; 4 nl/ml). The initial glucose concentrations of the medium were 11 mM (A) and 22 mM (B). Values were means with standard errors of the means shown by vertical bars, *n* 4. Mean values were significantly different compared with the respective control group: **P* ≤ 0·05, ***P* ≤ 0·01. Mean values were significantly different compared with the respective insulin group: †*P* ≤ 0·05, ††*P* ≤ 0·01. Mean values were significantly different compared with the respective EO group ‡*P* ≤ 0·05, ‡‡*P* ≤ 0·01. Mean value was significantly different between the water and sage tea gruops: §*P* ≤ 0·05. Mean value was almost significant compared with respective control group: ‖*P* = 0.573.

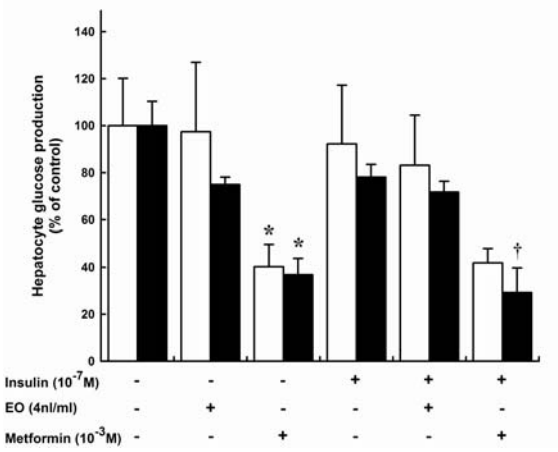

Fig. 3. Hepatocyte glucose production (24 h) by primary cultures of rat hepatocytes (isolated from streptozotocin-induced diabetic animals; □), and the effects of previous *in vivo* treatment with sage tea (for 14 d; ■) on hepatocyte responses to insulin (10^{-7} M), essential oil (EO; 4 nl/ml) and metformin (10^{-3} M). The initial glucose concentrations of the medium were 22 mM, and experiments were performed 3 h after plating. Values were means with standard errors of the means shown by vertical bars, *n* 4. Water-drinking rats: 100% = 8·4 (SEM 1·7) μmol glucose/mg protein, sage-tea-drinking rats: 100% = 8·7 (SEM 0·9) μmol glucose/mg protein. Mean values were significantly different compared with the respective control group: **P* ≤ 0·05. Mean values was significantly different compared with the respective insulin group: †*P* ≤ 0·05.

gluconeogenesis is active even when plasma glucose concentrations are already elevated, which further aggravates hyperglycaemia (Roden & Bernroider, 2003). By analogy with the effects of the drug metformin, used in the prevention and treatment of diabetes, the observed decrease in hepatocyte glucose production of sage-tea-drinking animals could be favourable, by

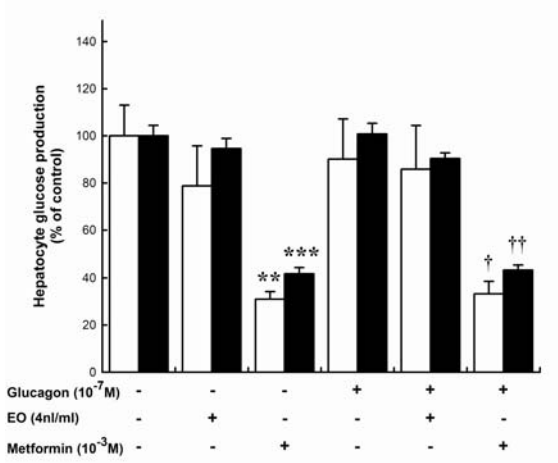

Fig. 4. Hepatocyte glucose production (24 h) by primary cultures of rat hepatocytes (isolated from streptozotocin-induced diabetic animals; □), and the effects of previous *in vivo* treatment with sage tea (for 14 d; ■) on hepatocyte responses to glucagon (10^{-7} M), essential oil (EO; 4 nl/ml) and metformin (10^{-3} M). The initial glucose concentration of the medium was 5·6 mM, and experiments were performed 3 h after plating. Values were means with standard errors of the means shown by vertical bars, *n* 4. Water-drinking rats: 100% = 7·9 (SEM 1·0) μmol glucose/mg protein, sage-tea-drinking rats: 100% = 7·6 (SEM 0·3) μmol glucose/mg protein). Mean values were significantly different compared with the respective control group: ***P*≤ 0·01, ****P* ≤ 0·001. Mean values were significantly different compared with the respective glucagon group: †*P* ≤ 0·05, ††*P* ≤ 0·001.

preventing the liver's contribution to hyperglycaemia in groups at risk. Metformin is a derivative of guanidine, the active compound of goat's rue (*Galega officinalis*; Williams & Pickup, 2004). It acts by reducing liver glucose production and increasing the action of insulin (Chiasson *et al*. 2002). Also, in the present experiments, metformin showed these effects in both hepatocytes isolated from normal and STZ diabetic rats.

Generally, sage-tea-drinking increased rat hepatocyte glucose consumption, decreased fasting gluconeogenesis and inhibited the stimulation of hepatic glucose production by glucagon. However, in spite of decreasing plasma glucose, sage tea did not, after an *in vivo* ipGTT in mice, improve glucose clearance, which suggests that sage tea did not increase the insulin response *in vivo*. In addition, the stimulatory effects of *in vitro* insulin on glucose consumption were observed only in co-incubations with EO. This suggests a role for EO in the increase in sensitivity to insulin. Increased insulin sensitivity has been suggested as a possible mechanism of action of other plant extracts with attributed hypoglycaemic activities (Li *et al*. 2004; Qin *et al*. 2004; Saxena & Vikram, 2004; Han *et al*. 2005). The presence of low amounts of EO in sage tea, about 4·8 mg/ml (Lima *et al*. 2004), could explain the lack of an increase in insulin sensitivity observed after tea-drinking both *in vivo* and *in vitro*. We cannot exclude the fact that higher doses of the tested compounds administered by either gavage or intraperitoneal injection could produce similar effects as *in vitro*.

Also, in STZ-induced diabetic rat hepatocytes, sage-tea-drinking and EO *in vitro* lead to no significant improvement in the response to insulin. The liver (and hepatocytes) usually suppresses glucose release in response to insulin (Klover & Mooney, 2004). In STZ rat hepatocytes, insulin administration failed to suppress glucose production. Previous studies have also indicated that insulin is incapable of stimulating glucose utilisation *in vitro* by hepatocytes from STZ-induced diabetic rats (Salhanick *et al*. 1983; Amatruda *et al*. 1984; Hussin & Skett, 1988). The insulin resistance imposed by STZ treatment was not reversed by sage tea and/or EO. The lack of effect of sage tea/EO on STZ hepatocytes seems to indicate that sage requires an intact insulin signalling pathway to produce its effects. In the STZ diabetic rat, stimulation of the hepatocytes with glucagon did not enhance gluconeogenesis. Others have also failed to significantly stimulate gluconeogenesis *in vitro* in hepatocytes from STZ-induced diabetic rats (Dunbar *et al*. 1989).

One possibility for the lack of glucagon stimulation of gluconeogenesis in diabetic hepatocytes is that gluconeogenesis, *in vivo*, had been maximally stimulated. Cells are, however, metabolically competent and respond to metformin with a decrease in glucose production. In STZ-treated rats, insulin deficiency increases gluconeogenesis through enhanced lactate and pyruvate uptake and flux through the enzyme phosphoenolpyruvate carboxykinase (Large & Beylot, 1999). Metformin has been shown to reduce substrate flux through this enzyme (Large & Beylot, 1999) and to inhibit phosphoenolpyruvate carboxykinase gene expression (Cheng *et al*. 2001; Yuan *et al*. 2002), thereby decreasing gluconeogenesis. This inhibition of gene expression seems to occur mainly through an insulin-independent pathway (Yuan *et al*. 2002). This agrees with the possibility that sage tea and/or sage EO requires an intact insulin signalling pathway to produce its effects, which were observed only in normal rats.

The reduction in fasting plasma glucose shown in healthy animals indicates the potential for sage extracts to help prevent type 2 diabetes through a metformin-like effect, mainly in people at risk of developing it, as is the case of those who present impaired glucose tolerance and impaired fasting glucose. Taking into consideration the high worldwide, and increasing, prevalence of type 2 diabetes and the high costs involved in its treatment, the primary prevention of this disease is an important issue (Lai, 2002; Costacou & Mayer-Davis, 2003; Jermendy, 2005). Considering that there is now substantial evidence that type 2 diabetes could be considered to be a preventable disease through changes in lifestyle that include, among others, dietary factors (Costacou & Mayer-Davis, 2003; Schernthaner, 2003; Simpson *et al*. 2003; Stoeckli & Keller, 2004; Jermendy, 2005), the search for preventive strategies should be actively promoted. Sage products can easily be considered as functional foods or food supplements that could have a beneficial impact in low-cost prevention strategies for diabetes. In addition, there are health benefits from the use of plant extracts as sources of antioxidants. With particular regard to the liver, sage tea has been shown not to have toxic effects and to improve liver glutathione levels (Lima *et al*. 2005). Among other effects, this may indirectly improve the liver-mediated insulin response *in vivo* (Guarino *et al*. 2003). We are currently undertaking a pilot study with human volunteers to test the effects on the control of glycaemia in individuals at risk of developing diabetes. Experiments with animal models of type 2 diabetes, such as the Zucker rat (Sreenan *et al*. 1996), could also provide additional information on the therapeutic effects of sage tea.

Acknowledgements

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Reprinted from *British Journal of Nutrition* (Cambridge University Press), 96, Lima CF, Azevedo MF, Araujo R, Fernandes-Ferreira M, Pereira-Wilson C, Metformin-like effects of *Salvia officinalis* (common sage): is it useful in diabetes prevention?, 326-333, Copyright (2006), with permission from the authors.

Chapter 7

Final Considerations

7.1. General discussion and conclusions

This section aims at making an integrated discussion of the most relevant results presented and discussed in detail in the previous chapters.

Salvia officinalis L is an aromatic and medicinal plant very popular worldwide with reputation in the treatment of different ailments. However, few pharmacological effects have been experimentally investigated, until today, in order to confirm their recognised benefits. Some studies have focused on their anti-inflammatory, anti-viral, estrogenic and memory-improving activities, and some experiments were already performed in patients, in addition to using cells and laboratory animals.

With the studies presented in this thesis, the main objective was to increase our knowledge about two attributed effects of *S. officinalis* – the antioxidant and antidiabetic effects. Regarding the first aim, the antioxidant power of sage is well known. However, the majority of the studies performed until the beginning of these studies used chemical and subcellular assays and, therefore, studies with cells and organisms were awaited. Regarding the second aim, few studies were performed to confirm the antidiabetic and/or hypoglycaemic activity of sage, and the ones performed were often inconclusive.

The antioxidant and antidiabetic studies were focused on the liver since sage compounds first pass by this organ after gastrointestinal absorption, which gives an opportunity to concentrate them. The liver is also a biotransforming organ, which renders it particularly susceptible to the toxic effects of xenobiotics. In addition, the liver is affected by several liver diseases where oxidative stress is involved. Therefore, cytoprotective activities relevant to the liver are of particular interest. These studies allowed also the observation of possible toxic effects of sage and its interactions with other drugs.

Regarding sage constituents, the studies were focused on the essential oil fraction (extract rich in low weight volatile compounds), a methanolic extract (extract rich in phenolic compounds) and a sage water extract prepared as a tea (extract rich in very polar compounds, which include also phenolic compounds). The last extract is also very important to study since it is the most common form of human consumption of this plant. Some major phenolic compounds of the extracts were also tested alone in some experiments (chapter 4).

The major studies reported, focused on the antioxidant effects of sage at cellular (hepatocyte) and organ (liver) levels. To assess the antioxidant effects of the sage extracts, conventional markers of cell oxidative stress were used, such as, lipid peroxidation, glutathione levels and DNA damage. The monitorisation of GSH levels seemed very important in view of its central role on oxidative stress defence and prevention of cell damage and death. Furthermore, the measurement of glutathione-related enzyme activities was, as much as

possible, performed in order to give a broader idea of the possible involved mechanisms of cell defence.

Firstly, the potential antioxidant effects of sage EO was tested in freshly isolated rat hepatocytes (chapter 2). Although sage EO is considered to have some antioxidant effects, these were to our knowledge never tested in cell systems. Our study demonstrated that sage EO does not have antioxidant effects in liver cells. On the contrary, at high concentrations (more than 2 µl/ml), sage EO induced significant cell death accompanied by GSH depletion, but not by lipid peroxidation. The GSH depletion induced by sage EO could be an explanation for its toxic effects. The lack of antioxidant effects of sage EO may be a result of the relative low concentration of compounds considered as antioxidants in this sage extract. This study revealed the toxic effects of high concentrations of sage EO to the liver besides their known neurotoxic effects due to its constitution in thujones and champhor.

Then, after an *in vivo* experiment with mice, where sage tea drinking (for 14 days) strengthened the liver antioxidant status (chapter 3), such as an improvement of GST activity and GSH levels as well as the prevention of lipid peroxidation and GSH depletion, the direct effects of a sage water (as a tea) extract and a methanolic extract were studied on HepG2 cells (chapter 4). Both extracts protected against cell death induced by *t*-BHP (a toxicant that induces oxidative stress), revealing the direct antioxidant effects of sage at cellular level. Sage was capable of acting at a cell critical parameter, namely GSH, preventing GSH depletion. A severe GSH depletion is known to mediate cell death by different mechanisms. The increase on GSSG/GSH ratio tends to decrease protein thiols that lead to severe toxicological implications, such as, disruption of cytoskeleton and calcium homeostasis as well as an increase of mitochondria permeability. Also, because GSH is an important intracellular antioxidant, GSH depletion leaves cells more vulnerable to oxidative stress. The studies presented in chapter 4 showed a dependence of the cytoprotective effects on the total content of phenolic compounds and the antiradical activity of the sage extracts. The results obtained with the phenolic compounds underscored the importance of the compound's lipophilicity, in addition to its antioxidant potential, for the overall biological activity. The lipophilicity of the natural antioxidants appeared to be of an even greater importance for their ability to protect DNA from oxidative damage.

Also in chapter 4, not only did sage extracts prevent GSH depletion in a situation of oxidative stress, they also increased basal GSH levels in HepG2 cells. This indicates a capacity of sage extracts to improve cell antioxidant defences. However, according to the results, this can not be attributed directly to the phenolic compounds content of sage extracts, which emphasises the importance of other types of compounds. The increase in basal GSH levels may be the result of induction of *de novo* glutathione synthesis. This is corroborated by the results in chapter 3, where sage tea, given *in vivo*, restored GSH levels *in vitro* in rat hepatocytes to higher values

than those obtained in controls. Sage tea was, in addition, capable of inducing significantly GST (chapter 3 and 5) as well as GPox, NADPH cytochrome P450 reductase and also, slightly, some CYP enzymes in mice liver (chapter 5). This leaves liver cells better prepared to face oxidative stress and toxicants. All these observations show the ability of sage to have indirect antioxidant effects, for example, by inducing the activity/expression levels of important enzymes and proteins in detoxification and antioxidant defence.

However, some of these effects of sage do not, in certain situations, result in protective effects. The ability of sage to increase the expression of certain proteins, such as CYP enzymes, can result in herb-drug interactions. In fact, that was what happened between sage tea and CCl_4. In chapter 5, and contrarily to what was expected, sage tea drinking (for 14 days) did not protect mice from CCl_4-induced hepatotoxicity. Instead, sage tea potentiated the toxicity of CCl_4 in the liver of both male and female mice. Since sage tea increased significantly the expression levels of CYP 2E1, the cytochrome that bioactivates CCl_4 into its toxic radicals, this effect can, at least in part, explain the observed herb-toxicant interaction between sage tea and CCl_4. Thus, these results draw attention to possible undesired herb-drug interactions between sage tea and drugs metabolised by the liver. However, because in this experiment sage tea replaced almost 100% the water consumed by the animals, the normal consumption of sage tea by humans (taking 1 or 2 cups of tea per day is, comparatively, a much smaller quantity than the one used in the assay) seems unlikely to result in dangerous or undesired interactions with other drugs. Nevertheless, if a phytomedicine with a reasonable dose of sage is taken over an extended period of time, an opportunity for enzyme induction is created, and mechanisms of interaction may take place.

Altogether, these results indicate that sage products may improve the antioxidant status of the liver that can be relevant in the face of oxidative stress. Sage extracts can be viewed as a possible therapeutical target of research, with potential for applications in the treatment of liver diseases where oxidative stress is known to be involved.

In chapter 6, some experiments were also performed regarding the antidiabetic effects of *S. officinalis*, mainly focusing on the liver. For that purpose, rat hepatocytes were isolated from both control and sage tea drinking rats and cultured *in vitro*. Primary cultures of rat hepatocytes were established both from normal fed animals (to induce glycolysis) and from normal overnight fasted animals (to induce gluconeogenesis); hepatocyte glucose consumption and hepatocyte glucose production were measured, respectively. The primary cultures established from normal fed animals were incubated with 11 mM and 22 mM of glucose to mimic postprandial and diabetic conditions, respectively. A similar experimental outline was also performed in primary cultures of hepatocytes isolated from streptozotocin-induced diabetic rats.

In experiments with mice (also in chapter 6), sage tea drinking was found to lower fasting plasma glucose but had no effect on glucose clearance in response to an intraperitoneal

glucose tolerance test. This fact indicated effects at liver level namely on gluconeogenesis. This was confirmed in the experiments using rat hepatocytes in culture, where sage tea drinking decreased significantly the glucagon-induced gluconeogenesis. In addition, sage tea drinking increased, after stimulation, glucose uptake capacity. Sage EO, added *in vitro* to the cultures, further increased hepatocyte sensitivity to insulin and inhibited gluconeogenesis. Overall these effects resemble those obtained with the pharmaceutical drug metformin, a known inhibitor of gluconeogenesis used in the treatment and prevention of type 2 *diabetes mellitus*. The results also stressed an important role of the EO fraction for the hypoglycaemic effects of sage. However, none of these effects were observed in cells isolated from diabetic animals. This could indicate the lack of antidiabetic effects of sage or an inappropriate animal model of type 2 diabetes.

In general, these results confirmed the hypoglycaemic effects of sage and shed some light on its possible mechanism of action. Although more research has to be done to study the therapeutic potential of sage in diabetes, the observed metformin-like effect of sage indicates a possible type 2 diabetes preventive potential, mainly in people at risk of developing it as is the case of those who present IGT. Moreover, due to the antioxidant effects of sage and because oxidative stress is involved in a variety of diabetic complications and possibly also in the onset of diabetes, the beneficial effects of sage through their antioxidant effects is a possibility that should be explored. Interestingly, an important function of GSH levels on glucose homeostasis has been suggested. Therefore, the positive effects of sage on GSH levels may also indirectly improve the liver mediated insulin response *in vivo*. Also, because GSH values are decreased in type 2 diabetes, sage could be helpful restoring the antioxidant defences in these patients, which are normally overwhelmed.

In conclusion, although more research efforts have to be made in order to apply sage products safely as a therapeutical tool for diabetes and liver diseases, such as hepatocellular carcinoma, viral and alcoholic hepatitis and non-alcoholic steatosis, they can be useful in preventive strategies. Sage products can be considered as a functional food or food supplements that could have a beneficial impact in low cost prevention strategies of diabetes and liver diseases. In addition, other types of diseases where it is known that oxidative stress is involved, such as cancer, neurodegenerative and cardiovascular diseases, may also be prevented by the regular consumption of sage. However, we should be aware that high doses of sage should not be taken over an extended period of time in combination with certain conventional medical drugs, since undesired herb-drugs interaction may take place.

7.2. Future perspectives

Before this work, *Salvia officinalis* already enjoyed its reputation as a strong antioxidant medicinal plant. However, that idea was based solely in chemical and subcellular experiments and on its known constituent antioxidant compounds, such as rosmarinic acid. In fact, due to its antioxidant activity, sage is used today as additive in the food processing and cosmetic industries. Based on these applications and on extrapolations from other examples, it has been considered that the consumption of *S. officinalis* have beneficial antioxidant effects for human beings. However, this had never been tested to confirm this idea.

The work performed here, in addition with other works done at the same time by different authors, confirmed for the first time the cellular and *in vivo* antioxidant effects of sage extracts. The circulating compounds in the body, after absorption, are in small concentrations and, many times as metabolites, different from the compounds ingested. As a result of extensive metabolisation and poor gastrointestinal absorption, the *in vitro* cellular effects may not be directly extrapolated to an *in vivo* situation, where bioavailability and metabolisation of the compounds have to be considered. In addition, an organism is a much more complex experimental model with many uncontrolled factors, playing in it roles that may cause unexpected interferences. Nevertheless, *in vitro* experiments, as the ones already performed in liver and neuronal cells, are useful to elucidate mechanisms of action. And, as observed in our studies, some of the *in vitro* effects are also, then, observed in the *in vivo* experiments. Therefore, these studies confirmed the attributed antioxidant effects of sage at cellular and organismal levels, which can explain the success of the ancient and traditional uses of sage in the treatment of several diseases.

These studies also shed some light on possible mechanisms of action of sage, such as an enhancement of phase II detoxifying and antioxidant enzymes and positive effects in the intracellular glutathione levels. However, some questions arise from our studies, which remain to be elucidated: is there an enhancement of *de novo* glutathione synthesis involved and responsible for the observed sage-induced increase in GSH levels? Are the increases in phase I and II enzymes, and possibly γ-glutamylcysteine synthetase, a key enzyme in *de novo* glutathione synthesis, regulated by the same mechanism, for example by the transcriptional regulation of the antioxidant response element? Or, are these different effects regulated by different mechanisms? And the antioxidant enzymes, such as GPox, is their enhancement managed directly by sage compounds or is it the result of indirect effects, for example, mediated through the induction of slight oxidative stress?

On the other hand, as observed by our preliminary studies (section 4.5.), *Salvia fruticosa* seems to have an even higher antioxidant effect than *S. officinalis*. This aromatic plant

also has a high content in EO but, contrarily to common sage, it is known to contain fewer toxic compounds, such as thujones. Therefore, the consumption of *S. fruticosa* has not been associated with undesirable effects due to the toxicity of the EO fraction. So, if *S. fruticosa* has higher antioxidant activity than common sage and its consumption is not so dangerous, this medicinal plant is probably of higher value and more investigations are awaited.

In the case of antidiabetic properties of *S. officinalis*, some experimental studies were already performed demonstrating some potential. However, although its hypoglycaemic effects were clearly shown, its therapeutical use against diabetes is far from clear. The use of a more suitable animal model of type 2 diabetes, such as the Zucker rat for example, could help elucidate the antidiabetic effects of sage. Nevertheless, in view of the metformin-like effect of sage, its use in the prevention of diabetes seems to be promising.

Another perspective that should be investigated related with diabetes is the sage potential to reduce and/or prevent post-diabetic complications. Since it is known that oxidative stress is involved in these complications, such as atherosclerosis, nephropathy, retinopathy and neuropathy, it is probable that the antioxidant effects of sage would have a beneficial impact. It is, in effect, an awaited and fruitful area of research. According with some theories, the antioxidant effects could also be helpful in the prevention of diabetes, which makes sage a good candidate to be tested.

In conclusion, in all the work reported in this thesis, a contribution was made to the elucidation of the biological (antioxidant and hypoglycaemic) effects of sage. In addition, the possible application of sage products, at least, in the prevention of liver diseases and diabetes is suggested. The use of sage products as a therapeutical tool for diabetes, liver diseases and possibly other oxidative stress-related diseases requires, however, more studies. In view of the increasing interest in the phytopharmaceutical products, this should be an area of increasing investment and research.

Appendix

Experimental Protocols

Appendix Index

Rat Hepatocyte Isolation and Use

P1

Experimental Protocol 1

Isolation of rat hepatocytes was performed by colagenase perfusion of the liver as previously described by Moldeus and collaborators (1978)[1] with some modifications.

Principle

The liver parenchyma (hepatocytes) is permeated by a collagenous fibrillar network, which provides a mechanical stabilization to the liver, embedded in an extracelular matrix composed by a complex mixture of proteoglycans and elastin[2,3]. The extracellular matrix, adhesion glycoproteins, Ca^{2+} ions and cell surface receptors play an important role in maintaining cell anchorage, shape, polarity and function[2]. Each hepatocyte is attached to, and communicates with, a number of neighbouring hepatocytes by means of junctional complexes[2]. The knowledge of these morphological aspects of the liver resulted in the modern methods of hepatocyte isolation and culture.

Therefore, the method used here involves the treatment of the hepatic tissue in 3 steps:

- **exposure to Ca^{2+}-free medium** - liver is perfused by the portal vein with an oxygenated Ca^{2+}-free balanced salt solution, which also contains a chelating agent for this ion, EGTA. Consequently, the extracellular concentration of Ca^{2+} decreases significantly resulting in an irreversible cleavage of the desmosomes (junctional complexes)[2,3].
- **collagen digestion** - liver is perfused by a second oxygenated balanced solution containing calcium and collagenase. This enzyme is a protease capable of a specific and hydrolytical cleavage of collagen molecules in their native conformation, which needs Ca^{2+} for its activity[3]. The addition of Ca^{2+} in this solution does not have effects on the cellular adhesion, since the cleavage of the junctional complexes is irreversible[2,3].
- **gentle mechanical dissociation** – after gentle disruption of the liver capsule, cells are dispersed in an appropriate

Materials required

Dissection plate

Cannula

Ligature

Peristaltic Pump

Connecting tubing

Scissors

Nylon mesh (100 µm)

Centrifuge

Carbogen gas (95% O_2; 5% CO_2)

Special reagents:

Collagenase (grade IV)

EGTA and albumin (defatted and crystalline)

Buffer Solutions

Buffer Solution 1 (BS1):

Hanks modified buffer supplemented with EGTA, albumin and Hepes, pH 7.4

137 mM NaCl

5.4 mM KCl

0.81 mM $MgSO_4$

0.34 mM Na_2HPO_4

0.44 mM KH_2PO_4

25 mM $NaHCO_3$

0.06 mM EGTA

0.67% (w/v) albumin

12.5 mM Hepes, pH 7.4

[1] Moldeus P, Hogberg J & Orrenius S, 1978. Isolation and use of liver cells. *Methods in Enzymology* **52**, 60-71.

[2] Puviani AC, Ottolenghi C, Tassinari B, Pazzi P & Morsiani E, 1998. An update on high-yield hepatocyte isolation methods and on the potential clinical use of isolated liver cells. *Comparative Biochemistry and Physiology* Part A **121**, 99-109.

[3] Berry MN, Edwards AM & Barritt GJ, 1991. Isolated hepatocytes: preparation, properties and applications. In: Burdon RH & van Knippenberg PH (Eds.), *Laboratory Techniques in Biochemistry and Molecular Biology*. Vol. 21, Elsevier Science Publishers B.V., Amsterdam.

Buffer Solutions

Buffer Solution 2 (BS2):

Hanks modified buffer supplemented with collagenase, $CaCl_2$ and Hepes, pH 7.4

137 mM NaCl

5.4 mM KCl

0.81 mM $MgSO_4$

0.34 mM Na_2HPO_4

0.44 mM KH_2PO_4

25 mM $NaHCO_3$

0.05% (w/v) collagase (grade IV)

4 mM $CaCl_2$

12.5 mM Hepes, pH 7.4

solution by gentle mechanical dissociation.

The obtained cellular suspension has, however, several non-parenchymal cells, making it, therefore, necessary to proceed to hepatocyte purification. Since hepatocytes have a higher density than non-parenchymal and dead cells, a highly enriched hepatocyte suspension is obtained after low-speed density gradient centrifugation.

Animals

From a surgical point of view, rats of 150-300 g body weight are the most convenient. This procedure can be applied to the preparation of hepatocytes from rats in either fed or fasted states and it is desirable to maintain consistency in regards to the strain, sex and weight of rats for reliable results. Additionally, a regular schedule for the surgical procedure is important. Moreover, for experiments relating to oxidative stress, the hepatocyte isolation procedure should be in the morning, where some intracellular antioxidants, such as GSH, are in higher concentration.

Apparatus

Liver can be perfused *in situ* or *ex situ* (Fig. 1). For the oxygenation of the solutions, a glass oxygenator reservoir is recommended (3a,b,c and d of Fig. 1). Alternatively, oxygenation can be achieved by bubbling carbogen directly in the solution.

Fig. 1.

1 – Thermostatted water bath

2 – Peristaltic pump

3a – Gas inlet

3b – Perfusion medium inlet

3c – Perfusion medium outlet (liver)

3d – Gas outlet and medium overflow to reservoir

4 – On/off tap

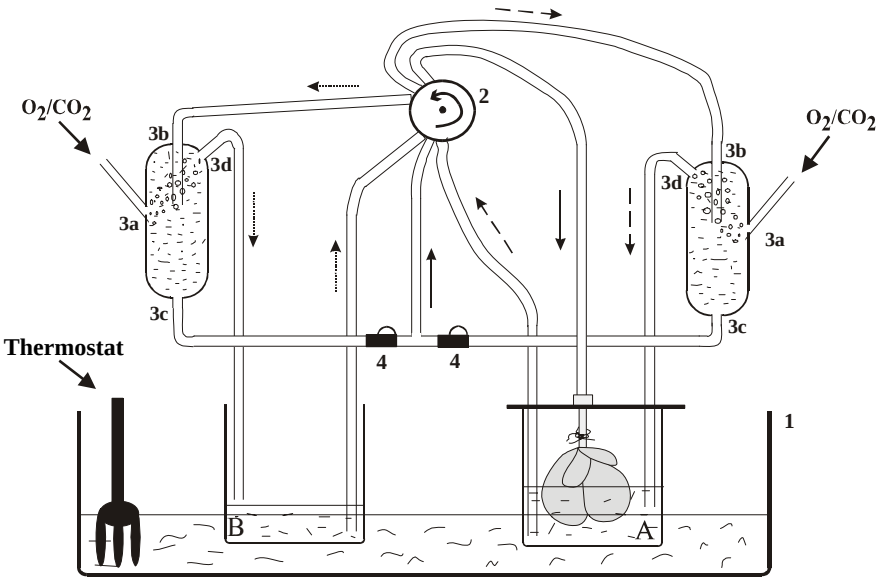


Fig.1. Schematic representation of perfusion apparatus (A – solution BS1; B – solution BS2).

Preparation of Stock Solutions

(i) Krebs-Henseleit 2 ×

Prepare a two-times concentrated stock balanced salt solution comprising 13.87 g NaCl, 0.711 g KCl, 0.323 g KH_2PO_4, 0.591 g $MgSO_4.7H_2O$, 0.760 g $CaCl_2.2H_2O$ dissolved in about 800 ml of H_2O. Bubble carbogen for about 5-10 min to exclude the following precipitation of calcium as $CaCO_3$. Then, add 4.18 g $NaHCO_3$ and make up the solution with water until 1000 ml. The solution is stable at 4ºC for a few days.

(ii) Hank 10 ×

Prepare a ten-times concentrated stock of balanced salt solution comprising 20 g NaCl, 1 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.12 g Na_2HPO_4, 0.15 g KH_2PO_4 dissolved in 250 ml of H_2O. The solution is stable at 4ºC for a few days.

(iii) CaCl2 5.88 %

Dissolve 5.88 g of $CaCl_2.2H_2O$ in 100 ml H_2O. Stable a 4ºC.

Buffer Solutions

Buffer Solution 3 (BS3):

Krebs-Henseleit buffer supplemented with Hepes, pH 7.4

119 mM NaCl

4.8 mM KCl

1.2 mM KH_2PO_4

1.2 mM $MgSO_4$

2.6 mM $CaCl_2$

25 mM $NaHCO_3$

12.5 mM Hepes, pH 7.4

Preparation of Working Solutions

BS1/2

Dissolve 0.63 g $NaHCO_3$ and 0.9 g Hepes in 30 ml Hanks stock (10×) and 270 ml H_2O. Bubble the solution with carbogen for about 2-3 min and adjust the pH to 7.4.

- **BS1** - dissolve 1.5 mg albumin and 50.2 mg EGTA in 220 ml of BS1/2 by stirring the solution for 15 min. Adjust the pH to 7.4.
- **BS2** - dissolve 20 mg collagenase in 800 µl $CaCl_2$ stock (5.88%) and 80 ml of BS1/2. Adjust the pH to 7.46.

BS3

Dissolve 0.9 g Hepes in 150 ml Krebs-Henseleit stock (2×) and 150 ml H_2O. Bubble the solution with carbogen for about 2-3 min and adjust the pH to 7.4.

Before the surgical procedure, BS1 and BS2 solutions should be warmed to 37ºC, while gassing with carbogen, in the working apparatus.

Notes

The working solutions should be prepared fresh daily just before the surgical procedure.

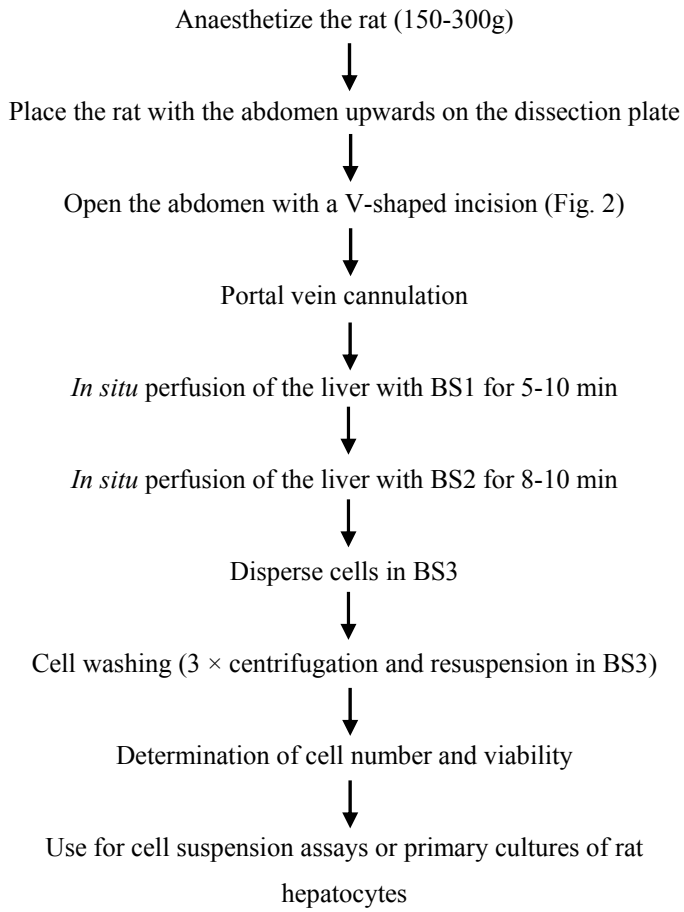
BS2) Collagenase (grade IV) should be added to the solution just after the cannulation of the portal vein has been succeeded. This avoids the lost collagenase (a high cost enzyme) in case of unsuccess.

Using our apparatus set, the thermostatted water bath should be to 39ºC so that the temperature of the solution flowing from the cannula is approximately 37ºC.

Protocol – Rat Hepatocyte Isolation

Notes

(I) Flow diagram



(II) Detailed protocol

1) Dose of pentobarbital: 60 mg sodium pentobarbitone/kg rat body weight.

Because this procedure ends with the death of the animal, anaesthesia with diethylether may be acceptable but requires training. Additionally, during the surgical procedure the anaesthesia must be maintained using cotton soaked with diethylether near the rat nose.

1) Anaesthetize the rat with intraperitoneal pentobarbital (60mg/kg) or diethylether (by inhalation). Place the unconscious animal, abdomen upwards, on the dissection plate securing the legs with rubber bands (Fig. 2). Open the abdomen with a V-shaped incision. Reflect the intestine to your right-hand side, thereby exposing the liver (Fig. 2). Gently push the two main lobes of the liver towards the animal's head to undercover the portal vein and, lying deeper, the inferior vena cava.

The portal vein is the major vessel that supplies the liver.

2) Before performing this point, with the aid of a small pair of curved forceps draw a cotton ligature under the portal vein. This will make easier and rapid to tight the cannula to the portal vein.

Using our pump (Gilson – Minipulse 3), tubing of 2.50 CC/M (Gilson) and the buffer temperature, to have a flow of approximately 5 ml/min, set to 15 rpm.

2) Puncture the portal vein 1.5 cm from the point where the vein branches to enter the liver lobes. Insert the cannula to lie about 0.5 cm proximal to the branch point with the pump delivering approximately 5 ml/min of BS1. Pull tight the ligature round the portal vein. After, cut the inferior vena cava, with a pair of scissors, below the level of the kidney. Finally, and *very*

importantly, puncture the diaphragm (near the liver) of the animal to kill the unconscious rat.

3) Set the pump to deliver approximately 10 ml/min of BS1. If the liver is too big, press the inferior vena cava proximal to the cut from time to time in order to perfuse all of the liver. Maintain the perfusion with BS1 for about 5-10 min.

4) Then, change the pumping solution to BS2 (which already contains collagenase), maintaining the flow at 10 ml/min. After approximately 8 min, the consistency of the liver will have become soft, moderately swollen and have a speckled appearance. If *gentle* pressure with a finger tip on a liver lobule results in fracture of the liver surface, digestion can be taken as complete.

5) At the end of the digestion period, remove the softened and disintegrated liver. With care pull it away from the ligamentous attachments to the mesentery with the help of scissors. Transfer the extirpated organ to a Petri plate containing BS3 and disrupt the liver capsule gently. Cells disperse in the BS3 solution by gentle mechanical dissociation.

Notes

3) Take great care to avoid air bubbles. Do not forget to continuously bubble carbogen in BS1 and BS2 in the thermostatted water bath.

Using our pump, tubing and the buffer temperature, to have a flow of approximately 10 ml/min, switch it for 26 rpm.

At this stage add the collagenase in BS2.

4) A higher yield of cells, with no apparent decrease in cell quality, can be obtained if perfusion continues until fissures form spontaneously in the liver without any applied pressure or manipulation.

Because of variation in the quality of collagenase, it is not feasible to prescribe an exact perfusion time and some experience is required in making a judgement as to when perfusion should be terminated.

5) Before using BS3, bubble it with carbogen for about 5 min.

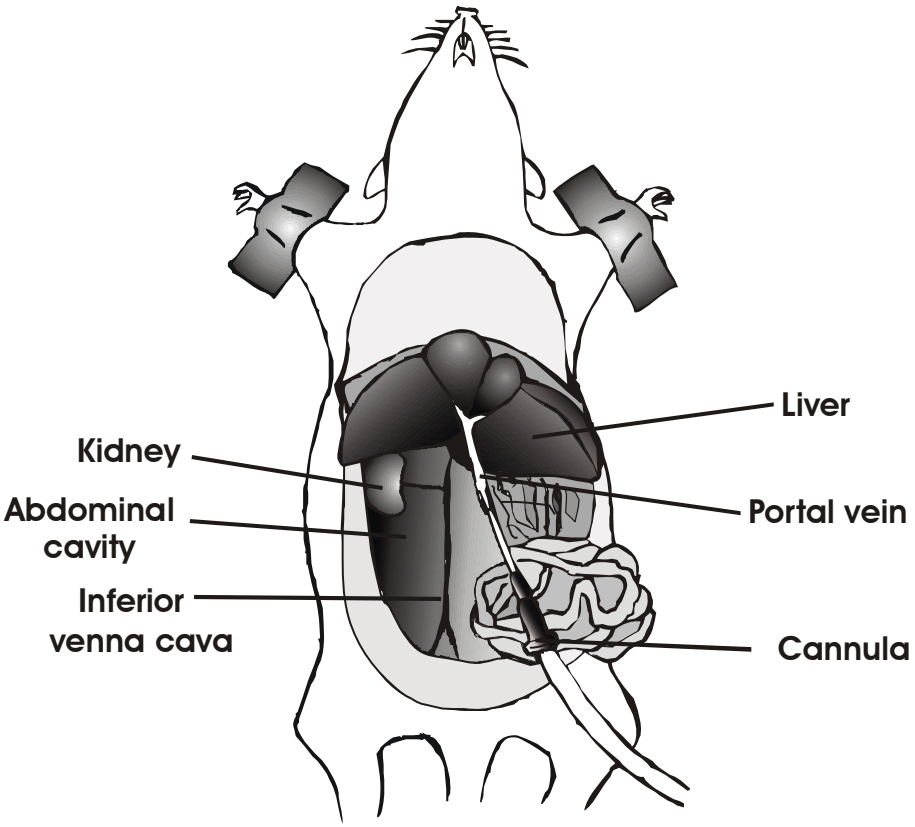


Fig.2. Schematic representation of the surgical procedure and cannulation of portal vein. Adapted from Berry et al.,1991[3].

Notes

6) BS3 should be on ice for the washing procedures.

Using our centrifuge (Heraeus Sepatech - Megafuge 1.0) and the respective rotor, 50 ×*g* is obtained at 500 rpm. However, centrifugations at 300 rpm can also be used.

After the third centrifugation the supernatants should be relatively clear. If not, wash the pellets again.

7) Cells must be maintained on ice.

The hepatocytes viability should be higher than 80%.

6) Pass cell suspension through a nylon mesh (100 µm pore-size) into a clean 100 ml beaker. Distribute the suspension between two ice-cold 50-ml centrifuge tubes and dilute the suspension to 50 ml in each tube. Centrifuge the tubes at 50 × *g* for 2 min. Aspirate the supernatants carefully and repeat the washing with BS3 and centrifuging procedures twice. At each step carefully resuspend each cell pellet in about 5 ml of BS3 by slowly tilting side ways the tube.

7) Combine the pellets in 10 ml of BS3 for determination of cell number and viability in a Neubauer chamber using trypan blue 0.4% (w/v).

Protocol – Hepatocyte Suspensions Assay

Before performing the experiments with the freshly isolated hepatocytes, pre-incubate them at 37ºC at least for 30 min. The experiments should be done within 4 hours after obtaining the hepatocyte suspension

Dilute the cells for the appropriate concentration in BS3 and incubate in 20-ml glass flasks at 37ºC in a reciprocating water-bath at 60-80 cycles/min, constantly gassing the top of each flask with carbogen.

Protocol – Primary Cultures of Rat Hepatocytes

i) From time to time gently resuspend the cells.

i) Add 2.5 ml of antibiotic/antimicotic solution 100 × (Sigma) to the hepatocyte suspension (final volume: 12.5-20 ml) and let be the cells in ice at least for 30 min.

ii) A concentration of 1×10^6 hepatocytes/ml is normally used.

ii) Centrifuge the necessary volume of the hepatocyte suspension to obtain the desire volume of culture medium with a certain concentration of hepatocytes.

iii) Use William's Medium E supplemented with 10% FBS (fetal bovine serum), 1% antibiotic/antimicotic solution (100 ×), 2.2 g sodium bicarbonate and 10 mM Hepes as culture medium. It can be also supplemented with 2 g of BSA (bovine serum albumin).

The insulin and dexamethasone helps the hepatocyte attachment.

iii) In the laminar flow chamber, add the necessary culture medium containing 10^{-9} M of insulin and 10^{-9} M dexamethasone to the hepatocytes pellet. Resuspend gently the hepatocytes.

iv) See protocol P20 for isolation of collagen and coating of culture plates.

iv) Seed the hepatocytes onto 6-well culture plates, previously coated with rat tail collagen, and incubate at 37ºC in a humidified incubator gassed with 5% CO_2/95% air.

v) Leave hepatocytes to attach for 3 h and replace the culture medium before performing the experiments.

Culture of HepG2 Cells and Use

P2

Experimental Protocol 2

HepG2 cells, a human hepatoma cell line, have been isolated from a liver biopsy of a male Caucasian aged 15 years with a well differentiated hepatocellular carcinoma. HepG2 cells are of epithelial morphology and have been grown successfully in large scale cultivation systems.

Principle

Animal cell lines, obtained from certain cancer cells and from genetic engineering, grow and replicate indefinitely in culture with the appropriate nutrients (and growth factors). They can be used *in vitro* for several kinds of experiments.

Preparation of Solutions

MEM

i) Add MEM powder (enough for 1 l of culture medium), 2.2 g $NaHCO_3$, 110 mg sodium pyruvate (cell culture tested) and 2.383 g Hepes (cell culture tested) into 890 ml of ultrapure water. Dissolve them by continuous agitation.

ii) Adjust pH between 7.2-7.3.

iii) Filter the medium into an autoclaved 1 l-flask using a 0.2 µm Filtropur unit (Sarstedt) in a laminar flow chamber. *All the operations from this point on should be carried out under strict aseptic conditions.*

iv) After filtration add 100 ml FBS and 10 ml antibiotic/antimicotic solution (100 ×) and keep the flask at 4ºC.

Cryoprotectant Medium

Add 5 ml of DMSO (sterile filtered – Sigma) to 95 ml of culture medium MEM.

For more information about this cell line see the ATCC (American Type Culture Collection) product information sheet for HepG2 – HB-8065 (www.atcc.org)

Materials required

Humidified CO_2 incubator

Laminar flow chamber

Room of biosafety level 1

Culture plates and flasks

Neubauer chamber

Special reagents:

Minimum essential medium Eagle (MEM) from Sigma

FBS (fetal bovine serum)

Antibiotic/antimicotic solution (100 ×)

Hepes (*e.g.*, # H4034, Sigma)

Solutions

Complete Grow Culture Medium (MEM), pH 7.4:

Minimum essential medium Eagle (Sigma) supplemented with 2.2 g sodium bicarbonate ($NaHCO_3$), 1 mM pyruvate, 10 mM Hepes, 10% FBS and 1% antibiotic/antimicotic solution (100 ×).

Cryoprotectant Medium

95% (v/v) MEM

5% (v/v) DMSO

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

Sterile filtered (0.2 µm)

Protocol – Defrosting Cells

Notes

Cells are frozen in vials in liquid nitrogen (liquid phase: -196ºC; vapour phase: -130ºC). *Be careful, liquid nitrogen is dangerous – cause severe burnings.*

1) Get the vial from liquid nitrogen immediately before the thawing procedure.

Thawing should be rapid (approximately 2 min).

1) Thaw the vial by gentle agitation in 37ºC water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.

2) Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3) It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture flask containing MEM be placed into the incubator for at least 15 min to allow the medium to reach its normal pH.

It is not necessary to remove the cryoprotectant agent if the vial is diluted in 15 ml of MEM.

3) Transfer the vial content to 75 cm^2 tissue culture flasks and dilute with MEM to 15 ml.

4) Change medium within 24 h after seeding.

4) Incubate the culture at 37ºC in a humidified incubator gassed with 5% CO_2/95% air.

Protocol – Subculturing

Volumes used in this protocol are for 75 cm^2 flasks; proportionally reduce or increase amount of dissociation medium for culture flasks of other sizes.

When cells are confluent, subculturing is necessary. In case of few cells being subcultured, HepG2 cells start to grow in islets (in 3 dimensions) and subculturing also becomes necessary.

1) Remove and discard culture medium.

2) Briefly rinse the cell layer with warm sterile PBS.

2) This removes all traces of serum which contains trypsin inhibitors.

3) Add 3 ml of Trypsin-EDTA solution and 1 ml of sterile PBS. Incubate at 37ºC.

3) Trypsin-EDTA solution (Sigma):

0.25% (w/v) trypsin-0.02% (w/v) EDTA in saline buffered solution

4) After 7-8 min observe cells under an inverted microscope. They should be rounded and cell layer begin being dispersed.

4) If necessary for cell detachment hit the flask. However this should be avoided since it clumps the cells.

5) Add 11 ml of MEM and dissociate cells by gently pipetting.

6) Add appropriate aliquots of cell suspension to new culture flasks.

6) If cells are to be seeded in culture plates for cell assays, determination of cell number in the cell suspension using the Neubauer chamber is necessary

Subcultivation Ratio: 1:4 to 1:6

or 3-4 × 10^6 cells/75 cm^2 flask

7) Incubate cultures at 37ºC in a humidified incubator gassed with 5% CO_2/95% air.

8) Renew MEM at least twice a week. After 1 week, cell layer are normally confluent and ready for subculturing.

Notes

Protocol – Freezing Cells

HepG2 cells can be stored as stocks in liquid nitrogen using Cryoprotectant Medium at density of 2-4 × 10^6 cells/ml in 1 ml-vials. Do not store cells at -80ºC, it will result in loss of viability.

1) Harvest cells in the same manner used for routine subculture.

2) Prepare cryoprotectant medium by allowing 1 ml for each vial to be frozen.

3) Centrifuge cell suspension at 100 ×*g* for 2 min.

3) Using our centrifuge (Heraeus Sepatech - Megafuge 1.0) and the respective rotor, 100 ×*g* is obtained at 800 rpm.

4) Resuspend the cell pellet in the cryoprotectant medium to give a final cell concentration of ~3 × 10^6 cells/ml and aliquot 1 ml into each sterile vial. The vials should be clearly marked.

5) Freeze the cells in a polystyrene box in a -80ºC freezer for 4-5 hours prior to transfer to liquid nitrogen.

5) During the freezing procedure, a cooling rate between 1-3ºC/min should be used. There are programmable freezers that perform this. However, polystyrene boxes or other commercial available boxes can instead be used in a -80ºC freezer.

During the studies for this thesis, mice were used in two experimental models: (1) intoxicated with CCl_4 as an *in vivo* model of hepatocellular injury; (2) to perform an intraperitoneal glucose tolerance test (ipGTT).

Animals

Male and female Balb/c mice (albino inbred strain) with 6-10 weeks of age were used and acclimated to the laboratory animal facilities in a regular light/dark cycle and constant temperature (20 ± 2 ºC) and humidity. Food and tap water were given *ad libitum* to the animals for at least one week before the start of the experiments.

CCl_4-induced hepatotoxicity

Principle

CCl_4 is a hepatotoxicant that induces damage effects in the liver upon its bioactivation mainly by cytochrome P450 2E1[1]. After bioactivation, the trichloromethyl ($^{\cdot}CCl_3$) and triclhoromethylperoxy ($CCl_3OO^{\cdot}$) radicals are formed, which 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[1].

The protective effects of an extract or compound against the CCl_4-induced hepatotoxicity can be studied given the extract/compound before or after administration of the toxicant. The extract can be given by gavage or intraperitoneal. To induce hepatotoxicity CCl_4 is administered by ip injection in a dose of 20 µl/kg dissolved in olive oil (0.25%, v/v)[2,3]. To observe the hepatic injury effects of CCl_4, the experiment should be stopped between 2-48 hours after toxicant administration[4].

[1] Weber LWD, Boll M & Stampfl A, 2003. Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. *Critical Reviews in Toxicology* **33**, 105-136.

[2] Mansour MA, 2000. Protective effects of thymoquinone and desferrioxamine against hepatotoxicity of carbon tetrachloride in mice. *Life Sciences* 66, 2583-2591.

[3] Chen JH, Tipoe GL, Liong EC, So HSH, Leung KM, Tom WM, Fung PCW & Nanji AA, 2004. Green tea polyphenols prevent toxin-induced hepatotoxicity in mice by down-regulating inducible nitric oxide-derived prooxidants. *American Journal of Clinical Nutrition* **80**, 742-751.

[4] Chung H, Hong DP, Jung JY, Kim HJ, Jang KS, Sheen YY, Ahn JI, Lee YS & Kong G, 2005. Comprehensive analysis of differential gene expression profiles on carbon tetrachloride-induced rat liver injury and regeneration. *Toxicology and Applied Pharmacology* **206**, 27-42.

Notes

1) Make a fresh solution of CCl_4 in olive oil before ip injections. Be careful with CCl_4 – it is very toxic.

2) Anaesthesia with diethylether can be used considering that this is a terminal experiment.

3) Freeze the liver immediately in liquid nitrogen and keep at -80ºC until use.

Protocol

1) After animal treatment with plant extract or pure compound, inject CCl_4 ip in a dose of 20 µl/kg body weight dissolved in olive oil (0.25%, v/v). Controls receive vehicle only (8 ml/kg).

2) After 24 h (other time points can be chosen), anaesthetise animals for blood collection. In an unconscious animal, collect blood from the eye artery after removal of the eyeball. Immediately after, sacrifice the mouse by dislocation of cervical vertebrae.

3) Collect the liver for histology and other determinations.

Intraperitoneal Glucose Tolerance Test (ipGTT)

Principle

A glucose tolerance test permormed in mice that excludes the effect of glucose absortion in the intestine.

Protocol

1) Glucose solution:

300 g/l in physiologic saline (NaCl 0.9%)

With a dose of 5.83 ml/kg, this gives a final dose of 1.75 g/kg bwt.

2) For plasma collection, use heparinised tubes.

1) After animal treatment with plant extract or pure compound, in 3 hours fasted mice, a glucose solution is injected ip in a dose of 5.83 ml/kg bwt. Controls receive vehicle only (physiologic solution).

2) After 45 min (other time points can be chosen for a time-course experiment) collect blood samples from the tail. In case of terminal experiment, blood can be collected as in the above protocol.

LDH Leakage

P4

Experimental Protocol 4

The LDH leakage was used as marker of cell membrane integrity to estimate cell viability.

Principle

When the cell membrane are damaged cytosolic enzymes pass into the extracellular medium. The measure of the enzyme leakage can give an estimation of cell damage (death) for a sample. The lactate dehydrogenase (LDH) is the most commonly used enzyme to measure cell membrane integrity. LDH is an enzyme located almost entirely in the cytoplasm which catalyses the reversible reduction of pyruvate to lactate in the last step of anaerobic glycolysis[1].

$$L\text{-lactate} + NAD^+ \overset{L\text{-LDH}}{\rightleftharpoons} \text{pyruvate} + NADH + H^+$$

LDH leakage can be given as the percentage of LDH activity in the extracellular medium from the total activity in the sample.

The LDH activity can be obtained by measuring the continuous oxidation of NADH (0.28 mM) to NAD^+ spectrophotometrically at 340 nm on a plate reader using pyruvate (0.32 mM) as substrate in 50 mM phosphate buffer (pH 7.4).

Protocol

Sample preparation

1) *Extracellular LDH*

Collect extracellular medium to a tube.

In the case of a cell suspension, centrifuge an aliquot (ex: 200 µl) at 10,000 rpm (~10,000 ×g) in a microcentrifuge for 1 min at room temperature. Transfer a portion of the supernatant to a clean tube.

2) Total LDH

In the case of a cell suspension, take an aliquot to a tube.

[1] Berry MN, Edwards AM & Barritt GJ, 1991. Isolated hepatocytes: preparation, properties and applications. In: Burdon RH & van Knippenberg PH (Eds.), *Laboratory Techniques in Biochemistry and Molecular Biology*. Vol. 21, Elsevier Science Publishers B.V., Amsterdam.

Materials required

96-well plates

Plate reader spectrophotometer

Sonicator

Special reagents:

NADH

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

Lysis Buffer

25 mM KH_2PO_4

2 mM $MgCl_2$

5 mM KCl

1 mM EDTA

1 mM EGTA, pH 7.5

100 µM PMSF (added fresh from a stock sol.)

Phosphate Buffer (PB)

50 mM KH_2PO_4, pH 7.4

Notes

1) Use a 1.5 ml tube.

Centrifuge normally used: Centrifuge 5415 C, Eppendorf.

2) If LDH leakage is to be presented as extracellular enzyme activity, this step is not performed.

Notes

4) Sonicator used: Ultrasonic Processor GEX 400. Sonicate at 20% of amplitude for 10 sec.

Stock Solutions

0.32 mM NADH

2.27 mg/10 ml PB (20 ml/plate)

Freshly prepared and stable for 3 h on ice.

7.36 mM Pyruvate

8.1 mg/10 ml PB (1 ml/plate)

Freeze in 1-ml aliquots at -20ºC. Maintain at 4ºC when used.

Notes

1) Use a repetitive pipette to pipette NADH and pyruvate into the wells.

2) This allows both consuming the pyruvate present in the sample (in case of cell culture medium) and the solution reaching 30ºC.

The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

3) Use the 'kinetic' function of SOFTmax Pro

4) Use only the linear part for the calculations.

In the case of a cell layer, total LDH is quantified as the sum of extracellular LDH plus the intracellular LDH. To measure intracellular LDH, rinse the cells with warm PBS. Then scrap the cell layer in PBS or Lysis Buffer and transfer it to a tube.

3) Then, keep samples at 4ºC if LDH is assayed on the same day or at -80ºC for later measurement of LDH activity

4) Before determining the LDH activity in cell suspension (total LDH) or cell scraps (intracellular LDH), lyse the cells in a sonicator to liberate all intracellular LDH.

Assay for LDH activity

1) In a 96-well plate, pipette to each well:

- 10-20 µl sample (make duplicates)
- 200 µl NADH (0.32 mM)

2) Incubate for 2 min at 30ºC in the plate reader.

3) Add as fast as possible 10 µl of pyruvate (7.36 mM) and read the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

4) A linear (negative) slope inferior to 100 mDO/min should be obtained. If not, dilute samples accordingly.

Express LDH leakage as percentage of extracellular LDH activity from total LDH activity taking into account dilutions and total volumes of extracellular medium and homogenisation medium with cell scraps.

To express LDH leakage as the activity of LDH in the extracellular medium use the following formula:

$$U/ml = \frac{Vmax \times 0.332}{1000 \times 6.22 \times V_{sample}}$$

U - µmol.min^{-1}

Vmax - slope in mDO.min^{-1}

V_{assay}/ℓ(lightpath) =constant (0.332) in ml.cm^{-1}

ε_{NADH} = 6.22 mM^{-1}.cm^{-1}

V_{sample} - volume of the sample used in the assay in ml (ex: 0.01 ml).

The activity can then be expressed per mg of protein (protein of the cell scraps).

MTT Assay

P5

Experimental Protocol 5

The MTT assay was used as marker of cell metabolic capacity to estimate cell viability.

Principle

Metabolically active cells have the capacity to reduce the soluble yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) in an insoluble purple formazan by the action of intracellular dehydrogenase enzymes, at the expense of reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilised and quantified spectrophotometrically and is proportional to viable cells[1].

Protocol

1) After cell incubation with the potential toxic compound for the desire time in a 24-cell culture plate (with 1ml of culture medium), add 100 µl of MTT stock solution (final concentration ~0.5 mg/ml) to each well.

2) Incubate at 37ºC for 2 hours in the humidified CO_2 incubator.

3) At the end of the incubation period, take off the medium and add 1 ml of acidic isopropanol to solubilise the purple formazan dye.

4) Carefully sonicate each well to solubilise completely the converted dye.

5) Transfer 200 µl of the dye solution of each well for a 96-well plate in duplicate.

6) Read the absorbance at 570 nm using 690 nm as reference in a plate reader spectrophotometer.

7) Express the results as % of sample absorbance in relation to the absorbance in the negative control.

[1] Mosmann T, 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**: 55-63.

Materials required

96-well plates

Plate reader spectrophotometer

Sonicator

Special reagents:

MTT

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

Sterile filtered (0.2 µm)

Organic Solvent

Acidic Isopropanol

0.04 M HCl in absolute isopropanol

Stock solution

MTT (5 mg/ml)

Add 50 mg of MTT to 10 ml of sterile PBS. Sonicate the solution if necessary. This solution can be kept at -20ºC for later use. Note: MTT is a light-sensitive compound.

Notes

1) Do not forget to use the appropriate controls: a negative control (without any compound added); a blank (without MTT added); and also a positive control (with a compound with known toxicity).

2) The time chosen can vary from 30 min to 4 h, depending on cell density and cell type.

Do not forget to include this time in the time action of the potential toxic compound.

4) Sonicator used: Ultrasonic Processor GEX 400. Sonicate at 20% of amplitude for 30 sec.

Calculations

1) Subtract the blank absorbance value from all other values. This can be done automatically in the SOFTmax Pro Software.

2) For each well express the absorbance as:

Abs 570 nm – Abs 690 nm

This can be also done automatically in the SOFTmax Pro Software.

3) Express the results as percentage of cell survival:

(absorbance treated wells / absorbance of control wells) × 100%

Dose response curves can be calculated for the compounds tested over a range of concentrations, enabling IC_{50} values to be obtained (*i.e.* concentration of chemical that reduces cell survival by 50%)

Notes

6) (*previous page*) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

Use the 'endpoint' function of SOFTmax Pro.

2) Formazan absorbs light at 570 nm but not at 690 nm. Absorbance at 690 nm results from cell debris and well imperfections.

Trypan Blue Staining

P6

Experimental Protocol 6

The trypan blue staining was used to measure cell viability of rat hepatocyte suspensions after isolation and HepG2 cells routinely in the subculturing procedures. It can also be used in other experiences with cells in suspension.

Principle

Trypan blue is an organic amine dye that is excluded by cells with intact membranes, whereas damaged cells readily take it up[1]. Trypan blue is negatively charged and it seems likely that the dye is excluded as a result of an energy-dependent maintenance by the cell of a plasma membrane potential (negative inside)[1]. Loss of this potential due to cell injury may allow penetration of trypan blue. The percentage of cells that exclude trypan blue from the total number of cells gives an estimation of cell viability in a sample.

Protocol

1) Prepare a haemocytometer with the coverslip.

2) Pipette into a 1.5 ml-tube 50 µl of trypan blue and 50 µl of cell suspension and mix gently with slow inversions of the tube. If cell suspension is too concentrated, dilute it with PBS appropriately.

3) Allow one drop of the mixed suspension to flow smoothly by capillary action under the coverslip of the haemocytometer, so as to fill the measuring compartment completely, yet not overflowing.

4) With a light microscope, count at least 200 cells using different chamber fields (and different counting squares) and calculate the percentage of viable cells (cells that exclude trypan blue). See Fig. 1.

5) Considering the dilutions made, calculate the concentration of viable cells per millilitre of suspension.

[1] Berry MN, Edwards AM & Barritt GJ, 1991. Isolated hepatocytes: preparation, properties and applications. In: Burdon RH & van Knippenberg PH (Eds.), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 21. Elsevier Science Publishers B.V., Amsterdam.

Materials required

Improved Neubauer haemocytometer

Light (inverted) microscope

Special reagents:

Trypan blue

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

Stock solution

Trypan Blue 0.04% (w/v))

Add 0.4 g trypan blue to 100 ml of PBS. Filter the solution with a 0.2 µm-membrane.

Notes

1) To mount the haemocytometer chamber lightly moisten the supporting ridges and apply the coverslip pressing firmly so that light interference patterns appear.

2) In each counting square the number of cells should be inferior to 100.

Notes

1) Example:

After counting 4 counting squares:

viable cells – 230

nonviable cells – 18

total cells – 248

viability = 230/248 ×100 = 92.7%

2) Example:

VC – 230/4 = 57.5

Dilution made:

50 µl cell suspension + 50 µl PBS

and then, 50 µl of above + 50 µl trypan blue

therefore, dilution factor – 2 × 2 = 4

[VC] = 57.5 × 4 /1×10^{-4} = 2.3×10^{6} cells/ml

Calculations

1) *Cell Viability*

i) In the same area (for example, 4 counting squares – Fig. 1), count the viable cells (cells that exclude trypan blue – bright cells) and the nonviable cells (cells stained in blue).

ii) Sum viable and nonviable cells for the total number of cells.

iii) Calculate viability as follows:

number of viable cells × 100 / number of total cells

2) *Concentration of viable cells in the suspension*

i) Calculate the average of viable cells (VC) per each counting square.

ii) Considering the volume of each counting square (shadowed square – Fig 1) to be 0.1 mm^3 (1 mm × 1 mm × 0.1 mm; the same as 1×10^{-4} ml) and considering the dilution made, calculate the concentration of viable cells per ml in the suspension as follows:

[VC] = VC × dilution factor / 1×10^{-4}

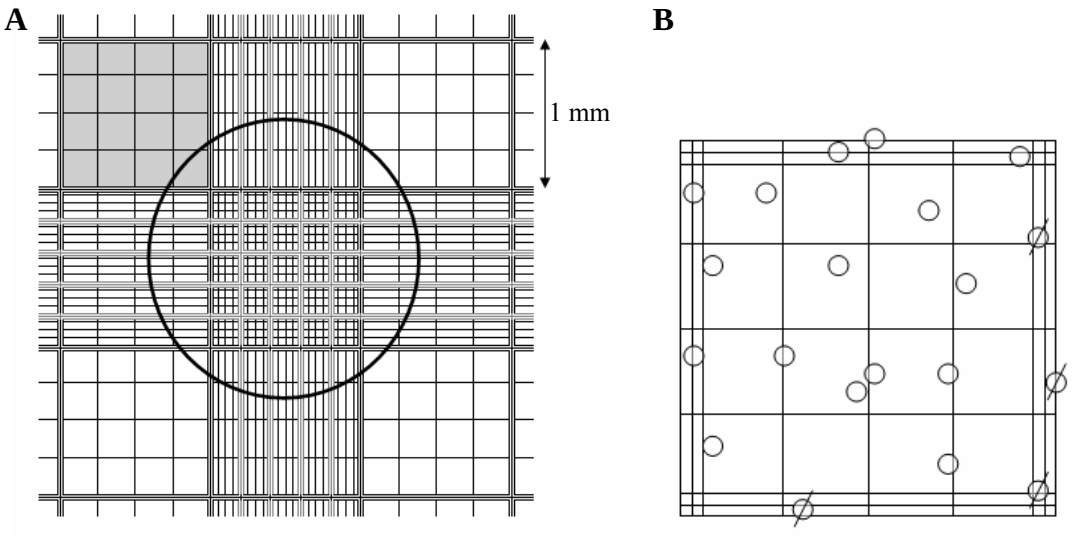


Fig. 1. Illustration of a haemocytometer chamber. **A)** General representation. The circle indicates approximately the microscopic field with an magnification of 100× (10× ocular plus 10× objective) The shadowed area represents the counting square (B). Since the depth of the chamber is 0.1 mm, each counting square have a volume of 0.1 ml; **B)** Magnification of the counting square (shadowed) of (A). The symbol O indicates cells that must be counted and the symbol ∅ indicates cells that must not. Therefore, only cells over two margins of the counting square must be counted.

TBARS (Lipid Peroxidation)

P7

Experimental Protocol 7

One of the targets for reactive species is the phospholipid bilayer, comprised of cellular and subcellular membranes, initiating the lipid peroxidation. Lipid peroxidation in biomembranes is believed to proceed via lipid peroxyl radical ($LOO^{\cdot}$)-mediated radical chain reaction, where lipid hydroperoxides (LOOH) accumulate in the membranes as primary oxidation products. Several different secondary oxidation products were also formed, which are used to estimate lipid peroxidation.

Principle

One of the secondary products formed and used to estimate lipid peroxidation is malondialdehyde (MDA). It can be measured by chromatographic techniques and both spectrophotometrically and fluorometrically after the reaction with thiobarbituric acid (TBA). Here, a spectrophometric method is described to determine lipid peroxidation, which measures the thiobarbituric acid reactive substances (TBARS). The reaction of MDA with TBA produces a red chromophore that can be detected at 535 nm[1].

Protocol

Sample preparation

1) *Cell Suspensions*. Add 250 µl of cell suspension to 500 µl of 10% (w/v) TCA, which is already in a 1.5 ml-tube. Make a blank tube with the buffer.

2) *Cell cultures*. Add 360 µl of culture medium to 60 µl of 50% (w/v) TCA, which is already in a 1.5 ml-tube. Make a blank tube with culture medium that was not in contact with cells.

3) If not measured immediately afterwards, keep the tubes at -80ºC for later measurement of TBARS.

[1] Ohkawa H, Ohishi, N & Yagi K, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* **95**, 351-358.

Materials required

96-well plates

Plate reader spectrophotometer

Water bath

Special reagents:

TBA

Solutions

Trichloroacetic acid (TCA) 10% (w/v)

Dissolve 10 g of TCA in a total volume of 100 ml of water

TCA 50% (w/v)

Dissolve 50 g of TCA in a total volume of 100 ml of water

TBA 1% (w/v)

Dissolve 200 mg of TCA in a total volume of 20 ml of water. Use a water bath with warm water to help dissolving TBA. This solution must be made just before use.

Notes

1) & 2) The final concentration of TCA should be around 6.75% (w/v) for precipitation of proteins before the TBARS assay. Therefore, if other volume ratios are required, make TCA solutions accordingly.

Notes

1) Centrifuge normally used:
Centrifuge 5415 C, Eppendorf.

3) The TBA solution must be prepared immediately before use. Do not let the solution cool too much; otherwise the TBA will precipitate again.

5) Do not immerse excessively the tubes; otherwise some water can enter in the tubes.

6) After cooling the tubes, do a short spin to them to collect any condensate at the bottom of the tube.

7) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

Use the 'endpoint' function of SOFTmax Pro.

Formula:

1000 – to give the results in nmol

A_{sample} – absorbance of the sample

A_{blank} - absorbance of the blank

156 = ε_{MDA} in $\mu mol^{-1}.ml.cm^{-1}$

0.904 = lightpath of 300 µl solution in a well of 96-well plate

TBARS assay

1) Centrifuge tubes at 10,000 rpm (~10,000 ×*g*) in a microcentrifuge for 1 min at room temperature.

2) Transfer 300 µl of supernatant to a clean 1.5 ml-tube.

3) Add 300 µl of TBA 1% (w/v).

4) Make a little pin hole in the cap of each tube.

5) Put the tubes floating in a boiling water bath for 10 min.

6) After cooling the tubes, transfer 300 µl of each sample/blank for a well of a 96-well plate.

7) Read the absorbance at 535 nm against the blank in a plate reader spectrophotometer.

Express lipid peroxidation as nmol MDA/mg protein using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}.cm^{-1}$. In the case of cell cultures, have into account dilutions and total volumes of extracellular medium and homogenisation medium with cell scraps, where the protein is measured.

Calculate nmol MDA/ml using the following formula:

$$\text{nmol MDA/ml} = \frac{1000 \times (A_{sample} - A_{blank})}{156 \times 0.904}$$

Then, express it per mg of protein (protein of cell suspensions or protein of the cell scraps in case of cell cultures).

Glutathione Levels

P8

Experimental Protocol 8

Glutathione is an intracellular antioxidant tripeptide that plays an important role in cell defence. Severe GSH (reduced glutathione) depletion leaves cells more vulnerable to oxidative damage, which causes progressive deterioration of macromolecules, cell structure and may lead to cell death[1]. Glutathione levels could therefore determine the cytotoxicity of a xenobiotic and its ultimate effects on cell survive. The ratio between GSH and oxidised glutathione (GSSG) are normally used to determine the cell's oxidative state.

Principle

There are different procedures (*e.g.*, chemical, enzymatic, chromatographic) for the determination of GSH and GSSG in biological samples. Here, an enzymatic method is described based in the methodology of Anderson (1985)[2].

To maintain initial GSH levels in the biological samples, they must be acidified quickly. Then several acids are used for deproteinisation, but 5-sulfosalicylic acid (SSA) (usually 5% w/v, final concentration) is preferable. Then, total glutathione levels (GSH + GSSG) can be measured enzymatically by the DTNB-GSSG reductase recycling assay. As indicated in the reaction (1), GSH is oxidised by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to give GSSG with stoichometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced back to GSH by the action of the highly specific glutathione reductase (GSSG reductase or GR) and NADPH (2). The rate of TNB formation is followed at 415 nm and is proportional to the sum of GSH and GSSG present in the sample.

$$2\ GSH + DTNB \rightarrow GSSG + TNB \qquad (1)$$

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2\ GSH + NADP^{+} \qquad (2)$$

To determine the GSSG levels in the biological samples, the previous methodology is used after treating GSH with 2-vinylpyridine. GSH levels are obtained by subtracting GSSG levels from the total GSH.

[1] Castell JV, Gomez-Lechon MJ, Ponsoda X & Bort R, 1997. In vitro investigation of the molecular mechanisms of hepatotoxicity. In: Castell JV & Gomez-Lechon MJ (Eds.), *In Vitro Methods in Pharmaceutical Research*, Academic Press, London, pp. 375-410.

[2] Anderson, 1985. Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology* **113**, 548-555.

Materials required

96-well plates

Plate reader spectrophotometer

Microtubes shaker device

Special reagents:

DTNB

NADPH (*e.g.*, # N1630, Sigma)

GR (*e.g.*, # B2633, Sigma)

SSA

2-vinylpyridine

GSH

GSSG

Solutions

SSA 10% (w/v)

Dissolve 10 g of SSA in a total volume of 100 ml of water

SSA 50% (w/v)

Dissolve 50 g of SSA in a total volume of 100 ml of water

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

Phosphate Buffer (PB)

6.3 mM EDTA

143 mM NaH_2PO_4, pH 7.5

Notes

1 & 2) The final concentration of SSA should be 5% (w/v) for precipitation of proteins before the assay. Therefore, if other volume ratios are required (*e.g.*, to concentrate or dilute the sample), make SSA solutions accordingly.

Assay Solutions

GSH and GSSG standards

Prepare the following GSH standards in SSA 5% (w/v): 2, 4, 8, 16, 32, 64 nmol GSH/ml

Prepare the following GSSG standards in SSA 5% (w/v): 1, 2, 4, 8, 16, 32 nmol GSSG/ml

Stock solutions with higher concentrations have to be prepared first. Then, freeze stocks and standards solutions for reuse.

DTNB 3 mM (Final Conc. 0.6 mM)

5,95 mg/ 5 ml PB (5 ml/96-well plate)

Prepare a fresh solution daily and maintain on ice.

NADPH 0.35 mM (F. Conc. 0.21 mM)

2,92 mg/ 10 ml PB (15 ml/96-well plate)

Prepare a fresh solution daily and maintain in ice. Prepare only 10% more solution than necessary – expensive compound!

GR 25 U/ml (F. Conc. 2 U/ml)

?? µl/ 1 ml PB (2 ml/96-well plate)

Prepare a fresh solution daily and maintain on ice. Prepare only 10% more solution than necessary – expensive enzyme!

? – Calculate for each enzyme lot. Each lot has a different concentration of enzyme.

Notes

1) Centrifuge normally used:
Centrifuge 5415 C, Eppendorf.

2) Use a repetitive pipette to pipette DTNB and NADPH into the wells.

3) The temperature of 30ºC allows the acquisition of reproducible measurements in both the Winter and Summer.

4) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

Use the 'kinetic' function of SOFTmax Pro.

Protocol

Sample preparation

1) *Cell Suspensions*. Add 200 µl of cell suspension to 200 µl of 10% (w/v) SSA, which is already in a 1.5 ml-tube.

2) *Cell cultures*. To measure GSSG, add 360 µl of culture medium to 40 µl of 50% (w/v) SSA, which is already in a 1.5 ml-tube. Then scrap the cells in 1 ml of PBS (a well from a 6-well plate) and add 360 µl of cell suspension to 40 µl of 50% (w/v) SSA to measure GSH levels.

3) Vortex the tubes.

4) If not measured immediately afterwards, keep the tubes at -80ºC for later measurement of glutathione levels.

DTNB-GSSG Reductase Recycling Assay

1) Centrifuge tubes at 10,000 rpm (~10,000 ×*g*) in a microcentrifuge for 1 min at room temperature and use the supernatant.

2) In a 96-well plate, pipette to each well:

- 30 µl of sample/standard (make duplicates)
- 50 µl DTNB (3 mM)
- 150 µl NADPH (0.35 mM)

3) Incubate for 15 min at 30ºC in the plate reader.

4) Add as fast as possible 20 µl of GR (25 U/ml) and read the absorbance at 425 nm on a plate reader every 10 sec for 3 min at 30ºC.

5) A linear (positive) slope should be obtained. If not, dilute samples accordingly. The concentration of your samples can be directly obtained using the standard curve (linear response) in the SOFTmax Pro.

6) Express glutathione levels as nmol GSH/mg protein, considering dilution factors. In the case of cell cultures, have into account dilutions and total volumes of extracellular medium and homogenisation medium with cell scraps, where the protein is measured.

Sample preparation for measuring GSSG

P8

1) Pipette into a 1.5-ml tube 100 µl of sample/standard (GSSG).

2) Under a fume wood, pipette into each tube 2 µl of 2-vinylpyridine and mix.

3) Then, also under a fume wood, pipette 10 µl of triethanolamine 50% (v/v) to the side of the tube and vigorously mix the solution.

4) Using a microtube shaker device, shake the tubes continuously for 1 hour at ambient temperature.

5) Finally, the derivatised samples are assayed as described above in the DTNB-GSSG reductase recycling assay.

Notes

2) 2-Vinylpyridine has a low vapor pressure and constant exposure may be irritating.

2 & 3) Use a repetitive pipette to pipette 2-vinylpyridine and triethanolamine into the tube.

5) Due to 2-vinylpyridine, pipette the samples in to the 96-well plate also under a fume wood.

At the end of the assay, fill the wells of the 96-well plate with HCl 1 N. Afterwards, get rid of all to the hazardous waste.

Glucose

P9

Experimental Protocol 9

Glucose is an important metabolite in the organism that is strictly regulated. The measurement of glucose is used for the diagnosis and monitoring of diabetes and other disorders of carbohydrate metabolism.

Principle

The concentration of glucose in a sample can be measured using a colorimetric enzymatic method[1]. Glucose oxidase (GOD) oxidises glucose to gluconic acid, with formation of H_2O_2. In the presence of peroxidase (POD), the H_2O_2 reacts with phenol and 4-aminoantipyrine, to produce a coloured complex (red) whose absorbance maximum is found at 505 nm[1]:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{POD}} \text{Coloured Complex} + H_2O$$

The absorbance at 505 nm is directly proportional to the concentration of glucose in the sample.
Here, is described an adaptation of the method Glucofix (Menarini) useful to be used in a plate reader spectrophotometer.

Protocol

1) In a 96-well plate, pipette to each well:

- 8 µl of sample/standard (make duplicates), in the case of your sample having less than 12 mM glucose.

or, - 4 µl of sample/standard (make duplicates), in the case of your sample having less than 24 mM glucose.

- 250 µl Kit reagent

2) Incubate for 15 min at 30ºC in the plate reader.

3) Read the absorbance at 505 nm against the blank in a plate reader spectrophotometer.

[1] Glucofix, A. Menarinini diagnostics, Firenze, Italy.

Materials required

96-well plates

Plate reader spectrophotometer

Solutions

Kit Reagent

Use as provided.

Kit Standard

Use as provided.

Notes

1) The sample can be any clear (without precipitates) solution.

Use a repetitive pipette to pipette Kit reagent into the wells.

2) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

3) Use the 'endpoint' function of SOFTmax Pro.

If the template is done accordingly, the program already subtracts the blank value to the samples and standard. This is important for subsequent calculations.

Notes

Formula:

Conc. Standard – concentration of the standard used. If you use the kit standard, the concentration is 5.56 mM or 100mg/dl.

A_{sample} – absorbance of the sample

$A_{standar}$ - absorbance of the standard

Use the absorbance of samples and standard with the blank value already subtracted.

Calculate glucose concentration using the standard with the following formula:

$$\text{Glucose [mM]} = \frac{A_{sample} \times \text{Conc. Standard}}{A_{standard}}$$

In the case of cell cultures, the glucose concentration can be then be expressed per mg of protein (protein of the cell scraps). For example, nmol glucose produced/mg protein or nmol glucose consumed/mg protein.

Total Protein

P10

Experimental Protocol 10

The measurement of total protein in samples is very important for normalizing the quantity of other metabolites and enzyme activities of the same sample per mg of protein. It is also important routinely in several biologic techniques, such as, western blotting. It also gives an idea when compared with a control situation if any treatment induce a general over-expression of proteins or decrease total soluble protein due to toxic effects.

Principle

The concentration of proteins in a sample can be measured spectrophotometrically using the Bradford Reagent[1]. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption at 595 nm is proportional to the protein present.

The Bradford Reagent is compatible with reducing agents (ex. DTT and 2-Mercaptoethanol). Reducing agents are often used to stabilise proteins in solution. Other protein assay procedures (Lowry and BCA) are not compatible with reducing agents. The Bradford Reagent should be used in place of these protein assays if reducing agents are present. However, the Bradford reagent is only compatible with low concentrations of detergents (see compatibility chart in ref. [1]). If the protein sample to be assayed has detergent(s) present in the buffer, it is suggested to use the BCA or Lowry protein determination procedure.

Here, is described an adaptation of the method explained in ref. [1] (Sigma) useful to be used in a plate reader spectrophotometer.

[1] Bradford Reagent, # B6916, Sigma, Missouri, USA.

Materials required

96-well plates

Plate reader spectrophotometer

Solutions

Bradford Reagent

Use as provided.

Protein Standard

Make a Standard Stock of 2 mg/ml with the solution where your protein is present – sample buffer (*e.g.* lysis buffer).

Dissolve 10 mg of bovine serum albumin (BSA) in 5 ml of sample buffer. Make aliquots of 500 µl and keep at -20ºC for later use.

Protocol

1) Gently mix the Bradford Reagent in the bottle:

2) Prepare protein standards in the sample buffer ranging from 0.125 - 2 mg/ml using a BSA standard (2 mg/ml).

3) In a 96-well plate, pipette to each well:

- 10 µl of sample/standard/blank (make duplicates).
- 250 µl Bradford Reagent

4) Let the samples incubate at room temperature for 10 minutes. Then measure the absorbance at 595 nm in the plate reader spectrophotometer. The protein-dye complex is stable up to 60 min.

5) The concentration of your samples can be directly obtained using the standard curve (quadratic response) in the SOFTmax Pro.

Notes

2) Prepare for example the following standards: 0.125, 0.25, 0.5, 1 and 2 mg/ml.

3) For the blank, pipette 10 µl of sample buffer.

Use a repetitive pipette to pipette Bradford Reagent into the wells.

4) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

Use the 'endpoint' function of SOFTmax Pro.

This method can also be used using only one standard concentration (500 µg/ml) instead of using a standard protein range. For that, you may use the following formula:

$$\text{Protein [µg/ml]} = \frac{A_{sample} \times \text{Conc. Standard}}{A_{standard}}$$

If the protein concentration of the unknown sample(s) is greater than 1 mg/ml, you must dilute the sample accordingly.

If protein concentration of your sample is quite low, you can increase the volume of your sample/standard/blank in the assay (*e.g.*, 20µl). However, you have to change the standard protein range accordingly. For example, to use 20 µl of sample, you should use a standard protein range of: 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml.

Formula:

Conc. Standard – concentration of the standard used: 500 µg/ml.

A_{sample} – absorbance of the sample

$A_{standar}$ - absorbance of the standard

Use the absorbance of the sample and standard with the blank value already subtracted.

Glutathione Reductase

P11

Experimental Protocol 11

Glutathione reductase (GR) is a flavoprotein catalysing the NADPH-dependent reduction of glutathione disulfide (GSSG – glutathione oxidised form) to GSH (glutathione – reduced form):

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

The reaction is essential for the maintenance of glutathione levels in reduced form. GSH has a major role as a reductant in oxidation-reduction processes and also in detoxification reactions.

Principle

The oxidation of NADPH is followed spectrophotometrically at 340 nm[1].

Here, a procedure based in the methodology of Carlberg & Mannervik (1985)[1] is described to measure the GR activity in a plate reader spectrophotometer. The assay is performed at 30ºC following NADPH oxidation at 340 nm in the presence of 3 mM GSSG, 0.12 mM NADPH, 2.5 mM EDTA, in 50 mM Hepes (pH 7.4).

Protocol

Sample preparation

Use a post-mitochondrial fraction of any cell homogenate. For that, centrifuge cell or tissue homogenates at 10,000 ×*g* for 10 min at 4ºC. Use supernatant as your sample.

Enzyme Soup

Just before the enzyme assay, make the following enzyme soup (25 ml/96-well plate):

[1] Carlberg I & Mannervik B, 1985. Glutathione reductase. *Methods in Enzymology* **113**, 484-490.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

NADPH (*e.g.*, # N1630, Sigma)

GSSG

Buffer Solutions

Hepes 50 mM

50 mM Hepes, pH 7.4

Assay Solutions

EDTA 50 mM (Final Conc. 2.5 mM)

18.6 mg/ 1 ml Hepes (1.5 ml/96-well plate)

GSSG 50 mM (Final Conc. 3 mM)

32.8 mg/ 1 ml Hepes (1.8 ml/96-well plate)

Maintain on ice. It can be be freezed for later use.

Starter Reaction Solution

NADPH 3.24 mM (F. Conc. 0.12 mM)

2,83 mg/ 1 ml Hepes (1 ml/96-well plate)

Prepare a fresh solution daily and maintain on ice. Prepare only 10% more solution than the necessary – expensive compound!

	(27)	(22)	(17)
EDTA 50 mM	1.35 ml	1.1 ml	1.02 ml
GSSG 50 mM	1.62 ml	1.32 ml	850 µl
Hepes	22 ml	17.58 ml	13.13 ml
Total	25 ml	20 ml	15 ml

Notes

Enzyme Soup

Number in brackets ( ): Volume in ml used for the calculations – it have in count the volume of the sample and the starter reaction solution.

Enzyme Assay

1 & 4) Use a repetitive pipette to pipette Enzyme Soup and NADPH.

1) In a 96-well plate, pipette to each well:

- 10 µl sample (make duplicates)
- 250 µl Enzyme Soup

2) The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

2) Incubate for 2 min at 30ºC in the plate reader.

3 & 4) Use the 'kinetic' function of SOFTmax Pro

3) Measure the non-specific activity by reading the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

4) Add as fast as possible 10 µl of NADPH (3.24 mM) and read the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

5) Use only the linear part for the calculations.

5) A linear (negative) slope inferior to 100 mDO/min should be obtained. If not, dilute samples accordingly.

To calculate GR activity, subtract the slope obtained from the non-specific activity (step 3) from the specific activity (step 4). This gives the Vmax for each sample. Then, the activity can be expressed as µmol NADPH oxidised per min per ml (U/ml) using the following formula:

$$U/ml = \frac{Vmax \times 0.332}{1000 \times 6.22 \times V_{sample}}$$

U - $\mu mol.min^{-1}$

Vmax - slope in $mDO.min^{-1}$

V_{assay}/ℓ (lightpath) =constant (0.332) in $ml.cm^{-1}$

$\varepsilon_{NADH} = 6.22\ mM^{-1}.cm^{-1}$

V_{sample} - volume of the sample used in the assay in ml (ex: 0.01 ml).

The specific activity of GR (U/mg protein) is then calculated dividing the above value per the value "mg protein/ml" - protein of the sample.

Glutathione Peroxidase

P12

Experimental Protocol 12

Glutathione peroxidase (GPox) is an antioxidant enzyme that reduces H_2O_2 (and other peroxides) to water in the presence of glutathione in reduced form (GSH):

$$2\ GSH + H_2O_2 \xrightarrow{GPox} 2\ H_2O + GSSG$$

Principle

Glutathione peroxidase catalyses the reduction of peroxides to water with oxidation of GSH to GSSG. GSSG can be then reduced back to GSH by GR with expense of NADPH:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

Therefore, GPox activity can indirectly be measured following the decrease of NADPH, *i.e.* oxidation to $NADP^+$, spectrophotometrically at 340 nm.

Here, a procedure based in the methodology of Flohe & Gunzler (1984) [1] and Martin-Aragon *et al.* (2001)[2] is described to measure the selenium-dependent and -independent GPox activity in a plate reader spectrophotometer. The assay is performed at 30ºC following NADPH oxidation at 340 nm in the presence of 1 mM GSH, 0.18 mM NADPH, 1 mM EDTA, 0.5 U/ml GR and 0.7 mM *t*-BOOH in 50 mM imidazole (pH 7.4).

Protocol

Sample preparation

Use a post-mitochondrial fraction of any cell homogenate. For that, centrifuge cell or tissue homogenates at 10,000 ×*g* for 10 min at 4ºC. Use supernatant as your sample.

Enzyme Soup

Just before the enzyme assay, prepare the following enzyme soup (25 ml/96-well plate):

[1] Flohe L & Gunzler WA, 1984. Assays of glutathione peroxidase. *Methods in Enzymology* **105**, 114-121.

[2] Martin-Aragon S, de las Heras B, Sanchez-Reus MI & Benedi J, 2001. Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. *Experimental and Toxicologic Pathology* **53**, 199-206.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

NADPH (*e.g.*, # N1630, Sigma)

GSH

Glutathione Reductase (GR) (*e.g.*, # B2633, Sigma)

tert-Butyl hydroperoxide (*t*-BOOH) (*e.g.*, # G3664, Sigma)

Buffer Solutions

Imidazole (Imdz.) 50 mM

50 mM Imidazole, pH 7.4

Assay Solutions

EDTA 50 mM (Final Conc. 1 mM)

18.6 mg/ 1 ml Imdz. (1 ml/96-well plate)

GSH 27 mM (Final Conc. 1 mM)

8.3 mg/ 1 ml Imdz. (1.1 ml/96-well plate)

Maintain on ice. It can be be freezed for later use.

NADPH 3.24 mM (F. Conc. 0.18 mM)

2,83 mg/ 1 ml Imdz (1.6 ml/96-well plate)

Prepare a fresh solution daily and maintain on ice. Prepare only 10% more solution than the necessary – expensive compound!

GR (F. Conc. 0.5 U/ml)

Use as provided. Calculate the necessary volume for enzyme lot. Each lot has a different concentration of enzyme.

	(27)	(22)	(17)
EDTA 50 mM	540 µl	440 µl	340 µl
GSH 27 mM	1 ml	815 µl	630 µl
NADPH 3.24 mM	1.5 ml	1.22 ml	944 µl
GR *	??	??	??
Imdz	22 ml	17.52 ml	13.09 ml
Total	25 ml	20 ml	15 ml

Notes

Enzyme Soup

Number in brackets ( ): Volume in ml used for the calculations – it have in count the volume of the sample and the starter reaction solution.

* ??: Depends of the lot of enzyme stock. Do the necessary calculations for each lot.

Starter Reaction Solution

18.9 mM t-BOOH (F. Conc. 0.7 mM)

2.43 µl/1 ml PB (1.2 ml/plate)

Prepare fresh.

Notes

1 & 4) Use a repetitive pipette to pipette Enzyme Soup and *t*-BOOH.

2) The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

3 & 4) Use the 'kinetic' function of SOFTmax Pro

5) Use only the linear part of the curve for the calculations.

Enzyme Assay

1) In a 96-well plate, pipette to each well:

- 10 µl sample (make duplicates)
- 250 µl Enzyme Soup

2) Incubate for 2 min at 30ºC in the plate reader.

3) Measure the non-specific activity by reading the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

4) Add as fast as possible 10 µl of *t*-BOOH (18.9 mM) and read the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

5) A linear (negative) slope inferior to 100 mDO/min should be obtained. If not, dilute samples accordingly.

To calculate GPox activity, subtract the slope obtained from the non-specific activity (step 3) from the specific activity (step 4). This gives the Vmax for each sample. Then, the activity can be expressed as µmol NADPH oxidised per min per ml (U/ml) using the following formula:

$$U/ml = \frac{Vmax \times 0.332}{1000 \times 6.22 \times V_{sample}}$$

U - $\mu mol.min^{-1}$

Vmax - slope in $mDO.min^{-1}$

V_{assay}/ℓ (lightpath) =constant (0.332) in $ml.cm^{-1}$

$\varepsilon_{NADPH} = 6.22\ mM^{-1}.cm^{-1}$

V_{sample} - volume of the sample used in the assay in ml (ex: 0.01 ml).

The specific activity of GPox (U/mg protein) is then calculated dividing the above value per the value "mg protein/ml" - protein of the sample.

Glutathione *S*-Transferase

P13

Experimental Protocol 13

Glutathione *S*-transferase (GST) is a phase II enzymes that participates in detoxification of many xenobiotics, electrophiles and reactive oxygen species through their conjugation to GSH.

Principle

The conjugation of aromatic substrates to GSH by GST is followed spectrophotometrically[1].

Here, a procedure based on the methodology of Habig *et al.* (1974)[1] is described to measure GST activity in a plate reader spectrophotometer using the aromatic substrate 1-chloro-2,4-dinitrobenzene (CDNB). The assay is performed at 30ºC following the formation of GSH conjugate at 340 nm in the presence of 1 mM GSH and 1 mM CDNB in 50 mM Hepes (pH 7.4). The activity is calculated using the extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ and expressed as µmol of conjugate produced per min per mg of protein.

Protocol

Sample preparation

Use a post-mitochondrial fraction of any cell homogenate. For that, centrifuge cell or tissue homogenates at 10,000 ×*g* for 10 min at 4ºC. Use supernatant as your sample.

Enzyme Soup

Just before the enzyme assay, make the following enzyme soup (25 ml/96-well plate):

	(27)	(22)	(17)
CDNB 27 mM	1 ml	815 µl	630 µl
Hepes	24 ml	19,2 ml	14.4 ml
Total	25 ml	20 ml	15 ml

[1] Habig WH, Pabst MJ & Jakoby WB, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* **249**, 7130-7139.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

1-chloro-2,4-dinitrobenzene (CDNB) (*e.g.*, # C6396, Sigma)

GSH

Buffer Solutions

Hepes 50 mM

50 mM Hepes, pH 7.4

Assay Solutions

CDNB 27 mM (Final Conc. 1 mM)

5.57 mg/ 1 ml ethanol (1.1 ml/96-well plate)

Starter Reaction Solution

GSH 27 mM (Final Conc. 1 mM)

8.3 mg/ 1 ml Hepes (1.1 ml/96-well plate)

Maintain on ice. It can be be freezed for later use.

Notes

Enzyme Soup

Number in brackets ( ): Volume in ml used for the calculations – it have in count the volume of the sample and the starter reaction solution.

Notes

1 & 4) Use a repetitive pipette to pipette Enzyme Soup and GSH.

2) The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

3 & 4) Use the 'kinetic' function of SOFTmax Pro

5) Use only the linear part of the curve for the calculations.

Enzyme Assay

1) In a 96-well plate, pipette to each well:

- 10 µl sample (make duplicates)

- 250 µl Enzyme Soup

2) Incubate for 2 min at 30ºC in the plate reader

3) Measure the non-specific activity by reading the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

4) Add as fast as possible 10 µl of GSH (27 mM) and read the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

5) A linear (positive) slope inferior to 200 mDO/min should be obtained. If not, dilute samples accordingly.

To calculate GST activity, subtract the slope obtained from the non-specific activity (step 3) from the specific activity (step 4). This gives the Vmax for each sample. Then, the activity can be expressed as µmol of conjugated produced per min per ml (U/ml) using the following formula:

$$U/ml = \frac{Vmax \times 0.332}{1000 \times 9.6 \times V_{sample}}$$

U - $\mu mol.min^{-1}$

Vmax - slope in $mDO.min^{-1}$

V_{assay}/ℓ(lightpath) =constant (0.332) in $ml.cm^{-1}$

$\varepsilon_{conjugated} = 9.6\ mM^{-1}.cm^{-1}$

V_{sample} - volume of the sample used in the assay in ml (ex: 0.01 ml).

The specific activity of GST (U/mg protein) is then calculated dividing the above value per the value "mg protein/ml" - protein of the sample.

Cytochrome P-450 (CYP) is regarded as the collective name of a superfamily of hemeproteins, which carry out the oxidative conversion of various exogenous and endogenous compounds, usually by hydroxylation, turning a hydrophobic compound into a more water soluble substance which can be excreted. Their principal function requires molecular oxygen, a supply of reducing equivalent from NADPH and a substrate[1]. Liver microsomes are rich in these enzymes.

Preparation of Liver Microsomes

Principle

CYP's are located within the cell in the membranes of endoplasmic reticulum. When the cell are homogenised in the appropriate buffer, the endoplasmic reticulum forms small vesicles in aqueous solution (microsomes) with a specific density that precipitate at 100,000 ×*g* by centrifugation. By differential centrifugation is possible to isolate the microsomes from the other constituents of the cell.

Here, an adaptation from the method used by Barbier *et al.* (2000)[2] is described in order to use low volumes of tissue homogenate (max. 1.5 ml).

Protocol

1) Prepare freshly the following homogenisation buffer (HB) for a total volume of 50 ml:

	Final Concentration	*Weight/Volume*
DTT	1 mM	7.72 mg
EDTA	1 mM	18.6 mg
Glycerol	20% (v/v)	10 ml
PMSF	0.1 mM	125 µl*
PB	160 mM	till 50 ml

Keep on ice until use.

[1] Rastogi S, Das M & Khanna SK, 2002. A novel approach to study the activity and stoichiometry simultaneously for microsomal pentoxyresorufin-O-dealkylase reaction. *FEBS Letters* **512**, 121-4.

[2] Barbier O, Lapoint H, el Alfy M, Hum DW & Belanger A, 2000. Cellular localization of uridine diphosphoglucuronosyltransferase 2B enzymes in the human prostate by *in situ* hybridization and immunohistochemistry. *The Journal of Clinical Endocrinology & Metabolism* **85**, 4819-4826

Materials required

Refrigerated Centrifuge

Ultracentrifuge

Mini-Beadbeater homogenizer

Buffer Solutions

Phosphate buffer (PB) 200 mM, pH 7.4

100 mM KH_2PO_4

100 mM K_2HPO_4, pH 7.4

Stable at 4ºC.

Notes

1) DTT - Dithiothreitol

PMSF - Phenylmethylsulfonyl fluoride

* From a stock of 40 mM PMSF in ethanol (kept at -20ºC)

2) In the appropriate tube (about 2 ml max) put some glass beads (1 mm diameter). Weigh the tube and put them on ice.

3) Put about 150-300 mg of liver tissue and weigh newly the tubes. Keep the tubes on ice and be fast as much as possible.

4) Homogenise the tissue in the ratio 1/5 (1+4) with HB two times for 20 sec at 42,000 rpm with the Mini-Beadbeater. (*e.g.*: 200 mg + 800 µl)

5) Centrifuge at 12,000 ×*g*, 20 min at 4ºC.

6) Centrifuge the supernatant at 105,000 ×*g*, 1 hour at 4ºC. For these small volumes use the adapter for 1.5/2 ml tubes.

7) Resuspend the pellet in 1 ml of HB (use a 200µl pipette and ultimately the ultrasound bath for 10sec), and centrifuge again at 105,000 ×*g*, 1 h at 4ºC.

8) Resuspend the pellet in about 500 µl of HB (use a 25G needle to resuspend it) and aliquot in 100 µl aliquots. Freeze in liquid nitrogen and keep them at -80ºC until use.

9) Use a small aliquot of the sample obtained above to measure the protein content. Preferably, measure the protein content on the same day of isolation using the Lowry method. Do not forget to make the protein standards in HB to eliminate possible interferences. If necessary, dilute samples also in HB.

Activities of CYP 1A and 2B Families

Principle

The activities of these two cytochrome families can be measured with fluorescent substrates using their specific dealkylation activity of different phenoxazones[3]:

CYP 1A: ethoxyresorufin-O-dealkylation (EROD)[3]

CYP 2B: pentoxyresorufin-O-dealkylation (PROD)[4]

Here, an adaptation of the method of Burk *et al.* (1985)[5] and Pearce *et al.* (1996)[6] is described. Briefly, the dealkylation of the specific substrates is made at 37ºC in phosphate buffer (100 mM, pH 7.4) containing specific enzyme substrate (see below), microsome protein (~200 µg/ml), 3 mM $MgCl_2$, 1 mM EDTA and a NADPH-generating system[3,6] or 250 µM NADPH[5].

At an exact time the reaction is stopped with ice cold acetone and the fluorescence measured with an excitation wavelength

Notes

3 & 4) All these operations (except weighing the tubes should be done in a 4ºC room. Make the calculations necessary to know the volume to add to each sample as fast as possible in order to prevent deterioration of the samples.

4) For each sample leave at least 5 min on ice before the second homogenisation procedure.

Homogenizer used: Mini-Beadbeater, Biospec Products, Bartlesville, OK, USA.

5) Centrifuged used: Sigma 2K15, B. Braun, West Germany.

6) Centrifuge used: Beckman XL-90 Ultracentrifuge, Ceckman Instruments, Inc., California, USA.

105,000 ×*g* ≈ 35,000 rpm with the rotor 90Ti in this centrifuge.

Before using the adapters, ask for Senior Researcher how to use them. The adapters must be filled with water and rigorously calibrated.

7) A white pellet, heavier, also usually appears and represents the glycogen. If possible discard it.

[3] Binda D, Lasserre-Bigot D, Bonet A, Thomassin M, Come MP, Guinchard C, Bars R, Jacqueson A & Richert L, 2003. Time course of cytochromes P450 decline during rat hepatocyte isolation and culture: effect of L-NAME. *Toxicology in Vitro* **17**, 59–67.

[4] Deserbac P, Vernevaut MF, Amiot MJ, Suschetet M & Siess MH 2001. Effects of a water-soluble extract of rosemary and its purified component rosmarinic acid on xenobiotic-metabolizing enzymes in rat liver. *Food and Chemical Toxicology* **39**, 109–117.

[5] Burk MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T & Mayer RT, 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochemical Pharmacology* **34**, 3337-3345.

[6] Pearce RE, McIntyre CJ, Madan A, Sanzgiri U, Draper AJ, Bullock PL, Cook DC, Burton LA, Latham J, Nevins C & Parkinson A, 1996. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Archives of Biochemistry and Biophysics* **331**, 145-169.

Materials required

Spectrofluorimeter
Thermostatted water bath

Special reagents:

Ethoxyresofurin (Resorufin ethyl ether)
(*e.g.*, # E3763, Sigma)

Pentoxyresorufin (Resorufin pentyl ether)
(*e.g.*, # P0928, Sigma)

(λEx) of 530 nm and emission wavelength (λEm) of 584 nm[6]. Zero time incubations serve as blanks.

The activity is presented as pmol substrate produced/min/mg protein using the molar extinction coefficient (ε) of 73.2 $mM^{-1}.cm^{-1}$ or by a standard curve with resorufin[1]. Usually the assay using a NADPH-generating system gives the higher activities and best results.

Protocol

1) Prepare freshly the following Enzyme Soup (25 ml/24 samples):

	Final Concentration	*Weight/Volume*
$MgCl_2$	7.5 mM	38.2 mg
EDTA	1 mM	9.3 mg
PB2	100 mM	till 25 ml
Substrate:		
EROD	5 µM	125 µl*
or PROD	10 µM	250 µl*

Keep on ice until use.

2) Prepare freshly one of the following Start Reaction Solution (1.25 ml/24 samples):

(a) NADPH

	Final Concentration	*Weight/Volume*
NADPH	250 µM	38.2 mg (5 mM)
PB2	100 mM	1.25 ml

or

(b) NADPH-generating System

	Final Concentration	*Weight/Volume*
NADP	500 µM	9.56 mg (10 mM)
G-6-P	1 mM	38 mg (100 mM)
G-6-PD	0.5 U/ml	??
PB2	100 mM	~1.25 ml

3) To a glass round bottom tube add:

- ~200 µg microsome protein (50 µl max.)
- 900µl Enzyme Soup

4) Stabilize the assay temperature at 37ºC in a thermostatted

Buffer Solutions

Phosphate Buffer 2 (PB2) 100 mM:

100 mM KH_2PO_4, pH 7.4

Stable at 4ºC.

Stock Solutions

Substrate Stock 1 (EROD) 1 mM:
Ethoxyresofurin

Substrate Stock 2 (PROD) 1mM:
Pentoxyresorufin

Prepare a 1 mM stock solution of the respective substrate (light sensitive) in DMSO (don't put in ice otherwise freeze immediately) and divide it into 100-200µl aliquots. Store at -20ºC.

Fluorescent standard

1 mM Resorufin (*e.g.*, # 230154, Aldrich)

Prepare a 1 mM stock solution of resorufin (light sensitive) in DMSO and divide it into aliquots. Store at -20ºC.

Stop Reaction Solution

Ice cold Acetone

Put about 25 ml acetone (enough for 24 samples) on ice before use.

Notes

1) * Pipette just prior use.

2) *Final Concentration*: final concentration in the assay (1 ml).

Number in brackets ( ): The real concentration made in the Start Reaction Solution.

??: Depends of the lot of enzyme stock. Do the necessary calculations for each lot. Do not forget that the real concentration in the NADPH-generating system must be 10 U/ml.

G-6-P: glucose-6-phosphate

G-6-PD: G-6-P dehydrogenase

3) To measure EROD activity use an Enzyme Soup with EROD substrate; to measure PROD activity use Enzyme Soup with PROD substrate.

water bath (*e.g.* 5 min).

5) Begin the reaction with 50 µl of Start Reaction Reagent and incubate also at 37ºC with frequent agitation of the tubes (agitation is very important).

6) After 20 min, stop the reaction with 2 ml of ice cold Stop Reaction Solution and put the tubes on ice. Do not forget to make a blank tube with no protein added.

7) Centrifuge the tubes at 4,000 rpm for 2 min.

8) Read the fluorescence in a spectrofluorimeter at λEx 530 nm and λEm 592 nm (put excitation and emission slits at 10nm).

9) Express the activity as pmol/min/mg protein using a standard curve with resorufin. Easier, you can pipette a known concentration of resorufin and see the change in fluorescence and extrapolate for your results. You should, however, previously tested you are using a concentration within the linear response of the standard curve. Do not forget to subtract the blank value to all samples and standard.

Therefore, you can use the corresponding pmol of resorufin for each sample and use in the following formula to have the CYP activity (CYP A):

$$\text{CYP A} = \text{Xpmol}/20/0.2 \text{ pmol/min/mg}$$

Notes

5) To initiate and stop the enzyme reaction in a set of tubes at precise time points, you should phase the operations for a set of samples (*e.g.* begin each tube of a set from half in half minute).

6) Protect the tubes as most as possible from light. Just after adding the Stop Reaction Solution, vortex the tube.

7) Centrifuged used: Kubota 2100, Kubota Corporation, Tokyo, Japan.

8) Spectrofluorimeter used: Luminescence Spectrometer LS 50, Perkin Elmer, Perkin-Elmer Ltd., Buckinghamshire, UK.

An λEm of 584 nm is recommended but an λEm of 592 nm was observed to be the peak of resorufin.

Xpmol: correspondent pmol of resorufin for the fluorescence value obtained for ach sample.

20: 20 min of the assay.

0.2: 0.2 mg of protein used in the assay.

Activity of CYP 2E1

Principle

The activity of this cytochrome can be measured spectrophotometrically by the conversion of *p*-nitrophenol (PNP) to 4-nitrocatechol followed at 480 nm using NADPH or NADPH-generating system[7].

Here, an adaptation of the method of Allis & Robinson (1994)[7] is described in order to be used in a plate reader spectrophotometer. Briefly, the conversion of the substrate is made at 37ºC in Hepes buffer (100 mM, pH 6.8) containing specific enzyme substrate, microsome protein (~300 µg/ml), and a NADPH-generating system. $MgCl_2$ is not included in the generating system or the assay mixture because it has been reported to inhibit the PNP hydroxylase reaction. All reagents are mixed

[7] Allis JW & Robinson BL, 1994. A kinetic assay for *p*-nitrophenol hydroxylase in rat liver microsomes. *Analytical Biochemistry* **219**, 49-52.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

p-nitrophenol (4- or *para*-nitrophenol) (*e.g.*, # 1048, Sigma)

Buffer Solutions

Hepes Buffer (HB) 100 mM:

100 mM Hepes, pH 6.8

Stable at 4ºC.

except for the NADPH-generating system and incubated at 37ºC for 5 min. The reaction is started by adding the generating system and incubated for 37ºC for an additional 5 min before recording the absorbance for 10 min. The second 5 min incubation it is desirable because a small decrease in absorbance, centered near 600 nm, interferes with the assay at 480 nm[7]. This interfering reaction is complete within 5 min and the assay for *p*-nitrophenol subsequently becomes linear. The solution of *p*-nitrophenol should be at room temperature and allowed to stand for at least one hour after preparation or chilling, to eliminate the absorbance changes of this solution. With this kinetic method, no additional processing of samples is required eliminating several potential sources of experimental error[7].

Stock Solutions

p-Nitrophenol (PNP) 1 mM

Dissolve 1.39 mg in 10 ml of HB.

Prepare in the assay day and keep the solution at room temperature at least for 1 hour.

Protocol

1) Prepare freshly the following Enzyme Soup (20 ml/45 samples in duplicate):

	Final Concentration	*Volume*
PNP	0.1 mM	2 ml
HB	100 mM	18 ml

Maintain at room temperature.

2) Prepare freshly the following Start Reaction Solution – NADPH-generating System (1 ml/45 samples in duplicate):

	Final Concentration	*Weight/Volume*
NADP	500 µM	9.95 mg (13 mM)
G-6-P	5 mM	39.5 mg (130 mM)
G-6-PD	1 U/ml	??
HB	100 mM	~1 ml

3) In a 96-well plate, pipette to each well:

- 300 µg microsome protein (make duplicates)
- 200 µl Enzyme Soup

4) Incubate for 5 min at 37ºC in the plate reader.

5) Add 10 µl of Start Reaction Solution (or only HB for blanks) and incubate also at 37ºC for more 5 min.

6) Read the absorbance at 480 nm on a plate reader every 20 sec for 10 min (at 37ºC).

Notes

1) PNP stock solution should had be done at least 1 hour above.

2) *Final Concentration*: final concentration in the assay (~ 260 µl).

Number in brackets ( ): The real concentration made in the Start Reaction Solution.

??: Depends of the lot of enzyme stock. Do the necessary calculations for each lot. Do not forget that the real concentration in the NADPH-generating system must be 26 U/ml.

G-6-P: glucose-6-phosphate

G-6-PD: G-6-P dehydrogenase

3 & 5) Use a repetitive pipette to pipette Enzyme Soup and Start Reaction Solution.

4) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

5) Make blanks for each sample.

6) Use the 'kinetic' function of SOFTmax Pro

Notes

7) A linear (positive) slope should be obtained.

Because it is difficult to see a linear response in this kinetic assay due to its low activity, alternatively, you can increase the incubation time and, because the response is linear, do the following:

1. Add 10 µl of Start Reaction Solution (or HB for blanks) and incubate also at 37ºC for more 5 min. Read the absorbance at 480 nm –> A_0 = Absorbance at 0 min.

2. Incubate for more half an hour mixing the plate every 5 min and read the absorbance again at 480 nm –> A_{30} = Absorbance at 30 min.

3. Calculate the slope manually: $\Delta A = (A_0 - A_{30})/30$.

7.3) Multiply this slope per 1000 to have the units in $mDO.min^{-1}$.

8) Express the activity as nmol/min/mg protein using the ε of 3.567 $mM^{-1}.cm^{-1}$.

A - $nmol.min^{-1}.mg$

Vmax - slope in $mDO.min^{-1}$

V_{assay}/ℓ(lightpath) =constant (0.332) in $ml.cm^{-1}$

$\varepsilon_{4\text{-nitrocatechol}} = 3.567\ mM^{-1}.cm^{-1}$

0.3 – protein used in the assay

To express the activity (A) in the sample use the following formula:

$$A = \frac{Vmax \times 0.332}{3.567 \times 0.3}$$

Note: Because Vmax is in $mDO.min^{-1}$, it is not necessary to multiply the value by 1000 to have the results in $nmol.min^{-1}.mg^{-1}$.

NADPH-Cytochrome P450 Reductase

P15

Experimental Protocol 15

The NADPH-cytochrome P450 reductase (CYPR) is an essential enzyme for microsomal P450-mediated monooxygenase activity, which by interaction with the different cytochrome P450's transfers the essential electron from NADPH.

Principle

CYPR activity is conveniently measured indirectly by its NADPH-cytochrome *c* reductase activity stimulated by high salt concentration conditions[1]. If the activity is assayed in the presence of mitochondria, KCN (1 mM) may be added as a precautionary measure to block non-microsomal activity. Instead of cytochrome *c*, the reduction of other compounds, such as tetrazoliums, may also be used to assay activity[1].

Here, a procedure based in the methodology of Phillips & Langdon (1962) described by Guengerich (1982)[1] is adapted to measure the CYPR activity in a plate reader spectrophotometer. The assay is performed at 30ºC following the reduction of cytochrome *c* at 550 nm in the presence of 100 µM NADPH and 40 µM cytochrome *c* in 300 mM phosphate buffer (pH 7.7).

The activity is calculated using the extinction coefficient of 21.1 $mM^{-1}.cm^{-1}$ and expressed as µmol of cytochrome *c* reduced per min per mg of protein.

Protocol

Sample preparation

Use a post-mitochondrial fraction of any cell homogenate. For that, centrifuge cell or tissue homogenates at 10,000 ×*g* for 10 min at 4ºC. Use supernatant as your sample. It can also be used the microsomal fraction (see P14).

[1] Guengerich FP, 1982. Microsomal enzymes involved in toxicology – analysis and separation. **In:** Hayes AW (Ed.), *Principles and Methods of Toxicology*, Raven Press, New York, pp. 609-634.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

NADPH (*e.g.*, # N1630, Sigma)

Cytochrome c (nitroblue tetrazolium can also be used)

Buffer Solutions

Phosphate Buffer (PB) 300 mM

300 mM KH_2PO_4, pH 7.7

Stable at 4ºC.

Assay Solutions

Cytochrome c 224 µM (Final C. 40 µM)

2.8 mg/ 1 ml PB (5 ml/96-well plate)

Prepare a fresh solution daily and maintain on ice. Prepare only 10% more solution than necessary – expensive compound!

NADPH 2.8 mM (F. Conc. 0.1 mM)

2, 3 mg/ 1 ml PB (1 ml/96-well plate)

Prepare a fresh solution daily and maintain on ice. Prepare only 10% more solution than necessary – expensive compound!

Notes

1 & 4) Use a repetitive pipette to pipette cytochrome *c*, PB and NADPH.

2) The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

3 & 4) Use the 'kinetic' function of SOFTmax Pro

5) Use only the linear part of the curve for the calculations.

U - µmol.min^{-1}.ml^{-1}

Vmax - slope in mDO.min^{-1}

V_{assay}/ℓ (lightpath) =constant (0.332) in ml.cm^{-1}

$\varepsilon_{reduced\ cytochrome\ c}$ = 21.1 mM^{-1}.cm^{-1}

V_{sample} - volume of the sample used in the assay in ml (ex: 0.02 ml).

Enzyme Assay

1) In a 96-well plate, pipette to each well:

- 20 µl sample (make duplicates)
- 50 µl cytochrome *c* (224 µM)
- 200 µl PB

2) Incubate for 3 min at 30ºC in the plate reader.

3) Measure the non-specific activity by reading the absorbance at 550 nm on a plate reader every 10 sec for 3 min.

4) Add as fast as possible 10 µl of NADPH (2.8 mM) and read the absorbance at 550 nm on a plate reader every 10 sec for 3 min.

5) A linear (positive) slope inferior to 200 mDO/min should be obtained. If not, dilute samples accordingly.

To calculate CYPR activity, subtract the slope obtained from the non-specific activity (step 3) from the specific activity (step 4). This gives the Vmax for each sample. Then, the activity can be expressed as µmol cytochrome *c* reduced per min per ml (U/ml) using the following formula:

$$U/ml = \frac{Vmax \times 0.332}{1000 \times 21.1 \times V_{sample}}$$

The specific activity of CYPR (U/mg protein) is then calculated dividing the above value per the value "mg protein/ml" - protein of the sample.

ALT and AST

P16

Experimental Protocol 16

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are both transaminase enzymes that are found mainly in the liver. After liver damage, high levels of these enzymes are present in plasma making them, therefore, good markers of liver injury.

Principle

ALT enzyme converts alanine and α-ketoglutarate to glutamate and pyruvate. On the other hand, AST converts aspartate and α-ketoglutarate to glutamate and oxaloacetate. If these enzyme processes are coupled with ones in which NADH is converted to the oxidised form, the activity of the transaminases can be indirectly measured following NADH oxidation spectrophotometrically at 340 nm[1].

To measure the ALT activity, the assay is performed at 30ºC following NADH oxidation at 340 nm in the presence of 200 mM *L*-alanine, 25 µM pyridoxalphosphate, 0.12 mM NADH, 12 U/ml *L*-lactacte dehydrogenase and 10.5 mM α-ketoglutarate in 50 mM imidazole (pH 7.4). To measure the AST activity, the assay is performed at 30ºC following NADH oxidation at 340 nm in the presence of 40 mM aspartate, 25 µM pyridoxalphosphate, 0.12 mM NADH, 8 U/ml *L*-malate dehydrogenase and 7 mM α-ketoglutarate in 50 mM imidazole (pH 7.4). The activities are expressed as µmol of substrate oxidised per min per liter of plasma/serum.

Protocol

Sample preparation

Use plasma or serum as your sample.

Enzyme Soup

Just before the enzyme assay, make the following enzyme soups for ALT and AST, respectively (25 ml/96-well plate):

[1] Plaa GL & Hewitt WR, 1982. Detection and evaluation of chemically induced liver injury. **In:** Hayes AW (Ed.), *Principles and Methods of Toxicology*, Raven Press, New York, pp. 407-445.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

L-Alanine

Aspartate

α-Ketoglutarate

Pyridoxalphosphate

Lactate dehydrogenase (LDH; free of ammonium sulphate; *e.g.* # 10127221001, Roche)

Malate dehydrogenase (MDH; in glycerol; *e.g.* # 10127248001, Roche)

Buffer Solutions

Imidazole (Imdz.) 50 mM

50 mM Imidazole, pH 7.4

Assay Solutions

Aspartate 1.08 M (Final Conc. 40 mM)

168 mg/ 1 ml Imdz. (1 ml/96-well plate)

Can be frozen once.

α-Ketoglutarate 283.5 mM

41.4 mg/ 1 ml Imdz. (1 ml/96-well plate)

Freshly prepared and stable for 3 h on ice.

NADH 2.4 mM (F. Conc. 0.12 mM)

8,51 mg/ 5 ml Imdz (1.5 ml/96-well plate)

Freshly prepared and stable for 3 h on ice.

Pyridoxalphosphate 5 mM (F.C.. 25 µM)

1.24 mg/ 1 ml Imdz (0.2 ml/96-well plate)

Unstable compound. Last solution to be prepared.

Notes

Final Conc.: final concentration in the assay (270 µl).

??: Depends of the lot of enzyme stock. Do the necessary calculations for each lot. Do not forget that you should use a total volume of 27 ml for the calculations in order to have in consideration the sample volume and the start reaction solution.

LDH: lactate dehydrogenase

MDH: malate dehydrogenase

(1) ALT

	Final Conc.	*Weight/Volume*
NADH (2.4 mM)	120 µM	1.35 ml
L-alanine	200 mM	481 mg
PyridoxalP. (5 mM)	25 µM	135 µl
LDH	12 U/ml	??
Imdz (50mM)	50 mM	23.5 ml

(2) AST

	Final Conc.	*Volume*
NADH (2.4 mM)	120 µM	1.35 ml
α-ketoglutarate	7 mM	667 µl
PyridoxalP. (5 mM)	25 µM	135 µl
MDH	8 U/ml	??
Imdz (50mM)	50 mM	22.85 ml

Enzyme Assay

1 & 4) Use a repetitive pipette to pipette Enzyme Soup and Start Reaction Solution.

1) In a 96-well plate, pipette to each well:

- 10 µl sample (make duplicates)
- 250 µl Enzyme Soup (ALT or AST)

2) The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

2) Incubate for 3 min at 30ºC in the plate reader

3) Measure the non-specific activity by reading the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

4) Use the 'kinetic' function of SOFTmax Pro

4) Add as fast as possible 10 µl of Start Reaction Solution [ALT: α-ketoglutarate (283.5 mM); or, AST: aspartate (1.08 M)] and read the absorbance at 340 nm on a plate reader every 10 sec for 3 min.

5) Use only the linear part of the curve for the calculations.

5) A linear (negative) slope inferior to 100 mDO/min should be obtained. If not, dilute samples accordingly.

U - µmol.min^{-1}

Vmax - slope in mDO.min^{-1}

V_{assay}/ℓ(lightpath) =constant (0.332) in ml.cm^{-1}

ε_{NADH} = 6.22 mM^{-1}.cm^{-1}

V_{sample} - volume of the sample used in the assay in ml (ex: 0.01 ml).

Because Vmax is in mDO.min^{-1}, it is not necessary to multiply the value by 1000 to have the results in U/L.

To calculate transaminase activities, subtract the slope obtained from the non-specific activity (step 3) from the specific activity (step 4). This gives the Vmax for each sample. Then, the activity can be expressed as µmol NADH oxidised per min per L of plasma/serum (U/L) using the following formula:

$$U/L = \frac{Vmax \times 0.332}{6.22 \times V_{sample}}$$

Comet Assay

P17

Experimental Protocol 17

The single cell gel electrophoresis assay or "comet assay" is a sensitive, quick and simple method for measuring DNA damage in single cells.

In general, the alkaline comet assay (the most used) consists of several stages beginning with isolation of cells, agarose embedding, lysis with high salt and detergent, alkaline unwinding and electrophoresis, neutralizing, staining and analysis. In the unwinding conditions used (pH > 13), the high alkalinity of the solution allows the separation of the DNA strands, which means that the assay detects DNA single-strand breaks and alkali labile sites in individuals cells, delaying repair sites of DNA[2].

Principle

When embedded, the cell forms a cavity in the agarose gel. Following lysis, when all the cellular proteins are stripped away and have dissolved through the gel, only the nuclear scaffold proteins remain and the DNA expands to occupy the cavity[1].

[1] Duthie SJ, 2003. The comet assay: protective effects of dietary antioxidants against oxidative DNA damage measured using alkaline single cell gel electrophoresis. **In:** Cutler RJ, Rodriguez H (Eds), *Critical Reviews of Oxidative Stress and Aging*, World Scientific, Singapore, pp. 309-323.

[2] Rojas E, Lopez MC & Valverde M, 1999. Single cell gel electrophoresis assay: methodology and applications. *Journal of Chromatography B* **722**, 225-224.

Materials required

Horizontal electrophoresis tank

Fluorescent microscope

Special reagents:

Low melting agarose

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

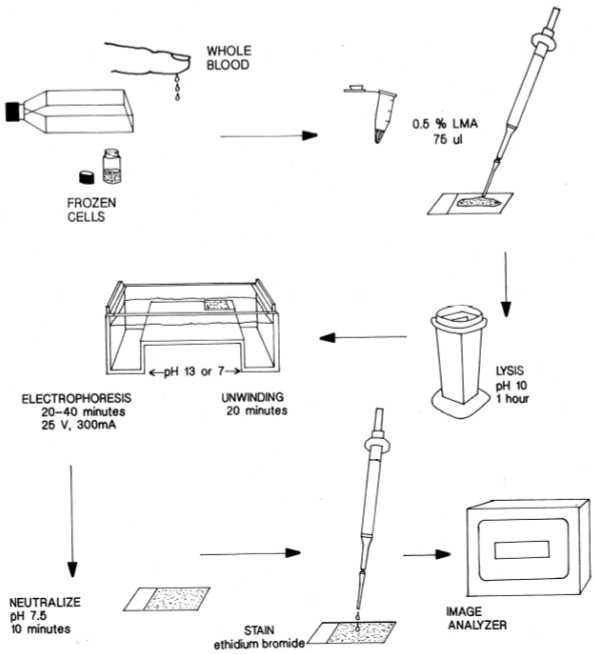


Fig. 1. Protocol outline of the comet assay. After embedding isolated cells in low melting agarose (LMA), cells are lysed for at least 1 hour. Then, DNA is unwound and electrophored in a horizontal electrophoresis tank and then neutralised. Finally, DNA is stained and visualised under a fluorescent microscope and the comet images analysed. Image taken from Rojas *et al.* (1999).

This core of supercoiled DNA is termed a "nucleoid". Following alkaline unwinding and electrophoresis, the negatively charged DNA is pulled towards the anode. However, only loops of DNA which contain breaks are released from the supercoiling and migrate in the gel. These loops extend from the head of the nucleoid to form the comet tail[1]. Comet images can be viewed and analyzed by fluorescence microscopy. The intensity of the comet tail reflects the extent of DNA breakage.

Here, a procedure based in the methodology of Klaud *et al.* (1996)[3], Uhl *et al.* (1999, 2000)[4,5] & Tice *et al.* (2000)[6] is described to perform the alkaline version of the comet assay with HepG2 cells.

[3] Klaud M, Eriksson S, Nygren J & Ahnstrom G, 1996. The comet assay: mechanisms and technical considerations. *Mutation Research* **363**, 89-96.

[4] Uhl M, Helma C & Knasmuller S, 1999. Single cell gel electrophoresis assay with human derived hepatoma (HepG2) cells. *Mutation Research* **441**, 215-224.

[5] Uhl M, Helma C & Knasmuller S, 2000. Evaluation of the single cell gel electrophoresis assay with human hepatoma Hep G2 cells. *Mutation Research* **468**, 213-225.

[6] Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC & Sasaki YF, 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environmental and Molecular Mutagenesis* **35**, 206-221.

Assay Solutions

Prepare the following assay solutions:

(1) Lysis Solution, pH 10

For 1 L:

	Final Conc.	*Weight*
NaCl	2.5 M	146.1 g
Na_2EDTA	100 mM	37.22 g
Tris Base	10 mM	1.211 g

NaOH to pH of 10 (approx 8.0 g)

Pure water added to give a final volume of 990 ml.

Store at 4ºC.

Add immediately prior to use:

TRITON X-100 1% (v/v) (*e.g.* 1 ml per 100 ml)

(2) Electrophoresis Buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13)

	Final Conc.	*Weight*
NaOH	3 N	120 g

Add distilled water to 1 L. Store at room temperature or 4ºC.

Na_2EDTA	200 mM	3.722 g

Add distilled water to 50 ml. Store at room temperature or 4ºC.

Make up Electrophoresis Buffer immediately prior to use. Use freshly made electrophoresis buffer for each set of gels run. For

Normal Melting Point Agarose 1% (w/v)

Dissolve 200 mg normal melting point (NMP) agarose in 20 ml of PBS, heat in microwave for 1-2 minutes at full power until dissolved. When required, melt immediately before use.

Low Melting Point Agarose 0.5% (w/v)

Dissolve 100 mg of low melting point (LMP) agarose in 20 ml of PBS, heat in microwave for 1-2 minutes at full power until dissolved. Aliquot 2 ml samples into cryotubes (more resistant to melting), and store in refrigerator (4ºC). When required, melt immediately before use and maintain at 37ºC in a thermostated water bath.

Ethidium Bromide Staining Solution

Prepare a working solution from a stock solution 10 mg/ml.

Dissolve 50 µl of stock solution in 50 ml of Tris-HCL to give a final concentration of 10 µg/ml. Store at 4ºC, in the dark.

Be aware, carcinogenic compound.

Notes

(2) With 300 mM NaOH, the pH is around 13. You do not need to check the pH.

1 L electrophoresis buffer, mix 100 ml 3N NaOH, 5 ml 200 mM EDTA, and 895 ml of distilled water.
The pH of this solution should be 13 or >13. Be aware of this solution, it's caustic.

Notes

(3) Neutralisation Buffer (Tris-HCl, pH 7.5)

For 500 ml:

	Final Conc.	*Weight*
Tris Base	0.4 M	24.23 g

Add distilled water near to 450 ml and adjust to pH 7.5 using concentrated HCl. Finally adjust the volume with water till 500 ml. Store at 4ºC.

Protocol

Slide Preparation

Dip conventional microscope slides (avoiding slides designated as super clean) in NMP agarose 1% (w/v) and then allowing the gel to dry horizontally at room temperature for 10 min. Take off bottom-side layer, label and dry the slides overnight at 37ºC. Use them within a few days.

Slide Preparation) Use the up-side layer, which did not contact with any surface.

Cell Preparation

1) Incubate 5×10^5 cells per well in a 6-well plate overnight.

2) Treat the cells as desired.

3) After the treatment, wash the cells with warm PBS.

3) Do not use a solution containing Ca and Mg to wash the cells.

4) Incubate the cells with warm 0.125% trypsin (in PBS) for 5 minutes.

4) You can dilute commercial trypsin solution 1:1 with PBS.

Do not exceed the 5 minutes.

5) Aspirate the trypsin medium and suspend the cells in 1 ml of PBS with the pipette.

5) The viability of the cell suspension should be greater than 80%. You can use the Trypan Blue Staining (P6) to see it.

6) Take off enough volume to another microtube in order to have about 50.000 cells.

Alkaline Single Cell Gel Electrophoresis

7) Centrifuge the cells for 1 minute at 5,000 rpm (~5,000 ×*g*).

7) Centrifuge normally used:

Centrifuge 5415 C, Eppendorf

8) Remove supernatant from centrifuged cells; resuspend cells in

8) Melt previously LMN agarose and maintain at 37ºC using a thermo stated water bath. Do not add the LMP agarose

Notes

8 cont.) immediately after melting it. Let first the temperature reaches 37ºC in the water bath.

9) Using a pencil, the label is not erased by the subsequent procedures.

9 & 10) Normally, two samples can be settled in each slide.

11) You may need only to add and dissolve Triton X-100.

12) You have to be careful with the agarose layer from this point forward, because it easily takes off from the slide.

For example, use the lids from multiwell plates as containers. The horizontal position prevents the layer from taking off.

16) Preferably put the slides with the labelled end towards the anode.

18) The voltage to be use depends of the distance between the anode and cathode. Use a ratio of 0.8 V/cm.

19) Use also, for example, the lids from multiwell plates.

21) Use also, for example, the lids from multiwell plates.

The slides can be kept at 4ºC for a week until analysis.

22) Be careful, carcinogenic solution. Use gloves when applying the solution and dry the excess (if some).

100 µl LMP agarose by gently pipetting.

9) Apply one drop of ~75µl LMP agarose/cell suspension on top of the base layer of NMP agarose. Label the slides with a pencil.

10) Cover each drop with a 22×22 mm coverslip and place the slides into fridge for 10 minutes.

11) Prepare the working lysis solution (add Triton X-100).

12) Gently slide off coverslips, place slides in the appropriate container and add lysing solution to fully immerse slides.

13) Incubate for 2 hours at 4ºC, in the dark to lyse the cells.

14) Meanwhile make up electrophoresis buffer, and pour into the electrophoresis tank at 4ºC.

15) After incubation, remove slides from the lysis solution, rise slides in distilled water and then in electrophoresis buffer.

16) Transfer slides into the electrophoresis chamber.

17) Allow the slides to incubate in the alkaline electrophoresis buffer for 40 minutes at 4ºC to allow the DNA to unwind.

18) Turn on power supply and set to 300 mA and 21 V (0.8 V/cm), run the electrophoresis for 20 minutes at 4ºC.

19) Remove slides from electrophoresis tank, and immerse in neutralisation buffer for 5 minutes.

20) Wash two more times for 5 min.

21) Fix (dehydrate) the slides for 5 minutes in 100% ethanol (or methanol), two times.

22) For analysis, apply 20 µl Ethidium Bromide Stainning Solution (working solution) to each sample and apply coverslips.

23) Evaluate DNA damage under a fluorescent microscope using the appropriate filter setting. Do at least 4 independent experiments.

Evaluation of DNA damage

For evaluation of DNA damage, observations should be made using a 20× or 40× objective (depending on the cell size) on a fluorescent microscope. One should avoid analysing cells at the

edges of the gel, where it has been observed that high levels of damage can be seen.

One of the methods of comet analysis is a quantitative method using image analysis programs. There are several image analysis systems commercially available for quantification of data, *e.g.* Komet analysis system developed by Kinetic Imaging Ltd., Comet Assay II developed by Perceptive Instruments, etc. There is one that is free and uses a public domain, which is well explained in the paper published by Helma C & Uhl M (2000)[7].

With this quantitative method, at least 50 cells per sample should be analysed. The results are normally expressed in terms of tail length, tail moment and % DNA in tail[7].

It is also possible to analyse comets semiquantitatively without image analysis software. A scheme has been developed by Collins AR *et al.* (1997)[see ref. 2] for visual scoring based on 5 recognisable classes of comet, from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). Each comet is given a value according to the class it is put into, so that an overall score can be derived from the gel (from 0 to 400). In this case, count 100 cells per slide. The analysis should be blind, *i.e.* without the knowledge of what sample is being analysed. For that use a code to label the samples that was unknown for the person that is analysing the comet images. Use only one person to analyse the comets.

Visit also the following websites:

www.geocities.com/cometassay/

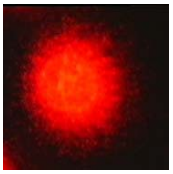
www.predictive-toxicology.org/comet/

[7] Helma C & Uhl M, 2000. A public domain image-analysis program for the single cell gel electrophoresis (comet) assay. *Mutation Research* **466**, 9-15.

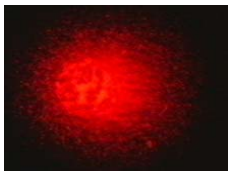
Examples of the five classes of the comet images that are used in the semiquantitative method of visual scoring:

Class 0

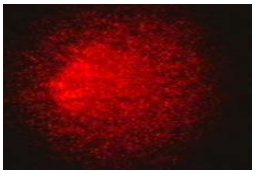
Class 1



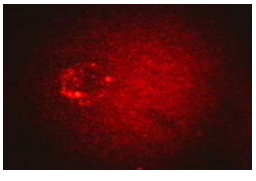
Class 2



Class 3



Class 4



Western Blot of CYP 2E1

P18

Experimental Protocol 18

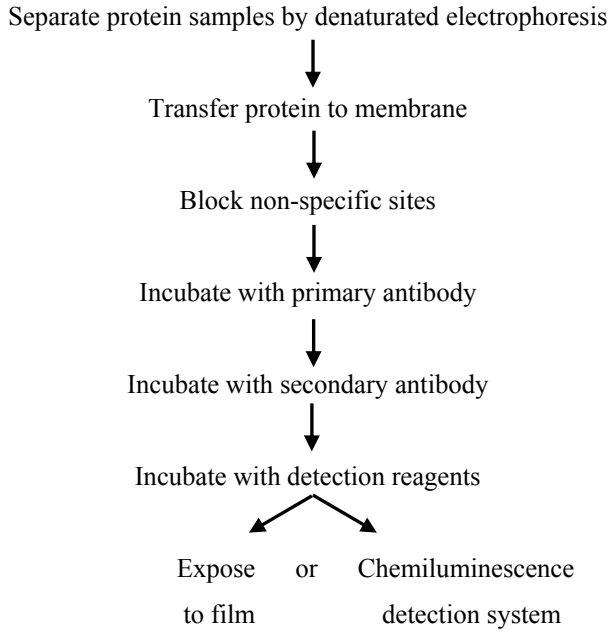
The cytochrome P450 2E1 (CYP 2E1) is involved in the oxidation of ethanol, acetone, acetaminophen, halothane, and other xenobiotics via a NADPH-dependent mechanism. In mammals, CYP 2E1 is constitutively expressed in many tissues but is found in highest concentrations in the liver. Within the liver, it is membrane bound in the smooth endoplasmic reticulum of hepatocytes[1].

Principle

This protein can be quantified by Western Blot using a specific antibody. For that, microsomal protein is separated in a denaturated discontinuous polyacrylamide gel by electrophoresis following the methodology of Laemmli (1970)[2]. Then, the separated proteins were electrotransferred to a membrane using the method of Towbin *et al.* (1979)[3] and the CYP 2E1 detected by using a specific antibody against this protein.

Protocol

Flow diagram



[1] Morgan K, French SW & Morgan TR, 2002. Production of a cytochrome P450 2E1 transgenic mouse and initial evaluation of alcoholic liver damage. *Hepatology* **36**, 122-134.

[2] Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

[3] Towbin H, Staehelin T & Gordon J, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350-4354.

Materials required

Mini-Protean III electrophoresis system (Bio-Rad Laboratories, Inc.)

Mini Trans-Blot System (submerged type) (Bio-Rad Laboratories, Inc.)

Polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences)

Roller Mixer SRT1 (Stuart, Barloworld Scientific Limited)

Special reagents:

Rabbit polyclonal antibody against CYP 2E1 protein (StressGen)

Horseradish peroxidase–labeled donkey anti-rabbit IgG antibody (Amersham Biosciences)

ECL reagents (Amersham Biosciences)

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

2× SDS Sample Buffer

125 mM Tris-HCl, pH 6.8

20% (v/v) Glycerol

4% (w/v) SDS

200 mM DTT

0.01% (w/v) Bromophenol blue

Buffer Solutions

Towbin Buffer

25 mM Tris base

192 mM glycine

20% (v/v) methanol

Blocking Buffer

5% (w/v) nonfat dry milk

0.05% (v/v) Tween 20

PBS

Incubation Buffer

1% (w/v) BSA (bovine serum albumin)

0.05% (v/v) Tween 20

PBS

Notes

2) Whereas 2× SDS Sample Buffer is not added to the samples, place the microtubes on ice.

3 & 4) These steps should be done immediately before the loading of protein samples in the polyacrylamide gel, for example during the polymerization of the stacking gel.

5) For details see for example the protocol present in: Ausubel FM et al, 1996. *Current Protocols in Molecular Biology*, Vol. 2, John Wiley & Sons, Inc, USA.

7) Run the electophoresis using the Towbin Buffer supplemented with 0.1% (w/v) SDS.

11) At no time should the apparatus or buffer be allowed to become hot. Use a cooling coil or run the transfer in a cold room (4ºC) to avoid the generation of gas bubbles in the sandwich.

14 & 16 & 18) Wash at room temperature with moderate agitation.

Sample preparation

1) Use microsomal protein as your sample (see P14).

2) Take 15 µg of protein to a microtube (preferably, it should be in a volume lower than 10 µl).

3) Add 10 µl of 2× SDS Sample Buffer and boil immediately the protein samples for 5 min.

4) Do a small spin to the microtubes to collect the condensate at the bottom of the tube.

Gel Electrophoresis – Reducing Conditions

5) Prepare a 12% (w/v) polyacrylamide discontinuous (with stacking gel) gel using, for example, the Mini-Protean III electrophoresis system.

6) Load protein samples and control (All Blue, Bio-Rad).

7) Start electrophoresis at 90 V for about 10 min and increase (after the dye front has moved into the separating gel) then for 180 V. Stop electrophoresis when the dye front reaches the bottom of the gel (around 50 min).

Protein Transfer

8) Transfer separated protein bands to PVDF membranes following manufacturer's instructions. Briefly:

9) Equilibrate gel and membrane in Towbin Buffer for 15 min.

10) Using a submerged transfer-type system (*e.g.* Mini Trans-Blot System, Bio-Rad) assemble the apparatus according to manufacturer's instructions.

11) Transfer at 4ºC (or use a cooling coil) for 1 h at 100 V, using Towbin Buffer.

Antibodies Incubations

13) Block non-specific binding sites on membrane with Blocking Buffer overnight at 4ºC.

14) Wash three times with PBS/0.05% (v/v) Tween 20 for 5 min per wash.

15) Incubate the membrane with the primary antibody (rabbit polyclonal antibody against CYP 2E1 protein) for 1 hour at room temperature using the Roller Mixer.

16) Wash three times with PBS/0.05% (v/v) Tween 20 for 5 min per wash.

17) Incubate the membrane with the secondary antibody (horseradish peroxidase–labelled donkey anti-rabbit IgG antibody) for 1 hour at room temperature using the Roller Mixer.

18) Wash three times with PBS/0.05% (v/v) Tween 20 for 5 min per wash.

Notes

15) Dilute 1:5,000 the antibody in 10 ml of Incubation Buffer supplemented with 0.05% (w/v) NaN_3 (sodium azide).

The use of sodium azide allows the reuse of the antibody solution, since the compound prevents the growth of microorganisms. For that, keep the solution at 4ºC.

17) Dilute 1:30,000 the antibody in 10 ml of Incubation Buffer. Do not use sodium azide, since it inhibits peroxidase.

Detection

19) Incubate membranes with ECL detection reagents for 1 min following manufacturer instructions.

20) Drain off excess detection reagent by holding the membrane gently with forceps and touching the edge against a tissue. Place the membrane, protein side up, between two sheets of suitable plastic clean surfaces and gently smooth out any air bubbles.

21) Place the wrapped blots, protein side up, in an X-ray film cassette and expose to an autoradiography film. Then develop the film

or

22) Place the wrapped blots, protein side up, under a chemiluminescence detection system and acquire the image.

22) Chemiluminescence detection system used: Chemi Doc XRS, Bio-Rad Laboratories, Inc.

Quantification

23) Quantify the bands in the developed film by densitometry using, for example, the SigmaScan Pro 5 program (SPSS Inc., San Rafael, CA, USA), or, if using the chemiluminescence detection system, with the attached program (Quantity One, Bio-Rad Laboratories, Inc.).

24) Express the results as Band Area Intensity (band area × band average intensity).

24) Using the Quantity One the value ‘Adj. Volume’ corresponds to the Band Area Intensity.

Antiradical Activity: DPPH and Superoxide Radical

Several methods to determine free radical scavenging (antiradical) activity have been reported, but those utilising the xanthine-xanthine oxidase system, phenazine methosulphate-NADH system and the reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) have received more attention[1].
Here, we describe two methods to determine antiradical activity of phenolic compounds and plant extracts: (1) against DPPH radical; and, (2) against superoxide radical using the phenazine methosulphate-NADH system

[1] Sanchez-Moreno C, Larrauri JA & Saura-Calixto F, 1998. A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture* **76**, 270-276.

(1) DPPH

Principle

The antiradical activity of phenolic compounds (PheOH) depends mainly on different structural features such as O-H bound dissociation energy, resonance delocalisation of the phenol radical (PheO˙) and steric hindrance derived from bulky groups substituting hydrogen in the aromatic ring[1]. The rate constants of the reaction of PheOH with free radicals will indicate the order of reactivity.

The main reaction involving DPPH would be[1]:

$$DPPH^{\cdot} + PheOH \rightarrow DPPHH +$$

$$[PheO^{\cdot}(I) \leftrightarrow PheO^{\cdot}(II) \leftrightarrow PheO^{\cdot}(III) \ldots]$$

where (I), (II), (III) . . . are resonance structures.

The concentration of antioxidant needed to decrease by 50% the initial substrate concentration (IC_{50}) is a parameter widely used to measure the antioxidant power. The lower the IC_{50} the higher the antioxidant power. Sanchez-Moreno *et al.* (1998) also introduced a new parameter, the antiradical efficiency (AE), which involves the potency (IC_{50}) and the reaction time (T_{IC50}):

$$AE = 1/(IC_{50} \times T_{IC50})$$

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

2,2-diphenyl-1-picrylhydrazyl (DPPH)
(*e.g.*, # D9132, Sigma)

Stock Solutions

DPPH 1 mM

3.94 mg/ 10 ml absolute ethanol

Light sensitive

Aliquot in 1.2 ml and keep at -20ºC.

Assay Solutions

DPPH 100 µM (Final Conc. 90 µM)

Dilute 1.1 ml of DPPH 1 mM in a total volume of 11 ml of absolute ethanol, immediately before use.

Light sensitive, maintain at room temperature.

(volume enough to test one compound/extract in the conditions described in the assay)

The lower the IC_{50}, the lower the T_{IC50} and the higher AE. T_{IC50} is defined as the time needed to reach the steady state at the concentration corresponding to IC_{50}[1]. For more details about this parameters see the paper of Sanchez-Moreno *et al.* (1998)[1].

Protocol

Sample preparation

The following assay is adapted in order to test 7 different concentrations of the compound/extract. Therefore, for each compound/extract chose 7 different concentrations to be tested. Then, immediately before the assay, make the corresponding 10 times more concentrated solutions (at least 200 µl each) dissolved in DMSO for each concentration chosen.

DPPH Assay

1) In a 96-well plate, pipette to each well:

- 19.4 µl sample/blank (DMSO) (6 wells each in a row)
- 175 µl DPPH (100 µM)/ absolute ethanol (pipette first the absolute ethanol)

2) Immediately after, read the absorbance at 515 nm on a plate reader every 20 sec for 20 min. (at room temperature)

3) After 20 min, if the discoloration of DPPH did not reach a steady state continue reading for more a 20 min-period.

Calculations

4) Take the absorbance from a time point where all concentrations reached the steady state. Use the same time point for each well.

5) Then, for each row, subtract the corresponding blank value (again use the same time point for blank as well samples).

6) Calculate the remaining DPPH for each well using the following formula:

$$\% \text{ DPPH} = Abs_{Cx} / Abs_{C0} \times 100$$

7) Using the GraphPad Prism (GraphPad Software Inc.), plot the

Notes

Sample preparation)

Example:

If you want to test the concentrations of 1, 2.5, 5, 10, 20, 35 and 50 µM of a compound, you have to make the following set of concentrations for the assay: 10, 25, 50, 100, 200, 350 and 500 µM.

The set of concentrations must include from 0% DPPH discoloration to 100% (or near). To know what the ideal set of concentrations is you have to do a preliminary experiment.

1) Use a repetitive pipette to pipette DPPH and absolute ethanol into the wells.

Follow the following scheme (use half of a 96 well plate):

C0	C0	C0	C0	C0	C0
C1	C1	C1	C1	C1	C1
C2	C2	C2	C2	C2	C2
C3	C3	C3	C3	C3	C3
C4	C4	C4	C4	C4	C4
C5	C5	C5	C5	C5	C5
C6	C6	C6	C6	C6	C6
C7	C7	C7	C7	C7	C7

C0 – blank - 19.4 µl DMSO

C1 – lower concentration of the compound/extract

……

C7 – higher concentration of the compound/extract

175 µl DPPH

175 µl absolute ethanol

2) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

Use the 'kinetic' function of SOFTmax Pro. Do not use the blank function for the blank column. As well samples, refer them as unknowns.

6) Abs_{Cx} – Absorbance of a well that contains tested compound/extract, where was added DPPH, in a chosen time point.

Abs_{C0} – Absorbance average of the row C0, where was added DPPH, in a chosen time point.

corresponding % DPPH for each compound/extract concentration (in logarithm) and take the IC_{50} (with the experimental setup outlined above for each compound/extract you have an independent experiment with 5 replicates).

8) Express IC_{50} value in terms of initial concentration of DPPH to make the results easily comparable with other published results.

9) You can also take the Hill slope value from the GraphPad Prism. Hill slope correspond to the graphically calculated slope from the plotted compound/extract concentration (in logarithm) versus the remaining DPPH concentration. The higher this value, the narrower the concentrations range from 0 to 100% of antiradical activity.

10) Calculate also Antiradical Efficiency (AE) using the method well described by Sanchez-Moreno *et al.* (1988)[1].

Notes

8) In case of a pure compound, express in mmol/mol DPPH by dividing the IC_{50} in µM by 0.09 (because we use 90 µM of DPPH in the assay)

In case of an extract, express in g/Kg DPPH by dividing the IC_{50} in µg/ml by 0.0355 (because 90 µM of DPPH in the assay correspond to 0.0355 × 10-3 Kg).

(2) Superoxide Radical

Principle

The antiradical activity against superoxide radical can be measured using a nonenzymatic assay using a phenazine methosulphate (PMS)-NADH system. Superoxide radicals were generated by the NADH/PMS system reducing the nitroblue tetrazolium (NBT) to a blue chromagen. Therefore, the production of superoxide radicals can be followed spectrophotometrically at 560 nm[2]. A compound/extract present in the assay with scavenging activity against superoxide radical will compete with NBT for superoxide radicals inhibiting, therefore the production of the blue chromagen. The superoxide dismutase (SOD) can be used as a positive control. The concentration of antioxidant needed to decrease by 50% the control's blue chromogen production (IC_{50}) is a parameter that can be used to measure the antioxidant power.

Here, a procedure based in the methodology of Valentao *et al.* (2001)[2] is described to measure superoxide scavenging activity of phenolic compounds/plant extracts in a plate reader spectrophotometer. The reaction mixture consists of NADH (166 µM), NBT (43 µM) and PMS (2.7 µM) in 19 mM

[2] Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM & Bastos ML, 2001. Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. *Journal of Agricultural and Food Chemistry* **49**, 3476-3479.

Materials required

96-well plates

Plate reader spectrophotometer

Special reagents:

Phenazine methosulphate (PMS)
(*e.g.*, # 68600, Fluka)

Nitroblue tetrazolium (NBT)

Buffer Solutions

Phosphate buffer (PB) 19 mM

19 mM KH_2PO_4, pH 7.4

Stock Solutions

PMS 7.05 mM

2.16 mg/ 1 ml PB

Highly light sensitive.

Aliquot in 20 µl and keep at -20ºC.

Assay Solutions

NBT 75 µM (Final Conc. 43.3 µM)

1.23 mg/ 20 ml PB (15 ml/96-well plate)

Light sensitive.

NADH 578 µM (Final Conc. 166 µM)

4.1 mg/10 ml PB (7.5 ml/96-well plate)

Freshly prepared and stable for 3 h on ice.

PMS 70.5 µM (Final Conc. 2.7 µM)

Dilute 10 µl of PMS 7.05 mM in 1 ml of PB. (1 ml/96-well plate)

Dilute only immediately before use. Highly light sensitive.

Notes

Sample preparation)

Example:

If you want to test the concentrations of 1, 2.5, 5, 10, 20, 35 and 50 µM of a compound, you have to make the following set of concentrations for the assay: 10, 25, 50, 100, 200, 350 and 500 µM.

The set of concentrations must include from 0% inhibition of blue chromogen production to 100% (or near). To know what the ideal set of concentrations is you have to do a preliminary experiment.

1) Follow the following scheme (use half of a 96 well plate):

C0	C0	C0	C0	C0	C0
C1	C1	C1	C1	C1	C1
C2	C2	C2	C2	C2	C2
C3	C3	C3	C3	C3	C3
C4	C4	C4	C4	C4	C4
C5	C5	C5	C5	C5	C5
C6	C6	C6	C6	C6	C6
C7	C7	C7	C7	C7	C7

C0 – blank - 26.1 µl DMSO

C1 – lower concentration of the compound/extract

……

C7 – higher concentration of the compound

Compound/Extract A

Compound/Extract B

1 & 3) Use a repetitive pipette to pipette NADH, NBT and PMS into the wells.

phosphate buffer, pH 7.4. Phenolic compounds were dissolved in DMSO. The production of blue chromogen is followed over time at 560 nm.

Protocol

Sample preparation

The following assay is adapted in order to test 7 different concentrations of the compound/extract. Therefore, for each compound/extract chose 7 different concentrations to be tested. Then, immediately before the assay, make the corresponding 10 times more concentrated solutions (at least 200 µl each) dissolved in DMSO for each concentration chosen.

Superoxide Assay

1) In a 96-well plate, pipette to each well:

- 26.1 µl sample/blank (DMSO) (3 wells each in a row)
- 75 µl NADH (578 µM)
- 150 µl NBT (75 µM)

2) Incubate for 3 min at 30ºC in the plate reader.

3) Add as fast as possible 10 µl of PMS (70.5 µM) and read the absorbance at 560 nm on a plate reader every 10 sec for 3 min.

4) A linear (positive) slope (approximately 110 $mDO.min^{-1}$) is obtained in the control (C0). If the compounds/extracts have superoxide scavenging activity, they should decrease the slope value in accordance to the concentration.

Calculations

5) Take the slope value (average) from each condition.

6) Calculate for each concentration the percentage of inhibition of blue chromogen production using the following formula:

$$\% \text{ inhibition} = S_{Cx} / S_{C0} \times 100$$

7) Using the GraphPad Prism (GraphPad Software Inc.), plot the corresponding % inhibition for each compound/extract concentration (in logarithm) and take the IC_{50} (with the

experimental setup outlined above for each compound/extract you should do three independent experiments with 3 replicates).

9) You can also take the Hill slope value from the GraphPad Prism. Hill slope correspond to the graphically calculated slope from the plotted compound/extract concentration (in logarithm) versus the % of inhibition of blue chromogen production. The higher this value, the narrower the concentrations range from 0 to 100% of antiradical activity.

Notes

2) Plate reader spectrophotometer used: SpectraMax 340 PC (Molecular Devices) in combination with its software SOFTmax Pro 4.0.

The temperature of 30ºC allows the acquisition of reproducible measurements in both Winter and Summer.

3) Use the 'kinetic' function of SOFTmax Pro.

6) S_{Cx} – Average slope value from a chosen concentration of the tested compound/extract.

S_{C0} – Average slope value from the control situation C0.

Coating with Collagen

P20

Experimental Protocol 20

The coating of surfaces with collagen is normally used in primary cultures of hepatocytes. Collagen isolated from rat tails is used to coat cell culture plates or flaks and then seeded with hepatocytes.

(1) Collagen Isolation

Principle

Collagen is soluble in liquid solution at low pH and salt concentration. Increasing the pH to 7.4 and increasing the ionic strength causes the collagen to gel.

Here, a procedure based in the methodology present in Freshney (1998)[1] is described to isolate collagen from rat tails. Rat tail tendons are dissolved in 0.5 M acetic acid and dialysed against PBS.

Protocol

1) Cut off rat tail and soak in 70% ethanol for 1 min.

2) Fracture progressively from the tip and pull out the tendons.

3) Dissolve tendons from 20 tails in 200 ml of 0.5 M sterile acetic acid. Stir overnight at 4ºC.

4) Filter through sterile muslin gauze.

5) Dialyse against 4 L sterile PBS (pH 7.4) for 24 hours at 4ºC with slow agitation.

6) Then, dialyse against 4 L sterile PBS (pH 4) for 24 hours at 4ºC with slow agitation.

7) Centrifuge to clarify at 50,000 ×*g* for 2 hours.

8) Keep the clarified collagen at -80ºC for later use.

[1] Freshney RI, 2000. *Culture of Animal Cells: a Manual of Basic Technique*, 4th Edition, Wiley-Liss, Inc, USA.

Materials required

Dialysis membranes

Buffer Solutions

PBS (Phosphate Buffered Saline)

137 mM NaCl

2.7 mM KCl

8.5 mM Na_2HPO_4

1.5 mM KH_2PO_4, pH 7.4

Sterilise by autoclaving.

Acetic acid 0.5 M

0.5 M $C_2H_4O_2$

Sterilise by autoclaving.

Notes

1) You can freeze the tails at -20ºC for later use.

2) Handle with sterile gloves and keep tendons sterile.

6) Adjust pH of PBS to 4.

7) Centrifuged used: Beckman Avanti J-25I (Beckman Instruments, Inc., USA) with the rotor JA-25.50.

(2) Collagen Coating

Principle

Collagen gel may be derivatised by carbodiimide to increase its adherence to the plastic substrate. This new type of collagen surface allows extended culture survival, improved microscopy and dry storage of coated culture dishes/flasks/culture plates[1].

Collagen is derivatised to plastic by a cross-linking reagent, 1-cyclohexyl-3-(2-morpholinoethyl)-carbidiimide-metho-*p*-toluenesulfonate (carbodiimide). In comparison to conventional ammonia-polymerised or adsorbed surfaces show superior culture viability and improved optical characteristics.

Here, a procedure based in the methodology present in Freshney (1998)[1] is described for a simple covalent bonding of collagen fibrils to active groups on tissue culture plastic. A large supply of coated dishes/flasks/plates can be prepared in a single 5-hours session and stored for later use.

Protocol

Materials required

Culture dishes/flasks/plates (could be without any special surface treatment)

Special reagents:

Carbodiimide (Cyanamide)
(*e.g.*, # C1920, Sigma)

Stock Solutions

Collagen (~500µg/ml)

Collagen solution diluted in acetic acid at protein concentration of approximately 500 µg/ml.

To know approximately the concentration of collagen in your solution (see above collagen isolation) dry 1 ml of collagen solution and weigh the dry powder.

Solutions

Ultra-Pure Water

Sterilise by autoclaving.

Notes

1) Normally, you can place a small granule of compound without weighing it.

You can use them immediately or seal them and store at 4ºC until used.

2) Each prepared tube will coat 15 35-mm culture dishes or 2.5 6-well culture plates or 5 25-ml culture flasks.

Do the following operations in a sterile laminar flow chamber:

1) Place about 2 mg of carbodiimide in each of several 15-ml sterile tube.

2) Add 14 ml of sterile ultra-pure water at room temperature to each tube containing carbodiimide to be used.

3) Vortex each tube for approximately 10 sec. and set aside.

4) Add 1 ml of stock collagen solution to one tube containing carbodiimide solution (~130 µg/ml) and rapidly vortex until uniform.

5) Rapidly transfer collagen-carbodiimide solution to dishes/flaks/plates, generously covering the bottoms.

5) Transfer approximately 1 ml in a 35-mm dish or 1 ml into a well of a 6-well culture plate or 3 ml in a 25-ml flask.

The rapidity of transfer minimises derivatisation to the solution tube and maximises early contact with the culture plastic.

6) Incubate dishes/flasks/plates at 25ºC for 3 hours.

7) Wash 3 times with sterile ultra-pure water.

8) Air dry at room temperature for 1 hour.

9) Sterilise under ultraviolet irradiation for 1 hour or overnight.

10) Use coated dishes/flaks/plates immediately or store dry at 4ºC for later use.

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