Rooibos tea (*Aspalathus linearis*) ameliorates the CCl₄-induced injury to mitochondrial respiratory function and energy production in rat liver

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**Abstract.** The rooibos tea (RT) is a source of valuable dietary dihydrochalcones – aspalathin, nothofagin and other polyphenols. Many *in vitro* and *in vivo* studies have shown that RT flavonoids have strong antioxidant effect and significantly reduce oxidative stress. We investigated the antioxidant activity and protective effect of an aqueous extract of RT on the liver mitochondria oxidative phosphorylation in rats with carbon tetrachloride-induced (CCl₄-induced) liver damage. Mitochondrial respiration and ATP production was determined amperometrically using a Clark-type oxygen electrode. We found significantly decreased parameters of oxidative phosphorylation in the group having received CCl₄ for 10 weeks. Simultaneous administration of RT increased oxygen uptake stimulated with ADP, and the rate of ATP generation in the mitochondria of rats, both having been impaired in rats treated with CCl₄ only. Treatment with RT significantly decreased CCl₄-induced elevated enzyme levels, improved capacity of the respiratory chain and energy production, presumably due to its potent and direct antioxidant activity, including inhibition of mitochondrial lipid peroxidation. Improved histological features support the view of antioxidant and membrane-stabilizing activity of RT. This fact may play a significant role in the protection of the liver from injury caused by known toxins, and from subsequent development of steatosis and fibrosis.

**Key words:** Rooibos tea — Carbon tetrachloride — Liver damage — Mitochondria — Oxidative phosphorylation

**Introduction**

Over the past decade, mounting data support the notion that mitochondrial dysfunctions as early and initiating events are involved in the etiology of different chronic pathological conditions in tissues and organs, such as liver, brain, or heart (Rector et al. 2010; Breuer et al. 2012; Ong and Gustafsson 2012). Among conditions associated with impaired mitochondrial function non-alcoholic fatty liver disease (NAFLD), as a multisystem disease and the most common cause of chronic liver disease worldwide, carries a substantial clinical burden as it affects several extra-hepatic organs and regulatory pathways, and is associated with an increased mortality (Le et al. 2017). The prevalence of NAFLD is approximately 20–30%, and parallels the continual rise of the obesity epidemic. NAFLD refers to a wide spectrum of liver diseases that, like alcoholic liver disease, range from simple steatosis, through non-alcoholic steatohepatitis (NASH) to fibrosis, and can eventually progress to cirrhosis associated with a 5–10-fold increase in mortality, mainly from cardiovascular disease, malignancy or end-stage liver disease.
NAFLD belongs to the mitochondrial diseases in whose pathogenesis the mitochondrial dysfunction plays a fundamental role (Pessaire and Fromenty 2005; Begriche et al. 2006). The liver is a vital organ in all mammals and is a major site for synthesis, metabolism, storage and redistribution of saccharides, proteins and lipids. Apart from that the liver mitochondria integrate these metabolic pathways and mediate apoptosis and necrosis, they fulfil an essential task in producing sufficient amount of energy in the form of ATP generated in the process of oxidative phosphorylation.

Under physiological conditions mitochondrial respiratory chain continually produces superoxide (O$_2^-$) as a by-product of normal respiration through the one-electron reduction of molecular oxygen. This production is kept under control by mitochondrial antioxidant systems (Begriche et al. 2013). However, excess free radical production originating from exogenous and/or endogenous sources may lead to mitochondrial overproduction of O$_2^-$, which initiates a range of damaging reactions through the production of hydrogen peroxide, ferrous iron, hydroxyl radical, and peroxynitrite. These oxidative events propagate, thus damaging lipids, proteins, and nucleic acids (Dalle-Donne et al. 2006; Al-Dalaen and Al-Qaitat 2014), which consequently leads to mitochondrial dysfunction. It has been suggested that it is the accumulation of damaged mitochondria presenting with abnormal reactive oxygen species (ROS) formation, glutathione (GSH) depletion, protein alkylation, and respiratory chain alterations that may contribute to the pathophysiology of a wide variety of chronic liver diseases. Depending on the nature and severity of exogenous noxae, the mitochondrial alterations may induce lipid accumulation, apoptosis, and/or necrosis leading to hepatic cytolysis and inflammation (Esposti et al. 2012). However, different types of stress can target directly or indirectly hepatocyte mitochondria, such as drugs, viruses, hypoxia condition, high levels of inflammatory cytokines, excess of β-oxidation, ectopic expression of cytochromes P450 etc. In such a case, overproduction of ROS may damage both mitochondrial and cellular biomacromolecules, as well as supramolecular structures. These cellular impairments can further favour the development of tissue lesions, such as steatohepatitis or hepatocellular carcinoma.

A proven and frequently used experimental model of free-radical injury to liver is a model in which rats are exposed to carbon tetrachloride (CCl$_4$). CCl$_4$ is one of the most widely used hepatic toxins for radical damage and induction of liver fibrosis/cirrhosis (Wu and Norton 1996; Vangavus et al. 2016). Reactive metabolites such as trichloromethyl radical (·CCl$_3$) or trichloromethylperoxy radical (·CCl$_3$O$_2$), formed by cytochrome P450, elicit production of ROS and cause lipid peroxidation, which is considered to be responsible for hepatocellular damage and enhanced production of connective tissue (Recknagel et al. 1989; Britton and Bacon 1994; Hernandez-Munoz et. al. 1994). Administration of CCl$_4$ to rats during a 5-week period induces above mentioned processes eventually leading to manifestation of liver fibrosis and cirrhosis with morphological and pathological features resembling patterns seen in humans (Tamayo 1983).

The need for inhibiting oxidative stress has promoted investigation of natural compounds with antioxidant properties. Polyphenols are the largest group of phytochemicals with over 8000 identified compounds. Polyphenols can be split into several subgroups, including flavonoids and non-flavonoids (e.g. lignans). Flavonoids are commonly found in plant-based food (fruits, vegetables, nuts, legumes, grains, cocoa powder, etc.) and beverages (such as tea, chocolate, coffee, and wine). Most data indicate a significant activity of flavonoids in the prevention of oxidative stress (Cao et al. 1997; Pereira et al. 2009; Van der Merwe et al. 2015; Waisundara and Hoon 2015; Domitrović and Potočniak 2016). The advantage of flavonoids is that they exhibit antioxidant activity in both hydrophilic and lipophilic media (Rice-Evans et al. 1996). Moreover, studies have shown that the polyphenols are able to penetrate tissues, particularly those in which they are metabolized such as intestine and liver (D’Archivio et al. 2007).

A herbal tea called rooibos tea (RT) is becoming popular partly because it is being marketed as a healthy beverage containing significant amounts of polyphenol antioxidants. The rooibos plant (Aspalathus linearis (Burm. f.) Dahlgren, Fabaceae) is an endemic South African fynbos species used for preparing traditional beverages for generations. Although in the early years, different Aspalathus species and ecotypes, were used to produce RT (Joubert and de Beer 2011), today only the Rocklands red type, native to the Pakhuis Pass area in the northern Cedarberg region of South Africa, is cultivated commercially and exported across the world by various companies, mainly from Clanwilliam and Wupperthal. It has been shown that major production areas of rooibos deliver herbal tea of similar phenolic and phenylpropenoic acid glucoside content (Joubert et al. 2016).

Rooibos contains a wide range of hydroxylated and polyhydroxylated phenolic compounds among which aspalathin, a C–C-linked dihydrochalcone glucoside, and aspalamin, a cyclic dihydrochalcone, are only isolated from rooibos. Rooibos is also one of the only three known sources of nothofagin, a 3-dehydroxydihydrochalcone glucoside. Other flavonoids include the C–C-linked β-D-glucopyranosides such as flavones orientin and iso-orientin as well as vitexin and isovitexin, both flavone analogues of nothofagin. The flavanones, dihydro-orientin and dihydroiso-orientin, as well as hemipherolin have also been isolated from rooibos. Other flavones isolated from rooibos include chrysoeriol, luteolin and luteolin-7-o-glucoside, while the flavonols quercetin, quercetin-3-orobinoside, hyperoside, isoquercitrin and rutin are also present. The presence of phenolic acids, lignans and
the coumarin, esculetin, as well as monomeric flavan-3-ol, (+)-catechin and oligomeric flavan-3-ol, procyanidin B3 and bis-fisetinidol-(4β,6:4β,8)-catechin, has also been detected in rooibos (Rabe et al. 1994; Joubert 1996; Kazuno et al. 2005; Joubert et al. 2008, Joubert and de Beer 2011, and reviewed in Ajuwon et al. 2015). These compounds serve as scavengers of free radicals. Tea prepared from fermented as well as from non-fermented rooibos leaves has been reported to reduce levels of ROS, such as superoxide radical (O$_2^−$) (Yoshikawa et al. 1990; Joubert et al. 2004), hydroxyl radical (•OH) (Yoshikawa et al. 1990; Joubert et al. 2005; Snijman et al. 2009) and alkyl-peroxyl radicals generated in the process of lipoperoxidation (Sano et al. 2003; Simpson et al. 2013). Besides antioxidant activities other biological and health-promoting effects have been shown for rooibos extracts including antimutagenic, anti-inflammatory, anti-diabetic, antimicrobial and hepatoprotective properties has been revealed (reviewed in Ajuwon et al. 2015).

Using the CCl$_4$-induced liver fibrosis model, we have previously demonstrated the hepatoprotective effects of RT in terms of significantly reduced fibrosis and steatosis induced by a ten-week intraperitoneal administration of CCl$_4$ to Wistar rats (Uličná et al. 2003, 2008) and accelerated healing in the phase of regeneration (Uličná et al. 2008). We hypothesized that if the above assumption about hepatoprotective effects of RT is true, there should be observable effects of RT on the function of liver mitochondria in the CCl$_4$-induced liver injury.

The purpose of this study was, therefore, to investigate in vivo effect of RT on mitochondrial bioenergetics in a model of CCl$_4$ liver damage, which, to our knowledge, has not been studied yet. A secondary outcome of the study was to evaluate the detrimental effect of 10-week administration of CCl$_4$ in terms of parameters of oxidative phosphorylation in the rat liver mitochondria. We executed a factorial experiment to determine what proportion of variation in the parameters of oxidative phosphorylation could be ascribed to RT when adjusting for adverse effect of CCl$_4$.

Materials and Methods

Chemicals

D(-) mannitol, saccharose, potassium chloride, ethylenedinitrilotetraacetic acid disodium salt (Titriplex III), potassium dihydrogen phosphate, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethansulfonate acid (HEPES) were obtained from Merck, L(+)-glutamic acid monosodium salt monohydrate, adenosine 5’diphosphate sodium salt (ADP), dextran, were obtained from Sigma. The remaining chemicals were of analytical grade purity and obtained from Centralchem.

Plant material

Rooibos (Aspalathus linearis) of the highest grade according to the South African regulations relating to quality standards for rooibos (Joubert and de Beer 2011) has been used to prepare aqueous extract. The extract was prepared daily by boiling 2.5 g dry tea in 1000 ml water for 20 min and cooling down to room temperature. Insoluble residue was separated and the aqueous extract was used in experiment as a substitute for drinking water.

Animals

Male Wistar rats were obtained from the Department of Toxicology and Laboratory Animal Breeding (Dobrá Voda, Slovakia). They were maintained at a 12 h light/dark cycle at a constant temperature of 20–24°C with a free access to tap water and food (the standard diet, KKZ-P/M, Dobrá Voda). All experiments were carried out according with the guidelines for the care and use of experimental animals, and were approved by the State Veterinary Administration of the Slovak Republic.

Experimental procedure

The animals were assigned randomly to one of four groups: C (the untreated control), RT+C (the group with free access to aqueous RT extract instead of tap water), CCl$_4$ (the group treated with carbon tetrachloride), RT+CCl$_4$ (the group treated with CCl$_4$ and, at the same time, with free access to RT extract). CCl$_4$ was applied intraperitoneally as a 50% solution in olive oil at a dose of 1 ml/kg twice a week for 10 weeks. To ensure identical experimental conditions, C and RT+C groups were administered with olive oil in the same manner as the experimental groups. Both groups, RT+C and RT+CCl$_4$, had free access to RT extract instead of tap water, starting a week before CCl$_4$ administration. During the experiment two animals in the CCl$_4$ and two animals in the RT+CCl$_4$ groups died. Ten weeks after CCl$_4$ administration had been started and 48 hours after the last treatment with CCl$_4$, the rats were anesthetized with thiopental at a dose of 80 mg/kg. Blood samples were taken from the abdominal aorta into heparinized tubes. The livers were removed and fresh liver tissue was immediately used for isolation of mitochondria. A part of right lobe was fixed for histological examination. The right lobe was fixed in 4% buffered formaldehyde solution and further processed for histological examination.

Histological examination

Paraffin sections were prepared and stained with hematoxylin and eosin to visualize general tissue morphology. Microscopic examination was done using a Leica DM 200 micro-
scope (Leica Germany) and images were observed under polarized light at magnification 200x. The percentage steatosis and fat droplet size were assessed using a histomorphometric method. The fibrosis extent was measured as a percentage of picrosirius-red-positive area in the microscopic field. The evaluation was performed by an expert liver pathologist (PJ) who was blinded to the allocation of the samples.

**Biochemical analysis**

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as concentrations of albumin (Alb) and total bilirubin (tBil) in plasma were evaluated by standard automated techniques using the Hitachi Analyzer Model 911 and adequate kits from the Roche Company (Switzerland).

**Isolation of mitochondria**

Mitochondria were isolated from freshly excised livers by differential centrifugation as described by Hogeboom (1955), with some modification. The isolation was performed according to Sammut et al. (1998), with the following modification: 225 mM mannitol, 75 mM saccharose, and 0.2 mM Triton X-100. Liver was minced in the isolation medium (pH 7.4) at 4°C, and homogenized using a teflon-to-glass homogenizer. The homogenate was centrifuged at 700 × g for 10 min, the supernatant was decanted and centrifuged at 5600 × g for 10 min. The mitochondrial pellet was washed twice with isolation medium. The resulting pellet was resuspended in the same medium to a final protein concentration of 20–40 mg/ml. All procedures were performed at 4°C. Proteins were determined according to Lowry et al. (1951).

**Measurement of mitochondrial function**

Respiratory function of the mitochondria was measured at 30°C by amperometric monitoring of oxygen consumption on an oxygraph Gilson 5/6 H (France) equipped with a Clark-type oxygen electrode. The incubation medium was prepared as described by Rouslin and Millard (1980) with a modification: 12.5 mM HEPES, 122 mM KCl, 3 mM KH₂PO₄, 0.5 mM Tritriplex III, and 2% dextran. Glutamate/malate at 2.5 mM was used as a NAD substrate. For assessing stimulated oxygen consumption, 500 nmol of ADP was added.

The following parameters of oxidative phosphorylation were determined in the liver mitochondria: respiratory control index (RCI) – the ratio of state 3 to state 4 indicates the dependence of the respiration rate on availability of ADP. Coefficient of oxidative phosphorylation (ADP:O) indicates how respiration is linked to phosphorylation. The rate of oxygen uptake by the mitochondria stimulated with ADP – state 3 [\( \text{O}_{2}(S_3) \)] expresses the velocity of oxygen consumption by mitochondria in the presence of ADP and substrate. The rate of basal oxygen uptake by mitochondria without ADP – state 4 [\( \text{O}_{2}(S_4) \)] denotes how fast oxygen is used by mitochondria in the presence of substrate only. The oxidative phosphorylation rate (OPR) determines the rate of ATP generation in state 3 (Estabrook 1967).

**Statistical analysis**

Data from the factorial experiment with the exposure (administration of CCl₄), and the treatment effect (RT) as the main factors were analysed using descriptive and inferential statistics. Results are presented as means ± SD (standard deviations) or median and interquartile ranges (from the lower 25% quartile Q₁ to the upper 75% quartile Q₃) in case of skewed data distribution. Differences in pre-post measurements on a sample were tested using the paired t-test and the Wilcoxon signed-rank test, respectively. Differences among the sample means were evaluated using analysis of variance (ANOVA) and the post hoc pairwise comparisons. In case of skewed distribution the results were compared with those obtained using the nonparametric ANOVA.

Statistical analyses were performed using StatsDirect 3.0.191 software (StatsDirect Ltd., Cheshire, UK). All presented p values were two sided. A value p < 0.05 was considered as significant in all statistical analyses.

**Results**

At the end of the experiment the mean body weights of C and CCl₄ groups were significantly different (Table 1). The body weights in groups C and C+RT significantly increased with the same increments, in comparison with their respective baseline values (both p = 0.002). On the other hand, the body weight in group CCl₄ decreased, but not significantly (p = 0.27). The weight in group RT+CCl₄ was moderately increased (p = 0.04). Further, CCl₄ administration resulted in a reduction in the body (p < 0.005) and the liver (p < 0.05) weights in comparison with C group at the end of the experiment. Group RT+CCl₄ had a decreased body weight (p < 0.005) in comparison with C group, but, unlike in CCl₄ group, the animals slightly put on weight during the experiment, which separated the animals having received RT+CCl₄ from those on CCl₄ only (p < 0.05).

Plasma concentrations of Alb, tBil and activity of ALT and AST are typically worsened by CCl₄-induced liver damage, as evidenced also in our study by markedly decreased concentration of Alb, increased concentration of tBil and increased activities of both aminotransferases (all p < 0.001). When the animals drank RT, damage to liver, reflected in high activities of aminotransferases and concentration of CCl₄,
Table 1. The effect of CCl4 and rooibos tea treatments on body, liver weight and plasma biochemical parameters

| Group               | C               | RT+C            | CCl4             | RT+CCl4          |
|---------------------|-----------------|-----------------|------------------|-----------------|
| Initial body weight (g) | 307 ± 19.47     | 300 ± 14.14     | 302 ± 21.50      | 298 ± 10.33     |
| Final body weight (g)  | 411 ± 19.97**   | 412 ± 13.58**   | 288 ± 23.12**    | 313 ± 18.89**   |
| Liver weight (g)      | 12.86 ± 0.98    | 13.05 ± 0.85    | 9.72 ± 3.24*     | 12.09 ± 2.16    |

Plasma

| Parameter          | C               | RT+C            | CCl4             | RT+CCl4          |
|--------------------|-----------------|-----------------|------------------|-----------------|
| Alb (g/l)          | 28.20 ± 1.32    | 29.14 ± 1.32    | 20.91 ± 1.79**   | 22.45 ± 2.24**  |
| ALT (μkat/l)       | 1.05 ± 0.12     | 0.92 ± 0.14*    | 17.46 ± 8.44**   | 10.25 ± 4.45**  |
| AST (μkat/l)       | 1.39 ± 0.23     | 1.33 ± 0.19     | 37.53 ± 15.15**  | 21.53 ± 5.28**  |
| tBil (μmol/l)      | 0.68 ± 0.46     | 0.56 ± 0.28     | 17.50 ± 7.45**   | 6.37 ± 4.23***  |

Data are expressed as the mean ± SD (standard deviation). Characteristics with skewed distributions are supplemented also by medians with lower and upper quartiles. C, control animals; RT+C, controls treated with rooibos tea; CCl4 animals treated with carbon tetrachloride; RT+CCl4, animals treated simultaneously with carbon tetrachloride and rooibos tea; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; tBil, total bilirubin. *p < 0.05, **p < 0.005 for comparisons between final and initial weights; *p < 0.05, **p < 0.005 for comparisons with C; #p < 0.05, ##p < 0.005 for comparisons between groups.

tBil due to exposure to CCl4, was significantly alleviated (all p < 0.001; Table 1).

Histological examination of the group samples at week 10 after treatment started showed marked differences in the micrographs taken for samples from animals that received CCl4 – when compared to the samples of control groups C (Fig. 1A) and RT+C (Fig. 1B), the tissues from intoxicated rats showed all signs of morphological abnormalities (p < 0.001).

Parameters of oxidative phosphorylation in liver mitochondria after CCl4 administration were significantly reduced (Fig. 2). RCI, ADP:O, QO2(S3) and OPR were decreased (p < 0.001) or worsened (QO2(S1); p = 0.087). In RT+CCl4 group these parameters were also decreased when compared with C group. Our findings have shown that RT consumption significantly improved RCI, QO2(S3), and OPR in liver mitochondria after CCl4 damage (all p < 0.001; Fig. 2).

When investigating the effect of treatment with RT on the markers of liver function, it might be of interest to study whether the observed strength of relationship differs between animals exposed to CCl4 and the controls. Therefore we also tested by means of analysis of variance the interaction between both factors, the exposure to CCl4 and the treatment with RT. We observed significant interaction effects in all the variables but body weight increments (all p < 0.025; Table 1). In the case of differences in body weight increments the interaction approached statistical significance (p = 0.086). For respiratory function parameters the most significant interactions were observed for RCI, QO2(S3) and OPR (p = 0.004, 0.0004 and 0.0002, respectively). The presence of these interactions can be seen in Figure 2 when comparing virtual lines connecting the respective means (C – RT+C and CCl4 – RT+CCl4 group). Apparently, the lines are non-parallel with the same pattern – higher (beneficial) effect in RT+CCl4 group and lower or none effect in RT+C group in all the above mentioned characteristics. Significant differences in the simple mean effects of RT indicate a higher effect for CCl4 group than that for C group consisted of healthy animals. Importantly, for non-significant findings, the RT administration did not lead to worsening in either characteristic.

Discussion

Attempts to improve bioenergetics in liver disease tend to focus on prevention of mitochondrial oxidative damage as a target, because mitochondrial energy production is thwarted by oxidative stress condition. It has been shown repeatedly that RT flavonoids can reduce oxidative stress. In order to simulate conditions of hepatic injury due to radicals, and thus induce oxidative stress, we chose a model of CCl4 damage. After 10-week exposure of rats to CCl4, we noticed a significant reduction in both, the body and liver weights, as compared to the controls, which was caused by weight stagnation over time in the affected animals. The increase in the body weight relative to the initial body weight in the
group of exposed animals, which were given extract of RT to drink instead of water, might be considered as a sign of favourable conditions.

Histopathological changes in the liver of rats subjected to a long-term (10-week) administration of CCl₄ confirmed observations from our previous studies using the same model (Uličná et al. 2003, 2008; Otrubová et al. 2018). Histomorphological and histometrical examination of the liver tissue revealed significant steatosis with a marked accumulation of lipid droplets, which was also evidenced by the determination of TAG levels using the spectrophotometric method. At the same time, the clinical picture was characterized by a visible fibrosis. We have shown that RT inhibited the development of CCl₄-induced steatosis as well as markedly protected liver tissue against CCl₄-induced fibrosis. Further, our findings of highly elevated plasma aminotransferases (ALT, AST) as well as an increased concentration of tBil and a decreased concentration of Alb in the plasma of rats treated with CCl₄ are consistent with the reported results on the hepatotoxic properties of CCl₄ - all the above mentioned parameters are typically impaired in fibrosis/cirrhosis condition (Le et al. 2017). Here, we found that administration of RT partly, but significantly reversed the abnormal increase in the aminotransferase activities and inhibited the production of tBil in CCl₄-treated rats, while its effect on the plasma Alb decrease was rather low. However, the reversal of increased serum levels of transaminases by RT indicated the protection of structural integrity of subcellular membranes thus preventing the leakage of intracellular enzymes.

As to the of CCl₄ toxicity towards liver mitochondria, we have shown that administration of CCl₄ caused damage to the respiratory function and energy production, which could be inferred from the deterioration of the oxidative phosphorylation parameters (RCI, ADP:O, QO₂(S₃) and OPR). The RCI generally reflects the ability of mitochondria to respond to ADP by increasing oxygen consumption. If reduced, it usually indicates impaired integrity of mitochondria. The observed decrease in RCI, together with decreased oxidative phosphorylation coefficient ADP:O indicated a less tight coupling of phosphorylation with oxidation. A sensitive indicator of the proper function of the respiratory chain is the rate at which the suspension of mitochondria consumes oxygen at ADP-stimulated respiration when the consumption is at its maximum – QO₂(S₃). Deteriorated respiratory chain function following administration of CCl₄ resulted in a decreased rate of oxygen consumption by mitochondria.

Figure 1. Photomicrographs of liver histology of rats treated with carbon tetrachloride (hepatotoxin) and rooibos tea (antioxidant) in a full factorial experiment lasting 10 weeks. A. Control group. B. Controls treated with rooibos tea (RT+C group). C. Animals treated with carbon tetrachloride (CCl₄ group). D. Animals treated simultaneously with carbon tetrachloride and rooibos tea (RT+CCl₄ group). Both control groups (A and B) showed essentially normal histology unlike groups treated with CCl₄ (C and D). However, abundant micro- and macrovesicular steatosis characteristic for CCl₄ group samples was visibly reduced in rooibos tea-treated rats (D) having presented vesicles smaller in shape and volume. Samples were stained with hematoxylin and eosin (H&E) and analysed at the same magnification (scale bar = 200 µm).
Rooibos tea ameliorates CCl₄-induced liver injury

when stimulated with ADP. The rate of oxidative phosphorylation (OPR), which determines the rate at which ATP is generated, has also been reduced to a great extent. Administration of the rooibos extract had beneficial effect on the parameters of oxidative phosphorylation as it significantly improved the function of the respiratory chain and the rate of ATP generation in liver mitochondria under a long-term (a 10-week) exposure to noxious CCl₄. Already a single necrogenic dose of CCl₄ causes a so-called chemical hepatectomy accompanied with significantly impaired respiratory function and reduced phosphorylation rate. Such kind of damage has been shown to be reversible (Uličná et al. 1994).

It is generally believed that oxidative stress can be mitigated by the intake of antioxidants. We have already shown that under such conditions, which were modeled also in the present study, flavonoids contained in RT significantly contribute to overall reduction in oxidative stress in CCl₄-damaged liver. One of the hallmarks of lipoperoxidation is the increased concentration of malondialdehyde (MDA) as an end-product of this process. Administration of CCl₄, which is a potent prooxidant, induces an increase in MDA levels in the liver tissue and aqueous extract of RT has been found to significantly lower MDA levels (Uličná et al. 2003, 2008).

The reduction of oxidative stress under these conditions has also been evidenced by coenzyme Q regeneration and subsequent decrease in the extent of lipoperoxidation in the liver (Kucharská et al. 2004). The beneficial effect of RT on MDA levels has also been demonstrated in another model of oxidative stress – streptozotocin-induced diabetes mel-

Figure 2. The effect of CCl₄ and rooibos tea treatments on the parameters of liver mitochondrial function (RCI, ADP:O, QO₂(S₃), QO₂(S₄), and OPR). Data are presented as the median (diamond sign), the lower-upper quartile range, minimal and maximal values for the both, control rats (open boxes) and for CCl₄-treated rats (filled boxes). Experimental groups: C, control animals; RT+C, controls treated with rooibos tea; CCl₄, animals treated with carbon tetrachloride; RT+CCl₄, animals treated simultaneously with carbon tetrachloride and rooibos tea. RCI, respiratory control index; ADP:O, coefficient ADP:O; QO₂(S₃), ADP-stimulated respiration rate in state 3; QO₂(S₄), basal respiration rate in state 4; OPR, oxidative phosphorylation rate. ⁎⁎ p < 0.005 for comparisons with C group, ǂǂ p < 0.005 for comparisons with CCl₄ group.
litus (DM). In this model, administration of RT has been significantly associated with decreased levels of advanced glycation end products and plasma MDA concentrations. Moreover, RT’s antioxidant effects at DM conditions have been reflected in a decrease in MDA concentration in the liver tissue and lens. A comparison of RT antioxidant properties with N-acetyl-L-cysteine, a medication powerful antioxidant, did not reveal any relevant difference (Uličná 2006).

Findings of another model study using tertiary butylhydroperoxide (t-BHP) as an exogenous inducer of oxidative stress in rats, have shown that supplementation with the fermented RT extract results in an inhibition of lipid peroxidation quantified as a significant reduction of t-BHP-induced elevated levels of conjugated dienes and MDA. The RT has also been found to positively affect the redox state by increasing the concentration of reduced glutathione (GSH), thereby increasing the ratio of GSH:GSSG in plasma and liver in rats. The RT partly reverses the abnormal increase in serum aminotransferases activities and attenuated histologically proven degenerative liver injury (Ajuwon et al. 2013). The hepatoprotective effect of RT has also been observed in the subsequent study by the same group (Ajuwon et al. 2014), in which the acute hepatic impairment was induced by a lipopolysaccharide Escherichia coli, serotype 0111: B4. The authors have found a significant reduction in pro-inflammatory marker levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6. These results suggest that aqueous rooibos extract attenuates lipopolysaccharide-induced liver injury presumably by modulating oxidative stress and suppressing pro-inflammatory cytokines formation.

Based on our findings and published studies we may assume that improving mitochondrial bioenergetics following RT administration might be associated with a direct reduction of oxidative stress. ROS production causes lipid peroxidation of mitochondrial membranes which can contribute to impaired mitochondrial function and perpetuate the ROS generation. Oxidative stress also triggers production of inflammatory cytokines, causing inflammation and fibrogenic response. This ultimately results in development of NASH (Rolo et al. 2012). Lipid peroxidation products directly attack and inactivate respiratory chain components, including cytochrome c oxidase, the terminal oxidase of the respiratory chain (Chen et al. 1998, 2000). Because of the critical role the respiratory chain plays in generating cellular energy, disorders that affect respiratory chain activity can interfere with the proper function of any organ system. Mechanisms by which RT polyphenols might act are not fully elucidated. RT polyphenols are likely to trap lipid peroxides by direct binding, or by acting as inhibitors of the lipid peroxidation cascade. The antioxidant condition of the cell could also be regulated by improving the glutathione redox status mediated by the capacity of RT flavonoids to increase (up-regulate) the mRNA expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of glutathione biosynthesis (Ajuwon et al. 2015).

Based on the results of these studies it can be assumed that the hepatoprotective effects of RT may occur as the result of stabilizing effect of its polyphenols on the cell membrane, as well as on the membranes of endoplasmic reticulum and mitochondria of hepatocytes. If the lipid membranes are protected from oxidative damage, the synthesis of proteins in the endoplasmic reticulum as well as production of energy in the mitochondria (i.e. ATP synthesis) remain preserved. Both these processes contribute to the regeneration of damaged liver tissue. In addition, emerging findings suggest a large number of potential mechanisms of action of polyphenols in preventing diseases, which may be independent of their conventional antioxidant activities, thus pointing to their pleiotropic health beneficial effects (D’Archivio et al. 2007).

Ultrastructural changes in the liver mitochondria involve the accumulation of fat in the organ. Electro-microscopic studies have shown that mitochondria in NAFLD are enlarged and swollen, their number is reduced, and that their matrix is hypodense with paracrystalline inclusions. Abnormalities in the liver mitochondria have been confirmed also in steatosis condition in humans (Caldwell et al. 1999) and other animal species (Rector et al. 2010). The reduction in liver mitochondria bioenergetics has been observed in 8 week-old rats with a high-fat-diet-induced liver steatosis (Uličná et al. 2012).

Findings of the experimental studies imply that dysfunctional mitochondrial bioenergetics in the liver is associated with the development and progression of steatosis. A significant increase in the concentration of triacylglycerols in the liver tissue occurs 24 hours after a single administration of CCl₄ (2.5 ml/kg). By that time, all parameters of oxidative phosphorylation in the liver are significantly impaired. After a 2-week auto-regeneration, the concentration of triacylglycerols (TAG) and oxidative phosphorylation parameters have been shown to approach the levels found in healthy controls (Uličná et al. 1994).

The development of fibrosis and massive liver steatosis after a long-term administration of CCl₄ is associated, as documented by our findings, with a significant deterioration in the mitochondrial bioenergetics. Using a factorial design, we were able to show that the effect of RT was directed to CCl₄-induced damage to liver where RT administration significantly improved parameters of oxidative phosphorylation in the liver mitochondria. Importantly, in the healthy liver when oxidative stress is absent, the RT influence was not apparent, neither as a protective, nor as an adverse effect.

Conclusion

In order to preserve cell existence, it is essential to supply the free energy required to drive all processes in the living
Rooibos tea ameliorates CCl_4-induced liver injury

system. Most often, the energy is delivered in the form of ATP produced in the mitochondria. Insufficient ATP production leads to disruption of cell functions and, in severe cases, to the cell death. In mitochondria damaged due to CCl_4 exposure in a model of oxidative stress, the reduction of energy production has serious pathophysiological consequences. Administration of RT, which contains a wide range of compounds with potent antioxidant properties, may improve respiratory chain function and ATP production. The observed positive influence of its administration on the mitochondrial bioenergetics can promote the use of RT as a hepatoprotector in patients diagnosed with liver disease, who may benefit from dietary treatment. There is ongoing research into the protective effect of rooibos on liver, and, in view of antioxidant and other possible medicinal properties of RT and its polyphenols, this healthy beverage deserves continuing studies both in prevention and in the supportive therapy for conditions where oxidative stress is present. The results of the study provide not only theoretical but also research-to-practice knowledge applicable in clinical practice.

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Conflict of interest. The authors declare that there is no conflict of interest.

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Rooibos tea ameliorates CCl4-induced liver injury

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