Ph.D thesis

Importance of herbal preparations in liver and bowel diseases

Erika Rapavi

Supervisor:
Dr. Anna Blázovics, Ph.D

Biochemical Research Group, II. Department of Medicine, Semmelweis University

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Herbal medicine has existed since prehistorical times and the primary form of medicine for approximately 80% of the world’s population today. Medicinal plants may be used directly as teas or extracts, or are applied in the production of the drugs. Several herbal ingredients are present in over-the-counter drugs. Herbal products were also used in public health care.

Plant polyphenols are common dietary components that have many potent biological properties. Many research laboratories reported the application of flavonoids as antibiotics and antidiarrheal, antiulcer, and anti-inflammatory agents, as well as in the treatment of diseases such as hypertension, vascular fragility, allergies, hypercholesterolemia, cardiovascular disease and cancer. Numerous epidemiological studies suggest that a decreased risk of heart disease and cancers of the breast, prostate, lung, colon, and stomach is associated with increased consumption of fruits and vegetables. The phenolic substances are secondary products of the plant metabolism. Their basic role is the defence of the plant cell from different damaging agents such as UV light, fungus, insects and viruses. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals. Mechanism of their antioxidant action can include suppressing reactive oxygen species formation, either by inhibition of enzymes or by chelating transition metal elements involved in free-radical production, intercepting reactive species, and upregulating or protecting antioxidant defence.

Despite the apparently beneficial health effects of flavonoids, several studies indicate their mutagenicity and genotoxicity in experimental systems. This may be due to their activity as pro-oxidants in generating free radicals that damage DNA. Additionally, high intakes of these compounds may potentiate other deleterious effects due to their diverse pharmacological properties, which may alter drug metabolism, modulate the activity of environmental genotoxicants, and alter the activity of other key metabolizing enzymes. Pro-oxidant or antioxidant activity of flavonoids is largely dependent upon the levels consumed, the redox state of their biological environment as well as the physiological conditions in the body.

**Specific aims of the study**

**In vitro investigations**

There is increasing evidence that antioxidants in human diet are greatly beneficial for the health and well-being. People gladly consume tea mixtures as a natural source of bioactive compounds. The quality, effectiveness, and the amount of active substances in herbal products vary according to different factors, for instance, sunlight, climate, soil, rainfall, as well as conditions of harvesting, storage, processing, transport, and preparation of infusion (amount of tea relative to water, infusion time and temperature, material of teapots, etc.). Of these various conditions, steeping time has been selected as the subject of our investigations. This study shows what can occur if the infusion time is not properly observed. Considering the different methods of making herbal tea applied in different cultures, which may lead to lower efficiency of the herbal tea, the purpose of the present study was to examine the changes in antioxidant activity and metal ion concentration attained in extracts of ‘Tieguanyin’ and ‘Mecsek’ herbal teas with applying different periods of steeping time. Additionally, partial phytochemical examination was carried out.

**Human in vitro studies**

Free radical production and disturbance in the redox status may modulate the expression of a variety of immune and inflammatory molecules. Antioxidants could inhibit the proliferation of T lymphocytes induced by mitogens. This is assumed to be due to their scavenging of free radicals, which may act as second messengers in the antigen-induced signalling cascade leading to cell proliferation.

The aim of our work was to investigate the effect of the infusion of dried calyx of *Hibiscus sabdariffa* L. and Beiqishen herbal tea in concentrations of 1, 10, 100 μg ml⁻¹ upon blast transformation of human peripheral blood lymphocytes. We studied whether the extract influences the level of spontaneous as well as mitogen (phytohemagglutinin and pokeweed mitogen)-induced human lymphocyte proliferation *in vitro*. Peripheral blood lymphocytes from heparinised venous blood of healthy subjects (n=17) were examined.

**Animal experiments**

Non-alcoholic fatty liver disease frequently occurring in human is more often related to hyperlipidaemia, obesity and diabetes. As is well known, free radical reactions and lipid peroxidation play an important role in the pathomechanism of fatty liver by increasing metabolic disorders in hyperlipidaemia. Naturally occurring compounds as regulators of redox
homeostasis are particular therapeutic importance in the treatment of the damage caused by free radicals and associated disorders. Numerous studies have shown that citrus flavonoids and their metabolites are potent antioxidants, and thus, are able to suppress many of the events of cancer and inflammation. Hesperidin (a flavanone glycoside) and diosmin (a flavone glycoside) found in citrus fruits are active ingredients of Detralex® (a purified flavonoid fraction composed of 90% diosmin and 10% hesperidin) used for the treatment of chronic venous insufficiency.

The prevalence of chronic venous insufficiency and non-alcoholic fatty liver disease is expected to rise with the increase in obesity, which is becoming an increasingly serious problem throughout the world. In addition, most patients with fatty liver show no symptoms or signs of liver disease at the time of diagnosis, therefore, both chronic venous insufficiency and fatty liver may develop in the same time. The effect of diosmin-hesperidin containing drug on the redox state of the fatty liver has, however, not been clarified so far. Therefore, in this study, male Wistar albino rats were fed a lipid-rich diet with or without 450 mg diosmin-50 mg hesperidin-containing drug (60 mg/kg body weight/day, per os) for 9 days to determine the impact of treatment on antioxidant defence system of the fatty liver.

The liver is an important target of the toxicity of drugs, xenobiotics and oxidative stress. Thioacetamide (TAA) is a thiono-sulfur-containing compound endowed with liver damaging and carcinogenic activity. TAA is often used in many studies to induce experimental liver necrosis in animal species. TAA was originally used to control the decay of oranges and then as a fungicide. Recently it is being employed in leather, textile, and paper industries as an accelerator in the vulcanization of buna rubber and as a stabilizer for motor fuels. Additionally, TAA was serves for many years as a replacement for hydrogen sulphide in the qualitative analysis of inorganic compounds. It is very poisonous material if swallowed or inhaled as well as is readily absorbed through the skin and may cause serious liver damage. Some authors reported that oxidative stress is involved in TAA-induced cell injury. There is no report available on the effect of diosmin-hesperidin containing drug on TAA-injured liver. In order to evaluate the impact of the drug on the redox balance of toxin-injured liver, Wistar albino rats were subjected to thioacetamide administration (500 mg TAA /l in their drinking water) with and without drug (425 mg/kg body weight/day). Animals were treated for 30 days.

Some authors reported that dietary phenolics exhibit pro-oxidant and cytotoxic properties under certain conditions. Based on these observations we examined the effect of high dosages of the diosmin-hesperidin drug (315 and 425 mg/body weight/day) on the redox state of liver in healthy animals, as well as on redoxi processes of liver regeneration in rats with experimental hepatic injury induced by lipid-rich diet and thioacetamide.

**MATERIALS AND METHODS**

**Materials**

‘Beiqishen’ Tea, ‘Tieguanyin Stomach’ Tea and ‘Mecsek Cleaning’ Tea were obtained through the commercial network. We used dried drug samples of hibisci flos bought at the marketplace of Cairo Khan-el-Khalili. Hibisci flos was identified in the Department of Pharmacognosy, Semmelweis University, where herbarium specimen is deposited. The drug consists of the dried calyx and epicalyx of *Hibiscus sabdariffa* L. var. ruber (Malvaceae) collected during the fornting period. Detralex® (a purified flavonoid fraction composed of 450 mg diosmin and 50 mg hesperidin) was obtained from Servier Laboratories (France).

**In vitro investigations**

**Preparation of aqueous extracts for biochemical measurements**

The samples (1 g) were infused with of 90-100 °C double distilled water (100 ml) and was covered. It was allowed to stand in test-tube for 5, 15, 30, 60, 120 min, then the extracts were filtered, the moisture was squeezed out, and the volume was made up to 100 ml.

**Partial phytochemical measurements**

- Determination of polyphenol content (Ph. Hg. VII.)
- Determination of tannin content (Ph. Hg. VII.)
- Determination of proanthocyanidins content (Szőke, É. and Kéry, Á. (Eds.), 2000)
- Determination of flavonoid content (DAB, 1996)
- Preparation of the active substances for gas chromatographic methods (Héthelyi et al, 2001)

**Analysis of elements** (Szentmihályi et al, 1999)

**Investigation of redoxi parameters**

- Total scavenger capacity (Blázovics et al, 1999)
- Hydrogen-donor ability (Hatano et al, 1988)
- Reducing power (Oyaizu, 1986)
- Cupper II-chelating activity (Shimada, 1992)
Total antioxidant status (*Randox diagnostic kit*)

**Human in vitro studies**

**Preparation of the infusion**
The drug (1 g) was infused with 100 ml of boiling, doubly distilled water and allowed to steep for 15 min., while it was covered. Then it was filtered, the moisture was squeezed out, and the volume was raised to 100 ml.

**Investigation of blast transformation of human peripheral blood lymphocytes** (*Gonzales-Cabello et al, 1987*)

**Animal experiments**
Male Wistar albino rats (n = 169) and female Wistar albino rats (n = 120) were obtained from TOXICOOP. Control animals were fed with standard chow (ssniff R/M-Z+H, Ssniff Spezialdiäten GmbH, Germany). Liver damage was produced in rats by fat rich diet or oral administration of 0.05 % thioacetamide solution. Fat rich diet contained 2.0% cholesterol, 20% sunflower oil and 0.5% cholic acid added to the standard food. Animals were treated with diosmin-hesperidin-containing drug (Detralex®) through a gastric tube parallel with feedings or were fed with this drug added to the standard chow.
The rats were killed by exsanguination via abdominal vein under Nembutal anaesthesia (35 mg/kg bw ip.). This study was appeared by the Regional Committee of Science and Research Ethics Semmelweis University, Permission Number TUKEB 24/1996.

**Biological samples**
- liver homogenate
- liver microsome
- duodenum, jejunum, ileum and colon homogenates
- serum
- plasma
- erythrocyte

**Metabolites and enzymes as markers of metabolism**
- Liver enzyme activities and main metabolite concentrations in serum
- Gluthathion-peroxidase activity in erythrocyte and liver homogenate (*Paglia és Valentine, 1967*)
- Superoxide-dismutase activity in erythrocyte and liver homogenate (*Randox diagnostic kit*)

**Enzyme activities in liver microsome**
- Cytocrome P450 content (*Greim, 1970*)
- Cytocrome b5 concentration (*Greim, 1970*)
- Cytocrome c reductase (*Masters et al, 1967*)
- CYP4501A activity (*Burke, 1985*)
- CYP4502E1 activity (*Reinke and Moyer, 1985*)
- FMO1 activity (*Pike et al, 2001*)

**Investigation of redoxi parameters in tissue**
- The conjugated diene concentration (*AOAC, 1984*)
- Total scavenger capacity (*Blázovics et al, 1999*)
- Hydrogen-donor ability (*Hatano et al, 1988*)
- Reducing power (*Oyaizu, 1986*)
- Free SH-group concentration (*Sedlak and Lindsay, 1968*)
- Malonaldehyde content (*Pyles, 1993*)

**Analysis of elements in tissue** (*Szentmihályi et al, 1999*)

**Histological examinations**
Midsections of the left lobes of the rat livers were processed for light microscopy. This processing consisted of fixing the specimens in a 6 % neutral buffered formalin, embedding the specimens in paraffin, making sections of 5 μm thickness, and staining with hematoxylin-eosin.

**Other methods**
- Protein content of biological samples (*Lowry et al, 1951*)
- Haematology
- Whole blood viscosity

**Statistical evaluation of the results** was performed using STATISTICA software (version 6.0; StatSoft Inc, USA), applying parametric Student’s *t*-test or non-parametric Wilcoxon test, depending on the distribution of the data.
RESULTS, THESIS

In the course of our investigations we applied biochemical, analytical and histological supplementary model systems in order to study the effect of herbal preparations on the redox homeostasis of the living organism. The in vitro and in vivo studies provided the following results:

1. Based on the partial phytochemical examination, in the case of calyx of Hibiscus sabdariffa L., ‘Beiqishen’, ‘Tieguanyin’ as well as ‘Mecsek’ tea mixture, the presence of polyphenol, flavonoid and tannin type molecules could be verified.

2. The results show that the aqueous extracts of the herbal teas examined have antioxidant properties as a function of concentration and steeping time in non-enzymatic system in vitro.

3. In animal experiments, it has been determined that treatment with the aqueous extracts of ‘Tieguanyin’ and ‘Mecsek’ tea mixture for a period of 21 days significantly affected the redox-homeostasis of the liver and plasma in healthy rats.

4. It has been established that three-week consumption of ‘Tieguanyin’ and ‘Mecsek’ herbal teas did not greatly influence the cytochrome P450 system.

5. We were first to report that, under experimental conditions, treatment with a drug containing diosmin (450 mg) and hesperidin (50 mg) in therapeutic dosage (60 mg/kg body weight/day) may improve the non-enzymatic antioxidant defensive system and the redox state in alimentary-induced fatty liver disease.

6. Treatment with the drug (containing diosmin-hesperidin) in the concentration applied (60 mg/kg body weight/day) had no significant effect on the serum lipid parameters in animals treated during the short-term experiment.

7. It has been verified that therapeutic dosage of the drug did not significantly affect the cytochrome P450 system in either healthy or sick animals.

8. Upon administration of a lipid-rich diet, the concentration of essential elements in the liver, plasma, and erythrocytes has changed. After treatment with a therapeutic dosage of the drug, these alterations approached the control value.

9. In healthy animals, the drug applied in a dose (425 mg/kg body weight/day) seven times as high as the therapeutic dosage of the drug containing diosmin and hesperidin significantly affected some of the redoxi parameters examined.

10. Pre- and post-treatment with high dosage (425 mg/kg body weight/day) of the drug caused haemolytic anaemia during the regeneration of alimentary induced fatty liver. This alteration was indicated by significantly lower concentration of RBC, HCT, and HGB and significantly increased the level of iron in plasma.

11. The data from our study show that treatment with a high dosage (425 mg/kg body weight/day) of the diosmin-hesperidin drug may enhance oxidative stress and hepatotoxicity induced by the thiacetamide in rats.

12. A dosage (315 mg/kg body weight/day) five times as high as the therapeutic dosage of the drug did not cause any change in the redox state of the toxin-injured liver during the recovery period.

13. Human in vitro investigations show that the infusion of the dried calyx of Hibiscus sabdariffa L. and ‘Beiqishen herbal tea have immunomodulating, cytostatic effect. Both samples as a function of concentration decreased mitogen-induced blastogenesis in normal subjects. These extracts did not affect, however, the level of spontaneous proliferation of human lymphocytes.
Original articles related to the thesis


Additional publications


Presentations published in journals or books


Rapavi, E., I. Kocsis, E. Fehér, E. Pintér, S. Bártkovits, A. Blázovics: Alteration in the free radical-antioxidant balance in small intestine mucosa of rats with early cirrhosis. Magyar Gastroenterológiai Társaság, 44.


**PRESENTATIONS/CONGRESS ABSTRACTS RELATED TO THE THESIS**  


Rapavi, E., Kocsis, I., Czinner, É. Stefanovits-Bányai, A. Blázovics: The effect of hesperidin and diosmin on ion concentration and antioxidant defense system in fatty liver in rats. Metal elements in environment, medicine and biology. 5th International Symposium, November 4-6, 2002, Timisoara, Roumania  


