Effect of hesperidin and naringin on the plasma lipid profile and plasma antioxidant activity in rats fed a cholesterol-containing diet

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Abstract: The objective of this study was to compare the influence of hesperidin and naringin, the main flavonones of orange and grapefruit, on plasma lipid profile and antioxidant activity in rats fed a cholesterol-containing diet. Sixty male Wistar rats were randomly divided into six groups of 10, named Control, Hesperidin, Naringin, Chol, Chol/Hesperidin and Chol/Naringin. The Control group was fed a basal diet (BD) and 1–2 mL of distilled water. To the BD of the other five groups were added 0.1–0.2 mg of hesperidin dissolved in 1–2 mL of distilled water (Hesperidin group), 0.46–0.92 mg of naringin in 1–2 mL of water (Naringin group), 1% of non-oxidised cholesterol (NOC) and 1–2 mL of water (Chol), 1% of NOC and 0.1–0.2 mg of hesperidin in 1–2 mL of water (Chol/Hesperidin), 1% of NOC and 0.46–0.92 mg of naringin in 1–2 mL of water (Chol/Naringin). After 30 days of the experiment it was found that the diets supplemented with hesperidin and naringin increased the plasma antioxidant activity. In conclusion, diets supplemented with hesperidin and naringin significantly hindered the increase in plasma lipid levels caused by cholesterol feeding. Hesperidin and naringin, bioactive compounds of citrus fruits, are powerful plasma lipid lowering and plasma antioxidant activity increasing flavonones.

Keywords: hesperidin; naringin; rats; plasma lipids; plasma antioxidant activity

INTRODUCTION

Consumption of diets rich in fruits and vegetables has been associated with reduced risk of some chronic diseases including the most dangerous, coronary atherosclerosis and cancer. As a consequence, consumption of such diets is inversely related to coronary atherosclerosis.[1,2] Citrus fruits are very popular among European and North American consumers. In the last 20 years our international team of biochemists, dieticians and cardiologists has intensively studied various citrus fruits *in vitro*, in experiments on laboratory animals and in investigations of patients.[3–5]

In the above-mentioned studies, as in investigations by other authors,[6,7] whole citrus fruits, their parts or juice were used. In spite of the fact that the flavonones hesperidin and naringin are predominant in oranges and grapefruits these bioactive compounds were less investigated.[8–12] So, Kroyer[10] has shown that hesperidin and naringin as well as their aglycones are responsible for the antioxidant activity of citrus peels. Also, Erlund *et al*.[11] indicate that these flavonones possess antioxidative and anticarcinogenic properties. Monforte *et al*.[12] reported that high consumption of hesperidin decreases serum cholesterol and triglycerides in rats.

Addition of citrus fruits to cholesterol-containing diets leads to hypocholesterolaemic effects and to a decrease in the content of total cholesterol in the liver during experiments on laboratory animals: to the cholesterol-lowering effect of citrus fruits in total.[4,5,9]

Mechanisms for the antioxidant activities of hesperidin, glucosyl hesperidin and naringin in rats with diet-induced hypercholesterolaemia have

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been described and various explanations given: direct antioxidant effect of hesperidin,[13] vasorelaxing properties,[14] effect caused by the improvement of very low density lipid (VLDL) metabolic abnormality, leading to the reduction of small dense LDL,[15] metal chelating properties and interactions with iron and copper ions,[16,17] and the low absorption of naringin at the upper intestinal level.[18] In spite of a number of reports on this subject some of questions have not been investigated, such as a possible cholesterol-lowering effect of the main flavonones of oranges and grapefruit. In order to answer this question it was decided to determine the bile flow before and at the end of the experiment. Therefore, in the present investigation hesperidin and naringin were used as antioxidants in *in vitro* studies, and these substances were as well as supplemented to the diets of rats in *in vivo* experiments.

As far as we know, there are no such comparative investigations of the main flavonones of oranges and grapefruit, including experiments *in vivo*.

MATERIALS AND METHODS

Hesperidin, naringin, Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid), and 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt (ABTS) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All reagents were of analytical grade. Deionised and distilled water was used throughout.

Sample preparation

Israeli Jaffa oranges (Shamouti) (*Citrus sinensis*) and Star Ruby (Sunrise) grapefruit (*Citrus paradisi*) of the same maturity degree harvested in 2003 were purchased from the same farmer. Juices of both fruits were prepared manually and prevented from oxidising by liquid nitrogen and equipment made from non-steel material. Then the contents of hesperidin and naringin were determined separately in orange and grapefruit juices, respectively. It was found that the concentration of hesperidin and naringin was 0.10 and 0.46 mg mL^{-1}, respectively. For the experiment *in vivo* two separate solutions were prepared in proportions of 0.10 and 0.46 mg of hesperidin and naringin dissolved in 1 mL of distilled water, respectively.

Determination of the total antioxidant potential

In this experiment the antioxidant potential of hesperidin and naringin were determined by two methods using $ABTS^{\bullet+}$ with $K_2S_2O_8$ and with MnO_2.

First method

The 2, 2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt ($ABTS^{\bullet+}$) radical cation was generated by the interaction of ABTS (250 μmol) and $K_2S_2O_8$ (40 μmol). After addition of 990 μL of $ABTS^{\bullet+}$ solution to 10 μL of Trolox standards (final concentration 0–20 μmol) in phosphate buffered saline (PBS), the absorbance was monitored exactly 1 and 6 min after the initial mixing at 734 nm.[19–21]

Second method

$ABTS^{\bullet+}$ was also prepared by passing a 5 mmol aqueous stock solution of ABTS through MnO_2 on a Whatman no. 5 filter paper. Excess MnO_2 was removed from the filtrate by passing it through a 0.2 μmol Whatman PVDF syringe filter. This solution was then diluted in a 5 mmol phosphate buffered saline, pH 7.4 to an absorbance of 0.70 at 734 nm. The percentage decrease of the absorbance in each method was calculated and plotted as a function of the concentration of the samples and of Trolox for the standard reference data.[21]

$ABTS^{\bullet+}$ with $K_2S_2O_8$ was also applied for determination of Trolox equivalent antioxidant capacity (TEAC) in plasma.

Rats and diets

The Animal Care Committee of the Warsaw Agricultural University approved this study. Wistar male rats ($n = 60$) with a mean weight of 110 g at the beginning of the study were provided by the Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences (Jablonna, Poland).

The rats were housed in plastic metabolic cages and were randomly divided into six diet groups of 10 and named Control, Hesperidin, Naringin, Chol, Chol/Hesperidin and Chol/Naringin. The rats of the Control group were fed basal diet (BD) and 1–2 mL of distilled water. The composition of the BD (in g kg^{-1}) was as follows: wheat starch, 693; casein, 150; peanut oil, 100; cellulise, 10; vitamin mixture, 10; and mineral mixture, 37. To the BD of the other five groups were added 0.1–0.2 mg of hesperidin dissolved in 1–2 mL of distilled water (Hesperidin group), 0.46–0.92 mg of naringin dissolved in 1–2 mL of distilled water (Naringin group), 1% of non-oxidised cholesterol (NOC) and 1–2 mL of distilled water (Chol group), 1% of NOC and 0.1–0.2 mg of hesperidin dissolved in 1–2 mL of distilled water (Chol/Hesperidin group), 1% of NOC and 0.46–0.92 mg of naringin dissolved in 1–2 mL of distilled water (Chol/Narigin group). The cholesterol batches were mixed carefully with the BD (1:99) just before the diets were offered to the rats. These diets contained, as percentages of energy, 68% carbohydrates, 23% protein and 9% fat. The calculated energy of the used diets was 39.53–40.03 kcal kg^{-1}, and the difference was not significant.

All rats were fed once a day at 10:00 h *ad libitum*. They had unrestricted access to drinking water. Hesperidin and naringin dissolved in distilled water were induced by intubation into the stomach. This feeding is more effective than one in which hesperidin and naringin could be mixed with BD, because these compounds were in the liquid state and therefore evaporation from the mixed diet was prevented. In order that the rats became used to the maximal

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quantity of the flavonones dissolved in distilled water, in the first 2 weeks every animal received only 1 mL day^{-1}; in the third week, 1.5 mL day^{-1}; and then to the end of the trial, 2 mL day^{-1}.[5] The feed intake and body gains were monitored daily. The amount of flavonones used was increased and in the last period of the experiment the rats of the Hesperidin, Naringin, Chol/Hesperidin and Chol/Naringin groups received 0.20 and 0.92 mg of hesperidin and naringin dissolved in 2 mL of distilled water, respectively.

It is generally accepted that the most reliable data for blood lipid metabolism can be obtained from fasting animals, 14–16 h after the last feed. Therefore, the feed was removed from the cages at 18:00 h the day before, and the samples were collected at 9:00 h the next day. The plasma was prepared and used for laboratory tests. Under general urethane narcosis (concentration of urethane was 1.8 g kg^{-1} body weight), the abdomen was opened to take samples of bile–pancreatic juice.[5]

Two time points were used in this experiment: before and after 30 days of different feeding. At these time points, a wide range of laboratory tests was performed. The plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TGs) were determined as previously described, without using coefficients of correlation.[5]

The collection of bile and the determination of the bile flow were carried out as previously described.[5]

Statistical analyses

To verify the statistical significance of the studied parameters, means (M) ± SD of samples that had been analysed five times were defined. When appropriate, differences between groups were tested by two-way ANOVA. The P values of <0.05 were considered significant.

RESULTS

In vitro experiments

Naringin and hesperidin showed different antioxidant activities in TEAC (μmol TE μmol^{-1}): 0.32 ± 0.02 and 0.99 ± 0.12 with $K_2S_2O_8$ and with MnO_2: 0.37 ± 0.03 and 1.11 ± 0.15. Our results correspond with those of other authors where it was shown that TEAC was 0.24 and 1.0 μmol TE μmol^{-1} for naringin and hesperidin, respectively.[19,20]

In vivo experiments

The addition of hesperidin, naringin or/and cholesterol to the diets did not lead to significant differences ($P > 0.05$) in feed consumption, body weight gains and feed efficiency (Table 1).

At baseline, the six diet groups did not differ from one another in plasma lipid concentrations (data not shown). The results of the changes after the experiment are summarised in Fig. 1. As can be seen, both flavonones supplemented diets in groups fed cholesterol significantly hindered the rise of total cholesterol (TC) and LDL-C, but did not influence the levels of HDL-C and TGs ($P > 0.05$).

The changes in the pancreatic–bile flow are shown in Fig. 2. As can be seen, addition of hesperidin and naringin to diets of rats fed cholesterol-free diets increases significantly the pancreatic–bile flow ($P < 0.05$).

The same index was significantly increased ($P < 0.05$) also in all three groups fed cholesterol-containing diets (Fig. 2).

At the end of the trial, an increase in the plasma antioxidant activity in both the Hesperidin and Naringin dietary groups was found (Fig. 3A): a significant increase in the Trolox equivalent values.

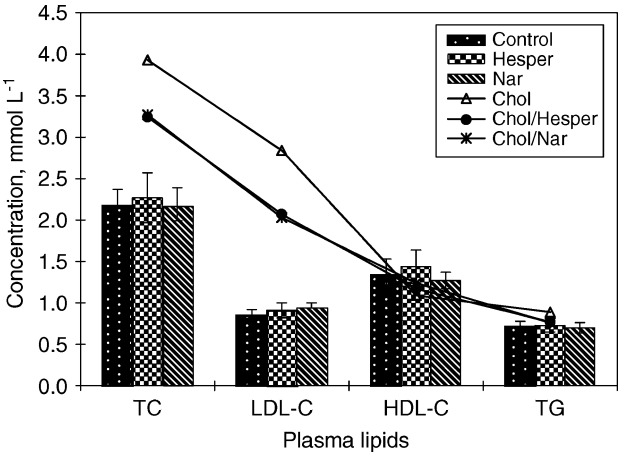

Figure 1. Changes in the plasma lipid levels after completion of the experiment ($n = 10$). Abbreviations: Chol, cholesterol; HDL, high density lipoprotein; Hesper, hesperidin; LDL, low density lipoprotein; Nar, naringin; TC, total cholesterol; TG, triglycerides.

Table 1. Weight gains, feed consumption and feed efficiency ratio in all six groups

Group	Weight gain (g day^{-1})	Feed consumption (g day^{-1})	Consumption by intubation (mL day^{-1})	Feed efficiency ratio
Control	4.38 ± 0.65^a	15.92 ± 1.84^a	Water 1–2 mL	0.274 ± 0.014^a
Cholesterol	3.88 ± 0.66^a	15.38 ± 1.67^a	Water 1–2 mL	0.251 ± 0.024^a
Hesperidin	3.82 ± 1.02^a	15.97 ± 1.19^a	Hesperidin in water 1–2 mL	0.236 ± 0.046^a
Naringin	3.75 ± 0.53^a	15.03 ± 1.34^a	Naringin 1–2 mL	0.248 ± 0.017^a
Chol/Hesper	3.91 ± 1.04^a	15.81 ± 1.12^a	Hesperidin in water 1–2mL	0.244 ± 0.056^a
Chol/Naringin	4.37 ± 0.86^a	15.71 ± 1.28^a	Naringin in 1–2 mL	0.278 ± 0.033^a

Values are means ± SD of five measurements. Means in columns without letters in common differ significantly ($P < 0.05$).
Chol, cholesterol; Hesper, hesperidin.

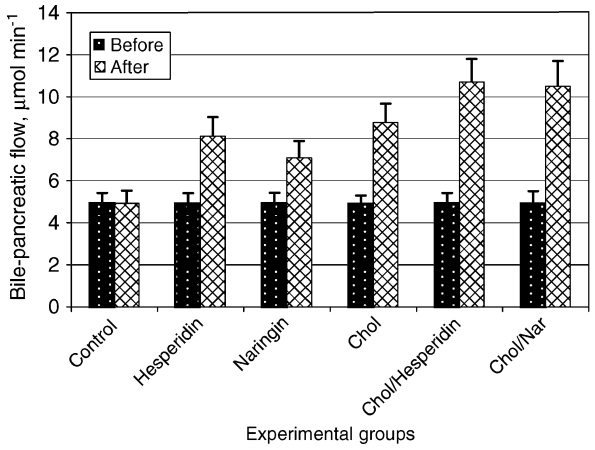

Figure 2. Changes in the pancreatic–bile flow ($n = 10$). Addition of hesperidin and naringin significantly increased the pancreatic–bile flow ($P < 0.05$).

However, the increase was significant ($P < 0.05$) only in the rats fed diet supplemented with hesperidin (Hesperidin group).

A decrease in the plasma antioxidant activity after completion of the trial was registered in all groups of rats fed cholesterol (Fig. 3B). However, this decrease was significant ($P < 0.05$) only in the rats of the Chol diet group (Fig. 3B).

Therefore, the addition of both flavonones to the diets of the Chol/Hesperidin and Chol/Naringin groups, respectively, significantly hindered the decrease in the plasma antioxidant activity.

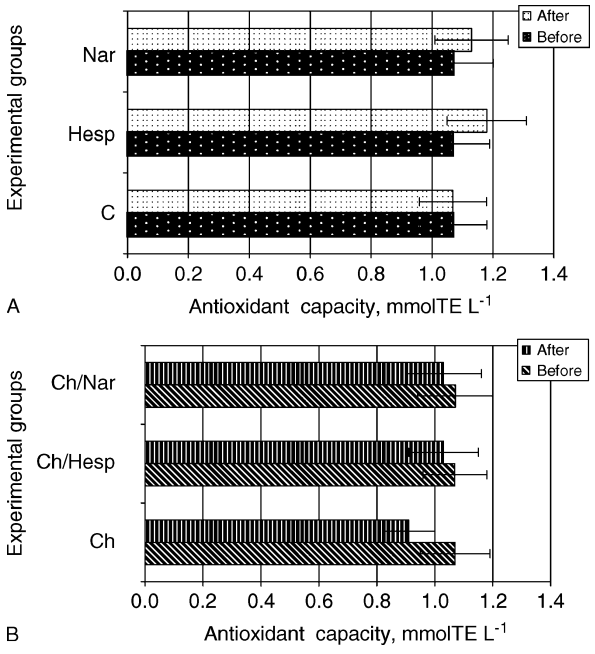

Figure 3. (A) Increase in plasma antioxidant activity in Hesperidin and Naringin diet groups ($n = 10$). However, the increase was significant only in rats of the Hesperidin diet group. (B) Decrease in plasma antioxidant activity in all groups of rats fed cholesterol after completion of the feeding period ($n = 10$). However, the decrease was significant only in rats of the Chol diet group. Ch, cholesterol; Hesp, hesperidin; Nar, naringin; C, control; TE, Trolox equivalent.

No significant changes were observed in all studied parameters in the rats of the Control group.

DISCUSSION

Based on the evidence that hesperidin and naringin are the most permanent flavonones of oranges and grapefruit, in the present investigation the possible plasma lipid-lowering and antioxidant activity of these substances was studied.[22,23] It was shown that supplementation of diets with both flavonones which were fed to the rats by intubation, similar to the method by which humans consume original juice, alters cholesterol and antioxidant status when rats are fed a diet high in cholesterol.[24] We wanted to determine if these flavonones, separately from other bioactive compounds of citrus fruits, would be able to exercise plasma lipid-lowering and antioxidant activity effects. It was found that the antioxidant potential of hesperidin was higher than of naringin. These results are in accord with the data of other researchers showing a protective effect of hesperidin in induced oxidative stress in rat liver and kidney. This protective effect of hesperidin can be correlated with its direct antioxidant effect.[13,19,20,22–24] We did not find significant differences in the feed intake, weight gains and feed efficiency ratio between the six diet groups, as previously.[5] At the end of the feeding period a plasma lipid-lowering effect was registered only in groups of rats fed cholesterol added diets supplemented with either hesperidin or naringin. Also these results are in accord with other data.[25,26] It was also reported that hesperidin significantly increases HDL and lowers cholesterol, LDL, total lipid and triglyceride plasma levels in normolipidaemic rats and in rats with diet- and Triton-induced hyperlipidaemia.[12] The results obtained can be compared with another experiment[25] which was conducted for 42 days with a 1% cholesterol diet with naringin supplementation of 0.1%, w/w in comparison with 30 days feeding used in the present report. In this experiment naringin did not significantly alter the levels of plasma triglycerides, however, the levels of plasma TC (3.80 ± 0.31 mmol L^{-1} vs. 2.61 ± 0.30 mmol L^{-1}, $P < 0.05$) were significantly lowered compared to those of the control. Similar results were obtained in the present report in the levels of TC (3.27 ± 0.5 mmol L^{-1} vs. 2.19 ± 0.2 mmol L^{-1}, $P < 0.05$). During the G-hesperidin administration period to the subjects at 500 mg dL^{-1} for 24 weeks serum TG level significantly decreased in the high-TG type.[15] The data obtained for the efficacy of hesperidin and naringin and their comparison showed that hesperidin had a higher antioxidant activity than naringin. The activity of hesperidin can be explained by the inhibitory effects of flavonoids on lipopolysaccharide (LPS)-induced nitric oxide production in macrophages.[27] The reported results of efficacy of naringin[28] were similar to those reported for hesperidin:[27] naringin was found to have blocked the LPS-induced transcriptional activity

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of tumour necrosis factor alpha (TNF-α). The comparison of naringin and hesperidin show that both of them display numerous biological effects: antioxidant, hypocholesteraemic, anti-atherogenic and favouring drug absorption: so, naringin was poorly absorbed by Caco-2 cells, according to its low value of apparent permeability coefficient. The results reviewed[18] indicated the involvement of P-glycoprotein capable of transporting naringin from the Caco-2 cell to the apical side. This phenomenon could explain, at least in part, the low absorption of this flavonone at the upper intestinal level. Both naringin and hesperidin in the case of a moderate or high consumption of orange juice, may represent an important part of the pool of total polyphenols present in plasma.[8]

Hesperidin and naringin supplementation did not affect the lipid levels in rats fed diets without cholesterol. These results were expected: it has already been demonstrated by other authors and in our previous experiments on laboratory animals and in investigations of patients in whom the hypolipidaemic effect of fruits and vegetables is evident only in animals fed cholesterol and in hypercholesterolaemic patients.[3,5,8,15,26] Long-term administration of hesperidin or glucosyl hesperidin for 25 weeks brings about an antihypertensive effect on spontaneously hypertensive rats and improves serum cholesterol composition.[29] The same results were obtained when fresh red grapefruit was compared with naringin. It was shown that the juice is preferable to naringin: it more effectively influences plasma lipid levels and plasma antioxidant activity and, therefore, could be used as a valuable supplement for disease-preventing diets.[4]

In our previous investigations of citrus fruits we found that their plasma lipid-lowering effect was real: diets supplemented with these natural products have increased the bile flow, the bile cholesterol and the biliary bile acids concentrations.[5] Also, in this investigation in groups of rats fed cholesterol diets supplemented with either hesperidin or naringin a significant increase in the bile–pancreatic flow was noted.

After completion of the trial a significant increase in the plasma antioxidant activity was found in both dietary groups fed added flavonones without cholesterol. In groups fed added cholesterol, a decrease in the plasma antioxidant activity was registered. However, the decrease in groups whose diets were enriched with either hesperidin or naringin (Chol/Hesperidin and Chol/Naringin) was significantly less than in the Chol group. Therefore, it was demonstrated that addition of flavonones hinders the decrease in the plasma antioxidant activity.

CONCLUSIONS

The flavonones hesperidin and naringin have relatively high antioxidant potential and therefore are powerful plasma lipid lowering substances only in rats with diet-induced hypercholesterolaemia. Hesperidin- and naringin-supplemented diets increase plasma antioxidant activity in groups of rats fed without cholesterol, and hindered the decrease in plasma antioxidant activity in rats with diet-induced hypercholesterolaemia. Hesperidin and naringin, separately from other bioactive compounds of citrus fruits, led to an increase in plasma lipid lowering and plasma antioxidant activity.

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