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Antioxidative and antidiabetic effects of naringin and curcumin: *In vitro* and *in vivo*

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Abstract

Context and purpose: The aim of the present study was to assess whether naringin or curcumin can influence oxidative stress induced DNA damage in mice with alloxan-induced diabetes. Naringin or curcumin preparations (50 mg kg^{-1}) were given intraperitoneally for 7 days. The antioxidant capacity of curcumin and naringin *in vitro* was evaluated using three assays which cover different aspects of antioxidant activity. In order to evaluate the effectiveness of naringin and curcuminin vivo we observed changes in body weight and survival of diabetic mice and used the comet and micronucleus assays.

Main findings: *In vitro* curcumin showed appreciable antioxidant properties, while naringin was much less effective. Naringin or curcumin administration to diabetic mice resulted in decreased DNA damage in lymphocytes and increased level of DNA damage in liver, kidney and reticulocytes. Administration of naringin and curcumin resulted in significant increase of the body weight and 100% survival of mice.

Summary and potential implications: Results suggests that antioxidant activity of naringin and curcumin leads to long time survival of diabetic mice and possible prevention of further oxidative damage, so they could be candidates for antidiabetic agent, but the precise targets of naringin and curcumin in diabetic mice are still to be clarified.

Keywords: diabetes; naringin; curcumin; antioxidant activity; DNA damage

Introduction

Diabetes mellitus, a disease with a high rate of increase, is a syndrome that continues to present one of the most urgent medical problems of the 21st Century in regard to the epidemic proportions it has reached [1].Oxidative stress plays an important role in the etiology of diabetes mellitus, initially characterized by a hyperglycemia [2]. Hyperglycemia not only generates more reactive oxygen species (ROS), but also attenuates anti-oxidative mechanisms through non-enzymatic glycosylation of anti-oxidant enzymes [3]. Diabetes and hyperglycemia are associated with the generation of ROS and can be sources of DNA damage via the oxidation of DNA bases and sugar-phosphate binding sites [4-6]. During this process, ROS causing oxidative damage particularly to heart, kidney, eyes, nerves, liver, small and large vessels, gastrointestinal and immunological system [6].

ROS have many important functions in metabolism. Besides being part in redox pathways, they are included in intracellular signaling and regulation, as well as in bacterial ingestion and killing by phagocytes [7]. However, excess in ROS levels can produce oxidative damage on cellular macromolecules, such as DNA and lipids in cellular membrane, impair protein function, and trigger cell death[8,5,6]. Consequently, ROS may contribute to pathogenesis and/or development of many diseases such as diabetes [6,9], cancer [7], and cardiovascular diseases [10]. It seems that some of those diseases can be prevented or reduced by intake of food rich in metabolites that can react with ROS and convert them into less reactive substances. Studies have been shown that many medicinal and nutritional plants are rich sources of polyphenols which, through their antioxidant activity, exert positive effects on human health [11,6]. It is important to find substances that have therapeutic function and not burden the body. Such components

could be naringin and curcumin because it was shown that polyphenols have preventive and protective effect on oxidative stress in diabetes and diabetic complications [12-14].

There are many types of natural polyphenols. Certain groups of polyphenols differ in number and arrangement of the hydroxyl groups, as well as by the nature and extent of alkylation. Flavanones, such as naringenin and its glycoside naringin, are highly reactive compounds and are present in citrus fruits and the medicinal herbs of Rutaceae, Rosaceae, and Leguminosae. Curcumin, on the other hand, has diarylheptanoid structure. It is the main polyphenol in turmeric (curry powder), and responsible for many health-related properties of the plant and food that contains it [15]. Plant phenolics are multifunctional antioxidants which can act as reducing agents, free radical scavengers, metal chelators, and singlet oxygen quenchers thus inhibiting processes that can lead to membrane damage, aging, heart disease, and cancer [3,15]. Thus, in order to evaluate the antioxidant capacity of plant materials *in vitro*, several methods and assays have been conducted such as FRAP (ferric reducing/antioxidant power), DPPH (2,2-diphenyl-1-picrylhydrazyl), TRPA (total reducing power ability), ORAC (oxygen radical absorbance capacity), LDL (low density lipoprotein) oxidation and others. However, different trends between the assays have been found [16,17]. Hence the use of several assays instead of a single one is desirable to properly evaluate and compare the antioxidant activity of natural polyphenols and plant extracts [18].

Despite the importance of naringin and curcumin for human health, relatively little is known about the mechanism of their action in regulating body weight and diabetes as well as their possibility to protect blood, liver, and kidney cells from alloxan-induced DNA damage. Therefore, the aim of this study was to compare the antioxidant activity of curcumin and naringin *in vitro*, as well as some of their antidiabetic effect *in vivo* on molecular cellular and organism level. Our results demonstrated that naringin was much less effective than curcumin *in vitro*, but administration of both to diabetic mice resulted in significant increase of body weight and 100% survival. In adition, administration of polyphenols to diabetic mice resulted in decreased DNA damage in lymphocytes and increased level of DNA damage in liver, kidney and reticulocytes.

Material and methods

Animals

Male and female CBA inbred mice 2 to 3 months old, weighing 20 to 25g were used in this study. Animals were obtained from Department of Animal Physiology, Faculty of Science, University of Zagreb and kept in individual cages during the experiment. They were fed a standard laboratory diet (4 RF 21, Mucedola, Settimo Milanese, Italy) at 12 h light:12 h dark cycle and tap water ad libitum. Maintenance and care of all experimental animals were carried out according to the guidelines in force in Republic of Croatia (Law on the Welfare of Animals, N.N. #19, 1999) and in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123. Study was approved by the ethical committee (Faculty of Science, University of Zagreb, Croatia).

Chemicals

Tween-40 (polyoxyethylene sorbitan monopalmitate), butylated hydroxyanisol (BHA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Chemical Co. (USA). Alloxan, acridine orange, ethidium bromide, Giemsa dye, low melting point (LMP), and normal melting point (NMP) agaroses were purchased from Sigma. Selected polyphenols; curcumin and naringin, were purchased from Aldrich Ch. Co. Inc. Milwaukee WI, USA and dissolved in saline solution. Test strips of blood glucose were from Betachek Visual (Sydney, Australia). All other used chemicals were laboratory-grade and were purchased from Kemika (Zagreb, Croatia).Perkin Elmer Lambda 25 spectrophotometer (Perkin Elmer, USA) was used for absorbance measurements in studies of antioxidant activity.

Experimental design

Fifty-two mice were randomly divided into four groups, as follows:

Group (i): control animals (normal, nondiabetic animals); received 0.5 mL saline solution with 0.5% v/v ethanol intraperitonealy (i.p.) per day by injection for 7 days;

Group (ii): diabetic controls; injected *i.v.* with alloxan in a single dose of 75 mg kg^{-1} body weight; these served as the untreated diabetic group; received 0.5mL saline solution with 0.5% v/v ethanol intraperitonealy (i.p.)per day by injection for 7 days;

Group (iii): received curcumin solution (0.5%v/v etOH) i.p. in a daily dose of 50 mg kg^{-1} for 7 days starting 2 days after alloxan injection; these served as the curcumin-treated diabetic group.

Group (iv): received naringin solution (0.5%v/v etOH)i.p. in a daily dose of 50 mg kg^{-1} for 7 days starting 2 days after alloxan injection; these served as the naringin-treated diabetic group.

Five mice from each group were used on the 9^{th} day after alloxan injection. The number of micronuclei in peripheral blood reticulocytes and comet assay parameters (percentage of DNA in tail and tail moment) in lymphocyte, liver and kidney cells were analyzed. Peripheral blood samples and tissue samples for micronucleus and comet assay were collected after the 7 days treatment with naringin or curcumin, on the 9^{th} day after alloxan injection.

The remaining animals, i.e., 8 animals of each group were used for the survival analysis and percentage of increased life span (% ILS).

Induction of experimental diabetes and determination of serum glucose level

Diabetes was induced in Swiss albino mice by a 0.5 mL of freshly prepared saline solution of alloxan monohydrate (75 mg kg^{-1}, i.v.). Two days after the administration of alloxan, the animals with blood glucose level above 20 mmol L^{-1} were selected for the study

(diabetic mice). Blood glucose level was determined by test strips of blood glucose (Betachek Visual blood glucose test strips, Sydney, Australia).

Determination of antioxidant capacity of the naringin or curcumin

In order to elucidate the mechanism by which naringin and curcumin exert their antioxidant effects their antioxidant activity was assessed using three different assays. Radical scavenging activity (RSA) was evaluated using DPPH free radicals. The concentration that scavenges 50% of DPPH of free radicals present in the solution was calculated (RSA EC50). The ability of naringin and curcumin to donate electron was assessed by using reducing power assay (RP). Compounds with reducing properties cause conversion of a ferricyanide complex to its ferrous form and the absorbance of the reaction mixture at 700 nm increases. The amount of the extract which causes the absorbance to reach the value of 0.5 was calculated (RP0.5). In β-carotene bleaching assay linoleic acid is converted to the linoleic free radical which reacts with β-carotene and causes its degradation according to pseudo-firstorder kinetics. Antioxidants are capable to react with free radical present in the solution thus reducing the rate of reaction. Antioxidant activity (ANT) in this assay was calculated using the bleaching rates of the solution with and without antioxidant. The ability of naringin and curcumin to chelate metal ions (ChA) was studied using ferrozine and Fe^{2+}ions. The complex between ferrozine and ferrous ion has maximum absorbance at 562 nm. The chelating agent present in the solution can remove iron from the complex with ferrozine causing a decrease in the absorbance. The concentration of analyte that chelates 50% of Fe^{2+} ions present in the solution was calculated (ChAEC50). The research protocols for the performed assays are thoroughly described in a previous study [19]

Survival analysis

Animal life span and the change in body mass of mice were monitored and evaluated as described in our previous research [8].

Peripheral blood micronucleus (MN) assay

Smear of peripheral blood from tail tip was prepared as described by Oršoli et al. [6].The smears were dried at room temperature and fixed in absolute methanol for 5 minutes. After fixation, slides were stained with acridine orange (AO) and washed with phosphate buffer (pH 6.8) twice.

The alkaline comet assay

To determine influence of alloxan-induced diabetes, naringin and curcumin on DNA (integrity), we used the single cell gel electrophoresis assay, also known as the comet assay. We used assay under alkaline conditions, basically as described by Singh et al. [20]. A total of one hundred randomly-captured comets from each slide (n=4) were examined using an epifluorescence microscope(Zeiss, Oberkochen, Germany) connected to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, Haverhill, Suffolk,UK). To quantify DNA damage, tail intensity (% of DNA in tail), and tail moment were evaluated.

Statistical analyses

The results were expressed as mean ± SEM and are representative of two independent experiments. Statistical comparisons for determination of antioxidant capacity were made using a Student's *t*-test. Statistical analyses were performed using the JMP V6 from SAS software (SAS Institute, Cary, NC, USA). The parameters of tail intensity and tail moment were evaluated using Statistica 5.0 package (StaSoft, Tulsa, USA). In order to normalize distribution and to equalize the variances, a logarithmic transformation of data was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post-hoc analysis of differences was done by Scheffé test. Survival curves were calculated by Kaplan-Meier method[21], and comparison between survival curves was made by log-rank test (= 5 %) [22]. Statistical significance of control and experimental groups in the micronucleus assay was evaluated by χ^2 test. The level of statistical significance was set at $P<0.05$.

Results

Antioxidant capacity of the extracts

Antioxidant effects of curcumin and naringin differ depending on the applied test. The scavenging effects of curcumin and naringin on DPPH free radicals are shown in Figure 1A, while the EC_{50} value is shown in Table 1. At the applied concentrations, RSA of the samples and the standard was increasing linearly ($r^2 \geq 0.99$). Although naringin demonstrated antiradical activity, it is reduced compared to the BHA. On the other hand, curcumin showed extremely effective radical activity comparable to BHA (Figure 1A). Both curcumin and BHA scavenged 50% of DPPH free radicals at concentration of below 10 $\mu g mL^{-1}$,showing no statistical differences in activity. With increased concentration of ascorbic acid and polyphenols the reducing power is also increased (Figure 1B). The increase tended to be linear and very low in case of the naringin, while the absorbance of reaction mixtures with curcumin and ascorbic acid remained relatively constant in higher concentrations. The increase of the reducing power of curcumin stops earlier and was lower than the activity of ascorbic acid and even lead to a decrease in its reducing power (Figure 1B). $EC_{1.0}$ values are presented in Table 1. Naringin and curcumin in the investigated concentrations (0–1000 $\mu g\ mL^{-1}$) demonstrated notable chelating ability towards ferrous ion, but curcumin showed higher ability like in the free radical scavenging activity test (Figure 1C). EC_{50} values are presented in Table 1.

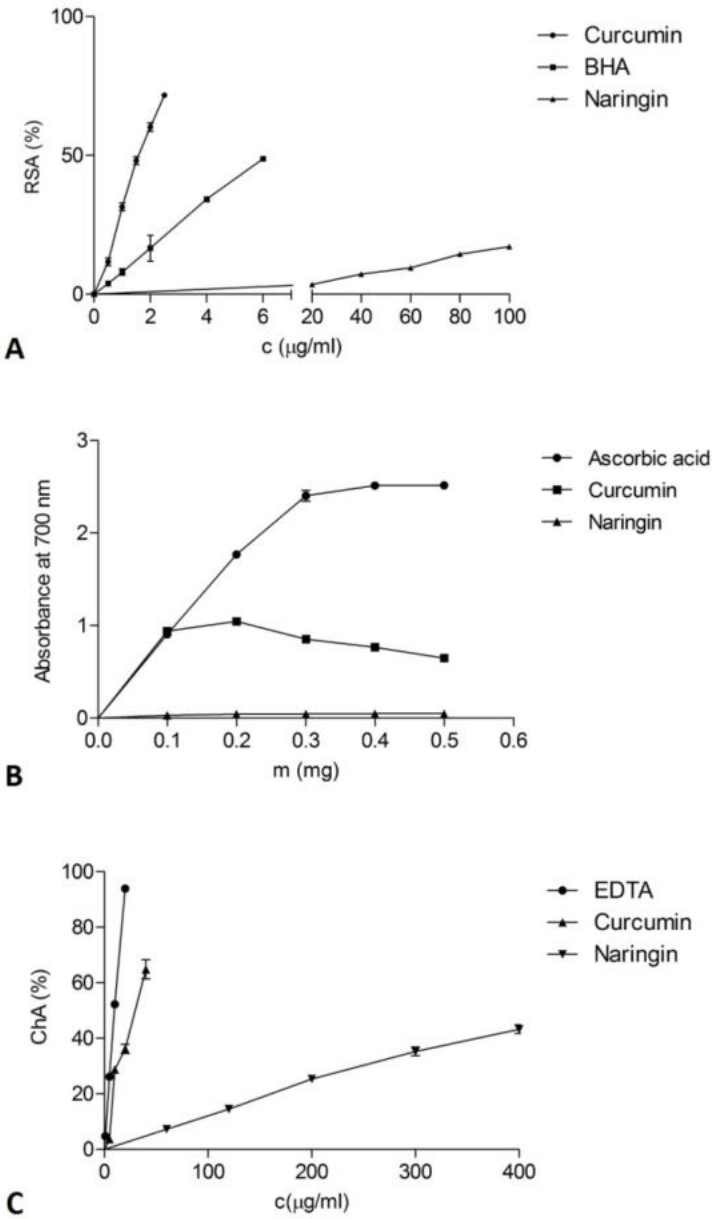

Figure 1.Antioxidant capacity of the naringin or curcumin and standards. Values are means ± SEM (n = 4).

A:DPPH radical scavenging activity (RSA) of curcumin, naringin and BHA.

B: Reducing power of curcumin, naringin and ascorbic acid.

C: Fe^{2+} chelating activities (ChA) of curcumin, naringin and EDTA.

Table 1.Radical scavenging activity, reducing power and metal chelating activity of curcumin and naringin.

Sample	RSA EC_{50} (µg/mL)	RP $EC_{1.0}$ (µg/mL)	ChA EC_{50} (µg/mL)
Curcumin	1.68 ± 0.006^{A}	0.302 ± 0.003^{A}	28.51 ± 0.398^{A}
Naringin	287.36 ± 7.650^{B}	7.967 ± 0.293^{B}	440.45 ± 4.013^{B}
Standard	$^{a}6.02 \pm 0.069^{A}$	$^{b}0.135 \pm 0.001^{A}$	$^{c}10.27 \pm 0.052^{C}$

The results are expressed as mean ± SEMand are representative oftwoo independent experiments.

A–C differences between extracts within column (samples not connected by the same capital letter are statistically different at $p< 0.05$, Student's *t*-test).
RSA EC_{50}: radical scavenging activity
RP $EC_{1.0}$: reducing power
ChA EC_{50}: metal chelating activity
[a]Standard: BHA; [b] Standard: ascorbic acid; [c] Standard: EDTA.

Effect of alloxan on blood glucose level

Blood glucose level was strongly elevated after administration of alloxan at dose of 75 mg kg^{-1}. On the 2nd day after treatment the average levels of blood glucose in each mouse were ranging between 20-30 mmol L^{-1} (data not shown).

Monitoring mass of the animals and the life span

The effects of diabetes caused by alloxan on the body weight of the mice during illness and the influence of curcumin and naringin are shown in Figure2.Decreasedweight of mice was recorded as the result of the diabetes development; decline was greatest between the first and third days, and after that, the animals treated with polyphenols have begun to recover and gain weight which nearly reaches weight of the healthy animals. After slight recover, animals with diabetes without treatment continued to lose weight.

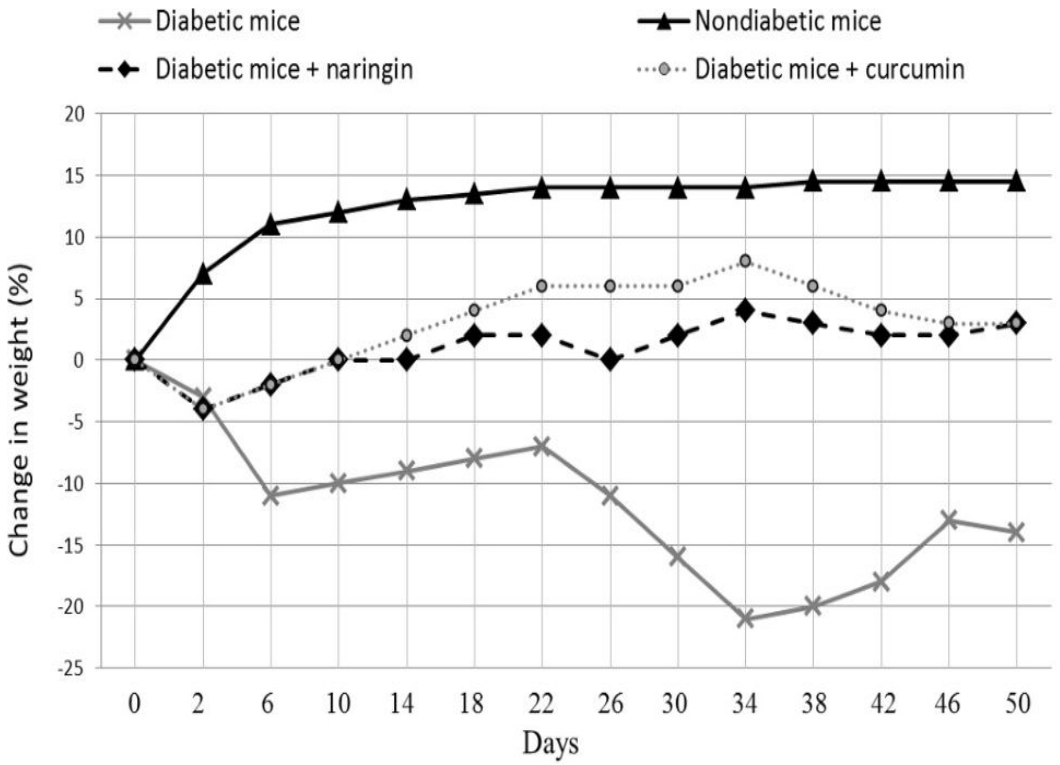

Figure 2. Effects of naringin or curcumin on the body weight of alloxan-induced diabetic mice.

Results are expressed as the means (n=8). Diabetic mice injected i.v. with alloxan in a single dose of 75 mg/kg body weight and served as untreated diabetic group. Untreated diabetic mice received 0.5 ml 0.5% etOH intraperitonealy (i.p.) for 7 consecutive days starting 2 days after alloxan injection. Control group of nondiabetic mice received 0.5 ml 0.5%v/v etOH intraperitonealy (i.p.) for 7 consecutive days. Diabetic mice treated with naringin or curcumin i.p. in a daily dose of 50 mg/kg for 7 days starting 2 days after alloxan injection.
During the experiment, all animals treated with polyphenols survived, whereas five animals died from the group of diabetic mice(data not shown). The rest of the animals from the diabetic group looked poor and sick, unlike animals with diabetes treated with curcumin or naringin that looked completely healthy.

The micronucleus test

Polyphenols increased the frequencies of micronucleus in blood reticulocytes in alloxan-induced diabetic mice. As shown in Figure 3,animals with diabetes have decreased number of micronucleus in reticulocytes compared with health mice. Treatment of diabetic mice with curcumin or naringin increased the frequencies of micronucleus in blood reticulocytes compared to diabetic mice without treatment.

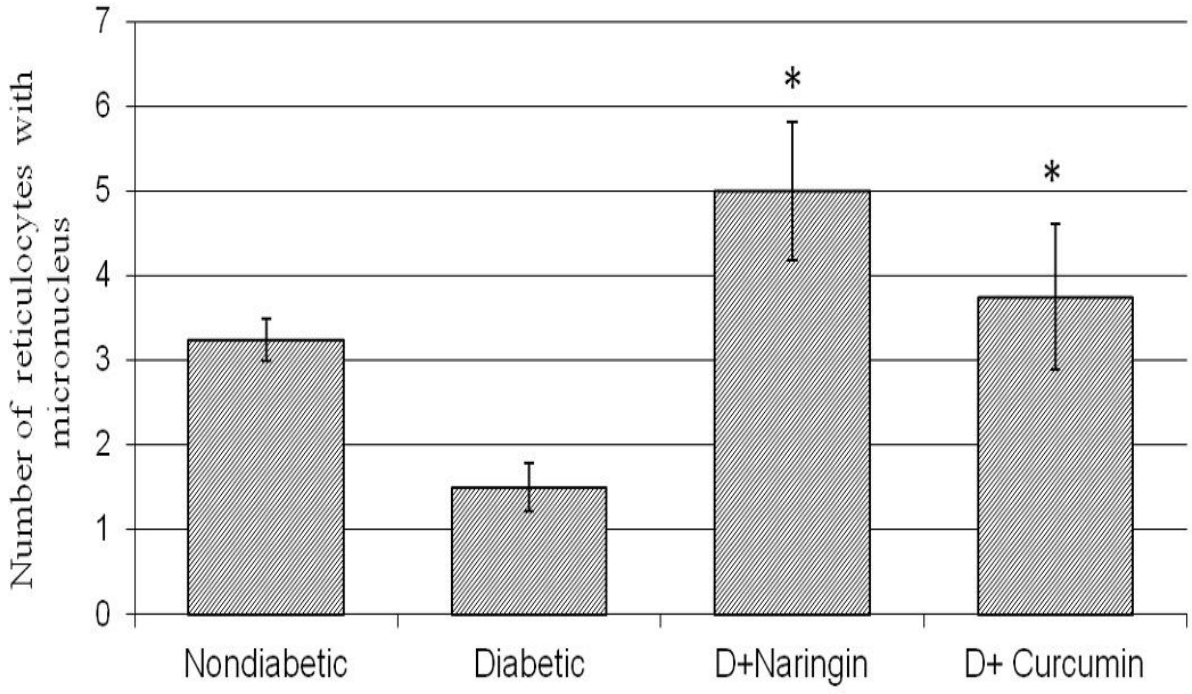

Figure 3.Effects of naringin or curcumin on number of reticulocytes with micronuclei in diabetic mice.
Results are expressed as the means± SEM of each group (n=8) and are representative of two independent experiments.

Nondiabetic mice (Nondiabetic) received 0.5 ml 0.5%v/v etOH intraperitonealy (i.p.) for 7 consecutive days. Diabetic mice injected i.v. with alloxan in a single dose of 75 mg/kg body weight. Untreated diabetic mice (Diabetic) received 0.5 ml 0.5%v/v etOH intraperitonealy (i.p.) for 7 consecutive days starting 2 days after alloxan injection. Diabetic mice treated with naringin (D+Naringin) or curcumin (D+Curcumin) received 0.5 ml (i.p.) of ethanolic polyphenol solution in a daily dose of 50 mg/kg for 7 days starting 2 days after alloxan (75 mg/kg) injection.
*Statistically significant difference between untreated diabetic mice and diabetic animals treated with naringin or curcumin (P<0.05).

The Alkaline Comet Assay

As shown in Figure 4alloxan-induced diabetes when measured in lymphocytes increased DNA damage compared to the control (nondiabetic) animals while both naringin and curcumin decreased DNA damage compared to the alloxan-induced diabetic animals. Different results were seen in liver and kidney cells where induced diabetes failed to increase DNA damage compared to the control animals. Naringin in liver cells significantly increased both comet parameters compared to the nondiabetic animals and those with diabetes, whereas in kidney cells no effect compared to both nondiabetic and diabetic animals were noticed. Curcumin significantly increased DNA damage compared to nondiabetic and diabetic animals in both cell types (P<0.05).

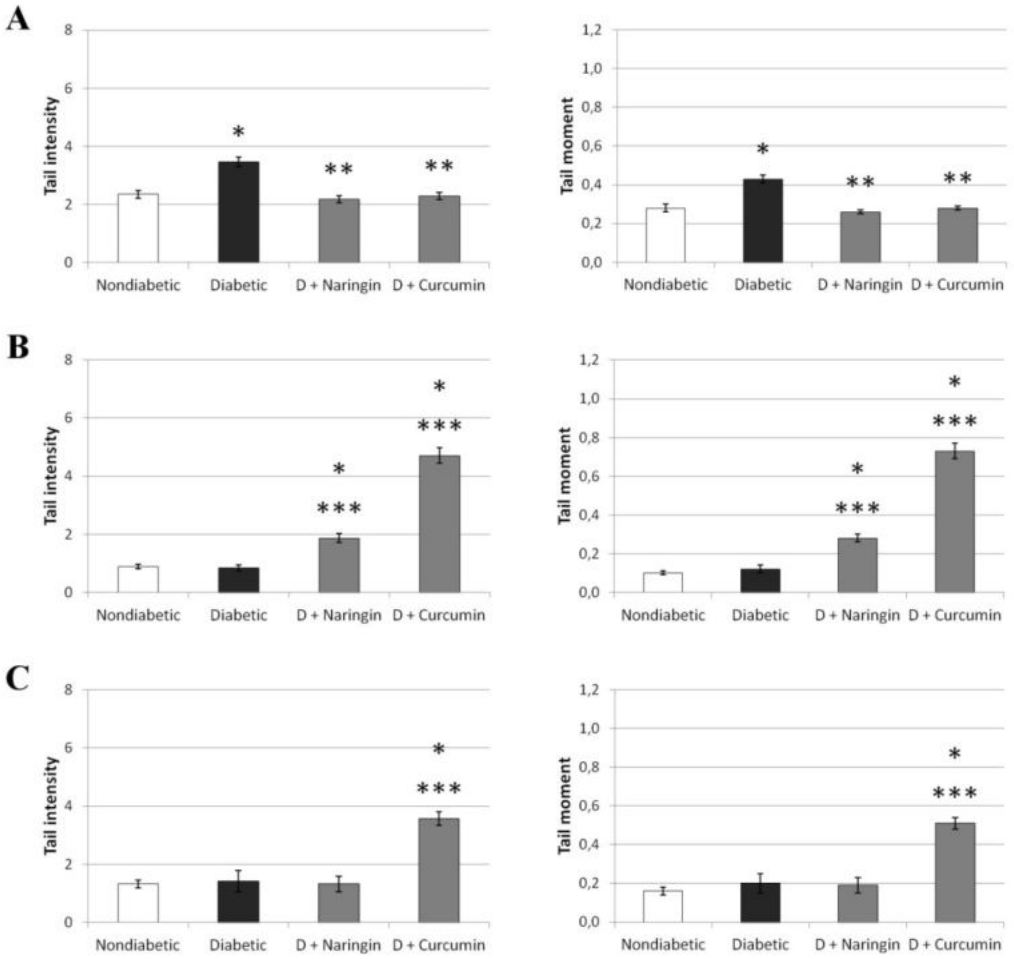

Figure 4.Effects of naringin or curcumin on alkaline comet assay parameters in mouse lymphocytes.
A:lymphocytes, B:liver cells, C: kidney cells Values are means ± SEM.

Nondiabetic mice (Nondiabetic) received 0.5 ml 0.5%v/v etOH intraperitonealy (i.p.) for 7 consecutive days, untreated diabetic mice (Diabetic) received 0.5 ml 0.5%v/v etOH intraperitonealy (i.p.) for 7 consecutive days starting 2 days after alloxan injection (75 mg/kg) and diabetic mice treated with naringin (D+Naringin) or curcumin (D+Curcumin) received 0.5 ml (i.p.) of ethanolic flavonoid solution in a daily dose of 50 mg/kg for 7 days starting 2 days after alloxan (75 mg/kg) injection. *Statistically significant increase compared to control (P<0.05), **Statistically significant decrease compared to alloxan treatment (P<0.05), ***Statistically significant increase compared to alloxan treatment (P<0.05)

Discussion

In the present work we investigated how the antioxidant capacity of curcumin and naringin in vitro coincides with their ability to protect cells of the kidneys, liver and blood against oxidative stress in alloxan-induced diabetes in mice.

Because of its complexity in antioxidant activity, it is important to use several assays instead of a single one. In this study, antioxidant activity of naringin and curcumin was investigated using three assays which cover different aspects of antioxidant activity. DPPH assay estimates the reactivity of electron- and/or hydrogen-donating antioxidants with free radicals, the reducing power of a compound estimates the ability to donate an electron, and thus cause the transformation of free radicals into a less reactive species and the chelating activity measures the ability of a substance to chelate Fe^{2+} ions which have a pivotal role in generation of hydroxyl radicals *in vivo*.

Antioxidant activities of curcumin and naringin varied depending on the applied test. The scavenging effects of curcumin and naringin on DPPH free radicals are shown in Figure 1A, while the EC_{50} value is shown in Table 1. At the applied concentrations, RSA of the samples and the standard was increasing linearly ($r^2 \geq 0.99$). The two polyphenols demonstrated appreciable scavenging properties against DPPH radicals, but while curcumin scavenged 50% of DPPH free radicals at concentration of below 10 μg mL^{-1},

the EC_{50} of naringinwas significantly higher, indicating lower activity. This could be explained by the greater number of conjugated bonds in the structure of curcumin, which are capable of stabilizing an unpaired electron by extended electron delocalization [15]. Literature reports suggest that the antioxidant activity of plants is associated with their reducing power, which terminates free radical chain reactions [23]. Reducing power of the curcumin and naringin was evaluated measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power of curcumin was still comparable to ascorbic acid (Figure 1B) and statistically higher than that of naringin which was negligible (Table 1). In the Fenton reaction, hydroxyl radical production is directly related to the concentration of iron or other transition ions. Since hydroxyl radicals are among the most harmful reactive oxygen species in biological systems, chelating activity of a compound contributes to its antioxidant properties. The chelating activity of the extracts in the tested concentrations increased linearly ($r^2 \geq 0.91$) (Figure 1C). Even though 5-hydroxy and 4-oxo groups of flavonoids is able to chelate ferrous ions [24], while the oxo groups in curcumin are susceptible to keto-enol tautomerism[25], the chelating activity of curcumin was significantly more pronounced than the activity of naringin (Table 1).

According to shown antioxidant activity of the naringin and curcumin (Figure 1, Table 1), increased body weight (Figure 2) and increased life span of the diabetic animals treated with naringinor curcumin it seems that highly damaged liver and kidney cells of the untreated diabetic mice undergo rapid deterioration so comet assay showed lower comet parameters in untreated diabetic mice compared to diabetic mice treated with naringin or curcumin. Furthermore, it is possible that the antioxidant ability of curcumin or naringin was crucial in preventing further oxidative damage, as well as in the repair of DNA. The reduced number of micronuclei in the untreated diabetic animals in relation to healthy mice (Figure 3) confirms our hypothesis which is supported by the results of previous research [5]. On the other hand, DNA damage in liver and kidneys as a result of treatment with curcumin could be explained with the known prooxidant ability of curcumin in high doses, especially in the presence of metal ions such as Fe^{2+} and/or Cu^{2+} as described by Chattopadhyay et al. [26]. Lower levels of DNA damage in the liver can be explained by the presence of large amounts of antioxidant enzymes like glutathione peroxidase, superoxide dismutase and catalase which activities can be elevated by the polyphenols [27,28].Different results observed in lymphocytes (Figure 4) may be due rapid restoration of white blood cells that is not the case in the liver and kidneys.

Conclusions

According to the increased body weight and increased life spanof the diabetic animals treated with naringin or curcumin it can be concluded that naringin and curcumin exerts antidiabetic effects in mice, possibly by attenuating the oxidative stress through scavenging of the ROS, by reducing power activity and by the partial improvement the repair process of DNA in kidney and liver tissues. On the basis of their positive effects naringin and curcumin, as well as their natural sources, such as grapefruit and turmeric, should be taken into account as candidates for antidiabetic agent but further studies are required to confirm this possibility.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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