Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v36i1.19839

Evaluation of the protective effect of guava fruits and leaves on oxidative stress

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ABSTRACT. Oxidative stress is an imbalance between reactive oxygen species and antioxidant capacity of action. Thus, research, alternative to mitigate the damaging effects of oxidative stress, improving the body´s antioxidant capacity, prevented the disease and its complicatons. The leaves and fruits of guava are rich in the antioxidants. This work aimed to study the effect of flour and ethanolic/acetone extracts leaves and fruits of guava on lipid oxidation in rats with hypercholesterolemic. The flour and extracts decreased leves of triglycerides and non – HDL cholesterol and increased in the HDL. Cholesterol levels decreased only leaves and fruits. The activity of GPx decreased in samples serum in all treatments and the SOD only the extracts were effective. There was no difference in levels the MDA in relation hypercholesterolemic control. For the samples of liver, there was no difference in activity SOD. In relation of activity GPx, only the flour was effective. There were differences in levels the MDA of the hypercholesterolemic animals treated with flour and extracts with the animals of the hypercholesterolemic control.

Keywords: antioxidants, enzymes, malonic dialdehyde.

Avaliação do efeito protetor de folhas e frutos da goiabeira no estresse oxidativo

RESUMO. O estresse oxidativo é um desequilíbrio entre as espécies reativas de oxigênio e capacidade antioxidante de ação. Dessa forma, pesquisas buscam alternativas para amenizar os efeitos prejudiciais do estresse oxidativo, melhorando a capacidade antioxidante do organismo, prevenido as enfermidades e suas complicações. As folhas e frutos de goiaba são ricos em antioxidantes. Este trabalho teve como objetivo estudar o efeito da farinha e folhas etanólicos/acetona extratos e frutos de goiabeira na oxidação lipídica em ratos com hipercolesterolemia. A farinha e extratos de folhas diminuiu de triglicérides e colesterol não - HDL e aumento do HDL. Os níveis de colesterol diminuiram apenas folhas e frutas. A actividade da GPx diminuiu em amostras de soro, em todos os tratamentos e a SOD apenas os extratos foram eficazes. Não houve nenhuma diferença nos níveis de MDA no controle da hipercolesterolemia relação. Para as amostras de fígado, não houve diferença na atividade da SOD. Na relação de GPx atividade, apenas a farinha foi eficaz. Houve diferenças nos níveis do MDA dos animais hipercolesterolêmicos tratados com farinha e extratos com os animais do controle hipercolesterolêmica.

Palavras-chave: antioxidantes, enzimas, dialdeído malônico.

Introduction

In recent decades cardiovascular diseases began to be the main cause of mortality in the developed and in the developing countries. The increase in the incidence of those diseases is due to changes in nutritional patterns and physical inactivity, enabling an increase in the prevalence of hyperlipidemia, considered one of the main risk factors due to the elevation of the plasmatic levels of total cholesterol and fractions, associated to the decrease in the HDL levels (BRUCKNER, 2008).

The excess cholesterol stimulates the production of reactive oxygen species (ROS), contributing to endothelial dysfunction, LDL modification and reduction of the antioxidant defense system, such as the decrease in the superoxide dismutase enzyme (SOD) and glutathione peroxidase activities (GPx) (LESTER et al., 2009). Those enzymes act together to inactivate ROS.

Excess ROS leads to the alteration in the signaling and redox control processes and/or molecular harm, a condition known as oxidative stress (SEIFRIED et al., 2007). As such, a defense system that impedes or minimize those species in the organism is important to reduce cardiovascular disease risk development.

Plants contain substances called antioxidants, characterized by the phenolic compounds, vitamin C and carotenoids that are capable of inactivating ROS and to increase LDL resistance to oxidation (GLADINE et al., 2007).

Taking into account the high cost of medicines and their lingering use, patients have resorted to the use of plants as an alternative treatment; however, those treatments have been used in an empirical manner by the population, their study being necessary to allow more reliable conclusions.

The tea of the leaves and the fruits of the guava tree are used in folk medicine for the treatment of glycemia, hypertension, gastrointestinal disturbances, cardiovascular diseases, and for anti-inflammator, hepatoprotective and anticancerous action (YUSOF; SAID, 2004; CHEN; YEN, 2007).

The objective of this work was to evaluate the effect of the leaves and fruits of the guava tree on the level of cholesterol and its fractions, antioxidant enzyme activity and to evaluate the lipoperoxidation.

Material and methods

Characterization of guava leaves and fruits

The leaves and fruits of the Pedro Sato guava tree cultivar were picked in the city of Lavras. The fruits were frozen and lyophilized for the obtaining of the flours. The leaves were put in an oven at 37°C, for five days. After that period they were macerated and triturated in a grinder for the obtaining of the flour. The fruits were identified by the College of Agriculture Lavras Herbarium, where the voucher specimen was deposited which received voucher number 26277.

The ethanol/acetone extracts of the flours were maintained under maceration in ethanol/acetone (70/30) at a 1:50 (w/v) proportion, for 24 hours and soon afterwards, filtered. The residue re-extracted. The two supernatants were combined, submitted to evaporation and, later lyophilized for the obtaining of the dry extracts (RUFINO et al., 2007).

The phenolic compounds were measured in the ethanol/acetone extracts and flours using the Folin-Denis reagent, which is reduced by the phenols, a blue colored complex in alkaline solution and measured at 760 nm (Association of Official Analytical Chemists - AOAC, 2005). The results were expressed as mg tannic acid, 1 g sample dry.

The antioxidant potential was determined by the DPPH method. Four dilutions were made from the extracts used for the determination of antioxidant potential. The ability to sequester DPPH (1.1-diphenyl-2-picrylhydrazyl) was performed according to the method described by Thaipong et al. (2006) with modifications. An 0.1 ml aliquots of each dilution were added to 3.9 mL of the DPPH solution in methanol (0.06 mM). At the end of 30 minutes the absorbance was measured at 515 nm and the ability to sequester the radical, expressed in percentage, was calculated relatively to the control (no antioxidant), according to the following expression:

$$\% \text{ sequestration} = \frac{\text{Absorbance of control} - \text{Absorbance sample}}{\text{Absorbance of control}} \times 100\%$$

Through the equation of the line, the concentrations (mg mL-1) required to inhibit 50% of DPPH • were calculated.

Biological analysis

The hypercholesterolemic diet was prepared using the commercial ration plus 0.50% cholesterol and 0.25% cholic acid (ROCHA et al., 2012).

The *in vivo* study was carried out according to the ethical principles in animal experimentation adopted by the Colégio Brasileiro de Experimentação Animal (COBEA) approved on 11/11/2010 by the animal research ethics committee of the Federal University of Alfenas, protocol number 326/2010. 50 male Wistar rats were used weighing 400 ± 50 g. The animals were divided in 10 groups with 5 animals in each group: non-hypercholesterolemic control (NC); hypercholesterolemic control (HC); non-hypercholesterolemic treated with the flour of the leaves (NFL); non-hypercholesterolemic treated with the flour of the fruits (NFF); hypercholesterolemic treated with the flour of the leaves (HFL); hypercholesterolemic treated with the flour of the fruits (HFF); non-hypercholesterolemic treated with extract of the leaves (NEL); non-hypercholesterolemic treated with extract of the fruits (NEF); hypercholesterolemic treated with extract of the leaves (HEL) and hypercholesterolemic treated with extract the fruits (HEF).

At the end of the experiment, the animals were anesthetized with Thiopental (35 mg Kg^{-1}) and the blood removed by the cardiac puncture technique. Soon afterwards the liver was removed and homogenized for the analyses.

The levels of total cholesterol and fractions were determined through Labtest® enzymatic-colorimetric kit. The non-HDL cholesterol levels were determined by the difference between the total cholesterol and HDL levels.

The SOD activity was determined considering that 1 unit of the enzyme is capable to produce 50% inhibition in the reaction (OYANAGUI, 1984). The

results were expressed in relation to the protein concentration. The GPx activity was determined incubated with NADPH reduced glutathione and glutathione redutase and t-butyl hydroperoxide (SINET et al., 1975). The results were expressed in relation to the protein concentration. The protein concentration was determined using the Bradford method.

The malonic dialdehyde levels (MDA) were estimated through the determination of the concentration of substances reactive to thiobarbituric acid, by high performance liquid chromatography. The determination of the MDA was conducted by HPLC in a C-18 column in reverse phase method with fluorescence detector with an excitation wavelength of 532 nm and emission at 553 nm. The elution was isocratic at a flow of 0.8 mL minute-1 with methanol/phosphate buffer mobile phase at a 50:50 v/v. The quantification was made by calibration with the 1.1.3.3-Tetramethoxypropane standard, for analysis of the whole of the respective areas (PUNCHARD; KELLY, 1996). All of the reagents used were of HPLC grade and the water used was purified by the Milli-Q system.

Statistical analysis

A completely randomized design in a 2 x 2 x 2 + 2 factorial outline was used. The statistical analyses were conducted using the Statistical Analysis System Institute (SAS, 1999). When significant, the Scott-Knott test was used for comparison of the averages.

Results and discussion

In Table 1 the phenolic compound content was higher for the extracts of leaves. In this study the phenolic compound content in the flour of the leaves (130.05 ± 6.71 mg g^{-1}) was inferior to those found in the literature (154.36 ± 2.97 mg g^{-1}) using the same extraction method in the guava leaves (OYANAGUI, 1984). The green tea herbs and rosemary are consumed in the form of tea have high levels of phenolic compounds and, consequently an expressive antioxidant activity. Literature data indicate that the phenolic compound content for those herbs were 149.27 ± 2.31 and 185.04 ± 4.99 mg g^{-1} for the green tea and rosemary, respectively (OYANAGUI, 1984). Those values are close to those found in this study for the flour of the guava tree leaves, suggesting that those leaves can be used as nutraceutics.

Table 1. Phenolic content and antioxidant potential in flours and ethanol/acetone extracts from guava leaves and fruits.

Parameters analyzed	Leaves		Fruits	
	Extract	Flour	Extract	Flour
Phenolic compounds (mg g^{-1})	369.89 ± 13.80	130.05 ± 6.71	14.66 ± 0.58	7.45 ± 0.16
DPPH (IC50% mg mL^{-1})	0.003 ± 0.0001	0.023 ± 0.0001	0.106 ± 0.01	0.438 ± 0.01

The phenolic compound content in the fruit flour, when transformed in fresh matter (1.50 mg g^{-1} FM), are in agreement with the levels found presented in the literature that varied between 1.24 to 1.87 mg g^{-1} FM (HASSIMOTO et al., 2005; MELO et al., 2006).

The liquid extract possesses a more apolar character than water, and thus that extraction form was more effective to extract the phenolic compounds in the leaves and fruits when compared the content found for the flours.

According to Table 1, it is observed that the extracts of the leaves and fruits presented higher phenolic compound content and, consequently higher antioxidant potential than the flours by the DPPH method. The same relationship can be noticed when comparinng leaves and fruits independent of the extraction procedure.

From the data of Table 2, it is observed a 109% increase in the total serum cholesterol levels of the HC with the NC, the difference being quite significant and indicating hypercholesterolemia. Those values corroborate with those from the literature (ROCHA et al., 2012; MACHADO et al., 2000). It is observed that the animals of HFL and HFF presented lower total serum cholesterol levels than the HC leading to a reduction of 29.93 and 35.18% in the total cholesterol levels. The same was not observed for the extracts of the leaves and fruits.

Table 2. Total serum cholesterol, triglycerides, HDL and non-HDL cholesterol.

Treatments	Cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})	non-HDL Cholesterol (mg dL^{-1})	HDL (mg dL^{-1})
[1]NFL	49.22 b	35.44 b	31.42 c	17.80 a
[2]NFF	46.12 b	37.55 b	26.92 c	20.00 a
[3]NEL	50.40 b	29.04 b	30.48 c	19.20 a
[4]NEF	47.26 b	30.10 b	28.86 c	18.40 a
[5]HFL	67.76 b	40.19 b	52.26 b	15.50 a
[6]HFF	62.68 b	42.00 b	47.88 c	14.80 a
[7]HEL	86.02 a	42.32 b	71.42 a	14.60 a
[8]HEF	77.20 a	49.79 b	58.00 b	19.20 a
[9]NC	46.23 b	37.02 b	30.43 c	15.80 a
[10]HC	96.70 a	68.00 a	88.50 a	8.20 b

Means followed by the same lowercase letter do not differ by Scott-Knott test (5% probability).[1]non-hypercholesterolemic control (NC); [2]hypercholesterolemic control (HC); [3]non-hypercholesterolemic treated with the flour of the leaves (NFL); [4]non-hypercholesterolemic treated with the flour of the fruits (NFF); [5]hypercholesterolemic treated with the flour of the leaves (HFL); [6]hypercholesterolemic treated with the flour of the fruits (HFF); [7]non-hypercholesterolemic treated with extract of the leaves (NEL); [8]non-hypercholesterolemic treated with extract of the fruits (NEF); [9]hypercholesterolemic treated with extract of the leaves (HEL); [10]hypercholesterolemic treated with extract the fruits (HEF).

All of the treatments exercised a positive effect on the triglyceride levels. There was a 59.10 to 73.22% reduction in the triglyceride levels in the animals treated with the flours and extracts.

The non-HDL cholesterol represent an estimate of the cholesterol concentration in the atherogenic lipoproteins (VLDL and LDL). It can be seen in Table 2 that the HC and HEL presented the highest non-HDL cholesterol levels, equal to 88.50 and 71.42 mg dL^{-1}, respectively, not having a significant difference between them. The animals of the HFL and HEF presented a significant reduction of the non-HDL cholesterol levels in relation to HC, however these were superior to NC. The highest reduction of the non-HDL cholesterol levels was in the animals of the HFF with a reduction of 45.90%.

The HDL is responsible for the inhibition of the cholesterol deposition in the arteries walls moderated by LDL and for the reverse transport of cholesterol, promoting the removal of the cholesterol from of the body cells to the liver (DUARTE et al., 2009). In Table 2, one can see that independent of the diet administered, all the animals treated with the samples presented significantly higher HDL concentrations than those of the HC.

The hypercholesterolemia significantly increased the SOD and GPx enzyme activity when compared to NC (Table 3). Similar results are observed in other works (CHENNI et al., 2007).

Table 3. Antioxidant enzyme activity and malonic dialdehyde content in serum samples.

Treatments	[1]SOD (U mg^{-1} of protein)	[2]GPx (μmol of NADPH mg^{-1} protein)	[3]MDA (μmol MDA mg^{-1} protein)
[4]NFL	5.256 b	1.86 x 10^{-13} c	2.04 x 10^{-3}
[5]NFF	5.064 b	1.49 x 10^{-13} c	1.59 x 10^{-3}
[6]NEL	5.038 b	2.12 x 10^{-13} c	2.13 x 10^{-3}
[7]NEF	5.140 b	1.57 x 10^{-13} c	1.99 x 10^{-3}
[8]HFL	5.348 b	1.56 x 10^{-13} c	1.45 x 10^{-3}
[9]HFF	5.164 b	3.29 x 10^{-13} b	1.49 x 10^{-3}
[10]HEL	5.560 a	2.81 x 10^{-13} b	1.97 x 10^{-3}
[11]HEF	5.720 a	2.56 x 10^{-13} b	1.43 x 10^{-3}
[12]NC	4.998 b	1.86 x 10^{-13} c	2.18 x 10^{-3}
[13]HC	6.184 a	4.58 x 10^{-13} a	2.30 x 10^{-3}

Means followed by the same lowercase letter do not differ by Scott-Knott test (5% probability). [1]superoxide dismutase enzyme (SOD); [2]glutathione peroxidase activities (GPx); [3]malonic dialdehyde levels (MDA); [4]non-hypercholesterolemic control (NC); [5]hypercholesterolemic control (HC); [6]non-hypercholesterolemic treated with the flour of the leaves (NFL); [7]non-hypercholesterolemic treated with the flour of the fruits (NFF); [8]hypercholesterolemic treated with the flour of the leaves (HFL); [9]hypercholesterolemic treated with the flour of the fruits (HFF); [10]non-hypercholesterolemic treated with extract of the leaves (NEL); [11]non-hypercholesterolemic treated with extract of the fruits (NEF); [12]hypercholesterolemic treated with extract of the leaves (HEL); [13]hypercholesterolemic treated with extract the fruits (HEF).

It can be seen that the SOD activity in the HFL and HFF did not differ significantly from the animals of the HC. In the same way, the cholesterol level presented in Table 2 for those animals did not differ from the HC. The animals of the HEL and HEF presented enzymatic activity similar to HC, suggesting that those extracts were not efficient in controlling the ROS generated.

In relation to the GPx activity, it is observed that all animals presented inferior enzymatic activity to the HC, meaning that in these groups there was a stabilization of the ROS generated.

When the MDA level is compared to the SOD and GPx enzymatic activity in the serum of the animals of the NC and HC, it is observed that there was an increase of the enzymatic activity in the HC while the MDA level was statistically similar among the different groups analyzed. These results can be attributed to a possible increase in the generation of ROS in the serum of the hypercholesterolemic animals.

Aerobic organisms respond to the increase in ROS formation, inducing repair and protection mechanisms through their antioxidante defense system (SAMPAIO; MORAES, 2010). It was demonstrated that there was a significant increase in the SOD and GPx activity in the serum of the animals treated with the hypercholesterolemic diet when compared to the NC. The activity of these enzymes represents the protection of the organism against the ROS (PUNCHARD; KELLY, 1996; BARREIROS et al., 2006). In spite of the existence of other unanalyzed antioxidants in this work (catalase, thioredoxin, glutathione and vitamin E), that could contribute to the protection against oxidative stress (RATNAM et al., 2006) the obtained results suggest that the antioxidant defense system was activated in a manner proportional to the need generated by the pro-oxidizing events, thus impeding the increase of the MDA concentration in the plasma of the hypercholesterolemic animals. The same has been related in various studies (CHENNI et al., 2007; MANTHA et al., 1996).

The treatment of the hypercholesterolemic animals with the extracts and flours was capable to reduce the activity of the SOD and GPx in the serum. These results can be attributed to the presence of antioxidant compounds, such as phenolic compounds, as observed in Table 1, that can act impeding the increase or sequestering ROS with a consequent reduction of the pro-oxidizing events. As such, the response of the organism in relation to the induction of the SOD and GPx serum activity would be lower than that observed in the hypercholesterolemic animals non-treated with the flours or extracts used in this study. Therefore, our results suggest that the flours of the leaves and fruits can be efficient in the control of oxidative stress in the serum of hypercholesterolemic animals.

It is observed that, unlike the results obtained in the serum, there was no significant difference in the

activity of the hepatic SOD activity among the analyzed groups (Table 4). However, when analyzing the GPx enzymatic activity it is observed that the animals of the HC group activity superior to that of the animals of the NC group. Among the different appraised treatments, it is observed that only the treatment with the fruit flour was capable to significantly reduce the GPx activity, at levels similar to those of the group NC.

Table 4. Antioxidant enzyme activity and malonic dialdehyde content in liver samples.

Treatments	[1]SOD (U mg^{-1} of protein)	[2]GPx (μmol of NADPH mg^{-1} protein)	[3]MDA (μmol MDA mg^{-1} protein)
[4]NFL	323.21	2.16×10^{-14} b	2.68×10^{-2} c
[5]NFF	328.20	3.10×10^{-14} b	3.17×10^{-2} c
[6]NEL	341.97	2.99×10^{-14} b	4.56×10^{-2} b
[7]NEF	345.73	3.73×10^{-14} b	3.12×10^{-2} c
[8]HFL	389.09	5.00×10^{-14} a	4.74×10^{-2} b
[9]HFF	356.02	3.68×10^{-14} b	3.07×10^{-2} c
[10]HEL	427.63	4.80×10^{-14} a	4.45×10^{-2} b
[11]HEF	395.25	4.54×10^{-14} a	5.27×10^{-2} b
[12]NC	371.56	2.97×10^{-14} b	2.91×10^{-2} c
[13]HC	457.32	5.60×10^{-14} a	7.97×10^{-2} a

Means followed by the same lowercase letter do not differ by Scott-Knott test (5% probability); [1]superoxide dismutase enzyme (SOD); [2]glutathione peroxidase activities (GPx), [3]malonic dialdehyde levels (MDA); [4]non-hypercholesterolemic control (NC); [5]hypercholesterolemic control (HC); [6]non-hypercholesterolemic treated with the flour of the leaves (NFL); [7]non-hypercholesterolemic treated with the flour of the fruits (NFF); [8]hypercholesterolemic treated with the flour of the leaves (HFL); [9]hypercholesterolemic treated with the flour of the fruits (HFF); [10]non-hypercholesterolemic treated with extract of the leaves (NEL); [11]non-hypercholesterolemic treated with extract of the fruits (NEF); [12]hypercholesterolemic treated with extract of the leaves (HEL); [13]hypercholesterolemic treated with extract the fruits (HEF).

The SOD and GPx isoformas found in the intracellular medium are different from those found in the extracellular liquids, thus being able to present a variation in the affinity for their respective substrata among the different compartments of the organism. Furthermore, the proportion between the content of antioxidant and pro-oxidizing substances is highly variable between the different organs and compartments of the organism. As such, the response of the antioxidant defense system to the increase of ROS can vary, not only in relation to the concentration, but also in function of the type of oxidizing species produced and the content of antioxidants present in the analyzed tissue (LIU et al., 2000).

The HC group presented a MDA level superior to CN, suggesting that, in spite of the GPx activity increase, this was not enough to contain the lipidic peroxidation in the liver of these animals.

These results corroborate with literature data that also demonstrated that a hypercholesterolemic diet was not capable to induce significant difference in the SOD activity, but it increased the GPx activityand the MDA level in rat liver samples (GOKKUSU; MASTAFAZADE, 2003).

It was observed that all the hypercholesterolemic animals treated with the extracts and flours presented a significant reduction of the hepatic MDA level in relation to the HC. Furthermore, the fruit flour was more effective in the reduction of the MDA concentrations leading to concentrations similar to those observed in the normolipidemic animals, which could justify the results observed in relation to the GPx activity in the liver of the hypercholesterolemic animals treated with this flour.

According to the results presented in Table 1, the flour of the fruits presented the lowest phenolic compound content and lowest antioxidant potential, suggesting that the phenolic compounds present in the other samples might have had their absorption limited because of their chemical composition.

The flours and ethanol/acetone extracts of the leaves and fruits of the guava tree exercised a hypocholesterolemic effect and they prevented the oxidative stress induced by the hypercholesterolemia in the liver and plasma of mice.

Accordingly, the leaves and fruits of Pedro Sato cultivar can be used as functional foods and raw material for the development of phytotherapeutics.

Conclusion

Flour and extracts ethanol / acetone from leaves and fruits of guava exerted hypocholesterolemic effect and prevented oxidative stress induced by hypercholesterolemia in the liver and plasma of rats.Thus, the leaves and fruit of the cultivar Pedro Sato can be used as functional foods and raw materials for the development of herbal medicines.

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Received on February 16, 2013.
Accepted on August 12, 2013.