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Lipid peroxidation inhibition by ethanolic extract and fractions from *Rhamnus sphaerosperma* var. *pubescens* (Reissek) M.C. Johnst. (Rhamnaceae)

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Abstract

Rhamnus sphaerosperma var. pubescens is a species native to Brazil, found in the southern region, and still there is no data in the literature about biological activities of this plant. Therefore, the aims of this study were to verify the antioxidant activity and determine the *ability to inhibit* lipid peroxidation of crude ethanolic extract and it fractions hexane, chloroform and ethyl acetate of the stem. The total antioxidant activity was evaluated by phosphomolybdenum complex method. And the *ability to inhibit* lipid peroxidation was evaluated, in vitro and in vivo experiments, by quantification of thiobarbituric acid reactive species formed from peroxidation induced. *The in vitro* test about lipoperoxidation, were used lipids obtained from egg yolk. And, for in vivo assay, the peroxidation was determined only for crude extract, using stomachs of female rats injured by administration of absolute ethanol. The samples of *Rhamnus sphaerosperma* var. pubescens demonstrated an important antioxidant capacity. In the phosphomolybdenum complex method, the chloroform fraction (86.4%) and ethyl acetate fraction (96.2%) showed the best activity than crude extract. For the in vitro induced lipid peroxidation, thechloroform (63.3%) and hexane (59.7%) fractions demonstrated high capacity in prevention of lipid oxidation. The crude extract showed effectiveness in both methods, and was used in the verification of inhibition of lipid peroxidation *in vivo*. In this test, the lesser dose tested, 20 mg/Kg, showed better effectiveness in inhibiting lipid oxidation, with reduction of 56.9%.Furthermore, was possible identify the stigmasterol and sitosterol as compounds of this plant. *Rhamnus sphaerosperma* var. pubescens stem possess potential antioxidant activity, reducing lipid peroxidation tested by in vitro and in vivo methods.

Keywords: *Rhamnus sphaerosperma* var. pubescens, lipid peroxidation, antioxidant, ethanolic extract, stigmasterol and sitosterol.

Introduction

Reactive oxygen species (ROS) and nitrogen (RNS) are formed in the organism by physiological and pathological processes. Therefore, the body has antioxidant mechanisms that control the performance of these species while avoiding tissue damage [1]. Excessive production of ROS and RNS causes an imbalance between oxidants and antioxidants mechanisms, causing oxidative processes in macromolecules, such as lipid peroxidation, resulting in tissue and cell damage, called oxidative stress, associated with the development and maintenance of various pathological conditions. Antioxidant agents are compounds that have the ability to revert or inhibit the oxidation of other substances, being explored as potential pharmacological agents for the treatment of these diseases [2].Vegetables are known to produce a diversity of substances with antioxidant capacity. The family Rhamnaceae is described by the therapeutic potential of their species. Specimens of the Rhamnus genus exhibit a pronounced antioxidant activity, such as radical scavenger and inhibition lipid peroxidation, as for example, R. alaternus and R. nakarai, species widely used in folk medicine [3-5]. *Rhamnus sphaerosperma* var. pubescens is a species notendemic, native in Brazil, found in the southern region. It is popularly known as “Cangica” and “Fruto-de-pombo”. And there are still no studies that describe their biological properties, thus, it have a great potential chemical and pharmacological because is framed in the genus Rhamnus. In this study we investigated the phytochemical composition of *Rhamnus sphaerosperma* var. pubscens stems, as well as, the antioxidant

activity *in vitro* and the inhibition of lipid peroxidation in animal model of tissue injury caused by ethanol.

Material and methods

Plant Material

The stems of *Rhamnus sphaerosperma* var. pubescens were collected in Curitiba-PR, in May 2011. The botanical identification was performed in Municipal Botanical Museum of Curitiba, Paraná. The voucher specimen was deposited under the number 243989.

Extraction and isolation

The dried plant material (2Kg) was submitted to ethanol extraction in Soxhlet apparatus modified. The crude ethanolic extract obtained was concentrated and subjected to liquid-liquid partitioning with hexane, chloroform and ethyl acetate. The hexane fraction was subjected to purification by chromatography on silica column (silica 60 Merck 0.063-0.200mm) with hexane, ethyl acetate and methanol. A crystalline sample was obtained. This sample was analyzed by nuclear magnetic resonance spectroscopy (NMR) of ^{1}H and ^{13}C.

Phosphomolibdenum spectrophotometric method

This analysis evaluates the antioxidant capacity of both Lipophilic and hydrophilic components. The fractions and ethanol extracts of stems were submitted to the Phosphomolibdenum spectrophotometric method, at a concentration of 200μg/mL. The antioxidant capacity of the samples was expressed in relative antioxidant activity (RAA) regarding rutin[6,7].

Determination of the inhibition of lipid peroxidation *in vitro*

This system evaluates the potential of different samples in inhibiting lipid peroxidation process. As source of lipids was used the gem of an egg, diluted to a concentration of 5% (w/v), in sodium dodecyl sulfate solution(0.55%). Then 0.5 mL of this solution of lipids was placed in contact with samples or standard butyl hydroxyl toluene (0.1mL, final concentration of 70μg/mL), the oxidizing agent 2,2'-azo-bis-2-amidinopropanochloride (50μL, 0.035M), 20% acetic acid (1.5mL, pH 3.5), distilled water (0.4mL) and After, the thiobarbituric acid (1.5mL, 0.4%).The reaction was carried out at 95ºC for 60 minutes. The organic phase was extracted with butanol (1.5mL) and the absorbance was read at 532nm in UV-Spectrophotometer Shimadzu® 1601[8].

Determination of the inhibition of lipid peroxidation *in vivo*

The determination of lipid peroxidation index was held in stomachs injured by the administration of absolute ethanol, of animals previously treated with crude ethanolic extract through the quantification of thiobarbituric acid reactive species.

Were used rats (*Rattusnorvegicus,* Wistar) adults, females, weighing between 150g and 300g (Protocol approved by the Ethics Committee of PUCPR,CEUA number 700).The animals (n=8) were fasted for 6 hours, with free access to water. The ethanolic extract of the stem was administered orally (gavage) in doses of 20, 100 and 500 mg/Kg, absolute ethanol (0.5ml) was also administered orally one hour after extract administration. The control group was treated with sodium chloride 0.9% (0.1mL/100 g).After an hour of administration of ethanol, the animals were killed with ketamine and xylasina and stomachs removed, weighed and homogenized with potassium phosphate buffer (200mM, pH 6.5). This homogenate (1500μl) was diluted in potassium chloride (500μl,0.15M). After that, sodium dodecyl sulfate (0.2mL, 8.1% w/v); acetic acid (1,5ml, 20%, pH 3.5) and thiobarbituric acid (1.5mL, 0.8% w/v) were added. The reaction was carried out at 95ºC for 60 minutes. And the organic phase was extracted with butanol (2.0mL) and the absorbance was read at 532nm in UV-Spectrophotometer Shimadzu® 1601. The results were expressed in respect of the amount of protein present in the sample[9,10].

Statistical analyses

Statistical analysis of the results were carried out through the evaluation of variance ANOVA followed by analysis of difference by Tukey test (using the software Statística 5.1).

Results

Identification of phytosterols

The identification of the substances was accomplished through the comparison of NMR data of literature[11].

NMR analysis of the sample, in $CDCl_3$, 200 MHz, showed the mixture of stigmasterol and sitosterol, both classified as phytosterols. The signs in 140.75 and 121.72 indicate the double bond between carbons C-5 and C-6 of both steroids. The double bond between C-22 and C-23, characteristic of stigmasterol, was identified by the signs 138.32 and 129,26. There was even a sign in 71.79, attributed to carbon C-3 of both sterols.

Stigmasterol: ^{13}C NMR (200 MHz, $CDCl_3$) in ppm: 37.23(C–1);31.88 (C–2);71.79 (C–3); 42.28 (C–4);140.75 (C–5);121.72 (C–6);31.88 (C–7);31.88 (C–8);50.11 (C–9);36.49 (C–10);21.06 (C–11);39.75 (C–12);42.28 (C–13);56.75 (C–14);24.29 (C–15);29.23 (C–16);56.03 (C–17);12.23 (C–18);19.38 (C–19);40.48(C–20);21.06(C–21);138.32(C–22);129.26(C–23);51.22 (C–24);31.63 (C–25);21.20 (C–26);19.01(C–27);25.39(C–28);12.23(C–29).

Sitosterol: ^{13}C NMR (200 MHz, $CDCl_3$) in ppm: 37.23 (C–1); 31.88 (C–2); 71.79(C–3);42.28(C–4); 140.75(C–5); 121.72(C–6); 31.63(C–7); 31.63(C–8); 50.11(C–9); 36.49 (C–10); 21.06 (C–11); 39.75 (C–12); 42.28 (C–13); 56.75 (C–14); 24.29 (C–15); 28.23(C–16);56.03 (C–17); 11.84 (C–18); 19.38 (C–19); 36.13 (C–20); 18.73 (C–21); 33.92(C–22); 26.04 (C–23); 45.81 (C–24); 29.12 (C–25); 19.80 (C–26); 19.01 (C–27); 23.04 (C–28); 12.23 (C–29).

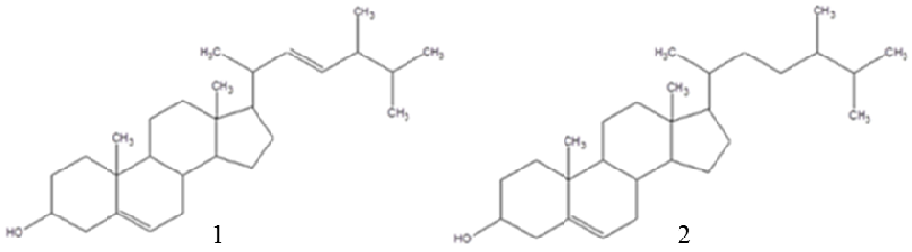

Figure 1.Phytosterols of *Rhamnus sphaerosperma* var. *pubescens*.1. Stigmasterol, 2. Sitosterol.

The identification of substances is necessary for characterization of vegetable, and still aims to establish components that may be responsible for their biological properties.

Antioxidant activity - Phosphomolybdenum Complex

Evaluation of antioxidant activity by phosphomolybdenum complex formation determines the total antioxidant capacity of a sample to an oxidation-reduction reaction[7]. The relative antioxidant activity (RAA) was determined by comparison with the standard rutin(figure2).

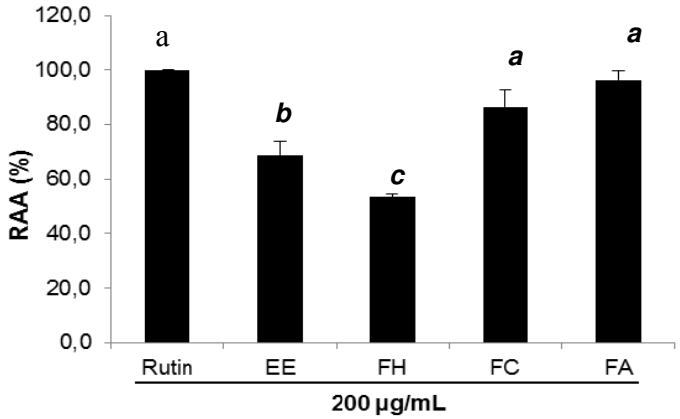

Figure 2.Antioxidant activity relative to rutin. (EE: ethanolic extract of the stem; FH: hexane fraction, FC: chloroform fraction; FA: ethyl acetate fraction). Different letters represent statistically considered for p 0.01.

The crude ethanolic extract of the stem has total antioxidant activity of 68.7% in relation to the activity of rutin, considered 100%. The chloroform fraction (86.4%) and ethyl acetate fraction (96.2%) showed the same activity of the standard, indicating that the purification of the extract with these solvents selects the compounds with the best capacity reduction in this method. On the other hand, the activity of hexane fraction was 53.2%, slightly lower than the EE, indicating that the extraction with hexane eliminates crude extract components that contribute to the antioxidant activity by this method.

Determination of lipid peroxidation inhibition index

This test is used to check the capacity of samples tested at inhibiting lipid peroxidation induced by an oxidizing agent. The results of *in vitro* and *in vivo* experiments are shown in figures 3 and 4, respectively. In testing *in vitro*, chloroform and hexane fractions of the stem demonstrated high capacity in prevention of lipid oxidation, 63.3% and 59.7%, higher standard result (51.1%), although the rate of inhibition of crude extract have been slightly lower (46.3%), both samples tested showed satisfactory results in this experiment.

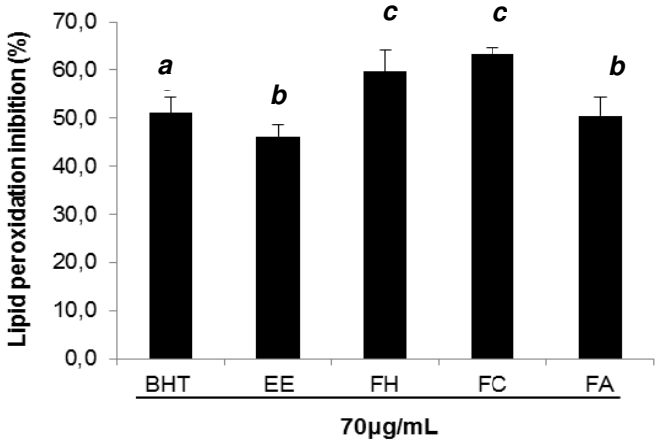

Figure 3. Inhibition of lipid peroxidation *in vitro*. (BHT: hidroxibutiltolueno; EE: crude ethanolic extract of the stem; FH: hexane fraction of the stalk, FC: chloroform fraction of the stem; FA: ethyl acetate fraction of the stem). Different letters indicate significant difference considered for p 0.05.

As the crude extract showed effectiveness in both methods was used in the verification of inhibition of lipoperoxidation *in vivo*. In this assay, the rate of peroxidation was determined by measurement of thiobarbituric acid reactive species formed in stomach injured by ethanol administration.

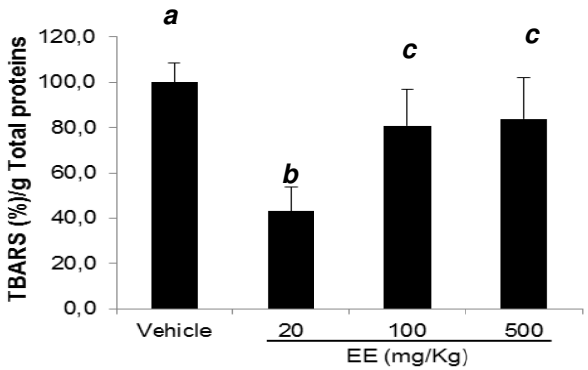

Figure 4. Inhibition of lipid peroxidation in vivo. (Vehicle = group treated with sodium chloride 0.9%; EE = ethanolic extract 20; 100 and 500 mg/Kg). Equal letters indicate equality statistics (statistical difference considered for p 0.05).

The ethanolic extract was tested in three concentrations (Figure 4). The lesser dose tested, 20 mg/Kg, showed better effectiveness in inhibiting lipid oxidation, with reduction of 56.9% compared to the control. The extract in the highest concentrations was not as effective, but also showed ability in preventing lipid peroxidation *in vivo*, both doses, 100 and 500 mg/Kg, with a reduction of 19.2 and 16.1%, respectively, showed a statistically significant difference with control, considered as 100% oxidation.

Discussion

The samples of *Rhamnus sphaerosperma* var. *pubescens* demonstrated an important antioxidant capacity in the methods used, consistent with studies conducted for other species of this genus, as *Rhamnus alaternus* and *Rhamnus nakaharai*, demonstrating its significant antioxidant activity, in various methods of analysis used [3-5].

Evaluation of antioxidant activity by the formation of the phosphomolybdenum complex allows evaluate lipophilic and hydrophilic components and determines the total antioxidant capacity of a sample to an oxidation reduction reaction. This test is based on the reduction of molybdenum in the presence of substances with antioxidant capacity [6]. In determining the total antioxidant activity, the samples showed a similar profile with rutin, a compound belonging to the class of flavonoids, which have significant antioxidant activity associated with several other properties, such as anti-inflammatory and antihyperglycaemic, for example[12]. Some flavonoids with antioxidant activity are described for *Rhamnus* species, like quercetin and kaempferol, found in in *Rhamnus nakaharai* [13]. Thus, the ability to inhibit or prevent oxidative damage can be associated with the treatment and prevention of diseases, especially those who own physiopathology associated with oxidative stress.

The inhibition of lipid peroxidation was evaluated by testing quantification of thiobarbituric acid reactive species formed from peroxidation induced by an oxidizing agent. The method is based on the reaction of malondialdehyde (MDA), formed by oxidation of lipids due to attack by oxidizing agents, with the thiobarbituric acid (TBA), demonstrating, indirectly, the amount of oxidized lipids [14]. In preliminary testing, the chloroform and hexane fractions showed better activity, with higher inhibition rate than BHT, in addition, the ethanolic extract also has activity near the standard, used in the *in vivo* test.

In the animal model of lipid peroxidation, the ethanolic extract showed greater effectiveness at the lowest dose tested in this experiment, the response was not dependent on the dose, since the highest doses were not as effective in reducing lipoperoxidation. This may indicate that the lowest concentration tested in this study already have maximum *in vivo* antioxidant activity of this extract.

Acute or chronic exposure to ethanol increases the production of ROS and RNS, lowers levels of cellular antioxidants and oxidative stress increases in various cells and tissues. The mechanisms by which alcohol cause cell injury is not yet fully defined, however, the role of lipid peroxidation has been explored in the development of oxidative stress and alcohol toxicity [15].

The antioxidant activity of a sample about ROS and RNS depends on factors determined by the chemical structure of the species involved. However, in reactions involving living organisms, many factors influence in the antioxidant activity, as metabolic rates of absorption, distribution and drug retention in tissues, such as the concentration and mobility of the sample in the environment of the reaction and interaction with other antioxidants [2].

Therefore, further studies are still needed to determine the antioxidant mechanism of action for this extract and establish safe doses for this use.

Conclusion

There is no data in the literature about the biological activities of *Rhamnus sphaerosperma* var. *pubescens*, therefore the obtained results stimulate the continuity of this study, aim identify the extract's antioxidant mechanism of action and other related properties of this plant.

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References

[1]. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free RadicBiol Med. 2001; 30(11):1191-212. URL:

http://dx.doi.org/10.1016/S0891-5849(01)00480-4.

[2]. Niki E. Assessment of antioxidant capacity *in vitro* and *in vivo*. Free RadicBiol Med. 2010; 49(4):503-515. URL: http://dx.doi.org/10.1016/j.freeradbiomed.2010.04.016.

[3]. Ammar RB, Sghaier MB, Boubaker J, Bhouri W, Naffeti A, Skandrani I, Bouhlel I, Kilani S, Ghedira K, Chekir-Ghedira L.Antioxidant activity and inhibition of aflatoxin B1-, nifuroxazide-, and sodium azide-induced mutagenicity by extracts from *Rhamnus alaternus* L. ChemBiol Interact. 2008;174(1):1-10. URL: http://dx.doi.org/10.1016/j.cbi.2008.04.006.

[4]. Ammar RB, Neffati A, Skandrani I, Sghaier MB, Bhouri W, Ghedira K, Chekir-Ghedira L. Anti-lipid peroxidation and induction of apoptosis in the erythroleukaemic cell line K562 by extracts from (Tunisian) *Rhamnus alaternus* L. (Rhamnaceae). Nat Prod Res. 2011; 25(11):1047-58.

[5]. Ng LT, Lin CC, Lu CM. Antioxidative effects of 6-Methoxysorigenin and Its Derivatives from *Rhamnus nakaharai.* Chem Pharm Bull. 2007;55(3):382-4. URL: http://dx.doi.org/10.1248/cpb.55.382.

[6]. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999; 269(2):337-41. URL: http://dx.doi.org/10.1006/abio.1999.4019.

[7]. Kalegari M, Miguel MD, Philippsen AF, Dias JFG, Zanin SMW, Lima CP, Miguel OG. Antibacterial, allelopathic and antioxidant activity of extracts and compounds from *Roureainduta* Planch. (Connaraceae).Journal of Applied Pharmaceutical Science. 2012; 2 (09):061-066.

[8]. Morais SM, Catunda-Junior FEA, Silva ARA, Martins-Neto JS, Rondina D, Cardoso JHL. Antioxidant activity of essential oils from Northeastern Brazilian Croton species. Quim Nova. 2006; 29(05):907-910.

[9]. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction.Anal Biochem. 1979; 95(2):351-8.

[10]. Maróstica-Junior MR, Silva TAAR, Franchi GC, Nowill A, Pastore GM, Hyslop S. Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil. Food Chemistry.2009; 116:8–12.

[11]. Kongduang D, Wungsintaweekul J, De-Eknamkul W. Biosynthesis of b-sitosterol and stigmasterol proceeds exclusively via the mevalonate pathway in cell suspensioncultures of *Croton stellatopilosus.* Tetrahedron Lett. 2008; 49(18):4067–4072. URL: http://dx.doi.org/10.1016/j.tetlet.2008.04.049.

[12]. Kamalakkannan N, Prince PS. Antihyperglycaemic and antioxidant effect of rutin, a polyphenolic flavonoid, in streptozotocin-induced diabetic wistar rats. Basic ClinPharmacolToxicol. 2006; 98(1):97-103.

[13]. Wei BL, Lu CM, Tsao LT, Wang JP, Lin CN. *In Vitro* Anti-Inflammatory Effects of Quercetin 3-O-Methyl Ether and Other Constituents from *Rhamnus* Species.Planta Med. 2001; 67(8):745-7.

[14]. Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared with thiobarbituric acid. Anal Biochem. 1997; 245(2):161-6. URL: http://dx.doi.org/10.1006/abio.1996.9990.

[15]. Pan JS, He SZ, Xu HZ, Zhan XJ, Yang XN, Xiao HM, Shi HX, Ren JL. Oxidative stress disturbs energy metabolism of mitochondria in ethanol-induced gastric mucosa injury. World J Gastroenterol. 2008; 14(38):5857-67. URL: http://www.wjgnet.com/1007-9327/14/5857.asp.