Universidade Estadual de Maringá
Centro de Ciências Agrárias
Programa de Pós-graduação em Ciência de Alimentos

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Inibição da lipase pancreática e da absorção intestinal de triacilglicerídeos por um extrato de casca de pinhão (*Araucaria angustifolia*) rico em taninos condensados

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Tese apresentada ao Programa de Pós-graduação em Ciência de Alimentos da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciência de Alimentos.

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BIOGRAFIA

Roselene Ferreira Oliveira, filha de Wilson Ferreira e Idazima Maciel Ferreira, nasceu em 07 de Julho de 1979, na cidade de Pitanga-PR. Em 12 de outubro de 2009, concluiu o curso de graduação em Tecnologia em Processamento de Alimentos vegetais pela Universidade Tecnológica Federal do Paraná-UTFPR-Câmpus Campo Mourão. O trabalho de conclusão de curso foi intitulado “Obtenção de ácido lático a partir de melaço de cana-de-açúcar e farinha de varredura”, orientado pela professora Dra. Mirela Vanin dos Santos Lima.

Durante a graduação, desenvolveu dois projetos de iniciação científica, sendo: 1º projeto: vinculado a Universidade Tecnológica Federal do Paraná – UTFPR, intitulado "Obtenção de ácido lático por fermentação de melaço de cana-de-açúcar e amido hidrolisado e suplementado", concluído no ano de 2008, orientado pela professora Dra Mirela Vanin dos Santos Lima;

2º projeto: vinculado a Universidade Tecnológica Federal do Paraná - UTFPR e Empresa de Biotecnologia - Ltda (Clean-up), intitulada "Desenvolvimento de tecnologia para produção de leitora e indicador biológico de resposta rápida", concluído no ano de 2009, orientado pelo professor Dr. Heron Lima de Oliveira.

Em 28 de fevereiro de 2012, concluiu o Mestrado no Programa de Pós-Graduação em Agronomia, área de concentração em Produção Vegetal, na Universidade Estadual de Maringá, com a dissertação intitulada “Características físico-químicas de goiabas minimamente processadas e armazenadas sob-refrigeração”, sob orientação do professor Dr Edmar Clemente.

Em Fevereiro de 2013, como bolsista DTI-C concluiu o projeto de Bioprospecção de fungos filamentosos produtores de holoenzimas com aplicação em biorrefinaria, sob orientação da professora Dra. Rosane Marina Peralta.

No mês de março de 2013 ingressou no doutorado no Programa de Pós-Graduação em Ciência de Alimentos sob-orientação da professora Dra. Rosane Marina Peralta.

Em abril de 2015 foi nomeada professora do Instituto Federal de Educação, Ciência e Tecnologia de Mato Grosso do Sul, Câmpus-Coxim, para ministrar aulas nos cursos pertencentes ao eixo da Produção Alimentícia.

Tem experiência nas áreas de Ciência e Tecnologia de Alimentos, atuando principalmente nos seguintes temas: Qualidade Pós-colheita, Bioquímica e Química de Alimentos.

Dedico

A Deus, minha família, amigos e a orientadora pelo apoio, força e incentivo.

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APRESENTAÇÃO

Em consonância com as regras do Programa de Pós-graduação em Ciência de Alimentos, esta tese está apresentada na forma de artigos científicos.

ARTIGO 1

Oliveira RO, Gonçalves GA, Dorneles FI, Koehnlein EA, Souza CGM, Bracht A, Peralta RM. Inhibition of pancreatic lipase and triacylglycerol intestinal absorption by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. Nutrients 2015, 7, 5601-5614; doi:10.3390/nu7075242. Fator de impacto JCR: 3,270

ARTIGO 2

Oliveira RO, Koehnlein EA, Kato CG, Nishida VS, Bracht A, Peralta RM. Biological activities and chemical constituents of *Araucaria angustifolia* - a review. Submetido ao periódico Journal of the Science of Food and Agriculture. Fator de impacto JCR: 1,714

GENERAL ABSTRACT

INTRODUCTION AND AIMS – Araucaria is a genus of evergreen coniferous trees in the family Araucariaceae. The genus Araucaria includes approximately nineteen species, all confined to the Southern Hemisphere. Two species occur in South America, *Araucaria angustifolia* and *Araucaria araucana*. *A. angustifolia* covers areas of the South and South East of Brazil and North East of Argentine. *A. araucana* is restricted to high mountain in the South of Argentina and Chile. Their seeds have been consumed from prehistoric times until today cooked in water, baked or as raw flour in regional dishes such as farofa, meat, rice, pancake, soup, gnocchi, mashed, cake, paçoca in Southern Brazil, Argentina and Chile. The aim of the first article work was to review the available literature on the chemical constituents of the seeds, seed coats and leaves of *A. angustifolia*, in addition to their nutritional and functional properties. This can be justified by the historical and cultural importance of both the *A. angustifolia* tree and its seeds (pinhão) and its largely unexplored potential for expanding production and marketing. The coat of the cooked or raw pinhão is usually discarded into the environment. It is estimated that approximately 10 tons of pinhão coats are discarded annually. As this coat takes a long time to decompose, several investigations have analyzed possible uses for it. Extracts of *A. angustifolia* seed coat rich in condensed tannin strongly inhibited both human salivary and porcine pancreatic α-amylase. The purpose of the second article was to characterize the possible inhibition of pancreatic lipase by a tannin rich extract obtained from the pinhão (*Araucaria angustifolia* seed) coat, based on the previous observation that this preparation inhibits α-amylases.

METHODS – Pinhão seeds used in this study were purchased in a local market (Maringá, PR, Brazil). The seeds used in this work were washed with tap water and dried at room temperature for 24 h. The coats of the seeds were removed and dried at 40 °C until constant weight. After drying, the seed coats were milled in to a fine powder. The powder was mixed with 70% ethanol (in water) at room temperature and maintained under agitation at 140 rpm for 3 h. Alcohol was eliminated using a rotary vacuum evaporator at 40 °C and the remaning solution subsequently was freeze-dried. The porcine pancreatic lipase was assayed using *p*-nitrophenyl-palmitate as the substrate. One enzyme unit was defined as 1 µmol of *p*-nitrofenol enzymatically released from the substrate per minute per mL. The intestinal absorption of triglycerides was tested by means of an oral olive oil tolerance test in mice. The mice were deprived of food for 18 h before the experiment. Pinhão coat extract solutions were administered orally with doses of 100, 250 and 500 mg per kg body weight. Olive oil was subsequently administered orally (5 mL per kg body weight). Before and at 1.5, 3.0, 4.5 and 6.0 h after this olive oil administration (or distilled water for the controls) blood samples from the tail vein were analyzed by means of an Accutrend Plus Roche triglycerides meter. Seven groups of mice (n = 3 per group) were utilized: 1) the positive control, only intragastric olive oil (5 mL per kg) administration; 2) the negative control, only tap water administration; 3) intragastric administration of olive oil plus orlistat (50 mg/kg); 4) intragastric administration of olive oil plus A. mearnsii tannin (500 mg/ kg); 5) intragastric administration of olive oil plus 100 mg/kg pinhão extract; 6) intragastric administration of olive oil plus 250 mg/Kg pinhão extract; 7) intragastric administration of olive oil plus 500 mg/Kg pinhão extract. Statistical analysis of the data was done by means of the Statistica program (Statsoft, 1998). Fitting of the rate

equations to the experimental initial rates was done by means of an iterative non-linear least-squares procedure using the Scientist software from MicroMath Scientific Software (Salt Lake City, UT). The procedure requires the introduction of preliminary estimates of each parameter. These preliminary estimates are improved by each successive iteration in which the squared difference between the calculated and experimental data is progressively diminished until it converges towards a minimum. The decision about the most adequate model (equation) was based on the model selection criterion (MSC) and on the standard deviations of the optimized parameters.

RESULTS AND DISCUSSION – Kinetic measurements of pancreatic lipase revealed that the pinhão coat tannins is an effective inhibitor. The inhibition was of the parabolic non-competitive type. The inhibition constants were equal to 332.7±146.1 and 321.2±93.0 µg/mL, respectively, corresponding roughly to the inhibitor concentration producing 50% inhibition ($[I]_{50}$). The pinhão extract was also effective in diminishing the plasma triglyceride levels in mice after an olive oil load; 50% diminution of the area under the plasma concentration versus time curve occurred at a dose of 250 mg/kg.

CONCLUSIONS - The results obtained in the present study revealed that the pinhão coat tannin is an effective inhibitor of pancreatic lipase. Consistently, it was also effective in diminishing the plasma triglyceride levels in mice after a load of olive oil. This is probably the consequence of an indirect inhibition of triglyceride absorption via inhibition of pancreatic lipase. For the pinhão coat tannin this is the second report of biological activity, the first one being a similar inhibition of the absorption of glucose derived from starch in consequence of an inhibitory action on alpha-amylase. All these actions are compatible with a potential anti-obesity action, as suggested for other polyphenol or tannin rich preparations.

KEY-WORS: *Araucaria angustifolia*, enzyme, lipase, obesity, pinhão, tannin.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – Araucária é um gênero de coníferas verdes da família Araucariaceae. O gênero Araucária inclui cerca de dezenove espécies, todas confinadas no Hemisfério Sul. Duas espécies ocorrem na América do Sul, *Araucária angustifolia* e *Araucária araucana*. *A. angustifolia* abrange áreas do Sul e do Sudeste do Brasil e Nordeste da Argentina. *A. araucana* é restrita a regiões montanhosas no sul da Argentina e Chile. Suas sementes são consumidas no sul do Brasil, Argentina e Chile desde os tempos pré-históricos até hoje cozidas em água, assadas ou na forma de farinha em pratos regionais, como farofa, carne, arroz, panqueca, sopa, nhoque, purê, bolo e paçoca. O objetivo do primeiro trabalho foi revisar a literatura disponível sobre os componentes químicos das sementes, casca das sementes e folhas da *A. angustifolia*, além das suas propriedades nutricionais e funcionais. Assim pode ser justificado pela importância histórica e cultural, tanto da árvore de *A. angustifolia* e sua semente (pinhão) quanto para expandir a sua produção e comercialização. A casca do pinhão cozida ou crua geralmente é descartada no meio ambiente. Estima-se que cerca de 10 toneladas de casca de pinhão são descartadas anualmente. Como esta casca leva muito tempo para se decompor, várias estudos buscam possíveis usos para ela. Em um estudo prévio, extratos de casca da semente de *A. angustifolia* rico em taninos condensados inibiram fortemente tanto a α-amilase salivar humana quanto à pancreática de porco. Desta forma, o objetivo do segundo artigo foi caracterizar a possível inibição da lipase pancreática pelo extrato rico em tanino obtido a partir da casca do pinhão (semente *Araucaria angustifolia*).

MÉTODOS – As sementes de pinhão utilizadas neste estudo foram adquiridas em mercado local (Maringá, PR, Brasil). As sementes foram lavadas com água da torneira e posteriormente secas em temperatura ambiente durante 24 h. As cascas das sementes foram removidas e secas a 40 °C até peso constante. Após a secagem, as cascas das sementes foram moídas até obtenção de um pó fino. A extração foi feita com etanol a 70% (em água) à temperatura ambiente sob agitação de 140 rpm por 3 horas. A extração foi repetida três vezes. O álcool foi eliminado usando um evaporador a vácuo rotativo a 40 °C e depois o extrato aquoso foi liofilizado. A atividade da lipase pancreática de porco foi testada utilizando *p*-nitrofenil palmitato como substrato. Uma unidade de enzima foi definida como um nmol de *p*-nitrofenol liberado enzimaticamente do substrato por minuto por mL. A absorção intestinal de triglicerídeos foi testada por meio de um teste oral de tolerância ao azeite de oliva em camundongos. Os camundongos foram privados de alimentos durante 18 h antes do experimento. Soluções de extrato da casca de Pinhão foram administrados por via oral em doses de 100, 250 e 500 mg por kg de peso corporal. O azeite foi subsequentemente administrado por via oral (5 mL por kg de peso corporal). No tempo zero e após 1,5, 3,0, 4,5 e 6,0 h da administração azeite (ou água destilada para os controles) amostras de sangue a partir da veia da cauda foram analisados por meio de um medidor de triglicerídeos *Accutrend plus Roche*. Foram utilizados sete grupos de camundongos (n = 3 por grupo): 1) controle positivo, onde administrou-se intragastricamente apenas o azeite (5 mL por kg); 2) o controle negativo, onde administrou-se apenas água; 3) grupo teste orlistat onde administrou-se intragastricamente azeite mais orlistat (50 mg / kg); 4) grupo onde administrou-se intragastricamente azeite mais tanino de *Acacia mearnsii* (500 mg/kg); 5) grupo teste onde administrou-se azeite mais 100 mg/kg de extrato de pinhão; 6) grupo teste onde administrou-se intragastricamente azeite mais 250 mg/Kg de extrato de pinhão; 7) grupo

teste onde administrou-se intragastricamente azeite mais 500 mg/Kg de extrato de pinhão. A análise estatística dos dados foi feita por meio do programa Statistica (Statsoft, 1998). As análises das velocidades iniciais foi realizada utilizando procedimento não linear iterativo de mínimos quadrados utilizando o software Scientist da MicroMath Scientific Software (Salt Lake City, UT). O procedimento exige a introdução de estimativas preliminares de cada parâmetro. Estas estimativas preliminares são melhoradas por cada iteração sucessiva em que a diferença de quadrados entre os dados calculados e experimentais é progressivamente diminuída até que converge para um mínimo. A decisão sobre o modelo mais adequado (equação) foi baseada no critério de seleção do modelo (MSC) e os desvios-padrão dos parâmetros otimizados.

RESULTADOS E DISCUSSÃO – As medidas cinéticas da lipase pancreática revelaram que o tanino da casca do pinhão é um inibidor eficaz. A inibição foi do tipo parabólica e não competitivo. As constantes de inibição foram igual a 332,7 ± 146,1 321,2 ± 93,0 e ug / ml, respectivamente, que corresponde aproximadamente à concentração de inibidor produzindo 50% de inibição ($[I]_{50}$). O extrato pinhão também foi eficaz em diminuir os níveis de triglicerídeos no plasma em camundongos após uma carga de azeite; 50% de diminuição da área sob a curva de concentração plasmática versus tempo ocorreu com uma dose de 250 mg/kg.

CONCLUSÕES – Os resultados obtidos no presente estudo revelaram que o tanino presente na casca do pinhão é um inibidor eficaz da lipase pancreática. Consistentemente, foi também eficaz na redução dos níveis de triglicerídeos do plasma em camundongos após uma carga de azeite. Isto é muito provavelmente a consequência de uma inibição indireta de absorção de triglicerídeos através da inibição da lipase pancreática. Para os taninos da casca do Pinhão este é o segundo relatório de uma atividade biológica, sendo o primeiro uma inibição semelhante da absorção de glicose derivada de amido em consequência de uma ação inibitória sobre a alfa-amilase. Todas estas ações são compatíveis com uma potencial ação anti-obesidade, como sugerido por outras preparações ricas em poli fenóis ou taninos.

PALAVRAS-CHAVES: *Araucaria angustifolia*, enzima, lipase, obesidade, pinhão, tanino.

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Biological activities and chemical constituents of *Araucaria angustifolia* — a review

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Abstract

Araucaria angustifolia (Bert.) O. Kuntze (*A. brasiliensis*), known as Paraná pine, is a conifer native to Southern Brazil. Its seeds, known as pinhão, have been consumed from prehistoric times until today cooked in water, baked or as raw flour in regional dishes in Southern Brazil and Argentina. They are very rich in amylaceous material, but also contain fibers, organic acids, lipids, oligosaccharides, lectins, phenolics, among other components. The seed coats are rich in proanthocyanidins and present antioxidant activity as well as inhibitory activity against amylases and lipases. The *A. angustifolia* leaves contain proanthocyanidins and bioflavonoids with several biological activities. This work presents details about the chemical composition of the various parts of the plant. Emphasis is given to the nutritional and functional properties of the seed.

Key words: *Araucaria angustifolia*, chemical composition, nutritional composition, bioactive compounds.

INTRODUCTION

Araucaria is a genus of evergreen coniferous trees in the family Araucariaceae. The genus *Araucaria* includes approximately nineteen species, all confined to the Southern Hemisphere. Two species occur in South America, *Araucaria angustifolia* and *Araucaria araucana*. *A. angustifolia* covers areas of the South and South East of

Brazil and North East of Argentina.[1] *A. araucana* is restricted to high mountains in the South of Argentine and Chile.[2] Their seeds have been consumed from prehistoric times until today cooked in water, baked or as raw flour in regional dishes such as farofa, meat, rice, pancake, soup, gnocchi, mashed, cake and paçoca in Southern Brazil, Argentina and Chile.

The aim of the present work was to review the available literature on the chemical constituents of the edible part of the seeds, seed coats and leaves of *A. angustifolia*, in addition to their nutritional and functional properties. This purpose can be justified by the historical and cultural importance of both the *A. angustifolia* tree and its seed (pinhão) and its largely unexplored potential for expanding production and marketing. When pertinent, a comparison with *Araucaria araucana* will be presented.

Araucaria angustifolia TREE

The conifer *Araucaria angustifolia* (Bert.) O. Kuntze (*Araucaria brasiliense*), popularly known as Paraná pine, Brazilian pine or simply "Araucaria", is the sole native gymnosperm of the Atlantic forest in Brazil and has great economic, cultural and social importance.[3,4] Originally, the natural forests of Araucaria occupied 185,000 km² in Brazil.[5,6] Timber exploration reduced dramatically this tree and nowadays only 2-4% of the original population still exists.[5] Due to this situation and the associated risks of extinction, the cultivation of the species has received strong encouragement from governmental agencies related to environment and agriculture.

With respect to morphology, *A. angustifolia* is characterized as a tall tree of 20-50 meters height, with a straight trunk 90-180 cm in diameter (Figure 1A). When young, the plant canopy is cone-shaped and, as it reaches adulthood, the canopy achieves the goblet shape due to the natural loss of the lower branches. Their acicula (leaves) are leathery, glabrous, acute-pungent and 3 to 6 cm in length.[6] *A. angustifolia* is a dioecious plant that has separate male and female trees, with pollination required for seed production. Pollination occurs from October to December, mainly by wind and the ripening of the pinecones takes place two years later. In natural populations, seed production usually occurs after 15 to 20 years of age, each tree producing annually from 40 to 200 cones.[7]

As will be described in detail in the next sections, the uses of the *Araucaria angustifolia* seed are diverse, ranging from the almond inside to the coat. The almond is consumed by different species of animals, especially rodents, as well as by humans and

has a high nutritional value. Indians of Southern Brazil (Caingang and Guarani) are used to eat pinhão since prehistoric times. Especially during winter, cooked or in the form of flour, the *A. angustifolia* seed often becomes the most important food for survival.[8] Nonwithstanding, information about the chemical composition and nutritional properties of the pinhão are still incomplete. Moreover, the use of the pinhão in the Brazilian cuisine is far from being widespread and intense due to the lack of methods for preserving it fresh and for industrial processing. Thus, techniques for conservation and sustainable use have been investigated to encourage its preservation and marketing during other periods of the year out of the production season. A more attractive market would encourage extraction and marketing by rural producers, promote cultivation and automatically discourage illegal logging.[9-11]

Besides the nutritional aspects, different parts of *A. angustifolia* are used in Brazilian folk medicine.[12] Tinctures extracted from the nodes are traditionally used orally or topically for the treatment of rheumatism and infusions of the nodes are used orally for the treatment of kidney diseases and sexually transmitted diseases. Infusions of the bark are used topically to treat muscular tensions and varicose veins, while the syrup produced with the resin is used for the treatment of respiratory infections. Infusions of the leaves are used to treat scrofula, fatigue and anemia and dyes are used for the treatment of wounds and herpes.[4,13]

THE PINHÃO, GENERAL INFORMATION

The flowering of *A. angustifolia* produces a set of seeds, which is called pine cone (Figure 1B). Each of these has a diameter of 10 to 25 cm and contains approximately 700-1200 scales with about 150 seeds with a weight ranging from 0.61 to 4.1 kg (Figure 1C).[14] In a pinecone of 2.3 kg (weight average) there are about 0.8 kg seeds (*pinhão*, plural *pinhões*). The edible part of the pinhão (almond) consists of a starchy mass with tougher texture when raw and soft after cooking. At its center, there is a filament of about 4/5 length occupied by the embryo, called filiform. The edible part is covered by two structures, an inner membrane firmly attached to the almond and an outer coat, highly resistant. Both the inner membrane and the outer coat have colors ranging from yellow to dark red and are removed after cooking.[15] The pinhão is about 3 to 8 cm in length, from 1 to 2.5 cm wide and weights in average 8.7 g (Figure 1C).

In Brazil, the pinhões are found in greater amounts from April to June.[9] For consumption the seeds are usually cooked in water or roasted. Roasting is usually done

on embers, and the procedure is usually known as *sapecada*. In addition to these forms of preparation, flours of raw or cooked pinhão are used in the preparation of regional dishes, cakes, breads and cookies. In regions where the *A. angustifolia* tree occurs, a common practice is the cooking in water, followed by preservation in salt and vinegar[15] (Figure 1D).

APPROXIMATE COMPOSITION AND NUTRITIONAL ASPECTS OF PINHÃO

The particular composition of each pinhão almond can result from variations in the stage of development, temperature and irrigation techniques or simply reflect genetic characteristics.[5] The accumulation of nutrients occurs during the dehydration of the seeds in the final stages of ripening, generally during the months from April to May, when the protein content increases. Table 1 shows the approximate composition of raw the seeds (almond) of *A. angustifolia* and *A. araucana*. The edible part of the seeds contains about 50% moisture. Starch is the main component, comprising around 31-36%. The content of lipids is very low, around 1.1-1.3%. Concerning fatty acids, 43% of them have a saturated chain, mainly palmitic acid (25%), followed by stearic, arachidic and behenic acids. Monounsaturated fatty acids comprise 14% (mainly oleic acid) and polyunsaturated fatty acids 29% (mainly linoleic acid).[16]

The *A. angustifolia* seeds have less protein than the *A. araucana* seeds, 3.57 and 7.80%, respectively. The amino acid composition of the proteins in the *A. angustifolia* seeds has already been determined.[15] After milling, the pinhão flour was dried at two temperatures, 50 and 80^{o} C. Both preparations are rich in glutamate and aspartate residues and contain minor amounts of histidine and cysteine (Table 2). Although the *A. angustifolia* seeds are considered essentially a source of starch, the pinhão flour is comparable to other protein sources used in the human diet: the amino acid composition resembles that observed for cereals, such as wheat and corn and is comparable to those of legumes, which contain lysine and histidine as limiting amino acids.[17] Additionally, the contents of phenylalanine, tryptophan and valine found the in pinhão flour were similar to those of casein and also presented higher contents of valine and methionine than soybean. For this reason, the pinhão flour was considered as an adequate substitute for up to 20% casein as a complementary source of protein in diets for growing rats.[15] The presence of trypsin inhibitor activity in the flour, particularly lectin (see further), limited the use of higher proportions of pinhão flour in the diet.

Concerning free amino acids, aspartic acid and glutamic acid are the most abundant in the mature seed.[5]

The content of soluble sugars in the *A. angustifolia* seed is relatively low (2.43%), especially after cooking (0.64%).[8] Glucose is the most common sugar (2.25%), followed by fructose (0.07%) and sucrose (0.11%). Upon cooking, 75% glucose, 55% fructose and 55% sucrose are lost. In *A. araucana* seeds the soluble sugar content is about 3 times greater (7.1%) than in *A. angustifolia* seeds.[18]

The *A. angustifolia* seed can be considered a good source of dietary fiber, comparable to legumes and vegetables, the content ranging from 5.7% to 17% after cooking.[8] The content is slightly lower in the raw seed. The higher fiber content is represented by insoluble fiber, which is greater in the cooked seed (5.17% vs. 4.26% in the raw seed), possibly due to the high amylose content of the pinhão starch. The latter can be transformed into resistant starch after cooking.[8] The fiber content of the seed of *A. araucana* (25.43%) is higher than that of *A. angustifolia*, but there is also a predominance of insoluble fiber.[18]

Table 3 shows the micronutrients evaluated in the edible part of raw and cooked *A. angustifolia* seeds. They can be considered a good source of magnesium and copper, and cooking with the coat protects them against mineral loss.[8]

PROPERTIES OF THE PINHÃO STARCH

Approximately 35% of the pinhão almond consists of starch, averaging 30% amylose in the raw seed.[19-21] Studies characterizing the pinhão starch concluded that its isolation is quite simple and that it is stable for a year at room temperature without changing color and flavor provided that the seeds are thoroughly cleaned.[19] The yield of the pinhão starch isolation was the same using fresh and frozen seeds (freezing may be required, because the pinhões are harvested once a year), namely 70%. Even when the content of proteins was low, there was no difficulty in separating them from starch. Low contamination by proteins is an important characteristic of starch preparations, since the technology used in the food industry has to be adapted to the content and type of protein. This characteristic suggests an advantageous use of the pinhão starch in the production of glucose and fructose syrups.

Compared to corn starch the pinhão starch has lower temperature and enthalpy for gelatinization, lower retrogradation degree and greater water absorption capacity, solubility and viscosity at low temperatures, and shows higher susceptibility to

amylolytic attack.[22] These properties result in advantages such as a soft texture, the possibility of being stored for long periods and the perspective of developing new products with thermolabile ingredients.[11,19,23]

Stahl et al.[11] evaluated the physical and chemical properties of phosphorylated pinhão starch in comparison with those of the phosphorylated corn starch. Phosphorylated starches are prepared by chemical methods in order to obtain clear pastes with greater consistency, freeze-thawing stability and high capacity of absorbing water (swelling), which can be used as stabilizers in foods such as fermented milk and ice cream. They can also be used as texture improvers and for water retention in cheese. It has been found that the native pinhão starch, when compared to native corn starch, presents a higher swelling power, increased solubility, reduced syneresis and a delayed loss of clarity of the paste during storage at 5°C. Phosphorylation of the pinhão starch yielded a starch phosphate with substitution levels of low, medium and high degrees (0.015, 0.07 and 0.14, respectively) similarly to corn starch (0.015, 0.07 and 0.12, respectively). For both, corn and pinhão starch, medium and high degrees of phosphorylation increased the cold water absorption capacity and clarity of the paste, decreased syneresis and induced a loss of birefringence to similar extents. The increase in swelling power and solubility caused by phosphorylation was more pronounced in corn starch than in the pinhão starch. The phosphorylated starch of low degree of substitution presented itself as a clear paste with swelling power and capacity of absorbing cold water similar to native starches, whereas phosphorylated starches of medium and high degrees of substitution were soluble without heating and formed pastes resistant to retrogradation.

Conforti and Lupano[20,21] compared the starch of seeds of *A. angustifolia* and *A. araucana* (Table 4). The amylose content of the starch of *A. angustifolia* (22.4%) is greater than that of *A. araucana* (17.3%), but similar to that of potato and corn starch. The authors reported that starch granules of both species are round or slightly oval, with a central hilum, and the gelatinization temperature of the *A. angustifolia* starch is higher than that of *A. araucana* starch, due to the higher amylose content of the former. The average size of the granules of *A. angustifolia* starch (12.2 μm) was larger than those of *A. araucana* starch (8.4 μm), which also presents more heterogeneous granules.

The most difficult step of the starch isolation process from the pinhão seed is the removal of the outer coat and especially the inner one, which is adhered to the almond and contains a high amount of phenolic compounds (345 mg catechin

equivalents/g fresh weight).[8,19] The incomplete removal of the inner coat can result in a starch with undesirable color due to the action of polyphenoloxidases (PPO), which use the phenolic compounds as substrates and are responsible for enzymatic browning. Dairot et al.[24] investigated the properties and characteristics of the polyphenoloxidase of the *A. angustifolia* seed in order to enable control of enzyme activity during the production of starch and other products that use raw seeds. The authors observed that this polyphenoloxidase showed optimal activity at pH 5.0, kept 46% activity at pH 4.0 and 72-63% at pH 6-8. In relation to temperature, the polyphenoloxidase showed optimal activity in the 30-35°C range when using catechol as the substrate. At 40 and 50°C, the polyphenoloxidase maintained 90 and 53% activity, respectively, suggesting a moderate thermal stability for the enzyme. Complete inactivation of polyphenoloxidase was found after boiling for 5 minutes. The study of how different compounds affect the activity of polyphenoloxidase revealed a diversity of effects: $MgCl_2$, $MgSO_4$ and $NiCl_2$ caused moderate inhibition, $AlCl_3$ and $ZnSO_4$ triggered stimulation, and $CaCl_2$ and $CuSO_4$ showed negligible effects. The addition of NaCl did not affect the activity of the polyphenoloxidase and the use of beta-mercaptoethanol, sodium metabisulfite and cysteine at the commonly used concentrations inhibited the enzyme. The chelating agent EDTA (ethylenediaminetetraacetic acid) caused a slight diminution of the activity, and the anionic detergent SDS (sodium dodecyl sulfate) completely inhibited the enzyme.

NEW TECHNOLOGIES FOR THE USE OF THE PINHÃO SEED

The flour of the edible part of the pinhão can be regarded as a new technological option in terms of raw material utilization and as a nutritional source for possible formulations of food products, including gluten-free breads[25] and as coating for β-carotene microencapsulation by freeze-drying.[26] No doubt that such products will add substantial value to the seed of *A. angustifolia*. However, due to the recalcitrant characteristics of the seeds, the viability of their effective use can get compromised by the drying processes such as those used with corn, rice, beans and others.[27] In this way, many studies have looked for storage alternatives aiming the pinhão conservation. Capella et al.[28] analyzed the chemical composition of the pinhão flour with respect to the conditions of pretreatment and dehydration as a technological option for food products. They observed that the drying time at 65°C required to achieve the moisture standards recommended by the Brazilian Sanitary Surveillance Agency (ANVISA) was approximately 5 hours for both raw and cooked seeds. The flour showed distinct

characteristics with regard to color, since the raw pinhão flour resembled the common wheat flour in relation to color and texture and the cooked pinhão flour showed a yellowish color and a higher density due to the incorporation of phenolic compounds of the coat and water adsorption. Drying caused losses with significant differences in most constituents as revealed by comparisons of the contents of cooked and raw seeds with their respective flours.[28] Nevertheless, the flour proved to be a good source of fibers (cooked 5.11% and raw 6.45%), proteins (3.41% and 3.07%) and lipids (5.14% and 6.39%). In some aspects, as highlighted by Capella et al. (25), the pinhão flours can be compared favorably to the wheat flour (2.7% fibers) and corn flour (3.83% lipids).

Some studies indicate that the pinhão starch is darker than that of corn and wheat due to the oxidation of phenolic compounds and the presence of phosphate, but even so, the use of the pinhão flour has been the object of various investigations aiming its use in the formulation of bakery products and others with promising results. Bezerra et al.[29] examined breads with different proportions of pinhão flour (0%, 5%, 10% and 25%) for their physico-chemical and sensory properties. Acceptance testing of the breads was performed with 38 untrained consumers, recruited among students and staff of the university in which the study was conducted. The authors used a 9 point-hedonic scale to evaluate the samples according to the flavor. The bread prepared with 5% pinhão flour showed a volume similar to the standard, unlike the breads with 10% and 25% that had a slightly smaller volume than the control. Furthermore, the authors found that the breads showed a compact structure, not crispy, of harder texture, small alveoli and a slightly yellow crumb. With respect to the preference of the panelists for the various breads in the sensory analysis no significant differences were detected. The physical and chemical analysis found that the bread with 25% pinhão flour presented a 0.3% fiber content, 10.17% proteins, 1.081 mg ascorbic acid, 1.85% ashes and 66.09% carbohydrates.

Helm et al.[30] developed two tagliatelle pasta formulations with 25% and 37.5% pinhão flour replacing wheat flour and subjected them to sensory analysis to assess attributes such as color, tenderness, flavor and odor. According to the authors, the formulations showed the characteristic flavor and aroma of pinhão, being appetizing, good looking, with good color, taste and odor. The time required for cooking the pasta was eight minutes. The authors suggested freezing as the method most suitable for marketing. The shelf life of the product was evaluated during a period of eight weeks, when changes were not observed in its characteristics. In their conclusions, the authors

highlighted that the final product experienced minimal organoleptic changes and a considerable increase in the protein content compared to the pasta without addition of pinhão flour.

Acorsi et al.[31] studied the feasibility of producing biscuits with 5, 10 and 20% pinhão flour (percentage of the total flour) in comparison to the control biscuit (no additions). The formulations were supplemented with wheat flour in inverse proportion to the added pinhão flour (100, 95, 90 and 80%), sugar (73 g/100 g), corn starch (45 g/100 g), fat (36 g/100 g), egg (34 g/100 g), grated coconut (9 g/100 g), baking powder (9 g/100 g) and milk (8 g/100 g). The chemical composition of the pinhão flour used by the authors contained 13.78% moisture, 79.12% carbohydrates, 2.14% ashes, 6.14% proteins and 0.9% fibers. Samples of biscuits with pinhão flour were subjected to acceptance testing using a hedonic scale. The samples used had three days of manufacture. According to the authors, the volumes of the biscuits with addition of pinhão flour and of those without pinhão flour were similar. The biscuits had a compact structure, except for the sample containing 10% pinhão flour, which had a crumbly structure. Compared with the control they had a coarser texture and a darker color. The flavor was considered pleasant by the tasters, especially the biscuit with 10% pinhão flour and no significant difference in acceptance between the control and pinhão flour supplemented biscuits was observed.

PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY

During cooking colored compounds present in the internal and external coats migrate not only to the water but also to the surface of the edible part of seed (almond).[8] This is revealed by both the astringent flavor and the brown color of both water and almond, the latter showing the brown color basically on its surface. By establishing a comparison with other commonly consumed foods, it is possible to observe that although the phenolic content is lower than that of oil seeds, which have a lipid content much higher than the seed, there are similarities with carbohydrate-rich foods, such as baked beans and potatoes, for example, as can be seen in Table 5. Both the raw and cooked pinhão almonds revealed to contain significant amounts of catechin, more specifically 17.5 mg/100 g seed (edible part) and 21.1 mg/100 g seed (edible part), respectively. The amounts of catechin in the pinhão seed are comparable to those found in catechin rich foods such as raw apples (9.0 mg/100 g), apricots (11.0 mg/100 g), grapes (17.6 mg/100 g) and blackberries (18.7 mg/100 g).[32,33] Gallic acid and quercetin

have also been identified. The raw almond contains 0.36 mg/100 g seed whereas in the cooked almond the content increases to 0.82 mg/100 g seed. The quercetin content also increases upon cooking, from 0.07 mg/100 g seed to 0.7 mg/100 g seed. The contents of protoanthocyanidins in the seed improved from 22.50 mg/100 g of seed to 2,035.00 mg/100 g seed after cooking. It is clear that cooking promotes migration of phenolics from the coat to the almond.[34] This migration is certainly responsible for the improvement in the antioxidant activity of seeds after cooking (Table 6).[34]

LECTINS

Lectins are a special group of proteins of non-immune origin able to agglutinate cells and precipitate glycoconjugates because of their property of binding reversibly to carbohydrates.[35] They exhibit a wide range of biological properties not yet fully elucidated. Datta et al.[36,37] purified two lectins from the pinhão seed with high binding capacity. A purified N-acetyl-D-glucosamine lectin specific of the pinhão seed presents agglutinating activity against rabbit erythrocytes.[35] It also presents significant antimicrobial activity against mainly Gram-negative bacteria due to its ability to form complexes with microbial glycoconjugates. The same authors also reported an action of the pinhão lectin against cellular acute inflammation when administered intravenously to rats with peritonitis and paw edema. Administration of 0.01, 0.1 and 1 mg/kg of the pinhão lectin significantly reduced the paw edema in a dose-dependent mode. This effect was suggested to involve a lectin domain, since incubation of the lectin with its ligand N-acetyl-D-glucosamine prevented its antiedematogenic activity, an action that was not observed upon incubation with mannose. In the carrageenan-induced peritonitis model, the pinhão lectin injected intravenously at the doses of 0.1 and 1 mg/kg inhibited neutrophil migration by 69% and 92%.

Mota et al.[38] studied the effects of a lectin from *A. angustifolia* seeds in a rat paw edema model and observed that intravenous injection of lectin (0.1-1 mg/kg) dose-dependently inhibited the increase in vascular permeability and edema induced by dextran. This action was due to the association of the lectin with the N-acetyl-glucosamine (Glyc-NAc) binding domain. The pinhão lectin also significantly inhibited the edema induced by serotonin (18%) and the polymer produced by the condensation of N-methyl-p-methoxyphenethylamine with formaldehyde (compound 48/80; (33%), but not the edema induced by histamine. In contrast, when applied via the subcutaneous route, lectin caused paw edema 1 hour later which was partially reversed by association

with Glyc-NAc (59%) or by prior intravenous administration of lectin (38.8%). This edematogenic activity of the pinhão lectin was significantly inhibited by pentoxifylline (44.4%) or dexamethasone (51%), but not by L-N-nitro-arginine or indomethacin, excluding the involvement of nitric oxide and prostaglandins.

The treatment of animals with the pinhão lectin as the sole anti-inflammatory agent (1 mg/kg IV) for 7 days did not affect the body weight of rats, liver, kidney, spleen and stomach, blood leukocyte count, urea, creatinine, or serum transaminase activity. Systemic toxicity was evident only with the administration of doses much higher (88.3mg/Kg) than those required for an anti-inflammatory action. Thus, the pinhão lectin exerts anti- and pro-edematogenic actions by interactions with its specific domain. These actions may share a common pathway involving both the activation and inhibition of inflammatory mediators of resident mastocytes.[38]

Vasconcelos et al.[39] studied the action of the pinhão lectin on the central nervous system of rats. The authors administered intraperitoneally lectin (at 0.1, 1 and 10 mg/kg) or saline (control) to male mice 30 minutes before administration of pentylenetetrazol (85 mg/kg, intraperitoneal injection), pilocarpine (400 mg/kg, subcutaneous injection) or strychnine (75 mg/kg, intraperitoneal injection). The following parameters were evaluated: latency of the first seizure or death, the percentage of convulsing animals and the percentage of surviving animals. Additionally the authors performed the open field test by administering intraperitoneally lectin (at concentrations of 0.1, 1 and 10 mg/kg), saline (control), diazepam (1 mg/kg) or flumazenil (1mg/kg). The authors concluded that the lectin of *A. angustifolia* exerts a depressant activity on the central nervous system, acting via a GABAergic mechanism. This conclusion is supported by the observations that the pinhão lectin was able to increase the latency for convulsions and death in models induced by pentylenetetrazol and strychnine and to reduce dose-dependently the locomotor activity in the field test, using diazepam as a positive control. The latter was reversed by pretreatment with flumazenil. These findings are interesting because they provide opportunities for the development of drugs with central depressant activity.

THE PINHÃO COAT

The coat of the cooked or raw pinhão is usually discarded into the environment. It is estimated that approximately 10 tons of pinhão coats are discarded annually.[40] As

this coat takes a long time to decompose, several investigations have analyzed possible uses for it.

Several authors[14,41-44] studied the use of the pinhão coat as an alternative to the adsorption of metal ions and dyes, potentially carcinogenic, from aqueous solutions in the treatment of industrial effluents. All studies argue that the pinhão coat can be a powerful and inexpensive tool for removing heavy metals and dyes in the treatment of effluents from tannery and metallurgical industries. According to Lima et al.[14] the intense brown color of the pinhão coat is due to the presence of tannins that possibly are primarily responsible for the adsorption of metal ions, such as copper, for example.

Besides this alternative of environmental protection, it is important to mention the content of phenolic compounds found in the pinhão coat. Henríquez et al.[18] found significant levels of phenolic compounds in the coat of *A. araucana* seeds, which are higher in the coat of the cooked seed (73.91 mg GAE/g dry weight). This differs from *A. angustifolia*, for which higher concentrations of phenolics were found in the raw coat (77,56 mg CE/g dry weight) than in the cooked coat (31,63 mg CE/g dry weight).[34] Silva et al.[45] concluded from Fourier transform-infrared spectroscopy analysis that the *A. angustifolia* seed coat tannin contains a higher proportion of procyanidins to prodelphinidins when compared to the tannin of *A. mearnsii*. Extracts of *A. angustifolia* seed coat rich in condensed tannin strongly inhibited both human salivary and porcine pancreatic α-amylase.[45] Inhibition of α-amylase resulted in delayed carbohydrate digestion and glucose absorption with attenuation of postprandial hyperglycemic excursions. The observation that the pinhão coat extract rich in tannins is an effective inhibitor of salivary and pancreatic α-amylases, suggests that it can be used to suppress postprandial hyperglycemia in diabetic patients. Furthermore, it was also suggested that the pinhão coat tannin could, in principle at least, be used to promote weight loss and to combat obesity, perhaps even as a kind of functional food. The possibility that the tannin rich extract could be active on other enzymes in addition to the α-amylases, as α-glucosidases, for example, can not be excluded.

Based on the previously mentioned observation that the pinhão coat extract inhibits amylases, Oliveira et al.[46] investigated a possible action on the pancreatic lipase. Inhibition was indeed found, thus confirming the previous hypothesis. The pinhão coat extract inhibits the pancreatic lipase by a non-competitive parabolic mechanism. Consistently, the pinhão coat extract was also effective in reducing plasma triglyceride levels in rats after an olive oil load. This observation is probably the

consequence of an indirect inhibition of triglyceride absorption via inhibition of pancreatic lipase. Taken together with the similar inhibition of glucose absorption after a starch load.[45] these effects represent a potential anti-obesity activity, as suggested for other polyphenols or preparations rich in tannin.

Mota et al.[47] studied three cosmetic formulations using pinhão coat extracts and observed that the antioxidant potential of the formulations were much higher compared with formulations without *A. angustifolia* extracts. In addition, the cosmetic formulation and gel showed high antioxidant. Michelon et al.[48] and Souza et al.[49] evaluated the *in vitro* and *in vivo* antioxidant activity as well as the antigenotoxic activity of bracts (undeveloped seeds) of *A. angustifolia* and observed that the aqueous extracts obtained under reflux (100^{o} C) for 15 min are rich in phenolics being catechin, epicatechin, quercetin, apigenin, and rutin the major polyphenols. This aqueous extract was able to scavenge DPPH radicals, and exhibited potent superoxide dismutase and catalase-like activities. The extract significantly protected MRC5 cells against H_2O_2-induced mortality and oxidative damage to lipids, proteins, and DNA.

LEAVES OF *A. angustifolia*

Studies have demonstrated that a fraction of the leaves of *A. angustifolia* containing biflavonoids was effective in protecting DNA from damage induced by ultraviolet radiation and in inhibiting lipid peroxidation.[49,50] In leaves of *A. angustifolia*, six main biflavones were identified : amentoflavone, mono-*O*-methylamentoflavone, di-*O*-methylamentoflavone, ginkgetin, tri-*O*-methyl amentoflavone and tetra-*O* methyl-amentoflavone[49,51] (Figure 2). The fraction rich in biflavonoids obtained from the leaves of *A. angustifolia* showed a greater ability to eliminate the singlet oxygen radical when compared with quercetin and ginkgetin. The same fraction also showed the greatest inhibition of single-stranded plasmid DNA breaks formation. On the other hand, the fraction rich in biflavonoids was not able to protect plasmid DNA against single strand breaks generated by the Fenton reaction, contrary to quercetin and rutin. Moreover, Souza et al.[49] and Yamaguchi et al.[51] observed that, similar to quercetin and rutin, the fraction rich in biflavonoids proved to be able of protecting liposomes against lipid peroxidation induced by ultraviolet (UV) radiation, which is responsible for damage caused on the skin.[49] Although the biological activity of the biflavone fraction obtained from the leaves of *A. angustifolia* is not as effective as quercetin, rutin, alpha-tocopherol and trolox in protecting against single DNA strand breaks it can nonwithstanding be

regarded as an excellent candidate for successful employment as antioxidant and sunscreen. Yamaguchi et al.[49] have demonstrated that the biflavonoids of the leaves from *A. angustifolia* were more efficient in preventing the formation of thymine cyclobutane dimers induced by UV-B radiation than the compounds commonly used in cosmetics, but were not as efficient under against UV-A radiation.

The substances isolated from the acicles of *A. angustifolia* were also identified in grimpas (dry branches that fall naturally from the trees) of this species[48], indicating a viable source of active compounds in a usually discarded material.

A study conducted by Freitas et al.[13] to confirm the popular use of leaves of *A. angustifolia* against Herpes Simplex Virus (HSV-1) showed that the ethyl acetate and *n*-butanol fractions obtained from the crude hydroalcoholic extract have the best antiherpetic activity. Chemical analysis of this subfraction revealed the presence of proanthocyanidins and biflavonoids (bilobetin, II-7-O-methyl-robustoflavona and cupressuflavone), but the authors reported that the proanthocyanidins are possibly the main responsible for the anti-HSV-1 activity.

MISCELLANEOUS COMPOUNDS FOUND IN VARIOUS PARTS OF *A. angustifolia*

In plant callus cultures the flavonoid compounds and phenyl propanoid derivatives E and Z isomers of octadecyl *p*-coumarate and octadecylferulate were identified.[51] In the stem of the seedling, amentoflavone biflavones (7,4',7"-tri-o- methyl amentoflavone, 7,4',4"-tri-*O*-methyl amentoflavone, 4',4"-di-*O*-methyl amentoflavone) were found, in the seedling roots, a trans-cumenic acid diterpene was identified, and from the wood of the adult plant, the compounds p-hydroxybenzaldehyde, coniferaldehyde, vanillin, cabreuvin and irisolidonisoflavones, and pinoresinol, eudesmin and lariciresinollignoids were isolated.[51]

CONCLUDING REMARKS

This review reveals that much is known about the chemical constituents of the edible part (almond) of the *A. angustifolia* seed and somewhat less about its nutritional properties, making it clear that more studies are necessary on this topic. Less is known about the composition of other parts of the seed and the whole plant, leaves for example. More studies are also needed with respect to the technological feasibility of using the pinhão almond, especially its starch, as an ingredient for the food industry.

The effective use of the pinhão flour as an alternative for the development of gluten-free products is also dependent on additional technological experimentation.

Finally, it is important to note that studies on *A. angustifolia*, a species threatened by extinction and with a particular socio-cultural value, must combine two important elements: the need for preservation of a typical ecosystem and the implementation of the *A. angustifolia* forests as a true economic alternative for local residents. The economic alternative includes, among others, the potentials of the *A. angustifolia* as a source of ingredients for the food industry and bioactive substances with functional properties. In addition, it is also important to highlight the feasibility of producing gluten-free foodstuffs for people with celiac disease, which deserve to be studied with respect to the nutritional value that the pinhão offers.

REFERENCES

1. Koch Z and Corrêa MC. *Araucária: A Floresta do Brasil Meridional.* Olhar Brasileiro: Curitiba, 2010. 167 p., second edition
2. Cardemil L, Riquelme A Expression of cell wall proteins in seeds and during early seedling growth of *Araucaria araucana* is a response to wound stress and is developmentally regulated. J Exp Bot **42**: 415–421 (1991)
3. Anjos A, Mazza MCM, Santos ACMC and Delfini LT. Modulation of acute inflammation by a chitin-binding lectin from *Araucaria angustifolia* seeds via mast cells. *Sci For* **66**:38-45 (2004).
4. Auler NMF, Reis MS, Guerra MP and Nodari RO The genetics and conservation of *Araucaria angustifolia:* I. Genetic structure and diversity of natural populations by means of non-adaptive variation in the State of Santa Catarina, Brazil. *Genet Mol Biol* **25** (3):329-338 (2002).
5. Astarita LV, Floh EIS and Handro W. Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*. *Biol Plant* **47** (1):53-59 (2003).
6. Carvalho PER. *Espécies florestais brasileiras: recomendações silviculturais, potencialidades e uso da madeira*. Colombo – PR, EMBRAPA, 1994. p. 70–78.
7. BRDE. Banco Regional de Desenvolvimento do Extremo Sul. *Cultivo da Araucaria angustifolia: análise de viabilidade econômico-financeira.* Agência de Florianópolis. Gerência de Planejamento, BRDE: Florianópolis, 2005. 53 p.
8. Cordenunsi BR, Menezes EW, Genovese MIS, Colli CL, Souza AGA and Lajolo FM. Chemical composition and glycemic index of Brazilian pine (*Araucaria angustifolia*) seeds. *J Agric Food Chem* **52**:3412-3416 (2004).
9. Amarante CVT, Mota CS, Megguer CA and Ide GM. Conservação pós-colheita de pinhões sementes de *Araucaria angustifolia* (Bertoloni) Otto Kuntze armazenados em diferentes temperaturas. *Ciênc Rural* **37** (2):346-351, 2007.
10. Balbuena TS, Silveira V, Junqueira M, Dias LLC, Santa-Catarina C, Shevchenkov A and Floha EIS. Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian Pine (*Araucaria angustifolia*). *J. Proteomics* **72**:337-352 (2009)
11. Stahl JA, Lobato LP, Bochi VC, Kubota EH, Gutkoskic LC and Emanuelli T. Physicochemical properties of pinhão (*Araucaria angustifolia*, Bert, O. Kuntze) starch phosphates. *LWT Food Sci Technol* **40**:1206-1214 (2007).

12. Aslam MS, Choudhary BA, Uzair M and Ijaz AS. Phytochemical and ethno-pharmacological review of the genus *Araucaria* – Review. *Trop J Pharm Res* **12**(4): 651-659 (2013).
13. Freitas AM, Almeida MTR, Andrighetti-Fröhner CR, Cardozo FTGS, Berardi CRM, Farias MR, Simões CMO. Antiviral activity-guided fractionation from *Araucaria angustifolia* leaves extract. *J Ethnopharmacol* **126**:512-517 (2009).
14. Lima EC, Royer B, Vaghetti JCP, Brasil JL, Simon NM, Santos-Jr, AA, Pavan FA, Dias SLP, Benvenutti EV and Silva EA. Adsorption of Cu(II) on *Araucaria angustifolia* wastes: Determination of the optimal conditions by statistic design of experiments. *J Hazard Mater* **140**:211-220 (2007).
15. Leite DMC, Jong EV, Noren CPZ and Brandelli A. Nutritional evaluation of *Araucaria angustifolia* seed flour as a protein complement for growing rats. *J Sci Food Agric* **88**:1166-1171 (2008).
16. TACO. *Tabela brasileira de composição de alimentos*. NEPA-UNICAMP: Campinas, 2006. 113p.
17. Young YR, Pellet PL. Plant proteins in relation to human protein and amino acid nutrition. *Am J Clin Nutr* **59**:1203S-1212S (1994)
18. Henríquez C, Escobar B, Figuerola F, Chiffelle I, Speisky H and Estévez AM. Characterization of pinon seed (*Araucaria araucana* (Mol) K. Koch) and the isolated starch from the seed. *Food Chem* **107**:592-601 (2008).
19. Bello-Pérez LA, Suárez-García FJ, Montealvo-Méndez G, Nascimento JRO, Lajolo FM and Cornedunsi BR. Isolation and characterization of starch from seeds of *Araucaria brasiliensis*: A novel starch for application in food industry. *Starch/Starke* 58:283-291 (2006).
20. Conforti PA and Lupano CE. Comparative study of the starch digestibility of *Araucaria angustifolia* and *Araucaria araucana s*eed flour.*Starch/Starke* **60**:192-198 (2008).
21. Conforti PA and Lupano CE Starch characterization of *Araucaria angustifolia* and *Araucaria araucana* seeds.*Starch/Starke* 59:284-289 (2007).
22. Wosiacki G and Cereda MP. Characterization of pinhão starch. Part III: hydration of the granules and susceptibility to enzymatic hydrolysis. *Starch/Starke* **41**:327-330 (1989).

23. Thys RCS, Aires AG, Marczak LDF and Norena CPZ. The effect of acid hydrolysis on the technological functional properties ofpinhão (*Araucaria brasiliensis*) starch. *Ciênc Tecnol Aliment* **33**(Supl. 1): 89-94 (2013).
24. Daroit DJ, Côrrea APF, Klug TV and Brandelli A. Partial purification and characterization of polyphenol oxidase from A*raucaria angustifolia* (Bert, O. Kuntze) seeds. *J Food Biochem* **34**:1216-1230 (2010).
25. Basso FM, Mangolim CS, Aguiar MF, Monteiro AR, Peralta RM, Matioli G. Potential use of cyclodextrin-glycosyltransferase enzyme in bread-making and the development of gluten-free breads with pinion and corn flours. *Int J Food Sci Nut.* **66**(3):275-81 (2015).
26. Spada JC, Noreñab CPZ, Marczaka LDF and Tessaro IC. Study on the stability of β-carotene microencapsulated with pinhão (*Araucaria angustifolia* seeds) starch. *Carbohydr Polym* **89**:1166–1173 (2012)
27. Fonseca SCL and Freire HB. Sementes Recalcitrantes: problemas na pós-colheita. *Bragantia Rev Ciênc Agron* **62**:297-303 (2003).
28. Capella ACV, Penteado, PTP and Balbi ME. Semente de *Araucaria angustifolia*: aspectos morfológicos e composição química da farinha. *B. CEPPA* **27**:135-142 (2009).
29. Bezerra JRMV, Gonzáles SM, Kopt C, Rigo M and Bastos RG. Elaboração de pães com farinha de pinhão. *RECEN* **8**:(1):69-81 (2006).
30. Helm CV, Faccin M and Santos MCA. Elaboração de massa alimentícia enriquecida com farinha de pinhão (*Araucaria angustifolia*). *RUBS* **1**:29-30 (2005).
31. Acorsi DM., Bezerra JRMV, Barão MZ and Rigo M. Viabilidade do processamento de biscoitos com farinha de pinhão. *Ambiência* **5** (2):207-212 (2009).
32. Han X, Shen T and Lou H. Dietary polyphenols and their biological significance. *Int J Mol* **8**:950-988 (2007)
33. USDA. Data base for the flavonoid content of selected food. March, 2003
34. Koehnlein EA, Carvajal AES, Koehnlein EM, Coelho-Moreira JS, Inácio FD, Castoldi R, Bracht,A and Peralta RM. Antioxidant activities and phenolic compounds of raw and cooked Brazilian pinhão (*Araucaria angustifolia*) seeds. *Afr J Food Sci* **6**:512-518 (2012).

35. Santi-Gadelha T, Gadelha CAA, Aragão KS, Oliveira CC, Mota MRL, Gomes RC, Pires AF, Toyama MH, Toyama DO, Alencar NMN, Criddle DN, Assreuy AMS and Cavada BS. Purification and biological effects of *Araucaria angustifolia* (Araucariaceae) seed lectin. *Biochem Biophys Res Commun* **350**:1050-1055 (2006).

36. Datta PK Figueiroa MODCR and Lajolo FMJ. Chemical modification and sugar binding properties of two major lectins from pinhão *(Araucaria brasiliensis)* seeds. *J Agric Food Chem* **41**:1851-1855 (1993).

37. Datta PK, Figueiroa MODCR and Lajolo FM. Purification and characterization of two major lectins from *Araucaria brasiliensis* syn. *Araucaria angustifolia* seeds (Pinhão).*Plant Physio.* **97**:856-862 (1991).

38. Mota MRL, Criddle DN, Alencar NMN, Gomes RC, Meireles AVP, Santi-Gadelha T, Gadelha CAA, Oliveira CC, Benevides RG, Cavada BS and Assreuy AMS. Modulation of acute inflammation by a chitin-binding lectin from *Araucaria angustifolia* seeds via mast cells. *Naunyn Schmiedebergs Arch Pharmacol* **374**:1-10 (2006).

39. Vasconcelos SM, Lima SR, Soares PM, Assreuy AMS, Sousa FCF, Lobato RFG, Vasconcelos GS, Santi-Gadelha T, Bezerra EHS, Cavada BS and Patrocínio MCA. Central action of *Araucaria angustifolia* seed lectin in mice. *Epilepsy Behav*. **15**:291-293 (2009).

40. Brasil JL, Eva RR, Milcharek CD, Martins LC, Pavan FA, Santos-Jr AA, Dias SLP, Dupont J, Norena CPZ and Lima EC Statistical design of experiments as a tool for optimizing the batch conditions to Cr(VI) biosorption on *Araucaria angustifolia* wastes. *J Hazard Mater* **133**:143-153 (2006).

41. Lima EC, Royer B, Vaghetti JCP, Simon NM, Cunha BM, Pavan FA, Benvenutti EV, Cataluna-Veses R and Airoldi C. Application of Brazilian pine-fruit shell as a biosorbent to removal of reactive red 194 textile dye from aqueous solution kinetics and equilibrium study. *J Hazard Mater* **155**:536-550 (2008).

42. Royer B, Cardoso NF, Lima EC, Vaghetti JCP, Simon NM, Calvete T and Vese RC. Applications of Brazilian pine-fruit shell in natural and carbonized forms as adsorbents to removal of methylene blue from aqueous solutions—kinetic and equilibrium study. *J. Hazard Mater* **164**:1213-1222 (2009).

43. Calvete T, Lima EC, Cardoso NF, Vaghetti JCP, Dias SL and Pavan FA. Application of carbon adsorbents prepared from the Brazilian pine-fruit-shell for the removal of Procion Red MX 3B from aqueous solution -Kinetic, equilibrium and thermodynamic studies. *Chem Eng J* **155**:627-636 (2009).
44. Calvete T, Lima EC, Cardoso NF, Vaghetti JCP, Dias SL and Pavan FA. Application of carbon adsorbents prepared from Brazilian-pine fruit shell for the removal of reactive orange 16 from aqueous solution: Kinetic, equilibrium, and thermodynamic studies. *J Environ Manage* **91**:1695-1706 (2010).
45. Silva SM, Koehnlein EA, Bracht A, Castoldi R, de Morais, GR, Baesso ML, Peralta RA, de Souza CGM, Sá-Nakanishi AB and Peralta RM. Inhibition of salivary and pancreatic α-amylases by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. *Food Res Int* **56**:1–8 (2014).
46. Oliveira RF, Gonçalves GA, Inácio FD, Koehnlein EA, Souza CGM, Bracht A and Peralta RM. Inhibition of pancreatic lipase and triacylglycerol intestinal absorption by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. *Nutrients* **7**:5601-5614 (2015).
47. Mota GST, Arantes AB, Sacchetti G, Spagnoletti A, Ziosi P, Scalambr, E and Vertuani S. Antioxidant activity of cosmetic formulations based on novel extracts from seeds of Brazilian Araucaria (Bertoll) Kuntze. *JCDSA* **4**:190-202 (2014).
48. Michelon F, Branco CS, Calloni C, Giazzon I, Agostini F, Spada PKW, Salvador M. *Araucaria angustifolia:* a potential nutraceutical with antioxidant and antimutagenic activities. *Curr Nutr Food Sci* **8**:155-158 (2012)
49. Souza MO, Branco CS, Sene J, DallAgnol R, Agostini F, Moura S and Salvador M. Antioxidant and antigenotoxic activities of the Brazilian pine *Araucaria angustifolia* (Bert.) O. Kuntze. *Antioxidants* **3**:24-37 (2014).
50. Yamaguchi LF, Kato MJ and Mascio PD. Biflavonoids from *Araucaria angustifolia* protect against DNA UV-induced damage. *Phytochemistry* **70**:615-620 (2009).
51. Yamaguchi LF, Vassão DG, Kato MJ and Mascio PD. Biflavonoids from Brazilian pine *Araucaria angustifolia* as potentials protective agents against DNA damage and lipoperoxidation. *Phytochemistry* **66**:2238-2247 (2005).
52. Fonseca FN, Ferreira AJS, Sartorelli P, Lopes NP, Floh EIS, Handro W and Kato MJ. Phenylpropanoid derivatives and biflavones at different stages of

differentiation and development of *Araucaria angustifolia*. *Phytochemistry* **5**:575-580 (2000).

53. Franco G. Tabela de composição química dos alimentos. 9ª ed. Atheneu: São Paulo, 2002.
54. Abe LT, Lajolo FM and Genovese MI. Comparison of phenol content and antioxidant capacity of nuts. *Ciênc Tecnol Aliment* **30** (Supl1):254-259 (2010).
55. Silva AG, Rocha LC and Brazaca SGC. Caracterização físico-química, digestibilidade protéica e atividade antioxidante de feijão comum (*Phaseolus vulgaris* L.). *Alim Nutr* **20**:591-598 (2009).
56. Shen Y, Jin L, Xiao P, Lu Y and Bao J. Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. *J Cereal Sci* **49**:106-111 (2009).
57. Faller ALK and Fialho E. Disponibilidade de polifenóis em frutas e hortaliças consumidas no Brasil. *Rev Saúde Públ* **43**:211-218 (2009).

Table 1. Approximate composition of the edible part (almond) of raw seeds of *A. angustifolia and A. araucana*[16].

	A. angustifolia g/100 g fresh weight	*A. araucana* g/100 g fresh weight
Moisture	49.50 ± 0.28	42.72 ± 0.28
Ashes	1.60 ± 0.07	2.15 ±0.01
Lipids	1.26 ± 0.07	1.11 ± 0.08
Proteins	3.57 ± 0.05	7.81 ± 0.35
Crude fiber	nd	4.51 ± 0.16
Reducing sugar	nd	0.59 ± 0.01
Insoluble fiber	4.26 ± 0.20	22.58 ± 2.61
Soluble fiber	0.63 ± 0.13	2.85 ± 0.62
Total starch	36.28 ± 0.11	31.34 ±1.60

Table 2. Amino acid composition (as mg amino acid g^{-1} crude protein) of pinhão flours.[5]

Amino acid	pinhão flour (dried at 50° C)	pinhão flour (dried at 80° C)	Pooled SEM
Ala	47.7	47.5	0.1
Arg	68.2	78.9	0.1
Asp	138.6	129.4	0.2
Cys	6.8	8.8	0.1
Glu	184.1	179.8	0.3
Gly	50.0	48.2	0.1
His	15.9	17.5	0.1
Ile	40.9	39.5	0.1
Leu	77.3	72.4	0.2
Lys	43.2	46.0	0.1
Met	25.0	24.2	0.1
Phe	56.8	54.8	0.1
Pro	50.0	48.2	0.1
Ser	54.5	52.6	0.1
Thr	40.9	39.5	0.1
Tyr	34.1	37.3	0.1
Trp	15.9	15.3	0.1
Val	68.2	68.0	0.1

Table 3. Composition of micronutrients per 100 g of the edible part of seeds of *A. angustifolia* raw and cooked (values wet basis).

Minerals and vitamins	Cordenunsi et al.[8]		TACO[16]	Franco[53]	
	raw	cooked	cooked	raw	cooked
Calcium (mg)	13	16	16	36	35
Magnesium (mg)	55	52	53	nd	nd
Manganese (mg)	nd	nd	0.41	nd	nd
Phosphorus (mg)	103	93	166	150	136
Irons (mg)	0.72	0.67	0.8	0.7	nd
Sodium (mg)	nd	nd	1	nd	nd
Potassium (mg)	nd	nd	727	nd	nd
Copper(mg)	0.26	0.23	0.18	nd	nd
Zinc (mg)	0.81	0.77	0.8	nd	nd
Retinol (µg)	nd	nd	nd	3	3
Thiamine(mg)	nd	nd	Tr	1.28	1.35
Riboflavin (mg)	nd	nd	Tr	0.23	0.24
Pyridoxine(mg)	nd	nd	Tr	nd	nd
Niacin (mg)	nd	nd	Tr	4.5	4.7
Vitamin C (mg)	nd	nd	27.7	23.1	13.9

nd – non-determined

Table 4. Proximate composition of isolated starch of *A. angustifolia* and *A. araucana* seeds (dry basis).[21]

Component (%)	*A. angustifolia*	*A. araucana*
Protein	3.25±0.17	3.40±0.29
Lipid	0.52±0.50	0.45±0.18
Ash	0.28±0.02	0.42±0.07
Carbohydrates[a]	95.95	95.73
Amylose[b]	22.4±1.90	17.30±1.40

[a]Estimate by difference; [b]calculated on starch basis

Table 5. Comparison of the content of phenolic compounds in the almonds of the *A. angustifolia* seeds and other foods.

Food	Phenolic compounds* (mg/100 g)	Phenolic compounds** (mg/100 g)	Reference
A. angustifolia- raw seed (fresh weight)	50±3	23±1	53
A. angustifolia cooked seed (fresh weight)		54±1	8
A. araucana raw seed (dry weight)	71±2		18
Raw carioca beans (fresh weight)		451±10	54
Cooked carioca beans (fresh weight)		49±0	54
Raw white rice (dry weight)	151.8±19.5		55
Potato (fresh weight)	31.5±2.1		56
Onion (fresh weight)	113.2±3.8		56
Raw Brazilian nut (fresh weight)	106±7		53
Raw cashew nut (fresh weight)	381±6		53
Roasted almond (fresh weight)	111±2		53
Raw walnut (fresh weight)	597±6		53
Raw chestnut/portuguese nut (fresh weight)	92±2		53
Roasted hazelnut (fresh weight)	114±3		53

(*)gallic acid equivalents; (**) catechin equivalents.

Table 6. Antioxidant activity of raw and cooked *A. angustifolia* seeds [34].

	Antioxidant activity			
Hydroalcoholic pinhão seed extracts	DPPH assay (µg/mL)	ABTS assay (µg/mL)	LPO inhibition (µg/mL)	Fe^{2+} chelating ability (µg/mL)
raw	870.7±30.8[a]	170.7±7.8[a]	55.9±4.7[a]	1,719±84.8[a]
cooked	112.6±5.4[b]	52.1±2.1[b]	53.8±4.5[a]	761±77.1[b]

EC_{50}= extract concentration producing 50% antioxidant activity; DPPH assay=1,1-diphenyl-2-picryl-hydrazyl assay; ABTS assay=2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid assay; LPO inhibition=β-carotene-linoleic acid assay;

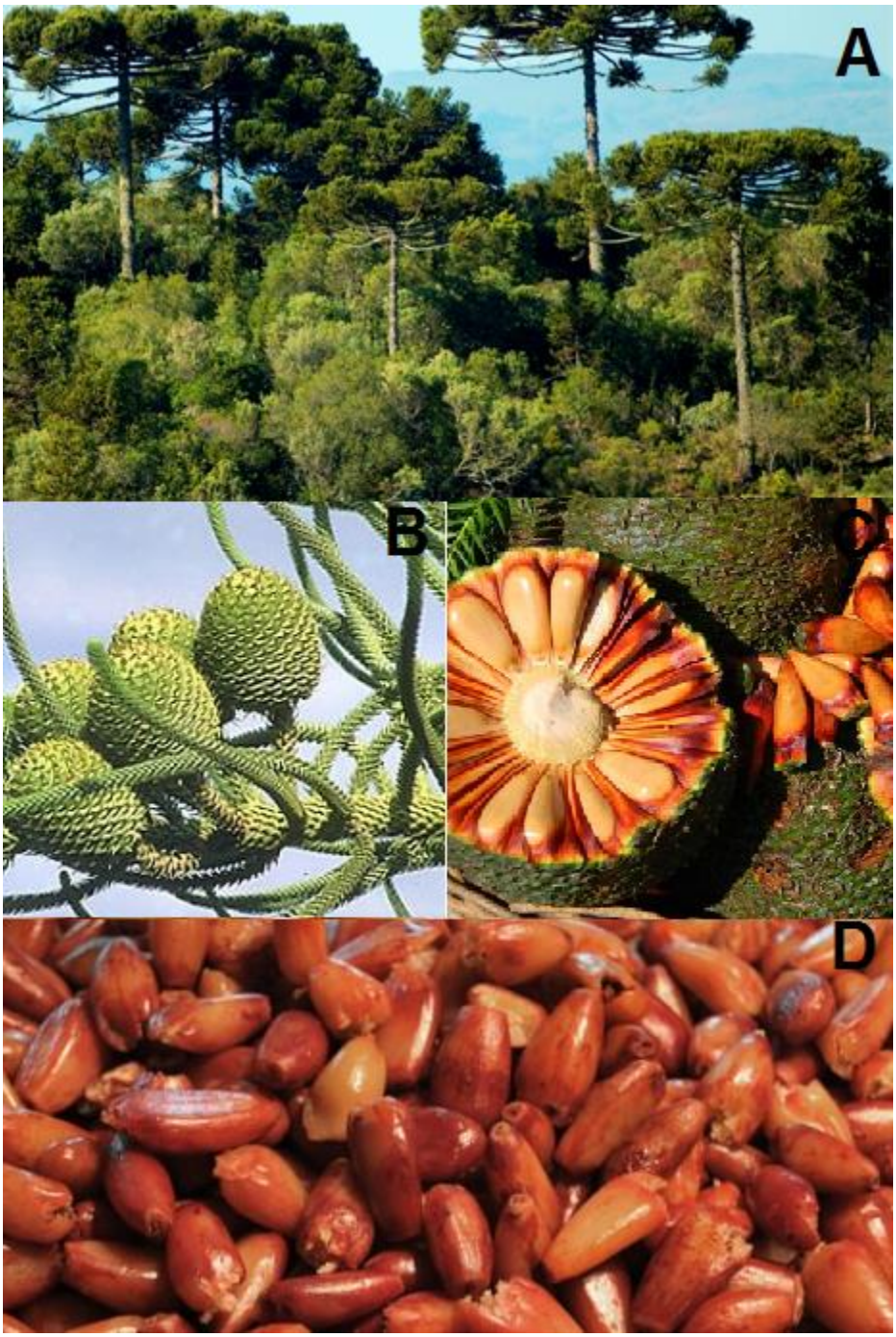

Figure 1. *Araucaria angustifolia*. (A) mature tree; (B) female cones or pine cones; (C) mature seeds; (D) cooked seed (edible part).

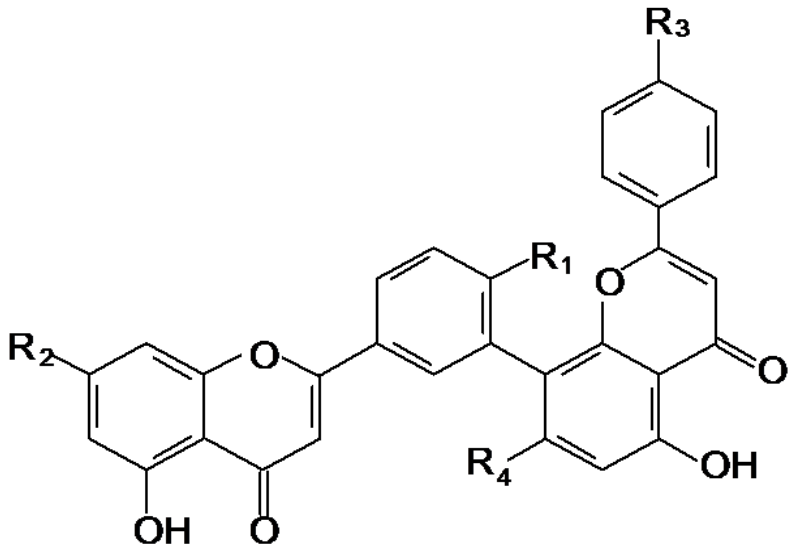

Figure 2. Six major biflavonoids present in *A. angustifolia* needles.

R_1=R_2=R_3=R_4=OH: amentoflavone;

mono-*O*-methylamentoflavone;

di-*O*-methylamentoflavone;

R_1=R_2=OMe, R_3=R_4=OH: ginkgetin; tri-*O*-methylamentoflavone;

R_1=R_2=R_3=R_4=OMe: tetra-*O*-methylamentoflavone

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Article

Inhibition of Pancreatic Lipase and Triacylglycerol Intestinal Absorption by a *Pinhão* Coat (*Araucaria angustifolia*) Extract Rich in Condensed Tannin

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Abstract: The purpose of the present work was to characterize the possible inhibition of pancreatic lipase by a tannin-rich extract obtained from the *pinhão* (*Araucaria angustifolia* seed) coat, based on the previous observation that this preparation inhibits α-amylases. Kinetic measurements of pancreatic lipase revealed that the *pinhão* coat tannin is an effective inhibitor. Inhibition was of the parabolic non-competitive type. The inhibition constants, $\overline{K}_{i1}$ and $\overline{K}_{i2}$, were equal to 332.7 ± 146.1 μg/mL and 321.2 ± 93.0 μg/mL, respectively, corresponding roughly to the inhibitor concentration producing 50% inhibition ($[I]_{50}$). Consistently, the *pinhão* coat extract was also effective at diminishing the plasma triglyceride levels in mice after an olive oil load; 50% diminution of the area under the plasma concentration *versus* the time curve occurred at a dose of 250 mg/kg. This observation is most probably the consequence of an indirect inhibition of triglyceride absorption via inhibition of pancreatic lipase. For the *pinhão* coat tannin, this is the second report of a biological activity, the first one being a similar inhibition of the absorption of glucose derived from starch as a consequence of an inhibitory action on α-amylases. Taken together,

these effects represent a potential anti-obesity action, as suggested for other polyphenol or tannin-rich preparations.

Keywords: tannins; lipase; enzyme; obesity

1. Introduction

Proanthocyanidins are the most common group of flavonoids in the Western diet. They are also known as condensed tannins, because they comprise a group of polyhydroxyflavan-3-ol oligomers and polymers of flavonol subunits linked by carbon to carbon bonds [1]. They have attracted great attention due to rapid growing evidence associating these compounds with a wide range of potential health benefits [2,3]. The opinion has been frequently expressed that the discovery of new materials rich in tannins with enzyme inhibitory properties could contribute to the development of new drugs useful in the control and treatment of diabetes, obesity and other physiological disorders [2,3]. *Pinhão* is the name generally given to the seed of the coniferous tree *Araucaria angustifolia* [4], which was once quite abundant in southern Brazil and which is still an important component of its flora. The *pinhão* seed is largely consumed as a food, but the coat is generally discarded. The latter, however, is rich in condensed tannins, belonging predominantly to the procyanidin class, which are chains of catechin, epicatechin and their gallic acid esters [5]. With respect to the *pinhão* coat tannins, it has been shown that an extract rich in these compounds (90% of its weight) is able to inhibit both salivary and pancreatic α-amylases [5]. As a consequence of these actions, the *pinhão* coat extract is also effective at diminishing the post-prandial glycemic levels in rats after starch administration. For these reasons, it was suggested that the *pinhão* coat extract could be used as an adjuvant in the suppression of postprandial hyperglycemia in diabetic patients [5].

The action of tannins or polyphenols in general is not restricted to the α-amylases or other types of glucosidases. Inhibition of these classes of enzymes is generally also associated with the inhibition of lipases, especially pancreatic lipase [6,7].It has been shown, for example, that a polyphenol preparation from the *Acacia mearnsii* bark is able to inhibit pancreatic lipase in addition tothe glucosidases maltase and sucrase [7]. By virtue of this observation and considering our previous finding of an inhibition of α-amylases by the *pinhão* coat extract rich in tannins [5], the logical hypothesis that can be formulated is that the tannins of the *pinhão* coat are equally able to inhibit pancreatic lipase or even other lipases. If this hypothesis is correct, the *pinhão* coat tannins should be equally able to affect lipid absorption *in vivo* [8]. Testing of these two hypotheses was precisely the purpose of the present work, which consisted of both *in vitro* experiments with the porcine pancreatic lipase and *in vivo* experiments with mice loaded intragastrically with olive oil. The results should contribute further to clarifying the potential usefulness of the *pinhão* coat, which is otherwise merely discarded as a waste product.

2. Experimental Section

2.1. Materials

Porcine pancreatic lipase (Type II) and orlistat were purchased from Sigma-Aldrich Co. The *A. mearnsii* bark tannin was purchased from Labsynth, Brazil. All other reagents were of the highest available grade.

2.2. Animals

Male healthy Swiss mice (35 g to 40 g, on average 4 weeks old) were used in all experiments. Each mouse was kept at room temperature (22 $\pm$ 1 °C) and humidity with a 12-h light/dark cycle. The experiments were approved by the Ethics Committee of Animal Experimentation of the University of Maringá.

2.3. Preparation of the Pinhão (A. angustifolia) Coat Extract

Pinhão seeds used in this study were purchased at a local market (Maringá, PR, Brazil) and prepared according to methods described elsewhere [5]. Briefly, the seeds used in this work were washed with tap water and dried at room temperature for 24 h. The coats of the seeds were removed and dried at 40 °C until constant weight. The seed coats corresponded to approximately 30% of the total weight. After drying, the seed coats were milled into a fine powder. The powder (100 g) was mixed with 300 mL of 70% ethanol (in water) at room temperature and maintained under agitation at 140 rpm for 3 h. The extractions were repeated three times. No increases in yield were achieved by further extractions. The combined mixtures were filtered through Whatman filter paper Number 1 and concentrated with a rotary vacuum evaporator at 40 °C to eliminate ethanol and finally freeze dried.

2.4. Pancreatic Lipase Activity and Kinetics

The porcine pancreatic lipase was assayed using *p*-nitrophenyl-palmitate as the substrate and spectrophotometrically recorded at 410 nm [9]. The substrate solution was prepared by suspending 20 mg of *p*-nitrophenyl palmitate in 10 mL of isopropanol. The suspension was sonicated until complete dissolution of *p*-nitrophenyl-palmitate. At the time of use, this stock solution was diluted with isopropanol to concentrations up to 0.5 mg/mL. The porcine pancreatic lipase was dissolved in Tris-HCL buffer (pH 8.0) at the concentration of 2 mg/mL. This suspension was centrifuged at 2000$\times$ *g* for five minutes and the supernatant used as the source of enzyme. The reaction mixture (2.4 mL) contained 100 mM Tris-HCl buffer (pH 8.2) and 530 µM substrate and was 25% isopropanol. After pre-warming the reaction mixture at 37 °C, the enzyme solution was added (0.1 mL), and the incubation was continued for 20 min at the same temperature. The reaction was stopped by transferring the reaction vessel to a bath of boiling water. After 10 min, the incubation was cooled to room temperature and centrifuged at 1500$\times$ *g* for 5 min. Absorbance of the supernatant at 410 nm was determined against a blank solution containing denatured enzyme. One enzyme unit was defined as 1 µmol of *p*-nitrophenol enzymatically released from the substrate per minute per mL. The kinetic measurements were done in the same way as described for the inhibition assays, except that the substrate concentration was varied by diluting the stock solution with isopropanol to the desired concentration, so that the isopropanol concentration was the same in each assay.

2.5. Oral Olive Oil Tolerance Tests in Mice

The intestinal absorption of triglycerides was tested by means of an oral olive oil tolerance test in mice [7]. The mice were deprived of food for 18 h before the experiment. *Pinhão* coat extract solutions were administered orally at doses of 100 mg, 250 mg and 500 mg per kg body weight. Olive oil was subsequently administered orally (5 mL per kg body weight). Before and at 1.5 h, 3.0 h, 4.5 h and 6.0 h after olive oil administration (or distilled water for the controls), blood samples from the tail vein were analyzed by means of an Accutrend Plus Roche triglycerides meter.

Seven groups of mice (n = 3 per group) were utilized: (1) the positive control, only intragastric olive oil (5 mL per kg) administration; (2) the negative control, only tap water administration; (3) intragastric administration of olive oil plus orlistat (50 mg/kg); (4) intragastric administration of olive oil plus *A. mearnsii* tannin (500 mg/kg); (5) intragastric administration of olive oil plus 100 mg/kg *Pinhão* extract; (6) intragastric administration of olive oil plus 250 mg/kg *Pinhão* coat extract; and (7) intragastric administration of olive oil plus 500 mg/kg *Pinhão* coat extract.

2.6. Calculations and Statistics

Statistical analysis of the data was done by means of the Statistica program (Statsoft, 1998, Tulsa, OK, USA). Fitting of the rate equations to the experimental initial rates was done by means of an iterative non-linear least-squares procedure using the Scientist software from MicroMath Scientific Software (Salt Lake City, UT, USA). The procedure requires the introduction of preliminary estimates of each parameter. These preliminary estimates are improved by each successive iteration in which the squared difference between the calculated and experimental data is progressively diminished until it converges towards a minimum [10,11].

The decision about the most adequate model (equation) was based on the model selection criterion (MSC) and on the standard deviations of the optimized parameters. The model selection criterion, which corresponds to the normalized Akaike information criterion [12], is defined as:

$$\mathrm{MSC} = \ln\left[\frac{\sum_{i=1}^{n} w_i\left(Y_{\mathrm{obs}_i} - \overline{Y}_{\mathrm{obs}}\right)^2}{\sum_{i=1}^{n} w_i\left(Y_{\mathrm{obs}_i} - Y_{\mathrm{cal}_i}\right)^2}\right] - \frac{2p}{n} \quad (1)$$

Y_{obs} are the experimental reaction rates, $\overline{Y}_{\mathrm{obs}}$ the mean experimental reaction rate, Y_{cal} the theoretically calculated reaction rate, w the weight of each experimental point, n the number of observations and p the number of parameters of the set of equations. In the present work, the model with the largest MSC value was considered the most appropriate, provided that the estimated parameters were positive.

3. Results and Discussion

3.1. Concentration Dependence of the Lipase Inhibition

In the first experiments, the hypothesis of an inhibitory action of the *pinhão* coat extract on pancreatic lipase was investigated by measuring the enzyme activity at a fixed substrate concentration

(530 μM *p*-nitrophenylpalmitate) and varying concentrations of the extract (up to 500 μg/mL). Parallel experiments were run with the *A. mearnsii* tannin (up to 500 μg/mL), as there is previous evidence that an *A. mearnsii* polyphenol preparation inhibits pancreatic lipase activity [7]. In Figure 1A, the relative rates were represented against the inhibitor concentration. It is apparent that the *pinhão* coat extract inhibited the enzyme with a clear concentration dependence, reaching 68% inhibition at the concentration of 500 μg/mL. The inhibition by the *A. mearnsii* tannin was reproduced in our experiments, with 35% inhibition at the concentration of 500 μg/mL. This is very close to the inhibition degree reported previously [7], but clearly less pronounced than that observed with the *pinhão* coat extract. Another difference between the actions of the *pinhão* coat extract and the *A. mearnsii* tannin is revealed by Figure 1B, in which the reciprocals of the reaction rates were represented against the respective concentrations. Whereas the inhibition caused by the *A. mearnsii* tannin was of the linear type, that caused by the *pinhão* coat extract was clearly parabolic. This has mechanistic implications and must be taken into account when further analyzing the inhibition caused by the *pinhão* coat extract in kinetic terms [13,14].

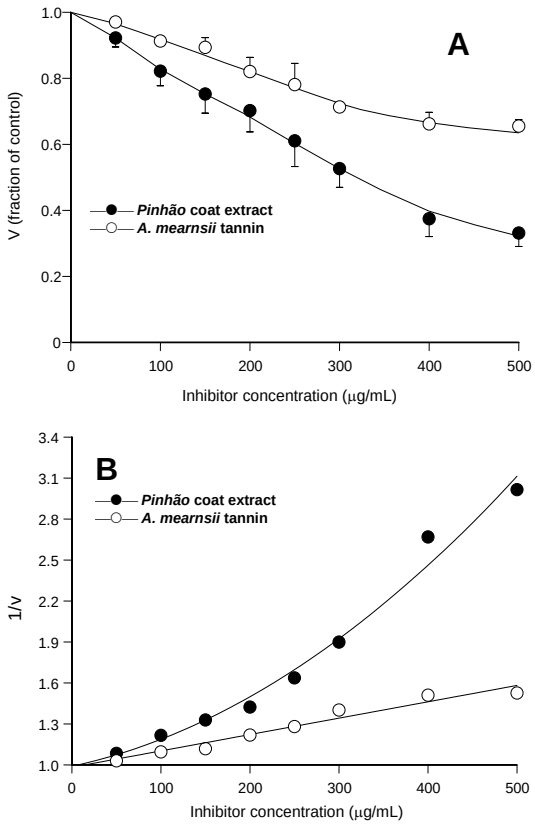

Figure 1. Inhibition of pancreatic lipase by *pinhão* coat extract and *A. mearnsii* tannin: concentration dependences. Initial reaction rates were measured as described in the Experimental Section. Each datum point represents the mean of three independent determinations: (**A**) relative reaction rates (v); (**B**) inverse reaction rates (1/v). The continuous lines in (B) represent the regression curves that were calculated after parabolic (A) and linear (B) regression analysis. The optimized equation for the *pinhão* coat extract was: $y = 0.989 + 0.00141 \cdot x + 0.00000567 \cdot x^2$ ($r = 0.992$); for the *A. mearnsii* tannin: $y = 0.982 + 0.00119 \cdot x$ ($r = 0.981$).

3.2. Kinetics of Pancreatic Lipase Inhibition

In order to characterize further the inhibition of pancreatic lipase by the *pinhão* coat extract, the activity of the enzyme was measured at different substrate and inhibitor concentrations. The results are summarized in Figure 2, which shows saturation curves that were progressively lowered as the *pinhão* coat extract was added at progressively increased concentrations. The saturation curves do not show any tendency of convergence at high substrate concentrations, which excludes the possibility of competitive inhibition. On the other hand, the saturation curve of pancreatic lipase in the absence of the inhibitor does not obey the simple Michaelis—Menten equation. This is revealed by the fact that the reaction rate ceased to increase when the substrate concentration was increased from 130 μM to 530 μM (Figure 1), revealing an apparent premature saturation. This particularity results in non-linear Lineweaver—Burk plots (not shown) with a concave up curve near the 1/v axis [13,14]. The latter phenomenon has been generally interpreted as substrate inhibition. Although it is not very pronounced in the concentration range investigated in the present work, it must be taken into account when analyzing the kinetics of the inhibition. Furthermore, consideration must also be given to the fact that the inhibition caused by the *pinhão* coat tannin was parabolic. This kind of relationship means that more than one inhibitor molecule binds to a single site in the enzyme, enhancing inhibition at higher concentrations to degrees that are above those expected when a single molecule binds to the site [13,14]. In principle, one may assume that the following complexes are formed in the presence of enzyme (E), substrate (S) and inhibitor (I): ES, ES_2, EI, ESI, ES_2I, EI_2, ESI_2 and ES_2I_2. If one assumes that (1) only the complex ES leads to product formation (*i.e.*, all other complexes are inhibitory), (2) binding of the second substrate molecule to the enzyme does not change its affinity to the inhibitor, (3) steady-state conditions hold for the substrate to enzyme interactions and that the (4) enzyme to inhibitor interactions are in equilibrium, the following equation can be derived for the reaction rate (v) dependence from both substrate ([S]) and inhibitor ([I]) concentrations [13,14]:

$$v = \frac{V_{\max}[S]}{K_M\left(1+\frac{[I]}{K_{i1}}+\frac{[I]^2}{K_{i1}K'_{i1}}\right)+[S]\left(1+\frac{[S]}{K_{iS}}\right)\left(1+\frac{[I]}{K_{i2}}+\frac{[I]^2}{K_{i2}K'_{i2}}\right)} \quad (2)$$

V_{max} is the maximal reaction rate, K_M the Michaelis constant and K_{iS} the substrate inhibition constant; K_{i1} and K'_{i1} are the inhibition constants for the EI andEI_2 complexes, respectively; K_{i2} the inhibition constants for the ESI and ES_2I complexes; and K'_{i2} the inhibition constant for the ESI_2 and ES_2I_2 complexes. These inhibition constants may be true dissociation constants under certain limiting conditions, but especially if more than one inhibitor is present in the *pinhão* coat extract, they are complex functions of several individual dissociation constants, but are still a measure of the potency of the inhibition [13,14]. It should be noted that the presence of more than one inhibitor does not invalidate Equation (2), provided that their concentrations are kept at constant ratios, as occurs when different amounts of the same extract are added [13,14]. Equation (2) was fitted to the whole experimental data set shown in Figure 2. Fitting was successful in that convergence between experimental and calculated data was reached quickly and positive values were obtained for all parameters. Table 1 lists the parameters obtained, which reveal consistent values for K_M, V_{max} and K_{iS} with relatively low standard deviations. The optimized values of the inhibition constants, however, indicate that Equation (2) might not be a good description of the data. Firstly, the standard deviations of the four inhibition constants greatly exceed

their optimized values, meaning that they cannot be determined with an acceptable precision. Moreover, the value of K_{i1} is exceptionally high, and the standard deviation exceeds it by orders of magnitude. Clearly, K_{i1} cannot be determined. This happens if the concentration of the complex EI is too low to be detected, which renders the $[I]/K_{i1}$ term in Equation (2) non-significant. The same applies to the K_{i2} value, which greatly reduces the significance of the $[I]/K_{i1}$ term in Equation (2) and also suggests that the concentrations of the ESI and ES_2I complexes are low. An alternative to Equation (2) would be Equation (3) in which binding of at least two molecules of the inhibitor is considered to occur practically at a single step, so that only binary complexes of the inhibitor with the various enzyme forms are considered, namely EI_2, ESI_2 and ES_2I_2 [15]:

$$v = \frac{V_{\max}[S]}{K_M\left(1+\frac{[I]^2}{(\overline{K}_{i1})^2}\right)+[S]\left(1+\frac{[S]}{K_{iS}}\right)\left(1+\frac{[I]^2}{(\overline{K}_{i2})^2}\right)} \quad (3)$$

In Equation (3), $\overline{K}_{i1}$ and $\overline{K}_{i2}$ are composite dissociation constants for the combination of at leasttwo inhibitor molecules with free enzyme or with substrate-complex enzyme, respectively. Fitting of Equation (3) produced more realistic parameters as revealed by the second column in Table 1. Figure 2, in turn, allows a graphical comparison of the experimental and calculated points, as the continuous lines were calculated according to Equation (3) using the optimized parameters. The K_M, V_{max} and K_{iS} values were close to those found when fitting Equation (2), but with smaller standard deviations. Equation (3) is no doubt a better description of the experimental data, as revealed by the smaller sum of squared deviations and greater model selection criterion, which are also given in the last two lines of Table 1. The $\overline{K}_{i1}$ and $\overline{K}_{i2}$ values, finally, are very close to each other, their values being within the range of the concentrations that were employed in the present experiments and their standard deviations being acceptable if one considers the complexity of the underlying phenomena. It should be noted that the similarity of the $\overline{K}_{i1}$ and $\overline{K}_{i2}$ values (they differ by only 3.4%) allows classification of the inhibition caused by the condensed tannin of the *pinhão* coat tannins as of the simple parabolic non-competitive type [13,14]. In this case, the value of the inhibition constants becomes also the approximate concentration of the condensed tannin that produces 50% inhibition at a fixed substrate concentration, *i.e.*, $[I]_{50} \approx \overline{K}_{i1} \approx \overline{K}_{i2}$. This postulate can be easily tested by examining the reaction rate *versus* inhibitor concentration curve in Figure 1A. Numerical interpolation yielded 316.3 μg/mL as the concentration producing 50% inhibition, a value that is very close to that predicted by the optimized inhibition constants.

The substrate inhibition caused by *p*-nitrophenyl palmitate was not very pronounced, as revealed by comparison of the K_M and K_{iS} values, the latter exceeding the former by a factor of 24.4 (see Table 1). However, it was consistently present in the four experimental curves that were measured in the present work, and if neglected, it would have interfered significantly with the interpretation of the results. It must be mentioned, however, that substrate inhibition is a frequently-reported phenomenon with pancreatic lipase [16], as well as with other lipases [17]. It should be noted, on the other hand, that the general kinetics and K_M values of lipases can be expected to vary considerably depending on the way in which the lipophilic substrate is solubilized and the general conditions of the assay. A K_M of 2.7 μM has been reported, for example, for pancreatic lipase hydrolyzing the same substrate used in the present work,

but in a medium containing 5 mM sodium deoxycholate in addition to 10% isopropanol and 50 mM phosphate buffer at pH 8.0 [18].

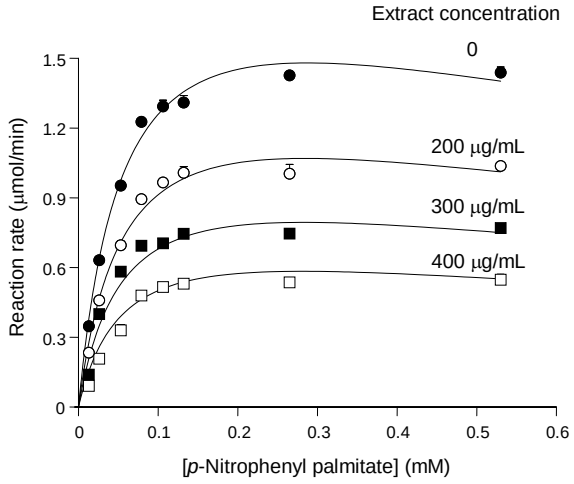

Figure 2. Substrate and inhibitor concentration dependences of the reaction rates of pancreatic lipase. Initial reaction rates, measured as described in the Experimental Section, were represented against the substrate concentration. The *pinhão* coat extract concentration in the assay medium is given on each curve. Each datum point represents the mean of at least three measurements. The error parameters are mean standard errors, which are not visible when smaller than the symbol size. The continuous lines represent the theoretical curves calculated according to Equation (3) with the optimized parameters listed in Table 1.

Table 1. Kinetic parameters of porcine pancreatic lipase obtained by fitting Equations (2) and (3) to the experimental data. Both equations were fitted simultaneously to the four substrate saturation curves obtained with the different *pinhão* coat extract concentrations shown in Figure 2. A non-linear least-squares procedure was used. The error terms correspond to standard deviations of the optimized parameters. The model selection criterion was 4.448, the correlation coefficient 0.995 and the sum of squared deviations 0.0541.

Parameters	**Optimized Values**	
	Equation (2)	**Equation (3)**
K_M (mM)	0.0583 ± 0.0077	0.0581 ± 0.0068
V_{max} (µmol· min^{-1})	2.094 ± 0.126	2.079 ± 0.112
K_{iS} (mM)	1.401 ± 0.337	1.418 ± 0.311
K_{i1} (µg· mL^{-1})	97,620 ± 15,080,211	—
K'_{i1} (µg· mL^{-1})	1.090 ± 168.991	—
K_{i2} (µg· mL^{-1})	1604.5 ± 1887.5	—
K'_{i2} (µg· mL^{-1})	82.19 ± 121.9	—
$\overline{K}_{i1}$ (µg· mL^{-1})	—	332.7 ± 146.1
$\overline{K}_{i2}$ (µg· mL^{-1})	—	321.2 ± 93.0
Sum of squared deviations	0.0614	0.0541
Model selection criterion	4.210	4.448

3.3. Effects on Triglyceride Absorption

The ability of the *pinhão* coat extract rich in tannins as an inhibitor of pancreatic lipase seems to be well demonstrated by the *in vitro* experiments. Confirmation of its effectiveness on the triglyceride hydrolysis *in vivo* can be obtained by means of experiments in which the blood levels of triglycerides are measured after an oral administration of these lipids. This test is based on the well-established notion that triglyceride hydrolysis is essential for its effective intestinal absorption along a mechanism that involves hydrolysis and re-esterification [8]. Figure 3 shows the results of the experiments in which an oral load of olive oil was applied to mice alone or in combination with three different doses of the *pinhão* coat extract, namely 100 mg/kg, 250 mg/kg and 500 mg/kg. The triglyceride levels were monitored during 6 h after administration. When water was given, the triglyceride levels remained more or less constant at around 115 mg/dL, providing a convenient base line for the 6-h period. Administration of olive oil alone caused a substantial rise in the plasma triglyceride levels, which reached values slightly above 400 mg/dL after 3 h. After six hours, they had almost declined to the basal levels. The triglyceride levels were reduced upon the *pinhão* coat extract administration, the effect being a function of the dose that was administered. This effect *versus* dose relationship can be seen in Figure 4 in which the areas under the dose-response curves were plotted against the *pinhão* extract doses. The base-line for calculating the areas under the curves in Figure 4 was provided by the plasma triglyceride levels measured after water administration (Figure 3). These areas have the dimensions of (g/dL × h), and they are a function of the lipid load that was effectively absorbed by the intestine. Figure 4 shows that the areas under the curves declined almost linearly with the extract dose that was administered. At the highest dose (500 mg/kg), it was diminished by 84% relative to the control (no extract administration); a 50% reduction can be expected at a dose around 258.9 mg/kg.

Figure 5 shows the results of positive control experiments in which orlistat (50 mg/kg) and *A. mearnsii* tannin (500 mg/kg) were administered to mice in the same manner as the *pinhão* coat extract. It is apparent that both orlistat (a classical lipase inhibitor) and the *A. mearnsii* tannin were quite effective at diminishing triglyceride absorption, the area under the corresponding curves representing less than 5% of the area under the control curve. This is not surprising for orlistat, which is a covalent inhibitor of pancreatic lipase [7], but is not necessarily expected from the *A. mearnsii* tannin, which is a much weaker lipase inhibitor than the *pinhão* coat tannin (see Figure 1). It should be mentioned that in this respect, the commercial *A. mearnsii* tannin preparation used in the present work was even more effective than the *A. mearnsii* polyphenol preparation used in previous studies [7], which diminished by 65% the area under the curve when a 500-mg/kg dose was administered in experiments similar to those of the present work. The difference between the *pinhão* coat tannin and the *A. mearnsii* tannin preparation in affecting triglyceride absorption may have several causes. The *pinhão* coat extract is certainly a better inhibitor of the *p*-nitrophenyl palmitate hydrolysis than the *A. mearnsii* tannin under the conditions of our assay. The latter, however, was done in the presence of isopropanol and with a non-natural substrate (*p*-nitrophenyl-palmitate). One cannot exclude that, under physiologic conditions, with the natural substrates (long-chain triglycerides)) and in the presence of bile acids and colipase, the *A. mearnsii* tannin reveals itself to be a better inhibitor than the *pinhão* coat extract. It should also be considered that the enzyme used in the *in vitro* assays was obtained from pig pancreas, and the *in vivo* experiments were done with mice. The actions of both the *A. mearnsii* tannin and the *pinhão* coat extract on both enzymes

can be different. Finally, it is possible that the mouse responds differently to both preparations. This includes different gastric movements, but also the possibility that the *A. mearnsii* tannin site of action is not restricted to pancreatic lipase. Irrespective of the real reason for the discrepancy, a definitive explanation depends on additional conclusive evidence.

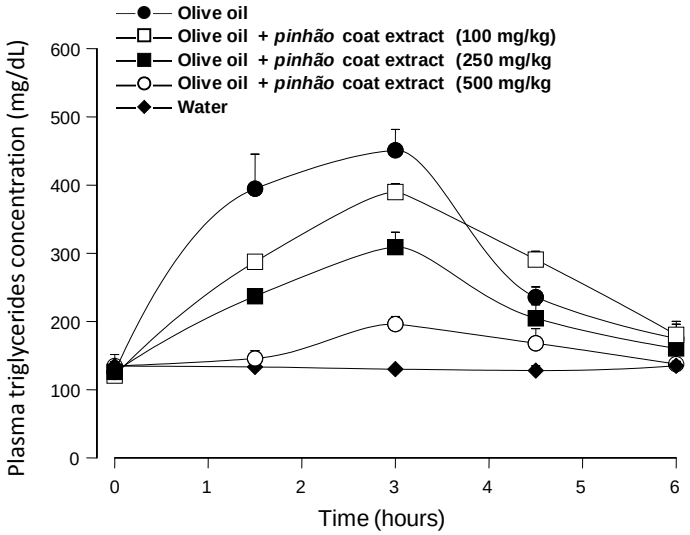

Figure 3. Blood triglyceride concentration profiles after intragastric olive oil loads in mice: the effect of the *pinhão* coat extract. The oral administration of olive oil was done immediately after the oral administration of three different doses of the *pinhão* coat extract. The doses that were administered are given on the graphs. Plasma triglycerides were measured as described in the Experimental Section. Each value represents the mean ± SD of three mice.

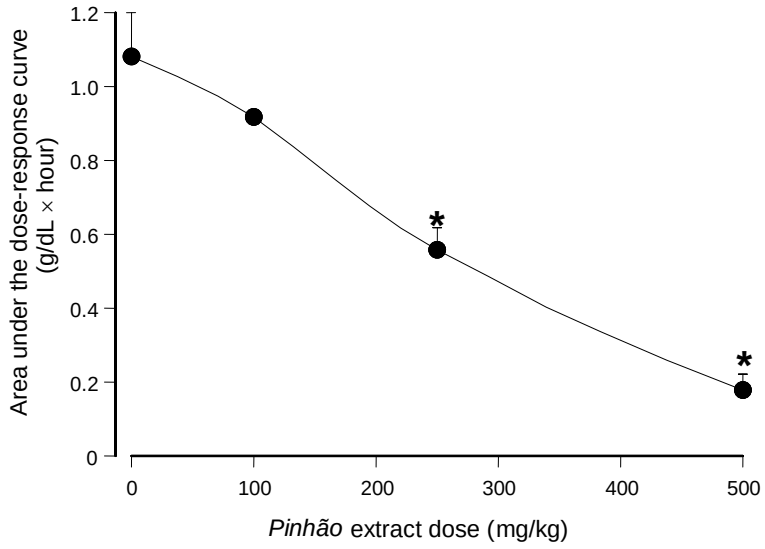

Figure 4. Dependence of the area under the plasma triglyceride concentration *versus* time on the administered *pinhão* coat extract dose. The areas were obtained from the results shown in Figure 3. Each value represents the mean ± SD of three mice. Asterisks indicate statistical significance relative to the control ($p \leqslant 0.05$ according to the Student–Newman–Keuls test).

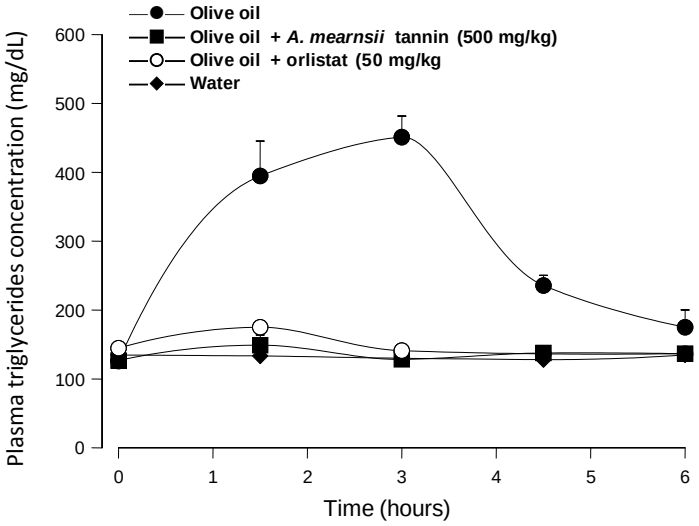

Figure 5. Blood triglyceride concentration profiles after intragastric olive oil loads in mice: The effects of orlistat and *A. mearnsii* tannin. The oral administration of olive oil was done immediately after the oral administration of orlistat and *A. mearnsii* tannin.The doses that were administered are given in the graphs. Plasma triglycerides were measured as described in the Experimental Section. Each value represents the mean ± SD of three mice.

A mechanism for the anti-obesity actions of an *A. mearnsii* polyphenol preparation has been proposed as consisting of a combination of lipase inhibition and maltase and sucrase inhibition [7]. The first action reduces triglyceride absorption and the second sugar absorption. A similar anti-obesity action can be proposed for the *pinhão* coat tannin based on the results obtained in the present work and on the inhibitory actions of the same preparation on both salivary and pancreatic α-amylases and on starch absorption that have been demonstrated in a previous work [5]. It remains to be investigated if absorbable constituents of the *pinhão* coat extract are able to alter the expression of obesity and diabetes-related genes in the adipose tissue, muscle and liver, as demonstrated to occur when mice are treated with an *A. mearnsii* polyphenol preparation [19]. On the other hand, it is important to emphasize that the actions demonstrated in the present work for the *pinhão* coat extract are similar not only to those proposed for the *A. mearnsii* polyphenols [7,19], but also for analogous constituents of other plants, such as apples [6] and several types of tea [20,21].

Inhibition of pancreatic lipase, as well as of α-amylases by the *pinhão* coat tannin requires binding to these enzymes, a phenomenon that was clearly demonstrated to exist for several combinations of tannins and proteins, including enzymes [22–24]. Binding of tannins to proteins involves both hydrophilic and hydrophobic interactions. It is non-specific in some cases and specific with a certain degree of cooperativity in others [22]. In the case of the *pinhão* coat condensed tannin, where procyanidins predominate [5], binding is a complex phenomenon, as indicated by the parabolic inhibition kinetics and probably facilitated by the numerous hydroxyl groups. These groups are probably responsible for the most important interactions at low concentrations [24]. At high concentrations, however, random hydrophobic stacking of the planar rings may occur between tannin and protein, as deduced for the wine tannins binding to saliva proteins based on nuclear magnetic resonance and molecular modeling [24].

4. Conclusions

The results obtained in the present study revealed that the *pinhão* coat tannin is an effective inhibitor of pancreatic lipase. Consistently, it was also effective at diminishing the plasma triglyceride levels in mice after a load of olive oil. This is most probably the consequence of an indirect inhibition of triglyceride absorption via inhibition of pancreatic lipase [8]. For the *pinhão* coat tannin, this is the second report of a biological activity, the first one being a similar inhibition of the absorption of glucose derived from starch as a consequence of an inhibitory action on α-amylase [5]. All of these actions are compatible with a potential anti-obesity action, as suggested for other polyphenol or tannin-rich preparations [5,6,20,21].

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Author Contributions

Roselene Ferreira Oliveira, Geferson Almeida Gonçalves, Fabíola Dorneles Inácio and Eloá Angélica Koehnlein conducted the experiments. Cristina Giatti Marques de Souza, Adelar Bracht and Rosane Marina Peralta planned the experiments. Adelar Bracht and Rosane Marina Peralta performed the calculations and wrote the article.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Shofield, P.; Mbugua, D.M.; Pell, A.N. Analysis of condensed tannins: A review. *Anim. Feed Sci. Tech.* **2001**, *91*, 21–40. [CrossRef]
2. Siqueira, C.F.Q.; Cabral, D.L.V.; Sobrinho, T.J.S.P.; Amorim, E.L.C.; Melo, J.G.; Araújo, T.A.S.; Albuquerque, U.P. Levels of tannins and flavonoids in medicinal plants: Evaluating bioprospecting strategies. *Evid. Based Complement. Alternat. Med.* **2012**. [CrossRef] [PubMed]
3. Yao, K.; He, Q.; Jia, D.Y.; Shi, B. The potential of wattle tannin extracts for fine use. *Nat. Prod. Res.* **2006**, *20*, 271–278. [CrossRef] [PubMed]
4. Cladera-Olivera, F.; Noreña, C.P.Z.; Pettermann, A.C.; Marczak, L.D.F. Influence of cooking in sorption isotherms of *pinhão* (*Araucaria angustifolia* seeds). *Latin Am. Appl. Res.* **2012**, *42*, 11–18.
5. Silva, S.M.; Koehnlein, E.A.; Bracht, A.; Castoldi, R.; Morais, G.R.; Baesso, M.L.; Peralta, R.A.; Souza, C.G.M.; Sá-Nakanishi, A.B.; Peralta, R.M. Inhibition of salivary and pancreatic α-amylases by a *pinhão* coat (*Araucaria angustifolia*) extract rich in condensed tannin. *Food Res. Int.* **2014**, *56*, 1–8. [CrossRef]

6. Sugiyama, H.; Akasome, Y.; Shoji, T.; Yamaguchi, A.; Yasue, M.; Kanda, T.; Ohtake, Y. Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. *J. Agric. Food Chem.* **2007**, *55*, 4604–4609. [CrossRef] [PubMed]
7. Ikarashi, N.; Takeda, R.; Ito, K.; Ochiai, W.; Sugiyama, K. The inhibition of lipase and glucosidase activities by *Acacia* polyphenol. *Evid. Based Complement. Alternat. Med.* **2011**. [CrossRef] [PubMed]
8. Ros, E. Intestinal absorption of triglycerides and cholesterol. Dietary and pharmacological inhibition to reduce cardiovascular risk. *Atherosclerosis* **2000**, *151*, 357–379.
9. Winkler, U.K.; Stuckmann, M. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.* **1979**, *138*, 663–670. [PubMed]
10. Wilkinson, G.N. Statistical estimations in enzyme kinetics. *Biochem. J.* **1961**, *80*, 324–332. [PubMed]
11. Cornish-Boaden, A. *Fundamentals of Enzyme Kinetics*; Springer Netherlands: Berlin, Germany, 1996.
12. Akaike, H. A new look at statistical model identification. *IEEE Trans. Automat. Control* **1973**, *19*, 716–723. [CrossRef]
13. Cleland, W.W. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: Nomenclature and theory. *Biochim. Biophys. Acta* **1963**, *67*, 173–187.
14. Plowman, K.M. *Enzyme Kinetics*; McGraw-Hill Book Company: New York, NY, USA, 1972.
15. Desseaux, V.; Koukiekolo, R.; Moreau, Y.; Santimone, M.; Marchis-Mouren, G. Mechanism of porcine pancreatic α-amylase: Inhibition of amylose and maltopentose hydrolysis by various inhibitors. *Biologia* **2002**, *57*, 163–170.
16. German, A.B.; Nekliudov, A.D.; Ivankin, A.N.; Berdutina, A.V. Reaction kinetics of hydrolysis of animal fat by pancreatic lipase. *Prikl. Biohim. Mikrobiol.* **2002**, *38*, 604–608.
17. Jansen, H.; Hülsmann, W.C. Long-chain acyl-CoA hydrolase activity in serum: Identity with clearing factor lipase. *Biochim. Biophys. Acta* **1973**, *296*, 241–248. [CrossRef]
18. Lewis, D.R.; Liu, D.J. Direct measurement of lipase inhibition by orlistat using a dissolution linked *in vitro* assay. *Clin. Pharmacol. Biopharm.* **2012**. [CrossRef] [PubMed]
19. Ikarashi, N.; Takeda, R.; Ito, K.; Ochiai, W.; Sugiyama, K. Anti-Obesity and anti-diabetic effects of Acacia polyphenol in obese diabetic KKAy mice fed high-fat diet. *Evid. Based Complement. Alternat. Med.* **2011**. [CrossRef] [PubMed]
20. Unno, T.; Tago, M.; Suzuki, Y.; Nozawa, A.; Sagesaka, Y.M.; Kakuda, T.; Egawa, K.; Kondo, K. Effect of tea catechins on postprandial plasma lipid responses in human subjects. *Br. J. Nutr.* **2005**, *93*, 543–547.
21. Gondoin, A.; Grussu, D.; Stewart, D.; McDougall, G.J. White and green tea polyphenols inhibit pancreatic lipase *in vitro*. *Food Res. Int.* **2010**, *43*, 1537–1544. [CrossRef]
22. Frazier, R.A.; Papadopoulou, A.; Mueller-Harvey, I.; Kissoon, D.; Green, R.J. Probing protein-tannin interactions by isothermal titration microcalorimetry. *J. Agric. Food Chem.* **2003**, *51*, 5189–5195. [CrossRef] [PubMed]

23. Barrett, A.; Ndou, T.; Hughey, C.A.; Straut, C.; Howell, A.; Dai, Z.; Keletunc, G. Inhibition of α-amylase and glucoamylase by tannins extracted from cocoa, pomegranates, cranberries, and grapes. *J. Agric. Food Chem.* **2013**, *61*, 1477–1486. [CrossRef] [PubMed]
24. Cala, O.; Pinau, N.; Simon, C.; Fouquet, E.; Laguerre, M.; Dufourc, E.J.; Pianet, I. NMR and molecular modeling of wine tannins binding to saliva proteins: Revisiting adstringency from molecular and colloidal prospects. *FASEB J.* **2010**, *24*, 4281–4290. [CrossRef] [PubMed]