**UNIVERSIDADE ESTADUAL DE MARINGÁ**
**CENTRO DE CIÊNCIAS AGRÁRIAS**
**Programa de Pós-Graduação em Ciência de Alimentos**

# Efeitos inibitórios de extrato de casca de pinhão (*Araucaria angustifolia*) nas alfa-amilases salivar e pancreática

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Maringá

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Dissertação apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de Mestre em Ciência de Alimentos, orientada pela Prof. Dra. Rosane Marina Peralta.

Maringá

2013

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

S586e

Silva, Simone Mariano da
Efeitos inibitórios de extrato de casca de pinhão (Araucária Angustifolia) nas alfa-amilases salivar e pancreática / Simone Mariano da Silva. -- Maringá, 2013.
33 f. : il., color., tabs., figs., grafs.

Orientador: Profª. Drª. Rosane Marina Peralta.
Dissertação (mestrado) - Universidade Estadual de Maringá, Centro de Ciências Agrárias, Programa de Pós-Graduação em Ciência de Alimentos, 2013.

1. Inibidores da α-amilase. 2. Diabetes. 3. Perda de peso. 4. Pinhão. 5. Taninos condensados. I. Peralta, Rosane Marina, orient. II. Universidade Estadual de Maringá. Centro de Ciências Agrárias. Programa de Pós-Graduação em Ciência de Alimentos. III. Título.

CDD 21.ed. 572.36

AHS

# AGRADECIMENTOS

Primeiramente e sem clichês, quero agradecer a Deus que sempre foi meu pai querido e meu amigo. Ele me capacitou nos dias em que nem eu acreditei em mim mesma, me trouxe paciência nos momentos de raiva, me deu ânimo quando me cansei, me fez prosseguir quando pensei em desistir, me trouxe paz no momento da tempestade, se fez presente quando me senti sozinha e em muitas vezes em lágrimas me confortou, me abraçou e meu deu colo. E hoje se faz presente mais uma vez me direcionando a cumprir o seu propósito em minha vida.

Agradeço a toda equipe do Laboratório de Bioquímica de Microrganismos da UEM, alunos, estagiários, professores e em especial a técnica Maria Aparecida (Pingo) que me auxiliou desde o primeiro dia que eu fui ao laboratório, me ensinando com sua infinita paciência. E a Alvina que em muitas vezes me fez rir e deixou meu dia mais feliz e que sem ela todo o trabalho do laboratório ficaria muito difícil.

A Profa. Dra. Rosane Marina Peralta pela disposição e paciência em me orientar nesse trabalho. Ao Prof. Dr. Adelar Abracht pela disposição em me auxiliar com a pesquisa e os dados finais.

Aos amigos do laboratório Fabiola, Carol, Josielle e Roselene que foram muito mais que amigas para mim. Cada uma foi em especial como uma mãe, como uma irmã, como uma amiga de infância, como alguém entre poucos que acreditaram e apostaram em mim. Serão pessoas que guardarei pra sempre comigo.

Quero agradecer imensamente a técnica Irene do biotério por ter me ajudado na última etapa da minha pesquisa com os ratinhos. Ela foi uma pessoa muito especial nesse processo e sem ela talvez não tivesse conseguido. Foi uma companheira, amiga e conselheira sempre me motivando. A Andréia que também foi peça fundamental nessa fase, sem ela também minha pesquisa *in vivo* talvez não saísse, obrigada de coração pela disposição e interesse em me ajudar.

Ao meu namorado Alexandre que no começo me ajudou a ter confiança, ter foco e correr atrás dos meus objetivos e vencer essa etapa. Obrigada pelo carinho, amor e afeto incomparável o grande amor da minha vida.

As minhas amigas Mayara e Juliana e minha irmã adorável Márcia Denise, que sei que nesse momento estão orgulhosas de mim, pois sabem o quanto isso é importante e o quão difícil foi chegar até aqui hoje.

Quero agradecer também aos meus pais, meu irmão e alguns familiares que de alguma forma me fizeram ser mais forte e provar que eu conseguiria começar e chegar até o fim.

A CAPES pela bolsa de pesquisa concedida ao Programa de Ciência de Alimentos da UEM.

# BIOGRAFIA

Simone Mariano da Silva nasceu em 28 de fevereiro de 1985 na cidade de Maringá -PR. Possui graduação em Nutrição pelo Centro Universitário de Maringá- CESUMAR, pós-graduação em Qualidade em Serviços de Alimentação pela Pontifícia Universidade Católica- PUC e aperfeiçoamento em Merenda escolar pela Universidade Federal do Paraná-UFPR. Tem experiência em alimentação do escolar, serviços de alimentação e nutrição clínica. Atualmente aluna de mestrado do Programa de Pós-graduação em Ciência de Alimentos da Universidade Estadual de Maringá, desenvolvendo sua dissertação de mestrado no Laboratório de Bioquímica de Microrganismos e trabalhando principalmente com o tema relacionado à inibição de amilase *in vitro* e *in vivo*.

# APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico.

S.M. Silva, E.A. Koehlein, A. Bracht, R. Castoldi, C.G.R.de Morais, M.L.Baesso,R.A. Peralta, R.M. Peralta. Inhibitory effects of a tannin rich preparation from pinhão (*Araucaria angustifolia*) coat on human salivary and porcine pancreatic alpha-amylases, submetido ao periódico científico Evidence-Based Complementary and Alternative Medicine.

# RESUMO GERAL

**INTRODUÇÃO E OBJETIVOS -** Taninos são moléculas consideradas como inibidoras de diversas hidrolases, incluindo as amilases. A descoberta de novos materiais ricos em taninos com propriedades inibidoras de amilases pode contribuir para a descoberta de novas drogas úteis para o controle e tratamento de diabetes, obesidade e outras desordens fisiológicas. *Araucaria angustifolia* é uma conífera nativa da América do Sul encontrada no Sul e Sudoeste do Brasil e Norte da Argentina. A casca da semente do pinhão é rica em taninos e até o momento, pouco explorada em estudos científicos. O objetivo deste trabalho foi investigar os possíveis efeitos de um extrato de casca de pinhão rico em taninos na atividade das amilases salivar e pancreática *in vitro* e *in vivo*. Todos os resultados obtidos com o extrato de casca de pinhão foram comparados com os obtidos utilizando-se um tanino comercial de *Acacia mearnsii* e acarbose, um bem conhecido inibidor de amilases.

**MÉTODOS -** $\alpha$-Amilase salivar humana e $\alpha$-amilase pancreática suína foram adquiridas da Sigma-Aldrich Co. Tanino comercial foi obtido junto à Labsynth. As sementes do pinhão foram cozidas em água sob pressão por 30 min. Após o cozimento, as sementes foram descascadas e as cascas secas foram trituradas até pó fino. Ao pó das sementes foi adicionado etanol a 70% em água e a mistura mantida sob agitação para extração dos taninos. O etanol foi eliminado por rota-evaporação e o material foi posteriormente liofilizado para eliminação da água. O pó seco obtido foi mantido em freezer até uso. Os experimentos cinéticos com a amilase salivar foram realizados a 40ºC em tampão Tris-HCl 50 mM, pH 8,0, contendo EDTA 10 mM e azida sódica 1 mM. Os experimentos com a amilase pancreática foram realizados em tampão fosfato 20 mM, pH 6,9 contendo NaCl 6,7 mM. Amido de batata foi utilizado como substrato. Sua hidrólise foi medida na ausência ou presença de um dos inibidores. Após 5 min, os açúcares redutores produzidos foram quantificados pelo método do ácido 3,5 dinitro-salicílico, utilizando maltose como padrão. Para o teste de tolerância oral ao amido, ratos machos Wistar (200-250 g) foram divididos em 5 grupos (n=4). Para os animais do grupo I (controle positivo), amido comercial de milho (1 g por kg de peso) foi administrado por gavagem. Os animais do grupo II (controle negativo) receberam água. Os animais do grupo III receberam amido comercial mais acarbose (50 mg/kg). Os animais do grupo IV receberam amido comercial mais tanino comercial (250 mg/kg). Os animais do grupo V receberam amido comercial mais extrato de casca de pinhão (250 mg/kg). A glicemia foi determinada antes da administração do amido (tempo zero) e após 15, 30, 45 e 60 min. As amostras de sangue foram obtidas da veia da cauda e a glicemia avaliada com auxílio de um glicosímetro (Accu-Chek® Active Glucose meter).

**PRINCIPAIS RESULTADOS -** A dependência da concentração dos inibidores foi avaliada a uma concentração fixa de amido variando-se as concentrações dos inibidores extrato de casca de pinhão, tanino comercial e acarbose. Todos os valores obtidos foram expressos como frações dos valores obtidos na ausência dos inibidores e plotados contra as concentrações dos inibidores. Os inversos das velocidades relativas foram representados para avaliar de forma preliminar se as inibições eram do tipo linear, parabólica ou hiperbólica. O efeito inibitório do extrato da casca do pinhão sobre a amilase salivar foi pequeno a baixas concentrações, mas acelerou a concentrações mais altas de forma que a dependência de 1/v da concentração foi parabólica pelo menos até 80 $\mu$g/mL. Para o tanino de *Acacia mearnsii* a atividade da enzima diminui quase que

linearmente até 500 μg/mL, resultando numa inibição parabólica. Para a acarbose, a dependência foi côncava até 8 μg/mL resultando em uma dependência linear de 1/v. A amilase pancreática foi mais sensível a todos os inibidores. Para o extrato de pinhão a inibição foi do tipo parabólico, mas foi linear para o tanino de *Acacia mearnsii.* Finalmente, foi do tipo parabólico com a acarbose. A análise cinética revelou para os três inibidores uma inibição do tipo mista. Para testar a efetividade dos inibidores *in vivo*, os níveis glicêmicos foram avaliados após o consumo de amido na presença ou ausência dos inibidores. Quando amido sozinho foi administrado a ratos em jejum, a glicemia elevou-se após 60 min. Para os ratos controle, os níveis glicêmicos permaneceram inalterados. Ambos acarbose e tanino de *A. mearnsii* preveniram consideravelmente a elevação da glicemia. O extrato de casca de pinhão teve um efeito similar, mas após 45 min, um aumento da glicemia foi observado, seguido de uma redução aos 60 min para valores próximos aos basais. Avaliações das áreas sobre as curvas permitem concluir que os três inibidores foram efetivos em prevenir a elevação da glicemia após ingestão de amido.

**DISCUSSÃO E CONCLUSÃO -** Os resultados obtidos neste estudo revelam que o extrato de casca de pinhão é um inibidor eficiente das amilases salivar e pancreática *in vitro.* O extrato de casca de pinhão foi eficiente também em reduzir a glicemia pós-prandial após administração de amido. O padrão cinético da inibição das amilases pelo extrato de casca de pinhão é complexo, e a natureza parabólica da inibição sugere a formação de complexos $EI_2$ e $ESI_2$. Complexidade similar foi detectada neste trabalho também para os inibidores tanino de *A. mearnsii* e acarbose. Comparando-se as constantes de inibição, a seguinte sequência pode ser escrita tanto para a amilase salivar quanto para a amilase pancreática: acarbose > extrato de casca de pinhão >tanino de *A. mearnsii*. O extrato de casca de pinhão pode ser útil para suprimir a hiperglicemia pós prandial em pacientes diabéticos. Ao reduzir a ingestão calórica pela inibição do metabolismo de carboidratos, o extrato de casca de pinhão pode, ao menos em princípio, promover a perda de peso e combater a obesidade. Para compreensão dos mecanismos inibitórios envolvidos, novos estudos visando a identificação dos principais componentes presentes no extrato estão em andamento.

**PALAVRAS-CHAVE:** inibidores da α-amilase; Diabetes; Perda de peso; Pinhão; Taninos condensados.

# GENERAL ABSTRACT

**INTRODUCTION AND AIMS -** Tannins are one of the most extensively studied molecules able to inhibit amylases. It is generally believed that the discovery of new materials rich in tannins with enzyme inhibitory properties can contribute for the discovery of new drugs useful in the control and treatment of diabetes, obesity and other physiological disorders. *Araucaria angustifolia* is a native conifer from South America, growing in southern and southeastern Brazil and northeastern Argentina. The *pinhão* coat is rich in tannins and up to now scarcely explored scientifically. The purpose of the present work was to investigate the possible effects of a pinhão coat extract rich in tannins on the activity of both the salivary and pancreatic $\alpha$-amylases under in vitro and in vivo conditions. All results obtained with the pinhão coat extract were compared with those obtained with a commercial tannin from *Acacia mearnsii* and with acarbose, a well known inhibitor of amylases.

**METHODS -** Human salivary $\alpha$-amylase and porcine pancreatic $\alpha$-amylase were obtained from Sigma-Aldrich Co. The commercial tannin was purchased from Labsynth, Brazil. Acarbose was supplied by Sigma-Aldrich Co. The whole *A. angustifolia* seeds were cooked in water for 30 min in a pressure pot. After cooking, the seed coats were trimmed off and milled until fine powder. The seed coat powder was mixed with 70% ethanol (in water) at room temperature and mantained under agitation. The mixtures were filtered through Whatman filter paper number 1. The filtrate was concentrated with a rotary vacuum evaporator at 40 °C to eliminate ethanol and finally freeze-dried. The freeze-dried powders were stored in freezer until use. The kinetic experiments with the human salivary $\alpha$-amylase were carried out at 40 $^{o}$C in 50 mM Tris-HCl buffer pH 8.0 containing 10 mM EDTA and 1 mM sodium azide. The experiments with the porcine pancreatic $\alpha$-amylase were carried in 20 mM phosphate buffer, pH 6.9, containing 6.7 mM NaCl. Potato starch (Sigma-Aldrich) was used as substrate. Substrate and one of the three inhibitors, acarbose, commercial tannin or pinhão coat extract were mixed and the reaction was initiated by adding the enzyme. The reaction was allowed to proceed for 5 min. The produced reducing sugars were assayed by the dinitrosalicylic acid method, using maltose as standard. Male healthy Wistar rats weighing 200–250 g were used in all *in vivo* experiments. For oral starch tolerance test, rats were divided into 5 groups (n = 4 rats per group). To group I (positive control) commercial corn starch (1 g per kg body weight) was administered intragastrically. Group II (negative control) received only tap water. Group III received intragastrically commercial corn starch plus acarbose (50 mg/kg). Group IV received intragastrically commercial corn starch plus tannic acid (250 mg/kg). Finally, group V received intragastrically commercial corn starch plus pinhão extract (250 mg/kg). Fasting blood glucose levels were determined before the administration of starch and amylase inhibitors (0 time). Later evaluations of blood glucose levels took place at 15, 30, 45 and 60 min. Blood samples from the tail vein were analyzed by means of a glucometer.

**MAIN RESULTS -** The inhibitor concentration dependences were measured at fixed starch concentrations and varying *pinhão* coat extract, *Acacia mearnsii* tannin and acarbose concentrations. All rates obtained in the presence of inhibitors were expressed as fractions of the rates measured in the absence of inhibitor (relative rates) and plotted against the inhibitor concentrations. In addition to the relative rates the inverse relative rates were also represented in

order to evaluate in a preliminary way if inhibition is linear or if it is of some other type (parabolic or hyperbolic). The inhibitory effect of the *pinhão* coat extract on the salivary α-amylase was small at low concentrations, but it accelerated at higher concentrations, so that the 1/v concentration dependence resulted in a parabolic relationship at least in the range up to 80 μg/mL. With the *Acacia mearnsii* tannin in the range up to 500 μg/mL the enzyme activity decreased almost linearly with the concentration. This feature also leads to a parabolic 1/v versus concentration plot. For acarbose the dependence was concave up in the range up to 8 μg/mL, resulting in a linear dependence of 1/v from the concentration. The porcine pancreatic α-amylase was more sensitive to all inhibitors, so that lower concentrations were used. The inhibition caused by the *pinhão* coat extract was again parabolic in the range up to 50 μg/mL. With the *Acacia mearnsii* tannin up to 300 μg/mL the enzyme activity decreased almost linearly with the concentration, resulting in a parabolic 1/v versus concentration dependence. With acarbose, on the other hand, the concave up rate versus concentration curve produced a linear 1/v versus concentration relationship. The kinetic analysis revealed the mixed type of inhibition for the three inhibitors. For testing the effectiveness of the inhibitors under in vivo conditions, the glycemic levels were measured after starch ingestion in the presence and absence of inhibitors. When starch alone was administered to fasted rats, the blood glucose levels increased after 60 minutes. In control rats (no starch administration) the blood glucose levels did not change. Both acarbose and the *A. mearnsii* tannin prevented considerably the blood glucose elevation. The pinhão coat extract had a similar effect, but after 45 min a transient increase in glycemy was found, followed by a diminution at 60 min to values close to the basal ones. Evaluations of the areas under the curves allow to conclude that all three inhibitors were effective in preventing the elevation of glycemy after starch ingestion.

**DISCUSSION AND CONCLUSION -** The results obtained in this study revelead that the pinhão coat extract is an efficient inhibitor of the salivary and pancreatic α-amylases *in vitro.* The pinhão coat extract was also efficient in reducing the post-prandial glycemy after starch administration to rats. The kinetic pattern of the inhibition of the α-amylases by the pinhão coat extract is complex, and the parabolic nature suggests the formation of the $EI_2$ and $ESI_2$ complexes. A similar complexity was found in the present work also for the inhibitors *A. mearnsii* tannin and acarbose. Comparison of the inhibitor constants leads to the following decreasing sequence of potency which is valid for both the salivary and pancreatic α-amylases: acarbose > pinhão coat extract > *A. mearnsii* tannin. The pinhão coat extract could be useful to supress the post-prandial hyperglycemia in diabetic patients. By reducing the caloric intake in consequence of the inhibition of carbohydrate transformation, the pinhão coat extract can, in principle at least, promote weight loss and to combat obesity. For understanding the inhibitory mechanisms involved, new studies aiming the identification of the main components of the extract are presently being conducted.

**Keywords:** α-amylase inhibitors; Diabetes; Pinhão, Condensed Tannins, Weight loss.

# Inhibition of salivary and pancreatic α-amylases by a condensed tannin from the pinhão coat (*Araucaria angustifolia)*

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## Abstract

The purpose of the present work was to investigate the possible inhibitory effect of a *pinhão* coat extract rich in tannin on the activity of α-amylases (human salivary and porcine pancreatic). Experiments with the classical α-amylase inhibitor acarbose and a commercial tannin from *Acacia mearnsii* were done for comparative purposes. Fourier transform-infrared spectroscopy analysis of the pinhão coat tannin revealed a higher proportion of procyanidins to prodelphinidins when compared to the *A. mearnsii* tannin. The *pinhão* coat tannin was an effective inhibitor of both human salivary and porcine pancreatic α-amylase. Inhibition was of the mixed S-parabolic I-parabolic type. For the human salivary α-amylase the inhibition constants were 56.88±5.74 and 103.27±11.85 μg/L; for the porcine pancreatic α-amylase the inhibition constants were smaller, namely, 20.25±1.97 and 46.79±4.57 μg/L. The decreasing potency sequence was: acarbose>*pinhão* coat tannin> *A. mearnsii* tannin. Similarly to acarbose and the *A. mearnsii* tannin, the *pinhão* coat tannin was also effective in diminishing the post-prandial glycemic levels in rats after starch administration. The inhibitory properties of the *pinhão* coat tannin indicate that it can be used to suppress postprandial hyperglycemia in diabetic patients. Considering that the inhibition of carbohydrate metabolism or absorption can decrease the caloric intake, the *pinhão* coat tannin could also, in principle at least, be used to promote weight loss and to combat obesity.

**Keywords:** *Araucaria angustifolia*, Amylase inhibitors, Condensed tannins, Diabetes, Pinhão, Weight loss

## 1. Introduction

Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are enzymes which catalyze the hydrolysis of the (α-1,4) glycosidic linkages in starch and various other oligosaccharides.[1] Human α-amylases of both salivary (HSA) and pancreatic origins (HPA) have been thoroughly studied from the viewpoint of clinical chemistry because they are important indicators in the evaluation of pancreas and salivary glands diseases. They received extensive biochemical and structural characterization. Furthermore, they are used as targets for drug design in attempts to treat several diseases such as diabetes mellitus, obesity, hyperlipidemia, and caries. For these diseases the control of carbohydrate digestion and monosaccharide absorption could be helpful to avoid further complications. For example, long term complications of diabetes mellitus include retinopathy, nephropathy, neuropathy, microangiopathy and increased risk of cardiovascular disease. At least potentially the control of carbohydrate digestion and monosaccharide absorption can be brought about by means of enzyme inhibitors and in this particular aspect α-amylase inhibitors are especially promising.[2]

Several molecules have been reported to possess α-amylase inhibitory activity. Among these molecules are flavonoids, polyphenolics, tannins, terpenes and cinnamic acid derivatives.[3-9] Acarbose, a pseudo-tetrasaccharide, has gained especial attention because it is a highly effective inhibitor of intestinal α-glucosidases, such as sucrase, maltase, glucoamylase, and an extremely potent α-amylase inhibitor.[10] The compound is in clinical use for treatment of noninsulin- and insulin-dependent diabetes mellitus since the 1990s. It lowers postprandial glucose elevation in diabetics thereby reducing carbohydrate digestion after meals.[11] Acarbose is not appreciably absorbed into the bloodstream and therefore its action is largely confined to the intestine. It must be noted, however, that acarbose is transformed by small and large intestine carbohydrases to give acarviosine–glucose and glucose. When these degradation products arrive in the large intestine the microbial fermentation of glucose may cause moderated diarrhea associated to flatulence.[3] Frequently such effects lead to therapy discontinuation a reason why the introduction of new α-amylase inhibitors is a matter of interest. For this reason, the consumption of plant-extracts may be a more acceptable source of amylase inhibitors due to their low cost and low incidence of grastrointestinal side effects.

Among the tannins, one of the most extensively studied concerning its enzyme inhibitory property is that one extracted from the bark of the black wattle tree (*Acacia mearnsii*). It is rich in unique catechin-like flavan-3-ols, such as robinetinidol and fisetinidol and is able to inhibit several enzymes such as α-amylases, α-glucosidases and lipases.[12,13] It is

generally believed that the discovery of new materials rich in tannin with enzyme inhibitory properties can contribute for the discovery of new drugs useful in the control and treatment of diabetes, obesity and other physiological disorders.[14,15]

*Araucaria angustifolia* is a native conifer from South America, growing in southern and southeastern Brazil and northeastern Argentina.[16] Natural populations or plantations of *A. angustifolia* in Brazil are distributed primarily in the southernmost states of Paraná, Santa Catarina and Rio Grande do Sul. The seed of *A. angustifolia*, named *pinhão*, is a seasonal product that is produced in the period from April to August.[17] The coat of the *pinhão* seed is usually discarded and it needs a considerable time to decompose. This waste is considered to be a material rich in polyphenols.[18,19] Application of the *pinhão* wastes loaded with congo red for Cu(II) removal from aqueous solutions has been proposed as an ecologically correct procedure.[18] On the other hand, to our knowledge, there have been no attempts of evaluating the potential of this material as an enzyme inhibitor. Such a property can be expected by virtue of its high tannin content. To fill this gap, the objective of the present study was to investigate the possible effects of a *pinhão* coat extract rich in tannins on α-amylases. For comparative purposes, similar experiments were also run with acarbose and a commercial tannin preparation obtained from *Acacia mearnsii*. Both *in vitro* and *in vivo* experiments were done. The former comprised also a more or less extensive kinetic characterization of the inhibitory effects.

## 2. Materials and Methods

### *2.1. Materials*

Human salivary α-amylase (Type IXA) and porcine pancreatic α-amylase (Type VI-B) were obtained from Sigma-Aldrich Co. Commercial tannin was purchased from LABSYNTH, Brazil. According to the supplier, this tannin was obtained from *Acacia mearnsii* bark. Acarbose (empirical formula $C_{25}H_{43}NO_{18}$, molecular weight 645) was supplied by Sigma-Aldrich Co.

### *2.2. Obtainment of pinhão (A. angustifolia) coat extract rich in tannins*

The whole *A. angustifolia* seeds (edible part plus coat) were cooked in water for 30 min in a pressure pot.[19] After cooking the seed coats were trimmed off and milled until fine powder. The seed coat powder (100 g) was mixed with 300 ml of 70% ethanol (in water) at room temperature and mantained under agitation at 140 rpm for 3 h. The mixtures were filtered through Whatman filter paper number 1. The filtrate was concentrated with a rotary

vacuum evaporator at 40 °C to eliminate ethanol and finally freeze-dried. The freeze-dried powders were stored in freezer until use.

*2.3. Comparison of tannin structures by Fourier transform-infrared spectroscopy (FTIR) analysis*

FTIR was used to study the functional groups and molecular structure of the pinhão extracts and compare to well-known condensed tannin from *Acacia mearnsii.* For analysis, 2 mg of each dried sample were mixed with 200 mg KBr of spectroscopic grade and made in the form of pellets at pressure of about 1 MPa. Sample spectra were obtained in triplicates using an average of 128 scans over the range between 500 $cm^{-1}$ and 3500 $cm^{-1}$ with a spectral resolution of 2 $cm^{-1}$. Peak height and area of Fourier transform infrared spectra were determined by Opus software version 6.5 normalized by maximum and minimum peaks

*2.4. Reaction rates measurements*

The kinetic experiments with the human salivary α-amylase were carried out at 40 $^{o}$C in 50 mmol/L Tris-HCl buffer pH 8.0 containing 10 mmol/L EDTA and 1 mmol/L sodium azide. The experiments with the porcine pancreatic α-amylase were carried in 20 mmol/L phosphate buffer, pH 6.9, containing 6.7 mmol/L NaCl. Potato starch (Sigma-Aldrich) was used as substrate. The substrate (0.05-1.0 g/100 mL) and one of the three inhibitors, acarbose, *A. mearnsii* tannin and pinhão coat tannin were mixed and the reaction was initiated by adding the enzyme. The reaction was allowed to proceed for 5 min. The produced reducing sugars were assayed by the dinitrosalicylic acid method, using maltose as standard.[21] The pH of the reaction medium was tested for all situations. No changes were detected during the incubation time.

*2.5. Animal experiments*

Male healthy Wistar rats weighing 200–250 g were used in all experiments. The rats were housed, fed and treated in accordance with the universally accepted guidelines for animal experimentation. Prior to the investigations, the animals were kept for one week under the standard environmental conditions. Throughout the experimentation period the rats were maintained in single cages and had access to standard pellet diet and water *ad libitum*. Food was withdrawn 18 h before the experiments. Blood glucose from cut tail tips was determined using Accu-Chek® Active Glucose meter.

*2.6. Oral starch tolerance test*

Rats were divided into 5 groups (n = 4 rats per group). To group I (positive control) commercial corn starch (1 g per kg body weight) was administered intragastrically. Group II (negative control) received only tap water. Group III received intragastrically commercial corn starch plus acarbose (50 mg/kg). Group IV received intragastrically commercial corn starch plus *A. mearnsii* tannin (250 mg/kg). Finally, group V received intragastrically commercial corn starch plus pinhão tannin (250 mg/kg). Fasting blood glucose levels were determined before the administration of starch and amylase inhibitors (0 time). Later evaluations of blood glucose levels took place at 15, 30, 45 and 60 min. Blood samples from the tail vein were analyzed by means of a glucometer.

*2.7. Calculations and statistical criteria*

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 procedures using the Scientist software from MicroMath Scientific Software (Salt Lake City, UT). The decision about the most adequate model (equation) was based on the model selection criterium (MSC) and on the stardard deviations of the optimized parameters. The model selection criterium, which corresponds to the normalized Akaike Information Criterium,[22] is defined as:

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

$Y_{obs}$ are the experimental reaction rates, $\overline{Y}_{obs}$ 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. When the MSC values differed by less than 5% the most appropriate model was considered that one yielding the smallest standard deviations for the estimated parameters.

## 3. Results

### *3.1. FTIR spectroscopy*

FTIR spectroscopy was used to compare the well known *Acacia mearnsii* tannin with the pinhão coat tannin from *Araucaria angustifolia* in the search of similarities and differences. Fig. 1 shows the FTIR spectra of both tannins. Analysis reveals many similarities. These include, for example, the peaks in the vicinity of 1650–1450 $cm^{-1}$ (aromatic rings), the 1382 $cm^{-1}$ absorption band (O–H plane deformation in polyphenols) and the peaks at the 1283–1247 $cm^{-1}$ range (aromatic C–O bond stretching).[23] The main difference between the *Acacia mearnsii* tannin and the pinhão coat tannin is the single peak at 1520 $cm^{-1}$ in the latter. This is likely to indicate that the pinhão coat tannin consists predominantly of procyanidin[23] whereas the double peak at about 1520 $cm^{-1}$ of the *Acacia mearnsii* tannin indicates prodelphinidin units. This conclusion is reinforced by the pronounced bands at 780–770 $cm^{-1}$ in the pinhão coat tannin spectrum meanwhile the *Acacia mearnsii* tannin spectrum shows bands near to 730 $cm^{-1}$.[24]

### *3.2. Concentration dependences of the α-amylases inhibition*

The inhibitor concentration dependences were measured at fixed starch concentrations and varying *pinhão* coat tannin, *Acacia mearnsii* tannin and acarbose concentrations. The results of these measurements are summarized in the six panels of Figure 1. All rates obtained in the presence of inhibitors were expressed as fractions of the rates measured in the absence of inhibitor (relative rates) and plotted against the inhibitor concentrations. In addition to the relative rates the inverse relative rates were also represented in order to evaluate in a preliminary way if inhibition is linear or if it is of some other type (parabolic or hyperbolic). The panels in the left of Figure 1 (A, C and E) refer to the data obtained with the human salivary α-amylase. The inhibitory effect of the *pinhão* coat extract was small at low concentrations (Panel A), but it accelerated at higher concentrations, so that the 1/v versus concentration dependence resulted in a parabolic relationship at least in the range up to 80 μg/mL. With the *Acacia mearnsii* tannin in the range up to 500 μg/mL the enzyme activity decreased almost linearly with the concentration (Panel C). This feature also leads to a parabolic 1/v versus concentration plot. For acarbose (panel E) the dependence was concave up in the range up to 8 μg/mL, resulting in a linear dependence of 1/v from the concentration.

The porcine pancreatic α-amylase (right panels in Figure 1) was more sensitive to all inhibitors, so that lower concentrations were used. The inhibition caused by the *pinhão* coat extract was again parabolic in the range up to 50 μg/mL (Panel B). With the *Acacia mearnsii* tannin up to 300 μg/mL the enzyme activity decreased almost linearly with the concentration, resulting in a parabolic 1/v versus concentration dependence (Panel D). With acarbose, on the other hand, the concave up rate versus concentration curve produced a linear 1/v versus concentration relationship (Panel F).

*3.3. Steady-state kinetics*

To extend the analysis further, initial reaction rates were measured by varying simultaneously the substrate and the inhibitor concentrations. The results of these experiments are summarized in Figure 2. In the graphs of Figure 2 the initial rates were represented against the substrate concentrations. For each inhibitor the rates at varying substrate concentrations were measured with two different inhibitor concentrations in addition to the condition of inhibitor absence (control curves). The inhibitor concentrations are indicated on each graph. Typical saturation curves were obtained with both enzymes, the human salivary α-amylase (panels A, C, E) and the porcine pancreatic α-amylase (panels B, D and F). Preliminary graphical analysis (double reciprocal plots; not shown) revealed that competitive and uncompetitive inhibitions can be both ruled out. For this reason, the subsequent analysis was focused on the mixed type of inhibition (non-competitive).[25,26]

Mixed slope-linear intercept-linear inhibition (S-linear I-linear) is described by equation (2):

$$v = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_{i1}}\right) + [S]\left(1 + \frac{[I]}{K_{i2}}\right)} \qquad (2)$$

The terms slope (S) and intercept (I) refer to the slopes and intercepts obtained in the double reciproval plots (I/v versus 1/[S] at different [I] concentrations.[25,26] In equation 2 $K_{i1}$ is the dissociation constant of the EI complex and $K_{i2}$ the dissociation constant of the ESI complex. This equation predicts linear 1/v versus [I] relationships, as indeed observed for the inhibition of the pancreatic α-amylase by the *A. mearnsii* tannin (Figure 1D) and the salivary α-amylase by acarbose (Figure 1E). The results of the numerical calculations in which equation (2) was fitted to the corresponding experimental curves can be evaluated from the theoretical curves

in Figure 2D (pancreatic α-amylase/*A. mearnsii* tannin) and Figure 2E (salivary α-amylase/acarbose). In each case the equation was fitted to the three experimental curves (no inhibition + 2 different inhibitor concentrations). This is a very sensitive procedure, because all three experimental curves must conform to theoretical curves calculated with the same values of four parameters ($V_{max}$, $K_M$, $K_{i1}$ and $K_{i2}$). The standard deviations of the estimated parameters are usually very high if agreement between theory and experiment is poor. This was not the case of the present analysis as revealed by the data in Table 1 (exp. 3 for salivary α-amylase/acarbose inhibition and exp. 5 for the pancreatic α-amylase/*A. mearnsii* tannin inhibition). The standard deviations of the $K_M$ and $V_{max}$ mean values are also relatively small.

Parabolic inhibition requires at least one $[I]^2$ term in the denominator of the rate equation [25,26], which is generated when at least one enzyme form binds two inhibitor molecules. When the $EI_2$ and $ESI_2$ complexes are formed in addition to the EI and ESI complexes, the inhibition is said to be of the S-parabolic I-parabolic type.[25,26] The pertinent rate equation presents factors containing $[I]^2$ terms multiplying both $K_M$ and [S]:

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

$K_{i1}$, $K'_{i1}$, $K_{i2}$, and $K'_{i2}$ are the dissociation constants of the EI, $EI_2$, ESI and $ESI_2$ complexes, respectively. A limiting case is the situation in which only the concentrations of the complexes $EI_2$ and $ESI_2$ are significant. In this case the true inhibition constants in equation (3), $K_{i1}$, $K'_{i1}$, $K_{i2}$, and $K'_{i1}$, cannot be determined because the terms $[I]/K_{i1}$ and $[I]/K_{i2}$ are negligible. In this case equation 4 applies:

$$v = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]^2}{(\overline{K}_{i1})^2}\right) + [S]\left(1 + \frac{[I]^2}{(\overline{K}_{i2})^2}\right)} \qquad (4)$$

In this rather especial case the inhibition constants that can be determined, $\overline{K}_{i1}$ and $\overline{K}_{i2}$, are composite dissociation constants although they still can be regarded as a measure of the inhibitor's capacity. The S-parabolic I-parabolic case of equations 3 and 4 must not necessarily hold and four other possibilities must be tested. Equation 5, for example, describes

S-linear I-parabolic inhibition in which the complexes EI and $ESI_2$ are the only ones that can be detected by kinetic measurements:

$$v = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_{i1}}\right) + [S]\left(1 + \frac{[I]^2}{(\overline{K}_{i2})^2}\right)} \qquad (5)$$

Taking all these considerations into account, 6 different equations were fitted to the four sets of experimental data for which parabolic inhibition was found, namely those referring to the inhibition of the salivary α-amylase by the *pinhão* coat extract (Figure 1A) and the *A. mearnsii* tannin (Figure 1C) and those referring to the inhibition of the pancreatic α-amylase by the *pinhão* coat extract (Figure 1B) and acarbose (Figure 1F). The results of the analyses were listed in Table 1. For the salivary α-amylase inhibition by the *A. mearnsii* tannin (exp. 2 in Table 1), equation 5 gave the best fit to the experimental data (see Figure 2C for comparing experimental and theoretical curves), indicating thus a S-linear I-parabolic type of inhibition in which only the EI and $ESI_2$ complexes are significant. This was indicated not only by the largest model selection criterium (Table 1) but also by the standard deviations of the fitted parameters. Attempts of fitting equation 3 or any other alternative equation containing different combinations of linear and parabolic factors invariably produced smaller model selection criterium values and larger standard deviations for the fitted parameters. In some cases these standard deviations were considerably larger than the fitted parameters itself. It should also be remarked that the $K_M$ value for the salivary α-amylase obtained in the calculations involving the *A. mearnsii* tannin inhibition is very close to the values obtained when analyzing the *pinhão* coat extract and the acarbose inhibitions (compare experiments 1, 2 and 3 in Table 1). This speaks in favour of the reliability of the calculations.

The best fits for the parabolic inhibitions of both the salivary and pancreatic enzymes by the *pinhão* coat extract was obtained with equation 4 (S-parabolic I-parabolic), indicating thus for both enzymes the significant formation of the complexes $EI_2$ and $ESI_2$. The goodness of the fits can be appreciated in Figure 2A and Figure 2B. All attempts of fitting equation 3 failed to produce reliable parameters as revealed by their large standard deviations. Other equations containing different combinations of linear and parabolic factors invariably produced smaller model selection criterium values and larger standard deviations for the fitted parameters.

For the pancreatic α-amylase inhibition by acarbose (exp. 6 in Table 1), the best fit was also obtained with equation 4 (S-parabolic I-parabolic), meaning a significant formation of the complexes $EI_2$ and $ESI_2$. The goodness of the fit can be appreciated in Figure 2F. Here again the attempts of fitting equation 3 failed to produce reliable parameters.

*3.4. Effects of the α-amylase inhibitors on the glycemic levels after starch administration to rats*

To test the effectiveness of the *pinhão* coat extract and the *A. mearnsii* tannin as inhibitors of starch hydrolysis in vivo, experiments were done in which the blood glucose levels were measured in rats after the administration of commercial corn starch. The *pinhão* coat extract and the *A. mearnsii* tannin were administered intragastrically as described in the Materials and Methods section. Single doses of 250 mg *A. mearnsii* tannin and 250 mg *pinhão* coat extract per kg were administered. For comparative purposes experiments were also done with acarbose (50 mg/kg). The mean results of the blood glucose measurements at the various times after the administration of starch and inhibitors are shown in Figure 3. When starch was administered alone the blood glucose levels increased rapidly and were still elevated at 60 minutes following administration. For the control rats the glycemic levels remained essentially constant. Both acarbose and the *A. mearnsii* tannin prevented to a considerable extent the elevation of the blood glucose levels. The *pinhão* coat extract had a similar effect, but at time 45 minutes an increase in blood glucose occurred, followed by a drop to much lower values at 60 minutes. This increase was reproducible as it occurred in all three experiments. A comparison of the effects of the inhibitors on a quantitative basis is allowed by the data in Table 2. The latter lists the areas between the glycemic curves obtained after the administration of starch (alone or with the inhibitors) and the basal glycemic curves. These areas can be regarded as a measure of the capacity of each inhibitor in slowing down starch hydrolysis in the intestinal tract. As revealed by Table 2 the effectiveness of both acarbose (50 mg/kg) and the *A. mearnsii* tannin (250 mg/kg) was practically the same. The *pinhão* coat extract was less effective, the main reason being, evidently, the glucose burst at time 45 minutes (Figure 3).

## 4. Discussion

Condensed tannins comprise a group of polyhydroxyflavan-3-ol oligomers and polymers linked by carbon-carbon bonds between flavanol subunits. The most common classes are the procyanidins, which are chains of catechin, epicatechin, and their gallic acid esters, and the

prodelphinidins, which consist of gallocatechin, epigallocatechin, and their galloylated derivatives as the monomeric units.[23] Condensed tannins have attracted great attention due to rapid growing evidence associating these compounds with a wide range of potential health benefits. Condensed tannins from *A. mearnsii* are well-characterized both chemically and with respect to their functional properties;. The same cannot be said about the tannins from pinhão coat.

The results obtained in the present study revealed that the *pinhão* coat tannin is richer in procyanidins when compared to the *Acacia* tannin and that it is an effective inhibitor of both the human salivary and the porcine pancreatic α-amylases. Consistently, the *pinhão* coat tannin was also effective in diminishing the post-prandial blood glucose levels in rats after starch administration. To our knowledge, there is no other report about biological effects of components extracted from the *pinhão* coat.

The kinetic pattern of the α-amylase inhibition by the *pinhão* coat extract is complex, the parabolic nature of the inhibition (more precisely mixed S-parabolic I-parabolic inhibition) suggesting the formation of $EI_2$ and $ESI_2$ complexes. Similar complexities have been also detected in the present work, however, for both the *A. mearnsii* tannin and the well-known inhibitor acarbose. It is worth to mention that in an earlier study, inhibition of the pancreatic α-amylase by acarbose was also found to be of the S-parabolic I-parabolic type.[27] In this study the substrate was amylose rather than starch and the inhibition was said to conform with equation 3 rather than equation 4. We have *a priori* no explanation for this difference, except for the fact that the substrates used in both studies were not exactly the same.

From a comparison of the inhibition constants listed in Table 1 for the various inhibitors investigated in the present study the following decreasing potency sequence can be written: acarbose > *pinhão* coat tannin > *A. mearnsii* tannin. This sequence is valid for both the salivary and pancreatic α-amylases. It should be remarked, however, that, on a weight basis, acarbose is approximately one order of magnitude more potent than the *pinhão* coat tannin which, in turn, is also approximately one order of magnitude more potent than the *A. mearnsii* tannin. In spite of these pronounced differences, the *pinhão* coat tannin and the *A. mearnsii* tannin were both effective in slowing down starch hydrolysis *in vivo* at doses that are far enough from the $DL_{50}$ values usually reported for tannins in general.[28]

In recent years, tannins have been reported as non-specific inhibitors of several hydrolytic enzymes such as lipases, α-glucosidases, α-amylases and invertase.[6-10,12,13, 29] Also, tannins possess antioxidant activity.[30] In this respect, recent work of our laboratory has

reported antioxidant activity of the edible part of the *pinhão*. This activity appears to be due, at least in part, to the migration of polyphenolics from coat to seed during cooking.[19] The inhibitory effects of the tannins are generally attributed to their ability of binding quite strongly to carbohydrates and proteins. Kandra *et al*.[31] suggested that the interaction between tannins, such as galloylated quinic acid, and the human α-amylase depends on the free hydroxyl groups on the tannins that are able to participate in hydrogen bonding. This seems a reasonable assumption, but it must be remarked that not all tannins are able to inhibit α-amylase.[3] Consequently, even though the presence of free hydroxyl groups can in principle favor the interaction of the tannins with proteins, the simple presence of those groups is not enough to ensure any inhibitory activity. An appropriate conformation of the inhibitor molecule combined with adequately positioned hydroxyl groups is perhaps important for optimizing the inhibitor-protein interactions.

Prolonged fasting and postprandial hyperglycaemia can contribute to elevated non enzymatic protein glycation or to toxic effects on the vascular endothelium. Consequently, maintenance of blood glucose homeostasis prevents the detrimental effects of hyperglycaemia.[32] Given the strong link between postprandial hyperglycaemia and diabetes complications, several herbal extracts have been studied to clarify their effectiveness in experimental animals and in *in vitro* bioassays. α-Amylase and α-glucosidase inhibitors are widely used oral agents for improving postprandial hyperglycaemia due to the lack of a hypoglycemic threat and, more important, to the prospect of blood glucose control without hyperinsulinemia and body weight gain.[33] Inhibition of α-glucosidase and α-amylase results in delayed carbohydrate digestion and glucose absorption with attenuation of postprandial hyperglycaemic excursions. The observations that the *pinhão* coat extract rich in tannins is an effective inhibitor of salivary and pancreatic α-amylases, comparable to *Acacia mearnsii* tannin, suggests that it can be used to suppress postprandial hyperglycemia in diabetic patients. This idea is reinforced by the observations that the extract was in fact able to lower blood glucose during starch digestion. Considering that the inhibition of carbohydrate metabolism or absorption can decrease the caloric intake, the *pinhão* coat tannin could also, in principle at least, be used to promote weight loss and to combat obesity. One can even not exclude the possibility that the extract could be active on other enzymes in addition to the α-amylases, as α-glucosidases, invertase and lipases, a possibility worth of further experimental work. In order to understand the inhibitory mechanisms more clearly, we are currently attempting to chemically characterize the *pinhão* coat tannin.

**Acknowledgments**

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico ,(CNPq) and Fundação Araucária for funding this study. Authors S.M. da Silva and R. Castoldi, thank Coordenação de Aperfeiçoamento do Pessoal do Ensino Superior (CAPES) and CNPq and for the financial support provided for their postgraduate studies in the Biological Sciences Program and in the Food Science Program of Universidade Estadual de Maringá. A. Bracht, M.L. Baesso and R.M. Peralta research grant recipients of CNPq. The authors thank M.A.F. Costa and A. Chaves by technical assistance

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**Table 1. Kinetic parameters of the inhibition of human salivary α-amylase and porcine pancreatic α-amylase by acarbose and tannins.** The parameters were those obtained by fitting equations 2, 4 or 5 to the experimental data shown in Figure 3. A non-linear least squares procedure was used. Details are given in the Materials and Methods section. The model selectium criterium was calculated according to equation 1. The error terms correspond to standard deviations of the optimized parameters.

| Exp. series number | Inhibitor | Type of inhibition | Type of inhibition constant | Inhibition constant *(μg/mL)* | Estimated $K_M$ *(g/100 ml)* | Estimated $V_{max}$ *(μmol/min)* | Model selection criterion |
|---|---|---|---|---|---|---|---|
| **Human salivary α-amylase** | | | | | | | |
| **1** | *Pinhão* coat tannin | Mixed S-parabolic I-parabolic (equation 4) | $\overline{K}_{i1}$<br>$\overline{K}_{i2}$ | 56.88±5.74<br>103.27±11.85 | 0.256±0.016 | 0.546±0.016 | 4.62 |
| **2** | *A. mearnsii* tannin | Mixed S-linear I-parabolic (equation 5) | $K_{i1}$<br>$\overline{K}_{i2}$ | 578.17±107.49<br>518.23±24.53 | 0.279±0.021 | 0.635±0.015 | 4.98 |
| **3** | Acarbose | Mixed linear (equation 2) | $K_{i1}$<br>$K_{i2}$ | 8.64±4.76<br>6.60±1.43 | 0.290±0.050 | 0.623±0.035 | 3.51 |
| **Porcine pancreatic α-amylase** | | | | | | | |
| **4** | *Pinhão* coat tannin | Mixed S-parabolic I-parabolic (equation 4) | $\overline{K}_{i1}$<br>$\overline{K}_{i2}$ | 20.25±1.97<br>46.79±4.57 | 0.107±0.009 | 0.490±0.01 | 4.28 |
| **5** | *A. mearnsii* tannin | Mixed linear (equation 2) | $K_{i1}$<br>$K_{i2}$ | 179.62±41.77<br>411.89±53.07 | 0.112±0.010 | 0.484±0.011 | 4.14 |
| **6** | Acarbose | Mixed S-parabolic I-parabolic (equation 4) | $\overline{K}_{i1}$<br>$\overline{K}_{i2}$ | 2.20±0.54<br>1.72±0.09 | 0.113±0.011 | 0.505±0.013 | 3.84 |

**Table 2. Areas between the glycemic curves after starch administration (starch alone or starch + α-amylase inhibitors) and the glycemic basal levels.** The areas were determined using the numerical integration procedures of the Scientist software from MicroMath Scientific Software (Salt Lake City, UT). The error terms correspond to standard errors of the means.

| **Conditions** | **Differential areas** *(mg/100 ml × minute)* |
|---|---|
| Starch alone | 2642.5±389.5 |
| Starch + *pinhão* coat tannin | 1573.8±118.4* |
| Starch + *A. mearnsii* tannin | 773.8±241.1* |
| Starch + acarbose | 716.3±111.2* |

*Statistically different from the starch alone condition ($p < 0.05$; Student-Newman-Keuls test).

**Figure legends**

Figure 1. FTIR spectrum of *A. mearnsii* tannin (red) and pinhão coat tannin (black)

Figure 2. Pinhão coat tannin (A,B), *Acacia mearnsii* tannin (C,D) and acarbose (E,F) concentration dependences of the salivary and pancreatic α-amylase inhibition. Initial reaction rates were measured as described in the Materials and Methods section. Each datum point represents the mean of three independent determinations. Legends: ●—●, reaction rates in the presence of the inhibitors relative to the reaction rates in the absence of inhibitors; ■—■, inverse of the relative reaction rates.

Figure 3. Substrate and inhibitor concentration dependences of the reaction rates of the salivary (A, C, E) and pancreatic α-amylase (B, D, F). Initial reaction rates were measured as described in the Materials and Methods section. Each datum point represents the mean of three independent determinations. Standard errors of the mean are shown unless their values are smaller than the symbol sizes. The solid lines were calculated by means of equation 2 (panels D and E), equation 4 (panels A, B and F) or equation 5 (panel C), using the optimized parameters given in Table 1.

Figure 4. Influence of α-amylase inhibitors on the glycemic levels of fasted rats during 60 minutes following starch administration. Blood samples from the tail vein were analyzed by means of a glucometer after intragastric starch administration (1 g per kg body weight). Each datum point represents the mean ± mean standard errors of four experiments. Experimental details are given in the Materials and Methods section.

**Figure 1**

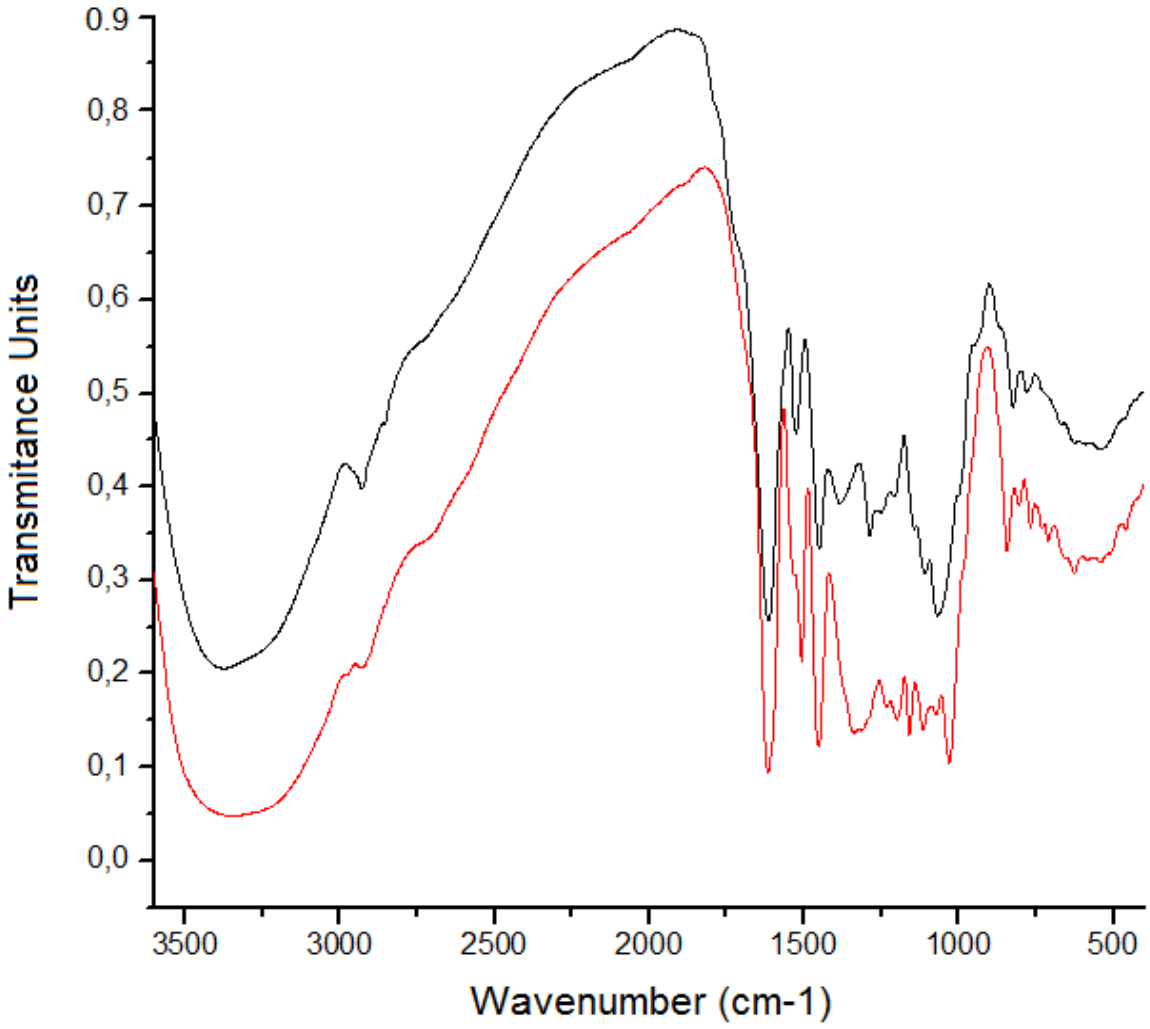

Transmitance Units
0.9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

**Figure 2**

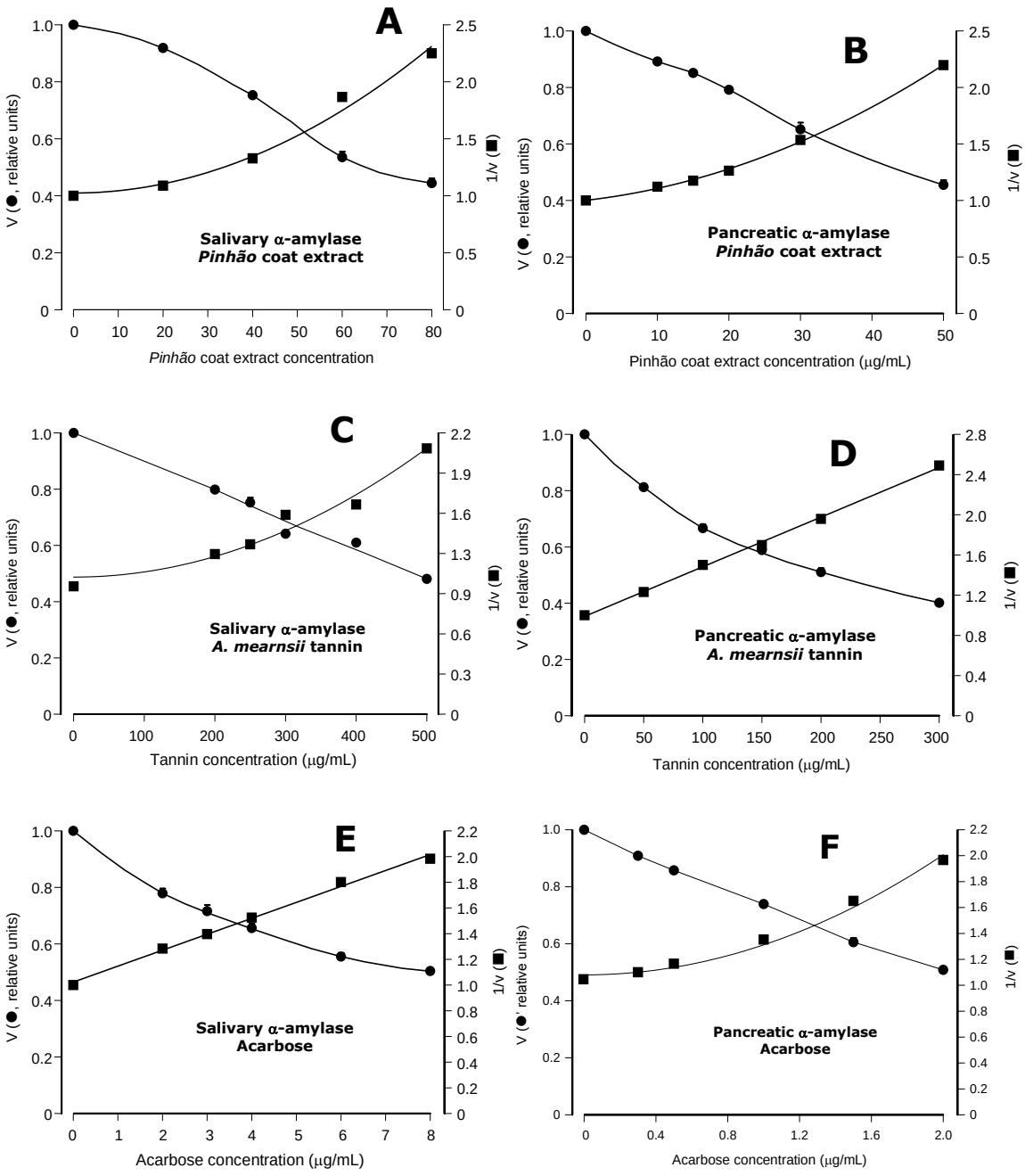

A
Salivary α-amylase
*Pinhão* coat extract
V (●, relative units)
1/v (■)
*Pinhão* coat extract concentration
B
Pancreatic α-amylase
*Pinhão* coat extract
Pinhão coat extract concentration (μg/mL)
C
Salivary α-amylase
*A. mearnsii* tannin
Tannin concentration (μg/mL)
D
Pancreatic α-amylase
*A. mearnsii* tannin
Tannin concentration (μg/mL)
E
Salivary α-amylase
Acarbose
Acarbose concentration (μg/mL)
F
Pancreatic α-amylase
Acarbose
Acarbose concentration (μg/mL)

**Figure 3**

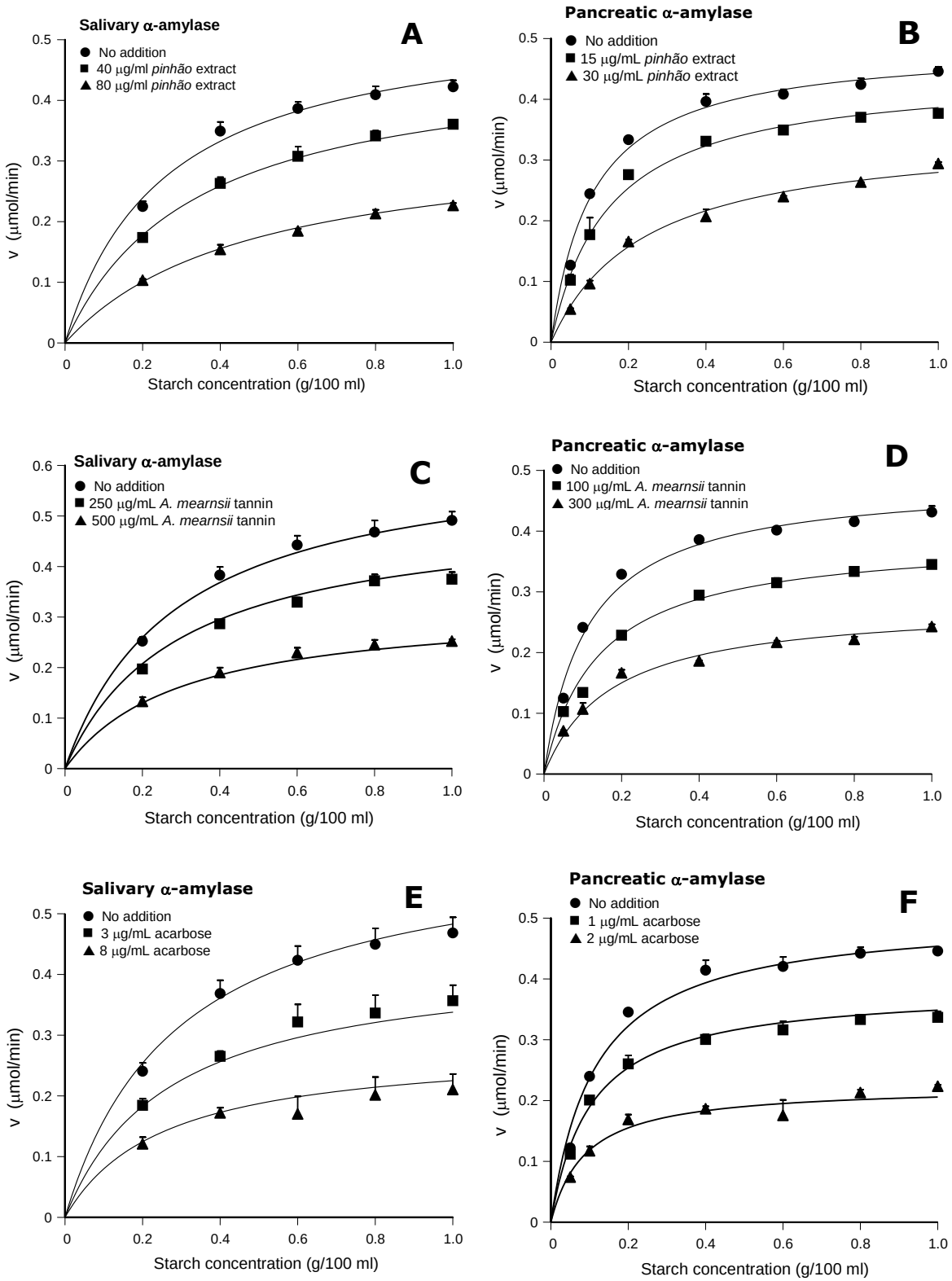
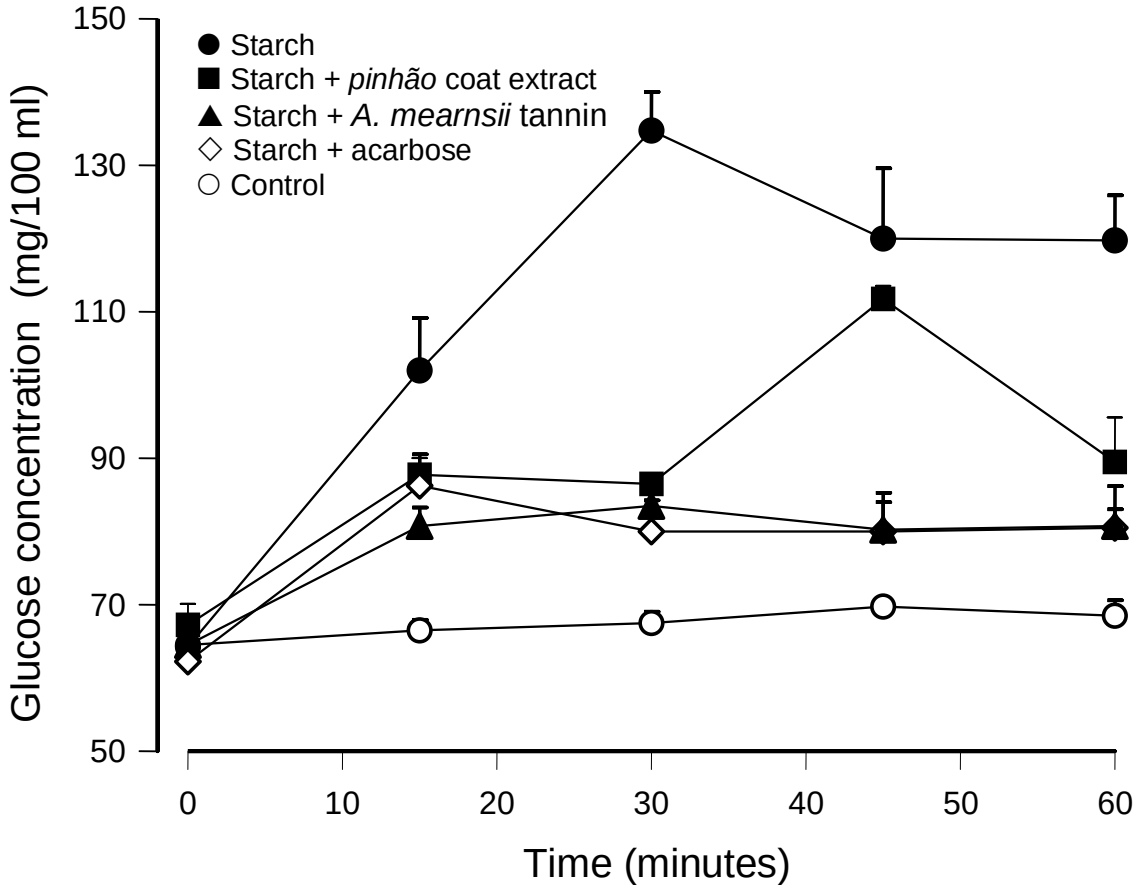

Salivary α-amylase
A
No addition
40 μg/ml pinhão extract
80 μg/ml pinhão extract
v (μmol/min)
Starch concentration (g/100 ml)
Pancreatic α-amylase
B
No addition
15 μg/mL pinhão extract
30 μg/mL pinhão extract
v (μmol/min)
Starch concentration (g/100 ml)
Salivary α-amylase
C
No addition
250 μg/mL A. mearnsii tannin
500 μg/mL A. mearnsii tannin
v (μmol/min)
Starch concentration (g/100 ml)
Pancreatic α-amylase
D
No addition
100 μg/mL A. mearnsii tannin
300 μg/mL A. mearnsii tannin
v (μmol/min)
Starch concentration (g/100 ml)
Salivary α-amylase
E
No addition
3 μg/mL acarbose
8 μg/mL acarbose
v (μmol/min)
Starch concentration (g/100 ml)
Pancreatic α-amylase
F
No addition
1 μg/mL acarbose
2 μg/mL acarbose
v (μmol/min)
Starch concentration (g/100 ml)

**Figure 4**


Starch
Starch + pinhão coat extract
Starch + A. mearnsii tannin
Starch + acarbose
Control
Glucose concentration (mg/100 ml)
150
130
110
90
70
50
0
10
20
30
40
50
60
Time (minutes)