Anais da Academia Brasileira de Ciências
ISSN: 0001-3765
aabc@abc.org.br
Academia Brasileira de Ciências
Brasil

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Anais da Academia Brasileira de Ciências, vol. 85, núm. 3, septiembre, 2013, pp. 1141-1148
Academia Brasileira de Ciências
Rio de Janeiro, Brasil

Anais da Academia Brasileira de Ciências (2013) 85(3): 1141-1148
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
www.scielo.br/aabc

Pouteria ramiflora extract inhibits salivary amylolytic activity and decreases glycemic level in mice

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Manuscript received on June 27, 2012; accepted for publication on February 27, 2013

ABSTRACT

In this study, extracts of plant species from the Cerrado biome were assessed in order to find potential inhibitors of human salivary alpha-amylase. The plants were collected and extracts were obtained from leaves, bark, and roots. We performed a preliminary phytochemical analysis and a screening for salivar alpha-amylase inhibitory activity. Only three botanical families (Sapotaceae, Sapindaceae and Flacourtiaceae) and 16 extracts showed a substantial inhibition (>75%) of alpha-amylase. The ethanolic extracts of *Pouteria ramiflora* obtained from stem barks and root barks decreased amylolytic activity above 95% at a final concentration of 20 µg/mL. Thus, adult male Swiss mice were treated orally with *P. ramiflora* in acute toxicity and glycemic control studies. Daily administration with 25, 50 and 100 mg/kg of aqueous extract of *P. ramiflora* for eight days can reduce significantly body weight and blood glucose level in mice. These data suggest that the crude polar extract of *P. ramiflora* decreases salivary amylolytic activity while lowering the blood levels of glucose.

Key words: alpha-amylase inhibition, hypoglycemia, *Pouteria ramiflora*, Sapotaceae.

INTRODUCTION

Recently, several studies of alpha-amylase inhibition have been conducted with the aim of discovering new potential drugs capable of reducing postprandial hyperglycemia that could be used in the treatment of diabetes mellitus type 2 and obesity. Inhibitors of this enzyme reduce postprandial hyperglycemia by delaying carbohydrate digestion and decrease intestinal glucose absorption (Ali et al. 2006, Funke and Melzig 2006, Gad et al. 2006, Kim et al. 2005, Shu et al. 2009). Plants continue to play an important role in the treatment of diabetes, particularly in developing countries (Marles and Farnsworth 1994, Oliveira et al. 2005). Medicinal plants from the Cerrado biome has been previously investigated by bioprospection (de Mesquita et al. 2009, Flausino et al. 2009, Mesquita et al. 2005, Napolitano et al. 2005, Rodrigues et al. 2006). However, few studies have investigated the biological activity of such medicinal plants extracts or their isolated components on the activity of alpha-amylase (Silva et al. 2007, 2009).

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Inhibition studies of alpha-amylase activity by natural products have been performed *in vitro* using human pancreatic alpha-amylase (HPA) (Ali et al. 2006, Conforti et al. 2005, Gad et al. 2006). Alternatively, as human salivary alpha-amylase (α-1,4-D-glucan-4-glucanohydrolases, EC 3.2.1.1, HSA) is highly homologous to HPA, and both proteins adopt very similar structures (Brayer et al. 1995, Ramasubbu et al. 2003), the use of HSA as an experimental model for *in vitro* inhibition studies with natural products has the advantage of saliva being ease and not invasive to collect. Previous studies have shown similar *in vitro* inhibition of HPA and HSA activities (Kim et al. 2005, McDougall et al. 2005).

The aims of this study are (1) to screen selected plants extracts from the Brazilian Cerrado for possible HSA inhibitory activity, and (2) to carry out a biological assay in mice to investigate any effects on glycemic and toxicity of the stem bark extract from *Pouteria ramiflora* (Mart.) Radlk.

MATERIALS AND METHODS

Collection and Preparation of the Plants for Screening

The plants were collected in the Cerrado biome, in the outskirts of Brasilia, Distrito Federal, Brazil, and were identified at the Department of Vegetable Anatomy, Institute of Biology, Universidade de Brasília (UnB). The voucher specimens were deposited in the Herbarium of UnB. Extracts were obtained from leaves, wood bark, stem bark and roots of the selected plants. Air-dried and powdered plant material were successively extracted with hexane and ethanol 95% by maceration. Crude extracts were obtained after evaporation of the solvents under reduced pressure at 40°C. Extracts were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, USA) at a concentration of 10 mg/mL. Stock solutions of each extract were prepared before experiments. Samples were stored at -20°C until analysis. Plant species investigated, part used, solvent and voucher numbers are presented in Table I.

Collection and Preparation of the *Pouteria ramiflora* Extract for *in vivo* Assay

Stem barks of *P. ramiflora* were collected in the Cerrado biome in the outskirts of Uberlândia, Minas Gerais, Brazil. The plant was botanically identified at the Institute of Biology, Universidade Federal de Uberlândia, and placed in the herbarium of the same institution (voucher specimen, HUFU, 45,535). The vegetables were dried at 37°C and then grounded in an electric mill. Aqueous solution of *P. ramiflora* (PrSBAE) was used for the extraction process that lasted eight days. After extraction, the solution was filtered and lyophilized. Extract was then solubilized in water for *in vitro* studies.

Preliminary TLC and HPLC Phytochemical Analysis

The TLC analysis was carried out using 60G silica gel plates (10 x 10 cm; 0.25 mm; Aldrich), eluted with BAW (n-butanol:acetic acid:water, 4:1:5, upper phase) or $CHCl_3$:MeOH (8:2) and stained with NP/PEG (natural products - polyethylene glycol reagent) and vanillin-sulphuric acid reagent in separated plates. All extracts were also analyzed in analytical HPLC (Shimadzu, Kyoto, Japan) with SupelcosilTM LC-18 column (250 x 4.6 mm; Supelco) and PDA detector. The mobile phase consisted of 0.1% aqueous acetic acid and methanol with a flow rate of 1 mL/min. The mobile phase composition began with 10% methanol, followed by linear increase of 66% in 32 min and returned to the initial condition in 35 min for the next run.

Screening for Alpha-Amylase Inhibitiory Activity

Alpha-amylase inhibition was performed using a commercial kit (Kit Analisa - Belo Horizonte, Minas Gerais, Brazil) based on starch-iodine color changes (Caraway 1959). Saliva was collected from nine individuals using a modified

method (Navazesh 1993). The pooled saliva was centrifuged at 14,000xg for 15 min at 4°C. The supernatant was diluted (500x) in phosphate buffered saline, pH 7.4. Extracts (10 mg/mL) were pre-incubated with the diluted saliva supernatant for 4 min 35 sec at 37°C. The final concentration of the plant extract in the incubation was 200 µg/mL. The reaction was started by addition of 10 µL of the supernatant solution in 250 µL of starch solution. The tubes were incubated at 37°C for 7 min 30 sec. The reaction was stopped by the addition of iodine. This assay was also carried out with acarbose (10 mg/mL) (provided by EMS S/A, São Paulo, Brazil) - a drug therapeutically used as alpha-glucosidase and alpha-amylase inhibitor (positive control). The final concentration of acarbose was 200 µg/mL. DMSO was used as a negative control (10 µL). The final concentration of the negative control in the incubation was 0.022 µg/mL.

The inhibition of the activity of HSA was expressed as a percentage in which alpha-amylase activity of the non-incubated saliva supernatant was considered 100%. Extracts that presented the highest inhibition rates were selected for a subsequent inhibitory assay at lower concentrations. For the secondary assay, the same method described before was used with increasing final concentration of the extract (20, 50 and 100 µg/mL) incubated with the diluted saliva supernatant.

Animals

Healthy adult male Swiss mice aged 7-8 weeks (weigh: 25-30g) were used for this study. Animals were housed under standard conditions (25°C, 12h light and 12h dark cycle) and fed with rat chow and water ad libitum. All procedures for handling, use and euthanasia of these animals followed carefully the resolutions proposed by the Brazilian Society of Science in Laboratory Animals and were approved by the Ethics Committee in Animal Research of the Universidade Federal de Uberlândia, Brazil (CEUA/UFU 060/10).

Acute Toxicity Study

Experiments were carried out as previously described (Brito 1994). In the acute toxicity study, animals were treated orally and by intraperitoneal injection with PrSBAE extract. Mice were given PrSBAE at doses of 50, 500 and 5,000 mg/kg daily for a period of 18 days (n=5). Animals were observed for 2h continuously and then hourly for 8h, and finally after every 24h up to 18 days for any physical signs of toxicity such as writhing, hypnosis, dyspnea or mortality.

Effects of the PrSBAE Extract on the Levels of Blood Glycaemia

Twenty mice were divided randomly in four groups and treated orally for eight days in the following manner: Group 1 (water, control), 2 (25 mg/kg of PrSBAE), 3 (50 mg/kg of PrSBAE), and 4 (100 mg/kg of PrSBAE). Treatments were administrated in the same time, once a day, in alternate days. After the eighth day, mice were fasted overnight (for 8h) and then were weighed and sacrificed. Glycemic level in blood serum was measured by a colorimetric enzymatic method (Labtest, Minas Gerais, Brazil).

Statistical Analysis

Statistical comparisons were made using one-way ANOVA followed by the Tukey test. *p*-values lower than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

One hundred and nine crude extracts were tested, of which 47 were ethanolic and 52 hexanic. In the initial screening, all extracts were prepared as hexanic and ethanolic crude extracts from different parts of 17 plant species belonging to six botanical families (Flacourtiaceae, Sapindaceae, Sapotaceae, Bignoniaceae, Asteraceae and Apocynaceae) (Table I). The percentages of inhibition of the HSA by the most effective extracts are shown in Table II. Only 16 extracts from six species belonging to the

botanical families, Sapotaceae, Flacourtiaceae and Sapindaceae, were found to have an inhibitory effect on HSA activity (greater than 75%), including four ethanolic extracts obtained from root bark, root wood and stem bark of *P. ramiflora*. The extracts were more effective to inhibit the activity of alpha-amylase *in vitro* than acarbose in the same concentration (200 μg/mL). We observed that ethanolic extracts presented more effective HSA inhibition activity than hexanic extracts. This result was also reported in other studies of HSA inhibition activity with plant extracts of different families from the Cerrado biome (de Souza et al. 2012, Silva et al. 2009).

Preliminary qualitative phytochemical analysis of the active extracts was performed to determine the probable type of compounds present in the extracts causing HSA inhibition. We visualize by TLC the components of only few extracts. Flavonoids were found in the ethanolic extract of the *P. torta* leaves and hexane extract of the *P. ramiflora* stem bark. Tannins and saponins were found in the ethanolic extract of the *Serjania lethalis* stem bark and root bark, and ethanolic extracts of the *P. ramiflora* root wood. Tannins were found in the ethanolic extracts of the *P. ramiflora* root bark. As we observed, previous studies have reported that the capacity of alpha-amylase inhibition by vegetal extracts is normally associated with polar compounds, as phenolic, tannins and triterpenoids compounds (Ali et al. 2006, Gad et al. 2006, Kandra et al. 2004).

The extracts that showed the highest inhibition in the screening test at a concentration of 200 μg/mL were subsequently investigated at concentrations of 100, 50 and 20 μg/mL. The *S. lethalis* stem wood

TABLE I
Family, plant species investigated, part used/solvent and voucher number.

Family	Species	Part used/sovent	Voucher
Apocynaceae	*Aspidosperma macrocarpa* Woodson	SW (E, H); SB (E, H); RW (E); RB (E, H); L (E)	(UB) 3692
	Himatantus obovatus (M. Arg) Woodson	RB (H);L (H)	(UB) 3678
	Hancornia pubescens (Nees & Mart.) M. Arg.	L (H); RW (H); RB (H)	(UB) 3677
Asteraceae	*Piptocarpha rotundifolia* (Less.) Baker	RW (E, H); L (E, H); RB (E, H); SB (H); SW (E)	(UB) 3676
Bignoniaceae	*Tabebuia caraiba* (Silva Manso) Benth. & Hook.f ex S. Moore	RB (E); SB (H); L (E, H); SW (E, H); SB (E); RB (H); RW (E)	(UB) 3701
	Anemopaegma arvense (Vell.) Stellf.	L (E, H); S (H); R (E, H); FS (E, H); S (E)	(UB) 3691
	Cybistax antisyphilitica (Mart.) Mart.	WS (E, H); L (E, H); SW (E, H); SB (E, H);	(UB) 3696
Flacourtiaceae	*Casearia sylvestris* SW. var. lingua (Camb.) Eichl.	SW (E, H); RB (E, H); SB (E, H); RW (H); R (E); L (E, H)	(UB) 3693
	Piptocarpha macropoda (DC.) Baker	SB (H); L (H)	(UB) 3708
Sapindaceae	*Cupania vernalis* Cambess	SB (E, H); RB (E, H); RW (H); SW (E, H); L (E, H); R (E)	(UB) 3695
	Matayba guianensis Aubl.	SB (E, H);SW (E, H); RB (E, H)	(UB) 3697
	Serjania lethalis A.St.Hil.	L (E, H); SW (E, H); SB (E, H); RB (E, H)	
	Magonia pubescens A. St.Hill.	L (H); SB (H)	(UB) 3702
Sapotaceae	*Chrysophyllum soboliferum* Rizzini	L (E, H)	(UB) 3733
	Pouteria ramiflora (Mart.) Radlk	L (E, H);RB (E, H); SW (E, H); RW (E); SB (E, H)	(UB) 3671
	Pouteria gardnerii (Mart. & Miq.) Baehni	L (E, H)	(UB) 3672)
	Pouteria torta Radlk.	L (E, H)	(UB) 3674

Part used: **L**, leaf; **SW**, stem wood; **SB**, stem bark; **RW**, root wood; **RB**, root bark; **R**, root; **FS**, fruit+seed; **S**, stem (bark+wood); **WS**, wood+stem bark. ***Extraction solvent*: H**, hexane; **E**, ethanol.

ethanolic extract and stem bark ethanolic extract (20 μg/mL) inhibited almost 40% of the HSA activity. The ethanolic extracts (20 μg/mL) of *Matayba guianenses* and *S. lethalis* root bark and *P. torta* leaves inhibited 70-80% of the HSA activity. Ethanolic extract of the *P. ramiflora* root bark and hexane extract of the *P. ramiflora* stem bark (20 μg/mL) showed a 95-100% of inhibition effect, respectively. Studies from the bark of *P. caimito* reveal the presence of the triterpenes (Ardon and Nakano 1973, Pellicciari et al. 1972). Seven polyphenolic compounds, gallic acid, (+)-gallocatechin, (+)-catechin, (-)-epicatechin, dihydromyricetin, (+)-catechin-3-O-gallate, and myricitrin, were isolated and identified from the fresh fruits of *P. campechiana*, *P. sapota* and *P. viridis* (Ma et al. 2004). These reports indicate that the capacity of alpha-amylase inhibition by genus *Pouteria* might be associated with such compounds.

We selected the stem bark aqueous extract of *P. ramiflora* (PrSBAE) for *in vivo* studies because it inhibited 92% of the HSA activity when it was diluted 1/10. In the acute toxicity test, mice treated with PrSBAE extract in doses higher than 500 mg/kg showed toxicity signals as hypnoses, dyspnea,

TABLE II
Plant species investigated, part used/solvent, yields in terms of dry starting material and percentage of inhibitory activity of crude extracts on human salivary alpha-amylase.

Species	Part used/sovent	Yield (%)	Inhibition (%)
Cupania vernalis Cambess	L (H)	7.82	57±50.1
	SW (E)	3.78	63±55.3
	RB (E)	-	94±5.6
	SB (E)	6.15	82±16.0
	R (E)	-	81±21.9
Matayba guianensis Aubl.	SB (E)	-	79±0.0
	RB (E)	-	86±9.1
Serjania lethalis A.St.Hil.	SB (H)	0.34	92±6.8
	SB (E)	3.20	98±1.5
	SW (E)	9.74	92±3.5
	RB (H)	0.46	66±14.0
	RB (E)	2.56	89±1.4
Casearia sylvestris SW. var. lingua (Camb.) Eichl.	RB (H)	2.25	58±50.9
	RB (E)	4.23	68±31.3
	SB (E)	14.97	90±5.8
	SW (E)	11.02	87±5.2
	L (H)	-	36±27.1
Pouteria ramiflora (Mart.) Radlk	L (E)	4.84	61±42.5
	RB (E)	8.65	96±4.9
	RW (E)	3.62	97±4.6
	SB (E)	-	77±19.9
	SW (H)	5.98	83±15.3
Pouteria torta Radlk.	L (E)	5.08	77±6.7
acarbose (positive control)	-	-	58±2.8
DMSO (negative control)	-	-	0±4.7

Part used: **L**, leaf; **SW**, stem wood; **SB**, stem bark; **RW**, root wood; **RB**, root bark. ***Extraction solvent***: **H**, hexane; **E**, ethanol. Results are represented as means of replicates±S.D.

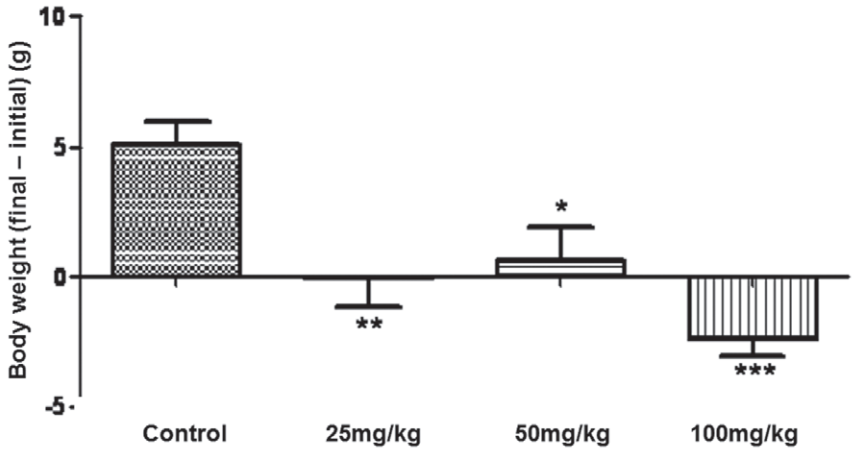

Figure 1 - The effect of administration of stem bark aqueous extract from *P. ramiflora* (PrSBAE) for eigth days on body weight loss in mice. Each column represents mean±S.E.M for five mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 different from control group.

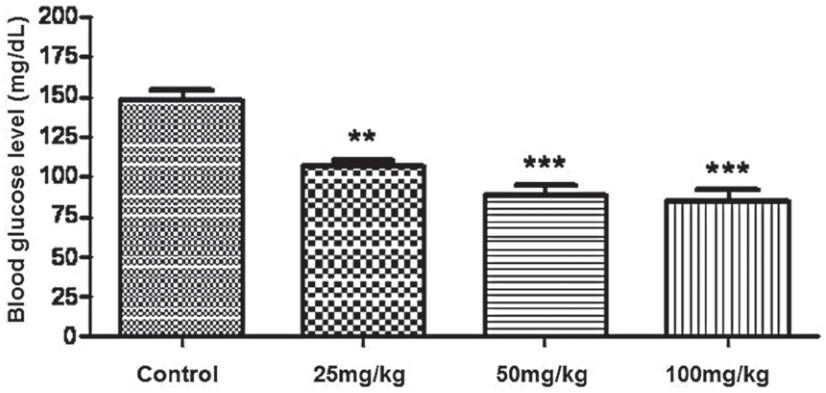

Figure 2 - The effect of administration of stem bark aqueous extract from *P. ramiflora* (PrSBAE) for eigth days on blood glucose level in mice. Each column represents mean±S.E.M for five mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 different from control group.

writhing. Two animals died. In mice that received 5,000 mg/kg of the extract we observed signals such as hypnosis, dyspnea. One animal died. When mice were treated orally and intraperitoneal with 100 mg/kg of the PrSBAE dose, they did not show any toxicity effect.

Weight and blood glucose level in mice treated with 25, 50 and 100 mg/kg of the PrSBAE extract are shown in figures 1 and 2. All administrated doses have shown a significant effect on weight loss when compared to the control group after eight days of treatment. Animals that received 50 and 100 mg/kg of extracts showed a significant reduction of the levels of blood glucose when compared to the control group. Mice treated with acarbose, a competitive inhibitor of intestinal alpha-glucosidases that slows the breakdown of sucrose and starch (Santeusanio and Compagnucci 1994), were not effective to reduce blood glucose level when compared with polyphenolic enriched crude plant extract after 30 min of treatment (Mai and Chuyen 2007). A previous study of our laboratory (Deconte et al. 2011) conducted in rats treated by 20 days with acarbose did not reveal difference of weight gain and glycemic level of non-diabetic and diabetic groups when compared with the respective controls. Heo et

al. (2009) showed that diphlorethohydroxycarmalol, isolated from Ishige okamurae, had inhibitory effects on alpha-glucosidase and alpha-amylase activities higher than those of acarbose. Therefore, our results showed that the *P. ramiflora* extracts were more effective to inhibit the activity of alpha-amylase *in vitro* than acarbose. The *in vivo* results suggest that PrSBAE reduction of glucose level of treated mice may be due to inhibition of carbohydrate-hydrolyzing enzymes.

Several plant extracts have high concentrations of tannins, that are compounds that may exert an anti-nutritional effect by interfering with gut function (Carbonaro et al. 2001) and reduce the glycemic response to carbohydrate foods in humans (Gin et al. 1999). In another study, it was investigated whether blood glucose reduction was due to reduced food intake in mice treated with the *Syzygium cumini* ethanol extract and tannic acid (Oliveira et al. 2005). In the study by Oliveira et al., it was hypothesized that polyphenolic compounds contribute to the reduction of food intake, body weight and the levels of blood glucose. Hence, we cannot exclude the possibility that the *P. ramiflora* extract may contain phenolic compounds and that its effects on weight reduction and the decrease in blood glucose are related to the dietary restriction associated with the inhibition of alpha-amylase.

In conclusion, the present study showed that *M. guianensis*, *S. lethalis*, *P. torta* and *P. ramiflora* inhibit alpha-amylase activity *in vitro* even at low concentrations. In addition, *P. ramiflora* bears positive effects on body weight and blood glucose levels.

ACKNOWLEDGMENTS

Grants to FSE from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, MG, Brasil). Fellowships to NMG from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil). We also thank Dr. Jose Elias de Paula (Botanical Garden of UnB) and Dra. Ana Angelica Almeida Barbosa (Botanical Garden of UFU) for approving the authenticity of the plants used in the research, and Dr. Paulo S. Pereira and Dra. Suzelei C. França (Department of Biotechnologyt, Ribeirao Preto University, UNAERP, Brazil) for their provision of TLC and HPLC preliminary phytochemical analysis.

RESUMO

Neste estudo, extratos de espécies de plantas do bioma Cerrado foram avaliados em busca de um potencial inibidor da enzima alfa-amilase salivar humana. As plantas foram coletadas e os extratos obtidos das folhas, da casca e da raiz. Nós realizamos uma análise fitoquímica preliminar e uma triagem destas plantas utilizando um ensaio de inibição da alfa-amilase salivar. Inicialmente, somente três famílias botânicas (Sapotaceae, Sapindaceae e Flacourtiaceae) e 16 extratos apresentaram inibição da alfa-amilase maior que 75% sendo que os extratos etanólicos da casca do caule e da raiz de *Pouteria ramiflora* tiveram mais de 95% de inibição para a concentração final de 20 μg/mL. Assim, camundongos Swiss adultos machos foram oralmente tratados com extrato aquoso de *P. ramiflora* para avaliar a toxicidade aguda e o controle glicêmico. A administração diária do extrato de *P. ramiflora* nas doses de 25, 50 e 100 mg/kg, por oito dias, foi capaz de reduzir o peso corporal e o nível glicêmico nos animais. Esses dados sugerem que o extrato polar de *P. ramiflora* diminui a atividade amilolítica da saliva, além disso, reduz os níveis de glicose sanguínea.

Palavras-chave: inibição da alfa-amilase, hipoglicemiante, *Pouteria ramiflora*, Sapotaceae.

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