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Artigo

Protease inhibition activity of extracts from Salicaceae species from Brazilian Cerrado and Atlantic Rain Forest and of an enriched fraction of clerodane diterpenes (casearins)

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RESUMO: "Atividade biológica de uma fração enriquecida de diterpenos clerodânicos (casearinas) e de extratos de espécies de Salicaceae do Cerrado e Mata Atlântica em um ensaio de inibição de proteases". Considerando o uso popular de *Casearia sylvestris* Sw., Salicaceae, para o tratamento de problemas gástricos e resultados pré-clínicos que mostraram potencial atividade anti-ulcerogênica, foi realizado um screening farmacológico para avaliar a atividade biológica de outras espécies de Salicaceae. Para isso, foi utilizado um ensaio de inibição de proteases como um modelo farmacológico molecular para screening de extratos com atividade anti-ulcerogênica. Os extratos etanólico e aquoso dos galhos e folhas de *C. gossypiosperma, C. decandra* e *C. rupestris* mostraram inibição da atividade da pepsina em aproximadamente 50% com a concentração de 1 µg/mL. Curiosamente, *C. obliquoa* e *Flacourtia ramontchi* não apresentaram atividade sobre a pepsina, mas seus extratos mais apolares mostraram atividade inibitória sobre a subtilisina. A fração enriquecida de diterpenos clerodânicos mostrou atividade inibitória (42,75%) sobre a pepsina com a concentração de 1 µg/mL, mas não sobre a subtilisina (23,76%). Os resultados obtidos com os extratos e folhas das espécies testadas mostraram um padrão de atividade diferente sobre os dois tipos de proteases, a pepsina e a subtilisina, as quais estão relacionadas com diferentes tipos de atividades biológicas. Ainda mais, os resultados com a fração enriquecida de diterpenos clerodânicos sugerem que estas substâncias podem estar relacionadas com a atividade do extrato bruto de *C. sylvestris.*

Unitermos: Salicaceae, *Casearia,* diterpenos clerodânicos, atividade anti-úlcera gástrica, inibição de proteases, ensaio de fluorescência.

ABSTRACT: Considering the traditional use of *Casearia sylvestris* Sw., Salicaceae, to threat gastric injuries and the pre-clinical studies showing its efficacy we aimed to screen other species to explore the biological activity of some species of this family. For this, we used a protease inhibition assay as a model for searching gastric anti-ulcer plant extracts. The ethanolic and aqueous extracts from branches and leafs of *C. gossypiosperma, C. decandra* and *C. rupestris* showed high percentage inhibition of pepsin, approximately 50%, with 1 µg/mL concentration. Curiously, *C. obliquoa* and *Flacourtia ramontchi* did not inhibit pepsin, but its most apolar extract showed inhibitory activity in the subtilisin assay. The enriched fraction of clerodane diterpenes inhibited the activity (42.75%) of pepsin with 1 ug/mL, but it did not inhibit subtilisin (23.76%). The results obtained with apolar and polar extracts from branches and leaves of some species of Salicaceae showed a different pattern of inhibition of two proteases, the aspartic pepsin and the serinic subtilisin, related with different biological activities. The results with the enriched fraction of clerodane diterpenes suggests that the activity observed with the *C. sylvestris* may be related with the presence of these substances in the crude extract.

Keywords: Salicaceae, *Casearia,* clerodane diterpenes, gastric anti-ulcer activity, protease inhibition, fluorescence assay.

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INTRODUCTION

Casearia sylvestris Sw. (Salicaceae) (APG II, 2003) is a traditional South-American medicinal plant used popularly as antiseptic, topical anesthetic, anti-ophidic venom, gastric anti-ulcer, and in weigh loss nutraceuticals formulas (Vieira Jr et al., 2009; Silva et al., 2006). Gastric anti-ulcer activities of the ethanolic extract of *C. sylvestris* have been described in animal models (Esteves et al., 2005; Basile et al., 1990) and its activity is attributed to its volatile oils, tannins and terpenes (Sertié et al., 2000). Other studies showed that the inhibitory activity of aqueous extract of *C. sylvestris* on metalloproteases is the main mechanism of action for its anti-ophidic venom properties (Silva et al., 2006; Borges et al., 2001). Considering, the lack of information about others species from the family Salicaceae we evaluate the inhibitory protease activity of *C. gossypiosperma, C. obliquoa, C. decandra, C. rupestris* and *Flacourtia ramontchi*. Additionally, the enriched fraction of casearins, clerodanic diterpenes from *C. sylvestris* with biological activity (Santos et al., 2009), were tested. Results from our laboratory showed that casearins showed antiulcerogenic activity *in vivo* (data not published).

For the pharmacological screening of extracts from the Salicaceae species, we used a Fluorescence Resonance Energy Transfer (FRET) based protease assay (Hirata et al., 2005) using the aspartic protease pepsin. It has been proposed that pepsin must be a promise target for the treatment of gastric injuries. Esophageal and gastric ulcer are due to the increased secretion of gastric acid and pepsin leading to auto digestion of gastric mucosa (Jainu & Devi, 2006; Tobey et al., 2001). Additionally, we performed a protease assay using the subtilisin, a serinic protease, for selective activity comparisons. Proteases are involved in many essential intracellular and extracellular physiological processes, but its critical role in the development of diseases is also well established. Therefore, proteases are perhaps the largest class of enzyme to be used as targets for structure based drug design with pepsin, elastase, renin and angiotensin-converting enzyme, as promise examples (Fear et al., 2007; Mittl & Grütter, 2006; Tobey et al., 2001).

As part of a research project focusing on phytochemical investigation of medicinal plants from the Brazilian's biomes Cerrado and Atlantic Rain Forest searching for new bioactivity compounds, we aimed to explore the potential inhibitory activity of the extracts of *Casearia* species based on its popular use for gastric injury.

MATERIAL AND METHODS

General experimental procedure

Fluorescence bioassay data were collected with a Multidetection microplate reader Synergy™ HT (Bio-Tek® Instruments, Inc. Winooski, Vermont, USA), with 360 nm excitation and 460 nm emission filters, and analyzed using a KC4 software (Bio-Tek® Instruments) and a Microsoft Windows XP. Equipments for phytochemical analysis were previously described (Santos et al., 2009).

Plant material

The leaf and branches of the specimens were collected at Campinas-SP, Brazil, in June 2007. Exsiccates were deposited at Instituto Agronômico de Campinas. The material was dried and triturated separately, and then submitted to extraction by maceration with solvents at room temperature. Extraction of enriched fraction of casearins B, D, L, O, X and caseargrewiin F were obtained as published (Santos et al., 2009).

Protease inhibition assays

Reagents

Pepsin from porcine gastric mucosa, Recombinant Type VIII Subtilisin Carlsberg, Arg-Glu-(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys-(DALBCYL)-Arg fluorogenic substrate (EDANS-DABCYL), DMSO spectrophometric grade were from Sigma-Aldrich, Sao Paulo, Brazil.

Procedure

Extracts samples (1 and 0.01 µg/mL) were pre-incubated with subtilisin (37 nM) or pepsin (1.7 nM) for 1 h and then, transferred to a black opaque microplate. The substrate EDANS-DABCYL (2 µM), prepared in the specific buffer for each protease, were automated injected. The final volume was 100 µL. Experiments were performed separately for each protease, which was prepared at the day of experiment. Reads were made for a period of 1 h, with 1 min intervals, and temperature controlled at 37 ºC. The mean, standard deviation and relative standard deviation (RSD) of triplicates and the % inhibition were calculated using the final fluorescence intensity measured.

Initially the extracts were dissolved in DMSO and the dilutions of samples tested were made in the respective buffer for each enzyme, i.e., 0.1 M sodium phosphate (pH 7.5) for subtilisin and 0.1 M sodium acetate (pH 4.4) for pepsin.

RESULTS AND DISCUSSION

As showed in the results presented in Table 1, ethanolic and aqueous extracts from branches and leafs of *Casearia sylvestris, C. gossypiosperma, C. decandra* and *C. rupestris* showed high percentage inhibition, approximately 50%, with the higher concentration used

(1 µg/mL). *C. obliquoa* did not show de same pattern of activity, but significantly inhibit pepsin (47.81%) and subtilisin (47.26%) with de hexanic fraction from leafs in the concentration of 1 µg/mL. Interestingly, hexanic fraction from branches of *C. decandra* and *Flacourtia ramontchi* also inhibit the activity of the serinic protease subitilisin, 47.56% and 32.84%, respectively. Additionally, *Flacourtia ramontchi* inhibit subtilisin with ethanolic extract from branches (50.30%) and leafs (44.14%) and aqueous extract from leafs (56.70%) in the concentration of 1 µg/mL. Hexanic extract from branches and the aqueous extract from leaves of *F. ramontchi* also inhibit pepsin, (48.29% and 43.77%, respectively), with the concentration of 1 µg/mL.

Table 1. Results (% inhibition (RSD)) obtained in FRET protease inhibition assays with enriched fraction of casearins from *Casearia sylvestris* (A) and extracts of *C. sylvestris*, (B), *C. gossypiosperma* (C), *C. obliquoa* (E), *C. decandra* (F), *C. rupestris* (G) and *Flacourtia ramontchi* (J). H, hexanic extract; E, ethanolic extract; A, aquous extract; b, branch; l, leaf.

	Subtilisin		Pepsin	
	1 µg/mL	0,01 µg/mL	1 µg/mL	0,01 µg/mL
A	23.76 (0.79)	7,40 (7,23)	42.75 (4.28)	28.15 (2.28)
BE l	25.52 (2.86)	8,18 (5,82)	36.57 (7.03)	7.97 (5.57)
CH b	18.43 (4.07)	22.05 (3.02)	23.82 (5.67)	-22,43 (2.87)
CH l	10.79 (7.85)	9.32 (2.84)	35.65 (2.15)	35.85 (1.27)
CE b	-105.40 (7.44)	-55.88 (9.82)	51.06 (0.35)	45.58 (2.37)
CE l	23.47 (0.77)	18.13 (8.07)	41.35 (3.00)	30.03 (2.43)
CA l	37.95 (3.86)	27.20 (1.22)	44.10 (2.61)	32.02 (1.75)
EH b	47.26 (9.48)	33.62 (5.65)	47.81 (5.51)	28.43 (0.60)
EE b	22.85 (14.65)	11.18 (3.20)	30.08 (3.72)	25.00 (5.38)
EE l	13.97 (3.16)	8.30 (9.53)	18.81 (3.46)	18.47 (5.06)
EA b	20.16 (0.40)	8.47 (8.63)	29.66 (6.13)	21.50 (3.14)
EA l	24.84 (2.80)	10.89 (6.54)	12.03 (0.03)	5.35 (2.54)
FH b	33.35 (2.84)	31.33 (13.33)	nd	nd
FH l	47.56 (3.16)	38.35 (9.61)	34.92 (0.07)	37.20 (3.98)
FE b	37.76 (2.18)	6.04 (3.80)	45.88 (6.75)	35.62 (2.29)
FE l	-32.71 (2.72)	-34.75 (0.95)	nd	30.72 (8.98)
FA b	54.20 (4.47)	43.22 (8.86)	49.32 (3.27)	36.50 (4.79)
FA l	17.10 (0.59)	0.29 (0.07)	41.02 (3.96)	35.75 (0.23)
GH b	14.25 (1.11)	17.75 (6.80)	47.91 (2.59)	0.86 (2.29)
GH l	27.74 (6.90)	23.66 (1.33)	27.14 (1.53)	17.22 (11.16)
GE b	48.86 (11.55)	44.06 (0.89)	48.87 (2.60)	44.49 (2.53)
GE l	22.87 (0.13)	23.85 (11.67)	30.54 (6.20)	15.30 (9.46)
GA b	34.04 (1.30)	32.09 (3.35)	50.78 (4.01)	41.74 (2.19)
GA l	49.55 (5.08)	40.54 (1.12)	44.45 (7.82)	42.27 (2.78)
JH b	32.84 (4.95)	38.68 (0.57)	48.29 (5.90)	35.43 (4.27)
JE b	50.30 (5.14)	40.68 (3.70)	31.67 (2.90)	15.82 (5.12)
JE l	44.14 (7.08)	32.88 (2.63)	13.44 (6.67)	13.66 (1.98)
JA b	12.54 (2.38)	18.11 (9.9)	35.50 (3.61)	34.13 (1.01)
JA l	56.70 (13.15)	29.64 (11.40)	43.77 (3.96)	36.00 (3.16)

nd – not determined

The results presented here showed some pattern of activity of the extracts tested. The most polar extracts from *C. sylvestris* and *C. gossypiosperma* specific inhibit the aspartic protease pepsin. Interestingly, the enriched fraction of casearins from the ethanolic extract of *C. sylvestris*, showed specific inhibitory activity of pepsin (42.75 %), but did not inhibit subtilisin (23.76 %). The apolar extract from *C. obliquoa, C. decandra* and *F. ramontchi* inhibit the serinic protease substilisin. The polar extracts of *C. decandra* and *F. ramontchi* showed inhibitory activity in different types of proteases, the serinic subtilisin and aspartic pepsin.

Considering the pharmacological interest in the use of pepsin as a molecular target to threat gastric ulcer (Jainu & Devi 2006; Tobey et al., 2001), the *in vivo* studies showing antiulcerogenic activity of the extracts of *Casearia sylvestris* (Esteves et al., 2005; Sertié et al., 2000; Basile et al., 1990) and its traditional use to threat gastric injuries (Silva et al., 2006), the results presented here suggests that this activity may be related to inhibition of pepsin. Additionally, the data obtained with the enriched fraction of clerodane diterpenes, the casearins, showed that these compounds may be involved with the antiulcerogenic activity of the extract of *C. sylvestris* and their mechanisms of actions are related to the inhibition of pepsin. The results showed that the ethanolic and aqueous extracts from other species of *Casearia* showed inhibitory activity of pepsin and phytochemistry studies have been performed in our laboratory to investigate the presence of clerodane diterpenes in this plants.

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