European Review for Medical and Pharmacological Sciences 2014; 18: 46-51

Effect of bauhinia bauhinioides kallikrein inhibitor on endothelial proliferation and intracellular calcium concentration

M. BILGIN[1,2], K.M. BURGAZLI[3], A. RAFIQ[1], M. MERICLILER[3], C. NEUHOF[1], M.L. OLIVA[4], M. PARAHULEVA[1], N. SOYDAN[1], O. DOERR[1], Y. ABDALLAH[1], A. ERDOGAN[1]

[1]Department of Cardiology and Angiology, Justus-Liebig-University of Giessen, Giessen, Germany
[2]Department of Radiology, Bezmialem Vakif University, Medical Faculty, Istanbul, Turkey
[3]Department of Internal Medicine and Angiology, Wuppertal Research and Medical Center, Wuppertal, Germany
[4]Departament of Biochemistry, Universidade Federal de São Paulo, São Paulo, Brazil

Abstract. – OBJECTIVES: Proteinase inhibitors act as a defensive system against predators e.g. insects, in plants. Bauhinia bauhinioides kallikrein inhibitor (BbKI) is a serine proteinase inhibitor, isolated from seeds of Bauhinia bauhinioides and is structurally similar to plant Kunitz-type inhibitors but lacks disulfide bridges. In this study we evaluated the antiproliferative effect of BbKI on endothelial cells and its impact on changes in membrane potential and intracellular calcium.

MATERIALS AND METHODS: HUVEC proliferation was significantly reduced by incubation with BbKI 50 and 100 µM 12% and 13%. Furthermore, BbKI (100 µM) exposure caused a significant increase in intracellular Ca^{2+} concentration by 35% as compared to untreated control.

RESULTS: The intracellular rise in calcium was not affected by the absence of extracellular calcium. BBKI also caused a significant change in the cell membrane potential but the antiproliferative effect was independent of changes in membrane potential.

CONCLUSIONS: BBKI has an antiproliferative effect on HUVEC, which is independent of the changes in membrane potential, and it causes an increase in intracellular Ca^{2+}.

Key Words:
Kallikrein inhibitor, Angiogenesis, Endothelial cells, Calcium, Hyperpolarization, Proliferation.

Introduction

Plant proteinase inhibitors act as a defensive system against predators such as insects. Bauhinia bauhinioides kallikrein inhibitor (BbKI), a serine proteinase inhibitor isolated from seeds of Bauhinia bauhinioides, is structurally similar to plant Kunitz-type inhibitors but lacks disulfide bridges. It has a single polypeptide chain with a molecular weight of 20 kDa[1]. Kunitz type proteinase inhibitors are present in the seeds of leguminosae subfamilies, which consist usually of single polypeptide chain but some of the inhibitors are also dimeric or double polypeptide chains[2,3]. It has been shown that BbKI can inhibit various animal proteolytic enzymes e.g. trypsin, chymotrypsin, human plasmin, plasma kallikrein and porcine pancreatic kallikrein[4]. Protease inhibitors work by reversibly binding or interacting with proteinases, thus, influencing catalytic activity[5]. Serine proteinase inhibitors act by binding tightly to the catalytic site[6,7].

In humans, kallikreins (tissue and plasma kallikrein) are serine proteases that liberate kinins (bradykinin and kallidin) from the kininogens. They are widely distributed in the body e.g. kidney, blood vessels, pancreas, gut, spleen, adrenal gland, plasma and neutrophils. In the cardiovascular system, the kallikrein-kinin system plays an important role in maintaining arterial blood pressure and vascular smooth muscle cell tone[8]. On the other hand, the serine proteases (e.g. kallikrein-kinin system) are reported to stimulate tumor invasion and angiogenesis[9]. It has been speculated that cancer cells utilize serine proteinases to access the local blood supply, migrate through the tissue matrix, and to move into and out of blood vessels. Higher gene expression of tissue kallikrein, a serine proteinase, is already reported in human adenocarcinomas and in cell lines from various cancerous tissues e.g. esophagus, kidney, prostate, colon, breast, pituitary, ovary, endometrium[10-15].

Corresponding Author: Ali Erdogan, MD; e-mail: ali.erdogan@innere.med.uni-giessen.de

Proteinase inhibition has been used to treat diseases involving massive release of proteolytic enzymes e.g aprotinin, a Kunitz serine protease inhibitor has been used to treat pancreatitis[16]. The current study aimed to investigate the effect of BbKI on human umbilical vein endothelial cell (HUVEC) proliferation, and the influence of Bb-KI on cell membrane potential changes and on intracellular calcium concentration.

Materials and Methods

Chemicals and Solutions

Bis-1,3-dibutylbarbituric acid-trimethine oxonol (DiBAC4(3)) and fluo-3 (AM) were both obtained from Molecular Probes (Leiden, Netherlands); 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester (BAP-TA-AM) from Merck KGaA (Darmstadt, Germany); fetal calf serum (FCS) from Biowest and Hank's balanced salt solution (HBSS) from PAA (Linz, Austria); endothelial basal medium and supplements from Promo Cell (Heidelberg, Germany); trypsin-EDTA (0.05%) from Sigma (Deisenhofen, Germany). Bauhinia bauhinioides kallikrein Inhibitor (BbKI) was a gift from ML Oliva. All other reagents and chemicals used in this study were of technical grade.

The HEPES-buffered bath solution was made of (in mmol/L): $CaCl_2$ 1.25; D-glucose 5.5; HEP-ES 10; KCl 5; $MgCl_2$ 0.5; NaCl 140; and Ca^{2+}-free buffer was made by reducing the amount of Ca^{+2} to 0.0 mmol/L, pH was adjusted to 7.3 with 1M NaOH.

Cell Isolation and Culture

HUVECs were isolated by collagenase digestion procedure and cultured. The method has been described previously by Jaffe et al[17]. Cells were cultured in endothelial cell growth medium enriched with 2% FCS and supplemented with growth factors and 1% of penicillin and streptomycin. The medium was exchanged every 2-3 days. All experiments were performed with cells from passage 3-4.

Endothelial Proliferation

The proliferation of HUVECs was studied by cell counting with a Neubauer chamber method as described previously Schaefer et al[18]. In summary, cells were seeded in 12-well plates (Becton Dickinson, Heidelberg, Germany), coated with 0.2% gelatine at a density of 10,000 cells/well. Cells were grown for 24 h in endothelial cell growth medium with 10% FCS followed by 24 h incubation in serum-free medium containing hydrocortisone (1 μg/ml) and gentamycin (50 μg/ml). After 24 h incubation the medium was replaced by endothelial cell growth medium with 2% FCS and the following modifications: BbKI (10-100 μmol/l) and/or valinomycin (1 μmol/l). After 48 h the cells were trypsinized and the cell number determined with a Neubauer chamber. The mean values of two counts per well were used for statistical analysis.

Measurement of the Membrane Potential

Changes in endothelial membrane potential were investigated using the fluorescence dye DiBAC4(3), which correlates with changing membrane potential. HUVECs were seeded on 24-well plates and grown for 2 days to confluence. The cells were loaded with DiBAC4(3) 0.5 μmol/l for 20 min at 37°C in the dark. Then the cells were stimulated with BbKI (10-100 μmol/l). Changes in fluorescence intensity were measured using a plate reader Genios (Tecan, Grödig, Austria). This plate reader measures changes in fluorescence, absorbance, and glow luminescence. This plate reader can detect fluorescence in a range of 340-700 nm. Fluorescence intensity changes were measured for 30 min in intervals of 30 seconds with an excitation wavelength of 485 nm and detection of the emitted light at 535 nm. The data was normalized to a parallel non-stimulated control. Fluorescence data was analysed and presented in percentage as compared to untreated control.

Calcium Measurement

Changes in Ca^{+2}i concentration were detected with the fluorescence probe fluo-3 (AM). HU-VECs were cultured on 24-well plates until they were confluent. The cells were loaded with fluo-3 (AM) (5 μmol/l) for 60 min at 37°C in the dark; the medium containing fluo-3 (AM) was replaced by HEPES-buffered bath solution for de-esterification for 25-30 minutes. Cells were stimulated with BbKI (10-100 μmol/l) and/or BAPTA-AM (10 μmol/l) and/or thapsigargin (10 μmol/l). BAPTA-AM was loaded 30 minutes before BbKI addition, to allow uptake and de-esterification, an untreated control was run in parallel. Changes in ffluorescence intensity of the calcium signal were measured using a plate reader Genios (Tecan, Grödig, Austria). This plate reader measures changes in fluorescence, absorbance, and

glow luminescence. This plate reader can detect fluorescence in a range of 340-700 nm. The excitation wavelength was 485 nm and emission was detected at 535 nm over 60 min at intervals of 60s. The data was normalized to parallel non-stimulated control.

Statistical Analysis

Results were analysed by means of ANOVA and post-hoc Tukey test (SPSS version 18.0; SPSS Inc. Chicago, IL, USA). Data are expressed as mean ± SEM. The significance level was $p < 0.05$.

Results

Concentration-Dependent Reduction of Cell Growth by BbKI

The effect of BbKI on HUVEC proliferation was studied by exposing the cells to BbKI (10-200 μmol/l) for 48 hours. BbKI caused a statistically significant reduction in cell proliferation in a concentration-dependent manner (Figure 1). As compared to untreated control the cell number decreased to 96 % ± 4.6 (10 μmol/l), 88% ± 2.5 (50 μmol/l), 87% ± 1.8 (100 μmol/l), and 73% ± 4.8 (200 μmol/l). This difference was statistically highly significant as compared to non-stimulated control for 50, 100 and 200 μmol/l.

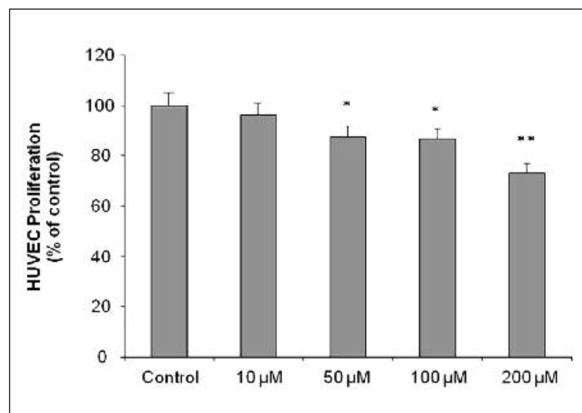

Figure 1. Effect of BbKI (10-200 μmol/l) on HUVEC proliferation. BbKI has an antiproliferative effect on HUVEC (n=12). The results are shown as percentage of untreated control. BbKI induced a significant (*) reduction in proliferation activity at 50 and 100 μmol/l and this effect was highly significant (**) at 200 μmol/l. The significance level was set as $p \leq 0.05$. The `*´ is for significance and `**´ shows high significance.

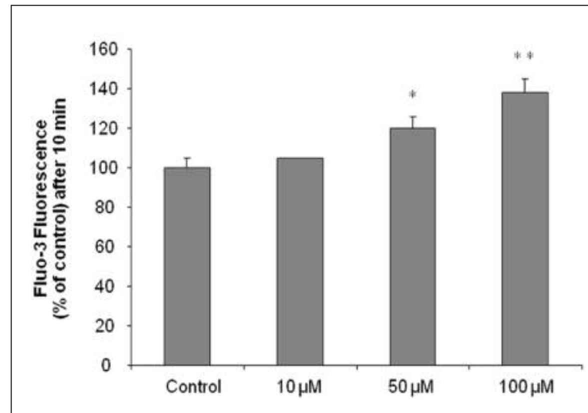

Figure 2. Effect of BbKI (10-100 μmol/l) on changes in cytosolic Ca^{2+}. BbKI (100 μmol/l) induces a highly significant increase of $[Ca^{2+}]i$. Calcium concentration was measured with fluo-3. Data are expressed as percentage of untreated control run in parallel 10 minutes after addition of BbKI. The significance level was set as $p \leq 0.05$. The `*´ is for significance and `**´ shows high significance.

Effect of BbKI on $[Ca^{+2}]i$

The BbKI-stimulated calcium signalling mechanism in HUVEC was analyzed by measuring the changes in cytosolic Ca^{+2} concentrations (Figure 2). As compared to the non-stimulated control after 10 minutes stimulation with BbKI caused a statistically significant increase in cytosolic Ca^{2+} of 79% ± 5.0, 85% ± 3.2 and 111%± 7.9 with 10, 50, and 100 μmol/l of BbKI, respectively. This rise in $[Ca^{+2}]i$ was completely absent in the presence of calcium chelator BAPTA (10 μmol/l). This rise in cytosolic Ca^{2+} was also observed when cells were incubated with calcium free bath solution, but this rise was absent in the presence of calcium chelator BAPTA (Figure 3). Further experiments with thapsigargin (10 μM), showed a highly significant increase of intracellular Ca^{2+} by 58% (in the presence of BBKI 100 μM) as compared to 35% increase with BBKI 100 μM (Figure 4). These results indicate that BbKI induces a significant calcium release from the endoplasmic reticulum.

BbKI Stimulation Causes Hyperpolarization of Endothelial Cells

Changes in the cellular membrane potential were analyzed by using a fluorescence probe DiBAC4(3). BbKI (100 μM) induced a significant reduction of membrane potential 8% ± 0.4 as compared to the parallel untreated control while incubation with 50 μmol/l caused a reduction of membrane potential by 7% ± 0.5 (Figure 5).

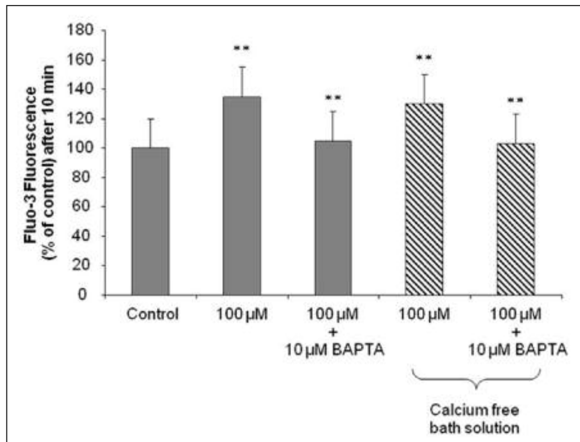

Figure 3. Effect of BbKI (100 μmol/l) in the presence of calcium chelator BAPTA on changes in cytosolic Ca^{2+}. BbKI (100 μmol/l) induces a highly significant increase of $[Ca^{2+}]i$. The effect of BBKI was abolished in the presence of BAPTA. Presence or absence of extracellular calcium had no significant effect. Calcium concentration was measured with Fluo-3. Data are expressed as percentage of untreated control run in parallel. The significance level was set as $p \leq 0.05$. The `*´ is for significance and `**´ shows high significance (n=20).

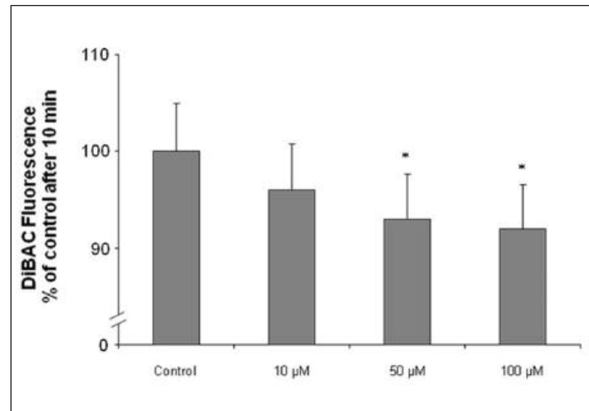

Figure 5. BBKI caused a significant change of the endothelial cell membrane potential. Data are shown as percentage of untreated control 10 minutes after addition of BbKI (10-100 μmol/l). The membrane potential was analysed using the fluorescence probe DiBac4(3). The significance level was set as $p \leq 0.05$. The `*´ is for significance (n=40).

BBKI Induced Antiproliferative Effect is Independent of Changes in Membrane Potential

Changes in cell membrane potential have no effect on antiproliferative activity of BBKI. This was studied by using valinomycin. There was no significant difference observed in antiproliferative activity of BBKI when cells were incubated with valinomycin (Figure 6).

Discussion

In this study we have shown the antiproliferative effect of BBKI, a serine protease inhibitor isolated from the seeds of Bauhinia bauhinioides. We

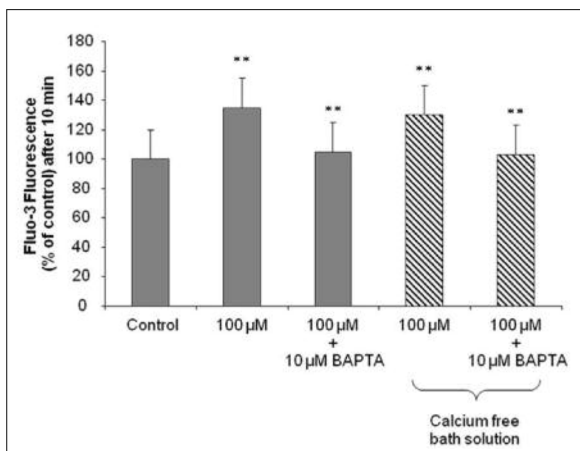

Figure 4. BbKI (100 μmol/l) induced a significant increase of $[Ca^{2+}]i$ by 36% as compared to untreated control after 10 min. Incubation of cells in the presence of thapsigargin (TG) 10 μM with BbKI shows 58% rise in intracellular Ca^{2+} as compared to untreated control. Data are expressed as percentage of untreated control run in parallel, 10 minutes after addition of BbKI. The significance level was set as $p \leq 0.05$. The `*´ is for significance and `**´ shows high significance (n=16).

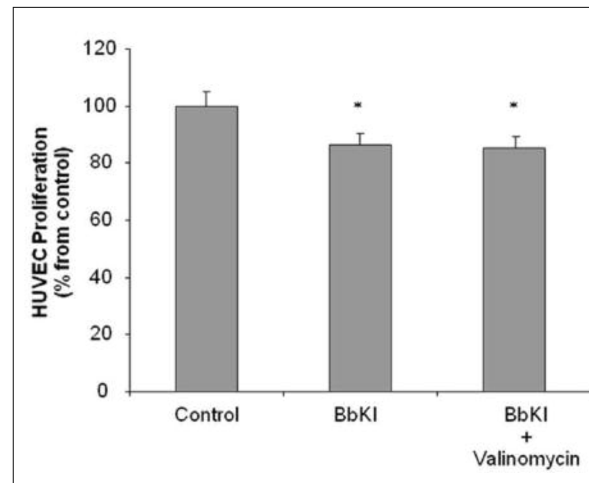

Figure 6. HUVEC proliferation inhibition by BbKI was independent of the BbKI-induced membrane potential changes. Proliferation was examined by cell counting with a Neubauer chamber in presence of BbKI (100 μmol/l) and valinomycin (1 μmol/l) and BbKI (100 μmol/l). Data are expressed as percentage of untreated control. The significance level was set as $p \leq 0.05$. The `*´ is for significance (n=6).

have shown that incubating HUVEC with different concentrations of BBKI has an antiproliferative effect. The kallikrein-kinin system is reported to play important role in vascular inflammatory responses e.g. vasodilation and vascular permeability. High expression of tissue kallikrein protein is reported to enhance tumorigenesis, cancer cell proliferation, angiogenesis, vascular permeability, and regulation of cancer cell invasion in different cancers e.g. endometrial, pituitary and prostate cancers[19]. It has been reported that tissue kallikrein inhibition by synthetic inhibitors e.g. FE999024 results in reduced metastasis and invasiveness of human breast cancer cells implanted in rat pulmonary vasculature[20]. Furthermore, kallistatin, an endogenous kallikrein inhibitor, is also reported to suppress tumour angiogenesis. It inhibits angiogenesis by suppressing the effect of VEGF, bFGF, and adhesion of endothelial cells[21].

In previous years it has been reported that the kallikrein-kinin system might play an important role in angiogenesis[14]. Several members of the tissue Kallikrein family e.g. KLK 5,6,7,8,9,10 and 11 are reported to play a vital role in tumor invasion, metastasis, and carcinogenesis[22]. Recently Gao et al[23] reported that tissue kallikrein promotes migration and invasion of prostatic cancer cells via protease activated receptor-1-dependent signalling. In this context inhibition of the kallikrein-kinin system through protease inhibitors from plants might lead to development of anti tumour therapies. Calcium is an important secondary messenger, which plays important role in various cellular functions e.g. migration, proliferation of endothelial cells. It has been shown[24] that various bioactive substances modulate release of calcium from intracellular stores, the endoplasmic reticulum, or influx of extracellular calcium across the plasma membrane. In this study we observed a significant increase in intracellular calcium. Further experiments with BAPTA and calcium containing, and calcium-free bath solutions demonstrated the release of calcium from endoplasmic reticulum. Previous studies show[25,26] that increased intracellular calcium stimulates Ca^{2+}-activated channels and can lead to hyperpolarization of the cell membrane. We here have shown that BBKI stimulation causes a significant change in the membrane potential. However, this change in membrane potential has no significant effect on antiproliferative activity of BBKI. Previously, we have shown[27,28] a role of increase of intracellular calcium and changes in the membrane potential in the antiproliferative activity of querectin and apigenin.

Conclusions

BBKI induces hyperpolarization of the endothelial cell membrane and causes an increase in intracellular Ca^{2+}. It also significantly reduces proliferative activity, which is independent of BBKI-induced hyperpolarization of the cell membrane. The antiproliferative effect of BBKI seems promising for the development of anticancer drugs. Further studies are required to understand the antiproliferative mechanisms of BBKI.

Acknowledgements

The authors gratefully thank Mrs. Christina Frey-Krug for her skilled technical assistance

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Adams DJ, Barakeh J, Laskey R, Van Breemen C. Ion channels and regulation of intracellular calcium in vascular endothelial cells. FASEB J 1989; 3: 2389-2400.
2) Berling R, Genell S, Ohlsson K. High-dose intraperitoneal aprotinin treatment of acute severe pancreatitis: a double-blind randomized multi-center trial. J Gastroenterol 1994; 29: 479-485.
3) Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol Rev 1992; 44: 1-80.
4) Bilgin M, Neuhof C, Doerr O, Benscheid U, Andrade SS, Most A, Abdallah Y, Parahuleva M, Guendez D, Oliva ML. Bauhinia bauhinioides cruzipain inhibitor reduces endothelial proliferation and induces an increase of the intracellular Ca^{2+} concentration. J Physiol Biochem 2010; 66: 283-290.
5) Bode W, Huber R. Natural protein proteinase inhibitors and their interaction with proteinases. Eur J Biochem 1992; 204: 433-451.
6) Borgoño CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 2004; 4: 876-890.
7) Chen LM, Richards GP, Chao L, Chao J. Molecular cloning, purification and in situ localization of human colon kallikrein. Biochem J 1995; 307: 481-486.
8) Clements J, Mukhtar A. Tissue kallikrein and the bradykinin B2 receptor are expressed in endometrial and prostate cancers. Immunopharmacology 1997; 36: 217-220.

9) Dlamini Z, Raidoo D, Bhoola K. Visualisation of tissue kallikrein and kinin receptors in oesophageal carcinoma. Immunopharmacology 1999; 43: 303-310.

10) Erdogan A, Most AK, Wienecke B, Fehsecke A, Leckband C, Voss R, Grebe MT, Tillmanns H, Schaefer CA, Kuhlmann CRW. Apigenin-induced nitric oxide production involves calcium-activated potassium channels and is responsible for antiangiogenic effects. J Thromb Haemost 2007; 5: 1774-1781.

11) Gao L, Smith RS, Chen LM, Chai KX, Chao L, Chao J. Tissue kallikrein promotes prostate cancer cell migration and invasion via a protease-activated receptor-1-dependent signaling pathway. Biol Chem 2010; 391: 803-812.

12) Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52: 2745-2756.

13) Jones TH, Figueroa CD, Smith C, Cullen DR, Bhoola KD. Characterization of a tissue kallikrein in human prolactin-secreting adenomas. J Endocrinol 1990; 124: 327-331.

14) Kataoka H, Itoh H, Koono M. Emerging multifunctional aspects of cellular serine proteinase inhibitors in tumor progression and tissue regeneration. Pathol Int 2002; 52: 89-102.

15) Krauchenco S, Nagem RA, da Silva JA, Marangoni S, Polikarpov I. Three-dimensional structure of an unusual Kunitz (STI) type trypsin inhibitor from Copaifera langsdorffii. Biochimie 2004; 86: 167-172.

16) Krauchenco S, Silva JA, Nagem RA, Brandão Neto JR, Forrer VP, Carmona E, Ferreira, Macedo MLR, Novello JC, Marangoni S, Polikarpov I. Crystallization and preliminary X-ray diffraction analysis of a novel trypsin inhibitor from seeds of Copaifera langsdorffii. Acta Crystallogr D Biol Crystallogr 2001; 57: 1316-1318.

17) Kuhlmann CR, Schaefer CA, Kosok C, Abdallah Y, Walther S, Lüdders DW, Neumann T, Tillmanns, Schafer C, Piper HM, Erdogan A. Quercetin-induced induction of the NO/cGMP pathway depends on Ca^{2+}-activated K+ channel-induced hyperpolarization-mediated Ca^{2+}-entry into cultured human endothelial cells. Planta Med 2005; 71: 520-524.

18) Laskowski M Jr, Kato I. Protein inhibitors of proteinases. Annu Rev Biochem 1980; 49: 593-626.

19) Mahabeer R, Bhoola KD. Kallikrein and kinin receptor genes. Pharmacol Ther 2000; 88: 77-89.

20) Miao RQ, Agata J, Chao L, Chao J. Kallistatin is a new inhibitor of angiogenesis and tumor growth. Blood 2002; 100: 3245-3252.

21) Nilius B, Droogmans G. Ion channels and their functional role in vascular endothelium. Physiol Rev 2001; 81: 1415-1459.

22) Oliva ML, Mendes CR, Santomauro-Vaz EM, Juliano MA, Mentele R, Auerswald EA, Sampaio MU, Sampaio CA. Bauhinia bauhinioides plasma kallikrein inhibitor: interaction with synthetic peptides and fluorogenic peptide substrates related to the reactive site sequence. Curr Med Chem 2001; 8: 977-984.

23) Oliva ML, Sampaio MU. Action of plant proteinase inhibitors on enzymes of physiopathological importance. An Acad Bras Cienc 2009; 81: 615-621.

24) Radisky ES, Kwan G, Karen Lu CJ, Koshland DE Jr. Binding, proteolytic, and crystallographic analyses of mutations at the protease-inhibitor interface of the subtilisin BPN'/chymotrypsin inhibitor 2 complex. Biochemistry 2004; 43: 13648-13656.

25) Rae F, Bulmer B, Nicol D, Clements J. The human tissue kallikreins (KLKs 1-3) and a novel KLK1 mRNA transcript are expressed in a renal cell carcinoma cDNA library. Immunopharmacology 1999; 45: 83-88.

26) Rehbock J, Buchinger P, Hermann A, Figueroa C. Identification of immunoreactive tissue kallikrein in human ductal breast carcinomas. J Cancer Res Clin Oncol 1995; 121: 64-68.

27) Schaefer CA, Kuhlmann CR, Gast C, Weiterer S, Li F, Most AK, Neumann T, Backenkohler U, Tillmanns H, Waldecker B, Wiecha J, Erdogan A. Statins prevent oxidized low-density lipoprotein- and lysophosphatidylcholine-induced proliferation of human endothelial cells. Vascul Pharmacol 2004; 41: 67-73.

28) Wolf WC, Evans DM, Chao L, Chao J. A synthetic tissue kallikrein inhibitor suppresses cancer cell invasiveness. Am J Pathol 2001; 159: 1797-1805.