Cajanus Cajan (l.) Millsp Aqueous Extracts against Melanoma Cell Line and their Proteases

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RESL designed the study, performed the statistical analysis, wrote the protocol, literature searches and wrote the first draft of the manuscript. Authors EMGFT and BRADS managed the analyses of the study. Authors APGAF and ALFS managed the melanoma cell line cultured. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Extract proteins with protease inhibitor (PI) activity from fresh organs of *Cajanus cajan*, using aqueous systems; study the activity of melanoma secreted proteases; investigate inhibitory effect of *C. cajan* extracts on melanoma proteases; and evaluated the effect of the extract, with the most protease inhibitor activity, on melanoma cell line (SK-MEL-28) viability.
Material and Methods: Extracts of *C. cajan* leaves, stems, and roots were prepared using different aqueous systems. Protein content was evaluated by Bradford method, protein profile by gel electrophoresis by Laemmli method and, extracts PI activity against trypsin, papain, and pepsin. Melanoma cell line was cultured in Dulbecco's medium, and secreted proteases was obtained from culture supernatant. Characterization of melanoma proteases included substrates activity, optimum pH, and effect of specific PIs and cations on protease activity. Anticancer activity was investigated

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using *C. cajan* extracts (containing PIs) and SK-MEL-28 extracellular fraction (containing proteases). Cytotoxic effect of extract was assayed on SK-MEL-28 cells line using methylthiazol tetrazolium method. CC-P was submitted to thin layer chromatography to identify alkaloids, coumarins, flavonoids, saponins and terpenes.

Results: *C. cajan* extracts showed different protein contents and protein profiles in electrophoresis analysis. *C. cajan* organs presented PIs activities against serine, cysteine, and aspartic proteases. Leaf extract prepared using phosphate buffer (CC-P) and stems extract prepared with water (CC-CA) had the best inhibitory activities against trypsin (~58%). Pepsin was the lowest inhibited (11-29%) and papain was the most inhibited (14-100%). Protease activity of melanoma fraction was the highest using casein as substrate, and two proteins with 150 and 100 kDa with gelatinase activity. These proteases has maximal activity at pH 7.0 and 9.0, and was importantly inhibited by benzamidine, 1,10-phenanthroline and EDTA, suggesting that serine and metalloproteases are secreted by SK-MEL-28 cells. CC-P was the most important inhibitor of melanoma proteases, and induced cytotoxicity on SK-MEL-28 cells in culture. Although there is correlation between melanoma protease inhibition and cell death, CC-P has secondary metabolites, as coumarins, flavonoids and terpenes that can have synergy of antitumor activity.

Conclusion: *C. cajan* extracts have serine, cysteine, and aspartic protease inhibitor activities. CC-P had the best inhibition on melanoma proteases and it was also cytotoxic to melanoma cell in culture. Therefore, these PIs can be important strategy for cancer treatment because tumor cells secrete proteases that are crucial to cancer progression.

1. INTRODUCTION

Cajanus cajan (L.) Millsp. (Fabaceae), commonly known as pigeon pea, is a perennial shrub native to western Africa and widely distributed in tropical and subtropical areas in over 90 countries around the world. *C. cajan* has been cultivated in Africa and India since ancient times and represents the main food staple [1,2]. In many parts of the world, it is a medicinal plant, and has been widely used as a folk medicine for the treatment of diabetes, skin conditions, hepatitis, malaria, anemia, measles, jaundice, dysentery, swelling and pain, parasites, and varicella, and it has been shown to have anti-inflammatory, antinociceptive, immuno-modulatory and antioxidant activities [3,4,5,6,7]. Many protease inhibitors (PIs) have been isolated, purified, and characterized from *Cajanus cajan* seeds [8,9,10] and the anticancer activity was also attributed to a PI found in seeds [11].

Proteases or peptidases catalyze the cleavage of peptide bonds in proteins and peptides and are found in all organisms, organs and organelles [12]. They participate in many essential physiological processes, such as amino acid assimilation for protein or organic molecule synthesis, cell death, differentiation, remodeling and growth of tissues and organs [13]. Tumors growth, metastasis dissemination, and cancer establishment, development, and promotion are driven by cytokines, growth factors, and proteases. These enzymes activate cytokines and signaling receptors by the cleavage of specific peptide bonds, alter the extracellular matrix (ECM) composition by removing proteins and proteoglycans to allow tumor invasion, suppress cell death pathways, activate survival pathways, stimulate the migration and invasion of tumor cells, and activate the production of inflammatory and angiogenic molecules [14,15].

Cancer proteases are important chemotherapeutic targets because specific inhibitors can prevent the development of new tumors and interfere with the progression of many types of tumors [16]. Thus, the targeting of PIs appears to be an important strategy for disease control [17]. PIs bind reversibly or irreversibly to the proteases, inhibiting and/or preventing their autoproteolysis. They can be synthetic, peptide mimetics, or natural, which are peptides that can be obtained from different sources, especially plants [18]. These inhibitors have been extensively investigated for cancers [19,20]. However, the new molecules, such as plant PIs, that are selective, effective, and accessible, remains a great challenge for anticancer therapies, and nature has always been an important source. In addition, the resistance of tumor cells to different treatments is the limiting factor of anticancer therapies [21].

Thus, the main objectives of this work were: extract proteins with PI activity from fresh organs of *Cajanus cajan*, using only aqueous systems; study the activity of melanoma cell line secreted proteases; investigate the inhibitory effect of *C. cajan* extracts on the melanoma cell line (SK-MEL-28) proteases; and evaluated the effect of the extract with the most protease inhibitor activity on melanoma cell line viability.

2. MATERIALS AND METHODS

2.1 Preparation of Aqueous *C. Cajan* Extracts

Fresh leaves (about 148 g for each extraction) were ground into a powder using liquid nitrogen, and proteins were extracted with different aqueous solvents, such as water and buffers (about 450 mL for each extraction), for 2 h at room temperature (24°C), with gentle stirring followed by centrifugation at 10,000 × *g* for 30min at 4°C. The supernatants were collected and lyophilized, resulting in three leaf extracts: an aqueous extract (CC-A) using only distilled water, a phosphate extract (CC-P) using 50 mM sodium phosphate at pH 6.5, and a Tris extract (CC-T) using 50 mM Tris-HCl at pH 7.5. Fresh stems (287.87 g) and roots (292.24 g) were homogenized in distilled water using a blender, and the supernatants obtained after centrifugation were lyophilized to give the stem (CC-CA) and root (CC-RA) aqueous extracts, respectively. The protein content was measured by the Bradford method to minimize the interference by plant alkaloids and polyphenols using BSA as a standard [22].

2.2 Polyacrylamide Gel Electrophoresis and Gelatin Gel Electrophoresis

The protein profiles of extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [23], and the gels were stained with Coomassie Blue R-250. For molecular mass characterization, Precision Plus Protein Standards (250-10 kDa) from Bio-Rad (Berkeley, CA, USA) were used as molecular mass standards. The enzymatic activity of SK-MEL-28 culture supernatant was first analyzed by gelatin substrate gel electrophoresis (gelatin-SDS-PAGE) under reducing conditions as previously described. Following electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h to remove SDS and incubated overnight at room temperature in different 50 mM buffers to allow for proteolysis. After 18 h, gels were stained with 0.1% (w/v) amide black and destained in methanol/acetic acid/distilled water (3:1:6, v/v/v).

2.3 Protease Inhibitory Activity of *C. Cajan* Extracts

Inhibitory assays were performed by pre-incubating of reference proteases trypsin, papain, and pepsin (1 mg/mL, Sigma-Aldrich) with 10 µg of protein from *C. cajan* extracts for 30min at room temperature. The reaction was started by the addition of casein substrate (0.5 mg/mL) to the buffer. The mixture was incubated at 24°C for 30min with slight agitation. The reactions were stopped with trichloroacetic acid and were centrifuged at 10,000 × *g* for 10min at 4°C, and the supernatants, which contained peptides from substrate hydrolysis, were collected, after which the absorbances were measured at 280 nm. Trypsin, papain and pepsin are representative proteases of the serine, cysteine and aspartic classes, respectively. The buffers used in the inhibition assays were Tris-HCl pH 8.5, sodium phosphate pH 5.5, and sodium acetate pH 4.5 for trypsin, papain, and pepsin, respectively. All buffers were used at 50 mM. Inhibition is expressed as residual activity (%) relative to the percentage of the appropriate control, for which the enzymes (trypsin, papain and pepsin) were assayed without the addition of the extracts [24].

The inhibitory activity of *C. cajan* extracts was assessed against SK-MEL-28 secreted proteases obtained from cell culture supernatant. First, 10 µg of protein from SK-MEL-28 extracellular fraction was incubated for 30min with 10 µg of protein *C. cajan* extracts in 50 mM Tris-HCl buffer pH 7.0. Then, casein substrate was added, and the reaction proceeded as described before. Controls were carried out in parallel using the same enzyme solutions without the extracts. Inhibition is expressed as a percentage of the control activity (100%).

2.4 Tumor Cell Line Cultivation and Extracellular Fraction Preparation

The culture of SK-MEL-28 cells, a human melanoma cell line that was established from a patient's axillary lymph node, acquired from Rio de Janeiro Cell Bank (BCRJ 0289). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin and

streptomycin). Three replications were included, and after the last harvest, the cells were discarded, collecting only the culture supernatant by centrifugation at 18 × g for 10min; the cell-free supernatant stored at -20°C. The SK-MEL-28 extracellular fraction was prepared from the culture supernatant. Initially, the supernatant was centrifuged at 10,000 × g for 30min at 4°C, collected and freeze dried, and kept at -4ºC until use. Protein content was measured by the Bradford method [22] using bovine serum albumin (BSA) as a standard.

2.5 Melanona Extracellular Fraction Protease Assays with Protein Substrates

Protease activity of melanona extracellular fraction obtained from SK-MEL-28 culture was evaluated using protein substrates, such as hemoglobin, BSA, casein and an esterolytic substrate N_α-p-Tosyl-L-arginine methyl ester hydrochloride ($_L$-TAME) from Sigma (St. Louis, MO, USA). Hemoglobin, BSA, $_L$-TAME (1 mg/mL) and casein (0.5 mg/mL) were dissolved in 50 mM of Tris HCl buffer with pH 7.0 and incubated for 30min at room temperature (24°C) with 10 µg of protein from the extracellular fraction. Trichloroacetic acid 10% (w/v) was used to stopped the reactions. These tubes were kept on ice for 10min and centrifuged at 10,000 × g for 10min at 4°C. The activity was mensured using UV spectrophotometer at 280 nm and described as specific activity, defined as U. min^{-1}. mg protein^{-1} [25].

2.6 Effect of Ph, Protease Inhibitors and Cations on Protease Activity of Melanoma Extracellular Fraction

The optimun pH assay was carried out by incubating 10 µg of protein from the SK-MEL-28 supernatant at room temperature with 0.5 mg/mL casein. Different buffers was used at 50 mM: sodium citrate (pH 4.0–6.5), Tris-HCl (pH 7.0–8.5), and sodium carbonate/ bicarbonate (pH 9.0–10.0). Reaction occurred for 30min at room temperature, and the hydrolysis of the substrate was determined by measuring the absorbance at 280 nm, and the specific activity was expressed as U. min^{-1}. mg protein^{-1} [26].

The types of SK-MEL-28 proteases were investigated using specific inhibitors for the known protease classes. Synthetic PIs at different concentrations of   were dissolved in

water [ethylene diamine tetraacetic acid (EDTA, 10 mM), benzamidine (Bza, 1 mM), iodacetamide (Ia, 100 µM)], dimethyl sulfoxide [phenyl methylsulfonyl fluoride (PMSF, 10 µM)], methanol [N-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 100 µM), and 1,10-phenanthroline (Pha, 10 mM)] in ethanol. These inhibitors were incubated with 10 µg of protein from the extracellular fraction for 30min at room temperature. The reaction was started by the addition of casein as substrate (0.5 mg/mL) for 15min, and the activity was measured as described above. Control reactions were carried out in parallel without inhibitors. Inhibition percentage activity was calculated in comparision with control activity results. All inhibitors were purchased from Sigma [24].

Ten micrograms of protein from each extract were incubated for 30min at 24°C with 10 mM of calcium, zinc, manganese, and magnesium chlorides before the substrate was added. The reactions were performed as described above. The residual activity was calculated by considering the protease activity in the absence of added ions as 100% [27].

2.7 Cytotoxicity Assay of *C. Cajan* Extract

The effect of extracts of *C. cajan* on the proliferation of the SK-MEL-28 melanoma cell line was evaluated in a molecular pharmacology laboratory (Farmanguinhos - FIOCRUZ). SK-MEL-28 cells were cultured in DMEM and maintained in the log growth phase. Twenty-four hours prior to treatment with the samples, 100 µL of the cell suspension (5 × 10^4 cells/mL) was added to each well of a 96-well plate, which was maintained in an oven with a 5% CO_2 atmosphere at 37°C. For the SK-MEL-28 cell line, treatment with the samples was performed at multiple concentrations, based on the amount of protein contained in the sample, as requested by the requester. The following concentrations were used in triplicate: 0.015 µg/mL, 0.15 µg/mL, 1,5 µg/mL, 15 µg/mL, and 150 µg/mL. Staurosporine at 5 µM (Sigma) was used as a positive inhibitor control. After 48 h, thiazolyl blue (MTT, Sigma) was added, and the plate was maintained in a CO_2 oven at 37°C for 4 h. After the incubation time, the supernatant was aspirated, and the formazan crystals, formed by the cellular activity, were solubilized with DMSO from VETEC (Rio de Janeiro, Brazil). After which the optical density was measured in a microplate reader at 540 nm (VictorX5; Perkin Elmer USA).

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2.8 Thin layer Chromatography of *C. Cajan* Extracts

The initial characterization of the presence of different classes of secondary metabolites present in the extracts was carried out through thin layer chromatographies. An amount of 2.5 mg of each extract was diluted in 0.5 mL of methanol or ethanol. The samples remained in ultrasound (UstraSonic Cleaner USC-1800A) for 5 min and then 10 µL were applied to the "spots" of silica gel 60 F254 chromatoplates (Merck). The methodology of Wagner and collaborators [28] was employed to identify the specific class of secondary metabolites and different mobile phases and developers were used: for alkaloids: toluene - ethyl acetate - diethylamine (70:20:10) and Draggendorf; coumarins: toluene - ether (1: 1) and ethanolic KOH developer; flavonoids: Ethyl acetate - formic acid - glacial acetic acid - water (100: 11: 11: 27) and NP/PEG developer; saponins: chloroform - methanol - water (64:50:10) and sulfuric vanillin developer; terpenoids: ethyl acetate - methanol - water (77: 15: 8) and sulfuric vanillin (or sulfuric anisaldehyde) developer.

3. RESULTS AND DISCUSSION

3.1 Characterization of Protein with PI Activity from *C. Cajan* Aqueous Extracts

The majority of plant extracts are prepared using organic solvents or a mixture of organic solvents with water to extract secondary metabolites that have hydrophobic characteristics. However, to extract proteins, which are polar molecules, has been employed water, buffers, or saline solutions [24,25]. As shown in Table 1, the *C. cajan* extracts presented different protein amounts. CC-RA, the root extract, had the lowest protein content, and CC-CA, the stem extract, had the highest. This results indicated that water was a good protein extracting agent of stem, however, it was not so good for extracting proteins from roots, whereas, using similar amounts of starting material, the water extracted about twice more proteins from stem than from roots. Species of Fabaceae family have higher protein contents in the organs, such as *Crotalaria spectabilis* [24] and *Canavalia ensiformis* [25]. On the other hand, *C. cajan* has a lower protein content than the other legumes; however, high levels of proteins can be found in their seeds that are extensively studied [29].

All works that reported the PI activity in *C. cajan* employed dry seeds or dry leaves, which were depigmented and defatted with several washes of acetone and hexane or acid acetic at pH 4.0 and, after removing the solvents, proteins are extracted with a combination of acetic acid, polyvinyl pyrrolidone (PVP), water, NaCl, or phosphate buffer and Tris HCl buffers during overnignt [8,10,11,30,31,32,33]. Differently to these protocols, the present methodology uses fresh organs, one reagent to extract proteins, is faster because has fewer steps to obtain the extracts. It is known that the use of dry organs, organic solvents and many steps to obtain the extracts can affect the structure and can interfere with the biological activity of protein [34,35].

PI activity from *C. cajan* aqueous extracts were investigated employing reference enzymes: trypsin, a bovine pancreas serine protease; pepsin, an aspartic protease from bovine stomach; and papain, a cysteine protease from *Carica papaya*. The highest level of inhibition was observed for papain, likely because papain is a plant protease (Table 1). All extracts inhibited the trypsin activity in different degrees, and the most pronounced inhibition was observed for CC-P and CC-CA. The lowest level of inhibition was observed for pepsin, probably because aspartic protease PIs are the rarest in nature [18]. These results suggest the presence of serine, cysteine, and aspartic protease PIs in *C. cajan* leaves, stems, and roots. Furthermore, indicate that aqueous extraction is effective in obtaining PI activity of *C. cajan.*

SDS-PAGE results demonstrated that the protein profiles of *C. cajan* leaf extracts were very similar under reducing and non-reducing conditions, with proteins between 270 and 39 kDa (Fig. 1). In all samples, a major band of approximately 58-50 kDa was observed, and it is possibly corresponding to ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), which is the most abundant enzyme in plants and the most abundant protein on the planet [36]. Stem and root extracts, however, showed a profile with a smaller number of protein bands than that observed in leaf extracts. CC-CA showed three major proteins: 47, 35, and 30 kDa. On the other hand, CC-RA also showed three major proteins but with different molecular weights: 60, 37 and 30 kDa. The band with refers to RuBisCO was not pronounced in both stem and root extracts, because this enzyme is predominantly found in leaves which is related to photosynthesis [36]. Although the *C. cajan* genome was almost

completely sequenced (72,7%), not all of proteins have been predicted. It is possible that more soluble proteins can be found in these *C. cajan* aqueous extracts, such as enzymes and their inhibitors. It is important to note that the sequence of at least fifteen PIs has been predicted in the *C. cajan* genome [37] and it is very possible that one or some of these PIs are in these extracts due to their activity on the reference proteases (Table 1). However, these *C. cajan* aqueous extracts haven't had their proteins sequenced yet.

3.2 Characterization of the Protease Activity from SK-MEL-28 Extracellular Fraction

The SK-MEL-28 culture supernatant contained many proteins from bovine calf serum, which was added for cell growth, and secreted proteins and enzymes from the melanoma cells. The Table 2 shows the protein contents and protease activity against L-TAME as substrate from SK-MEL-28 extracellular fraction proteases. The crude supernatant had about 3.60-times higher protein content than the fraction due to the loss of low water solubility proteins. On the other hand, protease activity was about 3.61-times higher in the fraction. The simple methodology employed preserved the structures and activities of secreted proteases.

Protease activity of the SK-MEL-28 extracellular fraction was assayed using soluble substrates and the highest activity was obtained for casein (Fig. 2A) and this protein was selected as the substrate for the remaining assays. SDS-PAGE containing gelatin is very useful to estimate the molecular weight of active proteases [22]. Two

protein bands with about 150 and 100 kDa from the SK-MEL-28 extracellular fraction had gelatinase activity (Fig. 2B). Monsky et al. 1994 reported the gelatinase activity of a 72-kDa seprase localized on invadopodia of SK-MEL-28, with is potential marker of invasiveness [38].

The maximal activity of secreted proteases from SK-MEL-28 was at pH 7.0 and 9.0 (Fig. 3A), suggesting the presence of serine and metalloproteases, because these enzymes have optimal activity in the neutral to alkaline pH range [37]. Specific inhibitors is one of the best strategies to study the catalytic mechanism of enzymes [12]. PMSF, general serine protease inhibitor, TPCK, chymotrypsin-like serine protease inhibitor, and benzamidine (Bza), trypsin-like serine protease inhibitor, bind to amino acid residue of the catalytic triad from serine proteases. On the other hand, 1,10 phenanthroline (Pha) and EDTA are chelating agents that sequester metal ions and interfere with metalloprotease activity, and iodacetamine (Ia) binds covalently with the thiol group of cysteine in cysteine proteases [39]. No inhibitor completely abolished the activity of SK-MEL-28 secreted proteases; however, the highest inhibition was observed with Bza (Fig. 3B), followed by Pha and EDTA, suggesting that serine and metalloproteases are secreted by SK-MEL-28 cells. Serine proteases are important virulence factors in tumors because they activate matrix metalloproteases, which degrade ECM to invade healthy tissues, release tumor growth factors from tissues, and thus contribute to tumor progression [14,15]. Metalloproteases require ions to modulate the catalysis [40] and SK-MEL-28 protease activity was weakly modulated by divalent cations (Fig. 3C).
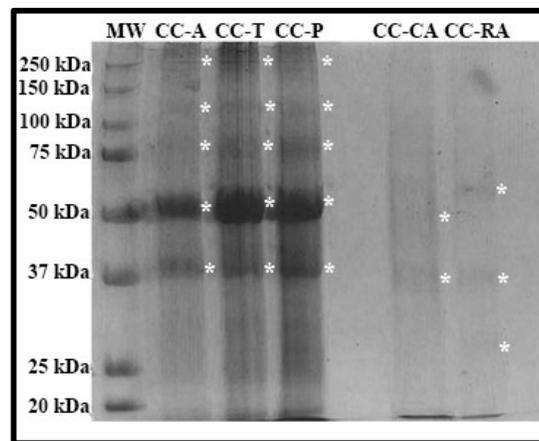

Fig. 1. SDS-PAGE 12 % of *C. cajan* extracts in non-reducing conditions

Table 1. Protein content and PI activity of *C. cajan* extracts against reference proteases

C. cajan extracts	Starting material(g)	Freeze-dried extract (g)	Protein (µg/mg)	Total protein(g)	Protein extraction yield (%)	Trypsin inhibition (%)	Pepsin inhibition (%)	Papain inhibition (%)
CC-A	148.80	4.97	147 ± 19.09	0.732 ± 0.09	0.49	0	29 ± 33.20	0
CC-P	148.30	3.97	140 ± 21.21	0.397 ± 0.08	0.27	58 ± 1.41	11 ± 0.71	81 ± 24.00
CC-T	148.10	6.52	120 ± 14.14	0.782 ± 0.09	0.41	30 ± 0.71	12 ± 2.12	14 ± 0.00
CC-CA	287.87	2.66	175 ± 31.11	0.465 ± 0.08	0.16	58 ± 9.19	29 ± 0.71	66 ± 0.71
CC-RA	292.24	5.14	68 ± 15.56	0.443 ± 0.08	0.15	48 ± 6.36	29 ± 0.71	100 ± 0.00

Table 2. Protein content and protease activity of SK-MEL-28 culture supernatant

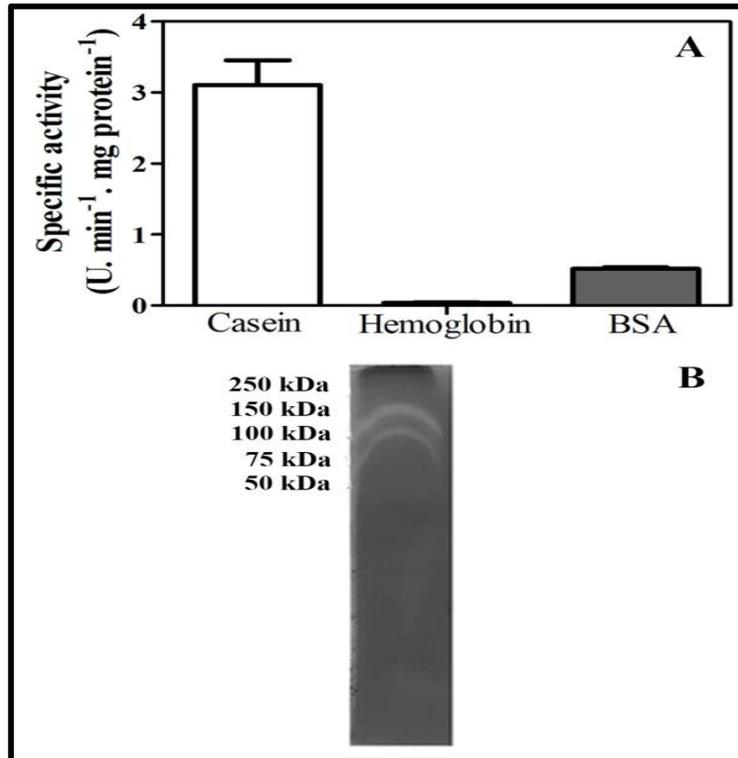| SK-MEL-28 culture supernatant	Volume/mass (mL/g)	Protein(µg)	Total protease activity (U × min^{-1} × mg^{-1} protein)
Crude supernatant	35.00 mL	1,228	1.3×10^{-2}
Freeze-dried fraction	0.58 g	341	4.7×10^{-2}

Fig. 2. Proteolytic activity of secreted proteases from the SK-MEL-28 extracellular fraction on protein substrates (A) and on Gelatin-SDS-PAGE 10%. The values of the standard molecular mass proteins (kDa) are located to the left side of the gel (B)
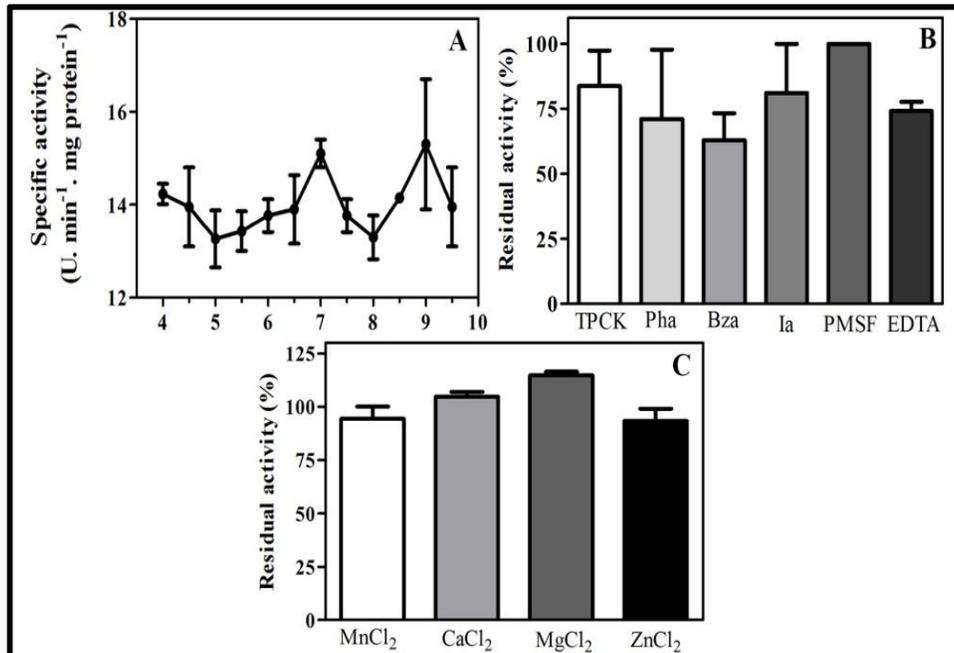

Fig. 3. Effect of pH (A), protease inhibitors (B) and cations (C) on the activity of SK-MEL-28 secreted proteases

3.3 Effect of *C. Cajan* Extracts on SK-MEL-28 Cells and their Secreted Proteases

The PI activities of the aqueous extracts from *C. cajan* against secreted proteases from the SK-MEL-28 extracellular fraction were evaluated. These enzymes were inhibited by aqueous extracts to different degrees (Fig. 4). CC-P was the most potent inhibitor and decreased the enzymatic activity to about 50% of the control value, followed by CC-A, which decreased the activity of the secreted proteases from the SK-MEL-28 extracellular fraction down to 33% of the control value, and this inhibitory effect of CC-P was dose-response manner (Fig. 5A). Thus, the increase in the extract protein concentration led to a significant decrease in the secreted protease activities. CC-P at 70 µg of protein completely abolished protease activity and CC-P and was then chosen to be evaluated for its cytotoxicity to melanoma cells in culture. CC-P at 15 µg of protein/mL reduced the growth of the SK-MEL-28 melanoma cell line by about 40% (Fig. 5B). Thus, this extract was considered cytotoxic to SK-MEL-28 cell growth, probably because it inhibited the activity of secreted serine proteases and metalloproteases of this cancer cells [41]. Proteases are crucial for tumor cell invasion and metastasis, that require the proteolytic degradation of MEC components, such as collagen, elastin, fibonectin, laminin, and proteoglycan [14,15]. These enzymes regulate the activities of growth factors/ cytokines and signaling receptors, suppress cell death pathways, and activate cell survival pathways, tumor-promoting inflammatory, angiogenic, and immune pathways [14,15,41,42]. The anti-cancer effect of purified polypeptide PI from *C. cajan* seeds has been reported against A549 cell line human lung carcinoma [11]. Besides, it is well known that PI activity from natural and synthetic inhibitors has expressive tumoricidal effects and interfere in cancer progression [16,19,20,21,43].

3.4 Characterization of Main Class Secondary Metabolites from *C. Cajan*

The secondary metabolites identification was performed by TLC using the CC-P that showed important inhibitory activity on melanoma secreted proteases, trypsin and papain. In this extract were detected coumarins, flavonoids and terpenoids, but not alkaloids and saponins, which are more non-polar substances (Fig. 6). TLC profile exhibited two blue bands with Rf 0.470 and 0.607 for coumarin (Fig. 6B), and for flavonoids was reveleaad four bands with Rf 0.551, 0.586, 0.603, 0.776 (Fig. 6C). Finally, the terpenoids analysis demonstrated four bands with Rf 0.063, 0.333, 0,397, 0.619 (Fig. 6E). Cajanuslactone is a coumarin obtained of *C. cajan* leaf using ethanolic extracts and has antibacterial activity [44]. *C. cajan* leaves are rich in flavonoids and was identified six flavones, two isoflavones, two flavonols, two flavanones, an isoflavanone and a single chalcone. These compounds showed several biological activities in *C. cajan* [45], and cajanol an isoflavanone isolated from roots, inhibited the growth of MCF-7 cell line human breast carcinoma in a time and dose-dependent manner [46]. If one of these bands are cajanuslactone, or cajanol further studies of mass spectroscopy are necessary to answer these questions.

Terpenoids have already been observed in *C. cajan* leaves extracts, but these molecules have not been identified [47,48]. TLC results constitute an initial investigation of which classes of secondary metabolites are found in *C. cajan* extracts. These aqueous extracts are not usually prepared to investigate the secondary metabolites, which are more hydrophobic molecules than polypeptides and proteins. Furthermore, extracts are complex mixtures, and it is important to have an idea about which classes of these metabolites are present, because they can have an additional biological effect. It is known, for example, that flavonoids are the chemical markers of legumes and have pronounced antioxidant and anti-inflamatory acitivity and have the potencial to inhibit the onset and development of many diseases, including cancers [49]. Although the melanoma secreted proteases and melanoma cells growth inhibition was a dose dependent of *C. cajan* extract protein concentration, in addition to PIs, CC-P also has some secondary metabolites that may have a synergistic effect on the final response, that is a possibly because the other mechanism than protease inhibition.
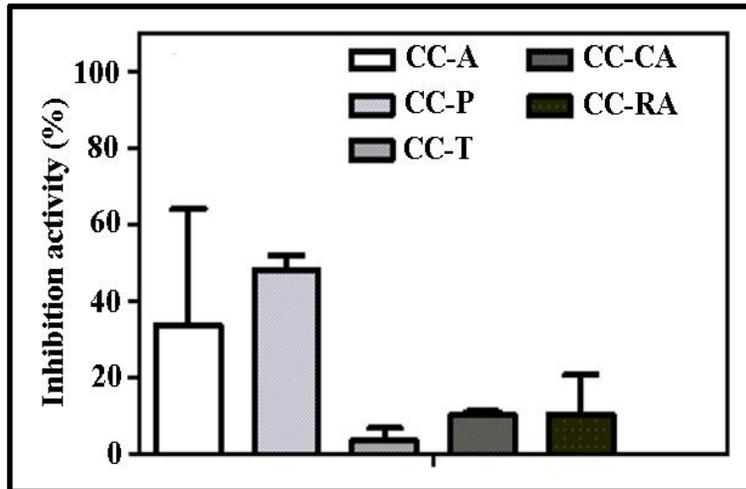
Fig.4. Residual activity of secreted proteases in SK-MEL-28 supernatant. The supernatant was pre-incubated with the extracts for 30min, and the enzymatic reaction was started by the addition of the casein substrate
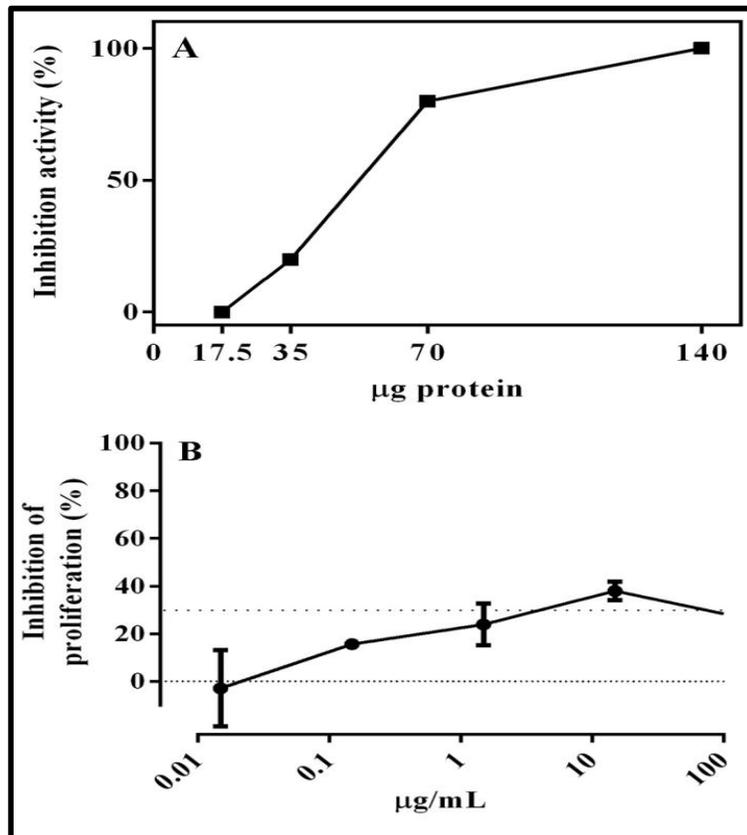

Fig. 5. Residual activity of SK-MEL-28 secreted proteases. The SK-MEL-28 extracellular fraction was pre-incubated with different protein concentration of CC-P for 30min, and the enzymatic reaction was started by the addition of casein substrate (A). Effects of CC-P on SK-MEL-28 melanoma cell growth. SK-MEL-28 cells were plated in 96 well plates and with *C. cajan* CC-P protease inhibitor, as described in methods (B)
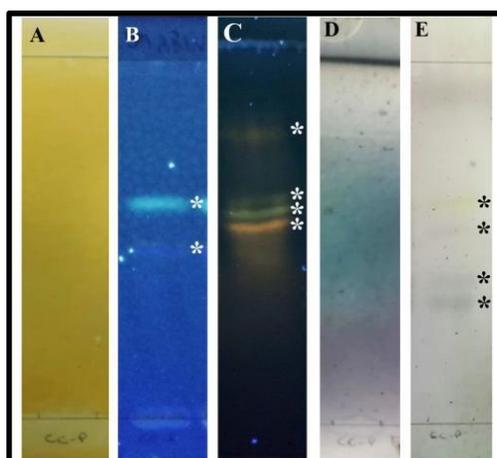
Fig. 6. Identification of alkaloids (A), coumarins (B), flavonoids (C), saponins (D) and terpenes (E) in CC-P from *C. Cajanus*

4. CONCLUSION

C. cajan leaf, stem, and root aqueous extracts are sources of proteins with PI activity and the protocol to obtain these proteins is simple, has low cost, and is effective in preserving the biological activity of extracted proteins. Therefore, this work was the first that identified cysteine, and aspartic PI activities in *C. cajan*, and PIs from roots and stem, because serine PIs are the most investigated in seeds of *C. cajan*. Besides, it was the first study which identified the anticancer activity in *C. cajan* leaf extract. All extracts inhibited the activities of melanoma secreted proteases, especially the leaf extract CC-P that was also cytotoxic to SK-MEL-28 cell growth. Further studies are necessary to clarify the antitumor activity of *C. cajan*; however, the secreted proteases from this cancer cells certainly contribute to melanoma progression, and these extracts, with PIs activities, represent potential candidates for anticancer adjuvant therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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