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Biochemical Characteristics and Healing Activity of *Bromelia laciniosa* Leaf Protease

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Abstract

Macambira plant (*Bromelia laciniosa* Mart. Ex Shult. & Schult. f.) is a species belonging to the family Bromeliaceae, which is distributed widely in the hinterlands of northeastern Brazil, from Bahia to Piauí states. The plants of this species contain an enzyme complex named bromelain, which has great potential for therapeutic applications. In the present study, bromelain derived from *Bromelia laciniosa* leaves was assessed for its thermodynamic

properties and *in vitro* cytotoxic and healing activities. Bromelain was extracted from the leaves and partitioned using ethanol. The partially purified bromelain was then characterized to determine the optimum pH and temperature and the kinetic and thermodynamic parameters. The partially purified bromelain derived from macambira leaves exhibited its highest activity at 47°C and pH 7, when the K_m and V_{max} values of 2.26 µM and 2.083 U/mL, respectively. The half-life of this bromelain at 47°C was 5.78 h. At 47°C, the Gibbs's free energy, enthalpy, and entropy values reached 89, 53.54, and –0.11 kJ/mol K, respectively. In the SDS-PAGE analysis, bromelain appeared as the main protein band, with an estimated molecular mass of 33 kDa. The *in vitro* cytotoxic activity and wound healing effects were determined using the MTT assay and Scratch assay, respectively. These assays were performed on the non-neoplastic cell lines L-929 and PBMC and the human tumor cell lines MCF-7, HCT-116, and NCI-H292. Bromelain exhibited a positive healing activity with a change rate similar to that observed for the control. In addition, bromelain exhibited cytotoxic activity against NCI-H292 tumor cells and chemopreventive potential. These results indicate that bromelain derived from macambira leaves may be utilized as a potential ingredient in pharmaceutical formulations, for various healing and chemopreventive purposes and as an alternative to the bromelain extracted from pineapple. In addition, the biochemical characteristics of this bromelain could find application in the food industry. Future studies should, therefore, focus on deciphering the action mechanisms underlying the activities of bromelain observed in the present study to further confirm and enhance its application potential for therapeutic purposes.

Keywords
Bromelain; human tumor cells; Macambira; NCI-H292; Scratch assay

1. Introduction

Proteases (proteinases, peptidases, or proteolytic enzymes) are a complex group of enzymes present in a wide range of organisms, including plants, animals, and microorganisms. These enzymes hydrolyze the peptide bonds in proteins and peptides, and are, therefore, considered the most important of all industrial enzymes. Moreover, the easy accessibility and wide distribution of this enzyme in nature have contributed further to its wide application [1-3]. Consequently, proteases account for approximately 60% of all enzymes used in the industry. The total market share of protease enzyme is estimated to reach approximately US $3 billion, while the annual growth rate (CAGR) of this enzyme is expected to reach 6.1% by 2024. However, despite the importance and extensive applicability of protease enzymes, the mode of action of this enzyme remains poorly understood to date.

Proteases are classified into six different categories based on their catalysis mechanism: aspartic, glutamic, metalloproteases, cysteine, serine, and threonine proteases. No study so far has reported the presence of glutamic proteases in mammals [4]. Bromelain, papain, and ficin are the main ones reported among phytoproteases.

Bromelain collectively refers to a protein (enzyme) family comprising sulfhydryl proteolytic enzymes and cysteine proteases [5]. Bromelain is generally extracted from pineapple (*Ananus comosus)* plant tissues, such as stalks, stems, fruits, and leaves. The effective application of bromelain in various industrial sectors has been investigated extensively in the last decades. Owing to the satisfactory results obtained in several of such investigations on bromelain, further studies exploring novel sources of bromelain continue to be reported to date [6].

Bromelain is considered one of the most important ones among the existing proteases owing to its range of applications in various industrial sectors, including food [meat breaking, beverage clarification, and protein processing (protein hydrolysate), among others], textile, leather, cosmetics, and detergent industries. In addition, bromelain has a particular significance in the pharmaceutical industry owing to its therapeutic effects, which have been reported in several previous studies [2, 7, 8].

Bromelain's potential therapeutic value is mainly associated with its biochemical and pharmacological properties [9, 10]. Several studies have attributed different therapeutic properties to this enzyme, including the anti-inflammatory action [11, 12], platelet aggregation inhibition [13], fibrinolytic and antithrombotic activity [14-16], antitumor action [17-19], cytokine and immunity modulation [20, 21], skin debridement property [22], drug absorption enhancement [23], mucolytic properties [24], digestion facilitation [25], and circulation and cardiovascular system improvement [26, 27]. Bromelain could also be utilized as a complementary therapy for COVID-19 and post-COVID-19 patients [16, 28]. Bromelain is also extensively used as an active ingredient in tooth-whitening dentifrices and skincare products [29]. Bromelain may also be used for promoting and accelerating wound healing indices and improving the biomechanical parameters of diabetic wounds [30-32].

Cutaneous wound healing is an essential physiological process that involves the collaboration of several cell types and their products [33]. The entire wound healing process may be divided into the following stages: inflammatory reaction, cell proliferation and synthesis of elements that would form the extracellular matrix, and the final stage of remodeling [34].

Although skin wound healing is a natural process, certain intrinsic and external factors may affect this natural ability of the skin and lead to non-healing lesions and chronic wounds, thereby directly impacting the health and the quality of life of the affected individual [31]. Chronic wounds develop due to a disturbed healing progression which might be attributed to various reasons, including concomitant diseases such as diabetes mellitus [35], peripheral vascular diseases [36, 37], chronic venous insufficiency [38], post-thrombotic syndrome, and chronic kidney failure [39], or neoplasms, malnutrition, and excessive cigarette smoking [40]. In this scenario, phytotherapy could be an interesting treatment option for cutaneous wounds, although high-quality investigations are warranted to corroborate the clinical effectiveness of this treatment in plants [31].

The plant family Bromeliaceae comprises numerous species, although only a few of these species have been investigated for therapeutic application potential so far. One such species is macambira (*Bromelia laciniosa* Mart. Ex Shult. & Schult. f.) [41], popularly known as *macambira, macambira-de-vaca*, and *macambira-de-Cachorro*. This species is distributed widely in the hinterlands of northeastern Brazil, from Bahia to Piauí states. Although the research reports on this species are scarce, macambira is considered one of the alternatives provided by the Caatinga biome for application as a complementary food for herds in the small goat, sheep, and pig breeding in northeastern Brazil. Macambira assists the breeders in reducing costs during the drought seasons

through the application of adequate and sustainable management strategies. Macambira shoot, which contains 4.9% protein, 2.8% starch, and 1.1% calcium, has been used as feed for both animals and humans during long periods of drought [42-44].

The pursuit of potential sources of phytoproteases is currently an interesting area of research. The use of plant proteases, however, remains relatively limited to papain and bromelain derived from papaya and pineapple plants [45-47], respectively. Carauá (*Ananas erectifolus* L. Smith), a plant belonging to Bromeliaceae and typical to the rain forests in Pará State, has also been investigated in this regard. There is substantial evidence of the presence of significant levels of Bromelain in Bromeliaceae plants and that the yields of purified bromelain achieved are adequate for using this protease as an additive in creams, lotions, and anti-inflammatory gels [48]. Buttazzoni et al. [49] evaluated the proteolytically active preparations produced from green and ripe *Bromelia laciniosa* fruits grown in Argentina and reported that ripe fruits were associated with a higher caseinolytic activity compared to the green ones.

In this regard, and based on the existing literature on the importance of bromelain in pharmaceutical and food industries, the present study investigated the potential enzymatic, anticancer, and wound-healing properties of *Bromelia laciniosa* Mart. Ex Shult. & Schult. f. plant through *in vitro* analysis.

2. Materials and Methods

2.1 Chemicals

Azocasein, bovine serum albumin, Coomassie Brilliant Blue R250, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and RPMI 1640 culture medium were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Thermo Fisher Scientific (Massachusetts, USA). Trichloroacetic acid was purchased from Dinâmica (São Paulo, Brazil). Potassium hydroxide was purchased from Química Moderna (São Paulo, Brazil). Ethanol was purchased from Neon (São Paulo, Brazil). The molecular weight standards were purchased from Bio-rad (Califórnia, USA). The human peripheral blood mononucleated cells (PBMC) were purchased from Merck KGaA (Darmstadt, Germany).

2.2 Plant Material

Macambira (*Bromelia laciniosa* Mart. ex Shult. & Schult. f.) leaves were collected in Serra Talhada (07°59'31"S and 38°17'54"W), Pernambuco State. The leaves were separated manually, followed by washing and excising the leaves into small pieces. A proportion of these cut pieces was used for producing exsiccates, which were subsequently subjected to botanical identification and deposited in the appropriate herbarium. The remaining leave pieces were used for producing the crude enzyme extract and then subjected to analysis. After the identification of the plant material, it was deposited in the Vasconcelos Sobrinho Herbarium at the Federal University of Pernambuco (UFPE) under the UFPE Herbarium no. 81.238. Finally, all procedures for accessing the genetic patrimony and the associated traditional knowledge were conducted, and the project was registered in SisGen (Register #AF785CB).

2.3 Bromelain Extraction

Bromelain was extracted from the macambira leaves through trituration using the 0.2 M sodium phosphate buffer at pH 7.0 [leaf:buffer ratio = 1:1 (w/v)] inside an industrial processor. Afterward, the extracted bromelain was filtered using filter paper and then centrifuged at 10,000 rpm for 20 min at 4°C to remove solid particles. This partially purified bromelain was stored in a freezer until used for analysis.

2.4 Enzyme Activity

The protease activity in the samples was determined using azocasein (Sigma Aldrich, St. Louis, MO, USA) as substrate [50]. The reaction mixture comprised 500 µL of azocasein at the concentration of 0.5% (w/v) in 20 mM sodium phosphate buffer (pH 7.0) and 500 µL of the crude enzyme extract. The reaction mixture was incubated at 47°C for 40 min. Afterward, 500 µL of 10% trichloroacetic acid was added to the mixture followed by centrifugation at 5500 rpm for 20 min at 4°C (Heraeus Multifuge X1RDatamed®). Next, 500 µL of the supernatant from the previous step of centrifugation was added to 500 µL of 500 mM potassium hydroxide. One unit of protease was defined as the amount of enzyme required to increase the absorbance per minute of the reaction by 0.001 when evaluated in a UV–Visible spectrophotometer (model Libra S22; Biochroms, Cambridge, England) at the wavelength of 430 nm. The denatured enzyme was used as the negative control.

2.5 Protein Content

Protein content was determined based on the Lowry method [51]. Bovine Serum Albumin was used as the protein standard. All protein content determinations were performed in triplicate.

2.6 Ethanol Precipitation

The protein in the crude enzyme extract was obtained using the ethanol precipitation method [52]. In brief, the ethanol solvent was cooled to 0°C and then added dropwise to the crude enzyme extract until the desired concentration (50%–90%, v/v) was reached. The components of the reaction mixture were allowed to remain in contact with each other for 1 h at 4°C and then centrifuged at 10,000 rpm for 20 min at 4°C. The obtained precipitate was resuspended in 20 mM sodium phosphate buffer at pH 7.0 and then lyophilized following the procedures recommended by the manufacturers of the lyophilizer (L101, Liotop®). The lyophilized protein was stored at –20°C in the freezer until used in subsequent analysis.

2.7 Biochemical and Thermodynamic Properties of the Partially Purified Bromelain

2.7.1 Effects of Temperature and pH on Bromelain Activity

In order to determine the optimum temperature, the activity of the partially purified bromelain was determined at different temperatures within the range of 27–97°C, for 40 min at each temperature. The optimum pH for Bromelain activity was determined by incubating the enzyme and substrate mixture at 47°C for 40 min under different pH conditions, using sodium citrate (pH 5.0–6.0), sodium phosphate (pH 7.0 and 8.0), and Tris-HCl (pH 9.0) buffers at the concentrations of 20

mM each. The Bromelain activity was estimated as described earlier. All evaluations were conducted in triplicate.

2.7.2 Thermal and pH Stability

Various aliquots of the enzyme were individually dissolved in different buffer solutions (20 mM) and then incubated at 47°C for different durations (0, 60, 120, and 180 min) to evaluate the pH stability of the enzyme. The residual Bromelain activity was then assessed for each duration relative to the activity of the reference sample at time 0 (= 100% activity). The activity was estimated as described earlier (subSection 2.3). All evaluations were performed in triplicate.

The partially purified Bromelain samples were placed in uniform-sized test tubes and then incubated in water at different temperatures (27–97°C) and different durations (0, 60, 120, and 180 min) to evaluate enzyme stability. After the heating process, all tubes were cooled in an ice bath, followed by the determination of the residual activity for each duration relative to the activity of the reference sample at time 0 (= 100% activity).

2.7.3 Kinetic Parameters

The kinetic parameters of the Bromelain samples during azocasein hydrolysis were determined by dissolving 500 µL of azocasein at different concentrations in the range of 0.42 to 2.1×10^5 M in 20 mM of sodium phosphate buffer (pH 7.0). Afterward, 500 µL of partially purified bromelain was added to the above solution, followed by incubation at 47°C for 40 min. The K_m and v_{max} values were determined from the Lineweaver-Burk plots.

2.7.4 Activation Energy and Temperature Quotient (Q_{10})

Activation energy (E_a) estimation was performed based on the slope ($-E_a/R$) of the V versus 1/T plot (for the temperature range of 7–47°C). In the slope equation, R is the universal gas constant (8.314 J/mol K), and T is the absolute temperature in Kelvin (K) [53]. The effect of temperature on the reaction rate was expressed in terms of temperature quotient (Q_{10}) [54]. Q_{10} was calculated using Equation 1 provided below:

$$Q_{10} = 10^{\frac{10}{Z}} \quad (1)$$

2.7.5 Kinetic and Thermodynamic Parameters for Thermal Inactivation of the Enzyme

The kinetic and thermodynamic parameters were calculated [55] by incubating the partially purified bromelain at temperatures ranging from 27–97°C for 180 min. The deactivation rate constant (*kd*) was calculated from the 'ln(A_0/A) versus time' graph plotted based on the experimental data, as represented by Equation 2 provided below:

$$ln\frac{A_0}{A} = -k_d \times t \quad (2)$$

In the above equation, t denotes time, A_0 denotes the initial enzyme activity, and A is the enzyme activity at a given time t. The partially purified Bromelain denaturation activation energy (E_{ad}) was calculated from the slope ($-E_{ad}/R$) of the Arrhenius plot [ln(k_d) vs. 1/T]. The apparent half-life ($t_{1/2}$)

of the enzyme was defined as the time at which the residual activity of the enzyme reached 50% of its initial activity, and is expressed by Equation 3:

$$t_{\frac{1}{2}} = \frac{ln2}{kd} \quad (3)$$

The *D* value denoting the time required for a 90% reduction in the initial enzyme activity at a specific temperature is associated with the first-order deactivation rate constant (*kd*) and was calculated using Equation 4 provided below:

$$D = \frac{2.3026}{kd} \quad (4)$$

The *Z* value was obtained by inverting the slope of the 'log (*D*) versus temperature (°C)' plot. The thermodynamic parameters of the partially purified bromelain were estimated using Equation 5 provided below:

$$kd = \frac{k_b \times T}{h} \times e^{-\frac{\Delta H}{RT}} \times e^{\frac{\Delta S}{R}} \quad (5)$$

In the above equation, k_b is the Boltzmann constant (1.38×10^{-23} J/K), T is the absolute temperature in Kelvin, and *h* is the Planck constant (6.63×10^{-34} J s). The Gibbs free energy, enthalpy, and entropy values were calculated using the following equations: $\Delta G_d = -R \times T \times \ln(k_d \times h/k_b \times T)$, $\Delta H_d = E_{ad} - R \times T$, and $\Delta S_d = (\Delta H_d - \Delta G_d)/T$.

2.8 Molecular Mass Determination Based on SDS-PAGE

Electrophoresis was performed using a polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) [56]. The gel was run for 90 min, followed by staining in Coomassie Brilliant Blue R250. The molecular weight standards used were myosin (293 kDa), phosphorylase B (97.4 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), Trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

2.9 Cytotoxic Activity Assessment

The cytotoxic activity of the enzyme was assessed using the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [57, 58]. The cells used in this *in vivo* assay were obtained from the Cell Culture Laboratory of the Department of Antibiotics at the Federal University of Pernambuco. The use of these cells was approved by the UFPE Research Ethics Committee (Opinion no. 1.858.432). The non-neoplastic cell lines used were L-929 (murine fibroblast) maintained in Dulbecco's Modified Eagle Medium (DMEM) culture medium) and human peripheral blood mononucleated cells (PBMC) that were resuspended in the RPMI 1640 culture medium. The human tumor cell lines used were MCF-7 (breast adenocarcinoma) and HCT-116 (human colorectal cancer) cell lines maintained in the RPMI 1640 culture medium and the NCI-H292 (human lung mucoepidermoid carcinoma) cell line maintained in DMEM. All culture media were supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin + streptomycin). The cells were incubated at 37°C in an oven with a humidified atmosphere enriched with 5% CO_2. The L-929, HCT-

116, and MCF-7 cells (10^5 cells/mL) were seeded in the wells of 96-well plates, followed by incubation for 24 h. Subsequently, the dissolved sample was added to all wells at concentrations ranging from 0.39 to 25 µg/mL. The doxorubicin drug was used as the standard in the concentration range of 0.156–10 µg/mL. After 72 h of incubation, 25 µL of MTT (5 mg/mL) was added to the wells. Next, after 3 h of incubation, the culture medium containing MTT was aspirated, followed by the addition of 100 µL DMSO to each well. Finally, the absorbance was measured at the wavelength of 560 nm in a microplate reader. All experiments were conducted in triplicate. The IC_{50} (concentration capable of inhibiting 50% growth compared to the negative control, µg/mL) value was calculated using GraphPad Prism 7.0 demo software.

2.10 Wound Healing Activity in vitro

The effect of bromelain on the proliferation and migration of human tumor cell lines was assessed in terms of the antiproliferative activity exhibited by the enzyme against these cells. The wound healing activity of the enzyme was assessed *in vitro* using the Scratch assay [59]. Fibroblast proliferation was investigated *in vitro* based on an assay conducted with L-929 fibroblasts (murine fibroblasts). In brief, fibroblasts were initially cultured in DMEM supplemented with 10% fetal bovine serum (FBS), followed by incubation at 37°C for 24 h under a 5% CO_2 atmosphere condition. The subcultures were performed using the trypsin-EDTA solution for detaching the adhered cells for resuspension into a fresh culture medium.

Afterward, the cultured fibroblasts (1.10^6 cells) were seeded into the wells of a 24-well plate and then incubated in the complete medium at 37°C and 5% CO_2 atmosphere. Subsequently, the culture medium was removed, and a straight line was drawn in the middle region of the plate with the aid of a sterile P200 pipette tip. This caused a rupture between the cells and resulted in a mechanical lesion. The wells were then washed with the culture medium to remove the debris created during the mechanical lesion. Afterward, partially purified bromelain was added to the well of the plate at different concentrations (12.5, 25, and 50 µg/mL) and incubated for different migration durations (0, 2, 4, 6, 8, 10, 12, and 24 h).

The images were captured using the digital camera attached to the inverted phase microscope that was used for visualizing the field of view containing the wound. Reference points were drawn using thin-tip markers on the outer side of the plate and also on the microscope stage.

2.11 Statistical Analysis

The Shapiro-Wilk test was performed to evaluate the normal hypothesis proposed regarding the variable investigated in the present study. The mean values recorded for the assessed samples were analyzed through non-parametric tests. The statistical difference between two groups was analyzed using Wilcoxon's test, while the statistical difference among three or more groups was analyzed using a one-way analysis of variance (ANOVA). The threshold of statistical significance was set at 5%. GraphPad Prism 5.01 software was employed for all statistical analyses.

3. Results

3.1 Ethanol Precipitation

Organic solvents are used as precipitating agents to facilitate the separation of unwanted enzymes/contaminants from the desired (target) enzyme/protein. This process also increases the concentration and specific activity of the desired enzyme. Table 1 presents the results of the ethanol precipitation-based partial purification process conducted for the extracted enzyme in the present study. The 70% concentration of ethanol resulted in the highest purification factor of 12.94 and a 94.12% yield. This value (4.8 U/mL) was close to the one recorded for the initial Bromelain activity in the crude extract (100%).

Table 1 Results of the ethanol precipitation-based partial purification of bromelain extracted from *Bromelia laciniosa* (UFPE N°. 81.238).

Concentration (%)	VA (U/mL)	TP (mg/mL)	SA (U/mg)	PF	Yield (%)
90	4.14 ±0.01	0.46	8.97	2.90 [d]	81.18
80	3.23 ±0.11	0.28	11.54	3.73 [c]	63.33
70	4.80 ±0.21	0.12	40.00	12.94 [a]	94.12
60	2.85 ±0.21	0.25	11.52	3.73 [c]	55.88
50	3.45 ±1.27	0.21	16.43	5.22 [b]	67.65
EB	5.10 ±0.85	1.65	3.090	100.00	100.00

C: Ethanol concentration; VA: Volumetric activity; TP; Total protein; SA: Specific activity; PF: Purification factor. Means followed by different letters in the column differ from each other, according to the Tukey Test at a 5% probability level.

3.2 Biochemical and Thermodynamic Properties of the Partially Purified Bromelain

The effects of temperature on enzyme activity were evaluated, and the results are presented in Figure 1. The relative activity of the partially purified bromelain extracted from macambira leaves ranged from 13.51% to 100%. The optimum activity (100%) of this bromelain was observed at 47°C. The results obtained at this temperature were different from and statistically superior to the ones recorded at other temperatures. After 57°C, the enzyme activity began declining from 97.30% and reaching 13.51% at 97°C. The enzyme stability was assessed for 180 min. While 72.083% of the original activity of bromelain was retained at 47°C, the activity decreased further as the temperature increased, reaching 30.43% at 97°C.

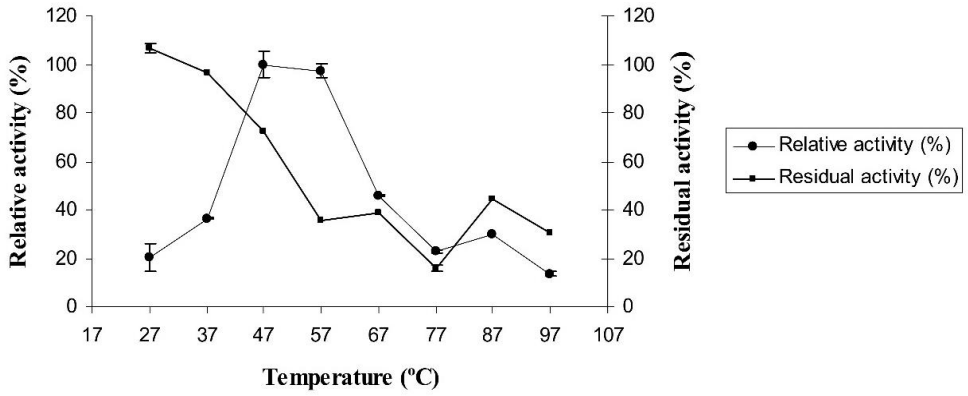

Figure 1 The effect of temperature on the (•) relative and (▪) residual activity of the bromelain extracted from *Bromelia laciniosa*. The reactions were conducted in 20 mM sodium phosphate buffer in the temperature range of 27–97°C for 40 min. The percentage residual activity of this bromelain was determined under standard assay conditions.

The effect of pH on the activity of the partially purified bromelain extracted from macambira leaves was investigated, and the results are presented in Figure 2. The relative enzyme activity ranged from 30.50% to 100%, while the optimum activity (100%) was observed at pH 7 (Figure 2). At pH 8, the activity of bromelain began declining from 72.37% and reached 61.40% at pH 9. The enzyme stability analysis revealed that the bromelain extracted from macambira leaves exhibited residual activity in the range of 12.90% (pH 5) to 100% (pH 6) after a 180-minute incubation. At the optimum pH (pH 7), bromelain lost 57.70% of its original activity. This could have happened due to autolysis, which often increases at neutral pH [60].

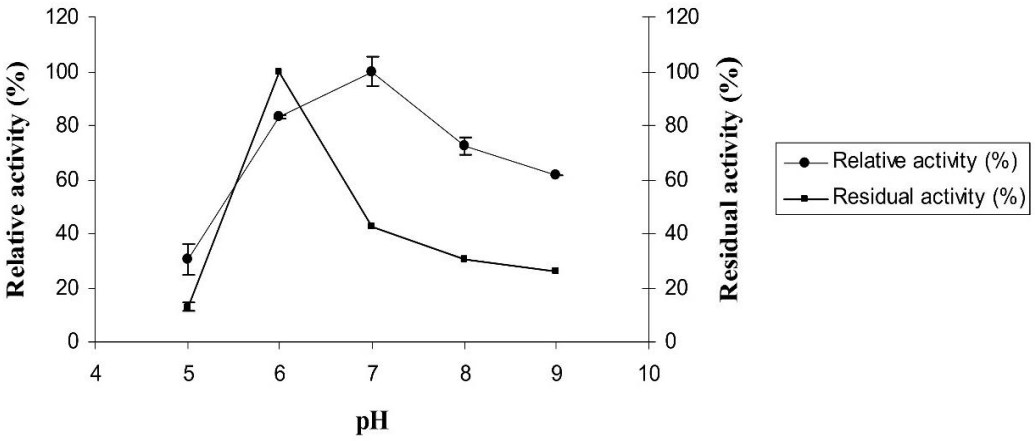

Figure 2 The effect of pH on the relative activity and residual activity of the bromelain extracted from *Bromelia laciniosa*. The reactions were conducted at 47°C for 40 min in the following buffer solutions (20 mM): sodium citrate (pH 5.0–6.0), sodium phosphate (pH 7.0 and 8.0), and Tris-HCl (pH 9.0). The percentage residual activity of this bromelain was determined under standard assay conditions.

The result described above is particularly important as the loss of enzyme activity and catalytic efficiency over time is a major issue encountered in the field of biotechnology [61].

The kinetic parameters K_m and V_{max} for the partially purified bromelain were determined using azocasein as substrate, and the values were 2.26 µM and 2.083 U/mL, respectively (Figure 3).

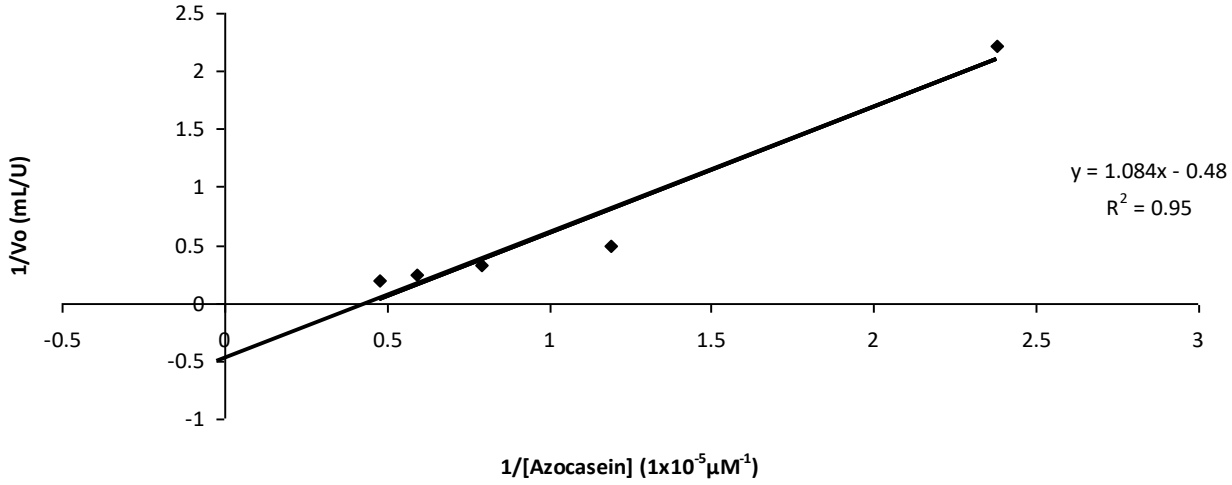

Figure 3 The Lineweaver-Burk plots for the hydrolysis of azocasein by the bromelain extracted from *Bromelia laciniosa*. The reaction was performed at 47°C and pH 7 for 40 min.

As depicted in Figure 4, the activation energy value estimated for the partially purified bromelain was 37 kJ/mol (8.84 kcal/mol).

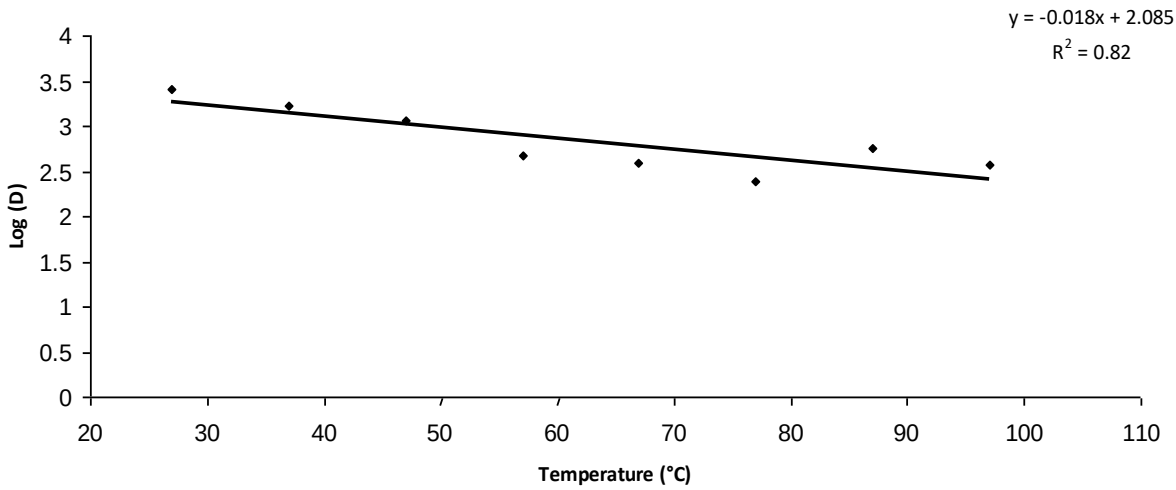

Figure 4 The Arrhenius plot generated for the azocasein hydrolysis activation energy according to the equation ln V = $-E_a/RT$.

The temperature coefficient recorded for the hydrolysis of azocasein by the bromelain extracted from macambira leaves was 1.51, which was within the defined limits.

According to the results presented in Table 2, the thermal denaturation kinetic constants (k_d) determined for the samples were inversely proportional to their respective half-lives. The constant k_d increased progressively, while the D values indicated that the evaluated enzyme became further

stabilized as the temperature approached 27°C (at this temperature, the D value was 42.64 h). The D value indicates the time required to reduce the initial enzyme activity by 90%. Furthermore, bromelain exhibited thermal resistance at its optimum temperature at 19.19 h. The half-life parameter indicates the time required to reduce the initial enzyme activity by 50% at a given temperature. The result data indicated that the half-life of bromelain decreased as the temperature increased, and a half-life of 5.78 h was recorded for this bromelain at its optimum temperature (47°C). These findings suggested that the bromelain extracted from macambira leaves has a potential for application in industries.

Table 2 Kinetic parameters of the thermal denaturation of the partially purified Bromelain from *Bromelia laciniosa*.

T (°C)	$t_{1/2}$ (h)	k_d (min)	Value D (h)
27	12.84	0.0009	42.64
37	8.25	0.0014	27.41
47	5.78	0.0020	19.19
57	2.36	0.0049	7.83
67	1.96	0.0059	6.50
77	1.23	0.0094	4.080
87	2.82	0.0041	9.36
97	1.83	0.0063	6.092

k_d = first-grade inactivation rate constant; $t_{1/2}$ = half-life values of the logarithmic plots from (relative residual activity) vs. incubation time; *D* = decimal reduction time.

The z-value recorded for bromelain in the present study was 55.56°C (Figure 5). Moreover, as the temperature was varied to either side of the z-value, the D value changed in a logarithmic manner. The determined z-value indicated that a wide temperature variation was necessary to affect enzyme stability.

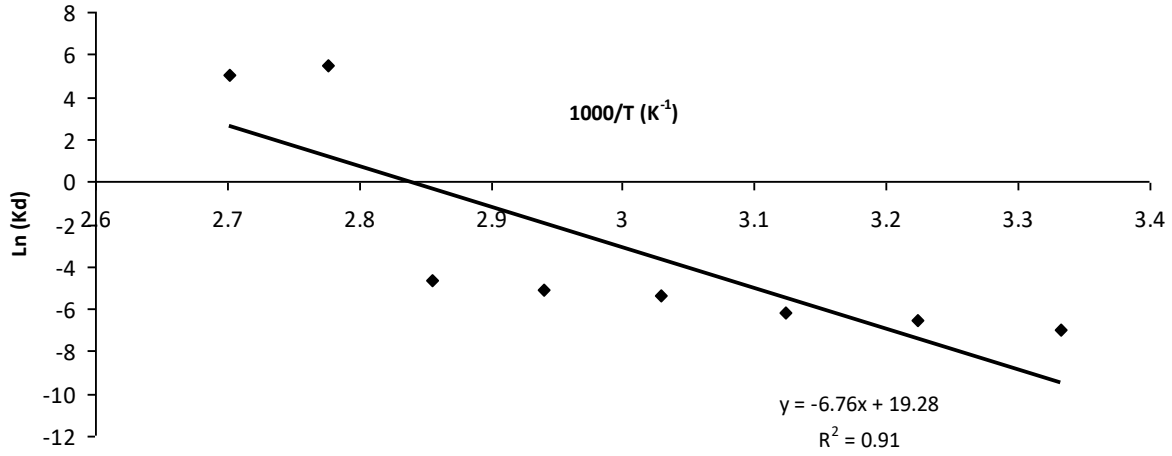

Figure 5 The thermal resistance curve plotted for the bromelain extracted from *Bromelia laciniosa* based on the thermal inactivation temperature.

The thermal inactivation of the partially purified bromelain was investigated at temperatures ranging from 27°C to 97°C. The results (Figure 6) revealed that the deactivation energy (E_{ad}) was 56.20 kJ/mol.

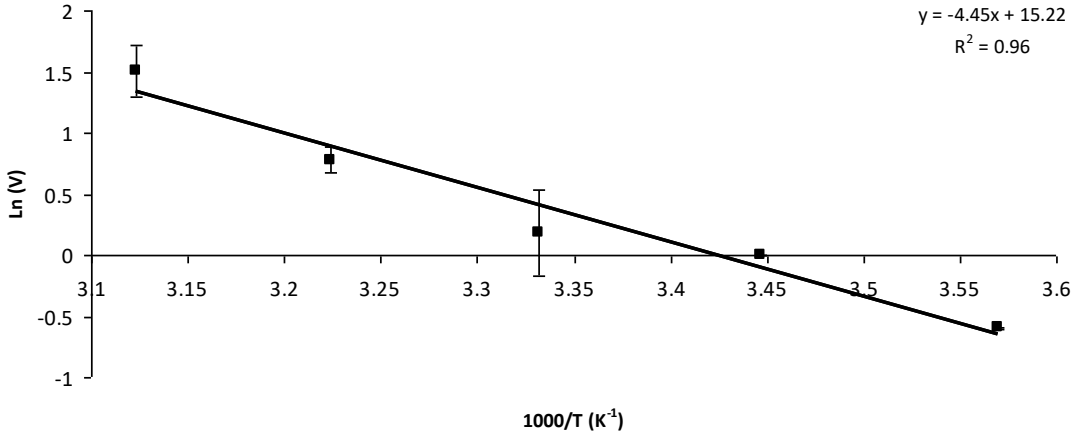

Figure 6 The Arrhenius plot used for calculating the irreversible thermal inactivation of the bromelain extracted from *Bromelia laciniosa.*

Table 3 presents the experimental values recorded for Gibbs free energy, enthalpy, and entropy. The ΔG_d data indicated that bromelain was denatured as the temperature was increased from 80.80 kJ/mol to 94.30 kJ/mol. The highest resistance to thermal unfolding was recorded at 27°C.

Table 3 Thermodynamic parameters of the thermal denaturation of the partially purified Bromelain from *Bromelia laciniosa*.

T (°C)	ΔH_d (kJ/mol)	ΔG_d (kJ/mol)	ΔS_d (kJ/mol·K)
27	53.70	91.025	-0.12
37	53.62	90.00	-0.12
47	53.54	89.19	-0.11
57	53.46	87.033	-0.10
67	53.37	86.65	-0.099
77	53.29	91.30	-0.11
87	53.21	87.70	-0.096
97	53.12	86.69	-0.091

ΔH_d = denaturation enthalpy; ΔG_d = free Gibbs energy; ΔS_d = irreversible inactivation entropy.

3.3 Molecular Weight Determination using SDS-PAGE

The findings of the SDS-PAGE electrophoresis revealed that the ethanol precipitation technique used for obtaining the enzyme could partially purify the Bromelain (Lane 3) in comparison to the crude enzyme extract (Lane 2). The analysis of the electrophoretic profile of the proteins of this partially purified bromelain extracted from *Bromelia laciniosa* leaves revealed two bands. One of these two bands was the major band with a probable molecular weight of 33 kDa, a value that was close to the one observed for the pineapple stem Bromelain (approximately 30 kDa) purchased from Sigma (USA).

3.4 Cytotoxic Activity Assessment

A cytotoxic assay was conducted to investigate the effects of the partially purified bromelain extracted from *B. laciniosa* leaves on normal cell lines (PBMC). The results are presented in Table 4. The Bromelain enzyme sample did not exhibit any cytotoxic activity against the investigated normal cell line in the concentration range of 0.39–25 µg/mL. The IC_{50} values were higher than the maximum concentration used in the present study. Figure 7 presents the cell death rate results for the enzyme in comparison to the corresponding values recorded for the cells subjected to doxorubicin. Considering the low cytotoxicity of Bromelain enzyme against normal cells in both present and previous studies, the effect of this bromelain on cancer cell cultures was investigated in the concentration range of 0.39–25 µg/mL. The determined IC_{50} values are listed in Table 5. The bromelain extracted from *B. laciniosa* exhibited cytotoxic activity against the tumor cell line NCI-H292, with an IC_{50} value of 7.87 µg/mL. Figure 8 presents the death rate results recorded for MCF-7, HCT-116, and NCI-H292 cell lines upon the enzymatic action.

Table 4 IC_{50} (µg/mL) values for bromelain against normal cell lines determined using the MTT assay.

Sample	PBMC	L-929	Murine macrophages
Bromelain	53.45 (34.11–110.10)	201.30 (138.90–322.40)	56.80 (37.94–105.90)
DOX	0.11 (0.06–0.17)	2.32 (2.00–2.68)	-

The data are presented as IC_{50} values and the respective cell confidence intervals for the PBMCs (human peripheral blood mononucleated cells), L-929 (murine fibroblasts), and murine macrophages (primary culture) treated with the partially purified bromelain relative to the control after 72 h of treatment. (–): Not tested.

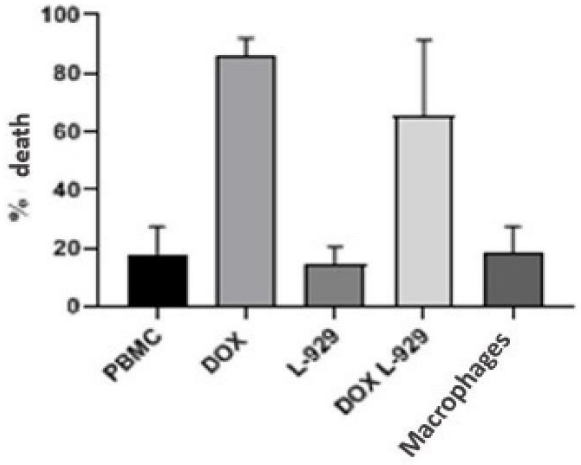

Figure 7 The death rates recorded for normal cells treated with the partially purified bromelain extracted from *Bromelia laciniosa*. PBMCs (human peripheral blood mononucleated cells), L-929 (murine fibroblast cells), and Bromelain enzyme-treated murine macrophages were compared to the control.

Table 5 IC_{50} (µg/mL) values for the bromelain extracted from *Bromelia laciniosa* against tumor cell lines, determined using the MTT assay.

Sample	MCF-7	HCT-116	NCI-H292
Bromelain	173.20 (87.90–573.80)	77.44 (37.06–192.31)	7.87 (5.86–11.28)
DOX	0.77 (0.59–0.95)	0.97 (0.87–1.07)	0.13 (0.06–0.23)

The data are presented as IC_{50} values and the respective cell confidence intervals for the MCF-7 (breast adenocarcinoma), HCT-116 (human colorectal cancer), and NCI-H292 (human lung mucoepidermoid carcinoma) cell lines treated with Bromelain enzyme relative to the control.

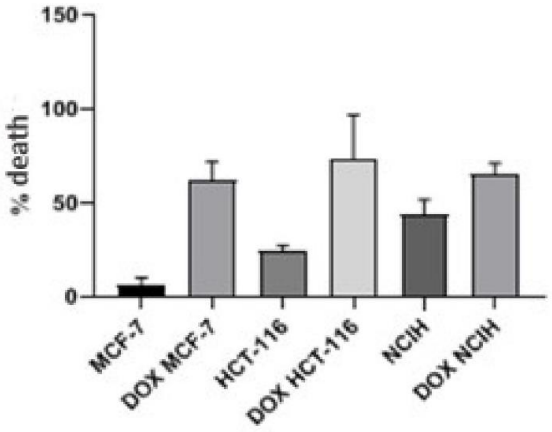

Figure 8 The death rates observed upon the application of the bromelain extracted from *Bromelia laciniosa* and doxorubicin to neoplastic cells, based on the MTT assay. MCF-7 (breast adenocarcinoma), HCT-116 (human colorectal cancer), and NCI-H292 (human lung mucoepidermoid carcinoma) cell lines were compared to the control after a 72-h treatment with bromelain or doxorubicin (DOX-positive control).

3.5 Wound Healing Activity in vitro

The cell migration of L-929 fibroblasts was investigated at the intervals of 0, 2, 4, 6, 8, 12, and 24 h, as well as upon using different concentrations of the enzyme. The results are presented in Table 6 and Figure 9. Cell migration of the L-929 fibroblasts subjected to the partially purified bromelain extracted from *B. laciniosa* was observed at the Bromelain concentration of 12.5 µg/mL. The migration rate increased as the Bromelain concentration increased. The migration rate recorded for the cells treated with 50 µg/mL of the partially purified bromelain was similar to that observed for the positive control, with the incision completely healed within 24 h.

Table 6 The Percentage (%) ±Standard deviation values for the migration of L-929 cells treated with the bromelain extracted from *Bromelia laciniosa*.

Sample	Time (h)						
(µg/mL)	**2**	**4**	**6**	**8**	**10**	**12**	**24**
Bromelain 12.5	8.12±2.76 ^{gA}	14.35±4.79 ^{fB}	52.71±8.42 ^{eB}	52.33±6.01 ^{dB}	54.00±5.73 ^{cC}	58.04±8.14 ^{bC}	62.18±3.84 ^{aC}
Bromelain 25	6.43±2.35 ^{gB}	9.60±2.42 ^{fC}	49.25±1.63 ^{eC}	51.14±3.11 ^{dC}	52.59±2.04 ^{cD}	56.00±2.04 ^{bD}	65.06±6.96 ^{aB}
Bromelain 50	3.22±1.65 ^{gD}	21.38±5.00 ^{fA}	54.30±8.42 ^{eA}	57.65±4.30 ^{dA}	60.70±3.78 ^{cB}	73.64±2.00 ^{bB}	100.00±0.00 ^{aA}
NC	3.10±0.55 ^{gE}	7.30±1.34 ^{fD}	45.63±3.63 ^{eD}	48.62±1.69 ^{dE}	49.03±1.09 ^{cE}	51.59±4.76 ^{bE}	58.63±5.85^{aD}
PC	3.43±0.89 ^{gC}	5.52±0.18 ^{fE}	45.49±1.34 ^{eE}	48.83±3.21 ^{dD}	64.62±5.74 ^{cA}	74.09±6.70 ^{bA}	100.00±0.00 ^{aA}

NC: negative control, without Bovine Fetal Serum (SFB); PC: positive control, with 10% SFB. Equal letters in the same row (lower case) and column (upper case) indicate that there was no statistically significant difference by the Tukey test ($p < 0.05$).

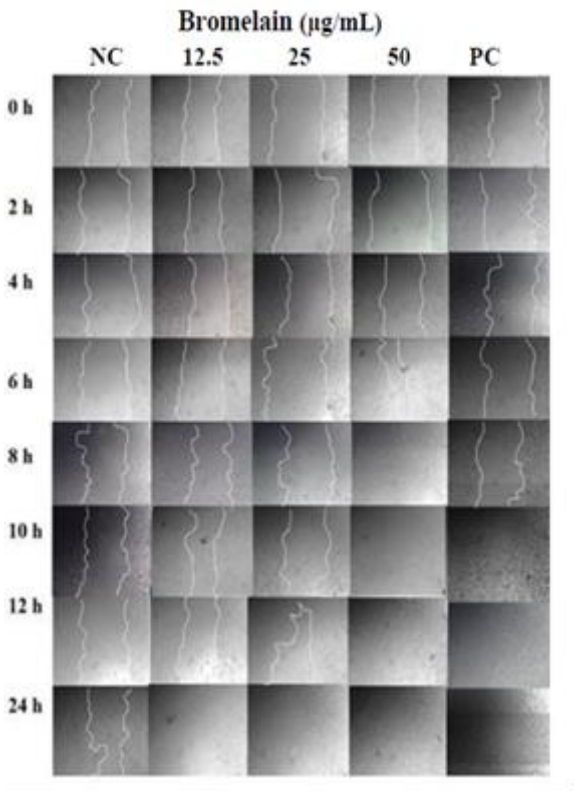

Figure 9 L-929 cell migration over a period of 24 h.

The above results evidenced that the partially purified bromelain stimulated fibroblast migration to the confluence area and closed the wound even when the medium supplemented with fetal bovine serum was not used as a growth factor.

4. Discussion

4.1 Ethanol Precipitation

Protein precipitation (through the addition of salt, organic solvent, non-ionic polymer, and metals, or by altering pH) is a key operational process among the practical methods used for recovering and purifying proteins from dilute solutions on a large scale. Organic solvents, such as ethanol, methanol, propanol, and ketones, are used widely for protein precipitation [62-64]. Bromelain is traditionally isolated using chemical precipitation which often uses a solvent mixture comprising 50% ammonium sulfate, 80% acetone, and 60% ethanol. The bromelain recovered using these methods exhibits approximately 85% activity at a purification factor ranging from 3 to 4.90 [65]. Ethanol precipitation is currently considered an outdated technique. It is nonetheless being used widely in the recovery and partial purification of enzymes. The reasons for its popularity include its easy operation, reproducibility, and minimum equipment requirements. In addition, ethanol is an inexpensive organic solvent produced widely in Brazil and is recoverable using a simple distillation process [66].

Ethanol, however, may alter the secondary, tertiary, or quaternary structure of the enzyme by distorting the binding sites between the amino acid residues arranged along the catalytic site [67]. In addition, ethanol may lead to enzyme aggregation [68]. Therefore, the bromelain to be used in industrial processes must be protected from the aggregation phenomenon, which is influenced by pH and ionic strength. The further the pH of the medium is from the isoelectric point of the protein, the lesser significant is the aggregation phenomenon. The aggregation phenomenon may even be entirely prevented under optimal pH and temperature conditions [69, 70]. Moreover, the enzymatic activity also depends on the protein structure and dynamics, as well as on the physical properties of the medium [71].

Proteins are dynamic entities exhibiting diverse molecular motions at a different time and length scales. Molecular motions play a fundamental role in the optimal functioning of an enzyme. However, whether a direct association exists between enzyme dynamics and the activity of the enzyme is yet to be established due to the remarkable complexity of biosystems. Structure and dynamics are two prime aspects among the various factors collectively governing enzyme activity [72]. It is reported that Bromelain dynamics around the enzyme's active site in the microsecond timescale could be a key factor in the regulatory process of the proteolytic activity of this enzyme. A strong association reportedly exists between the active site dynamics and the proteolytic activity of the enzyme. According to Das et al. [71], the compact structural conformation and flexibility of the active site in bromelain favor its proteolytic activity. Therefore, it was inferred that the tertiary structure of bromelain extracted from *Bromelia laciniosa* in the present study was not affected by the precipitation process, as evidenced by the retained enzyme activity of the partially purified Bromelain fractions.

In a previous study on the bromelain isolated from pineapple core (*Ananas comusus*) and purified using ethanol-based fractionation, the highest specific activity of the enzyme was observed in the 30%–60% ethanol fraction (fraction 2–3.107 U/mg). In addition, this fraction had a protein content of 61.25 mg and a degree of purity 155 times higher than that of the original crude enzyme [13]. In another study, an ethanol-based Bromelain purification process was applied to pineapple waste [67], which resulted in a higher purification factor (2.28) and yield (98%) compared to the use of 70%

ethanol-based purification. In addition, the authors confirmed the maintenance of the tertiary and secondary structure of the enzymes, as well as the respective activities of these structures, using SDS-PAGE and native-PAGE electrophoresis, spectrofluorimetry, circular dichroism, and FTIR analysis. In 2017, Wu et al. [73] concluded that this technique (Aqueous biphasic system) enabled a further convenient Bromelain extraction process. Furthermore, after purifying the bromelain extracted from pineapple stem using a two-phase aqueous system, the authors reported achieving a purification factor of 16.3 and a 55.6% yield for bromelain.

4.2 Biochemical and Thermodynamic Properties of the Partially Purified Bromelain

Although *Bromelia laciniosa* is also reported to produce extracellular proteases [49, 74], most studies reported in the literature are focused mainly on exploring pineapple-derived proteases. In a study, the Bromelain enzymes extracted from different parts of pineapple plants cultivated in Nigeria presented similar results [75]. The authors reported that the maximum enzyme activity in all samples from different plant regions was observed at 40°C. Sarkar et al. (2017) [76] investigated a pineapple-derived Bromelain and reported that its optimal activity occurred at 50°C. Nurhidayat et al. (2018) [77] reported that the highest activity of the investigated pineapple-derived bromelain occurred at 37°C. It is noteworthy that the bromelain extracted from macambira leaves in the present study exhibited a remarkably stable activity in the temperature range of 27–97°C during a 180-min (3 h) temperature treatment.

In the optimal pH-assessing studies, the best activities recorded so far for the bromelain extracted from *Bromelia fastuosa* and pineapple fruits were observed at pH 7.0 and 7.5, respectively [78, 79]. Miranda et al. (2017) [80] assessed the bromelain extracted from different parts of the pineapple plant, such as the stem, crown, peel, pulp, and leaves. The authors reported the maximum Bromelain activity at pH 6 for all plant parts. In 1986, Priolo et al. [74] evaluated the stability of the bromelain extracted from macambira fruits cultivated in Argentina, reporting that the best stability was achieved at the optimal pH of 6.4 [74]. This finding was consistent with the findings of the present study. Moreover, in the previous study, the activity of the investigated enzyme did not decrease during a 20-min incubation period. In another study, the bromelain extracted from pineapple stem lost only 0.2% of its initial activity, at pH 5, after 7 h at 40°C. In addition, 43.30% of the initial activity of the above enzyme was lost at pH 6 during the same period [81].

The bromelain extracted from pineapple pulp presented a K_m value of 0.037 mM and a V_{max} value of 3.8 U/mL in azocasein hydrolysis [82]. The bromelain extracted from white carauá cultivated using the same substrate presented a K_m value of 185.18 µM and a V_{max} value of 158.73 U/mL [83]. In comparison to these data, the bromelain analyzed in the present study exhibited a higher affinity for azocasein. However, the kinetic constants depend on the reaction parameters, which are, in turn, affected by both extreme and moderate substrate concentrations [84].

The protease extracted from *Jatropha curcas* reportedly exhibited an activation energy value of approximately 0.57 kJ/mol [85]. In comparison to this result, the bromelain extracted from macambira leaves in the present study had a greater energy barrier, which led to a faster enzymatic reaction. However, these results were achieved using casein as the substrate, and the Q_{10} of the enzymatic reactions often remained between 1 and 2. Any deviations in these values could indicate the influence of other factors [54]. In a previous study, the bromelain extracted from macambira leaves exhibited optimal biocatalytic performance within the investigated temperature range in

comparison to the half-life ($t_{1/2}$) recorded for pineapple-derived bromelain at different temperatures (45, 55, 65, and 75°C). According to Zhou et al. (2021) [86], the biocatalyst (Bromelain) presented half-life ($t_{1/2}$) values ranging from 0.087 h to 1.36 h. In two other studies, the denaturation activation energy determined for the bromelain extracted from pineapple stem (26.51 kJ/mol [87]) and that recorded for commercial bromelain (38.26 kJ/mol [88]) were lower than the corresponding ones recorded in the present study. This observation suggests that an irreversible Bromelain inactivation is achieved with greater energy and in a longer duration. On the other hand, pineapple pulp Bromelain presented a denaturing energy value of 313.18 kJ/mol [89]. It is worth emphasizing that casein was used as the substrate in all the aforestated studies.

Bromelain extracted from macambira leaves exhibited good stability throughout the investigated temperature range, a fact that could also be observed in the recorded D values (6.092 to 42.64 h). The ΔH and ΔS values recorded for bromelain in the present study varied little, indicating low enzyme denaturation and reasonable thermal and conformational stability [90]. Furthermore, considering that the strength of a non-covalent bond is 5.4 kJ/mol [91], the transition state formation of the bromelain extracted from macambira leaves was followed by the rupture of approximately 10 bonds at the optimum temperature of the enzyme. According to the negative activation entropy value, it was inferred that the structure of the enzyme–substrate complex at the transition state was more ordered compared to the native enzyme [92].

The biochemical characterization of enzymes is of significance in assessing their biotechnological potential. The properties of bromelain, including its temperature and stability profiles, substrate specificity, optimum pH, and kinetic and thermodynamic properties, would enable predicting its successful application in certain specific sectors or for specific processes. The main limitation encountered in the biotechnological application of enzymes is enzyme denaturation. Therefore, understanding the mechanisms underlying enzyme inactivation is important [93]. Investigations should consider evaluating the enzyme activity and stability at different temperatures, salt concentrations, optimal pH values, media types, and incubation periods when using proteases in industrial applications. For instance, proteases consumed in the pharmaceutical sector reportedly differ from those used in the food and detergent sectors [94-96]. The results obtained in such investigations would contribute to advancements in production, consumption, diversification of properties, and income generation.

4.3 Molecular Weight Determination using SDS-PAGE

After SDS-PAGE electrophoresis, the macambira bromelain appeared as the main protein band, with an estimated molecular mass of 33 kDa. However, the actual molecular weight may range from 23.97 kDa to 66 kDa, as reported by different authors for the Bromelain enzymes extracted from pineapple fruit and stem (24.5 to 66 kDa) and *Bromelia fastuosa* fruits (25 kDa), the protease derived from *Ficus Benjamina latex* (23.97 kDa), and the ficin derived from *Ficus johannis* (25 kDa) [82,97-101].

4.4 Cytotoxic Evaluation

In a previous study, the cytotoxic effect of pineapple Bromelain on NIH-3T3 fibroblast cultures was investigated for 24 and 48 h, and the results revealed an LD_{50} value of 0.0957 g/mL after 24 h and 0.0511 g/mL after 48 h [101]. The PBMC cells incubated with bromelain exhibited significantly

increased proliferative response compared to the responses observed for non-stimulated cells. Moreover, the cell proliferation activity was dose-dependent, with the strongest proliferation observed at the Bromelain concentrations of 10 µg/mL and 100 µg/mL. In addition, bromelain also induced the secretion of macrophage/monocyte-derived IL-6, GM-CSF, TNFα, and IFN-γ cytokines [102].

Bromelain's cytotoxicity has also been investigated in human gastric carcinoma (AGS), human prostate carcinoma (PC3), and human breast adenocarcinoma (MCF7) cell lines [103]. The enzyme was observed to inhibit cell line growth at concentrations above 75 µg/mL. An IC_{50} value of 65 µg/mL was recorded for AGS and MCF7 cells, while an IC_{50} value of 60 µg/mL was recorded for PC3 cells.

Furthermore, bromelain extracted from Queen and Kew pineapple fruits (through acetone-based precipitation) was evaluated for its cytotoxicity [104] against human myeloid leukemia (K562), human hepatocellular carcinoma (HepG2), adenocarcinoma (MCF-7 breast), human colorectal carcinoma (HCT 116), murine sarcoma (Sarcoma 180), and murine skin melanoma (B16F10) cell lines. It was reported that the enzyme exhibited a dose-dependent cytotoxic effect (0 to 50 µg/mL) during the 24-h study period. Another study evaluated and compared the *in vitro* performance of free and encapsulated bromelain [19]. Free Bromelain showed weak antiproliferative effects against U251 (glioma), MCF7 (breast), OVCAR-03 (ovarian), PC-3 (prostate), HT-29 (colon adenocarcinoma), and K-562 (chronic myeloid leukemia) cell lines after 48 h of its application.

Studies have reported that the mechanism underlying this cytotoxic effect of bromelain on tumor cells is associated with its de-protective action in neoplastic cells. While acting on the tumor cells, bromelain also facilitates the degradation of the fibrin clots covering the tumor, thereby allowing the immune system to fight the neoplastic cells. The factors that serve as stimuli for neoplastic cell progression, in addition to fibrin, include the induction of adhesion molecules, angiogenesis, and cellular activation. Therefore, it is speculated that bromelain's fibrinolytic and anticoagulant activities could have a relevant effect on fibrin and tumor cell coagulation, in addition to enhancing the immune defenses of an individual and reducing metastasis [105]. However, bromelain is effective mostly at the early stages of tumor development [106].

Similarly, the bromelain extracted from macambira leaves has also been demonstrated to exhibit antineoplastic activity in different types of neoplasms, and could, therefore, serve as a strong candidate for application in antineoplastic therapy, either alone or in combination with other substances. However, further investigation on the mechanism of action of this bromelain is warranted for its appropriate application.

4.5 Wound Healing Activity in vitro

In the present study, the most used soft tissue-healing models, namely, the fibroblasts cells, were used in the wound healing activity assessments. The results of the *in vitro* assessment revealed that the bromelain extracted from macambira leaves enabled cell migration similar to that observed in the positive control, i.e., complete healing was achieved within 24 h. A previous study that assessed the *in vivo* healing activity of the pineapple peel protein extract reported no significant difference in the lesion histology results between the control and treatment groups [107]. Fathi et al. (2020) [32] assessed the effect of bromelain on the various aspects of the wound healing process in type-1 diabetic rats. The authors reported that bromelain caused significant wound contraction and also reduced the formation of the granulation tissue by the 7^{th} day after treatment. In addition,

Bromelain application resulted in significantly increased fibroblasts by the 5th day after treatment, which again decreased by the 7th day after treatment. On the other hand, Miranda et al. (2021) [108] developed membranes composed of carboxymethyl cellulose/acetylated arrowroot starch incorporated with bromelain extract-loaded nanoparticles (NPs) and liposomes (LIPs). In the healing potential assessments, no edema was revealed in the histological analysis of the animals treated with these membranes incorporated with bromelain-loaded NPs and LIPs on the 14th day after treatment.

Although a few studies have stated bromelain's potential as a healing substance [109, 110], the number of experimental studies focused on investigating the underlying action mechanism remains low. It is speculated that bromelain serves as a healing process-acceleration agent, plasminogen activator, and stimulator of the production of plasmin serum protease. In addition, bromelain's action could be based on the promotion of the alignment of collagen fibers, which facilitates uniform tissue growth. Bromelain action could also be based on the acceleration of blood perfusion and partial oxygen pressure recovery in wound tissues and the control of the expression of the tumor necrosis factor-α (TNF-α). The above-stated mechanisms also improve the allergic and systemic inflammation processes, thereby assisting in mitigating pain [105]. Once the anti-inflammatory prostaglandins are activated, neutrophils are attracted to the wound site, where these cells produce free radicals to assist in the killing of pathogens. Afterward, neutrophils are gradually replaced by macrophages, which secrete cytokines and growth factors, in addition to contributing to angiogenesis, fibroplasia, and extracellular matrix synthesis, all of which play important roles in the transition to the next stage of healing, i.e., the proliferative stage [111].

The results of the present study demonstrated that the bromelain extracted from macambira leaves exhibited a significant healing activity and could, therefore, be utilized as an effective health supplement to both promote and accelerate the wound healing indices.

5. Conclusions

The enzyme activity analysis of the Bromelain enzyme extract revealed that macambira leaves are a satisfactory source of bromelain. The present study is the first one to report the thermodynamic parameters of partially purified bromelain extracted from macambira leaves (*Bromelia laciniosa)* along with its partial purification and application in cytotoxic and healing activity.

The bromelain extracted from *Bromelia laciniosa* was not affected by the precipitation process. Moreover, ethanol precipitation resulted in a high purification factor using just a one-step precipitation process. The investigated enzyme exhibited good stability in a wide range of temperatures and pH values. The molecular weight of the bromelain extracted from macambira leaves was estimated to be 33 kDa using SDS-PAGE. The little variation observed in the enthalpy and entropy values for this bromelain indicated low enzyme denaturation and reasonable thermal and conformational stability. The observed properties of the investigated bromelain suggested its potential relevance to various industrial applications, such as meat tenderization and protein processing (as protein hydrolysates), particularly owing to its catalytic thermodynamic properties and high thermostability.

The bromelain extracted from *B. laciniosa* also exhibited cytotoxic activity against the tumor cell line NCI-H292, with an IC_{50} value of 7.87 μg/mL. The *in vitro* wound healing activity assessment

revealed that this bromelain could enable cell migration similar to that observed in the positive control. Moreover, this bromelain could also completely heal the wound within 24 h, which reinforces its therapeutic potential, although further investigation is necessary for validation of these findings.

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Author Contributions

D.J.N.S., and L.L.A.N: Manuscript writing, revision, and data interpretation. D.J.N.S., L.L.A.N., C.E.S.S., and E.F.O.B: Performing experiments, data collection and analysis; TCCL, TGS and ARS: Study design, manuscript editing, and supervision. All authors have read and approved the final manuscript.

Competing Interests

The authors declare that they have no conflicts of interest.

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