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Novel Stable Protease Inhibitor from *Phoenix dactylifera (L.)* Flowers with Antimicrobial and Antitumoral Activities

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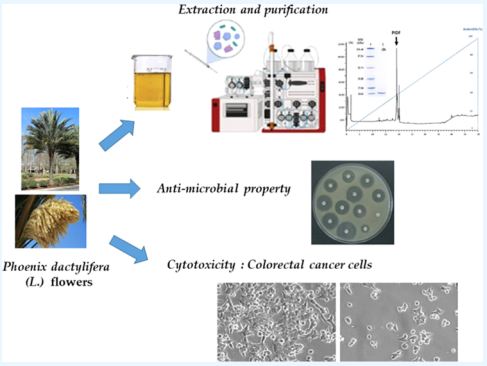
ABSTRACT: A novel protease inhibitor isolated from date palm *Phoenix dactylifera (L.)* flowers (PIDF) was purified and characterized. A heat and acidic treatment step followed by ethanol precipitation and reverse-phase high-performance chromatography was applied to purify this natural protease inhibitor to homogeneity with a single band of about 19 kDa. The stability study depicted that PIDF was fully stable at 40 °C and retained 65% of its initial activity after heating at 50 °C for 24 h. Its thermal stability at 70 °C was markedly enhanced by adding calcium, bovine serum albumin, and sorbitol as well as by metal divalent cations, especially Mg^{2+} and Hg^{2+}. This protease inhibitor showed high inhibitory activity against therapeutic proteases, including pepsin, trypsin, chymotrypsin, and collagenase, and acted as a potent inhibitor of some commercial microbial proteases from *Aspergillus oryzae*, *Bacillus. sp*, and *Bacillus licheniformis*. Moreover, a potent antibacterial spectrum against Gram (+) and Gram (−) bacterial strains and an efficient antifungal effect were observed. Its cytotoxicity toward human colorectal cancer cell LoVo and HCT-116 lines suggested that PIDF could serve as a new therapeutic target inhibiting human colorectal cancer.



1. INTRODUCTION

Proteolysis is an important process in several biological pathway signals, post-translational processing, catabolism, and various pathological mechanisms. Indeed, inflammation, apoptosis, blood clotting, and various neurological diseases involve proteases by their damaging overexpression due to the loss of regulation of corresponding genes. Several natural, specific, and selective protease inhibitors (PIs) are well characterized for majorly regulating proteolytic activities.[1] PIs are small molecules that form stoichiometric high-affinity complexes by binding to the active-site proteases and inhibiting their hydrolytic activity.[2] The main physiological function of endogenous PIs is the prevention of unregulated proteolysis through activation of coenzymes and release of biologically active polypeptides. PIs are widely distributed and have been isolated and characterized from a large number of organisms, including microorganisms, animals, and especially plants.[3]

Plant PIs (PPIs) were found in the aerial parts of plants (leaves, flowers and roots) as well as in storage tissues, such as tubers and seeds.[1] They act as specific inhibitors against digestive enzymes of phytophagous herbivorous insects, pests, and fungal pathogens.[4] PPIs are classified according to the catalytic site of the target proteases, which are serine, cysteine, aspartic, and metalloproteases into four classes including aspartic protease inhibitors (pepstatins), serine protease inhibitors (serpins), cysteine protease inhibitors (cystatins), and metallocarboxy protease inhibitors.[2]

Most PPIs are serine protease inhibitors and are widely found in the plant kingdom. According to amino-acid sequences, disulfide bridge content, three-dimensional structures, and especially thermal stability and resistance to denaturing agents, PPIs were classified into seven distinct families: Kunitz, Bowman−Birk, potato inhibitors, squash, cereal, and mustard. More than 48 families of PPIs were identified based on the sequence homologies of their inhibitor domains. Simple inhibitors are the designed PPIs with a single inhibitor unit, and complex inhibitors are relative to PPIs with multiple inhibitor units. The most common Kunitz-type protease inhibitors target specifically serine, cysteine, and aspartyl proteases.[2] They are characterized by a molecular mass of around 20 kDa, two S−S bridges, and a structural fold formed by 12 antiparallel β-strands with long interconnecting loops and an α-helix.[5]

Apart from their solubility in aqueous solutions and resistance to proteolysis, several PPIs were described to be highly stable under extreme pH and temperature conditions and toward strong denaturing agents such as detergents and

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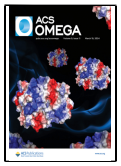

surfactants. This stability was attributed to the formation of intramolecular disulfide bonds or the presence of many aromatic residues. Various biological activities *in vitro* and *in vivo* have been also reported among them, including antimicrobial, antiviral, pesticidal, antitumoral, and anticoagulant properties.[3] These properties make PPIs very useful tools in numerous potential applications, especially pharmaceutical research that requires acid tolerance of orally administered drugs to avoid acid gastric degradation. In all biotechnological applications, high stability allows for reduction of the transportation and storage costs of any product.[3] PPIs are described to be effective in preventive and therapeutic effects on several cancers, as well as on inflammation and a large number of human diseases.[6]

The date palm (*Phoenix dactylifera* (*L.*)), belonging to the *Arecaceae* family, is distributed mostly in the Arabian Gulf from Saudi Arabia, Pakistan, North Africa, India, and America.[7] Separate plants with unisexual inflorescences of staminate or pistillate flowers called "male and female" palms, respectively, characterize this dioecious species.[8] Recently, Karra et al.[9] reported that male date palms provide about 1575 tonnes/year of flowers in Tunisia. These agro resources have been widely used for production of a tonic drink with antidiabetic and antioxidant effects. Although male date palm flowers are rich in proteins (14.8%) and then constitute available agro resources that can be valorized, it was not described until now as a protease inhibitor source.[9] In this context, the current study reported the purification and biochemical characterization of a new serine protease inhibitor named PIDF from date palm flowers *P. dactylifera (L.)* as well as the evaluation of its antimicrobial and antitumoral properties.

2. RESULTS AND DISCUSSION

2.1. Solvent Optimization for PIDF Extraction. The extraction medium plays a major role in the complete protein extraction from various sources. Hence, different solvents at different pH values were used for extracting *PIDF* from date palm flowers. Maximal protein concentration and protease inhibitor activity (57.8%) were obtained using a phosphate buffer of 0.1 M at pH 7.0 (Table 1). However, alkaline or acidic solutions showed a low yield of inhibitor extraction. Therefore, phosphate buffer (0.1 M) at pH 7.0 was used in all subsequent experiments. In the same line, several studies showed that phosphate buffer facilitated the maximal release of proteins, especially protease inhibitors from plant parts into the solvent. In fact, 0.1 M phosphate buffer (pH 7.6) was reported to be a good solvent for the maximal extraction of proteins from *Cajanus cajan* seeds with high trypsin inhibitory activity.[10] Likewise, Kansal et al.[11] showed that phosphate buffer (50 mM) at pH 7.6 generated the highest extraction yield of the trypsin inhibitor from chickpea seed flour. Recently, novel cysteine protease and trypsin inhibitors have been isolated using 50 mM phosphate buffer at pH 7.6 from *Juglans regia* and *Cassia leiandra* seeds, respectively.[12−14]

Table 1. Inhibitor Activity of PIDF in Various Extraction Media[a]

extraction medium	trypsin inhibition (%)
NaCl 15%	3.7 ± 0.82
NaOH 0.2 M	1.5 ± 0.43
HCl 0.05%	8 ± 0.67
phosphate buffer 0.1 M, pH 7.0	57.8 ± 2.75

[a]Results are expressed as mean ± SD (n = 3), and ± represents means of three independent experiments.

2.2. PIDF Purification. PIDF was purified from date palm flowers according to the described protocol in Section 3. After the heat and acidic treatments of the crude extract, ethanol fractionation followed by a C18 reverse-phase high-pressure liquid chromatography (RP-HPLC) was performed. The peak retention time of 19 min, corresponding to 40% acetonitrile elution, indicated pure PIDF with protease inhibitory activity measured under optimum conditions of pH and temperature (Figure 1A). PIDF was purified with a recovery of 18.8% and a purification factor of 38.1 (Table 2). The SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) profile showed a single band with an apparent molecular mass of about 19 kDa (Figure 1B). This finding was comparable with molecular masses of several purified PIs from leaves of *Moringa oleifera* and *Rhamnus frangula* that range between 22 and 24 kDa.[10,15] Purified PIDF exhibited a specific activity of 1803.8 IU/mg determined under standards assay according to Kunitz et al.[16] This specific activity is higher than that of the trypsin inhibitor purified from *C. leiandra* seeds (239 IU/mg),[13] while the trypsin inhibitor from *Chenopodium quinoa* seeds and the cysteine protease inhibitor from *J. regia* exhibit a specific activity of only 5.033 and 3.31 IU/mg, respectively.[12,17]

2.3. PIDF Biochemical Characterization. *2.3.1. Effect of pH on PIDF Activity and Stability.* The physicochemical stability of molecules with low molecular weight, such as PIs, offers the advantage of using them in a wide range of applications, especially in the food industry, agricultural biotechnology, and pharmaceutical sciences. Accordingly, in order to determine the optimum pH of PIDF activity, the percentage of inhibition under standard conditions was measured at several pH values ranging from 2.0 to 12. As shown in Figure 2A, a maximal activity of about 86% was reached at pH 8. PIDF retained more than 50% of its inhibitory activity at pH intervals ranging from 6.0 and 9.0. Figure 2A shows that PIDF exhibited about 85% of its inhibitor activity after incubation at pH 7.0−9.0 values. This pH stability, which might be associated with the rigidity of the PIDF tridimensional structure, could be considered as an important characteristic of the studied protease inhibitor in biotechnology-based applications. Indeed, a high content of cysteine residues forming disulfide bridges confers resistance in acidic and alkalic solutions of various proteins.[13] The residual activity of 50% observed after incubation at pH 5.0 and 10.0 (Figure 2A) could be attributed to the change of the tridimensional conformation that slightly affected the inhibitor activity. Similar results were found with a serine protease inhibitor extracted from *Acacia Senegal* seeds that was stable in pH ranging from 6.0 to 9.0 and retains 90% of its inhibitory activity at pH 9.0 versus 80% at pH 5.0.[18] Khan et al. showed that a cysteine protease inhibitor purified from *J. regia* had an optimal pH of about 7.6 and was inactive in acidic (pH 3.0) and alkaline (pH 10.0) conditions.[12] PI purified from *Lathyrus sativus* seeds was described to be stable in pH 9.0−11.0 with a maximal activity at pH 11.0.[19]

Figure 2B shows PIDF activity and stability profiles at various temperatures. At 40 °C, PIDF exhibited a maximum inhibitory activity of 84%. Only 31% of inhibition was observed at 20 °C, which could be explained by PIDF rigidity and lesser collisions between protease and the inhibitor.[12] At 30 or 50 °C, this activity was 66 or 48%, respectively (Figure

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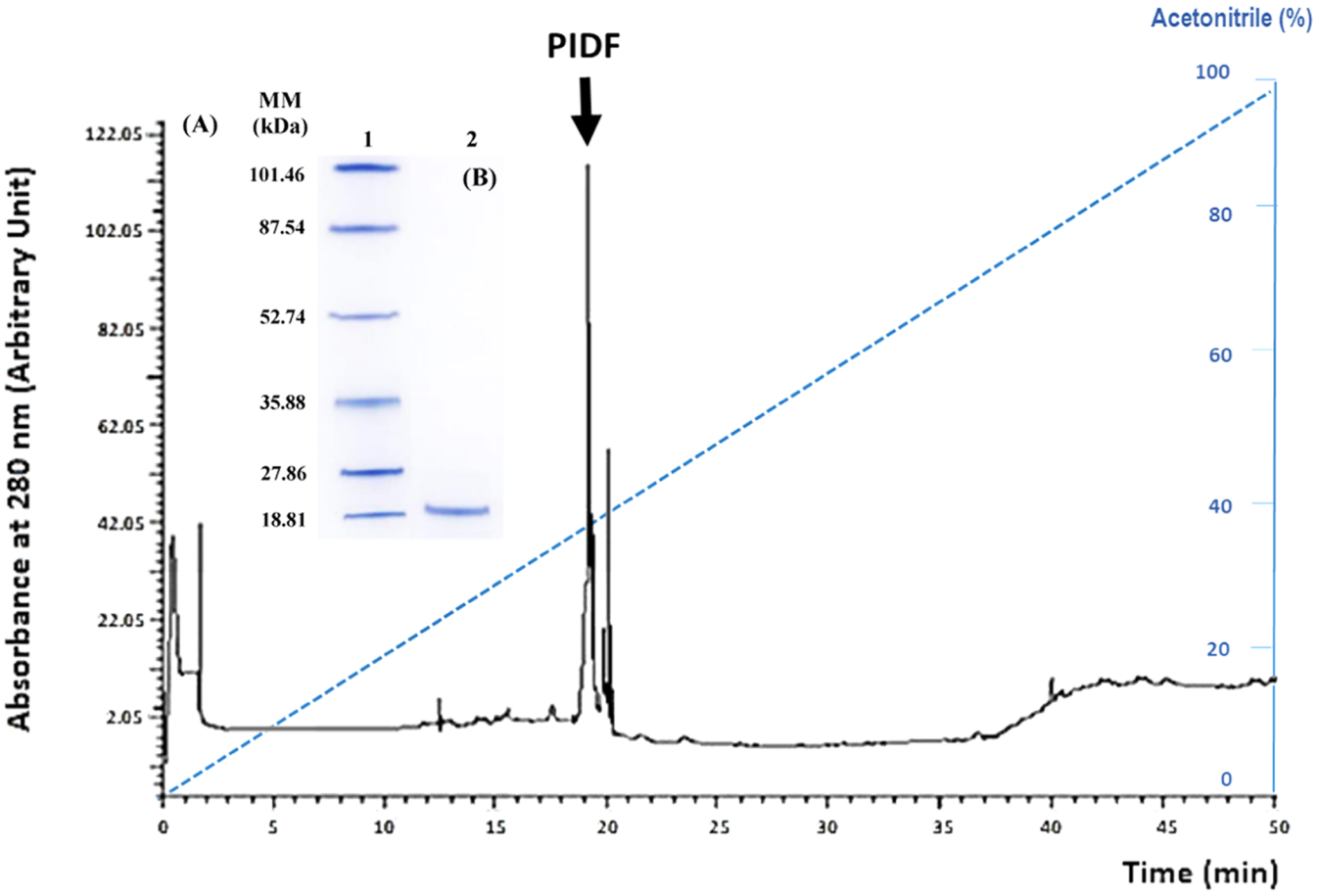

Figure 1. Purification of PIDF: (A) Chromatography of RP-HPLC on a C18 column and (B) SDS-PAGE profile of purified PIDF. Lane 1: molecular weight markers and lane 2: purified PIDF obtained after the RP-HPLC step.

Table 2. PIDF Purification Table

purification step	total activity (IU)	protein (mg)	specific activity (IU/mg)	activity recovery (%)	purification factor
crude extract	9550	202	47.3	100	1
heat and acidic treatment (70 °C, pH 3.0, 5 min)	7500	45	166.6	78.5	3.5
ethanol fractionation (40−85%)	5750	15	383.3	60.2	8.1
RP-HPLC	2345	1.3	1803.8	18.8	38.1

2B). However, PIDF lost its inhibitory potential at higher temperatures. Besides, PIDF was fully stable at a temperature interval between 20 and 45 °C with an inhibitory activity of more than 80%, while it was fully inactive at 70 °C due to the destruction of intra- and intermolecular hydrogen bonds (Figure 2B). Indeed, at high temperatures, the protein tertiary structure is unfolded and structural stabilizing interactions are no longer closely aligned.[20] Similar temperature activity and stability profiles were reported with several PIs, such as PI purified from *J. regia*,[12] *C. leiandra*,[13] and *Cassia grandis*.[21]

2.3.2. Effect of Addition of Stabilizers on PIDF Protease Inhibition Activity. It is well documented that the most important feature of peptides used in several biotechnological applications is the enhancement of thermal stability.[22] Several molecules are known to be additives that protect proteins against high-temperature inactivation by stabilizing the thermally unfolded proteins.[22] To enhance the thermal stability of the purified PIDF at high temperatures, the effect of various thermal stabilizer additives (bovine serum albumin (BSA), casein, starch, sucrose (1%), sorbitol, glycerol, (10%) $CaCl_2$, cysteine hydrochloride, poly(ethylene glycol) PEG 8000, urea (10 mM), and glycine (1 M)) was carried out at 70 °C (Figure 3).

As shown in Figure 3, BSA, starch, and sucrose at a 1% concentration as well as sorbitol at 10% and $CaCl_2$ at 10 mM improved PIDF stability at 70 °C up to 80%, compared to the sample without a stabilizer. However, a reduction of the PIDF inhibitory potential was observed with glycerol, glycine, PEG, urea, and casein (Figure 3). Therefore, PIDF thermal stability was promoted in the following order by $CaCl_2$ (inhibitory activity of 86.5% ± 2.12) > BSA (74% ± 4.2) > sorbitol (68.5% ± 2.12) > starch (60% ± 2.8) > sucrose (51.5% ± 3.5). This property was also established with trypsin inhibitors isolated from white cranberry beans and *Cyamopsis tetragonoloba* seeds in the presence of $CaCl_2$ and glycerol as additives stabilizers.[23,24] According to Bijina et al.,[10] the enhancement of thermal stability with $CaCl_2$ can be related to the ability of calcium ions to stabilize proteins through specific and nonspecific bindings within the protein molecule, preventing its unfolding at high temperatures.[10]

2.3.3. Effect of Addition of Metal Ions on PIDF Activity. Monovalent and divalent metal ions are known among agents that maintain the tertiary integrity of protease inhibitors.[10] The

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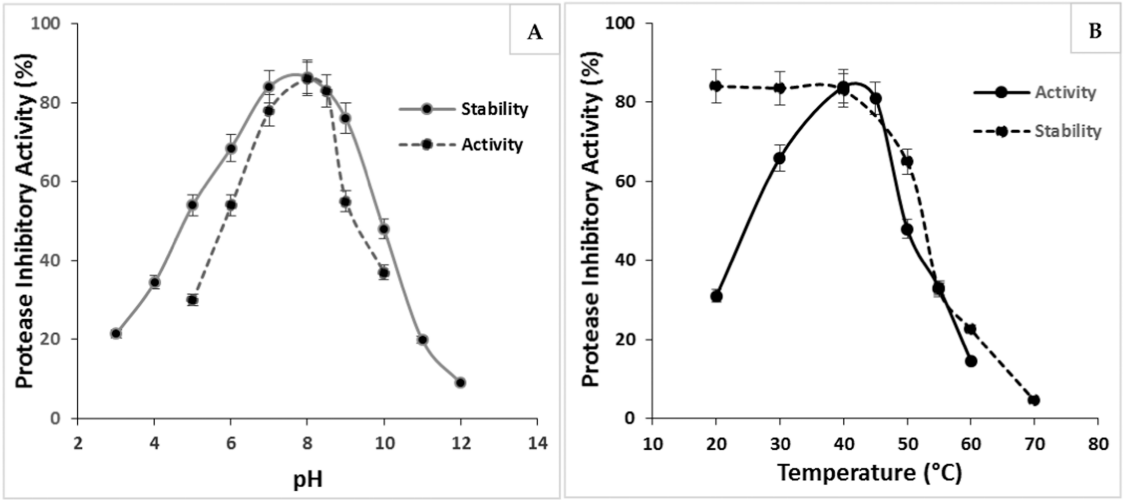

Figure 2. Stability and activity profiles of purified PIDF toward (A) pH and (B) temperature. The inhibitor activity was measured using trypsin as a substrate. Results are expressed as mean ± SD (n = 3), and ± represents means of three independent experiments.

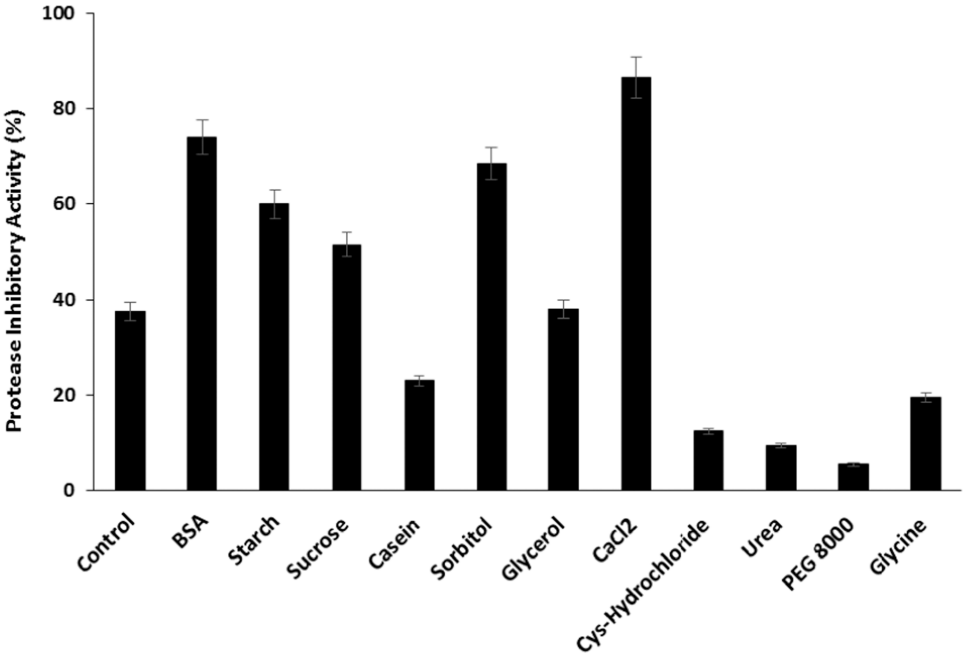

Figure 3. Variation of PIDF inhibitory activity toward various stabilizers at 70 °C. PIDF activity was tested under standard assay conditions (pH 8.0 and 40 °C) after 2 h of incubation at 70 °C in the presence of several thermostabilizers: (BSA), casein, starch, sucrose (1%), sorbitol, glycerol, (10%) $CaCl_2$, cysteine hydrochloride, PEG 8000, urea (10 mM) and glycine (1 M). Results are expressed as mean ± SD (n = 3), and ± represents means of three independent experiments.

impact of several metal ions at 1 and 10 mM on the PIFF inhibitor activity is illustrated in Figure 4. Bivalent cations Ca^{2+}, Hg^{2+}, Mg^{2+}, and Zn^{2+} at 1 and 10 mM remarkably improved the PIDF activity. Indeed, Mg^{2+} and Hg^{2+}, followed by Zn^{2+}, were the most effective bivalent cations at 10 mM, probably by stabilizing the three-dimensional PIDF structure (Figure 4). A similar behavior was described by the *M. oleifera* protease inhibitor, where 1 mM Zn^{2+} and 10 mM Hg^{2+} enhanced the protease inhibitor activity up to 31 and 64%, respectively. This enhancement was related to the binding of metal ions into the protein in a reversible mechanism, resulting in maintaining conformational stability.[10] A serine protease inhibitor purified from *Rhamnus frangula* showed that Mg^{2+} stabilizes its spatial conformation.[15] The reduction of the inhibitory potential of PIDF in the presence of Cd^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+} could be explained by the interference of these cations with charged amino-acid residues located in the active-site region of the inhibitor, leading to a decrease in its efficiency for binding with the protease.[23]

2.3.4. Effect of PIDF on Therapeutic and Commercial Protease Activity. The prevention and treatment of several human diseases is in continuous progression through using natural products such as PIs that are an efficient pharmacological tool to treat infectious and systemic diseases. Apart from their great interest in studies of protein−protein interactions, PIs were useful in pharmacotherapeutics for drug design and allowed for the prevention of the spreading of pathogenic agents that cause several diseases, including

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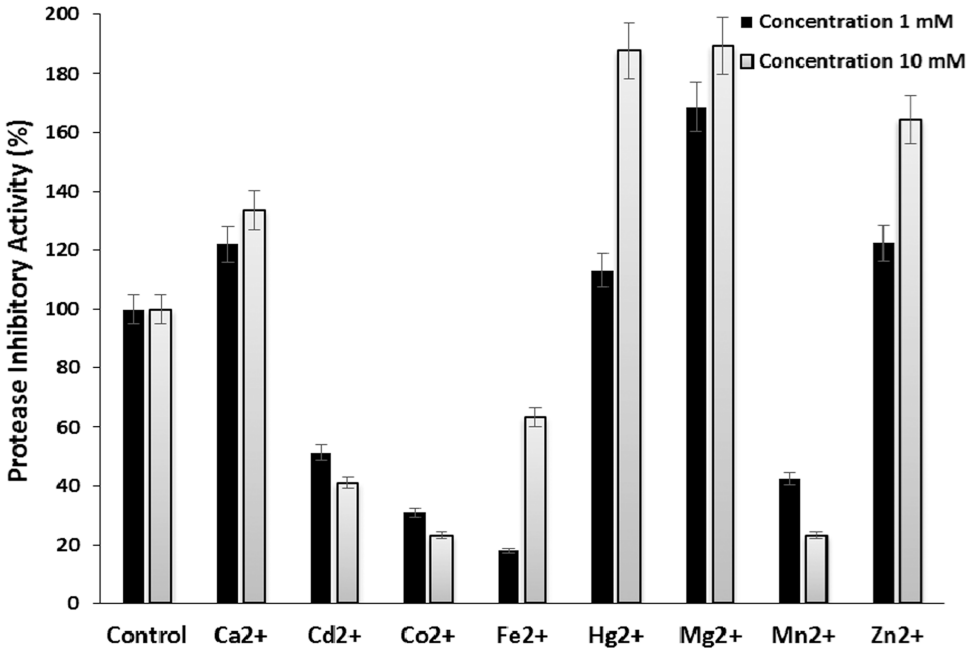

Figure 4. Effect of different metal cations on PIDF inhibitor activity at 1 and 10 mM concentrations. PIDF activity was tested under standard assay conditions (pH 8.0 and 40 °C). Control represents 100% of the PIDF inhibitor activity under standard assay conditions without any metal in the presence of 1 mM EGTA as a cation chelator. Results are expressed as mean ± SD (n = 3), and ± represents means of three independent experiments.

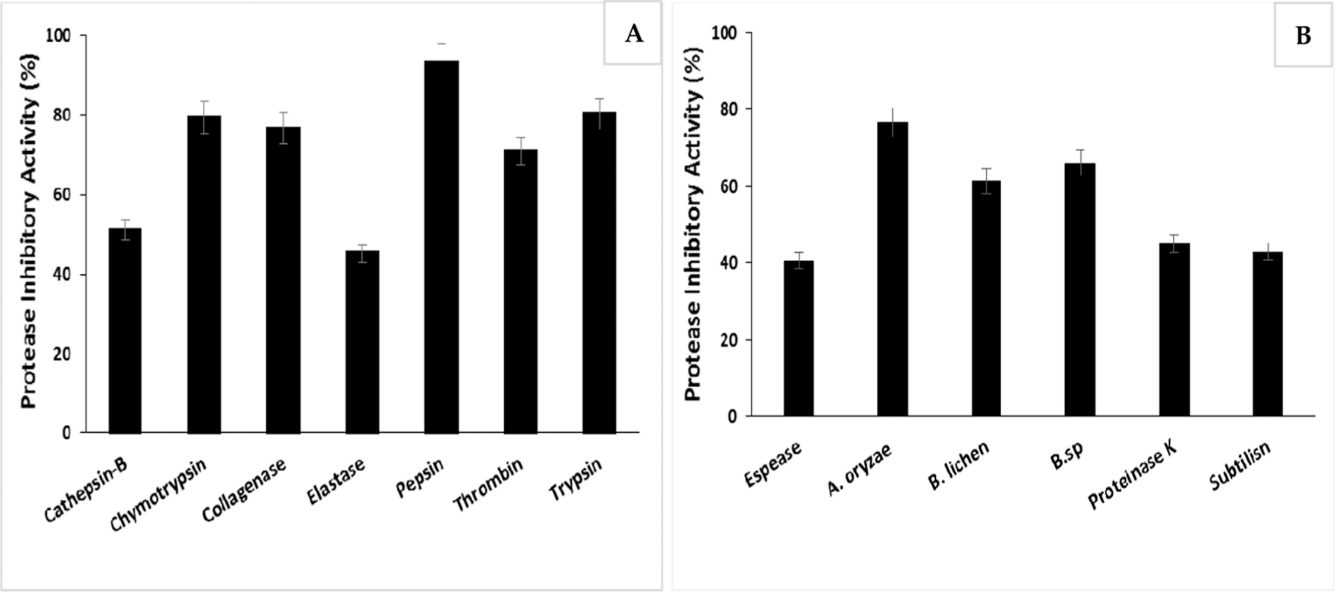

Figure 5. PIDF Inhibitory activity against (A) pharmaceutically and (B) commercially available proteases. Results are expressed as mean ± SD (n = 3), and ± represents means of three independent experiments.

hepatitis, cancer, malaria, and others.[3] Therefore, the evaluation of substrate specificity and selectivity by testing several commercial proteases can offer a strategy for site-specific targeted drug development. As shown in Figure 5A, PIDF was more efficient toward pepsin than trypsin, with inhibitory activities of 93.3 and 80.3%, respectively. Although PIDF specificities toward chymotrypsin and collagenase were similar (79.3 and 76.6%, respectively), it exhibited different affinity with thrombin (71%), elastase (45%), and cathepsin B (48%). The PIDF specificity toward pepsin could support its potential application in patients' treatment with ulcer diseases since it has been proven that pepsin is implicated in gastric ulcers.[25] Its recorded affinity toward thrombin (71%) made it a potential candidate to be used as an anticoagulant agent. A similar protease-specific behavior toward trypsin and chymotrypsin was observed with a serine protease inhibitor from *R. frangula* (RfIP1).[15] The highest affinity toward elastase (94% inhibitory activity), followed by trypsin and chymotrypsin (82 and 78%, respectively), was described with the protease inhibitor purified from *Conyza dioscoridis* (PDInhibitor). PDInhibitor shows inhibitory activities of about 79, 78, 73, and 42% against chymotrypsin, collagenase, thrombin, and

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pepsine, respectively.[26] whereas, ClTI, a trypsin inhibitor purified from *C. leiandra* seeds, showed a specific activity of 239 U/mg only toward trypsin as a substrate.[13]

Proteolysis is the most important step of a typical infection cycle of many bacterial pathogens. Indeed, secreted proteases facilitate the efficient dissemination within the host by degradation of the host's protein membranes.[27] Therefore, their inhibition offers the potential for antimicrobial drug development.[28] In line with this, PIDF-specific inhibition of some microbial pathogen proteases was investigated. As illustrated in Figure 5B, proteases from *Aspergillus oryzae* (*A. oryzae*), followed by *Bacillus* sp (*B.* sp) and *Bacillus licheniformis* (*B. lichen*), were inhibited effectively by PIDF with 77, 66, and 61%, respectively. Comparable findings have been reported by Bijina et al., with PI from *M. oleifera* leaves that exhibited an inhibitory effect on *B. lichen* and *A. oryzae* commercial proteases.[10]

2.4. Biological Activities. *2.4.1. Antimicrobial Activity of PIDF.* One of the most serious problems for the biomedical field is the growth of resistant bacteria that reduce the efficacy of antibiotics and then generate the antibiotic-resistance crisis. Therefore, looking for natural therapeutic alternatives in place of antibiotics is an interesting research field. Therefore, the potential use of PIs as new antimicrobial agents is promising, not only for their antimicrobial activity but also because their specific protease targets as crucial factors in microbial pathogenesis.[3]

The antimicrobial potential of PIDF was investigated toward several bacterial and fungal strains by measuring the inhibition zone. Table 3 shows that PIDF exhibited an efficient antimicrobial effect against tested Gram (−) and Gram (+) bacterial strains as well as fungal strains. Indeed, significant inhibition zones ranging from 10 to 22.5 mm were observed with all of the tested bacterial strains. It seemed that PIDF was clearly more efficient against *Bacillus subtilis* (*B. subtilis*) (22 mm), *Bacillus cereus* (*B. cereus*) (19 mm), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (22.5 mm). Since *P. aeruginosa* is the third most common cause of nosocomial infection and has developed a resistance to various antibiotics through mutations and adaptations,[29] the potential inhibition of its regulatory proteolysis system by PIDF could cause attenuation of virulence infection. The inhibitory effect against *Bacillus* strains has great importance since *Bacillus* are among the bacterial genus that became more resistant against some antibiotics by synthesizing several enterotoxins causing enteritis and septicemia.[30]

Table 3. Antimicrobial Activities of Purified PIDF against Different Gram (+) and Gram (−) Bacterial and Fungal Strains Using the Agar Diffusion Assay[a]

pathogens	inhibition zone (mm)	
	PIDF	ampicillin
Bacteria Gram (+)		
Bacillus cereus (ATCC 14579)	19 ± 1.41	18 ± 1.4
Bacillus subtilis (ATCC 6633)	22.5 ± 0.7	20 ± 1.6
Enterococcus faecalis (ATCC 29122)	14 ± 1.41	24.2 ± 0.7
Staphylococcus epidermidis (ATCC 14990)	10 ± 1.41	21.5 ± 1.4
Staphylococcus aureus (ATCC 25923)	11.5 ± 0.7	26 ± 0.5
Bacteria Gram (−)		
Escherichia coli (ATCC 25966)	13 ± 1.41	22.6 ± 1.5
Klebsiella pneumonia (ATCC 700603)	15.5 ± 0.7	14 ± 1.5
Pseudomonas aeruginosa (ATCC 27853)	22 ± 1.41	20.5 ± 0.5
Salmonella enteric (ATCC 43972)	18 ± 1.41	17 ± 0.6
Fungal strains		
Aspergillus niger	10.5 ± 0.7	
Botrytis cinerea	8.5 ± 0.7	
Fusarium solani	14 ± 1.41	
Penicillium digitatum	16.5 ± 0.7	

[a]Results are expressed as mean ± SD (n = 3), and ± represent means of three independent experiments.

This antibacterial behavior was also described with several plants' PIs. Indeed, RfIP1 displayed an inhibition zone of 24 and 25 mm against *B. subtilis* and *B. ceureus*, respectively.[15] The cysteine PI from *J. regia* WCPI, at 20 mM, was characterized by a higher bactericidal activity against *Erwinia carotovora*, a causative infectious agent in plants, and *Agrobacterium tumefaciens*, an economically significant plant pathogen.[12] The purified PDInhibitor was effective against Gram (+) and Gram (−) bacteria with IC_{50} values varying from 13 to 20 μg/mL.[26]

An efficient PIDF antifungal effect was also observed with inhibition zones ranging from 8.5 to 16.5 mm in this increasing flowing order *Botrytis cinerea* (*B. cinerea*) (8.5 mm) > *Aspergillus niger* (*A. niger*) (10.5 mm) > *Fusarium solani* (*F. solani*) (14 mm) > *Penicillium digitatum* (*P. digitatum*) (16.5 mm).

Using the same fungal strains, PDInhibitor exhibited a high antifungal effect with an IC_{50} value of about 4.05 μg/mL against *B. cinerea* and *A. niger*. This inhibitor was less efficient toward *P. digitatum* (IC_{50} 6.7 μg/mL) and *F. solani* (IC_{50} 14 μg/mL).[26] In contrast, ClTI purified from *C. leiandra* showed potent fungicidal activity against *C. albicans* at 2.1 μM with severe deformities in the *Candida* cell morphology. Indeed, ClTI induced perforations in the *Candida* cell surface and lysis, leading to the extravasation of the cytoplasmic fluid.[14]

These findings underlined that plant PIs could act as potent inhibitors of the growth of bacteria and fungi, making them excellent tools in the development of new antimicrobial agents for medical applications.

2.4.2. Cytotoxicity Effect on Human Colorectal Cancer Cell Lines. Proteases are key factors in tumor progression at both primary and metastatic sites. Metastases and tumoral invasion and progression are promoted by overexpression of proteolytic enzymes in the tumor or surrounding tissues through degradation of the extracellular and intracellular matrix.[31] Therefore, protease involvement in cancer is promising for cancer drug design.[32] Recently, protease inhibitors, especially those from plants, have been used as therapeutic agents in many clinical applications. Plant PIs are interesting for drug designs for their high stability and substrate selectivity.

Colorectal cancer is considered to be responsible for most of the cancer-related deaths worldwide, and treating this type of cancer has become a major focus of research. Several PPIs exhibited high potential in protection against inflammatory disorders and colorectal cancer development within the mammalian gastrointestinal tract.[33]

In this line, the cytotoxic effect of PIDF toward two human colorectal cancer cell lines (HCT-116 and LoVo) was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay. Cells were treated by PIDF concentrations ranging from 10 to 100 μg/mL. RfIP1 from *Rhamnus frangula* was used, at the same concentrations, as a positive control for a comparative study.[15]

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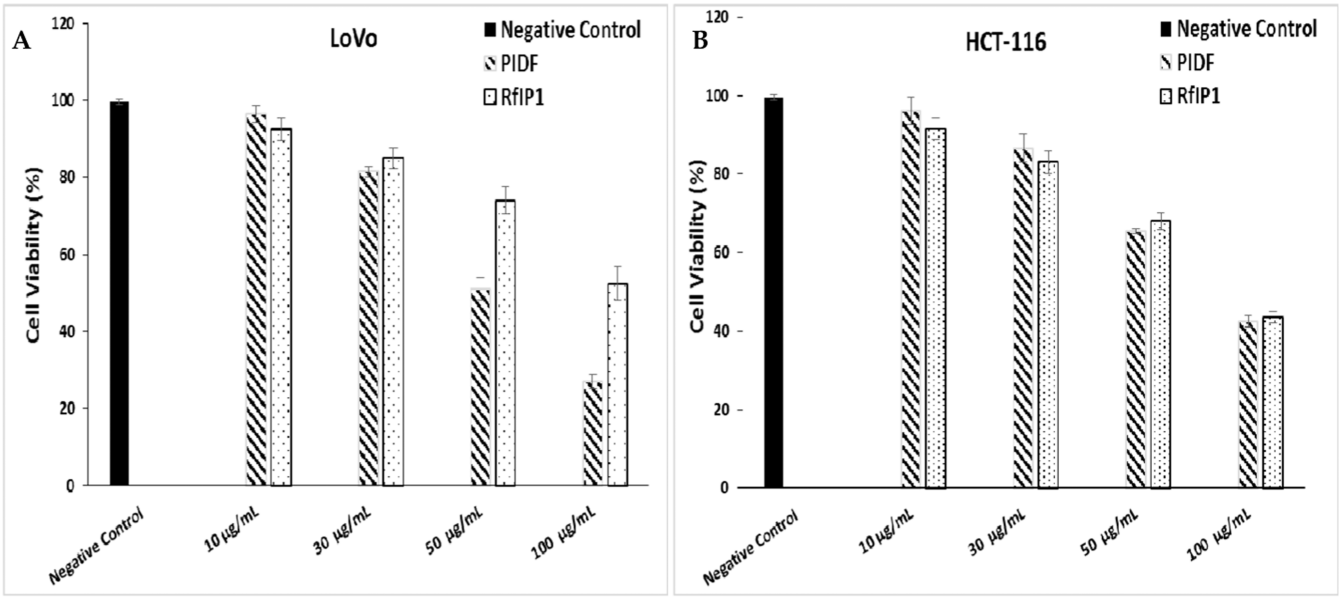

Figure 6. PIDF cytotoxic potential against human colorectal (A) LoVo and (B) HCT-116 cell lines. Cells were treated with different concentrations of PIDF for 24 h from 10 to 100 μg/mL. Cell viability was quantified using the MTT assay. Results are expressed as mean ± SD (n = 3), and ± represents means of three independent experiments.

Results presented in Figure 6A,B showed that PIDF displayed a significant cytotoxic effect against the two cell lines (LoVo and HCT-116) in a dose-dependent manner. Compared to RfIP1, a similar effect of PIDF was observed against HCT-116 cells (Figure 6A), while it was slightly more efficient against LoVo cells (Figure 6B). Indeed, at 100 μg/mL, the percentage of cell viability was 42.5 and 27% toward HCT-116 and LoVo cells, respectively (Figure 6), whereas IC_{50} values of 45.2 and 50 μg/mL were recorded for LoVo and HCT-116 cells, respectively. These results encouraged using PIDF as a potent anticolorectal cancer drug although similar findings were previously described with various PPIs. For instance, the purified PDInhibitor, at 200 μg/mL, reduced the HCT-116 and LoVo human cell line viability up to 27 and 52%, respectively.[26] Likewise, Bowman−Birk inhibitors purified from soybean inhibited, in a significant concentration- and time-dependent manner, the proliferation of colorectal human adenocarcinoma cells HT29.[34]

3. MATERIALS AND METHODS

3.1. Materials and Chemicals. White flowers are manually raised from a freshly opened male spa of date palm trees from Tunisia's southern region. The samples were washed with distilled water, dried at room temperature in the shade for about 7 days, and ground.

A chromatography material C18 reverse-phase column (100 mm × 2 mm) connected to a dual-wavelength UV−visible detector (Model 2487), SDS-PAGE, a spectrophotometer, a rotary shaker, a CO_2-humidified incubator, and a centrifuge were obtained from Bio-Rad (Hercules, CA).

Chemicals were obtained from commercial sources. Potato dextrose agar medium, phosphate-buffered saline (PBS, H 7.4), NaCl, Bradford reagent, ethanol, acetonitrile, sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), β-mercaptoethanol, Coomassie brilliant blue R-250, casein, trypsin, trichloroacetic acid, bovine serum albumin, $CaCl_2$, cysteine hydrochloride, glycerol, glycine, PEG 8000, sorbitol, starch, sucrose, and urea were obtained from Bio-Rad (Hercules, CA). Dulbecco's modified Eagle medium containing 15% fetal bovine serum, ampicillin, and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was purchased from Life Technologies, Paisley, U.K.

Commercial proteases Esperase (Novozyme, P5860), proteinase K (P2308), subtilisin (P5380), and those obtained from *A. oryzae* (P6110), *Bacillus licheniformis* (P4860), and Bacillus sp. (P3111) as well as proteases with therapeutic importance, including elastase (E8140), cathepsin B (C6286), chymotrypsin (C3142), collagenase (C2674), and thrombin (T7513), were obtained from Sigma-Aldrich.

3.2. Methods. *3.2.1. Extraction of the Protease Inhibitor.* Extraction of the protease inhibitor from date palm flowers was performed according to the Bijina et al.[10] method using different solvents (0.05 M HCl, 0.2% NaOH, 15% NaCl or 0.1 M phosphate buffer at pH 7.0). The ground sample (25 g) was mixed with 100 mL of each extraction solvent and was stirred in a shaker incubator at 200 rpm for 4 h at room temperature. After refrigerated centrifugation for 15 min at 12,000 rpm of the mixture, the collected supernatant was used to assess inhibitory activities after protein quantification. Thereby, the PIDF extracting solvent showing the highest trypsin inhibitory activity was selected for subsequent steps of purification of this crude extract.

3.2.2. Purification of PIDF. The crude extract (~9500 IU) was first heated for 5 min at 70 °C; then, denatured proteins were removed by centrifugation (12,000 rpm, 30 min), and the resulting supernatant was subjected to an acidic treatment for 5 min at pH 3.0, followed by a cold ethanol precipitation (1 V/4 V). After centrifugation at 12,000 rpm for 30 min, the pellet was suspended in 10 mL of phosphate buffer (0.1 M, pH 7.0). The sample was then loaded on a C18 reverse-phase column (100 mm × 2 mm) connected to a dual-wavelength UV−visible detector (Model 2487). Elution was performed with a mobile phase of an increasing gradient of acetonitrile from 0 to 100% at a flow rate of 0.5 mL/min, and proteins containing fractions were identified at a wavelength of 280 nm.

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3.2.3. Protein Analysis. The total soluble protein content was determined according to the Bradford[35] method by measuring optical density at 595 nm and using BSA as a standard. The molecular weight and protease inhibitor homogeneity were estimated by SDS-PAGE using a polyacrylamide solution at 15%.

3.2.4. Protease Inhibitor Activity. The activity of the protease inhibitor was measured according to the standard method developed by Kunitz et al.[16] A casein solution at 1% as a substrate was prepared using 0.1 M phosphate buffer (pH 7.0). The assay was performed by incubating the PIDF sample (1 mL) with a trypsin solution (1000 Units/mg) at an equal volume after its preincubation for 15 min at 37 °C. Then, 2 mL of 1% casein was added, and the mixture was incubated for 30 min at 37 °C. Afterward, the trypsin reaction was stopped by adding 2.5 mL of 5% trichloroacetic acid. The mixture underwent refrigerated centrifugation for 15 min at 12,000 rpm, and the absorbance value was measured at 280 nm using a spectrophotometer. Tests were performed in triplicate. The protease inhibitor activity (PIU) was evaluated in terms of the number of absorbance units after casein hydrolysis by trypsin per minute and calculated as follows: Activity (PIU) = DO (280 nm) × F × 5/0.0055 × T, where F is the dilution factor, T is the time reaction (min), and 0.0055 is the slope of the calibration curve DO/protein concentration.

$$\% \text{ inhibition} = [(\text{ACTcontrol} - \text{ACTreaction}) / \text{ACTcontrol}] \times 100$$

where ACTcontrol is the protease activity without PIDF and ACTreaction is the protease activity after preincubation with PIDF.

3.2.5. Characterization of PIDF. 3.2.5.1. Temperature and pH Profiles. The effect of pH and temperature on PIDF activity was assessed by measuring the inhibition of protease activity in suitable buffers ranging from 3.0 to 12.0 and at several temperatures ranging from 20 to 60 °C, respectively. To investigate the pH stability, purified PIDF (0.4 mg/mL) was incubated in various buffers for 24 h. Then, the reaction mixture was subject to refrigerated centrifugation at 12,000 rpm for 15 min, and the supernatant was used to measure the percentage of inhibition under standard conditions (37 °C, pH 7.0). The thermal stability of PIDF (0.4 mg/mL) was performed by incubating the PIDF at various temperatures (20−70 °C) for 24 h, followed by refrigerated centrifugation. All experiments were performed in triplicate.

3.2.5.2. Effect of Some Additives and Metal Ions on PIDF Stability. To improve its thermostability, PIDF was incubated for 2 h at 70 °C with different additives such as BSA, $CaCl_2$, casein, cysteine hydrochloride, glycerol, glycine, PEG 8000, sorbitol, starch, sucrose, and urea according to the method reported previously by Bacha et al.[15] Similarly, the divalent ion effect on PIDF stability was assessed by incubating the purified PIDF with several metal ions in 1 or 10 mM concentration at 37 °C for 1 h, and the % of inhibition was measured at standard conditions. The inhibitor activity of the control was measured in the presence of 1 mM chelator EGTA

3.2.5.3. PIDF Inhibitory Activity in Therapeutic and Commercial Proteases. In order to understand the protease inhibitor interactions, the potential inhibitory effects of PIDF against some commercially and therapeutic available proteases were explored, as previously reported by Bacha et al.[15]

3.2.6. Antimicrobial Effect of PIDF. The susceptibility of Gram (+) and Gram (−) bacterial strains toward purified PIDF was investigated by using the agar well diffusion method. The plate surface inoculation was carried out by spreading 0.2 mL of a microbial inoculum (10^6 CFU) over the entire agar surface, followed by loading wells with different volumes of the PIDF solution (1 mg/mL). Then, the agar plates were incubated overnight at 37 °C. The PIDF diffused into medium agar and inhibited the growth of the tested microbial strain. The negative and positive controls were prepared using sodium acetate buffer and ampicillin, respectively. The ability of PIDF to inhibit various bacteria growth was evaluated by measuring the diameter of zone inhibition around each well, while the impact of PIDF on different fungi's development was assessed using the poisoned medium method. The study evaluated the inhibition of mycelial growth on medium PDA by adding PIDF in comparison to the sample control.

3.2.7. Colorectal Cancer Cell Cytotoxicity. The cell lines used in this study were HCT-116 and LoVo (human colorectal cancer). They were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle medium containing 15% fetal bovine serum at 37 °C in a 5% CO_2-humidified incubator for 24 h. The MTT assay was used for assessing the cell metabolic activity. Cell viability was calculated as the mean ± SD (n = 3) and was expressed as a relative percentage of the OD values measured at 550 nm for PIDF-treated cells relative to positive (pure *Frangula* inhibitor) and negative controls. The cell viability was determined at 600 nm for PIDF-treated cells, and the control was expressed as a relative percentage of the OD values (cell viability ratio (%) = (OD treated/OD untreated) × 100%). The results are shown as the mean ± SD (n = 3). The cells (4×10^4 in each well) were incubated in a 96-well plate at 37 °C for 24 h in the absence or presence of different concentrations of PIDF, previously diluted in the culture medium. Then, 20 μL of MTT (5 mg/mL in PBS) was added to the cells and incubated at 37 °C for 4 h. Finally, the medium was carefully removed from each well and replaced with an equal volume of a saline solution (50:50). To dissolve the formazan crystals, the preparations were mixed thoroughly using a shaker before measuring the absorbance at 550 nm.

4. CONCLUSIONS

The present study showed that date palm (*P. dactylifera (L.)*) flowers could be a source of protease inhibitors. The purified PIDF revealed a molecular mass of about 19 kDa and was stable within a large pH range of 6.0 and 9.0. Besides, the stability at 70 °C was enhanced by adding calcium, BSA, and sorbitol as well as by metal divalent cations, especially Mg^{2+} and Hg^{2+}. We also reported that PIDF exhibited high inhibitory activity against both therapeutic proteases such as pepsin, trypsin, chymotrypsin, and collagenase and some commercially microbial proteases from *A. oryzae*, *B.* sp, and *B. lichen*. Furthermore, an efficient antibacterial activity against Gram (+) and Gram (−) bacterial trains as well as an antifungal property of the pure PIDF were also demonstrated. Besides, an antiproliferative potential of the studied PIDF toward two human colorectal cancer cell lines (LoVo and HCT-116) in a dose-dependent manner was also described. Collectively, PIDF could serve as a good therapeutic targeted drug for colorectal cancer. However, further investigations are required to determine the mechanism of this novel protease inhibitor.

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Author Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by I.J., N.K., A.B.B., M.A., H.H., and H.G. The first draft of the manuscript was written by N.K., H.G., and A.B.B. All authors have read and agreed to the published version of the manuscript.

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REFERENCES

(1) Habib, H.; Fazili, K. M. Plant protease inhibitors: a defense strategy in plants. *Biotechnol. Mol. Biol. Rev.* **2007**, *2* (3), 68−85.

(2) Rachel, K. V.; Sirisha, G. V. A review of protease inhibitors from different sources. *Int. J. Appl., Phys. Bio-Chem. Res.* **2014**, *4*, 1−18.

(3) Cotabarren, J.; Lufrano, D.; Parisi, M. G.; Obregón, W. D. Biotechnological, biomedical, and agronomical applications of plant protease inhibitors with high stability: A systematic review. *Plant Sci.* **2020**, *292*, No. 110398.

(4) Marathe, K. R.; Patil, R. H.; Vishwakarma, K. S.; Chaudhari, A. B.; Maheshwari, V. L. Protease inhibitors and their applications: An overview. *Stud. Nat. Prod. Chem.* **2019**, *62*, 211−242.

(5) Khamrui, S.; Dasgupta, J.; Dattagupta, J. K.; Sen, U. Single mutation at P1 of a chymotrypsin inhibitor changes it to a trypsin inhibitor: X-ray structural (2.15 Å) and biochemical basis. *Biochim. Biophys. Acta, Proteins Proteomics* **2005**, *1752* (1), 65−72.

(6) Srikanth, S.; Chen, Z. Plant protease inhibitors in therapeutics-focus on cancer therapy. *Front. Pharmacol.* **2016**, *7*, No. 470.

(7) Ghori, W.; Saba, N.; Jawaid, M.; Asim, M. *A Review on Date Palm (Phoenix dactylifera) Fibers and Its Polymer Composites*, IOP conference series: materials science and engineering; IOP Publishing, 2018012009.

(8) Salomon-Torres, R.; Krueger, R.; Garcia-Vazquez, J. P.; Villa-Angulo, R.; Villa-Angulo, C.; Ortiz-Uribe, N.; Sol-Uribe, J. A.; Samaniego-Sandoval, L. Date palm pollen: Features, production, extraction and pollination methods. *Agronomy* **2021**, *11* (3), 504.

(9) Karra, S.; Sebii, H.; Bouaziz, M. A.; Blecker, C.; Attia, H.; Besbes, S. Male date palm flower powder: Effect of incorporation on physico-chemical, textural, and sensory quality of biscuits. *J. Food Process. Preserv.* **2020**, *44* (9), No. e14687.

(10) Bijina, B.; Chellappan, S.; Basheer, S. M.; Elyas, K.; Bahkali, A. H.; Chandrasekaran, M. Protease inhibitor from *Moringa oleifera* leaves: Isolation, purification, and characterization. *Process Biochem.* **2011**, *46* (12), 2291−2300.

(11) Kansal, R.; Kumar, M.; Kuhar, K.; Gupta, R. N.; Subrahmanyam, B.; Koundal, K. R.; Gupta, V. K. Purification and characterization of trypsin inhibitor from *Cicer arietinum L.* and its efficacy against *Helicoverpa armigera*. *Braz. J. Plant Physiol.* **2008**, *20*, 313−322.

(12) Khan, A. A.; Fazili, A. B. A.; Bhat, S. A.; Bhat, W. F.; Asghar, M. N.; Khan, M. S.; Bano, B. Purification, characterization and studies of a novel cysteine protease inhibitor from *Juglans regia*: Implications as a potential biopesticide. *J. King Saud Univ., Sci.* **2022**, *34* (3), No. 101829.

(13) Dias, L. P.; Oliveira, J. T.; Rocha-Bezerra, L. C.; Sousa, D. O.; Costa, H. P.; Araujo, N. M.; Carvalho, A. F.; Tabosa, P. M.; Monteiro-Moreira, A. C.; Lobo, M. D. A trypsin inhibitor purified from *Cassia leiandra* seeds has insecticidal activity against *Aedes aegypti*. *Process Biochem.* **2017**, *57*, 228−238.

(14) Araújo, N. M.; Dias, L. P.; Costa, H. P.; Sousa, D. O.; Vasconcelos, I. M.; de Morais, G. A.; Oliveira, J. T. ClTI, a Kunitz trypsin inhibitor purified from *Cassia leiandra* Benth. seeds, exerts a candidicidal effect on *Candida albicans* by inducing oxidative stress and necrosis. *Biochim. Biophys. Acta, Biomembr.* **2019**, *1861* (11), No. 183032.

(15) Bacha, A. B.; Jemel, I.; Moubayed, N. M.; Abdelmalek, I. B. Purification and characterization of a newly serine protease inhibitor from *Rhamnus frangula* with potential for use as therapeutic drug. *3 Biotech.* **2017**, *7*, No. 148.

(16) Kunitz, M. Crystalline soybean trypsin inhibitor: II. General properties. *J. Gen. Physiol.* **1947**, *30* (4), 291−310.

(17) Pesoti, A. R.; Oliveira, B. M. d.; Oliveira, A. C. d.; Pompeu, D. G.; Gonçalves, D. B.; Marangoni, S.; Silva, J. A. d.; Granjeiro, P. A. Extraction, purification and characterization of inhibitor of trypsin from *Chenopodium quinoa* seeds. *Food Sci. Technol.* **2015**, *35*, 588−597.

(18) Babu, S. R.; Subrahmanyam, B. Bio-potency of serine proteinase inhibitors from Acacia senegal seeds on digestive proteinases, larval growth and development of *Helicoverpa armigera* (Hübner). *Pestic. Biochem. Physiol.* **2010**, *98* (3), 349−358.

(19) Aghaali, N.; Ghadamyari, M.; Hosseininaveh, V.; Riseh, N. S. Protease inhibitor from the crude extract of plant seeds affects the digestive proteases in *Hyphantria cunea* (Lep.: Arctiidae). *J. Plant Prot. Res.* **2013**, *53* (4), 338−346.

(20) Iyer, P. V.; Ananthanarayan, L. Enzyme stability and stabilization—aqueous and non-aqueous environment. *Process Biochem.* **2008**, *43* (10), 1019−1032.

(21) Brandao-Costa, R. M.; Araujo, V. F.; Porto, A. L. F. CgTI, a novel thermostable Kunitz trypsin-inhibitor purified from *Cassia grandis* seeds: Purification, characterization and termiticidal activity. *Int. J. Biol. Macromol.* **2018**, *118*, 2296−2306.

(22) Pandhare, J.; Zog, K.; Deshpande, V. V. Differential stabilities of alkaline protease inhibitors from actinomycetes: effect of various additives on thermostability. *Bioresour. Technol.* **2002**, *84* (2), 165−169.

(23) Puntambekar, A.; Dake, M. Protease inhibitor from white cranberry beans (*Phaseolus vulgaris*): Isolation, purification and characterization. *Int. J. Pharm. Pharm. Sci.* **2017**, *9* (9), 190−198.

(24) Patidar, P.; Golani, M.; Hajela, S.; Hajela, K. Impact of oxidizing, reducing, and stabilizing agents on the inhibitory properties of *Cyamopsis tetragonoloba* trypsin inhibitor. *J. Appl. Biol. Biotechnol.* **2020**, *8* (5), 68−71.
(25) Salman, H. A.; Yaakop, A. S.; Al-Mustafa, A.; Tarawneh, K.; Aladaileh, S.; Al-Rimawi, F.; Alakhras, F.; Abu-Lafi, S.; Al Zarzour, R.; Wahab, H. The dual impact of Jordanian *Ephedra alte* for inhibiting pepsin and treating microbial infections. *Saudi J. Biol. Sci.* **2021**, *28* (11), 6245−6253.
(26) Karray, A.; Alonazi, M.; Smaoui, S.; Michaud, P.; Soliman, D.; Ben Bacha, A. Purification and biochemical characterization of a new protease inhibitor from *Conyza dioscoridis* with antimicrobial, antifungal and cytotoxic effects. *Molecules* **2020**, *25* (22), 5452.
(27) Figaj, D.; Ambroziak, P.; Przepiora, T.; Skorko-Glonek, J. The role of proteases in the virulence of plant pathogenic bacteria. *Int. J. Mol. Sci.* **2019**, *20* (3), 672.
(28) Culp, E.; Wright, G. D. Bacterial proteases, untapped antimicrobial drug targets. *J. Antibiot.* **2017**, *70* (4), 366−377.
(29) Kirthika, P.; Lloren, K. K. S.; Jawalagatti, V.; Lee, J. H. Structure, Substrate Specificity and Role of Lon Protease in Bacterial Pathogenesis and Survival. *Int. J. Mol. Sci.* **2023**, *24* (4), 3422.
(30) Hajji, M.; Jarraya, R.; Lassoued, I.; Masmoudi, O.; Damak, M.; Nasri, M. GC/MS and LC/MS analysis, and antioxidant and antimicrobial activities of various solvent extracts from *Mirabilis jalapa* tubers. *Process Biochem.* **2010**, *45* (9), 1486−1493.
(31) Eatemadi, A.; Aiyelabegan, H. T.; Negahdari, B.; Mazlomi, M. A.; Daraee, H.; Daraee, N.; Eatemadi, R.; Sadroddiny, E. Role of protease and protease inhibitors in cancer pathogenesis and treatment. *Biomed. Pharmacotherapy* **2017**, *86*, 221−231.
(32) Rakashanda, S.; Rana, F.; Rafiq, S.; Masood, A.; Amin, S. Role of proteases in cancer: A review. *Biotechnol. Mol. Biol. Rev.* **2012**, *7* (4), 90−101.
(33) Caccialupi, P.; Ceci, L. R.; Siciliano, R. A.; Pignone, D.; Clemente, A.; Sonnante, G. Bowman-Birk inhibitors in lentil: Heterologous expression, functional characterisation and anti-proliferative properties in human colon cancer cells. *Food Chem.* **2010**, *120* (4), 1058−1066.
(34) Clemente, A.; del Carmen Arques, M. Bowman-Birk inhibitors from legumes as colorectal chemopreventive agents. *World J. Gastroenterol.: WJG* **2014**, *20* (30), 10305.
(35) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

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