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REVIEW ARTICLE

Trends and Prospects of Plant Proteases in Therapeutics

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Abstract: The main function of proteases in any living organism is the cleavage of proteins resulting in the degradation of damaged, misfolded and potentially harmful proteins and therefore providing the cell with amino acids essential for the synthesis of new proteins. Besides this main function, proteases may play an important role as signal molecules and participate in numerous protein cascades to maintain the vital processes of an organism. Plant proteases are no exception to this rule. Moreover, in contrast to human-encoded enzymes, many plant proteases possess exceptional features such as higher stability, unique substrate specificity and a wide pH range for enzymatic activity. These valuable features make plant-derived proteolytic enzymes suitable for many biomedical applications, and furthermore, the plants can serve as factories for protein production. Plant proteases are already applied in the treatment of several pathological conditions in the human organism. Some of the enzymes possess antitumour**,** antibacterial and antifungal activity. The collagenolytic activity of plant proteases determines important medical applications such as the healing of wounds and burn debridement. Plant proteases may affect blood coagulation processes and can be applied in the treatment of digestive disorders. The present review summarizes recent advances and possible applications for plant proteases in biomedicine, and proposes further development of plant-derived proteolytic enzymes in the biotechnology and pharmaceutical industries.

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1. INTRODUCTION

The major function of proteases in plants, as well as in other organisms, is the cleavage of proteins. The degradation of damaged, misfolded and potentially harmful proteins provides the cell with amino acids essential for the synthesis of new proteins. In addition, the selective destruction of regulatory proteins by the ubiquitin/proteasome system controls the key aspects of the growth, development and protection of plants. On the other hand, proteases are responsible for the post-translational modifications of proteins by limited proteolysis that are necessary particularly for the regulation of protein localization and enzymatic activities [1]. Thus, proteolytic enzymes are involved in all aspects of plant life, such as regulated cell death [2], photosynthesis [3], immune response [4], embryogenesis [5], and others. Proteases are specifically expressed in space and time and accumulate in the various compartments of the plant cell [6]. Proteases are commonly expressed in the form of zymogens – precursors which play a critical role in the folding, stabilization and function of the protease [7].

Depending on the location of the peptide bond and the cleavage which is catalysed by the enzyme, prote-

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ases are usually divided into three groups: the aminopeptidases and carboxypeptidases which hydrolyse the peptide bonds located at the N- or C-terminus of the polypeptide, respectively, resulting in the cleavage of one amino acid, di- or tripeptides and endopeptidases, which hydrolyse bonds remote from; the end of the amino acid sequences [8]. The mechanism of action of proteases is based on carbonyl-group polarization of the substrate peptide bond by stabilizing the oxygen in an oxyanion hole, making the carbon atom more susceptible to nucleophilic attack [6]. The role of a nucleophile can be performed by various amino acid residues or metal ions, and according to these differences, proteases are usually divided into seven classes: serine proteases, metalloproteases, cysteine proteases, aspartic proteases, asparagine proteases, threonine proteases and glutamate proteases [9, 10].

Proteases in plants play an important physiological role in a variety of biological processes. The family of subtilases is the most studied among the serine proteases. These have been shown to participate in microsporogenesis in the anthers of higher plants [11], in hypersensitivity reactions [12], symbiosis [13] and in regulated cell death [14]. However, in the regulated cell death of plants, other families of proteases are involved (for example, cysteine protease exhibiting caspase-like activity) [15]. Plants do not have macrophages, and therefore dying cells in plants degrade their materials themselves through the lytic system of defence and development regulated by certain proteases (*e.g.*, cysteine protease vacuolar processing enzyme (VPE)) [16]. Thus, it is clear that proteases related to different families may take part in one process, or vice versa, one protease can be involved in different processes within different compartments [17].

It is worth mentioning that cysteine proteases play a defensive role in most laticifers in latex/rubber-producing plants, and production of such proteases is strongly connected to the differentiation of plant cells into laticifers [18]. Laticifers form a network of vessels throughout the plant organism, and damage to laticifers inevitably leads to latex release. Latex proteases have been shown to be active against phytopathogenic fungi [19], and it has been suggested that these proteases protect the plant by sanitizing and sealing the wounded areas on the tree [20].

Many proteases are involved in plant growth and development. Matrix metalloproteases participate in the remodelling of the extracellular matrix [21], and similarly to animals, the 26S proteasome of *Arabidopsis* includes T1-family threonine protease and regulates signal transduction [22]. The precise role of most of the plant aspartic proteases has hitherto not been elucidated, and their biological functions are still hypothetical. Nonetheless, based on the existing data, it can be assumed that aspartic proteases are implicated in the processes of ageing [23], response to stress [24] and reproduction in plants [25]. Glutamate and asparagine proteases have not been sufficiently studied to determine their role in plants.

It is evident that proteases of different families are involved in distinct processes and act in concert. Plants possess not only basic but also unique features: they are autotrophic organisms; their cells contain a cell wall and unique organelles such as chloroplasts and central vacuoles. Moreover, plant physiology differs greatly from the physiology of other organisms, and plant genomes encode special protein products supporting unique plant features. Plant proteases are also interesting due to their wide range of working temperatures and pH levels [26]. Therefore, plants could be a perfect source of proteins, including proteases possessing different activities. This makes plants suitable for a broad range of uses in biotechnology and medicine. Here, we review current advances in the employment of plant proteases in some biomedical applications.

2. PHARMACOLOGICAL APPLICATIONS OF PLANT PROTEASES

2.1. Antitumour Activity

Plants are a natural source of many antitumour agents. Nowadays, 60% of antitumour pharmaceuticals are obtained from natural sources [27]. Extracts of different plants frequently exhibit antitumour activity and are used broadly in the practice of ethnomedicine. However, exact knowledge regarding their content and their mechanisms of action still does not exist. The hydrophilic extract derived from the Baltic brown seaweed *Fucus vesiculosus* was shown *in vitro* to contain pharmacologically active substances effective against pancreatic cancer, which is known as an aggressive type of cancer with a poor five-year survival rate [28]. *F. vesiculosus* extracts strongly inhibited the cell cycles of proliferating pancreatic cell lines, but not those of non-malignant resting T-cells and terminally differentiated cells of the pancreas *in vitro*. The effect was enhanced in combination with the inhibitors of autophagy. *Hibiscus sabdariffa* extract was shown to be active against multiple myeloma cells and oral squamous cell carcinomas *in vitro* [29], *Melissa officinalis* extracts were effective against hormone-dependent cancers *in vitro* [30], leaf extract of *Cynara*

scolymus exerted broad antitumour effects on mesothelioma cell lines both *in vitro* and *in vivo* [31], *Phyllostachys edulis* extracts induced apoptosis in osteosarcoma cells *in vitro* [32], *etc.*

In some cases, proteases presented in plant extracts are proposed as the major components responsible for the antitumour activity. Thus, such activity was shown for aqueous extract of *Carica papaya*, cultivated in the tropical zones of the globe: *C. papaya* is the source of latex and contains 15% dry matter, which is formed mainly by cysteine proteases such as papain, chymopapain, caricain and glycil endopeptidase [33]. Papain is known for its mitogenic properties [34]: it induces proliferation of various parenchymatous organ cells of the rat *in vitro* [35] and reduces cytoplasmic spreading and cell-cell interactions (reduces the number of surface proteins) of secondary chick embryo fibroblasts *in vitro* [36]. However, *C. papaya* extracts significantly inhibit the growth of tumour cell lines *in vitro* by mediating a Th1-type shift in the human immune system. The extracts were also suggested to activate immunomodulatory genes [37]. Papain was shown to have an antitumour effect on the growth rate, metastasis and invasiveness of Lewis lung carcinoma and B16 melanoma cells *in vivo* [38]. Along with DNAse I, aimed at destroying the circulating cell-free DNA, a mixture of proteases including papain, chymotrypsin and trypsin was suggested as an antitumour composition following *in vivo* investigations [39].

Another cysteine protease that is suggested to possess antitumour activity is bromelain, extracted from the waste of pineapple *Ananas comosus*. Bromelain is represented by the group of proteolytic enzymes located in different parts of the pineapple: stem bromelain (EC 3.4.22.32), and other forms of bromelain present in fruit juice, peel, core, crown and leaves [40]. Stem bromelain is commercially available and is most often used in studies and referred to as "bromelain". Bromelain was shown to possess antimetastatic properties and to cleave receptors *in vitro* – the surface protein α3β1 integrin and the hyaluronan receptor CD44 – involved in glioma cell invasion [41]. An antitumour effect was observed *in vitro* with cholangiocarcinoma: bromelain and the above-mentioned papain inhibited NFκB/AMPK signalling, which resulted in a decrease in the proliferation, invasion and migration of carcinoma cells [42]. Bromelain also exerts antiproliferative and proapoptotic effects in colorectal carcinoma cells *in vitro*, suggesting chemopreventive actions in colon carcinogenesis [43].

At present, the precise mechanisms of bromelain and papain action on tumour progression are not yet fully understood. However, papain and bromelain are thought to act in a similar way. They influence tumour progression in several directions (Fig. **1**). Firstly, they decrease the amounts of surface proteins and adhesion molecules on host CD4+, CD8+ T-cells and macrophages through proteolytic cleavage [40, 44]. Secondly, papain and bromelain are known to bind irreversibly to antiproteases such as α2-macroglobulin or α1-antitrypsin [45], which stimulates the synthesis of more antiproteases by macrophages, which in turn inactivates tumour proteases essential for tumour development and metastasis, such as cathepsins [46]. Thirdly, papain and bromelain may decrease the amounts of cytokines, *e.g.*, TNF-β, thus promoting the immunosuppression of the host and providing tumour escape. Plant protease-antiprotease complexes bind to cytokines such as IL-1, IL-6, IFN-γ and TNF-β and lead them out from the tumour, providing an antitumour and anti-inflammatory effect (Fig. **1**) [40]. In addition, tumours block the immune system factors which allow the tumour cells to protect themselves from being recognized by antibodies and immune cells. Blocking factors are soluble surface antigens bound to antibodies inhibiting macrophages, monocytes and natural killer cells. Exogenous proteolytic enzymes such as papain and bromelain reduce the amount of these blocking factors [40].

Proteolytic enzymes including P1G10 from *Carica candamarcensis* were shown to possess antitumour activity manifested in anti-angiogenesis, loss of cell adhesion and apoptosis in murine melanoma B16F1 *in vitro* [47].

Another cysteine protease from *Bromelia fastuosa*, fastuosain, was shown to possess antitumour effects both *in vitro* and *in vivo* [48]. Its protective action works similarly to papain action in B16 melanoma cells through the proteolysis of CD44 molecules and the effect on the production of antiproteases [49].

At present, it is obvious that anticancer enzymatic therapy assays based on plant proteases cannot replace conventional cancer treatment strategies such as surgery, radiotherapy, chemotherapy and hormone therapy, despite the fact that such conventional strategies have serious limitations and side effects [45]. Nonetheless, it is an alternative prospect which could be used together with traditional methods of cancer treatment, and there is considerable potential for further improvement.

2.2. Blood Coagulation

Coagulation (or clotting) is the process of blood-clot formation by a set of enzymatic reactions, giving

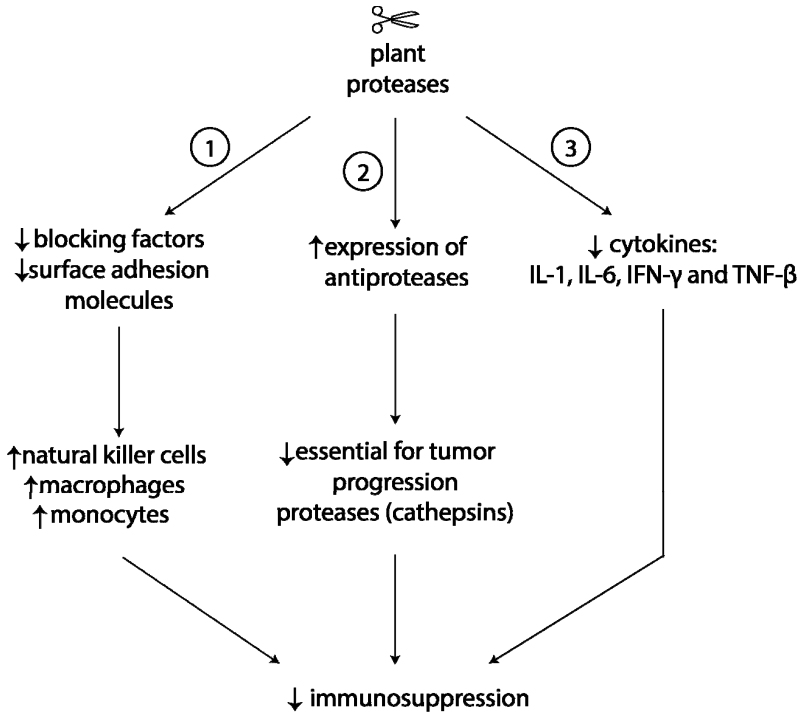

Fig. (1). Schematic representation of papain and bromelain antitumour action. The enzymes act in three directions: 1 - papain and bromelain cleave surface CD molecules of CD4+, CD8+ T-cells and macrophages and blocking factors emitted by the tumour, and release monocytes, macrophages and NK cells from the action of these blocking factors, 2 – plant proteases bind to antiproteases enhancing the expression of antiproteases by macrophages, and thus reducing the amount of cathepsins which are essential for tumour development and metastasis and, 3 – plant proteases decrease the amount of pro-inflammatory cytokines.

protection from unstoppable bleeding [50]. Fibrinolysis is the process of fibrin degradation into soluble fragments performed by the cascade of enzymatic reactions resulting in the dissolution of the clot or thrombus – a process referred to as thrombolysis [51]. Under normal conditions coagulation and fibrinolysis are balanced, but when there is a pathological abnormality, then the ratio of coagulation to fibrinolysis becomes unbalanced, leading to unstoppable bleeding or thromboembolic disorders. Thus, fibrin is an essential protein in both systems and is present in the bloodstream in its precursor form fibrinogen. Fibrinogen (factor I) is a dimeric glycoprotein which is present in human blood plasma and is essential for haemostasis, wound healing, inflammation, angiogenesis and other biological functions [52]. It is a soluble protein, but its conversion into monomeric fibrin leads to the formation of insoluble fibrin clots. Each subunit contains three polypeptide chains Aα, Bβ and γ; structurally, two peripheral (D) and one central domain (E) can be distinguished [52]. Fibrinogen cleavage may be induced by plasmin or thrombin (Fig. **2**) circulating in blood, resulting in fibrinogen degradation or in the formation of fibrin clots, respectively. Fibrin clots, in turn, can be degraded by plasmin.

2.2.1. Procoagulant Properties

Although procoagulant drugs based on pure plant proteases have not yet been developed, many plant extracts containing proteases are widely used in traditional medicine to stop bleeding and for wound healing. Thus, the procoagulant properties of the proteases currently constitute a subject of interest. There are a few plant latex proteases and protein fractions with procoagulant properties that reduce clotting time (Table **1**). In clinical practice, the activated partial thromboplastin time (APTT), the thrombin time (TT) and the prothrombin time (PT) are the most frequently used methods for assessing blood coagulation. The APTT measures the activity of the intrinsic system and the common pathway of the coagulation system, while the PT assesses the function of the extrinsic system and the common pathway.

The proteases presented in Table **1**, except Nivulian-II, showed to have *in vitro* fibrinogenolytic activity. Purified fibrinogen hydrolysis leading to clot formation means that fibrinogen cleavage is performed in a thrombin-like manner by all proteases except for those from the *Zingiberaceae* family, ficin, latex protease from *Wrightia tinctoria* and latex glycosilated

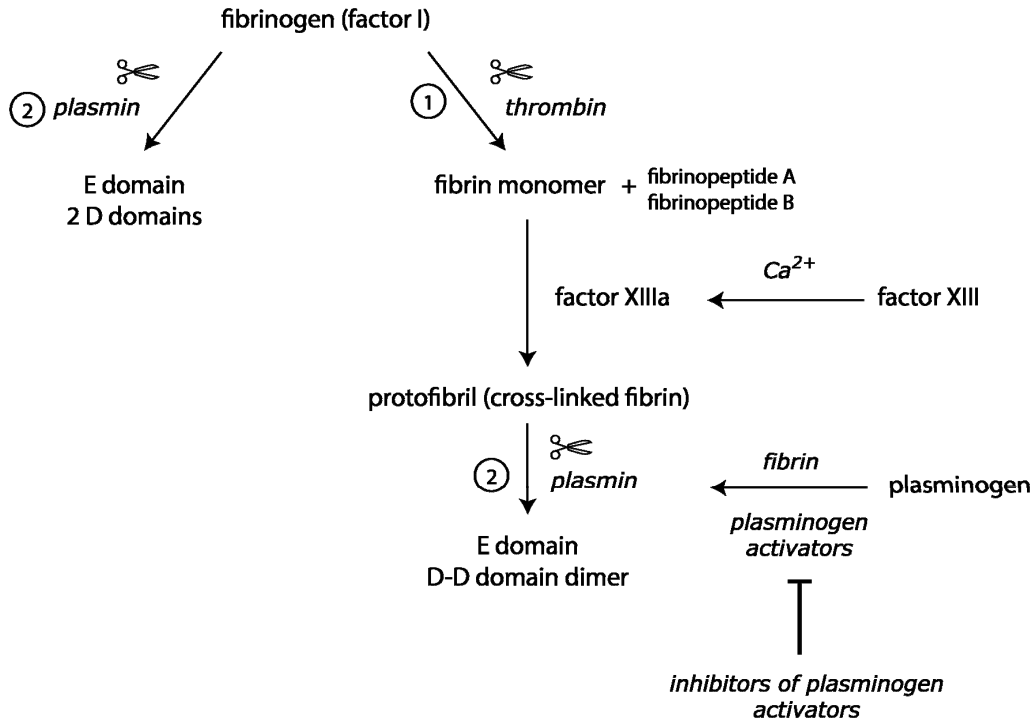

Fig. (2). Schematic representation of fibrinogen processing by plasmin and thrombin resulting in fibrin clot formation (1), and its lysis by plasmin (2). The plant proteases described possess plasmin-like and/or thrombin-like activity. This means that such proteases could act as plasmin and/or thrombin in the human body and affect the coagulation process through fibrin and/or fibrinogen cleavage, resulting either in clot formation or clot lysis. The scissors sign indicates the possible involvement of proteolytic activity of exogenous plant proteases and their replacement of the endogenous proteases plasmin and thrombin.

protease (LGP) from *Synadenium grantii*. Pergularain e I from *Pergularia extensa* is the only protease with seen cleavage of fragments similar to fibrinopeptides A and B, usually produced by thrombin cleavage in plasma leading to clot formation in the presence of Ca^{2+} and transglutaminase (factor XIII) [53, 54].

Most plant proteases also revealed fibrinolytic properties. Proteases from the *Asclepiadaceae* family were found to have both plasmin-like and thrombin-like activities [55, 56]. Some proteases (from *Calotropis procera*, *Cnidoscolus urens* and *S. grantii*) exhibited decreased APTT and unchanged PT, suggesting the possible preferential activation of coagulation factors of the intrinsic pathway [55, 57, 58]. Furthermore, it was found that some proteases are involved not only in fibrino(geno)lysis, but also in the activation of the enzymes from the previous steps of the coagulation cascade. Ficin decreases both the APTT and the PT in normal human plasma and in plasma which is deficient in coagulation factors, but hardly decreases the APTT in FX-deficient (factor X-deficient) plasma and the PT in FX- and FV-deficient (factor V-deficient) plasma at all. In plasma reconstituted with the FX values of APTT and PT, it is indistinguishable from normal plasma. No PT decrease was obtained by ficin in the prothrombin-deficient plasma, suggesting the exception of direct cleavage of fibrinogen by ficin. Ficin proteases (23.2 kDa and 23.5 kDa) affect blood coagulation by FX activation with identical cleavage patterns, depending on the presence of Ca^{2+} and Mn^{2+} [59]. Papain, another protease, exhibits factor XIIIa-like activity, inducing cross-linking in a highly purified fibrinogen preparation. Papain is not able to activate prothrombin to thrombin, thus it cleaves fibrinogen directly in thrombin-like manner forming soft clots [60].

The data summarized in Table **1** show the haemostatic potency of plant latex proteases and protein extracts in stopping bleeding and healing wounds.

2.2.2. Anticoagulant Properties

The anticoagulant properties of plant proteases and proteolytic extracts provide increased coagulation time in human plasma. They digest fibrinogen with different patterns and effectiveness: some proteases showed complete fibrinogen hydrolysis, while others showed only partial hydrolysis of fibrinogen. Most of the reviewed proteases possess direct fibrinolytic activity and the ability to lyse blood clots (Table **2**). A few plant proteases reveal antiplatelet activity. For some proteases, the prolonged APTT and the insignificantly

Table 1. Procoagulant plant proteases and plant extracts.

Plant Protease and its Source	Type of Proteases	Fibrinogenolytic Activity *in vitro*	Fibrinolytic Activity *in vitro*	Fibrin Clot Formation	Refs.
Papain from *C. papaya*	Cysteine	Thrombin-like	Thrombin-like	Papain-fibrin exhibited γ-chain cross-linking	[75]
Ficin from *Ficus carica*	Cysteine	No direct fibrinogen cleavage	ND*	Decreased APTT and PT in normal plasma	[59]
Crude proteases from *Artocarpus altilis*	Cysteine	ND	ND	Reduced clotting time in platelet poor plasma	[76]
Protease fraction from *Curcuma aromatica*	Serine	Complete hydrolysis of all subunits of human fibrinogen (not thrombin-like)	ND	Strong procoagulant activity	[77]
Protease fraction from *Curcuma longa*	Serine		ND		
Protease fraction from *Curcuma caesia*	Serine		ND		
Protease fraction from *Curcuma amada*	Serine	Partial hydrolysis, γ-resistant (not thrombin-like)	ND		
Protease fraction from *Curcuma zedoria*	Cysteine and serine		ND		
Pergularain e I from *P. extensa*	Cysteine	Thrombin-like	Plasmin-like	Decreased APTT, unchanged PT: possible interference with intrinsic pathway	[54]
Protease from *Asclepias curassavica*	Cysteine				[56]
Protease from *Cynanchum puciflorum*	Cysteine				[56]
Protease from *C. gigantea*	Cysteine				[55]
Proteins LPPII and LPPIII from *C. procera* latex	Cysteine		Plasmin-like, partial γ-dimer hydrolysis		[78]
Protease from *W. tinctoria*	Serine	Complete fibrinogen hydrolysis (no thrombin-like activity)	Complete fibrin hydrolysis	Reduced recalcification time of clot formation in citrated plasma	[79]
Crude proteases from *Tabernaemontana divaricata* latex	Cysteine	Thrombin-like	Plasmin-like fibrin lysis in blood clots	Reduced clotting time in platelet poor plasma	[76]
CgLP PI from *Cryptostegia grandiflora*	Cysteine	Complete fibrinogen hydrolysis	Plasmin-like, but not complete: digested γ-γ dimer, while α and β-chains were resistant	Reduced clotting time in citrated plasma	[80]
LGP from *S. grantii*	Serine	Complete fibrinogen hydrolysis	Complete fibrin hydrolysis in plasma and whole blood clots	Reduced recalcification time and unchanged PT in citrated human plasma	[58]
Nivulian-II from *Europhobia nivulia*	Cysteine	ND	ND	Decreased the coagulation time of whole blood in mice	[81]
Protein fraction F 1.0 from *C. urens*	Cysteine	Partial fibrinogen hydrolysis	Complete hydrolysis fibrin plasma clots at high concentrations	Reduced APTT and unchanged PT in citrated plasma: possible interference with intrinsic pathway	[57]
Protease isoforms from *Cucumis sativus*	Serine, cysteine, metalloproteases	Complete fibrinogen hydrolysis	Complete fibrin hydrolysis	Decreased clotting time	[82]

*ND – not defined.

Table 2. Anticoagulant plant proteases and plant extracts.

Plant Protease and its Source	Type of Proteases	Fibrinogenolytic Activity *in vitro*	Fibrinolytic Activity *in vitro*	Fibrin Clot Formation	Refs.
Crinumin from *Crinum asiaticum*	Serine	ND*	Plasmin-like	ND	[61]
ATFE-I from *Allium tuberosum*	Serine	Partial fibrinogen hydrolysis, γ-resistant	ND	ND	[83, 84]
ATFE-II from *A. tuberosum*	Cysteine	Aα-chain cleavage only	ND	ND	[83, 84]
Dimeric protease from *Spirodela polyrhiza*	Serine	Partial fibrinogen hydrolysis, γ-resistant	present	Prolonged APTT, PT	[85]
Kitamase from *Aster yomena*	Metalloprotease	Aα-chain complete and partial Bβ-chain cleavage	Complete fibrin clot hydrolysis, comparable to u-PA	Increased APTT and PT, increased bleeding time	[62]
Protease from *P. japonicas*	Serine	Complete fibrinogen hydrolysis	Complete fibrin clot hydrolysis, comparable to u-PA	Prolonged APTT and PT, slightly increased bleeding time	[63]
Glycosilated Eumiliin from *Europhobia milii Var. hisloii*	Cysteine	Partial fibrinogen hydrolysis, γ-resistant *in vivo*: inhibited fibrinogenolytic activity	ND	ND	[86]
Glycosilated Hirtin from *Europhobia hirta*	Serine	Partial fibrinogen hydrolysis, γ-resistant, thrombin-like	Complete α- chain and partial β and γ- chains fibrin hydrolysis	Prolonged clotting time of citrated plasma	[87]
Dimeric glycoslated protease EuP-82 from *Europhobia cf. lactea*	Serine	Complete fibrinogen hydrolysis at ph 10-11.	ND	ND	[88]
AMP48 from *A. heterophyllus*	Serine	Complete Aα- chain and partial Bβ and γ- chains hydrolysis at ph 8.8	Complete α- chain and partial β and γ- chains fibrin hydrolysis	ND	[89]
Protein seed extract from *A. heterophyllus*	Serine and cysteine	Complete fibrinogen hydrolysis	Complete hydrolysis of α-polymer and α-chain, β- and γ-chain resistant; no plasminogen activation	Prolonged the clotting time APTT, unchanged PT in citrated human plasma, inhibits intrinsic/common pathway	[90]
P1G10 protein fraction from *C. candamarcensis*	Cysteine	Complete fibrinogen hydrolysis	Present	Prolonged APTT, PT and TT	[91]
Protease StSBTc-3 from *S. tuberosum*	Serine	Complete Bβ-chain and partial Aα-and γ-chains hydrolysis	Dose-dependent clot dissociation	Prolonged TT	[92]
Bromelain from *A. comosus*	Cysteine	Complete fibrinogen hydrolysis	u-PA-like	Increased APTT and PT, slightly increased coagulation time	[64]
CIP-I and CIP-II enzyme isoforms from *Codium intricatum*	Serine	Complete fibrinogen hydrolysis	ND	ND	[93]
CLP protease from *C. latum*	Serine	Complete fibrinogen hydrolysis	Plasmin-like	Not thrombin-like	[66]
FI protease from *C. divaricatum*	ND	Complete fibrinogen hydrolysis	ND	Present	[70]
CDP FII protease from *C. divaricatum*	Serine	Complete fibrinogen hydrolysis	Complete fibrin hydrolysis	Not thrombin-like	[70]
Codiase from *C. fragile*	Serine	Complete fibrinogen hydrolysis	Complete fibrin hydrolysis, whole blood clots lysis *in vitro* (u-PA-like)	Prolonged APTT, PT and unchanged TT, prolonged tail bleeding time	[65]
Protease from *C. costata*	Serine	Complete fibrinogen hydrolysis	Complete fibrin hydrolysis (plasmin- like), whole blood clots lysis (u-PA-like)	Prolonged APTT, PT, unchanged TT, inhibits intrinsic/common pathway	[67]

*ND – not defined.

changed PT, (*e.g.*, proteases from *Costaria costata and Artocarpus heterophyllusprotein* seed extract) may be related to the inhibition of the intrinsic and/or common pathway of coagulation. This suggests that the extrinsic pathway could not be completely inhibited by these proteases.

A few latex proteases showed to possess thrombolytic activity (Table **2**). Some plant proteases prevent or decrease the induced thrombus formation in a mouse model. Members of the *Asteraceae* family revealed fibrinolytic activity comparable with urokinase-type plasmin activators (u-Pa) [61-63]. A fibrin cleavage pattern similar to that of u-Pa (both direct and indirect hydrolysis by plasminogen activation to plasmin) is attributed to the bromelain from *A. comosus* [64], codiase from *Codium fragile* [65], CLP protease from *Codium latum* [66] and protease from *C. costata* [67]. These features make these proteases competitive for use as thrombolytic agents. The proteases from *Petasites japonicus* and codiase from *C. fragile* that protect mice with carrageenan-induced thrombosis with greater efficacy than u-Pa, and show no haemorrhagic activity, seem to be especially promising. Codiase is also shown to exhibit antiplatelet activity [65]. Bromelain is known for its ability to activate factor XII and prekallikrein, and to hydrolyse cholesterol [68].

Dual (procoagulant and anticoagulant) activity is shown in protein-rich extracts from *Bromeliaceae* species, such as *Pseudananas macrodontes* (Pm), *Bromelia balansae* (Bb) and *Bromelia hieronymi* (Bh), in comparison with commercial stem bromelain. All extracts show the same ability to hydrolyse all fibrinogen subunits. Fibrin clotting suggests time-dependent thrombin-like activity. Procoagulant properties are revealed at low concentrations of Pm, Bh and bromelain by APTT and PT reduction, but at high concentrations all protease extracts show prolonged PT and APTT (anticoagulant properties). The fibrinolytic activity of Pm, Bb and Bh is lower than the activity of bromelain at the assayed concentrations [69]. Interestingly, proteases from the alga *Codium divaricatum* have both fibrino(geno)lytic enzyme (CDP or FII) and fibrinogen clotting enzyme (FI) [70]. It is interesting to note that the coagulation cascade is not only affected by the proteolytic action of plant proteins. Even though it has no proteolytic activity, heteromultimeric glycoprotein (HSGPL1) from *A. heterophyllus* affects the intrinsic coagulation pathway as a serine protease inhibitor, and has been shown to reduce the activity of human blood coagulation factors XIa and α-XIIa [71].

Plant proteases from both higher plants and algae possess anticoagulant and antithrombotic activities, as summarized in Table **2**.

2.3. Wound Healing

A wound is a trauma where there has been an infringement of epithelial integrity. There are four sequential yet overlapping phases in the classic model of wound healing: haemostasis, inflammation, proliferation and remodeling. These phases include the formation of a fibrin clot, the establishment of immune barriers, the dissolution of the fibrin clot, the removal of dead tissue, angiogenesis, extracellular matrix synthesis and the development of a new epithelium. The efficiency of latex use is associated with its large number of bioactive components, including proteases with specific activity, considering that pH values in the wounded area vary from 5.0 or lower (superficial) to 8.0 (full thickness) [72]. The haemostatic effect of proteases is based on the activation of zymogen FX through proteolysis which leads to its activated form FXa (serine protease), which in turn mediates the conversion of prothrombin to thrombin [73]. It has been shown that the ability to cleave FX is characteristic of papain and ficin [50], however, Ca^{2+} and Mn^{2+} are essential for stabilizing the FXa: their absence results in continued cleavage of FXa by ficin [59].

Proteases from the latex of *C. gigantea* are capable of cleaving fibrinogen to form fibrin fibres, which are the basis of blood clots. However, they cause bleeding at high concentrations, since they also have fibrinolytic activity [74].

Such thrombin-like activity is also characteristic of the glycoprotein from latex of *S. grantii* and *W. tinctoria* latex proteases which have been characterized as serine proteases, and isolated the cysteine protease pergularain e I from the latex of *P. extensa* [58, 94, 95]. Moreover, *in vitro* studies on a modified mixed lymphocyte culture demonstrated that bromelain and papain contributed to an increased production of IL-6, which is an important participant in the immune response to injury [96]. However, these results are not consistent with the studies on bromelain activities after oral administration [97].

2.4. Debridement of Burns

The presence of dead tissue is one of the most important infection factors in burns, and specifically in third-degree burns, as it is a source of intoxication and degradation, creating favourable conditions for many pathogenic bacteria. Therefore, an important task is to

remove the dead tissue early without affecting healthy skin. Currently, burns are usually treated by a combination of surgical and chemical treatments. Chemical treatment that avoids the surgical disadvantages (such as pain and blood loss) needs more time and repeated exposures to remove necrotic tissue. Since the middle of the last century, scientists have studied the enzymes from different sources which could be applied to the enzymatic treatment of burns [98]. In particular, there have been many studies explaining the use of proteases with bacterial origins, mainly from *Clostridium histolyticum* and *Bacillus subtilis* [99, 100] and also plant proteases, mostly the above-mentioned papain and bromelain.

Plant proteases used in burn debridement hydrolyse skin proteins such as collagen type I (chain A for papain) and devitalized and necrotic tissue. The pH of a burn area varies from 7.9 to 8.5 [101]. Papain is active over the wide pH range of 3.0 to 12.0, and shows maximum activity at pH 4.0-7.0 and temperature 37°C [102], whereas bromelain breaks down substrates over a pH range of 5.5 to 8.5 [103]. Papain was one of the first enzymes to be investigated for its use in the treatment of burns. A mixture of papain, urea and chlorophyllin was sold as Panafil, a drug which has already been taken off the market [104]. However, the use of an alternative drug based on urea and papain, called Debridace, may result in such adverse effects as high fever and excruciating pain [105]. Debricin based on ficin effectively degrades dead tissues in burn areas [106].

Bromelain is one of the most effective chemical means of treating burns [107]. In 1960, Klein further studied the use of bromelain in the treatment of burns begun by [103]. According to *in vitro* studies, bromelain was the most effective of the enzymes studied at that time [108]. Bromelain quickly and effectively degraded third-degree burn eschar fragments at temperatures from 37˚C to 38˚C [103]. Further studies led to the development of a purification method for bromelain, which later began to be used in the effective debriding mixture patented under the name Debridase [109]. The effective removal of necrotic tissue within four hours by Debridase also contributed to a more rapid re-epithelialization of damaged skin and a reduction in the wound area requiring transplantation [110, 111]. Furthermore, it was shown that an increased efficiency of eschar debridement could be obtained by combining Debridase with ultrasound [112]. Recently, a new drug for burn debridement based on bromelain has been developed, called NexoBrid, which also has high efficiency in the removal of burn eschar [113, 114]. Studies comparing the efficacy and safety of Debridase and NexoBrid, have not yet been conducted.

Two other sulfhydryl proteases, ananain and comosain, also present in the juice of stems of the pineapple, but in minor amounts, have been shown to successfully digest necrotic tissue [115]. However, probably due to the low enzyme yield in the purification, there have been far fewer trials of application of these enzymes than of Debridase application.

Recently, actinidin, a protease from kiwi fruit which was previously used as a meat tenderizer, was first investigated in relation to burn debridement. It was revealed that actinidin exhibits bactericidal properties and reduces wounds after debridement in the treatment of burns [116].

P1G10 from *C. candamarcensis*, as mentioned above, acts as a mitogen and stimulates wound healing of skin after experimentally induced burns [117]. This enzyme is a potential candidate for future clinical applications: it was the first known plant protease that exerts a proliferative effect on mammalian cells [118].

Cysteine protease from wheat, *Triticum aestivum*, Triticain-α, was recently reported to possess collagenolytic activity, *i.e.*, to effectively digest bovine dermis collagen at 37°C in the pH range 3.5 to 6.5 [119]. Due to this property, Triticain-α is a potential candidate therapeutic agent for wound healing and burn debridement assays.

2.5. Oral Healthcare

Caries is one of the most common diseases of the oral cavity and is characterized by a pH less than or equal to 5.5. Chemomechanical caries removal is based on the preliminary softening of the affected dentin, followed by scraping. Scraping not only removes the smear layer, but also demineralizes the top few micrometres exposing collagen fibres, underneath which the dentin is located. There are three types of collagen fibres: collagen types I, III and V. Collagen proteins form networks, which must be hydrolysed during caries removal [120]. The first formulations for chemomechanical dental caries removal were based on sodium hypochlorite (NaOCl), sodium hydroxide (NaOH) and sodium chloride (NaCl) [121, 122]. Later, in Brazil, a preparation called Papacarie was developed, which was based on papain, chloramine and toluidine blue [123]. Papain breaks up the partially degraded collagen molecules of the damaged tissue as there is no antiprotease activity, and in this way, preserves demineralized den-

tin. Moreover, papain reduces tissue repair time. Papacarie is characterized by antibiotic (mainly regarding *Streptococcus mutans* and *Lactobacillus*), and anti-inflammatory properties [123]. Papacarie gel is a less-invasive caries treatment method, and as it reduces the need for anaesthesia, is used for children [124]. In 2013, Indian scientists proposed a new, cheaper drug for chemomechanical caries removal, named Carie Care. Carie Care is similar to Papacarie in composition, but differs due to the addition of essential oils from plants [125]. When these products were compared, it was shown that Papacarie removes caries faster and has a more potent antibacterial effect [126]. This contradicts the *in vitro* studies [127]. Similarly, bromelain is widely used in dentistry because of its collagenolytic activity. It was examined for deproteinizing dentin and led to the removal of the collagen network [120]. In addition, bromelain and papain gel is used for deproteinization before orthodontic bracket attachment [128].

Tooth whitening is another use of plant proteases. It has been shown that papain and bromelain have bleaching properties, as they remove the protein element of plaque [129, 130]. However, since papain and bromelain are enzymes, the shelf life of the paste is limited.

2.6. Antimicrobial Properties

2.6.1. Antibacterial Properties

Several plant proteases possess antibacterial activity. Papain and bromelain showed bactericidal and effective inhibitory activity against the gram-positive bacterium *Alicyclobacillus sp*. Papain is also known for its antibacterial activity against several *B. subtilis* strains and enteropathogens such as *Enterobacter cloacae, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* [131, 132]. Considering the optimal pH values for growth of these bacteria, enzymes possessing antimicrobial activity need to be active in the pH range 2.0 to 9.5, and this correlates with the papain activity diapason (pH 3.0 to 12.0) [102].

It is probable that the inhibitory activity of papain and bromelain against bacteria is associated with their three-dimensional structure and with amidase and esterase but not with proteolytic activity [133]. Bound to leupeptin, these proteases showed the same levels of antibacterial activity, implying that an absence of proteolytic activity does not affect the antibacterial properties of the enzymes. It was shown that papain and bromelain synergistically interact with each other and with nisin - a polycyclic antibacterial peptide [133]. However, the precise mechanism for the antibacterial action of papain and bromelain is still not clear. In addition, papain was shown to scavenge 1,1-diphenyl-2-picrylhydrazyl hydroxyl and superoxide radicals and to reduce H_2O_2-induced oxidative stress in *E.coli* strains [134]. Antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853 was demonstrated for AMP48 (antimicrobial activity of a 48 kDa protease) from *A. heterophyllus* latex [135], altering its shape and size, but the precise mechanism has not yet been investigated.

2.6.2. Antifungal Properties

Antifungal properties of two aspartic proteases from *Solanum tuberosum* (StAP1 and StAP3) against *Fusarium solani* spores and *Phytophthora infestans* cysts have been reported [136, 137]. According to the studies, StAP1 reduced the viability of *F. solani* spores in a dose-dependent manner and was about twice as effective as StAP3. Furthermore, the inhibition of *P. infestans* germination by 50% needed an approximately 70-fold lower StAP1 concentration than StAP3 [137]. This is significantly lower than the concentrations previously reported for potato proteins active against *P. infestans* [138]. This study revealed that StAPs cause membrane permeabilization in spores and hyphae of *F. solani* and cysts of *P. infestans*. The results of the research suggest that the electrostatic interaction between StAPs and the negatively charged membrane components and/or specific receptors could be the initial step of StAPs' antimicrobial activity mechanism [137]. Further investigation showed the effect of deglycosylation on the antifungal activity of both StAPs and resulted in lower values for dgStAPs than those obtained for native forms at all concentrations and times assayed, which seems to be a result of conformational changes [139]. To sum up, native StAP1 seems to be a more potent antimicrobial agent than StAP3.

Antiproliferative properties of zingipain, a cysteine protease from *Zingiber ottensii* Valeton rhizomes, against fungi, bacteria, and human malignant cell lines have been described [140]. However, the antifungal activity of zingipain against three tested phytopathogenic fungal species *Exserohilum turicicum, Fusarium oxysporum* and *Corynespora cassiicola* was considered weak. The precise mechanism of zingipain antifungal and antiproliferative activity has yet to be investigated.

Strong antifungal activity against *Candida albicans,* which is one of the most common human pathogens,

and no activity against the less pathogenic *Candida tropicalis* was also demonstrated by AMP48 [135].

2.7. Digestion Disorders

2.7.1. Gastrointestinal Infections and Ulcers

Plant proteases are used in the treatment of gastrointestinal infections caused by nematodes. Both humans and animals in the agriculture industry suffer from such infections all over the world. Some cysteine proteases from plants possess anthelmintic activity [141]: they are able to degrade cuticles of nematodes at pH 7.4. It was shown that purified ficin, papain, chymopapain, extracts of Egyptian milkweed latex and extracts containing bromelain are effective against the nematodes *Heligmosomoides polygyrus* compared to the control, pyrantel tartrate, which has proven anthelmintic activity [142]. Natural anthelmintic preparations are a good alternative to synthetic drugs, to which nematodes have already acquired resistance.

Papain and cysteine proteinases from *Carica candamarcensis* exert a protective effect in a gastric ulcer rat model [143]: they restore the equilibrium of imbalanced compounds such as HCl, ethanol, pepsinogen and protective agents such as bicarbonate, prostaglandins, *etc.* [144]. P1G10 acts as a mitogen on fibroblast and epithelial cells and stimulates angiogenesis and wound healing in gastric and cutaneous ulcer models [47]. Bromelain also resulted in rapid symptom improvement in the treatment of ulcerative colitis [145].

2.7.2. Coeliac Disease

The potential enzymatic treatment of coeliac disease (CD) involves the use of the cysteine protease Triticain-α from *T. aestivum*. [119, 146]. CD is an autoimmune disorder in genetically predisposed people carrying HLA-DQ2/DQ8 alleles, and is associated with incomplete exogenous degradation of gluten – a sum of seed storage proteins of grains such as wheat, barley, rye and oats [147]. The histopathological symptoms of coeliac disease include malabsorption of nutrients by the small intestine, atrophy of villi, crypt hyperplasia and increased infiltration by intraepithelial lymphocytes [148]. At present, only one approach for the treatment of CD has proven efficiency, *i.e.*, a gluten-free diet (GFD) implying avoidance of gluten-containing foods. However, despite its efficacy GFD has its own disadvantages [149]. Thus, a promising approach for the treatment of CD is enzymatic therapy aimed at increasing insufficient proteolytic activity or substituting for the defective enzyme [150, 151]. Triticain-α functions in wheat germinating seeds and possesses glutenase activity [119, 152]. Triticain-α has been shown to effectively cleave major gluten-derived toxic peptides. This enzyme is active in a pH range of 3.4 to 6.5, and is stable to pepsin-mediated proteolysis, which makes the oral administration of this enzyme possible in the course of coeliac disease treatment.

Another protease composition used in CD treatment is ALV003, based on a mixture of barley-derived glutenase EP-B2 and prolyl endopeptidase (PEP) from *Sphingomonas capsulata* (SC-PEP) [153]. This glutenase mixture was shown to successfully attenuate gluten-induced mucosal injury in patients with CD. Currently, ALV003 is undergoing clinical trials.

2.7.3. Other Gastrointestinal Conditions

Plant extracts are often used for the treatment of gastrointestinal diseases. For example, solon (*Sophoradin* extract), amaranth-seed extract, grapefruit-seed extract and capsaicin (extract of chilli pepper) were investigated for gastroprotective effects on acute gastric lesions induced by corrosive concentrations of ethanol [154]. Due to the broad range of optimal pH values, bromelain could be used both in the stomach and in the small intestine. Bromelain has been used as a digestive drug in cases of exocrine pancreas insufficiency [155], and could replace pepsin and trypsin. Bromelain, when combined with ox bile and pancreatin is very effective in lowering stool fat excretion in pancreatic steatorrhoea patients. It also increases uptake of radioactive sulphur by 50% and glucosamine by 30-90%, enhancing gastric mucosa healing [156]. Bromelain inhibits the attachment of enterotoxin to the large and small intestine thus preventing enterotoxin-induced diarrhoea [157]. It also exhibits anti-inflammatory properties in inflammatory bowel disease (IBD), decreasing the clinical and histological severity of IBD in mice [158].

In addition, cysteine proteases and cellulases are used for the treatment of phytobezoars, emerging in agricultural animals' digestive systems [159].

2.8. Other Applications

As seen above, bromelain is the one of the most common plant enzymes used in industry and medicine. Among other things, elucidating the therapeutic capacities of bromelain in the treatment of cardiovascular diseases in animals and humans has been reported [160]. In comparison with the control, bromelain treatment of animals provided higher left ventricular functional recovery throughout reperfusion, increased aortic flow, a reduction of infarct size and degree of apoptosis and

reduction of thrombosis formation. It also showed the cardioprotective effect and amelioration of rejection-induced arterial wall remodeling. Bromelain was also tested on patients with hypertension, history of stroke or heart attack and thrombophlebitis. It resulted in the reduction of blood platelets and reduced thrombus formation. Reduction of platelet aggregation was also shown in patients with myocardial infarction: bromelain treatment resulted in the functional recovery of the heart through limiting myocardial injury in ischaemia experiments, increasing aortic flow and reducing the infarct size [161]. Furthermore, oral administration of bromelain showed no significant side effects [162], suggesting that eight times the maximum recommended dosage was still tolerable [161]. These data indicate that bromelain is a prospective plant protease for the treatment of cardiovascular diseases and that additional long-term trials of bromelain and its side effects are required.

In addition, bromelain was used in the treatment of oedema and ecchymosis in facial injuries [163], especially in the treatment of boxers' traumas [164] and in the post-operative treatment of arthrotomies of the knee and facial injuries [165]. Bromelain revealed anti-inflammatory and analgesic properties in mild to acute knee pain and improved well-being in an open study [166]. Different dosages of bromelain were examined in clinical trials of knee and shoulder osteoarthritis [167]. Bromelain is considered as a food supplement that may provide an alternative treatment to nonsteroidal anti-inflammatory drugs (NSAIDs) [158]. It is also active against sinusitis [160]. As well as bromelain, papain prevents trauma inflammations. The reduction of postoperative oedema, swelling and ecchymosis in nasal plastic surgery by papain has also been reported [168]. The anti-oedematous effect is likely to be connected to binding to antiproteases (α2-macroglobulin) [40]. Papain is widely used in studies *in vivo* as an agent that induces osteoarthrosis [169] and reversible damage of cartilage and may cause irreversible damage to epiphyseal plates [170].

The combination of bromelain, trypsin and rutin results in a significant and similar reduction in pain and inflammation to diclofenac, due to the different substrate specificity of these enzymes [162]. For example, trypsin enhances fibrinolysis, and papain and bromelain bind to the pro-inflammatory cytokines IL-1, IFN-γ and TNF-α or modulate immunity through the proteolysis of cell-surface CD molecules and consequent CD2-mediated T-cell stimulation, and papain reduces the IL-2 and IL-4 levels [163, 26]. Rutin is a flavonoid compound that inhibits hyaluronidase in connective tissues, and the blockage of ATPases, phospholipases, cyclooxygenases and lipoxygenases, and thus provides an additional oedema-protective effect [162].

Another cysteine protease from *C. papaya* – chymopapain – dissolves herniated nuclear material, and has become the basis of the invention of the chemonucleolysis procedure for the treatment of sciatica as a consequence of disc herniation. Chemonucleolysis is considered to be a more effective and safer procedure than chymopapain injection and open discectomy. The only disadvantage of this procedure is the possible allergic reaction to papain or papaya, which can easily be predicted [171]. The cysteine protease from ginger, *Zingiber officinale*, produces the novel gelatin hydrolysate, inducing enhanced absorption of hydroxyproline and reduction of joint pain and blood-sugar levels [172].

Generally, plant extracts are traditionally used in the treatment of a wide range of diseases. *Ginkgo biloba* extract was shown to ameliorate the cardiac remodeling induced by acute myocardial infarction: the mechanism partly involved regulating the expression of TGF-β, MMP-2 and MMP-9 [173]. Plant extracts of *Lactuca scariola* and *Artemisia absinthium* both demonstrated analgesic properties [174]. Extracts from red clover (*Trifolium pratense*), soybean (*Glycine max*), black cohosh (*Cimicifuga racemosa*) [175], chasteberry (*Vitex agnus-castus*), hops (*Humulus lupulus*), Asian ginseng (*Panax ginseng*), North American ginseng (*Panax quinquefolius*), dong quai (*Angelica sinensis*) and liquorice (*Glycyrrhiza glabra*) [176] are used in the hormonal substitution therapy of menopausal symptoms. Medication containing *Withania somnifera, Banksia serrata, Z. officinale* and *C. longa* extracts offered some improvements compared with placebos in knee osteoarthritis patients [177].

Furthermore, in 1995 there was a suggestion that plant proteases such as papain and bromelain could be used in cosmetic formulations. Papain and bromelain break down the collagen proteins under physiological conditions in the stratum corneum cells, and reveal a softer and younger layer of the skin. These mixtures have been proposed for the treatment of skin diseases, skin laxity, wrinkles and dry skin. Alternative formulations are based on organic and inorganic acids, steroids, fungicides, antibiotics and anti-inflammatory agents. Their use led to some temporary relief from the disease, but long-term relief from symptoms has not been confirmed [178]. Even though the extracts of various

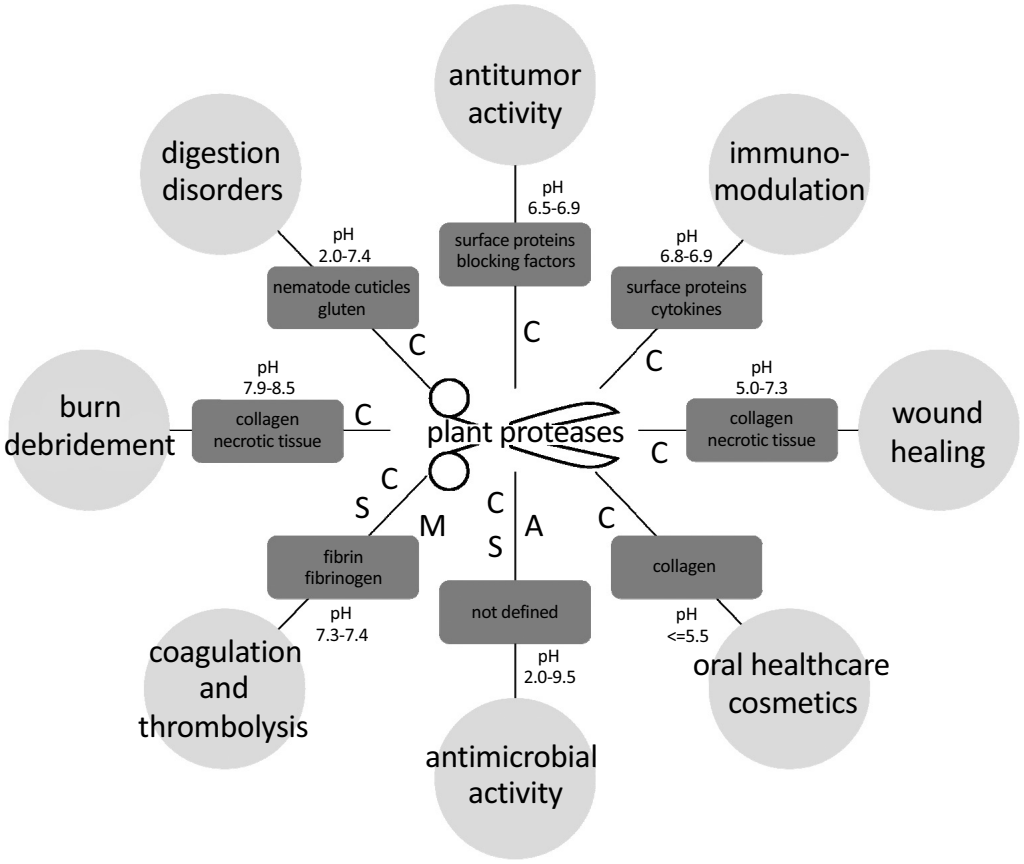

Fig. (3). Contribution of different families in a variety of applications of plant-derived proteases in biomedicine. Rectangles contain substrates cleaved by plant proteases for each biomedical application and pH values at the affected site. C – cysteine proteases, S – serine proteases, A – aspartic proteases, M – metalloproteases.

plants, and their purified components have been used for a sufficiently long time in the cosmetic industry, applying plant proteases in this department is rare, which is why there are so few studies on this topic.

3. FUTURE OUTLOOK

At present, papain and bromelain are the most widely used plant proteases in biomedicine, and some commercially available drugs exist. These enzymes are used in wound healing and burn debridement due to their collagenolytic activity and they are the basis of such drugs as Debridace (papain) [179], NexoBrid (bromelain) [114] and Debricin (ficin) [106]. Examples of papain-based and urea-based ointments are Debridace (Virchow Healthcare, India), Accuzyme (Healthpoint, Ltd., USA) and the composition of papain-urea-chlorophyll known as Panafil (Healthpoint, Ltd., USA). Papain is also used in oral healthcare: collagen fibrils can be partially degraded by papain gel (Papacarie and Carie Care (India)) during the procedure of chemomechanical removal of caries [180]. Wobenzym (Douglas Laboratories, USA) is a mixture of different proteases and also contains bromelain and papain. It is targeted to support healthy joint, immune and circulatory systems [181]. Chymopapain solution is used for desolation of herniated nuclear material [171]. In addition, Table **3** summarizes data on clinical trials conducted worldwide of drugs based on plant proteases (adapted from the site http://clinicaltrials.gov/).

Plant proteases participate in numerous cascades and play critical roles as signalling molecules in maintaining the organism's vital functions. It should be taken into account that some plant proteases are very specific to their target substrate. Thus, they are appropriate subjects for targeted therapy. Moreover, we might perhaps assume that certain plant proteases, as potent thrombolytic agents, could possibly be delivered to the thrombus site in a similar way to tissue-type plasminogen activator (tPA). For example, to affect only plasminogen at the thrombus site, the method based on heparin blockage and protamine release of cation-modified tPA was used [182]. Modified tPA was efficiently delivered to the thrombus by introducing a fibrin clot-specific antibody in the heparin/protamine drug delivery system (ATTEMPTS). Later, this approach was improved and developed without any

Table 3. Summarized data on clinical trials of drugs based on plant proteases according to http://clinicaltrials.gov/.

Plant Protease	Title of the Study	NCT Number	Conditions	Status
Papain	Chemomechanical Caries Removal Using Papain Gel	NCT01811420	Dental Caries	Completed
	Assessment of the Safety and Efficacy of DER-MASTREAM™ - ENZYSTREAM™ System for the Treatment of Chronic Venous Ulcers	NCT00485329	Lower Extremity Chronic Venous Ulcers	Completed
	Effective Treatments for Jellyfish Stings	NCT02015195	Jellyfish Stings	Completed
	A Comparative Study of Papacarie® and the Conventional Method for Dental Caries Treatment	NCT01641861	Dental Caries Secondary Dental Caries Personal Satisfaction	Completed
	Discomfort Evaluation During Atraumatic Restorative Treatment in Children	NCT03143387	Dental Caries Primary Teeth Post Dental Restoration	Not yet recruiting
	Adverse Event Risk Assessment in the Use of Nitrous Oxide for Pediatric Dental Patients	NCT02909816	Dental Carie; Orthodontics	Recruiting
	Efficacy and Safety of Inhaled Budesonide in Very Preterm Infants at Risk for Bronchopulmonary Dysplasia	NCT01035190	Bronchopulmonary Dysplasia	Completed
Papain, bromelain	The Safety of Four Different Dose Levels of Wobenzym in HIV-Positive Patients	NCT00002311	HIV Infections	Completed
Bromelain, comosain	Bromelain, Comosain as a New Drug for Treating and Preventing Various Types of Cancer in the Humans	NCT02340845	Cancer	Unknown status
Bromelain	Bromelain and Cardiovascular Risk Factors in Diabetes	NCT01524159	Cardiovascular Disease	Completed
	A Study to Evaluate the Efficacy and Safety of NexoBrid in Children With Thermal Burns Compared the Standard of Care	NCT02278718	Thermal Burns	Recruiting
	A Study to Evaluate the Efficacy and Safety of NexoBrid in Subjects With Thermal Burns	NCT02148705	Thermal Burns	Recruiting
	Bromelin, a Compound Prepared With Ananas Comocus Extract and Honey, for Acute Cough	NCT01356693	Cough, Acute	Unknown status
	Complementary Therapies for the Reduction of Side Effects During Chemotherapy for Breast Cancer	NCT00160901	Breast Cancer	Completed
	Feasibility Study: Enzymatic Debridement in Patients With Partial Thickness Burns	NCT00898521	Burn	Unknown status
	Effects of Dietary Supplements on Response to Air Pollution	NCT01488656	Inflammation	Completed
	Does a Nutritional Supplement Increase Vitality, Energy and Perceived Well Being?	NCT01427426	Healthy	Completed
	In Vivo Inhibition Profile of CYP2C9 by Pineapple Juice	NCT01649492	Healthy	Unknown status
EP-B2 (ALV003)	Safety and Efficacy of ALV003 for the Treatment of Celiac Disease	NCT00959114	Celiac Disease	Completed
	A Phase 1, Study of the Safety and Tolerability of ALV003 in Healthy Adult Volunteers and Subjects With Well-Controlled Celiac Disease	NCT00626184		Completed

(Table 3) contd….

Plant Protease	Title of the Study	NCT Number	Conditions	Status
	Safety and Efficacy of Varying Methods of ALV003 Administration for the Treatment of Celiac Disease	NCT01255696		Completed
	Evaluation of the Efficacy and Safety of ALV003 in Symptomatic in Celiac Disease Patients	NCT01917630		Unknown status
	Study of ALV003 in Healthy Adult Volunteers and Subjects With Well-Controlled Celiac Disease Following a Gluten-Containing Meal	NCT00669825		Completed
	Effects of Gluten Digestion With ALV003	NCT00859391		Completed
	Evaluation of Patient Reported Outcome Instruments in Celiac Disease Patients	NCT01560169		Completed

associated side effects: an enzyme is delivered to the target site in an inactive form (prodrug) and then released in an active form with the use of protamine – the so-called heparin antagonist [183]. Some of the reviewed plant proteases used for debridement of burns and wound healing, possess collagenolytic activity, and thus, could be possible antifibrotic drugs that could be delivered to fibrosis sites via immunoliposomes. This method was used for the targeted delivery of deferoxamine (DFO) – an antifibrotic drug – into the fibrosis site with the use of immunoliposomes. The single-chain Fv (scFv) antibody fragment specifically targets FAP (fibroblast activation protein) resulting in significant reduction in collagen deposition.

Nevertheless, plant-derived compounds are used for targeted delivery. For example, anisomelic acid which induces apoptosis in cervical cancer cells was delivered with the use of a folic acid-targeted system using chitosan-coated rod-shaped mesoporous silica particles (Chitosan-NR-MSP) [184]. The internalization of the particle occurs after binding to a cancerous folate receptor in the SiHa and HeLa cell lines and inducing apoptosis.

Plant-derived and animal proteins are used as coating materials for nanoparticles. However, animal proteins such as albumin, gelatin and collagen are hydrophilic, and thus they are unstable in aqueous environments, which leads to the fast release of the payload. Moreover, animal proteins may transmit diseases, for example, bovine spongiform encephalopathy [185]. In addition, recombinant animal proteins are expensive and productivity is low compared with naturally derived plant proteins [186]. The latter are more water-stable due to their higher content of hydrophobic amino acid residues and they need no external cross-linking.

Though plant extracts have been widely used since ancient times, they were replaced by enzymes of bacterial origin because of the latter's low cost and large-scale production. In recent years, plant proteases have become popular again in biotechnology and in the pharmaceutical industry. There are many plant proteases which have potential for pharmaceutical and biotechnological applications, *e.g.*, crinumin – a chymotrypsin-like serine protease from *Crinum asiaticum L.* latex, which has easy availability, a simple purification procedure, high yield, stability and activity in adverse conditions [187]. Neriifolin S is another prospective serine protease from *Euphorbia neriifolia L.* with broad substrate specificity. The enzyme is active over a wide range of pH (6.0 to 10.5) and temperature (20–60°C). Neriifolin exhibits milk-clotting activity and is resistant to autodigestion at higher concentrations [188]. Indicain is a dimeric serine protease from *Morus indica cv. K2*. It may find applications in the pharma industry due to its unique properties: the enzyme has unique antigenic determinants; it is stable with respect to pH, strong denaturants, temperature and organic solvents [189]. Another protease is wrightin from the latex of the plant *Wrightia tinctoria (Roxb.) R. Br.* The enzyme is very stable over a broad range of pH from 5.0 to 11.5, has an optimum temperature of 70°C and remains active in the presence of various denaturants, surfactants, organic solvents and metal ions [190]. Cardosins and cyprosins are well-known milk-clotting enzymes from *Cynara cardunculus* [191]. The metalloprotease cotinifolin from *Euphorbia cotinifolia L.* offers easy availability and a simple purification procedure which makes the enzyme a good system for biophysical study, and for biotechnological and industrial applications [192].

However, plant proteases have both advantages and disadvantages. On the one hand, it is not always a single enzyme, but a group of enzymes that must be isolated, for example, papain and bromelain. Thus, researchers often deal with almost the whole plant extracts resulting in broad specificity and a lack of direc-

tion in their actions. On the other hand, plant proteases are commercially acceptable for recombinant protein production for human therapeutics, vaccine antigens, industrial enzymes and nutraceuticals [193] Fig. (**3**). Moreover, they are often active over a range of temperatures and pH values. Plant proteases usually exhibit low toxicity and induce no resistance in the human body compared with, for example, synthetic anthelmintic drugs. Thus, taking all the summarized data into account, we have attempted to formulate the requirements for an "ideal" plant protease. It must be specific and directed, and the mechanism of protease action must be elucidated. At the same time, it must have low toxicity and be easily purified from a suitable source. In addition, the protease should be easily modified using bioengineering methods to improve its capacities. At present, with the use of molecular modeling approaches, novel improved three-dimensional structures can be obtained based on existing protein structures. There are many prediction algorithms which may help to improve a protein. For example, modern approaches such as EVfold and EVcouplings predict co-evolved residues interacting within the protein structure (EVfold) or between different protein molecules (EVcomplex) [194]. Predicted residues could be mutated, which could result in changes and improvements to protein properties, which could then be tested *in vivo*. Artificial evolution is another way to enhance protein capabilities: random mutations are introduced into a known three-dimensional structure of a protein-substrate complex, and these mutations are then assessed and tested experimentally in order to confirm their profitability, resulting in the emergence of a novel enzyme with unique properties [195].

CONCLUSION

The data presented in this review indicate that proteases from different plants are widely used in the treatment of diverse diseases. Some plant proteases exhibit antitumour effects and antimicrobial activities. These proteins are widely used in wound healing, burns debridement, blood coagulation and thrombolysis processes, digestion disorders and oral healthcare. Plant proteases are capable of immunological modulation. Thus, the multiple directions of action of plant proteases make them essential for modern medicine.

It is worth mentioning that the proteases described are mainly part of the family of cysteine proteases. Most of them possess unique properties and can be used under different conditions. Based on the data presented, it may be concluded that papain, bromelain and ficin are the best-developed plant proteases at the industrial level, and even though the "ideal" plant protease still does not exist, the emergence of new proteases with better properties is inevitable.

LIST OF ABBREVIATIONS

ADP	=	Adenosine diphosphate
APTT	=	Activated partial thromboplastin time
ATPS	=	Aqueous two phase system
CD	=	Celiac disease
CNS	=	Central nervous system
DFO	=	Deferoxamine
DNA	=	Deoxyribonucleic acid
FAP	=	Fibroblast activation protein
FV	=	Factor V
FX	=	Factor X
GFD	=	Gluten free diet
HLA	=	Human leukocyte antigen
IBD	=	Inflammatory bowel disease
IFN-γ	=	Interferon γ
IL	=	Interleukin
LDL	=	Low-density lipoprotein
LGP	=	Latex glycosilated protease
MMP-2, -9	=	Matrix metalloproteinase-2, -9
NK cells	=	Natural killer cells
NSAID	=	Nonsteroidal anti-inflammatory drug
PEI	=	Polyethylenimine
PT	=	Prothrombin time
TGF-β	=	Transforming growth factor β
TNF-β	=	Tumor necrosis factor β
tPA	=	Tissue type plasminogen activator
TT	=	Thrombin time
u-PA	=	Urokinase-type plasminogen activator
VPE	=	Vacuolar processing enzyme

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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