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Research Article

Plant protease inhibitors and their antiviral activities - Potent therapeutics for SARS CoV-2

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Abstract Protease inhibitors are highly active diverse family of poly(peptides) that are generally present in high concentrations in the storage tissues of the plants such as seeds and tubers. They play important roles in the regulation of proteases and the defence mechanism of plants against pathogens and display antimicrobial, antitumor and antiviral properties. Protease inhibitors have proved to be pharmacologically efficient tools in curing infections and systemic diseases via control of proteolysis. Recently, the outbreak of coronavirus (COVID-19) from Wuhan city of China has caused a global pandemic which has put the entire world on a standstill. Although the entire world has diverted all their efforts in finding an appropriate preventive and cure strategy, yet till date no success has been obtained. Since various viral diseases have been successfully cured by inhibition of viral proteases which are necessary for proteolytic processing of polyproteins, the inhibition of the proteases present on the surface of SARS-CoV-2 using protease inhibitors could prove to be fruitful in the treatment of this disease. This review gives a detail information of several natural protease inhibitors present in plants and their antiviral potential. The phytomolecules may be used for prophylaxis and effective therapeutics for the ongoing COVID-19 disease.

Keywords: Plant protease inhibitors; COVID-19; serpins; antiviral natural compounds; therapeutics

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Key highlights

- Plants are natural sources of protease inhibitors (PIs).
- In plants, PIs are known to act in the defence mechanism against pathogens.
- Plant PIs have been known to possess antiviral activities against several pathogenic viruses such as HIV, Hepatitis C virus and human cytomegalovirus (HCMV).
- Plant PIs can inhibit the main protease (Mpro or 3CL) of SARS-CoV-2 essential for processing of the polyproteins of the virus into functional proteins.
- Plant PIs also act as inhibitory molecules against

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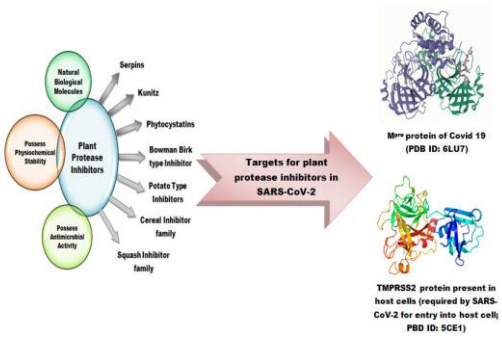
TMPRSS2, a transmembrane protein present on the host cell, required by the virus to enter into the host cell.



Graphical abstract

Introduction

Proteases (peptidases or proteinases) are one of the most important class of hydrolytic enzymes with discernible roles in several physiological and biochemical processes. Even though these enzymes are essential for maintenance and survival of their host as they are involved in signal transduction, protein-protein interaction, inflammatory response, protein catabolism, blood coagulation and digestion, their regulation is very crucial as they as they can be potentially harmful [1.2].

Protease inhibitors (PIs) are highly active compounds which are involved in important physiological reactions related to metabolism, cell physiology and regulation of proteolytic action. In a number of biological pursuits like blood clotting, apoptosis, hormone processing and inflammation, the PIs are now treated as very important signaling molecules [3]. They are widely distributed in plants and animals. In plants, PIs are present as small proteins in the storage tissues such as seeds and tubers in high concentrations and in other tissues they exist in low concentrations. Plant PIs act as storage proteins in the form of nitrogen sources, are also involved in modulation of enzymatic processes, regulation of apoptosis and defence mechanism against animals, insects and microorganisms [4]. Plant PIs possess a notable resistance to heat treatment and a high stability against alterations in ionic strength, pH, proteolysis as well as denaturing agents due of the high content of cysteine residues in disulfide bridges [5]. Several recent investigations report novel biologic activities for plant PIs such as antimicrobial activities, anticoagulant activities, antioxidant action as well as inhibition of tumor-cell growth; thus marking them potent molecules for inactivating proteases involved in several human diseases like arthritis, pancreatitis, thrombosis, emphysema, hypertension, cardiovascular morbidities, neurodegenerative diseases (such as Alzheimer's disease) and muscular dystrophy. They have been employed in several fields of biotechnology and agriculture and control of the spread of several pathogens that cause life threatening diseases like cancer, AIDS, hepatitis, malaria and various others have proved to be prevented by using plant PIs in drug design [5]. In order to be used as therapeutics in humans, the PIs should be capable of inhibiting each of the major intestinal proteases, such as pancreatic trypsin, α-chymotrypsin, as well as elastase and must be nontoxic, too. PIs are being commercially used for deterrence of protease-induced perianal dermatitis and several nontoxic PIs have been isolated and purified from barley seeds, cabbage leaves and *Streptomyces* [6].

PIs are found in plants belonging to a variety of systematic groups especially those belonging to the *Solanaceae* family harbour several high levels of PIs. [6]. In plants, PIs were first discovered as chymotrypsin and trypsin inhibitors in tomatoes infected with *Phytophthora infestans* and were correlated to plant resistance to pathogens [7]. Later serine PIs of 20-24 kDa were found in potato tubers in response to infection with *P. infestans* and mechanical wounding [8,9].

Classification of plant protease inhibitors

On the basis of primary and tertiary structure, including the number and position of disulphide bonds and active sites, PIs can be classified in four groups according to the class of proteases they inhibit: serine protease, cysteine protease, metallocarboxy-protease or aspartic protease [10]. Based on structural and biochemical properties, plant PIs have also been classified as serpins and Bowman-Birk serine (BBIs), cysteine, potato type I and type II PIs, cereal trypsin/α-amylase, mustard trypsin, squash inhibitors, metallocarboxypeptidase and soybean trypsin (Kunitz) inhibitors (Table 1). On the basis of their amino acid similarities and the structures obtained, 48 identified plant PIs have been grouped into 26 related superfamilies (or clans). According to the MEROPS database, the inhibitors have 82 family members [2]. Different classes of plant PIs exhibit different mechanisms through which they interact with the target proteases. Some of the PIs utilize an irreversible inhibition of proteolytic activity (e.g. serpins) while most of them exhibit a canonical-competitive inhibition mode via 'substrate-like' binding to the catalytic domain of the targeted protease (e.g., BBIs and Kunitz inhibitors) or they make use of a non-catalytically competitive inhibition (e.g. cystatins or mustard-type PI) else they may act via a mixed mode, where the primary competitive binding to the active site is supported by a secondary binding event [e.g. metalloprotease inhibitors; 11].

Table 1: Plant protease inhibitors of different families.

Family	Protease inhibitor	Plant source	Characteristic	Reference
Serpins	At-serpin 1	*Arabidopsis thaliana*	Acts against metacaspase *in vivo* and plays role in plant immunity	[32]
	OSZa-d	Oat (*Avena sativa* L.) grain	OSZa and OSZb are efficient inhibitors of pancreatic elastase. OSZb is an inhibitor of chymotrypsin whereas OSZc is a fast inhibitor of trypsin. Together they display a broader activity against the digestive serine proteinases than the other serpins from rye, barley or wheat.	[33]

	CmPS-1 *(Cucurbita maxima* phloem serpin-1)	*Cucurbita maxima*	Possess anti-elastase activity. May impart resistance against bacteria, insects and phytoplasma.	[18,34]
	WSZI	*Triticum aestivum*	Inhibits chymotrypsin and Cathepsin G	[11]
	HorvuZx (BSZx)	*Hordeum vulgare*	Inhibits trypsin, chymotrypsin, Factor Xa, thrombin, Factor VIIa, plasma kallikarein and leukocyte elastase.	[11]
Kunitz	Kunitz trypsin inhibitor	*Artocarpus Integrifolia* (Jackfruit)	Inhibits elastase, trypsin and chymotrypsin. However, it displays a very poor action on *Streptomyces caespitosus* and *Aspergillus oryzae* proteases	[35]
	Tamarind Kunitz inhibitor	*Tamarindus indica*	Inhibits trypsin and Factor Xa	[11]
	SKTI-3	*Glycine max*	Inhibits plasmin, human Factor XIIa, plasmin kallikrein, trypsin, chymoreypsin	[11]
	PdKI-2	*Pithecelobium dumosum* seed	Inhibits trypsin as well as papain, a cysteine protease. Active against digestive enzyme of larvae from diverse orders and hence can be used as a potent insect antifeedant	[36]
	Kunitz inhibitor CPTI	*Cicer arietinum*	Show differential inhibitory activity against trypsin, chymotrypsin, *H. armigera* gut proteases and bacterial protease(s)	[37]
	PFTI	*Plathymenia foliolosa*	Inhibits bovine trypsin and bovine chymotrypsin. Exhibits significant inhibitory activity against on larval midgut proteases of *A. kuehniella* and *D. saccharalis*	[38]

	PCP1 6.6 and PCPI 8.3	*Solanum tuberosum*	Possesses inhibitory action against cathepsin B, H and L. Also inhibits dipeptidyl peptidase I and Clostripain.	[11]
Bowman birk type inhibitors (BBI)	Soybean BBI (Isotype 2-II; 8 kDa)	*G. max*	Inhibits trypsin, chymotrypsin, mast cell chymase, cathepsin G, matriptase, leukocyte elastase, duadenase	[11]
	BTCI	*Vigna unguiculata* (black eyed pea)	Trypsin/chymotrypsin inhibitor. Moderately active against the digestive chymotrypsin of adult insects	[39]
	AsPIs	*Acacia Senegal* seeds	Highly active against serine proteases. Possess remarkable inhibitory activity towards total gut proteolytic enzymes followed by trypsin and chymotrypsin and retards the growth and development of *H. armigera*	[40]
	BI-I (seven isotypes I-VII)	*Ananas comosus*	Possesses inhibitory activity against trypsin, papain, bormelain, cathepsin L and chymotrypsin	[11]
Phytocystatin	Oryzacystatin I and II	*Oryza sativa*	Inhibits cathepsin B, Hand L and Legumain	[41,42]
	SQAPI	*Cucurbita maxima*	Inhibits pepsin proteases	[43]
	Corn cystatin-I	*Zea mays*	Inhibits Cathepsin H and L	[44]
Potato inhibitor family	CI-1	*H. vulgare*	Inhibitor of trypsin, chymotrypsin, subtilin and neutrophil elastase	[45]
	PSI- 1.1	*Capsicum annuum*	Trypsin and chymotrypsin inhibitor	[11]
	TI-II	*Solanum lycopersicum*	Inhibitor of trypsin, chymotrypsin and subtilisin	[46]
	PI-2	*S. tuberosum*	Trypsin and chymotrypsin inhibitor	[11]
Cereal inhibitor and squash inhibitor	Corn Hageman factor inhibitor	*Z. mays*	Inhibitors of serine proteases and α amylase	[47]
	RATI	*Eleusine coracana* (ragi)	Inhibitors of serine proteases and α amylase	[48]

family	BTI-CMe	*H. vulgare* (barley)	Trypsin inhibitor; Exhibits in vitro inhibition of trypsin-like proteases of the gut extracts of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae).	[18]

Serine PIs or serpins constitute a major class of plant PIs, which have been classified into more than 20 families. Serpins are mainly found in plants belonging to the *Solanaceae, Fabaceae, Euphorbiaceae, Poaceae,* and *Cucurbitaceae* families [12.13] with most of these being isolated from barley grain, wheat grain, rye, wild oats, pumpkin and *A. thaliana* [4]. In plants they are responsible for controlling protein synthesis and turnover besides physiological functions such as fertilization, growth & development, digestion, cell signaling or migration, immune defense, wound healing and disease progression. They play crucial role(s) in the pathogenesis and/or host tissue penetration of a number of diseases, such as cardiopulmonary disease and emphysema [14]. Serpins display a distinctive mechanism of irreversible inhibition termed as "suicide substrate" mechanism rather than the standard reversible inhibition mechanism followed by other PIs. They are metastable proteins with a molecular weight usually higher than 40 kDa [15].

A report demonstrated that in serine PIs, the 'reactive sites' are mutating faster than amino acids in rest of the proteins, implying that their roles in defense against microorganisms (and insects) may exert a strong selection pressure on these proteins to conserve the reactive sites and that this selection may be related to plant defense [16]. Serine PIs also called serpins inhibit both serine and cysteine proteases [17]. Although several serpins with inhibitory activity against caspases and papain like cysteine proteases have been reported but they predominantly act against trypsin like serine proteinases [18].

Another important class of PIs is the inhibitors of the cysteine proteases (cystatins or phytocustatins) which range in molecular weight from 10 kDa to 23 kDa. They inhibit cysteine proteases in a non-catalytically competent manner (i.e. although they do not bind to proteases in a strictly substrate-like manner but they still block access to the catalytic site; [11]. Cystatins regulate endogenous and heterologous cysteine proteases in a variety of physiological processes such as abiotic stress tolerance, protection against insects and nematodes via inhibition of digestive enzymes in their gut, regulation of peptidase activity during apoptosis, protection of cytosolic metabolism from intracellular peptidases released by incidental rupturing of protein bodies. They have been isolated and characterized from a number of vegetables and crop plants such as cabbage, apple, papaya, avocado, carrot, cowpea, ambrosia, castano, seeds of wheat, maize, sunflower, soybean, sugarcane, rice etc [5].

The Kunitz and BBI have been observed in the leguminous family and they generally range in size from 18-24 and

5-16 kDa, respectively. Both of them function via competitive inhibition of protease using the standard mechanism of substrate like binding to the catalytic site of the protease. Kuntiz inhibitors are known to function in the regulation of physiological homeostasis and in inhibition of pathogenic proteases while the expression of BBIs in plants is strongly induced by pathogenic invasion [11]. Other than this, a few aspartate and metalloprotease inhibitors have been reported which are isolated from potato tubers, sunflower flowers, barley and thistle (*Cynara cardunculus)*. Metalloprotease are highly compact and stable proteins in nature because of the high content of disulphide bonds in them [5].

Antiviral potential of plant PIs

According to various reports, serine PIs in plants provide protection against various pests and pathogens. In most of the pathogenic organisms like bacteria, fungi, viruses, insects and vertebrates, proteases comprise around 1-5% of the genome among which majority of the functions are performed by serine proteases [14]. The NS3 protein of Hepatitis C virus (HCV) is a chymotrypsin like protein which contains a serine protease domain that is responsible for processing of the HCV polyprotein. The Human cytomegalovirus (HCMV) contains a Ser-His-His catalytic triad and therefore is a serine protease which is essential for capsid formation during viral replication [19,20]. Therefore, serpins can be effectively utilized to attenuate such serine proteases thereby providing protection against a wide variety of pathogens. Novel antiviral strategies include targeting either host or viral accessory protein to ultimately block viral replication or inhibit cellular proteins necessary for the virus life cycle. Proteolytic cleavage of the precursor hemagglutinin (HA0) into HA1 and HA2 subunits by host proteases is essential for fusion of HA with the endosomal membrane and thus represents an essential step for viral infection [14]. The trypsin PIs from the leaf extract of *Capsicum baccatum* var. pendulum inoculated with Pepper yellow mosaic virus (PepYMV) significantly reduced the yellow mosaic viral infection [21]. The *Cucumis metuliferus* serine PIs (*CmSPI*) gene when overexpressed and silenced in *Nicotiana benthamiana* and *Cucumis metuliferus* displayed potyvirus resistance and synchronous development of potato ring spot viral symptoms, respectively [14]. The sunflower trypsin inhibitor (TI) from *Helianthus annuus* is the smallest known Bowman birk type inhibitors (BBI) which has been explored as a model peptide for drug design [22-24]. Various plant PIs displaying antiviral activity have been previously reported (Table 2).

Table 2: Prominent plant protease inhibitors with antiviral activity.

Protease inhibitor	Plant source	Antiviral activity	Reference
Capsicum baccatum trypsin protease inhibitor	*Capsicum baccatum* var. Pendulum	Reduction in the yellow mosaic virus infection of *Capsicum baccatum*	[21]
BSKTI (Kunitz trypsin protease	*G. max* cv.Dull Black seeds	Anti HIV-1 reverse transcriptase activity	[27]

inhibitor)			
BvvTI (Kunitz trypsin protease inhibitor)	*B. variegate* seeds	Anti HIV-1 reverse transcriptase activity as well as antitumor activity against human nasopharyngeal cells	[25]
KBTI (Kunitz trypsin protease inhibitor)	*G. max* seeds	Anti HIV-1 reverse transcriptase activity as well as antitumor activity against human nasopharyngeal cells, breast cancer cells and hepatoma cells	[26]
Coumarin derivatives (aspartyl protease inhibitor)	Fruits such as (bilberry, cloudberry), green tea, chicory, soy, higher plants such as Rutaceae and Umbelliferone, stem bark of *Calophyllum dispar* (Clusiaceae)	Act against aspartyl proteases of retroviruses such as HIV. Potential therapeutic for malaria, Q fever, mycoplasmosis and, nucleoplasmosis	[28,29]
CmSPI (*Cucumis metuliferus* serine protease inhibitor)	*Cucumis metuliferus*	Overexpression of the gene provides resistance to potyvirus in *Nicotiana benthamiana*; Silencing of the CmSPI gene in *Cucumis metuliferus* results in development of potato ring spot viral symptoms	[14]
Novel trypsin-chymotrypsin inhibitor	*Vicia faba* (bakla) seeds	Anti HIV-1 reverse transcriptase activity as well as antifungal activity against *Mycosphaerella arachidicola* and *Physalospora piricola*.	[30]
Chymotrypsin inhibitor	*Acacia confusa* seeds	Anti HIV-1 reverse transcriptase activity	[49]

The Kunitz trypsin inhibitors isolated from *B. variegate* and *G. max* seeds termed BvvTI and KBTI, respectively, display significant activity against the HIV-1 reverse transcriptase. They also possessed anti-tumor activity against the human nasopharyngeal cancer cells, human breast cancer cells and hepatoma cells [25,26]. Another Kunitz trypsin inhibitor, BSKT1 isolated from *G. max* cv. Dull black seeds also possessed anti HIV-1 reverse

transcriptase activity [27]. According to the invention of Domagala et al., the derivatives of coumarin which is found in fruits (bilberry and cloudberry), green tea, chicory, soy, higher plants such as Rutaceae and Umbelliferone as well as the stem bark of *Calophyllum dispar* (Clusiaceae) are inhibitors of aspartyl proteases, especially the aspartyl proteases of retroviruses such as HIV and hence can be expected to be used as an antiviral agent in the treatment of retroviral infections. They also have been found to be potential therapeutics for treatment of malaria, mycoplasmosis, Q fever and mononucleosis [28,29]. Ye and Ng in 2002 isolated a novel trypsin chymotrypsin inhibitor from *Vicia faba* (commonly known as bakla in India) seeds which displayed anti HIV-1 reverse transcriptase activity as well as antifungal activity against *Mycosphaerella arachidicola* and *Physalospora piricola* [30]. A novel, fairly stable Kunitz trypsin inhibitor of serpin family was isolated from *Allium sativum* (garlic) by Shamsi and colleagues which could act as a potential non toxic therapeutic against a number of viral diseases [31].

The COVID-19 pandemic

In December 2019, the city of Wuhan, the capital of Hubei province in China, reported the outbreak of a pulmonary disease caused by a novel strain of coronavirus and since then the virus has spread globally [50]. The spread of 2019-nCoV, now officially known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still progressing world over despite of the severe containment measures being taken [51]. The virus consists of an RNA genome which is 82% identical to the SARS coronavirus (SARS-CoV) and both viruses belong to clade b of the genus *Betacoronavirus* and hence it has been named as SARS-CoV-2 and the disease caused by SARS-CoV-2 is called COVID-19 [52, 53]. Although less is known about the origin of the virus but on the basis of the sequence of the viral genome and the evolutionary analysis, bats have been suspected as their natural hosts and it has been supposed that in humans SARS-CoV-2 might have been transmitted from bats via some unknown intermediate host [54]. Within humans, the disease is transmitted by inhalation or contact with infected droplets released by an infected person and the incubation period ranges from 2 to 14 d. The symptoms usually consist of fever, cough, sore throat, breathlessness, fatigue, malaise etc. Although the disease is mild in most people; but in some (usually the elderly and those with comorbidities), it may advance to pneumonia, acute respiratory distress syndrome (ARDS) and multi organ dysfunction. Many people are asymptomatic. The case fatality rate is estimated to range from 2 to 3%. It was listed as a potential global health emergency by WHO due to high mortality, high basic reproduction number and lack of clinically approved drugs and vaccines for COVID-19. India too has reported more than 92,700,00 of coronavirus cases along with 1,35,000 deaths all over the country till Nov. 26, 2020.

The replication cycle of the SARS-CoV-2 virus has been illustrated (Figure 1) to focus on therapeutics for efficient neutralization of virus or inhibition of some intervening virus adsorption or replication step(s). For entry into the host cell, the viral S protein binds to the host cellular receptor angiotensin converting enzyme 2 (ACE2). The binding requires the host cell surface associated trans-membrane protease serine 2 (TMPRSS) for cleavage

of the trimeric S protein [54]. After binding of the S protein and ACE2, there occurs a conformational change in the S protein which facilitates the fusion of the viral envelope with the host cell's membrane through endosomal pathway. After entry into the host cell, the virus un-coats itself and releases its RNA, which is replicated and translates into viral replicase polyproteins. The polyproteins are then processed into functional proteins by the main protease of SARS-CoV-2, M^{pro} also called as 3CL protease. The viral proteins and the genomic RNA subsequently assemble into virions in the endoplasmic reticulum and Golgi and subsequently released out of the cell [55].

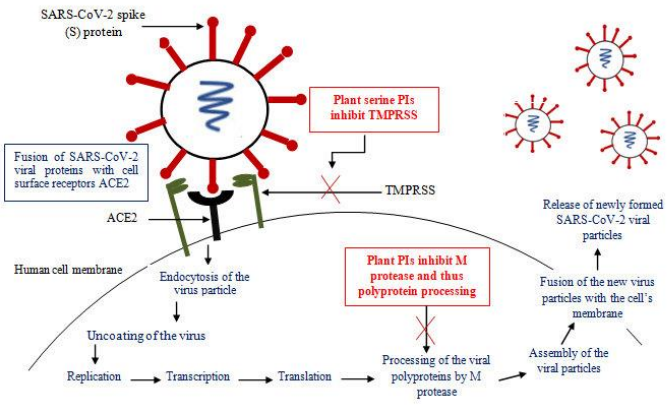


Figure 1: Schematic representation of replication cycle of the SARS-CoV-2 and the potent inhibitory effects of plant PIs on its replication in human cells.

Potent inhibitory effects of plant PIs on SARS CoV-19

Most of the nation's world-wide have been diverting their best efforts for the implementation of appropriate preventive and control strategies to deal with SARS CoV-19. Neither vaccines nor direct-acting antiviral drugs are available for the treatment of human and animal coronavirus infections [56]. The inhibition of viral proteases necessary for proteolytic processing of polyproteins has been a successful strategy in the pharmacological treatment of HIV and HCV, respectively, proving the potential of PIs for the treatment of viral infections. Similarly, the main protease of SARS-CoV-2, M^{pro} or 3CL is thought to be essential for viral replication and therefore, is regarded as promising target for plant PIs and antiviral pharmacotherapy [Figure 1; 57]. Inhibiting the activity of this enzyme would block viral replication in the infected host cells. Since no human proteases with similar cleavage specificity are known, inhibitors are unlikely to be toxic. Approved PIs including disulfiram, lopinavir and ritonavir have been reported to be active against SARS and MERS. Disulfiram, an approved drug to treat alcohol dependence, has been reported to inhibit the papain-like protease of MERS and SARS in cell cultures *in vitro*, but clinical evidence is lacking. According to the observation of Baden and colleagues, lopinavir–ritonavir combination did not seem to be highly effective in patients with COVID-19 [58] and adverse gastrointestinal effects were seen in approximately 13% of the patients [59]. Since better effective therapies for COVID-19 is the demand of the moment and plant PIs may prove to be potential therapeutic agents by inhibiting this main protease of the virus.

As described before, TMPRSS plays a major role in 2019-nCoV infection as it is the main protease which allows the fusion of the virus particles with human cells. Hence, because TMPRSS is required by the COVID-19 virus to enter into the human cells, the inhibition of this protease by non toxic plant serine PIs may prove to be

potential treatment options in 2019-nCoV infection [Figure 1; 60].

Conclusion

The review suggests that PIs are widely distributed in several plants where they play important role(s) in providing defense against pathogenic diseases. The plant PIs have been classified into different families on the basis of their structural similarity and protease inhibited. Because of their non toxic nature and fairly good stability, they have been employed in several biotechnological and pharmaceutical applications. The PIs are effective tools in inhibiting proteases associated with a number of diseases. They are also highly efficient in inhibiting viral proteases, they can be employed as a potential therapeutic in the treatment of the ongoing COVID-19 pandemic which has been declared by the WHO as a global emergency. Further docking and *in vivo* studies are required for finding the possible use of these plant PIs in the treatment of COVID-19.

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Conflict of Interest

Both the authors declare that they have no conflict of interest among themselves at their place of work or with the institution.

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