Review Paper

Plant protease inhibitors as a potential source for novel therapeutic agents: a review

ABSTRACT

Most of the currently available therapeutic agents, particularly for cardiovascular disorders and cancers are very expensive and induce some serious side effects. Some of these drugs have also become less effective due to the emergence of antibiotic resistance. There is a necessity and great demand for the development of novel efficacious plant-based agents that are of pharmacologically effective. In this connection, this review focuses on therapeutic potential of plant protease inhibitors. Protease inhibitors are of a particular concern at present due to their potent ability to inhibit protease enzymes that are involved in pathogenesis of various human diseases. In addition to their function as protein-degrading enzymes, protease inhibitors are now well-known for their capability to involve in many biological activities as signaling molecules. Plant protease inhibitors are also engaged in several physiological and pathological processes, such as blood clotting, inflammation, immune regulation, apoptosis and carcinogenesis. Therefore, isolation of plant protease inhibitors and evaluation of their therapeutic capacity against chronic human diseases have become a major research interest. Nevertheless, protease inhibitor content and protease specificity vary significantly even in the same plant species depending on the geographical location and environmental factors. Consequently, it is important to identify potent therapeutic potential of each plant protease inhibitor on human health individually.

Keywords: Plant-based drugs, Proteases inhibitors, Therapeutic capacity, Chronic human diseases

1. INTRODUCTION

Plants are naturally well-off with diverse health promoting properties including excellent nutritional and medicinal potentials and play a crucial role in human life since their existence [1]. A considerable percentage of world populations still rely entirely on plants for their nutritional and medicinal needs. Plant parts have been served as the primary raw material for synthesis of many drugs in pharmaceutical industry thus far [2]. From prehistoric times, plants and plant extracts have been used by traditional medical practitioners due to their promising curative capacities [3]. Treatment methods use in Ayurveda, Unani, Homeopathy and Allopathic systems were primarily built on plant-based medication [1].

Epidemiological studies and clinical trials have revealed that plant-based diet is also as a key factor for disease prevention. It has been identified that consumption of diets that are rich in fruits, vegetables and legumes decrease the occurrence of chronic diseases including cancer [4]. For example, incidence of cancer, particularly colon, prostate and breast cancers are significantly less among people who consume large amounts of cereals and pulses including legumes [5]. Likewise, Block [6] reported that probability of having cancers among

people who consume plant-based diets is approximately one-half when compared to the people who consume less amounts of fruits and vegetables. Tuso and Ismail [7] stated that plant-based diet also decreases the chances of having diabetes and heart diseases as well as reduces the possibilities of developing unhealthy conditions like obesity and high blood pressure. It also improves the health status of people who are suffering from diabetes, heart diseases, obesity and high blood pressure. In this context, World Health Organization has recognized medicinal plants as a reliable natural source to discover new drugs and highly recommended to explore new therapeutically effective agents from herbs to treat chronic diseases [8].

Researchers have identified that the therapeutic potential exhibited by plants are mainly due to large array of natural compounds present in them [9]. Among those natural compounds, most of the secondary metabolites are well-studied at biochemical and molecular levels and their therapeutic capabilities have well-recognized. In addition to phytoconstituents, broad spectrum of other compounds that accounts for the medicinal property of plants is identified. Among them, low molecular weight proteins including protease inhibitors (PIs) are one of the most important groups of such compounds [1].

Protease inhibitors are a group of proteins that hinder the function of proteases, the enzymes that are capable of catalyzing the breakdown of proteins via a process known as proteolysis [10]. Presence of PIs in plants is well identified since 1938 and several plants have been screened for presence of PIs [11]. As PIs occur naturally in angiosperms as well as in gymnosperms, they are one of the most abundant types of proteins found in plant kingdom [12]. PIs are small proteins with majority of them having the molecular mass of 8-20 kDa [13]. They are found plentifully in storage organs like tubers and seeds, comprising about 10 % of the overall protein content of plants [14]. However, they also occur in other parts of the plants including leaves [15]. Many types of naturally occurring plant PIs have been isolated and purified from various plants across the globe to evaluate their nutritional and medicinal properties [16].

Many researchers have recently focused on therapeutic capability of PIs present in medicinal plants. It has been identified that plant PIs are effective for inactivating target proteases that are involved in pathogenesis of various human diseases including cardiovascular disorders, cancers, HIV-AIDs, neurodegenerative diseases, inflammatory conditions, ulcerative colitis, pancreatitis and osteoporosis [1,17, 18,19]. Therefore, in future, PIs isolated from plants may add to the existing drug lists or even may replace some of the expensive compounds utilize in drug preparation in pharmaceutical industry at present [1]. As a result, screening of plants for PIs and evaluation of their therapeutic potential have become an important field of study. This review emphasizes on the therapeutic potential of plant PIs for the treatment of chronic human diseases and some of their applications in clinical trials as novel drugs.

2. FAMILIES OF PLANT PROTEASE INHIBITORS

Protease inhibitors are categorized into several groups either by the type of protease they inhibit or by their mechanism of action [20]. Major families of plant PIs include Bowman-Birk Inhibitors (BBI), Serine Protease Inhibitors, Soybean Trypsin Inhibitors (Kunitz), Cereal Trypsin/α-Amylase Inhibitors, Cysteine Protease Inhibitors, Potato Type I and Type II Inhibitors, Metallocarboxypeptidase Inhibitors, Serpin Inhibitors, Mustard Trypsin Inhibitors and Squash Inhibitors [15]. Among them, BBI and kunitz are widely distributed in legume seeds and have been isolated and extensively studied on their biophysical, physicochemical and therapeutic properties [14]. Depending on the type of proteases they inhibit, PIs are broadly grouped into four classes known as Cysteine, Serine, Metallocarboxy and Aspartic.

Among them, PIs that inhibit cysteine, serine and metallocarboxy proteases are found ubiquitously in plants, whereas PIs active against aspartic proteases have not been found in seeds [21]. All those different types of plant PIs play significant role in plants.

3. EFFECT OF PLANT PROTEASE INHIBITORS ON DISEASE CONTROL AND HUMAN HEALTH PROMOTION

Proteases are proteolytic enzymes that involved in many biological processes of living organisms including regulation of activities of other proteins, modulation of protein-protein interactions, generation of new bioactive molecules, contribution to the processing of cellular information and magnification of molecular signals, etc [22]. Hence, they engage in important biological activities of living organisms, such as cell proliferation and differentiation, tissue remodeling, DNA replication and transcription, wound healing, immunity, inflammation, blood coagulation, necrosis, apoptosis, etc [26]. Nevertheless, imbalance of proteolytic activities results in severe adverse pathological conditions including cancer, neurodegenerative disorders, cardiovascular diseases and inflammatory diseases [20]. Thus, proteases play a key role in pathogenesis of many chronic human diseases. The activity of proteases can be regulated by inhibitors and the process of proteolytic degradation that exhibit variable degrees of affinity with those proteases [24]. Therefore, PIs have been recognized as useful agents to control the progression of such human diseases by inactivating target proteases that are involved in pathogenic processes [1].

In this connection, many plant PIs are explored extensively in pharmaceutical industry for their ability to inhibit or regulate proteases that are engaged in disease progression. Despite their role as protein-degrading enzymes, currently PIs have also been recognized as essential mediators in several physiological and pathological processes, such as inflammation, blood clotting, apoptosis and endocrine activities [1]. Further, diverse plant PIs are known to be efficient against cardiovascular disorders, inflammatory conditions, osteoporosis, neurodegenerative diseases and many viral and parasitic infections [25, 1]. It is well-known that the preventive role of plant PIs against the progression of human diseases is largely depend on their bioactivities.

Previous studies have revealed that plant PIs exhibit various biological activities, such as anti-oxidant, anti-cancer, anti-inflammatory, anti-microbial, etc, suggesting the therapeutic importance of plant PIs for the treatment of chronic diseases. Trypsin PI has been recognized for its high anti-oxidant and free radical scavenging activities that could possibly be useful in preventing oxidative stress mediated diseases including cancers [26]. BBI demonstrates its anti-inflammatory activity through suppression of functions of proteases, such as trypsin, leukocyte elastase and human cathepsin G, which are released from human inflammatory cells [27, 28, 29].

Due to PIs capacity to inhibit bacterial proteases that are involved in vital physiological activities, they have also been recognized as potent anti-bacterial agents [30]. Fistulin, a PI present in the leaves of *Cassia fistula* demonstrated anti-bacterial activity against wide range of pathogenic microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Klebsiella pneumonia* [31]. Besides, trypsin inhibitor detected from *Moringa oleifera* flowers (MoFTI) demonstrated potent anti-bacterial activity against broad spectrum of both Gram-negative and Gram-positive bacteria [32]. Despite the anti-bacterial activity, Kunitz PI isolated from *Nicotiana tabacum* (NtKTI1) expressed potent anti-fungal activity against *Rhizoctonia solani* and moderate activity towards *Rhizopus nigricans* and *Phytophthora parasitica* var. *nicotianae* [33]. Further, trypsin inhibitor present in *Pseudostellaria heterophylla* also showed anti-fungal potential against *Fusarium oxysporum* [34].

Protease inhibitors have also identified as useful agents for immunosuppressive activities [35] and interestingly, they have also been evaluated comprehensively for their capacity to serve as anti-tumor agents against several human cancers. For instance, plant BBI is believed as a therapeutically important agent for cancer prevention due to its potential to inhibit cancer initiation irrevocably especially at the initial stages [36. 37]. Further, Yavelow *et al.* [38] and Fields *et al.* [37] reported that BBIs also have an ability to stop malignant transformation *in vitro*.

Importantly, several plant PIs are currently under the assessment of human clinical studies, such as blood coagulation, immune regulation, platelet aggregation and anti-carcinogenesis [39, 40]. The above evidence suggests that plant PIs possibly exhibit a significant therapeutic potential against multiple chronic diseases. Thus, PIs are a promising source for the development of new therapeutic agents. Yet, the most extensively studied plant PIs origin from Poaceae, Fabaceae and Solanaceae plant families [41]. Among the plants, potato, pineapple, squash, wheat, buckwheat, chickpea, pigeonpea, bean, barley, millet, groundnut and corn are recognized as good sources of PIs [1]. Besides, it has been identified that these naturally occurring PIs differ in protease specificity and several types of PIs might present in a single plant [1, 41, 42]. Additionally, PIs isolated from various plants showed different physicochemical properties and bioactivities. As a result, it is important to identify potent therapeutic potential of each plant PI on human health individually.

4. THERAPEUTIC IMPORTANCE OF DIFFERENT GROUPS OF PLANT PROTEASE INHIBITORS

Evaluation of plant PIs for their ability to combat against various clinical diseases has begun in 1950's [43]. Since then, researchers have isolated and purified PIs from diverse plant families and studied them for their medicinal potentials using both *in vitro* and *in vivo* techniques. Among them, BBI, serine, kunitz, trypsin and cysteine PIs have been examined extensively for their applications in biotechnology and biomedicine as novel therapeutic agents for human illnesses [42, 44]. Besides, mainly Fabaceae, Poaceae, Solanaceae, Malvaceae, Rutaceae and Moringaceae plant families have been studied comprehensively for PIs to date [41, 45]. Evidence from previous research has revealed that various types of plant PIs play a role in disease prevention and management due their potent biological activities.

4.1. BBI and BBI-type PIs

Da Costa Souza *et al.* [46] have purified a BBI-type PI from seeds of *Vigna unguiculata* and it is termed as the black-eyed pea trypsin/chymotrypsin inhibitor (BTCI). It has been identified that BTCI has an effect on the catalytic activities of MCF-7 breast cancer cells and purified 20S proteasomes in which BTCI mainly crosses the breast cancer cell membranes and restrain the proteasomes present in the cytoplasm and nucleus. As a result of the above activities, BTCI has been recognized as a PI with a potential to inhibit trypsin, chymotrypsin and caspase activities of 20S proteasomes [46].

BBI and BBI-type PIs have been isolated from some other leguminous seeds as well, particularly from *Glycine max* L. and *Cicer arietinum* L. BBIs present in both plants have exhibited high level of inhibitory activity against trypsin and chymotrypsin [19, 47]. An elevated level of trypsin is found in ovarian and colorectal tumors, and therefore inhibitory activity against trypsin makes those plant PIs to act as possible candidates for anti-cancer agents. Imbalance levels of trypsin and chymotrypsin are also responsible for pancreatitis and inhibitory activity against those proteolytic enzymes possibly can reduce the development of pancreatitis. Additionally, BBI-type PI isolated from *Cicer arietinum* L. has an

ability to considerably inhibit the progression of breast cancer (MDAMB-231) and prostate cancer (LNCaP) cells [19].

In addition to the inhibition of trypsin and chymotrypsin, BBIs present in seeds of *Glycine max* L. showed an ability to decrease proteolytic activities of other proteases, such as cathepsin G, elastase, chymase, urokinase protein activator, serine protease-dependent matrix metalloproteinases, PI3 kinase and mitogen activated protein kinase. It also mediated the up regulation of expression of connexin 43 [48]. An *in vivo* study conducted in rodents showed that BBI therapy of *Glycine max* L. has a significant suppressive activity on inflammation in anal gland and colon after subjected to tumorigenic agents [49]. BBIs are also effective against development of colorectal carcinomas with no any adverse outcomes or side effects given the fact that they can diminish the activation and rapidity of such malignancies even at very low concentrations of BBIs in the diet of dimethylhydrazine (DMH) using rat models [39]. Moreover, BBIs of *Glycine max* L. are capable of suppressing the progression of prostate cancer cells [50]. Recent studies have also revealed the potential of BBI to prevent the development of prostate tumors via its anti-proliferative activity by stimulating connexin 43 present in the gap junctions of transgenic rats [51, 52]. Further, *Glycine max* L. BBI exerts its anti-cancer effect against breast cancer due to its capacity to involve in proteasome inhibition [53].

Despite the application of BBI in animal models, it has also been used in several clinical trials to understand its therapeutic importance in human diseases. For instance, clinical trials were conducted using BBI as a treatment in patients with prostate cancer, lung cancer, ulcerative colitis, oral leukoplakia, encephalomyelitis, gingivitis and multiple sclerosis. In those human trials, BBI was given in the form of Bowman-Birk inhibitors concentrate (BBIC), an investigational novel drug prepared from a concentrated soybean protein extract with high content of BBI. Each patient was administered with varying concentrations of BBIC doses per day with the highest dosage up to 1066 chymotrypsin inhibitory units (C.I.U) [54]. None of the completed clinical trials of phase I and phase II-a reported toxic effect, antibody neutralization effect or any other adverse outcome of BBIC [55, 56]. Interestingly, BBIC has exhibited a dose-related response against oral leukoplakia and prostate cancer [56]. Similarly, Malkowicz *et al.* [57] conducted a randomized and double-blinded phase I human trial among 19 men who were with an early stage of prostate cancer. This study showed that six months treatment of BBIC decreases the amounts of prostate-specific antigen (PSA), which is clinically important indicator for the diagnosis of prostate cancer. BBI has also exhibited a potent anti-inflammatory activity without any toxicity in patients who have ulcerative colitis [18]. Even with the limited number of clinical trials completed up to date, BBI has been suggested as an excellent therapeutic agent against common chronic diseases. At present, various other PIs are also subjected to assessment in clinical trials to evaluate their therapeutic activity against chronic human diseases [1].

4.2. Serine PIs

Serine PIs are also proven as one of the prime candidates for novel therapeutics in drug discovery due to their ability to control proteolysis. In fact, serine plant PIs have been found to be effective in treating inflammatory diseases, respiratory disorders, cardiovascular diseases, HIV-AIDS, immune diseases, neurodegenerative disorders (i.e., Alzheimer disease) and cancers [58, 59]. At present, eight classes of serine plant PIs have been identified on the basis of the sequence of their amino acid chains [60] and among them, soybean-derived serine PI, PI 1 and PI 2 have been well-examined [61, 62]. Almost all of the formerly examined serine PIs were isolated from Cucurbitaceae, Euphorbiaceae, Fabaceae, Poaceae and Solanaceae plant families [59]. At present, other plant families are also being explored for serine PIs.

Bacha *et al.* [59] have isolated serine PI from the leaves of *Rhamnus frangula* (RfIP1) that belongs to the family Rhamnaceae. It has been identified that serine PI from *Rhamnus frangula* exhibited high level of inhibitory activity against some pharmacologically important proteases, such as chymotrypsin, thrombin, trypsin, cathepsin B and collagenase. The ability of RfIP1 to inhibit thrombin allows it to act as a potent anti-coagulant agent. Cathepsin B is associated with tumor invasion, and thereby the capability of RfIP1 in restraining cathepsin B exhibited its ability to act as an anti-tumor agent. Besides, cathepsin B is known as the most harmful and toxic protease in many protozoan infections including Leishmaniasis [63]. Thus, RfIP1 is also capable of playing protective role against parasitic growth and recognized as a novel therapeutic agent for Leishmaniasis [64]. RfIP1 also possessed an *in vitro* anti-bacterial activity against broad spectrum of Gram-positive and Gram-negative bacteria. Accordingly, RfIP1 can be used as a potent drug in pharmaceutical industry due to its therapeutic importance as an anti-coagulant and anti-cancer.

4.3. Kunitz and Kunitz-type inhibitors

Kunitz and kunitz-type inhibitors (KTIs) occur naturally in wide range of plants. KTIs are about 8-22 kDa proteins that contain reactive site for trypsin and two disulphide bonds [65]. Neuhof *et al.* [66] stated that KTIs detected from seeds of *Bauhinia bauhinioides* and *Bauhinia rufa* have an ability to decrease the formation of edema in perfused rabbit lungs. Wang and Ng [34] have isolated and purified a Kunitz-type trypsin inhibitor with a molecular mass of 20.5 kDa from roots of *Pseudostellaria heterophylla* and it has expressed anti-fungal and trypsin inhibitory activities comparable to soybean trypsin inhibitor. Additionally, KTIs are also capable of preventing cell invasiveness via reducing extracellular signal transduction in human ovarian cancer cells [67, 68].

Nakahata *et al.* [69] have screened *Enterolobium contortisiliquum*, which belongs to the family Fabaceae for PIs. From *Enterolobium contortisiliquum* seeds, they have purified a trypsin inhibitor (EcTI) of molecular mass of 20 kDa and it has been recognized as a KTI. EcTI has demonstrated an inhibitory activity for clinically important proteases, such as trypsin, chymotrypsin, plasmin and plasma kallikrein. It has also demonstrated an inhibitory activity against gastric cancer cells via controlling integrin-dependent signal transduction pathways [70]. Importantly, it is capable of inhibiting human cancer cell lines namely breast (SkBr-3 and MCF-7), leukemia (K562 and THP-1) and colorectal (HCT116 and HT29), in addition to its effect on human mesenchymal stem cells (hMSCs) and primary fibroblasts. Furthermore, EcTI is identified as a cytotoxic agent against cancer cells, which is not harmful for normal body tissue [69].

4.4. Cysteine PIs

Like most of the other PIs, cysteine PIs are also widespread in plant kingdom. They occur in seeds of several monocotyledonous including rice (*oryzacystatin*) and maize as well as in dicotyledonous plants, such as apple fruit [71, 72, 73, 74, 75, 76]. Researchers have identified cysteine PIs as an important tool for the utilization in pharmaceutical industry due to their potent health benefits. Obayomi *et al.* [77] purified cysteine PI from *Tetracarpidium conophorum* which is competitively inhibit papain, a proteolytic enzyme that aids in digestion. The biological activities and physicochemical properties illustrated by cysteine PI suggest it as an effective therapeutic agent for the treatment of clinical conditions, such as cancer, neurodegenerative diseases and atherosclerosis.

4.5. Novel plant PIs

In addition to known plant PIs, researches have also focused on purifying novel PIs from plants. For example, CGPIs is one of such PIs extracted from *Coccinia grandis* leaves with a molecular mass of 14.3 kDa [78]. CGPIs have exhibited a significant inhibitory effect for chymotrypsin and bovine pancreatic trypsin. The CGPIs demonstrated a dose-dependent inhibitory activity against the progression of colon cancer. Further, CGPIs also capable of hindering sporulation and mycelial growth in infective microorganisms including both bacteria and fungus, such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Proteus vulgaris*, *Aspergillus flavus*, *Mucorindicus*, *Cryptococcus neoformans*, *Candid aalbicans* and *Pencilliumnotatum* [78].

Four different types of new PIs were purified from the seeds of *Lavatera cashmeriana* and they were termed as LC-pi I, II, III and IV. According to their molecular size, these four types of PIs have been identified as KTIs [79]. These PIs were found to be involving in inhibiting trypsin, chymotrypsin and elastase. All of them have exhibited *in vitro* anti-cancer effect on colon (HCT-116), leukemia (THP-1) and lung (NCIH322) cancer cell lines [79, 80, 81]. Besides, LC-pi I has also showed potent inhibitory activity against prostate and breast cancer cell lines due to its capacity to hinder elastase, trypsin and chymotrypsin [81]. Further, LC-pi I has demonstrated a significant anti-bacterial activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [80].

Protease inhibitors isolated from mature leaves and seeds of *Moringa oleifera* showed inhibitory activity against proteases that are of pathological importance, such as trypsin, chymotrypsin, elastate, thrombin, cathepsin B, papain and cysteine. They have also demonstrated complete inhibitory activity against proteases isolated from *Bacillus licheniformis* and *Aspergillus oryzae* [45]. Thereby, *Moringa oleifere* PI controls a wide range of protease mediated activities including fibrinolysis, tissue remodeling, aging and development of cancer [24, 58, 82]. The affinity of *Moringa oleifere* PI towards thrombin expressed its potential to act as an anti-coagulant. Further, trypsin inhibitor detected from *Moringa oleifera* flowers (MoFTI) demonstrated potent anti-bacterial activity against broad spectrum of Gram-negative bacteria including, *Escherichia coli*, *Proteus mirabilis* and *Salmonella enteritidis*, and Gram-positive bacteria, such as *Bacillus subtilis*, *Enterococcus faecalis* and *Staphylococcus aureus* [32]. Bijina *et al.* [45] also reported the possible use of PIs isolated from *Moringa oleifera* in sea food preservation. As a result, *Moringa oleifera* is a promising source for the development of useful drugs in pharmaceutical industry.

Some researchers have studied on anti-HIV effect of PIs from plant sources using pepsin as a substitute for HIV protease. In this context, a potent anti HIV-1 protease activity of *Adhatodavasica* was detected using pepsin [83]. Besides, water extracts of *Sesbania grandiflora* leaves and flowers have also demonstrated mild anti-HIV activity [84]. Another study also stated the inhibitory potential of PIs isolated from *Terminalia arjuna* (fruit and bark), *Terminalia chebula* (arial parts and fruit) and *Terminalia horrida* (resin) of Combretaceae family against HIV-1 protease [85].

A study conducted in Sri Lanka on five medicinal plants, such as *Mangifera zeylanica*, *Sesbania grandiflora*, *Terminalia bellerica*, *Terminalia catappa* and *Terminalia chebula* revealed the presence of therapeutically important PIs. In this study, pepsin and trypsin were used as model enzymes to examine aspartic and serine protease inhibitory activities, respectively. Among the tested extracts, the highest inhibitory activity against pepsin and trypsin was detected from the water extracts of the bark of *Terminalia catappa* and *Sesbania grandiflora*. Additionally, inhibitory activities exhibited by those PIs remained stable with varying temperature [86].

5. CONCLUSION AND FUTURE RECOMMENDATIONS

Plant PIs are recognized as very effective curative agents for various human diseases because of their potent ability to inhibit proteases that are involved in the process of pathogenesis. In addition, therapeutically important plant PIs can also be provided naturally through routine diet containing legumes, soybean, rice, potato, etc. Depending on the geographical location and environmental factors, PI content and activity may differ even in the same plant species. Therefore, there is a necessity to conduct further studies to identify possible plant sources for therapeutically important PIs in various geographical locations and also important to characterize isolated PIs and assess their therapeutic applications individually. Investigation of PIs from diverse plant families may lead to discover novel PIs with different pharmacological and therapeutic importance. Identification and scientifically validation of the therapeutic effect of plant PIs could also be useful for people to identify plants that are rich in nutritional and medicinal properties.

CONSENT

Not applicable

ETHICAL APPROVAL

Not applicable

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