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Research Article

EVALUATION OF PHENOL CONTENT, ANTIOXIDANT, AND PROTEINASE INHIBITORY ACTIVITY OF PLANT DERIVED PROTEASE INHIBITORS OF EIGHT ANTI-DIABETIC PLANTS

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ABSTRACT

Objective: The objective of this study was to partially purified from the eight known anti-diabetic plants and evaluated for the total phenol, protease inhibitory (PI) activity, and antioxidant activity.

Methods: PI activity was performed using casein as substrate. The total phenolic content was estimated by folin-ciocatechu reagent method. The antioxidant activity was estimated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) model and hydrogen peroxide scavenging activity.

Results: The results shown that *Syzygium cumini* has the highest total phenol and protein content. *Solanum tuberosum* has the highest percentage PI activity. In DPPH model of antioxidant in SC has the highest inhibitory activity, whereas in the hydrogen peroxide scavenging activity *Momardica charantia* has the maximum inhibition.

Conclusion: These data once again prove that phenolic compounds have statistically significant antioxidant activity. Among all the PIs ST has maximum inhibition activity. To conclude, all these PIs can be an excellent source of antioxidants.

Keywords: Antioxidant, Plant protease inhibitor, Phenolic, Hydrogen peroxide, Protein content.

INTRODUCTION

Protease inhibitors (PIs) are common in the plant kingdom. Plant PIs (PPIs) is generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants [1]. They are also induced in plants in response to injury or attack by insects or pathogens. In plants, these PIs acts as anti-metabolic proteins, which interfere with the digestive process of insects [2,3]. PIs were classified into serine, cysteine, aspartate, and metallocarboxy PIs. PIs that are active against all the mechanistic classes of proteases have also been described in plants [1]. PIs act by forming to form stable complexes with target proteases, blocking, altering, or preventing access to the enzyme active site. PPIs also interfere with the growth and development of many phytophagous insects [1]. This PPI was also proved to be beneficial in the treatment of pancreatitis, shock, allergy. It has proven that intake of this PPI has decreased the incidence of breast, colon and prostatic cancers intake of this PPI have decreased the incidence of cancer [4]. This PPI is very active in various cellular responses such as cell signaling, digestion, differentiation, and apotopsis [5,6].

Phenols are the organic compounds with multiple -OH groups in their molecular structure [7,8]. Polyphenols are capable of suppressing the cholesterol, blood pressure levels, and incidence of pathogens. Its free radical scavenging activity has proven to have anti-mutagenic and also in proving various lifestyle diseases such as ulcerative collitus, colorectal cancer, diabetic complications, and anti-atherogenic [9].

New compounds or natural extracts possessing antioxidant activity can be used as sources of different valuable agents. These antioxidants play an important role protecting the tissues from oxidative stress. Imbalance in the oxidatant and antioxidatant levels will certainly lead to many chronic diseases such as diabetes mellitus, hepatoprotective, cardiovascular diseases, and many other. There are many studies stating that pheolic compounds posses antioxidant acitivity. This study mainly focuses on identification of novel antioxidants from plant source and giving a new source for protein derivatives for oxidative stress diseases.

Eight plants were selected (*Allium sativum* [AS], *Zinziber officinalis* [ZO], *Solanum tuberosum* [ST], *Glycine max* [GM], *Syzigium cumini* [SC], *Momardica charantia* [MC], *Solanum lycopersicum* [SL], and *Curcuma longa* [CL]) for screening the antioxidant activity. Phenolic content and antioxidant activities have been already discussed for these plants. However, there has been no study comparative investigation of total phenol, antioxidant, and PI activity in partially purified PPIs that will lead to evaluate their use as natural antioxidants.

METHODS

All the eight plants were collected from the local fields of Belagavi, Karnataka. The plants were authenticated by the Dr. Harsha Hegde, Scientist-C, RMRC (ICMR), Belagavi and voucher number was collected. The seeds of GM, SC, MC, SL and tubers of ST and rhizomes of CL, ZO and bulb of AS were collected in vinyl bags, washed with the water and dried in the room temperature for 1 day. Tannic acid (Himedia), casein (Himedia), 1,1-diphenyl-2-picrylhydrazil (DPPH), Hydrogen Peroxide (H_2O_2) (S D fine chemicals) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other chemicals used were of 99% or greater purity.

Partial purification of PPI

About 25 g of the collected plants were homogenized in the 100 ml of 0.1 M phosphate buffer (pH7.0). The solution was then filtered through the 4 layered cheese cloth. Then, the solution was then centrifuged (Kubato cooling centrifuge) at 10,000 rpm for 15 minutes at 4°C. The supernatants were collected and fractioned by ammonium sulfate (30%, 60%, 90%) and dialyzed by the cellulose membrane (12-14 kDa, Himedia) for 24 hrs at 4°C. The dialyzed material was then lyophillized and stored in –80°C until further use. All the procedures were performed 4°C to minimize the enzymatic loss.

Proteolysis assay

In this method, the trichloroacetic acid (TCA) soluble fractions formed by action of trypsin on the protein substrate casein were measured by the change in absorbance at 750 nm. The residual caseinolytic activity of the trypsin in the presence of inhibitor, at 37°C was used as a

measure of inhibitory activity, Appropriate blanks for enzyme, inhibitor, and substrate were also included in the assay along with the test [10].

Proteolysis method is modified of (Bijina *et al*., 2011) 100 µL of trypsin (0.5 mg/ml) was preincubated with 100 µL of suitable dilution of PI at 37°C for 15 minutes. To the above mixture 200 µL of 1% casein was added and incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 250 µL of 0.44 M TCA solution. The reaction mixture was transferred to centrifuge tube, and the precipitated protein was removed by centrifugation, at 10,000 rpm for 15 minutes (Sigma, Germany). The 100 µL of the supernatant was diluted with 500 µL of sodium carbonate (10%) and 100 µL of Folin–Ciocaltechu reagent (FCR) (50%), the absorbance was read at 750 nm in 96 well plate ELISA plate reader UV-visible spectrophotometer (Thermo Scientific) against appropriate blanks.

The TCA soluble peptide fractions of casein formed by the action of trypsin in the presence and absence of inhibitor was quantified by comparing with tyrosine as standard. One unit of trypsin activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions. One unit of inhibitory activity was defined as the decrease by one unit of absorbance of TCA soluble casein hydrolysis product liberated by the trypsin action at 750 nm per minute at 37°C in the given assay volume. The PI activity was expressed in terms of percent inhibition and it was calculated as:

Inhibitory activity (%) = (A–B)/A × 100

(A = Amount of tyrosine released without inhibitor; B = Amount of tyrosine released with inhibitor)

The specific activity, residual inhibitory activity and units/ml were also calculated for the all the 8 plants.

Analysis of the phenol content

The phenol content was measured using FCR a modified method of [12] in 96 well plates. A 0.1 mL extract (2-10% w/v) was mixed with 0.1 ml FCR, and 0.3 mL of 7.5% Na_2CO_3 was added to the mixture and vortexed. The mixture was then incubated for 30 minutes in the dark. The blue color absorbance of the reaction was observed at 685 nm. The tannic acid (0.5-3 µg/ml) was used as standard. The results were triplicated.

DPPH scavenging activity

DPPH assay is a most popular model for screening antioxidant activity. DPPH is characterized as the stable free radical by virtue of the delocalization of spare electrons over the molecule as the whole so that the molecule does not dimerize, as would be the case most other free radicals. The delocalization of the electron also gives rise deep violet color, observed at 517 nm using UV-visible spectrophotometer (Thermo Scientific Elisa plate reader). 0.1 mM DPPH solution was prepared by dissolving in 80% methanol [13].

This is the modified method of Lu and Foo [14]. The 1 mg/ml of stock solutions of PPI were prepared in alcohol. Different concentrations (10-40 µg/ml) were diluted with Tris HCl and add100 µL of the DPPH is added in 96 well plates. The mixed solution was left to stand in a dark place at room temperature for 30 minutes, after which the absorbance was measured at 517 nm. Experiments were triplicated, and the results were expressed as the % radical scavenging activity,

DPPH free radical scavenging capacity (%) = (A0–A1)/A0 × 100

(A0 = Control group's absorbance, A1 = Experiment group's absorbance).

Hydrogen peroxide scavenging (H_2O_2) assay

Evaluation of hydrogen peroxide scavenging activity is important because the human being exposed to H_2O_2 indirectly via environment (0.28 mg/kg/day). It is decomposed into water and oxygen after entering into the body; this may lead to production of hydroxyl radical (OH*) further initiate lipid peroxidation and cause DNA damage. 40 mM of hydrogen peroxide solution prepared in phosphate buffer (50 mM, pH7.4). Extracts (20-60 µg/ml) were dissolved in distilled water and added to hydrogen peroxide and incubated for 10 minutes. The abservances were read at 230 nm in UV-visible ELISA plate reader [15].

Percentage of H_2O_2 scavenging assay (%) = (A0–A1)/A0 × 100

(A0 = Control group's absorbance, A1 = Experiment group's absorbance).

Statistical analysis

Statistical analysis was performed using graph pad prism. The data were expressed as the mean value±standard error of the mean.

RESULTS

PPI is widely distributed in the plant kingdom and highly concentrated in the tubers, seeds and aerial parts. They are well studied in the families such as Graminacea, Solanaceae, and Cucurbitaceae. Eight plants were chosen for the partial purification PPI, it was shown that 60-90% ammonium sulfate saturation produced highest percentage yield in SC (22.13±3.20), GM (13.75±2.40) and SL (11.35±2.61), whereas the lowest percentage yield was observed in CL (2.16±0.05) and AS (6.57±1.42). The data illustrated in Table 1.

The PI activity is estimated by the ability of PI in digestion of casein by trypsin. Among all the purified compounds ST (83.77%) has the highest inhibitory activity followed by the ZO (73.22%) and the least inhibitory activity in the MC (24%).

The content of phenolic compounds was calculated as mg/ml of tannin standard. The amount of total phenols in the PPI of the above plants varied from (3.67 to 0.66 mg/ml). The phenolic content in the PPI are SC>GM>SL>MC>AS>ST>ZO>CL. The data is shown in Table 1.

Free radical scavenging is the accepted mechanism for antioxidant to inhibit lipid peroxidation. DPPH can be used to evaluate the antioxidant activity of many compounds in a very short time. The Table 2, most of the PPI's (40 µg/ml) exhibited concentration (10-40 µg/ml) dependent inhibition activity. Among them, MC (79.76%), SC (78.4%) and SL (77.4%) showed similar inhibitory activities. The lowest inhibitory activity was shown in AS (22.4%), followed by CL (24.4%). Data is illustrated in Table 2 and graphically in Fig. 1.

It is observed that all the PPIs are capable of scavenging hydrogen peroxide in the concentration dependent (20-60 µg/ml). Most of the PPI expressed more than 60% inhibition except the CL and ST. The percentage of scavenging hydrogen peroxide with 60 µg/ml follows MC (79.42%) and SC (79.17%) lowest is observed in the CL (21.12%) and ST (33.51%). The scavenging ability of various PPI with hydrogen peroxide is given in Table 3 and graphically in Fig. 2.

DISCUSSION

The human body is a complex system which counteracts the harmful effects of the reactive oxygen species (ROS) generated.

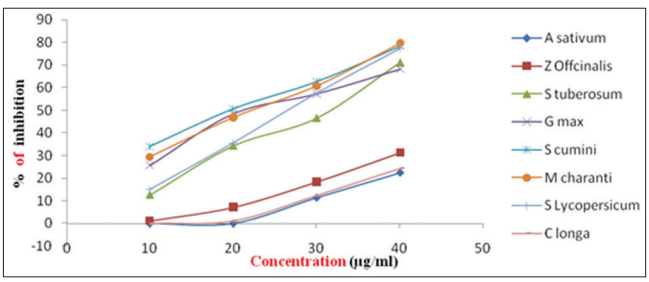

Fig. 1: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of of different concentrations (10-40 µg/ml) of eight anti-diabetic plants derived protease inhibitors in the phosphate buffer extract. Values are expressed as percentage inhibition (n=3)

Table 1: Protein estimation, percentage inhibitory activity and total phenolic content of the eight anti-diabetic plant derived protease inhibitors

S. No	Plants	Protein estimation (mg/ml)	% inhibitory activity	Specific activity (%/mg)	Total phenol content (mg/ml)	% yield
1	AS	6.57±1.42	65.44	9.96	1.09±0.67	1.24
2	ZO	7.38±2.12	73.22	9.92	0.85±0.55	10.74
3	ST	3.9±1.40	83.77	21.48	0.96±0.48	1.09
4	GM	13.75±2.40	26.33	1.915	2.94±0.10	32.41
5	SC	22.13±3.20	30.88	1.395	3.67±0.24	35.78
6	MC	9.53±1.55	24.00	2.51	1.86±0.37	20.42
7	SL	11.35±2.61	27.88	2.45	2.10±0.65	14.39
8	CL	2.16±0.05	52.11	24.12	0.66±0.41	2.45

Each value is the mean±standard deviation of three replicate analyses (n=3), AS: *Allium sativum*, ZO: *Zinziber officinalis*, ST: *Solanum tuberosum*, GM: *Glycine max*, SC: *Syzigium cumini*, MC: *Momardica charantia*, SL: *Solanum lycopersicum*, CL: *Curcuma longa*

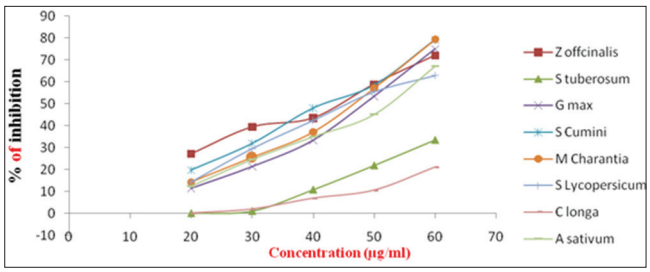

Fig. 2: Hydrogen peroxide scavenging activity of different concentrations (20-60 µg/ml) of eight anti-diabetic plants derived protease inhibitors in the phosphate buffer extract. Values are expressed as percentage inhibition (n=3)

Enzymatic and non-enzymatic antioxidants help in removal of the ROS. Antioxidants have a very important role in the maintaining oxidative stress. These generated free radicals may further lead to cause the large number of the diseases such as diabetes mellitus, Alzheimer disease [16], cancer [17], cardiovascular complications [18], neural disorders [19], cognitive impairment [20], and gastric ulcers [21]. By dietary intake of the antioxidants may give a protection from the free radicals. It was already proved that dietary intake of antioxidants may protect and postpone from the degenerative disorders. Since these dietary supplements are having substantial amounts of antioxidant activity may replace the synthetic compounds. Plants are reported to have large quantities of the polyphenolic content [22]. These poly phenolic compounds have been substantially explored in the food industry, for their activity in delaying the lipid oxidation as well as the boosting the quality of life with food and nutritional values [23].

PIs have already been in the market for treatment of various complications such as HIV, cancer, and diabetes mellitus. [24] We have made a unique approach by isolating the PIs from the plants that are already proven to be anti-diabetic. These chosen anti-diabetic plants are regular food supplements. Hence, we made an attempt to partially purify PPI and screen for the antioxidant activity, PI and estimation of total phenolic content. Phenols are chemical compounds with (-OH) groups in their structures. They are already proven to posses antioxidant activity because of the hydroxyl groups. Plants are the rich source of phenols in the storage tissues [13,25].

PIs are distributed in all the plant kingdom among them serine PIs are well studied class of inhibitors. Most of them are characterized from the Gramineae, Leguminosae and Solanaceae. They are generally 4-85 kDa with the majority in the range of 8to 20 kDa [26]. They have a high content of cysteine residues that form the sulfide bridges and confer resistance to heat, pH and proteolysis. Most of the PIs are synthesized in response to stress conditions like pathogens, insects wounding and environmental stress like salt. The main function of PPI's is thought to

Table 2: Scavenging activity of Plant protease inhibitors on DPPH

Plant name	Concentrations(µg/ml)	% inhibition
A. sativum	10	-
	20	-
	30	11.4±2.3
	40	22.4±1.5
Z. officinalis	10	1.1±3.6
	20	7.1±2.7
	30	18.3±1.9
	40	31.2±2.3
S. tuberosum	10	12.6±1.8
	20	34.3±4.3
	30	46.5±5.3
	40	41.0±3.7
G. max	10	25.7±2.4
	20	48.4±3.7
	30	57.2±4.9
	40	68.1±3.1
S. cumini	10	34.0±5.1
	20	50.7±3.3
	30	62.7±4.8
	40	78.4±3.4
M. charntia	10	29.4±2.2
	20	46.8±3.1
	30	60.8±3.8
	40	79.7±2.1
S. lycopersicum	10	15.0±2.4
	20	35.6±3.2
	30	57.6±1.2
	40	77.4±4.5
C. longa	10	-
	20	1.1±3.8
	30	12.4±2.7
	40	24.4±3.6

Values are expressed in mean+SD for the triplicate samples. *A. sativum: Allium sativum, Z. officinalis: Zinziber officinalis, S. tuberosum: Solanum tuberosum, G. max: Glycine max, S. cumini: Syzigium cumini, M. charntia: Momardica charantia, S. lycopersicum: Solanum lycopersicum, C. longa: Curcuma longa*

be defense and regulation of endogenous proteins [27,28]. The activity of PPI is mainly due to the formation of stable complex with the target proteases, blocking, altering or preventing the access to the enzyme site [29].

In this study, PPI was partially purified by ammonium sulfate precipitation (0%, 30%, 60% and 90%) and estimated for the proteolysis assay using casein as substrate, phenolic content (FCR method), and antioxidant activity (DPPH and H_2O_2). The percentage yield was higher in the GM and SC and lowest in the AS and CL. Among them, the ST species have the highest proteolytic activity, lowest in the MC. Phenolic content and protein content was higher in the SC and lesser in the CL and ST, respectively.

Table 3: Scavenging activity of Plant protease inhibitors on hydrogen peroxide

Plant name	Concentrations (µg/ml)	% inhibition
A. sativum	20	12.3±1.2
	30	24.5±3.2
	40	34.9±4.4
	50	45.0±2.5
	60	67.0±3.8
Z. officinalis	20	27.2±1.5
	30	39.4±3.9
	40	43.5±2.4
	50	58.9±3.6
	60	72.0±4.1
S. tuberosum	20	-
	30	1.0±1.8
	40	10.8±2.9
	50	21.8±2.4
	60	33.5±3.1
G. max	20	11.4±4.4
	30	21.4±3.8
	40	33.3±3.9
	50	53.3±4.1
	60	74.9±2.5
S. cumini	20	19.8±1.3
	30	31.9±2.8
	40	48.0±3.2
	50	58.4±4.6
	60	79.1±3.7
M. charntia	20	14.2±2.4
	30	25.4±3.6
	40	37.0±4.2
	50	57.1±2.9
	60	79.4±3.8
S. lycopersicum	20	14.3±3.2
	30	29.5±1.4
	40	42.3±2.4
	50	55.3±3.3
	60	62.7±3.2
C. longa	20	-
	30	1.8±2.4
	40	6.8±3.5
	50	10.5±2.9
	60	21.1±4.2

Values are expressed in mean+SD for the triplicate samples, *A. sativum: Allium sativum, Z. officinalis: Zinziber officinalis, S. tuberosum: Solanum tuberosum, G. max: Glycine max, S. cumini: Syzigium cumini, M. charntia: Momardica charantia, S. lycopersicum: Solanum lycopersicum, C. longa: Curcuma longa*

The phenolic compounds are known to act as antioxidants by reducing oxidative damage [30]. They act by trapping free radicals directly or by scavenging them through a series of reactions [31]. Phenols play an important role in stabilizing lipid peroxidation by their antioxidant activity [32]. The redox properties play an important role in adsorbing and neutralizing free radicals quenching singlet and triplet oxygen or decomposing peroxides [23]. In the DPPH model, the highest inhibitory activity was observed in the seeds of MC followed by the SC, the lowest activity was CL. These SC results were already been fruit extracts of the same plant. The SC plant contains higher levels of phenols, and was showing good antioxidant activity [33]. MC is also well known for anti-diabetic activity as well as antioxidant activity because of the presence of greater amount of phenols [34].

In the H_2O_2 model, MC and SC shown similar results, whereas the lowest inhibitory activity is shown by the CL and then followed by the ST. The MC and SC have higher levels of the pheolic content. These phenolic compounds are known to have good antioxidant activity [33,34]. These data once again prove that phenolic content and antioxidant activity are correlated. These findings suggest that PPI can also be used as nutritional supplements as well as the antioxidant activity, similar results were observed in many plants [35].

CONCLUSION

We made an attempt at finding a new source of proteins with antioxidant activity. First, proteins were partially purified from the eight different sources of plants in the Belagavi district and analyzed for phenol content, antioxidant activity and proteinase inhibitory activities of these plants. Considering these results, PPI may serve as a new antioxidant activity moieties. Further studies may be carried out in elucidating the mechanism of actions of these PPI as new mechanism for the antioxidants.

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