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ORIGINAL ARTICLE

In vitro Antioxidant and Antidiabetic Activity of Oligopeptides Derived from Different Mulberry (*Morus alba* L.) Cultivars

Suchisree Jha, Saran Kumar Gupta, Phalguni Bhattacharyya[1], Amitava Ghosh[2], Palash Mandal

Department of Botany, University of North Bengal, Siliguri, [1]Department of Botany, Malda College, University of Gour Banga, Malda, [2]Department of Botany, Asutosh College, University of Calcutta, Kolkata, West Bengal, India

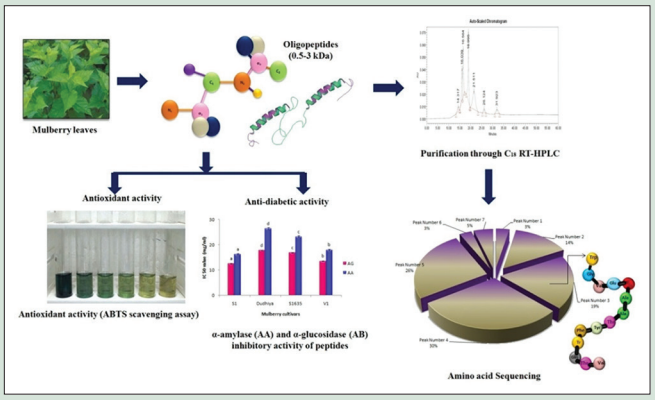
ABSTRACT

Background: Several plant peptides have been known to exhibit various pharmacological activities. However, the bioactivity of mulberry peptides is not yet reported. **Objective:** In the present study, the peptides from different mulberry cultivars, namely S1, V1, S1635, and Dudhiya, were isolated and assessed for their *in vitro* free radical scavenging capacity and inhibitory activity of two key enzymes (α-amylase [AA] and α-glucosidase [AG]) associated with type-II diabetes. **Materials and Methods:** The 0.5–3 kDa ranged oligopeptides were isolated using cation exchange chromatography and ultrafiltration system and further characterized by high-performance liquid chromatography and protein sequencer. The peptides were investigated for their *in vitro* antioxidant potential by 1,1-diphenyl-2 picrylhydrazyl, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and nitric oxide scavenging capacity and reducing power, metal chelating, and anti-lipid peroxidation activity along with *in vitro* antidiabetic activity by AA and AG inhibition. **Results:** The results revealed that the peptides were found to possess significant free radical scavenging as well as AA and AG inhibitory activity in a dose-dependent manner. Overall, the peptide isolated from S1 cultivar exhibited the most promising therapeutic potential. Further, the variation in amino acid composition of the oligopeptides could be associated with the observed variation in their bioactivity. **Conclusion:** These natural peptides may constitute an important part of the antioxidant defense system as well as antidiabetic agent and further could be used for the formulation of functional food and nutraceuticals.

Key words: Antidiabetic, antioxidant, high-performance liquid chromatography, mulberry, oligopeptide, ultrafiltration

SUMMARY

- The 0.5–3 kDa ranged oligopeptides were isolated from mulberry leaves by cation exchange chromatography and ultrafiltration system and further characterized by high-performance liquid chromatography and protein sequencer. The peptides were evaluated for their *in vitro* antioxidant and antidiabetic activity. The peptides were found to exhibit potential free radical scavenging as well as α-amylase and α-glucosidase inhibitory activity. The variation in amino acid composition of the oligopeptides could be associated with the observed variation in their bioactivity.



Abbreviation Used: HPLC: High-performance liquid chromatography, PMSF: Phenyl methyl sulfonyl fluoride; IAA: Indoleacetic acid, ABA: Abscisic acid, GA3: Gibberellic acid, DPPH: 1,1-diphenyl-2 picrylhydrazyl, ABTS: 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, AG: α-glucosidase, AA: α-amylase, DNSA: 3,5-Dinitrosalicylic acid, AAE: Ascorbic acid equivalent, TBARS: Thiobarbituric acid-reactive species.

Correspondence:
Dr. Palash Mandal,
Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory,
University of North Bengal, Siliguri - 734 013,
West Bengal, India.
E-mail: nbubotanypm@gmail.com

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INTRODUCTION

Since ancient times, human beings have been dependent on the plants for their existence. Before civilization, plant served human as a source of food and shelter, but their requirement grew parallely with the advancement of civilization. On the other hand, the global prevalence of many diseases, including diabetes, has risen gradually due to improper lifestyle, unhealthy food, and stressful daily life. Oxidative stress is one of the major factors for generating diabetes. Diabetes mellitus is a metabolic chronic disease in which there are high blood sugar levels. Diabetes occurs when either sufficient insulin hormone has not produced in the body (Type I) or body cannot react to insulin in proper way (Type II).[1] Therefore, two things are important for maintaining diabetes; first, to sustain normal blood glucose levels, and second, to reduce oxidative stress in the cellular system. Oxidative stress generates reactive oxygen species (ROS) which is not only involved in induction of diabetes but also creates complexity in long-term diabetic treatment.[2] Antioxidant components are capable to scavenge the free radicals and help in the prevention of diabetes.[3] Mulberry plant is used as traditional medicine in several countries including India, and modern science brings a lot of information regarding bioactive compounds present in mulberry and their effects on human and animal system.[4] The antioxidant property of mulberry leaves and their effect on glucose metabolism along with α-glucosidase (AG) inhibitor activity have been studied by previous authors.[5]

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Unfortunately, the synthetic antioxidant and antidiabetic pharmaceutical drugs have been negatively perceived by nutritionists and researchers due to their side effects coupled with expensive cost.[6] Hence, the search for natural and affordable bioactive compounds which can improve insulin action and lower blood glucose levels becomes pertinent. Consequently, studies on the beneficial health benefits of plants have been significantly increased the interest of researchers on understanding the preventive and protective actions of plants against prevailing chronic diseases.[7] In recent times, several naturally occurring bioactive plant peptides have been isolated, and they are also found to be involved in the regulation of different physiological processes in plants. Several authors have claimed the potential antioxidant property of peptides isolated from various plants, such as buckwheat, alfalfa leaves, wheat, and mung bean, antidiabetic property, and hypotensive activity.[7-11]

However, numerous works have been done on different molecular weight peptides of various plants' source reporting their physiological and biochemical roles; there still exists a dearth about the antioxidant and antidiabetic potential of oligopeptides which is yet to be revealed. Considering the aforementioned facts, the present study is an attempt for isolation and high-performance liquid chromatography (HPLC) and protein sequencer-based characterization of oligopeptides from different mulberry cultivars and assessment of *in vitro* antioxidant and antidiabetic activity of these target peptides.

MATERIALS AND METHODS

Isolation and purification of peptides

Mature mulberry leaves of four different cultivars, namely S1, S1635, V1, and Dudhiya, were collected. Leaf pieces were crushed in the presence of liquid N_2. The aqueous leaf extract was centrifuged at 10,000 rpm for 30 min in the presence of protease inhibitor (phenyl methyl sulfonyl fluoride) at 4°C. The supernatant was passed through separate cation and anion exchange resin (Dowe × 50 and Dowe × 1, Sigma Chemical Co., USA), filled inside two-glass column (60 cm × 2.9 cm, 1.6 mEq/ml). Finally, solutions were filtered through Millipore ultrafiltration system with Amicon filters 3 and 0.5 kDa cut-off with 1.5 kg/cm^2 N_2 gas pressure.

High-performance liquid chromatography analysis and peptide sequencing

Partially purified peptides were passed through C_{18} reverse-phase (RP)-PLC, Waters™ 486 RP column (3.9 mm × 150 mm) with 515 HPLC pump. Methanol (10%) was used as a running solvent, HPLC was run for 60 min with a flow rate of 0.5–1.0 mL/min under 4000 psi pump pressure, and the absorbance was 250 nm. The peak area and peak height of peptide(s) appeared at different retention time (RT) were measured separately. Sequencing was performed through Shimadzu PPSQ-31A automated protein sequencer (reactor temperature: 60°C, column temperature: 37°C, and mobile phase: 10% methanol) with 15-cycle operation. After each cycle of Edman degradation, the phenylthiohydantoin derivatives were recognized through Shimadzu UV-Vis SPD-20A detector with detecting wavelength at 289 nm.

Animal material

Goat liver used in lipid peroxidation assay was obtained from close by slaughterhouse, and the experiment was conducted within 1 h of slay.

1,1-diphenyl-2 picrylhydrazyl-based free radical scavenging activity

The free radical scavenging capacity of the sample extracts against 1,1-diphenyl-2 picrylhydrazyl (DPPH)-free radical was determined as suggested by Blois.[12]

2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation(s) decolorization assay

2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation(s) scavenging activity was performed according to Re *et al.* method with slight modification.[13]

Nitric oxide scavenging assay

Nitric oxide scavenging ability of different cultivar peptides were measured using Greiss reagent.[14]

Metal chelating activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured following the method of Dinis *et al.*[15] with slight modification.

Lipid peroxidation assay

Lipid peroxidation induced by Fe^{2+} in goat liver homogenates was carried out following the method suggested by Bouchet *et al.*[16]

Reducing power assay

The reducing power of the peptides was evaluated by assessing the activity of the peptides to reduce ferric chloride as mentioned by Oyaizu.[17]

α-glucosidase inhibition assay

The AG inhibitory activity of peptides was estimated according to the method of Jung *et al.*[18] Peptide was mixed with AG enzyme (0.1 U/ml) in the 1:2 ratio and the reaction mixture was incubated at 37°C for 30 min. Equal volume of AG enzyme pNPG (5 mM) was added in reaction mixture and again incubated at same conditions. Then, 100 mM sodium carbonate was added with above reaction mixture and incubated at 37°C for 20 min. The absorbance was taken at 405 nm in the respect with control. The AG inhibitory activity was estimated by measuring the concentration required to inhibit 50% of the enzyme activity.

α-amylase inhibition assay

The α-amylase (AA) inhibition potential of the peptides was evaluated by standard spectrophotometric method.[19] 0.5 ml of aqueous peptide extract was reacted with AA solution (0.5 ml) and incubated for 5 min at 37°C. Then, 1% starch solution (0.5 ml) was added and further incubated for 10 min. To the above-obtained reaction mixture, 1 ml of 3,5-dinitrosalicylic acid reagent was added reaction termination. Later, the mixture was heated in a hot water bath till the color of reaction mixture changed to orange-red. After change in color, the mixture was cooled to room temperature and diluted up to 5 ml with distilled water. The absorbance was recorded at 540 nm. The AA inhibitory activity was determined by measuring the concentration required to inhibit 50% of the enzyme activity.

Statistical analysis

Differences and interaction between low molecular weight mulberry peptides and antioxidant and antidiabetic potentiality were determined by two-way analysis of variance. Separation of mean was performed by Duncan's multiple range test (DMRT) at $P < 0.05$.

RESULTS

The HPLC profile of peptides isolated from different mulberry cultivars clearly revealed a specific pattern according to their stationary and mobile phase interaction. Based on the RT, isolated heterogeneous oligopeptides exhibited different peak pattern. HPLC analysis also was performed

with HPLC peptide standard mixture (Sigma-Aldrich: H2016-1VL) for comparison [Figure 1]. In case of S1, five peaks were detected [Figure 1]; each peak denotes the presence of single or multiple smaller peptides in extracted crude sample. Dudhiya had 10 peaks whereas seven peaks were detected for both S1635 and V1 peptides [Figure 2]. The measurement of the amount or concentration of peptide in the sample was conducted by two different ways, one by determination of the peak height from the baseline and second by detection of peak area. The area and height of the S1, Dudhiya, S1635, and V1 peptide peak appeared in HPLC detector are represented in Figures 3-5.

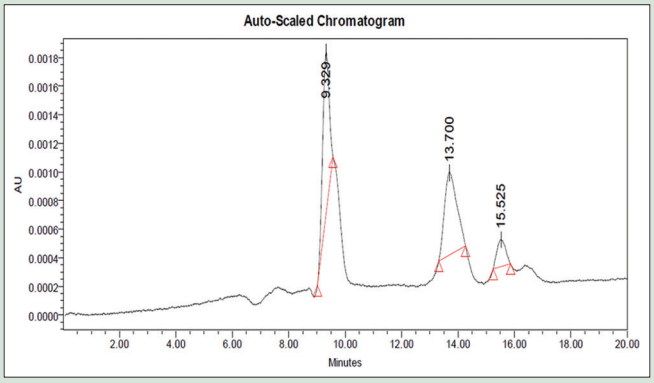

Figure 1: High-performance liquid chromatography generated auto-scaled chromatogram of peptide mixture (Methionine Enkephalin, Leucine Enkephalin and Angiotensin II: as a standard peptide mixture)

The free radical scavenging ability of the peptides of different mulberry peptides was evaluated and expressed in terms of IC_{50} values as presented in Table 1. The result revealed that the peptides of all four cultivars scavenged DPPH and ABTS radicals in a concentration-dependent manner. The IC_{50} values of various peptides for DPPH and ABTS radicals ranged from 322.67 to 876.92 μg/mL and 141.29 to 256.59 μg/mL, respectively [Table 1]. Peptide of S1635 exhibited the highest DPPH scavenging capacity (322.67 μg/ml) whereas S1 peptide showed the most potent ABTS scavenging ability (141.29 μg/mL). Further, the nitric oxide scavenging activity of the peptides was found between 5.11 and 176.38 μg/mL, with S1 being the best. Similarly, a significant difference in the metal chelating ability of the mulberry peptides was observed with S1 (169.55 μg/mL) being most efficient while Dudhiya with least activity (328.57 μg/mL). In addition, the antilipid peroxidation activity of these peptides was studied by evaluating their capacity to inhibit the production of malondialdehyde (MDA, lipid peroxidation) in liver homogenate in the presence of ferrous ion. The result revealed that in the presence of mulberry peptides inhibited the MDA production with the IC_{50} value ranging from 202.3 to 315.5 μg/mL again S1 peptide being most potent [Table 1]. The reducing power of the peptides was expressed

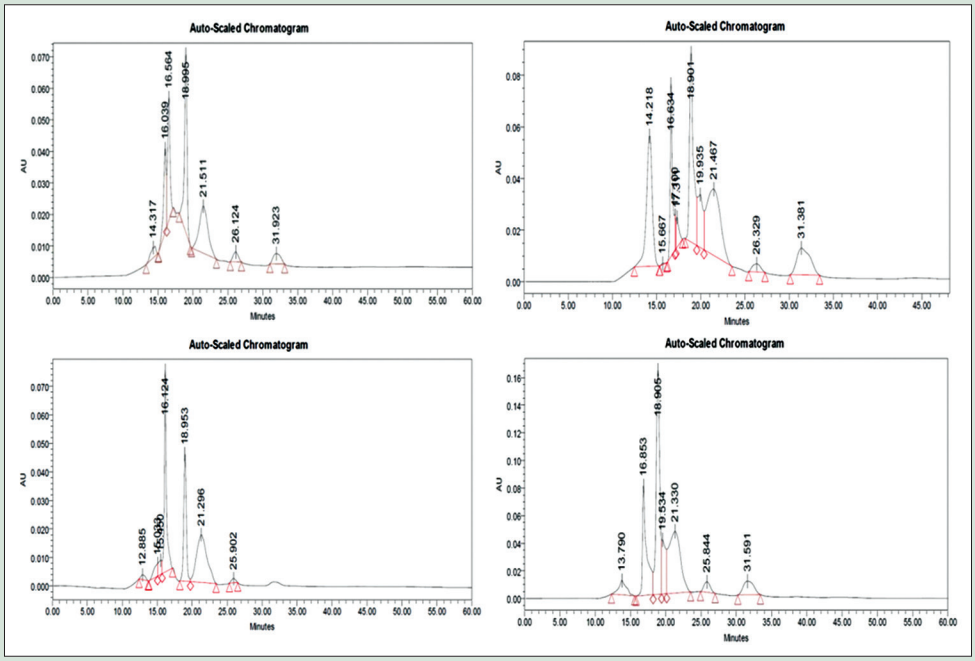

Figure 2: High-performance liquid chromatography-generated autoscaled chromatogram of peptide (s) isolated from S1, Dudhiya, S1635, and V1 leaves

Table 1: Antioxidant activity of peptides isolated from leaves of different mulberry cultivars

Cultivars	DPPH (μg/mL)	ABTS (μg/mL)	NO (μg/mL)	MC (μg/mL)	ALP (μg/mL)	RP (AAE/g)
S1	698.74±1.92^{c}	143.55±1.3^{a}	5.11±1.86^{a}	169.55±1.61^{a}	202.3±2.4^{a}	258.99±1.31^{a}
Dudhiya	876.92±3.05^{d}	256.59±3.72^{c}	111.74±2.95^{b}	328.57±2.06^{d}	315.5±4.6^{d}	222.53±1.82^{b}
S1635	322.67±2.12^{a}	141.29±2.64^{a}	172±1.91^{c}	183.42±1.36^{b}	226.4±2.1^{b}	24.32±0.97^{d}
V1	676.21±1.98^{b}	209.63±2.45^{b}	176.38±1.83^{c}	227.57±1.11^{c}	263.5±3.3^{c}	166.83±1.72^{c}

Results are expressed as Mean±SEM of triplicate determinations. Values with different letters (a, b, c, and d) differ significantly ($P \leq 0.05$) by DMRT. DPPH: DPPH scavenging activity; ABTS: ABTS radical scavenging activity; NO: Nitric oxide scavenging activity, MC: Metal chelating activity ALP: Antilipid peroxidation activity; RP: Reducing power; SEM: Standard error of mean, DMRT: Duncan's multiple range test

as ascorbic acid equivalent (AAE), and the S1 peptide displayed the highest reducing power among the cultivars investigated (258.99 AAE/g).

The mulberry peptides were found to inhibit the activity of both the enzymes AA and AG in a concentration-dependent manner [Figure 6]. However, the IC_{50} values revealed that S1 peptide displayed the best inhibitory activity (16.25 μg/mL) compared to other cultivar peptides. Further, the AG inhibitory of S1 peptides (12.56 μg/mL) was found to be significantly higher followed by V1, Dudhiya, and S1635.

Considering the prominent therapeutic potential of S1 peptide, its amino acid sequencing was done which revealed that the peptides contained aromatic amino acids. The sequence of S1 peptides includes 14 amino acids which are "WGVENAATYFWQTV" with 100% reliability observed after fourth cycle of analysis in Try-His-Lys-Ala followed by Ala-Try-Glu-Gly and Ala-Try-Pro-Asp as well as Try, Asp, Lys, and Gly [Figure 3].

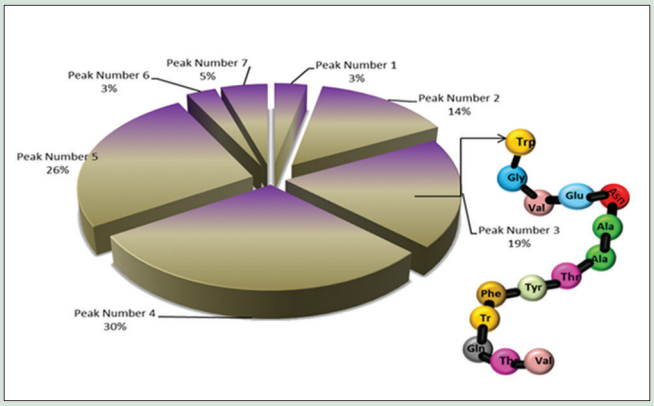

Figure 3: High-performance liquid chromatography-generated autoscaled peak area of low molecular weight (0.5–3 kDa) peptides isolated from young leaves of S1 mulberry cultivars with amino acid sequence of fraction number 3

DISCUSSION

The evaluation of antioxidant activity is considered as an important parameter for assessing the nutritional and therapeutic potential of plants.[20] The antioxidant activity of the different mulberry cultivars was exemplified by their free radical DPPH, ABTS, and nitric oxide activity and reducing power and metal chelating ability. The free radical scavenging activity (FRSA) was expressed in terms of IC_{50} values. Further, significant FRSA observed in the mulberry cultivars might be attributed to their hydrogen donating ability which may be responsible for the neutralization of these free radicals. Since then, it is reported that the biological activity of peptide depends on their amino acid composition.[21] Peptides containing aromatic amino acid (*Try* and *Phe*) are reported to exhibit strong antioxidant activity.[22] Some amino acids in the presence of their aromatic side chain such as *Trp* and *Tyr* (indolic and phenolic group, respectively) act as strong free radical scavenger.[23] Saiga *et al.* stated that the presence of hydrophobic amino acids such as *Phe* and *Ala*, in peptide, shows promising FRSA.[24] Accordingly, the noble S1 peptide exhibiting the best FRSA was found to possess these amino acids (*Phe, Tyr, Trp,* and *Ala*) in its peptide sequence. Considering the above-cited reports, it is probable that the quenching of these unstable radicals may be the consequence of the reaction of these amino acids of mulberry extract with these radicals.

The reducing power of the mulberry cultivars was evaluated by their ability to reduce ferric (Fe^{3}+) to ferrous (Fe^{2}+). The reducing capacity of a bioactive compound serves as a significant indicator of its antioxidant potentiality.[25] Our results were in accordance to Tang *et al.* and Xie *et al.*, where the antioxidant activity of plant peptides has been successfully established.[8,9,26]

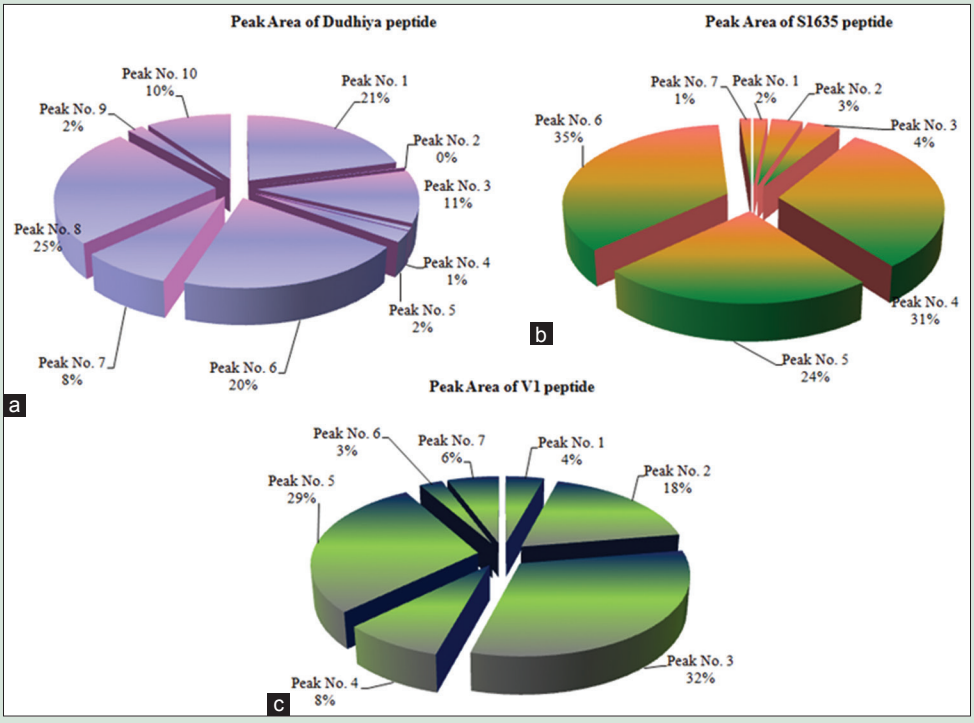

Figure 4: High-performance liquid chromatography-generated autoscaled peak area of low molecular weight (0.5–3 kDa) peptides isolated from mature leaves of V1 mulberry leaves. (a) Peak area of Dudhiya peptide, (b) peak area of S1635 peptide and (c) peak area of V1 peptide

Table 2: Two-way analysis of variance analysis between low molecular weight peptides and antioxidant activity

Source of variation	SS	df	MS	*F*	*P*	*F* crit
Peptides	100,380.9	3	33460.31	2.937,801	0.076431	3.490,295
Antioxidant activity	793,805.9	4	198451.5	17.42,396	6.15E-05*	3.259,167
Error	136,674.9	12	11389.58			
Total	1030,862	19				

*Significant at 0.01 level. SS: Sum of squares; MS: Mean square

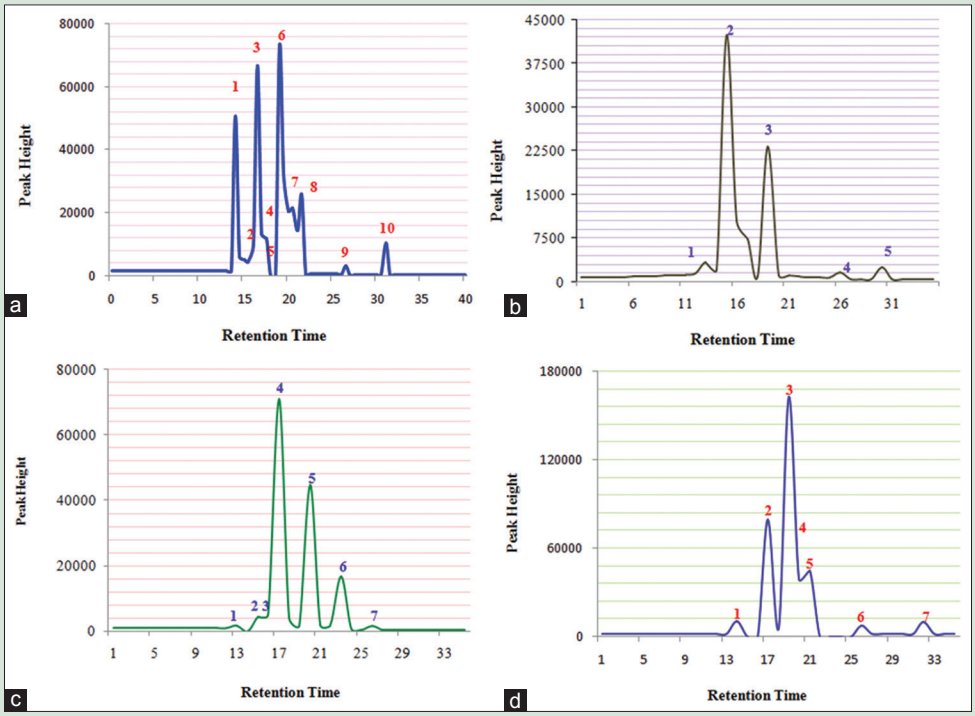

Figure 5: High-performance liquid chromatography-generated autoscaled peak height of low molecular weight (0.5–3 kDa) peptides isolated from mature leaves of (a): S1; (b): Dudhiya; (c): S1635, and (d): V1 mulberry leaves

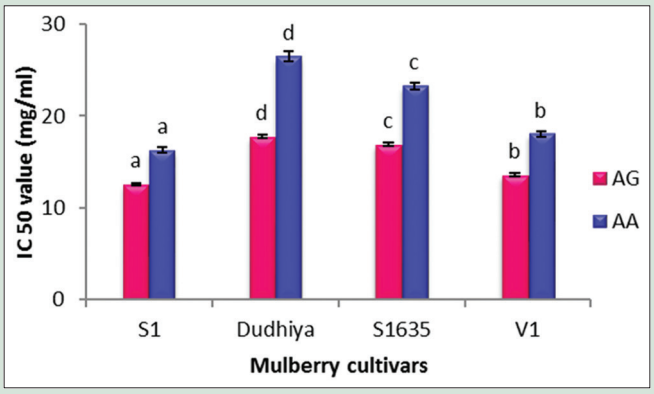

Figure 6: α-amylase and α-glucosidase inhibitory activity of peptides isolated from leaves of different mulberry cultivars. Results are expressed as mean ± standard error of mean of triplicate determinations. Values with different letters (a, b, c, and d) differ significantly ($P \leq 0.05$) by Duncan's multiple range test

Another important antioxidant mechanism includes the chelation of transition metal ions (copper and ferrous ions), which prevents their involvement in Fenton and Haber–Weiss reactions further inhibiting the generation of highly reactive hydroxyl radicals.[27] The bioactive compounds efficient in ferrous ion chelation helps in mobilization of iron present in cellular system by forming soluble and stable complexes that are removed along with the excretory products.[28] Meanwhile, the observed chelating property of mulberry peptides in the present work indicates significant therapeutic potential. In addition, considerable variation in the antioxidant activity of mulberry peptides was observed among different cultivars [Table 2] with S1 exhibiting highest and Dudhiya with lowest activity. In agreement to our observation, Zou *et al.* reported that the antioxidant activity of mulberry leaves depends on mulberry cultivars.[29]

Another effect of free radicals on the cellular system is peroxidation of lipid, thus leading to membrane disruption. The mulberry peptides were found to inhibit lipid peroxidation in liver homogenate as evident from the significant reduction in the MDA content in the presence of peptides in reaction mixture. The components having antioxidative nature are known to inhibit peroxidation of lipid membranes, thus indicating the protective role of these peptides in maintaining normal cellular functioning.[30]

One major therapeutic approach for the treatment of diabetic disorder is to minimize postprandial hyperglycemia. This can be achieved by the inhibition of two key enzymes (AA and AG) responsible for release of glucose by the breakdown of carbohydrates in the digestive tract.[31] Consequently, inhibitors of these key enzymes lead to reduction of glucose absorption rate, thus controlling the postprandial

hyperglycemia. Therefore, the evaluation of these enzyme inhibitory activities of the mulberry peptides becomes essential for assessment of their antidiabetic potential. The hypoglycemic property of mulberry leaf extracts has been reported in animal model system in previous studies.[1,32] The mulberry peptides exhibited potential enzyme inhibitory activity of both AA and AG in a concentration-dependent manner, the S1 peptide being the most potent among the studied cultivars. Likewise, previously, Estrada-Salas *et al.* have reported potent antidiabetic activity in canary seed peptides.[11]

ROS generated during the metabolic processes are considered a major source for the oxidative stress in the living system.[33] The increased level of ROS in the cellular system has been associated with degradation of pancreatic β-cells, leading to type I diabetes as well as onset of type II diabetes by insulin resistance.[34] Accordingly, the antidiabetic property of a sample can be correlated with their antioxidant activity attributed to their metal ion chelation and reducing nature.[3] Moreover, huge amount of thiobarbituric acid-reactive species were reported in the plasma and the tissue of diabetic rats than in healthy rats, which further indicates the probable role of antilipid peroxidative property of mulberry peptides in prevention of diabetic disorders.[35] As stated by Finefrock *et al.*, most of the enzymes utilize the transition metal ions as a cofactor for their catalytic activity; hence, chelation of these metal ions would result in inhibitory impact on their catalytic activity.[36] As it has been earlier mentioned that the mulberry peptides exhibited pronounced metal chelating activity, consequently, it can be stated that the amino acid composition of these peptides is probably responsible for its inhibitory effect on these enzymes. Such cohesiveness between antioxidant antidiabetic activities has been established by various authors in their respective investigation.[3,30] Therefore, the application of these bioactive mulberry peptides in the management of glucose metabolism and alleviation of oxidative stress-mediated complications might be a beneficial approach in the diabetes treatment.

CONCLUSION

It can be concluded that the potential antidiabetic activity of the mulberry peptides could be attributed to their antioxidant nature, which is the probable cause for inhibition of the enzymatic activity of two key enzymes (AA and AG). Meanwhile, the variation in the antioxidant and antidiabetic activity of isolated peptides could be the consequence of the observed variation in amino acid composition in different mulberry cultivars. Further, S1 peptides were found to be the most potent among the cultivars studied and could be implemented in the formulation of functional foods and nutraceuticals which may serve as efficient approach toward diabetic complication management.

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Conflicts of interest

There are no conflicts of interest.

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