Chapter – VI

Characterization of bioactive peptide

6.1 INTRODUCTION

Peptides are nothing but smaller proteins, which biologically are consisting of short chains of amino acids linked by peptide bond or amide bond. Peptides are considered as important molecules with diverse biological activity. Several peptides are isolated from varieties of plant sources *viz.* soybean (Moure *et al.,* 2006), wheat (Zhu *et al.,* 2006), peanut (Jamdar *et al.,* 2010) and *Amaranthus* (Alcantara Quintana *et al.,* 2015). Approximately 238 plant peptides already had been isolated, among them 61 are placed under antimicrobial plant peptides (Hammami, 2009). The most of the plant peptides have 20 to 67 amino acids residues. However, sufficient scientific reports were not available regarding plant oligopeptide characterization, sequence analysis, proteolytic processing or associated biological information.

To date, reversed phase high performance liquid chromatography (RP- HPLC) has become an essential, well established tool for analysis and purification of proteins and peptides. Separation of peptides also was done on a thin-layer chromatography (TLC) plate (Reisinger *et al.,* 1992). The primary structure of proteins or peptides can be analyzed by Edman degradation or mass spectrometry. Proteins/peptides are either identified by database searching with their respective masses (peptide mass fingerprinting) or sequence of amino acids present in target sample (Deutzmann, 2004). Therefore, the aim of this chapter is to purify and characterize the low molecular weight mulberry peptides through which the functional aspects of these oligopeptides can be speculated.

6.2 MATERIAL AND METHODS

6.2.1 Isolation and purification of low molecular weight peptide(s)

Mention in section 4.2.1

6.2.2 HPLC analysis and amino acid sequencing

The semi purified concentrated peptide(s) from different maturity status of leaves were passed through C18 HPLC, Waters™ 486 reverse phase column in 10% Methanol as running solvent fitted with 515 HPLC pump, running time 60 min, absorbance at 250 nm, column length 3.9 mm × 150 mm, injection volume 20 µL, flow rate 0.5-1.0 mL/min, pump pressure 4000 psi., and purified. The peptide(s) appeared at different retention time were repeatedly tried and purified, concentrated and collected in deep freeze under -20°C. Each peak was isolated with their retention time and re-injected into the column to check its repetitive occurrence.

Sequencing was performed through Shimadzu PPSQ-31A automated protein sequencer with 15 cycles operation, reactor temperature 60˚C, column temperature 37˚C with mobile phase by 10% methanol. HPLC characterization of PTHs made use of a steel-walled C_{18} analytical column. After each cycle of Edman degradation, the PTH-derivatives were identified through Shimadzu UV-Vis SPD-20A Detector with detecting wavelength at 289 nm. System integrator calibrated the maximum probable sequence of amino acids.

6.2.3 TLC based peptides separation

Peptide separation was performed by TLC methods (Reisinger *et al.,* 1992) with some modification. Silica gel-60 F_{254} chromatographic plates were used for TLC. The peptides sample was spotted into 8 cm × 2 cm (height × width) silica plate with 3 mm thickness. The spots were allowed for drying and the silica plate was placed in a previously saturated solvent chamber. To make a saturated TLC chamber, solvent mixture was poured into TLC chamber and kept it for 30 minutes as undisturbed condition. The plate was eluted within chamber by a solvent mixture of n-butanol, acetic acid, water 3:1:1 respectively (by volume). The plate was immersed into chamber in such a way that the spot line remains above the solvent. The plate was allowed to run within chamber for capillary action to draw the solvent up through the plate and the solvent run immediately was stopped before it reached the terminal line (1 cm from the end). Plate was dried in a hot air oven for 10 min and immersed into freshly prepared ninhydrin stain. After staining, plate was again dried at 100˚C for 5-10 minutes. Ninhydrin reacts with amino acids present in separate peptides and make them visible. Immediately, the TLC plate was scanned (Bio-Rad scanner). The Rf (relative font) values and relative density of the bands of different peptides was analyzed by Image Lab (Bio-Rad) software.

6.3 RESULT AND DISCUSSION

TLC analysis was performed with low molecular weight (LMW) especially 0.5-3 kDa oligopeptides because strong bioactivity was acquired with LMW peptide fractions (result shown in chapter IV and V). After the spraying of 0.25% ninhydrin reagent different colored bands were appeared in TLC plate. Ninhydrin hydrate with amino groups present in protein or peptides and produced Riemann's purple. When amino acids and small peptides react with ninhydrin, somewhat different colour was developed, presumably because of reaction pattern (slow reaction or unfavorable equilibrium) or formation of byproducts (Mendel, 2004).

A Ninhydrin spray was used to develop approximately sixty ninhydrin-positive compounds on TLC (Dent, 1948). Thompson and Morris (1959) described a sensitive paper chromatographic analysis of amino acids present in plant sample through ninhydrin reagent. Now ninhydrin spray are widely used with both paper and silica gel plates in forensic science (LaPorte and Ramotowski, 2003; Schwarz and Frerichs, 2002); environmental chemistry (Nagaraja *et al.,* 2002) and food chemistry (Pavia *et al.,* 2000), clinical chemistry, pharmacology and toxicology (Abdellatef and Khalil, 2003; Kim *et al.,* 2002; Moulin *et al.,* 2002).

Figure 6.1-6.7 shows representative digital picture of oligopeptide pattern on TLC plate and respective graphical analysis. Rf values and relative density of each bands present on TLC plate were determined through Image-Lab (Bio red) software analysis. Bands with various color and different Rf values denote the specific amino acid present in N-terminal end of small peptides or nature of α-amino acid of peptides chain or poly-functional amino acids present in peptides, presumably because ninhydrin reacts with N-terminal end and/or α-amino acid of peptides chain (Friedman and Williams, 1973 and 1974).

Pattern of oligopeptides differ with maturity stages of mulberry leaves. Peptides isolated from both young and mature S1 mulberry leaves (S1Y and S1M respectively) had six bands with various Rf values on TLC plate whereas, peptides isolated from senescence S1 mulberry leaves (S1S) exhibited five bands (Figure 6.1-A). Reddish violet bands with Rf-0.258 (S1Y) and Rf-0.274 (S1M) was observed in peptides isolated from young and mature leaves. Relative density (Figure 6.1-B) of reddish band was decreased in S1M peptides in comparison with S1Y. While, reddish violet band was totally absent between Rf 0.25 to 0.28 in case of peptides (S1S) isolated from senescence leaves. Instead, a brownish violet colored band with Rf-0.229 was observed in S1S. This observation revealed that, peptide chain responsible for reddish violet bands were gradually degenerated with the maturation of S1 mulberry leaves.

Table 6.1 showed a general account of colors formed by amino acid on TLC plate with ninhydrin spray and tentative Rf values in n-propanol water. Peptides isolated from Young and mature leaves of S1635 mulberry cultivars (S1635 Y and S1635 M respectively) had six numbers of bands each on TLC plate with various Rf values. Though the numbers of bands were same in S1635Y and S1635M but the nature of peptides might differ, presumably because in S1635Y a reddish pink band was observed with Rf-0.197 (band no. 2), whereas reddish violet bands with Rf-0.234 (band no. 2) was found in S1635M peptides (Figure 6.2-A). Pale pink colored bands (band no. 1) were found in

both S1635Y (Rf-0.11) and S1635M peptides (Rf-0.125), while it is completely absent in S1635S peptide. In case of S1635S sample, first band (band no. 1) appeared with Rf value 0.248 and shows pale pink color. Relative density of peptides isolated from mature leaves was higher in comparison with the rest two S1635 peptides (Figure 6.2-B). Another significant difference was observed in S1635Y, where pinkish band was observed with Rf-0.365 (band no.4). However, yellow bands with Rf-0.383 (band no.4) in S1635M and reddish orange bands with Rf-0.385 (band no.3) in S1635S were obtained in TLC plate after heating.

Peptides isolated from young (V1Y) and mature (V1M) leaves of V1 mulberry cultivars had seven numbers of bands on TLC plates, while peptides isolated from senescence (V1S) leaves had six numbers of bands. Brownish violet bands with Rf-0.63 (V1Y) and Rf-0.616 (V1M) was observed which was completely absent in V1S (Figure 6.3). This kind of observation reveled that some peptides might be degraded with the increasing foliar maturation.

Similar prototype was established on TLC plate, in case of peptides isolated from young (Dudhiya Y) and mature (Dudhiya M) leaves of Dudhiya mulberry cultivars. Band number 2 showed deep reddish violet in Dudhiya Y and Dudhiya M peptides while it was brownish violet in case of peptides isolated from senescence leaves (Figure 6.4). Peptides isolated from mature Dudhiya leaves exhibited higher relative density than others (Figure 6.4-B).

TLC based analysis revealed that the amino acid present in peptides chain (especially, α-amino acid or N-terminal end) varied with the variation of maturity stages of leaves. Few peptides were damaged gradually with the increasing foliar age. Comparative study revealed that nature of oligopeptides also varied with the variation of mulberry cultivars (Figure 6.5-6.7). The Rf values were separated in each peptides isolated from different mulberry cultivars. The difference of molecular weight of peptides might be responsible for shifting the Rf values of peptides. The Rf values of peptide bands on TLC plates varied from the standard Rf values (Table 6.1) of the single amino acid because of number of amino acids present in a peptides chain could vary widely due to which their partition co-efficient in between stationary and mobile phase might be altered.

Basak *et al.* (2005) used sulfur compounds with ninhydrin in the detection of amino acids on TLC plate. Different amino acids appeared with diverse colors in TLC plate after reacting with ninhydrin reagents and the Rf values of amino acids exhibited between 0.02 to 0.60 (Sahana *et al.,* 2011). Sinhababu (2013) developed separate colored bands for amino acid detection on TLC plate by

using ninhydrin reagents developed with *para*-bromobenzoic acid. Antimicrobial synthetic peptides also had been detected through TLC based separation (Jaskiewicz *et al.,* 2016).

HPLC analysis was performed with those peptides which exhibited better silkworm feeding response and antioxidant activities at LMW condition. Though, peptides from Dudhiya leaves did not show better activity but HPLC of these peptides were performed for preparing comparative accounts between higher and lower most bioactive peptides (S1 and Dudhiya peptides respectively). HPLC analysis also was performed with HPLC peptide standard mixture (Sigma-Aldrich: H2016-1VL) for make a comparison (Figure 6.8). The HPLC chromatogram profile of LMW peptides isolated from different mulberry sources clearly revealed a specific pattern according to their stationary and mobile phase interaction. Based on the retention time, isolated heterogeneous oligopeptides from different maturity stages of mulberry leaves exhibited different peak pattern in HPLC profile. In the case of S1-Y and S1-S, seven peaks were clearly identified, whereas five peaks were detected with S1-M (Figure 6.9-6.11). Each peak denotes presence of single or multiple smaller peptides in extracted crude peptides. Figure 6.12 showed comparative appearance of different peaks at various retention time (RT) of S1 peptides (S1-M and S1-S). Peptides isolated from Dudhiya young, mature and senescence leaves had 9, 10 and 6 peaks respectively at different retention time (Figure 6.13-6.15). Figure 6.16 denotes comparative appearance of peaks at different RT of Dudhiya peptides. Seven peaks were detected with both S1635-M and V1-M peptides (Figure 6.17 and 6.18). The measurement of the amount or concentration of peptides in the sample is conducted by two different ways, one by determination of the peak height from the baseline and the secondly by detection of peak area. Sometimes, a linear relationship was found in between height or area and the peptide concentration. For a comparative study, the peak area and peak height of the peptides appeared in HPLC chromatogram detector was calculated and are represented in Figure 6.19-6.27. Dauly *et al.* (2006) evaluated the use of RP-HPLC for characterization of the fractions of intact proteins as separation technique. Mant *et al.* (2007) used HPLC technique to purification and characterization of peptides.

Comparative profile of peptides was evaluated for determining the changes of HPLC peaks during maturation and senescence of mulberry leaves. At the time of maturation, non-overlapping peak 2 of HPLC of Dudhiya genotype might be responsible for alteration of bioactivity, whereas during senescence, peptides isolated from Dudhiya genotype comprising greater abundance might contribute inhibitory function as decoded from HPLC chromatogram.

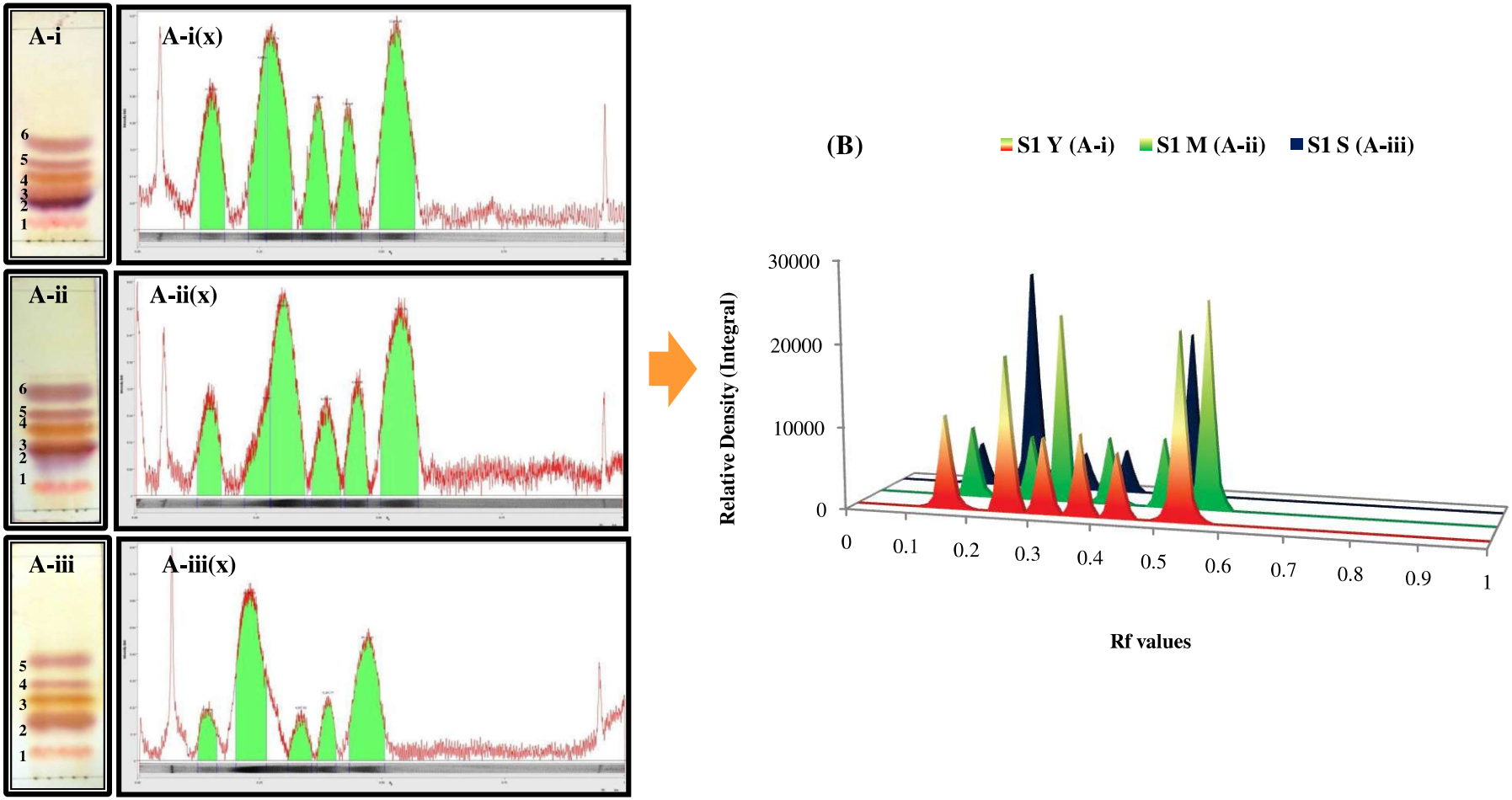

Figure 6.1: (A): Digital image of oligopeptide separation on TLC plate [peptide isolated from young (**A-i**), mature (**A-ii**), and senescence (**A-iii**) S1 mulberry leaves] and auto generated chromatogram (Image lab: Bio Red) of peptides separation on TLC plate [**A-i(x)**, **A-ii(x)** and **A-iii(x)** for peptides isolated from young, mature and senescence S1 mulberry leaves respectively). (**B**): Chromatogram depicting the relative density of peptides on TLC plates.

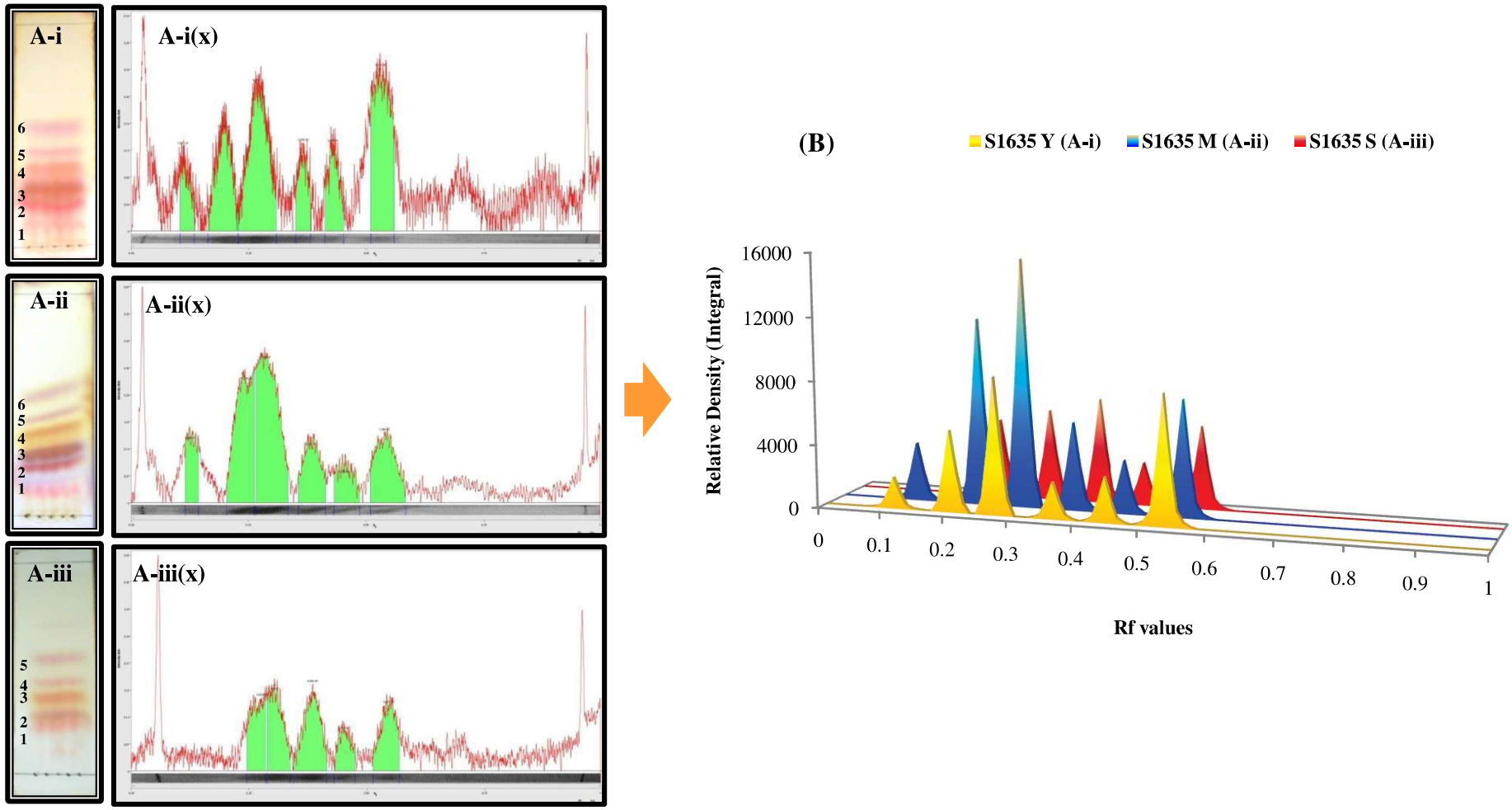

Figure 6.2: (A): Digital image of oligopeptide separation on TLC plate (peptide isolated from young (**A-i**), mature (**A-ii**), and senescence (**A-iii**) S1635 mulberry leaves and auto generated chromatogram (Image lab: Bio Red) of peptides separation on TLC plate [**A-i(x)**, **A-ii(x)** and **A-iii(x)** for peptides isolated from young, mature and senescence S1635 mulberry leaves respectively). **(B)**: Chromatogram depicting the relative density of peptides on TLC plates.

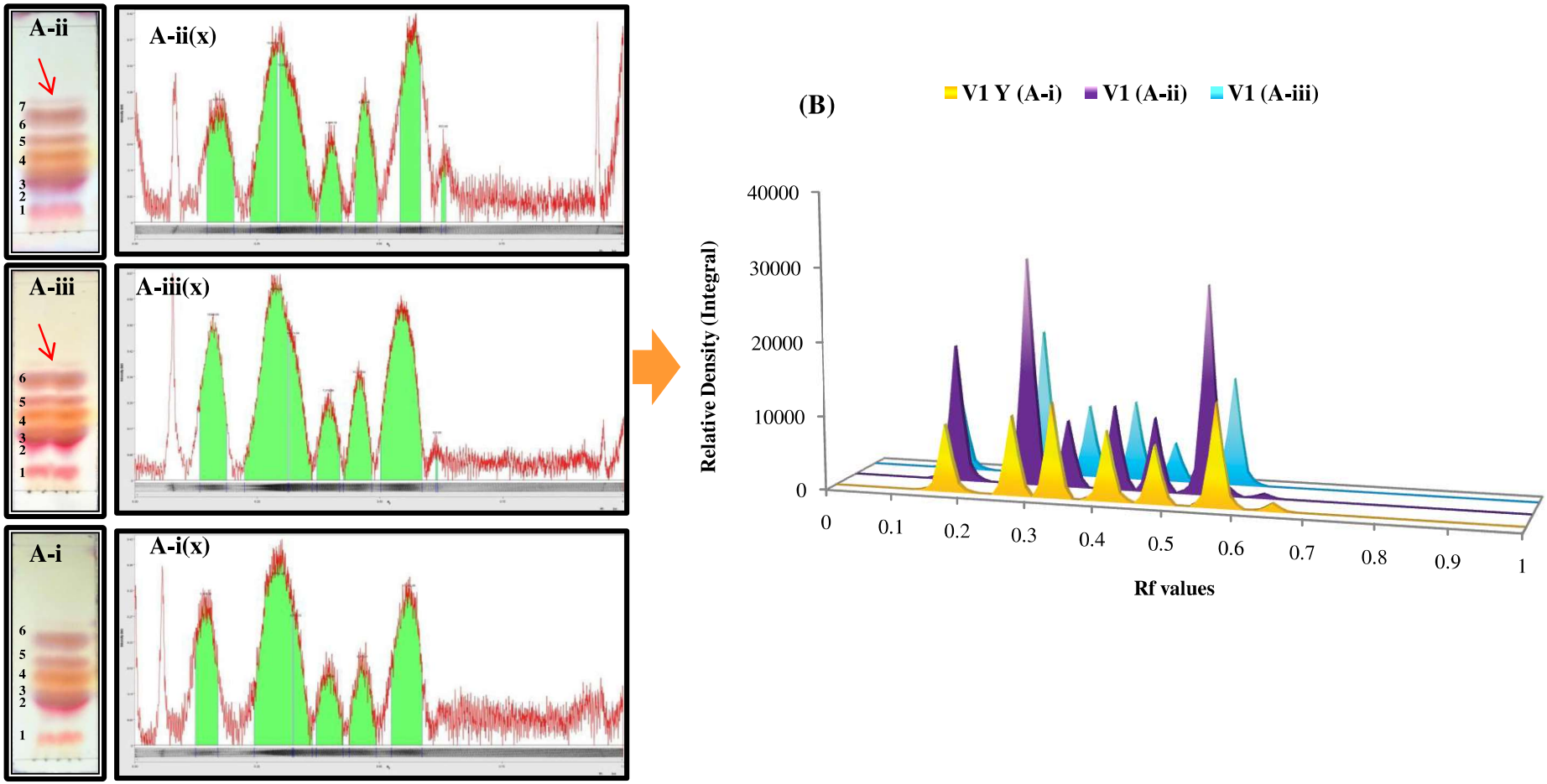

Figure 6.3: (A): Digital image of oligopeptide separation on TLC plate (peptide isolated from young (**A-i**), mature (**A-ii**), and senescence (**A-iii**) V1 mulberry leaves and auto generated chromatogram (Image lab: Bio Red) of peptides separation on TLC plate [**A-i(x)**, **A-ii(x)** and **A-iii(x)** for peptides isolated from young, mature and senescence V1 mulberry leaves respectively). **(B)**: Chromatogram depicting the relative density of peptides on TLC plates.

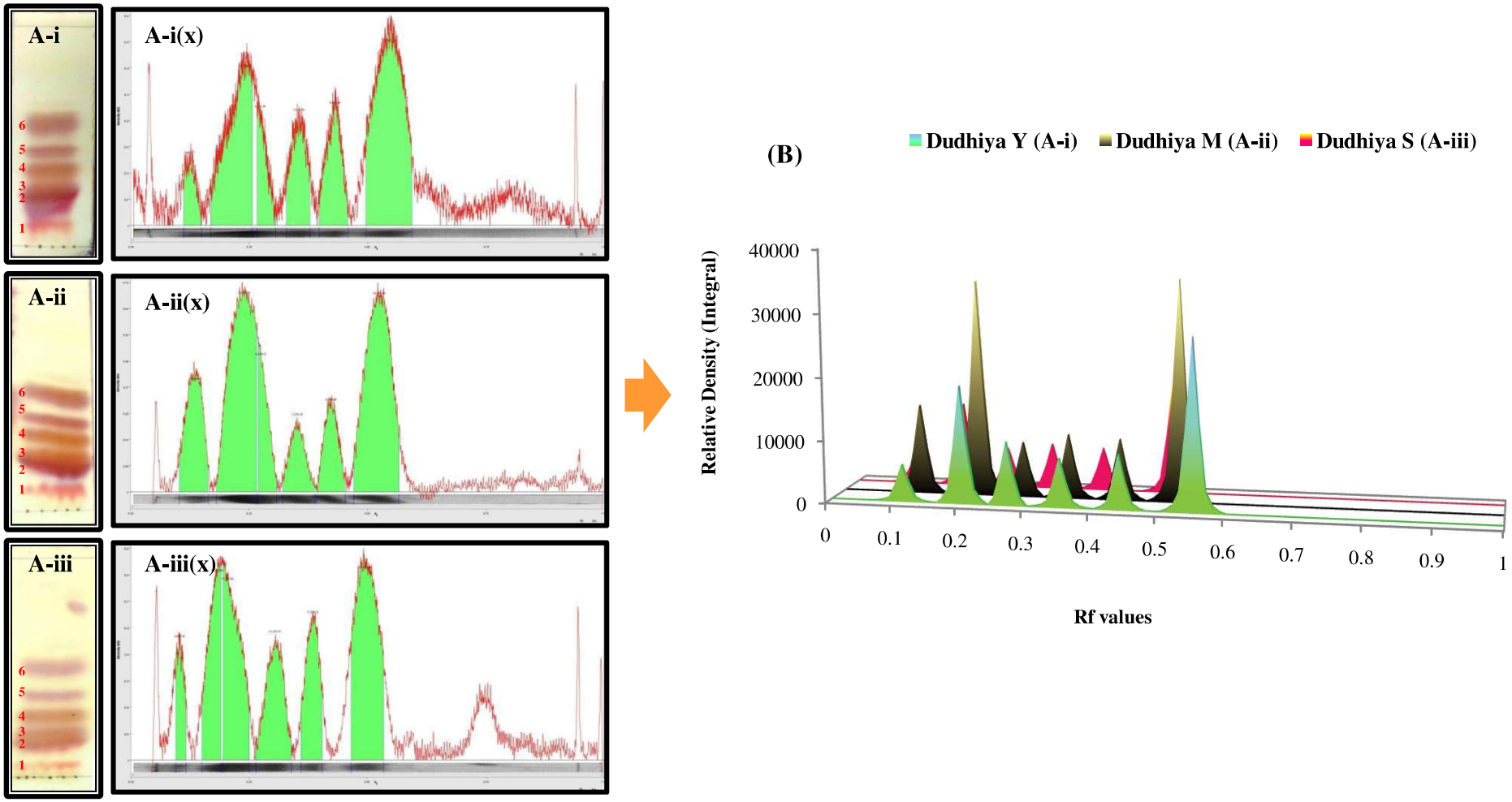

Figure 6.4: (A): Digital image of oligopeptide separation on TLC plate (peptide isolated from young (**A-i**), mature (**A-ii**), and senescence (**A-iii**) Dudhiya mulberry leaves and auto generated chromatogram (Image lab: Bio Red) of peptides separation on TLC plate [**A-i(x)**, **A-ii(x)** and **A-iii(x)** for peptides isolated from young, mature and senescence Dudhiya mulberry leaves respectively). **(B)**: Chromatogram depicting the relative density of peptides on TLC plates.

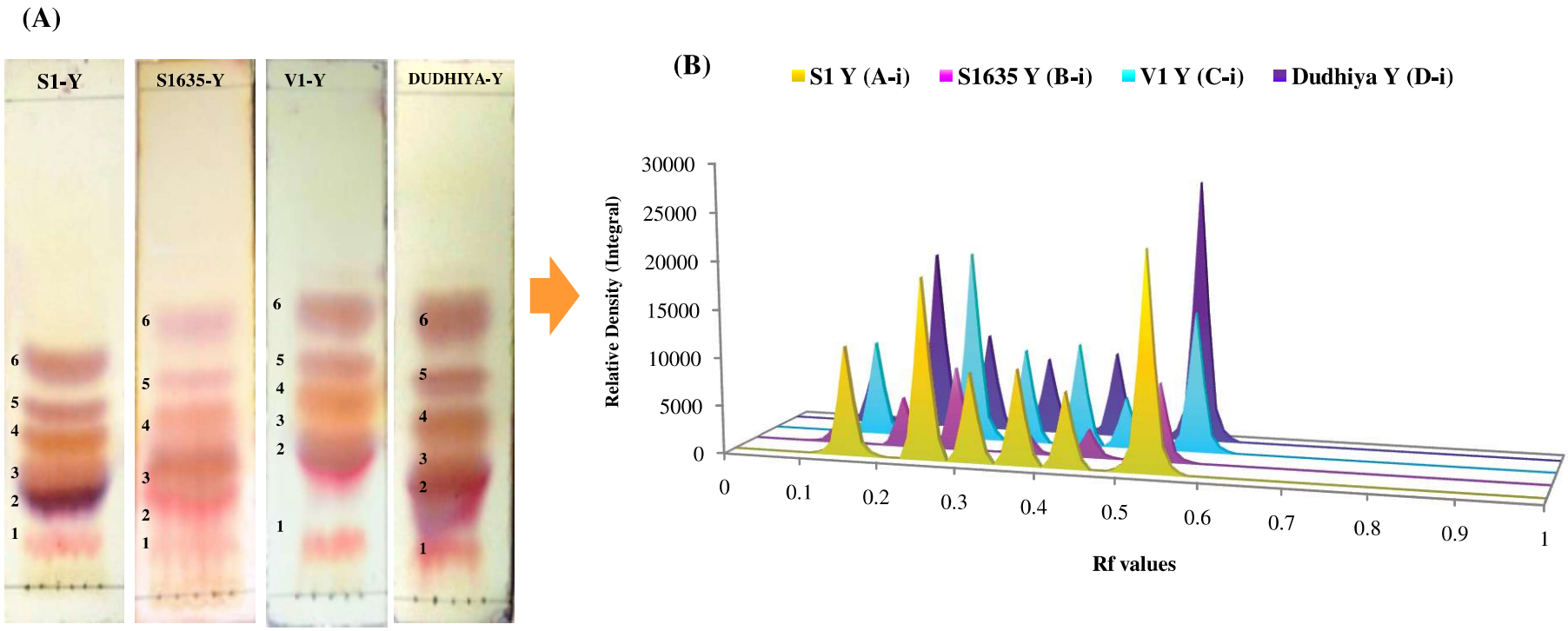

Figure 6.5: (A): A comparative study of oligopeptide separation on TLC plate (peptide isolated from young leaves of S1, S1635, V1 and Dudhiya mulberry leaves). **(B):** Chromatogram depicting the relative density of peptides on TLC plates.

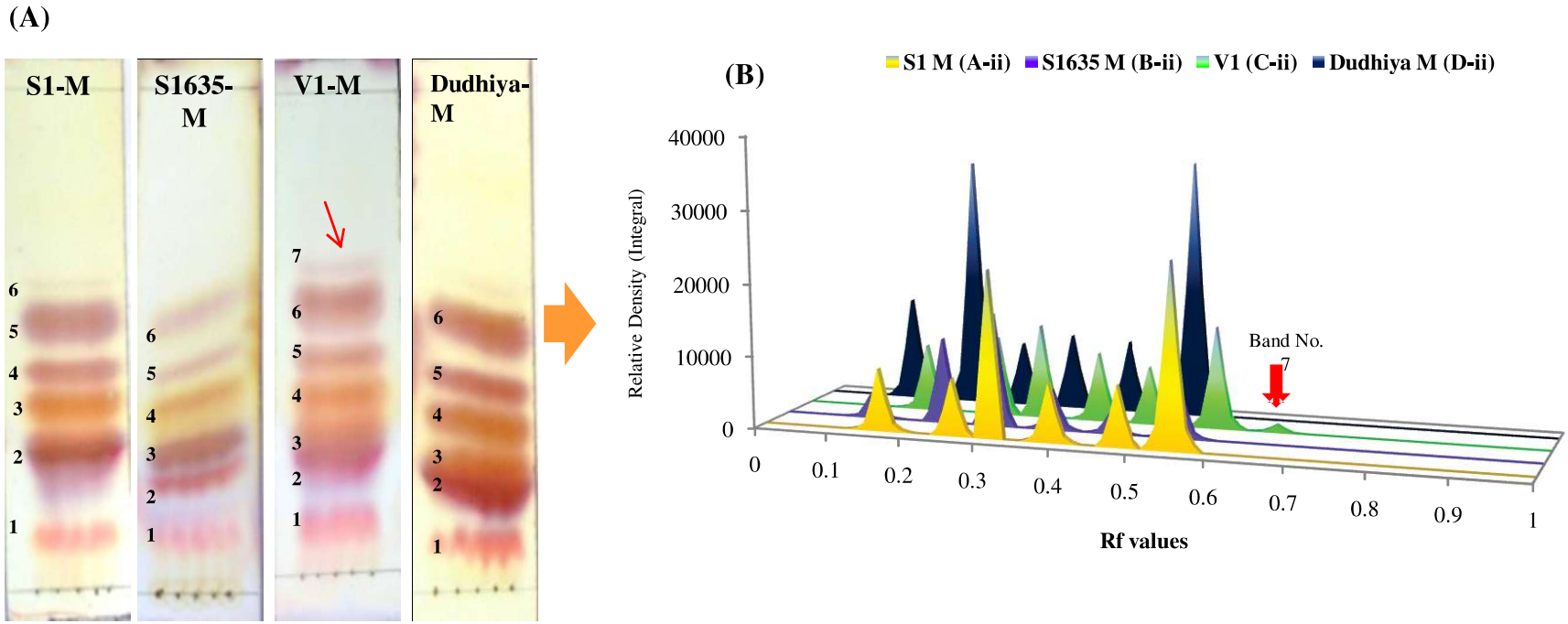

Figure 6.6: (A): A comparative study of oligopeptide separation on TLC plate (peptide isolated from mature leaves of S1, S1635, V1 and Dudhiya mulberry leaves). **(B):** Chromatogram depicting the relative density of peptides on TLC plates.

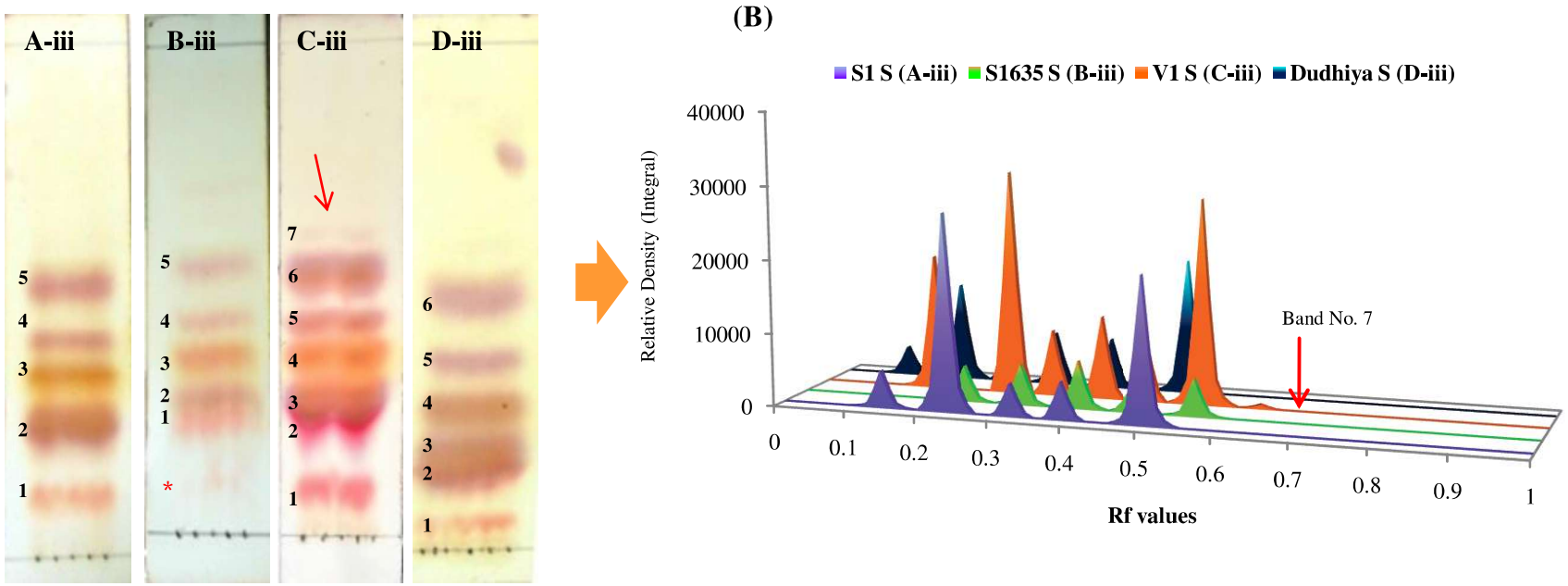

Figure 6.7: (A): A comparative study of oligopeptide separation on TLC plate (peptide isolated from senescence leaves of S1, S1635, V1 and Dudhiya mulberry leaves). **(B):** Chromatogram depicting the relative density of peptides on TLC plates.

* Bands between Rf- 0.1-0.2 was absent.

Table 6.1: Colours developed by amino acids on TLC plates with ninhydrin reagent and their Rf values in n-propanol-water solvent system (Sahana *et al.*, 2011)

Amino acid	Colour observed after heating	Standard Colours	Approximate Rf values
Arginine	Blood red		0.16
Leucine			0.55
Cysteine	Lilac/Pale pink		0.32
Histidine	Dark orange		0.2
Isoleucine			0.53
Glutamine	Pink		0.15
Lysine	Brownish violet		0.12
Tyrosine			0.57
Hydroxyproline			0.34
Asparagine	Reddish pink		0.14
Tryptophan	Deep violet		0.62
Threonine			0.37
Phenylalanine	Violet		0.58
Aspartic acid			0.33
Serine	Brick red		0.35
Alanine			0.37
Glutamic acid	Reddish violet		0.35
Valine			0.45
Methionine	Deep reddish violet		0.51
Glycine	Reddish orange		0.32
Proline	Yellow		0.26

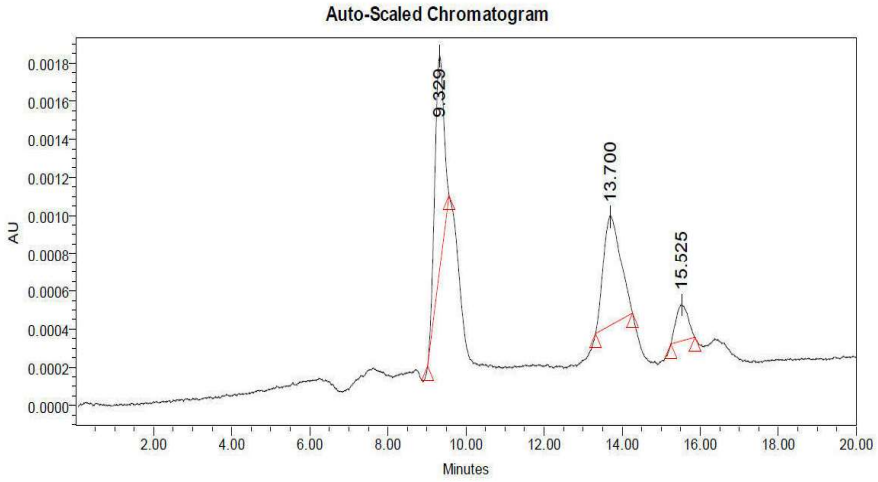

Figure. 6.8: HPLC generated auto-scaled chromatogram of peptide mixture (Methionine Enkephalin, Leucine Enkephalin and Angiotensin II: as a standard peptide mixture)

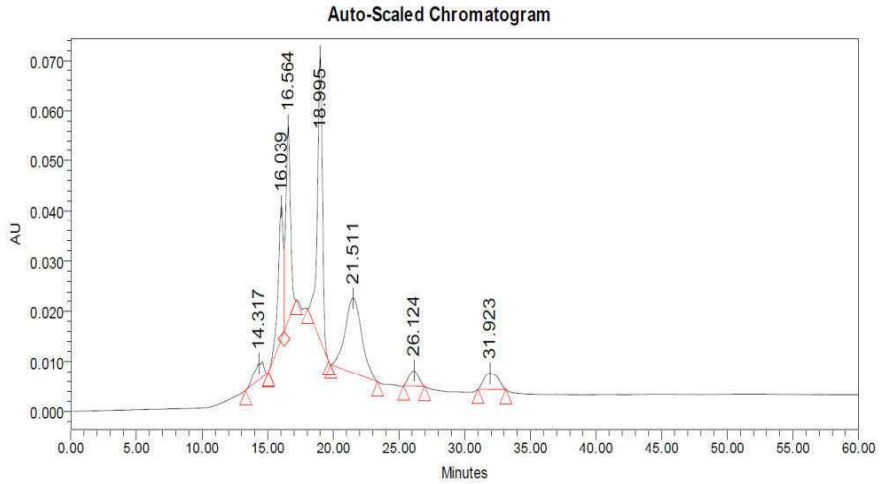

Figure. 6.9: HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 young leaves

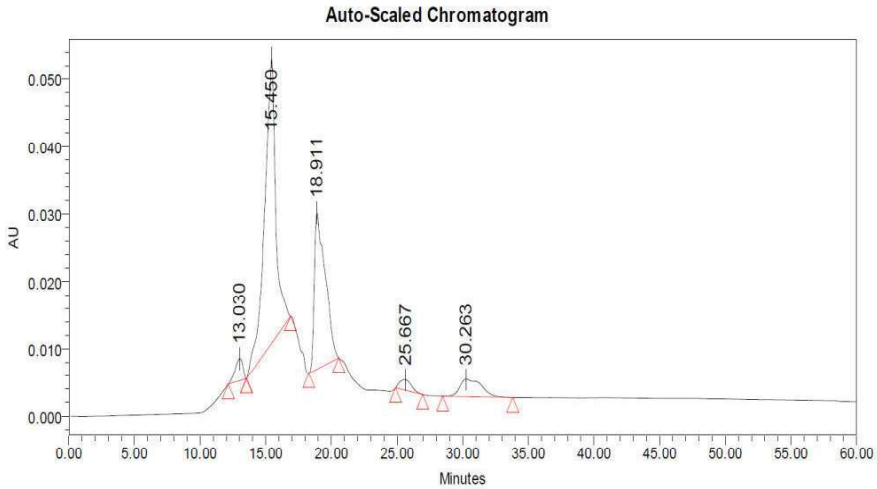

Figure. 6.10: HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 mature leaves

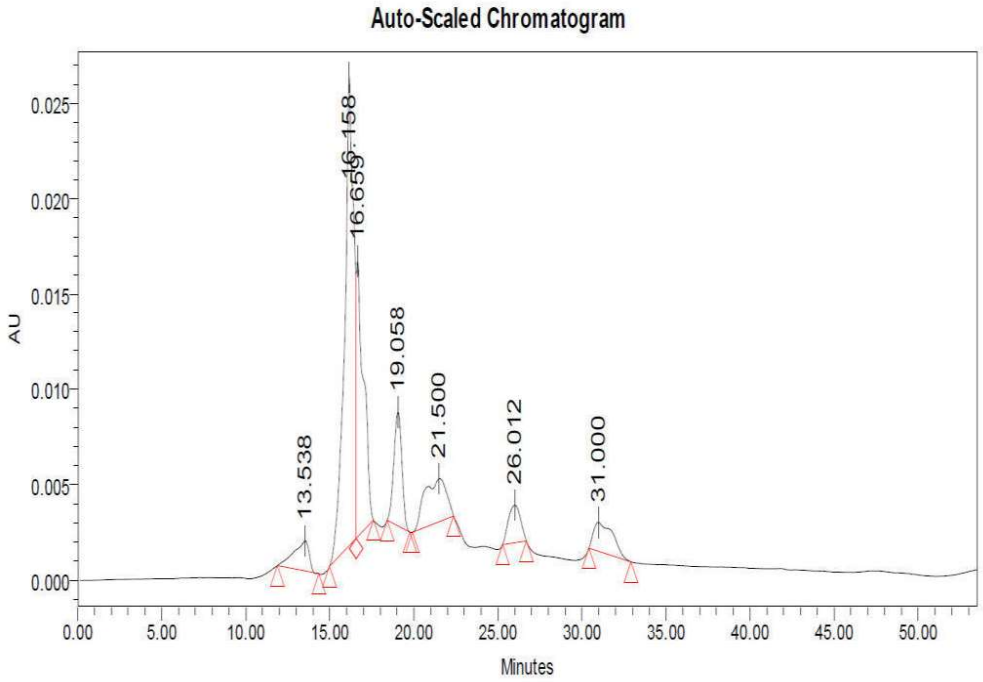

Figure. 6.11: HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 senescence leaves

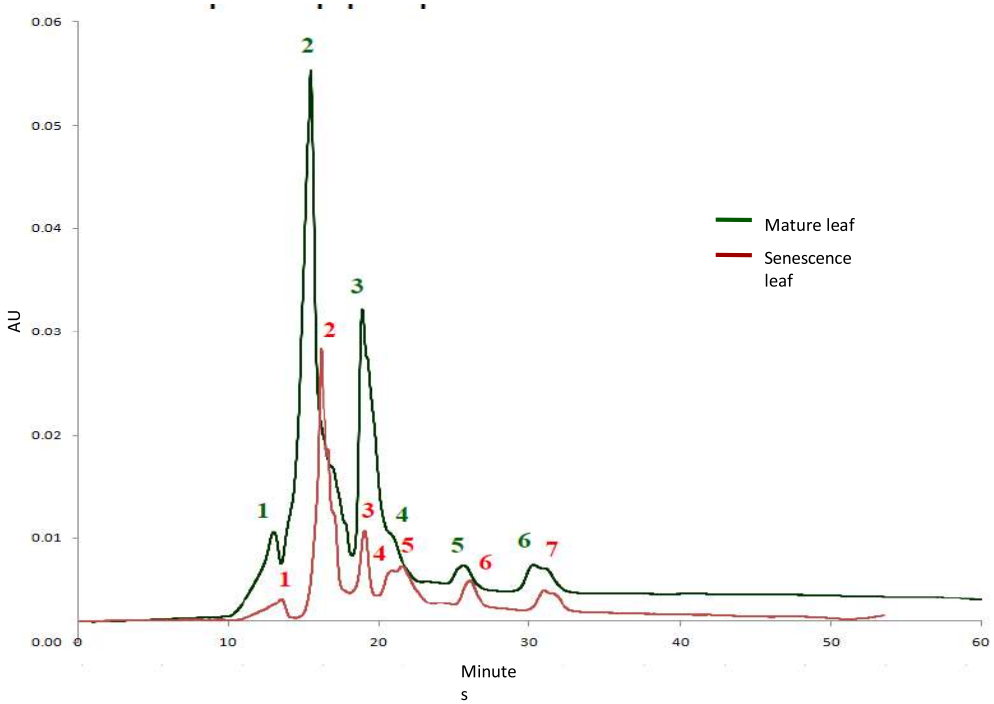

Figure. 6.12: Comparative peptide profile: Mature vs. senescence leaf of S1 mulberry variety

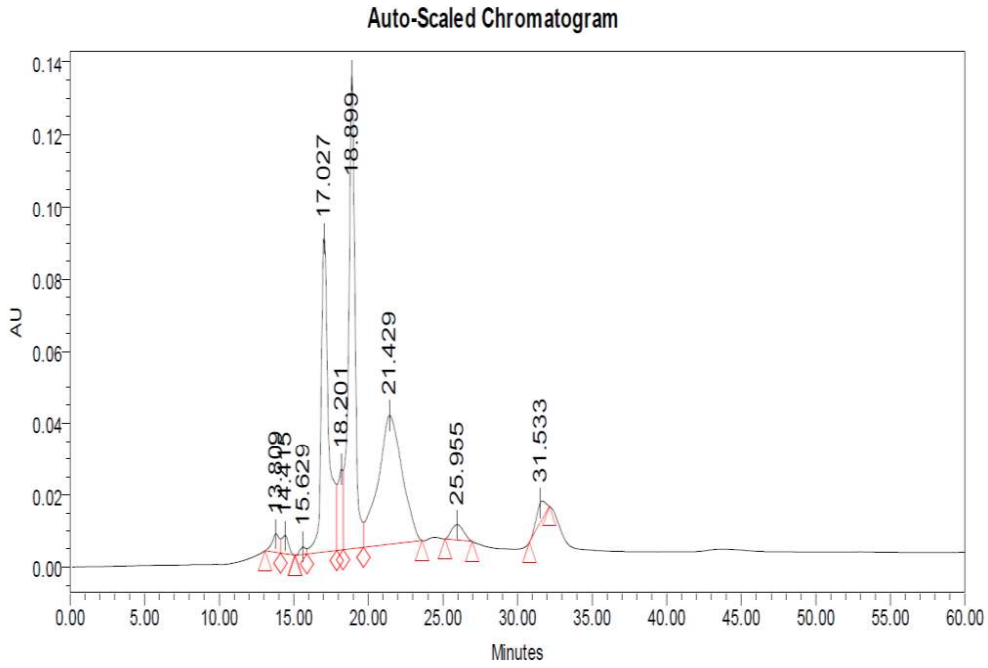

Figure. 6.13: HPLC generated auto-scaled chromatogram of peptide(s) isolated from Dudhiya young leaves

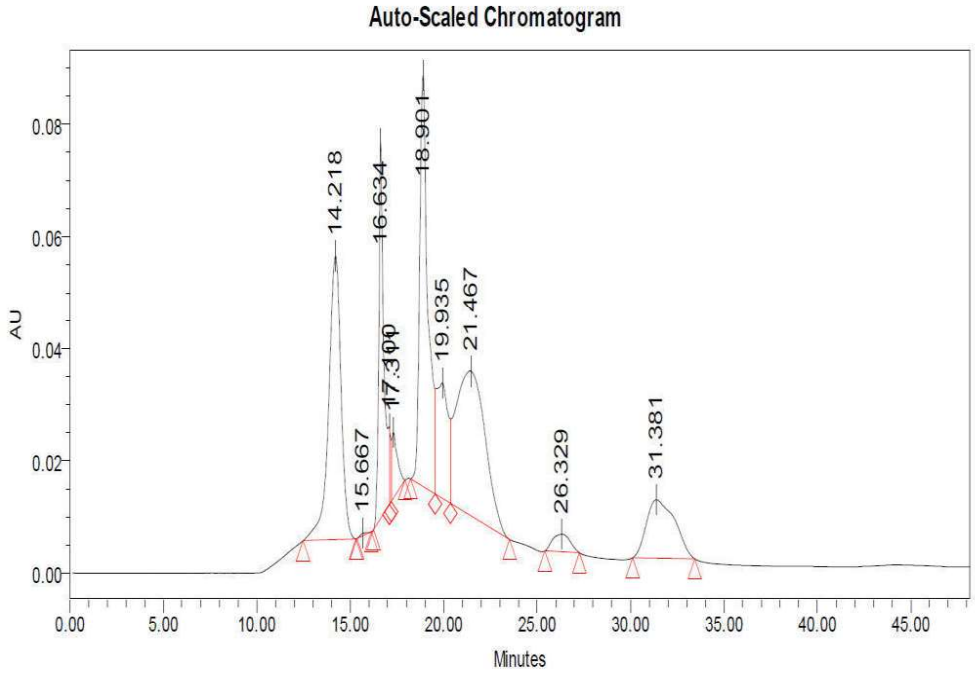

Figure. 6.14: HPLC generated auto-scaled chromatogram of peptide(s) isolated from Dudhiya mature leaves

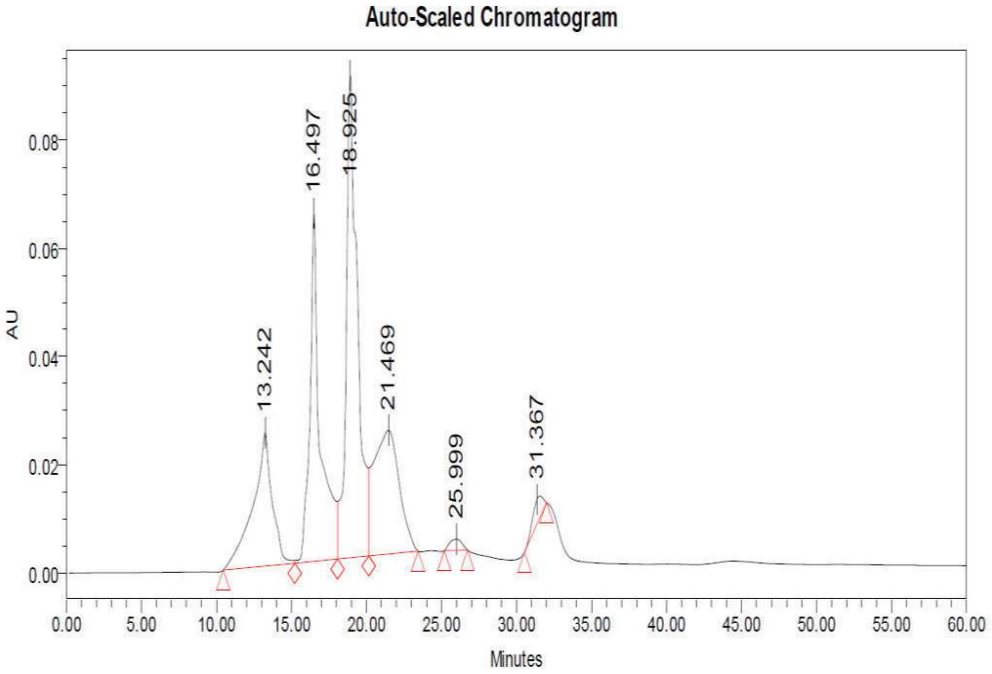

Figure. 6.15: HPLC generated auto-scaled chromatogram of peptide(s) isolated from Dudhiya senescence leaves

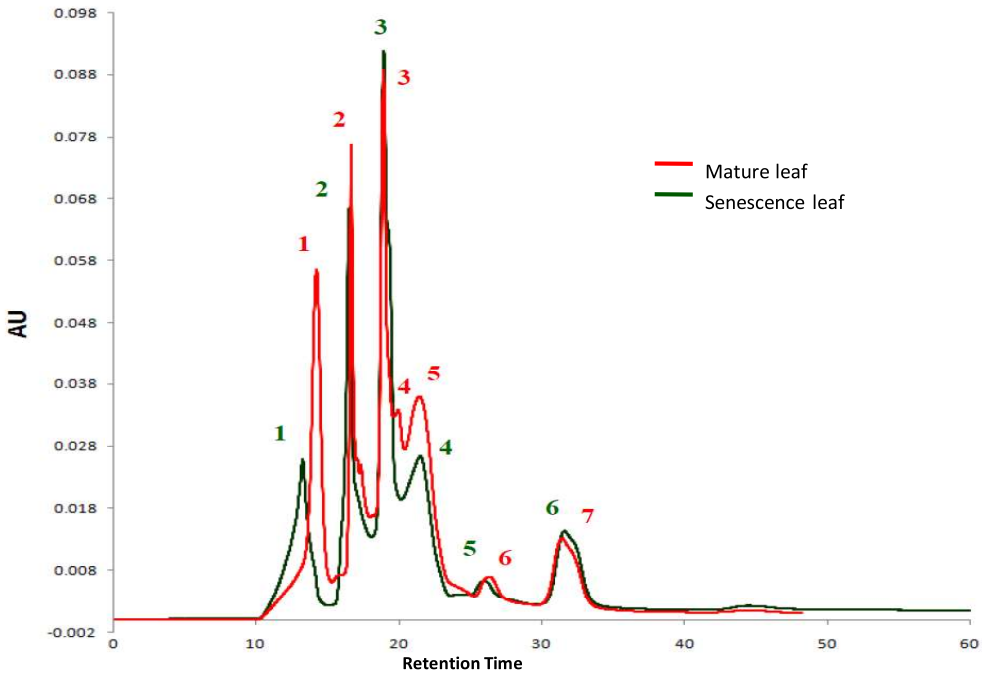

Figure. 6.16: Comparative peptide profile: Mature vs. senescence leaf of Dudhiya mulberry variety

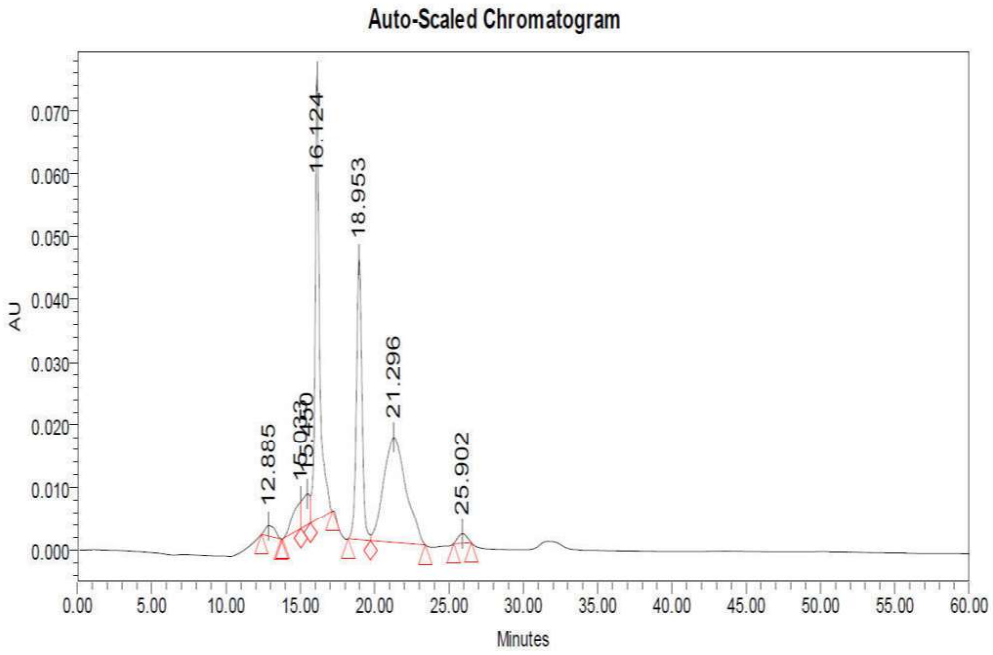

Figure. 6.17: HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1635 mature leaves

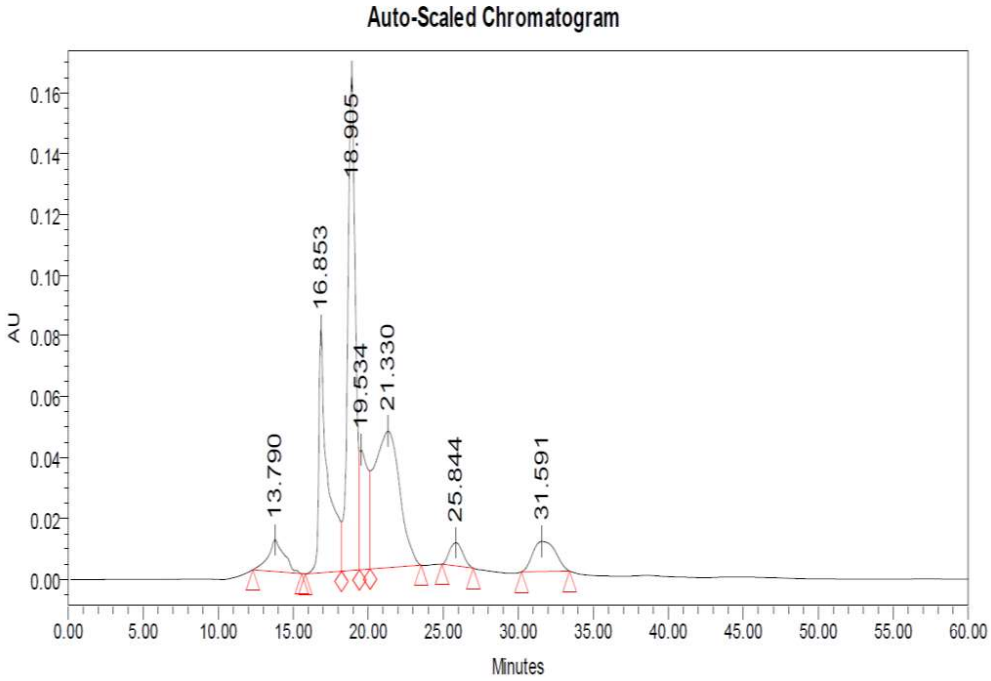

Figure. 6.18: HPLC generated auto-scaled chromatogram of peptide(s) isolated from V1 mature leaves

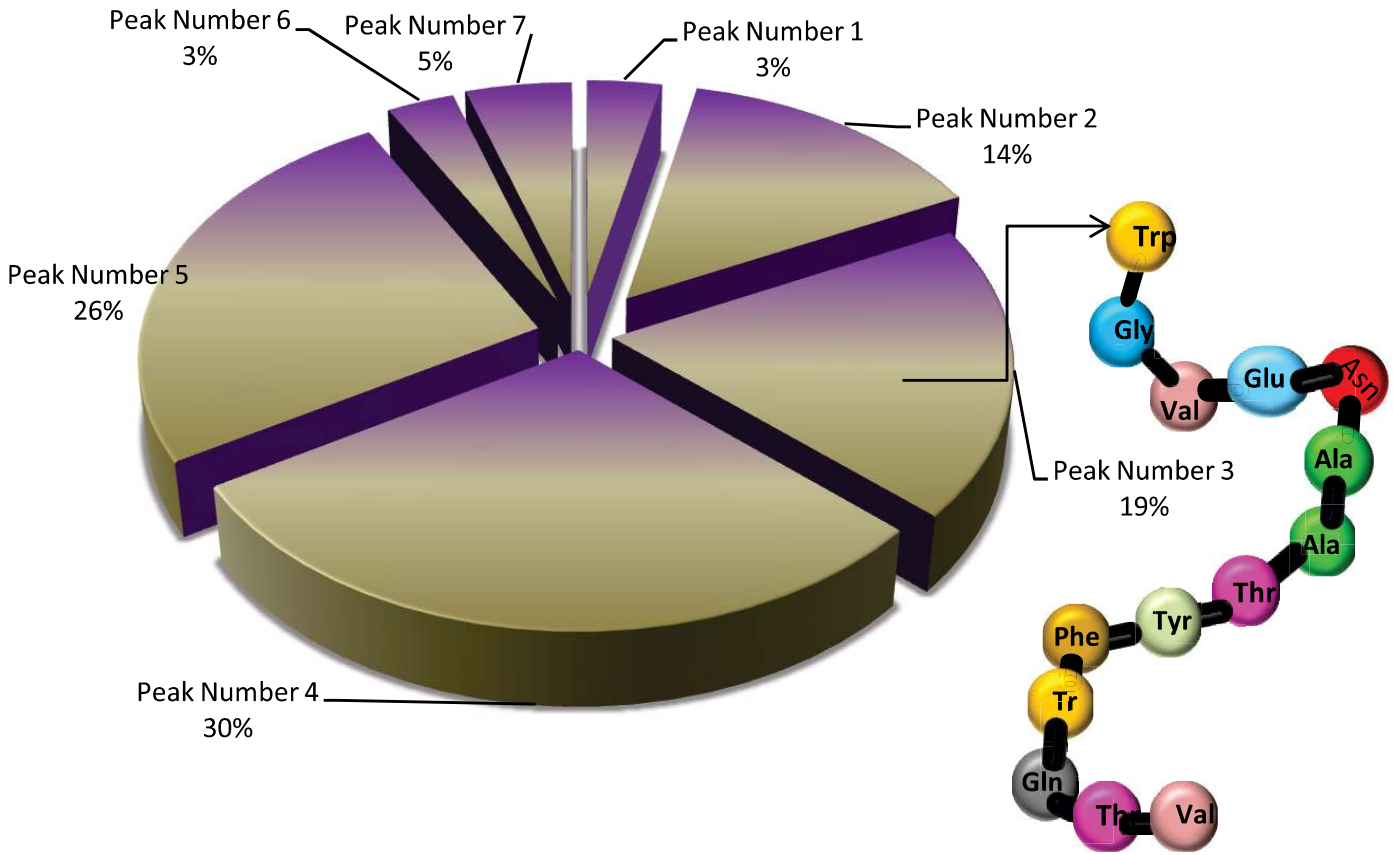

Figure 6.19: HPLC generated auto-scaled peak area of low molecular weight (0.5-3 kDa) peptides isolated from young leaves of S1 mulberry cultivars with amino acid sequence of fraction number 3

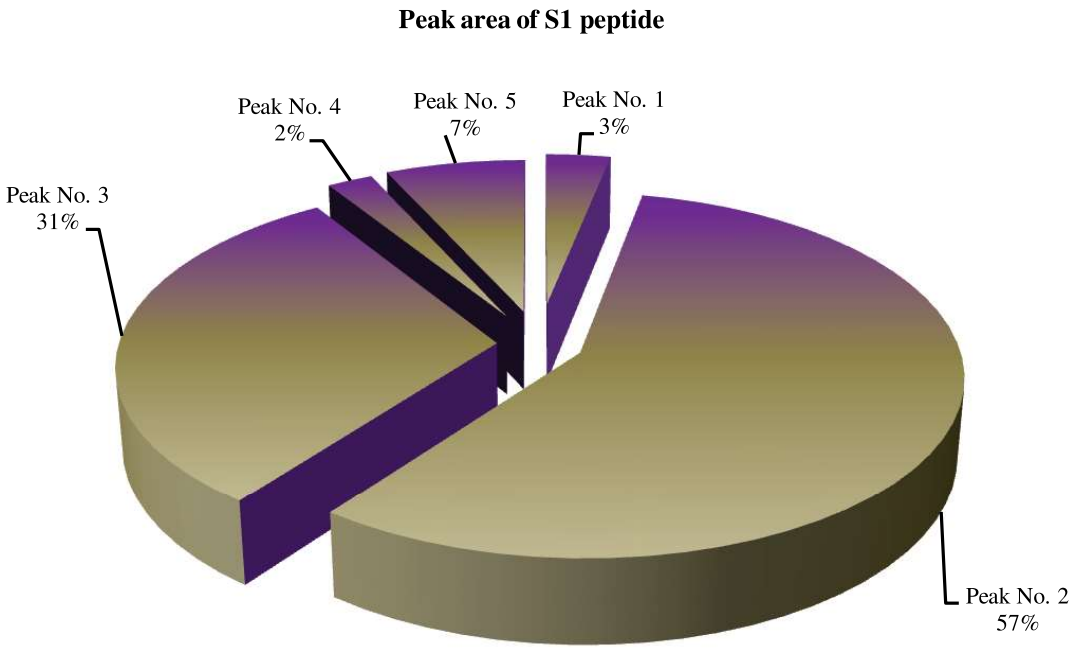

Figure. 6.20: HPLC generated auto-scaled peak area of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of S1 mulberry leaves

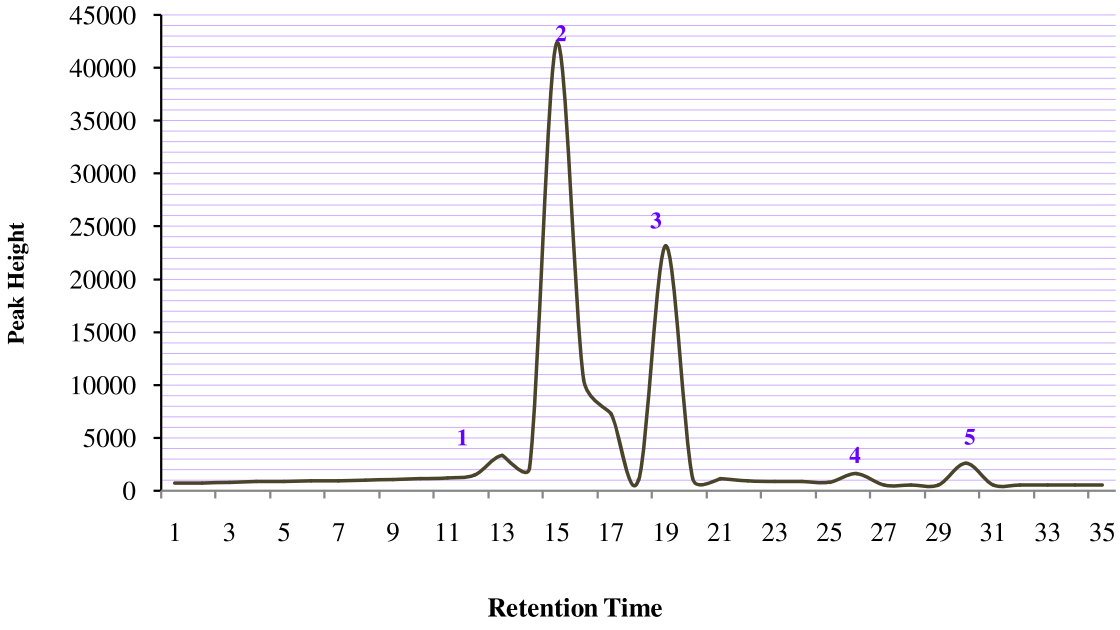

Figure. 6.21: HPLC generated auto-scaled peak height of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of S1 mulberry leaves

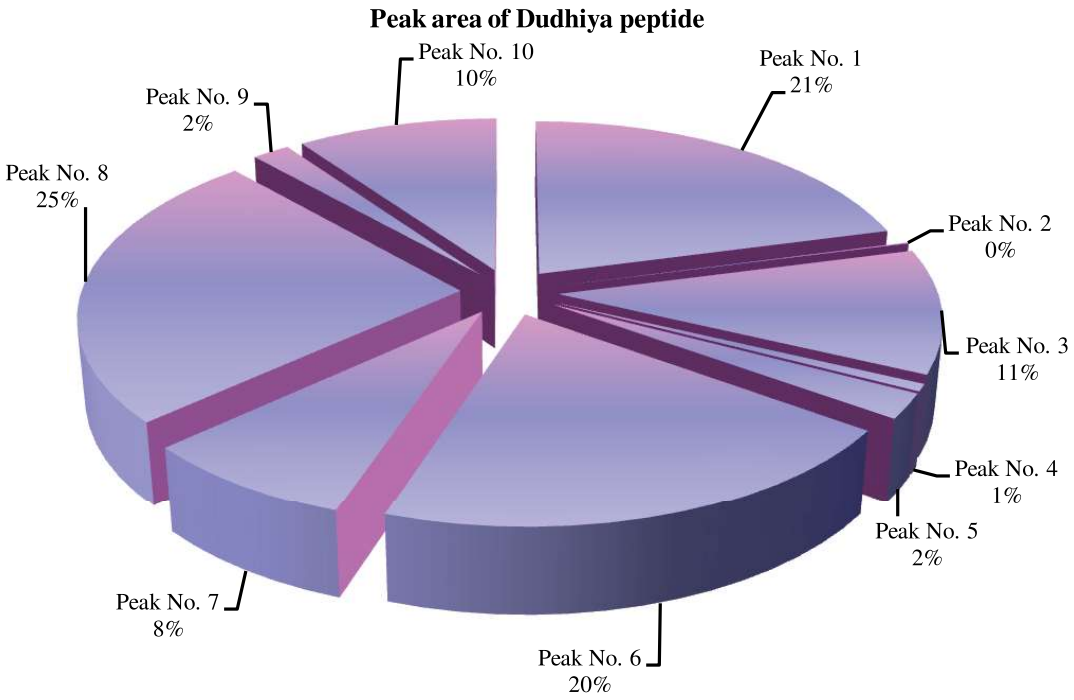

Figure. 6.22: HPLC generated auto-scaled peak area of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of Dudhiya mulberry leaves

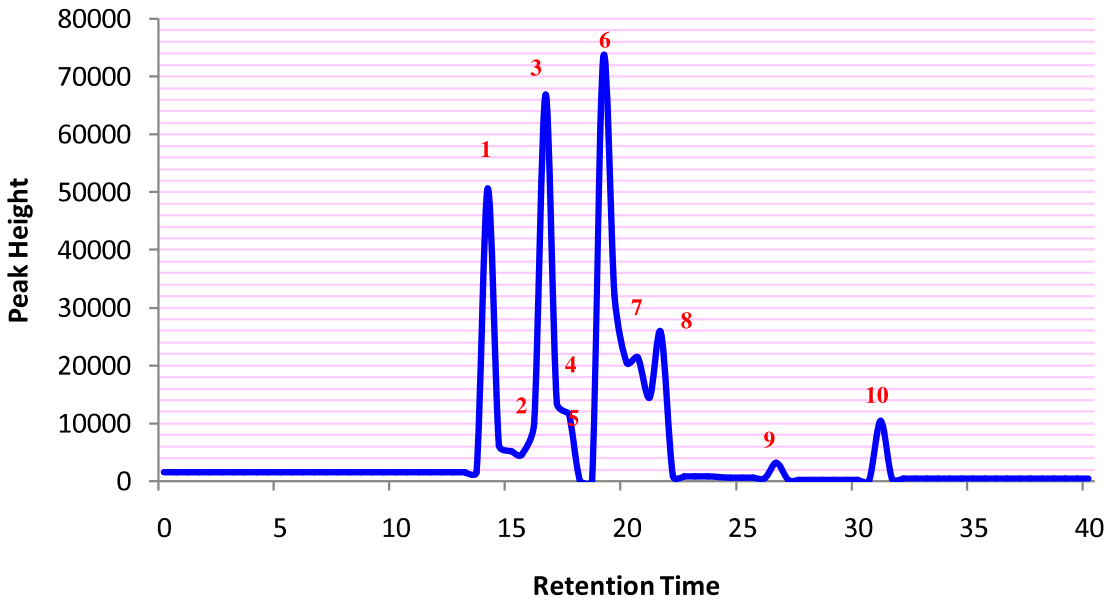

Figure. 6.23: HPLC generated auto-scaled peak height of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of Dudhiya mulberry leaves

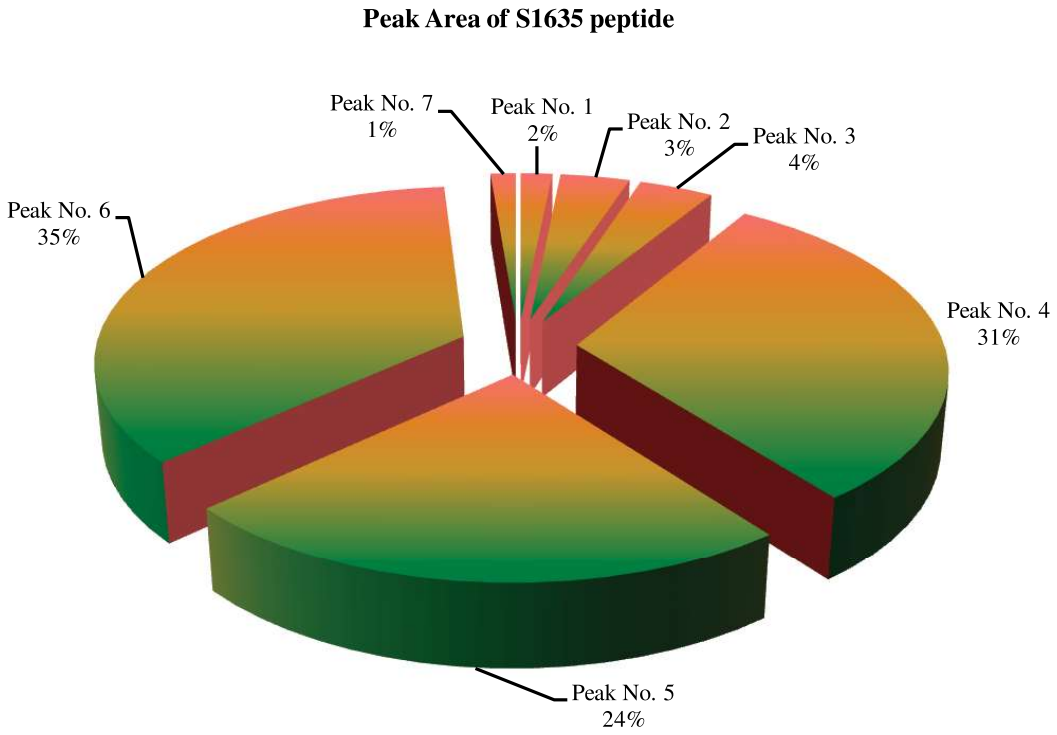

Figure. 6.24: HPLC generated auto-scaled peak area of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of S1635 mulberry leaves

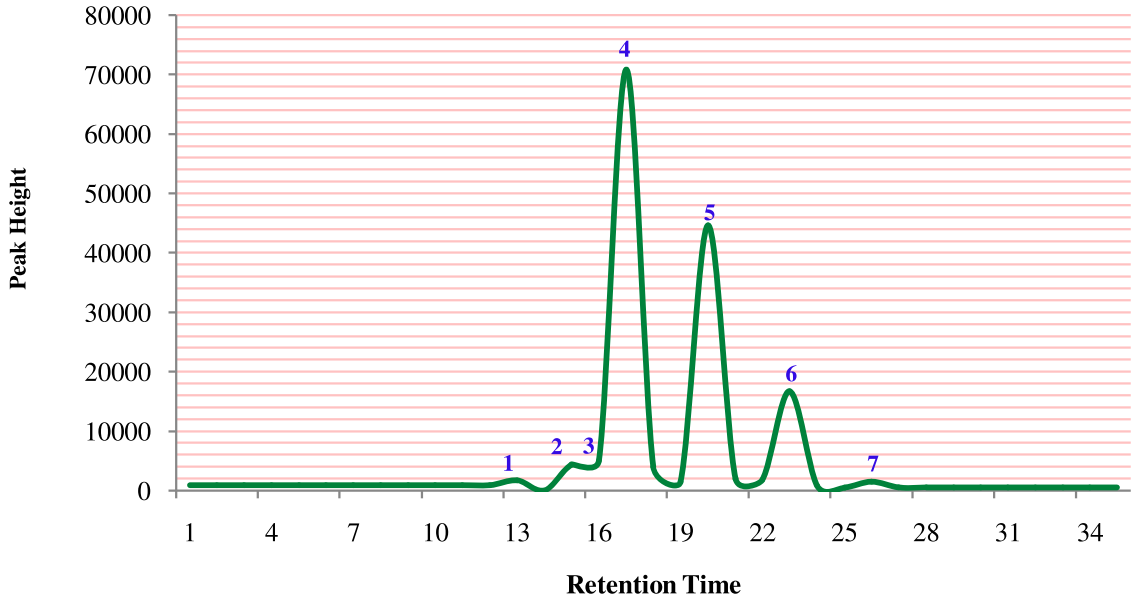

Figure. 6.25: HPLC generated auto-scaled peak height of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of S1635 mulberry leaves

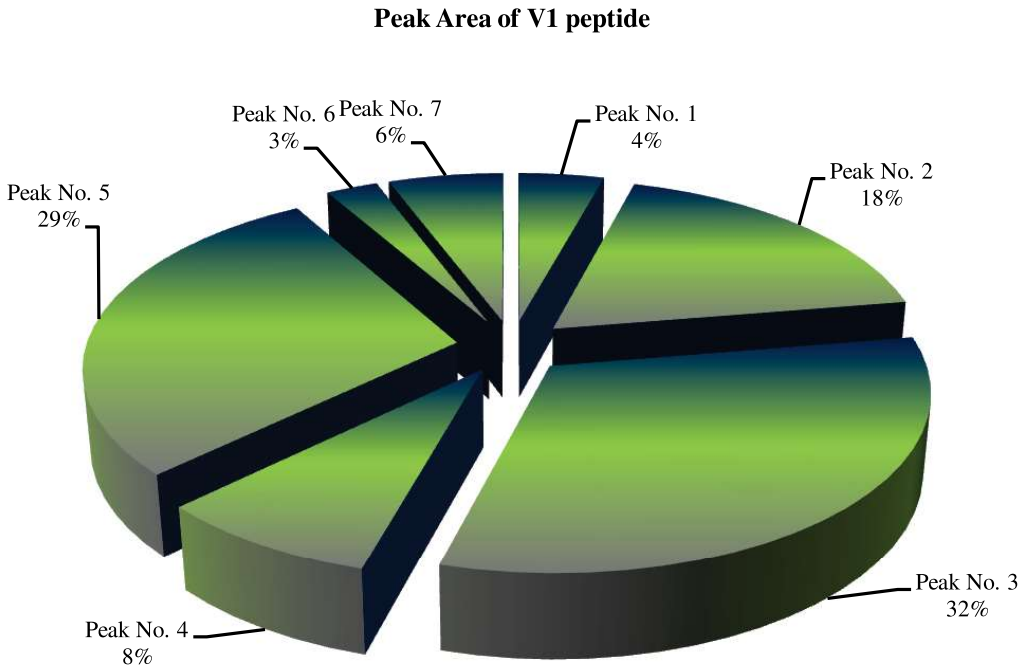

Figure. 6.26: HPLC generated auto-scaled peak area of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of V1 mulberry leaves

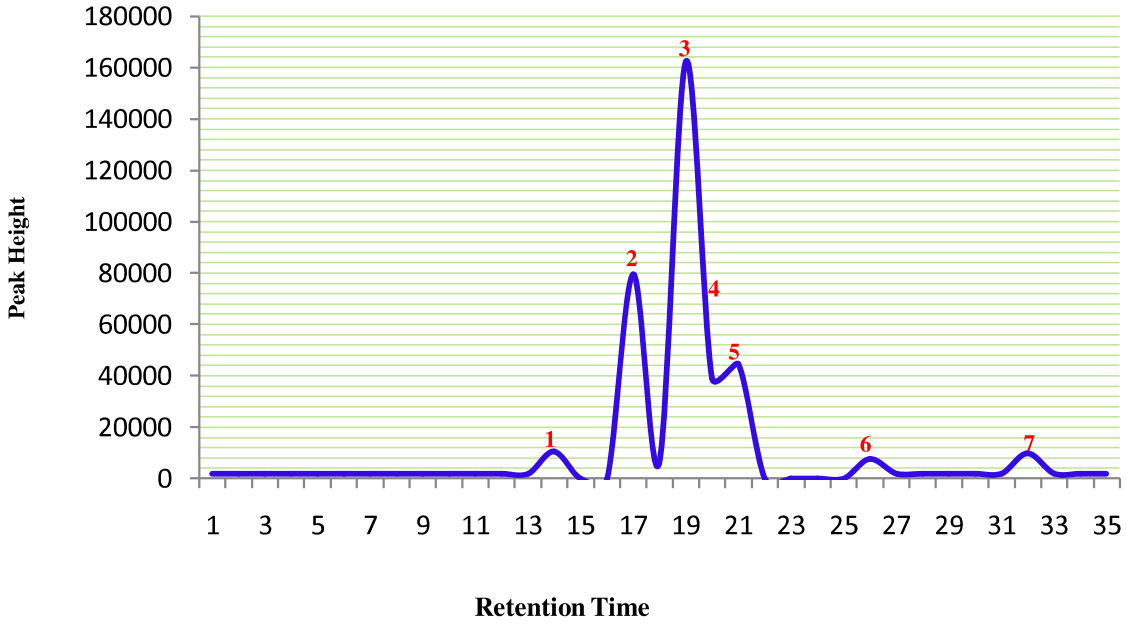

Figure. 6.27: HPLC generated auto-scaled peak height of low molecular weight (0.5-3 kDa) peptides isolated from mature leaves of V1 mulberry leaves

Table 6.2 Mulberry peptides (isolated from S1 mature leaves) sequence similarity with other peptide or protein sequence (NCBI data base)

Sl. No.	Name of the protein	Source	Sequence similarity	Score	Identity	Positive	Gap	Expected function
1.	Cathepsin B precursor	*Bombyx mori*	WGVENAATYFW WGVEN----Y-W WGVENDNKY-W	22.3 bit (45)	7/11 (64%)	7/11 (63%)	1/11 (9%)	Involved in the bulk degradation of intracellular and endocytosed proteins
2.	Bombyxin B-8 precursor	*Bombyx mori*	ATYFWQ A-YFWQ APYFWQ	21.0 bit (42)	5/6 (83%)	5/6 (83%)	0/6 (0%)	Helps in insect molting and metamorphosis. It plays important roles in sugar metabolisms, ovarian development and growth of insects
3.	Ribosephosphate pyrophosphokinase-3	*Solanum tuberosum*	ENAATYFW----------QTV EN----TYFW----------QTV ENGMTYFWITDSCPQTV	26.5 bits (55)	9/17 (53%)	9/17 (52%)	6/17 (35%)	Involved in the synthesis of purine and pyrimidine, Cofactor likes NAD, NADP and amino acid *His* and *Try*
4.	Ribosephosphate pyrophosphokinase-3	*Lycopersicon esculentum*	ENAATYFW------QTV EN-----TYFW------QTV ENGMTYFWITDSCPQTV	26.5 bits (55)	9/17 (53%)	9/17(52 %)	6/17 (35%)	Involved in the synthesis of purine and pyrimidine, Cofactor likes NAD, NADP and amino acid *His* and *Try*
5.	Methyltransferase-like protein	*Fragaria vesca subsp. vesca*	WGVENAATYFW WGV-------AT--FW WGVTHVATFFW	26.5 bits (55)	7/11 (64%)	8/11 (72%)	0/11 (0%)	Has a regulatory role in protein-protein interaction, protein-DNA interaction and protein activation

The sequence of S1 peptides includes 14 amino acids which are "WGVENAATYFWQTV" with 100% reliability observed after 4th cycle of analysis is Try-His-Lys-Ala- followed by Ala-Try-Glu-Gly and Ala- Try- Pro-Asp as well as Try, Asp, Lys and Gly. On the other hand Dudhia mature peptides had 15 amino acids namely Trp-Glu-Trp-Phe-Ser-Trp-Ser-Glu-Trp-Met-Trp-Trp-Trp-Met-Trp (WEWFSWSEWMWWWMW) with 87.7% reliability after 4th cycle.

Psi-Blast analysis from the protein database (NCBI) of isolated peptide sequences showed partial identity especially with developmental proteins of *Bombyx mori* and other plant species like *Lycopersicon esculentum*, *Fragaria vesca* etc as demonstrated in Table 6.2. The S1 peptide raction exhibited sequence similarity with the Cathepsin-B precursor and Bombyxin-B8 precursor, both obtained from *B. mori* and in case of Cathepsin B the sequence similarity was identical at 64% with an appearance bit source of 22.3 and a possible gap in amino acid was 9%. Bombyxin B precursor on the other hand showed 83% identical sequences. Biological activity of peptide depends on their amino acid composition (Korhonen and Pihlanto, 2003). Peptide containing aromatic amino acid (*Try* and *Phe*) had strong antioxidant activity (Comfort *et al.,* 2011). Some amino acids in presence of their aromatic side chain like *Trp* and *Tyr* (indolic and phenolic group respectively) acts as strong free radical scavenger (Guo *et al.,* 2009). Saiga *et al.* (2003) stated that the presence of hydrophobic amino acids like *Phe, Ala*, in peptide shows higher free radical scavenging activity. Our noble S1 peptide contains *Phe* an aromatic amino acid, *Tyr* a heavy amino acid, and also hydrophobic (*Phe, Ala*) amino acid which are all responsible for high antioxidant activity of peptides. Thus, it can be stated that the existence of specific amino acids in peptides are responsible for enhanced antioxidant activity (data shown in chapter V).

6.4 CONCLUSION

Low molecular weight (0.5-3 kDa) mulberry peptides are diverse peptides differing in their amino acid composition and that display wide scale of various free-radical scavenging potentiality. The amino acid composition differs with the variation of mulberry genotypes. Profile study of bioactive peptides through thin layer chromatography revealed that several small peptides were degenerated during maturation of mulberry leaves. Low molecular weight oligopeptides were extensively purified by C18 RP-HPLC. The formation of multiple peaks for compositionally pure peptide sample might be attributed due to presence of multiple smaller peptide chains which was denaturised and separated during separation process using RP-HPLC. Complex oligopeptides was separated and picomolar-fetomolar amount of peptide sample was collected for amino acid sequencing. Peptides isolated from

S1 mulberry leaves had fourteen amino acids whereas, peptides isolated from Dudhiya had fifteen numbers of amino acids. The fewer number of amino acid might be agreed with the lower molecular weight of isolated sample. The presence of aromatic, hydrophobic and heavy amino acids like Tyr, Phe, Ala were responsible for antioxidant potentiality of isolated mulberry peptides. The present study reflects the general explanation related with the behaviour that mulberry peptides had a role in oxidative stress management in silkworm larval growth and development (mention in chapter V).