RESULTS AND DISCUSSION

Mulberry (*Bombyx mori*) and Eri (*Philosamia ricini*) silkworm larvae are basically reared for obtaining cocoons and silk production. Healthy 5th instar larvae were used as biological material in the present study for the induction of antimicrobial peptides (AMPs). Healths of silkworm larvae are determined in terms of rearing performance.

5.1 Rearing performance of Silkworm larvae:

Rearing performance were determined by using many parameters like larval weight, pupal weight, cocoon weight, cocoon shell ratio, silk filament length (Ruth *et al.*, 2014), moulting period, larval duration, larval width, effective rate of rearing (ERR), Denier (Kumar *et al.*, 2014), peduncle, width of shell, shell thickness, fecundity (Kavane, 2014), moth emergence (Shivashankar and Chandan, 2014), larval length, pupal width, consumption rate, food utilization rate, digestibility rate, food consumption index, growth rate of larval, spinning days (Kumar and Balasubramanian, 2014), ingesta, digesta, excreta (Kedir *et al.*, 2014), % Reliability, Renditta (Yogananda *et al.*, 2013), Neatness, Boil off ratio (Gowda *et al.*, 2013), larval mortality, pupal mortality (Fazli *et al.*, 2013), % Pupation (Rahmathulla, 2012), hatchability, survival rate (Kedir *et al.*, 2013), moth weight, moth wing expansion, moth body length (Wankhade *et al.*, 2014) etc. All these parameters have significance in sericulture and analyzed outputs will help for better rearing with huge yield of silk. In the present study healthy 5th instar larvae were used, hence performance was determined of hatching and larval period only. Rearing performance of these larvae were determined by studying days required for hatching, duration required for completion of instar, duration required for completion of moults, larval weight and larval length. Along with these numerical values few morphological changes were also noticed.

In general 11 to 14 days were required for hatching of silkworm eggs by using artificial methods (Ye and Hu, 1996). In the insects diapause is a extraordinary adaptation to avoid the unfavorable environmental conditions during the egg hatching (Manjula and Hurkadli, 1995). If diapause eggs are left under natural condition, embryos grow up to certain stage and growth ceases to enter into diapauses (Veda *et al.*, 1997). Without any type of delay spring generation diapause eggs will produce offspring, whereas summer generated diapause eggs hibernated throughout autumn and winter and then hatched in the forth coming spring (Kubota *et al.*, 1979; Manjula and Hurkadli, 1995). A multivoltine race does not generally require treatment for diapauses breaking. If
diapauses will be subjected to an artificial treatment for a proper period, it is possible to break the diapause (Rahman and Ahmed, 1989; Saheb et al., 1991; Hurkadli, 1997). Various physical and chemical means are used for breaking the diapauses (Veda et al., 1997). Physical means viz. temperature and friction treatment, hot water, air pressure, electric treatment, ultraviolet treatment, supersonic treatment etc. were performed to break diapauses. Under the chemical means HCl treatment, nitric acid treatment, sulphuric acid treatment, enzyme treatment, ozone treatment and perchloride treatment etc. have been induced to break the diapauses (Rahman and Ahmed, 1989; Saheb et al., 1991; Hurkadli, 1997; Veda et al., 1997). Most influenced and routine method for artificial hatching is acid treatment (Krishnaswami et al., 1973).

Present study showed Mulberry and Eri silkworm larvae as five instars in its larval period interspersed with four moults. Greedily all the instar period larvae are feeding on leaves of mulberry (mulberry silkworm) or castor (Eri silkworm). Feeding is stopped by all larvae during the moulting period. Larvae cast off their old skin and take rest by keeping head held up during moulting. Shining bodies of instar larvae become translucent, loose and wrinkled; hence overall appearance is quite dull in the moults. Knowingly or unknowingly these moults are just to be slowly searching something and secret sticky substance.

5.1.1 Morphological changes during hatching of Mulberry Silkworm:

First step of rearing is hatching of eggs. These eggs were obtained as egg cards. Small tiny eggs or Disease Free Layings (DFLs) were 1 to 1.3 mm in lengths and 0.9 to 1.2 mm widths. After incubation for 8 to 10 days, blue coloured head pigments were observed through the egg shell (eye spot stage). On the subsequent day, whole eggs were appeared bluish-black through the egg shell (blue egg stage). Larvae were hatched at the morning of successive day when black-box were kept in brighten light in between 7 am to 9 am. Newly hatched larvae were black or dark brown in color, whereas smoother and lighter (Pale or gloomy) colors were observed as maturation progresses. Hatching period required 10 days.

5.1.2 Morphological changes during hatching of Eri Silkworm:

Eri silkworm eggs are ovoid, dull white with 1.5mm lengths to 1.0 mm width. Color of eye spot and blue egg stage are different in Eri silkworm. Previous to hatching color of the shell changes from whitish to yellowish, then ashy and finally blackish. These tiny worms come out leaving empty white colored egg shell. Eggs usually hatch in the morning between 7am to 10am. Larvae were greenish yellow and body as showed
efficiently roofed by hairs, hence appearance look like hairy caterpillars. At the end of 3\textsuperscript{rd} day larvae were pure yellow and in the 3\textsuperscript{rd} instar i.e. mature stage it becomes yellow, translucent and covered with white powdery substance. Hatching period was 11 days for Eri silkworm respectively.

Natural or artificial Diapauses are broken either by natural means or artificial methods results into hatching of silkworm eggs within 10 to 11 days (Rajan \textit{et al.}, 1996). Different methods of hatching such as Black pot, Black box, Black paper bag, News paper bag, Zero energy chamber and open try have showed hatching of \textit{Bombyx mori} eggs within 9 to 10 days (Sudhakar \textit{et al.}, 1999). Generally duration of hatching in Eri silkworm is 10 days (Krishnaswami \textit{et al.}, 1988). Duration of hatching in mulberry and Eri silkworms were showed similarity with above mentioned results of researchers, which concludes that incubation of eggs were performed properly by maintaining better incubation conditions.

5.1.3 Duration-wise performance in Mulberry silkworm larvae:

First instar and 1\textsuperscript{st} moulting of mulberry silkworm larvae were completed within 3 days and 18 hours, where as 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th} and 5\textsuperscript{th} instars were completed in 3, 4, 5 and 7 days respectively. Second, third and forth moultings were completed within 25, 27 and 32 hours respectively. Late mouls were taking longer time to enter into instar (Table 5.1).

<table>
<thead>
<tr>
<th>Mulberry silkworm stages</th>
<th>1\textsuperscript{st}</th>
<th>2\textsuperscript{nd}</th>
<th>3\textsuperscript{rd}</th>
<th>4\textsuperscript{th}</th>
<th>5\textsuperscript{th}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instar duration (Days)</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Molt’s duration (Hours)</td>
<td>18</td>
<td>23</td>
<td>23</td>
<td>27</td>
<td>spinning</td>
</tr>
</tbody>
</table>

In the past study effect of two mulberry varieties showed 24 days were required for completion of larval period (Kumar \textit{et al.}, 2013). In the past study carried out by using five improved mulberry varieties as a feed 3\textsuperscript{rd}, 4\textsuperscript{th} and 5\textsuperscript{th} instars and 2\textsuperscript{nd} and 3\textsuperscript{rd} moulting were also studied for its larval duration. Larval duration of 3\textsuperscript{rd} instar larvae were seen in between 3.01 to 3.15 days, 4\textsuperscript{th} instar 4.02 to 4.19 days and 5\textsuperscript{th} instar 6.84 to 7.12 days. Similarly in the past study moulting as 2\textsuperscript{nd} moult duration were required as 36.38 to 36.75 hours and 3\textsuperscript{rd} molting period of 35.38 to 38.25 hours (Kumar \textit{et al.}, 2014). Rearing performance of three way cross breeds have showed larval duration 21.25 to 22.75 days (Gowda \textit{et al.}, 2013). During the study of rearing performance in the long and short rainy locations larval duration were 26.53 to 33.47 days respectively as mentioned by Nguku \textit{et al.}, 2009. Temperate mulberry varieties feeding and spring
rearing had showed results of duration required for instar and moulting of mulberry silkworm. Duration required for larvae to complete the instar was as follows, 1<sup>st</sup> instar (5 to 6 days), 2<sup>nd</sup> instar (5 to 6 days), 3<sup>rd</sup> instar (5 to 5.5 days), 4<sup>th</sup> instar (6.5 to 7 days) and 5<sup>th</sup> instar (8.5 to 9 days). Duration required for larvae to remain in moulting stage as, 1<sup>st</sup> moulting (1.5 days), 2<sup>nd</sup> moulting (1 day), 3<sup>rd</sup> moulting (1 to 2 days) and 4<sup>th</sup> moulting (2 to 2.5 days). Total feeding days were 32 to 34 days, while moulting and spinning required 5 to 7 days (Singh et al., 2012). Feeding of admixture of different mulberry leaves were resulted into growth of larvae showed as 24.23 to 27.33 days larval duration (Kalshetti et al., 2014). In general 25 to 31 days were required for complete larval cycle only (Kalita and Dutta, 2014). In present study the approximate larval duration of mulberry silkworm were completed within 26 days, as compared with the above said researchers, even duration wise study of mulberry silkworm larvae’s instars and moults were also showed quite better performance.

5.1.4 Duration-wise performance in Eri silkworm larvae:

First instar and 1<sup>st</sup> moulting of mulberry silkworm larvae were completed within 3 days and 22 hours, whereas 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> instars were completed in 4, 4, 5 and 6 days respectively. Second, third and fourth moults were completed within 25, 27 and 32 hours respectively as shown in the table.5.2.

<table>
<thead>
<tr>
<th>Mulberry silkworm stages</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt;</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt;</th>
<th>4&lt;sup&gt;th&lt;/sup&gt;</th>
<th>5&lt;sup&gt;th&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instar duration (Days)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Molt’s duration (Hours)</td>
<td>22</td>
<td>25</td>
<td>27</td>
<td>32</td>
<td>spinning</td>
</tr>
</tbody>
</table>

In the past review feeding of different castor genotypes to the Eri silkworm were showing 24.33 to 25 days larval duration (Kedir et al., 2013). October to November rearing of Eri silkworm at the Vidarbha (Maharashtra) were showed variation in life cycle duration with its race viz. greenish blue (40.04 days), yellow zebra (40.06 days), yellow plain (40.16 days) and greenish blue plain of 40.18 days (Wankhade et al., 2014). Autumn rearing of Eri silkworm on castor, tapioca, barera and papaya showed change in different larval durations as 20, 21.12, 22 and 24 days respectively, while spring rearing also showed variation as in castor (19 days), tapioca (21 days), barera (22.12 days) and papaya as 23 days (Kumar and Gangwar, 2010). Rearing of Eri in monsoon season at Uttar Pradesh showed 19.12 to 20 days larval duration (Kumar and Elangovan, 2010). Effect of seasonal variation and different host plants at Tamilnadu showed that variation
in larval duration in Eri silkworm. Throughout the year 19 to 25 days were required for larval growth and 2.5 to 3 days were taken during moulting. Time required for completion of 1st instar (3.5 to 4 days), 2nd instar (4 to 4.5 days), 3rd instar (3.5 to 4 days), 4th instar (4.5 to 5 days) and 5th instar of 6.5 to 7.5 days (Subramanianan et al., 2013). Effect of independent and sequential feeding of different host plants to Eri silkworm showed variation in larval duration from 19 to 24.67 days (Venu and Munirajappa, 2013). Study at agro climatic conditions of Western Odisha showed that races also shows variation in larval duration viz. Borduar (24 days), Titabar (22 days) and 21 day for Mendipathar (Ray et al., 2010). Host plants were showing effect on rearing of Eri silkworm such as castor (19.25 days), tapioca (20.50 days), papaya (22 days) and 22 days on jatropha (Kumar and Elangovan, 2010a). In the previous study the efficacy of castor genotypes for rearing of Eri silkworm showed at 1st instar requires 4 to 4.5 days, 2nd instar 3.5 to 4 days, 3rd instar 4 to 4.5 days, 4th instar 4.25 to 4.5 days and 5th instar 7 to 7.5 days i.e. total larval duration required as 22.75 to 25 days (Chandrashekhar et al., 2012). Host plants and seasonal variation study showed the variation in larval duration as spring (February to March) 17 to 22.33 days, summer (June to July) 17.33 to 22 days, autumn (September to October) 20 to 23.33 days and winter (November to January) 17 to 22.33 days (Deka et al., 2011). In general larval duration required for completion of life cycle of Eri is 19 to 29 days (Kalita and Dutta, 2014). The present studies, hatching of silkworm larvae were required approximate 26 days for completion of larval duration previous to entering into spinning stage. Great rearing performance as similarly showed by Eri silkworm larvae with respect to duration required for completion of instars and molts.

5.1.5 Rearing performance based on lengths of Mulberry silkworm larvae:

In the present study 4.86±0.85 cm/larva average lengths of mulberry silkworm were observed (Table 5.3). In past study feeding of five improved varieties to the mulberry silkworm has resulted into variation in larval length as 6.44 to 6.61 cm/larva (Kumar et al., 2014). In the earlier experimental study effect of mulberry leaves and leaves treated with spirulina were showed average length 6.42 cm/larva (Valantina et al., 2014). In general Bombyx mori are showing length approximately 4 cm (Kalita and Dutta, 2014). In the present study lengths of mulberry silkworm larvae were showed within the range, which concludes better rearing performance.
Table 5.3: Lengths of fifth instar Mulberry silkworm larvae

<table>
<thead>
<tr>
<th>Lengths of Mulberry silkworm larvae (cm/larva)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
</tr>
<tr>
<td>4.1</td>
</tr>
<tr>
<td>3.1</td>
</tr>
</tbody>
</table>

Mean: 4.86, Mean Deviation: 0.8586, Sample variance: 22.8819, Stand Deviation: 4.7835, Coefficient of Variation: 98.4261

5.1.6 Rearing performance based on lengths of Eri silkworm larvae:

In the present study average length of Eri silkworm 5th instar larvae were 7.01±0.42 cm/larva (Table 5.4). Past study resulted in the morphological differences of *Samia ricini*, *Samia canningi* and their cross were showed variation in the 5th instar larval length as 8.5, 7.1 and 9.5 cm/larva (Dulur *et al*., 2011). Seasonal variations were profound influence on larval length viz. winter (7.34 to 7.8 cm/larva), rainy (6.54 to 7.20 cm/larva) and in summer 6.54 to 6.88 cm/larva (Renuka and Shamitha, 2014). In general different races of mature (5th instar) Eri silkworm were showed average length in between 6.9 to 8 cm/larva (Kalita and Dutta, 2014). In our study lengths of Eri silkworm larvae were matching with results of other research workers. It concludes that rearing performance was quite better and healthy silkworms were reared.

Table 5.4: Lengths of fifth instar Eri silkworm larvae

<table>
<thead>
<tr>
<th>Lengths of Eri silkworm larvae (cm/larva)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.8</td>
</tr>
<tr>
<td>6.6</td>
</tr>
<tr>
<td>7.8</td>
</tr>
</tbody>
</table>

Mean: 7.0133, Mean Deviation: 0.42, Sample variance: 5.4744, Stand Deviation: 2.3397, Coefficient of Variation: 8.0681

5.1.7 Rearing performance based on weights of Mulberry silkworm larvae:

Average weight of 5th instar mulberry silkworms were 3.99±0.66 g/larva (Table 5.5). In previous study feeding of mulberry silkworm with local mulberry variety and Chakmajra variety in spring season has showed 4.62 and 5.05 g/larva weight of silkworm (Kumar *et al*., 2013). Feeding on three different mulberry genotypes showed 4.13 to 4.88 g/larva weight (Ruth *et al*., 2014). Five germplasm mulberry varieties also have showed effect on 5th instar larval weight from 2.62 to 3.90 g/larva (Yogananda *et al*., 2013). Feeding with five improved mulberry varieties has showed variation in larval weight as 3.27 to 3.45 g/larva (Kumar *et al*., 2014). Rearing performance of three way cross breed
mulberry silkworms have showed 1.67 to 1.87 g/larva larval weight (Gowda et al., 2013).

Table 5.5: Weights of fifth instar Mulberry silkworm larvae

<table>
<thead>
<tr>
<th>Weights of Mulberry silkworm larvae (g/larva)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.55</td>
</tr>
<tr>
<td>5.05</td>
</tr>
<tr>
<td>4.11</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>3.9976</td>
</tr>
</tbody>
</table>

As per previous study Bivoltine six mulberry silkworm varieties grown at short rain and long rain location have also affected larval weight from 3.33 to 4.72 g/larva (Nguku et al., 2009). Rearing performance of bivoltine hybrid silkworm feed on temperate mulberry varieties in spring season has showed 4.7 to 4.9 g/larva larval weight (Singh et al., 2012). Weight of mulberry silkworm were also affected by Spirulina treated MR2 mulberry leaves and the average weight of larva were 2.62 g/larva (Valantina et al., 2014). Feeding of admixture of different mulberry leaves to the mulberry silkworm has showed changed larval weight as 3.6 to 3.9 g/larva (Kalshetti et al., 2014). In general larval weight of 5th instar larvae of mulberry silkworm were 2.78 g/larva (Kalita and Dutta, 2014). Weights of reared 5th instar larvae were approximately 4 g/larva which concludes that feeding were carried out using good quality mulberry leaves. Larval lengths were greater than average lengths of larvae discussed in above said research work, that is rearing was quite successful.

5.1.8 Rearing performance based on weights of Eri silkworm larvae:

Average weights of 5th instar Eri silkworms were 5.88±0.99 g/larva (Table 5.6). In the past reports feeding with different castor leaves has showed variation in the weight of Eri silkworms as 7.6 to 8.20 g/larva (Kedir et al., 2013). Rearing of Eri silkworm at Vidarbha region of Maharashtra during month of October to November have showed different weights of larva viz. greenish blue zebra (7.33 g/larva), yellow zebra (7.25 g/larva), yellow plain (6.68 g/larva) and greenish blue plain of 6.66 g/larva (Wankhade et al., 2014). Effect of different food plants on the weight of 5th instar Eri silkworm were showing variation according to season and food plants used viz. in autumn season (September to October) feeding on castor (7.45 g/larva), tapioca (6.82 g/larva), barrera (6.05 g/larva) and papaya (5.35 g/larva), while reared in spring season (February to March) also affect the weight of 5th instar larvae viz. feeding on castor (7.6 g/larva),

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tapioca (6.8 g/larva), barera (6.25 g/larva) and papaya 6.65 g/larva (Kumar and Gangwar, 2010). Feeding of eight different castor varieties to the Eri silkworm has given rise to 5th instar larvae having larval weight of 6.71 to 7.34 g/larva (Sarmah et al., 2011). During the Monsoon season rearing of Eri silkworm in Uttar Pradesh has showed larval weight of 6.52 to 7.10 g/larva (Kumar and Elangovan, 2010).

### Table 5.6: Weights of fifth instar Eri silkworm larvae

<table>
<thead>
<tr>
<th>Weights of Eri silkworm larvae (g/larva)</th>
<th>Means</th>
<th>Mean Deviation</th>
<th>Sample Variance</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.43</td>
<td>4.20</td>
<td>3.92</td>
<td>4.40</td>
<td>7.27</td>
<td>6.33</td>
</tr>
<tr>
<td>7.21</td>
<td>7.26</td>
<td>5.65</td>
<td>4.70</td>
<td>5.55</td>
<td>3.67</td>
</tr>
<tr>
<td>5.31</td>
<td>7.10</td>
<td>6.76</td>
<td>7.12</td>
<td>5.47</td>
<td>6.75</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Deviation</td>
<td></td>
<td>0.9948</td>
<td>30.7150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Variance</td>
<td></td>
<td>3.07150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>5.5421</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient of Variation</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Various six strains of Eri silkworm up on rearing in autumn and spring has showed variation in 5th instar larval weight as 6.11 to 6.57 g/larva and 5.42 to 6.56 g/larva respectively (Sharma and Jogen, 2013). During the rearing performance study of Eri silkworm with respective to the seasonal variation had showed variation in larval weight viz. April to May (5.09 g/larva), May to June (7.02 g/larva), July to August (6.46 g/larva), August to September (6.66 g/larva), October to November (7.32 g/larva), November to January (5.98 g/larva) and February to March of 5.92 g/larva (Sarmah et al., 2012a). In the relational study of rearing of Eri silkworm throughout the year, there were change in larval weight viz. effect of bed cleaning (6.30 to 6.80 g/larva), effect of feeding (5.90 to 6.87 g/larva), effect of population density (6 to 7.27 g/larva) and influence of rearing methodology as 6.2 to 7.6 g/larva (Subramanianan et al., 2013). Larval weight of Eri silkworm were also varied in accordance to seasonal variation winter (5.53 to 7.74 g/larva), rainy (4.60 to 4.84 g/larva) and summer as 3.43 to 3.69 g/larva (Renuka and Shamitha, 2014). In the previous study the effect of food plants and seasonal variation again proved that there was change in larval weight viz. spring (9.01 to 10.60 g/larva), summer (9.41 to 11g/larva) and late summer 8.50 to 9.04 g/larva (Baruah, 2012). Influence of independent and sequential feeding of different food plants had showed changed larval weight from 5.78 to 7.53 g/larva (Venu and Munirajappa, 2013).

Effect of castor food plant has showed variation in larval weight with respect to season viz. autumn (5.92 g/larva), winter (5.67 g/larva), spring (6.07 g/larva) and summer (5.68 g/larva), while castor replaced by kesseru in the autumn (4.97 g/larva), winter (4.90 g/larva), spring (4.20 g/larva) and in summer 4.85 g/larva (Singh et al.,
Rearing performance of Eri silkworm in agroclimatic conditions of Western Odisha has shown variation in larval weight with respect to race viz. Borduar (9.8 g/larva), Titabar (9.5 g/larva) and Mendipathar with 9.2 g/larva (Ray et al., 2010). Various food plants also affect the larval weight of 5th instar larvae as castor (7.38 g/larva), tapioca (6.45 g/larva), papaya (6.18 g/larva) and jatropha with 5.55 g/larva (Kumar and Elangovan, 2010a). Various castor genotypes when used as food plants for the growth of Eri silkworm also affect the larval weight from 6.22 to 7.52 g/larva (Chandrashekhar et al., 2012). Seasonal variation has an effect on larval weight spring (February to March), summer (June to July), autumn (September to October) and winter (November to January) as g/larva 6.05 to 7.38, 5.53 to 6.71, 5.60 to 6.48 and 4.90 to 6.47 respectively (Deka et al., 2011). Different varieties of *Samia ricini*, *Samia canningi* and their crosses had showed variation in larval weight from 5.8 to 7.9 g/larva (Dulur et al., 2011). In general larval weight of *Philosamia ricini* was up to 5.5 to 15 g/larva (Kalita and Dutta, 2014). In our study weights of reared 5th instar larvae were approximately 6 g/larva which concludes that feeding were carried out properly using good quality castor leaves. Larval lengths were matching with average lengths of larvae discussed in above mentioned research work, that is rearing performance were resulted better.

### 5.2 Immune challenging to silkworm larvae:

In the present study *Bombyx mori* and *Philosamia ricini* 5th instar larvae were used for immune challenging experiment. Two fold diluted suspensions of *Escherichia coli* (NCIM 2137) and *Staphylococcus aureus* (NCIM 2672) were mixed to 1:1 ratio and 5μl were induced aseptically into hemocoel of each *Bombyx mori* and *Philosamia ricini* larva. In the previous study silkworm larvae and pupa of *Bombyx mori* has been used for lysozyme study. In the earlier study peptidoglycan induced and non-induced hemolymph of silkworm larvae were observed for lysozyme and cecropin activity. Immune challenging has showed 4.4 to 7.3 times enhanced lysozyme activity and cecropin activity were also observed, which is almost absent in healthy silkworm (Isao et al., 1994). Experiments with three lepidopteran larvae (*Galleria mellonella*, *Bombyx mori* and *Agrius convolvuli*) has showed increased lysozyme activity upon immune challenging by *E. coli*, these lysozymes has showed anti-Gram negative activity, which were absent in chick lysozymes (Kyung et al., 2002). Bacterial clearance in the hemolymph was observed by immune stimulation with *E. coli* treated mulberry leaves upon feeding to *Bombyx mori* (Thangamalar et al., 2010). Infection to the *Bombyx mori* by *S. aureus, E. coli* and *P. pastoris* were showed expression of antimicrobial peptide
synthesis, which was studied by isolation of mRNA and its expression in *E. coli* using recombinant DNA technology (Wanying et al., 2011). Purified peptidoglycan from *E. coli* and *Bacillus megaterium* were also as an inducer of antimicrobial peptides synthesis in *Bombyx mori* and peptidoglycan recognition were also showed to be independent of prophenoloxidase activity (Isao et al., 1992). Experiments with *Bombyx mori* and *M. sexta* revealed that spatzle were required for effective induction of antimicrobial peptide synthesis (Yang et al., 2007).

Antimicrobial activity was showed enhanced activity in pupae of *Samia cynthia* (Eri silkworm) after immune challenging by *E. coli* and *E. cloacae* (Hans et al., 1974). Immune challenging with *B. licheniformis* peptidoglycan, *E. coli* LPS and Zymosan were resulted into induction of antimicrobial peptide attacin in the *Samia cynthia ricini* Eri silkworm (Keiko et al., 2002). Induction of peptidoglycan recognition protein genes were isolated from fat body of *Samia cynthia ricini* upon challenging by PGN, LPS, Laminarin, Zymosan and Oligo chitin (Kazuhiko et al., 2007) and *B. licheniformis* and its PGN (Hiroko et al., 2007). Hemolin immune protein of lepidopteran were isolated from *Samia cynthia ricini* by inducing with linear PGN (*M. luteus*), LPS (*E. coli*), Laminarin (*Laminaria digitata*), Zymosan (*S. cerevisiae*), Oligo citin and Curdlan. Immune protein prophenoloxidase-activating proteinase were induced for the production by challenging with β-1,3-Glucan in *Samia cynthia ricini* (Yanyuan et al., 2007).

Antibacterial low molecular weight three proteins were isolated from *Antheraea assama* (Muga silkworm) after immune challenging by non-pathogenic *Pseudomonas aeruginosa* (Yjotsna et al., 2005). Immune challenging by *Enterococcus faecium* has induced synthesis of moricin analogue (antimicrobial peptide) in hemolymph of *Spodoptera litura* (Yuki et al., 2005). Immune stimulation by *E. coli* and phenanthroline has induced synthesis of cecropin B and D in Chinese Oak silk moth *Antheraea pernyi* (Xiang-ming et al., 1982).

### 5.2.1 Effect of immune challenging on hemolymph protein content:

In the present study hemolymph protein estimation of 5th instar larvae were performed to study the changes in the protein concentration among healthy and infected larvae. In the healthy 5th instar mulberry silkworm 39.3±1.56 mg/ml protein concentration were estimated from the hemolymph, whereas in the Eri silkworm larvae protein concentration were estimated as 15.6±1.2 mg/ml. After 24 hours of induction immune challenged 5th instar larvae were showed decreased protein concentration as mulberry silkworm 26.8±1.04 mg/ml and Eri silkworm 9.8±0.64 mg/ml (Table 5.7).
Table 5.7: Changes in hemolymph protein concentration

<table>
<thead>
<tr>
<th>Sr. Nos.</th>
<th>Protein content (mg/ml)</th>
<th>Mulberry silkworm larvae</th>
<th>Eri silkworm larvae</th>
<th>Healthy</th>
<th>Challenged</th>
<th>Healthy</th>
<th>Challenged</th>
</tr>
</thead>
<tbody>
<tr>
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<td>25</td>
<td>14</td>
<td>9</td>
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<td>9.1656</td>
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<td>0.006</td>
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<td></td>
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<td>0.0171</td>
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<td></td>
</tr>
</tbody>
</table>

In the past study of infection by BmIFV (*Bombyx mori* Infectious Flacherie Virus) hemolymph protein were decreased by 50% within 12 days over the control hemolymph protein. Three *Bombyx mori* races were showed variation in hemolymph protein concentration as 34.23, 38.20 and 40.12 mg/ml (Yogananda et al., 2014). Enhanced protein level observed in the hemolymph of mulberry silkworm when treated with phytojuvenoid and it has showed 21.58 mg/ml of protein (Srivastava and Upadhyay, 2014). As growth progresses there were increase in protein concentration from 6.31 to 44.11 mg/ml within 12 days, while infection with BmIFV resulted into decreased protein concentration from 6.31 to 22.42 mg/ml (Mamatha and Balavenkatasubbaiah, 2014). Untreated 5th instar Eri silkworm larvae showed 15.1 mg/ml hemolymph protein, while low temperature has induced the enhanced protein concentration as 17.2 to 25.5 mg/ml as time progresses up to 7th day of post treatment (Singh et al., 2010).

Infection study using entomopathogenic nematode to the *Spodoptera littoralis* larvae also affected the protein concentration *viz.* healthy larvae had 17.47 mg/ml protein in the hemolymph, while post infection protein concentration were decreased in *Steinernema feltiae* infection (12.1 mg/ml), *Steinernema riobrave* (9.95 mg/ml) infection and *Heterorhabditis bacteriophora* infection as 8.13 mg/ml (Naglaa et al., 2014).
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Infection by *B. thuringiensis* to *Plodia interpunctella* larvae also showed influence on the hemolymph protein concentration. Untreated hemolymph of said larvae as control crude sample and control TCA precipitated sample were showing 68 mg/ml and 50.4 mg/ml protein concentration respectively, while post infection hemolymph protein were decreased as treated crude sample (58.67 mg/ml) and treated TCA precipitated sample contained 44.8 mg/ml (Aboul-Ela *et al.*, 1991).

*B. thuringiensis* infection to the *Achoea janata* L. were decreased in the protein concentration in hemolymph (Govindarajan *et al.*, 1976), while *S. littoralis* larvae with similar infection showed increased protein content (Slama *et al.*, 1983). Infection by microbial agent to the *Manduca sexta* larvae has changed the protein profiling of midgut and hemolymph (Rupp and Spence, 1984). Infection with nuclear polyhedrosis virus to the 6th instar larvae of *Peridroma saucia* showed hypoproteinemic effect (Martignoni and Milstead, 1964; Martignoni and Milstead, 1966).

Higher molecular weight polypeptides has rise to synthesis of complex proteins. It plays a vital role in the formation of structures and to do various biological functions. When carbohydrates and lipids are not available (adverse condition) protein will also utilized as energy source. Modification or modulation of proteins during stress condition induces alternative pathways in the tissues (Bano *et al.*, 1981; Assem and Hanke, 1983). Very few references are available explaining the relation of infection and its effect on protein metabolism. Presence of high protein content is the indication of better metabolic activity in the tissue. Higher protein content in the whole body parts of silkworm is because of feeding of tender leaves instead of medium or coarse leaves (Hisao, 1994). Accumulation of biomolecules is mostly dependant on leaves quality and quantity consumed by silkworm (Ito and Arai, 1963). Absorption of food constituents’ *viz.* proteins and amino acids from digested mulberry is mostly depending on midgut of larvae (Seo *et al.*, 1985). In the castor 18.23 to 28.74 mg/g protein content were showed on the basis of dry weight (Manjunath and Sannappa, 2012). Diseased larvae stopped the feeding i.e. consumption of organic molecules decreased, hence inactive and small sized larvae were showed in the immune challenged group. Near about 25 to 30 % mortality were also noticed. Infection in the hemocoel also affect the midgut because larvae have open circularity system *i.e.* hemolymph has directly comes in contact with midgut, it will results into infection to gut. Infected larvae showed dysentery and vomiting of gut juice, hence diseased larvae became dull, soft and flaccid previous to death. In the general observation lipid content and carbohydrate content mostly increased during the infection.
If these two biomolecules increases protein content should be declined, similar results were observed in our study conducted with mulberry and Eri silkworm.

5.2.2 Mortality rate due to immune experiments:

In the present study immune challenging were resulted into mortality of larvae viz. mulberry silkworm 28% mortality and 25.5% mortality in Eri silkworm within 24 hours post infection (Table 5.8).

Table 5.8: Mortality in immune challenged Silkworm larvae

<table>
<thead>
<tr>
<th>Mulberry silkworm larvae</th>
<th>Eri silkworm larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Initial larvae</td>
<td>Number of live larvae after immune challenge</td>
</tr>
<tr>
<td>200</td>
<td>144</td>
</tr>
<tr>
<td>100%</td>
<td>72%</td>
</tr>
</tbody>
</table>

% Mortality = 28% Mortality = 25.5

In the earlier mortality study with tasar silkworm (*Antheraea mylitta*) has showed the mortality increases in the outdoor rearing comparative to indoor. Bacteria, viruses, fungi, pebrine, pests, rain fall and shifting of larvae were also responsible for mortality (Kumar and Shamitha, 2013). Infection by microsporidiosis (Pebrine) caused by Nosema species to the tropical tasar silkworm (*Antheraea mylitta*) showed 20 to 28% mortality post infection (Velide and Rao, 2011). Infection by microsporidiosis (Pebrine) caused by Nosema species to the tropical tasar silkworm (*Antheraea mylitta*) showed 5% mortality post infection (Velide and Bhagavanula, 2012). Infection by *Beauveria bassiana* (*1 x 10^5* conidia/ml) to the *Bombyx mori* before and after moulting resulted into 4.62 to 90% mortality (Chandrasekharan and Nataraju, 2011).

Pebrine infected tasar silkworm larvae showed mortality in all instars and also in the rainy, autumn as well as winter season (Mishra et al., 1992). Second and third instar mosquito (*Anopheles stephensi*) larvae were required 31.6 x 10^4 and 7.2 x 10^1 spores of *Beauveria bassiana* as LD50 dose respectively (Geetha and Balaraman, 1999). Infection of *Nosema bombycis* and cross-infection of *N. mylitta* on *Bombyx mori* with respect to temperature and relative humidity showed 36.66 to 57.58% mortality (Satadal and Buddhadeb, 2008). If infection by viral polyhedral occlusion bodies (OBs) to the larvae of *Bombyx mori* as dose of 1300, 1800 and 2000 OBs/larva resulted in mortality as 17.5, 20 and 23% respectively (Khurad et al., 2005). Infection with AdorGV to the *Adoxophyes orana* larvae viz. 1<sup>st</sup> instar to 5<sup>th</sup> instar, showed decreased mortality as 100% (1<sup>st</sup> instar), 96% (2<sup>nd</sup> instar), 72% (3<sup>rd</sup> instar), 40% (4<sup>th</sup> instar) and 12% in 5<sup>th</sup> instar (Karel, 2007). The total mortality in *Bombyx mori* caused by the individual infection of
BmNPV were 10.33 to 55.67% under different concentrations and using with S. faecalis / S aureus, it caused 9 to 56.33% mortality (Selvakumar and Datta, 2013). Second instar Helicoverpa armigera larvae were treated with Bacillus thuringiensis strains recorded mortality in the range of 16.67 to 94.44% (Lalitha et al., 2012).

5.3 Differential protein expression analysis by SDS-PAGE Electrophoresis:

In the present study four hemolymph samples were separated on SDS-PAGE electrophoresis. Hemolymph samples of the control or healthy Mulberry (Bombyx mori) silkworm larvae were used as control (BC) and immune challenged or treated samples (BT), whereas control or healthy hemolymph samples of Eri (Philosamia ricini) silkworm larvae were used as control (PC) and treated sample (PT). Separation of proteins were showed at range of 201.2 kDa to 17 kDa were not clearly observed for differential study as shown in (Fig. 5.1).

![Figure 5.1: SDS-PAGE electrophoresis of hemolymph samples](image-url)

In the earlier study two different biliverdin-binding proteins, designated BBP-I (24 kDa) and BBP-II (48 kDa), were purified by using SDS-PAGE from the larval hemolymph of the Eri silkworm, Samia cynthia ricini (Hitoshi, 1998). Recombinant B. mori cecropins and moricins were differentially separated by Tricine-SDS-PAGE electrophoresis (Wanying et al., 2011). In the past study regarding immune system of
Chinese silk moth (*Antheraea pernyi*) and Cecropia moth, it showed the pupae produces P4 and P5 immune proteins like Cecropia. The immune system in the Chinese oak silk moth, *Antheraea pernyi*, has been compared with that of the Cecropia moth which has been characterized earlier. The pupae produced a set of immune proteins with P4 and P5 as major labeled components similar to that earlier found in Cecropia. The major antibacterial factors in A. pernyi were cecropin D (Xiang-ming *et al*., 1982). Peptidoglycan recognition proteins (20 kDa) were differentially separated in the *Samia cynthia ricini* using SDS-PAGE electrophoresis (Hiroko *et al*., 2007). In the *Bombyx mori* differential protein expression studies were performed by earlier researchers to study the protein profile of different larval instars (Yoshihiro and Okitsugu, 1990) as well as healthy and immune challenged hemolymph samples (Abraham *et al*., 1995). In the present study (healthy) control and immune challenged (treated) hemolymph samples were practiced for separation and differential protein expression analysis, but proper resolutions were not observed in separation, hence more advanced 2 Dimensional Gel electrophoresis were used for further study.

5.4 Differential protein expression analysis by 2D Gel Electrophoresis:

In the present study total four samples as BC, BT, PC and PT were separated on 2D gel electrophoresis and images were obtained using simple computer scanner HP Scanjet G4010. Gel images were then analyzed using Image Master 2D Platinum 7 Software. Expression of two more spots were observed in treated sample of *Bombyx mori* BT (Fig. 5.3) those were missing in control sample BC (Fig. 5.2). As per image Master 2D Platinum software these differential spots were designated as BT53 and BT66, which were produced due to immune challenging experiment and response given by larvae against microorganism. As per results provided by software molecular weight and pI of BT53 and BT66 were showed 28 kDa (pI 4.08) and 98 kDa (pI 9.44) respectively (Fig. 5.3).

In the present differential study of PC and PT samples of *Philosamia ricini* one extra spot was observed in PT sample (Fig. 5.5) more than PC sample (Fig. 5.4). As per image Master 2D Platinum software differentiation spot were designated as PT355. PT355 were showed 96 kDa molecular weight and 9.40 pI. Differentially expressed PT355 protein is the result of indication due to response of larvae upon immune challenge.
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Figure 5.2: 2D Gel of *Bombyx mori* control (BC) hemolymph sample

Figure 5.3: 2D Gel of *Bombyx mori* treated (BT) hemolymph sample
Chapter 5: Results and Discussion

Figure 5.4: 2D Gel of *Philosamia ricini* control (PC) hemolymph sample

Figure 5.5: 2D Gel of *Philosamia ricini* treated (PT) hemolymph sample
In the past study 2D Gel electrophoresis were performed to analyze the changes in protein expression in the *Galleria mellonella* larvae, which were immune challenged by phosphate buffer saline and four different concentrations of glucan (Peter et al., 2010). In the past study of sugarcane borer (*Diatraea saccharalis*) healthy and immune challenged by *Escherichia coli* and *Bacillus subtilis* hemolymph samples were separated using 2D Gel electrophoresis and two extra proteins (HDs1 and HDs2) were showed in immune challenged hemolymph (Silva et al., 2010). In the present study on the basis of 2D gel analysis two spots BT53 and BT66 were observed more in treated sample (BT) than the control sample (BC) of *Bombyx mori*, whereas PT355 single spot were differentiated in PC and PT samples of *Philosamia ricini*. Infection by mixture of *E. coli* and *S. aureus* to the silkworm larvae were resulted into expression of two newer proteins in *Bombyx mori* and one of *Philosamia ricini*. In the past study expression of proteins or AMPs as an immune response in many insects as well as *Drosophila* species (Vierstraete et al., 2004; Guedes et al., 2003; Levy et al., 2004) and *Bombyx mori* (Wang et al., 2004) were determined using the 2D Gel electrophoresis.

### 5.5 First phase purification of *Bombyx mori* samples:

In the present study first phase purification were performed by using RP-HPLC and eluted fractions were collected at every two min interval, whereas peaks were recorded at 215 nm. Some clearly resolved and significant height showing peak values were observed in the control sample of *Bombyx mori* as shown in the chromatogram. Elution time and approximate peak height of control sample were listed below as 3.317 min (1860.550 mAU), 3.580 min (1881.309 mAU), 4.04 min (1726.917 mAU), 4.393 min (1798.156 mAU), 7.759 min (1264.011 mAU), 12.108 min (130.731 mAU), 19.074 min (407.095 mAU), 52.622 min (191.439 mAU) and 60.801 min (665.172 mAU) respectively, Similarly treated sample as showed with peaks of 3.499 min (1830.191 mAU), 4.08 min (1670.082 mAU), 4.413 min (1845.599 mAU), 7.876 min (1284.749 mAU), 11.100 min (128.509 mAU), 12.244 min (147.448 mAU), 19.154 min (606.138 mAU), 38.700 min (694.718 mAU), 52.586 min (178.638 mAU) and 60.794 min (653.067 mAU). In the comparative study BT samples were showed two more peaks at 11.100 min and 38.700 min more than BC (Table 5.9).
### Table 5.9: First phase purification of *Bombyx mori* samples

<table>
<thead>
<tr>
<th>Elution time (min)</th>
<th>~ Peak Height (mAU)</th>
<th>Elution time (min)</th>
<th>~ Peak Height (mAU)</th>
</tr>
</thead>
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<tr>
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5.6 First phase purification of *Philosamia ricini* samples:

In the present study first phase purification were performed using RP-HPLC and eluted fractions were collected at every two min interval, whereas peaks were recorded at 215 nm. Some clearly resolved and potent height showing peak values were observed in the control sample of *Philosamia ricini* as shown in the chromatogram. Elution time and approximate peak height of control samples were listed below as 3.288 min (1832.320 mAU), 4.368 min (1550.365 mAU), 6.892 min (518.070 mAU), 8.044 min (896.357 mAU), 14.719 min (1207.384 mAU), 26.205 min (244.587 mAU), 31.390 min (1500.263 mAU), 52.550 min (233.318 mAU) and 60.717 min (559.360 mAU) respectively. In case of treated sample the peaks results showed as 3.351 min (1850.732 mAU), 4.059 min (1678.837 mAU), 4.387 min (1763.513 mAU), 6.916 min (341.211 mAU), 8.134 min (352.736 mAU), 11.982 min (255.937 mAU), 14.696 min (1733.276 mAU), 18.362 min (471.234 mAU), 26.118 min (263.043 mAU), 31.387 min (1492.413 mAU), 52.534 min (304.504 mAU) and 60.709 min (577.268 mAU). In the comparative study PT samples were showed two more peaks at 11.982 min and 18.362 min more than PC (Table 5.10).

HPLC purification methods were mostly performed for the purification of AMPs. In the past study for the purification C-18 column and Acetonitrile/TFA solvent system were performed for first phase purification of AMP (Pavliňa et al., 2011). Some other researchers also performed first phase purification of immune induced proteins those were listed as columns and solvent systems used separated by slash (/) as C-18 ODS-80TM/ Acetonitrile-Water (Eiko et al., 2011), Cation-Exchange/ Ammonium acetate Buffere (Jean et al., 1989), CM-Cellulose/ Ammonium acetate Buffer/NaCl (Yoshihiro and Okitsugu, 1990), CM-Toyopearl/ Ammonium acetate Buffer (Keiko et al., 2002), CM-Trisacryl/ Ammonium acetate Buffer (Jean-Luc et al., 1988), FPLC G-75/ Potassium Phosphate Buffer (Sunayana et al., 2014) , Gel filtration G-50/ Ammonium acetate Buffer (Kyung et al., 2002), Phenyl-SepharoseG-25, G-50, G-100/ Sodium Phosphate Buffer/NaCl or Ammonium acetate Buffer (Hadi et al., 2013; Jie and Zheng-wang, 2010) and Sep-Pack C-18/ Acetonitrile/TFA or Tris Buffer (Lester et al., 1999; Jun-Ho et al., 2012). In our study C-18 column and Acetonitrile/TFA solvent system were performed during the first phase purification of immune induced and control hemolymph proteins prior to antimicrobial susceptibility testing.
Table 5.10: First phase purification of *Philosamia ricini* samples

<table>
<thead>
<tr>
<th>Elution time (min)</th>
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<td></td>
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<td><strong>18.362</strong></td>
<td><strong>471.234</strong></td>
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<td>244.587</td>
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<td>263.043</td>
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<td>31.390</td>
<td>1500.263</td>
<td>31.387</td>
<td>1492.413</td>
</tr>
<tr>
<td>52.550</td>
<td>233.318</td>
<td>52.534</td>
<td>304.504</td>
</tr>
<tr>
<td>60.717</td>
<td>559.360</td>
<td>60.709</td>
<td>577.268</td>
</tr>
</tbody>
</table>
5.7 Antimicrobial Susceptibility Testing (AST) of silkworm samples:

5.7.1 AST of *Bombyx mori* control hemolymph samples:

In the present study *Bombyx mori* control hemolymph samples are designated as BC. Total 35 fractions (each of two min) obtained after RP-HPLC purification were screened to detect antimicrobial competence. BC13, BC18, BC23, BC25 and BC28 were demonstrated antimicrobial potential, whereas BC28 was lost its activity after storage. These results were not presented in the following sections.

In the present study growth of *Escherichia coli* (NCIM 2137) were inhibited by BC18, BC23 and BC25 as 17, 11 and 39mm zone of growth inhibition respectively, where as standard antibiotic Streptomycin HLS\(^{300}\) (Himedia 300 mcg/disk) showed 25mm (Table 5.11 and Fig. 5.6). BC25 fraction and antibiotic Streptomycin HLS\(^{300}\) were showed similar activity on *Pseudomonas aeruginosa* (NCIM 2074) as 35mm zone of growth inhibition (Table 5.11 and Fig. 5.7). BC25 fraction showed 23mm zone of bacterial growth inhibition on *Staphylococcus aureus* (NCIM 2672), whereas 45mm were showed by Streptomycin HLS\(^{300}\) (Table 5.11 and Fig. 5.8). BC13, BC18, BC25 and Streptomycin HLS\(^{300}\) showed antimicrobial action against *Streptococcus faecalis* (NCIM 2405) as 10, 19, 39 and 30mm zone of growth inhibition respectively (Table 5.11 and Fig. 5.9) All the fractions did not show antimicrobial action against *Aspergillus niger*, whereas Standard Antifungal Clotrimazole CC\(^{10}\) showed 11mm zone of growth inhibition (Table 5.11). BC25 fraction showed 28mm zone of growth inhibition against *Candida albicans* though Clotrimazole CC\(^{10}\) did not show growth inhibition. Protein concentrations and elution time of fractions were mentioned in the Table 5.11.

**Table 5.11: AST of *Bombyx mori* control hemolymph samples**

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Zone of growth inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
<td>BC13</td>
</tr>
<tr>
<td>Protein Conc. (μg/ml)</td>
<td></td>
</tr>
<tr>
<td>BC13</td>
<td>5.053</td>
</tr>
<tr>
<td>HPLC Elution time (min)</td>
<td>25-27</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (NCIM 2137)</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (NCIM 2074)</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (NCIM 2672)</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em> (NCIM 2405)</td>
<td>10</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>-</td>
</tr>
</tbody>
</table>

**SAB:** Disk of Standard Antibiotic Streptomycin HLS\(^{300}\) (Himedia 300 mcg/disk).

**SAF:** Disk of Standard Antifungal Clotrimazole CC\(^{10}\) (Himedia 10 mcg/disk).

- : No inhibition of growth.
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Figure 5.6: AST of BC fractions on *Escherichia coli*

Figure 5.7: AST of BC fractions on *Pseudomonas aeruginosa*

Figure 5.8: AST of BC fractions on *Staphylococcus aureus*

Figure 5.9: AST of BC fractions on *Streptococcus faecalis*

Figure 5.10: AST of BC fractions on *Candida albicans*
5.7.1 AST of *Bombyx mori* treated hemolymph samples:

In the present study *Bombyx mori* treated or immune challenged hemolymph samples are denoted as BT. Thirty five eluted fractions of RP-HPLC were collected every two min and used for screening of antimicrobial activity. Five fractions showed antimicrobial potential. BT25 and BT28 fractions missed antimicrobial potential during storage hence were kept away from the discussion. BT2, BT13, BT18 and Streptomycin HLS<sup>300</sup> were showed activity against *Escherichia coli* (NCIM 2137) as 34, 36, 39 and 25 mm zone of growth inhibition (Table 5.12 and Fig. 5.11). Growth of *Pseudomonas aeruginosa* (NCIM 2074) was inhibited as 25, 26, 25 and 35 mm by BT2, BT13, BT18 and Streptomycin HLS<sup>300</sup> respectively (Table 5.12 and Fig. 5.12), whereas same fractions showed inhibition of *Staphylococcus aureus* (NCIM 2672) as 42, 46, 45 and 45 mm respectively (Table 5.12 and Fig. 5.13) Growth of *Streptococcus faecalis* (NCIM 2405) were also inhibited by BT2, BT13, BT18 and Streptomycin HLS<sup>300</sup> with 34, 31, 37 and 30 mm zones of inhibition respectively (Table 5.12 and Fig. 5.14). Clotrimazole CC<sup>10</sup> has showed 11 mm zone of growth inhibition against *Aspergillus niger*, whereas BT samples has showed absence of inhibition (Table 5.12 and Fig. 5.15) Growth of *Candida albicans* were inhibited due to the activity of BT2, BT13 and BT18 as 16, 22 and 21 mm zone of inhibition, whereas Clotrimazole CC<sup>10</sup> were unable to do the inhibition activity (Table 5.12 and Fig. 5.16).

**Table 5.12: AST of *Bombyx mori* treated hemolymph samples**

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Zone of growth inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
<td>BT2</td>
</tr>
<tr>
<td>Protein Conc. (μg/ml)</td>
<td>4.023</td>
</tr>
<tr>
<td>HPLC Elution time (min)</td>
<td>3-5</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (NCIM 2137)</td>
<td>34</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (NCIM 2074)</td>
<td>25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (NCIM 2672)</td>
<td>42</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em> (NCIM 2405)</td>
<td>34</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>16</td>
</tr>
</tbody>
</table>

**SAB:** Disk of Standard Antibiotic Streptomycin HLS<sup>300</sup> (Himedia 300 mcg/disk).
**SAF:** Disk of Standard Antifungal Clotrimazole CC<sup>10</sup> (Himedia 10 mcg/disk).

- : No inhibition of growth.
Figure 5.11: AST of BT fractions on *Escherichia coli*

Figure 5.12: AST of BT fractions on *Pseudomonas aeruginosa*

Figure 5.13: AST of BT fractions on *Staphylococcus aureus*

Figure 5.14: AST of BT fractions on *Streptococcus faecalis*

Figure 5.15: AST of BT fractions on *Aspergillus niger*

Figure 5.16: AST of BT fractions on *Candida albicans*
5.7.3 AST of *Philosamia ricini* hemolymph samples:

In the present study control and treated hemolymph samples of *Philosamia ricini* were denoted as PC and PT respectively. In the primary screening of RP-HPLC fractions out of 70 samples only four PC2, PC26, PT13 and PT19 samples showed activity against *Escherichia coli* (NCIM 2137) as 15, 16, 26 and 10mm zone of growth inhibition respectively (Table 5.13 and Fig. 5.17). All other microorganism used in present study were found resistant to the said fractions, whereas Streptomycin HLS\textsuperscript{300} showed antimicrobial activity against *Escherichia coli* (NCIM 2137), *Pseudomonas aeruginosa* (NCIM 2074), *Staphylococcus aureus* (NCIM 2672) and *Streptococcus faecalis* (NCIM 2405) as shown in table 5.13. Clotrimazole CC\textsuperscript{10} showed effect on *Aspergillus niger* as 11mm zone of growth inhibition, while *Candida albicans* were resistant to the same (Table 5.13).

**Table 5.13: AST of *Philosamia ricini* hemolymph samples**

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Zone of growth inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
<td>PC2</td>
</tr>
<tr>
<td>Protein Conc. (μg/ml)</td>
<td>3.08</td>
</tr>
<tr>
<td>HPLC Elution time (min)</td>
<td>3-5</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (NCIM 2137)</td>
<td>15</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (NCIM 2074)</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (NCIM 2672)</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em> (NCIM 2405)</td>
<td>-</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>-</td>
</tr>
</tbody>
</table>

SAB: Disk of Standard Antibiotic Streptomycin HLS\textsuperscript{300} (Himedia 300 mcg/disk).
SAF: Disk of Standard Antifungal Clotrimazole CC\textsuperscript{10} (Himedia 10 mcg/disk).
- : No inhibition of growth.

![Figure 5.17: AST of PC & PT fractions on *Escherichia coli*](image-url)
Antimicrobial Susceptibility Testing (AST) was performed by many researchers to determine the activity of AMPs against bacteria, fungi, viruses and protozoa. AST methods used by these researchers were Well Diffusion Assay (Pawel et al., 2010; Ru et al., 2012; Mingming et al., 2013), Disk Diffusion Assay (Samuthirapandian et al., 2010; Sunayana et al., 2014), Agar Overlay Assay (Aliahmadi et al., 2011), Radial Diffusion Assay (Jun-Ho et al., 2012; Pavlina et al., 2011; Masoud et al., 2014), Colony Count Assay (Kyung et al., 2002), Liquid Growth Inhibition Assay (Jie and Zheng-wang, 2010; Pavlina et al., 2011), Bactericidal Assay (Tommy et al., 2010), Macro-broth Dilution Assay (Masoud et al., 2014) and Micro-broth Dilution Assay (Eiko et al., 2011; Xiang-Jun et al., 2012). In the present study Well and disk diffusion assay were performed in same agar plate as HPLC fractions were loaded into wells and standard antibacterial and antifungal disks were used as reference.

In the present study BC and PC samples showed antimicrobial activity, though these were the non-induced hemolymph samples, hence these samples should contain constitutive AMPs. In the past research some of the constitutive AMPs isolated from various multicellular organisms showed antibacterial activity. Penaeidin-5 isolated from Black Tiger Shrimp was showed MIC 20-200 μM (Shao-Yang et al., 2006). Similarly some other source organisms, AMP isolated and its MIC range were given as below separated by slash (/) Blue Crab/ Callinectin/ MIC 1.44 μM (Lester et al., 1999). , Cattle Tick/ Ixodidin/ MIC 0.12-0.24 μM (Andrea et al., 2006), Crab/ AMP/ IZD 14-17 mm (Rameshkumar et al., 2009), Female Hard Tick/ Amblyomma/ MIC 7.5-30 μM (Ren et al., 2004), Frog/ Brevinin/ MIC 6.3-100 μM (Eiko et al., 2011a), Fruit fly/ SK84/ MIC 4-8 μM (Jie and Zheng-wang, 2010), Leaves of Coriandrum sativum/ PlamtaricinC5/ MIC 35-86 μg/ml (Masoud et al., 2014), Lobster/ CAP/ MIC 1.28 mg/ml (Andrea et al., 2008), Royal Jelly of Honeybee/ Jelleines/ MIC 0.1-100 μM (Renato et al., 2004), Seeds of Benincasa hispida/ Hispidalin/ IZD 21-29 mm (40 μg/ml) (Sunayana et al., 2014), Solitary Eumenine Wasp/ Eumenitin/ MIC 7.5-60 μM (Marisa et al., 2011) and Spider/ Ctenidins/ MIC 2.5-5 μM (Tommy et al., 2010). In the present study BC13, BC18, BC23 and BC25 (Bombyx mori) samples and PC2 and PC26 (Philosamia ricini) samples were showed antibacterial activity as a constitutive AMPs.

Some of the already studied constitutive AMPs also showed antifungal activity. These antifungal AMPs, source organism used for isolatation and its MIC range were presented as below separated by slash (/) Penaeidin-5/ Black Tiger Shrimp/ MIC 5-20 μM (Shao-Yang et al., 2006), Lumbricin/ Earthworm/ MIC 8-25 μg/ml (Ju et al., 1998),
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Palustrin/ Frog/ MIC 100 μM, Nigrocin/ Frog/ MIC 50 μM (Eiko et al., 2011a), MAF-1/ Hausefly/ MIC 3.8-4.2 μM (Ping et al., 2009), PlantaricinC5/ Leaves of Coriandrum sativum/ MIC 62-86 μg/ml (Masoud et al., 2014), Eumenitin/ Solidary Eumenine Wasp/ MIC 7.5 μM (Marisa et al., 2011), Pelteobagrin/ Yellow Catfish/ MIC 64-256 μg/ml (Yueju, 2011). In the present study only BC25 of Bombyx mori has showed activity against Candida albicans as constitutive AMP, whereas PC samples of Philosamia ricini did not show antifungal activity.

In the previous studies induced AMPs were isolated from different multicellular organism belonging from various taxonomic classes. Induced AMPs BmcecB6 and Bmmor isolated from mulberry silkworm showed antibacterial activity with 0.625-2.5 μM MIC (Wanying et al., 2011), whereas another AMP Lebocin showed 4 to 48mm zone of bacterial growth inhibition (Seiichi and Minoru, 1995). Oak Silk Moth Cecropins AMPs showed antibacterial activity as LC 0.21-88 μM (Xiang-ming et al., 1982). Previously studied AMPs isolated from various taxa were listed below as name of AMP, source organism used for isolation and its antibacterial activity as Tenecin4/ Beetle/ MIC 0.4-5 μg/ml (Jun-Ho et al., 2012), VpBD/ Clam/ MIC 1.64-26.26 μM (Jianmin et al., 2010), Maximin BK/ Frog/ MIC 8.1-19 μg/ml (Hadi et al., 2013), Brevinin/ Frog/ MIC 6.3-100 μM (Eiko et al., 2011a), Bmkbpp/ Scorpion/ MIC 2.3-68.2 μM (Xian-Chun et al., 2012), StCT/ Scorpion/ MIC 12.5-100 μg/ml (Wenyong et al., 2010), Ns-D/ Seeds of Nigella sativa/ IZD 6-15 mm 0.4μg/μl (Eugene et al., 2011), ASABF/ Sponge/ MIC 3.5-17.5 μg/ml (Matthias et al., 2012), Pelteobagrin/ Yellow Catfish/ MIC 2-256 μg/ml (Yueju, 2011). In the present study BT and PT samples were induced AMPs as these were isolated from immune challenged silkworm larvae. BT2, BT13 and BT18 (Bombyx mori) and PT13 and PT19 (Philosamia ricini) samples were showed better antibacterial activity when compared with the BC and PC samples. Induced AMPs showed such type of better antibacterial activity at microgram concentration.

In the past study some of AMPs showed to be antifungal in nature. Antifungal AMP, source organism and its antifungal activity were listed below as Pseudin-2/ Frog/ MIC 8-64 μM (Seong-Cheol et al., 2011), Maximin BK/ Frog/ MIC 26-36 μg/ml (Hadi et al., 2013), Palustrin/ Frog/ MIC 100 μM (Eiko et al., 2011), Nigrocin/ Frog/ MIC 50 μM (Eiko et al., 2011a), Bmkbpp/ Scorpion/ IC50 0.2-3.1 μM (Xian-Chun et al., 2012), Ns-D/ Seeds of Nigella sativa/ IC50 1.8-27.4 μg/ml (Eugene et al., 2011), ASABF/ Sponge/ MIC 8.5-13 μg/ml (Matthias et al., 2011), Leb/ Tobacco hornworm/ MIC 200
μM (Xiang-Jun et al., 2012), Pelteobagrin/ Yellow Catfish/ MIC 64-256 μg/ml (Yueju, 2011). In the present study BT and PT samples were designated due to immune treated samples. BT2, BT13 and BT18 samples showed better activity against Candida albicans, while PT sample as well as antibiotic Clotrimazole CC\textsuperscript{10} has not shown antifungal property against the strain of Candida albicans.

5.8 Second phase RP-HPLC purification of immune induced proteins:

In the present study second phase purification of only treated samples with antimicrobial activity were considered. Three samples of Bombyx mori as BT2, BT13 and BT18, while single sample PT13 of Philosamia ricini were purified using RP-HPLC. PT19 sample of Philosamia ricini was rejected from final purification due to 0.145 μg/ml protein conc. In the final purification of BT2 three peak values were found as 1a, 1b and 1c (Fig. 5.18), similarly BT13 also showed three peak values as 2a, 2b and 2c (Fig. 5.18). Sample BT18 and PT13 showed two peak values as (3a, 3b) and (4a, 4b) respectively (Fig. 5.18). Eluted fractions were manually collected and more peak height showed fractions were used for protein verification. As shown in table 5.14 BT2 (1a), BT13 (2a), BT18 (3a) and PT13 (4b) samples were selected for further study. Designation of peak, area, height, width, area% and symmetry of all peaks were listed in the Table 5.14.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peaks</th>
<th>Time (min)</th>
<th>Area (mAU*s)</th>
<th>Height (mAU)</th>
<th>Width (min)</th>
<th>Area%</th>
<th>Symmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT2</td>
<td>1a</td>
<td>3.516</td>
<td>2538.1</td>
<td>60.4</td>
<td>0.7004</td>
<td>4.297</td>
<td>0.575</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>4.307</td>
<td>572.7</td>
<td>17.5</td>
<td>0.5207</td>
<td>0.928</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>5.217</td>
<td>310.8</td>
<td>9.8</td>
<td>0.4455</td>
<td>0.526</td>
<td>0.323</td>
</tr>
<tr>
<td>BT13</td>
<td>2a</td>
<td>3.307</td>
<td>99</td>
<td>3.2</td>
<td>0.5165</td>
<td>1.524</td>
<td>0.794</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>4.057</td>
<td>92.4</td>
<td>2.6</td>
<td>0.6008</td>
<td>1.422</td>
<td>0.841</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>5.222</td>
<td>105.6</td>
<td>2.1</td>
<td>0.8331</td>
<td>1.625</td>
<td>1.007</td>
</tr>
<tr>
<td>BT18</td>
<td>3a</td>
<td>3.241</td>
<td>57.9</td>
<td>2.6</td>
<td>0.324</td>
<td>0.105</td>
<td>0.605</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>4.12</td>
<td>50.5</td>
<td>1.8</td>
<td>0.3595</td>
<td>0.092</td>
<td>0.701</td>
</tr>
<tr>
<td>PT13</td>
<td>4a</td>
<td>3.228</td>
<td>19.5</td>
<td>1.1</td>
<td>0.3055</td>
<td>0.036</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>4.18</td>
<td>35.1</td>
<td>1.5</td>
<td>0.3884</td>
<td>0.064</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 5.18: Second phase purification of immune induced proteins
In the earlier studies researchers were used up to five phase purification of AMPs using HPLC techniques (Eiko et al., 2011a). Second phase purifications were performed by using C-18 (Pavlina et al., 2011), Cation-exchange (Seiichi and Minoru, 1995), Sep-pack (Lester et al., 1999), Gel-permeation g-75 or g-100 (Abraham et al., 1995), Heparine-Hitrap-Sepharose (Eugene et al., 2011), Ion-exchange (Kyung et al., 2002), ODS pack (Jun-Ho et al., 2012), Q-Sepharose (Hitoshi, 1998), RP-HPLC (Pawel et al., 2010, Jie and Zheng-wang, 2010, Ru et al., 2012, Sunayana et al., 2014) and size-exclusion (Jane et al., 1997) columns. Solvent systems preferred by these research workers were as Ammonium Hydroxide Acetonitrile Buffer, Methanol/Trifluroacetic acid, Acetonitrile/TFA, Acetonitrile/Water, Ammonium acetate Buffer, Phosphate Buffer, Phosphate Buffer/NaCl, Tris-HCl/NaCl, Aqueous NaCl, Aqueous Trifluroacetic acid, Aqueous Isopropanol etc. In the present study mostly used RP-HPLC methodology were practiced with C-18 column and Acetonitrile/TFA solvent system using gradient elution as a final purification prior to verification of presence of proteins by SDS-PAGE electrophoresis.

5.9 Verification of purified immune induced proteins by SDS-PAGE:

Samples collected from final purification were separated on SDS-PAGE electrophoresis for verification of presence of proteinaceous compounds. Samples loaded on SDS-PAGE were listed as BT2 (1a), BT13 (2a), BT18 (3a) and PT13 (4b). In the bracket peak designation were given but for the simplicity only BT2, BT13, BT18 and PT13 designations were considered for further study. Proteins of BT2 sample were separated in between the 34.4 to 48 kDa as compared with standard protein markers. BT13 sample were resolved as band in between 6.4 to 17 kDa, whereas BT18 samples band were determined in between 48 to 74.1 kDa. PT13 sample were as showed separation between ranges of 74.1 to 114.3 kDa respectively. As per the figure molecular weights of separated bands should be as BT2 (34.4 to 48 KD), BT13 (6.4 to 17 KD), BT18 (48 to 74.1 KD) and PT13 (74.1 to 114.3 KD) respectively (Fig. 5.19).

HPLC is the mostly preferred technique for the isolation of AMPs. A peak showed by HPLC analysis doesn’t mean these peaks will be of proteins; hence its verification is necessary. As per availability, reliability, confidence limit and convenience HPLC purification should be carried out up to five stages or phases but its purity of separation should be confirmed by using protein electrophoresis techniques. In the past studies verification or confirmation of proteinaceous fractions of HPLC from any suitable phase were performed by using Native-PAGE (Mariola, 2004), SDS-PAGE
(Samuthirapandian et al., 2010; Aliahmadi et al., 2011; Iwona et al., 2014), Acid Urea-PAGE (Jone et al., 2010; Sarita et al., 2011), Tricine-SDS-PAGE (Jun-Ho et al., 2012; Aliahmadi et al., 2011; Hadi et al., 2013; Sunayana et al., 2014), 2D Gel (Silva et al., 2010; Peter et al., 2010) and Capillary Zone electrophoresis (Lopez et al., 2003). In the present study authentication of proteinaceous fractions obtained from second phase RP-HPLC were carried using SDS-PAGE electrophoresis. SDS-PAGE resolved each protein bands as a single band for BT2, BT13, BT18 and PT13 samples, which confirms the better purification of second phase RP-HPLC and proteinaceous nature of fraction also.

Figure 5.19: Verification of immune induced proteins by SDS-PAGE

5.10 MALDI-TOF/TOF analysis of immune induced proteins:

Proteins verified using SDS-PAGE electrophoresis were characterized by MALDI-TOF/TOF to obtain the sequences based on Peptide Mass Fingerprinting (PMF). Three samples of Bombyx mori as BT2, BT13 and BT18, whereas single sample of Philosamia ricini were performed for Peptide Mass Fingerprinting (PMF) analysis.
5.10.1 MALDI-TOF/TOF analysis of BT2:

In the present study, characterization of BT2 sample were performed using MALDI-TOF/TOF analysis subsequent to the final purification and verification by RP-HPLC and SDS-PAGE electrophoresis respectively. In the MASCOT analysis total 16 values of peptides obtained after trypsin digestion and MALDI-TOF/TOF analysis were searched on the basis of m/z ratio, though only 8 found matching with database. Peptides were matching with MB21L_DROME of SwissProt database with 23% sequence coverage and 62 score. MB21L_DROME is the mab-21-like protein originally isolated from *Drosophila melanogaster* (CG4766). Molecular weight (observed) of first matched peptide as showed 2383.4583 Dalton, which starts at position 1st and ends at 20th position. Matched peptides along with its position and molecular weight in Dalton (D) were showed as 153 to 167 (1707.4333 D), 157 to 167 (1234.4420 D), 166 to 171 (842.3292 D), 170 to 181 (1434.4891 D), 258 to 267 (1277.4612 D), 309 to 323 (1838.5492 D) and 325 to 337 (1638.5342 D). Actual amino acid sequences of these matched sequences were represented in the respective column. The molecular weights (Dalton) of non-matching sequences were listed as 768.3813, 1179.3785, 1229.5316, 1357.4513, 1487.4585, 1851.5497, 1307.4285 and 2871.7959. Finally sequence coverage of BT2 was obtained having 368 amino acids, 42336 D molecular weight and 8.77 pI (Table 5.14).

5.10.2 MALDI-TOF/TOF analysis of BT13:

In the present study of MASCOT analysis total 20 values of peptides obtained after trypsin digestion and MALDI-TOF/TOF analysis were searched on the basis of m/z ratio, though only 7 showed matching with database. Peptides were matching with GM15525 of NCBInr database with 63% sequence coverage and 68 score. GM1552 is the protein originally isolated from *Drosophila sechellia* (GM1552). Molecular weight (observed) of first matched peptide as showed 2398.5107 Dalton, which starts at position 1st and ends at 20th position. Matched peptides along with its position and molecular weight in Dalton (D) were showed as 9 to 31 (2704.5864 D), 38 to 51 (1657.4520 D), 46 to 60 (1707.4237 D), 52 to 58 (797.2114 D), 72 to 86 (1838.5332 D) and 75 to 88 (1638.5256 D). Actual amino acid sequences of these matched sequences were represented in the respective column. The molecular weights (Dalton) of non-matching 13 sequences were listed as 700.2470, 704.2305, 713.2766, 768.3732, 882.3810, 1179.3706, 1277.4547, 1307.4174, 1320.3243, 1493.4286, 1791.3550, 1851.5416 and
2383.4473. Finally, the sequence coverage of BT13 was obtained having 111 amino acids, 12991 D molecular weight and 9.94 pI (Table 5.16).

**Table 5.15: Peptide Mass Fingerprinting of BT2**

<table>
<thead>
<tr>
<th>Position</th>
<th>Start</th>
<th>End</th>
<th>Molecular Weights (Daltons)</th>
<th>Matching Peptide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>2383.4583</td>
<td>2382.4510</td>
<td>2382.1139</td>
</tr>
<tr>
<td>153</td>
<td>167</td>
<td>1707.4333</td>
<td>1706.4260</td>
<td>1706.8695</td>
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Table 5.16: Peptide Mass Fingerprinting of BT13

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SEQUENCE 111AA; 12991 MW; pI 9.94
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5.10.3 MALDI-TOF/TOF analysis of BT18:

In the present study of MASCOT analysis total 16 values of peptides obtained after trypsin digestion and MALDI-TOF/TOF analysis were searched on the basis of m/z ratio, though only 9 showed matching with database. Peptides were matching with G6PD_DROYA of SwissProt database with 23% sequence coverage and 58 score. G6PD_DROYA is the Glucose-6-phosphate 1-dehydrogenase fragment of enzyme originally isolated from Drosophila yakuba. Molecular weight (observed) of first matched peptide as showed 1993.5486 Dalton, which starts at position 50th and ends at 64th position. Matched peptides along with its position and molecular weight in Dalton (D) were found as 56 to 61 (700.2477 D), 110 to 129 (2398.4966 D), 204 to 213 (1234.4266 D), 370 to 392 (2704.5681 D), 387 to 406 (2383.4385 D), 403 to 426 (2871.7722 D), 439 to 453 (1838.5228 D) and 502 to 507 (704.2374 D). Actual amino acid sequences of these matched sequences were represented in the respective column. The molecular weights (Dalton) of non-matching 7 sequences were listed as 768.3516, 882.3658, 1082.3784, 1434.4707, 1487.4388, 1493.4186 and 1657.4438. Finally sequence coverage of BT18 was obtained with 518 amino acids, 59938 D molecular weight and 6.32 pI (Table 5.17).

5.10.4 MALDI-TOF/TOF analysis of PT13:

In the present study of MASCOT analysis total 20 values of peptides obtained after trypsin digestion and MALDI-TOF/TOF analysis were searched on the basis of m/z ratio, though only 7 showed matching with database. Peptides were matching with RBF_DROME of SwissProt database with 13% sequence coverage and 50 score. RBF_DROME are the Retinoblastoma family proteins originally isolated from Drosophila melanogaster (RBF). Molecular weight (observed) of first matched peptide as showed 2704.5256 Dalton, which starts at position 58th and ends at 59th position. Matched peptides along with its position and molecular weight in Dalton (D) were showed as 95 to 108 (1707.3864 D), 154 to 168 (1838.4950 D), 277 to 297 (2398.4553 D), 298 to 311 (1638.4863 D), 656 to 661 (768.3532 D) and 755 to 774 (2383.3987 D). Actual amino acid sequences of these matched sequences were represented in the respective column. The molecular weights (Dalton) of non-matching 13 sequences were listed as 700.2388, 713.2642, 735.2906, 814.2574, 850.3359, 1179.3445, 1307.3901, 1320.2994, 1487.4128, 1493.3978, 1657.4200, 1851.5022 and 2500.6685. Finally sequence coverage of PT13 was obtained as 845 amino acids, 96826 D molecular weight and 6.14 pI (Table 5.18).
Table 5.17: Peptide Mass Fingerprinting of BT18

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SEQUENCE 518 AA; 59938 MW; pI 6.32
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109
### Table 5.18: Peptide Mass Fingerprinting of PT13

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SEQUENCE 845 AA; 96826 MW; pI 6.14

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HGKCSYIKLD DICWFRLFCA KNQKPSNTVD LVTSSMLMIC CDLIIYNNVL AEKRTDLINP

KFGSNPSMT ELEDPRHNPAC ILSNFCDMTE EAAMAKATTF RQIMSFFQAC STYGNKDTM

QLLILNNFE NNLKLHNNIY EQYVLVSVGFE DERILSAYDA GEHTALNDQS LRPFVPTLTR

KQDLPQQPAM AGDKFEPFVPN ATNNVQKGLA PGRITEPTDP VKQAGEEVIA KLSIIIEEIE

QKFGLHKYST EAKSRQGLAK SSFFYLDQGI LQAEIRKPD IDLRKLLVQK VSLIFVINITL

MACCVELMLE ARKTELFPPW VLDCFSLASAF EQFIIEEEVVH RGHSEGCQLN RSLIKHLNSI

EBTCLERLAW ARNSTVVEMI ARALFLPLPTW LMVNLKRAAG PLQIFLRKVY LGLWRILQKL

CSELSLCEK PSITWHRFDE SIHETHLMDK RHRDLQNHMC AIYIYIVRKR MEDFKSFIDM

RAYRNSQFQAV NSVYRFEVIPD INEDGEPKVK DIHFPYNHTY VLMLAQFVYD YLNVTPOVSG

RASDLQSLPH KERAAPQPK VTQSHSLFVS QM3KNETIQQS PQOQVSFCR SPAKDLQAMN

EKVRGKGKRMN SFGDEPGLGT MAETKRSKIS QVKAVMDDPE LQSAEEXTAV TTEGCVGEGE

GEHET
In the previous literature study of MALDI TOF/TOF instrument and Peptide Mass Fingerprinting (PMF) analysis were performed to characterize the AMP Maximin Bk of *Bufo kavirensis* skin secretion (Hadi *et al*., 2013). AMP Hispidalin (molecular mass 5.7KDa with 49 amino acid residues) of *Benincasa hispida* Seeds were also characterized using MALDI-TOF analysis (Sunayana *et al*., 2014). In the earlier study Electrospray Ionization-Mass Spectrometric (ESI–MS) analyses were performed with matrix assisted Laser Desorption/Ionisation-Time of flight Mass Spectrometry (MALDI-TOF–MS) for the characterization of ctenidins of spider *Cupiennius salei* (Tommy *et al*., 2010). AMP Bb-AMP4 isolated from *Bellamya bengalensis* (fresh water snail) were characterized using Tandem Mass Spectrometry MS/MS (Samiran *et al*., 2011). AMP SK84 isolated from *Drosophila virilis* were analyzed to obtain the N-terminal and C-terminal amino acid sequence of the 9 kDa peptide by Edman degradation (Jie and Zheng-wang, 2010). In the present study characterization was performed of precursor proteins using MALDI-TOF/TOF and PMF analysis. Sequence coverage of obtained four samples as BT2, BT13, BT18 and PT13 were denoted as JSBbT2, JSBbT13, JSBbT18 and JSBpT13 respectively prior to bioinformatics analysis in the present study. Three immune induced proteins as JSBbT2, JSBbT13 and JSBbT18 (*Bombyx mori*) and JSBpT13 of *Philosamia ricini* were characterized to know the amino acid sequence, molecular weight and pI.

5.11 Homology modeling of propeptides:

In the present study homology modeling of primary amino acid sequences derived from MALDI-TOF/TOF analysis were performed by using SWISS-MODEL workspace for predicting the 3D structures of bioactive propeptides as JSBbT2, JSBbT13, JSBbT18 and JSBpT13 (Marco *et al*., 2014; Arnold *et al*., 2006; Benkert *et al*., 2011). In the earlier studies homology modeling of AMPs were performed as Bb-AMP4 (Samiran *et al*., 2011), Radish defensins (Eugene *et al*., 2011), Defensin-A, Defensin-B and Defensin-C of *Aedes aegypti* (Dhananjeyan *et al*., 2008) and pediocinCP2 of *Pediococcus acidilactici* for the 3D structure prediction and structure function relationship studies. CPHmodels, MODELLER, SWISS-MODEL, Phyre2, HHpred, LOMETS, MODBASE, Robetta, chunk-TASSER, I-TASSER, PSiFR, ESyPred3D, PMP, PSI (Protein model portal), ProModel, SCWRL4, LOOPP, RaptorX, 3DJIGSAW, CPHModel, ESyPred3D, GeneSilico, Geno3D, HHpred, LOMETS (Meta-server combining 9 different programs), MODELLER (ModWeb: A Server for Protein
Structure Modeling), Phyre and Phyre2, Protinfo, ROBETTA, BHAGEERATH-H, SWISS-MODEL and TIP-STRUCTFAST tools can be used for structure building of AMPs with homologous structures from database proteins.

5.11.1 Homology modeling of JSBbT2:

JSBbT2 has showed ≤ 23.05 % sequence identity with monomer or homo-dimer cyclic GMP-AMP synthase. Sequence coverage for this template has 0.88% and sequence similarity has only 0.33%. Along with this template protein mitochondrial dynamic protein, uncharacterized protein, 2’-5’-oligoadenylate synthase, CCA-adding enzyme, tRNA nucleotidyl transferase etc. were used as template for model building of JSBbT2 (Fig. 5.20).

5.11.2 Homology modeling of JSBbT13:

JSBbT13 model prediction has showed ≤ 0.63 % coverage and 0.35 % sequence similarity with Matrix protein 1. Other model peptides Fibrinogen, smooth muscle tropomyosin etc. were also considered for model building of JSBbT13 (Fig. 5.21).

5.11.3 Homology modeling of JSBbT18:

JSBbT18 has showed ≤ 65.06% sequence identity with monomer or homo-dimer cyclic Glucose-6-Phosphate 1-Dehydrogenase, but its binding site not conserved, biologically not relevant *i.e.* this said propeptide not considered as an enzyme. Sequence coverage for this template 0.94% and sequence similarity only 0.49%. Along with this template protein Galactose/Lactose metabolism regulatory protein GAL80, Glucose-Fructose Oxidoreductase, precursor form of Glucose-Fructose Oxidoreductase, Sugar 3-Ketoreductase, HIV-1 tat interactive protein 2, putative export protein and Tor inhibition protein etc. were used as template for model building of JSBbT18 (Fig. 5.22).

5.11.4 Homology modeling of JSBpT13:

Immune induced propeptide JSBpT13 obtained from *Philosamia ricini* has showed sequence identity ≤ 38.76% with Retinoblastoma-associated protein having monomer oligo-state orientation. Only ≤ 0.40% sequence similarity has observed with ≤ 0.73% coverage. These were higher values matched with Retinoblastoma-associated protein, along with this Retinoblastoma Pocket, Transcription initiation factor IIB, Origin recognition complex subunit 6, various Cyclins, CCND3 protein, RNA polymerase II holoenzyme cyclin-like subunit etc. were used for 3D structure prediction. As 100% coverage has not predicted and only ≤ 0.34 sequence similarity were matching protein should be newer one (Fig. 5.23).
Chapter 5: Results and Discussion

5.1 Protein phylogeny of propeptides:

Various tools are available for the construction of protein or nucleotide based phylogeny such as Archaeopteryx, EvolView, ETE toolkit, Hypergeny, InfoViz Tree Tools, iTOL-interactive Tree Of Life, TreeVector, jsPhyloSVG, JStree, OneZoom, Phylodendron, PhyloExplorer, Phyloviewer, PhyloWidget, TRED, Treedraw, TreeViz, T-REX (Webserver) etc. In the present study Blastp of NCBI were used for construction of phylogeny to study the relatedness of immune induced proteins with database proteins. In the past study relatedness of some AMPs were performed using phylogenetic tree for maximin Bk (Hadi et al., 2013), zebra mussel defensin Dpd (Wei and Mohamed, 2010), Immulectin-1-2 (Ayako et al., 2006), lysizyme (Archana et al., 2007) and peritrophins (Xin-Jun et al., 2006).
5.12.1 Protein phylogeny of JSBbT2:

Protein phylogeny of propeptide JSBbT2 as showing close matches with CG4766 protein (*Drosophila melanogaster*) and GK19727 (*Drosophila willistoni*), while other insect proteins or peptides were showing relatedness viz. hypothetical protein phum_PHUM258430 (*Pediculus humanus corporis*), mab-21 protein or its isoforms from *Papilio xuthus*, *Cerapachys biroi* and *Apis dorsata* as well as it has matching with AGAP001033-PA-like protein of *Anopheles sinensis* (Fig. 5.24).

![Protein phylogeny of JSBbT2](image)

**Figure 5.24: Blastp based phylogeny of JSBbT2**

5.12.2 Protein phylogeny of JSBbT13:

Protein phylogeny of propeptide JSBbT13 has closely matches with proteins as G15525 (*Drosophila sechellia*), G124495 (*Drosophila mojavensis*), GD25030 (*Drosophila simulans*), G11550 (*Drosophila yakuba*), GF11833 (*Drosophila ananassae*) and GG20013 of *Drosophila erecta* (Fig. 5.25).
5.12.3 Protein phylogeny of JSBbT18:

Protein phylogeny of propeptide JSBbT18 has closely matches with proteins from EMBL as CAT03361.1 (Aphis gossypii), CAS92004.1 (Drosophila melanogaster), CAS91779.1 (Drosophila melanogaster), CBL86135.1 (Aphis mellifera), CBL86136.1 (Nasonia vitripennis) and CAS91955.1 of Drosophila melanogaster (Fig. 5.26).
5.12.4 Protein phylogeny of JSBpT13:
Protein phylogeny of propetide JSBpT13 has matching maximally with retinoblastoma family protein obtained from *Drosophila melanogaster, Musca domestica, Bombyx mori, Cerapachys biroi* and *Apis dorsata* as well as it showed similarity with GG12742 (*Drosophila erecta*), P130 (*Aedes aegypti*) and hypothetical protein and_005973 of *Anopheles darlingi* (Fig. 5.27).

![Phylogenetic tree of JSBpT13](image)

**Figure 5.27: Blastp based phylogeny of JSBpT13**

5.13 AMPer matching of propeptides:
AMPer a database and automated discovery tool were used for similarity search with known mature and propeptide database AMPs, which were used for classification or grouping of unknown AMP. It is based on a BLAST search, which is performed between query sequence and corresponding Swiss-Prot proteins. Positive match result will be given, if query sequence passes the conditions like matching with HMMs and feature annotation agrees to at least 90% of its length as well as best matching peptide should be at least 50% identical to the Swiss-Prot proteins (Christopher *et al.*, 2007). JSBbT2 has matched with mature peptide Metalnikowin and Pyrrhocoricin AMPs, which are belonging from AMPer group 123. These two AMPs were showing lowest HMM E-
value 0.0058. JSBbT13 has not showed any type of similarity with mature or propeptide AMPs of AMPer database. JSBbT18 is showing similarity with two mature peptides and three propeptides from AMPer database. Two matching mature peptides were Mast cell degranulating peptide (AMPer group 50 and lowest HMM E-value 0.0086) and Rhinocerosin or Holotricin (AMPer group 63 and lowest HMM E-value 0.00022). Three matching propeptides were Gallinacin-1 (AMPer group 11 and lowest HMM E-value 0.0018), Lebocin (AMPer group 20 and lowest HMM E-value 0.0068) and Neutrophil defensin or Defensin 5 (AMPer group 32 and lowest HMM E-value 0.0011). JSBpT13 propeptide has showed similarly with mature peptide Beta-defensin and Dolabellanin (AMPer group 61 and lowest HMM E-value 0.00013), Spingerin (AMPer group 85 and lowest HMM E-value 0.00084), Defensin (AMPer group 98 and lowest HMM E-value 0.0071), Cysteine-rich antifungal protein (AMPer group 133 and lowest HMM 0.0054) and Beta-defensin (AMPer group 145 and lowest HMM E-value 0.001). Similarly JSBpT13 also was showing matching with propeptide Styelin (AMPer group 21 and lowest HMM E-value 0.0024). JSBpT13 when BLSAT matches to AMPer propeptide Beta-defensin and Dolabellanin (AMPer group 61), it was showing BLAST E-value 0.003 and 50% identity. This information can be used for the classification of propeptides. Present study propeptides can be classified under the groups of AMPer database as JSBbT2 group 123, JSBbT18 groups 11, 20, 32, 50 and 63, JSBpT13 groups 11, 20, 21, 32, 61, 85, 98, 133 and 145, whereas JSBbT13 cannot be classified using AMPer database prediction (Table 5.19).

Table 5.19: Propeptide matching with AMPer Database AMPs

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5.14 Epitopes in propeptides:

If AMPs were antigenic in nature it will induce the synthesis of antibodies in vertebrate body. First entry of AMP may be excised from antibody response, but subsequent entry of same AMP may result to undergo influence of immediate antibody response, hence antigenic determinants predictions were performed for the present study. JSBbT2, JSBbT13 and JSBbT18 propeptides of *Bombyx mori* showed 16, 3 and 18 antigenic determinants respectively (Table 5.20).

**Table 5.20: Antigenic determinants in *Bombyx mori* propeptides**

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<th>Propeptides</th>
<th>Start</th>
<th>Sequences of antigenic determinants</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSBbT2</td>
<td>36</td>
<td>AIREICKIVQDILKEVEL</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>EPRFISSLVEC</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>EGVEVIS</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>EFEIVLYLNQ</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>TLPGCAVLKL</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>MSLWVEF</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>TASYGLLS</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>137</td>
<td>RFQTLVAQACDKSVYR</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>169</td>
<td>RERFVVQITPAFKCSG</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>187</td>
<td>PRSAAHWPVPHPLPWPHPNIVA</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>213</td>
<td>GFDLLSKESVI</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>237</td>
<td>DAWVLSF</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>255</td>
<td>CRRRCRLSML</td>
<td>263</td>
</tr>
<tr>
<td></td>
<td>267</td>
<td>RDRHLDLPGNPISAYHLKLLLLEYCEK</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>310</td>
<td>NGIFQLISCLQYRRCPHYFLPAL</td>
<td>333</td>
</tr>
<tr>
<td></td>
<td>340</td>
<td>SPSALEQAAAKQVWR</td>
<td>353</td>
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<td>JSBbT13</td>
<td>5</td>
<td>PIIRSILSHFRGCTIRSYLVVLDP</td>
<td>28</td>
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<tr>
<td></td>
<td>32</td>
<td>IEKQLKLE</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>TERGVLDLRHELALKQK</td>
<td>60</td>
</tr>
<tr>
<td>JSBbT18</td>
<td>4</td>
<td>ALDLIHKLSKSPTMVEC</td>
<td>21</td>
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<tr>
<td></td>
<td>28</td>
<td>IPHTFVIF</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>AKKKIYPKLWW</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>RDDLPLKTIFCGYARSMLTVDSIKECLPYMKVQS</td>
<td>90</td>
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<tr>
<td></td>
<td>115</td>
<td>GFELLNQ</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>NRIIFYLALPPSVFEETVNIKQICMSVCGWNRVIIE</td>
<td>168</td>
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<tr>
<td></td>
<td>177</td>
<td>SSQALSDHLAALFHEDQLYRIDHY</td>
<td>200</td>
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<td></td>
<td>227</td>
<td>NIASVLITF</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>258</td>
<td>MQNHJLQILSLVAMEKPVSCHP</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>285</td>
<td>EKVJKLKSIEET</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>298</td>
<td>LKDMVGLQYLYG</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>334</td>
<td>PTYALGVLK</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td>349</td>
<td>QGVFILRC</td>
<td>357</td>
</tr>
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<td></td>
<td>366</td>
<td>AEVRQYQDVPG</td>
<td>377</td>
</tr>
<tr>
<td></td>
<td>387</td>
<td>NELVIRVQPGELY</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>427</td>
<td>YKDSYLPDAYELILDVFCGSQMHFVRS</td>
<td>454</td>
</tr>
<tr>
<td></td>
<td>462</td>
<td>RIFTPLHQ</td>
<td>470</td>
</tr>
<tr>
<td></td>
<td>475</td>
<td>HIRPITYQ</td>
<td>482</td>
</tr>
</tbody>
</table>
JSBpT13 was showing 31 antigenic determinants. The epitopes of *Philosamia ricini* propeptide JSBpT13 were presented in table 5.21. In the present study antigenic characters of AMPs were studied, which the first evidence is reporting in our study as compared with earlier studies. Antigenic AMPs will be inactivated by vertebrate immune response; hence it should be also determined along with other properties of AMPs.

**Table 5.21: Antigenic determinants in *Philosamia ricini* propeptide**

<table>
<thead>
<tr>
<th>Propeptide</th>
<th>Start</th>
<th>Sequences of antigenic determinants</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSBpT13</td>
<td>9</td>
<td>LGAEVVSGGLVA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>GYETYLEVSQR</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>SHWMCCAIYATACRR</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>GQNAVVKGNCVSLNNLLRCCKMSIYEF</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>MIFSCPP</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>169</td>
<td>HSKYISLHG</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>AHGKCSYIKLDDICWRLFLCAK</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>207</td>
<td>NTVDLVTSYNLMICCIDLIYNNVLA</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>HNPHCILSN</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>284</td>
<td>MSSFQAS</td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>314</td>
<td>KSLNISYEQYVLSV</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>SLRPPVTP</td>
<td>357</td>
</tr>
<tr>
<td></td>
<td>384</td>
<td>NVKQLSA</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>405</td>
<td>GEEVIAKLSIIE</td>
<td>417</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td>EQKFLAK</td>
<td>426</td>
</tr>
<tr>
<td></td>
<td>434</td>
<td>SRFQLAKSFFFYLLDQILQ</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>460</td>
<td>DIDLKRLLVQKVSLVIFNITLMACCVELVLEAYKTE</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>497</td>
<td>KFPWVLDCFSISAFAEFQKIIEIVVRH</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td>524</td>
<td>SHEGCLNRSLIKHL</td>
<td>537</td>
</tr>
<tr>
<td></td>
<td>561</td>
<td>ASAOPLPTWLMVNL</td>
<td>575</td>
</tr>
<tr>
<td></td>
<td>577</td>
<td>RAAAGPLQIFLRKYYLLGW</td>
<td>594</td>
</tr>
<tr>
<td></td>
<td>596</td>
<td>RIQKLCSELSLCEK</td>
<td>609</td>
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<tr>
<td></td>
<td>613</td>
<td>SIWHIFFEH</td>
<td>620</td>
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<tr>
<td></td>
<td>636</td>
<td>QNIMCAIYIYIR</td>
<td>647</td>
</tr>
<tr>
<td></td>
<td>666</td>
<td>OPQAVNSVYREVFI</td>
<td>679</td>
</tr>
<tr>
<td></td>
<td>688</td>
<td>KVKKDIHIFYNHTYVPLMRQFVIDYNVTIP</td>
<td>716</td>
</tr>
<tr>
<td></td>
<td>723</td>
<td>SDLQLSP</td>
<td>729</td>
</tr>
<tr>
<td></td>
<td>737</td>
<td>QPKKVTQSHSLFVS</td>
<td>750</td>
</tr>
<tr>
<td></td>
<td>761</td>
<td>PNQMVYFRCRSPAKD</td>
<td>775</td>
</tr>
<tr>
<td></td>
<td>808</td>
<td>KISQVKA</td>
<td>814</td>
</tr>
<tr>
<td></td>
<td>831</td>
<td>TTEGCVG</td>
<td>837</td>
</tr>
</tbody>
</table>

### 5.15 Toxicity of propeptides:

In the present study of toxicity testing JSBbT13 propeptide were found totally free from toxic amino acid residues. Propeptide JSBbT2 has showed toxic stretch from 281 to 284 amino acid residues, while 285 to 308 amino acids were trailing residues falling in toxic stretch. Propeptide JSBbT18 also showed toxic stretch at 281 amino acid...
residues, whereas 282 to 290 amino acids showed as trailing residues falling in toxic stretch. Similarly JSBpT13 has showed a total eight toxic stretches and trailing residues in toxic stretch as shown in table 5.22. During drug designing toxicity of AMPs should be determined. In the previous study AMP tyrocidine showed toxic to human blood cells (Rammelkamp and Weinstein, 1942). Removal of C-terminal 15 residues of melittin (Subbalakshmi et al., 1999) and HP 2-20 (Park et al., 2007) AMPs exhibited 300 times less cytotoxicity to rat and human respectively compared with its original forms. Length of AMP is also responsible for cytotoxicity. Amino acid composition also contributes to the cytotoxicity of AMPs (Nell et al., 2006). Replacing Asn and Gln by two Arg residues in the LL37 AMP resulted into formation of P60.4 AMP having less cytotoxicity on eukaryotic cells (Goblyos et al., 2013).

Table 5.22: Toxicity testing of propeptides

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>Toxic Stretch</th>
<th>Trailing Residues falling in Toxic stretch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>JSBbT2</td>
<td>281</td>
<td>284</td>
</tr>
<tr>
<td>JSBbT13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>JSBbT18</td>
<td>0</td>
<td>281</td>
</tr>
<tr>
<td>JSBpT13</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>197</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>216</td>
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<td></td>
<td>218</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>482</td>
</tr>
</tbody>
</table>

5.16 Allergic potential of propeptides:

Four peptides were predicted for allergen testing and showed not to be an allergen, while showing ≤ 6% probability to be an allergen (Table 5.23). If Deduced AMPs are allergens it will cause inflammation as well as severe hypersensitivity, hence to avoid this allergic reactions determination of allergic potential should be performed during the drug discovery. In the present study AMPs and their allergic potentials were determined is the first evidence of such study as per literature survey.

Table 5.23: Allergy testing of propeptides

<table>
<thead>
<tr>
<th>Propeptide</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>% probability to be an allergen</td>
<td>5.6</td>
<td>1.6</td>
<td>4.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Prediction</td>
<td>Presumably none allergic propeptides</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.17 Deduced AMPs from propeptides:

Many AMPs were predicted by the AntiBP tool, but highest score showing initial two AMPs were selected for further study obtained from each propeptide. AMPs JSBbT2 (315) and JSBbT2 (122) were deduced from JSBbT2, whereas AMPs JSBbT13 (78) and JSBbT13 (95) were assumed from propeptide JSBbT13. Propeptide JSBbT18 were used for deduction of JSBbT18 (507) and JSBbT18 (39), similarly JSBpT13 (191) and JSBbT13 (182) were deduced from JSBpT13 propeptide of Philosamia ricini. Antimicrobial scores, starting as well end position of deduced AMPs are listed in mentioned in table 5.24.

Table 5.24: AMPs deduced from propeptides

<table>
<thead>
<tr>
<th>ProPeptide</th>
<th>Start point</th>
<th>AMP sequence</th>
<th>End point</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSBbT2</td>
<td>315</td>
<td>NH2-QLISCLQYRRCPHYF</td>
<td>329</td>
<td>1.801</td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>NH2-FITASGYSRKRKIRA</td>
<td>136</td>
<td>1.726</td>
</tr>
<tr>
<td>JSBbT13</td>
<td>78</td>
<td>NH2-FDLKVINSNRGRKERK</td>
<td>92</td>
<td>1.876</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>NH2-RRPPADKSKPKVGDF</td>
<td>109</td>
<td>1.849</td>
</tr>
<tr>
<td>JSBbT18</td>
<td>507</td>
<td>NH2-FKYSGSYKWHGGKAA</td>
<td>521</td>
<td>1.521</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>NH2-FVIFGASDLAKKKI</td>
<td>53</td>
<td>1.440</td>
</tr>
<tr>
<td>JSBpT13</td>
<td>191</td>
<td>NH2-DICWRLFLCAKNQKP</td>
<td>205</td>
<td>2.473</td>
</tr>
<tr>
<td></td>
<td>182</td>
<td>NH2-GKCSYIKLDDICWRL</td>
<td>196</td>
<td>1.752</td>
</tr>
</tbody>
</table>

5.17.1 Sequence homology of deduced AMPs:

In the present study darkly shaded amino acids were highly conserved when compared with less conserved and faintly shaded residues. Alignment studies were performed by ClustalW online available tool and shades as well as pretty printing look were given by BoxShade tool. Multiple alignment and sequence homology studies don’t show any fixed type of conserved amino acid pattern as shown in Fig. 5.28. In the previous study conserved amino acids, similarity and relationship study were performed using multiple alignment of AMPs as Esculentin-1 and 2, Palastrin-2, Brevinin-2, Nigrocin-2 (Eiko et al., 2011), Selective plant defensins (Eugene et al., 2011), LLP and lysozyme (Archana et al., 2007), peritrophins (Xin-Jun et al., 2006) using various online or offline software tools.

Table 5.28: Sequence homology of deduced AMPs

<table>
<thead>
<tr>
<th>ProPeptide</th>
<th>AMP sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT13 (191)</td>
<td>1 -- DICWRLFLCAKNQKP</td>
</tr>
<tr>
<td>PT13 (182)</td>
<td>1 -- GCSYIKLDDICWRL</td>
</tr>
<tr>
<td>BT2 (122)</td>
<td>1 FIT -- ASGYSRKRKIRA</td>
</tr>
<tr>
<td>BT18 (39)</td>
<td>1 FVIFGASDLAKKKI --</td>
</tr>
<tr>
<td>BT2 (315)</td>
<td>1 QLISCLQYRRCPHYF --</td>
</tr>
<tr>
<td>BT13 (78)</td>
<td>1 FDLYVNS-NRGKEREK</td>
</tr>
<tr>
<td>BT18 (507)</td>
<td>1 FKYSGSYKWHGGKAA --</td>
</tr>
<tr>
<td>BT13 (95)</td>
<td>1 -- RRPPADKSKPKVGDF</td>
</tr>
</tbody>
</table>
5.18 Epitopes and toxic regions in deduced AMPs:

All the AMPs derived from JSBbT2 propeptide were under the influence of Antigenic determinants those were represented by bold alphabets. AMPs deduced from propeptide SBbT13 and JSBbT18 were none antigenic, whereas AMP JSBpT13 (191) and JSBpT13 (182) showed antigenic property. Only JSBpT13 (191) AMP has showed toxicity due to amino acids 197 to 201 as well as 202 to 205 amino acids were under the influence of toxic stretch (Table 5.25).

Table 5.25: Antigenic and Toxic regions in simulated AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>Start point</th>
<th>AMP sequence</th>
<th>End point</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSBbT2</td>
<td>315</td>
<td>NH$_2$-QLISCLQYRRCPHYF</td>
<td>329</td>
<td>1.801</td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>NH$_2$-FITASGYLSSRKIRA</td>
<td>136</td>
<td>1.726</td>
</tr>
<tr>
<td>JSBbT13</td>
<td>78</td>
<td>NH$_2$-FDLVKSNRGRK</td>
<td>92</td>
<td>1.876</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>NH$_2$-RRPADDKSPKVGD</td>
<td>109</td>
<td>1.849</td>
</tr>
<tr>
<td>JSBbT18</td>
<td>507</td>
<td>NH$_2$-FKYSGYKWHGGKA</td>
<td>521</td>
<td>1.521</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>NH$_2$-FVIFGASGLAK</td>
<td>53</td>
<td>1.440</td>
</tr>
<tr>
<td>JSBpT13</td>
<td>191</td>
<td>NH$_2$-DICWRLFLCAKNQKP</td>
<td>205</td>
<td>2.473</td>
</tr>
<tr>
<td></td>
<td>182</td>
<td>NH$_2$-GKCSYIKLDDICWRL</td>
<td>196</td>
<td>1.752</td>
</tr>
</tbody>
</table>


5.19 Physico-chemical properties of deduced AMPs:

5.19.1 Length:

Activity of AMP is based on its length because minimum 7-8 amino acids are needed to orient in amphipathic structure with hydrophilic and hydrophobic faces on both sides of AMP molecule. As per the barrel-stave model at least 8 amino acid β-sheet AMP and 22 amino acid α-helical AMPs can transverse bacterial plasma membrane (Westerhoff et al., 1989). Length of AMP has been also associated with toxicity *i.e.* longer the AMP higher is the toxicity to Erythrocytes. AMPs are oligopeptides having varying lengths from few to over hundreds of amino acids (Ali and Dacheng, 2013). These AMPs were deduced by using AntiBP tool, hence lengths of all are 15 amino acids long (Table 5.26).
Table 5.26: Lengths of deduced AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPS</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>Number of amino acids</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Mol. Wt. (Dalton)</td>
<td>1927.2</td>
<td>1669.9</td>
<td>1847.1</td>
<td>1697.9</td>
</tr>
</tbody>
</table>

5.19.2 Amino acid rich peptides:

Many antimicrobial peptides were rich for particular type of single or two amino acids and known as rich peptides, hence amino acid composition were determined for all twelve deduced AMPs. Those peptides have > 20% particular type of amino acid/s can be considered as rich peptides. Deduced AMPs JSBbT2 (122), JSBbT13 (78), JSBbT13 (95), JSBbT18 (507) and JSBbT18 (39) possess 20% single or two amino acid residues as shown in Table 5.27.

Table 5.27: Amino acid composition of deduced AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPS</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>Ala(A) %</td>
<td>0</td>
<td>13.3</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>Arg(R) %</td>
<td>13.3</td>
<td>13.3</td>
<td>20</td>
<td>13.3</td>
</tr>
<tr>
<td>Asn(N) %</td>
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<td>0</td>
<td>13.3</td>
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<tr>
<td>Asp(D) %</td>
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<td>6.7</td>
<td>13.3</td>
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<td>Cys(C) %</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Gln(Q) %</td>
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<td>Glu(E) %</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Gly(G) %</td>
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<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
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<tr>
<td>Ile(I) %</td>
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<td>0</td>
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<tr>
<td>Leu(L) %</td>
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<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
</tr>
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<td>Lys(L) %</td>
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<td>20</td>
</tr>
<tr>
<td>Met(M) %</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Phe(F) %</td>
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<td>6.7</td>
<td>6.7</td>
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</tr>
<tr>
<td>Pro(P) %</td>
<td>6.7</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Ser(S) %</td>
<td>6.7</td>
<td>20</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Thr(T) %</td>
<td>0</td>
<td>6.7</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Trp(W) %</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Tyr(Y) %</td>
<td>13.3</td>
<td>6.7</td>
<td>0</td>
<td>13.3</td>
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<tr>
<td>Val(V) %</td>
<td>0</td>
<td>0</td>
<td>6.7</td>
<td>6.7</td>
</tr>
</tbody>
</table>
In the previous study generally Proline rich AMPs initially isolated from intestine of Sus scrofa (Agerberth et al., 1991) followed by D. melanogaster (Bulet et al., 1993), European sap-sucking bug Pyrrhocoris apterus (Cociancich et al., 1994), Apis mellifera (Casteels-Josson et al., 1993) and Myrmecia gulosa (Mackintosh et al., 1998). These AMPs have been further grouped as Glycine/Proline rich and Alanine/Proline rich. Straight forwarded relation was not observed with its mechanism of action and richness of Proline amino acid. In 1991, it was evidenced that Glycine rich AMPs were longer than others and possess 25 to 50% Glycines (Bulet et al., 1991). In aqueous environment structures were disordered which will be self ordered upon contact with artificial membranes (Bruston et al., 2007). Helix having Glycine ridge at one side leads to multimerization using helix-helix interaction within the bacterial membrane (Zangger et al., 2008). Typical example of tryptophan rich AMP (indolicin) was isolated from neutrophils of Bos taurus. These linear AMPs with no disulfide bridge cannot orients into secondary structures in water, whereas proximity with lipid micelles result into wedge shaped structure (Rozek et al., 2000). Higher affinity were observed for 25% tryptophan rich AMP with neutral POPC and anionic POPG vesicles (Hsu et al., 2005). A global cationic amphipathic helical 25% Histidine containing AMPs triggers membrane disruption by parallel orientation with plasma membrane surface (Mason et al., 2009). These deduced AMPs of Bombyx mori can be grouped as serine rich peptide, Arginine/lysine rich peptide, lysine/Proline rich peptides, Glycine/Lysine rich peptide and lysine rich peptides.

5.19.3 Isoelectric point:

Isoelectric point (pI) is the pH where there is zero net charge on an AMP; hence it will affect the solubility of AMP as it precipitates and also loses its biological functions. In the present study Isoelectric points of deduced AMPs were in between 8.03 to 11. Isoelectric points of JSBbT2 (122), JSBbT13 (78), JSBbT13 (95), JSBbT18 (507) and JSBbT18 (39) are near to 10, whereas JSBbT2 (315), JSBpT13 (191) and JSBpT13 (182) possess pI below the nine (Table 5.28). In general pI of majority of AMPs should be near to pH 10 similar to detergents or emulsifying agents like soaps, which assists the mechanism of action for interacting with lipid bilayer of biological membrane (Torrent et al., 2011). On the basis of pI values JSBbT2 (122), JSBbT13 (78), JSBbT13 (95), JSBbT18 (507) and JSBbT18 (39) can be concluded as better interactive with lipid bilayer of target cell than the JSBbT2 (315), JSBpT13 (191) and JSBpT13 (182) peptides.
5.19.4 Instability index:

The instability index is estimate of stability of AMP in a test tube. Instability index <40 is the indication of stability of AMPs (Wadood et al., 2014; Guruprasad et al., 1990). In our study deduced AMPs JSbbT18 (39) and JSbbT13 (78) showed great in vitro stability due to -1.99 and 0.58 instability index values respectively. Instability index of JSbpT13 (182), JSbbT18 (507), JSbpT13 (191) and JSbbT2 (122) are 8.14, 10.75, 15.07 and 23.69 respectively; hence these are moderately stable AMPs. JSbbT13 (95) and JSbbT2 (315) are unstable AMPs due to 87.67 and 129.66 instability values respectively (Table 5.28).

Table 5.28: pI, Instability index, Aliphatic index and GRAVY of AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSbbT2</th>
<th>JSbbT13</th>
<th>JSbbT18</th>
<th>JSbpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPs</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>pI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.9</td>
<td>11</td>
<td>11</td>
<td>10.28</td>
</tr>
<tr>
<td>Instability index</td>
<td>129.66</td>
<td>23.69</td>
<td>0.58</td>
<td>87.67</td>
</tr>
<tr>
<td>Aliphatic index</td>
<td>78</td>
<td>91.33</td>
<td>45.33</td>
<td>26</td>
</tr>
<tr>
<td>GRAVY&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.287</td>
<td>0.10</td>
<td>-1.973</td>
<td>-1.660</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup>: Isoelectric point and <sup>b</sup>: Grand average of hydropathicity

5.19.5 Aliphatic index:

Relative volume occupied towards aliphatic side chains (Alanine, Valine, Isoleucine and Leucine) of AMP is aliphatic index. Aliphatic index plays positive role in the great thermal stability of globular protein. In present study all deduced AMPs showed positive aliphatic index though values are differing from each other due to composition and distribution of hydrophobic amino acids. JSbbT18 (507), JSbbT13 (95) and JSbbT13 (78) are less stable in the wide range temperature due to 13.33, 26 and 45.33 aliphatic indexes respectively. Moderate thermo-stability can be achieved by JSbbT2 (315), JSbpT13 (191) and JSbbT2 (122) due to 78, 84.67 and 91.33 aliphatic index values respectively, while somewhat greater stability observed in JSbbT18 (39) and JSbpT13 (182) because of 110.67 and 104 aliphatic index values (Table 5.28). Aliphatic index >70 were showed in almost AMPs and indicates stability for wider range of temperature (Vanlalhruaia, 2014). Positive aliphatic index indicates enhanced thermo-stability of globular protein (Ikai, 1980).
5.19.6 GRAVY:

Grand Average of Hydropathy (GRAVY) value of AMP is calculated as the sum of hydropathy values of all the amino acids, divided by the number of amino acid residues in the complete sequence. Positive and negative GRAVY is indication of hydrophobicity and hydrophilicity respectively (Kyte and Doolittle, 1982; Andrea et al., 2006a). In our study JSBbT2 (122) and JSBbT18 (39) has showed positive GRAVY as 0.10 and 0.627 respectively, hence shows less affinity towards the hydrophilic regions of phospholipid bilayer, whereas JSBpT13 (182), JSBpT13 (191), JSBbT2 (315), JSBbT18 (507), JSBbT13 (95) and JSBbT13 (78) are hydrophilic GRAVY values as -0.073, -0.240, -0.287, -0.987, -1.660 and -1.973 respectively. Negative GRAVY showing deduced AMPs may attract towards the hydrophilic head group of phospholipid bilayer and then afterwards will penetrates using hydrophobic residues of AMPs in the trans-membrane lipid layer of plasma membrane of target cells (Table 5.2).

5.19.7 In vivo half-life:

Time taken to disappear half the amount of protein after its synthesis or entry into the cell is considered as half-life. This prediction is based on "N-end rule" for three model organisms as human reticulocytes, yeast and E.coli (Varshavsky, 1997). According to this rule N-terminal residues of a protein plays significant role for maintaining its stability in vivo (Tobias et al., 1991). In the present estimated half-life of deduced AMPs in the reticulocytes of human are predicted using bioinformatics tool. JSBpT13 (182) posses 30 hours in vivo half-life, whereas JSBbT2 (122), JSBbT13 (78), JSBbT18 (507), JSBbT18 (39) and JSBpT13 (191) have 1.1 hours only. JSBbT13 (95) and JSBbT2 (315) showed 1.1 and 0.8 hours in vivo half-life respectively. These values have significance for catabolic study of AMP in the human body. Only JSBpT13 will persist for longer time in the body i.e. catabolic rate is very slow, while other AMPs will create burden on excretory system and cannot shown effect for longer time (Table 5.26). In the present study half-life of deduced AMPs were also estimated in yeast cell and E. coli. Present study will be beneficial during the synthesis of AMPs using rDNA technology. Only JSBpT13 (182) can be synthesized in the yeast due to its better half-life of 30 hours, whereas JSBpT13 (191) and JSBpT13 (182) can be synthesized in the E. coli due to half-life >10 hours. Remaining AMPs of present study cannot be synthesized in yeast as well as E. coli as half-life values are <10 min (Table 5.29).
5.19.8 Net charge:

Sum of ionizable amino acid residues at particular pH will give rise to anionic or cationic net surface charge to AMPs. All deduced AMPs of present study were cationic in nature. JSBpT13 (182) poses +1 net charge. JSBbT2 (315), JSBbT18 (39) and JSBpT13 (191) have +2 net charge, whereas JSBbT2 (122), JSBbT13 (95) and JSBbT18 (507) have +3 net charge. Highest +4 net charge were observed in JSBbT13 (78). The cationicity property is due to the content of Arginine plus lysine and Aspartic acid plus Glutamic acid. Ratio of Arginine/lysine to Aspartic acid/Glutamic acid is responsible for net charge of AMPs viz. ratio 2:3 gives +1 charge to JSBpT13 (182), ratio 0:2 gives +2 charge to JSBbT2 (315), ratio 1:3 gives +2 charge to JSBbT18 (39) and JSBpT13 (191), ratio 0:3 gives +3 charge to JSBbT2 (122) and JSBbT18 (507), ratio 2:5 gives +3 charge to JSBbT13 (95) and in JSBbT13 (78) ratio 2:6 gives +4 net charge to AMP (Table 5.30).

Maximum AMPs were cationic due to rich amount of lysine, Arginine and rarely aspartic acid or Glutamic acid. Most of AMPs possess +2 to +9 net charge, while naturally isolated have +4 to +6 cationicity (Giangaspero et al., 2001). Bacterial and other microbes surface have been negatively charged hence cationic AMPs can easily approach towards the plasma membrane of bacteria (Yeaman and Yount, 2003). Higher the cationicity greater is the antimicrobial activity but the present relation were 100% true because enhanced cationicity beyond the limit may reduce the mechanism of action or increases cytotoxicity (Dathe et al., 2001). Presently discussed all AMPs can attract to the surface of negatively charged bacteria easily.

Table 5.29: *In vivo* half-life of AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPs</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>Reticulocyte</td>
<td>0.8</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Yeast</td>
<td>10 min</td>
<td>3 min</td>
<td>3 min</td>
<td>2 min</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>10</td>
<td>2 min</td>
<td>2 min</td>
<td>2 min</td>
</tr>
</tbody>
</table>

Table 5.30: Net charge of deduced AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
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</thead>
<tbody>
<tr>
<td>AMPs</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>Net Charge</td>
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<td>4</td>
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<tr>
<td>Asp + Glu</td>
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<td>0</td>
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<td>2</td>
</tr>
<tr>
<td>Arg + Lys</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>
5.19.9 Hydrophobicity:

Amino acids having non-polar nature were responsible for giving hydrophobicity to the AMPs. AMPs were allowed to partition within the microbial cell membrane is because of hydrophobicity. Percent hydrophobic residues observed in the deduced AMPs of present study are 20% of JSBbT13 (78) and JSBbT13 (95), 26% of JSBbT18 (507), 40% of JSBbT2 (315) and JSBbT2 (122), 46% JSBpT13 (182) and in JSBbT18 (39) and JSBpT13 (191) 53% hydrophobic amino acid residues were observed as shown in Table 5.31. Enhanced hydrophobicity beyond the certain limit will cause serious problem of mammalian cell toxicity and forgiveness of antimicrobial activity (Yeaman and Yount, 2003). Some time increased hydrophobicity may be responsible for more hemolytic activity (Wieprecht et al., 1997) and less hydrophobicity for declined antimicrobial activity (Chen et al., 2007). As per the above said general statements JSBbT13 (78), JSBbT13 (95) and JSBbT18 (507) may be less effective AMPs due to <30% hydrophobic residues, whereas remaining AMPs of present study will show moderate antimicrobial activity.

5.19.10 Boman index:

According to Boman index for AMPs, there is strong correlation in between index itself, its physicochemical properties and biological functions (Boman, 2003). Boman index is the ability of AMP to bind with other proteins or peptides. In our study Boman index is determined for deduced AMPs viz. JSBbT18 (39) -0.09 kcal/mol, JSBbT18 (507) 1.1 kcal/mol, JSBpT13 (191) 1.49 kcal/mol, JSBbT2 (122) 1.73 kcal/mol, JSBbT2 (315) 1.93 kcal/mol, JSBpT13 (678) 2.31 kcal/mol, JSBbT13 (95) 3.83 kcal/mol and JSBbT13 (78) 5.38 kcal/mol respectively. AMP JSBbT18 (39) is less interactive with other proteins/peptides i.e. its biological activity is very poor, though it shows less side effects. Deduced AMPs JSBbT13 (78) and JSBbT13 (95) are better AMPs due to 5.38 and 3.83 kcal/mol Boman index respectively i.e. interact with extreme range of proteins, hence shows multi-functionality. Other AMPs of the present study are moderately bioactive (Table 5.31).

5.19.11 Half-life in gut environment:

Oral dose of drug were one of the best routes for drug delivery because of its low cost and minimum infection prone to inappropriate uses as well as that were readily accepted by the patients. Values of half-life of AMPs in the intestinal environment are given in seconds and prediction was in terms of stability as high, normal or low. In the present study deduced AMPs JSBbT13 (78) and JSBbT2 (122) has showed normal
stability due to 0.56 and 0.97 sec. half-life in gut environment, where as higher stability showed in JSBbT2 (315), JSBbT18 (507), JSBpT13 (191), JSBpT13 (182) and JSBbT13 (95) having 1.012, 1.457, 1.94, 2.01 and 2.30 sec. half-life respectively (Table 5.31). Physicochemical properties of AMPs viz. larger molecular weight, higher susceptibility to proteases and other enzymes, problems in renal as well hepatic clearance are the problems, hence maintaining its stability is difficult task (Sharma et al., 2014).

Table 5.31: Hydrophobicity, Boman index and Half-life of deduced AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPs</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>% hydrophobic residues</td>
<td>40</td>
<td>40</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Boman Index (kcal/mol)</td>
<td>1.93</td>
<td>1.73</td>
<td>5.38</td>
<td>3.83</td>
</tr>
<tr>
<td>Half life (Second)</td>
<td>1.012</td>
<td>0.97</td>
<td>0.56</td>
<td>2.30</td>
</tr>
<tr>
<td>Stability</td>
<td>High</td>
<td>Normal</td>
<td>Normal</td>
<td>High</td>
</tr>
</tbody>
</table>

5.19.12 Cell penetrating ability:

Structures and their mechanism of action regarding AMPs have vast array of diversity. AMPs should interact with bacterial cell surface which will induce perturbations in the plasma membrane. It will release cytoplasmic contents into environment by disrupting electrochemical gradients of membrane through structural changes, pores formation or membrane solubilisation. Support Vector Machine (SVM) scores of JSBbT2 (315) and JSBpT13 (191) are only positive with 0.04 and 0.01 scores respectively, hence can penetrate the cell membrane easily. Other AMPs of the present study are non-cell penetrating peptides due to negative SVM scores. Barrel-stave (Baumann and Mueller, 1974), Toroidal pore (Ludtke et al., 1996), In-plane diffusion (Leontiadou et al., 2006), Carpet (Oren and Shai, 1998), Electroporation (Tieleman, 2004) and Sinking raft (Pokorny and Almeida, 2005) models were explained for mechanism of AMPs by using pore formation in target cell membrane. Negative value of SVM score indicates very poor cell penetration ability and positive values for good cell penetrating ability. The values when becomes more positive it will result into cell penetration ability of AMP. JSBbT2 (315) and JSBpT13 (191) are moderate cell penetrating AMPs (Table 5.32).

Table 5.32: Cell penetrating ability of deduced AMPs

<table>
<thead>
<tr>
<th>Propeptides</th>
<th>JSBbT2</th>
<th>JSBbT13</th>
<th>JSBbT18</th>
<th>JSBpT13</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVM Score</td>
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<td>-0.04</td>
<td>-0.01</td>
<td>-0.13</td>
</tr>
<tr>
<td>Penetration ability</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>AMPs</td>
<td>315</td>
<td>122</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>SVM Score</td>
<td>0.04</td>
<td>-0.04</td>
<td>-0.01</td>
<td>-0.13</td>
</tr>
<tr>
<td>Penetration ability</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
5.19.13 Secondary structures of deduced AMPs:

AMPs are grouped on the basis of their secondary structure as α-Helix, β-Sheet and Complex. Deduced AMPs JSBbT2 (315), JSBbT2 (122) and JSBpT13 (191) are predicted as α-Helix AMPs in our present study. JSBbT13 (78) and JSBpT13 (182) are predicted as β-Sheet AMPs, whereas JSBbT18 (39) grouped in complex AMPs as it contains α-Helix as well as β-Sheets. Two AMPs of our study named JSBbT13 (95) and JSBbT18 (507) doesn’t possess α-Helix and β-Sheets, hence cannot be grouped under any class based on its secondary structure (Fig. 5.29).

![Figure 5.29: Secondary structures of deduced AMPs](image-url)
Initially α-Helix AMPs were identified and characterized. These are nonstructural peptides in aqueous environment, while adopt α-helix upon interaction with biological membranes, which allows insertion and cause permeabilization of the target cell membrane (Bechinger et al., 1993). Most significant and studied examples of this class are the Cecropins, Magainins and Melittins. A single β-hairpin structure displaying AMPs are kept under β-sheet group. These AMPs have simple secondary structures due to presence of single or double disulfide bridges. These structures have been already detected in aqueous environment; though are further stabilized upon interaction with bacterial membrane. Tachyplesin (Kawano et al., 1990) and Protegrins (Fahrner et al., 1996) are the representative examples of β-sheet AMPs. Complex AMPs has properties of α-helix and β-sheet amino acid residues’ orientation. These AMPs show either α-helix or β-sheet forms, None of AMP belongs to complex group. In the past study various tools were used for the prediction of secondary structures of AMPs as RaptorX-SS8, NetSurfP, Jpred, Meta-PP, PREDATOR, PredictProtein, PSIPRED, SymPred, YASSPP, PSSpred etc. In the present study secondary structures were predicted using SAS tool, which were used for classification and for study the functionality of AMP based on its structure.

5.19.14 Helix Wheel Diagrams of deduced AMPs:

Previously many software tools were used for study of AMPs, their helix pattern, hydrophobic movement with respect to scale and angle of Eumenitin-R, Eumenitin-F, EMP-ER, EMP-EF AMPs (Marisa et al., 2011). Pelteobagrin (Yueju, 2011) and Pleurocidin (Woo and Dong, 2008).

Schiffer and Edmundson (1967) represented the helix amphiphilicity as 2D ‘helical wheel’ diagram, which shows projections down to the helix axis and their relative orientation with amino acid residues. Hydrophobic movement was explained by Eisenberg, Weiss and Terwilliger (Eisenberg et al., 1982), which is a quantitative measurement of amphiphilicity perpendicular to the axis of secondary structure segments. Even distribution of hydrophilic and hydrophobic amino acids gives rise to less hydrophobic movement scale, while if arrangement of hydrophobic amino acid residues as well as hydrophilic amino acid residues are in the opposite pole on the sides of helix, hydrophobic movement scale increases. Angle of hydrophobic movement is the site where secondary structure comes in contact with cell membrane phospholipid. Hydrophobic movement is a determination of peptide’s amphipathicity, which is an
average of vectorial sum of all amino acid residues within ideal helix (Yeaman and Yount, 2003).

Shapes and colors represent nature of amino acid residues as circles (Hydrophilic), diamonds (Hydrophobic), triangles (Potentially negative), pentagon (Potentially positive), dark green (most hydrophobic), faint green (decreased hydrophobicity), yellow (nil hydrophobicity), pure red (uncharged hydrophilic), faint red (decreased hydrophilicity) and light blue (potentially charged). At the centre of each wheel diagram hydrophobic movement scale (HM scale) and hydrophobic movement angle (HM angle) are separated by sign @ in the Figure 5.30.

Probability of AMPs to act as trans-membrane peptides generally should possess lower hydrophobic moment values than 0.2. Hydrophobic movement scales of present study AMPs are >0.2, hence may be less penetration ability in the plasma membrane of target cell and are not trans-membrane peptides. Polar angle or hydrophobic movement angle of present study AMPs are viz. JSBbT2 (315) 291.1°, JSBbT2 (122) 54.1°, JSBbT13 (78) 267.4°, JSBbT13 (95) 25.8°, JSBbT18 (507) 325.4°, JSBbT18 (39) 294.6°, JSBpT13 (191) 222.6° and JSBpT13 (182) 17°, hence JSBpT13 (182) and JSBbT13 (95) have better and faster cell permabilization ability with shorter half-life (Fig. 5.30).

Higher permeabilizations as well as hemolytic activity were obtained by peptides those have greater hydrophobic movement scale. Hydrophobic movement scales doesn’t straight forwardly correlates to all peptide secondary structures because of uneven distribution of hydrophobic as well as hydrophilic amino acid residues, hence ideal helices were not formed with membranes (Dathe and Wieprecht, 1999). Binding of AMP requires hydrophobic surface that could penetrate in cell membrane core made up from non-polar acyl (fatty acids) chains and hydrophilic surface should remain attached to polar head of phospholipid. Cell membrane binding and perturbation of peptide were considered as polar angle. AMPs having smaller polar angle has greater hydrophobic facets, which is directly proportional to better permabilization and faster pore forming ability, but these have shorter half-life compared with larger polar angle (Yeaman and Yount, 2003).
Chapter 5: Results and Discussion

Figure 5.30: Helix Wheel Diagrams of deduced AMPs
5.20 Sequence homology of AMPs with APD2 database AMPs:

In this study conserved amino acid patterns, % similarity, structural relations, source organisms used, biological activity and MIC of deduced AMPs are discussed based on sequence alignment with homologous AMPs of APD database.

5.20.1 Sequence homology of JSBbT2 (315):

In present study Cysteine (C) amino acid residues showed to be mostly conserved among these six homologous AMP sequences. JSBbT2 (315) is matching with Scolopin 1 (AP01509), Panitide L2 (AP02173), Siamycin II (AP00590), Dybowskin-2 (AP00565) and Tricyclic peptide RP (AP00028) with ≤33.33% similarity. JSBbT2 (315) is helix type of AMP, whereas Siamycin II and Tricyclic peptide RP are beta sheet AMPs. Structure of Scolopin 1 and Dybowskin-2 are unknown to the database, while Panitide L2 is bridge type of AMP. Source organisms used for isolation of these AMPs are centipede venoms, monocot plant, Streptomyces and frog. JSBbT2 (315) and Scolopin 1 are showing anti-gram positive, anti-gram negative and antifungal activity. Anti-gram negative activity was similarly showed in Panitide L2, whereas Siamycin II and Tricyclic peptide RP showed antiviral activity. MIC of four AMPs are unknown to the database, while Panitide L2 has 2.5 μM MIC (Table 5.33). Silkworm AMP JSBbT2 (315) is matching with AMPs derived from various taxonomic groups and maximum properties are not resembling with each other.

Table 5.33: Sequence homology of JSBbT2 (315)

<table>
<thead>
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<th>Antimicrobial Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD ID</td>
<td>Name</td>
</tr>
<tr>
<td>AP01509</td>
<td>Scolopin 1</td>
</tr>
<tr>
<td>AP02173</td>
<td>Panitide L2</td>
</tr>
<tr>
<td>AP00590</td>
<td>Siamycin II</td>
</tr>
<tr>
<td>AP00565</td>
<td>Dybowskin-2</td>
</tr>
<tr>
<td>AP00028</td>
<td>Tricyclic peptide RP</td>
</tr>
<tr>
<td>% similarity</td>
<td>33.33</td>
</tr>
<tr>
<td>Structure</td>
<td>Unknown</td>
</tr>
<tr>
<td>Source</td>
<td>centipede venoms</td>
</tr>
<tr>
<td></td>
<td>monocot plant</td>
</tr>
<tr>
<td></td>
<td>Streptomyces</td>
</tr>
<tr>
<td>Activity</td>
<td>AP/AN/AF</td>
</tr>
<tr>
<td>MIC</td>
<td>2.5 μM</td>
</tr>
<tr>
<td>Reference</td>
<td>Peng et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Nguyen et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Constantine et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Kim et al., 2007</td>
</tr>
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<td></td>
<td>Helynck et al., 1993</td>
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<td></td>
<td>AP000590</td>
</tr>
<tr>
<td></td>
<td>AP00028</td>
</tr>
<tr>
<td></td>
<td>AP02173</td>
</tr>
<tr>
<td></td>
<td>JSBbT2 (315)</td>
</tr>
<tr>
<td></td>
<td>AP00565</td>
</tr>
<tr>
<td></td>
<td>AP01509</td>
</tr>
</tbody>
</table>

AP: Anti-gram positive, AN: Anti-gram negative, AV: Antiviral, AF: Antifungal, AC: Anticancer
5.20.2 Sequence homology of JSBbT2 (122):

In the present study Phenylalanine (F), Isoleucine (I), Glycine (G), Leucine (L) and Serine (S) amino acid residues were showing to be most conserved among these homologous AMP sequences. Deduced AMP JSBbT2 (122) is matching with Bmkn2 (AP01978), Temporin-SN4 (AP02275), Temporin-1KM (AP02222), Brevinin-1TSa (AP00588) and Temporin-SN2 (AP02273) with ≤44.44% similarity. JSBbT2 (122) is helix type of AMP, whereas structures of Bmkn2 (AP01978), Temporin-SN4 (AP02275), Temporin-1KM (AP02222), Brevinin-1TSa (AP00588) and Temporin-SN2 (AP02273) are unknown to database. Source organisms used for isolation of these AMPs are scorpion venom and frog. JSBbT2 (122), Temporin-1KM and Brevinin-1TSa are showing anti-gram positive, anti-gram negative and antifungal activity. Anti-gram positive and anti-gram negative activity also showed by Bmkn2, whereas Temporin-SN4 showed anti-gram positive and antifungal activity. Temporin-SN2 is showing anti-gram positive activity only. MIC of four AMPs are unknown to the database, while Temporin-1KM has 2 to 4 μM MIC (Table 5.34). Only sequence homology doesn’t mean AMPs and it should also show the similarity with respect to conserved amino acid patterns, structure, source organisms used and activity of AMPs. As per the above discussion silkworm AMP JSBbT2 (122) can be said conserved like frog’s AMPs.

Table 5.34: Sequence homology of JSBbT2 (122)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Antimicrobial Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD ID</td>
<td>AP01978</td>
</tr>
<tr>
<td>Name</td>
<td>Bmkn2</td>
</tr>
<tr>
<td>% similarity</td>
<td>44.44</td>
</tr>
<tr>
<td>Structure</td>
<td>Unknown</td>
</tr>
<tr>
<td>Source</td>
<td>scorpion venom</td>
</tr>
<tr>
<td>Activity</td>
<td>AP/AN</td>
</tr>
<tr>
<td>MIC</td>
<td>-</td>
</tr>
<tr>
<td>Reference</td>
<td>Zeng et al., 2004</td>
</tr>
</tbody>
</table>

| APD ID       | AP02275                |
| Name         | Temporin-SN4           |
| % similarity | 43.75                  |
| Structure    | Unknown                |
| Source       | Frog                   |
| Activity     | AP/AF                  |
| MIC          | -                      |
| Reference    | Yang et al., 2013      |

| APD ID       | AP02222                |
| Name         | Temporin-1KM           |
| % similarity | 43.75                  |
| Structure    | Unknown                |
| Source       | Frog                   |
| Activity     | AP/AN/AF               |
| MIC          | 2-4 μM                 |
| Reference    | Song et al., 2013      |

| APD ID       | AP00588                |
| Name         | Brevinin-1TSa          |
| % similarity | 41.66                  |
| Structure    | Unknown                |
| Source       | Frog                   |
| Activity     | AP/AN/AF               |
| MIC          | -                      |
| Reference    | Conlon et al., 2006    |

| APD ID       | AP02273                |
| Name         | Temporin-SN2           |
| % similarity | 41.17                  |
| Structure    | Unknown                |
| Source       | Frog                   |
| Activity     | AP/AN                  |
| MIC          | -                      |
| Reference    | Yang et al., 2013      |

| JSBbT2 (122) | FITASGLSLRKIRA-------- |
| AP02222      | FIPVLSGLLL---------    |
| AP01978      | FIGAIRLLLLFK---      |
| AP02275      | FITGLISGLMAL--------- |
| AP00588      | FIGSIVGALPSLISKIRN   |

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal
5.20.3 Sequence homology of JSBbT13 (78):

In our study Phenylalanine (F) and Serine (S) amino acid residues have showed predominant conserved among these homologous AMP sequences. Deduced AMP JSBbT13 (78) is matching with Trichoplaxin (AP02437), Temproin-1KM (AP02222), Temporin-1CEa (AP01794), Odorranain-G1 (AP01296) and Temporin 1OLa (AP00871) with ≤36.36% similarity. JSBbT13 (78) is beta sheet type of AMP, whereas structures of Trichoplaxin (AP02437), Temporin-1CEa (AP01794) and Temporin 1OLa (AP00871) are helix AMPs. Source organisms used for isolation of these AMPs are *Trichoplax adhaerens* and frog. JSBbT13 (78), Trichoplaxin, Temproin-1KM and Odorranain-G1 are showing anti-gram positive, anti-gram negative and antifungal activity. Anti-gram negative and anticancer activity was shown by Temporin-1CEa, whereas activity of Temporin 1OLa is unknown. MIC of three AMPs are unknown to the database, while Trichoplaxin and Temproin-1KM are showing 3 to 12.5 and 2 to 4 μM MIC (Table 5.35). Only sequence homology doesn’t mean AMPs should also show the similarity with respect to conserved amino acid patterns, structure, source organisms used and activity of AMPs. As per the above discussion silkworm AMP JSBbT13 (78) can be said conserved like frog’s AMPs.

### Table 5.35: Sequence homology of JSBbT13 (78)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Antimicrobial Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD ID</td>
<td>Name</td>
</tr>
<tr>
<td></td>
<td>Trichoplaxin</td>
</tr>
<tr>
<td></td>
<td>Temproin-1KM</td>
</tr>
<tr>
<td></td>
<td>Temporin-1CEa</td>
</tr>
<tr>
<td></td>
<td>Odorranain-G1</td>
</tr>
<tr>
<td></td>
<td>Temporin 1OLa</td>
</tr>
<tr>
<td>% similarity</td>
<td>AP02437</td>
</tr>
<tr>
<td></td>
<td>36.36</td>
</tr>
<tr>
<td>Structure</td>
<td>Helix</td>
</tr>
<tr>
<td>Source</td>
<td>Trichoplax adhaerens</td>
</tr>
<tr>
<td></td>
<td>Frog</td>
</tr>
<tr>
<td>Activity</td>
<td>AP/AN/AF</td>
</tr>
<tr>
<td></td>
<td>AP/AN/AF</td>
</tr>
<tr>
<td></td>
<td>AP/AC</td>
</tr>
<tr>
<td></td>
<td>AP/AN/AF</td>
</tr>
<tr>
<td>MIC</td>
<td>3-12.5 μM</td>
</tr>
<tr>
<td></td>
<td>2-4 μM</td>
</tr>
<tr>
<td>Reference</td>
<td>Simunic <em>et al.</em>, 2014</td>
</tr>
<tr>
<td></td>
<td>Song <em>et al.</em>, 2013</td>
</tr>
<tr>
<td></td>
<td>Wang <em>et al.</em>, 2011</td>
</tr>
<tr>
<td></td>
<td>Li <em>et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>Conlon <em>et al.</em>, 2007</td>
</tr>
</tbody>
</table>

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal, AC: Anticancer
5.2.4 Sequence homology of JSBbT13 (95):

Arginine (R), Proline (P), Lysine (K) and Glycine (G) amino acid residues were showed conserved in the homologous AMP sequences. Deduced AMP JSBbT13 (95) is matching with Hyposin-H2 (AP00903), Hyposin-H1 (AP00902), Temporin-CPa (AP01496), Bradykinin (AP01473) and Histone H2B-1 (AP00338) with ≤40% similarity. JSBbT13 (95) cannot be grouped under helix, beta or complex AMP, whereas structures of Hyposin-H2 (AP00903), Hyposin-H1 (AP00902), Temporin-CPa (AP01496), Bradykinin (AP01473) and Histone H2B-1 (AP00338) AMPs are unknown to database. Source organisms used for isolation of these AMPs are amphibian, human, trout and frog. JSBbT13 (95), Temporin-CPa, Bradykinin and Histone H2B-1 are showing anti-gram positive, anti-gram negative and antifungal activity, whereas activity of Hyposin-H2 and Hyposin-H1 is unknown. MIC of four AMPs are unknown to the database, while Temporin-CPa is showing 25 to 50 μM MIC (Table 5.36). Only sequence homology doesn’t mean AMPs and it should also show the similarity with respect to conserved amino acid patterns, structure, source organisms used and activity of AMPs. Silkworm AMP JSBbT13 (95) is matching with AMPs derived from various and wide taxonomic groups and maximum properties are not resembling with each other.

Table 5.36: Sequence homology of JSBbT13 (95)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Antimicrobial Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD ID</td>
<td>Name</td>
</tr>
<tr>
<td>AP00903</td>
<td>Hyposin-H2</td>
</tr>
<tr>
<td>AP00902</td>
<td>Hyposin-H1</td>
</tr>
<tr>
<td>AP01496</td>
<td>Temporin-CPa</td>
</tr>
<tr>
<td>AP01473</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>AP00338</td>
<td>Histone H2B-1</td>
</tr>
</tbody>
</table>

|                |                         |              |           |         |        |     |                        |
| JSBbT13 (95)   |                         |              |           |         |        |     |                        |

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal
5.2.0.5 Sequence homology of JSBbT18 (507):

In the present study Glycine (G), Tryptophan (W), Lysine (K) and Alanine (A) amino acid were conserved residues in the homologous AMP sequences. Deduced AMP JSBbT18 (507) is matching with Trichoplaxin (AP02437), Temporin-SN3 (AP02274), UyCT2 (AP02125), P-05 (AP02392) and UyCT1 (AP02124) with ≤41.17% similarity. JSBbT18 (507) cannot be grouped under helix, beta or complex AMP, whereas structures of Trichoplaxin (AP02437) is helix and structure of other AMPs as Temporin-SN3 (AP02274), UyCT2 (AP02125), P-05 (AP02392) and UyCT1 (AP02124) are unknown to database. Source organisms used for isolation of these AMPs are *Trichoplax adhaerens*, *Ciona intestinalis*, Scorpion venom and frog. JSBbT18 (507) and Trichoplaxin are showing anti-gram positive, anti-gram negative and antifungal activity, whereas activity of Temporin-SN3 is unknown. P-05 and UyCT1 are showing anti-gram positive and anti-gram negative activity. MIC of two AMPs are unknown to the database, while Trichoplaxin, UyCT2, and UyCT1 have 3 to 50, 25 to 40 and 10 to 15 μM MIC respectively (Table 5.37). Silkworm AMP JSBbT18 (507) is matching with AMPs derived from various and wide taxonomic groups and maximum properties are not resembling with each other.

**Table 5.37: Sequence homology of JSBbT18 (507)**

<table>
<thead>
<tr>
<th>Properties</th>
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<tbody>
<tr>
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<td>AP02437</td>
</tr>
<tr>
<td>Name</td>
<td>Trichoplaxin</td>
</tr>
<tr>
<td>% similarity</td>
<td>41.17</td>
</tr>
<tr>
<td>Structure</td>
<td>helix</td>
</tr>
<tr>
<td>Source</td>
<td><em>Trichoplax adhaerens</em></td>
</tr>
<tr>
<td>Activity</td>
<td>AP/AN/AF</td>
</tr>
<tr>
<td>MIC</td>
<td>3-50 μM</td>
</tr>
</tbody>
</table>

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal
5.20.6 Sequence homology of JSBbT18 (39):

Phenylalanine (F), Glycine (G), Alanine (A), Leucine (L) and Lysine (K) amino acid residues are most conserved sequences in JSBbT18 (39) deduced AMP. Deduced AMP JSBbT18 (39) is matching with Meucin-13 (AP01544), Temporin-RN1 (AP01951), Temporin-SN1 (AP02272), Temporin-PTa (AP01434) and Temporin-SN4 (AP02275) with ≤47.05% similarity. JSBbT18 (39) can’t be grouped under complex AMP, whereas structures of Meucin-13 (AP01544) and Temporin-PTa (AP01434) are helix AMPs. Structures of Temporin-RN1 (AP01951), Temporin-SN1 (AP02272) and Temporin-SN4 (AP02275) are unknown to database. Source organisms used for isolation of these AMPs are *Mesobuthus eupeus*, *Hylarana picturata*, *Hylarana spinulosa* and frog. JSBbT18 (39), Meucin-13 and Temporin-RN1 are showing anti-gram positive, anti-gram negative and antifungal activity, whereas Temporin-PTa showed anti-gram positive, anti-gram negative and antiviral activity. Activity of Temporin-SN1 and Temporin-SN4 is unknown. MIC of four AMPs are unknown to the database, while Temporin-RN1 and Temporin-PTa have 4.7 and 3.1 to 25 μM MIC respectively (Table 5.38). Silkworm AMP JSBbT18 (39) is matching with AMPs derived from various and wide taxonomic groups and maximum properties are not resembling with each other.

Table 5.38: Sequence homology of JSBbT18 (39)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Antimicrobial Peptides</th>
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</thead>
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<tr>
<td>APD ID</td>
<td>AP01544</td>
</tr>
<tr>
<td>Name</td>
<td>Meucin-13</td>
</tr>
<tr>
<td>% similarity</td>
<td>47.05</td>
</tr>
<tr>
<td>Structure</td>
<td>helix</td>
</tr>
<tr>
<td>Source</td>
<td><em>Mesobuthus eupeus</em></td>
</tr>
<tr>
<td>Activity</td>
<td>AP/AN/AF</td>
</tr>
<tr>
<td>MIC</td>
<td>4.7 μg/ml</td>
</tr>
<tr>
<td>Reference</td>
<td>Gao <em>et al.</em>, 2009</td>
</tr>
</tbody>
</table>

| APD ID          | AP01951                |
| Name            | Temporin-RN1           |
| % similarity    | 45                     |
| Structure       | Unknown                |
| Source          | frog                   |
| Activity        | AP/AN/AF               |
| MIC             | -                      |
| Reference       | Ma *et al.*, 2010      |

| APD ID          | AP02272                |
| Name            | Temporin-SN1           |
| % similarity    | 44.44                  |
| Structure       | Unknown                |
| Source          | frog                   |
| Activity        | -                      |
| MIC             | -                      |
| Reference       | Yang *et al.*, 2013    |

| APD ID          | AP01434                |
| Name            | Temporin-PTa           |
| % similarity    | 44.44                  |
| Structure       | helix                  |
| Source          | Hylarana picturata     |
| Activity        | AP/AN/AV               |
| MIC             | 3.1-25 μM              |
| Reference       | Conlon *et al.*, 2008  |

| APD ID          | AP02275                |
| Name            | Temporin-SN4           |
| % similarity    | 43.75                  |
| Structure       | Unknown                |
| Source          | Hylarana spinulosa     |
| Activity        | -                      |
| MIC             | -                      |
| Reference       | Yang *et al.*, 2013    |

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal, AV: Antiviral
5.20.7 Sequence homology of JSBpT13 (191):

The sequence homology of JSBpT13 (191) showed that amino acid Cysteine (C) and Alanine (A) residues were conserved in homologous AMP sequences. Deduced AMP JSBpT13 (191) is matching with Polybia-MPI (AP00541), Japonicin-1Npa (AP01577), Tolworthcin 524 (AP02410), Hylin a1 (AP01331) and Pleurain-J1 (AP02267) with ≤41.17% similarity. JSBpT13 (191) can grouped under helix AMP, whereas structures of Polybia-MPI (AP00541), Japonicin-1Npa (AP01577) and Hylin a1 (AP01331) are also helix AMPs. Structures of Tolworthcin 524 (AP02410) and Pleurain-J1 (AP02267) are unknown to database. Source organisms used for isolation of these AMPs are Wasp venom, Bacillus and frog. JSBpT13 (191), Hylin a1 and Pleurain-J1 are showing anti-gram positive, anti-gram negative and antifungal activity, whereas Polybia-MPI showed anti-gram positive, anti-gram negative, antifungal and antiviral activity. Japonicin-1Npa only showed anti-gram positive activity, while Tolworthcin 524 shows anti gram positive as well as anti-gram negative activity. MIC of single AMP is unknown to the database, while Japonicin-1Npa, Tolworthcin 524, Hylin a1 and Pleurain-J1 are showing 37.5 to 80 μg/ml, 13 to 264 U, 8 to 64 μM and 6.3 to 50 μM MIC respectively (Table 5.39). Silkworm AMP JSBpT13 (191) is matching with AMPs derived from various and wide taxonomic groups and maximum properties are not resembling with each other.

Table 5.39: Sequence homology of JSBpT13 (191)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Antimicrobial Peptides</th>
</tr>
</thead>
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<tr>
<td>APD ID</td>
<td>AP00541</td>
</tr>
<tr>
<td>Name</td>
<td>Polybia-MPI</td>
</tr>
<tr>
<td>% similarity</td>
<td>41.17</td>
</tr>
<tr>
<td>Structure</td>
<td>Helix</td>
</tr>
<tr>
<td>Source</td>
<td>Wasp venom</td>
</tr>
<tr>
<td>Activity</td>
<td>AP/AN/AF/AV</td>
</tr>
<tr>
<td>MIC</td>
<td>37.5-80 μg/ml</td>
</tr>
<tr>
<td>Reference</td>
<td>Souza et al., 2005</td>
</tr>
<tr>
<td>JSBpT13 (191)</td>
<td>1 - - - - - - D-ICWLRLFLCALKNQKP--</td>
</tr>
<tr>
<td>AP00541</td>
<td>1 - - - - - - I-DWKKLLDAAKQIL--</td>
</tr>
<tr>
<td>AP02410</td>
<td>1 - - - - - - DWTCSGDIVCAAACS----</td>
</tr>
<tr>
<td>AP02267</td>
<td>1 FIPGLRLFATVVPTVCAINKLPPG</td>
</tr>
<tr>
<td>AP01331</td>
<td>1 - - - - - - IFGAILPLALKNLIK--</td>
</tr>
<tr>
<td>AP01577</td>
<td>1 - - - - - - FLLFP-LMCIQGKCC--</td>
</tr>
</tbody>
</table>

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal, AV: Antiviral
5.20.8 Sequence homology of JSBpT13 (182):

The deduced AMP JSBpT13 (182) is matching with Hylaranin-L2 (AP02421), Odorranain-U1 (AP01307), Halictine 1 (AP01922), Tigerinin-4 (AP00306) and Tigerinin-3 (AP00305) with ≤41.17% similarity. JSBpT13 (182) can grouped under beta AMP, whereas structures of Odorranain-U1 (AP01307) and Halictine 1 (AP01922) are helix AMPs. Structures of Hylaranin-L2 (AP02421), Tigerinin-4 (AP00306) and Tigerinin-3 (AP00305) are unknown to database. The most conserved amino acid are Glycine (G), Isoleucine (I), Leucine (L) and Cysteine (C) as showed in sequence homology study. Source organisms used for isolation of these AMPs are bee venom and frog. JSBpT13 (182) and Hylaranin-L2 are showing anti-gram positive, anti-gram negative and antifungal activity, whereas Odorranain-U1, Tigerinin-4 and Tigerinin-3 showed anti-gram positive and anti-gram negative activity. Halictine 1 shows anti-gram positive, anti-gram negative, antifungal and anticancer activity. MIC of four AMP is unknown to the database, while Hylaranin-L2 and Halictine 1 are showing 4.3 to 34 and 0.8 to 49 μM MIC respectively (Table 5.40). Silkworm AMP JSBpT13 (182) is matching with AMPs derived from various and wide taxonomic groups and maximum properties are not resembling with each other.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Antimicrobial Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD ID</td>
<td>Name</td>
</tr>
<tr>
<td>AP02421</td>
<td>Hylaranin-L2</td>
</tr>
<tr>
<td>AP01307</td>
<td>Odorranain-U1</td>
</tr>
<tr>
<td>AP01922</td>
<td>Halictine 1</td>
</tr>
<tr>
<td>AP00306</td>
<td>Tigerinin-4</td>
</tr>
<tr>
<td>AP00305</td>
<td>Tigerinin-3</td>
</tr>
</tbody>
</table>

Table 5.40: Sequence homology of JSBpT13 (182)

AP: Anti-gram positive, AN: Anti-gram negative, AF: Antifungal, AV: Antiviral