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# *In vitro* Bioefficacy of Different Antimicrobial Peptides Against Different Pathovers of *Xanthomonas* Using Paper Disc and Micro-Dilution Broth Method

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***In vitro* bioefficacies of the different antimicrobial peptides (AMPs) were tested against three bacteria using paper disc and micro-dilution broth method. Nine different cationic AMPs viz. D4E1, PEP11, ESF1, ESF4, ESF5, ESF6, ESF12, ESF13 and ESF17 exhibiting variable amino acid compositions and charges, were used for the present investigation. Among the various AMPs tested against *Xanthomonas axonopodis* pv. *punicae* (*Xap*), *Xanthomonas axonopodis* pv. *citri (*Xac*)* and *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) ,the peptide D4E1 (net charge + 8) was the most effective AMP against all the three *Xanthomonas* sp. followed by PEP11, ESF12 and ESF1 having net charge +4. The peptide ESF13 was not effective against all the three bacteria while ESF4 was not effective against *Xap* and *Xac* whereas ESF6 was also not effective against *Xac*.AntiMicrobial peptides when tested *in vitro* through micro-dilution broth method showed that the inhibition growth per cent against Xap was greater than 90% ($MIC_{90}$) for D4E1, PEP11, ESF17, ESF5 and ESF12. For Xac, D4E1, PEP11, ESF17, ESF5, ESF6, ESF1 and ESF12 showed the inhibition growth percent greater than 90% ($MIC_{90}$). For Xam,the inhibition growth per cent was seen greater than 90% ($MIC_{90}$) for D4E1, PEP11, ESF17, ESF1 and ESF12. The most promising peptide D4E1 exhibited better stability against endogenous proteases which recorded only 10.08 % hemolysis at a very high i.e.100 $\mu$M concentration.The D4E1 peptides appears to be a promising candidate to be explanted through gene manipulation to control bacterial blight of pomegranate.**

**Keywords:** Anti-Microbial Peptides, Bioefficacy, *Xanthomonus* sp., Inhibition.

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*, Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* and bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* are the important diseases of respective crop.

The disease was found to attack all the above ground parts. Although, the farmers have adopted all the available and possible protection measures, management of the disease cannot be done effectively. Among these, pomegranate is an important dry land horticulture fruit crop which recently has completely reformed economy of the dry land farmers. But now a days this crop has number of problems amongst this bacterial blight or oily spot is most important one. Bacterial blight caused by *Xanthomonas* axonopodis pv. *punicae* has emerged as a major constraint in pomegranate production in India. The prospects of developing cultivars with resistance to bacterial blight through conventional breeding are limited, as no resistance source is available in the available gene pool. The

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control of bacterial pathogen as on today through use of chemicals is having limited success and is difficult to operate on a large scale.

Antimicrobial peptides (AMPs) are active against a wide range of plant pathogenic bacteria. Identification of the effective cationic peptides inhibiting the activity of the *X. axonopodis* pv. *punicae* can help in synthesis of a gene encoding such antimicrobial peptides by taking into consideration the codon usage in pomegranate. Such synthetic genes can be used for *Agrobacterium*-mediated transformation of pomegranate. This is definitely going to add technological advancement in the strategic area of biotic stress tolerance. Transgene cassette thus developed can be studied in a model expression system before being attempted for *Agrobacterium*-mediated transformation. Transgenic pomegranate expressing antimicrobial peptides may help in controlling bacterial blight which at present is a threat to pomegranate cultivation across India.

At present, there are few reports describing the inhibiting activity of the different *Xanthomonas* spp.. In view of this, the present study was undertaken to screen the efficacy of cationic antimicrobial peptides against different pathovors of *Xanthomonas*.

## MATERIALS AND METHODS

The laboratory glasswares, equipments and chemicals used for different studies were of analytical grade and standard firms viz. Hi-Media Laboratories Pvt. Ltd., Mumbai (India); Qualigens laboratories Mumbai, SD Fine-Chem Limited, Mumbai and RFCL Ltd., New Delhi (India).The peptides used in the experiment were PEP11, D4E1, ESF1, ESF4, ESF5, ESF6, ESF12, ESF13, ESF17 and were synthesized and purified to > 95% purity. Infected leaves and fruit samples of respective crop plants showing typical symptoms of oily spot of pomegranate, citrus canker and bacterial blight of cotton were collected for isolation of bacterium Nutrient agar with the addition of sucrose (NAS) medium was used for isolation and maintenance of the bacterium. The composition of media was : Peptone: 5 g, Beef extract: 3 g, Sucrose: 20 g, Agar: 20 g, distilled water: 1 litre, pH 7.0.

The infected plant parts were washed thoroughly in tap water and used for isolation, the plant parts were cut into small pieces and were surface sterilized with 1:1000 (0.1%) mercuric chloride ($HgCl_2$) solution for one minute and washed three times serially in sterile distilled water to remove the traces of mercuric chloride. These small pieces were then macerated or cut into much smaller pieces with the help of sterilized blade/ scalpel in a sterile petridisc containing few drops of sterile distilled water for around 15-20 min so as to allow the bacteria to diffuse out. The bacterial ooze was streaked with the sterilized bacteriological inoculating needle on sterilized NAS medium filled in a sterilized petridisces. The inoculated plates were incubated in the inverted position for a period of 2-3 days at 28± $2^0$C and observed for development of well separated, typical, light yellow coloured bacterial colonies resembling *Xanthomonas* sp.

The suspected bacterial colonies were picked up with the help of sterilized bacteriological inoculation loop and streaked onto the surface of NAS sterilized petriplates. The inoculated plates were incubated at 28± $2^0$C for 72 hr. Observations were made for the development of well separated typical, translucent, bright yellow, smooth and mucoid colonies, such pure colonies were further streaked and maintained on to the NAS slants. The bacterial cultures thus obtained were stored in the refrigerator at $5^0$C and periodically sub-cultured to maintain the viability of organism. Pathogenicity test of respective bacterium was taken under glasshouse condition The organisms were re-isolated from artificially inoculated leaves and fruits of pomegranate plants, leaves of citrus and cotton showing typical symptoms of the disease. The re-isolation were carried out on NAS medium and observed for the growth of the bacterium.

The filter paper disc method (or zone of inhibition method) and microtitre plate micro dilution method (or automated colorimetric method) were used for the *in vitro* evaluation.

In filter paper disc method (or zone of inhibition method), a sterile Whatman filter paper disc (0.5 cm) was impregnated with the respective peptides of different concentrations and were placed on the bacterial lawn prepared by pouring the freshly prepared luke warm sterilized NAS media containing the respective bacterial suspension in a petriplates. It is then incubated in B.O.D. chamber

at 28 ± $2^0$C and after 2 and 5 days of incubation, the inhibition zone were recorded in each treatment; the presence of a clear zone of inhibition surrounding the disc is indicative of inhibitory activity against the bacteria. An experiment with nine anti-microbial peptides and one control (sterilized distilled water) were carried out. The inhibition per centages of the bacteria were calculated by using the formula described as below:

$$\text{Per cent Inhibition} = \frac{\text{Mean of the inhibition zone (mm)}}{90} \times 100$$

The antibacterial activity of the antimicrobial peptides were tested by automatically determining the microbial growth by optical density measurement at 630 nm.

The dilution of the synthetic peptides were made on ELISA reader to obtain a final concentration of 500 ppm and the wells of microtitre plate was made upto a final volume of 200 µl where the volume of the sample reading contained 20 µl of specific peptides + 20 µl of bacterial suspension + 160 µl NAS broth. Similarly volume of positive (+ve) control contained 20 µl of distilled water + 20 µl of bacterial suspension + 160 µl NAS broth while the volume of negative (-ve) control contained 20 µl of distilled water + 20 µl of peptide + 160 µl NAS broth. Corrected values were obtained by subtracting the absorbances of –ve control at specific hour from the absorbances of the sample at specific hr. The recording of the absorbances were done at an interval of 12 hr by incubating the microtitre plates at 28±$2^0$C with 20 sec. shaking before the absorbance measurement for a period of 48 hr.(Monroc et. al 2006)

The growth inhibition percentage were calculated by using the formula as Percent Inhibition = 1 - (O.D. or absorbances of corrected value at specific hr/O.D. or absorbances of positive control at specific hour) x 100 with the little modification as that given by Thomas *et al.* (2006). Three replicates for each bacterial strain were performed. Positive controls contained bacterial suspension instead of peptide and negative controls contained peptides without bacterial suspension.

Hemolytic activity of the three promising peptides was evaluated by determining hemoglobin release from erythrocyte suspension of fresh cow blood sample The percentage hemolysis was calculated using the formula.

$H = 100 \times [(O_p - O_b)/(O_m - O_b)]$ Where,

$O_p$ : Density for given peptide concentration

$O_b$ : Density for buffer

$O_m$ : Density for SDS positive control

## RESULTS AND DISCUSSION

Antimicrobial peptides (AMPs) are active against a wide range of plant pathogenic bacteria with no hazardous effect over the plant and environment (Stotz *et al.*2013). Among the various AMPs tested against *Xanthomonas axonopodis* pv. *punicae* (*Xap*), the data revealed that the peptide D4E1 (net charge + 8) was statistically the most effective AMPs with the maximum growth inhibition per cent of 13.88 % (12.50 mm), followed by PEP11 (net charge +4) with the growth inhibition per cent of 12.77 % (11.50 mm). Their efficacies was then succeeded by ESF12 (net charge +4) and ESF1 (net charge +4) (Table1). Similarly, the various AMPs tested against *Xanthomonas axonopodis* pv. *citri*., the data indicates that D4E1 with all concentration was the most effective AMPs with the highest growth inhibition per cent of 11.67 % (10.50 mm) and it was followed by PEP11 with the per cent inhibition of 9.17 % (8.25 mm). The effectiveness was then succeeded by ESF12 and ESF1 which showed the comparable growth per cent inhibition with D4E1. (Table2).From the results obtained for *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), the revealed that the AMP D4E1 was the most effective peptide, which showed the maximum growth inhibition per centage of 11.67 % (10.50 mm) and it was followed by PEP11, ESF12 and ESF1 which showed the comparable effectiveness with the per cent growth inhibition of 8.61 % (7.75 mm), 8.33 % (7.50 mm) and 8.05 % (7.25 mm) respectively (Table3).

Thus from these results, it was obvious that D4E1 is the most effective peptide. The efficacy of D4E1 was more because it carries more +ve net charge as compared to the other AMPs. ESF13 was not effective against all the three bacteria while ESF4 was not effective against *Xap* and *Xac* whereas ESF6 was also not effective against *Xac*. As the *Xanthomonas* spp. tested were gram negative bacteria i.e. the microbial membrane carries

anionic charge that facilitates binding of more cationic peptides and thereby controlled by this peptide was more effective (Bele'n *et al.*, 2007, Power and Hancoc 2003). Rehman and Khanum (2011) investigated the two active anti-microbial peptides using disc diffusion method. Astafieva *et al.* (2012) tested the antibacterial activity of the three anti-microbial against *Pseudomonas syringae*, *Bacillus subtilis*, *Clavibacter michiganense* sub sp. *michiganense*, and *Xanthomonas campestris* through radial diffusion assay (paper disc method)..Although there are less information available that indicates the effectiveness of different AMPs in controlling bacterial blight of pomegranate, citrus canker and bacterial blight of cotton. The above results are in agreement with Ballweber *et al.* (2002) and Oard *et al.* (2004), where they showed the effectiveness of the various AMPs against several bacterial and fungal pathogens.

From the results obtained for the different AMPs by Micro-dilution Microplate Broth method

**Table 1.** Details of the anti-microbial peptides (AMPs) used

| S. no. | AMPs | Peptide sequences | Net charge | Conc. (µM) | Source |
|---|---|---|---|---|---|
| 1. | D4E1 | FKLRAKIKVRLRAKIKL | +8 | 2.40<br>0.96<br>0.48 | Cecropin |
| 2. | PEP11 | WKLFKKILKVL | +4 | 3.53<br>1.41<br>0.70 | Cecropin-melittin hybrid |
| 3. | ESF1 | MASRAAGLAARLARLALRAL | +4 | 2.44 | Magainin |
| 4. | ESF4 | MASQAAGLAAQLAQLALQAL | -1 | 2.80 | Magainin |
| 5. | ESF5 | MASRAAGLARRLARLARRAL | +6 | 2.29 | Magainin |
| 6. | ESF6 | MAARAAGLAARLAALALRAL | +3 | 2.56 | Magainin |
| 7. | ESF12 | MASRAAGLAARLARLALR | +4 | 0.54<br>1.07<br>2.68 | Magainin |
| 8. | ESF13 | MASDAAGLAADLADLALDAL | -1 | 2.65 | Magainin |
| 9. | ESF17 | ASRAAGLAARLARLALR | +3 | 2.88 | Magainin |

**Table 2.** *In vitro* effect of different anti-microbial peptides against *Xanthomonas axonopodis* pv. *punicae* by filter paper disc method

| Treat. No. | Name of peptide | Concentration (mM) | Zone of inhibition (mm) | Growth inhibition (%) |
|---|---|---|---|---|
| $T_1$ | D4E1 | 2.40 | 12.50 | 13.88 |
| $T_2$ | PEP11 | 3.53 | 11.50 | 12.77 |
| $T_3$ | ESF1 | 2.44 | 7.25 | 8.05 |
| $T_4$ | ESF4 | 2.8 | 0.00 | 0.00 |
| $T_5$ | ESF5 | 2.29 | 7.25 | 8.05 |
| $T_6$ | ESF6 | 2.56 | 6.25 | 6.94 |
| $T_7$ | ESF12 | 2.68 | 8.50 | 9.44 |
| $T_8$ | ESF13 | 2.65 | 0.00 | 0.00 |
| $T_9$ | ESF17 | 2.88 | 7.75 | 8.05 |
| $T_{10}$ | Water | - | 0.00 | 0.00 |
| S.E. + | | 0.26 | 0.29 | |
| C.D. at 5 % | | 0.74 | 0.83 | |

against *Xanthomonas axonopodis* pv. *punicae*, the growth inhibitory per cent was observed greater than 90 per cent (i.e., MIC $_{90}$) for the peptides D4E1(98.13), PEP11(98.32), ESF17(96.81), ESF1(93.55), ESF5(96.10) and ESF12(97.99). However, ESF6, ESF4, ESF13 showed the growth inhibitory per cent of 81.64, 32.55 and 67.73 respectively which were lesser than 90 per cent (MIC less than $MIC_{90}$). The least growth inhibitory per cent was observed for ESF 4 (32.55) and ESF 13(67.73)(Table4).From the data obtained against *Xanthomonas axonopodis* pv. *citri*, it revealed that the growth inhibitory per cent was seen greater than 90 per cent (i.e., $MIC_{90}$) for the peptides D4E1 (99.11), ESF12 (99.36), PEP11 (94.94), ESF17 (94.73), ESF1 (97.15), ESF5 (96.40) and ESF6 (99.92). However, ESF4 (7.05) and ESF 13 (11.34) showed the growth inhibitory per cent lesser than 90 per cent (MIC less than $MIC_{90}$) with the least one for ESF4. (Table5) .The results of the present study obtained against *Xanthomonas axonopodis* pv. *malvacaerum*, the data revealed that the growth inhibitory per cent was seen greater than 90 per cent (i.e., $MIC_{90}$) for the peptides D4E1 (95.28), PEP11 (95.44), ESF17 (92.14) and ESF1 (93.40). However, ESF5 (88.36), ESF12 (85.69) and ESF6 (80.82) showed the growth inhibitory per cent lesser than 90 per cent (MIC lesser than $MIC_{90}$).

**Table 3.** *In vitro* effect of different anti-microbial peptides against *Xanthomonas axonopodis* pv. *citri* by filter paper disc method

| Treat. No. | Name of peptide | Concentration (mM) | Zone of inhibition (mm) | Growth inhibition (%) |
|---|---|---|---|---|
| $T_1$ | D4E1 | 2.40 | 10.50 | 11.67 |
| $T_2$ | PEP11 | 3.53 | 8.25 | 9.17 |
| $T_3$ | ESF1 | 2.44 | 7.00 | 7.78 |
| $T_4$ | ESF4 | 2.8 | 0.00 | 0.00 |
| $T_5$ | ESF5 | 2.29 | 6.50 | 7.22 |
| $T_6$ | ESF6 | 2.56 | 0.00 | 0.00 |
| $T_7$ | ESF12 | 2.68 | 7.25 | 8.05 |
| $T_8$ | ESF13 | 2.65 | 0.00 | 0.00 |
| $T_9$ | ESF17 | 2.88 | 6.25 | 6.94 |
| $T_{10}$ | Water | - | 0.00 | 0.00 |
| S.E. + | | 0.23 | 0.25 | |
| C.D. at 5 % | | 0.66 | 0.73 | |

**Table 4.** *In vitro* effect of different synthetic anti-microbial peptides against *Xanthomonas axonopodis* pv. *malvacearum* by filter paper disc method

| Treatment | Name of peptide | Concentration (mM) | Zone of inhibition (mm) | Growth inhibition (%) |
|---|---|---|---|---|
| $T_1$ | D4E1 | 2.40 | 10.50 | 11.67 |
| $T_2$ | PEP11 | 3.53 | 7.75 | 8.61 |
| $T_3$ | ESF1 | 2.44 | 7.25 | 8.05 |
| $T_4$ | ESF4 | 2.8 | 5.75 | 6.39 |
| $T_5$ | ESF5 | 2.29 | 6.50 | 7.22 |
| $T_6$ | ESF6 | 2.56 | 5.75 | 6.39 |
| $T_7$ | ESF12 | 2.68 | 7.50 | 8.33 |
| $T_8$ | ESF13 | 2.65 | 0.00 | 0.00 |
| $T_9$ | ESF17 | 2.88 | 6.25 | 6.94 |
| $T_{10}$ | Water | - | 0.00 | 0.00 |
| S.E. + | | 0.24 | 0.27 | |
| C.D. at 5 % | | 0.70 | 0.77 | |

The least growth inhibitory per cent was observed for ESF 4 (28.37) and ESF 13 (20.43) (Table6).

Thus from the above results it is seen that the peptides when tested *in vitro* through micro-dilution broth method at 500 ppm concentration , the inhibition of growth against *Xap* was greater than 90 per cent ($MIC_{90}$) for the peptides D4E1, PEP11, ESF17, ESF5 and ESF12. Whereas for *Xac*, it was recorded in D4E1, PEP11, ESF17, ESF5, ESF6, ESF1 and ESF12 peptides. The inhibition growth per cent greater than 90 ($MIC_{90}$) for *Xam* was recorded in peptides D4E1, PEP11, ESF17 and ESF1. Thus, it is showed that D4E1, PEP11, ESF17, ESF5, ESF12 and ESF1 were the most effective peptides against all the pathogens. Powell *et al.* (1995) also recorded similar results for *in vitro* bioassays of synthetic and natural peptides and they reported that ESF1, ESF5, ESF6 and

**Table 5.** *In vitro* efficacy of the different anti-microbial peptides on the growth of *Xanthomonas axonopodis* pv. *punicae* using 96-well microtiter plate method

| Peptide/ Conc. (mM) | Hours | Sample reading (A) | -ve control (B) | +ve control (C) | Corrected value (A-B) | Growth inhibition (%) |
|---|---|---|---|---|---|---|
| D4E1 | 12 | 0.168 | 0.086 | 0.188 | 0.082 | 56.38 |
| (2.40) | 24 | 0.127 | 0.080 | 0.298 | 0.047 | 84.23 |
| | 36 | 0.128 | 0.088 | 0.365 | 0.040 | 89.04 |
| | 48 | 0.103 | 0.092 | 0.589 | 0.011 | 98.13 |
| PEP11(3.53) | 12 | 0.107 | 0.101 | 0.188 | 0.006 | 96.81 |
| | 24 | 0.105 | 0.100 | 0.298 | 0.005 | 98.32 |
| | 36 | 0.122 | 0.098 | 0.365 | 0.024 | 93.42 |
| | 48 | 0.119 | 0.105 | 0.589 | 0.014 | 97.62 |
| ESF17 | 12 | 0.062 | 0.056 | 0.188 | 0.006 | 96.81 |
| (2.88) | 24 | 0.078 | 0.057 | 0.298 | 0.021 | 92.95 |
| | 36 | 0.104 | 0.057 | 0.365 | 0.047 | 87.12 |
| | 48 | 0.277 | 0.057 | 0.589 | 0.220 | 62.65 |
| ESF1 | 12 | 0.304 | 0.198 | 0.188 | 0.106 | 43.62 |
| (2.44) | 24 | 0.292 | 0.189 | 0.298 | 0.103 | 65.44 |
| | 36 | 0.292 | 0.209 | 0.365 | 0.083 | 77.26 |
| | 48 | 0.283 | 0.245 | 0.589 | 0.038 | 93.55 |
| ESF5 | 12 | 0.155 | 0.114 | 0.188 | 0.041 | 78.19 |
| (2.29) | 24 | 0.136 | 0.115 | 0.298 | 0.021 | 92.95 |
| | 36 | 0.132 | 0.109 | 0.365 | 0.023 | 93.70 |
| | 48 | 0.150 | 0.127 | 0.589 | 0.023 | 96.10 |
| ESF12 | 12 | 0.104 | 0.099 | 0.188 | 0.005 | 97.34 |
| (2.68) | 24 | 0.105 | 0.099 | 0.298 | 0.006 | 97.99 |
| | 36 | 0.096 | 0.084 | 0.365 | 0.012 | 96.71 |
| | 48 | 0.099 | 0.086 | 0.589 | 0.013 | 97.79 |
| ESF6 | 12 | 0.186 | 0.126 | 0.188 | 0.060 | 68.09 |
| (2.56) | 24 | 0.182 | 0.125 | 0.298 | 0.057 | 80.87 |
| | 36 | 0.200 | 0.133 | 0.365 | 0.067 | 81.64 |
| | 48 | 0.309 | 0.127 | 0.589 | 0.182 | 69.10 |
| ESF4 | 12 | 0.217 | 0.073 | 0.188 | 0.144 | 23.40 |
| (2.58) | 24 | 0.290 | 0.089 | 0.298 | 0.201 | 32.55 |
| | 36 | 0.393 | 0.130 | 0.365 | 0.263 | 27.95 |
| | 48 | 0.695 | 0.255 | 0.589 | 0.440 | 25.30 |
| ESF13 | 12 | 0.272 | 0.196 | 0.188 | 0.076 | 56.57 |
| (2.65) | 24 | 1.012 | 0.797 | 0.298 | 0.215 | 27.85 |
| | 36 | 1.065 | 0.833 | 0.365 | 0.232 | 36.44 |
| | 48 | 1.031 | 0.842 | 0.589 | 0.189 | 67.71 |

ESF12 peptides were inhibitory to the growth of the bacteria and fungi. The Badosa *et al.* (2007) tested the *in vitro* inhibitory growth of a 125-member library of synthetic linear undecapeptides by automatically determining the microbial growth through optical density measurement of the microtitre plate at 600 nm.

The most promising peptides viz. D4E1, PEP-11 and ESF-12 at 25, 50 and 100 µM concentration recorded less than 3, 7 and 11 per cent hemolysis, respectively Per cent hemolysis recorded for the promising peptides at 25 µM concentration was less than 3 per cent (table7). Whereas the most promising peptides D4E1 at highest concentration (100 µM) recorded less than 15 % hemolysis (10.08 %) this is acceptable to the eukaryotic cells of the mammals. The toxicity to eukaryotic cells of the most active peptide was

**Table 6.** *In vitro* efficacy of the different anti-microbial peptides on the growth of *Xanthomonas axonopodis* pv. *citri* using 96-well microtiter plate method

| Peptide/ Conc. (mM) | Hours | Sample reading (A) | -ve control (B) | +ve control (C) | Corrected value (A-B) | Growth inhibition (%) |
|---|---|---|---|---|---|---|
| D4E1 | 12 | 0.153 | 0.082 | 0.529 | 0.071 | 86.58 |
| (2.40) | 24 | 0.114 | 0.085 | 0.949 | 0.029 | 96.94 |
| | 36 | 0.118 | 0.103 | 1.177 | 0.015 | 98.73 |
| | 48 | 0.078 | 0.067 | 1.242 | 0.011 | 99.11 |
| PEP11 | | | | | | |
| (3.53) | 12 | 0.136 | 0.100 | 0.529 | 0.036 | 93.19 |
| | 24 | 0.153 | 0.105 | 0.949 | 0.048 | 94.94 |
| | 36 | 0.217 | 0.138 | 1.177 | 0.079 | 93.29 |
| | 48 | 0.263 | 0.154 | 1.242 | 0.109 | 91.22 |
| ESF17 | 12 | 0.078 | 0.057 | 0.529 | 0.021 | 96.03 |
| (2.88) | 24 | 0.114 | 0.059 | 0.949 | 0.055 | 94.20 |
| | 36 | 0.252 | 0.190 | 1.177 | 0.062 | 94.73 |
| | 48 | 0.447 | 0.341 | 1.242 | 0.106 | 91.47 |
| ESF1 | 12 | 0.486 | 0.315 | 0.529 | 0.171 | 67.67 |
| (2.44) | 24 | 0.437 | 0.306 | 0.949 | 0.131 | 86.20 |
| | 36 | 0.461 | 0.298 | 1.177 | 0.163 | 86.15 |
| | 48 | 0.288 | 0.253 | 1.242 | 0.035 | 97.15 |
| ESF5 | 12 | 0.134 | 0.115 | 0.529 | 0.019 | 96.40 |
| (2.29) | 24 | 0.281 | 0.150 | 0.949 | 0.131 | 86.20 |
| | 36 | 1.004 | 0.143 | 1.177 | 0.861 | 26.85 |
| | 48 | 0.694 | 0.138 | 1.242 | 0.556 | 55.23 |
| ESF12 | 12 | 0.117 | 0.113 | 0.529 | 0.004 | 99.24 |
| (2.68) | 24 | 0.106 | 0.104 | 0.949 | 0.002 | 99.79 |
| | 36 | 0.099 | 0.087 | 1.177 | 0.012 | 98.98 |
| | 48 | 0.095 | 0.087 | 1.242 | 0.008 | 99.36 |
| ESF6 | 12 | 0.065 | 0.059 | 0.529 | 0.006 | 98.87 |
| (2.56) | 24 | 0.102 | 0.095 | 0.949 | 0.007 | 99.26 |
| | 36 | 0.621 | 0.620 | 1.177 | 0.001 | 99.92 |
| | 48 | 1.442 | 1.388 | 1.242 | 0.054 | 95.65 |
| ESF4 | 12 | 0.562 | 0.098 | 0.529 | 0.464 | 12.29 |
| (2.58) | 24 | 0.949 | 0.221 | 0.949 | 0.751 | 20.86 |
| | 36 | 1.315 | 0.261 | 1.177 | 1.094 | 07.05 |
| | 48 | 1.408 | 0.061 | 1.242 | 1.147 | 07.65 |
| ESF13 | 12 | 0.530 | 0.276 | 0.529 | 0.469 | 11.34 |
| (2.65) | 24 | 1.044 | 0.533 | 0.949 | 0.768 | 19.07 |
| | 36 | 1.404 | 1.384 | 1.177 | 0.871 | 26.00 |
| | 48 | 1.923 | 0.057 | 1.242 | 0.539 | 56.60 |

determined as the ability to lyse erythrocytes in comparison to SDS.

Badosa *et al.* ( 2007) the hemolytic activity for D4E1 peptide in mammalian cells showed low toxicity at the concentration used for phytopathogen elimination and excellent results for *in vitro* control of proteobacteria by the D4E1 peptide was reported by Stover *et al.* (2013). Attilio *et al.* (2013) proved the stability of the most promising peptide D4E1 by producing transgenic sweet orange with D4E1 gene driven by the AtPP2 promoter. Hence, in future D4E1 peptide could be used to create the resistance against *Xanthomonas axonopodis* pv. *punicae* in pomegranate by *Agrobacterium*-mediated transformation.

**Table 7.** *In vitro* efficacy of the different anti-microbial peptides on the growth of *Xanthomonas axonopodis* pv. *malvacearum* using 96-well microtiter plate method

| Peptide/ Conc. (mM) | Hours | Sample reading (A) | -ve control (B) | +ve control (C) | Corrected value (A-B) | Growth inhibition (%) |
|---|---|---|---|---|---|---|
| D4E1 | 12 | 0.273 | 0.097 | 0.359 | 0.176 | 50.97 |
| (2.40) | 24 | 0.192 | 0.112 | 0.416 | 0.080 | 80.77 |
| | 36 | 0.219 | 0.093 | 0.435 | 0.126 | 71.03 |
| | 48 | 0.113 | 0.083 | 0.636 | 0.030 | 95.28 |
| PEP11 | | | | | | |
| (3.53) | 12 | 0.181 | 0.103 | 0.359 | 0.078 | 78.27 |
| | 24 | 0.118 | 0.095 | 0.416 | 0.023 | 94.47 |
| | 36 | 0.170 | 0.142 | 0.435 | 0.028 | 93.56 |
| | 48 | 0.129 | 0.100 | 0.636 | 0.029 | 95.44 |
| ESF17 | 12 | 0.094 | 0.060 | 0.359 | 0.034 | 90.53 |
| (2.88) | 24 | 0.099 | 0.060 | 0.416 | 0.039 | 90.63 |
| | 36 | 0.103 | 0.058 | 0.435 | 0.045 | 89.66 |
| | 48 | 0.106 | 0.056 | 0.636 | 0.050 | 92.14 |
| ESF1 | 12 | 0.339 | 0.214 | 0.359 | 0.125 | 65.18 |
| (2.44) | 24 | 0.340 | 0.214 | 0.416 | 0.126 | 69.71 |
| | 36 | 0.368 | 0.247 | 0.435 | 0.121 | 72.18 |
| | 48 | 0.404 | 0.362 | 0.636 | 0.042 | 93.40 |
| ESF5 | 12 | 0.240 | 0.184 | 0.359 | 0.056 | 84.40 |
| (2.29) | 24 | 0.255 | 0.179 | 0.416 | 0.076 | 81.73 |
| | 36 | 0.280 | 0.179 | 0.435 | 0.101 | 76.78 |
| | 48 | 0.248 | 0.174 | 0.636 | 0.074 | 88.36 |
| ESF12 | 12 | 0.206 | 0.106 | 0.359 | 0.100 | 72.14 |
| (2.68) | 24 | 0.243 | 0.106 | 0.416 | 0.137 | 67.07 |
| | 36 | 0.239 | 0.099 | 0.435 | 0.140 | 67.82 |
| | 48 | 0.184 | 0.093 | 0.636 | 0.091 | 85.69 |
| ESF6 | 12 | 0.220 | 0.119 | 0.359 | 0.101 | 71.87 |
| (2.56) | 24 | 0.221 | 0.117 | 0.416 | 0.104 | 75.00 |
| | 36 | 0.218 | 0.114 | 0.435 | 0.104 | 76.09 |
| | 48 | 0.233 | 0.111 | 0.636 | 0.122 | 80.82 |
| ESF4 | 12 | 0.313 | 0.070 | 0.359 | 0.243 | 32.31 |
| (2.58) | 24 | 0.372 | 0.074 | 0.416 | 0.298 | 28.37 |
| | 36 | 0.641 | 0.344 | 0.435 | 0.297 | 31.72 |
| | 48 | 1.256 | 0.849 | 0.636 | 0.407 | 36.01 |
| ESF13 | 12 | 0.333 | 0.059 | 0.359 | 0.274 | 23.68 |
| (2.65) | 24 | 0.394 | 0.063 | 0.416 | 0.331 | 20.43 |
| | 36 | 0.645 | 0.362 | 0.435 | 0.283 | 34.94 |
| | 48 | 1.573 | 1.252 | 0.636 | 0.321 | 49.53 |

**Table 8.** Hemolysis of promising peptides

| Treat. No. | Treatments | Hemolysis (%) |
|---|---|---|
| $T_1$ | D4E1 – 25 µM | 1.88 |
| $T_2$ | D4E1 – 50 µM | 4.57 |
| $T_3$ | D4E1 – 100 µM | 10.08 |
| $T_4$ | ESF-12 – 25 µM | 2.91 |
| $T_5$ | ESF-12 – 50 µM | 6.62 |
| $T_6$ | ESF-12 – 100 µM | 11.30 |
| $T_7$ | PEP-11 – 25 µM | 2.06 |
| $T_8$ | PEP-11 – 50 µM | 4.92 |
| $T_9$ | PEP-11 – 100 µM | 13.84 |
| | S.E. + | 0.652 |
| | C.D. at 5 % | 1.938 |

## CONCLUSIONS

Among the nine peptides tested *in v*itro through paper disc method, D4E1 PEP-11 and ESF-12 were the most effective against all pathogens viz. *Xap*, *Xac* and *Xam*. ESF 13 was not effective against all the three bacteria, ESF6 was not effective against *Xac* while ESF4 was not effective against *Xap* and *Xac*. The antimicrobial peptides when tested *in vitro* through micro-dilution broth method against all *Xanthomonas* spp., D4E1, PEP11, ESF17 and ESF12 had shown the maximum inhibition of bacterial growth. The anti-microbial peptides that can be used effectively against *Xanthomonas* spp. in crop improvement programme are D4E1, PEP11, ESF17 and ESF12. Three promising peptides viz. D4E1, PEP-11 and ESF-12 at 25, 50 and 100 µM concentration recorded less than 3, 7 and 11 per cent hemolysis, respectively, which is acceptable for eukaryotic cells.

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