Influence of *Peganum harmala* peptides on the transcriptional activity of biofilm related genes in sensitive and resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract: Microbial biofilms have gathered interest in recent years as they have become the major cause of nosocomial infections. The abuse and misuse of antibiotics have created a selective pressure that results in widespread formation of resistant bacterial strains and a need to devise novel plant based antimicrobials. In this study, antimicrobial peptides were isolated from *Peganum harmala* and their effect was examined on biofilm related colonization genes of *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from burn and surgical wounds. Results showed that in *P. aeruginosa* isolated from burn wound, the expression of flagellar gene (*flgK),* pilin gene (*pilA*) and fimbriae gene (*cupA1*) was significantly down-regulated indicating that *Peganum harmala* antimicrobial peptides (PhAMP) damage locomotors of planktonic cells by affecting the gene expression while in resistant biofilm cells, the expression of *flgK, cupA1* and polysaccharide synthesis gene (*pslA*) was enhanced in the presence of PhAMP. In *P. aeruginosa* isolated from surgical wounds which was more sensitive*;* the expression of *flgK, pilA, cupA1* and *pslA* was significantly down-regulated in biofilms and planktonic cells in the presence of PhAMP thus disrupting locomotors of planktonic as well as biofilm cells. In *S. aureus* isolated from burn wounds*;* the expression of capsular polysaccharide synthesis gene (*CPS5*) and inter cellular adhesion gene (*icaA*) was significantly up-regulated in biofilms as well as in planktonic cells in response to PhAMP stress showing resistance mechanism. Thus these genes can be used as efficient resistance markers for bacterial pathogens against antimicrobial agents.

Keywords: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, biofilm, planktonic cells.

INTRODUCTION

Many bacteria live in nature through developing biofilms on surfaces. Biofilm is a bacterial structural association which is embedded in self-made polymer matrix made up of polysaccharide, protein and extra cellular DNA. Biofilm formation is a multi-step process, where first step is the attachment of planktonic cells reversibly to the surface by the help of flagella and pili converting it into irreversible attachment. Weak interactive forces are present (Banin *et al*., 2005). Second step is matrix and micro colony formation. Exopolysaccharides (EPS) that consists of lipids, nucleic acids, lipopolysaccharides and proteins are secreted outside the bacterial cells forming a micro colony (Høiby *et al.*, 2011). This EPS acts as diffusion barrier for certain harmful materials and form a three dimensional structure (Flemming and Wingender 2001). This matrix leads to the maturation of biofilms (Fuqua *et al.*, 1994). Non-motile bacteria form a stalk like appearance while motile organisms form a Mushroom shaped complex architecture. At the end, dispersal of biofilms are done by the help of several factors like nutrient depletion, oxygen and presence of toxic compounds (Tsuneda *et al.,* 2003).

The drugs traditionally have been developed to kill planktonic bacteria. However, it is now unraveled that planktonic bacteria are more susceptible to antimicrobial chemicals designed to kill them than are biofilm bacteria (Flemming and Wingender, 2010, Høiby *et al.*, 2010) and many of the infections plaguing humans are actually caused by bacteria in the biofilm mode of growth, not the planktonic mode (Alfred B. Cunningham 2008). Facing the antibiotic limitations, there is an increasing requirement for the innovation and formation of antibacterial compounds that present novel properties for effectively controlling as well as managing infectious bacterial diseases (Cegelski *et al.,* 2008). Peganum is a small genus that belongs to the family Zygophylaceae and is mainly present in the Mediterranean region, Central Asia, North Africa and also present in Australia and America. From the ancient times, *Peganum harmala* was known to be a significant medicinal plant (Niroumand *et al.*, 2015) and it was used in universal medicine (Sharaf *et al.*, 1997). Seed extracts of *P. harmala* have been proven useful against cancer cell lines (Wang *et al.,* 2017), possess anti parasitic activity (Moazeni *et al.,* 2017) and anti-viral activity (Moradi *et al.*, 2017).

Antimicrobial peptides are small molecules that contain antimicrobial activity and are an important part of innate immune system (Hancock and Diamond 2000). Normally these molecules consist of 10-50 amino acid subunits and

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are grouped based on size, amino acid composition as well as conformation (Nakatsuji and Gallo, 2012). In a previous study (Khalid *et al.,* 2018), we explored the antimicrobial potential of *P. harmala* peptides in biofilm and planktonic cell growth inhibition. This study aims at the molecular mechanism of biofilm inhibition caused by PhAMP in *P. aeruginosa* and *S. aureus* strains of varying sensitivities.

MATERIALS AND METHODS

Growth of bacterial biofilms and planktonic cells

Pseudomonas aeruginosa and *Staphylococcus aureus* were isolated from burn and surgical wounds (Khalid *et al.,* 2018). Overnight cultures were prepared by inoculating a loop full of glycerol stock in 5ml Luria-Bertani (LB) medium and incubated at 37°C with shaking at 150 rpm for 16h. One ml of overnight bacterial culture was pelleted down by centrifugation at 13000rpm for 1 min and re-suspended in sterile LB broth so that the OD_{600} of the cultures was maintained between 0.02-0.04 via spectrophotometer OPTIMA® SP-300. Eight biological replicates of each strain were dispensed in 24 well cell culture polystyrene plates. For this, 1ml of inoculation culture was added in microtiter plate containing 13mm cell culture coverslip. To study the effect of *Peganum harmala* antimicrobial peptide (PhAMP), 18.5µg/ml PhAMP was added in each well and incubated at 37°C with shaking for 16 hours.

RNA extraction and cDNA synthesis

Three ml of bacterial planktonic cells were collected for RNA isolation. Biofilm containing discs from culture plates were gently dipped in distilled water to get rid of planktonic cells and then transferred to phosphate buffer (PBS) and vortexed. Bacterial biofilm cells were quantified by spectrophotometer. Both the bacterial planktonic and biofilm cells were pellet down by centrifugation for 10 minutes at 8000rpm and supernatant was discarded. RNA was extracted from pellets by using RNeasy Mini kit (QIAGEN) as previously described (Qaisar *et al.,* 2013). On-column DNase treatment was performed using QIAGEN RNase-free DNase Set Kit to get rid of DNA present in RNA samples. Total RNA was eluted in diethyl pyro carbonate (DEPC) treated water and stored at -70°C. One µg isolated RNA was reverse transcribed using Thermo Scientific Revert Aid First Strand cDNA Synthesis kit (Fermentas).

Real-time RT-PCR for estimation of gene expression

Real-time PCR (RT-PCR) was performed to check the expression levels of various genes that were linked to biofilm formation. Gene specific primers were designed using primer3 software and the sequences are given in the Table 1. 30S rRNA (*rpsL*) genes was used as an internal control for normalization of RNA quantities as previously described (Qaisar *et al.,* 2016). SYBER-Green (Thermo Scientific) master mix was used as a reaction mixture for detection of PCR product. Three replicates of each sample were used and reaction was placed in the PikoReal qPCR (Thermo Scientific). Normalization and analysis of data was performed using PikoReal Software 2.2 (Thermo Scientific). Statistical analysis and plotting of data was performed using PRISM GraphPad software (Kruczek *et al.,* 2014). 2-way Analysis of Variance (ANOVA) was used to find the significance while Bonferroni post-test was used to compare the expression between control and PhAMP treated samples.

RESULTS

PhAMP reduces the expression of bacterial locomotion related genes in planktonic cells of P. aeruginosa

Planktonic cells are the unicellular, free-swimming bacteria capable of converting into biofilm cells through up or down-regulation of certain genes. Expression of bacterial locomotor genes i.e. flagellar *(flgK),* pilin *(pilA) and* fimbriae *(cupA1)* gene *and* polysaccharide synthesis gene *(pslA)* was examined under the treatment of PhAMP to check its effect on planktonic cells. In the presence of PhAMP, *cupA1* showed 6 fold, *flgK* 1.5 fold and *pilA* gene showed 2.8 fold decrease in expression as compared to control (fig. 1). This reduction in expression of locomotion related genes indicates that planktonic cells are susceptible to PhAMP as transcription of these important genes is significantly reduced in the presence of PhAMP. Expression of *pslA* gene did not change significantly.

Expression of biofilm related genes was enhanced in P. aeruginosa burn wound isolate

Burn wounds are prone to bacterial infections which is the main cause of mortality and morbidity in burn patients. Bacterial biofilms are formed by the attachment of bacteria to the organic surfaces through their flagella, pilli and fimbriae. We tested the expression of bacterial flagellar gene (*flgK*), pilin gene (*pilA*), fimbriae gene (*cupA1*) and polysaccharide synthesis gene (pslA) to study the effect of PhAMP on these organs. In the presence of PhAMP, cupA1 gene showed 20 fold, *flgK* gene 4 fold, *pilA* gene 2 fold and pslA gene 27 fold increased in the expression as compared to the control (fig. 2). This enhancement in transcript levels of biofilm related genes in response to PhAMP leads to the addition of biofilm as previously evidenced (Khalid *et al* 2018). It is assumed that bacteria sense the presence of antibacterial agent PhAMP in the environment and prepare themselves to switch to the more resistant mode of life style (biofilm) by enhancing the formation of pilin, fimbriae, flagella and polysaccharides. This resistance mechanism is only evident in *P. aeruginosa* isolate which has improved resistance against PhAMP.

Table 1: Gene specific primers used for real time RT-PCR

Bacterial Strain	Gene Name	Primer ID	5'…3' sequence	Primer Tm	Product size
P. aeruginosa	*pslA*	*pslA*(forward)	CTGTTCCTGCTGTACTACCCC	62°	240bp
		pslA(reverse)	CTTGCTGCTGAGGTAGGGAAA		
	cupA1	*cupA1*(forward)	CGGCAAACACTATCACATTCA	58°	211bp
		cupA1(reverse)	AACAGGGTGGTGAAATGCTC		
	pilA	*pilA*(forward)	GATCGAACTGATGATCGTGGT	60°	214bp
		pilA(reverse)	GACATATGTTTCGGTCGCAGT		
	flgK	*flgK*(forward)	CGATACCGTCAACAAGCAACT	60°	169bp
		flgK(reverse)	CTTGCTGGTATCGGTGATGTT		
S. aureus	*CPS5*	*CPS5*(forward)	CGGTACAGCAGTTAAAGTCGC	60°	170bp
		CPS5(reverse)	TTGAACCCAATACAGGCAATCC		
	icaA	*icaA*(forward)	CCAGAAACATTGGGAGGTCTT	60°	102bp
		icaA(reverse)	CCTTTTCGTTTTCATTGTGCT		

Expression of bacterial locomotor genes in planktonic cells of P. aeruginosa isolated from surgical wounds

P. aeruginosa isolated from surgical wound exhibited bigger zone of inhibition by antimicrobial peptides from *Peganum harmala* (PhAMP) as compared to the burn wound isolate of the same species (Khalid *et al.*, 2018). In order to explore the mechanism of biofilm inhibition in sensitive strain, we tested the effect of PhAMP on the expression of biofilm related genes; *flgK*, *pilA*, *cupA1* and *pslA* of *P. aeruginosa* isolated from surgical wounds. In the presence of PhAMP, *cupA1* gene exhibited 2.4 fold, *flgK* 1.5 fold and *pilA* gene 1.4 fold decreased in expression as compared to control (fig. 3) and *pslA* gene showed non-significant differences. A reduction in planktonic cell growth was also observed in surgical wound pathogen (Khalid *et al.*, 2018) as locomotors necessary for the acquisition of nutrients are significantly down-regulated.

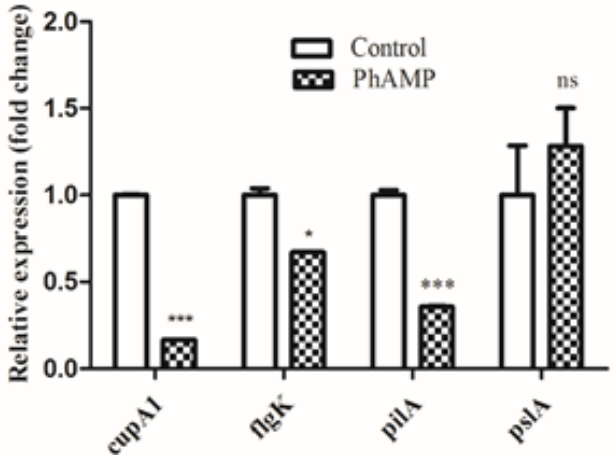

Fig. 1: Expression of *P. aeruginosa* locomotion related genes is significantly reduced by *P. harmala* anti-microbial peptides (PhAMP) in planktonic cells: ns indicates that p-value is more than 0.05, *** indicates that p-value is less than or equal to 0.001 and * indicates that p-value is less than 0.05. Error bars represent standard deviation (SD) among replicated samples.

Expression of biofilm related genes is reduced in P. aeruginosa surgical wound isolate

Biofilm formation plays a significant role in the survival of bacterial pathogens in diverse environmental conditions by providing refuge to the individual cells. Biofilm formation was inhibited in surgical wound isolate but enhanced in burn wound isolate on treatment with PhAMP (Khalid *et al.*, 2018) indicating the sensitivity of surgical wound isolate towards PhAMP. We tested the expression of bacterial *flgK*, *pilA*, *cupA1* and *pslA* genes to study the mechanism of action of PhAMP in biofilm inhibition. In the presence of PhAMP, *cupA1* gene exhibited 3.4 fold, *flgK* gene 2.3 fold, *pilA* 2.8 fold and *pslA* 2.6 fold decrease in the expression as compared to control (fig. 4). Results showed that biofilm formation in *P. aeruginosa* is inhibited (Khalid *et al.*, 2018) as locomotors are destroyed and the secretion of polysaccharide is decreased by PhAMP. However, biofilm inhibition was not evident in the burn wound isolate because the transcription of these important genes was not inhibited, rather enhanced (fig. 2).

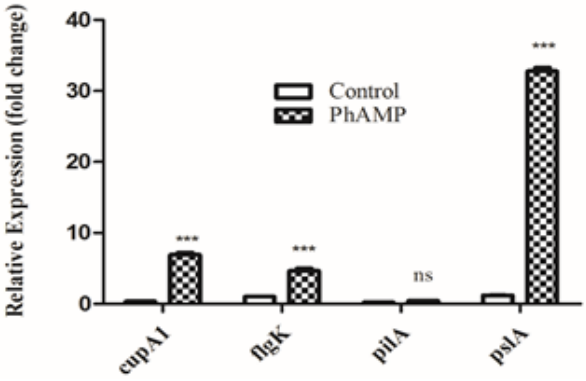

Fig. 2: Expression of biofilm related genes is enhanced in the presence of *P. harmala* anti-microbial peptides

(PhAMP) in *P. aeruginosa* burn wound isolate. ***indicates that the p-value is less than or equal to 0.001 and ns indicates that p-value is more than 0.05. Error bars represent standard deviation (SD) among triplicates.

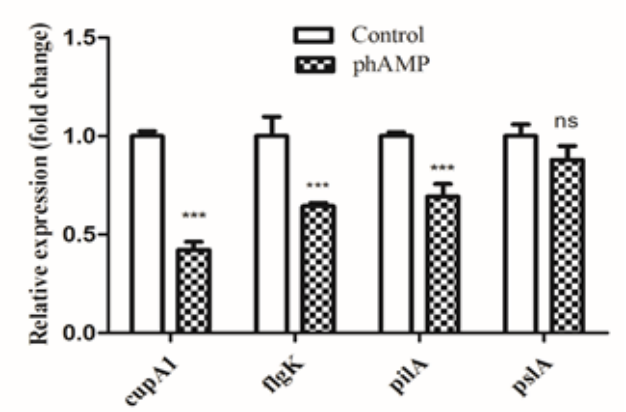

Fig. 3: Effect of *P. harmala* anti-microbial peptides (PhAMP) on Planktonic cell growth in *P. aeruginosa* isolated from surgical wounds: ***indicates that p-value is less than or equal to 0.001 and ns represents p-value more than 0.05. Error bars represent standard deviation (SD) between control and treatment samples.

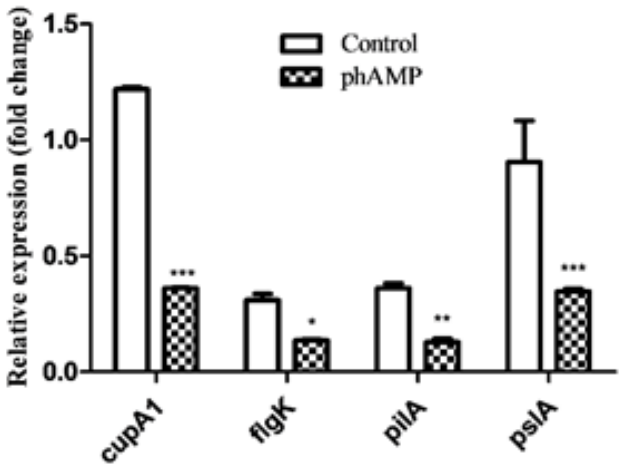

Fig. 4: Effect of *P. harmala* anti-microbial peptides (PhAMP) on Biofilm formation in *P. aeruginosa* isolated from surgical wounds: ***indicates that p-value is less than or equal to 0.001, **indicates that p-value is less than 0.01 and *indicates that p-value is less than 0.05. Error bars represent standard deviation (SD) among triplicates.

Expression of bacterial colonization genes is enhanced in planktonic and biofilm cells of Staphylococcus aureus burn wound isolate

S. aureus is capable of colonizing surfaces of various kinds, leading to the formation of biofilms. Expression of bacterial colonization genes i.e. capsular polysaccharide gene *(CPS5) and* intercellular adhesion gene *(icaA)* was examined under the treatment of PhAMP to check its effect on planktonic and biofilm cells separately. In the presence of PhAMP, the expression of *CPS5* gene was enhanced to 72 fold as compared to control likewise *icaA* expression was enhanced to 35 fold (fig. 5a). The result showed that on treatment with PhAMP, planktonic cells sense the stress condition and prepare themselves to go in the colonization mode by enhancing the expression of genes which lead to biofilm formation. In the biofilm cells, the expression of *CPS5* gene was enhanced to 3.7 fold while *icaA* expression was enhanced to 19 fold as compared to control (fig. 5b). Biofilm formation in *S. aureus* isolated from burn wound was also enhanced in the presence of PhAMP (Khalid *et al.*, 2018) as capsular polysaccharide synthesis and intercellular adhesion genes are significantly up-regulated thus increasing the micro colony formation.

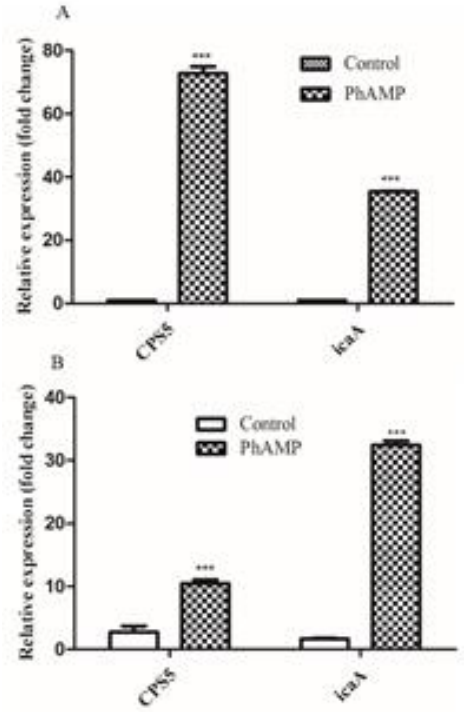

Fig. 5: Effect of *P. harmala* anti-microbial peptides (PhAMP) on colonization related genes of *S. aureus* isolated from burn wounds (a) Planktonic (b) Biofilm cells: ***indicates that p-value is less than or equal to 0.001. Error bars represent standard deviation (SD) among replicated samples.

DISCUSSION

Transcriptional dynamics of locomotion related genes *flgK* and *pilA*, surface attachment gene *cupA* and polysaccharide synthesis genes *pslA* of *P. aeruginosa* are involved in biofilm formation. Up-regulation of these genes enhanced biofilm formation and down-regulation inhibit biofilm (figs. 2 and 4). While in *S. aureus* polysaccharide synthesis gene *CPS5* and cell-cell adhesion gene *icaA* are directly involved in switching

from planktonic mode to biofilm mode of growth (fig. 5). These genes can be used as molecular markers for determining the resistance of bacteria against antibiotics. Next generation sequencing is needed to explore the source of adaptive resistance of *P. aeruginosa* against PhAMP in burn wound isolate.

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REFERENCES

Alfred B, Cunningham JEL and Rockford JR (2008). Bacteria in a biofilm have different characteristics than the same bacteria in isolation. Biofilms: The Hyper Text Book. Montana State University, Bozeman, MT. p.1800

Banin E, Vasil ML and Greenberg EP (2005). Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc. Natl. Acad. Sci. USA*, **102**(31): 11076-11081.

Cegelski L, Marshall GR, Eldridge GR and Hultgren SJ (2008). The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.,* **6**(1): 17.

Flemming HC and Wingender J (2001). Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects. *Water Sci. Technol.* **43**(6): 1-8.

Flemming HC and Wingender J (2010). The biofilm matrix. *Nat. Rev. Microbiol.*, **8**(9): 623-633.

Fuqua WC, Winans SC and Greenberg EP (1994). Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.,* **176**(2): 269-275.

Hancock RE and Diamond G (2000). The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.,* **8**(9): 402-410.

Høiby N, Ciofu O and Bjarnsholt T (2010). Pseudomonas aeruginosa biofilms in cystic fibrosis. *Future Microbiol.,* **5**(11): 1663-1674.

Høiby N, Ciofu O, Johansen HK, Song ZJ, Moser C, Jensen PØ, Molin S, Givskov M, Tolker Nielsen T and Bjarnsholt T (2011). The clinical impact of bacterial biofilms. *Int. J. Oral Sci.* **3**(2): 55.

Khalid R, Jaffar Q, Tayyeb A and Qaisar U (2018). *Peganum harmala* peptides (PhAMP) impede bacterial growth and biofilm formation in burn and surgical wound pathogens. *Pak. J. Pharm. Sci.,* **31**(6): 2597-2605.

Kruczek C, Qaisar U, Colmer Hamood JA and Hamood AN (2014). Serum influences the expression of *Pseudomonas aeruginosa* quorum sensing genes and QS controlled virulence genes during early and late stages of growth. *Microbiol. Open*, **3**(1): 64-79.

Moazeni M, Ardakani ZSS, Saharkhiz MJ, Jalaei J, Khademolhoseini AA, Abad SSE and Alavi AM (2017). *In vitro* ovicidal activity of *Peganum harmala* seeds extract on the eggs of *Fasciola hepatica*. *J. Parasitic Diseases*, **41**(2): 467-472.

Moradi M-T, Karimi A, Rafieian-Kopaei M and Fotouhi F (2017). *In vitro* antiviral effects of Peganum harmala seed extract and its total alkaloids against influenza virus. *Microb. Pathog.* **110**: 42-49.

Nakatsuji T and Gallo RL (2012). Antimicrobial Peptides: Old Molecules with New Ideas. *J. Invest. Dermatol.*, **132**(3): 887-895.

Qaisar U, Kruczek CJ, Azeem M, Javaid N, Colmer-Hamood JA and Hamood AN (2016). The *Pseudomonas aeruginosa* extracellular secondary metabolite, Paerucumarin, chelates iron and is not localized to extracellular membrane vesicles. *J. Microbiol.,* **54**(8): 573-581.

Qaisar U, Luo L, Haley CL, Brady SF, Carty NL, Colmer-Hamood JA and Hamood AN (2013). The PVC operon regulates the expression of the Pseudomonas aeruginosa fimbrial chaperone/usher pathway (cup) genes. *PloS one*, **8**(4): e62735.

Sharaf M, El-Ansari MA, Matlin SA and Saleh NA (1997). Four flavonoid glycosides from *Peganum harmala*. *Phytochemistry*, **44**(3): 533-536.

Tsuneda S, Aikawa H, Hayashi H, Yuasa A and Hirata A (2003). Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiol. Lett.,* **223**(2): 287-292.

Wang KB, Li DH, Bao Y, Cao F, Wang WJ, Lin C, Bin W, Bai J, Pei YH and Jing YK (2017). Structurally diverse alkaloids from the seeds of Peganum harmala. *J. Nat. Prod.* **80**(2): 551-559.