Cibtech Journal of Pharmaceutical Sciences ISSN: 2319–3891 (Online)
An Online International Journal Available at http://www.cibtech.org/cjps.htm
2012 Vol. 1 (1) April-June, pp.22-35/Akhtar et al.
Research

ANTIMICROBIAL SPECTRUM OF FLUORESCENT PSEUDOMONAD, R-62 AGAINST BACTERIA

***Nabila Akhtar[1], M. Sajjad Khan[2] and M. D. Kharya[2]**
[1]Department of Molecular Research, Bhopal memorial hospital and research center, Bhopal (M.P)
[2]Department of Pharmaceutical Sciences, H. S. Gour University, Sagar (M.P)
**Author for Correspondence*

ABSTRACT
Secondary metabolites from microorganisms are common sources of antibiotics. However, recent increases in bacterial infections, decreasing availability of potent antibiotics and an increasing bacterial resistance to available antibiotics requires the search for new antibacterial compounds to be broadened. In this study we investigated the antimicrobial properties of two secondary metabolites from fluorescent pseudomonads (R-62), a lipodepsipeptide produced by *Pseudomonas syringae*, which contains two primary types of glycolipids produced by *Pseudomonas aeruginosa*. Human pathogens, food spoilage organisms and fermentative bacteria from both gram-positive and gram-negative classes were tested to determine the inhibitory potential of these compounds. The rate of antimicrobial action was determined by monitoring the rate of uptake of propidium iodide during exposure of R-62. R-62 compound is able to disrupt the membrane of all the gram-positive bacteria and inhibit the formation of cell wall, the rhamnolipids acting significantly faster (3-4 times depending upon the organism tested, $p<0.05$) than R-62 and inhibit them. Inhibition was confirmed by the microbroth dilution method and both the compounds inhibited all the gram-positive organisms tested, as well as *Flavobacterium devorans* with MICs ranging from 3-32µg/ml. Both compounds inhibited *Mycobacterium smegmatis*, *Bacillus subtilis* spores *and Clostridium sporogenes* spores with an MIC of 4µg/ml. Interestingly, these compounds acted synergistically to inhibit *Listeria monocytogenes*, thereby lowering the MIC for *L. monocytogenes*. No toxicity was observed during exposure of these compounds to mouse enteroendocrine, human embryonic kidney and human lung fibroblasts cells. Combined above data concluded that both these compounds have potential to be used as / and can be used as antibacterial compounds.

Key Words: *Antimicrobial, MICs, Syringopeptin, Rhamnolipids*

INTRODUCTION
The ability to control infections due to microorganisms has been one of the single most profound developments in the history of health science. The initial success of antibiotics is now marred by the emergence of resistant organisms (Ang *et al.,* 2004). Antibiotic resistance is a complex problem exacerbated owing to the versatility of the microbes, overuse of antibiotics and the lack of patients completing the prescribed dosage (Ang *et al.,* 2004). Among many issues of antibiotic resistance, one of the most troubling issues is the establishment of vancomycin resistant organisms (Richet *et al.,* 2001). The solution to this complex problem remains to be identified, but discovery of new compounds is essential in solving this issue. One source of new antibiotics is the secondary metabolites of bacteria with different modes of action. The antimicrobial potential of secondary metabolites of fluorescent pseudomonads has been studied extensively (Bender *et al.,* 1999, Budzikiewicz 1993 and Leisinger and Margraff 1979). Among the known antibacterial compounds, the most well characterized compounds are produced by *Pseudomonas syringae (P. syringae)* and *Pseudomonas aeruginosa (P. aeruginosa)*. The ability of these organisms to inhibit competing microorganisms via a myriad of mechanisms has inspired

the search for new compounds from these and other pseudomonads (Bender *et al.,* 1999, Budzikiewicz, 1993 and Leisinger and Margraff 1979). Many pathovars of *P. syringae* produce non-specific toxins (e.g. syringomycin, syringopeptin, coronatine, phaseolotoxin, syringotoxin and tabtoxin) that increase the virulence of the organism to the host plant (Bender *et al.,* 1999). Some of these toxins have antimicrobial and antifungal properties (Bender *et al.,* 1999) which fuels their use as biocontrol agents (22). The most promising antibacterial activity is shown by pseudomonad's (Bender *et al.,* 1999, Buber *et al., 2002*, Lavermicocca *et al.,* 1997 and Volksch and Weingart 1998). In nature, pseudomonad's because electrolyte leakage by forming pores in the plasma membrane of plant cells, thereby promoting transmembrane ion flux that leads to plant necrosis (Iacobellis *et al.,* 1992). Pseudomonads also have biosurfactant properties with a low critical micelle concentration (0.4-0.9 mm) (Dalla *et al.,* 2003) that may aid in the spread of the organisms on the plant surface (Bender *et al.,* 1999). Additionally pseudomonads along with syringomycin induce stomatal closure in plants, thereby preventing entry of other pathogens, which effectively reduces competition by other organisms on the plant surface (Giorgio *et al.,* 1996). Pseudomonad's are the cyclic lipodepsipeptides and are produced by many strains of *P. syringae* (Adetuyi *et al.,* 1995, Grgurina *et al.,* 2002, Scholz *et al.,* 2001 and Volksch and Weingart 1998). Currently, five different Pseudomonad's have been identified which vary in the length and number of fatty acid chain and composition of amino acids in the peptide moiety (Adetuyi *et al.,* 1995, Ballio *et al.,* 1995, Bender *et al.,* 1999, Grgurina *et al.,* 2002 and Isogai *et al.,* 1995). Pseudomonad, R-62 contain amino acids with the N-terminal being acylated with either 3-hydroxydecanoic or 3-hydroxydodecanoic acid to 2, 3-dehydro-2-aminobutyric acid (Ballio *et al.,* 1995, Bender *et al.,* 1999 and Isogai *et al.,* 1995). An eight-member lactone ring is formed due to the ester bond between allothreonine and C-terminal tyrosine (Ballio *et al.,* 1995, Bender *et al.,* 1999 and Isogai *et al.,* 1995). A high percentage of hydrophobic amino acids are found in R-62 with the peptide being composed of d-amino acids primarily (Ballio *et al.,* 1995, Bender *et al.,* 1999 and Isogai *et al.,* 1995). The peptide sequence of the pseudomonad's vary from strain to strain (Ballio *et al.,* 1995, Bender *et al.,* 1999 and Isogai *et al.,* 1995) and mechanism of bacterial inhibition is unknown however, initial studies indicate that Pseudomonad's form pores in the cell membrane (Agner *et al.,* 2002, Hutchison and Gross 1997, Iacobellis *et al.,* 1992, Dalla *et al.,* 2003 and Szabo *et al.,* 2004). It is hypothesized that R-62 molecules adsorb onto the cell membrane via the hydrophobic acyl chain inserted between the phospholipids of the membrane. Once the adsorbed monomers form aggregates of sufficient concentration, a pore is formed (Dalla *et al.,* 2003, Hutchison and Gross 1997). However, this mode of action remains to be proven, as does the minimum number of R-62 molecules required for pore formation. *P. aeruginosa* is the epitome of opportunistic pathogens in humans, but the wide interest in this organism is not only stems but also a wide catabolic potential and the array of compounds with antibiotic activity that it produces (Budzikiewicz 1993). Cell free culture supernatants from *P. aeruginosa* were extensively used in therapy of diphtheria, influenza and meningitis and it exhibited enzymatic properties called as pyocyanase (Leisinger and Margraff 1979). Subsequently, nearly 50 antimicrobial substances have been characterized from fluorescent pseudomonads (Budzikiewicz 1993). This study is focused on the antimicrobial properties of pseudomonad's which are glycolipids produced by some strains of *P. aeruginosa* (Benincasa *et al.,* 2004, Budzikiewicz 1993, Maier and Soberon-Chavez, 2004 and Rendell *et al.,* 1990). In *in-vivo* R-62 prevent macrophage phagocytosis of the organism by bringing about structural changes in the macrophages so they cannot associate with the bacteria (McClure and Schiller 2003). The target of R-62 against bacteria is the cell membrane for an action (Abalos *et al.,* 2001); presumably the surfactant properties of the compound are responsible for the permeabilization effect on the cell surface. In this study, we

hypothesized that Pseudomonad's inhibit a wide spectrum of bacteria without causing toxicity to mammalian cell lines.

MATERIALS AND METHODS

Purification of Antimicrobial Pseudomonad

Pseudomonad (R-62) was produced and purified from *P. syringae* as described by Bidwai *et al.,* In brief, the culture was grown to stationary phase for 10^0 standing culture at room temperature (~25°C). After collection of the supernatant R-62 was extracted with acidified acetone, concentrated through rotavapor (R215, Buchi), purified to homogeneity by reverse phase HPLC (VP series, Simadzu) and lyophilized through lab lyophilizer (LD85) for storage at 4°C until further use. Purity and the molecular weight of the compound were verified by MALDI-TOF analysis. Commercial Pseudomonad samples were obtained as 25.1% aqueous solution (product JBR-425) from Jeneil Biotech, Inc. (Saukville, WI). The purity and molecular weight of the R-62 were determined using MALDI-TOF at the Center for Integrated BioSystems.

Determination of Rate of Antimicrobial Action and the Minimum Inhibitory Concentrations (MICs) against Bacteria

The antimicrobial action for each compound was initially determined by the rate of uptake of propidium iodide (PI) (Fluropure grade, Molecular Probes, Inc.; Eugene, OR) as previously described (Haughland 2002). In brief, all cultures were grown overnight in their respective optimal growth medium and temperature from freezer vials (Table 1.0). Each culture was sub-cultured twice, harvested in mid log phase, washed with saline and adjusted to an OD600 of 0.25 in saline. PI with an excitation and emission wavelength of 535 and 617nm respectively, were added to the culture suspension at a final concentration of 10µM. Each organism was treated with 2.2ml final volume of 50 µg/ml R-62, the increase in fluorescence was measured with a Shimadzu RF-1501 spectrophotoflurometer at 15 s intervals for a maximum period of 120 min. Saline was added in place of R-62 as a negative control. All inhibition experiments were done in three biological replicates. The rate of antimicrobial action was expressed as the inhibition rate (IR) (Eq. 1). The curve fitting was done using Origin Pro Ver. 7.0 (Natick, MA).

IR = ((Log RFU/ (Time)) – C) / Time (when d Log RFU/ dT > 0) (Eq. 1)

Where RFU = relative fluorescent units; and C = Y intercept

Table 1a: List of Yeast strains used for antimicrobial screening and their growth conditions.

Organism	Strain	Temperature (°C)	Oxygen Demand/Medium
Brettonomyces bruxellensis	12021	30	Aerobic/ Nutrient agar
Candida vini	12043	33	
Pichia fermentans	12142	37	
Saccharomyces luduigi	12033	30	
Metschinikowia pulcherrima	12022	37	
Kloeckera apiculata	12111	33	

The MIC for the organisms was determined by microbroth dilution method as prescribed by the National Committee for Clinical Laboratory Standards (NCCLS) (Woods *et al.*, 1995). The microorganisms were prepared as described above and resuspended in their optimal growth media (Table 1.0) to ~10^5 CFU/ml containing R-62 at 8.0 µg/ml in a total volume of 550 µl were tested in a 48-well plate (Corning, NY).

The plates were incubated in optimal growth conditions for the respective organism and monitored for an increase in OD600 after 48 h by a Perkin-Elmer (HTS 7000) plate reader (Downers Grove, IL). A positive control (inhibition of growth) Polymyxin B (Sigma-Aldrich) for Gram-negative organisms, Penicillin G (Sigma-
Aldrich) for the Gram-positive organisms and a negative control (no inhibition of growth) Rifampicin (Sigma-Aldrich) for *M. smegmatis, E. faecalis* and *S. aureus* at 1000 µg/ml were included using saline in the assay for each compound. The least concentration at which there was no increase in OD over 48 h was reported as the MIC. Each MIC was determined in two biological replicates with triplicate tests per replication. The triplicates were averaged for each replicate reported. The activity was measured by exposing *L. monocytogenes* to R-62 at a concentration of 8.0 µg/ml and the PI uptake was monitored. (Table-2)

Table 1b: List of bacteria used for antimicrobial screening and their growth conditions.

Organism	Strain	Temperature (°C)	Oxygen Demand	Medium
Aeromonas caviae	13137	30	Aerobic	Nutrient agar
Bacillus cereus	10987	30	Aerobic	Nutrient agar
Bacillus subtilis	23857	26	Aerobic	Nutrient agar
Bacillus megaterium	14581	30	Aerobic	NB
Brevibacterium linens	BL1	37	Aerobic	TSB
Citrobacter fruendii	MGE	37	Aerobic	Nutrient agar
Clostridium sporogenes	11811	37	Anaerobic	Reinforced
Enterobacter aerogenes	10000	30	Aerobic	Clostridial
Enterococcus faecalis	13048	37	Aerobic	Medium
Erwinia herbicola	700802	37	Aerobic	Nutrient agar
Eschereschia coli	33243	37	Aerobic	BHI
Eschereschia coli H7:0157	K12	37	Aerobic	Nutrient agar
Flavobacterium devorans	35150	30	Aerobic	Nutrient agar
Klebsiella pneumoniae	10829	37	Aerobic	Nutrient agar
Lactobacillus plantarum	700721	37	Microaerophilic	Nutrient agar
Lactococcus lactis	8014	37	Microaerophilic	NB
Listeria innocua	4355	30	Microaerophilic	MRS
Listeria monocytogenes	IL1403	37	Aerobic	MRS
Micrococcus luteus	33090	37	Aerobic	Ellikers Broth
Mycobacterium smegmatis	43251	30	Aerobic	BHI
Salmonella typhimurium	21102	37	Aerobic	BHI
Salmonella enteridis	14468	37	Aerobic	BHI
Staphylococcus aureus	13076	37	Aerobic	Luria Broth
Streptococcus mutans	700931	37	Aerobic	Nutrient agar
Streptococcus suis	700699	37	Aerobic	TSB
Streptococcus agalacticae	891591	37	Aerobic	BHI
***Bacillus subtilis* (spores)**	700610	37	Aerobic	BHI
***Clostridium sporogenes*(spores)**	12403 11437	37	Anaerobic	BHI Reinforced

Determination of Cytotoxicity in Cell Culture

Toxicity of the R-62 to mammalian cells was assayed in cell culture using mouse enteroendocrine cells (STC-1) (Vincent *et al.,* 2001), human embryonic kidney cells (HEK 293; ATCC CRL-1573) and human lung fibroblasts (LL47; ATCC CCL-135). Each cell line was subjected to R-62 at the observed MIC (e.g. 4 µg/ml and 8 µg/ml). The human embryonic kidney cells and human lung fibroblasts were grown as per the ATCC recommendation, while the mouse enteroendocrine cells were grown as described by Vincent *et al.,*

Table 2: MICs (Two biological replicates done in duplicate) and mean Iinhibitory rates (IR's) (2 biological replicates) of SP 25A and R-62 against screened organisms. (ND represents Not Determined).

Genus	IR R-62 (8.0µg/ml)	MIC(µg/ml)	IR SP 25A (8.0µg/ml)	MIC(µg/ml)
Bacillus megaterium	1.043	4	0.005	3
Listeria innocua	0.014	5	0.005	3
Listeria monocytogenes	0.032	6	0.005	3
Bacillus cereus	0.834	4	0.004	4
Bacillus subtilis	1.807	4	0.006	4
Clostridium sporogenes	0.698	4	0.008	4
Flavobacterium devorans	0.518	16	0.002	4
Lactococcus lactis	1.219	4	0.008	4
Micrococcus luteus	0.183	8	0.006	4
Mycobacterium smegmatis	ND	4	ND	4
Streptococcus mutans	0.164	4	0.003	4
Streptococcus suis	1.018	4	0.006	4
Bacillus subtilis (spores)	ND	4	ND	4
Clostridium sporogenes(spores)	ND	4	ND	4
Enterococcus faecalis	0.482	>60	0.001	8
Lactobacillus acidophilus	0.196	16	0.003	8
Staphylococcus aureus	0.894	>60	0.003	8
Streptococcus agalacticae	1.073	4	0.004	8
Lactobacillus plantarum	0.287	32	0.003	16
Aeromonas caviae	0.000	>60	0.000	>50
Citrobacter fruendii	0.000	>60	0.000	>50
Enterobacter aerogenes	0.000	>60	0.000	>50
Erwinia herbicola	0.000	>60	0.000	>50
Escherichia coli K12	0.000	>60	0.000	>50
Klebsiellapneumoniae	0.000	>60	0.000	>50
Salmonella typhimurium	0.000	>60	0.000	>50
Salmonella enteridis	0.000	>60	0.000	>50
Brevibacterium linens	0.512	ND	0.009	ND
Escherichia coli	0.000	ND	0.000	ND

Media and serum were purchased from HyClone Laboratories (Logan, UT). Triton and syringopeptin (SP25A) were purchased from Sigma Aldrich and were used as internal control. All cells were grown in

10% fetal bovine serum (FBS). The number of total and dead cells was counted after 0, 24, 48, 72 and 96 h using a Nucleocounter automated cell counting system (New Brunswick; Edison, NJ). In brief, cells (STC 1 - 200,000 cells/well, HEK 293 - 200,000 cells/well and LL47 - 100,000 cells/well) were Incubated with an appropriate medium for 24 h prior to addition of fresh media containing the antimicrobial compounds. After addition of the antimicrobial compound, the cell cultures were incubated at 37°C with 5% CO2 for 0, 24, 48, 72 and 96 h.

Cells were harvested by trypsinization using 0.25 % trypsin-EDTA for 2 min. The trypsin was neutralized by addition of 200 µl of serum containing fresh medium. The cells were harvested and transferred to 1.5 ml tubes, centrifuged (3-5 min at <100 x g) and resuspended in 200 µl of fresh medium. Subsequently, for total cell count 100 µl of the cell suspension was added to the Lysis buffer (Reagent A100 in the starting kit (Cat No. M1293-0020, New Brunswick Scientific) for 30 s, which was stabilized using 100 µl of Reagent B. A positive control of completely lysed cells by lysing all the cells with triton was used along with a negative control using sterile PBS (pH 7.4). For dead cell count 100 µl of cell lysate was counted without lysis buffer. All cell counts were obtained using the Nucleocounter automated cell counting system and data were reported as the percent of cell death. The toxicity testing was done in biological replicates with triplicate wells per replication and was averaged before reporting the reading. (Table-3)

Table 3a: Cytotoxicity study of R-62 to STC-1 with RPMI 1640 media in culture represent number of cell w.r.t. 4 and 8mcg concentrations respectively

Time interval	Control (STC-1)	R-62 with STC-1		SP25A		Triton with STC-1	
		4mcg	8mcg	4mcg	8mcg	4mcg	8mcg
0	2	2	2	2	2	2	2
24	5.82	3.78	3.41	3.11	2.77	1.97	1.23
48	8.92	7.47	6.17	7.02	5.43	1.23	0.912
72	14.4	8.63	7.9	7.96	6.31	0.88	0.47
96	48.3	33.7	31.89	29	27.19	0.39	0.11

Table 3b: Cytotoxicity study of R-62 to HEK-293 with RPMI 1640 media in culture represent number of cell w.r.t 4 and 8mcg concentrations respectively

Time interval	Control	R-62 with HEK-293		SP25A		Triton with HEK-293	
		4mcg	8mcg	4mcg	8mcg	4mcg	8mcg
0	2	2	2	2	2	2	2
24	3.72	2.73	1.86	3.11	2.76	1.51	0.96
48	5.47	4.19	2.57	4.98	3.89	0.98	0.43
72	11.04	7.45	4.99	10.2	9.09	0.49	0.19
96	23.1	16.76	11.21	21.87	19.1	0.13	0.04

Table 3c: Cytotoxicity study of R-62 to LL-47 with RPMI 1640 media in culture represent number of cell w.r.t 4 and 8mcg concentrations respectively

Time interval	Control	R-62 with LL-47		SP25A		Triton with LL-47	
		4mcg	8mcg	4mcg	8mcg	4mcg	8mcg
0	1	1	1	1	1	1	1
24	2.13	1.67	1.1	1.98	1.7	0.64	0.43
48	4.12	2.43	1.96	2.65	2.12	0.39	0.21
72	7.94	3.86	2.34	4.45	3.96	0.19	0.1
96	15.3	4.93	2.61	8.16	6.7	0.09	0.02

RESULTS

Compound Purity

After purification R-62 was subjected to MALDI-TOF and HPLC analysis to confirm the purity of the fractionated compound. HPLC analysis (Figure 2) revealed a single peak, as did MALDI TOF (Figure 1). This single major peak had a molecular weight of 2,400.37 Da, which was in agreement with the reported size. (Monti *et al.*, 2001)

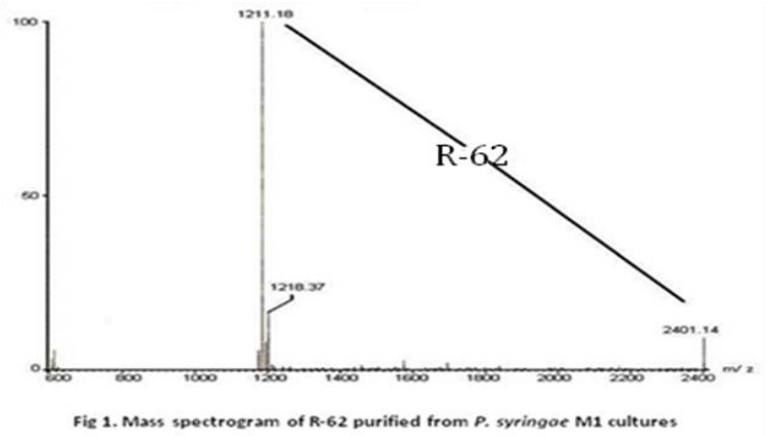

Fig 1. Mass spectrogram of R-62 purified from *P. syringae* M1 cultures

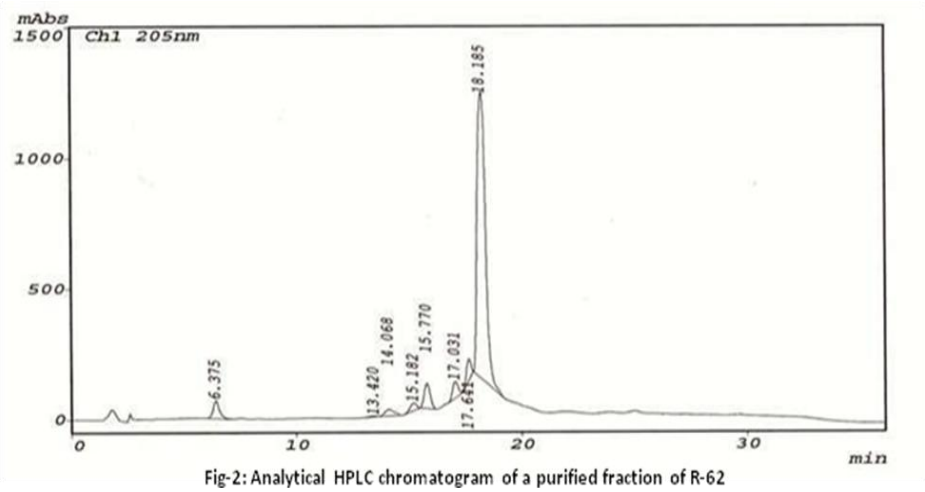

Fig-2: Analytical HPLC chromatogram of a purified fraction of R-62

Bacterial Inhibition by R-62

Cell lysis was measured through PI, a membrane impermeant nucleic acid stain, as a probe to monitor cell membrane integrity during cellular exposure to both the compounds (Haughland 2002).

As such, the rate of PI accumulation (Eq. 1) was used to compare the inhibitory rate for each organism tested (Figure 3). R-62 was not inhibitory to any gram-negative organisms except *F. devorans*, while it inhibited all gram-positive organisms tested. Also, R-62 did not inhibit the growth of any yeast (*Brettonomyces bruxellensis, Candida vini, Pichia fermentans, Saccharomyces luduigi, Metschinikowia pulcherrima, Kloeckera apiculata)*. The greatest rate of inhibition was found for *Brevibacterium linens*, while *E. faecalis* had the slowest rate of inhibition.

Cellular Toxicity

Three mammalian cell lines were used to assess cytotoxic effects of R-62 was observed at different concentrations ranges from 10 to 100µg/ml. No significant ($p>0.05$) cytotoxicity was observed at 0, 24, 48, 72 and 96 h after treatment for each concentration. (Figure 4a, 4b and 4c) While a small amount of lysis was observed, it was not above background. Cells treated with triton (positive control) showed 100% lysis after 96 hrs incubation results no such compound affect the cell membrane.

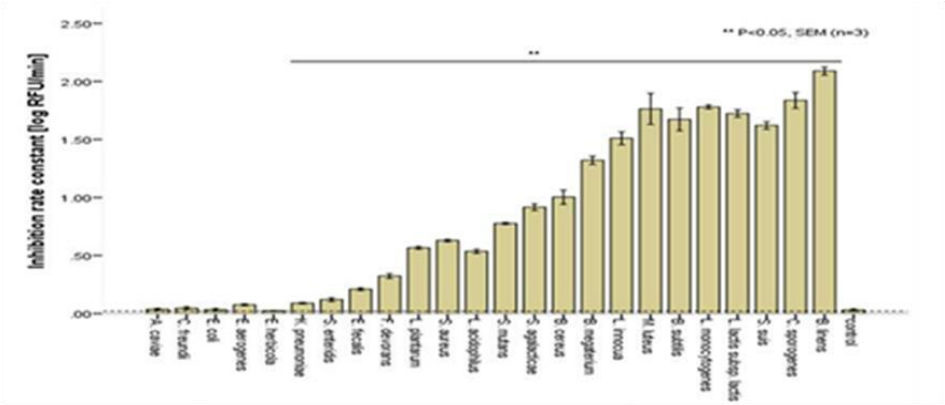

Fig. 3: Inhibition rate for R-62 at 8.0 µg/ml. The error bars represent SEM from two biological replicates, each replicate consist n=3.

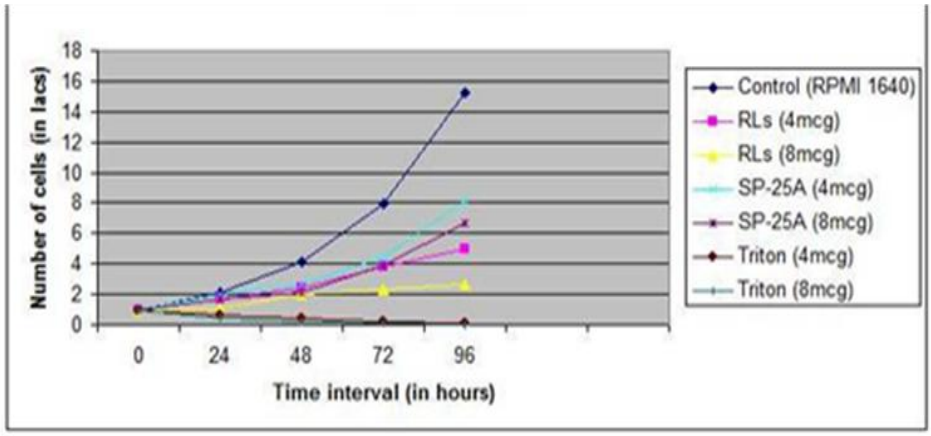

Fig. 4a Comparative cytotoxic effects of R-62 against LL-47 cells

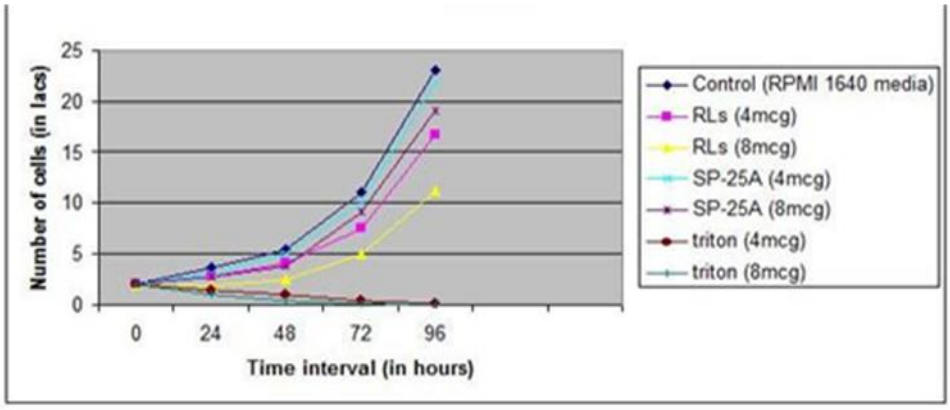

Fig. 4b Comparative cytotoxic effects of R-62 against HEK-293 cells

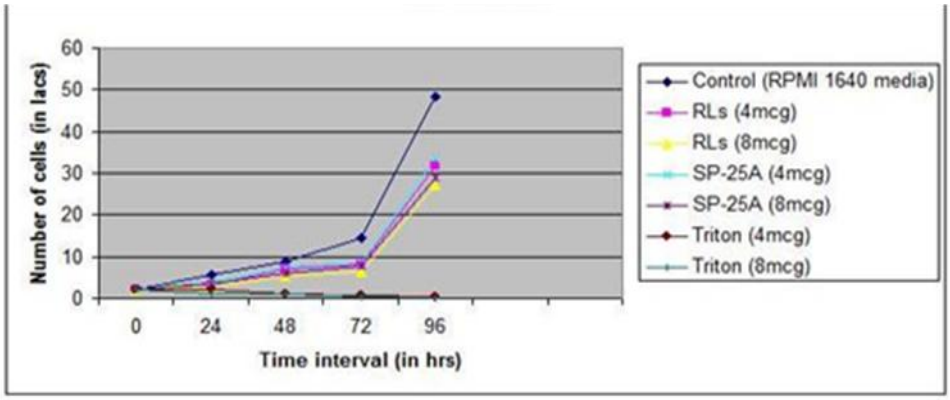

Fig. 4c Comparative cytotoxic effects of R-62 against STC-1 cells

DISCSUSSION

Compound Purity

After purification R-62 subjected to MALDI-TOF and HPLC analysis (Figure 1, 2) revealed a single peak similar to MALDI TOF. This single major peak in both analyses had a molecular weight of 2,400.37 Da, which was in agreement with the reported size. (Monti *et al.*, 2001) (Figure 2) MS analysis revealed Rhamnose-C10-C10 (MW = 503.31). These observations are in agreement with the product data sheet. The bacterial inhibition by R-62 is thought that these compounds target the cell membrane, inducing lysis (Bender *et al.*, 1999, Dalla *et al.*, 2003 and Lang, 1993). This study used PI, a membrane impermeant nucleic acid stain, as a probe to monitor cell membrane integrity during cellular exposure to both the compounds (Haughland, 2002). Accumulation of PI is directly correlated to increasing exposure time for each compound, indicating that the compounds compromised the cell membrane. R-62 was not inhibitory to any gram-negative organisms except *F. devorans*, while it inhibited all gram-positive organisms tested. The rate of action was distributed differently relative to R-62, but the same Gram-reacting organisms had the same inhibition for each compound. Depending upon the species, R-62 is 3 to 433 times faster in compromising the cell membrane as compared to other rhamnolipids, the difference being highest for enterococci and lowest being for the *Listeria* species while the rate of action indicates the speed to compromise the membrane, an indication of MIC is required to demonstrate the inhibition of growth. The

MIC of R-62 was tested at a dose of 8.0μg/ml for 48 hours where inhibition of spore germination from *Bacillus* and *Clostridium* was initiated at 4μg/ml. This is the first anti-spore activity reporting by this compound and the source organisms is capable to inhibit the growth of *M. smegmatis* at 4μg/ml. Lavermicocca *et al.,* measured the antibacterial activity of R-62 using six organisms. No inhibition was observed for the three Gram-negative organisms, even at 120 μg/ml. However, all three gram-positive bacteria were inhibited (*Micrococcus luteus*, *Bacillus megaterium* and *Rhodococcus facians*) while the overall observations are in agreement between this study and Lavermicocca *et al.,* the exact concentrations are not comparable due to differences in methodology. In this study it was observed that R-62 inhibited *M. smegmatis*, a surrogate organism for *M. tuberculii*, at 8 μg/ml.

The literature contains conflicting reports on the spectrum of activity for rhamnolipids. This may be attributed to the fact that different groups have used R-62 mixtures with different compositions R-62 homologues. For example, Abalos *et al.,* (2001) Benincasa *et al.* (2004*) and* Haba *et al.,* (2003) reported that the mixtures of rhamnolipids are active against both grampositive and gram-negative bacteria. It was observed that rhamnolipids were active against gram-positive bacteria and only one gram-negative bacterium (*F. devorans*) at <60 μg/ml. Kim *et al.,* reported the ability of R-62 to lyse zoospores from *Phytophthora capsici* within 1 min at a concentrations <50 μg/ml. Conversely, R-62 inhibited bacterial spore germination in *B. subtilis* and *C. sporogenes* at 8 μg/ml. Woo *et al.,* (2002) reported synergistic activity of pseudomonad's with fungal cell wall degrading enzymes to inhibit fungal pathogens. Also, there have been reports of synergism between cationic pore forming peptides (Kobayashi 2002), but there has been no report of synergism between a lipodepsipeptide and a glycolipid. The importance of this finding is best refined for use in specific applications and is therefore beyond the scope of this work. The observations for cellular toxicity indicate that neither concentration compromised the host membrane. Menestrina *et al.,* reported an IC50 value of 8.88 μg/ml of R-62 for RBC hemolysis, where a possible explanation of this observation is that RBC's lacks an endo-membrane, which is thought to play a central role in the rapid resealing response in event of plasma membrane disruption (McNeil *et al.,* 2003). Although, R-62 have shown a different inhibitory pattern addressing its varying activity against different type of strains tested. One of the basic aims of this study was to determine the use of R-62 against multiple drug resistant strains which was done using *E. faecalis* and *S. aureus* that are gentamicin/vancomycin/teicoplanin resistant. We observed that R-62 had a higher inhibitory action than other rhamnolipids, yet R-62 were unable to inhibit growth at 60 μg/ml. This is similar to the concept described by Wu *et al.,* who found that cationic peptides were not correlated with the ability to permeabilize the cell membrane and the antimicrobial activity where several inferences can be made from this lack of correlation. The biochemical changes brought about by R-62 were overcome by a stress response that repaired the compromised membrane, but they could not repair the changes brought about by R-62. This reveals that either the R-62 have a differing mode of action on the cell membrane or R-62 has multiple modes of action (i.e. it may act on multiple cellular targets) however in some instances the membrane repair in response to cationic peptides as reported by Wu *et al.,* (1999). It was observed that some peptides did not depolarize the cell membrane at MIC concentrations, suggesting that at these MICs bacteria repaired their cell membrane and that a mechanism other than membrane disruption leads to cell death. In this study, we observed R-62 to disrupt the membrane enough to uptake PI, yet the organisms retained the ability to replicate. This study also demonstrated the ability of R-62 to compromise the membrane of gram-positive bacteria with MICs of ≤8 μg/ml. Considering the inhibition of multiple drug resistant enterococci and staphylococci, *Mycobacterium*, *Bacillus* spores and the lack of toxicity towards

mammalian cells makes R-62 a very promising therapeutic agent, which needs to be confirmed with in vivo studies.

CONCLUSION

R-62 was purified and analysed through MALDI-TOF and HPLC analysis revealed the relative concentration of R-62. R-62 was found to be inhibitory against all gram-positive organisms whereas, potent against *B. subtilis*. It was found to be highly active at minimum concentration (4µg/ml) but no inhibition was observed for the gram-negative organisms, even at high concentration of 120µg/ml. The inhibitory effect of R-62 was found to be significant ($p<0.05$) than the rate of inhibition of R-62 against varying concentrations was tested and achieved the varying level of inhibition. No significant ($p>0.05$) cytotoxicity was observed till 72 h after treatment at either concentration of 4 and 8µg/ml. It was observed that due to the lack of toxicity towards mammalian cells makes R-62 a very promising therapeutic agent.

ACKNOWLEDGEMENTS

We thank for the administration and research department of 'Bhopal Memorial Hospital and Research Center' Bhopal for providing the facility of their advanced labs to perform this innovative work.

REFERENCES

Abalos AP, Infante MR, Casals M, Garcý´F and Manresa A (2001). Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir* **17** 1367-1371.

Adetuyi FC, Isogai A, Giorgio DD, Ballio A and Takemoto JY (1995). Saprophytic Pseudomonas syringae strain M1 of wheat produces cyclic lipodepsipeptides. FEMS Microbiology Letters **131** 63-7.

Agner G, Kaulin YA, Gurnev PA, Szabo Z, Schagina LV, Takemoto JY and Blasko K (2000). Membrane-permeabilizing activities of cyclic lipodepsipeptides, syringopeptin 22A and syringomycin E from *Pseudomonas syringae* pv. *syringae* in human red blood cells and in bilayer lipid membranes. *Bioelectrochemistry* **52** 161-7.

Ang JY, Ezike E and Asmar BI (2004). Antibacterial resistance. *Indian Journal of Pediatrics* **71** 229-39.

Ballio AF, Bossa DDG, Nola AD, Manetti C, Paci M, Scaloni A and Segre AL (1995). Solution conformation of the *Pseudomonas syringae* pv. *syringae* phytotoxic lipodepsipeptide syringopeptin 25-A, Two dimensional NMR, distance geometry and molecular dynamics. *European Journal of Biochemistry* **234** 747-58.

Bender CL, Alarcon-Chaidez F and Gross DC (1999). Pseudomonas syringae phytotoxins: mode of action, regulation and biosynthesis by peptide and polyketide synthetases. *Microbiology Reviews* **63** 266-92.

Benincasa M, Abalos A, Oliveira I and Manresa A (2004). Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. Antonie Van Leeuwenhoek. **85** 1-8.

Beom SK (2000). In vivo control and in vitro antifungal activity of rhamnolipid B, a glycolipid antibiotic, against *Phytophthora capsici* and *Colletotrichum orbiculare*. *Pest Management Science* **56** 1029-1035.

Bidwai AP, Bachmann RC and Takemoto JY (1987). Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin - stimulation of red beet plasma-membrane ATPase activity. *Plant Physiology* **83** 39-43.

Buber E, Stindl A, Acan NL, Kocagoz T and Zocher R (2002). Antimycobacterial activity of lipodepsipeptides produced by *Pseudomonas syringae*. pv. *syringae* B359. *Nature Protocol Letters* **16** 419-423.

Budzikiewicz H (1993). Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiology Reviews* **10** 209-28.

Dalla Serra MGM, Carpaneto A, Gambale F, Fogliano V and Ballio A (2003). Molecular mechanisms of action of syringopeptins, antifungal peptides from Pseudomonas syringae pv. syringae. *In:* Pore-forming peptides and proteins, *Edited by* Gianfranco Menestrina and M. Dalla Sera (Taylor and Francis Group, London, UK) 272-295.

Dalla SM, Fagiuoli G, Nordera P, Bernhart I, Volpe CDD Giorgio DD, Ballio A and Menestrina G (1999). The interaction of lipodepsipeptide toxins from Pseudomonas syringae pv. syringae with biological and model membranes: a comparison of syringotoxin, syringomycin and two syringopeptins. Molecular Plant Microbe. *Interact* **12** 391-400.

Giorgio DD, Camoni L, Mott KA, Takemoto JY and Ballio A (1996). Syringopeptins, *Pseudomonas syringae* pv *syringae* phytotoxins, resemble syringomycin in closing stomata. *Plant Pathology* **45** 564-571.

Menestrina G, Coraiola M, Fogliano V, Fiore A, Grgurina I, Carpaneto A, Gambale F and Serra MD (2003). Antimicrobial lipodepsipeptides from *Pseudomonas* spp: a comparison of their activity on model membranes, *In* N.S. Iacobellis (ed.), Pseudomonas syringae and related pathogens: biology and genetics. Kluwer Academic Publishers, Norwell, MA 185-198.

Grgurina I, Mariotti F, Fogliano V, Gallo M, Scaloni A, Iacobellis NS, Cantore PL, Mannina L, Castelli VV, Greco ML and Graniti A (2002). A new syringopeptin produced by bean strains of *Pseudomonas syringae* pv. *syringae*. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology **1597** 81-89.

Haba E, Pinazo A, Jauregui O, Espuny MJ, Infante MR and Manresa A (2003) Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. *Biotechnology and Bioengineering* **81** 316-22.

Haughland RP (2002). Handbook of fluorescent probes and research chemicals, 9th ed. Molecular Probes, Inc. Eugene, Oreg 193-207.

Hutchison ML and Gross DC (1997). Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae*: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Molecular Plant Microbe Interaction* **10** 347-54.

Iacobellis NS, Lavermicocca P, Grgurina I, Simmaco M and Ballio A (1992) Phytotoxic properties of *Pseudomonas syringae* pv *syringae* Toxins, Physiol and Mol. *Plant Pathology*. **40** 107-116.

Isogai A, Iguchi H, Nakayama J, Kusai A, Takemoto JY and Suzuki A (1995). Structural analysis of new syringopeptins by tandem mass spectrometry. *Bioscience, Biotechnology and Biochemistry* **59** 1374-6.

Janisiewicz WJ, Jeffers SN (1997). Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage. *Crop Protection* **16** 629-633.

Kobayashi S (2002). Bacteria-selective synergism between the antimicrobial peptides magainin 2 and tachyplesin I: toward cocktail therapy. *Yakugaku Zasshi* **122** 967-73.

Kohler T, Curty LK, Barja F, Delden CV and Pechere JC (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *Journal of Bacteriology* **182** 5990-6.

Lang SWF (1993). Biological activity of biosurfactants. In Kosaric N, (edited).Biosurfactant properties and applications, Marcel Dekker, New York **48** 251–269.

Lavermicocca P, Iacobellis NS, Simmaco M and Graniti A (1997). Biological properties and spectrum of activity of *Pseudomonas syringae* pv. *syringae* toxins. *Physiology and Molecular Plant Pathology* **50** 129-140.

Leisinger T and R Margraff (1979). Secondary metabolites of the fluorescent pseudomonads. *Microbiology Reviews* **43** 422-42.

Dalla M, Serra GM, Carpaneto A, Gambale F, Fogliano V and Ballio A (2003). Molecular mechanisms of action of syringopeptins, antifungal peptides from Pseudomonas syringae pv. syringae. In Gianfranco Menestrina and M. Dalla Sera, Pore-forming peptides and proteins Taylor and Francis Group, London, UK 272-295.

Maier RM and Soberon-Chavez G (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Applied Microbiology and Biotechnology* **54** 625-33.

Mata-Sandoval JC, Karns J and Torrents A (2001). Effect of nutritional and environmental conditions on the production and composition of rhamnolipids by *P. aeruginosa* UG2. *Microbiology Research* **155** 249-56.

McClure CD and Schiller NL (2003). Inhibition of macrophage phagocytosis by *Pseudomonas aeruginosa* rhamnolipids in vitro and in vivo. *Current Microbiology.* **33** 109-17.

McNeil PL, Miyake K and Vogel SS (2003). The endomembrane requirement for cell surface repair. Proc. National Academy of Science. U S A **100** 4592-7.

Monti SM, Gallo M, Ferracane R, Borrelli RC, Ritieni A, Greco ML, Graniti A and Fogliano V (2001). Analysis of bacterial lipodepsipeptides by matrix-assisted laser desorption/ionisation time-of-flight and high-performance liquid chromatography with electrospray mass spectrometry. Rapid Communication *Mass Spectrum* **15** 623-8.

Noordman WH and Janssen DB (2002). Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. *Applied Environmental Microbiology* **68** 4502-8.

Rendell NB, Taylor GW, Somerville M, Todd H, Wilson R and Cole PJ (1990). Characterisation of *Pseudomonas* rhamnolipids. *Biochemistry Biophysics Acta* **1045** 189-93.

Richet HM, Mohammed J, McDonald LC and Jarvis WR (2001). Building communication networks: international network for the study and prevention of emerging antimicrobial resistance. *Emerging Infectious Disease* **7** 319-22.

Vincent Wu S, Moon Yang NR, Steven H, Young James, Sinnett-Smith and Enrique Rozengurt. (2001). Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells. Proc. National Academy Science **99** 2392-2397.

Scholz-Schroeder BK, Hutchison ML, Grgurina I and Gross DC (2001). The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of sypA and syrB1 biosynthesis mutant analysis. *Molecular Plant Microbe Interact* **14** 336-48.

Sim L, Ward OP and Li ZY (1997). Production and characterisation of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. *Journal of Industrial Microbiology and Biotechnology* **19** 232-8.

Szabo Z, Budai M, Blasko K and Grof P (2004). Molecular dynamics of the cyclic lipodepsipeptides' action on model membranes: effects of syringopeptin22A, syringomycin E and syringotoxin studied by EPR technique. *Biochemistry, Biophysics Acta* **1660** 118-30.

Szabo Z, Grof P, Schagina LV, Gurnev PA, Takemoto JY, Matyus E and K. Blasko (2002). Syringotoxin pore formation and inactivation in human red blood cell and model bilayer lipid membranes. *Biochemistry, Biophysics Acta* **1567** 143-9.

Volksch B and Weingart H (1998). Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. *Journal of Basic Microbiology* **38** 135-45.

Woo S, Fogliano V, Scala F and Lorito M (2002). Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. *Antonie Van Leeuwenhoek* **81** 353-6.

Woods GL, WJA (1995). Antibacterial susceptibility tests: Dilution and disk diffusion methods. *In* P.R. Murray (edited) *Manual of Clinical Microbiology* 6th edition. ASM Press, Herndon,VA 1327-1341

Wu M, Maier E, Benz R and Hancock RE (1999). Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* **38** 7235-42.