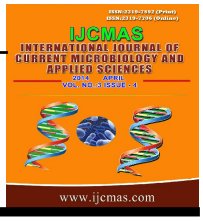
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 4 (2014) pp. 801-812**
http://www.ijcmas.com



Original Research Article

Detection of *Mycobacterium Smegmatis* Biofilm and its Control by Natural Agents

Syed H. Abidi[1,2*], Khalid Ahmed[1], Sikander K. Sherwani[2,3], Nazia Bibi[2], and Shahana U. Kazmi[2]

[1]Department of Biological and Biomedical Sciences, Aga Khan University, Karachi-Pakistan
[2]Immunology and Infectious Diseases Research Lab (IIDRL), Department of Microbiology, University of Karachi, Karachi-Pakistan
[3]Department of Microbiology, Federal Urdu University of Arts, Science and Technology, Karachi-Pakistan
Corresponding author

Keywords

Biofilm, *Myco-bacterium smegmatis*, Plant extracts

A B S T R A C T

Study of biofilms in *Mycobacterium smegmatis* can aid in understanding biofilm formation in other pathogenic Mycobacteria like *Mycobacterium tuberculosis*. Biofilms may contribute in therapeutic failure by protecting the bacteria from effective penetration and action of antimicrobial agents and immune factors and consequently leading to inefficient clearance of pathogens, relapse of infection, and selection and propagation of drug-resistant Mycobacteria. Biofilm formation by *Mycobacterium smegmatis* was evaluated using various qualitative and quantitative techniques. Subsequently, in an attempt to search for effective biofilm controlling natural agents, antimicrobial and antibiofilm activity of five different plant extracts (*Azadirachta indica, Hippophae rhamnoides, Juglans regia, Vaccinium oxycoccos*, and Spices) was tested, using disk diffusion, microbroth dilution and quantitative spectrometric methods, respectively. *Mycobacterium smegmatis* exhibited strong biofilm production, when analyzed both qualitatively and quantitatively. The test of efficacy of plant extracts as antimicrobial and antibiofilm agents revealed that the extracts of Spices and *Vaccinium oxycoccos* (Cranberry) exhibited efficient antimicrobial potential, while extract of *Azadirachta indica* (Neem) was most efficient in reducing and removing *M. smegmatis* biofilms. Our study supports the use of plant extracts as effective natural agents to control *Mycobacterium smegmatis* biofilms. These results might be extrapolated to other pathogenic Mycobacteria, which may help in efficient control of biofilm forming Mycobacteria.

Introduction

Many microbial species have evolved to survive in stressful environments by self-assembling in highly organized, surface attached, and matrix encapsulated

structures called biofilms (Hall-Stoodley and Stoodley 2005). Growth in communities appears to be a preferred survival strategy of microbes, and is achieved through genetic components that regulate surface attachment, intercellular communications, and synthesis of extracellular polymeric substances (EPS) (Kolter 1998; Henke and Bassler 2004). The tolerance to environmental stress is likely facilitated by EPS, and perhaps by the physiological adaptation of individual bacilli to heterogeneous microenvironments within the complex architecture of biofilms (Branda, Vik et al. 2005).

Mycobacterium smegmatis with its short generation time and low biosafety requirements serves as an appropriate model to study Mycobacteria in general (Reyrat and Kahn 2001). *Mycobacterium smegmatis* also share some of its virulence gene homologues with *Mycobacterium tuberculosis* (Reyrat and Kahn 2001; Gopalaswamy, Narayanan et al. 2008; Altaf, Miller et al. 2010; Pacheco, Hsu et al. 2013). Therefore, the better understanding of *Mycobacterium smegmatis* might be helpful to understand different aspects of other highly virulent Mycobacteria.

Biofilm formation has been described as an important factor having significant implications in pathogen virulence (Shi, Fu et al. 2011; Lebeaux, Chauhan et al. 2013). Biofilms protect the pathogens from inhibitory effect of antibiotics and immune cells, because it prevents their effective penetration (Ojha, Anand et al. 2005; Begun, Gaiani et al. 2007; Ojha, Trivelli et al. 2010). In cases like Mycobacterium Tuberculosis, successful treatment is a long task with viable chances of infection relapse, if pathogen is not completely eliminated from the body. In such scenarios, biofilms can cause failure of therapy because biofilm-forming communities are often drug-resistant and hard to clear (Ojha, Baughn et al. 2008). Although biofilm formation has not been fully described in *Mycobacterium tuberculosis*; other Mycobacteria like, *M. avium*, *M. marinum*, and *M. smegmatis*, have been described as potent biofilm producers (Shi, Fu et al. 2011). Therefore, development of proper biofilm detection techniques and identification of potent biofilm controlling agents in Mycobacteria models such as *M. smegmatis* holds a significant value in designing effective biofilm control strategies against other Mycobacteria of clinical significance.

Several efficient qualitative and quantitative techniques have been described for rapid and efficient detection of biofilms (Merritt, Kadouri et al. 2005; Hannig, Follo et al. 2009), and simultaneous screening of different agents for their anti-biofilm potential (Pitts, Hamilton et al. 2003), where, many of these anti-biofilm agents reported are synthetic or of chemical origin (Chen and Stewart 2000; Cserhati, Forgacs et al. 2002). In the current study, both qualitative and quantitative techniques have been utilized for the detection of *M. smegmatis* biofilms. Additionally, an efficient quantitative spectroscopic technique was also employed to analyze the potential of several plant extracts in simultaneously reducing *M. smegmatis* biofilm formation and removing pre-formed biofilms.

Materials and Methods

Mycobacterium smegmatis culture

The culture of *Mycobacterium smegmatis* was obtained from laboratory's culture

bank and maintained on Trypton Soy Agar (Sigma-Aldrich) plates at 37 ^{0}C.

Evaluation of Biofilm Forming Potential

The biofilm formation potential of *Mycobacterium smegmatis* was evaluated by following methods: 1) Air Liquid Interface Assay, 2) Scanning Electron Microscopy, and 3) Microtitre-plate method.

Air Liquid Interface Assay

The *Mycobacterium smegmatis* biofilm was microscopically visualized using Air liquid interface assay as described previously (Merritt, Kadouri et al. 2005; Abidi, Sherwani et al. 2013). Briefly, the culture of *Mycobacterium smegmatis* was 1:100 diluted in 3 ml Tryptone Soy Broth (TSB; Sigma-Aldrich). Subsequently, 300 µl of each diluted culture was pipetted into each well of a 12-well, flat-bottom plates. The plates were covered with lid and incubate at 37°C for 48 hour, in a position that they were making a 45° angle to the surface of incubator. After incubation, the cultures were aspirated, and the wells were gently washed twice by adding 400 µl sterile TSB medium. After two washes, 200 µl of TSB medium was added to each well. The plate was laid flat on the stage of an inverted microscope (Olympus, Japan) and biofilm formation was visualized. The pictures of the biofilm were taken using a digital camera (Canon-A450, Malaysia).

Scanning Electron Microscopy (SEM)

The biofilm formation by Mycobacterium smegmatis was also visualized using Scanning electron microscopy. Briefly, Mycobacterium smegmatis culture was inoculated in polystyrene tube containing 5 ml Tryptone Soy Broth (TSB) and incubated for 24 hours at 37 ^{0}C. After incubation, 2 ml of 20% glucose was added to the culture tube and incubation was further carried out for 24 hours at 37 ^{0}C. Subsequently, the contents of the tube were decanted and the tube was washed with PBS (pH 7) and air-dried. Dried tube was fixed using 5ml of 99% Methanol. Methanol was discarded and the tube was stained with 5ml Crystal Violet (0.1%) for approximately 20 minutes. Afterwards, the stain was removed and tube was washed with distilled water. The tube was then air-dried in an inverted position. Prior to electron microscopy, the tube was cut, with help of a sterile blade, into sections of about 2 mm. The stained sections were placed on a gold coater (SEM JEOL JFC-1500 Quick auto sputter) and coated with the gold film up to 300 Armstrong. The sample was then placed in a sample chamber of the microscope (SEM JEOL JSM-6380A) and scanning was performed under different magnifications ranging from 1500x to 3000x, at a voltage of 15kV (Ganderton, Chawla et al. 1992).

Microtitre-plate method

Mycobacterium smegmatis biofilm forming potential was quantitatively analyzed using microtitre-plate method as described previously (O'Toole, Pratt et al. 1999; O'Toole, Kaplan et al. 2000; Merritt, Kadouri et al. 2005). Briefly, *Mycobacterium smegmatis* culture was inoculated in 3–5 ml TSB and incubated for 24 hours at 37°C. After incubation, cultures were 1:100 diluted in the TSB, and 100 µl of each diluted culture was pipetted in each well of 96-well flat-bottom microtitre plate (non-tissue culture treated, Sigma-Aldrich). Plates was covered and incubated at 37°C for 48 hours. After incubation, contents of the well were aspirated out and the wells were

washed thoroughly with PBS. Subsequently, the wells were stained for 10 min by adding 125 ul of 0.1% Crystal Violet (w/v) solution to each well. Afterwards, the stain was removed and the plate was washed with clean tap water and left to air dry. Subsequently, 200 µl of 95% Ethanol was added to each stained well and plates were incubated for 10 to 15 min at room temperature. Contents of each well were mixed by pipetting, and then 125 µl of the Crystal Violet/Ethanol solution was transferred from each well to a separate well of an optically clear flat-bottom 96-well plate. Optical densities (OD) of each of these 125-µl samples were measured at 630 nm using spectrophotometer (Starfax 2100, Awareness Technology Inc). Experiment was performed in duplicate.

Antimicrobial and Antibiofilm activity of Plant extracts

Antimicrobial activity of several plant extracts has been well documented against many pathogens (Mahesh and Satish 2008; Das, Tiwari et al. 2010; Bibi, Nisa et al. 2011). In this study, we evaluated the antimicrobial and–antibiofilm activity of five different plants extracts against *Mycobacterium smegmatis* (Table 1).

Preparation of aqueous plant extracts

The 5% aqueous extracts of plants (Table 1), were used in the study. Briefly, 2.5 g dried leaves/bark/powder were soaked into 50 ml autoclaved distilled water and boiled for three minutes for three times with two minutes interval between each boiling time. Subsequently, the extract or supernatant was collected in a separate 50ml tube and centrifuged for 15 minutes at 5000 rpm. The clear supernatant obtained was collected and filter sterilized using 0.2 um filter (Micropore filters), and stored at -20 ^{0}C. In this study, maximum a week old extracts were used, otherwise fresh extracts were prepared. For Cranberry extracts, 5 ml of pure concentrate was poured in 95 ml sterile distilled water to yield 5 % solution.

Determination of antimicrobial potential of plant extracts

The antimicrobial potential of plant extracts against *Mycobacterium smegmatis* culture was examined by Disk Diffusion method (Binutu and Lajubutu 1994). Briefly, *Mycobacterium smegmatis* culture was inoculated in 5ml Mueller-Hilton Broth at incubated at 37 ^{0}C for two hours. After the incubation, microbial lawn was prepared by pouring 100 µl of culture on the Mueller-Hilton Agar plates. Disks soaked with 30 ul of plant extracts (Table 1) were placed onto the lawn and plates were incubated at 37 ^{0}C for 24-48 hours. Antimicrobial potential of the plant extracts were evaluated on the basis of appearance and size of the zone of inhibition.

Determination of Minimum inhibitory concentration (MIC) of plant extracts

Minimum inhibitory concentration (MIC) of the plant extracts were determined by Micro Broth Dilution method using 96-well microtitre plate (Aboaba, Smith et al. 2006). Briefly, *Mycobacterium smegmatis* culture was inoculated in 5ml Mueller-Hilton Broth at incubated at 37 ^{0}C for two hours. Simultaneously, 1 mg/ml stock solutions of plant extracts (Table 1) were prepared in sterile distilled water, and two fold serial dilutions of plant extracts were made in 100 µl Mueller-Hilton broth to achieve a final concentration of 4 µg/ml in the last well. Subsequently, 10 µl of two hour old culture was added to each well.

One well served as a medium control while other served as culture control. The plates were incubated for 24 hours at 37 ^{0}C. The MIC was considered as the lowest concentration of the well that exhibited no visible bacterial growth.

Reduction and Removal of *Mycobacterium smegmatis* Biofilms

A quantitative spectrophotometric method, as described by Pitts, et al., (Pitts, Hamilton et al. 2003), was used, with modification, to measure the biofilm disinfection and removal efficacy of the plant extracts. This method allows a rapid detection of concentration–dependent anti-biofilm activity of various agents (Pitts, Hamilton et al. 2003). The experiment was performed in two ways. In first experiment, biofilm reduction potential of the plant extracts was evaluated. Briefly, *Mycobacterium smegmatis* culture was inoculated in 5-ml TSB and grown to stationary phase. The culture was diluted 1:100 in the Tryptone Soy Broth (TSB) and 100 µl of diluted culture was pipette in 12 different wells i.e. two wells for plant extracts, one for blank (B) and one for control (C), in a fresh 96-well, non-tissue culture treated microtiter plate. Hundred microliter of each extract – to be evaluated for antibiofilm potential – was inoculated in each well; and plate was covered and incubated at 37 ^{0}C for 24 hours.

In the second experiment, potential of plant extracts to remove pre-formed biofilm was evaluated. Briefly, microtitre plate was inoculated as mentioned above, and incubated at 37 ^{0}C for 24 hours. After incubation, plates were washed with sterile water to remove planktonic cells and 200 µl of each plant extract was inoculated in each well. Plates were then incubated for a period of 1.5 hour.

After incubation, four small trays were set up in a series, and 1 to 2 inches of autoclaved tap water was added to the last three, while first tray served as waste. Planktonic bacteria were removed from the microtiter plates by vigorously shaking the plates over the waste tray. Wells were washed, by submerging the plates in the first water tray and then emptied over waste tray by vigorous shaking. Subsequently, for biofilm staining, 125 ul of 0.1% crystal violet solution was added to each well and incubated for 10 min at room temperature. Following incubation, the stain was emptied over the waste tray and plates were washed consecutively in each of the next two water trays with vigorous shaking to remove all liquid. Subsequently, the plates were inverted and vigorously tapped on paper towels to remove all the contents and left to air dry. Finally, the dye was solubilized by adding 200 µl of 95% ethanol to plate, and incubating the plate for 10 to 15 min at room temperature. In the next step, contents of each well were mixed by repeated pipetting, and then 125 µl of the crystal violet-ethanol solution was transferred from each well to a separate well of a new optically clear flat-bottom 96-well plate. Optical densities (OD) of each of these 125-µl samples were measured at a wavelength 630 nm.

Measurement of anti-biofilm efficacy called Percentage Reduction/Removal was calculated from blank, control and test OD, using equation:

Percentage Reduction/Removal = [(*C*-*B*) - (*T*-*B*) / (*C*-*B*)]*100%

Where *B* = absorbance of blank (no biofilm, no treatment), *C* = absorbance of control (biofilm, no treatment) and *T* = absorbance of test (biofilm and treatment)

Results and Discussion

Qualitative and quantitative analysis of *Mycobacterium smegmatis* biofilm

In our study, Air-liquid assay and Scanning Electron Microscopy (SEM) was used to qualitatively analyze the biofilm formation by *Mycobacterium smegmatis*. In the Air-liquid assay, dense matt (biofilm) formation and strong aggregation by the *Mycobacterium smegmatis* was observed (Figure 1). Similarly, scanning electron microscopy revealed that the *Mycobacterium smegmatis* exhibits strong cellular aggregation and dense matt (biofilm) formation, as evident at 1500x (Figure 2a) and 3700x magnification (Figure 2b).

The quantitative biofilm analysis also revealed *Mycobacterium smegmatis* isolate as the dominant biofilm former with an OD_{630} of 0.79.

Antimicrobial activity of plant extracts against *Mycobacterium smegmatis*

In our study, antimicrobial potential of 5 different plant extracts (Table 1) was evaluated against *Mycobacterium smegmatis*. The results revealed that extracts of Spices and *Vaccinium oxycoccos*, with zones of inhibition of 12mm, respectively, were found to be effective against *Mycobacterium smegmatis* (Table 2). Minimum inhibitory concentration (MIC) values of Spices and *Vaccinium oxycoccos* were 500 ug/ml and 1000 ug/ml, respectively (Table 2). The extracts of *Juglans regia, Hippophae rhamnoides* and *Azadirachta indica* exhibited no antimicrobial activity against *Mycobacterium smegmatis* (Table 2).

Biofilm Reduction and Removal potential of plant extracts

In this study, biofilm reduction and removal potential of five different plant extracts were evaluated. The extracts of *Azadirachta indica, Vaccinium oxycoccos* and Spices displayed substantial biofilm reduction potential, which was 173%, 28% and 26%, respectively (Figure 3a). Similarly, extracts of *Azadirachta indica, Hippophae rhamnoides,* and *Vaccinium oxycoccos* exhibited significant biofilm removal potential, which was 32%, 13% and 12%, respectively (Figure 3b). In our study, we evaluated biofilm forming potential of *Mycobacterium smegmatis* and employed a quantitative spectroscopic technique to analyze the antibiofilm potential of several plant extracts in simultaneously reducing *M. smegmatis* biofilm formation and removing pre-formed biofilms. The results identified *Mycobacterium smegmatis* as a dominant biofilm former, which supports earlier studies that *M smegmatis* has a strong propensity to form biofilms (Ojha, Anand et al. 2005; Shi, Fu et al. 2011).

Mycobacterium smegmatis has been implicated as a causative agent of peritoneal dialysis-related peritonitis and venous catheter-associated bacteremia, which has been partly attributed to the biofilm forming potential of the organism (Chang, Tsay et al. 2009; Jiang, Senanayake et al. 2011). It has also been used as a model to study *Mycobacterium tuberculosis,* because it has been shown to have the fastest growth rate of biofilm formation compared with other *Mycobacterium* species such as *M. phlei* and *M kansasii* (Bonkat, Bachmann et al. 2012).

Table.1 List of plant extracts tested for reducing and removing *Mycobacterium smegmatis* biofilms

Botanical Name	Local Name	Part used	Extract	Concentration
Azadirachta indica	Neem	Fresh Leaves	Aqueous	5 %
Hippophae rhamnoides	Sea buckthorn	Dried Berries	Aqueous	5 %
Juglans regia	Dandasa	Dried Bark	Aqueous	5 %
Vaccinium oxycoccos	Cranberry	Juice	Pure concentrate	5 %
-	Culinary Spices	Grounded powder	Aqueous	5 %

Table.2 Antimicrobial activity of five different plants extracts: The table shows antimicrobial activity of plants extracts measured in terms of zone of inhibition (mm) and Minimum Inhibitory concentration (MIC).

Plant Extracts	Zone of Inhibition (mm)	Minimum Inhibitory Concentration (µg/ml)
Spices	12	1000
Vaccinium oxycoccos	12	500
Juglans regia	0	NI
Hippophae rhamnoides	0	NI
Azadirachta indica	0	NI

Figure.1 Qualitative analysis of biofilm formation by *Mycobacterium smegmatis*: Biofilm formation by *Mycobacterium smegmatis* as observed under inverted microscope using Air-liquid interface assay. Dense matt formation and microbial aggregation is clearly evident in the wells.

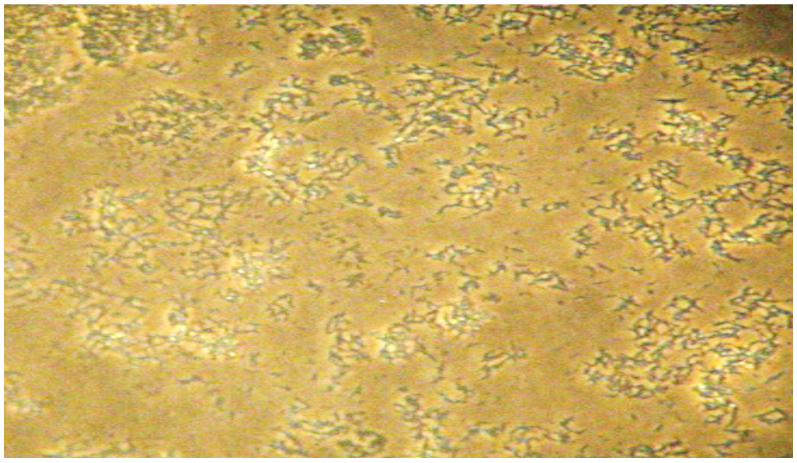


Figure.2 Scanning electron microscopic analysis of biofilm formation by *Mycobacterium smegmatis*: Dense matt formation and microbial aggregation is clearly evident at A) 1500x, and B) 3,700x magnification.

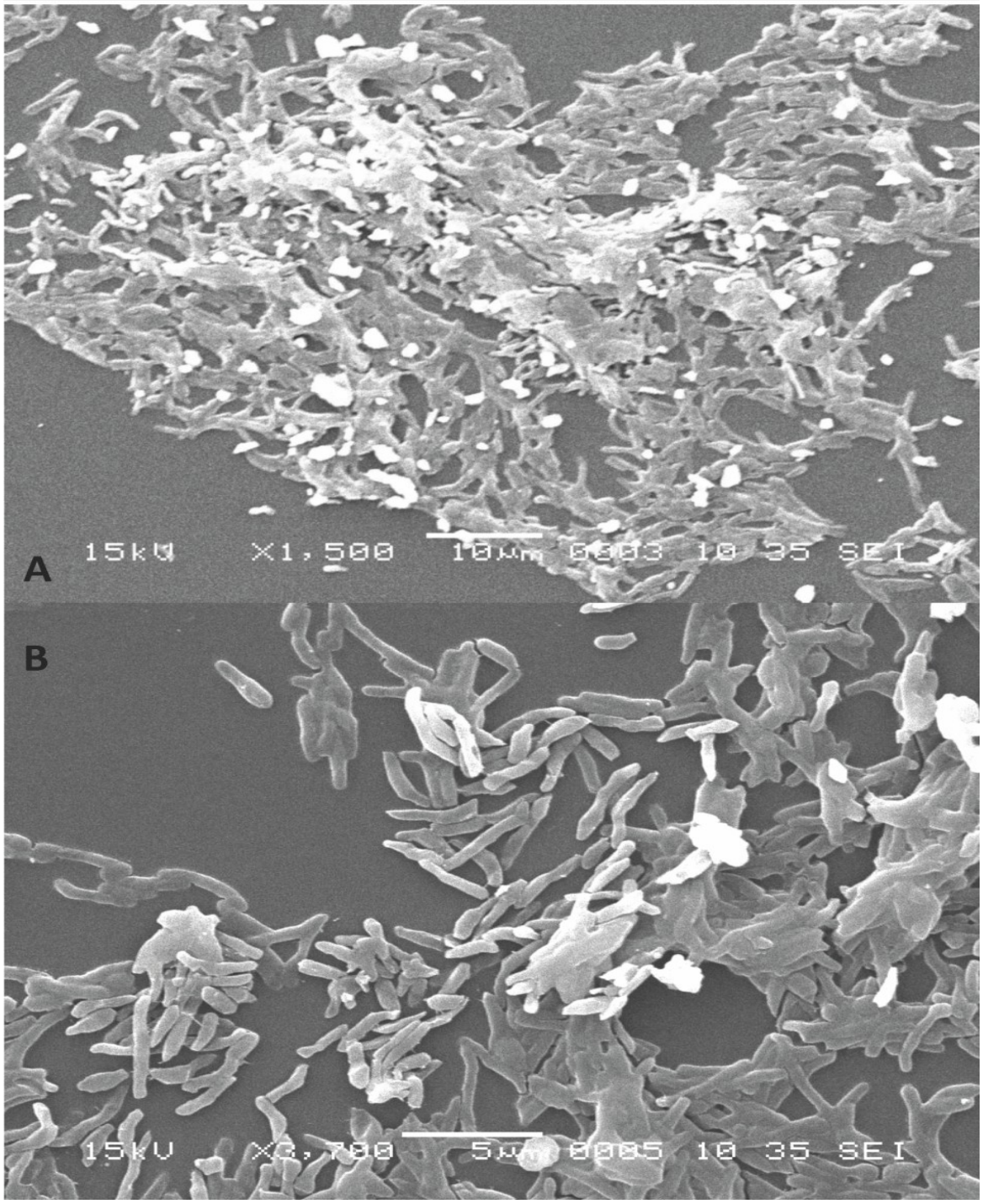


Figure.3 Potential of various plant extracts to reduce and remove *Mycobacterium smegmatis* biofilms: Graph showing A) percent reduction in biofilm formation, and B) percent removal of pre-formed biofilms. Error bars represent standard error of mean.

Figure 3a:

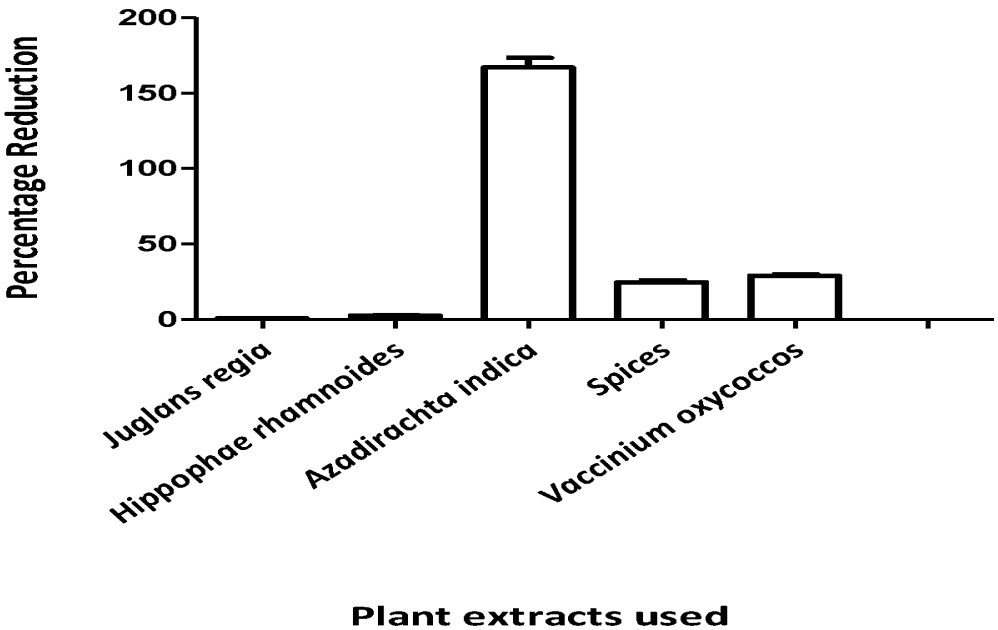
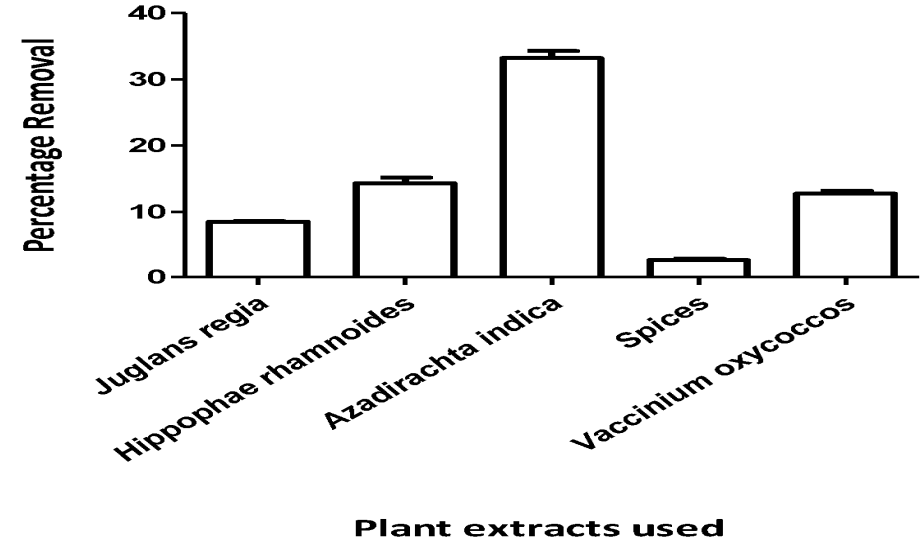


Figure 3b:



Air-liquid interface was performed in polystyrene wells as has been described by Ojha, *et al.*, [6], which gives a rough estimation of biofilm forming potential of the pathogen (Merritt, Kadouri et al. 2005). *M. smegmatis* appeared to effectively adhere to plate wells and form biofilms. This observation was further confirmed by Scanning Electron Microscopy, which gives an ultra-magnified visualization of different patterns of microbial adherence and biofilm formation (Hannig, Follo et al. 2009).

In the next stage, we tested several plant extracts for their anti-biofilm potential (Table 1 and 2). The inhibitory potential was tested against both biofilm disinfection/reduction and biofilm removal, by using a quantitative microtitre plate technique employing crystal violet dye as biomass indicator (Pitts, Hamilton et al. 2003; Silva, Pires et al. 2012). This technique is a sensitive and rapid method to determine concentration-dependent efficacy of different test agents. Our results indicated several of the plant extracts were effective in both reducing biofilm formation and removing pre-formed biofilms with 36% to 56% biofilm reduction and 34% to 35% biofilm removal.

The extracts of *Azadirachta indica (*Neem*), Vaccinium oxycoccos* (Cranberry*), Hippophae rhamnoides* (Sea buckthorn) and Spices displayed a substantial anti-biofilm potential. *Vaccinium oxycoccos* extracts have been known to contain fructose and other polymeric compounds as active agents (Ofek, Goldhar et al. 1991). These active compounds have the potential to inhibit adhesins and prevent matrix formation. Spices - mixture of different plants - have Terpenoids as active compound, which has an active ability to disrupt cellular membranes (Cowan 1999). *Azadirachta indica* contains more than 140 active compounds which are grouped into isoprenoids and nonisoprenoids (Subapriya and Nagini 2005). The terpenoids and essential oils possessed by the *Azadirachta indica* aqueous extracts can actively disrupt cellular membranes (Cowan 1999). Flavonoids are present as active agents in *Hippophae rhamnoides* extracts (Teng, Lu et al. 2006), which forms complexes with cell wall and inhibits enzyme activity. They also binds to adhesins and inhibit matrix formation (Cowan 1999). The biofilm removal or reduction potential of the plant extracts can be through the mechanisms mentioned thereof, which are attributed to their constituent active compounds. These plants can be studies for the identification of the active anti-biofilm compounds.

In conclusion, we have shown and corroborated earlier studies that *M. smegmatis* forms peculiar biofilms which can be used to further our understanding of biofilm formation in of *Mycobacterium tuberculosis* and devising effective control measures. Furthermore, we have tested easily available traditional plant extracts and proved their anti-biofilm potential, which can be tapped by identifying active antibiofilm agents.

Acknowledgement

All consumables were obtained from Immunology and Infectious Diseases Research Laboratory.

References

Abidi, S. H., S. K. Sherwani, et al. (2013). "Drug resistance profile and biofilm forming potential of Pseudomonas Aeruginosa isolated from contact lenses in karachi-Pakistan." BMC Ophthalmol 13(1): 57.

Aboaba, O. O., S. I. Smith, et al. (2006). "Antibacterial Effect of Edible Plant Extract on Escherichia coli 0157:H7." Pakistan Journal of Nutrition 5(4): 325-327.

Altaf, M., C. H. Miller, et al. (2010). "Evaluation of the Mycobacterium smegmatis and BCG models for the discovery of Mycobacterium tuberculosis inhibitors." Tuberculosis 90(6): 333-337.

Begun, J., J. M. Gaiani, et al. (2007). "Staphylococcal biofilm exopolysaccharide protects against Caenorhabditis elegans immune defenses." PLoS pathogens 3(4): e57.

Bibi, Y., S. Nisa, et al. (2011). "Antibacterial activity of some selected medicinal plants of Pakistan." BMC Complement Altern Med 11: 52.

Binutu, O. A. and B. A. Lajubutu (1994). "Antimicrobial potentials of some plant species of the Bignoniaceae family." Afr J Med Med Sci 23(3): 269-273.

Bonkat, G., A. Bachmann, et al. (2012). "Growth of mycobacteria in urine determined by isothermal microcalorimetry: implications for urogenital tuberculosis and other mycobacterial infections." Urology 80(5): 1163 e1169-1112.

Branda, S. S., S. Vik, et al. (2005). "Biofilms: the matrix revisited." Trends Microbiol 13(1): 20-26.

Chang, C. Y., R. W. Tsay, et al. (2009). "Venous catheter-associated bacteremia caused by rapidly growing mycobacteria at a medical center in central Taiwan." J Microbiol Immunol Infect 42(4): 343-350.

Chen, X. and P. S. Stewart (2000). "Biofilm removal caused by chemical treatments." Water Research 34(17): 4229–4233.

Cowan, M. M. (1999). "Plant products as antimicrobial agents." Clin Microbiol Rev 12(4): 564-582.

Cserhati, T., E. Forgacs, et al. (2002). "Biological activity and environmental impact of anionic surfactants." Environ Int 28(5): 337-348.

Das, K., R. K. S. Tiwari, et al. (2010). "Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends." Journal of Medicinal Plants Research 4(2): 104-111.

Ganderton, L., J. Chawla, et al. (1992). "Scanning electron microscopy of bacterial biofilms on indwelling bladder catheters." Eur J Clin Microbiol Infect Dis 11(9): 789-796.

Gopalaswamy, R., S. Narayanan, et al. (2008). "Mycobacterium smegmatis biofilm formation and sliding motility are affected by the serine/threonine protein kinase PknF." FEMS Microbiol Lett 278(1): 121-127.

Hall-Stoodley, L. and P. Stoodley (2005). "Biofilm formation and dispersal and the transmission of human pathogens." Trends Microbiol 13(1): 7-10.

Hannig, C., M. Follo, et al. (2009). "Visualization of adherent micro-organisms using different techniques." J Med Microbiol 59(Pt 1): 1-7.

Henke, J. M. and B. L. Bassler (2004). "Bacterial social engagements." Trends Cell Biol 14(11): 648-656.

Jiang, S. H., S. Senanayake, et al. (2011). "Peritoneal dialysis-related peritonitis due to Mycobacterium smegmatis." Perit Dial Int 31(2): 215-216.

Kolter, R. L., R. (1998). "One for all and all for one." Science 280: 226-227.

Lebeaux, D., A. Chauhan, et al. (2013). "From in vitro to in vivo Models of Bacterial Biofilm-Related Infections." Pathogens 2(2): 288-356.

Mahesh, B. and S. Satish (2008). "Antimicrobial Activity of Some Important Medicinal Plant Against Plant and Human Pathogens." World Journal of Agricultural Sciences 4(S): 839-843.

Merritt, J. H., D. E. Kadouri, et al. (2005). "Growing and analyzing static biofilms." Curr Protoc Microbiol Chapter 1: Unit 1B 1.

O'Toole, G., H. B. Kaplan, et al. (2000). "Biofilm formation as microbial

development." Annu Rev Microbiol 54: 49-79.

O'Toole, G. A., L. A. Pratt, et al. (1999). "Genetic approaches to study of biofilms." Methods Enzymol 310: 91-109.

Ofek, I., J. Goldhar, et al. (1991). "Anti-Escherichia coli adhesin activity of cranberry and blueberry juices." N Engl J Med 324(22): 1599.

Ojha, A., M. Anand, et al. (2005). "GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria." Cell 123(5): 861-873.

Ojha, A. K., A. D. Baughn, et al. (2008). "Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria." Molecular microbiology 69(1): 164-174.

Ojha, A. K., X. Trivelli, et al. (2010). "Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms." J Biol Chem 285(23): 17380-17389.

Pacheco, S. A., F. F. Hsu, et al. (2013). "MmpL11 Protein Transports Mycolic Acid-containing Lipids to the Mycobacterial Cell Wall and Contributes to Biofilm Formation in Mycobacterium smegmatis." J Biol Chem 288(33): 24213-24222.

Pitts, B., M. A. Hamilton, et al. (2003). "A microtiter-plate screening method for biofilm disinfection and removal." J Microbiol Methods 54(2): 269-276.

Reyrat, J. M. and D. Kahn (2001). "Mycobacterium smegmatis: an absurd model for tuberculosis?" Trends Microbiol 9(10): 472-474.

Shi, T., T. Fu, et al. (2011). "Polyphosphate deficiency affects the sliding motility and biofilm formation of Mycobacterium smegmatis." Curr Microbiol 63(5): 470-476.

Silva, S., P. Pires, et al. (2012). "The effect of silver nanoparticles and nystatin on mixed biofilms of Candida glabrata and Candida albicans on acrylic." Med Mycol.

Subapriya, R. and S. Nagini (2005). "Medicinal properties of neem leaves: a review." Curr Med Chem Anticancer Agents 5(2): 149-146.

Teng, B. S., Y. H. Lu, et al. (2006). "In vitro anti-tumor activity of isorhamnetin isolated from Hippophae rhamnoides L. against BEL-7402 cells." Pharmacol Res 54(3): 186-194.