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Antibiofilm and Antimicrobial activity of the trans-Himalayan Medicinal Plant' Extracts; Bacterial Resistance Elixir

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Abstract

The social behaviour of microbial life, the biofilm has an inordinate impact on socio-economic structure. Bio-medically multiple determinants of pathogenicity and resistance to bactericidal antibiotic in the biofilm. Socio-anthropologically lack of identical global biomedical model and irrational prescription of antibiotics. This scenario to see the biofilm problem have evolved the need for a holistic approach to solving them. Latterly, phytochemicals have been exploited to fight out the microbial resistance and also complimented with prescribed medicine. The methanol extract of has the lowest MIC (0.015 mg/ml). At multiple MIC, the plant extracts resulted in reducing the bacterial count up to 99.9% (R^2 = 0.89 to 0.93) within 4-18 h. The plant extracts significantly inhibited biofilm formation at MIC/16 ($p < 0.001$) and also, eradicated 5-day old biofilm at 64 MIC ($p < 0.001$). This work incisively showed the antimicrobial potential of the medicinal plant extract and role in inhibition & removal of biofilms. Potent antimicrobial activity, antibiofilm potential and biosafety of medicinal plants extract reveal the prospective active principle for the biofilms, resistome jeopardy and drug development.

Keywords: Antimicrobial, Biofilm, Biopharmaceuticals, *Pseudomonas* and Resistances.

Introduction

Phylogenesis, variation, and adaptation in microbial cells have brought in, the drug resistance and biofilms deportment. Biofilms are the thin but robust layer of mucilage adhering to the solid surface containing the community of microorganisms and other components (Dunne 2002; Götz 2002; Karatan& Watnick 2009). They are 10-1000 times more resistance to the antimicrobial agent of all origin; biological, chemical, natural and synthetic (Mah and O'Toole 2001; Flemming and Wingender 2010). Biofilms are typically implicated to the chronic infections leading to the persistent infections Costerton *et al.,*(1999) with serious clinical manifestation Donlan and Costerton(2002) in contrast to the planktonic bacteria involved in the acute processes. This is directly related to microbiome hence affect the holobiont and is

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apprehensive. This has direct impact on socioeconomic structure because of longer hospital stay and expensive second-line antimicrobial drugs.

Biofilm formation proceeds in steps (adherence to maturation). Various genetic, physiological and environmental factors promote biofilm formation and drug resistance in bacteria (Stoodley *et al*., 2002; Gupta *et al*., 2016). Aggregation of peptides into functional amyloids are the important component of bacterial biofilm Hobley*et al.,*(2015) and resistance. Amyloids contribute to biofilm assembly & integrity Schwartz *et al.,*(2012) and virulence trait by both Gram-positive (Romero *et al.,* 2010; Marinelli*et al.,* 2016) & Gram-negative bacterial strains (Dueholm*et al.,* 2010; Gerven*et al.,* 2015; Zeng *et al.,* 2015). Self-assembled and thermodynamically favourable amyloid fibres are highly resistant tochemical and physical perturbations Gerven*et al.,*(2015). Multiple determinants of pathogenicity (polysaccharides, extracellular DNA, proteins, lipids etc.) and prevention to access of antimicrobials for the bacterial cells in biofilms, makes the plethora of dire.

Resistance to bactericidal beta-lactam antibiotic is very common, recently in a study by Mir *et al*., (2016) extended spectrum β-lactamase enzyme producing bacterial strain has been found in nature showing the resistance to third generation cephalosporin (cefotaxime). Combination therapies and potentiation of the antimicrobial agents has over the years become the most effective strategies to bring around the infections caused by drug-resistant bacteria and biofilms. (Yadav *et al.,* 2015; Chu *et al.,* 2016; Lee *et al.,* 2016; Zhou *et al.,* 2016). The phytochemicals already have demonstrated robust antibacterial and antibiofilm potential, also synergistic activity with antibiotics. Simões*et al.,* (2009) reviewed on antimicrobial, antibiofilm activity of phytochemicals and discussed the various mechanism of action. In addition to this, phytochemical coating inhibits surface adhesion in bacteria Trentin *et al.,*(2015), inhibit surface colonisation Payne *et al.,*(2013), intricate with Ca^{+2} and obstruct the biofilm formation Lee *et al.,*(2016), blocks production of quorum-sensing signal molecules as well as extracellular virulence factors Harjai*et al.,*(2010), impair curli assembly Serra *et al.,*(2016) and inhibit amyloid synthesis Chu *et al.,*(2016). Plants selected in present study comprises the rich amount of pharmaceutical and nutraceutical alkaloids, flavonoids, glycosides, lignin, saponins, tannins, terpenoid and volatile/essential oils (Chaurasia*et al.,* 2012; Lin *et al.,* 2013; Pandey *et al.,* 2013; Sultan *et al.,* 2016; Kumar *et al.,* 2016) and most of them are antibacterial. However, identification of active compound(s) in plant extract and its mechanism of action need extensive investigations. Thus, only a few organic biofilm inhibitors have been identified.

The lacuna; beyond the laboratory science of infection' cure & control which is not typical from the science of action of bio-medically important medicinal plants, but in practice, they work contrarily. Possibly because hospitals are not the identical clone of the global biomedical model Geest&Finkler (2004) because of different cultures, customs, mores, and tradition of societies which create different medical views. Additionally, access to technical facilities and availability of essential medicine (limits/confine) the diagnostic and therapeutic traditions which sometimes lead to the irrational prescription of antibiotics Podosky (2015) and hence results in antimicrobial resistance in the body. However, medicinal plants have an important role in the health care system of Asian & African countries (Ngo *et al.,* 2013; Mokgobi 2014; Mukherjee *et al.,* 2016; Zhou *et al.,* 2016). A very large population of the developed countries are adopting the medicinal plants as an alternative in addition to the prescribed medication because these are immuno-stimulant, biologically safe, easily

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available, can prevent the emergence of resistance, provide a broader spectrum of activity than monotherapy regime and induce placebo on both patient and practitioner Moerman (2000). Secondary metabolites and compound derived from natural product are the most desirable therapeutic agent in the modern medicinal system Harvey *et al.,*(2015) but, only 1% plant-derived antimicrobial agent are approved from FDA Newman and Cragg (2016) and mostly others remains under trails (Li and Vederas 2009; Butler *et al.,* 2014). While traditional and alternative medicinal system is majorly dependent on the plant's products. In this perspective use of safe alternative, the natural product (medicinal plant) can be projected as holistic overture and may be the milestone in lieu of biomedical and socio-anthropological ailing. This muse expresses the needs of phytochemical research. This study focuses on the *in vitro* antimicrobial and antibiofilm activity of medicinal plants.

Materials and methods

Bacterial Strains, Chemicals, and Media

Escherichia coli ATCC 3222, *Escherichia coli* ATCC 25922, *Escherichia coli* 4315, *Staphylococcus aureus* MTCC 96, *Pseudomonas aeruginosa* PAO-1, *Salmonella typhi Ty*2, *E coli* CI-3850, *E. coli* CL1, *E. coli* II, *E. coli* III, *Staphylococcus aureus* 737, *Staphylococcus epidermidis* 101, *Staphylococcus saprophyticus* 7443, *Staphylococcus saprophyticus* 102, *Staphylococcus aureus* MRSA 20 and *Staphylococcus aureus* MRSA105 are used and procured from the clinical laboratory, Panjab University, Chandigarh; PGIMER, Chandigarh and CSIR-IMTECH, Chandigarh. All the chemicals and reagents used in the current study are of analytical grade.

Preparation of Antimicrobial Agents

Dried roots and rhizomes of medicinal plants were pulverized. Then extracted for 24 h using organic (acetone & methanol) solvent in Soxhlet apparatus. Thereafter, reducing the extract in Soxhlet apparatus, extract were dried to solidify or till the solvent is completely evaporated in incubator at 45° C. The stock (500 mg/ml) were prepared in 10% ethanol/DMSO for *Berginia liulata* (BL), *Discorea deltoid* (DD), *Jurinia dolomeacea* (JD), *Picrorahiza kurroa* (PK), *Podophyllum hexandrum*(PH), *Rheum astrale* (RH), *Saussurea costus* (SC), *Selenium veginatum*(SV), *Vellerina jatamansi*(VJ). Aqueous extraction is done by suspending grounded plant material into hot water and kept at room temperature overnight, then filtered with Whattman filter paper no.1 and reduced to dry in the incubator, and further dissolved in sterile normal saline and used. Cefotaxime and 10% ethanol/DMSO were included as the control.

Determination of Antibacterial Activity, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Qualitative antibacterial activity of plant extracts was determined by modification in method of Tagg and McGiven (1971), briefly by swabbing log phase (10^6 CFU/ml) bacterial culture on Mueller-Hinton agar plate, 9 mm well were punched and 50 µl (100 mg/ml water extract, 40 mg/ml acetone extract and 30 mg/ml methanol extract) extract was added. Keeping the plates undisturbed till extract is absorbed completely in agar, plates were inverted and incubated for 24 h at 37° C. Then diameter of the zone of inhibition was measured.

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MIC was determined according to CLIS(2012) guidelines, by broth macro dilution method briefly, aliquots of 1ml of the plant extract was serially two-fold diluted in sugar tube containing 1 ml Luria broth, then 10µl of log phase (10^6 CFU/ml) bacterial culture was added and incubated at 37° C. Next day, the tubes were examined visually for growth (turbidity) and no growth (no turbidity). The highest dilution inhibiting the growth was taken as MIC. A loopful from the highest dilution streaked on nutrient agar plates which did not show any bacterial growth after overnight incubation was taken as MBC. Experiments were carried out in triplicates.

Time-Kill Study

A log phase grown culture (10^6 c.f.u. /ml) in Luria broth was supplemented with plant extract at the concentration of 0.5×, 1×, 2×, and 4× MIC then incubated at 37° C. Viable count were determined by a spread plate method. Briefly, the sample was collected at the various time interval (0 h-24 h) and diluted tenfold. Each dilution was spread on the nutrient agar plate with the help of glass spreader and CFU were counted after 24 h. The experiment was carried out in triplicate and the results were presented as mean log10 CFU of organism ± SEM.

Inhibition of Biofilm Formation at Subinhibitory Concentration (SIC) of Water Extract and Methanol Extract of Selected Medicinal Plants

The experiment was performed according to the protocol of Karaolis*et al.,* (2005) with slight modifications. Briefly, by adding 200 µl Luria broth in the microtiter plate 20 µl of test antibacterial agents at SIC was added, then 10 µl of inoculum (10^6 CFU/ml) was added to each well except for negative control. After incubation for 24 h at 37° C, the well was emptied and washed with PBS, further quantification of biofilm was done according to protocol define by Stepanovic*et al.,* (2007). The surface attached cell was fixed with 200 µl methanol for 15 min then emptied and dried at room temperature. Subsequently, the plate was stained with 200 µl filtered 0.1% w/v crystal violet stain for 1 min. each well then washed twice with 200 µl sterile distilled water. Then by adding 33% (200 µl) glacial acetic acid non-adherent crystal violet from the cell adhered to the substratum was dissolved and measure at A_{620} against blank of 33% glacial acetic acid. The relative percentage of biofilm was defined as: {(mean A_{620} of control - mean A_{620} of test well)/mean A_{620} of control well} ×100. Where control is (A_{620} of well with media and extract - A_{620} of well with media only) and test is (A_{620} of well with media, extract and inoculum - A_{620} of well with media and inoculum only).

Effect of Methanol Extract of Selected Plants, Cefotaxime and Combination of both on five-day Established Biofilm

Established biofilm was grown as described by Saising *et al.,* (2011). Two hundred microliter of growing culture (10^6 CFU /ml) was transferred to a 96 well microtiter plate and incubated at 37° C for 24 h and 5 days. For 5-day biofilm, the planktonic cell was removed and fresh Luria broth was added daily. After 5 day incubation, the well was rinsed twice with PBS. Then by adding Luria broth (200 µl) and 20 µl of the test agent at the respective concentration to each well and incubated at 37° C for 24 h. To determine the synergy between plant extract and antibiotic on sessile bacteria multiple FIC of extract and antibiotic was taken as the test agent. After incubation, the medium was removed and washed twice with PBS. Viable cells in biofilm were then quantified by crystal violet (as describes earlier) assay and MTT assay. For MTT assay 200µl PBS was added to each well and then 10µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml; Sigma) was added and further incubated at 37°

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C for 2 h. The insoluble purple formazan was obtained by cleavage of MTT by the dehydrogenase enzyme of the living cell. The formazan crystal was dissolved in DMSO and the A_{620} was determined.

The removal percentage of biofilm was defined as: {(mean A_{620} of control well - mean A_{620} of treated well)/ mean A_{620} control well} × 100.

Statistical Analysis

Statistical analysis on data obtained from experimentation was performed using analysis of variance with Graph prism pad 5.0 software. Comparison between mean values was carried out by two-way ANOVA & Bonferroni's post-test. To demonstrate the association among the biologically related variables regression was applied to the data. The difference was considered significant at $p \leq 0.05$.

Results

Antimicrobial potential of medicinal plant(s) extract(s)

The methanol extract presented maximum antibacterial activity followed by acetone extract and then aqueous extract. The order of antibacterial activity of acetone and methanol extracts was maximum in PH followed by VJ, SV then PK against all the bacterial strains used in this study. While the pattern of activity of water extract was found totally different, the most active were SV followed by PK, VJ, and then PH.

The sensitivity pattern of different bacterial strain against plant extract was in order SA > SS > MRSA > EC > PAO1, while for cefotaxime was EC > SA > MRSA > SS > ST > PAO1. The zone of inhibition was found to be wider with acetone (11 mm - 30 mm) and methanol (12 mm – 31 mm) extracts against all test bacterial strains as compared the zone of inhibition observed with water extracts (10 mm-16 mm) (Table1). The zones of inhibition were narrower against all bacterial strains with *Picrorahiza kurroa* extracts. Also the zone of inhibition was wider for Gram-positive bacterial strain then the Gram-negative bacterial strains.

MICs and MBCs Values

The MIC and MBC value of methanol extract of test plant against the standard and clinical bacterial isolates ranged from 0.015 to 3.906 mg/ml. Gram-positive bacterial strain was more sensitive to bacterial strains then the Gram-negative bacterial strains. Interestingly MIC and MBC of *Podophyllum hexandrum* ranged 0.03 to 0.122 mg/ml, which were similar to those of cefotaxime (Table 2).

Death Rate Kinetics of Test Bacterial Strain Supplemented with Extract

Statistical regression analysis of bacterial killing at varying extract MICs in growth media, with time was the best fit in non-linear correlation (Figure 1). The coefficient of multiple determination (global/shared value) (R^2) ranged from 0.8833 (EC in PK) to 0.9370 (SA in PH) in 24 h for all the combination of extract and bacterial strain. Significant increase in death rate kinetics observed at 4×MIC, 2×MIC ($p < 0.001$) and MIC ($p < 0.01$), while non-significant at 0.5×MIC when compared to control. Furthermore, the number of the viable cell of SA after exposure to methanol extract of PH at a concentration of 4 × MIC decreased by 99.9% within 4 h whilst it took 7 h to 18 h to reduce the same number of cell in other combination at the same concentration.

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Inhibition of Biofilm Formation and their Susceptibility to the SIC of Methanol and Water Extract of Medicinal Plants

Test plant(s) extract(s) used in the study has linearly hampered the biofilm formation significantly at MIC/2, MIC/4 and MIC/16 ($p<0.001$) (Figure 2). At MIC/2 PH has inhibited the maximum (≤ 79%) cell adhesion for all the test bacterial strains, while at lower concentration (MIC/64) it was active against Gram-negative only. It is interesting to note that at the MIC/64 extract of PK was active against Gram-positive bacterial strains only. In current study maximum inhibition in cell adhesion was for PAO1 and minimum for SS. Inhibitory action of water extract were in similar order but at higher concentration of extract.

5-day Old Biofilm Removed at Multiple MIC of Plant Extract & Antibiotic and Their Synergistic Activity

The established biofilm was offed ($p < 0.001$) linearly at the higher concentration in dose dependent manner using the extract of selected plant(s) and cefotaxime (Figure 3). Where the methanol extract of PH has reduced the formed biofilm in all test bacterial strain significantly ($p < 0.001$) than the control antibiotic cefotaxime and other test plant extract used in the study. Biofilm formed by the clinical strain of MRSA were offed by more than 70% comparing to control (non-treated cell) at 64 MIC of PH extract. The survival of 5-day biofilm cell of *E. coli* was maximum (83%) at 64 MIC of PK. Biofilm reduction activity has been observed enhanced when shifting to higher MIC i.e. from 8 MIC to 16 MIC & 32 MIC.

Discussion

Plant secondary metabolites have the potential antimicrobial and antibiofilm activity alone and also act as synergist to other secondary metabolites, thus enhance the activity (Saising *et al.,* 2011; Yadav *et al.,* 2015; Chu *et al.,* 2016; Lee *et al.,* 2016;). This gives the credence to present study and correlates well with our previous studies (Tiwari *et al.,* 2005; Kiran *et al.,* 2010; Sabharwal and Tiwari 2014).

Most biofilm infections are chronic infections. Thus biofilm characteristic is closely related to pathogenicity and difficult to treat with routine antibiotics. Plant secondary metabolites are formed in plants in response to microbial infection and *in vitro* experiments shows their potent antibacterial activity. It is, therefore, the reason that medicinal plants are studied extensively for the last two decades as therapeutic agents for various human ailments including biofilms (Butler *et al.,* 2014; Harvey *et al.,* 2015), but a very few plant-derived antimicrobial agents have been approved by FDA Patridge*et al.,*(2016).

The active molecules from selected plants viz. *Valleriana jatamansi* (jatamansic acid, valeranone, patchouli alcohol etc.), *Podophyllum hexandrum* (Podophylloresine, D-allose, phthalic acid, kaemferol etc.), *Picrorahiza kurroa* (gallic acid, picroside, apocynin, kutkoside etc.) and *Selinum veginatum* (selinidin, angelisin, selinone, orosenol, lomantin etc.) are grouped under natural phenolics & polyphenols (quinones, flavones, flavonoids flavonols, tannins and coumarins), terpenoids, essential oils, resins, alkaloids, lectin, polypeptides and other molecules. These active molecules possess multiple therapeutic effects on cancer, pathogenic infections, diabetes, arthritis, hepatic diseases, bowel syndrome, and central nervous system (CNS) disorders. Traditionally, the selected plants were used to treat warts, wound healing, remedy for insomnia, antispasmodic, purgative, carminative and also used for aromatherapy (Chaurasia*et al.,* 2012; Lin *et al.,* 2013; Pandey *et al.,* 2013; Batra*et al.,* 2016;

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Kumar *et al.,* 2016; Sultan *et al.,* 2016). Furthermore, selected plant extracts have the potent antimicrobial activity (Kumar and Singh 2011; Ratheeet *al.,* 2012). Thus, corroborating the results for antibacterial activity of selected plant extracts, we further investigated their antibiofilm activity. Moreover, the slight difference in plant extract activity depends on the sample collection site and the method of extraction.

Yields of extraction and antibacterial activities of organic extracts were higher than the aqueous extracts (Table 1 & 2). This signifies the presence of lipophilic active components in plant material. The lipophilic compounds (secondary metabolite) from plant extracts can pass through the cell wall and cytoplasmic membrane, disrupting the structure of different layers of polysaccharides, fatty acids, and phospholipids, permeabilizing the cell membrane and resulting in cell lysis Burt (2004). The crude extract has the versatility of bioactive molecules in them which inhibit the range of pathways in bacterial cells Santos *et al.,*(2016). The high activity of plant extract against Gram-positive than the Gram-negative (Table 1 & 2) gives the account of the role of efflux pump and cell wall components in the Gram-negative bacteria. These results are in line with Silva *et al.,* (2016). Proteomic studies done on bacterial cell treated with medicinal plant extract suggest that the changes in cell wall biosynthesis, cell division hindrance, protein degradation, stress responses and cell surface antigen modulation in bacterial cell are the reason of the antimicrobial and antibiofilm activity imparted by the phytochemicals (Ajiboyee*t al.,* 2016; Das *et al.,* 2016; Santos *et al.,* 2016; Thakur *et al.,* 2016).

Biofilm assays revealed that the extracts used in the present study can inhibit the biofilm formation significantly ($p < 0.001$) at SIC (Figure 2) but had the non-significant effect on bacterial growth (Figure 1). This may be due to the attachment of active molecules in our extracts onto the bacterial cell surface adhesion receptors, which may further inhibit or interferes with the signalling molecules responsible for biofilm formation. It can also be attributed to the fact that it could inhibit the other virulence factors responsible for biofilm formation. As discussed earlier, plant crude extract has the versatility of bioactive molecules which can inhibit the range of bacterial pathways. Correlation of bacterial growth behaviour in plant extract enriched media with time was found to be concentration dependent during the investigation of assays for the biofilm inhibition & removal of biofilm and cell death rate kinetics. These results support Lee *et al.,*(2016).The biofilm forming ability of bacterial strains was reported to be mediated by amyloid-like protein, these are shared the feature of many microbial communities (curli in *E. coli* and *Fap* fimbriae in *Pseudomonas aeruginosa* etc.) featuring biofilm matrices. Congo red staining for amyloidosis Chu *et al.,*(2016) indicated that extracts have affected functional amyloid synthesis till the lower concentration of MIC/16 in most of the cases when the extract was added to the growth media (unpublished results). Thus, it can be believed that the extracts used in the present study inhibit the factor responsible for biofilm assembly and dis-assembly.

Invulnerable methods of treatment and medication are there. For the rational prescription of antibiotic which is completely dependent on the microbiological report (if facility available) or otherwise on the basis of epidemiological studies. Where alternative methods; treatment with the medicinal plant is the two-way process, rather than targeting only bacterial cells, it also stimulate the immune system of the host. Medicinal plant given supplementary with antibiotic reduces the MIC dose of both, hence reduce the risk of resistance and dose-dependent cytotoxicity. In practice, a very large population of both old world and new world uses plant products in addition to the prescribed medication. Use of medicinal plant induces

placebo effect on both patient and practitioner Moearman (2000); contribution to the behavioural aspect which is beyond the scope of infectious disease & microbiology. This can achieve the sustainable success over bacterial resistance and public health by contributing toward the antimicrobial stewardship program.

This recitation gives the account for antimicrobial & antibiofilm activity of test agents (natural products).In this perspective, use of safe alternative can be projected as treatment overture for resistance jeopardy. However, improvement in science skills for the identification of active compounds and *in vitro* laboratory studies of the medicinal plant to check the safety & efficacy remains the issue of great concern. Furthermore, ascertainment of the amyloid formation and deciphering the mechanism of action attributed by the molecule would be the potential strategies for the development of newer antimicrobial and antibiofilm agent for future.

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Conflict of interest

Authors declare no conflicts of interest.

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Table 1: Diameter of zone of inhibition ±SD of medicinal plants extracts from different extractants against common pathogens.

Plant	Extractants	Zone of inhibition (mm)				
		E. coli 25922	*P. aeruginosa* PAO-1	*S. saprophyticus* 7443	*S. aureus* 96	*MRSA 20*
Podophyllum hexandrum	Acetone	21.66±2.02	18.67±0.66	26.66±1.45	30.67±0.33	24.66±1.76
	Methanol	22..33±0.88	19.33±0.88	28±1.15	31±0.88	25±1.2
	Aqueous	11.66±0.88	9.66±0.66	15.66±0.33	16.66±0.88	13±0.57
Valleriana jatamansi	Acetone	21±1.15	20.33±1.20	25.33±1.45	28.66±2.18	23.66±1.45
	Methanol	21.66±1.2	18.66±0.66	26.66±0.88	30±0.57	24±0.88
	Aqueous	11.33±1.2	11±1.15	14.33±0.66	16.66±0.33	11.66±0.66
Selenium veginatum	Acetone	20.667±0.88	18.33±0.88	24.66±0.33	25.33±0.33	22±1.52

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	Methanol	21.33±0.88	18.66±0.88	26.33±0.88	30.33±0.88	23.33±1.2
	Aqueous	10.33±0.66	10±1.52	15.33±0.88	16.33±0.66	11.33±0.88
Picrorahiza kurroa	Acetone	10.33±0.88	11±1.55	16±1.52	19.66±0.88	12.66±1.2
	Methanol	14.33±0.33	12.33±0.88	18.66±1.2	19.33±0.88	18.33±0.33
	Aqueous	9.33±0.33	7.66±0.66	11.66±1.2	13.66±0.88	10.66±0.88

Table 2: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of medicinal plants extracts and cefotaxime against selected bacterial strains.

Herbs	Extractants	MIC/MBC (mg/ ml)				
		E coli 25922	*P. aeruginosa* PAO-1	*S. saprophyticus* 7443	*S. aureus* 96	*MRSA 20*
Podophyllum hexandrum	Acetone	0.976/3.906	3.906/8.812	0.244/0.976	0.122/0.976	0.488/1.953
	Methanol	0.06/0.122	0.122/0.486	0.03/0.122	0.015/0.030	0.03/0.122
	Aqueous	125/250	250/500	62.5/125	31.25/62.5	125/250
Valleriana jatamansi	Acetone	1.953/3.906	7.812/15.625	0.488/1.953	0.244/0.976	0.976/1.953
	Methanol	0.488/1.953	1.953/7.812	0.122/0.486	0.060/0.244	0.122/0.244
	Aqueous	62.5/250	125/250	31.25/125	31.25/125	62.5/125
Selenium veginatum	Acetone	1.953/3.906	7.812/7.812	0.486/1.953	0.244/0.976	0.976/1.953
	Methanol	0.976/3.906	1.953/3.906	0.244/1.953	0.122/0.244	0.486/1.953
	Aqueous	31.25/31.25	62.5/125	15.625/31.25	15.625/31.25	31.25/62.5
Picrorahiza kurroa	Acetone	7.812/15.625	15.625/31.25	1.953/7.812	1.953/7.812	3.906/7.812
	Methanol	1.953/7.812	3.906/15.625	0.488/1.953	0.244/0.976	0.488/1.953
	Aqueous	31.25/125	62.5/125	15.625/125	15.625/31.25	31.25/125
Cephotaxime (µg/ml)		0.122/0.122	15.6/31.25	0.976/1.953	0.244/0.488	0.488/0.976

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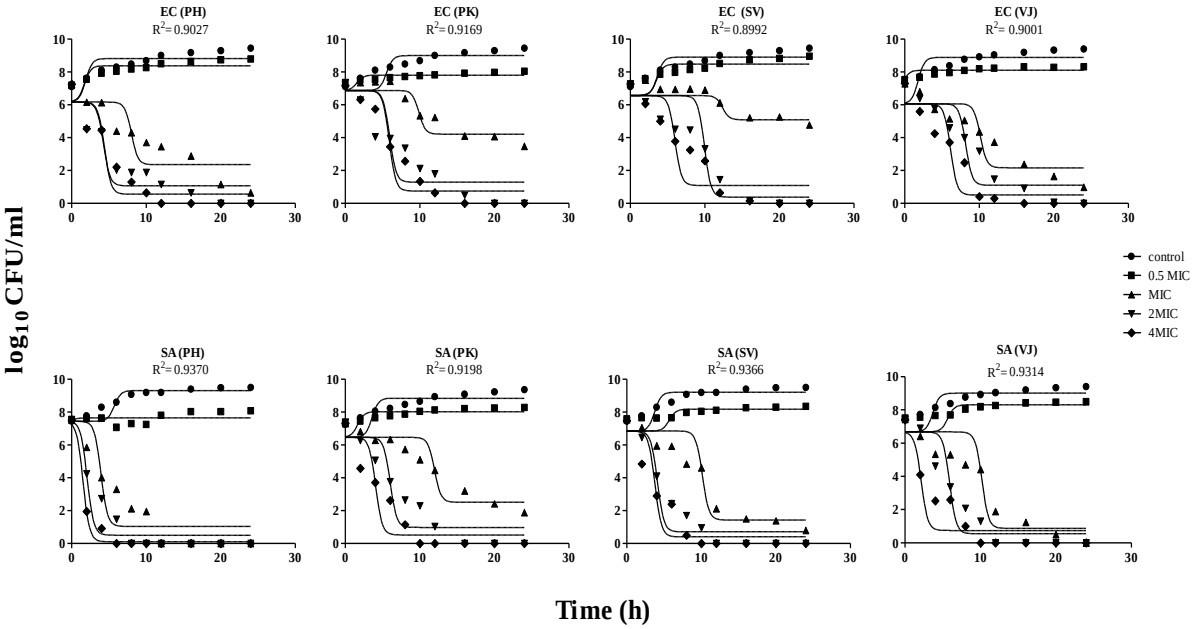

Figure 1: Non-linearity of death rate kinetics of test bacterial strains cultured in Luria broth supplemented with methanol extract of medicinal plants at 0.5×MIC, MIC, 2×MIC & 4×MIC of respective pathogen with time.

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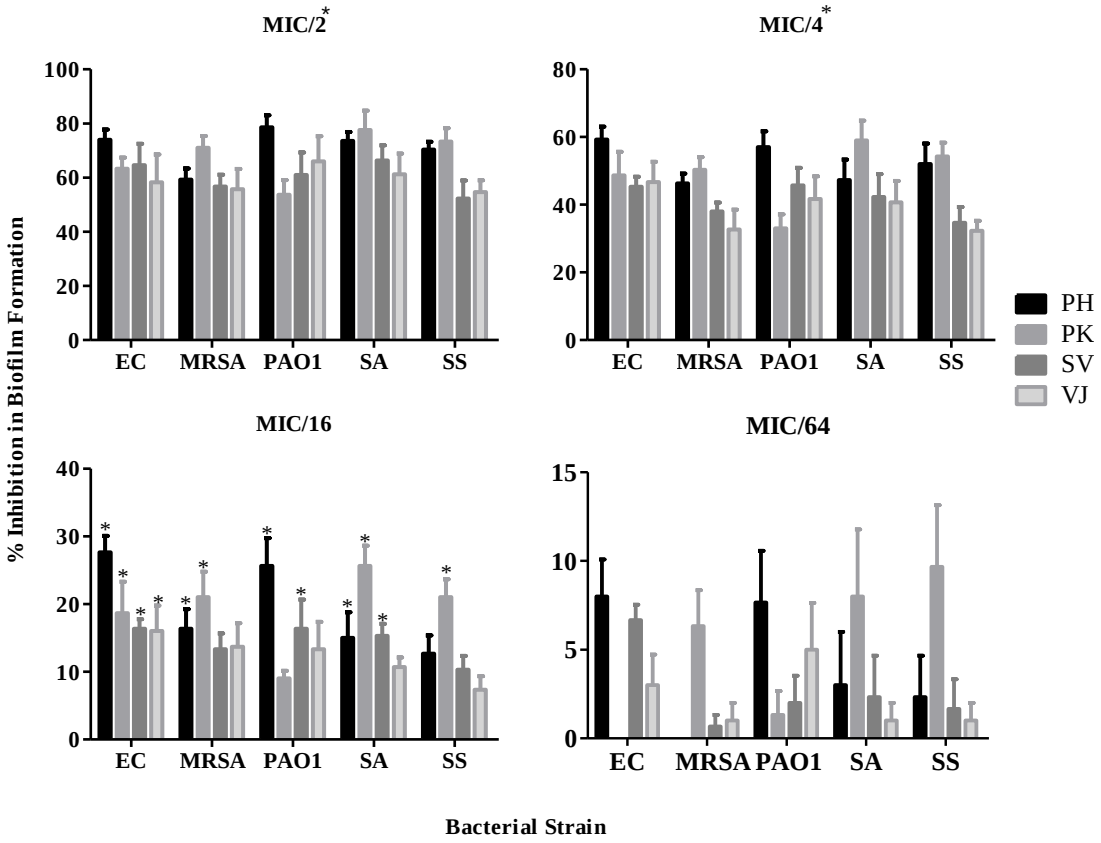

Figure 2: Inhibition in biofilm formation at Subinhibitory concentration (SIC) of methanol extract of medicinal plants. *Significance was determined at $p < 0.05$ when compared to control having no agent.

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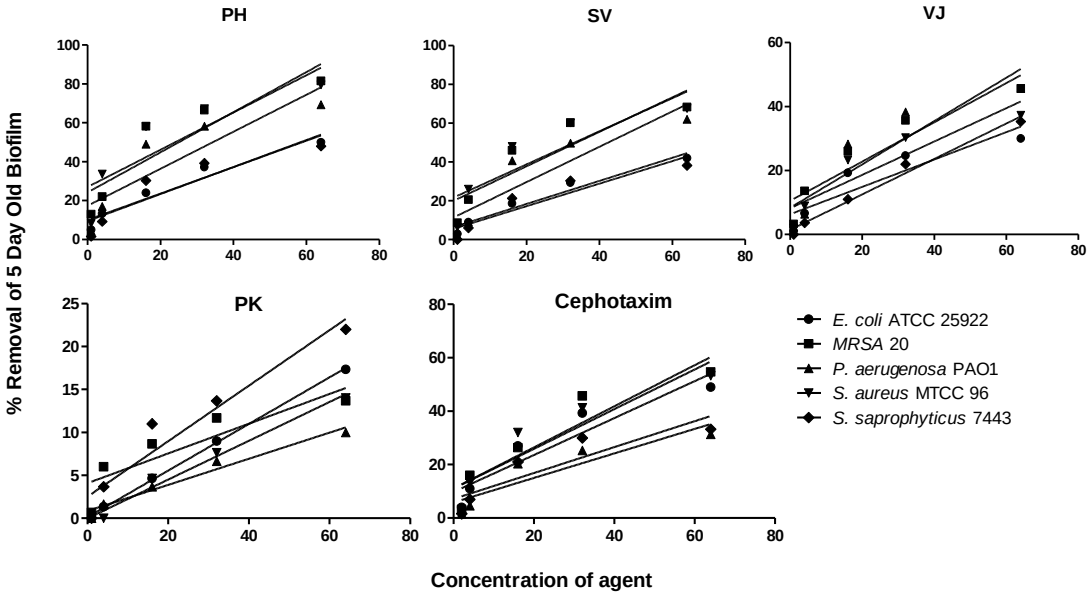

Figure 3: Removal of 5 day old biofilm formed by test bacterial strain at multiple MIC of methanol extract of medicinal plants.