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In vitro antibacterial activity of *Fumaria indica* (H.) pugsley and *Silybum marianum* L. against planktonic and biofilm form of *Pseudomonas aeruginosa*

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Abstract

Present study investigated antibacterial potential of aerial partsof *Fumaria indica* and*Silybummarianum* against planktonic and biofilm form of 3 clinical strains P1, P2 and P3 of *Pseudomonas aeruginosa*. Antibacterial activity against planktonic form was investigated by Well diffusion method. The antibiofilm activity was assessed by using Pellicle inhibition (PI) and Congo red assay (CR). Crude methanolic extracts of *Fumaria indica*showed good antibacterial activity against *P. aeruginosa* with maximum 15.8mm zone of inhibition (ZOI) against P2 strain of *P. aeruginosa* and minimum 13.2mm zone of inhibition against P3 strain. *Silybummarianum*showed maximum 13.3mm zone of inhibition and minimum 12.6mm ZOI against P2 and P3 strains of *P. aeruginosa* respectively.Furthermore, *F. indica* showed a moderate (+++) to weak (++++) antibiofilm activity against all tested strains of *P. aeruginosa* in Pellicle inhibition assay, while *S. marianum* possessed from moderate to no activity against tested bacterial strains, respectively. In Congo red assay *F. indica* showed a strong antibiofilm effect as compared to *S. marianum*. Based on these results it may be concluded that leaf extractof *Fumaria indica*possessed a good to moderate antibacterial activityagainst planktonic and biofilm form of *P. aeruginosa*. While *Silybummarianum*possessed moderate to weak antibacterial potential against *P. aeruginosa*.

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Introduction

The phenomena of biofilm formation involved bacterial communities sheathed in extracellular polymeric substances (EPS) (Karatan and Watnick, 2009). Biofilm form of life represents a defensive style of bacterial growth that enables bacterial communities to stay alive in unfavourable environmental conditions. Biofilms can form on different varieties of surfaces and are common in natural, hospital and industrial settings. Different studies have shown that biofilms are responsible for approximately 60% human infections (Begun *et al.*, 2007). These bacterial communities in biofilm form are different physiologically from planktonic form. Bacteria live in biofilm mode possessed increased resistance to antibiotics (Davies *et al.*, 1998; Hall-Stoodley *et al.*, 2003). The exhibition of resistance to antibiotics results in a growing need to find novel antibacterial drugs that are safe and also able to control infections caused by bacterial biofilms (Cegelski *et al.*, 2008).

P. aeruginosa, an important opportunistic organism is well-known to be a key human, pathogen responsible for various infections, predominantly in patients with cystic fibrosis and immunocompromised persons (Deep *et al.*, 2011). Moreover, *P. aeruginosa* have the ability to form biofilms in a variety of environments resulting in chronic bacterial infections (Hoiby *et al.*, 2010; Bjarnsholt, 2013).

*Fumaria indica (*Hausskn.)Pugsleyis an annual, small, leafy and highly branched flowering herb that usually grows upto 5-25cm in length. It belongs to family Fumariaceae and consists of 14 genera and nearly 400 species. It is locally named as Krachay or Paprha (Pashto) and is traditionally used for a variety of purposes (Table 1) such as a blood purifier, for removal of pimples, and to relieve heals and palms inflammation (Ahmad *et al.*, 2011). Ahmad *et al.*, (2015) reported the Ethnopharmacological data of 46 anti-hypertensive plants species belonging to different families including *F.indica* of region Dir lower Pakistan. Rathi *et al.*, (2008) isolated an alkaloid called protopine from *F. indica* and it possessed a significant hepatoprotectants potential as to standard drug silymarine. Phytochemical investigations of *F.indica* showed that it consists of alkaloids such as fuyuziphine, alpha-hydrastine (Pandey *et al.*, 2008).

Silybummarianum(L.) Gaertn. belongs to family Asteraceae and is an annual or biennial herb. *S. marianum* is local to the Mediterranean regions but it is now naturalized throughout the world. It is also present abundantly in Pakistan especially in Khyber Pukhtoonkhwa (KPK) and Punjab province (Bisset, 1994). It is locally known as Worajakai (Table 1) and the flower is traditionally used for the treatment of tuberculosis and jaundice (Ahmad *et al.*, 2011). Evren and Yurtcu, (2015) studied the antibacterial, antiadherent and biofilm potential of silymarin obtain from *Silybummarianum* on standard gram-positive and gram-negative bacterial strains. They concluded that silymarin was not very effective against gram negative strains. Greenlee *et al.*, (2007) reported that the major component of traditional milk thistle extract is silymarin and is present in concentration from 4 to 6% Kroll *et al.*, (2007).

This study provides a comprehensive data set on the *in vitro* antibacterial potential of the methanolic fractions of *Fumaria indica* (*F. indica*) and *Silybummarianum*(*S. marianum*) leaves against different clinical strains of *P.aeruginosa*.

Materials and methods

The leaves of *F. indica* and *S. marianum*as shown in Figure 1 were collected from Chakdara, Lower Dir Khyber Pakhtunkhwa, Pakistan. The selected plant specimens were identified by Dr Abdul Majid, Lecturer, Department of Botany, Hazara University Mansehra. The voucher specimens were mounted in herbarium sheet and were kept in Hazara University Herbarium. The shade-dried leaves of plants (1kg) were chopped into fine powder. Methanolic extract of each plant sample was prepared by soaking in methanol for 3 days with vigorous agitation. The

extracts were then filtered and solvents were evaporated by using a rotary evaporator and stored at 4°C. This process of extraction was done 3 times in order to get maximum extraction. The crude methanolic extracts were subjected tomicrobial bioassays.

Test microorganisms

The pre-identified bacterial strains used in the current study were collected from Pathology Laboratory of Pakistan Institute of Medical Sciences, Islamabad (PIMS), Pakistan. Initially, three Gram negative clinical strains of *Pseudomonas aeruginosa* P1, P2 and P3 were used. The bacterial cultures were inoculated individually and kept at 37°C overnight in a shaker incubator at 150rpm. The bacterial cell number was adjusted to approximately 10^8 CFU/ml (colony forming unit per ml).

Antibacterial test using the agar diffusion method (well)

A preliminary evaluation of the antibacterial activity of crude methanolic extracts (5-15mg/ml) was determined by agar diffusion method (Walter *et al.*, 2011). DMSO was used as a negative control. The antibacterial activity was determined by measuring the diametersin 'mm' of inhibition zone.

Pellicle inhibition assay

The antibiofilm effect of *F. indica* and *S. marianum* crude methanolic extracts were performed by pellicle inhibition assay according to the method of Joshua *et al.*, 2006 with modifications. In this assay four different concentrations (15mg/ml, 12.5 mg/ml, 10mg/ml, 7.5mg/) of each crude extracts was used against three clinical bacterial strains (P1, P2, P3) of *Pseudomonas aeruginosa*. Test tubes were prepared pipeting 6ml of Mueller Hinton broth (MH) medium in each testubelabelled P1, P2 and P3 respectively. Then 60µl of bacterial inoculum and 100µl of extract of required concentration was added to each tube. Positive control contains MH medium and bacteria while negative control contains MH media and plant extract only. The tubes were incubated at room temperature for seven days without agitation. After incubation the effects of plant extracts on pellicle was represented by (+) signs and was evaluated as - no biofilm, + significant biofilm inhibition, ++ good biofilm inhibition, +++ moderate biofilm inhibition; ++++ weak biofilm inhibition.

Congo red assay

Congo red assay was performed for crude methanolic extracts of *F.indica* and *S. marianum* against all three bacterial strains (P1, P2 and P3) in order to study their effect on the production of polysaccharides, which are responsible for the formation of biofilm. Congo red agar plates were prepared by adding 40µl/ml Congo red dye, 10g/L tryptone and 20µl/ml coomassie brilliant blue. Overnight cultures of P1, P2 and P3 (2.5 µL) strains in Mueller Hinten broth medium with crude methanolic extract (15mg/ml) of each plant and with no extract was transferred onto the congo red agar plates and was kept for 4 days at room temperature (Merritt *et al.*, 2007). Without plant extracts, P3 strain of *P. aeruginosa* formed a very dark red colour colony as compared to P1 and P2 strains, which showed that this strain have a high tendency to form biofilm due to high production of polysaccharides.

Results and discussion

Plants have been used as a traditional medicine since antiquity and a large numbers of antimicrobial drugs have been obtained from it on the basis of their traditional use as a medicine (Cragg and Newman, 2002). In our study we used the methanol extracts of *F. indica* and *S. marianum* to determine its antibacterial potential against planktonic and biofilm form of *P. aeruginosa*. In various studies it have been reported that methanol extract of plants exhibited good antibacterial activities (Kang *et al.,* 2011; Parekh *et al.*, 2005).

Antibacterial effect of crude methanolic extracts of F. indica and S. marianumagainst planktonic form of P. aeruginosa

In antibacterial activity of crude methanolic leaves extracts of *F. indica* and *S. marianum*against planktonic form of *P. aeruginosa*,*F. indica*

showedmaximum activity against P2 strain with zone of inhibition (ZOI) from 15.4mm to 10.7mm at different concentrations, while against P1 strain a ZOI from 13.8mm to 9.5mm was observed. Against P3 strain, minimum antibacterial activity was calculated with ZOI from 13.2 mm to 9.2 mm as compared to P1 and P2 strains as shown in Figure 2A.

Table 1. Traditional use of *Fumaria indica* and *Silybummarianum*. (Ahmad *et al*., 2011).

Names	Local name	Part used	Family	Traditional uses
Fumaria indica	Krachay	Whole Plant	Fumariaceae	Blood purifier, removal of pimples, relieve heals and palms inflammation
Silybummarianum	Worajakai	Flower	Asteraceae	Tuberculosis and Jaundice

Table 2. Pellicle inhibition of *F. indica* against different strains of *P. aeruginosa* after 7 days of incubation. (n=3)

Concentration (mg/ml)	P1	P2	P3	P1+	P2+	P3+
15	+++	+++	+++	+++++	+++++	+++++
12.5	+++	+++	+++	+++++	+++++	+++++
10	++++	++++	++++	+++++	+++++	+++++
7.5	++++	++++	++++	+++++	+++++	+++++

P1+P2+P3 represents +ve control to the respective P1, P2, and P3 strains of *P. aeruginosa*.

In our finding, *F. indica* showed good antibacterial activity against planktonic form of *P. aeruginosa* strains tested. Khan *et al*., 2014 reported a moderate activity of methanol extract of *F. indica* against *P. aeruginosa*. For *S. marianum,* maximum antibacterial activity was recorded against P2 strain with ZOI 13.3mm to 9.3mm, while against P1 and P3 strains, 13.1mm to 9.1 mm and 12.6 mm to 9.8mm zones of inhibition were calculated as shown in Figure 2B. Furthermore, P3 strain showed more resistance to the herbal treatment. So from the results we found that crude methanol extract of *S. marianum*showed moderate activity as compared to *F. indica*. Evren and Yurtcu, 2015reported thatSilymarin, a major component of *S. marianum* showedno activity against ATCC strains of *P. aeruginosa*.

Table 3. Pellicle inhibition of *S. marianum* against different strains of *P. aeruginosa* after 7 days of incubation. (n=3).

Concentration (mg/ml)	P1	P2	P3	P1+	P2+	P3+
15	+++	+++	+++	+++++	+++++	+++++
12.5	++++	++++	++++	+++++	+++++	+++++
10	++++	++++	++++	+++++	+++++	+++++
7.5	+++++	+++++	+++++	+++++	+++++	+++++

P1+P2+P3 represents +ve control to the respective P1, P2, and P3 strains of *P. aeruginosa*.

This variation in activity may be due to the source of strains used. Also the plant extracts showed inhibition in a concentration dependent manner and thus the antibacterial activity increases with the increase in plant extract concentration.

Antibiofilm activity of crude methanolic extracts of F. indica and S. marianumin different clinical strains of P. aeruginosa

Antibiofilm activity of *F. indica* and *S. marianum* was recorded by pellicle inhibition at different concentrations as shown in (Table 2). A moderate antibiofilm effect (+++) was observed for *F. indica* against pellicle formation of all *pseudomonas* strains

(P1, P2, P3) at 15-12.5mg/ml concentration. From the results it is clear that with increase in concentration from 15 to 12.5mg/ml there is no effect on biofilm inhibition indicating12.5mg/ml to be the minimum affective dose for all the tested strains. It has been suggested that if low concentration of antibiotics or other drugs is able to prevent initial adherence of bacteria to surfaces, the subsequent step of biofilm formation would also be inhibited (Furneri *et al.* 2003). Atconcentration 10mg/ml and 7.5mg/ml, a weak activity (++++) against P1, P2 and P3 biofilm was observed.

Fig. 1. (A) *F. indica* (B) *S. marianum*.

For *S. marianum*, moderate antibiofilm (+++) activity was observed against all tested strains at 15mg/ml as shown in (Table 3). At 12.5mg/ml and 10mg/ml concentration, a weak (++++) antibiofilm effect was recorded against all tested strains. Meanwhile, at concentration 7.5mg/ml, no biofilm (+++++) inhibition was observed.

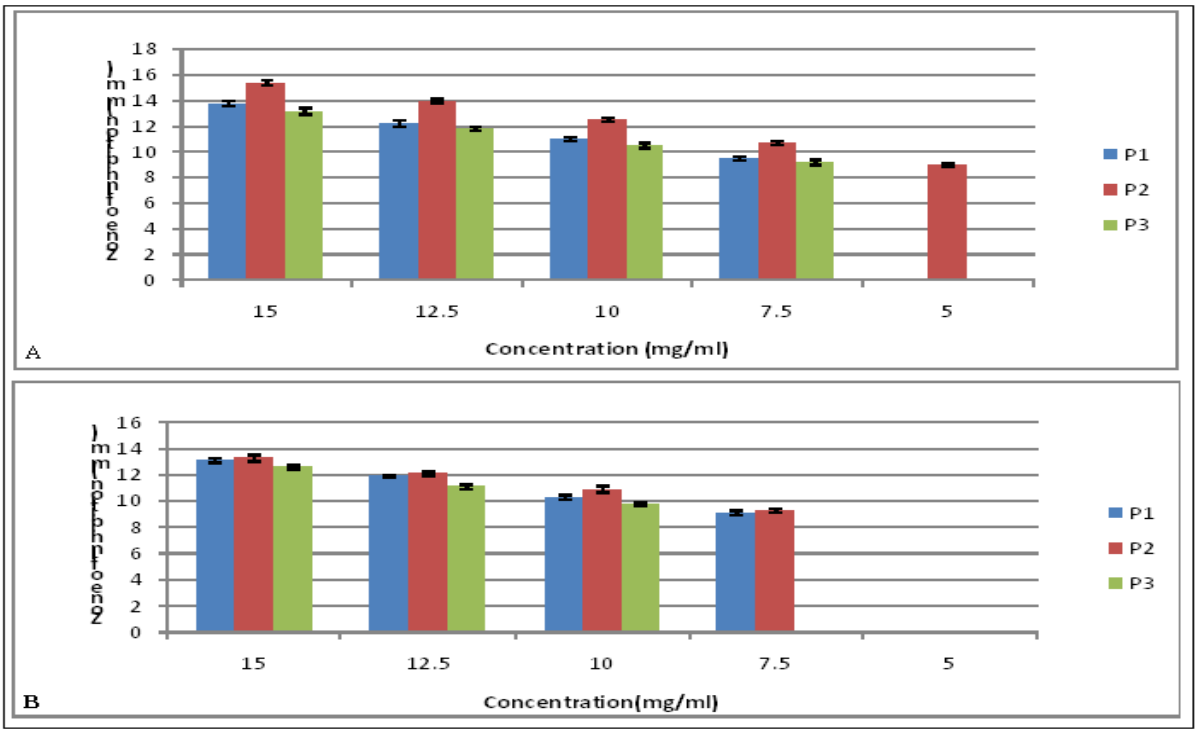

Fig. 2. Zone of Inhibition (mm) against different *P. aeruginosa* strains by the crude methanolic extracts of *F. indica* (A) and *S. marianum*(B) after 24h incubation. Data represent as mean ±standard error. (n=3)

The affect of plant extracts to inhibit cell attachment is the confirmation of previous reports where it was found that inhibition of cell attachment to a substrate is easier to achieve than inhibiting the growth of an already established biofilm (Cerca *et al*. 2005). So in our findings, a moderate to weak antibiofilm activity in pellicle inhibition assay was observed for *F. indica* at different concentrations, while for *S. marianum*, weak to no activity was recorded respectively. No data is available on pellicle inhibition for *F. indica* and *S. marianum*. Pattiyathanee *et al*., 2009 reported that curcumin extract inhibit pellicle biofilm formation in *Helicobacter pylori.*

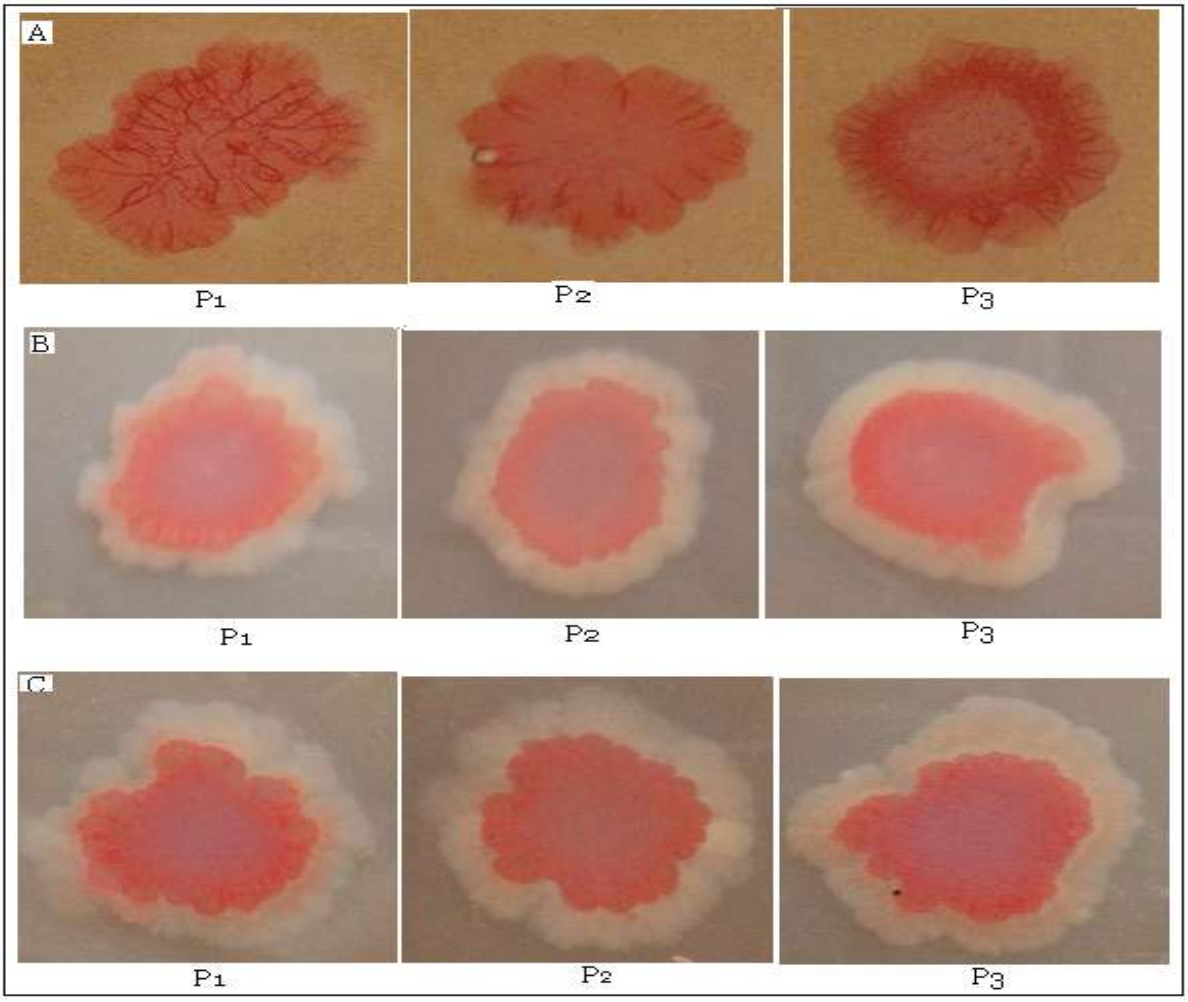

Fig. 2. Effect of extracts (15mg/ml) on biofilm formation (A) strains grown without plant extract (B) strains grown with crude *F. indica* extract (C) strains grown with crude *S. marianum* extract.

Antibiofilm activity of F. indica and S. marianum by Congo red assay

The antibiofilm effect of *F.indica* and *S. marianum* at 15mg/ml was recorded and a moderate effect on the colour of the bacterial colony was observed for both plants as compared to the respective controls against all tested strains of *P. aeruginosa*. This indicates that it effects the polysaccharide production in these bacterial strains and in turn effecting biofilm formation as shown in Figure 2 (A, B and C).

Using congo red assay, we have found that without plant extract maximum polysaccharide production was found in P3 strain with bright red colour as compared to P1 and P2 strain (Figure 2 A).

Both crude extracts of *F. indica* and *S. marianum* have clearly inhibited the production of polysaccharide and in turn inhibits biofilm formation which result in light red colour colonies. From Figure 2 B and C it is clear that inhibition of polysaccharide

by crude extract of *F. Indica* is comparatively more than *S. Marianum*.No data is available on congo red assay for *F. indica* and *S. marianum*. Kim and Park, 2013 reported that ginger extract effect the morphology and colour of *P. aeruginosa* (PA14) forming biofilm by using congo red assay. In both pellicle and congo red assay there exist a pattern of increase in resistance (Sandasi *et al*., 2010) as compared to the planktonic counterparts of *P. aeruginosa*tested in well diffusion method. As it is known that bacteria in biofilm mode showed more resistant than in planktonic form (Hall-Stoodley*et al*., 2003). Here also in our experiment we report that in biofilm assays of *F. indica* and *S. marianum* a dose dependent biofilm inhibition was recorded.

Taken together, we reported for the first time the antibacterial potential of the methanolicleaves extracts of *F. indica* and *S. marianum*against planktonic and biofilm form against clinical strains of *P. aeruginosa*. Moreover, the moderate to weak antibiofilm activity in pellicle inhibition and congo red assay indicates a pattern of increase in resistance as compared to the planktonic counterparts (Sandasi*et al*., 2009) in disc diffusion method.

These findings showed that *F. indica* possessed good antibacterial potential as compared to *S. marianum* and need further study in order to be used in control strategies against biofilm formation in *P. aeruginosa*.

Conflict of interest

The authors have no conflict of interest to disclose.

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