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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**Key words:** Antibacterial activity, *Fumaria indica*, *Silybum marianum*, *Pseudomonas aeruginosa*, Biofilm, Planktonic.

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**Abstract**

Present study investigated antibacterial potential of aerial parts of *Fumaria indica* and *Silybum marianum* 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. *Silybum marianum* 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 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 extract of *Fumaria indica* possessed a good to moderate antibacterial activity against planktonic and biofilm form of *P. aeruginosa*. While *Silybum marianum* 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 (Holby et al., 2010; Bjarnsholt, 2013).

_Fumaria indica_ (Hausskn.) Pugsley is an annual, small, leafy and highly branched flowering herb that usually grows up to 5-25 cm 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_ 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 to microbial 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 diameters in ‘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 testlablabelled 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. marianum* against 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*,
showed maximum activity against P2 strain with zone of inhibition (ZOI) from 15.4 mm to 10.7 mm at different concentrations, while against P1 strain a ZOI from 13.8 mm to 9.5 mm 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 *Silybum marianum*. (Ahmad *et al.*, 2011).

<table>
<thead>
<tr>
<th>Names</th>
<th>Local name</th>
<th>Part used</th>
<th>Family</th>
<th>Traditional uses</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fumaria indica</em></td>
<td>Krachay</td>
<td>Whole Plant</td>
<td>Fumariaceae</td>
<td>Blood purifier, removal of pimples, relieve heals and palms inflammation</td>
</tr>
<tr>
<td><em>Silybum marianum</em></td>
<td>Worajakai</td>
<td>Flower</td>
<td>Asteraceae</td>
<td>Tuberculosis and Jaundice</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P1+</th>
<th>P2+</th>
<th>P3+</th>
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<tr>
<td>15</td>
<td>+++</td>
<td>+++</td>
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<td>12.5</td>
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<td>10</td>
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<td>7.5</td>
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</tbody>
</table>

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 13.3 mm to 9.3 mm, while against P1 and P3 strains, 13.1 mm to 9.1 mm and 12.6 mm to 9.8 mm 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, 2015 reported that Silymarin, a major component of *S. marianum* showed no 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).

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P1+</th>
<th>P2+</th>
<th>P3+</th>
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<tbody>
<tr>
<td>15</td>
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</tbody>
</table>

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. marianum* 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 indicating 12.5mg/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). At concentration 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*.

![Figure 2](image)

*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. aeruginosat*ested 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 methanolic extracts of *F. indica* and *S. marianum* against planktonic and biofilm form against clinical strains of *P. aeruginosat*ested. 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 (Sandasiet 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. aeruginosat*.

Conflict of interest
The authors have no conflict of interest to disclose.

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