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Research Article

Antibacterial and antibiofilm potential of leaves extracts of *Mirabilis jalapa* L. and *Ajuga bracteosa* wall. against *Pseudomonas aeruginosa*

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Abstract

In the Present study the antibacterial and antibiofilm potential of *Mirabilis jalapa* and *Ajuga bracteosa* crude methanolic extracts of leaves against P1, P2 and P3 clinical strains of *Pseudomonas aeruginosa* was explored. Antibacterial activity was investigated by agar well diffusion method and the antibiofilm potential was determined by Anti-Pellicle assay and exopolysaccharide inhibition (EI) by Congo red (CR) assay. In antibacterial assay, *M. jalapa* extract showed good antibacterial activity against *P. aeruginosa* with maximum 13.5 mm inhibition zone (ZI) against P2 strain and minimum 12.8 mm ZI against P3 strain of *P. aeruginosa* as compared to *A. bracteosa* which showed a maximum 13.4 mm ZI and a minimum of 12.7 mm against both P2 and P3 strains of *P. aeruginosa*, respectively. Both *M. Jalapa and A. bracteosa* extracts showed moderate (+++) and weak (++++) effect on pellicle inhibition against the tested strains of *P. aeruginosa*. In CR assay, a moderate antibiofilm effect was recorded on EI for both plants. Based on the results it was concluded that methanol extracts of *M. jalapa and A. bracteosa* possessed good antibacterial activity and moderate antibiofilm potential against *P. aeruginosa*.

Keywords: Agar well diffusion assay; *Ajuga bracteosa*; *Mirabilis jalapa*; Natural products; Opportunistic pathogen

Introduction

Many of the bacterial species have survived in unfavourable conditions by attaching each other and often cells adhere to surfaces to form highly organized matrix sheathed structure known as biofilm **[1, 2]**. The

structure of biofilms shields the microbes from the effect of antimicrobial agents and immune cells, as it stops their penetration. It has been reported that biofilms are involved in about 60% of human infections [3]. The bacterial species that form the biofilm structure are physiologically different from their planktonic counterparts. Bacteria survive in biofilm form exhibited increased resistance to antibiotics [2, 4]. Therefore, the emergence of resistance to antibiotics resulted in the enhance need of natural antibacterial drugs active against microorganisms involved in biofilm formation and spared of infections [5]. *Pseudomonas aeruginosa* (*P. aeruginosa*) has emerged as a key opportunistic pathogen involved in the production of various virulence factors responsible for the infections especially in peoples with compromised immunity, burn wounds and cystic fibrosis [6, 7]. *P. aeruginosa* have this remarkable ability to live as biofilms and results in chronic infections and also showed increased resistance to antimicrobials agents [8].

Mirabilis jalapa (M. jalapa) is a perennial herb that most often grows upto 1.5 m in length and has beautiful red, yellow or white flowers. It belongs to Nyctaginaceae family that has 30 genera and 290 species. It is represented by 5 genera and 10 or 11 species in Pakistan [9]. Locally it is known as Gule Badi (Pashto). Traditionally the leaves of *M. jalapa* has been used against root tubers, abscess, pain and typhoid [10]. It has been reported that seeds of *M. jalapa* possessed antibacterial activity [11]. The anti-inflammatory potential of *M. jalapa* leaves has also been reported [12, 13].

Ajuga bracteosa (A. bracteosa) belongs to family Labiatae and usually grows up to 5-50 cm. It is a perennial herb and is distributed across eastern part of Afghanistan, Pakistan, Kashmir, Bhutan, China and Malaysia [14]. Its local name is Butey or Khwaga Bhootey (Pashto) and has been used traditionally (Table 1) to relive abdominal pain, to cure itching and pimples, jaundice, respiratory diseases and hypertension [10, 15]. Previously the antibacterial potential of essential oils of *A. bracteosa* leaves has been reported [16, 17]. This data provides comprehensive information on the *in vitro* antibacterial and antibiofilm potential of crude methanolic extracts of *M. jalapa* and *A. bracteosa* leaves against *P. aeruginosa*.

Materials and methods

Plant extraction

The aerial parts of *M. jalapa and A. bracteosa* were collected from the area of Shaheed abad, Lower Dir, Khyber Pakhtunkhwa, Pakistan as shown in Figure 1. The plant specimens collected were then identified by Dr. Uzma Khan, Associate professor, Department of Botany, Hazara University Mansehra. The voucher specimens of both collected plants were placed in Hazara University Herbarium. The shade-dried leaves of plants (1kg) were crushed into powder and the methanol crude extract of both plants were prepared by keeping it in methanol solvent for 3 days on vigorous shaking. The extracts present in methanol were then filtered and methanol was evaporated via rotary evaporation process and the semi-solid extracts of both plants were stored at 4°C. The extraction process was repeated 3-4 times in order to get maximum plants extraction. The crude methanol extracts of plants were then used for further processing.

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Figure 1. **(A) *M. jalapa*** **(B) *A. bracteosa***

Bacterial strains

The bacterial strains of *P. aeruginosa* used in the study were pre-identified and were collected from Pakistan Institute of Medical Sciences (PIMS) Pathology Laboratory Islamabad, Pakistan. The bacterial cultures were first inoculated individually and kept at 37°C overnight in a shaker incubator at 150rpm. The bacterial cell number was standardized to approximately 10^8 CFU/ml.

Antibacterial activity

The antibacterial activity of *M. jalapa* and *A. bracteosa* crude methanol extracts was determined by a modified agar well diffusion method used by Walter *et al*. **[18]**. Four different extract concentrations (5-15 mg/ml) were used in this assay. The antibacterial activity of each extract was determined by measuring the diameter of zone of inhibition (ZOI) in millimeter (mm). The experiment was performed in triplicate.

Antipellicle assay

The antipellicle potential of *M. jalapa* and *A. bracteosa* was determined according to modified method of Joshua *et al*. **[19]**. Test tubes were labelled and prepared by pipeting 5 ml of Nutrient broth (NB) medium in each test tube respectively. Then 60 µl of tested bacterial inoculum and 100 µl of methanol extract of each plant were added to the respective tubes. The test tubes were then placed at room temperature for seven days without agitation. Positive (NB media + bacteria) and negative controls (NB media + plant extract) were used. The effect of plant extracts on pellicle was represented by (+ve and -ve) signs and was expressed as –ve for no biofilm, +ve for significant biofilm inhibition (completely disrupting pellicle layer), ++ve for good biofilm inhibition (partial disruption of pellicle layer), +++ve for moderate biofilm inhibition (moderate disruption), ++++ve weak biofilm inhibition (thin pellicle layer) and +++++ve no biofilm inhibition (thick pellicle layer).

Exopolysaccharide inhibition assay

The Exopolysaccharide inhibition assay was performed for crude extracts of both *M. jalapa* and *A. bracteosa* against three tested strains of *P. aeruginosa*. In this assay Congo red agar plates were prepared by adding 40 µg/ml Congo red dye, 10 g/l tryptone and 20 µg/ml coomassie brilliant blue. Then (2.5 µl) of inoculum of each strain in nutrient broth with crude methanolic extract (15 mg/ml) of both plants and with no extract (control) was transfer into the CR agar plates and were kept for 4 days at room temperature **[20]**. In the absence of plant extracts, P3 strain of *P. aeruginosa* formed a dark red color bacterial colonies as compared to P1 and P2 strains, which showed that this strain have a higher tendency to form biofilm due to increase production of polysaccharides. When extracts were used, a color change in the bacterial colonies was recorded and was consider as polysaccharides inhibition and antibiofilm effect.

Results and discussion

Numerous studies have been reported that numbers of plant extracts were used to screen out their antimicrobial potential **[21,**

22]. The advantages of biofilm formation are numerous for bacteria. Bacteria that involved in the formation of biofilms are protected from host, antibiotics and thus exhibited increased drug resistance [23, 25]. In this study the crude methanol extracts of *M. jalapa* and *A. bracteosa* were used to evaluate its antibacterial and antibiofilm potential against *P. aeruginosa*. It has been reported that using methanol for extraction of plants showed encouraging antimicrobial properties [17].

During antibacterial assay, the methanol extract of *M. jalapa* exhibited higher antibacterial activity as compared to *A. bracteosa* against *P. aeruginosa*. It showed maximum activity against P2 tested strain with zone of inhibition (ZOI) from 13.5 mm to 9.7 mm at different concentrations; against P1 strain ZOI varies from 13.2 mm to 9.7 mm and against P3 strain a lowest antibacterial effect was observed with ZOI from 12.8 mm to 9.2 mm respectively, as shown in Figure 2. In current findings, *M. jalapa* exhibited good antibacterial activity at higher concentrations (15-10 mg/ml) against *P. aeruginosa* and it was observed that it showed antibacterial strength in a concentration dependent way. Others researchers also reported a moderate antibacterial potential of *M. jalapa* seeds against different bacterial strains used and they found a low activity against Gram's negative strains as compared to Gram's positive strains [11, 26]. *A. bracteosa* also showed strong antibacterial potential against P2 strain with ZOI 13.4 mm to 9.8 mm, whereas against P1 and P3, 13.2 mm to 9.4 mm and 12.7 mm to 9.0 mm inhibition zone was observed, respectively as shown in Figure 3. At concentration 5 mg/ml, both plant extracts were inactive against all three strains tested. In antibacterial activity assay, P3 bacterial strain showed increased resistance to the plant extracts as compared to P1 and P2 strains moreover, it was found that *A. bracteosa* showed low activity as compared to *M. jalapa*. It has been documented that hexane extract of *A. bracteosa* showed no antibacterial activity [27]. The antibacterial potential of methanol and acetone extract of *A. bracteosa* essential oil from leaves has been studied and concluded that acetone extract was more active against Gram negative bacterial strains than methanol extract and vice versa [17]. This fluctuation in antibacterial potential may be due to the source of bacterial strains used. *A. bracteosa* also showed bacterial inhibition in a concentration dependent way. The antibacterial potential of *A. bracteosa* is due to the presence of biologically active alkaloids, flavonoids, tannins and phenolic compounds [17].

In antibiofilm assay, the effect of *M. jalapa* and *A. bracteosa* methanolic extracts was observed on inhibition of pellicle layer at different concentrations as shown in (Table 1 and 2). A moderate disruption (+++) of pellicle layer was recorded for both *M. jalapa and A. bracteosa* against all 3 *P. aeruginosa* strains tested at 15-12.5 mg/ml concentrations. Results revealed that with increase in concentration from 12.5 to 15 mg/ml there is no increase in antipellicle effect signifying 12.5 mg/ml to be the lowest effective dose for all the tested strains. It has been documented that when low concentration of antibiotics or other antimicrobial drugs is able to prevent initial adherence of bacteria to surfaces, the steps of mature biofilm formation would also be effected [16]. A weak antipellicle activity (++++) was observed for both plants against P1, P2 and P3 strains biofilm formation from concentration 10 mg/ml to 7.5 mg/ml, respectively. During antipellicle activity, the extracts inhibition of bacterial cell attachment confirmed that prevention of bacterial attachment to surface is easier than mature biofilm [17]. In the current findings,

a moderate to weak antibiofilm activity in pellicle inhibition assay was observed for *M. jalapa* and *A. bracteosa* at different concentrations. There is no data exist on the pellicle inhibition for *M. jalapa* and *A. bracteosa*. The effect of plant extracts on pellicle inhibition has been previously reported [28].

The antibiofilm potential of *M. jalapa* and *A. bracteosa* on exopolysaccharide production in Congo red assay was recorded at 15 mg/ml and a moderate antibiofilm effect was observed. The changes in color of the bacterial colonies due to extracts was recorded and compared to controls used against the tested strains as shown in Figure 4 (A, B and C). In CR assay, without plant extract, maximum polysaccharide production was observed in P3 strain forming dark red color colonies as compared to P1 and P2 strain (Figure 4 A). It was observed that the extracts clearly suppressed exopolysaccharide production, form light red color bacterial colonies and as a result inhibited biofilm formation. There is no data present on biofilm inhibition by CR assay for *A. bracteosa* and *M. jalapa*. However, the antibiofilm effects of plants extracts by CR assay have been previously reported [17]. In antibacterial and antibiofilm assays, an increase in resistance was found to extracts in biofilm mode [29]. It is well known that bacteria in biofilm form showed more resistant to antibacterial agents [30].

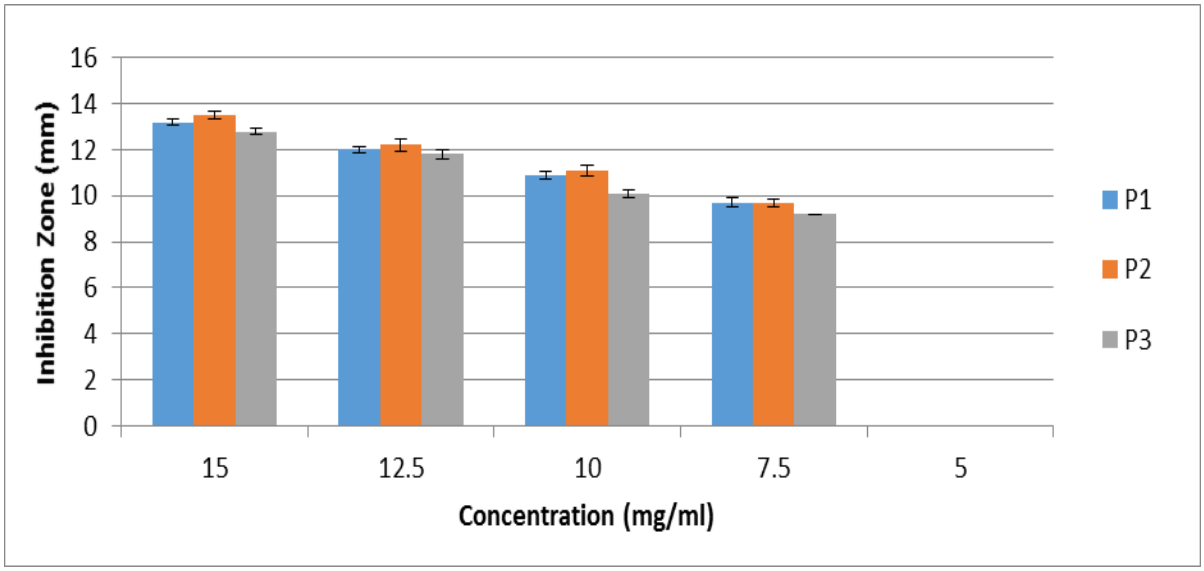


Figure 2. Inhibition zone (mm) against different strains of *P. aeruginosa* by the methanolic extract of *M. jalapa* after 24h incubation. Data represent as mean ±standard error. (n=3)

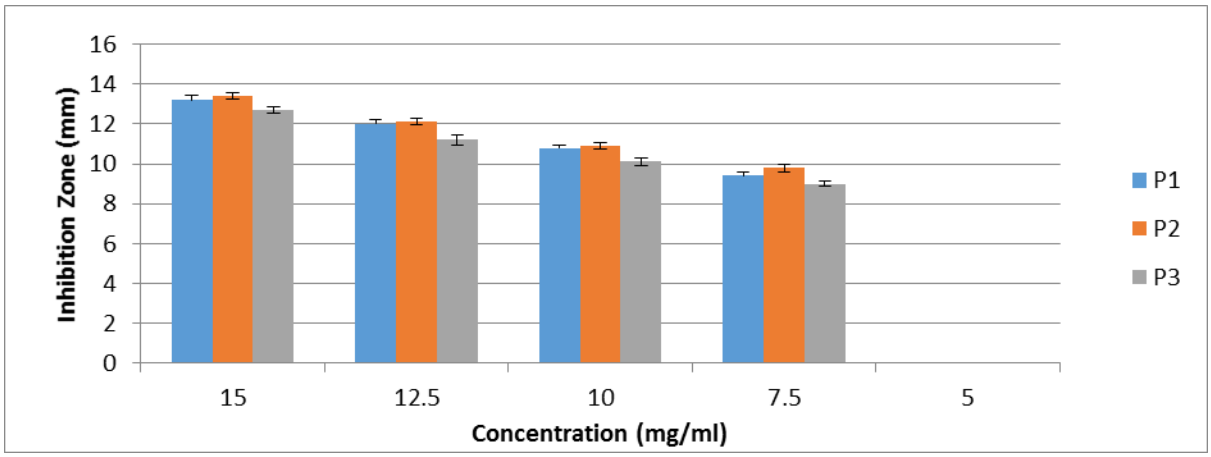


Figure 3. Inhibition zone (mm) against different strains of *P. aeruginosa* by the methanolic extract of *A. bracteosa* after 24h incubation. Data represent as mean standard error. (n=3)

Table 1. Antipellicle activity of *M. jalapa* methanol extract against different *P. aeruginosa* strains after 7 days of incubation

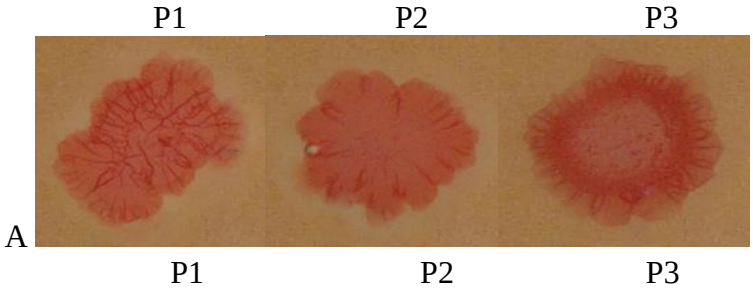
Concentration (mg/ml)	P1 strain	P2 strain	P3 strain
Positive control	+++++	+++++	+++++
15	+++	+++	+++
12.5	+++	+++	+++
10	++++	++++	++++
7.5	++++	++++	++++

+++++= extract free, +++, ++++ = moderate and weak disruption of pellicle layer

Table 2. Antipellicle activity of *A. bracteosa* methanol extract against different *P. aeruginosa* strains after 7 days of incubation

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

+++++= extract free, +++, ++++ = moderate and weak disruption of pellicle layer



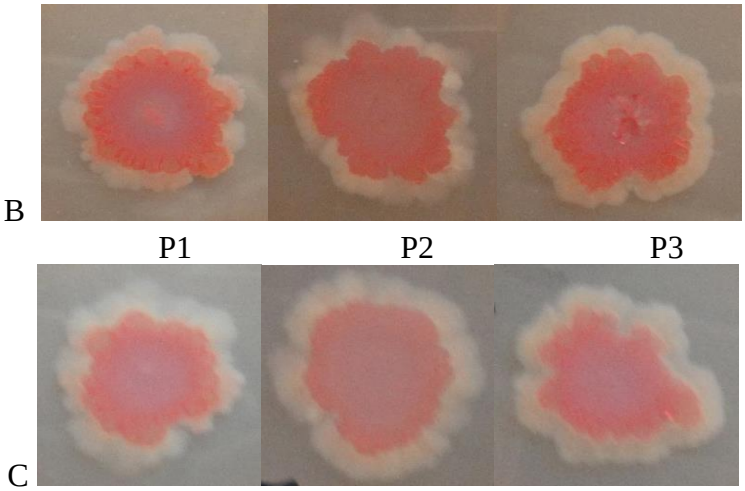


Figure 4. Effect of extracts (15 mg/ml) on exopolysaccharide inhibition (A) bacterial strains without plant extract (B) bacterial strains with *M. jalapa* methanol extract (C) bacterial strains with *A. bracteosa* methanol extract

Conclusion

From the results it is concluded that the methanolic extracts of *M. jalapa* and *A. bracteosa* leaves exhibited antibacterial and antibiofilm potential against clinical strains of *P. aeruginosa*. Further study is needed to isolate antibacterial compounds in order to use it for control strategies against *P. aeruginosa* biofilm formation in bacterial infections.

Authors' contributions

Conceived and designed the experiments: I Khan, Khan U & Khan W, Performed the Experiments: I Khan, Analyzed the Data: I Khan, MA Nawaz, M Subhan & S Pervez, Contributed reagents/ materials/ analysis tools: I khan, AK Jan & S Ahmad, Wrote the paper: I Khan, K Khan, MA Nawaz.

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