

INHIBITION OF PYOCYANIN PRODUCTION IN *PSEUDOMONAS AERUGINOSA* BY NATURAL ANTIMICROBIAL COMPOUNDS FROM HERBAL EXTRACTSPREETI JAIN¹, SUMAN KUMARI¹, ASHISH MALIK^{2*}¹Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India. ²Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India. Email: Ashishmalik31@gmail.com

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ABSTRACT

Objective: *Pseudomonas aeruginosa* produces a range of metabolites including pyocyanin that enhance its ability to resist antibiotics and becomes capable of surviving adverse conditions.

Methods: In this report, eight plants (extracted with five solvents) were screened for antibacterial activity against *P. aeruginosa* by microbroth dilution method. Afterward tested for inhibition of pyocyanin in presence and absence of plant extracts.

Result: Among these *Digera muricata* and *Solanum quitoense* exhibited good antibacterial potential. Inhibition of pyocyanin is identified as a potential anti-virulence strategy. Therefore, acetone extract of six plants exhibited minimum inhibitory concentration (MIC) ≤ 3.125 mg/ml and methanol extract of three plants exhibited MIC ≤ 6.25 mg/ml was used to check pyocyanin inhibition in *P. aeruginosa*. In acetone extracts, significant pyocyanin inhibition was found in *Ipomoea pesticides* and *Citrullus colocynthis*. In methanol extracts, *C. colocynthis* and *D. muricata* showed considerable pyocyanin inhibition.

Conclusion: Overall result indicates that the best antimicrobial compound (growth inhibitor) may not be best inhibitor of pyocyanin biosynthesis or vice-versa. Moreover, *I. pesticides*, *C. colocynthis*, and *D. muricata* seem to contain compounds which inhibit the growth of bacteria as well as the biosynthesis of pyocyanin.

Keywords: *Pseudomonas aeruginosa*, Pyocyanin, Antibacterial activity, Chloroform extraction assay, Herbal extract.

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous bacterium involved in numerous pathogenic infections in immunocompromised individuals [1]. *P. aeruginosa* infection is difficult to eradicate, due to antibiotic resistance developed with the increased and indiscriminate use of antibiotics [2]. Virulence factors present in *P. aeruginosa* damage the host tissues and increase the survival ability of this bacterium [3]. Therefore, inhibition of virulence factors has been identified as a potential new therapeutic approach to treat *P. aeruginosa* infections. Inhibition of virulence factors attenuates the pathogenicity of the bacterium, and the host immune system can clear the infection. Pyocyanin is one of the many virulence factors produced by *P. aeruginosa*. Pyocyanin pigment is a toxin [4] and its biosynthesis is regulated by quorum sensing system of bacteria [5]. Therefore, inhibition of pyocyanin is identified as a potential anti-virulence strategy and interest has been developed in the discovery of compounds which inhibit pyocyanin biosynthesis.

Health-care demands new antibacterial agents that are more potent [6]. Herbal medicines have been increasingly recognized as an alternative form of health care, therefore, the screening of medicinal plants for anti-virulence factors compounds is becoming important. Therefore, in this study, *Leucas aspera*, *Ipomoea pesticides*, *Citrullus colocynthis*, *Digera muricata*, *Gomphrena celosioides*, *Helianthus annuus*, *Solanum quitoense*, and *Alternanthera pungens* plants were collected (Table 1) and have been screened against *P. aeruginosa* for their antimicrobial activity and pyocyanin biosynthesis inhibition capability.

METHODS

Chemicals and apparatus

Chloroform, acetone, sterile distilled water, dimethyl sulfoxide (Hi-media), hydrochloride (HCL), ampicillin, nutrient broth, ethanol,

resazurin dye, autoclave (Hicon), laminar flow (Metrex), incubator shaker (Remi), Halo DB 20 spectrophotometer (Dynamica), sphinx vortex shaker (Tarsons), water bath (Hicon), centrifuge (Remi), and 96-well plates.

Microorganism

Lyophilized culture of *P. aeruginosa* was obtained from National Dairy Research Institute, Karnal in September 2013. Cultures were revived in nutrient broth and used for antimicrobial microbroth dilution assay and pyocyanin production assay under the influence of antimicrobial extracts.

Plant material

Eight medicinal plants were collected from the local area and their natural habitat Rohtak, Haryana, India during September 2012 and February 2013 (Table 1). The *L. aspera* (leaves), *I. pesticides* (leaves), *C. colocynthis* (fruit and leaves), *D. muricata* (leaves), *G. celosioides* (whole), *Helianthus annuus* (whole), *S. quitoense* (leaves), and *A. pungens* (whole) were collected and identified from Department of Botany, M. D. University, Rohtak (India). Plant identifications were reconfirmed from the flora of Haryana.

Preparation of extracts

Plant materials were washed with distilled water three times and air dried by keeping in the shade for 3 weeks. The properly dried plant materials were crushed and ground to fine powder. For each plant/plant part, 100 g of material was extracted consecutively for three times for 72 hrs with five different solvents (100 ml each) in ascending order of polarity, i.e., hexane, chloroform, acetone, methanol, and water. The combined extracts were filtered, and solvents were evaporated to dryness at room temperature to yield a crude extracts. The extracts were stored at -20°C till further use [7].

Table 1: List of plants used and their ayurvedic uses

Plant name	Voucher specimen no	Family name	Part used	Ayurvedic/traditional use and reference
<i>D. muricata</i> (Lesua)	125/2012	Amaranthaceae	Leaves	Used for treatment of kidney stone and urinary tract disorder [6]
<i>A. pungens</i> (Khaki)	126/2012	Amaranthaceae	Whole plant	Diuretic properties, gonorrhoea [8]
<i>G. celosioides</i> (prostrate globe - amaranth)	127/2012	Amaranthaceae	Whole plant	Liver disease [9]
<i>S. quitoense</i> (Naranjilla)	128/2012	Solanaceae	Fruits	To make beverages, also have nutritional value [10]
<i>I. pesticides</i> (Panchpatia)	156/2013	Convolvulaceae	Leaves	Treatment of skin disorder
<i>C. colocynthis</i> (Bitter cucumber)	157/2013	Cucurbitaceae	Fruit and whole plant	As most violent purgative drug, as energy source and as oilseeds [11]
<i>H. annuus</i> (Sunflower)	158/2013	Asteraceae	Seeds, leaves, roots	Antioxidant, anti-inflammatory and diuretic properties [12]
<i>L. aspera</i> (Goma madhupati)	159/2013	Labiatae	Flower and root	External application mostly for skin snake bite and wounds [13]

D. muricata: *Digera muricata*, *A. pungens*: *Alternanthera pungens*, *G. celosioides*: *Gomphrena celosioides*, *S. quitoense*: *Solanum quitoense*, *I. pesticides*: *Ipomoea pesticides*, *C. colocynthis*: *Citrullus colocynthis*, *H. annuus*: *Helianthus annuus*, *L. aspera*: *Leucas aspera*

Preparation of inoculum

Using aseptic techniques, bacterial culture was transferred in 100 ml nutrient broth and incubated overnight at 37°C. After 15-18 hrs of incubation, the culture was centrifuged at 4,000 rpm for 5 minutes, the supernatant was discarded, and a clean sample of bacteria was prepared. The pellet was re-suspended using 20 ml of double distilled water and centrifuged again at 4,000 rpm for 5 minutes. This step was repeated until the supernatant becomes clear. The optical density of that bacterial suspension was measured spectrophotometrically at 600 nm, and serial dilutions were made till the optical density becomes 0.6. The actual numbers of colony forming units were determined from the viability graph. The required dilution factor was calculated and the dilution made to obtain a concentration of 10⁶ cells/ml.

In vitro antibacterial screening

Antibacterial activities of the extracts were determined by the microbroth dilution assay in 96-well culture plates [14]. Stock solution of 50 mg/ml concentration was prepared in dimethyl sulfoxide (DMSO) or sterile water (the hexane/petroleum ether, chloroform plant extracts are dissolved in 10% [v/v] DMSO, acetone and methanol extracts of plants are dissolved in 5% [v/v] DMSO). Autoclaved nutrient broth (100 µl) was added to the wells of the culture plates, and the first row of microtiter plate was filled with 100 µl of the test material. Two-fold serial dilutions of test extracts were made. 20 µl of 2 X resazurin solutions was added as an indicator in each well. Finally, a volume of 10 µl was taken from bacterial suspension and then added to each well to achieve a final concentration of 5×10⁶ CFU/ml. To avoid the dehydration of bacterial culture, each plate was wrapped loosely with cling film. The experiment was run in duplicate. Proper controls were kept for each experiment. The plates were incubated at 37°C and examined for change in color after 18 hrs for the growth of test bacteria. The extract was considered to be active if the wells appear clear without any visible growth of bacteria and the result is expressed as minimum inhibitory concentration (MIC).

Pyocyanin inhibition assay

Pyocyanin quantification was done by chloroform extraction method [15]. Cultures were treated with plant extracts having maximum MIC grown in triplicates and pyocyanin assay was also run in triplicate. *P. aeruginosa* was incubated with plant extracts for different time periods (8 hrs, 10 hrs, and 12 hrs). Three sets of 18 culture tubes were prepared having 4.5 ml sterilized nutrient broth in each. To these tubes, 0.5 ml plant extract (concentration having half of MIC) was added. Three sets of control culture tubes were also prepared having 5 ml broth in each tube. To each tube, 20 µl of bacterial inoculum was added. After incubation for indicated time periods, cultures were centrifuged at 10000 rpm for 10 minutes, and the supernatant was

collected. To 5 ml supernatant 3 ml of chloroform was added and vortexed for 20 seconds. The samples were centrifuged for 10 minutes at 5000 rpm, and 3 ml of the resulting blue layer formed at the bottom (chloroform + pyocyanin) is transferred to a new tube. 2 ml of 0.2 M HCl was added to each tube and vortexed for 20 seconds. The sample was again centrifuged for 2 minutes at 5000 rpm, and 1 ml of the pink layer is transferred to the cuvettes. HCl (0.2 M) is used as a blank. Spectrophotometric measurements were done at 520 nm. Pyocyanin concentration (µg/ml) is calculated by multiplying the OD value at 520 nm with 17.072.

RESULTS AND DISCUSSION

Yield % of plant extracts is given in Table 2 the text.

Antibacterial activity

About 40 extracts of eight plants were screened for their antimicrobial potential against *P. aeruginosa*, and six plants were found to exhibit considerable MIC. MIC of all the extracts is given in Table 3.

Ampicillin and Streptomycin were used as positive control shows MIC 125 and 250 µg/ml, respectively. *L. aspera* has been reported to treat skin infections, snake bite, and wound healing was also found to be inactive against *P. aeruginosa*. The *H. annuus* was also found to be inactive against *P. aeruginosa*. *Ipomoea pesticides* showed week MIC in the range 6.25-12.5 mg/ml in accordance with its use in traditional medicine. Interestingly, *D. muricata* and *Solanum quitoense* were never reported for antimicrobial activity in traditional medicine or elsewhere exhibited strong antimicrobial activity. Hexane extracts of the plants exhibited the highest potential while chloroform and acetone extracts have considerable MIC. Aqueous extracts were not found to have antimicrobial activity. The acetone and methanol extracts of the plants exhibited MIC in the range of 1.56-6.25 mg/ml were further used for pyocyanin inhibition assay.

Pyocyanin inhibition

Recent reports showed that pyocyanin provides strong support to increase the virulence of *P. aeruginosa*. Various *in vitro* studies demonstrated that pyocyanin interferes with multiple cellular functions. Therefore, devising strategies to inhibit pyocyanin biosynthesis and neutralize its toxicity may be helpful in the treatment of cystic fibrosis and other infections caused by *P. aeruginosa*. In this study, pyocyanin was quantified in control and cultures of *P. aeruginosa* treated with sub-MIC of antimicrobial extracts. Pyocyanin concentrations found in control and acetone and methanol extracts treated cultures (at different time period) are given in Table 4.

Table 2: Yield % of plant extracts

Plant extract	Hexane yield %	Chloroform yield %	Acetone yield %	Methanol yield %	Water yield %
<i>D. muricata</i>	0.072	1.67	1.68	7.42	18.01
<i>A. pungens</i>	0.17	0.42	0.56	3.44	1.750
<i>G. celosioides</i>	0.2	1.10	0.61	4.11	4.34
<i>S. quitoense</i>	0.2	0.315	2.33	1.027	1.236
<i>I. pesticides</i>	0.17	1.95	0.6	2.94	3.5
<i>C. colocynthis</i>	1.55	3.124	3.34	13.78	6.45
<i>H. annuus</i>	0.2	1.82	2.15	6.29	11.81
<i>L. aspera</i>	0.48	4.01	1.72	4.71	3.25

D. muricata: *Digera muricata*, *A. pungens*: *Alternanthera pungens*, *G. celosioides*: *Gomphrena celosioides*, *S. quitoense*: *Solanum quitoense*, *I. pesticides*: *Ipomoea pesticides*, *C. colocynthis*: *Citrullus colocynthis*, *H. annuus*: *Helianthus annuus*, *L. aspera*: *Leucas aspera*

Table 3: MIC (mg/ml) of plant extracts of against *P. aeruginosa* (-) represents the absence of activity in the respective plants

Plant extract	Hexane (mg/ml)	Chloroform (mg/ml)	Acetone (mg/ml)	Methanol (mg/ml)	Water (mg/ml)
<i>D. muricata</i>	0.781	1.56	1.56	6.25	12.5
<i>A. pungens</i>	3.125	3.125	3.125	6.25	-
<i>G. celosioides</i>	3.125	3.125	6.25	12.5	-
<i>S. quitoense</i>	1.56	1.56	1.56	12.5	-
<i>I. pesticides</i>	6.25	12.5	6.25	-	-
<i>C. colocynthis</i>	3.125	3.125	3.125	6.25	-
<i>H. annuus</i>	12.5	12.5	12.5	12.5	-
<i>L. aspera</i>	12.5	12.5	12.5	12.5	-

P. aeruginosa: *Pseudomonas aeruginosa*, *D. muricata*: *Digera muricata*, *A. pungens*: *Alternanthera pungens*, *G. celosioides*: *Gomphrena celosioides*, *S. quitoense*: *Solanum quitoense*, *I. pesticides*: *Ipomoea pesticides*, *C. colocynthis*: *Citrullus colocynthis*, *H. annuus*: *Helianthus annuus*, *L. aspera*: *Leucas aspera*, MIC: Minimum inhibitory concentration

Pyocyanin production was found to be inhibited in treated cultures with *I. pesticides*, *D. muricata*, and *G. celosioides* acetone extracts as compared to control cultures after 8 hrs. Whereas, *A. pungens*, *S. quitoense*, and *C. colocynthis* inhibited pyocyanin production after 10 hrs surprisingly, *G. celosioides* and *I. pesticides* extracts which initially did not exhibit any effect, found to be most effective to block pyocyanin biosynthesis to 0.00 and 0.0239 $\mu\text{L/ml}$ after 10 hr incubation. *D. muricata* which exhibited best MIC among the plants studied did not block pyocyanin synthesis as compared to other plants.

Methanol extracts of *A. pungens*, *D. muricata*, and *C. colocynthis* exhibited better MIC, therefore, pyocyanin was estimated in cultures treated with Sub-MIC of these extracts. Methanol extract of *C. colocynthis* exhibited better inhibition at 8 hrs. Cultures treated with these extracts found to have significantly depleted levels of pyocyanin after 12 hrs incubation (Table 4). Comparison of all these activities is shown in graph (Fig. 1).

Overall, results indicate that blockage of pyocyanin synthesis and bacterial growth inhibition is the separate processes. The best antimicrobial compound may not be best the inhibitor of pyocyanin biosynthesis or *vice-versa*. Moreover, *I. pesticides*, *C. colocynthis*, and *D. muricata* seem to contain compounds which inhibit the growth of bacteria as well as the biosynthesis of pyocyanin. Extracts found active can be further explored to search active molecules which can be screened for combinatorial therapies with antibiotics to incorporate into existing regimens for the treatment against *P. aeruginosa*.

CONCLUSION

This report is the first comprehensive study of herbal inhibition of pyocyanin in *P. aeruginosa*. Interestingly, inhibition of pyocyanin synthesis and bacterial growth is the separate processes. The best antimicrobial compound may not be best inhibitor of pyocyanin biosynthesis or *vice-versa*. Among all acetone extracts of selected plants, *D. muricata* and *S. quitoense* exhibited significant antibacterial activity but does not showed best pyocyanin inhibition. This study gives a preliminary idea for extraction of the new active compound and use of these plants in combination with antibiotics to combat antibiotics resistance against *P. aeruginosa*.

Table 4: Pyocyanin quantification in *P. aeruginosa* culture treated with acetone and methanol plant extracts by chloroform extraction method

Plant extract	8 hrs ($\mu\text{L/ml}$)	10 hrs ($\mu\text{L/ml}$)	12 hrs ($\mu\text{L/ml}$)
Control	1.46819 \pm 0.0744	1.56493 \pm 0.0355	1.6446 \pm 0.0355
Acetone plant extract			
<i>D. muricata</i>	1.81646 \pm 0.0418	0.41144 \pm 0.0235	0.16901 \pm 0.0137
<i>A. pungens</i>	0.34485 \pm 0.0215	0.21511 \pm 0.0175	0.12633 \pm 0.0142
<i>G. celosioides</i>	3.05589 \pm 0.1456	0.0239 \pm 0.0085	0.00171 \pm 0.0045
<i>S. quitoense</i>	0.25437 \pm 0.0195	1.38283 \pm 0.0422	0.01878 \pm 0.0096
<i>I. pesticides</i>	1.70379 \pm 0.0376	0.00154 \pm 0.0032	0.0000
<i>C. colocynthis</i>	0.7597 \pm 0.0083	0.0939 \pm 0.0121	0.0239 \pm 0.0085
Methanol plant extracts			
<i>D. muricata</i>	1.53989 \pm 0.0365	0.12975 \pm 0.0142	0.00171 \pm 0.0045
<i>C. colocynthis</i>	0.15536 \pm 0.0175	0.01195 \pm 0.0096	0.00000
<i>A. pungens</i>	1.3429 \pm 0.0232	0.2388 \pm 0.0871	0.00212 \pm 0.0085

D. muricata: *Digera muricata*, *A. pungens*: *Alternanthera pungens*, *G. celosioides*: *Gomphrena celosioides*, *S. quitoense*: *Solanum quitoense*, *I. pesticides*: *Ipomoea pesticides*, *C. colocynthis*: *Citrullus colocynthis*, *H. annuus*: *Helianthus annuus*, *L. aspera*: *Leucas aspera*

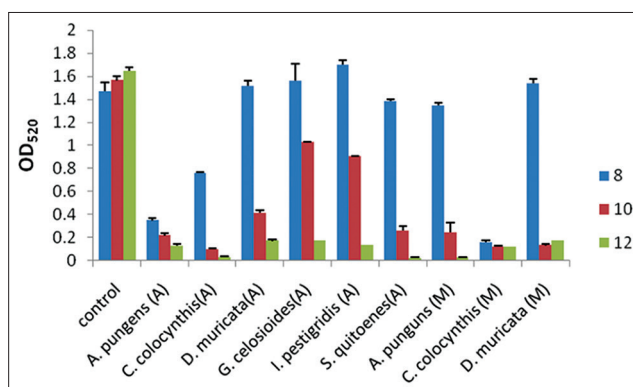


Fig. 1: Comparison of pyocyanin inhibition activities of different plants

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