

Evaluating the anti- *Pseudomonas Aeruginosa* Efficacy and Potential Cytotoxicity of *Nerium Oleander* Alcoholic Extract

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Abstract

Nerium oleander known as oleander has belonged to the poisonous plants its habitat in a tropical and subtropical region. The chemical analysis with GC-Mass of the alcoholic extract of oleander leaves revealed that this plant has many chemical compounds more than 80 compounds and high-peaks about 29 compounds which are represented by alkaloids, phenol, terpenes, and fatty acid. HPLC analysis showed many essential oils that have many biological effects.

To evaluate the antibacterial activity of the alcoholic extract of *N. oleander* against locally isolated *Pseudomonas aeruginosa* the broth micro-dilution method was adapted to different concentrations were started from 3.9 to 1000 mg/ml. The results revealed that the alcoholic extract has antibacterial activity depending on the concentration and bacterial strain.

When treating the onion root cells as a model of the study to find the cellular toxic effect the results showed that treatment with an alcoholic extract of oleander leaves with concentrations (control, 150, 300, 600, and 1200 mg/ml) for four hrs., the mitotic index (MI) was decreased to less than 50%, when treated with high concentrations 600, 1200 mg/ml and many cases of chromosomal aberration were shown, such as the abnormal shape of the cells with a percentage about 1 and 2.8 % at the concentrations 600 and 1200 mg/ml respectively, as well as the multinucleated cells recorded the highest percentage at the concentrations 1200 mg/ml with 14.4%, and nuclear lesion (chromatin degradation) were seen in a large percentage at the interphase were recorded 13.66, 25.42, and 32.3% at the concentrations 300, 600, and 1200 mg/ml respectively.

Keywords- antibacterial activity, mitotic index, chromosomal aberration, oleander.

Introduction

The overuse of antibiotics has become an essential factor for increasing the emergence of multi-drug resistance (MDR) bacterial isolates, which become a hazard to public health. Besides, the accessible antimicrobial agents, occasionally, do not affect on these bacteria⁽¹⁾. There are many approaches to remedy and control the infection caused by MDR strains of bacteria, with emphasis on the use of active phytochemical compounds from the herbal plant which serve as a defense mechanism against these bacteria^(2,3). The burn wound is a susceptible site of opportunistic colonization by organisms of endogenous and exogenous origin such as *P. aeruginosa*. The traditional association of *P. aeruginosa* with burn infections is very strong;

burn infections and associated sepsis are the cause of the high rate of fatalities in seriously burned patients.⁽⁴⁾ However, *P. aeruginosa* outbreaks in burn units are still associated with high death rates around 60%⁽⁵⁾. According to World Health Organization assessments that 80% of the people in developing countries depend on traditional therapies and use extracts of the plant with an extensive variety of secondary compounds as the main medicinal source to treat numerous infectious diseases, especially these compounds have lower side effects compared to antibiotics^(6,7). One of these plants *N. oleander* known as oleander (Apocynaceae), one such herb, has been introduced to Iraq as an ornamental plant growing originally in the Mediterranean region, subtropical Asia, and distributed in many parts of the world⁽⁸⁾. *Nerium* is an important medicinal plant

in folk medicine⁽⁹⁾. This plant has pharmacological applications due to produces of secondary metabolites such as alkaloids, flavonoids, and steroids⁽¹⁰⁾. Also, have potentially toxic effects that belong to all parts of oleander are toxic containing oleandrin and other cardiac glycosides⁽¹¹⁾. *N. oleander* extracts have significant antibacterial effects⁽¹²⁾, cytotoxic effect.⁽¹³⁾, cardiotoxic and diuretic in edema^(14,15)

Materials and Methods

Microorganisms:

Twenty isolates of *P. aeruginosa* were isolated from burn swabs, obtained from Microbiology laboratory at Department of Biology, College of Science, University of Baghdad These isolates were previously identified by subculturing on to 5% blood agar, McConkey and ceramide agar, and the important biochemical tests such as gram stain, oxidase test, catalase test, pigment production and growth at 42°C.⁽¹⁶⁾ The PCR technique was adopted to confirm the identification via amplification of a fragment of *16SrRNA* gene as described by⁽¹⁷⁾. These isolates were stored in glycerol vials at deep freeze by lab staff.

Bacterial suspension preparation

Few separate pure colonies, grown on nutrient agar, were taken to a sterile normal saline tube with turbidity adjusted to approximately 1.5×10^8 CFU/ml in comparison to McFarland turbidity standard (tube no. 0.5).

Antibiotics susceptibility test

P. aeruginosa isolates were tested against the following antibiotics: ceftazidime (30µg/disk), Imipenem (10µg/disk), Gentamicin (10µg/disk), Amikacin (30µg/disk), and Ciprofloxacin (5 µg/disk) to detect the multidrug-resistant isolates, this test was done by disk diffusion method⁽¹⁸⁾

Antibacterial assay

Micro-dilution method

To determine the minimal inhibitory concentration (MIC) of alcoholic extract of *N. oleander* against *P.*

aeruginosa isolates, the broth microdilution protocol mentioned by^(19,20) was followed, the double serial dilutions of alcoholic extract were started from 3.9 to 1000 mg/ml which prepared from a stock solution (5000 mg/ml) using Mueller Hinton broth as a diluent. Further, one hundred microliters of each dilution were distributed in 96 well cell culture plates. Control wells contained bacteria-free growth media plus DMSO (sterility control). All wells were inoculated with 10 µl of a bacterial suspension. All trials were repeated in triplicate. Subsequently, the microtiter plates were incubated, aerobically, at 37°C for 24 hrs.

Plant collection

This work was carried out in the Laboratory of Biology department, College of Science, University of Baghdad during the period 1/12/2019 - 1/5/2020. The leaves of the *N. oleander* were cut into small pieces and then placed in the oven for drying at 40-50°C for 5 days, and then placed in the mill until it became a powder.^(21,22,23)

Phytochemical analysis

1-Gas chromatography-mass spectroscopy (GC-MS) analysis

GC-MS analysis was carried out on a GC – mass 5977A Series Agilent system autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions^(24,25,26).

Results and Discussion

Susceptibility of bacterial to antibiotics

In this study 20 isolates of *P. aeruginosa* were submitted to antibiotic susceptibility test by disk diffusion test, this technique was done by using five different antibiotics: ceftazidime, Imipenem, Gentamicin, Amikacin, and Ciprofloxacin to detected the multidrug-resistant isolates (MDR), the results revealed 12 (60%) isolates were resistant to all these antibiotics and this reflected as MDR depending on breakpoints of CLSI⁽²⁷⁾. (Table 1).

Table1. Susceptibility of bacteria to antibiotics Resistance profile of *P. aeruginosa* against five antibiotics S-Susceptible, I-Intermediate, R-Resistant.

Isolates code	Ceftazidime 30µg/disk	Imipenem 10µg/disk	Gentamicin 10 µg/disk	Amikacin 30µg/disk	Ciprofloxacin 5 µg/disk
Ps1	R	R	R	R	R
Ps2	S	S	S	S	R
Ps3	S	S	S	S	I
Ps4	S	S	S	S	S
Ps5	S	S	S	S	S
Ps6	R	R	R	R	R
Ps7	R	R	I	R	R
Ps8	S	S	S	S	S
Ps9	S	R	R	S	R
Ps10	S	S	S	S	S
Ps11	R	R	R	R	R
Ps12	R	R	R	R	R
Ps13	R	R	R	R	R
Ps14	R	R	R	R	R
Ps15	R	R	R	R	R
Ps16	R	R	R	R	R
Ps17	R	R	R	R	R
Ps18	R	R	R	R	R
Ps19	R	R	R	R	R
Ps20	R	R	R	R	R

Antibacterial activity of *N. oleander* extract against *P. aeruginosa*

Our results revealed that the extract of *N. oleander* has antibacterial activity at different concentrations. However, non-MDR *P. aeruginosa* isolates (ps2, ps3, ps4, ps5, ps7, ps8, ps9, ps10) were sensitive to (62.5 mg/ml) .whilst, all MDR *P. aeruginosa* except four isolates were inhibited at MIC 500 mg/ml, and the MIC value for the remaining four isolates (ps1, ps6, ps15, ps18) was 250 mg/ml. According to GC-MS and HPLC analysis

of *N. oleander* ethanol extracts shown that the existence of secondary metabolites such as alkaloids, cardiac glycosides, phenolic compounds, flavonoids, tannins, essential oil, terpenes, and saponins. These compounds are considered as the main groups of antimicrobial compounds in plants^(28,29). The alcoholic extract of *N. oleander* leaves showed the highest antimicrobial action against *P. aeruginosa* at 900 mg/ml while the lowest level of the extract was 500 mg/ml⁽³⁰⁾. Also according to⁽³¹⁾. results showed the aqueous and ethanol extracts

of *N. oleander* have antimicrobial activity against all bacteria isolated from burns infection, The minimum inhibitory concentration of the aqueous extracts ranged between 25- 100 mg/ml, while the ethanol extract ranged between 25 -50 mg/ml on the test organisms. The results reported by⁽³²⁾. found that ethanol extract of *N. oleander* had a deterrent effect in all concentrations on *Salmonella Typhi*. The MIC of the ethanol extract was 128 mg/ml and the minimum bactericidal concentration (MBC) was 256 mg/ml. but aquatic and alcoholic extracts were not effective on the standard strain *Listeria monocytogenes*.

Phytochemical detection

1- GC-MASS analysis

The GC-MS chromatogram of the 83 peaks of the compounds was detected. Chromatogram GC-MS analysis of the alcoholic extract of *N. oleander* showed

the presence of 29 major peaks and the components corresponding to the peaks were determined as Dodecanoic acid lauric acid, eicosene, alkaloid, E-14-Hexadecenal, Heptadecene, Octadecene 2, Tetradecanoic acid, Olic acid, Bicyclic 3.1.1 heptane, Cycloheptan,1,2-dimethyl- acetate, Trichloromethan Eugenol, Phenol, glyceride, ctadecyne, phytol, acetate, Hexadecanoic acid, methyl ester, methyl ester, palmitic acid, 9-Octadecenoic acid (9Z)-, Stearic acid, methyl ester, and Methyl stearate. These results are in along with⁽³³⁾ they found that chemical compositions of the leave ethanol extract of *N. oleander* were investigated using gas chromatography-mass spectroscopy (GC-MS).

2- HPLC analysis revealed that leaves extract of *N. oleander* has different types of essential oils such as α -picene, caryophyllene, chamazulene, and p-cymene (Table 2).

Table 2. HPLC analysis of leaves extract of *N. oleander*

	Compounds	Ret. Time of Test (min)	Area of Test	Ret. Time of standard (min)	Area of Standard	conc. Of test
1	α -picene	3.615	7294017.5	3.232	1639319.625	22.24708772
2	caryophyllene	4.473	9149397	4.273	5078418.5	9.008116405
3	chamazulene	4.998	4903959	4.932	2144605	11.43324528
4	p-cymene	6.265	22130402	6.498	12517030	8.840117025

Cytotoxic effects of oleander alcoholic extracts on the cell division of onion root tips *Allium cepa in vivo*:

Mitotic index (MI)

Mitotic index reduced significantly at all oleander leaves extract concentrations, recording 7, 6, 4.4, 2.8, and 2.7% for control, 150, 300,600,1200 mg/ml) respectively Figures 3. The mitotic index decreased less than 50% in (600 and 1200 mg/ml) recoding (38.35, 36.98%) respectively also as shown in figure 1 and table 2. The

decrease in a mitotic index is due to the extracts have some substances that cause a reduction in the mitotic index to 50% or less have toxic or semi lethal effects such as Phenol, octadecadienoic acid, Oleic acid glyceride, ctadecyne, phytol, acetate, Hexadecanoic acid, methyl ester, Palmitic acid, methyl ester, n-hexadecanoic acid that interaction with DNA topoisomerase-I⁽³⁴⁾

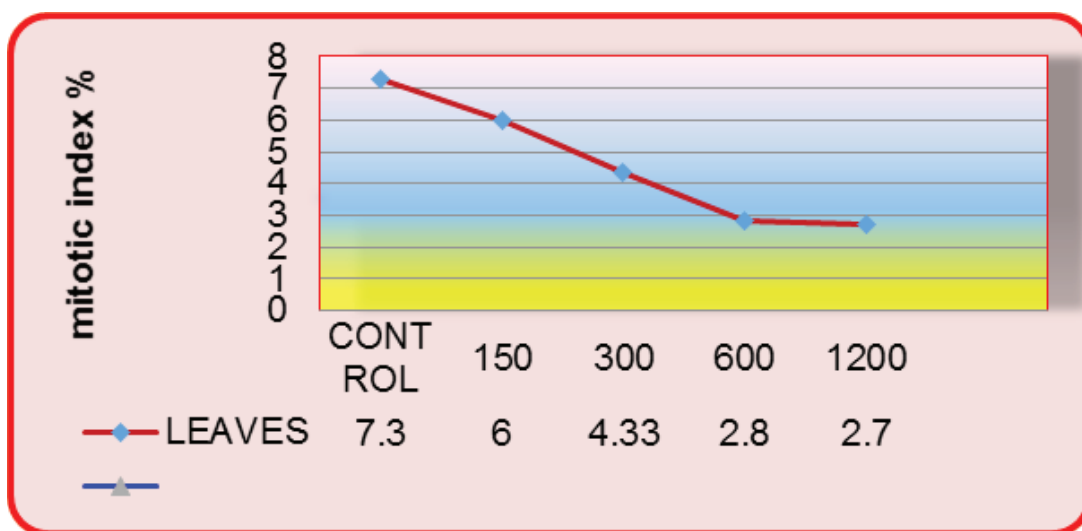


Figure 1. Mitotic index%

Prophase

Prophase index recording (40.62, 35.49, 42.3, 49.01 and 44.64%) in (control, 150, 300, 600, 1200 mg/ml) respectively as shown in table 1 and table 2. Some abnormal cells attributed in this phase such as abnormal cell shape as shown in Figure 2.

Metaphase

Metaphase index recording 25.16, 30.84, 31.93, 24.85, 18.29% in control, 150, 300, 600, 1200 mg/ml respectively, (table 3).

Anaphase and Telophase

Anaphase was decreased significantly recording 82.68, 75.12, 85.07, and 47.96% in 150, 300, 600, 1200 mg/ml respectively (table 3 and 4).

Using of onion test to evaluate the cytotoxic effects of the alcoholic extract of oleander leaves under study

revealed the presence of several cytological aberrations in cells were multinucleated cells, abnormal cell shape, and nuclear lesion (chromatin lesion) compared with control as shown in figures 3-A, B, C, D. This test considered as the important test *in vivo* where the roots grow in direct contact with the substance of interest enabling possible damage to DNA of humans to be predicted. The results showed that higher concentrations were able to inhibit cell division significantly as shown in Figure 2, indicating the toxicity of the tested concentration in *Allium* root tip cells⁽³⁵⁾. Single and multiple lesions were the most frequent aberrations in prophase and interphase cells. Lesions are the result of inhibition in the S phase of the cell cycle where duplication of DNA strands happens. Formation of prophase lesions are common in irradiated cells, but the site of lesions or its repair mechanisms are not yet explored in detail⁽³⁶⁾.

Table 3. Mitotic index, phases, and percentage of abnormality in *A. cepa* roots at different concentrations of alcoholic extract of oleander leaf after four hours.

Concentration mg/ml	MI%	Phase index %				Chromosomal aberration %		
		Prophase	Metaphase	Anaphase	Telophase	Nuclear-lesion	Multi-nucleate	Abnormal cell shape
Control	7.3	40.62	25.16	10.05	22.42	0.00	0.00	0.00
150	6	35.94	30.84	8.31	24.88	0.00	0.00	0.00
300	4.43	42.3	31.93	7.55	18.15	13.66	0.00	0.00
600	2.8	49.01	25.85	8.55	19.15	25.42	3	1
1200	2.7	44.64	18.29	4.82	32.24	30.30	14.4	2.8

Table 4. Mitotic division (%control) and the phases in the onion root tips were treated with different concentrations of alcoholic extract of oleander leaves.

Concentration mg/ml	MI%	Prophase%	Metaphase%	Anaphase%	Telophase%
150	82.19	88.47	122.57	82.68	110.97
300	60.68	104.13	126.90	75.12	80.95
600	38.35	120.65	102.74	85.07	85.41
1200	36.98	109.89	72.69	47.96	143.80

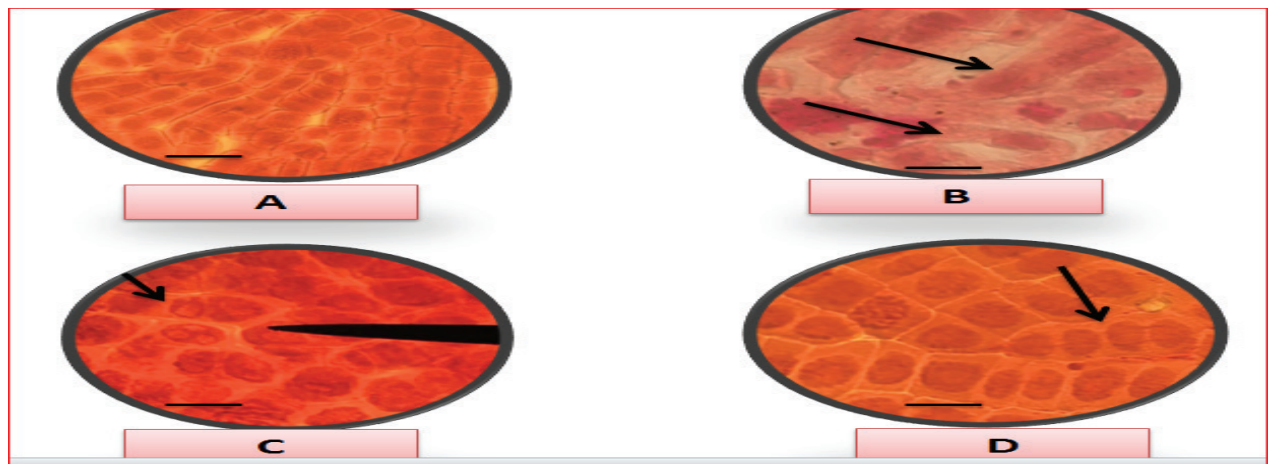


Figure 2. *A. cepa* root tips treated with alcoholic extract of oleander leaves, A: normal cells in control, B: abnormal cells shape treated with high concentration, C: single and multi-nuclear lesion, and D: multinucleated cells. 1000x, this segment represents 5 micron

Conclusion

Through these results were obtained from this study, it was found that the alcoholic extract of oleander is very toxic at high concentrations, because it contains many chemical compounds such as alkaloids especially N-hexadecanoic acid that has cytotoxic potential.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

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