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Research Article
Determination of Oleandrin Levels by HPLC-DAD in Vegetal Material Collected Throughout Algeria and the Study of Some Influencing Factors

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Abstract

Background: Oleander is a widespread evergreen shrub belonging to the family of Apocynaceae, it can grow to reach 20 feet high, the plant contains glycosides in all parts with different levels. Intoxications usually occur after the ingestion of oleander's material and are featured by a scope of clinical symptoms ranging from digestive to cardiac signs and causing in some cases death. **Objective:** The main toxic is oleandrin, its levels vary considerably, the study aims to evaluate the levels of oleandrin in samples of oleander's (*Nerium oleander* L.) material collected from different regions of Algeria and the study of some factors that may influence it. **Materials and Methods:** The plant material mainly leaves but also flowers and branches were collected across 25 sites throughout Algeria, oleandrin, extracted by liquid-liquid extraction and analyzed by high performance liquid chromatography coupled to a diode array detector (HPLC-DAD), the samples were divided into groups and statistical tests (parametric and non-parametric) were performed. **Results:** The results show that leaves contain the paramount levels varying from 0.022-0.549%. Whereas, flowers and branches oleandrin levels range respectively from 0.005-0.025 and 0.004-0.062%. **Conclusion:** Significant variability was noted, related to the plant "Variety", height and habitat and certain conditions such as exposure to light or the nature of the soil.

Key words: Oleandrin, *Nerium oleander*, influencing factors, variety

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nerium oleander L. is an evergreen shrub that belongs to Apocynaceae, it grows up to 6 m high[1] and bears yellow to dark green ternate, sessile, lanceolate, leathery, entire, whorled, narrowed at the base leaves of 5-20 cm long[2,3]. Some cultivars have white or yellow variegated leaves[3]. The flowers are about 1-3 inches in diameter[4] may be single or double in some cultivars, the colors vary from light or dark pink, lilac, crimson, purple, salmon, apricot, copper, orange, yellow and white[3-5].

The color and type of the flowers are used to distinguish different varieties; likewise, the presence of variegated leaves and the growth habits can also be differentiating criteria[3]. All parts of the plant whether fresh, dried or boiled are toxic due to cardenolides glycosides[6].

The main toxic is represented by oleandrin, a steroidal glycoside belonging to cardenolides, formed by the bond between an aglycon: Oleandrigenin and a sugar: Oleandrose[7]. It has a mechanism of action similar to digitalis[8].

According to data from the Algerian poisoning control center, oleander is responsible for 6% of poisoning within those due to plants ingestion, after ingestion of the plant material the symptoms are mainly cardiac and gastrointestinal, signs include: Nausea, vomiting, salivation, colic and diarrhea[9], sinus bradycardia is the most common cardiovascular sign and it can evolve to atrioventricular block (AVB) and asystole. Arrhythmias, tachycardia and ventricular fibrillation can occur in severe cases[10]. The treatment can be symptomatic and antidotal (Digibind, DigiFab)[11].

Poisoning might be intentional (attempt to suicide) or accidental, resulting from the use of the plant material as folk medicine for treating gangrene, eczema, headache, toothache, cold, scars, hair loss, skin lesions and for abortion[12,13]. It could be also used for cardiovascular diseases, diuresis disorders, menstrual disorders as a laxative, insecticidal, fungicidal, for the treatment of ringworm and warts as well as for snake bites[14]. Oleandrin is currently used orally as a diuretic and cardiotonic, it is listed for these properties in the Russian Pharmacopoeia[15] and constitutes a field of research in neurology (neuroprotection against ischemic damage)[16] and oncology: As an anticancer[17,18].

Various methods have been developed for the identification of oleandrin in plant material by colorimetry (Kedde reaction)[19], infrared or ultraviolet spectroscopy[20], the quantification of oleandrin, however, requires the use of a chromatographic technique (HPLC, HPTLC)[21,22], HPLC coupled with mass spectroscopy permits the quantification of oleandrin in biological matrixes[23].

In order to determine the levels of oleandrin in oleander's material throughout the Algerian territory and the study of some factors that may influence it, a rapid and practical method was developed based on a liquid-liquid extraction and HPLC-DAD analysis.

MATERIALS AND METHODS

Instrumentation: The apparatus was a Waters 2695 equipped with a quaternary pump, detection was made with diode array detector and data acquisition were performed using the software "Waters Empower®" analysis were conducted with the column: Symmetry® C18, 3.5 µm, 4.6×75 mm, guard columns XBridge TM C18 5 µm, 4.6×20 mm.

The extraction was performed using the following: A rotary evaporator: Buchi R215 (Maximum pressure: 4 bar), an ultrasonic bath, a centrifuge, acrodisks: iso-disk TM×25 mm filters 0.45 µm, a vortex.

Pretreatment of vegetal material and weighing were performed by an oven, an electric grinder, a copper mortar. A balance Kern ALS 220-4 N max 220 g, d = 0.1 mg.

Chemicals and solvents:

- Oleandrin standard: CAS number: 465-16-7, purchased from Phytolab GmbH and Co.KG, Vestenbergsgreuth, Germany, Quantity = 10 mg, Batch: 6922, chromatographic purity = 97.48%
- Methanol supra pure, HPLC grade (Sigma-Aldrich, batch N°: SZBC170AV), methanol HiPerSolv (CHROMANORM, batch N°: 20864.320), methanol HPLC grade (Scharlau, batch N°: 13087014)
- Acetonitrile HPLC grade (Scharlau, batch N°: 12063603), acetonitrile HPLC grade (Panreac, batch N°: 361881.1612)
- Ultrapure water provided by the laboratory

Plant material: About 148 samples of leaves, 4 of flowers and 3 samples of branches were collected between August 15th and September 10th from different regions of Algeria.

Sampling was done on the basis of some factors that can influence the contents of oleandrin namely: The "Variety", the height of the shrubs and the biotope (Nature of the soil, pollution and light exposure). The samples of RLL group are excluded from the study of the oleandrin content depending on varieties, height and regions, because growing under specific conditions and presenting low; misleading levels, they are used to study the influence of the biotope.

Fig. 1(a-g): Morphological features of the studied "varieties", (a) Variety 1, (b) Variety 2, (c) Variety 3, (d) Variety 4, (e) Variety 5, (f) Variety 6 and (g) Variety 7

Distribution by morphological variability ("variety"): The exact nomenclature of varieties is not taken into account due to the absolute need for a genomic fingerprinting to allow the accurate genetic identification[3]. Failing that, the classification of "Varieties" is referred to that reported in the study, which limits it to the morphological features and the color of the plant vegetal material especially flowers and leaves[3-25].

Collected samples were classified in "Varieties" according to the following criteria (Table 1):

- Flowers color: Red, deep pink, pale pink, white
- Flowers type: Single or double
- Leaves color: Completely green or green with yellow spots

Wild shrubs are those that grow naturally especially along the wadis[26] and belong exclusively to the "Variety 1", whereas, the other varieties are ornamental cultivars (Fig. 1).

Distribution by the height of the shrubs: The height 2 m was selected as a target point separating the group of shrubs with height inferior to 2 m and shrubs group whose height is superior to 2 m. The study was limited to "Variety 1" shrubs due to its large sampling size.

Distribution according to the biotope: Solely the samples of "Variety 1" are kept for the study of the biotope influence (Table 2).

Table 1: Morphological characteristics and nature of oleander varieties

Variety	Nature of the shrubs	Morphological characteristics
1	Wild	Pale pink, single flowers
2	Ornamental	Deep pink, double flowers
3	Ornamental	Green leaves with yellow spots, flowers similar to those of variety 2
4	Ornamental	White, single flowers
5	Ornamental	Pale pink, single flowers morphologically similar to those of variety 4
6	Ornamental	White, double flowers
7	Ornamental	Red double flowers

Table 2: Distribution of the samples of variety 1 according to the biotope

Factors	Features of the biotope	Size
Soil nature	Rocky soil	4
Pollution	Shrubs within 500 m from a cement plant	12
Light exposition	Shrubs with reduced exposure to light (growing under a bridge)	5
Control group	Samples from shrubs that have an optimum exposure to light, no exposed to pollution and growing on a non-rocky soil	83

Distribution by geographic regions: Samples from shrubs of "Variety 1" are compared by geographical regions (East, Center, West and South).

Pre-treatment of samples: The collected plant material underwent immediately drying at 80°C for 40 min[21-26] and stored in paper bags to be protected from light and moisture. The day of the assay, the plant material is pulverized using a copper mortar and an electric mill to produce a fine homogeneous powder.

Extraction: One gram of the plant material powder is extracted into glass tubes with 10 mL of a methanol-water solution (9.1) (v/v), the mixture is stirred vigorously using a vortex and then placed in an ultrasonic bath for 30 min. The mixture was then, centrifuged at 4000 rpm for 3 min and the supernatant was recovered. The pellet is extracted another time with 10 mL of methanol-water solution (9.1) (v/v) and underwent the same steps above-mentioned.

The combination of the two supernatants is then evaporated to dryness using a rotary evaporator at temperature 45°C and 240 rpm. The dry residue is recovered with 10 mL of a water-acetonitrile mixture (8.2) (v/v) then filtered through HPLC acrodiscs (porosity = 0.45 µm)[27]. The extract micrometer was diluted to 1/5 in a methanol-acetonitrile mixture (v/v) and injected into the HPLC apparatus. The method extraction yield varies between 95.38 and 102.48% with a mean of 100.23%.

Method development and optimization: Oleandrin standard was dissolved in the mixture methanol/acetonitrile (1/1) (v/v). A suitable chromatographic separation of oleandrin is obtained, with a mobile phase of 37% acetonitrile and 63% water, a flow rate of 1.3 mL min^{-1} and an injection volume of 20 µL with a column temperature of 30°C. Analysis time was 8 min and the absorption maximum wavelength of oleandrin is 217 nm (Table 3). The retention time was 6.74 min and a relative standard deviation of 1.84% (Fig. 2).

Table 3: Conditions of the analytical method

Optimisation parameters	Conditions
Mobile phase composition	Acetonitrile/eau: 37/63
Flow	1.3 mL min^{-1}
Injection mode	Automatique
Injection volume	20 µL
Column	C18
Column temperature	30°C
Detector	Diode array
Absorbtion wavelength	217 nm

Statistical tests: Statistical tests were performed using the software SPSS version 17, the calculations and tests of validations were performed with the software AVA.

Method validation: The method is validated according to the SFSTP validation guidelines of 1992. The new recommendations (2003 and 2006) were also taken into account[28,29].

Oleandrin contents in oleander plant material are hugely heterogeneous, therefore, the analytical validation was performed upon two ranges of calibration curves: One representing low concentrations: 1, 2.5, 5, 7.5 and 10 mg L^{-1} which would be used for the determination of oleandrin low concentrations (mainly for the branches and flowers) and the other for high concentrations: 5, 20, 35, 50, 75 and 100 mg L^{-1} referred to as "Curve for high concentrations" (mainly for leaves) (Table 4).

Oleandrin is quantified by two approaches: Normal calibration (Calibration curve prepared using external standards) and the method of standard additions (Standard solutions added to the samples). For normal calibration, the determination is made directly from the calibration curve,

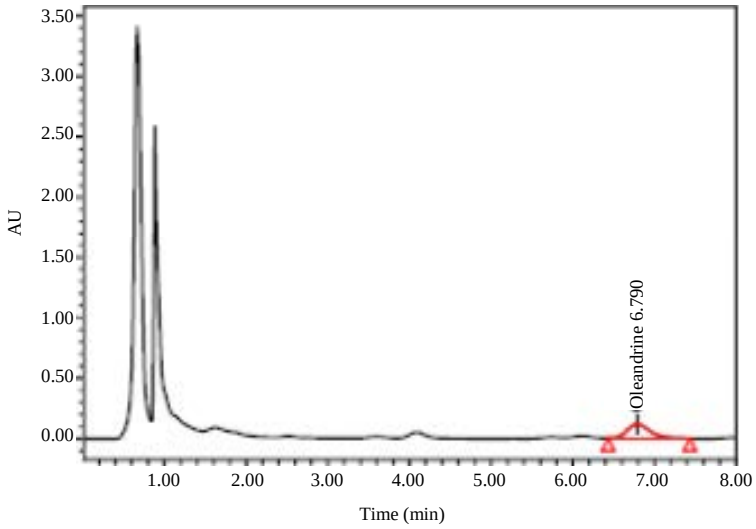

Fig. 2: Chromatogram of the sample N°5 (+5 ppm) from the site Annaba

Table 4: Validation performances of the analytical method

		Performances	
Validation criteria		Low concentration calibration curve	High concentration calibration curve
Extraction yield		100,228%	
Analysis range		[1-10] mg L^{-1}	[5-100] mg L^{-1}
Linearity	Equation of the line correlation coefficient	Y = 20665X+2391.4	Y = 21885X-911.08
		0.999	0.998
	Lower limit of detection	0.1 mg L^{-1}	
	Lower limit of quantification	0.2 mg L^{-1}	
Trueness	Recovery (%)	100,13 [99-101,26]	100,01 [96,87-103,15]
	Relative bias (%)	0.13	0.01
Precision	Coefficient of variation of repeatability	2.6 (<5%)	0.71 (<5%)
	Coefficient of variation of intermediate precision	2.5 (<5%)	1.08 (<5%)
Accuracy	(Trueness+precision)	2.63 (<5%)	1.09 (<5%)
Specificity		Purity angle (0.155) <Purity threshold (0.359)	

meanwhile, for the standard addition method, a calibration curve is plotted by the addition of three concentration levels (1, 2.5 and 5 mg L^{-1}) to the sample whose concentration in oleandrin is unknown. For this approach, the concentration of oleandrin in the sample is determined by reading into the calibration curve of standard additions:

$$y = ax+b$$

where, x is the concentration of oleandrin in mg L^{-1}. The absolute value of x when y = 0 corresponds to the targeted concentration.

The results obtained with the standard additions method are retained for the following, insofar as the results obtained with both methods are so close.

Oleandrin levels in oleander material: Oleandrin content (ppm) in plant material is expressed by the formula:

$$X\ (ppm) = 50 \times C$$

Where:

X : Content of oleandrin in ppm

C : Oleandrin concentration in mg L^{-1}, read directly from the calibration curve of standard additions

RESULTS AND DISCUSSION

Oleandrin levels in leaves

Whole sample: Medians (Fig. 3), means and standard deviations of concentrations of oleandrin in the leaves by city and site of collection are given in the Table 5. Data analysis of all leaves samples (148 samples) gives a mean of 1480.19 ppm, median of 1075.30 ppm, a standard deviation of 1148.82 ppm with minimum and maximum values, respectively of 229.76 and 5490.41 ppm.

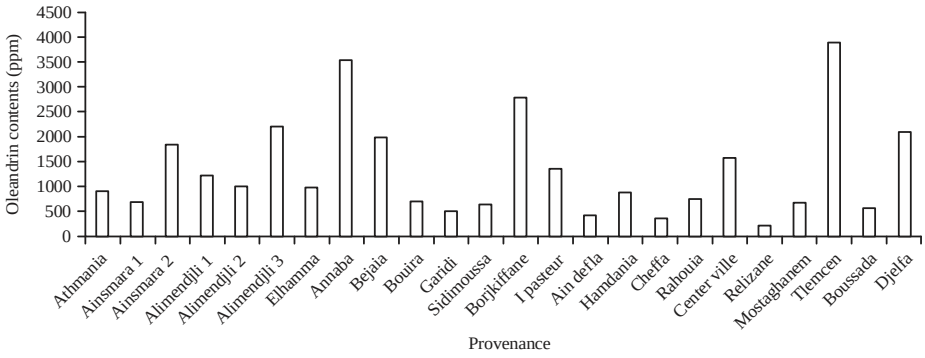

Fig. 3: Medians of oleandrin contents according to the site of collection

Table 5: Statistical description of oleandrin levels (ppm) in the leaves according to the site of collection

City	Site	Mean	Median	Standard deviation	Minimum value	Maximum value	Sample size
Constantine	Athmania	965,76	924,35	406,02	376,94	1664,97	8
	Ainsmara1	869,13	721,71	461,25	495,79	1590,97	5
	Ainsmara2	1817,24	1868,32	244,6	1551,13	2032,28	3
	Ali mendjli1	1263,44	1248,24	116,36	1141,2	1472,96	7
	Ali mendjli2	1083,68	1052,99	171,05	916,74	1358,37	5
	Ali mendjli3	2223,68	2223,68	464,9	1894,95	2552,42	2
	Elhamma	963,08	1006,5	111,2	726,65	1053,85	7
	Total	1161,48	1053,85	449,45			37
Annaba		3897,51	3567,01	908,52	2864,22	5490,41	8
Béjaia		2115,94	2018,39	256,78	1822,82	2538,96	7
Bouira		935,88	726,54	397,05	576,82	1513,06	5
Alger	Garidi	580,08	533,78	308,74	270,5	939,3	5
	Sidi moussa	671,38	670,18	70,75	573,26	765,73	5
	Borj kiffane	3205,98	2810	967,83	2088,75	5151 ,51	15
	I pasteur	1577,86	1396,94	563,34	1048,72	3060,95	12
	Total	1980,58	1642,85	1298,41			37
Ain defla		474,54	450,99	179,87	285,98	806,93	6
Médéa	Hamdania	892,34	900,47	188,49	603,78	1102,92	7
	Cheffa	461,09	399,64	273,58	229,76	815,3	4
	Total	735,52	815,3	301,84			11
Chlef		714,61	660,03	361,73	333,16	1232,22	6
Tiaret	Rahouia	915,51	765,19	381,11	639,92	1350,45	3
	Centre ville	1816,23	1611,55	361,27	1603,77	2233,38	3
	Total	1365,87	1477,11	594,72	639,92	2233,38	6
Rélizane		302,63	235	136,22	231,34	544,77	5
Mostaghanem		710,49	691,7	82,16	640,91	817,64	4
Tlemcen		3918,45	3913,41	508,26	3412,72	4429,22	3
Boussada		653,07	599,61	305,78	294	1075 ,76	10
Djelfa		2025,89	2126,09	455,47	1528,65	2422,93	3

A significant disparity in the means and medians based on the collecting site is noted. The highest mean and median are observed for samples from the city of Tlemcen, while the lowest mean and median are observed for the samples from the city of Relizane, the highest oleandrin contents correspond to a sample from the city of Annaba and the lowest content is reported in Cheffa site.

In a similar study, oleandrin contents in leaves from plants collected throughout different sites were found to range from 70-4250 ppm[21]. This huge disparity may be explained by the influence of the following factors.

Oleandrin levels by "Variety" (strains): Samples of the "Varieties" 4 and 5 have close medians (Table 6), they are assembled in one group in order to perform the statistical tests. Mann whiteney test was used to compare between the groups of varieties. No significant difference noted between the medians of the "Varieties" 4.5 (median = 3567.01 ppm) and 7 (4448.73 ppm) same for medians of "Varieties" 2 (2324.97 ppm) and 6 (1950.3 ppm). Conversely, a significant statistical difference is observed between the medians of the "Varieties":

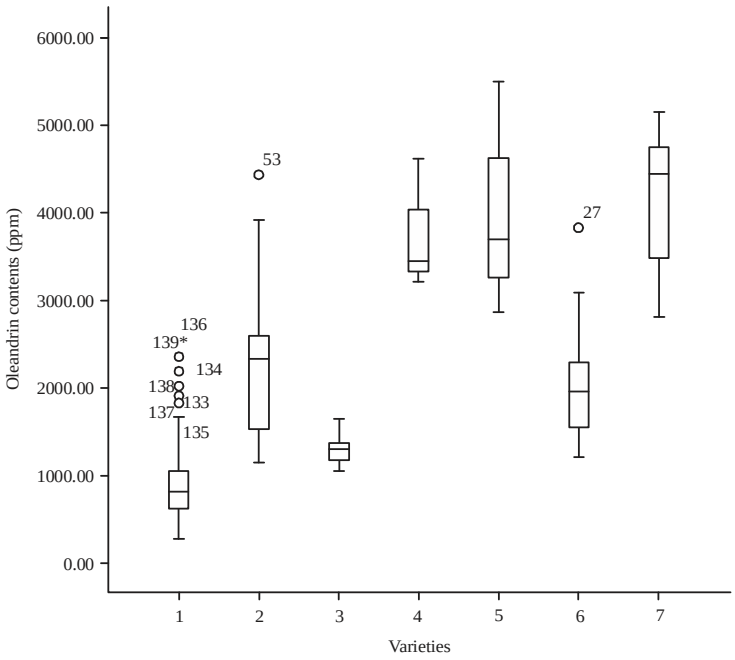

Fig. 4: Oleandrin levels according to "varieties"

Table 6: Statistical description of oleandrin contents by varieties

Variety	Mean	Median	Standard deviation	Minimal value	Maximal value	Size
1	909,4	817,64	484,01	270,5	2538,96	83
2	2302,42	2324,97	939,11	1141,2	4429,22	22
3	1295,35	1294,02	198,87	1048,72	1642,85	7
4	3757,95	3446,12	750,8	3213,31	4614,42	3
5	3981,25	3687,9	1067,35	2864,22	5490,41	5
6	2049,47	1950,3	718,4	1205,6	3822,81	14
7	4127,11	4448,73	961,58	2810	5151,51	5
4,5	3897,51	3567,01	908,52			8

Table 7: Oleandrin levels by height

Height	Mean	Median	Standard deviation	Minimal value	Maximal value	Size
<2 m	775,58	806,93	314,24	270,5	1664,97	35
>2 m	1006,98	867,19	560,55	294	2538,96	48

- 1 and 2: U = 107, exact significance = 2.20 e^{-10}
- 1 and 3: U = 93, exact significance = 0003
- 2 and 3: U = 24, exact significance = 0.005
- 2 and 4.5: U = 18, exact significance = 0.001
- 2 and 7: U = 8, exact significance = 0.003

"Varieties" are ranged in the following descending order: "Variety 7" (4448.73 ppm), "Variété 5" (3687.9 ppm), "Variety 4" (3446.12 ppm), "Variety 2" (2324.97 ppm), "Variety 6" (1950.3 ppm), "Variety 3" (1294.02 ppm), "Variety 1" (817.64 ppm) (Fig. 4).

Comparing to similar studies, in a study conducted by Karawya *et al.*[30] varieties with deep pink and red flowers are characterized by higher levels of oleandrin compared to varieties with white flowers.

Oleandrin levels according to the height of the shrub: Within "Variety 1" samples, oleandrin contents in shrubs whose height is superior to 2 m (mean = 1006.98 ppm) are higher than in those with height inferior to 2m (775.58 ppm). Student t-test revealed a significant difference with p = 0.03 (Table 7, Fig. 5).

Oleandrin levels according to the biotope: Samples from shrubs that grow on rocky ground, those with exposure to light is reduced and those exposed to pollution are compared to a control group (Table 8, Fig. 6). Samples from "Rélizane" were collected from shrubs growing along a river under a bridge (reduced exposure to light). Low oleandrin levels were found in these samples.

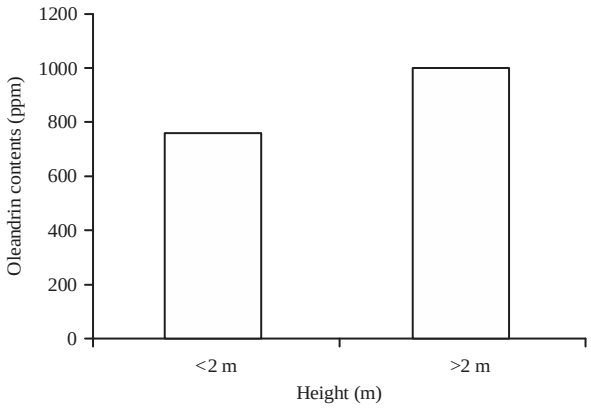

Fig. 5: Medians of oleandrin contents according to the height of the shrub

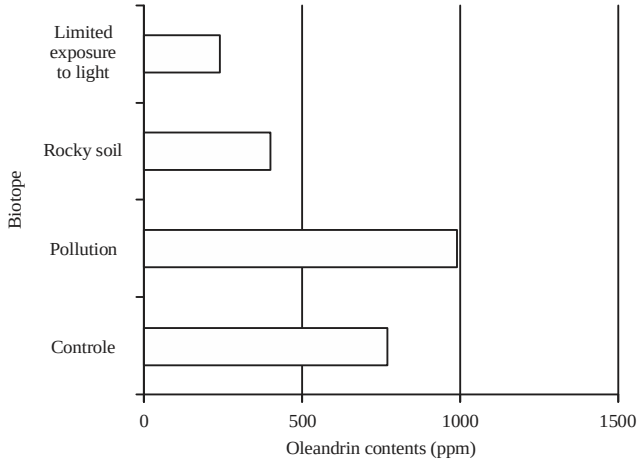

Fig. 6: Medians of oleandrin contents according to the biotope

Table 8: Oleandrin levels by biotope

Biotope	Mean	Median	Standard deviation	Minimal value	Maximal value	Size
Control	902,24	765,72	513,77	270,5	2538,96	71
Pollution	951,75	987,25	253,51	576,82	1513,06	12
Rocky soil	461,09	399,64	273,58	229,76	815,3	4
Limited exposure to light	302,63	235	136,22	231,34	544,77	5

Table 9: Groupe RLL

Group	Mean	Median	Standard deviation	Minimal value	Maximal value	Size
RLL	375,05	261,77	210,52	229,76	815,3	9

The group of shrubs g r owing on a rocky ground (Cheffa site) and those with limited light exposure have close values (means and medians) which allows to group them in order to increase the sample size and permit the statistical test, the group is named RLL (Table 9).

A statistically significant difference is observed between the medians of the group RLL and the group of control (U = 71, asymptotic significance of 1.54 e^{-4}), On the other hand, no statistically significant difference noted between the "Pollution" group and the control group.

It is concluded that light exposure and the nature of soil are determining factors of oleandrin contents, in fact, in a study probing the influence of the soil on the production of Cardenolides by Digitalis obscura, the contents of Cardenolides in plants showed significant negative correlations with the levels of phosphorus in the plants and those of copper in the soil, suggesting that these elements may affect the cardenolides biosynthesis[31].

In another study, *Nerium oleander* shrubs were found in an extreme acidic environment (Riotinto, Spain) where

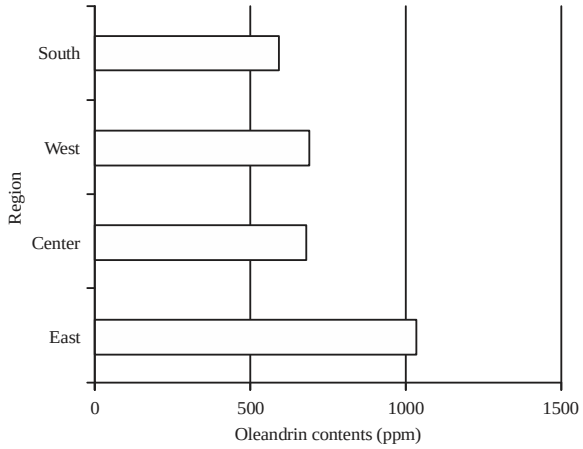

Fig. 7: Oleandrin contents medians by regions

Table 10: Oleandrin levels by regions

Region	Mean	Median	Standard deviation	Minimal value	Maximal value	Size
East	1220,1	1040,11	565,07	376,94	2538,96	32
East truncated of Bejaia's samples	969,27	1006,5	310,38	376,94	1664,97	25
Center	715,37	691,44	293,03	270,5	1513,06	28
West	759,7	693,16	297,17	333,16	1350,45	13
South	653,07	599,61	305,78	294	1075,76	10

the soil, water and sediments are characterized by a pH<3, with high concentrations of S, Fe, Cu and various other metals. This study confirms the *Nerium oleander*'s resistance to stress caused by pollution[32].

In our study, samples from two distinct regions (Bouira and El Hamma) were collected from shrubs within 500 m of a cement factory showed oleandrin levels similar to those of unexposed shrubs.

Oleandrin levels by geographical region: The Kruskal-Wallis test (non-parametric) was used to compare between oeandrin levels of groups of shrubs from different regions (East, Centrer, West and South). A statistically significant difference was observed between the medians of oleandrin contents from East, Center, West and South regions (Chi-square = 20.07, degree of freedom = 3 and asymptotic significance = 1.63 e^{-4}), the significant difference is maintained even with the withdrawal of samples from the city Bejaia that have a higher median (asymptotic significance = 0.01) (Table 10). By contrast, no statistically significant difference was found between the oleandrin levels of the regions: Centre, West and South.

The study showed higher oleandrin levels in shrubs from the eastern region compared to the other regions (Centre, West and South) (Fig. 7). Soil characteristics and environmental conditions may explain this difference.

Table 11: Oleandrin levels in flowers and branchs

Provenance and variety	Vegetal material	Oleandrin contents (ppm)
I Pasteur (variety 6)	White flowers	53,38
	Branches	42,42
Bordj kiffane (variety 2)	Pink fowers	198,64
	Branches	85,33
Bordj el kiffane (variety 7)	Red flowers	258,09
	Branches	624,90
Bejaïa (variety 1)	Pink flowers	69,84

For the influence o f seasons that it have not been able to study, data from the study show that the highest concentrations of oleandrin were found during the spring[24-33].

Oleandrin levels in flowers and branches: The following (Table 11) shows oleandrin contents (ppm) of *Nerium oleander*'s flowers and branches samples.The results confirm the data showed in the study by which the leaves contain the highest oleandrin contents. Indeed, roots and seeds contain the highest contents of cardiac glycosides and lowest concentrations of oleandrin. In contrast to leaves that have the highest contents of oleandrin and the lowest in cardiac glycosides[24].

The limited size of flowers and branches samples may not permit conclusions, further in-depth studies may provide more information.

CONCLUSION

The study it has conducted confirms the important variability of oleandrin levels in oleander plant material.

It resulted, corroborating the study that the levels of oleandrin vary depending on the part of the plant with higher contents in the leaves.

Significant variability is also reported depending on "Varieties", a parameter which is related to the genetic variability between the wild shrub and different cultivars differentiated by color and morphology, nevertheless the study should be deepened by the determination of oleandrin levels on varieties classified on the basis of DNA fingerprinting. The plant height and some habitat conditions such as exposure to light or the nature of soil are also factors that may influence the levels of oleandrin.

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