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Phytochemical screening and analysis of heavy metals of *Nerium oleander* (L.) leaves

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Abstract: The main objective of this research is studying phytochemical screening of the extracts of polluted *Nerium oleander* (L.), and unpolluted *Nerium oleander* (L.) collected from Meknes region (Morocco), and determination of metals concentration that can have harmful effects on human health. In addition, the study provides scientific data.

Studies have shown that the two extracts of polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.) plant are rich in flavonoids, catechic and gallic tannins. In contrast, anthracene derivatives and flavonoids are absent. On the other hand, the comparative analysis of the results of atomic absorption spectrometry showed that the two plants of polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.) contain a fairly high content of Na, Ca and Mg while the Pb concentration has exceeded the standard given by the WHO. While lithium and iron are present in low concentrations.

Keywords: Heavy metals; *Nerium oleander* (L.); Contamination; Pollution; Alkaloids; Tannins; Glycosides; Flavonoids.

1. Introduction

Motor vehicle, road traffic, and infrastructure are the major source of heavy metals that are released in the environment. The main metallic pollutants are lead that is emitted by the exhaust gases of engines, copper and cadmium coming from brake linings, and zinc that is present in tires of the cars and lubricants [1]. These pollutants accumulate in ecosystems and beyond certain thresholds become toxic to humans and the environment. Likewise, the chemical compounds or elements released into the atmosphere which result from human activities and which can cause damage to live organisms are considered as air pollutants [2].

Currently, much research has focused on determining the content of these chemical substrates in plants, particularly in urban areas. This determination was made by atomic absorption spectroscopy. The latter is a method used to measure the concentration of some metals such as Fe, Cu, Al, Pb, Ca, Zn, etc.

Heavy metals entering the environment come from natural sources and anthropogenic sources. Their inputs may be the result of spills directly into marine and freshwater ecosystems, an indirect pathway as in the case of dry, wet landfills, agricultural run-off, and road traffic [2].

Metals from anthropogenic inputs are present in reactive chemical forms and thus carry much higher risks than naturally occurring metals that are most often immobilized in relatively inert forms [3]. Since the plants are grown on the soil, this latter is an essential factor in their formation and growth. Therefore, pollution by trace elements has a significant impact on soil, air, and plants [4].

Since 1970, a great deal of research has been conducted on using plants as bioindicators and bio accumulators of atmospheric pollution [5].

Trees used as bioindicators of atmospheric pollution are plane trees (*Platanusorientalis*), carob (*Ceratoniasiliqua*) [6,7], Aleppo pine (*Pinushalepensis*) [8], and *Nerium oleander* (L.) [9]. These plants are referred to as sensitive trees.

This work aims to show the importance of studying the bioaccumulation of metals by plants in order to

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assess environmental pollution and its relation with specific sources of pollution like road traffic.

Nerium oleander (L.) (Apocynaceae family), is an ornamental plant in Mediterranean regions of Africa and Europe with a large distribution in tropical and subtropical parts of the world [10]. Various studies have demonstrated that *Nerium oleander* (L.) presents a large spectrum of beneficial biological activities. Tea infusate made from the leaves, flowers, is used in traditional medicine to trait some diseases such as heart failure, leprosy, malaria, ringworms, and indigestion [10-12].

Nerium oleander (L.) can be used in another way; for example, insecticide, antibacterial, antioxidant, and antineoplastic [13-16].

Nerium oleander (L.) is rich on flavonoids (14.43%), alkaloids (0.30%), glycosides (0.30%), steroids, tannic acid (14.5%), saponins (71.00%), and triterpenoids (7.00%). The total amount of tannins, vitamin C, and phenols are also considerably present in leaves (600, 430, and 600mg/g respectively). In *Nerium oleander* (L.) extract, the quantity of K, Cr, and Zn are greater. However, the concentration of Mn and Fe is higher in leaves, and there is no particular difference in the amount of Cu, Co, Ca, Na, and Mg in the leaves and gemmomodified extract of *Nerium oleander* (L.) [17]. A wide variety of compound obtained from plants are recognized as secondary metabolites. About 34 volatile components, presenting 93.22% of the whole composition, were identified in the *Nerium oleander* (L.) oil obtained from flowers was found to be rich in constituents; digitoxigenin (11.24%), α-pinene (5.53%), amorphane (8.12%), calarene (5.11%), β-phellandrene (4.83%), limonene (5.02%), terpinen-4-ol (3.97%), isoledene (2.93%), sabinene (3.21%), 3-carene (2.55%), β-pinene (2.02%) cymen-8-ol (1.66%), and humulene (2.28%) [14]. The popular values of *Nerium oleander* (L.) are due to two glycosides (oleandrin and neriin), an alkaloid (having cardio-stimulatory effect), and three glycosides (gentiobiosylnerigoside, gentiobiosyl-beaumontoside, and gentiobiosyl-oleandrin) obtained from leaves [18].

2. Material and methods

2.1. Plants

The plant studied was collected in March from two sampling sites. The first one is a double-track near Cheikh El Kamel in Meknes. This road is polluted because of the large vehicles traffic (Figure 1).

Figure 1. Location of polluted ***Nerium oleander* (L.)**

The second site is an area located in outskirts of Meknes, further from road traffic. In this site, *Nerium oleander* (L.) plant is growing spontaneously (Figure 2).

Figure 2. ***Nerium oleander* (L.)** obtained from the unpolluted area

2.2. Preparation of the plants

After collecting the plants, we separate leaves and stems, and then leaves are dried in the shade for 15 days. Leaves obtained are ground to obtain a fine powder.

2.3. Phytochemical study

Phytochemical study of *Nerium oleander* (L.) leaves revealed many secondary metabolites such as alkaloids, tannins, glycosides, and flavonoids. It involves performing characterization reactions (staining and precipitation) in tubes to check the presence or absence of these chemical families in *Nerium oleander* (L.) extracts. Indeed, solutions have been prepared to carry out the following analysis:

- Maceration in sulfuric acid 5% (S_1) :

10g of *Nerium oleander* (L.) powder and 50 mL of the 10% diluted sulfuric acid were introduced in a 250 mL Erlenmeyer. The preparation was stirred and let to macerate for 24 hours at room temperature of the laboratory. Then we filtered and washed with water to obtain 50 mL of the filtrate.

- Infusion 5% (S_2) :

5g of *Nerium oleander* (L.) powder was put in an Erlenmeyer containing 100 mL of boiling water. After 15 minutes, the mixture was filtered and adjusted to 100 mL.

- Decoction 1% (S_3) :

100 mL of distilled water was put in an Erlenmeyer, and then added 1g of *Nerium oleander* (L.) powder. The mixture was boiled for 15 min, then filtered and adjusted to 100 mL after cooling.

2.3.1. Alkaloids

In a test tube into which we introduced 1 mL of S_1. We added 5 drops of Mayer's reagent (1.358g of $HgCl_2$ in 60mL of water + 5g of KI in 10 mL of water and then adjusted the solution to 100 mL with distilled water). After 15 minutes, the presence of the alkaloids is denoted by the appearance of a precipitate.

2.3.2. Tannins

In a test tube, we introduced 5 mL of S_2 and 1 mL of 1% aqueous solution of $FeCl_3$. The presence of gallic or catechin tannin results in the development of a greenish or blackish blue coloration. Therefore, to differentiate between tannins, we used Stiasny reagent, whose principle is the following:

- Catechetical tannin

To 30 mL of the infused solution, we added 15 mL of Stiasny reagent (10 mL of 40% formalin + 5 mL of concentrated hydrochloric acid). We heated in a water bath at 90°C for 15 minutes. The precipitate obtained indicates their presence.

- Gallic tannins

We filtered and saturated the filtrate with powdered sodium acetate. The development of a dark blue after addition of few drops of 1% $FeCl_3$ shows their presence.

2.3.3. Flavonoids

To 5 mL of the S_2 solution, 5 mL of 10% H_2SO_4 and 5 mL of NH_4OH diluted to half were added. The presence of anthocyanin results in an accentuation of the coloring by acidification, and then they turn to purplish blue by alkalinization.

Reaction to Cyanidin:

5 mL of S_2 and 5 mL of hydrochloric alcohol (alcohol (95%), distilled water, concentrated hydrochloric acid) were prepared. In a test tube, 1 mL of isoamyl alcohol and some magnesium chips were put. A pink-orange color (flavones) or purplish-pink (flavanones) or cherry red (flavonols) in the supernatant layer of the mixture indicates the presence of free flavonoid (genin). Then we made the cyanidine reaction without adding magnesium and then heat for 15 minutes in a water bath. The development of a cherry red, purplish, or reddish-brown color indicates the presence of leucoanthocyanins and catechol, respectively.

2.3.4. Anthracene Derivatives

In a test tube, 1g of plant powder and 10 mL of $CHCl_3$ were introduced, and we close the tube. We heated on a steam bath for 1 minute, then filtered and made up to 10 mL with chloroform.

Hydrolysate:

The residue of powder extracted with chloroform was treated with 10 mL of water and 1 mL of concentrated HCl. After heating in a boiling water bath for 15 minutes, the solution was cooled under a stream of water and filtered. The filtrate thus obtained was made up to 10 mL with distilled water.

2.4. Infrared spectroscopy analysis

Infrared spectroscopy is a method of qualitative analysis that characterizes the main functions of an organic molecule. Infrared radiation is electromagnetic radiation with a longer wavelength than visible light but shorter than that of the microwave. The infrared range is between 4000 cm^{-1} and 400 cm^{-1} corresponds to the vibration energy domain of the bonds. The device used in this analysis is of the JASCO IFTR 4000 type.

2.5. Thermogravimetric Analysis (ATD/ATG)

5 mg of the plant powder was put in Thermodifferential Analysis machine, the machine used was 60 Shimadzu workstation performing simultaneously DTA/ TGA, this analysis measures the loss of mass as according to the temperature, and the temperature rise speed was 20°C/min for 29 min 15 s, note that this process is linear.

2.6. Determination of metals by atomic absorption spectrophotometry (SAA)

The S.A.A technique is most commonly used for the determination of metallic trace elements. These were measured by the following apparatus: AA-7000 SHIMADZU.

For calcination assay, 6g of the sample was put in a porcelain dish at 600ºC in a muffle furnace for six hours. The ash obtained is mineralized with 75% HNO_3 in a beaker for four hours. Then the solution was filtered on Wattman filter paper. The metal elements were then assayed by flame atomic absorption spectrophotometry.

The machine was adjusted according to the wavelength corresponding to the maximum absorption of each element to analyze, which is sprayed inside the apparatus in a flame produced by a gaseous mixture (acetylene/air).

3. Results and discussion

3.1. Phytochemical Screening

In order to identify different groups of secondary metabolites in the aqueous extract of both plants of *Nerium oleander* (L.) obtained from polluted and unpolluted area, phytochemical screening was carried out by conducting a set of characterization reactions of different chemical compounds, namely: flavonoids, saponins, tannins, alkaloids, anthocyanins (Table 1). The results of the chemical characterization are classified according to the different observation criteria that we noted as follows:

++++: Very positive reaction; +++: Positive reaction; ++: Moderately positive reaction; +: Doubtful reaction; -: Negative test.

Table 1. Results of phytochemical screening of polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.).

Secondary metabolites			Unpolluted *Nerium oleander* (L.)	Polluted *Nerium oleander* (L.)
Alkaloids			-	-
Tannins	**Free tannins**		++	++
	Catechin tannins		+	+
	Gallic tannins		+++	+++
Favonoids	**Flavones**		-	-
	Anthocyanins		-	-
Derivatives anthracene	**Anthracene free**		-	-
	Anthracene combined	**O-heteroside**	-	-
		O-heteroside genine	-	-
		C-heteroside	-	-

The preliminary evaluation of the phytochemical composition on the various preparations of the hydroalcoholic extracts of leaves of polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.) revealed the presence of free tannins, catechin tannins and gallic tannins. This study showed that both plants do not contain anthracene derivatives and flavonoids.

3.2. IR analysis of unpolluted *Nerium oleander* (L.) at different temperatures: 110°C (gross), 300°C and 600°C

At this stage, we performed a spectroscopic analysis of the plant calcined at different temperatures: 110°C, 300°C and 600°C.

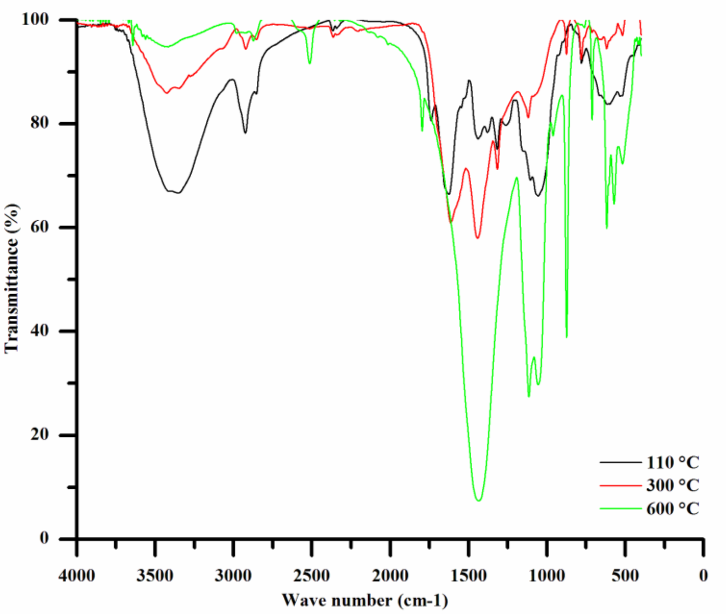

Figure 3. IR spectrum (KBr) of unpolluted *Nerium oleander* (L.) at different temperatures: 110°C, 300°C and 600°C

The examination of the infrared spectrum (Figure 3) of unpolluted *Nerium oleander* (L.) shows the appearance of a broad and intense band around 3368 cm^{-1} corresponding to the valence vibration band of the OH groups of the alcohol. Another band appears at about 293 cm^{-1} relatives to the vibration band of aliphatic (C-H) elongations. Similarly, a band of average intensity around 1635 cm^{-1} is recorded, attributable to the extensional vibrations of the C=C bonds. The IR spectrum of the sample taken at T=300°C shows the disappearance of the characteristic bands with the appearance of a new broad and fine band at 1449 cm^{-1} attributable to calcinite. Similarly, the IR spectrum of the sample, which has been taken at T=600°C, shows the appearance of other bands around 1078 cm^{-1} and 870 cm^{-1} that can be attributed to SiO_2 and quartz, respectively.

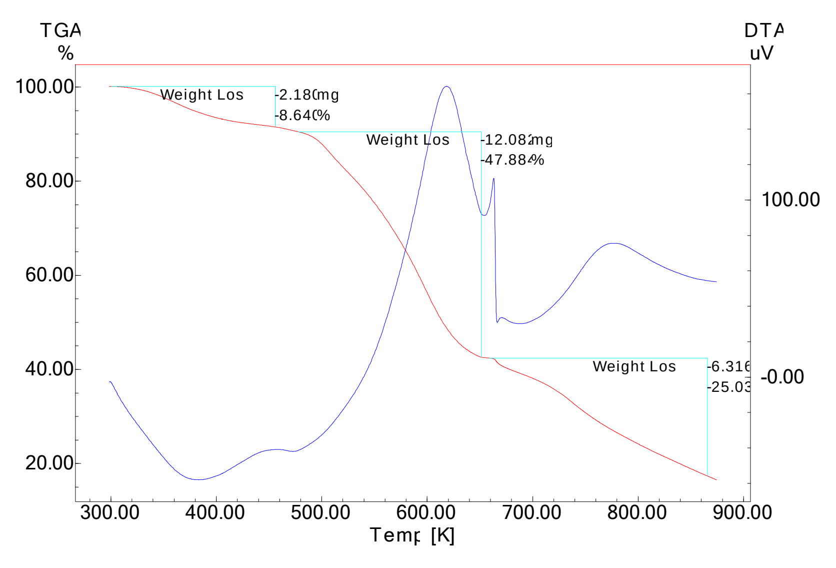

Figure 4. ATD/ATG spectrum of unpolluted *Nerium oleander* (L.)

To follow the loss of mass of the sample during its rise in temperature, we used a thermogravimetric analysis (ATD) device of the Shimadzu thermal analysis type. The curves recorded for a temperature range from 0°C to 700°C. The heating rate is 10°C/min. The sample of about 25.56mg was placed in an alumina boat. The temperatures relating to the maximum degradation rates were evaluated. Figure 4 shows the thermogram obtained.

Table 2. Variation in mass lost of *Nerium oleander* (L.) obtained from the **unpolluted** area according to the temperature.

Plant	Step	Temperature range	A loss of mass (%)
Nerium oleander (L.) **obtained from** unpolluted **area**	1	27-110°C	8.64
	2	110-300°C	56.52
	3	300-600°C	81.55

The study of thermal degradation of unpolluted *Nerium oleander* (L.) gave three areas of mass loss:

- Between 27°C and 110°C; 8.64% of the total mass was lost. This is due to the evaporation of water molecules in the plant.
- Between 110°C and 300°C; 47.88% of the total mass was lost, this is related to the partial degradation of organic molecules.
- At last, between 300°C and 600°C; 25.03% of the total mass was lost, this is due to the complete degradation of organic molecules. Only the mineral elements remain.

The thermogram clearly shows that the sample loses approximately 81.55% of its total mass. The plant is therefore thermally stable up to 600°C. To explain this loss of mass at different temperature ranges, the sample was carried in a muffle furnace at different temperatures: 110°C, 300°C, and 600°C for calcination, loss of masses was recorded in Table 3.

Table 3. Loss of mass during the calcination of unpolluted *Nerium oleander* (L.).

Temperature (°C)	m_i (g)	m_f (g)	Mass lost (%)
110	6.04	5.58	7.62
300	6.04	1.42	76.49
600	6.04	0.55	90.89

m_i : initial mass; m_f : final mass

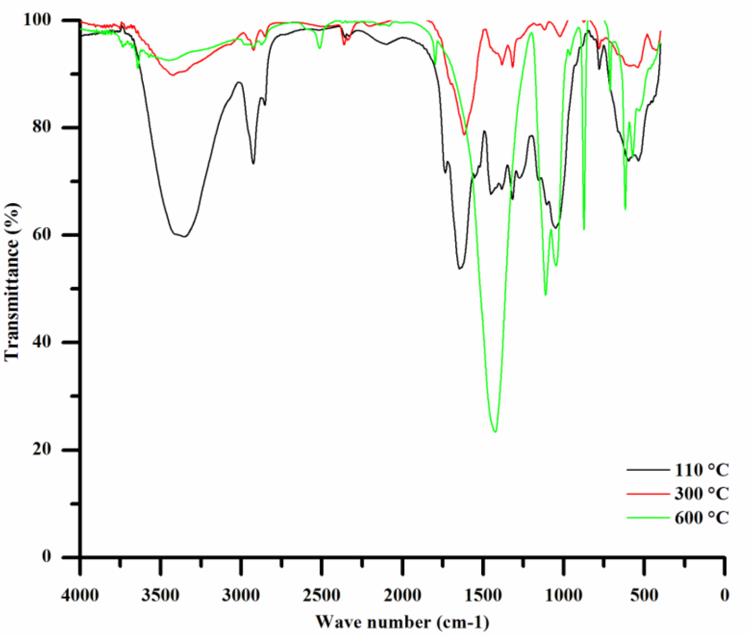

Figure 5. IR spectra (KBr) of polluted *Nerium oleander* (L.) at different temperatures

The calcination results show a concordance with the values found by the ATG since more than half of the organic matter is lost at 300°C, in particular at 600°C. Concerning *Nerium oleander* (L.) obtained from polluted area, the same analysis: IR, ATD, ATG and SAA were done. The infrared spectra of *Nerium oleander* (L.) calcined at three temperatures: 110°C, 300°C and 600°C, were recorded in Figure 5.

Figure 5 represents the infrared spectra of *Nerium oleander* (L.) plant obtained from the polluted area. At 600°C the plant lost all organic molecules, only some mineral elements remain, such as quartz, calcite, and silicon oxide.

Thermogravimetric analysis (GTA) of *Nerium oleander* (L.) polluted shows mass lost at 110°C, 300°C and 600°C (Figure 6).

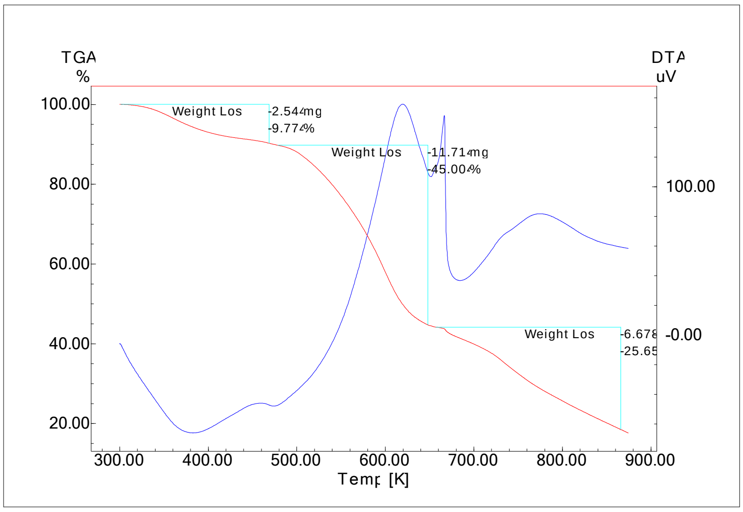

Figure 6. ATD/ATG Spectrum of polluted *Nerium oleander* (L.)

The thermogram shows three intervals of mass lost according to the temperature (Table 4).

Table 4. Mass Lost of polluted *Nerium oleander* (L.) according to temperature.

Plant	Step	Temperature range (°C)	Mass lost (%)
Nerium oleander (L.) obtained from unpolluted area	1	27-110	9.77
	2	110-300	54.77
	3	300-600	80.41

- Between 27°C and 110°C, 9.77% of the total mass was lost. This loss is due to evaporation of water molecules.
- Between 110°C and 300°C; 45.00% of the total mass was lost; this is due to the partial degradation of organic molecules.
- Between 300°C and 600°C, 25.64% of the total mass was lost. In this step, only mineral elements exist.

The thermogram shows that the plant lost approximately 80.41% of its total mass. Loss of mass was stabilized at 600°C. To explain this loss of mass at different temperature ranges, the sample was then carried in a muffle furnace at different temperatures: 110°C, 300°C, and 600°C for calcination. The mass lost were recorded in table 5.

Table 5. Mass Lost During Calcination of *Nerium oleander* (L.) obtained from the polluted area.

Temperature (°C)	m_i (g)	m_f (g)	Loss of mass (%)
110	6.56	5.56	15.24
300	6.56	2.71	58.69
600	6.56	0.55	91.62

m_i : initial mass; m_f : final mass

The results of the calcination confirm approximately the values obtained by ATG. It was noted that more than half of the organic matter is lost at 300°C, in particular at 600°C.

3.3. Determination of heavy metals by atomic absorption spectrometry

To determine the metal content of *Nerium oleander* (L.) obtained from polluted and unpolluted area, these samples, which were calcined at 600°C and treated with nitric acid, were analyzed by flame atomic absorption spectrometer. The concentrations of the minerals were determined based on their calibration curves. The results are shown in Table 6.

Table 6. Concentration of some minerals in polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.).

Metal	Content of unpolluted *Nerium oleander* (L.) (mg/kg)	Content of polluted *Nerium oleander* (L.) (mg/kg)	Normal Content in plants according to WHO (mg/kg) [19]	Heavy metal content in human body (mg/kg) [20]
Cu	0.5355	0.9536	10	1
Zn	5.1533	15.1817	50	33
Pb	1.9288	2.3567	10	-
Fe	0.0348	0.0592	20	60
Cd	0.0001	0.0005	0.3	-
Na	41.7640	42.3632	-	800
Ca	321.3503	334.4723	-	19000
Li	0.0904	0.0183	-	-
Mg	121.0151	100.7464	-	270

Comparative analysis of the results of atomic absorption spectrometry shows a high content of Na, Ca, and Mg in polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.). Regarding Cu and Zn, it has been found that their concentrations in the two plants are below the critical limits given by the World Health Organization (WHO). However, the concentration of the element Zn is more important in the plant polluted (15.1817 mg/kg). We also note the absence of Cd in both plants, while Lithium and Iron are present with low concentration in both polluted and unpolluted plants.

For Pb, its concentration is higher in *Nerium oleander* (L.) polluted (2.3567mg/kg) than unpolluted one. This is due to the exhaust gases from vehicles. These results are in agreement with the literature where it is shown that *Nerium oleander* (L.) has an intense concentration of Pb and a low concentration of Fe [21].

4. Conclusion

Phytochemical screening has shown that *Nerium oleander* (L.) obtained from the unpolluted and polluted area are rich in flavonoids, catechic and gallic tannins while anthracene derivatives and flavonoids are absent.

Analysis by infrared spectroscopy has shown that both plants contain alkanes, alkenes, alcohol functional group, and other minerals such as calcite, quartz, and SiO_2.

Comparative analysis of the results of atomic absorption spectrometry has shown that *Nerium oleander* (L.) plants contain a high content of Na, Ca and Mg; while Lithium and Iron are in low concentrations in polluted *Nerium oleander* (L.) and unpolluted *Nerium oleander* (L.).

From these results, we can conclude that metals concentration is higher in *Nerium oleander* (L.) obtained from the polluted area than *Nerium oleander* (L.) obtained from unpolluted area, this is due to road traffic in sampling site. However, the concentrations are still less than WHO limits.

Also for some metals concentration (Cu, Pb, Fe) in *Nerium oleander* (L.) obtained from unpolluted area, is almost the same concentration found in *Nerium oleander* (L.) obtained from polluted area, so there are other factors which are responsible for transmitting heavy metals to the plants (soil, water, etc.)

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