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CHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITY OF "*NERIUM OLEANDER*" LEAVES

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ABSTRACT

The phenolic products of medicinal plants have a great pharmacological interest. This product gives the powers of medicinal plants. They are the source of several active principles widely used in modern medicine. The use of *Nerium oleander* in Moroccan traditional medicine is very common. Few studies have focused on the chemical analysis and phenolic compounds of this plant. For this, we investigated the mineral composition and phenolic combination of the leaves *oleander* and the study of the antioxidant activity. The mineral analysis shows a very high level of potassium and protein. The biochemical studies revealed a very high quantity of polyphenols in the leaves. Thus, the HPLC analysis of the phenolic fraction shows great variability of substances. The cinnamic acid is the majors compounds identified in the phenolic fraction. The other compounds identified are catechin, epicatechine, chlorogenic acid. This present study which is made for the first time showed a very important antioxidant effect, the value of IC50 (The half maximal inhibitory concentration of DPPH) is 0,43 mg mL^{-1} for the phenolic fraction. On the other hand, the antioxydant activity of the organic extract, the methanolique fraction, n-butanolique fraction and the decoction, has a percentage of inhibition of DPPH over than 90% at a concentration of µg/mL. IC50% values are respectively 0,005 mg mL^{-1}; 0,018 mg mL^{-1} and 0,005 mg mL^{-1}.

Keywords: *Nerium Oleander*, Polyphenols, HPLC, Antioxydant Activity

1. INTRODUCTION

The therapeutic use of plants is very old and evolves with the history of humanity. The secondary metabolites give them the first place in pharmacology. Among these products, we find the polyphenols which are an important family of active principles (Munin and Edwards-Levy, 2011). In addition to their antioxidant and their very marked antimicrobial activity, the polyphenols have other activities such as vasculoprotectrice activity (tanins, anthraquinones, flavonoides), anti-inflammatory and anti-tumor (Xia *et al*., 2010).

Oxidative stress in animal cells is often associated with an overproduction of O_2 or H_2O_2. It causes damage to the biological molecules. The accumulation of this damage is the cause of various diseases. The body has several defenses against oxidative stress. Among these means are cited enzymes, proteins chelating and transition metals (Perez-Matute *et al*., 2012). There are other ways to wrestling against oxidative stress such as biological antioxidants. These are biological substances exogenous that protect biological systems against the deleterious effects of oxidative stress (Perez-Matute *et al*., 2012). The polyphenols are one of these biological substances. Their antioxidant properties are attributed to the ability of these natural compounds to scavenge free radicals such as radicals hydroxyles (OH•) and superoxydes (O_2•) (Popovici *et al*., 2009).

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Moreover, the *oleander* "*Nerium oleander*" is a plant used in Mediterranean and Asian traditional medicine. The several ethnobotanical studies show the importance of *N. oleander* in the treatment of several diseases. Recipes prepared with *N. oleander* are described in cases of ulcers and even to induce abortion (Hseini and Kahouadji, 2007). In some Moroccan areas, the leaves are used in maceration and in external friction tale scabies, lice, hair loss, diabetes and toothache (Lahsissene *et al*., 2009).

The objective of this study was firstly to accomplish a phytochemical investigation of the leaves of *N. oleander* from two areas of Marrakech. For this, a mineral analysis of leaves was completed. Moreover an extraction and assay of the phenolic compounds by spectrophotometry and HPLC were carried out. Similarly, the evaluation of the antioxidant activity of this fraction by the method of DPPH was made; allowing defining extracts with antioxidant activity. These extracts are first exhausted with organic solvents of increasing polarity.

2. MATERIALS AND METHODS

2.1. Plant Material

Leaf harvesting of *N. oleander* was conducted in January. Sampling was conducted in two areas of the city of Marrakech. In the Ourika valley located 40 km south and characterized by a semi-arid climate and temperature 17,6°C and in the gardens of the city of Marrakech. Samples transported to the laboratory has been cured at room temperature away from sunlight and then finely ground.

2.2. Methods

2.2.1. Mineral Composition

2.2.1.1. Content on Ashes

The content of ashes was determined by calcinations of 1 g of powder of plant material to 105°C. After 4 hours, the rate of ash is obtained by weighing samples.

2.2.1.2. Dose of Mineral Elements

0.1 g of ash obtained was dissolved in 0.6 mL of 1N HCl. After evaporation, it is added to 5 mL of distilled water. The determination of phosphate was performed by the colorimetric method, based on the properties of molybdates which in the presence of phosphorus in acid medium, give birth to a phosphomolybdic complex. The dosage of potassium and sodium were realized by using the flame spectrometry. The concentrations were calculated using the calibration ranges from 10 to 50 mg L^{-1}. For phosphorus, the reference range is prepared in the same conditions with KH2PO4 concentrations ranging from 0 to 0.8 mg L^{-1}.

2.2.1.3. Dose of Nitrogen

The determination of nitrogen consists on a mineralization of organic nitrogen in to ammoniacal nitrogen by concentrated sulfuric acid in warm and in the presence of a catalyst of mineralization (mixture of potassium sulphate, copper sulphate and selenium which makes the action of the acid more effectively by increasing the boiling temperature). Titration of the distillate was directed by H2SO4 (N/14).

Total nitrogen = N (Ve-Vt)x1.4/p. Ve: Volume of the sample, Vt: Volume control, N: Normality of sulfuric acid, P: Taking of tests.

2.2.1.4. Protein Dose

The protein percentage is calculated from the total nitrogen content by the following relation % Protein = % total nitrogen * 6,25.

2.2.2. Extraction and Purification of Polyphenols

The polyphenol extraction is made according to the method described by Macheix *et al*. (1974). This method consists in macerating 10 g of plant powder in methanol-water (160/40: v/v). After filtration, the aqueous-alcoholic extracts undergo vacuum evaporation. The aqueous phase is depigmented three times with hexane to remove nonpolar compounds. The aqueous phase is extracted with ethyl acetate (25 mL: 3 times). The organic phase was concentrated under vacuum. It is then recovered with 2 mL of methanol.

2.2.3. Determination of Polyphenols

The content of phenolic compounds was estimated by the Folin-Ciocalteu method. About 20 µL of the methanol extract is added to 1745 mL of distilled water and 250 µL of diluted Folin reagent 1/3. After stirring, 500 mL of sodium carbonate solution at 20% is added. The tubes are incubated at a temperature of 40°C for one hour. The absorbance was measured at 760 nm. The quantity of polyphenols was deduced from the calibration curve established with caffeic acid at concentrations ranging from 0 to 50 g. The results are expressed as mg of caffeic acid equivalent per gram of dry weight.

2.2.4. Analysis by High Performance Liquid Chromatography (HPLC)

The chromatographic analysis of phenolic compounds in leaves of *N. oleander* was performed by

a HPLC system KNUAER, conducted of: Automatic injector, autosumpler 3950, manager 5050 PDA detector diode 2800 (Diode Array Detector: UV + Vis) and pump 1000. The column used for the separation of type Eurospher II 100-5 C 18, 250×4.6 mm precolumn. The temperature was maintained at 25°C. The injection volume is 10 µL. The mobile phase consisted of Acetonitrile (A) and the ultra-pure water (B) at pH 2.6 adjusted with orthophosphoric acid, initially 5/95% and following the gradient in the table. The flow rate is 1 mL min^{-1}. The total time of analysis was 60 min. the peaks were identified using 15 standards: Catechine; epicatechine; chlorogenic acid cafeic acide; rutine; protocatechine acid; galic aid; cinnamic acide; fereulic acid; p coumaric; shikimic acid; vanillic acid; quinonic acid; dihydroxybenzoic acid and quercitine.

2.2.5. Extraction with Organic Solvents

The extracts were obtained after depletion coarse with methanol at room temperature for 12H. The resulting liquid is evaporated under vacuum. The dry residue is dissolved in 300 mL of distilled water heated. This phase is exhausted successively with solvents of increasing polarity: Hexane, dichloromethane, ethyl acetate and finally with n-butanol. The aqueous extract is obtained by a decoction of the 10g plant powder in 100 mL of water. The resulting solutions were evaporated until dry residue. They were stored at 4°C.

2.2.6. Evaluation of the Antioxidant Activity of Organic Extracts and Phenolics Compounds

The detection of anti-radical substances of the extracts was performed by the method of DPPH (Sgherri *et al*., 2012). About 100 µL of each extract is placed in a tube which was added 900 µL of DPPH solution (0.004% in methanol). After shaking, the tubes are placed in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. The percentage inhibition of free radical DPPH (I%) was calculated by the following formula:

$$I\ \% = (A\ blanc - A\ sample / A\ blanc) \times 100$$

Evaluation of antioxidant activity is also estimated by IC50 value corresponds to the minimum concentration of the extract can reduce 50% of DPPH. It is determined graphically.

2.2.7. Statistical Study

The results are expressed as mean ± standard deviation. They are subjected to analysis of variance ANOVA (SPSS 10.0 for Windows). Means are considered significantly different when $p \leq 0.05$.

3. RESULTS

3.1. Mineral Analysis

3.1.1. Ash Content, Potassium, Sodium and Phosphorus

The mineral analysis of *N. oleander* leaves showed high levels of ash and potassium (**Fig. 1**). For cons, the sodium and phosphorus levels are very low. The leaves of *N. oleander* gardens of Marrakech have concentrations higher than the region of Ourika.

3.1.2. Nitrogen Content and Protein

The leaf analysis (**Fig. 2**) in terms of their nitrogen concentrations and protein showed an average of nitrogen 0.97±0.004 mg g^{-1} for the region of Marrakech which is significantly higher ($P = 0.03$) than that of Ourika (0.17±0.001 mg g^{-1} MVS). Protein levels are significantly elevated.

For the following studies, only the results of the fraction of Marrakech are presented. The other fraction has similar results.

3.1.3. HPLC Analysis

Figure 3 show that the phenolic extract of the leaves of *oleander* has several deerived phenolics. The major peak corresponds to the cinnamic acid in comparison with standards used (retention time of 43.9 min). The compounds identifed in comparison with standrads used are: chlorogenic acid, rutin, catechin the epicatechin, quercitin, quenonic acid and shikimic acid.

3.2. Evaluation of the Antioxidant Activity of Organic Extracts and Phenolics from the Leaves of *N. oleander*

Figure 4 shows a significant increase in percentage inhibition of DPPH according to the concentration of phenolic extract. The fraction of *N. oleander* study presents a powerful anti-radical activity. This activity is comparable to that of ascorbic acid (vitamin C). Statistical analysis did not allow to unsaddle a significant difference between the values of the two fractions. Indeed, the IC50 value is 0.43 mg/g ±0.05 for *N. oleander* and 0.58 mg/g ±0.05 for ascorbic acid.

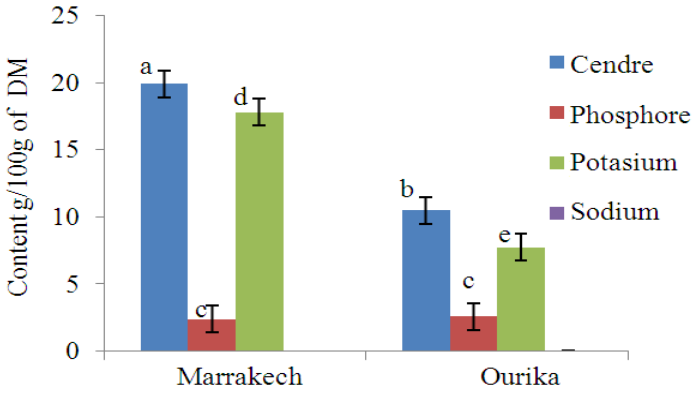


Fig. 1. Levels ash, sodium, phosphorus and potassium expressed in g/100 g of dry leaves *N. oleander*

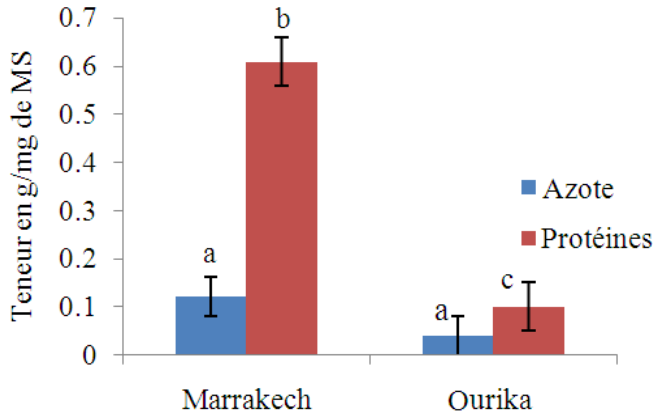
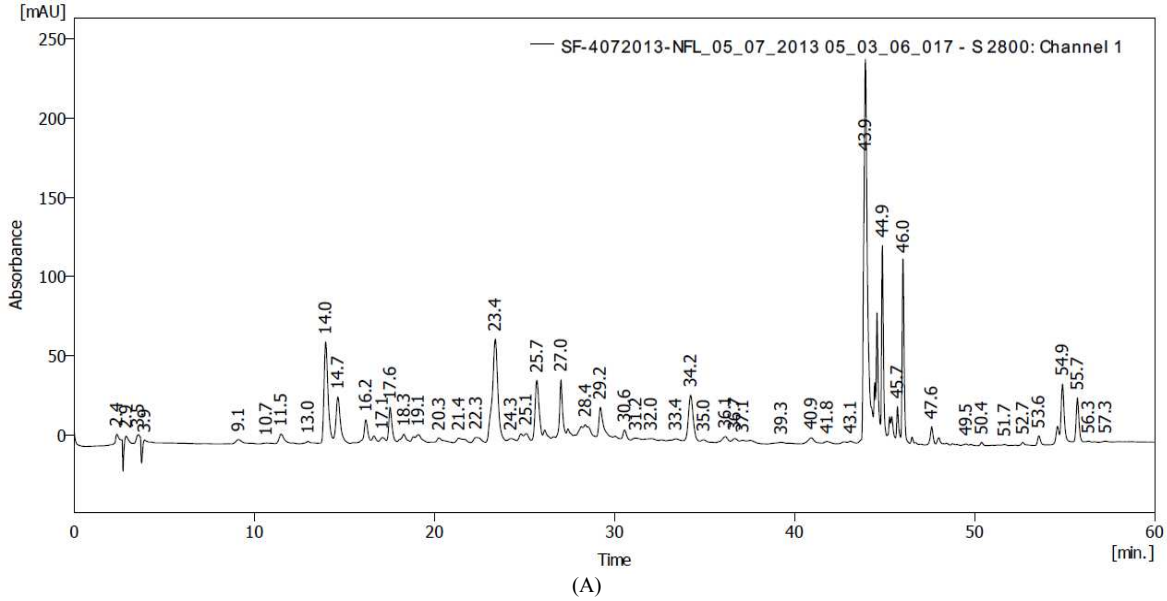


Fig. 2. Concentration of total nitrogen and leaf protein in g/mg of dry matter in both regions (Marrakech and Ourika)



(A)

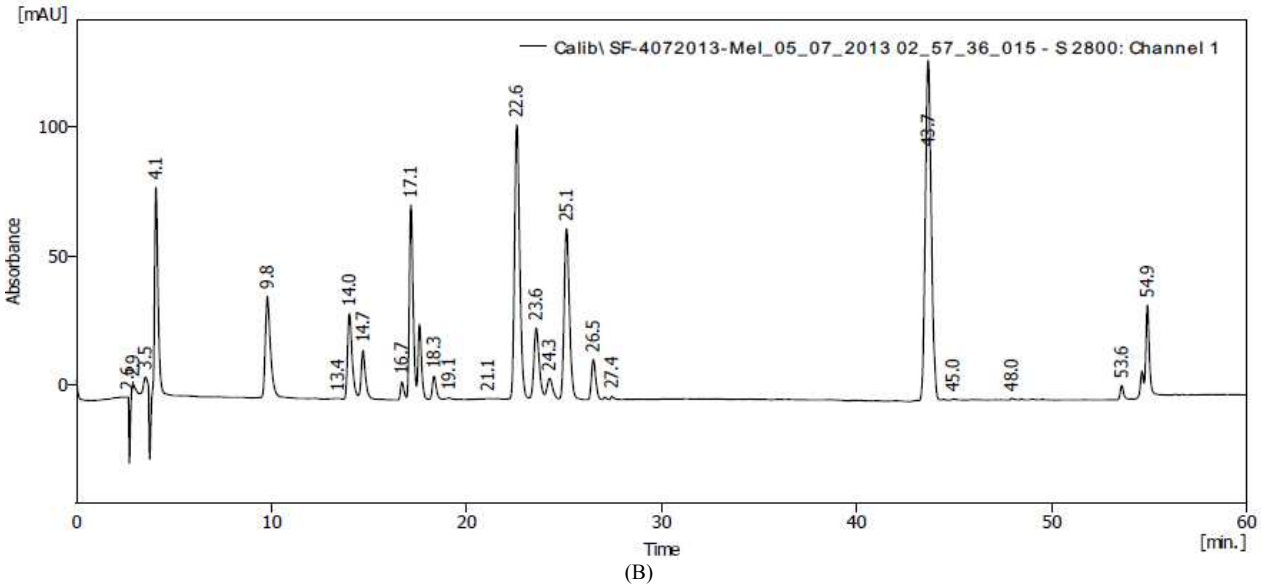


Fig. 3. (A) Chromatographic profiles of phenolic compounds from the leaves of Nerium from the garden. (B) profile of standards mixture: catechine, epicatechine, chlorogenic acid, cafeic acide, rutine, protocatechine acid, galic acid, cinnamic acide, fereulic acid, p coumaric, shikimic acid, vanillic acid; quinonic acid, dihydroxybenzoic acid and quercitin

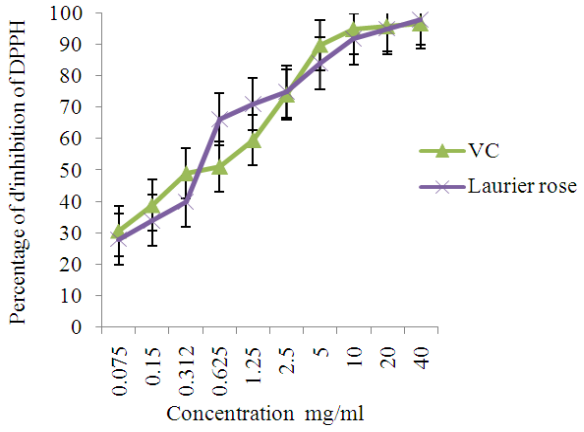


Fig. 4. Antioxidant activity of the phenolic fraction of *oleander* and ascorbic acid (DPPH% inhibition) versus different concentrations of phenolic extracts

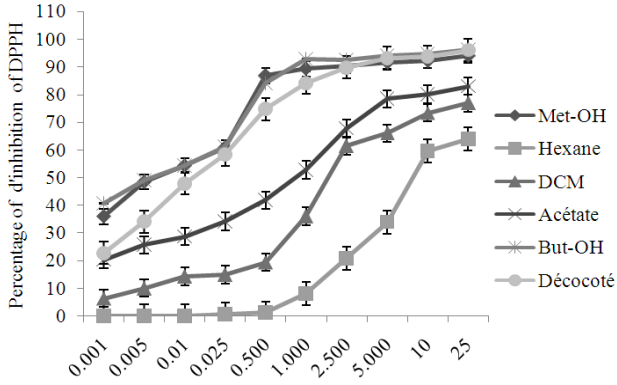


Fig. 5. Antioxidant activity (% inhibition of DPPH) under different concentrations of the extracts of the leaves of *N. oleander*

4. DISCUSSION

The antioxidant activity of different extracts of *N. oleander* is shown in **Fig. 5**. It demonstrates an increase in inhibition of DPPH as the extract concentration. The methanol extract, N-butanol and the decoction as a percentage of inhibition of DPPH with higher values that exceed 90% at a concentration of 5 mg mL^{-1}. The IC50% values most marked is: Methanol and decocted with N-butanol values 0.005±0.000, 0.018±0.006, 0.005±0.000, respectively.

This study shows that leaf *N. oleander* has significant content levels of potassium and protein. The value of potassium is very high compared to those reported by Ennabili (2008) who studied the content of this compound in *N. oleander*. The same authors reported greater nitrogen content of what is shown in this study. Polyphenols are the compounds most important secondary metabolites, through their antioxidant and antimicrobial activity. This study has

highlighted, for the first time, that the leaves of *N. oleander* have a very large quantity of total polyphenols. The chromatographic profile of the phenolic extract shows that these fractions have multiple different types of compounds. The major compounds were cinnamic acid. Cinnamic acid is often characteristic of a plant species or even of a particular organ or tissue of that plant. Previous studies have shown the pharmacological properties of cinnamic acid including hepatoprotective, antioxidant activity and anti-diabetic activities (Adisakwattana *et al*., 2012). The antiradical capacity, proven earlier in this study, *oleander* is due to this compound. The roles mentioned could explain the use of this plant in traditional Moroccan medicine. In addition, this plant could be a source of drugs recommended in principle health problems mentioned above due to its availability and its wide geographic dispersion in Morocco and other countries.

Since antiquity, natural products, especially those of plant origin have always been an important source of therapeutic agents. Recent data from the pharmaceutical industry show that natural products represent a valuable source for the production of new chemical entities. Indeed, Reactive Oxygen Species (ROS) released by the human body are eliminated by molecules with antioxidant properties. Antioxidants are found in all plants (Muanda *et al*., 2011). The results obtained highlight thevery importante antioxidant activity of phenolic fractions very important. Another study by Rajendran (2011) showed that the essential oils of laurel have a very large quantity of saturated flavonoid and have a very important antioxidant activity. The organic extracts are an inexhaustible source because of their high yields. Evaluation of antioxidant potential of extracts of leaves *N. oleander* and the values of IC 50% found can be separated into two groups: the first group, which includes methanolic decoction and N-butanol extracts, is a very strong activity with IC 50% less than 8 mg mL^{-1}, much lower than the value of phenolics (430 mg mL^{-1}). This is partly comparable with the values reported by Mohadjerani (2012) found that the methanol extract has the most potent reducing agents. Govind *et al*. (2011) also recorded remarkable antioxidant effect of the extract. The second group is the ethyl acetate extracts, dichloromethane and hexane 50% CI which is between 0.5 and 6 mg mL^{-1}. The antioxidant capacity of the first group may be due to the presence of phenolic compounds.

5. CONCLUSION

The use of plants that contain bioactive compounds is steadily increasing. The primary objective of this study was firstly, to analyze mineral *oleander*. On the other hand, highlight the antioxidant capacity of the phenolic fraction and organic extracts and define the phenolic composition of *N. oleander* by spectrophotometry and HPLC. In the first part of this study, mineral analysis has highlighted significant wealth *N. oleander* in potassium and nitrogen. The calculation of the protein shows a wealth of *oleander* leaves. This study showed, for the first time, a very important anti radical activity in relation to phenolic compounds. This activity is comparable to that recorded by ascorbic acid. Thus, *oleander* has a very important source of antioxidants, they would protect the human body against several diseases. HPLC analysis of phenolic compounds helped to a very important wealth in various phenolic compounds. Products identified are cinnamic acide, chlorogenic acid, rutin, catechine, epicatechin, quercitin and quenonique acid, the majority element, presents very interesting virtues. This product is originally a very important role of *oleander* and explains the use and position of the plant in Moroccan pharmacopoeia. Other compounds separated by HPLC should also be identified in order to prove the advantage of *oleander* use in traditional medicine.

Alongside this study, an analysis of the phenolic fraction by HPLC-MS is done to separate the compound identified. Also a study of the antibacterial and antifungal activity of organic extracts of *N. oleander* is underway.

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