

Abstract

This study compared antioxidant (DPPH, ABTS, FC) and anticarcinogenic (MTT test) activities of three hydroethanolic extracts from different morphological parts of *Nerium oleander* lin, (leaves (LE), pink (PFE) and white flowers (WFE)). All of the extracts exhibited different antioxidant and anticarcinogenic activities based on their antioxidant presence in the extract. Principal component analysis (PCA) was applied to understand the relationship between the antioxidant and anticarcinogenic activity, and to gain insight into the similarities and differences between the three extracts. Basing on the set data, this analysis clearly indicate that WFE have more potent antioxidant activity and a potential to inhibit HT29 colorectal cancer cell growth than LE and PFE respectively. The FTIR analysis indicated the presence of various functional groups such as alcohol, phenol, alkane derivatives, aromatic compound, that are responsible for the biological properties of studied extracts.

Keywords: *Nerium oleander*, antioxydant activity, anticarcinigenic activity, PCA analysis, FTIR analysis

1.Introduction

Oxidative stress in animal cells causes damage to biological molecules. The accumulation of such damage is the cause of various diseases, particularly cancer (Koss-Mikołajczyk, 2019^a). Cancer is a major public health problem worldwide. Its treatment and prevention are difficult because of the different forms of occurrence that result from it. (Stevigny, Bailly et Quetin-Leclercq, 2005). Antioxidants are the first choice of chemoprevention when it comes to cancer. Many synthetic antioxidants are known, but their use is gradually being restricted because of their potential toxicity^[1] Therefore there is a need for more effective, less toxic and profitable antioxidants. For this reason, there has been an

increasing demand for antioxidants of plant origin [2]. In recent years, many researchers have been trying to find and isolate antioxidants from medicinal plants with known anticancer properties (Koss-Mikołajczyk, 2019^b, Koss-Mikołajczyk, 2019^c, **Popovici et al., 2009**),

Nerium Oleander Lin is an evergreen medicinal plant belonging to the Apocynaceae family. It is used in traditional Mediterranean and Asian medicine. It is the only species currently classified in the genus *nerium*. The flowers of this plant are produced in terminal cluster of about 5 cm in diameter with five petals and different colours. *N. oleander* has been described as highly toxic to humans, animals and certain insects due to the presence of cardiac glycosides mainly nerine and oleander at a high concentration with digitalilike activity (**SD Langford and PJ Boor , 1996; PK Mazumder, 1994; Radford DJ et al; 1986**). However, despite its toxicity, a range of pharmacological activities has been described: antiulcer (**Sabira, 1998**), hepatoprotective[3], anticarcinogenic[4], anti-diarrheal and cytotoxic activities[5] larvicides (**Raveen, 2014**), anthelmintic[6]. *N. oleander* leaves exhibit cardiotonic, diaphoretic, diuretic, anticancer, antibacterial [7] and antifungal activities (**Wang et al 2000**). The bark is used as an expectorant, cardiac tonic, diuretic, emetic and diaphoretic (**Patel et al. 2010**), plus steroids which have medical properties such as anti-inflammatory, hepatoprotective, potentially immune, antipyretic, antioxidant, antifungal, anticancer and anti-HIV properties [8]. Terpenes, flavonoids and polyphenols have also been described as active pharmacological components of *N. oleander* (**Goraksh JH. 2016**). Previous studies showed that *Nerium oleander* flowers are emetic, diaphoretic, cardiotonic, diuretic, expectorant and sternutatory (**Asha & Chakraborty 2010**), as well as the cardioprotective and antioxidant potential of ENO against myocardial oxidative damage induced by isoproterenol in experimental rats [9, 10]. **WHO???** and his colleagues were able to extract the essential oil from *N.oleander* pink flowers and show its anti-tumor activity on the Ehrlich Ascites Carcinoma (EAC) cell lines. A wide range of polyphenols is present in *N. oleander*, as

revealed by[11].Cinnamic acid was the main component and otherswere: epicatechin, catechin and chlorogenic acid. Other pharmacologically active compounds which are cardiac glycosides have been identified such as nerine, oleandrin, cardenolides, gentiobiosyl, oleanine and odoroside [12].

Previous studies focused mainly on the leaves of *N. oleander*, moreover, most studies on the anticarcinogenic effects of have been conducted with individual phenolic compounds and the potential synergistic effects of the compounds and their interactions in extract were rarely evaluated and studied [13].Therefore, in the present work we investigated chemical compositionof extracts from *N. oleander* leaves and flowers using FTIR analysis and evaluated their antioxidant andanticarcinogenic effects *in vitro*.Principal Component Analysis (PCA) was applied to understand therelationship between the antioxidant and anticarcinogenic activities measured.The extracts was obtained by microwave assisted extraction (MAE), using the extraction conditions optimized previously by AYOUAZ, Oliveira-Alves [14] from *N. oleander* leaves.

2.Materials and methods

2.1.Chemicals and reagents

The following chemicals and biochemicals were used: disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid (HCl) and chloride aluminium ($\text{AlCl}_3, 6\text{H}_2\text{O}$) were purchased from Prolabo (Loire, France); 2,2-azinobis-(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid)diammonium salt (ABTS), 1-diphenyl-2-picrylhydrazyl (DPPH), thiazolyl blue tetrazolium bromide (MTT), phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin–streptomycin solution and McCoy’s medium were from Sigma Aldrich (Stenenheim, Germany). Folin–Ciocalteu’s phenol reagent (FC) was from Merck (Darmstadt, Germany). Water was purified with a QPLUS185 system from Millipore (Bedford, MA,

USA). Standard antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma–Aldrich (USA)

2.2. Plant material

Fresh leaves and flowers of *N. oleander* were collected from the Kherrata region in Algeria (36°29'34''N; 5°16'39''E) at an altitude of (600m). The samples were dried in an oven at 40°C until the stabilization of their weights. The samples were then pulverized using an electric grinder and sieved through a standard 250µm-125µm sieve, the powder was stored in airtight bags until further use.

2.3. Microwave-assisted extraction (MAE procedure)

N. oleander powder was extracted using a microwave oven (Samsung Model NN-S674MF, Kuala Lumpur, Malaysia) with a working frequency of 2450 kHz. The oven was adjusted to condense the vapours emitted during extraction in order to obtain a constant sample. The control of the irradiation system in the oven is digital, as well as for the power which is adjustable between (100W-900W). The extraction conditions used have already been optimised in laboratory in our previous work [14]. Briefly, 1 g of powder has been weighed in a 100 ml flask and mixed with a volume of 20 ml of 35 % ethanol. The suspension was then microwaved (500W, 60s). The crude extract was retrieved by volumetric flasks and filtered through filter paper, then processed for later use at 4°C. The extraction solvent (ethanol) has been chosen based on the results of our preliminary study.

2.4. FTIR spectrum analysis

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in tested

compounds. The FTIR spectrum was used to classify the functional groups of the active components based on the peak value in the infrared radiation region. *N. oleander* aqueous extracts were combined with KBr, salt, using a mortar and pestle, and compressed into a thin pellet and infrared spectrum and peak values were recorded on a Perkin Elmer FTIR spectrometer, between 4000–400 cm⁻¹.

2.5. Extraction yield

The percentage extraction yield (%) was calculated by the following formula:

$$Yd = (PC / PD) \times 100$$

PC: weight of crude extract, PD: weight of the dry plant powder.

2.6. Antioxidant activity

Antioxidant activities of the plant extracts (LE, WFE and PFE) were studied according to the methods previously described by (Koss-Mikołajczyk, Kusznierevich [15], Koss-Mikołajczyk, 2019^d). Three spectrophotometric tests (DPHH, ABTS and FC) were used to evaluate the *in vitro* antioxidant capacity of ethanolic extracts from *N. oleander*.

For the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity, a calibration curve was prepared using Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) as a standard. Briefly, after the adjustment of the absorbance of DPPH on 1.00 ± 0.02, 1 mL of DPPH was added into each well of 48 wells plate. Next 30 μl of plant extract or standard solution was added in 3 repetitions, the plate was then left at room temperature for 15 min. After incubation the absorbance was measured, at λ = 515 nm. The obtained results were expressed as Trolox Equivalents (TE μmol / mL).

The Free radical-scavenging activity (ABTS) is based on the ability of an antioxidant to stabilize the cationic radical ABTS⁺ of blue green coloration by transforming it into colourless

ABTS. Briefly, 1ml of ABTS has been applied to each well of 48well plate. Next, 10ul of plant extracts or standard solutions (Trolox) has been added andthe plate has been left at room temperature for 15min.After incubation the absorbance was measured, at $\lambda = 734$ nm.The obtained results were expressed as Trolox Equivalents (TE $\mu\text{mol} / \text{mL}$)

For the antioxidant activity measured by Folin–Ciocalteu reagent(FC) 1 ml of FC reagent was applied to each well of 48-well plate and 0.1 ml (100 μl) of plant extracts or standard solution (Trolox)was applied and the plate was left at room temperature for 15 minutes. After incubation the absorbance was measured, at $\lambda = 750$ nm.The obtained results were expressed as Trolox Equivalents (TE $\mu\text{mol} / \text{mL}$)

2.7. Evaluation cytotoxic activity

2.7.1. Cell culture

In the Smart Cell Incubator (Heal Force), human colon adenocarcinoma (HT29) cells from the ATCC cell culture set (USA) were grown at 37°C in humidified atmosphere containing 5 % CO₂. The cells were grown in McCoy's medium supplemented with L-glutamine (2 mol / L), sodium pyruvate (200 g / L), fetal bovine serum (100 mL / L), and antibiotics – Penicilin (10000 U / L)and streptomycin 100 g / L).HT29 cells were used to evaluate the cytotoxic activity of the tested extracts.

2.7.2.Cytotoxic activity towards HT29 cells

In order to evaluate cytotoxic activity of tested plant extracts HT29 cells were seeded in 96-well tissue culture plates (about 10,000 cells / well in 0.15mL of the medium) and allowed to settle at 37 °C for 24 h. They were then treated for 6 or 24 h with studied plant extracts (0.05mL of different concentrations 1–15 % v/v in PBS).The cytotoxic effect of tested plant extracts was determined using MTT assay as described previously by (Koss-

Mikołajczyk, Kusznierewicz [15], Koss-Mikołajczyk, 2015) Plant extracts' growth inhibitory effect was expressed as a percentage of untreated cell growth (% control).

2.8. Statistical analysis

All analyzes we carried out in triplicate and the results were expressed as means $\pm$ standard deviations (SD). The correlation coefficient of the different properties was determined using Excel 2016. PCA transforms the original measured variables into new uncorrelated variables called principal components. The first component describes most of the variation in the data. The second principal component is orthogonal and covers much of the remaining variation. Analyzed data was standardized to account for the different magnitude so the responses, and parameters contribute equally to the data set variance and to the principal component calculation.

3. Results and discussion

3.1. FTIR fingerprinting analysis for functional groups identification

Results of FTIR spectroscopic studies have revealed the presence of various chemical constituents in extracts of *N. oleander* with different peak values (Table 2 and Fig.2) corresponding to 3200, 2900, 1600, 1010 and 796 cm⁻¹ stretching frequency. The IR stretching frequency at 3200 cm⁻¹ correspond to OH group, which confirm the presence of phenolic compounds in the extracts (LE, WFE, PFE), our previous study confirmed the presence of polyphenols in leave and red flower extracts of *N. oleander* [14]

The peak 2900 cm⁻¹ confirms the presence of an alkane derivative, which corresponds to the -CH alkyl group. The extracts exhibited the characteristic absorption bands were exhibited at 2900, 1010 cm⁻¹ for C=OH group. A peak at 1600 cm⁻¹ and 796 correspond to (C=C) aromatic group, and CH aromatic group respectively.

3.2. Extraction yield

The extracts studied in this work were obtained by microwave assisted extraction. We find that the yields of hydro-ethanolic extraction vary considerably. WFE clearly gives the best yield (26%,) followed by PFE (21%) and the lowest yield is that of LE (20%). Several factors can influence the performance of the extraction, such as particle size, temperature, extraction time, the nature of the solvent, the hydroalcoholic mixtures as solvent give satisfactory results in an extraction process [16].

3.3. Antioxidant activity

Most of the work published on *N. oleander* is limited to evaluating the antioxidant capacity of its essential oils. Some studies revealed that ethanolic and methanolic extracts of *N. oleander* flowers show an effective antioxidant activity, which includes the reducing power, DPPH, FRAP, ORAC, CUPRAC [14, 17]. Vinayagam and Sudha [18] carried out a study on the methanolic extract of the flowers and leaves *N. indicum* in which the activity is expressed as a percentage inhibition of the free radical. The study found that the inhibition level of free DPPH radicals for leaves and flowers was 72.8% and 67% respectively, and that superoxide anion radical flowers scavenging activity was 66% compared to leaves 25%. Similarly, lipid peroxidation in flowers exhibited greater activity than leaves. The results clearly indicate that the methanol extracts of *N. indicum* flowers have more potent antioxidant activity than its leaves. In contrast, white flowers have not been the subject of any chemical or biological study.

In this study three different spectrophotometric methods were performed to estimate the antioxidant activity of the extracts: DPPH, ABTS and FC. The antioxidant activities were expressed as Trolox equivalents (TE $\mu\text{mol} / \text{mL}$). In FC test (Table 1), LE showed the highest antioxidant activity (427.87 ± 10.42 TE $\mu\text{mol} / \text{mL}$), followed by WFE (.....) and the lowest was showed by PFE (356.3 ± 3.9 TE $\mu\text{mol} / \text{mL}$). In DPPH test it was WFE that exhibited highest DPPH radical scavenging activity (212.68 ± 27.43 TE $\mu\text{mol} / \text{mL}$) from all tested

extracts. Similarly, the highest ABTS radical scavenging activity was showed by WFE(399.40 ± 52.02 TE umol / mL).

Pearson's correlation analysis revealed that results obtained by FC test showed positive correlations with DPPH radical scavenging activity and ABTS radical scavenging activity. The test also revealed a strong positive correlation between DPPH and ABTS.

FTIR analysis revealed the presence of OH and C-OH alcohol groups, which may correspond to polyphenols. Phenolic compounds are very important plant constituents with several biological functions, including antioxidant activity due to their ability to recover radicals because of their OH groups [18].

3.4. Evaluation of anticarcinogenic activity

Human colon adenocarcinoma (HT29) cells were used to evaluate the cytotoxic effect of WFE, PFE and LE from *N. oleander*. Different concentration of extracts were tested for the ability to inhibit the growth of HT29 cells. **Fig.1** shows anticarcinogenic effect of tested plant extracts at various doses (0.1, 0.02, 0.5, 0.75, 1 mg / mL) against HT29 cells for 24h using the MTT test. The MTT cell proliferation test measures the rate of cell proliferation and conversely, the reduction in cell viability. The in vitro results concluded that the tested plant extracts have a dose-dependent inhibitory effect on HT29 cell growth, and the inhibition of cell growth is directly proportional to the dose.

A very weak correlation exists between FC and the capacity to inhibit the proliferation of HT29 cells with the C5 concentration, while a very strong positive correlation exists between DPPH and ABTS with the capacity to inhibit HT29 cancer cells with the concentration C5 and C6. This indicates that in the extracts studied, the bioactive molecules ensuring the scavenging power of free radicals (DPPH and ABTS), are themselves responsible for the inhibition of the proliferation of HT29 cells. This leads us to conclusion that, while the

concentration of the phenolic compounds is an important factor but it is not always sufficient, there is another criterion relating to the phenolic compounds to be taken into account in the interpretation of the activity.

The tested plant extracts showed the ability to inhibit cell growth in a dose-dependent manner. To the best of our knowledge, the anticarcinogenic activity of the extract from *N. oleander* white flower has never been studied before. The published work on the biological activity of *N. oleander* is limited to the evaluation of the antiproliferative capacity of cardiac glucoside or individual polyphenol. The FTIR analysis revealed the presence of the OH and C-OH alcohol groups which may correspond to polyphenols, our previous study [14] revealed the presence of polyphenols such as quercetin, chlorogenic acid, and certain glycosidic flavonoids in *N. oleander* leaves extract which may be responsible for the anticarcinogenic activity. Secondary metabolites such as phenolics have a powerful power to pierce free radicals [19]. Natural antioxidants increase the antioxidant capacity of plasma and reduce the risk of certain diseases such as cancer, heart disease and stroke. The study by Namian, Talebi [20] showed that the methanolic extracts of *N. oleander* showed to inhibit the proliferation of three different cancer cell lines T47D, HepG-2 and K562. The authors suggested that this effect could be related is linked to a cardiac glycoside, oleandrin, which is the main active compound in the fight against cancer in *N. oleander*.

3.4. Principal component analysis

Principal component analysis (PCA) was performed to study the correlation between the indices of evaluation of antioxidant (DPPH, ABTS, FC) and anticarcinogenic activities measured in *N. oleander* leaves, pink and white flowers. Relations between primary variables and obtained main components is shown graphically in Fig. 3. Each variable is represented by a vector.

Principal component analysis (PCA) is the statistical tool used to explain differentiation between samples and to obtain more information on the variables that mainly influence the sample similarities and differences. This procedure extracts the dominant patterns in the data matrix in terms of a complementary set of scores and loading plots. PCA permits us to achieve a reduction in dimensionality, data exploration for finding relationship between objects, an estimation of the correlation structure of the variables and an investigation of how many components (a linear combination of original features) are necessary to explain the greater part of variance with a minimum loss of information [21].

PCA results revealed that the first two principal components accounted for 100 % of the total variance: PC1 was responsible for 62.34 % and PC2 described 37.66 % of the data variability. The values of HT29C5, DPPH and ABTS (0.998, 0.993, 0.993) are strongly correlated with first principal component (PC1) followed by HT29C6 and FC with values 0.831 and 0.449, respectively, while HT29C4, HT29C2 and HT29C3 (0.992, 0.941 and -0.066) are correlated with second component (PC2). Pearson's correlation analysis showed also that FC showed correlations with DPPH radical scavenging activity, ABTS radical scavenging activity, and with activity to inhibit HT29 cancer cell proliferation with the C5 concentration, indicating that these compounds contribute significantly to the antioxidant capacity, while its correlations were negative ($P > 0.05$) with the activity of inhibiting the proliferation of HT29 cancer cells with the concentrations C2, C3, C5 and C6. In addition, the DPPH radical scavenging activity, the ABTS radical scavenging activity and the activity of inhibiting the proliferation of HT29 with C5, C6 as well as the radical scavenging activity ABTS with the activity of inhibiting the proliferation of HT29 with C5 and C6, are strongly positively correlated with each other.

As shown in **Fig.3**, almost all input variables are located near the circle, which means that most of the information contained in these variables is carried by the main components. The

fact of placing variables next to each other indicates a strong positive correlation. We can observe from the graph that the angle between the value of DPPH and ABTS as well as the value of DPPH and HT29C5 is very small, which indicates that these factors are positively correlated. Vectors perpendicular to each other indicate a lack of correlation (DPPH and HT29C4).

Conclusion

Based on the research, using principal component analysis (PCA), we visualized the relationships of the investigated phytochemical parameters of three extracts from *N.oleander* (WFE, PFE and LE). The results showed that PFE has more potent biological activity compared to LE and WFE. *N. Oleander* flowers can therefore be a good source of natural antioxidant and anticancer agents and as well as a possible pharmaceutical supplement.

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Conflict of interest

All authors confirm they have no conflict to declare

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