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ORIGINAL ARTICLE

Anticarcinogenic and Antioxidant Activities of Leaves and Flowers Hydroalcoholic Extracts of *Nerium oleander L.*: PCA Analysis and Phytochemical Content by FTIR Spectroscopy

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ABSTRACT

Aims: The aim of this work is the evaluation the anticarcinogenic effect on HT29 cancer cells lines and antioxidant effect of three extracts from different morphological parts (leaves (LE), pink flowers (PFE) and white flowers (WFE)) of *Nerium oleander lin*.

Methods: This research provides the anticarcinogenic activity of extracts from *N. oleander* white flowers. Principal Component Analysis (PCA) was applied to estimate the relationship between antioxidant and anticarcinogenic activities, and to further understand the similarities and differences between the extracts.

Results: All of the extracts exhibited different antioxidant (ABTS, DPPH and FC) and anticarcinogenic activities. FTIR analysis designed the existence of various functional groups in extracts confirming the presence of alcohol, alkanes, amino, aromatic compound and primary alcohol. The PCA results show that the WFE extract exhibits a greater antioxidant activity with a potent potential for inhibiting the cell growth of colorectal cancer HT29 than the two other extracts (LE and PFE).

Conclusions: *Nerium oleander* may therefore be an excellent source of natural antioxidant and anticancer agents, as well as a possible pharmaceutical supplement.

Keywords: *Nerium oleander*, antioxidant activity, anticarcinogenic activity, PCA analysis, FTIR analysis.

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1 INTRODUCTION

Oxidative stress in animal cells can cause damage to biological molecules, and leads to several pathologies, especially cancerous diseases which are among the major public health issues in the world [1]; because cancer treatment and prevention become challenging due to the different forms of occurrence that result [2]. Antioxidant molecules are considered the first choice in cancer chemoprevention, and numerous synthetic antioxidants are well studied, making their utilization restricted due to their potential toxicity [3]. Therefore, there is a need for more effective, for this reason the demand for natural and plant-based antioxidants has increased [4-6].

In recent years, several researchers have attempted to find performant methods aiming to isolate antioxidants from medicinal plants exhibiting anticancer properties [7, 8]. *N. oleander* is a species of shrubs or small trees belonging to the family *Apocinaceae,* utilized in conventional Asian and Mediterranean medicine. This species is currently the unique recognized species belonging to the genus Nerium. This plant

produces terminal clusters of five-petalled blooms with varying colors that measure about 5 cm in diameter. Because *N. oleander* contains cardiac glycosides, particularly nerine and oleander at high concentrations of digitalin linear activity, it has been found to be highly hazardous for humans, animals, and some insects. [9]. However, despite its toxicity, a range of pharmacological activities has been described: antiulcer [10], hepatoprotective [11], anticarcinogenic [12, 13] anti-diarrheal, cytotoxic [14], larvicides [15] and anthelmintic activities [16]. *N. oleander* leaves exhibit cardiotonic, diuretic diaphoretic, antibacterial, anticancer [17], and antifungal activities [18]. The bark is used as an expectorant, cardiac tonic, diuretic, emetic and diaphoretic [19], plus steroids which possess medical properties such as anticancer anti-inflammatory, potentially immune, hepatoprotective, antioxidant, antipyretic, antifungal, and anti-HIV properties [20], terpenes, flavonoids and polyphenols have also been known as active pharmacological components of *N. oleander* [21]. Furthermore, previous studies have shown that *N. oleander* flowers are diaphoretic, emetic, diuretic, cardiotonic, sternutatory and expectorant [22]. The cardioprotective and antioxidant properties of *Nerium oleander* extract against rat experimental models of isoproterenol-induced myocardial oxidative damage [23, 24]. A wide range of polyphenols is found in *N. oleander*, as revealed by Sebeia et al. [25]. Cinnamic acid was the main constituent and others were: catechin, epicatechin, and chlorogenic acid. Other pharmacologically active compounds which are cardiac glycosides have been identified such as nerine, oleandrin, cardenolides, gentiobiosyl, oleanine and odoroside [26].

Early research has mainly centered on the *N. oleander* leaves. To the best of our knowledge, the current study provides for the first time the anticarcinogenic activity of extracts from *N. oleander* white flowers. In our earlier studies, chlorogenic acid and its derivatives, quercetin, and kaempferol glycosylated derivatives were the primary compounds found in the ethanolic extract of *N. oleander* leaves. [27]. In this investigation, we intended to studying the chemical composition of *N. oleander* extracts (leaves (LE), pink flowers (PFE), and white flowers (WFE)) by using FTIR analysis and evaluated *in vitro* its potential anticarcinogenic and antioxidant effects. Principal Component Analysis (PCA) was used to analyze the connection between the antioxidant and anticarcinogenic activities that were measured. Under previously perfected extraction conditions, microwave-assisted extraction (MAE) was used to get extracts of the leaves, pink flowers, and white flowers [27].

2 Material and Methods

2.1 Chemicals and Reagents

The following chemicals and biochemicals were used: disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid (HCl) and trichloride aluminum ($AlCl_3$, $6H_2O$) were purchased from Prolabo (Loire, France); 2,2-azinobis-(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid)diammonium salt (ABTS), 1.1-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 purchased from Sigma Aldrich (Steninheim, Germany). Folin–Ciocalteu's phenol reagent (FC) was furnished by 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 flowers and leaves from *N. oleander* were recovered at an altitude of (600m) from the Kherrata region in Algeria (36° 29'34 " N; 5° 16'39' 'E). The samples were dried at 40 °C in an oven until their weight was stabilized. Using an electric grinder, the samples were then pulverized and sieved through a regular 250 μm – 125 μm sieves and the powder was deposited in waterproof bags before further usage.

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. To obtain a consistent sample, the oven was calibrated to condense the vapors that were released throughout the extraction. Digital technology was used to regulate the oven's irradiation system and to switch between different power levels (100 W – 900 W). The extraction conditions used have already been optimized in the laboratory in our earlier work [27]. Briefly, 1 g of sample was weighed in a 100 mL flask and mixed with a volume of 20 ml of 35 % ethanol. The suspension was then microwaved (500 W, 60 s). For further processing at 4 °C, the crude extract was collected using volumetric flasks and filtered using filter paper. The extraction solvent (ethanol) was selected based on the results of our preliminary study [27].

2.4 FTIR Spectrum Analysis

Probably the most effective method for defining the types of chemical bonds (functional groups) present in tested compounds is the Fourier Transform Infrared Spectrophotometer (FTIR). On a Perkin Elmer FTIR spectrometer, peak values between 4000 and 400 cm^{-1} were observed using *N. oleander* aqueous extracts that had been combined with potassium bromide (KBr), salt, and a mortar and pestle. Based on the peak value, the functional groups of the active components in the infrared radiation region were identified using the FTIR spectrum.

2.5 Extraction Yield

Equation (1) was used to calculate the percentage extraction yield (%):

$$Yd = (PC / PD) \times 100 \ldots\ldots (1)$$

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

2.6 Antioxidant Activity

The antioxidant activities of plant extracts (LE, WFE and PFE) were assessed using the techniques outlined by Koss et al. [28] and Koss et al. [29]. Three spectrophotometric tests (DPPH, ABTS and FC) were carried out to evaluate the antioxidant capacity *in vitro* of hydro-ethanolic extracts of *N. oleander*.

DPPH assay: 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity measures the antioxidant activity of compounds capable of transferring hydrogen atoms. A calibration curve was prepared using Trolox (6-hydroxy-2,5,7,8-tetramethylchro mane-2-carboxylic acid) as a standard ($y = - 0.0008x + 0.4956$, $R^2 = 0.9998$) from 31.25 µg/mL to 1.0 mg/mL. After adjusting the absorbance of DPPH to 1.00 ± 0.02, 100 µL of the sample was added to 1 mL of 5 mmol L^{-1} DPPH- dissolved in ethanol. The samples were incubated for 30 min at room temperature. Each sample was measured in triplicate, absorbances were measured at 515 nm, and results were reported as TE µmol / mL of sample.

ABTS assay: The action of free radical scavenging (ABTS) is based on an antioxidant's ability to stabilize cationic radical ABTS blue-green coloring by turning it into colorless ABTS. To generate a radical stock solution, ABTS powder was dissolved in an aqueous solution of $Na_2S_2O_8$ (4.45 mmol L^{-1}) at a concentration of 7 mmol/L and allowed to stand in the dark at room temperature for 24 hrs. Before measurements, the stock solution of ABTS was diluted in water until the absorbance reached 0.8 ± 0.05 at λ=734. Then, 100 µL of different extracts were added to 3.9 mL of ABTS solution. The absorbance was then measured at 745 nm after 6 min of incubation at temperature. The equivalent capacity was measured using a Trolox curve for the ABTS assay (standard curve equation: $y = - 0.0009 x + 0.4836$, $R^2 = 0.9978$ from 31.25 µg/mL to 500 µg/mL), and the results were presented in TE µmol / mL of sample.

FC assay: Commercial FC reagent was diluted with water in a ratio of 1:9 (v/v). 100 µL of each sample was added to the FC reagent solution (500 µL). After 5 min, aqueous sodium carbonate solution (7.5 % w/v) was added to reach a final reaction mixture volume of 1 mL. The reagents were mixed and the absorbance was measured at 755 nm after 15 min. The results were calculated using a Trolox calibration curve as a standard.

2.7 Evaluation of Cytotoxic Activity

Cell Culture

In a humidified atmosphere containing 5 % of CO_2 in the Smart Cell Incubator (Heal Force), the human colon adenocarcinoma (HT29) cells from the ATCC cell culture set (USA) were grown at 37 °C. In McCoy medium supplemented with antibiotics such as L-glutamine (2 mol / L), sodium pyruvate (200 g / L), fetal bovine serum (100 mL / L) and penicillin (10000 U / L) and streptomycin 100 g / L. Aiming to evaluate the cytotoxic activity of the extracts tested, HT29 cells were used.

Cytotoxic Activity

HT29 cells were plated into 96-well tissue culture plates (approximately 10.000 cells / well in medium 0.15 mL) and allowed to settle at 37 °C for 24 h. Then, they received treatment for 6 to 24 h with investigated plant extracts (0.05 mL of various doses of 1 – 15 % v/v in PBS). The cytotoxic effect was measured using the MTT assay as mentioned above[28] and [30]. The Plant extracts' ability to suppress growth was quantified as a percentage of untreated cell growth. (% control).

2.8 Statistical Analysis

All analyses we performed in triplicate and the results were reported as means ± standard deviations (SD). Using Excel 2016, the correlation coefficient of the various properties was calculated. The original measured variables are transformed by PCA into new, uncorrelated variables termed main components. In the first chapter, much of the variability in the data is explained. The second key variable is orthogonal and encompasses a great deal of the remaining difference. In order to account for the various magnitudes, The studied data were standardized in order to ensure that both the responses and the parameters contribute equally to the variance of the data set and the estimation of the primary variable. PCA analysis was performed using Statistica V.8 software.

3 Results and Discussion

3.1 FTIR Fingerprinting Analysis for Functional Groups Identification

The findings of FTIR spectroscopic analysis have shown the presence of different chemical constituents in *N. oleander* extracts with various peak values (Table 1 and Figure 1) corresponding to 3200, 2900, 1600, 1010 and 796 cm^{-1} stretching frequency. The IR stretching frequency at 3200 cm^{-1} corresponds to the OH group, which confirms the presence of phenolic compounds in the extracts (LE, WFE, PFE). Our previous study confirmed the presence of polyphenols in leave and pink flower extracts of *N. oleander* [27].

The pic 2900 cm^{-1} confirms the presence of an alkane derivative, which corresponds to the -CH alkyl group. At 2900, 1010 cm^{-1} for the C=OH group, the extracts exhibited the characteristic absorption bands. A peak of 1600 cm^{-1} and 796 is equal to the aromatic group (C = C) and the aromatic group CH.

Table 1. FTIR Peak Values of Three Extracts of *N. oleander* (LE, WFE, and PFE)

Frequency (cm^{-1})	Group	Frequency (cm^{-1})	Group	Frequency (cm^{-1})	Group
3200	OH	3200	OH	3200	OH
2900	CH alkyl	2900	CH alkyle	2900	CH alkyl
1600	C=C aromatic	1600	C=C aromatic	1600	C=C aromatic
1010	C-OH primary alcohol	1010	C-OH primary	1010	C-OH
796	CH aromatic	796	CH aromatic	796	CH aromatic

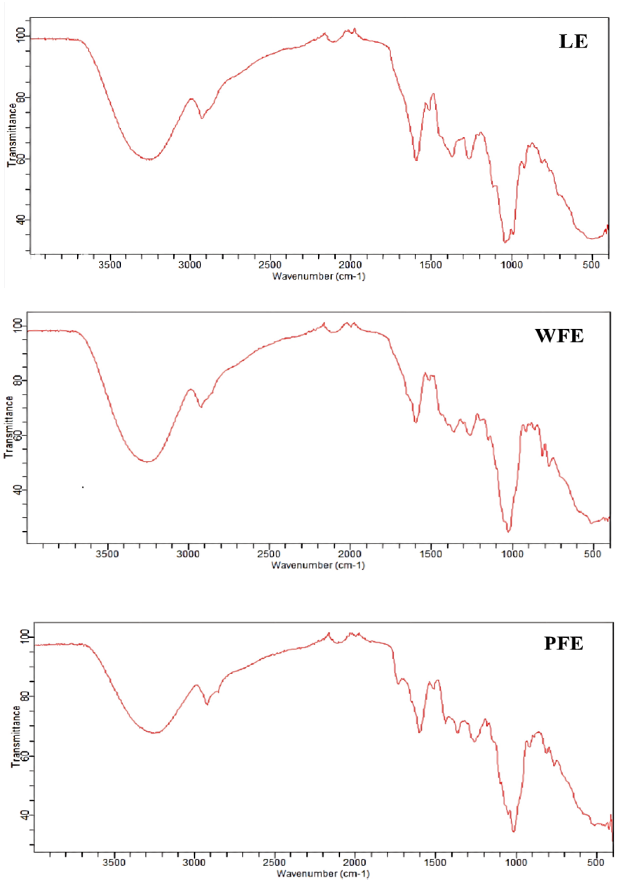


Figure 1. FTIR Spectra of Extracts of *N. oleander* (LE: Leaves; PFE: Pink Flowers; White Flowers (WFE)

3.2 Extraction Yield

In this study, the studied extracts were obtained through microwave-assisted extraction. We found that the yields of hydro-ethanolic extraction vary considerably. WFE clearly provides the best yield (26 %) followed by PFE (21 %). Several factors can influence the performance of the extraction, such as the particle size, the temperature, the extraction time, the nature and the concentration of the solvent which provide satisfactory results in an extraction process.

3.3 Antioxidant Activity

In the current study, three different spectrophotometric techniques were carried out to estimate the antioxidant activity of the extracts: ABTS, DPPH, and FC. The antioxidant activities were expressed as µmole Trolox equivalent per milliliter (µmol TE / mL. The results are shown in Figure 2.

No significant difference was noticed between the three extracts (pink flowers, white flowers, and leaves) for the reduction of DDPH and ABTS radicals, while a significant difference was observed for the pink flowers extract via FC activity.

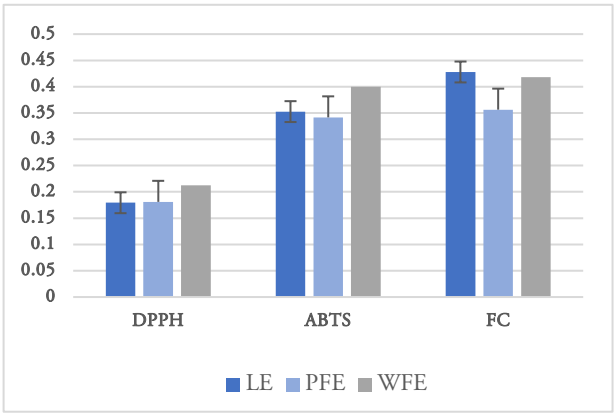


Figure 2. Results of Antioxidant Tests of Extracts of Leaves, Pink Flowers and White Flowers of *N. oleander* (The Absence of Significant Variances ($p < 0.05$) between Values is Denoted by the Same Letters. (LE: leaves; PFE: pink flowers; white flowers (WFE)

Pearson's correlation analysis revealed that the results obtained by the FC test were positively correlated with DPPH and ABTS activities (cf. Table 2). The test also revealed a strong positive correlation between DPPH and ABTS.

Most of the studies published on *N. oleander* are limited to evaluating the antioxidant capacity of its essential oils. Some studies revealed that ethanolic and methanolic extracts of *N. oleander* flowers display an effective antioxidant activity. This activity includes the reducing power, DPPH, FRAP, ORAC,

Table 2. Correlation Coefficients between Assays

Variables	FC	DPPH	ABTS	HT29C2	HT29C3	HT29C4	HT29C5	HT29C6
FC	1							
DPPH	0.338*	1						
ABTS	0.549*	0.972**	1					
HT29C2	-0.993**	-0.224	-0.446	1				
HT29C3	-0.389*	-0.999**	-0.984**	0.277	1			
HT29C4	-0.831**	0.242	0.009	0.891**	-0.189	1		
HT29C5	0.389*	0.999**	0.984**	-0.277	-1.00**	0.189	1	
HT29C6	-0.124	0.892*	0.762**	0.240	-0.866**	0.655**	0.866**	1

DPPH radical scavenging activity; ABTS radical cation scavenging activity; FC: Folin ciacalteu, HT29C: anticarcinogenic activity with concentration (C1, C2, C3, C4, C5 and C6).

and CUPRAC [27, 31, 32] carried out a research on the methanolic extract of *N. indicum* in which the activity was expressed as 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 flower scavenging activity was 66 % compared to leaves 25 %. Likewise, lipid peroxidation demonstrated superior activity in the flowers than in the leaves. The findings clearly demonstrated that the extracts of methanol *N. indicum* flowers possessed an antioxidant activity that is more potent than their leaves. White flowers, by comparison, were not the target of any chemical or biological analysis.

The FTIR analysis revealed the presence of OH and C-OH alcohol groups, which may correspond to polyphenols. Phenolic chemicals are essential parts of plants that serve a variety of biological purposes, including antioxidant activity due to their ability to recover radicals due to their OH groups [32].

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 extract concentrations were checked for the capacity to inhibit HT29 cell growth. Figure 3 shows anticarcinogenic effect of tested plant extracts at various doses (0.1, 0.02, 0.5, 0.75, and 1 mg/mL) against HT29 cells for 24 hours using the MTT test. The rate of cell multiplication and, conversely, the decline in cell viability were both measured by the MTT cell proliferation test. The *in vitro* results confirmed that the inhibition of HT29 cell growth, for the three (3) extracts, was directly proportional. The inhibition of HT29 cell proliferation of the leaf extract (LE), pink flower extract (PFE) and white flower extract (WFE) was respectively 56 %, 63 % and 67 % for a concentration of 1 mg/mL of dry extract.

A weak correlation was noticed between FC and the capacity to prevent the proliferation of HT29 cells with the C5 concentration, while a strong positive correlation was recorded between DPPH and ABTS with the capacity to inhibit HT29 cancer cells with the concentration C5 and C6 (cf. Table 2). This indicates that in the studied extracts, the bioactive molecules, ensuring the scavenging power of free radicals (DPPH and ABTS), were responsible for the inhibition of the proliferation of HT29 cells. This leads us to the conclusion that, while the concentration of the phenolic compounds constitutes a significant factor but it is not always sufficient, there is a further criterion relating to the phenolic compounds to be taken into account in the interpretation of the activity.

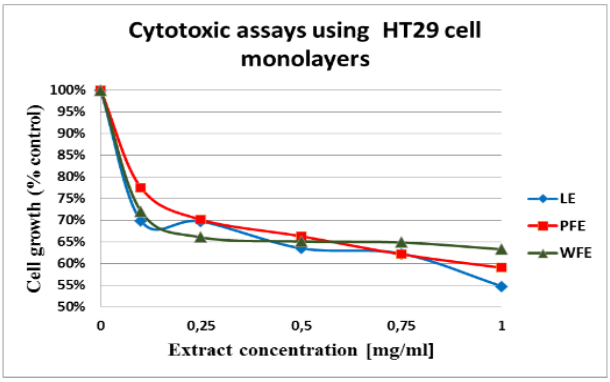


Figure 3. Cytotoxic Essays Using HT29 Cell Monolayers After 24 hours of Incubation with Extract of Leaves (LE), Pink Flowers (PFE), and White Flowers (WFE)

The FTIR analysis revealed the presence of the OH and C-OH alcohol groups which may correspond to polyphenols, our previous study [13, 27] indicated the presence of polyphenols such as quercetin, chlorogenic acid, and certain glycosidase flavonoids in *N. oleander* leaves extract which might be responsible for the anticarcinogenic activity. Secondary metabolites, such as phenolic components, possess a powerful capacity to pierce free radicals. Natural antioxidants increase plasma's antioxidant capacity, lowering the risk of diseases such as cancer, heart disease, and stroke [33].

Three different cancer cell lines – T47D, HepG-2, and K562 – were shown in the study to be inhibited in their proliferation by the leaves and flowers' methanolic extracts of *N. oleander*. The authors suggested that this effect could be related to a cardiac glycoside, oleandrin, being the main active compound in the fight against cancer in *N. oleander* [33].

3.5 Principal Component Analysis

Principal Component Analysis (PCA) was used to study the correlation between the indices of evaluation of antioxidant (DPPH, ABTS, and FC) and anticarcinogenic activities measured in *N. oleander* leaves, pink, and white flowers. The relationships between primary variables and the key components obtained are shown graphically in Figure 4. A vector represents any element.

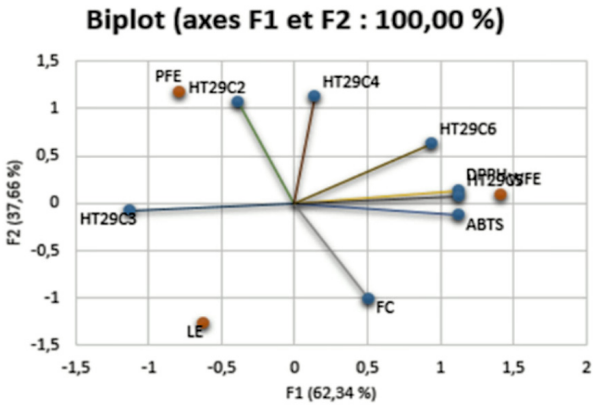


Figure 4. The principal Component Analysis (PCA) Revealing Correlation with PC1 and PC2 between Three Extracts of *N. oleander* of Phytochemical Parameters (WFE, PFE and LE)

The PCA represents a powerful statistical method to elucidate the sample separation and to obtain additional data on variables especially the sample's similarities and differences. This method can provide insight into the correlation of a group of variables and not just between two variables in a complementary set of scores and loading diagrams. Referring to such techniques, will allow the extraction of more dominant trend of information. PCA enables the reduction of dimensionality, data exploration to identify relationships between objects, estimation of the correlation structure of the variables, and investigation of the number of components (a linear combination of original features) necessary to explain the majority of the variance with the least amount of information lost [33]. Results obtained through PCA analysis showed that 100 % of the overall variance was accounted for by the first two main components, where 62.34 % were responsible for PC1 and 37.66% of the data variability was expressed by PC2. HT29C5, DPPH and ABTS (0.998, 0.993, 0.993) were strongly correlated with the first main component (PC1), followed by HT29C6 and FC with values of 0.831 and 0.449, respectively. While, the second component (PC2) was correlated with HT29C4, HT29C2, and HT29C3 (0.992,0.941 and -0.066). Pearson's correlation analysis revealed that FC, correlated with the activity of DPPH radical scavenging, the activity of ABTS radical scavenging, and the activity of inhibiting HT29 cancer cell proliferation with C5 concentration, 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, C54 and C6. Furthermore, a significant positive correlation exists between the proliferative inhibition activity of HT29 cells (C5 and C6) and the radical scavenging activities of ABTS and DPPH, as well as between the proliferative inhibition activity of HT29 cells (C5 and C6) and ABTS.

Almost all input variables are located near the circle, which means that the key components bear much of the information found in these variables. The fact that variables are located next to each other shows a clear positive correlation. From the graph, we can underline that the angle between the value of DPPH and ABTS as well as the value of DPPH and HT29C5 is quite minor, implying that these factors are positively correlated. Vectors perpendicular to each other show a lack of similitude (DPPH and HT29C4). We can deduce from the graph via PCA analysis that the white flower extract displayed a better antioxidant effect and antiproliferative potential to prevent the growth of HT29 cells compared to the other two extracts (PFE and WFE).

4 Conclusion

Based on the comparison of antioxidant and anticarcinogenic activities of the three extracts from *Nerium oleander* using PCA analysis, the results showed that WFE has more potent biological activity than LE and PFE. *Nerium oleander* may therefore be an excellent source of natural antioxidant and anticancer agents, as well as a possible pharmaceutical supplement.

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