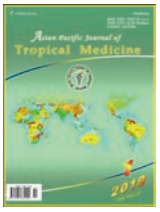

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# Cytotoxic, antioxidant and antimicrobial activities of *Nerium oleander* collected in Morocco

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ABSTRACT

**Objective:** To investigate the cytotoxic, antioxidant and antibacterial activities of ethanolic and aqueous crude extracts of *Nerium oleander* (*N. oleander*) leaves. **Methods:** Cytotoxic activities were evaluated by WST-1 bioassay on two human cancer cell lines, namely human colon adenocarcinoma cell line HT-29 and human breast cancer cell line MDA-MB-231. The antioxidant property of *N. oleander* extracts was assessed by DPPH scavenging and β-carotene bleaching tests. The agar disc diffusion method was used for the determination of antimicrobial activity against different strains. **Results:** Using cell viability indices, the WST-1 test revealed that both extracts reduced cell viability in both cell lines. Our results also showed that aqueous extract was more active than ethanolic extract, with $IC_{50}$ values of (1.67±0.22) μg/mL and (2.36±0.44) μg/mL on MDA-MB-231 cells, and (2.89±0.35) μg/mL and (5.09±0.52) μg/mL on HT29 cells, respectively. The study of the antioxidant activity showed that *N. oleander* extracts had a considerable scavenging capacity and exerted a significant preventive effect against the oxidation of β-carotene by the peroxide radicals. However, the antibacterial test showed that both ethanolic and aqueous extracts of *N. oleander* had a moderate antibacterial effect limited only to Gram-positive bacteria. **Conclusions:** Our results shows that *N. oleander* aqueous and ethanolic extracts have significant cytotoxic activities against tumor cell lines and possesses a strong antioxidant capacity, suggesting the presence of active compounds in *N. oleander* leaves that could be a potential source of phytochemicals with high medicinal value to be used in cancer treatment and prevention.

## 1. Introduction

Overtime, plants were used to treat different diseases and were the major source of therapeutic agents. Currently there are thousands of species of plants with a high diversity of chemical compounds with therapeutic properties. During the last decades, a great interest was given to medicinal plants, widely used in folk medicine for cancer treatment, leading to discoveries of many antitumor agents[1].

*Nerium oleander* (*N. oleander*) L., also named *Nerium indicum* Mill. and *Nerium odorum* Ait, and locally called "Defla", is an evergreen shrub or small tree typically growing to 2-6 m in height, belonging

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to the Apocynaceae family. This plant is widely distributed in the Mediterranean region, subtropical Asia and the southwestern part of the United States[2]. This species is often grown in gardens as an ornamental plant due to its beautiful and fragrant flowers, fence and wind-break[3]. Leaves are in pairs of three or whorled, very green, leathery and are narrowly elliptic to linear entire. Flowers grow in clusters in terminal branches, each 2.5-5.0 cm; funnel-shaped with five lobes, fragrant and show various colors from pink to red, white, peach and yellow[4].

*N. oleander* L. is well known for its medicinal use. Various parts of the plant have been used mainly as a heart tonic, as diuretic and in the treatment of swelling, eye and skin diseases. Other studies have reported that *N. oleander* possesses a wide range of biological activities including cardiotonic, anti-inflammatory, antibacterial, anticancer, cytotoxic, antiplatelet aggregation and central nervous system depressant activities[5].

Chemical investigations of *N. olenader* highlighted the presence of many bioactive compounds grouped as pregnanes[6], cardenolides[7-9], phenolics, cardiac glycosides[10,11], alkaloids, tannins, flavonoids and terpenoids[12].

The present study planned to evaluate antibacterial, antioxidant and cytotoxic activities of the aqueous and ethanolic extracts of *N. oleander* harvested in Morocco.

## 2. Material and methods

### 2.1. Preparation of plants extracts

Leaves of *N. oleander* were collected in the city of Rabat, Morocco. The leaves had been thoroughly cleaned of unwanted material, dried for a week at ambient temperature and then ground finely. Aqueous and ethanol extractions were performed for three nights under agitation. The mixture was filtered and concentrated in vacuum at 45 ℃ to obtain a green paste, then stored at 4 ℃ for further use. Extracts were dissolved in dimethyl sulfoxide (DMSO) and conserved at –20 ℃ until use.

### 2.2. Cell culture

Cell lines used in this study included MDA-MB-231, a human breast cell line, and HT-29, a human colon cell line. Cancerous cell lines were cultured in DMEM medium supplemented with 1% glutamine (Gibco), 10% fetal calf serum (Gibco) and 1 % of mixture of streptomycin/penicillin. Cells were maintained at 37 ℃ with 5% $CO_2$ and 95% humidity. The cells were fed until confluent and expanded by trypsinisation, and then sub-cultured at lower numbers in new culture flasks. The viability was checked by trypan blue and if it was more than 95%, the experiments were performed.

### 2.3. Cell proliferation (WST-1) assay

Cell viability was estimated on the basis of mitochondrial metabolic activity using WST-1 which is disodium mono{4-[3- (4-iodophenyl)-2-(4-nitrophenyl)-2H-tetrazol]-3-ium-5-yl] benzene-1,3-disulfonate} assay as described by Piastowska-Ciesielska[13]. Exponentially growing MDA-MB-231 and HT-29 cells were seeded on 96-well microplates at a density of 8 000 cells per well. After 24 h, the culture medium was replaced by an experimental one with different concentrations of the extracts (initially dissolved in DMSO). Narrow range concentrations tested in this study varied from 0.75 μg/mL to 20 μg/mL, obtained after diluting stock solutions with media so that the final concentrations of DMSO did not exceed 0.05%. Each concentration/assay was performed in duplicate. The cells were then re-incubated for 72 h. Then, 100 μL of the medium were aspirated from each well, 10 μL of WST-1 reagent was added and the plate was re-incubated for further 4 h.

Cell viability was assessed by absorbance reading of each well at 450 nm using a Wallac Victor X3 multiplate reader. Data were expressed as percentages of absorbance between treated and control wells. In these tests, medium without extract and mitomycin C were used as negative and positive controls, respectively (data not showed).

### 2.4. Antibacterial effect

#### 2.4.1. Bacterial strains

In order to determine the antibacterial activity of *N. oleander* extracts, 6 bacterial strains were used; 3 Gram-positive bacteria: *Enterococcus faecalis (E. faecalis), Listeria monocytogenes (L. monocytogenes)* and *Staphylococcus aureus (S. aureus)*, and 3 Gram-negative bacteria: *Escherichia coli (E. coli )*, *Pseudomonas aeruginosa (P. aeruginosa )* and *Salmonella typhimurium*. Bacterial growth was carried out at 37 ℃ in a liquid medium (peptone water) and on Müeller-Hinton agar (MHA) as a solid medium.

#### 2.4.2. Preparation of bacterial suspensions

To prepare the bacterial suspensions, four to five well-isolated colonies were selected and emulsified in a tube containing sterile distilled water to have turbidity near that of McFarland 0.5 standards, the suspensions were then diluted in tubes containing sterile distilled water to have a final concentration of $10^7$. An inoculum of 100 μL of each bacterial suspension was plotted in petri dishes, each containing 20 mL of MHA medium, and left hanging for 5 min before inserting the discs.

#### 2.4.3. Disc diffusion method (Aromatogram)

The experimental protocol consisted on depositing a sterile Wattman paper disk (0.5 cm in diameter) impregnated with 20 μL of extract dissolved in 0.05% of DMSO. The dishes were left for 1 h at room temperature and then incubated at 37 ℃ for 18 h to 24 h. After incubation, the radius of the inhibition zone was measured in millimeters (from the center of the disk to the limit of the inhibition zone).

### 2.5. Antioxidant effect

#### 2.5.1. DPPH radicals

The hydrogen atoms or electrons donation ability of the plant extracts were measured using the bleaching of a purple-colored methanol solution of DPPH[14]. Aqueous and ethanolic extracts were tested at concentrations ranging from 0.312 5 mg/mL to 10 mg/mL. Briefly, 150 μL of each concentration was deposited in the wells of a 96-well microtiter plate and then 50 μL of 1 mM DPPH were added to each well. The microplates were kept at room temperature in the dark for 30 min. The absorbance was then measured at 517 nm. BHT, a standard antioxidant, was used as a positive control. The percentage of DPPH trapping activity was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where A stands for absorbance, control represents purple-colored methanol solution of DPPH and sample is the plant extract tested at different concentrations. The concentration providing 50% inhibition ($IC_{50}$) calculated from the dose response graph.

#### 2.5.2. Bleaching of beta-carotene

β-carotene bleaching assay was carried out according to the method reported by Lu *et al.*[15]. Briefly, 0.14 mg of β-carotene was dissolved in 0.7 mL of chloroform. Then, 200 μL of the mixture was putted into a flask containing 1.4 mg of linoleic acid and 14 mg of Tween 40, the solution was vigorously stirred and the chloroform was then removed by incubation of the solution at 50 ℃ for 2 h. After the chloroform had evaporated, distilled water was added to form an emulsion and 200 μL of this emulsion were added to the wells of a microtitration plate supplemented with 8 μL of different concentrations of the extracts ranging from 0.312 5 mg/mL to 10 mg/mL. The absorbance of the β-carotene emulsion was measured at 470 nm, immediately (T0) and after incubation at 50 ℃ for 120 min (T120).

The antioxidant activity is expressed as a percentage inhibition with reference to the control according to the following equation:

$$\% \text{ Inhibition} = \frac{A_{sample\ at\ T120} - A_{control\ at\ T120}}{A_{sample\ at\ T0} - A_{control\ at\ T0}} \times 100\%$$

### 2.6. Statistical analysis

Data are presented as means ± SD of assays performed in triplicate. Statistical analysis for the cytotoxicity assay was performed by Student's *t*-test. Statistical analyses of DPPH and β-carotene results were performed by Levene test. Significant differences are obtained when *P* value was <0.05. The statistical analysis was performed using Excel Stat.

## 3. Results

### 3.1. Cytotoxic effect of N. oleander extracts

The WST-1 test revealed that ethanol and aqueous extracts reduced the cell viability in both HT-29 and MDA-MB231 cells in a dose-dependent manner after 72 h of treatment (Figure 1). Compared with the respective non-treated cells, progressive decrease in cell viability in HT-29 and MDA-MB231 cells were found with increasing concentrations of the extracts. Moreover, aqueous extract showed higher cytotoxic activity in both MDA-MB231 and HT29 cell lines. Indeed, in breast cancer cell line MDA-MB231, the $IC_{50}$ obtained with the aqueous extract was (1.67±0.22) μg/mL, slightly lower than the $IC_{50}$ obtained with the ethanolic extract [(2.36 ± 0.44) μg/mL). In colon cancer cells HT29, the aqueous and ethanol extracts showed $IC_{50}$ values of (2.89±0.35) μg/mL and (5.09±0.52) μg/mL, respectively. Statistical analysis showed that the differences between cell viability obtained with tested extracts and negative controls were all highly significant (*P*<0.001).

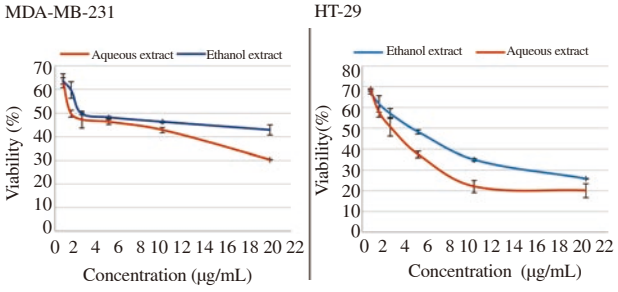


**Figure 1.** Cytotoxic effect of ethanol and aqueous extracts of *N. oleander*'s leaves against MDA-MB-231 and HT-29 cell lines.

Cells were incubated for 72 h with different concentrations of the plant extracts (ranged from 0.75 μg/mL to 20 μg/mL for both extracts).

### 3.2. Antibacterial effect

The disc diffusion method showed that the antibacterial activity of ethanol and aqueous extracts of *N. oleander* was variable according to the tested bacteria. The results indicated that both ethanolic and aqueous extracts were active on *E. faecalis* 471 strains with a diameter of inhibition zone reaching (5.3±0.6) mm and (10.0±1.2) mm, respectively. Whereas, *L. monocytogenes* was sensitive to only the aqueous extract with a diameter of inhibition zone of (4.0± 1.0) mm. The other tested strains: *E. coli*, *S. aureus* CECT 476, *S. typhimurium* and *P. aeruginosa* were resistant to both extracts.

### 3.3. Antioxidant effect

#### 3.3.1. DPPH activity

In this study, *N. oleander* ethanol and aqueous extracts were investigated for their antioxidant activity with DPPH scavenging assay. Results of the antioxidant power of *N. oleander* extracts compared to the standard product were reported in Table 1. The aqueous extract showed a high antioxidant activity and all the used

**Table 1**

Scavenging activities of different concentrations of *N. oleander*'s ethanol and aqueous extracts on the DPPH radical (%).

| Extract (mg/mL) | 0.312 5 | 0.625 | 1.25 | 2.5 | 5 | 10 | *P* | *F* |
|---|---|---|---|---|---|---|---|---|
| Aqueous extract | 76.71 ± 2.18 | 84.54 ± 2.18 | 90.26 ± 1.66 | 91.54 ± 2.05 | 92.67 ± 1.21 | 93.17 ± 0.62 | 0.10 | 2.21 |
| Ethanol extract | 2.41 ± 1.38 | 52.91 ± 1.14 | 73.50 ± 1.09 | 84.74 ± 0.46 | 87.75 ± 2.18 | 87.25 ± 0.46 | 0.05 | 1.94 |
| BHT | 86.57 ± 1.04 | 89.32 ± 3.95 | 93.92 ± 1.39 | 94.18 ± 1.73 | 95.18 ± 1.23 | 98.01 ± 3.45 | - | - |

Results are expressed in percentage of scavenging DPPH radical. The *P* value refers to the comparaison of extract results with BHT results. We used Fisher test for this study.

**Table 2**

β -carotene bleaching assay at 470 nm in presence of *N. oleander* extracts and BHT (%).

| Extract (mg/mL) | 0.312 5 | 0.625 | 1.25 | 2.5 | 5 | 10 | *P* | *F* |
|---|---|---|---|---|---|---|---|---|
| Aqueous Extract | 65.23 ± 4.89 | 66.08 ± 6.11 | 69.94 ± 4.94 | 72.41 ± 3.95 | 76.54 ± 4.61 | 79.37 ± 2.03 | 0.37 | 1.13 |
| Ethanol Extract | 49.95 ± 5.81 | 56.12 ± 3.52 | 57.38 ± 6.48 | 58.16 ± 4.91 | 58.29 ± 9.08 | 61.89 ± 6.36 | 0.08 | 1.91 |
| BHT | 68.98 ± 3.86 | 81.98 ± 2.27 | 83.19 ± 1.76 | 84.08 ± 1.69 | 87.85 ± 1.66 | 87.92 ± 2.25 | - | - |

Results are expressed as percentage of inhibition of β -carotene oxidation.

concentrations had a powerful effect (above 50%), similar to that obtained with BHT, used as a reference molecule ($F$=2.21, $P$=0.10). However, the ethanolic extract showed a dose dependent activity giving an $IC_{50}$ of (2.200 ± 0.014) mg/mL. Levene test analysis showed a significant difference between ethanolic extract and BHT activities ($IC_{50}$=3.48 μg/mL) ($P$=0.05).

### *3.3.2. β -carotene test*

The bleaching or discoloration of β -carotene in the presence of the two extracts and standard molecule (BHT) are represented in Table 2. β -carotene bleaching assay clearly showed that both aqueous and ethanolic extracts of *N. oleander*, as well as BHT, exerted a significant preventive effect against the oxidation of β -carotene by the peroxide radicals. Moreover, all tested concentrations of both ethanolic and aqueous extracts highlighted an important antioxidant power as compared to BHT. Statistical analysis showed no significant differences between aqueous and ethanolic extracts, and BHT activities.

## 4. Discussion

### *4.1. Cytotoxic activity of N. oleander extracts*

In this study, both ethanolic and aqueous extracts of *N. oleander* exhibited high cytotoxic activities on breast cancer MDA-MB231 and colon cancer HT29 cell lines and obtained $IC_{50}$ ranged from 1.67 μg/mL to 5.09 μg/mL. The cytotoxic activity of *N. oleander* extracts was evaluated on different cancer cell lines and results converge to their high cytotoxic power. Calderón-Montaño *et al.* has reported that *N. oleander* extract had a potent effect on A549 lung cancer cell line and the cytotoxicity was significantly higher than that obtained on nonmalignant cell line, with a potency and selectivity similar to those of the anticancer drug cisplatin[16]. Other studies have reported a cytotoxic activity of *N. oleander* and derived molecules on HL60 leukemia cell lines, DU145 and PC3 prostate cancer cell lines, MCF-7 breast cancer cell line, WI-38 human fibroblast cells, HepG2 liver tumor cells, and PC12 pheochromocytoma cells[17-21].

The interesting cytotoxicity of *N. oleander* extracts toward cell lines is mainly due to the presence of cardiac glycosides, widely studied for their potential anticancer activity. Evaluation of the activity of oleandrin, the major glycoside isolated from *N. oleander*, on human pancreatic tumor cells PANC-1 shows that this molecule is able to stop cell proliferation of PANC-1 cells and to arrests cells at $G_2$/M stage of cell cycle[18].

A supercritical $CO_2$ extract of *N. oleander* (PBI-05204), containing oleandrin, was tested on a human pancreatic cancer Panc-1 orthotopic model and results clearly showed that 25% of mice treated with PBI-05204 (40 mg/kg) for 6 weeks showed dissectible tumor at the end of the treatment period whereas all control mice exhibited tumors by the end of treatment. Moreover, treatment with only 20 mg/mL of PBI-05204 was associated with a significant reduction of average tumor weight[22]. Recently, PBI-05204 was tested in patients with advanced solid tumors, as a first-in-human phase Ⅰ study, and is currently under the phase Ⅱ trial[23]. Other glycosides include odorside, neritaloside and the aglyconeoleandrigenin[24,25]. The Anvirzel™, obtained from *N. oleander* and containing oleandrin, odorside, neritaloside and the aglycone oleanderi genin have shown an interesting anticancer effects against many cancer cell lines and was subject to phase Ⅰ trial[26].

### *4.2. Antibacterial activity*

The *in vitro* antimicrobial activity of *N. oleander* aqueous extract inhibited the growth of two bacterial strains, producing a zone diameter of inhibition of (10.0±1.2) mm on *E. faecalis* 471, and (4.0±1.0) mm on *L. monocytogenes* showing better activity than the ethanol extract which inhibited the growth of just *E. faecalis* 471 with (5.3±0.6) mm as the inhibition zone diameter. None of the extracts had an effect on the Gram negative bacterial strains. In this study, aqueous and ethanolic extracts were active only on some Gram positive bacteria. This could be due to the presence of cell envelope that could be non-permeable to the tested plant extracts and restrict their penetration in the bacteria. Indeed, many studies have reported that Gram-negative bacteria are often more resistant to plant-derived antimicrobials, compared to Gram-positive

bacteria[27,28].

Worldwide, limited data are available regarding the evaluation of the antibacterial activity of *N. oleander* extracts. Sharma *et al.* have investigated different extracts of *N. oleander* against *B. pumilus, B. subtilis, S. aureus* and *E. coli*[3]. All tested bacteria were sensitive to methanolic and ethanolic extracts of *N. oleander* roots and leaves, but resistant to all chloroform extracts. The methanolic extract highlighted a marked antibacterial effect whereas the ethanolic one has showed a moderate antibacterial activity against almost all tested bacteria[3]. For the same purpose, other studies have focused on the evaluation of antibacterial effect of extracts and essential oils of *N. oleander* flowers on both Gram positive and Gram negative bacteria[27-29]. Of particular interest, Derwich *et al.* have shown that essential oils from *N. oleander* flowers was active on *E. coli*, *P. aeruginosa* and *S. aureus* with inhibition zones of 28.89 mm, 18.22 mm and 6.32 mm, respectively[30]. On the other hand, Namian *et al.* have shown interesting antibacterial effect of hexanic, dichloromethane and methanolic extract of both leaves and flowers *E. coli, E. carotovora, S. aureus, B. cereus* and *B. pumillus* with inhibition zones ranging from 8 to 19 mm[31]. Difference in antibacterial effect of *N. oleander* extracts highlights the chemical difference between plant sampling and solvents used for the extraction.

### *4.3. Antioxidant activity*

*N. oleander* ethanol and aqueous extracts were also investigated for their antioxidant activity with DPPH scavenging assay and the bleaching of β-carotene test. Results clearly showed the high antioxidant power of *N. oleander* extracts compared to the standard product. The $IC_{50}$ obtained with the ethanol extract was (2.200 ± 0.014) mg/L whereas all tested concentrations of the aqueous extract gave similar results compared to BHT, with an effect above 50%. Moreover, the β-carotene bleaching assay showed that the two *N. oleander* extracts exerted a significant preventive effect against the oxidation of β-carotene.

The high antioxidant power of *N. oleander* extracts was already reported and discussed. Singhal *et al.* have evaluated the antioxidant activity of the methanolic extract of *N. oleander* flowers, using various tests, including reducing power, lipid peroxidation and DPPH, and reported a strong antioxidant power and obtained results were equivalent to those obtained with BHT which was used as a reference molecule[32]. These results were comforted by other studies showing a considerable scavenging capacity and a high reducing power of different *N. olenader* extracts[31-33].

Interestingly, all these studies have reported that the high antioxidant power of *N. oleander* is mainly due to the presence and the diversity of phenolic compounds in the plant. The main phenolic compounds reported in *N. oleander* are rutin, catechine, epicatechin, quercitin and quenonique acid, and are reported in both leaves and flowers[34]. It's widely accepted that phenolic compounds have a strong antioxidant activity, including free radical scavenging and reducing power[35].

The present study was very informative highlighting interesting antioxidant and cytotoxic effects of methanolic and ethanolic ectracts of *N. oleander* leaves, and other assays including (1) assessment of chemical composition; (2) identification and isolation of components responsible of the related activities; (3) study of pathways implicated in the observed effects. Moreover, *N. oleander* is well known for its high toxicity and is considered poisonous to human beings[32]. Therefore, *in vivo* assays will be of a great interest to evaluate pharmacological effects and to determine sub-toxic doses to explore the antiproliferative and/or anticancer activities.

In conclusion, the present study gives evidence that *N. oleander* extracts exert a potent cytotoxic activity on both MDA-MB-231 and HT-29 human cell lines and have an effective antioxidant activity, suggesting the presence of active compounds in *N. oleander* leaves that could be a potential source of phytochemicals with high medicinal value to be used in cancer treatment and prevention.

## Conflict of interest statement

The authors declare that they have no conflicts of interest.

## Foundation project

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