Original Article

Screening of Biological Activities (Antioxidant, Antibacterial and Antitumor) of *Nerium oleander* Leaf and Flower Extracts

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ABSTRACT

Oleander (*Nerium oleander* L. Apocyanaceae) is an evergreen shrub, widely use for medicinal purposes. The objective of this work is to investigate the antioxidant, antibacterial and cytotoxic effects of the plant leaves (L) and flowers (F) extracts. These activities had been evaluated by DPPH, agar disc diffusion, agar dilution and MTT, respectively. Our results showed that methanol extracts (L & F) have high antioxidant activity with RC_{50} value of 0.27, 0.2mg/mL respectively, dichloromethane (DCM) and methanol extracts (L & F) showed strong antibacterial activity against both gram negative and gram positive bacteria. MTT assay exhibited that dichloromethane extracts (L & F) have high cytotoxic effects against $T_{47}D$, HepG-2 and K562 cell lines with IC50 value of 57.77, 233.42µg/mL, 55.90, 108.31µg/mL, 70.03, 102.31µg/mL, respectively.

Keywords: *Nerium oleander*, medicinal plants, cytotoxic activity, antibacterial activity, herbal medicine.

INTRODUCTION

Nerium oleander L. (Apocyanaceae) is an evergreen shrub distributed in the Mediterranean region and subtropical Asia[1,2]. People use medicinal plant for many years as a treatment. The use of traditional medicine is still strong in the cure of ailments[3]. Plant-derived compounds introduce potential sources for new antioxidant, antibiotics and anticancer agents[4]. Herbal medicine is use widely in the world, Since better cultural acceptability, better compatibility with the human body and fewer side effects[5,6]. All plants containing compounds are important, these compounds are mainly secondary metabolites include Steroides, Alkaloides, Tanins and Phenol compounds that are synthesized and assymbeled in all parts or specific parts of the plant. The *Nerium oleander* is a small evergreen shrub of 2-5m in hight, the leaves are 5-20cm long. The flowers are white, pink, red or yellow. All

parts of the Oleander are poisonous to humans, animal and certain insects[7]. *Nerium oleander* produce secondary metabolites that some of them have pharmacological interest. The important pharmacological activities are antinociceptive, anti-inflammatory, antibacterial, anticancer and CNS depressant activity[7]. Different parts of the plant are reputed as therapeutic agents in the treatment of leprosy, swellings, eye and skin diseases. The methanolic extracts showed anti-inflammatory activity, inhibitory activity against the induction of the intercellular adhesion molecule-1, anticancer activity and cell growth inhibitory activity[8,9]. The aim of the present study is in vitro screening of antioxidant, antibacterial and a cytotoxic activity of the extracts of *Nerium oleander* leaves.

MATERIALS AND METHODS

Plant Material

Plant materials were collected from Kermanshah in west of Iran, situated between 33° 36° 15° north latitude and 45° 24° 30° east altitude. A sample of this plant has been deposited at the Herbarium for Medicinal Plants at the Faculty of science of the university of Mohaghegh Ardabili (No: 1389-1).

Plant collection

Fresh plant parts were collected, dried and powdered under sterile conditions. The plant leaves and flowers were soxhlet extracted with n-Hexane (Hex), dichloromethane (DCM) and methanol (Met), respectively. The extracts were dried in vacuum[10,11].

Antioxidant assay

Serial dilutions were prepared with the stock solutions (1mg/mL) of the plant extracts to reach concentrations 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0078, 0.0039 and 0.0019 mg/mL. The solvent for all the solutions was methanol. Diluted solutions (5mL each) were mixed with 5mg of 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma) and spent 30 min for any reaction to occur. The UV absorbance was recorded at 517nm. The RC_{50} value that is the concentration of the text extracts that decrease 50% of free radical concentration was measured as mg/mL[11, 12].

Antibacterial assay

The extracts obtained from leaves and flowers were studied for antibacterial activity. The antibacterial study of n-Hex, DCM and Met extracts were evaluated by using agar disc diffusion, agar dilution and determination of Minimum Inhibitory Concentration (MIC). The antibacterial activity of the plant extracts was determined against *Escherichia coli* (PTCC 1047), *Staphylococcus epidermis* (PTCC 1114), *Staphylococcus aureus* (PTCC 1112), *Bacillus cereus* (PTCC 1247), *Erwinia carotovora* (PTCC 1675), *Bacillus pumillus* (PTCC 1319). For the disc diffusion method the filter paper discs (6mm in diameter) were soaked with 30µ.L of stock solution of the extracts (100mg/mL), also the sterilized petri dishes were labeled with the bacterial names, that had been previously inoculated with the tested microorganisms. Then filter paper discs placed on to these agar plates. The plate incubated at 37°C for 24 h. After the incubation time all the plates were examined for the presence of zones of inhibition as a property of antibacterial activity. The diameter of inhibition zones were measured in millimeters. Two different commercially available antibiotics discs, Gentamicine (10 mcg per disc) and nitroforantoine (300 mcg per disc) used as positive control. The MIC of the extracts against the tested microorganisms was determined by the agar dilution method[13,14].

Cytotoxicity assay

$T_{47}D$: human breast cancer (Pasteur, C203), HepG-2: human hepatocellular carcinoma (Pasteur, C124) and K562: human chronic myeloid Leukemia (Pasteur, C122) cell lines were used to study the cytotoxic activity of *Nerium oleander* leaf and flower extracts. Cells were grown in RPMI 1640 (Gibco, No 51800-019) medium, supplemented With 10% foetal calf serum (FCS), 100unit/mL penicillin, 100µg/mL streptomycin and maintained at 37°C in 5% Co_2 incubator. For testing, cells were washed by PBS (phosphate buffer saline) and harvested by tripsinozation and plated in 96-well plates at a density of 1×10^4 cell/well in 200µL medium and incubated for 24 h at 37°C in the incubator.

Next, the medium was removed and cells were treated with a FCS-free medium containing different concentrations of plant extracts. The concentrations were 25, 50, 100, 200µg/mL for Met and Hex extracts and 12.5, 25, 50, 100 µg/mL for DCM extracts (L). Afterward plates were incubated for time periods of 24, 48 and 72 h. The cytotoxicity of plant extracts was determined by MTT assay. This assay measures the metabolism of 3-(4,5-dimethyl thiazol-2yl)-2, 5-biphenyl tetrazolium bromide to form an insoluble formazan precipitated by mitochondrial dehydrogenases only present in viable cells. Four hours before completion of the incubation times, the medium was removed and then 180µL FCS-free medium and 20µL of 2.5mg/mL MTT were added to each well then plates were incubated again for 2-4 h completing the incubation time. After that, the supernatants were removed and 200µL DMSO was added to each well. Plates were shaken for 5 min. The absorbance at 660nm was measured using ELISA microtiter plate reader. Using wells without cells as blank[15].

Morphological observation

Morphology of three cell lines was initially observed under inverted microscope before performing all experiments. Apoptotic morphology of them was further observed by acridine orange/ethidium bromide (AO/EB) double staining.$T_{47}D$, HepG-2 and K562 cells were seeded at a density of 5_10^5 cells/well and 2.5_10^5 cells into $25cm^2$ flask, respectively. The cells were incubated without or with different concentration of the extracts (15 and 20µg/mL) after 24 h adherence. Following 24 and 48 h incubation, cells were washed with PBS and harvested by trypsine/EDTA. They were resuspended in culture medium at a density of 1_10^7 cells /mL. The cells were stained with 0.1 mg/mL AO and 0.1 mg/mL EB (Sigma) in PBS, then immediately examined under a fluorescence photomicroscope[16].

RESULTS

The DPPH assay showed that Met extracts of the plant leaves and flowers have high antioxidant activity and could scavenge free radicals with RC_{50} value of 0.27, 0.20mg/mL respectively, DCM extracts (L & F) indicated strong effect with RC_{50} value of 0.74, 0.51mg/mL, and Hex extracts (L & F) exhibited weak activity with RC_{50} value of 2.54, 2.53mg/mL respectively.

Antibacterial activity of oleander extracts was evaluated by agar disc diffusion and agar dilution against gram-positive and gram-negative bacteria. The results are summarized in (Table 1,2). The data indicated that DCM and Met extracts (L & F) showed strong antibacterial activity against all the tested bacteria, with inhibition zones of 14-18, 12-16mm and MIC values of 0.006-1, 0.006-1mg/mL for DCM extracts (L & F) respectively, for the Met extracts (L & F) inhibition zones are between 15-19, 13-16mm and MIC values of 0.006-1 , 0.006-1mg/mL.

MTT assay showed that DCM extracts (L & F) have high cytotoxic effects against $T_{47}D$, HepG-2 and K562 cell lines, Met extracts (L & F) showed rather strong activity on three cell lines and Hex extracts (L & F) have weak cytotoxic effects.(Table 3).

Morphological observations of $T_{47}D$, HepG-2 and K562 were performed by acridine orange/ethidium bromide (AO/EB) double staining. According to our results it seems that effective compounds of the extracts act via induce of apoptotic death on cancer cells.

The extracts of *Nerium oleander* leaf & flower had significant antioxidant, antibacterial and cytotoxic activity. These biological activities are because of the presence of secondary metabolites that can produce in all parts or special parts of the plant. Antioxidant activity is because of Phenolic compounds that decrease the concentration of radicals. Also antibacterial effects are due to from component such as Terpens, Phenolic compounds. According to the previous study the mechanism of this activity is disrupt the permeability barrier of cell membrane and the accompanying loss of chemiosmotic control. The major active compound in cancer fighting and boosting in *Nerium oleander* is a cardiac glycoside, oleandrin. Morphological studies of treated cells indicated that cytotoxic effect of oleandrin is via induce of apoptotic death.

DISCUSSION

Plants have provided a source of inspiration for novel drug compounds as plants-derived medicines have made significant contribution towards human health. Phytomedicines can be used for the treatment of diseases. Plant based drugs have enormous therapeutical potential as they can serve the purpose without any side effects that are often associated with synthetic drugs. There for it is suggested that further work be performed on the isolation and identification of the active compound from *Nerium oleander* plant. The results indicated that *N.oleander* leaf and flower extracts could be considered as a plant derived antibiotics, antioxidant and chemotherapeutic agents[3,17].

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Table 1. Antibacterial activity of the extracts of *N.oleander* leaf. Using agar disc diffusion (mm) and agar dilution methods (MIC mg/ml)

Extract	n-Hex		DCM		Met		AB	
Bacteria	Disc Diffusion	MIC	Disc Diffusion	MIC	Disc Diffusion	MIC	GM	FM
E.coli	14	0.5	17	0.05	16	0.025	17	15
E.carotovora	15	0.006	16	0.003	19	0.0015	24	20
S.epidermidis	-	0.1	-	0.025	-	0.025	0	11
S.aureus	14	-	18	0.0125	17	0.025	0	11
B.cereus	13	-	18	0.0125	17	0.025	25	28
B.pumillus	12	0.05	14	0.0125	15	0.025	30	26

Notes: Diameter of inhibition zone including disc diameter of 6mm.
MIC: minimum inhibition concentration (as mg/mL).

Table 2. Antibacterial activity of the extracts of *N.oleander* flowers. Using agar disc diffusion (mm) and agar dilution methods (MIC mg/ml)

Extract	n-Hex		DCM		Met		AB	
Bacteria	Disc Diffusion	MIC	Disc Diffusion	MIC	Disc Diffusion	MIC	GM	FM
E.coli	-	-	14	0.5	15	0.1	17	15
E.carotovora	10	0.0125	16	0.006	14	0.006	24	20
S.epidermidis	-	0.5	-	0.5	-	0.1	0	11
S.aureus	9	-	13	-	16	0.5	25	28
B.cereus	8	-	12	0.1	13	0.1	19	22
B.pumillus	11	0.5	15	0.1	13.5	0.1	30	26

Table 3. IC_{50} value (µg/ml) of different extracts of *N.oleander* leaf & flower extracts on three cell lines

Extracts	$T_{47}D$ 72(h)	HepG2 72(h)	K562 72(h)
Hex(L)	242.59	120.01	113.52
Hex(f)	266.48	121.43	119.89
DCM(L)	57.77	55.90	70.03
DCM(f)	233.42	108.31	102.31
Met(L)	257	121.81	109
Met(f)	273	134	118.29

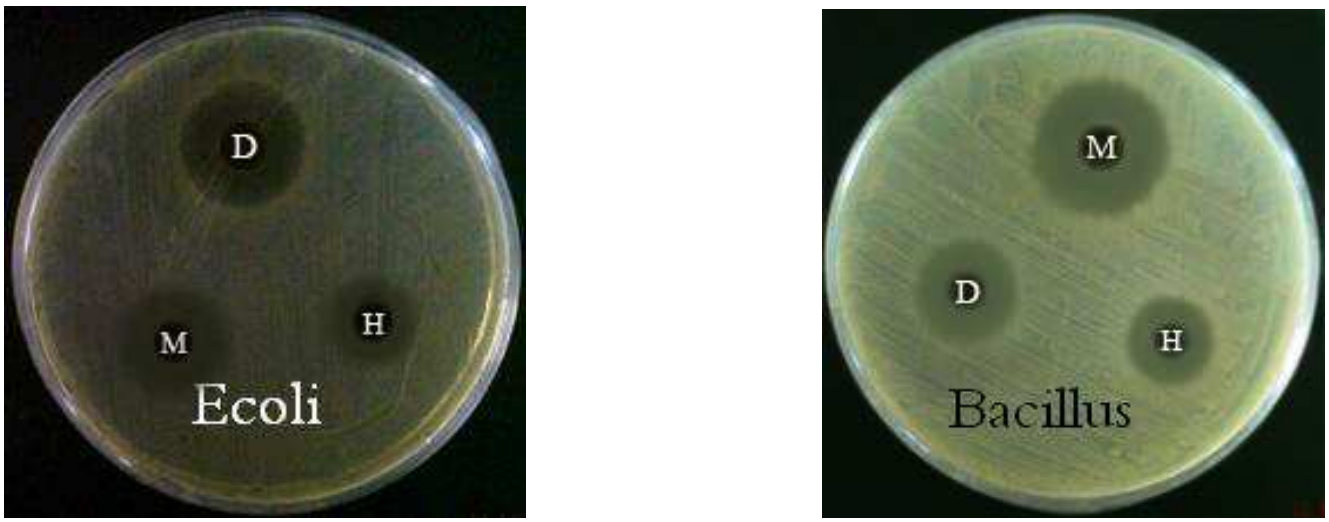

Figure.1. Antibacterial activity of the extracts of N.oleander leaf on E. coli and Bacillus Cereus. DCM (D), Met (M), Hexan (H).