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PHYTOCHEMICALS, ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF *NERIUM OLEANDER* L.

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Abstract

Nerium oleander L. is used as conventional medicine for the management of various diseases. This investigation looked into the toxicity, antibacterial and phytochemical profiles of *N. oleander* leaf extract. Different concentrations of *N. oleander* methanolic leaf extract were applied to Vero and HeLa cells to determine the cytotoxic activity of the plant. The Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, USA) was used to assess the viability of the cells. After 48 hrs, LD_{50} values of the methanolic extract were determined to be LD_{50} of 9 mg/ml against Vero cell and 46.5 mg/ml on HeLa cell, respectively. The present investigation showed the potential cytotoxic effect of *N. oleander* extract on Vero cells and anti-cancerous effects on HeLa cells through the induction of death cells. The antibacterial activity of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Bacillus subtilis* showed inhibition zones of 10, 8, 7, and 2 mm, respectively. The results of the investigation showed that leaves of *N. oleander* contain tannins, reducing sugar, glycosides, and terpenoids.

Introduction

Medicinal plants are crucial to the health and strength of individuals and communities. The great majority of pharmaceuticals are made from compounds that are derived from secondary metabolites found in herbs (Twaij and Hasan 2022). These secondary metabolites have a wide range of functions, including antiviral, antimalarial, analgesic, diuretic, anthelmintic, antibacterial, anticancer, anti-inflammatory, antifungal, antiallergic, and antimutagenic (Çilesizoğlu *et al.* 2022). In recent years, there has been a lot of interest in medicinal plants, which are commonly used in traditional medicine to treat cancer. This has led to the discovery of numerous antitumor agents (Greenwell and Rahman 2015).

Nerium oleander L. belongs to Apocynaceae family. This species naturalized in the Mediterranean region and subtropical Asia but also growing in the United States, Australia, China, and Middle East countries (El-Sawi *et al.* 2010). Different parts of the *N. oleander* plant have historically been used to treat skin cancer, asthma, dysmenorrheal, epilepsy, malaria, scabies, abortifacients, eczema, sores, warts, corns, ringworm, herpes, and psoriasis (Kumar *et al.* 2013). The bark and leaves have emetic, diuretic, expectorant, heart-tonic, and diaphoretic properties (Patel *et al.* 2010). Root oil is used to treat skin conditions and leprosy (Garg *et al.* 2010). Chemical investigations of *N. oleander* showed the presence of numerous bioactive compounds grouped ascardenolides, phenolics, cardiac glycosides, alkaloids, tannins, flavonoids, and terpenoids (Çilesizoğlu *et al.* 2022).

The present study was planned to evaluate the antibacterial activity, phytochemical screening, and cytotoxic activities of the methanolic extract of *N. oleander*.

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Materials and Methods

Fresh leaves of *N. oleander* were collected from the Gazipur and Dhaka University areas. The herbarium specimens were authenticated by Bangladesh National Herbarium (DACB) and Jagannath University Herbarium. A voucher specimen with the accession number DACB 87048 has been officially recorded and archived by the Bangladesh National Herbarium.

The collected leaves were cleaned using clean tap water. Drying leaves were crushed finely in a machine grinder. The powdered plant materials were stored at room temperature in sealed bottles.

Hundred grams of the powdered leaves of the species were soaked in 1000 ml (Ajax AR grade) methanol for specific days in an air-tight container at room temperature (23-25^{o}C). After filtering through filter paper (Whatman No. 1), the obtained extract was air dried to remove the solvent. The final pellet was then kept in an airtight screw-cap tube at 4°C.

Pellets were dissolved in 2.5% dimethyl sulfoxide (DMSO) for the extraction process and then filtered through a 0.45-µm Millipore filter to ensure sterility. After that, they were diluted to the appropriate concentrations using distilled water for the antibacterial assays and culture medium for the cytotoxicity tests.

The qualitative phytochemical screening of plant extract was performed to identify the main classes of compounds. Tests for alkaloids and tannins were conducted on extract that dissolved in 2.5% DMSO, and reducing sugar, glycosides, terpenoids, flavonoids, saponins, steroids, and volatile oils were done on plant extract dissolving in ethanol following standard procedures (Odebiyi and Sofowora 1978, Trease and Evans 1989, Dohou *et al.* 2003, Talukdar and Chaudhary 2010, Alamzeb *et al.* 2013, Thusa and Mulmi 2017).

Gram-positive and Gram-negative bacterial isolates *i.e., Salmonella typhi, Citrobacter, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus cereus, B. subtilis,* and *Staphylococcus aureus* were collected from the Food Analysis and Research Laboratory, Centre for Advanced Research in Sciences, University of Dhaka. Mueller Hinton agar and bacteriological peptone were utilized for the culture of bacteria. The antibacterial properties of the extract were assessed using the disc diffusion method (Bauer *et al.* 1966).

HeLa and Vero cell lines were cultured in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% foetal bovine serum (FBS), 0.2% gentamycin, and 1% penicillin-streptomycin (1:1). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. Once a monolayer had developed on the flask, the cells were subcultured. Trypsin was used to detach the cells, and the reaction was inhibited by adding complete medium.

HeLa cells (2×10^4/100 µl) and Vero cells (1.5×10^4/100 µl) were seeded onto a 96-well plate and incubated at 37°C plus 5% CO_2, and the trypan blue dye assay was used to observe the living and dead cells prior to the addition of samples. Trypan blue is an essential dye used to estimate the number of viable cells in a population. Cell death typically occurs due to the conditions and medium concentration. The survival rate of cells was calculated using the formula:

$$\% \text{ survivability of cells} = (\text{Live cell count/total cell count}) \times 100$$

After dissolving the sample plant extract in 2.5% DMSO, it was filtered. Then these were diluted to desired concentrations with a culture medium for cytotoxicity. The next day, 25 µL of filtered sample was added to each well. After 48 hours of incubation, cytotoxicity was assessed using an inverted light microscope. For each sample, duplicate wells were used.

Results and Discussion

Phytochemical screening of plant extract showed the presence or absence of alkaloids, tannins, reducing sugar, glycosides, terpenoids, flavonoids, saponins, steroids, and volatile oils. The present results of these analyses showed that leaves of *N. oleander* contain tannins, reducing

sugar, glycosides, and terpenoids and moderately contain steroids, while alkaloids, flavonoids, saponins, and volatile oils were absent in the tested plant extract. Similarly, Çilesizoğlu *et al.* (2022) reported the presence of tannins, reducing sugar, glycosides, and terpenoids in *N. oleander*, while the alkaloid and saponin were not detected in the methanol extract of this plant. Using *N. oleander* leaf extract, Bhuvaneshwari *et al*. (2007) conducted qualitative analyses and found that the extract contained terpenoids, alkaloids, cardiac glycosides, saponins, and tannins. When *Nerium* leaves were extracted using benzene and alcohol, Rajendra *et al.* (2013) found that the extract contained phenolic compounds, alkaloids, tannins, flavonoids, and cardiac glycosides. They also reported negative results for anthraquinone glycoside and carbohydrate.

Out of the eight bacterial species analyzed for antibacterial effect, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Salmonella typhi*, and *Bacillus subtilis* showed better zones of inhibition of 10, 8, 7 and 2 mm, respectively. The present investigation revealed that the leaf extract of *N. oleander* showed inhibitory activity against 5 tested organisms (Table 1). This showed that the natural products from the leaves of *N. oleander* have certain dynamic standards responsible for antibacterial activity. Since the growth of *S. aureus*, *P. aeruginosa*, and *S. typhi* is controlled by *N. oleander*, it indicates that they could inhibit the activity of bacteria that cause diarrhoea, UTI, and typhoid, respectively. Saranya *et al*. (2017) observed that the ethanol extract of *N. oleander* was more active against *P. aeruginosa, Salmonella, S. aureus, E. coli*, followed by *B. subtilis*.

Table 1. Antibacterial activity of leaf extract of *Nerium oleander*.

Bacteria	Inhibition zone (mm)
Gram positive	
Bacillus subtilis	4
B. cereus	0
Staphylococcus aureus	10
Gram negative	
Citrobacter sp.	0
Escherichia coli	2
Salmonella typhi	7
Klebsiella pneumoniae	0
Pseudomonas aeruginosa	8

Sharma *et al.* (2010) have studied the effects of various *N. oleander* extract on *B. pumilus, B. subtilis, S. aureus*, and *E. coli*. While all of the chloroform extract showed resistance against the tested bacteria, they were all sensitive to the methanolic and ethanolic extract of the roots and leaves of *N. oleander*. The methanolic extract demonstrated a strong antibacterial effect, while the ethanolic extract demonstrated a moderate level of antibacterial activity against nearly all of the tested bacteria. Significantly, Derwich *et al.* (2010) demonstrated that the essential oils extracted from *N. oleander* flowers exhibited activity against *E. coli, P. aeruginosa, and S. aureus*, exhibiting inhibition zones of 28.89 mm, 18.22 mm, and 6.32 mm in that order.

To determine the cytotoxicity of plant extract, the survivability of cells on HeLa and Vero cell lines was investigated. Where, 2.5% DMSO was served as control. *N. oleander* extract showed the highest cytotoxicity on HeLa and Vero cell lines. That means 95% of cancer cells died as well as 80% of normal cells died. In the case of *N. oleander* leaf extract, LD_{50} of 46.5 mg/ml indicated that 50% of the test population exposed to the leaf extract of *N. oleander* at a concentration of 46.5 mg/ml would be expected to die cervical cancer (HeLa) cells after 48 hrs. In the case of Vero cells,

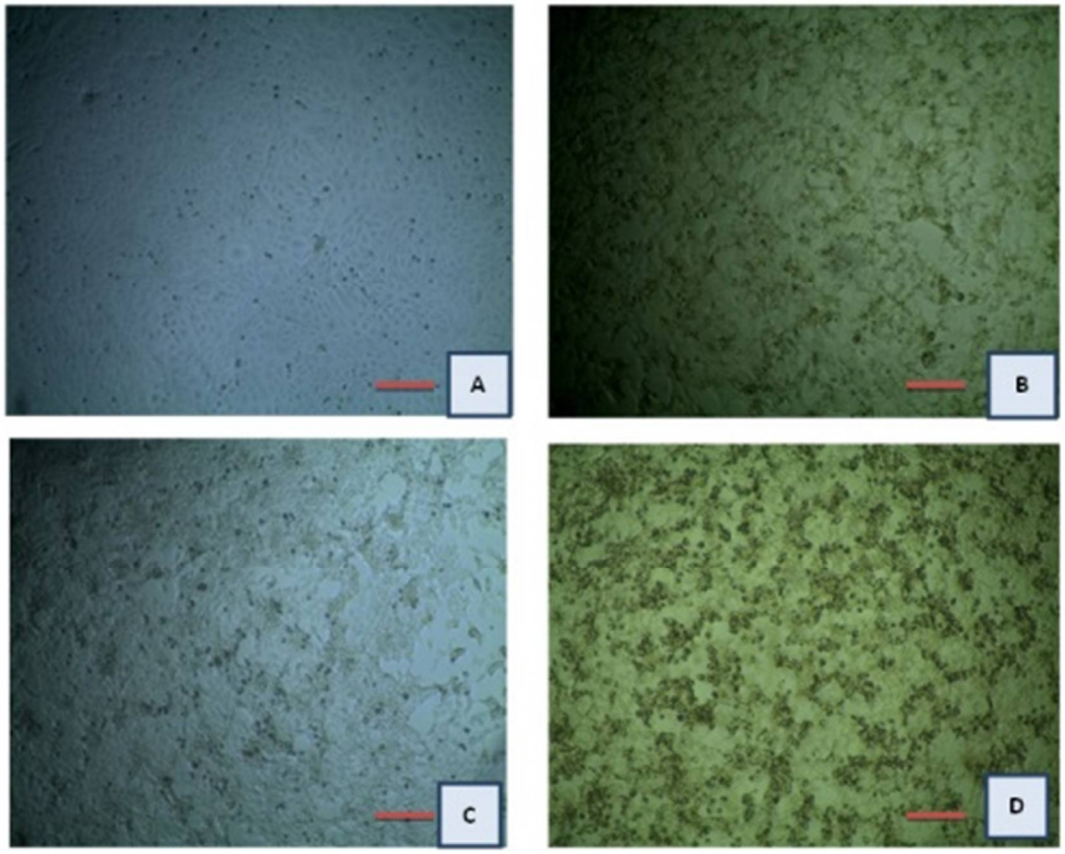

Fig. 1. Cytotoxic activity of cell free extract of selected bacteria on the HeLa and Vero cell line. A: Untreated control of Vero cells, B: treated with methanol extract of *N. oleander* showing cytotoxicity, C: untreated control of HeLa cells and D: treated with *N. oleander* plant extract showing reduced cell size and many floating dead cancerous cells. 1 Bar = 20 µm

LD_{50} of 9 mg/ml indicated that 50% of the tested normal Vero cells exposed to the leaf extract of *N. oleander* at a concentration of 9 mg/ml would be expected to die cells after 48 hrs. In this research, leaves extract of *N. oleander* showed more significant cytotoxicity against HeLa and Vero cell lines. Results are depicted in Table 2. The microscopic observations uncovered that the number of dead cells grew in proportion to the increasing concentrations of the extract treatment in both cell lines. Cells additionally showed cellular atrophy, cell shrinkage because of loss of water and cellular content, condensation of the cytoplasm and nucleus, loss of cell-cell contacts, and tissue disintegration denoting cell death (Fig. 1). Namian *et al.* (2013) evaluated the cytotoxicity of dichloromethane extract of leaf and flower of *N. oleander* by MTT assay. These had high cytotoxic effects against human breast cancer ($T_{47}D$), human hepatocellular carcinoma (HepG-2) and human chronic myeloid leukemia (K562) cell lines. According to Newman *et al.* (2007), the primary cause of the intriguing cytotoxicity of *N. oleander* extract on cell lines is the presence of cardiac glycosides, which have been extensively researched for possible anticancer properties.

Table 2. Growth inhibition percentages recorded at screening concentrations of 1g/ml of 2.5% DMSO extract on HeLa and Vero cell lines.

Sample	Survival of cells		Remarks
	HeLa	Vero	
Solvent (-)	100%	100%	Cytotoxicity was observed on the HeLa and Vero cell lines. The LD_{50} values for the HeLa and Vero cell lines were 46 mg/ml and 9 mg/ml, respectively.
Solvent (+)	>95%	>95%	
N. oleander	<5%	10-20%	

The results of this study revealed that different potential phytochemicals were present in the leaf extract of *N. oleander* plant. This plant also showed significant cytotoxicity on both normal cells and human cervical cancer cells. Therefore, the plant is great contender for additional chemical investigation to isolate the active constituents.

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