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Study on phytochemical, antibacterial, antifungal, antioxidant and anticancer activity of *Nerium oleander* L.

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Abstract

Plants have provided a source of inspiration for novel drug compounds as plants derived medicine have made significant contribution to human health. Phytomedicine can be used for the treatment of many diseases. The objectives of this work are to investigate the phytochemical, antioxidant, antimicrobial and cytotoxic effects of the plant *Nerium oleander* floral extracts. These activities had been evaluated by DPPH, disk diffusion, and MTT assay respectively. The result of phytochemical analysis showed the presence of flavonoids, terpenoids, cardiac glycoside, saponins, phenolic compounds in different solvents. Acetone extract from *Nerium oleander* flower showed antimicrobial and antifungal activity. Free radical scavenging activities of the extracts was evaluated using DPPH method. The results showed that antioxidant activity 97.78µg/ml respectively. Anticancer activity was measure by MTT assay. Acetone extract showed significant toxicity to the triple negative breast cancer cell line (MDA-MB-231) at the dose level tested. The extract of *Nerium oleander* flower had significant antioxidant, antimicrobial and cytotoxic activity.

Key works : *Nerium oleander,* Phytochemical, Antibacterial, Antifungal and Anticancer activity.

Plants have contributed many novel compounds for preventive and curative medicine in modern science. The plants interact to the stressful environments by physiological adaptation and altering the biochemical profile of plant tissues and producing a spectrum of secondary metabolites[7]. The medicinal plants include a various type of plants used in herbalist and some plants have a medicinal activity. These medicinal plants consider rich sources of ingredients and can be used in drug development and synthesis[6]. Ayurveda is the

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most ancient science of life having a health approach. The preparation of medicines pharmacy and parts of this science, are involved from a very rudimentary form in India, around 20,000 medicinal plants have been recorded[2].

Nerium oleander L. possess important pharmacological activities like anti-inflammatory, antibacterial, anticancer, antinociceptive and CNS depressant activity[4]. This plant is used as traditional medicine. Its treatment of several disease is described in cases of ulcers and even to induce abortion. The leaves a reused in external friction tale scabies, hair loss, diabetes and toothache. Few studies focus on the chemical analysis and phenolic compounds of this plant. The biochemical studies a very high quantity of polyphenols in the leaves[1]. In this recent study dried fresh flowers of the plant extract have been reported to have anti-inflammatory activity. It is also shown that the floral extract has antibacterial, antifungal, antioxidant and anticancer activity.

Nerium oleander was collected from the local market in Coimbatore. The flower was washed properly and it was air dried. This dried material was grinded using grinder. 25gm of air dried powered was taken in 150ml of acetone, methanol and chloroform. Plugged with aluminium foil and then kept on rotary shaker at 14rpm for 48 hours. The supernatant collected and the solvent was filtered with whatmann filter paper. The solvent was evaporated and stored at 4°C.

Test for flavonoids (Lead acetate test) :

One ml of extract was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids[4].

*Test for Terpenoids (*Salkowski *test) :*

To 1ml of extract was added 2ml of chloroform. Concentrated H_2SO_4 was added to form a layer. A reddish-brown colour of the interface indicates the presence of terpenoids[7].

*Test for Cardiac Glycosides (*Keller Killiani *Test) :*

One ml of extract was mixed few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added and observed for the formation of two layers. Lower reddish-brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides[9].

Test for Phenolic compounds (Ferric chloride test) :

Extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols[12].

Test for Saponins (Foam test) :

One ml of plant extract was shaken with 2ml of distilled water then agitated. In 15minutes formation of foam indicates Saponins[10].

Test for Tannins :

Few drops of 0.1% ferric chloride was added to 1ml of sample and observed for the

appearance of brownish dark blue colour for the presence of tannins[8].

Test for Proteins (Ninhydrin test) :

One ml of the extract and 5% of Ninhydrin reagent was added and kept boiling waterbathfor1-2minutes. Appearance of violet colour indicates the presence of proteins.

Antmicrobial activity :

The antimicrobial activity of the flower extract *Nerium oleander* was carried out. The flower extract was screened for antimicrobial and antifungal activities.

Antibacterial Assay :

Method: Disc diffusion method

Organisms :

- *Bacillus subtilis*
- *Salmonella typhi*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*

Media : Nutrient agar

The extract obtained from flowers was 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. *Bacillus subtilis*, *Salmonella typhi, Pseudomonas aeruginosa, and Staphylococcus aureus*. For the disc diffusion method, the filter paper discs (6mm in diameter) were soaked with 25µ. Lof stock solution of the extracts (50, 75, 100mg/mL). The sterilized petri dish was labelled with the bacterial names that had been previously inoculated with the test microorganisms. Then filter paper discs were placed onto these agar plates.

The plate was incubated at 37ºC 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 was measured in millimetre. The available antibiotics disc, Tetracycline (10 mcg per was disc used as positive control. The MIC of the extracts against the tested microorganisms were determined by the agar dilution method[9]. (Figs. 1,4 & 7).

Antifungal Assay :

Method: Disc diffusion method

Organisms: *Aspergillus parasiticus,*

Aspergillus niger

Media: Sabouraud dextrose agar

Filter paper discs of (6mm diameter) were soaked with extracts. The petri dish was sterilized and labeled with fungal names and also labeled with flower solvents used. The sterilized sabouraud dextrose agar (SDA) medium was poured into the petri dishes containing fungal suspension. The media was allowed to solidify in the room temperature. The filter paper disc which was soaked with different concentration of extracts was placed in Petri dishes at their labelled positions. A plate washed then Incubatedat 37°C for 48hours and the Inhibitory zone of each disc was measured[14]. (Figs. 5,6 & 8).

3. Antioxidantactivity :

DPPH *assay :* The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical method. The stock solution was prepared by dissolving 24 mg DPPH in 100mlmethanol and stored at -20°C until needed. The working solution was obtained by mixing 10 ml of stock solution with 45 ml of methanol to obtain an absorbance of1.1±0.02 units at 515nm using the spectrometer. 150 µL of extract at different concentration (20-100µg/ ml) was allowed to react with 2850µL of the DPPH solution for 2 hr inthe dark. The absorbance of the solutions obtained was measured at 515nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the scavenge the DPPH radical was calculated using the following equation.

% of DPPH of radical scavenging activity = (control OD-Sample OD/ Control OD) ×100
Where control was the absorbance of the control reaction and test was the absorbance in the presence of extracts[12].

Anticancer activity, Apoptotic Induction Studies :

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms lead to characteristic cell changes (morphology) and death. A cell initiates intracellular apoptotic signalling in response to a stress, which bring about cell suicide. The studies on the ability of various compounds to induce apoptotic incancer cells results in the identification of anticancer/anti prolifer ativeactiv ityinthem.

Various assay methods are available for the detection of this activity of which 2common assays were demonstrated. A common and well-established anticancer drug -5Fluorouracil (5FU) was used to demonstrate the apoptotic induction in the cultured MDA-MB-231(Triple Negative Breast cancer cell line)

Methods :

Type of cells: MDA-MB-231(Triple Negative Breast cancer cell line)
Sample: *Nerium oleander* (Acetone extract)
Test conducted: MTT (Methyl Thiazole Tetrazolium) assay
No of replicates: Triplicate
Dose :10 µl

MTT assay :

The MTT proliferation measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The yellow Tetrazolium MTT(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide) is reduced by the action of dehydrogenise enzymes to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophoto metrically at570nm. The MTT reagent yields low background absorbance value in the absence of cells. The formazan crystals, upon dissolution in to appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and can be measured. Since reduction of MTT can only occur in metabolically active

cells the level of activity is a measure of viability of cells. Three thousand cells/well were seeded in 96-wellplatesin DMEM supplemented with 10% FBS. After 24 hours, the cells were exposed to 10µl concentrations of 5FU against untreated normal controls. Following 24-hour incubation with the 5FU, MTT reagent was added in a 10% equivalent volume to that of the total culture volume and further incubated for 4hours.

After the incubation, the media contained in the wells were removed by tapping and the solubilization solution in an equal volume to that of the culture volume was added. This was kept a side for 30 minutes in dark at room temperature. Finally, absorbance was read at 570nm in an ELISA reader[2].

Percentage of Inhibition was calculated as per the formula:

$$\% \text{ Inhibition} = 100 - \frac{\text{Abs. Of Control Group}}{\text{Abs. Of Test Group}} \text{ X100}$$

The phytochemical active compound of *Nerium oleander* was qualitatively analyzed for flower extract and the results are presented in (Table-1). In these screening different types of results in different solvent extracts. The qualitative phytochemical investigation of aqueous and acetone methanol and chloroform extracts of flower of *Nerium oleander* showed the presence of active chemical constituents such as flavonoids, Cardiac Glycosides, Proteins, Phenolic compounds, Saponins, Terpenoids and Tannins.

1) *Antimicrobial activity :*

This work revealed antimicrobial activity of acetone extract of plant, Nerium *oleander* against different microorganism. The flower of plant of Nerium *oleander* was tested their antimicrobial activity against four bacteria and two fungi.

The study shows the pharmacological importance of flower of *Nerium oleander* showing anti microbial and antifungal activity and thoubs anteaters traditional medicinal use.

Table-1. Qualitative phytochemical analysis of *Nerium oleander* Flower extract

Qualitative phytochemical analysis of *Nerium oleander* flower extract				
Chemical test	Acetone extract	Methanol extract	Chloroform extract	Aqueous extract
Flavonoid (Lead acetate test)	+	_	+	_
Terpenoids (Chloroform test)	+	+	_	_
Cardiac glycoside (Keller-killianitest)	+	+	+	_
Phenolic Compound (Ferric chloride test)	+	+	_	+
Saponins (Foam test)	_	+	+	_
Proteins (Ninhydrin)	+	+	+	_
Tannins	+	+	_	_

Note: (+) Positive (-)Negative

Nerium oleander was studied by measuring zone of inhibition formed around the agar disc and the results are given in (Table-3) The acetone extract showed antibacterial activity against (*Stapyhlcoccus aureus*, *Salmonella typhi, Bacillus subtilis*, *Pseduomonas aeruginosa* and antifungal activity against *Aspergillus parasiticus*, *Aspergillus niger*

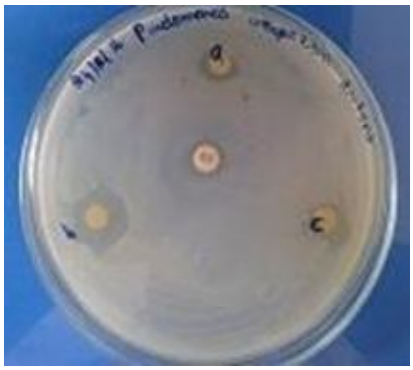


Figure 1. Antibacterial activity of *Nerium oleander* against *Pseudomonas aeruginosa*

Figure 2. Antibacterial activity of *Nerium oleander* against *Staphylococcus aureus*

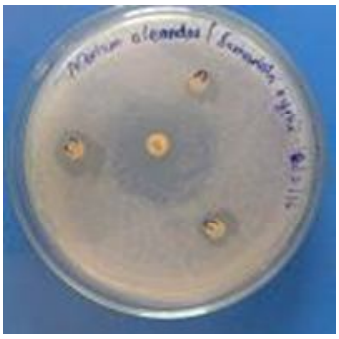


Figure 3. Antibacterial activity of *Nerium oleander* against *Salmonella typhi*

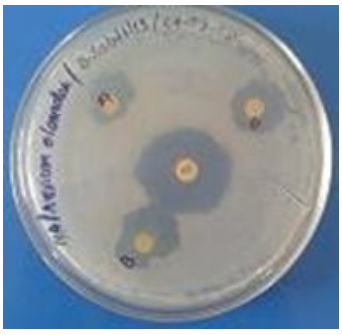


Figure 4. Antibacterial activity of *Nerium oleander* against *Bacillus subtilis*

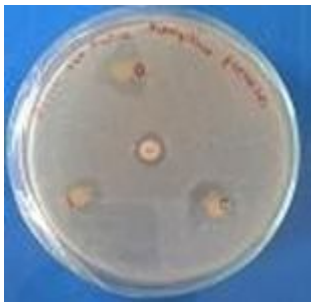


Figure 5. Antifungal activity of *Nerium oleander* against *Aspergillus parasiticus*

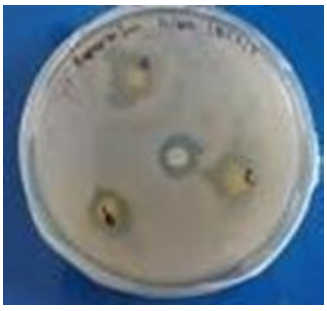


Figure 6. Antifungal activity of *Nerium oleander* against *Aspergillus niger*

Table-2. Antibacterial activity (zone of inhibition)

S. No	Organisms	Concentration			
		50%	75%	100%	Control
1.	*Pseduomonas aeruginosa*	1.5	2	2.7	3
2.	*Stapthylococcus aureus*	1	1	1.5	1.5
3.	*Salmonella typhi*	1	1.5	2	1
4.	*Bacillus subtilis*	1.5	2	3	3

Table-3. Antifungal activity (Zone of inhibition)

S. No	Organisms	Concentration			
		50%	75%	100%	Control
1.	*Aspergillus niger*	1.5	1.5	2.6	1.6
2.	*Aspergillus parasiticus*	1.5	1.8	2	1

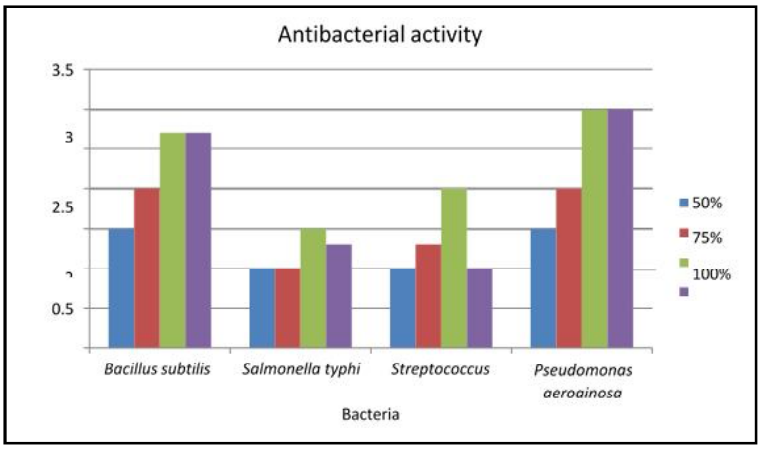


Figure 7. Antibacterial activity of *Nerium oleander* against selected bacteria

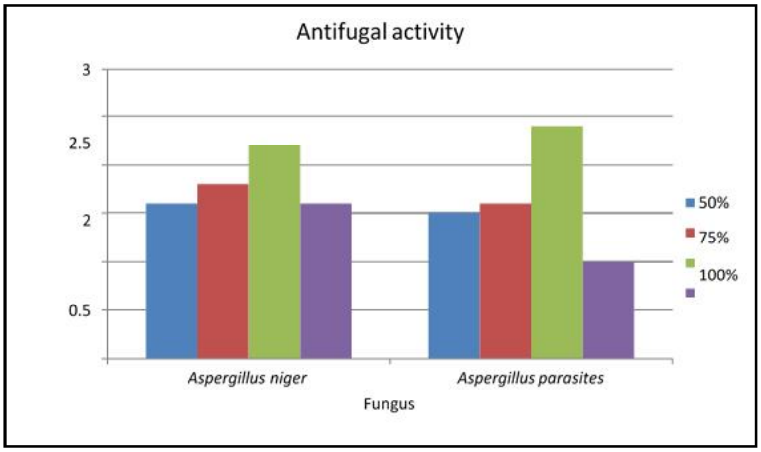


Figure 8. Antifungal activity of *Nerium oleander* against selected fungi

The acetone extract showed good Anti-microbial activity against bacterial species *Bacillus subtilis, Psuedomonas aeruginosa,* fungal species *Aspergillus niger, Aspergillus parasiticus.*

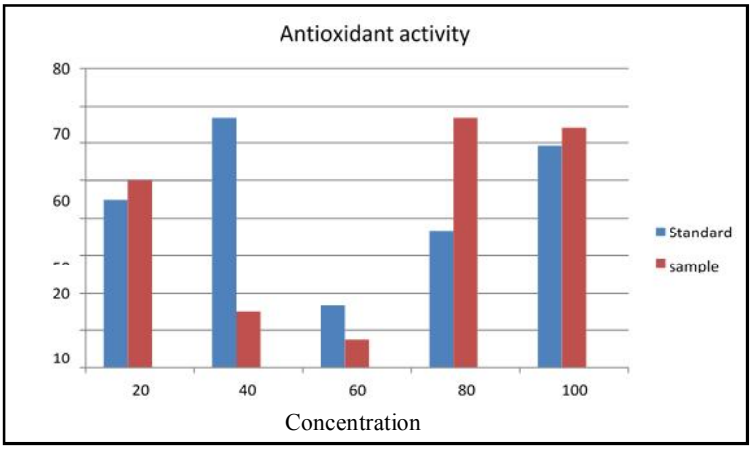
2) *Antioxidant activity :*

The DPPH antioxidant activity for the acetone extracts was determined and IC_{50} values were calculated. The percentage inhibition of scavenging activity was shown in (Table-4) and a plate Different concentration of the acetone extracts wasanalyzedshowed IC_{50} value.

The DPPH assay showed that acetone extracts of the plant flowers have high antioxidant activity and could scavenge free radical with IC_{50} value of 97.78µg/ml respectively. (Fig. 9).

Table-4. Antioxidant activity of *Nerium oleander*

S.No	Concentration	% of inhibition			Average	IC_{50}
Standard						
1	20	47.5	42.5	45	45	6.948
2	40	70	62.5	67.5	66.667	
3	60	20	12.5	17.5	16.667	
4	80	40	32.5	37.5	36.667	
5	100	62.5	55	60	59.167	
Sample						
S.No	Concentration	% of inhibition			Average	IC_{50}
1	20	52.5	47.5	50	50	97.78
2	40	17.5	12.5	15	15	
3	60	10	5	7.5	7.5	
4	80	70	62.5	67.5	66.667	
5	100	67.5	60	65	64.167	



Graph 9. Antioxidant of *Nerium oleander*

3) *Anti-cancer activity :*

Trypan blue staining :

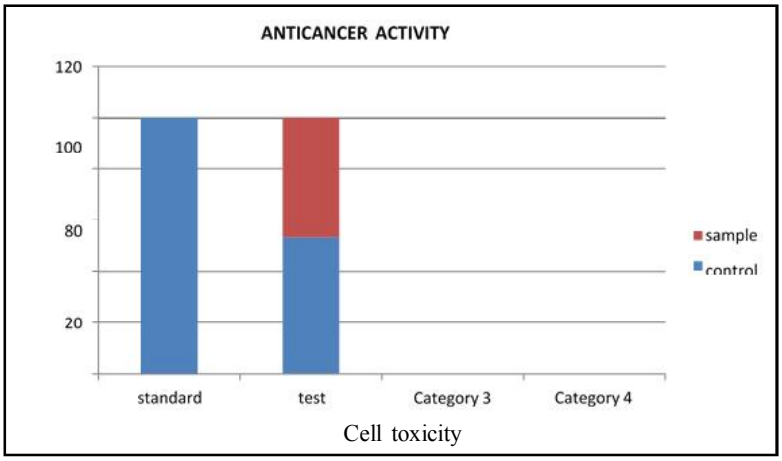
The cytotoxic activity of plant extract in normal liver cell showed maximum viability of cells at 10 µl concentration and other concentration showed decreasing viability with increasing concentration. And highest viability concentration is taken as optimal dosage concentration, which taken for MTT assay.

MTT assay :

The acetone extract of exhibit invitro cytotoxicity againstMDA-MB-231 (Triple Negative Breast cancer cell line). but it was found to be safe in normal cells. The cancer cell showed 53.23% viability after treating with the extract and the untreated control showed 100% viability. (Fig. 10).

Table-5. Anticancer activity of *Nerium oleander* flower acetone extract

Treatment group	% of viability
Control	100
Sample	53.23



Graph 10. MTT assay in MDA- MB 231 Cell line

The acetone extract showed significant toxicity to the triple negative breast cancer cell line (MDA-MB-231) at the dose level tested. This indicates that the extract has good anticancer activity against triple negative breast cancer.

Anticancer activity was measure by MTT assay. Acetone extract showed significant toxicity to the triple negative breast cancer cell line (MDA-MB-231) at the dose level tested. This indicates that the extract has good anticancer activity against triple negative breast cancer.

The extract of *Nerium oleander* flower had significant antioxidant, antimicrobial and cytotoxic activity. These biological activities are because of the presence of secondary metabolites. Morphological studies of treated cells indicated that cytotoxic effect of oleanderin is via induce of apoptotic death.

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