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Antioxidant and Anticancer Activity Assessment of *N. Oleander* on the Hacat Skin Cancer Cell

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The study aimed to use in-vitro DPPH and the HaCaT skin cancer cell line method to examine hydroalcoholic leaf extract's antioxidant and anticancer properties from Nerium oleander. The hydroalcoholic extract of Nerium oleander was prepared to assess antioxidants and anti-cancers using the in-vitro DPPH and MTT assay method against the HaCaT skin cancer cell line. The antioxidant activity of Nerium oleander hydroalcoholic leaves extract was found to be IC50 =896.9μg/ml, equivalent to IC50= 10.93μg/ml of ascorbic acid, and IC50 = 91.49±0.181μg/ml effective against the HaCaT skin cancer cell line. Further research against carcinogenesis from the hydroalcoholic extract of Nerium oleander leaves, which demonstrated therapeutic potential against cancer cells, can yield significant results. The bioactive chemicals of Nerium oleander leaves may be beneficial for treating skin cancer. Limitations: The study's emphasis on in-vitro tests, which might not accurately capture the nuances of in-vivo settings, has limitations. To confirm the extract's potential for medicinal use, more research should examine its safety and efficacy in animal models and human trials.

Keywords: Hydroalcoholic extract; HaCaT skin cancer cell line; N. oleander; Skin cancer.

One of the leading causes of death worldwide is cancer. Thus, there is ongoing pressure to discover new bioactive substances from natural sources. Since tumour cells are becoming resistant to currently prescribed medications like vinca alkaloids and taxanes, there is an urgent need for new anticancer drugs[1, 2]. *N. oleander* is an evergreen dogbane plant in the Apocynaceae family. Currently, it is the only species officially acknowledged as a member of the genus *Nerium*. It is also known as the oleander because of its slight similarity to the unrelated olive Olea. The most common plant is the oleander, whose seductive blossoms pose a particular risk of accidental consumption[3]. *N. oleander* has long been considered a poisonous plant due to the potential for specific compounds to be dangerous when ingested in large quantities, particularly by animals. *N. oleander* has excellent medicinal benefits and is used to cure many conditions, including ringworm, asthma, epilepsy, leprosy etc. It is also used to induce abortions, and research on its potential to treat cancer is still ongoing[4–7].

The phytochemical content and toxicity of leaf and flower extracts from *N. oleander* L. gathered in the Turkish province of Giresun are examined in this study. Similar studies are encouraged by the results, which emphasize

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the necessity for more investigation into the toxicity and phytochemical profiles of plants[8]. The cardiotoxicity and beneficial inotropic effects of hydroalcoholic extracts of *N. oleander* were investigated for potential arrhythmogenic effects; these findings are essential for comprehending the plant's influence on cardiomyocyte electrophysiology[9]. *N. oleander's* supercritical CO_2 extract suppresses the growth of human pancreatic cancer by blocking the PI3K/mTOR pathway, demonstrating the anti-proliferative properties of oleandrin in an orthotopic Panc-1 model and in vitro[10]. A suggested Phase II dosage of 0.2255 mg/kg/day for *N. oleander's*, an oleander-derived substance, is indicated for patients with advanced solid tumors. It suppresses Akt, FGF-2, NF-êB, and p70S6K[11]. The capacity of oleandrin and N. oleander to block HTLV-1 transmission was investigated; this suggests that they may have broad antiviral potential by interfering with the formation of virological synapses and the incorporation of viral envelope glycoprotein into mature particles[12]. Oleandrin, which is extracted from *N. oleander* leaves, has anti-inflammatory and anti-tumor properties. In animal models, it inhibits TPA-induced tumor promotion, making it a possible chemopreventive drug against skin cancer[13]. Therefore we select this plant to evaluate *N. oleander* hydroalcoholic extract's anticancer potential using the in vitro approach on the HaCaT skin cancer cell line.

MATERIAL AND METHODS

Chemicals like DPPH (SRL Chem Cat no. SR-29128), Methanol (S.D. fine Cat no. 10930lC250), and Ascorbic Acid (S.D. fine F13A/0413/1106/62) were purchased, and the remaining chemicals used were analytical grade.

Collection of plants and analytical reagents

The university's medicinal garden in Ayodhya, Uttar Pradesh, India, produced *N. oleander* leaves, which Dr. A. K. Shukla, an assistant professor, verified in the botany department of K. S. Saket P.G. University Ayodhya. The Institutional Animal Ethics Committee authorized and reviewed the experimental approach before the animals were gathered by CPCSEA, New Delhi, and regulations and treated with the utmost care. Animal studies were conducted at Bhupal Nobles' University in Udaipur, Rajasthan, India.

Extraction

We gathered, cleaned, dried, and ground up the leaves of the *N. oleander* plant. The N. oleander leaf coarse powder was soaked for 15 days in a hydroalcoholic solvent. The hydroalcoholic extract was concentrated using a rotary evaporator until it was dry, and it was then kept in an airtight container for further use[14–16].

Screening for phytochemicals

The official procedures were employed to complete phytochemical screening[17–19].

Evaluation of antioxidant activity

0.1 ml of 0.1 mM DPPH solution was placed in a 96-well plate, and 5il of a separate stock of the test drug (as mentioned in the Excel sheet) was added. The reaction was set up in triplicate, and blank duplicates were made using 0.2 ml of DMSO/Methanol and five il of a chemical at various doses (as mentioned in the Excel sheet). In the dark, the plate was incubated for 30 minutes. After incubation, a microplate reader (iMark, BioRad) was used to measure the decolourization at 495 nm. Twenty microliters of deionized water in a reaction mixture were used as the control. About the control, the scavenging activity was expressed as "% inhibition and was used to calculate IC-50.

Calculations

((Abs Control- Abs Sample)/Abs Control)×100 is the DPPH Scavenging activity.

Maintenance of cell lines

The skin cancer cell line HaCaT was acquired from NCCS in Pune, India. The cells were kept in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% antibiotic-antimycotic solution in a CO2 incubator at 5% CO_2, 18–20% O2, and 370°C. The cells were subcultured every two days.

Background of the study

A colourimetric test called 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) is used to measure cytotoxicity and cell growth. It reduces the yellow-coloured, water-soluble tetrazolium dye MTT to formazan crystals. Live cells produce mitochondrial lactate dehydrogenase, which breaks MTT into insoluble formazan crystals. When dissolved in a suitable solvent, these crystals take

on a purple hue, the intensity of which can be detected spectrophotometrically at 570 nm and is correlated with the number of viable cells[20-22].

***In-vitro* anticancer activity assessment (MTT assay)**

In order to evaluate the possible cytotoxicity of the materials presented against HaCaT cells, a typical cell line generated from adult human skin and cultivated on specialized plates, we used the MTT assay as a methodological approach. Following this, 10,000 cells were deposited onto each plate and given a 24-hour window to multiply in a regulated setting with high humidity and temperature. A DMEM medium supplemented with different chemicals was used to promote cellular proliferation. The test materials (hydroalcoholic extract) were added to the cells at different concentrations after the incubation period, as specified in an Excel document that was provided. The cells were exposed for a further twenty-four hours, then treated with MTT solution and incubated for two more hours. The absorbance was then measured with a spectrophotometric device at 540 nm and 660 nm. Graph Pad Prism-6 software was used for quantitative analysis to calculate the IC-50 value, which is the concentration of the chemical needed to cause a 50% drop in cell viability. Equation Y = Mx + C, where Y is the viability, M is the slope, and C is the y-intercept, was used to use linear regression analysis to obtain this important statistic. The viability graph allows the values for Y = 50, M, and C to be determined. Additionally, an AmScope digital camera (10 MP Aptima CMOS) and an inverted microscope (Olympus eK2) were used to examine the cells under a microscope. The acquired images made it easier to assess cellular morphology thoroughly.The formula [(Absorbance of treated cells / Absorbance of untreated cells) * 100] was employed to quantify cell viability as a percentage. This computation was integral in ascertaining the relative impact of the materials on cell survival.

RESULTS AND DISCUSSION

Screening for phytochemicals

After conducting a phytochemical screening test on the test sample, it was discovered that the hydroalcoholic extract of *Nerium oleander* included alkaloids, glycosides, tannins, flavonoids, triterpenoids, and saponins.

Antioxidant property

Plant materials were tested for antioxidant activity, and the mean, standard deviation, and SEM values were obtained. Next, determine the percentage by which the plant extract's scavenging action is inhibited. The experimental findings are displayed in the table 1 below.

Table 1 shows that the assessment of antioxidant property of plant extract was performed 4 times and calculated the mean value, standard deviation and standard error of the mean that increased the accuracy of results.

Antioxidant property (DPPH scavenging) was observed in the sample (IC50 =896.9µg/ml), which was found equivalent to (IC50= 10.93µg/ml) of ascorbic acid [22]. The amount of a plant extract (896ìg/ml) required to 50% suppress or inhibit concentration for free radicals (scavenging activity).

Table 1. Antioxidant property (DPPH scavenging) observed in different samples

	Final Replicate Value					Stats		
Sample Conc.	1	2	3	4	Sample Conc.	Mean	SD	SEM
0	-4.76563	-1.17187	2.109375	3.828125	0	8.77E-15	3.794227	1.897114
10	-0.39063	-1.32812	1.171875	-1.17187	10	-0.42969	1.14376	0.57188
50	0.390625	0.390625	0.546875	3.359375	50	1.171875	1.460192	0.730096
100	6.015625	3.828125	4.296875	5.703125	100	4.960938	1.062615	0.531307
125	12.5	11.09375	11.25	15.15625	125	12.5	1.879335	0.939668
250	21.64063	21.17188	22.26563	21.48438	250	21.64063	0.459988	0.229994
500	30.07813	36.17188	36.01563	35.70313	500	34.49219	2.949154	1.474577
1000	54.84375	49.21875	50.625	54.0625	1000	52.1875	2.697293	1,348,647

Unexpected results

The antioxidant property of plant extract was determined by DPPH scavenging method. The results was found in the sample (IC50 =896.9µg/ml), which was found equivalent to (IC50= 10.93µg/ml) of ascorbic acid. The results of the DPPH Scavenging Assay suggested that plant material may contain bioactive chemicals that could shield the skin from free radicals. These results also indicated that plant having potential bioactive compounds and it is able to eradicate many other diseases. Therefore need to do more research work on this plant and development of novel topical dosage form.

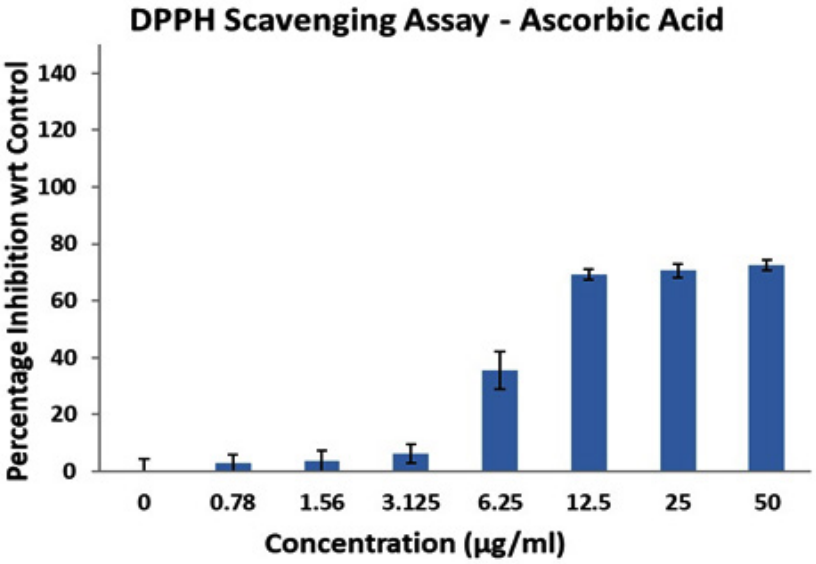

Fig. 1. DPPH Scavenging Assay-Ascorbic Acid

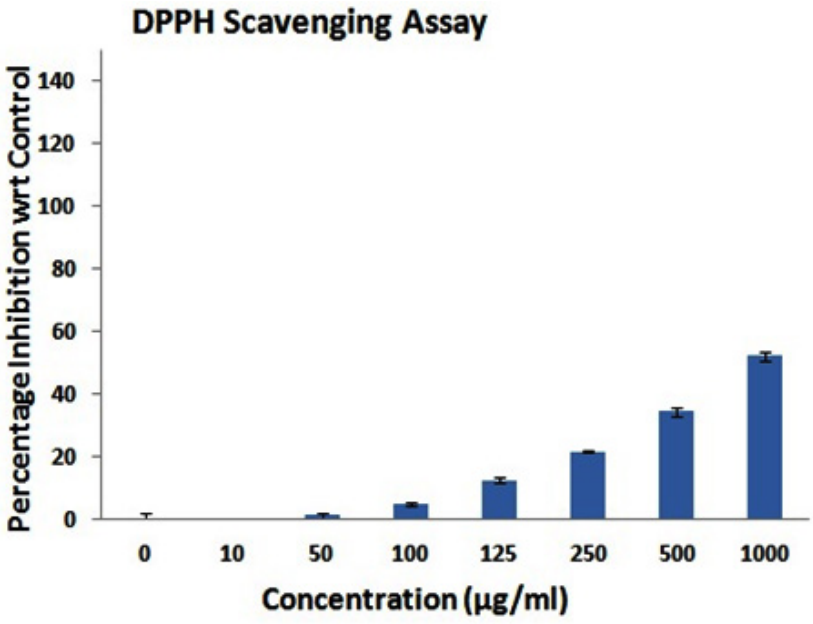

Fig. 2. DPPH Scavenging Assay observed in the sample

Nerium oleander hydroalcoholic extract was investigated for its potential anticancer properties. We employed an *in-vitro* testing technique, wherein we treated HaCaT skin cancer cells with varying material concentrations (10, 50, 100, 250, 500, and 1000 µg/ml). The findings demonstrated the efficacy of Test Compound S1, particularly at low dosages, in destroying malignant cancer cells. Because S1 in the extract had low IC50 values on the skin cancer cell line HaCaT, it demonstrated high potential in the battle against skin cancer. The study showed that S1 may inhibit the growth of human skin cancer cells. More investigation is required to learn how S1 combats cancer cells. This involves employing P.I. labelling to examine the cell cycle, Annexin V/PI staining to examine apoptosis, and evaluating the expression of proteins linked to cell death, such as caspase 3, 7, 9, Bcl2, p53, and ROS. These extra experiments will aid in our comprehension of the mechanisms underlying S1's possible anticancer effects in the laboratory. The comprehensive findings of our investigation are displayed in Figures 3 to 8 respectively.

In this work, we investigated the ability of a hydroalcoholic extract from the *Nerium oleander* plant to combat cancer. The hydroalcoholic extract's anticancer solid properties were observed in skin cancer cells (HaCaT). More in-depth study is required to comprehend its exact components and operation. This can significantly advance skin cancer diagnosis, treatment, and management.

The IC50 values of the test substances, S1, against the skin cancer cell line HaCaT following a 24-hour incubation period.

Fig. 3. MTT assay-HaCaT-control

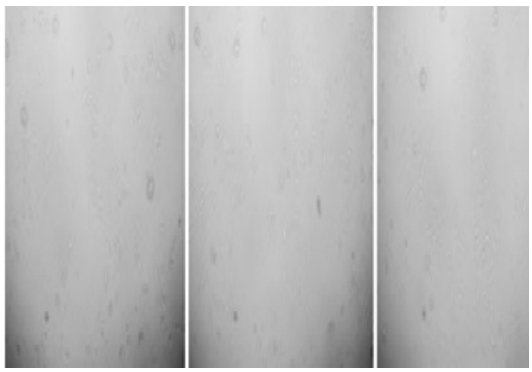

Fig. 4. MTT assay-HaCaT- Treated (100µg/ml)

Fig. 5. MTT assay-HaCaT-treated (250µg/ml)

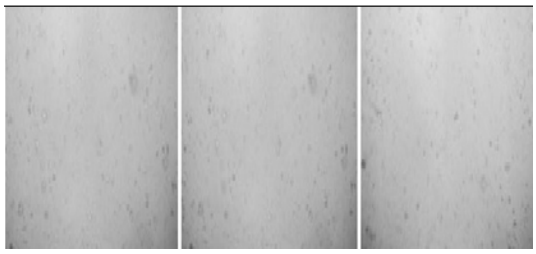

Fig. 6. MTT assay-HaCaT-treated (500µg/ml)

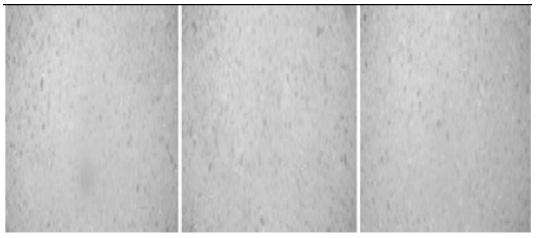

Fig. 7. MTT assay-HaCaT-treated (1000µg/ml)

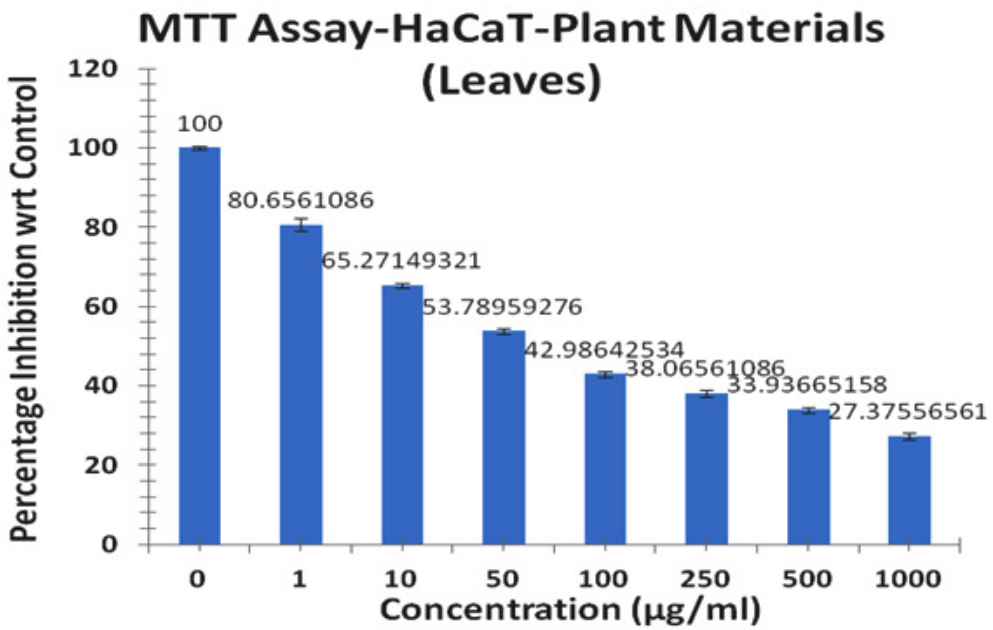

Fig. 8. MTT assay-HaCaT- *Nerium oleander* hydroalcoholic extract for anticancer activity assessment

A plant extract of 91.49 ìg/ml was 50% effective in killing the growth of cancerous cells (anticancer activity). The results of the MTT assay suggested that plant material may contain bioactive chemicals that could shield the skin from carcinogenic agents.

It has been found that *N. oleander's* extract suppresses the growth of human pancreatic cancer by blocking the PI3K/mTOR pathway, suppresses Akt, FGF-2, NF-êB, and p70S6K and *N. oleander's* extract bioactive compound Oleandrin, which is extracted from *N. oleander* leaves, has anti-inflammatory and anti-tumor properties. In animal models, it inhibits TPA-induced tumor promotion, making it a possible chemopreventive drug against skin cancer[7-13].

The HaCaT skin cancer cell line is included in the study title, which could restrict how broadly the results can be applied. Although utilizing a particular cell line can yield important information about how Nerium oleander hydroalcoholic extract affects skin cancer cells, it might not be a reliable indicator of how it affects other cancer cell types or cancer in living things. Furthermore, results from in vitro research may not translate directly into clinical settings since it is impossible to reproduce the intricate interactions within a real creature fully. Furthermore, although in vitro research helps screen possible therapeutic compounds in advance, it ignores crucial aspects such as metabolism, pharmacokinetics, and toxicity necessary to determine whether a material is suitable for use in humans. Therefore, we need to do more research to identify critical results.

CONCLUSION

Antioxidant and anticancer activity assessments of *Nerium oleander* hydroalcoholic extracts using the in vitro approach on the HaCaT skin cancer cell line showed that the bioactive chemicals of *N. oleander* leaves may be beneficial for treating skin cancer. However, further research is needed to elucidate the specific bioactive component responsible for the observed anticancer activity of leaf extract from *Nerium oleander*.

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Authors Contribution

The primary author of this article, Manish Kumar Yadav, conducted the research for it. The remaining writers each made an equal contribution, including formatting and drafting.

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Ethics Approval Statement

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Conflict of interest

Nil.

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