PRELIMINARY PHYTOCHEMICAL ANALYSIS OF THE FLOWERS OF *NERIUM OLEANDER* L.

Project submitted

To

MAHATMA GANDHI UNIVERSITY

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BACHELOR OF SCIENCE IN BOTANY

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CERTIFICATE

This is to certify that this project work entitled "**Preliminary Phytochemical Analysis of the Flowers of *Nerium oleander* L.**" is a bonafide piece of project work done by **KAVYA SUDHAN (Reg No.: 210021022653), AKSHAY SURESH (Reg No: 210021022643), JITHIN P.J (Reg No: 210021022670)** in the Department of Botany, Bharata Mata College, Thrikkakara under my guidance and supervision for the award of Degree of Bachelor of Science in Botany during the academic year 2021-2024. This work has not previously formed the basis for the award at any other similar title of any other university or board.

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DECLARATION

I hereby declare that this project entitled "**Preliminary Phytochemical Analysis of the Flowers of *Nerium oleander* L.**" is the result of work carried out by me under the guidance of **Dr. Abin Kurian,** Department of Botany, Bharata Mata College, Thrikkakara. This work has not formed on the basis for the award at any other similar title of any other university of board.

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CONTENTS

INTRODUCTION

Plants are crucial for life on Earth and are vital to people's livelihoods. They have been a source of medicinal compounds for centuries, with the plant kingdom containing a vast array of active ingredients used to manage many diseases. The importance of herbs in treating human ailments is significant, leading to the widespread use of medicinal plants with curative properties. It's estimated that 14-28% of higher plant species have medicinal uses, and 74% of pharmacologically active plant-derived components were discovered through ethnomedicinal practices. Traditional medicines, primarily plant extracts, are relied upon by nearly 80% of the world's population for primary healthcare (Sajem and Gosai, 2006).

Herbal and natural products have been part of folk medicine worldwide for centuries. They tend to have fewer adverse reactions compared to modern pharmaceuticals, making them appealing to the public and healthcare institutions as cost-effective alternatives to synthetic drugs (Nair *et al*., 2005). Herbal medicines are highly sought after worldwide for primary healthcare due to their wide range of biological and medicinal activities, along with their high safety margins and lower costs. The use of medicinal plants stems from a long history of combating illnesses, leading to the discovery of drugs in various plant parts. Increased awareness of the side effects and complications associated with chemical and synthetic medicines, cosmetics, and health supplements has further fuelled the use of herbal products globally. These products are increasingly used, often in a self-prescribed manner, in both Eastern and Western societies.

Herbal medications are extensively sought after globally for basic healthcare due to their vast spectrum of biological and therapeutic activities, together with their high safety margins and reduced prices (Saravanan *et al*., 2015). The usage of medicinal plants derives from a long tradition of combating ailments, leading to the discovery of medications in diverse plant components. Increased knowledge of the adverse effects and risks associated with chemical and synthetic medications, cosmetics, and health supplements has further spurred the usage of herbal products internationally. These products are being utilized, frequently in a self-prescribed way, in both Eastern and Western countries (Basu *et al*., 2013)

The pharmacological effects of plant materials are generally owing to their secondary products, which are often a mixture of metabolites rather than a single substance. These effects are unique to certain plant species or groups, reflecting the different composition of secondary products in each plant. Plant essential oils and extracts, utilized for millennia in numerous

purposes, including food preservation and medicine, should be scientifically explored to increase healthcare quality. Medicinal plants include phytochemicals such alkaloids, flavonoids, polyphenols, and glycosides, which display diverse pharmacological actions. Screening these bioactive chemicals has led to the identification of novel pharmaceutical medicines with protective and therapeutic actions against a range of diseases (Parekh and Chanda, 2005; Jadon and Dixit, 2014).

Pharmacopoeial standards alone are insufficient to guarantee the quality of plant materials due to the often-poor condition of received materials, hindering effective microscopic examination. Thus, chemical methods, instrumental analysis, and thin-layer chromatography are necessary to assess plant material quality. Non-standardized extraction procedures can degrade phytochemicals and cause variations, leading to a lack of reproducibility (Pandey and Tripathi, 2014).

***Nerium oleander* L.**, is an evergreen shrub from the Apocynaceae family, widely grown ornamentally in warm temperate and subtropical areas worldwide. While it is generally known as oleander because of its similarity to the olive tree. Native to the Mediterranean area and subtropical Asia, this plant is endemic to the India-Pakistan peninsula. It grows throughout the Himalayas from Nepal to Kashmir up to a height of 1950m, it is also found in Afghanistan, Baluchistan, and gardens across India (Chaudhary *et al*., 2015). The plant has narrow, dark green leaves that are 5 to 20 cm long and come to a sharp tip. Its flowers, which can be various colours including lilac, salmon, pink, purple, and white, are clustered at the ends of branches and have five petals. The fruit is a long, narrow follicle that opens to release fluffy seeds. The plant can be propagated from seed and shows significant variability in seedling populations (Sinha and Biswas, 2016).

Various components of the plant, including the leaves, roots, and root bark, have been utilized in traditional medicine to treat a range of diseases. The leaves of the white-flowered species were suggested by Charka for external use in chronic and severe skin disorders like leprosy, while Sushruta utilized the plant in medicinal pastes for treating ailments such as alopecia. The root, when powdered and combined with water, was utilized to treat venereal infections. Additionally, the powdered leaves were used as a snuff to cure epilepsy. However, it is vital to know that all portions of the plant, especially the roots, are exceedingly deadly if swallowed. Studies have indicated that tinctures prepared from oleander flowers display

cardiotonic effects, whereas the roots contain CNS-active and spasmolytic qualities (Praveen *et al.,* 2012; Khare, 2004).

The significance of the current study lies in the absence of detailed phytochemical studies on the analysis of the flowers of *N. oleander,* even though extensive studies were conducted on other plant parts. Hence, preliminary phytochemical analysis of the flowers of *N. oleander* was investigated in the current study using chromatographic techniques to analyse its phytochemical potential.

OBJECTIVES OF THE STUDY

- Preparation of the different flower extracts of *Nerium oleander* for phytochemical analysis
- Preliminary phytochemical analysis of the flower extracts of *Nerium oleander*
- Standardization of the solvent systems for the chromatographic studies.
- Phytochemical investigation of the flower extracts of *Nerium oleander* using Thin Layer Chromatography (TLC).

REVIEW OF LITERATURE

According to the World Health Organization (WHO, 1977), a medicinal plant is one whose parts contain chemicals that can be used for healing reasons or are precursors for useful drug synthesis. This term separates plants with known therapeutic qualities and constituents from those considered medicinal but not thoroughly investigated. The word 'herbal drug' refers to plant parts (leaves, flowers, seeds, roots, barks, stems, etc.) used for making medicines (Irchhaiya *et al*., 2015). WHO (2001) further describes medicinal plants as herbal preparations made by putting plant materials to extraction, fractionation, purification, concentration, or other processes, for direct consumption or as a base for herbal products. Medicinal plants contain active ingredients used to cure diseases or reduce pain (Okigbo *et al*., 2008).

Phytochemicals, derived from plants, exhibit various biological activities and have been used in traditional medicine for centuries. About 70% of modern medicines have direct or indirect origins in plants. These compounds include antibiotics, antifungals, antivirals, antitumor agents, and compounds that deter germination, protecting plants from pathogens, insects, and predators. Plants also produce UV-absorbing compounds to shield leaves from sunlight damage. Due to the complexity of these compounds, they are often challenging to synthesize in labs, highlighting the ongoing importance of phytochemicals in drug discovery (Newman and Cragg, 2014; Lalrinzuali *et al*., 2015).

In optimization studies, the choice and strength of solvent are frequently identified as critical parameters. Surprisingly, the ratio of solvent to sample did not greatly alter the extraction efficiency, suggesting that huge amounts of solvents may be unnecessary. Each optimal extraction procedure is tailored to the plant being studied. Factors like temperature, solvent choice, and agitation speed can increase extraction but may also damage substances if not carefully regulated. Therefore, picking approaches with fewer influential factors is wise. However, for purity concerns, advanced extraction procedures like ASE should be considered (Azwanida, 2015).

Nerium oleander has a long history of use in ancient texts and folklore. Various parts of the plant, including flowers, leaf juice, bark, and latex, have been utilized for treating microbial and fungal infections. The plant is also employed as a therapeutic agent for various ailments. Leaves and bark are used as expectorants, heart tonics, diuretics, emetics, and diaphoretics. Roots, when boiled in water, are beneficial for skin complaints, herpes, and ringworm infections. Small doses of leaf juice are used for snake and venomous bites. Juice

from young leaves treats eye diseases, while root paste is used for ulceration, hemorrhoids, cancer, and leprosy. Oil from the root is applied to leprosy and skin diseases. Leaf decoction is used externally for scabies and to reduce swellings. Leaves and flowers act as emetics, diaphoretics, cardiotonics, diuretics, expectorants, and sternutatories (Patel *et al.,* 2010)

2.1 Phytochemical evaluation

Siham *et al*. (2014) found high levels of polyphenols in Nerium oleander leaves. HPLC analysis revealed cinnamic acid as the major component, along with epicatechin, catechin, and chlorogenic acid. The aqueous leaf extract yielded 2.3% crude polysaccharides, predominantly pectic polysaccharides composed of arabinose, galacturonic acid, galactose, and rhamnose (Sinha & Biswas, 2016). Neriucoumaric and isoneriucoumaric acids, two new coumaryl oxy-triterpenoids, were isolated from fresh leaves. Preliminary screening showed the presence of carbohydrates, flavonoids, alkaloids, steroids, cardiac glycosides, and tannins (Yadav *et al*., 2013). Nerium oleander leaves also contain oleanderocinoic acid, flavonoid glycosides, quercetin-5-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside, kaempferol-5-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside, and oleandigoside, which exhibited growth inhibitory and cytotoxic activities against MCF-7 human breast cancer cells (Siddiqui *et al*., 2012).

Sharma *et al*. (2012) isolated two new compounds, heptacosane-3-enyl-5-hydroxyhexanoate and 4-oxooctyl-2-hydroxyundecanoate, from stems. A polysaccharide fraction isolated from flower extracts contained L-rhamnose, L-galactose, and D-galacturonic acid (Qun *et al*., 2010). Additionally, cytotoxic pentacyclic triterpenoids, ciskarenin and trans-karenin, were reported in N. oleander leaves (Siddiqui *et al*., 1995). Oleanderoic acid and oleanderen, a new labdane diterpene and triterpene, were isolated from fresh leaves (Siddiqui *et al*., 1987). Kaneroside and neriumoside, cardiac glycosides, were isolated from fresh, undried winter leaves (Siddiqui *et al*., 1986).

2.2 Pharmacological studies

Huq *et al*. (1999) discovered a new cardenolide, 12β-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide, in the roots of Nerium oleander, which exhibited antibacterial and digoxin-like cardiac activities. Hussain & Gorsi (2011) demonstrated the potent antibacterial and antifungal properties of the ethanolic extract of both the plant's roots and leaves. Tannu *et al*. (2011) observed the antimicrobial activity of stem extracts on Pseudomonas aeruginosa and Bacillus subtilis in Wistar albino rats. Wong *et al*. (2013) found N. oleander effective against

Staphylococcus aureus. Jude (2013) noted the plant's strong inhibitory effects against Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, and Escherichia coli. Jeyachandran *et al*. (2010) reported that the methanolic extract exhibited a significant zone of inhibition (28 mm) against S. typhi. Swai *et al*. (2010) found Bacillus subtilis more sensitive than Gram-negative bacteria.

Malik *et al*. (2015) demonstrated that the ethanolic extract of N. oleander leaves exhibited the highest bactericidal activity against Pseudomonas aeruginosa at a concentration of 900 mg/ml. Hadizadeh *et al*. (2009) reported that ethanolic flower extracts showed antifungal activity against Fusarium oxysporum, Alternaria alternata, Fusarium solani, and Rizoctonia solani, with the best inhibition against F. oxysporum and F. solani. Derwich *et al*. (2010) found that essential oil extracted from N. oleander flowers exhibited in vitro antibacterial activity against Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus, with minimum inhibitory concentrations ranging from 1.45 to 5.10 mg/ml.

Ali and colleagues (2009) extracted essential oil from oleander flowers, which exhibited antitumor activity against Ehrlich Ascites Carcinoma (EAC) cell lines. Pathak et al. (2000) investigated the effects of different concentrations of Anvirzel (1.0 ng/ml to 500 microgram/ml) or Oleandrin (0.01 ng/ml to 50 microgram/ml) on continuously treated and pulse-treated/recovery cell cultures. Both compounds induced cell death in human cancer cells but not in murine cancer cells.

MATERIALS AND METHODS

3.1 Collection and processing of plant material.

The dried flowers of *Nerium oleander* L. were used for the current study. The fresh flowers of *Nerium oleander* were collected from their natural localities of Ernakulam and were authenticated. The fresh flowers collected were shade dried for two weeks in room temperature. After drying, the flowers were ground into fine powder using a mechanical blender and transferred into airtight glass containers with proper labelling and stored at ambient temperature for further studies **(Plate: 1).**

3.2 Preparation of flower extracts

N. oleander flower extracts were prepared using two different solvents such as Chloroform and Methanol. The cold extraction method was used to prepare both extracts. 5g of dried and powdered flower was taken in a conical flask (500ml) and 200 ml of chloroform was added for the preparation of the chloroform extract. Similarly, methanol flower extract of *N. oleander* was also prepared using the same methodology. The two conical flasks were kept in a rotary shaker for 3 days at room temperature. The extracts thus obtained were filtered using Whatman No. 1 filter paper. The extracts were later transferred to air tight glass containers, labelled and stored in refrigerator for the further analysis.

3.3 Qualitative preliminary phytochemical analysis.

To identify the presence of various classes of metabolites, different preliminary analysis was conducted.

3.3.1 Test for Alkaloids

3.3.1.1 Mayer's test: To a few ml of sample extract, two drops of Mayer's reagent are added along the sides of the tube. Appearance of white creamy precipitate indicates the presence of alkaloids (Tiwari *et al*., 2011).

3.3.1.2 Wagner's test: A few drops of Wagner's reagent are added to few ml of extract along the sides of the tube. A reddish-brown precipitate confirms the test as positive (Tiwari *et al*., 2011).

3.3.2 Test for Coumarins

Coumarins test: To two ml of sample extract, three ml 10% sodium hydroxide solution is added along the sides of the test tube. Appearance of yellow coloured solution indicates the presence of coumarins (Jayaprakash and Sangeetha, 2015).

3.3.3 Test for Flavonoids

3.3.3.1 Alkaline reagent test: 2 ml of 2% sodium hydroxide solution was mixed with plant crude extract, intensive yellow colour was formed, which turned colourless on addition of dilute acid (Jaradat *et al*., 2015).

3.3.3.2 Lead acetate test: To 2 ml of extract, few drops of lead acetate solution was mixed. Formation of yellow precipitate (Tiwari *et al*., 2011).

3.3.4 Test of Glycosides

Keller Killiani's test: To 2 ml extract glacial acetic acid is added along the sides of the test tubes and one drop of 5% ferric chloride solution added. Reddish brown colour appears at the junction of 2 layers and upper layer appears bluish green (Singh and Bag, 2013).

3.3.5 Test of Phenol

Ferric chloride test: To 2-3 ml of extract a few drops of 5% ferric chloride solution was added, presence of deep blue-black colour (Santhi and Sengottuvel, 2016).

3.3.6 Test for Quinones

Quinones test: To 2-3 ml of sample extract 3 ml hydrochloric acid is added. Appearance of yellow colour (Harborne, 1999).

3.3.7 Test for Saponins

Foam test: 2 ml sample is taken in a test tube to which 4 ml distilled water is added, mix well and vigorously. Indicates the formation of foam at the top of the sample (Hossain *et al*., 2013).

3.3.8 Test for Steroids

Salkowski's test: To 2 ml of extract 2ml chloroform is added. 2 ml concentrated sulphuric acid is added along the sides of the test tube. Chloroform layer appears red colour and acid layer shows greenish yellow fluorescence (Joseph *et al*., 2013).

3.3.9 Test for Tannins

Braymer's test: 2-3 ml extract is diluted by adding 2 ml of distilled water. To which 2-3 drops of 5% ferric chloride is added. Appearance of black green or bluish colour (Rishikesh *et al*., 2013).

3.3.10 Test for Terpenes

Copper acetate test: 2 ml extract is dissolved in distilled water, to which 3-4 drops copper acetate solution is added and mixed, results in the production of emerald green (Morsy, 2014).

3.3.11 Tests for Carbohydrates

Molish's test: To 2 ml of plant sample extract, two drops of alcoholic solution of α- naphthol was added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates (Banu & Cathrine, 2015).

3.3.12 Tests for Reducing sugars

3.3.12.1 Fehling's test: Fehling A and Fehling B reagents are mixed and few drops of extract was added and boiled. A brick red coloured precipitate of cuprous oxide forms, if reducing sugars present (Joseph *et al*., 2013).

3.3.12.2 Benedict's test: 0.5ml of aqueous extract of the plant material was taken in a test tube. 5ml of Benedict's solution was added to the test tube, boiled for 5 minutes and allowed to cool spontaneously. A red color precipitate of cuprous oxide was formed in the presence of a reducing sugar (Rishikesh *et al*., 2013).

3.4 Solvent system standardisation

The solvent systems for the chromatographic analysis of chloroform and methanol were standardised by comparing the trials of different solvent system combinations.

3.4.1 Standardisation of Chloroform

The solvent system required to be used for the TLC analysis of chloroform extract of flower was standardised by conducting different TLC trials by 10 different ratios of low polar solvents such as Toluene and Ethyl acetate combinations.

Trials	Solvent system
T_1	100 % Toluene
T_2	Toluene : Ethyl acetate ; 9 : 1 ratio
T_3	Toluene : Ethyl acetate ; 8 : 2 ratio
T_4	Toluene : Ethyl acetate ; 7 : 3 ratio
T_5	Toluene : Ethyl acetate ; 6 : 4 ratio
T_6	Toluene : Ethyl acetate ; 5 : 5 ratio
T_7	Toluene : Ethyl acetate ; 4 : 6 ratio
T_8	Toluene : Ethyl acetate ; 3 : 7 ratio
T_9	Toluene : Ethyl acetate ; 2 : 8 ratio
T_{10}	Toluene : Ethyl acetate ; 1 : 9 ratio

3.4.2 Standardisation of methanol

The methanol extract solvent system was standardised by 8 different TLC trials of Toluene, Ethyl acetate and Methanol combination ratios by increasing the ethyl acetate concentration. The polarity of the solvent system was increased for the standardisation of methanol sample.

Trials	Solvent system
T_1	Toluene : Ethyl acetate : Methanol ; 9:1:1 ratio
T_2	Toluene : Ethyl acetate : Methanol ; 8:2:1 ratio
T_3	Toluene : Ethyl acetate : Methanol ; 7:3:1 ratio
T_4	Toluene : Ethyl acetate : Methanol ; 6:4:1 ratio

T_5	Toluene : Ethyl acetate : Methanol ; 5:5:1 ratio
T_6	Toluene : Ethyl acetate : Methanol ; 4:6:1 ratio
T_7	Toluene : Ethyl acetate : Methanol ; 3:7:1 ratio
T_8	Toluene : Ethyl acetate : Methanol ; 2:8:1 ratio

3.5 Thin Layer Chromatographic studies

TLC analysis was performed on Aluminium backed pre-coated Merck silica gel plate 60 F_{254} plate (10 cm x 3 cm Merck, Germany). The origin was marked at 1cm from the bottom of the TLC plate. The TLC plates were activated and the test solutions was applied on to the TLC plate of uniform thickness of 0.2 mm in the form of bands with a width of 10 mm using a 2 mL capillary tube (Camag, Switzerland). The plates were then developed in their respective standardized solvent systems in a twin trough chamber previously saturated with solvent for 30 min to a distance of 9 cm. The plates were then treated at 100° C for 5 minutes before visualization.

3.5.1 Solvent system

Toluene: Ethyl acetate (8:2) was used as the solvent system for the chloroform extract.

Toluene: Ethyl acetate: Methanol (7:3:1) was used for the Methanol extract.

3.5.2 Visualization

Observed the plate under UV light at 254 nm and 365 nm in the UV visualization chamber (Remi, India). Plate was also visualised in the visible light.

3.6 Chemicals and reagents used in the study

Solvents such as Methanol, Chloroform, Toluene, Ethyl acetate was purchased from Merck and Nice was used. All other chemicals employed were of standard analytical grade from Merck, India.

Plate 1: A: Habit of *Nerium oleander*. **B:** Fresh flower of *N. oleander*. **C:** Dried and powdered flower of *N. oleander*.

RESULT AND DISCUSSION

4.1 Preliminary phytochemical analysis

The preliminary phytochemical analysis of the chloroform and methanol flower extracts of *N. oleander* was done using different phytochemical tests. The potential phytochemicals present in the two extracts were analysed and characteristic differences were observed regarding the chemical composition of different extracts.

4.1.1 Chloroform extract

The results of the phytochemical analysis of the Chloroform extract using different phytochemical tests showed the presence of some major classes of phytoconstituents **(Table 1).**

Table-1 Preliminary phytochemical tests for Chloroform extract

Sl. No.	Phytochemical constituents	Name of test	Inference
1	Alkaloids	Mayer's test Wagner's test	+ +++
2	Coumarins	Coumarins test	+
3	Flavonoids	Alkaline reagent test Lead acetate test	+ +
4	Glycosides	Keller Killiani's test	+++
5	Phenols	Ferric chloride test	-
6	Quinones	Quinones test	-
7	Saponins	Foam test	+
8	Steroids	Salkowski test	+++
9	Tannins	Braymer's test	-
10	Terpenoids	Copper acetate test	-
11	Carbohydrates	Molish's test	_
12	Reducing sugar	Fehling's test Benedict's test	++ +

(+) Present, (-) Absent

The phytochemical constituents such as alkaloids, coumarins, flavonoids, glycosides, saponins, steroids, and reducing sugars were present. Whereas, phenols, quinones, tannins, terpenoids and carbohydrates were absent.

4.1.2 Methanol extract

The preliminary phytochemical analysis of the methanol extract to detect the presence of potential classes of phytoconstituents was done using different phytochemical tests **(Table 2).**

Table-2 Preliminary phytochemical tests for Methanol extract

Sl. No.	Phytochemical constituents	Name of test	Inference
1	Alkaloids	Mayer's test Wagner's test	+++ +++
2	Coumarins	Coumarins test	+++
3	Flavonoids	Alkaline reagent test Lead acetate test	++ ++
4	Glycosides	Keller Killiani's test	+
5	Phenols	Ferric chloride test	+++
6	Quinones	Quinones test	++
7	Saponins	Foam test	+
8	Steroids	Salkowski test	+++
9	Tannins	Braymer's test	+++
10	Terpenoids	Copper acetate test	++
11	Carbohydrates	Molish's test	+++
12	Reducing sugar	Fehling's test Benedict's test	- -

(+) Present, (-) Absent

In methanol extract, all of the phytochemical constituents such as alkaloids, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins, terpenoids and

carbohydrates were present except reducing sugar were observed. Majority of these class of compounds were detected significantly in the phytochemical screening of the methanol extract.

4.1.3 Comparative preliminary phytochemical analysis

The preliminary phytochemical analysis of chloroform extract and methanol extract confirmed the presence of various classes of phytoconstituents. Six different phytoconstituents were observed in the preliminary phytochemical analysis of chloroform extract and eleven different class of compounds were observed in the methanol extract. Alkaloids, coumarins, flavonoids, glycosides, steroids and saponin were found to be present in both chloroform and methanol extracts. Alkaloids, glycosides, steroids and reducing sugars had the highest intensities in chloroform extract. All of the phytochemical tests conducted in methanol extract had the best results and the class of compounds such as, alkaloids, coumarins, phenols, steroids and tannins in turn, could be considered as the major class of compounds in relation to their respective abundances. Phenols, quinones, tannins and terpenoids present in the methanol extract were not observed in the phytochemical screening of the chloroform extract and similarly, the reducing sugars present in the chloroform extract was absent in the methanol extract. The comparative analysis showed that the methanol extract had more phytoconstituents as compared to that of the chloroform extract.

Chaudhary *et al*., (2015) studied the phytochemical screening of various leaf extracts of *N. oleander* using various solvents such as petroleum ether, chloroform, methanol and aqueous extract showed the presence of alkaloids, tannins, carbohydrates, steroidal glycosides etc. The methanol and aqueous extracts were found to have the highest number of phytochemicals similar to that of the current study. Bameta *et al.,* (2017) carried out the preliminary phytochemical analysis of the leaves and stem extracts of *N. oleander* using methanol, ethanol, chloroform and petroleum ether. Among the seven phytochemical tests done on leaf extracts, reducing sugar, phenol, steroid, and coumarin were discovered, whereas saponin, sugar, and tannin were not present. Methanolic and petroleum ether extracts indicated the presence of reducing sugar, steroids, and coumarin, whereas chloroform and ethanol extracts showed reducing sugar and phenol, respectively. Stem extracts proved positive for all phytochemicals. Ethanol extracts displayed the most array of phytochemicals (tannin, phenol, saponin, coumarin), followed by methanol (coumarin, saponin) and chloroform (reducing sugar). Petroleum ether extract did not exhibit any phytochemical components.

Water and methanol extracts of *N. oleander* leaf and flowers were analysed qualitatively for phytochemicals by Cilesizoglu et al., (2022). The leaf extract showed positive results for cardiac glycosides, saponins, tannins, and alkaloids in both water and methanol extracts, except for tannins in the methanol extract. Cardiac glycosides were present in both extracts, with a higher concentration in the methanol extract. Saponins were detected in both extracts, with the water extract showing denser foam formation, indicating a higher saponin content. Tannins were present in the water extract but not in the methanol extract. Alkaloids were more intense in the methanol extract, but both extracts tested positive for alkaloids. Redha, (2020) analysed *N. oleander* leaves and flowers for phytochemicals, revealing the presence of phenols, tannins, flavonoids, coumarins, sterols, triterpenes, alkaloids, and phlobatannins. The dried semi-powdered samples were extracted using various solvents, and the total phenolic and flavonoid contents were determined. The alcoholic and aqueous extracts showed the highest phenolic content, while the alcoholic extracts had the highest flavonoid content.

The qualitative preliminary phytochemical analysis of the methanol extracts of the flowers of *N. oleander* was investigated by De Britto *et al*., (2011) and observed the presence of phytochemicals such as alkaloids, flavonoids, phenolic compounds, steroids and terpenoids. Pandeeswari *et al*., (2022) studied the acetone and petroleum ether extracts of *N. oleander* red flowers which exhibited a higher presence of phytochemical groups. Tannins, flavonoids, quinones, phenols, and coumarins were identified in all extracts. The red flowers from the acetone and ethyl acetate extracts contained tannins, saponins, flavonoids, quinones, terpenes, phenols, and coumarins. These phytochemicals have previously been noted for their toxic effects on mosquito eggs. Similar works were reported by Choudhary and Singh, (2018) in *N. indicum*. The phytochemical examination of several N. indicum leaf extracts revealed the presence of glycosides, terpenoids, and flavonoids in each extract. The aqueous extract has the greatest diversity of elements, including glycosides, alkaloids, tannins, terpenoids, flavonoids, saponins, phenols, and quinones. In comparison, the Hexane extract exhibited the least variability. The aqueous extract contained only phenols and quinones. Phlobatannins, carbohydrates, and amino acids were not present in the aqueous extract. Saponins and tannins were detected in both aqueous and ethanolic extracts. Amino acids were only discovered in the ether extract, whereas phenols were found only in the acetone extract. Carbohydrate was found in all extracts excepts the aqueous extract.

4.2 Chromatographic studies

Thin Layer Chromatography was used for the analysis of the phytochemical composition of flower extracts of *N. oleander*. The TLC profile was developed for the chloroform and methanol extracts of *N. oleander* flower. The solvent systems for the different extracts were standardized. Disparities were observed in terms of the number of bands and band intensity of the TLC profile developed for different extracts. These disparities in turn show the qualitative and quantitative deviations in chemical constituents.

4.2.1 Chloroform extract

TLC profile was developed for the flower extracts of *N.oleander.* The best solvent system for the separation of chloroform fraction was toluene: ethyl acetate mobile phase combination in the ratio 8:2. The developed plate was visualized at different wavelengths.

4.2.1.1 At 254 nm

A total of five compounds were found to be present in the chloroform extract at 254 nm. The bands were observed with an Rf value of 0.08, 0.2, 0.25, 0.76 and 0.96 respectively and all the five compounds were of relatively low polarity in response to their observed Rf values (**Table 3 and Plate 2).**

Table-3 TLC analysis of Chloroform flower extracts of *N. oleander* at 254 nm

Sl. No.	Rf	Band colour
1	0.08	Black
2	0.2	Light Black
3	0.25	Light Black
4	0.76	Black
5	0.96	Black

4.2.1.2 At 365 nm

The chloroform extract visualized at 365 nm had a total of five compounds. The compound with Rf value of 0.27 was found to be the most prominent band with high intensity and band width with distinct fluorescent blue in colour. The other major bands observed were with the Rf values of 0.05, 0.21, 0.85 and 0.96. **(Table 4 and Plate 2).**

Table-4 TLC analysis of Chloroform flower extracts of *N. oleander* at 365 nm

Sl. No.	Rf	Band colour
1	0.05	Pale Blue
2	0.21	Light Yellow
3	0.27	Florescent Blue
4	0.85	Light Blue
5	0.96	Light Blue

4.2.1.3 At visible light

TLC profiling of chloroform extract at visible light showed that there are no distinct observable bands. Hence, all the compounds in the chloroform flower extract of *N. oleander* are UV active compounds **(Plate 2).**

4.2.2 Methanol extract

TLC profile was developed for the methanol flower extracts of *N. oleander.* The best solvent system for the separation of methanol fraction was standardised as toluene: ethyl acetate: methanol mobile phase combination in the ratio 7:3:1. The developed plate was visualized at different wavelengths and Rf values are calculated.

4.2.2.1 At 254 nm

A total of twelve compounds were found to be present in the methanol flower extract at 254 nm. The bands were observed with a calculated Rf value of .06, 0.1, 0.12, 0.27, 0.33, 0.4, 0.48, 0.5, 0.56, 0.66, 0.72 and 0.96 respectively. Among the 12 different bands, the bands with Rf value of 0.06, 0.48, 0.72 and 0.96 were the bands with high band intensities and band width. The compounds with Rf value at 0.96 and 0.72 were dark blue in colour and were more

UV active. The different phytoconstituents were evenly separated in the TLC profiling of the methanol flower extracts **(Table 5 and Plate 2).**

Table-5 TLC analysis of Methanol flower extracts of *N. oleander* at 254 nm

Sl. No.	Rf	Band colour
1	0.06	Blue Black
2	0.1	Black
3	0.12	Black
4	0.27	Light Grey
5	0.33	Black
6	0.4	Black
7	0.48	Black
8	0.5	Black
9	0.56	Black
10	0.66	Black
11	0.72	Dark Blue
12	0.96	Blue Black

4.2.2.2 At 365 nm

The methanol extract visualized at 365 nm had a total of eight compounds. The compounds with Rf values of 0.68 was found to be the most prominent blue fluorescent band with high intensity and band width. The compounds with Rf values of 0.05 and 0.96 were the other two prominent bands with dark blue and light fluorescent blue in colour respectively. The compounds with Rf values of 0.2, 0.33, 0.37, 0.43 and 0.48 were also observed in the TLC analysis of the methanol flower extract **(Table 6 and Plate 2).**

Table-6 TLC analysis of Methanol flower extracts of *N. oleander* at 365 nm

Sl. No.	Rf	Band colour
1	0.05	Dark blue
2	0.2	Light Yellow
3	0.33	Light blue
4	0.37	Pale yellow
5	0.43	Light Yellow
6	0.48	Pale Blue
7	0.68	Deep Fluorescent Blue
8	0.96	Light Fluorescent blue

4.2.1.3 At visible light

TLC plate of methanol extract at visible light had only a single compound with a Rf value of 0.06 with a pale-yellow colour **(Table 7 and Plate 2).**

Table-7 TLC analysis of Methanol flower extracts of *N. oleander* at visible light

Sl. No.	Rf	Band colour
1	0.06	Pale yellow

4.2.3 Comparative TLC analysis of chloroform and methanol extracts.

The comparative analysis of the TLC profiles of chloroform and methanol extracts based on developed chromatographic plates showed that the Rf value of the bands which have the highest intensities in the developed chromatographic plates also possess a significant band width. The Rf values of the developed plates are manually calculated.

The comparative TLC analysis showed that a total of 31 distinct bands were observed among the two *N. oleander* flower extracts. The maximum number of phytoconstituents was found to be present in the methanol extract. A total of 21 different phytoconstituents were present in the methanol extract. The chloroform extract had a total of 10 compounds. Methanol flower extracts of *N. oleander* at 254 nm showed the highest number of bands (12) among all of the visualisation studies. Similarly, the flower extracts of *N. oleander* at visible light had the lowest number of bands. The chloroform extract at visible light no characteristic bands and that of methanol extract had a single band. The comparative analysis showed that the phytoconstituents present in the flowers of *N. oleander* tends to be more on a high polarity as the majority of the bands with high intensities and band width were observed in the methanol extract and compounds were evenly separated in the TLC profiling.

The TLC profiling of N. oleander stem extract was examined by Shinde *et al*., (2012). The solvent systems were standardised as ethyl acetate: methanol: water (100:13:10) and petroleum ether: ethyl acetate (10:1) for chloroform extracts. Ethyl acetate: formic acid: Toluene (4.5:0.75:2.5) system was standardized for methanol extract. Chloroform extract revealed bright blue and dark blue hue for oleander glycoside and purpurea glycoside correspondingly. Methanolic and chloroform extract also revealed pink and violet shade indicate terpenoids and steroids respectively. Praveen *et al*., (2012) studied the accelerated solvent extraction of *N. oleander* with chloroform produced clean extracts of leaves, flowers and twigs. the separation of *N. oleander* components was effective using a mobile phase of chloroform-acetone-acetic acid 8.5:1:0.5 (v/v), resulting in three bands with RF values of 0.18, 0.31, and 0.49. The maximum absorption wavelength was determined to be 275 nm. Densitometric profiling, successive use of various spray reagents, and characterization by 1H-NMR confirmed the presence of oleandrin in both the plant and autopsy samples.

An HPTLC method was established by Turkmen *et al*., (2013) to detect and quantify oleandrin in plant material and biological samples. The RF value of oleandrin was determined to be 0.24 ± 0.01 using the specified mobile phase. The RF values obtained from serum and urine samples, as well as from the compound isolated from the plant, showed absorption maxima at 275 nm, confirming the presence of oleandrin in these samples. Arasaretnam *et al.,* (2022) conducted investigations on the leaf and bark extracts utilizing solvents such as petroleum ether, dichloromethane, ethyl acetate and methanol. They hypothesized that the existence of phytochemicals in *N. oleander* is impacted by factors such as the type of plant, the

portion of the plant examined, the solvent and its polarity, the method of extraction, and the polarity of the phytochemicals themselves. The qualitative phytochemical study of *N. oleander* leaves demonstrated a greater presence of phytochemicals compared to other parts of the plant. The availability of these phytochemicals varied depending on the polarity of the solvent employed for extraction, with certain compounds being more soluble in low polarity solvents and others in polar solvents.

Madaci *et al*., (2022) investigated the variety of phenolic compounds in *N. oleander* leaves, identify major flavonoids, and studied their biological properties. Various extraction methods and solvents were tested, leading to the isolation of different phases. These phases were then analysed using chromatography and spectral analysis, resulting in the identification and purification of five compounds such as, apigenin, kaemphferol, quercetin, quercetin 3OR and luteolin. Phytochemical screening of *N. oleander* leaves was conducted by Alfonso *et al*., (2004), revealing the presence of cardio-tonic glycosides, flavonoids, steroids-terpenoids, saponins, and reducing sugars. The toxic cardenolide oleandrine, responsible for the species' allelopathic activity, was quantified by fractionating leaf extracts using silica gel column and thin layer chromatography, followed by fraction purification through recrystallizations and colorimetric determination. The leaves of *N. oleander* were found to contain 0.157% oleandrine.

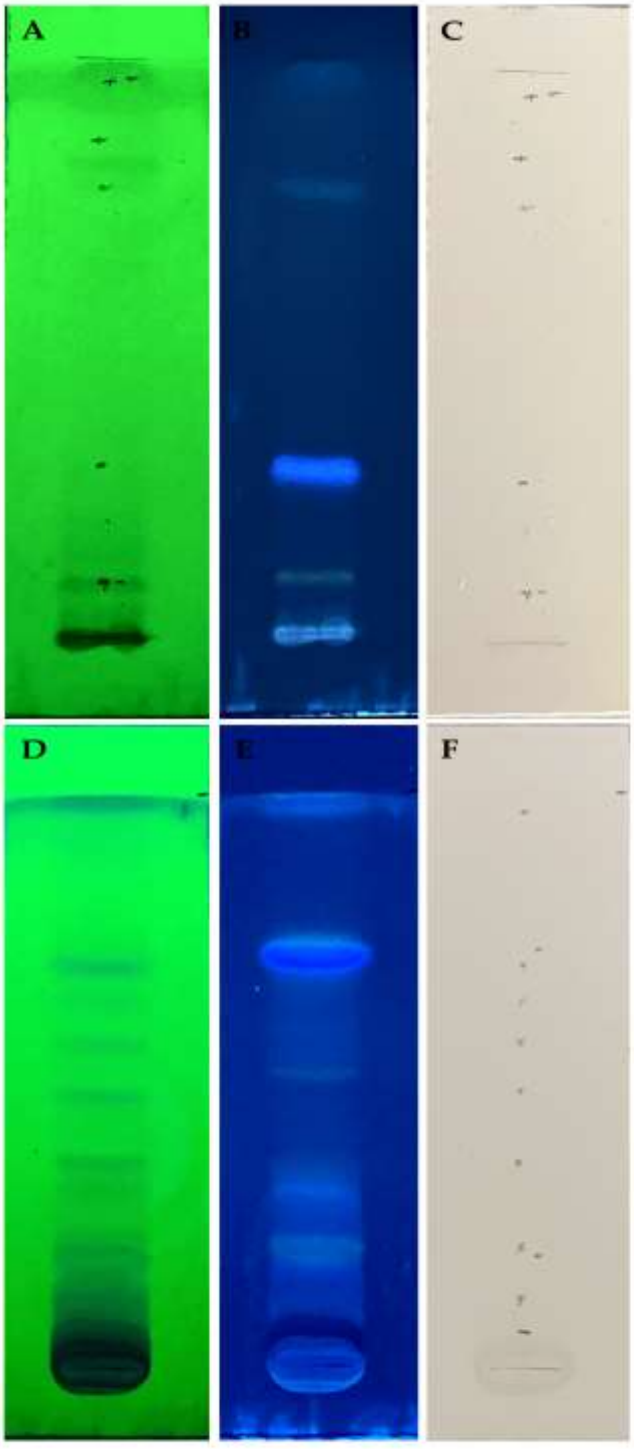

Plate 2: TLC profile of ***Nerium oleander*** **flower. A,B & C:** TLC chromatogram of the Chloroform extract under 254nm, 365nm and visible light respectively. **D,E &F:** TLC chromatogram of Methanol extract under 254nm, 365nm and visible light respectively

SUMMARY AND CONCLUSION

Since the dawn of human civilization, people have used plants and their derivatives as medicines. Plants can produce and store a wide array of small molecules known as secondary metabolites. In modern times, researchers extensively study these secondary metabolites, once unknown for their biological activities, as potential medicinal compounds. Alkaloids, steroids, and saponins are the major phytochemicals of interest, along with a variety of other naturally occurring compounds such as flavonoids, tannins, terpenoids, and essential oils.

In the context of increasing demand for herbal medicine, scientific validation of traditional knowledge about medicinal plants is essential. While traditional practices often use entire herbs, including leaves, stems, and roots, the medicinal properties of flowers are also significant and can effectively treat various ailments. There is a pressing need to further investigate the medicinal potential of flowers to address a wide range of health issues. The present study was undertaken to investigate the phytochemical constitution of the flowers of *Nerium oleander*. *Nerium oleander* L., is a perennial flowering plant from the Apocynaceae family. Traditionally, it has been utilized in India for treating cancer, leprosy, and skin conditions. It is abundant in essential phytochemicals like oleandrin, digitoxingenin, quercetin, α-tocopherol and ursolic acid.

The preliminary phytochemical analysis of chloroform and methanol flower extracts of *N. oleander* extract confirmed the presence of various classes of phytoconstituents, chloroform extract had 6 and methanol extract had 11 different class of compounds. Alkaloids, coumarins, flavonoids, glycosides, steroids and saponin were found to be present in both chloroform and methanol extracts. All of the phytochemical tests conducted in methanol extract had the best results and the class of compounds such as, alkaloids, coumarins, phenols, steroids and tannins in turn, could be considered as the major class of compounds in relation to their respective abundances. The comparative analysis showed that the methanol extract had more phytoconstituents as compared to that of the chloroform extract.

TLC profiling of the flower extracts of *N. oleander* had a total of 31 distinct bands. Disparities were observed in terms of number of bands and band intensity of the TLC profiles developed for flower extracts. The Rf value of the bands which have the highest intensities in the developed chromatographic plates tends to be the abundant compound in the extracts. In the comparative analysis of the extracts, the methanol extract contained the most phytoconstituents, with 21 different compounds, while the chloroform extract had 10.

Methanol extracts showed the highest number of bands (12) under 254 nm light, while visible light revealed the fewest bands. The chloroform extract under visible light showed no characteristic bands, while the methanol extract had one. The analysis suggested that the flower's phytoconstituents are predominantly polar, as seen in the methanol extract with well-separated compounds.

In conclusion, the present study was successful in carrying out a preliminary phytochemical screening of the potential phytoconstituents in the flowers of *N. oleander* using chromatographic techniques. The isolation and characterization of these bioactive compounds through detailed chromatographic and spectroscopic analysis of the flowers of *N. oleander* in turn validate the potential pharmacological activities they possess. Further research could explore the potential of leveraging the toxicity of *N. oleander* for pharmacological benefits.

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