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ISOLATION, CHARACTERIZATION AND INVITRO ANTIOXIDANT ACTIVITY OF BIOACTIVE CONSTITUENTS FROM NERIUM OLEANDER FLOWERS

Pokala Mounika*[1], Sana Sultana[2], Mamindla Navya Sri[3], Mathangi Raghu Varma[4], Muppidi Navya Sri[5], Thorrem Ashwini[6]

Department of Pharmaceutical Analysis &Quality Assurance , Vaageswari College of Pharmacy, Karimnagar 505527, Telangana, India.
Corresponding author E- mail: mounika.pokala27@gmail.com

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ABSTRACT

Nerium Oleander is a medicinal flowering plant tradionally studied for its Phytochemical Investigation and Biological properties. In this present study we aim to perform Column Chromatography, UV visible Spectroscopy and Antioxidant activity. The extraction of bio active compounds from Nerium oleander flowers is commonly carried out using Soxhlet apparatus, which enables continuous solvent extraction and improves the recovery of phytoconstituents such as flavonoids, alkaloids, glycosides, terpenoids and phenolic compounds. Column chromatography performed using silica gels stationary phase and solvents such as hexane, ethyl acetate and methanol as mobile phase. the obtained extract is further analysed by using UV-Visible spectroscopy to identify the presence of major phytochemical compounds based on there characteristic absorption peaks within the ultraviolet and visible wavelength region. the wavelength range commonly used for analyzing Nerium oleandar flower extract for UV region 200-400nm, visible region 400-800nm.thin layer chromatography is a simple and analytical procedure which is used for the separation and identification of phytochemicals present in the extract to perform the antioxidant activity it refers to the ability of a substance to prevent or slow down damage caused by free radicals and reactive oxygen species in biological material. the antioxidant potential of the flower extract evaluated by the hydrogen peroxide scavenging activity. thus, this study states that *Nerium* oleander flower extract contains important phytochemical constituents and promising antioxidant properties with potential pharmaceutical importance.

INTRODUCTION:

Nerium oleander is an evergreen ornamental and medicinal plant that belongs to the *Apocynaceaefamily.* It is widely distributed in tropical and subtropical regions and it is known for its attractive flowers, rich phytochemical compositon and pharmacological properties[1].The plant is commonly known as oleander and produces flowers in various colors such as pink white red and yellow. The flower contains many bioactive phytoconstituents such as flavanoids,, phenolics, tannins, alkaloids, saponins, cardiac glycosides and terpenoids. Among these constituents cardiac glycosides like oleandrin considered pharmacologically important but highly toxic,these compounds are responsible for several pharmacological

activities like anticancer, anti-inflammatory, anti oxidant, anti microbial and analgesic activity[3]. As *Nerium oleander* is a highly toxic plant and should be handled carefully during extraction and laboratory studies proper safety precautions are essesntial while working with the plant material and extracts.[11]

In Soxhlet Extraction method the dried flower materials is extracted using the soxhlet apparatus with suitable solvent methanol or ethanol to obtain its bioactive phytoconstituents from the plantmaterail.Soxhlet extraction is a continuous solid-liquid extraction technique in which the dried powder of flowers is repeatedly extracted with solvent under the reflux condition[13]. The apparatus consists of a round bottom flask, extraction chamber and condenser. During the process,The solvent is heated to form vapours, through which it is condensed and passes through the sample material for the extraction of the desired compounds and it is done after the repeated cycles[14]. This method mainly provides the efficeant amount of the extract using limited amount of the solvent (methanol or ethanol)This method is highly valued because it allows repeated washing of the sample with fresh solvent, leading to improved extraction yield and purity of compounds[15].

Phytochemical Screening is an important method to identify whether the Biologically active compounds are present in the extract.The Nerium oleander flower extract also contains some of the compounds such as Alkaloids, cardiac glycosides, flavanoids and phenolic compound etc[3].These are the Important compounds because it contains the Biological Activity. So to confirm the presence of the phytoconstituents present in the extract we perform the phytochemical screening test[4]. The Alkaloids present in the extract can be detected by the Mayer's test or Dragendroff's reagent test, where the formation of a reddish-brown precipitate indicates the presence of Alkaloids[9].

Nerium oleander flower extracts are commonly analysed using Thin Layer Chromatography (TLC).It is a simple and rapid technique used to separate and identify phytochemicals. Silica gel is coated on a plate act as the stationary phase while solvent system acts as the mobile phase. Based on Polarity of the compounds[10]. TLC is widely used to detect phytoconstituents in plant extracts such as Phenolics, flavanoids, tannins, cardiac glycosides and saponins. The separated spots are identified by their respective retention factor (Rf value) and visualized under UV-light or by spraying reagents[2].

UV-Visible Spectroscopy is a simple and rapid analytical technique used to identify the presence of phytoconstituents in plant extracts based on their absorption at ultraviolet and visible light[5].The flower of Nerium Oleander contain various bioactive Phytochemicals such as flavonoids, phenolic compounds, alkaloids, tannins and cardiac glycosides exhibit characteristic absorption peaks at specific wavelengths.These Phytochemicals are mainly Responsible for the biological and pharmacological activity[6]. Such as Antioxidant Activity.Therefore, UV-Visible spectroscophy is an important method for the characterization of bioactive phytoconstituents present in the *Nerium Oleander*flower extract and supports further phytochemical and pharmacological investigations[9].

The antioxidant activity of flower extract is carried out using DPPH radical scavenging assay, Hydrogen peroxide scavenging assay [H_2O_2], nitric oxide scavenging assay, reducing power Assay and ABTS radical scavenging Assay that determines the ability of the extract to reduce oxidative stress[7]. The ability ofthe plant extract to scavenge hydrogen peroxide is measured spectrophotometrically. Hydrogen peroxide is weak oxidizing agent which generates highly reactive hydroxyl radicals inside cells which leads to oxidative stress and cellular damage[12]. The phenolic and flavonoid compounds present in Nerium oleander flowers donate electrons to neutralize hydrogen peroxide and reduce oxidative damage. Hence, the hydrogen peroxide scavenging assay is useful for assessing the antioxidant potential of flower extract[11].

MATERIALS AND METHODS

Materials: We obtained Nerium oleander flowers from Algunoor,karimnagar. Ascorbic acid bought from Neighbourhood Market. Chemicals and reagents are from Molychem,Mumbai and Finar chemicals, Gujarat,India.

Instruments: Double beam UV-Visible Spectrophotometer model UV-1800 schimadzu, Sonicator citizen industries, Electronic Balance kshitij industries.

Nerium oleander

Soxhlet Extraction

Soxhlet extraction method is commonly used for the efficient extraction of bioactive compounds from Nerium oleander flowers.It is carried out in a soxhlet apparatus, In this method the fresh flowers of Nerium oleander is collected, washed properly to remove all the dust particles and impurities and shade dried for atleast a week until the petals gets dried completely.The dried flowers are then grinded to obtain a fine powder. The 45gms of the powdered flower material is placed inside a thimble made up of a filter paperand then placed in it by connecting a Round Bottom flask with the solvent methanol 500ml and then transferred it in the heating mantle at 54 and a reflux condenser which is connected at the top, and run tap water continuously untill the end of the process the water flowed into it to avoid hazards.The condensed solvent dissolves the phytochemicals present in the flower powder, and once the chamber fills the siphon level, the extract-containing solvent flows back into the flask.The process is continued for 6-8 hours for obtaining 15-20 cycles to extract whole contituents from the powder. (As shown in figure 1) Now remove the round bottom flask and collect the solvent for 1-2 days to obtain thick consistency.(As shown in figure 2).Since Nerium oleander is highly toxic, proper safety precautions such as wearing gloves, and mask should be followed during the procedure.

Phytochemical Screening

Phytochemical screening is an important preliminary step in the study of medicial plants to identify the presence of bioactive phytoconstituents such as flavanoids, cardiac glycosides which are responsible for their therapeutic properties such as Antioxidant, Anticancer, Anti-inflammatory, Anti microbial and analgesic activity. Phytochemical tests are carried out on the flower extract of *Nerium oleander*collected through the soxhlet extraction process.Phytochemical tests are mainly done to determine the presence of these compounds and to evaluate its medicial potential.The Alkaloids present in the extract can be detected using Mayer's test after adding this reagent to the extract the color of the extract turns into reddish-brown color which indicates the presence of the Alkaloids positive results. Tannins are detected by adding ferric chloride solution to the extract blue or green color was observed. Saponins are identified using a foam test in which the froth formation occurs which indicates the presence of saponins. Cardiac Glycosides can be identified using keller-killiani test, which produces a brown ring indicates the presence of cardiac glycoside compond.while Phenolic compounds can be detected by ferric cholride reaction in which the bluish-black or dark green coloration indicates the presence of phenolic compound.

Meanwhile Column Chromatograhy was also performed using silica gel as stationary phase and mobile phase with various solvents such as Hexane, Ethyl acetate and Methanol. Based upon their polarity and affinity towards the Stationary phase and Mobile Phase column chromatography helps to isolate and characterize the bioactive compounds such as Flavonoids, which mainly contributes in the biological and pharmacological activity such as Antioxidant Activity.

Thin Layer Chromatography

Thin layer chromatographyis a simple and rapid analytical technique used to separate, identify and monitor compounds in a mixture. *TLC* is mainly used to separate and visualize different bioactive phytochemical constituents present in the Nerium oleander flower extract. It can be used as screening technique to observe the presence of compounds such as phenolic compounds.The precoated silica gel TLC sheet of 10X10 cm was cut with the scissors. The baseline was drawn at 2cm distance with a pencil and the

extract was subjected on the base line using glass capillary tubes. The solvent system of mobile phase was prepared by using n-Butanol:Water:Acetic acid at the ratio of 4:5:1 and it was allowed to kept for the saturation for 15-20 minutes. The extract subjected to TLC plate was kept in the solvent system chamber and allowed to run the solvent to the 70 percent of the sheet then remove the TLC sheet from the solvent system and allow to dry it. Spray the freshly prepared vanillin reagent to detect the compounds present in them. The UV-Cabinet is used to observe the spots of compounds. The movement of the active compounds was expressed by their retention factor (RF).

UV-Visible Spectroscopy

UV-Visible Spectroscopy is an analytical method which is mainly used to identify the phytoconstituents present in the plant material. Prepare astock solution from the Nerim oleander flower Extract by using Methanol as solvent. Dissolve10mg (0.01g) of Nerium Oleander flower extract in 10mlof methanol which is (1000µg/ml)Primary stock A Solution. From stock A take 1ml of sample in each 3 quartz and makeup with 10ml of methanol label it as secondary stock solutions as 3 of them are of100µg/ml. Now make dilutions from the secondary stock of 2 quartzby adding 1ml of sample and makeup with 10ml of methanol which is (10µg/ml)again take 2ml of sample and makeup with 10ml of methanol (20µg/ml)again add 3ml of sample and makeup with 10ml of methanol (30µg/ml)now take 4ml of sample and makeup with 10ml of methanol(40µg/ml) And atlast take 5ml of sample from secondary stock solution and add makeup the volume with 10ml of methanol (50µg/ml).All the dilutions were placed on the sonicator for 10minutes,it helps in the proper dissolvation of the extract into the solvent as well as helps to remove all the air bubbles from the dilutions. The primary stock solution of Pure Nerium Oleander flower extract along with secondary stock dilutions is observed under the UV-Visible spectroscopy, we use methanol as a blank and then tranfer the samples one by one into a quartz cuvette and then scan the sample in a UV-Visible Spectrometer within the wavelength of 200-800nm. Record the absorption peaks and corresponding absorption values. Now compare the observed peaks with reported values to know the phytochemical constituents present in the Extract.

After separating the individual phytochemical constituents by performing column chromatography method by using stationary phase as silica gel and mobile phase with solvents such as Hexane ethyl acetate and methanol. The obtained each compound such as phenolics and flavonoids are also further analysedby using UV-Visible spectroscopy for this, Take 1ml of phenolic compound and makeup the volume with 10ml of Methanol and for Flavonoids also take 1ml of extract and makeup the volume with 10ml of Methanol at 200-400nm. Now identify the presence of major phytochemical compounds based on there characteristic absorption peaks and absorbance values within the wavelength region.

Antioxidant Activity

Antioxidant are compounds which protects from oxidative damage caused by free radicals and reactive oxygen species (ROS) In a biological system. Among various ROS, Hydrogen peroxide (H_2O_2) which is an important non-radical oxidizing agent. It can penetrate biological membranes and generate highly reactive hydroxyl radicals, leading to cellular damage. So, it is considered as an important mechanism of antioxidant defense.

Hydrogen Peroxide Scavenging Assay- H_2O_2

The hydrogen peroxide scavenging assay is widely used in-vitro method for evaluating the Antioxidant potential of plant extracts. *Nerium oleander flowers*. Flowers contains various phytoconstituents and secondary metabolites which exhibits antioxidant properties . Antioxidants present in the extract neutralizes the hydrogen peroxide, so the absorbance is decreased and measured spectrophotometrically at 230nm. Hence, it determines the ability of *Nerium oleander*flow extracts to scavange hydrogen peroxide and evalute their antioxidant Activity.

Preparation of Solutions:

- Prepare 40mm hydrogen peroxide in phosphate buffer withPH 7.4
- Dissolve flower extract in methanol to obtain different concentrations such as 20,40,60,80,100 microgram per ml.
- Prepare Ascorbic acid solution in the same concentrations as flower extract and this is used as standard.

PROCEDURE

- Add 2 mL of 40 mM H_2O_2 to 1 mL of flower extract at different concentrations in separate test tubes.
- Add 2 mL of 40 mM H_2O_2 to 1 mL of ascorbic acid at different concentrations in separate test tubes and mix thoroughly,.
- Incubate at room temperature for 10 min, Measure the absorbance at 230 nm against a phosphate buffer blank (without H_2O_2).

Fig1: Soxhlet extraction

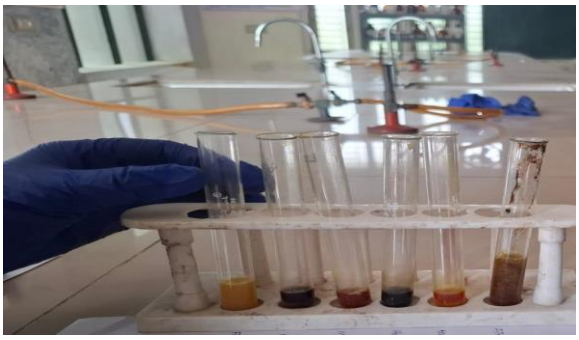


Fig2(a): Phytochemical screening

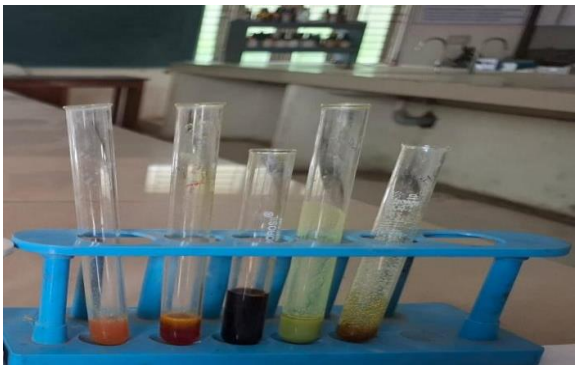


Fig2(b): Phytochemical screening

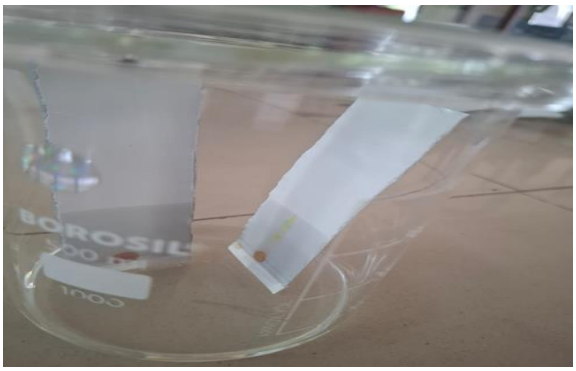


Fig3: Solvent System used in TLC

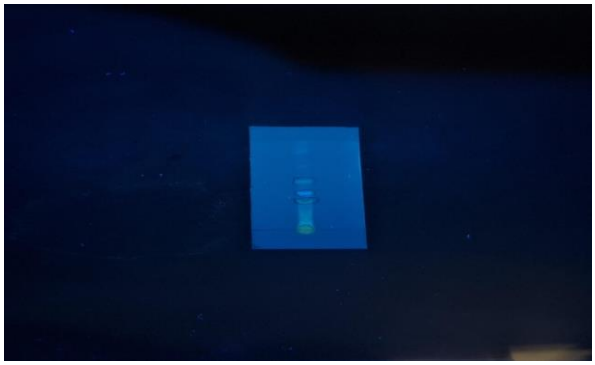


Fig 4: Detection of TLC under UV cabinet

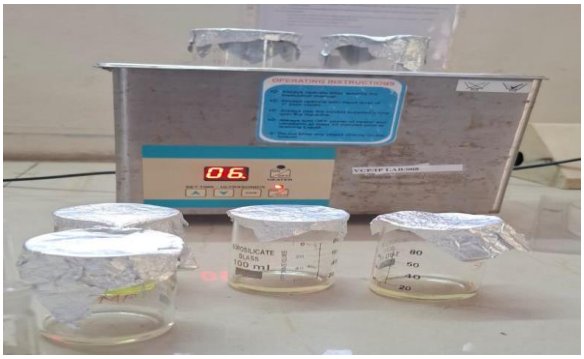


Fig 5: Ultrasonication

RESULTS: The Results were Tabulated and given below.

TABLE 1:Phytochemical Evaluation of *Nerium Oleander* Flower

S:No	Compounds	Tests	Observations	Result
1	Alkaloids	Wagner's test	Reddish-Brown ring	+ve
2	Phenolics	Gelatin	White ppt	+ve
3	Flavonoids	Lead acetate	Yellow ppt	+ve
4	Cardiac glycosides	Killer-kilani test	Brown ring	+ve
5	Saponins	Foam test	Persistant foam	+ve

+ve indicates presence of compounds and –ve indicates absence of compounds

TABLE 2 -Thin layer chromatography of Nerium oleander flower (phenolic compounds)

Stationary Phase	Silica Gel
Mobile Phase	Toulene: Ethyl Acetate: Formic Acid
Ratio	5:4:1
Detecting agent	Vanillin Sulphuric Acid, UV Cabinet
Rf Value	0.46

Below Fig 6&7 Methanolic flower extract of Nerium oleander has shown the characteristic absorption peaks in the UV region, indicating the presence of phytochemicals

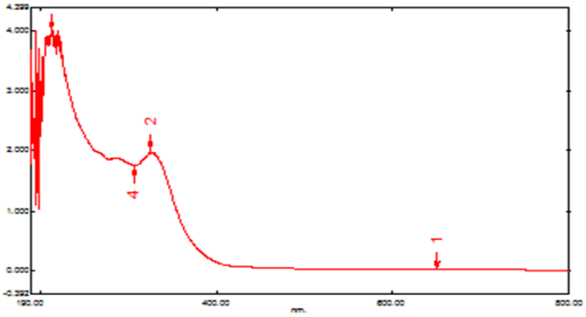


Fig 6 UV-Visible spectrum of *Nerium oleander* flower extract

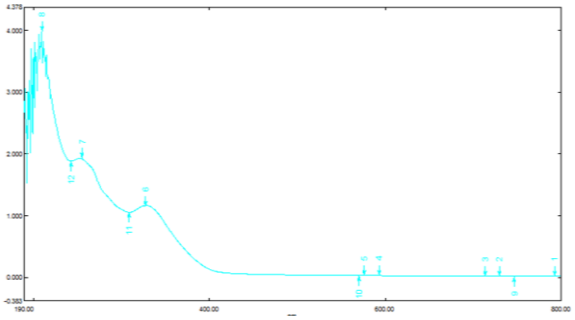


Fig-7 UV-Visible spectrum of phenolic compound

TABLE 3: Hydrogen Peroxide Assay

Concentrations in microgram/ml	Absorbance value of extract at 230nm	Absorbance values of Ascorbic Acid at 230nm
20	0.595	0.455
40	0.682	0.488
60	0.753	0.544
80	0.834	0.594
100	0.968	0.708
control	0.490	0.490

Table 4: IC_{50} values of Antioxidant activity

Method used	Absorbance of Extract	Absorbance of Ascorbic Acid
Hydrogen Peroxide Assay	97.1%	44.1%

Fig 8-Concentrations Vs Absorbances values of extract and ascorbic acid at 230nm

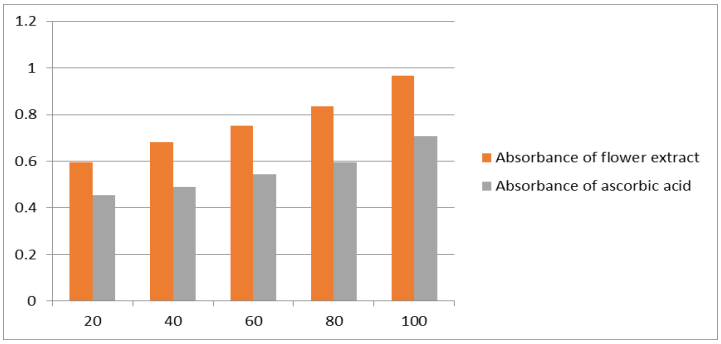


Fig -9 Absorbance versus concentration standard curve of *Nerium oleander* flower extract

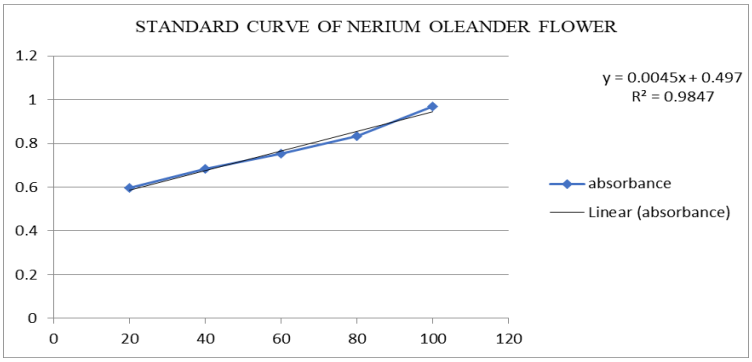
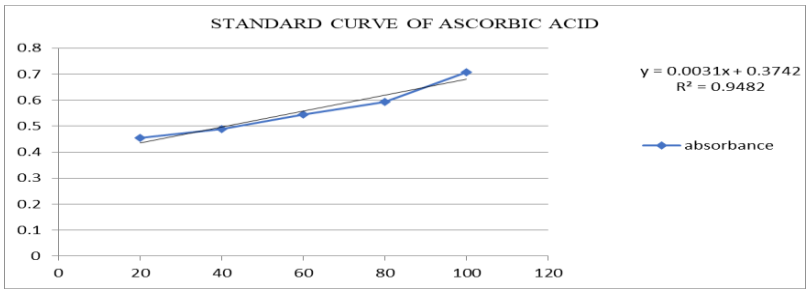


Fig -10 Absorbance versus concentration standard curve of Ascorbic acid



DISCUSSION

1.Soxhlet Extraction of *Nerium oleander* flowers

The present investigation validated that the dried flower of the Nerium oleander was successfully recovered a variety of bioactive phytoconstituents from Soxhlet extraction method using methanol as a solvent. After completion of extraction for upto 6-8hrs the methanolic extract is obtained then the solvent is removed under reduced pressure,and a yellowish brown color with a characteristic aromatic flowral odour, or slighly pungent was observed which indicates the presence of the bioactive phytochemical constituents.The repeated extraction of the plant material resulting in the enhanced extraction of the both moderately polar and non-polar compounds.The obtained extraction is further subjected for the phytochemical Screening.

2. Phytochemical Screening

Phytochemical screening of the Methanolic extract of *Nerium oleander* flowersrevealed the presence of the various bioactive secondary metabolites. The extract tested positive for Alkaloids, Flavonoids, Saponins, Cardiac glycosides and Phenolic compounds. These phytochemical Constituents are known to contribute to the biological and pharmacological activities.The presence flavonoids, phenolics and tannins may contains the Antioxidant Activity. The column chromatography was also performed using silica gel as stationary phase and mobile phase with solvents such as Hexane, ethyl acetate and methanolresulted in the separation of the bioactive compounds such as pigment phenolics which mainly contributes in the Antioxidant Activity.

3.Thin layer chromatography

Thin layer chromatography was performed mainly to separate and identify the Bioactive phytochemical compounds present in the Methanolic flower extract of the Nerium oleander. The chromatographic separation was carried out on silica gel precoated TLC plates using mobile phase containing n-Butanol:Water:Acetic acid (4:5:1). After this the plates were visualized under UV light and sprayed with vanillin reagent to detect the separated compounds.TheTLC profile revealed the presence of several distinct spots indicating the presence of the multiple phytochemical constituents in the extract. The observed spots exhibits different retention factor (RF) values, indicating the presence of compounds with different polarities. The separated bands indicates the phenolicsssand other secondary metabolites which contributes to Antioxidant Activity.

4. UV-Visible Spectroscopy

The UV-Visible spectrascopy of the methanolic flower extract of Nerium oleander has shown the characteristic absorption peaks in the UV region, indicating the presence of phytochemicals such as Flavonoids, Alkaloids and Phenolic compounds. The absorption values and peaks that indicating that the exract contains bioactive constituents which is responsible for AntioxidantActivity.

5. Antioxidant Activity -Hydrogen Peroxide Scavenging Assay

The methanolic extract of Nerium oleander flower showed concentration dependent hydrogen peroxide scavenging activity. The increased concentration from 20-100 microgram per ml showed increase in percentage scavenging activity. The hydrogen peroxide scavenging assay absorbance was checked at 230nms and the results are presented in Table 3.The IC^{50} values of antioxidant activity are given in table 4. The bar charts of absorbance of extract ans asorbic acid are presented.

CONCLUSION

From the above study we have concluded that the Nerium oleander flower extract contains the bioactive secondary metabolities such as Alkaloids, Flavonoids, Saponins, Cardiac glycosides and phenolic compounds.These compounds are identified by performing the phytochemical tests according to the standard procedures and recorded the results. The separation of compounds are carried out by the column chromatography by which the phenolic compound was separated it mainly contributes in the Antioxidant Activity. TLC was performed to the fractions, observed under the UV-light and their retention factor (Rf) was calculated and compared with the standard values. UV Spectroscopy is performed to confirm the presence of the phytochemical conctituents of different nanometers of wavelength in the extract. The phenolic compounds separated from the column chromatography is observed under UV Spectroscopy and confirmed by comparing the nanometer peak wavelength with the extract. The observed phytoconstituents are responsible for the Antioxidant activity. The Antioxidant Activity was performed after confirming the presence of phenolic compounds in UV Spectroscopy by Hydrogen peroxide scavenging Assay to observe the potency of *Nerium oleander* flower extract compared with the standard Ascorbic Acid.

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