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Phytochemical, pharmacognostical estimation and antioxidant potental of nerium indicum mill. stem extracts

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Abstract
The objective of this study was to carry out phytochemical and pharmacognostic and quantitative evaluation of Stem of nerium indicumm mill. Nerium is a distinct genus of evergreen coniferous tree from the family Apocynaceae, containing only one species, Nerium indicum (Kaner). Stem and powdered microscopy revealed useful diagnostic features. Phytochemical testing of varies extracts revealed the presence of glycosides, flavonoids, tannins, phenolic compounds, carbohydrates etc., which was further confirmed by TLC. The total phenolic and flavonoid content of plant extracts was expressed as Gallic acid equivalents and as rutin equivalents respectively. Total phenolic content and flavonoid contents was found to be more in ethanolic extract of stem as compared to ethyl acetated extract respectively. In-vitro Antioxidant activity study indicates that ethyl acetated extract has the more antioxidant effect (98.89% inhibition of free radicals at 100µg/ml) as compared to ethanol extract. Accordingly safe experimental dose was calculated as ≤ 200mg/kg & was used accordingly for further screening of extracts.

Keywords: nerium indicum, standardization, antioxidant activity

Introduction
Medicinal plants have been defined in many ways, the most accepted definition as given by the Agricultural and Natural Resource Development being, "Plants that are recognized by people to have reliable and effective medicinal values, are commonly used in treating and preventing specific ailments and diseases, and play an essential role in health care". (Vijayalakshmi S et. al, 2016).

The World Health Organization (WHO) estimates that 4 billion people (80 % of the world population) presently use herbal medicine as a part of primary health care. (Suriyavathana M et. al, 2010).

Herbal medicines have already formed the basis of therapeutic use in developing countries, and also seen an increase in the use by developed world as well. This is mainly because these herbs/plants are relatively economical, easily available and their uses were decided on ancestral experience. In addition, synthetic drugs are not only expensive and inadequate for the treatment of disease, but also faced with adulteration and side effects. Therefore, medicinal plants have been useful in the development of new drugs and continue to play valuable role in the drug discovery process *Nerium indicum Mill* is already traditionally as folk medicine to treat a number of illnesses.

Materials and Method
Plant collection and extraction
The fresh stem of plant Nerium indicum was collected from known source that is from Visava garden Nanded. The Morphological study & microscopic characters of the plant was identified and further authenticated by Dr. S. S. Bodke, HOD, Dept. of Botany, Yeshwant Mahavidyalaya, Nanded. Plant was authenticated as Nerium indicum (apocynaceae). Voucher No. NCP/M.pharm/H-5 was allotted to the plant herbarium.

Fig 1: Nerium indicum Mill.

The fresh stem of plant Nerium indicum was subjected to shade drying and further crushed to coarse powder, and then the powder is passed through the mesh 14 and stored in air tight container for further use which is subjected to ethanol and ethyl acetated solvents by continuous hot extraction method.

Preparation of plant extract
Successive solvent extraction (Soxhlet extraction) will be employed for extraction. Solvents may be selected from non-polar to polar nature like petroleum ether, ethyl acetate, and ethanol etc. Solvent will be selected by considering nature of phytoconstituents present in plant material. Study of literature survey revealed that leaves are aromatic and contain proteins, carbohydrates, fiber, flavonoids, quercetin, kaempferol on the basis of literature The extraction method selected for extraction of Nerium indicum Mill. Stem is

Continuous hot extraction method using Soxhlet apparatus for ethanol & ethyl acetated as solvents.

Pharmacognostic evaluation

Morphological study of plant material

Organoleptic properties of Nerium indicum Mill. leaves were studied

1. Colour: green
2. Odour: odourless.
3. Stem: Shrub, lower portions woody, aerial.
4. Leave: Cauline and ramal, whorled with three leaves in each whorl.
5. Flower: Bracteate, bracteolate, pedicellate, complete Root
6. Inflorescence: Terminal, dichasial cyme or panicle cyme.
7. Fruit: Drupe.
8. Seed: Capsule spreading seeds (pods)

On the extremes of the branches there are also small oval cones [6-8mm in length]. Microscopic study of powderd Drug

Powder of the drug shows presence of fibers, calcium oxalate crystals, unicellular trichoms, sclerides, and oil cell.

TCL Fingerprinting

Preparation of TLC Plates

It is a technique in which a solute undergoes distribution two phases, stationary phase acting through adsorption and a mobile phase in form of liquid. The adsorbent is relatively thin, uniform layer of dry finely powdered material applied to glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on basis of partition or a combination of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent. Identification can be effected by observation of spots of identical Rf value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of spots usually serves for semi quantitative estimation

Phytochemical Evaluation

Total Polyphenolic Content

Procedure 4 ml of Folin-Ciocalteu reagent was mixed with 1 ml of extract solution, this solution mixture was kept on standing for 5 min & then 5 ml of sodium carbonate was added to it. The absorbance of reaction mixture was measured against blank (without extract) at 765 nm using UV-Visible spectrophotometer. Gallic acid was used as standard for determination of total polyphenol content of extract. The calibration curve was drawn using various concentrations of gallic acid (50, 100, 150, 200, 250 µg/ml).

Total Flavonoid Content

Procedure 1ml of extract solution was mixed with 4 ml of distilled water & 0.3 ml of NaNo2. After 5 min 0.3 ml of AlCl3 & 2 ml of NaOH was added, at last total volume was made up to 10 ml with distilled water. The solution was Mixed well & absorbance of the solution mixture was measured at 510 nm against prepared blank (without extract). Rutin was used as standard for determination of total flavonoid content of extracts. The calibration curve was drawn using various concentrations of rutin (100, 200, 300, 400, 500 µg/ml).

***In vitro* Anti-Oxidant Activity**

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. The antioxidant activity of Nerium indicum was determined by DPPH free radical scavenging assay method.

Preparation of test solution

Nerium indicum extracts (Ethyl acetate and Ethanolic) 50mg was separately dissolved in 50ml of methanol from which different concentration of 25µg/ml, 50µg/ ml, 75µg/ml, 100µg/ml, 125µg/ml were prepared.

Preparation of Standard solution:

Standard i.e. rutin ascorbic acid, gallic acid(1mg) was dissolved in 1ml of methanol from which different concentration of, 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml125µg/ml were prepared**.**

Methods

2, 2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) Activity

Principle

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the nonradical form DPPH-H. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm. The free radical scavenging activity can be measured by using 2, 2diphenyl-1-picrylhydrazyl.

Procedure

Different concentrations (25, 50, 75,100,125µg/ml) of both the extracts were prepared with methanol and 1ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 30 min. After 30 min, the absorbance of the mixtures was measured at 517 nm. 1ml ofDPPH and 1ml of methonal was taken as control

$$(\%)\ \text{Scavenging activity} = \frac{\text{Control absorbance-Test absorbance}}{} \times 100$$

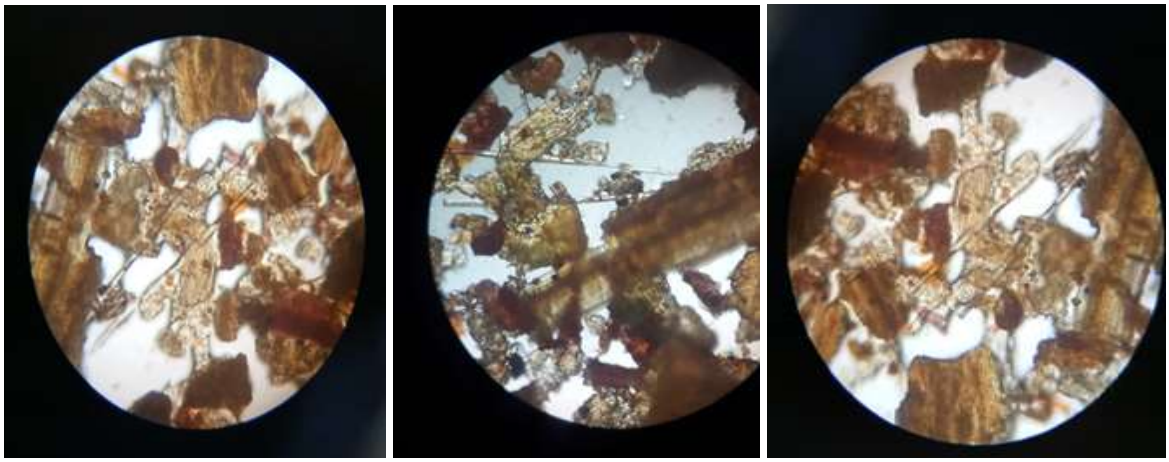
Results

Microscopical description of Nerium indicum Mill. Stem.

Fig 2: T. S. of Nerium indicum Mill. Stem.

Microscopic study of powderd Drug

Powder of the drug shows Presence of fibers, calcium oxalate crystals, unicellular trichoms, sclerides, and oil cell.



(a) Trichomes (b) Sclerides (c) Fibers

Fig 3: Powder characteristics of Nerium indicum Mill. Stem

Table 1: Preliminary Phytochemical Evaluation of Ethanolic & ethyl acetated extract of Nerium indicum Mill.

Sr. No	Test	Ethanolic extrat	Ethyl acetated extract
1	Flavanoids		
	a)Shinoda test	(+)	(+)
	b)Lead acetate test	(+)	(+)
	c)Zinc and HCL	(+)	(+)
2	Glycoside		
	a) Borntragers test	(-)	(-)
	b) Modifide borntragers test	(-)	(-)
3	Tannins		
	a) Ferric chloride test:	(+)	(+)
	b) Lead Acetate test	(+)	(+)
4	Amino acid		
	a) Millon's test:	(-)	(-)
	b) Ninhydrine test:	(-)	(-)
5	Carbohydrate		
	a) Molish test:	(+)	(+)
	b) Barfoeds test	(+)	(+)
6	Alkaloid		
	a) Hager's test:	(-)	(-)
	b) Murexoid test:	(-)	(-)
	c) Tannic acid test:	(-)	(-)
7	Triterpenoids		
	a) Salkowiski test:	(+)	(+)
	b) Liebermann-starch	(-)	(-)

(+): present (-) absent

Thin layer chromatography.

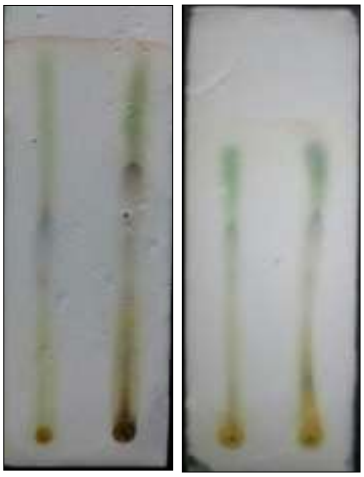


Fig 4: TLC of Ethanolic & Ethyl acetated extracts of plant Nerium indicum Mill.stem

Sr.no	Extracts	Solvent sysyem	Rf values and colours
1	Ethanolic extract	Chloroform:	
		Acetone:	0.43(Blue spot)
		Toluene: Methanol:	0.30(pink spot)
		Ethyl acetate	
		(1:0.5:7:0.5:1)	0.18(green spot)
2	Ethyl acetated extract	Ethyl acetate:	
		methanol:	0.71(Blue spot)
		Toluene:	0.60(pink spot)
		Hexane:	0.34(Yellow spot)
		Acetone	
		(1:0.5:7:1:0.5)	

Ethanolic extract of *Nerium indicum Mil* stem when subjected to TLC screening showed the presence of Rf 0.43(Blue spot) 0.30(pink spot) 0.18
(green spot) in the solvent system Chloroform: Acetone: Benzene: Methanol: Ethyl acetate (1:0.5:7:0.5:1) &Ethyl acetated Extract shows presence of Rf 0.71 (Blue spot) 0.60 (pink spot) 0.34 (green spot) in the solvent system Ethyl acetate: Methanol: Toluene: Chloroform: Acetone: (1:0.5:7:1:0.5).

Total phenolic content

The total phenolic content in the *Nerium indicum Mill*. Stem extracts using folin-coicalteus reagent is expressed in terms of gallic acid equivalent.

The values obtained for the conc. of total phenols are expressed as mg/gm.

Table 5: total phenolic content

Sr no.	Extracts	Concentration (ug/mg)	Absorbance	TPC
1	Standard	100	0.124	31
2	Petroleum ether	100	0.168	32.35
3	Ethanol	100	0232	42
4	Ethyl acetated	100	0.246	6.15

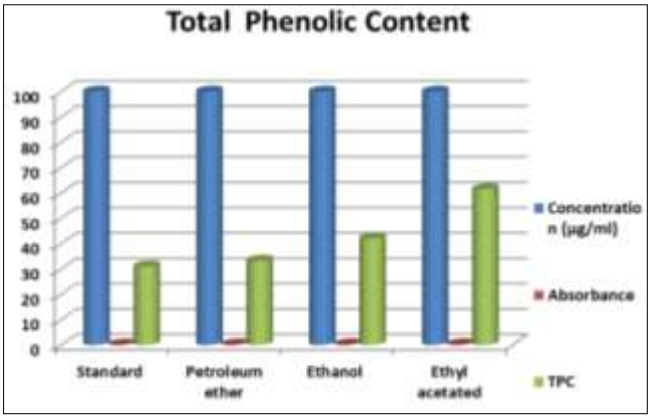


Fig 5: Total Phenolic content.

Total flavonoid content

The concentration of flavonoids in *Nerium indicum Mill.* Stem extract was determined using spectrophotometric method with aluminum chloride.

The content of flavonoids was expressed in terms of rutin equivalent**.**

Table 6: Total Flavonoid content.

Sr. no	Extracts	Concentration (ug/mg)	Absorbance	TFC
1	Petroleum ether	100	0.146	35.60
2	Ethanol	100	0.174	42.43
4	Ethyl acetated	100	0.146	45.60

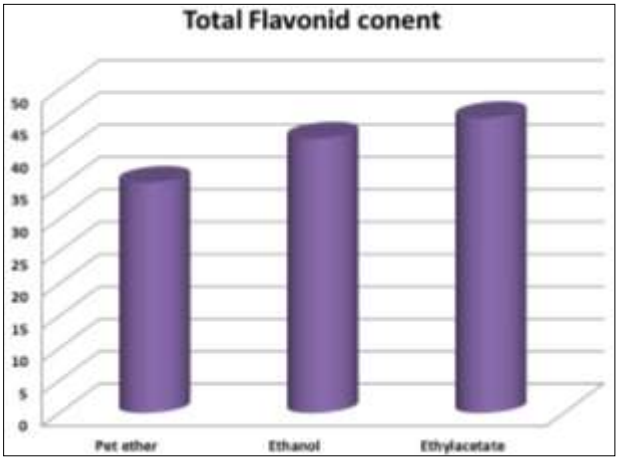


Fig 6: Total Flavonid content

***In-vitro* Anti-oxidant studies**

In-vitro Anti-oxidant study of *Nerium indicum Mill.* Stem includes following anti-oxidant method, DPPH (2, 2-dipheny 1, 1-picrylhydrazyl) radical scavenging activity.

Table 7: DPPH radical scavenging activity of Ethanolic extract of Nerium indicum Mill. Stem.

Sr. No.	Concentrations µg/ml	Absorbance of extract (Mean)	% Scavenging activity of extract	Absorbance of Standard Ascorbic acid	% Scavenging activity of Ascorbic acid
1	25	0.128	73.71	0.018	96.30
2	50	0.096	80.28	0.012	97.53
3	75	0.041	91.58	0.0091	98.13

4	100	0.027	94.45	0.0084	98.27
5	125	0.024	95.07	0.0063	98.70

Each values represents as means S.E.M; n =3

The Ethanolic extract shows 95.07 present scavenging activities at 125µg g/ml concentration while standard ascorbic acid at 125µg g/ml concentration shows 98.7 percent scavenging activity.

Table 8: DPPH radical scavenging activity of ethyl acetated extract of Nerium indicum Mill. Stem.

Sr. No.	Concentrations µg/ml	Absorbance of extract (Mean)	% Scavenging activity of extract	Absorbance of Standard Ascorbic acid	% Scavenging activity of Ascorbic acid
1	25	0.098	79.87	0.018	96.30
2	50	0.043	91.17	0.012	97.53
3	75	0.032	93.42	0.0091	98.13
4	100	0.016	96.71	0.0084	98.27
5	125	0.014	97.12	0.0063	98.70

Each values represents as means S.E.M; n =3

The ethyl acetated extract shows 97.12 present scavenging activities at 125µg g/ml concentration while standard ascorbic acid at 125µg g/ml concentration shows 98.7 percent scavenging activity.

Table 9: DPPH radical scavenging activity of Ethanolic & ethyl acetated extracts of *Nerium indicum Mill.* Stem.

Extracts	% Scavenging activity				
	Concentrations (µg/ml)				
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	125 µg/ml
Ethanolic	73.71	80.28	91.58	94.45	95.07
Ethyl acetated	79.87	91.17	93.42	96.71	97.12
Standard Ascorbic Acid	96.30	97.53	98.13	98.27	98.70

Each values represents as means S.E.M.±; n=3

From the above table it reveals that both the extract i.e. Ethanolic & ethyl acetated Extracts shows anti-oxidant potential. Ethanolic extract having 97.07 percent scavenging activity, ethyl acetated extract having 97.71 percent scavenging activity, while Ascorbic acid shows 98.7 percent scavenging activity at 125 g/ml concentration respectively. The Ethyl acetated extract of plant and Standard Ascorbic acid has almost equivalent anti-oxidant potential.

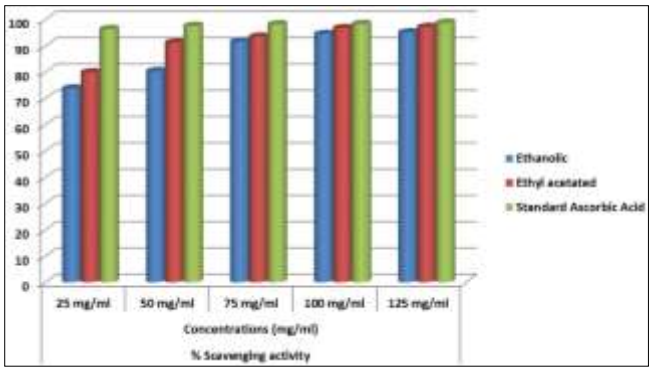


Fig 7: DPPH radical scavenging activity.

Discussion

Phytochemical investigation of plant helps in identification of nature of chemical constituents present. Ethanol extract showed the presences of mainly glycosides, carbohydrates, etc. and ethyl acetated extract showed the presences of glycosides, carbohydrates, tannins and flavonoids etc.

TLC for Ethanolic extract &ethyl acetated extract of *Nerium indicum* was carried out by using solvent system Chloroform: Acetone: Toluene: Methanol: Ethyl acetate; & Ethylacetate: methanol: Toluene: Chloroform: Hexane: Acetone; respectively, spots were observed under UV, which helps in identification of specific chemical constituents present in the extracts of Nerium indicum.

In DPPH radical scavenging activity, both the extract i.e. Ethanolic (95.05%) & ethyl acetated (97.12%) shows percent scavenging activity, while Ascorbic acid shows 98.7% scavenging activity at 125 µg/ml concentration respectively. The extract Ethylacetate and Ascorbic acid has almost same anti-oxidant potential.

Acute toxicity studies (OECD 423: Acute Oral Toxicity-Class Method) conducted by researchers revealed that the graded doses administration of extracts (up to a dose of 2000 mg/kg) did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, coma and in appearance of the animals. No death was recorded up to the dose of 2000 mg/kg body weight. The result of such studies showed that in single dose; the plant extracts had no adverse effect, indicating that the medium lethal dose (LD50) could be greater than 2000 mg/kg body weight in mice. Accordingly safe experimental dose was calculated as ≤ 200mg/kg & was used accordingly for further screening of extracts.

Conclusion

Different extracts of *Nerium indicum mill*. Stem showed presence of various phytoconstituents such as alkaloids, glycoside, flavonids, tannis and phenolic compounds.

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