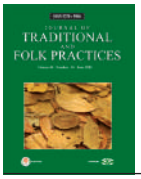

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Evaluation of pharmacognostic and phytochemical profile of *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. leaves - a lesser known ethnomedicinal plant from Western Ghats, India

A L Aneeshkumar[1], E A Siril[1*], S R Suja[2] and S Rajasekharan[2]

[1]Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram 695 581, Kerala, India
[2]Ethnomedicine and Ethnopharmacology Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695 562, Kerala, India
* easiril@yahoo.com

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Abstract

Neurocalyx calycinus (R. Br. ex Benn.) Rob. is a woody stemmed herb found in evergreen forests of Western Ghats of India and Sri Lanka. The Cholanaickan tribe of South India has been using the leaves of *N. calycinus* for the treatment of wounds, inflammations and various skin diseases. However, no scientific reports are available to fix the identity of true plant material to ensure its quality, purity, antioxidant properties and phytochemical constituents. Due to its ethnobotanical importance, the objectives of the present study have been designed to evaluate the identity of *N. calycinus* leaves through necessary pharmacognosticand phytochemical studies. The determination of salient diagnostic features such as morphological analysis, quantitative microscopy, microscopic powder analysis, physicochemical constants and fluorescence behaviour were carried out. High Performance Thin Layer Chromatography (HPTLC) and Gas Chromatography-Mass Spectrometry (GC–MS) fingerprint profiles were developed using ethanol extract for qualitative and quantitative standardization of the plant sample. Stomatal study revealed paracytic types of stomata on abaxial surface only. Phytochemical screening showed that alkaloids, steroids, phenolic compounds, glycosides and saponins were found in the ethanol extract. The thin layer chromatography studies of ethanol extract revealed that toluene: ethyl acetate (1:1) solvent system is suitable for maximum separation. The HPTLC fingerprint profile at 254nm, 366nm, 425nm showed major chemical constituents. GC-MS analysis indicated the presence vitamin E and its precursor compounds. The information obtained from the present study is the first of its kind, which can be used for the proper identification of the species for further pharmacological and therapeutical evaluation, and also assist its standardization with regard to quality and purity.

Keywords: *Neurocalyx calycinus,*Cholanaickan tribe, Ethnobotany, HPTLC, GC/MS, Pharmacognosy

1. Introduction

Neurocalyx Hook. (Family: Rubiaceae) is an endemic genus of south Western Ghats of India and Sri Lanka (Bremer, 1979). The genus *Neurocalyx* includes five species, of which two are seen in South India (*N. bremeri*, *N. calycinus*) and the remaining *N. championii*, *N. gardneri* and *N. zeylanicus* are found in Sri Lanka (Bremer, 1987; Viswanathan *et al*., 2005). *Neurocalyx calycinus* (R. Br. ex Benn.)

Rob. (syn. *Argostemma calycinum* R. Br. ex. Benn., *Neurocalyx capitata* Benth. ex Hook. F., *Neurocalyx hookerianus* Wight, *Neurocalyx wightii* Arn) is a woody stemmed herb grows upto 15 - 20 inches and dispersed on rocky crevices on the banks of streams in the tropical wet evergreen forest of southern India.

Cholanaickans are one of the oldest native communities of Kerala State, India. They are most primitive and vanishing tribes living in the Karulai and Chungathara forest ranges in Nilambur, Malappuram district (Mathur, 2013). They are one of the last remaining hunter-gatherer tribes of south India, living in rock shelters or crude huts beside brooks (Menon, 1996). This tribal community use fresh leaves of the plant for the treatment of various ailments such as inflammation, skin diseases and wounds (Saradamma *et al*., 1987).

The first step towards ensuring the quality of a starting material is the authentication followed by creating numerical values of standards for comparison. Pharmacognostical parameters for easy identification like leaf constants, microscopy and physicochemical analyses are few of the basic protocol for the standardisation of herbals. The evaluation of a crude drug is an integral part of establishing its correct identity. Before including any crude drugin herbal pharmacopoeia, pharmacognostic parameters and standards must be established. The present investigation aims phytochemical and pharmacognostic evaluation of the drug plant *N. calycinus* to establish its botanical identity and detect adulteration if any in the raw drug samples.

2. Materials and Methods

2.1. Collection of plant material

The plant *N. calycinus* was collected from the evergreen forest streams of the upper hill of Athirumala (Latitude 8°37’28”N Longitude 77°12’53”E, Altitude 800-1200m asl) in Thiruvananthapuram, Kerala, India during the summer season. The plant was authenticated by taxonomists at Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, India, where the voucher specimen is deposited (TBGT 57049/dated 16/11/2013). The leaves including petiole were segmented and were shade dried. After the completion of dehydration (within 10-14 days) the leaves were powdered to suitable size with the help of a multipurpose grinder and is stored in air tight amber containers. Both fresh sample and leaf powder was subjected to pharmacognostic and phytochemical analysis.

2.2. Chemicals

All reagents, chemicals and solvents were of analytical grade and were purchased from HiMedia Laboratories, India.

2.3. Morphological analysis

The morphological features of fresh leaves including petioles of *N. calycinus* were examined for both qualitative and quantitative traits. Colors were identified as per the horticultural color chart (Wilson, 1938).

2.4. Microscopical analysis

2.4.1. Quantitative stomatal characters

Epidermal peels of *N. calycinus* leaves were collected by Jeffery’s method (Patil and Patil, 1987). Small segments of leaves were incubated in Jeffery’s solution. The peels were stored in 30 % alcohol, stained with 1 % aqueous Safranin and mounted in dilute glycerin. The epidermal peels were analyzed by trinocular research microscope (BX 51 Olympus, Japan) and the microphotographs of the stomatal complex were taken by an image analysis software (Micro-image Olympus, Japan).

2.4.2. Determination of leaf constants

A piece of the leaf was cut and boiled in chloral hydrate solution. Upper and lower epidermis were peeled out and mounted in glycerin on a glass slide. The slide was observed under trinocular research microscope (BX 51 Olympus, Japan). The preparation was subjected to the determination of various leaf constants such as palisade ratio (Kokate *et al*., 1995), stomatal index (Salisburry and Ross, 1991), total number of vein – islet and total number of vein termination (Evans, 2009).

2.4.3. Anatomical characterization

Free hand sections were taken from the petiole and leaves by using a razor blade and stained with 1% aqueous safranin. Semi-permanent preparations were made by mounting in glycerin. The preparations were analyzed by trinocular research microscope and the microphotographs were taken. Descriptive terms of the anatomical features of the sections were also recorded (Esau, 1960; 1964).

2.4.4. Powder analysis

Shade dried, coarsely powdered samples were used for micro characterization. The specimen for examination was prepared by placing a little amount of powder on a slide, and then added 1-2 drops of phloroglucinol and a drop of conc. HCl, then covered it with a cover slip, draw off the liquid from one side of the slide with filter paper, and then added 1-2 drops of chloral hydrate solution from the other side of the slide, and the lignified elements were stained by Crimson red (Brain and Turner, 1975).

2.5. Physico-chemical analysis

2.5.1. Organoleptic analysis

The organoleptic characteristics of powered samples such as appearance, color in daylight, odour and taste were studied.

2.5.2. Determination of physical constants

The procedure recommended in Indian Pharmacopoeia (1985; 1996) were followed in the determination of loss on drying at 105°C, swelling index, foaming index, crude fiber content, pH of 1 % solution and foreign organic matters.

2.5.3. Determination of ash values

The residue of the powdered samples after incineration contains predominantly inorganic salts known as ash. The determination of the percentage of total ash, acid insoluble ash, water soluble ash, and sulphated ash were studied in accordance with the standard procedures recommended in Indian Pharmacopoeia (1985; 1996).

2.5.4. Determination of extractive values

The percentage of extractive values in different solvents according to their increasing polarity namely petroleum ether (60 – 80 ºC), benzene, chloroform, ethanol (95 %), methanol and water were calculated by individual extraction of the leaf powder (15 gm, 80 µm mesh sizes) in a Soxhlet extractor for 10 hours each (Kokate, 1994).

2.5.5. Fluorescence property

Plant powder showed various fluorescence behaviour when they areexposed to ultraviolet radiation. Fluorescence characteristics of the leaf powder as such and after treating them with chemical reagents such as sodium hydroxide, potassium hydroxide, iron (III) chloride, sulfuric acid, ammonia, hydrochloric acid, nitric acid, sodium carbonate, ethyl alcohol, silver nitrate and acetic acidwere observed in daylight as well as under ultraviolet radiation at short UV – 254 nm and long UV – 365 nm (Chase and Pratt, 1949; Kokoshi *et al*., 1958).

2.5.6. Preliminary phytochemical screening

Qualitative phytochemical analysis was carried out in n-hexane, chloroform, ethanol and aqueous extracts of leaves of *N. calycinus* using standard procedures (Sofowora, 1982; Harborne, 1998).

2.6. Optimization of thin layer chromatography solvent system

For the optimization of thin layer chromato graphy (TLC) fingerprint profile, several solvent systems were tried (Wagner and Bladt, 1996). The leaf extracts were spotted on to the silica gel 60 F_{254} precoated TLC plates (Merck, Darmstadt, Germany). The chromatograms were observed under both visible and UV (at 254 nm and 366 nm) light and were photographed. The Retention Factor (R_f) of the bands was calculated using the formula;

R_f = Distance travelled by the substance (cm)/Distance travelled by the mobile phase (cm).

2.7. High performance thin layer chromatography profiling

The ethanol extract (5 mg/mL) was spotted as 6 mm bands in 5, 10 and 15 µL aliquots with a Camag 100 µL Hamilton syringe on a silica gel precoated aluminum G60 F_{254} TLC plate (10 × 10 cm with 250 µm thickness) using an automated sample applicator, CAMAG Linomat V applicator. The laboratory was kept at 23 ± 2 °C with a relative humidity of 30–60 %. The TLC Plates were developed using toluene: ethyl acetate (6:1) as the mobile phase in a 24 × 15 × 8 cm custom-made glass twin trough chamber. The plates were kept for development, to a migration distance of 90 mm and were allowed to dry at ambient conditions in a ventilated fume hood for 10 min. Plates were scanned at 254 nm, 366 nm, and 425 nm using the UV scanner CAMAG Scanner type 3 (Camag, Switzerland) band width 6 mm, slit dimension 4.00 × 0.30 nm, scanning speed 20 nm/sec and source of radiation was deuterium lamp. The R_f and peak area were interpreted by using the software Win-CATS software version 1.4.3. Quantitative evaluation was done via peak areas.

2.8. Gas chromatography mass spectrometry analysis

The phytochemical screening of ethanol extract was performed using a Varian GC CP-3800 system comprising a Combi PAL autosampler and a Gas Chromatograph interfaced to a Mass Spectrometer (Saturn 2200 MS) equipped with a VF-5ms (equivalent to 5 % phenyl/95 % dimethylpolysiloxane, low bleed, highly inert) - a Factor Four GC capillary column, (0.25 mm × 30 m ID × 0.25 µm df). For GC-MS detection, an electron ionization system was operated in the electron impact mode with ionization energy of 70 eV. Helium gas (99.999 %) was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 µL was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 280°C, and the oven temperature was programmed from 60°C (isothermal for 2 min), with an increase of 5°C/min to 260°C, then 20°C/min to 300°C, holding with a 6min isothermal at 300°C. Mass spectra were taken at 70eV; a scan interval of 0.5s and mass range from 40 to 600 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 50 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was ion trap and the interpretation on mass-spectrum was conducted using the database of National Institute Standard and Technology (NIST ver. 2.1). The specta of the unknown components were compared with the spectaof known components stored in the NIST library. The name, molecular weight, Chemical Abstracts Service (CAS) number, amount (%) of the components of the test materials was ascertained.

3. Results and Discussion

3.1. Morphological analysis

Leaves simple, opposite decussate and crowded towards the tip of the branch (Fig. 1A). The lamina (Fig. 1B-C) is dorsi-ventral and hypostomatic nature (Table 1). The midrib is partially differentiated in the ventral surface; margin is entire.

3.2. Microscopical analysis

The quantitative microscopic features of leaves such as stomatal characters, vein-islet number, vein termination number (Table 2), stomatal index (Table 3) and palisade ratio (Table 4) were determined and charted.

Transverse section of *N. calycinus* leaf petiole was winged (Fig. 1) and wavy in outline with a depression on adaxial surface.The ground tissue was differentiated into outer four to five layers of parenchyma cells with multi-cellular pointed trichomes followed by an inner layer of collenchyma cells (Fig. 2A and C). Vascular strands of the petiole formed as a large median arc with two adaxial lateral traces. The median bundle consisted of xylem elements arranged in radial rows and phloem, exterior to the xylem (Fig. 2B and D). Starch grains were detected on the epidermal and hypodermal region at the proximal front of petiole (Fig. 2E). Petiole showed abundant calcium oxalate needle-like crystals in the ground parenchyma cells (Fig. 2F).

Fig. 1. Habit and leaf morphology of *Neurocalyx calycinus* with pendulous inflorescence. **A.** Habit, leaves are crowded towards the tip of the branch; **B.** Abaxial surface showed prominent midrib and veins; **C.** Adaxial surface waxy in texture

Table 1. Qualitative and quantitative morphological characteristics of leaves of *N. calycinus*

Morphological characteristics	**Observation**
Petiole	Attenuate
Petiole color	Spinach green 0960
Petiole length	1.5 – 3.2cm (2.25 ± 0.16)
Petiole width	1.1 – 2.7mm (2.20 ± 0.14)
Petiole shape	Cylindrical
Petiole hairs	Hirtellous
Leaf length	27.5-41.0cm (34.63 ± 1.22)
Leaf width	5.7 – 9.4cm (7.46 ± 0.27)
L / W of leaf	3.82 - 5.55 (4.67 ± 0.14)
Laminar shape	Lanceolate
Laminar symmetry	Symmetrical
Dorsal surface	Smooth shining, Spinach green 0960/1
Ventral surface	Pubescent, Downy, Sage green 000861/1
Venation	Pinnately reticulate
Inter secondary vein	Weak
Apex shape	Acuminate
Base shape	Acute
Base angle	Acute
Odour	Characteristic
Taste	No characteristic taste

Values are mean (N = 12) ± SE

Table 2. Quantitative analysis of stomatal characters of the leaves of *N. calycinus*

Sl. No.	Variables	Range	Mean
1	Length of guard cell	19.44 - 24.25µm	21.69 ± 0.42
2	Width of guard cell	5.67 - 7.72µm	6.73 ± 0.19
3	L/W of guard cell	3.01 - 3.68	3.24 ± 0.14
4	Length of subsidiary cell	36.40 - 46.33µm	40.55 ± 0.83
5	Width of subsidiary cell	4.87 - 13.11µm	8.80 ± 0.65
6	L/W of subsidiary cell	3.53 - 7.91	4.90 ± 0.40
7	Length of stomatal complex	42.79 - 56.38µm	51.29 ± 1.17
8	Width of stomatal complex	30.94 - 56.62µm	47.90 ± 2.21
9	L/W of Stomatal complex	0.91 - 1.55	1.09 ± 0.05
10	Vein termination number	2.8 – 3.9	3.4 ± 0.2
11	Vein islet number	8.5 – 10.3	9.8 ± 0.3

Values are mean (N = 12) ± SE

Table 3. Determination of stomatal index on abaxial surface of leaves of *N. calycinus*

Leaf portion		Range	Mean
Leaf apex	Margin	26.21 - 34.78	30.19 ± 1.12
	Middle	23.28 - 34.09	29.02 ± 1.55
Leaf centre	Margin	27.62 - 33.91	32.11 ± 1.44
	Middle	28.57 - 36.27	31.54 ± 1.10
Leaf base	Margin	20.97 - 29.85	26.04 ± 1.48
	Middle	18.06 - 28.99	25.21 ± 1.71

Values are mean (N = 12) ± SE

Table 4. Determination of palisade ratio of leaves of *N. calycinus*

Leaf portion	Range	Mean
Leaf apex	10.4 – 12.3	11.60 ± 0.23
Leaf centre	11.2 – 12.7	12.02 ± 0.19
Leaf base	8.4 – 10.4	8.82 ± 0.25

Values are mean (N = 12) ± SE

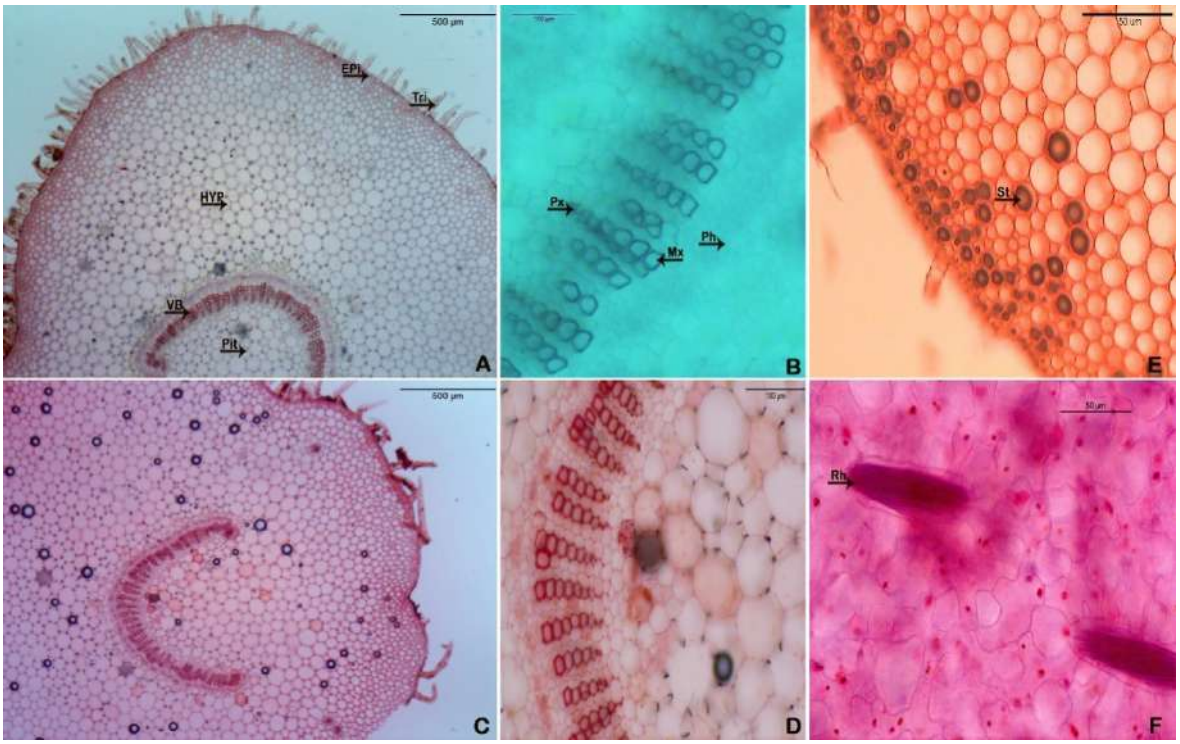

Fig. 2. T S of distal and proximal front of leaf petiole of *N. calycinus*. **A.** Distal front ground plan; **B.** vascular bundle of distal front showing proto and meta xylem; **C.** proximal front ground plan; **D.** dvascular bundle of proximal front showing proto and meta xylem; **E.** deposition of starch grains on the epidermal and hypodermal region; **F.** deposition of needle like raphide crystals. Tri. Trichome; Epi. Epidermis; Hyp. Hypodermis; VB. Vascular bundle; Pit. Pith; Px. Protoxylem; Mx. Metaxylem; Ph. Phloem; St. Starch grains; Rh. Raphide crystals.

The midrib is broadly hemispherical and prominent on the abaxial side with a short lump on the adaxial side. Multicellular unbranched trichomes consisting of single rows of cells were seen on the abaxial side of the midrib and the trichomes were not uniform in length.The epidermis was covered with cuticle, followed by four to five layers of compact parenchyma cells on both sides of the midrib, followed by collenchymatous hypodermis. The vascular strand on the midrib was seen as a wide discontinuous arc on the abaxial side (Fig. 3A, C and E). Xylem elements were in radial rows. Protoxylem and metaxylem were seen towards the upper and lower epidermal cells respectively, which were less lignified (Fig. 3B, D and F). Phloem was seen towards the lower epidermis. Glandular trichomes are present in the epidermal layers (Fig. 3G).Starch depositions were found in the hypodermal region (Fig. 3H).

The epidermal cells of the lamina were square shaped with thin cuticle. Laminar hairs and trichomes (Fig. 4B) were seen on the lower epidermis.It was followed by mesophyll cells, differentiated into the upper palisade tissue which is cylindrical, compact and occupies one-third thickness of lamina.The lower spongy parenchyma cells were loosely arranged and four to five layered (Fig. 4A).The lateral vascular bundles of the veins were small and collateral with thin bundle sheath.The ground tissue was parenchymatous and compact. Stomata were paracytic (Rubiaceous type),abundant and confined mainly towards the lower epidermis (Fig. 4C-D).

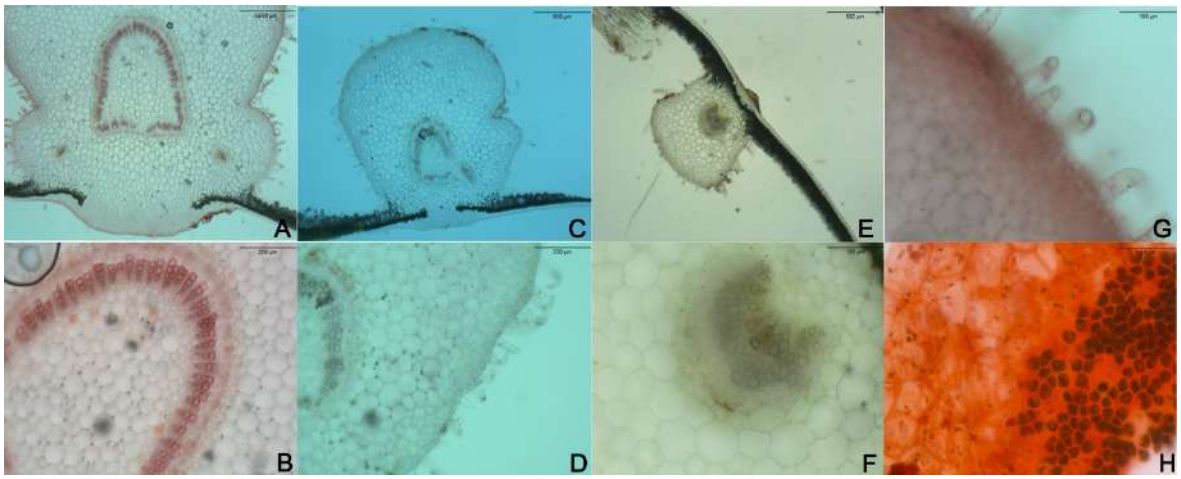

Fig. 3. T S of base, middle and apex region of leaf midrib of *N. calycinus*.**A.** Leaf base ground plan; **B.** Vascular bundle of leaf base; **C.** Ground plan of leaf's middle portion; **D.** Vascular bundle of leaf's middle portion; **E.** Leaf apex; **F.** Vascular bundle of leaf apex; **G.** Glandular trichomes; **H.** Starch depositions.

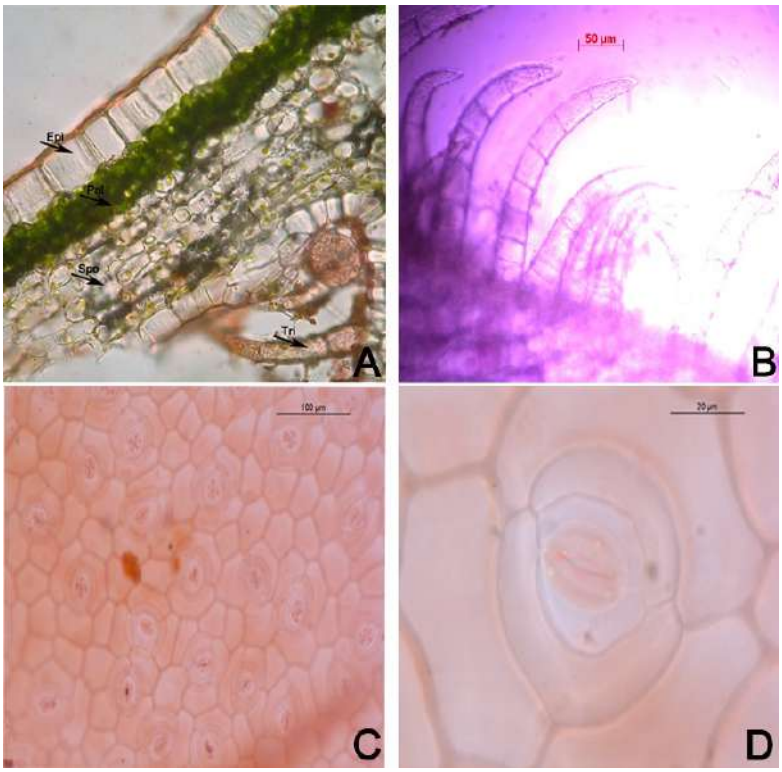

Fig. 4. T.S of lamina of *N. calycinus*. **A.** a portion of leaf lamina; **B.** Auto florescence of leaf epidermal trichomes by phase contrast microscope; **C** and **D.** Paracytic stomata. Epi.Epidermis; Pal. Palisade mesophyll cells; Spo. Spongy mesophyll cells; Tri. Trichomes.

The microscopic observation of the leaf powder of *N. calycinus* showed the following structures: multicellular trichomes, spiral vessels, starch grains of simple and clustered types, paracytic stomata and angular parenchyma cells (Fig. 5A-H).

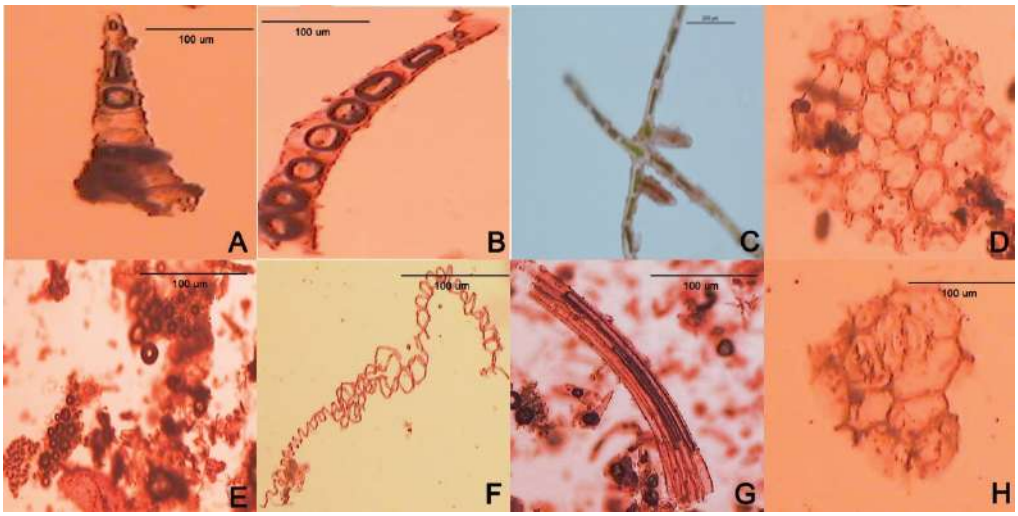

Fig. 5. Microscopic examination of leaf powder of *N. calycinus*.**A-C;** Trichomes; **D.** Parenchyma cells; **E.** Starch grains; **F.** Xylem vessels with spirally thickened walls; **G.** Xylem tracheids; **H.** Stomata.

3.3. Physico–chemical analysis

The leaf powder has a green colour, a characteristic odour and taste.The powder is coarse in texture and not much free flowing.The physicochemical standards like the percentage of total ash, acid insoluble ash, water soluble ash, crude fibre content, foaming index, swelling index and loss on drying at 105 °C were determined (Table 5). The percentage of individual (Table 6) and successive extractive values, consistency and colour were noted (Table 7). The fluorescence behaviour of the leaf powder of *N. calycinus* with different chemical reagent was also noted (Table 8).

Table 5. Physico chemical parameters of powdered leaves of *N. calycinus*

Parameters	**Value (% w/w)**
Foreign organic matters	Nil
Foaming index	< 100
P^H (1% solution)	6.9 ± 0.08
Swelling index	11.17 ± 0.09
Loss on drying at 105°C	21.31 ± 0.06
Moisture content	17.31 ± 0.05
Crude fiber content	23.26 ± 0.19
Total ash	9.12 ± 0.08
Acid insoluble ash	0.03 ± 0.01
Acid soluble ash	9.17 ± 0.01
Water soluble ash	4.91 ± 0.04
Water insoluble ash	4.27 ± 0.074
Sulphated ash	12.67 ± 0.01

Values are mean (N = 3) ± SE

Table 6. Individual extractive values of powdered leaves of *N. calycinus*

Solvent	**Consistency**	**Colour**	**Yield (%w/w)**
Petroleum ether	Sticky	Willow green 000862/1	0.72 ± 0.03
Benzene	Sticky	Parsley green 00962/3	1.62 ± 0.03
Chloroform	Sticky mass	Ivy green 0001060/2	2.14 ± 0.05
Ethanol	Sticky mass	Ivy green 0001060	6.50 ± 0.18
Methanol	Semi solid	Leek green 000858	10.87 ± 0.04
Water	Solid	Maroon 1030	16.23 ± 0.10

Values are mean (N = 3) ± SE

Table 7. Successive extractive values of powderedleaves of *N. calycinus*

Solvent	Consistency	Colour	Yield (% w/w)
Petroleum ether	Sticky	Willow green 000862/1	0.75
Benzene	Sticky	Sage green 000861	0.66
Chloroform	Sticky mass	Ivy green 0001060	0.50
Ethanol	Sticky mass	Leek green 000858/2	1.88
Methanol	Semi solid	Leek green 000858/2	3.63
Water	Solid	Maroon 1030/3	10.07

Table 8. Fluorescence analysis of leaf powder of *N. calycinus*

Treatment	Normal light	Short UV (254nm)	Long UV (365nm)
Dry powder	Willow green (000862/2)	Willow green (000862/3)	Sage green (000861/3)
Powder + D. Water	Willow green (000862/1)	Lavender green (000761)	Ivy green (0001060/1)
Powder + 50% NaOH	Fern green (0862/2)	Lavender green (000761/1)	Sage green (000861/1)
Powder +50% KOH	Willow green (000862/1)	Langite green (53)	Ivy green (0001060/2)
Powder + 5% $FeCl_3$	Pastel Levender 440	Wisteria blue 640	Sea lavender violet 637
Powder + Conc. H_2SO_4	Sage green (000861)	Leek green (858)	Ivy green (0001060)
Powder + 50% H_2SO_4	Sage green (00086/3)	Fern green (0862)	Ivy green (0001060/2)
Powder + Dil.NH_3	Lavender green 000761	Willow green 000862/ 2	Ivy green (0001060/1)
Powder + Conc. HCl	Willow green (000862/2)	Ivy green (0001060/1)	Ivy green (0001060/1)
Powder + 50% HCl	Willow green (000862/2)	Spinach green (0862/2)	Ivy green (0001060/2)
Powder + Conc.HNO_3	Maize yellow (607/3)	Fern green (0862/2)	Ivy green (0001060/2)
Powder + 50% HNO_3	Fern green (0862/3)	Parsley green (00962)	Ivy green (0001060/1)
Powder + Na_2CO_3	Leek green 000858/1	Fern green (0862/1)	Ivy green (0001060)
Powder + Alco. 50% KOH	Willow green (000862)	Sage green (000861)	Rhodamine pink (527)
Powder + $AgNO_3$	Leek green (000858/2)	Parsley green (00962/2)	Ivy green (0001060/3)
Powder + Acetic acid	Spinach green (0862/2)	Parsley green (00962/3)	Rose pink (427)

3.3.1. Preliminary phytochemical screening

Phytochemical screening of various solvent extracts of leaf of *N. calycinus* confirmed the presence of primary metabolites such as carbohydrates, proteins and aminoacids and secondary metabolites such as alkaloids, steroids, phenolic compounds, glycosides, saponins and the absence of anthraquinones, gums, mucilages and volatile oils (Table 9).

Table 9. Qualitative phytochemical analysis of extracts of *N. calycinus* leaves

Phytoconstituents		Aqueous	n-hexane	Chloroform	Ethanol
Carbohydrates	Molish's test	+ + +	-	-	+ + +
	Fehling's test	+ + +	-	+ +	+ +
Proteins	Millon's test	+ +	-	-	+
	Biuret test	+ +		+	+
Alkaloids	Hager's reagent	-	-	+	+ + +
	Dragendorff's reagent	-	-	-	+
Glycosides	Keller – Kiliani test	-	-	-	+ + +
	Kedde test	-	-	-	+ +
Phenolic compounds	Ferric chloride test	+ +	-	+ +	+ + +
	Lead acetate test	+ +	-	-	+ +
	Shinoda's test	-	-	-	+ +
Phytosterols	Salkowski reaction	-	+ +	-	+
Fixed oils and fats	Spot test	-	+	-	-
Saponins	Foam test	+	-	-	+
Gum and mucilage	Alcohol 95% test	-	-	-	-
Volatile oils	Steam distillation	-	-	-	-
Iridoids	Trim- Hill reagent test	-	-	-	+ + +
Anthraquinones	Chloroform - 10% ammonia test	-	-	-	-

+ = slightly present, + + = moderately present, + + + = highly present,–= absent. All the tests were carried out three times. Observations were based on the colour intensity and precipitation with appropriate reagents.

3.4. TLC standardization

Probable number and types of phyto components present in the extract were established using TLC. Initially, several solvent systems were tried from which increased resolution was obtained to get the maximum separation on plates. Solvent combination toluene: ethyl acetate (6:1) showed the maximum separation of bands in the ethanol extract of leaf of *N. calycinus*.The R_f values were 0.25; 0.27; 0.28; 0.30; 0.34; 0.38; 0.40; 0.53; 0.60; 0.65; 0.72; 0.74; 0.79; 0.83; 0.87; 0.91. The chromatogram thus generated was duly documented (Fig. 6).

3.5. HPTLC profiling

HPTLC chromatographic studies of the ethanol extract of leaves of *N. calycinus* showed 14 phyto-constituents at 254 nm. Among these, four R_F value were prominent 0.56, 0.28, 0.71, 0.66 and the areas were 27.24%, 25.84%, 15.37%, 10.86% respectively. Scanning under 366nm revealed the presence of fourteen phyto constituents among these,$3R_F$value are prominent 0.55, 0.29, 0.71 and the areas were 31.18%, 23.42%, 20.44% respectively. 13 spots of phyto constituents were revealed in visible light at 425nm. Out of these $4R_F$values were pronounced 0.55, 0.28, 0.70, 0.22 and the areas were 33.68%, 22.96%, 13.90%, 11.01% respectively (Fig.7 and Table 10).

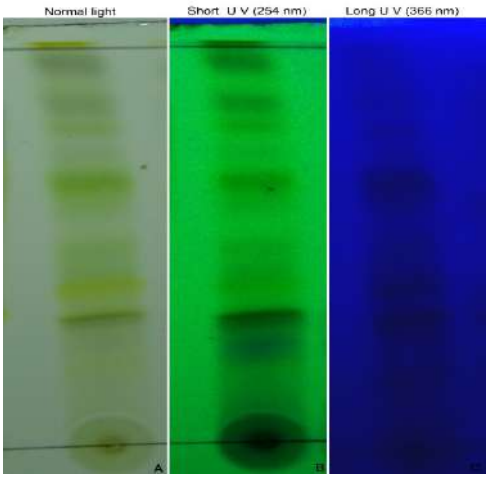

Fig. 6. Thin layer chromatogram fingerprint of ethanol extract of *N. calycinus* leaves in the solvent system toluene: ethyl acetate (6:1) without derivatization. A. normal fluorescent light; B. Short UV (254 nm); C. Long UV (366 nm).

Table 10. Relative percentage area and R_F values of the separated compounds of the ethanolextracts of *N. calycinus* leaves at 254 nm, 366 nm and 425 nm in high performance thin layer chromatograph studies

254 nm			**366 nm**			**425 nm**		
Peak	**Max Position in R_f**	**Area %**	**Peak**	**Max Position in R_f**	**Area %**	**Peak**	**Max Position in R_f**	**Area %**
1	0.04	4.55	1	0.01	0.30	1	0.01	0.14
2	0.07	2.88	2	0.05	5.97	2	0.05	5.79
3	0.12	0.90	3	0.07	1.18	3	0.10	0.14
4	0.15	2.67	4	0.10	0.24	4	0.12	1.12
5	0.17	0.63	5	0.12	1.71	5	0.17	4.19
6	0.22	7.06	6	0.15	1.63	6	0.22	11.01
7	0.28	25.84	7	0.17	0.50	7	0.28	22.96
8	0.39	0.38	8	0.22	6.02	8	0.36	1.89
9	0.43	0.40	9	0.29	23.42	9	0.39	3.07
10	0.48	0.95	10	0.39	1.43	10	0.43	1.38
11	0.56	27.24	11	0.42	0.77	11	0.55	33.68
12	0.66	10.86	12	0.55	34.18	12	0.62	0.72
13	0.71	15.37	13	0.62	2.20	13	0.70	13.90
14	0.80	0.26	14	0.71	20.44			

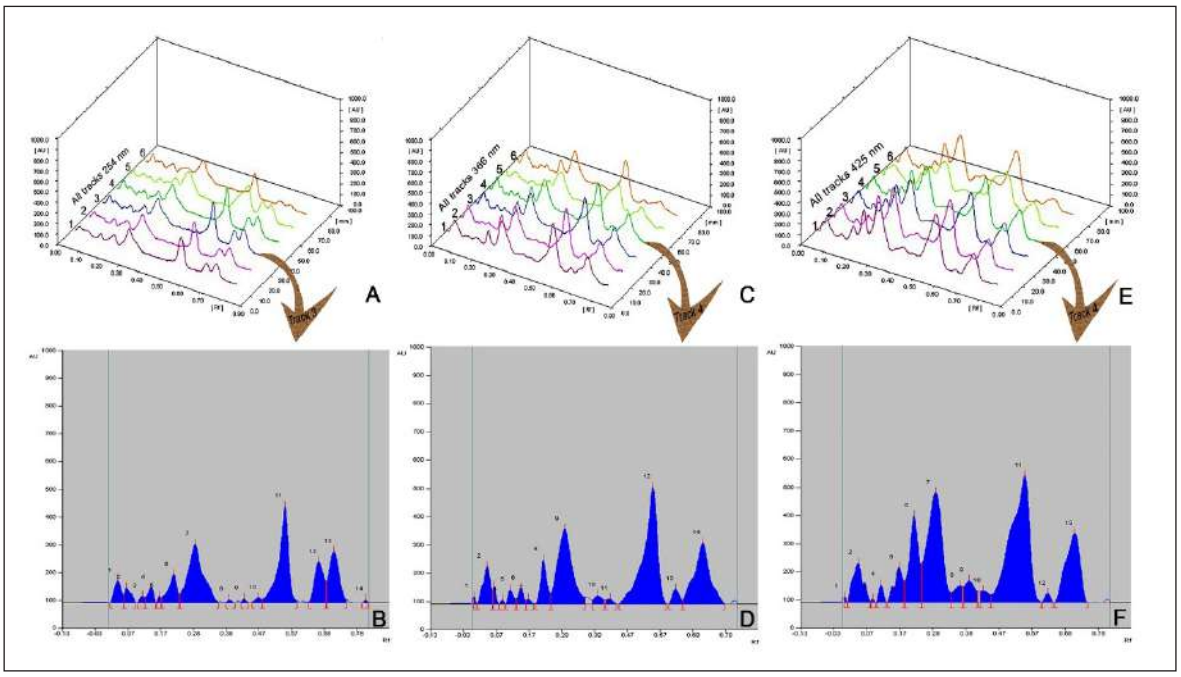

Fig.7. High performance thin layer chromatogram of ethanol extract of *N. calycinus* leaves in the solvent system toluene: ethyl acetate (6:1).tracks 1-2 contains 5 µL, 3-4 contains 10 µL and 5-6 contains 15 µL of extract. A, C and E.3 dimensional densitometric chromatogram at 254, 366 and 425 nm respectively showing different peaks of phytoconstituents; B (track 3), D (track 4) and F (track 4). chromatogram of track 3 has been shown under 254, 366 and 425 nm wavelength respectively.

3.6 GC/MS analysis

The GC-MS analysis of crude ethanol extract of leaves of *N. Calycinus* showed 37 peaks (phytoconstituents). The retention time (RT) and concentration (peak area %) of each peak were detected and tabulated (Fig. 8 and Table 11). The mass spectrum of major phyto constituents were compared with those available in database library (NIST ver.2.1). Based on the library search, 32 peaks and their corresponding mass spectra were tentatively resembled to that of respective standards. The remaining five peaks were unmatched to the library search. This attributes to occurrence of identified undocumented compound or a new compound, further NMR based spectral studies is the way to elucidate the information of that compound.

Table 11. Phytochemicals identified in the ethanol extracts of leaves of *N. calycinus* by GC-MS

Peak	RT (Min.)	Name of the compound	CAS No.	Peak area (%)
1	9.80	Propane, 1,1,3-triethoxy-	7789-92-6	2.5
2	12.11	Benzofuran, 2-ethenyl-	7522-79-4	2.8
3	13.36	Isoquinaldamide	1436-44-8	0.7
4	14.32	Benzaldehyde, 3-methyl-	620-23-5	0.8
5	16.63	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	1450-72-2	7.3
6	18.77	Tetradecane, 2,6,10-trimethyl-	14905-56-7	0.3

7	19.95	2-Azafluorenone	5061-91-6	0.3
8	21.10	Pyrazolidine-3,5-dione, 4-phenyl-	23876-79-1	0.1
9	21.54	Phenol, 2,5-bis(1,1-dimethylethyl)-	5875-45-6	0.6
10	22.95	tert-Butyl-4-hydroxy anisole	-	1.3
11	23.64	2,2-Dimethylpropanoic acid, 4-hexadecyl	-	0.5
12	25.78	1-{2-[3-(2-Acetyloxiran-2-yl)-1,1-dimeth	-	0.8
13	27.27	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	1620-98-0	2.3
14	27.74	Cyclopentanone, 3,3-dimethyl-2-(3-methyl	88725-86-4	0.5
15	28.06	1,3-Benzenediol, 5-pentadecyl-	158-56-3	4.4
16	28.778	2(1H)-Naphthalenone, octahydro-4a methyl	54594-42-2	0.5
17	29.35	1,2-Benzenedicarboxylic acid, butyl 2-me	17851-53-5	3.6
18	31.25	Dibutyl phthalate	84-74-2	4.6
19	31.57	Palmitic anhydride	623-65-4	4.9
20	31.91	Hexadecanoic acid, ethyl ester	628-97-7	1.2
21	33.78	9,12-Octadecadienoic acid (Z,Z)-, 2,3-di	2277-28-3	0.4
22	34.08	Phytol	150-86-7	7.0
23	34.81	9,12-Octadecadienoic acid (Z,Z)-, 2,3-di	2277-28-3	18.2
24	34.90	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(h	3443-84-3	7.5
25	35.21	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2	56051-53-7	0.6
26	41.09	Phthalic acid, monocyclohexyl ester	7517-36-4	5.0
27	44.05	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,	54159-46-5	1.7
28	44.47	Pregn-3,17,21-triol-20-one, 3,9-epoxy-3-	106502-85-6	0.7
29	46.92	Vitamin E	10191-41-0	9
30	48.28	Campesterol	474-62-4	0.9
31	48.61	Cholesta-22,24-dien-5-ol, 4,4-dimethyl-	-	1.4
32	49.42	gamma.-Sitosterol	83-47-6	4.2
Unidentified peaks				
1	27.74			1.0
2	31.03			1.1
3	35.21			8.4
4	35.55			0.2
5	44.31			0.7

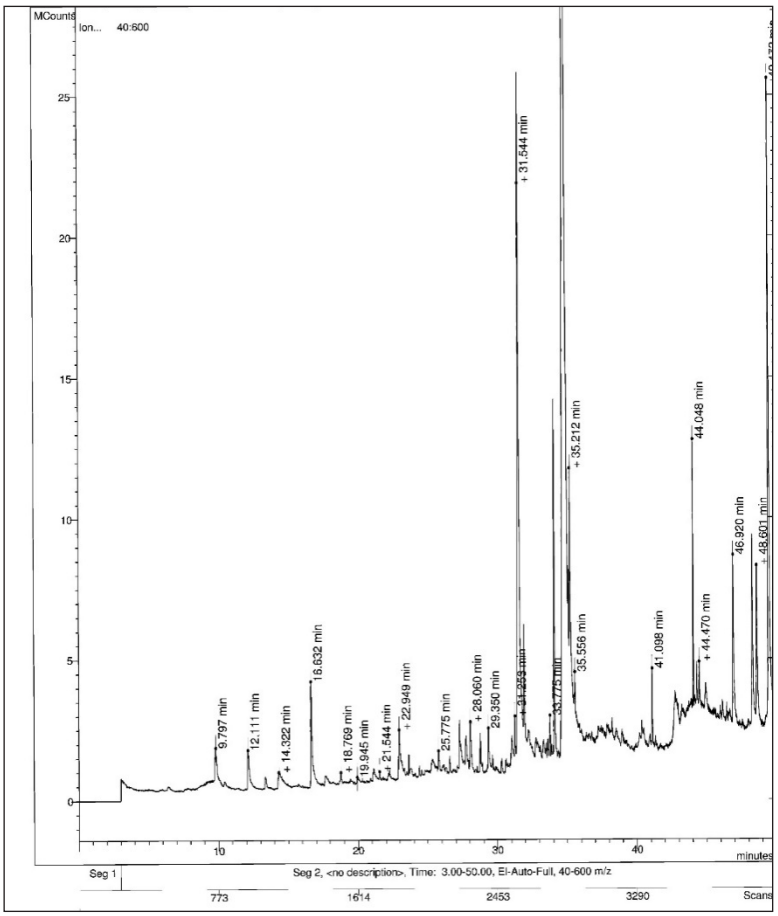

Fig. 8. GC-MS total ion current (TIC) chromatogram of ethanol extract of leaves of *N. calycinus*.

The morphological features are useful in rapid identification of a plant material.*N. calycinus* grows up to 20 to 40 cm in height.The macroscopic features of *N. calycinus* will be useful for its correct identification and to discriminate from its adulterants for pharmaceutical industries.

The quantitative determination of microscopic features of leaves will be useful for setting standards for crude drugs. These constants will also help in differentiating closely related species. When a leaf drug is adulterated with its allied species hardly differing morphologically, the quantitative microscopy may serve the purpose of identification. For instance, four closely related species of *Datura* (*D. stramonium*, *D. tatula*, *D. laevis* and *D. innoxia*) were distinguished by their stomatal number (Trease and Evans, 1985). The leaf of two species of *Catharanthus* was identified by quantitative microscopy such as stomatal index, palisade ratio and veinlet termination number (Chaudhury, 1963). The total length of veinlets per unit area of the leaf surface is a specific character of a specific plant which has direct physiological significance and is not related to the age of the plant (Aman *et al*., 2005). In *N. Calycinus,* the veinlet number is in the range of 8.5 – 10.3 is an important key identification feature of leaf from its neighboring genus.

Anatomical characters are useful to identify plant parts. *Rauwolfia serpentina* (Sarpgandha) species were distinguished from two other species such as *R. micrantha* and *R. canescens* through their anatomical features of roots (Youngken, 1954; Sulochana, 1959). Some important anatomical characters can be used to establish the real affinities of the genera in a larger taxonomic group. The presence of

hypostomatic leaves, straight-walled epidermal cells, paracytic stomata, dorsi-ventral mesophyll and the collateral vascular system in *N. calycinus* leaves reflects the general anatomical characters of the family Rubiaceae (Metcalfe and Chalk, 1950). Also, the presence of smooth cuticle, thin walled epidermal cells, multicellular pointed unbranched trichomes with varying length were found in other Rubiaceous species. *N. calycinus* also showed the key features of Rubioideae like an increase in diameter of the hypodermal cells towards the palisade parenchyma which is two to three layered bundles of needle-like raphides, presence of large and loose aggregates of randomly orientated needle-like crystals in its chlorenchyma. True raphides, defined by the International Association of Wood Anatomists Committee (1989) as a bundle of parallel needles, are a noteworthy feature of Rubiaceae.

The taste, texture, odour and colour are specific to each plant; organoleptic analysis of a crude drug powder will be helpful in the identification of the powder from the other adulterant powders. Evaluation of physicochemical parameters is an indispensable part in raw drug standardisation for maintaining the consistency and quality of herbal formulations.

The commonly applied parameter for the detection of impurities and adulteration of the drug is done by estimation of ash values, which establishes the quality and purity of the plant material for the preparation of a drug. Ash value can also detect the nature of the material added to the drug for adulteration (Jahan *et al*., 2008; Mulla and Swamy, 2010). In the present study, total ash value (9.12) is higher than acid insoluble (0.03) and water soluble (4.91) ash, which may be due to high content of carbonates, phosphates, silicates and silica. Water soluble ash is the water-soluble portion of the total ash (Vaghasiya, *et al*., 2008; Dave, *et al*., 2010) and is used to estimate the amount of inorganic compound present in drugs. The acid insoluble ash is constituted by silica and indicates contamination with earthy materials.

The extractive values in different organic solvents are based on the quantity, which is soluble in them. It makes a valuable test to determine the quality of the drug, and any variation in the chemical constituents may cause a change in the extractive values. Thus, it helps in the determination of the adulteration and is an index of the purity of crude extract for the clinical studies. The extractive value of *N. calycinus*, was therefore determined by individual and successive hot extraction method in different solvents according to the increasing polarity. In both extractions, universal solvent water gave the highest extractive value. This may be due to the presence of more amount of polar compounds than nonpolar ones. The variation in the extractive values may be possible due to the presence of the specific compound, according to the solubility, soil condition, atmospheric condition and water content of the sample (Jahan *et al*., 2008; Thomas, *et al*., 2008).

When chemical and physical methods are inapplicable, as often happens with the powdered drugs, the plants material may be identified from their adulterants by fluorescence study. Fluorescence is an important phenomenon exhibited by various chemical constituents present in plant material. In most cases, the herbal materials used for the preparation of drugs were used in powder form and adulteration of the powder is very common. However, fluorescence characteristic of any powder drug is very distinctive, and its distinguishing features will help in the determination of an adulterant in drug powder (Jahan *et al*., 2008). Previously, Ceylon Cinnamon was easily distinguished from Chinese and Saigon variety by their characteristic fluorescence (Hashmi and Singh, 2003). Such approach was also made in the roots of *Derris elliptica*, *D. malaccensis a*nd *Lonchocarpus nicou* (Wallis, 2005).

Preliminary phytochemical screening is a way to understand the major phytocompounds present in the plant. The presence of these biochemical compound depends upon the solvent used for the extraction and the part of the plant used for the study (Indian Pharmacopoeia, 1985). The key interactions between plant habit and their habitat including biotic and abiotic environment plays a

significant role in the production of metabolites. The main reason for the anti-inflammation and wound healing activity of theplants is due to the presence of chemical constituents which are mainly soluble in the polar, semipolar, nonpolar solvents.

Thin layer chromatography (TLC) is a technique used for the separation and identification of various components present in the crude extract. The separation is based on the differences in adsorption coefficients of the individual components of a mixture. Components which are strongly adsorbed in the stationary phase moves up less readily than those which are adsorbed to a lesser extent, leading to the separation of the compounds.

High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of crude plant extracts. A complete chemical profiling of the extract can be analyzed using different wavelengths of light (Herbal-ahp.org, 2018). HPTLC studies of ethanol extract revealed that 13-14 numbers of phyto constituents that were present in each of the three different wavelengths. Among the three different wavelengths, R_F value 0.55 at 254 nm and 425 nm showed 31.08 and 33.68 area percentage respectively, but, R_f value of 0.56 at 366 nm showed 27.24 area percentage. The 425 nm showed the maximum number of components compared to 254 nm and 366 nm. These fingerprint profiles showed the major chemical constituents in the crude extract along with their R_f values that would serves as a criterion for further investigation on the medicinal properties of this plant.

Standardization and characterization of herbal drugs is a topic of continuous scientific interest in the herbal drug industry. Gas chromatography provides an effective resolution for separating components from the crude mixture of compounds. Compounds with a lower molecular weight will elute out earlier than compounds with higher molecular weights due to differences in boiling points. Identification of these separated compounds can be accomplished through their characteristic molecular finger prints and mass spectra (Adams, 2004).The ethanol leaf extracts of *N. calycinus* were used for the GC-MS analysis. Since there are no available standards for this plant compounds, the results were only qualitative. The constituents were identified after comparison with those available in the NIST database attached to the GC-MS instrument.

4. Conclusion

The plant, *N. calycinus* may be explored as a promising source for the development of a potential drug. Therefore, it is recommended that the extraction and purification of such phytochemicals be very valuable in the preparation of drugs of various types.The result of these investigations could, therefore, serve as a basis for proper identification, collection and investigation of the plant.The macro and micro morphological features of the leaf described distinguishes it from other plants.

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Conflict of interest

The authors declare that there is no conflict of interest.

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