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Phytochemical Evaluation of Ttabernaemontana Divaricata (Plum) Plant

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Abstract: ***This work presents the basic concepts and practical methods used in the pharmacognostic and phytochemical evaluation of medicinal plants, with particular attention to Tabernaemontana divaricata. It outlines the importance of macroscopy, microscopy, physicochemical analysis, preliminary phytochemical screening, extraction techniques, chromatographic methods, and spectroscopic tools for the identification and characterization of plant constituents. The article also provides a detailed botanical profile of T. divaricata, including its taxonomy, habitat, morphology, cultivation, chemical constituents, and traditional medicinal uses. In addition, the materials, analytical instruments, plant collection, authentication process, and preparation of leaf samples for laboratory investigation are described, forming a foundation for future studies on the isolation and evaluation of bioactive compounds from this medicinal plant.***

Keywords: Pharmacognosy, Tabernaemontana divaricata, Phytochemical screening, Medicinal plants, Chromatography

1. Introduction

The plant has been used as a remedy in ancient. A complete understanding of medicinal plants involves a number of disciplines, including commerce, botany, horticulture, chemistry, enzymology, genetics, quality control and pharmacology. Pharmacognosy is not any one of these per se, but seeks to embrace them as a unified whole to better understand and utilise medicinal plants.

1.1 Importance of Macroscopy, Microscopy and Physicochemical Evaluation [1, 2, 3, 4]

Nowadays, pharmaceutical businesses start processing of medicinal and aromatic plants in their formula by using extraction of active components. Standardisation of crude drugs plays a completely important role in identifying the purity and pleasant of crude capsules. The prevailing investigation reveals standardisation, which includes moisture content, overall ash, acid-insoluble ash, water-soluble ash, water soluble extractive free, alcohol soluble extractive value, phytochemical screening, and in addition isolation and identification of phytoconstituent.

Macroscopy gives information about external features of plants, which helps to identify plant material. Microscopy includes the internal structure of every part of plant material. Example like leaves is flat, thin, green appendages to the stem. It includes tissue system i.e. epidermis, mesophyll, staller tissue. It also includes stomata, subsidiary cells, epidermal cells, trichomes, etc.

1.2 Preliminary Screening Importance

Preliminary phytochemical evaluation is the step before and after extraction to predict which phytoconstituent may be present in the extract and after means to confirm the phytoconstituent present in extract.

1.3 Extraction and Isolation of compounds (5, 6, 7, 8, 9, 10, 11, 12)

The overall strategies of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot non-stop extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, countercurrent extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents).

Soxhlet extractor

Soxhlet type of extraction performed with the help of soxhlet apparatus. This assembly was invented in 1897 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. However, a Soxhlet extractor is not limited to the extraction of lipids. Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. After extraction there is isolation of phytocostituent performed for identification of compounds.

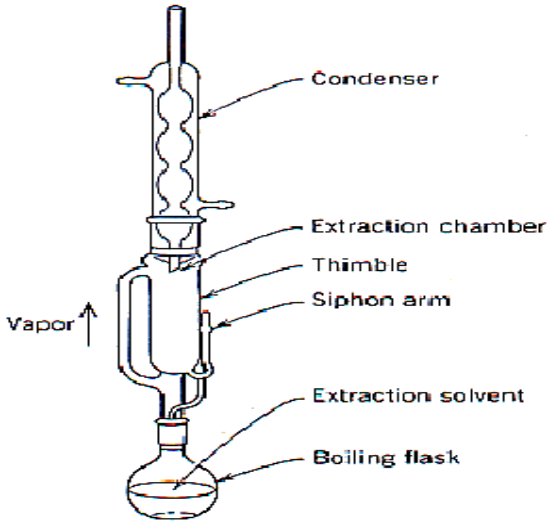


Figure 1: Soxhlet Extractor assembly

1.4 Chromatography (13, 14)

Chromatography technique is also included in the phytochemical evaluation parameter. Chromatography can be used to separate mixtures of colored compounds. Mixtures that are suitable for separation by chromatography include inks, dyes and coloring agents in food. The chromatography

technique is used for the isolation of phytoconstituents from plant material by using polar as well as non- polar solvent.

Types of Chromatography

(a) Paper Chromatography

The principle involved partition chromatography wherein the substances are distributed or partitioned between liquid phases. One phase is water, which is held in the pores of the filter paper used; and other is the mobile phase which moves over the paper. The compounds in the mixture get separated due to differences in their affinity towards water (in stationary phase) and mobile phase solvents during the movement of mobile phase under the capillary action of pores in the paper.

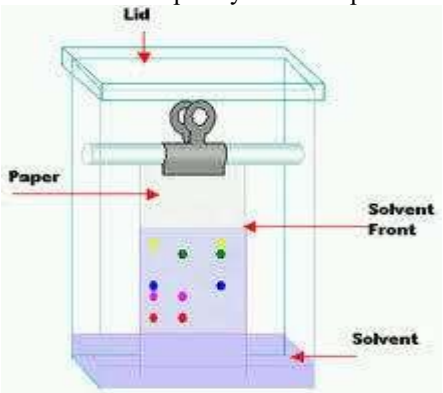


Figure 2: Paper Chromatography

(b)Thin Layer Chromatography

The separation depends on the relative affinity of compounds towards stationary and the mobile phase. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or characters are identified by means of suitable detection techniques.

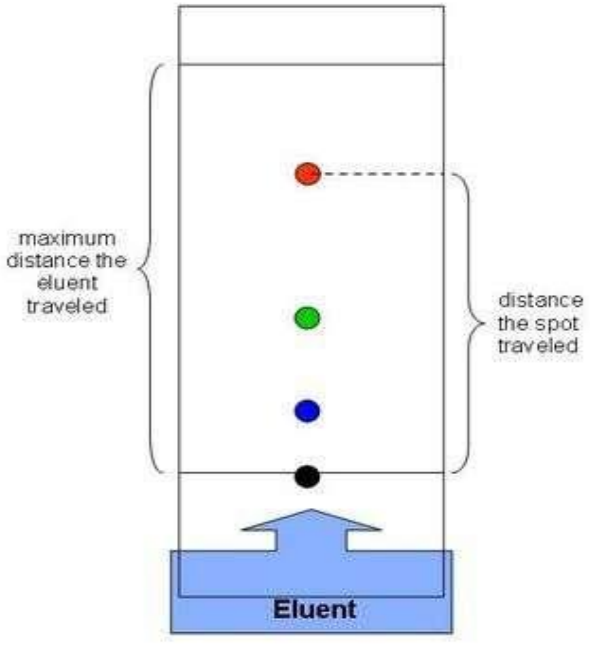


Figure 3: Thin layer chromatography

Column Chromatography

Column chromatography is a common technique used to separate individual compounds from a mixture. Column chromatography can be used on both a small or large scale to isolate and purify material. Column Chromatography consists of two phases: one mobile phase and one contiguous stationery phase. The stationery phase is solid and the mobile phase is liquid. The compound mixture moves along with the mobile phase through stationery phase and separates depending on the different degree of adhesion (to the silica) of each component in the sample or the compound mixture.

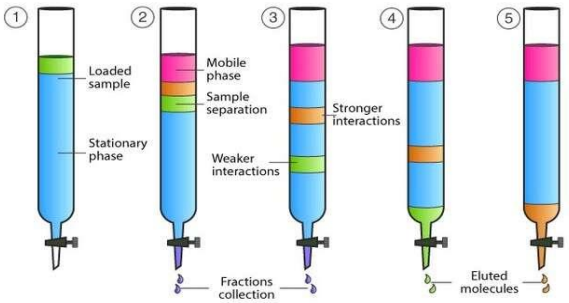


Figure 4: Column Chromatography

1.5 Spectrophotometric Methods (16)

Uv- Visible Spectrophotometry

UV-visible spectrometry having essential components like source of radiant energy, lenses, mirror, silts, monochromator system, sample holder, and container to hold sample. Power supply sources should be of stable type resisting voltage fluctuations.

***For ultraviolet region*:** The hydrogen lamp and deuterium lamp are the most common sources of ultraviolet radiation. In hydrogen lamp, a pair of electrodes is enclosed in a glass tube provided with silica or quartz window (for ultraviolet radiation to transmit through) and is filled with hydrogen gas at low pressure. When current is passed through these electrodes maintained at high voltage, discharge of electron occurs which excites hydrogen gas to high energy states. The electrons return to their ground state and emit radiation in the region of 180-350 nm. When deuterium replaces hydrogen, similar excitation and relaxation of gas results in emission of ultraviolet region, but the intensity is of high order.

***For visible region*:** A glass enclosed tungsten filament incandescent lamp is most widely used sources for visible and infrared region. The filament is heated by a stabilized power supply or by storage battery. It is available in various sizes and shapes suitable for fixing in the instrument and operates from 3-220 volts.The tungsten lamp emits continuous radiation in the region of 350-2500 nm. About 15 cm of radiant energy falls within visible region. The source of radiation being inexpensive is very common in most spectrophotometers.

Infrared Spectrometry

The infrared absorption spectrometry is concerned with the absorption of infrared radiation by a molecule and exhibits characteristic absorption spectra. The infrared region of the electromagnetic spectrum extends from the red end of visible spectrum to the microwave region of electromagnetic

spectrum (0.8-200 μ). The most useful range is between 4000-400 cm^{-1}. This region is often called the fundamental region from the stand point of both application and instrumentation. The IR spectrum is usually subdivided into the spectrum of near infrared, middle and far infrared region, the limits of which are given below.

Table 1: IR spectrum regions

Region	Wavelength (λ) range μ	Wave number (ū) range cm^{-1}
Near IR	0.78-2.5	12800-4000
Middle IR	2.5-50	4000-200
Far IR	16-200	625-10
Infrared	2.5-16	4000-625 (most useful range)

Examination of Infrared spectrum

Recorded infrared spectrum shows number of absorption peak, some fine, some broad with varying intensities in its complete region (4000-650 cm^{-1}). The identification of unknown organic compound is usually done by examining certain group frequencies. From the number of absorption peaks, some show distinctly the presence of particular group which helps in identification of a compound. Some important regions which are examined are described below:

a) ***The region between 4000-1400 cm^{-1}*:** This broad group shows presence or absence of many groups in the molecule. The important groups accounted for include NH, OH, C=O, C=C, C=N etc. The presence of aromatic nucleus (2000-1670) and hydrogen bonding O-H, N-H, etc. are encountered in this region.
b) ***The region between 1400-900 cm^{-1}*:** This is commonly known as finger print region. This region accounts for many absorption bands characteristic of functional groups. Since, location of functional groups can be attributed in this region termed as finger print region. Absorption bands due to bending vibrations of different groups as well as stretching vibrations of C-C, C-O, and C-N are observed.
c) ***The region below 900 cm^{-1}*:** This region from 900-650 cm^{-1} gives few but sharps bands which can be accounted for the specific groups. The presence of an aromatic nucleus is indicated in 1000-625 cm^{-1} region. The N-H, C-H rocking is also seen in this region.

The infrared spectrophotometer is a very important tool used in qualitative identification and quantitative estimation of many drugs and chemicals.

1.6 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) is a form of absorption spectrometry. It is concerned with the absorption of certain energy by a spinning nucleus in a magnetic field when irradiated by certain energy radiation perpendicular to it. It thus permits identification of atomic configurations in a molecule. Absorption occurs when nuclei undergoes transitions from one alignment in the applied field to the opposite one. The proton of hydrogen gives proton magnetic resonance (PMR) while that of the carbon-13 isotope is called C^{13} NMR. The wavelengths and energy required in NMR spectroscopy are far different from that of UV visible and IR spectrophotometry. In NMR spectroscopy, radio frequency waves are used which are of long wavelength and therefore have less energy associated with them. These do not affect the electronic, vibrational or rotational levels of molecule. They are able to interact with nuclei of certain atoms when exposed to strong magnetic field.

1.7 Mass Spectrometry

Mass spectrometry dealing with many analytical problems in organic and inorganic chemistry like determining stable isotopes, examining ionization phenomena, studying free radicals, etc. It is also used by physicists, in space research and biomedical research. The development of mass spectrometry began when Wines (1898), demonstrated that the beam of positive ions could be deflected by both electric and magnetic fields. The instrumentation and techniques of mass spectrometry have steadily progressed since the work by Thomson (1905), Aston and Dumpster (1979), who built the first mass spectrometer. The mass spectrometer is an instrument in which the substance in gaseous or vapor state is bombarded with a beam of electrons, to form positively charged ions (cations) which are further sorted according to their mass to charge ratio to record their masses and relative abundances.

2. Plant Profile

Plant profile[17, 18]

Profile of Tabernaemontana divaricata (doubled petaled flower) plant

Figure 5: Photo of Tabernamontana Divaricata (doubled petaled flower) plant

Vernacular name
Common name: Crape jasmine
Hindi name: Chandni
Telugu name: Nandivardhanamu
Kannadam name: Nandibatlu
Malayalam name: Kutampale
Tamil name: Nandiyavattai
Synonym: Chandni, crape jasmine, florepleno.

Taxonomical Classification
Kingdom: Plantae
Division: Angiosperms
Class: Eudicots
Subclass: Asterids
Order: Gentianales
Family: Apocynaceae
Subfamily: Rauvolfioideae

Genus: Tabernaemontana Plum.ex L. 1753

Habitat
It has a pan-tropical distribution, found in Asia, Africa, Australia, North America, South America, and an extensive collection of oceanic islands.

Plant Description
Those flowers are evergreen shrubs and small trees developing to at least one–15 m tall. The leaves are contrary, three–25 cm long, with milky sap; subsequently its miles one of the various plant genera commonly called "milkwood". The plants are fragrant, white, 1–5 cm in diameter. The cultivar t. divaricata cv. 'plena', with doubled-petaled flowers, is a popular houseplant.

Some contributors of the genus Tabernaemontana are used as components to some variations of the psychedelic drink ayahuasca. The genus is thought to incorporate ibogaine (e.g. in bëcchëte, t. undulate) conolidineand voacangine (namely in t. Africana).

Leaves: Leaves are evergreen, rectangular-shaped, easy, glossy, and contrary in arrangement, a median of four to six inches lengthy, and darkish green in shade. It has pinnate veins, milky sap, and entire margins.

Flowers: The white plants are double, waxy, frilly, very Tabernaemontanadivaricata 'Flore pleno' leaves close up fragrant (especially at night time), and a pair of to two 1/2 inches throughout. It blooms all through the years in hotter areas with a heavier flowering inside the spring and from spring to fall someplace else.

Fruits: fruit are two to a few inch lengthy orange/purple pods.

Cultivation

***Hardiness*:** It is hardy in USDA zones nine to eleven. it can be grown in a few regions of zone eight with the same old iciness killing to the ground and regrowth in the spring.

***Mild*:** Full sun for best flowering and compactness, however it's going to tolerate partial color with fewer plant lives. Tabernaemontanadivaricata 'Flore pleno' flower buds. Until a site is completely exposed, light conditions will alternate during the day and even in the course of the year. The northern and eastern facets of a residence receive the least amount of mild, with the northern publicity being the shadiest. The western and southern sides of a house receive the most mild and are taken into consideration the hottest exposures due to intense afternoon solar.

***Salt*:** Slight salt spray tolerance – it's going to tolerate some spray, however it is nice to have greater protection inclusive of a fence or constructing as a further barrier in the back of the primary row of plantings or the primary dunes.

***Soil*:** It prefers a fertile soil; however it's going to grow on different nicely-tired soils in the pH variety of 5.five to six.5. It can show off a few chlorosis on alkaline soils.

***Watering*:** It has mild drought tolerance as soon as mounted, so some water is wanted for survival in dry spells. It will obviously appearance higher and flower higher with irrigation for the duration of dry spells.

***Propagation*:** Softwood cuttings taken in warm weather and positioned below mist

***Pruning*:** Pinch when young to sell bushiness. Prune as needed to control size or preserve form. Kinds of pruning encompass: pinching, thinning, shearing and rejuvenating. Pinching is getting rid of the stem hints of a young plant to sell branching. Doing this avoids the need for more severe pruning later on. Thinning involves disposing of entire branches returned to the trunk. This may be carried out to open up the indoors of a plant to let greater mild in and to boom air stream that can reduce down on plant sickness. The great manner to start thinning is to start through putting off lifeless or diseased wood. Shearing is leveling the surface of a shrub the usage of hand or electric powered shears. That is achieved to preserve the favored form of a hedge or topiary. Rejuvenating is removal of antique branches or the general discount of the scale of a shrub to repair its unique shape and size.

***Fertilizing*:** It has no unique fertilizer wishes. Except a soil test indicates in any other case, a slow-release balanced analysis fertilizer applied in keeping with the product label will work.

Chemical Constituent
Phenols, Flavonoids, Proteins, Alkaloids, Steroids like secondary metabolites present in Tabernaemontana divaricata leaf part of plant. Bisindole alkaloids present in divaricata species specifically in flower part.

Uses
1) The leaves have milky juice that heal wounds and irritation, vegetation are used for pores and skin illnesses.
2) Roots are aphrodisiac, tonic .purgative, astringent to the bowels and tonic to brain.
3) Useful in paralysis and strangury, alcoholic and aqueous extract own enormous anti-inflammatory and analgesic homes.

3. Materials and Instruments

Materials
All chemicals used in study were of analytical grade.

Table 2: List of chemicals

S No.	Materials/Chemicals	Sources/Suppliers
1	Ethanol	Loba Chemicals, Mumbai
2	Ethylacetate	Loba Chemicals, Mumbai
3	Methanol	Loba Chemicals, Mumbai
4	Chloroform	Loba Chemicals, Mumbai
5	Glacial acetic acid	Loba Chemicals, Mumbai
6	Benzene	Loba Chemicals, Mumbai
7	Butanol	Loba Chemicals, Mumbai
8	Silica gel 70-230 mesh for column chromatography	Loba Chemicals, Mumbai
9	Silica gel 60 F_{254} percolated TLC plate (20×20)	Loba Chemicals, Mumbai

Instruments

Table 3: List of Instruments

Instruments/ Equipment	Manufactures	Model No.
Digital Balance	Shimazdu	AUW220D
Glassware	Borosil and J-Sil	-
Sieves	Retch	-
Compound microscope	Microlux-16	CM/L-9010256
UV cabinet	Shanti scientific	-
IR Spectrophotometer	Shimazdu	84005 IR
UV spectrophotometer	Shimazdu	-
NMR spectrophotometer	Bruker	-
LC-MS spectrophotometer	Agilent MSD	LC-QTOF MS/MS

4. Experimental and Results

4.1 Collection And Authentication (2, 4, 5, 22,)

The leaf part of Tabernaemontana divaricata (T. divaricata) plant was collected from garden area of Government College of pharmacy Amravati. The plant was authenticated by head of botany department (Dr.J.A.Tidke) Sant Gadge baba Amravati University, Amravati, Maharashtra, India. A voucher specimen no.API/2018/I was deposited at the department of botany Sant Gadge Baba Amravati University for future reference.

4.2 Drying and Size Reduction of Plant Material

The leaf part of T. divaricata plant was washed with water to remove dust particle. Then after washing leaves were kept under shade for drying up to two weeks. After completing drying process leaves are converted into coarse powder.

4.3 Morphology of Plant

Organoleptic characters
Colour-Green above and pale in beneath
Odour- Characteristic
Taste- Characteristic
Macroscopy
Apex- Acuminata
Margin-Slightly Wavy
Veins- Reticulate, anastomosing towards margin.
Shape- Ovate or oblong (1-15) cm long.
Base- symmetrical, petiole (1-3cm).
Extra feature: upper surface is shiny, midrib prominent on lower surface.
Microscopy of T.S

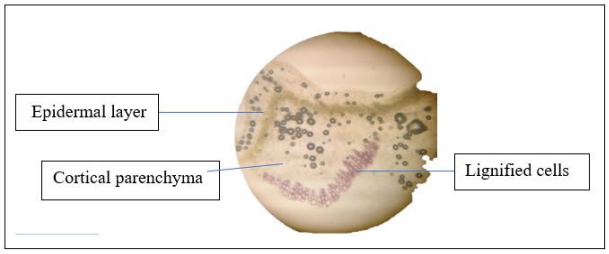


Figure 6: T.S of Tabernaemontana divaricata leaf part

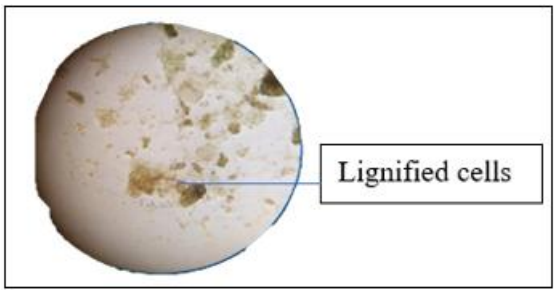


Figure 7: T.S. of T. divaricata leaf part

Powder Microscopy

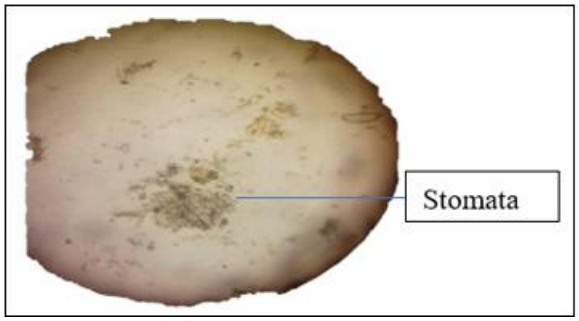


Figure 8: Powder microscopy of T. divaricata leaf part

4.4 Physicochemical Evaluation

Loss on Drying: 0.024
Ash value
Total ash value: 0.88%
Water soluble ash value: 0.66%
Acid soluble ash value: 0.04%
pH: 6.0
Extractive value
***Alcoholic extractive value*: 8%**
***Water soluble extractive value*: 94.8%**

4.5 Preliminary Phytochemical Screening

Table 4: Preliminary phytochemical screening

S. No	Chemical Test	In aqueous extract	In alcoholic extract
1	Test for carbohydrates Molisch test	 +	 +
2	Test for Alkaloids i)Wagner test ii)Hager test iii)Mayer's test	 + + -	 + + -
3	Test for tannins i)Lead test ii)5% FeCl3 iii)Bromine Water	 + - +	 + - +
4	Test for steroids i)Salkowski test ii)Liebermann burchard test	 - -	 - -
5	Test for Glycosides i)Cardiac glycosides test ii)Antraquinone test	 - -	 - +
6	Test for amino acids	-	-
7	Test for flavonoids i) Sulphuric acid test	 -	 +
8	Test for phenols	+	+

4.6 Soxhlet Extraction of dried and powdered plant material

Dried coarse powders of leaf part of plant were extracted in Soxhlet apparatus by using ethanol as a solvent.1000 gm quantity of powder required 22 days for completing extraction process. The powder material kept in muslin bag to avoid blocking of thimble. Everyday minimum 50gm powder material was extracted. After completion of extraction all extracts were collected and distillation performed for concentrating the extracts. One fourth portion of extracts concentrated and proceed for liquid-liquid fractionation.

Figure 9: Soxhlet extraction of plant material of T. divaricate

4.7 Liquid-Liquid fractionation

The conc. ethanolic extract(350ml) of T. divaricata leaf part of plant were mixed with n-hexane and extracted in successive volumes(50ml, 25ml, 25ml) using separating funnel. The n-hexane layer (organic layer) was separated and aqueous layer was subjected for successive extraction with n-hexane. The same procedure repeated for 4-5 times and all aqueous layer were combined and concentrated by distillation. The conc.aqueous layer extract evaporated until dryness in oven at 50°C temperature.

Figure 10: Seprating funnel showing seprated chemicals in the organic and aqueous phase

4.8 Column chromatography

Silica column chromatographic fractionation and isolation of chemical constituent from aqueous fraction of ethanolic extract of leaf part of T. divaricata

Absorbent: Silica gel (70-230mm mesh size) for column, chromatography activated at 105°c for 2 hrs.
Dimension: 45cm in length and 3cm bore size. (Internal diameter)
Length of absorbent packed: 30 cm
Elution: Gradient

Silica Column preparation

The silica gel (200-400 mesh size) was used as stationary phase. Column was prepared by using silica gel sulary in chloroform solvent. The prepared silica gel sulary devoid of any air bubble poured in column and suitably packed the column. The chloroform solvent passed through column initially.

Sample loading

The aqueous fraction collected from ethanolic extract of T. divaricata was mixed with 50ml methanol and 10g silica added in it and evaporated it until dryness in oven at 50°c temperature. The dried sample was loaded on to the surface of column.

Silica column chromatographic fractionation and Isolation of compounds from aqueous fraction of ethanolic extract of T. divaricata

The isolation of compounds from aqueous fraction of ethanolic extract of T. divaricata was fractionated through gradient elution technique with different proportion of chloroform, methanol as mobile phase. Initially elution carried out through 100% chloroform and by increasing polarity with methanol means carried out with mobile phase CHCl3: MeOH (99:1, 97:3, 90:10, 80:20, 75:25, and 70:30). The mobile phase continues until sepration of same constituent performed which was confirmed by performing TLC profile monitoring elute. The eluent of volume 50ml collected from silica column having elution rate 1-2ml/min. The fractions collected in 100ml conical flask, conc. and evaporated and performed TLC profile. Those fractions showed same profile were combined and concentrated.

Figure 11: Column chromatography of aqueous layer of ethanolic extract of T. divaricata (leaf Part).

4.9 Silica column chromatographic sepration of compounds from aqueous fraction of ethanolic extract of T. divaricata

Table 5: Details of different fractions obtained from aqueous layer of ethanolic extract of T. divaricata after silica column chromatography.

S. No	Fractions	Mobile phase (for column)	UV_{254}	UV_{365}	TLC profile (R_f value)
1	1	chloroform: methanol (99:1)	Blue	Bright white	1
2	2				
3	3				
4	4				
5	5	chloroform: methanol (97:3)		Light white	1,0.9
6	6				0.8
7	7			Light blue	0.5
8	8				
9	9				0.5
10	10				
11	11				
12	12	Chloroform: methanol (90:10)		Bright blue	0.8
13	13				
14	14				
15	15			Pale blue	0.5
16	16	Chloroform: methanol (80:20)			
17	17				
18	18			Bright blue	0.7
19	19				
20	20				
21	21				
22	22			Pale blue	0.5
23	23	Chloroform: methanol (80:20)	Blue	Pale blue	0.5
24	24				
25	25			Bright blue	0.5
26	26				
27	27				
28	28		Blue	Bright blue	0.9, 1
29	29				
30	30			Bright blue	0.2
31	31				
32	32				
33	33				
34	34				
35	35				
36	36				
37	37				
38	38				
39	39				
40	40				
41	41				
42	42				
43	43			Bright blue	0.2, 0.5
44	44				
45	45				
46	46				
47	47				
48	48				
49	49				
50	50				
51	51				
52	52				
53	53				
54	54	chloroform: methanol (75:25)			
55	55				
56	56				
57	57		Blue		
58	58				
59	59				
60	60			Bright blue	0.2
61	61				
62	62				
63	63				
64	64				
65	65				
66	66				
67	67				
68	68			Bright blue	0.5
69	69				
70	70				
71	71				
72	72				
73	73			Bright blue	0.2
74	74				
75	75				
76	76		Blue	Bright blue	0.2
77	77				
78	78				
79	79				
80	80			Bright blue	0.3
81	81				
82	82				
83	83				
84	84	Chloroform: methanol (75:25)			
85	85				
86	86				
87	87				
88	88				
89	89				
90	90				

TLC profile of (1-90) fractions isolated from aqueous fraction of ethanolic extract of T. divaricata

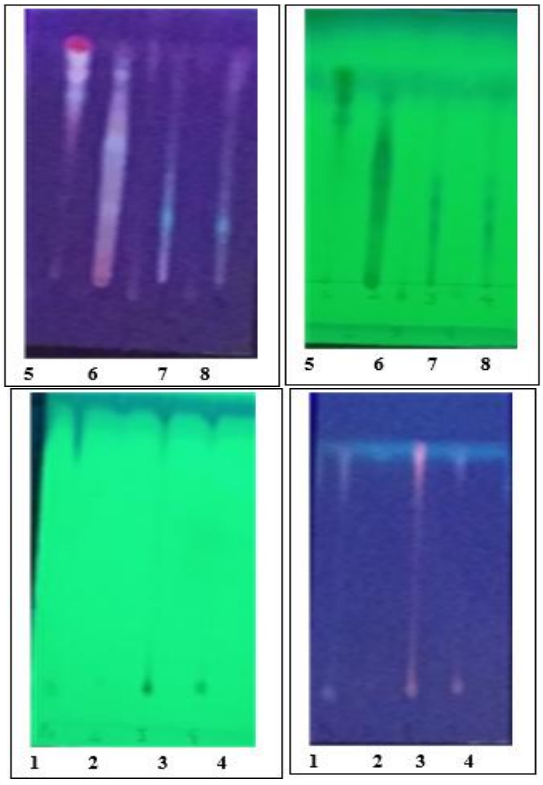


Figure 12: TLC profile of fractions (1-8) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254 nm and 365 nm)

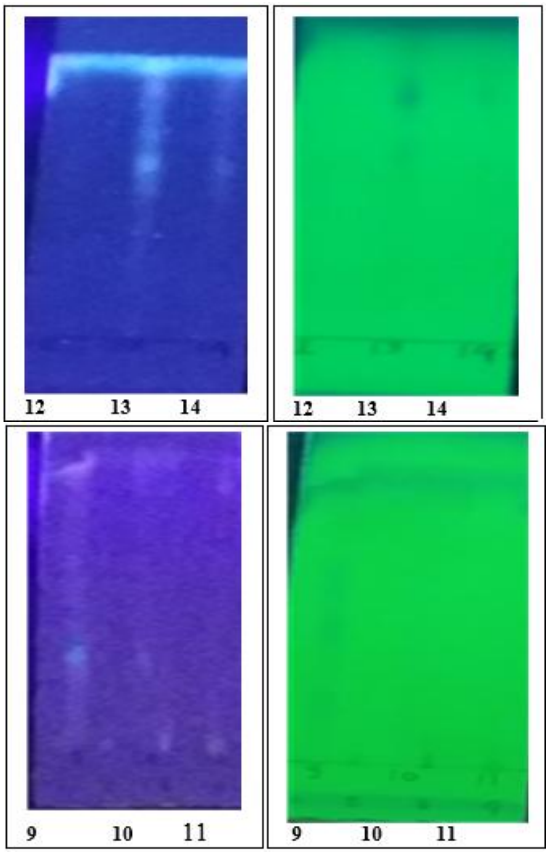


Figure 13: TLC profile of fractions (9-14) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254 nm and 365 nm)

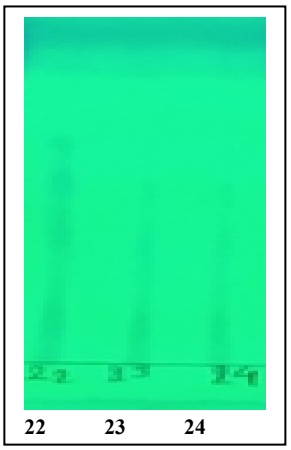


Figure 14: TLC profile of fractions (15-21) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254 nm and 365 nm)

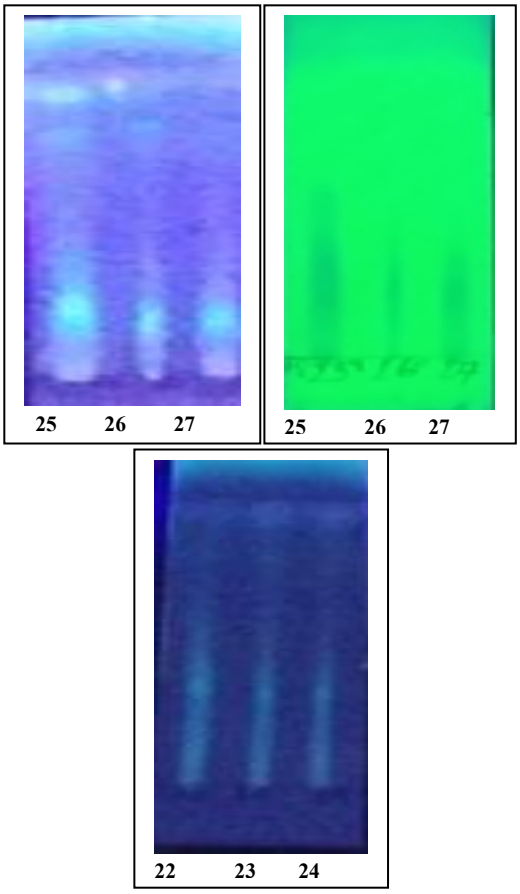
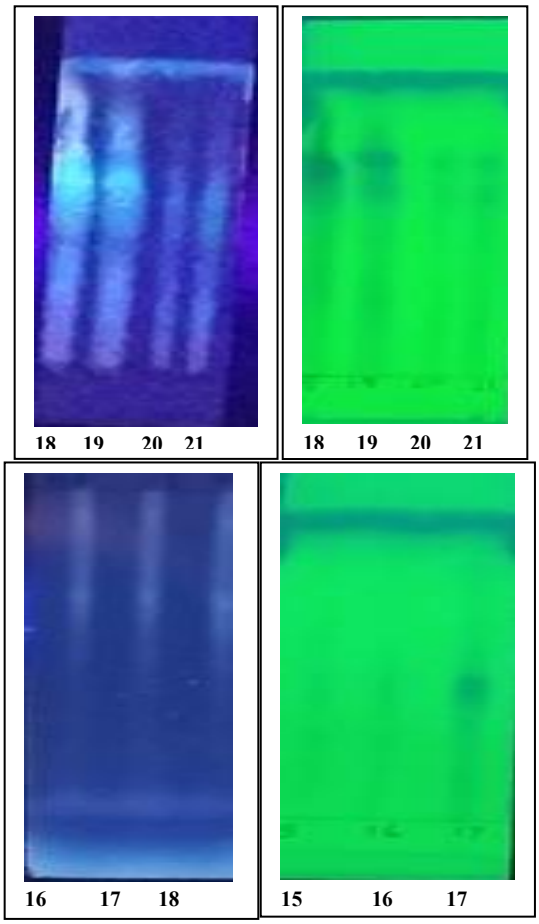


Figure 15: TLC profile of fractions (22-27) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254 nm and 365nm).



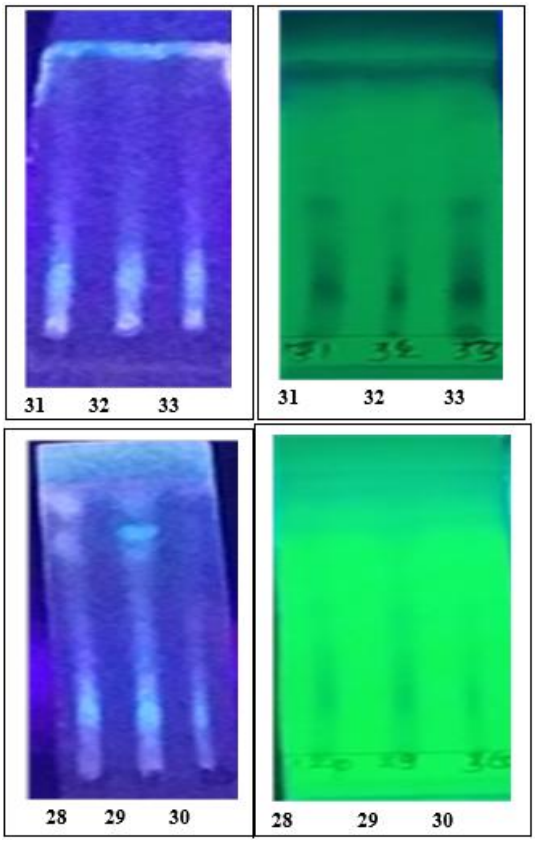


Figure 16: TLC profile of fractions (28-33) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254 nm and 365nm).

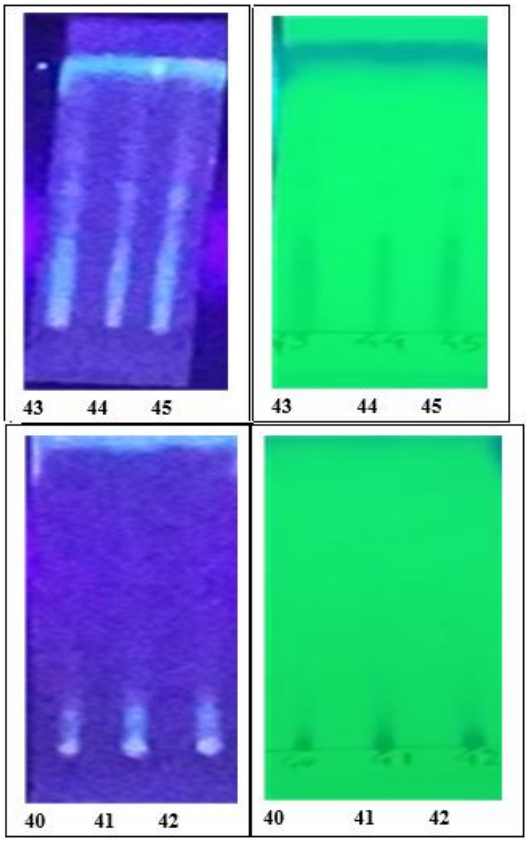


Figure 18: TLC profile of fractions (40-45) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

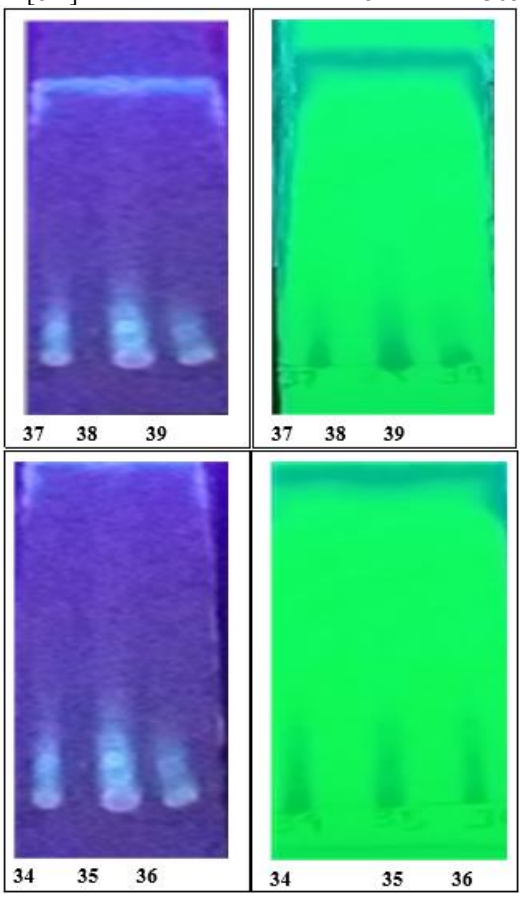


Figure 17: TLC profile of fractions (34-39) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

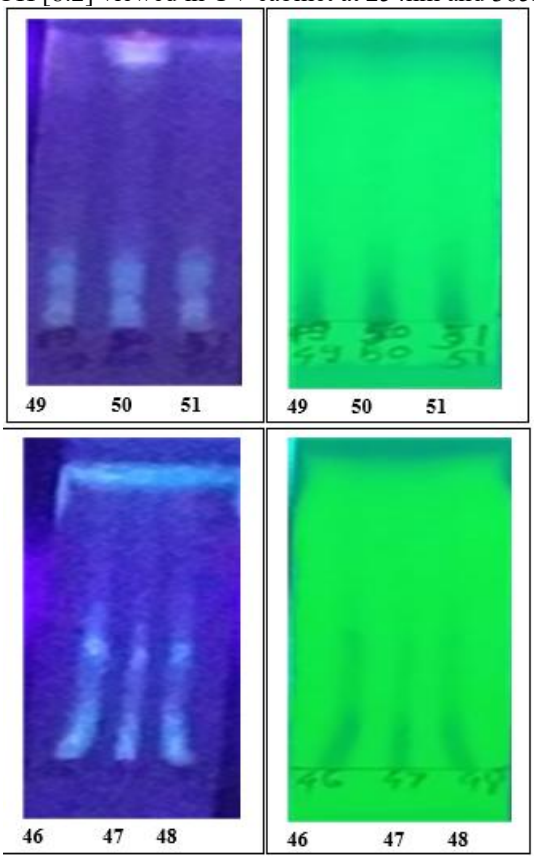


Figure 19: TLC profile of fractions (46-51) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

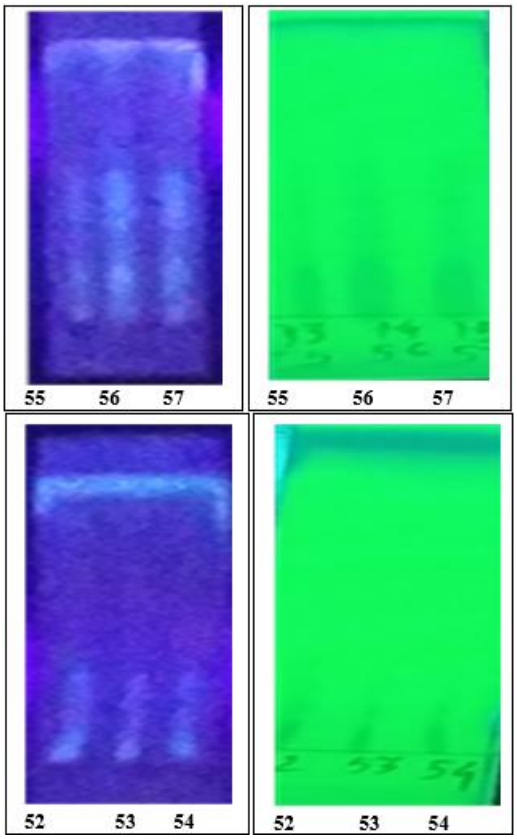


Figure 20: TLC profile of fractions (52-57) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

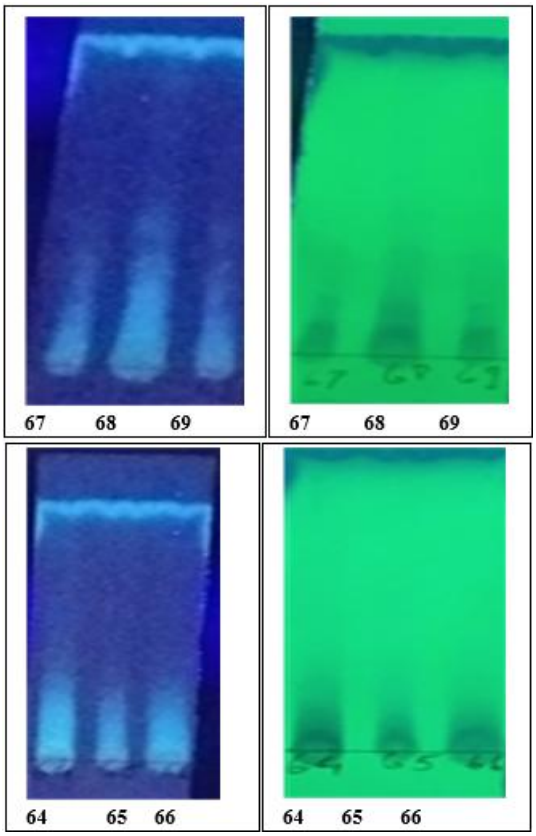


Figure 22: TLC profile of fractions (64-69) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCL3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

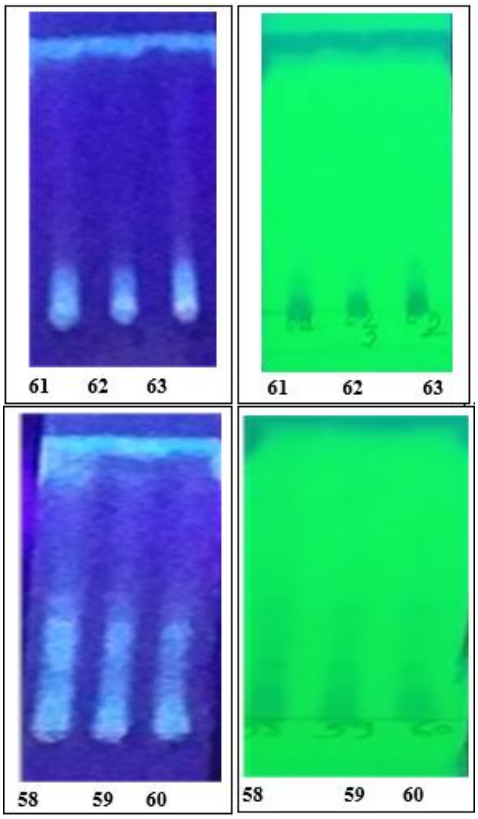


Figure 21: TLC profile of fractions (58-63) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

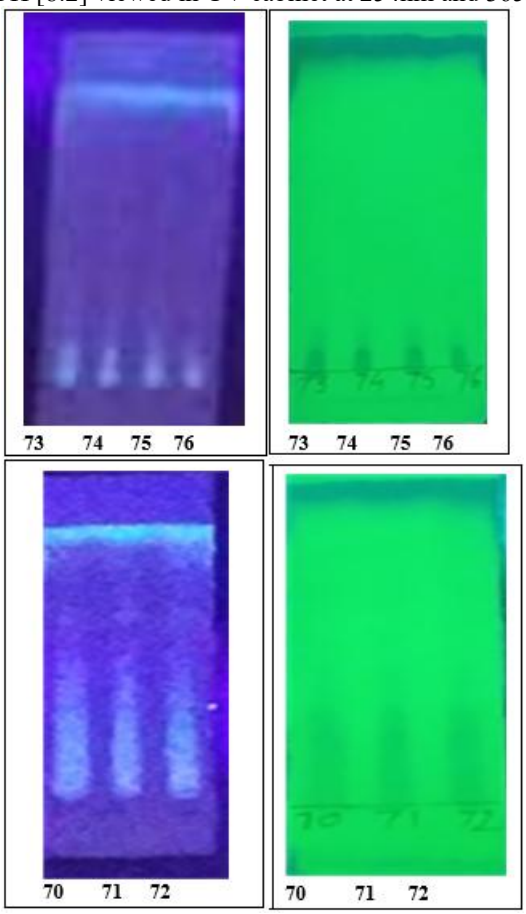


Figure 23: TLC profile of fractions (70-76) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCL3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm)

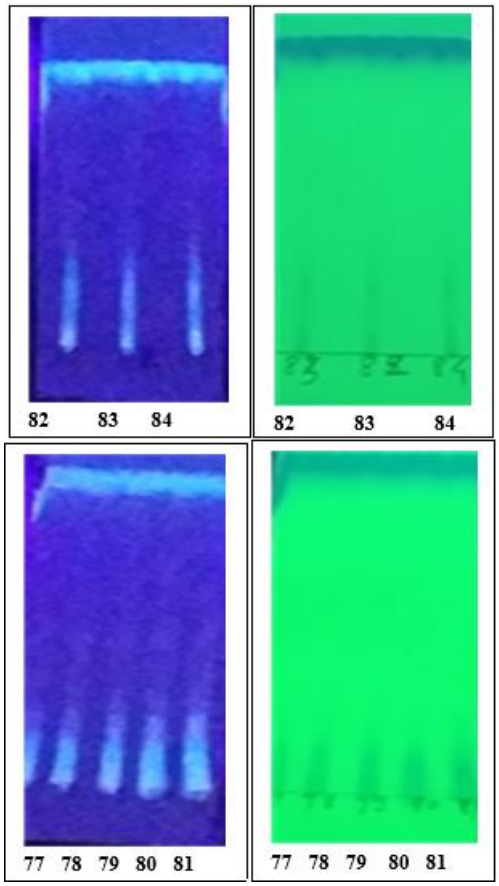


Figure 24: TLC profile of fractions (77-84) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm).

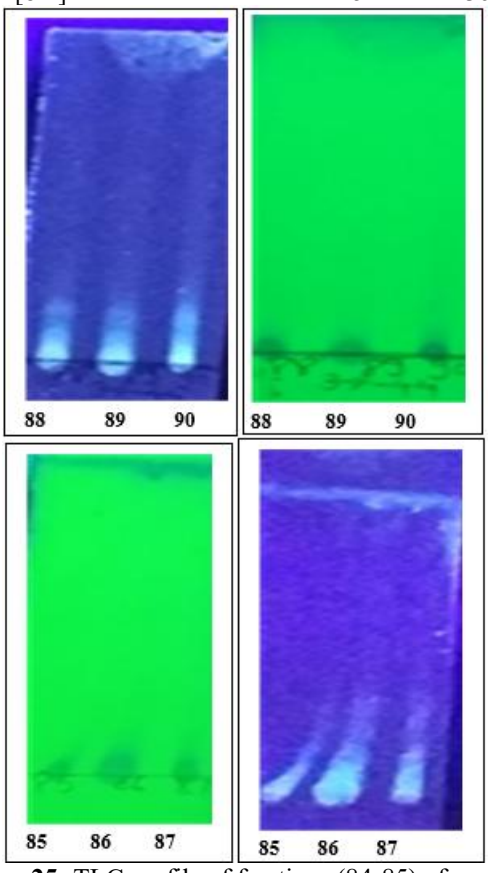


Figure 25: TLC profile of fractions (84-85) of aqueous fraction of ethanolic extract of T. divaricata (MP: CHCl3; MeOH [8:2] viewed in UV cabinet at 254nm and 365nm).

4.10 TLC analysis of combined fractions after processing fractions isolated from aqueous layer of ethanolic extract of T. divaricata

Table 6: Details of combined fractions obtained from aqueous layer of ethanolic extract of T. divaricata after silica column chromatography.

S. No	Fractions (comb & conc.)	Mobile phase(for TLC)	Spots	UV_{254}	UV_{365}	R_f
1	1-4	$CHCl_3$: Me(99:1)	1	Blue	Light blue Light blue pink Light blue	1 1 1 1
2	7-9	$CHCl_3$: Me	1	Blue	Bright blue Light blue	0.3
3	10-14	-	1 2	Grey.blue -	Light white, Bright blue Bright blue	0- 0.1,1 0.6
4	15-17	-	1 2	Grey blue -	Light blue Light blue	0.1 0.4
5	18-21	-	1 2	Blue grey -	Bright blue Light blue	0.2 0.6
6	22-24	-	1	Grey	Bright blue	0.4
7	25-27	-	1 2 3	Blue - -	Bright blue Light blue Bright blue	0.2 0.8 1
8	28	-	1 2 3	Blue - -	Bright blue Light blue Light blue	0.2 0.9 1
9	29-30	-	1 2	Grey -	Bright blue Light blue Light blue	0.2 0.9
10	31-48	-	1 2	Grey -	Bright blue Light blue	0.2 0.5
11	50	-	1 2	Grey -	Bright blue Light blue	0.2 1
12	49,51-54	-	1	Grey -	Bright blue Bright blue	0.2 0.5
13	55-57	-	1 2	Grey -	Bright blue Bright blue	0.2 0.5
14	58-60	-	1 2 3	Blue - -	Bright blue - -	0.1 0.4 0.9
15	61-63	-	1	Grey	Bright blue	0.2
16	61-69	-	1	Grey	-	-
17	70-75	-	1	-	-	-
18	76-84	-	1	-	-	-
19	85-90	-	1	-	-	-

4.11 TLC profile of combined fractions after processing fractions isolated from aqueous layer of ethanolic extract of T. divaricata

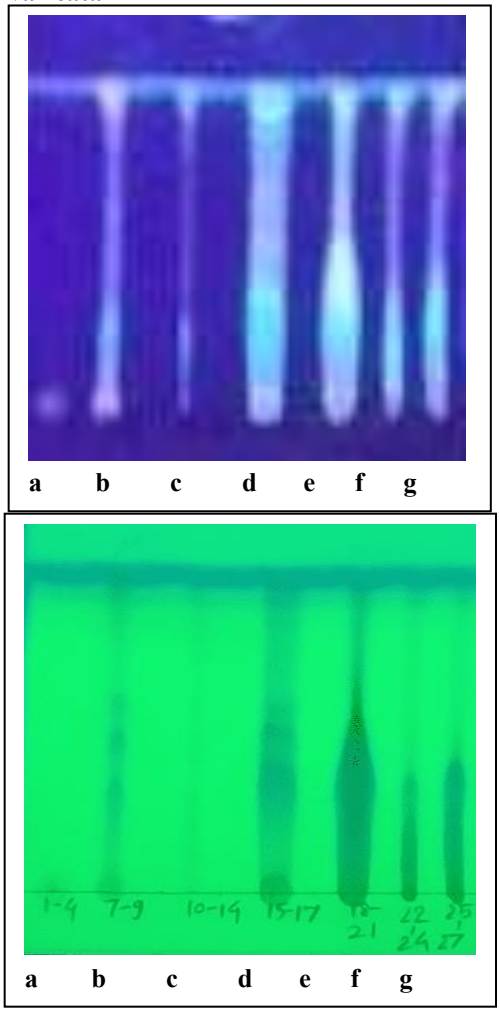
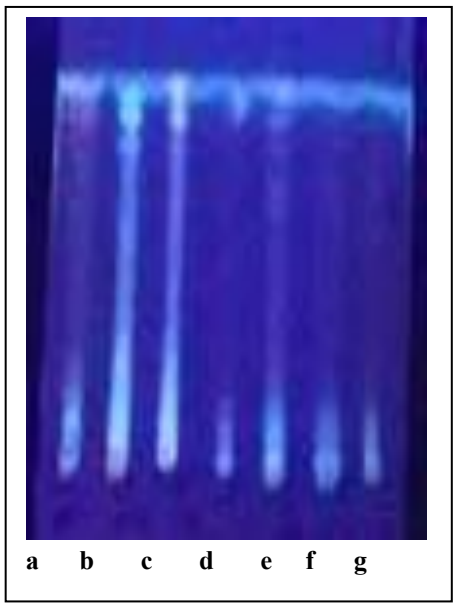


Figure 26: Photograph of TLC profile of combined fractions (a:1-4,b:7-9,c:10-14,d:15-17,e:18-21,f:22-24,g:h:25-27) after processing fractions isolated from aqueous layer of ethanolic extract of T. divaricata. (TLC viewed in UV cabinet at 254nm and 365nm).



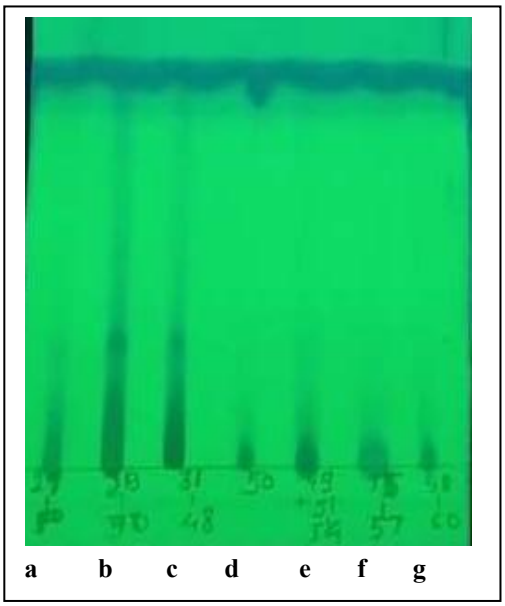


Figure 27: Photograph of TLC profile of combined fractions (a:28,b:29-30,c:31-48,d:50,e:49+51-54,f:55-57,g:58-60) after processing fractions isolated from aqueous layer of ethanolic extract of T. divaricata. (TLC viewed in UV cabinet at 254nm and 365nm).

4.12 Rechromatography

Silica column rechromatographic fractionation and isolation of chemical constituent from 5,6th fraction obtained from aqueous fraction of ethanolic extract of leaf part of T. divaricata

Absorbent: Silica gel (70-230mm mesh size) for column, chromatography activated at 105°c for 2 hrs.
Dimension: 30cm in length and 2cm bore size. (Internal diameter)
Length of absorbent packed: 20cm
Elution: Gradient

Silica Column preparation

The silica gel (200-400 mesh size) was used as stationary phase. Column was prepared by using silica gel sulary in chloroform solvent. The prepared silica gel sulary devoid of any air bubble poured in column and suitably packed the column. The chloroform solvent passed through column initially.

Sample loading

The 5,6th fraction collected from silica column of aqueous fraction of ethanolic extract of T. divaricata were evaporated until dryness and 30ml methanol and 1g silica added in it and evaporated it until dryness in oven at 55°c temperature. The dried sample was loaded on to the surface of column.

Silica column rechromatographic fractionation and Isolation of compounds from 5,6th fraction obtained from silica column of aqueous fraction of ethanolic extract of T. divaricate

The isolation of compounds from aqueous fraction of ethanolic extract of T. divaricata was fractionated through gradient elution technique with different proportion of chloroform, methanol as mobile phase. Initially elution carried out through 100% chloroform and by increasing polarity with methanol means proceed with mobile phase

CHCl3: Me (99:5). The mobile phase continues until sepration of same constituent performed which was confirmed by performing TLC profile by monitoring elute. The eluent of (25ml) volume collected having elution rate 1-2ml/min. The fractions collected in 100ml conical flask, conc. and evaporated and performed TLC profile. Those fractions showed same profile were combined and concentrated.

Figure 28: Rechromatography of 5,6th fraction obtained from silica column of aqueous fraction of ethanolic extract of T. divaricata (leaf Part

Table 7: Details of different rechromatographic fractions obtained from 5,6th fraction from silica column of aqueous layer of ethanolic extract of T. divaricata.

Sr. no	Fractions	Mobile phase (for column)	UV_{254}	UV_{365}	Rf value	Fraction contains same constituent combined & conc.
1	1	chloroform: methanol (95:5)	Grey	Bright blue	1	Fraction (1-3) comb. & conc.
2	2					
3	3					
4	4		Dark blue	Bright white	1, 0.9	Fraction (4-6) comb. & conc.
5	5					
6	6					
7	7		Grey	Light blue	1	Fraction (7-9) comb. & conc.
8	8					
9	9					
10	10		Grey	Light blue		Fraction (10-31) comb. & conc.
11	11					
12	12					
13	13		Grey	Light blue	1	Fraction (13-18) comb. & conc.
14	14					
15	15					
16	16					
17	17					
18	18					
19	19	chloroform: methanol (95:5)	Grey	Light blue	1	Fraction (19-31) comb. & conc.
20	20					
21	21					
22	22					
23	23					
24	24					
25	25					
26	26					
27	27					
28	28					
29	29					
30	30					

TLC profile of (1-31) fractions isolated from 5,6th fraction from silica column of aqueous fraction of ethanolic extract of T. divaricate

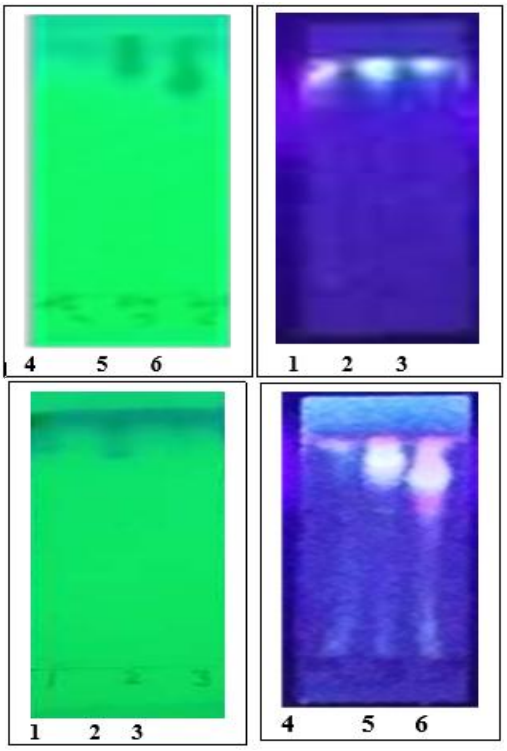


Figure 29: TLC fractions (1-9) of rechromatography from silica column of aqueous fraction of ethanolic extract of T. divaricata (MP:CHCl3:MeOH [8:2] viewed in UV cabinet at 254nm and 365nm).

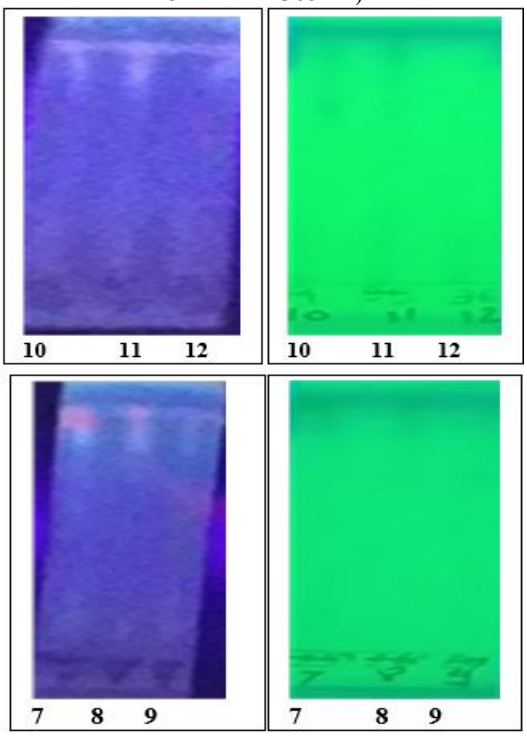


Figure 30: TLC fractions (7-12) of rechromatograhy from silica column of aqueous fraction of ethanolic extract of T. divaricata (MP:CHCl3:MeOH [8:2] viewed in UV cabinet at 254nm and 365nm).

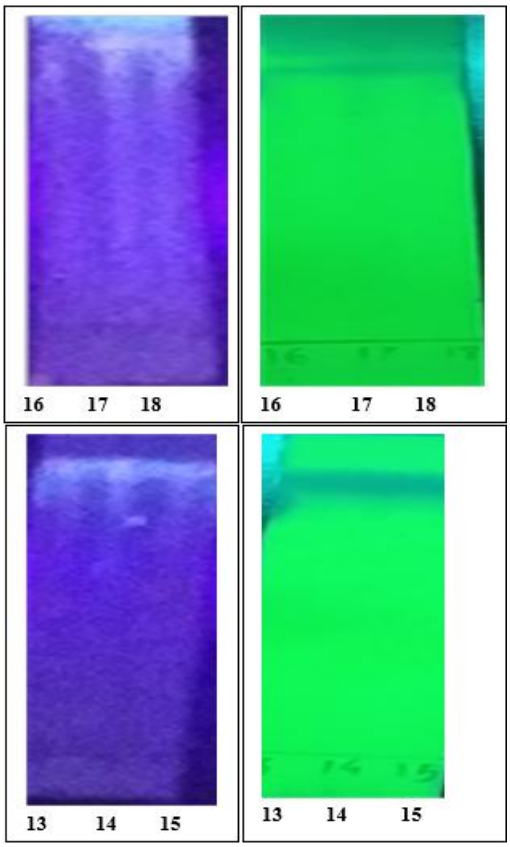


Figure 31: TLC profile of fractions (13-18) of rechromatography from silica column of aqueous layer of ethanolic extract of T. divaricata. (MP:CHCl3:MeOH [8:2] viewed in UV cabinet at 365 nm)

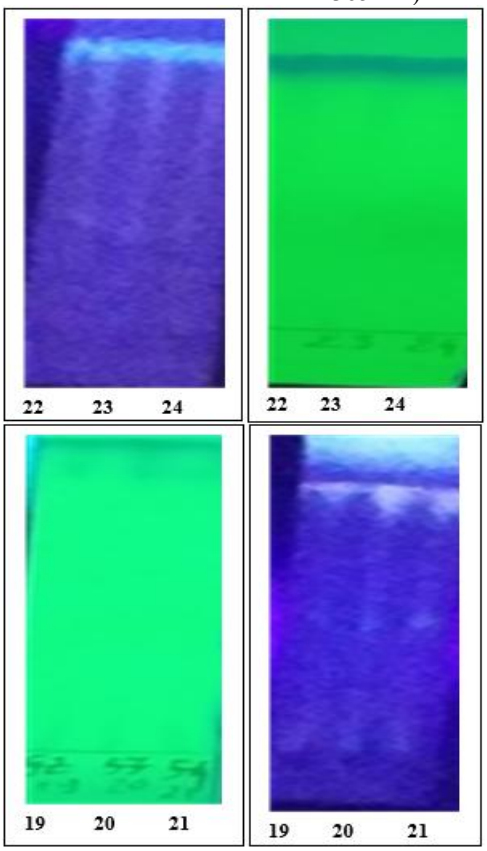


Figure 32: TLC profile of fractions(19-24) of rechromatography from silica column of aqueous layer of ethanolic extract of T. divaricata. (MP:CHCl3:MeOH [8:2] viewed in UV cabinet at 365 nm)

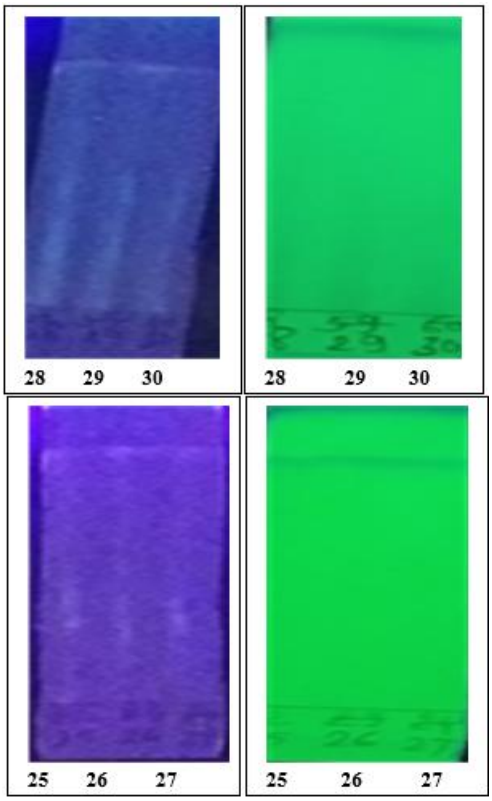
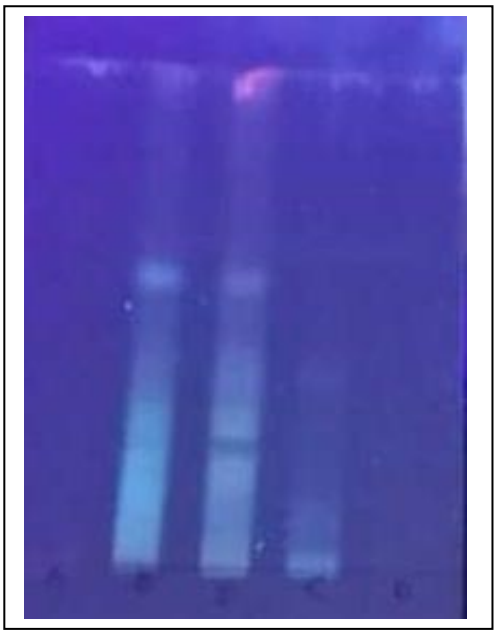


Figure 33: TLC profile of fractions(25-30) after processing fractions isolated from aqueous layer of ethanolic extract of T. divaricata. (MP:CHCl3:MeOH [8:2] viewed in UV cabinet at 365 nm)

4.13 TLC analysis of isolated and purified compunds of aqueous fraction of ethanolic extract of T. divarticate finalised for spectral characterization studies

Table 8: Isolated and purified componds with Rf value

Sr No.	Fractions	UV_{254}	UV_{365}	Rf
1	1-4	Grey	Bright blue	1
2	29-30	Grey	Bright blue	0.9,1
3	49-69	Grey	Bright white	1
4	77-84	Grey	Bright blue	1



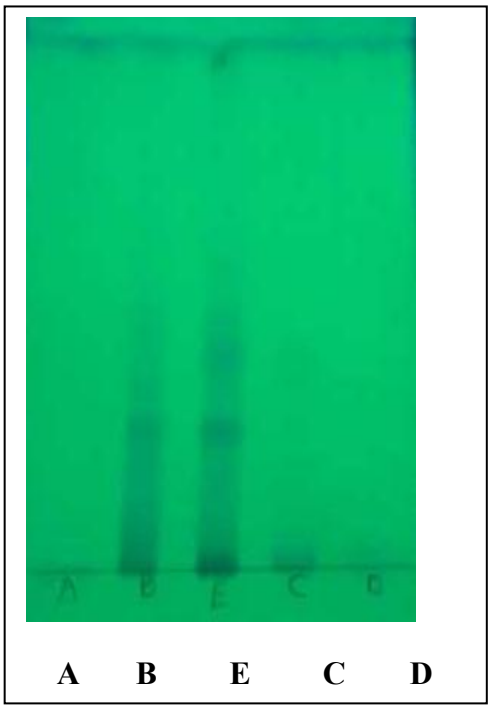


Figure 34: Photograph of TLC profile of isolated compounds from fractions of silica column (A: 1-4, B: 29-30, C: 49-69, D: 77-84, E: extract) of aqueous layer of ethanolic extract of T. divaricata viewed in UV (I-254nm, II-365nm) in mobile phase (Ethyl acetate:MeOH:Formic acid: Water [10:1:0.5:1])

4.14 TLC analysis of isolated fractions from aqueous layer of ethanolic extract of T. divaricata for obtaining purified compounds.

TLC analysis of isolated and purified compound

Compound 1 (Fraction 1-4)

Mixture of fractions (1-4) which already evaporated kept in refrigerator for freez drying. After freez drying chloroform amd methanol added in proportion (8:2) to solubilized dried fraction. Again the sample get evaporated untill dryness and we get purifed compound which prcoceed for spectral characterization.

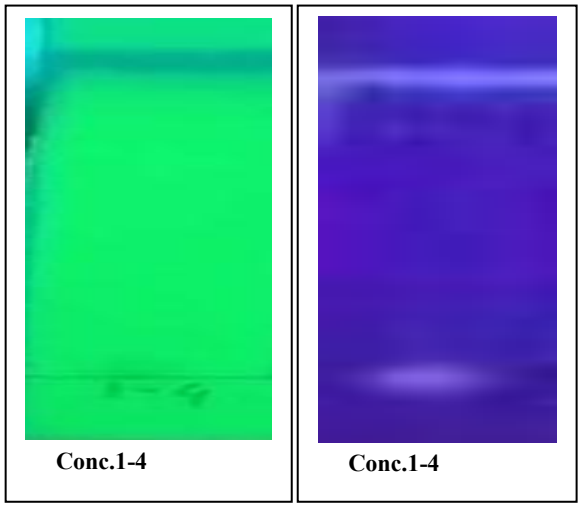


Figure 35: TLC profile of isolated and purified compound 1 viewed under 254 nm and 365nm.(mobile phase: CHCl3:MeOH [8:2]).

4.15 Spectral characterization of isolated and purified compounds using spectroscopic techniques.

UV Spectroscopic study

Determination of λmax

The sample solution was prepared by dissolving 1 mg of compounds in (1 ml) of chloroform as well as methanol as per there solubilty (10µg/ml). These solution was scanned one by one using 100 double beam spectrophotometer for the range (200-400) nm. The UV spectrum and wavelength of maximum absorption were recorded.

Fourier transform infra red (FT-IR) Spectroscopy

A FT-IR spectrum of compounds were taken by triturating sample with KBr in glass mortar (1:10) using fourier transform Infra red spectrophotometer (varian carry 640 IR) with diffuse reflectance principle. The spectrum was taken over a frequency range 4000-500 cm^{-1}.

Nuclear Magnetic Resonace (NMR) Spectroscopy

The structural elucidation of the compounds were conducted using proton and carbon NMR (^{1}H and C^{13} NMR). ^{1}H and C^{13} NMR spectra were recorded on Bruker 500 MHz in deuterated methanol (MeOD). Chemical shifts are reprted as values in ppm to TMS as an internal standard.

4.16 UV spectroscopic analysis of isolated and purified compound 1 from aqueous fraction of ethanolic extract of T. divaricata

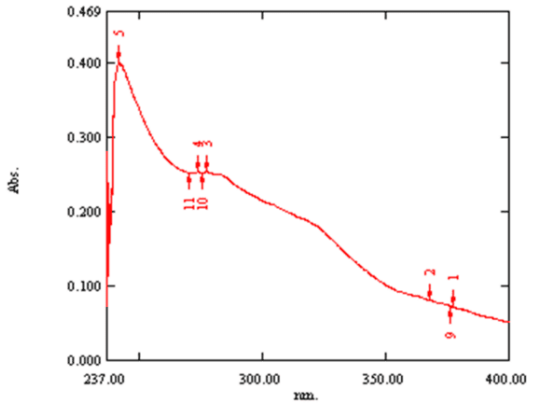


Figure 36: UV spectrum of isolated and purifed compund1 The Spectrum shows absorbance at λmax value at 238nm wavelength.

4.17 FT-IR spectroscopic analysis of isolated and purified compound 1 from aqueous fraction of ethanolic extract of T. divaricata

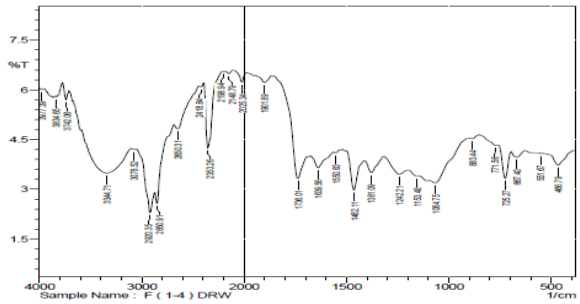


Figure 37: FT-IR spectrum of isolated and purified compound 1.

5.20 ^{1}H NMR spectroscopic analysis of isolated and purified compound 1 from aqueous fraction of ethanolic extract of T. divaricata

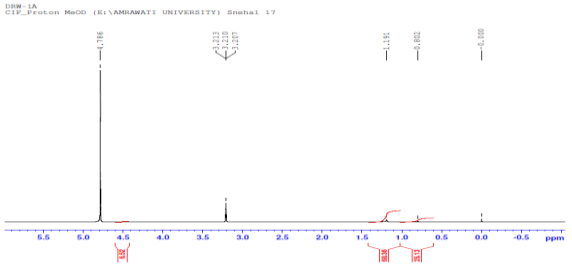


Figure 38: 1H NMR spectrum of isolated and purified compound 1 (in solvent DeOH) 500 Hz.

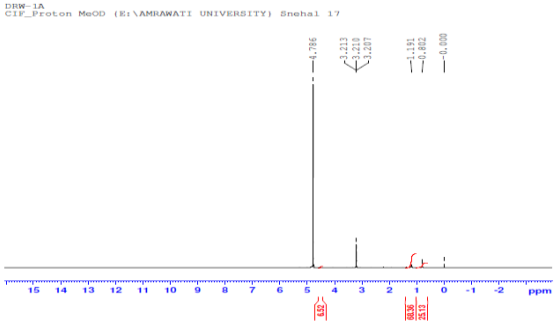


Figure 39: 1H NMR spectrum of isolated and purified compound 1 (in DeOH solvent) at 500 Hz.

4.18 ^{C13} NMR spectroscopic analysis of isolated and purified compound 1 from aqueous fraction of ethanolic extract of T. divaricata

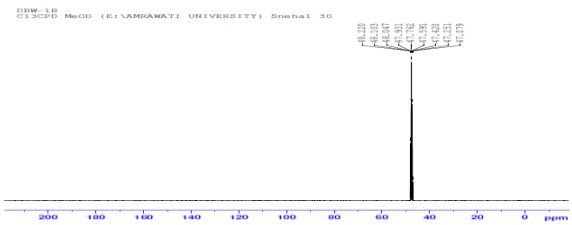


Figure 40: C13 NMR spectrum of isolated and purified compound 1 (in DeOH solvent) at 500 Hz.

4.19 TLC analysis of isolated and purified compound

Compound 2 (Fraction 49-69)

Mixture of fractions (49-69) was kept in refigerator after addition of chloroform for overnight. After overnight freezing of mixture next liquid liquid fractionationation performed adding chloroform, methanol. Methanolic fractionation seprated out and stored in glass bottle for spectral characterization studies.

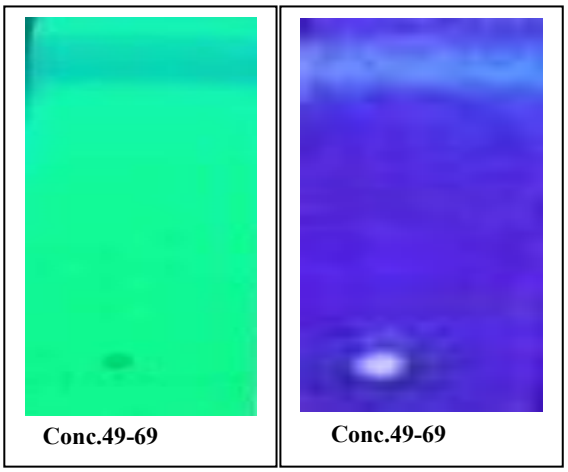


Figure 41: TLC profile of isolated and purified compound 2 viewed under 254 nm and 365nm.(Mobile phase: Ethyl acetate: MeOH: Water [7:2.5:0.5]).

4.20 UV spectroscopic analysis of isolated and purified compound 2 from aqueous fraction of ethanolic extract of T. divaricata

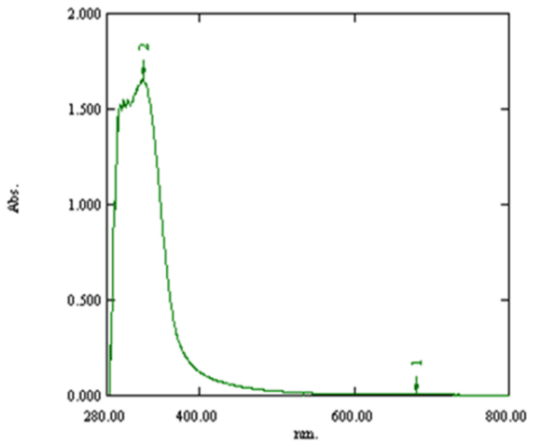


Figure 42: UV spectrum of isolated and purified compound 2

The Spectrum shows absorbance at λmax value of 350 nm wavelength.

4.21 FT-IR spectroscopic analysis of isolated and purified compound 2 from aqueous fraction of ethanolic extract of T. divaricata

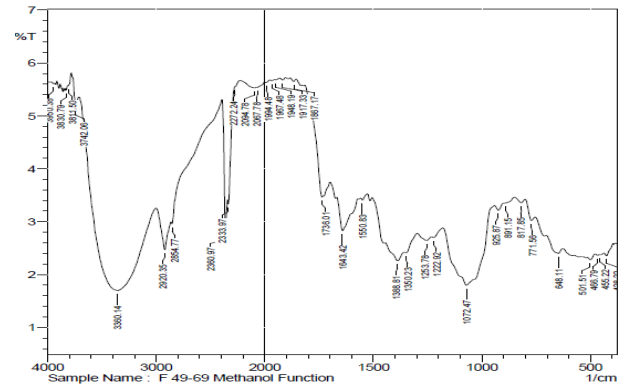


Figure 43: FT-IR spectrum of isolated and purified compound 2.

4.22 1 H NMR spectroscopic analysis of isolated and purified compound 2 from aqueous fraction of ethanolic extract of T. divaricata

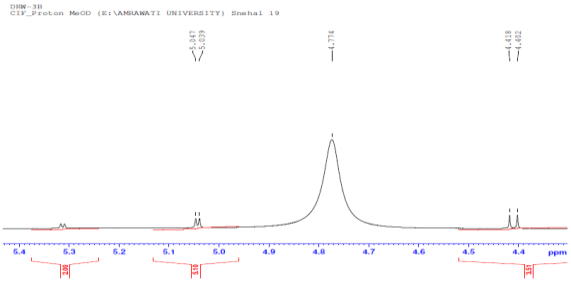


Figure 44: 1H NMR spectrum of isolated and purified compound 2 (in DeOH solvent) at 500 Hz.

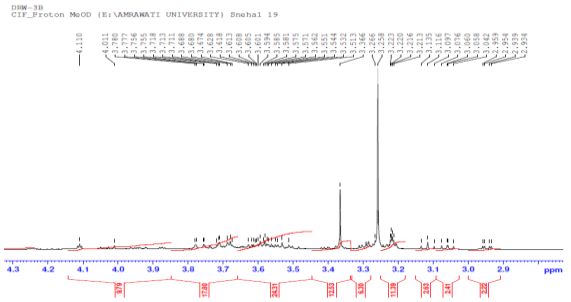


Figure 45: 1H NMR spectrum of isolated and purified compound 2 (in DeOH solvent) at 500 Hz.

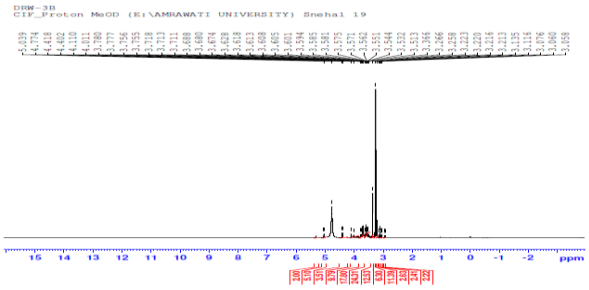


Figure 46: 1H NMR spectrum of isolated and purified compound 2 (in DeOH solvent).

4.23 C^{13} NMR spectroscopic analysis of isolated and purifed compound 2 from aqueous fraction of ethanolic extract of T. divaricata

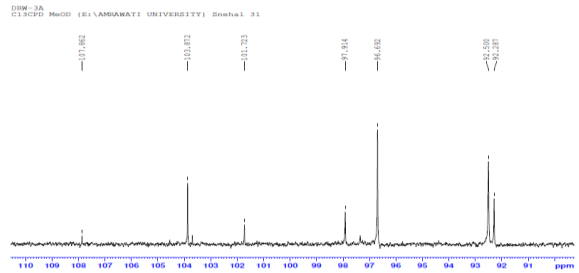
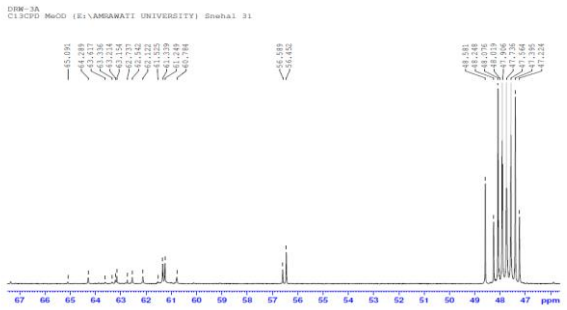


Figure 47: C13 NMR spectrum analysis of isolated and purified compound 2 (in DeOH solvent) at 500 Hz.



NMRFigure 48: C13 NMR spectrum analysis of isolated and purified compound 2 (in DeOH solvent)

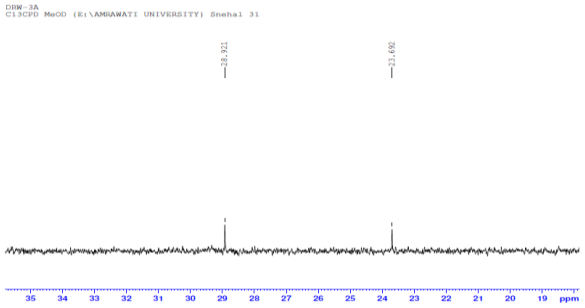


Figure 49: C13 NMR spectrum of isolated and purified comound 2 (in DeoH solvent) at 500 Hz.

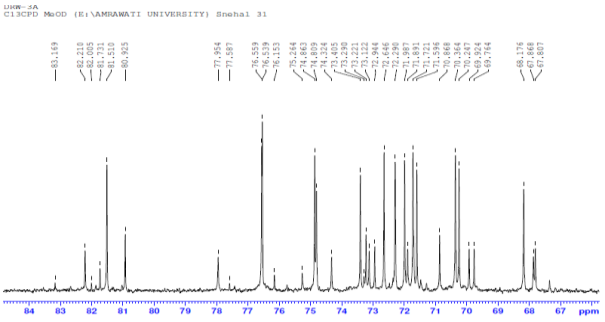


Figure 50: C13 NMR spectrum of isolated and purified compound 2 (in DeOH solvent) at 500 Hz.

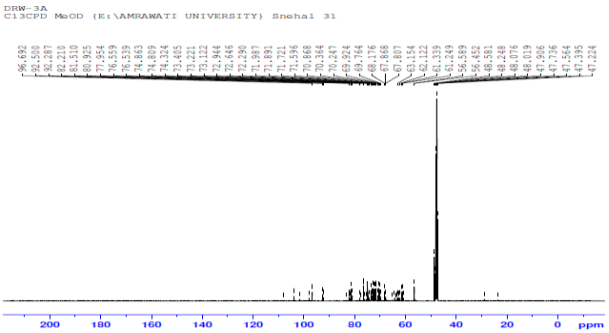


Figure 51: C13 NMR spectrum of isolated and purified of compound 2 (in DeOH solvent) at 500 Hz.

4.24 TLC analysis of isolated and purified compound

Compound 3 (Fraction 77-84)

Mixture of isolated fractions (77-84) produced crystalline compound at room temperature(38°C). Crystalline compound seprated out and kept in glass bottle for spectral caracterization studies.

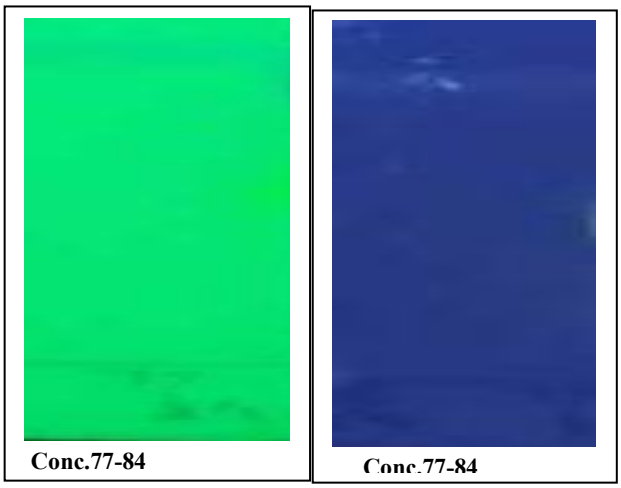


Figure 52: TLC profile of isolated and purified compound 4 viewed under 254 nm and 365nm. (Mobile phase: Ethyl acetate: MeOH: Water [7:2.5:0.5]).

4.25 UV spectroscopic analysis of isolated and purified compound 3 from aqueous fraction of ethanolic extract of T. divaricata

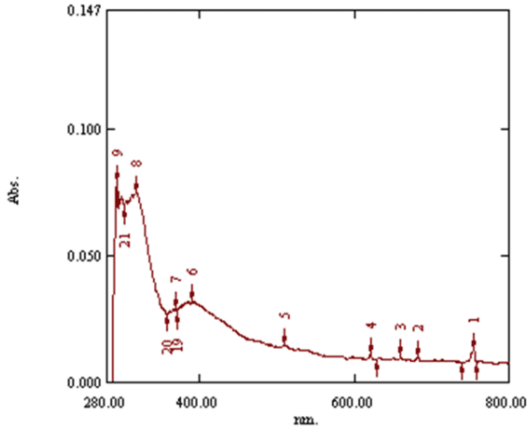


Figure 53: UV spectrum of isolated and purified compound 3

The Spectrum shows absorbance at λmax value of 290nm, 310nm wavelength

5.25 FT-IR spectroscopic analysis of isolated and purified compound 3 from aqueous fraction of ethanolic extract of T. divaricata

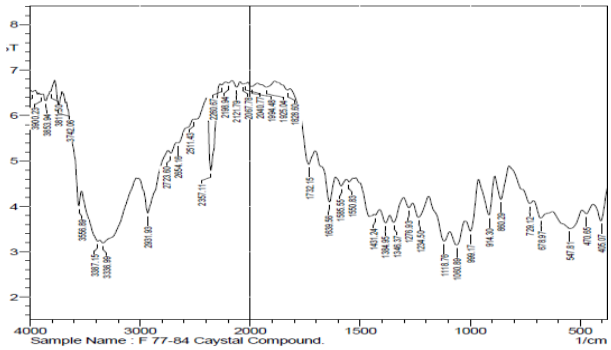


Figure 54: FT-IR spectrum of isolated and purified compound 3.

5.26 ^{1}H NMR spectroscopic analysis of isolated and purified comound 3 from aqueous fraction of ethanolic extract of T. divaricata

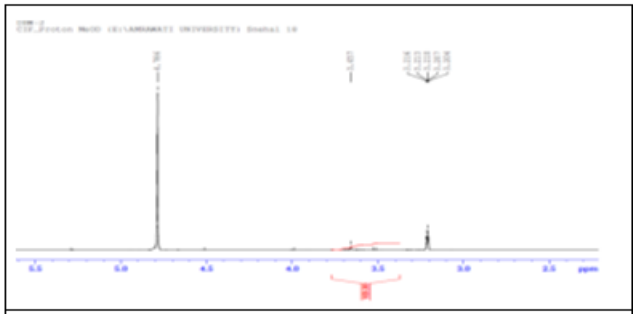


Figure 55: 1H NMR spectrum of isolated and purified compound 3 (in DeOH solvent) at 500 Hz.

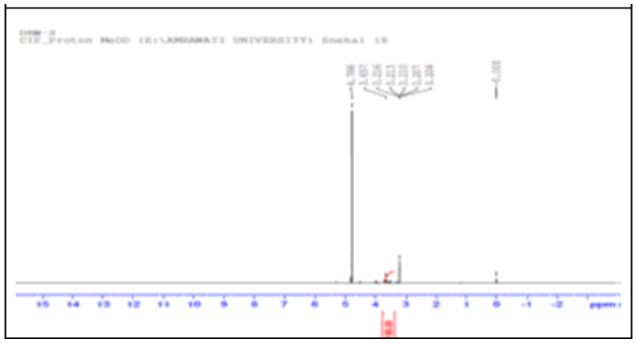


Figure 56: C13 NMR spectrum of isolated and purified compound 3 (in DeOH solvent) at 500 Hz.

5. Discussion

Tabernaemontana divaricata belongs to apocynaceae family contains secondary metabolites like alkaloids, tannins, saponins, flavonoids etc. [20, 24]

This work undergone through phytochemical evaluation studies. Evaluation parameter includes microscopy, macroscopy, powder microscopy, physicochemical evaluation and preliminary screening. This help to study plant profile and to predict primary or secondary metabolites present in it.

Microscopy of *T. divaricata* leaf part (transverse section) has shown lignified cells, cortical parenchyma, epidermal layer and crystals. In powder microscopy anisocytic stomata had observed.

Physicochemical evaluation parameter has shown loss on drying: 0.024, ash value (Total ash value: 0.88%, Water soluble ash value: 0.66%, Acid soluble ash value: 0.04%). pH: 6. Extractive value (alcoholic: 8%, water soluble: 94.8%). Preliminary phytochemical screening of alcoholic and aqueous extract of leaf part of *T. divaricata* has shown positive test for carbohydrates, alkaloids, tannins and phenolic compounds.

For isolation of compound, soxhlet type of extraction performed by using ethanol solvent. It is polar solvent in which almost phytocostituent get solubilized. Chloroform and methanol solvent used in this work to isolate compounds by

increasing proportion of methanol. Methanol is highly polar solvent by increasing methanol proportion polarity increased. The isolated compounds were identified by chromatographic and spectral characterization like UV, IR, NMR (^1H, ^{13}C) and mass spectroscopy.

Compound 1

The compound 1 isolated and purified from faction (1-4) of aqueous fraction of ethanolic extract of T. divaricata in mobile phase CHCl3: MeOH (99:1). It was greenish powder. Its UV spectrum report showed absorbance at λmax value 238 nm wavelength (Fig. 36).

FT-IR spectrum of compound 1(Fig. 37) showed presence of aliphatic primary amine band at 3344.71cm-1, alkene band at 2920.35cm-1, nitro compound band at 1550.83cm-1, aldehyde band at 1736.01cm-1, conjugated alkene band at 1639.56cm-1 suggesting presence of aliphatic compound.

1H NMR spectrum of compound1(Fig. 38) has shown solvent front (DeoH) signal at δ 4.786, and some other signals on upfield i.e. δ 3.213, δ 3.210, δ 3.207, δ1.191, δ 0.802 represent aliphatic proton.

C^{13} NMR spectrum of compound1 (fig.28) has shown solvent front (DeoH) signal at δ 47.62.There was absence of carbon signals.

Compound 2

The compound 2 isolated and purified from fraction (49-69) of aqueous fraction of ethanolic extract of T. divaricata in mobile phase $CHCl_3$: MeOH (80:20). It was pale brownish in colour. Its UV spectra report showed absorbance at λmax value 350 nm wavelength.

FT-IR spectrum of compound 2(Fig. 43) showed presence of aliphatic amine band at 3360.14cm-1, alkene band at 2920.35 cm-1, aldehyde band at 1736.01cm-1, cyclic alkene band at 1643.42cm-1, halo compound band at 891.85cm-1.

1H NMR spectrum of compound 1(Fig. 45) has shown solvent front (DeoH) signal at δ 3.551cm-1.There were some other signals on downfield that i.e. δ 3.581cm-1, 3.575cm-1 represent aromatic proton.

C13 NMR spectrum of compound 2(Fig. 47) has shown presence of number of signals like δ 107.862cm-1, δ 103.872cm-1, δ 101.723cm-1, δ 97.912cm-1, δ 96.692cm-1, δ 92.500cm-1, δ 92.287 cm-1. It showed complexity of compounds.

Compound 3

The compound isolated and purified from fraction (77-84) of aqueous fraction of ethanolic extract of T. divaricata of mobile phase $CHCl_3$: MeOH (75:25). It was obtained in crystalline form. UV spectrum report showed absorbance at λmax value 290 and 310 nm wavelengths.

FT-IR spectrum of compound 3(Fig. 54) has shown aliphatic primary amine band at 3387.15cm-1, alkane band at 2931.93cm-1, aromatic compound band at 2723.60cm-1, ester band at 1732.15cm-1, alkene band at 1639.56cm-1.

1H NMR spectrum of compound 3 (Fig. 55) has shown solvent front (DeoH) signal at δ 4.786 cm-1. Some other signals i.e. δ3.656 cm-1, δ 3.3210cm-1. 1H NMR spectrum has shown signals at δ 5.32 (1H, d, J= 4 Hz), [J -coupling constant, d- doublet]. At δ 4.42 (1H,d,J=0.016 Hz).

6. Summary

The present work undergone morphological characters, microscopy of leaf part (T.S), powder microscopy, physicochemical evaluation like loss on drying, ash value (acid soluble, water insoluble, total ash), pH, extractive value (alcoholic, water soluble) of coarse powder of leaf part of *T. divaricata*. Preliminary screening of powder of leaf part of T. divaricata plant was performed in both aqueous and alcoholic solvent.

Extraction of coarse powder of leaves of T. divaricata plant performed using ethanol as solvent through soxhlet apparatus. Liquid-Liquid fractionation of extract performed with n-hexane solvent and aqueous fraction separated out through separating funnel. Isolation of purified compounds performed with the help of column chromatography by using silica column. Chloroform, methanol used in silica column as mobile phase.

Spectral characterization of isolated and purified compound performed through UV, FT-IR, NMR (C^{13}, ^1H) spectrophotometric method and spectral data interpreted. UV spectra showed absorbance at λmax wavelength. FT-IR spectra showed presence of hydroxyl, carbonyl and ester stretching. But in case of NMR spectra, ^1H and C^{13} NMR has shown complexity of peaks which difficult to interpret compounds. However it may help in further studies in phytochemical evaluation of plant material.

7. Conclusion

This work was undergone to isolate, purify and characterize phytochemicals from the ethanolic extract of *T. divaricata* leaves. The extract of *T. divaricata* showed different fluorescent compounds as revealed by TLC studies. However, these compounds when isolated by silica column chromatography, they were separated in the mixture owing to their similar chemical and polarity nature. A compound from the fraction (1-4) was isolated in a pure state, however, its interpretation could not possible, might be due to absence of 1H- and 13C-NMR signals in the spectra. This compound was studied on preliminary level by UV-vis spectroscopy and FTIR spectroscopy suggesting that the compound is aromatic. This work may be useful to further carry out phytochemical study on the particular plant material.

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