Chapter -2

2. DEVELOPMENT OF METHODS FOR STANDARDIZATION OF SELECTED PLANTS

2. Experimental

Present section deals with detailed description of methods employed for carrying out different studies, categorized into following headings.

2.1. Collection and identification of plant material

Whole plants of *E. echinatus* were collected from the outfield of Gulbarga city, Karnataka, India and whole plants of *T. glaberrima* were obtained from the local market of Vadodara city Gujarat, India. The selected plant materials were authenticated in the Botany Department of The M. S. University of Baroda, Vadodara, India. Voucher specimens (No: Pharmacy/EE/05-06/01/SP; No: Pharmacy/TG/05-06/02/SP) have been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

2.2. Preparation of powdered material

The different parts of the plants (roots of *E. echinatus* and aerial parts of *T. glaberrima*) were first properly cleaned and then dried under shade. The shade dried plant materials were then subjected to size reduction using a mechanical pulverizer to coarse powder which was used in further studies.

2.3. Preliminary phytochemical studies

2.3.1. Preparation of extracts

Petroleum ether (60 - 80°) extract

The coarsely powdered shade dried plant material (100 g) was extracted with petroleum ether (60 – 80°) by hot extraction process (Soxhlet) for 4 hours. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo*.

Benzene extract

The marc left after petroleum ether extraction was dried in air and extracted with benzene by hot extraction process (Soxhlet) for 4 hours. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo*.

Chloroform extract

The marc left after benzene extraction was dried in air and extracted with chloroform by hot extraction process (Soxhlet) for 4 hours. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo*.

Ethyl acetate extract

The marc left after extraction with chloroform was dried in air and extracted with ethyl acetate by hot extraction process (Soxhlet) for 4 hours. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo*.

Methanol extract

The marc left after extraction with ethyl acetate was dried in air and extracted with ethanol (70%) by hot extraction process (Soxhlet) for 4 hours. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo*.

Aqueous Extract

The marc left after methanol extraction was dried and extracted with chloroform water by maceration process. After completion of extraction, the solvent was removed by evaporation and the residue was concentrated *in vacuo*.

Color, consistency, fluorescence behavior and percentage yield of the extracts were noted. Fluorescence behavior of the extracts was observed and recorded in daylight and under ultraviolet light. In brief to carryout the fluorescence studies, the extracts or fluorescent principle was adsorbed on Whatmann filter paper and observed under UV-visible light (Chase and

Pratt, 1949). The extracts were preserved under vacuum for further phytochemical studies.

2.3.2. Preliminary phytochemical analysis of successive extracts

Following qualitative chemical tests were carried out on vacuum dried successive extracts of the roots of *E. echinatus* and aerial parts of *T. glaberrima* to identify the presence of various chemical constituents (Kokate, 2005; Ansari, 2007; Houghton and Raman, 1998).

- **Test for alkaloids**
  - Mayer's reagent
  - Dragendorff reagent
  - Wagner's reagent
  - Hager's reagent
- **Test for steroids / terpenoids**
  - Liebermann Burchard test
  - Salkowaski test
- **Test for saponins**
  - Foam test
- **Test for glycosides (anthraquinones)**
  - Borntrager test
  - Modified Borntrager's test
- **Test for tannins and phenolic compounds**
  - Dilute ferric chloride solution
  - Lead acetate solution
  - Test with gelatin solution
- **Test for flavonoids**
  - Shinoda test (Mg/HCl test- Magnesium turnings test)
- **Test for fixed oils and fat**
  - Stain test
  - Saponification test
- **Test for carbohydrates**

- Molisch's test
- Fehling's test
- Benedict's test

- **Test for proteins and amino acids**
  - Biuret test
  - Ninhydrin test
- **Test for gums and mucilage**
  - Precipitation with alcohol
  - Molisch's test

2.3.3. TLC profile of the extracts obtained by successive solvent extraction

All the successive extracts of selected plant materials were subjected to TLC studies using various solvent systems (Table 2.1) to determine presence of various phytoconstituents. The R_f values of observed compounds were noted for all the extracts.

2.4. Standardization parameters for the selected plant materials

1. **Pharmacognostical parameters**

   Macroscopical studies

   Microscopical studies

   Determination of physico chemical constants

   Fluorescence studies

2. **Phytochemical parameters**

   HPTLC finger print profile for the methanol extracts of the selected plant materials.

   Determination of phenolic content

   Determination of the total flavonoid content

3. **Standards for in-organic elements including the heavy metals.**
4. **Isolation of genomic DNA and its finger printing for the selected plant materials.**

Table 2.1 Different solvent systems used for recording the TLC profiles of successive extracts of the selected plant materials.

Successive Extracts	Solvent system 1	Solvent system 2	Solvent system 3
E. echinatus			
Petroleum ether	**A**	**B**	**C**
Benzene	**A**	**D**	**B**
Chloroform	**A**	**E**	**B**
Ethyl acetate	**A**	**B**	--
Methanol	**F**	--	--
T. glaberrima			
Petroleum ether	**A**	**G**	**H**
Benzene	**A**	**G**	**I**
Chloroform	**A**	**I**	**J**
Ethyl acetate	**K**	**L**	**F**
Methanol	**L**	**F**	--

A. Toluene: chloroform: ethyl acetate (10:2:1)

B. Chloroform: methanol: formic acid (98:2:2)

C. Chloroform: methanol (9.6:0.2)

D. Chloroform 100%

E. Chloroform: ethyl acetate (9:1)

F. Butanol: glacial acetic acid: water (10:1:2.5)

G. Toluene: chloroform: methanol (0.5:0.3:0.7)

H. Toluene: ethyl acetate (93:7)

I. Toluene: ethyl acetate: diethyl amine (70:20:10)

J. Ethyl acetate: methanol: water (100:13.5:10)

K. Chloroform: methanol: ammonia (8:4:0.15)

L. Toluene: ethyl acetate: formic acid: methanol (6:6:1.6:0.4)

2.4.1. Pharmacognostical parameters

Macroscopical studies

Macroscopical examinations of the selected parts (roots of *E. echinatus* and aerial parts of *T. glaberrima*) of the plants were carried out using the reported methods in standard texts (Wallis, 1985; Mukherjee, 2002).

Microscopical studies

Microscopy of whole drug: The plant materials were preserved in a mixture of solvents containing formalin, acetic acid and alcohol (70 % v/v) (5:5:90) for microscopical studies; transverse sections (T.S) of the different organs of plant material were taken using a hand microtome, satisfactory sections were selected, stained with different stains and examined for the various components (Mukherjee, 2002). Permanent slides were prepared as per the method described by Johansen (1940) and microphotographs of the sections were made at different magnifications, depending upon the anatomical details to be brought out, using Olympus BX 40 microscope attached with Olympus DP12 digital camera.

Lignified elements: For observing the lignified tissues the sections were treated with a mixture of phloroglucinol-concentrated hydrochloric acid (1:1) on a slide, drain off the reagent and were mounted in glycerin-water mixture. Lignified elements take pink or red color (Lala, 1981).
Starch: The presence of starch was examined by treating the sections of the plant materials (or powder) with N/50 iodine solution. Starch grains stain blue color (Lala, 1981).

Microscopy of powdered crude material: For examining the characters of powder material, little amount of powder was placed in glycerin-water mixture on a slide and observed under microscope. The powder was pretreated, wherever necessary, with suitable clearing reagents (Lala, 1981;

Mukherjee, 2002) and the presence of different elements was examined by treating with different reagents as explained in the above section.

Proximate analysis

Crude drugs were subjected to determination of physico chemical constants using reported methods (Indian Pharmacopoeia, 1996; WHO, 1998). Following determinations were made.

i. Loss on drying: An excess of water in medicinal plant material will encourage microbial growth and deterioration following hydrolysis. Limits for water content should therefore be set for every given plant material. Loss on drying was determined as mentioned below.

Accurately weighed (5 g) plant material was placed in a weighing bottle which was previously dried and tared. The samples were dried at 100 - 105 °C until two consecutive weighing do not differ by more than 5 mg. The loss in weight was calculated with reference to the air dried plant material.

ii. Total ash: Controlled incineration of plant drugs results in an ash residue. It usually represents the mixture of inorganic salts and silica naturally occurring in the plant drugs and adhering to it, but it may also include inorganic matter added for the purpose of adulteration.

For determining the total ash about 2 g of the powdered drug was weighed accurately in a weighed silica crucible and spread as a fine layer at the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450 °C until free from carbon then cooled and weighed. The procedure was repeated till a constant weight was obtained. The percentage of the total ash was calculated with reference to the air-dried drug.

iii. Acid insoluble ash: The ash obtained above (total ash) was boiled with 25 ml of hydrochloric acid for 5 minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a weighed silica crucible, ignited, cooled and weighed. The

procedure was repeated to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

iv. Water soluble ash: The total ash (obtained as above) was boiled for 5 minutes with 25 ml of hot water. The insoluble matter was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a weighed silica crucible and ignited at a temperature not exceeding 450 °C. The procedure was repeated till a constant weight was obtained. The weight of the insoluble matter was deducted from the weight of the total ash. The difference in weight was considered as water soluble ash. The percentage of water soluble ash was calculated with reference to air dried drug.

v. Alcohol soluble extractives: Extraction of plant (drug) material with solvent yields a solution of different components and the composition of this solution will depend upon the constituents present in the drug and the solvent used.
Method: Powdered plant material (5 g) was macerated with 100 ml of methanol (90% v/v) in a closed flask for 24 hours. The contents of the flask were shaken for first six hours and then set aside for 18 hours. The contents were filtered and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 °C and weighed. The percentage of methanol soluble extractive was calculated with reference to the air dried plant material.

vi. Water soluble extractives: Method: Powdered plant material (5 g) was macerated with 100 ml of chloroform water in a closed flask for 24 hours. Contents of the flask were shaken for first six hours and then set aside for 18 hours. The contents were filtered, and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 °C and weighed. Percentage of water soluble extractive as calculated with reference to the air dried plant material.

2.4.2. Phytochemical parameters

2.4.2.1. HPTLC finger print profile for the methanol extracts of the selected plant materials

Three solvent systems of different polarity were developed for methanol extract of the selected plants so as to resolve polar, medium polar and non polar components in the extract on a HPTLC plate. The characteristic finger print profiles for various chemical constituents in each extract under UV light of different wavelengths (UV-254, UV-366) and after derivatization with suitable reagents were recorded on a CAMAG-HPTLC system.

Sample solutions

Powdered plant materials (10 g) of *E. echinatus* and *T. glaberrima* were extracted separately with methanol (3 X 50 ml) under reflux on a water bath for 30 minutes. Extracts were filtered, combined and concentrated to dryness. A stock solution (10 mg/ml) of each extract was prepared in methanol and was used for further studies.

Chromatographic conditions

Stationary phase : Precoated silica gel 60 F_{254} TLC plate (10 X 10 cm or 20 X 10 cm)(E. Merck, Cat. No.1.05554.0007)

Solvent system : See Table 2.2

Procedure

Suitably diluted stock solutions were spotted on TLC plates (Merck) with the help of CAMAG Linomat V applicator as 8 mm bands. Then the plates were developed to a height of 80 mm using different solvent systems in a twin trough chamber (which is previously equilibrated with vapours of mobile phase for a period of 20 minutes).

Table 2.2 Solvent systems used for recording the HPTLC finger print profiles of methanol extract of the selected plant materials.

Extract	Solvent system 1	Solvent system 2	Solvent system 3
Methanol Extract of *E. echinatus*	**A**	**B**	**C**
Methanol extract of *T. glaberrima*	**A**	**B**	**D**

A. Toluene: chloroform: ethyl acetate: acetic acid (10:2:1:0.4)

B. Toluene: ethyl acetate: formic acid: methanol (6:6:1.6:0.4)

C. Butanol: glacial acetic acid: water (10:1:2.5)

D. Toluene: ethyl acetate: di ethylamine (7:2:1)

Detection

The developed plates were dried in air and then scanned under UV light at 254 nm and 366 nm using TLC Scanner 3 (CAMAG). The plates were derivatized, wherever necessary, with suitable reagents (Stahl, 1969; Wagner, 1996) to detect the presence of specific chemical constituents and the characteristic peaks of the detected compounds were recorded at 540 nm. The photographs were taken with the help of Reprostar 3 (CAMAG) digital camera.

2.4.2.2. Determination of total phenolic content

Total phenolic content was determined according to the method described by Singleton and Rossi (1965).

Reagents: Gallic acid, Folin Ciocalteu reagent, 20% sodium carbonate, distilled water.

Preparation of test solution

Accurately weighed (2 g) coarse powder of the plant material was refluxed with (4 X 25 ml) methanol (95%) for 30 minutes on a water bath. The extracts were collected and the volume was reduced. Finally the volume was made up to 25 ml with methanol (95%).

Preparation of standard solution

A stock solution of the gallic acid containing 1 mg/ml was prepared by dissolving accurately weighed amount of standard gallic acid (Loba Chemie) in methanol. The stock solution was diluted further to get the solutions containing different concentrations of gallic acid (100 - 500 µg/ml).

Preparation of calibration curve

From the diluted stock solutions a suitable quantity (0.5 ml) was taken into a 25 ml volumetric flask, and 10 ml of distilled water and 1.5 ml of Folin Ciocalteu reagents were added. The mixture was allowed to stand for

5 minutes and then mixed with 4 ml of 20% sodium carbonate. Distilled water was then added to make the total volume 25 ml. The mixture was kept for 30 minutes and the absorbance of blue color developed was recorded on a spectrophotometer (Shimadzu UV 1700) at 765 nm against reagent blank.

Method

Similarly test solutions of the plant materials (0.5 ml) in triplicate were processed as above and the percentage of total phenolics was calculated from the calibration curve of gallic acid. Results were expressed as percentage of gallic acid.

2.4.2.3. Determination of the total flavonoid content

Flavonoids with various biological activities are considered as one of the key components in the plants. Therefore a quantitative determination of flavonoids was conducted according to the method described by Chang et al., (2002). The method includes the formation of acid stable complexes with C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols.

Reagents

Aluminum chloride (10%), Potassium acetate (1M), Quercetin (Hi Media), etc.

Preparation of test solution

Accurately weighed (2 g) coarse powder of the plant material was refluxed with (4 X 25 ml) methanol (95%) for 30 minutes on a water bath. The extracts were collected and the volume was reduced. Finally make up the volume to 25 ml with methanol (95%).

Preparation of standard solution

A stock solution containing 10 mg/ml was prepared by dissolving accurately weighed standard quercetin in methanol (95%). Solutions of different

concentrations (20, 40, 80, 160 and 320 µg/ml) were then obtained by diluting the stock solution with methanol.

Preparation of calibration curve

The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm on a spectrophotometer. A blank solution was prepared by substituting the aluminum chloride with same amount of distilled water.

Method

Similarly test solutions of the plant materials (0.5 ml) in triplicate were reacted with aluminum chloride as described above for calibration curve so as to determine the flavonoid content.

2.4.3 Estimation of in-organic elements including the heavy metals

Elemental content of the selected plant materials (roots of *E. echinatus* and aerial parts of *T. glaberrima*) were estimated on atomic absorption spectrophotometer at Vaibhav analytical laboratory, Ahmedabad.

Instrumentation

The atomic absorption spectrophotometer (AAS) (SYSTRONIC 128), coupled with hydride generator and hollow cathode lamps for different elements including heavy metals was used.

Chemicals and reagents

Concentrated HCl and concentrated HNO_3 of AR grade, distilled de-ionized water was used for all analytical work. All the glassware's were washed with 2% extraction solution (conc. HCl: conc. HNO_3 (10:3)), soaked in 3N HCl for 24 hours, and rinsed with distilled de-ionized water before use.

Table 2.3. Different standards used for estimation of various elements and their working range on AAS.

Metal/ Element	Standard's used	Working range (ppm)	Wavelength (nm)
Sodium	NaCl	20 – 80	766.5
Potassium	KCl	20 – 80	589.6
Iron	$Fe(NH_4)_2(SO_4)_2$	2 – 8	248.3
Copper	$CuSO_4$	1 – 5	324.7
Zinc	$ZnSO_4$	0.1 - 0.5	213.9
Manganese	$MnSO_4$	1 – 5	279.8
Lead	$PbNO_3$	2 – 8	217.0
Cadmium	$CdSO_4$	--	228.8
Arsenic	As_2O_3	--	Visible light

Preparation of test solutions

Accurately weighed (10 g) amount of the powdered plant material was digested by boiling with extraction solution, containing conc. HCl and conc. HNO_3 (10:3) for a period of 15 - 20 minutes. Filter and make up the volume of filtrate to 100 ml with distilled water. Further dilutions of the test solution, if required, were prepared to obtain the concentration of a particular metal in the working range of its standard metal.

Preparation of calibration curves

Standard solutions of the metals to be estimated were obtained commercially from E-Merck. The standard solutions were diluted sufficiently as per Indian Pharmacopoeia (1996) to obtain a solution of required concentration (Table 2.3).

2.4.4. Finger print profile of the isolated genomic DNA of selected plants

Based on the specificity of the genotype of a system, a particular DNA profile can be ascribed to a particular organism. This profile is as unique as a fingerprint; it is specific to that individual. This concept of fingerprinting has been increasingly applied in the past few decades to determine the ancestry of plants, animals and other microorganisms.

Genotypic characterization of plant species and strains is useful as most plants, though belonging to the same genus and species, may show considerable variation between strains. Additional motivation for using DNA fingerprinting on commercial herbal drugs is the availability of intact genomic DNA from plant samples even after they are processed. Fingerprinting of DNA is dictated by several factors; sequence or restriction site data, taxonomic level of study, the level at which the study is being done (species, genera, etc.), robustness and reproducibility of the method, effectiveness in terms of cost and time etc.

Plant material: Samples of fresh young, tender, un-bruised leaves of *E. echinatus* and *T. glaberrima* were collected in the early morning from the out skirts of the Baroda city and were used for isolation of the genomic DNA.

Reagents and solutions

1. Extraction buffer (EB): consisting of 2% hexa-decyl-trimethyl ammonium bromide (C-TAB) (w/v), 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidone.
2. A mixture of chloroform: iso-amyl alcohol ($CHCl_3$: IAA) (24:1).
3. CTAB solution: CTAB (10%) solution.
4. CTAB precipitation buffer (CPB): 1% CTAB, 50 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8).
5. Salt TE buffer (HTE): 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 1 M NaCl.
6. TE buffer (LTE): 1 mM Tris-HCl (pH 8), 0.1 mM EDTA (pH 8).
7. Ethanol: Ethanol (95% and 90%).
8. RNase solution: 3.5 mg/ml of bovine pancreatic RNase A

DNA extraction

All steps were carried out at room temperature unless stated otherwise; extraction was carried out as per the method described by Sangwan et al (1998).

Harvested leaves were rinsed with autoclaved water and dried by using a blotting paper. Leaves (2 g) were ground to a fine powder in presence of liquid nitrogen and then extracted in 3 volumes of EB by incubating at 65 °C for 30 minutes. Then it was subjected to centrifugation at 10,000 *X g* for 15 minutes, supernatant was collected and extracted with equal volumes of chloroform and iso-amyl alcohol mixture (24:1), upper aqueous phase was collected after centrifugation.

To the aqueous phase one-tenth volume of warm (55 °C) 10% CTAB solution was added and re-extracted with chloroform iso-amyl alcohol mixture as

above. To the clear aqueous phase at least 1.5 volume of CPB was added, mixed gently to observe precipitate which was collected by centrifugation. Pellet was dissolved in HTE and the DNA was precipitated by adding 2 volumes of ethanol. Pellet was again dissolved in LTE and incubated with 100 mg/ml of DNase free RNase A for one hour at 30 °C.

Then it was extracted with chloroform-iso-amyl alcohol mixture and one-tenth volume of 3 M sodium acetate was added. It was then precipitated with two volumes of ethanol; obtained pellet was washed with 90% ethanol, and dried under vacuum. The precipitate was re-hydrated in 100 – 200 µl of LTE for at least 1 hour.

Gel electrophoresis of the DNA

Requirements: Lambda DNA (Sigma chemicals, USA), marker DNA with 10 kb strand, agarose gel (0.5%) treated with ethydium bromide.

Method: The sample DNA in 10 mM Tris-HCl pH 8, 1mM EDTA, 20% (w/v) glycerol, and 0.5% (w/v) bromophenol blue were electrophoresed in 0.5% agarose gel. Gels that had been cast in 0.5X TBE buffer (40 mM Tris-borate pH 8.0, 1 mM EDTA) containing 0.25 µg/ml ethidium bromide, were run in the same buffer at 100 V for 2 hours. DNA samples were visualized and digitally recorded using a gel documentation system (Sambrook and Russel, 2001).

2.5. Results

2.5.1 Preliminary phytochemical analysis of *E. echinatus* and *T. glaberrima*

The percentage yield, color and consistency of the various successive extracts obtained were recorded (Table 2.4). The yield from polar solvents (methanol) was higher than that observed with non polar solvents (petroleum ether) for aerial parts of *T. glaberrima* where as, in case of *E. echinatus* the yield was almost equal. These extracts showed different colors when observed under day light and in UV light of different wavelength at 254 nm and 366 nm (Table 2.5).

2.5.2. Preliminary phytochemical screening

Preliminary phytochemical analysis revealed the presence of phytosterols, terpenoids and phenolic compounds as constituents in the roots of *E. echinatus* where as alkaloids, phytosterols, terpenoids, tannins, phenolic compounds and amino acids in aerial parts of *T. glaberrima*. The presence of carbohydrates and fatty materials and absence of anthraquinones was noticed in both the plants. Results are summarized in table 2.6.

2.5.3. TLC profiles of the extracts obtained by successive solvent extraction

The successive extracts were subjected to TLC to confirm the presence of phytoconstituents and the R_f values of the compounds were then recorded (Table 2.7 and 2.8).

2.5.4. Standardization parameters

2.5.4.1. Pharmacognostic parameters for *E. echinatus*

a. Macroscopic studies

The shade dried intact roots of *E. echinatus* (Figure 2.1) are 30 - 50 cm long with a diameter of 0.5 – 1 cm. Outer surface is grayish brown in color with

Table 2.4. Color, consistency and percentage yield of successive extracts of selected plant materials

Sl.No.	Solvents Used	Plant materials			
		Roots of *E. echinatus*		**Aerial parts of *T. glaberrima***	
		Color & Consistency	**Yield (% w/w)**	**Color & Consistency**	**Yield (% w/w)**
1	Petroleum ether (60-80°)	Brownish yellow; highly sticky, soft mass	5.04	Dark green; sticky soft semisolid mass	1.67
2	Benzene	Yellowish brown; semisolid	0.58	Light green; semisolid mass	1.10
3	Chloroform	Brown; hard semisolid mass	0.20	Light green; dry flakes	0.44
4	Ethyl acetate	Brown; semisolid mass, oily	0.33	Light green; soft semisolid mass	2.19
5	Methanol	Yellowish brown; soft semi solid	5.47	Dark greenish; soft semi solid mass	12.08
6	Water	Dark brown; dry flakes	1.57	Brown; dry flakes	6.54

Table 2.5. Fluorescence studies of successive extracts of the selected plant materials

Sl. No.	Solvent extracts	Roots of *E. echinatus*			Aerial parts of *T. glaberrima*		
			UV light			UV light	
		Day light	254 nm	366 nm	Day light	254 nm	366 nm
1	Petroleum ether	Yellowish brown	Yellowish green	Fluorescent green	Dark green	Dark green	Dark brown
2	Benzene	Yellow	Yellow	Yellowish brown	Light green	Light green	Brown
3	Chloroform	Light yellow	Light yellow	Brown	Light green	Light green	Brown
4	Acetone	Brown	Brown	Dark brown	Light green	Greenish yellow	Light brown
5	Alcohol	Yellowish	Yellowish	Light blue	Dark green	Greenish yellow	Yellowish brown
6	Water	Earthy brown	Light violet	Dark brown	Brown	Light green	Greenish yellow

Table 2.6. Qualitative chemical analysis of successive extracts of selected plant materials

Class of compounds	Plant materials											
	Roots of *E. echinatus*						Aerial parts of *T. glaberrima*					
	P	B	C	E	M	W	P	B	C	E	M	W
Alkaloids	-	-	-	-	-	-	-	-	+	+	+	-
Steroids/terpenoids	+	+	+	+	-	-	+	+	+	+	-	-
Saponins	-	-	-	-	-	-	-	-	-	-	+	-
Flavonoids	-	-	-	-	-	-	-	-	-	+	+	-
Phenolics & tannins	-	-	-	+	+	+	-	-	+	+	+	+
Carbohydrates	-	-	-	-	+	+	-	-		+	+	+
Proteins & amino acids	-	-	-	-	-	-	-	-	-	-	-	+
Fixed oils/fats	+	-	-	-	-	-	+	+	-	-	-	-

P : Petroleum ether extract
B : Benzene extract
C : Chloroform extract
E : Ethyl acetate extract
M : Methanol extract
W : Water extract
+ : Present
- : Absent

Table 2.7. TLC profile of the secondary metabolites detected in successive extracts of the roots of *E. echinatus*

Class of compound	Detection reagent	Solvent system	R_f values of the detected bands on TLC in various extracts				
			P	B	C	E	M
Phytosterols/ terpenoids	Anisaldehyde -H_2SO_4 reagent	A	0.06, 0.12, 0.16, 0.25, 0.31, 0.46, 0.51, 0.59, 0.64, 0.65, 0.68, 0.76, 0.83, 0.94	0.05, 0.08, 0.11, 0.15, 0.25, 0.30, 0.45, 0.63, 0.82, 0.95, 0.98	0.05, 0.73, 0.94	0.02, 0.04, 0.08, 0.16, 0.38, 0.76, 0.93	--
Phenolics & tannins	Alcoholic $FeCl_3$	B	--	--	--	0.23, 0.36, 0.95	0.5, 0.19, 0.30, 0.52, 0.58, 0.93

A. Toluene: chloroform: ethyl acetate (10:2:1) **B.** Butanol: glacial acetic acid: water (10:1:2.5)

P : Petroleum ether extract B : Benzene extract C : Chloroform extract
E : Ethyl acetate extract M : Methanol extract
+ : Present - : Absence

Table 2.8. TLC profile of the secondary metabolites detected in successive extracts of the aerial parts of *T. glaberrima*

Class of compound	Detection reagent	Solvent system	Extracts				
			P	B	C	E	M
Alkaloids	Dragendorff Reagent	**3, 5, 2** for C,E,M respectively	--	--	0.04, 0.10, 0.14	0.13	0.1, 0.2, 0.3
Phytosterols/ terpenoids	Anisaldehyde-H_2SO_4 reagent	**1**	0.35, 0.41, 0.5, 0.56, 0.58, 0.63, 0.72, 0.82, 0.91, 0.97	0.05, 0.21, 0.32, 0.36, 0.45, 0.5, 0.52, 0.68, 0.82, 0.97	0.15, 0.21, 0.25, 0.32, 0.39, 0.58, 0.72, 0.78, 0.85	--	--
Phenolics & tannins	5% Alcoholic $FeCl_3$	**2, 4, 5,** for M, C, E, respectively	--	--	0.06, 0.18, 0.30, 0.51	0.11, 0.35, 0.85	0.16, 0.37, 0.58, 0.63, 0.72

1. Toluene: chloroform: ethyl acetate (10:2:1) **2.** Butanol: glacial acetic acid: water (10:1:2.5) **3.** Toluene: ethyl acetae: diethyl amine (70:20:10) **4.** Ethyl acetate: methanol: water (100:13.5:10): **5.** Chloroform: methanol: ammonia (8:4:0.15)

P : Petroleum ether extract B : Benzene extract C : Chloroform extract

E : Ethyl acetate extract M : Methanol extract

+ : Present - : Absence

Figure 2.1. Roots of *Echinops echinatus* Roxb

long longitudinal wrinkles and small rootlets in the lower region. The wood is smooth and yellowish white.

b. Microscopic studies

Microscopic characters of the roots

Transverse section of *E. echinatus* root is circular in outline and shows the following regions (Figure 2.2).

Periderm: In young root the epidermis is single layered with unicellular hairs where as in old roots the epidermis is replaced with periderm. Periderm is 6 - 8 layers, thick and is not clearly distinguished into phellem, phellogen and phelloderm.

Cortex: Followed by periderm is a homogenous cortex comprising of 3 - 5 layers of thin walled large parenchymatous cells. The cortical cells are devoid of any cellular inclusions.

Endodermis: A distinct, single layer of endodermis separates the cortical region from vascular region and shows the presence of casparian thickening.

Vascular bundle: occupies more or less the central region and is separated into xylem and phloem by few layers of cambium. The xylem cylinder consists of patches of xylem included by parenchyma cells which are elongated thin walled and closely packed. Within the xylem patch medullary rays of 2 - 4 layers extend from the primary xylem to up to the outer phloem region. The xylem consists of few vessels and tracheids. Phloem cells are thin walled and are devoid of starch grains. Pericycle is made up of parenchyma cells.

Microscopic characters of the stem

The T.S of the stem is circular in outline and shows the following regions (Figure 2.3).

Epidermis: It is single layered and shows the presence of thick cuticle. The epidermal cells are cuboidal, occasionally uniseriate multicellular trichomes emerge out from the epidermal cells.

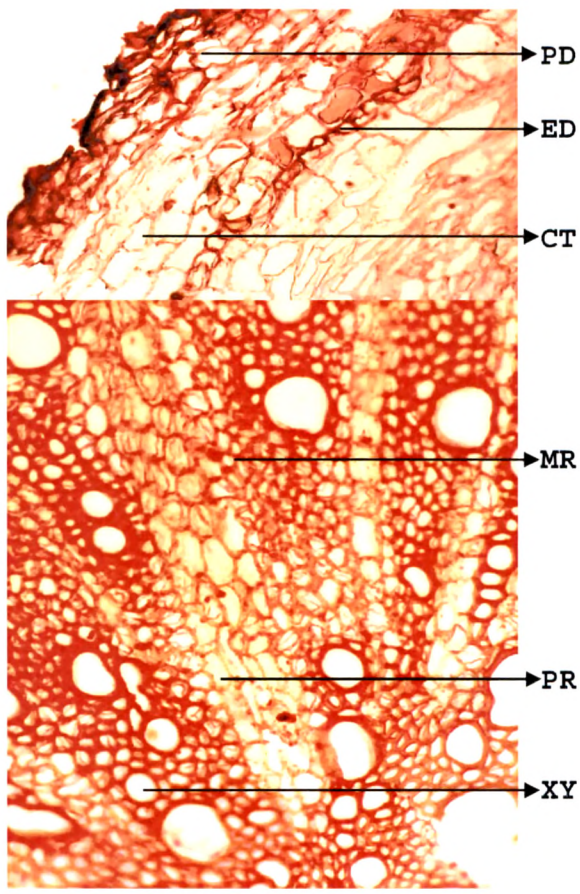


Figure 2.2. Microscopy of the roots of *E. echinatus* (X10) (PD, Periderm; ED, endodermis; CT, cortex; MR, bilayered medullary rays; PR, parenchyma inclusion; XY, secondary xylem).

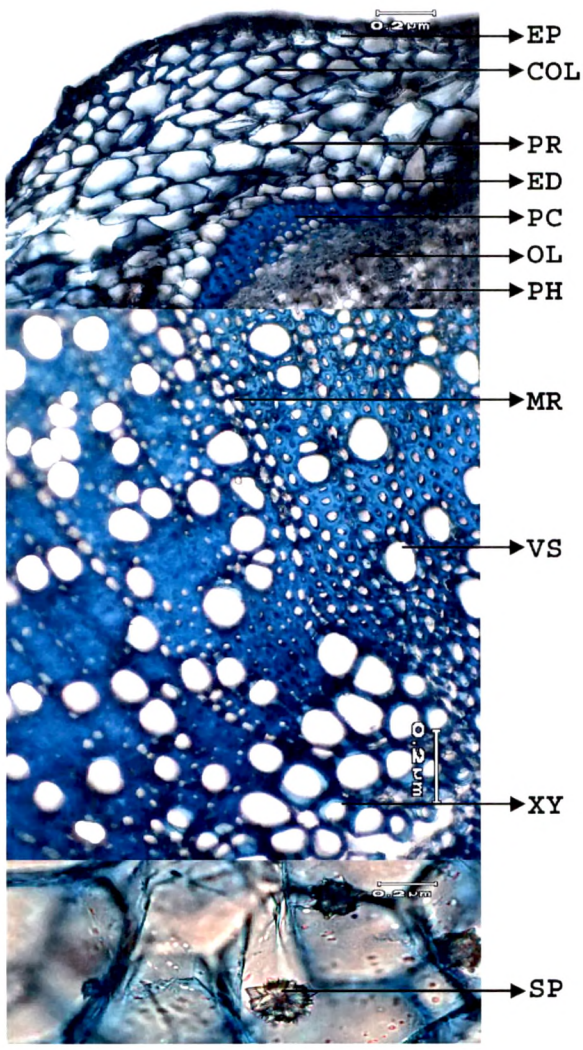


Figure 2.3. Microscopy of the stem of *E. echinatus*. (EP, epidermis; COL, collenchyma; PR, parenchyma; ED, endodermis; PC, pericycle; OL, oil globules; PH, phloem; MR, medullary rays; VS, vessels; XY, xylem (X10), SP, spheraphides (X40)).

Cortex: is heterogeneous having outer 4 - 5 layers of collenchyma cells and inner 3 - 4 layers of parenchyma cells. Parenchyma cells are compactly arranged.

Endodermis: separates the cortical region from the vascular region. Endodermis is single layered followed by a heterogeneous pericycle. Pericycle is made up of patches of sclerenchyma alternating with parenchyma cells. Parenchyma cells apparently show the presence of oil globules.

Vascular bundle: Phloem region shows the isolated sclerids, single leaf trace bundle separates out from the axial vasculature and traverse into the leaf base. Xylem forms a continuous cylinder it consists of vessels of large lumen, tracheids and xylem parenchyma.

Medullary rays: 1 - 2 layers of medullary rays traverse from the primary xylem to up to the outer phloem region. Primary vascular bundles are distinct and 19 - 20 in number.

Pith: comprises of small rounded compactly arranged parenchyma cells. Large number of pith cells shows the presence of spheraphides.

Microscopic characters of the leaf

Transverse section of the leaf shows dorsiventral nature of the leaf. Following are the important tissues in the lamina and the midrib regions (Figure 2.4).

Lamina: Upper epidermis is single layered with rectangular cells having a thick cuticle on outer walls. Lower epidermis is identical to upper epidermis. A number of uniseriate, multicellular trichomes cover the entire lower region of the lamina.

The uniseriate trichomes are very long with all the cells collapsed except the 4 - 6 cells at the base and close to the epidermis. Mesophyll is differentiated into upper palisade and lower spongy parenchyma.

Midrib: The midrib region is 3 - 5 ridged. Each ridge shows the presence of a vascular bundle. Below the upper epidermis and above the lower epidermis a patch of 4 - 5 layered collenchyma is seen. Rest of the midrib region is

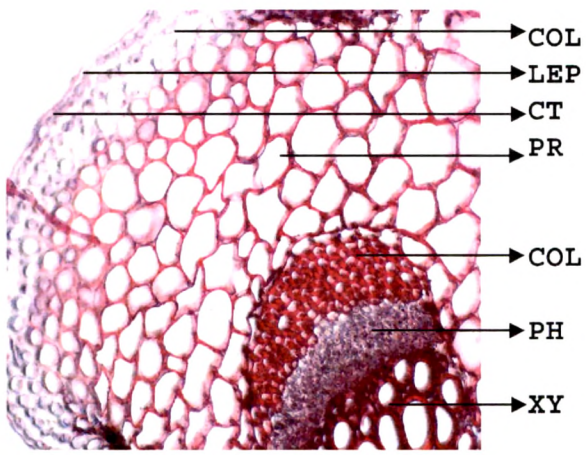


Figure 2.4. Microscopy of the leaf of *E. echinatus*. (COL, collenchyma; LEP, lower epidermis; CT, cuticle; PR, parenchyma; PH, phloem; XY, xylem (X 10)).

covered by rounded parenchyma cells having intracellular spaces. Vascular bundles are embedded in the parenchymatous tissue; each vascular bundle is caped on either side by a patch of sclerenchyma. Vascular bundles are endarch in nature.

Powder study of the roots of *E. echinatus*

The various diagnostic characters of the root powder are depicted in figure 2.5.

Cork: Thick rectangular cells placed in radial serration with thick suberin deposition.

Vessel elements: Large number of vessel elements either in entire or fragmented form showing various types of thickening like helical, simple pitted, bordered alternate pitting are found, though helical thickening predominates among all.

Tracheids: Occasionally few xylem parenchyma cells and tracheids creep in the powder.

2.5.4.2. Pharmacognostic parameters for *T. glaberrima*

a. Macroscopic studies

Various macroscopical features of the aerial parts of *T. glaberrima* are described in chapter 1, section 1.4.2. The shade dried intact roots are brown in color with wrinkled surface, 20 - 30 cm long and 0.3 – 0.6 cm in diameter, internal surface is white-cream colored.

b. Microscopic studies

Microscopic characters of the roots

Transverse section of *T. glaberrima* root is circular in outline and shows the following regions (Figure 2.6).

Periderm: The outermost cortical region is replaced by cork. It consists of 2 - 3 layers of tangentially elongated cells filled with brownish matter. Phellogen is 3 - 4 layered, immediately below the cork; cells are tangentially elongated and slightly compressed due to pressure exerted by secondary tissues formed inside.

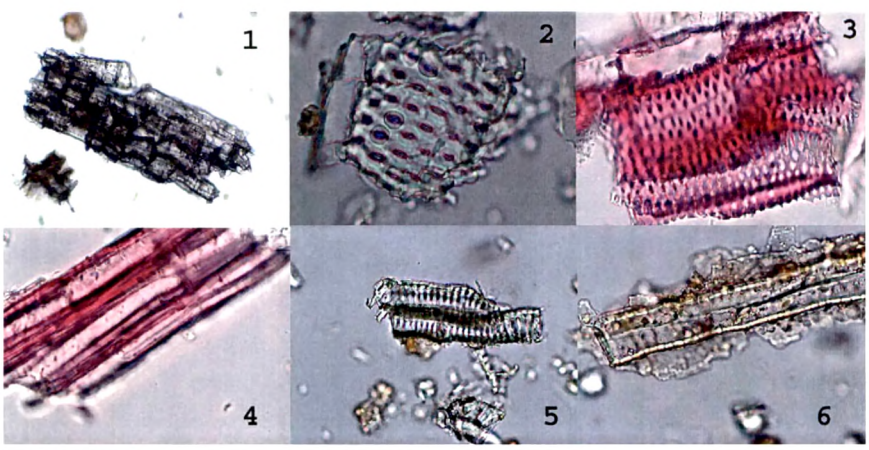


Figure 2.5. Diagnostic features for the powder microscopy of the roots of *E. echinatus* (X40). (1) cork cells; (2) vessels with alternate pitting; (3) vessels with simple pitting; (4) tracheids; (5) vessels with helical thickening; (6) xylem parenchyma.

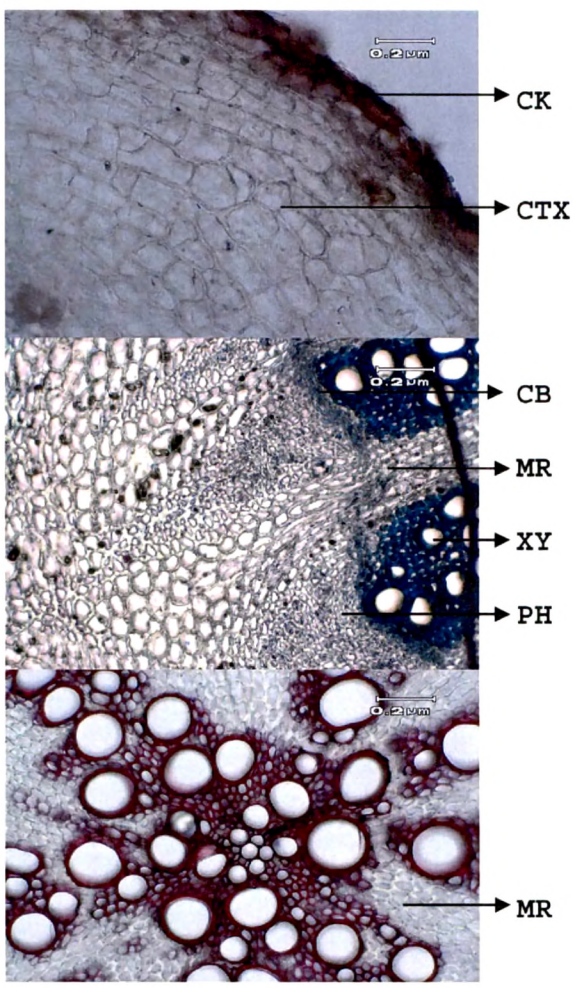


Figure 2.6. Microscopy of the roots of *T. glaberrima* (X10) (CK, cork; CTX, cortex; CB, cambium; MR, medullary rays; XY, secondary xylem; PH, phloem).

Cortex: Followed by periderm is a homogenous layer of cortex, comprising 6 - 8 layers of thin walled polyhedral parenchyma with small intercellular spaces. The cortical cells are devoid of any cellular inclusions.

Vascular bundle: The primary xylem bundles have tapering arms of protoxylem and centrally located large metaxylem. The secondary vascular cylinder appears in the form of ridges and furrows and does not lead to formation of complete cylinder. Large amount of parenchymatous cells are produced which leads to the development of discrete secondary vascular bundles with very wide parenchymatous medullary rays (8 - 10 layers), medullary cells intrude the vascular cylinder.

Cambium: Few layers of cambium separate the xylem and phloem.

The xylem rays form ribbon like sheets of tissue traversing radially like secondary xylem and are continuous through the cambium into secondary phloem.

Microscopic characters of the stem

T.S of the stem shows ridges and furrows. Following are the different regions of the T.S (Figure 2.7).

Epidermis: Outline of the stem shows the presence of ridges and furrows. Epidermis is single layered with very thick cuticle followed by 7 - 8 layers of collenchyma below the ridges and furrows.

Chlorenchyma: followed by collenchyma are 3 - 4 layers of Chlorenchyma.

Vascular bundles: around 12 - 13 collateral, discrete vascular bundles are arranged in a ring form. The vascular bundles are endarch in nature and caped by sclerenchymatous pericyclic fibers. Endodermis is not very distinct, vessel elements are radially arranged one above the other. Secondary growth does not lead to the formation of a complete cylinder of vascular tissue and shows the presence of lignified parenchyma between the vascular bundles. Parenchymatous tissue facing towards the pith region of the vascular bundle also appears to be lignified.

Pith: is large made up of round parenchymatous cells.

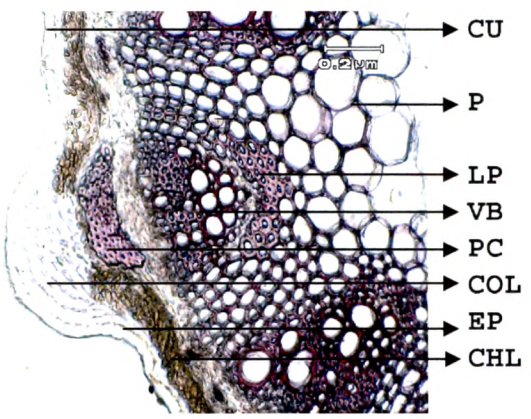


Figure 2.7. Microscopy of the stem of *T. glaberrima* (X10). CU, cuticle; P, pith; LP, lignified parenchyma; VB, vascular bundle; PC, pericycle; COL, collenchyma; EP, epidermis; CHL, Chlorenchyma.

Microscopic characters of the leaf

T.S shows isobilateral nature of the leaf. Following are the important tissues in the lamina and the midrib regions (Figure 2.8).

Lamina

Epidermis: It is single layered with rounded cells, the outer walls of which are covered by a cuticle. Uniseriate multicellular trichomes are present on the lower epidermis. The size of trichome cells reduce from the base to the tip.

Mesophyll: A distinct differentiation of mesophyll into palisade and spongy parenchyma can be seen. Palisade is divided into upper palisade (3 layers) and lower palisade (2 layers), with rod shaped compactly arranged cells, the length of palisade cells increases towards the spongy tissue. Spongy parenchyma consists of 2 - 3 layers of loosely arranged parenchyma cells showing the presence of lignified vascular strands. Palisade tissue extends into the midrib region.

Midrib The mesophyll tissue extends almost till midway of the midrib. A single collateral vascular bundle is placed in the central portion traversing the midrib with the phloem facing towards the lower epidermis. The vascular bundle is caped by collenchymatous tissue on its either side. Surrounding the vascular bundle is rounded thin walled parenchyma. Two layers of collenchymatous cells are present below upper and above the lower epidermis, thus collenchyma occurs on both dorsal and ventral sides.

Surface preparation shows the presence of anisocytic stomata, each stomata is surrounded by 4 - 5 subsidiary cells. The subsidiary cells appear similar to the associated epidermal cells which are of same size with straight walls or slightly arched.

Powder study of the aerial parts of *T. glaberrima*

Vascular elements: Large number of vessel elements either in entire or fragment form showing all kinds of thickening like helical, annular and pitted are found, though helical thickening predominates (Figure 2.9).

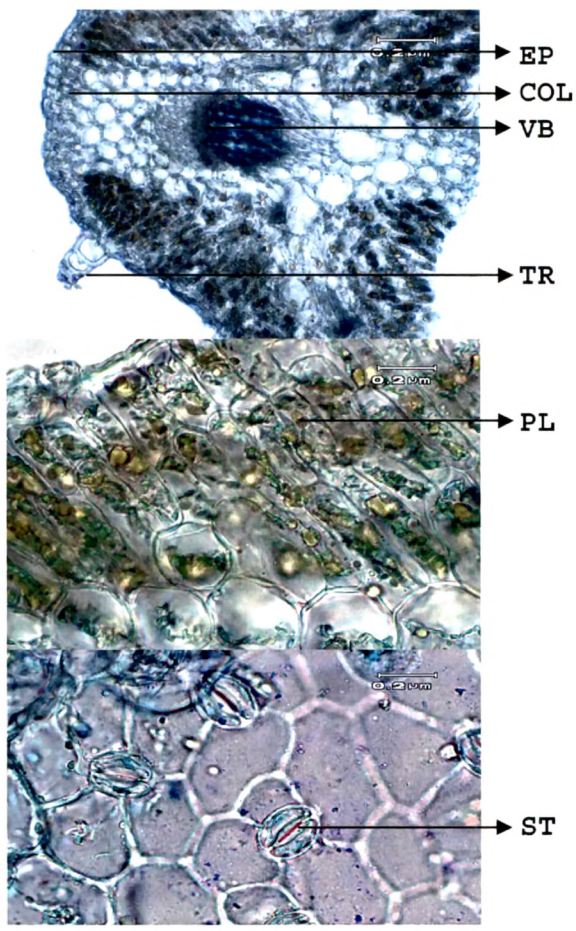


Figure 2.8. Microscopy of the leaf of *T. glaberrima*. EP, epidermis; COL, collenchyma; VB, vascular bundle; TR, trichome (X10). PL, palisade (X40). ST, stomata (X40).

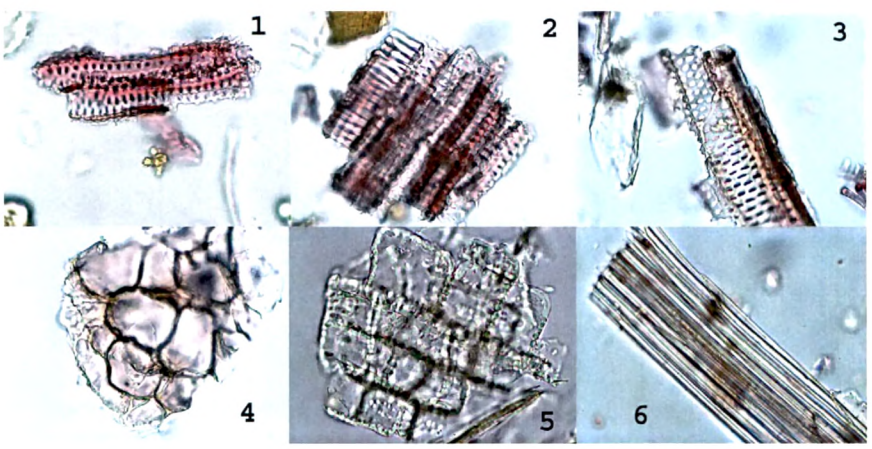


Figure 2.9. Diagnostic features for the powder microscopy of aerial parts of *T. glaberrima* (X40). 1-3, vascular elements; 4, cortical parenchyma; 5, parenchyma with plasmodesmata; 6, fibers.

Parenchyma: cortical parenchyma consisting of thin walled polyhedral cells with intra cellular spaces. Typical parenchymatous cells showing the presence of plasmodesmata are seen occasionally.

Fibers: long non lignified fibers of uniform thickness appear in bundle form or occasionally in single.

Tracheids: Fragments of tracheids with linear pits are common in the powder.

Trichomes and anisocytic stomata associated with epidermis are seen rarely in the field.

2.5.4.3. Proximate analysis

Various physico chemical constants viz., ash values, extractive values and moisture content were determined for both the roots of *E. echinatus* and the aerial parts *T. glaberrima;* the values are expressed as the mean of triplicate readings and are depicted in histograms (Figure 2.10 and 2.11).

2.5.4.4. Fluorescence studies

A number of crude drugs contain substances, which shows fluorescence in UV-visible range, this characteristic of the crude drug is used as a means of their identification. Fluorescent properties exhibited by the powder of both the plant materials viz., *E. echinatus* and *T. glaberrima* are recorded under day light and in UV light at 254 nm and 366 nm (Table 2.9).

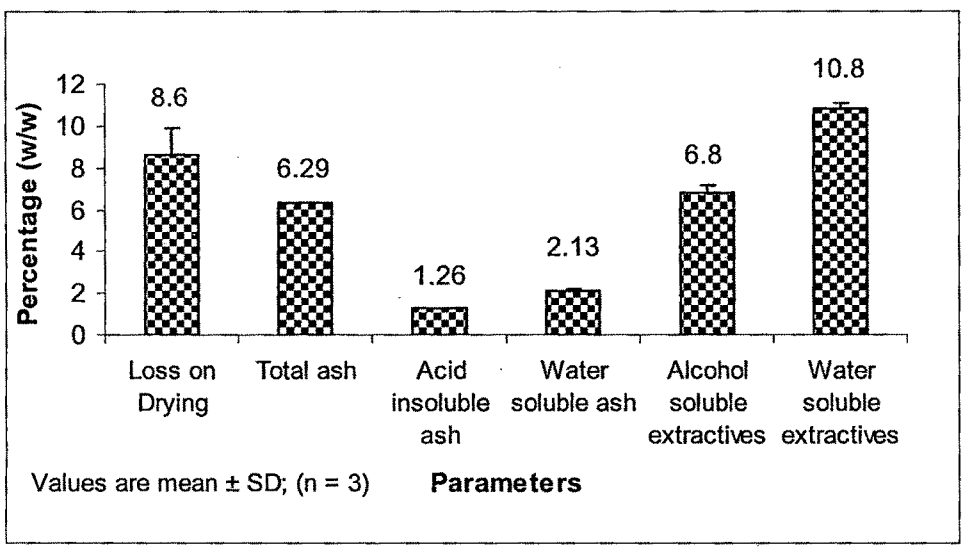


Figure 2.10. Physico chemical constants for the roots of *E. echinatus*.

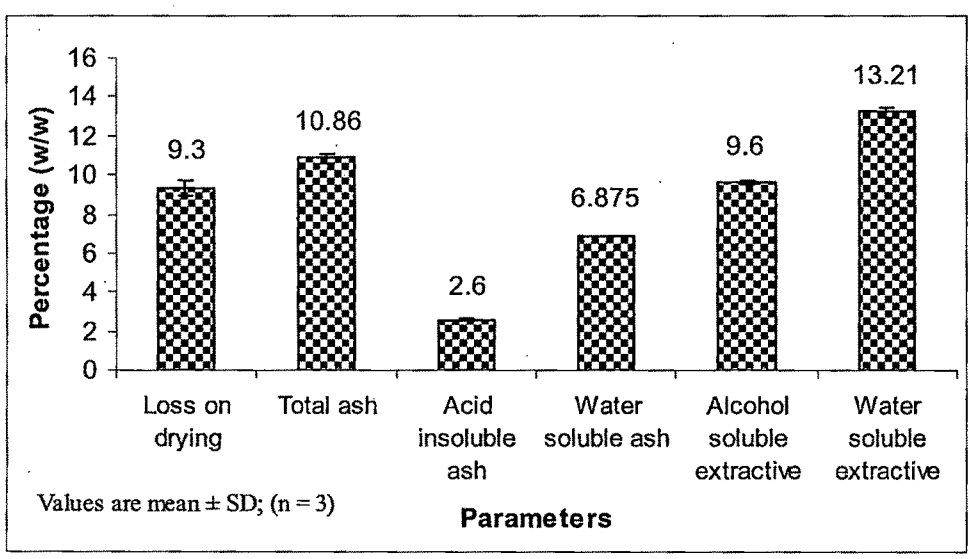


Figure 2.11. Physico chemical constants for aerial parts of *T. glaberrima.*

Table 2.9. Fluorescence analysis of the powder material of *E. echinatus* roots and *T. glaberrima* aerial parts

Sl. No.	Treatment	Roots of *E. echinatus*			Aerial parts of *T. glaberrima*		
			UV light			UV light	
		Day light	254 nm	366 nm	Day light	254 nm	366 nm
1	Powder as such	Light brown	Brown	Brown	Grayish green	Yellowish green	Brown
2	Powder in methanol	Light brown	Brown	Yellowish brown	Light yellow	Light green	Brick red
3	Powder + 1 N NaOH (Aqueous)	Reddish brown	Greenish brown	Blackish brown	Yellow	Green	Yellowish brown
4	Powder + 1 N NaOH (Alcoholic)	Light yellow	Yellow	Greenish yellow	Light yellow	Light green	Brick red
5	Powder + 1 N HCl	Brownish cream	Light brown	Yellowish brown	Light yellow	Light green	Yellowish green
6	Powder + 50% H_2SO_4	Blackish brown	Green	Brown	Green	Dark green	Blackish brown

2.5.4.5 Phytochemical parameters

a. HPTLC finger print profile for the methanol extracts of selected plants

Preliminary phytochemical screening showed the presence of terpenoids, phytosterols and phenolic compounds in *E. echinatus* and alkaloids, terpenoids and phenolic compounds in *T. glaberrima*, all of which can be extracted using methanol as solvent. Therefore total methanol extract containing the above constituents was prepared from both the plant materials and used for finger print studies. Compounds of varying polarity in the methanol extract were separated using various solvent systems on TLC. The HPTLC finger print profile comprising of typical spectra, R_f values, UV λ_{max} and relative percentage of the separated compounds were then recorded.

In case of methanol extract of *E. echinatus*, solvent system 1 (Table 2.2) was used to resolve non polar compounds and the separated compounds (steroids and terpenoids) were detected by derivatization with anisaldehyde sulphuric acid (Table 2.10; Figure 2.12). Where as solvent system 2 and 3 (Table 2.2) were used to resolve medium polar and polar compounds respectively and the separated compounds (mainly phenolics) were detected by derivatization with alcoholic $FeCl_3$ (Table 2.11; Figure 2.13 and 2.14).

In case of *T. glaberrima*, solvent system 1 (Table 2.2) was used to resolve non polar compounds and the separated compounds (phytosterols and terpenoids) were detected by derivatization with anisaldehyde sulphuric acid (Table 2.12; Figure 2.15). Solvent system 2 (Table 2.2) was used to resolve polar compounds (phenolics) and the separated compounds were detected by derivatization with alcoholic $FeCl_3$ (Table 2.13; Figure 2.16). Solvent system 3 was used to resolve the alkaloids which were detected by derivatization with Dragendorff reagent (Table 2.14; Figure 2.17). In all the cases, after derivatization, the plates were scanned densitometrically at 540 nm (Figure 2.18 and 2.19).

Table 2.10. R_f values, relative percentage and absorption maxima of the separated compounds in methanol extract of the roots of *E. echinatus*.

Peak	Solvent 1							
	254 nm			366 nm			540 nm	
	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %
1	0.01	1.64	295	0.05	12.42	653	0.06	22.18
2	0.05	1.84	209	0.09	1.66	697	0.13	3.82
3	0.19	4.43	211	0.14	0.88	697	0.23	12.14
4	0.23	4.35	660	0.39	7.12	655	0.38	8.98
5	0.49	4.15	--	0.47	1.65	693	0.79	18.48
6	0.64	11.45	700	0.56	16.75	698	0.92	11.41
7	0.79	17.94	699	0.65	21.61	697	0.97	22.99
8	0.92	54.20	359	0.70	3.95	694	--	--
9	--	--	--	0.93	33.96	366	--	--

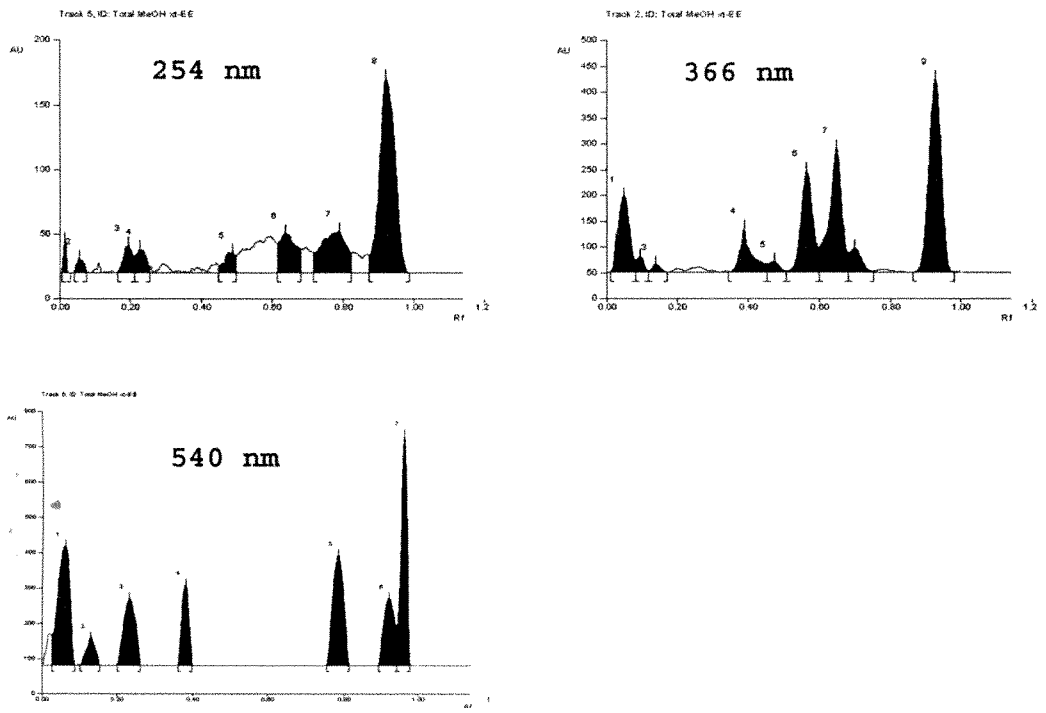


Figure 2.12. HPTLC chromatograms of methanol extract of the roots of *E. echinatus* in solvent system 1 after densitometric scan under UV 254 nm & 366 nm and at 540 nm.

Table 2.11. R_f values, relative percentage and absorption maxima of the separated compounds in methanol extract of the roots of *E. echinatus*.

Peak	Solvent 2								Solvent 3			
	254 nm			366 nm			540 nm		254 nm		540 nm	
	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.07	13.45	331	0.03	0.72	--	0.09	20.14	0.04	3.26	0.04	2.96
2	0.17	35.4	328	0.11	2.99	329	0.18	34.48	0.16	7.70	0.14	6.65
3	0.24	30.5	332	0.22	4.11	330	0.27	29.98	0.23	9.30	0.24	7.54
4	0.61	7.47	634	0.31	4.21	467	0.40	1.62	0.44	29.86	0.39	10.87
5	0.66	9.07	322	0.53	9.69	216	0.43	1.72	0.67	36.85	0.43	16.47
6	0.94	4.11	--	0.58	6.51	220	0.64	5.11	0.87	10.50	0.69	39.82
7	--	--	--	0.64	11.33	318	0.69	4.63	0.95	2.53	0.91	15.69
8	--	--	--	0.67	12.77	322	0.97	2.31	--	--	--	--
9	--	--	--	0.75	14.86	219	--	--	--	--	--	--
10	--	--	--	0.86	10.91	660	--	--	--	--	--	--
11	--	--	--	0.90	9.25	655	--	--	--	--	--	--
12	--	--	--	0.95	12.64	366	--	--	--	--	--	--

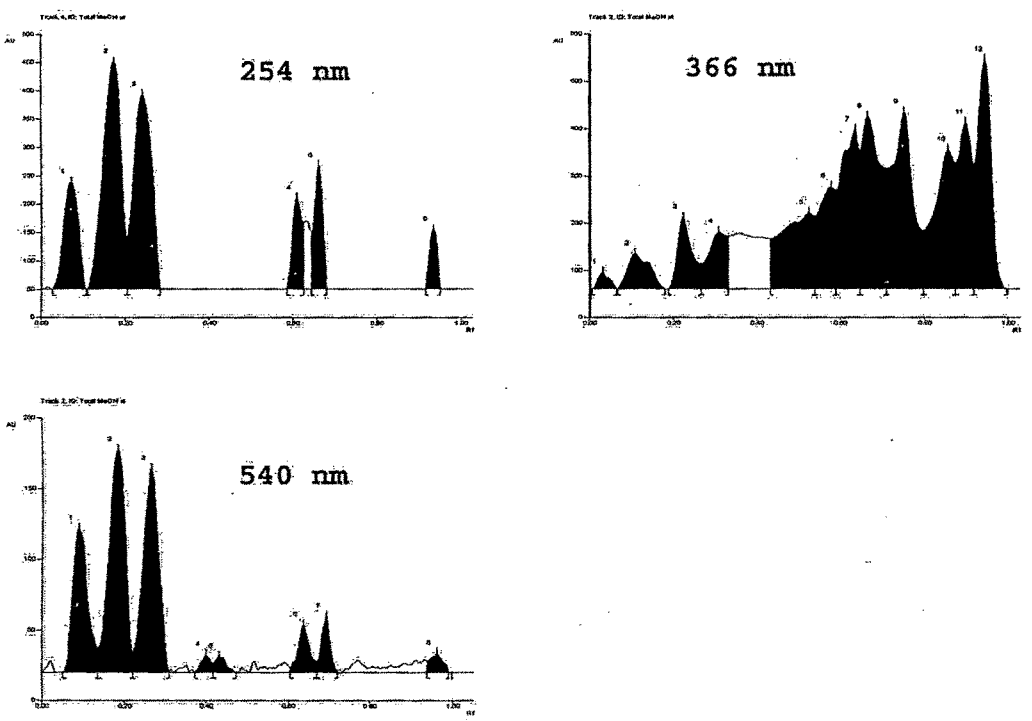


Figure 2.13. HPTLC chromatograms of methanol extract of the roots of *E. echinatus* in solvent system 2 after densitometric scan under UV 254 nm & 366 nm and at 540 nm.

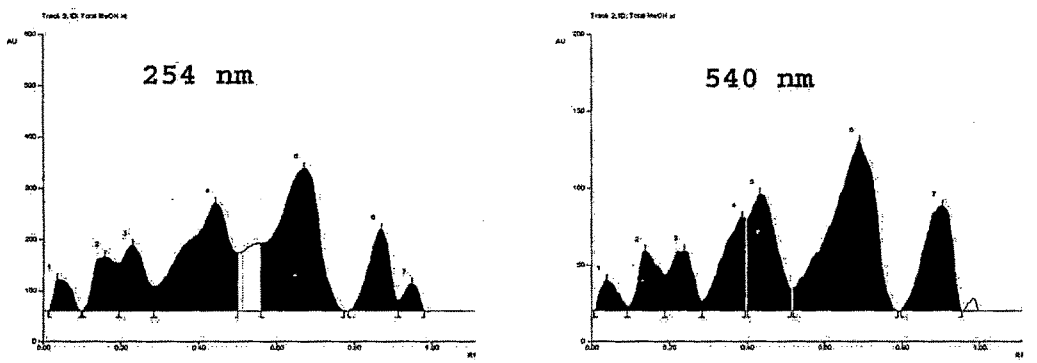


Figure 2.14. HPTLC chromatogram of methanol extract of *E. echinatus* roots in solvent system 3 after densitometric scan under UV 254 nm & 366 nm and at 540 nm.

Table 2.12. R_f values, relative percentage and absorption maxima of the separated compounds of the methanol extract of the aerial parts of *T. glaberrima*.

Peak	Solvent 1							
	254 nm			366 nm			540 nm	
	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %
1	0.02	6.49	288	0.01	5.46	289	0.02	5.84
2	0.06	0.33	342	0.07	7.02	341	0.14	1.79
3	0.14	5.46	323	0.10	5.55	344	0.18	0.29
4	0.17	5.61	350	0.14	4.65	323	0.30	16.83
5	0.22	14.70	357	0.20	6.39	352	0.32	8.88
6	0.28	20.89	295	0.32	17.02	290	0.37	10.85
7	0.31	16.76	290	0.39	24.99	200	0.43	11.89
8	0.38	3.95	415	0.47	7.22	200	0.45	8.38
9	0.45	3.56	200	0.52	10.63	409	0.49	14.65
10	0.48	0.83	200	0.56	5.28	413	0.58	2.12
11	0.52	4.95	409	0.71	2.81	200	0.66	0.91
12	0.57	0.94	413	0.96	2.98	200	0.88	2.32
13	0.90	2.34	200	--	--	--	0.90	2.75
14	0.97	13.19	200	--	--	--	0.98	12.50

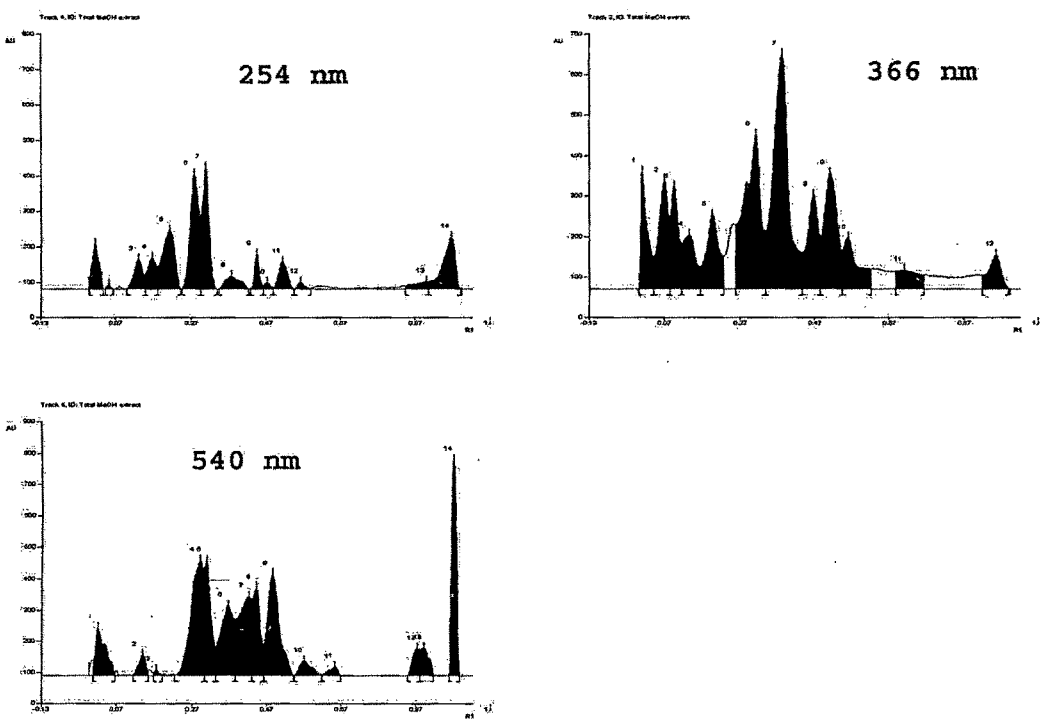


Figure 2.15. HPTLC chromatograms of methanol extract of the aerial parts of *T. glaberrima* aerial in solvent system 1 after densitometric scan under UV 254 nm & 366 nm and at 540 nm.

Table 2.13. R_f values, relative percentage and absorption maxima of the separated compounds of the methanol extract of the aerial parts of *T. glaberrima*.

Peak	Solvent 2							
	254 nm			366 nm			540 nm	
	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %
1	0.13	6.74	254	0.02	3.84	700	0.12	10.60
2	0.19	1.32	200	0.25	8.33	587	0.32	2.52
3	0.31	0.79	--	0.40	11.57	587	0.65	51.25
4	0.40	4.48	308	0.46	13.28	587	0.69	34.72
5	0.53	14.02	312	0.56	15.15	587	0.99	0.91
6	0.59	15.72	358	0.61	3.01	700	--	--
7	0.62	18.40	341	0.64	0.20	700	--	--
8	0.69	14.96	200	0.68	8.36	587	--	--
9	0.87	13.45	200	0.75	6.44	587	--	--
10	0.91	10.11	200	0.83	8.72	587	--	--
11	--	--		0.89	13.87	700	--	--
12	--	--		0.92	7.22	700	--	--

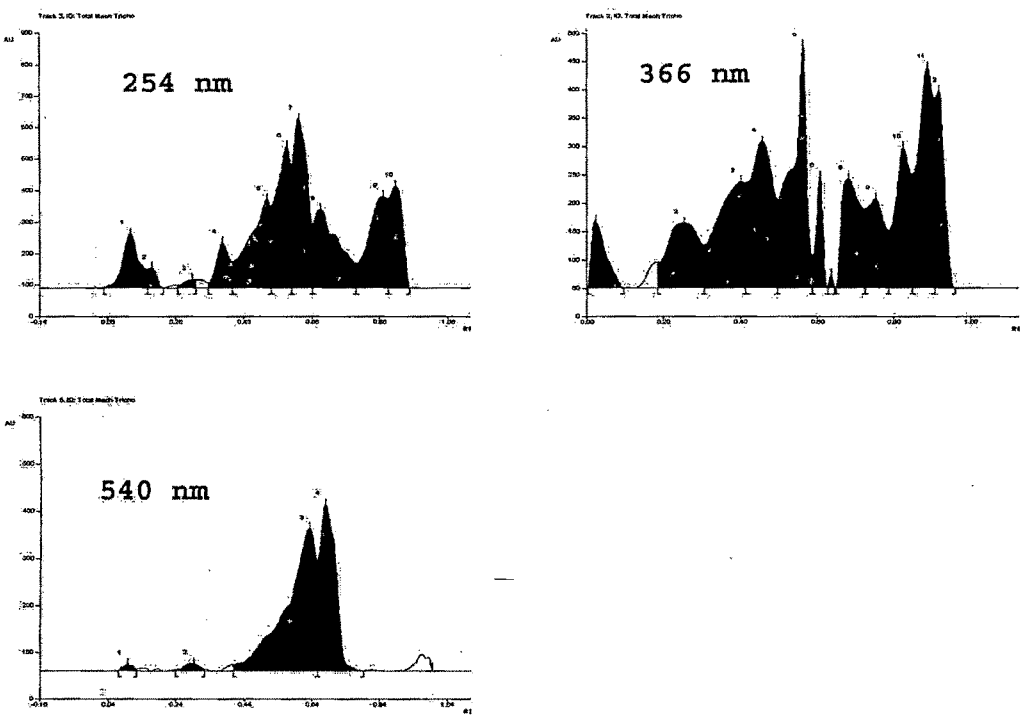


Figure 2.16. HPTLC chromatograms of methanol extract of the aerial parts of *T. glaberrima* in solvent system 2 after densitometric scan under UV 254 nm & 366 nm and at 540 nm.

Table 2.14. R_f values, relative percentage and absorption maxima of the separated compounds of the methanol extract of the aerial parts of *T. glaberrima*.

Peak	Solvent 3							
	254 nm			366 nm			540 nm	
	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %	UV λ_{max}	Max R_f	Relative %
1	0.03	5.96	660	0.11	0.59	--	0.12	5.16
2	0.11	0.71	260	0.18	5.34	180	0.21	21.16
3	0.15	0.90	242	0.24	9.0	154	0.29	1.75
4	0.17	0.64	174	0.33	0.63	115	0.35	4.74
5	0.23	2.51	333	0.39	2.01	96	0.51	1.87
6	0.35	3.14	98	0.46	6.61	100	0.54	3.99
7	0.43	4.89	174	0.51	5.76	98	0.65	12.08
8	0.65	2.08	127	0.65	8.81	129	0.71	20.36
9	0.68	3.07	262	0.67	6.67	270	0.82	25.64
10	0.74	12.67	288	0.74	17.20	297	0.91	1.90
11	0.80	10.85	156	0.86	36.42	393	0.94	1.35
12	0.86	21.60	392	0.94	0.97	527	--	--
13	0.94	30.99	481	--	--	--	--	--

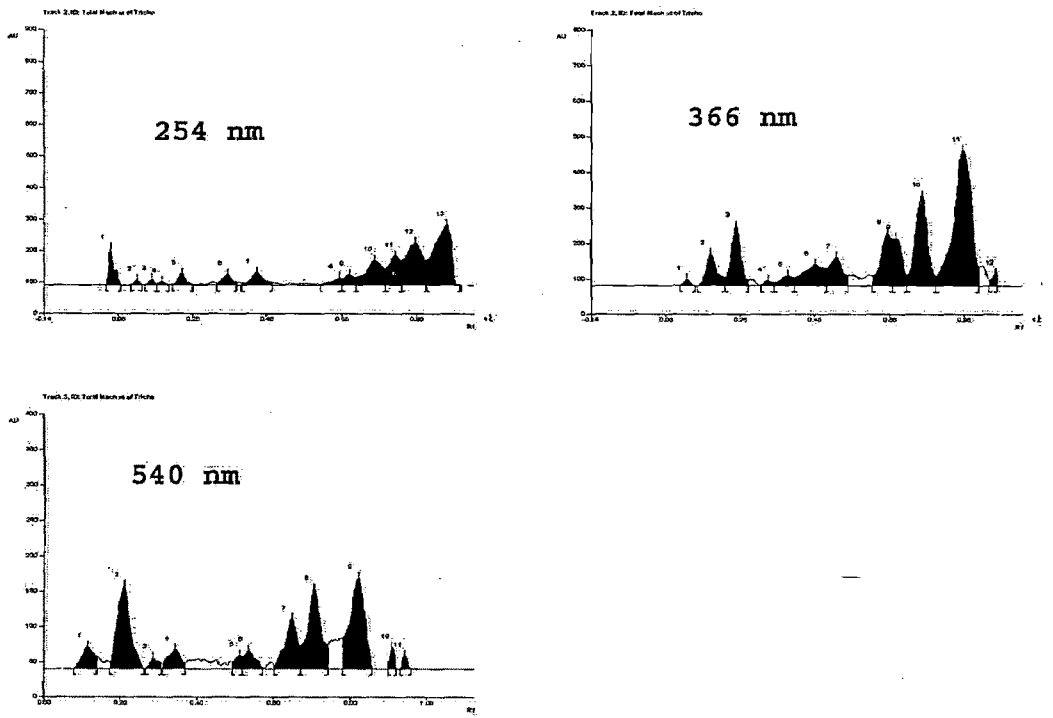


Figure 2.17. HPTLC chromatograms of methanol extract of the aerial parts of *T. glaberrima* in solvent system 3 after densitometric scan under UV 254 nm & 366 nm and at 540 nm.

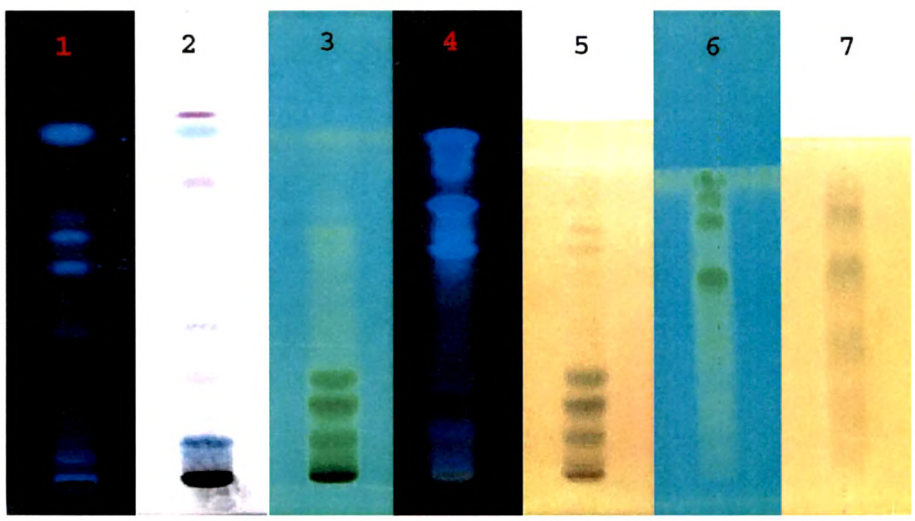


Figure 2.18. Separation of components in methanol extract of the roots of *E. echinatus* on HPTLC layers using different solvents.

1-2: Solvent system A

3-5: Solvent system B

6-7: Solvent system C

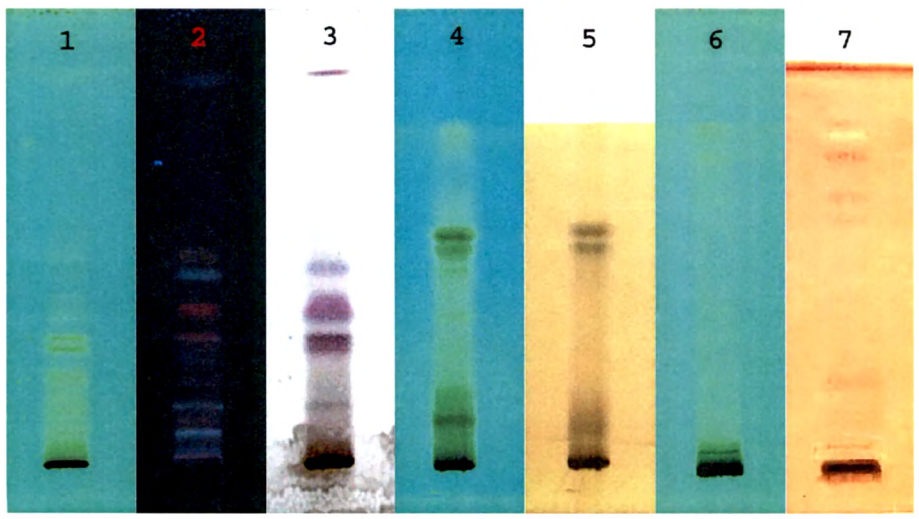


Figure 2.19. Separation of components in methanol extract of the aerial parts of *T. glaberrima* on HPTLC layers using different solvents.

1-3: Solvent system A

4-5: Solvent system B

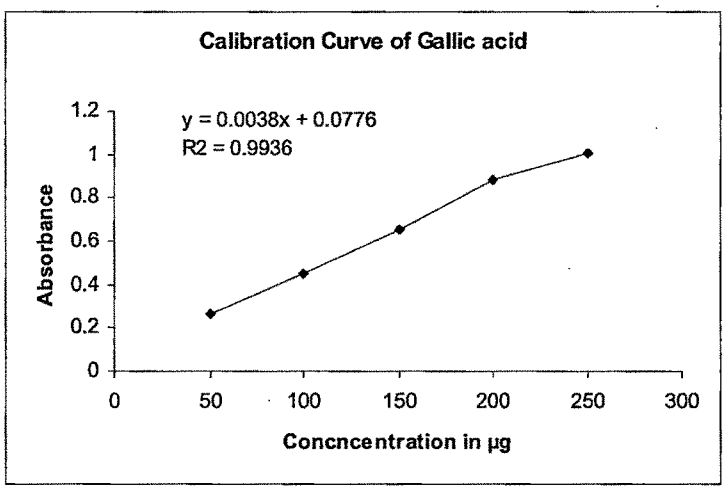
6-7: Solvent system D

b. Determination of Total phenolic and flavonoid content

Total content of phenolics and flavonoids were determined for both the plant materials. Total phenolic content in the samples was calculated from the calibration curve of gallic acid (Figure 2.20). The calibration curve prepared was found to be linear within the selected concentration range (50-250 µg) with a correlation coefficient of 0.993. A quantitative estimation by spectrophotometry showed a high content of total phenolics in the plant materials viz., 0.51 % w/w and 0.44 % w/w in the roots of *E. echinatus* and in the aerial parts of *T. glaberrima* respectively (Table 2.15). Similarly the calibration curve for quercetin was found to be linear within the concentration range of 20–320 µg with a correlation coefficient of 0.999 (Figure 2.20). The flavonoid content in both the plant materials was found to be 0.62 %w/w and 0.28 %w/w respectively for the roots of *E. echinatus* and in the aerial parts of *T. glaberrima* (Table 2.15).

2.5.4.6. Estimation of in-organic elements including the heavy metals

Contamination of the medicinal plant materials with heavy metals can cause chronic or acute poisoning; therefore it has become necessary that all the starting materials should be ensured for their heavy metal content including other necessary inorganic elements. Elemental analysis was carried out in both the selected plant materials to determine the presence of three heavy metals (viz., Lead, Cadmium and Arsenic) and six other inorganic elements viz., Sodium, Potassium, Zinc, Manganese, Copper and Iron. Elemental content was calculated from the calibration curves of the respective elements (Figure 2.21, 2.22, 2.23 and 2.24). The calibration curves prepared were found to be linear within the selected concentration range for each metal. From the results it was found that, the plant materials are devoid of arsenic and cadmium where as trace amounts of lead were found to be present in both the plant materials (0.01 mg/g for *E. echinatus* and 0.02 mg/g for *T. glaberrima*), which were within the limit of WHO specification (10 mg/kg of ADI) for dried plant materials. Both the plant materials showed the presence of macronutrients and micronutrients (Table 2.16).



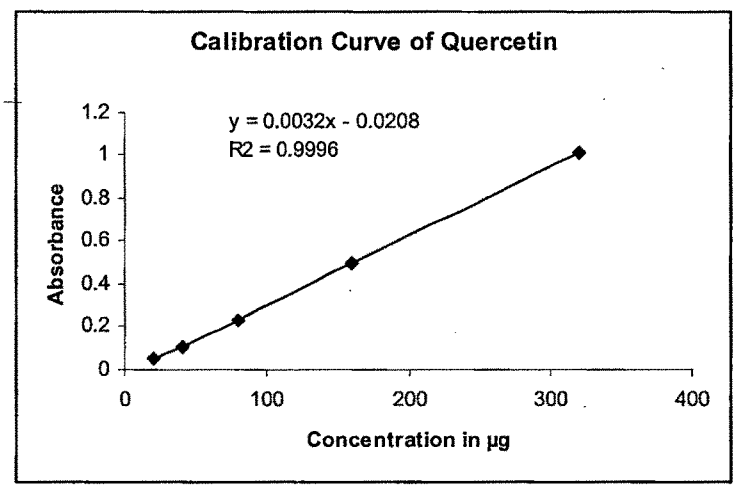


Figure 2.20. Calibration curve of gallic acid and quercetin used for quantification of total phenolic and flavonoid contents.

Table 2.15. Total phenolic and flavonoid content in the roots of *E. echinatus* and aerial parts of *T. glaberrima*

Plant material	Total Phenolic content (% w/w)	Total Flavonoid content (% w/w)
Roots of *E. echinatus*	0.51 ± 0.01	0.62 ± 0.01
Aerial parts of *T. glaberrima*	0.44 ± 0.01	0.28 ± 0.01

Values are mean ± SD; (n = 3)

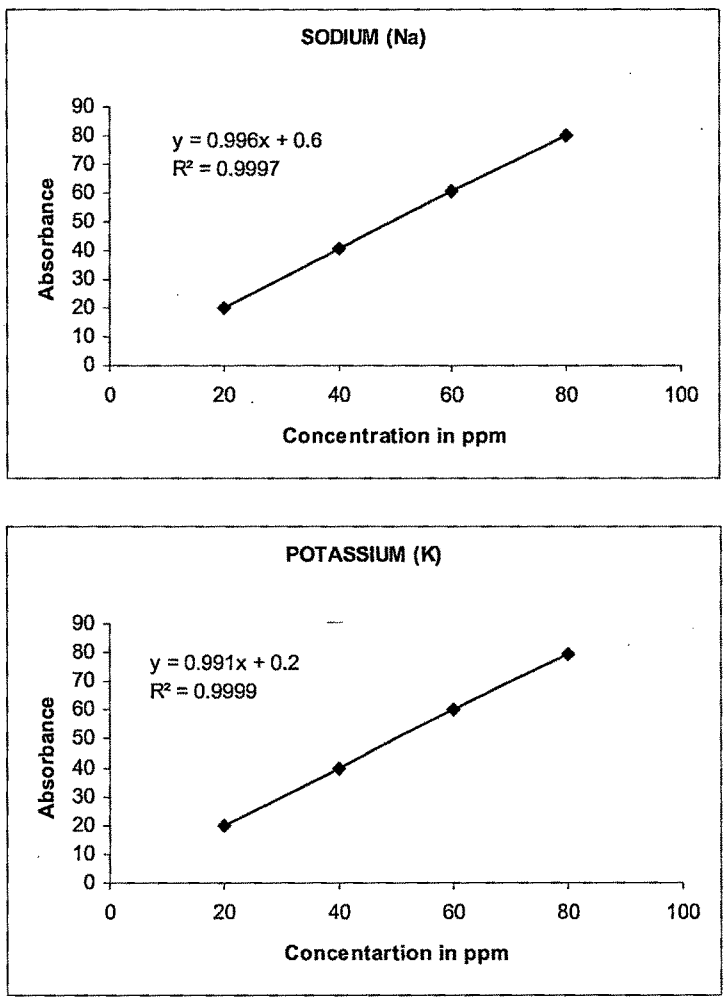
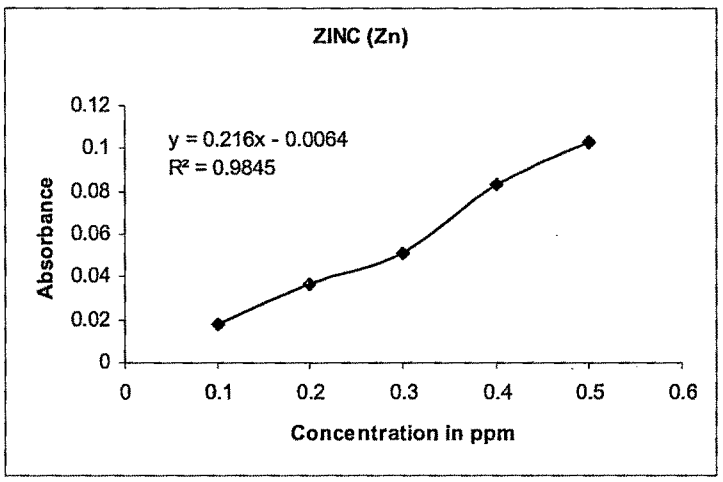


Figure 2.21. Calibration curve of Sodium and Potassium



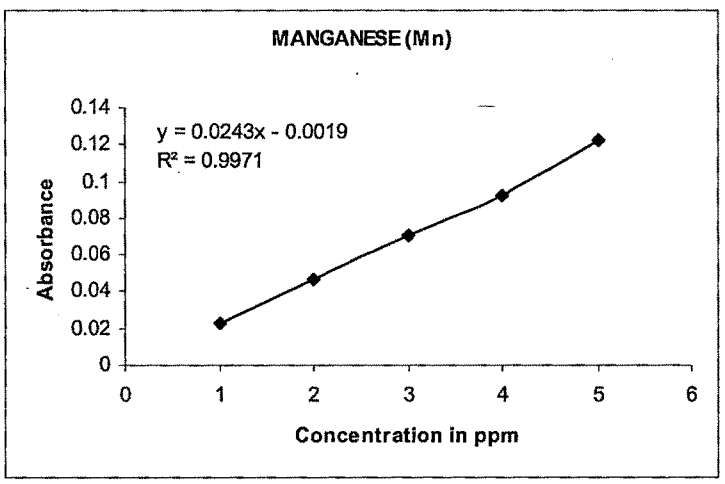


Figure 2.22. Calibration curve of Zinc and Manganese

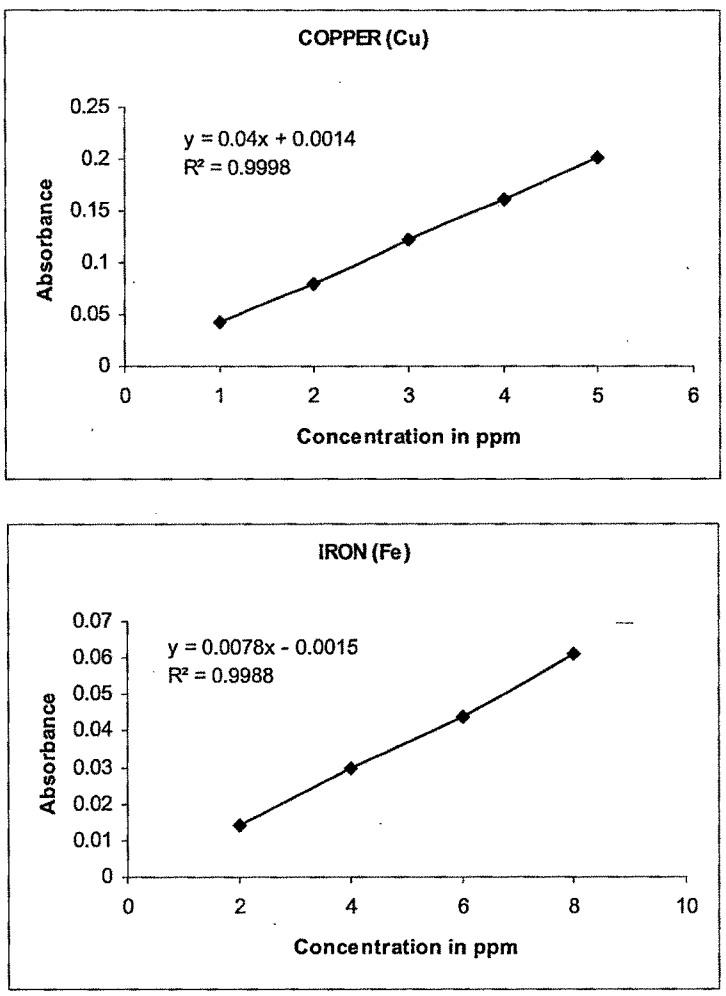


Figure 2.23. Calibration curve of Copper and Iron

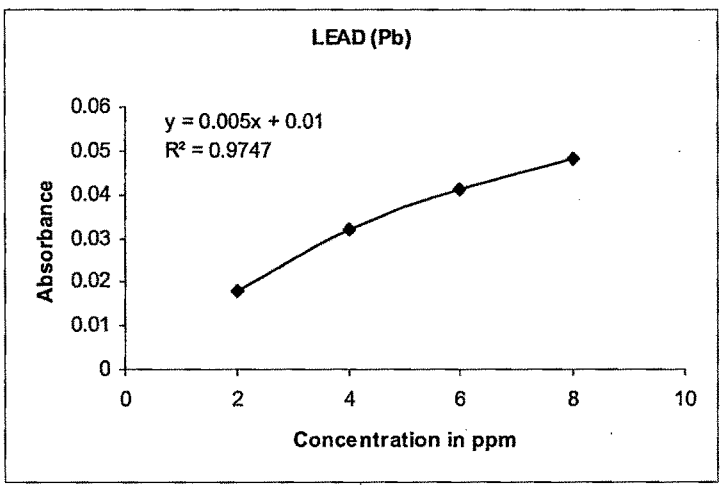


Figure 2.24. Calibration curve of Lead

Table 2.16. Content of heavy metals and other inorganic elements in the roots of *E. echinatus* and aerial parts of *T. glaberrima*

Elements	*E. echinatus*		*T. glaberrima*	
	ppm	**mg/g**	**ppm**	**mg/g**
Sodium	1247.81	1.24	4513.18	4.51
Potassium	10374.9	10.37	13159.68	13.15
Zinc	108.28	0.108	52.72	0.052
Manganese	45.89	0.045	27.86	0.027
Copper	0.917	0.0009	12.6	0.01
Iron	2424.66	2.42	3529.89	3.52
Lead	11.41	0.01	21.67	0.02

2.5.4.8. Isolation of genomic DNA and its finger print studies

Isolation of genomic DNA was carried out using the young leaves. Using younger leaves instead of older ones reduces nucleic acid contamination by plant metabolites that are expected to interfere with solubilization of precipitated nucleic acids.

The extraction process involves, first of all, breaking or digesting away cell walls in order to release the cellular constituents. This is followed by disruption of the cell membranes to release the DNA into the extraction buffer. This is normally achieved by using detergents such as cetyl-trimethyl-ammonium-bromide (CTAB). The released DNA should be protected from endogenous nuclease. EDTA is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases, for this purpose. The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and pigments which may interfere with the extracted DNA and difficult to separate. Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat treated RNase A. Polyvinylpyrrolidone (PVP) was added to remove polyphenols. Polysaccharides are reported to interfere in the quantification of nucleic acids by spectrophotometric methods (Wilkie et al., 1993). NaCl at concentrations of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson et al., 1993).

The isolated genomic DNA's were subjected to electrophoresis along with λ-DNA and a marker of 10kbp. Fire type bands were obtained from the electrophoresis (Figure 2.27). The bands of genomic DNA's from *E. echinatus* and *T. glaberrima* were found to be in high purity and are identical with that of λ-DNA (a standard reference used for comparing the genomic DNA).

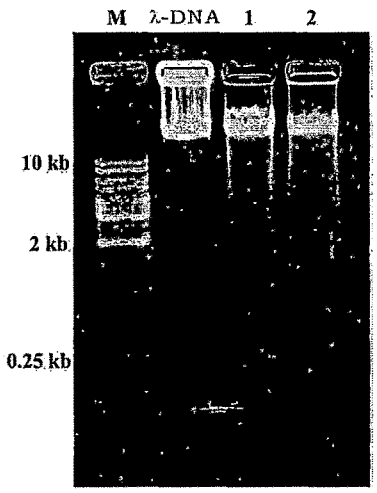


Figure 2.25. Gel photograph of purified DNA of *E. echinatus* (1) and *T. glaberrima* (2) along with a 10 kbps molecular weight marker (M) and λ-DNA.

The obtained data can be used to authenticate 'Brahmadandi'. However further studies using RAPD and PCR-RFLP etc, along with suitable primers may help in differentiating the plants within the species or other morphologically similar plants.

2.6. Discussion

In ethnomedicinal practices the traditional healers use *E. echinatus* and *T. glaberrima* as 'Brahmadandi' in the treatment of various ailments especially in reproductive disorders (See Chapter 1; section 1.41 and 1.42). Both the plants are reported to be sold under the common trade name *'Brahmadandi'* in various Indian markets (Issar, 1974). It has also been reported that there exists around 10 - 12 species of the genus *Tricholepis* in India (Chaudhary and Pandey, 2001). The whole herbs can easily be differentiated by morphological and floral arrangements, but it becomes difficult to differentiate particular organs of the plants (such as roots or aerial parts), which are sold in market in their mutilated form. Therefore some diagnostic features have been evolved to identify and to differentiate the roots of *E. echinatus* and aerial parts of *T. glaberrima* from other crude drugs and adulterants.

Microscopic evaluation of medicinal herbs is an indispensable and cost effective tool in the conventional analytical pharmacognosy for the identification of medicinal herbs (Wallis, 1965). Use of microscopic characteristics has been the mainstay of classical pharmacognosy and remains as one of the essential component of the modern monograph. In this regard the important microscopic features of the roots, stems and leaves have been documented for both *E. echinatus* and *T. glaberrima*. Transverse section (T.S) of the roots of *E. echinatus* showed the presence of an endodermis having casparian thickening which is not seen in the T.S of the roots of *T. glaberrima*. Presence of bilayered medullary rays and parenchyma inclusions in the xylem cylinder are the other features of the T. S of *E echinatus* which are also not seen in the T.S of the roots

T. glaberrima. Unlike the T.S of the roots of *E. echinatus*, T.S of the roots of *T. glaberrima* showed a clear differentiation of the periderm and a more widely spread cortical region.

T.S of the stems of *E. echinatus* is circular in outline where as that of *T. glaberrima* shows the presence of ridges and furrows. Collenchyma is much wider in the T.S of the stems of *T. glaberrima* compared to that in the T.S of *E. echinatus*. T.S of the stems of *T. glaberrima* showed the presence of 3 – 4 layer of chlorenchyma, which is not seen in the T.S of the stems of *E. echinatus*. A clear endodermis, presence of leaf trace bundles and large number of cluster crystals of calcium oxalate are the other characteristic features of the stems of *E. echinatus*. Whereas in case of *T. glaberrima* the endodermis is not very distinct and neither the leaf trace bundles nor the cluster crystals are seen.

In case of *E. echinatus*, T.S of the leaf showed the presence of very long, uniseriate, collapsed trichomes, a 3 – 5 ridged midrib with each ridge showing the presence of a vascular bundle and the presence of patches of sclerenchyma on either side of the vascular bundle. Whereas the T.S of the leaves of *T. glaberrima* showed the presence of short multicellular trichomes in which size of the cells reduces from the base to the tip. Unlike the *E. echinatus* the leaves of *T. glaberrima* showed the presence of a single collateral vascular bundle in the central portion of the midrib.

Powder characters will be of great value for identifying the powdered crude drugs. Roots of the *E. echinatus* showed the presence a large number of vessel elements as the dominating characters in powder whereas the aerial parts of *T. glaberrima* showed a large number of vessel elements, typical parenchyma cells showing the presence of plasmodesmata and non lignified fibers appearing in bundle as the dominating diagnostic features.

However, when the same organs of closely allied taxa are used as traditional medicines and when the substitution occurs with similar organs of other plant materials, microscopy alone may be inadequate for the purpose of an unequivocal diagnosis. Also microscopy does not reveal much about the deterioration of the crude drug. Thus many of the modern herbal pharmacopoeias and bodies governing the regulations on herbal medicines like WHO have included the TLC as a powerful tool for true identification of the plant material in terms of its chemical constituents (WHO, 1998).

In the recent years, HPTLC has emerged as a powerful tool for the establishment of TLC fingerprint profile (IHP, 2002; Pandey et al, 2006). The parameters to be considered to establish a complete TLC fingerprint profiles includes distinctive pattern of chromatogram, the migration distances of the compounds separated (R_f), the bands as observed with naked eye, as examined under UV (254 and 366nm), the UV absorption spectra of the resolved compounds, densitometric/ fluorimetric measurements of the resolved compounds for the calculation of their relative percentage and finally response to several reagents during derivatization.

The main advantage of the fingerprint techniques is that the herbal drugs can be authenticated especially when the active principles are not known or when chemical markers are not available for the analysis. In the absence of known chemical markers the distinctive TLC fingerprint profile would form a characteristic identity for the drug which can be used to ascertain the quality of the herbal drug. Moreover, in those cases where chemical markers/biomarkers are known and method of analysis established for those compounds, it is still advisable and essential to develop fingerprint profiles to further characterize the herbal drug, since it is believed and in certain cases established that many compounds other than the marker compounds present in the herb have a role in the final therapeutic activity of the drug (Bruneton, 1995; Pugh and Sambo, 1988).

Thus the HPTLC finger print profiles of the important chemical constituents in the methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* have been established. For this purpose three different solvent systems of varying polarity were used so that the compounds of different polarity can be resolved out. A complete HPTLC finger print profile of the resolved compounds comprising of the typical spectra, R_f value, UV absorption maxima and the percentage proportion of the individual components in the extract are recorded and documented. The reported data may be of great value as a reference standard for evaluation of both the plant materials.

Determination of inorganic elements, including the trace elements and heavy metals has gained outstanding importance in the life sciences. Elements present even at minimal concentrations in biological and environmental matrices, in fact, can exert fundamental influence on vital functions in the human body proportionally to their amounts.

All the living organisms require inorganic elements for their growth and survival. Medicinal plants contain considerable amounts of mineral constituents; in particular, the presence of essential elements (Mg, Mn, Zn, and many others) is a prerequisite for correct growth and development of plants. Inorganic elements in plants also plays vital role in the accumulation of secondary metabolites such as alkaloids, glycosides, terpenoids, phenolic compounds etc (Andrijany, 1998; Lokova et al., 1998) as they are responsible for the activity of a number of enzymatic systems, which in turn regulate the metabolic pathways leading to the synthesis of these compounds.

The recent reports (Saper et al., 2004) on the potential deleterious effects of some Ayurvedic medicines, due to presence of unacceptable levels of metals and metalloids such as Lead, Arsenic, Mercury etc, and their possible chronic toxicological effects, has caused much concern not only among the

herbal practitioners, but also among large population who still depend on the medicinal plants for their healthcare needs. Thus it has become necessary that all the medicinal plant materials should be ensured for their content of heavy metals and other inorganic elements. The plant materials under study viz., roots of *E. echinatus* and aerial parts of *T. glaberrima* were tested for the presence of heavy metals and other essential inorganic elements. The heavy metal content was found to be within the limits of WHO specification.

Genotypic characterization of plant species and strains is useful as most plants, though belonging to the same genus and species, may show considerable variation between strains. DNA fingerprinting of herbal drugs, though still in its early years, seems to be a promising tool for the authentication of medicinal plant species and for ensuring better quality herbs and nutraceuticals. Fingerprinting of plant DNA is dictated by several factors; sequence or restriction site data, taxonomic level of study, the level at which the study is being done (species, genera, etc.), robustness and reproducibility of the method, effectiveness in terms of cost and time, and availability of DNA.

Additional motivation for using DNA fingerprinting on commercial herbal drugs is the availability of intact genomic DNA from plant samples after they are processed. Adulterants can be distinguished even in processed samples, enabling the authentication of the drug (Mihalov et al., 2000). Studies have reported the genotyping of several medicinal plants, and have made available their DNA fingerprints.

In the present studies an attempt has been made to record the finger print pattern of the isolated genomic DNA from *E. echinatus* and *T. glaberrima* (the two different species). However further studies using RAPD (random amplified polymorphic DNA), PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphisms), are necessary for differentiating

the plants within the species or other morphologically similar oriental medicines.

The pharmacognostical studies carried out on both the plants therefore serve as valuable tool and provide suitable standards for the identification of the plant materials. Phytochemical screening and the TLC profile of detected phytoconstituents may also serve as reference standards. The important features for both the plant materials are summarized in following tables.

Table 2.17. Some important features of phytochemical studies of both plants

Parameters		***E. echinatus***	***T. glaberrima***	**Comments**
Yield of successive Petroleum ether extract		5.04 %	1.67 %	*E. echinatus* is rich in non polar compounds
Yield of Successive water extract		1.57 %	6.54 %	*T. glaberrima* is rich in polar compounds
TLC profile of petroleum ether extract.		13-14 pink to purple bands	4-5 pink to purple bands	More number of non polar compounds in *E. echinatus*
Chemical screening	Terpenoids	Detected	Detected	Alkaloids were detected only in *T. glaberrima*
	Alkaloids	Alkaloids were not detected	Alkaloids were detected	

Table 2.18. Some important morphological features of both plants

Parameter	*E. echinatus*	*T. glaberrima*
Whole plant	Plant is a much branched and rigid.	Plant is quite glabrous; stem erect, slender.
Leaves	Leaves are sessile, 7.5-12.5 cm long; sinuate and spinescent, the spines often 2.5 cm long	Leaves sessile 2.5 - 6.3 cm long; spinous – serriate.
Inflorescence	Balls of the heads are round normally 2.5-3.8 cm in diameter	Heads are ovoid, 1.5 – 2 cm long.
Roots	30 - 50 cm long with a diameter of 0.5 – 1 cm.	20 - 30 cm long and 0.3 – 0.6 cm in diameter.

Table 2.19. Comparative microscopic features of the different organs of both plants

Parts of the plant	Parameter	*E. echinatus*	*T. glaberrima*
Roots	Endodermis with casparian thickening	Present	Not seen
	Bilayered medullary rays and parenchyma inclusions in the xylem cylinder	Present	Not seen
	Cortical region	3-5 layered	Wider cortical region 6-8 layered.
	Periderm	Not differentiated	Cortex and phellogen are clearly seen.
Stems	T.S of the stem	Circular in outline	Shows ridges and furrows.
	Collenchyma	Short	Wide
	Chlorenchyma	Not seen	3-4 layered
	Leaf trace bundles	Seen frequently	Not seen
	Cluster crystals of calcium oxalate	Many	Not seen
Leaves	Trichomes	Long, uniseriate, collapsed trichomes	Short uniseriate trichomes
	Midrib	Shows 3-5 ridges	Single ridge
	Vascular bundle	3-5 vascular bundles	Single centrally located vascular bundle

Table 2.20. Some important features of powder microscopy of both plant materials

Parameter	**Roots of *E. echinatus***	**Aerial parts of *T. glaberrima***
Cork cells	Presence of cork cells	Not seen
Parenchyma	Not seen	Cortical parenchyma with polyhedral cells
Vessel elements	Vessel elements with helical, simple pitted, bordered alternate pitting arrangements	Vessel elements with helical, annular and pitted thickenings
Tracheids	Tracheids are seen	Not seen
Plasmodesmata	Not seen	Parenchymatous cells showing the presence of plasmodesmata
Fibers	Not seen	Long non lignified fibers in bundle.

Table 2.21. Physico chemical constants of both plant materials

Parameter		Roots of *E. echinatus*	Aerial parts of *T. glaberrima*
Total ash value		6.29	10.86
Extractive value	Alcohol	6.8	9.6
	water	10.8	13.2

Table 2.22. Finger print pattern of the methanol extracts of both plant materials

Parameters	Roots of *E. echinatus*	Aerial parts *T. glaberrima*	Comments
Separation of terpenoids (non polar compounds)	6-7 pink to purple bands	3-4 pink to purple color bands	*E. echinatus* shows more terpenoids
Separation of phenolic (polar)compounds	7-8 bluish black color bands	4-5 bluish black bands are seen	*E. echinatus* shows more phenolic compounds
Separation of Alkaloids	Not detected	10-11 orange colored bands are seen	Alkaloids were not detected in *E. echinatus*

Table 2.23. Phenolic and flavonoid content in both plant materials

Compounds	**Roots of *E. echinatus***	**Aerial parts *T. glaberrima***	**Comments**
Total phenolic content	0.51%	0.44%	*E. echinatus* was found to contain more phenolic compounds.
Total flavonoid content	0.62%	0.28%	

Table 2.24. Content of heavy metal and other inorganic elements in both plant materials

Compounds	**Roots of *E. echinatus***	**Aerial parts *T. glaberrima***	**Comments**
Sodium (mg/g)	1.24	4.51	Essential inorganic elements were found to be high in *T. glaberrima* than the *E. echinatus*. Copper content in *T. glaberrima* was found to almost 100 folds higher than that in *E. echinatus*. Detected heavy metal is within the limit of WHO specification.
Potassium (mg/g)	10.3	13.1	
Copper (mg/g)	0.0009	0.01	
Iron (mg/g)	2.42	3.52	
Lead (mg/g)	0.01	0.02	

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