

RESEARCH ARTICLE

Pharmacognostical Standardization and HPTLC fingerprint of *Tribulus terrestris* LU.Subasini¹, S.Thenmozhi^{2*}, M.Dhanalakshmi², G.Victor Rajamanickam³ and G.P.Dubey⁴¹Department of Pharmacology, Management and Science University, Selangor, Malaysia²Department of Pharmacognosy, Swamy Vivekanandha College of Pharmacy, Thiruchengode – 637 205, Namakkal (D.T), Tamil nadu, India³Centre for Advanced Research, Vel's University, Pallavaram, Chennai-117, Tamil nadu, India.⁴Banaras Hindu University, Varanasi, UP, India

Received 01 Dec 2016; Revised 12 Feb 2017; Accepted 23 Feb 2017

ABSTRACT

This herb is prostrate, annual or biennial having a length up to 90 cm. Leaves paripinnate; leaflets 4-5 pairs, subequal, 2-4 cm in length oblong to linear-oblong, mucronate, pubescent on both surfaces. This trailing plant is common in sandy soil, found throughout India, ascending to 3300 m in Himalaya, particularly common in dry and hotter parts of the country viz., Rajasthan, Gujarat, Deccan, and Andhra Pradesh. It is a traditional medicinal plant, which is valued for its hypertensive, CNS stimulant, spasmogenic, analgesic, vasodepressant, muscle relaxant, cardiogenic, diuretic, antiurolithiatic, antibacterial, antifungal, antimicrobial, molluscicidal, cytotoxic activity on FL-cells, hepatoprotective, cytoprotective and anticonvulsant. The current study was therefore carried out to provide requisite pharmacognostic details of whole plant of *Tribulus terrestris* L. Pharmacognostic evaluation included examination of morphological and microscopical characters; physicochemical properties, phytochemical analysis and HPTLC finger print. Phytochemical screening reported the presence of tannins, alkaloids, glycosides, steroidal compounds. The R_f values are detected at 254 nm and 366 nm by qualitative densitometric HPTLC fingerprint, can be used as identifying marker for Hydro alcoholic extract. The present studies will the information with respect to identification and authentication of crude drug.

Keywords: *Tribulus terrestris* L., Pharmacognosy, HPTLC finger print, Tannins.**INTRODUCTION**

Tribulus terrestris L. (Family: *Zygophyllaceae*) (Syn: Puncture vine) its flowers and fruits almost throughout the year. The flowering starts within 20-35 days and the fruit mature in 14 days after the formation of seed. Flowers axillary or leaf opposed, pale yellow to yellow, solitary on peduncles shorter than the leaves 1.5 cm across petals yellow in colour. Number of chemical constituents has already been identified. Some of the chemicals are here brought to notice. They are sapogenin with pyroketone ring (diosgenin), gitogenin and hecogenins, chlorogenin, diosgenin and its acetate, astragalins, dioscin, furostanol glycoside and spirosterol^[1]. The roots and fruits are sweet, cooling, diuretic, aphrodisiac, emollient, appetizer, digestive, anthelmintic, expectorant, anodyne, anti-inflammatory, alterant, laxative, cardiogenic, styptic, lithontriptic and tonic. They are useful in strangury, dysuria, gonorrhoea, gleet, chronic cystitis, urinary

disorders, renal and vesical calculi, anorexia, dyspepsia, helminthiasis, cough, asthma, consumption, inflammations and cardiac disorders^[2]. Therefore, present investigation of *Tribulus terrestris* L. whole plant is taken up to establish pharmacognostic profile of the leaves, stem, root and HPTLC finger print which will help in crude drug identification as well as in standardization of the quality and purity.

MATERIALS AND METHODS

Herbarium of *Tribulus terrestris* L. was prepared and authenticated in multcentres such as Rabinat Herbarium, St. Joseph College, Trichy, St. Xavier's College, Palayamkottai and Botanical survey, CCRAS Unit, Chennai and Govt. Medical College, Palayamkottai. The voucher specimens are deposited in the CARISM herbarium and maintained (0038/s/2007). Fresh whole plants of *Tribulus terrestris* L. were collected from Thuraiyur, Trichy, washed under running tap

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water and blotted dry for further studies. The whole plant was dried for about two weeks, ground into powder and used for further analysis. Physicochemical constants such as the percentage of total ash, acid insoluble ash, water soluble ash, water soluble and alcohol soluble extractive values were calculated according to the methods described by Mukherjee^[3]. Preliminary phytochemical analysis of powdered drug was performed as described by Khandelwal^[4] and Kokate^[5]. A qualitative densitometric HPTLC analysis was performed for the development of characteristic finger print profile using different solvents according to polarity. 5µl of whole plant extract was spotted on pre-coated silica gel G60F254 TLC plates (Merck) with the help of CAMAG Linomat V applicator. The plates were then developed in glass twin trough chamber (20 cm×10 cm) presaturated with mobile phase. The developed plates were scanned using TLC Scanner 3.

Elemental Analysis by AAS

After calibrating the instrument with prepared working standard, the digested liquid samples solution is subjected to analysis of Fe, Cu, Mn, Zn, Ni, Mg, Mo, etc., by AAS flame/Graphite furnace with specific instrumental conditions as given by instruments manufacturer. Introduce the solution into flame, record the reading, using the mean of the three readings and quantified the concentration of the metals in the given samples against the standard calibration curve obtained from Concentration vs. Absorbance of the prepared known concentration on the day of the analysis.

After calibrating the instrument with prepared working standard, the 10 ml of digested liquid sample is pipetted out to a specific container of Mercury Hydride system analyzer followed by adding 1.5% HCl of 10 ml as diluent for each flask and blank, 3% of NaBH₄ solution in 1% of NaOH is run through the reaction flask to quartz cell without heating against the calibration curve obtained from concentration Vs absorbance of the prepared known concentration on the day of the analysis.

RESULTS

Macroscopic characters

This herb is prostrate, annual or biennial having a length of up to 90 cm. Leaves paripinnate; leaflets 4-5 pairs, subequal, 2-4 cm in length oblong to linear-oblong, mucronate, pubescent on both

surfaces. Flowers axillary or leaf opposed, pale yellow to yellow, solitary on peduncles shorter than the leaves 1.5 cm across petals yellow in colour obovate 7.5 X 4.5 mm. Fruits globose, consisting of 5 woody mericarps with 2 long and 2 short spines. Seeds are many in each coccus. The carpels or cocci of the fruit resemble a cloven hoof of the cow. Drug consist of root, 7-18 cm long and 0.3-0.7 cm in diameter, slender, cylindrical, fibrous, frequently branched bearing a number of small rootlets, tough, woody and yellow light brown in colour, surface becomes rough due to presence of small nodules, fracture fibrous, odour aromatic, taste; sweetish and astringent (**Fig 1**)



Fig 1: *Tribulus terrestris* L. whole plant

Microscopic characters

T.S. of Root:

The root is whitish in color. The root contains outer epidermis, cortex, phloem and xylem regions. Epidermis is of 2-3 cells layered with rectangular parenchyma cells. The cells are golden yellow in color (when stained with IKI some of the cortex cells contain phenols (appear golden yellow to brown color) (**Fig 2**).

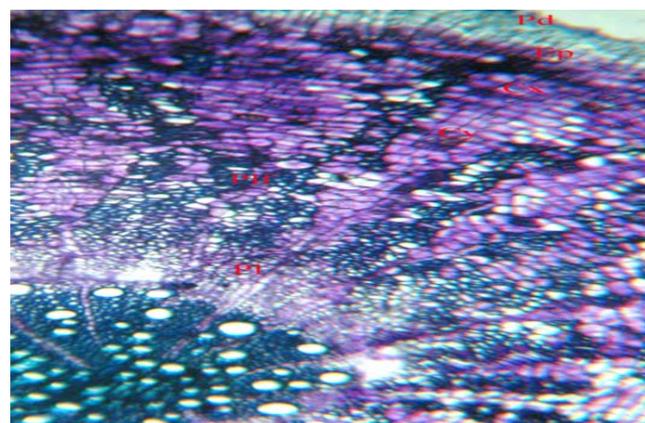


Fig 2: T.S. of Root

Cortex: 3-4 cells thickness, some of the cortex cells containing starch grains appear black in colour (stained with IKI). Cortex regions have patches of sclerenchyma cells and have thick cell

walls that appear blue in colour (stained with TBO).

Phloem: The phloem cells are polygonal and irregular in shape and size. Some of the phloem cells contain cystoliths (calcium carbonate crystals) (Fig 3).

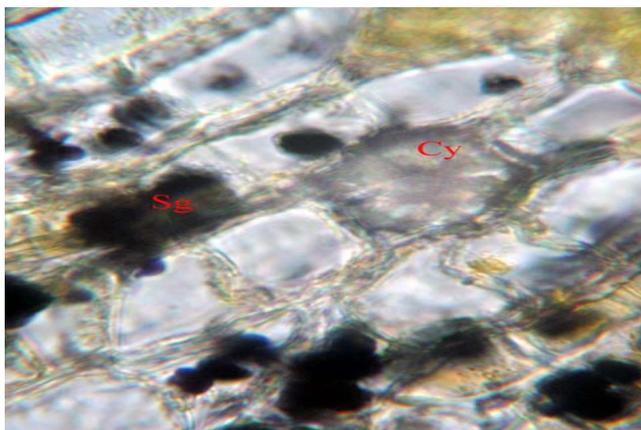


Fig 3: Phloem

Some secondary cortex cells of phloem; secondary phloem divided into two zones, outer zone characterized by presence of numerous phloem fibres with a few sieve tubes slightly collapsed, inner zone frequently parenchymatous, devoid of fibres often showing sieve tubes and companion cells; phloem rays distinct, few cells get converted into fibres in outer region; cambium 3-5 layered; wood composed of vessels, tracheids, parenchyma and fibres and traversed by medullary rays; vessels scattered, arranged in singles or doubles towards inner side, in groups of three to four on outer side having bordered pits; tracheids long, narrow with simple pits.

Mesocarp 6-10 layers of large parenchymatous cells, rosette of calcium oxalate crystals abundantly present; mesocarp followed by 3-4 compact layers of small cells containing prismatic crystals of calcium oxalate (Fig 4).

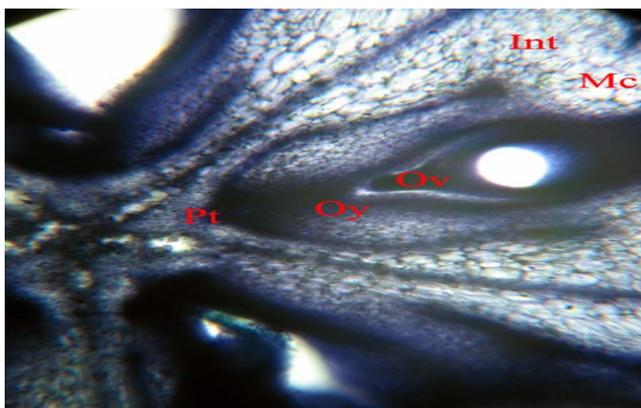


Fig 4: T.S. of Stem

Xylem parenchyma rectangular or slightly elongated with simple pits and reticulate

thickening; xylem fibres few; tracheids elongated with simple pits; medullary rays heterogenous, 1-4 cells wide; starch grains and rosette crystals of calcium oxalate present in secondary cortex, phloem and medullary rays cells; few prismatic crystals also present in xylem ray cells. Transverse section of fruit shows small epidermal cells of each coccus rectangular; unicellular trichomes in abundance (Fig 6-8).

Fig 6: T.S. of Leaf (Fibres)

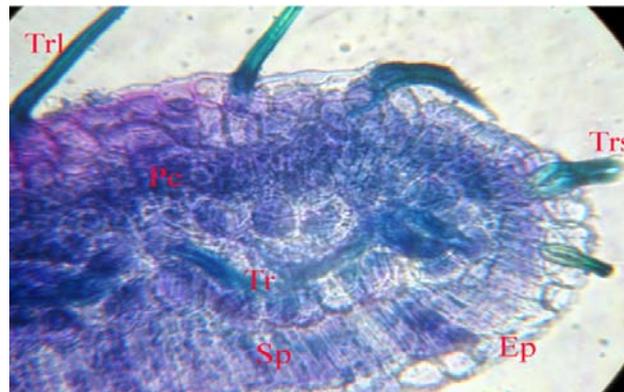


Fig 7: T.S. of Leaf (Trichomes)

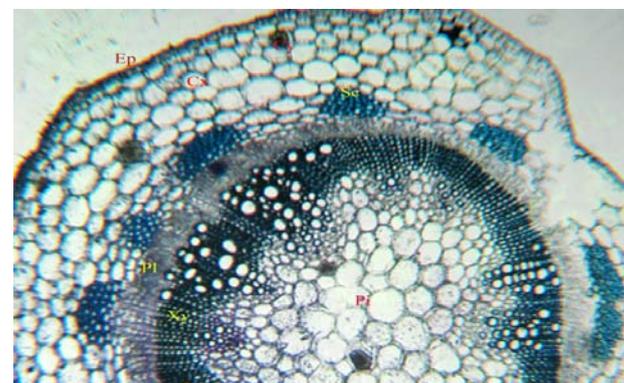


Fig 8: T.S. of Leaf

Xylem: The vessel elements are broad. Xylem parenchyma is 1-2 cell layered. The xylem cells are irregular in shape. Sometimes the pith regions contain cystolith (calcium crystals). Some of the pith region contains starch grains (Fig 4 and 5).

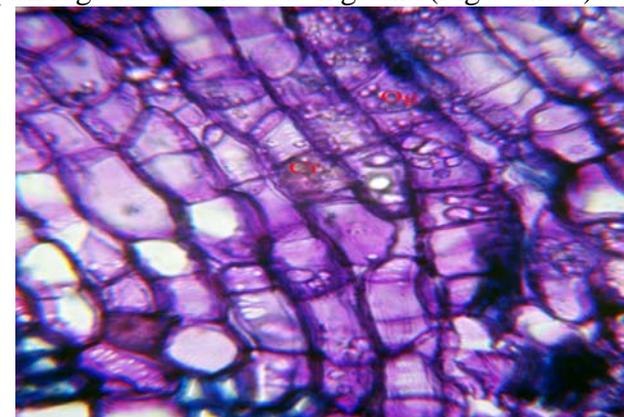


Fig 5: T.S. of Seed

T.S. of Fruit: The fruits are greenish in color, 5 locular. Each locules have tubecles. The tubecles are joined together by a thin two cell layered septum. The fruits are dehiscent. Some of the tubecles are spiny. The spines are made up of compact elongated longitudinal cells. Two spines are large, straight and lengthy while others are small and curved. The fruit contains three different types of trichomes. One is lengthy and straight, the second is short and straight and the third one is short and curved. The T.S. of the fruits shows five locules. The septum cells are thin and are 2 cells layer in thickness with non-lignified cell wall. Each locule contains ovules covered by integuments and micropylar regions. The Basal regions of the integuments are made up of oval, spherical parenchymatous cells. The middle region contains large polygonal cells. The epidermis of the integuments consists of short rectangular cells. Each locule containing 4 seeds is arranged in rows and the seeds are yellow in colour. The seeds have axial placentation. The

central portion of the locules contains cystolith (calcium carbonate crystals) (Fig 5).

Physico-chemical Parameters

Ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (metallic salts and silica). This value varies within fairly wide limits and is therefore an important parameter for the purpose of evaluation of crude drugs [3-4]. Therefore percentage of total ash, acid insoluble ash and water soluble ash were carried out. The extraction of any crude drug with a particular solvent yields a solution containing different phyto-constituents. Extractive value is also useful for evaluation of crude drug, which gives an idea about the nature of the chemical constituents present in a crude drug and is useful for the estimation of specific constituents [5] results are tabulated in (Table 1).

Table 1: Proximate Analysis [6]

| S. No | Raw drug | Total Ash (%w/w) | Water Insoluble Ash (% w/w) | Acid Insoluble Ash (% w/w) | Crude Fibre Content (%w/w) | Water Soluble Extract (% w/w) | Ethanol Soluble Extract (%w/w) |
|-------|------------------------------|------------------|-----------------------------|----------------------------|----------------------------|-------------------------------|--------------------------------|
| 1 | <i>Tribulus terrestris</i> L | 5.2 | 2.4 | 1.1 | 14.8 | 10.58 | 14.36 |

Preliminary Phytochemical studies

The whole plant powder was extracted with hydroalcoholic in the ratio of 70:30. These extract was tested for presence of different phytoconstituents by qualitative and quantitative methods [7-12]. The results of phytochemical analysis are tabulated in (Table 2 & 3).

Table 2: Qualitative Phytochemical Analysis of Hydroalcoholic Extract

| S. No | Phytoconstituents | <i>Tribulus Terrestris</i> L Extract |
|-------|-------------------|--------------------------------------|
| 1 | Alkaloids | + |
| 2 | Carbohydrate | + |
| 3 | Phytosterol | + |
| 4 | Protein | + |
| 5 | Glycosides | + |
| 6 | Flavonoids | + |
| 7 | Saponins | ± |
| 8 | Volatile oil | - |

Table 3 Quantitative Estimation of Phyto-Constituents in Raw Drugs

| S. No | Plants | Total Phenolics (mg/100 gm) | Total Tannins (mg/100gm) | Total Proteins (µg/100gm) | Vitamin C (µg/100gm) | Vitamin E (µg/100gm) | Total Carbohydrate (mg/100gm) |
|-------|--------|-----------------------------|--------------------------|---------------------------|----------------------|----------------------|-------------------------------|
| 1 | TT | 7.04±0.3 | 5.00±0.1 | 35.0±0.9 | 0.19±0.1 | 0.21±0.5 | 725±4.2 |

(Values are mean ± SD)

Table 3 (a): Estimation of active constituents of TT

| S. No | Name of the Phytoconstituents | <i>Tribulus Terrestris</i> L. |
|-------|-------------------------------|-------------------------------|
| 1 | Glycosides | 47.7739 % |
| 2 | Alkaloids | 0.7306 % |
| 3 | Flavonoids | 43.1895 % |
| 4 | Tannins | 41.8186 % |
| 5 | Fixed oil | 3.2366 % |
| 6 | Resins | 40.8522 % |
| 7 | Bitters | - |
| 8 | Vitamin C | 4.1414 mg |

HPTLC finger print

Chromatography was performed on a 10x3cm pre-coated HPTLC Silica gel 60F₂₅₄ plate. The plates were washed by methanol and activated at 60^o C

for 5 minutes prior to chromatography. Samples were applied on to the plate of 6mm band using CAMAG Linomat V applicator. The slit dimension was kept at 5 mm x 0.45 mm and 20 mm/s scanning speed was employed. The mobile phase was chosen after trial and error and 10 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 10 cm x 10 cm twin glass chamber saturated with the mobile phase. The mixture was spotted using CAMAG Linomat V applicator. *Tribulus terrestris* Linn. Plant extract contains 10 compounds in mobile phase CHCl₃:CH₃OH

(4.5:0.5) ratio among the observed peaks at R_f value of 0.88 and 0.66 are found to have greater area such as 41532.7 and 21492.4. This area is directly proportional to quantity of the compound

present in the extract. This area is directly proportional to quantity of the compound present in the extract (**Table 4, Fig 1a & 2b**).

Table 4: HPTLC fingerprint shows 10 peaks in the Extract the R_f values & area

| Peaks | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-------|--------|--------|--------|---------|--------|--------|---------|--------|---------|---------|
| R_f | 0.11 | 0.20 | 0.29 | 0.34 | 0.48 | 0.57 | 0.66 | 0.72 | 0.78 | 0.88 |
| Area | 1414.4 | 7877.2 | 4875.8 | 18287.5 | 4757.7 | 7968.6 | 21492.4 | 4290.6 | 22071.1 | 41532.7 |

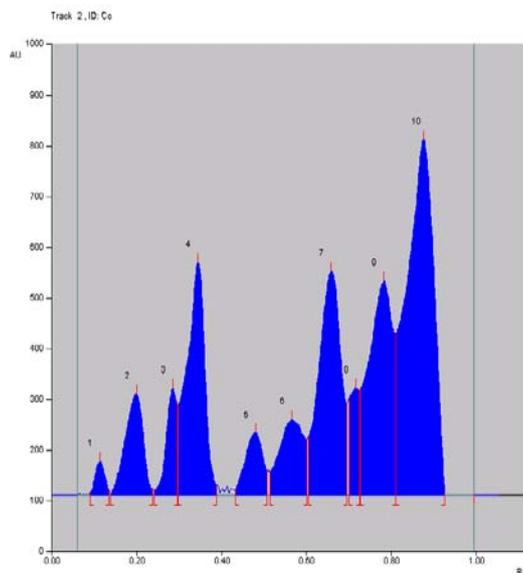


Fig 1a: Peak display at 10 µl 254nm – TT

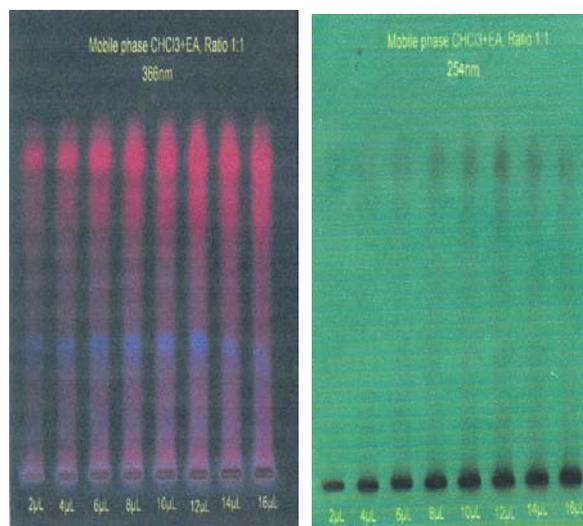


Fig 1b: Photo documentation for TT

Elemental analysis

The various mineral elements are generally being imbibed into the plants from the soil, water and atmosphere. The level of mineral elements in plant varies depending upon the environmental factors and the type of plant itself. Among plant types growing in the same environment, fungi lichen and mosses accumulate more metals than the others. For a particular species, the concentration level generally decreases in the order root > stem > leaves > fruit > seed when the source of the mineral element is only the soil. Moreover the concentration of elements also varies with the age of the plant.

Mineral elements are more useful to man than being harmful. Human body requires mineral elements to certain extent. At the same time, when it crosses the limit, it becomes toxic and degenerate the system. High level of toxic elements occur in medicinal preparations either when they are used as active ingredients as in the case of Pb and Hg in some Chinese, Mexican and Indian medicines^[8] or when the plants are grown in polluted areas fertilizers, such as near roadways, metal mining and smelting operations and when one uses fertilizer containing cadmium

and organic mercury or lead based pesticides, and contaminated irrigation water^[9]. Hence, analysis of various mineral/metal elements is imperative in the use of plants as drugs. Inorganic micronutrients include Fe, Cu, Zn, Mn, Co, Mo, V, B, Cl, I, Br and Na. They are important as catalyzing metabolic reactions and in osmoregulation. They are required in optimum quantities for better growth of the plant but when supplied in excess, it is turning to be harmful. Results of the micronutrients and trace elements are given in the (**Table 5 & 6**). In view of the criticism provided for the traditional drugs on the ground of metal toxicity, the extract, which is going to be tested for the drug is brought under the observation of elemental analysis. The values are very much within the limits of WHO except aluminium that are also an element of useful one for the metabolism. As there is no alarming presence of heavy metals in the extracts, the extract has been taken up for further acute toxicity studies.

Table 5: Elemental Analysis using Flame Photometry

| Plant | Na (mg/l) | Ca (mg/l) | K (mg/l) | Li (mg/l) |
|-------|-----------|-----------|----------|-----------|
| TT | 1.15 | 390.97 | 39.09 | 0.35 |

Table 6: Distribution of Elements in *Tribulus Terrestris* L.

| Plant Code | Fe | Cu | Mn | Ni | Zn | Co | Cr | Al |
|------------|----|----|----|----|----|----|----|----|
| | | | | | | | | |

| | | | | | | | | |
|------------|--------|--------|--------|--------|--------|--------|--------|---------|
| Raw plant | 0.6675 | 0.1320 | 0.1635 | 0.2025 | 0.6520 | 0.1695 | 0.3660 | 8.8750 |
| Ashes | 0.7040 | 0.5240 | 0.5520 | 0.2240 | 0.3040 | 0.1080 | 0.0960 | 11.8760 |
| HAE | 0.1830 | 0.0210 | 0.0390 | 0.0545 | 0.0485 | 0.0670 | 0.2000 | 2.3490 |
| Plant Code | V | Mo | Pb | Cd | Hg | As | Se | |
| Raw plant | 0.1120 | 1.5600 | 0.7440 | 0.1036 | 0.0042 | 0.0009 | 0.0027 | |
| Ashes | 0.0019 | 0.0052 | 0.0221 | 0.0144 | 0.0029 | 0.0002 | 0.0027 | |
| HAE | 1.1200 | 0.1980 | 0.2040 | 0.0495 | 0.0159 | 0.0012 | 0.0044 | |

Any plant is likely to have some elements or others in low or high quantity. The quantity depends on the soil nature and the environmental conditions. In the present study, the concentration of various elements in raw plants, the ashes of different plants, the aqueous extracts and in hydroalcoholic extracts has been determined by using flame photometry in Table 5 using AAS and the same is tabulated in Table 6.

Heavy metals and toxic elements

The toxicity sequence for heavy metals varies with the taxonomical group of plants; for flowering plants the sequence observed is Hg > Pb > Cu > Cd > Cr > Ni > Zn; for algae it is Hg > Cu > Fe > Cr > Zn > Ni > Co > Mn and for fungi the observed sequence is Ag > Hg > Cu > Cd > Cr > Ni > Co > Zn > Fe. Many species belonging to Rubiaceae are aluminium accumulators. In fact, aluminium accumulators are woodier than herbaceous. Aluminium interacts with a number of other elements including calcium, fluorine, iron, magnesium, phosphorous and strontium and when ingested in excess it can reduce their absorption^[10].

DISCUSSION

Tribulus terrestris L. is currently being used in the treatment of various disease conditions without standardization. The standardization of a crude drug is an integral part of establishing its correct identity. For inclusion of a crude drug in Pharmacopoeia, pharmacognostic parameters and standards must be established. The results of these investigations could, therefore, serve as a basis for proper identification, collection and investigation of the plant.

As observed in the transverse section of leaves, stem, root and fruits (seed). The root contains outer epidermis, cortex, phloem and xylem regions. Epidermis is of 2-3 cells layered with rectangular parenchyma cells. The fruit contains three different types of trichomes. One is lengthy and straight, the second is short and straight and the third one is short and curved. The T.S. of the fruits shows five locules.

Equally important in the evaluation of the crude drugs, is the ash value, water soluble ash value

and acid insoluble ash value determination. The total ash is particularly important in the evaluation of purity of drugs, i.e the presence or absence of foreign organic matter such as metallic salts and silica^[4]. The total ash value, water soluble value and acid insoluble value of *T.terrestris* is 5.2 %, 2.4 % and 1.1 % respectively. Since the ash value is constant for the given drug, this value is one of the diagnostic parameter of the drug. Extractive values are primarily useful for the determination of exhausted or adulterated drugs. It revealing presence large amount of water soluble constituents and secondary metabolites in the whole plant. Presence or absence of certain important compounds in an extract is determined by colour reaction of the compound with specific chemicals. This procedure is a simple preliminary pre-requisite before going for detailed phytochemical investigation. Various tests have been conducted qualitatively and quantitatively to find out the presence or absence of bioactive compounds. Different chemical compounds such as carbohydrate, protein, amino acids, phenol, tannins, flavonoids, alkaloids, steroids and glycosides^[7-12] are detected in the whole plant extract which could make the plant useful for treating different ailments as having a potential of providing useful drugs for human use. HPTLC fingerprint profile along with their Rf values and percentage proportion were recorded, which would serve as a reference standard for the scientist engaged in research on the medicinal properties of plant. From the above investigation, the results obtained, for from both raw drug and hydroalcoholic extract shows that the heavy metals are well within permissible limits and thus the safety and efficacy of the *T.Terrestris* plant is proved. Mineral elements are more useful to man than being harmful. Human body requires mineral elements to certain extent. At the same time, when it crosses the limit, it becomes toxic and degenerate the system^[13]. High level of toxic elements occur in medicinal preparations either when they are used as active ingredients as in the case of Pb and Hg in some Chinese, Mexican and Indian medicines^[14] or when the plants are grown in polluted areas fertilizers, such as near roadways, metal mining and smelting operations

and when one uses fertilizer containing cadmium and organic mercury or lead based pesticides, and contaminated irrigation water^[15]. Hence, analysis of various mineral/metal elements is imperative in the use of plants as drugs.

The various morphological, microscopical, physicochemical, phytochemical and elemental analyses developed in these studies will help for botanical identification and standardization of the drug in crude form.

CONCLUSION

Thus the organoleptic, microscopic characters, physico-chemical, preliminary phytochemical, elemental analysis and HPTLC fingerprint screening can be used as a diagnostic tool for the correct identification of the plant. The adulterants if any in this plant material can be easily identified by using these results.

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