Qualitative and Quantitative Analysis of Phytoconstituents and their Therapeutic Potential in *Tribulus terrestris*

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Abstract

Tribulus terrestris is an aggressive colonizing species widespread in temperate and tropical regions. The plant is known for its therapeutic potential and has been used in traditional medicinal systems since ages. It probably originated from the Sahara Desert region of Africa and later on spread to dry temperate regions of the world. Tribulus is a perennial, procumbent herb bearing silky hair on green shoot. The present study aims at finding the therapeutic potential of T. terrestris through phytochemical analysis. Crude methanolic extract of dried Tribulus fruits was prepared and stored in powdered form and used for all assays. Thin Layer Chromatography (TLC), Total Phenolic content, Total Flavonoid content, Free Radical Scavenging assay and GC-MS were carried out for the qualitative and quantitative estimation and identification of biologically significant phytocomponents.

The results obtained in the current study greatly support the emergence of T. terrestris (TT) as a source of immense medicinal and therapeutic potential. Different parts of the plant are widely being used to treat various ailments and there have been reports of successful animal and clinical trials. However, the shortcomings still lie in the fact that phytoconstituents of the species vary with changing geographical and climatic conditions. Hence, further research needs to be carried out for standardization and gaining deeper insights into molecular diversity.

Keywords: *Tribulus terrestris,* phytochemistry, total phenolic content, ethnobotany, GC-MS.

Introduction

T. terrestris is a perennial creeper, predominant in temperate and tropical regions of Central and South Europe, Asia, South Africa, South and West Australia and parts of North and South America[9,16]. This aggressive and hardy invasive species is well adapted to arid climatic conditions where only few plant species survive. It is well known for its therapeutic potential and can be traced upto 5,000 years back, mainly in Indian and Chinese traditional medicine practices. Different parts of the plants are used for the treatment of eye disorders, bloating, fluid retention, pathological leucorrhoea, sexual dysfunction and as diuretic, stimulant and anti-urolithiatic[8,13]. A herbal tonic prepared from the plant is used for eye and throat disorders and diarrhoea in South Africa[12].

Bulgarian Folk medicine reports use of *T. terrestris* for blood purification and in haemorrhoids[13]. *Tribulus*, also known as puncture vine, yellow vine, Devil's horn, is popular by several regional names in different parts of India such as *Gokhru*/*Gokshura* in Bengal, *Beta-Gokhru* in Gujrat, *Negalumullu*, *Neringil, Nerunjimullu, Sirunerunji, Chiripalleru* etc., in South India, *Kokullak* in Ladakh and *Bhakra* in Punjab. This study aims at finding the therapeutic potential of *T. terrestris* through phytochemical analysis. Several bioassays were carried out to determine the applications and the bioactive compounds that may be responsible for the results obtained.

Origin: The species is considered native to the Mediterranean and Eurasia. It probably originated from the Sahara desert region of Africa and later on spread to dry temperate regions of the world as alien or invasive species[4,9].

Habitat and Distribution: *T. terrestris* is the most common of the 25 species of genus Tribulus. It is a noxious weed, native to warm and dry temperate and tropical regions of Eurasia, Africa and Australia. Well adapted to light textures soils, it can grow in several soil types, generally found as weed in cultivated crops, pastures and other unused land areas[13]. It is commonly found throughout India and has even been reported to grow at an altitude of 11,000 ft in Western Tibet. It is growing in all continents except Antarctica[4].

Classification

Kingdom	Plantae
Division	Phanerogams
Subdivision	Angiospermae
Class	Dicotyledonae
Subclass	Polypetalae
Series	Disciflorae
Order	Geraniales
Family	Zygophyllaceae
Genus	*Tribulus*
Species	*terrestris* Linn.

Plant Characteristics: *Tribulus* is perennial, procumbent, bearing silky hair on young green shoots that can grow upto 30-70 cm long. Simple or profusely branched stems exhibit opposite phyllotaxy with unequal leaves. Stomata are anomocytic type with no subsidiary cells present around guard cells. Small stipules along with five to seven pairs of

leaflets are present in the axil of smaller leaf of each pair. Small pentamerous flowers, 4-5 mm in diameter, are attached to lobed annular disc. Petals are yellow in colour and slightly longer than sepals. The inner floral whorls consist of ten stamens and carpel having ovary with five cells. Fruits are woody burr type with five wedge shaped segments. Spiny nature of fruits protects against grazing. Upto 5 yellow-coloured seeds are enclosed in woody stellate carpels. Upto 2000 seeds may be present in each plant. Roots are brown coloured, fibrous and cylindrical[5]. The morphology of *T. terrestris* has been reported to be extremely variable in terms of hairiness and basal spines[10].

Material and Methods

Plant Collection and Drying: Complete *T. terrestris* plants along with roots were collected from natural habitat during fruiting season. Specimen was submitted to Herbarium, Department of Botany, University of rajasthan and RUBL number (RUBL211799) was obtained for specimen identification. Plants were washed and fruits were hand-picked. Fruits were washed thrice in plain water and shade dried for 20 days. Dried fruits were crushed into coarse particles using metallic mortar pestle and further ground into fine powder with the help of mixer-grinder. Powdered form was stored in phyta-jar.

Extract Preparation: Soxhlet and Ultrasonic-Assisted Extraction (UAE) methods were employed for preparation of crude methanolic extracts from Tribulus powder. Soxhlet extraction was carried out using the method of Subramaniam and Nagarjan. Hundred grams of powder were extracted in 300 mL of methanol for 24 hours. UAE was done for 20 minutes at 50°C. The liquid extracts were filtered and the filtrates were kept at room temperature for drying and later stored in glass vials at 4°C for further use[21].

Phytochemical Screening: Aqueous solution of dried methanolic extract was used to test for the presence of secondary metabolites/phytochemicals using standard reactions as listed by Das and Gezici[7]. All the reactions were done in triplicate.

Alkaloids: Test sample was acidified with 2% HCl at 60°C for two hours and later cooled and filtered. Formation of white precipitate on adding 2 mL of either modified Mayer's reagent or Wagner reagent indicated presence of alkaloids. Development of orange colour on spraying Dragendorff's reagent on TLC plate of crude extract further confirms the presence of alkaloids.

Flavonoids: Appearance of yellow colour on addition of dilute ammonia solution to test sample followed by conc. H_2SO_4 indicated presence of flavonoids. Yellow colour appears on adding NaOH to test sample and disappears on addition of acid. This indicates presence of flavonoids.

Steroids: Chloroform and conc. H_2SO_4 were added to test sample and test tube shaken well. Appearance of red colour in chloroform layer and greenish yellow in acid layer indicates steroidal presence. Addition of acetic anhydride to test sample followed by heating and subsequent cooling results in formation of blue colour, indicating presence of steroidal compounds.

Phenolic Compounds: Few drops of ferric chloride in test sample result in formation of bluish-black colour indicates presenting of phenolic compounds. Yellow coloured precipitate is formed on adding few drops of lead acetate to test sample. This indicates presence of phenolic compounds.

General test for Saponins: Formation of froth on shaking aqueous solution of sample indicates saponin presence.

General Test for Tannins: Aqueous solution of extract was heated and filtered. Addition of ferric chloride to filtrate results in dark green colour formation.

Thin Layer Chromatography (TLC): Repeated TLC runs were carried out using solvents of varying polarity as mobile phase in order to obtain spots for compounds of different chemical nature. Silica gel G was used as adsorbent. Retention factors were calculated for all spots using the formula:

$$R_f = \frac{\text{Distance Travelled by Spot}}{\text{Distance Travelled by Solvent}}$$

Compounds were identified with the help of *Phytochemical Methods:* A Guide to Modern techniques of Plant Analysis[24].

Biological Assays

Total Phenolic Content (TPC): TPC of the crude methanolic extract was estimated using the conventional Folin-Ciocalteu method using gallic acid as standard and with slight modifications[20]. 0.5 mL of 1 mg/mL crude aqueous extract was taken in a test tube and made 3 mL with distilled water. 0.5 mL of Folin-Ciocalteu reagent was added and left to sit for 3 min. This was followed by adding 2 mL of 20% sodium carbonate solution. Calibration tubes were prepared from a stock solution of 1mg/mL of gallic acid ranging from 120 µg/mL upto 600 µg/mL with an interval of 120 µg/mL. 0.5 ml of calibration standards was taken in different test tubes and same protocol that of test sample was followed for each tube. A blank tube was also prepared with distilled water keeping all other steps same.

The reaction tubes were kept in dark for 120 min with intermittent shaking. Later, absorbence was measured at 765 nm. Calibration curve was plotted and TPC of *T. terrestris* was calculated with the help of the standard curve.

Total Flavonoid Content (TFC): TFC was estimated using a previously described aluminium chloride method[17] with slight modifications. Briefly, 0.5 mL of 10 mg/mL crude methanolic plant extract was taken in test tube and made 2 mL with methanol. 0.1 mL of 10% $AlCl_3$ was added to the

test tube followed by 0.1 mL of 1M potassium acetate. Lastly, 2.8 mL distilled water was added. Blank was prepared by using 0.1 mL of distilled water in place of aluminium chloride. Quercetin was used as standard for making calibration curve. Dilutions of 6.25, 12.5, 25, 50, 80 and 100 µg/mL of quercetin were prepared in methanol and aliquots were prepared in similar manner as test sample. The reaction mixtures were incubated for 30 min with intermittent shaking and absorbence was measured at 415 nm. TFC was expressed in terms of mg of quercetin equivalent

Antioxidant Assay: Anti-oxidant assay was carried out following the well-established DPPH Assay as previously described[21] with slight modifications. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) and ascorbic acid as standard were acquired from Sigma-Aldrich and Merck respectively. Methanolic solutions of plant extract and DPPH stock were used. 0.1 mL of 10 mg/mL test sample and 0.1 mL of 10mM standard solution were taken in test tubes containing 3.9 mL of 0.06mM DPPH. Blank was prepared with 0.1 mL methanol in DPPH solution. The reaction mixtures were wrapped in aluminium foil and incubated for 15 min in dark. Absorbance was measured at 515 nm at intervals of 15 min. Complete experimental procedure was carried out in dim light. Percent DPPH radical scavenging was calculated by the formula:

$$\% \text{ DPPH Radical Scavenging} = \frac{(\text{Abs. of Control} - \text{Abs.of test Sample})}{\text{Abs. of Control}} \times 100$$

GC-MS: GC- Mass Spectroscopy was employed for phytochemical profiling of *T. terrestris* in order to identify the marker compounds and bioactive principles that may be responsible for the biological activities and therapeutic potential of the plant. Dilute ethanolic solution of dried plant extract was prepared and subjected to GC-MS in a single quadrupole GCMS QP-2020 Plus (Shimdzu, India). Sample was run in a 30 m long Rxi-5Sil MS (Crossbond, 5% diphenyl/ 95% dimethyl polysiloxane) column having internal diameter of 0.25mm and film thickness of 0.25 m. Sample was injected at 230°C in split mode with split ratio of 1:10 and column flow rate of carrier gas (He) at 1.24mL min^{-1} maintained throughout the run. Oven temperature was initially maintained at 40°C for 5 min and later on at 280°C. The temperatures of ion source and interface were both maintained at 280°C and the solvent cut time was set at 2.5 min to prevent the detector from being saturated with solvent ions during MS. The mass scan spanned from m/z 40 to m/z 400. The mass spectra for each separated compound were recorded followed by identification with the help of NIST17 library database.

Results

The dried and powdered plant material was subjected to two types of extraction procedures *viz.,* Soxhlet and Ultrasonic Assisted Extraction (UAE) in order to compare the efficiency of the two extraction procedures. Methanol was used as solvent in both cases and several parameters were compared as mentioned in table I. UAE proved to be a more efficient extraction method in this case as a higher amount (0.084 g) of extract per gram of plant material was obtained through it against 0.0693 g of extract per gram of plant material obtained through Soxhlet extraction.

UAE also proved to be highly time efficient since it required only 30 minutes for the results while Soxhlet was run for about 24 hours. However, a higher solvent efficiency was observed in Soxhlet extraction where 500 mL of solvent was used for extraction of 100g of plant material against 100 mL of solvent consumed for extraction of 10g plant material in UAE.

Crude extracts prepared through both extraction procedures were separately subjected to various phytochemical screening procedures. Initially, preliminary tests for the detection of secondary metabolites including alkaloids, flavonoids, steroidal saponins, phenolic compounds and tannins, were carried out and both types of extracts showed positive responses for all as summarised in table II.

Thin Layer Chromatography (TLC): The extracts were then subjected to chromatographic resolution using various solvents for identification of different categories of probable compounds. A total of 18 compounds were identified through various spot resolution methods and calculation of retention factor (R_f). A summary is provided in table III along with the chemical nature of the compounds and solvents used.

Table I
Comparative study of extraction procedures.

S.N.	Property	Soxhlet Extraction	Ultrasonic Extraction
1	Weight of PM	100 g	10 g
2	Vol of Solvent	500 mL	100 mL
3	Solvent/g of material	5 mL	10 mL
4	Dry wt. of extract	6.93 g	0.84 g
5	Wt. of extract / g of PM	0.0693 g	0.084 g
6	Time Taken	24 Hours	30 min
5	Solvent Used	Methanol	Methanol
6	Texture of extract	Fine Powder	Fine Powder

Total Phenolic Content (TPC): Total phenolic contents of both extracts were estimated by plotting standard curve of concentration of gallic acid (µg/mL) against absorbance taken at 765 nm. The UAE extract showed a considerably higher phenolic content (70.458 µg/mL) as compared to the Soxhlet extract (65.104 µg/mL). The standard curve values are as shown in table IV and depicted in fig. I along with extract concentrations.

Total Flavonoid Content: TFC of both extracts was also calculated by plotting standard curve of various concentrations of quercetin (µg/mL) against absorbance taken at 415 nm. The corresponding concentrations of extracts were calculated using the graph based on the absorbance recorded. Similar to TPC, higher TFC was observed in UAE extract (10.955 µg/mL) as compared to Soxhlet extract (9.32 µg/mL) as depicted in fig. II. The absorbance and corresponding concentration values for the quercetin and TT extracts are given in table V.

Anti-Oxidant Assay: Percentage Radical Scavenging Activity (% RSA) of the given extracts was calculated through DPPH Assay. The absorbance at 515 nm for blank, standard and both extracts was taken at 0, 15 and 30 min of reaction and the % RSA of extracts was calculated using the formula:

$$\% \text{ DPPH Radical Scavenging} = \frac{(\text{Abs. of Control} - \text{Abs.of test Sample})}{\text{Abs. of Control}} \times 100$$

It was observed that TT shows high anti-oxidant potential. On comparison of the two extracts, the anti-oxidant activity of UAE extract (approx. 61%) was found to be significantly higher i.e. more than double that of Soxhlet extract (approx. 30%) immediately after the reaction at 0 min. After 15 min, %RSA of both extracts was lowered, however, UAE extract still showed higher antioxidant potential (43.6 %) than that of Soxhlet extract (23.3%). At 30 min, both extracts showed almost equal values with UAE extract having 23% and Soxhlet extract having 22% RSA. Graph was plotted to compare the anti- oxidant potential of both extracts at different time intervals as shown in fig. III. Table VI depicts values of absorbance of all reaction mixtures at 515 nm and %RSA values of both types of TT extracts.

Table II
Preliminary detection of Secondary Metabolites

S.N.	Secondary Metabolites	Soxhlet Extract	UAE Extract
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Steroidal Compounds	+	+
4	Phenolic Compounds	+	+
5	Saponins	+	+
6	Tannins	+	+

Table III
Probable compounds identified through TLC

S.N.	Probable Compound	Chemical Nature	Solvent Used
1	Methoxy-cinnamic Acid	Carboxylic Acid	BAW
2	Glycine/Serine	Amino Acid	BAW
3	Kaempferol	Flavonoid	BAW
4	Apigenin	Flavonoid	Forestal
5	Chrysoeriol	Flavonoid	Forestal
6	Myricetin	Flavonoid	Forestal
7	Tricin	Flavonoid	Forestal
8	Gitogenin	Steroidal Saponin	Chloroform:Ethyl Acetate
9	Diosgenin	Steroidal Sapogenin	Chloroform:Ethyl Acetate
10	Tigogenin	Steroidal Sapogenin	Chloroform:Ethyl Acetate
11	SQDG	Glycoglycerilipid	CHCl3:MeOH:Acetic Acid:Water
12	Diacylgalactosylglycerol	Glycoglycerilipid	CHCl3:MeOH:Acetic Acid:Water
13	Phosphatidylethanolamine	Phospholipid	CHCl3:MeOH:Acetic Acid:Water
14	Triacylglycerol	Triglyceride	CHCl3:MeOH:Acetic Acid:Water
15	Gallic Acid	Phenolic Acid	Ethyl Acetate:Benzene
16	Phloroglucinol	Phenolic Acid	Ethyl Acetate:Benzene
17	Vanillic Acid	Phenolic Acid	Ethyl Acetate:Benzene
18	Protocatechuic/Gentisic Acid	Phenolic Acid	Ethyl Acetate:Benzene

Table IV
TPC standard curve values

Sample	Absorbance at 765 nm	Concentration (µg/mL)
1	0	Blank
2	0	40
3	0.0428	60
4	0.113	80
5	0.2663	100
6	0.3195	120
7	0.4638	140
8	0.6205	160
9	0.7782	180
10	0.8264	200
11	0.9409	220
12	0.9409	240
TT-S	0.1397	65.104
TT-U	0.1654	70.458
TT-S: *Tribulus terrestris*-Soxhlet Extract TT-U: *Tribulus terrestris* -Ultrasonic Extract		

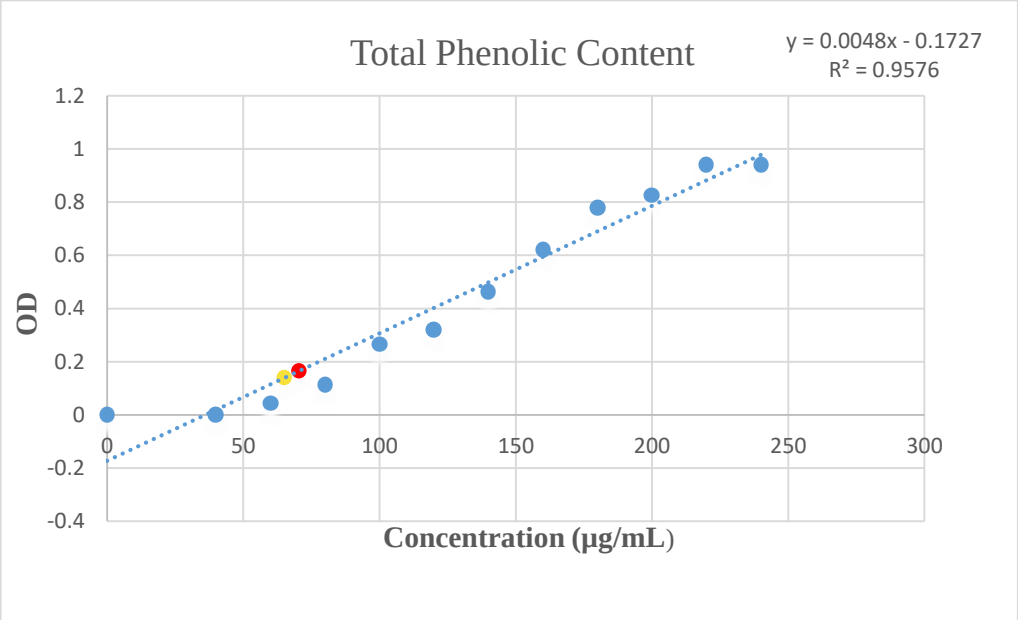

Fig. I: TPC standard curve depicting concentration of UAE and Soxhlet extracts

Table V
TFC Standard Curve Values

Sample	Absorbance at 415 nm	Concentration (µg/mL)
1	0	Blank
2	0.0653	6.25
3	0.1902	12.5
4	0.3967	25
5	0.7884	50
6	1.2813	80
7	1.5366	100
TT-S	0.1397	9.32
TT-U	0.1654	10.955
TT-S: *Tribulus terrestris*-Soxhlet Extract TT-U: *Tribulus terrestris* -Ultrasonic Extract		

GC-MS: GC-MS for TT was carried out to analyse the phytochemical constitution and to identify compounds/metabolites that may be responsible for the biological activities as previously described. At the end of the run, sixty-four compounds were listed along with their retention time, peak area, area percentage, height percentage and similarity index was used for identification from NIST Mass Spectral Libraries. Highest area percentage (43.37%) was octadecadienoic acid followed by n-hexadecanoic acid. Many of the compounds identified were organic acids which may have significant roles in biological cycles and metabolism. Some other metabolites belonging to flavonoids, phenols, alkaloids and steroidal saponins which have been reported to exhibit significant biological activities, were identified and are listed in tables VII and VIII.

Discussion

Tribulus terrestris is generally considered a weed and its aggressive colonizing nature is well known. Despite efforts to control the spread, its practical potency is gradually burgeoning. TT is generally regarded as safe in high doses and there have been no reports of adverse effects in animal and/or clinical trials.

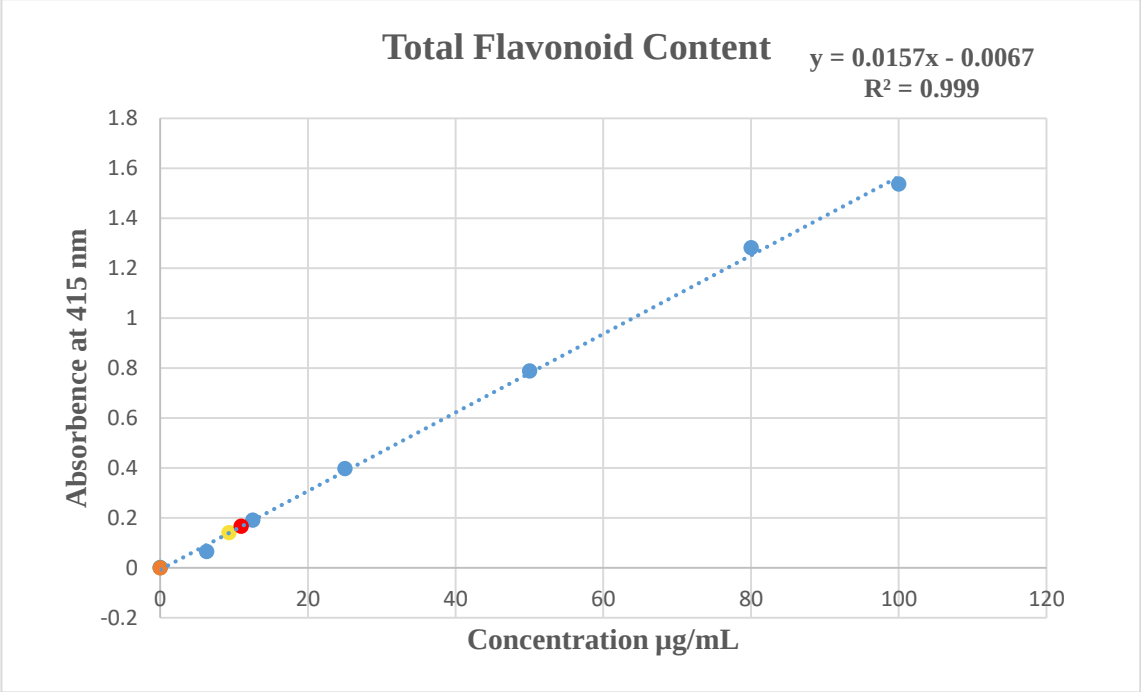

Fig. II: TFC standard curve depicting concentrations and Absorbance values of Quercetin and UAE and Soxhlet extracts

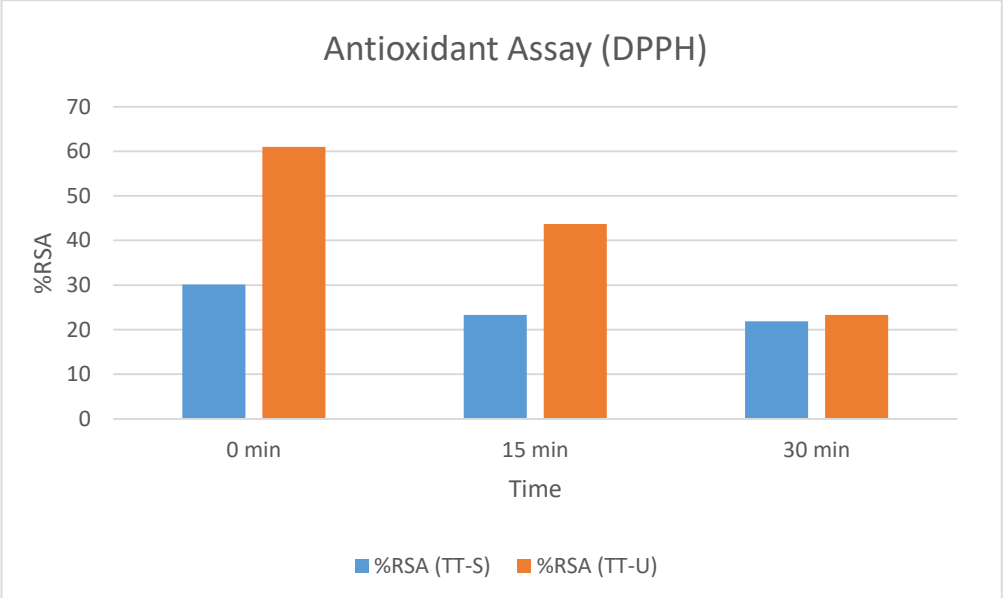

Fig. III: Comparative Analysis of anti-oxidant potentials of Soxhlet and UAE extracts of *Tribulus terrestris*

Table VI
DPPH Assay

	Absorbance at 515 nm				%RSA	
Time/Sample	**Blank**	**Standard (10mg/mL)**	**TT-S**	**TT-U**	**TT-S**	**TT-U**
0 min	0	-1.5441	-1.0792	-0.6021	30.10815362	61.0064115
15 min	0	-1.349	-1.035	-0.7597	23.27650111	43.68421053
30 min	0	-0.6734	-0.526	-0.5166	21.88892189	23.28482328
% RSA : Percentage Radical Scavenging Activity; TT-S: Soxhlet extract of *Tribulus terrestris*; TT-U: Ultrasonic Assisted Extract of *Tribulus terrestris.*						

Table VII
Compounds identified from GC-MS

S.N.	RT (min)	Compound	Peak Area (%)	SI Value	Chemical Formula
1	19.21	Azaleic Acid	0.51	95	$C_9H_{16}O_4$
2	25.51	Phytol	0.07	79	$C_{20}H_{40}O$
3	30.15	ɣ-Sitosterol	0.49	80	$C_{29}H_{50}O$
4	23.58	Palmitoleic Acid	0.07	90	$C_{16}H_{30}O_2$
5	13.37	Cinnamaldehyde	0.05	86	C_9H_8O
6	12.77	Cyclohexane	0.12	89	$C_{12}H_{24}$
7	24.32	Tetratetracontane	0.07	83	$C_{44}H_{90}$
8	27.39	Hentriacontane	0.38	88	$C_{31}H_{64}$
9	27.57	Eicosatrienoic Acid	1.04	79	$C_{20}H_{34}O_2$
10	30.15	β-Sitosterol	0.49	77	$C_{29}H_{50}O$
RT=Retention Time; SI= Similarity Index					

Table VIII
Biological Activities of Metabolites

S.N.	Compound	Biological Role
1	Azaleic Acid	Rosacea, Melasma, Acne vulgaris[19]
2	Phytol	Antitumor, Anticonvulsant, Antinociceptive[14]
3	ɣ-Sitosterol	Immunomudulatory, Antidiabetic, Antinociceptive[1]
4	Palmitoleic Acid	Increases Insulin sensitivity, Decreases risk of diabetes[3]
5	Cinnamaldehyde	Antimicrobial, Antioxidant, Anticancer, CNS Disorders, Arthritis[11,23]
6	Cyclohexane	Antimicrobial[22]
7	Tetratetracontane	Antibacterial and Cytoprotective[2]
8	Hentriacontane	Anti-tumor[15]
9	Eicosatrienoic Acid	Anti-Inflammatory[5]
10	β-Sitosterol	Immuno-Modulatory, anti-cancer,used for heart diseases, hypercholesterolemia, tuberculosis, cervical cancer[18]

Its extract has been reported to be effective in breathing disorders, urinary dysfunction, oxidative stress management exhibiting antihypertensive, antitumor, cytotoxic and vasodilatory properties. The growing medicinal and pharmaceutical significance of TT may be attributed to wide assembly of steroidal saponins which have also been reported to exhibit hypoglycemic and hypolipidemic effects in animal trials[13]. On the basis of recent scientific studies and in view of the immense therapeutic potential that the herb holds, it is only natural to gain deeper insights of its phytochemistry and active principles that may be responsible for its biological activities. Given the observations from present study and existing literature, it is clear that the plant is host to several biologically significant substances such as saponins, alkaloids, flavonoids, cinnamic acid amides. They may however, vary qualitatively and quantitively depending on part of herb used and geographical and prevalent climatic conditions.

Several metabolites such as phytosteroids, flavonoids, alkaloids and glycosides are reported to be prevalent in fruit and root while leaf tissue is rich in saponins and derivatives like diosgenin, gitogenin and chlorogenin. These differences are also responsible for the product-to-product variations

seen in market products that is why standardization of such products is extremely crucial for public utility. Libilov, a formulation of the saponin fraction of *T. terrestris* is reported to have stimulating effect on spermatogenesis through testosterone secretion.

Scientific studies have also revealed effect on TT saponin in improved coronary circulation by means of coronary artery dilation. The alcoholic extracts of TT growing in certain regions of the world have also been reported to exhibit antimicrobial activities. Its efficacy has been reported against vitiligo[13]. TT has long been used in Indian medicinal system against impotency and cardiovascular disorders in the form of crude saponin formulations.

Conclusion

In order to explain the variations in phytoconstituents, correlation studies between morphology and phytochemistry will be required. Additional investigations for biological significance of individual compounds may be able to shed greater light on the potential of this ever-emerging medicinal herb. Also, little information is available on molecular basis of biodiversity and molecular markers[6].

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