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EFFECT OF SEASONAL AND GEOGRAPHICAL VARIATION ON THE PHYTOCONSTITUENTS AND MEDICINAL PROPERTIES OF *TRIBULUS TERRESTRIS*

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Abstract:
Seasonal and geographical variations have impact on secondary plant metabolites in medicinal plants. In the present study an attempt was made to evaluate the phytochemical variation and its effect on the medicinal properties of Tribulus terrestris which was collected from two different seasons (Summer and winter) and from two different geographical regions i.e. Rafha, and Riyadh, Saudi Arabia (hot desert climate) and Bangalore, India (tropical savanna climate).The amount of Phenolic acids and flavonoids was evaluated by Folin ciocalteu method and Aluminum chloride colorimetric method respectively. Anti-oxidant activity was carried out for all the extracts by the DPPH method .The results revealed that the amount of phenolic acid and flavonoid was found to be higher i.e. 428 µgm and 295 µgm respectively in TT1-S which was in the sample collected during summer from the Rafha region. To further investigate the best season of collection of samples in Rafha, HPLC analysis was carried out for TT1-S and TT1 –W extracts using rutin and gallic acid as the standards; the amount of rutin and gallic acid was found to be higher in TT1-S. Furthermore analgesic activity was carried out for these extracts and the sample collected during summer exhibited significant activity when compared to the sample collected during winter. The results of this study give evidence that the season and geographical variations bring about a change in the plant metabolites which in turn affect the medicinal properties .From the results it is evident that the best season to collect the plant is during summer in Rafha as the amount of phytoconstituents is found to be more during this season

Key words: *Tribulus terrestris, seasonal, geographical, phytoconstituents, medicinal properties*

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INTRODUCTION:

The variability in the phytoconstituents in medicinal herbs due to genetic, cultural and environmental factors has made the use of herbal medicines more challenging. Season and geographical variations has impact on secondary plant metabolites (active principles) in medicinal plants. The therapeutic efficacy of the plants varies during different times or seasons of the year. The quantity of the phytoconstituents varies quantitatively at different seasons of the year and the majority of plants are usually collected during season when the herbs are at peak maturity and concentration [1]. Literature in the ancient Ayurvedic texts has mentioned the awareness of the physicians about relation between period of collection and distribution of active principles [2]. Secondary metabolites play a major role in the adaptation of plants to the environment. Environmental factors like temperature, humidity, light intensity, etc influence the growth of a plant and secondary metabolite production [3]. Literature reveals that temperature stress can affect the secondary metabolites and other compounds that plants produce which are usually the basis for their medicinal activity [4,5]. Many researchers have demonstrated that plants collected from different geographical regions are diverse in the chemical composition[6,7]

Tribulus terrestris is an annual or perennial herb, belonging to the family Zygophyllaceae .It is commonly referred to as English plantain, buckhorn plantain, and lamb's tongue. In Arabic it is called by various names some of them being Darisa , dacn-ash-sheikh; kharshoom-an-naga; kotaba.

The plant is native to warm temperate and tropical regions of the Old World in Southern Europe, southern Asia, throughout Africa, and Australia. It can survive even in desert climate [8]. It is known for its use in the traditional medicine of many countries for treatment of cardiac diseases, edema, skin itch, acute conjunctivitis, headache, vertigo, edema, abdominal distention, anthelmentic, vitiligo .It is also used in the folk medicine of India in Ayurveda for its anti-urolithiatic, diuretic and aphrodisiac properties [8-11]. Local Bedouin use the plant in the treatment of urinary disorders, impotency, and liver diseases. The seeds are used to treat hemorrhages, kidney stone and gout. The fruit is considered to be tonic, diuretic, aphrodisiac and hepatoprotective .The other activities reported are in infertility therapy antioxidant, tective , anti-inflammatory, antispasmodic, anti-hyperglycemic, cardio protective, antitumor [12-19]

In the present study an attempt was made to evaluate the phytochemical variation in the plants collected from two different seasons(Summer and winter) and from two different geographical regions i.e. Rafha and Riyadh, Saudi Arabia (hot desert climate) and Bangalore, India(tropical savanna climate).

Rafha is a town in the Northern Border province of Saudi Arabia close to the border with Iraq. It is located at around 29°38′19″N 43°30′5″E. It is the second largest city in the Northern Borders Province (also known as Al-Hudud ash Shamaliyah). Köppen-Geiger climate classification system classifies its climate as hot desert [20].

Bangalore is the capital of South Indian state of Karnataka, India; at a height of over 900 m (3,000 ft) above sea level, Bangalore is known for its pleasant climate throughout the year. It is located at 12.97°N 77.56°E. It has a tropical savanna climate (Köppen climate classification) [21]

MATERIALS AND METHODS:

1. Collection of the plant:

Tribulus terrestris which are wildly grown ,were collected from (a)Rafha, Northern Border Province Saudi Arabia, (b) Bangalore, Karnataka, India; in two seasons i.e. summer and winter in 2016 and one sample was collected from(c) Riyadh province (winter).The samples were shade dried, pulverized and stored in the research lab of Faculty of Pharmacy,Rafha, Northern Border University . Hence forth the sample collected from Rafha, Saudi Arabia will be referred to as TT1 [TT1-S (Collected in summer) and TT1-W (collected in winter)], Sample collected from Bangalore, India will be referred to as TT2 [TT2-S (Collected in summer) and TT2-W (collected in winter)], while the sample collected from Riyadh is TT3 [TT3-W (collected in winter)]

2. Extraction of the plant material:

The different samples of the plant were subjected to extraction with methanol. Extracts were collected, evaporated and vacuum dried.

3. Phytochemical analysis:

3(a) Preliminary phyto chemical analysis: The various extracts of the plant were subjected to qualitative analysis for the phytoconstituents like alkaloids, carbohydrates, glycosides, steroids, tannins, proteins, amino acids and flavonoids as per the standard procedures[22].

3(b) Determination of total phenolic content in *Tribulus terrestris* [23]

The total phenolic content was estimated by the Folin ciocalteu method for all the extracts. Different concentrations of gallic acid were prepared using methanol for plotting the standard graph. For the test samples 1ml each was pipetted out and 9 ml of distilled water was added. A blank was maintained by taking 10 ml of distilled water and to this 1 ml of Folin ciocalteu reagent was added. Mixed well, after five minutes 10 ml of 7% standard sodium carbonate solution was added to the flasks. Made up the volume

of all the flasks up to 25 ml with distilled water. The absorbance was recorded at 750 nm in the UV spectrometer. A standard graph plotting absorbance versus the concentration was obtained from the standard gallic acid. The total phenolic content was expressed as gallic acid equivalent.

3(c) Determination of flavonoids in *Tribulus terrestris*

The flavonoid content of the plant extracts was evaluated by the Aluminum chloride colorimetric method [23].The sample of the plant extract was mixed with 1.5 ml of methanol, 0.1 ml of 1M potassium acetate 0.1 ml of 10 % aluminum chloride and 2.8 ml of distilled water .The absorbance of this mixture was measured at 415 nm. A calibration curve for the standard quercitin was obtained by taking different dilutions. The total flavonoid contents were calculated as quercitin equivalent from the calibration curve by plotting the absorbance versus concentration.

3(d).Colorimetric estimation of condensed tannins by vanillin assay[23]:

Aliquots of the standard Catechin in the range of 50-350 µg/ml were transferred to two sets of tubes and the volume in each of the tubes was brought to 1 ml by methanol. The tubes were incubated at 30° C in a water bath. Five ml of working reagent was added at an interval of 1 min to one set of the tubes and 5 ml of 4 % HCl was added to the second set at intervals of 1.0 min (blank).The samples were kept in the water bath for 20 min and the absorbance was recorded at 500 nm. The absorbance of the blank was subtracted from that of the sample containing vanillin reagent. The same procedure was followed for the sample. The amount of condensed tannins contents were calculated as Catechin equivalent from the calibration curve of standard Catechin by plotting the absorbance versus concentration.

3(e).HPLC Analysis

Shimadzu High Performance Liquid Chromatographic system equipped with LC-10ATVP pump, a rheodyne injector, SPD M10AVP Photo Diode Array Detector in combination withCLASS-VP 6.12 SP5 integration software.Running conditions included: injection volume 50 µl, mobile phase, Acetonitrle : Phosphate buffer(0.005M) pH3, ratio of 70:30,rate, 1 ml/min, detection at 254nm, Elution Type: Isocratic. Gallic and rutin were used as standards and were identified by comparing the chromatographic peaks with the retention time of the standards.

4.Medicinal properties:

4(a)Anti-oxidant activity [24]:

The anti-oxidant activity of the extracts was evaluated by using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH). The stock solution of the extracts was prepared in methanol. The working solutions of the extracts were prepared using suitable dilutions. The anti-oxidant activity was determined based on the radical scavenging effect of the stable 1,1-Diphenyl-2-picryl-hydrazyl.DPPH was prepared as 0.002 % solution in methanol and mixed with 1ml of both the standard and the samples. The prepared solutions were kept in the dark for ½ an hour and the absorbance was measured at 517 nm.

The % absorbance was calculated using the formula:

$$\% \text{ Absorbance} = \frac{A - B \times 100}{A}$$

Where A is the absorbance of the blank and B is the absorbance of the sample.

4(b) Analgesic activity

The analgesic activity of TT1-S and TT1-W was evaluated using Eddy's hot plate [23]. Rats of either sex weighing 250-275 g were used. The animals were divided into groups; Group 1 served as the control, group 2 as standard and group 3 and 4 were used for the higher and lower dose of TT1-S while group 5 and 6 for TT1-W .The animals were placed on the hot plate and the time taken for licking was be recorded.

RESULTS AND DISCUSSION:

Tribulus terrestris were collected from the geographical regions as shown in table 1. They were shade dried in the research lab of Faculty of Pharmacy, pulverized and stored until further use

Table 1: Geographical details:

Sample	Place	Latitude	Longitude	Altitude	Climate
1TT	Rafha -Saudi Arabia	29°38′19″N	43°30′5″E	508m	Hot desert
TT2	Bangalore –India	12.97°N	77.56°E	900 m	Tropical savanna climate
TT3	Riyadh-Saudi Arabia	24.7136° N	46.6753° E	612 m	Hot desert

Table 2: Amount of total phenolics, flavonoids and condensed tannins in the extracts

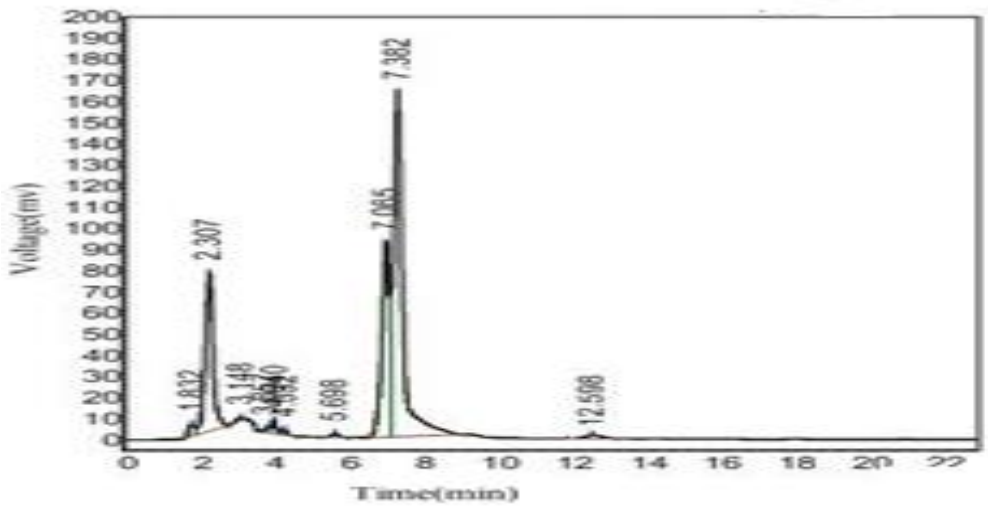
Extract	Total phenolics µgm	Total flavonoid Mgm	Condensed tannins µgm
TT 1-S	428	295	212
TT 1-W	195	206	138
TT 2-S	325	218	282
TT2-W	238	124	230
TT3 -W	186	210	200

The extracts were subjected to preliminary phytochemical analysis which revealed that the plant under investigation contained alkaloids, glycosides, tannins, flavonoids, saponins, sterols, carbohydrates and proteins. The deserts plants almost contain variety of secondary metabolites like flavonoids and phenolic acids to protect themselves from herbivores. Thus it was valuable and interesting to perform a comparative study selecting a plant that grows in a desert as well as tropical climate and also to study the variation in the amount of phytoconstituents due to this change in the geographical terrain and at the same time identifying the ideal season for collection. Literature survey reveals that plant metabolites like phenolic compounds, flavonoids, tannins sterols etc. play an important role in many of the activities some of which being the anti-oxidant and analgesic activities [25]. The bioactive phytochemicals and other secondary metabolites are particularly prone to qualitative and quantitative variations depending on several factors like temperature, season of collection, developmental stages, geographical variation etc. Secondary metabolites play an important role in the adaptation of plants to the environment and in overcoming stress conditions. Drought, high salinity, and freezing temperatures are environmental conditions that cause adverse effects on the growth and metabolite production of the plant [26]. With an aim to study the difference in the phytoconstituents due to variation in season of collection and the geographical source the total phenolic acids, total flavonoids and condensed tannin content were evaluated. The amount of total phenolics, flavonoids and condensed tannins in the extracts are as shown in table 2.

The amount of total phenolic acid was evaluated by the Folin Ciocaltteu method. It is one of the important methods for the quantification of plant phenolics, it measures the amount of the substance (sample) that is needed to inhibit the oxidation of the reagent i.e. Folin Ciocaltteu reagent. It is evident from the results of the phytochemical analysis that the amount of phytoconstituents like flavonoids and phenolic acids were significantly higher in the plant collect during summer (TT1-S) from the desert region of Rafha, Saudi Arabia. The sample collected from Rafha and Riyadh Province during winter (TT1-W and TT3-W respectively) did not show any significant variation in the amount of phenolic acids and flavonoids this could probably be because of similarity in the climatic conditions. The amount of phenolic acid was found to be more in TT1-S, i.e. 428 µgm, followed by TT2-S, i.e. 325 µgm of gallic acid. Similarly the amount of flavonoids present in TT1-S was the highest 295 µgm when compared to the other extracts. The higher content of phenolic acids and flavonids in TT 1-S maybe attributed to the adaptation of plants to stress in the desert area. Literature reveals that the plants those grow on low nutrient soils and under harsh climatic conditions (extreme temperatures, intense solar radiation, and water scarcity) are specifically susceptible to oxidant stress. Hence they have efficient defense systems. It has been reported that the desert plants seem to particularly adapt themselves so as to protect them against the ravages of a harsh environment [27]. The amount of condensed tannins was found to be more in TT2-S i.e. 282 µgm while there was no significant difference in the amount of condensed tannins in the other extracts. A similar study conducted in Israel reported higher content of anthocyanins, a class of flavonoids in pomegranates from the desert climate when compared to those from the Mediterranean climate [28].

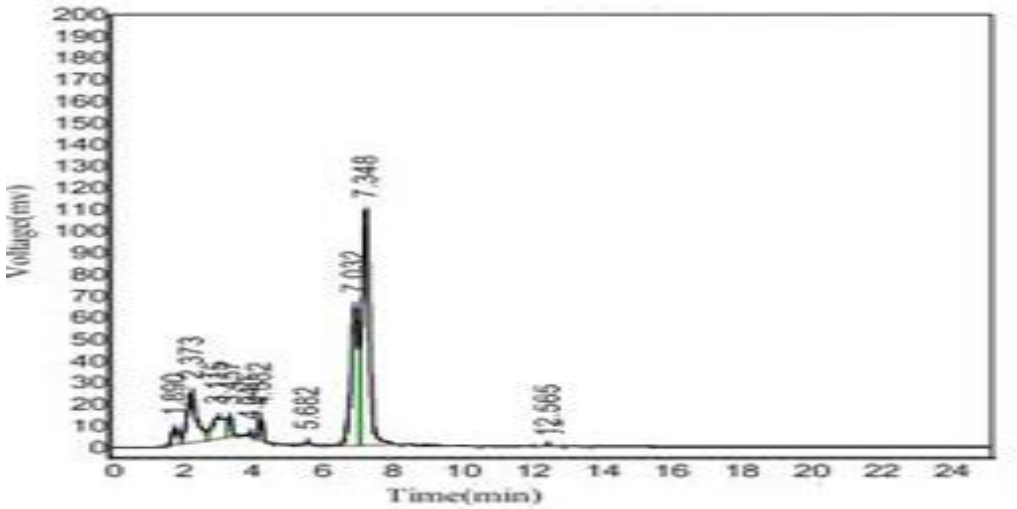
High performance liquid chromatography is one of the important and popular methods for analysis of plant extract. Each phytoconstituent has a characteristic peak and is detected by the detector. The HPLC analysis was carried out using rutin and gallic acid as biomarkers representing the two important class of phytoconstituents i.e. flavonoids and phenolic acids. Rutin has been reported to be one of the important bioactive phytoconstituents in *Tribulus Terrestris* [29] .TT1-S and TT1-W extracts were selected for HPLC analysis as the interest of our research lies in finding out the period of collection of this plant in Rafha, Saudi Arabia.

HPLC analysis of the two extracts has shown a difference in the amount of gallic acid and rutin. Rutin showed a retention peak at 2.3 while gallic acid showed a retention peak at 7.3.In the HPLC chromatogram of the methanolic extract of TT1-S the area under the curve for rutin was found to be 1122640 and for gallic acid it was found to be 2819753, while that for TT1-W the area under the curve for rutin was found to be 466571 and for gallic acid was found to be 1607811. The result of the HPLC analysis reveals that the amount of rutin and gallic acid was found to be more in the TT1-S when compared to TT1-W. The results indicate that the season of plant growth influences the amount of chemical constituents and summer season was ideal for the collection of the plant as the amount of phytoconstituents was more during this season.



Peak Id	Ret time	Height	Area
Rutin	2.307	74549	1122640
Gallic acid	7.382	163494	2819753

1Fig-HPLC chromatogram of TT1**- S**



Peak Id	Ret time	Height	Area
Rutin	2.373	23085	466571
Gallic acid	7.348	108223	1607811

Fig-2 HPLC chromatogram of TT1-W

Table-3:Anti-oxidant activity of the extracts:

µg/m	TT1-S		TT1-W		2T1-S		TT2-W		TT3-W	
	Abs	% Inh	Abs	%Inh	Abs	%Inh	Abs	%Inh	Abs	% Inh
10	0.030	90.32	0.061	80.32	0.040	87.09	0.045	85.48	0.070	77.41
20	0.027	91.29	0.056	81.93	0.038	87.74	0.040	87.09	0.065	79.03
40	0.023	92.58	0.051	83.54	0.035	88.70	0.041	86.77	0.063	79.67
80	0.019	93.87	0.048	84.51	0.030	90.32	0.037	88.06	0.054	82.58
100	0.019	93.87	0.047	84.83	0.025	91.93	0.030	90.32	0.050	83.87
120	0.018	94.19	0.044	85.80	0.021	93.22	0.028	90.96	0.044	85.80
140	0.017	94.51	0.041	86.77	0.020	93.54	0.025	91.93	0.042	86.45
180	0.017	94.51	0.038	87.74	0.020	93.54	0.024	92.25	0.041	86.77
200	0.016	94.83	0.037	88.06	0.019	93.87	0.021	93.22	0.041	86.77

Analgesic activity was evaluated by Eddy's hot plate method for the extracts TT1-S and TT1-W .The results are as shown in table 3.

Table 4: Analgesic activity of the methanolic extracts of TT1-S and TT1-W:

	Reaction time in seconds					
Groups	Dose	0 min	15 min	30 min	60 min	120 min
Control		3.12±0.51	2.87±0.76	2.74±0.75	2.46±0.42	2.67±0.39
Indomethacin	2mg/ml	3.09±0.15	4.3±0.36	±1.10*7.02	6.9±0.49*	6.68±0.82
TT1-S	250mg	2.59±0.21	3.9±0.58	5.54±0.73	6.78 ±0.66*	6.66±0.96
	500mg	2.82±0.70	7.5±0.62*	8.9±0.28**	8.78±0.51**	±0.48*7.43
TT1-W	250mg	2.63±0.29	4.69±1.09	5.99±1.03	5.07±1.46	4.55±1.21
	500mg	2.98±0.30	7.24±0.27*	8.1±1.01**	±0.87**8.3	7.33±1.57

All values are mean ± SEM, n=5, *P<0.05 indicates significant and **P<0.001 is extremely significant when compared with control

Taking forward the research to co-relate the effect of phytoconstituents on medicinal properties. Anti-oxidant activity was evaluated for all the five extracts by DPPH method. The absorbance and % inhibition of the extracts are as shown in the table-3.The results revealed that TT1-S showed the best antioxidant activity.

The analgesic activity was evaluated by Eddy hot plate method. .Two doses were selected i.e. 250 mg/kg (lower dose) and 500 mg/kg (higher). The extracts at the higher dose showed activity with in the first 15 mts and lasted for about 120 mts.TT1-S showed the highest activity at 30 mts which lasted for about 120mts, while TT1-W showed highest activity at 60 minutes . The hot plate model for analgesic activity has been used to study centrally acting analgesics. In these models, sensory nerves sensitize the nociceptors and the involvement of endogenous substances such as prostaglandins are minimized. Therefore it can be suggested that these extracts may be acting centrally. This method significantly inhibited the two measureable behavioral components of this method i.e. paw licking and jumping; which are considered to be supraspinally integrated indicating that the extracts may be acting on the supraspinal level.

CONCLUSION:

From the results of our studies it was evident that the season of collection and geographical source of the plants play an important role in the phytoconstituents of plants; this in turn affects the medicinal properties of the plant. The amount of phytoconstituents was more in the plant collected from the desert region. And the best season for collection is summer as the amount of phytoconstituents present in this sample was more when compared to the sample collected during winter season.

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