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Variation in phytochemical constituents of *Thymus linearis* Benth different regions of Himachal Pradesh, India

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Abstract
Secondary metabolites, commonly known as phytochemicals, can be extracted from fruits, leaves, bark and roots of floral components and are responsible for biosynthesis of endogenous compounds through specialized proteins found in plants. These metabolites are not only helpful in promoting human health but also crucial for plants themselves as they enhance the plant immune system which leads to predator avoidance. IHR (Indian Himalayan Region) is a natural storehouse of such valuable medicinal flora. Some of the medicinal herbs are very lesser known in spite being having tremendous curative measures. One such important herb is *Thymus linearis* Benth. L., which comes under the genus *Thymus* and family *Labiateae*. *T. linearis* commonly known as "Van Ajwain" is a perennial herb that has the centre of origin in the Northern and Central European region, and naturally grows in the Himalayan Forest under wild conditions. This herb has an immense therapeutic utilization and is used in preparations of natural herbal remedies such as syrups, tinctures, infusions, decoctions, tea, and oil as this species has antiseptic, anthelmintic, carminative, expectorant and sedative characteristics.
So, the current investigation mostly focuses on the quantification of four major phytochemicals i.e., total phenolic compounds, alkaloids, saponin and total flavonoid in the samples of *T. linearis* collected from different sites of Himachal Pradesh. The quantification value shows a great variation of 0.815%±0.50, 45.359±0.50 mg GAE/g, 1.48%±0.50 and 3.578±0.50 mg QE/g for alkaloid, total phenolic compound, saponin and flavonoid respectively. A number of physiographic, physiognomic and edaphic factors may be responsible for such variation in Phyto-chemical constituents of *T. linearis* in different geographic regions of Himachal Pradesh.

Keywords: IHR, quantitative analysis, biosynthesis, predator avoidance, anthelmintic

Introduction
Indian Himalayan Region (IHR) is a natural storehouse of valuable medicinal flora, most of which are very lesser known despite having tremendous medicinal uses. Around 1748 species are identified to show medicinal values in the Indian Himalayas, some of these are found throughout the Himalayan regions while some are endemic to specific physio-geographical regions [1, 2]. One such important floral genus is *Thymus* (Labiateae), widely distributed in temperate zones and comprises about 350 species worldwide [3].
Thymus linearis (wild thyme) belonging to the family Labiatae (Lamiaceae) is an aromatic perennial shrub commonly known as wild/creeping thyme and grows commonly in the Himalayan regions of India. Wild thyme grows best on dry, stony ground, open sandy heaths, and grasslands [4]. This species has its center of origin in the Northern and Central European region and is the native species of Austria, Baltic States, Belarus, Belgium, Buryatiya, Central European Rus, Czechoslovakia, Denmark, East European Russia, Finland, France, Germany, Great Britain, Hungary, Netherlands, North European Russi, Northwest European R, Norway, Poland, Sweden, Ukraine and West Siberia (https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names: 30066276-2). In Himachal Pradesh, the vernacular name of this species is "Van Ajwain" or "Jungli Ajwain". It is also known as Breckland thyme, however, its specific name "serpyllum" is derived from the Greek word meaning "to creep," because of wild thyme's trailing habit [4].
T. linearis is an aromatic, hairy, more or less procumbent, about 6-12 inches long creeping shrub. Its leaves are nearly sessile, oblong-ovate, and obtuse, usually from 0.4- 1cm in length to 0.2-0.4cm in width. Leaves have small hair-like structures in their base and appear like the rosette at the top of the stem.

Flowers are small, present in whorls, having hairy calyx, bilabiate with upper lip broad and 3-lobed while the lower lip has two lobes. Corolla is gamopetalous and the corolla tube is as long as the calyx. Stamens are four in number, nearly equal and protruding [5].

Secondary metabolites are organic molecules produced by living organisms that are not directly involved in the normal growth and development of the organism while they are considered as an integral part of the metabolism of a living being. They are mostly classified based on their biosynthesis [6]. Secondary metabolites mostly play a role in the defence mechanisms protecting them from herbivores. Along with this, they are used by humans as medicines, food flavourings, pharmaceuticals, fragrances, colours, biopesticides, food additives and drugs etc. [7]. The major secondary metabolites of *T. linearis* such as Thymol and Carvacrol belong to the class of phenolic compounds.

The medicinal properties of *T. linearis* have been extensively used in traditional medicines for centuries. The aerial parts particularly leaf in fresh and dried conditions possess certain healing properties due to the presence of essential oils [4]. In recent years interest has been increased in Ethnomedicinal, phytochemical, and pharmacological studies in *T. linearis* as it serves as it is widely used in many pharmaceutical and chemical industries for different formulations such as syrups, tinctures, infusions, decoctions, tea and oil [4]. Wild thyme is commonly used by local people for various functions such as antiseptic, anthelmintic, carminative, expectorant, sedative, and tonic [4]. *T. linearis* is an important source of medicinal substances with antioxidant [8], antimicrobial [9], anti-fungal [10], antitumor [11] properties and their effective medicinal application, as well as use in pharmaceutical, food, and cosmetic industries. The widespread use of *T. linearis* dates back to ancient Egypt, where they were used for making perfumed balms, for embalming, and for medical purposes [4]. Along with that, thyme was used to treat asthma and loosen congestion in the throat & stomach along with that it can also relieve rheumatism, gout, and sciatica [12]. Decoction sweetened with honey is good against pulmonary diseases [13]. The extract of the plant also showed anti-diabetic, antimicrobial, antifungal, and antioxidant activities [8, 9, 10, 14-16]. *T. linearis* contains thymol as an active compound that is used as antiseptics, mouthwashes, and gargles and has been used as a disinfectant [13]. The increased demand for natural products instead of synthetic chemicals from consumers has inspired researchers toward medicinal and aromatic plants among which *T. linearis* is one important species [4].

So, the current investigation mostly focuses on the quantification of three major phytochemicals such as total phenolic compound, total alkaloid content and saponin contents of the samples collected from different sites of Himachal Pradesh, which comes under North-West Himalaya.

Materials and Methods

Study Area: The current study has been conducted throughout the state of Himachal Pradesh (Fig. 1). It includes parts of the Trans and Northwest Himalaya (30°22′40″-33°12′40″N to 75°45′55″- 79°04′20″ E) and covers 55,673 km^2 area. Altitudinal variations of the state are found between 350m-7025m. In this study, *T. linearis* samples were collected from the altitudinal range between 1820m - 3625m (Table 1).

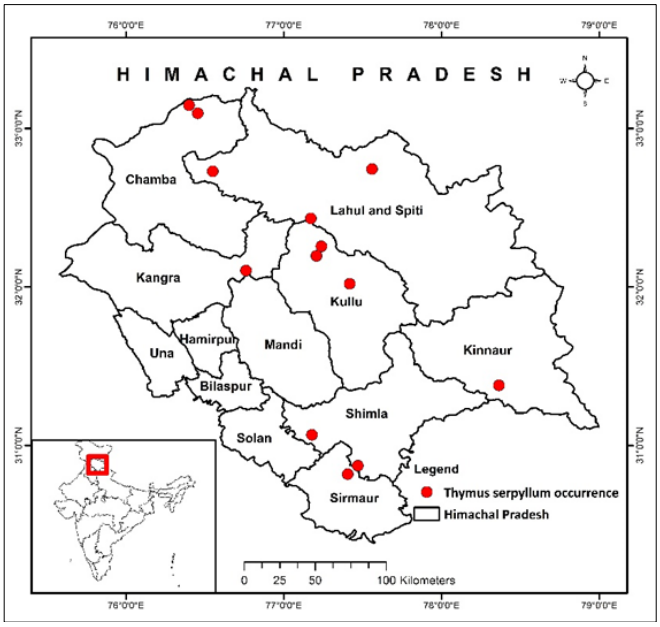

Fig 1: Occurrence of *Thymus linearis* in the study area, Himachal Pradesh

Table 1: Different sample collection sites in Himachal Pradesh

Source	Forest Division	District	Altitude (amsl)
Nohradhar	Renuka	Sirmaur	1520
Tipra	Chopal	Shimla	1938
Baragran	Joginder Nagar	Kangra	2300
Sargheen	Shimla	Shimla	2354
Tauk	Parbati	Kullu	2528
Rajgundha	Hamirpur WL	Kangra	2535
Hamta	Kullu	Kullu	2815
Jagatsukh	Kullu	Kullu	2923
Sural Bhatori	Pangi	Chamba	3100
Hudan Bhatori	Pangi	Chamba	3200
Churdhar	Shimla WL	Sirmaur	3215
Gramphu	Lahaul (Keylong)	Lahaul and Spiti	3325
Udaipur	Lahaul (Keylong)	Lahaul and Spiti	3405
Rakcham	Sarahan WL	Kinnaur	3457
Palmo	Lahaul (Keylong)	Lahaul and Spiti	3625

Collection of plant material and processing: The aerial parts of the targeted species were used for the phytochemical screening. The samples were collected from fifteen (15) sources of *T. linearis* in Himachal Pradesh. After the collection of plant parts, they were dried in the room with slight exposure to light. *T. linearis* aerial parts were dried for 4-5 weeks. After drying, the plant parts were ground to obtain powdered sample. This ground plant material was used for analysis purposes.

Qualitative Analysis of Alkaloid, Total Phenolic Compounds and Saponin Contents

Plant Extract Preparation: Methanolic/Ethanolic extract of samples was used for qualitative as well as quantitative analysis. For qualitative analysis, 50 mg of the powdered plant material was dissolved in 5 mL methanol and then sonicated for 45 minutes at 40°C and then centrifuged for 10 min at 1,000 rpm. The clear supernatant was collected and stored for further analysis [17].

Test for Alkaloid: The detection of alkaloids was done through Wagner's test [18]. 1ml of the extract was treated with a few drops of Wagner's reagent i.e., Iodine in Potassium Iodide. The formation of brownish/reddish precipitates indicates the presence of alkaloids.

Test for Phenol: Ferric chloride test [19] was used for the detection of phenolic compounds. Plant extracts were treated with a few drops of Ferric chloride reagent (2% $FeCl_3$ in 95% ethanol). The formation of stable greenish colour indicates the presence of phenolic compounds.

Test for Saponin: Foam test: Extracts were taken and 20 ml of distilled water was added and shaken for 15 min in a graduated cylinder. A layer of stable foam indicates the presence of saponin [20].

Test for Flavonoid: Detection of flavonoids in the plant sample was done using the method given by Hossain *et al.* in 2013 [21]. 1 ml methanolic extract was taken in a test tube and added few drops of dilute NaOH solution. An intense yellow colour appeared in the test tube. It became colourless with the addition of a few drops of dilute acid that will indicate the presence of flavonoids.

Quantitative analysis of Alkaloid and Total Phenol
Quantitative analysis
Alkaloid content estimation: The alkaloid content of the dried plant material was estimated by Harborne Method [22]. Weighed 2.5g of powdered sample. Mixed the sample in 200ml of 10% acetic acid in methanol. Covered the solution and kept for 4 hrs. Filtered the solution using Whatman filter paper no. 42 in a vacuum filter and the extract was concentrated on water bath to one-quarter of its original volume. Added Ammonium Hydroxide dropwise until the precipitation was completed. The whole solution was filtered using the above filtration setup and the supernatant was discarded and precipitated washed with diluted Ammonium hydroxide and filtered. The residue was dried in the oven and weighed. The weight of alkaloid was calculated by the difference in the initial and final weight of the filter paper
Alkaloid percentage was calculated as a percentage per unit dry weight by using the formula:

$$\text{Alkaloid (\%)} = \frac{\text{Weight of alkaloid content extracted}}{\text{weight of powdered sample used}} \times 100$$

Total Phenolic Content estimation: In the first step of total phenol estimation, the calibration curve of gallic acid was made. The standard solution of Gallic acid was prepared by dissolving 1mg of gallic acid in 100mL of distilled water. This was the stock solution (10µg/mL). From this stock solution, different samples of gallic acids were made with concentrations i.e., took 1mL, 2mL, 3mL, 4mL and 5mL from stock solution in different test tubes and diluted to make the final volume as 5mL. After dilution, the concentrations of these solutions were 2µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL respectively (Table 2). Noted absorbance for these five concentrations of gallic acid through a spectrophotometer. Plotted the concentrations against absorbance.

Table 2: Standard Gallic Acid solution

Test Sample	Concentration (ug/ml)	Preparation
Standard GA 1	2	1mL(stock)+ 4mL (dilution)
Standard GA 2	4	2mL(stock)+ 3mL (dilution)
Standard GA 3	6	3mL(stock)+ 2mL (dilution)
Standard GA 4	8	4mL(stock)+ 1mL (dilution)
Standard GA 5	10	5mL(stock)+ 0mL (dilution)

The total phenol content (TPC) of plant extract was estimated through Folin-Ciocalteu reagent following the method described by Chandra in 2014 [17] with slight modifications. 10 mg of powdered sample weighed and mixed in 5ml of Methanol. After mixing sonicated for 45 minutes at 40°c followed by 10000 rpm for 10 min. Clear supernatant was collected and stored as stock solution. 0.2 ml of sample solution was taken and dissolved in 0.6ml of distilled water. Now 0.2 ml of FCR reagent was added and allowed to stand for 5 min. 1 ml of 20% Na_2CO_3 was added. Final volume was made 3 ml by adding distilled water. The solution now obtained was kept in dark for 30 min. and again centrifuged. Absorbance was recorded at 650 nm. Estimation of TPC was done based on standard curve of gallic acid. A standard curve of gallic acid concentration was prepared. Results were calculated using the following formula [23],
Total Phenol Content = GAE×V× DF/W
GAE - Concentration of Gallic acid established from the calibration curve in mg/ml
V- Total volume of sample (ml)
DF- Dilution Factor
W- Sample weight (g)

Saponin Content Estimation: Saponin content was estimated using the method of Mir *et al.* in 2016 [20]. 20 g of plant sample was dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55 ºC. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 ºC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven at a constant weight and the content (percentage) of saponin was calculated [24].

Total flavonoid content estimation: The total flavonoid content of methanolic extracts of all samples was estimated using the method described by Neupane and Lamichhane (2020) [25]. The plant samples were extracted using methanol and the extract prepared was again dissolved in methanol to obtain an extract solution of 500 µg/mL. After that, 300 µL extract of this extract was taken in a test tube and added in 3.4 mL aqueous methanol (30% v/v). Then, 150 µL aqueous Sodium nitrite solution (0.5 M) was added followed by 150 µL Aluminium chloride solution (0.3 M) in each test tube.

After 5 min, 1 mL of NaOH solution (1 M) was added and the content was mixed properly. The absorbance of the solution was measured at 506 nm using UV-visible spectrophotometer against a blank (Prepared with a similar procedure but replacing plant extract with an equal volume of methanol). The standard curve of Quercetin was prepared with a similar procedure at different concentrations and the absorbance of each source was compared with this standard curve values. Absorbances were used to calculate the flavonoid content in mg Quercetin Equivalent/gm dry weight of the plant sample.

Results and Discussions

Quantitative Estimation of Phytochemicals

Alkaloid Content: In the qualitative test, the presence of brownish precipitates in *T. linearis* indicated the presence of alkaloids in the sample. The alkaloid percentage of different sites of Himachal Pradesh is listed in Table 3. The alkaloid content per unit dry weight of *T. linearis* rhizomes was estimated 0.171% to 1.96%. The *T. linearis* sample collected from Huddan Bhatori showed the maximum alkaloid percentage (1.96%) while the sample collected from Gramphu showed the minimum content (0.171%) (Fig 2). A very low correlation was seen in the estimated alkaloid content and altitude with the correlation coefficient, r = -0.09.

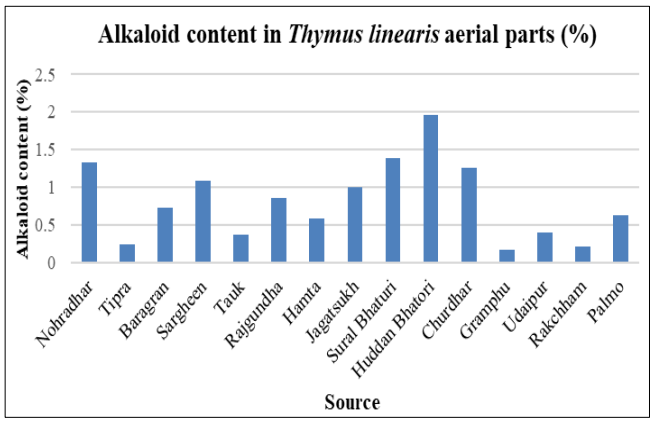

Fig 2: Showing the percentage of alkaloid content in the aerial parts of *T. linearis*

Total Phenol Content: The appearance of a greenish colour in *T. linearis* rhizomes sample through qualitative test indicated the presence of phenolic compounds in the sample. By plotting the absorbance of different concentrations of gallic acid (Table 3), the calibration curve of gallic acid was made and obtained the regression equation y=0.0638x (after keeping the intercept =0), R^2 = 0.9949 (Fig. 3). This equation was used for the calculation of total phenolic content in plant samples by plotting Standard Gallic acid curve.

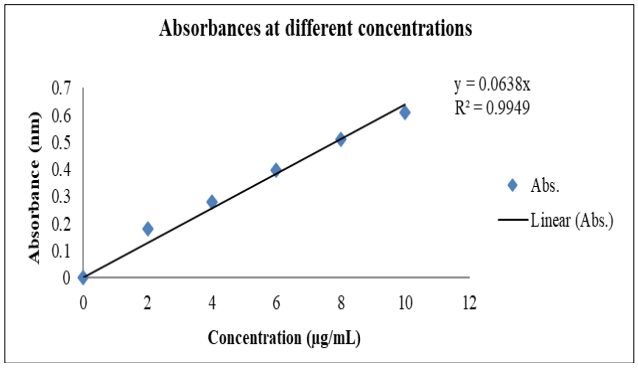

Fig 3: Calibration curve of gallic acid

Table 3: Absorbance of standard gallic acid solution

Test Sample	Concentration (ug/ml)	Absorbance (nm)
GA 1	2	0.18
GA 2	4	0.28
GA 3	6	0.39
GA 4	8	0.51
GA 5	10	0.61

The total Phenolic content of *T. linearis* was found 24.497 to 56.850 mg GAE/g (Table 4). The maximum phenol content in the plants was seen in the samples collected from Sargheen (56.850 mg GAE/g) whereas the minimum was recorded for the samples of Sural Bhatori (24.497 mg GAE/g). The correlation between the estimated total phenolic content and altitude was seen very low with the correlation coefficient value of r = -0.229 showing a negative correlation.

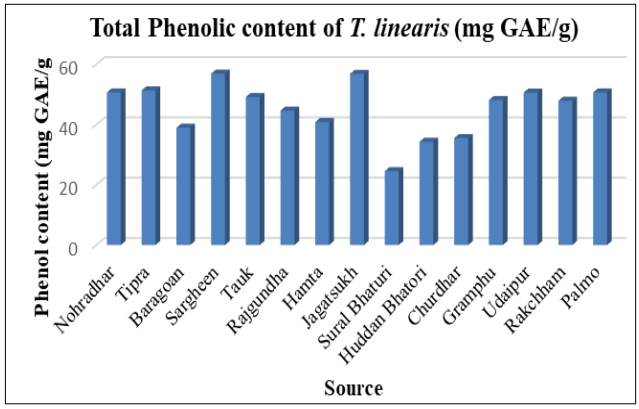

Fig 4: Total phenol content in the roots of *Thymus linearis*

Saponin Content: The formation of foam in qualitative test indicates the presence of saponin. So, the saponin content quantification of *T. linearis* was estimated 0.471% to 4.283%. Statistical analysis showed that the saponin content of the sample collected from Baragran (4.283%) and Palmo (3.103%) is significantly different and is the highest among all. Whereas the saponin contents of samples collected from Gramphu are estimated to be the least in quantity (i.e., 0.471%). No correlation was seen between the estimated content of saponin and altitude (r = -0.01).

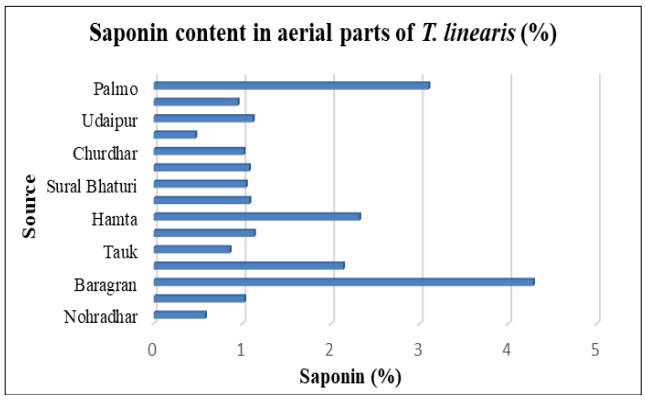

Fig 5: Saponin content in the roots of *Thymus linearis*

Flavonoid Content

After the confirmation of the presence of flavonoid in the *T. linearis* through qualitative test, further quantitative estimation was done. By plotting the absorbance of different concentrations of Quercetin (Table 4), the calibration curve of Quercetin was made and obtained the regression equation y =

0.2961x (after keeping the intercept=0), R^2 = 0.9948 (Fig 6). This equation was used for the calculation of total flavonoid content in plant samples by plotting the standard Quercetin curve.

Table 4: Different concentrations of Quercetin and their absorbance to prepare the standard curve of Quercetin

Test Sample	Concentration (ug/mL)	Absorbance (nm)
Q 1	0.1	0.14
Q 2	0.5	0.17
Q 3	1.0	0.33
Q 4	2.5	0.75
Q 5	5.0	1.46

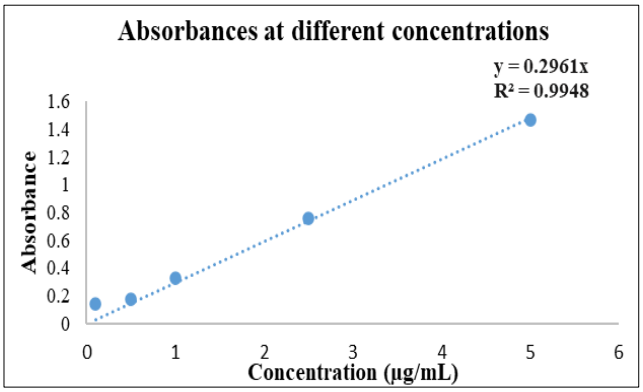

Fig 6: Calibration curve of Gallic acid

The flavonoid content in the *T. linearis* was found 1.51 to 6.70 mg Quercetin Equivalent/g. The content was estimated as maximum (6.70 mg Quercetin Equivalent/g) in the sample collected from the Churdhar region of Sirmaur district of Himachal Pradesh. The value of the correlation coefficient between estimated flavonoid content and altitude was found r = 0.237 showing a low positive correlation between these two entities.

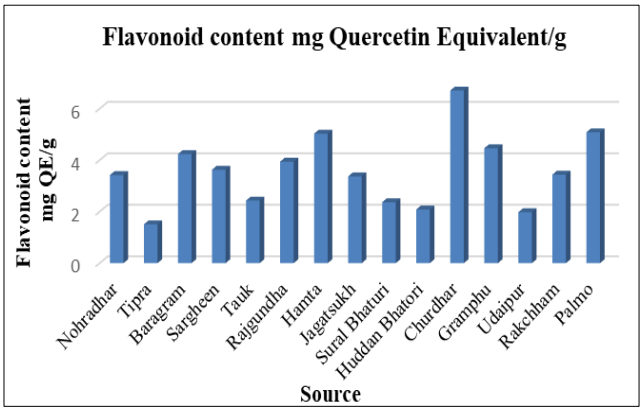

Fig 7: Total flavonoid content in aerial parts of *T. linearis*

Conclusion

In this study, an attempt was made to evaluate the phytochemical contents in the targeted species of *Thymus linearis* both qualitatively and quantitively. It can be recommended that the phytochemical quantification showed a great variation in different sites of Himachal Pradesh. Four major groups of phytochemicals (Phenol, Alkaloid, Saponin and Flavonoid) were detected in the species by qualitative test and by following the prescribed methodology the quantification of the active compounds was conducted. From the quantification data, it can be concluded that all the classes of phytoconstituents estimated in the study, showed a great variation in content i.e., ranges for phenol, alkaloid, saponin and flavonoid from 0.815%±0.50, 45.359±0.50 mg GAE/g, 1.48%±0.50 and 3.578±0.50 mg QE/g respectively. The huge variation in the content of phytoconstituents may be the result of various factors affecting the phytochemical variation in the species starting from the altitude, climate and other edaphic factors, which is a matter of further study. From the experiment, a very low negative correlation of alkaloid and saponin was seen with altitude. A slight positive correlation was observed between the flavonoid and altitude while a very low negative correlation was found between the total phenolic content and altitude. However, we can conclude that alkaloid and flavonoid content was maximum in higher altitudes (near 3200 m MSL) whereas Phenol and Saponin content were recorded maximum in the middle altitudinal range of 2000m to 2500 m MSL.

Acknowledgement

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Table 5: Phytochemical variation amongst different sources of *T. linearis* in H.P.

Sources	Alkaloid (%)	Phenol (mg GAE/g)	Saponin (%)	Flavonoid mg Quercetin/g
Nohradhar	1.329	50.633	0.581	3.42
Tipra	0.240	51.310	1.024	1.51
Baragram	0.730	38.910	4.283	4.24
Sargheen	1.081	56.850	2.140	3.63
Tauk	0.376	49.110	0.859	2.43
Rajgundha	0.856	44.614	1.134	3.94
Hamta	0.592	40.810	2.323	5.03
Jagatsukh	1.002	56.783	1.083	3.37
Sural Bhaturi	1.385	24.497	1.043	2.37
Huddan Bhatori	1.960	34.240	1.077	2.08
Churdhar	1.266	35.487	1.017	6.70
Gramphu	0.171	48.090	0.471	4.46
Udaipur	0.396	50.583	1.121	1.98
Rakchham	0.218	47.863	0.949	3.44
Palmo	0.624	50.610	3.103	5.08
Cd at 5%	0.35	2.50	2.52	0.86
SEM±	0.17	1.22	0.24	0.42

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