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The Role of Habitat Elevation in Artemisia Species Chemotype and Artemisinin Production

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Abstract: We set out to discover if chemotypes exist in Artemisia species, how artemisinin concentration changes with habitat height, and whether there are any new plant sources of artemisinin that may replace Artemisia annua. Research Tools and Procedures: Seven species, spanning three distinct height ranges, were chosen for this investigation. We used high-performance liquid chromatography to quantify artemisinin after extracting it from the leaves, stems, and roots of every species of Artemisia. End result: The artemisinin content of various Artemisia species was shown to be significantly affected by their height variation. The artemisinin content of leaves increased at height II in three species of Artemisia: Artemisia moorcroftiana, Artemisia vestita, and Artemisia roxburghiana var. roxburghiana, with values of 0.09%, 0.08%, and 0.07% of dry weight, respectively. Artemisia vulgaris had a high artemisinin concentration at heights II and III (0.06% and 0.07% of dry weight, respectively), in contrast to Artemisia sieversiana, which exhibited a low artemisinin content at height I (0.08% of dry weight). Findings: This research found that chemotypes exist in Artemisia species and that plant geographical habitat height impacts artemisinin production.

Keywords: Artemisinin, chemotype, ecotype, and height variation are some of the keywords regarding alternative species.

INTRODUCTION

The Asteraceae family annual plant Artemisia annua, which has a long history of use in Chinese medicine for the treatment of malaria, produces artemisinin, a potent weapon against parasites that have developed resistance to traditional treatments. [1] Optimal effectiveness in combating this illness and lowering transmission rates is now achieved by combining artemisinin with other medications. [2] in Artemisia is most abundant in cold and temperate zones in North America and Eurasia, especially in the temperate belt of Asia. The following nations are significant: Spain, Argentina, France, America, Bulgaria, France, and Hungary. the third Notably, it is suggested that northwest Asian mountain territories (such as mesothermic subarctic or semi-humid forest steps near Urals) encompassed genus origins based on modern distribution patterns relevant to fossil data;[4] primary types being situated mostly in the north temperate. This suggestion is mainly based on the modern distribution of *Artemisia* and its allies, with their primary types mostly lying in north temperate Asia, as well as on fossil and paleogeographic data.

The amount of artemisinin in different Artemisia species is reportedly unpredictable and may change over time, even within the same plant. The natural population of A. annua, on the other hand, contains very little artemisinin (0.01%-0.15%). [5] The artemisinin content of plants in this species may vary due to factors such as cross-pollinating, with larger concentrations seen during full bloom. Other factors that greatly impact the production levels of mature plants include seasonal variations and geographical location. Thus, variations in the amount of artemisinin found in A. annua leaves are probably attributable to variances in harvest time or different cultivars employed, as well as environmental stress factors like light exposure or nutrient availability, which might affect the productivity of these plants.

There has been debate in the literature on which plant parts have the greatest concentration of artemisinin. There is ten times more artemisinin in flowers than in leaves, say the researchers. [6] Nonetheless, there are some who argue that the leaves contain about 89% of the total, with the tallest region reaching a maximum of 41.7% at 50 cm, almost double the amount found in the lower portions. There is a wide range of out-of-leaf collection rates among frequently cultivated accessions, from 0.02% to 1.38%, as reported in the literature. These results point to diverse results, thus researchers are looking at measuring levels in different parts of plants at different altitudes (roots, stems, and leaves). Distinct total content distributions depending on leaf components were found in lowland and highland locations when compared. This sparked more studies with the goal of improving yields worldwide. Despite their heavy reliance, institutions in Pakistan's northern hilly areas have mostly ignored biodiversity reserves, highlighting an opportunity to promote ecological and economic advantages. Everyone agrees that it's critical to stress sustainability via appropriate activities that don't upset the ecological equilibrium. The significance of teaching children about different viewpoints on human relationships, including living in harmony with the natural world, is highlighted by this method. As a result, it has risen to the forefront of educational discourse, calling for multidisciplinary groups to investigate and use the best scientific methods. Creative solutions may be fostered via the integration of conventional knowledge with culturally aware approaches through these tactics. The artemisinin concentration of Artemisia annua, a plant known for its powerful antimalarial qualities, has been shown to vary according to its growing height. Several studies have shown that artemisinin levels are directly correlated with elevation; plants grown in certain altitude ranges tend to have much higher concentrations of this bioactive component. Due to variations in environmental conditions such soil composition, sunshine exposure, and temperature, it is generally believed that A. annua specimens with higher heights have higher levels of this important chemical. When it comes to drug manufacturing, increasing yields is of the utmost importance, and altitude gradients play a significant role in this endeavor by influencing physiological processes associated with secondary metabolite production, such as the pathways that lead to substances like artemisinin.

Materials and Methods

Plants collection

Seven *Artemisia* species that occur naturally in Northern Pakistan were gathered from various hilly locations [Figures 1 and 2]. All seven species were collected at three different elevations, where their population was observed to be highly dense. The elevations of the sampling points (in feet) and the height groups (categorized as I, II and III) of the seven *Artemisia* species are presented in Table 1. A taxonomist identified these specimens using the Flora of Pakistan reference book at Quaid-i-Azam University's Department of Plant Sciences located in Islamabad, Pakistan, by comparing them with already designated herbarium sheets for each individual *Artemisia* spieces kept within a preserved collection of dried plants housed there. We selected these particular seven wild-grown *Artemisia* varieties so we could measure artemisinin concentration levels present throughout leaves', stems' or root systems harvested from three elevation-based strata as measured against height I (<5000 ft), height II (between 5000–6000 ft) and finally climatic zone given name Height III; which occurs above an altitude greater than six thousand feet above sea level.

Preparation of extracts

The herbs were utilized to extract artemisinin using the method as reported earlier with modifications.[7] First, their weights were measured, and then, they were ground with a mortar and pestle until a homogenous mixture was produced in 5 ml of high-performance liquid chromatography (HPLC) grade toluene. The samples underwent sonication for half an hour over ice, preventing any possible evaporation due to overheating before being centrifuged (2000 g at −8°C) for 20 min so that artemisinin-rich toluene could be separated from cellular debris. Next, the supernatant was extracted and preserved within dram vials while pellets containing cell debris were resuspended in fresh 5 ml amounts, as seen fit by each researcher, and sonicated again before centrifuge treatments through which heavy particles settled into these pallets. The supernatant was carefully removed, and the cellular debris was resuspended in toluene. The mixture was vortexed and centrifuged again to extract more artemisinin from the material. The retrieved supernatants were amalgamated to yield pooled extracts for subsequent HPLC analysis. Subsequently, the extracts were air-dried to ensure retention during storage, facilitated by gradual temperature reduction to minimize the risk of fragmentation, and then stored under optimal freezing conditions (-20°C).

The yield was estimated using the following formula:

$$\% \text{ Yield} = \frac{\text{Weight of dried extract} \times 100}{\text{Weight of plant sample}}$$

Quantification of artemisinin

The quantification of artemisinin in the dried n-Hexane extracts was performed using HPLC following the protocol advised earlier with modifications.[7] An Agilent Technologies Zorbax SB C18 column (150 mm × 4.6 mm × 5 μm) was utilized as the stationary phase with a mobile phase flow rate of 1 mL/min. The Diode Array Detector (G1315B-DAD) indicated maximum absorbance at 260 nm and the retention/ elution time peak for artemisinin occurred after approximately 12 min. Artemisinin identification employed an authentic

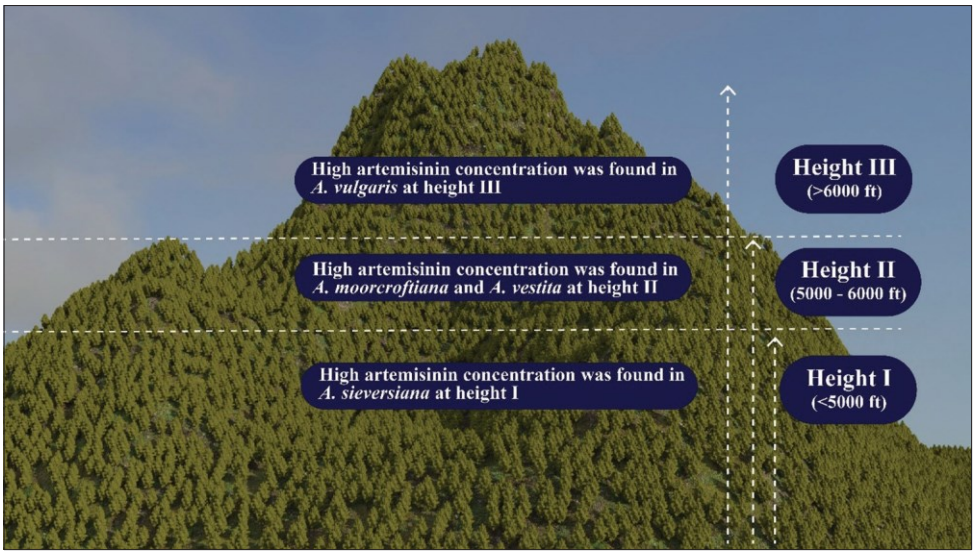

Figure 1: Geographical distribution of plant species collected from different heights (graphical abstract). *A. vulgaris: Artemisia vulgaris, A. moorcroftiana: Artemisia moorcroftiana, A. vestita: Artemisia vestita, A. sieversiana: Artemisia sieversiana*

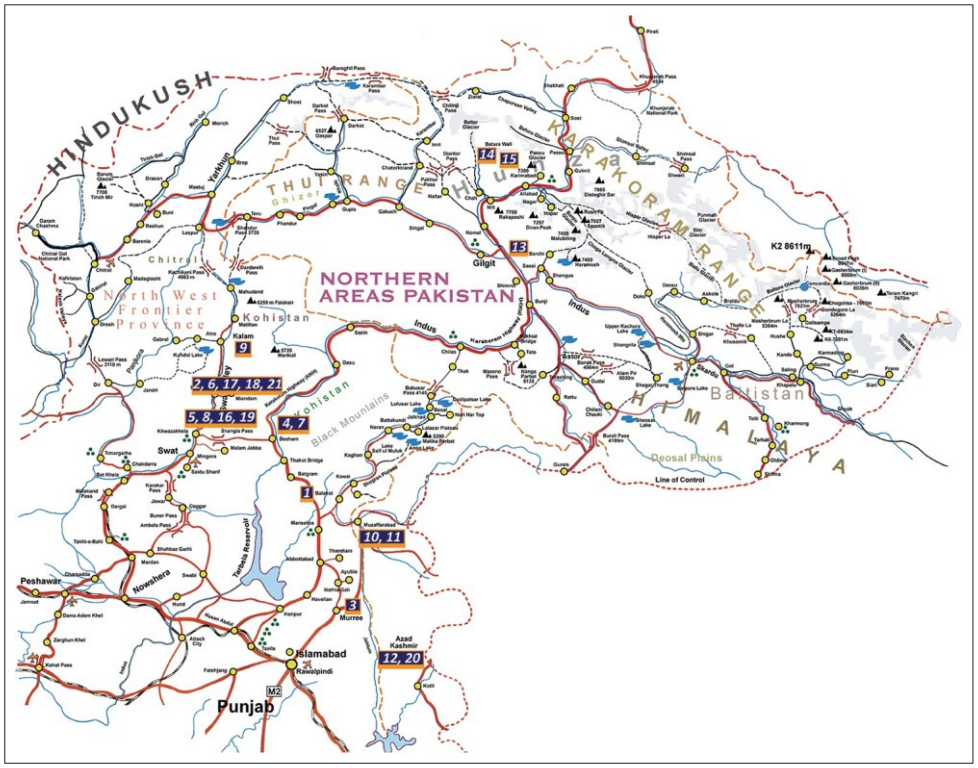

Figure 2: Geographical map of Northern Pakistan showing plant collection points

standard of the compound while injection volumes for both samples and standards were fixed at 20 µL each. Six dilutions of standard solution from concentrations ranging between 0 and 500 µg/mL were then injected one by one into HPLC before obtaining a calibration curve by plotting chromatographic peak area (mAU) against concentration. Data analysis showed that this linear response depicted in Figure 3 had an excellent correlation coefficient $R = 0.9994$ due to the successful application of linear regression analysis on the equation obtained from the consulting calibration curve.

Statistical analysis

All the experimental data were analyzed statistically using analysis of variance and Fisher's least significant difference (LSD) test.

RESULTS

The altitude of the plant habitat had a significant effect (α level 0.05) on the artemisinin content of leaves and stems of the same *Artemisia* species growing at three different spots. The

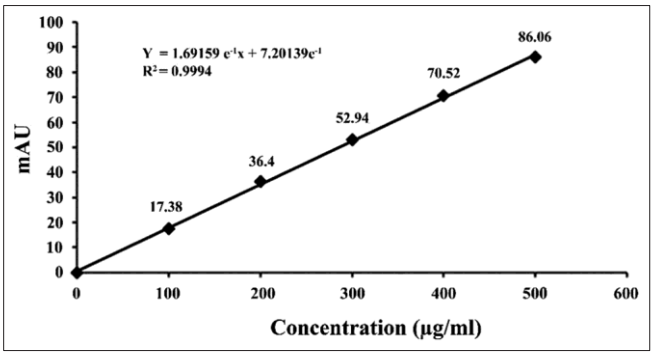

Figure 3: Calibration curve of standard artemisinin

effect of altitude was more prominent in the leaves and stem artemisinin content in five *Artemisia* species, i.e., *Artemisia vulgaris*, *Artemisia moorcroftiana*, *Artemisia roxburghiana var. roxburghiana*, and *Artemisia vestita*. All of the Artemisia species we looked at have rather modest artemisinin concentrations in their roots. Sampling at height II revealed the highest concentration of artemisinin in the roots. A. vestita, A. moorcroftiana, and A. roxburghiana var. roxburghiana had very high artemisinin content in its leaves when measured at height II. Similarly, Artemisia vulgaris at heights II and III and Artemisia sieversiana at height I both have high artemisinin leaf content. In addition, the artemisinin concentration of the leaves was twice as high as that of the same species obtained from lower elevations [Figure 4]. At height II, the leaves of A. moorcroftiana had the highest artemisinin content (0.09% ±0.01%), whereas the leaves of A. sieversiana and A. vestita, collected at heights I and II, respectively, had the second-highest artemisinin concentration (0.08% ± 0.01%). Leaves of A. vulgaris taken at height III had the highest concentration of artemisinin at 0.07% ±0.01%, as shown in Figure 4. The complete plant sample of A. sieversiana grown at height I had the highest average amount of artemisinin. At height II, A. vestita and A. moorcroftiana showed the second-highest average artemisinin concentration. A. vulgaris samples collected at height II had the third greatest concentration of artemisinin, whereas A. roxburghiana var. roxburghiana samples obtained at height III had the same result [Figure 5]. Figure 6 displays the findings of the calculation of the average artemisinin content in the leaves, stems, and roots. According to our research, the concentration of artemisinin in the leaves was higher than in the stems and roots at all plant habitat heights, reaching a peak at height II (Figure 6). At all three of their habitat heights, we found that the artemisinin distribution patterns in the stems and roots were quite comparable to those in the leaves.

Discussion

Artemisinin has been an effective remedy against different pathogenic fungi of plants such as *Rhizoctonia cerealis,*

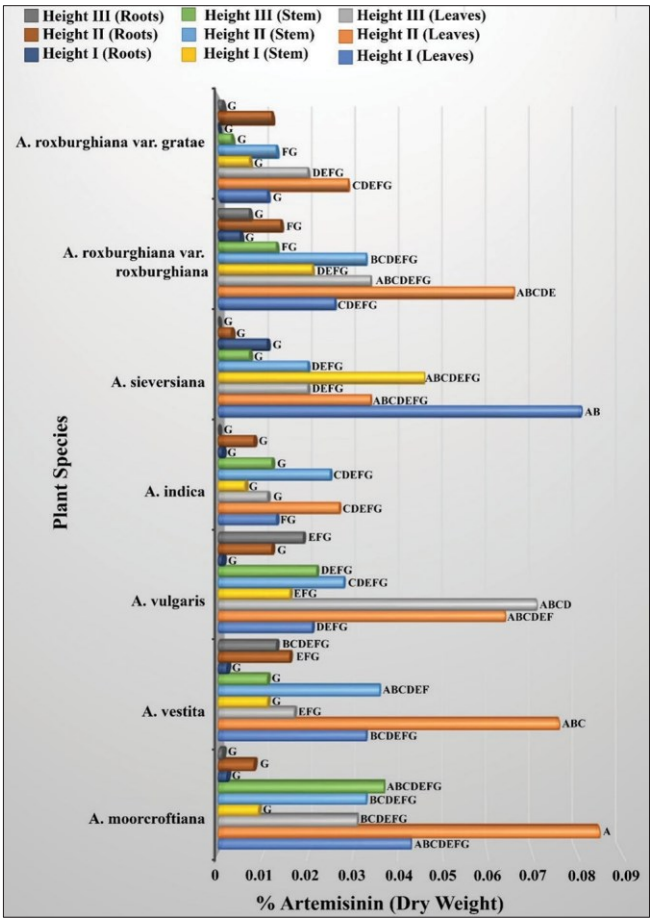

Figure 4: The content of artemisinin in the leaves, stems, and roots of the seven *Artemisia* species collected from three different altitudes (alphabetic on the bars are ranking orders after analysis of variance and least significant difference). *A. moorcroftiana: Artemisia moorcroftiana, A. vestita: Artemisia vestita, A. vulgaris: Artemisia vulgaris, A. indica: Artemisia indica, A. sieversiana: Artemisia sieversiana, A. roxburghiana: Artemisia roxburghiana*

Gaeumannomyces graminis var. tritici, Verticillium dahlia, and *Gerlachia nivalis,* suggesting a potential protective role of artemisinin against plant diseases.[8] Amusingly, in an era when the scientific community is striving hard to search for novel compounds with more specificity to their cellular and molecular targets, artemisinin stands prominent with its diverse molecular targets. Since the time artemisinin was recognized for its potent antimalarial properties, there has been an extensive scientific trial of artemisinin to investigate its other pharmacological properties. For instance, it has shown significant cytotoxic and antiproliferative properties against cancer cells, anti-infective characteristics against schistosomiases, and antiviral effects against certain viral particles such as hepatitis B, hepatitis C, human cytomegalovirus, and bovine diarrhea virus. The thing that is more exciting about artemisinin is its diverse bioactivity spectrum that includes protozoans such as *Trypanosoma*,[9] *Toxoplasma gondii*, *Leishmania*,[10] *Fungi*,[11] some *Trematodes*,[12] yeast, and bacteria.[13]

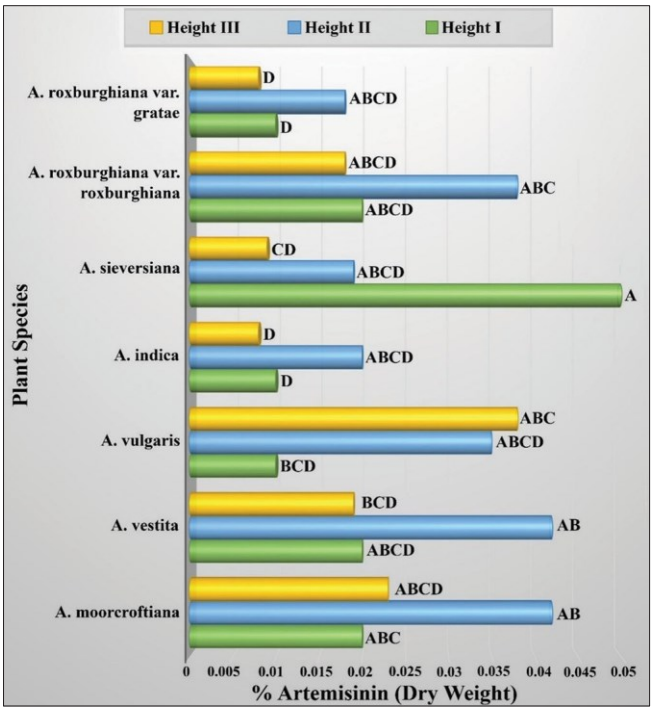

Figure 5: The variation in the average artemisinin content of the seven *Artemisia* species according to their height (alphabetic on the bars are ranking orders after analysis of variance and least significant difference). *A. moorcroftiana: Artemisia moorcroftiana, A. vestita: Artemisia vestita, A. vulgaris: Artemisia vulgaris, A. indica: Artemisia indica, A. sieversiana: Artemisia sieversiana, A. roxburghiana: Artemisia roxburghiana*

Various methods for producing artemisinin in vitro have been documented, perhaps due to its possible medical uses. [8] Nevertheless, when it comes to synthesizing artemisinin on a big scale, none of these intricate systems provide a more feasible approach. One of the most prominent commercial sources of artemisinin is the herbal method of extracting it from A. annua, even though its production may be adequately achieved by genetic engineering. [15] Its yield from A. annua might be anywhere from 0.01% to 1.5% of its dry weight, according to reports. two sources: [16,17] Because A. annua naturally has a lower artemisinin concentration, it is essential to utilize other Artemisia species for their concealed artemisinin content. Previous research has shown that additional Artemisia species do, in fact, contain artemisinin. [6] The primary objective of this research was to determine the effect of altitude on the artemisinin content by statistically and qualitatively analyzing the artemisinin content in different Artemisia species grown at different elevations in Pakistan.

The extraction of artemisinin from the herbal samples utilized toluene as one of the most widely exploited laboratory techniques. It is worth mentioning here that our findings regarding the artemisinin content may differ from its previously reported concentrations owing to the different extraction methods. We extracted 2 g of *A. annua* leaf powder in 50 mL of methanol, followed by 45-min sonication and 3-min centrifugation at 12,000 rpm (yielding 0.03%–0.71%

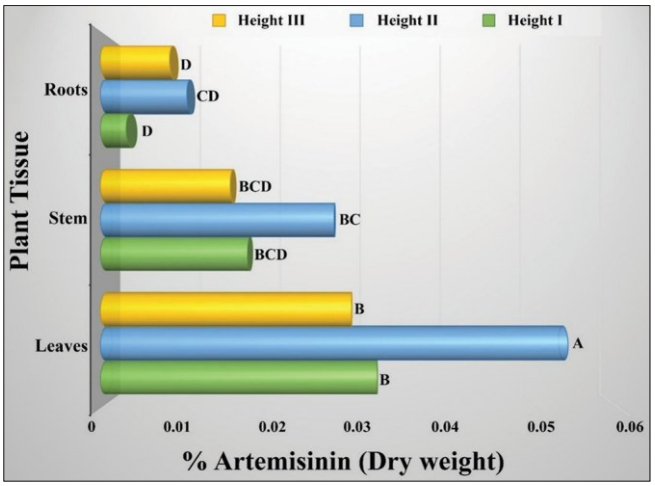

Figure 6: The impact of height on the mean artemisinin content of the leaves, stems, and roots of all seven *Artemisia* species at three different heights (alphabetic on the bars are ranking orders after analysis of variance and least significant difference)

artemisinin). We then proceeded to extract 0.003% artemisinin from 1 g of powdered A. annua roots in 3 mL of toluene by letting it sit at room temperature for 30 minutes. Another option is to use a Soxhlet apparatus to extract artemisinin from 5 g of powdered A. annua leaves. We used 200 mL of petroleum ether and heated it to 60°C for 6 hours, giving 0.652% artemisinin. In addition, we obtained 0.0226%-0.785% artemisinin by refluxing 0.5 g of powdered A. annua leaves in 50 mL of n-hexane at 75°C for 1 hour. As an alternative, we macerated 1 gram of fresh plant material in 6 milliliters of chloroform for one minute, which resulted in a concentration of 0.068% artemisinin. The amount of artemisinin in leaves is significantly affected by the plant's height, according to our research. While samples taken from 6500-foot-altitude leaves yielded 0.70% artemisinin per dry mass, those taken from 5400-foot-altitude leaves yielded 0.63%. These findings largely corroborate previous research that has shown that height has a comparable effect on the concentration of artemisinin in the leaves. The amount of artemisinin in the leaves of A. roxburghiana and A. indica changed little but noticeably as a function of height. However, the amount of artemisinin in their leaves was significantly affected by their height. Figure 6 shows that various Artemisia species were shown to generate greater artemisinin at height II, which was between 5,000 and 6,000 feet. Contrary to previous reports, our data show that the artemisinin concentration of the leaves is lower at 5400 feet than at 6500 levels.

A chemotype refers to a plant with noticeable chemical composition of secondary metabolites, characterized by minor epigenetic and genetic modifications with little or no effect on the morphology of the plant. The previously published scientific reports have highlighted prominent variations in artemisinin content among *A. annua* plants from Germany, China, the USA, Yugoslavia, and Vietnam. It was concluded that different biosynthetic precursors of artemisinin, for

Table 1: Seven *Artemisia* species collected from the three altitudes of Northern Pakistan

Plant scientific names	Height group	Height (ft)	Sampling point
A. moorcroftiana	I	4960	But Pul, Balakot
A. moorcroftiana	II	5804	Miandam
A. moorcroftiana	III	6511	Bhurban, Murree
A. vestita	I	1981	Yadgar Center, Basham
A. vestita	II	5363	Barhkhar Gai, Shangla
A. vestita	III	6104	Miandam
A. vulgaris	I	2829	Rawal Bridge, Basham
A. vulgaris	II	5825	Topseen, Shangla
A. vulgaris	III	8025	Mutaltan, Kalam
A. indica	I	3174	Budgran, Muzaffarabad
A. indica	II	5174	Chakar, Muzaffarabad
A. indica	III	6016	Rawalakot
A. sieversiana	I	4734	Deenoor, Gilgit
A. sieversiana	II	5901	Hasanabad, Hunza
A. sieversiana	III	7739	Kareemabad, Hunza
A. roxburghiana var. roxburghiana	I	3831	Dhery, Shangla
A. roxburghiana var. roxburghiana	II	5600	Kherabad, Miandam
A. roxburghiana var. roxburghiana	III	9480	Bahrain Road, Miandam
A. roxburghiana var. gratae	I	3831	Basham Road, Shangla
A. roxburghiana var. gratae	II	5416	Supply Stop, Rawalakot
A. roxburghiana var. gratae	III	9480	Bahreen Road, Minadam

A. moorcroftiana: Artemisia moorcroftiana, A. vestita: Artemisia vestita, A. vulgaris: Artemisia vulgaris, A. indica: Artemisia indica, A. sieversiana: Artemisia sieversiana, A. roxburghiana: Artemisia roxburghiana

instance, artemisinic and dihydroartemisinic acids, produced remarkable variability in *A. annua* from different geographical origins. These findings suggested the presence of different chemotypes within *A. annua*. In the current study, we have investigated the impact of altitude on the artemisinin content of the seven *Artemisia* species. These species exhibited distinct chemotypes, with a significant impact of their respective geographical conditions on their artemisinin contents. Our findings are quite in agreement with the previously published data that affirms substantial differences in artemisinin content, even within the same species, inferable to the climatological conditions.[18,19]

As evident from the results presented in Figure 4, the height of various geographical locations produced a significant impact on the *in vivo* production of artemisinin in the leaves of five *Artemisia* species, namely, *A. vestita, A. moorcroftiana, A. sieversiana, A. vulgaris,* and *A. roxburghiana var. roxburghiana*. These findings affirm the existence of distinct chemotypes within these five *Artemisia* species. Our findings corroborate with a number of previously published reports. For instance, a substantial difference was observed in three key components – *Artemisia* ketone, 1,8-cineole, and camphor – of *A. annua* based on their global phytogeographic locations. Likewise, other studies evaluated two distinct chemotypes with relatively different artemisinin contents. In addition, researchers reported four chemotypes in *Artemisia absinthium* with a prominent difference in their essential oil composition. These alterations within the artemisinin content were attributed to natural factors such as latitude, altitude, and soil type, as well as the day length, flowering time, drying time, cultivation methods, and storage conditions.

CONCLUSION

A. annua was not the only Artemisia species found in northern Pakistan that contained artemisinin. Additionally, seven species were examined to determine the impact of habitat height on artemisinin production and chemotype presence or absence. The results showed that five Artemisia species exhibit chemotypes, and that Artemisia species grown at 5000-6000 feet (height II) yield the highest leaf artemisinin concentration. The present research found that chemotype occurs among Artemisia species and that plant geographical habitat height impacts artemisinin production.

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