**Spatio-temporal heterogeneity in phytochemicals and proximate composition of *Agave sisalana* (sisal) adapted to different ecological zones of the Punjab, Pakistan**

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**Abstract**

 This study was designed to determine proximate composition and phytochemical heterogeneity in *Agave sisalana* due to varying environmental conditionsatfive districts, i.e., Chakwal, Khushab, Rawalpindi, Faisalabad, and Layyah in Punjab, Pakistan. Plant sampling was carried out across two years (2017-18 and 2018-19) during mid-spring, summer, autumn, and winter seasons. Autumn season reflected saponins, tannins, and flavonoids in higher concentration during 2018-19, while steroids and terpenoids were found in higher concentration during spring 2018-19. The spatio-temporal variations in proximate analysis were more apparent in different samples collected from different districts. Data recorded from Khushab district during autumn season reflected higher composition of proximate analysis and phytochemical contents as compared to other seasons. Overall, the spatial differences in phytochemicals concentration were strongly associated with environmental conditions prevailing in different seasons in selected districts.

**Keywords.** Sisal,phytochemical, seasons, saponins, steroids, terpenoids, tannins, flavonoid

**Introduction:**

Sisal (*Agave sisalana* Perrine) is a xerophytic, perennial, hapaxanthic monocotyledonous, succulent plant. It yields hard fiber having a high commercial value, and is 6th most important fiber crop in the world. Approximately 75% of global production of hard natural fibers originates from sisal plant (Davis and Long, 2015; Duarte *et al.,* 2018; Nikam *et al*., 2019). It is currently cultivated on a large scale in Mexico (native), Central America, Brazil, Asia, and Africa (Coleman‐Derr *et al*., 2016). It is a monocarpic plant form an inflorescence after 6 to 9 years and then dies ([Asfaw, 2011](https://www.frontiersin.org/articles/10.3389/fmicb.2018.01227/full#B4)). Its reproduction is mainly asexual via suckers originating from the rhizomes and bulbils (Monja-Mio *et al*., 2019). It constitutes various biochemicals, phytochemicals including some sugars (Arrizon *et al*., 2010), alkaloidal amines, flavonoids, [sterols](https://www.sciencedirect.com/topics/food-science/sterol), steroidal [alkaloids](https://www.sciencedirect.com/topics/food-science/alkaloid), [sapogenins](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/sapogenin), glycosides, terpenoids, tannins, saponins, and flavonoids ([Debnath](https://www.sciencedirect.com/science/article/pii/S0926669017304314?casa_token=vpsUxwrmmU8AAAAA:eb51nO7w0h9MZNn2qnPN4VhDy5UqcdmHeQvTQuKyRGLSggEKZLJ-xRm9_3YjRbJlpBZ4iiXA" \l "bib0080) *[et al](https://www.sciencedirect.com/science/article/pii/S0926669017304314?casa_token=vpsUxwrmmU8AAAAA:eb51nO7w0h9MZNn2qnPN4VhDy5UqcdmHeQvTQuKyRGLSggEKZLJ-xRm9_3YjRbJlpBZ4iiXA" \l "bib0080)*[., 2010](https://www.sciencedirect.com/science/article/pii/S0926669017304314?casa_token=vpsUxwrmmU8AAAAA:eb51nO7w0h9MZNn2qnPN4VhDy5UqcdmHeQvTQuKyRGLSggEKZLJ-xRm9_3YjRbJlpBZ4iiXA" \l "bib0080); Ajayi *et al.,* 2011), which may perform analgesic, anti-inflammatory, anthelmintic, gastroprotective, antioxidant, antiviral, antimycotic, antibacterial, antituberculosis, bactericidal and insecticidal activities ([Hamissa](https://www.sciencedirect.com/science/article/pii/S0926669017304314?casa_token=vpsUxwrmmU8AAAAA:eb51nO7w0h9MZNn2qnPN4VhDy5UqcdmHeQvTQuKyRGLSggEKZLJ-xRm9_3YjRbJlpBZ4iiXA" \l "bib0035) *[et al](https://www.sciencedirect.com/science/article/pii/S0926669017304314?casa_token=vpsUxwrmmU8AAAAA:eb51nO7w0h9MZNn2qnPN4VhDy5UqcdmHeQvTQuKyRGLSggEKZLJ-xRm9_3YjRbJlpBZ4iiXA" \l "bib0035)*[., 2012](https://www.sciencedirect.com/science/article/pii/S0926669017304314?casa_token=vpsUxwrmmU8AAAAA:eb51nO7w0h9MZNn2qnPN4VhDy5UqcdmHeQvTQuKyRGLSggEKZLJ-xRm9_3YjRbJlpBZ4iiXA" \l "bib0035); Viel *et al*., 2017).

Proximate analysis revealed that sisal pulp may have a crude protein up to 7.3% and fiber of 15.2%, respectively (Gebremariam and Machin (2008). Its fiber has high durability, strength, ability to stretch, resistance to deteriorate, interaction with dyestuff (Gonzalez *et al*. 2015). Main product derived from this fiber includes biodegradable yarn used in handicrafts, production of upholstery and tequilas, ropes of various utilities, pulp for cellulose industry, decorative carpets, animal feed, organic [fertilizer](https://www.sciencedirect.com/topics/materials-science/fertilizer), biofertilizers, and bagging (Elanchezhian *et al*., 2018; de Melo *et al*., 2019). The aggregations observed in SEM micrographs of sisal bundles are attributed to CaC2O4·H2O crystals responsible for the high calcium content of the sisal ashes (Benitez-Guerrero *et al*., 2017.

The spatial variability in time due to variations in climatic conditions through the years (van Leeuwen, 2010). Su *et al*., (2018) reported that plant growth and nutrient status changed with spatiotemporal variability. Sisal utilizes the crassulacean acid metabolism (CAM) pathway for fixation of CO2 ([Abraham *et al*., 2016](https://www.frontiersin.org/articles/10.3389/fmicb.2018.01227/full#B3)), through thick leaves having a waxy cuticle layer, and they are arranged around the stem in a spiral shape which forms a rosette that helps in water retention (Holtum *et al*., 2014; Davis *et al*., 2017) and also provides an adaptive advantage in a vast range of geographical zones because of its resistance to abiotic and biotic factors such as extreme temperatures, drought, salinity, and pathogens (Tamayo-Ordóñez *et al*., 2016; Sarwar *et al*., 2020). The information regarding the effect of spatiotemporal variability on the proximate analysis and phytochemicals of sisal is still scarce and scanty and provokes new research demands. Based on this, a study was conducted to explore the proximate analysis and to know phytochemicals in sisal collected from different ecoregions of the Punjab, Pakistan, which could provide the precision management of soil nutrients, planting environment suitable for fiber quality, and to explore upcoming possibilities for improving phytochemicals in sisal; having prime importance in the medical industry.

**Materials and Methods**

Sisal (*Agave sisalana* Perrine) plants were collected from three sites of all five selected districts of the Punjab, Pakistan, (Table 1). Three natural vegetation sites were identified in each selected district and surveyed randomly to collect plant samples during mid of all four seasons (spring, summer, winter, and autumn) for two years. Phytochemical aspects of sisal plant samples were determined using appropriate analytical techniques.

Table 1: Geographical indicators of eco-regions in Punjab, Pakistan.

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Fig. 1. Weather data of two years and four seasons during 2017-19 from study districts

**Phytochemical Screening**

**Saponin determination**

Saponin content was determined by using the prescribed protocol of Nahapetian and Bassiri, (1975).

 **Determination of total flavonoid content**

Using the method of Chen *et al*. (1993) flavonoid contents were determined.

**Estimation of Steroids**

Steroids were estimated by the method described by Madhu *et al*. (2016).

**Triterpenoid**

Triterpenoids were measured through a modified method by Chang and Lin (2012).

**Tannins determination**

Tannins were measured by method of Kavitha and Indira (2016).

**Proximate analysis**

The proximate analysis of samples was carried out by the AOAC (2010) procedure and formula.

The data were analyzed statistically by Fisher’s Analysis of Variance technique using honestly significant difference (HSD) as mentioned by Steel *et al*. (1997) with the help of Statistix version 8.1 software.

**Results**

Saponins contents in sisal leave elucidated significant (P<0.01), seasonal effects, while variations among Locations and the interaction among Season and locations were non-significant (P>0.05), during 2017 and 2018. Temporal interaction between seasons and years was also found statistically significant. For years, saponins were insignificantly different during the year 2017-18, while significant variation in saponin contents of sisal leaves were found during the year 2018-19. Similarly, analysis revealed a greater accumulation of saponin content during autumn 2018 which was significantly higher than the saponin values recorded during the remaining seasons and years. The highest value of saponin (2.44%) was recorded in autumn-2018 while the lowest value (0.26%) was recorded during spring-2017 (Fig.2a)

Steroid contents determined in sisal leaves showed significant Season effects during 2018, while non-significant (P>0.05) seasonal variations were seen during 2017. Besides, variations among Locations, and the interaction among Seasons and locations was non-significant during 2017 and 2018. The highest steroid contents (3.1%) were recorded during spring 2019 followed by that in the autumn season. Lowest steroid contents (0.82%) were recorded during autumn 2017 (Fig. 2b). Samples collected from Faisalabad and Rawalpindi revealed a profoundly higher concentration of steroids, while the lowest steroids content was observed in sisal samples collected from district Layyah.

The flavonoids varied among the Seasons x Locations, and their interactions were found insignificant (P>0.05) during both years (2017-2018). Fig. 2c. depicted that a significantly higher concentration of flavonoids was recorded during year 2018-2019 compared to the 2017-18 experimental year. On the other hand, flavonoids were found significantly higher (7.20 mg g-1) during autumn 2018 followed by (6.52 mg g-1) during summer 2018. Sisal plants contained more flavonoids during summer and autumn 2018 conditions, but non-significant difference was found across the seasons during year 2017. Samples collected from Chakwal and Rawalpindi districts revealed a profoundly higher concentration of flavonoids (5.37 mg g-1) and (5.34 mg g-1), respectively while the lowest content (5.0 mg g-1) was observed in samples of district Layyah (Fig.2c).

Non-significant (P>0.05) variation was recorded in individual effects of Season and Location during 2017, while significant (P<0.01) differences were recorded during 2018. Interaction between Seasons x Locations during 2017 and 2018 was non-significant (P>0.05). Besides, terpenoids were found significantly higher during 2019 spring followed by 2018 autumn. For years **×** Locations, significantly higher concentrations of terpenoids were recorded during years 2018-2019 as compared to 2017-18, and at locations of Chakwal, Khushab, and Rawalpindi whereas Faisalabad and Layyah locations were found with lesser terpenoids contents in the sisal plants native to these sites. Samples collected from Khushab district exhibited profoundly higher concentrations of terpenoids (2.539 mg UA/g) during autumn 2018 while the lowest value of terpenoids (1.3075 mg UA/g) was also observed in Khushab site collected during the year, 2017 (Fig.2d).

Significant (P<0.01) variation was recorded in individual effects of Season, while non-significant (P>0.05) in Season and Locations and their interaction during 2017, while the individual effect of factors and the interaction among them was Significant (P<0.01) during 2018. whereas, results obtained from different locations were non-significant for sisal leaf tannins contents, while, it was significantly higher (2.22 mg of tannic acid/g) when assessed during summer, 2018 followed by value (2.08 mg of tannic acid/g) in samples of sisal leaves obtained and assessed during autumn 2018. Sisal plants accumulated more tannins during summer and autumn 2018, however, their concentrations during experimental year 2017-18 were comparatively less (Fig.2f).

**Proximate analysis**

Ash content Significantly (P<0.01) varied in individual effects of Season, but non-significant (P>0.05) in Season and Locations and their interaction during 2017, while individual effect of factors and the interaction among them was Significant (P<0.01) during 2018. Results elaborated the highest ash contents in sisal leaves from District Rawalpindi (3.03) during spring season followed by Khushab (2.81) in spring, all the districts depicted statistically different values for all seasons and locations. Data exhibited lower values for all sites during winter season. The least value (0.90) was observed during winter season for Faisalabad site which remained at par for all locations. Results for years interaction with seasons depicted significant variability for interaction as depicted in Fig.3a. The summer season of year 2018-19 expressed higher ash content (3.48) followed by spring (3.32) which was at par, but autumn and winter seasons followed respectively in descending order. The Fig.2a elaborated the significant variation of year 2017-18 and 2018-19. The least value of ash content (1.05) was observed during winter season 2017-18 and similar trend recorded for all seasons of year 2018-19.

 Interaction across the seasons i.e. (Locations × Seasons) was found to be significant (P<0.01) during 2017 and 2018. Results depicted the greatest accumulation of water in sisal leaves were from District Khushab (94.10) during spring season followed by Rawalpindi (93.71) in winter, All the districts were statistically similar. Data exhibited lower values for Faisalabad and Layyah sites during autumn season but statistically they were at par. Sisal leaf samples elaborated the similar trend in all seasons except autumn for all sites. The least value (82.33) was observed during autumn season at district Layyah site which remained at par with autumn season for Faisalabad location. Data pertaining to years interaction with seasons exhibited significant variability (Fig.2b). Spring season of year 2018-19 depicted higher moisture content (94.64) followed by winter, summer, and autumn seasons respectively. The Fig.3b revealed the significant variation of year 2017-18 and 2018-19. The least value of moisture content (87.57) was obtained in autumn 2017-18 and similar was recorded for year 2018-19 except the autumn seasons, however other seasons depicted similar trend during year 2017-18.

 Interaction across Locations × Seasons was found significant (P<0.01) during 2017 and 2018. Crude protein content exposed showed highest value (16.99) during winter 2018-19 in district Rawalpindi while it remained statistically at par for district Khushab for winter 2018-19. For year 2017-18 variable trend was observed for all four seasons Spring, summer, winter and autumn, on the other hand year 2018-19 also expressed significant variation for all seasons. Synthesis of crude protein expressed better results with a descending trend during year 2017-18 for *Agave sisalana* in winter, summer, spring, and autumn for year 2017-18, respectively. The lowest value (9.37) was recorded for Layyah site during autumn 2017-18 (Fig.3c).

 Interaction across the seasons i.e. (Locations × Seasons) was non-significant (P>0.01) during 2017 and 2018. Results elaborated the highest crude fiber contents in sisal leaves collected from District Layyah (14.07) during summer season followed by similar trend across all districts. Data exhibited lower values for all sites during autumn season. The least value (9.39) was observed during autumn season at district Layyah site followed by Faisalabad, Rawalpindi, Chakwal and Khushab site respectively. The data for years interaction with seasons depicted significant variability, and summer season of year 2018-19 exposed higher crude fiber content (14.71) followed by spring (13.218) and winter (12.96) which were at par statistically for year 2018-19, the autumn and winter seasons of both years remained similar. The least value of crude fiber content (10.71) was observed in autumn season of both years (Fig.3d)

**Discussion:**

This study showed that secondary metabolites present in the leaf juice were rich in tannins, saponins, flavonoids, steroids, and terpenoids. Ajayi *et al.* (2011) reported extensive prevalence of a variety of phytochemicals, saponins, tannins, flavonoids, and steroids in sisal juice. Saponins, tannins, and flavonoids were in higher concentration during autumn 2018-19. Szakiel *et al*. (2011) reported that temperature is a very important factor influencing phytochemical synthesis in plants. However, temperature extremes can be the most prominent cause of environmental stress, the elevated temperature was found to reduce photosynthetic rates (by 52% as compared to control plants), stomatal conductance (by 60%) that may indirectly affect the synthesis of phytochemicals in sisal.

The winter season with the lowest soil and air temperatures, higher relative humidity, and higher soil water availability accumulated less flavonoids. Similar findings were reported by [Mogren](https://www.sciencedirect.com/science/article/pii/S0308814610007478?casa_token=GuQ1abvEP3oAAAAA:apv6m2patmjSdP7kD4svu6PFLJEufeAkVzLo0bvtQ3bokplnigVh043UmJwN98sXpqB_fm7Vfn_J" \l "bib18) *[et al](https://www.sciencedirect.com/science/article/pii/S0308814610007478?casa_token=GuQ1abvEP3oAAAAA:apv6m2patmjSdP7kD4svu6PFLJEufeAkVzLo0bvtQ3bokplnigVh043UmJwN98sXpqB_fm7Vfn_J" \l "bib18)*[. (2006)](https://www.sciencedirect.com/science/article/pii/S0308814610007478?casa_token=GuQ1abvEP3oAAAAA:apv6m2patmjSdP7kD4svu6PFLJEufeAkVzLo0bvtQ3bokplnigVh043UmJwN98sXpqB_fm7Vfn_J" \l "bib18) , who found a high correlation between global radiation and quercetin levels in onion bulbs. Some results indicate that the synthesis of saponins and other phytochemicals is higher in response to stress, suggesting that these compounds could be involved in the adaptation of the plant to survive under adverse soil and climatic conditions (Copaja *et al*., 2003).

Steroids and terpenoids were high in spring 2018-19, moreover, the terpenoids were found dominant across the years. Conformity of the above results was reported by Onwuliri and Wonany (2005). As per the present study results, summer and autumn seasons were found to affect tannins significantly in sisal leaves. Such a response could be an inference of increased humidity during mid to late summers with a relevant impact on sisal growth in early autumn. Similarly, autumn and summer seasons also triggered the flavonoid concentrations under the arid conditions of Chakwal and Rawalpindi districts whereas, regions with lesser aridity but more soil fertility, i.e., Faisalabad and Khushab followed them. Moderate to high temperatures in autumn with instance of humidity resulted in more prevalence of flavonoids, however, winter low temperatures reduced flavonoids concentration in the leaves of sisal.

 Tannins and flavonoids were among significant phenolic compounds because of their weighty molecular mass and affinity towards water for solubilization and performance as chaperons to retain moisture. These tannins act as antioxidants by sustaining proteins and sugars under adverse conditions. Hence, they reduce their availability for degradation by active oxygen species (AOS) or microbes. High summer and autumn temperatures may cause degradation of protein and lipids in addition to the flaccidity of sisal leaves. Nevertheless, they can sustain their growth by accumulating exceptional concentrations of tannins for their adaptability under local conditions, particularly having limited water availability (Jouany and Morgavi., 2007).

 As far as the ash contents of sisal leaves are concerned, they ranged between 0.4% to 0.92%. Sisal leaves reportedly increase hydrophilicity under certain drier conditions with the help of higher ash contents and vice versa. In addition, their incorporation at a certain maturity or in the form of bulbils drop, and addition of nonviable bulbils in the soil will help chock the soil pore spaces and help sustain water, a sort of mulch (Nair, 2019). Similarly, in present study, lower values for all sites during winter season was observed. The least value (0.90) was observed during winter season at district Faisalabad which remained at par with winter season values for all locations. Results for years interaction with seasons depicted significant variability for interaction as in (Fig.3a). Crude protein referred to quality of leaves of sisal with respect to its dietary handling for animal consumption as well as for pharmaceutical uses. Temporal and spatial variations were reflected in the crude protein assays as they are highly degradable under high temperature and dry conditions (Verhoeven *et al*., 2018). Leaf fiber is the chief remarkable motive after leaf pulp for which it is adapted to a variety of climatic conditions (Barreto *et al*., 2011). Fiber tensile strength, fiber length, leaf yield and leaf length data showed significant result at P<0.0001. In conclusion, keeping in view various phytochemical components are present in *Agave sisalana* it can be grown successfully in marginal lands for commercial extraction of these chemicals. In crux, spatio-temporal variations are key source of understanding its availability at different sites in the Punjab, Pakistan.

 

**Fig.2:** Photochemical analysis ofsisal leaves as affected by different seasons of years and location. S (season), L (locations).

 **Fig.3:** Proximate analysis ofsisal leaves as affected by different seasons of years and location. S (season), L (locations).



Fig.4. Correlation matrix among different response variables during 2017 and 2018. Blue circle represents a positive correlation, while red for negative correlation. The red triangle showed strong positive correlation among different parameters. Sap (saponins); Ter (terpenoids); WC (water content), CF (crude fiber), Tan (Tannins); Ste (steroids), CP (crude proteins), Flavo (Flavonoids).

 Data concerning correlation matrix during 2017 represents the strong linear correlation between Sap and Ter. In addition, CP, Ste, Tan, CF, WC showed strong relations among each other while negative with Ter and sap. Whilst, Flavo and Ash content showed no/ or non-significant correlation with all response variables.  During 2018, CP, CF, and WC showed a positive correlation with each other. Tan and Ash content showed a positive relation with CP and CF, while non-significant relation was seen with WC. Besides, Ste showed a positive relation with CP, a negative with WC, and no relationship with CF. Moreover, Sap, Ter and Flavo showed negative relation with CF, CW, and CP.  In-depth, Ter and Sap are negatively correlated with CP and CF during both experimental years, while strong negative relation was seen in 2017 as compared to 2018, which may represent adjustment of secondary metabolites with fiber content during more harsh environmental conditions of 2018**.**

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