THE LEAF OF THE MEDICINAL PLANT *JUSTICIA WYNAADENSIS* UNDERGOES SEASONAL SHIFTS IN ITS PHENOLIC, FLAVONOID, CAROTENOID, AND MINERAL COMPOSITION

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ABSTRACT

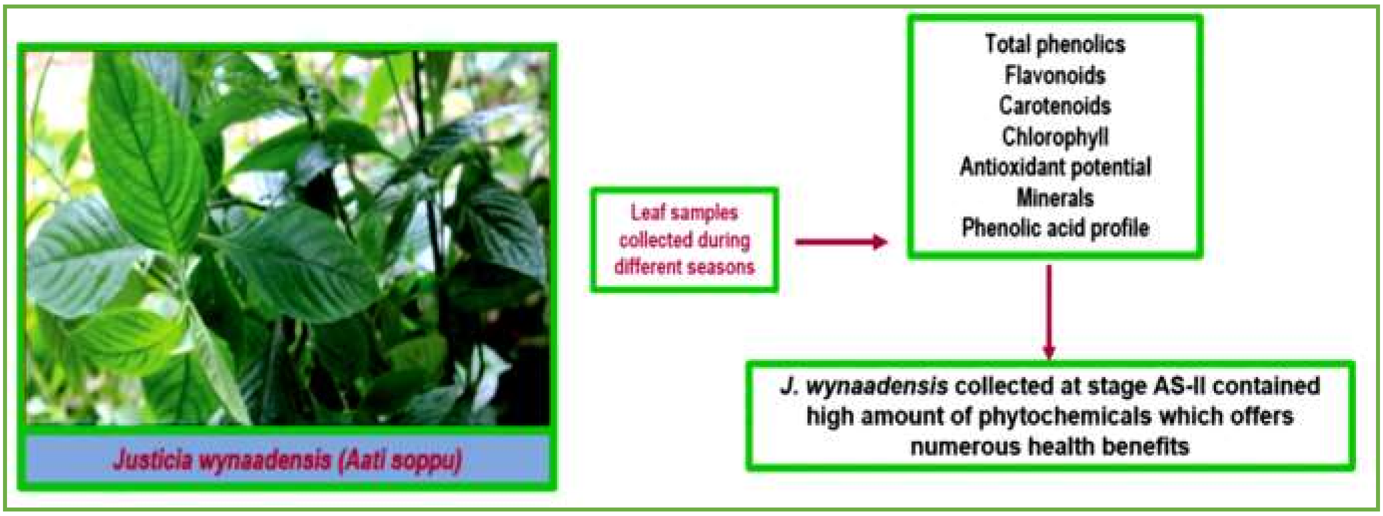
Aim: *This research looked at the variations in phenolics, flavonoids, carotenoids and mineral elements in the leaf extract of the medicinal plant J. wynaadensis (Aati soppu) over the monsoon, pre-monsoon, and post-monsoon periods.*
Methodology: *Three separate collections of J. wynaadensis leaves were made: (i) in the first week of July, at the start of the monsoon (AS-I); (ii) in the middle of the monsoon (AS-II) and (iii) after the monsoon (AS-III) in the first week of September. Total chlorophyll, carotenoids and phenolics, flavonoids with antioxidant potential, and mineral concentrations were measured in the samples taken. Using the UPLC-H class combined with TQD-MS/MS, we were able to identify and quantify certain phenolic acids.*
Results: *The biochemical composition of J. wynaadensis samples taken at different times of the year was very variable. The amount of phenolics (388.1 mg GAE 100 g), flavonoids (287.1 mg CE 100 g), and carotenoids (2.40 g–1 CE 100 g) in the AS-II samples were substantially higher than in the pre-and post-monsoon samples. Salicylic acid (2455–4747 g/g f.w.t.) was found to have the highest concentration of the phenolic acids measured, followed by caffeic acid (350 g/g f.w.t.). Calcium was second most common (263–442 mg 100g), but potassium was most common (240–1–1–1 mg 100g). Except for zinc, the levels of most of the microminerals dramatically dropped from the beginning of the monsoon to the conclusion. The iron content of the leaf samples was high, ranging from 2.37 to 3.313 mg per 100g.*
Interpretation: *The health advantages of J. wynaadensis are due in large part to the high levels of phenolics and flavonoids seen in samples obtained during the AS-II growth phase. The healing effects of Aati soppu gathered in AS-II are likely due to the higher concentration of bioactive phytochemicals like salicylic acid.*

Keywords: *Justicia wynaadensis*, Kodagu, Medicinal palnt.

INTRODUCTION

India is home to between 3,000 and 5,000 plant species, making it one of the world's most biodiverse countries (Zeven and de Wet, 1982). Of these, around 1,000 are cultivated for human consumption. Major biodiversity hot zones, including many edible and medicinal plant species, may be found in and around the Western Ghats. People in these regions have long relied on traditional foods as a source of nutrition and health care (Pandey et al., 2013). The locals only harvest and use certain plants at certain times of the year or for special events. The practice of eating plants has a rich cultural history (Greeshma and Sridhar, 2016).

The stem of the *Justicia* plant is just 2-3 meters in length, and its nodes are spaced far apart. The oppositely placed, elliptic-lance-shaped leaves are 5–10 cm long, with a petiole just 1-2 cm long. The leaves have 6–8 pairs of veins. The flowers bloom in pairs on drooping spikes 5–10 cm in length. The ovate bracts are between three and five mm in length. Both the sepals and the filaments of the two stamens are long and thin. The stigma is split into two parts, and the style is thread-like. The plant blooms from December to February and can be found growing wild in the area and in some people's gardens. During the monsoon's dark and wet months, the plant undergoes a remarkable change. According to research conducted by Nital *et al.* (2015), these leaves contain a total 18 different types of herbal medications that are beneficial to human health. It has been hypothesized that it reduces cholesterol and cholesterol esters in cells. It is widely held that adhering to this habit will help people maintain good health throughout the year. During the cold, damp and gloomy monsoon months, it creates heat and stabilizes the body temperature, ultimately improving blood circulation. It is said to deworm human beings and provide protection from the flu and the cold. Having one glass of *Justicia* plant tea daily has been shown to reduce the severity of urinary tract infections (Bhagya *et al*., 2013; Dsouza and Nanjaiah, 2018). Saponins, vitamins, carbohydrates, quinines, flavonoids, antioxidants, *etc*. are all plentiful in *Justicia wynaadensis*. Fever, skin illness, ulcers, wounds, fractures, leucorrhea, inflammation, tumors and a few other ailments are well treated.



Subbiah and Norman (2002) found that the content of cholesterol and cholesteryl ester in human macrophage cell lines was reduced and that this had a unique inhibitory effect on the uptake of oxidized low-density lipoproteins. The presence of phenolics, flavonoids, catalase and peroxidase enzyme activities contribute to the powerful antioxidant activity (Sudha *et al*., 2011). Due to its content of alkaloids, steroids, flavonoids, and cardiac glycosides, *J. wynaadensis* has anti-inflammatory properties on par with diclofenac (Vidyabharathi, 2012). Methanolic extracts of *J. wynaadensis* have been shown to contain phenolics, flavonoids and alkaloids, all of which have potential uses as natural antioxidants and as dietary supplements (John *et al*., 2013; John *et al*., 2014).
Twenty-four compounds, including dihydro coumarin, phytol, palmitic acid, linoleic acid, stearic acid, squalene, and phytosterols like campesterol and stigmasterol, were identified by GC/MS analysis of methanolic extracts of *Justicia* leaves and stems conducted by Ponnamma and Manjunath (2012). According to the results of the study conducted by Dsouza and Nanjaia (2018), the 3,3,4-trihydroxy flavone isolated from *J. wynaad*ensis has the potential to serve as a source of antimicrobial agents for treating a wide range of illnesses. Antioxidant activity in the leaf extracts was on par with that of reference compounds including BHT, ascorbic acid, quercetin, curcumin, and mannitol.
Several *in vitro* tests (Vandana and Shanti, 2017; Chavan *et al*., 2019) have shown that *J. wynaadensis* leaf extract is highly antioxidant and free radical scavenging. Although data on the health benefits and phytochemical composition of *J. wynaadensis* are available, little is known about how these phytochemical components, such as phenolics, flavonoids, and antioxidant capacity, vary with the seasons. The purpose of this research was to examine the variation in phenolics, flavonoids, carotenoids, and mineral elements in *J. wynaadensis* over the monsoon, pre-monsoon, and post-monsoon periods.

MATERIALS AND METHODS

Sample collection: The leaf samples were gathered in 2017 at three different times of the year: (AS-I) at the beginning of the monsoon, in the first week of July; (AS-II) in the middle of the monsoon, in the first week of August; and (AS-III) after the monsoon, in the first week of September. Several biochemical and mineral studies were performed on the collected materials.

Determination of total phenolics, flavonoids and antioxidant potential: We extracted about 5 g of freshly obtained materials with 80% aqueous ethanol, centrifuged the extract at 6000 rpm for 15 minutes at 4°C, and collected the supernatant in brown reagent bottles (to prevent light exposure).
Two more solvent extractions were performed on the residue, and the combined extracts were then analyzed for total phenolics, total flavonoid concentration, and antioxidant potential. The Folin-Ciocalteu test was used to calculate the total phenolic content of the extract (Singleton *et al*., 1999).
The total phenol content was calculated using gallic acid as an internal standard and the results were represented as milligrams (mg) of gallic acid

equivalent per one hundred grams (g) of fresh leaf weight. The total flavonoid content was calculated as the catechin equivalent per 100 g of sample using the aluminium chloride method (Zhishen *et al.,* 1999), with catechin (0–50 g) as the standard. Both the radical scavenging activity (measured with DPPH) and the reducing power (measured with FRAP) contributed to the overall antioxidant potential. Both the FRAP assay (as described by Benzie and Strain, 1996) and the 1,1'-diphenyl-12-picrylhydrazyl (DPPH) free radical scavenging activity assay (developed by Brand-Williams *et al*., 1995) were performed. The results were reported in terms of the antioxidant capacity (AEAC) per 100 g of fresh leaves, with ascorbic acid serving as the benchmark in both cases.

Identification and quantification of phenolic acids using UPLC-H class coupled with TQD-MS/MS: For the purpose of identifying and quantifying phenolic acids, ultra-pressure liquid chromatography-mass spectrometry (UPLC-MS/MS) was used. The ionization source is electrospray ionization (ESI), and the instrumentation is a tandem (triple) quadrupole detector (TQD) MS/MS system from Waters Inc., USA (Accubity UPLC-H class). The column temperature was kept at 25 °C, and a Waters UPLC BEH-C18 column (2.1 x 50 mm) with 1.7 m particles was utilized, guarded by a Waters Vanguard BEH-C-18 with 1.7 m particles.

Using MS with ESI ionization and then the Multiple Reaction Monitoring technique, the eluted compounds were identified and their -ve estimates were calculated (Arivalagan *et al*., 2018). According to Chen *et al*. (2001), 80% methanol was used to extract the various phenolic acids. The extracted sample was filtered through a 0.2-m nylon filter before being injected into an LC-MS/MS system and subsequently eluted with a mobile phase containing 0.1% formic acid in water (A) and 0.2% formic acid in methanol (B) on a gradient from 0 to 18 minutes.

RESULTS AND DISCUSSION

The levels of phenolics, flavonoids, carotenoids, and mineral components in *J. wynaadensis* were tracked over time and throughout harvesting windows during the monsoons. Locals use the leaves of *J. wynaadensis* for both culinary and medicinal purposes in early August due to traditional beliefs that the plant has wound-healing capabilities. Most of the metrics examined indicated statistically significant differences among collection periods. There was a statistically significant difference between the times of sample collection and the total phenolic content, which ranged from 323.8 to 388.2 mg GAE per 100 grams.

Table 1: Temporal changes in the level of phenolics, flavonoids, carotenoids, chlorophyll and antioxidant potential in *Justicia wynaadensis* leaf samples collected during monsoon (AS-II), pre (AS-I) and post monsoon (AS-III) seasons.

Parameters	AS-I	AS-II	AS-III	CD
TPC (mg GAE 100 g^{-1})	323.8^{b}±6.18	388.2^{a}±9.09	366.5^{a}±19.91	31
DPPH (mg AEAC 100 g)	138^{c}±23.8	287^{a}±12.1	216^{b}±18.1	33.6
DPPH (mg AEAC 100 g	103.6±1.89	117.2±17.36	133.9±31.57	ns
FRAP (mg AEAC 100 g)	3.29^{a}±0.01	3.11^{b}±0.01	3.35^{a}±0.06	0.07
Total carotenoids (µg β-CE 100 g)	1.69^{b}±0.09	2.40^{a}±0.20	0.501^{c}±0.01	0.26
Chlorophyll a (mg g)	4.90^{a}±1.27	2.07^{b}±0.23	0.623^{b}±0.01	1.35
Chlorophyll b (mg g)	2.91^{a}±1.72	1.09^{ab}±0.11	0.279^{b}±0.01	1.8
Total Chlorophyll (mg g)	7.81^{a}±0.57	3.16^{b}±0.33	0.902^{c}±0.01	0.69

The data reported is the mean standard deviation (in terms of 100 g of fresh sample) from three separate tests. Total Phenolic Content, Total Flavonoid Content, Gallic Acid Equivalent, Catechin Equivalent, Ascorbic Acid Equivalent Antioxidant Capacity, and Beta-Carotene Equivalent are some of the terms used to describe the antioxidant potential of a given food or drink. Different letters in each row after the mean value are substantially different at the 5% level of significance, according to Duncan's Multiple Range Test (CD-Critical Difference at the 5% level of significance).

Three independent experiments were performed and data are presented as mean ± SD in a 100 g fresh sample. TPC-Total Phenolic Content; TFC- Total Flavonoid Content; GAE- Gallic Acid Equivalent; CE-Catechin Equivalent; AEAC – Ascorbic acid Equivalent Antioxidant Capacity; β-CE- β-carotene

Equivalent. CD- Critical Difference at 5% level of significance; Different letters in each row after the mean value are significantly different at a 5% level of significance according to Duncan's Multiple Range Test Compared to samples obtained at the start of the -1 monsoon, those collected on day 17 of Aadi masa (AS-II) had a significantly higher level of phenolics (388.2 mg GAE 100 g) (Table 1). From AS-I to AS-II, we saw a 20 per cent increase in phenolic content, followed by a 6 per cent decrease in AS-III. Like phenolics, flavonoid concentration was significantly higher in AS-II (287 mg CE -1 100 g) than in AS-I (138 mg 100 g) (Table 1). Flavonoid content ranged from 138 mg to 287 mg CE per 100 grams.

Table 2: Temporal changes in the phenolic acid constituents (µg g^{-1} f.wt.) in *Justicia wynaadensis* leaf samples collected during monsoon (AS-II), pre (AS-I) and post monsoon (AS-III) seasons.

Phenolic acids	AS-I	AS-II	AS-III	% Increase in AS-II over AS-I	% Decrease in AS-II over AS-I
p-OH Benzoic acid	2.28	1.49	2.26	-	34.65
t-Cinnamic acid	28.4	2.37	26.3	-	91.6
2,4-OH Benzoic acid	7.92	5.76	0.720	-	27.27
Gentisic acid	6.21	12.4	13.0	99.84	-
Proto catechuic acid	113	15.1	39.3	-	86.62
o-Coumaric acid	398	678	135	70.31	-
p-Coumaric acid	357	8.23	3.22	-	97.7
Vanillic acid	15.6	6.30	7.56	-	59.72
Gallic acid	152	60.0	29.4	-	60.57
Caffeic acid	350	795	552	126.94	-
Sinapic acid	1.77	4.26	2.25	140.68	-
Ferulic acid	598	526	363	-	-
Salicylic acid	3301	4747	2455	43.78	-
Ellagic acid	25.1	15.5	12.4	-	38.2

Increase in the phenolic content was observed from AS-I to AS-II, which declined about 6% in AS-III. Flavonoid content varied between 138 mg–287 mg CE 100 g^{-1}, and similar to phenolics, flavonoid content was also significantly high in AS-II (287 mg CE 100 g^{-1}) compared to AS-I (138 mg 100 g^{-1}).

The flavonoid concentration increased by 108.4% from AS-I to AS-II, while it dropped by roughly 33.3% in the post-monsoon season sample. These findings show that *J. wynaadensis* harvested during AS-II had an exceptionally high concentration of phenolics and flavonoids, which have many positive health effects and are consumed by the public at this time. Bioavailability limits the protective effects of polyphenol-rich diets against chronic pathological illnesses such as cancer, diabetes, cardiovascular disease and ageing, as described in a review by Pandey and Rizvi (2009). However, the antioxidant activity did not show any clear trends. Table 1 shows that its health-promoting and functional dietary components have a total antioxidant activity of between 3.11 and -1.33 in the FRAP assay and between 103.56 and 133.91 mg AEAC per 100 grams in the DPPH assay.

Leaf samples included anywhere from 0.501 g (CE 100 g) (AS-III) to 2.40 g (CE 100 g) (AS-II) of total carotenoids. AS-II (2.40 g -1 CE 100 g) (when it is used as a curative) saw a 42% increase in its level in the leaf samples compared to AS-I, and then AS-III saw a precipitous 80% fall (Table 1). Carotenoids are powerful antioxidants, and beta-carotene is a key building block for vitamin A, which is vital for maintaining strong immune systems, clear eyesight, and supple skin and mucous membranes (Nkondjock & Ghadirian, 2004). Chlorophyll has been demonstrated to have anti-inflammatory, antioxidant, anticancer, and wound healing capabilities (Paranagama *et al*., 2003); however, the quantity of chlorophyll a, chlorophyll b, and total chlorophyll drastically decreased from ASI to ASIII. Two mono hydroxyl benzoic acid derivatives (p-OH benzoic acid and salicylic acid) and four dihydroxy benzoic acid derivatives (protocatechuic acid, vanillic acid, 2,4-dihydroxy benzoic acid, and gentisic acid) were found in *J. wynaadensis* leaves, along with one trihydroxy benzoic acid derivative (gallic acid) and six hydroxycinnamic acid derivatives (o-coumaric acid). The highest concentration of phenolic acid measured was salicylic acid, which was between -1 and -1 at 2455 and 4747 micrograms per gram, followed by caffeic acid (350 to 795 micrograms per gram), o- and -1 coumaric acid (135 to 678 micrograms per gram), and ferulic acid (363 to 598 micrograms per gram).

Table 3: Temporal changes in the mineral constituents (mg) in *Justicia wynaadensis* (mg 100 g-1 f. wt.) leaf samples collected during monsoon (AS-II), pre (AS-I) and post monsoon (AS-III) seasons

Minerals	AS-I	AS-II	AS-III	CD
Nitrogen	370^{b}±7.4	467^{a}±9.3	485^{a}±10.0	17.2
Phosphorus	8.75^{c}±0.18	28.8^{b}±0.58	42.5^{a}±0.85	1.20
Potassium	278^{a}±5.6	276^{a}±5.5	240^{b}±4.8	10.6
Calcium	442^{a}±8.8	271^{b}±5.4	263^{b}±5.3	13.4
Magnesium	40.5^{a}b±0.81	39.0^{b}±0.78	42.0^{a}±0.84	1.62
Iron	3.13^{a}±0.063	2.37^{c}±0.047	2.79^{b}±0.056	0.11
Manganese	0.320^{a}±0.006	0.210^{c}±0.004	0.230^{b}±0.005	0.01
Copper	0.320^{a}±0.006	0.215^{b}±0.004	0.175^{c}±0.004	0.01
Zinc	0.325^{c}±0.007	0.340^{b}±0.007	0.390^{a}±0.008	0.01

Three independent experiments were performed, and data are presented as mean ± SD. CD- Critical Difference at 5% level of significance; Different letters in each row after the mean value are significantly different at a 5% level of significance according to Duncan's Multiple Range Test.

There were noticeable variations in the phenolic acid content of *J. wynaadensis* between samples taken before and after the monsoon. The concentration of salicylic acid in ASII was 43.8% higher than in ASI, and then it dropped to 48.3% lower in AS III. Extreme downpouring, *i.e.*, the largest rainfall for three weeks in July compared to other months, when temperature and relative humidity prevail optimally, may account for the rise in salicylic acid concentration during the transition from AS-I to AS-II.

Salicylic acid, a bioactive phytochemical found in higher concentrations in *J. wynaadensis* samples collected during ASII, has been used to remove the epidermis of the skin to treat warts, calluses, psoriasis, dandruff, acne, ringworm, and ichthyosis (Arif, 2015; Bikowski, 2004; Saoji and Madke, 2021). Salicylic acid has been shown to be effective as a keratolytic, comedolytic, and bacteriostatic agent (Madan and Levitt, 2014; Bosund *et al*., 1960), causing the epidermis to shed its cells more quickly, opening blocked pores, and neutralizing bacteria. Natural hydroxy acids, such as salicylic acid, have been used in the skincare business of the cosmeceutical industry for anti-ageing, free radical scavenging, and hyperpigmentation treatment (Kornhauser *et al*., 2012). According to local wisdom, the healing powers of *J. wynaadensis* are at their peak in the month of August, when its salicylic acid level is highest and it is most commonly used as a food. According to labelling research, phenylalanine ammonia-lyase (PAL), a major regulator of the phenylpropanoid pathway, converts cinnamate into salicylic acid *via* o-coumarate or benzoate. Salicylic acid is synthesized from cinnamate *via* benzoate in tomato, tobacco, rice, potato, and cucumber (Chen *et al*., 2009); in sunflower, potato and pea, salicylic acid is formed from benzoate; in *Primula acaulis* and Gaultheria procumbens, salicylic acid is formed *via* both o-coumarate and benzoate.

Labelling studies are necessary to confirm, but the reduction in cinnamic acid and benzoic acid in ASII may be interpreted as evidence that these are the immediate precursors for the synthesis of salicylic acid in *J. wynaadensis* leaves, which spikes in August as the monsoon approaches its end. Caffeic acid, ferulic acid, and o-coumaric acid were the other major phenolic acids in *J. wynaadensis*; like salicylic acid, they increased in ASII by 126.9% (2.3 times) and 70.3% (1.7 times), respectively, compared to ASI, and then declined in ASIII. Caffeic acid, a polyphenol that makes up the majority of hydroxyl cinnamic acid in the human diet, is made by plants through a process called secondary metabolism. Antibacterial, antiviral, antioxidant, anti-inflammatory, anti-atherosclerotic, immune-stimulatory, antidiabetic, cardioprotective, anti-proliferative, hepatoprotective, and anticancer actions are just some of the physiological effects of caffeic acid and its derivatives. Caffeic acid protects plant leaves from UV-B radiation and aids in the plant's defence mechanism against predators, pests, and diseases (Espndola *et al*., 2019). Accelerated biosynthesis of the end product caffeic acid by the endogenous shikimate pathway has been demonstrated in other plants (Silva *et al*., 2014; Rodrigues *et al.,* 2015), which may explain why the immediate precursors of caffeic acid, t-cinnamic and p-coumaric acids, decreased by 91.6 and 97.7%, respectively, in ASII. The levels of ferulic acid in ASI and ASII were similar, but in ASIII they dropped. Because of its increased lipophilicity, the hydroxyl cinnamic acid, ferulic acid, is able to penetrate the skin more effectively. Both ferulic acid and caffeic acid derivatives have been shown to have antibacterial activity by disrupting the bacterial and fungal cell membranes, leading to cytoplasmic membrane leakage and eventual cell death. To wit: Taofiq *et al.,* 2017. Competitive inhibition of tyrosinase by ferulic acid has been shown to reduce melanin production in the skin (Kumar and Pruthi, 2014). Further evidence suggests that the presence of these phenolic acids may contribute to the observed anti-inflammatory effect in the skin by *J. wynaadensis* leaves when their content is significantly higher in August (ASII). Ferulic and caffeic acids have proven cosmeceutical and commercial potential as protective ingredients against UV-induced skin ageing and UVB-induced skin erythema (Taofiq *et al*., 2017). The molecular mechanism through which hydroxyl cinnamic acids, ferulic acid, p-coumaric acid, caffeic acid, and chlorogenic acid and its derivatives exert their anti-inflammatory actions was reviewed by Alam *et al*. (2016). Sinapic acid is biosynthesized from caffeic acid *via* intermediary ferulic acid and is abundant in fruits and vegetables (Silambarasan *et al.,* 2016). It has been linked to antioxidant and anti-inflammatory effects. Chlorophyll a, b, and total Ellagic acid, gallic acid, vanillic acid, protocatechuic acid, copper, calcium, manganese, iron, ferulic acid, 2,4-dihydroxybenzoic acid, and potassium are all abundant in samples taken at the beginning of the monsoon (AS-I) stage.

TPC, TFC, caffeic acid, sinapic acid, salicylic acid, coumaric acid, carotenoids, ferulic acid, 2,4-dihydroxybenzoic acid, and potassium were also highly concentrated in AS-II, which occupied the third quarter. Since potassium, ferulic acid, and 2,4-dihydroxybenzoic acid all appeared in both the AS-I and AS-II samples, it is clear that they were present at elevated levels during both collection periods. The final section of the scatter plot included data from the AS-III sampling stage. The AS-II is high in TPC and TFC, as well as carotenoids, salicylic acid, and other phenolic acids, which give the human body resistance against a wide range of diseases, as shown by the biplot.

The phenolics, flavonoids, and carotenoids content of *J. wynaadensis* leaves was significantly higher during the monsoon (AS-II) season, which may contribute to its health advantages. Salicylic acid, found in large quantities in *J. wynaadensis,* has been included in various medications for its therapeutic effects on skin conditions. Thus, the therapeutic effects of *J. wynaadensis* can be related to a dramatic shift in the photochemistry of the plant's leaves during the first week of August.

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