Article

Metabolomic Analysis for Compositional Differences of Bush Tea (*Athrixia phylicoides* DC.) Subjected to Seasonal Dynamics

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Abstract: Bush tea (*Athrixia phylicoides* DC.) is a South African indigenous herbal tea rich in secondary metabolites with medicinal significance. However, studies on the effects of seasonal changes on bush tea and quantification of its metabolites using untargeted approach have not been explored. Therefore, this study's objective was to investigate the effects of seasonal variation on the metabolites of bush tea using LC-MS/MS (Liquid Chromatography with Tandem Mass Spectrometry) and ¹H NMR (High-Resolution Proton Nuclear Magnetic Resonance) techniques. Bush tea leaves were plucked once in each season namely, autumn (March to May), winter (June to August), spring (September to November), and summer (December to February). The primary metabolites including non-essential amino acids, organic acids, and vitamins in bush tea were predominantly elevated during summer and spring. The ¹H NMR-based metabolomic analysis revealed that bush tea metabolome was strongly affected by seasonal variations, using the partial least squares-discriminant analysis (PLS-DA) which demonstrated four distinct groups of seasons. Similarly, the ¹H NMR based metabolic profiling of bush tea subjected to different seasons resulted in putative annotation of six phenolic compounds which included rutin, 4-(hydroxyphenyl) propyl coumarate, caffeic acid, hymenoxin, quercetin, and kaempferol. The phenolics and primary metabolites differed remarkably per season with enhanced accumulation observed in the summer and spring harvested bush tea. The LC-MS and ¹H NMR metabolomics analysis suggests that summer and spring can be recommended as preferred plucking seasons for bush tea leaves and twigs.

Keywords: bush tea; seasonal variation; *Athrixia phylicoides*; tea metabolites; phenolics

1. Introduction

Bush tea (*Athrixia phylicoides* DC.) is a South African indigenous aromatic and perennial, leafy shrub which has a distinguished history of usage by the native people for decades [1]. Although scientific studies have demonstrated the potential of bush tea as an herbal tea comprising secondary metabolites with beneficial effect and pharmacological properties [2,3], its commercialization is still at its infancy. Bush tea has been reported to contain several polyphenols, sesquiterpenes, coumarins, flavonoids, alkaloids, polysaccharides, amino acids, lipids, vitamins, and inorganic elements [4,5].

Tea metabolome has shown to be influenced by both abiotic and biotic factors [6]. The presence of active compounds and the therapeutic efficacy of tea plants are associated with changing

climatic conditions resulting from seasonal variations [7,8]. Seasonal fluctuations expose plants to different temperature levels [9], light intensity, and precipitation [6], which affect concentrations of tea phytochemicals and metabolic pathways responsible for the enhancement of metabolites [10]. Environmental factors such as light, influence the biosynthesis of phenolic compounds like catechin in black tea (*Camellia sinensis*) [6]. Low temperature induced the biosynthesis of catechins [8] while high temperature accelerated leaf growth, diluting the contents of secondary metabolites [9]. Precipitation was found to be negatively associated with the accumulation of all catechin fractions in *C. sinensis* [6]. In bush tea, Mudau et al. [11] demonstrated seasonal variation effects on selected phytochemicals, reporting that the plant's total antioxidants content were increased during summer and winter, condensed tannins during autumn (4.82%), while total polyphenols were increased in winter (51.1 mg·g⁻¹).

Seasonal changes induce different stresses in plants; the thermal stress during warmer seasons enhanced the antioxidant properties and availability of carbohydrates [12], while the solar radiation stress was found to influence the production of phenolic compounds [13]. Consequently, these stresses influence secondary metabolic products of tea plants since they are synthesized under stressful conditions to protect the plant cells from oxidation, protein denaturation, and damage [14]. It is therefore critical to assess teas harvested at different seasons to identify appropriate plucking seasons when the plants have accumulated optimum concentrations of secondary metabolites with pharmaceutical significance [15,16].

Several studies have demonstrated the use metabolomics analysis through proton nuclear magnetic resonance spectroscopy (¹H NMR) [17,18], gas chromatography-mass spectrometry (GC-MS) [19], and ultra-performance liquid chromatography quadrupole-time of flight mass spectrometry (UPLCQ-TOF MS) in various teas [20,21]. These metabolomics studies coupled with chemometrics showed a more comprehensive representation of associations between the environmental factors [20,22,23], growing conditions [24], and tea processing conditions [25] with tea chemical profile. However, data on seasonal effects on metabolites accumulation in bush tea are lacking. The lack of data is accentuated by the fact that bush tea is traditionally picked from the wild. Trials on a small scale are underway since the concept of domesticating wild plant to balance the biodiversity became a norm due to biodiversity laws in many parts of the world. The herbal teas are becoming a thriving business since the demand is exceeding the supply. Hence, the objective of the study was to determine the effects of seasonal changes on the metabolites of bush tea using LC-MS/MS and ¹H NMR techniques under cultivated environment.

2. Materials and Methods

2.1. Experimental Site

The trial was conducted at the Agricultural Research Council, Vegetable and Ornamental Plant Institute, situated about 25 km north of central Pretoria on the Moloto/KwaMhlanga Road (R573), GPS coordinate 25°59'' S; 28°35'' E, 1168 m altitude. The farm covers approximately 4000 ha, of which only 650 ha is under irrigation. The bush tea was grown under 40% shade net and drip irrigation at a frequency of 3.78 liters per hour for every 15 s (3 times a day) throughout the study.

2.2. Experimental Design and Treatments

One-year-old pruned bush tea plants were grown under shade net in three parallel blocks of 11 m. The tea plants were plucked once in each season in a one-year cycle and the seasons included autumn/fall (March to May), winter (June to August), spring (September to November), and summer (December to February).

2.3. Sample Preparation

Mature leaf and twig samples were harvested from bush tea plants and air-dried at room temperature in the shade to constant weight [26] (Samples were then ground to a powder using a benchtop grinder and stored in glass vials below -50°C until extraction).

2.4. LC-MS/MS Triple Quadrupole Mass Spectrometer

Fifty milligrams of each sample (five replicates for each treatment) were weighed into a 2 mL Eppendorf tube. One milliliter of the solvent (75% LC-MS grade methanol) was added to the samples and vortexed for one minute, and then sonicated for 30 min. Samples were then centrifuged for 15 min at 10,000 rpm. Seven hundred microliters of the supernatant were then pipetted into HPLC (High Performance Liquid Chromatography) vials, and metal caps with rubber septa were secured with a crimper.

The supernatant was injected into an ultra-high-performance liquid chromatography (UHPLC coupled to a high-resolution triple quadrupole MS (Shimadzu Model 8040, Shimadzu, Kyoto, Japan). Metabolites were separated and detected using ultra C18 AQC column (100 mm $\times$ 2.1 mm i.d., 3 μm particle) and maintained at 35°C . The mobile phase consisted of 0.1% formic acid in water (solvent A) and LC-MS grade methanol (solvent B), at a flow rate of 0.3 mL/min. Gradient elution applied was: 85% A: 15% B to 65% A: 35% B in 4 min, changed to 50% A: 50% B for 2 min, then to 20% A: 80% B for 1 min, and back to the initial ratio (85% A: 15% B to 65% A: 35% B) for 0.5 min. Furthermore, the UHPLC system was interfaced with a Xevo G2 QT of MS (Shimadzu, Kyoto, Japan), then applied with a source—ESI positive mode; capillary voltage—3 kV; cone voltage 30 V; calibration—sodium formate; lock spray—leucine enkephalin, and mass scan range at 200–1500 m/z . The data acquisition was performed using LabSolutions software LC-MS Ver.5.82 (Shimadzu, Kyoto, Japan) and LabSolutions database.

2.5. ^1H NMR Data Acquisition

Nuclear magnetic resonance spectral data were obtained using a 600 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA, USA), with 20 scans recorded. The sample preparation, data acquisition, analysis, mining, and processing were done as described by Maree and Viljoen [27]. The powdered bush tea samples (50 mg) were weighed into 2 mL Eppendorf tube for extraction and analysis. A 1.0 mL solvent mixture of 1:1 methanol D₄ and deuterium oxide (D₂O) (pH 6.0) was added. The mixture was vortexed at room temperature for 1 min and ultra-sonicated for 15 min to break the cell membranes, and then centrifuged for 5 min. Approximately 0.5 mL of the supernatant was transferred into NMR tubes for analysis.

All ^1H spectra were subjected to baseline and phase correction using MestReNova 12.0 (Mestrelab Research S.L., Santiago de Compostela, Spain). The spectral regions (0–10.00 ppm) were divided into 0.04 ppm bins, converted to American Standard Code for Information Interchange (ASCII) format and imported to Microsoft[®] Excel 2010 (Microsoft Corporation, Redmond, Washington). MetaboAnalyst with Pareto scaling method was used to perform the multivariate data analysis.

2.6. Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA), carried out using STATISTICA (StaSoft Inc., Tulsa, OK, USA) package (2011) to analyze the integrated peak area. Mean separation was done using Tukey's Honest Significant Difference (HSD) at $p < 0.05$. The supervised partial least squares-discriminant analysis (PLS-DA) was carried out using MetaboAnalyst 4.0 (<http://metaboanalyst.ca>). The heatmap was constructed using hierarchical cluster analysis (Euclidean distance and clustering algorithms as Ward's linkage) in MetaboAnalyst software.

3. Results

3.1. Seasonal Variation on Bush Tea Primary Metabolites Using LC-MS/MS

The heatmap revealed distinct groups of primary metabolites of bush tea harvested at different seasons indicating that tea metabolome patterns are dependent on seasonal changes. The heatmap comprises of two main clusters with the second cluster further divided into two. Bush tea harvested in summer grouped in the first cluster on the left. The second cluster was divided into two groups, and the autumn and winter plucked bush tea gathered together in one sub-cluster while spring plucked bush tea clustered and dominated the sub-cluster on the right (Figure 1).

Figure 1. The heatmap for seasonal variation of bush tea primary metabolites identified from LC-MS/MS (Liquid Chromatography with Tandem Mass Spectrometry) triple quadrupole on bush tea (*A. phylicoides* DC.) using unsupervised hierarchical clustering. The row represents the primary metabolites and column represent seasons.

The LC-MS/MS Triple Quadrupole detected different groups of primary metabolites and statistical analysis of their integrated peak revealed significant differences among the seasons (Table 1). For essential amino acids, the summer harvested bush tea samples significantly exhibited the highest threonine peak area (8.73×10^5), and the lowest area was recorded in the autumn bush tea plants with 3.29×10^5 peak area. Harvested leaves and twigs exhibited higher methionine sulfoxide in winter (2.80×10^5) and low in autumn (1.45×10^5). Both leucine and isoleucine were high in the winter and low in the summer bush tea leaves and twigs.

Table 1. Effects of seasonal variations on peak intensities of primary metabolites from bush tea (*A. phylicoides* DC.).

Season	Autumn	Winter	Spring	Summer
(Peak Intensities)				
Essential amino acids				
Threonine	$3.29 \times 10^5 \pm 0.42 \times 10^5$ b	$3.83 \times 10^5 \pm 0.75 \times 10^5$ b	$8.73 \times 10^5 \pm 1.34 \times 10^5$ a	$8.65 \times 10^5 \pm 0.41 \times 10^5$ a
Methionine sulfoxide	$1.45 \times 10^5 \pm 0.28 \times 10^5$ c	$2.80 \times 10^5 \pm 0.47 \times 10^5$ a	$2.20 \times 10^5 \pm 0.13 \times 10^5$ b	$1.54 \times 10^5 \pm 0.07 \times 10^5$ c
Isoleucine	$3.24 \times 10^4 \pm 1.13 \times 10^4$ a	$3.58 \times 10^4 \pm 0.93 \times 10^4$ a	$2.01 \times 10^4 \pm 0.13 \times 10^4$ b	0.0 ± 0.0 c
Leucine	$3.49 \times 10^4 \pm 1.28 \times 10^4$ b	$3.85 \times 10^4 \pm 0.39 \times 10^4$ a	$0.59 \times 10^4 \pm 0.04 \times 10^4$ c	0.0 ± 0.0 d
Conditional and non-essential amino acids				
Asparagine	$0.37 \times 10^6 \pm 0.06 \times 10^6$ b	$0.11 \times 10^6 \pm 0.02 \times 10^6$ c	$1.51 \times 10^6 \pm 0.22 \times 10^6$ a	0.0 ± 0.0 d
Aspartic acid	$1.46 \times 10^5 \pm 0.19 \times 10^5$ bc	$1.27 \times 10^5 \pm 0.12 \times 10^5$ c	$3.23 \times 10^5 \pm 0.43 \times 10^5$ a	$1.65 \times 10^5 \pm 0.09 \times 10^5$ b
Serine	$1.46 \times 10^5 \pm 0.21 \times 10^5$ c	$0.99 \times 10^5 \pm 0.09 \times 10^5$ d	$3.82 \times 10^5 \pm 0.41 \times 10^5$ a	$2.06 \times 10^5 \pm 0.12 \times 10^5$ b
Alanine	$5.16 \times 10^5 \pm 0.77 \times 10^5$ c	$4.42 \times 10^5 \pm 0.61 \times 10^5$ c	$9.91 \times 10^5 \pm 1.34 \times 10^5$ b	$11.93 \times 10^5 \pm 2.47 \times 10^5$ a
Glutamic acid	$1.42 \times 10^6 \pm 0.21 \times 10^6$ c	$1.32 \times 10^6 \pm 0.16 \times 10^6$ c	$2.46 \times 10^6 \pm 0.27 \times 10^6$ a	$1.69 \times 10^6 \pm 0.14 \times 10^6$ b
Glycine	$0.99 \times 10^4 \pm 0.09 \times 10^4$ c	$1.16 \times 10^4 \pm 0.34 \times 10^4$ c	$2.57 \times 10^4 \pm 0.29 \times 10^4$ a	$1.63 \times 10^4 \pm 0.58 \times 10^4$ b
Proline	$3.48 \times 10^7 \pm 0.07 \times 10^7$ b	$2.75 \times 10^7 \pm 0.47 \times 10^7$ c	$10.99 \times 10^7 \pm 1.69 \times 10^7$ a	$9.87 \times 10^7 \pm 0.56 \times 10^7$ a
Organic acids				
Malic acid	$7.71 \times 10^5 \pm 1.71 \times 10^5$ c	$13.95 \times 10^5 \pm 2.93 \times 10^5$ a	$10.25 \times 10^5 \pm 0.79 \times 10^5$ b	$11.28 \times 10^5 \pm 0.85 \times 10^5$ b
Pyruvic acid	$1.06 \times 10^3 \pm 0.47 \times 10^3$ c	$1.30 \times 10^3 \pm 0.82 \times 10^3$ b	$1.01 \times 10^3 \pm 0.66 \times 10^3$ c	$1.78 \times 10^3 \pm 0.41 \times 10^3$ a
Lactic acid	$4.83 \times 10^4 \pm 1.41 \times 10^4$ c	$7.99 \times 10^4 \pm 3.1 \times 10^4$ b	$7.03 \times 10^4 \pm 1.34 \times 10^4$ b	$12.28 \times 10^4 \pm 1.05 \times 10^4$ a
Citric acid	$14.22 \times 10^4 \pm 4.85 \times 10^5$ c	$22.34 \times 10^5 \pm 6.45 \times 10^5$ b	$15.41 \times 10^5 \pm 2.52 \times 10^5$ c	$36.27 \times 10^5 \pm 7.59 \times 10^5$ a
Succinic acid	$2.78 \times 10^4 \pm 0.83 \times 10^4$ c	$4.04 \times 10^4 \pm 1.28 \times 10^4$ b	$2.69 \times 10^4 \pm 0.23 \times 10^4$ c	$5.62 \times 10^4 \pm 0.35 \times 10^4$ a
Fumaric acid	$4.20 \times 10^4 \pm 1.33 \times 10^4$ c	$4.65 \times 10^4 \pm 2.19 \times 10^4$ c	$10.03 \times 10^4 \pm 2.38 \times 10^4$ b	$20.97 \times 10^4 \pm 1.32 \times 10^4$ a
Vitamins				
Nicotinic acid	$1.98 \times 10^4 \pm 0.56 \times 10^4$ b	$1.21 \times 10^4 \pm 0.46 \times 10^4$ c	$9.25 \times 10^4 \pm 1.51 \times 10^4$ a	$8.60 \times 10^4 \pm 0.65 \times 10^4$ a
Niacin amide	$6.84 \times 10^4 \pm 1.49 \times 10^4$ c	$9.54 \times 10^4 \pm 1.86 \times 10^4$ b	$9.49 \times 10^4 \pm 0.84 \times 10^4$ b	$15.00 \times 10^4 \pm 1.65 \times 10^4$ a
Pantothenic acid	$2.19 \times 10^4 \pm 0.41 \times 10^4$ c	$2.91 \times 10^4 \pm 0.69 \times 10^4$ b	$6.92 \times 10^4 \pm 1.21 \times 10^4$ a	$6.74 \times 10^4 \pm 0.57 \times 10^4$ a

Values are means $\pm$ standard errors. Means in the same row with different letters are significantly ($p < 0.05$) different using Tukey's Honest Significant Test.

Non-essential amino acids were also evaluated for their accumulation in each season (Table 1). The spring harvested bush tea samples showed high integrated peak area of asparagine (1.51×10^6) while summer exhibited low peak area (0.0). The intensity of aspartic acid was high in the spring (3.23×10^5), followed by summer (1.65×10^5) and autumn (1.46×10^5) bush tea plants, and low in the winter (1.27×10^5) harvested tea plants. A similar trend was observed with compound serine. Alanine was found to be high in the summer (11.93×10^5), followed by spring (9.91×10^5), then autumn (5.16×10^5) and low area under peak were recorded in the winter samples (4.42×10^5).

The conditional amino acids were also evaluated, and the high peak area of glutamic acid was recorded in the spring samples with 2.46×10^6 and low in the winter harvested bush tea samples with 1.32×10^6 . Bush tea harvested during spring exhibited high peak area of glycine (2.57×10^4) while low peak area (0.99×10^3) was detected in the autumn sampled plants. Proline area under peak was high in the spring samples with 10.99×10^7 and low in the winter samples with 2.75×10^7 .

Malic acid was high in winter with the peak area of 13.95×10^5 and low in the autumn bush tea leaves and twigs with area of 7.72×10^5 . A similar trend was observed on pyruvic acid, lactic acid, citric acid, fumaric acid and succinic acid, where all the mentioned organic acids were high in summer and low in autumn.

Nicotinic acid, also known as vitamin B₃ showed high peak area in spring, followed by summer and autumn but low in autumn. Niacinamide (NAA), another form of vitamin B₃, was high in the summer samples (15.00×10^4) and low in the winter bush tea samples (6.84×10^4). The high intensity of pantothenic acid (vitamin B₅) was detected in the spring plucked leaves and twigs (6.92×10^4) low area under peak was recorded in the autumn (2.19×10^4) (Table 1).

3.2. The Effect of Harvest Season on Bush Tea Metabolome Using ^1H NMR

The ^1H NMR metabolites peak intensities of bush tea harvested in four different seasons (autumn, winter, spring, and summer) were clearly distinguished using a co-variance of data the PLS-DA (Figure 2). The seasons differentiated from each other on the first two-component PLS-DA scores plot by the principal component t(1) (70.9%), principal component t(2) (2.8%), and principal component t(3) (13.9%). The PLS-DA revealed four distinct groups, bush tea harvested in winter grouped at the top towards the right side of the PLS-DA score plot while the autumn plucked bush tea aligned together towards the right. The summer harvested bush tea clustered together in the middle of the PLS-DA score plot while the spring plucked grouped toward the bottom right side.

Figure 2. Partial least squares-discriminant analysis (PLS-DA) component score plot for ^1H NMR (High-Resolution Proton Nuclear Magnetic Resonance) peak intensities of 20 bush tea (*A. phyllicoides* DC.) samples subjected to seasonal variation.

3.3. Annotation of Bush Tea Metabolites from ^1H NMR Spectrum

Figure 3 presents representative one dimensional ^1H NMR fingerprints of bush tea samples collected over four seasons. The spectra allow for the annotation of phenolic compounds 1–6 upon the comparison of their respective chemical shifts with literature values. The annotated compounds

included rutin with peaks at δ_H 7.64, 6.38, 6.18, 3.34, and 3.82 ppm (rutinose sugar peaks) and 1.06 ppm (rhamnose-CH₃), which was supported with the anomeric protons observed as doublets at δ_H 4.55 and 5.09 ppm [28,29]. Peaks corresponding to (4-hydroxyphenyl) propyl coumarate (δ_H 7.54 ($J = 15.9$ Hz), 7.51, 6.76, 6.68, 6.32 ($J = 15.9$ Hz), 4.08, 2.69, and 2.01 ppm) [30] and caffeic acid (7.62 ppm ($J = 15.78$ Hz), 7.03, 6.99, 6.92, and 6.38 ppm ($J = 15.78$ Hz) [31] were also observed. In addition, the polymethoxylated flavone, hymenoxin (δ_H 7.59, 7.44, 7.06, 6.60, 4.05, 4.02, 3.99, and 3.97 ppm) [32], quercetin (7.66, 7.54, 6.88, 6.41, and 6.20 ppm) [33] and kaempferol (8.08, 6.92, 6.45, and 6.19 ppm) were also identified [34]. The strong doublet at 5.41 ($J = 3.84$ Hz) is characteristic of sucrose while the upfield resonance around δ_H 0.87 is characteristic of the terminal methyl groups of fatty acids. This is further supported by δ_H 1.25, 1.30 ppm which are assignable to repeating methylene and δ_H 2.00 ppm for allylic methylene units of the fatty acids [35,36].

Figure 3. (A–C) ^1H NMR spectra (600 MHz; TSP = 0.00 ppm) of bush tea harvested in four seasons showing annotated compounds: (1) rutin, (2) (4-hydroxyphenyl) propyl coumarate, (3) hymenoxin, (4) caffeic acid, (5) quercetin, (6) kaempferol, sucrose, and fatty acids.

To evaluate seasonal variations of the selected compounds, characteristic chemical shifts of compounds were selectively plotted against their respective intensities (Figure 4), due to overlaps in the basic resonances of the phenolic protons and those of the coumaric acid derivatives.

Figure 4. The effect of season on peak intensities of compounds annotated from bush tea. Peak intensity values per chemical shift with the same letters are not significantly different at $p \leq 0.05$ using Tukey's Honest Significant Test, ns = non-significant. The error bars on the figure represent mean standard error.

4. Discussion

Determination of the best harvesting periods or seasons yielding maximum concentrations of phytochemicals is fundamental for improved tea quality. Data that compares seasonal responses on metabolites in bush tea is lacking. Hence, this study investigated the dependence of bush tea metabolites on seasonal changes through metabolomics approach and found differences in the accumulation of various metabolites in each season. The essential amino acids were highly accumulated in the winter harvested bush tea. This could be attributed to the acclimation of tea plants to cold winter season as a mechanism for cold tolerance. The synthesis of proteins (conditional and non-essential amino acids) were favored by spring conditions while the production of organic acids and vitamins (vitamin B₃ and B₅) in bush tea plants were enhanced by summer conditions. Thermal stress, known to influence metabolic regulation [37,38], could be responsible for higher proteins and vitamins during spring and summer season.

The ¹H NMR-based metabolomics method is a convenient means to explore the link between tea metabolome and climatic variables [24]. Hence, there was a need to ascertain the metabolic dependence of bush tea on seasonal dynamics through the ¹H NMR chemometrics data analysis. The strong dependence of bush tea on seasons were illustrated in the PLS-DA and the heatmap. The changing phytochemical pattern in tea plants is based on the metabolic regulation within the cell, which is affected by many factors, including temperature and water.

The annotated phenolic compounds namely rutin, (4-hydroxyphenyl) propyl coumarate, caffeic acid, quercetin, kaempferol, and hymenoxin have been previously isolated from bush tea extracts [2,3,30]. Therefore, this study provides a novel discovery of how each of the select compounds is affected by seasonal dynamics to understand the picking windows of bush tea in South Africa.

The selected phenolic peaks of bush tea flavonols (rutin, kaempferol, and quercetin) were significantly influenced by seasonal changes (Figure 4). Rutin and quercetin were elevated in summer while kaempferol was increased in spring. The biosynthesis of these phenolic compounds in summer harvested bush tea leaves could be induced by warmer temperatures, stronger sunlight, and longer daytime length [9,39]. Hence, the autumn and winter harvested bush tea recorded low intensities of rutin, kaempferol, and quercetin.

The leaves and twigs of bush tea plucked in winter, which is a slow-growing or dormant period had higher intensity of caffeic acid and lower in autumn. The cold stress during winter might have effectively stimulated the production of this phenolic compound in tea plant cells [14]. Hence, there was a significant increase during the lower temperature season. This is consistent with findings by Król et al. [40] who reported an increased caffeic acid in grapevine under cold stress. The intensities of 4-(Hydroxyphenyl) propyl coumarate were elevated in the summer plucked bush tea leaves and twigs and reduced in the autumn harvested bush tea. Accumulation of this coumarate during summer may be attributed to the higher precipitation levels and warmer temperatures which are known to enhance induction of the biosynthesis of total phenolics [41]. Hymenoxin was increased in the spring plucked bush tea leaves and twigs and reduced in autumn. In the experimental site, spring climatic conditions are also characterized by warm to hot temperature and low to high rainfall levels and day length, thus, the accumulation of hymenoxin in this season could be a result of precipitation and sunlight exposure which is associated with elevation of concentration of phenolic compounds [42].

Seasonal comparison of the ¹H NMR chemical shifts peak intensities of the sugars and fatty acids were also analyzed statistically. Generally, fatty acids were higher in the spring and summer plucked bush tea leaves and lower in the autumn plucked bush tea leaves. Our study suggests that summer and spring enhanced primary and secondary metabolites, and this is consistent with previous studies which recommended summer as the best season for enhanced total antioxidants [11] and polyphenolic content [43].

5. Conclusions

The study demonstrated that changes in the chemical profile pattern of bush tea are associated with seasonal variations influenced by climatic conditions in each season. Concentrations of non-essential amino acids, organic acids and vitamins in bush tea were increased in summer and spring, respectively. The PLS-DA further confirmed the dependence bush teas secondary metabolites on seasons. The six phenolic compounds annotated in this study include rutin, 4-(hydroxyphenyl) propyl coumarate, caffeic acid, hymenoxin, quercetin, and kaempferol. The intensities of the selected phenolics, sugars and fatty acids varied significantly per season with higher intensities in the summer and spring plucked bush tea leaves and twigs. Based on the LC-MS and ¹H NMR metabolomics analysis, summer and spring can be recommended as preferred plucking seasons for bush tea leaves and twigs. A further study is, however, recommended to quantitatively determine the seasonal variation of the full spectrum of the bush tea compounds.

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