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Posted Date: 15 April 2026

doi: 10.20944/preprints202604.1070.v1

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Article

Seasonal and Organ-Specific Variations of Alkaloids in *Buxus obtusifolia* (Mildbr.) Hutch: A Multivariate LC/MS Study

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Abstract

Buxus obtusifolia (Mildbr.) Hutch is an evergreen shrub endemic to East Africa and is traditionally used to treat chest ailments. Our recent investigation on dichloromethane leaves extract of this species yielded several aminosteroid alkaloids, some of which demonstrated promising *in vitro* antiprotozoal activity. Given that abiotic factors are known to influence the biosynthesis and accumulation of plant secondary metabolites, this study aimed to investigate seasonal and organ-specific variability in the alkaloid profile of *B. obtusifolia*. Consequently, leaf and twig samples were collected each month from the same population over a period of one year and analyzed using UHPLC/+ESI-QqTOF-MS/MS. The resulting data were processed with Bruker MetaboScape to generate a bucket table of variables <*t_R*:*m/z*> from the MS chromatograms. Principal component analysis (PCA) was subsequently used to characterize variations in the metabolite profile. Evaluation of the first three principal components (PC1–PC3) from the scores and loadings plots revealed clear differences between leaves and twigs, as well as minimal seasonal trends. A volcano plot was used to further analyze the differences between the two organs. In total, 15 aminosteroid alkaloids were identified as key contributors to these differences. This represents the first seasonal and organ-specific phytochemical variability investigation in *B. obtusifolia*. Thus, this study offered valuable insights into some abiotic factors influencing phytochemical profile of this plant, as well as the optimal harvest period for targeted isolation of bioactive aminosteroids.

Keywords: *Buxus obtusifolia* (Mildbr.) hutch; buxaceae; aminosteroid alkaloids; seasonal variation; multivariate data analysis (MVDA); principal component analysis (PCA); volcano plot; liquid chromatography-mass spectrometry

1. Introduction

Buxus obtusifolia (Mildbr.) Hutch is an evergreen shrub endemic to Kenya and Tanzania, and holds a significant ethnomedicinal value among some East African communities who utilize its root decoctions to treat chest ailments [1]. Our recent phytochemical investigation of the species' leaves led to identification of 24 aminosteroid alkaloids. Notably, some of these compounds exhibited promising *in vitro* antiprotozoal activity against *Trypanosoma brucei rhodesiense* (*Tbr*), the causative agent of East African Human Trypanosomiasis and *Plasmodium falciparum* (*Pf*), the primary parasite responsible for tropical Malaria [2]. It is widely established that abiotic factors such as fluctuating temperatures, light intensity, nutrient availability, and water stress influence growth as well as biosynthesis and accumulation of active principles in medicinal plants [3,4]. Consequently, these

environmental variations can significantly alter the overall therapeutic efficacy of the resulting herbal materials. Numerous studies have demonstrated that the total alkaloid content in both higher plants [5–7] and their associated endophytic fungi [8,9] is greatly influenced by environmental factors. Using a multivariate data analysis (MVDA) approach, our group has recently evaluated seasonal, organ-specific and location variability in the alkaloid profiles of *Buxus sempervirens* var. *arborescens* and *suffruticosa* [10], and *Pachysandra terminalis* [11]. These investigations identified various aminosteroid alkaloids as the key contributors to the observed chemical differences among the plant organs (leaves, twigs and flowers) across seasons.

Our recent study on *B. obtusifolia* represented the first phytochemical and pharmacological investigation on this species [2]. Accordingly, to the best of our knowledge, no previous studies have examined seasonal and organ-specific variability in its phytochemical profile. The aim of the present study was therefore to investigate the variations in the alkaloid content of *B. obtusifolia*, considering both the seasonal dynamics over a full annual vegetation cycle as well as differences between the primary aerial organs. Samples of the leaves and twigs harvested monthly for one year were prepared and analyzed using Ultra High Performance Liquid Chromatography/positive Mode-Electrospray Ionization Quadrupole Time-of-Flight-Tandem Mass Spectrometry (UHPLC/+ESI-QqTOF-MS/MS, henceforth abbreviated LC/MS). In order to extract meaningful information from the resulting complex LC/MS data matrix, principal component analysis (PCA) and volcano plot were employed to identify the characteristic aminosteroid alkaloids responsible for seasonal variation and differences between leaves and twigs.

2. Results

2.1. Sample Preparation, LC/MS Characterization, and Data Preprocessing

To establish a seasonal profile, the leaves and twigs of *B. obtusifolia* were collected monthly over a period of one year (February 2024–January 2025). The air-dried plant materials were separately extracted with dichloromethane to obtain 24 extracts which were subsequently analyzed using LC/MS. The resulting LC/MS raw data were processed using MetaboScape 3.0, where molecular features were extracted to generate a bucket table of 2195 $\langle t_R:m/z \rangle$ variables from 84 MS chromatograms (24×3 measurements for leaf and twig samples and 12 measurements for a quality control sample). In order to identify seasonal trends, this complex dataset was imported to ProfileAnalysis 2.1 and evaluated using two multivariate statistical methods, principal component analysis (PCA) and volcano plot.

2.2. Quantification of Annual Variability of Previously Isolated *B. obtusifolia* Aminosteroid Alkaloids

Our recent investigation reported 24 aminosteroids (1–24, Supplementary Materials, Figure S1) along with their antiprotozoal activity from the leaves of *B. obtusifolia* [2]. By comparing their exact retention times and mass-to-charge ratios $\langle t_R:m/z \rangle$, variables corresponding to each of the previously isolated compounds were traced within the bucket table, and their relative abundance across the 24 samples was assessed. In order to track the relative quantities of these constituents throughout an annual vegetative cycle, bar charts were constructed in Microsoft Excel to visualize the MS signal intensities across the sampling period. For each feature, the mean signal intensity of the two most reproducible replicate measurements per month was calculated, with standard deviation represented as error bars. Samples were arranged chronologically from February 2024 to January 2025, allowing for a comparative analysis of each compound across the year. For instance, Figure 1 shows bar chart plots of 29-trimethoxybenzoyloxy cycloprotobuxoline-C (5, A) and obtusiepoamine-A (21, B) with promising bioactivity [2], representing the 9 β ,10 β -cyclo-5 α -pregnane and 9 (10→19) *abeo*-5 α -pregnane derivatives, respectively.

On average, both compounds are more abundant in the leaves (red bars) than in the twigs (green bars), showing the leaves as the preferred starting material for their isolation. Furthermore, 29-trimethoxybenzoyloxy cycloprotobuxoline-C (5), which exhibited the strongest antiplasmodial

activity (IC_{50} = 0.5 μ mol/L vs. 5.9 μ mol/L for cytotoxicity; selectivity index, SI = 12) in our previous work [2], showed the highest concentration in January. In contrast, obtusiepoxamine-A (**21**), which showed moderate antitrypanosomal activity (IC_{50} = 2.2 μ mol/L) and the highest selectivity index (SI = 16) among the 9 (10→19) *abeo*-5 α -pregnane derivatives [2], reached its peak concentration in March. Additionally, both compounds **5** and **21** exhibited similar but minimally elevated concentrations, occurring in February–March for **5** and January–February for **21**, with both compounds also showing high levels in December. Overall, it can be deduced from these plots that the optimal time to harvest the leaves for isolating these two compounds would be from December to March.

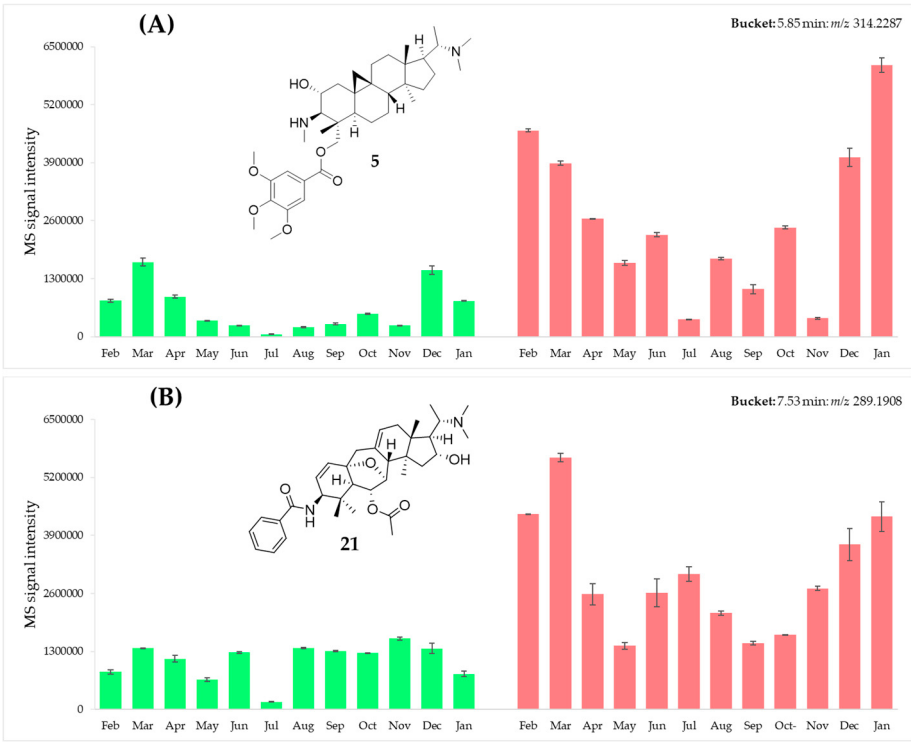


Figure 1. Monthly profiles of 29-trimethoxybenzoyloxy cycloprotobuxoline-C (**5**, A) (5.85 min; m/z 314.2287) and obtusiepoxamine-A (**21**, B) (7.53 min; m/z 289.1908). Bar charts are the mean signal intensities ($\pm$ SD) of two replicate measurements across twelve monthly samples (February 2024–January 2025) of *B. obtusifolia* twigs (green, n = 24) and leaves (red, n = 24).

Using the same criteria, the optimal harvest period for all *B. obtusifolia* aminosteroid alkaloids reported in our recent study [2] was determined (Supplementary Materials, Figure S2). The results (Table 1) indicated that, except compounds **11**, **14** and **16**, all the other alkaloids exhibit their peak concentrations during the first quarter of the year. Additionally, except for compounds **8a** + **8b**, **11**, and **24**, the rest of the compounds were mainly found in higher quantities in the leaves compared to the twigs.

Table 1. Optimum harvesting period and preferred starting material for previously reported aminosteroids from *B. obtusifolia*. The compounds are numbered according to our previous publication [2].

Compound	Name	Bucket < t_R : m/z >	Type of Ion	Month with Maximum Content	Organ
1	Cycloprotobuxoline-C	5.06 min: m/z 209.2021	[M + 2H] ²⁺	March	Leaves > twigs

2	Cycloprotobuxoline-C <i>N</i> ₂₀ -oxide	4.63 min: <i>m/z</i> 217.1995	[M + 2H] ²⁺	March	Leaves > twigs
3	16α-Hydroxycycloprotobuxoline-C	4.74 min: <i>m/z</i> 217.1996	[M + 2H] ²⁺	April	Leaves > twigs
4	Cycloprotobuxoline-D	5.05 min: <i>m/z</i> 202.1941	[M + 2H] ²⁺	March	Leaves > twigs
5	29-Trimethoxybenzoyloxy cycloprotobuxoline-C	5.85 min: <i>m/z</i> 314.2287	[M + 2H] ²⁺	January	Leaves > twigs
6	<i>N</i> ₃ -Demethylcycloprotobuxoline-C	4.84 min: <i>m/z</i> 403.3730	[M + H] ⁺	April	Leaves > twigs
7	16α-Hydroxy- <i>N</i> ₃ - demethylcycloprotobuxoline-C	5.66 min: <i>m/z</i> 419.3675	[M + H] ⁺	February	Leaves > twigs
8a + 8b	Cycloprotobuxoline-D <i>N</i> ₃ - <i>trans</i> - (8a) and cycloprotobuxoline-D <i>N</i> ₃ - <i>cis</i> (8b) -formamide	6.46 min: <i>m/z</i> 431.3647	[M + H] ⁺	April	Leaves ≈ twigs
9a + 9b	16α-Hydroxycycloprotobuxoline-C <i>N</i> ₃ - <i>trans</i> -formamide (9a) and 16α- hydroxycycloprotobuxoline- C <i>N</i> ₃ - <i>cis</i> -formamide (9b)	7.76 min: <i>m/z</i> 461.3553	[M + H] ⁺	March	Leaves > twigs
10	Cyclonataminol	4.89 min: <i>m/z</i> 223.1997	[M + 2H] ²⁺	March	Leaves > twigs
11	<i>N</i> ₃ -Demethyl cyclonataminol	4.70 min: <i>m/z</i> 216.1917	[M + 2H] ²⁺	June	Leaves ≈ twigs
12	Deoxycyclovirobuxeine-B	5.58 min: <i>m/z</i> 200.1962	[M + 2H] ²⁺	January	Leaves > twigs
13	Cyclovirobuxeine-A	5.25 min: <i>m/z</i> 215.2018	[M + 2H] ²⁺	February	Leaves > twigs
14	Cyclovirobuxeine-B	5.29 min: <i>m/z</i> 208.1937	[M + 2H] ²⁺	December	Leaves > twigs
15	<i>N</i> ₂₀ -Demethyl deoxycyclobuxoxazine A	5.42 min: <i>m/z</i> 208.2079	[M + 2H] ²⁺	January	Leaves > twigs
16	Obtusibuxeine A	6.72 min: <i>m/z</i> 374.3053	[M + H] ⁺	October	Leaves >> twigs
17	<i>O</i> ¹⁰ -Obtusifuranamine-A	6.30 min: <i>m/z</i> 593.3572	[M + H] ⁺	February	Leaves > twigs
18	<i>O</i> ¹⁰ -Obtusifuranamine-B	7.03 min: <i>m/z</i> 655.3697	[M + H] ⁺	March	Leaves > twigs

19	16-Deoxy- <i>O</i> ¹⁰ -obtusifuranamine-B	7.55 min: <i>m/z</i> 639.3775	[M + H] ⁺	April	Leaves > twigs
20	<i>O</i> ² -Natafuranamine	6.74 min: <i>m/z</i> 593.3559	[M + H] ⁺	January	Leaves > twigs
21	Obtusiepoxamine-A	7.53 min: <i>m/z</i> 289.1908	M + 2H] ²⁺	March	Leaves > twigs
22	Obtusidienolamine-A	7.04 min: <i>m/z</i> 579.3763	[M + H] ⁺	January	Leaves > twigs
23	Deoxyobtusidienolamine-A	7.23 min: <i>m/z</i> 563.3813	[M + H] ⁺	February	Leaves > twigs
24	Obtusiaminocyclin	5.92 min: <i>m/z</i> 368.2594	[M + H] ⁺	January	Leaves ≈ twigs

To correlate the optimum harvesting period with environmental conditions during the sampling period, we analyzed two abiotic factors, that is, temperature and precipitation. Abiotic stress, particularly drought and elevated temperatures have been widely demonstrated to induce the accumulation of nitrogen-containing plant secondary metabolites, including alkaloids [12,13]. This is consistent with our observations, which showed accumulation of most compounds between January and March, coinciding with high temperatures (>30 °C) and low precipitation (Figure 2). Physiologically, drought and heat stress can trigger stress-response pathways, such as abscisic acid (ABA) signaling and oxidative stress mechanisms, which in turn upregulate the genes responsible for secondary metabolite biosynthesis [14,15]. Consequently, water-stressed plants often exhibit higher levels of defensive alkaloids that may contribute to stress tolerance or herbivore deterrence [16]. Furthermore, it is worth noting that reduced plant growth under water-limited conditions may lead to higher relative metabolite concentrations when expressed on a dry-weight basis.

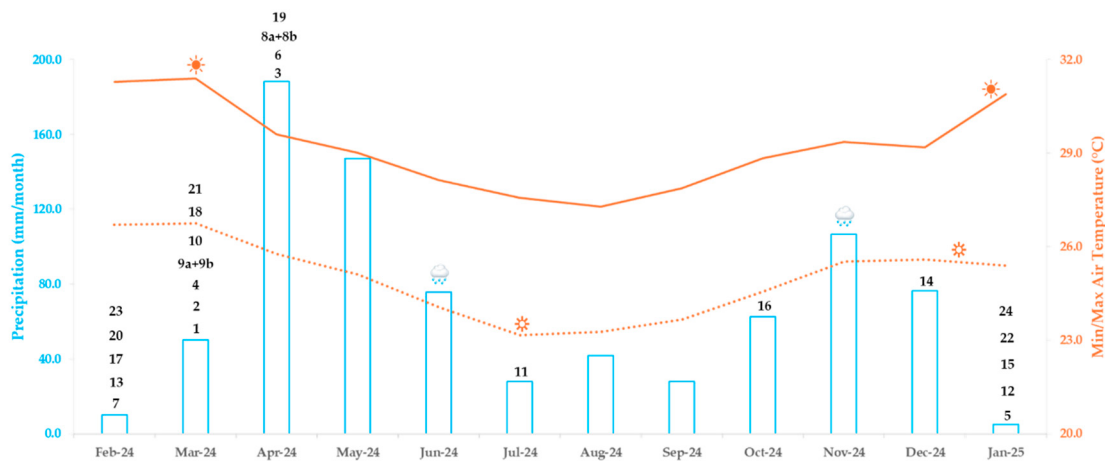


Figure 2. Months with maximum content of the previously isolated aminosteroid alkaloids (1–24) from the leaves of *B. obtusifolia* across a one-year cycle, correlated with monthly weather data. Weather parameters from February to December 2024 are gridded data provided by the Kenya Meteorological Department, while those for January 2025 were estimated from a correlation plot between satellite-derived data and the gridded dataset (blue bars = monthly precipitation; orange solid line = monthly maximum temperature; orange dotted line = monthly minimum temperature). Compound numbers correspond to those used in the text and in Table 1.

2.3. Principal Component Analysis (PCA)

Principal component analysis (PCA) is a widely-known unsupervised multivariate statistical method used to reduce the dimensionality of complex datasets while retaining the maximum possible variance [17]. PCA simplifies data interpretation with minimal loss of information by transforming a large set of correlated variables into a smaller number of orthogonal, uncorrelated variables known as principal components (PCs). The first principal component (PC1) captures the highest degree of variability, while subsequent components (PC2, PC3, amongst others) explain the remaining variance in descending order [18]. Following the methodology established in previous studies by our group [10,11], PCA models were calculated and cross-validated using three different scaling approaches (Level, Unit Variance, and Pareto scaling) as well as analysis of the unscaled data. When compared, all four approaches produced models that effectively discriminated between leaves and twigs samples of *B. obtusifolia* (Supplementary Materials, Figure S3). The Pareto-scaled model was selected for further analysis, as it provides the most balanced representation of variance between high and low abundance metabolites, as previously described by both Szabó and Schmidt and Schäfer et al. of our group [10,11].

Using ProfileAnalysis 2.1, samples are visualized in a score plot, which shows their relative positions in the PC space and enables the identification of natural groupings or trends. The corresponding loadings plot is used to determine which original variables contribute most significantly to each principal component. The results of the PCA model obtained with the Pareto-scaled data are presented in Figure 3.

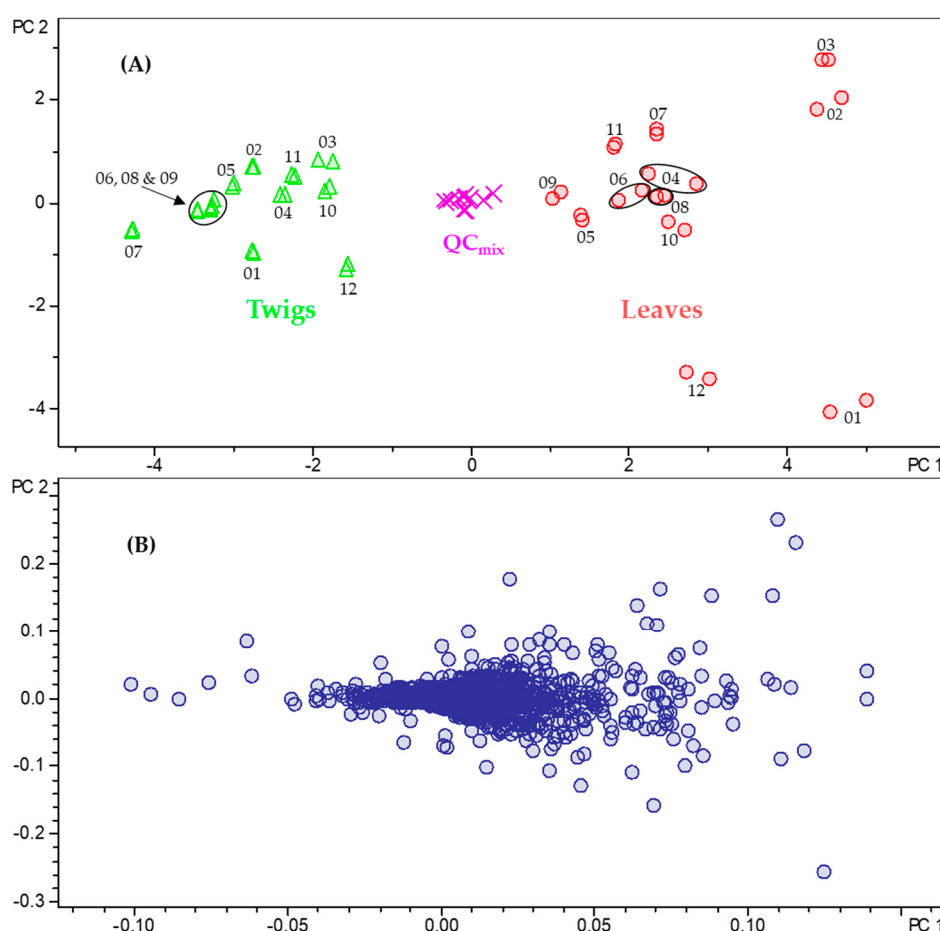


Figure 3. PCA model showing the scores (A) and loadings (B) plots based on Pareto-scaled data for the annual metabolite profile of *B. obtusifolia*. PC2 (10.0% of the total variance) is plotted against PC1 (43.2% of the total variance). In the scores plot (A), green triangles = twigs, red dots = leaves, pink crosses = quality control (QC_{mix}). Numerical values (01–12) indicate the months from January to December.

The scores plot (A), displaying the second PC (PC2) plotted against the first PC (PC1), clearly demonstrates a separation between the two organs of *B. obtusifolia*. PC1 differentiates between the leaf and twig samples and accounts for 43.2% of the total variance, while PC2 explains an additional 10.0% of the variance and reflects the temporal variability of the samples. Together, the PC2 vs. PC1 scores plot captures 53.2% of the total variance in the bucket table. The quality control (QC_{mix}), consisting of an equal mixture of all samples, is located in the middle of the plot as expected. The associated loadings plot (B) shows the distribution of bucket variables $\langle t_R:m/z \rangle$ within the PC coordinate system. Variables characteristic of the leaves are located on the right (high positive values on PC1), whereas those characteristic of twigs are on the left (strongly negative values on PC1). Consequently, these variables represent the primary contributors to the observed differences between the two plant organs. Interestingly, PC2 appears to reflect a relatively large difference between December and January (negative scores on PC2) and February and March (positive scores on PC2).

2.4. Identification of Aminosteroids Contributing to the Chemical Differences Between the Organs and Temporal Variation

The loadings plot (Figure 4) was examined to extract the molecular masses of the most influential bucket variables, to identify the aminosteroids responsible for differences between leaf and twig samples. The molecular formulae of these variables were determined by analyzing the corresponding +ESI-QqTOF MS/MS spectra, and compared with literature data as well as previously isolated compounds from *B. obtusifolia* [2]. Furthermore, the fragmentation patterns of these variables were evaluated and compared with diagnostic fragments characteristic of the steroidal skeletons found in *Sarcococca* [19], *Pachysandra* [20], and *Buxus* [21], all members of the Buxaceae family. These diagnostic fragments have been previously compiled and described in earlier studies of our group [10,11].

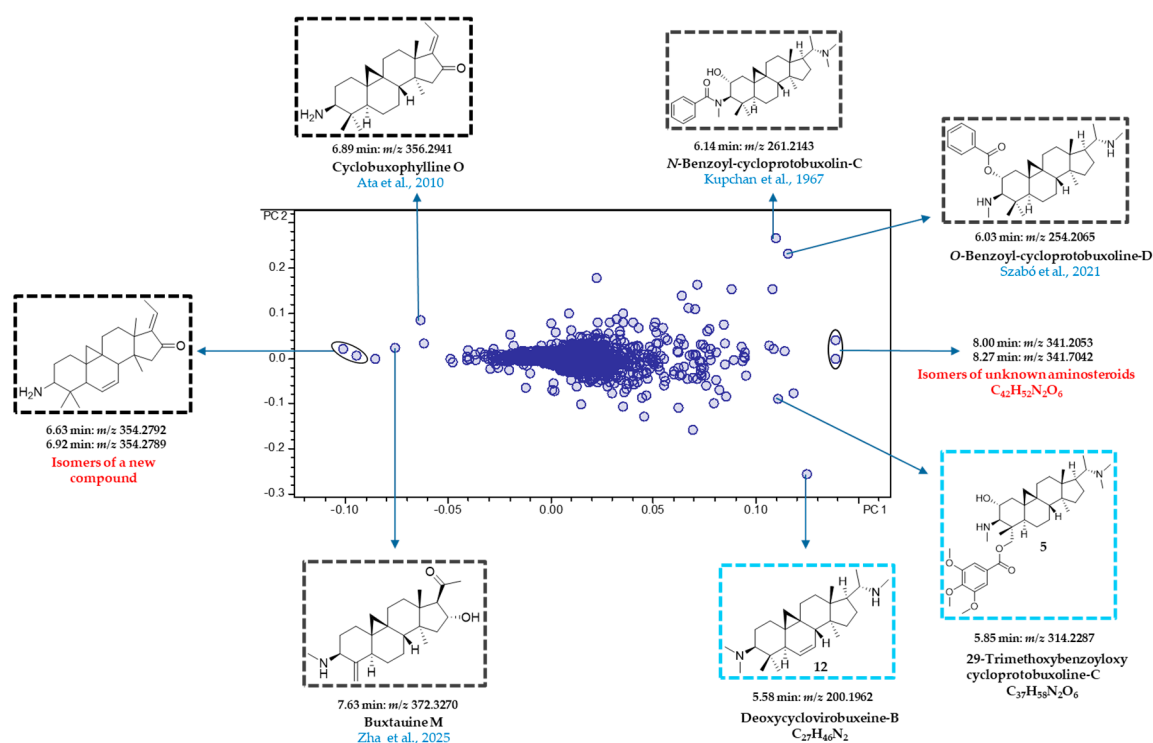


Figure 4. PCA Loadings plot (PC2 vs. PC1) of the organ-specific metabolite profile of *Buxus obtusifolia*. Compounds highlighted in blue were previously isolated in our study [2], while those in black were tentatively identified based on their molecular masses using the SciFinder and LOTUS databases. The cited references in the figure are Kupchan et al., 1967 [22], Ata et al., 2010 [23], Szabó et al., 2021 [24], and Zha et al., 2025 [25].

Among the leaf-specific compounds, 29-trimethoxybenzoyloxy cycloprotobuxoline-C (**5**) (5.85 min: m/z 314.2287) and deoxycyclovibuxine-B (**12**) (5.58 min: m/z 200.1962) were identified as

characteristic constituents of the leaves of *B. obtusifolia*. Notably, these two compounds demonstrated the highest antiplasmodial ($IC_{50} = 0.5 \mu\text{mol/L}$ for compound **5**) and antitrypanosomal activity ($IC_{50} = 0.8 \mu\text{mol/L}$ for compound **12**) in our recent study on the leaves of this species [2]. Correlation with the scores plot (Figure 3A) further indicates that deoxycyclovirobuxine-B (**12**) is most strongly associated with leaf samples collected in January and December. Similarly, *O*-benzoyl-cycloprotobuxoline-D (6.03 min: m/z 254.2065) and *N*-benzoyl-cycloprotobuxolin-C (6.14 min: m/z 261.2143) were also identified as characteristic compounds of the leaves. The former was previously reported from *Buxus sempervirens* L. by Szabó et al. [24], while the latter was described from the same species by Kupchan et al. [22]. Based on the positions of the corresponding buckets in the loadings plot (Figure 4), the two compounds are characteristic of samples collected in February and March (Figure 3A). Cyclobuxophylline O (6.89 min: m/z 356.2941) and buxtauine M (7.63 min: m/z 372.3270) were identified as characteristic compounds of the twigs of *B. obtusifolia*. Finally, all the 24 compounds described in our previous study from this species could be located in the PC2 vs. PC1 loadings plot (Supplementary Materials, Figure S4). Among these, compounds **1**, **2**, **4**, **5**, **10**, **12**, **16**, **21**, **22** were found to significantly influence the organ-specific PCA model.

2.5. Seasonal Differences in the Annual Alkaloid Profile of *Buxus obtusifolia*

To further investigate the seasonal variability of the alkaloid profile of *B. obtusifolia*, the third principal component (PC3) was plotted against the first (PC1) (Figure 5). Since PC3 accounts for an additional 10.0% of the variance, PC3 vs. PC1 scores plot captures a total of 60.2% of the overall variance.

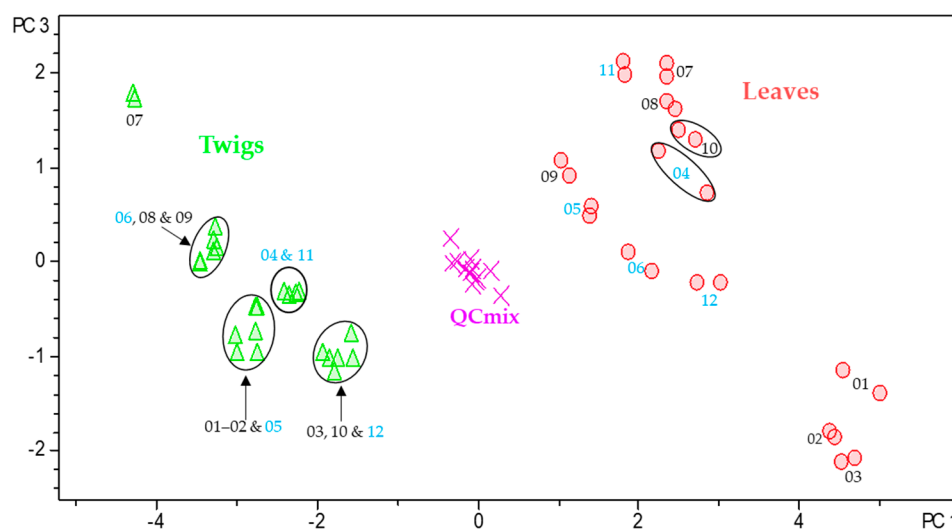


Figure 5. PCA scores plot (PC3 vs. PC1) of the seasonal metabolite profile of *Buxus obtusifolia*. Numerical values (01–12) indicate the months from January to December. Months highlighted in blue and black correspond to the rainy and dry seasons, respectively.

Based on monthly precipitation data provided by the Kenya Meteorological Department (Figure 2), samples were classified into rainy and dry seasons. Months with an average precipitation $> 70 \text{ mm}$ (April, May, June, November and December) were designated as the “rainy season,” while the remaining months were considered as the “dry season.” Given that sampling was conducted in a tropical region, temperature variation across the study period was minimal, except January–March, when average monthly temperatures exceeded 30°C (Figure 2). This relatively stable temperature pattern likely explains the absence of a clear and consistent seasonal separation among the samples of the two organs. However, the leaf samples collected during the warmest months (January–March) cluster in the lower right region of the scores plot (Figure 5), indicating that the corresponding bucket

variables are characteristic of the dry and warmest season. In contrast, the twig samples collected in July appear distinctly separated in the upper left region of the scores plot.

Accordingly, cycloprotobuxoline-C (**1**) (5.06 min:*m/z* 209.2021), 29-trimethoxybenzoyloxy cycloprotobuxoline-C (**5**) (5.85 min:*m/z* 314.2287), and obtusiepoxamine-A (**21**) (7.53 min:*m/z* 289.1908) (Figure 6) previously isolated in our study [2], were identified as characteristic of leaf samples from the warmest season. In addition, a previously undescribed aminosteroid with the molecular formula $C_{23}H_{39}NO_2$ (7.68 min:*m/z* 362.3075) was found to be characteristic of twig samples collected during the dry and coolest month of July.

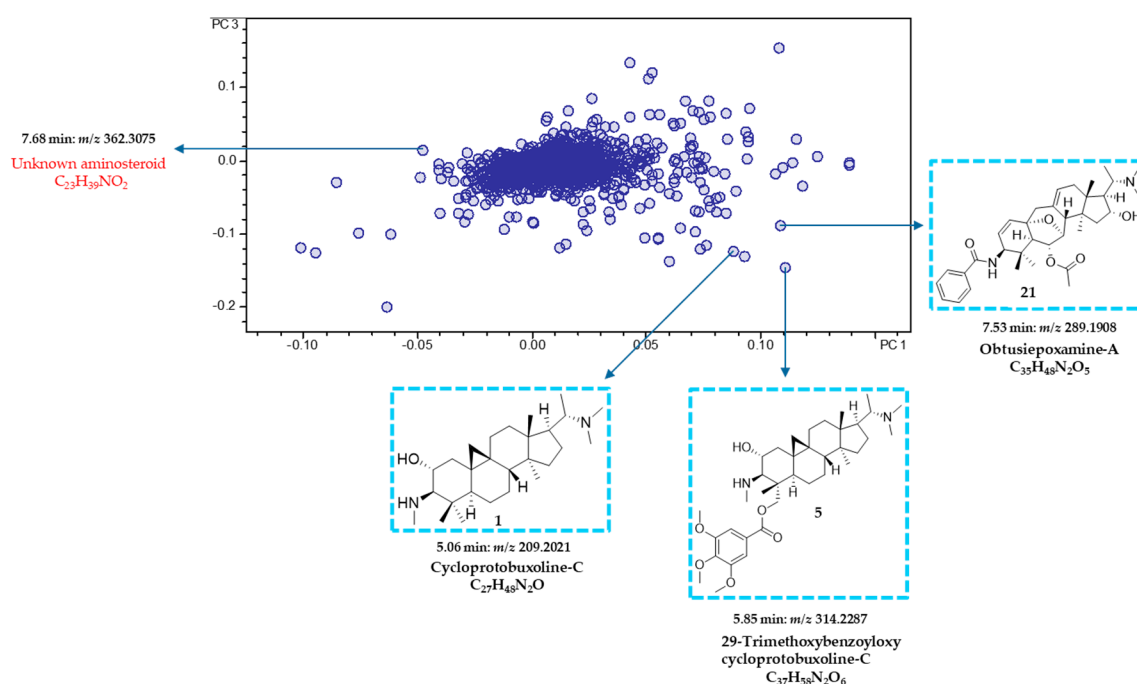


Figure 6. PCA loadings plot (PC3 vs. PC1) of the seasonal metabolite profile of *Buxus obtusifolia*. Compounds highlighted in blue were previously isolated in our study [2].

2.6. Volcano Plot-Based Comparison of Leaves and Twigs Alkaloid Profile

The two PCA models (PC2 vs. PC1 and PC3 vs. PC1) revealed a clear separation between the leaf and twig samples of *B. obtusifolia* and highlighted the major compounds contributing to this separation. However, compounds present in low concentrations are normally overlooked due to their limited influence on PCA models and are therefore difficult to identify through this approach alone. To complement the PCA results, a volcano plot based on direct pairwise t-test comparison between the two organs was performed. Each bucket was individually evaluated by plotting the statistical significance of the difference between the leaves and twigs ($-\log_{10}(p\text{-value})$) against the magnitude of change ($\log_2(\text{fold change})$). This allowed visualization of both the magnitude and significance of concentration differences without less abundant compounds being dominated by the more abundant ones. From Figure 7, bucket variables corresponding to leaf compounds occur to the far right on the x-axis, while those occurring mainly in the twigs are on the left side of the x-axis. Out of the 2,195 variables, 1,190 showed statistically significant differences between leaves and twigs ($p\text{-value} < 0.05$, $|\log_2 \text{fold change}| \geq 1$), with 510 features concentrated in the twigs (green bubbles) and 680 concentrated in the leaves (red bubbles). Notably, all variables with the highest PCA loadings (PC2 vs. PC1, Figure 4) were also statistically significant in the volcano plot, although they were not necessarily among the most differentially expressed variables. This finding is consistent with observations reported for *B. sempervirens* in the earlier study by our group [10].

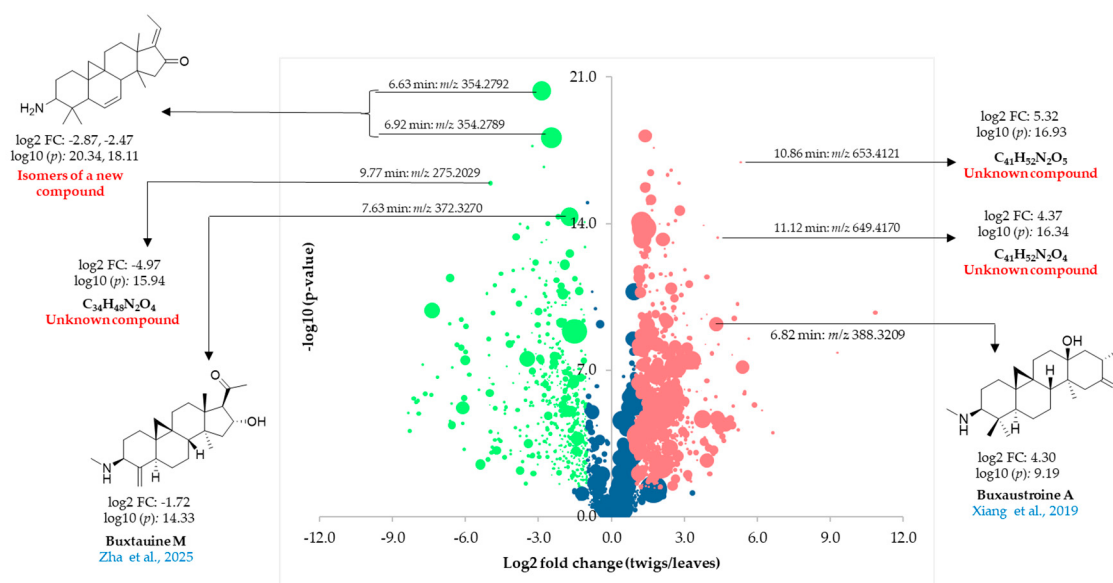


Figure 7. Volcano plot of twig and leaf annual samples from *B. obtusifolia*. Data points represent molecular features defined by their $\langle tr:m/z \rangle$ plotted as the mean log2 fold change against $-\log_{10}(p\text{-value})$, and bubble sizes are proportional to the maximum signal intensity observed across all 24 samples. Significant features in twig and leaf samples are highlighted in green and red, respectively, while non-significant features are shown in blue. Significance thresholds were set at $|\log_2 \text{fold change}| \geq 1$ and $p\text{-value} < 0.05$. The cited references in the figure are Zha et al., 2025 [25] and Xiang et al., 2019 [26].

Among the significant compounds, buxtauine M (7.63 min: m/z 372.3270) and the two new isomers (6.63 min: m/z 354.2792 and 6.92 min: m/z 354.2789), all of which had been identified as characteristic compounds of the twigs of *B. obtusifolia* (Figure 4), were additionally detected to be significantly more concentrated in the twigs than in the leaves (Figure 7). The volcano plot also highlighted another minor variable belonging to a previously unknown compound (9.77 min: m/z 275.2029) with a high log2(fold-change) of -4.97 as a characteristic compound of the twigs. Conversely, buxastroine A (6.82 min: m/z 388.3209) was shown to be in significantly higher concentration in the leaves compared to the twigs. Two additional variables (10.86 min: m/z 653.4121 and 11.12 min: m/z 649.4170) exhibited particularly substantial log2(fold-changes) of 5.32 and 4.37, respectively. Based on their molecular mass, these variables were attributed to minor, previously unreported aminosteroids that are concentrated in the leaves.

3. Materials and Methods

3.1. Plant Material Processing and Extraction

The aerial parts of *Buxus obtusifolia* were harvested monthly from the same population site described in our previous study [2], over a period of one year (February 2024–January 2025). Identification of the plant material was done by Mr. Patrick Mutiso, a taxonomist from the Faculty of Science and Technology, University of Nairobi. The voucher specimens were deposited at both the University of Nairobi Herbarium (UoN_JM 2022_002) and at the Institute of Pharmaceutical Biology and Phytochemistry, University of Münster (IPBP 916 - TS_JM_2022_002). To complement the seasonal alkaloid variation, organ-specific variability was also evaluated by separately processing the leaves and twigs. The plant materials were air-dried under shade at room temperature to a constant weight and pulverized into fine powder using a mill. For each sample, 5 g of the powdered material was exhaustively extracted with dichloromethane (2 × 30 mL) under continuous agitation on a magnetic stirrer for 30 min. The obtained extracts were combined and thoroughly evaporated under reduced pressure at 40 °C.

3.2. UHPLC/+ESI-QqTOF-MS/MS-Analysis

The resulting extracts were analyzed using the previously described LC/MS method by our research group [11], with minor changes. The extracts were dissolved in methanol at a concentration of 10 mg/mL and analysed using a Bruker Daltonics micrOTOFQII time-of-flight mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Chromatographic separation was achieved using a binary gradient of H₂O (+0.1% formic acid; A) and Acetonitrile (+0.1% formic acid; B) at a flow rate of 0.4 mL/min and column temperature of 40 °C. The elution profile described in our recent article [2] was used: 0–1.88 min: linear from 15% B to 30% B; 1.88–7.88 min: linear from 30% B to 33% B; 7.88–9.9 min: linear from 33% B to 50% B; 9.9–9.93 min: linear from 50% B to 100% B; 9.93–15.88: isocratic 100% B; 15.88–15.98 min: linear from 100% B to 15% B; 15.98–20.0 min: isocratic 15% B. In order to monitor injection precision and instrument sensitivity, 10 µL of papaverine (0.25 mg/mL) was added to each sample as an internal standard. For quality control, 20 µL of each sample was pooled into a collective LC/MS vial (QCMix) and measured after every six samples to assess sensitivity shifts and peak alignment consistency. Additionally, a methanol blank was measured after every 20 samples to monitor any carry-over of analytes. To obtain more information about the fragmentation patterns, some representative samples were initially subjected to auto MS/MS analysis at a collision energy of 20 eV. These initial runs also served to saturate the column matrix, ensuring reproducible retention times throughout the sequence. Following the matrix saturation measurements, the complete sample set was analyzed in triplicate using full-scan mode without intermediate recording of fragment spectra to obtain maximum number of data points per peak. An injection volume of 2 µL was employed for all analyses. The robustness of the dataset was ensured by measuring the samples in three distinct sequences: once sequentially from January 2025 to December 2024, followed by a randomized sequence and concluding with a sequential repetition.

3.3. Preprocessing of LC/MS Data

The raw LC/MS data from 84 sample measurements (24×3 measurements for leaf and twig samples and 12 measurements for a quality control sample) were processed using MetaboScape 3.0 software (Bruker Daltonics, Bremen, Germany). Molecular features were extracted to generate a comprehensive bucket table of $\langle t_R:m/z \rangle$ variables. The sample groups were defined by organ (leaves and twigs) and harvest month (January–December). The filter parameters were defined as follows: minimum features for extraction, 20/84 (accounting for molecular features detected in at least 20/84 samples during extraction); presence of features, 20/84 (representing features only present in at least 20/84); filter features by occurrence in groups, month 10% (accounting for features occurring in at least 10% of the samples within each group). The peak detection parameters were set as: intensity threshold, 1000 counts; minimum peak length, 8 spectra; minimum peak length (recursive), 7 spectra; retention time range, 1–15 min; mass range, m/z 50–1500. Ion deconvolution parameters were set as: EIC correlation, 0.7; primary ions, $[M + H]^+$; seed ions, $[M + 2H]^{2+}$, $[M + Na]^+$, $[M + K]^+$; common ions, $[M + H - H_2O]^+$. The resulting bucket table containing 2195 variables x 84 analyses, was exported to Bruker ProfileAnalysis 2.1 software (Bruker Daltonics, Bremen, Germany) for multivariate statistical evaluation.

3.4. Principal Component Analysis (PCA) Modelling

Prior to PCA modelling, the 2195 bucket variables' intensity values of each analysis were normalized to those of the internal standard papaverine (t_R 5.84 min: m/z 340.1580) in the respective run. To optimize the statistical output, three different scaling approaches were evaluated, such as level, unit variance, Pareto, as well as analysis of the unscaled data (Supplementary Materials, Figure S3). All PCA models were cross-validated using leave-one-out cross-validation to ensure robustness of the resulting data. While all four approaches demonstrated good sample separation, the Pareto-scaled model was selected for further analysis due to its superior representation of the variance between high and low abundance metabolites.

3.5. Volcano Plot

Using the bucket table exported from Mataboscape, a t-test was performed in Microsoft Excel to compare mean signal intensities between twig and leaf samples of *B. obtusifolia*. Log2 fold change and $-\log_{10}(p\text{-value})$ were calculated for each bucket and visualized as a bubble volcano plot, with bubble size proportional to the maximum signal intensity observed across all samples. Significance thresholds were set at $|\log_2 \text{fold change}| \geq 1$ and $p\text{-value} < 0.05$.

4. Conclusions

The present study investigated variations in the alkaloid content of the leaf and twigs samples of *B. obtusifolia* over a full annual vegetation cycle using a multivariate data analysis approach. Principal component analysis and volcano plot of the LC/MS dataset revealed clear organ-specific differences in alkaloid profiles, along with some minimal trends in seasonal metabolite variation. In total, 15 compounds were identified as key contributors to the observed patterns. Additionally, among the 24 aminosteroids described in our previous study from this species, compounds **1, 2, 4, 5, 10, 12, 16, 21, 22** exhibited the most profound organ-specific differences as reflected in the PCA model. These findings provide valuable insights into the phytochemical dynamics of *B. obtusifolia*, and offer a basis for the targeted isolation of bioactive aminosteroids in future pharmacological studies.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Chemical structures of aminosteroids previously isolated from *Buxus obtusifolia* in our recent study [2]; Figure S2: Monthly profiles of compounds **1–4, 6–20, 22–24**; Figure S3: PCA models of the LC/MS data of *B. obtusifolia* samples with different scaling methods; Figure S4: Loadings plot of PC2 vs. PC1 with assignment of all 24 aminosteroids described in our previous study [2]. Figure S5–S35: Spectral data of aminosteroids identified in this study.

Author Contributions: Conceptualization, T.J.S.; investigation, J.W.M.; formal analysis, J.S.; data curation, J.W.M. and J.S.; writing—original draft preparation, J.W.M and T.J.S.; writing—review and editing, J.S.; L.K.O. and N.M.K.; supervision, T.J.S.; project administration, T.J.S.; funding acquisition, L.K.O.; N.M.K. and T.J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received external funding in the form of a doctoral fellowship (October 2022 to September 2025) for Mr. Justus Wambua Mukavi from the Kenyan government, through National Research Fund-Kenya, in cooperation with the German Academic Exchange Service (NRF-DAAD). Mr. Mukavi is also grateful for a doctoral completion scholarship (October 2025 to June 2026) from Apothekerstiftung Westfalen-Lippe. External funding was received from Apothekerstiftung Westfalen-Lippe under the project title: “Struktur-Wirkungsbeziehungen für die antiprotozoale Aktivität von Aminosteroiden und Amino-nortriterpenen aus Buxaceae und Apocynaceae”.

Data Availability Statement: All original data for this study are detailed in the article and Supplementary Materials. The raw data supporting the findings of this study are available from the authors upon request.

Acknowledgments: J.W.M is most grateful to the Kenyan government, through National Research Fund - Kenya, in cooperation with the German Academic Exchange Service (NRF-DAAD) and Apothekerstiftung Westfalen-Lippe for a doctoral fellowship to Mr. Justus Mukavi at the University of Münster, Germany. The authors very cordially thank Apothekerstiftung Westfalen-Lippe for the financial support of this study. J.W.M thanks Dr. Andrea Gerdemann (Institute of Food Chemistry, Münster) for her help with processing LC/MS data using MetaboScape. The authors thank the Kenya forest service (KFS) for providing the plant material and Mr. Patrick Mutiso of the University of Nairobi (Kenya) for the plant identification. This work is part of the activities of the Research Network Natural Products against Neglected Diseases (ResNet NPND, see www.resnetnpnd.org).

Conflicts of Interest: The authors declare no conflict of interest.

Author Statement: This work is part of the doctoral thesis of Justus W. Mukavi.

Abbreviations

The following abbreviations are used in this manuscript:

<i>B. obtusifolia</i>	<i>Buxus obtusifolia</i>
UHPLC/+ESI-	Ultra-high-performance liquid chromatography electrospray
QqTOF-MS/MS	ionization quadrupole time of flight mass spectrometer
LC/MS	Shortform for UHPLC/+ESI-QqTOF-MS/MS
QC	Quality control
t_R	Retention time
Min	Minutes
MVDA	Multivariate data analysis
PCA	Principal component analysis
PC	Principal component
ABA	Absciscic acid

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