Effect of Timing and Rates of Nitrogen Application on Yield, Chemical Compositions, Pharmacologic Activities, and Cytotoxicity of Herbal Bush Tea (*Athrixia phyllicoides* DC.)

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Abstract. Bush tea is a popular South African herbal and medicinal tea with the potential for commercialization. The objective of the study was to investigate the effects of different rates of applied nitrogen (N) and timing (early and late) of N application on yield, chemical composition, pharmacologic activity, and cytotoxicity of bush tea. Factorial treatments consisted of timed N application rates (0, 75, 150, and 225 kg·ha⁻¹) for both early and late N application. The treatments were arranged in a randomized complete block design with five replicates. The results show a significant positive response of bush tea fresh and dry leaf and twig weight, chlorophyll, leaf tissue N, total polyphenols, and total flavonoids in response to applied N rates, regardless of the timing of N application, reaching a maximum at 225 kg·ha⁻¹ of N. Later, regardless of the timing of N application, total tannins and total antioxidant activity increased with increasing applied N from 0 to 225 kg·ha⁻¹, reaching a maximum of 150 kg·ha⁻¹ N. The results of this study suggest that, with the exception of antimicrobial activity, most of the parameters recorded increased with increasing rates of N applied. The results further denote that parameters recorded were consistently greater on early applied N compared with late applied N. However, there was no significant difference between the minimum inhibition concentration (MIC) and minimum microbicide concentration (MMC) of early and late applied N. Bush tea applied with 75 N and 150 N had a significant MIC value (3.1 mg·mL⁻¹) for *Escherichia coli* and *Klebsiella pneumonia* compared with 6.3 mg·mL⁻¹ reached at 225 kg·ha⁻¹ N. Among N rates applied, the MIC for *Klebsiella oxytoca*, *Proteus vulgaris*, *Salmonella typhi*, *Serratia marcescens*, and *Staphylococcus aureus* were not significantly different. Hence, N rates applied did not have a significant effect on bush tea MMC values of all microbial species tested. The cytotoxicity of bush tea leaf and twigs harvested from early and late N application were significantly reduced with increasing nitrogen levels reaching a maximum at 225 kg·ha⁻¹. There was a wide variation of compounds despite rates of N applied as well as timing of application, with most compounds such as norfenfluramine, phytol, caryophyllene, propylene glycol, α -copaene, and squalene detected in greater quantities.

Bush tea is a South African herbal tea with the potential for commercialization. The commercialization of bush tea is supported by a report of the toxicologic assessment of bush tea conducted by Chellan et al. (2008). They have reported widespread consumption

of bush tea in South Africa. Mudau et al. (2007) also reported that commercialization of bush tea can provide a healthy beverage alternative to caffeine-containing teas. The plant is used popularly as an herbal tea and a medicinal plant for the treatment of different ailments (Nchabeleng et al., 2013).

Naturally, bush tea grows at different altitudes with different rainfall regimes and soil characteristics (Nchabeleng et al., 2012). Therefore, to achieve good-quality bush tea, it is important to study the nutritional requirement of field-grown bush tea plants. Fertilizers play a critical role in determining horticultural crop yield, quality, and nutritional content (Martinez-Ballestra et al., 2008). In general,

all major quality attributes in horticultural crops, including visual quality and taste, are influenced directly by N availability (Locascio et al., 1984). In green tea production, fertilizer is a major agro-input for the production of high-yield and high-quality tea (Woldegebriel, 2007). Fertilizers improve the nutritional status of both soil and plants (Njogu et al., 2014). Because tea production is a perennial monoculture, a well-balanced fertilizer is necessary throughout the year (Hamid et al., 2014).

The production of secondary metabolites in medicinal plants has been reported to be influenced by genetics and cultivation conditions, such as climate, plant density, and the use of fertilizers (Baranauskiene et al., 2003). Among fertilizers, N is the most important nutrient for crop production because it promotes both yield and quality of plant secondary metabolites (Sifola and Barbieri, 2006). The reason for extensive N application is that the quality of green tea is known to correlate directly with the concentration of primary compounds related to N application (Watanabe, 1995). In addition, N is a well-known essential element for plant growth and it plays a regulatory role in the synthesis of secondary metabolites, such as phenolic compounds (Ruan et al., 2010). Conversely, Bryant et al. (1983) hypothesized that high N levels in plant tissues contribute to the formation of more amino acids and proteins for growth in relation to defense compounds such as secondary metabolites.

Previous studies demonstrated that early-applied N levels ranging from 75 to 225 kg·ha⁻¹ improved growth and quality in cultivated bush tea (Tshivhandekano et al., 2017). However, data are lacking to demonstrate the effect of timing and rates of N application on bush tea. Thus, this study aimed at investigating the effect of applied N rates and timing of N application on yield, chemical compositions, pharmacologic activity, and cytotoxicity of bush tea.

Materials and Methods

Experimental site. The experiments were conducted at the Tshwane University of Technology (TUT) experimental farm, which is situated north of Pretoria (lat. 25°36'03.2"S; long. 28°13'30.9"E). The trials were conducted beginning in spring (Sept. 2016) to the end of Nov. 2016. The plants were allowed to resprout and the trial was repeated beginning in the summer (Dec. 2016) until the end of the summer season (Feb. 2017). The resprouted shoots were fertilized in the same manner as the initial plants. Weather data are presented in Table 1; this was the best time to cultivate bush tea in South Africa (Mudau et al., 2006, 2007).

Five soil samples were collected per horizon (A and B) from different spots on the plot the trial was conducted. Each soil sample collected was packed separately in different marked brown paper bags. The initial soil chemical analyses were conducted using the procedure described by Hanlon et al. (1994). The soil contained 0.001 mg·kg⁻¹ nitrate-N.

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Other soil chemical characteristics are presented in Table 2.

Experimental design and treatment details. Factorial treatments consisted of timed N application rates (0, 75, 150, and 225 kg·ha⁻¹) for both early and late N application. Applied N rates were 0, 75, 150, and 225 kg·ha⁻¹ per plot of 36 plants spaced at 0.5 × 0.5 m. The N rates were timely (early and late) manually broadcasted once off. Early (3 weeks after plant establishment) and late (6 weeks after plant establishment) N application was conducted in a randomized complete block design with five single plants per treatment replicated five times. Limestone ammonium nitrate (28% N) was used as an N fertilizer source. All plants received 75 kg·ha⁻¹ phosphorus (P) and 45 kg·ha⁻¹ potassium (K). The fertilizer source for P was single superphosphate (10.5%); for K, it was potassium chloride (50%) [Ocean Agriculture (PTY) LTD, Muldersdrift, South Africa]. Micronutrients were foliar applied as recommended by Maedza et al. (2017). Two liters of water per plant was applied every third day using a drip irrigation system.

Plant material. The plant materials were collected from Haenerstburg (lat. 23°56' S; long. 29°54' E; 890 m a.s.l.) in Limpopo Province, South Africa. Plants were propagated by using cuttings as described by Mudau et al. (2006). Bush tea cuttings 7 to 8 cm long were planted as described by Maudu et al. (2012), with sand used as the growth media. The cuttings were placed on the mist bed and allowed to sprout and root. The well-matured bush tea that hardened in the shade nets after propagation were later transplanted to the TUT experimental farm for the open field trial, using a plant spacing of 0.5 × 0.5 m.

Parameters recorded. Biomass (fresh and dry weight of leaf and twigs), chlorophyll content, leaf tissue N, total polyphenols, total flavonoids, total tannins, antioxidant activity, antimicrobial activity, cytotoxicity, and detected compounds were documented.

Determination of chlorophyll content. Chlorophyll was measured once every month during the growing seasons with a portable Minolta chlorophyll meter (SPAD-502; Spectrum Technologies, Inc., Plainfield, IL).

Determination of fresh weight and dry weight. The average fresh and dry weights were determined using standard level precision balances. Fresh weight was measured immediately after harvest whereas dry weight was measured after 2 weeks of freeze drying.

Harvest and extraction of bush tea leaf and twig samples. The bush tea leaves and twigs were harvested using the current traditional method of cutting the plant from the stem to allow resprouting. The bush tea leaves and twigs from both early and late applied N plots were all harvested at the end of spring (Nov. 2016) and the end of summer (end of Feb. 2017). During harvest, the leaves and twigs were packed in marked brown paper bags. After harvest, bush tea leaves and twigs were transported immediately to the science campus of the University of South Africa in Florida (lat. 5.26°9'501"S, long. 27°54'113"E), where they were weighed and freeze-dried for 2 weeks. One hundred grams of blended leaf materials was soaked in 150 mL ethanol and shaken at room temperature for 24 h. The ethanol supernatant was filtered and then evaporated on a rotary evaporator under reduced pressure at 37 °C. The extract was stored in the cold room at 5 °C, after which it was subjected to total polyphenol, total flavonoid, total tannin, antioxidant activity, antimicrobial activity, and cytotoxicity analyses.

Leaf and twig tissue N analyses. The freeze-dried bush tea leaves and twigs harvested from both early and late applied N were finely ground to pass through a 20-mesh screen. A sample of 0.2 g was digested at 37 °C for 1 h in 100-mL tubes containing 4 mL concentrated sulfuric acid, 2 mL 30% hydrogen peroxide, and 2.5 g of catalyst, which was comprised of a powdered mixture of 15 g copper sulfate, 250 g potassium sulfate, and stearic acid (Anon, 1972). After digestion, 100 mL distilled water was added to each sample and the hydrated samples were filtered through Whatman® No. 2 filter paper. Filtered samples were bottled and stored at -20 °C before analysis. Nitrogen concentrations were determined in thawed samples using an Auto-Analyser (Anon,

1972) connected to a Sanplus Segmented Flow Analysis System (Skalar, Netherlands) and were expressed as percent N dry weight.

Determination of total polyphenol content. The total polyphenol content was determined by colorimetric assay (Mohammed and Manan, 2015). Bush tea leaf and twig extracts (200 µL) was added to 800 µL distilled water followed by 100 µL Folin-Ciocalteu. The mixtures were incubated for 3 h at room temperature. A total of 300 µL sodium carbonate (Na₂CO₃) (20% w/v) was added into the mixture and incubated for 2 h in the dark at room temperature. After 2 h, the absorbance was recorded with a Metertech ultraviolet/VIS SP8001 spectrometer (Metertech, Taipei, Taiwan) at 765 nm. A blank was prepared with distilled water. A gallic acid standard curve was prepared from 0 to 200 mg·L⁻¹ using the same method. The total phenolic contents were calculated from the prepared standard curve of 0–200 mg gallic acid equivalent (GAE) per gram dry matter; total phenolic content was expressed in milligrams GAE equivalent to g dry matter.

Determination of total flavonoid content. Flavonoid content was analyzed by colorimetric analysis (Mohammed and Manan, 2015). A mixture of 200 µL bush tea leaf and twig extracts was added to 150 µL sodium nitrite (5% w/v) and was incubated for 6 min at room temperature. After incubation, 150 µL aluminum chloride hexahydrate (10% w/v) was added and incubated for another 6 min at room temperature. After the second incubation, 800 µL sodium hydroxide (NaOH; 10% w/v) solution was added and incubated at room temperature for 15 min. For a negative control, distilled water replaced the extract. Absorbance was read by using a Metertech ultraviolet/VIS SP8001 spectrometer at 510 nm. Before total flavonoid content was determined, a standard curve of quercetin dissolved in 80% ethanol was prepared from 0 to 500 µg·mL⁻¹. Total flavonoid was expressed in milligrams quercetin equivalent gram dry matter.

Determination of total tannin content. The total tannin content was determined by the Folin-Ciocalteu assay method (Mohammed and Manan, 2015). Bush tea leaf and twig extracts of 100 µL were added to 750 µL distilled water, 500 µL Folin-Ciocalteu reagents, and 1000 µL 35% Na₂CO₃. The mixture was diluted with distilled water to 10 mL and then shaken. After shaking, the mixture was incubated for 30 min at room temperature and subsequently read at 725 nm with a Metertech ultraviolet/VIS SP8001 spectrometer. Gallic acid standard solutions were prepared using the same method. Distilled water replaced the extract and was used as a negative control. The total tannin content

Table 1. Weather data for 2016–17 at the Tshwane University of Technology experimental farm, Pretoria, Gauteng Province, South Africa.

Month (2016–17)	Avg temperature (°C)		Avg relative humidity (%)	Avg rainfall (mm)
	Maximum	Minimum		
Sept.	28.21	8.96	42.99	37.65
Oct.	29.84	13.27	49.49	58.13
Nov.	29.43	15.21	57.48	66.15
Dec.	29.6	16.3	60.93	106.95
Jan.	29.65	17	66.15	157.76
Feb.	30.13	16.52	64.19	87.08

Table 2. Chemical characteristics of soils collected from the Tshwane University of Technology experimental farm during the 2016–17 growing season.

Horizon	Mg (cmol·kg ⁻¹)	CEC (cmol·kg ⁻¹)	P (mg·kg ⁻¹)	pH (H ₂ O)	Org C (%)	Fe (mg·kg ⁻¹)	Mn (mg·kg ⁻¹)	Cu (mg·kg ⁻¹)	Zn (mg·kg ⁻¹)	Cl (%)	Na (cmol·kg ⁻¹)	K (cmol·kg ⁻¹)	Ca (cmol·kg ⁻¹)
A, 0–250 mm	5.346	26.007	1.12	7.32	1.3	10.54	12.14	2.37	3.1	42.2	0.285	0.776	11.991
B, 250–700 mm	5.25	24.357	0.43	7.7	0.79	12.5	14.46	2.08	1.79	51.1	0.294	0.21	10.881

CEC = cation exchange capacity; Org = organic.

was calculated based on the prepared standard curve with 0–100 mg gallic acid and presented as GAE per gram dry matter.

Determination of antioxidant activity. The method described by Du Toit et al. (2001) was used to determine the total antioxidant activity of bush tea leaf and twig extracts. Vitamin C was used as the standard. The plant extract (2 mg) was dissolved in 200 μL ethanol to yield a stock concentration of 10 $\text{mg}\cdot\text{mL}^{-1}$; whereas 2 mg vitamin C was dissolved in 1 mL ethanol to give a stock concentration of 2 $\text{mg}\cdot\text{mL}^{-1}$ to serve as the positive control. A solution of 1,1-diphenyl-2-picrylhydrazyl was prepared by dissolving 20 mg in 500 mL ethanol to yield a stock concentration of 0.04 $\text{mg}\cdot\text{mL}^{-1}$. All samples were tested in triplicate using 96-well microplates. The startup concentration for the test samples in the first row was 500 $\mu\text{g}\cdot\text{mL}^{-1}$, which was a composition of distilled water and the extract, whereas the positive control (vitamin C) was a mixture of distilled water and vitamin C with a final concentration of 100 $\mu\text{g}\cdot\text{mL}^{-1}$ (in the first row). Blank controls were prepared by adding ethanol to distilled water; negative controls contained test samples and distilled water. These were serially diluted from the first row to the last row, and 1-diphenyl-2-picrylhydrazyl was added into all the wells except the negative controls. The plates were covered in foil and incubated for 30 min. The absorbencies were read with an enzyme-linked immunosorbent assay (ELISA) plate reader (KC junior version 4.0; Bio Tek Instruments) at 515 nm, and the absorbance values were analyzed with Graph Pad Prism 4.0. For antioxidant activity analyses, the sum of squares was partitioned into linear or quadratic polynomial orthogonal contrasts by using the Statistical Analyses System, version 9.4 (SAS Institute, Cary, NC).

Antimicrobial assay. The microdilution technique of using 96-well microplates, as described by Eloff (1998), was used to obtain the MIC (measured in milligrams per milliliter) and MMC (measured in milligrams per milliliter) values of the bush tea leaf and twig ethanol extracts against the selected microorganisms under study. MIC is defined as the greatest dilution or least concentration of the extracts that inhibit growth of organisms (Sen and Batra, 2012). MMC was recorded as the lowest concentration of extract that inhibited 100% growth of microorganisms (Cohen et al., 1998). Extracts were serially diluted in the 96-well plate and the final concentration of extracts and ciprofloxacin (positive control) ranged from 0.196 to 25 $\text{mg}\cdot\text{mL}^{-1}$. The 24-h-old microorganisms were added into the 96-well plates and incubated for 24 h at 37 °C. MIC was determined by adding 40 μL of 0.2 $\text{mg}\cdot\text{mL}^{-1}$ *p*-iodonitrotetrazolium violet (Sigma-Aldrich, South Africa) to microplate wells which were then incubated at 37 °C for 24 h. After incubation, MMC was tested by adding 50 μL of the suspensions from the wells, which did not show any growth after incubation during MIC assays, to 150 μL fresh broth. These suspensions were reincubated at 37 °C for another 24 h. Last,

40 μL of 0.2 $\text{mg}\cdot\text{mL}^{-1}$ *p*-iodonitrotetrazolium violet was added to the microplate wells and incubated at 37 °C for 2 h to complete the MMC concentration assay.

Microbial species used. The microbial strains used were the Gram-negative bacteria *Escherichia coli* (ATCC 8739), *Klebsiella oxytoca* (ATCC 49131), *Proteus vulgaris* (ATCC 6380), *Serratia marcescens* (ATCC 13880), *Salmonella typhi* (ATCC 14028), and *Klebsiella pneumonia* (ATCC 13883); the Gram-positive bacteria were *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 25931). The selected microorganisms were grown in casein–peptone soy agar medium (Merck SA (Pty) Ltd., Modderfontein, Johannesburg). The McFarland standard was used to record the bacterial concentration of 4×10^7 (Dey et al., 2010).

XTT cytotoxicity assay. The cytotoxicity of bush tea leaf and twig extracts were tested on human embryonic kidney 293 cells. One hundred microliters of human embryonic kidney cell suspensions (1×10^5 cells/mL) were added to the inner wells of a 96-well plate; 200 μL of incomplete medium [Dulbecco's Modified Eagle's Medium (DMEM)] was added to the outer wells. The plates were incubated for 24 h to allow the cells to attach to the base of the plate. Serial dilution of the plant extracts [already dissolved in dimethyl sulfoxide (DMSO)], the positive control (actinomycin D) and negative (vehicle) control DMSO with the complete medium (10% fetal bovine serum and DMEM) and penicillin/streptomycin were carried out in a 24-well plate to give eight different concentrations of each sample and a final volume of 1 mL/well. One hundred microliters of each concentration from the 24-well plate were added to the 96-well plate in triplicate, and there was a triplicate medium control and a DMSO control for each extract. A reference plate was prepared to account for the color of the plant extracts. This plate contained the plant extracts and medium in duplicate but no cells. The plates were incubated for 72 h, after which 50 μL XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reagent from Roche Diagnostics GmbH (Randburg, Johannesburg) was added to all the wells and they were incubated for about 2 h 30 min. After incubation, the plates were read on an ELISA microplate reader with KC Junior version 4.0 software to read the

absorbance at 450 nm and 690 nm as the reference wavelengths. Graph Pad Prism 4.0 was used to analyze the data.

Gas chromatography linked to mass spectrometry. Bush tea samples (one leaf per sample) were analyzed using headspace solid-phase microextraction gas chromatography linked to mass spectrometry using the method of Musarurwa et al. (2010), but oven ramping temperatures and injection ratios were modified. The headspace solid-phase microextraction of leaves were performed with Supelco SPME fibers [DVB/Carboxen/PDMS, StableFlex (Supelco)]. Leaves were placed directly into a 20-mL headspace vial, then sealed with an aluminum-coated silicone rubber septum. Volatiles were extracted at 70 °C for 15 min. Gas chromatography was performed with a Waters GCT Premier AS 2000 coupled to a mass spectrometer equipped with an HP5 column (30 m, 0.25 mm i.d., 0.25 μL film thicknesses). Temperatures were set at 250 °C for both the injection (splitless injection) and the ion source temperature. Helium was used as the carrier gas (1 $\text{mL}\cdot\text{min}^{-1}$). The temperature ramp regime was initiated by heating at 5 °C for 3 min, followed by an oven ramp to 142 °C at 5 °C $\cdot\text{min}^{-1}$, and a second ramp of 1 °C $\cdot\text{min}^{-1}$ up to 240 °C. A mass scanning range of 40 to 550 $\text{m}\cdot\text{z}^{-1}$ (perfluorotri-n-butylamine as mass reference) was used, and mass spectra were recorded at two scans. The National Institute of Standards and Technology library was used for the tentative identification of compounds.

Statistical analyses. Data were subjected to analysis of variance by using the general linear model of the Statistical Analyses System, version 9.4 (SAS Institute). Treatment sum of squares was partitioned orthogonally to determine the effects of control vs. treatment and early treatment vs. late treatment. In both seasons, data were pooled and mean separation was done using Duncan's multiple range test. For gas chromatography linked to mass spectrometry, Kovats indices were calculated from an alkane series to verify compound names.

Results

Biomass (fresh and dried weight). The results in Table 3 demonstrated that, regardless of the timing of N application, as N application rates increased from 0 to 225 $\text{kg}\cdot\text{ha}^{-1}$, bush

Table 3. Bush tea mean fresh weight, dry weight, and leaf chlorophyll value in response to applied N rates and timing of N application.

Applied N ($\text{kg}\cdot\text{ha}^{-1}$)	Chlorophyll (SPAD value)	Fresh wt (g)	Dry wt (g)
Early application			
0	34.57 f	85 f	33 d
75	42.33 d	110 e	60 c
150	47.23 b	165 b	103 a
225	50.03 a	170 a	108 a
Late application			
75	39.96 e	105 e	42 c
150	45.27 c	130 d	55 cd
225	47.87 b	147 c	82 b
Contrast: Control vs. treatment	0.0001	0.0001	0.0001
Contrast: Early vs. late	0.0001	0.0001	0.0001

Means with the same letters in a column are not significantly different at $P < 0.0001$.

tea leaf and twig fresh and dry weights increased significantly, reaching a maximum at 225 kg·ha⁻¹ N. In addition, with the exception of the fresh weight of bush tea treated with 75 kg·ha⁻¹ N, early N applied bush tea resulted in a significantly greater biomass compared with late applied N. A strong correlation was recorded between fresh weight and dry weight with leaf and twig tissue N, total polyphenol content, total flavonoid content, total tannin content, total antioxidant activity, and cytotoxicity of both early and late N applied bush tea (Tables 4 and 5). However, there was no relationship (<50%) between fresh weight and antioxidant activity, as well as dry weight and antioxidant activity on late N applied bush tea.

Chlorophyll. The results of chlorophyll (SPAD values) presented in Table 3, show that, regardless of the timing of N application, as applied N rates increased from 0 to 225 kg·ha⁻¹, chlorophyll values also increased significantly, reaching a maximum at 225 kg·ha⁻¹. Bush tea chlorophyll was significantly greater on early N applied rates compared with late N rates applied. Chlorophyll of both early

and late N applied bush tea had a strong relationship with leaf and twig tissue N, fresh weight, dry weight, polyphenol content, flavonoid content, tannin content, and cytotoxicity (Tables 4 and 5). There was a weak relationship between chlorophyll and antioxidant activities in bush tea leaves and twigs.

Leaf tissue N. The percentage of leaf and twig tissue N of both early and late N applied bush tea increased with increasing N rates from 0 to 225 kg·ha⁻¹ (Table 6). The bush tea leaf and twig tissue N of both early and late applied N increased significantly with increasing rates of N, reaching a maximum at an N rate of 225 kg·ha⁻¹ for early applied N and 150 kg·ha⁻¹ for late applied N. However, early applied N to bush tea leaves and twigs had significantly greater tissue N compared with late applied N bush tea. The results denote that early applied N rates increased significantly bush tea leaf and twig tissue N compared with late applied N rates. The bush tea leaf and twig tissue N of both early and late applied N exhibited a strong relationship with chlorophyll, fresh weight, dried weight, polyphenol content, flavonoid content, tannin

content, antioxidant activity, and cytotoxicity (Tables 4 and 5).

Polyphenol content. Regardless of the timing of N application, as applied N levels increased from 0 to 225 kg·ha⁻¹, the production of bush tea total polyphenols (Table 6) increased significantly, reaching a maximum at 225 kg·ha⁻¹. The results suggest that bush tea from early applied N levels had a significantly greater total polyphenol content compared with late applied N rates. The results further suggest that bush tea total polyphenol content had a strong relationship with leaf and twig tissue N, chlorophyll, fresh weight, dry weight, flavonoid content, tannin content, and cytotoxicity (Tables 4 and 5). However, bush tea total polyphenol content exhibited weak relationships with tannin content and antioxidant activity in early applied N, and in antioxidant activity in late applied N.

Flavonoid content. The bush tea total flavonoid content of both early and late applied N increased with increasing applied N from 0 to 225 kg·ha⁻¹, reaching a maximum at 225 kg·ha⁻¹ N (Table 6). The results

Table 4. Correlations of bush tea parameters recorded for early applied N.

	Leaf tissue N (%)	Chlorophyll (SPAD values)	Fresh wt (g)	Dry wt (g)	Total polyphenols (GAE/g dry matter)	Total flavonoids (QE/g dry matter)	Total tannins (GAE/g dry matter)	Total antioxidant activity (IC ₅₀)
Chlorophyll	0.96**							
Fresh weight	0.91**	0.97**						
Dried mass	0.85**	0.94**	0.99**					
Polyphenols	0.94**	0.99**	0.92**	0.88**				
Flavonoids	0.99**	0.98**	0.93**	0.87**	0.98**			
Tannins	0.78*	0.62*	0.67*	0.60*	0.53	0.68*		
Antioxidant	0.59	0.52	0.67*	0.66*	0.38	0.50	0.88**	
Cytotoxicity	0.97**	0.86**	0.81**	0.72*	0.84**	0.93	0.86**	0.61*

Significant at * $P \leq 0.05$ or ** $P \leq 0.01$.

GAE = gallic acid equivalent; QE = quercetin equivalent.

Table 5. Correlations of bush tea parameters recorded for late applied N.

	Leaf tissue N (%)	Chlorophyll (SPAD values)	Fresh wt (g)	Dry wt (g)	Total polyphenols (GAE/g dry matter)	Total flavonoids (QE/g dry matter)	Total tannins (GAE/g dry matter)	Total antioxidant activity (IC ₅₀)
Chlorophyll	0.96**							
Fresh weight	0.95**	0.99**						
Dry weight	0.85**	0.92**	0.95**					
Polyphenols	0.98**	0.93**	0.94**	0.89**				
Flavonoids	0.78*	0.88**	0.91**	0.99**	0.83**			
Tannins	0.83**	0.91**	0.86**	0.69*	0.72*	0.64*		
Antioxidant	0.68*	0.51	0.46	0.20	0.59	0.08	0.60*	
Cytotoxicity	0.79*	0.87**	0.81**	0.63*	0.67*	0.58	1.00**	0.61*

Significant at * $P \leq 0.05$ or ** $P \leq 0.01$.

GAE = gallic acid equivalent; QE = quercetin equivalent.

Table 6. Response of bush tea leaf and twig tissue N and chemical compositions to applied N rates and the timing of N application.

Applied N (kg·ha ⁻¹)	Leaf tissue N (%)	Total polyphenols (GAE/g dry matter)	Total flavonoids (QE/g dry matter)	Total tannins (GAE/g dry matter)	Total antioxidant activity (IC ₅₀)
Early application					
0	1.67 ± 0.24 d	50.93 ± 0.12 e	0.67 ± 0.09 f	0.53 ± 0.03 f	36.26 ± 1.51 f
75	1.95 ± 0.27 b	64.06 ± 0.29 c	1.21 ± 0.37 c	1.79 ± 0.0 a	51.25 ± 1.88 c
150	2.06 ± 0.18 a	65.14 ± 0.47 b	1.43 ± 0.43 b	1.99 ± 0.01 a	55.32 ± 0.89 a
225	2.07 ± 0.29 a	66.99 ± 0.00 a	1.58 ± 0.47 a	1.74 ± 3.0 b	51.91 ± 9.76 c
Late application					
75	1.71 ± 0.23 d	50.95 ± 0.49 e	0.74 ± 0.22 e	1.42 ± 0.01 e	37.71 ± 2.05 e
150	1.88 ± 0.29 c	62.36 ± 0.10 d	0.79 ± 0.28 d	1.71 ± 0.01 c	53.17 ± 3.12 b
225	1.88 ± 0.21 c	64.49 ± 0.18 c	1.02 ± 0.38 c	1.61 ± 0.35 d	39.16 ± 0.69 e
Contrast: Control vs. treatments	0.001	0.001	0.001	0.001	0.001
Contrast: Early vs. late treatments	0.001	0.001	0.001	0.001	0.001

Means with the same letters within the column are not significantly different using Duncan's multiple range test at 1%.

GAE = gallic acid equivalent; QE = quercetin equivalent.

demonstrate that early applied N rates increased significantly bush tea total flavonoid content compared with late applied N rates. Early N applied bush tea total flavonoid content had strong relationships with leaf and twig tissue N, fresh weight, dry weight, total polyphenol content, total flavonoid content, tannin content, and cytotoxicity (Table 4). However bush tea total flavonoid content had a weak relationship with antioxidant activity. Similar results were recorded on late applied N bush tea, with additional weak relationships of bush tea total flavonoid content and cytotoxicity (Table 5).

Tannin content. Table 6 shows the results of total tannin content of bush tea leaves and twigs harvested from early and late applied N. The results suggest that as N levels increased from 0 to 225 kg·ha⁻¹, both early and late applied N total tannin content increased, reaching a maximum at 150 kg·ha⁻¹ N. It is evident that early N application increased bush tea leaf and twig total tannin content significantly compared with late applied N. Early N applied bush tea total tannin content

had strong relationships with leaf and twig tissue N, fresh weight, dry weight, total flavonoid content, total tannin content, total antioxidant activity, and cytotoxicity (Table 4). However early applied N bush tea total tannin content had a weak relationship with total polyphenol content. Late applied N bush tea total tannin content exhibited a strong relationship with all parameters recorded (Table 5).

Antioxidant activity. The total antioxidant activity of bush tea leaves and twigs harvested from early and late applied N results are presented in Table 6. The results suggest that as the applied N rate increased from 0 to 225 kg·ha⁻¹, early and late N application increased the total antioxidant activity significantly, reaching a maximum at 150 kg·ha⁻¹ N. The results suggest that early N application influenced total antioxidant activity significantly compared with late N application. Early N applied bush tea antioxidant activity had strong relationships with bush tea fresh weight, dried weight, and total tannin content (Table 4). Late applied N exhibited strong relationships with bush tea total antioxidant activity, leaf and twig tissue N, and total tannin content (Table 5). Total antioxidant activity of early N applied bush tea had weak relationships with leaf and twig tissue N, chlorophyll, total polyphenol content, and total flavonoid content. There were weak relationships between late applied N bush tea total antioxidant activity and chlorophyll, fresh weight, dry weight, total polyphenol content, and total flavonoid content.

Cytotoxicity. The cytotoxicity results (Table 7) are expressed as the maximum effective concentration required to inhibit 50% of the human embryonic kidney cells (IC₅₀, measured in micrograms per milliliter). Regardless of the timing of N level application, the cytotoxicity results of bush tea leaves and twigs suggests a significant increase in bush tea nontoxicity with increasing N rates. The

nontoxicity of early and late N applied bush tea leaves and twigs reached a maximum at 150 kg·ha⁻¹ N. Therefore, the results imply that the nontoxicity of bush tea is not affected significantly by the timing of N application and the rates of N applied. Regardless of the timing of N application, bush tea nontoxicity had a strong interrelation with all parameters recorded (Tables 4 and 5).

Antimicrobial activity. MIC and MMC values of early and late N applied bush tea leaves and twigs were not significantly different. Table 8 presents MIC values of both early and late applied N; Table 9 presents MMC values of both early and late applied N. Bush tea applied with 75 N and 150 N had a significant MIC value (3.1 mg·mL⁻¹) for *E. coli* and *K. pneumonia* compared with 6.3 mg·mL⁻¹ reached at 225 kg·ha⁻¹ N. Among applied N rates, MIC for *K. oxytoca*, *P. vulgaris*, *S. typhi*, *S. marcescens*, and *S. aureus* were not significantly different. Hence, applied N rates did not have a significant effect on bush tea MMC values of all microbial species tested. Bush tea MIC values ranged from 3.1 to 12.0 mg·mL⁻¹, whereas MMC values ranged from 6.3 to 25.0 mg·mL⁻¹ according to the microbial species used.

Compounds. Volatile compounds detected in bush tea from both early and late applied N by using gas chromatography linked to mass spectrometry varied (Table 10). Thus, it suggests that applied N and timing of N application influenced the variation of compounds detected as well as the relative abundance of compounds. Six compounds (phytol, caryophyllene, norfenfluramine, propylene glycol, α -copaene, and squalene) were found to be significantly greater in the control, early applied nitrogen rate of 150 kg·ha⁻¹, and late applied nitrogen of 75 kg·ha⁻¹ and 225 kg·ha⁻¹. It was observed that early applied N consistently had more compounds detected compared with the late N treatments.

Table 7. Bush tea cytotoxicity in relation to applied N rates and timing (early and late) of applied N.

Applied N (kg·ha ⁻¹)	IC ₅₀ (μg·mL ⁻¹)	R ²
Early application		
0	128.8 b	0.94
75	214.9 c	0.19
150	237.1 a	0.63
225	222.3 b	0.76
Actinomycin D	93.2 d	0.72
Late application		
75	213.8 c	0.23
150	236.9 a	0.54
225	221.5 b	0.44
Control vs. treatment	0.0001	
Early vs. late	0.0001	

Means with the same letters within the column are not significantly different when using Duncan's multiple range test at 1%.

Table 8. Bush tea leaf and twig minimum inhibition concentrations in response to applied N rates.

Applied N (kg·ha ⁻¹)	Minimum inhibition concn of bush tea (mg·mL ⁻¹)						
	<i>Escherichia coli</i>	<i>Klebsiella oxytoca</i>	<i>Klebsiella pneumoniae</i>	<i>Proteus vulgaris</i>	<i>Salmonella typhi</i>	<i>Serratia marcescens</i>	<i>Staphylococcus aureus</i>
0	3.1 b	6.3 b	3.1 b	6.3 b	12.5 b	12.5 b	6.3 b
75	3.1 b	6.3 b	3.1 b	6.3 b	12.5 b	12.5 b	6.3 b
150	3.1 b	6.3 b	3.1 b	6.3 b	12.5 b	12.5 b	6.3 b
225	6.3 c	6.3 b	6.3 b	6.3 b	12.5 b	12.5 b	6.3 b
Ciprofloxacin	0.196 a	0.196 a	0.196 a	0.196 a	0.196 a	0.196 a	1.96 a
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Means with the same letters within the column are not significantly different when using Duncan's multiple range test at 1%.

Table 9. Bush tea leaf and twig minimum microbicide concentrations in response to applied N rates.

Applied N (kg·ha ⁻¹)	Minimum microbicide concn of bush tea (mg·mL ⁻¹)						
	<i>Escherichia coli</i>	<i>Klebsiella oxytoca</i>	<i>Klebsiella pneumoniae</i>	<i>Proteus vulgaris</i>	<i>Salmonella typhi</i>	<i>Serratia marcescens</i>	<i>Staphylococcus aureus</i>
0	6.3 b	12.5 b	6.3 b	25.0 b	25.0 b	25.0 b	12.5 b
75	6.3 b	12.5 b	6.3 b	25.0 b	25.0 b	25.0 b	12.5 b
150	6.3 b	12.5 b	6.3 b	25.0 b	25.0 b	25.0 b	12.5 b
225	6.3 b	12.5 b	6.3 b	25.0 b	25.0 b	25.0 b	12.5 b
Ciprofloxacin	0.196 a	0.196 a	0.196 a	0.196 a	0.196 a	0.196 a	1.96 a
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Means with the same letters within the column are not significantly different when using Duncan's multiple range test at 1%.

Discussion

From this study, it was evident that early N application yielded significantly more bush tea biomass compared with late applied N. Nitrogen application increases overall plant growth (Wang et al., 2012). Similarly, N application increased bush tea biomass (Mudau et al., 2005). Nitrogen had been found to be the most important yield-increasing fertilizer, with the greatest yield of 872 kg·ha⁻¹ obtained with 200 kg·ha⁻¹ N applied in *Camellia sinensis* (Sitienei et al., 2013). However, proper N supply is required to optimize vegetative and reproductive growth and, consequently, overall yields (Lawlor, 2002).

High N availability for plants resulted in increased chlorophyll, photosynthesis, and primary growth of plant (Stefanelli et al., 2010). It was found that N application increased *Labisia* leaf chlorophyll (Ibrahim et al., 2011). Similarly, the level of chlorophyll a + b in leaf of *Lavandula angustifolia* was reported to increase from 4.36 to 5.48 mg·g⁻¹, respectively, at the rates of 50 and 200 kg·ha⁻¹ N (Biesiada et al., 2008). In addition, it was

Table 10. Compounds detected and their abundant concentrations on bush tea leaf and twig in response to applied N rates.

Compounds	Concn (kg·ha ⁻¹)						
	Early applied N				Late applied N		
	Control	75	150	225	75	150	225
Alpha-copaene	38.89	10.63	0	10.13	0	0	0
1,2,3-Propanetriol, 1-acetate	0	0	0	10.13	0	0	0
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	41.54	28.79	0	0	26.58	0	0
1H-Pyrrole-2,5-dione, 3-ethenyl-4-methyl-	0	28.50	0	0	22.88	0	33.02
2-Methoxy-4-vinylphenol	77.11	25.68	0	14.97	0	86099	15.15
2-Pyrrolidinone, 1-methyl-	0	0	0	13.92	0	0	0
α-Cadinol	0	0	0	0	15.70	0	19.74
Acetic acid	10.66	24.48	0	37.95	13.69	87.76	39.60
Acetic acid, methyl ester	0	0	0	62.09	0	0	62.09
Acetic anhydride	0	19.23	0	0	0	0	73.03
Acetophenone	28.14	0	0	0	51.03	77.46	0
α-copaene	10.46	36.34**	0	0	35.28**	0	28.37*
Allo-Inositol	15.00	48.55	49.46	0	0	0	0
α-Ocimene	0	0	0	49.97	0	0	13.03
Benzaldehyde	0	0	0	31.95	18.25	28.09	64.49
Benzene acetic acid	0	0	0	73.38	0	64.57	0
Benzyl alcohol	0	0	0	48.66	16.66	31.60	0
Butanoic acid	0	43.07	0	28.62	0	14.03	0
Butanoic acid, 4-hydroxy-	30.12	67.47	78.09	0	53.06	35.38	37.43
Butyrolactone	0	0	0	10.07	0	0	0
Caryophyllene	14.02	86.14**	65.28**	38.01**	11.71	91.780**	11.22
Dibutyl phthalate	0	0	1.672	0	0	0	0
dl-α-Tocopherol	0	18.49	12.91	20.49	0	0	0
D-Limonene	60.45	70.11	11.15	63.25	22.23	0	19.67
Eicosane	26.14	22.27	13.06	0	72.14	0	65.70
Ethane, nitro-	12.31	56.59	0	0	29.60	0	0
Ethyl acetoacetate	13.93	24.35	60.51	39.52	0	0	0
Ethyl orthoformate	0	0	0	0	98.63	41.01	88.38
Eucalyptol	0	10.52	63.64	11.92	0	0	0
Eugenol	0	0	28.93	0	0	0	20.69
Furfural	0	0	0	11.97	50.89	0	18.78
Hexacosane	27.81	61.43	0	99.54	0	0	0
Hexanoic acid	0	15.77	0	0	0	0	0
Indolizine	0	0	0	0	58.62	0	18.79
Inositol	0	51.13	61.16	42.56	38.91	0	64.89
L-Inositol	0	59.62	19.93	45.56	0	0	0
Methane sulfonyl chloride	0	0	0	40.18	19.78	27.39	28.11
Muco-inositol	31.88	12.14	24.49	41.24	0	0	47.89
Neo-inositol	50.64	85.08	0	20.18	14.07	0	0
Nitrous acid	0	0	0	15.18	0	0	0
Norfenfluramine	10.68	12.99*	19.83**	38.97**	29.60**	19.60**	33.76**
Octacosanol	30.18	12.49	12.34	0	39.35	0	68.03
Octadecanoic acid	12.63	33.91	58.54	74.82	0	10.99	29.33
o-Xylene	55.17	0	568490	0	15.32	0	15.329
p-Cymene	35.32	53.39	0	67.82	11.65	29.38	23.36
Pentanoic acid	0	0	86.99	0	0	54.26	0
Phenol	30.57	0	98.29	89.78	84.59	10.39	0
Phthalic anhydride	72.40	44.56	0	0	0	0	0
Phytol	0	17.97*	18.45*	36.12**	23.86**	26.08**	56.45**
Propylene glycol	0	0	0	61.38*	25.75	0	31.25*
Salicylic acid	0	0	0	27.45	0	0	0
Squalene	12.15	0	55.89**	56.53**	49.43**	25.03**	33.80**
Styrene	0	18.92	31.70	0	0	23.25	0
Terpineol	0	46.76	58.09	0	0	0	0
Tetracosane	0	0	70.79	0	0	15.14	27.46
Tetradecanoic acid	0	0	15.23	77.18	0	13.86	19.15
Tetraethyl silicate	0	0	0	0	0	24.16	19.96
Undecane	0	0	0	0	0	54.68	83.64
Vanillin	0	0	0	73.51	0	0	51.84

Relative abundance followed by asterisks are significant at * $P < 0.05$ and ** $P < 0.01$.

reported that as N levels increased in an ascending order of $0 > 90 > 180 > 270 \text{ kg·ha}^{-1}$ N, the net photosynthesis rate of *Labisia pumila* simultaneously increased steadily (Ibrahim et al., 2011). The results of the current study support the results of Ibrahim et al. (2011), who revealed that as N rate increased from 0 to 225 kg·ha^{-1} N, leaf tissue N also increased considerably. The N uptake of *Camellia sinensis* increased with increased N

application (Okano et al., 1997). Bush tea leaf tissue N increased with increased N (Mudau et al., 2005). The current results concur with the findings of Mudau et al. (2006), who reported a quadratic response of leaf tissue N in potted bush tea cultivated under 50% shade net. In addition, Cechin and Fumis (2004) reported that increased N availability also resulted in greater sunflower leaf N content. According to Ibrahim et al. (2011), the increase in leaf tissue

N in response to increased N application might be the result of intensification of nitrate content in the leaf.

It has been reported that, regardless of season addition of N fertilizer, resulted in a significant ($P \leq 0.001$) increased concentration of total polyphenols in bush tea leaf (Mudau et al., 2006). The increase in the concentration of total polyphenol mineral nutrition was also reported in tea (Owour,

1989; Owour and Odhiambo, 1994). However, the results of the current study disagree with the observations made by Ibrahim et al. (2011), that total polyphenol content and total flavonoid content decreased in leaves with increasing N fertilization from 90 to 180 kg·ha⁻¹ N and then 270 kg·ha⁻¹ N by 42%, 43%, and 57%, respectively, compared with non-N fertilization. In addition, a high N supply has been reported to decrease the concentrations of *Chrysanthemum morifolium* total flavonoids by 18% to 35% (Liu et al., 2010).

The current results exhibited a trend similar to that reported by Chabeli et al. (2008): Under 50% shade net, potted bush tea total tannin content increased with increasing N application, reaching a maximum at 300 kg·ha⁻¹ N. However, the difference is that, with the current results, N plateaued at 150 kg·ha⁻¹, which is in contrast to the results of Chabeli et al. (2008), who reported that N plateaued at 300 kg·ha⁻¹.

The current results of total antioxidant activity concurs with Mogotlane et al. (2007), who reported that the amount of total antioxidant activity in bush tea leaf was greatest at 300 kg·ha⁻¹ N. The nontoxicity results support the results reported previously by Mavundza et al. (2010), that quercetin compound extracted from bush tea exhibited minimal toxicity effect (IC₅₀ value, 81.38 ± 0.33 µg·mL⁻¹). The IC₅₀ value of all the bush tea extracts was reported to be greater than that of actinomycin D, which was used as a positive control. Considering that the values of these extracts of bush tea were greater than that of actinomycin and greater than 100 µg·mL⁻¹, bush tea should be considered nontoxic (Patel et al., 2010).

The relationship between bush tea leaf and twig N and total polyphenol content, total flavonoid content, total tannin content, total antioxidant activity, and cytotoxicity presents a sharp contrast with the carbon nutrient balance hypothesis. The hypothesis postulates that carbon-based compounds decrease with elevated nutrient availability (Bryant et al., 1983). It is hypothesized that plants respond to varying levels of resource availability in a physiologically passive manner driven by simple mass action (Hamilton et al., 2001; Nitao et al., 2002). Palumbo et al. (2007) argued that phenotypic plasticity in secondary metabolite production is influenced by resource availability and selective pressures that might be genetically regulated. On the other side, the current results are in support of the findings of Ibrahim et al. (2011), who reported a positive relationship between leaf tissue N and chlorophyll, photosynthesis, and biomass.

The results of this study are consistent with the results reported by Tshivhandekano et al. (2014) on wild harvested bush tea. They found that bush tea has antimicrobial activity in different zones (MIC range, 1.56–12.50 mg·mL⁻¹; MMC range, 0.78–12.50 mg·mL⁻¹), depending on the microbial species tested. In contrast, Thembo et al. (2010) reported that an MIC value greater than 1.6 mg·mL⁻¹ is

classified as weak. Therefore, the current study showed that, regardless of treatments being applied as early and late, bush tea leaves and twigs had weak antimicrobial activity on tested bacteria strains based on MIC and MMC values. In addition, the results are in contrast with Osuagwu and Edeoga (2010), who reported significantly increased antimicrobial activity of the leaves of *Ocimum gratissimum* and *Gongronema latifolium* through fertilizer treatment. Further to the weak antimicrobial activity, the current results are in contrast to the report on widespread uses of bush tea as treatment of infection, suggesting that it may have strong antimicrobial activity (Tshivhandekano et al., 2014).

Various compounds such as norfenfluramine, phytol, caryophyllene, propylene glycol, α-copaene, and squalene were detected in both early and late N applied bush tea. The compounds detected have different pharmaceutical properties. Norfenfluramine is generally used as an appetite depressant (Rothman et al., 2003), whereas phytol is an aromatic ingredient used in many fragrance compounds and it may be found in cosmetic and non-cosmetic products (McGinty et al., 2010). Caryophyllene has been found to demonstrate selective antibacterial activity against *S. aureus* (Dahham et al., 2015). Propylene glycol is a commonly used solvent for oral, intravenous, and topical pharmaceutical agents (Lim et al., 2014). α-Copaene is found in the inner bark essential oil of *Kielmeyera coriaca* Mart. and has remarkable antimicrobial activity against Gram-positive and Gram-negative bacteria (de Carla et al., 2015). Squalene is a naturally occurring polyprenyl compound known primarily for its key role as an intermediate in cholesterol synthesis. Its primary therapeutic use is as an adjunctive therapy in a variety of cancers (Kelly, 1999). The results are in support of the findings by Maedza et al. (2017), who reported that both qualitative and quantitative variation of bush tea chemical constituents were influenced by foliar application of different minerals. The results do not show any linear or quadratic increase of compounds based on the timing of N application or N application rates. Ibrahim et al. (2011) reported that application of N greater than 90 kg·ha⁻¹ reduced the level of secondary metabolites produced on *Labisia pumila*.

In conclusion, except for antimicrobial activity, all other parameters recorded increased with increasing rates of N from 0 to 225 kg·ha⁻¹ regardless of the timing of application. However, it was evident that early N application significantly improved most parameters recorded compared with late applied N. There were no significant differences between MIC and MMC values of bush tea leaves and twigs harvested from early and late applied N. The MIC values ranged between 3.1 and 12.5 mg·mL⁻¹ whereas MMC values ranged between 6.3 and 25 mg·mL⁻¹. The metabolite profiles using gas chromatography linked to mass spectrometry and liquid chromatography linked to mass spectrometry revealed variation in compounds found and their relative abundance

based on level of applied N and timing of N application. The major compounds found were norfenfluramine, phytol, caryophyllene, propylene glycol, α-copaene and squalene.

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