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 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 for both early and late N application. The results further denote that study suggest that, with the exception of antimicrobial activity, most of the parameters recorded were consistently greater on early applied N compared with late applied N. The MIC for Proteus vulgaris and Klebsiella pneumonia reached a maximum of 150 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 the soil contained 0.001 mg·L⁻¹ nitrate-N. 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.

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 Challan 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 (Baranauksiene 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.

Factorial treatments consisted of timed N application rates (0, 75, 150, and 225 kg·ha⁻¹) for both early and late N application rates, reaching a maximum at 225 kg·ha⁻¹ N. 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.

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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 used 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 Haenertsburg (lat. 23°56' S; long. 29°54' E; 890 m a.s.l.) in Limpopo Province, South Africa. Plants were propagated by cutting and used as described by Maudau et al. (2006). Bush tea cuttings 7 to 8 cm long were planted as described by Maudau 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 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 (lat. 25°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.

**Determination of total polyphenol content.** Flavonoid content was analyzed by colorimetric analysis (Mohammed and Manan, 2015). A mixture of 200 mL bush tea leaf and twig extracts was added to 150 mL sodium nitrite (5% w/v) and was incubated for 6 min at room temperature. After incubation, 150 mL aluminum chloride hexahydrate (10% w/v) was added and incubated for another 6 min at room temperature. The second incubation, 800 mL 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 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 was determined by the CEC (cmol·kg⁻¹) connected to a Sanplus Segmented Flow Analysis System (Skalar, Netherlands) and were expressed as percent N dry weight.
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 mg·mL⁻¹; whereas 2 mg vitamin C was dissolved in 1 mL ethanol to give a stock concentration of 2 mg·mL⁻¹ to serve as the positive control. A solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was prepared by dissolving 20 mg in 500 mL ethanol to yield a stock concentration of 0.04 mg·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 μg·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 μg·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 prepared by dissolving 20 mg in 500 mL ethanol to yield a stock concentration of 0.04 mg·mL⁻¹.

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 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 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.

**Thermoassay.** 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 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 mg·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 mg·mL⁻¹ p-iodonitrotetrazolium violet (Sigma-Alrich, South Africa) to the microplate wells which were then incubated at 37 °C for 2 h. After incubation, MMC was tested by adding 50 μL of the suspensions from the wells, which did not show any growth after incubation for MIC assays, to 150 mL fresh broth. These suspensions were reincubated at 37 °C for another 24 h. Last, 40 μL of 0.2 mg·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 × 10⁹ (Dey et al., 2010).

**XTT cytotoxicity assay.** The cytotoxicity of bush tea leaf and twig extracts was tested on human embryonic kidney 293 cells. One hundred microliters of human embryonic kidney cell suspensions (1 × 10⁵ 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)] was performed to account for the color of the DMSO. The positive control (acetylimidocid 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 Musaruwra 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 mL·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·min⁻¹, and a second ramp of 1 °C·min⁻¹ up to 240 °C. A mass scanning range of 40 to 550 m/z (perfluorotrimethylene 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 alkanic 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 kg·ha⁻¹, bush

| Applied N (kg·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 c        | 60 c       |
| 150                 | 47.23 b                  | 165 b        | 103 a      |
| 225                 | 50.03 a                  | 170 a        | 108 a      |
| Late application    |                          |              |            |
| 75                  | 39.96 c                  | 105 c        | 42 c       |
| 150                 | 45.27 c                  | 130 d        | 55 cd      |
| 225                 | 47.37 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, dry weight, polyphenol content, flavonoid content, tannin content, 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 N 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.

| Component          | 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 (IC₅₀) |
|--------------------|-------------------|---------------------------|--------------|------------|-------------------------------------|-----------------------------------|-------------------------------|----------------------------------|
| Chlorophyll        | 0.96**            | 0.97**                    |              |            |                                     |                                   |                               | 0.86**                           |
| Fresh weight       | 0.91**            | 0.97**                    |              |            |                                     |                                   |                               | 0.69**                           |
| Dry mass           | 0.85**            | 0.94**                    | 0.90**       |            |                                     |                                   |                               | 0.88**                           |
| Polyphenols        | 0.94**            | 0.99**                    | 0.93**       | 0.88**     |                                     |                                   |                               | 0.98**                           |
| Flavonoids         | 0.99**            | 0.98**                    | 0.93**       | 0.98**     |                                     |                                   |                               | 0.98**                           |
| Tannins            | 0.78*             | 0.62*                     | 0.67*        | 0.60*      | 0.53                                | 0.68*                             |                               | 0.88**                           |
| Antioxidant        | 0.59              | 0.52                      | 0.67*        | 0.66*      | 0.38                                | 0.50                              | 0.93                           | 0.86**                           |
| Cytotoxicity       | 0.97**            | 0.86**                    | 0.81**       | 0.72*      | 0.84**                              | 0.93                              | 0.61*                          | 0.61**                           |

Significant at *P ≤ 0.05 or **P = 0.01.

GAE = gallic acid equivalent; QE = quercetin equivalent.

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

| Component          | 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 (IC₅₀) |
|--------------------|-------------------|---------------------------|--------------|------------|-------------------------------------|-----------------------------------|-------------------------------|----------------------------------|
| Chlorophyll        | 0.96**            | 0.99**                    |              |            |                                     |                                   |                               |                                  |
| Fresh weight       | 0.95**            | 0.99**                    |              |            |                                     |                                   |                               |                                  |
| Dry mass           | 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 ≤ 0.05 or **P = 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 (IC₅₀) |
|---------------------|-------------------|-------------------------------------|-----------------------------------|-------------------------------|----------------------------------|
| Early application    |                   |                                     |                                   |                               |                                  |
| 0                   | 1.67 ± 0.24 d     | 50.93 ± 0.12 c                      | 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 e                     | 1.79 ± 0.0 a                   | 51.25 ± 1.88 e                   |
| 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 c                      | 0.74 ± 0.22 e                     | 1.42 ± 0.01 c                  | 37.71 ± 2.05 c                   |
| 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: 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.

**Polphenol 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 N 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
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, 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 are presented in Table 6. The results suggest that as N level increased from 0 to 225 kg·ha⁻¹, both early and late applied N 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 polyphenol content, total flavonoid content and cytotoxicity (Table 5).

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

| Applied N (kg·ha⁻¹) | IC₅₀ (µg·mL⁻¹) | R² |
|---------------------|----------------|----|
| 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%

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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 (µg·mL⁻¹) |
|---------------------|-----------------------------------------------|
|                     | Escherichia coli | Klebsiella oxytoca | Klebsiella pneumoniae | Proteus vulgaris | Salmonella typhi | Serratia marcescens | Staphylococcus aureus |
| 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                 | 12.5 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 b                |
| P value             | 0.0001          | 0.0001             | 0.0001                | 0.0001          | 0.0001           | 0.0001              | 0.0001                |

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

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

| Applied N (kg·ha⁻¹) | Minimum microbicidal concn of bush tea (µg·mL⁻¹) |
|---------------------|-----------------------------------------------|
|                     | Escherichia coli | Klebsiella oxytoca | Klebsiella pneumoniae | Proteus vulgaris | Salmonella typhi | Serratia marcescens | Staphylococcus aureus |
| 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 and 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
reported that as N levels increased in an ascending order of 0 > 90 > 180 > 270 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 < 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, Table 10. Compounds detected and their abundant concentrations on bush tea leaf and twig in response to applied N rates.

| Compounds                      | Early applied N | Late applied N |
|-------------------------------|-----------------|----------------|
|                               | Conc (kg·ha⁻¹)  | Conc (kg·ha⁻¹) |
|                               | 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         |
| 1,6-Octatriene, 3,7-dimethyl-, (Z)- | 41.54 28.79 0 0 26.58 | 0 0 0     |
| 1H-Pyrole-2,5-dione, 3-ethyl-4-methyl- | 0 28.50 0 0 22.88 | 0 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 0 | 0 0 0          |
| α-Cadinol                    | 0 0 0 0 | 0 0 0         |
| Aceto phenone                 | 10.66 24.48 0 37.95 | 13.69 87.89 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    |
| Butanoic acid                 | 28.14 0 0 51.03 | 77.46 0      |
| 1,2,3-Propanetriol, 1-acetate | 10.46 36.34** 0 0 | 35.28** 0 28.71* |
| t-Occimene                    | 15.00 48.55 0 49.46 | 0 0 0       |
| 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 |
| Nitrous acid                  | 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 0 1.672 | 0 0 0        |
| dl-α-Tocopherol               | 0 0 0 12.19 | 20.49 0 0     |
| D-Limonene                    | 60.45 70.11 0 11.15 | 63.25 22.23 0 |
| Eicosane                      | 26.14 22.27 0 13.06 | 72.14 65.70 0 |
| Ethane, nitro-                | 12.31 56.59 0 0 | 29.60 0 0     |
| Ethyl acetocetate             | 13.93 24.35 0 60.51 | 39.52 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 0 28.93 | 0 0 0        |
| 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-Isositol                    | 0 0 0 19.93 | 45.56 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 0 |
| 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 | 0 0 54.26 |
| Phenol                        | 30.57 0 89.29 89.78 | 84.59 10.39 0 |
| Phthlic 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 | 0 0 0        |
| Terpineol                     | 0 46.76 58.09 0 0 | 0 0 0       |
| Tetracosane                   | 0 0 70.79 0 0 | 0 0 15.14 27.46 |
| Tetra decanoic acid           | 0 0 15.23 77.18 | 0 13.86 19.15 |
| Tetraethyl silicate           | 0 0 0 0 | 0 0 24.16 19.96 |
| Undecane                      | 0 0 0 0 | 54.68 83.64 0 |
| 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.
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 (Tshihvandekano et al., 2014).

Various compounds such as norfenfluramine, phytol, caryophyllene, propylene glycol, *α*-c copeaene, 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 (McCinty 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 (Linn et al., 2014). *α*-C copeaene is found in the inner bark essential oil of *Kielmeyera coriacea* Mart and has remarkable antimicrobial activity against Gram-positive and Gram-negative bacteria (de Carla et al., 2015). Squalene is a naturally occurring polyphenol 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 N and timing of application. Ibrahim et al. (2011) reported that application of N greater than 90 kg·ha⁻¹ reduced the level of secondary metabolites produced on *Labiis 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*-c copeaene and squalene.

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