Antimicrobial Activity, Total Phenolic and Ascorbic Acid Content of *Terminalia ferdinandiana* Leaves at Various Stages Of Maturity

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Abstract
This work investigated the effect of leaf development (maturity) on morphology, antimicrobial activity, total phenolic (TPC) and ascorbic acid content in leaves of *Terminalia ferdinandiana*, an endemic plant of Australia. The results of this study indicated that total ascorbic acid was in the range of 23.0 to 35.5 mg/100 g dry weight (DW), showing an increase with advance of maturity. TPC in water and methanolic extracts were in the range of 237.3 - 598.6 and 210.3 - 319.6 mg Gallic acid equivalent (GAE)/ g DW, respectively. Leaf extracts exhibited pronounced inhibitory activity towards *Staphylococcus aureus* where total ascorbic acid and TPC were positively correlated with the observed antimicrobial activity. These results indicated that leaves extracts might be used as an alternative to synthetic antimicrobial agents, with a great potential for application as an environmentally friendly sanitizer in the hospitality and healthcare industries.

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Introduction

*Terminalia ferdinandiana* (Exell, Combretaceae), is an endemic plant of Australia, with edible fruits that are extremely rich in antioxidant compounds.\(^1\)\(^-\)\(^6\) Like its counter parts from the genus *Terminalia*, this plant has a rich history of being utilised as a traditional medicine by the Australian aboriginal communities. Fruits of this plant have been used to prepare various ailments for the cure of headaches, to alleviating symptoms of colds and flu and as an antiseptic.\(^1\)\(^,\)\(^8\)

This plant has been also used for its medicinal properties in the same way as *T. carpentariae*, another Australia native *Terminalia* species.\(^9\)

Recent studies have reported on a number of bioactive properties of *T. ferdinandiana* which support many of the traditional medicinal claims of this plant by the Australian Aboriginal communities.\(^10\)\(^-\)\(^12\) Some of these reports also indicated that polar solvent extracts from *T. ferdinandiana* fruit were effective in inhibiting both Gram-positive and Gram-negative bacteria.\(^10\)\(^-\)\(^12\)

Protective effects of *T. ferdinandiana* fruit extracts on oxidative stress and inflammatory pathways have been also reported by other authors.\(^13\)\(^-\)\(^14\) The potent biological activity observed in *T. ferdinandiana* attributed to the presence of enhanced levels of antioxidant bioactive compounds.\(^15\)\(^-\)\(^16\) Some of the bioactive phytochemicals detected in *T. ferdinandiana* fruit and leaves include ascorbic acid, ellagic acid, gallic acid, \(\alpha\)-tocopherol, ethyl gallate, chebulic acid, corilagin, hydroxycinnamic acid, lutein, tannins, chebulagic acid, exifone, punicalin, castalagin, appanone A-7 methyl ether, xanthotoxin, phthalane, saponins, flavonoids, and terpenes.\(^1\)\(^-\)\(^19\) Elevated antioxidant activity was confirmed by early studies by Netzel and collaborators (2007) who measured the antiradical properties by the TROLOX Equivalent Antioxidant Capacity (TEAC) assay.\(^15\)

The antioxidant capacity of 567 T ferdinandiana fruit from 45 geographic sites was also reported demonstrating the important antioxidant activity of this plant.\(^2\)\(^,\)\(^20\)\(^-\)\(^21\) Recently, TPC of methanolic extracts of *T. ferdinandiana* fruits and leaf (obtained by accelerated solvent extraction) have been reported to be 12.2 and 11.7 g GAE/100 g DW.\(^22\)

One of the most prominent antioxidant phytochemicals present in *T. ferdinandiana* is ascorbic acid or vitamin C, which is essential for human health. Due to its strong antioxidant properties, ascorbic acid neutralizes reactive oxygen species, prevents the generation of new free radicals by suppressing relevant molecular pathway and assists in the recycling of other antioxidants.\(^20\)\(^-\)\(^21\) Vitamin C also plays an important immunomodulatory functions such as regulation of macrophage activity, reduction of inflammatory mediators, and imparting direct bacteriostatic effect at high concentrations.\(^22\)

High levels of ascorbic acid (>14% DW) have been reported in *T. ferdinandiana* fruits.\(^5\)\(^,\)\(^6\) Other studies have also reported a wide range of ascorbic acid levels in *T. ferdinandiana* fruits (3.5 - 5.9% FW) and in the range of 0.1 - 5.3% FW.\(^1\)\(^4\)\(^,\)\(^5\)\(^2\)\(^3\) Levels of ascorbic acid in the *T. ferdinandiana* fruit were observed at significantly higher concentrations than other well-known natural sources of ascorbic acid like citrus fruit (0.5% FW ascorbic acid),\(^2\)\(^4\) Acerola fruit (1.0 and 1.4% FW)\(^2\)\(^4\)\(^-\)\(^2\)\(^5\) and Camu-Camu (1.8% FW).\(^2\)\(^4\)\(^-\)\(^2\)\(^5\)

As the consumer awareness on the health promoting activities of the *T. ferdinandiana* fruit and its products, increasing demands for new applications are driving food industry to find novel applications of *T. ferdinandiana* as a functional food ingredient. Currently, only frozen puree and freeze-dried powder of *T. ferdinandiana* fruit are commercially available; however, anecdotal evidences and recent studies have indicated that other tissues such as leaves, seed coats and kernels could also be used for food and other applications.\(^22\)\(^,\)\(^26\) The leaves of *T. ferdinandiana* could be a great candidate for such novel applications. Anecdotally, there are accounts of using broken *T. ferdinandiana* leaves for cleaning by scrubbing hands within the Indigenous Australians indicating potential antimicrobial efficacy of the leaves. A recent study, evaluated the antimicrobial activity of extracts were prepared from different *T. ferdinandiana* tissues including fruits, leaves, seedcoats, and barks. It was observed that both fruit and leaf extract exhibited superior antimicrobial activity, against common foodborne bacteria.\(^22\)\(^,\)\(^26\)

As *T. ferdinandiana* is a semi-deciduous tree, it drops its leaves in the dry season, and spouts new leaves at the beginning of the wet season. The characteristic morphology of the leaves should make them easier to harvest than fruit. Leaves of *Terminalia* plants are usually spirally organised (often crowded) at the
ends of the branches, sometimes on short shoots. In fact, the genus name ‘Terminalia’ comes from the Latin word ‘Terminus’ relating to the fact that the leaves are located at the very tips of the shoots. Mature *T. ferdinandiana* leaves are usually large in size, making it possible to collect larger volume of raw material. Although several studies have shown the bioactive potentials of *T. ferdinandiana* leaves, information on the effect of maturity of leaves on their bioactive compounds or bioactivity is not available.

The objective of this work was to provide with information on morphology, antimicrobial activity, total phenolic and ascorbic acid content in *T. ferdinandiana* leaves over different stages of maturity.

**Materials and Methods**

**Plant Material**

Fieldwork for this study was conducted in undeveloped bushland in the northwestern corner of the Charles Darwin University, Casuarina campus (Darwin, Northern Territory, Australia) (see Figure 1). The study site is within one kilometre of the coastal shoreline, comprising open savanna woodland with incipient monsoon forest along old streamlines and is representative of *T. ferdinandiana* habitat. The mean annual rainfall from 1995 to 2020 was 1768 mm and mean daily temperatures ranged from a minimum of 23.2 to a maximum of 32.1°C (Bureau of Meteorology 2020).

Leaf samples were harvested from November 2017 to June 2018. A total of 15 different maturity stages of leaves (10 leaves per stage) were collected from 10 individual trees (AT1 to AT10). Stage 1 to 4 contain immature leaves; stage 5 to 10 contain mature leaves and stage 11 to 15 contain senescing leaves. Leaves of stages 12 to 15 were collected from fewer than 10 individual trees as some of the trees had dropped their leaves ahead of others. Details of the harvesting time are provided in Table 1. For the current study, 5 maturity stages, were selected from 3 individual trees (10 leaves per stage per tree) for analysis. The selected maturity stages for the current study allowed us to look at leaves at immature stage (e.g. stages 2 and 3), mature stage (e.g. stage 6 and 10) and senescing stage (e.g. stage 15) (see Figure 2).

**Morphological Parameters**

Length and width of 10 individual leaves of each maturity stage from three individual trees were measured using adigital calliper (Craftright Engineering Works, Jiangsu, China) followed by measuring the weight on laboratory scales (Sartorius CP224 Sanalytical balance with readability precision 0.01g, Gottingen, Germany). The leaves were frozen at -80°C and then freeze dried at -50°C for 48h.
(CSK Climatek, Darra, QLD, Australia). After freeze drying, around 2 g of dry leaves were ground using a Retsch MM400 ball mill (Retsch GmbH, Haan, Germany) at a speed of 30 Hz for 1 min.

Table 1: Time of collection of *Terminalia ferdinandiana* leaf samples

| Maturity stages          | Time of collection |
|--------------------------|--------------------|
| Immature leaves*         | 1 14 Nov 2017      |
|                          | 2 14 Nov 2017      |
|                          | 3 14 Nov 2017      |
|                          | 4 14 Nov 2017      |
| Mature leaves            | 5 9 Jan 2018       |
|                          | 6 14 Feb 2018      |
|                          | 7 12 Mar 2018      |
|                          | 8 14 Apr 2018      |
|                          | 9 14 May 2018      |
| Senescing leaves         | 10 21 May 2018     |
|                          | 11 30 May 2018     |
|                          | 12** 6 Jun 2018    |
|                          | 13*** 13 Jun 2018  |
|                          | 14*** 21 Jun 2018  |
|                          | 15*** 26 Jun 2018  |

*These samples were collected on the same time point as the newly sprouted leaves of stage 1. Leaves were divided into stage 2, 3 and 4 on the increase in sizes.

**Leaves from stage 12 were collected from 6 individual trees (10 leaves per tree).

***Leaves from stage 13, 14 and 15 were collected from 4 individual trees (10 leaves per tree).

Fig. 2: The selected maturity stages of the current study. Stages 2 and 3 represent the immature leaves, stages 6 and 10 represent the mature leaves and stage 15 represent senescing leaves

Extraction of Bioactive Constituents

Bioactive constituents from *T. ferdinandiana* leaves were extracted by either methanol or deionized water. Triplicate samples (approx. 0.5 g) of the freeze-dried powders were accurately weighed into separate centrifuge tubes and individually blended with 5 ml of each solvent. After 30 seconds of vortex-mixing, the tubes were sonicated in an ultrasonic bath (Elma
Schmidbauer GmbH, Ruiselede, Belgium) for 5 min at room temperature, followed by another 5 min of gentle agitation. The slurry was subsequently centrifuged at 3900 rpm for 5 min using an Eppendorf 5180 centrifuge (Eppendorf, Hamburg, Germany). The supernatant was carefully transferred and collected while the residues extracted twice again. After 3 times of extractions, a total volume of 15 ml of the crude extracts were combined and evaporated in a rotatory evaporator (Genevac Ltd, Ipswich, Suffolk, England) at 40°C. The dried extracts were freshly reconstituted in 5 ml of aqueous 20% v/v methanol (for methanol extracts) or deionized water (for water extracts) prior to analysis of antimicrobial activities. The reconstitute extract was stored at biomedical freezer (MDF U5312, PHCbi, Panasonic) and daily monitored using a digital thermometer.

Supplementary 2: Representative photos show (B) the inhibition of methanol extracts and (A) antibiotic solution (10 µl of Penicillin and streptomycin at 1g each/10 mL methanol) against *Staphylococcus aureus* and negative control 20% methanol.

**Antimicrobial screening**

A total of three pathogenic microbial strains were tested in this study to evaluate the antimicrobial activity of *T. ferdinandiana* leaf extracts: *Staphylococcus aureus* NTCC 6571, a Gram-positive bacteria; *Escherichia coli* NTCC 9001, a Gram-negative bacteria and Candida albicans ATCC 90028 a fungi. The bacterial strains were purchased from American Type Culture Collection, USA or National Collection of Type Cultures (NCTC), UK. Well diffusion assay was used to evaluate the antimicrobial activity of the leaf extracts followed the method published by Phan and collaborators (2019). The inhibition zone was measured using a 150 mm Digital Calliper (Craftright Engineering Works, Jiangsu, China). MIC will be suggested for further investigations using a widen ranges of microorganisms. All plates were incubated overnight in triplicate (see Supplementary material S2).

**Total Phenolic Content (TPC)**

The total phenolic content (TPC) of the samples was determined using the Folin Ciocalteau (FC) method using a micro-plate reader (Sunrise Tecan, Maennedorf, Switzerland). Gallic acid standards ranging from 21 to 105 mg/L were prepared to establish the standard curve for quantification of TPC in the extract. TPC was expressed as mg gallic acid equivalents per gram of sample in dry weight (mg GAE/g DW).

**Extraction and Analysis of Ascorbic Acid**

Measurement of vitamin C content in *T. ferdinandiana* leaf extracts was conducted by utilizing ultra-high-performance liquid chromatographic–photodiode array (UHPLC-PDA) methodology (See chromatogram S1).

**Data Analysis**

Descriptive statistics (average, minimum and maximum values, and standard deviation), principal component analysis (PCA) and multiple linear regression (MLR) were applied to inspect the relevant and interpretable structure in the data set associated with the variables measured in the leaf samples at the different maturities. The optimum number of components in both PCA and MLR analysis was determined internal cross validation.
The Unscrambler software (version 10.5, CAMO, Norway) was used to develop the PCA and MLR regression models. Samples were standardised using the pre-processing $1/SD$.\(^{31}\)

### Supplementary 1: UHPLC-PDA acquired at 245 nm of (A) ascorbic acid standard and (B) the leaf extract

### Table 2: Morphological data for *Terminalia ferdinandiana* leaves at various stages of maturity

| Tree ID | Maturity stage | Length (mm) | Width (mm) | Weight (g) |
|---------|----------------|-------------|------------|------------|
| AT2     | 2              | 58.7± 6.5   | 36.8± 5.1  | 0.4 ± 0.1  |
|         | 3              | 112.5± 8.6  | 95.5± 10.9 | 1.9 ± 0.3  |
|         | 6              | 159.6± 16.5 | 123.4± 9.6 | 4.0 ± 0.9  |
|         | 10             | 149.5± 18.0 | 122.6± 15.8| 3.4 ± 0.6  |
|         | 15             | 124.8± 30.0 | 106.3± 16.4| 2.4 ± 0.9  |
| AT5     | 2              | 80.5± 7.7   | 50.9± 7.1  | 0.8 ± 0.2  |
|         | 3              | 133.4± 20.4 | 98.9± 18.1 | 2.5 ± 0.8  |
|         | 6              | 184.6± 17.1 | 126.3± 16.1| 5.1 ± 0.8  |
|         | 10             | 162.4± 24.1 | 118.1± 27.7| 3.6 ± 1.1  |
|         | 15             | 189.4± 28.4 | 123.9± 17.1| 4.1 ± 1.1  |
| AT9     | 2              | 73± 19.9    | 46.2± 12.2 | 1.2 ± 0.8  |
|         | 3              | 138.1± 25.5 | 91.4± 15.8 | 2.5 ± 0.9  |
|         | 6              | 187± 23.8   | 136.1± 14.8| 5.0 ± 1.0  |
|         | 10             | 168.9± 48.4 | 119.7± 21.5| 3.6 ± 1.7  |
|         | 15             | 135.3± 17.7 | 109.9± 19.0| 3.6 ± 1.3  |

Results are mean ± SD ($n = 10$)

AT indicate the tree ID of the individual trees collected from the study site at Charles Darwin University, Darwin, Northern Territory, Australia.

### Results and Discussion

The current study analysed *T. ferdinandiana* leaves at 5 maturity stages namely immature leaves at stages 2 and 3; mature leaves at stages 6 and 10, and senescing leaves at stage 15. Leaves from stages 1 to 4 were collected at the same time as
the freshly sprouted leaves (e.g. stage 1). These immature leaves were then divided into 4 stages according to their sizes. From stages 5 to 15, leaves were collected at different time points and grouped according to their stage of development/maturity (Table 1).

Morphological analysis showed that at maturity stage 2, irrespective of the tree, leaves had the smallest length and width, both of which showed a gradual increase in the leaves of stage 3. However, leaves at maturity stages 6, 10 and 15 did not have a clear trend and showed high variability in length, width and weight (Table 2). Tree-to-tree variability has been also observed in *T. ferdinandiana* fruits. The high degree of variability observed in the leaves and fruits could be attributed to the fact *T. ferdinandiana* is a wild harvested plant and often undergoes wide cross-pollination. Interestingly, the variability of the morphological parameters seemed to decrease with the maturity stage of the leaves which is indicated by a lower coefficient of variation (% CV) in the morphological parameters measured (Table 5).

| Tree ID | Maturity stage | Total ascorbic acid (mg/100 g DW) | Total polyphenol content (mg GAE/g DW) |
|---------|----------------|-------------------------------------|----------------------------------------|
|         |                | Methanolic extracts | Water extracts  | Methanolic extracts | Water extracts |
| AT2     | 2              | ND         | 533.4 ± 1.5       | 263.1 ± 0.8 |
|         | 3              | ND         | 598.6 ± 1.4       | 301.9 ± 1.3 |
|         | 6              | 23.0 ± 0.1 | 279.8 ± 2.6       | 253.9 ± 2.2 |
|         | 10             | 29.6 ± 0.5 | 384.9 ± 2.4       | 274.9 ± 0.5 |
|         | 15             | 34.8 ± 0.3 | 407.5 ± 1.9       | 295.9 ± 0.6 |
| AT5     | 2              | ND         | 294.8 ± 1.5       | 222.2 ± 0.5 |
|         | 3              | ND         | 330.3 ± 0.8       | 231.8 ± 0.6 |
|         | 6              | 32.3 ± 0.8 | 237.3 ± 1.6       | 210.3 ± 2.1 |
|         | 10             | 34.9 ± 0.3 | 283.9 ± 0.9       | 220.3 ± 2.5 |
|         | 15             | 35.5 ± 0.1 | 247.5 ± 2.1       | 215.6 ± 0.4 |
| AT9     | 2              | ND         | 388.7 ± 2.3       | 276.2 ± 0.3 |
|         | 3              | ND         | 579.6 ± 0.3       | 319.6 ± 0.2 |
|         | 6              | 22.5 ± 0.4 | 359.8 ± 1.0       | 258.4 ± 0.2 |
|         | 10             | 32.6 ± 0.5 | 323.8 ± 0.9       | 259.0 ± 0.4 |
|         | 15             | 34.7 ± 0.7 | 379.9 ± 0.7       | 283.2 ± 0.5 |

Results are mean ± SD (n = 3)

The ascorbic acid levels determined in leaves at maturity stage 6, 10 and 15 were between the ranges of 22.5 to 34.8 mg/100 g DW (Table 3) whereas no peak of ascorbic acid was observed in the UHPLC-PDA chromatogram for leaves sourced from stages 2 and 3, respectively. This data suggested that levels of ascorbic acid in these immature leaves might be present either at a relatively low concentration or lower than the limit of detection (LOD = 0.1 parts per million) of the method used for the analysis. The lower levels of ascorbic acid in *T. ferdinandiana* leaves compared to fruits have been previously reported by other authors. It is well known that ascorbic acid is an important bioactive compound having important roles in the plant (e.g. redox reactions, cofactor of enzymes, photosynthesis, hormone biosynthesis, antioxidant function). Variation in the ascorbic acid content has been reported among different tissues and organs in the same tree by other authors. High concentration of ascorbic acid was found in tissues such as leaves and flowers compared with those photosynthetically
active such as stems and roots where higher concentrations are present in the meristematic tissues and reproductive organs (e.g. flowers, young fruits). It has been also reported that the concentration of ascorbic acid might be affected by the environment and developmental stages of the organ (i.e. mature vs immature fruits). These factors might explain the observed variations in ascorbic acid in the leaf samples analysed.

The total phenolic content (TPC) of the leaf extracts ranged from 210.3 to 598.6 mg/g DW. The TPC values varied from 237.3 to 598.6 mg GAE/g DW in the methanolic extracts and from 210.3 to 319.6 mg GAE/g DW in the water extracts. TPC was higher in the leaf methanolic extracts compared to the water extracts. Similar results were reported by Akter and collaborators (2019). TPC showed a gradual decrease with the advance of maturity, with the highest levels observed in leaves at stage 3 irrespective of the tree in both the methanolic and water extracts (Table 3).

**Table 4: Inhibition zones (mm) of methanol and water extracts against *S. aureus.***

| Tree ID | Maturity stage | Inhibition zones (mm) | Methanolic extracts | Water extracts |
|---------|----------------|-----------------------|---------------------|----------------|
| AT2     | 2              | 23.9 ± 0.8            | 21.8 ± 0.3          |
| 3       | 27.5 ± 0.5     | 24.9 ± 0.9            |                     |
| 6       | 24.9 ± 0.9     | 23.1 ± 0.9            |                     |
| 10      | 26.3 ± 0.3     | 24.2 ± 0.6            |                     |
| 15      | 27.2 ± 0.6     | 25.5 ± 0.4            |                     |
| AT5     | 2              | 23.6 ± 1.5            | 21.8 ± 1.3          |
| 3       | 29.1 ± 0.4     | 23.3 ± 0.6            |                     |
| 6       | 24.4 ± 0.4     | 22.6 ± 0.7            |                     |
| 10      | 26.1 ± 0.1     | 23.8 ± 0.4            |                     |
| 15      | 27.7 ± 0.2     | 25.3 ± 0.4            |                     |
| AT9     | 2              | 24.7 ± 0.4            | 21.2 ± 0.7          |
| 3       | 28.3 ± 1.0     | 24.9 ± 0.8            |                     |
| 6       | 25.7 ± 0.4     | 23.6 ± 0.7            |                     |
| 10      | 26.6 ± 0.9     | 24.2 ± 0.7            |                     |
| 15      | 27.2 ± 0.7     | 26.7 ± 0.9            |                     |
| Antibacterial control* | 53.3 ± 0.2 | 52.8 ± 0.31 |
| Solvent control** | - | - | |

Results are mean ± SD (n = 3)

*1 µg penicillin and streptomycin was used as antibacterial control.
**Solvent controls included 20% (v/v) aqueous methanol for methanolic extracts and water for water extracts.

The antimicrobial analysis showed that the methanolic and water extracts of *T. ferdinandiana* leaves have strong inhibitory efficacy (e.g. inhibition zone > 13 mm) against the Gram-positive *S. aureus*, but no inhibition against the Gram-negative *E. coli* and *C. albicans* (Table 4). The antimicrobial efficacy in terms of inhibitory zones were in the range of 23.6 to 29.1 mm and 21.2 to 26.7 mm for methanolic and water extracts, respectively. A recent study has also reported that methanolic and aqueous
leaf extracts of *T. ferdinandiana* were good inhibitors of the Gram positive *Bacillus anthracis*, the etiological agent of anthrax.\textsuperscript{19} Akter and collaborators (2019) observed that the methanolic and water extracts of *T. ferdinandiana* leaves inhibited both Gram positive and negative bacteria.\textsuperscript{26} The observed difference in the antimicrobial efficacy of *T. ferdinandiana* leaf extracts might be associated with the difference in the type of bioactive compounds extracted and their extent of release from the sample matrix as a result of the extraction method used and geographical location of the studied plant material.\textsuperscript{38-39}

Pearson correlation showed strong positive correlation between levels of ascorbic acid and antimicrobial inhibition exhibited by methanolic (Pearson r = 0.60) and water (Pearson r = 0.55) extracts (Table 5). This relationship indicated that ascorbic acid might be a contributing factor, in the observed antimicrobial activity of *T. ferdinandiana* leaf extracts. In fact, a number of studies have reported the antimicrobial efficacy of ascorbic acid against a number of pathogenic bacteria including *S. aureus*, *E. coli*, *Helicobacter pylori*, *Campylobacter jejuni* and *Mycobacterium tuberculosis*.\textsuperscript{40-42}

The TPC of methanolic and water extracts were negatively correlated to total ascorbic acid content (Pearson r = -0.047 and -0.11 respectively). This could be explained by the fact that ascorbic acid may not be the primary contributor to TPC of *T. ferdinandiana*.\textsuperscript{43} As mentioned before, *T. ferdinandiana* fruits and leaves are also rich sources of bioactive antioxidant compounds other than ascorbic acid, such as ellagic acid, gallic acid and \( \alpha \)-tocopherol.\textsuperscript{1-6} Significant positive correlation was observed between the morphological parameters, TPC and antimicrobial inhibition, indicating that with maturity the level of phenolics and antimicrobial efficacy increases (Table 5).

The data was also analysed by principal component analysis (PCA). This method transforms a group of highly correlated variables into new data sets called principal components (PC). Then, data was interpreted using the scores and loadings. This algorithm of PCA reduces the dimensionality of the data but retains most of the variation in the data set, which increases interpretability simultaneously minimizing loss of information.\textsuperscript{31} It was observed a variation between samples obtained from different trees (Figure 3A) and maturity stages (Figure 3B), indicating the existence of natural variability between the trees. The PCA loadings allowed to interpret which variables might influence the separation between leaves (Figure 4). Most of the differences observed between the samples were found to be explained by the morphological parameters, level of ascorbic acid and antimicrobial efficacy. Further more, loadings in PC1 explained the differences in maturity stages where high and positive loadings corresponded well with all chemical variables such as ascorbic acid and TPC.

### Table 5: Descriptive statistics of the variables measured in the leaf samples at different maturity stages

|                          | Mean  | SD   | CV (%) |
|--------------------------|-------|------|--------|
| Length (mm)              | 137.2 | 45.8 | 33.3   |
| Width (mm)               | 100.4 | 34.1 | 34     |
| Weight (g)               | 2.9   | 1.6  | 55     |
| Total ascorbic (mg/100 g dry weight) | 31.1  | 4.8  | 15.5   |
| TPC methanolic extracts (mg GAE/g DW) | 375   | 11   | 2.9    |
| TPC water extracts (mg GAE/g DW) | 259   | 3.2  | 1.2    |
| Inhibition methanolic extracts | 26.2  | 1.6  | 6      |
| Inhibition water extracts | 23.7  | 1.4  | 6      |
Fig. 3: Principal component analysis (PCA) (A) individual trees and (B) maturity stages of the leaves. The symbols indicate individual subjects.

Fig. 4: Loading plots of principal components. Blue, red and green indicate PC-1, 2 and 3, respectively. Variables include: length, width and weight of leaves, TPC and antimicrobial activity of methanolic (MeOH) extracts, TPC and antimicrobial activity of water extracts.

Fig. 5: Multiple linear regression results. Coefficients of regression derived from the multiple linear regression model for length, width and weight of leaves, total ascorbic acid content, TPC and antimicrobial activity of methanolic (MeOH) extracts, TPC and antimicrobial activity of water extracts.

Multiple linear regression (MLR) analysis indicated that 97% of the variance in maturity of *T. ferdinandiana* leaves could be explained by the model reported in Figure 5A. The regression coefficients showed that ascorbic acid had a large influence in explaining maturity stages of the leaves, followed by both antimicrobial efficacy and TPC of the water extracts (Figure 5B). This indicates that in addition to the
presence of bioactive compounds (e.g. ascorbic acid) other parameters such as antimicrobial activity and TPC might also vary with leaf maturity. Therefore, the concentration of ascorbic acid and TPC might be used as biomarkers to monitor leaf maturity in *T. ferdinandiana*.

**Conclusion**

For the first time, information on morphology, antimicrobial activity, total phenolic and ascorbic acid content in *Terminalia ferdinandiana* leaves was reported. Variation in the morphological parameters, TPC and ascorbic acid content with advancement of maturity was observed in the samples analysed. Both PCA and MLR analyses indicated that effect of individual trees and maturity stages, where the concentration of ascorbic acid explains the variability in maturity among leaves. However, the data in this study was not based on a large number of biological samples, and hence, is not sufficient to describe the effect of maturity on nutritional composition of *T. ferdinandiana* leaves. The use of statistical techniques such as PCA and MLR regression, allowed us to obtain additional information from the data set allowing for a better interpretation of the differences associated with maturity. Results from this study also indicated the pronounced inhibitory effect of *T. ferdinandiana* against *S. aureus*. Currently only the *T. ferdinandiana* fruit (as a freeze-dried powder and puree) is commercially available as a functional food ingredient, whereas, leaves or any other tissues, are not used for any industry applications. Leaf extracts showed promise as antimicrobial agent, suggesting that might be used as alternative to synthetic antimicrobial agents. Further studies will be recommended on *T. ferdinandiana* leaves, adding a large number of biological samples, trees and maturities, bioactive compounds, and anti-nutritional compounds.

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**Conflict of Interest**

The authors do not have any conflict of interest.

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