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Enhanced nutritional and phytochemical profiles of selected underutilized fruits, vegetables, and legumes

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Underutilized crops with enhanced nutrition and health value could be reintroduced to the diet as a promising intervention to meet global food and nutrition challenges. Considering the alarming rise in noncommunicable diseases and persistent poverty, dietary choices must be re-evaluated. Underutilized crops contribute to a nutrient-dense, economically feasible, and sustainable diets and can be included as functional ingredients for the nutritional enrichment of processed foods. This review aims to provide a critical analysis of nutritional and phytochemical analysis of selected underutilized fruits, vegetables, and legumes. The following underutilized fruits (Kakadu plum and Natal plum), vegetables (Moringa and Nightshade), and legume (wattle seeds) have been selected based on popularity, environmental importance, cultural value, nutrient and phytochemical content, and economic potential.

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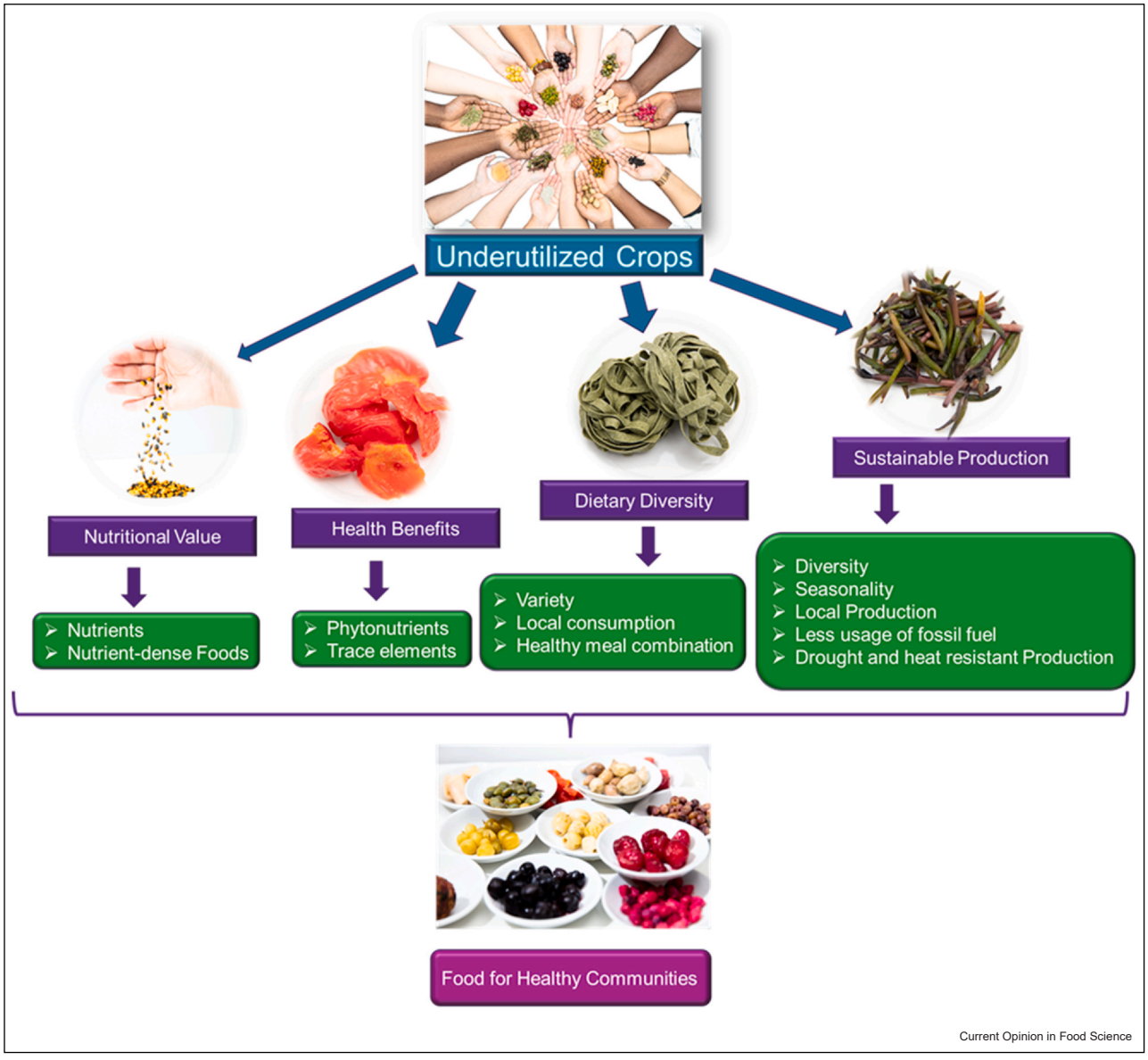
Introduction

Food and nutrition security are two challenges that are faced by an increasing global population estimated to reach 9.7 billion by 2050. The COVID-19 pandemic has exacerbated this situation, starting from 2020, the number of people facing hunger increased and was between 720 and 811 million [1]. An inability to access adequate, healthy, and nutritious food constitutes food insecurity. Food insecurity has a hidden component known as micronutrient deficiencies, which are also critical factors in food insecurity [2]. Whether it is a developed or developing country, the triple burden of malnutrition, undernourishment, micronutrient deficiency, and overnutrition is affecting different strata of society. Research has shown that underutilized food crops reduce food insecurity [3] and can mitigate malnutrition and 'hidden hunger'. Deficiencies of micronutrients, especially iron and zinc, are nutritional problems that affect over 2 billion people globally [4]. Underutilized species are often described in agriculture literature as 'forgotten', 'neglected', and orphaned [5•]. Many underutilized fruits are rich in carbohydrates, proteins, and fats, but are rarely consumed as part of a regular diet [6]. Diet diversity is a cornerstone of a healthy diet, and a modest strategy is to improve health through the utilization of indigenous fruit and vegetables [7], refer to Figure 1, it provides a summary of the value of underutilized crops in building healthy communities in terms of diet, climate, and biodiversity, all of which are important factors for sustainable food production. Affordability and accessibility of food products play a crucial role in achieving food security [7]. To eradicate hunger, poverty, and reduce the burden of malnutrition around the world, nutrition analyses of underutilized crops are vital for future research, planning, commercialization, and utilization [6]. Underutilized crops have become an important resource for climate-change adaptation in most countries [8].

In addition, diet-related diseases are a major cause of disease and health inequity in rural areas. Chronic noncommunicable diseases (NCDs) such as type-2 diabetes, cardiovascular disease, and cancer are on the rise and more prevalent among consumers who consume highly processed high-fat, high-sugar-convenient foods [9]. Phytochemicals are biologically active compounds that have non-nutritive and health-protective properties [10]. Furthermore, underutilized crops are known to possess high antioxidant properties [11]. Moreover, postharvest processing technologies can be applied to release the complex minerals and enhance their bioavailability, bioaccessibility, and bioactivity [12]. This article presents a comprehensive review with an in-depth analysis of the newest research findings on nutritional and phytochemical profiles of selected underutilized fruits, vegetables, and legumes. Kakadu plum (*Terminalia ferdinandiana*), is one of the fruits with the highest content of vitamin C and a rich source of phenolic acids and tannins. Kakadu plum is endemic to Australia, wild-harvested, and is an Aboriginal owned and led value

Figure 1



Benefits of underutilized foods for healthy communities.

chain and a popular fruit among communities. It has significant commercial applications in food, beverage, and supplement industries as a functional ingredient due to these properties [13]. Natal plum (*Carissa macrocarpa*), a rich source of cyanidin derivatives, is eaten fresh and used in value-added products for diet diversity, has commercial potential to develop value chains in remote communities in South Africa [14]. Moringa (*Moringa oleifera*) known for its high content of vitamin A, iron, zinc, polyphenols, carotenoids, and glucosinolates is used in food formulations for health [15]. African nightshade (*Solanum* species) is an African Indigenous Leafy Vegetable, a good source of proteins, minerals, and vitamins (beta- carotene) is used in different food preparations, including fermentation [16,17], and wattle seeds (*Acacia coriacea*), a legume with high protein, fiber, zinc, and iron, a promising legume to develop functional foods and ingredients [18].

Nutrient-dense crops

Food comprises nutrients that are essential for body function, including the macronutrients and the micronutrients. Modern lifestyles and affordability to purchase foods rich in micronutrients have resulted in people consuming foods that are energy dense, which can lead to the development of noncommunicable diseases [12].

Table 1

Comparison of wattle seed flour (*Acacia coriacea*) with staple/conventional foods.

Food crop	Energy (kJ/ 100 g DW)	Protein (g/100 g DW)	Fat (g/100 g DW)	Carbohydrate (g/100g DW)	Dietary fiber (g/ 100 g DW)	Iron (mg/ 100g DW)	Reference
[a]*Acacia coriacea* (wattle seed flour)	1310	22.5	9.8	13.7	41.4	5.1	[18]
Triticum aestivum Wheat-flour whole grain	1448	15.1	2.7	71.0	10.6	3.9	[19]
Cicer arietinum L. Chickpeas dry	1581	20.5	6.0	63.0	12.2	4.3	[20]

[a] Wattle-seed whole-grain flour.

As an intervention, healthy diets can be developed by incorporating underutilized crops to increase the availability of micro- and macronutrients and meet the daily calorie requirements [17].

As an example, legumes such as wattle seed and chickpea, both belonging to the Fabaceae family, were compared with whole-grain wheat flour, a staple cereal consumed by most people. Based on the comparison, both legumes are excellent sources of protein, almost twice as much as whole-grain wheat flour (Table 1). The major advantage of wattle seeds over wheat and chickpeas is that wattle seeds are low in carbohydrates and high in fiber [18]. Wattle seeds (*Acacia coriacea*) are therefore an attractive addition to diets that aim to lower carbohydrate intakes while increasing fiber and protein levels, thereby targeting a population at risk for type-2 diabetics. Additionally, the fat content of *Acacia coriacea* is four times higher than that of wheat [19] and 1.6 times that of chickpeas [20]. In comparison with chickpeas and wheat, wattle seed contains a higher level of Fe. Thus, the wattle seed has the potential to become a nutritionally beneficial legume if used in a healthy diet and must be promoted as a nutrient-dense, underutilized legume to contribute to dietary diversity. The genus Acacia, also known as wattle, is distributed in warm temperate, tropical, and subtropical regions in the world and comprises over 1350 species. It can be grown in arid regions and is a rain-fed crop and therefore can be sustainably produced [21].

Moringa fresh leaves (100 g) contain approximately four times more vitamin A than a carrot, four times more vitamin C than an orange, higher calcium than carrot, banana, and orange, and higher potassium than carrot and orange (Table 2) [22–24]. Dried Moringa leaves have been used in different food formulations. The addition of moringa leaf powder to wheat flour from 0% to 10% w/w considerably increased the protein from 13.6 to 14.6 g/100 g dry weight (DW), ash from 1.9 to 2.5 g/100 g DW, and fiber content from 13.7 to 19.0 g/100 g DW of the bread samples [15]. *M. oleifera* leaves contain between 25.79 and 31.03 mg of zinc per kg, which is the recommended daily intake of zinc [22]. Moreover, moringa leaves have a low calorific value, making them a useful addition to any obese diet [22]. The beta-carotene content in African nightshade (*S. nigrum*) is higher than the commonly consumed leafy vegetables (2.8–3.6 mg/ 100 g fresh weight (FW)) listed in the USDA National Nutrient Database [19]. African nightshade leaves are an important source of B vitamins, mainly thiamine (0.08–0.35 mg/100 g FW), riboflavin (0.17–0.19 mg/100 g FW), and folate (12–56 μg/100 g FW) [25].

Kakadu plum (*Terminalia ferdinandiana*) fruits have higher vitamin-C contents (2300–3150 mg/100 g FW) compared with citrus fruits (70 mg/100 g FW) [26•]. Blackberries (72.5 mg/100 g FW) and blueberries

Table 2

Comparison of nutritional composition of moringa fresh leaves with commonly consumed food crops.

Food crop (fresh weight)	Vitamin A (mg/100 g)	Vitamin C (mg/100 g)	Potassium (mg/100 g)	Calcium (mg/100 g)	Reference
Moringa leaves	6.78	220	259	440	[23,24]
Carrot	1.8-	2.2	210	33	[19,24]
Bananas	0.001	12.3	326	5	[19]
Orange	-	59.1	166	43	[19]

Table 3

Phytochemical profiles of selected underutilized fruits and vegetables.

Underutilized fruits and vegetables	Phytochemical profiles	References
Terminalia ferdinandiana (Kakadu plum)	Phenolic acids and tannins — Chebulinic acid, Chebulagic acid, Corilagin, Elarcarpusin, Helioscopin B, 3,4,6-tri-o-galloyl-β-D-glucose, Ellagic acid, Gallic acid, and Punicalagin	[26•,28,31,44]
Moringa oleifera	Flavonoids, glucosinolate, phenolic acids, alkaloids, phytosterols, rutin, quercetin, rhamnetin, kaempferol, apigenin, myricetin, 4-*O*-(α-*L*-rhamnopyranosyloxy)-benzylglucosinolate, ellagic acid, ferulic acid, caffeic acid, *o*-coumaric acid, and chlorogenic acid. Marumoside A, marumoside, B pyrrolemarumine-4″-*O*-α-*L*-rhamnopyranoside, β-sitosterol, campesterol, and stigmasterol	[15,23•,29,37,39]
Carissa macrocarpa (Natal plum)	Phenolic acids, flavonoids, cyanidin-3-o-glucoside, catechin, Epicatechin, caffeic acid, luteolin, apigenin, quinic acid, quercetin-3-O-rhamnosylglucoside, chlorogenic acid, gallic acid, caffeic acid, protocatechuic acid, 4-hydroxybenzoic acid, dicaffeoyltartaric acid, P-coumaric acid, ferulic acid, vanillic acid, pyrogallol, cyanidin-3-O-β-glucopyranoside, cyanidin-3-O-β_sambubioside, cyanidin-3-o-glucoside, quercetin-3-O-rhamnosyl galactoside, quercetin-3-O-rhamnosylglucoside, Eriodictyol 7-O-glucoside, and Naringenin 4-O-glucoside	[34,40,41]
Solanum retroflexum (African Nightshade)	Kaempferol derivatives: kaempferol-3-*O*-sinapoyldihexoside–hexoside, kaempferol-3-*O*-rutinoside, kaempferol-dihexoside, and rutin	[30,42,43]

(13.3 mg/100 g FW), contain lower levels of vitamin C than Natal plum (*Carissa macrocarpa*) (115.75 mg/100 g FW) [27].

Phytochemical-rich foods

Polyphenols, carotenoids, phytosterols, and glucosinolates (secondary metabolites) are some of the most prevalent phytochemicals found in plant food with health benefits [9]. Most of the known and identified phytochemicals in Kakadu plum [28], moringa [29], Natal plum [27], and African Nightshade [30] are given in Table 3. In comparison with strawberries (5.5 mg/100 g DW) and boysenberries (4.8 mg/100 g DW), the Kakadu plum (621.7 mg/100 g DW) showed the highest level of free ellagic acid [26•], suggesting that it is a good source of ellagic acid and can be taken as a food supplement. Furthermore, punicalagin (hydrolyzable tannins) content in Kakadu plums was 74 mg/100 g of fruit [31]. Punicalagin contents in pomegranate juice ranged from 20 to 1350 mg/L [32]. Punicalagin is known for its antioxidant, antimicrobial, and anti-inflammatory properties [31]. The phytochemicals in underutilized crops have antioxidant properties, are effective at scavenging free radicals, and exhibit anti-inflammatory properties, as well as positive health benefits [33]. The antioxidant properties of underutilized crops can be affected due to different maturity stages at harvest, or during postharvest processing or storage. The ferric reducing antioxidant power (FRAP) activity in the Natal plum fruit increased significantly with maturity (deep-red stage) [27]. The concentrations of all phenolic compounds mentioned in Table 3 were the highest in mature fruits (deep-red stage) [27]. The Natal plum fruits stored at 2 °C accumulated anthocyanins (cyanidin derivatives) that enhanced their chilling resistance in comparison with those stored at higher temperatures (4 and 10 °C) [34]. Furthermore, fruit stored at 2 °C for 12 days showed higher antioxidant activity (FRAP). Therefore, storing Natal plum at 2 °C for 12 days can be recommended if the fruit is used as a functional ingredient [34].

International Diabetes Federation projects that 783 million people will have diabetes by 2045 [35]. Vargas-Sánchez et al. in their review indicated that quercetin and kaempferol, and the phenolic acids chlorogenic acid and caffeoylquinic acid are found in moringa leaves. In the small intestine (duodenum and jejunum), these compounds appear to inhibit sodium–glucose transporter-1 and thus reduce intestinal glucose absorption [36]. Moringa leaves are a rich source of flavonoids quercetin and kaempferol, and the phenolic acids chlorogenic acid and caffeoylquinic acid. Various animal studies demonstrated that *Moringa oleifera* leaves lowered blood-sugar levels, suggesting that this product

could be used in place of metformin, a drug commonly used to treat type-2 diabetes [37]. In a study by Kushwaha et al. [38], blood-glucose levels of postmenopausal women decreased significantly after consumption of 7 g of moringa leaf powder over a period of 3 months. In addition, moringa leaves are rich in glucosinolates, 4-O-(a-L-rhamnopyranosyloxy)-benzylglucosinolate (glucomoringin) [39]. In some epidemiological, animal, and clinical studies, there is evidence that dietary glucosinolates and their cognate isothiocyanates contribute to protection against NCDs by inducing phase-2 cytoprotective enzymes via Keap1–Nrf2–ARE interaction [39].

The combined consumption of Natal plums and Marula nuts increased the bioaccessibility of cyanidin-3-O-β-sambubioside and cyanidin-3-O-glucoside by 80.2% and 71.9%, respectively, and increased the α-glucosidase inhibition capacity [40]. Furthermore, the choice of a solvent system affects the phenol content of the fruit during extraction. Ethanol is safe for human consumption and is an excellent solvent for extracting free and bound phenolic compounds from underutilized crops into food as functional ingredients [41].

Solanum retroflexum (African Nightshade) is the most consumed underutilized vegetable in Southern Africa. Stir-fried African nightshade had the highest antioxidant activity and inhibition of α-glucosidase [42]. A positive correlation was shown between α-glucosidase activity and rutin, chlorogenic acid, caffeoylmalic acid, or quercetin-3-O-xylosyl-rutinoside in stir-fried nightshade leaves [42]. Furthermore, boiling enhanced the concentration of contained myricetin, quercetin-3-O-robinoside, 3,4-dicaffeoylquinic acid, 3-caffeoylquinic acid, and rutin in black nightshade (*Solanum nigrum*) [43]. After *in vitro* digestion of black nightshade, phenolics were reduced; however, bioactivity was retained, especially in preventing oxidative stress in Caco-2 cells, indicating their ability to reduce oxidative stress-related digestive disorders [43].

Products made from Kakadu plums are commercially available. The fruit is sold fresh or frozen, or processed into puree or powder, and used in the food and beverage industry, cosmetics, and supplement industries [13]. Studies on cell viability are crucial to determining the safety and toxicity of underutilized crops. Differentiated Caco-2, HT29–MTX–E12, and HepG2 cells were tested with the ethanolic extract of the Kakadu plum and proved less toxic and safe for consumption [44].

To assess the safety of the products and ingredients of the underutilized crops, a comprehensive risk assessment is required. Nevertheless, the consumption of phytochemicals is still not regulated and incorporated into national and international standards, and there is a need to address this issue due to the increased promotion of phytochemicals as beneficial to health. From a market and consumer perspective, the government or those responsible for regulations should look at health claims that are evidence-based.

Strategies to promote utilization and consumption

The challenges faced by these underutilized crops are climate change, loss of genetic diversity and traditional knowledge, diminished value due to lack of research and development, lack of infrastructure and genetic material, intense urbanization, poor communication, and absence of legal frameworks, policies, and national programs [45]. To promote the utilization of these crops, there should be more research on the nutritional and health value of these crops and their cultural connection. A recent paper [46•] provides information on databases for agricultural crop diversification. The data include various datasets on nutritional composition of crops, yield, and other factors. An excellent collation of B-vitamin nutritional data of edible-plant species was put together from eight national and regional food-composition databases generating information on edible-plant diversity for B vitamins and conservation priority for future generations [47•]. It increases dietary diversity as part of other agricultural development policies that aim to improve nutrition and health. It also complies with sustainable development goals food and nutrition security, health, and poverty. Governments and relevant international agencies should have policies on utilization of these crops by clearly mapping out what is available in different regions and a pathway to bring these underutilized crops to the market by codesigning these transformative pathways with relevant stakeholders.

Further studies on gut microbial biotransformation to understand the nutritional and biological activities of generated metabolites and potential health benefits are needed [48,49]. In addition, the possible toxicity due to microbial breakdown of phytochemical biotransformation should be investigated [50•]. For the underutilized fruits and vegetables to be included in national databases, more clinical studies should be conducted to evaluate their health benefits and safety. Especially, the impact of antinutrients on the bioaccessibility of Fe must be investigated. The appropriate agroprocessing techniques to facilitate the elimination of antinutritive compounds need to be investigated. To link underutilized fruits and vegetables with the supply chain and to generate revenues, it is also crucial to implement the best postharvest management practices. A lack of guidelines regarding water use and production has been cited as bottlenecks for the promotion of these underutilized vegetables. Thus, guidelines for horticulture production of the underutilized vegetables using state-

of-the-art agronomy-management techniques are needed for mass propagation and adoption.

Conclusion

The underutilized fruits (Kakadu plum and Natal plum), vegetables (Moringa and Night Shade), and legume (wattle seeds) exemplify the potential for underutilized crops. There are over 7000 edible plants that are available for interventions promoting consumption, however, only a fraction of them are commonly consumed and there is limited knowledge of the nutritional and micronutrient profiles. With growing interest in using underutilized food crops in diet diversity, it is critical to not only understand their nutritional and phytochemical properties, but also bioaccessibility during digestion, and biological activities. To date, efforts on investigations are mainly focused on moringa and African leafy vegetables, but there are many other underutilized crops that need to be profiled for nutritional and phytochemical content to determine their potential as functional foods. Meanwhile, limited studies have revealed quantitative loss of phytonutrient compounds during postharvest processing and changes in biological activity. In addition, the ability to characterize nutritional and specific phytochemicals derived through postharvest processing remains limited. The available literature on the influences on the bioaccessibility of phytochemicals from various underutilized food crops and their bioactivities is currently increasing. While numerous *in vitro* studies have been conducted, clinical trials are inadequate and partly hinder the translation of results into product commercialization. Furthermore, the interaction between antinutrients and phytonutrients in underutilized crops must be explored and the interference must be reduced by adopting appropriate processing technologies and fostering interactions with gut microbiota critical to their physiological effect. These underutilized crops have been used traditionally by communities for thousands of years due to their sustainable production practices, nutritional, and health value, and demonstrate the immense capacity to contribute to diet diversity.

Conflict of interest statement

Nothing declared.

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Antioxidant Rich Extracts of *Terminalia ferdinandiana* Inhibit the Growth of Foodborne Bacteria

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Abstract: *Terminalia ferdinandiana* (Kakadu plum) is a native Australian plant containing phytochemicals with antioxidant capacity. In the search for alternatives to synthetic preservatives, antioxidants from plants and herbs are increasingly being investigated for the preservation of food. In this study, extracts were prepared from *Terminalia ferdinandiana* fruit, leaves, seedcoats, and bark using different solvents. Hydrolysable and condensed tannin contents in the extracts were determined, as well as antioxidant capacity, by measuring the total phenolic content (TPC) and free radical scavenging activity using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Total phenolic content was higher in the fruits and barks with methanol extracts, containing the highest TPC, hydrolysable tannins, and DPPH-free radical scavenging capacity (12.2 ± 2.8 g/100 g dry weight (DW), 55 ± 2 mg/100 g DW, and 93% respectively). Saponins and condensed tannins were highest in bark extracts (7.0 ± 0.2 and 6.5 ± 0.7 g/100 g DW). The antimicrobial activity of extracts from fruit and leaves showed larger zones of inhibition, compared to seedcoats and barks, against the foodborne bacteria *Listeria monocytogenes*, *Bacillus cereus,* Methicillin resistant *Staphylococcus aureus*, and clinical isolates of *Pseudomonas aeruginosa*. The minimum inhibitory concentration and minimum bactericidal concentration in response to the different extracts ranged from 1.0 to 3.0 mg/mL. Scanning electron microscopy images of the treated bacteria showed morphological changes, leading to cell death. These results suggest that antioxidant rich extracts of *Terminalia ferdinandiana* fruits and leaves have potential applications as natural antimicrobials in food preservation.

Keywords: Kakadu plum; *Terminalia ferdinandiana*; antioxidants; antimicrobial activity; food preservation; phytochemicals; polyphenols

1. Introduction

Antioxidants from plants and herbs are progressively being used as alternatives to synthetic antioxidants (like butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate) to preserve food [1]. The use of synthetic antioxidants is tightly regulated due to the health risks such as potential organ toxicity and carcinogenicity associated with overuse [2]. In addition to being used as natural food preservatives, the use of plant antioxidants as functional food ingredients and/or supplements is also growing, based on new findings regarding their potential biological activities [3]. In particular, plant phytochemicals have received substantial research attention based on their ability to act as reducing agents, hydrogen donors, and singlet and triplet oxygen quenchers [4]. Food safety is an important international concern, as food spoilage, due to bacteria and fungi, causes considerable economic loss worldwide [5]. Due to recent outbreaks of emerging pathogens, such as *Listeria monocytogenes*,

and rising worldwide impacts of foodborne illness, consumer concerns over food safety and food formulation have increased [1,5], along with the demand for non-toxic natural food preservatives [5]. Many plant extracts possess antimicrobial activity, however inherent variations in bioactivity and concentration places some limitations on the use of plant extracts in food products [6]. However, plant phytochemicals, such as polyphenols, alkaloids, and polypeptides, are known to retain the microbiological and chemical quality of fresh and processed foods [7].

Terminalia ferdinandiana Exell., commonly called Kakadu plum, billy goat plum, gubinge, or salty plum, is a native flowering Australian plant from the Combretaceae family [8]. This endemic Australian, semi-deciduous plant grows in the tropical rangelands of the Northern territory, the Kimberley area of Western Australia, and in some northern parts of Queensland (Figure 1) [9,10]. The fruits are smooth-skinned, fleshy ovoid drupes with a short beak that become yellow-green when ripe (Figure 1). There are about 250 species of the genus *Terminalia* from the family Combretaceae growing in tropical regions across the globe [11]. Among them, approximately 30 species or subspecies of *Terminalia* are endemic to Australia [9,10]. A high degree of phytochemical variability exists amongst different species and subspecies due to genetic diversity, soil and climate conditions, fruit ripening stage, storage, and other post-harvest conditions [12].

Figure 1. (**A**) Distribution of *Terminalia ferdinandiana* from the Australasian Virtual Herbarium website. (https://avh.ala.org.au) showing ● West Australian, ● Northern Territory and ● Queensland locations. (**B**) Mature tree; (**C**) leaves and fruits; (**D**) seeds; and (**E**) bark.

Due to increasing commercial demand from local and international food industries, *T. ferdinandiana* products are often stored for long periods. Subsequently, the bioactivity and safety of these products must be ensured following long-term storage. A study by Sultanbawa et al. [13] investigated the safe storage of *T. ferdinandiana* extracts and not only confirmed the retention of bioactivity over a period of 18 months in frozen storage (−20 °C), but also identified chemical markers for determining the end-of-storage life of *T. ferdinandiana* extracts.

Studies focused on the bioactivities of the various phytochemicals in *T. ferdinandiana* fruit and leaves have identified ellagic acid, gallic acid, ethyl gallate, chebulic acid, corilagin, hydroxycinnamic acid, ascorbic acid, α-tocopherol, lutein, tannins, chebulagic acid, exifone, punicalin, castalagin,

appanone A-7 methyl ether, xanthotoxin, and phthalane [14–16]. The objective of the present study was to determine the potential of *T. ferdinandiana* extracts as antioxidants and antimicrobial agents in food preservation.

2. Materials and Methods

2.1. Chemicals

Methanol anhydrous (99.8%), ethyl alcohol (pure), acetone (HPLC grade ≥99.9%), n-hexane (99%), Folin–Ciocalteu's phenol reagent, gallic acid monohydrate (American Chemical Society (ACS) reagent), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), n-butanol anhydrous (99.8%), potassium phosphate tribasic (regent grade ≥98%), vanillin, sulfuric acid (99.9%), saponin from Quillaja bark (sapogenin content ≥10%), potassium iodate (99.7–100.4%), tannic acid, and catechin analytical standard were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Sodium carbonate anhydrous was obtained from Chem-supply, Bedford St, Gillman, South Australia, AU; chloroform (high purity solvent) and trolox were obtained from Merck KGaA, Darmstadt, Germany; HCl (Trace metal grade) was obtained from Fisher Scientific, United States Fisher HealthCare, Veterans Memorial Dr. Houston, Texas, USA. Standard plate count agar (American Public Health Association) (PCA) (CM0463), potato dextrose agar (PDA) (CM 0139), nutrient broth (CM 001), and tryptone soya yeast extract broth (TYSEB) (CM 129B) were purchased from Oxoid Ltd, Basingstoke, UK. Grade AA (6 mm) discs were purchased from GE Healthcare Life Sciences, Whatman, UK.

2.2. Sample Collection and Processing

Ripe and mature fruits of *T. ferdinandiana* (total harvest of 5000 kg) were collected from over 600 trees, from native bushland covering a land area of 20,000 km^2 in the Northern Territory, Australia, in 2015. A voucher specimen, AQ522453, was deposited at the Queensland Herbarium. A portion of the collected fruits were processed by Sunshine Tropical Fruit Products, Nambour, Queensland, Australia, to provide a seedless puree, along with the separated seeds, which were stored at −80 °C until further analysis. The puree was then freeze-dried and milled to provide a uniform powder that was stored at −20 °C and used throughout this study. The freeze-dried puree will be referred to as fruits/fruit extract. The frozen seeds were thawed and washed several times with double distilled water to remove the pulp residue. The seeds were then oven-dried for 48 h at 40 °C. After drying, the seeds were individually cracked using an Engineers vice size 125 (DAWN tools and Vices Pty Ltd, Heidelberg West, Victoria, Australia) to release the kernels from the seedcoats. The seedcoats were processed and analyzed separately in a previous study [17]. The separated seedcoats were hammer milled and used for this study. Leaves and bark were also collected from the same region during the same fruit harvest and were freeze-dried and milled. The milled freeze-dried powders of leaves and bark were used throughout this study.

2.3. Preparation of Kakadu Plum Extracts

Accelerated solvent extraction (ASE) (Dionex ASE 200 system, Dionex Corp., Sunnyvale, CA, USA) was performed to prepare the extracts for antioxidant and antimicrobial assays [18]. Briefly, 10 mL stainless steel extraction cells were assembled and fitted with a 27 mm cell filter at the bottom end. Aliquots (1.0 g) of freeze-dried powders of fruits and leaves, and dried powders of seedcoats of *T. ferdinandiana* were mixed with diatomaceous earth (approximately four to five times the weight of the powders to fill the cells completely) and placed in the cells. Five different solvents were used: methanol, ethanol, acetone, hexane, and distilled water. The ASE unit was operated under the following conditions: 60 °C for methanol and ethanol, 50 °C for acetone and hexane, and 75 °C for distilled water; preheat 5 min; static time 5 min, eight extraction cycles, rinse volume 25% with fresh extraction solvent; and purged with 150 psi for 60 sec. The cells containing the samples were prefilled with the respective extraction solvent, pressurized, and heated with the extracts collected into 60 mL amber glass vials.

MiVac sample concentrator (GeneVac Inc., New York, NY, USA) was used to concentrate and dry the extracts. The temperatures for solvent evaporation were controlled as follows: methanol and ethanol extracts at 50 °C, acetone and hexane extracts at 45 °C, and water extracts at 70 °C. The concentrated extracts were weighed and stored at −20 °C until analysis.

2.4. Antioxidant Capacity

2.4.1. Total Phenolic Content

The total phenolic content (TPC) of the various solvent extracts of *T. ferdinandiana* tissues was determined by spectrometry using the Folin–Ciocalteu reagent [19]. The extracts (25 µL) were added to the 96-well plate and 125 µL freshly prepared Folin–Ciocalteu reagent and 125 µL sodium carbonate (7.5% *w*/*v*) were also added to the wells. The mixture was incubated in the dark for 30 min at room temperature. Absorbance was measured at 750 nm using a Tecan Microplate Reader (Tecan Infinite M200, Tecan Trading AG, Mannedorf, Switzerland) with Magellan Software (version 6.4, Tecan Trading AG). Results were expressed as g gallic acid equivalents (GAE)/100 g dry weight (DW).

2.4.2. DPPH Radical Scavenging Activity

The DPPH radical scavenging activity assay was performed as per the previously described method [19]. Methanol (200 µL) was used as blank. Control wells contained 100 µL of methanol and 100 µL of DPPH (0.15 mM). Samples (100 µL) and 100 µL of DPPH (0.15 mM) were added to the appropriate wells. The plates were shaken for 15 sec and incubated for 40 min at room temperature and were kept in the dark. Trolox standards at different concentrations (5–35 µM/L) were treated as samples. Absorbance was measured at 517 nm using a Tecan Microplate Reader with the percentage radical scavenging activity of each extract calculated from the standard curve, using the formula described previously [20]; Percentage of radical scavenging activity = ($Control_{Abs}$-$Sample_{Abs}$ / $Control_{Abs}$) × 100%.

2.5. Determination of Total Saponin Content

Saponin extracts were prepared as previously described by Xi, et al. [21], with modifications. Briefly, 0.5 g powdered *T. ferdinandiana* fruits, leaves, seedcoats, and barks were extracted three times with 80% ethanol at a ratio of 1:10 *w*/*v* under reflux at 80 °C for 1 h. The combined alcohol extract was concentrated, suspended in distilled water, and then partitioned successively with chloroform (ratio 1:3 *v*/*v*) and n-butanol saturated with water (ratio 1:3 *v*/*v*, three times). The n-butanol extract was combined and evaporated using a rotary evaporator at 60 °C to give a solid residue. Prior to the assay, the extracts were solubilized in 0.5 M phosphate buffer (pH 7.4). The total saponin content of each extract was determined using the method described by Xi et al. [21]. The extracts (50 µL) were mixed with 500 µL of vanillin (8% *w*/*v*) and 5 mL of sulphuric acid (72% *w*/*v*). The mixture was then incubated for 10 min at 60 °C and cooled in an ice water bath for 15 min. The absorbance was read at 538 nm. Saponin from quillaja bark was used as a reference standard, with the total saponin content of each extract expressed as g quillaja saponin equivalents (QSE)/100 g DW).

2.6. Determination of Condensed Tannin Content

The condensed tannins in the *T. ferdinandiana* tissues were determined using the vanillin/HCl assay described by Ahmed, et al. [22]. The extract (500 µL) was mixed with 3 mL of vanillin reagent containing 4% concentrated HCl and 0.5% vanillin in methanol in a 15 mL falcon tube. The mixture was allowed to stand for 15 min at room temperature and the absorbance was then recorded at 500 nm. Methanol was used as the blank. An appropriate standard curve was prepared using catechin, with concentrations ranging from 0.02 to 2.5 mg/mL *w*/*v*. The amount of condensed tannins in the extracts was expressed as g catechin equivalents (CaE)/100 g DW.

2.7. Determination of Hydrolysable Tannin

The hydrolysable tannins in the *T. ferdinandiana* tissue extracts were determined using the potassium iodate assay previously described by Hoang, et al. [23]. Briefly, 50 µL of 1 mg/mL *w/v* extract was added to a 96-well plate with 150 µL of 2.5% *w/v* potassium iodate. Absorbance was measured at 550 nm after 15 min, using a Tecan Microplate Reader (Tecan Infinite M200) with Magellan Software (version 6.4). Tannic acid was used as a standard and results were expressed as mg tannic acid equivalents (TAE)/100 g DW.

2.8. Antimicrobial Activity

2.8.1. Foodborne Microorganisms

Foodborne microorganisms, including pathogenic and clinical isolates, were chosen for this study. A total of 4 g positive bacteria—*Staphylococcus aureus* (NCTC 6571) (National Collection of Type Cultures, Health Protection Agency Centre for Infection, London, UK), methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates (CI) (Royal Brisbane and Women's Hospital, Herston, Queensland, AU), *Bacillus cereus* (ATCC 10876) (Microbiologics Inc., St. Cloud, MN, USA), and *Listeria monocytogenes* (ATCC 19111) (The University of Queensland, Brisbane, AU)—and 2 g negative bacteria—*Pseudomonas aeruginosa* (ATCC 10145) (Microbiologics Inc., St. Cloud, MN, USA), and *Pseudomonas aeruginosa* clinical isolates (CI) (ATCC 9001) (Royal Brisbane and Women's Hospital, Herston, Queensland, AU)—were tested. Bacteria were maintained on plate count agar (PCA) medium at 4 °C and sub-cultured on PCA medium at 37 °C for 24 h.

2.8.2. Disc Diffusion Assay

The ASE of fruits, leaves, seedcoats, and barks of *T. ferdinandiana* were diluted with 20% ethanol to prepare a final concentration 10 mg/mL *w/v* extract, except for the water extracts, which were diluted with reverse osmosis (RO) water. The zone of inhibition was determined using the Kirby–Bauer assay modified by Dussault, et al. [24]. The zone of inhibition was measured using a digital calliper 150 mm (ALDI, Australia). RO water and 20% ethanol were used as the negative controls. A standard antibiotic solution, oxytetracycline (0.06 mg/mL; Sigma-Aldrich, St. Louis, MN, USA), was used as positive control. All experiments were performed in triplicate and the antimicrobial activity was evaluated by measuring the inhibition zones against the tested microorganisms.

2.8.3. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined by the microplate dilution method [25]. Different concentrations of the extracts were prepared using 20% ethanol and added to the microtiter wells to obtain final concentrations of 4, 3.5, 3, 2.5, 2, 1.5, 1, and 0.5 mg/mL. Nutrient broth (NB) (200 µL) was used as a control to ensure the broth was sterile, whilst 50 µL bacterial culture (1×10^5 colony forming unit (CFU)/mL, as determined by colony counting after serial dilution and plating) and 150 µL nutrient broth were used as the negative control. Aliquots (100 µL) of extracts (1–8 mg/mL) were added to a 96-well microplate. A total of 50 µL bacterial culture and 50 µL NB were also added to the wells to make the final volume 200 µL. Six replicates were prepared for each concentration of the extracts and the positive antibiotic control solution (0.0625 mg/mL oxytetracycline). The microplates were incubated at 37 °C with visual observation of bacterial growth performed after 24 h. The MIC values were identified as the minimum concentration at which no visible bacterial growth was recorded [18]. The MBC was observed as the lowest concentration that completely inhibited the bacteria. A 50 µL aliquot from all wells showing no visible bacterial growth in the MIC assay [26] was applied to PCA plates and incubated at 37 °C for 24 h. The MBCs of the extracts were measured by observing the viability of the initial bacterial inoculum.

2.8.4. Scanning Electron Microscopy

The methicillin resistant *S. aureus*, clinical isolates of *P. aeruginosa*, *L. monocytogenes*, and *B. Cereus* strains were grown for 7 h in tryptone soya yeast extract broth (TSYEB) at 37 °C. Methanolic ASE of *T. ferdinandiana* fruits and leaves were reconstituted in 75 µL 20% *v/v* ethanol, added to 1 mL bacteria and broth samples, and incubated for 24 h at 37 °C. The negative control was comprised of 75 µL 20% *v/v* ethanol. The samples and controls were washed three times in sterile phosphate buffered saline and fixed in 3% *v/v* glutaraldehyde [27]. Glutaraldehyde-fixed samples were fixed again in 1% *v/v* osmium tetroxide and dehydrated with ethanol. Samples were adhered to coverslips coated with poly-L-lysine (1 mg/mL) and dehydrated in the same manner, before being dried in a critical point dryer (Tousimis Research Corporation, Rockville, MD, USA) according to manufacturer's instructions. Coverslips were attached to stubs with double-sided carbon tabs and coated with gold using a sputter coater (Agar Scientific Ltd, Essex, UK), following the manufacturer's instructions. Samples were imaged in a Jeol Neoscope JCM 5000 (Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV and for high resolution images in a Jeol JSM 7100F (Jeol Ltd., Tokyo, Japan) field emission scanning electron microscopy (SEM) at an accelerating voltage of 1 kV.

2.9. Statistical Analysis

All values were expressed as mean ± SD (n = 3). Statistical analysis of the results was performed using two-way ANOVA, followed by Tukey's multiple comparison post hoc tests, with significant differences observed at $p < 0.05$ using GraphPad Prism version 8 (La Jolla, CA, USA).

3. Results and Discussion

3.1. Extraction Yields

The ASE yields from the *T. ferdinandiana tissues* are presented in Figure 2 and vary depending upon the plant tissue and solvent used. The yield variations observed from the same tissues following different ASE methods could be attributed to the varying polarities of compounds present in the various tissues [1]. A yield of 45% was achieved from fruit and bark powders using methanol extraction, whilst ethanol produced extract yields of 59% and 26% from barks and fruit powders, respectively. Water extraction produced similar yields from fruits, leaves, and barks, ranging from 30–40%. Acetone and hexane produced comparatively lower extract yields to other solvents, except from leaves, where acetone produced an extract yield similar to ethanol. The variable yields achieved by the different ASE methods could be due to the solubility of the phytochemicals in the different tissues, as well as the duration, temperature, and pH of the extraction conditions, along with the particle size of the sample and the solvent-to-sample ratio [28].

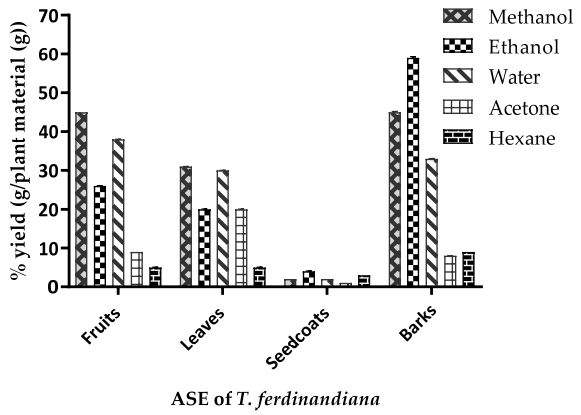

Figure 2. Yield (%) of the accelerated solvent extraction (ASE) of *Terminalia ferdinandiana*. Results are shown as the mean of triplicate experiments ± SD.

3.2. Antioxidant Capacity

3.2.1. Total Phenolic Content

The total phenolic contents (TPC) of all extracts are presented in Table 1 and ranged from 0.04–24 g GAE/100 g DW. In the *T. ferdinandiana* fruits extracts, TPC ranged from 0.38–12 g/100 g DW. The barks and leaves also contained high TPC, ranging from 0.04–2 g/100 g DW. The highest TPC was measured in methanol ASE from the fruit and leaves, followed by ethanol > acetone > water > hexane. In the bark ASE, ethanol produced the highest TPC, followed by methanol > water > acetone > hexane. The seedcoat extracts produced the lowest TPC overall, however had similar trends to the bark extracts, with ethanol > methanol ≈ water > acetone > hexane.

Table 1. Total phenolic contents in *T. ferdinandiana* tissues.

	Total Phenolic Content (GAE g/100 g DW)			
	Fruits	**Leaves**	**Seedcoats**	**Barks**
Methanol	12.2 ± 2.9 $^{a, w}$	11.7 ± 0.5 $^{a, w}$	0.2 ± 0.0 ^{x}	18.0 ± 2.0 $^{a, y}$
Ethanol	11.6 ± 1.0 $^{a, w}$	8.8 ± 0.5 $^{b, x}$	0.3 ± 0.0 ^{y}	23.5 ± 0.5 $^{b, z}$
Water	5.2 ± 0.2 $^{b, w}$	4.2 ± 0.4 $^{b, c, x}$	0.2 ± 0.0 ^{y}	6.7 ± 0.2 $^{c, w}$
Acetone	8.0 ± 0.2 $^{b, w}$	5.2 ± 0.2 $^{c, w}$	0.1 ± 0.0 ^{x}	3.5 ± 0.0 $^{d, z}$
Hexane	0.4 ± 0.0 ^{c}	0.2 ± 0.0 ^{d}	ND	0.04 ± 0.0 ^{e}

Results are expressed as mean ± SD; (n = 3). Mean values of each column with different letters are significantly different (at $p < 0.05$). a, b, c, d, e; denote significant differences of extraction solvents within same tissue. w, x, y, z; denote significant differences of the same extraction solvent across tissues.

The variation in TPC observed in the different extracts could be due to the variable solubility of polyphenols in different solvents, as well as the complex structure of the cellular macromolecules within different plant tissues. The enrichment of phenolic compounds in the extracts also depended on the solvent and the process of extraction [29]. For example, it is possible that hexane primarily extracted non-polar components, such as chlorophyll, waxes, and terpenoids from the tissues producing the lowest TPC. Regardless of the method used to prepare extracts from the different *T. ferdinandiana* tissues, the TPC content in the extracts indicates enrichment with phenolic compounds, which are potent scavengers of free radicals in vitro, and are believed to provide in vivo antioxidant protection against biomolecule damage and peroxidation of cellular membranes [30].

3.2.2. DPPH radical Scavenging Capacity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical widely accepted as a tool for estimating the free radical-scavenging capacity of an antioxidant. The effect of an antioxidant on DPPH radical scavenging is determined by the ability of the antioxidant to donate hydrogen [31]. The ASE extracts of *T. ferdinandiana* tissues were assayed for DPPH radical scavenging capacity. Results are presented in Table 2, showing that ASE extracts from the different tissues produced similar results, except for hexane ASE, which showed very low radical scavenging activity. Plant antioxidants are mostly water-soluble and present as glycosides located in the cell vacuole [32]. This is consistent with the findings in Table 2, where polar solvents produced extracts with greater antioxidant potential than non-polar solvents.

Table 2. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of *T. ferdinandiana* tissues.

	DPPH Radical Scavenging Activity (%)			
Accelerated solvent extracts (ASE) of *T. ferdinandiana*	**Fruits**	**Leaves**	**Seedcoats**	**Barks**
Methanol	93.4 ± 0.3 $^{a, x}$	89.4 ± 0.5 $^{a, x}$	93.0 ± 0.2 $^{a, x}$	84.7 ± 0.1 $^{a, y}$
Ethanol	94.3 ± 0.1 ^{a,x}	91.8 ± 0.2 ^{a,y}	88.0 ± 0.2 ^{b,y}	85.8 ± 0.2 ^{a,z}
Water	93.7 ± 0.2 $^{a, x}$	84.0 ± 0.5 $^{b, y}$	90.9 ± 0.2 $^{b, x}$	79.6 ± 0.2 $^{b, y}$
Acetone	91.5 ± 0.4 $^{a, w}$	79.2 ± 0.3 $^{c, x}$	74.2 ± 0.3 $^{c, x}$	85.5 ± 0.3 $^{a, y}$
Hexane	12.9 ± 0.9 $^{b, w}$	68.7 ± 0.4 $^{d, x}$	2.1 ± 1.3 $^{d, y}$	77.5 ± 3.8 $^{b, z}$

Results are expressed as mean ± SD; (n = 3). Mean values of each column with different letters are significantly different (at $p < 0.05$). a, b, c, d; denote significant differences of extraction solvents within same tissue. w, x, y, z; denote significant differences of the same extraction solvent across tissues.

3.3. Determination of Total Saponins

Saponins are bitter tasting, water-soluble triterpenoids found in various plants. Saponins have been found to possess in vitro anti-inflammatory activities [33], however the bitter taste may limit applications when present in higher quantities. The increased consumer demand for natural products with beneficial physicochemical and biological properties makes steroidal and triterpenoid saponins promising compounds for industrial applications [34]. The major sugar moieties of saponins are glucose, arabinose, galactose, glucuronic acid, xylose, and rhamnose [35]. Saponin-containing plants used for human consumption include soybeans, pulses, peas, chickpea, lentils, oats, potatoes, pepper, tomatoes, onions, garlic, tea, asparagus, cucumber, pumpkins, squash, gourds, melons, watermelons, sugar beet, yam, sunflower, and cassava [36,37]. The saponin content of lentils ranges from 3.7 to 4.6 g/kg, green peas 11 g/kg, chickpeas 60 g/kg, oats 1 g/kg and spinach 47 g/kg DW [37]. The saponin contents of *T. ferdinandiana* tissues are presented in Table 3. The slightly bitter taste of *T. ferdinandiana* fruits might be due to the presence of low amounts of saponins. Barks were highest in saponins, whereas no saponins could be detected in the seedcoats.

3.4. Determination of Condensed and Hydrolysable Tannins

Tannins are higher molecular weight polyphenolics, found mostly in plants used as food and feed [38]. Tannins are usually divided into two groups—hydrolysable and condensed tannins. The degree of tannin polymerization has been found to directly correlate with radical scavenging capacity [39,40]. The antioxidant effects of tannins are mostly attributed to free radical scavenging capacity, chelation of transition metals, inhibition of pro-oxidative enzymes, and lipid peroxidation [39]. Tannins have varying degrees of hydroxylation, and their molecular size is sufficient to form complexes with proteins [41].

Condensed tannin contents of *T. ferdinandiana* tissues are presented in Table 3, showing that bark has the highest content compared to leaves, which have the lowest content. Fruits were found to contain 0.8 g condensed tannins/100 g DW. The hydrolysable tannin contents of *T. ferdinandiana* tissues are shown in Table 4. The hydrolysable tannin content of the tissues ranges between 0.1 and 120 mg/100 g DW of the plant material. Leaves contain more tannins than fruits for all solvent extracts. Very low levels of tannins were observed in all seedcoat extracts, except for the acetone ASE. The highest amount of hydrolysable tannins was found in leaves (Table 4). These results suggest that the level of hydrolysable tannins is greatly influenced by the tissue type, solvents (different polarities), and extraction conditions (Table 4). The results of the present study indicate that tannins in *T. ferdinandiana* fruit extracts were predominantly condensed tannins.

Using regression analyses, the correlations between TPC, DPPH radical scavenging values, saponin content, and the condensed and hydrolysable tannin content of the fruits, leaves, seedcoats, and barks were explored. The antioxidant capacity and tannin content of the fruits were positively correlated with the antioxidant capacity and tannin content of the leaves (pearson $R^2 = 0.9736$, $p < 0.0001$), seedcoats

(pearson R^2 = 0.6886, p = 0.0001), and barks (pearson R^2 = 0.7728, $p < 0.0001$). The saponins content of the tissues was positively correlated with the condensed tannins (pearson R^2=0.9922, $p < 0.05$).

Table 3. Saponins and condensed tannins in *T. ferdinandiana* tissues.

T. ferdinandiana Tissues	Saponin Content (QSE g/100 g DW)	Condensed Tannin Content (CaE g/100 g DW)
Fruits	0.4 ^{a} ± 0.0	0.8 ^{a} ± 0.1
Leaves	0.3 ± 0.1	0.02 ^{b} ± 0.0
Seedcoats	ND	0.1 ^{a} ± 0.0
Barks	7.0 ^{b} ± 0.2	7.0 ^{c} ± 0.7

Results are expressed as mean ± SD; (n = 3). ND = not detected. Mean values of each column with different letters are significantly different ($p < 0.05$).

Table 4. Hydrolysable tannins in *T. ferdinandiana* tissues.

	Hydrolysable Tannin Content (TAE mg/100 g DW)			
ASE	**Fruits**	**Leaves**	**Seedcoats**	**Barks**
Methanol	55.3 ± 1.6 ^{a,w}	120.8 ± 2.3 ^{a,x}	0.9 ± 0.0 ^{a,y}	16.5 ± 0.2 ^{a,z}
Ethanol	33.3 ± 0.8 ^{b,w}	81.4 ± 1.4 ^{b,x}	1.42 ± 0.0 ^{a,y}	20.4 ± 0.2 ^{b,z}
Water	7.5 ± 0.4 ^{c,w}	52.0 ± 0.5 ^{c,x}	1.42 ± 0.1 ^{a,y}	13.6 ± 0.0 ^{c,z}
Acetone	10.8 ± 0.7 ^{d,w}	66.5 ± 1.1 ^{d,x}	27.1 ± 1.8 ^{b,y}	4.9 ± 0.0 ^{d,z}
Hexane	0.1 ± 0.1 ^{e,w}	3.1 ± 0.2 ^{e,x}	2.6 ± 0.2 ^{a,x}	0.2 ± 0.4 ^{e,w}

Results are expressed as mean ± SD; (n = 3). Mean values of each column with different letters are significantly different ($p < 0.05$). a, b, c, d, e; denote significant differences of extraction solvents within same tissue. w, x, y, z; denote significant differences of the same extraction solvent across tissues.

3.5. Antimicrobial Activity

3.5.1. Disc Diffusion Assay

Plant derived antimicrobials can effectively reduce or inhibit pathogenic and spoilage microorganisms and have the potential to be an alternative to synthetic antimicrobials [6]. The use of natural antimicrobial agents in food processing to extend the shelf-life of food products is well documented [6]. Consumer concern over synthetic preservatives in food products has contributed to the search for preservatives from natural sources. The antimicrobial activities of extracts from *T. ferdinandiana* tissues prepared with different solvents were determined against different microorganisms, with the inhibition zone measured in mm, and presented in Table 5 and illustrated in Figure 3. Overall, methanol extracts were found to be the most effective against the organisms tested and showed a broad spectrum of antimicrobial activity against the tested bacteria. The antimicrobial activity of the methanol extracts was similar to the acetone extracts, whilst water extracts from fruit, leaves, and bark were found to be active against *S. aureus*, MRSA, *P. aeruginosa* CI, and *B. cereus.* Fruit and leaf extracts were found to have similar zones of inhibition against the tested organisms, with MRSA, *L. monocytogenes*, and *B. cereus* the most sensitive bacteria among those tested. *S. aureus* was inhibited less compared to MRSA. Seedcoat extracts were found to be the least active against the microorganisms tested.

Herbal remedies formulated from whole plants are gaining more interest, as they are safer than synthetic options. The antimicrobial activity from *T. ferdinandiana* extracts against different microbial strains supports the scientific rationality of using plants/plant tissue in traditional medicine [42]. The inhibition of the growth of six bacterial strains by the fruit and leaf extracts could be due to the presence of antioxidant phytochemicals, mainly polyphenols, in the extracts. The *T. ferdinandiana* results support several other studies, showing the antimicrobial activity of plant extracts due to the presence of polyphenolic compounds in the extracts [26,43]. Polyphenols, particularly tannins and flavonols, are known to possess antimicrobial activity and can suppress the growth of microorganisms

by various mechanisms, such as the inhibition of biofilm formation, host-ligand adhesion reduction, and the neutralization of bacterial toxins [44].

In the present study, we found that *T. ferdinandiana* tissue extracts are high in TPC and tannins. Other species of Terminalia plants, such as *Terminalia arjuna*, *Terminalia bellerica*, *Terminalia chebula*, *Terminalia sambesiaca*, *Terminalia Kaiserana* and *Terminalia sericia,* are also high in tannins and other polyphenols [45–47]. Previous reports on the antimicrobial properties of Terminalia plants were supported by the presence of a vast range of phytochemicals, including polyphenols and tannins [48–50]. Tannins inhibit bacterial growth by binding to bacterial enzymes and interfering with phosphorylation, and sometimes forming complexes with transition metal ions, which are important for bacterial growth [51].

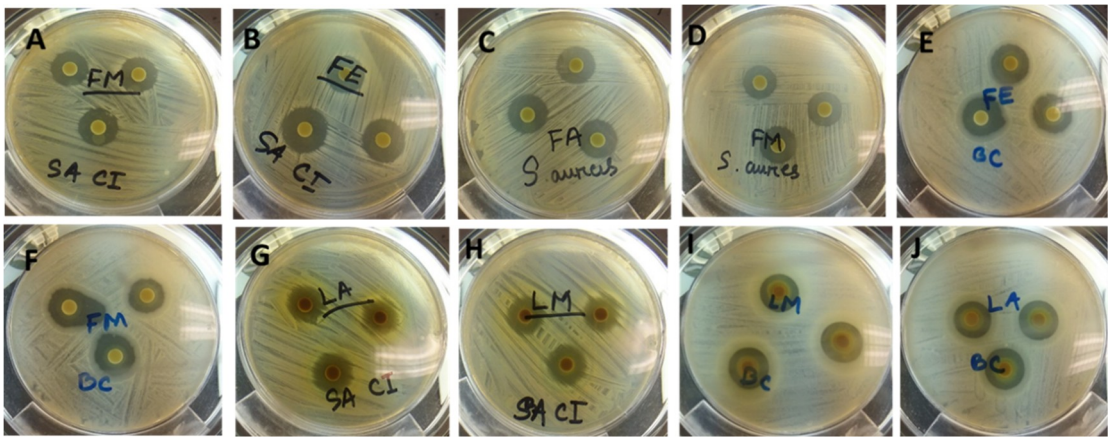

Figure 3. Antimicrobial activity (zones of inhibition) of ASE extracts of *T. ferdinandiana* tissues against various organisms. FM and FE against MRSA (A and B), FA and FM against *Staphylococcus aureus* (C and D), FE and FM against *Bacillus cereus* (E and F), LA and LM against MRSA (G and H), LM and LA against *B. cereus* (I and J). FM; Fruit methanol extract, FE; Fruit ethanol extract, FA; Fruit acetone extract, LA; Leaf acetone extract, LM; Leaf methanol extract; MRSA; Methicillin resistant *Staphylococcus aureus.*

Table 5. Antimicrobial activity of the extracts of *T. ferdinandiana* tissues.

ASE Extraction Solvent	*T. ferdinandiana* Tissues	Zone of Inhibition (in mm)					
		S. aureus	MRSA	*Pseudomonas aeruginosa*	*P. aeruginosa* (CI)	*B. cereus*	*Listeria monocytogenes*
Methanol	**Fruits**	13.8 ± 0.3 $^{a, w}$	16.4 ± 0.0 $^{a, x}$	–	11.2 ± 0.0 $^{a, w}$	16.4 ± 0.9 $^{a, x}$	20.4 ± 2.0 $^{a, y}$
	Leaves	–	15.2 ± 0.4 $^{a, w}$	–	14.6 ± 1.5 $^{b, w}$	16.0 ± 0.6 ^{a,w}	21.3 ± 0.2 $^{a, x}$
	Seedcoats	–	8.8 ± 0.0 $^{b, w}$	–	–	11.5 ± 0.6 $^{b, x}$	11.4 ± 0.8 $^{b, x}$
	Barks	11.6 ± 0.4 ^{a}	12.0 ± 0.8 ^{c}	–	–	12.8 ± 0.3 ^{b}	–
Water	**Fruits**	–	–	–	12.9 ± 1.4 ^{a}	–	–
	Leaves	–	13.3 ± 1.4 $^{c, w}$	–	10.7 ± 0.8 $^{a, x}$	–	–
	Seedcoats	–	–	–	–	–	–
	Barks	10.8 ± 1.4 ^{a}	11.6 ± 0.5 ^{c}	–	–	11.1 ± 0.4 ^{b}	–
Ethanol	**Fruits**	–	17.1 ± 0.1 ^{a}	–	–	17.8 ± 0.6 ^{a}	18.5 ± 0.5 ^{a}
	Leaves	–	14.6 ± 0.2 $^{a, w}$	–	–	16.5 ± 1.0 $^{a, x}$	20.0 ± 0.6 $^{a, y}$
	Seedcoats	–	–	–	–	9.8 ± 0.8 ^{c}	10.8 ± 0.6 ^{b}
	Barks	12.1 ± 0.3 ^{a}	12.7 ± 0.9^{c}	–	–	13.2 ± 1.1 ^{b}	–
Acetone	**Fruits**	16.7 ± 0.5 $^{b, w}$	16.6 ± 0.3 $^{a, w}$	–	13.3 ± 0.1 $^{b, x}$	18.4 ± 1.5 $^{a, y}$	20.5 ± 0.4 $^{a, z}$
	Leaves	–	15.7 ± 0.9 $^{a, w}$	8.7 ± 0.8 $^{a, x}$	14.1 ± 0.1 $^{b, w}$	16.1 ± 0.3 $^{a, w}$	21.0 ± 1.3 $^{a, y}$
	Seedcoats	–	–	–	–	–	–
	Barks	15.0 ± 1.0 $^{b, w}$	11.0 ± 1.5 $^{c, x}$	–	–	15.7 ± 0.6 $^{a, w}$	15.3 ± 1.8 $^{c, w}$
Oxytetracycline (0.25 mg/mL)		33.9 ± 0.0 $^{c, w}$	29.7 ± 1.9 $^{d, w}$	13.8 ± 0.5 $^{b, x}$	18.1 ± 0.6 $^{c, y}$	17.3 ± 1.5 $^{a, y}$	33.3 ± 0.4 $^{d, w}$

Results are expressed as mean ± SD; (n = 3). Mean values of each column are significantly different ($p < 0.05$). (–) denotes that no zone of inhibition was observed. Criteria for antimicrobial activity: <10 mm = weak, 10–15 mm = moderate, and >15 mm = strong. Mean values of each column with different letters are significantly different ($p < 0.05$). a, b, c, d; denote significant differences of extraction solvents within same tissue. w, x, y, z; denote significant differences of the same extraction solvent across tissues. Controls (reverse osmosis (RO) water and 20% ethanol) did not show any zone of inhibition.

3.5.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC values of the extracts of *T. ferdinandiana* tissues against the tested microbial strains are shown in Table 6. In general, the MIC and MBC values of the extracts against the tested microorganisms ranged from 1.0 mg extract/mL to 3.0 mg/mL, with *L. monocytogenes*, *B. cereus*, and MRSA the most sensitive among the tested microorganisms. Different tissues extracted with different solvents showed variable inhibitory effects. For example, the MIC of *S. aureus* was 1 mg/mL against bark ethanol extracts, whereas the MIC in response to bark water extracts was 3 mg/mL. The MIC of *P. aeruginosa*, *P. aeruginosa* CI, *L. monocytogenes,* and *B. cereus* ranged from 1 to 2 mg/mL of extracts, however for *S. aureus* and MRSA, 3 mg/mL was needed for inhibition.

Overall, the ethanol and acetone extracts were the most effective at inhibiting the growth of the microorganisms compared to methanol and water extracts. It is interesting to note that even though the antioxidant capacity and phenolic content of the methanol and water extracts was found to be higher than the ethanol extracts, the acetone extracts showed lower antioxidant capacity and phenolic contents overall. The phytochemicals responsible for antioxidant capacity may not be the only compounds contributing to antimicrobial activity, it is possible that other phytochemicals with antimicrobial potentials are exerting antimicrobial activities.

Table 6. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts of *T. ferdinandiana* tissues.

Tested Microorganisms	**MIC (mg/mL)**													
	FM	**LM**	**SM**	**BM**	**FW**	**LW**	**BW**	**FE**	**LE**	**SE**	**BE**	**FA**	**LA**	**BA**
Staphylococcus aureus	1.5			2			3				1	1		2
MRSA	3	2.5		1			2.5	3	3		3	3		1
Pseudomonas aeruginosa	1				1	1			1			1		
***Pseudomonas aeruginosa* CI**						1			1			1		
Bacillus cereus	1.5	1	1	1.5			1	1	1	1	1		1	1
Listeria monocytogenes	1	1	1					1	2	1		1		1
	MBC (mg/mL)													
	FM	**LM**	**SM**	**BM**	**FW**	**LW**	**BW**	**FE**	**LE**	**SE**	**BE**	**FA**	**LA**	**BA**
Staphylococcus aureus	1.5			1			2				1	1		2
MRSA	3	3		1			2	2	3		2	3		1
Pseudomonas aeruginosa	1				1	1			1			1		
***Pseudomonas aeruginosa* CI**						1			1			1		
Bacillus cereus	1.5	1	1	1				1	1	1	1		1	1
Listeria monocytogenes	1	1	1					1	2	1		1		1

MIC and MBC values ranges between 1–3 mg/mL. CI—clinical isolates, FM—fruit methanol, LM—leaf methanol, SM—seedcoat methanol, BM—bark methanol, FW—fruit water, LW—leaf water, SW—seedcoat water, BW—bark water, FE—fruit ethanol, LE—leaf ethanol, SE—seedcoat ethanol, BE—bark ethanol, FA—fruit acetone, LA—leaf acetone, BA—bark acetone. Controls did not inhibit the growth of any of the tested microorganisms.

3.5.3. Scanning Electron Microscopy

The antimicrobial effects of *T. ferdinandiana* fruit and leaf extracts on the morphology of MRSA, *B. cereus*, *L. monocytogenes,* and *P. aeruginosa* CI cells were determined by scanning electron microscopy, as illustrated in Figures 4 and 5. All bacterial cells treated with the extracts at the MIC were damaged compared to the control cells (20% *v*/*v* ethanol). The control cells had a smooth surface, with the outer layer of the bacteria relatively intact (Figure 4A,D and Figure 5A,D). By contrast, the damaging effects of the fruit and leaf extracts on bacterial cell walls were evident compared to the appearance of the control cells (Figure 4B,C,E,F and Figure 5B,C,E,F). Almost all the bacterial cells treated with the fruit and leaf extracts showed the disintegration of the outermost layer and, in some cases, the outermost layer had disappeared (Figures 4F and 5E,F).

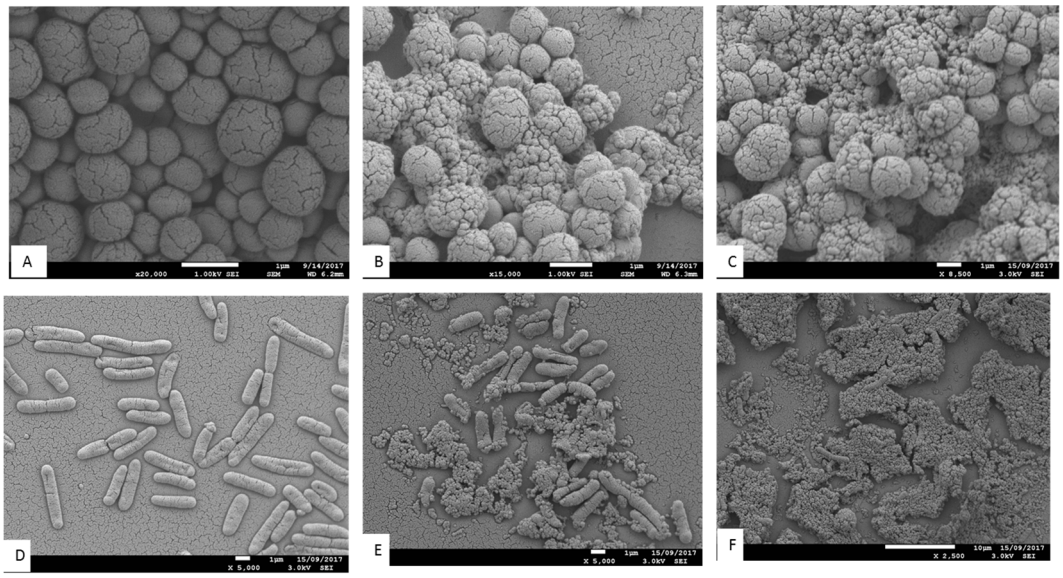

Figure 4. Antimicrobial activity of methanolic ASE of *T. ferdinandiana* fruits and leaves. Scanning electron microscopy (SEM) images of methicillin resistant *Staphylococcus aureus* control (**A**), effect of fruit extracts (**B**), effect of leaf extracts (**C**), and clinical isolates of *Pseudomonas aeruginosa* control (**D**), effect of fruit extracts (**E**) and effect of leaf extracts (**F**). Samples were imaged in a Jeol JSM 7100F field emission SEM at an accelerating voltage of 1 kV.

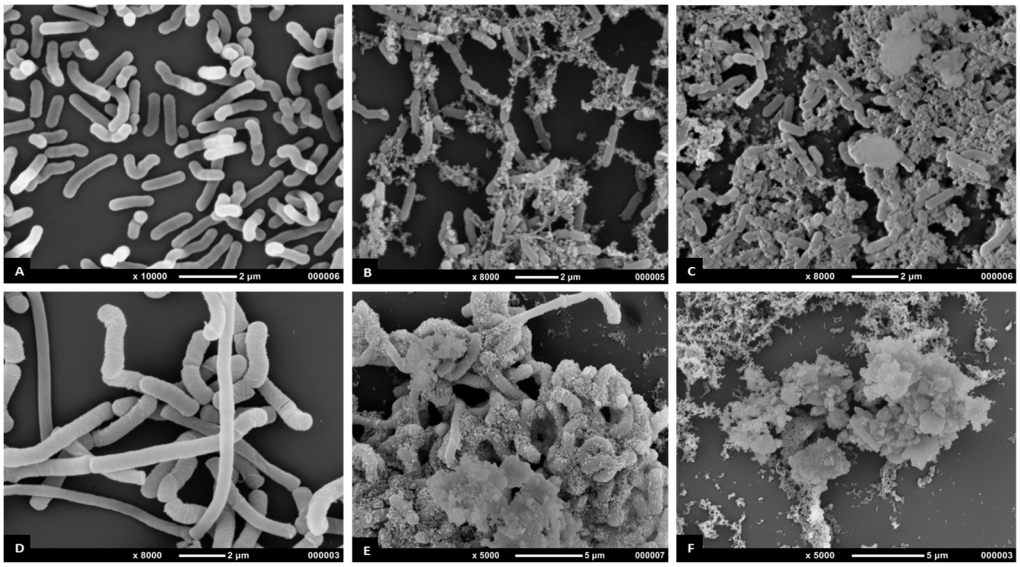

Figure 5. Antimicrobial activity of methanolic ASE of *T. ferdinandiana* fruits and leaves. SEM images of *Listeria monocytogenes* treated with control (**A**), fruit extracts (**B**), and leaf extracts (**C**), and *Bacillus cereus* treated with control (**D**), fruit extracts (**E**), and leaf extracts (**F**). Samples were imaged in a Jeol Neoscope JCM 5000 at an accelerating voltage of 10 kV.

The antimicrobial mechanisms or exact target sites for natural antimicrobials have not been identified yet and warrant further investigation [6]. However, it is thought that terpenoids and phenolics are involved in membrane disruption, phenolic acids and flavonoids cause metal chelation, coumarin interferes with the genetic material, and alkaloids inhibit the growth of microorganisms [52]. Phytochemicals are also reported to be involved in membrane disruption and, in turn, cause leakage of cellular content [53]. It was observed that plant phytochemicals interfere with active transport mechanisms and possibly dissipate cellular energy in adenosine triphosphate (ATP) form [54].

In Figure 5B,C, some of the extract-treated *L. monocytogenes* cells underwent splitting, a change in cell morphology due to deep wrinkling and distortion. Therefore, it is postulated that fruit and leave methanol extracts have antimicrobial activity against *L. monocytogenes*. Antioxidative polyphenols might have been involved in causing lesions in the cytoplasmic membrane, which in turn may have caused leakage of intracellular contents, impairment of microbial enzymes, and potentially cell death [53]. This evidence suggests that *T. ferdinandiana* fruits extracts may effectively inhibit *L. monocytogenes* in food products.

To visualize the effects of *T. ferdinandiana* fruit and leaf methanol extracts, SEM images of *B. cereus* cells treated with MIC doses of extracts were taken and are presented in Figure 5. The fruit and leaf extracts altered the cell morphology (Figure 5E,F) in comparison to controls (Figure 5D). The control bacterial cells appeared whole and distinct from one another, whilst the bacterial cells treated with both the fruit and leaf extracts were deformed. In particular, the cell wall of *B. cereus* treated with leaf extracts appeared to be degraded (Figure 5F).

A change in cell morphology was observed in *P. aeruginosa* clinical isolates incubated with *T. ferdinandiana* fruit and leaf extracts, as shown in Figure 4E and 4F. The cell surface morphology of *P. aeruginosa* control cells was intact and smooth (Figure 4D) compared to cells incubated with the MIC of *T. ferdinandiana* fruit extracts, which changed to granular with the appearance of blisters (Figure 4E). Treatment with leaf extracts was even more pronounced, as evidenced by the loss of cellular orientation (Figure 4F). These results suggest that *T. ferdinandiana* leaf extracts are more active than fruit extracts in promoting *P. aeruginosa* cell death caused by cell membrane disintegration and cell atrophy, indicating that the active compounds present in *T. ferdinandiana* leaf extracts may act on the cell membrane or extracellular proteins, resulting in the inhibition of bacterial cell growth.

Scanning electron microscopy images of MRSA (Figure 4B,C) treated with *T. ferdinandiana* extracts also showed partial disintegration of the bacterial cell surfaces and reduced residual cellular content. Cell surfaces also appeared rougher after *T. ferdinandiana* extract treatment. The potent antimicrobial activity observed in the *T. ferdinandiana* extracts in the present study can therefore be attributed to the presence of numerous phytochemicals in the plant, especially ascorbic and ellagic acid, as previously reported [15]. In the presence of *T. ferdinandiana* extracts, bacterial cells grew as isolated colonies, compared to control cells. The antimicrobial activity of plants is mostly attributed to their principal phenolic components, which exhibit significant bactericidal activity against MRSA. A reaction between phenolic compounds and bacterial membrane proteins was suggested to be involved in their antimicrobial action, which can weaken the cell wall or damage the cytoplasmic membrane directly [55].

These results indicate that antimicrobial compounds are contained in *T. ferdinandiana* leaves and fruit and act by damaging bacterial cell walls or inducing cell lysis. It is possible that the antimicrobial compounds present in *T. ferdinandiana* extracts readily enter the cells through these lesions, whilst also facilitating the leakage of cell contents. That is, when microbial cell walls or membranes become compromised, possibly by interacting with phenolic compounds, low molecular weight substances, such as K^+ and PO_4^{3-}, tend to leach out first, followed by the loss of other intracellular molecules, such as proteins, DNA, RNA, and other higher molecular weight materials [56]. These antimicrobial compounds may even react with bacterial DNA, ultimately resulting in cell death. Some researchers have reported that bioactive compounds derived from plants have antimicrobial effects on cells through reduced oxygen uptake, reduced cellular growth, inhibition of lipid, protein, and nucleic acid synthesis, changes in the lipid profile of the cell membrane, and inhibition of microbial cell wall synthesis. Cox et al. [57] reported that slight changes in the structural integrity of cell membranes can affect cell metabolism and lead to cell death.

A wide variety of phenolic compounds, including tannins, gallic acid, ellagic acid, corilagin, geraniin, tannic acid, punicalagin, castalagin, and punicalin, have been reported to be present in the *Terminalia* genus [58]. Antimicrobial activity of these compounds has also been reported against a number of microorganisms, such as MRSA, *S. aureus*, *P. aeruginosa*, Genus *vibrio*, *Escherichia coli*,

Candida Albicans, and *Aspergillus fumigatus* [59]. Previous reports on the phytochemicals present in *T. ferdinandiana* include gallic acid, apionic acid, gluconolactone, chebulic acid, ferulic acid, exifone, corilagin, punicalin, castalagin, and chebulagic acid [14,60]. High levels of ellagic acid and ascorbic acid have also been reported in *T. ferdinandiana* [15]. *T. ferdinandiana* fruit is currently marketed commercially as a functional ingredient in the form of a freeze-dried powder in the food industry, however, other tissues such as leaves have not yet been considered as functional (food) ingredients.

4. Conclusions

The contamination of food by microorganisms is a worldwide public health problem. To avoid these problems, plant-derived natural preservatives could offer a safer alternative. To date, this is the first study to extensively investigate the antimicrobial properties of *T. ferdinandiana* extracts, revealing that extracts of *T. ferdinandiana* fruit and leaves possess significant in vitro antimicrobial properties against common foodborne bacteria. The antimicrobial properties of this plant were also supported by the presence of significant antioxidant and tannin contents. Overall, the results of our present study showed that *T. ferdinandiana* fruit and leaves have great potential as natural preservatives in the food industry. However, further research on the bioactive compounds present in *T. ferdinandiana* extracts is needed to determine the compounds responsible for the antimicrobial properties.

Author Contributions: S.A. performed the experiments; collected, analysed and interpreted the data and drafted the manuscript. S.A.O., Y.S., M.T.F., M.E.N. and U.T. conceived and designed the experiments, checked and approved the results and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that there is no competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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High throughput screening to determine the antibacterial activity of *Terminalia ferdinandiana* (Kakadu plum): A proof of concept

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ABSTRACT

Different types of susceptibility tests are available to identify antimicrobial activity, including the disc agar diffusion and broth micro-dilution methods. In recent years, high throughput screening (HTS) methods have been considered and evaluated as an efficient method to rapidly monitor the antimicrobial potential of a wide range of plant products. The objective of this study was to test the ability of a 96-well plate reader as HTS method to evaluate the antimicrobial potential of extracts of *Terminalia ferdinandiana* (Kakadu plum). The main changes observed in the UV-VIS spectra of the bacteria samples were related to the biochemical and chemical compounds that might originate from the effect of the *T. ferdinandiana* extracts and the bacteria. Partial least squares discriminant analysis (PLS-DA) allowed the correct classification of samples according to the concentration of extract added to the culture (e.g. high, medium and low). The results of this study indicated that might be possible to record changes in the UV-VIS spectra associated with the interactions between bacteria and *T. ferdinandiana* extracts using a 96-well plate reader. The method was able to detect or differentiate between live and dead bacteria based on the UV-VIS spectra as a function of the addition of the *T. ferdinandiana* extracts.

1. Introduction

Microbiologists play an important role in identifying the drugs that will be most effective in the treatment of clinical infections, in defining the antibiotic resistance profiles of microorganisms found in the environment and to monitor the prevalence of microorganism in foods (Negi, 2012). Recently, an increase in the resistance of bacteria to antibiotics has been reported by several authors (Yoneyama and Katsumata, 2006). Therefore, it is necessary to evaluate the sensitivity of microorganisms to a range of antimicrobial agents using rapid detection methods (Negi, 2012).

Different types of susceptibility tests are available to screen for antimicrobial potential, including the utilization of the agar disc diffusion (disk and well diffusion methods) and broth micro-dilution methods, as well as other commercial tests that determine the minimal inhibitory and bactericidal concentrations (Negi, 2012; Akter et al., 2019).

The agar disc diffusion method is the most commonly used method worldwide, mainly owing to its low cost and simplicity (Akter et al., 2019; Negi, 2012). In this method, each disc or well contains an antimicrobial agent which will form an inhibition zone where the microorganism is not able to grow (Akter et al., 2019; Negi, 2012). The size (diameter) of the inhibition zone is used to classify the strains as resistant (R), intermediate (I), or sensitive (S) (Rodloff et al., 2008). The measurement of the zone of inhibition is not standardised in agar disc diffusion assay and can be subjective due to visual observation and based on the technical staff conducting the assay (Akter et al., 2019; Negi, 2012). The use of automated approaches for objective measurements can reduce the number of errors and improve the accuracy of susceptibility tests (Negi, 2012).

Recently, high throughput screening (HTS) methods have been considered as an efficient method to rapidly evaluate the antibacterial potential in a wide range of natural products (De La Fuente et al., 2006). Rapid assays that evaluate changes in cellular functions appear to be essential for characterizing different bioactive compounds and their potential role as antimicrobial compounds derived from plant products

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(Brusotti et al., 2014).

With the increased use of antibiotics new and more resistant strains of bacteria have come to fruition (Gould and Bal, 2013). The rapid capacity of bacteria to grow and evolve in response to antibiotics has resulted in the need for fast and low-cost monitoring tools to quantify and qualify the levels of contamination, as well as to generate targeted therapeutics for the type of strain in question. There is a need for the development of these tools for multiple organisms in a variety of matrices; exacerbated with superbugs and the 2020 COVID-19 pandemic crisis (Kost, 2020). The last few decades have witnessed an increasing interest in the application of the so called 'rapid analytical methods or high throughput techniques (Mishra et al., 2008).

Terminalia ferdinandiana (commonly known as Kakadu plum, Gubinge, Bush plum, Billy goat plum and Salty plum) is a semi-deciduous tree of the family Combretaceae an endemic plant to Australia (Konczak et al., 2010; Williams et al., 2016; Akter et al., 2018). The fruit has been used as a food source by Indigenous people in the northern regions of Australia for thousands of years (Akter et al., 2018). The high concentration in some bioactive compounds such as vitamin C and polyphenol compounds determine that this plant has high antibacterial potential (Akter et al., 2018; Akter et al., 2019). Extracts (e.g. methanol) of this plant have been reported to have effective antibacterial properties (Akter et al., 2018 and Akter et al., 2019).

The objective of this study was to evaluate the use of a 96-well plate reader a HTS method as a proof of concept to monitor the antibacterial potential of the extracts of *T. ferdinandiana*.

2. Materials and methods

2.1. Plant material collection and preparation of extracts

T. ferdinandiana fruits were sourced from the Aboriginal Community in Western Australia in 2020. The fruits were washed and freeze-dried (ScanVac CoolSafe Superior Touch, LabGear Australia, QLD, Australia) and ball-milled (Retsch MM400, Metrohm Australia Pty Ltd., NSW, Australia) to provide uniform powder. Samples were stored at −20 °C before use. The powder (2 g) was extracted with ethanol (8 mL) and a stock concentration of 250 mg/mL was used as antibacterial agent.

2.2. Microbial strains and culture conditions

Staphylococcus aureus (NCTC 6571), *Escherichia coli* (NCTC 9001) (National Collection of Type Cultures, Health Protection Agency Centre for Infection, London, UK) and methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates (Royal Brisbane and Women's Hospital, Herston, Queensland, Australia) were used in this study. Bacteria were maintained on standard plate count agar medium (CM0463) (Oxoid LTD, Basingstoke, Hampshire, England) at 4 °C and sub-cultured on plate count agar medium at 37 °C for 24 h. The standard plate count agar medium (23.5 g) was dissolved in 1 L of distilled water. The medium was brought to boil to dissolve completely. The medium was then dispensed into bottles and sterilized by autoclaving at 121 °C for 15 min before use. The cultures were diluted in tryptone soy yeast extract broth (TSYEB) to an absorbance reading of 0.5 at 540 nm using a spectrometer (Genesys 20, ThermoScientific, Australia) to obtain an inoculum of 105 colony forming unit (CFU)/mL.

2.3. Microbial assay

Three different concentrations of *Terminalia ferdinandiana* extracts were prepared using sterile distilled water and added to the 96-well plates (clear, flat bottom) (Nest Biotech Co., Ltd) to obtain final concentrations of 15.63, 6.25 and 3.13 mg/mL. Nutrient broth (NB) (200 μL) was used as a control to ensure the broth was sterile, whilst 50 μL bacterial culture (10^5 (CFU)/mL) and 150 μL nutrient broth were used as the negative control. Aliquots (50 μL) of extracts were added to the 96-well microplate. A total of 50 μL bacterial culture and 100 μL NB were also added to the wells to make the final volume 200 μL. Four replicates were performed for each concentration of the extracts. The 96-well plates were read at 0, 4 and 24 h of incubation at 37 °C. Table 1 summarises the experimental conditions followed in this study.

2.4. Kinetic loop and spectral absorbance analysis

Varioskan LUX multimode microplate reader equipped with a Xenon flash lamp (Thermo Fisher Scientific, Seventeen Miles Rock, Queensland, Australia) was used to read the well plates. The kinetic loop was set for 15 min with a reading interval of 5 min and total number of readings was set to 4 (total 25 min). The spectral absorbance was recorded in the range of 200 to 1000 nm with a wavelength interval of 10 nm (step). The measurement time of each well was set at 100 ms. The microplate and spectra collection were controlled using the SkanIt microplate reader software version 6.0.1.6 (Thermo Scientific; thermoscientific.com).

2.5. Data analysis

The spectra data were exported from the SkanIt microplate reader software in *.xlsx format to the Unscrambler software (Version X, CAMO ASA, Oslo, Norway) where chemometric analysis and spectral preprocessing was carried out. Before any data analysis, spectra were processed using the Savitzky-Golay transformation (second derivative, 21-point smoothing, 2nd polynomial order) (Savitzky and Golay, 1964). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were carried out to identify trends in the spectra and to classify the samples. PLS-DA was used to classify samples according to type of microorganism and treatment. In this study, samples were assigned with a dummy number linked with the treatment applied to the samples (1 = low, 2 = medium and 3 = high concentration of *T. ferdinandiana* extracts). Similarly, samples were linked with the type of microorganism used to carry out the experiments. Full cross-validation was used to evaluate the models and to minimise the effect of overfitting during model development (PCA and PLS-DA) (Bureau et al., 2019; Cozzolino, 2020).

3. Results and discussion

The automated recordings of bacterial growth with a 96-well microplate reader is a useful tool to study microorganisms in the presence of natural products as antimicrobial activity as reported by other authors (Rufián-Henares and Morales, 2008). Although this technique has been used in food applications, it has not been explored to evaluate the antimicrobial activity of natural plant products.

Table 1
Table indicating the treatments (concentration), control and microorganisms used for the high throughput-screening assay:

Name of Microorganisms	Concentrations of KP extracts			Contamination control	Negative control
	High (mg/mL)	Medium (mg/mL)	Low (mg/mL)		
Staphylococcus aureus	15.63	6.25	3.13	Nutrient Broth	*S. aureus* + Nutrient broth
Escherichia coli	15.63	6.25	3.13	Nutrient Broth	*E. coli* + Nutrient Broth
Methicillin resistant *Staphylococcus aureus* (MRSA)	15.63	6.25	3.13	Nutrient Broth	MRSA + Nutrient broth

Fig. 1 shows the second derivative UV-VIS spectra of the samples collected at different incubation times. The main changes observed in the spectra were related with biochemical and chemical compounds that might originate from the interactions or effects of the *T. ferdinandiana* extracts with the bacteria. These spectral changes were observed at the following wavelengths 370 nm, 390 nm, and 410 nm. Information contained in these wavelengths was associated with DNA, and with the occurrence of amino acids and proteins, as reported by other authors (Hu et al., 2017). A decrease in the absorbance values was observed between 370 nm and 410 nm directly associated with the increase in concentration of *T. ferdinandiana* extracts added to the broth. The changes in absorbance at these wavelengths indicated that the extracts have an effect on the growth of the bacteria. The main absorbance around 350 nm was present in the control samples (e.g. broth).

Fig. 2 shows the principal component score plot of the Gram positive and negative samples incubated with the addition of three levels of *T. ferdinandiana* extract as antibacterial agent. The first two principal components explained 99% of the variation in the data set. Panel A highlights the effect of the bacteria while Panel B the effect of the concentration of the *T. ferdinandiana* extracts. A clear trend or direction in the PCA scores showed that separation between the bacteria is mainly related with the addition of the extracts. This trend is mainly observed along PC1 (92% of the variation). As indicated by the PC loadings, wavelength at 400 nm explained the main differences in PC1 while wavelength are 380 nm and 450 nm explains the differences observed in PC2 (Fig. 3).

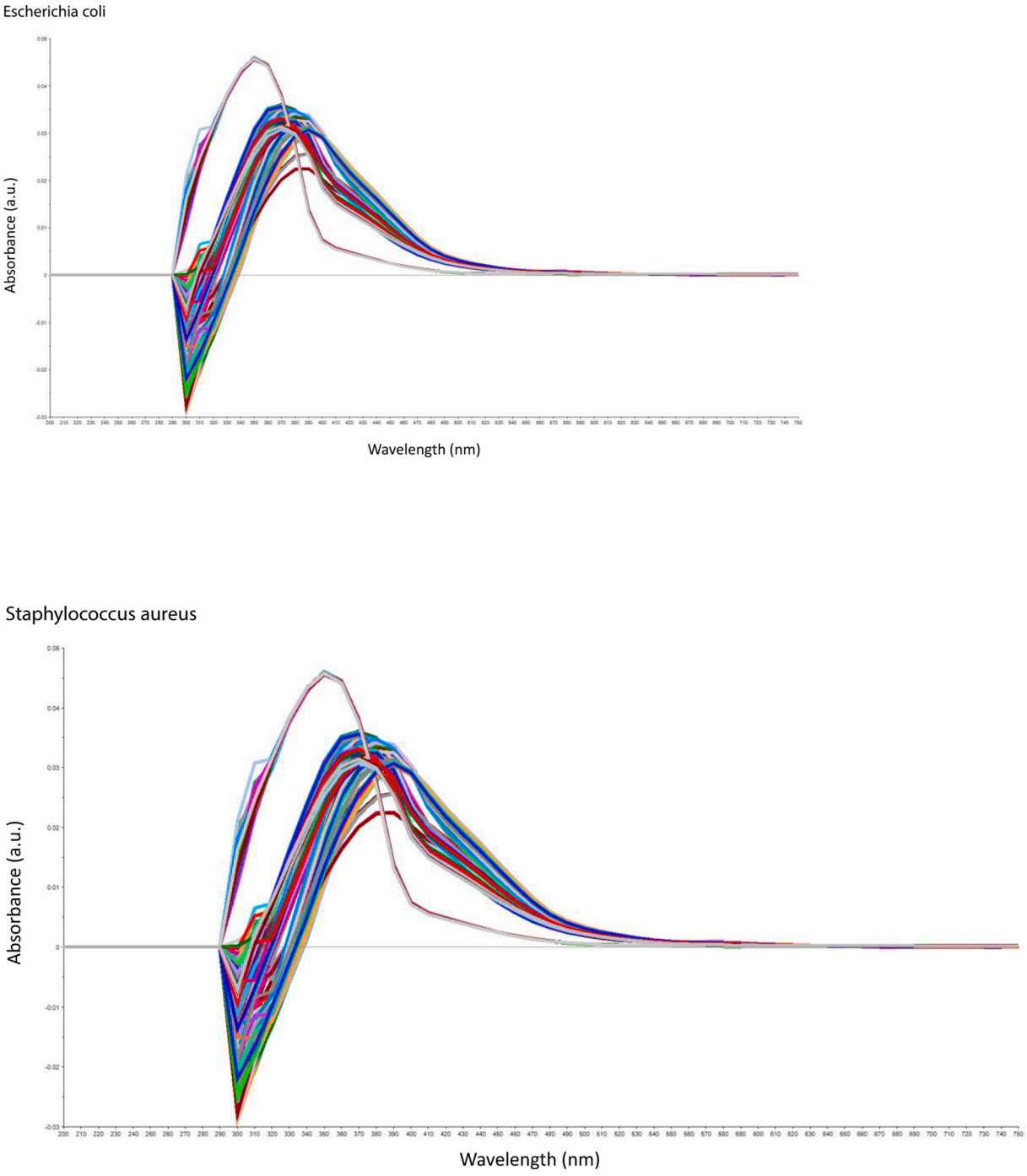
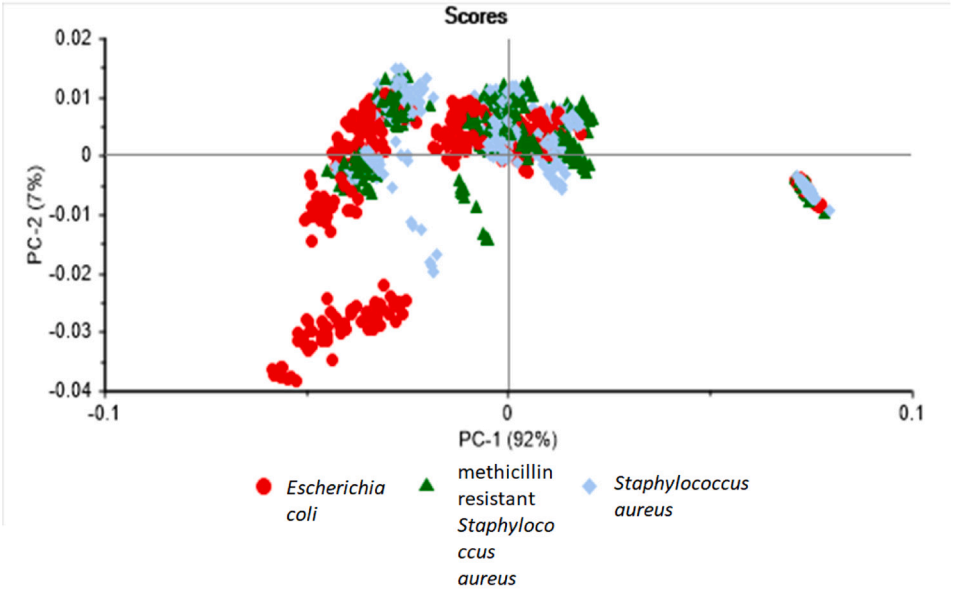


Fig. 1. Second derivative of the UV-VIS spectra of the bacteria samples with the addition of *T. ferdinandiana* extracts.

Panel A



Panel B

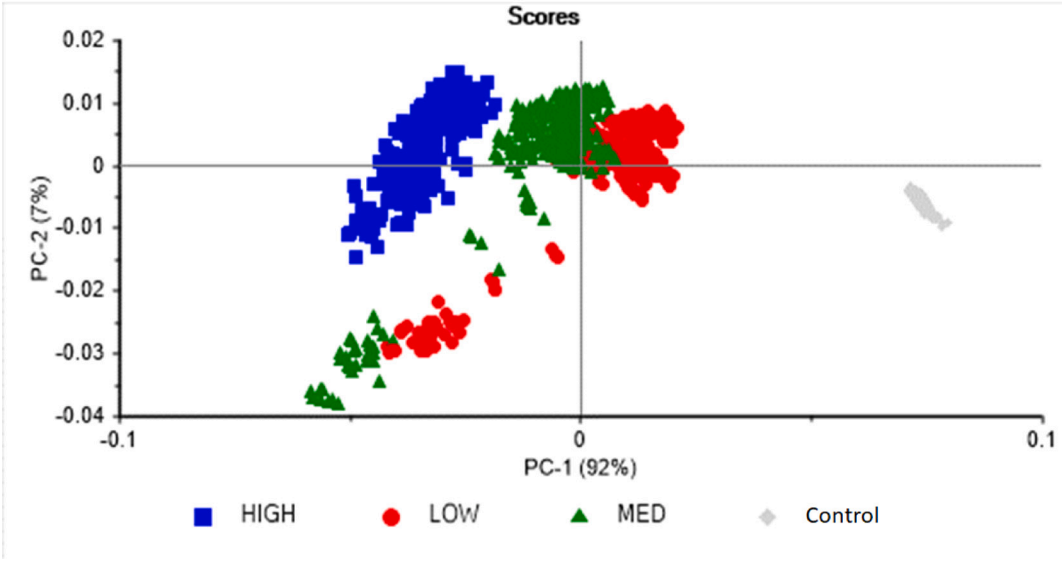


Fig. 2. Principal component score plot of bacteria samples analysed using UV-VIS with the addition of different levels of *T. ferdinandiana* extracts. Panel A samples labelled by type of microorganism, Panel B samples labelled by the concentration of *T. ferdinandiana* added to the culture.

Table 2 shows the statistics obtained from the PLS-DA regression models used to predict both bacteria type and concentration of *T. ferdinandiana* extracts. Excellent calibration statistics were obtained for the prediction of concentration of *T. ferdinandiana* extracts to the bacteria. In all the cases, the coefficients of determination (R^2) were higher than 0.95. However, the calibration models for the prediction of bacteria type (all samples) as well as the prediction of Gram-positive vs Gram-negative bacteria did not produce good calibration models. The best calibration ($R^2 = 0.52$) was obtained when the PLS models were used to predict Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. These results suggested that the system can easily monitor the different concentrations of *T. ferdinandiana* extracts in the broth.

Previous studies have demonstrated the ability of *T. ferdinandiana* fruit extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria (Akter et al., 2019). Reports have also demonstrated that this fruit contains high levels of antioxidants (Mohanty and Cock, 2012). It has been reported by other authors that *T. ferdinandiana* fruits has the highest recorded concentrations of ascorbic acid of any fruit in the world (Netzel et al., 2007). However, it is unlikely that ascorbic acid alone is responsible for the broad antibacterial activity where an interaction between ascorbic acid and polyphenols might be the explanation for its antibacterial activity. Previous studies have demonstrated that ascorbic acid alone displays only weak antibacterial activity towards *E. coli* and *S. aureus*, even at relatively high concentrations (Mohanty

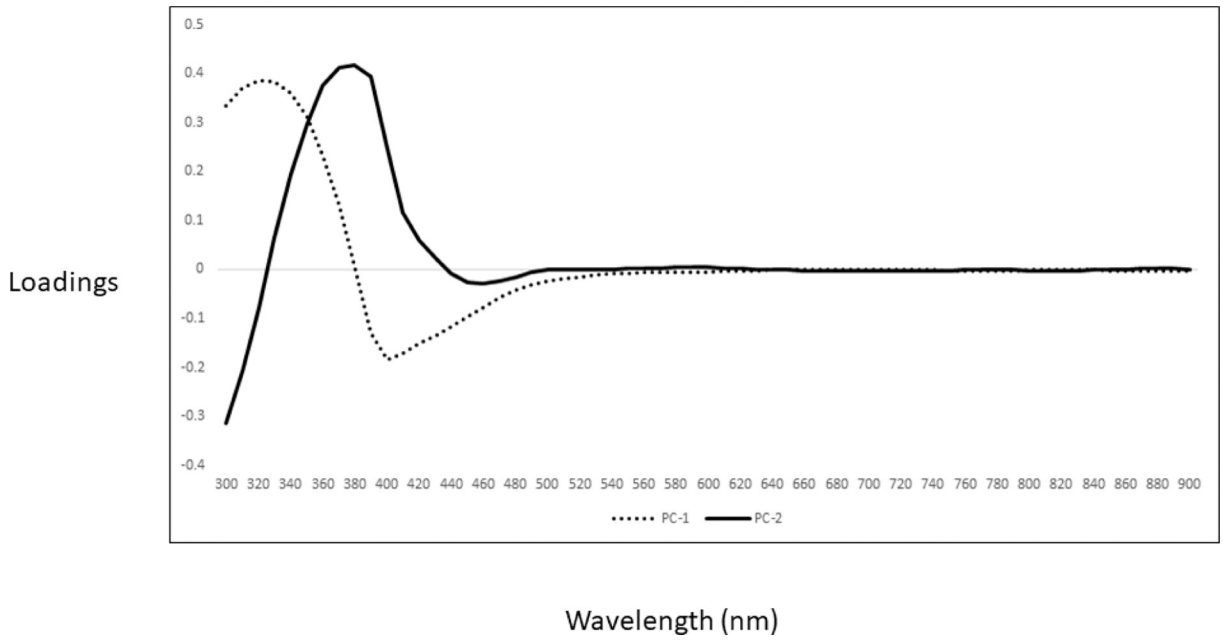


Fig. 3. Loadings derived from the principal component analysis of bacteria samples analysed using UV-VIS with the addition of different levels of *T. ferdinandiana* extracts.

and Cock, 2012). Instead, if ascorbic acid is involved in the antibacterial bioactivities reported here, it is more likely that it works in a synergistic manner with other *T. ferdinandiana* extract phytochemicals. Ascorbic acid has previously been shown to enhance the antibacterial activity of other polyphenolic compounds through an inhibition of the oxidation of these polyphenols (Mohanty and Cock, 2012). Previous studies by Akter and collaborators reported that methanol extracts were found to be the most effective against similar bacteria and showed a broad spectrum of antibacterial activity against the tested bacteria (Akter et al., 2019). The inhibition of the growth of bacterial strains by the fruit and leaf extracts could be due to the presence of antioxidant phytochemicals, mainly polyphenols, in the extracts (Akter et al., 2019).

The results from this study confirmed those previously reported that indicated the antimicrobial compounds available in *T. ferdinandiana* extracts might interfere with the bacterial cell walls and induce cell lysis. The presence of phenolic compounds and other low molecular weight compounds (e.g. K^+ and PO_4^{3-}) might also be responsible in enhancing the loss of other intracellular molecules, such as proteins, DNA, RNA, and other higher molecular weight materials. These antimicrobial compounds may even react with bacterial DNA, ultimately resulting in cell death (Akter et al., 2019).

Considering that the bacteria used in this study are food spoilage microorganism and pathogens for human, the results are of particular relevance. It is difficult to relate the antimicrobial activity of natural bioactive products as having antimicrobial activity. These results are of importance as it describes a rapid methodology to evaluate the antimicrobial activity of natural products using a 96-well plate reader system.

Table 2
Partial least squares discriminant (PLS-DA) analysis classification of the bacteria samples with the addition of *T. ferdinandiana* extracts.

	n	R^2	SECV	bias	slope	LV
Concentration all samples	864	0.98	0.67	0.004	0.98	9
Concentration *E. coli*	288	0.96	1.05	−0.06	0.96	8
Concentration MRSA	288	0.96	0.96	0.01	0.96	5
Concentration *S. aureus*	288	0.94	1.24	−0.001	0.94	3
Bacteria type all	1008	0.40	0.65	−0.0005	0.37	12
Bacteria Grams + vs -	672	0.52	0.34	−0.0006	0.53	12

R^2: coefficient of determination; SECV: standard error in cross validation; LV: latent variables.

4. Conclusion

The results of this study indicated that it is possible to record the UV-VIS spectra of the interaction between bacteria and *T. ferdinandiana* extracts using a 96-well plate reader. The method was able to detect or differentiate between live and dead bacteria based on the UV-VIS spectra as a function of the addition of the *T. ferdinandiana* extracts. Further studies are required to determine antimicrobial activity of different plant extracts and commercial antibiotic compounds against a range of microorganisms.

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Declaration of Competing Interest

The authors declares no conflict of interest.

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Hydrolysable tannins in *Terminalia ferdinandiana* Exell fruit powder and comparison of their functional properties from different solvent extracts

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ABSTRACT

This study identified and quantified hydrolysable tannins (HTs) in *Terminalia ferdinandiana* Exell (Kakadu plum) fruit, freeze dried powder extracted with 80% aqueous acetone (AA) and 80% aqueous acidified ethanol (AAE), using UHPLC–Q/Orbitrap/MS/MS. The vitamin C and ellagic acid were quantified by UHPLC-PDA. A total of seven HTs were identified: corilagin, 3,4,6-tri-O-galloyl-β-D-glucose, elaeocarpusin, chebulinic acid, chebulagic acid, helioscopin B, and punicalagin, with five classified as ellagitannins. The two extracts AA and AAE, comprised of gallic acid (2.5 and 2.2 mg/g DW), punicalagins α and β (2.8 and 1.3 mg/g DW), respectively, and both contained ellagic acid (~4 g/100 g DW). These extracts showed high antioxidant properties and strong antimicrobial effects against methicillin-resistant *Staphylococcus aureus* clinical isolate, *Staphylococcus aureus*, and *Shewanella putrefaciens*. These results suggest that Kakadu plum fruit is a rich, edible source of ellagitannins, ellagic acid and vitamin C with potential applications in food, cosmetic and nutraceutical industries.

1. Introduction

In recent decades, consumer awareness about the strong association between the consumption of fruits, vegetables and cereals and the reduction of risk of chronic non-communicable diseases (CNDs) has risen (Vargova et al., 2019). Epidemiological surveys have linked the health-promoting attributes of fruits, vegetables and cereals to the presence of (poly) phenolic compounds (Cory et al., 2018) with several supportive *in vitro* and *in vivo* studies (Fraga-Corral et al., 2020; Richmond et al., 2019). As (poly) phenolic compounds are present in many food sources, the total daily intake of the average person of these compounds are safe, but can also be considerable (1–2 g/day) depending on the food products ingested, (e.g. fresh, processed, blended) and the individuals' overall diet. (Ganesan & Xu, 2017; Landete, 2011).

(Poly) phenolic compounds have a wide range of structurally different molecules, such as flavonoids, stilbenes, lignans, tannins and phenolic acids, all of which have a phenolic nucleus in common (Ganesan & Xu, 2017). Among them, flavonoids are the most extensively studied of the more than 8,000 (poly) phenolic compounds isolated from fruits and vegetables (Cory et al., 2018). An important, but neglected, group among the (poly) phenolic compounds are tannins. Recent reports on the beneficial effects of tannins in the prevention and treatment of various pathological conditions (Smeriglio et al., 2017), have substantially enhanced scientific interest in tannins, which has consequently recently, become an emerging field in nutrition (Evtyugin et al., 2020).

Tannins are high molecular weight (500 up to 20,000 Daltons), natural (poly) phenolic compounds found in a variety of plant foods. Two classes commonly identified are: hydrolyzable tannins (HTs), specific to dicotyledonous angiosperms; and condensed tannins, ubiquitous in plants (Fraga-Corral et al., 2020). HTs contain both central sugar and hydroxyl groups, esterified by either hexahydroxydiphenic acid (HHDP) or gallic acid, which hydrolyse to produce ellagic acid - as in ellagitannins (ETs) and gallic acid - produced by gallotannins (GTs) (Clifford & Scalbert, 2000). Recently, nearly 500 HTs have been characterized, with ETs constituting the largest group (Fraga-Corral et al., 2020). These ETs, GTs and their markers (ellagic acid and gallic acid) are ingested by humans through plants, and plant based foods (Konczak et al., 2014; Netzel et al., 2007). However, the majority of HTs characterization to

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date has been carried out on the non-edible parts of the examined plants which are typically not consumed or placed in food products (Clifford & Scalbert, 2000), leading to limited information on the HTs in food products or plant ingredients, such as Kakadu Plum (KP) fruit powder. Consequently, plant products rich in HTs are now increasingly being studied for applications in the functional food industries (Evtyugin et al., 2020).

The Combretaceae plant family comprises plants commonly used in tropical and subtropical regions, as both food and for medicinal value (Mohanty & Cock, 2012). Approximately 250 species in the family belong to the genus *Terminalia* (Wright et al., 2019). About 28 *Terminalia* species are endemic to Australia (Mohanty & Cock, 2012) while 24 species are found in India (Amalraj & Gopi, 2017). The *Terminalia* species are widely used in the functional and nutraceutical industries owing to the therapeutically important composition of HTs and as a unique edible source of ellagic acid and ETs (Konczak et al., 2014; Pellati et al., 2013).

Terminalia ferdinandiana Exell, commonly known as Kakadu Plum (KP), is a native semi-deciduous Australian tree growing in the Northern Territory and Western Australia (Kimberley region) (Mohanty & Cock, 2012). It is has been a key species for Aboriginal Australians for thousands of years, from which native foods and medicines are sourced (Richmond et al., 2019); its fruits are consumed as food (Konczak et al., 2014) and its bark and leaves have been used to treat different ailments by Aboriginal Australians in accordance with their Traditional Knowledge of medicincinal uses of plants (Chaliha et al., 2017; Gorman et al., 2006).

In recent years, KP fruit extracts have shown anti-inflammatory (Chaliha & Sultanbawa, 2019), antioxidant (Konczak et al., 2014), anticancer (Shalom & Cock, 2018), and antibacterial (Akter, Netzel, et al., 2019; Cheesman et al., 2019) activity. Nutritive components (e.g. minerals, vitamins, sugars) as well as several bioactive secondary metabolites (Konczak et al., 2014; Williams et al., 2014) have also been reported in this fruit. Kakadu Plum particularly represents an exceptional source of vitamin C (Brand et al., 1982), a unique edible source of ellagic acid and ETs (Konczak et al., 2014; Williams et al., 2016, 2014). These exceptional properties have increased the commercial value of wild harvested KP. Approximately 17 tons of KP fruits are harvested annually (Richmond et al., 2019) across Australia, and KP products are widely marketed as ingredients of food supplements, preservatives, cosmetics, anti-aging and beverage products (Konczak et al., 2014; Richmond et al., 2019).

Notably, most (poly) phenolic compounds in plant material are commonly recovered by using organic solvents that might remain in both the ingredients and finished products. These residual solvents can sometimes be hazardous to human health, as well as the environment, having being classified by the International Conference on Harmonization (ICH) into Class I, Class II, and Class III (FDA, 2012) substances. Water, ethanol, acetone, and methanol are commonly used for the extraction of HTs (Williams et al., 2016). However, HTs are poorly soluble in water, and methanol is excluded for use in most products due to its inherent toxicity. Hence, the present study focused on identification and quantification of HTs in extracts prepared from Class III solvents, including ethanol and acetone, which are recommended due to a lower risk to human health (FDA, 2012). The use of aqueous solutions of these organic solvents have been proven to provide higher ETs yields than when the solvents are used in absolute concentration, as reported in previous studies (Konczak et al., 2014; Williams et al., 2016). Importantly, it has also been reported that some alcoholic solvents cleave GTs bonds at neutral pH (Mueller-Harvey, 2001). Therefore, 80% ethanol containing 0.1% HCl was selected to prevent this undesired reaction.

The present study aimed to identify and quantify HTs present in the freeze-dried KP fruit powder extracts, and evaluate their antimicrobal and antioxidant properties.

2. Materials and methods

2.1. Materials and chemicals

Commercial freeze-dried KP fruit powder, from the Northern Territory in Australia (Batch Number 16145) was purchased from Traditional Homeland Enterprise (T.H.E., Victoria, Australia). The samples were stored at −20 °C until analysis.

Polyphenol standards (ellagic acid, gallic acid, punicalagin α/β, chebulinic acid, L-ascorbic acid) were analytical grade with purity >99% and supplied by Sigma Aldrich (Castle Hill, New South Wales, Australia). HPLC grade organic solvents (ethyl acetate, ethanol, acetone, methanol, acetonitrile), formic acid, reagents (Folin–Ciocalteu's phenol and 2, 2-diphenyl-1-picrylhydrazyl (DPPH)) were also sourced from Sigma Aldrich. Sodium carbonate anhydrous were supplied by Chem-supply (Bedford St, Gillman, South Australia).

Standard plate count agar (PCA-CM0463) and Mueller-Hinton agar (MHA) were obtained from Oxoid Ltd (Basingstoke, UK). The bacterial strains were from the American Type Culture Collection (ATCC; Oxoid Ltd, Basingstoke, UK) and the National Collection of Type Cultures (NCTC; Health Protection Agency Centre for Infection, London, UK), and clinical isolate was from the Royal Brisbane and Women's Hospital (Herston, Queensland, Australia). They included Gram-positive: *Staphylococcus aureus* (NCTC 6571), Methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates (CIII) (MRSA3) (18/10/2012), *Bacillus cereus* (ATCC 10876), and Gram-negative: *Escherichia coli* (NCTC 9001), *Shewanella putrefaciens* (ATCC 49138). These bacteria were selected based on ability to cause spoilage in food, particularly in seafood and meat (*Shewanella putrefaciens)*, as indicator organisms for poor hygienic practices (*Escherichia coli*) and the potential to cause food poisoning (*Bacillus cereus, Staphylococcus aureus*) and disease (Methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates).

2.2. Identification and quantification of hydrolysable tannins by UHPLC-Mass spectrometry (MS)

2.2.1. Extraction of polyphenols

The samples were extracted according to Phan et al. (Phan et al., 2019) with slight modifications. Approximately 500 mg of KP powder (in triplicate) was extracted with 80% aqueous acetone, acetone, 80% aqueous acidified ethanol and ethanol on a reciprocating shaker (RP1812, Paton Scientific, Adelaide, SA, Australia) (15 min, 200 rpm). The mixtures were placed in a sonication bath for 15 min, then centrifuged at (3900 rpm, 10 min; Eppendorf Centrifuge 5804, Hamburg, Germany). The supernatants were collected and filtered through 0.2 μm PTFE filters (Millipore, USA) prior to UHPLC-Q/Orbitrap/MS/MS analysis.

2.2.2. UHPLC–Q/Orbitrap/MS/MS analysis

A Thermo high resolution Q Exactive focus mass spectrometer (Thermo Fisher Scientific Australia Pty Ltd., Melbourne, VIC, Australia), equipped with a DIONEX Ultimate 3000 UHPLC-UV/Vis, was used for analysis following the method previously described by Phan et al., (2019) and Simirgiotis et al., (2016). The compounds were separated on a Waters HSS T3 analytical column (150 × 2.1 mm i.d; 1.8 μm) maintained at 40 °C, with 0.1% formic acid in Milli-Q water as mobile phase A and acetonitrile containing 0.1% formic acid as mobile phase B. The flow rate was 0.4 mL/min with gradient elution for B as follows: 1% B isocratic elution for 3 min, 45% B over 15 min, and 100% B by 18 min. The column was re-equilibrated for 7 min before the next injection. The injection volume was 0.6 μL and the UV chromatogram was monitored at 250 and 360 nm.

The Q-Exactive mass spectrometer was operated in negative ionization mode with full MS and all-ion-fragmentation (AIF) scans at a resolving power of 70,000 full width at half maximum. A scan range of *m/z* 100–1500 and 80–1200 was applied for the full MS and AIF scans,

respectively. The automatic gain control (AGC) was set at 3E6 and the injection time to 200 ms, the ions were filtered by the quadrupole, operating at an isolation window of 4.0 *m/z*. The Thermo Xcalibur™ software (Thermo Fisher Scientific) was used for data acquisition.

2.3. Extraction and analysis of ellagic acid

The extraction and analysis of ellagic acid and ETs were carried out according to the method described previously in Williams et al (2014) and Singh et al (2016) with slight modifications.

2.3.1. Extraction of free and total ellagic acid

Triplicate samples (~100 mg) were mixed with 5 mL of the extracting solvents including 80% aqueous acetone and 80% aqueous acidified ethanol, vortexed and sonicated for 10 min. The mixture was centrifuged (≈3220 × g, 5 min at 20 °C; Eppendorf Centrifuge 5810 R, Hamburg Germany) and the supernatant was collected and combined (referred as extract A). Next, the pellets were re-extracted 3 times using ethanol (for 80% aqueous acidified ethanol) or acetone (for 80% aqueous acetone) following the procedure described above and referred as extract B. All the extractions were conducted in triplicate. Two millilitres of the extract A were added into a 5 mL Reacti-Therm (Fisher Scientific, Bellefonte, PA, USA) vial containing a stirring bar, and subjected to overnight hydrolysis of ETs released in the extract A at 90 °C using 2 M HCl to determine the total ellagic acid (referred as extract C) (Williams et al., 2014).

2.3.2. UPLC-PDA analysis

Ellagic acid released in the extracts A, B and C was analysed using a Waters Acquity™ UPLC-PDA System. The separation was carried out using a Waters BEH Shield RP C18 column (100x2.1 mm i.d; 1.7 μm) at 35 °C. The mobile phases included 0.1% formic acid in Milli-Q water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). The flow rate was 0.3 mL/min with gradient elution for mobile phase B as follows: 35% B isocratic elution for 5 min, 50% B over 10 min and 100% B by 15 min. The column was re-equilibrated for 7 min before the next injection. An aliquot of 2 μL of sample was injected and the PDA detector was monitored from 190 to 400 nm with a rate of 2.5 Hz. The chromatogram was extracted at 254 nm. An external calibration curve of ellagic acid was prepared to quantify the levels of free ellagic acid (extracts A and B) and the total ellagic acid (extracts B and C).

2.4. Determination of vitamin C content

Extraction and analysis of vitamin C, including L-ascorbic acid (L-AA) and dehydroascorbic acid (DHAA), in the KP powder followed the method previously described by Phan et al (Phan et al., 2019) adapted from Spinola et al (Spínola et al., 2012) and Campos et al (Campos et al., 2009). A Waters Acquity™ UPLC-PDA System (Waters, Milford, MA, USA) was used for the anaysis. A Waters HSS-T3 analytical column (100 × 2.1 mm i.d; 1.8 μm) maintained at 25 °C was used to seperate the compound with an isocractic elution (0.1% formic acid as moble phase) at a flow rate of 0.25 mL/min. An external calibration curve of standard L-AA (192.5 μg/mL to 0.77 μg/mL) was used to quantify vitamin C content.

2.5. Determination of antimicrobial activity

The 80% aqueous acetone and 80% aqueous acidified ethanol extracts (described in section 2.2.1) were concentrated using a miVac sample Duo concentrator (Genevac Ltd., Ipswich, UK) and lyophilized at −40 °C, under vacuum (-100 kPa) (Lindner & May Ltd, Windsor, Brisbane). The freeze-dried extract was freshly reconstituted in 20% ethanol to give two different concentrations of 20 and 50 mg/mL before antimicrobial analysis.

Food and disease related microorganisms including Gram-positive and Gram-negative bacteria were used for the antimicrobial test employing a well diffusion assay as previously reported (Phan et al., 2019). A mixture of penicillin and streptomycin (1 μg each/10 mL prepared in Milli-Q water) (Gibco, Life Technologies, Melbourne, VIC, Australia) was also included in the assay as a positive control.

Additionally, the minimum inhibitory concentration (MIC) was determined by the microplate dilution method using flat bottom 96-well sterile microtiter plates (Sultanbawa et al., 2009). Only the most sensitive strains from the agar well diffusion assay results were assessed for MIC and MBC tests. Briefly, a loop of culture from the *S. aureus*, *S. putrefaciens* and MRSA3 slope was placed in 10 mL of tryptone soy yeast extract broth (TSYEB) and incubated at 37 °C for 24 h. This culture was adjusted to an absorbance reading of 0.5 at 600 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Sydney, Australia) in TSYEB broth and further diluted to obtain 10^5 cfu/mL, using peptone and TSYEB. The MIC was evaluated by placing 100 μL extracts (prepared to obtain the final concentration of 4, 3, 2, 1 and 0.5 mg/mL), 50 μL nutrient broth (NB), and 50 μL inoculum (10^5 cfu/mL), respectively, in the extract wells. In parallel, each negative control well contained 200 μL NB alone while the positive control well contained 50 μL bacterial inoculum and 150 μL NB. Then, the microplates were incubated at 37 °C and the MIC values were determined as the minimum concentration at which no visible bacterial growth was observed. To determine the minimum bactericidal concentrations (MBCs), a 50 μL aliquot from all wells showing no visible bacterial growth in the MIC assay was applied to MH plates and incubated at 37 °C for 24 h. The lowest concentration of the extract that showed no growth was taken as the MBC (Akter, Netzel, et al., 2019).

2.6. Total phenolic content (TPC) and DPPH radical scavenging assay

Antioxidant properties of the extracts 80% aqueous acetone and 80% aqueous acidified ethanol (described in section 2.2.1) were assessed employing TPC and DPPH radical scavenging assays. TPC was determined using a Folin–Ciocalteu assay (Singleton & Rossi, 1965), using a micro-plate absorbance reader (Infinite M200, Tecan Austria GmbH, 5082 Grodig, Austria) monitored at 700 nm. TPC was quantified using an external standard curve of gallic acid (12–120 μg/mL) and expressed as gallic acid equivalents (GAE) per g in dry weight (DW).

The DPPH radical scavenging assay was performed according to the method previously reported by Moore & Yu (Moore & Yu, 2007) using a micro-plate absorbance reader (Infinite M200, Tecan Austria GmbH, 5082 Grodig, Austria) monitored at 517 nm. The blank and control were methanol and 0.15 mM DPPH solution, respectively. The percentage of DPPH radical scavenging was calculated as follows:

$$\%\ DPPH\ inhibition = ((\text{Control absorbance-Sample absorbance})/\text{Control absorbance}) * 100.$$

The pellets obtained from the extraction for the TPC and DPPH analysis (section 2.2.1) were also subjected to alkaline hydrolysis to release bound phenolic compounds using 3 M NaOH. The samples were mixed on a reciprocating shaker (RP1812, Paton Scientific, Adelaide, SA, Australia) for 1 h at 200 rpm. Then, the hydrolysate was extracted with ethyl acetate, as previously reported (Phan et al., 2019). The precipitates obtained after ethyl acetate evaporation were redissolved in 5 mL ethanol and subjected to TPC assay for quantification of bound TPC.

2.7. Statistical analysis

All the experiments were carried out in triplicate and the results represent the mean of triplicate measurements ± standard error of the mean (SEM). The mean difference between extracts was compared by one-way ANOVA, followed by Tukey post hoc test using SPSS statistical package version 20.0 software (SPSS Inc. Chicago, IL, USA). A value of $p < 0.05$ was considered as significant.

3. Results and discussion

3.1. Identification and quantification of hydrolysable tannins in different extracts

The present study reported HTs found in KP fruit powder using different extract solvents including 80% aqueous acetone and 80% aqueous acidified ethanol, ethanol and acetone. The retention time (Rt) and mass spectral features of major peaks extracted from UHPLC-Q/Orbitrap/MS/MS are displayed in Table 1, and Supplementary Figs. S1-9. A total of nine compounds were identified: two peaks (1 and 9) corresponding to phenolic acids, five peaks (2, 3, 5, 6, and 8) could be identified as ETs, and two peaks (4 and 7) belong to GTs. Additionally, four (poly) phenolic compounds (peaks 1, 2, 8 and 9) were confirmed with the aid of authentic standards, while the remaining five compounds were identified by comparing the deprotonated molecular ion peaks $[M-H]^-$ and MS^2 fragments with data from literature. These results showed that ellagic acid was the main peak (Rt = 11.76 min; Fig. S1), supporting previous studies (Chaliha et al., 2017; Konczak et al., 2014) which found that KP fruit is rich in this compound.

Peak 4 released an $[M-H]^-$ at *m/z* 635 and $[2\,M-H]^-$ at *m/z* 1271.2 (Table 1). This compound additionally showed a characteristic fragment $[M-H-170]^-$ at *m/z* 465 which resulted from the loss of gallic acid and this could be identified as 3, 4, 6-tri-O-galloyl-β-D-glucose (MW 636.5 g/mol), which is in agreement with a previous report on *Terminalia chebula* Retz fruit (Pellati et al., 2013). Peak 3, eluted at Rt 10.39 min, presented characteristic ETs like mass fragments associated with loss of gallic acid $[M-H-170]^-$ and galloylglucose group $[M-H-170-162]^-$. Literature related to ETs also reported that the compound with an $[M-H]^-$ ion at *m/z* 633.07 and respective mass fragments of $[M-H-170]^-$, *m/z* 463 and $[2\,M-H]^-$ at 1267.14 was identified as corilagin (MW 634.5 g/mole) in *T. chebula* ayurvedic decoction, and chestnut (Pellati et al., 2013; Venter et al., 2019). Peak 5 could be identified as chebulagic acid as this compound produced a deprotonated molecular ion $[M-H]^-$ at *m/z* 953.09 together with an additional fragment at *m/z* 301 confirming the existence of HHDP- characteristic group of ETs. Similar MS^2 data was also reported for chebulagic acid in *T. chebula* (Pellati et al., 2013). Peak 6 had the $[M-H]^-$ and MS^2 fragmentations (*m/z* at 554, 463, 301) that are typical of elaeocarpusin as previously characterised by Yang and colleagues (Yang et al., 2012). Conversely, analysis of mass spectra revealed a deprotonated molecular ion peak at *m/z* 1111 eluted after 11.54 min (peak 7). This compound was tentatively identified as helioscopin B based on the information retrieved from *Euphorbia helioscopia* L study (Lee et al., 1990). Additionally, the co-elution of ellagic acid and chebulinic acid was observed (Rt 11.75 and 11.76 min) (Figs. S1 and 2) and their identification were performed based on the Rt, and mass features of the corresponding authentic standards.

Previous studies have isolated and structurally confirmed common HTs (punicalagin, corilagin, 1,3,6-trigalloyl glucose, elaeocarpusin, chebulagic acid, helioscopin B and chebulinic acid) by HPLC/QTOF/MS and UHPLC-triple quadrupole MS/MS from the methanolic extract of KP fruits (Akter, et al., 2019; Cheesman et al., 2019; Mohanty, 2016). In parallel, ETs including punicalagin, chebulagic acid, corilagin, punicalin, and chebulinic acid were identified in KP leaves (Courtney et al., 2015). Aligned with these findings, our study also identified identical (poly) phenolic compounds in the commercial freeze-dried KP fruit powder extracts.

HTs are typically present in many of the plant families which hold economic importance (Evtyugin et al., 2020). For instance, punicalagin, corilagin, and 3, 4, 6-tri-*O*-galloyl-*β*-D-glucose are found in high concentrations in pomegranate (Wang et al., 2018). The majority of the currently identified HTs were frequently reported in a number of *Terminalia* species (Chandrasekhar et al., 2018; Pellati et al., 2013), except for the HTs elaeocarpusin and helioscopin B. Some of these *Terminalia* are popular as functional food and nutraceutical agents. For instance, *T. chebula* fruit extracts retained high levels of 3, 4, 6-tri-*O*-galloyl-*β*-D-glucose, punicalagin, corilagin, chebulinic acid, and chebulagic acid showed to have neuroprotective, anti-inflammatory and anticancer activities (Chandrasekhar et al., 2018; Pellati et al., 2013).

Further semi-quantitative analysis of the identified HTs, based on GAE (Table 2), indicated that corilagin (503.6 ± 57 mg GAE/100 g DW in 80% aqueous acetone , 424.3 ± 35 mg GAE/100 g DW in 80% aqueous acidified ethanol) was the dominant ($p < 0.05$) HT in the functional KP fruit extracts, followed by 3,4,6-tri-*O*-galloyl-*β*-D-glucose, elaeocarpusin and chebulinic acid. The levels of chebulagic acid and helioscopin B were also substantial. The analysed content of these HTs, where KP fruits exhibited high corilagin contents, were in agreement with the previous reports (Cheesman et al., 2019; Mohanty, 2016). More so, quantification of punicalagins (α and β), using authentic standard, also showed that 80% aqueous acetone and 80% aqueous acidified ethanol gave high levels of punicalagin (281.2 ± 16.6 mg/ 100 g DW and 129.1 ± 0.1 mg/g DW respectively), whereas it was not detected or present at trace amounts in the ethanol and acetone extracts. Conversely, the present KP extracts recovered a significant level of gallic acid that ranged from 53 to 253 mg/100 g DW (Table 2). In particular, the 80% aqueous acetone and 80% aqueous acidified ethanol extracts exhibited almost three times as much gallic acid as previously reported in KP fruits (Konczak et al., 2014); 2 to 21 times the amount of gallic acid found in the peel of six pomegranate cultivars (0.12 to 1.14 mg/g DW) (Yan et al., 2017). The increased gallic acid in this study, could be attributed to the gallic acid obtained from the hydrolysis of gallotannins. As a result, the present study further assessed functional properties of

Table 1
Tentatively identified hydrolysable tannins in KP fruit 80% aqueous acidified ethanol extract employing UHPLC-Q/Orbitrap/MS/MS.

Peaks	Rt (min)	$[M-H]^-$ *m/z*	MS/MS (*m/z*)	Tentatively identified/ confirmed compounds	Molecular weight (g/mole)	References
1	4.23	169.01	125.02	Gallic acid ($C_7H_6O_5$)	170.12	Confirmed by standard
2	9.72	1083.18	600.99, 541.03, 301	Punicalagin ($C_{48}H_{28}O_{30}$)	1,084.71	Confirmed by standard
3	10.39	633.07	1267.14 $[2\,M-H]^-$, 463, 338.96, 301, 275.02	Corilagin ($C_{27}H_{22}O_{18}$)	634.5	(Pellati et al., 2013; Venter et al., 2019)
4	10.67	635.1	1271.2 $[2\,M-H]^-$, 465.07, 461.17, 317.04, 169.01	3,4,6-Tri-O-galloyl-β-D-glucose ($C_{27}H_{24}O_{18}$)	636.5	(Pellati et al., 2013; Venter et al., 2019)
5	11.10	953.09	663.51, 476.04, 301.09, 168.99, 125.02	Chebulagic acid ($C_{41}H_{30}O_{27}$)	954.66	(Pellati et al., 2013)
6	11.17	1109.10	554.05 $[M-2H]^{2-}$, 463.05, 301, 175.02	Elaeocarpusin ($C_{47}H_{34}O_{32}$)	1110	(Yang et al., 2012)
7	11.54	1111.11	937.08, 767.08, 617.08, 604.51, 555.05, 173.08, 169.01	Helioscopin B ($C_{47}H_{36}O_{32}$)	1112	(Lee et al., 1990)
8	11.75	955.11	785, 477.05, 465.07, 617.08, 169.01, 125.02	Chebulinic acid ($C_{41}H_{32}O_{27}$)	956.67	Confirmed by standard and (Pellati et al., 2013; Sobeh et al., 2019)
9	11.76	301	145.03	Ellagic acid ($C_{14}H_6O_8$)	302.197	Confirmed by standard

Rt: Retention time.

Table 2
The contents of hydrolysable tannins in KP fruit extracts.

Extract	Hydrolysable tannins contents (mg GAE/100 g DW)						Gallic acid (mg/100 g DW)	Punicalagin α/β (mg/100 g DW)
	Chebulinic acid	Chebulagic acid	Corilagin	Elaeocarpusin	Helioscopin B	3,4,6-tri-o-galloyl-β-D-glucose		
AA	101.2 ± 6.1^{ABa}	50.0 ± 2.5^{Aa}	503.6 ± 57.0^{Ca}	130.7 ± 2.2^{ABa}	31.5 ± 2.8^{Aa}	212.0 ± 12.8^{Ba}	253.2 ± 6.7^{a}	281.2 ± 16.6^{a}
AAE	94.6 ± 4.0^{ABa}	37.6 ± 4.0^{ABb}	424.3 ± 35.0 ^{Da}	104.0 ± 7.6^{BCb}	24.1 ± 2.9^{Aa}	173.4 ± 13.3^{Ca}	216.6 ± 21.6^{a}	129.1 ± 0.1^{b}
E	10.5 ± 2.5^{ABb}	1.4 ± 0.4^{Ac}	106.7 ± 17.4^{Cb}	3.3 ± 0.9^{Ac}	1.4 ± 0.4^{Ab}	39.8 ± 9.5^{Bb}	113.1 ± 22.4^{b}	ND
A	5.6 ± 0.8^{Ab}	1.0 ± 0.2^{Ac}	42.5 ± 8.0^{Bb}	1.5 ± 0.3^{Ac}	0.6 ± 0.04^{Ab}	16.2 ± 2.8^{Ab}	53.4 ± 9.0^{b}	ND

Values are expressed as mean ± SEM (n = 3). Mean comparison using One-Way ANOVA followed by Tukey's Post Hoc analysis except for punicalagin. Different capital letters (A-F) in the same row for phenolic contents indicate significant difference ($p < 0.05$), Different small letters (a-c) in the same column indicate significant difference ($p < 0.05$). mg GAE/100 g DW: milligram gallic acid equivalent per 100 g dry weight, E: ethanol, A: acetone, ND: not detected.

different solvent extracts from KP fruit powder mainly focusing on antimicrobial activities and antioxidant capacity.

3.2. Quantification of ellagic acid and vitamin C

Ellagic acid and vitamin C are key antioxidant functional elements widely reported in KP fruits. A summary in Table 3 shows the level of ellagic acid that exists as free form ranging from 811.1 ± 17.5 to 889.1 ± 2.8 mg/ 100 g DW. These results are in accordance with previous findings (Williams et al., 2016) on the aqueous acetone extract of commercial KP fruits (856.50 mg/ 100 g DW) and free ellagic acid content found in *T. chebula* fruits (8 mg/g DW) (Pellati et al., 2013). However, these amounts are lower than the average free ellagic acid contents of KP fruits collected from Northern Territory (Konczak et al., 2014), which clearly reflects the influence of genetic diversity, growing region, soil, processing, and the specific collection times during harvest season (Konczak et al., 2014; Williams et al., 2016).

Ellagic acid is the main phytochemical responsible for the functional and nutraceutical properties of strawberries (da Silva Pinto et al., 2008), pomegranates, walnuts, longan seeds, and mango kernels (Chaliha & Sultanbawa, 2019; Evtyugin et al., 2020; Landete, 2011). However, these plant foods have variable proportions of free ellagic acid because the ellagic acid accumulates predominantly in the form of ETs (Williams et al., 2014). In some instances however, the level of free ellagic acid could exceed 50% of the total ellagic acid content (Williams et al., 2016). Thus, food samples must be hydrolysed to release conjugated ellagic acid from tannin compounds so that total ellagic acid can be measured, and ETs can be indirectly estimated as ellagic acid equivalent by subtracting the amount of free ellagic acid from the total ellagic acid after hydrolysis.

Recently, Evtyugin and co-authors (Evtyugin et al., 2020) have reviewed the levels of free and total ellagic acid present in various fruits, seeds and nuts following the above mentioned analysis approach. The review showed that the highest free ellagic acid amounts were reported in Camu-camu fruit flour (764.9 mg/Kg DW) and pomegranate peel (637.7 mg/Kg DW), results which are lower than our findings in KP fruit powder, as displayed in Table 3. More importantly, the ellagic acid found to exist dominantly as ETs in the analysed KP samples as acid hydrolysis of ETs, released up to 80% and 76% conjugated ellagic acid in 80% aqueous acetone and 80% aqueous acidified ethanol extracts, respectively (Table 3). It is also important to note that the free to total ellagic acid ratio, of 20% in 80% aqueous acetone and 24% in 80% aqueous acidified ethanol were found in the present study: this is up to eight times higher than the free ellagic acid proportion in strawberry fruits of Piedade cultivar grown in the Sao Paulo state in Brazil (da Silva Pinto et al., 2008), and New Zealand grown boysenberries (Williams et al., 2014).

The above results demonstrated that ellagic acid levels in KP fruits are much higher than levels found in many plant foods including berries and pomegranates (Evtyugin et al., 2020). The unique composition may arise from the selective pressure of the growing environment, exposure to light and / or limited nutrients (Gorman et al., 2020). Many tropical plants encounter stress early in their young and maturing stages and subsequently elicit the phenylpropanoids pathway, enabling the biosynthesis of secondary metabolites, including polyphenols, to protect themselves (Toscano et al., 2019). In addition, the vitamin C in KP (Fig. 1) might indirectly contribute to the observed high level of ellagic acid and ETs by reducing oxidative inactivation during times of stress (Williams et al., 2014).

The forms of ellagic acid can influence the antioxidant properties of ETs-rich plants because the higher number of hydroxyl functioning in ETs, donate hydrogen atoms, which neutralize reactive radicals (Landete, 2011). These effects have been observed by the strong antioxidant capacity of the studied extracts (Table 3) that might in part be related to the level of ETs, 2814.4 ± 100.1 mg EAE/100 g DW 80% aqueous acidified ethanol to 3221.8 ± 49.5 mg EAE/g DW 80% aqueous acetone.

Furthermore, a significant difference ($P < 0.05$) in the concentrations of free ellagic acid and total ellagic acid were noted between the extracting solvents (Table 3). Aqueous acetone gave the maximum total ellagic acid (4032.9 ± 39.4 mg/100 g DW) and ETs (3221.8 ± 49.5 mg EAE/ 100 g DW) that significantly differed from the 80% aqueous acidified ethanol extracts (total ellagic acid = 3703.5 ± 97.4 mg/100 g DW and ETs = 2814.4 ± 100.1 mg/100 g DW). In contrast, the 80% aqueous acidified ethanol showed higher efficiency in retaining free ellagic acid compared to 80% aqueous acetone ($p < 0.05$).

L-ascorbic acid (L-AA), dehydroascorbic acid (DHAA) and total

Table 3
The total phenolic (TPC), percentage of DPPH radical scavenging and ellagic acid contents in the freeze-dried KP fruits powder.

Extracts	Ellagic acid			TPC (mg GAE/g DW)			% DPPH radical scavenging capacity
	FEA (mg/ 100 g DW)	ETs (mg EAE/ 100 g DW) &	TEA (mg/ 100 g DW)	Free	Bound	Total	
AAE	889.1 ± 2.8^{a}	2814.4 ± 100.1^{a}	3703.5 ± 97.4^{a}	170.7 ± 4.3^{a}	1.5 ± 0.1^{a}	172.2 ± 4.5	61.9 ± 1.1^{a}
AA	811.1 ± 17.5^{b}	3221.8 ± 49.5^{b}	4032.9 ± 39.4^{b}	142.9 ± 7.8^{a}	0.6 ± 0.02^{b}	143.5 ± 7.9	64.5 ± 1.8^{a}

Values are expressed as mean ± SEM, n = 3. Mean comparison using One-Way ANOVA followed by Tukey's Post Hoc analysis. Mean with different letters in the same column indicate significant difference ($p < 0.05$). AA, AAE represent 80% aqueous acetone, 80% aqueous acidified ethanol, respectively; mg EAE: milligram ellagic acid equivalent; mg GAE/g DW: milligram gallic acid equivalent per gram dry weight, FEA:- free ellagic acid, TEA:- total ellagic acid &calculation adopted from Williams et al (2014).

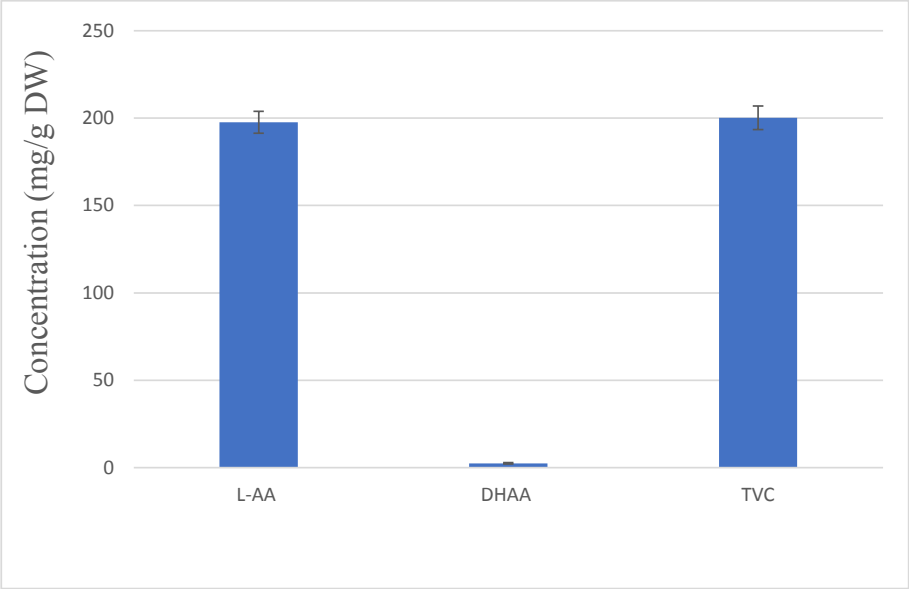

Fig. 1. Vitamin C contents of the freeze-dried powder. Values are presented as mean ± SEM of triplicate measurements. TVC: total vitamin C contents.

vitamin C results are presented in Fig. 1. The KP sample had 197.6 ± 6.3 mg/g DW L-AA, which is comparable with a previous report on KP fruits from CSIRO Site, Northern Territory (NT) (197 ± 59 mg/g DW) (Konczak et al., 2014), and Kakadu plum puree processed in other parts of the Northern Territory (191.8 mg/ g DW) (Chaliha et al., 2017). However, the L-AA of commercial KP freeze dried powder (118.6 ± 18.9 mg/g DW) is lower (Williams et al., 2014) than these previous reports. Despite the variability of vitamin C among the KP fruits, the overall high levels make it a unique dietary source of vitamin C. While there is no confirmed reason for the variability the difference in genetic make-up and growing environment might have favoured high vitamin C production in KP (Carr & Rowe, 2020; Gorman et al., 2020; Toscano et al., 2019).

Humans cannot synthesize Vitamin C, which our bodies use for growth and repair of tissues, and for its high antioxidant activity (Campos et al., 2009; Spínola et al., 2012). Vitamin C includes all compounds exhibiting equivalent biological activity to L-AA, including its oxidation products (DHAA) (Spínola et al., 2012). Since over 85% of humans' dietary vitamin C is obtained from fruit and vegetables (Spínola et al., 2012), it is important to measure both L-AA and DHAA to accurately measure the total vitamin C contents in different foods and food ingredients. The analysed KP fruits contained a total vitamin C content of 200.1 ± 6.7 mg/g DW. Dehydroascorbic acid (DHAA) was only 1.3% of the total vitamin C content (Fig. 1) similar to many horticultural crops where DHAA represents less than 10% (Lee & Kader, 2000).

Additionally, countries have different guidelines for optimal dietary intake of vitamin C based on its role in preventing scurvy. In United States and Canada, the Recommended Dietary Allowance (RDA) is at 90 mg/day and 75 mg/day for men and women, respectively (Fenech et al., 2019). However, encouraging people to attain these recommended levels through adequate intake from fresh fruits and vegetables, where at least one component has high vitamin C per serving of food levels (Carr & Rowe, 2020; Lee & Kader, 2000), is still quite challenging. Adding KP as a functional ingredient to foods to meet these dietary requirements has significant potential.

3.3. Antimicrobial properties of different extracts

Microbial growth inhibitions by 80% aqueous acetone and 80% aqueous acidified ethanol extracts, prepared at 20 and 50 mg/mL, are given in Table 4. The extracts were effective growth inhibitors of MRSA3, *S. aureus* and *S. putrefaciens* which can cause disease and food spoilage (Bouarab Chibane et al., 2019; Dickmann et al., 2017). The two extracts with a MIC and MBC of 3 and 4 mg/mL respectively, showed antimicrobial activity against *S. putrefaciens*, MRSA3 and *S. aureus* refer to Table 4.The current results also revealed that *E. coli* was resistant whereas strong inhibition (≥13 mm) of MRSA3 growth has been observed at each tested dose (20 mg/mL and 50 mg/mL). This is consistent with a previous study on KP (Akter, et al., 2019; Chaliha et al., 2020; Cheesman et al., 2019; Wright et al., 2019) where the fruit and leaf extracts showed inhibitory effects on the growth of MRSA, *S. aureus* and *S. putrefaciens,* but failed to inhibit *E. coli* growth. Similarly, the crude extracts of *T. bellerica* fruit and *T. catappa* leaves showed strong inhibition against *S. aureus* (Allyn et al., 2018; Elizabeth, 2005). Interestingly, our results and the previous findings demonstrated the sensitivity of Gram- positive bacteria in comparison to Gram-negative bacteria. Both methicillin resistant and sensitive *S. aureus* strains to KP products gives an important insight to the antibacterial action mechanisms of the extracts. The Gram-negative (e.g. *E. coli*) resistance is notable in numerous studies because the thick outer lipid layer around the bacterial cell wall restricts the influx of bioactive extracts (Teixeira et al., 2013).

The growth inhibitory effects of the extracts at each concentration (20 mg/mL and 50 mg/mL) did not significantly vary ($P > 0.05$) among MRSA3, *S. aureus* and *S. putrefaciens*. The strong inhibition zones produced by 20 mg/mL dose on MRSA3 were 15.1 ± 0.3 and 13.3 ± 0.4 mm for 80% aqueous acetone and 80% aqueous acidified ethanol extracts, respectively, while the 50 mg/mL of each extract showed respective zones of inhibition at 19.8 ± 0.2 and 16.9 ± 0.5 mm. Both extracts exhibited strong inhibition (x ≥ 13 mm) against *S. aureus* and *S. putrefaciens* growth at 50 mg/ mL concentration. Different responses have also been revealed when the sensitivity of the tested bacteria was assessed. The order of sensitivity at 20 mg/mL dose of each extract was MRSA3 > *S. putrefaciens* > *S. aureus* > *B. cereus* > *E.coli.* The 50 mg/mL concentration inhibited the growth in the following order: *S. aureus* > MRSA3 > *S. putrefaciens* > *B. cereus* > *E.coli.* The 20 mg/mL dose of 80% aqueous acetone extract significantly inhibited ($p < 0.05$) MRSA3 growth compared to its effect on *B. cereus* and *E. coli* while the 50 mg/mL dose showed a significant ($p < 0.05$) MRSA3 inhibitory activity *in* comparison to S. *putrefaciens, B. cereus* and *E. coli*.

The results of the present study showed that ellagic acid, gallic acid,

Table 4
The zone of inhibition (mm), the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of KP fruits extracts against tested microorganisms (zone of inhibition is to the nearest mm).

Microbes	20 mg/ml		50 mg/ml		Standard	Ethanol (20%, v/v)	MIC (mg/ml)		MBC (mg/ml)	
	AAE	AA	AAE	AA			AAE	AA	AAE	AA
B. cereus	5.0 ± 0.2^{aA} +	5.2 ± 0.1^{aA} +	6.4 ± 0.8^{aA} +	11.0 ± 0.3^{aA} ++	22 ± 0.1	N	ND	ND	ND	ND
E. coli	4.5 ± 0.1^{a} -	N	5.1 ± 0.6^{aA} +	1.5 ± 0.6^{bA} -	19.5 ± 0.5	N	ND	ND	ND	ND
MRSA3	13.3 ± 0.4^{bA} +++	15.1 ± 0.3^{bA} +++	16.9 ± 0.5^{bA} +++	19.8 ± 0.2^{cB} +++	22.5 ± 1.5	N	3	3	4	4
S. aureus	10.2 ± 0.1^{bA} ++	9.6 ± 0.7^{cA} ++	17.6 ± 1.5^{bA} +++	20.6 ± 0.1^{cA} +++	39 ± 2.0	N	3	3	4	4
S. put	11.4 ± 1.2^{bA} ++	10.9 ± 0.8^{cA} ++	15.7 ± 1.2^{bA} +++	14.2 ± 0.8^{dA} +++	25.5 ± 0.5	N	3	3	4	4

Values are expressed as mean ± SEM of triplicate measurements. AAE: − 80% aqueous acidified ethanol, AA: − 80% aqueous acetone, *B. cereus*: - *Bacillus cereus*, *E. coli*: – *Escherichia coli*, *MRASA3*: – Methicillin resistant *Staphylococcus aureus* clinical isolate, *S. aureus:* – *Staphylococcus aureus, S. putrefaciens Shewanella putrefaciens,* N: zero zone of inhibition, (-) no activity ($x < 5$ mm), (+) weak activity ($5 \leq x \leq 6$ mm), (++) moderate activity ($6 < x \leq 12$ mm), (+++) strong activity ($x \geq 13$ mm), where × is zone of inhibition. Standard is a mixture of penicillin and streptomycin.
Different capital letters in the same row and Different small letters in the same column under each tested dose indicate significant difference ($p < 0.05$). ND not determined, as only the most sensitive strains from the agar diffusion assay were selected for MIC and MBC assessments.

and ETs are abundant in KP fruits. These compounds displayed strong antibacterial activities (Li et al., 2018). There are also several similar reports (Evtyugin et al., 2020; Francisco Javier et al., 2020; Savic et al., 2019) showing plants that contain ETs and ellagic acid exhibited potent anti-microbial activity. Similarly, Bobinaitė and colleague (Bobinaitė et al., 2013) found that raspberry marc extracts with the highest level of TPC and ETs displayed the highest inhibitory effect against Gram-positive bacteria. Likewise, the KP fruit extracts showed inhibitory and bactericidal effects against Gram-positive bacteria and this most probably is associated with, but not limited to the identified HTs. The antimicrobial efficacy differences between this study and others reported in the literature could be due to the different assays used to determine antimicrobial properties of the tested extracts rather than the starting material (freeze dried KP fruit powder). Finally, the extracts maybe used as a safe and non-toxic natural preservative substituting synthetic antimicrobials to maintain quality and safety of food.

3.4. TPC and antioxidant activity of different extracts

The TPC and DPPH radical scavenging capacity of the functional extracts is summarized in Table 3. The results demonstrate that the extracts gave high TPC (up to 171 mg GAE/ g DW) and displayed more than 60% DPPH radical scavenging power.

In a previous study conducted by Konczak et al (Konczak et al., 2014) on KP fruits, it was shown that 80% aqueous acidified methanol gave the highest TPC, which ranged from 121.5 to 505.2 mg GAE/g DW. The authors also showed that the extract retained a low level of flavonoids (0.53 to 2.08 mg rutin equivalent/g DW), hydroxycinnamic acid (0.3 to 1.6 mg caffeic acid equivalent/g DW) and proanthocyanidins (0.01 to 1.97 mg catechin equivalent/g DW). These results found by Konczak et al (Konczak et al., 2014) affirm the dominance of HTs and ellagic acid as the main (poly) phenolic compounds in KP fruits, similar to the current assay (Tables 1 and 3).

HTs and ellagic acid are well known, natural antioxidants (Landete, 2011). In addition to their important nutritional and health contributions; these antioxidant properties, expressed as TPC and DPPH radical scavenging (Table 3), are used in the food industry to prolong the stability of foods, particularly for the prevention of lipid oxidation in food products (Akter, Netzel, et al., 2019; Konczak et al., 2014). Hence, these properties are another mechanism by which KP has potential application in food preservation and shelf life extension. Moreover, these properties have prompted the use of antioxidant rich KP fruit powder as a functional ingredient to prevent the impact of diseases attributed to oxidation processes.

The TPC of the residue left after extraction was also determined for the first time and displayed in Table 3 as a marker of bound phenolics. The pellet left after 80% aqueous acetone extraction gave the lowest TPC (0.6 ± 0.02 mg GAE/g DW) while the 80% aqueous acidified ethanol extracts residue had the highest (1.5 ± 0.1 mg GAE/g DW). The yield of each residue was significantly different ($p < 0.05$) showing the significant level of bound (poly) phenolics inaccessible to solvents during extraction. These compounds are non-absorbable but hydrolysed in the intestine and biotransformed by colon microbes into small bioaccessible metabolites whose health benefits have been recognized in several studies (Landete, 2011).

4. Conclusions

Nine (poly) phenolic compounds were identified in 80% aqueous acidified ethanol extracts and 80% aqueous acetone functional extracts of the KP fruit powder. They were identified as ETs (5 peaks), GTs (2 peaks), and phenolic acids (2 peaks). The main HTs identified in the two extracts were corilagin ($p < 0.05$) followed by 3, 4, 6-tri-*O*-galloyl-*β*-D-glucose, elaeocarpusin, chebulinic acid, chebulagic acid, and helioscopin B, respectively. Furthermore, 80% aqueous acetone and 80% aqueous acidified ethanol extracts retained significant levels of gallic acid and punicalagin. Ellagic acid, the main bioactive compound in KP, was also retained at a high level (~4 g/ 100 g DW) in the two studied extracts. Further analysis of these ellagic acid and ETs-rich extracts demonstrated the strong antimicrobial activities against MRSA3, *S. aureus* and *S. putrefaciens* growth. The extracts also exhibited high TPC and DPPH radical scavenging capacity. Significantly, a high total vitamin C (200.1 ± 6.7 mg/g DW) in the freeze-dried KP powder was shown, demonstrating that KP fruit possesses multiple health benefits. Overall, the present findings showed that the 80% aqueous acetone and 80% aqueous acidified ethanol extracts of freeze-dried KP fruit powder are a rich source of ETs, vitamin C and ellagic acid, providing a wide choice for potential application in diet diversity, shelf life extension, and as a functional ingredient to be used in the nutraceutical, cosmetic, beverage and pharmaceutical industries.

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CRediT authorship contribution statement

Eshetu M. Bobasa: Methodology, Investigation, Data curation, Writing - original draft. **Anh Dao Thi Phan:** Conceptualization, Methodology, Data curation, Writing - original draft. **Michael E. Netzel:** Supervision, Writing - review & editing. **Daniel Cozzolino:** Visualization, Data curation, Supervision, Writing - review & editing. **Yasmina Sultanbawa:** Conceptualization, Investigation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129833.

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Effects of drying methods and maltodextrin on vitamin C and quality of *Terminalia ferdinandiana* fruit powder, an emerging Australian functional food ingredient

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Abstract

BACKGROUND: *Terminalia ferdinandiana*, common name Kakadu plum (KP), fruit is a valuable source of vitamin C, and its concentration can be used as a quality index of KP products, such as dried fruit powder. The present study investigated the effects of two drying methods (freeze-drying and oven-drying) and the addition of maltodextrin (0–25%) on vitamin C, Maillard products, and overall quality of KP fruit powder.

RESULTS: Freeze-drying was a better dehydration technique than oven-drying in retaining vitamin C, reducing the formation of non-enzymatic browning and oxidation products, and improving powder colour ($P < 0.05$). Non-enzymatic browning products (furfural and 5-hydroxymethyl furfural) were generated in the oven-dried samples as a function of heating and high water activity. Maltodextrin acted as a vitamin C stabilizer in protecting vitamin C from oxidation, and significantly improved the colour attributes of the final dry products. Incorporation of 10–15% maltodextrin could reduce the percentage loss of vitamin C from 8.1% to 3.4% and 18.9% to 11.4% (compared with the control) during freeze-drying and oven-drying, respectively. Scanning electron micrographs revealed differences in the microstructures of the KP powder processed by the two drying methods with different levels of maltodextrin. Multivariate data analysis (principal component analysis) showed separation between the oven-dried and freeze-dried samples, and also suggested that addition of maltodextrin of 7.5–10% and 10–15% are effective for preserving vitamin C and other quality properties of the freeze- and oven-dried KP powder samples, respectively.

CONCLUSION: The results obtained are important for the KP industry, including Indigenous enterprises, in selecting the most appropriate drying method for KP fruit in terms of quality and sustainability.

Supporting information may be found in the online version of this article.

Keywords: *Terminalia ferdinandiana*; Kakadu plum; maltodextrin; ascorbic acid oxidation; non-enzymatic browning; drying techniques

INTRODUCTION

Terminalia ferdinandiana, common name Kakadu plum (KP), belonging to the family Combretaceae, is endemic to Australia and has a long history of traditional medicinal applications and food cuisine by the Australian Indigenous people.[1] *T. ferdinandiana* fruit is an excellent source of vitamin C (ascorbic acid (AA)), containing up to 320 g kg^{-1} dry weight (DW) or 53 g kg^{-1} fresh weight (FW),[2,3] which is significantly higher than other common fruit sources of AA, such as orange ~0.53 g kg^{-1} FW,[4] apple ~0.56 g kg^{-1} FW,[5], and feijoa ~0.63 g kg^{-1} FW.[6] AA is essential for human health owing to its biological functions as a cofactor for enzymes involved in wound healing processes and collagen synthesis, as well as its antioxidant properties.[7] The recommended dietary intake of AA in Australia and New Zealand is 45 mg per day for adults.[8] This means that a daily consumption of only 0.8–1.6 g fresh KP fruit – calculated on the reported AA content of 27.4–53 g kg^{-1} in fresh fruit[3] – would be necessary to fulfil the recommended dietary intake of AA.

There is a growing demand for natural food ingredients from plant sources with bioactive properties for applications in the food and nutraceutical industries. *T. ferdinandiana* fruit is

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commercially used in Australia as a fresh/frozen fruit, frozen puree, and dried fruit powder and can be 'classified' as an emerging functional ingredient. KP fruit powder is one of the most common commercially used products, as it has advantages of long shelf life, ease of transport, and being odourless. For the production of KP powder, freeze-drying and oven-drying have been applied, with both having advantages and disadvantages. Freeze-drying is considered as one of the most effective drying methods to obtain a premium-quality product with high retention of bioactive compounds and nutrients; however, it is costly, time-consuming, and more complex in terms of operation than the oven-drying method.[9,10] Although oven-drying is one of the most economically, convenient, and easy-to-do processes, exposure to high temperature in the presence of oxygen can cause significant losses of heat-labile nutrients and bioactive compounds, and can induce the disintegration of food structure and undesirable changes in sensory attributes of the final products.[9] This is particularly true for *T. ferdinandiana* fruit with extremely high levels of AA – a highly unstable and sensitive compound to oxygen, temperature, light exposure, pH, metal ions, and high water activity.[11,12]

Degradation of AA in dry products is a main reason causing loss of nutrients during drying and storage, as reported previously in processed KP[12] and other dried fruits.[13,14] The degradation of AA is a complex process and can occur via two principal pathways: oxidative and non-oxidative degradations.[15,16] In the presence of oxygen, AA is quickly oxidized to form dehydroascorbic acid (DHAA) and 2,3-diketogulonic acid as main intermediates. As DHAA is highly unstable, it can be further degraded and subjected to intermolecular rearrangement reactions to form a variety of five-membered aromatic ring products (C5-products) such as 2-furoic acid, 3-hydroxy-2-pyrone, and 5-methyl-3,4-dihydroxytetrone.[15–17] In contrast, under anaerobic, strong acid-catalysed, and low moisture content condition, both AA and DHAA together with dicarbonyl groups generated during the AA degradation are precursors of numerous chemical reactions to form off-flavour and brownish-coloured compounds.[16,18,19] A strong relationship between AA degradation and the formation of off-flavour and brownish-coloured compounds was reported in orange juice and West Indian cherry juice respectively.[18,19] Among the AA degradation products, furfural was determined as one of the main compounds derived from non-oxidative degradation, especially in concentrated fruit juices, after heat treatment and storage at high temperature (>38 °C).[20,21] Besides furfural, 5-hydroxymethyl furfural (5-HMF), the most undesirable of Maillard products, is also generated during drying processes and considerably affects the sensory attributes and quality of the dry products.[22]

KP fruit is a valuable source of AA, and its retention can be used as a quality index of dried KP products. Therefore, it is important to monitor the drying process and to develop protocols to minimize the loss of AA during processing and storage. Maltodextrin has been widely applied as a carrier for spray-dried food materials that are difficult to dry through the capacity to reduce the sugar-caused stickiness.[23] Moreover, maltodextrin has shown the ability to preserve AA and antioxidant capacity in several dried food products during drying and long-term storage, such as camu-camu,[24] acerola powder,[25] and amla juice powder.[26] Possessing a film-forming capacity and appropriate physical properties, maltodextrin can protect AA,[11,25,27] and other susceptible bioactive compounds like carotenoids,[28] against exposure to heat, light, and oxidation.

Although quality changes associated with drying of fruit products have been reported, there is still limited information, and no study has reported the effects of different drying methods and maltodextrin on the degradation of AA and quality of KP powder, an understudied native fruit. The present study aimed to investigate the effects of two different drying methods (freeze-drying and oven-drying) and the addition of maltodextrin at different concentrations as a carrier on the stability of AA, Maillard products, and physico-chemical parameters of KP powder. The results obtained will add more information to the literature and suggest to the KP industry, including Indigenous enterprises, the most appropriate drying method and suitable levels of maltodextrin for KP powder production in terms of quality and sustainability.

MATERIAL AND METHODS

Chemicals and reagents

L-AA, 5-HMF, furfural, and furoic acid (HPLC grade) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All other chemicals and solvents (HPLC grade) were sourced from Merck (Darmstadt, Germany) or Sigma-Aldrich.

KP powder production

Commercial frozen additive-free KP puree (~15 kg), purchased from Traditional Homeland Enterprises Holding Co Pty Ltd (Morwell, VIC, Australia), was thawed at 4 °C overnight and used for KP powder production. Maltodextrin (food grade) with dextrose equivalent (DE) of 17–19 from Manildra Group (Gladesville, NSW, Australia) was completely pre-dissolved in water before adding to the KP puree at different levels from 5% to 25% (w/w). Maltodextrin at the specific DE of 17–19 was selected based on its physical–chemical properties, including high viscosity, solubility, and hygroscopicity, which are suitable for fruit powder production.[29] The blends (~500 g) were homogenized for 4 min at maximum speed using a high-speed homogenizer (Ultra-Turrax® T25; IKA, Königswinter, Germany). The homogeneous mixtures were smeared into rectangular stainless steel trays (50 cm × 30 cm × 5 mm, length × width × depth) and subsequently subjected to either freeze-drying (−48 °C, 0.390 hPa) for 7 days to obtain a moisture content of ~1% in the final freeze-dried product (Lab Gear SCANVAC, Brisbane, QLD, Australia) or oven-drying (45 ± 2 °C, air velocity at 1.0 ± 0.1 m s^{-1}, humidity 30 ± 1.8%; Steridium, Brisbane, QLD, Australia) for 3–4 days until the weight of the final products was unchanged within 2 h (Table 1). The KP puree without maltodextrin was processed under the same conditions as the treated samples and included as a control. After drying, the samples were ground into a fine powder using a blender (Waring®8010/8011; Waring Laboratory, Sydney, NSW, Australia). The powdered samples were passed through a 200 μm sieve to obtain a uniform particle size and stored in air-tight containers at −80 °C for further analysis. All experiments were conducted in duplicate.

Analysis of AA

Extraction and analysis of vitamin C (AA) were conducted according to the method described previously.[6] Briefly, 200 mg of powdered sample or 1 g of puree was extracted with 3% metaphosphoric acid containing 8% acetic acid (v/v) and 1 mmol L^{-1} ethylenediaminetetraacetic acid. The reduction of DHAA, which was also present in the extracts/samples, to L-AA was performed following the method of Spinola *et al.*[30] prior to ultra-performance liquid chromatography coupled with photodiode array (UPLC-PDA) analysis. Vitamin C (L-AA + DHAA) was determined using a Waters Acquity™ UPLC-PDA system (Waters,

Table 1. Physico-chemical characteristics and drying time of the Kakadu plum (KP) puree and the KP blends with added maltodextrin at different levels

Samples	Maltodextrin (%, w/w)	Moisture content (g/100 g)	Total soluble solids (°Bx)	pH	Drying time (days)	
					Freeze-dried	Oven-dried
KP blends	5	90.3 ± 0.2 b	7.8 ± 0.2 f	3.37 ± 0.01 a	7	3
	7.5	88.7 ± 0.1 c	9.6 ± 0.1 e	3.36 ± 0.02 a		
	10	87.1 ± 0.1 d	11.4 ± 0.2 d	3.36 ± 0.01 a		
	15	84.1 ± 0.3 e	14.7 ± 0.1 c	3.36 ± 0.03 a		
	20	80.7 ± 0.1 f	18.2 ± 0.4 b	3.36 ± 0.01 a		4
	25	77.7 ± 0.1 g	21.4 ± 0.3 a	3.36 ± 0.01 a		
KP puree (control)	0	93.3 ± 0.2 a	4.4 ± 0.1 g	3.37 ± 0.01 a	7	3

Data presented as mean plus/minus standard deviation ($n = 4$); Different letters in the same columns indicate significant differences at $\alpha = 0.05$.

Milford, MA, USA) and a Waters HSS-T3 column (150 mm × 2.1 mm i.d.; 1.8 µm) at 25 °C with aqueous 0.1% formic acid as mobile phase (0.3 mL min^{-1}) and isocratic elution. L-AA was detected at 245 nm, identified and quantified by comparison with a commercial standard. An external calibration curve of L-AA was used for quantification, and vitamin C results are expressed as grams per kilogram DW. The extraction was conducted in duplicate.

Analysis of furoic acid, furfural, and 5-HMF

Extraction and analysis of furoic acid, furfural, and 5-HMF were conducted following the methods described by Yuan and Chen[16] and Korbel *et al.*,[20] with modifications. Briefly, 200 mg sample was homogenized with 50% methanol (v/v) using a vortex and subsequently placed in an ultrasonication bath (Elma Transsonic Digital T840 DH; Elma GmbH, Singen, Germany) for 30 min at room temperature, followed by centrifugation at 1800 × *g* for 10 min (Eppendorf Centrifuge 5804; Eppendorf, Hamburg, Germany). Supernatants were retained, and residues were re-extracted twice following the procedure already described. The supernatants were combined and injected into the Waters UPLC-PDA system. The compounds were separated on a Waters HSS-T3 column (150 mm × 2.1 mm i.d.; 1.8 µm) maintained at 25 °C using 95% aqueous acetonitrile containing 0.1% formic acid (v/v) as mobile phase (0.3 mL min^{-1}) and isocratic elution. The compound identification and quantification were conducted at 250 nm for furoic acid and 280 nm for furfural and 5-HMF, using an external standard-mix calibration. The results are expressed as milligrams per kilogram DW. The extraction was conducted in duplicate.

Browning index

Browning index values of the extracts (obtained as in previous section) were determined following Righetto and Netto,[18] using a microplate absorbance reader (Sunrise Tecan, Männedorf, Switzerland) at 420 nm. The browning index was expressed as absorbance at 420 nm/g DW of KP powder.

Total soluble solids and pH

The KP blends (~10 g) were mixed with an equivalent amount of water using a vortex and centrifuged at 1800 × *g* for 5 min at room temperature (Eppendorf Centrifuge 5804). The supernatants were collected and total soluble solids (degrees Brix) and pH measurements were made using a digital (0–85%) refractometer (Atago, Tokyo, Japan) and a Metrohm Karl Fischer pH meter (Metrohm, Herisau, Switzerland) respectively. The measurements were conducted in duplicate.

Chroma colour

A hand-held colorimeter (Konica Minolta CR-400; Thermo Fisher Scientific Pty Ltd, Brisbane, QLD, Australia) was used for measuring the colour properties (lightness L^*, red to green a^*, and blue to yellow b^*) of the KP powder samples. The measurements were conducted in duplicate by directly placing the colorimeter sensor on the surface of the samples to minimize the dispersion of light to the surroundings. To facilitate comparison in colour attributes between the samples having maltodextrin and the control, total colour differences ΔE were generated according to

$$\Delta E = \left[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\right]^{1/2}$$

where ΔL, Δa, and Δb are respectively the differences in lightness–darkness value, red–green axis, and yellow–blue axis between the added maltodextrin samples and the control.

Moisture and water activity

Moisture content was determined according to AOAC method 934.01.[31] For water activity, ~ 2 g of the powdered samples was placed in a standard measuring cup and the water activity was determined using a LabTouch-a_w water-activity meter (Novasina AG, Lachen, Switzerland) at a constant temperature of 25 ± 1 °C and an average stable scanning mode. The measurements were conducted in duplicate.

Scanning electron microscopy

The powdered samples were sprinkled on adhesive tapes and attached to scanning electron microscopy (SEM) specimen holders, followed by coating with argon. The microstructure of the samples was observed using a JSM-5000 NEOSCOPE scanning electron microscope (Jeol, Tokyo, Japan) operated at an accelerating voltage of 10 kV. Multiple SEM images were captured at different positions between 4400× and 5500× magnification.

Statistical analysis

Data were analysed using a general linear model with interaction, followed by Tukey's method of multiple comparison using Minitab 17 for Windows (Minitab Inc., State College, PA, USA). A *P*-value of ≤0.05 was used to determine significant differences. Pearson's correlation coefficient analysis was also applied to test

the strength of linear associations between variables (measured parameters) using Minitab 17. Principal component analysis (PCA) was used to analyse the data using a more holistic approach. The PCA models were developed using The Unscrambler X (Camo Analytics AS, Oslo, Norway) with leave-one-out cross-validation. In total, 11 variables consisting of all measured parameters were normalized (1/standard deviation) prior to generating the PCA models.

RESULTS AND DISCUSSION

Physico-chemical characteristics of the KP blend mixtures and the puree

Table 1 presents the moisture and total soluble solid contents (degrees Brix) of the blends and the control sample. Statistically significant ($P < 0.05$) differences were observed among the samples as a consequence of the addition of maltodextrin. The data showed an increase in total soluble solids and a significant drop in the moisture content as a direct effect of maltodextrin addition. The pH was unchanged, independently of the addition of maltodextrin ($P > 0.05$; Table 1). Under the conditions of the present study, the samples with higher maltodextrin levels (20% and 25%) allocated to the oven-drying treatment took longer to dry until unchanged weight within 2 h (4 days) compared with those having less than 20% maltodextrin (3 days). This might be explained by a rise in thickening and cohesive forces within the KP blend matrix derived from the starch-like properties of maltodextrin.[32] The differences in the physico-chemical parameters and heating exposure times among the treatments indicated potential differences in AA retention and oxidation behaviour, as well as in the quality of the final dry products that are discussed in the following sections.

Moisture content and water activity

Statistically significant ($P < 0.05$) differences in the moisture content and water activity a_w were observed among the samples studied (Fig. 1). The freeze-drying process has shown more effectiveness in removing water from the KP puree. The freeze-dried samples had significantly ($P < 0.05$) lower moisture contents and a_w (below 1% and 0.1 respectively) than the oven-dried samples (moisture contents of 3.5–7.1% and a_w of 0.31–0.58) (Fig. 1). Although the difference in the moisture content between the two drying treatments was maximal (7%), a larger variation in the a_w values was observed (below 0.1 *versus* 0.31–0.58). High water activity may affect the powder stability and product shelf life, as a lower a_w may inhibit microbial activity and minimize chemical reactions. Compared with the control, the addition of maltodextrin increased water absorption of the KP blends, which might lead to higher moisture content and consequently a longer drying time observed in the oven-dried samples. However, this phenomenon was not observed in the freeze-dried samples as this process allowed water removal from frozen materials through sublimation under a reduced pressure (Fig. 1).[33]

Colour appearance

The colour characteristics (L^*, a^*, and b^*) of the powdered samples were significantly ($P < 0.05$) different, which was related to the effect of both temperature and maltodextrin addition (Fig. 2). The oven-dried samples had lower L^* and higher a^* and b^* values than the freeze-dried samples. This could be due to the higher temperature used in the oven drying (45 °C) enabling the formation of yellow–brownish compounds through non-enzymatic browning reaction,[20, 22] resulting in the higher b^* values observed for the oven-dried powder ($P < 0.05$; Fig. 2(C)). Incorporating maltodextrin into the KP puree could also improve the colour quality of the final dried products. An increase in L^* (lightness) and decreases in a^* (red–green) and b^* values (blue–yellow) were observed in the samples containing maltodextrin compared with the controls, particularly the freeze-dried samples ($P < 0.05$; Fig. 2 (A)–(C)). Despite maltodextrin having been added to the KP puree, the oven-dried samples showed no significant changes in a^* and b^* values in comparison with the control ($P < 0.05$, Fig. 2(B), (C)), probably due to the drying being undertaken at high temperature. Several workers have also reported an improvement in the colour quality of several fruit juice powder products incorporating maltodextrin and processed by freeze-drying[33] or spray drying.[26] The total colour difference present in Fig. 2(D) also reflects obvious changes in the overall colour attributes of the KP powder products containing maltodextrin compared with the control.

Microstructure of the powdered KP samples

Representative SEM micrographs (Fig. 3) show different irregular microstructures of the powdered samples obtained from the two drying methods with different maltodextrin levels. There were several holes distributed evenly in the microstructure of the freeze-dried sample without maltodextrin (Fig. 3(A)). However, these holes did not appear in the microstructures of the other samples analysed. The freezing process performed at −80 °C before the freeze-drying resulted in the formation of ice crystals, which were subsequently sublimed during freeze-drying. Consequently, this caused the formation of tiny holes on the particle surface. These holes can be the space filled by the KP fruit fibres. Similar observations have been reported in freeze-dried blackberry powder.[34] The powder particles containing holes might favour the oxidation of AA as a result of a combined effect of free circulation of oxygen within the sample matrix and an increase in exposure area to oxygen. The results from SEM clearly showed the effect of the two drying methods on the microstructure of the KP dry products. The oven-dried samples showed dense and shrinkage microstructures (Fig. 3(E)–(H)). In contrast, the freeze-dried samples with added maltodextrin had a fine, smooth, and loosening microstructure (Fig. 3(B)–(D)), particularly the samples containing high levels of maltodextrin (10–25%). Consequently, it might help to reduce AA oxidation as maltodextrin has shown a potential to entrap bioactive compounds.[35] The presence of cracks, dents, and roughness on the dried particles' surfaces may adversely affect the flow ability and reconstitution properties.[36]

AA degradation

The original AA content of the KP puree used in the present study was 212 ± 2 g kg^{-1} DW (21.2 g kg^{-1} FW with 90% moisture content). Therefore, monitoring the loss of this bioactive compound and maintaining high levels are of importance for the quality of KP powder. In this study, AA was highly unstable and sensitive to heat, as it was degraded during the sample preparation and drying processes. Statistically significant ($P < 0.05$) differences in the reduction of AA were observed from 212 ± 2 g kg^{-1} to 196 ± 5 g kg^{-1} and 173 ± 2 g kg^{-1} for the freeze- and oven-dried samples respectively (Fig. 4(A)). The oven-drying method caused the percentage loss of AA to be approximately three times higher than that after freeze-drying (14.4–18.9% *versus* 3.3–8.1% DW respectively; Fig. 4(B)), indicating the effect of drying methods (e.g. high temperature) on AA degradation. Sommano *et al.*[12] reported the percentage loss of AA in KP fruits at

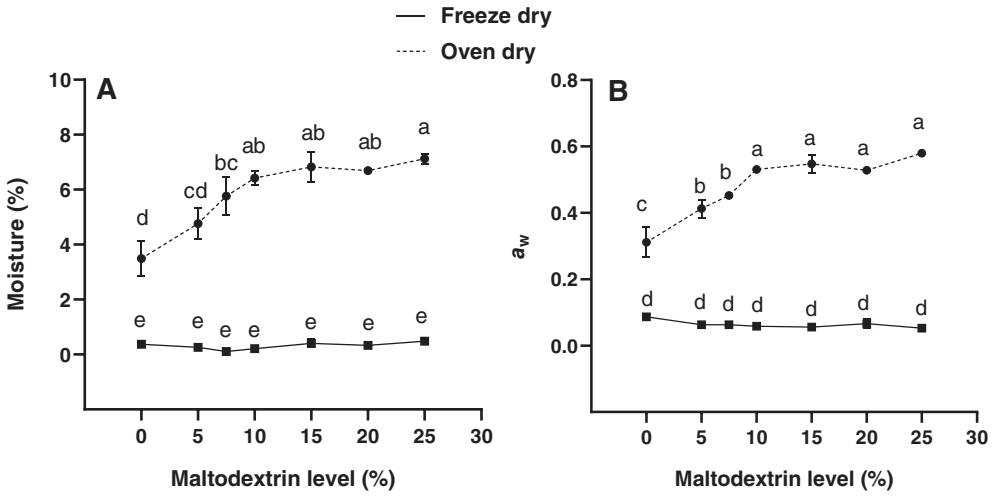

Figure 1. (A) Moisture content and (B) water activity of Kakadu plum powdered samples processed by freeze-drying and oven-drying. Different letters indicate significant differences at $\alpha = 0.05$.

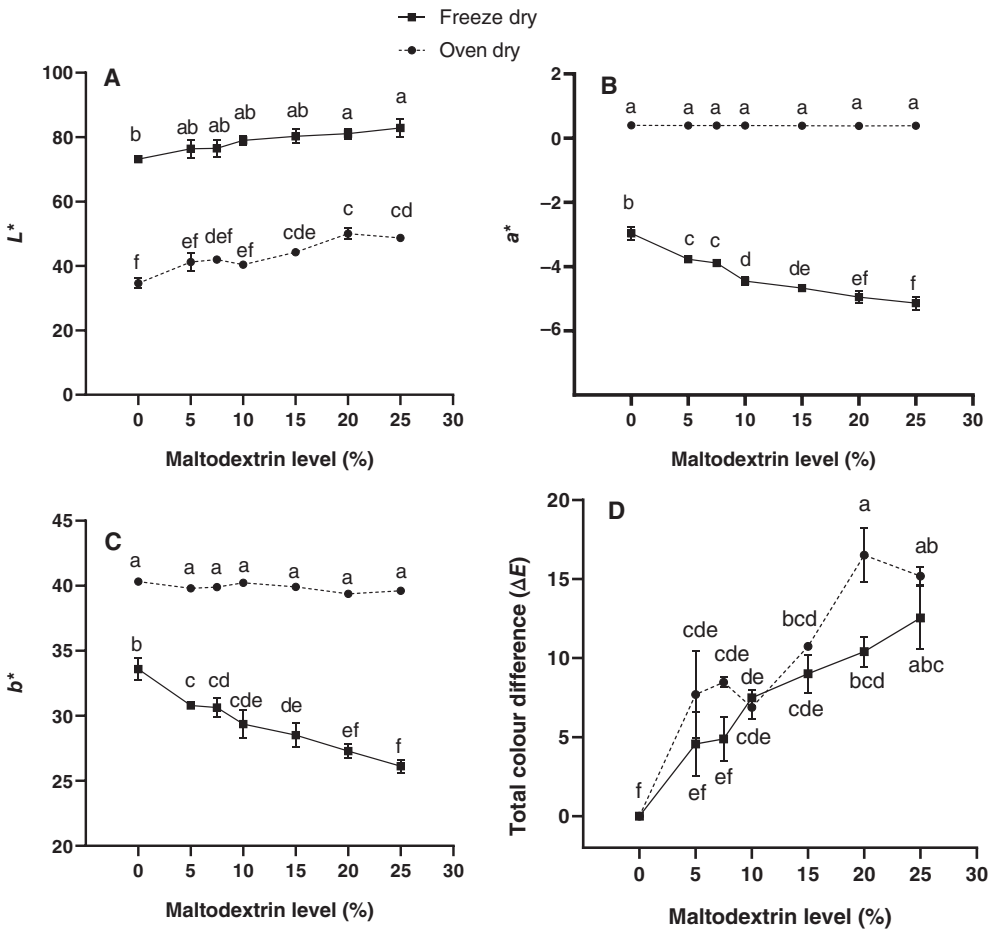

Figure 2. Colour characteristics of Kakadu plum powdered samples processed by freeze-drying and oven-drying: (A) L^*, (B) a^*, (C) b^*, and (D) total colour difference. Different letters indicate significant differences at $\alpha = 0.05$.

13.6% after freeze-drying. The results obtained from this study are in agreement with previous studies that reported a considerable reduction of AA in many types of dried fruits and vegetables processed by either freeze-drying or oven-drying, with more AA loss occurring when samples were dehydrated using the oven-drying method.[13,37]

The results of Pearson's correlation coefficient test (Supporting Information Table S1) show a positive correlation ($R > 0.8$, $P < 0.001$) between the reduction of AA and the formation of its degradation products (furoic acid and furfural). As shown in Fig. 4, the more AA was lost, the greater the formation of furoic

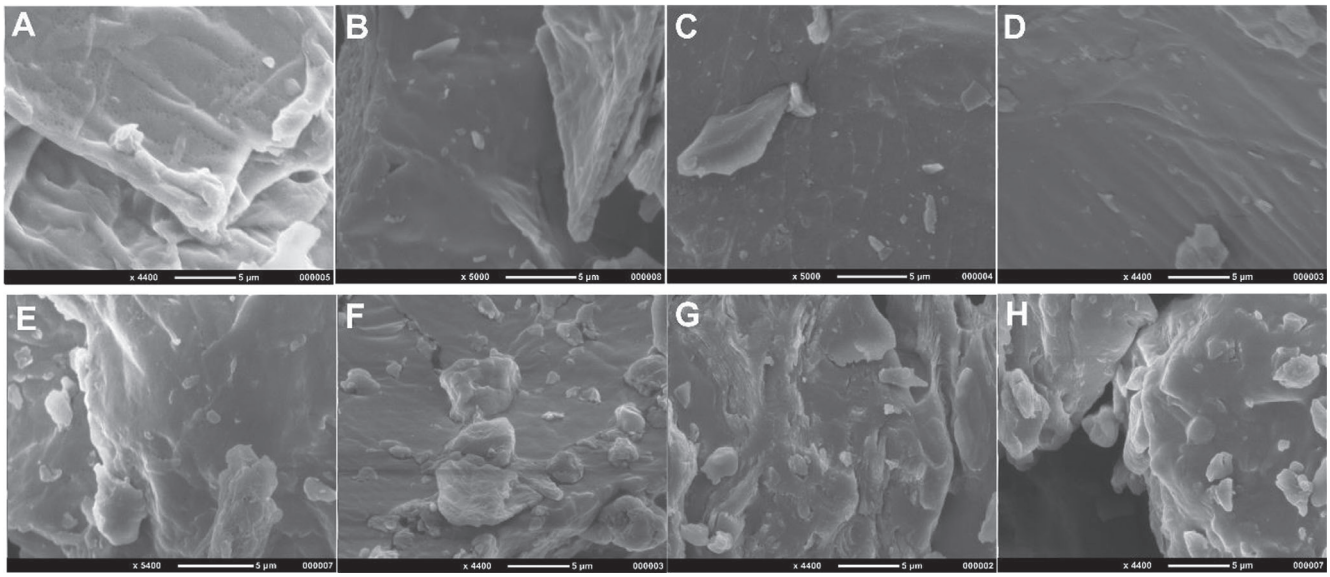

Figure 3. Representative scanning electron micrographs of (A–D) freeze-dried and (E–H) oven-dried Kakadu plum powdered samples containing different maltodextrin levels: (A) 0%, (B) 5%, (C) 10%, (D) 25%, (E) 0%, (F) 5%, (G) 10%, and (H) 25%. Scale bars: 5 μm; original magnification 4400–5400×.

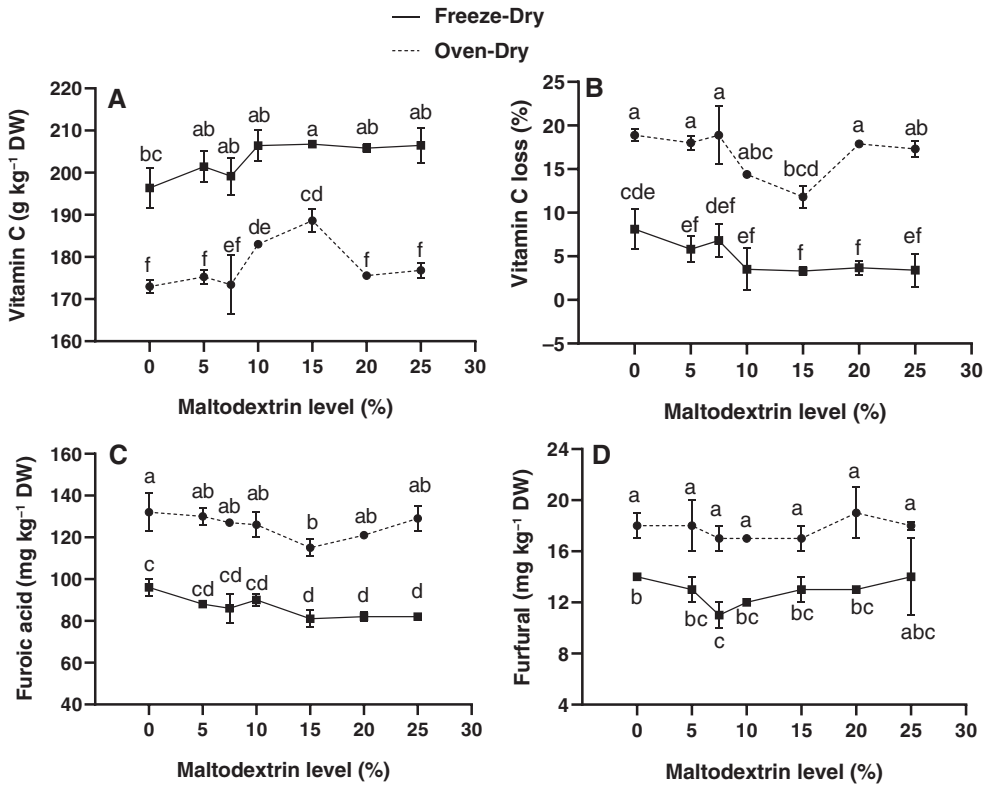

Figure 4. (A) Vitamin C content, (B) the percentage loss of vitamin C, and the formation of (C) furoic acid and (D) furfural. Different letters indicate significant differences at $\alpha = 0.05$.

acid and furfural. Comparison of the two drying methods showed that freeze-drying led to more retained AA and consequently resulted in a lower amount of the corresponding degradation products. Furoic acid and furfural have been reported as the main C5-compounds that could be generated during the degradation of AA in the presence or absence of oxygen in a low acidic environment (KP fruit puree with pH 3.37, Table 1), as demonstrated previously by other workers.[16,18,19] Furthermore, furoic acid (81–32 mg kg^{-1} DW; Fig. 4(C)) was produced at much higher levels than furfural (11–-19 mg kg^{-1} DW; Fig. 4(D)), suggesting that, under the conditions of the present study, the oxidative reaction of AA to form furoic acid might be the main pathway, whereas non-oxidative degradation to yield furfural was a secondary pathway. In a simplified model system employing an aqueous AA solution, Sawamura *et al.*[17] and Kimoto

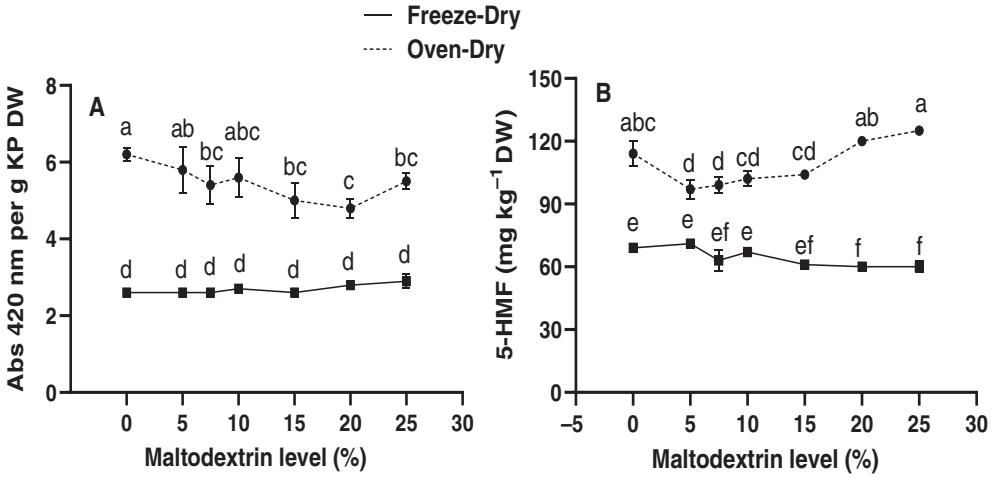

Figure 5. (A) Browning index (optical density at 420 nm) and the formation of 5-hydroxymethyl furfural (5-HMF) in Kakadu plum powdered samples. Different letters indicate significant differences at $\alpha = 0.05$.

et al.[15] demonstrated furoic acid as one of the main degradation products of AA after it had been oxidized to DHAA.

Figure 4(A) and (B) show the effect of maltodextrin in preserving AA in the two drying processes. Compared with the control samples, adding maltodextrin significantly ($P < 0.05$) reduced the percentage loss of AA from 8.1% to 3.3% in the freeze-dried samples and from 18.9% to 14.4% in the oven-dried samples, particularly at maltodextrin levels of 10–15%. However, incorporation of maltodextrin at higher levels, up to 20–25%, did not significantly result in a further reduction in the percentage loss of AA as observed in the freeze-dried samples ($P > 0.05$; Fig. 4(B)). Nevertheless, this trend was not observed for the oven-dried treatment, as more AA was lost in the samples containing 20–25% maltodextrin than in those having 10–15%. This could be explained by the longer drying time needed for these samples (Table 1). On the other hand, adding <10% maltodextrin did not significantly ($P > 0.05$) improve AA retention in the final dry products processed by the two drying methods (Fig. 4(A), (B)). A similar trend in the effects of different maltodextrin levels on the formation of furoic acid was also observed, as shown in Fig. 4(C). The results obtained suggest that maltodextrin addition at 10–15% is appropriate and effective in minimizing the losses of AA during dehydration of the KP puree.

The physical properties of maltodextrin, such as film-forming and thickening ability,[32] might be responsible for reducing the level of dissolved oxygen within the KP blend matrix, and hence might help to protect AA from oxidation.[21,38] The ability of maltodextrin to protect AA during storage of freeze-dried orange juice powder has been reported.[33] That study also reported no significant effect of maltodextrin addition at a low level (2%) in preserving AA in orange juice powder produced by freeze-drying.[33] This supports our findings that addition of <10% maltodextrin did not significantly ($P > 0.05$) reduce the loss of AA compared with the control samples (Fig. 4(A), (B)).

Browning index and the formation of non-enzymatic browning products

Figure 5 and Supporting Information Table S1 show a positive correlation ($R = 0.64$, $P < 0.001$) between browning index and the formation of 5-HMF, a well-known compound formed during dehydration processes by the Maillard reaction. Factors such as low a_w, low moisture content, acidic environment, temperature, and heating duration potentially affect the formation of 5-HMF.[20 22] In this study, the oven-drying was operated at a higher temperature, which caused a significantly ($P < 0.05$) higher level of 5-HMF and browning index values than the freeze-drying process did. This suggests that freeze-drying improved the quality of the KP powder, as 5-HMF negatively impacts the colour and overall flavour attributes of food products. Furthermore, the freeze-drying could decrease a_w to below 0.1, whereas the oven-drying process resulted in higher a_w values in the range 0.3–0.58 (Fig. 1(B)), which is the optimum for the Maillard reaction.[20] The Pearson correlation (Supporting Information Table S1) showed a positive correlation between 5-HMF, furfural, browning index, and a_w ($R = 0.62–0.84$, $P < 0.001$), suggesting that non-enzymatic browning products (furfural and 5-HMF) generated in the oven-dried samples associated with the function of heating and high water activity.

Similar to the results of AA, maltodextrin addition significantly ($P < 0.05$) reduced the formation of 5-HMF and the browning index for both drying methods, depending on the levels of maltodextrin (Fig. 5). The oven-dried samples containing 5–10% maltodextrin had lower 5-HMF concentrations ($P < 0.05$) than the control did. However, 5-HMF and browning index started to increase in the oven-dried samples having higher maltodextrin levels of up to 20–25%, probably due to the effect of heating duration (4 days; Table 1). In contrast, for the freeze-dried samples, the high levels of added maltodextrin (i.e. 20–25%) significantly ($P < 0.05$) reduced the formation of 5-HMF compared with the control (Fig. 5(B)). Interestingly, although the freeze-drying was operated in the frozen condition, the formation of 5-HMF still occurred. This phenomenon has been demonstrated and explained previously by Fitzpatrick *et al.*[39] by the freeze-drying mediating the formation of 5-HMF.

Principal component analysis

PCA was utilized to visualize the effects of the two drying methods and maltodextrin addition to the powdered KP samples (Fig. 6). The PCA scores plot (Fig. 6(A)) explains 96% of the total variability (PC-1 92% and PC-2 4%) in the data set. Two distinctive groups of samples associated with the drying method (i.e. oven-dry and freeze-dry) can be observed. In addition, a separation between samples with different maltodextrin levels belonging to the oven-dry treatment was observed, suggesting that

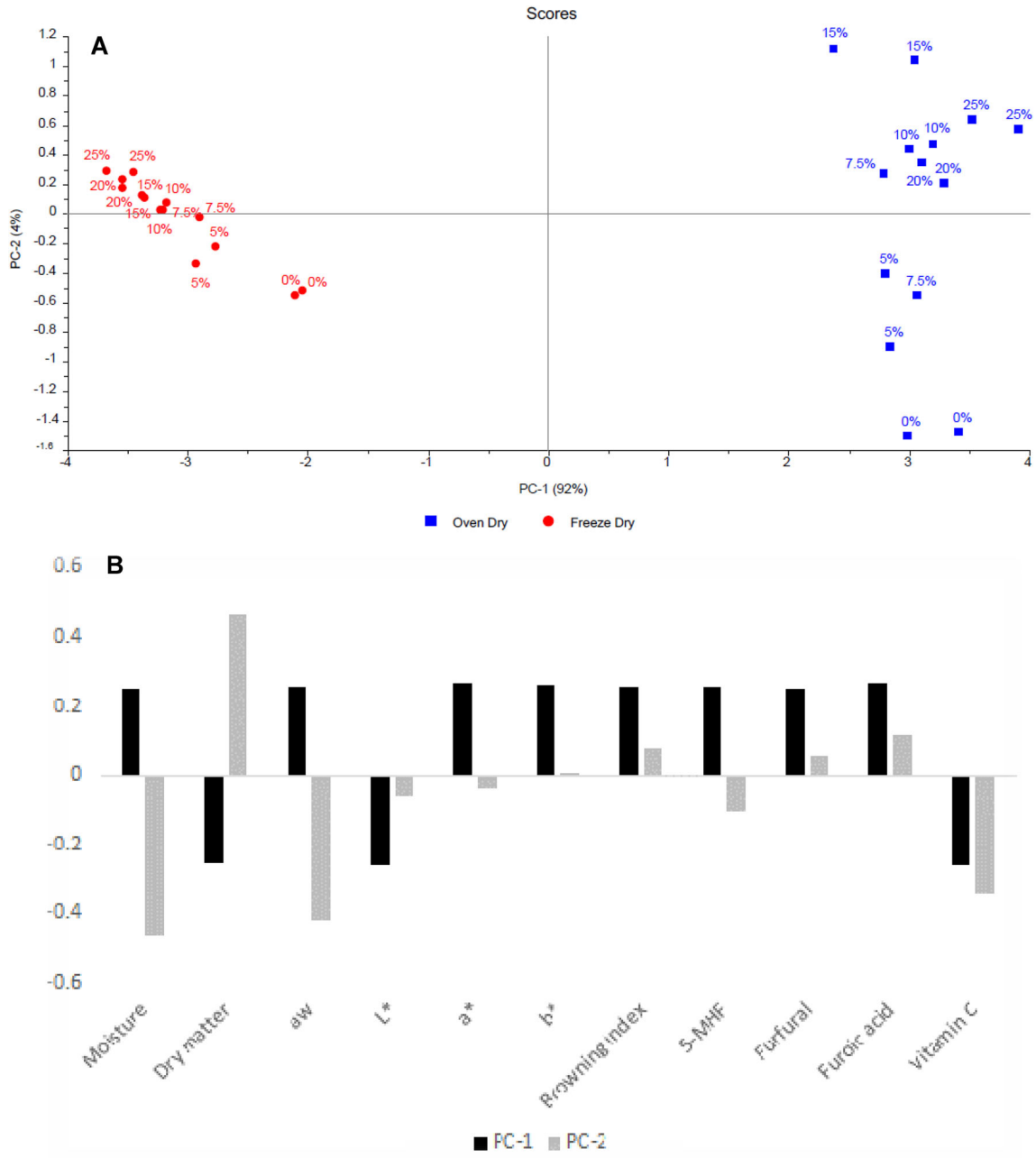

Figure 6. (A) Principal component analysis scores and (B) loadings plots showing the effects of drying methods and different maltodextrin levels.

maltodextrin had a greater effect than with the freeze-dried treatment. The resulting trend also suggested that the different maltodextrin levels, and in particular the addition of maltodextrin at 7.5–10% and 10–15%, might be sufficient to improve the AA retention and quality of the KP powder processed by freeze-drying and oven-drying respectively (Fig. 6(A)). The loadings derived from the PCA (Fig. 6(B)) indicated that all variables contribute to explain the separation between the oven-dried and freeze-dried samples along the PC-1, whereas moisture, water activity, and AA explain the observed differences between the samples containing different maltodextrin levels along the PC-2. Furthermore, AA and colour parameter L^* positively correlated with the freeze-dried samples, whereas moisture, water activity, colour parameters a^* and b^*, and degradation products 5-HMF, furoic acid, and furfural are positively correlated with the oven-dried samples. Overall, the PCA confirmed the high variability in the samples generated by oven-drying.

CONCLUSIONS

This study demonstrated the effects of two drying methods and the addition of maltodextrin on AA and the quality of powdered KP samples. The results also indicated that freeze-drying is the better processing technique for maintaining a high level of AA in KP powder, as well as for minimizing the formation of undesirable compounds. In addition, the results of this study demonstrated the potential of utilizing maltodextrin as an AA stabilizer, contributing to the improvement of colour appearance of the KP powdered samples processed by freeze-drying and oven-

drying. The results obtained not only added to the current knowledge about the degradation of AA during drying, but also provided useful insights in order to develop protocols for processing of KP powder. These protocols are of great benefit to the industry, as they will guarantee the maintenance of the high-quality standards for this novel functional food ingredient. Further studies employing different types of polysaccharides and detailed storage trials are necessary in order to obtain a comprehensive understanding on the effect of maltodextrin as an additive to protect AA from oxidation during storage. Furthermore, studies focusing on the changes of ellagic acid and other bioactive compounds in KP fruit during drying with the addition of maltodextrin should be done in the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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ORIGINAL PAPER

Proximate composition, functional and antimicrobial properties of wild harvest *Terminalia carpentariae* fruit

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Abstract

Terminalia hadleyana (subsp. carpentariae C. T. White) is native to Northern Australia where fruits of this plant have been used as a traditional food by the Australian Indigenous people. The aim of this study was to evaluate the morphology, chemical composition, functional (e.g. vitamin C, phenolic content) and antimicrobial properties of *T. carpentariae* fruits, harvested from the wild at full maturity. Variability has been observed in both fruit morphology (size and weight) and chemical composition. Proximate analysis showed that these fruits have high concentration of dietary fibre (DF) (51.2 g/100 gDW), and minerals such as K (1780 mg/100 g DW), Ca (373 mg/100 g DW) and Mg (150 mg/100 g DW). High levels of total phenolic content (TPC) (11,392 mg GAE/100 g DW) and vitamin C (11,046 mg/100 g DW) were also observed. Fruit extracts also showed inhibitory effects against the growth of foodborne microorganisms such as *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens*. Overall, these results contribute to provide with relevant information of the potential of *T. carpentariae* fruit as a functional ingredient to the Australian Indigenous communities and the emerging Australian native food industry.

Keywords *Terminalia carpentariae* · Phytochemicals · Hydrolysable tannins · Polyphenols · Antimicrobial activity · Antioxidant · Wild harvested

Introduction

The genus *Terminalia* comprises of more than 250 species, and it is the second-largest genus in the family Combretaceae [1]. Plants from the genus *Terminalia* are mainly distributed in tropical and subtropical regions of America, Australia, Asia, and Africa [2, 3]. More than 30 species or subspecies grow in Australia, including the well-known *Terminalia ferdinandiana* (commonly known as Kakadu plum) [4]. Both fruits and leaves of *T. ferdinandiana* have been reported to possess strongest radical scavenging activity and the highest levels of antioxidant compounds compared with other native fruits found in Australia [5].

Terminalia hadleyana (subsp. *carpentariae* C. T. White), belongs to the family Combretaceae, popularly known as wild peach, and is native to Northern Australia [6]. This plant is a shrub or small tree with mottled yellow, grey or orange bark up to 10 m high [6]. The cambium layer of *T. carpentariae* contains a series of bioactive phytocompounds, including saponins, tannins, flavonoids, and triterpenoids exerting antioxidant and antimicrobial activities [7]. More than 20 compounds have been identified in the leaf of *T. carpentariae* exhibiting high inhibitory effects against *Bacillus anthracis* [8]. Most of the plants of the genus *Terminalia* have a long history of usage in medicinal applications worldwide [1] where several bioactive phytochemicals (> 300) have been found [9]. Pharmacological studies have reported the importance of these bioactive phytochemicals in liver and kidney protection [10–13], and having antibacterial [14], antifungal [15, 16], antiparasitic [17], anti-inflammatory [18, 19], and anti-obesity activities [20, 27]. Among these bioactive compounds, tannins, including ellagitannins, gallotannins, dimeric- and trimeric tannins were reported as the main bioactive compounds in most of the *Terminalia* species [9].

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In addition to tannins, vitamin C, a strong natural antioxidant, has been also documented as an important bioactive compound in many scientific studies of *Terminalia* [5, 21]. For example, fruits of *T. ferdinandiana* contain exceptional levels of vitamin C up to 22,000 mg/100 g DW [21]. The high concentration of this vitamin in fruits of *Terminalia* has been reported to be approximately 900-fold higher than blueberries where in oranges and grapefruits the concentration of this vitamin only reaches around 0.5% of the dry weight of the fruit [2, 22].

It has been also reported that different plant extracts (e.g. methanolic and water) of the genus *Terminalia* exhibited antimicrobial properties and consequently some of these species have been used in traditional medicine. Fruits of both *T. ferdinandiana* and *chebula* have been reported to exhibit antibacterial properties against both gram-positive and gram-negative bacteria [23, 24]. Other species of *Terminalia* such as *australis*, *brownii* and *mollis* have been also reported to exhibit high antifungal activity [25, 26].

Although, the fruits and other plant parts of this plant have been traditionally used as food or medicine by the Australian Indigenous people for thousands of years, limited scientific reports have been published on the composition, antioxidant and antimicrobial properties of *T. carpentariae* fruits. Besides, no research has been reported on the identification and quantification of phytochemicals of *T. carpentariae* fruits and their potential biological functions.

The aim of this study was to evaluate the morphology, chemical composition, functional (e.g. vitamin C, phenolic content) and antimicrobial properties of *T. carpentariae* fruits, harvested from the wild, at full maturity.

Materials and methods

Sample collection and processing

Fruit samples used in this study were harvested at full maturity in December 2019 from individual trees grown in the wild in East Arnhem Land (Northern Territory, Australia). Individual fruit samples were freeze-dried at − 48 °C for 72 hs (CSK Climatek, Darra, Queensland, Australia). Then, the pulp and seed of the fruit samples were manually separated, followed by milling the seedless pulp fruit samples into a fine powder using a mixer mill MM 400 (Retsch GmbH, Haan, Germany). Samples were stored at – 35 °C until further analysis.

Proximate and mineral analysis

Proximate and minerals analysis were performed in a commercial laboratory (Symbio Alliance Laboratories, Brisbane, QLD, Australia). Analysis were carried out using accredited in-house or standard AOAC methods. Crude protein (CP) was determined using AOAC method 990.03, total fat (TF) using AOAC method 991.36, ash using AOAC method 923.03. Saturated (SFA), monounsaturated (MFA), polyunsaturated (PUFA), trans fats (TFA), dietary fiber (DF) and carbohydrates (CHO) were determined using in-house methods (Symbio Alliance Laboratories, Australia), and minerals were determined by Inductively Coupled Plasma (ICP)—mass spectrometry. Moisture (M) content was determined according to AOAC method 934.01.

Morphology

Individual fruit samples (n = 100) were randomly selected and thawed at room temperature where the length and width were recorded using a 150 mm Digital Calliper (Craftright Engineering Works, Jiangsu, China). The weight of whole fruit, pulp and seeds, was recorded using a laboratory scale (Sartorius CP224S, Gottingen, Germany).

Determination of total soluble solids, pH and titratable acidity

The pulp of fruit samples (n = 20) was finely ground into a puree and diluted (1:10; w/v) with Milli-Q water (18 M Ω cm at 23 °C) (Millipore, USA). Centrifuged supernatant was retained for total soluble solid (TSS) measurement using an OPTi@ Digital Handheld Refractometer (Xylem, UK). The pH was recorded on the diluted puree using a pH meter (Metrohm, NSW, Australia). The titratable acidity (TA) was determined by titrating with 0.1 N NaOH using an automatic titration unit (Metrohm, NSW, Australia).

Extraction and analysis of bioactive compounds

Fruit bioactive compounds were extracted using an aqueous methanol 80% (v/v) solution. Freeze-dried fruit powder (approx. 2 g) samples were homogenized using 20 ml of 80% methanol, followed by sonication in an ultrasonic bath (Elma Schmidbauer GmbH, Ruiselede, Belgium) for 15 min. The tubes were continuously shaken on a reciprocating shaker (RP1812, Paton Scientific, Adelaide, SA, Australia) for another 15 min, followed by centrifuging the slurry at 3900 rpm for 10 min using Eppendorf 5180 centrifuge (Eppendorf, Hamburg, Germany). The residues were re-extracted twice where a total volume of 30 ml of the supernatant was kept at – 35 °C for further analysis. The remaining supernatant was evaporated to dryness and later used to determine antimicrobial activity. DIONEX Ultimate 3000 UHPLC system hyphenated with a Thermo high resolution Q Exactive focus mass spectrometer (Thermo Fisher Scientific Australia Pty Ltd., Melbourne, VIC, Australia) was used for analysis as described previously [23, 28].

Quantification of compounds was performed following the method described previously by Bobasa and collaborators [23]. The LCMS system was monitored using Xcalibur™ 4.1 software, while TraceFinder™ 4.1 software was used for data processing. The external calibration curves of gallic acid, ellagic acid, corilagin and 3,4,6-Tri-O-galloyl-β-D-glucose ranging from 0.1 to 50 ppm were used for the equivalent quantification of phenolic compounds [23, 28].

Determination of total phenolic content, DPPH free radical scavenging and vitamin C

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay described elsewhere [29, 30]. TPC were expressed as gallic acid equivalents per gram DW of sample (mg GAE/100 g DW) [29, 30]. The radical scavenging activity was determined using a DPPH free radical scavenging assay following the method described elsewhere [29, 30]. The results were expressed as Trolox equivalents (TE) per 100 g DW of sample (mM TE /100 g DW). Extraction and determination of vitamin C was conducted using a UHPLC-PDA analysis following the method previously described by Chaliha and collaborators [30].

Antimicrobial activity

To determine the antimicrobial activity of *T. carpentariae* fruit extracts, different foodborne microorganisms were used. The selected microorganisms used in this study were Gram-positive bacteria (*Staphylococcus aureus* NCTC 6571, *Listeria monocytogenes* ATCC 19,111 and methicillin-resistant *Staphylococcus aureus* (MRSA), Gram-negative bacteria (*Escherichia coli* NCTC 9001, *Pseudomonas aeruginosa* ATCC 10,145 and *Pseudomonas aeruginosa* clinical isolates ATCC 9001 and *Shewanella putrefaciens* ATCC 49,138) and fungi (*Aspergillus niger* ATCC 16,888, *Aspergillus flavus* ATCC 20,025 and *Penicillium chrysogenum* ATCC 10,106). The dry extract was freshly reconstituted in 20% (v/v) ethanol to obtain a concentration of 50 mg/ml. A disc diffusion assay was used to evaluate the antimicrobial activity of the fruit extracts as described elsewhere [29]. An aqueous 20% (v/v) ethanol was included to test the effects caused by the solvent as the negative control. 2 mg/ml Oxytetracycline hydrochloride (Sigma-Aldrich, USA) and 1 mg/ml Amphotericin B (Sigma-Aldrich, USA), antibiotic and antifungal substances, were also included as positive controls. The inoculated plates were incubated at 37 °C for 24 h, whilst the inoculated fungal plates were incubated at 25 °C for 48 h. After incubation, the diameter of the inhibition zones was measured using a 150 mm Digital Calliper. The antimicrobial activity results were expressed as strong (> 13 mm), moderate (6–12 mm), weak (≥ 5 mm) and no activity (< 5 mm) [29, 31, 32].

Statistical analysis

The results were expressed as means ± standard deviation (SD) and calculated using GraphPad Prism version 9.0 (San Diego, CA, USA). All the analysis were done in triplicate. Antimicrobial activity was statistically analysed using one-way ANOVA and means compared with Tukey's multiple comparison post hoc tests ($p < 0.05$).

Results and discussion

Morphology, moisture, titratable acidity and total soluble solids

Table 1 reports the morphological parameters measured in the fully mature and wild harvest *T. carpentariae* samples. Published data on fruits from other species such as *T. ferdinandiana* was added for comparison purposes only [21]. The results showed an effect of the species on the morphological characteristics of the fruits (e.g. size). However, the proportion of seed and pulp was relatively similar between the two species (86.6 vs 87%) analysed. Variability in the morphological parameters between individual fruit samples was also observed (weight and size), reflecting the effect of tree variability in the wild. This variability could be also attributed to abiotic effects such as microclimate (e.g. rainfall and temperature), soil, and elevation, as reported by other authors [30, 33, 34]. In this study, fruit samples were collected from two distinctive geographical locations identified as Arnhem Land plateau and lowlands. These regions differ in altitude and this issue might contribute to explain the observed variations in the fruit samples analysed [30, 33, 34]. Table 1 also shows the results for M, pH, TA, and TSS, measured in the *T. carpentariae* fruit samples. Overall, the M, TA and TSS content in the *T. carpentariae* fruit samples analysed is lower than those from *T. ferdinandiana.*

Proximate and mineral composition

Proximate and mineral composition of the *T. carpentariae* fruit samples analysed is reported in Tables 1 and 2, respectively. The CP content (7.2 g/100 g DW) in *T. carpentariae* fruit samples was higher than *T. ferdinandiana* fruits (4.7 g/100 g DW). The TF content of *T. carpentariae* samples was considered low (1.6 g/100 g DW), with most of the fat comprising of PUFA (1.2 g/100 g DW). It was observed that the concentration of PUFA is twice the amount reported for *T. ferdinandiana* (0.6 g/100 g DW), which could be beneficial in reducing the risks of cardiovascular diseases and cancer as reported by other authors [35]. Fruit samples of *T. carpentariae* have a high amount of DF (51.2 g/100 g DW) compared to *T. ferdinandiana*

Table 1 Descriptive statistics for morphology and chemical composition determined in the set of *T. carpentariae* fruit samples

	T. carpentariae	*T. ferdinandiana*[a]
Morphology		
Length (mm)	33.1 ± 3.8 (11.4%)	NA
Width (mm)	19.3 ± 1.9 (9.9%)	NA
Weight (g)	4.5 ± 1.0 (22.9%)	NA
Length range (mm)	21.5–41.2	16.4–37.4
Width range (mm)	11.0–23.6	8.0–20.1
Weight range (g)	2.2–7.3	1.0–4.6
% seed	13.4	13
% pulp	86.6	87
pH	3.7 ± 0.1 (3.1%)	3.8–4.0
TA	1.5 ± 0.2 (1.5%)	3.0–4.8
M (% w/w)	79.2 ± 2.2 (2.8%)	83–87
TSS (°Brix)	11.1 ± 2.2 (20.0%)	19.3–41.1
Vit C[#]	11,046 ± 572 (5.2%)	22,000
L-AA[#]	9,807 ± 344 (3.5%)	NA
DAA[#]	1,239 ± 228	NA
Proximate analysis[$]		
CP	7.2	4.7
TF	1.6	0.9
SFA	0.4	0.3
MFA	0.1	< 0.1
PUFA	1	0.6
TFA	< 0.01	< 0.01
Ash	4.5	5.5
DF	51.2	45.9
CHO	32.9	37

Please note that *T. ferdinandiana* was added for comparative purposes

In brackets coefficient of variation (SD/mean × 100); *TA* titratable acidity, *M* moisture, *TSS* total soluble solids, *CP* crude protein, *L-AA* L-ascorbic acid, *DAA* dehydeoxyascorbic acid, *Vit C* vitamin C, *TF* total fat, *SFA* saturated fatty acids, *MFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *TFA* trans fatty acids, *DF* dietary fiber, *CHO* carbohydrates

NA data not available

[a]*T. ferdinandiana* data was sourced from Sultanbawa et al. (2018) and Phan et al. (2020)

[#](mg/100 g DW)

[$](g/100 g DW)

(45.9 g/100 g DW). It was found that the concentration of K, Mg and Zn in the *T. carpentariae* fruit samples analysed was lower than those reported for other species of *Terminalia* [36], while the other minerals analysed in the fruit samples were found at higher concentrations (Table 2). In this study, the concentration of K in the fruit samples was the highest (1780 mg/100 g DW), followed by Ca (373 mg/100 g DW) and Mg (150 mg/100 g DW).

Total phenolic content, DPPH free radical scavenging and vitamin C

The content of vitamin C in the *T. carpentariae* fruit samples analysed was found at a moderate level (11,046 mg/100 g DW) (see Table 1). The concentration of vitamin C in this fruit is comparatively higher than other Australian native fruits such as desert lime (188.6 mg/100 g DW), Davidson's plum (30 mg/100 g DW) and bush tomato (17 mg/100 g DW) [37], however, is lower compared to fruits of *T. ferdinandiana* (22,000 mg/100 g DW [21]. It has been also reported that the concentration of vitamin C (ascorbic acid) varies among the different species of *Terminalia* where a concentration of this vitamin of 797.9 mg/100 g DW and 0.874 mg/100 g DW was reported for *T. chebula* and *catappa*, respectively [38, 39]. This variability may be associated with the accumulation of ascorbic acid in plants, that is influenced by cultivar or species, environment, time of harvest, and post-harvest storage conditions [30].

The TPC in the fruit samples analysed was11,392.9 mg GAE/100 g DW (see Table 3). The content of TPC in these fruits is considered higher than other *Terminalia* species, such as *T. chebula* (40 mg GAE/100 g DW) [40] and *T. catappa* (1005 mg GAE/100 g DW) [41], however, lower than *T. ferdinandiana* fruit (range 37,610 to 50,520 mg GAE/100 g DW) [21] and *T. bellirica* (18,871 GAE mg/100 g DW) [42]. *T. carpentariae* exhibited a high radical scavenging activity with an EC50 value of 12.9 µg/ml. In a recent study, Dharmaratne and collaborators [42] reported higher EC50 values in fruits of *T. bellirica* (6.99 ± 0.15 µg/ml) while Abdulkadir [43] reported weaker free radical scavenging activity in ripe fruits of *T. catappa* (95.99 µg/ml).

Antimicrobial activity

Table 3 reports the antimicrobial activity (shown as inhibition zones) of the *T. carpentariae* fruits extracted (80% methanol) against the foodborne microorganisms selected. Ethanol 20% (negative control) did not show any zone of inhibition against any of the tested microorganisms. The methanolic extracts of *T. carpentariae* showed strong antibacterial activity against the gram-negative *S. putrefaciens*, and moderate activity against *P. aeruginosa* ($p < 0.05$). However, no inhibitory effects against mold, *L. monocytogenes* and *E. coli* were observed. These results suggested that extracts from *T. carpentariae* fruits are more susceptible to bacteria than yeast or fungi, which might be suitable for natural antibacterial applications. Previous studies using other *Terminalia* species have also reported similar trends [29, 44–48]. For example, fruits of *T. chebula* did not exhibit any inhibitory activity against fungi [44]. Extracts of *T. carpentariae* have also showed strong antibacterial activity against gram-positive *S. aureus* and MRSA (Table 3), indicating

Table 2 Content of mineral and trace elements in the set of *T. carpentariae* fruit samples analysed

	T. carpentariae	*T. ferdinandiana*	Nutritional information*
Sodium (Na)	101	40	1300 mg/day AI
Potassium (K)	1780	2717	4.7 g/day AI
Magnesium (Mg)	150	203	350 mg/day EAR
Calcium (Ca)	373	295	1200 mg/day AI
Iron (Fe)	11	1.7	8 mg/day RDA
Zinc (Zn)	1.1	2.15	11 mg/day RDA
Manganese (Mn)	8.8	5.1	2.3 mg/day AI
Cobalt (Co)	0.18	0.01	NA
Cooper (Cu)	3.84	1.4	900 µg/day AI
Nickel (Ni)	0.28	0.49	NA
Chromium (Cr)	0.37	0.07	35 µg/day AI

Please note that *T. ferdinandiana* and nutritional information were added for comparative purposes

T. ferdinandiana data sourced from Sultanbawa et al. (2018)

*Nutritional information was referred to Meyers et al. (2006); (NA) no data available; *AI* adequate intake, *EAR* estimated average requirement, *RDA* recommended dietary allowance. Units; mg/100 g DW

Table 3 (A) Total phenolic content, DPPH free radical capacity, 50% effective concentration (EC50) of DPPH radical scavenging and (B) antimicrobial activity of *T. carpentariae* fruit samples analysed

A		Average ± SD	CV (%)
	TPC (mg GAE/100 g DW)	11,392.9 ± 258.2	2.3
	DPPH (mg TE/g DW)	219.9 ± 1.6	0.7
	EC_{50} (µg/ml DW)	12.9	–
B	Zone of inhibition (mm)	Average ± SD	CV (%)
	S. aureus	20.4 ± 0.8^{a}	4.0
	L. monocytogenes	NI	NI
	MRSA	22.5 ± 0.3^{a}	1.2
	P. aeruginosa	8.2 ± 0.4^{b}	4.4
	P. aeruginosa (CI)	NI	NI
	E. coli	NI	NI
	S. putrefaciens	21.2 ± 0.4^{a}	2.0
	A. niger	NI	NI
	A. flavus	NI	NI
	P. chrysogenum	NI	NI

MRSA methicillin-resistant *Staphylococcus aureus*, Results are means ± SD (n = 3); *CV* coefficient of variation, *NI* no inhibition, different letters (a, b) indicated statistically significant differences at the level ($p \leq 0.05$)

that these fruit extracts have the potential to inhibit bacteria which are resistant to antibiotics. The results of the present study are also in agreement with those reported by other authors using *T. ferdinandiana* [29, 44–48]. Previous reports have also indicated that methanolic extracts of *T. ferdinandiana* were effective as an antibacterial agent against *E. coli* [29, 44, 45]. However, this might be explained by differences in the strain of *E. coli* selected to conduct the present study. Moreover, *P. aeruginosa* and *E. coli* have been considered among the most clinically challenging species to be inhibited or killed by some class of antibiotics [46–48]. Due to the distinctive composition of the outer membrane of gram-negative bacteria, they are more resistant to several antibiotics compared to gram-positive bacteria [46–48]. In our present study, the extracts of *T. carpentariae* showed no zone of inhibition against *E. coli* which might be due to its structure. The strain selected by Cock and Mohanty [45] was more susceptible than the one utilised in the present study. Extracts of *T. carpentariae* were not effective against *L. monocytogenes* while they have shown an inhibitory activity against *P. aeruginosa*. Previous authors have also reported high antimicrobial activity of *T. ferdinandiana* extracts against *L. Monocytogenes* but no inhibition against *P. aeruginosa* [29, 44, 45]. Other authors have also suggested the

synergic effects of vitamin C as antimicrobial agent [4, 29, 44, 45]. Fruit samples of *T. carpentariae* have lower vitamin C than *T. ferdinandiana*, and these might explain the observed differences in antibacterial activity between these two species. In addition, Mohanty and Cock [4] have stated that vitamin C might indirectly improve the antibacterial activity of polyphenolic compounds by inhibiting the oxidation of these compounds. However, results presented in this study are preliminary and require further research to determine the exact mechanism of antimicrobial action of the extracts of *T. carpentariae.*

Phytochemical profiling

Individual phenolic compounds were determined in the *T. carpentariae* fruit samples and reported in both Fig. 1 and supplementary Table. Approximately, a total of 15 phenolic compounds were identified by comparing the mass spectra (MS1 and MS2) with available analytical standards and the scientific literature [49–53]. The compounds identified in Fig. 1 included organic acids (Peak 1), gallotannins (peaks 2, 3, and 4), ellagitannins (peaks 6, 7, 8, 9, and 10), flavone (peaks 14 and 16) and flavone derivatives (peak 11, 12, 13, and 15) [23, 49].

Peak 1 and 2 corresponds with ascorbic acid and gallic acid standards, respectively. Peaks 3, 4 and 7 might be associated with gallotannins [49–53]. Peaks 3 and 4, yield similar molecular ions and could be identified as digalloyl hexoside and its isomer [49–53]. In this study, peak 5 was not identified while peak 6 was identified as corilagin. Peaks 7 and 8, are associated with chebulagic acid as reported by other authors [23, 49–52]. Peak 9 is tentatively identified as avicularin [49–53] while peak 10 was identified as ellagic acid [23, 49–52]. Peak 11 was identified as 5,6-dihydroxy-3′,4′,7-trimethoxy-flavone derivative compounds [23, 49–52]. Peak 14 was identified as tricin [49–53] while peak 16 tentatively identified as 5,6-dihydroxy-3′,4′,7-trimethoxy-flavone [49–53].

It has been reported that ellagitannins exhibited high potential inhibitory effects against bacterial growth and antioxidant properties [13]. Different authors have found that ellagitannins are present in the fruit of different *Terminalia* species, such as *T. bellerica*, *chebula*, *horrida* and *ferdinandiana* [23, 49]. In this study, corilagin was considered the most important phenolic compound found in the fruit of *T. carpentariae*. These results also agreed with those reported by other authors in fruits of *T. bellirica* [19] and *T. ferdinandiana* [23].

Conclusion

This is the first study to investigate the morphology, chemical composition, phytochemical profiling, antioxidant and antimicrobial activity of *T. carpentariae* fruits. Large variability has been observed in both the morphology (e.g.

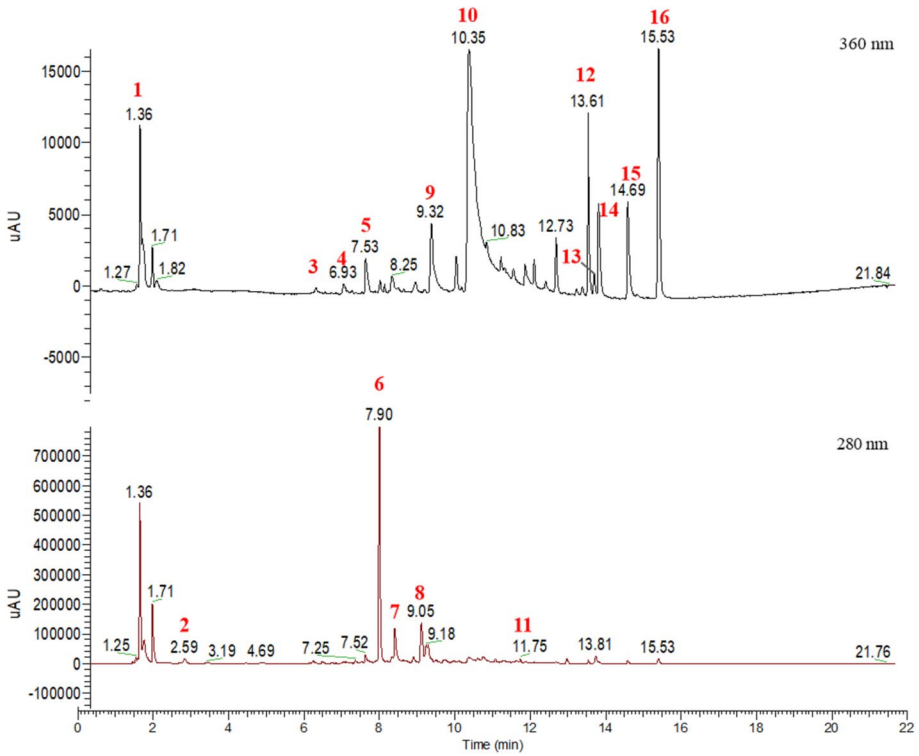

Fig. 1 HPLC–UV chromatogram of *T. carpentariae* fruit samples collected at 280 and 360 nm. The peak number is described in the text and supplementary Table

weight and size) and chemical composition as these fruits were harvested from the wild. Proximate analysis indicated that these fruits are a good source of CP and DF, with high potential as alternative source of minerals such as K, Ca and Mg. The identified phenolic compounds and high levels of vitamin C supported the evidence of the antioxidant and antimicrobial properties of this fruit (high levels of total phenolic content and free radical scavenging capacity). Extracts of *T. carpentariae* exhibited high inhibitory effects against Gram-positive bacteria. These results will contribute to provide with the relevant information of the potential of *T. carpentariae* fruit as a functional ingredient to the Australian Indigenous communities and the emerging Australian native food industry.

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Data availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Code availability Not applicable.

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