# CHARACTERISATION AND PROFILING OF BIOACTIVE CONSTITUENTS, NUTRIENTS AND MINERALS IN MARULA WINE DURING THE FERMENTATION PERIOD

by

**PERPETUA MANTATI TEBEILA**

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SUPERVISOR: PROF KLM MOGANEDI

CO-SUPERVISOR: PROF P MASOKO

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**DECLARATION**

I **PERPETUA MANTATI TEBEILA** solemnly declare that the thesis hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy in Microbiology has not been previously submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Signature: PM Tebeila Date: 13/02/2022

**DEDICATION**

This work is dedicated to my family, grandmother Perpetoah Mantati Malapane. Thank you for carrying our name with such dignity and passing on the baton to me. I am truly honoured. Continue to rest in peace.

To my parents, Reneilwe and Rankwane Tebeila without whom I would not be alive to do this. Thank you for your support.

To my siblings, John and Mogotladi Tebeila whose love for me gives me strength. I love you so much.

To my nieces and nephews, precious and dearly loved. Go for your dreams.

To my late aunt and uncle, Mapula and Mr Masinga, I will forever be fond of the sweet names you called me. You were my inspiration and safe place during my younger days. Thank you so much.

To my friends and prayer partners. I am most favoured to have you in my life.

To God almighty, Jesus Christ my Lord and saviour. I owe you my very life.

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## CONFERENCES AND PROCEEDINGS

### Paper presentations

Tebeila P.M., Moganedi K.L.M., Masoko P. 2018. Characterization of physicochemical and bioactive properties of marula wine produced by Moshira villagers in the Sekhukhune District. Faculty of Science and Agriculture (FSA) Research Day. Fusion Boutique Hotel, Polokwane. (Won first prize, PhD presenter)

### Poster presentations

Tebeila P.M., Phiri A., Mokobane D., Moganedi K.L.M. 2018. Intrinsic and extrinsic factors that are important in marula fruit wine and for its production. Indigenous plant use forum (IPUF)(Oudtshoorn).

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| ATCC | American Type Culture Collection |
| CFU | Colony forming units |
| CLSI | Clinical and Laboratory Institute |
| GC-FID | Gas Chromatography-Flame Ionization Detector |
| GC-MS | Gas chromatography-mass spectrometry |
| TREC | Turfloop Research Ethics Committee |
| WHO | World Health Organization |
| BAW | Butanol/ acetic acid/ water |
| CEF | Chloroform/ethyl acetate/formic acid |
| DPPH | 2,2, diphenyl-1-picrylhydrazyl |
| EC50 | Half maximal effective concentrations |
| EMW | Ethyl acetate/methanol/ water |
| FRAP | Ferric reducing antioxidant power |
| GC | Gas chromatography |
| INT | ρ-Iodinitrotetrazolium salts |
| MIC | Minimum inhibitory concentration |
| $NaNO_2$ | Sodium nitrite |
| NCCLS | National Committee for Clinical Laboratory Standards |
| UV/ VIS | Ultraviolet/Visible |
| UL | University of Limpopo |
| SK | Sekhukhune |
| TO | The Oaks |
| SAWS | South African Weather Services |
| ARC | Agricultural Research Council (ARC) |

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# Chapter 1

## 1.1 Introduction

*Sclerocarya birrea* subsp*. caffra* (Marula) is widespread throughout the semi-arid deciduous savannah of sub-Saharan Africa (Shackleton et al., 2002) and occurs in eighteen countries in other parts of Africa (Hall et al., 2000). The tree has been imported to Israel and successfully domesticated in the Negev and southern Arava Valley (Nerd and Mizrahi, 1993; Nerd and Mizrahi, 2000).

Marula is a multipurpose tree that forms part of the diet, culture, and tradition of rural communities in southern Africa (Wynberg et al., 2002). The ripened fruits are eaten raw and used to make jam, jelly, and marula wine (commonly called marula beer) (Shone, 1979; Pierre, 2002; Bille and Steppich, 2003). The kernels are eaten or extracted for their oil. The bark, leaves and roots are used to treat various human ailments such as dysentery, fevers, malaria, diarrhoea, stomach ailments, rheumatism, sore eyes, gangrenous rectitis, infertility, headaches, toothache, and body pains (Mander, 1998; Ojewole et al., 2010).

It is noteworthy that the most common marula fruit product is marula wine. The wine is produced as a huge enterprise during the fruiting season in countries such as Botswana, South Africa, Swaziland, Namibia, and Zimbabwe (Mojeremane and Tshwenyane, 2004). This small-scale commercialisation generates income for rural and poor community members thus improving their livelihood. For example, some community traders in Limpopo province, South Africa charge R150 for a 20 L brew, R10 for a 2 L bottle and R5 for a 500 mL (Daily Sun, 2018).

The wine is produced through spontaneous fermentation of a diluted marula fruit juice. The process is mediated by native microbiota found on the fruit and equipment that are used. It is then consumed from as early as the fourth day to whenever it is no longer palatable, which is mostly from the $8^{th}$ day of production. It is common practise in Swaziland to place marula wine in clay pots and store it underground for consumption at a later stage as a matured drink known as Ummbela (Ntshangase, 2016).

The nutritional compositions of the marula fruits and juice have been extensively documented (Hiwilepo-van Hal et al., 2014). Marula fruits have a lipid content of 50 – 85% dry weight

(Eromosele et al., 1991; Leakey, 1999), a fatty acid composition as high as 47% dry weight (Glew et al., 2004) and protein content of 3600 mg/100 g dry weight with an abundance of sulphur-containing amino acids like methionine and cysteine (Mariod and Abdelwahab, 2012). The juice has a carbohydrate fraction (consisting mainly of sucrose, glucose, and fructose) of 7 to 14% of the fresh weight (Gous et al., 1988); a vitamin C content ranging from 62 mg/100 g (Carr, 1957) to more than 2,100 mg/100 g (Eromosele et al., 1991; Hillman et al., 2008). Minerals such as calcium, magnesium, potassium, and phosphorus (Eromosele et al., 1991; Borochov- Neori et al., 2008), ash content of 0.09 g/100 g (Taylor, 1985) and moisture content between 82 and 93% (Shone, 1979; Gous et al., 1988) have also been reported. Nevertheless, the biochemical evolution of the nutrients from fruit juice state through fermentation to wine product state has not been widely documented. This is important when different varieties of wine with good nutrient content are desired.

The juice has further been reported to contain soluble phenolics at 56 g/100 g (Borochov-Neori et al., 2008). These phenolics possess biological activities such as anti-microbial, anti-atherogenic and antioxidant activities (Ndhlala et al., 2007; Borochov-Neori et al., 2008; Hiwilepo-van Hal et al., 2014). The reported soluble phenolics fractions include hydrolysable tannins, catechins and hydroxycinnamic acid derivatives (Borochov-Neori et al., 2015).

The phytochemical and nutritional composition of marula wine is sparsely documented. The composition of marula wine includes small amounts of protein and amino acids (Steinkraus, 1983), 28% less vitamin C than in the juice; a third or more of total polyphenols and antioxidant activity retained from marula juice when fermented at temperatures between 30 $^{o}$C and 40 $^{o}$C and 2–5% alcohol content (Dlamini and Dube, 2008; Hiwilepo-van Hal et al., 2013). The soluble phenolics identified through preliminary investigations included gallic acid, catechin, ascorbic acid and epicatechin (Hiwilepo-van Hal et al., 2013).

The nutrient composition can be influenced by environmental conditions. The geographical origin of the marula tree, climate, and soil type in which it grows influences the concentration of minerals (Gous et al., 1988), vitamin C (Leakey, 1999), moisture (Gous et al., 1988), sugars (Leakey, 1999), phenolics and flavonoids (Hillman et al., 2008; Alothman et al., 2009) the tree produces. In addition, genetic variations in the trees and anthropogenic factors in the areas where trees grow may also influence these chemical properties (Hiwilepo-van Hal et al., 2013). However, one species, *Sclerocarya birrea (*Hoscht*)* subsp*. caffra* (Sond.) kokwaro

of marula tree exists in South Africa and the tree is abundantly found in the Limpopo and Mpumalanga provinces (Moganedi et al., 2011). This infers that the diversity observed in nutritional and medicinal characteristics of the tree is due to epigenetic factors. The sampling areas chosen for this research study were Sekhukhune- Moshira in Fetakgomo- Greater Tubatse municipality, The Oaks in Maruleng municipality and University in Polokwane municipality. The municipalities are encircled in figure 1.1.


**Figure 1.1**: Map of Study areas where marula fruits and/or marula wine were collected.

Notwithstanding the health and nutritional value of marula fruit juice, not much has been documented about the properties of marula wine. Noting the socio-cultural and economic importance of marula wine and its widespread consumption among the youth and elders, it is important to know and document the health and nutritive characteristics of the resulting wine. Knowledge of how these evolve during the fermentation process is important for the valuation of the marula wine due to its importance in African communities. Biochemical transformations occur during the fermentation of marula juice (Hiwilepo-van Hal et al., 2013) and these will notably affect the presence, appearance, and disappearance of desirable medicinal and nutritional compounds.

### 1.2 The study aim

The aim of the study was to investigate the fruit quality and the presence and changes in bioactive constituents, nutrients and minerals in fermenting marula juice and wine during the fermentation period. Furthermore, soil and water compositions were analysed.

### 1.3 The study objectives

The following measurable steps were taken to achieve the aim.

i. To characterise the ripe marula fruit properties and the environmental conditions in which they are harvested.
ii. To perform qualitative analysis of secondary metabolites in the marula fruit juice and fermenting wines.
iii. To screen for antioxidant and antibacterial activities of extracts from marula fruit juice and fermenting wines.
iv. To quantify the ash and dietary fibre contents of marula fruit juice and fermenting wines.
v. To determine the contents of minerals, sugar, protein and lipids in the marula fruit juice and fermenting wines.
vi. To measure the moisture content, clarity and acidity and alcohol levels of the wines.

## Chapter 2

### 2.1 Literature review

#### 2.1.1 Marula tree and its uses

Many indigenous plants of Africa have long formed an integral part of the diet of the rural communities, medicine, culture, livelihoods, and spirituality (Fox and Young, 1982; Shackleton et al., 2002). *Sclerocarya birrea (*Hoscht*)* subsp*. caffra* (Sond.) Kokwaro, belonging to the family Anacardiaceae is a popular indigenous tree throughout the semi-arid deciduous savannas of much of sub-Saharan Africa*. S. birrea*, commonly known as marula tree is a medium-sized tree that grows well in clay soils or sandy loam soils and is common in areas receiving 200 –1370 mm of rainfall annually (Orwa et al*.*, 2009). It can grow to heights of between 7 and 18 m, with grey creviced bark, a short taproot of 2.4 m, lateral roots that can reach up to 30 m (Orwa et al., 2009), stout branchlets and pale foliage. The tree produces green fruits which are plum-like, 3 – 4 cm in diameter and approximately 15 – 25 g in weight. When ripened, the fruit turns pale yellow with juicy mucilaginous flesh. The outer skin of the fruit has a rather pungent, apple-like odour and its flavour has been described as resembling some other fruits such as mango (Coates, 1977; Shackleton et al*.*, 2002; Wynberg et al., 2002; Ojewole, 2006).

All parts of the marula tree can be used, making it a non-timber forest product (NTFP) that contributes largely to livelihoods, culture and increasingly to economic emancipation among rural communities in southern Africa. Indeed, many uses for marula have been predominantly domestic or at most extended only to local communities. The scope of use is determined, among various areas, in one or more countries by tradition, the abundance of the trees and to what extend the tree has been commercialised. Marula wood is used for fuel wood, fencing poles, or to carve out utensils such as spoons, rough seats/stools, food trays for pigs, hoe handles, catapults, pestles and mortars (Shackleton and Shackleton, 2002). Of widespread usage are the marula fruits which are eaten raw or used to prepare juice, porridge, jam, jelly, dry fruit rolls and also fermented to make alcoholic beverages such as beer, wine and Amarula cream liqueur (Weinert et al., 1990; Mizrahi and Nerd, 1996; van Wyk et al., 2002; Mojeremane and Tshwenyane, 2004; Hillman et al., 2008; Viljoen et al., 2008). The kernels can be eaten fresh or roasted, pressed for oil that is used for cooking, preserving meat, skincare and treatment of leather or fed to livestock (Shackleton et al*.*, 2002; Mokgolodi et al*.*, 2011). The skin of marula fruits can be dried to use as a substitute for coffee, cooked as a relish or used during droughts as fodder for livestock (Mojeremane and Tshwenyane, 2004).

The roots, bark and leaves are used for medicinal preparations used to treat a range of ailments (Mariod and Abdelwahab, 2012). Studies indicate the consumption of fresh fruit, kernels, and the production of beer/ wine as the three popular uses of marula fruits across different areas (Shackleton and Shackleton, 2002). By far the most prevalent use, however, is the production of marula beer/wine, as far as we have observed in literature, nearly 1814.37 kg of marula fruits – equating to about 150-350 L of beer/wine consumed per household each season in Namibia and South Africa (Shackleton, 2004). During the 2019 Marula Festival hosted in the Limpopo province, 12 000 L of the brew was sold to the Limpopo department of Economic Development, Environment and Tourism (SowetanLive, 2021).

### 2.1.2 Marula tree and its economic importance

Apart from subsistence use, marula beer/wine is produced and sold in Botswana, South Africa, Swaziland, Namibia, and Zimbabwe (Mojeremane and Tshwenyane, 2004). This small-scale commercialisation generates income for rural and poor community members thus improving their livelihood. For example, some community traders in Limpopo province, South Africa charge R150 for a 20 L brew, R10 for a 2 L bottle and R5 for a 500 mL (Daily Sun, 2018).

In South Africa, marula fresh fruits are collected and sold to Mirma/Distell Pty Ltd which produces Amarula Cream liqueur (Nine, 2006), and to Mine Workers Development Agency and Marula Natural Project Trust, which produces fruit pulp as well as essential and edible oils (*Marula - Company Profile - How we do it*, 2020). Community members make about R13 per 25 kg and R30 per 50 kg of fruit sold to Distell factory (*Marula, the fruit of their labour - Review*, 2020). Recently, several companies trading in marula-based products have emerged. PortiaM (est. 2011) is among the leading skincare range companies produced from marula. This company uplifts youth and women in rural communities through local projects conducted under its foundation (*Home - Portia M Skin Solutions - Marula Oil For skin*, 2020). Marula guys (est. 2018) are a multidisciplinary company offering marula products ranging from food and beverages, skincare products and activated carbon for industrial applications - activated carbons prepared from marula stones can adsorb heavy metals from aqueous solutions or wastewater (Misihairabgwi et al., 2014). This company also pays local community members to harvest the fruit. In Namibia, Eudafano Women's Cooperative (est. 1996) is a leading producer of marula oil for international markets (Eudafano, 2016). By 2016 this company was composed of 26 associations and 2,500 women harvesting and processing fruit with an income of about $ 104,712 per member (Eudafano, 2016).

The importance of marula as a tool for development and economic advancement of rural communities has led to several initiatives. The most popular ones include the uMthayi Marula Festival, held in the Kwa-Ngwanase district, KwaZulu Natal (https://www.kzndard.gov.za, 2020) and the South African annual Marula festival, known also as The Festival of The First Fruit – 'Ku LumaNguva'/'Go Loma Morula' held in Phalaborwa, Limpopo province. These festivals attract tourists from southern Africa and generate profit that is invested back into the communities. The South African Annual Marula festival generated over 30 million during the 2020 celebration (www.limpopomarulafest.co.za, 2020). Furthermore, the Limpopo province government is establishing a multi-million-rand marula industrial hub in Phalaborwa, complete with processing facilities, a research centre, and agri-business support services for rural farmers and small businesses wanting to get into the marula value chain. This facility will also be used to host the South African annual Marula festival (*Limpopo plans multi-million rand marula hub*, 2020). Perhaps the most progressive of the initiatives is the potential to develop a regional marula sector for southern Africa which was the main agenda point in the regional marula workshop held in Namibia in 2019. The benefits of having a marula sector are numerous and include among others, rural development, job creation and new export markets (*Report on the first regional workshop on a Marula sector development plan 2*, 2020). However, as with many other commercialised products, marula products must comply with EU food and cosmetic regulations and get approval for safe consumption to successfully penetrate global markets. To this end, a scientific basis for claimed marula benefits and safe use must be developed and articulated at industry, consumer, and policy level. This task is achievable as more research on marula is being publicised and the knowledge base is steadily increasing.

### 2.1.3 Medicinal value of marula and its wine

The bulk of the published research on marula (*S. birrea*) is on the medicinal uses of its bark, root and leaves (Mariod and Abdelwahab, 2012). In South Africa, Tanzania, Ghana, Zimbabwe and Niger the stem-bark, roots and leaves of marula tree are used for cold and flu, sore throat/ mouth, malaria and fever, diarrhoea and dysentery, stomach ailments, vomiting, headaches, toothache, backache and body pains, infertility, schistosomiasis, epilepsy, diabetes mellitus, proctitis, ulcers, inflammation, arthritis, hypertension, skin diseases, fungal infections, snake poison, sore eye, pharyngitis, splenomegaly, goitre and haemorrhoids (Watt and Breyer-Brandwiik, 1962; Gelfand et al.,1985; Hutchings et al., 1996; van Wyk et al., 1997; Mshana et al., 2000; Eloff, 2001; van Wyk et al., 2002; Wezel, 2002;

Ojewole, 2003; Hamza et al., 2006; Mathabe et al., 2006; Ojewole, 2006; Dimo et al., 2007; Gondwe et al., 2008; Masoko et al., 2008; van Wyk, 2008; Maroyi, 2013; Chauke et al., 2015). Pharmacological studies on these plant parts (both *in vitro* and *in vivo*) have been conducted to lend scientific support to their traditional use (Ojewole et al., 2010). The researchers reported the presence of secondary metabolites or phytochemicals produced by marula (Ndhlala et al., 2007; Borochov-Neori et al., 2008; Masoko et al., 2008; Manzo et al., 2017). Roots, bark and leaf extracts have been characterised for phytochemicals of which nine phenolic compounds namely, gallic acid, catechin, epicatechin, epicatechin-3-O-gallate, epigallocatechin-3-O- gallate, quercetin-3-O-arabinoside, quercetin-3-O-rhamno-side, quercetin-3-O-glucoside and procyanidin B2 were identified and confirmed (Russo et al., 2013). Several other studies on the biological properties of marula tree included a standard screening test for the presence of a group of phytochemicals such as tannins, saponins, flavonoids and alkaloids (Kutama et al., 2013; Manzo et al., 2017; Mai et al., 2019). The consensus among researchers is that these non-nutritional phytochemicals are responsible for most of the biological activities of marula extracts. These phytochemicals include sesquiterpene hydrocarbons (Pretorius et al., 1985), caffeic acid, vanillic acid, p-hydroxybenzaldehyde, ferulic acid, p-hydroxybenzoic acid and p-coumaric acid (Ndhlala et al., 2007), derivatives of hydrolysable tannins, catechins and hydroxycinnamic acid (Borochov-Neori et al., 2008), gallic acid and epicatechin (Hiwilepo van-Hal, 2013).

A larger volume of the published research on biological activities of marula provides evidence for its antioxidant and antimicrobial properties (Mariod et al., 2012; Russo et al., 2013; Tanih and Ndip, 2012). Antioxidant capacity has been reported in fruit and juice extracts (Borochov-Neori et al., 2008; Hillman et al., 2008; Hiwilepo van-Hal, 2013). Notably, the antioxidant capacity hereby reported results from the synergistic action of the non-nutritional phytochemicals and ascorbic acid with up to 70% of it attributed only to the presence of ascorbic acid (Owusu-Apenten, 2003). The antioxidant capacity of marula juice was reported in the ranges between 141 mg/100 mL (Hillman et al., 2008) to 2960 mg/100 mL (Mdluli and Owusu-Apenten, 2002). This range is much higher than 44 – 76 mg/100 mL and 44–132 mg/100 mL ascorbic acid equivalents reported for orange pomegranate respectively (Hillman et al., 2008). Hiwilepo-van Hal et al. (2013) reported antioxidant capacity of marula wine as 0.011 ± 0.003 µmol/g (TEAC equivalent) with about 80% of it retained from the juice after fermentation (Hiwilepo-van Hal, 2013).

The antimicrobial activity has been reported in different extracts of the stem bark against *Bacillus subtilis* (Moyo, 2009), *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis* and *Escherichia coli* (Eloff, 2001; MacGaw et al., 2007; Moyo, 2009; Kutama et al., 2013; Manzo et al., 2017; Louis et al., 2018; Mai et al., 2019), *Salmonella typhi* (Manzo et al., 2017), *Klebsiella pneumoniae* (Mai et al., 2019), *Cryptococcus albidus* and *Candida parapsilosis* (Masoko et al., 2008) and *Candida albicans* (Runyoro et al., 2006; Moyo, 2009). Leaves extracts showed activity against *E. faecalis*, *E. coli* and *P. aeruginosa* and *S. aureus* (Eloff, 2001; Kutama et al., 2013) and the different extracts of the root against *Candida albicans* (Hamza et al., 2006; Louis et al., 2018), *Candida glabrata, Candida parapsilosis, Candida tropicalis, Candida kruseii* and *Cryptococcus neoformans* (Hamza et al., 2006).

The Antioxidant activity has been shown in stem bark extracts (Mariod et al., 2008; Masoko et al., 2008; Moyo, 2009; Tanih and Ndip, 2012; Russo et al., 2013; Lall and Sharma, 2014) in leaf extracts (Braca et al., 2003; Mariod et al., 2008; Moyo, 2009; Russo et al., 2013), in root extracts (Russo et al., 2013; Armentano et al., 2015; Akoto et al., 2020) and kernel oil cake (Mariod et al., 2008).

Other pharmacological activities described include anti-inflammatory properties of stem bark (Ojewole, 2003) and root extracts (Akoto et al., 2020), anti-diarrhoeal effects of stem bark extracts (McGaw et al., 2007, McGaw and Eloff, 2008), anti-diabetic properties of stem bark (Ojewole, 2004; Musabayane et al., 2006; Gondwe et al., 2008; Makom Ndifossap et al., 2010; Mohammed et al., 2014), root and leaf extracts (Mohammed et al., 2014), anti-cancer effects of stem bark (MacGaw et al., 2007; Tanih and Ndip, 2013; Mai et al., 2019;), kernel oil (Mariod et al., 2005) and root extracts (Armentano et al., 2015), anthelmintic activities of stem bark (MacGaw et al., 2007) and root extracts (Akoto et al., 2020), antiplasmodial effects of bark and leaf extracts (Gathirwa et al., 2008); antihypertensive properties of bark and leaf extracts (Ojewole, 2006) as well as anticonvulsant effects of stem-bark in mice (Ojewole, 2006). These reports provide evidence of the health-effects exerted by the marula tree and its parts. Since the biological activities listed above occur as a result of the presence and actions of secondary metabolites produced by the plant, the fruits and juice may likely contain some of the active metabolites.

### 2.1.4 Nutritional and non-nutritional value of marula tree

The nutritional compositions of marula seed, fruits and juice have been extensively documented (Hiwilepo- van Hal et al., 2014). Marula seed has a lipid content that varies from 40 – 85% dry weight (Arnold et al., 1985; Eromosele et al., 1991; Leakey, 1999; Mizrah et al., 1999; Francis and Tahir, 2016; Magaia and Skog, 2017) with quantities as high as 19.5 g/100 g dry weight reported (Glew et al., 1997), a major fraction of which is triacylglycerol, representing 76.5% of the total lipid, followed by phospholipids at 12.5% and diacylglycerol at 5.6% (Mariod, 2005); a fatty acid composition (80% being unsaturated) accounting for 47 mg/g dry weight of the seed (Glew et al., 2004; Magaia and Skog, 2017) two thirds of which is oleic acid, followed by stearic, palmitic, linoleic and archidonic acids (Ogbobe, 1992; Mariod et al., 2004; Mariod et al., 2008; Magaia and Skog, 2017) protein content varying from 5.6% to 36.4% dry weight (Glew et al., 1997; Glew et al., 2004; Francis and Tahir, 2016) with glutamic acid as the most abundant amino acid at 25.7% followed by arginine 13.9%, aspartic acid, valine, glycine, phenylalanine, leucine and alanine (Magaia and Skog, 2017); crude fibre content of 2.4% comprising uronic acids 82.4%, glucose 10.5%, arabinose 3.9% and xylose 3.2% (Francis and Tahir, 2016; Magaia and Skog, 2017); energy value of approximately 2699 – 2703 kJ/100 g (Wynberg et al., 2002); minerals including phosphorus (212 mg/100 g), calcium (156 –808 mg/100 g), magnesium (193 – 462 mg/100 g), potassium (601 mg/100 g), iron (2.8 mg/100 g), sodium, zinc and copper (Mojeremane, 2004; Borochov-neori et al., 2008). The moisture content of raw seed powder was a low 4.17% (Francis and Tahir, 2016).

Marula fruits have a lipid content of 50 – 85% dry weight (Eromosele et al., 1991; Leakey, 1999), a fatty acid composition as high as 47% dry weight (Glew et al., 2004) and protein content of 3310 – 3600 mg/100 g dry weight with an abundance of sulphur-containing amino acids like methionine and cysteine (Mariod and Abdelwahab, 2012; Muhammad et al., 2011). The juice has a carbohydrate fraction of 7 to 14% fresh weight (Gous et al., 1988) and 90.35% dry weight which consists mainly of sucrose at 6160 mg/100 g dry weight, glucose at 1700 mg/100 g dry weight and fructose (Muhammad et al., 2011), dietary fibre of 0.7 g/100 g (Borochov-neori et al., 2008); a vitamin C content ranging from 62 mg/100 g (Carr, 1957) to more than 2,100 mg/100 g (Eromosele et al., 1991; Hillman et al., 2008; Muhammad et al., 2011; Hiwilepo-van Hal et al., 2012). This level is about four times higher compared to orange and pomegranate juices (Hillman et al., 2008). Minerals such as calcium (51.73 mg/100 $cm^3$), magnesium (24.53 mg/100 $cm^3$), potassium (44.54 mg/100 $cm^3$), sodium (14.88 mg/100 $cm^3$), iron (8.83 mg/100 $cm^3$), manganese, zinc, copper, nickel, chromium and phosphorus

(Borochov- Neori et al., 2008; Eromosele et al., 1991; Muhammad et al., 2011); ash content of 0.09g /100g to 5g/100g (Taylor, 1985; Muhammad et al., 2011) and moisture content between 82 and 93% (Shone, 1979; Gous et al., 1988) have also been reported. The phenolic content of marula juice ranges from 700 to 2500 mg GAE/100 g dry weight (Hillman et al., 2008). This is more than the phenolic content of guava, pineapple, and banana (Alothman et al., 2009). The nutritional composition of marula fruits and juice demonstrates its great potential for developing a nutritional wine especially if fermentation does not significantly deplete the important minerals and protein.

### 2.1.5 Nutritional and non-nutritional value of marula wine

In comparison with other non-grape and indigenous wines or beers, the nutritional and non-nutritional constituency of marula wine is scarcely reported. Studies carried out by Dlamini and Dube (2008) and Hiwilepo-van Hal et al. (2013) provide the bulk of the information available on marula wine. Marula wine is usually consumed after 4 days of fermentation and the alcohol level ranges between 2 and 5.5% (Dlamini and Dube, 2008; Mokgolodi et al., 2011; Hiwilepo-van Hal et al., 2013). This level of alcohol is higher than that of banana wine 1.37% (Idise, 2011), comparable to that of palm wine 1 – 6% (Santiago-Urbina and Rui´z-Tera´, 2014), but lower than that of mango wine 8.9 – 9.5% (Musyimi et al., 2014). The sugar content of marula wine at 8.4% was higher than 5.4% of mango wine but lower than 10 –12% in palm wine. Marula wine pH can reach 3.44 which is more acidic than palm wine (Panda et al., 2014) at pH 5.59 but comparable to pH 3.3 and pH 3.52 shown in banana wine (Akubor et al., 2003) and jackfruit (Panda et al., 2016) wine. The ascorbic acid level of marula wine ranged from 96 mg/100 g (Dlamini and Dube, 2008) to 159 mg/100 ml (Hiwilepo-van Hal et al., 2013) which is much lower than 1780 mg/100 ml in jackfruit wine (Panda et al., 2016) but higher than 10 –19 mg/100 ml (Okechukwu et al., 1984) in palm wine and 80 mg/100 ml (Panda et al., 2014) in bael fruit wine. The total phenolic content (TPC) of marula wine ranges from 690 ± 30 mg GAE/100ml when fermented at room temperature to 960 ± 130 mg GAE/100 ml when fermented at 40 ºC. These were comparable to 930 mg GAE/100 ml (Panda et al., 2014) reported in bael fruit wine and higher than 22.06 mg GAE/100 ml reported in cagaita wine, 12.70 mg GAE/100 ml in jabuticaba wine and 2.8 mg GAE/100 ml pitaya wine (De Souza et al., 2018). The information on the marula wine has many gaps especially with regards to the nutritional properties such that it is hard to deduce whether the wine is as nutritious as the juice or fruits. However, the reported ascorbic acid demonstrates that the wine retains a bulk of it from the juice.

### 2.1.6 Potential of marula wine as a nutritive low alcoholic drink

Recently, increased demands for cash income, diversification of livelihood strategies and the recognition by external agencies of the potential of indigenous plants as an option for promoting development and conservation have led to the increased commercialisation of many previous subsistence resources (Arnold and Ruiz Perez 1998; Belcher and Ruiz Perez 2001). These resources include subtropical and tropical fruits which are abundant in Africa. Among these is marula whose subsistent use has increasingly evolved into a small enterprise in most rural areas. The use of marula products had largely been sustained by folkloric tales, traditional beliefs, and customs rather than scientific evidence. Nevertheless, more traders have made a profit in the past 20 years than ever before. Lack of information on, among other things, the nutritional and medicinal properties of marula has resulted in delayed large-scale commercialisation of its products. Over the past decade, however, marula oil has penetrated global markets yielding estimated profits of ~ $39 million by the end of 2019, and likely to grow at a steady pace to reach ~ $60 million by the end of 2029 (https://www.globenewswire.com). Even more profitable is the Amarula cream liqueur manufactured by Distell Group Holdings Ltd which sold about 1 million 9 litre cases worldwide in 2019 (https://www.statista.com). The global adoption of marula oil has been linked to its composition of fatty acids, vitamins C and E, antioxidants and minerals (https://www.conserve-energy-future.com/benefits-marula-oil.php, 2020). These properties are indeed among the list of functional ingredients in functional foods and beverages that are increasingly sort after by consumers worldwide. Notwithstanding the increased use of marula oil in the cosmetic industry, marula beverages such as wine have the potential to penetrate the functional food and beverage industry and increase the overall market value of marula. Currently, beverages are by far the most active functional food category because of convenience and possibility to meet consumer demands for container contents, size, shape, and appearance, as well as ease of distribution and storage for refrigerated and shelf-stable products (Huma Bader-Ul-Ain et al., 2019). Moreover, they have excellent potential as carrier vehicles for delivering nutrients and bioactive compounds (Kumar et al., 2015). Grape wine is a well-studied and widely used functional beverage leading fruit wines in the functional beverage industry. However, consumer demands for functional beverages, based on sources like fruits and vegetables, have expanded the market for non-grape fruit wines. In response, research on grape wine properties that make it functional has been conducted for fruit such as cranberry, sweet cherry, pomegranate, and blueberry. The results have shown that these fruits can be made into functional beverages. The properties include among others total

phenolics, flavonoids, antioxidant activity and elemental composition (Dey et al., 2009). From the documented findings on marula thus far its fruit and juice contain some of these properties at concentrations equal or above those seen in grapes and other fruits (Vinci et al., 1995; Glew et al., 2004). For example, properties such as vitamin C and antioxidant capacity have a retention capacity post-fermentation of 72% and 80% respectively (Dlamini and Dube, 2008; Hiwilepo van Hal et al., 2014).

### 2.1.7 The importance of this study

The nutrient and non-nutrient compositions reviewed above varied from area to area based on differences in the place of origin, soil, climate, genotypes and environmental conditions during production, ripening stage of the fruits and the time that lapsed after harvesting (Hiwilepo-van Hal et al., 2013). It is acknowledged that the geographical origin of the marula tree, climate, and soil type in which it grows influences the concentration of minerals (Gous et al., 1988), vitamin C (Leakey, 1999), moisture (Gous et al., 1988), sugars (Leakey, 1999), phenolics and flavonoids (Hillman et al., 2008; Alothman et al., 2009) it produces. Also, genetic variations in the trees and anthropogenic factors in the areas where trees grow may influence these properties (Hiwilepo-van Hal et al., 2013). However, one species, *Sclerocarya birrea* (Hoscht) subsp. *caffra* (Sond.) Kokwaro of marula tree exists in South Africa and the tree is abundantly found in Limpopo and Mpumalanga provinces (Moganedi et al., 2011). This infers that the diversity observed in nutritional and medicinal characteristics of the tree is due to epigenetic factors.

It is noteworthy that the most common marula fruit product is marula wine and has a high socio-economic value in African communities. Nevertheless, the biochemical evolution of the nutrients from fruit juice state through fermentation to wine product state has not been widely documented. Numerous chemical transformations occur during biochemical reactions in the fermentation of marula juice (Hiwilepo-van Hal et al., 2013) and these will notably affect the presence, appearance, and disappearance of desirable medicinal and nutritional compounds. Noting the socio-cultural and economic importance of marula wine and its widespread consumption among the youth and elders, it is important to know and document the evolution and characteristics that result. Agreeably, marula juice is a good source of health-promoting components based on the nutritive and biological properties. If these properties are retained or increased during fermentation of the juice, the resulting wine would have potential as a nutraceutical drink.

### 2.1.8 Conclusion

From the literature reviewed above, there is a knowledge gap in the nutritional and non-nutritional composition of marula wine. This study profiled the presence of nutrients and non-nutritive constituents of the wine and their evolution over the fermentation period. The compounds of interest are alcohol, organic acids, proteins, lipids, ash and minerals, sugars, and phenolic compounds. Furthermore, the biological activities of phenolic compounds contained in the wine will be measured and tracked throughout.

## CHAPTER 3

## Determination of marula fruit properties and the environmental factors at selected localities where marula wine is widely produced

### 3.1 Abstract

Physicochemical properties of marula fruits, *Sclerocarya birrea* (Hoscht) subsp. *caffra* (Sond.) Kokwaro were investigated. The fruits were collected from The Oaks, Sekhukhune and the University of Limpopo, in Limpopo Province, South Africa. Physical parameters such as fruit mass, fruit length, fruit width, peel mass, kernel mass and juice yield were determined using a laboratory balance and vernier calliper and chemical constituents such as fruit pH, total soluble solids and titratable acid were determined with a calibrated pH meter, a refractometer and by titration method with 0.1 M NaOH respectively. Information on the climate and soil type for all the areas were sourced from national databases. Overall marula fruits in this study were of the average fruit mass, length and width of 29.59 g, 3.29 cm and 3.96 cm respectively. The marula juice was acidic containing on average 0.49 titratable acid at pH of 3.65, with about 10.58 °Brix sugar content. There were significant ($p = 0.000$) variations among the fruit samples in all physicochemical properties. The Oaks had the largest fruits attaining the mean fruit mass of 37.59 g ± 3.98, peel mass of 14.94 g ± 1.68, kernel mass of 11.71 g ± 1.77, fruit length of 3.53 cm ± 0.19 and fruit width of 4.29 g ± 0.19. Sekhukhune fruit differed from University of Limpopo only in fruit length, fruit width, juice mass ($p = 0.000$) and total soluble solids ($p = 0.04$). Sekhukhune fruits produced 9.73 ml ± 2.12 of juice, which represented 31.38% of the fruit mass. This yield was significantly higher than all the other areas. However, the University of Limpopo had the highest total soluble solids and titratable acid % ratio (35.34:1) attaining better ripening and maturity. This suggests that TSS/TA ratio should be used to select appropriate fruits to use in winemaking. The areas had similar climatic conditions except at higher temperatures. The Oaks and Sekhukhune experienced hotter days than the University of Limpopo ($p = 0.000$, t-value = 5.777). The effect of this temperature difference on the physicochemical properties of marula fruits and juice could not be established. Thus, although the marula fruits and juice from the different sampling areas varied in their physicochemical properties, these variations could not be linked to any differences in the climate and environmental factors of the sampling areas.

Keywords: fruits, juice, physical properties, juice yield, chemical properties, TSS/TA ratio, environmental factors

## 3.2 Introduction

*Sclerocarya birrea* of the Anacardiaceae family, usually referred to as marula (Figure 3.1) is a naturally occurring tree throughout Africa. *S. birrea* is divided into three subspecies namely *birrea* which is endemic to Western Africa, *multifoliolata* mainly found in Tanzania and *caffra* (Sond.) Kokwaro which is the more popular subspecies found in east tropical Africa (Kenya, Tanzania); south tropical Africa (Angola, Malawi, Mozambique, Zambia, and Zimbabwe); southern Africa (Botswana, Namibia, South Africa and Swaziland) and the huge island nation of Madagascar (Flora Zambesiaca 1966; Fox and Norwood Young 1982; Arnold and de Wet 1993; Hall et al., 2000; SEPASAL 2001). The genus has also been introduced to Australia, India (Tamil Nadu), Israel, Mauritius, Oman and Réunion (Hall, 2002).

The genus name *Sclerocarya* is derived from the Greek word *skleros,* meaning hard, and *karyon*, meaning a nut which refers to the hard stone of the fruit (Shone, 1979). The species name *birrea* is derived from *birr* which is the common name for the tree in Senegal, West Africa (Palmer and Pitman 1972). The name *"caffra*" was derived from the Hebrew word, 'kafri' meaning a 'countryman' and 'Kaffaria' the descriptive name given to the southeast part of South Africa today known as Eastern Cape (Orwa et al, 2009). The term or name marula, adopted as an imported word into English and Afrikaans is widely used for the species across southern African languages such as lobedu, Pedi and Tswana (Shackleton et al., 2003).

Numerous imprecise habitat requirements have been observed for both the growth and distribution of marula. Generally, the tree grows well in warm, dry and frost-free climates within the mean annual ranges of 19 – 35 $^{o}$C and 0 – 1500 mm in temperature and rainfall respectively (Peters, 1988; Orwa et al., 2009) moderate to low elevations between 0 – 4 500 m (Hall et al., 2000; Taylor et al., 1995); wooded grassland, riverine woodland and bushland vegetation (Jama et al., 2008; Orwa et al., 2009); well-drained and high salt soils such as sandy and a mixture of sand, silt and clay called loams (Duke, 1989; Hall et al., 2000; DFSC, 2003). Growth has been reported at high altitudes where temperatures may drop below the freezing point for a very short period in winter (Marula Net Database, 2003), rocky soils such as granite, laterite and basaltic clays, clear stands and frequently hills and slopes (Moss and Taylor, 1983; Lewis, 1987; DFSC, 2003; Orwa et al., 2009). Swampy and clay soils, dense stands or population forests and frost do not support the growth and spread of marula (von Breitenbach 1965; Palmer and Pitman 1972; Shone, 1979; Johnson and Johnson, 1993).

**Figure 3.1**: The marula tree. Own picture was taken at Sekhukhune Moshira in March 2018.

The main use of marula fruits is in processing them into wine. For brewing, the marula fruits skins are cut open using a cow horn or stainless-steel fork and squeezed to extract their juice (den Adel 2002; Hiwilepo van-Hal, 2013). However, the squeezing process produces low juice yields (about 20%) because part of the flesh is attached to the skin and the kernel (Gous et al., 1988). The low juice yield can be compounded if the fruits used are not fully ripened. The fruits can be selected based on their colour, size, firmness, and taste. These parameters can be assessed both by physical evaluation and chemical testing. Suárez et al. (2012) reported that the physicochemical characteristics for ripe fruits were a firmness of 2.5 kg/cm$^2$, sweet to very sweet taste of sugar level ~11°Brix at ~pH 4.4, as well as 'green-yellow/ yellow colour analysed using RGB scale as R: 205 – 224, G: 215 – 221 and B: 72 – 125. The perception of ripeness is more closely associated with the reduction of an acidic taste than sugar content i.e., the TSS/TA ratio determines the delectability of the juice (Litz, 1997).

The geographical origin of the marula tree, soil type, climate, genetic and anthropogenic factors present in the area in which the tree grows influence the concentration of minerals (Gous et al., 1988), vitamin C (Leakey, 1999), moisture (Gous et al., 1988), sugars (Leakey, 1999), phenolics and flavonoids (Hillman et al., 2008; Alothman et al., 2009) it produces.

However, the phenotypic characteristics of a tree are often stable throughout its lifespan when growing in an environment of constant climatic conditions (Moganedi, 2011). Temperature is known to affect both photosynthesis and respiration and their ratio must be high to achieve high yield. Higher temperatures induce stress to the plant through the inactivation of enzymes (Moretti et al., 2010). However, with a combination of higher carbon dioxide levels and heat induce production of more bioactive compounds such as phenols and ascorbic acid (DaMatta et al., 2010). Rain and humidity during the growing season have several adverse effects. Rain and cool weather during bloom can reduce bee activity and cross-pollination and thus diminish the crop load. Rain and high humidity favour fungus and bacterial diseases that can decrease the crop yield (Jaynes, 1969). According to Shone (1979), the marula tree is best suited to the drier areas receiving an annual rainfall of between 250 mm and 800 mm per annum.

One species, *Sclerocarya birrea* (A. Rich.) Hochst, subsp. *caffra* (Sond.) Kokwaro of marula tree exists in South Africa (Moganedi et al., 2011). However, there is high genetic diversity among trees growing in Limpopo province to those in Mpumalanga province. This is markedly lower within trees growing in the Limpopo Province. This diversity provides a basis for the phenotypic variation observed in marula. However, epigenetic influences and/or their interactions with genetic properties may also result in variations in the physicochemical properties.

This chapter reports on the assessment of the ripe marula fruit and the environmental conditions in which they were harvested.

## 3.3 Materials and methods

### 3.3.1 Collection of fruits

Green to yellow-green fruits were collected from the University of Limpopo grounds (23°53'11.4"S 29°44'16.4"E) and bought from community partners trading in marula products from two separate areas, The Oaks (24°21'44.6"S 30°40'31.0"E) and Sekhukhune (24°20'35.8"S 30°05'33.1"E). These fruits were placed in a cool place until ripened. The fruits were collected from all three areas between February and March 2018.

#### 3.3.1.1 Sampling

For each of the three areas, forty ripened fruits were picked from the stored ones, labelled and assessed for both physical and chemical properties.

### 3.3.2 Assessment of the physical properties of the marula fruit

3.3.2.1 Fruit colour

The RGB (red, green and blue) colour code chart was used. Each fruit was observed against natural light. Two assessors, male and female evaluated the fruit by turning it upside down and around to derive an overall impression of the colour shade of the fruit. A colour chart was used to estimate the colour range coded in the chart as variations of red, green and blue. The hex colour codes were then allocated to each fruit. The corresponding colour names were determined using RGB colour code chart (www.rgbcolorcode.com). To estimate the sample fruit colour, percentages were calculated where the occurrence of a certain colour was taken out of the sample, i.e.

$$\frac{the\ size\ of\ specific\ colour}{the\ sample\ size} \times 100$$

3.3.2.2 *Size*

A vernier calliper was used to measure the diameter and length of each fruit. The length was measured from the proximal (adjacent to the stalk) end to the distal end of the fruit and the diameter determined between the opposite smooth ends of the fruit. The values were presented in centimetres.

3.3.2.3 The overall weight of fruits

The fruits were individually weighed on a calibrated laboratory weighing balance (Radwag PS750/C/2). A clean weighing boat was placed on the weighing balance and tared before each measurement was taken. All measurements were recorded in grams (g).

3.3.2.4 Juice extraction and weighing

A puncture was made on the skin of each fruit. The juice was extracted into a 50 mL plastic beaker by squeezing the peel and the kernel. The amount of juice was weighed using a calibrated laboratory weighing balance (Radwag PS750/C/2). All measurements were recorded in grams (g).

3.3.2.5 Kernel size

The kernels of each fruit were transferred into clean weighing boats and weighed using a calibrated laboratory weighing balance (Radwag PS750/C/2). The weight of the weighing boat was automatically subtracted by taring the weighing balance before each measurement was taken. All measurements were recorded in grams (g).

3.3.2.6 Peel size

Peels removed from each fruit were transferred into clean weighing boats and weighed using a calibrated laboratory weighing balance (Radwag PS750/C/2). The weight of the weighing boat was automatically subtracted by taring the weighing balance before each measurement was taken. All measurements were recorded in grams (g).

3.3.2.7 Juice proportion

To determine was portion of a fruit weight was only juice, the following formula (Petje, 2008) was used:

Juice percentage (%) = Fruit mass(g) – (Peel and kernel mass (g)) * 100/Fruit weight (g)

### 3.3.3 Assessment of chemical properties of the marula fruit

3.3.3.1 pH

The pH of the marula fruit juice was determined using a calibrated pH meter (Crison).

3.3.3.2 Titratable acidity

Titratable acidity (TA) was calculated from titrating 0.1 M sodium hydroxide (NaOH) into a 10 mL volume of the marula fruit pulp and using phenolphthalein as an indicator. A persistent pink colour was used as a cut-off point for the addition of NaOH. The results were recorded in millilitre (mL).

Calculations for TA % (Petje, 2008) were carried out as follows:

$$\frac{volume\ of\ alkali\ (ml) \times normality\ of\ alkali \times 7.5}{volume\ of\ sample\ (ml)}$$

3.3.3.3 Total soluble solids (as °Brix)

Total soluble solids of the juice from each fruit were determined using a calibrated hand refractometer (ATC Atago). A few drops of each fruit juice were placed on the refractometer prism and the daylight plate was closed to spread the juice across the prism without any dry spots or bubbles. The refractometer was then pointed toward natural light and the scale was viewed through the eyepiece. The °Brix value (sugar content) was recorded. Distilled water

and a paper towel were used to rinse and dry the prism of the refractometer after each reading.

A ratio of total soluble solids to titratable acids was determined to assess fruit ripeness using the following formula: TSS/ TA ratio = Total soluble solid (°Brix value)/ Titratable acid

### 3.3.4 Environmental conditions of localities where marula fruits were harvested

A literature search was performed to obtain information on the types or textures of soil found in the areas from which marula was collected. Information on the temperature and rainfall was obtained via email on 24 April 2018 from the South African Weather Service (SAWS). Oudestad station (25°10'51.6"S 29°20'20.4"E at altitude 953 m) was used to collect climate data for The Oaks and Sekhukhune. The University of Limpopo data was obtained from Polokwane station (23°51'25.2"S 29°27'03.6"E at altitude 1226 m). Data on the soil was obtained via email on 21 April 2021 from Agricultural Research Council (ARC).

### 3.3.5 Statistical analysis

Descriptive statistics (average, range, minimum and maximum, standard deviation) was used to analyse marula fruit juice percentage (%), titratable acid percentage (%), and total soluble solids/ acid percentage (%) ratio. For inferential statistics, one-way ANOVA ($p< 0.05$) with multiple comparisons and independent t-tests were performed using IBM SPSS software.

## 3.4 Results

Three samples of 40 marula fruits in each were collected from the University of Limpopo, The Oaks and Sekhukhune (Moshira village). These fruits were assessed for physicochemical properties and the data derived thereof analysed statistically. The bulk of the results are presented in tables starting with descriptive statistics and ending with information on the environmental conditions.

### 3.4.1 Colour of marula fruits

The predominant colour of the ripe marula fruits was canary yellow (63.33%) followed by laser lemon (22.23%). The University of Limpopo had the most canary-coloured fruits (Table 3.1).

**Table 3.1**: The colour of the ripe marula fruits.

| Sample area | RGB scale | Colour | Percentage (%) |
|---|---|---|---|
| **The Oaks (TO)** | 255: 255: 153 | Canary yellow | 63.34 |
| | 255: 255: 204 | Cream | 33.33 |
| | 255: 255: 102 | Laser lemon | 3.33 |
| **Sekhukhune (SK)** | 255: 255: 153 | Canary yellow | 50.00 |
| | 255: 255: 102 | Laser lemon | 46.70 |
| | 255: 255: 204 | Cream | 3.33 |
| **University of Limpopo (UL)** | 255: 255: 153 | Canary yellow | 76.67 |
| | 255: 255: 102 | Laser lemon | 16.67 |
| | 255: 255: 204 | Cream | 6.66 |

### 3.4.2 Physical properties of marula fruits

Data regarding physical characteristics (fruit mass, fruit length and fruit width) of marula fruit from the three sample areas are summarised statistically in Table 3.2.

**Table 3.2**: Physical properties of marula fruits.

| Measurement | TO fruit mass (g) | SK fruit mass (g) | UL fruit mass (g) | TO fruit length (cm) | SK fruit length (cm) | UL fruit length (cm) | TO fruit width (cm) | SK fruit width (cm) | UL fruit width (cm) |
|---|---|---|---|---|---|---|---|---|---|
| **Minimum** | 27.99 | 18.00 | 15.30 | 3.14 | 2.80 | 2.54 | 3.86 | 3.14 | 3.22 |
| **Maximum** | 44.60 | 34.56 | 36.67 | 3.82 | 3.94 | 3.63 | 4.66 | 4.27 | 4.14 |
| **Mean** | **37.59** | 26.05 | 25.14 | **3.53** | 3.32 | 3.02 | **4.29** | 3.88 | **3.70** |
| **Standard Deviation** | 3.986 | 4.219 | 5.903 | 0.186 | 0.266 | 0.280 | 0.192 | 0.276 | 0.272 |

*****TO= The Oaks; *SK= Sekhukhune; *UL= University of Limpopo

#### 3.4.2.1 Fruit mass

The highest and lowest fruit mass were recorded for The Oaks ( $\overline{x}$ 37.59 g) and University of Limpopo ( $\overline{x}$ 25.14 g). The fruit masses between the marula fruits from The Oaks, Sekhukhune and UL was significantly different (p = 0.000), with the fruits from The Oaks being larger than those from both the Sekhukhune ($\overline{x}$ 26.05 ± 4.21 g; p = 0.000) and the University of Limpopo ($\overline{x}$ 25.14 ± 5.90 g; p = 0.000). There was, however, no significant

difference in fruit mass between Sekhukhune and the University of Limpopo (p = 0.711, appendices 1 and 1a).

3.4.2.2 Fruit Length

The Oaks marula had the biggest fruit in terms of length ($\overline{x}$ 3.53 ± 0.18 cm). This length was significantly bigger compared to that of Sekhukhune ($\overline{x}$ 3.32 ± 0.26; p = 0.004) and University of Limpopo ($\overline{x}$ 3.02 ± 0.28; p = 0.000). There was also a statistically significant difference between Sekhukhune and the University of Limpopo (p = 0.004, appendices 2 and 2a).

3.4.2.3 Fruit width

There was a statistically significant difference in width of fruits between areas (p = 0.000). A Tukey HSD post hoc test indicated that the width of marula from UL was the smallest compared to Sekhukhune ($\overline{x}$ 3.88 ± 0.27cm; 0.015) and The Oaks ($\overline{x}$ 4.29 ± 0.19 cm, appendices 3 and 3a).

### 3.4.3 Physical properties of marula fruit parts

The averages, standard deviation and other descriptive statistical variables calculated from marula parts (fruit peels, fruit kernels and fruit juice) data are summarised in Table 3.3.

**Table 3.3**: Physical properties of marula fruit parts.

| Measurement | TO fruit peels (g) | SK fruit peels (g) | UL fruit peels (g) | TO fruit kernels (g) | SK fruit kernels (g) | UL fruit kernels (g) | TO fruit juice (g) | SK fruit juice (g) | UL fruit juice (g) |
|---|---|---|---|---|---|---|---|---|---|
| **Minimum** | 10.97 | **5.61** | 6.33 | 8.23 | **4.84** | 3.82 | 8.17 | **5.80** | 2.22 |
| **Maximum** | 18.34 | **13.41** | 13.98 | 15.45 | **11.10** | 11.00 | 15.67 | **13.99** | 11.01 |
| **Mean** | 14.94 | **9.93** | 10.35 | 11.71 | **7.94** | 7.00 | 11.79 | **9.73** | 6.48 |
| **Standard Deviation** | 1.689 | 1.588 | 2.068 | 1.771 | 1.610 | 2.149 | 2.011 | 2.128 | 2.396 |

*TO= The Oaks; *SK= Sekhukhune; *UL= University of Limpopo

3.4.3.1 Fruit peels

The Oaks had the highest peel mass ($\overline{x}$ 14.94 ± 1.68 g), which was significantly different from the University of Limpopo ($\overline{x}$ 10.35 ± 2.068) and Sekhukhune ($\overline{x}$ 9.93 ± 1.58) both at p-value of 0.000. There was no difference in peel mass between the University of Limpopo and Sekhukhune (p = 0.56) and with Sekhukhune fruits as well (p = 0.06, appendices 4 and 4a).

3.3.3.2 Fruit kernels

The Oaks fruits had the biggest kernels with an average mass of 11.71 g and this was significantly different to the other two areas (p = 0.000). Furthermore, the analysis revealed the difference (p = 0.000) between Sekhukhune ($\overline{x}$ 7.94 ± 1.61 g) and University of Limpopo ($\overline{x}$ 7 ± 2.14 g, appendices 5 and 5a).

3.4.3.3 Fruit juice

On estimation, only about 30% of marula fruit constituted the juice. Notwithstanding the kernel, juice yield was directly proportional to peel mass. Sekhukhune fruits yielded more juice (31.38%) followed by University of Limpopo fruits (30.94%). The difference in juice mass was statistically significant (p = 0.000, appendices 6 and 6a) between all the three groups.

### 3.4.4 Chemical properties of marula fruits

The average chemical properties of marula fruit sampled for this study are summarised in Table 3.4 below. On average the marula fruits were acidic and contained an average of 0.49 titratable acid at a pH of 3.65, with about 10.58 °Brix sugar content.

**Table 3.4**: Chemical properties of marula fruits.

| Measurement | TO fruit pH | SK fruit pH | UL fruit pH | TO fruit TSS (°Brix) | SK fruit TSS (°Brix) | UL fruit TSS (°Brix) | TO fruit titratable acid | SK fruit titratable acid | UL fruit titratable acid |
|---|---|---|---|---|---|---|---|---|---|
| **Minimum** | 2.87 | 3.63 | 2.99 | 7.80 | 6.60 | 8.60 | 0.37 | 0.12 | 0.08 |
| **Maximum** | 3.71 | 4.44 | 4.71 | 13.00 | 14.60 | 13.00 | 1.42 | 1.09 | 0.47 |
| **Mean** | 3.15 | 4.01 | 3.80 | 10.55 | 10.21 | 10.99 | 0.96 | 0.29 | 0.23 |
| **Standard Deviation** | 0.186 | 0.225 | 0.432 | 1.085 | 1.942 | 1.020 | 0.29 | 0.18 | 0.088 |

***TO= The Oaks; *SK= Sekhukhune; *UL= University of Limpopo; TSS= Total soluble solids**

3.4.4.1 Fruit acidity

- *pH*

The Oaks marula fruits had the lowest average pH at 3.15. At this pH, these marula fruits were significantly more acidic than the University of Limpopo ($\overline{x}$ 3.80) and Sekhukhune ($\overline{x}$ 4.01) at p value of 0.000, appendices 7 and 7a.

- *Titratable acidity*

Marula fruits from The Oaks had a much higher titratable acid than that of Sekhukhune and the University of Limpopo. A Welch's ANOVA determined a significant difference (p = 0.000) between The Oaks and both Sekhukhune and the University of Limpopo. There was however no statistically significant difference (p = 0.233, appendices 8 and 8a) between Sekhukhune and the University of Limpopo.

There was an inverse proportionality between the pH and the amount of organic acids (titratable acidity) in the juice. The pH measure is relatively higher in the juice wherein the level of titratable acidity is lower, inferring a concentration factor influence on the pH of the juice.

3.4.4.2 Total soluble sugars (°Brix*)*

University of Limpopo marula fruit juice had the highest soluble sugar content ($\overline{x}$ 10.99 ± 1.02), and the difference was significant (p = 0.038, appendices 9 and 9a) compared to Sekhukhune juice.

A slight correlation ($r^2$ = 0.17) was found between total soluble solids and juice yield of fruits sampled from Sekhukhune, suggesting a low influence of dilution factor on the sugar content of the juice.

### 3.4.5 Parameters of ripe marula fruits

The chemical ripeness of marula fruit was determined using higher pH and higher TSS/ TA ratio. University of Limpopo marula showed a TSS/ TA four-fold higher than that of The Oaks (Table 3.5).

**Table 3.5**: Parameters of ripe marula fruits.

| Sample area | Colour (RGB scale) | Total soluble solid (°Brix) | pH | TSS/ TA ratio |
|---|---|---|---|---|
| **The Oaks** | Canary and cream | 10.55 | 3.15 | 8.18: 1 |
| **Sekhukhune** | Canary and laser lemon | 10.21 | 4.01 | 28.36: 1 |
| **University of Limpopo** | Canary | 10.99 | 3.80 | **35.34: 1** |

The higher TSS/TA ratio is a measure of the degree of ripeness of the fruit, i.e., the amount of organic acids is important for determining the degree of ripeness of the fruit.

### 3.4.6 Environmental conditions

Data on climate and soil type were obtained for the assessment of environmental factors on the physicochemical properties of the fruits.

3.4.6.1 Climatic conditions

Average temperature and rainfall for the three sampling areas during the period when marula fruits were collected are tabulated (Table 3.6).

The Oaks and Sekhukhune had significantly higher temperatures than the University of Limpopo ($p = 0.000$, appendix 10). There was no significant difference in rainfall between all the three areas ($p = 0.803$).

**Table 3.6**: Climatic conditions and soil type of the sampling areas during the collection of marula fruits.

| Sampling area | Temperature Min/Max (° C) | Rainfall (mm) | *Soil type/ texture | **Characteristics of soil types |
|---|---|---|---|---|
| **The Oaks (TO)** | 17.4/ 31.1 | 3.4 | Hutton loamy coarse sand (86-100% sand, 0-14% silt and 0-10% clay). Coarse sand texture in the grey topsoil. Lime is rare. | Sand: very low nutrients and poor water holding capacity<br><br>Silt: more fertile and better water holding capacity than sand<br><br>Clay: retains water but has less penetrative property for air and water. Does not support plant growth<br><br>Loam: a mixture of clay, sand and silt. Moderate amount of nutrients and water retentive capacity. |
| **Sekhukhune (SK)** | 17.4/ 31.1 | 3.4 | Hutton sandy loam (50-70% sand, 0-50% silt, 0-20% clay). Has 2-10 % stones for 25-75 mm of the top layer. | |
| **University of Limpopo (UL)** | 17.3/ 27.8° C | 2.9 | Hutton sandy clay loam (65% sand, 30% clay and 5% silt). | |

* Classification of soil type derived from http://www.fao.org; ** https://byjus.com/biology/types-of-soil/ (2021)

The area at the University of Limpopo receives less rainfall and the soil type is composed of more clay which has high retentive power for water but low penetrative capacity for moisture comparatively. This could contribute to the juice yield of marula fruits wherein The Oaks and Sekhukhune areas produced more juice and showed to receive better rainfall (Table 3.6).

## 3.5 Discussion

The intent of this chapter was to assess the properties of ripe marula fruits and the environmental conditions in which they were harvested, with the aim of establishing any relationship between the different environmental factors and the differences observed in the physicochemical properties of the marula fruits.

Overall, the average fruit mass, length and width were 29.59 g, 3.29 cm and 3.96 cm respectively. Generally, these parameters are varied between different localities within and across different areas in southern and South Africa. Fruit weight from as low as 20.1 g to 34.14 g were reported elsewhere (Leakey et al., 2005; Petje, 2008; Suárez et al., 2012). The fruit lengths observed in this study were naturally smaller than Namibian marula fruits at 3.75 cm to 4 cm and larger in width than Namibian fruits at 3.7 cm (Leakey et al., 2005; Suárez et

al., 2012). Notwithstanding any possible influence by climatic and environmental factors, the genetic characteristics seem to be highly contributing to the physical characteristics of the marula fruits since intraspecific differences were observed in characteristics such as fruit size and interspecific similarities were apparent in terms of fruit width from different regions (Leakey et al., 2005; Suárez et al., 2012).

Adding to the mass and size of the fruits were the peel, kernel and least of all the juice. These characteristics were also variable between different localities in South Africa and those recorded for Namibia (Leakey et al., 2005; Petje, 2008). Generally, the larger fruits had the larger peels and kernels and the least juice. However, juice yield was directly proportional to peel mass which contributed more to the overall fruit mass with correlations of $r^2 = 0.4$, $r^2 = 0.04$ and $r^2 = 0.03$ for the University of Limpopo, Sekhukhune and The Oaks respectively. This trend differed from that observed by Leakey and colleagues (2005) where juice mass contributed more to the overall mass of the fruit. Ahmad et al. (2018) also found that pulp contributed 53% to the fruit mass of mangoes.

The predominant colour for ripe fruits in this study was canary yellow, RGB 255: 255: 153 mostly observed with the fruits from the University of Limpopo. The soluble sugar content found in these fruits was significantly higher than the fruits from the other areas. However, these fruits also had the lowest titratable acid and balanced pH. Furthermore, fruits sampled from Sekhukhune had a weak correlation between total soluble solids and juice yield implying that dilution factor had little influence on the sugar content of the juice. Ripeness is decided based on several factors such as the colour of the fruit, higher pH, lower titratable acid and higher soluble sugar content (www.unece.org, 2021). Suárez and colleagues (2012) classed marula fruits as ripe when within a pH range of 4.2 to 4.4 and displaying sugar content of approximately 11.00 °Brix. University of Limpopo fruits displayed an average sugar content of 10.99°Brix but had a slightly lower average pH of 3.80 which brought them short of the criteria of ripeness recommended by Suárez and colleagues (2012). Overall marula fruits in this study were acidic, had titratable acidity of 0.49 on average and pH of 3.65, with about 10.58 °Brix sugar content. Low alcohol fruit wine, which is wine with less than 10% ABV (Puckette, 2021), has been made from Bael and mango fruit juice with the sugar content of 20°Brix. The wines had an alcohol volume of 7.9% and 5% respectively (Sharma et al., 2007; Varakuma et al., 2011). Marula wine with an alcohol volume between 2 and 5% have been reported (Dlamini and Dube, 2008; Mokgolodi et al., 2011; Hiwilepo-van Hal et al., 2013).

Barring the high acidity, the marula fruits had good sugar content to produce a low alcohol wine.

TSS/TA ratio is a fruit testing procedure used to determine how far along fruits have ripened. A higher value indicates better maturity and ripening (www.agric.wa.gov.au, 2021). The fruits from the University of Limpopo showed to have a higher TSS/TA value and thus better ripening. Although there was no significant difference in the sugar content between any of the areas, The Oaks marula fruits had the lowest TSS/TA ratio due to its high titratable acidity of 0.96. This low ratio emphasises the importance of a good balance between acidity and sugar content in obtaining sweet and good flavoured fruits. Studies reported that an increase in perceived sweetness of the fruits is linked to a decrease in acidity rather than an increase in sugar content (Litz, 1997; Durmaz et al., 2010; Suárez et al., 2012). The TSS/TA ratio has been calculated for several fruits and juices such as ber walliati at 36:1 (Parshant, 2015); 7 to 9:1 for oranges and mandarins (www.yara.us); 26.2:1 for Jonagold apple juice (Mendes da Silva et al., 2019); and 36.57:1 for table grape (Daniels et al., 2019). Thus, although the University of Limpopo fruits are smaller in mass and size, and yield slightly lower volumes of juice, they have a balanced chemical profile with a higher sugar content to acid ratio and intermediate pH. Sekhukhune marula fruits also showed a good TSS/TA ratio of 28.36:1; coupled with a better juice yield makes them a good choice for wine and other product development.

Statistical analysis comparing means of physical and chemical properties of fruits obtained in the three areas used in this study indicates that although the fruits are of the same species and originate from the same province (Moganedi et al., 2011), there are marked differences in their composition. Fruits from The Oaks were particularly different from those of Sekhukhune and the University of Limpopo in all properties except sugar content. This highly significant difference between trees of different locations and/or populations has been observed with *Trichoscypha acuminata* “Gabon grape” (Tsobeng et al., 2020). The Oaks fruits and fruit parts were markedly larger than Sekhukhune and the University of Limpopo. Sekhukhune fruit differed from UL only in fruit length, fruit width and juice mass. This trend was observed with South African marula fruits from the Limpopo and Gauteng provinces where the fruits differed significantly in their fruit mass, skin mass and pulp mass (Leakey et al., 2005) than fruits sampled from Malawi and Mozambique (Mkwezalamba et al., 2015). Fruits from Sekhukhune and the University of Limpopo had fewer variations, differing only in

fruit length, fruit width and juice mass. Interestingly, Moganedi et al. (2011) observed a high genetic similarity between marula trees across the Limpopo and Mpumalanga province. These variations observed in this study thus allude that the variations are due to minor differences between multiple genetic loci noting that these characteristics such as sweetness, yield and size of the fruits are quantitative (multilocus) traits (Cirilli et al., 2021).

Researchers have specified that variations in soil and climate can affect the occurrence and concentration of nutritional and biochemical components and physicochemical properties in marula and other indigenous fruits (Haque et al., 1970; Leakey, 1999; Hillman et al., 2008; Alothman et al., 2009). Climate information obtained for analysis in this study showed variation at a single factor. The Oaks and Sekhukhune climate differed from that of the University of Limpopo only in high temperature. Temperature and rainfall from The Oaks and Sekhukhune were obtained from the same station thereby undermining their contributions towards the differences in their fruit compositions. If hot weather is considered a factor in the differences in physicochemical composition between The Oaks and the University of Limpopo, it can only be argued for fruit length, fruit width and juice mass as observed with Sekhukhune fruits. Nevertheless, the temperatures reported for the three areas all fall within the reported range of 19 – 35 $^{o}$C (Orwa et al., 2009). Any association between the climate conditions and the differences observed in fruit compositions between the fruits from Sekhukhune and The Oaks could not be established since the data obtained in this study for these two areas is collected from the same weather station, Oudestad. Unless better and discriminatory climate data can be obtained, a combination of other factors may be involved. Nonetheless, elevated temperatures can block the ripening and maturing processes of fruits (Hribar and Vidrih, 2015) and lead to the production of low-quality grapes in terms of size and taste (Coombe (1987); Hale and Buttrose (1974).

Marula trees grow adequately in well-drained and high salt soils such as sandy and a mixture of sand, silt and clay called loams (Duke, 1989; Hall et al., 2000; DFSC, 2003). The estimated types of soil textures from the areas were loamy coarse sand, sandy loam and sandy clay loam for The Oaks, Sekhukhune and University of Limpopo respectively, and they fit the criteria of soils described above. The area at the University of Limpopo has more clay in its soil which retains more water for plants to use, however, this water is held tightly in the micropores such that the plants cannot access it (Easton, 2021). The area around Sekhukhune and The Oaks drains more readily and allow water to penetrate the soil and get absorbed in the roots of the trees. The advantage then for sandy soils as those in

Sekhukhune and The Oaks apart from the extra rainfall received, is the higher amount of water that is accessible to the plant. This could contribute to the higher juice yield observed for fruits obtained from Sekhukhune. Notwithstanding other factors, the largest fruit size of fruits from The Oaks and Sekhukhune could be attributed to soil drainage. The Oaks fruits were significantly more acidic which could be attributed to the deficiency in lime reported for its soil (Goulding, 2016). Information on the soil pH and all other properties was not readily available. The effect of soil properties such as salinity and soil depth on the yield and fruit maturity of pears are documented. Soil salinity had a direct relationship with fruit size and titratable acidity, while soils with shallow depth produced fruits with high levels of flesh firmness and soluble solids in pears (Aruani et al., 2014).

### 3.6 Conclusion and Recommendations

Our analysis established that marula fruits from the areas selected in this study are significantly different between themselves and from marula fruits found in other areas or countries based on their physical and chemical properties. The peels and kernels constituted the bulk of the fruit mass with the juice accounting for 30% of the fruits. The fruit size however did not affect the sugar content. The key observation of the study was in the acidity and sugar content ratio. The bigger fruits had higher acid levels resulting in a lower TSS/TA ratio which made them less ideal to use in wine production. Based on the ripe fruit parameters, the fruits from the University of Limpopo followed by Sekhukhune were better selections for marula wine production.

Factors sponsoring the differences in the fruit composition are still undefined as the information environmental conditions of the sampling areas obtained and analysed for this study was insufficient. Where temperature differences were observed only fruit length, fruit width and juice mass were involved. However, these parameters were different also for areas with the same temperature. The soils from which the fruits were grown and harvested, although different in their textures, were not adequately described. Indeed, the soils with more drainage also supported the growth of larger fruits, however, there is no conclusive evidence to establish this observation. Although several researchers insist that climate and soil type affect the composition of fruits, this remains a challenge to establish without targeted research controlling these parameters. Furthermore, these factors could be acting together and with genetic variations to produce the differences.

Further studies are required to elucidate the factors that produce variations in fruit compositions. Understanding the variations in the soil can inform its effect on the fruit composition. The climate requirements for the growth of wild marula are quite broad and will be difficult to study without controlled cultivation. Extensive research in which temperature and water supply are varied and compared could provide insight into the effects they have on the quality of the fruits. This information would assist winemakers select fruits with better physicochemical properties such as higher juice yield and full ripeness for production of quality wines with higher alcohol content.

## CHAPTER 4

## Qualitative and quantitative analyses of secondary metabolites in the marula fruit juice and wine

### 4.1 Abstract

Marula wine is revered for its nutritive and health properties. The information on health properties exists mostly about the marula fruit juice and there is very little on how much is retained in the marula wine, including the changes that occur during the fermentation process. The focus of this study was to measure and track the changes in composition and quantities of secondary metabolites in fresh marula juice and fermenting marula wine over time. Qualitative screening for the presence of saponins, phenols, tannins, steroids, terpenoids, alkaloids, phlobatannins, flavonoids and cardiac glycosides was conducted on dried juice and wine samples using rapid chemical tests. The profile of the phenolic compounds was determined using the Thin Layer Chromatography with silica gel plates that were developed in solvent systems of varying polarity. Ultra-violet light and vanillin sulphuric acid were used to visualise profiles of the phenolic compounds present in the extracts. The secondary metabolites flavonoids, phenolics and tannins were quantified using the Folin-Ciocalteu reagents and aluminium chloride method. Additionally, the individual secondary metabolites were identified and quantified using Ultra- High Performance Liquid Chromatography. All the juice and wine samples showed the presence of saponins, tannins, terpenoids, steroids, cardiac glycosides, and phenols. The polar solvent system showed better profiles of the compounds present in the extracts when visualised using vanillic sulphuric acid and UV light absorption. The marula wines had higher concentrations of total flavonoids, phenols and tannins when compared to the marula juice. There was however no significant difference ($p > 0.05$) in the concentrations of these metabolites throughout the fermentation period. University of Limpopo and Sekhukhune wines had significantly higher tannin and phenolic contents ($p < 0.0001$), with Sekhukhune wine having significantly higher flavonoid content ($p =0.005$) as well. The wines had more phenolic acids than flavonoids. The University of Limpopo had more luteolin (1.63 µg/mL), gallic acid (5.66 µg/mL) and slightly more taxifolin (1.78 µg/mL) and vanillic acid (7.05 µg/mL) whereas Sekhukhune had more apigenin (0.69 µg/mL), Kaempferol (0.33 µg/mL) and quercetin (0.23 µg/mL). The changes and differences in the content of phenolic compounds in the wines from juice to wine and area to area were brought about by microorganisms such as *Lactobacillus species*, *Lactobacillus plantarum*, non-*Saccharomyces (Hanseniaspora guilliermondii)* and *Saccharomyces cerevisiae*.

Different combinations of yeasts responsible for fermenting wine produce variability in the phenolic composition and/or content of the resulting wine. The University of Limpopo and Sekhukhune wines had better profiles of secondary metabolites and showed that marula wine can be a good source of active secondary metabolites.

Keywords: Wine, Thin layer chromatography, Folin-Ciocalteu reagents, aluminium chloride method, Ultra- High Performance Liquid Chromatography, tannins, phenolic acids, flavonoids

## 4.2 Introduction

Fruit-bearing trees have long been recognised for their contribution to human diet and nutrition. However, in recent times they have been increasingly acknowledged for their dietary phytochemicals or the composition of their bioactive compounds (Wallace et al., 2019). The trees, fruits or products thereof which provide humanity with good nutrition and health benefits have been defined as nutraceutical. With the rising demand for food and the recent scourge of Covid-19, nutraceuticals such as green tea supplements, Vitamin B12, Hemp-derived cannabidiol have gained importance. The search for alternative sources for food that is nutritious and with good health properties has led to an increased focus on indigenous plants. Dietary bioactive compounds derived from indigenous plants have been tested and proven beneficial to human health (Lupton et al., 2014). Among over 5000 dietary bioactive compounds identified to date, polyphenols and carotenoids are the most widely studied (Liu, 2013; Casas et al., 2018) of which phenolic acids (including hydroxybenzoic and hydroxycinnamic acids), hydrolysable and condensed tannins, and flavonoids are most important (King and Young, 1999).

Polyphenols are the biggest group of phytochemicals classified according to the chemical structures of the aglycones and divided into four subgroups, namely, phenolic acids, flavonoids, polyphenolic amides, and other polyphenols (Tsao, 2010). Phenolic acids and flavonoids cover most of the important and well-known dietary and medicinal phytochemicals (Lin et al., 2016). Phenolic acids are non-flavonoid polyphenolic compounds which can be further divided into two main types, benzoic acid (Figure 4.1) and cinnamic acid (Figure 4.2) derivatives based on C1–C6 and C3–C6 backbones (Tsao, 2010).

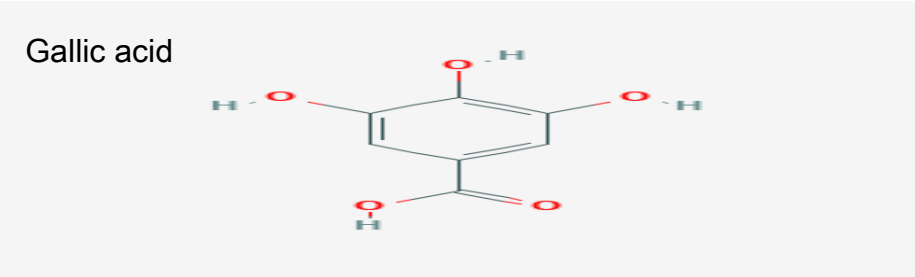


**Figure 4.1**: A typical benzoic acid. Source: PUBCHEM NCBI.

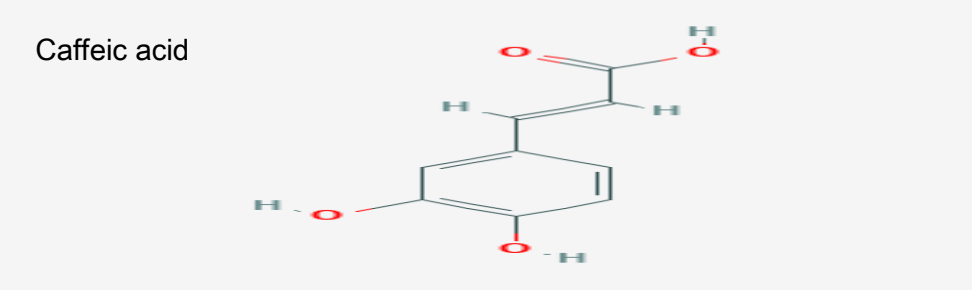


**Figure 4.2**: A typical cinnamic acid. Source: PUBCHEM NCBI.

Flavonoids (Figure 4.3) are low molecular weight polyphenolic substances based on a 15-carbon skeleton and consists of two aryl rings (A- and B-rings) that are connected by a heterocyclic pyran ring (C-ring) and form the $C_6$–$C_3$–$C_6$ flavan nucleus (Catarino et al., 2016).

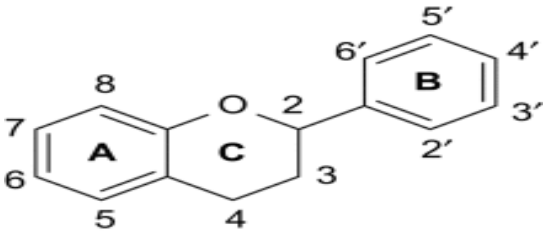


**Figure 4.3**: Basic structure of flavonoids. Source: Catarino et al. (2016).

The main subclasses of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins. Other flavonoid groups which are minor components of the diet are dihydroflavones, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones (Crozier et al., 2006).

In phytochemistry, secondary metabolites such as alkaloids, terpenoids, steroids, tannins, saponins, cardiac glycosides, flavonoids and phlobatannins are tested using rapid chemical tests described by Trease and Evans (1983). These tests are performed as a screening process for the detection of known nutraceutical phytochemicals. Upon receiving positive results, several qualitative and quantitative assays are performed to extract, isolate, identify and characterise the phytochemicals present in the sample (Harborne, 1973; Stalikas, 2007). Sample preparation ahead of the above tests is important due to the variability of the chemistry of the phytochemicals such as polarity, acidity, number of hydroxyl groups and aromatic rings, concentration levels and complexity of the matrix (Stalikas, 2007). The

methods of sample preparation are just as variable and include drying, filtration and centrifugation which are followed, when necessary, by one or more extraction procedures such as liquid-liquid extraction (LLE) and solid-liquid extraction using solvents such as ethanol, acetone or methanol, or a mixture with water (Ross et al., 2009). Solid-phase extraction (SPE) and column chromatography are used to further separate or isolate the compounds in a sample, (Stalikas, 2007). Analysis of the prepared sample is mostly done using chromatographic techniques such as Thin Layer Chromatography (TLC) and variations of liquid chromatography (High Performance Liquid Chromatography) which are coupled with chemicals and ultraviolet or infra-red lights to enable visualisation of compounds and their detection as they elute through a column (Harborne, 1973; Stalikas, 2007). Total phenolic, flavonoid and tannin contents in samples are usually quantified through UV/Vis spectrophotometric assays using reactions of phytochemicals with Folin–Ciocalteu reagents and aluminium chloride ($AlCl_3$), and their absorbance are determined at wavelengths ranging from 510 nm to 725 nm (Singleton and Rossi, 1965; Lamaison and Carnet, 1990).

In the southern African region, the phytochemical composition of numerous food-producing plants has been studied and these include *Dovyalis caffra* Kei apple (Taher et al., 2018), *Carpobrotus edulis* Hottentot-fig (Castaneda-Loaiza et al., 2020) and *Sclerocarya birrea* marula (Ndhlala et al., 2006; Borochov-Neori et al., 2008). Among these, marula is the most commercially significant. According to a recent review by Pfukwa et al. (2020), Hottentot-fig peel extracts had a total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC) of 272.82 mg gallic acid equivalents (GAE)/g dry weight DW, 1.58 mg quercetin equivalents (QE)/g DW and 20.3 mg catechin equivalents (CE)/g DW respectively (Castaneda-Loaiza et al., 2020). The phytochemical composition of these extracts included syringic acid-O-hexoside, feruloyl hexose isomers 1, 2 and 3, ferulic acid, quercetin-di-O-hexoside, isoferulic acid, kaempferol-O-(rhamnosyl) hexoside isomer 1, hyperoside, isoquercitrin, rutin, azelaic acid, astragalin, isorhamnetin-O-hexoside isomer 1, quercetin, kaempferol, isorhamnetin, methoxy-trihydroxyflavone, flavokawain C and emodin (Castaneda-Loaiza et al., 2020). Kei apple fruit extracts had a total phenolic content and total flavonoid content of 2901 mg gallic acid equivalents (GAE) /100 g and 1371 mg quercetin equivalents (QE)/100 g respectively (Taher et al., 2018). The phytochemical compounds identified in the Kei apple extracts were chlorogenic acid, pyrogallol protocatechuic acid, catechin, epicatechin and hesperidin, ellargic acid, ferulic acid, syringic acid, p-coumaric acid, vanillic acid and 3-methoxy-4-hydroxyphenyl propionic acid (Mpai et al., 2018; Taher et al.,

2018). Marula fruit extracts evaluated by Ndhlala et al. (2006) had total phenolic content and total flavonoid content of 2262 µg gallic acid equivalents (GAE)/g and 202 µg catechin equivalents (CE)/ g, respectively. The fruit pulp and juice contained sesquiterpene hydrocarbons (Pretorius et al., 1985), caffeic acid, vanillic acid, p-hydroxybenzaldehyde, ferulic acid, p-hydroxybenzoic acid, p-coumaric acid (Ndhlala et al., 2007), derivatives of hydrolysable tannins, catechins, and hydroxycinnamic acid (Borochov-Neori et al., 2008), gallic acid and epicatechin (Hiwilepo van-Hal, 2013). The total phenolic content of marula juice was 267 mg dL−1. These biological constituents asset the nutraceutical potential of food products made from these plant resources. In the case of marula wine, this will be the case if these bioactive compounds are retained and remain stable during the fermentation process and storage period.

In comparison with other non-grape and indigenous wines or beers, the nutritional and non-nutritional constituency of marula wine is scarcely reported. The total phenolic content of marula wine ranges from 690 ± 30 mg GAE/100 when fermented at room temperature to 960 ± 130 mg GAE/100 mL when fermented at 40 ºC. These were comparable to 930 mg GAE/100 mL (Panda et al., 2014) reported in bael fruit wine and higher than 22.06 mg GAE/100 mL reported in cagaita wine, 12.70mg GAE/100mL in jabuticaba wine and 2.8 mg GAE/100 mL in pitaya wine (De Souza et al., 2018).

The evolution and profile of secondary metabolites in marula wine has not been widely reported. Thus, this study focussed on measuring and tracking the changes in composition and quantities of secondary metabolites in fresh juice and wine throughout the fermentation period.

## 4.3 Materials and Methods

### 4.3.1. Fruits and wine collection

A 50 kg box full of green to yellow-green fruits were collected between February and March 2018 from the University of Limpopo grounds (-25.6150; 28.0166) and placed in a cool place until ripened. Overnight fermenting wine prepared in 20 L plastic buckets were purchased from community partners in The Oaks (-24.36239; 30.67527) and Sekhukhune (-24.34329; 30.09252) and transported to the laboratory in March 2018. The buckets were stored at room temperature (25 – 30 $^{o}$C) to continue fermenting over 20 days.

### 4.3.2. Juice preparation

A puncture was made on the skin of each fruit. The juice was extracted into a 9 L plastic bucket by squeezing the peel and the kernel. The juice was then transferred into lockable plastic bags and stored at -20 $^{o}$C until analysis commenced.

### 4.3.3. Laboratory wine preparation (University of Limpopo wine)

The traditional recipe and process of producing marula wine was followed. An equal volume (10 L) of tap water was added to the marula juice. The bucket was tightly closed and placed at room temperature overnight. A glob of a layer that had formed overnight on the surface of the juice was removed by filtering the juice using a strainer. The fermenting wine was placed at room temperature to continue fermenting over 20 days.

### 4.3.4. Wine sampling

Duplicate samples of wines were collected at 2 days intervals and stored in sterile 2 mL and 50 mL centrifuge tubes and 1 L lockable plastic bags at -20 $^{o}$C until analysis commenced.

### 4.3.5. Sample preparation and extraction

The laboratory and purchased wines as well as undiluted juice extracted from the University of Limpopo fruits were separately filtered through a Whatman No.1 filter paper, transferred into pre-weighed slant bottles and freeze-dried. All the dried samples were reconstituted in distilled water to concentrations of 10 mg/mL and 20 mg/mL for separate tests.

### 4.3.6. Analysis of secondary metabolites

Secondary metabolites were analysed through qualitative and quantitative analytic methods.

4.3.6.1 Qualitative fingerprinting of secondary metabolites

Thin layer chromatography (TLC) was used according to Kotze and Eloff (2002). Thin layer chromatography (TLC) plates (Merck, Silica gel F245) were spotted with 10 µL of each of the

extracts reconstituted to 20 mg/mL and left to dry. The plates were developed in saturated TLC tanks using mobile phases of decreasing polarity, BAW (butanol/ acetic acid/ water) (3:2:2), EMW (ethyl acetate/ methanol/ water) (10:5.4:4), CEF (chloroform/ ethyl acetate/ formic acid) (10:8:2) (Merck, technical grade). The plates were removed from the tanks when the mobile phases had reached the solvent-front and were air-dried in a fume hood cabinet.

Once the separation of the constituent compounds was completed, ultraviolet (UV) light active compounds were visualised at wavelengths of 254 and 365 nm, then followed by spraying the TLC plates with vanillin-sulphuric acid reagent [0.1 g vanillin (Sigma Aldrich) prepared in 28 mL methanol (Merk, technical grade) and 1 mL sulphuric acid (Sigma Aldrich) to visualise the other compounds and heated until colour development at 110 °C.

#### 4.3.6.2 Screening of secondary metabolites

The juice and wines were screened for various categories of secondary metabolites.

##### 4.3.6.2.1. Alkaloids

Juice and wine extracts (0.2 g) were suspended in 5 mL of hydrochloric acid (HCl) and 5 drops of Drangendoff's reagent were added. A colour change was observed to draw an inference. A colour change to orange red indicated the presence of alkaloids (Harborne, 1973).

##### 4.3.6.2.2. Terpenoids

Salkowski test was used to detect terpenoids. A volume of 5 mL of the aqueous extract was mixed with 2 mL of 99% chloroform and 3 mL of concentrated sulphuric acid to form a layer. A red-brown coloration of the interface of the formed layer indicated the presence of terpenoids (Borokini and Omotayo, 2012).

##### 4.3.6.2.3. Steroids

A 2 mL volume of acetic anhydride was added to 0.5 g dried juice and wine, followed by the addition of 2 mL of sulphuric acid. The samples were observed for a blue-greenish colour change indicative of the presence of the steroids (Borokini and Omotayo, 2012).

##### 4.3.6.2.4. Flavonoids

Diluted ammonia solution (5 mL) was added to an aqueous filtrate of the juice and wine extracts. Concentrated sulphuric (2mL) acid was added. A temporary yellow colour change was observed to ascertain the presence of flavonoids (Borokini and Omotayo, 2012).

4.3.6.2.5. Cardiac glycosides

The presence of cardiac glycosides in the marula juice and wines was determined using the Keller-Killani test. Glacial acetic (2 mL) acid was added to 0.5 g of dried extracts. A few drops of 1% aqueous ferric chloride were added followed by cautiously adding 1 mL of concentrated sulphuric acid. The appearance of a brown ring at the interface between two layers was indicative of the presence of cardiac glycosides (Borokini and Omotayo, 2012).

4.3.6.2.6. Tannins

Dried juice and wine extracts (0.5 g) were re-suspended in 5 mL of distilled water. The solutions were heat treated to boiling point and cooled to room temperature. Once cool, 1 mL was transferred into a clean test tube and a few drops of 1% aqueous ferric chloride solution were added. A blue-black colouration of the solution indicated the presence of tannins (Trease and Evans, 1989).

4.3.6.2.7. Phlobatannins

The dried juice and wines (0.3 g) were dissolved in 10 mL of distilled water and filtered with Whatman No.1 filter paper. The filtrates were boiled with 2% hydrochloric acid (HCl) solution. The formation of a coloured precipitate was indicative of the presence of Phlobatannins (Borokini and Omotayo, 2012).

4.3.6.2.8 Saponins

The froth test was used to detect saponins according to the method of Odebiyi and Sofowora (1978). Juice and wine extracts (0.5 g) were re-suspended in 30 mL of tap water. The mixture was vigorously shaken and heated to about 100 °C. The formation of a persistent froth indicated the presence of saponins.

4.3.6.2.9 Phenols

Dried marula juice and wines (0.5 g) were dissolved in 1 mL of distilled water. This was followed by the addition of a few drops of 10% aqueous ferric chloride solution. Blue-green colouration indicated the presence of phenols (Pasto and Johnson, 1979).

### 4.3.7. Quantitative analysis of secondary metabolites

Total phenolic, flavonoid and tannin contents of the marula juice and wines were determined. These secondary metabolites were found in fruits and plants that have antioxidant, antimicrobial (Ghasemzadeh and Ghasemzadeh, 2011; Medini et al., 2014) and other biological activities.

### 4.3.8. Determination of total phenolic content

The Folin-Ciocalteu reagent method (Dewanto et al., 2002) was used to measure the total phenolic content (TPC) of aqueous extracts juice and wines that were concentrated to 10 mg/mL. Ten microliters of the extracts were diluted with 490 µL of distilled water and 0.25 mL of Folin-Ciocalteu reagent was added into each test tube. To cease the reaction, 1.25 mL sodium carbonate ($Na_2CO_3$) was added, and the mixture was incubated in the dark at room temperature (25 $^oC$) for 30 minutes. After the incubation the absorbance of the mixture was determined using an ultraviolet/visible (UV/VIS) spectrophotometer at 725 nm. Tannic acid standard was prepared as the extracts above at various concentrations of 1.25, 0.63, 0.31, 0.16, 0.08 mg/mL. Distilled water was used as blank instead of the extracts. The absorbance readings obtained for the tannic acid standard were used to plot a standard curve and the results were expressed as milligram of tannic acid equivalence/gram of extract (mg of TAE/g extract). The experiment was conducted in triplicates and independently repeated three times.

### 4.3.9. Determination of total tannin content

The Folin-Ciocalteu method described by Tambe and Bhambar (2014) with minor modifications was used to determine the total tannin content (TTC) in the juice and wine extracts. An aliquot of 50 µL of 10 mg/mL of the juice and wine extracts were mixed into 4 mL of distilled water. The Folin-Ciocalteu reagent (250µL) was added to the mixture and vortexed. To this mixture, 500µL of 35% sodium carbonate ($Na_2CO_3$) in distilled water was added. The mixture was made up to 10 mL with distilled water, shaken and kept in the dark at room temperature (25 $^oC$) for 30 minutes. Gallic acid was used as a standard at different concentrations (1.0, 0.5, 0.25, 0.125, 0.625 mg/mL) which were prepared in the same manner as the extracts. The absorbance for the solutions was measured at 725 nm using a UV/VIS spectrophotometer against a blank that was prepared in the same manner as the test solutions without adding any extract. Tannin content was expressed as milligram gallic acid equivalence/gram of extract (mg GAE/g extract). The experiment was conducted in triplicates and independently repeated.

### 4.3.10. Determination of total flavonoid content

Total flavonoids (TFC) were measured by a colorimetric assay according to Tambe and Bhambar (2014). An aliquot of 100 µL of 10 mg/mL of the juice and wine aqueous extracts were mixed into 4.9 mL of distilled water. To this mixture, 300 µL of 5% sodium nitrite ($NaNO_2$)

in distilled water was added and the mixture was incubated for 5 minutes at room temperature (25$^{o}$C). Following incubation, 300 µL of 10% aluminium chloride ($AlCl_3$) in distilled water was added. This mixture was again incubated for 5 minutes at room temperature. Two milliliters of 1 M sodium hydroxide (NaOH) were added, and the total volume of the mixture made up to 10mL using distilled water. Quercetin was used as a standard at different concentrations (500, 250, 125, 62.5, 31.5 µg/mL) which were prepared in the same manner as the extracts. The absorbance of the experimental samples and the standard were determined using a UV/VIS spectrophotometer at a wavelength of 510 nm. For the blank 100 µL of distilled water was added instead of the extracts. The total flavonoid content of the samples was expressed as milligram quercetin equivalence/ gram of extract (mg QE/g extract). The experiment was conducted in triplicates and independently repeated three times.

### 4.3.11 Analysis of phenolic acids and flavonoids

The marula juice from the University of Limpopo and all wine samples from all three sampling areas (2 mL) were filtered using 0.45 µm syringe filters. Quantification of the constituent phenolic acids and flavonoids was performed on an ultra-high pressure liquid chromatography (UHPLC) system (Shimadzu, Kyoto, Japan) equipped with a degassing unit (DGU-403), binary pumps (LC-40B XR), solvent delivery module (LC-40B XR), auto-sampler (SIL-40C XR), column oven (CTO-40C), and a diode array detector (DAD) (SPD-M40). A 2 µL volume of the extract was injected into the system and separation was carried out on a Raptor C18 column (2.7 µm x 100 mm x 2.1 mm ID, Restek, Bellefonte, USA) at an oven temperature of 40 $^{o}$C. Mobile phase A which consisted of 1% formic acid in Milli-Q water and B, was 1% formic acid in a mixture of 50% methanol and acetonitrile, with the total run time being 15 minutes. Individual phenolic compounds, apigenin, caffeic acid, gallic acid, ferulic acid, kaempferol, luteolin, p-coumaric acid, quercetin, sinapic acid, taxifolin and vanillic acid (Sigma Aldrich, Johannesburg, South Africa) were identified using retention times of analytical standards and quantified through extrapolation from the calibration curves of the analytical standards at different concentrations (2.5, 5, 10, 20, 40, 80, and 160 µg/mL).

### 4.3.12 Statistical analysis

Descriptive statistics (average, standard deviation, percentage difference) were calculated. For inferential statistics, one-way ANOVA ($p< 0.05$) with multiple comparisons and independent t-tests were performed using IBM SPSS software.

## 4.4 Results

### 4.4.1. Qualitative fingerprinting of secondary metabolites

Thin layer chromatography using mobile phases of varying polarities was used to separate phytochemicals found in wine extracts sampled every second day over a 16-day period. Separated compounds were visualised using UV light (Figure 4.1) and vanillin- sulphuric acid (Figure 4.2).

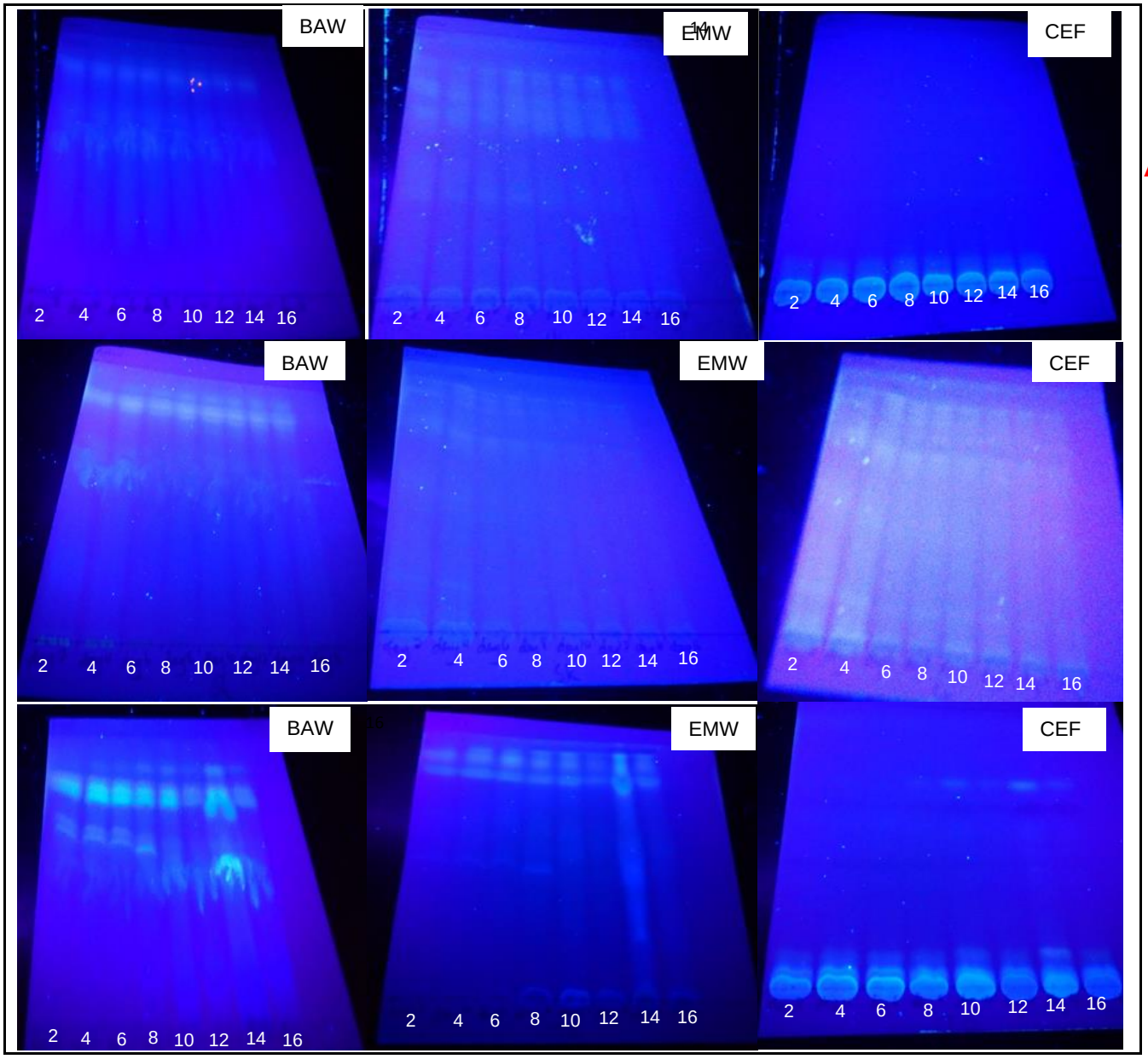


**Figure 4.4**: TLC profile of water extracts of the University of Limpopo (A), Sekhukhune (B) and The Oaks (C) marula wine developed in solvent systems of varying polarities viewed under UV light at 254 nm. The numbers indicate the sampling interval in days.

University of Limpopo samples were best visualised on chromatograms developed in EMW mobile system whereas Sekhukhune and The Oaks samples were best visualised on chromatograms developed in CEF and BAW mobile systems, respectively.

Using the ethanol: methanol: water (EMW) polar mobile system most compounds were separated between retention factor values 0.6 to 0.9 (Fig. 4.4). The number of compounds were six and four from day 2 to 16 in the University of Limpopo and Sekhukhune wine extracts respectively whereas the number dropped from four to three on days 12, 14 and 16 in The Oaks extracts. Thus, University of Limpopo had more different UV light 365 nm reactive compounds than all the other areas. Furthermore, fermentation had little or no effect on the presence of these compounds.

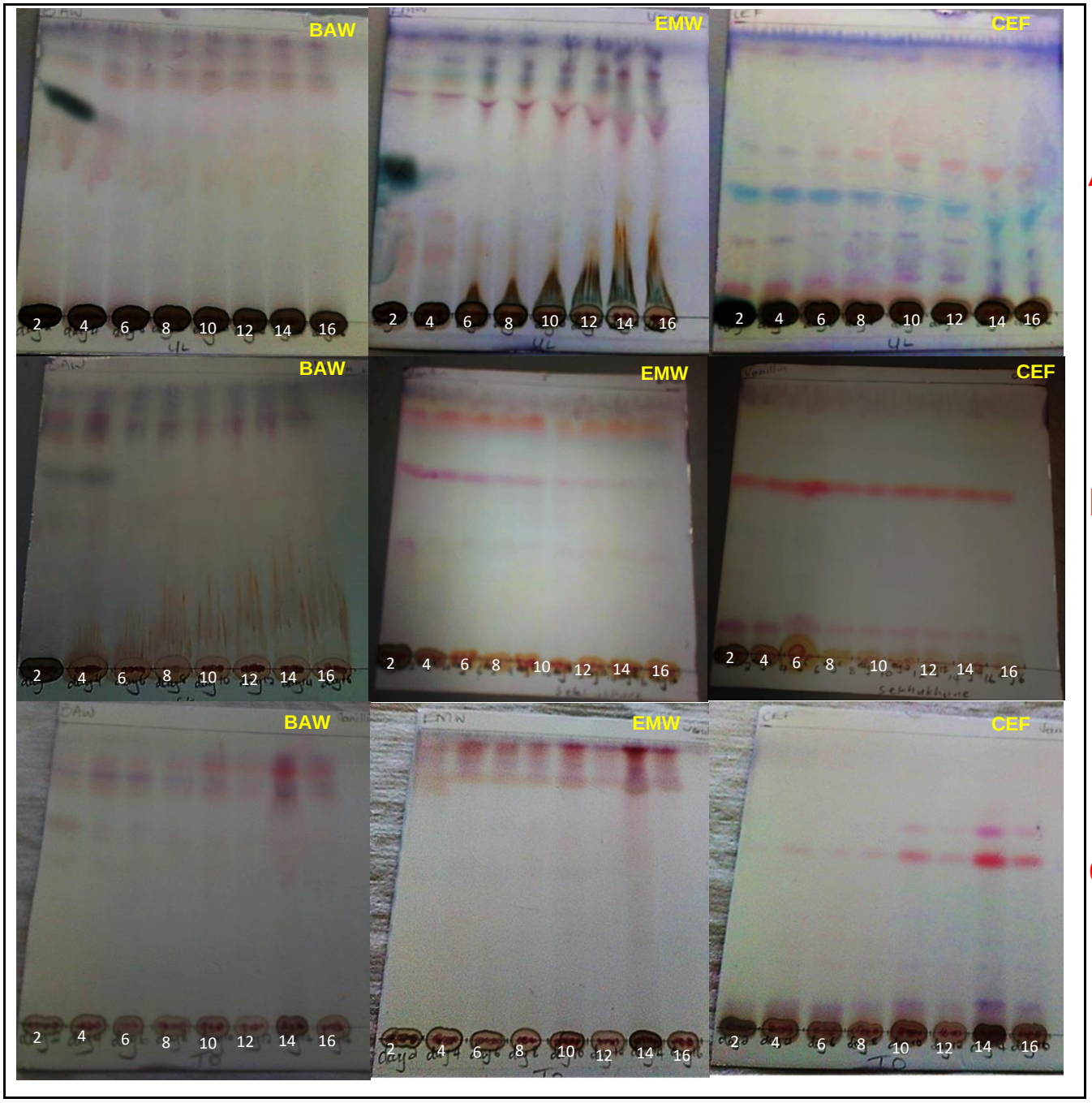


**Figure 4.5**: TLC profile of water extracts of the University of Limpopo (A), Sekhukhune (B) and The Oaks (C) marula wine developed in solvent systems of varying polarities sprayed with vanillin-sulphuric acid. The numbers indicate the sampling interval in days.

The two mobile phases such as chloroform: ethyl acetate: formic acid (CEF) and ethanol: methanol: water (EMW) produced the best profile of compounds visualised with vanillin-sulphuric acid reagent (Fig. 4.5). There were different compounds or classes of compounds that were observed through this detection system. By order of decreasing count, University of Limpopo wine had purple/ bluish- purple and orange compounds; Sekhukhune wine had

orange and purple/ bluish- purple compounds whereas The Oaks had pink, purple and orange compounds. The pink and orange compounds were bright and intense colour for all the areas when compared to the blue ones. Notably, the compounds were present from the start to the end of the fermentation period.

### 4.4.2. Secondary metabolites screened in the juice and wine extracts

The juice and wines were screened for various categories of secondary metabolites using rapid chemical tests (Table 4.1) that helps determine the presence of different metabolites based on characteristic colour changes occurring during or after reactions with the chemicals used. Marula juice from the University of Limpopo and the wines from all three areas contained saponins, tannins, terpenoids, steroids, cardiac glycosides, and phenols.

**Table 4.1**: Secondary metabolites present in the marula juices and wines. These are consensus observations for all the three areas.

| Secondary metabolites | Undiluted fresh Juice | Day 2 wine | Day 4 wine | Day 6 wine | Day 8 wine | Day 10 wine | Day 12 wine | Day 14 wine | Day 16 wine |
|---|---|---|---|---|---|---|---|---|---|
| **Alkaloids** | - | - | - | - | - | - | - | - | - |
| **Phenols** | + | + | + | + | + | + | + | + | + |
| **Terpenoids** | + | + | + | + | + | + | + | + | + |
| **Cardiac glycosides** | + | + | + | + | + | + | + | + | + |
| **Flavonoids** | - | - | - | - | - | - | - | - | - |
| **Steroids** | + | + | + | + | + | + | + | + | + |
| **Saponins** | + | + | + | + | + | + | + | + | + |
| **Tannins** | + | + | + | + | + | + | + | + | + |
| **Phlobatannins** | - | - | - | - | - | - | - | - | - |

(+) = detected, (-) = not detected

The undiluted fresh juice and all the wines from all three areas contained most secondary metabolites except alkaloids, flavonoids and phlobatannins.

### 4.4.3. Quantitative analysis of secondary metabolites

The juice and wine extracts were measured for the total concentrations of phenolics, flavonoids and tannins (Table 4.2).

**Table 4.2**: Quantified total phenolic, flavonoid and tannin contents of marula juice and wines from University of Limpopo, and Sekhukhune and The Oaks.

| Sampling days | Sampling areas | Tannin content (mg of GAE/g of sample) | Phenolic content (mg of TAE/g of sample) | Flavonoid content (mg of QE/g of sample) |
|---|---|---|---|---|
| Day 0 (Undiluted fresh juice) | UL | **1.74±0.40** | **179.82±0.71** | **0.12±0.13** |
| **Day 2** | UL | **15.29±0.20** | **963.57±0.59** | **0.21±0.04** |
| | SK | **8.80±0.05** | **500.39±0.78** | **1.16±0.08** |
| | TO | **0.57±0.04** | **1.59±0.59** | **0.41±0.11** |
| **Day 4** | UL | **6.81±0.29** | **344.51±0.80** | **0.13±0.05** |
| | SK | **18.15±0.26** | **927.10±0.76** | **2.63±0.09** |
| | TO | **0.54±0.03** | **-** | **0.41±0.13** |
| **Day 6** | UL | **8.23±0.18** | **414.37±0.69** | **0.16±0.06** |
| | SK | **6.29±0.25** | **334.87±0.69** | **0.84±0.04** |
| | TO | **0.82±0.02** | **-** | **0.39±0.11** |
| **Day 8** | UL | **7.90±0.29** | **397.99±0.91** | **0.23±0.36** |
| | SK | **12.14±0.22** | **664.45±0.61** | **1.44±0.05** |
| | TO | **0.68±0.04** | **1.44±0.42** | **0.43±0.08** |
| **Day 10** | UL | **8.32±0.28** | **453.50±0.65** | **0.18±0.05** |
| | SK | **8.17±0.35** | **448.11±0.66** | **1.02±0.08** |
| | TO | **0.48±0.01** | **3.42±0.75** | **0.39±0.05** |
| **Day 12** | UL | **10.10±0.22** | **557.24±0.77** | **0.34±0.36** |
| | SK | **8.08±0.22** | **457.26±0.79** | **0.75±0.08** |
| | TO | **0.56±0.02** | **1.83±0.47** | **0.46±0.05** |
| **Day 14** | UL | **6.79±0.22** | **368.94±0.61** | **0.19±0.85** |
| | SK | **8.33±0.04** | **455.72±0.65** | **0.81±0.07** |
| | TO | **0.57±0.02** | **1.59±0.47** | **0.51±0.08** |
| **Day 16** | UL | **7.28±0.10** | **402.38±0.87** | **0.24±0.05** |
| | SK | **8.90±0.39** | **509.11±0.66** | **1.09±0.09** |
| | TO | **0.52±0.03** | **4.87±0.75** | **0.48±0.05** |

*(-) represents no detectable concentration

Fresh undiluted juice from University of Limpopo had lower concentrations of all the compounds compared to the wine fermented for 2 days and represented a notable but not statistically significant increase of over eight-fold ($p = 0.102$), four-fold ($p = 0.155$) and one-fold ($p = 0.158$) of the original contents of total tannins, total phenolics and total flavonoids. The fermentation process of the University of Limpopo wine decreased the total tannin and

total phenolic contents from day 2 of fermentation to day 16 by 1.5% while the total flavonoid content increased slightly by 14%. From day 2 to day 16 of Sekhukhune wine fermentation, there was a 1.1% and 1.8% increase in the content of total tannins and total phenolics. The total flavonoid content however decreased by 6%. Sharp fluctuations were observed in the total phenolic content of day 4 wines of both Sekhukhune (46%) and the University of Limpopo (64%) wines. The Oaks wine showed an 8% decrease in total tannin content, 67% and 14% increases in total phenolic and flavonoid contents from day 2 to day 16 of fermentation.

The wines from Sekhukhune and the University of Limpopo had comparable concentrations of total tannin and total phenolic compounds, p values 0.906 and 0.968 respectively. Sekhukhune wine had more total phenolics by the 16$^{th}$ day of fermentation which represented 21% more than the University of Limpopo. The concentration of total phenolics in The Oaks wine was too low to be detected on days four and six, albeit total tannin and total flavonoids were measured. The Oaks wine had comparable total flavonoid content to the University of Limpopo wine, p = 0.447. Sekhukhune wine however had significantly higher total flavonoid content among all the areas, p = 0.0076.

### 4.4.4. Phenolic compounds

Liquid chromatography was used to identify and quantify the individual phenolic compounds in the juice and wines. The compounds were identified using retention times of analytical standards and quantified through extrapolation from standard curve at different concentrations of the standards (Table 4.3).

**Table 4.3**: Quantified phenolic acids and flavonoids found in the marula wines.

| | Concentration (µg/mL) | | |
|---|---|---|---|
| **Flavonoids** | **The Oaks** | **University of Limpopo** | **Sekhukhune** |
| Apigenin | - | 0.09 | 0.69 |
| Kaempferol | - | 0.02 | 0.33 |
| Luteolin | - | 1.63 | 0.53 |
| Quercetin | - | 0.03 | 0.23 |
| Taxifolin | - | 1.78 | 1.60 |
| **Phenolic acids** | | | |
| Caffeic acid | - | 0.71 | 0.53 |
| Ferulic acid | - | 0.90 | 0.34 |
| Gallic acid | - | 5.66 | 2.54 |
| p-Coumaric acid | - | 0.66 | 0.41 |
| Sinapic acid | - | 0.52 | 0.07 |
| Vanillic acid | - | 7.05 | 6.74 |

*(-) = not detected

The most abundant flavonoid and phenolic acid found in the wines were taxifolin and vanillic acid respectively. The wines had more phenolic acids than flavonoids. The University of Limpopo had more luteolin (1.63 µg/mL), gallic acid (5.66 µg/mL) and slightly more taxifolin (1.78 µg/mL) and vanillic acid (7.05 µg/mL) whereas Sekhukhune had more apigenin (0.69 µg/mL), Kaempferol (0.33 µg/mL) and quercetin (0.23 µg/mL). The Oaks had no detectable amount of the selected compounds analysed.

## 4.5 Discussion

Thin layer chromatography is a method of separating compounds in a mixture using a solid sheet coated with an adsorbent as the stationary phase and a mixture of solvents as the mobile phase. The separation is achieved based on the relative affinity between the stationary and mobile phases of the compounds ("Thin Layer Chromatography (TLC)", 2021). In our study, aluminum plates coated with monochromatic fluorescent substance 254 nm silica gel (Merck, Silica gel $F_{254}$) were spotted with aqueous extract of marula juice and wine and developed in saturated tanks of three solvent systems (BAW, EMW and CEF). Compounds that contain either pi bonds or atoms with non-bonding orbitals (Clarke, 2021) can either fluoresce or quench fluorescence when they absorb light in the region from 200 - 800 nm which is where the spectra are measured. Within the spectra, saturated compounds absorb shorter wavelengths and create dark spots behind a fluorescent background whereas compounds with longer conjugated systems of pi electrons absorb longer wavelengths and create bright spots (Org Chem Text, 2021). The compounds found in all the wines could not absorb the 254 nm wavelength. Rather, the compounds absorbed light closer to blue light region (400 to 525 nm) indicating that they contained longer conjugated systems of pi electrons. These compounds could be polyphenols which have been reported as containing pi-conjugated systems with hydroxyl-phenolic groups (Hassane et al., 2012). Caffeic acid, ferulic acid, gallic acid, p-coumaric acid, sinapic acid, kaempferol, quercetin were isolated from the marula wine and these are polyphenols also found in red and white grape wine (Zoecklain et al., 1995; Tian et al., 2009) and in cherry, blueberry, blackberry and raspberry wines (Cakar et al., 2016).

The Oaks UV light reactive compounds diminished towards the last 6 days of fermentation possibly degraded by the action of lactic acid bacteria and spoilage microorganisms such as *Gluconobacter* spp. and *Acetobacter* spp. (Alimardani-Theuil et al., 2011). Lactic acid bacteria produce phenolic acid decarboxylate and vinylphenol reductase that metabolise hydroxycinnamic acids into vinylphenols (Santamaría et al., 2018).

Vanillin sulphuric acid is a universal chromogenic reagent which is used for the detection of steroids, terpenoids, phenols, catechin, tannins, flavonoids, and cardiac glycosides (Jork et al., 1990). The purple/ bluish purple, orange and pink coloured bands or spots observed on the chromatograms visualised using vanillin sulphuric acid could be terpenoids, flavonoids and tannins (Taganna et al., 2011; Ahmed et al., 2012).

Various chemical tests were conducted to identify and confirm the presence of different classes of phenolic compounds present in the wine and juice extracts. Tannins, saponins, cardiac glycosides, phenols, terpenoids, steroids tested were detected. These provided preliminary evidence of the presence of health-promoting groups of plant phenolic compounds in the marula juice and wines. A recent review reported the presence and pharmacological effects of these compounds in various beverages, fruits and vegetables (Shahidi and Ambigaipalan, 2015). These compounds, depending on the composition of the components of the diet and their bioavailability (Scalbert and Williamson, 2000), manifest extensive biological properties such as anti-allergenic, anti-artherogenic, anti-cancer, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Middleton et al., 2000; Puupponen-Pimiä et al., 2001; Manach et al., 2005; Khan and Mukhtar, 2010).

The concentrations of tannins, phenols and flavonoids for the University of Limpopo, Sekhukhune and The Oaks wines were determined. In reference to the marula juice from the University of Limpopo which contained tannins of 1.74 ± 0.40 mg of GAE/g of sample, phenols of 179.82 ± 0.71 mg of TAE/g of sample and 0.12 ± 0.13 mg of QE/g of sample, the wine had higher concentrations of all compounds. This indicated that fermentation resulted in the production of these compounds. This phenomenon has been observed during grape fermentation and was attributed to the action of microorganisms such as *Lactobacillus* species, *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts (Lingxi and Baoshan, 2019; Morata et al., 2020). The enzymes and metabolites of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts were found to influence extraction of phenolic compounds from grapes (Bakker and Timberlake, 1997; Alimardani-Theuil et al., 2011). In addition, *Lactobacillus plantarum* 75 strains were reported to increase the total polyphenol content in fermented nightshade leaves compared to the raw nightshade leaves (Degrain et al., 2020). Typically, during marula fermentation *Hanseniaspora guilliermondii* and *Lactobacillus* species (including *L. planturum*) dominate the initial stages of fermentation whereas *Saccharomyces cerevisiae* and *Acetobacter* species dominate the latter stages (Phiri, 2018; Maluleke, 2019). The notable increase in the concentration of phenolic compounds from juice to wine could be as a result of the *Hanseniaspora guilliermondii* and *Lactobacillus* species. The fluctuations occurring at different intervals, from day 2 to day 16 of fermentation of all the wines from all the areas such as the relatively sharp increase in total phenolic content (46%) in Sekhukhune day 4 wine or the even sharper decrease of 64% in University of Limpopo day 4 wine could

have resulted from simultaneous occurrence of biological reactions, such as enzyme facilitated decarboxylation and esterification, as well as alkaline/acid hydrolysis of phenolic compounds observed with other fermentation studies (Adebo and Medina-Meza, 2020; Leornard et al., 2021). In red wine fermentation, microorganisms such as *Bacillus subtilis*, *S. cerevisiae* and *L. plantarum* produce enzymes such as phenolic acid decarboxylase, p-coumaric acid decarboxylase (Smit et al., 2003), ferulic acid decarboxylase and phenyl acrylic acid decarboxylase (Mukai et al., 2010) that can decarboxylate phenyl acrylic acids, p-coumaric, ferulic, caffeic acid and cinnamic acid into volatile phenols that can create off flavours in the wine. The enzyme tannin acyl hydrolase produced by *L. plantarum* catalyses the hydrolysis of hydrolysable tannins and gallic acid esters to produce gallic acid and glucose (Lekha and Lonsane, 1997; Vaquero et al., 2004).

It is evident however that the wines contain the various compounds all the way through to the end of fermentation. It may be that with better control of the microorganisms used in fermentation, the concentrations of total phenolics, total tannins and total flavonoids can be optimised.

By comparing the total phenolic, flavonoid and tannin contents among the wines from the University of Limpopo, Sekhukhune and The Oaks, The Oaks wine had comparable total flavonoid content to University of Limpopo wine while Sekhukhune wine significantly edged the two areas. No significant difference was observed between Sekhukhune and The University of Limpopo in their phenolic and tannin contents. The differences observed in the concentration of the phenolic compounds from area to area could be caused by various factors. Findings have shown that grape variety, growing condition, winemaking method, and yeast strain selection are among the many factors that bring variations in grape wines (Downey et al., 2006; Morata et al., 2019). As shown in Chapter 3, only one species, *Sclerocarya birrea* (Hoscht) subsp. *caffra* (Sond.) Kokwaro of marula tree exists in South Africa (Moganedi et al., 2011). The three areas chosen for this study are all in the Limpopo Province and the genetic diversity among trees is markedly low as inferred from the intraspecific variations detected by Moganedi et al. (2011). Also, results from Chapter 3, have shown that the environmental conditions of the three areas differ only in temperature. However, this could not justify the differences in phenolic compounds since, The Oaks and Sekhukhune both had the same temperature and yet significantly different contents for all compounds. The water and soil types could not be compared due to insufficient information.

If winemaking procedures were to contribute to the differences in the contents, it would only be in terms of handling. However, the recipes for making the wine were fairly the same (personal communication with community partners). Interesting differences were noted in the analysis of the microbial composition of Sekhukhune and University of Limpopo wines as studies by Phiri (2018). The microbial loads for bacteria and yeasts for Sekhukhune were much higher than those of the University of Limpopo throughout the fermentation period. Secondly, the abundance of *Hanseniaspora guilliermondii* in the University of Limpopo wine was between 80 and 90% for days 2 – 4 compared to 50 – 60% in the Sekhukhune wine. *Saccharomyces cerevisiae* was present in the Sekhukhune wine at 20 – 30% of the microbial population during the initial stages of fermentation. Thus, the impact on phenolic compounds from the initial stages of fermentation are different for Sekhukhune where the *Saccharomyces cerevisiae and Hanseniaspora guilliermondii* act simultaneously. All these variations in the composition and abundance of microorganisms provide a basis for the differences in the tannin, phenolic and flavonoid contents.

In a Namibian study of marula wine, fermentation was performed on juice containing 1130 ± 40 mg GAE/100 mL of phenolics for 8 days. This content dropped to 690 ± 30 mg GAE/100 mL representing 61% of the initial content. Compared to the University of Limpopo wine, the content increased by 121% by the 8$^{th}$ day of fermentation and 123% by the 16$^{th}$ day from 179.82 ± 0.71 mg of TAE/g of sample in the undiluted juice to 397.99 mg of TAE/g of sample and 402.38 mg of TAE/g of sample respectively. The variations in the juice content could be explained by the climate, the extraction solvent used and most importantly the calibration standard (Lamien-Meda et al., 2008) whereas difference in the effect of fermentation could be because the fruits contain different types of phenolic compounds and strains of microorganisms.

Tropical edible fruits, leafy vegetables, honey, and fruit wines are sources of dietary polyphenols (Miean and Mohamed, 2001; Ljevar et al., 2016; Kekelidze et al., 2018; Bento-Silva et al., 2020). The wines in our study contained flavanonol (taxifolin), flavones (luteolin and apigenin), flavonols (kaempferol and quercetin), hydroxybenzoic acids (vanillic acid and gallic acid) and hydroxycinnamic acids (*p*-coumaric acid, ferulic acid, caffeic acid and sinapic acid). Compounds such as *p*-coumaric acid, ferulic acid, caffeic acid and vanillic acid were isolated in the marula juice (Ndhlala et al., 2007) and gallic acid was previously isolated in the wine (Hiwilepo van-Hal, 2013). Munoz-Bernal et al. (2020) reported scenarios wherein

some polyphenolic compounds are carried from the raw juice through fermentations while others are produced from microbial activity during the fermentation process. The phenolic compounds from the University of Limpopo juice could not be identified, however it is possible that some of the polyphenolic compounds identified in the wine were carried through fermentation from the juice. The compounds with notable concentrations in the University of Limpopo wine were luteolin (1.63 µg/mL), gallic acid (5.66 µg/mL) taxifolin (1.78 µg/mL), vanillic acid (7.05 µg/mL) and in Sekhukhune wine, apigenin (0.69 µg/mL), kaempferol (0.33 µg/mL) and quercetin (0.23 µg/mL) (1.78 µg/mL). The compounds that have been reported elsewhere to possess antioxidant and antimicrobial activities include taxifolin (Topal et al., 2016), vanillic (Alves et al., 2016, Qian et al., 2019), gallic acid (Yang et al., 2020), luteolin (Zhang et al., 2017; Zhang et al., 2018), apigenin (Seydi et al., 2016), kaempferol and quercetin (Espley et al., 2014). Flavones are potent antioxidants (Panche et al., 2016) and hydroxycinnamic acids important antimicrobial agents (Yang et al., 2020). Their notable presence in the wines provide evidence for the nutraceutical potential of marula wine.

### 4.6 Conclusion and recommendations

Our results show that marula juice and wine contain phenolic compounds that are important for human health. The phenolic compounds increase from juice and persist throughout fermentation although at fluctuating concentrations. The individual compounds found in the wine include antioxidant and antibacterial agents such as taxifolin, vanillic, gallic acid, luteolin, apigenin. The concentration of phenolic contents varied amongst areas due to microorganisms that are present from the juice and evolve as fermentation progresses. However, we could not establish any correlation between the variable results between areas and environmental factors. Furthermore, our results were not entirely comparable to other studies as we used different standards and extraction methods for our analysis. Water was used as the extractant to represent the actual components of the juice and wines. In light of the different analytical methods for the determination of phenolic compounds, from extractants to standard used, the results of one study may not be comparable to another and the need for standard analytical methods for wines is imminent. As the wines used in this study were produced through spontaneous fermentation, the results may be between different batches based on the evolution of wild microorganisms in the fruits and consequently the wines. The starter culture-based wine may provide a better understanding of the relationship between microorganisms used as inocula, the bioactive metabolites such as phenolic compounds produced and the unique sensorial properties thereof.

## CHAPTER 5

## Antioxidant and antimicrobial activities of marula juice and wines

### 5.1 Abstract

*Sclerocarya birrea* (Marula) stem bark, root, leaves, and kernel oil are well known for their medicinal and therapeutic properties. Studies profiling the biological activities of marula juice and wine produced from the juice are not as widely reported. Given the popularity of marula wine among marula tree users and its potential for commercialisation, it is important to study its medicinal properties. The aim, therefore, was to measure and track the changes in the biological activities of marula juice and wine during fermentation. Thin layer chromatography (TLC) technique was used to screen for possible antioxidant and antibacterial compounds in aqueous extracts of the juice and wines. Antioxidant capacity was assessed by quantifying the DPPH free radical scavenging activity and ferric reducing power of the extracts. Minimum inhibitory concentrations of the extracts required to inhibit the growth of selected pathogenic bacteria were determined to quantify their antibacterial activity. Overall, the juice and wine had antioxidant activity albeit much lower than ascorbic acid. The free radical scavenging activity increased as fermentation progressed with the University of Limpopo wine activity still increasing on day 16 at $EC_{50}$ 140.48 ± 0.64. Ferric reducing power was relatively higher on days 2 and 14 at 625 µg/mL of the extracts indicating that fermentation improves antioxidant activity. There was also a positive correlation between scavenging activity and ferric reducing power and the wine total phenolic content. Notable antibacterial activity was seen in the later stages of fermentation and against all tested pathogens the lowest inhibitory concentration of which was 1.25 mg/mL. The biological activities observed in the wines could be attributed in part to the presence of flavonoids and hydroxycinnamic acids, apigenin, caffeic acid, gallic acid, ferulic acid, kaempferol, luteolin, p-coumaric acid, quercetin, sinapic acid, taxifolin, and vanillic acid detected in marula wines. This asserts that marula wine possesses antioxidant and antibacterial properties.

Keywords: Wine, track, thin layer chromatography, antioxidant activity, DPPH radical scavenging activity, ferric reducing power, antibacterial activity, minimum inhibitory concentration

## 5.2 Introduction

Plant secondary metabolites, also known as phytochemicals, possess biological activities that are important in human health. The biological activity of phytochemicals describes their capacity to achieve a defined biological effect on a target (Jackson et al., 2007) and is measured using a biological assay in terms of potency or the concentration of phytochemical compound needed to produce the effect (Pelikan, 2004). These biological activities include among others antioxidant, antimicrobial, anticancer, antidiabetic, anti-inflammatory, and cardio-protective properties (Habauzit and Morand, 2012; Ahumada-Santos et al., 2013; Ambriz-Pérez et al., 2016; Preethi et al., 2016; Ahangarpour, 2019). There are various biological assays to measure and determine the different biological activities. For the measurement of antioxidant activity, chemical-based methods such as radical/ROS-based scavenging assays (the trolox equivalent antioxidant capacity (TEAC/ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) assays, cupric reducing antioxidant capacity (CUPRAC) assay and non-radical redox potential-based assays (ferric reducing antioxidant power (FRAP) are widely used (Danet, 2021). In TEAC/ABTS assay, the antioxidant capacity is measured as the ability of an analysed sample to diminish the colour intensity of a blue-green coloured radical cation, 2,2′-azino-bis(3-ethylbenzothiazole-6-sulphonate) (ABTS•+). This free radical is soluble in water and alcoholic mix with a wavelength maximum of 734 nm. TEAC/ABTS assay allows the evaluation of synthetic as well as natural antioxidants such as phenols, thiols, flavonoids, amino acids, carotenoids, and vitamin C (Schaich et al., 2015). The DPPH assay is based on the measurement of the scavenging capacity of antioxidants towards the deep purple coloured stable free radical 2,2-diphenyl-1-picrylhydrazyl, DPPH•. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine resulting in the decrease of the absorbance of DPPH• radical at a wavelength of 517 nm and a loss of a violet colour in the solution (Contreras-Guzman and Strong, 1982; Schaich et al., 2015). Using a known antioxidant as a standard, the percentage DPPH scavenging effect is calculated using the equation 4.1 below.

$$\% \text{ Inhibition} = \frac{A0 - A1}{A0} \times 100 \qquad \text{(equation 4.1)}$$

where A0 represents absorbance of control; A1 represents the absorbance of standard.

This assay may be used in aqueous and nonpolar organic solvents to assess both hydrophilic and lipophilic antioxidants (Prior et al., 2005). It is a rapid, simple, inexpensive and widely used method to quantify antioxidants in complex biological systems, for solid or liquid samples such as wheat grain and bran, vegetables, herbs, edible seed oils, flours, juice and wines (Vaher et al., 2010; Kim et al., 2017; de Souza et al., 2018; Wahyono et al., 2020). FRAP assay is based on the capacity of antioxidants to reduce ferric 2,4,6-tripyridyl-striazine complex $[Fe^{3+}-(TPTZ)2]^{3+}$ to the blue-coloured ferrous complex $[Fe^{2+}-(TPTZ)2]^{2+}$. The reduction is measured spectrophotometrically as an increase in absorption at wavelengths 593nm and 700nm. The antioxidant capacity is expressed as μM $Fe^{2+}$ equivalents or as a standard antioxidant equivalent (Benzie and Strain, 1999; Gulcin, 2020). FRAP has been applied to assess the antioxidant capacity of beverages such as fruit juices (orange, lemon, apple, pineapple, pear, grapefruit, and tropical fruits); alcoholic drinks (beer, rum, whiskey, grappa, cognac, and red, rosé and white wines); oils such as olive, extra virgin olive, sunflower, corn, soybean and peanut; fruit and vegetables such as grapefruit, orange, pear, honeydew and cantaloupe melons, pineapple, black and white grapes, fig, prickly pear, black and green olives, mushroom, yellow onion, chili and red bell peppers, potato, pumpkin, spinach, and sauce tomatoes (Pellegrini et al., 2003). To conclusively measure the antioxidant activity of compounds within a matrix or extract, a combination of assays is recommended. The choice of assay is based on antioxidant structure and properties (such as mechanism of action), solubility of active compound, partition co-efficient and the solvent system (Danet, 2021).

There are three categories of methods currently employed in research laboratories for the investigation of antimicrobial activities exhibited by plants or plant chemicals. The disk diffusion method (Bauer et al., 1966) is mostly used for qualitative assessment where results indicate that a bacterial isolate is either susceptible, intermediate, or resistant. Bioautography is the other screening method that uses thin layer chromatography to screen for the antimicrobial activity of separated substances such as phytochemicals (Choma and Grzelak, 2011). It uses *p*-iodonitrotetrazolium violet as an indicator of microbial growth where clear white zones against a purple background on the TLC plate indicate antimicrobial activity of the compound (Das, 2010). These screening methods are preferred because although they are simple, cheap, timesaving and require no sophisticated equipment, they still give higher sensitivity than any other methods (Choma and Grzelak, 2011). The better quantitative test is the minimal inhibitory

concentration (MIC) method which measures the lowest concentration of an antimicrobial agent/ sample to which a bacterial isolate is susceptible (Tenover, 2009) and categorises the effects of the agent as either bacteriostatic or bactericidal (Masoko et al., 2005). Coupled with the Iodonitrotetrazolium chloride (INT), the lowest concentration at which there is no reduction to a pink colour is regarded as the MIC value (Eloff, 1998). MIC methods are used to determine susceptibilities of bacteria to drugs especially in resistance surveillance, when a quantitative result is required for clinical management and as used in this study for the comparative testing of new antimicrobial agents (EUCAST, 2003).

A larger volume of the published research on the biological activities of marula provides evidence for its antioxidant and antimicrobial properties. Antioxidant activity has been shown in stem bark extracts (Mariod et al., 2008; Masoko et al., 2008; Moyo, 2009; Tanih and Ndip, 2012; Russo et al., 2013; Lall and Sharma, 2014) in leaf extracts (Braca et al., 2003; Mariod et al., 2008; Moyo, 2009; Russo et al., 2013); in root extracts (Russo et al., 2013; Armentano et al., 2015; Akoto et al., 2020) and kernel oil cake (Mariod et al., 2008). Antioxidant capacity has been reported as well in fruit and juice extracts (Borochov-Neori et al., 2008; Hillman et al., 2008; Hiwilepo van-Hal et al., 2013).

Notably, the antioxidant capacity hereby reported results from the synergistic action of the non-nutritional phytochemicals and ascorbic acid with up to 70% of it attributed only to the presence of ascorbic acid (Owusu-Apenten, 2003). The antioxidant capacity reported for marula juice ranges between 141 mg/100 mL (Hillman et al., 2008) to 2960 mg/100 mL (Mdluli and Owusu-Apenten, 2002). This range is much higher than 44 – 76 mg/100 mL and 44 –132 mg/100 mL ascorbic acid equivalents reported for orange pomegranate respectively (Hillman et al., 2008). Hiwilepo-van Hal et al. (2013) reported antioxidant capacity of marula wine as 0.011 ± 0.003µmol/g (TEAC equivalent) with about 80% of it retained from the juice after fermentation (Hiwilepo-van Hal et al., 2014). This level of interest in the health properties of marula asserts its use in traditional medicine. This can potentially provide a good background for marketing the marula wine as a nutraceutical alcoholic drink.

The antimicrobial activity has been reported in different extracts of the stem bark against different classes of microorganisms such as *Bacillus subtilis* (Moyo, 2009); *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis* and *Escherichia coli* (Eloff, 2001; MacGaw et al., 2007; Moyo, 2009; Kutama et al., 2013;

Manzo et al., 2017; Louis et al., 2018; Mai et al., 2019), *Salmonella typhi* (Manzo et al., 2017), *Klebsiella pneumoniae* (Mai et al., 2019), *Cryptococcus albidus* and *Candida parapsilosis* (Masoko et al., 2008) and *C. albicans* (Runyoro et al., 2006; Moyo, 2009); in different extracts of the leaves against *E. faecalis*, *E. coli* and *P. aeruginosa* and *S. aureus* (Eloff, 2001; Kutama et al., 2013); and in different extracts of the roots against *Candida albicans* (Hamza et al., 2006; Louis et al., 2018)*, Candida glabrata, Candida parapsilosis, Candida tropicalis, Candida kruseii* and *Cryptococcus neoformans* (Hamza et al., 2006). The antimicrobial properties demonstrate the potential of marula and its parts as natural sources of antimicrobial agents.

The antibacterial and antioxidant properties of marula juice and wine have not been widely studied. This exploration focussed on assessing antibacterial activity against known pathogens and antioxidant activity resulting from the action of phytochemicals produced or present in the marula juice and wine.

## 5.3 Materials and methods

### 5.3.1. Sample preparation

Aliquots of 5 mL juice and wines were separately filtered into pre-weighed slant bottles and freeze-dried for three days. The dried samples were reconstituted to concentrations of 10 mg/mL and 20 mg/mL in distilled water. The samples were stored at -20°C and thawed before use in the assays described below.

### 5.3.2 Qualitative antioxidant activity

Thin layer chromatography (TLC) plates sprayed with 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were used to screen for possible antioxidant compounds in the juice and wines. Briefly, 10 µL of each of the samples at 20 mg/mL were spotted on the TLC plates and developed in saturated TLC tanks using mobile phases of decreasing polarity (Chapter 4, section 4.5.2.1). The developed plates were removed from the tanks and allowed to air-dry. Thereafter, DPPH solution (0.2% w/v in methanol) was sprayed onto the chromatograms. The presence of antioxidant activity was indicated by the development of yellow bands against a purple background (Deby and Margotteaux, 1970).

### 5.3.3 Quantitative antioxidant activity by Free radical scavenging activity assay

Free radical scavenging activity of the juice and wines was quantified using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) as described by Leong and Shui (2002). Juice, wine and known antioxidant L-ascorbic acid at different concentrations (250 – 15.63 µg/mL) were

prepared to a volume of 1 mL solution. Then 1 mL of 0.2 mmol/L DPPH- methanol solution was added to each solution and vortexed. The prepared solutions were incubated in the dark for 30 minutes. A mixture of 1 mL 0.2 mmol/L DPPH and 1mL of distilled water was used as the control. After incubation, the solutions were analysed on a UV/VIS spectrophotometer at the wavelength of 517 nm. The experiment was conducted in triplicates and independently repeated three times.

The percentage antioxidant potential was calculated using equation 4.1. The antioxidant activity was expressed as the half maximal effective concentration ($EC_{50}$).

### 5.3.4 Quantitative antioxidant activity assay by ferric reducing power

The ferric reducing capacity of extracts was investigated by using the potassium ferricyanide-ferric chloride method (Tundis et al., 2013). Briefly, 2.5 mL of L-Ascorbic acid, juice and wine extracts at different concentrations (625 – 39 µg/mL), 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%) were mixed and vortexed. These were then incubated at 50°C for 20 minutes. After incubation, 2.5 mL of 10% (w/v in distilled water) trichloroacetic acid was added followed by centrifugation at 3000 rpm for 10 minutes. The supernatant (5 mL) was mixed with 5 mL of distilled water and 1 mL of ferric chloride ($FeCl_3$) (0.1% w/v in distilled water). The solutions were analysed on a UV/VIS spectrophotometer at the wavelength of 700nm. A blank containing all the chemicals was prepared using distilled water instead of the extracts. The experiment was conducted in triplicates and independently repeated three times. The sample concentration providing 0.5 of absorbance ($EC_{50}$) was calculated by plotting absorbance against the corresponding sample concentration.

### 5.3.5 Determination of the antibacterial activity

5.3.5.1 Test microorganisms

American type culture collection (ATCC) specimens that were recommended for antibacterial activity testing by the United States National Committee for Clinical Laboratory Standards (NCCLS, 1990) were used. These included *Enterococcus faecalis* (ATCC 21212), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 27853) and isolated bacteria endogenous to marula fruits, namely, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter cloacae*. The bacteria were maintained on nutrient agar at 4 °C. The bacterial densities were as follows: *S. aureus*, 1.5 x $10^{10}$ colony-forming units (CFU/mL); *E. faecalis* at 3.1

x $10^8$ CFU/mL; *P. aeruginosa* at 2.9 x $10^8$ CFU/mL; *E. coli* at 2.1 x $10^{11}$ CFU/mL; *K. oxytoca* at 1.8 x $10^9$ CFU/mL, *E. cloacae*, 3.8 x $10^8$ CFU/mL*; K. pneumoniae*, 2.7 x $10^9$ CFU/mL.

5.3.5.2 Qualitative antibacterial activity by TLC- Bioautography

The antibacterial activities of the juice and wines were qualitatively determined using a slightly modified bioautography method described by Begue and Kline (1972). The wine extracts (20 mg/mL) were spotted the TLC plates and the chromatograms were developed as described in section 4.5.2.1*,* chapter 3**.** Thereafter, the chromatograms were completely air-dried for two weeks and sprayed with overnight bacterial culture using a spray-gun, until the plates were moistened. The moist chromatograms were incubated for 24 hours at 37 $^oC$. After incubation, 2.0 mg/mL of *p*-iodonitrotetrazolium chloride (INT) solution was sprayed onto the chromatograms and re-incubated for 30 minutes at 37 $^oC$. Clear zones against a purple background indicated the presence of compounds in the extracts that inhibited microbial growth.

5.3.5.3 Quantitative Antibacterial Activity Assay by Microbroth Dilution Method

Antibacterial activity was quantified by determining the minimum inhibitory concentration (MIC) of the juice and wine extract at 10 mg/mL using the microdilution method developed by Eloff (1998). Sterile distilled water (100 µL) was added to the wells of a 96 well microtiter plate. The water extracts (100 µL) were serially diluted with distilled water in the 96 well microtiter plates to obtain concentrations from 2.5 mg/mL to 0.02 mg/mL. After diluting the extracts, each microorganism culture (100 µL) was separately added to each well. The microtiter plates were covered with a sterile laboratory plastic wrap and incubated for 24 hours at 37 °C. Following incubation, 40 µL of 0.2 mg/mL of *p*-iodonitrotetrazolium chloride (INT) solution was added to each well of the microtiter plates and incubated for another 30 minutes. Chloramphenicol and sterile distilled water were used as positive and negative controls respectively. Bacterial growth was seen in wells showing a purple colour. The lowest concentration at which clear wells were seen indicated the presence of a compound in the extract that inhibited microbial growth. The extracts were assayed in triplicates.

#### 5.3.5.4 Statistical analysis

Descriptive statistics (average, standard deviation) were calculated. For inferential statistics, one-way ANOVA ($p < 0.05$) with multiple comparisons and independent t-tests were performed using IBM SPSS software.

## 5.4 Results

Aqueous extracts of juice and wine were prepared and tested for the presence and quantity of antioxidant and antibacterial activities. Qualitative assays were conducted to screen for the presence of the activities While the active compounds detected in the extracts were quantified using several quantitative assays. The results are presented in figures, tables and graphs starting with those obtained qualitatively.

### 5.4.1. Qualitative DPPH assay on TLC

The chromatograms below (Figure 5.1) demonstrate separated compounds of aqueous juice and wine extracts that can neutralise or quench the purple-coloured DPPH free radical via transferring hydrogen atoms or electrons. The interactions between the radicals and antioxidants decrease the concentration of the purple radical in the reaction mixture and produces the yellow bands seen against a purple background.

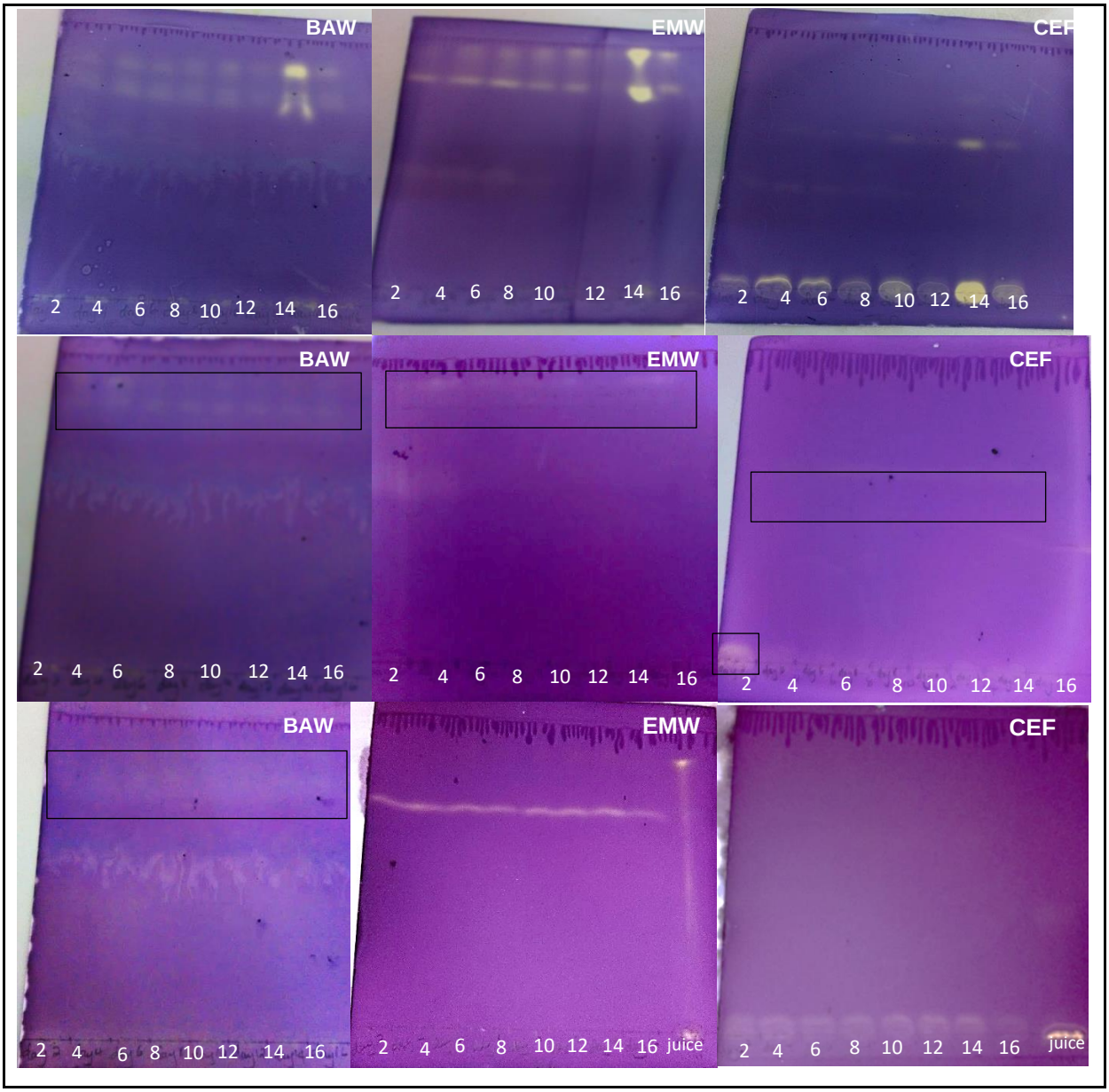


**Figure 5.1**: Chromatograms demonstrating the antioxidant activity of The Oaks (A), Sekhukhune (B) and the University of Limpopo (C) wine aqueous extracts. The yellow colour indicates antioxidant activity.

The active compounds found were predominantly polar with clear profiles developed in the EMW mobile system. The active compounds in the juice extract are positioned higher on the chromatogram than those of the wine suggesting that they may be different. The antioxidant composition was present in the undiluted fruit juice and in all the wines. The Oaks wine extracts had around three active bands, at $R_f$ values 0.6, 0.8 and 0.9 which was more than the single band observed in both the University of Limpopo and Sekhukhune wine extracts. Sekhukhune wine had radical scavenging compounds, active

from day 2 to day 16 of fermentation at $R_f$ value 0.9 on chromatograms developed in EMW mobile phase and $R_f$ value 0.6 on chromatograms developed in CEF mobile phase. The University of Limpopo wine had active compounds throughout the fermentation period at $R_f$ value 0.7. The fresh undiluted juice extract formed a tail from poor separation in the EMW mobile phase. The spot (loaded extract) on the origin, the tail and the aggregated band of compounds at $R_f$ value 0.9 showed activity.

### 5.4.2 Half maximal effective concentrations ($EC_{50}$) towards DPPH inhibition

The free radical scavenging activity of the juice and wine extracts was quantified by determining the concentration of the extracts that was required to scavenge half of the DPPH free radical. In principle, lower $EC_{50}$ values indicate good scavenging activity. Table 5.1 below represents the concentrations of the aqueous extracts of the juice and wines from the selected areas.

**Table 5.1**: The free radical scavenging activity (EC50 values) of aqueous juice and wine extracts.

| **Extract** | **DPPH radical scavenging activity ($EC_{50}$) concentrations** |
|---|---|
| University of Limpopo Day 0 (fresh juice) | 116.84±0.08 |
| Day 2 wines: The Oaks **|** Sekhukhune \| University of Limpopo | 570.76±0.11 \| 455.77±0.18 \| 219.46±0.06 |
| Day 4 wines: The Oaks \| Sekhukhune \| University of Limpopo | 476.55±0.11 \| 412.43±0.22 \| 118.71±0.08 |
| Day 6 wines: The Oaks \| Sekhukhune \| University of Limpopo | 1398.51±0.15 \| 869.78±0.18 \| 469.77±0.31 |
| Day 8 wines: The Oaks \| Sekhukhune \| University of Limpopo | 462.09±0.16 \| 269.72±0.16 \| 528.38±0.67 |
| Day 10 wines: The Oaks \| Sekhukhune \| University of Limpopo | 990.43±0.14 \| 425.92±0.13 \| 335.35±0.30 |
| Day 12 wines: The Oaks \| Sekhukhune \| University of Limpopo | 4072.86±0.11 \| 393.25±0.19 \| 406.19±0.37 |
| Day 14 wines: The Oaks \| Sekhukhune \| University of Limpopo | 7510. 91±0.11 \| 541.43±0.11 \| 293.39±0.36 |
| Day 16 wines: The Oaks \| Sekhukhune \| University of Limpopo | 11218.39±0.13 \| 960.21±0.40 \| 140.48±0.64 |
| **L-ascorbic acid** | **22.68±0.12** |

Predominantly, the wines had lower scavenging activity when compared to the standard, L-ascorbic acid. University of Limpopo wine had the most potent radical scavenging activity throughout fermentation when compared to the other areas, and this was still increasing on day 16. The radical scavenging activity of Sekhukhune and The Oaks wines decreased as fermentation progressed reaching their lowest highest $EC_{50}$ concentrations on day 16. Day 4 wine from the University of Limpopo had the most antioxidant activity, whereas wines from Sekhukhune and The Oaks performed best on day 8. University of Limpopo Day 16 wine had the highest activity of all wines at the latter stage of fermentation.

### 5.4.3 Ferric reducing power of juice and wine aqueous extracts

Ferric ion reduction, an alternative method used for the estimation of antioxidant activity of extracts was performed on the juice and wine aqueous extracts. The juice and wine extracts had inferior reducing power compared to L- ascorbic acid as seen in figure 5.2. The juice extracts had comparable reducing activity as the University of Limpopo wine extracts, inferior only to the day 2 and day 10 extracts. The University of Limpopo wine

extracts had the highest activity, followed by Sekhukhune extracts which had comparable activities on days 14 and 16. There are visible spikes in activity on days 2, 8 and 14 for extracts from all areas, however, day 10 University of Limpopo increases whereas the other two areas declined.

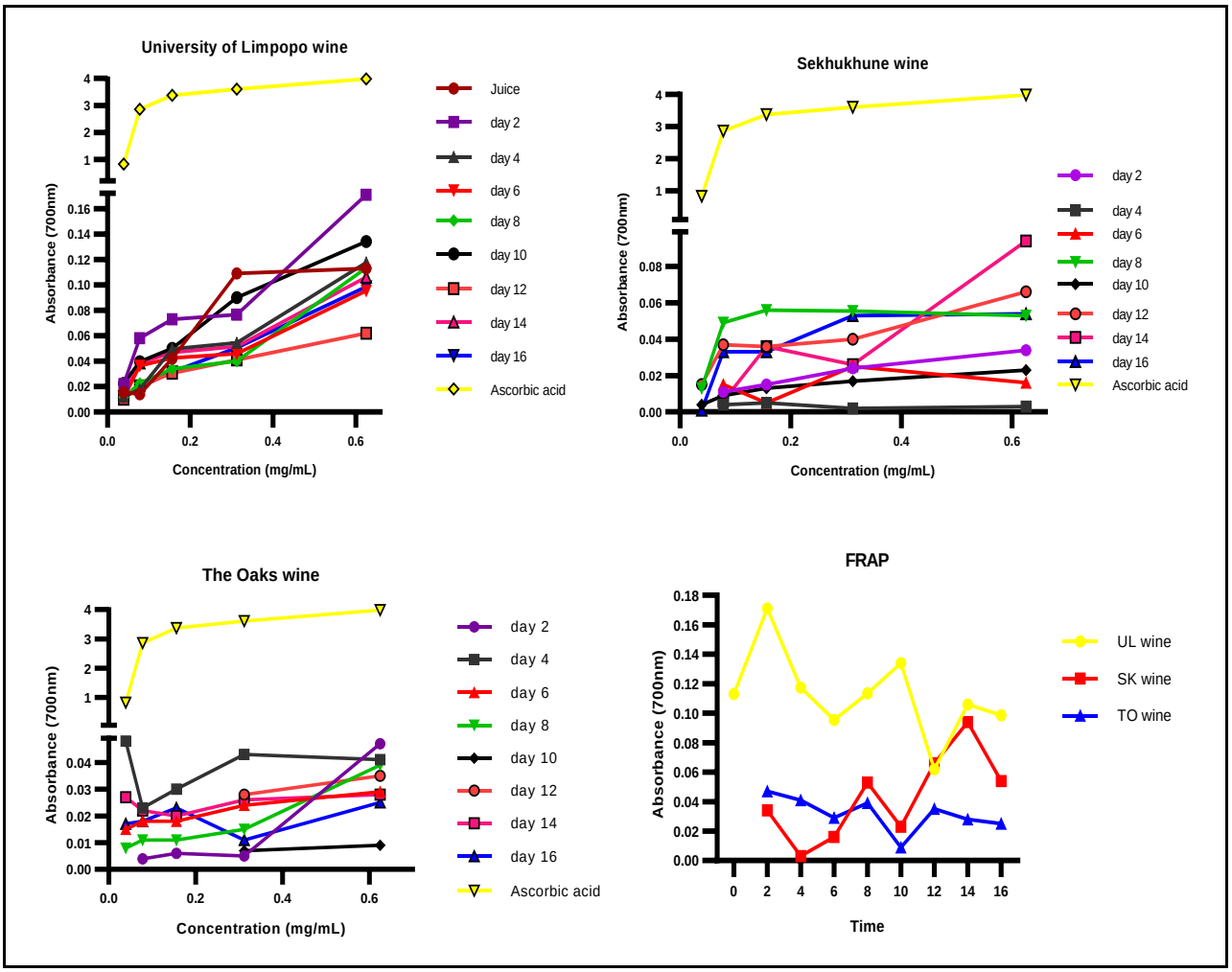


**Figure 5.2**: The ferric reducing power of University of Limpopo, Sekhukhune and The Oaks juice and wine aqueous extracts.

The reducing power was observed throughout the 16-day fermentation period. Expectedly, absorbance increased as concentration increased indicating that ferric reducing power increased with increasing concentrations of the wine extracts.

### 5.4.4 Antibacterial activity of marula juice and wine

5.4.4.1 Bioautography assay

Bioautography was used to localise and discriminate antibacterial compounds in the juice and wine extracts. The chromatograms below illustrate phytochemicals found in the marula wines from Sekhukhune and the University of Limpopo, with antibacterial activity

against ATCC strains of *Staphylococcus aureus* (Figure 5.3A) and *Enterococcus faecalis* (Figure 5.3B) respectively. Sekhukhune wine extracts were active from days 10 – 16 at $R_f$ value 0.15 and the University of Limpopo wines were active from days 12 –16 at $R_f$ value 0.06. Only Sekhukhune and the University of Limpopo wines were active and that only against Gram-positive bacteria. The endogenous bacteria showed no sensitivity to the wine antibacterial compounds.

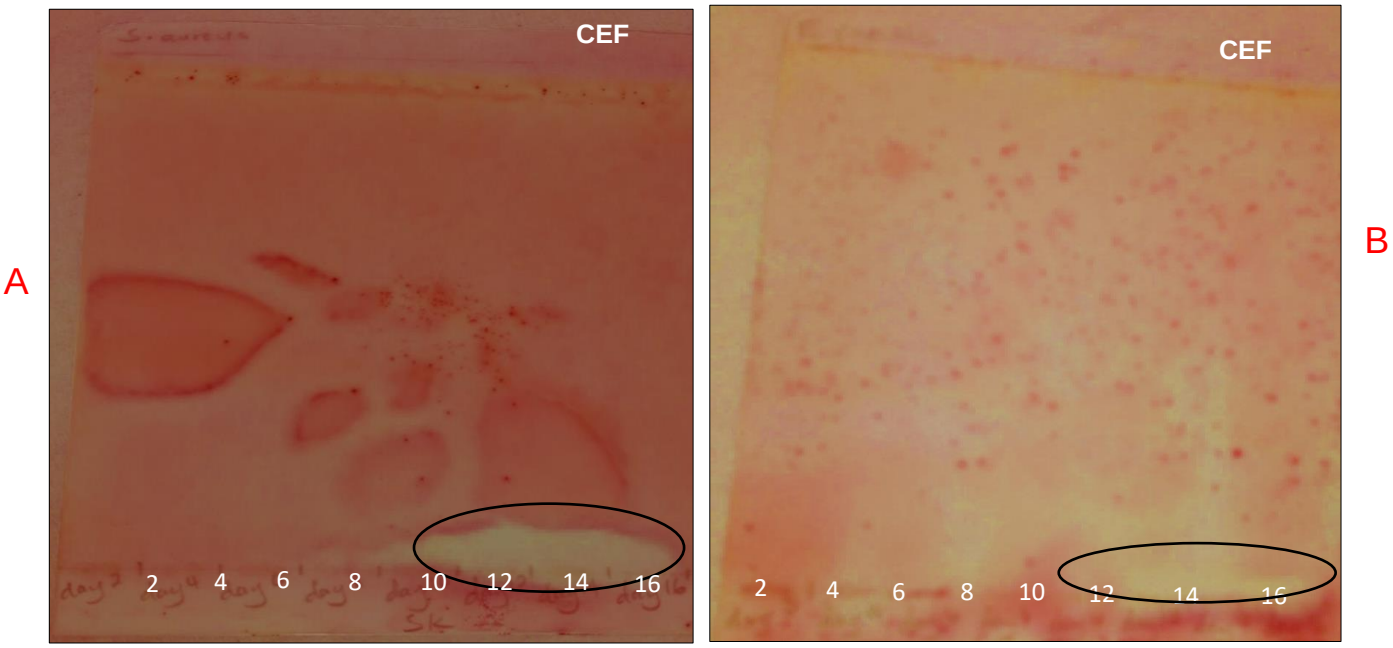


**Figure 5.3**: Bioautography of aqueous Sekhukhune and the University of Limpopo marula wine extracts separated by CEF mobile system and sprayed with overnight *S. aureus* culture (A) and *E. faecalis* culture (B).

5.4.4.2 Broth Micro-dilution assay

The micro-dilution assay was used to determine the minimum concentration (MIC) of the extracts (mg/mL) that was able to inhibit the microbial growth of Type strains and endogenous bacteria previously isolated from marula wines. Table 5.2 below shows the MIC values of all marula juice and wines ran parallel to a known antibiotic, chloramphenicol.

**Table 5.2:** The Minimum inhibitory concentration (MIC) of marula juice and wine extracts (mg/mL) against selected pathogenic bacteria.

| MIC value (mg/mL) | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Wine samples (day intervals) | | | | | | | | | | | | | | | | | | | | | | | | | |
| Bacteria | 0 | | | 2 | | | 4 | | | 6 | | | 8 | | | 10 | | | 12 | | | 14 | | | 16 | | | Chloramp-henicol |
| | UL | TO | SK | UL | TO | SK | UL | TO | SK | UL | TO | SK | UL | TO | SK | UL | TO | SK | UL | TO | SK | UL | TO | SK | UL | TO | SK | |
| *E. coli* | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | 2.5 | ND | 2.5 | 2.5 | ND | 2.5 | ND | ND | 2.5 | 0.02 |
| *E. faecalis* | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | ND | ND | ND | 0.02 |
| *S. aureus* | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 1.25 | ND | ND | 1.25 | ND | ND | 1.25 | ND | ND | 0.02 |
| *P. aeruginosa* | ND | ND | ND | ND | 2.5 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | ND | ND | ND | ND | ND | ND | 0.02 |
| *K. oxytoca* | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | ND | ND | ND | 0.02 |
| *K. pneumoniae* | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 2.5 | ND | ND | 2.5 | ND | ND | ND | ND | ND | ND | ND | ND | 0.02 |
| *E. cloacae* | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 1.25 | ND | 2.5 | 1.25 | ND | 2.5 | 1.25 | ND | ND | ND | ND | ND | 0.04 |

Keys: ND: Not detected

Generally, the wine showed inferior antibacterial activity when compared to the known antibiotic. However, better antibacterial activity was seen in the later stages of fermentation and against *S. aureus* and *E. cloacae,* MIC values 1.25-2.5 mg/mL. University of Limpopo wine edged the other wines showing much better activity from day 10 to 16 against all but *E. coli* and *K. oxytoca*. Day 12 University of Limpopo wine showed inhibition against all the bacteria. The Oaks wine showed inhibition only at days 2 and 4 of fermentation. These results showed antibacterial activity against both Gram-positive and Gram-negative bacteria that was not detected through bioautography. The compounds must have acted synergistically to exert the augmented antibacterial activity.

## 5.5 Discussion

Qualitative and quantitative assays were used to screen and quantify the antioxidant activity and antibacterial activities of the marula juice and wines. Generally, active compounds for antioxidant activity were separated more with the polar mobile phase (EMW) and some in the intermediate phase (CEF). This suggested that the compounds in the extracts were mostly polar polyphenols (Tsao, 2010). The compounds at $R_f$ value 0.6 in The Oaks wine diminish from day 10 to 16. It is possible that the compound that diminishes may be responsible for the scavenging activity observed or that there may be more than one compound observed at $R_f$ value 0.6. The $EC_{50}$ values were relatively lower between day 2 and 10 corroborating the presence of a synergistic activity of compounds in the wine. The fading of compounds from day 12 may be due to the presence of microorganisms such *Gluconobacter* spp. and *Acetobacter spp*. (Alimardani-Theuil et al., 2011) and some lactic acid bacteria occurring in the latter stages of fermentation which that metabolise hydroxycinnamic acids into vinylphenols (Zuehlke et al., 2013; Santamaría et al., 2018). The active compounds at $R_f$ values 0.8 and 0.9, purple when visualised by vanillin sulphuric acid and possibly terpenoids could have been carried through the fermentation or produced from the action of microorganisms such as *S. cerevisiae* which facilitates the production of terpenoids (Carsanba et al., 2021). Terpenoids have reported antioxidant activity (Yang et al., 2020). Sekhukhune wine had radical scavenging compounds, active from day 2 to day 16 of fermentation**.** These compounds that were orange when visualised with vanillin sulphuric acid are possibly flavonoids (Taganna et al., 2011) which have reported antioxidant activity (Panche et al., 2016). This is corroborated by a significantly higher flavonoid content was found in the wine. Comparing by the lower $EC_{50}$ values obtained in the Sekhukhune wine than The Oaks wine, the content of flavonoids is more important than that of terpenoids in the antioxidant activity of Sekhukhune marula wine. The University of Limpopo wine had active compounds throughout the fermentation period which were purple when visualised with vanillin sulphuric acid. These compounds are also possibly terpenoids with reported antioxidant as seen with The Oaks wine. However, the University of Limpopo had more antioxidant activity indicated by lower $EC_{50}$ values. It is possible then that the University of Limpopo wine had other antioxidant compounds which acted synergistically as seen with Cannabis flavonoids and terpenoids (Baron, 2018). The antioxidant activity observed on the spot and at $R_f$ value 0.9, much higher than the $R_f$ value observed in the wine indicated the possibility that the juice contained more compounds which can only be separated using a

different combination of solvents. Also, the compounds found on the spot could have antioxidant activity only when they act together and not as separated compounds.

Antioxidant activity generally has direct proportional correlation with concentrations of total phenolics in different plant and fruit extracts. Hiwilepo van- Hal and colleagues (2013) found a positive correlation between antioxidant activity and ascorbic acid ($r^2 = 0.59$) and phenolic content ($r^2 = 0.64$) (Hiwilepo van- Hal et al., 2013). There were weak correlations between the $EC_{50}$ values and total phenolics, flavonoids and tannins for the University of Limpopo, ($r^2 = 0.01, 0.13. 0.06$) and The Oaks, ($r^2 = 0.37, 0.07$) respectively. Sekhukhune wines showed positive correlations between DPPH % inhibition and total phenolics, flavonoids and tannins of $r^2 = 0.22$, $r^2 = 0.12$ and $r^2 = 0.20$ respectively. Among the antioxidants which were identified and quantified, the following were observed: flavones, luteolin and apigenin in the University of Limpopo and Sekhukhune wines. These are reported potent antioxidants second only to catechin (Panche et al., 2016). Their presence could be responsible for much of the antioxidant activity observed in the wines. It is plausible however from the weak correlations with the quantified phenolic compounds that the scavenging activity of the wines could be overestimated due to interferences from other reducing compounds. Dlamini and Dube (2008) and Hiwilepo van- Hal and colleagues (2013) indicated that marula wine is a good source of ascorbic acid that retains 70-72% of the content found in the juice after four days of fermentation. Therefore, the activity observed in the wines could be largely contributed by ascorbic acid.

As antioxidant capacity cannot be determined using a single assay, Ferric reducing power assay which primarily measures the ability of antioxidants to directly reduce a free radical by electron transfer was performed. Generally, the ferric reducing power increased with an increase in the concentration of all the extracts. L-ascorbic acid used as the standard for the assay had considerably higher reducing ability than all the extracts. It is worth noting that FRAP results can vary tremendously depending on the reaction time and the polarity of the extractant used. Phenols such as ferulic acid, quercetin, caffeic acid and ascorbic acid extracted in water require longer reaction time, even up several hours to obtain their representative reducing ability (Prior et al., 2005). There was however a noticeable increase in the reducing ability from an average of 0.06 mg/mL in the undiluted fresh juice to 0.08 mg/mL in the wine due to the biochemical reactions between phenolic compounds and microorganisms. The reducing power in the wines increased with several fluctuations.

Fluctuations obtained corresponded to observed fluctuations in total tannin content. Total tannins increased with the reducing power in the University of Limpopo and vice versa in Sekhukhune and The Oaks wines. Although, catechin or other tannin compounds with potent antioxidant activity (Gu et al., 2008) were not isolated and identified in this study, Hiwilepo van-Hal (2013) reported these in the marula wine. Gallic acid which can be found both as a free state and as a constituent of tannins, is an important phenolic compound in both red and white grape wine (Waterhouse, 2002) with antioxidant activity (Fitzpatrick and Woldemariam, 2017). It was found in our wines as a free state compound. It is possible however that our wine has catechin or its derivatives. Further studies to identify individual compounds present in the wines are required.

There was a positive correlation between reducing power and total phenolic content ($r^2$=0.26), total tannin content ($r^2$=0.16) as well as total flavonoid content ($r^2$=0.09) in the university of Limpopo wine. The positive correlation indicated that the ferric reducing power of the University of Limpopo wine increased with the content and concentration of phenolic compounds. On the other hand, Sekhukhune wine had slightly higher concentrations of apigenin, a potent antioxidant; kaempferol and quercetin. The lower reducing power of The Oaks wine had a positive correlation ($r^2$=0.36) with total flavonoid content further pointing out that the flavonoids in marula wine are strong antioxidants albeit present in low concentrations. The individual compounds could not be detected and identified. A stronger positive correlation was found between total phenols and reducing power in five red, five white and one rose wine ($r^2$=0.99) (Paixao et al., 2007).

The antibacterial activity of the juice and wine was tested using p-iodonitrotetrazolium violet for bioautography and for microbroth Dilution against ATCC strain bacterial pathogens and possible pathogenic bacteria isolated from marula wine.

The marula wines possessed antibacterial compounds with broad-spectrum activity, acting against both Gram-negative and Gram-positive bacteria. Although some compounds had the ability to exert antibacterial activity independently, a bulk of the activity was exerted in the conjugative form with the other compounds. Antibacterial activity exerted by the action of individual or a combination of compounds has been reported in wines (Radovanović et al., 2014). The antibacterial activity is likely due to the flavonoids and phenolic acids quantified and identified which included flavonoids and phenolic acids that have reported antimicrobial activity against different microorganisms, especially the hydroxycinnamic acids such as

caffeic acid, ferulic acid and p-Coumaric acid (Jamir et al., 2019; Yang et al., 2020). Radovanović et al. (2014) found strong correlation between the contents of caffeic acid, gallic acids, resveratrol, quercetin, quercetin-3-glucoside, catechin and gallic acids and antimicrobial activity (Radovanović et al., 2014) while Papadopoulou et al. (2005) found a positive correlation between phenolic content and the antimicrobial activity of red and white wines (Papadopoulou et al., 2005). With an increase in the presence and quantity of these phenolic compounds, an increase in antibacterial activity was observed. This could explain the better activity showed by the University of Limpopo wine. The wines also contained classes of phenolic compounds from the start to the end of fermentation all with reported antimicrobial activity. Sekhukhune and the University of Limpopo wines had comparable and significantly higher total phenolic content through to the last day of fermentation than The Oaks wine. The lack of antibacterial activity in The Oaks wine could be attributed to its relatively low content of phenolics whereas the antibacterial activity observed for the two other areas could be attributed to the higher phenolic content. The phenolic compounds found in the marula wine preserve and enhance its safety for human consumption.

### 5.6 Conclusion and recommendations

Marula wine contains antioxidants and antibacterial agents. The active compounds were extractable in water and transformed through biochemical reactions facilitated by microorganisms in the fermenting wine. The compounds form part of various classes of medicinally important compounds such as phenolics, tannins and flavonoids. Positive correlations were found between their presence and DPPH scavenging activity as well as reducing power. This showed that the antioxidant activities of the active compounds followed both the SET and HAT mechanisms of radical neutralisation. Interestingly, the antibacterial compounds of the wine emerged strongly at the latter stages of fermentation, a positive attribute for the development of wine with a longer shelf life. This narrative is reflective of wine produced through spontaneous fermentation with a cocktail of wild microorganisms participating in the fermentation, flavouring and spoilage processes. Research on a starter culture wine, using pasteurised juice and where growth parameters and microorganisms are controlled will provide clearer information on the compounds formed and their biological activities.

## Chapter 6

## Changes in the physicochemical and nutritional properties of the marula juice and the fermenting marula wine

### 6.1 Abstract

Marula tree (*Sclerocarya birrea* subsp*. caffra*) is a wild plant that is indigenous to many parts of sub- Saharan Africa, and it has multitude of uses. Its fruits are most used in preparations of food and alcoholic drinks. The nutritional properties of its products are scarcely studied. This study investigated the presence and evolution of chemicals and nutrients of the marula fruit juice and fermenting wines from The Oaks, Sekhukhune and University Limpopo. The pH, alcohol, sugars, titratable acidity, total acidity and organic acids were analysed with Fourier transformed infrared spectroscopy (FTIR) and the protein content was measured using QuantiPro BCA Assay Kit and lipids extracted with chloroform-methanol solvents. The mineral composition was determined using inductively coupled plasma emission spectroscopy (ICPE) whereas the total mineral content (ash content) was determined using dry ashing method. The juice contained the highest total sugar (53.60 g/L), citric acid (4.57 g/L) and tartaric acid (1.40 g/L) compared to all the wines at averages of 0.68 g/L and 0.45 g/L respectively. The wines contained more glucose (7.31 g/L) than fructose (0.86 g/L), with higher malic and lactic acids at the pH range of 3.33 – 4.21. The overall alcohol content for the wines averaged 1.50% for The Oaks, 1.86% for Sekhukhune and 3.10% for University of Limpopo, the highest concentration of which was observed in the University of Limpopo wine. The juice had lower concentrations of protein and higher concentrations of crude fat than day 2 wine. The University of Limpopo wine contained significantly higher protein content at 85.77 1.50 µg/L. The total mineral content started high at 181.1 g/L and 453.1 g/L in The Oaks and University of Limpopo wines while Sekhukhune wine gradually increased from 177.6 g/L to 297.7 g/L by the 12$^{th}$ day of fermentation. Potassium, magnesium, calcium, and sodium were the most abundant minerals found in all the wines from day 2 to day 16 of fermentation at averages of 53.01 g/L, 14.13 g/L, 9.38 g/L and 7.40 g/L respectively. Sekhukhune wine had significantly higher concentrations of arsenic, chromium, magnesium, sodium, phosphorus, and lead whereas University of Limpopo wine had significantly higher concentrations of zinc ($p< 0.05$). The lead content however was higher than the recommended level of 0.15 mg/L. The marula wine retained small amounts of proteins, acceptable concentrations of organic acids and minerals that positively contribute to its organoleptic and nutritional properties. This makes the marula wine a good candidate for a low alcohol nutritious drink.

Keywords: Wine, juice, proteins, FTIR, ICPE, organic acids, sugars, minerals, lipids, alcohol

## 6.2 Introduction

Wild fruit trees are naturally occurring resources of food with nutritional and health benefits to humans. South Africa is home to numerous fruit trees which have been identified, studied, and protected under the National Forest Act, 1998 (Act No. 84 of 1998). Among these trees, *Sclerocarya birrea* subsp. *caffra*, known as marula, is widely distributed and grows abundantly in the Eastern Cape, Limpopo, Mpumalanga, and KwaZulu Natal provinces (Shackleton et al., 2001). The tree has become an integral part of the traditions and culture, celebrations, and livelihoods of many South Africans. Marula tree produces green plum sized fruits which turn pale-yellow, thick, and very juicy with tart, sweet and refreshing taste. The fruit pulp or juice is also processed into fruit drinks, nectars and teas, alcoholic beverages such as marula beer and Amarula cream, wines, liqueurs, and punches (Mojeremane and Tshwenyane, 2004; Hiwilepo-van Hal et al., 2013). The nutritional value of marula fruit juice has been largely associated with the high vitamin C content that ranges between 62 mg/100 g (Carr, 1957) and more than 2,100 mg/100 g (Eromosele et al., 1991; Hillman et al., 2008; Muhammad et al., 2011; Hiwilepo-van Hal et al., 2012). This level is about four times higher compared to orange and pomegranate juices (Hillman et al., 2008). However, the juice possesses more nutritional properties that provide the basis for its importance in human diet. It has a carbohydrate fraction of 7 to 14% fresh weight (Gous et al., 1988) and 90.35% dry weight which consists mainly of sucrose at 6160 mg/100g dry weight, glucose at 1700 mg/100g dry weight and fructose (Muhammad et al., 2011); dietary fibre of 0.7 g/100 g (Borochov-neori et al., 2008); moisture content between 82 and 93% (Shone, 1979; Gous et al., 1988); ash content of 0.09 g/100g to 5 g/100g (Taylor, 1985; Muhammad et al., 2011) and minerals such as calcium (51.73 mg/100cm$^3$), magnesium (24.53 mg/100cm$^3$), potassium (44.54 mg/100cm$^3$), sodium (14.88 mg/100cm$^3$), iron (8.83 mg/100cm$^3$), manganese, zinc, copper, nickel, chromium and phosphorus (Eromosele et al., 1991; Borochov- Neori et al., 2008; Muhammad et al., 2011). Furthermore, a lipid content of 13.5 g/100g dry weight (Glew et al., 1997) and protein content of 3.3 to 3.6 g/100g dry weight (Muhammad et al., 2011; Mariod and Abdelwahab, 2012) have been reported in the fruits. The nutritional composition reported varied from study to study due to variations in genetic, environmental factors, fruit ripeness and analytical methods used (Leakey, 1999; Hillman et al., 2008).

The marula wine that is fermented for 4 to 8 days has an alcohol level between 2 and 5.5% (Dlamini and Dube, 2008; Mokgolodi et al., 2011; Hiwilepo-van Hal et al., 2013). This level of

alcohol is higher than that of banana wine 1.37% (Idise, 2011), comparable to that of palm wine 1 – 6% (Santiago-Urbina and Rui´z-Tera´, 2014), but lower than that of mango wine 8.9 – 9.5% (Musyimi et al., 2014). The sugar content of the marula wine was 8.4% which was higher than 5.4% of mango wine but lower than 10 –12% in palm wine. Marula wine pH can reach 3.44 which is more acidic than palm wine at pH 5.59 (Panda et al., 2014) but comparable to pH 3.3 and pH 3.52 reported for banana wine (Akubor et al., 2003) and jackfruit (Panda et al., 2016) wine respectively. The marula juice reportedly contains a high amount of ascorbic acid. Interestingly, the wine contains comparable levels of ascorbic acid level that ranges from 96 mg/100 mL (Dlamini and Dube, 2008) to 159 mg/100 mL (Hiwilepo-van Hal et al., 2013). This is lower than 1780 mg/100 mL in jackfruit wine (Panda et al., 2016) but higher than 10 –19 mg/100mL (Okechukwu et al., 1984) in palm wine and 80 mg/100 mL (Panda et al., 2014) in bael fruit wine. The wine also contains vitamin B and provides energy to supplement low carbohydrate diets (Baxter, 2000). The wines were analysed only at one point, on the $4^{th}$ or $8^{th}$ day of fermentation. The wine can serve as an energy source for diets low in carbohydrates. The information on the nutritional benefits of marula wine is still scanty, with most reports on the vitamin C and sugar contents. A comprehensive nutritional profile needs to be studied to explore the potential of marula wine as a nutritional beverage.

The Dietary Guidelines for Americans (DGA) provides extensive recommendations for the consumption of nutritional beverages such as milk, 100% fruit and vegetable juices and various teas and coffee. Most of these beverages provide non-nutritional bioactive compounds such as flavonoids along with minerals and vitamins and are therefore classified as functional beverages (Huma Bader-Ul-Ain et al., 2019; Mario et al., 2020). Fruits such as grapes, cranberry, sweet cherry, pomegranate, and blueberry have nutritional properties that are also found in their wines (Dey et al., 2009). This has expanded the functional beverage category by fruit wines provided these are consumed moderately, ≤1 drink for women and ≤2 drinks for men (US HHS and USDA, 2015). From the documented findings on marula thus far its fruit and juice contain some of these properties at concentrations equal or above those reported in grapes and other fruits (Vinci et al., 1995; Glew et al., 2004). There is therefore potential for marula wine to be made into a functional beverage.

The intent of this chapter was to profile the nutritional properties of marula juice and fermenting wines throughout 16 days. The parameters were analysed every second day of fermentation to track their evolution. The aim was achieved through the following objectives:

i. To measure the pH, total acidity, organic acids and alcohol levels of the marula juice and wine

ii. To determine the contents of sugar, protein and lipids in the marula juice and wine.

iii. To quantify the ash content and determine the constituent minerals in marula juice and wine.

## 6.3 Materials and methods

### 6.3.1. Fruits and wine collection

The green to yellow-green fruits were collected from the University of Limpopo grounds (-25.6150; 28.0166) into a 50kg box and placed in a cool place until ripened. Overnight fermenting wine prepared in 20 L plastic buckets were purchased from community partners in The Oaks (-24.36239; 30.67527) and Sekhukhune (-24.34329; 30.09252) and transported to the laboratory. The buckets were stored at room temperature (25 – 30 $^{o}$C) to continue fermenting over 20 days.

### 6.3.2. Juice preparation

A puncture was made on the skin of each fruit. The juice was extracted into a 9 L plastic bucket by squeezing the peel and the kernel. The juice was then transferred into lockable plastic bags and stored at -20 $^{o}$C until analysis commenced.

### 6.3.3. Laboratory wine preparation (University of Limpopo wine)

The traditional recipe and process of producing marula wine was followed. Briefly, an equal volume (10 L) of tap water was added to the marula juice. The bucket was closed tightly and placed at room temperature overnight. A glob of a layer that had formed overnight on the surface of the juice was removed by filtering the juice using a strainer. The fermenting wine was placed at room temperature to continue fermenting over 20 days.

### 6.3.4. Wine sampling

Duplicate samples of wines were collected at 2 days intervals and stored in sterile 2 mL and 50 mL centrifuge tubes and 1 L lockable plastic bags at -20 $^{o}$C until analysis commenced.

### 6.3.5. Sample preparation

The laboratory and purchased wines as well as undiluted juice extracted from the University of Limpopo fruits were separately filtered through a Whatman No.1 filter paper and transferred into clean 50 mL centrifuge bottles.

### 6.3.6. Determination of physicochemical properties

Marula wine samples were sent to the Agriculture Research Council-Infruitec-Nietvoorbij laboratory for chemical analysis. The wines were analysed on the ALPHA II Wine analyzer (Bruker Optics, Germany) instrument used for the investigation of finished wines, must or juices. The machine uses a Fourier Transform Infrared (FTIR) technique where the measurement is performed on the Attenuated Total Reflectance (ATR) element. Wine samples were defrosted and mixed thoroughly to obtain a homogenous mixture prior to analysis at 4 °C. Wines were analysed for pH, alcohol, sugars, titratable acidity, total acidity.

### 6.3.7. Determination of protein content

The BSA kit (Sigma) was used, and the manufacturer's instructions were followed to prepare the stock solution of the protein, the six protein standards and the blank. Absorbance was measured at 562nm with the UV/Vis spectrophotometer. The results were used to draw a standard curve. To measure the protein content in the juice and wines, stock solutions at 30 µg/mL were prepared and diluted within a linear range of 0.5 µg/mL and 30µg/mL. These were mixed by vortexing and incubated as indicated by the manufacturer. The absorbance was read at 562nm. The protein content was determined by extrapolation using the protein standard curve. The results were reported in µg/L.

### 6.3.8. Determination of crude fat

Samples of undiluted juice and each of the wines were extracted with chloroform:methanol (1:2 v/v) method (Folch et al., 1957). Briefly, A 3 mL test sample was mixed with 3.7 mL chloroform and 7.5 mL methanol in a test tube and incubated in a shaker incubator for an hour at 25 $^{o}$C. After incubation, 3.75 mL of chloroform and methanol were added and left to stand for 5 minutes at room temperature. Two distinct layers formed, and the top layer was carefully removed and discarded. A 3.68 mL volume of wash mixture of chloroform:methanol:water (3: 48: 47 v/v) was added to the bottom layer. The top layer that formed was again carefully removed and discarded. The wash step was repeated twice. The fatty layer that remained was transferred into pre-weighed vials and placed under a fan to evaporate the solvents. After drying, the vials were weighed again.

The following formula was used to calculate the obtained crude fat:

% Crude fat = (W2 – W1) x 100/S,

where W1= weight of empty vial (g), W2= weight of vial and extracted fat (g) and S= Weight of sample.

The crude fat was reported in g/L.

### 6.3.9. Determination of ash content

The AOAC (2005) method was used for the analysis of the total mineral content. Porcelain crucibles were washed and placed in a furnace at 600 $^{o}$C for 30 minutes to remove any contaminants. They were then cooled in a desiccator for another 30 minutes. Thongs were used to handle the crucibles. Each crucible was then weighed on a weighing balance, the weighing balance was then tared, and 5 g of the wine sample was added into each crucible. The crucibles were then labelled and placed in a furnace for 16 hours at 600 $^{o}$C. The crucibles were then removed from the furnace with thongs and cooled in a desiccator. Each crucible was then weighed again, and the dry sample mass was determined.

The moisture content of each sample was determined by burning 2 g of each sample on the moisture content machine. The moisture content together with the dry sample mass was used to determine the mineral (ash) content in percentage form which was then converted to g/L.

### 6.3.10. Determination of mineral composition

The wine sample was digested in an acid solution where 7.5 mL nitric acid and 1.5 mL hydrogen peroxide were added to 5 mL of the wine sample in a digestion vessel. The solution was mixed by shaking the digestion vessel carefully and digested the samples in a microwave mineral digester which took 15 minutes. Thereafter, the digestion vessels were cooled in the laminar flow for 10 minutes. The vessels were then opened in a laminar flow ensuring that the opening of the vessel is facing the laminar flow to prevent contact with the acid. The contents from digestion were then transferred to 50 mL centrifuge tubes and distilled water was added to the contents up to 50 mL. The 25 contents were then run using the Shimadzu Inductively Coupled Plasma Emission Spectroscopy (ICPE-9000) (Poitevin, 2012).

### 6.3.11. Statistical analysis

Descriptive statistics (average, range, and percentage difference) were determined using Microsoft Excel. For inferential statistics, one-way ANOVA ($p < 0.05$) with multiple comparisons and independent t-tests were performed using GraphPad Prism 8. Microsoft Excel was used to calculate correlation co-efficient between variables.

## 6.4 Results

The analysis of different chemicals and nutritional components of the marula juice and wine is presented graphically in this chapter.

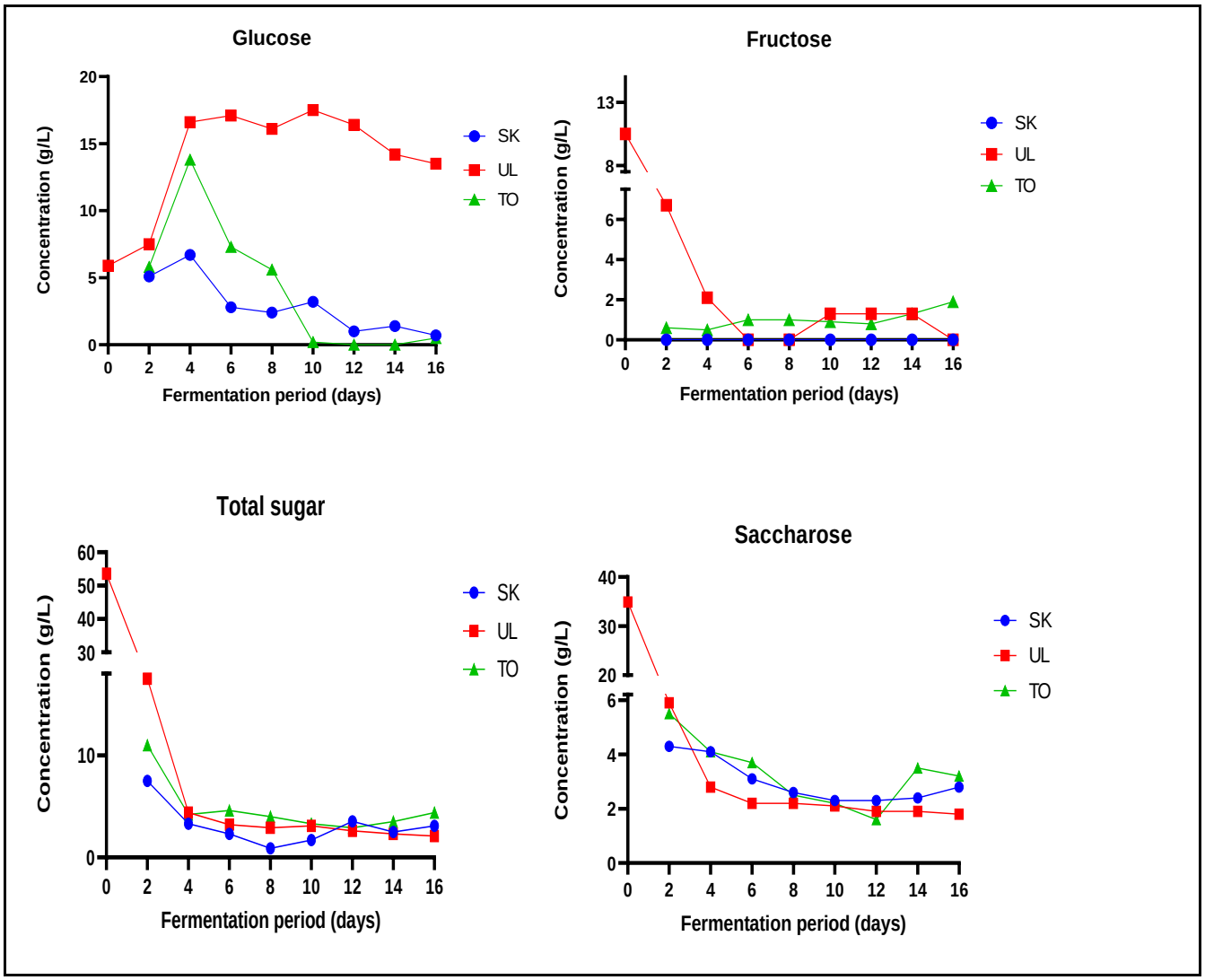


**Figure 6.1**: Sugar levels in the University of Limpopo juice (UL day 0), the Sekhukhune (SK), University of Limpopo (UL) and The Oaks fermenting wines.

The University of Limpopo fresh juice contained 53.6 g/L total sugar, of which fructose and glucose represented 10.5 g/L and 5.9 g/L respectively. The bulk of the sugar (34.9 g/L) was saccharose (Fig. 6.1). The glucose concentrations of the wines had opposing pattern. There was a 91% and 86% decrease in the concentration of glucose from day 2 to day 16 of fermentation in The Oaks and Sekhukhune wines while there was a 56% increase in the University wine over the same period. Sekhukhune wine had no fructose whereas its concentration was completely depleted in the University of Limpopo by day 16. There was a strong correlation between the concentrations of fructose and total sugar ($r^2 = 0.9$) as well as saccharose and total sugar ($r^2 = 0.99$) in the University of Limpopo wine. Positive correlations in the Sekhukhune and The Oaks wines were found between saccharose and total sugar concentrations as $r^2 = 0.49$ and $r^2 = 0.69$ respectively.

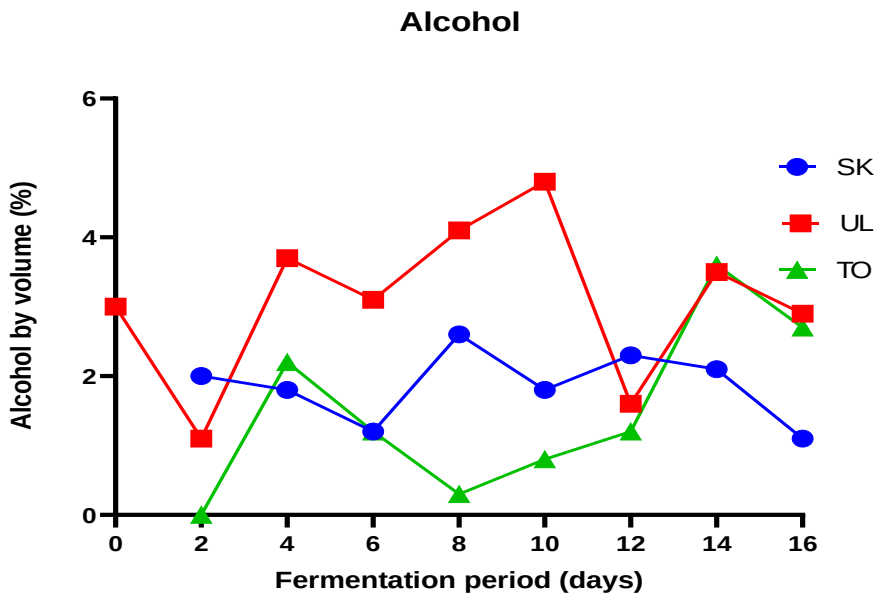


**Figure 6.2**: Alcohol levels in the University of Limpopo juice and the Sekhukhune (SK) and University of Limpopo (UL) and The Oaks fermenting wines.

The alcohol concentration of the wines peaked at different intervals of fermentation (Fig. 6.2). University of Limpopo and Sekhukhune wines obtained their highest concentrations of 4.40% and 2.60% on day 8 respectively while The Oaks wine had more alcohol, 3.60% on day 14. The overall alcohol content was less than 5%. The University of Limpopo had significantly more alcohol than the other two areas ($p = 0.005$) while the other two contained similar

concentrations, p = 0.806. The Oaks wine contained 0% alcohol on day 2 and it corresponded to the high total sugar. The higher alcohol content in the University of Limpopo wine correlates with the higher consumption of sugars as compared to the other two wines (Figs. 6.1 and 6.2).

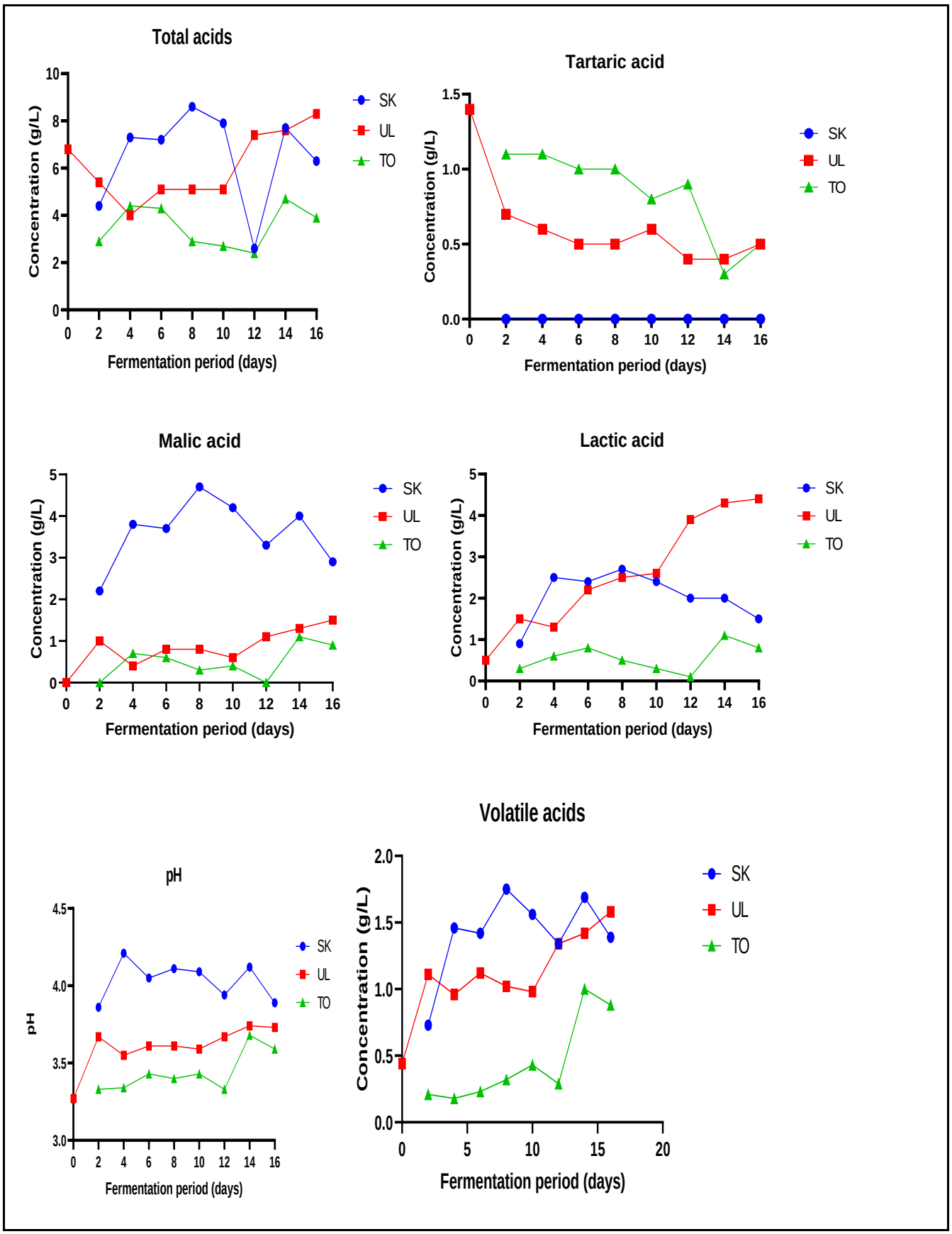


**Figure 6.3:** Acid levels in the University of Limpopo juice and the Sekhukhune (SK) and University of Limpopo (UL) and The Oaks fermenting wines.

The marula juice UL day 0 (Figure 6.3) had tartaric and citric acids which represented 87.8% of the total acidity. In general, the wines increased in acidity from day 2 to day 16 of fermentation. The Sekhukhune wine showed higher total acidity with moderately higher levels of volatile acids, malic and lactic acids. This is contrary to the acid levels observed in The Oaks wine. Citric acid, which showed to decline as fermentation progressed in all the wines, had comparable levels ($p > 0.05$). Of interest were the steady increase in the amount of volatile acids which hovered below 0.5 g/L for The Oaks wine, and largely below and around 1.5 g/L for University of Limpopo and Sekhukhune wines respectively. Similarly, the total acidity, which represents organic acids, stabilised around 8 g/L for the Sekhukhune wine and was still gradually increasing to levels around 8 g/L for the University of Limpopo wine. Generally, the trends are similar for all the wines with the major difference being the amounts obtained for each wine. In addition, two of the three primary acids in wines such as tartaric acid and citric acid were present in the marula juice as well.

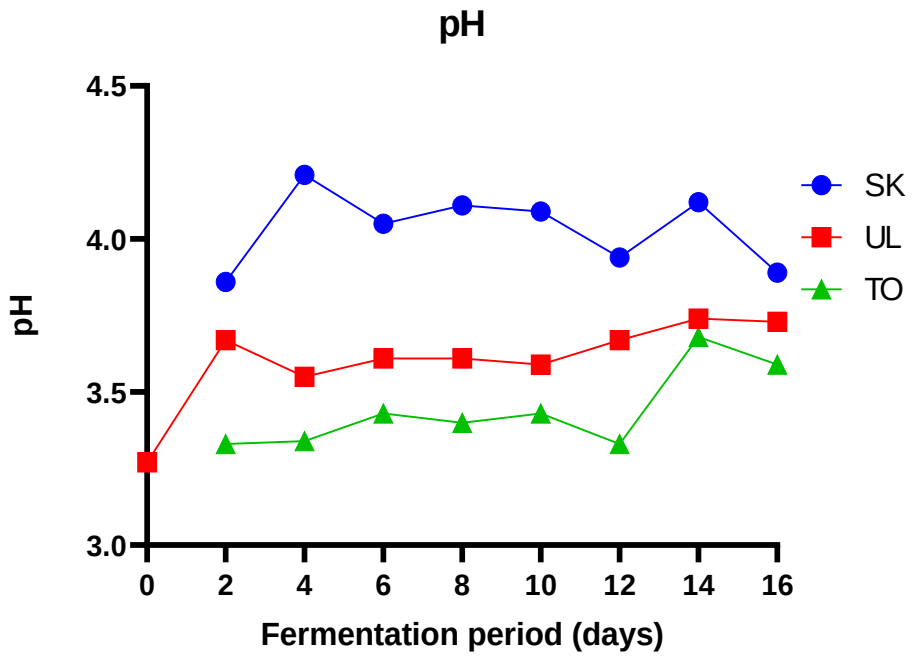


**Figure 6.4**: pH of the University of Limpopo juice and the Sekhukhune (SK) and University of Limpopo (UL) and The Oaks fermenting wines.

The pH of all the wines (Fig. 6.4) did not change drastically from day 2 to day 16 of fermentation. The patterns corresponded more to the changes in lactic acid concentrations in University of Limpopo and The Oaks wines, while the little to no presence of citric acid effected the significantly higher pH of Sekhukhune wine ($p < 0.000$). The high pH in Sekhukhune wine corroborated its low total acidity.

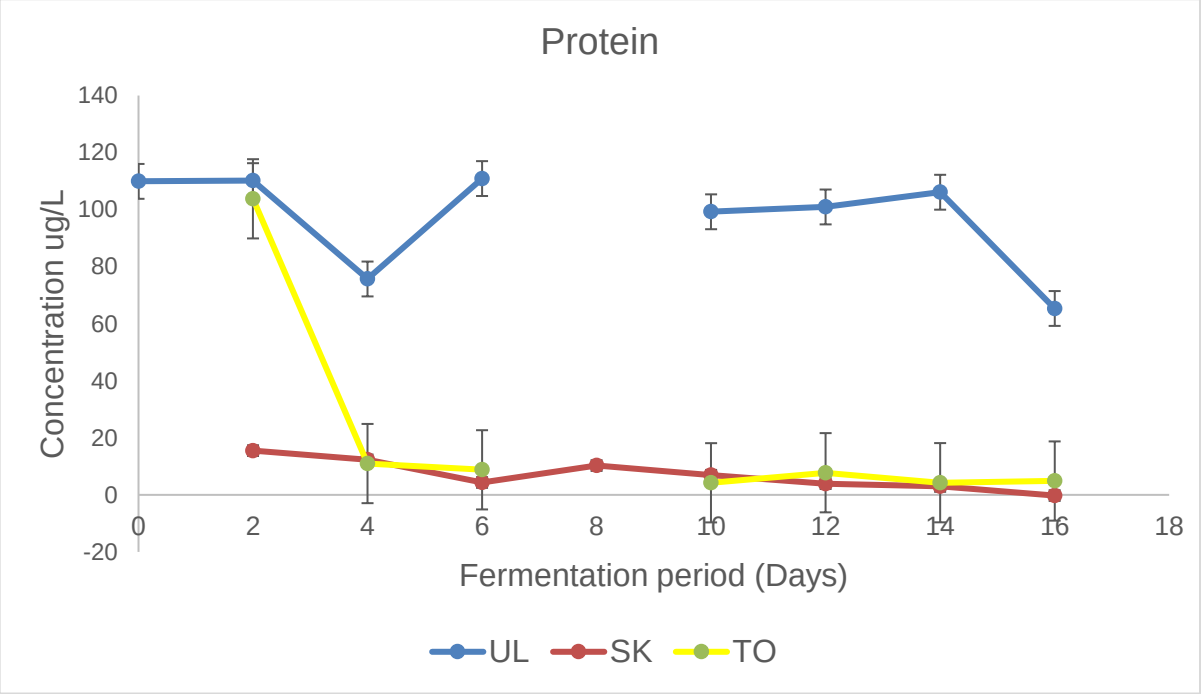


**Figure 6.5**: Protein content in the University of Limpopo juice and the Sekhukhune (SK) and University of Limpopo (UL) and The Oaks fermenting wines.

The University of Limpopo wine contained significantly more protein (Fig. 6.5) than Sekhukhune and The Oaks ($p = 0.000$). The protein content of The Oaks and Sekhukhune were comparable ($p = 0.308$). The protein content of The Oaks and Sekhukhune wines remained relatively low from day 2 to 16. Generally, the protein content decreased significantly in all the wines from day 2 to day 16, $p < 0.05$.

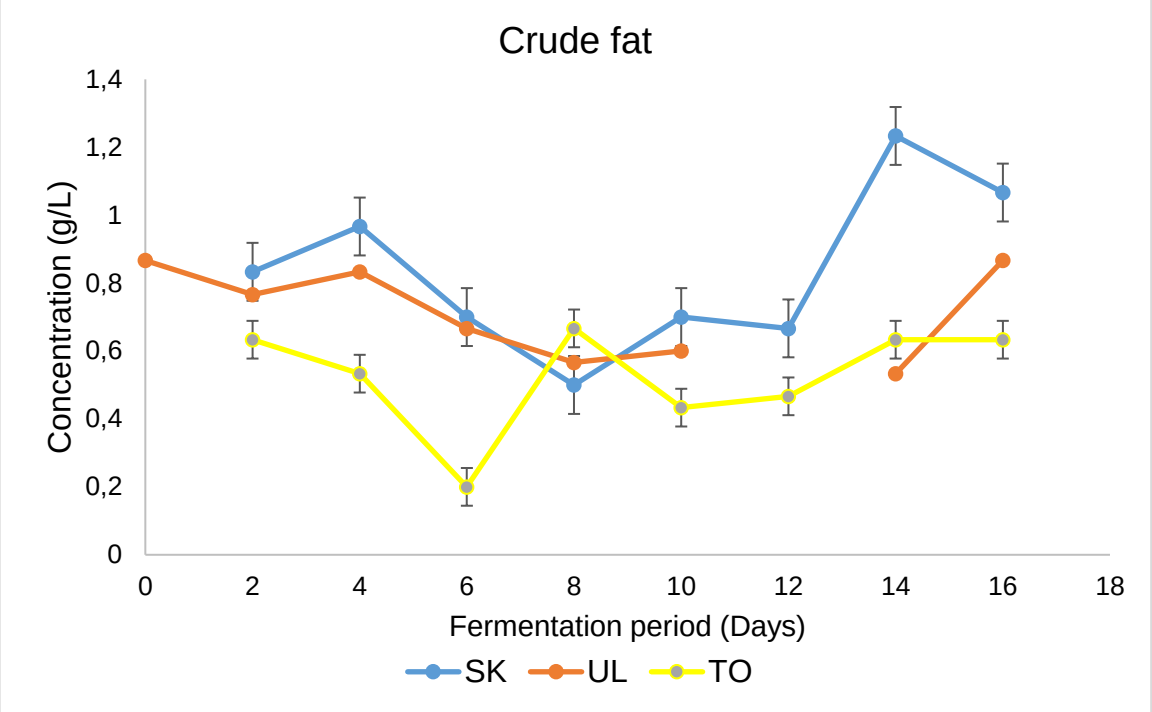


**Figure 6.6**: Crude fat content in the University of Limpopo juice and the Sekhukhune (SK) and University of Limpopo (UL) and The Oaks fermenting wines.

Crude fat content increased by 22% in the Sekhukhune wine between day 2 and day 16 of fermentation while no differences were recorded by The Oaks and University of Limpopo on the same intervals (Fig. 6.6). An 11% decrease in the crude fat was observed from the fresh undiluted juice to the day 2 wine of University of Limpopo. Sekhukhune had significantly higher crude fat content than The Oaks ($p = 0.021$) however, no significant difference was observed compared to the University of Limpopo, p figure= 0.834. Nevertheless, the amount of crude fat in the fermenting marula wines was low throughout.

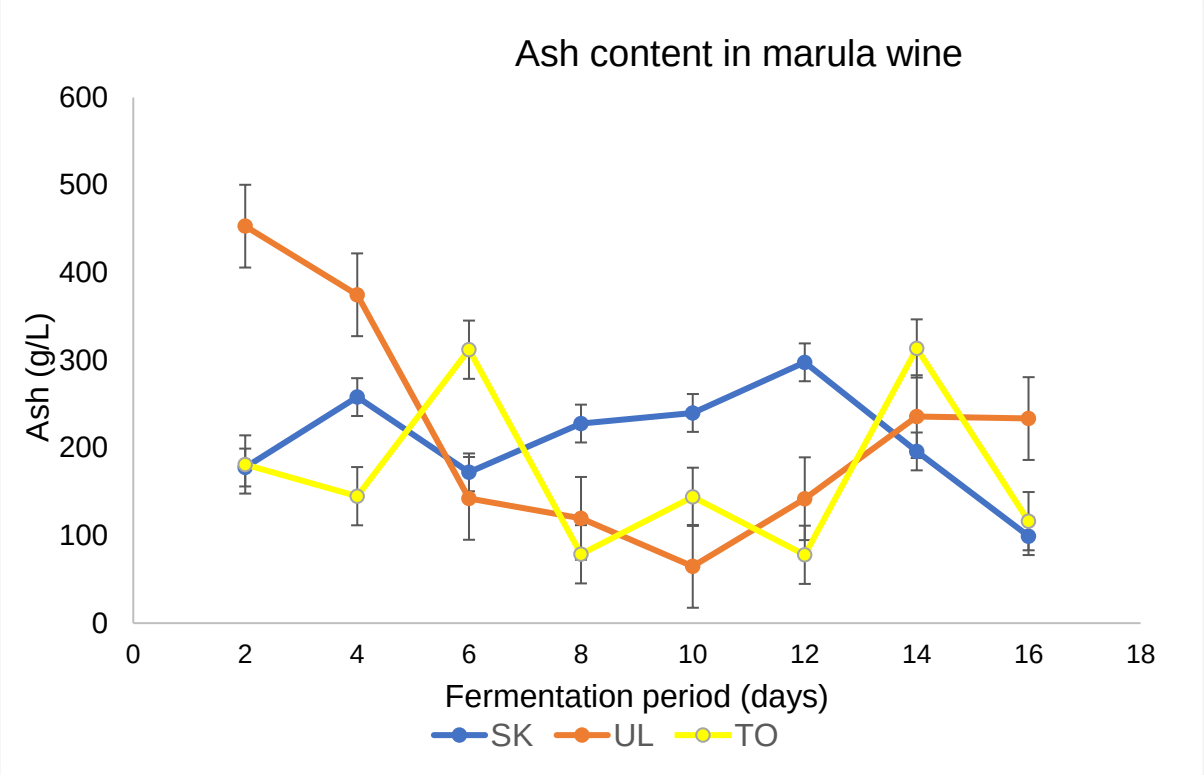


**Figure 6.7**: Ash content in the Sekhukhune (SK), University of Limpopo (UL) and The Oaks fermenting wines.

Contrasting patterns were observed for ash content during the fermentation of the wines from the three areas. The total mineral content in University of Limpopo and The Oaks wines started high at 453.1 g/L and 181.1 g/L and decreased to 142 g/L and 78 g/L on day 12 respectively while SK wine started low at 177.6 g/L and gradually increased to 235.7 g/L at day 12. Generally, the total mineral content was gradually decreasing as fermentation progressed, although the slight drop from the start to the end of fermentation study period was non-significant ($p > 0.05$).

**Table 6.1**: Mineral composition in the Sekhukhune, The Oaks and University of Limpopo wines.

| Sample Name | As mg/L | Ca mg/L | Cr mg/L | Fe mg/L | Mg mg/L | Na mg/L | P mg/L | Pb mg/L | Zn mg/L | Cu mg/L | K mg/L |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SK DAY 2 | 5.8 | 14.7 | 0,35 | 0.52 | 25.5 | 11.2 | 17.9 | 1.26 | 0.15 | 0.38 | 96 |
| SK DAY 4 | 4.12 | 12.5 | -0,01 | 0.17 | 22.2 | 10.2 | 10.4 | 0.75 | 0.42 | 0.16 | 14.1 |
| SK DAY 6 | 4.82 | 13.7 | 0.19 | 1.18 | 24.3 | 10.7 | 13.7 | 1.11 | 0.25 | 0.33 | 98.3 |
| SK DAY 8 | 4.51 | 14.4 | 0.2 | 0.3 | 25.2 | 9.82 | 13.3 | 1.17 | 0.39 | 0.22 | 99.1 |
| SK DAY 10 | 4.72 | 11.4 | 0.18 | 0.32 | 21.8 | 9.12 | 15.3 | 1.04 | 0.16 | 0.23 | 105 |
| SK DAY 12 | 3.81 | 10.8 | -0.01 | 0.32 | 21.4 | 8.56 | 11.5 | 0.83 | 0.45 | 0.28 | 86.7 |
| SK DAY 14 | 2.61 | 10.1 | -0.18 | 0.17 | 18.3 | 8.02 | 9.26 | 0.65 | 0.55 | 0.15 | 29.7 |
| SK DAY 16 | 4.03 | 10.3 | -0.11 | 0.15 | 17.8 | 8.68 | 10.9 | 0.76 | 0.54 | 0.19 | 54.4 |
| **Average** | **4.30** | **12.24** | **0.07** | **0.39** | **22.06** | **9.54** | **12.78** | **0.95** | **0.36** | **0.24** | **72.91** |
| TO DAY 2 | 1.62 | 6.6 | -0.64 | -0.19 | 9.77 | 4.84 | 3.74 | 0.11 | 0.62 | 0.18 | 35.9 |
| TO DAY 4 | 1,58 | 5.85 | -0.47 | -0.35 | 8.34 | 6.38 | 3.49 | 0.05 | 0.62 | 0.17 | 47.2 |
| TO DAY 6 | 2.82 | 7.37 | -0.29 | 0.41 | 9.42 | 7.09 | 4.49 | 0.33 | 0.41 | 0.46 | 41.1 |
| TO DAY 8 | 0.87 | 4.8 | -0.75 | -0.51 | 6.53 | 4.68 | 2.66 | 0.17 | 0.84 | 0.3 | 42.3 |
| TO DAY 10 | 2.3 | 6.15 | -0.47 | -0.48 | 8.39 | 7.24 | 4.46 | 0.28 | 0.66 | 0.15 | 0.6 |
| TO DAY 12 | 0.92 | 4.32 | -0.69 | 0.08 | 5.55 | 5 | 2.64 | 0.06 | 0.68 | 0.21 | 87.6 |
| TO DAY 14 | 1.39 | 7.24 | -0.58 | -0.35 | 10.6 | 6.72 | 3.97 | 0.28 | 0.58 | 0.18 | 43.2 |
| TO DAY 16 | 1.42 | 4.86 | -0.56 | -0.35 | 7.13 | 4.15 | 4.39 | 0.15 | 0.63 | 0.18 | 53.5 |
| **Average** | **1.61** | **5.90** | **-0.56** | **-0.23** | **8.22** | **5.76** | **3.73** | **0.18** | **0.63** | **0.23** | **43.93** |
| UL DAY 2 | 2.37 | 11.4 | -0.33 | 0.30 | 13.5 | 7.09 | 5.65 | 0.54 | 2.57 | 0.17 | 32.8 |
| UL DAY 4 | 2.11 | 11.4 | -0.33 | 0.26 | 12.5 | 7.87 | 8.76 | 0.30 | 2.24 | 0.12 | 8.66 |
| UL DAY 6 | 1.4 | 9.52 | -0.39 | 0.51 | 11.5 | 6.02 | 6.04 | 0.22 | 1.92 | 0.19 | 32 |
| UL DAY 8 | 2.64 | 10.1 | -0.27 | 0.49 | 12.1 | 7.56 | 6.31 | 0.74 | 2.36 | 0.15 | 35.4 |
| UL DAY 10 | 2.15 | 7.47 | -0.31 | 1.62 | 10.7 | 6.74 | 7.23 | 0.15 | 1.7 | 0.18 | 71 |
| UL DAY 12 | 2,41 | 12 | -0.28 | 0.13 | 14.1 | 6.71 | 6.96 | 0.50 | 2.56 | 0.23 | 55.1 |
| UL DAY 14 | 1.99 | 8.26 | -0.44 | 0.09 | 10.5 | 6.39 | 6.43 | 0.27 | 1.7 | 0.22 | 52.1 |
| UL DAY 16 | 2.37 | 9.88 | -0.31 | -0.14 | 11.9 | 6.99 | 6.53 | 0.54 | 2.16 | 0.38 | 50.5 |
| **Average** | **2.18** | **10** | **-0.33** | **0.41** | **12.1** | **6.92** | **6.74** | **0.41** | **2.15** | **0.21** | **42.19** |

Key: SK- Sekhukhune; TO- The Oaks; UL- University of Limpopo

Calcium, magnesium, sodium, phosphorus and potassium were the most abundant of the selected minerals analysed in all the three wines, with potassium and magnesium being present in higher amounts than the other minerals. In contrast, the amounts of chromium, lead, iron, zinc, and copper were low to undetectable in all the wines (Table 6.1). The Sekhukhune wine had relatively higher amounts of minerals when compared to The Oaks and University of Limpopo. The minerals in the Sekhukhune wine all started high from day 2 and decreased by day 16, and this is congruent with the trend observed in figure 6.7. The concentration of all the minerals fluctuated and differed significantly ($p < 0.05$) except that of

copper (p= 0.146) in The Oaks wine as well as arsenic (p= 0.773) and lead (p= 5) in the University of Limpopo wine. The University of Limpopo wine had significantly more phosphorus, copper, and potassium while The Oaks wine had more phosphorus, lead, zinc and potassium by the end of fermentation. Sekhukhune wine had significantly higher concentrations of arsenic, chromium, magnesium, sodium, phosphorus and lead, p< 0.05 whereas University of Limpopo wine had significantly higher concentrations of zinc. The concentrations of calcium and iron were comparable in wines from University of Limpopo and Sekhukhune, whereas lead, sodium, and arsenic had comparable concentrations in wines from The Oaks and University of Limpopo. There was no statistical difference in the concentrations of copper (p= 0.705) and potassium (0.057) in the wines from all the areas.

### 6.5 Discussion

Natural fermentation occurs spontaneously in fruits through the action of endogenous microorganisms found in the fruit skins, seeds and juice when exposed to conducive conditions. Yeasts metabolise readily available simple and energy-rich sugars such as glucose and fructose into alcohol, glycerol, and carbon dioxide (Saranraj et al., 2017). This process preserves most of the sugar's energy in a form of alcohol. In our study, marula juice was spontaneously fermented into wine over a 16-day period. Alcohol production was monitored against sugar consumption throughout fermentation. Fruit juices with low sugar content ferment and produce low alcohol beverages, although the type and performance of homofermentative yeasts present in the wine contribute (Mestre et al., 2019). In addition, high citric acid levels during fermentation can lead to a slower yeast growth rate (Nielsen and Arneborg, 2007) and hydrolysis of alcohol by bacterial fermentation that produces acetic acid and acetaldehyde (McGovern et al., 2004; Ghosh et al., 2012).

The yeast strains *Saccharomyces cerevisiae*, *Haeniaspora guilliermondii*, *Rhodotorula mucilaginosa*, *Issatchenkia terricola* have been reported to dominate the fermenting marula wine (Phiri, 2018). Most of these yeasts are non-*Saccharomyces* yeasts that typically produce less alcohol as they require higher contents of high-energy sugars to produce small amounts of ethanol (Gonzalez et al., 2013). Furthermore, the yeasts are fructophilic and as observed in this study, fructose was utilised faster and at smaller concentrations than glucose. However, higher alcohol production corresponded with the increase in glucose levels when *S. cerevisiae,* an efficient glucose consumer*,* dominated other yeasts. The lower fructose concentration and the proportional ratios of non-*Saccharomyces and S. cerevisiae* yeasts

(Phiri thesis 2018) contributed more to the low alcohol level of both the Sekhukhune and The Oaks wines.

Bacteria are responsible for most of the changes in total acidity in wines. The total acidity of the fruit refers largely to the concentration of malic, citric, and tartaric acids reported in grapes (Defilippi et al., 2009). The marula juice had malic and citric acids which represented 87.8% of the total acidity. In the wine, however, other organic acids such as acetic acid and lactic acid were synthesised through the action of lactic acid and acetic acid bacteria (Ghosh et al., 2012; Saranraj et al., 2017; Byrne and Howell, 2021). A cocktail of lactic acid and acetic acid bacteria, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Gluconobacter oxydans*, *Acetobacter aceti* and *Acetobacter pasteurianus* were previously isolated from marula wine throughout the fermentation period (Phiri, 2018; Maluleke, 2019). The Oaks and the University of Limpopo wines had higher levels of citric acid, much higher than 0.5-1 g/L reported for fermenting grape wine (Kalathenos et al*.,* 1995), and citric acid has been reported to slow down the growth rate of yeasts and thus delays alcoholic fermentation (Nielsen and Arneborg, 2007). Heterofermentative lactic acid bacteria such as *L. fermentum* and *L. plantarum* breakdown citric acid into lactic acid (Lonvaud-Funel 1999; Lefeber et al., 2011) and this conversion is evident in this study from the observed inverse relationship between these two acids. Regardless, the lactic acid concentrations matched the normal average of between 1 and 3 g/L reported in grape wine (Boulton et al., 1996) except in the latter stages of fermentation in the University of Limpopo wine. The volatile acidity observed in this study matches the amounts acceptable in white grape wine of 0.2 g/L or less and it is calculated as acetic acid (OIV, 2010). Higher levels of acetic acid produce off-flavours and gives off a vinegary aroma.

The criteria for analysing wine include residual sugar level, acidity and alcohol. The South African wine law mandates the labelling of wines to include among other things the alcohol content and sweet/dry descriptions (Sawis, 2021). The chemical profile reported here indicates that the total sugar, total acidity and alcohol content of marula wine after 16 days of fermentation averages around 3.2 g/L, 6.1 g/L and 2.2% respectively. This profile matches that of dry wine, whose residual sugar should be between 0 and 4 g/L (What is Residual Sugar in Wine? | Wine Folly, 2021) and is well within the regulation range of 4.0 – 8.0 g/L for total acidity (Rajković et al., 2007). With all the chemical parameters well within regulation, ultimately the real test of the quality of the wines requires a sensorial panel of experts and consumers.

The organic acids lactic acid, tartaric acids and malic acid do not only contribute to the organoleptic properties of the wine but also have reported antimicrobial activity particularly in low pH conditions (Ricke, 2003). Mugochi et al. (1999) reported the antimicrobial activity of marula wine against *Salmonells enteriditis*, *Shigella sonnei* and *Shigella flexneri*, which was linked to its high lactic acid content. The University of Limpopo wine had higher concentrations of lactic acid towards the end of fermentation than both Sekhukhune and The Oaks. This could further explain the higher antibacterial activity of this wine. Malic acid as well was reported to have antibacterial activity against *E. coli (*Raybaudi-Massilia et al., 2009). This could explain the antibacterial activity observed for Sekhukhune wine against *E. coli* possibly. Overall, the antimicrobial activity of wines has been attributed to the synergistic effect of organic acids, ethanol and low pH wherein the low pH was a major contributing factor (Moretro and Daeschel, 2004).

lipidMarula juice recorded 109.9 µg/L protein content which was much lower than the 3600 mg/100 g dry weight (Glew et al., 1997) reported in literature. Variations in protein content of grapes have been attributed to grape variety (Sarmento et al., 2001), the maturity of the grape berry at harvest, which is proportional to the protein content, region, climate conditions as well as soil type (Murphey et al., 1989). Factors such as region, climate conditions, soil and time that lapsed before analysis of samples have been noted for the differences in marula protein content (Hiwilepo van-Hal et al., 2013). A combination of these factors could explain the big difference in the protein content of marula. The range of proteins in the marula wines was 17.7 – 111 µg/L in University of Limpopo wine, 0.3 – 15.5 µg/L in Sekhukhune wine and 4.3 – 105 µg/L in The Oaks wine. Protein levels in grape wine usually range from 15 to 300 mg/L (Waters et al., 2005). The wines had very low concentrations of proteins by comparison. *Saccharomyces cerevisiae* can hydrolyse the proteins from the fruit on one side and similarly contribute to protein content of the wine through autolysis of *Saccharomyces cerevisiae* (Ferreira et al., 2002). The high TSS: Acid ratio, that signify better ripening of the fruits can contribute to the higher protein content in marula wine as observed with the University of Limpopo wine in chapter 3. This is probable since protein content in grape berries generally increases during ripening (Tian et al., 2019). A minimum of 0.80g protein per kg body weight per day is recommended to meet the functional needs of a human body such as promoting skeletal-muscle, protein accretion and physical strength (IOM, 2005). Although, the protein levels are low to provide the daily requirement, their presence in the wine provides good basis for its consumption.

Crude fat was extracted from the juice and wines with a chloroform and methanol mixture and using water to protect the lipids against oxidation. Marula fresh undiluted juice from University of Limpopo contained 0.87 g/L of crude fats which was higher than 13.5 g/100g dry weight found in the pulp by Glew et al. (1997). Total lipid concentration of 2.8 g/L was reported in Sauvignon Blanc grape juice of which the majority were complex lipids that *S. cerevisiae* cannot breakdown. The increase in crude fat content from day 2 of fermentation could be due to lipolytic bacteria such as *Lactobacillus plantarum* present in the marula wine (Kenneally et al., 1998; Phiri, 2018) or production by autolysis of yeasts that released fatty acids along with glycerol into the wines (Liu and Kokare, 2017; Claus and Mojsov, 2018). The glycerol is however not fermentable and is used by yeast to prevent dehydration (Aslankoohi et al., 2015). Increased lipolysis has been associated with formation of aroma compounds (Tumanov et al., 2018) which can improve the appeal of the wine and improve its consumption. The marula wine still contained high fat content by the end of fermentation indicating low fat utilisation.

Overall, all the marula wines contained a higher total mineral content than what is commonly found in wines in the range of 1- 5 g/L (Sáiz-Abajo et al., 2006; Steidl, 2010). The wines contained abundant concentrations of potassium, magnesium, calcium, and sodium which have already been previously reported as the dominant minerals in marula juice (Borochov-Neori et al., 2008). These minerals are part of macronutrients which are required in larger quantities in the human diet and were present in fluctuating concentrations most of which decreased from the start to the end of fermentation. The reduction of these minerals could have been due to crystallisation, precipitation, and utilisation by yeasts (Jedlička et al., 2014). Calcium enhances the taste and aroma of the wine whereas higher concentrations of magnesium may add bitterness to it, and potassium affects the acid content and the pH wine and softens its taste (Mlček et al., 2018). Generally, wines can contain 60 to 1000 mg/L of phosphorus, 160 – 2500 mg/L of potassium, 100 – 220 mg/L of calcium and 50 – 2000 mg/L of magnesium (Fic, 2015). The concentrations of these minerals in the marula wines were well below the range found in grape wine, with the Sekhukhune wine having the highest concentrations of all the macronutrients except potassium compared to wines from the other two areas. The wines also contained micronutrients such as chromium, lead, copper, iron, and zinc. These are required in smaller concentrations as most are toxic metals and can spoil the aroma, colour, and taste of the wine (Mlček et al., 2018). Lead for instance is a contaminant that could get into the wine from the soil due to emissions, agricultural chemicals,

and industrial pollution (Fic, 2015), whereas iron causes wine turbidity and browning (Jackson, 2008). The recommended lead content in wine is 0.15 mg/L and the iron content can range between 0.3 – 10 mg/L (Kraus, 1999; OIV, 2016). The lead content of marula wine was much higher than the recommended level and is probably due polluted air. The iron content was well within the common range. The differences in the mineral content from area to area could be attributed to environmental factors such as origin of tree, soil type, soil fertility and climatic conditions (Hiwilepo van-Hal et al., 2013).

### 6.6 Conclusion and recommendations

The nutritional profile of marula wine edged that of the fresh juice in the protein content, the higher energy in the form of alcohol and the accumulation of organic acids. The total fat content was similar in both the juice and the wine. The wines consumed more fructose than glucose and produced less than 5% of alcohol. However, the balance of sugar and alcohol make for acid in the University of Limpopo good tasting wine, especially with the higher lactic acid content. The wine is a source of important minerals for both the quality of the wine and the nutritional benefits. The protein content was lower than that of grape wine which in white wine helps decrease the chances of haze formation. Marula wine is a good way to preserve the nutritional properties of marula fruits which can be enjoyed when beyond the fruiting season.

The fat content however remained high at the end of fermentation. This indicated that the fermenting yeast struggled to utilise it. The high glucose content and low alcohol production point to fructophilic nature of the fermenting yeast and its low affinity for glucose. Research involving co-fermentation with isolated lipolytic microorganisms may investigate how the total fat and fatty acid contents are affected. The low affinity for glucose consumption shown by fermenting yeast should also be investigated and the outcome used to increase alcohol production.

The lead content of the wines was higher than the recommended level which could make the wines unsafe. Research into how it accumulates in the wine and how it can be removed or reduced is required.

## Chapter 7

### 7.1 General discussion and conclusion

South Africa is home to numerous fruit trees which have been identified, studied, and protected under the National Forest Act, 1998 (Act No. 84 of 1998). *Sclerocarya birrea* subsp. *caffra*, known as marula, is of keen interest in this study, mainly because it is an African indigenous wild tree and due to its socio-economic and cultural importance among African communities across the continent of Africa. Several products have been produced over the years from the different parts of the tree; however, the majority are still sold locally within African communities. These include jam, jelly, marula fruit beer (wine), sculptures and fruit rolls. A few have entered the mainstream market and these include products made from marula fruit oil such as Portia*M* products (*Home - Portia M Skin Solutions - Marula Oil For skin*, 2020). In addition, there are several products infused with marula in the continent which includes Marula fruit wine cocktail, Four Cousin Marula Dream Cream Liqueur, African Secret Marula Cream Liqueur, Nature Own Cape Marula, Cape Africa NV Marula Cream, Serengeti Cream Liqueur, Marula fruit, African Craft Premium Din-Marula, Flowstone Marula Gin. Of all the mentioned marula products, there is none which falls under the categories of wines and is produced solely from the marula fruit juice.

The traditional marula wine is of keen interest in this study due to socio-economic status and great economic potential of this beverage. A good market already exists for marula wine, especially in Africa among Africans, mainly due to the long-standing relationship and importance of the traditional brew in the African culture and traditional practices. There is a good prospect of developing the marula wine into a first African wine produced from indigenous fruits of Africa and this can contribute to the sustainable development goal on improving the socio-economic status of the African people. Production of the brew in traditional setting is marred by challenges such as quick spoilage and inconsistency of quality between batches and localities. Currently, the enterprise only thrives during the marula season. Advancing and exploring the commercialisation of marula wine would require that the wine complies with quality and information prescripts which are standards that the wine industry must meet in terms of the product quality and stability (Sawis, 2021). There is a dearth of knowledge on the chemical, nutritional and stability of marula wine. Anecdotal information exists on the chemical and nutrient contents, and bioactive components of the marula juice, whereas there is scanty information on the chemical properties that contribute to the organoleptic characteristics of the wine. Thus, there is a gap of knowledge on the

characterisation of the fruit, profiling of the wine parameters and a full understanding of the chemical dynamics that take place during the fermentation and storage phases in the production of the marula wine. This knowledge will guide the improvement of the shelf-life and enhancement of wine quality and stability during the production of a commercial marula wine. The phytochemical and nutritional composition of marula wine is sparsely documented. Furthermore, the dynamics that occur in their evolution during fermentation are unknown. This is primarily important because of the need to produce food and beverages that are nutrient rich and have fewer preservatives (Pfukwa et al., 2020). The current study sought to generate some of this missing knowledge.

The findings in this study have elucidated on the importance of combining physical and chemical attributes when selecting fruit to produce wine. Our findings showed that larger sized fruits do not translate into better juice yield nor better-quality fruits. Rather, the level of ripeness attained by the fruits in terms of chemical properties such as sugar content and acidity were in fact more important in determining the fruit quality. Notwithstanding the variations brought on by the different localities, determination of the sugar content to titratable percentage ratio of the juice gave a good measure of the level of ripeness of the marula fruit as it was shown that marula fruits with lower acid content attained better ripeness. The riper fruits also had better juice yield which could be attributed to the production of pectinase by *S. cerevisiae* in order that there may be more simple sugars in the fruits for its metabolism (Singh, 2018). More acidic fruits would not support the growth of these microorganisms and in turn the fruits would taste sour. Most elderly women who produce marula wine often combine visual assessment of the fruit for its peel colour with tasting, to determine how sweet the fruits are. Should a fruit turn out bad, they would complain about a sour taste which is attributable to acids such as citric acid found in the fruit juice.

By comparing the content of biologically active compounds of the wine to that of the juice, certain phenolic compounds found in the marula juice in this study increased albeit insignificantly in the fermenting wine. This phenomenon, facilitated by endogenous microorganisms, has been observed in fermentations of grape wine (Munoz-Bernal et al., 2020). The compounds isolated included gallic acid, taxifolin, vanillic acid, apigenin, kaempferol and quercetin, all with reported antioxidant and antibacterial activities (Panche et al., 2016; Yang et al., 2020). Beverages such as rooibos and black teas, and also grape wine have gained great market value due to the presence of these phenolic compounds. Their presence in the marula wine, not only at the beginning of fermentation but also beyond the

usual six to eight days during which the traditional wine is still palatable, indicates the potential of developing a wine with a longer shelf-life which would preserve and carry the health compounds beyond marula fruiting season. A positive correlation was also observed between the presence of phenolic compounds and both the antioxidant and antibacterial activities. This demonstrated further the pharmaceutical potential of the marula wine. In general, the antioxidant activity of our wines deteriorated at the later stages of fermentation while the antibacterial activity improved. In as far as health components are concerned, the wine would benefit the consumer regardless of the time of consumption. Moreover, the composition of organic acids, pH and ethanol could exert a synergistic and protective role to the consumer against foodborne pathogens. The wines however had lower content of protein at the latter stages of fermentation, which was much lower than the recommended daily intake of 0.80 g/kg and the concentrations in grape wine (Waters et al., 2005; IOM, 2005). Calcium, magnesium, sodium, phosphorus, and potassium were dominant in the marula wines. These too were lower in their concentration compared to grape wine (Fic, 2015). However, these minerals are important to human health and body functions such that a natural resource such as the wine can only benefit its consumers.

The variations observed in the compositions of nutrients and bioactive compounds from one area to another which could arise from various factors, although these could not be determined through our investigation. There appears to be a link between fruit quality with regards to the nutrient content in the juice, which in turn determines the quality of wine. The lower the nutrient supply to microorganisms in the fruit, the lesser their activity which as far as our findings show directly impacts on the production of phenolic compounds and proteins. Thus, the nutrient and bioactive composition of marula wine can be optimised through better selection of fruits. Although grape wine contains most of the compounds tested for in this study, it is important to note that marula wine has not been as widely studied. Furthermore, grape wine fermentation has been optimised to produce wines with the desired chemical, sensorial and nutrient profile. The grapes are also cultivated in vineyards where the optimum conditions for high quality fruits are controlled and maintained. At this stage marula wines are produced without any control. The presence alone of bioactive compounds and nutritional properties in the wine points to the natural potential of the fruits and endogenous microorganisms. To produce a competent nutraceutical, standard fruit quality parameters that will inform the selection of fruits, the addition of a sugar into the juice as a nutrient source for

the microorganisms and the use of a combination of microorganisms as inoculum should be explored.

This study has provided base information about the important health related compounds in the marula wine. This comes at a time when the demand for natural sources of nutritional and therapeutic foods and drinks have skyrocketed. As most of the beverages already on the market may be highly priced and inaccessible to people in impoverished areas, the wine could serve as a readily available alternative. Furthermore, the popularity of marula wine has greatly increased with the inception of the annual marula festival, a platform where the wine and its heritage are celebrated. The results of this study can further expand its market by providing a scientific background behind the traditional and experiential knowledge passed on through generations. The claimed healing properties of the marula wine could very well be attributed to the presence of the bioactive compounds and synergistic action of chemicals reported here.

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# Appendices

## Appendix 1

WELCH ANOVA

Fruit mass (g)

| | Statistic[a] | df1 | df2 | Sig. |
|---|---|---|---|---|
| Welch | 100.849 | 2 | 76.279 | .000 |
| Brown-Forsythe | 84.302 | 2 | 102.716 | .000 |

## Appendix 1a

Multiple Comparisons

Dependent Variable: Fruit mass (g)

Games-Howell

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Mass | (J) Mass | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| TO | SK | 11.53925* | .91774 | .000 | 9.3464 | 13.7321 |
| | UL | 12.44525* | 1.12616 | .000 | 9.7473 | 15.1432 |
| SK | TO | -11.53925* | .91774 | .000 | -13.7321 | -9.3464 |
| | UL | .90600 | 1.14717 | .711 | -1.8405 | 3.6525 |
| UL | TO | -12.44525* | 1.12616 | .000 | -15.1432 | -9.7473 |
| | SK | -.90600 | 1.14717 | .711 | -3.6525 | 1.8405 |

*. The mean difference is significant at the 0.05 level.

## Appendix 2

ANOVA

Fruit length (cm)

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3.928 | 2 | 1.964 | 32.067 | .000 |
| Within Groups | 5.328 | 87 | .061 | | |
| Total | 9.256 | 89 | | | |

## Appendix 2a

Multiple Comparisons

Dependent Variable: Fruit length (cm)

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Size | (J) Size | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| TO | SK | .20900* | .06390 | .004 | .0566 | .3614 |
| | UL | .50900* | .06390 | .000 | .3566 | .6614 |
| SK | TO | -.20900* | .06390 | .004 | -.3614 | -.0566 |
| | UL | .30000* | .06390 | .000 | .1476 | .4524 |
| UL | TO | -.50900* | .06390 | .000 | -.6614 | -.3566 |
| | SK | -.30000* | .06390 | .000 | -.4524 | -.1476 |

*. The mean difference is significant at the 0.05 level.

## Appendix 3

ANOVA

Fruit width (cm)

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 5.464 | 2 | 2.732 | 43.777 | .000 |
| Within Groups | 5.430 | 87 | .062 | | |
| Total | 10.894 | 89 | | | |

## Appendix 3a

Multiple Comparisons

Dependent Variable: Fruit width (cm)

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Size | (J) Size | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| TO | SK | .40633* | .06450 | .000 | .2525 | .5601 |
| | UL | .58967* | .06450 | .000 | .4359 | .7435 |
| SK | TO | -.40633* | .06450 | .000 | -.5601 | -.2525 |
| | UL | .18333* | .06450 | .015 | .0295 | .3371 |
| UL | TO | -.58967* | .06450 | .000 | -.7435 | -.4359 |
| | SK | -.18333* | .06450 | .015 | -.3371 | -.0295 |

*. The mean difference is significant at the 0.05 level.

## Appendix 4

WELCH ANOVA

Fruit peel (g)

| | Statistic[a] | df1 | df2 | Sig. |
|---|---|---|---|---|
| Welch | 105.601 | 2 | 77.155 | .000 |
| Brown-Forsythe | 95.885 | 2 | 110.806 | .000 |

a. Asymptotically F distributed.

## Appendix 4a

Multiple Comparisons

Dependent Variable: Fruit peels (g)

Games-Howell

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Mass | (J) Mass | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| TO | SK | 5.00825* | .36659 | .000 | 4.1323 | 5.8842 |
| | UL | 4.58325* | .42217 | .000 | 3.5738 | 5.5927 |
| SK | TO | -5.00825* | .36659 | .000 | -5.8842 | -4.1323 |
| | UL | -.42500 | .41223 | .560 | -1.4112 | .5612 |
| UL | TO | -4.58325* | .42217 | .000 | -5.5927 | -3.5738 |
| | SK | .42500 | .41223 | .560 | -.5612 | 1.4112 |

## Appendix 5

ANOVA

Fruit kernel (g)

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 495.888 | 2 | 247.944 | 71.888 | .000 |
| Within Groups | 403.536 | 117 | 3.449 | | |
| Total | 899.424 | 119 | | | |

## Appendix 5a

Multiple Comparisons

Dependent Variable: Fruit kernel (g)

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Mass | (J) Mass | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| TO | SK | 3.76575* | .41527 | .000 | 2.7799 | 4.7516 |
| | UL | 4.70425* | .41527 | .000 | 3.7184 | 5.6901 |
| SK | TO | -3.76575* | .41527 | .000 | -4.7516 | -2.7799 |
| | UL | .93850 | .41527 | .066 | -.0473 | 1.9243 |
| UL | TO | -4.70425* | .41527 | .000 | -5.6901 | -3.7184 |
| | SK | -.93850 | .41527 | .066 | -1.9243 | .0473 |

*. The mean difference is significant at the 0.05 level.

## Appendix 6

ANOVA

Fruit juice (g)

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 573.946 | 2 | 286.973 | 60.153 | .000 |
| Within Groups | 558.171 | 117 | 4.771 | | |
| Total | 1132.118 | 119 | | | |

## Appendix 6a

Multiple Comparisons

Dependent Variable: Fruit juice (g)

Tukey HSD

| | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| (I) Mass | (J) Mass | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| TO | SK | 2.06500* | .48840 | .000 | .9056 | 3.2244 |
| | UL | 5.31325* | .48840 | .000 | 4.1538 | 6.4727 |
| SK | TO | -2.06500* | .48840 | .000 | -3.2244 | -.9056 |
| | UL | 3.24825* | .48840 | .000 | 2.0888 | 4.4077 |
| UL | TO | -5.31325* | .48840 | .000 | -6.4727 | -4.1538 |
| | SK | -3.24825* | .48840 | .000 | -4.4077 | -2.0888 |

*. The mean difference is significant at the 0.05 level.

## Appendix 7

WELCH ANOVA

Fruit pH

| | Statistic[a] | df1 | df2 | Sig. |
|---|---|---|---|---|
| Welch | 179.745 | 2 | 72.757 | .000 |
| Brown-Forsythe | 88.850 | 2 | 74.821 | .000 |

a. Asymptotically F distributed.

Dependent Variable: Fruit pH

Games-Howell

| (I) Measurement | (J) Measurement | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| TO | SK | -.85750* | .04620 | .000 | -.9680 | -.7470 |
| | UL | -.65650* | .07432 | .000 | -.8357 | -.4773 |
| SK | TO | .85750* | .04620 | .000 | .7470 | .9680 |
| | UL | .20100* | .07696 | .030 | .0160 | .3860 |
| UL | TO | .65650* | .07432 | .000 | .4773 | .8357 |
| | SK | -.20100* | .07696 | .030 | -.3860 | -.0160 |

*. The mean difference is significant at the 0.05 level.

## Appendix 8

WELCH ANOVA

Titratable acidity

| | Statistic[a] | df1 | df2 | Sig. |
|---|---|---|---|---|
| Welch | 111.671 | 2 | 64.576 | .000 |
| Brown-Forsythe | 155.457 | 2 | 73.230 | .000 |

a. Asymptotically F distributed.

## Appendix 8a

Multiple Comparisons

Dependent Variable: Titratable acidity

Games-Howell

| (I) Measurement | (J) Measurement | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| TO | SK | .67775* | .05488 | .000 | .5461 | .8094 |
| | UL | .73075* | .04865 | .000 | .6129 | .8486 |
| SK | TO | -.67775* | .05488 | .000 | -.8094 | -.5461 |
| | UL | .05300 | .03208 | .233 | -.0242 | .1302 |
| UL | TO | -.73075* | .04865 | .000 | -.8486 | -.6129 |
| | SK | -.05300 | .03208 | .233 | -.1302 | .0242 |

*. The mean difference is significant at the 0.05 level.

## Appendix 9

**Fruit TSS**

| ANOVA summary | |
|---|---|
| F | 3,102 |
| P value | 0,0487 |
| P value summary | * |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0,05036 |

## Appendix 9a

| Tukey's multiple comparisons test | Mean Diff, | 95,00% CI of diff, | Below threshold? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| TO TSS vs. SK TSS | 0,3450 | -0,4050 to 1,095 | No | ns | 0,5209 |
| TO TSS vs. UL TSS | -0,4400 | -1,190 to 0,3100 | No | ns | 0,3480 |
| SK TSS vs. UL TSS | -0,7850 | -1,535 to -0,03505 | Yes | * | 0,0379 |

## Appendix 10

Independent Samples Test Maximum temperature

| | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 95% Confidence Interval of the Difference | |
| | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Equal variances assumed | .703 | .404 | 5.777 | 116 | .000 | 3.34576 | .57918 | 2.19863 | 4.49290 |
| Equal variances not assumed | | | 5.777 | 112.305 | .000 | 3.34576 | .57918 | 2.19823 | 4.49329 |