POLYPHENOLS, ASCORBATE AND ANTIOXIDANT CAPACITY OF THE KEI-APPLE (*Dovyalis caffra*)

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To God who is over all and through all and in all. Your grace was sufficient even for this...

To Dr. Du Toit Loots, my promoter and valued contributor. Your generous input, guidance and the many hours you spent on this project means a lot to me. Thank you for freely sharing your vast store of knowledge so patiently with me.

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SUMMARY

Motivation

There is a close relationship between the susceptibility to disease and nutritional state, in the sense that an adequate diet enhances resistance to disease. There is an increasing interest in this beneficial relationship among scientists, food manufacturers and consumers. The trend is moving towards functional foods and their specific health benefits.

The results of numerous epidemiological studies and recent clinical trails provide consistent evidence that diets rich in fruits and vegetables can reduce the risk of chronic diseases. These protective effects are mediated through multiple groups of beneficial nutrients contained in the fruits and vegetables, one of these being polyphenol antioxidants. The intake of the polyphenols plays an important role in the reduction and prevention of coronary heart disease (CHD), cardiovascular disease and cancer, as a consequence of their associated antioxidant properties.

Fruits contain an array of polyphenols with antioxidant capacity. Polyphenols may be classified in two broad groups namely: flavonoids and non-flavonoids. Flavonoid subgroups in fruits are further grouped as catechins, anthocyanins, procyanidins and flavonol among others. Phenolic acids occur as hydroxylated derivatives of benzoic acid and cinnamic acid, and are classified as non-flavonoids. Polyphenols have redox properties allowing them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, and thus contribute to the antioxidant capacity of fruits and vegetables. Because of the numerous beneficial effects attributed to these antioxidants, there is renewed interest in finding vegetal species with high phenolic content and relevant biological activities.

In view of the importance of these substances towards health and food chemistry, this study will focus on the polyphenol and Vitamin C characterisation and quantification of an indigenous South African fruit, the Kei-apple *(Dovyalis caffra)*, thought to have antioxidant properties. Due to the fact that polyphenol content influences the colour, taste and possible health benefits of the fruit / processed food product, this study will supply valuable information to industry in choosing the best fruit processing methods to attain the desired end product. The exploitation of indigenous South African fruits (Marula and Kei-apple) is receiving increasing prominence, not only due to their health benefits, but also the opportunities these present to rural based economics. Furthermore, this research will serve as a platform for further research on the Kei-apple and other indigenous South African fruits with possible health benefits.

Aims

The overall aim of this study is the quantification and characterisation of various nutritionally important antioxidants (polyphenols and ascorbate) in the Kei-apple fruit in its entirety, as well as in its individual fruit components (peel, flesh and seeds). In addition, the total antioxidant capacity of the entire fruit and the various fruit components will be determined in the unfractionated and fractionated fruit extracts. Gas chromatography coupled mass spectrometry (GC-MS) characterisation of the individual polyphenol components will also be analyzed in order to speculate on possible specific health benefits which the Kei-apple may posses.

Methods

The study was designed to ensure that a representative fruit sample was collected. Approximately 100 kg Kei-apples were picked in the month of November 2004 from the Bloemhof area in South Africa. A sample of 50 fruits was rinsed and separated into the various components (peel, flesh and seeds). An additional 50 fruits were randomly selected, cleaned and used in their entirety for data representative of the entire fruit. The sample extracts were

prepared, after being grounded and lyophilized, by a method described by Kähkönen *et al.* (1999) using 70% aqueous acetone. The C_{18}-fractionation on the fruit and separated fruit components resulted in four fractions containing (1) phenolic acids; (2) procyanidins, catechins and anthocyanin monomers; (3) flavonols and (4) anthocyanin polymers.

The total polyphenol content of the fruit and fruit components as well as the above mentioned C_{18}-fractions were determined by Folin-Ciocalteu's method (Singleton & Rossi, 1965). Both free and total ascorbate concentrations in these samples were determined as described by Beutler (1984), in addition to total sugar content of these via standard methods. Apart from their nutritional interest, both these measurements are necessary for the correction of the total polyphenol concentrations. The total antioxidant capacity of the entire fruit and various fruit components was determined by measuring the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) of the unfractionated and fractionated extracts. Using GC-MS analysis, the various individual polyhenol compounds contributing to the total polyphenol content of the Kei-apple was separated, identified and quantified.

This quantitative data was captured and statistically analysed. The analysis of variation was performed using the Tukey Honest Significant Difference test for *post-hoc* comparison. ORAC, FRAP and polyphenol Pearson correlation analyses were performed using Statistica (Statsoft Inc., Tulsa, Oklahoma, USA) with significance set at $P \leq 0.05$.

Results and discussion

This study determined the presence of various nutritionally important antioxidants (polyphenols and ascorbate), the total antioxidant capacity in the entire fruit as well as in the individual fruit components (peel, flesh and seeds) and their polyphenol sub group fractions.

Total phenol content: The Kei-apple, in its entirety, has a polyphenol concentration of 943 ± 20.3 mg GAE/100g dry weight. Comparison of the individual fruit components showed the seeds to have the highest total polyphenol concentration with 1990 ± 31.3 mg GAE/100g dry weight, followed by that of the peel, 1126 ± 45.8 mg GAE/100g dry weight and then that of the flesh, 521 ± 1.01 mg GAE/100g dry weight.

Total, L-ascorbic (ASC) and L-dehydroascobic (DHA) concentration: The total ascorbate of Kei-apple fruit is 517 ± 0.92 mg/100g dry weight. In contrast to the polyphenol content, the flesh of the Kei-apple had significantly the highest concentration of total ascorbate 778 ± 1.20 mg/100g dry weight, L-ascorbic 241 ± 21.0 mg/100g dry weight, as well as L-dehydroascobic 537 ± 22.2 mg/100g dry weight. The ratio of L-ascorbic acid/total ascorbate for the flesh, entire fruit, peel and seed is 0.31, 0.43, 0.49, 0.95, respectively, indicating the seeds are the most stable source of biologically active Vitamin C, with 95% of the total ascorbate occurring as L-ascorbate. This is also in line with the total polyphenol content of these components, confirming a polyphenol sparing effect on ascorbate.

C_{18}-fractionation extracts: Solid phase (C_{18}) fractionation of the Kei-apple fruit and fruit components showed that the fruit, peels and seeds consist predominantly of phenolic acids, followed by procyanidin, catechin and anthocyanin monomers and thereafter varying amounts of anthocyanin polymers and flavonols.

Antioxidant capacity: The antioxidant capacity of the entire fruit and individual fruit components as determined by ORAC, (r=0.76) and FRAP, (r=0.95) significantly correlated with the total polyphenol content, as well as to each other (r=0.88), indicating both to be good predictors of antioxidant capacity.

GC-MS polyphenol characterisation of the Kei-apple: Caffeic acid and hydro-*p*-coumaric acid were seen to be the phenolic acids occurring in the highest concentrations in the Kei-apple fruit. The majority of these are concentrated in the flesh and in the case of caffeic acid, also in the peel. The order of predominance of other major non-flavonoid components in the whole fruit analysis are *m*-hydroxybenzoic acid > *p*-hydroxyphenyl acetic acid > 3-methoxy-4-hydroxyphenylpropionic acid > *p*-coumaric acid. The peel of the Kei-apple, apart from caffeic acid, has exceptionally high concentrations of ferulic acid and also serves as a source of protocatechuic acid. Syringic acid was most prominent in the seeds. Although the total flavonoid concentration in the Kei-apple was low, taxifolin and catechin were identified and the seeds almost entirely accounting for these.

Conclusion

From this study it was concluded the Kei-apple is a rich source of antioxidant compounds (polyphenols and ascorbate), with a strong antioxidant capacity, and hence may be associated with health promotion properties, particularly in the prevention of cancer, cardiovascular disease, and neurodegeneration. Additionally, due to the increased scientific and commercial interest in this fruit, it is essential to take into consideration the various factors (agronomic, genomic, pre- and post harvest condition and processing) and tissues. This might affect the chemical composition of the final marketed product, which may play a significant role in determining the polyphenol and ascorbate composition and bioactivity of these compounds during food processing procedures. Hence, the polyphenol composition of the various fruit components should be taken into consideration when selecting a method of fruit processing into the desired end product.

Key words

Kei-apple; polyphenols; ascorbate; antioxidant capacity; gas chromatography mass-spectrometry.

OPSOMMING

Motivering

Daar is 'n noue verwantskap tussen die vatbaarheid vir infeksies en voedingstatus, aangesien 'n gebalanseerde dieet en goeie algemene toestand weerstandbiedendheid teen infeksies verhoog. Hierdie voordelige eienskappe wek toenemende belangstelling onder wetenskaplikes, voedselvervaardigers en verbruikers, omdat die mark na funksionele voedsel en hulle spesifieke gesondheidsvoordele neig.

Die resultate van talle epidemiologiese studies en kliniese toetse toon dat 'n dieet wat ryk is aan vrugte en groente, die risiko van chroniese siektes kan verlaag. Die beskermende effek word gereguleer deur verskeie voordelige chemiese stowwe wat in vrugte en groente voorkom. Een van hierdie groepe verbindings is die polifenol (karbolsuur) antioksidante. Die inname van polifenole speel 'n belangrike rol in die verlaging van en beskerming teen koronêre hartsiektes (CHD), kardiovaskulêre hartsiektes en kanker weens hulle antioksidant eienskappe.

Vrugte bevat 'n groot verskeidenheid van polifenole met antioksidantkapasiteit. Polifenole word in twee groepe naamlik flavonoïede en nie-flavonoïede geklassifiseer. Flavonoïede word verder in katesjiene, antosianien, prosianidien en flavonole onderverdeel. Karbolsure kom voor as hidroksiliese derivate van bensoësuur en kaneelsuur en word as nie-flavonoïede geklassifiseer. Polifenole bevat redokseienskappe wat hulle instaat stel om as reduserende agente, waterstof skenkers en singlet suurstofonderdrukkers op te tree en daardeur bydra tot die antioksidantkapasiteit van vrugte en groente. Weens die verskeidenheid voordelige eienskappe wat toegeskryf word aan die antioksidante is daar 'n verhoogde belangstelling in die soeke na nuwe plantspesies met hoë fenoliese konsentrasie en relevante biologiese aktiwiteit.

In die lig van die belangrikheid van hierdie substanse vir gesondheid en voedselchemie, fokus hierdie studie op die polifenol en vitamien C karakterisering en kwantifisering van 'n inheemse Suid-Afrikaanse vrug, die Kei-appel *(Dovyalis caffra)*, wat vermoedelik antioksidant eienskappe besit. Omdat die polifenol inhoud die kleur, smaak en moontlike gesondheidsvoordele van die vrug of verwerkte produkte mag beïnvloed, voorsien hierdie studie waardevolle inligting aan die industrie om die beste keuse te maak vir voorbereiding en verwerkingsmetodes om die geskikte eindproduk te verkry. Die ontginning van inheemse vrugte in Suid-Afrika soos die Maroela en Kei-appel, ontvang al hoe meer aandag, nie net as gevolg van die moontlike gesondheidsvoordele wat dit inhou nie, maar ook die geleenthede wat dit bied vir die landelike ekonomie. Voorts dien die navorsing as 'n basis vir verdere navorsing op die Kei-appel en ander inheemse Suid-Afrikaanse vrugte wat moontlike gesondheidsvoordele inhou.

Doelwitte

Die primêre doel van die studie is die kwantifisering en karakterisering van verskeie belangrike antioksidante soos polifenole en askorbien in die totale Kei-apple vrug asook die individuele komponente (skil, vlees en sade). Daarbenewens word ten doel gestel om die totale antioksidantkapasiteit van die hele vrug en die individuele komponente gefraksioneerde en ongefraksioneerde te bepaal asook om gaschromatografie met gekoppelde massaspektrometrie (GC-MS) te gebruik vir die karakterisering van die individuele polifenole om te spekuleer oor die moontlike gesondheidsvoordele waaroor die Kei-appel mag beskik.

Metodes

Die studie is so ontwerp om 'n verteenwoordigende vrugtemonster te verseker. Ongeveer 100 kg Kei-appels is in November 2004 in die Bloemhof omgewing in Suid-Afrika gepluk. 'n Monster van 50 vrugte is skoongemaak en in die verskeie komponente (skil, vlees en sade) verdeel. 'n Ekstra 50 vrugte is lukraak gekies, skoongemaak en in hulle geheel gebruik om te verseker dat

die data verteenwoordigend is van die hele vrug. Nadat die monsters fyngemaal en gevriesdroog is, is die monsterekstrakte voorberei met 'n metode soos beskryf deur Kähkönen *et al.* (1999) deur gebruik te maak van 70% waterige asetoon oplossing. C_{18}-fraksionering (soliede fase ekstraksie) is op die verskeie komponente gedoen en die gevolg was vier fraksie bestaande uit (1) fenoliese suur (karbolsure); (2) prosianidien, katesjiene en antosianien monomere; (3) flavonole en (4) antosianien polimere.

Die totale polifenol konsentrasie in die verskillende ekstrakte en C_{18}-ekstrak fraksies is deur die Folin-Ciocalteu-metode bepaal (Singleton & Rossi, 1965). Vry- en totale askorbiensuur-konsentrasies is bepaal deur die metode soos beskryf by Beutler (1984), sowel as die totale suikers in die verskillende ekstrakte. Beide die metings is noodsaaklik om 'n gekorrigeerde polifenol konsentrasie te verkry. Die totale antioksidantkapasiteit van die heel vrug en die individuele komponente is bepaal deur die "Oxygen Radical Absorbance Capacity" (ORAC) en "Ferric Reducing Antioxidant Power" (FRAP) van die gefraksioneerde en ongefraksioneerde ekstrakte. Deur gebruik te maak van GC-MS analise is die onderskeie individuele polifenol komponente, wat bydra tot die totale polifenolinhoud van die Kei-appel, geidentifiseer en gekwantifiseer.

Die kwantitatiewe data is op die rekenaar ingelees en statisties ontleed. Variansieanalise is uitgevoer en die "Tukey Honest Significant Difference" toets is gebruik vir *post-hoc* vergelyking. Pearson korrelasiekoeffisiënte tussen ORAC, FRAP en die polifenolkonsentrasies is uitgevoer deur van Statistica (Statsoft Inc., Tulsa, Oklahoma, USA) gebruik te maak. Statisties betekenisvolle verskille is aanvaar indien $P \leq 0.05$.

Resultate en bespreking

In die studie is die verskillende belangrike antioksidante (polifenole en askorbien) en die totale

antioksidantkapasiteit in die hele vrug, sowel as in die individuele komponente (skil, vlees en sade) en hulle polifenol sub-groep fraksies bepaal.

Totale polifenolinhoud: Die totale Kei-appel vrug het 'n polifenolkonsentrasie van 943 ± 20.3 mg GAE/100g droëmassa. Deur die individuele komponente met mekaar te vergelyk is gevind dat die saad die hoogste totale polifenolkonsentrasie het met 1990 ± 31.3 mg GAE/100g droëmassa, gevolg deur die skil 1126 ± 45.8 mg GAE/100g droëmassa en die vlees 521 ± 1.01 mg GAE/100g droëmassa.

Totale, L-askorbiensuur (ASC) en L-dehidroaskorbiensuur (DHA) konsentrasie: Die totale Kei-appel vrug toon 'n totale askorbiensuurkonsentrasie van 517 ± 0.92 mg/100g droëmassa. In teenstelling met die polifenolinhoud, het die vlees van die Kei-appel beduidend die hoogste konsentrasie totale askorbiensuur, 778 ± 1.20 mg/100g droëmassa, L-askorbiensuur, 241 ± 21.0 mg/100g droëmassa en L-dehidroaskorbiensuur, 537 ± 22.2 mg/100g droëmassa. Die verhouding van L-askorbiensuur/totale askorbiensuur vir die vlees, total vrug, skil en saad is 0.31, 0.43, 0.49, 0.95 onderskeidelik, wat 'n aanduiding is dat die saad die mees stabiele bron van aktiewe vitamien C is, waarvan 95% van die totale askorbiensuur as L-askorbiensuur voorkom. Dit is ook in ooreen-stemming met die totale polifenolinhoud van die komponente, wat die polifenol se besparingeffek op askorbiensuur bevestig.

Polifenol C_{18}-fraksionering: Die C_{18}-fraksionering van die Kei-appel vrug en die individuele komponente bestaan oorwegend uit fenoliese sure, gevolg deur prosianidien, katesjiene en antosianien monomere, antosianien polimere en flavonole in variërende hoeveelhede.

Antioxidant kapasiteit: Die antioksidantkapasiteit van die total vrug en die individuele komponente soos bepaal deur ORAC, (r=0.76) en FRAP, (r=0.95) korreleer goed met die totale

polifenolinhoud en met mekaar (r=0.88) en dui daarop dat beide goeie aanduiders van antioksidantkapasiteit is.

GC-MS karakterisering van die polifenole in die Kei-appel: Kaffeïensuur en hidro-*p*-kumaarsuur is die fenoliese sure wat in die hoogste konsentrasie in die Kei-appel vrug voorkom. Die meeste hiervan is in die vlees gekonsentreer, en in die geval van die kaffeïensuur, ook in die skil. Die rangorde van die ander belangrikste nie-flavonoïede in die heel vrug is *m*-hidroksibensoësuur > *p*-hidroksifenielasynsuur > 3-metoksi-4-hidroksifenielpropionsuur > *p*-kumaarsuur. Afgesien van die kaffeïensuur in die skil, is feruliensuur in uitsonderlike hoë konsentrasie teenwoordig en dit dien ook as bron vir protokatesjoeësuur. Seringsuur is oorheersend in die saad teenwoordig. Alhoewel die totale flavonoied konsentrasie in dei Kei-appel laag was, is taksifolien en katesjien slegs in die saad geïdentifiseer.

Gevolgtrekking

Uit die studie word afgelei dat die Kei-appel 'n ryk bron van plant gederivatiseerde antioksidantkomponente (polifenole en askorbiensuur) is, met sterk antioksidantkapasiteit wat geassosieer word met gesondheidseienskappe, spesifiek in die voorkoming van kanker, kardiovaskulêre siektes en neurodegenerasie. As gevolg van die verhoogde wetenskaplike en kommersiële belangstelling in die vrug, is dit belangrik om die volgende faktore in ag te neem (agronomiese, genomiese, voor- en naoes toestande en die verwerking) asook ook die weefsels wat die chemiese samestelling van plantaardige voedsel mag beïnvloed, en wat 'n betekenisvolle rol speel in die bepaling van polifenole en askorbiensuur en die bioaktiwiteit van die komponente gedurende voedselverwerking. Om 'n spesifieke eindproduk te verkry moet die polifenol-samestelling van die verskillende vrugte komponente in ag geneem word wanneer 'n metode gekies word vir die verwerking van vrugte.

Sleutelwoorde

Kei-appel; polifenol (karbolsuur); askorbiensuur; antioksidantkapasiteit; gaschromatografie – massaspektrometrie.

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LIST OF ABBREVIATIONS

°C	Degree Celsuis
°C/min	Degree Celsuis per minute
AA	Ascorbic acid equivalents
ANOVA	Analysis of the variance
AOAC	Official Methods of Analysis of the Association of Official Analytical Chemists
ApoA-I	Apolipoprotein A
ApoB	Apolipoprotein B
ASC	Ascorbate
BSTFA	Bis(trimethylsilyl) trifloroacetamide
C_{18}	Carbon 18
CHD	Coronary heart disease
DHA	Dehydroascorbate
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
eV	Electron Volt
FAO	Food and Agriculture Organisation
Fe	Iron
FRAP	Ferric reducing antioxidant power
g	Gram
g/g	Gram per gram
g/kg	Gram per kilogram
GAE	Gallic acid equivalents
GC-MS	Gas chromatography coupled mass spectrometric

HCl	Hydrochloric acid
HMG-CoA	3-hydroxy-3-methylglutaryl-co-enzyme A
Kg	Kilogram
LDL	Low density lipoprotein
m	Meta
mg GAE/100g	Milligram Gallic acid equivalents per 100g
mg	Milligram
mg/100g	Milligram per 100g
mg/kg	Milligram per kilogram
mg/L	Milligram per liter
mg/mL	Milligram per millilitre
min	Minute
mL	Millilitre
mL/min	Millilitre per minute
mM AA/100g	Millimole ascorbic acid equivalent per 100g
mm	Millimetre
Mm	Millimole
mmole TE/100g	Millimole Trolox equivalent per 100g
n	Amount of samples
N	Normality
NF-κB	Nuclear transcription factor - κB
nm	Nanometre
nM	nanomolar
ORAC	Oxygen radical absorbance capacity
p	Para
PGJ	Purple grape juice

r	Correlation coefficient
rpm	Revolution per minute
SD	Standard deviation
TE	Trolox equivalents
TMCS	Trimethylchlorosilane
TNF-ά	Tumor necrosis factor- ά
UV	Ultraviolet
α	Alfa
β	Beta
κ	Kappa
µL	Microliter
µM	Micromolar

CHAPTER 1

PREFACE

PREFACE

1. TITLE

Polyphenols, ascorbate and antioxidant capacity of the Kei-apple (*Dovyalis caffra*).

2. HYPOTHESIS

The Kei-apple, a fruit growing wild in the eastern regions of South Africa, is possibly a rich source of antioxidants (polyphenols and vitamin C), with associated health benefits.

3. AIMS AND OBJECTIVES

To quantify and characterize the polyphenols, ascorbate and total antioxidant capacity of the Kei-apple fruit as well as in the individual fruit components (peels, flesh and seeds) of the Kei-apple, in order to determine its value as a functional food.

4. STRUCTURE OF THE MINI-DISSERTATION

This mini-dissertation is presented in article format.

Chapter 2 consists of a literature review giving an overview of the published, available data on the issues relevant to this topic. These include: the importance of polyphenols and antioxidant vitamins in the diet; the synthesis and functions of polyphenols; classification of polyphenols; biological effects of polyphenols and antioxidant vitamins on human health and the effects of fruit processing on these antioxidants. The references used in this review are listed throughout the text and the complete reference list is at the end of the chapter.

Chapter 3 consists of a manuscript on the polyphenol, ascorbate and antioxidant capacity of the Kei-apple. This manuscript has been prepared for submission to the journal "Food

Chemistry". The article is has been formatted according to the publisher's guidelines given at the end of this chapter.

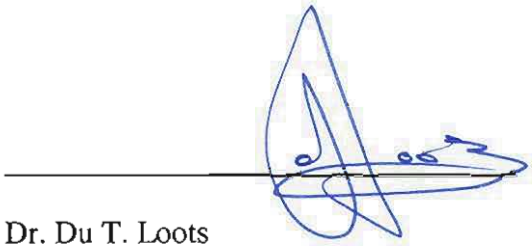
Chapter 4 consists of a summary of the results of the study, as well as concluding remarks and recommendations to the various interest groups involved in the field of functional foods, namely nutrition experts, regulators, researchers and the industry.

5. AUTHORS' CONTRIBUTIONS

The study reported in this dissertation was planned and executed by four researchers and the contribution of each is listed in the table below. A statement from the co-authors is also included, confirming their role in the study and giving their permission for the inclusion of the article in this dissertation.

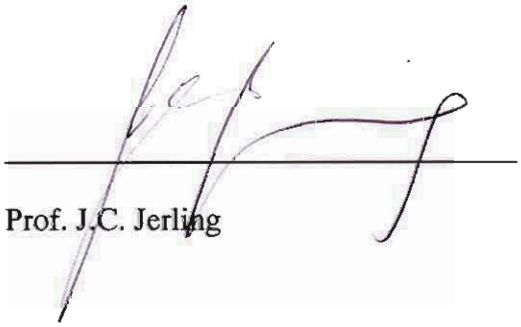
NAME	ROLE IN THE STUDY
Ms T de Beer Hons B.Sc Biochemistry	Responsible for the literature searches, study design, experimental analyses, result interpretation and text drafting.
Dr Du T. Loots PhD. (Biochemistry) Supervisor.	Guidance in all processes of the study design, experimental analyses, result interpretation and text drafting.
Dr F. van der Westshuizen PhD. (Biochemistry)	Guidance in analysis of ORAC/FRAP.
Prof. J.C. Jerling PhD. (Physiology) Co-supervisor	Guidance in statistical analysis, and text drafting.

I declare that I have approved the above-mentioned article, that my role in the study, as indicated above, is representative of my actual contribution and that I hereby give my consent that it may be published as part of the M.Sc. mini-dissertation of Ms T. de Beer.



Dr. Du T. Loots

Dr. F. van der Westshuizen



Prof. J.C. Jerling

CHAPTER 2

LITERATURE REVIEW

- **REASONS FOR THE IMPORTANCE OF POLYPHENOLS IN THE DIET;**
- **THE SYNTHESIS AND FUNCTIONS OF POLYPHENOLS;**
- **CLASSIFICATION OF POLYPHENOLS;**
- **BIOLOGICAL EFFECTS OF POLYPHENOLS ON HUMAN HEALTH AND**
- **EFFECTS OF FRUIT PROCESSING ON POLYPHENOLS.**

LITERATURE REVIEW

1. INTRODUCTION

It is evident from the literature that there is a justifiable concern over the health status of the world's population and the negative impact that diet and lifestyle changes are having on many disease profiles. Special concern is cited for countries, with a rapid urbanisation, such as South Africa, which are dealing with problems of poverty, malnutrition and both chronic and infectious disease.

In response to these concerns, the various health benefits of polyphenols have recently received much attention, as a result of their possible impact on human health, by means of their biological activity in cancer, cardiovascular diseases and neurodegeneration. Dietary factors may play a role in up to 35% of all human cancers (Love & Sayed, 2001). This beneficial effect is thought to be a result of the high content of antioxidants, ascorbate (vitamin C), vitamin E and carotenoids in these foods (Pederson *et al.,* 2000). As a result of this, focus on the role of polyphenolics as a source of dietary antioxidants has increased (Robards & Antolovich, 1997).

Polyphenolic compounds are a useful addition to an overall healthy diet. In theory, eating plenty of fruit and vegetables each day is an ideal way of overcoming micronutrient deficiencies, providing antioxidants and ensuring a diet high in fibre. The people in South Africa do not achieve the recommended daily intake of 5 portions (400 g) of fruit and vegetables. Reasons for this are affordability, availability and taste preferences (Love &

Sayed, 2001). To encourage the use of affordable and available alternative fruits, the use of indigenous fruits is currently being promoted.

Therefore, the trend of the future in nutrition is to move towards functional foods with specific health benefits which play an important role in maintaining human health (Van der Sluis *et al.,* 2002). The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease (CHD) (Gaziano, 2000), cardiovascular disease (Olas *et al.,* 2002) and cancer (Gee & Johnson, 2001) is continuously raising interest among scientists, food manufacturers and consumers.

Plant foods contain not only macro and micro-nutrients (*e.g.* protein, fat, carbohydrates, fibre and micro-nutrients such as vitamins and minerals) but also large numbers of non-nutrient compounds called phytochemicals (Mahan & Escott-Stump, 2003). The major classes of phyto-chemicals include the terpenes, polyphenols and thiols (Mahan & Escott-Stump, 2003). Polyphenolic compounds are phytochemicals that are ubiquitous in vegetables and fruits and their juices (Liu *et al.,* 2002). The term "phenolic compounds" refers to substances that possess an aromatic ring bearing one or more hydroxyl substitutes (Morton *et al.,* 2000). Due to their chemical structure, these compounds are powerful antioxidants and are considered necessary for cellular metabolism in plants (Leighton & Inés, 1999). Polyphenols are the most abundant group of plant phenolic compounds, known to provide much of the flavour, colour and taste in fruits, vegetables, seeds, and other parts of the plant (Billot *et al.,* 1990). Presence of polyphenols in plant foods is largely influenced by genetic factors and environmental conditions. Other factors such as germination, degree of ripeness, variety, processing and storage also influence the content of plant phenolics (Urquiaga & Leighton, 2000; Bravo, 1998).

The numerous beneficial effects attributed to polyphenols have given rise to new interest in finding vegetal species with high polyphenol content and relevant biological activity (Miranda-Rottmann *et al.*, 2002; Galli *et al.,* 2002; Ferguson, 2001; Middleton *et al.,* 2000). Furthermore, polyphenols are the subject of intense scientific research focusing on the prevention or treatment of diseases. Various health benefits of polyphenols have been associated with their antioxidant, antibacterial, anti-mutagenic, anti-inflammatory and anti-allergenic properties (Billot *et al.*, 1990). *In vitro* studies, demonstrate that polyphenols are more powerful antioxidants than vitamin C and E (Leighton & Inés, 1999). It has been reported that the majority of the antioxidant capacity of fruits and vegetables may come from these compounds (Liu *et al.,* 2002). Results of the numerous epidemiological studies and recent clinical trails provide consistent evidence that diets rich in fruits and vegetables can reduce the risk of chronic diseases (Van der Sluis *et al.,* 2002).

As a result of the importance of these substances to health and food chemistry, this study will focus on the polyphenol and vitamin C content of a native South African fruit, the Kei-apple *(Dovyalis caffra)*. Exploitation of the indigenous South African fruits (marula and Kei-apple) is receiving increasing prominence due to their health benefits and the opportunities this presents to rural based economics. Hence, evaluation of these fruits for processing is essential to successful exploitation.

2. POLYPHENOLS

2.1 Classification

Phytochemicals are grouped into classes on the basis of their similar protective functions and individual physical and chemical characteristics (Mahan & Escott-Stump, 2003). Polyphenols

are classified in two broad classes namely the flavonoids and non-flavonoids (Harborne, 1980). The differentiation between these classes is based on the number and the nature of subsequent groups attached to the rings (Robards & Antolovich, 1997). The range of known flavonoids is vast, currently exceeding 5000 in numbers (Harborne, 1994).

2.1.1 Flavonoids

Flavonoids are mainly found in the woody and external parts of the plants, such as the skin, seeds and flowers (Kähkönen *et al.,* 1999). Unfortunately, it is these parts that are usually discarded during food preparation (Miller & Ruiz-Larrea, 2002). Flavonoids represent the most common and widely distributed group of plant phenolics. Their common structure is that of diphenylpropanes (C_6-C_3-C_6), figure 1, which consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle.

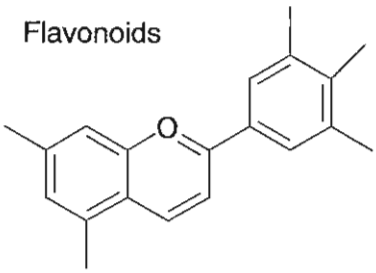


Figure 1: Chemical structure of flavonoids (Manach *et al.*, 2004).

Structural variation within the rings (Figure 2) sub-divides the flavonoids into several families: flavonols, flavones, flavanols, isoflavones, anthocyanidins and others (Cao *et al.,* 1997). These families are grouped together because of their structural similarities (Robards & Antolovich, 1997). These flavonoids often occur as glycosides, glycosylation rendering the molecule more water soluble and less reactive towards free radicals. The sugar most commonly involved in glycoside formation is glucose, although galactose, rhaminose, xylose and arabinose, as well as disaccharides such as rutinose (Miller & Ruiz-Larrea, 2002), also

occur.

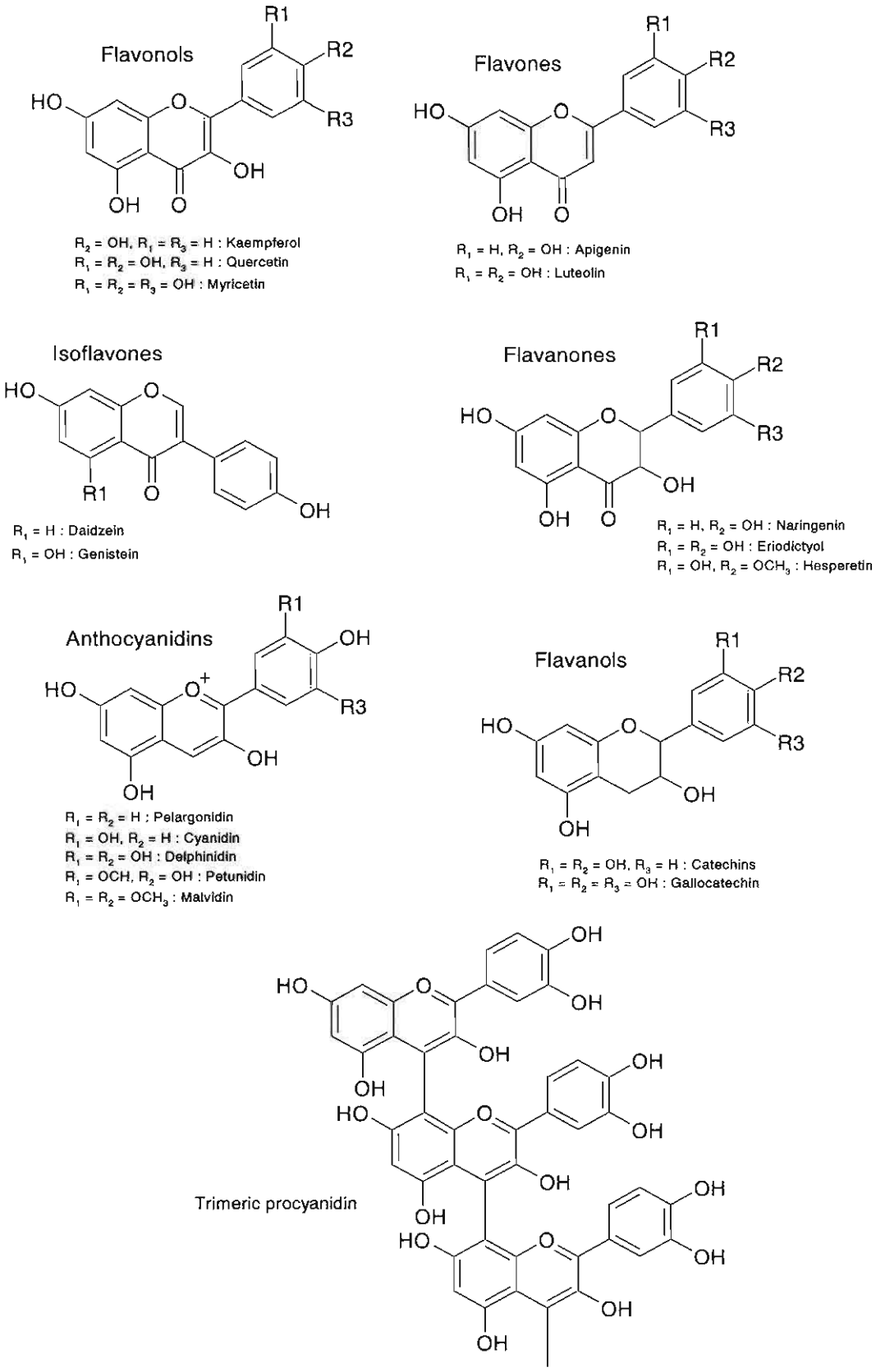


Figure 2: Chemical structures of flavonoids (Manach *et al.*, 2004).

The flavonoid variants are all related by a common biosynthetic pathway, incorporating precursors from both the shikimate and the acetate-malonate pathways (Robards & Antolovich, 1997). Flavonoid biosynthesis involves the interaction of at least five different pathways namely the glycolytic pathway, pentose phosphate pathway, shikimate pathway, the general phenylpropnoid metabolism (producing activated cinnamic acid derivatives and lignin) and the diverse specific flavonoid pathways (Forkmann, 1994; Hrazdina, 1994; Heller, 1994). Further modification occurs at various stages, resulting in an alteration in the extent of hydroxylation, methylation, isoprenylation, dimerisation and glycosylation (producing - O - or C glycosides) (Robards & Antolovich, 1997).

2.1.2 Non-flavonoids

Non-flavonoids can be divided into two classes of phenolic acids: derivates of benzoic acid and derivates of cinnamic acids (Manach *et al.,* 2004). Phenolic acids are important secondary plant metabolites and are widely distributed in fruits, vegetables, teas and related beverages (Merken & Beecher, 2000; King & Young, 1999; Tanaka, 1999; Okuda *et al.,* 1995; Haslam, 1981, 1977). It has been reported that these non-flavonoids have health promoting effects such as antioxidant, anti-tumor and anti-carcinogenic activities (Rapisarda *et al.,* 1999; Hatano, 1995; Okuda *et al.,* 1992; Okuda *et al.,*1984).

These compounds function as antioxidants by metal chelation and scavenging free radicals. Due to their ideal structural chemistry for free radical scavenging activity, they have been shown to be more effective antioxidants *in vitro* than vitamin E and C on the same molar basis (Rice-Evans *et al.,* 1997).

2.1.2.1 Hydroxybenzoic acids

Hydroxybenzoic acids are found in all plant material, and are particularly abundant in acidic tasting fruit. Their actual distribution however depends on the species, cultivar and ripeness of the fruit in question (Miller & Ruiz-Larrea, 2002). Free gallic acid for example has an antioxidant activity three times that of vitamin C or vitamin E (Rice-Evans *et al.*, 1997). This indicates that its three hydroxyl groups can function independently as electron acceptors. Ellagic acid (or digallic acid) is another hydroxybenzoic acid and is found in very high quantities in raspberries, strawberries and blackberries. These berries contain more than fifteen times the amount of ellagic acid than other fruits (Miller & Ruiz-Larrea, 2002). Figure 3, shows the chemical structure of benzoic acids.

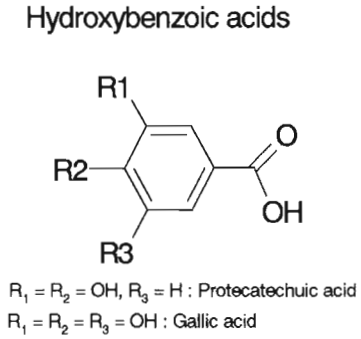


Figure 3: Chemical structures of hydroxybenzioc acid (Manach *et al.*, 2004).

2.1.2.2 Hydroxycinnamic acids

The most abundant hydroxycinnamic acids are p-coumaric, caffeic acid and ferulic acid (Figure 4). These occur most frequently as simple esters with hydroxy carboxylic acids or glucose (Manach *et al.*, 2005). Hydroxycinnamic acids are also highly acidic compounds, and are more abundant and diverse, with a far higher dietary intake than hydroxybenzoic acids. They are distributed throughout all parts of a fruit, with the highest concentrations in the outer extremities of ripe fruits (Manach *et al.*, 2004).

Hydroxycinnamic acids are hydroxylated derivates of cinnamic acid and have been shown to inhibit LDL oxidation *in vitro* (Meyer *et al.,* 1998). Fruits containing high amounts of hydroxycinnamic acids are blueberries, kiwis, plums, cherries and apples, in concentrations ranging between 0.5-2 g/kg fresh weight (Manach *et al.,* 2004). Chlorogenic acid is the most frequently encountered caffeoyl ester and can be found in coffee and many fruits and vegetables (Scalbert & Williamson, 2000). This hydroxycinnamic acid is a good substrate for the enzyme polyphenoloxidase that plays a role in the browning of fruit caused by damage (Van der Sluis *et al.,* 2002).

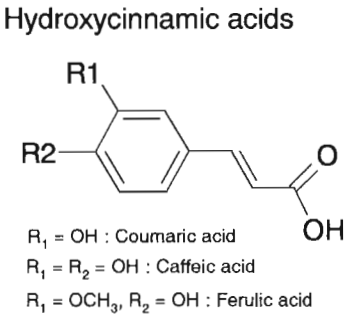


Figure 4: Chemical structure of hydroxycinnamic acids (Manach *et al.,* 2004)

2.2 Biological effects of polyphenols

Flavonoids have important effects in plants biochemistry and physiology acting as antioxidants, enzyme inhibitors, precursors of toxic substances, pigments and light screens (Middleton *et al.,* 2000). *In vitro* and *in vivo* studies showed polyphenols to have well recognized antioxidant capacity (Yoshida *et al.,* 2000). These antioxidant properties have been attributed to their capacity to scavenge reactive oxygen and nitrogen species as well as to chelate redox active metals (Oteiza *et al.,* 2005). These antioxidant functions contribute to the biological effects that may have an impact on human health. This is demonstrated by *in vitro* and *in vivo* studies of their biological activities on chronic diseases and cancer (Keen *et al.,* 2005). In general, however, this section on the biological effects will focus on the

interaction of polyphenols with living organisms and the interpretation of the *in vitro* studies for the same purpose, hence describing the possible therapeutic uses of these compounds against chronic diseases such as cardiovascular disease, coronary heart disease and cancer.

2.2.1 Functions of polyphenols in plants

Phenols are universal components of vascular plant material. Different distribution patterns can be observed in the same plant, thus leaves, stems, roots, fruits, and seeds, may have varying constituents (Harborne, 2000). In the apple for example, phenolic compounds tend to be concentrated in the skin (Harborne, 2000). It has been reported that the skin of apples and mangoes contain about two to four times the polyphenolic content than their pulp (Imeh & Khokhar, 2002). Grapes on the other hand, store only 30% of their polyphenolic content in the skin; the remaining 70% is concentrated in the seeds (Waterhouse *et al.,* quoted by Imeh & Khokhar, 2002).

Phenolic compounds play a role in the structural stability of plant material as their chemical structures enables them to form a variety of ester and ether cross linkages. It is therefore not surprising that phenolic compounds are present in abundance wherever vascular bundles or structural features of plant are found (Morton *et al.,* 2000). Phenolic compounds are synthesized in plants from L-tyrosine or L-phenylalanine via the shikimic acid pathway (Miller & Ruiz-Larrea, 2002). The polyketide pathway is also a source of phenolic compounds (Morton *et al.,* 2000). Humans and animals cannot synthesize the enzymes necessary for the shikimic acid pathway and therefore cannot manufacture phenols or break down the phenolic ring. This is the reason why phenols accumulate in human and animal tissue (Miller & Ruiz-Larrea, 2002). Plants are thought to manufacture phenols for a variety of reasons but mainly do so as part of their response to stress (Miller & Ruiz-Larrea, 2002).

Plant leaves have shown an increase in flavonoid synthesis after being treated with damaging levels of ultraviolet-B radiation (Harborne, 2000).

Phenolic compounds are further closely associated with the sensory and nutritional quality of foods, contributing directly or indirectly to desirable or undesirable aromas and tastes (Shahidi & Naczk, 1995). In low concentrations, phenolics may protect food from oxidative deterioration, however, at high concentrations, they (or their oxidation products) may participate in discoloration of foods, by interaction with proteins, carbohydrates and minerals (Robarbs *et al.,* 1999). Furthermore, phenolic compounds have anti-bacterial, anti-viral, anti-fungal and antioxidant actions (Miller & Ruiz-Larrea, 2002).

2.2.2 Functions of polyphenols in humans

The biological activities of polyphenols in humans includes anti-inflammatory (Read, 1995), anti-allergic, anti-carcinogenic, anti-hypertensive and anti-arthritic activities (Ficarra *et al.,* 1995). The antimicrobial property of polyphenolic compounds is well documented (Chung *et al.,* 1998).

2.2.2.1 Antioxidant effects

Polyphenols exhibit a wide range of biological effects as a consequence of their related antioxidant properties (Mojžišová & Kuchta, 2001). They can exert their antioxidant activities by acting as free radical scavengers (Sato *et al.,* 1996), metal chelators, inhibiting lipid peroxidation (Cook & Samman, 1996) and by their involvement in various physiological activities such as anti-inflammatory, anti-allergic, anti-carcinogenic, anti-hypertensive and anti-arthritic activities (Middleton, 1996; Dakora, 1995; Raghavan *et al.,* 1995; Das *et al.,* 1994; Leibovitz & Meuller, 1993; Thompson, 1993).

The best described property of polyphenols is their role as antioxidants in the body (Nijveldt *et al.,* 2001). The flavones (apple skins, berries, cranberries and grapes) and catechins (red wine and tea) are the best described flavonoids for protecting the body against reactive oxygen species. Free radicals and reactive oxygen species are produced during normal oxygen metabolism or are induced by exogenous damage (Nijveldt *et al.,* 2001) for example cigarette smoke, environmental pollutants, radiation, ultra-violet light, certain drugs, ozone (Langseth, 1995) and exercise (Mojžišová & Kuchta, 2001). According to Halliwell (1994), if there is an imbalance between the production or exposure to reactive oxygen and nitrogen species, and *in vivo* antioxidant defence mechanisms and pro-oxidants (Urquiaga & Leighton, 2000), then a state of oxidative stress may occur. Body cells and tissue are continuously threatened by the damage caused by free radicals (De Groot, 1994). The mechanism and sequence of events by which free radicals interfere with normal cell functions remains unclear. Research indicates that lipid peroxidation may be one of the most detrimental effects of oxidative stress, as it results in cellular membrane damage. Furthermore, free radicals are able to attract various inflammatory mediators which contribute to a general inflammatory response and tissue damage (Nijveldt *et al.,* 2001).

According to Halliwell (1995), living organisms have developed several effective mechanisms to protect themselves from reactive oxygen species. The body's antioxidant–defence mechanism includes enzymes such as superoxide dismutase (Mojžišová & Kuchta, 2001), catalase and glutathione peroxidase (Langseth, 1995). Non-enzymatic counterparts *e.g.* ascorbic acid, α-tocopherol and gluthathione also play a role. Polyphenols fit into the body's antioxidant-defence mechanism by having a possible additive effect to the endogenous scavenging mechanisms (Nijveldt *et al.,* 2001).

- **Direct radical scavenging activity**

Polyphenols stabilize reactive oxygen species by direct reaction with them. This reaction results in a more stable, less reactive oxidized compound (Nijveldt *et al.,* 2001). The reactive species known to be scavenged by polyphenols include superoxides, hydroxyl radicals, nitric oxide, nitrogen dioxide, ozone, hypochlorous acid, peroxynitrites and nitrous acid (Halliwell, 2000). Polyphenols occur in dietary plants as glycosides. After ingestion, glycosides are converted to aglycones, by enzymatic deconjugation and bacterial action (Amakura *et al.,* 2000). These aglycones show stronger radical scavenging activity than their glycoside equivalents (Amakura *et al.,* 2000).

- **Iron chelating activity**

The metal chelating effects of polyphenols *in vitro* suggest that they can play an important role in the prevention and protection of certain diseases (Nijveldt *et al.,* 2001; Yoshino & Murakami, 1998; Morel *et al.,* 1994; Fraga *et al.,* 1987). Metal chelation has been often considered as a minor mechanism in the antioxidant actions of polyphenols (Fraga *et al.,* 1987). Apart from scavenging free radicals, antioxidants can prevent oxygen radical dependent damage *in vivo* by blocking radical formation in animals. The mechanism through which this occurs may be by removing free radical precursors (superoxide and hydrogen peroxide), or by reaction with transition metals. Binding of iron to phenolic antioxidants can suppress the accessibility of the iron to oxygen molecules, by oxidizing ferrous iron to the ferric state and consequently result in the prevention of the hydroxyl radical formation (Yoshino & Murakami, 1998). This means that metal coordination by polyphenolics may be its most effective antioxidant action and recent studies showed that the iron-chelating activity of some flavonoids is closely related to their antioxidant action (Morel *et al.,* 1994). Yoshino & Murakami (1998) found that flavonoids show an antioxidant effect by enhancing iron

oxidation, while non-flavonoids can reduce iron and form Fe^{2+}-polyphenol complexes. Additionally, non-flavonoids (protocatechuic acid and clorogenic acid) show an inhibitory effect on lipid peroxidation possibly by their metal chelation ability (Nijveldt *et al.,* 2001).

- **Lipid Peroxidation**

Lipid peroxidation plays a key role in the development of atherosclerosis and other chronic diseases (Salonen *et al.,* 1997). Free radicals oxidize the polyunsaturated fatty acids in lipoproteins, especially in low density lipoproteins (LDL), which leads to cell death. These oxidation reactions can be prevented by polyphenol antioxidants (Mursu *et al.,* 2005).

In humans, polyphenol supplementation studies resulted in inconsistent findings in lipid peroxidation. Different methods of assessing the resistance of LDL to oxidation, could partly explain the inconsistency (Mursu *et al.,* 2005). According to Hodgson *et al.* (2000) LDL may not be appropriate for the studying of the effects of polyphenolic compounds as flavonoids are hydrophilic of nature and may not accumulate sufficiently with LDL to inhibit the oxidation. They may however act sufficiently in the hydrophilic fraction *e.g.* on the surface of the lipoprotein particles (Mursu *et al.,* 2005). Mursu and co-workers found that polyphenol-rich phloem increased *in vitro* oxidation resistance of serum lipids and radical scavenging activity in a dose depended manner.

In the study done by Aviram *et al.* (1994), comparing the effects of red wine to that of white wine, the red wine resulted in a 20% reduction in plasma lipid peroxidation in the presence of a free radical-generating system, and a 46% reduction in LDL lipid peroxidation in response to copper ions. While the white wine showed an increase of 33% and 57% in lipid peroxidation in the plasma and LDL respectively. A study done on humans by Pérez *et al.*

(2002) to determine the long-term effects of vitamin E and a combination of vitamin C and E, showed a reduction in 7ß-hydroxycholesterol (a marker for lipid peroxidation), with a enhancement in the oxidation resistance of isolated lipoproteins and total serum lipids. Freese *et al.* (2002) however found no decrease in lipid peroxidation with a high intake of fruit and vegetables in their subjects.

2.2.2.2 Polyphenols and disease

Potter (1997) reviewed 200 epidemiological studies, the majority of which showed a protective effect from increased fruit and vegetable consumption (Urquiaga & Leighton, 2000). When the roles of individual antioxidants *e.g.* vitamins A, E and carotenoids, were examined by epidemiological studies or supplementation trails, the results were not as clear cut as those obtained for fruit and vegetables and were often disappointing. Potter's conclusion was that fruit and vegetables provide the best polypharamacy against the development of a chronic disease, considering that they contain a vast array of antioxidant components such as polyphenols which may act synergistically.

An increasing body of epidemiological evidence on the oncoprotective properties of polyphenols supports the concept that a diet rich in fruits and vegetables, promotes health by preventing and delaying cardiovascular disease, coronary heart disease and neurogenerative disorders (Keen *et al.,* 2005; Kampa *et al.,* 2004). Diets rich in fruits and vegetables, such as vegetarian and Mediterranean diets, contain large quantities of polyphenols and have been associated with a diminished risk in a number of diseases. There is however no accurate information available on the dietary intake of polyphenols in these diets as their content in plant foods varies greatly, even among cultivars of the same species.

In order to satisfy the growing demand of consumers for products with an adequate content of bioactive components with associated health benefits, it is necessary to quantify polyphenol concentrations in food products. The possible health benefits of polyphenols will be discussed in detail below.

2.2.2.3 Polyphenols and heart disease

Basic science, clinical observation and epidemiological studies have all contributed to current evidence of the role of antioxidants in the protection of the vascular system (Nijveldt *et al.*, 2001).

Flavonoids can exert their antioxidant activity various mechanisms by quenching reactive oxygen and nitrogen species and hence potentially modifying the destructive mechanisms relevant to cardiovascular diseases (Mojžišová & Kuchta, 2001). Possible mechanisms through which flavonoids can reduce cardiovascular risk are by inhibiting of oxidation the low-density lipoproteins and platelet aggregation. These mechanisms are known to interact in all stages of the atherosclerotic process (Berliner & Heinecke, 1996). The mechanism to counteract the reactive oxygen species includes the binding of metal ions needed for catalysis of reactive oxygen species generation, the up-regulation of endogenous antioxidant enzymes and the repair of oxidative damage to biomolecules (Morton *et al.*, 2000).

Atherosclerosis is the main cause of coronary heart disease (CHD) and results in occlusion of the arteries carrying blood to the heart and consequently ischemic damage. It is also a multifactoral disease that is the primary cause of deaths world wide (Mojžišová & Kuchta, 2001). Clinical studies done by Hertog *et al.* (1995); Hertog *et al.* (1993) and Knekt *et al.* (1996) on humans showed that flavonoids might reduce the risk of coronary heart disease.

Epidemiological studies suggest that the increased levels of polyphenol intake may be associated with reduced CHD risk (Hollman *et al.,* 1996; Knekt *et al.,* 1996; Hertog *et al.,* 1993).

Several *in vitro* and *in vivo* (humans and animal) studies suggested that another mechanism for the reduction of CHD, is the cholesterol hypothesis of atherogenesis, whereby antioxidants may inhibit the oxidation of LDL cholesterol, reduce platelet aggregation or reduce ischaemic damage (Laughton *et al.,* 1991; de Whalley *et al.,* 1990). Many phenolic compounds have been shown to have antioxidant activity *in vitro* and several observational studies support their role in potentially protecting against cardiovascular disease (Morton *et al.,* 2000). Grape juice has been found to be a potent inhibitor of LDL oxidation and Pearson *et al.* (1999), found that 6 commercial brands of apple juice and apple fractions also inhibited *in vitro* LDL oxidation. Although polyphenols are seen to inhibit adhesion, aggregation and secretion of blood platelets *in vitro,* most results available in the literature have concentrated solely on their effect on aggregation (Beretz, 2000).

The role of purple grapes on the inhibition of platelet function was investigated by Freedman *et al.* (2001). They found that the drinking of purple grape juice (PGJ) by healthy volunteer subjects led to a dose dependent inhibition of aggregation. It was recently found by Keevil *et al.* (2000), that purple grape juice decreases platelet aggregation but neither the orange nor the grapefruit juice resulted in any platelet aggregation inhibition. The difference in the platelet inhibitory effect between purple grape juice and orange or grapefruit juice may be due to the different classes of flavonoid compounds each one contains. Thus the flavonols in grape juice may be strong platelet aggregation inhibitors, while the flavonols in citrus fruit may have little or no effect on platelet aggregation. Additionally, the polyphenolic concentrations are also

higher in purple grape juice. The same citrus fruit juice doses may thus have too low a total phenolic content to achieve the same effect (Keevil *et al.,* 2000). Olas *et al.* (2002), investigated resveratrol, a phytoalexin found in grapes. Their results showed and confirmed that resveratrol inhibits the biological activity of blood platelets. Therefore the platelet inhibiting effects of the polyphenolic compounds in grape juice and other juices may decrease the rate of development of the atherosclerotic narrowing of coronary and other arteries (Folts, 1998).

Epidemiological evidence however has its limitations in the sense that it is not possible to isolate any single polyphenol or subclass as being more strongly associated with the risk of CHD than another. Alternatively, different dietary sources of these may be associated with the risk of CHD. Another limitation is that higher polyphenol intakes may only be a surrogate for other uncontrolled dietary and lifestyle practices that are inversely associated with the risk of CHD. A precise, aetiological mechanism by which polyphenols reduce the risk of CHD has yet to be established on both epidemiological and clinical settings. Available epidemiological evidence does however support the possibility that polyphenols, as well as major subclasses of flavonoids may have a protective effect on the risk of CHD (Gaziano, 2000).

However, before general recommendations about dietary intake can be made, the specific mechanisms of how these compounds may affect cardiovascular health and disease need to be uncovered (Morton *et al.,* 2000).

2.2.2.4 Polyphenols and cancer

Accordingly to Nijveldt *et al.* (2001) polyphenols may play a role in the prevention of cancer, due to their toxic effects on cancer and immortalized cells. The body's antioxidant systems are frequently inadequate and damage from reactive oxygen species are proposed to be involved in carcinogenesis (Loft & Poulsen, 1997). Reactive oxygen species can damage DNA and lead to mutations. If these changes appear in critical genes, such as tumor suppressor genes, initiation or progression of cancer may result (Nijveldt *et al.,* 2001).

Antioxidant polyphenols have been reported to inhibit carcinogenesis. Some flavonoids (fisetin, apigenin and luteolin) are potent inhibitors of cell proliferation (Nijveldt *et al.,* 2001). Various studies have tested either individual polyphenolic compounds or fruit extracts with regards to cancer. An *in vitro* study showed quercitin to inhibit proliferation of colonic epithelial tumor cells in mice (Deschner *et al.,* 1991). Another study using an apple polyphenol extract resulted in colon and liver cancer cell growth inhibition in a dose dependent manner (Eberhardt *et al.,* 2000). Additionally, studies done with raspberry extracts of concentrations > 10 mg/mL, also showed inhibition of cell proliferation in a dose dependent manner (Liu *et al.,* 2002). This study however determined that although the pigment content of raspberries affected their antioxidant activity, it had no effect on its ability to inhibit cell proliferation. It is therefore assumed that polyphenols other than anthocyanins in the raspberries are responsible for the inhibition of tumour cells (Liu *et al.,* 2002).

Several types of polyphenols (phenolic acids, hydrolysable tannins and flavonoids) show anti-carcinogenic and anti-mutagenic effects and are thought to do so through a number of mechanisms:

1. by interfering with several steps leading to the development of malignant tumours,

2. inactivating carcinogens,
3. inhibiting the expression of mutant genes and the activity of enzymes involved in the activation of procarcinogens, and
4. activating enzymatic systems involved in the detoxification of xenobotics (Bravo, 1998), and
5. several studies have shown that in addition to their antioxidant protective effect on DNA and gene expression, polyphenols, particular flavonoids, inhibit the initiation, promotion and progression of tumors (Urquiaga & Leighton, 2000).

The above mentioned biological effects of polyphenols are thought to be because of their inherent antioxidant capacity (Bidlack, 1999). Despite these positive effects however, some polyphenols have been reported to be mutagenic in microbial assays, and co-carcinogens or promoters in inducing skin carcinogenesis in the presence of other carcinogens (Chung *et al.*, 1998). This latter possibility warrants further research.

There are hundreds of polyphenols with antioxidant activity that are potential contributors to the antioxidant mechanism in humans and animals. These compounds are excellent candidates to explain the health benefits of diets rich in fruits and vegetables. However, there is still not enough information regarding food composition, bioavailability, interaction with other food components and their biological effects, to adequately explain the associated health benefits (Institute of Medicine, 1998, Robards & Antolovich, 1997). There is however enough evidence to suggest that some of these compounds will be absorbed in sufficiently high concentrations to have a physiologically beneficial contribution to health (Morton *et al.*, 2000).

Epidemiologic studies are a useful tool in the evaluation of human health effects of long-term exposure to physiologic concentrations of polyhenols. Unfortunately, reliable data on polyphenol contents of foods are scarce. Comprehensive data is available on only the flavonoid subclasses: flavonols, flavones, and catechins (Arts & Hollman, 2005). Although, epidemiological studies have shown that diets rich in plant foods protect humans against degenerative diseases such as cancer, the results are conflicting (Manach *et al.,* 2005; Diplock *et al.,* 1998). Of the several cancers studied, protective effects have only been reported for lung cancer in relation to flavonol and flavone intake (Arts & Hollman, 2005). The conflicting results in these studies may arise from the insufficient control of confounders, such as socioeconomic and lifestyle factors as well as through inadequate methodology to define dietary intake assessment and food composition (Mckay & Blumberg, 2002). To address the drawbacks of previous studies, a carefully designed approach should be undertaken to ensure that all confounders have been taken into consideration during the assessment.

2.3 The influence of fruit processing for juice production on polyphenol content

The influence of the various stages of the food production chain on the occurrence and stability of polyphenols in the consumed product are important. Cultivation methods, the choice of raw materials, industrial processing (extraction process and subsequently biochemical and chemical reaction), storage, distribution and final processing by the consumer may all affect the final concentrations and the bioactivity of polyphenols in these products (Van der Sluis *et al.,* 2002). All phenolic compounds are highly unstable and rapidly transformed into various reaction products when plant cells are damaged during fruit processing (Cheynier, 2005). As with fruit peeling, dehulling of legume seeds, decortication and bolting of cereals can result in the loss of some of the polyphenol content. Peeling of

fruit can eliminate a significant portion of polyphenols as these substances are often present in higher concentrations in the outer parts of fruits and vegetables. Grinding of plant tissues may lead to oxidative degradation of polyphenols as a result of cellular decompartmentation and contact between cytoplasmic polyphenol oxidase and phenolic substrates present in the vacuoles. Polyphenols are then transformed into brown pigments that are polymerized to different degrees (Manach *et al.,* 2004). Due to industry's interest in the Kei-apple for the fruit juice market, various industrial methods used for juice preparation will be discussed in the context of polyphenol content of the final juice product.

2.3.1 Maceration

Maceration operations facilitate diffusion of polyphenols into juice. This maceration accounts for the fact that the polyphenol content of red wines are up to 10 times higher than that of white wines (Manach *et al.,* 2004). The different maceration methods include: crushing, pressing, diffusion, extraction and centrifuging. For refractory material, pre-treatment with a macerating enzyme, with or without heating to 60°C and holding for up to 40 min. can greatly increase juice yields. Even without macerating enzyme addition, heating to 70°C softens the fruit, inactivates native enzymes and reduces microbial load to produce satisfactory juice yields. Disadvantages of this are that delicate flavours can be destroyed and unacceptable darkening due to enzymatic and non-enzymatic browning may occur. Rapid heating and cooling prior to juicing can overcome some of these quality problems. Mashed fruit and purees may be batch heated to optimum macerating enzyme temperature (55-60°C) in open steam-jacketed kettles with stirring and then cooled. This heating and cooling can be accomplished by swept surface heat exchangers or thermal screws (a hollow steam-heated auger in a steam-jacketed trough). Steam or cooling fluid flowing through both auger and trough are able to effectively and continuously heat or cool the material (FAO, 2001).

There are many fruit specific ways to extract juice. The sizes can range from kitchen to industrial scale depending upon the volume, end use and raw materials. A compromise between juice yield and quality dictated the juicing procedure and what additional processing steps are chosen (deaeration, clarification, peeling etc.) is discussed below.

2.3.1.1 Crushing

Juice extraction equipment for crushing ranges from hand operated crushers to mechanical extractors for higher outputs. With soft or comminuted fruit, a cone screw expresser or paddle pulper fitted with appropriate screens serves to separate the juice from particulate matter. Where skin or seed shattering is a problem, brush paddles can replace metal bars. Two pulpers in series with screens of 1 to 0.2 mm can effectively clean up many types of juices (FAO, 2001).

Fruit with unpalatable skins and seeds must be treated more cautiously than ones which can be completely pulverized. It is possible to minimize extraction of skin and seed components by a crushing regime that mashes fruit carefully peeled and deseeded or cored prior to juicing. Contour peelers, such as those used for apples can be adapted to a range of fruits that are sufficiently firm and uniform to facilitate the rotary peeling action. Flavonols are however located mainly in the skin and seeds, and removal of these components prior to juicing affects the final concentrations of these in the juice product (Dawes & Keene, 1999).

2.3.1.2 Pressing

Presses are the most common and the most traditional methods of juice extraction. Factors contributing to the efficiency of pressing include the viscosity of the juice, the resistance to deformation of the solid phase of the pulp, pulp porosity and the applied pressure (Beveridge,

1997). These parameters depend on the nature of the pulp and are subject to change during the pressing process. Several types of presses are available: traditional rack and cloth-, screw-, Bucher-Guyer horizontal- and belt presses (Kader, 1992). Prior to extraction, fruits and vegetables are ground to reduce particle size, break the material into small pieces and open cells to release the juice. The degree of grinding depends on the material and its condition (Beveridge, 1997). Water solubility influences the concentration of the flavonoids in the juice produced via this method of juice extraction (Van der Sluis *et al.,* 2002).

2.3.1.3 Diffusion extraction

Fruit to be extracted must be sliced thinly depending on the character of the fruit. This process relies on heat for diffusion of soluble solids from the fruit. Soluble solid diffusion rates increase with temperature. This also allows for a decrease in enzyme activity such as polyphenol oxidase, a decreased oxygen solubility and less darkening of both slices and extracted juice. Heating however destroys polyphenols and lowers the content of these in the final juice mixture. However, compared to pressed juices, tannin levels are higher, giving a distinctive sharp sour and astringent taste. Total cinnamics extracted is also higher. At higher temperature of 63°C monomeric procyanidins, catechins and epicatechins are also easily extracted (Beveridge, 1997) without destruction.

2.3.1.4 Infusion extraction

Another method of extracting water-soluble components from fruits and other plant material is infusion extraction. The flesh is comminuted with added water to dissolve solids that are then separated from the pulp. Multiple extractions with temperature, pH adjustments and enzyme treatment (as discussed in detail under 2.3.2.2) can extract practically all-soluble solids. A counter-current flow, where fresh solvent (usually water) is applied at the last stage

of spent pulp, ensures that all juice solids are extracted. In this case, subsequent concentration of the extract is necessary to return it to the initial fruit °Brix (FAO, 2001).

2.3.1.5 Steam extraction

With soft, readily extractable fruits, a steam extraction system has potential. An early example is a home extraction unit of Scandinavian origin. The fruit is placed into a strainer that drains concentrically away from an inner container with boiling water. Rising steam condenses in the fruit compartment or on the fruit, heating it and leaching out soluble components that drain away from the pulp. The use of high temperatures in a larger scale semi-continuous unit of French design, allows for oxidative enzyme inactivation, pasteurization and oxygen exclusion during extraction. Thus the juice has unusually good colour and flavour retention. The pulp or press residue of high value fruits can be extracted in this manner with water or other solvents to yield extracts containing pigments, nutrients, nutraceuticals, essences, or other useful by-products (FAO, 2001). The heating may however have a negative effect on the polyphenols extracted. Anthocyanins are very sensitive to heat. Therefore, during the extraction process of the juice the minimum heat should be applied for the shortest time (Markakis, 1982).

2.3.1.6 Centrifugation

The ability of the decanter, in combination with appropriate enzyme systems to extract juices form heated mashes, placed this technique at the heart of several juice manufactures. Careful high temperature, short time thermal treatments of fruit substrates, allow for destruction of enzymes that generate production of brown pigments. However, during the extraction of grapes, the flavonoid and iron levels were seen to be lowered (Beveridge, 1997). Rapid methods such as centrifugation and filtration are also able to produce clear juice. A

continuous or a decanting centrifuge with automatic desludging, in order to produce a clear or nearly clear juice, is quite effective. A fine mesh shaker screen is often used to further remove particulate matter (FAO, 2001). Due to the speed of the process and enclosed system, the oxidation of phenolic components is avoided. The pomace that comes from the decanter has a brighter colour compared to a press (Pecoroni, 1996).

The more soluble phenolics occur in the outer tissues of fruits than in their inner tissues (Antolovich *et al.,* 2000). Anthocyanins are more water-soluble than flavonols, and are contributing to the colour of the fruit, by accumulating in the vacuoles of the peel (Van der Sluis *et al.,* 2002). Anthocyanins are also unstable in chemical and physical processing. Catechins and anthocyanins are sensitive to oxidation, but with the enclosed system oxidation of anthocyanins is prevented (Robards & Antolovich, 1997). During centrifugation, the solids are separated form the liquid resulting in less flavonols as these occur in higher concentrations in the peel and seeds (Van der Sluis *et al.,* 2002). Due to the low solubility of phenolic compounds and their uneven distribution in the tissues, the centrifugation technique is ineffective in extracting all the phenolic compounds. During the production of olive oil, a 2-phase and 3-phase decanter system is used. In the 3-phase decanter, polyphenol compounds are lost as a result of the vast amount water used (Antolovich *et al.,* 2000).

2.3.2 Additional processing steps

2.3.2.1 Deaeration

In many of the above mentioned steps, the fruit and juice is subjected to considerable aeration and the inclusion of oxygen can promote enzymatic browning (Markakis, 1982). Therefore care should be taken to perform these steps rapidly, at low as possible temperatures and/or

protect the material from oxygen. Sometimes preheating is used to inactivate natural and/or added enzymes, provided that rapid cooling follows.

Deaeration can be accomplished by either flash heating the juice in a vacuum chamber or saturating the juice with an inert gas. Nitrogen or carbon dioxide can be bubbled through the juice prior to storing in an inert atmosphere. Clearly, once air is removed or replaced by the inert gas, the juice must be protected from the atmosphere in all subsequent processing steps that follow. A compromise however of the flash heating is that it results in the removal of some desirable volatile aromas, which the use of the inert gas prevents (FAO, 2001).

2.3.2.2 Enzymatic oxidation of polyphenols

In apples and apple products, the most important groups of flavonoids are the catechins, flavonols and anthocyanins (Lister *et al.,* 1994). Catechins are oxidation sensitive, and together with chlorogenic acid, serve as a substrate for the enzyme polyphenol oxidase. This enzyme plays a role in the browning of fruits which have been damaged. Flavonols are less water soluble than anthocyanins and are not a desired substrates for this enzyme (Van der Sluis *et al.,* 2002). Despite this apple juice is seen to have a far lower flavonoid concentration than unprocessed apples. Commercially available apple juice has only 2.5 mg/L quercitin, while the concentrations in unprocessed apples are about 36 ± 19 mg/kg (Van der Sluis *et al.,* 2002). Large losses of hydroxycinnamic acids in processed apple juices can be attributed to the oxidation of these phenolic compounds by the same enzyme (Dawes & Keene, 1999).

Enzymatic treatment of the pulp is often used before pressing to prevent browning. Before the addition of pectolytic enzymes, the pulp can be aerated to allow complete oxidation of these polyphenols. This process however affects the final antioxidant activity of the juice

(Van der Sluis *et al.,* 2002). The pectinolytic enzymes used during the processing also hydrolyse hydroxycinnamic acid esters (Manach *et al.,* 2004). In the evaluation of the effects of the different processing methods on the flavonoid content and antioxidant activity, Van der Sluis and co-workers found a 2-30 fold reduction of flavonoids in the apple juice compared to fresh apples.

2.3.2.3 Clarification and stabilization of fruit juice

Fruit juice preparation often involves clarification or stabilization steps aimed at removing certain flavonoids responsible for the discoloration and haze formation, consequently leading to a low polyphenol content (Dawes & Keene, 1999).

3. KEI APPLE

The numerous beneficial effects attributed to phenolics have raised interest in finding new vegetal species with high phenolic content and relevant biological activity. Exploitation of indigenous South African fruits is receiving more and more prominence due to the opportunities it presents in preventing malnutrition and the burden of diseases.

Kei-apple has a frank taste and subsequently thought to be rich in polyphenolic compounds which are well known for their possible health benefits, but no confirmation of this has yet been reported. Therefore, the Kei-apple, as an indigenous fruit native to the Kei River of southwest Africa, is being evaluated to determine its nutritional value. In addition to the health benefits which these South African indigenous fruits may possess, the possible economic benefits which the Kei-apple and other indigenous fruits (such as the Marula) may hold for South African economy cannot be ignored. Figure 5, shows the Kei-apple in its

different stages of ripeness. Whereas, figure 6, is an example of a ripe Kei-apple cut in half.

Figure 5: Kei-apple in different stages of ripeness.

Figure 6: A ripe Kei-apple cut in half.

The Kei-apple *Dovyalis caffra,* belongs to the family Flacourtiaceae and is also known as *umkokolo* in Africa. It is native to the Kei River of south western Africa and occur abundantly, growing wild in the eastern regions of South Africa. The nearly round bright yellow fruit has a tough skin and an apricot-textured, juicy, highly acidic flesh with 5-15 seeds arranged in double rings in the centre. The fruit is however considered too acidic to be

eaten directly from the tree. It is normally served as a dessert, where it is cut in half, peeled, seeded, sprinkled with sugar and allowed to stand for a few hours before serving. It is also added into fruit salads, made into syrups, shortcake, jam and jelly. Under-ripe Kei-apples may also be harvested.

Very little is however known about the nutritional value of the fruit. It has been reported that a ripe fruit consists of 83 mg/100g ascorbic acid and 3.7% pectin. Scientists in Egypt reported 15 amino acids: alanine, arganine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, valine, phenylalanine, praline and trace amounts of serine and threonine (Morton, 1987). Due to the amount of interest in both the health and economic potential of this fruit, research identifying its nutritional components is needed. This research will focus on quantifying and characterizing the polyphenols and vitamin C in the Kei-apple, as well as determining its antioxidant capacity, in order to predict its role in the prevention of diseases related to oxidative stress.

The competing demands of taste and health play an important role in the food industry as the major determinant of food selection is taste. Foods that are bitter, acid, or astringent tend to be rejected by consumers, and this may have a negative economic effect. Polyphenolic compounds are responsible for the above mentioned tastes. These polyphenolic compounds in plant foods are influenced by genetic factors and environmental conditions (Drewnowski & Gomez-Carneros, 2000). By knowing the concentrations and the types of polyphenolic compounds in the fruits, it enables the food industry to remove the polyphenolic compounds that give the bitter taste by selective breeding of new and less bitter cultivars. This information also assists in ensuring that a functional food is developed, with the necessary polyphenolic compounds, which are bio-available, and affect the biological activities having

an impact on health. Therefore, the necessary industry should take note of the importance of the nutritional values of indigenous fruit and take steps to preserve their nutritional value in the development of these products.

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CHAPTER 3

ARTICLE

POLYPHENOLS, ASCORBATE AND ANTIOXIDANT CAPACITY OF THE KEI APPLE *(DOVYALIS CAFFRA)*

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Polyphenols, Ascorbate and Antioxidant Capacity of the Kei-apple (*Dovyalis caffra*)

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Abstract

Polyphenol and ascorbate characterisation of the Kei-apple (*Dovyalis caffra*) and its various fruit components (peels, flesh and seeds) were investigated and compared. Both the total ascorbate and polyphenol content of the fruit were high (517 mg/100g and 943 mg GAE/100g dry weight respectively) in comparison to other fruits previously reported. The majority of the polyphenols were identified as phenolic acids. The seeds of the fruit had the highest polyphenol content (1990 mg GAE/100g dry weight) followed by the peel and flesh components. Antioxidant capacity analyses using ORAC and FRAP showed these to correlate significantly to the polyphenol concentrations (r=0.76 and r=0.95 respectively). GC-MS analyses indicated caffeic acid to be the major component of the entire fruit and flesh, ferulic acid the major component in the peels, and syringic acid the major component in the seeds. Other notable polyphenols identified in high concentrations included 3-methoxy-4-hydroxyphenylpropionic, *p*-coumaric acid, *m*-hydroxybenzoic, *p*-hydroxyphenylacetic acid, vannilic acid and hydro *p*-coumaric acid. These results not only support the putative high antioxidant value linked to this fruit, but also better define this potential in terms of the major antioxidants that exist in the Kei-apple fruit and its various fruit components.

Key words: Kei-apple, polyphenols, ascorbate, antioxidant capacity, GC-MS

1. Introduction

The various health benefits of polyphenols that have been associated with their antioxidant, anti-bacterial, anti-inflammatory and anti-allergenic properties, have recently received much attention as a result of their impact on human health (Scalbert, Manach, Morand, Rémésy & Jiménez, 2005). Polyphenolic compounds are phytochemicals that are ubiquitous in

vegetables, fruits and their juices (Pearson, Tan, German, Davis & Gershwin, 1999). Factors such as ripening, environment, genetics and processing, may result in a loss or enrichment of some of these compounds (Imeh & Khokhar, 2002; Scalbert & Williamson, 2000). Valued as antioxidants, polyphenols are a useful addition to an overall healthy diet. The exploitation of indigenous South African fruits (Marula and Kei-apple) is receiving more and more prominence, not only due to the opportunities these present to rural based economics, but also their potential role in terms of human health.

The Kei-apple, *Dovyalis caffra,* belongs to the family Flacourtiaceae and is also known as "umkokolo" in Africa. It is native to the Kei River of South-west Africa and occurs abundantly, growing wild in the eastern regions of South Africa. The nearly round bright yellow fruit has a tough skin and an apricot textured, juicy, highly acidic flesh with 5 - 15 seeds arranged in double rings in the centre. Very little is known about the nutritional value of this fruit. It has been reported that the ripe fruit contains 83 mg/100g ascorbic acid and 3.7% pectin (Morton, 1987). The fruit is thought to be rich in polyphenols, due to its astringent taste, but no verification of this has been reported.

In this study various nutritionally important antioxidants (polyphenols and ascorbate) in the Kei-apple fruit and its individual fruit components (peels, flesh and seeds) were quantified and characterized. In addition, the total antioxidant capacity of the entire fruit and various fruit components were determined by measuring the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) of the unfractionated and C_{18}-fractionated extracts. Gas chromatography coupled mass spectrometric (GC-MS) characterisation of the individual polyphenol components is described and the major components discussed in the light of their possible health benefits.

2. Materials and methods

2.1. Materials

Approximately 100 kg of Kei-apples were picked in the month of November 2004 in the Bloemhof area in South Africa. All fruits where immediately stored at -85°C until sample preparation. A sample of 50 fruits was rinsed and separated into the various fruit components (peels, flesh and seeds). An additional 50 fruits were randomly selected, cleaned and used in their entirety to collect data which would be representative of the whole fruit. The above mentioned samples were lyophilised and stored at -85°C until extraction.

2.2. Methods

2.2.1 Preparation of extracts

After being grounded by mortar and pestle, lyophilised samples were weighed into a centrifuge tube (500 mg) and polyphenolics extracted as described by Kähkönen *et al.* (1999). All the extracts were done in triplicate. Briefly, 10 mL of 70% aqueous acetone (Merck, USA) was added, and each sample was homogenized for 1 min. Tubes were centrifuged at 3000 rpm for 15 min., and the clear supernatant was collected. This procedure was repeated with another 10 mL of 70% aqueous acetone. Supernatants were combined and dried under nitrogen and stored at -85°C until further analysis. The solid residue was resuspended in double distilled water.

2.2.2. C_{18}-fractionation

C_{18}-fractionation of the above extracts was done on adapting a method described by Oszmianski, Ramos & Bourzeix (1988). This method is based on multiple elutions at different pH values with three different mobile phases and results in four fractions containing (1) phenolic acids; (2) procyanidins, catechins and anthocyanin monomers; (3) flavonols and (4) anthocyanin polymers. Briefly, 1 mL of sample was introduced into preconditioned C_{18} cartridges (Sigma-Aldrich, USA) and the various fractions eluted at the specified pH values using 5 mL of the described elution solvents (Oszmianski *et al.,* 1988).

2.2.3. *Determination of total polyphenols*

The total polyphenol content in the various extracts and C_{18}-fractions were determined according to the Folin-Ciocalteu method (Singleton & Rossi, 1965). Unfractionated extracts were diluted 16 times prior to analysis. Fractionated samples were used as collected from the C_{18}-columns. Samples, 200 µL, were introduced into test tubes followed by 1 mL Folin-Ciocalteu's reagent (Sigma-Aldrich, USA). This was allowed to stand for 8 min. at room temperature. Next, 0.8 mL sodium carbonate (7.5%) (Sigma-Alrich, USA) was added, mixed and allowed to stand for 30 min. Absorption was measured at 765 nm (Shimadizu UV-1601 Spectrophotometer). Total phenolic content was expressed as gallic acid (Sigma-Aldrich, USA) equivalents (GAE) in milligram per litre (mg/L). As a result of the contribution of ascorbic acid and sugars to the response of the Folin-Ciocalteu assay, corrections for these factors were done as described by Asami, Hong, Barret & Mitchell (2003), and Slinkard & Singleton (1977), respectively. Mean values of polyphenol content were expressed as mg gallic acid equivalents (GAE)/100g wet and dry weight ± standard deviation (n=3).

2.2.4. Ascorbate determination

Ascorbate (ASC), dehydroascorbate (DHA) and total ascorbate (ASC+ DHA) concentrations were determined in the extracts spectrophotometrically at 578 nm (Shimadizu UV-1601 Spectrophotometer) using a method described by Beutler (1984). Mean values of ascorbate content were expressed as mg/100g wet and dry mass ± standard deviation (n=3).

2.2.5. Total sugar determination

The total sugar content was determined in the different extracts using the Official Methods of Analysis of The Association of Official Analytical Chemists (AOAC) International – 16th edition. The sugar content for the entire fruit, flesh, peel, seed extracts was 0.56 g/g, 0.67 g/g, 0.24 g/g and 0.43 g/g dry weight respectively, and was used for the correction of the polyphenol concentrations determined via the Folin–Ciocalteu assay.

2.2.6. Oxygen Radical Absorbance Capacity (ORAC)

The fruit extracts were prepared as described earlier and the further extractions as well as oxygen radical absorbance (ORAC) capacity analyses of hydrophilic and lipophylic compounds were performed essentially as described by Prior *et al.* (2003). The analysis of lipophylic compounds was aided by the addition of randomly methylated β-cyclodextrin (gift from Dr R Prior), as a solubility enhancer as described by Huang, Ou, Hampsch-Woodill, Flanagan & Deemer (2002). Briefly, in a volume of 200 µL, the reaction contained fluorescein (Sigma-Aldrich, USA), (56 nM), as a target for free radical attack by 2,2'-azobis (2-amidino-propane) dihydrochloride (Sigma-Aldrich, USA), (240 nM). A BioTEK

fluorescence plate reader (FL-600, UK) was used and the decay of fluorescence of fluorescein (excitation 485 nm, emission 520 nm) was measured every 5 min. for 2 hours at 37°C. Costar black opaque (96-well) plates were used in the assays. Trolox (Sigma-Aldrich, USA) was used as standard at a range between 0-20 μM with a polynomial (2^{nd} order) curve fit analysis. Mean values of antioxidant capacities were expressed as mmoles Trolox equivalent (TE)/100g wet and dry mass ± standards deviation (n=3).

2.2.7. Ferric Reducing Antioxidant Power (FRAP)

FRAP values were determined essentially as described previously (Benzie & Strain, 1999). Briefly, the reduction of a Fe^{3+} - 2,3,5-triphenyltetrazolium (Sigma-Aldrich, USA) complex in the assay by the antioxidants in the samples was monitored at 593 nm. L-ascorbic acid (Sigma-Aldrich, USA), (AA) was used as a standard and the FRAP of the samples were expressed as mean mmol ascorbic acid equivalents (mM AA)/100g wet and dry mass ± standard deviation (n=3).

2.2.8. GC-MS analysis

Extraction and derivatisation of a 0.25 g of lyophilised sample (entire fruit, peels, seeds and flesh) were done in triplicate by modifying of the method previously used (Loots, Mienie, Berg & Van der Schyf, 2002). To the sample, 3 mL sodium acetate buffer (pH 5.6) and 100 μL glucoronidase arylsulphatase (Sigma-Aldrich, USA) were added. Then the samples were incubated in a shaking water bath overnight at 37°C. The mixture was acidified with 5N HCl to a pH < 2 and 100 μL of a 3 mM solution of 3-phenylbutyric acid (internal standard) (Sigma-Aldrich, USA) and extracted twice, first with 6 mL ethyl acetate (Burdick & Jackson,

USA), followed by 3 mL ethyl ether (Sigma-Aldrich, USA) by sonification for 10 min. Approximately 2 mg of anhydrous Na_2SO_4 (Sigma-Aldrich, USA) was added to each tube to remove any water that may still be present in the samples. The samples were then centrifuged for 2 min. at 2000 rpm. The solution was decanted from the pellet into a small Kimax tube and dried under a nitrogen stream. The dry extract was derivatised at 70°C for 30 min. with 100 µl bis (trimethylsilyl) trifloroacetamide (BSTFA) (Sigma-Aldrich, USA) and 20 µL trimethylchlorosilane (TMCS) (Sigma-Aldrich, USA).

An Agilent 6890 GC ported to a 5973 Mass Selective detector (California, USA) was used for identification and quantification of individual polyphenols. For the acquisition of an electron ionisation mass spectrum, an ion source temperature of 200°C and electron energy of 70 eV were used. The gas chromatograph was equipped with a SE-30 capillary column (Chemetrix, USA), a split/splitless injection piece (250°C) and a direct GC-MS coupling (260°C). The 10:1 split injection (0.6 µL) was used during the MS analysis. Helium (1 mL/min.) was used as the carrier gas. An oven temperature of 100°C, isometric for 1 min., was used as an initial temperature after which a rise of 10°C/min. was continued until a temperature of 200°C was reached. This was followed by a temperature increase of 15°C/min. until a final temperature of 300°C was reached. This temperature was then maintained for a further 5 min.

2.2.9. Statistical analysis

Data is presented as mean ± SD of triplicate determinations. Significant differences between the various fruit components were calculated by analysis of the variance (ANOVA) using the Tukey Honest Significant Difference test for post-hoc analysis. Pearson correlation coefficients between ORAC, FRAP and the total polyphenol content were calculated using Statistica (Statsoft Inc., Tulsa, Oklahoma, USA) with significance set at $p \leq 0.05$.

3. Results and discussion

3.1 Total polyphenol content

There was a significant difference in the total polyphenol concentration between the fruit, flesh, peel and seed fractions ($p < 0.05$), as can be seen in Table 1. The entire fruit had a polyphenol concentration of 943 ± 20.3 mg GAE/100g dry weight. Comparison of the individual fruit components showed the seeds to have significantly the highest total polyphenol concentration, followed by that of the peels and flesh. Comparing the polyphenol concentrations of the C_{18}-fractions of the various fruit components, the peel had significantly the highest phenolic acids, while the seeds had significantly the highest procyanidin, catechin and anthocyanin monomers as well as anthocyanin polymers.

Compared to findings of other fruits reported in the literature, the Kei–apple had a considerably higher level of total phenols (225 ± 4.84 mg GAE/100g wet weight), for example berry species, *Rubus* raspberries (126-402 mg/100g wet weight), *Rubus* species blackberries (472-678 mg/100g wet weight) and *Rubus* hybrid blackberries (80-230 mg/100g wet weight) (Moyer, Hummer, Finn, Frei & Wrolstad, 2002), which are known for their high polyphenol contents. Other fruit comparisons showed the Kei-apple to have similar levels of polyphenols to that of strawberries (1480-2370 mg/100g dry weight), apples (1190-1210 mg/100g dry weight) and gooseberries (1240 mg/100g dry weight). It did however seem that the Kei-apple had approximately half the total polyphenol concentration of that of raspberries (2390 mg/100g dry weight), cranberries (2120 mg/100g dry weight), bilberries (2970 mg/100g dry weight) and whortleberries (2870 mg/100g dry weight) as reported by Kähkönen *et al.* (1999). One should, however, bear in mind that direct comparisons with the literature with regards to total polyphenol concentrations, might be misleading due to variations in

analytical methods and fruit cultivars used. Accurate comparisons of this nature could only be made if the various fruits were analysed alongside one another using the same analytical techniques. A previous comparison of Kei-apple juice to that of other fruit juices, (strawberry, red grape and orange), prepared under identical conditions (Loots, Van der Westhuizen & Jerling, 2006), however, showed the Kei-apple polyphenol and ascorbate concentrations to be significantly higher than these fruit, with a comparably low DHA concentration.

3.2 Ascorbate content

As indicated in Table 1, the Kei-apple had a total ascorbate value of 517 ± 0.92 mg/100g dry weight of which 223 mg/100g dry weight is as L-ASC and 294 mg/100g dry weight is as DHA. Comparing the individual fruit components, the flesh of the Kei-apple had significantly the highest concentration of total ascorbate 778 ± 1.20 mg/100g dry weight followed by that of the peel and the seed fractions, 298 ± 7.30 mg/100g dry weight and 158 ± 7.89 mg/100g dry weight, respectively. Furthermore, the flesh had a significantly higher concentration of L-ASC than that of the peel and seed fractions. The seeds, however, showed 95% of the total ASC to be L-ASC with very little DHA, where as the flesh fractions total ascorbate consisted primarily of DHA (~70%). The total ascorbate content of the different components decreased in the order: flesh, peels and seeds. Franke, Custer, Arakaki & Murphy (2004) confirmed a wide variation between the total ascorbate content of different tissues in the same species. Comparing the ascorbate values of the Kei-apple (123 mg/100g wet weight) and that of other fruits reported in the literature, including Kiwifruit (25.5-205 mg/100g wet weight), strawberries (31.6-31.7 mg/100g wet weight), oranges (Navels) (54.1-62.4 mg/100g wet weight), apples with skin (3.0-3.5 mg/100g wet weight) and grapefruit

(41.3-52 mg/100g wet weight) (Franke *et al.,* 2004; Nishiyama, Yamashita, Yamanaka, Shimohashi, Fukuda & Oota, 2004), confirmed that this fruit is an excellent source of vitamin C. This data also supports previous findings in Kei-apple juice (Loots *et al.,* 2006). L-ascorbate was however unstable and reported to be easily oxidised to L-dehydroascorbate due to the reaction with oxygen, in the presence of heavy metal ions and light (Nishiyama, *et al.,* 2004), which in turn had been shown to have little anti-scorbutic activity (Otsuka, Kurata & Arakawa, 1996). The ratio of L-ascorbic acid/total ascorbate of the flesh, entire fruit, peels and seeds were 0.31, 0.43, 0.49 and 0.95, respectively. This coincided with the polyphenol content of the various fruit components, also showing an increasing order of flesh, fruit, peels and seeds (Table 1), confirming the role of polyphenols in ascorbate stability (May, Qu, Whitesell & Cobb, 1996).

3.3 C_{18}-fractionation extracts

Solid phase (C_{18}) fractionation of the entire fruit and three fruit components showed the majority of the polyphenols to be phenolic acids, followed by procyanidin, catechin and anthocyanin monomers and then anthocyanin polymers and flavonols (except for the flesh having higher flavonols than anthocyanin polymers). Comparing the three fruit components, phenolic acids occurred significantly at the highest concentration in the peels (603 ± 3.01 mg GAE/100g dry weight), whereas the seeds showed significantly the highest anthocyanin polymers (240 ± 10.9 mg GAE/100g dry weight) as well as procyanidins, catechins and anthocyanin monomers (295 ± 1.85 mg GAE/100g dry weight). Although non-significant, the flesh showed the highest flavonol concentration.

As shown in Table 1, the polyphenols were not evenly distributed qualitatively and quantitatively between the different components, similarly to the report by Imeh *et al.* (2002).

As shown previously, phenolic acids and procyanidins were associated with an astringent taste in fruits (Loots *et al.,* 2006; Park & Cha, 2003). The flavonol content was almost equally divided between the different components and was the lowest subgroup of polyphenols detected. Maturity of the fruits could influence the flavonol concentration (Park *et al.,* 2003) and it would be interesting to see if Kei-apples with various grades of ripeness show changes in their flavonol content not only in the entire fruit, but also in the various fruit components.

3.4 Antioxidant capacity of the unfractionated and fractionated samples

The antioxidant capacities of the unfractionated samples were determined and are summarised in Table 3. The entire fruit extract showed an ORAC value of 4.64 ± 0.30 mmole TE/100g dry weight and a FRAP value of 8.54 ± 0.13 mmole AA/100g dry weight. By comparing the antioxidant capacity of the different fruit components showed that the seeds had significantly the highest antioxidant capacity followed by the peels and flesh. Antioxidant capacity of the C_{18}-fractionated fruit components indicated that the phenolic acids of all the various components, were the main contributors to the total antioxidant capacity of the fruit (Table 5). Comparing the antioxidant capacity (determined by ORAC and FRAP) of the Kei-apple fruit to that of *Vaccinium* genotypes (blueberries), *Rubus* species (black berries) and the *Ribes* genotypes (gooseberries) (Moyer *et al.*, 2002) showed it to have a higher antioxidant capacity despite similar polyphenol concentrations. This might be explained by the higher concentration of ascorbate in the Kei-apple, compared to these fruits.

3.5 Correlations of ORAC, FRAP and polyphenol content

Analysis of Kei-apple juice by Loots *et al.* (2006) and berries by Moyer *et al.* (2002), showed the polyphenol concentrations of these to correlate well with their antioxidant capacities, as determined by ORAC and FRAP analyses. Additionally, they showed both methods to be good predictors of antioxidant function (Loots *et al.*, 2006; Moyer *et al.*, 2002). Our results confirmed this with ORAC (r=0.76) and FRAP (r=0.95) values significantly correlating with the total polyphenol content and ORAC and FRAP values correlating significantly with each other (r=0.88). Ou *et al.* (2002), however, showed discrepancies when correlating these parameters. Total polyphenol content was however not the only factor influencing antioxidant capacity. Other factors like structural arrangement, number, position of hydroxyl groups, double bonds and aromatic rings in addition to other antioxidants such as ascorbic acid might also influence the antioxidant capacity (Rice-Evans, Miller & Paganga, 1996). An example of this was the anthocyanin polymer fraction of the flesh which had a higher ORAC value (Table 4), despite it having a lower concentration of polyphenols than that of the procyanidins, catechins and anthocyanin monomers.

Furthermore, this study, and previous findings on Kei-apple juice (Loots *et al.,* 2006) and berries (Moyer *et al.,* 2002), showed FRAP to be a better predictor of antioxidant capacity than ORAC. It should be noted that ORAC and FRAP measure antioxidant capacity *via* two different mechanisms, i.e. radical scavenging and ferric reducing power, respectively, which might influence the variations seen between these analyses. The various individual polyphenol components of the mixture might have stronger free radical scavenging abilities than ferric ion reducing power or vice versa, thus explaining the small variations seen when comparing these two measures for antioxidant capacity.

3.6 GC-MS polyphenol characterisation of the Kei-apple and its components

A summary of the identified polyphenol compounds in the Kei-apple by GC-MS is given in Table 6. Although the non-flavonoid - hydrocinnamic acids were distributed in all parts of the fruit, the highest concentrations of these occurred in the peels and flesh. Caffeic acid and hydro-*p*-coumaric acid was seen to be the phenolic acid occurring in the highest concentrations in the Kei-apple fruit (27.9 ± 1.78 mg/100g dry weight and 22.0 ± 0.73 mg/100g dry weight, respectively). The majority of these were seen to be concentrated in the flesh and in the case of caffeic acid and in the peels. The order of predominance of other major non-flavonoid components in the whole fruit analysis were *m*-hydroxybenzoic acid > *p*-hydroxyphenyl acetic acid > 3-methoxy-4-hydroxyphenylpropionic acid > *p*-coumaric acid. The Kei-apple peel had, apart from caffeic acid, high concentrations of ferulic acid (485 ± 21.0 mg/100g dry weight) and also served as a source of protocatechuic acid (125 ± 5.78 mg/100g dry weight). Syringic acid was most prominent in the seeds (80.7 ± 1.57 mg/100g dry weight). Although the total flavonoid concentration in the Kei-apple was low, taxifolin and catechin were identified, with the seeds almost entirely accounting for these compounds.

An increasing body of epidemiological evidence on the oncoprotective properties of exogenous antioxidants supported the concept that a diet rich in fruit promotes health by preventing and delaying cardiovascular disease, coronary heart disease and neurogenerative disorders (Kampa *et al.,* 2004; Keen, Holt, Oteiza, Fraga & Schmitz, 2005). According to Kampa *et al.* (2004), exogenous antioxidants belong to distinct classes, for example phenolic acids or flavonoids. Characterisation of the individual polyphenols in the Kei-apple showed that the non-flavonoid, caffeic acid, was the most prominent phenolic acid in the fruit. This phenolic acid had been associated with certain beneficial effects such as anti-genotoxicity,

prevention of cancers and to have pro-apoptotic effects (Kampa *et al.,* 2004). The possible mechanisms through which caffeic acid possess these putatively beneficial effects included interaction with arylhydrocarbon/xenobiotic receptors, inhibition of NF-$_K$B as well as increased nitric oxide (Safe, 2001; Kim, Gazourian, Quadri, Romieu-Mourez, Sherr & Sonenshein, 2000; Mortensen, Skouv, Hougaard & Larsson; 1999). Phenolic acids such as caffeic acid also have intrinsic free radical scavenging activity (Kampa *et al.,* 2004). Monoamine oxidase and 5-lipoxygenase that were implicated in various neurogenerative diseases were inhibited by caffeic acid (Loots *et al.,* 2006). According to Mansouri, Makris & Ketalas (2005), benzoic acid derivatives were strong hydrogen peroxide quenchers. As a result of the limited distribution of hydroxybenzioc acids in foods, nutritionists had not given much attention to this compound (Manach, Williamson, Morand, Scalbert & Rémésy, 2005). Phenolic compounds, particularly flavonoids had been shown to possess important antioxidant activity towards radicals, which could damage life essential molecules such as nucleic acids and proteins. The flavanonol, taxifolin, showed anti-proliferative effects and was seen to inhibit carcinogenic cell growth (Gee & Johnson, 2001) and the production of TNF-ά (Ueda, Yamazaki & Yamazaki, 2004), hence its anti-cancer properties. Taxifolin further influenced lipid production, affecting HMG-CoA reductase activity, a key enzyme in cholesterol biosynthesis, and apolipoproteins, apoA-I and apoB synthesis and secretion (Theriault, Wang, Van Iderstine, Chen, Franke & Adeli, 2000), hence its function in the possible prevention of cardiovascular diseases.

4. Conclusion

From this study we reported the qualitative and quantitative properties of Kei-apple in terms of the plant derived antioxidant compounds, polyphenols and ascorbate, in various sections of

the fruit. It was concluded that the Kei-apple is a rich source of these plant derived antioxidant compounds with a relatively high antioxidant capacity which might be associated with health promotion properties. However, due to the increased scientific and commercial interest in this fruit, it is essential to take into consideration the various factors (agronomic, genomic, and post-harvest condition and processing) and tissues that might affect the chemical composition of plant foods. These aspects might play a significant role in determining the polyphenol and ascorbate composition and bioactivity of these compounds during food processing procedures.

Abbreviations used

ASC, ascorbate; DHA, dehydroascorbate; SD, standard deviation; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; AOAC, Association of Official Analytical Chemists; TE, trolox equivalents; AA, ascorbic acid; GC-MS, gas chromatography mass spectrometry; BSTFA, bis(trimethylsilyl) trifloroacetamide; TMCS, trimethylchlorosilane.

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Table 1. Total polyphenols content and total ascorbate, ascorbate and dehydroascorbate concentrations on the basis of dry and wet (fresh) weight determined in Kei-apple components.

	Total polyphenols (mg GAE/100g)[a]		Total ascorbate (mg /100g)		Ascorbate (mg/100g)		Dehydroascorbate (mg/100g)	
Extract	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight
Fruit	943 ± 20.3[¥]	225 ± 4.84[§]	517 ± 0.92[×]	123 ± 0.22[†ᵒ$]	223 ± 4.77[fΩ]	53.1 ± 1.14[rᵻ]	294 ± 5.65[Δ]	70.2 ± 1.35[δ]
Flesh	521 ± 1.01[¥]	111 ± 0.21[§]	778 ± 1.20[×]	165 ± 0.23[†ᵉt]	241 ± 21.0[δι]	51.2 ± 4.47[€∞]	537 ± 22.2[Δ]	114 ± 4.72[δ]
Peel	1126 ± 45.8[¥]	330 ± 13.4[§]	298 ± 7.30[×]	87.3 ± 2.14[ᵒ•]	146 ± 8.56[ΩΩδ]	42.9 ± 2.51[rϵJ]	152 ± 1.61[Δ]	44.4 ± 0.47[δ]
Seed	1990 ± 31.3[¥]	1076 ± 16.9[§]	158 ± 7.89[×]	85.2 ± 4.26[$ t]	149 ± 6.34[fι]	80.5 ± 3.42[£∞J]	8.76 ± 3.64[Δ]	4.73 ± 1.97[δ]

[a]GAE/100g: gallic acid equivalents per 100 g.

Means with a symbol in common differ significantly from each other ($p \leq 0.05$).

Table 2. Concentrations of various solid phase C_{18} polyphenol fractions of Kei-apple components, determined by the Folin-Ciocalteu method.

Components	Fraction 1 (mg GAE/100g)[a]		Fraction 2 (mg GAE/100g)[a]		Fraction 3 (mg GAE/100g)[a]		Fraction 4 (mg GAE/100g)[a]	
	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight
Fruit	496 ± 9.38$^{\times}$	118 ± 2.24$^{\Delta}$	193 ± 7.39$^{\circ\int}$	46.1 ± 1.76†	29.7 ± 0.39	7.08 ± 0.09$^{\$}$	145 ± 4.89^{f}	34.6 ± 1.17$^{\Omega}$
Flesh	325 ± 9.54$^{\times}$	69.1 ± 2.03$^{\Delta}$	77.3 ± 11.9$^{\circ\delta t}$	16.4 ± 2.54†	34.4 ± 5.03	7.32 ± 1.07*	25.4 ± 2.46^{f}	5.41 ± 0.52$^{\Omega}$
Peel	603 ± 3.01$^{\times}$	177 ± 0.88$^{\Delta}$	185 ± 8.05$^{\delta}$	54.2 ± 2.36†	30.2 ± 2.65	8.85 ± 0.78$^{\iota}$	78.1 ± 6.64^{f}	22.9 ± 1.95$^{\Omega}$
Seed	571 ± 14.8$^{\times}$	309 ± 7.97$^{\Delta}$	295 ± 1.85$^{\int t}$	159 ± 1.00†	27.1 ± 5.56	14.7 ± 3.01$^{\$*\iota}$	240 ± 10.9^{f}	130 ± 5.89$^{\Omega}$

[a]GAE/100g: gallic acid equivalents per 100 g.

Fraction 1: Phenolic acids.

Fraction 2: Procyanidins, catechins and anthocyanin monomers.

Fraction 3: Flavonols.

Fraction 4: Anthocyanin polymers.

Means with a symbol in common differ significantly from each other ($p \leq 0.05$).

Table 3. Antioxidant capacity values of the unfractionated fraction of the Kei-apple components determined by ORAC and FRAP method.

Components	Unfractionated (ORAC)[a]		Unfractionated (FRAP)[b]	
	Dry weight	Wet weight	Dry weight	Wet weight
Fruit	4.64 ± 0.30	1.11 ± 0.072$^{\iota*}$	8.54 ± 0.13$^{\mathrm{t}\beta}$	2.04 ± 0.03$^{\alpha\Delta x}$
Flesh	4.63 ± 0.19	0.99 ± 0.040$^{\$\circ}$	5.32 ± 0.13$^{\Omega f\beta}$	1.13 ± 0.027$^{\alpha\infty\epsilon}$
Peel	4.84 ± 0.24	1.42 ± 0.071$^{*\circ]}$	8.98 ± 0.62^{f†}	2.63 ± 0.18$^{\times\epsilon r}$
Seed	4.90 ± 0.20	2.65 ± 0.11$^{\iota\$]}$	11.0 ± 0.40$^{\mathrm{t}\,\Omega\dagger}$	5.97 ± 0.22$^{\Delta\infty r}$

[a] mmole Trolox equivalent (TE) per 100 g wet and dry mass ± standard deviation (n=3).

[b] mmole ascorbic acid equivalent (mM AA) per 100 g wet and dry mass ± standard deviation (n=3).

Means with a symbol in common differ significantly from each other ($p \leq 0.05$).

Table 4. Antioxidant capacity values of the fractions of Kei-apple components determined by the ORAC method.

Components	Fraction 1		Fraction 2		Fraction 3		Fraction 4	
	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight
Fruit	4.13 ± 0.24	0.98 ± 0.06$^{\text{rε}}$	1.31 ± 0.02$^{\infty}$	0.31 ± 0.004$^{\text{t}}$	0.62 ± 0.04$^{\$\text{*}}$	0.15 ± 0.009$^{£¥\alpha}$	1.2 ± 0.1$^{\text{J}\alpha\beta}$	0.3 ± 0.03$^{\Phi\Lambda}$
Flesh	4.33 ± 0.08$^{\Omega}$	0.92 ± 0.02$^{\times\dagger}$	1.03 ± 0.26$^{\Delta}$	0.22 ± 0.06$^{\text{J}}$	0.05 ± 0.05$^{\$\text{ι}}$	0.01 ± 0.01$^{£\square}$	1.68 ± 0.12$^{\kappa\alpha}$	0.36 ± 0.025$^{\varphi\S}$
Peel	4.27 ± 0.14	1.25 ± 0.04$^{\epsilon\times f}$	0.98 ± 0.27$^{\delta}$	0.29 ± 0.08°	0.05 ± 0.02$^{\text{*}\#}$	0.01 ± 0.004$^{\alpha\text{♀}}$	0.0008 ± 0.0002$^{\text{J}\,\kappa\Sigma}$	0.0002 ± 0.00007$^{\Lambda\S\text{Þ}}$
Seed	3.88 ± 0.13$^{\Omega}$	2.10 ± 0.07$^{\text{r}\dagger f}$	2.35 ± 0.14$^{\infty\Delta\delta}$	1.27 ± 0.08$^{\text{t}\text{J}\circ}$	0.69 ± 0.16$^{\text{ι}\#}$	0.4 ± 0.08$^{¥\square\text{♀}}$	1.84 ± 0.28$^{\Sigma\beta}$	0.99 ± 0.15$^{\Phi\varphi\text{Þ}}$

mmole Trolox equivalent (TE) per 100 g wet and dry mass ± standard deviation (n=3).

Fraction 1: Phenolic acids.

Fraction 2: Procyanidins, catechins and anthocyanin monomers.

Fraction 3: Flavonols.

Fraction 4: Anthocyanin polymers.

Means with a symbol in common differ significantly from each other ($p \leq 0.05$).

Table 5. Antioxidant capacity values of the fractions of Kei-apple components determined by the FRAP method.

Components	Fraction 1		Fraction 2		Fraction 3		Fraction 4	
	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight
Fruit	4.6 ± 0.09	1.1 ± 0.02	1.06 ± 0.10$^{\S\#}$	0.25 ± 0.025$^{¥\pounds}$	0.032 ± 0.0068	0.076 ± 0.0016^{Þ}	0.54 ± 0.085$^{\neq}$	0.13 ± 0.020†
Flesh	3.76 ± 0.11*	0.80 ± 0.023°	1.05 ± 0.14$^{\int\Lambda}$	0.22 ± 0.030$^{\alpha\ddagger}$	BD	BD	0.30 ± 0.017$^{\infty}$	0.065 ± 0.0036$^{\lambda}$
Peel	5.36 ± 0.30*	1.57 ± 0.089°	1.62 ± 0.039$^{\#\Lambda}$	0.47 ± 0.012$^{¥\bullet}$	BD	BD	0.39 ± 0.10$^{\Omega}$	0.12 ± 0.031$^{\times}$
Seed	4.44 ± 0.29*	2.40 ± 0.16°	1.70 ± 0.057$^{\S\int}$	0.92 ± 0.031$^{\pounds\,\alpha\bullet}$	0.18 ± 0.037	0.099 ± 0.020^{Þ}	1.41 ± 0.26$^{\neq\infty\Omega}$	0.76 ± 0.14$^{\dagger\,\lambda\times}$

mmole ascorbic acid equivalent (mM AA) per 100 g wet and dry mass ± standard deviation (n=3).

Fraction 1: Phenolic acids.

Fraction 2: Procyanidins, catechins and anthocyanin monomers.

Fraction 3: Flavonols.

Fraction 4: Anthocyanin polymers.

Means with a symbol in common differ significantly from each other ($p \leq 0.05$). BD: below detection limit.

Table 6. GC-MS polyphenol concentrations as determined in the different Kei-apple fruit components.

Class	Compound	Fruit	Flesh	Peel	Seed
Non-Flavonoids					
• Hydoxybenzoic acids	*m*-Hydroxybenzoic acid	10.5 ± 0.87	49.9 ± 2.39	7.01 ± 0.59	5.64 ± 0.33
	Vanillic acid	4.51 ± 0.21	26.7 ± 0.98	2.85 ± 0.14	2.08 ± 0.01
	Resorcylic acid	3.18 ± 0.20	Not detected	0.81 ± 0.05	Not detected
	Protocatechuic acid	4.76 ± 0.39	125 ± 5.78	14.8 ± 0.75	1.88 ± 0.45
	Syringic	3.45 ± 0.19	Not detected	Not detected	80.7 ± 1.57
• Hydroxycinnamic acids	*p*-Coumaric	8.29 ± 0.64	57.2 ± 2.32	2.78 ± 0.08	11.7 ± 0.79
	Ferulic acid	0.84 ± 0.03	Not detected	485 ± 21.0	0.56 ± 0.02
	Caffeic acid	27.9 ± 1.78	292 ± 11.0	191 ± 5.98	9.23 ± 0.74
	Isoferrullic	Not detected	15.1 ± 0.87	1.55 ± 0.10	Not detected
• Hydroxyhydrocinnamic acids c	Hydro-p-coumaric acid	22.0 ± 0.73	132 ± 7.88	Not detected	2.96 ± 0.09
• Hydroxyphenylacetic acids	*p*-hydroxyphenyl acetic acid	9.31 ± 0.44	35.6 ± 0.26	4.63 ± 0.32	2.10 ± 0.10
	3-methoxy-4-hydroxyphenylpropionic acid	9.20 ± 0.63	60.4 ± 1.27	Not detected	1.60 ± 0.06

Flavonoids					
• Catechins	Catechin	6.42 ± 0.27	Not detected	Not detected	5.88 ± 0.28
• *Flavanonols*	Taxifolin	10.9 ± 0.77	Not detected	Not detected	8.19 ± 0.54

Concentration expressed as mean ± standard deviation (n=3) as mg/100g dry weight.

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CHAPTER 4

CONCLUSIONS & RECOMMENDATIONS

CONCLUSIONS AND RECOMMENDATIONS

INTRODUCTION

The main findings of the study reported in this mini-dissertation will be summarised in this final chapter, together with some general conclusions and recommendations to those involved (government, industry, researchers, nutrition experts) in the field of functional foods. Only a general discussion and conclusion will be provided in this chapter, as each of the results of the study are presented, discussed and interpreted in conjunction with a summary of the available published literature in the preceding chapters.

SUMMARY OF MAIN FINDINGS

Total phenols

- The total polyphenol concentration of the Kei-apple is 943 ± 20.3 mg GAE/100g dry weight.
- In the C_{18}-fractions, the seeds have significantly the highest polyphenols, consisting predominantly of phenolic acids.
- There is a significant difference in the total polyphenol concentration between the different fractions (peels, flesh, seeds and entire fruit).
- The total polyphenol concentration decreased in the different components in the order of seeds > peels > flesh.

Total, L-ascorbic (ASC) and L-dehydroascorbic (DHA) concentration

- The entire fruit had a total ascorbate value of 517 ± 0.92 mg/100g dry weight (123 ± 0.22 mg/100g wet weight) of which 223 ± 4.77 mg/100g dry weight was L-ascorbate and 294 ± 5.65 mg/100g dry weight was L-dehydroascorbate.

- The total ascorbate concentration between the individual components showed a significant difference.
- The total ascorbate concentration decreased in the individual components in the order of flesh > peels > seeds.
- The ratio of L-ascorbate/total ascorbate for the flesh, whole fruit, peels and seeds is 0.31; 0.43; 0.49; 0.95. This is in line with the polyphenol concentration of these components confirming a polyphenol sparing effect on vitamin C.

C_{18}-fractionation extracts

- The entire fruit, peels and seeds consist predominantly of phenolic acids > procyanidin, catechin and anthocyanin monomers > anthocyanin polymers > flavonols, while the flesh consists predominantly of phenolic acids > procyanidin, catechin and anthocyanin monomers > flavonols > anthocyanin polymers.
- By comparing the three fruit components, the phenolic acids significantly occur in the highest concentrations in the peel, whereas the seeds showed significantly the highest incidence of anthocyanin polymers as well as procyanidins, catechins and anthocyanin monomers.
- The astringent taste can be attributed to the high concentrations of phenolic acids and procyanidins detected in the Kei-apple.

Antioxidant capacity

- The Kei-apple fruit had an ORAC of 4.64 ± 0.30 mmole Trolox equivalent (TE) per 100 g dry weight (1.11 ± 0.072 mmole Trolox equivalent (TE) per 100 g wet weight) and a FRAP of 8.54 ± 0.13 mmole Trolox equivalent (TE) per 100 g dry weight (2.04 ± 0.03 mmole Trolox equivalent (TE) per 100 g wet weight).

Correlation between antioxidant capacity markers (ORAC & FRAP) and polyphenol content

- In the combined samples the ORAC (r=0.76) and FRAP (r=0.95) correlate significantly with the polyphenol concentration.
- The ORAC and FRAP values showed a significant correlation (r=0.88) indicating both to be good predictors of antioxidant capacity in the Kei-apple.

GC-MS polyphenol characterisation of the Kei-apple:

Polyphenols quantified and identified in the individual components and in the entire fruit.

- Caffeic acid (27.9 ± 1.78 mg/100g dry weight) is the most prominent polyphenol in the Kei-apple followed by hydro-*p*-coumaric acid (22.0 ± 0.73 mg/100g dry weight) and *m*-hydroxybenzoic acid (10.5 ± 0.87 mg/100g dry weight).
- When comparing the individual fruit fractions, flesh predominantly consists of caffeic acid (292 ± 11.0 mg/100g dry weight) > hydro-*p*-coumaric acid (132 ± 7.88 mg/100g dry weight) > protocatechuic acid (125 ± 5.78 mg/100g dry weight). The most prominent polyphenols in the Kei-apple peels are ferulic acid, 485 ± 21.0 mg/100g dry weight, followed by once again, caffeic acid at 191 ± 5.98 mg/100g dry weight.
- Syringic acid (80.7 ± 1.57 mg/100g dry weight) and *p*-coumaric acid (11.7 ± 0.79 mg/100g dry weight) are the non-flavonoids that are the most prominent in the seeds.
- Although the total flavonoid concentrations were low, taxifolin and catechin occurred in relatively high concentrations in the seeds. Neither taxifolin nor catechin was detected in either the flesh or peel.

- Due to the fact that caffeic acid is the most prominent compound in the Kei-apple, this fruit's role in cancer prevention may be of further interest.

CONCLUDING RECOMMENDATIONS

This study concludes that the Kei-apple and its various fruit components are a rich source of antioxidant compounds (polyphenols and vitamin C) with high antioxidant capacity.

This information is vital to industries involved in fruit processing in order to select fruit processing procedures which will ultimately develop a product with the highest potential for health benefits. In addition this research will serve as a platform for further research on the Kei-apple and other indigenous South African fruits with possible health benefits. Another challenge to the industry may be to develop methods by which the polyphenols remaining in the pomace after extraction, are extracted and later added to the final juice product.

The development of functional foods is not only the domain of the food technologists and marketers. It is essential to involve all the parties e.g. the scientist, public health nutrition experts, consumer scientists, communication specialists and industry during the development process. To ensure that a product with the highest health benefits is developed, all the various factors (agronomic, genomic, pre- and post-harvest condition and processing) and tissues that may affect the chemical composition of plant foods must be taken into consideration before selecting the processing procedures for the desired end product.

To select the most suitable fruit processing method in acquiring a Kei-apple juice with the highest potential impact on human health, the results obtained in this study should be taken into consideration. The uneven distribution of polyphenols and ascorbate in the peel, flesh and seeds make it impossible to eliminate any of these components, as this will result in a loss of some of the polyphenols and ascorbate which are essential for health and a possible selling

point for a Kei-apple product (Manach *et al.,* 2004). Overall, caffeic acid is the most prominent phenolic acid in the fruit. When comparing the different components: ferulic acid was the most prominent polyphenol in the peel, caffeic acid in the flesh and syringic acid and the two flavonoids, taxifolin and catechin, in the seeds. Due to the fact that caffeic acid and taxifolin are associated with playing a role in the prevention of cancer and cardiovascular disease, it is wise not to lose this polyphenol portion during fruit processing (Kampa *et al.,* 2004; Gee & Johnson, 2001; Theriault *et al.,* 2000). Maceration operations are recommended as it facilitates diffusion of polyphenols from the seed and peels factions into the juice.

In closing, the research done in this study provides vital information regarding beneficial nutrients found in the Kei-apple. In order to maximise the health benefits of the Kei-apple products, various processing methods can be used to maximise the polyphenol, vitamin C content of the final product by inclusion of peels and seeds into these procedures due to their high polyphenol contents. In addition, due to the strong presence of caffeic acid in the Kei-apple, and its associations to cancer prevention, further research into the use of the Kei-apple in cancer prevention may be of significance.

POSSIBILITIES FOR FURTURE RESEARCH

- Further research may be aimed at determining the effect of location, fruit ripeness, soil type and rainfall, on the level of polyphenols and vitamin C in this fruit.
- Research on other factors such as pre- and post-harvest conditions as well as processing can also add significant value in the determination of the chemical composition of the Kei-apple.

- Value might be added by breeding techniques or genetic modification of the polyphenol and/or the organic acid profiles of the Kei-apple, in order to increase its palatability.
- Due to the strong presence of caffeic acid and other polyphenols in the Kei-apple, further research into the use of the Kei-apple in cancer prevention, haemostatic factors, phase one enzyme activity and oxidative stress related diseases might be of significance.

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