

Phenolic compounds in aqueous extracts of  
marama bean [*Tylosema esculentum* (Burchell) A.  
Schreiber] seed coat, sorghum (*Sorghum bicolor*  
L. Moench) bran and their bioactive properties

By  
Jeremiah Sijubo Shelembe

Submitted in partial fulfilment of the requirements for the degree  
PhD Food Science  
in the  
Department of Food Science  
Faculty of Natural and Agricultural Sciences  
University of Pretoria  
South Africa

May 2012

## DECLARATION

---

I Jeremiah S. Shelembe declare that the thesis, which I hereby submit for the degree PhD Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:.....

DATE: May 2012

## ACKNOWLEDGEMENTS

---

I would like to express my sincere thanks and acknowledgement to the following:

Dr K. G. Duodu, my supervisor, for his guidance, advice and encouragement for the successful execution of this research and compilation of this thesis.

Professor Amanda Minnaar and Professor Megan Bester, my co-supervisors, for their valuable contribution towards the research and review of the contents of this thesis.

Dr Duncan Cromaty for his assistance and guidance in using the HPLC-MS system for this study.

Staff and fellow students in the Department of Food Science, Department of Anatomy and Department of Pharmacology of the University of Pretoria for their constructive discussions and sharing of ideas.

My wife Cynthia, and my children Nondumiso, Sandile, Ncamiso, Nosipho, Ntokozo and Nandi for their love and patience during my studies.

My mother, brothers and sisters for their moral support.

## ABSTRACT

---

Phenolic compounds in aqueous extracts of marama bean [*Tylosema esculentum* (Burchell) A. Schreiber] seed coat, sorghum (*Sorghum bicolor* L. Moench) bran and their bioactive properties

By

**Jeremiah S. Shelembe**

**Supervisor:** Dr. K. G. Duodu  
**Co-supervisors:** Prof A. Minnaar and Prof. M. Bester  
**Department:** Food Science  
**Degree:** PhD Food Science

The marama bean is an underutilised legume growing wild in the arid and semi arid regions of Southern Africa. Sorghum is an important cereal crop in arid regions of the world. The seed coats of marama beans and bran of sorghum contain antioxidant phenolic compounds with potential health benefits. Aqueous extracts for possible use as antioxidant functional food ingredient were prepared from seed coats of marama beans and bran of condensed tannin sorghum by extracting with water (water extract) or with water acidified to pH 2 (acidified water extract). Aqueous extraction was used in order to obtain an extract free from organic solvents, safe for human consumption, and containing compounds representative of those that are available under aqueous conditions of the gastrointestinal tract. The extracts were analysed for total phenolic content, total flavonoid content, condensed tannin content and protein precipitation capacity using spectrophotometric methods and for individual phenolic compounds using HPLC-MS. Proanthocyanidins were characterised using thiolysis degradation in conjunction with HPLC-MS. The antioxidant activities of the extracts were measured using ABTS, DPPH and ORAC assays. Extracts were also evaluated for protective effect against free radical induced human erythrocytes haemolysis, oxidative DNA damage and human LDL oxidation.

Extracts from marama bean seed coats had significantly higher total phenolic content, total flavonoid content, condensed tannin content, protein precipitation capacity and phenolic compound concentration than equivalent extracts from condensed tannin sorghum bran. Three phenolic acids and three flavonols esterified to gallic acid were identified in the extracts from marama bean seed coats. Extracts from condensed tannin sorghum bran had six major phenolic acids, two phenolic aldehydes and three flavanones. Proanthocyanidins in extracts from marama bean seed coats were predominantly highly galloylated prodelphinidins while those in extracts from condensed tannin sorghum bran were procyanidins.

Extracts from marama bean seed coats had higher antioxidant activity and protective effects against free radical induced erythrocyte haemolysis and LDL oxidation compared to equivalent extracts from condensed sorghum bran. Extracts from condensed tannin sorghum bran showed some protective effect against oxidative DNA damage. However, extracts from marama bean seed coats gave inconclusive results probably due to prodelphinidins binding to DNA. Extraction of marama bean seed coats under acidic condition resulted in reduction in phenolic compound content, antioxidant activity and protective effect against erythrocyte haemolysis, possibly due to co-precipitation of phenolic compounds with interpolymer complex precipitate formed between highly galloylated condensed tannins and cell wall polysaccharides at pH 2. In contrast, extraction of condensed tannin sorghum bran under the acidic condition resulted in an extract with significantly higher phenolic content and protective effect against erythrocyte haemolysis than the water extract possibly due to enhanced extraction of free and esterified phenolics and release of bound phenolic compounds. Water extracts and acidified water extracts (from marama bean seed coats or sorghum bran) did not show significant differences in their protective effect against oxidative DNA damage and LDL oxidation.

Extraction under acidic condition may be the preferred method for sorghum bran because it increases recovery of phenolic compounds, but not for marama bean seed coats because it causes reduction in phenolic compound content. The findings of this study show that the extracts have a potential to reduce oxidative stress which is implicated in many chronic diseases such as neurodegenerative diseases, cancer and cardiovascular disease. The extracts can be used in the development of functional foods with potential health benefits.

## TABLE OF CONTENTS

---

Title page.....	i
DECLARATION.....	ii
ACKNOWLEDGEMENTS .....	iii
ABSTRACT.....	iv
LIST OF TABLES .....	x
LIST OF FIGURES .....	xii
<b>1. INTRODUCTION .....</b>	<b>1</b>
<b>2. LITERATURE REVIEW .....</b>	<b>3</b>
2.1.1. The marama bean .....	3
2.1.2. Chemistry of plant phenolic compounds and their content in marama bean seed coats and condensed tannin sorghum bran.....	4
2.1.2.1. Phenolic compounds.....	4
2.1.2.2. Phenolic acids .....	5
2.1.2.3. Flavonoids .....	8
2.1.2.4. Tannins .....	12
2.1.3. Extraction of phenolic compounds.....	15
2.1.4. Antioxidant activity and structure relationship .....	17
2.1.5. <i>In vitro</i> antioxidant activity of legume extracts .....	20
2.1.6. Oxidative stress and chronic diseases .....	22
2.1.7. Protective effect of polyphenolic antioxidants against chronic diseases ..	23
2.1.8. Methods for the evaluation of antioxidant activity of extracts.....	26
2.1.9. Gaps in knowledge.....	29
<b>3. HYPOTHESES AND OBJECTIVES .....</b>	<b>31</b>
3.1. Hypotheses .....	31
3.2. Objectives.....	31
<b>4. RESEARCH.....</b>	<b>33</b>
<b>4.1. Characterization of phenolic acid and flavonoid compounds in aqueous     extracts prepared from marama bean seed coats and condensed tannin sorghum     bran</b>	
4.1.1. Abstract .....	34
4.1.2. Introduction .....	35

4.1.3.	Materials and methods .....	36
4.1.3.1.	Materials .....	36
4.1.3.2.	Methods .....	37
4.1.3.2.1.	Sample preparation .....	37
4.1.3.2.2.	Preparation of extracts .....	37
4.1.3.2.3.	Total phenolic content .....	38
4.1.3.2.4.	Total flavonoid content.....	38
4.1.3.2.5.	Characterization of phenolic acids and flavonoids in aqueous extracts by HPLC-MS .....	39
4.1.3.3.	Statistical analysis.....	40
4.1.4.	Results and discussion.....	40
4.1.4.1.	Yield, total phenolic and total flavonoid content of aqueous extracts .....	40
4.1.4.2.	Characterization of phenolic acid and flavonoid compounds by HPLC-MS .....	44
4.1.4.2.1.	Marama bean seed coats .....	44
4.1.4.2.2.	Condensed tannin sorghum bran .....	53
4.1.5.	Conclusion.....	60
<b>4.2.</b>	<b>Characterization of proanthocyanidins in aqueous extracts from marama bean seed coats and condensed tannin sorghum bran .....</b>	<b>62</b>
4.2.1.	Abstract .....	63
4.2.2.	Introduction.....	64
4.2.3.	Materials and methods .....	65
4.2.3.1.	Materials .....	65
4.2.3.2.	Methods .....	66
4.2.3.2.1.	Sample preparation .....	66
4.2.3.2.2.	Determination of condensed tannin content by vanillin-HCl method.....	66
4.2.3.2.3.	Determination of condensed tannin content by Butanol-HCl assay.....	67
4.2.3.2.4.	Determination of protein precipitation capacity ....	68
4.2.3.2.5.	Identification of terminal and extension units in proanthocyanidins by HPLC-MS .....	69

4.2.3.3.	Statistical analysis.....	71
4.2.4.	Results and discussion.....	72
4.2.4.1.	Condensed tannin content and protein precipitation capacity of methanolic extracts.....	72
4.2.4.2.	Condensed tannin content and protein precipitation capacity of aqueous extracts .....	73
4.2.4.3.	Characterization of condensed tannins in extracts.....	75
4.2.4.3.1.	Marama bean seed coat proanthocyanidins .....	75
4.2.4.3.2.	Condensed tannin sorghum bran proanthocyanidins. ....	79
4.2.4.3.3.	Flavan-3-ol composition and mean degree of polymerization of proanthocyanidins in extracts.....	82
4.2.5.	Conclusions .....	85
<b>4.3.</b>	<b><i>In vitro</i> antioxidant activity and protective effect of aqueous extracts prepared from marama bean seed coat and condensed tannin sorghum bran against oxidative damage of biological molecules .....</b>	<b>86</b>
4.3.1.	Abstract .....	87
4.3.2.	Introduction .....	88
4.3.3.	Materials and methods .....	89
4.3.3.1.	Materials .....	89
4.3.3.2.	Methods .....	90
4.3.3.2.1.	Sample preparation and extraction .....	90
4.3.3.2.2.	ABTS free radical scavenging assay .....	90
4.3.3.2.3.	DPPH free radical scavenging assay .....	91
4.3.3.2.4.	Oxygen radical absorbance capacity assay (ORAC). ....	92
4.3.3.2.5.	AAPH-mediated red blood cell haemolysis assay.....	93
4.3.3.2.6.	AAPH-mediated oxidative DNA damage .....	94
4.3.3.2.7.	Copper-catalyzed human low density lipoprotein oxidation .....	94
4.3.3.3.	Statistical analysis.....	95
4.3.4.	Results and discussion.....	95
4.3.4.1.	Antioxidant capacity .....	95





4.3.4.2.	Effect of extracts on AAPH-mediated red blood cell haemolysis	98
4.3.4.3.	Effect of aqueous extracts on AAPH-mediated oxidative DNA damage	101
4.3.4.4.	Effect of extracts on copper-catalyzed human LDL oxidation.....	106
4.3.5.	Conclusions .....	110
<b>5.</b>	<b>GENERAL DISCUSSION.....</b>	<b>112</b>
5.1.	Discussion of methods used .....	112
5.2.	Discussion of study results.....	122
<b>6.</b>	<b>CONCLUSIONS AND RECOMMENDATIONS .....</b>	<b>138</b>
<b>7.</b>	<b>REFERENCES .....</b>	<b>140</b>
<b>8.</b>	<b>PUBLICATIONS.....</b>	<b>162</b>

## LIST OF TABLES

---

Table 2.1.1. Flavonoid subclasses and substitution pattern (structures shown in Fig. 2.1.3) of representative compounds .....	10
Table 2.1.2. Major flavonoids reported in sorghum grain .....	11
Table 4.1.1. Effect of extraction under acidic condition on yield, total phenolic and total flavonoid content of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran .....	41
Table 4.1.2. Mass spectra and UV data for selected phenolic acid and flavonoid standard compounds.....	45
Table 4.1.3. Phenolic acid and flavonoid compounds identified in aqueous extracts prepared from marama bean seed coats.....	51
Table 4.1.4. Effect of extraction under acidic condition on phenolic acid and flavonoid compounds in aqueous extracts from marama bean seed coat .....	52
Table 4.1.5. Mass spectra and UV data of phenolic compounds identified in aqueous extracts from condensed tannin sorghum bran .....	57
Table 4.1.6. Effect of extraction under acidic condition on phenolic compounds in aqueous extracts from condensed tannin sorghum bran.....	59
Table 4.2.1. Condensed tannin content and protein precipitation capacity of methanol extracts from marama bean seed coat and condensed tannin sorghum bran .....	72
Table 4.2.2. Effect of extraction under acidic condition on condensed tannin content and protein precipitation capacity of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran .....	74
Table 4.2.3. Mass spectra data of thiolysis degradation products of proanthocyanidins in extracts from marama bean seed coats .....	78
Table 4.2.4. Mass spectra data of thiolysis degradation products of proanthocyanidins in extracts from condensed tannin sorghum bran .....	81
Table 4.2.5. Constitutive unit composition (relative percentage by peak area) of proanthocyanidins in polymer (FIII) and oligomer (FII) fractions from aqueous extracts from marama bean seed coat and condensed tannin sorghum bran .....	83

Table 4.3.1. Effect of extraction under acidic condition on antioxidant activity of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran.....	96
Table 4.3.2. Pearson's correlation coefficients between antioxidant activities and phenolic contents determined with different methods .....	98

## LIST OF FIGURES

---

Figure 2.1.1. Marama bean plant (A) and marama bean pod with seeds (B) .....	3
Figure 2.1.2. Chemical structure and substitution pattern of representative phenolic acids. ....	6
Figure 2.1.3. Generic structure of flavonoid compounds. ....	8
Figure 2.1.4. Basic chemical structures of flavonoid subclasses. ....	9
Figure 2.1.5. Hydrolysable tannin structure.....	13
Figure 2.1.6. Proanthocyanidin chemical structures showing the different linkages. ....	14
Figure 2.1.7. Catechol and pyrogallol representative structures.....	18
Figure 2.1.8. Flavonoid structure showing functional groups that affect antioxidant activity.	19
Figure 4.1.1. Appearance of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran after treatment; A) Marama water extract; B) Marama acidified water extract; C) Sorghum water extract; D) Sorghum acidified water extract. ....	42
Figure 4.1.2. HPLC chromatogram recorded at 280 nm and total ion current (TIC) chromatogram of phenolic compounds in A) water and B) acidified water extracts from marama bean seed coats.. ....	48
Figure 4.1.3. Mass spectra of phenolic acid and flavonoid compounds in aqueous extracts from marama bean seed coats.....	49
Figure 4.1.4. HPLC chromatogram recorded at 280 nm and total ion current (TIC) chromatogram of phenolic compound profile of A) water and B) acidified water extracts from condensed tannin sorghum bran. ....	54
Figure 4.1.5. Mass spectra of phenolic acid and aldehyde compounds in water extracts prepared from condensed tannin sorghum bran. ....	55
Figure 4.1.6. Mass spectra of flavonoid compounds in hydrolysed water extracts from condensed tannin sorghum bran. ....	56
Figure 4.2.1. Total ion current chromatogram and HPLC chromatogram recorded at 280 nm of thiolysis degradation products of proanthocyanidins in polymer fraction (FIII) from extracts prepared from marama beans seed coats.....	76
Figure 4.2.2. Mass spectra of thiolysis degradation products of proanthocyanidins in acetone extracts from marama bean seed coats.....	77

Figure 4.2.3. Total current ion and HPLC chromatograms of thiolysis degradation products of proanthocyanidins in polymer fraction from extracts prepared from condensed tannin sorghum bran. ....	80
Figure 4.2.4. Mass spectra of thiolysis degradation products of proanthocyanidins in acetone extracts from condensed tannin sorghum bran. ....	81
Figure 4.3.1. Effect of water and acidified water extracts prepared from marama bean seed coats on AAPH-mediated human red blood cell haemolysis. ....	99
Figure 4.3.2. Effect of water and acidified water extracts from condensed tannin sorghum bran on AAPH-mediated human red blood cell haemolysis. ....	100
Figure 4.3.3. Effect of water and acidified water extracts from marama bean seed coats and condensed tannin sorghum bran on AAPH-induced oxidative supercoiled plasmid pBR 322 DNA damage. ....	102
Figure 4.3.4. Effect of water and acidified water extracts from marama bean seed coats on AAPH-induced oxidative supercoiled plasmid pBR 322 DNA damage. ....	103
Figure 4.3.5. Effect of water and acidified water extracts (200 µg/ml) from condensed tannin sorghum bran on AAPH-induced oxidative DNA damage. ....	105
Figure 4.3.6. Effect of water and acidified water extracts prepared from marama bean seed coats on copper-catalyzed LDL-oxidation determined with the thiobarbituric (TBA) assay. ....	106
Figure 4.3.7. LDL oxidation kinetic curves showing how two extracts with significantly different antioxidant concentration could exhibit similar TBARS levels. ....	108
Figure 4.3.8. Effect of aqueous extracts prepared from condensed tannin sorghum bran on copper-catalyzed LDL oxidation using thiobarbituric assay (TBA assay). ....	109
Figure 5.1.1. Thiolysis degradation of proanthocyanidins in the presence of toluene- $\alpha$ -thiol. ....	117
Figure 5.2.1. Chemical structures of phenolic acid and flavonoid compounds identified in aqueous extracts from marama bean seed coats. ....	123
Figure 5.2.2. Chemical structures of phenolic compounds identified in aqueous extracts prepared from condensed tannin sorghum bran. ....	125
Figure 5.2.3. Proposed heteropolyflavan polymer structure of proanthocyanidins in extracts from marama bean seed coats. ....	128

Figure 5.2.4. Chemical structure of proanthocyanidin polymer in extracts from condensed tannin sorghum bran.....129

Figure 5.2.5. Hydrogen bonding between highly galloylated condensed tannins and cell wall polysaccharides to form interpolymer complex precipitate at low pH ...130

Figure 5.2.6. Mitigation of oxidative stress caused by reactive oxygen species by antioxidants shown as 1 to 6. AH, antioxidant and A•, oxidized antioxidant.....133

Figure 5.2.7. Summary of the protective effect of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran against induced oxidative damage of biomembrane, DNA and LDL as biomarkers of oxidative stress.....135