



**BAMBARA GROUNDNUT (*VIGNA SUBTERRANEAN*) FROM MPUMALANGA PROVINCE
OF SOUTH AFRICA: PHYTOCHEMICAL AND ANTIMICROBIAL PROPERTIES OF
SEEDS AND PRODUCT EXTRACTS**

by

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DECLARATION

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ABSTRACT

Bambara groundnut (*Vigna subterranea*) an indigenous legume cultivated in Sub-Saharan Africa has been proclaimed to have medicinal properties from communities and in rural areas. However, there is not enough scientific information to validate these claims. Therefore, this study aimed to identify possible medicinal properties of Bambara groundnut (BGN), by analysing the phytochemical and antimicrobial properties of BGN seed and product extracts from Mpumalanga province within South Africa. The BGN extracts (70% methanol, 70% ethanol, milli-Q water) from seeds and products (milk and yoghurt) were screened for the presence of alkaloids, flavonoids, phenols, riboflavin and thiamine using analytical laboratory methods for basic screening, high-performance liquid chromatography (HPLC) and gas chromatography (GC) for quantification. The antimicrobial activity involved direct bioautography and minimum inhibitory concentration (MIC) against six antibiotic-resistant microorganisms, *Acinetobacter baumannii* ATCC 19606^T, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. *aureus* ATCC 33591 and *Candida albicans* ATCC 24433. For the seed extracts, flavonoids and phenols were highly concentrated in the red and brown hulls of BGN compared to whole and dehulled BGN. Organic solvents in comparison to water yielded the highest concentration of flavonoids, whilst water yielded the highest concentration for phenols. Flavonoid compounds that were detected at the highest concentrations were rutin (24.458 ± 0.234 mg.g⁻¹, brown hull extracted with 70% methanol), quercetin (0.070 ± 0.043 mg.g⁻¹, red hull extracted with 70% methanol), kaempferol (0.391 ± 0.161 mg.g⁻¹; brown hull extracted with 70% ethanol) and myricetin (1.800 ± 0.771 mg.g⁻¹; red hull extracted with 70% methanol). For phenol compounds, gallic acid (0.009 ± 0.004 mg.g⁻¹; brown hull extracted with milli-Q water), catechin (0.026 ± 0.041 mg.g⁻¹; brown hull extracted with milli-Q water), methyl gallate (0.008 ± 0.013 mg.g⁻¹; brown whole extracted with milli-Q water), chlorogenic acid (0.115 ± 0.199 mg.g⁻¹; brown hull extracted with milli-Q water) and ellagic acid (0.105 ± 0.082 mg.g⁻¹; red hull extracted with milli-Q water) were detected. Vitamins B₁ and B₂ (riboflavin and thiamine) were mostly present in milli-Q water extracts. Black-eye hull had the highest concentration of thiamine (vitamin B₁) and riboflavin (vitamin B₂) consisting of 0.072 mg.g⁻¹ (extracted with milli-Q water) and 0.002 mg.g⁻¹ (extracted with 70% ethanol and 70% methanol). Red and brown hull extracts from organic solvents (70% ethanol and 70% methanol) showed the highest antimicrobial activity, whereas the whole, dehulled and hulls (black-eye and brown-eye) extracts had no antimicrobial activity. As for BGN products extracts, flavonoid compounds that were detected at the highest concentrations were rutin (5.694 mg.g⁻¹, whole BGN milk, milli-Q water), quercetin (0.703 mg.g⁻¹, whole BGN yoghurt, milli-Q water) and myricetin (0.987 mg.g⁻¹, whole BGN yoghurt, 70% ethanol). Additionally highest

concentrations of phenol components were gallic acid (0.009 mg.g⁻¹, dehulled BGN yoghurt, 70% methanol) and ellagic acid (0.353 mg.g⁻¹, whole BGN yoghurt, 70% methanol). Thiamine and riboflavin was not detected for either BGN milk or BGN yoghurt. Antimicrobial activity of BGN seed extracts on *C. albicans* ATCC 24433 displayed the highest cell reduction of 55.8% (brown hull, 70% methanol) at 100 µg.mL⁻¹, *P. aeruginosa* ATCC 27853 cell reduction of 43.9% (red hull, 70% ethanol) at 30 µg.mL⁻¹, *E. faecalis* ATCC 29212 cell reduction of 42.9% (brown hull, 70% ethanol) at 50 µg.mL⁻¹, *S. aureus* subsp. *aureus* ATCC 33591 cell reduction of 22.7% (red hull, 70% ethanol) at 5 µg.mL⁻¹ while *K. pneumoniae* subsp. *pneumoniae* ATCC 700603 a cell reduction of 30.8% (red hull, 70% ethanol) at 20 µg.mL⁻¹. No activity was observed against *A. baumannii* ATCC 19606^T under the conditions tested. As for antimicrobial activity of BGN product extracts, no activity was observed against the selected microorganism strains under the conditions tested. Lastly GC-MS analysis of BGN seed extracts showed the presence of alkaloid compounds (e.g. 9-Octadecenamide, (Z)-, ethanol 1-methoxy- benzoate, 1-Monolinoleoylglycerol trimethylsilyl and ether butylated hydroxytoluene), with potential to act as antimicrobial agents and antioxidants.

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DEDICATION

I'd like to dedicate this thesis to the Almighty, my loving family (Mrs Fahiema Dyers, Mr Faizel Harris, Mr Zaid Dyers, Ms Tharwat Harris and Ms Aanisah Harris) as well as my friends who have always stood by my side and supported me throughout this entire journey. As without them, I would not have been where I am today.

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GLOSSARY

BGN = Bambara groundnut

BGNH = Bambara groundnut hulls

DBGN = Dehulled Bambara groundnut

DBGNM = Dehulled Bambara groundnut milk

DBGNY = Dehulled Bambara groundnut yoghurt

Phytochemical = plant based chemical with biologically active compounds

WBGN = Whole Bambara groundnut

WBGNM = Whole Bambara groundnut milk

WBGNY = Whole Bambara groundnut yoghurt

CHAPTER 1

MOTIVATION OF THE STUDY

1.1 Introduction

Legume seed plants are one of the leading sources of food protein in the world. Legumes provide nutritional value and are of economic significance to the world's population (Baoua *et al.*, 2014). They play an important role in dietary composition of human nutrition for low income consumers from developing countries as well as provide essential source of protein for vegetarians, especially vegans. Legumes usually considered as the "poor man's" meat, contain a good source of slow release carbohydrates, as well as high protein content (Tharanathan & Mahadevamma, 2003).

Several proteins and peptides from legume seeds can be included into the category of nutraceuticals (Carbonaro *et al.*, 2014). The term "nutraceuticals" is a combination of the words "nutrition" and "pharmaceutical", and can be defined as food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease. The interest in plant-based nutraceuticals and products containing nutraceuticals has grown significantly in recent years due to the vast number of beneficial properties attributed to the high content of phytochemicals. These phytochemicals are non-nutritive compounds found in plants and have been shown to have disease-preventive properties (López-Gutiérrez *et al.*, 2015).

It is reported by Scio *et al.* (2012) that infectious disease is one of the worlds' leading causes of premature death, killing almost 50 000 people daily. In recent years, drug resistance of human pathogenic bacteria has been commonly reported from all over the world (Scio *et al.*, 2012). Subsequently, a substantial rise in infectious diseases has led to an increase in mortality rates, due to pathogenic microbes having an increased resistance to antimicrobial agents/drugs and with indiscriminate use of synthetic antibiotics, thus creating a constant search for antimicrobials particularly of natural origin. Medicinal plants contain a variety of bioactive compounds having healing/therapeutic potential. Ajiboye & Oyejobi (2017) mentions that according to the World Health Organization, a medicinal plant is any plant which in one or more of its organ contains constituents that can be used for the synthesis of useful drugs. Additionally, medicinal plants contain biologically active chemical constituents or phytochemicals such as saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds which have curative properties (Ajiboye & Oyejobi, 2017). There have been reports of plants being used traditionally as antimicrobials (Vats *et al.*, 2012). Hence, with increased incidences of resistance from bacteria to antibiotics, natural products from plants could be considered as interesting alternatives. Some plant extracts and its phytochemicals are known to exhibit antimicrobial activity, and can be of great significance in therapeutic treatments (Scio *et al.*, 2012).

Bambara groundnut [BGN] (*Vigna subterranea*), an indigenous legume, is cultivated in sustainable agricultural systems in sub-Saharan Africa and is primarily grown for its seeds (Karunaratne *et al.*, 2010). The legume provides a source of protein in many regions of Africa where it is commonly grown by locals to sustain their families (Mwale *et al.*, 2007). The BGN protein composition (16-25%) is highly comparable or superior to other legumes, providing an important supplement to cereal-based diets (Karunaratne *et al.*, 2010).

Bambara groundnut has been the focus of extensive research in the past decade as a functional food alternative, resulting in products developed from Bambara groundnut as well as characterizing its physicochemical and phytochemical potential. It is reported by Nyau *et al.* (2015) that BGN from Zambia contain antioxidant activities that are similar to legumes such as lentils, common beans and chickpeas. Bambara groundnuts from Nigeria showed the presence of phytochemicals specifically flavonoids, tannins and alkaloids (Olaleye *et al.*, 2013). Additionally, Olaleye *et al.* (2013) reports that the alkaloids in BGN have analgesic properties, with the same effect as painkillers or pain relievers.

Further applications of BGN include, the physicochemical properties of BGN fibres valuable to the food industry as thickening agents, stabilisers, health ingredients as well as cryoprotectants in frozen dairy products (Maphosa & Jideani, 2015). Recent studies have proven BGN to be promising in improving food security through its food products (milk, yoghurt, fibre). Milk from BGN has been reported with acceptable physical attributes and comparable to existing plant milk (Hardy & Jideani, 2017). The nutritional profile of BGN milk is high enough to sustain the growth of probiotics (Murevanhema & Jideani, 2013) leading to the development of BGN probiotic beverage or yoghurt. Also, Bambara groundnut (sourced in Nigeria) yoghurt was more acceptable compared to soy yoghurt (Murevanhema & Jideani, 2013; Falade *et al.* 2015). These findings indicate that BGN have the potential for use in the nutraceutical industry and its consumption could possibly offer some health benefits (Nyau *et al.*, 2015).

The medicinal use of BGN is based on information obtained from communities in several parts of Africa, where this crop is reportedly used for the treatment of various ailments. The water boiled from the maize and pulse mixture is ingested to treat diarrhoea. Anon (2011) reported that raw BGN can be chewed and swallowed to treat nausea suffered by pregnant women, while in Kenya, the Luo tribe use BGN to cure diarrhoea (Mkandawire, 2007). The traditional utilization of BGN to treat several ailments is notable and therefore creates an opportunity for detailed scientific study on the pharmaceutical value of the crop (Mølgaard *et al.*, 2011). BGN hulls have been reported to have quantities of phenolic compounds. In addition, the hulls are an inexpensive source of nutraceuticals and functional ingredients (Klompong & Benjakul, 2015). According to Mbagwu *et al.* (2011) the phytochemical screening carried out on whole BGN (sourced from Idemili in Anambara State,

Nigeria) showed the presence of valuable phytochemicals and contained a high percentage of alkaloids compared to other legumes.

However, not enough is known about the phytochemicals and antimicrobial properties of Bambara groundnut seeds and products from Mpumalanga Province within South Africa. As this would provide yet another means of highlighting the potential of BGN as an underutilised legume and tap into ways of encouraging more sustained production and use of BGN (Jideani & Diedericks, 2014). In this study, BGN seeds, BGN milk and BGN yoghurt were extracted with solvents (70% methanol, 70% ethanol and milli-Q water) and analysed for the presence of phytochemicals and antimicrobial activity.

1.2 Statement of the Research Problem

The medicinal role of BGN is largely based on information obtained from communities in several parts of Africa, where this crop is reportedly responsible and useful in the treatment of various ailments. The traditional uses of BGN to treat several ailments are notable, and present a gap for detailed study on the pharmaceutical value of the crop. Though, there is no scientific evidence to validate the medicinal properties of BGN, it is important to evaluate the phytochemical and antimicrobial properties of Bambara groundnut (*Vigna subterranea*) seeds and products (milk and yoghurt) from Mpumalanga province, South Africa extracted with solvents (70% methanol, 70% ethanol and milli-Q water). Analysing active compounds from BGN seed extracts, BGN milk and BGN yoghurt may lead to the discovery of its medicinal value (Murevanhema & Jideani, 2013; Klompong & Benjakul, 2015; Nyau *et al.*, 2015)

1.3 Broad Objectives of the Study

The aim of this study was to evaluate the phytochemical and antimicrobial properties of Bambara groundnut extracts and products (milk and yoghurt) from Mpumalanga province, South Africa.

1.4 Specific Objectives of the Study

Specific objectives were to:

1. Identify some phytochemicals in five whole, dehulled and hull BGN seeds (black, black-eye, brown, brown-eye and red) based on extraction solvent extract.
2. Identify some phytochemicals in BGN milk and yoghurt based on extraction solvent.
3. Determine the antimicrobial potential of each BGN seed (black, black-eye, brown, brown-eye and red) based on extraction solvent (ethanol, methanol and water).
4. Determine the antimicrobial potential of BGN milk and BGN yoghurt.

1.5 Hypotheses

The following hypotheses of the study were:

1. It is hypothesized that BGN seed extract, milk and yoghurt extracts will contain some phytochemicals and will exhibit anti-microbial activity.
2. The different solvents will differ in the phytochemicals extracted and their antimicrobial activity
3. The BGN seeds will differ in their phytochemicals and antimicrobial activity

1.6 Delineation of the Research

The Bambara groundnut [BGN] seeds used in this study were purchased from Mpumalanga province in South Africa. Five BGN seed varieties will be utilized for extraction, namely black, black-eye, brown, brown-eye and red. Three extraction solvents will be used: ethanol, methanol and water. A combination of all listed five BGN varieties will be used for the production of BGN milk and BGN yoghurt.

1.7 Significance/Importance of the Study

It is expected that BGN extracts and products used in this study will highlight its potential use as a functional food due to its phytochemical and antimicrobial content as well as increasing the value and overall importance of BGN as an underutilized legume in South Africa. Furthermore, these bioactive (phytochemicals and antimicrobials) compounds found in BGN could be used as therapeutics as a functional food for disease prevention and in time allow for BGN and BGN products enter the pharmaceutical market. The research study will align with the grand challenge of “Farmer to Pharma” Ten-Year Plan for South Africa. Lastly this study will result in human capital development.

1.8 Expected Outcomes

It is expected that the study will highlight the potential of BGN as a functional food due to its phytochemical and antimicrobial content as well as increasing the value in South Africa. Finally, the completion of this study will increase human capacity building, through Master’s qualification.

1.9 Thesis Overview

Chapter 1 is the introduction and motivation of the study. Chapter 2 looks at literature research of BGN background and application, phytochemicals found from plant origin, importance of antimicrobial activity and pathogenic resistance. Chapter 3 is the first research chapter looking at some phytochemical properties of BGN seeds extracts and identifying individual seed colour phytochemicals. Chapter 4 is the second research chapter, specifically looking at the phytochemical properties in BGN products milk and yoghurt.

Chapter 5 is the study of antimicrobial properties of BGN seed extracts, BGN milk and BGN yoghurt. Lastly, chapter 6 concludes with a general summary and recommendations of the study.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The risk of hunger has become a major concern globally. Therefore new sources of food must be obtained to support the constant increase of the human population. To help sustain a balance between the steadily increasing population and agricultural productivity, plant materials especially high in protein seeds should be utilized as components in food stuffs. The essential role legumes play in the diets of many populations in protein-deficient countries has been emphasized by numerous authors. This has incited research on various traits or factors of legume utilization (Sefa-Dedeh & Yiadom-Farkye, 1988). In the world presently one of the few but major growing concerns experienced in developing countries is how to increase the shortage of protein in the diets of a large segment of the population (Baoua *et al*, 2014). Plant materials high in protein will therefore be the key to contribute to the diets of the population in developing countries, for instance one of the high protein plant seeds grown in sub-Saharan Africa is Bambara groundnut (*Vigna subterranea* (L.) Verdc) (Karunaratne *et al*, 2010).

2.2 Bambara Groundnut Background

The botanical name of Bambara groundnut is *Voandzeia subterranean* (L.) *thouars*, synonyms of *Vigna subterranea* and belongs to the plantae of the family of *Fabaceae* and sub family of *Faboidea*. The common names of Bambara groundnut are Okpa (Nigeria Igbo), Gurujia (Nigeria, Hausa), Njugo bean (South Africa), Nzama (Malawi), Ntoyo (Ci Bembia) or Katoyo (Zambia) (Okonkwo & Opara, 2010). A few other names are, Congo groundnut, Congo goober, Madagascar groundnut, earth pea, baffin pea, voandzou, nzama (Malawi), indhlubu, and underground bean (Stephens, 2012).

Bambara groundnut (BGN) is a legume plant seed with the importance as being a source of protein in many regions of Africa where it is mainly grown by the local rural population for the sustenance of their families. Its protein content is comparable or even superior to other legumes, making Bambara groundnut a worthy supplement for cereal-based diets (Mwale *et al*, 2007). Bambara groundnut provides a significant source of protein and carbohydrates to the rural population and provides an additional income to subsistence farmers (Sesay, 2009). The BGN fruits are produced underground similar to peanut (*Arachis hypogea*). The pods are hard, wrinkled when dried and each pod contains at least one or two seeds. The colour of the BGN seed differs, ranging from black to white and may be speckled with various colours. Bambara groundnut grows well in and is adapted to both barren and wet climates as well as also tolerates poor soil in which case most other legumes crops will not survive (Onimawo *et al*, 1998). Bambara groundnut also contains high lysine

content which complements the low lysine content in cereals. It fixes nitrogen in the soil through nodulation, and thus reducing the need for application of fertilizers. It is a drought, pest, disease resistant crop and it also reduces pests in the field (Tibe *et al.*, 2007).

2.3 Nutritional Content of Bambara Groundnut

As seen in Table 2.1 Bambara groundnut provides a range of numerous advantages including the fact that it is a protein rich food with more than 50% carbohydrate content (Onimawo *et al.*, 1998). The energy value of Bambara groundnut seed is greater than that of various other grain legumes (Adu-Dapaah & Sangwan, 2004; Okonkwo & Opara, 2010).

Table 2.1 Nutritional composition of BGN (Onimawo *et al.*, 1998; Adu-Dapaah & Sangwan, 2004; Okonkwo & Opara, 2010)

Carbohydrate	49 – 63.5%
Protein	15 – 25%
Fat	4.5 – 7.4%
Fibre	3.2 – 4.4%
Ash	0.7%
Cholesterol	0.01%

2.4 Utilisation and Consumption of Bambara Groundnuts

Legume species like the bambara groundnut, kidney bean, lima bean, pigeon pea and jack bean which show remarkable adaptation to tropical conditions are less commonly used by the people due to lack of adequate information on their nutritional potentials. Of late, however, because of the severity of the protein deficit, significant interest has been incited on the utilization of these relatively neglected legume sources for human food and as components for livestock feed (Apata & Ologhobo, 1994).

The crop has become a point of interest for utilization and cultivation in the dry savannah zones. In Botswana Bambara groundnut is cultivated by 90% of the farmers of which about 63% farmers grew the crop for consumption, 12% for sale and 25% for both consumption and sale (Jideani & Mpotokwana, 2009).

Bambara groundnuts are consumed in various ways in both immature and fully matured form, whilst immature, the Bambara groundnut seeds may be consumed fresh (raw), grilled or even boiled before consumption. These seeds are also more palatable when compared to the mature seeds which are hard. To soften the mature seeds and render them more pleasant tasting and sweet, the seeds are boiled or roasted (Stephens, 2012). Immature seeds are normally consumed as a snack by boiling the fresh seed with salt or roasting the seeds, and may also be pounded with or without hulls and boiled into a stiff

porridge. Mature seeds may be consumed as is by boiling in water, or it is often ground into flour and consumed as porridge by mixing the flour with butter or oil (Jideani & Diedericks, 2014).

Other traditional uses of bambara, namely, is made into a relish mixed with onions, tomatoes and oil, milled into flour and used to make small flat cakes or biscuits, mixed with cereals and used to make a porridge, mixed with maize to make a very filling porridge, boiled and eaten together with plantains or roasted and eaten as a snack (De Kock, 2015). Some seeds are cooked in pods and eaten as a snack and cooked with samp and eaten as a main meal, and it takes roughly 2-3 hours to cook. In Botswana it is grown as an intercrop (growing two species in proximity) with cereal crops such as maize, sorghum and millet and is rarely grown as a sole crop. This crop has diverse uses, providing nutritious food, animal feed, and a source of income for the rural dwellers. However, the digestion and bioavailability of the nutrients in the Bambara seeds for animal and human nutrition is limited by anti-nutrients such as trypsin inhibitors and condensed tannins (Tibe *et al.*, 2007).

2.5 Bambara Groundnut Products and Functionality

It is a legume of African origin and used locally as a vegetable and can be consumed at any stages of maturation (Atiku *et al.*, 2004; Okonkwo & Opara, 2010). It is reported by Boateng *et al.* (2013) that the solubility observed for the protein isolate of BGN promotes their potential use in beverages namely vegetable milk for lactose intolerant individuals. Bambara groundnut has potential for integration into various human foods, whereby providing useful plant proteins. Characteristics of BGN such as high water and oil absorption of protein isolates is an indication that they will do well in terms of physicochemical properties in the development of vegetable milk (Boateng *et al.*, 2013).

Food products such as low-fat spread, meat replacers, milks and emulsifiers have been developed from soy, peanut, and cowpea, which have received financial support from government and international organisations, however not enough similar research has been done on Bambara groundnut (Boateng *et al.*, 2013). Bambara groundnut seeds can be used to create vegetable milk that is comparable to soy milk (Jonah *et al.*, 2010; Bamshaiye *et al.*, 2011). Tests regarding protein functionality of BGN seed indicate that it can compete with or replace other conventional flours in a range of processed products. Bambara groundnut makes a complete food, as it contains sufficient quantities of proteins, carbohydrates and lipid (Massawe *et al.*, 2005). Shelf stable patented products were previously developed by Murevanhema & Jideani (2013) and Hardy & Jideani (2016) from BGN, namely milk and probiotic beverage, demonstrating that BGN is more than suitable for producing lactose intolerant or vegetarian substitutes for dairy milk and yoghurt. Milk produced from Bambara groundnut was preferred to that of other pulses (cowpea, pigeon pea and soybean) due to its flavour and colour (Alakali & Satimehin, 2007). Milk from Bambara groundnut is consumed

in several ways (Oluwole *et al.*, 2007). The milk is made by soaking Bambara groundnut overnight, homogenizing and get rid of insoluble material, producing a milky liquid. The beany taste can be removed by dry-frying the beans after soaking but before homogenization (Brough *et al.*, 1993; Hillocks *et al.*, 2012). Bambara milk produced in this way, were more preferred by the taste panel (Brough *et al.*, 1993). An assessment of BGN milk and milks prepared from cowpea, pigeon pea and soybean were made using sensory and instrumental analysis. Sensory analysis exhibited that all milks were acceptable, with Bambara groundnut ranking first in the trial. The lighter colour of Bambara groundnut milk was more acceptable to the taste panel but there was no correlation between viscosity measured on a viscometer and viscosity perceived by the taste panel (Brough *et al.*, 1993).

2.6 Plant Phytochemicals

The diverse usage of herbal remedies and health care preparation, such as those described in the ancient text has been traced to the occurrence of natural product with medicinal properties. In modern times, the focus has been shifted back to plants as sources of therapeutic agents due to their more favorable characteristics. These characteristics among others are reduced cost, relative lower probability of adverse reactions compared to contemporary conventional pharmaceuticals (Buvanewari *et al.*, 2011). For a long period of time (prehistoric ages), plants have been a source of motivation for novel drug compounds, as plant based medicines have made large contributions to human health and well-being. With the increasing occurrence of microorganisms developing resistance to antibiotics, there is an need to develop new antimicrobial compounds which are nontoxic and easily affordable (Seth & Sarin, 2010; Santosh *et al.*, 2013).

Phytochemicals from many indigenous plants are increasingly recognized as important antimicrobial and anticancer therapies. Tropical areas like Africa are the main sources of patentable plant products and have indigenous populations with traditional medicinal knowledge (Avrelija & Walter, 2010). The medicinal value lies in chemical substances that produce a definite physiological action on the human body. It is mentioned in previous studies by Espín *et al.* (2007) that consuming foods derived from plant origin results in a variety of health benefits. The few important bioactive constituents of plants are namely alkaloids, tannins, flavonoids, and phenolic compounds. Phytochemicals exhibit a wide range of biological effects as a consequence of their antioxidant properties, several types of polyphenols, namely phenolic acid, hydrolysable tannins and flavonoids show anticarcinogenic and anti-mutagenic effects (Okwu, 2005).

Diets that have an abundance of foods containing phytochemicals are protective against a variety of diseases (Okwu, 2005). Quite a few of these indigenous medicinal plants are used as spices and food plants (Edeoga *et al.*, 2005). Consequently, consumers are

increasing their preferences for the consumption of nutraceuticals due to their health benefits (López-Gutiérrez *et al.*, 2015).

Nutraceuticals have appeared in the pharmaceutical market in various forms (capsules, pills, powders, vials etc.) containing food bioactive compounds. The phytochemicals contain several groups of polyphenols some examples are anthocyanins, proanthocyanidins, flavanones, isoflavones etc. (Espín *et al.*, 2007).

Legumes are the main protein sources of diet, they are high in vitamins namely thiamine, riboflavin and niacin which help to release energy from nutrients. Legumes contain non-nutritive compounds that are anti-nutrients such as trypsin inhibitor, phytate, and saponin which are glycoside composed of a lipid soluble glycone that consists of either a sterol or a triterpenoid which are phytochemicals (Mbagwu *et al.*, 2011).

2.6.1 Alkaloids

Alkaloid is a basic, nitrogen-containing compound usually found in higher plants (plants of relatively complex or advanced characteristics). Alkaloids by definition are organic compounds consisting of carbon atom skeletons that also include nitrogen (Lansky *et al.*, 2013). It is mentioned by Panter (2004) who elaborates on Pelletier definition of alkaloid is that “an alkaloid is a cyclic compound containing nitrogen in a negative oxidation state which is of limited distribution in living organisms”. Panter (2004) concludes that the definition includes alkaloids with nitrogen as part of a heterocyclic system and extra cyclic bound nitrogen.

Several thousand alkaloids have been identified and characterized in plants. Over 20% of higher-plant families contain alkaloids, therefore alkaloids are the most ubiquitous and significant class of plant toxins (Panter, 2004). Although it has been said that alkaloids are generally waste by-products of plant metabolism, they may play a role in binding ionic nitrogen needed by seedlings. Alkaloids were originally related with the plant kingdom, however progressively more alkaloid compounds are being discovered in microorganisms, fungi, marine invertebrates, insects, and higher animals (Roberts and Wink, 1998; Muzquiz, (2000). Numerous of these alkaloids have led to the discovery of new pharmaceuticals and novel treatments for diseases in humans and animals. Quinolizidine alkaloids are generally found in the genera of the Fabaceae (legume) family. This family is enormous in addition to being commercially important, and contains hundreds of quinolizidine alkaloids (Panter, 2004).

Lansky *et al.* (2013) reported that alkaloids are among the most physiologically active classes of compounds found in plants, often affecting neurological and other vital physiological functions in animals. Various alkaloids have been used for years in medicine and some are still prominent drugs today. Hence this group of compounds has had great importance in many fields of scientific endeavour and continues to be of great interest today

(Muzquiz, (2000). They generally tend to have strong actions on a huge variety of body tissues. An example of an action of alkaloid is caffeine, when present in tea and coffee is responsible for their stimulating action on the nervous system. Other popular and well known medicinal alkaloids are quinine, codeine, morphine, nicotine, etc. (Mukhopadhyay, 2000).

Plants containing alkaloids are an intrinsic part of the regular Western diet (Koleva *et al.*, 2012). Alkaloids are considered anti-nutritional because commonly they have a bitter taste which could limit food intake and some alkaloids have toxic effects. Furthermore, they have medicinal uses, thus the interest in screening for alkaloids in plants could be due to their potential medical and pharmacologic uses (Rodriguez *et al.*, 1998).

2.6.2 Flavonoids in Plants

Flavonoids also known as bioflavonoids, are a class of plant secondary metabolites which are present in photosynthesizing cells and are usually found in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey (Shohaib *et al.*, 2011; Marques *et al.*, 2012). Flavonoids are polyphenolic compounds that are present and scattered widely in the plant kingdom and include several subclasses, such as flavonols, flavones, flavanols, anthocyanins, flavanones and isoflavones as defined in Figure 2.1 (Xu & Lee, 2001).

The intake of flavonoid-rich foods potentially has beneficial effects on health, with different classes of flavonoids having different effects on disease risk. It is mentioned by Peluso & Palmery (2015) that the relationship between flavonoid intake and risk of disease appeared to be non-linear. Additionally, unlike vitamins, there are no symptoms of deficiency for flavonoids. Thus, our body treats them like other xenobiotics. Organic xenobiotics consist of a set of chemical compounds which span a wide variety of characteristics. There is no recommended daily allowance (RDA) for flavonoids. Flavonoids could act both as drugs and pro-drugs with pharmacological and toxicological promiscuity (Peluso & Palmery, 2015). Evidence suggested by Di Carlo *et al.* (1999) that inadequate intake of certain nutrients prompt humans to chronic degenerative diseases. Particularly it was confirmed that intake of an adequate diet rich in vegetable and fruit reduces the likelihood of cardiovascular diseases, but the exact mechanisms for this protective effect are inadequately understood (Cao *et al.*, 1997).

Cao *et al.* (1997) also states that the biological and pharmacological effects of a flavonoid compound may depend upon its behaviour as either an antioxidant or a pro-oxidant. However, increased circulating antioxidants are believed to be important. This is supported by recent trials mentioned by Di Carlo *et al.* (1999) reporting that the intake of antioxidant flavonols predict a reduced rate of coronary heart disease mortality in elderly men. Epidemiological studies show that dietary consumption of foods containing flavonoids, quercetin, catechin and epicatechin particularly red wine as well as in fruits and vegetables, are inversely associated with subsequent coronary heart disease (Di Carlo *et al.*, 1999).

Skibola & Smith (2000) mentioned that because flavonoids are widely distributed in edible plants and beverages and thus have previously been used in traditional medicine. Therefore, they are more likely to have minimal toxicity. Furthermore, flavonoids have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity, antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity (Cushnie & Lamb, 2005).

One of the main functions of flavonoids and related polyphenols is their role in protecting plants against microbial invasion due to their widespread ability to inhibit spore germination of plant pathogens. There is a constant rising interest in plant flavonoids for treating human diseases and especially for controlling the immunodeficiency virus which is the causative agent of AIDS (Harbone & Williams, 2000).

2.6.3 Riboflavin

Riboflavin (vitamin B2) is a water-soluble member of the vitamin B complex. The extraction of riboflavin and thiamine from foodstuff matrices for analytical purposes generally involves their separation from protein by acid hydrolysis generally with hydrochloric acid, followed by enzymatic reaction, although this latter process is sometimes omitted and proteins are sometimes removed by precipitation with trichloroacetic acid (Sánchez-Machado *et al.*, 2004). Both vitamins can be quantified in foodstuffs by using high-performance liquid chromatography (HPLC) with either UV detection or fluorescence detection. In the case of using fluorescence detector, riboflavin needs no derivatization because of its natural fluorescence, but thiamine requires prior oxidation to thiochrome by reaction with potassium hexacyanoferrate. Methods using fluorescence detection are considered generally faster, more sensitive, and more specific than those using UV detection (Sánchez-Machado *et al.*, 2004). Riboflavin is distinctive among the water-soluble vitamins in that milk and dairy products provide the greatest contribution to its intake in Western diets. Further food groups like meat and fish are also good sources of riboflavin as well as certain fruit and vegetables, particularly dark-green vegetables encompass high concentrations (Powers, 2003). Biochemical symptoms of depletion appear within only a few days of dietary deficiency. In western countries, poor riboflavin intake appears to be of most concern, despite the variety of riboflavin rich foods available. There is evidence that deprived riboflavin status interferes with iron handling and contributes to the cause of anaemia when iron intakes are low. Various mechanisms for this have been proposed, including effects on the gastrointestinal tract that might compromise the handling of other nutrients. Riboflavin deficiency has been considered as a possible risk factor for cancer, although there has not been satisfactorily established in humans (Powers, 2003).

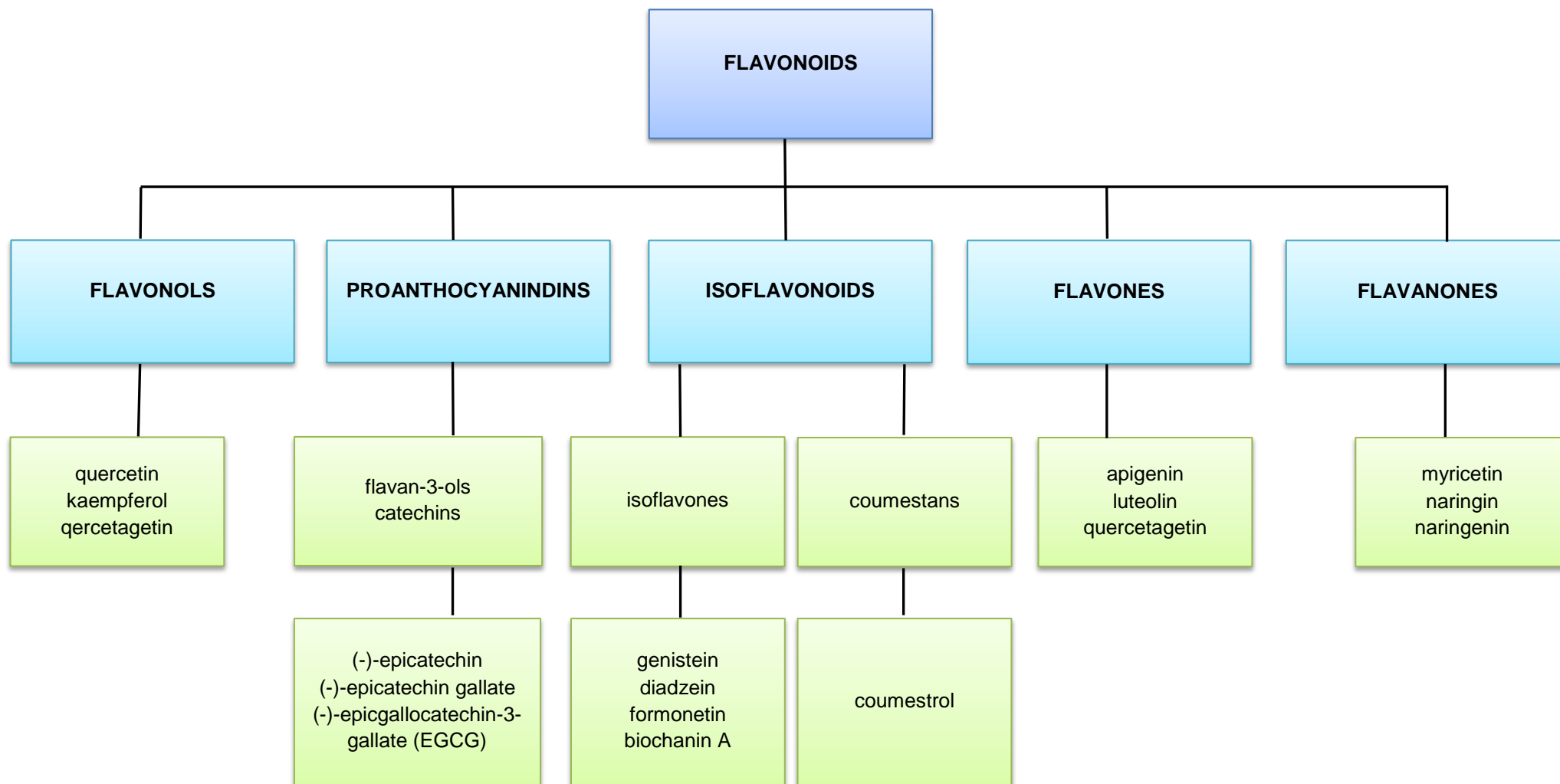


Figure 2.1 Family of major dietary flavonoid groups (Skibola & Smith, 2000).

2.6.4 Tannins

The word 'tannin' is derived from the French 'tanin', and it is used for a variety of natural polyphenols. Tannins are widely dispersed in the plant kingdom. It is claimed these polyphenolics have beneficial effects on health as antioxidant, antimicrobial, anti-inflammatory, antiviral and as anti-carcinogen agent (Gudej & Tomczyk, 2004). Large tannin concentrations are found in nearly every part of the plant, namely the bark, wood, leaves, fruit, roots, and seed (Khanbabaee & Van Ree, 2001; Riedl & Hagerman, 2001). Tannins were later defined as high molecular weight polyphenols that precipitate protein from solution, and found in higher plants including many plants used as foods and feed (Hartzfeld *et al.*, 2002; Riedl & Hagerman, 2001).

The rise in tannin production of the plant can be associated with a sickness or ill condition. It is therefore assumed that the biological role in the plant of the various types of tannin is related to protection against infection, insects, or animal herbivory. The tannins appear as light yellow or white amorphous/shapeless powders or shiny, nearly colourless, loose masses, with a characteristic strange smell and astringent taste (Khanbabaee & Van Ree, 2001).

Tannins are plant secondary substances produced during metabolism that were originally recognized because they interact strongly with collagen, thereby converting animal skin to leather, a process also known as "tanning". Tannins are known to have healing properties due to their ability to promote protein precipitation, which gives rise to a protective layer that can improve the regenerative process (De Souza *et al.*, 2008). Hydrolyzable tannins comprise of simple phenolic acids for instance gallic acid esterified to polyols, typically glucose, whereas condensed tannins are polymers of flavonoid units (Riedl & Hagerman, 2001).

Recent research by Tibe *et al.* (2007) indicated that condensed tannins in low concentrations have beneficial effects in animal and human nutrition and health. Condensed tannins are polyphenolic substances extensively distributed in plants, particularly in legumes and due to their large structure are known to inhibit protein digestibility (Tibe *et al.*, 2007). It does this by forming irreversible complexes with proteins, thereby reducing the bioavailability of amino acids (Tibe *et al.*, 2007). Condensed tannins (otherwise labeled as proanthocyanidins) are oligomers and polymers of flavan-3-ol units, which are often, linked either via C4 C6 or C4 C8 bonds (B-type *proanthocyanidins*). The most frequent condensed tannins occurring in plant tissues are procyanidins. Procyanidins are typical condensed tannins composed of catechin or epicatechin and may contain gallic acid esters (Riedl & Hagerman, 2001). Condensed tannins are known to be able to interact with biological systems through the induction of some physiological effects, such as antioxidant, anti-allergy, anti-hypertensive, as well as antimicrobial activities (Romani *et al.*, 2006).

2.6.5 Thiamine

Vitamin B1, also known as thiamine, is one of 8 B vitamins. B vitamins, often referred to as B complex vitamins assist the body to change carbohydrates into glucose, which is used to produce energy. They also aid the body to metabolize fats and protein. B complex vitamins are needed for healthy skin, hair, eyes, liver and help the nervous system function properly as well as needed for good brain function (Gibson & Blass, 2007).

Thiamine is a water-soluble vitamin that is essential for average cell function, growth and development (Lockman *et al.*, 2003). All B vitamins are water-soluble and the body does not store them. Like other B complex vitamins, thiamine is often referred to as an “anti-stress” vitamin due to the possibility of strengthening the immune system and improves the body's ability to handle stressful conditions. It is named B1 because it was the first B vitamin discovered (Gibson & Blass, 2007).

Thiamine is necessary for all tissues and is found in high concentrations in the skeletal muscle, heart, liver, kidneys and brain. The active form of thiamine is thiamine diphosphate. It serves as a cofactor for several enzymes involved primarily in carbohydrate catabolism. Thiamine exists in both plants and animals and plays a crucial role in certain metabolic reactions. The body requires it to form adenosine triphosphate (ATP), which every cell of the body uses for energy (Ehrlich, 2013). The average daily intake of thiamine is 0.5 mg. The major sources of dietary thiamine are whole grain cereals and cereal products, vegetables, meat and milk products. Refined rice, sugar, alcohol, fat and other refined foods are poor sources. The cooking process may possibly result in substantial loss of thiamine. Cooking losses of thiamine are greatest following the boiling of rice and green vegetables. Thiamine is generally preserved after frying and microwaving, due to the short transit times. Tea and raw fish contain anti-thiaminases that can hydrolytically destroy the vitamin in the gut (Lynch & Young, 2000; Ismail *et al.*, 2013). Thiamine deficiency is relatively uncommon in western society and only mostly seen in alcoholics due to a combination of poor diet and inhibition of thiamine absorption in the gut by ethanol. Thiamine deficiency is also relatively common with chronic vomiting, gastrointestinal disease, anorexia, total parenteral nutrition, and in the elderly. Patients with chronic heart failure on long term treatment with diuretics have been shown to be susceptible to mild deficiency leading to impairment of cardiac function. This is believed to be the urinary loss in combination with reduced thiamine uptake in cardiac cells (Lynch & Young, 2000).

Due to thiamine relatively short storage time, thiamine deficiency can occur within 10 days and more serious deficiency occurs within 21 days if intake is stopped (Lynch & Young, 2000). The state of severe depletion of thiamine is seen in patients in 18 days, but the most common cause of thiamine deficiency in prosperous countries is alcoholism. Alcohol affects thiamine uptake and other aspects of thiamine utilization, and these effects may contribute to the occurrence of thiamine deficiency in alcoholics. Considerable efforts are made to

understand the genetic and biochemical determinants of inter-individual differences in susceptibility, to development of thiamine deficiency-related disorders (Singleton & Martin, 2001).

It's uncommon to be lacking in thiamine, though alcoholics, people with Crohn's disease, anorexia, and those undergoing kidney dialysis may be deficient. Symptoms of thiamine deficiency are fatigue, irritability, depression and stomach discomfort. Individuals with thiamine deficiency have trouble processing carbohydrates which allows a substance called pyruvic acid to build up in their bloodstream, resulting in a loss of mental alertness, troubled breathing, and heart damage as well as major disease known as beriberi (Ehrlich, 2013).

The major diseases of thiamine shortage in humans contain cardiovascular (wet beriberi) and nervous (dry beriberi, or neuropathy and or Wernicke-Korsakoff syndrome) systems. The most important necessity of thiamine is to treat beriberi, which is caused by lack of sufficient thiamine in a diet. Beriberi symptoms include swelling, tingling, or burning sensation in the hands and feet, confusion, trouble breathing due to fluid in the lungs, and uncontrolled eye movements called nystagmus. Individuals in the developed countries usually don't get beriberi due to foods for example cereals and breads are fortified with vitamin B1 (Ehrlich, 2013; Otemuyiwa & Adewusi, 2013).

Wernicke-Korsakoff condition is a brain disorder also caused by thiamine deficiency. Wernicke-Korsakoff is essentially two disorders: Wernicke's disease includes impairment to nerves in the central and peripheral nervous systems and caused by malnutrition due to alcoholism. Korsakoff syndrome is characterized by memory difficulties and nerve damage. However, high dosage of thiamine can improve muscle coordination and confusion, but rarely improves memory loss (Ehrlich, 2013; Otemuyiwa & Adewusi, 2013).

2.7 Pathogens and Antimicrobials

Amongst the known foodborne pathogens, recently there have been more pathogens identified, suggesting that a lot has to be learned about pathogens (Tauxe, 2002). Foodborne disease remains considerable as one in four Americans is projected to have a significant foodborne illness each year (Tauxe, 2002). Pathogens are nearly present everywhere, they are potentially threatening bacteria in foods, soil and in water and has historically been the result of unwarranted deaths and illness. Present trends in nutrition and food technology are increasing the stresses on food microbiologist to ensure a safe food supply. In the last decades bacterial pathogens encountered to human illness are through consumption of undercooked or minimally processed ready-to-eat meats, dairy products, or fruits and vegetables (Mandal *et al.*, 2011). The majority of these illnesses are not accounted for by known pathogens, so more must remain to be discovered (Tauxe, 2002).

Over 70% of pathogens found in US hospitals have grown resistant to at least one antibiotic, resulting in mortality of more than 14,000 patients annually from nosocomial (diseases originating from hospital) infections. In order to solve the antimicrobial resistance issue, one of the effective approaches could be the discovery and development of new antimicrobial agents that provide clinical significant value as well as originating from natural resources. It is important to discover new antimicrobial agents in the pharmaceutical market in order to replace currently available antimicrobials (Khanam *et al.*, 2014).

Plant products with antimicrobial properties have obtained emphasis for possible application in food production in order to prevent bacterial and fungal growth (Coz *et al.*, 2010). The food industry in general generates a large amount of bio-waste depending on product type, which is rich in bioactive compounds that could be potential source of antioxidant, antimicrobial or other bioactive compounds (Kanatt *et al.*, 2011). Phytochemicals will find their way into the collection of prescribed antimicrobial drugs. On average, two or three antibiotics derived from microorganisms are launched each year. New sources, especially plant sources, are also being investigated.

The public is becoming increasingly aware of problems with the over prescription and abuse of traditional antibiotics. Furthermore, many people are interested in having more independence over their medical care. A multitude of plant compounds is readily available over-the-counter from herbal suppliers and natural-food stores, in addition self-medication with these substances is commonplace (Cowan, 1999). The development of multi-drug resistant bacterial strains throughout the world limits the effectiveness of modern drugs and limits treatment thus leading to prolonged infections (Prasad *et al.*, 2008). For over 80% of the world's population, especially in the developing world traditional medicine from plant extracts carry on providing health coverage (Igbinosa *et al.*, 2009).

Antimicrobial resistance threatens the effectiveness of treatment of infections and is regarded a public health issue with national and global magnitudes. Antibiotics play a critical role in reducing infectious diseases all over the world. However, it should be considered that the curative power of infectious disease by drugs or drug efficacy is not infinite (Ndiokubwayo *et al.*, 2013). Antimicrobial resistance frequently occurs in microorganisms that are likely to be transmitted in the community such as organisms causing pneumonia, diarrheal diseases, tuberculosis, sexually transmitted diseases and malaria. Drug resistance has drastically increased the costs of fighting tuberculosis and malaria (Ndiokubwayo *et al.*, 2013).

There has been a growing incidence of multiple resistances in pathogenic microorganisms in recent years largely due to haphazard use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Kritzing *et al.*, 2004; Parekh & Chanda, 2007). Due to problems with toxicity of existing antimicrobial agents as well as the emergence of drug-resistant strains plant extracts can be exploited as an

alternative mechanism to control these pathogens. Several of these plant extracts contain compounds that have an inhibitory effect on harmful bacterial and fungal human pathogens (Kritzinger *et al.*, 2004).

The understanding of issues related to antimicrobial resistance and its magnitude are disadvantaged by insufficient data as surveillance of drug resistance is limited to a few countries resulting in incomplete data on the true extent of this problem (Ndiokubwayo *et al.*, 2013). Immune compromised patients in hospital are vulnerable to nosocomial infections caused by opportunistic pathogens which are foodborne related.

2.8 Nosocomial Bacteria

Nosocomial infections can be defined as infections occurring within hospital admission, discharge or an operation and affect 10% of patients admitted to hospital (Kamboj & Sepkowitz, 2009). Annually nosocomial infections results in high mortality (5000 deaths) in immunosuppressed patients with an incredibly high cost to the National Health Service (Urrea *et al.*, 2003). Methicillin resistant *Staphylococcus aureus* is a major cause of nosocomial infection leading to a wide range of diseases (Samak *et al.*, 2012).

Nosocomial infection is a major problem in hospitals worldwide and the occurrence is two to threefold higher in developing countries (Naidu *et al.*, 2014). On average, a patient suffering from a nosocomial infection spent roughly twice as long in hospital admission when compared to uninfected patient as well as incurring additional costs. Intensive care units (ICU) patients have the highest occurrence of nosocomial infections in hospital setting (Inweregbu *et al.*, 2005; Naidu *et al.*, 2014). Intensive care unit patients are at larger risk of developing nosocomial infections due to numerous reasons, namely presence of underlying diseases, longer stay, invasive diagnostic and monitoring procedures performed, impaired host defences, and colonization by resistant microorganisms (Ak *et al.*, 2011). There have been numerous efforts in many countries to control nosocomial infections, with the goal of lowering and stabilising the rates (Mireya *et al.*, 2007). The following microorganism strains all exhibit nosocomial properties namely, *Acinetobacter baumannii*, *Candida albicans*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

2.8.1 *Acinetobacter baumannii*

Acinetobacter baumannii an emerging opportunistic nosocomial pathogen, with gradually global prevalence, is a non-fermentative, gram-negative, conditional pathogenic bacterium, which can colonize and survive in low-moisture environments for prolonged period under a wide range of environmental conditions such as hospital environment and human skin (Wisplinghoff *et al.*, 2008; Dent *et al.*, 2010; Zhao *et al.*, 2015). When motionless, *A. baumannii* appears as a coccobacillus, though during growth it takes on a rod form (Dent *et*

al., 2010). *A. baumannii* also found in environments such as soil and water and in food products, namely vegetables, fish, meat, and raw bulk tank milk (Rafei *et al.*, 2015). Unlike the conventional pathogens that are commonly implicated in foodborne illnesses, members of the genus *Acinetobacter* are rarely associated with diarrheal disease, perhaps due to the difficulty in isolating these Gram-negative bacteria from food sources. Yet, several species of *Acinetobacter*, especially *A. baumannii*, have many of the characteristics associated with successful pathogens and exhibit an ability to acquire the multiple-drug resistance (MDR) phenotype (Amorim & Nascimento, 2017).

Major epidemiological features of these organisms include their propensity for clonal spread, their involvement in hospital outbreaks, and their resistance to multiple antimicrobial agents (Wisplinghoff *et al.*, 2008). *A. baumannii* is responsible for a variety of nosocomial infections, namely nosocomial pneumonia, blood infection, urinary tract infection, surgical wound infection, etc., especially for patients in intensive care unit (Wisplinghoff *et al.*, 2008; Peleg *et al.*, 2009).

This microorganism has clinical significance due to its remarkable ability to gain resistance factors, making it one of the organisms threatening the current antibiotics. It has been reported by Peleg *et al.* (2009) that *A. baumannii* strains resistant to all known antibiotics, suggesting that the international health care community should be on a lookout for the organism. The increasing risk of antibiotic resistance in microbes impacting on the patient results has been recognized as a challenge for treatment of clinical infection with broad spectrum antibiotics use (Zhao *et al.*, 2015).

A. baumannii is in general considered harmless nonetheless they cause various infectious diseases in compromised patients with immunodeficiency. This bacterium easily acquires drug resistance, thereby inducing a natural transformation in order to capture the DNA fragments present in the extracellular space and to incorporate this DNA into the chromosomal DNA, resulting in mutation of the organism. As a result, multidrug-resistant strains of *A. Baumannii* have become an important problem in hospital-acquired infections (Kamoshida *et al.*, 2014). *A. baumannii* has the ability to survive for prolonged periods throughout a hospital environment (Maragakis & Perl, 2008). The organism commonly targets the most vulnerable hospitalized patients, those who are critically ill with breaches in skin integrity and airway protection (Peleg *et al.*, 2009).

Multidrug resistant *A. baumannii* is an important cause of hospital acquired infection and has been shown to increase mortality and length of stay (Dent *et al.*, 2010). Multidrug resistant *A. baumannii* is often associated with co-infection by other virulent pathogens (Dent *et al.*, 2010). Multidrug-resistant *A. baumannii* causes infections that include pneumonia, meningitis, urinary tract infection, and wound infection (Maragakis & Perl, 2008). Risk factors for colonization or infection from the organism include prolonged length of hospital stay, exposure to an intensive care unit (ICU), receipt of mechanical ventilation, colonization

pressure, exposure to antimicrobial agents, recent surgery, invasive procedures, and underlying severity of illness (Maragakis & Perl, 2008).

2.8.2 *Candida albicans*

Candida albicans is an opportunistic pathogen that can cause local and systemic infections in susceptible persons, commonly affecting low immune compromised patients and those undergoing prolonged antibiotic treatment. Yet, the information available on plants, particularly medicinal plants, active against this yeast species has, until recently, has not resulted in effective formulations for humans or animal use (Duarte *et al.* 2005). Thereby creating a gap to utilise medicinal plants or foods to act as an anti-fungal against this pathogen.

Candida albicans, is a di-morphic yeast-like fungus. *C. albicans* which resides in the mucocutaneous cavities of skin, intestine of humans, can cause infections under altered physiological and pathological conditions (Manohar *et al.*, 2001). The increasing antimicrobial resistance of pathogens isolated from humans and animals, combined with the increasing awareness of the consumers on chemical substances used as food preservatives, necessitates research for more efficient antimicrobials with fewer side-effects on human health (Papadopoulou *et al.*, 2005).

One of the common diseases linked with *Candida albicans* is Irritable bowel syndrome (IBS) and is the most common gastroenterological disorder in clinical practice in Western society. It is now known that many patients with IBS have specific food intolerances. Diets have enabled many patients to control their symptoms but limited diets are expensive and socially inconvenient. *Candida albicans* has been implicated in antibiotic-associated diarrhoea in elderly patients and it has been suggested that food intolerance may be a consequence of the overgrowth of *C. albicans* in the gut (Middleton *et al.*, 1992).

2.8.3 *Enterococcus faecalis*

Enterococci belong to the lactic acid bacteria (LAB) and they are of importance in foods due to their involvement in food spoilage and fermentations, as well as their utilisation as probiotics. However, they are also important nosocomial pathogens that cause infections and some strains are resistant to many antibiotics (Franz *et al.*, 2011). *Enterococci* can spoil processed meats however, they are also important for ripening and aroma development of certain traditional cheeses and sausages, especially those produced in the Mediterranean area (Franz *et al.*, 2004). Opportunistic enterococcal infections have usually been perceived as severe clinical threats due to rising occurrence of antibiotic resistance and lateral transfer of resistance traits (Thomas *et al.*, 2009).

Enterococcus faecalis is a natural inhabitant of the gastrointestinal tract also a known cause of infective endocarditis. The organism has recently emerged as a significant

nosocomial pathogen. The bacterium *E. faecalis* is a gram-positive, facultative anaerobe, and a normal inhabitant of the human intestinal tract (Hubble *et al.*, 2003).

Interest in enterococci is due to their prominence in multidrug-resistant nosocomial infections, which are difficult to control or treat (Nallapareddy *et al.*, 2005; Bourgogne *et al.*, 2008). The enterococci are able to thrive in environments with high antibiotic usage due to both their intrinsic resistance to antibiotics and their aptitude for substitution of genetic information, whereby allowing enterococci to gain and share resistance determinants. Enterococcal infections are mainly caused by *E. faecalis* and *E. faecium*. These species are generally harmless commensals, with some enterococci being marketed in Europe to alleviate symptoms of irritable bowel syndrome and recurrent chronic sinusitis or bronchitis (Bourgogne *et al.*, 2008).

2.8.4 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a colonizing opportunistic pathogen of humans and animals, and a common contaminant of retail meat. In animals, *K. pneumoniae* causes disease in cows, horses, and companion animals. In humans, *K. pneumoniae* frequently colonizes the gut and periodically causes extraintestinal infections (Davis *et al.*, 2015). *Klebsiella pneumoniae* is the most common gram-negative bacterium causing community-acquired pneumonia and up to 5% of community-acquired urinary tract infections (Cano *et al.*, 2009). *Klebsiella* genus is defined as containing gram-negative bacilli of the family *Enterobacteriaceae*, non-motile, usually encapsulated and rod-shaped (Otman *et al.*, 2007).

K. pneumoniae is among the bacteria that most readily develop resistance mechanisms to multiple classes of antibiotics, and the prevalence of drug resistance is increasing at an alarming rate. *K. pneumoniae* is an emerging pathogen associated with significant mortality, thereby creating an urgent need to develop strategies for prevention and infection control (Yu *et al.*, 2007; Gasink *et al.*, 2009).

K. pneumoniae a member of the human intestine flora is regularly associated with nosocomial infection. Certain diseases such as malignancy, cirrhosis, biliary tract disorders, diabetes mellitus, and alcoholism may impair an individual's immune defences and therefore increase the risk of *K. pneumoniae* infection. The organism is the second most common cause of gram-negative bacteraemia (bacteria present in blood) after *Escherichia coli* it also causes significant morbidity and mortality in general populations (Tsai *et al.*, 2010). *Klebsiella* species are among the most common pathogens recovered in intensive care units. *Klebsiella pneumoniae* is also among the bacteria that most readily grow resistance to antibiotics. *K. pneumoniae* is a major challenge in antimicrobial resistance (Gasink *et al.*, 2009).

2.8.5 *Pseudomonas aeruginosa*

Pseudomonas species are present everywhere and isolated from a multiplicity of sources including drinking water, domestic wild animals, humans, plants and also from a variety of foods (Abd El-Aziz, 2015)). *Pseudomonas aeruginosa* is a gram-negative opportunistic pathogen and often accountable for incurable infections in immunocompromised individuals (Hentzer *et al.*, 2002). *P. aeruginosa* presents a serious health risk challenge for treatment of both public acquired and nosocomial infections, and variety of the appropriate antibiotic to initiate therapy is essential to improving the clinical outcome (Lister *et al.*, 2009). Yet, the most appropriate antibiotic is difficult due to the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, even whilst treating an infection. *P. aeruginosa* can endure for long periods under the selective pressure imposed by the immune system and the antibiotic treatment. This is due to growth and adaptive evolution facilitated by genetic variation (Ciofu *et al.*, 2010).

Studies by Lister *et al.* (2009) have exhibited that infections caused by drug-resistant *P. aeruginosa* are linked to significant increases in morbidity, mortality, need for surgical intervention, extent of hospital stay and chronic care, as well as overall cost of treating the infection. The development of resistance during the course of therapy/health treatment, leads to a complication which has been shown to double the length of hospitalization and overall cost of patient care (Lister *et al.*, 2009). *P. aeruginosa* is responsible for infections causing millions of cases each year in the community and 10–15% of all healthcare associated infections (De Simone *et al.*, 2014).

P. aeruginosa is usually acquired from the environment. Contaminated respiratory care equipment, irrigating solutions, catheters, infusions, cosmetics, dilute antiseptics, cleaning solutions, and even soaps act as a catalyst of transmission (Yetkin *et al.*, 2006).

2.8.6 *Staphylococcus aureus*

Staphylococcal food-borne disease (SFD) is one of the most common food-borne diseases worldwide resulting from the contamination of food by preformed *S. aureus* enterotoxins (Kadariya *et al.*, 2014). Outbreak investigations have found that poor food handling practices in the retail industry account for the majority of SFD outbreaks. However, several studies have documented prevalence of *S. aureus* in many food products including raw retail meat indicating that consumers are at potential risk of *S. aureus* colonization and subsequent infection (Kadariya *et al.*, 2014).

Staphylococcus is pathogenic to humans and further mammals. Initially they were separated into two groups on the foundation of their ability to clot blood plasma (the coagulase reaction). The coagulase-positive *staphylococci* were found to be the pathogenic species *Staphylococcus aureus*. The coagulase-negative *staphylococci* are known to comprise over 30 other species. The coagulase-negative *staphylococci* are common

commensals (characterized by a symbiotic relationship in which one species is benefited while the other is unaffected) of skin, though some species can cause infections. Coagulase is an indicator for *S. aureus* but there is no direct evidence that it is a virulence factor (Kuroda *et al.*, 2001).

Virulence results from the combined effect of many factors expressed during infection. Antibodies will neutralize *staphylococci* toxins and enzymes, but vaccines are not available. *Staphylococci* are common causes of infections associated with indwelling medical devices. These are tough to treat with antibiotics alone and frequently require removal of the device (Kuroda *et al.*, 2001).

S. aureus is one of the main causes of public acquired and hospital acquired infections. It produces plentiful toxins that cause unique disease such as toxic shock syndrome, *staphylococci* scarlet fever, surgical wound infection, and septicaemia in addition to acquired resistance to practically all antibiotics (Kuroda *et al.*, 2001; Samak *et al.*, 2012; Harastani *et al.*, 2014).

S. aureus can be isolated from the skin of 5–30% of individuals, mainly from intertriginous (two skin areas may touch or rub together) areas and persistent nasal carriage of the organism is present in 20% of adults. *S. aureus* is able to secrete exotoxins with super antigenic properties (Breuer *et al.*, 2004).

The increasing occurrence of methicillin resistant *S. aureus* and its ability to spread in the hospitals and the community creates a major challenge for infection control (Harastani *et al.*, 2014). Methicillin resistant *S. aureus*, a multidrug resistant variation of usual *S. aureus* has become a substantial public health issue during the past decade, due to a significant rise in the frequency of methicillin resistant *S. aureus* isolated from patients with complicated infections. Antibiotic resistance decreases the health treatment effectiveness of antibiotics used to treat variety of bacterial infections. This has demanded the use of new antimicrobial substances from other sources including plants (Samak *et al.*, 2012).

2.9 Bioautography

Bioautography is an effective and inexpensive technique for the phytochemical analysis of plant extracts to identify and characterize bioactive compounds. It can be performed both in developed laboratories as well as in small research laboratories which have minimum access to sophisticated equipment. Despite having sophisticated online high-performance liquid chromatography coupled bioassays, bioautography offers a simple, rapid and inexpensive method for the chemical and biological screening of complex plant extracts, with subsequent bioassay-guided isolation (Dewanjee *et al.*, 2014).

Bioautography is a microbiological screening method frequently used for the detection of antimicrobial activity. The inhibitory activities for antimicrobials, antioxidants and enzymes can be performed on TLC bioautography (Horváth *et al.*, 2010). The screening can

be defined as the first procedure and is applied to an analysed sample in order to establish the presence or absence of given analytes. Simply speaking, it is a measurement providing a “yes/no” response. Generally screening methods tend to have higher sensitivity than other methods (Cheng & Wu, 2013). Screening methods are simple, cheap, efficient and do not require sophisticated equipment. Bioautography screening methods are based on the biological activities, e.g. anti-bacterial, anti-fungal etc. of the tested substances.

This detection method can be successfully combined with other chromatography techniques, namely thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), over pressured layer chromatography (OPLC) and planar electro chromatography (Choma & Grzelak, 2011). It gives very quick access for detection and localization of the active chemicals in a complex plant extract (Tasdemir *et al.*, 2004). Bioassays are defined as tests which are used to detect the organic activity of an extract or substance isolated from an extract, obtained from a living organism. Accurate bioautography involves growing an organism on the TLC plate, while autography is the application of a chemical method to detect a biological effect or process (Horváth *et al.*, 2010). This method is different with work using agar dishes, which do not distinguish between active and inactive components found together in the zones of inhibition. In this case, only the bioactive sum of a sample is indicated, and not the activities of single compounds. A number of samples can be treated at the same time when compared to HPLC. Chromatography of the samples is under strictly identical conditions (Horváth *et al.*, 2010).

Thin layer chromatography has an additional advantage over HPLC in that the organic mobile phase, which might cause inactivation of enzymes or living organisms, is evaporated and cannot impede the detection (Marston, 2011). HPLC on-line bioassays have to be compatible with the eluent. After the separation of sample components on TLC, these products are immobilized on the plate. They are easily accessed because they are open and, furthermore, they are available for slow manipulations, such as incubation of bacterial cell cultures (Marston, 2011).

A study done by Jesionek *et al.* (2015) investigated plant components of *Hypericum perforatum* L. tincture by Thin layer chromatography – direct bioautography (TLC-DB) using nine bacterial strains: *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *S. epidermidis*, *Pseudomonas syringae* pv. *maculicola*, *Xanthomonas campestris* pv. *vesicatoria*, and *Aliivibrio fischeri*. Results showed compounds with the widest range of antimicrobial activity were isolated using semipreparative TLC and identified the phytochemicals as apigenin, 3,8'-biapigenin, quercetin, kaempferol, and linolenic acid by TLC, HPLC-diode array detection, and HPLC/MS/MS techniques.

With regards to HPLC assays, there is a constant flow of eluent and any interactions with chemicals or organisms have to be fast. The core applications of TLC bioautography are the fast screening of a large number of samples for bioactivity and in the target-directed

isolation of active compounds since TLC only involves an amount of product on the plate and cannot deal with concentrations, it is only a semi-quantitative method (Marston, 2011).

There are different approaches for bioautography to localize antimicrobial activity on a TLC chromatogram, namely agar diffusion or contact bioautography, direct bioautography and immersion or agar overlay bioautography. Those assays supply a quick screen for new antimicrobial compounds through bioassay-guided isolation (Patil *et al.*, 2013). Direct bioautography in combination with thin layer chromatographic (TLC) separation is a rapid and sensitive screening method for the detection of antimicrobial compounds (Horváth *et al.*, 2002). Test microorganism cultures are capable of growing directly on the TLC plate, therefore each step of the assay is performed on the sorbent. Similarly, to usual antimicrobial screening methods, TLC-bioautography must be carried out under controlled conditions since the experimental conditions, namely solvent, sample application, resolution of compounds, type of microorganism and incubation time may influence the result (Horváth *et al.*, 2002).

Planar chromatography is an effective technique for separating certain classes of mixed compounds of biological interest. In higher plants there are various substances which may have antimicrobial effect. Bioautography is a method to localize antibacterial activity on a chromatogram (Horváth *et al.*, 2002).

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CHAPTER 3

BAMBARA GROUNDNUT (*VIGNA SUBTERRANEAN*) FROM MPUMALANGA PROVINCE OF SOUTH AFRICA: PHYTOCHEMICAL PROPERTIES OF SEED EXTRACTS

Abstract

Bambara groundnut an indigenous legume cultivated in Sub-Saharan Africa has been proclaimed to have medicinal properties from communities and in rural areas. However, there is not enough scientific information to validate these claims. Therefore, this study aimed to identify possible medicinal properties in Bambara groundnut, whereby analysing the phytochemical properties of Bambara groundnut (*Vigna subterranea*) (whole, dehulled and hull) seed extracts from Mpumalanga province within South Africa. The BGN extracts from solvents 70% methanol, 70% ethanol and milli-Q water were screened for the presence of certain phytochemicals, specifically alkaloids, flavonoids, phenols, riboflavin and thiamine using analytical laboratory methods for basic screening, and high-performance liquid chromatography (HPLC) and gas chromatography (GC) for quantification. Flavonoids and phenols were highly concentrated in the red and brown hulls of BGN compared to whole and dehulled BGN. Organic solvents in comparison to water yielded the highest concentration of flavonoids, whilst water yielded the highest concentration for phenols. Flavonoid compounds that were detected at the highest concentrations were rutin ($24.458 \pm 0.234 \text{ mg.g}^{-1}$, brown hull extracted with 70% methanol), quercetin ($0.070 \pm 0.043 \text{ mg.g}^{-1}$, red hull extracted with 70% methanol), kaempferol ($0.391 \pm 0.161 \text{ mg.g}^{-1}$; brown hull extracted with 70% ethanol) and myricetin ($1.800 \pm 0.771 \text{ mg.g}^{-1}$; red hull extracted with 70% methanol). For phenolic compounds, gallic acid ($0.009 \pm 0.004 \text{ mg.g}^{-1}$; brown hull extracted with milli-Q water), catechin ($0.026 \pm 0.041 \text{ mg.g}^{-1}$; brown hull extracted with milli-Q water), methyl gallate ($0.008 \pm 0.013 \text{ mg.g}^{-1}$; brown whole extracted with milli-Q water), chlorogenic acid ($0.115 \pm 0.199 \text{ mg.g}^{-1}$; brown hull extracted with milli-Q water) and ellagic acid ($0.105 \pm 0.082 \text{ mg.g}^{-1}$; red hull extracted with milli-Q water) were detected. Vitamins B₁ and B₂ (riboflavin and thiamine) were mostly present in milli-Q water extracts. Black-eye hull had the highest concentration of thiamine (vitamin B₁) and riboflavin (vitamin B₂) consisting of 0.072 mg.g^{-1} (extracted with milli-Q water) and 0.002 mg.g^{-1} (extracted with 70% ethanol and 70% methanol). GC-MS analysis showed the presence of alkaloid compounds (e.g. 9-Octadecenamide, (Z)-, ethanol 1-methoxy- benzoate, 1-Monolinoleoylglycerol trimethylsilyl and ether butylated hydroxytoluene), with potential to act as antimicrobial agents and antioxidants.

3.1 Introduction

The use of plants as primary health remedies due to their pharmacological properties is relatively typical practice (Patra *et al.*, 2011). In developing countries, people with low financial compensation from small isolated villages and native communities, use traditional medicine which is usually plant based, for the treatment of common infections. These plants are ingested as elixirs, teas or juice preparations to treat respiratory infections. Ointments made from plants are applied directly on the infected wounds or burns. As soon as the general public from these remote communities' contract infectious diseases, they are usually treated by traditional healers or shaman's due to their expertise in procedures of making diagnoses, treating wounds and creating herbal medicines (Rojas *et al.*, 2006). The plants used in traditional medicine remain as a great source of natural antioxidants, antimicrobials and possibly anticancer agents that may serve as leads for the development of new potent antibiotics to which pathogenic strains are not resistant (Abu-Shanab *et al.*, 2004; Rojas *et al.*, 2006; Patra *et al.*, 2011; Iwu *et al.*, 2014). Furthermore, the traditional healers state that patients of these villages and communities have reduced risk to infectious diseases from resistant pathogens than patients from urban areas treated with synthetic antibiotics. Subsequently patients treated in hospital environment have a higher risk of contracting a nosocomial infection (Rojas *et al.*, 2006; Mireya *et al.*, 2007).

Bambara groundnut (*Vigna subteranea*) [BGN] an indigenous African crop is grown across the continent from Senegal to Kenya and from the Sahara to South Africa (Mpotokwane *et al.*, 2008; Eltayeb *et al.*, 2011; Jideani & Diedericks, 2014; Nyau *et al.*, 2014). The pods are hard and wrinkled when dry. Each pod contains one or two seeds. The colour of the seed varies from black, brown or red and may be mottled with various colours (Onimawo *et al.*, 1998; Jideani & Diedericks, 2014). Before consumption, the hulls are removed and generally regarded as waste. However, BGN hulls have been reported to have quantities of phenolic compounds. In addition, the hulls are an inexpensive source of nutraceuticals and functional ingredients (Klompong & Benjakul, 2015).

The medicinal use of BGN is based on information obtained from communities in several parts of Africa, where this crop is reportedly used for the treatment of various ailments. The water boiled from the maize and pulse mixture is ingested to treat diarrhoea. Anon (2011) reported that raw BGN can be chewed and swallowed to treat nausea suffered by pregnant women, while in Kenya, the Luo tribe use BGN to cure diarrhoea (Mkandawire, 2007).

The traditional utilization of BGN to treat several ailments is notable and therefore creates an opportunity for detailed scientific study on the pharmaceutical value of the crop (Mølgaard *et al.*, 2011). According to Mbagwu *et al.* (2011) the phytochemical screening carried out on whole BGN (sourced from Idemili in Anambara State, Nigeria) showed the presence of valuable phytochemicals and contained a high percentage of alkaloids compared to other legumes. Furthermore, Nyau *et al.* (2015) reported that whole BGN

(Lusaka, Zambia) possessed antioxidant activities. These findings point toward Bambara groundnuts having the potential as a functional food, with medicinal properties. However, there is limited research with regards to phytochemical properties on whole, dehulled (endosperm) and hulls of Bambara groundnut for all seed colours (black, black-eye, brown, brown-eye and red) from South Africa. Therefore, in the present study the aim was to evaluate the phytochemical properties of BGN extracts from Mpumalanga province in South Africa.

3.2 Materials and Methods

3.2.1 Source of BGN, chemical reagents and equipment

Bambara groundnuts were purchased from Mpumalanga Province, South Africa. The BGN seeds were sorted into their respective colours as seen in Figure 3.1 according to different testa and hilum colours as black, black-eye, brown, brown-eye and red.

The chemicals used in this study were of HPLC and analytical grade. Reagents were prepared according to standard analytical procedures: Methanol Chromosolv® for HPLC \geq 99.9% (Sigma-Aldrich), Acetonitrile LiChrosolv® LC-MS \geq 99.9% (Merck), Acetic acid (glacial) \geq 99% (Merck) and Ethanol 99% AR (Merck). Milli-Q water ($18.2 \text{ M}\Omega\cdot\text{cm}^{-1}$), purified using the Milli-Q water purification system (Millipore, Microsep, South Africa) were used for making dilutions and solutions. All prepared reagents were stored at 18 – 20°C in dark environment to prevent deterioration or contamination.

All equipment utilized was from the Department of Food Science and Technology based at the Cape Peninsula University of Technology, South Africa.

3.2.2 Production of whole, dehulled and hull Bambara groundnut flour

Upon receiving the BGN, it was sorted into its respective colours. The black, black-eye, brown, brown-eye and red colours of whole BGN (WBGN) were milled separately into flour with a sieve size of 250 μm sieve size using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) and packed and sealed in clear plastic bags. The whole Bambara groundnut flour (WBGNF) of each variety was stored in a refrigerator at 4 – 6°C until further analysis.

The WBGN seeds were dried at 40°C in an industrial oven dryer (Geiger and Klotzebücher, Cape Town, South Africa) for 48 h. A Corona® manual grain was used to mill BGN to allow easier manual removal of hulls to produce dehulled Bambara groundnut (DBGN). The black, black-eye, brown, brown-eye and red DBGN and their respective hulls were milled into flour using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) with



A



B



C



D



E

Figure 3.1 Various BGN colours used in the study, A = black-eye, B = brown-eye, C = black, D = red, E = brown

250 µm sieve size, packed and sealed in clear plastic bags. The dehulled BGN flour (DBGNF) of each BGN seed colour was stored in a refrigerator at 4-6°C until further analysis.

The black, black-eye, brown, brown-eye and red BGN hulls were each milled into flour to form BGN hull flour (BGNHF) using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) sieved with 250 µm sieve size, packed in clear plastic bags and stored in a refrigerator at 4-6°C until further analysis.

3.2.3 Production of whole, dehulled and hull Bambara groundnut extracts

The 70% methanol, 70% ethanol and milli-Q water extracts were prepared using Ultrasound-Assisted Extraction (UAE) from the different BGN seed flours (WBGNF, DBGNF and BGNHF). Approximately 15 g of each seed flour (WBGNF, DBGNF and BGNHF) separately in 150 mL of each solvents (70% methanol, 70% ethanol and milli-Q water) was sonicated for 30 minutes at 25°C using the Lasec SA 2510 Branson ultrasound bath 42 kHz ± 6%, USA. After extraction, the mixture was centrifuged at a speed of 15316 x g for 15 min at 4°C in a Beckman rotar JA-14 in Beckman Coulter Avanti J-E centrifuge, USA. The resulting supernatant of the 70% ethanol and 70% methanol solvent mixture was concentrated to 30 mL by evaporation under pressure in a rotary evaporator (Buchi RE 011 model, Switzerland) at 40°C to remove residual ethanol and methanol (Nyau *et al.*, 2014). The supernatant of the milli-Q water solvent mixture was syringe filtered directly into 5 mL freeze drier vials. The extracts of whole BGN (WBGN), dehulled BGN (DBGN) and BGN hull (BGNH) per solvent (70% methanol, 70% ethanol and milli-Q water) were then frozen at -80°C and freeze dried to obtain a powdered extract using the BenchTop Pro with Omnitronics freeze dryer, United Scientific, Germany. The freeze-dried extracts were stored at -4°C and subjected to phytochemical screening and antimicrobial activity (Figure 3.2).

3.3 Qualitative Phytochemical Analysis

Freeze-dried extracts of WBGN, DBGN, and BGNH per BGN seed colour underwent preliminary phytochemical analysis to determine whether specific phytochemicals were present.

3.3.1 Qualitative analysis for tannins/polyphenols

The method consisted of adding 1 mL of freshly prepared 10% (m.v⁻¹) KOH solution to 1 g of individual extracts of WBGN, DBGN and BGNH. If an opaque precipitate appeared, it indicated the presence of tannins (Santosh *et al.*, 2013).

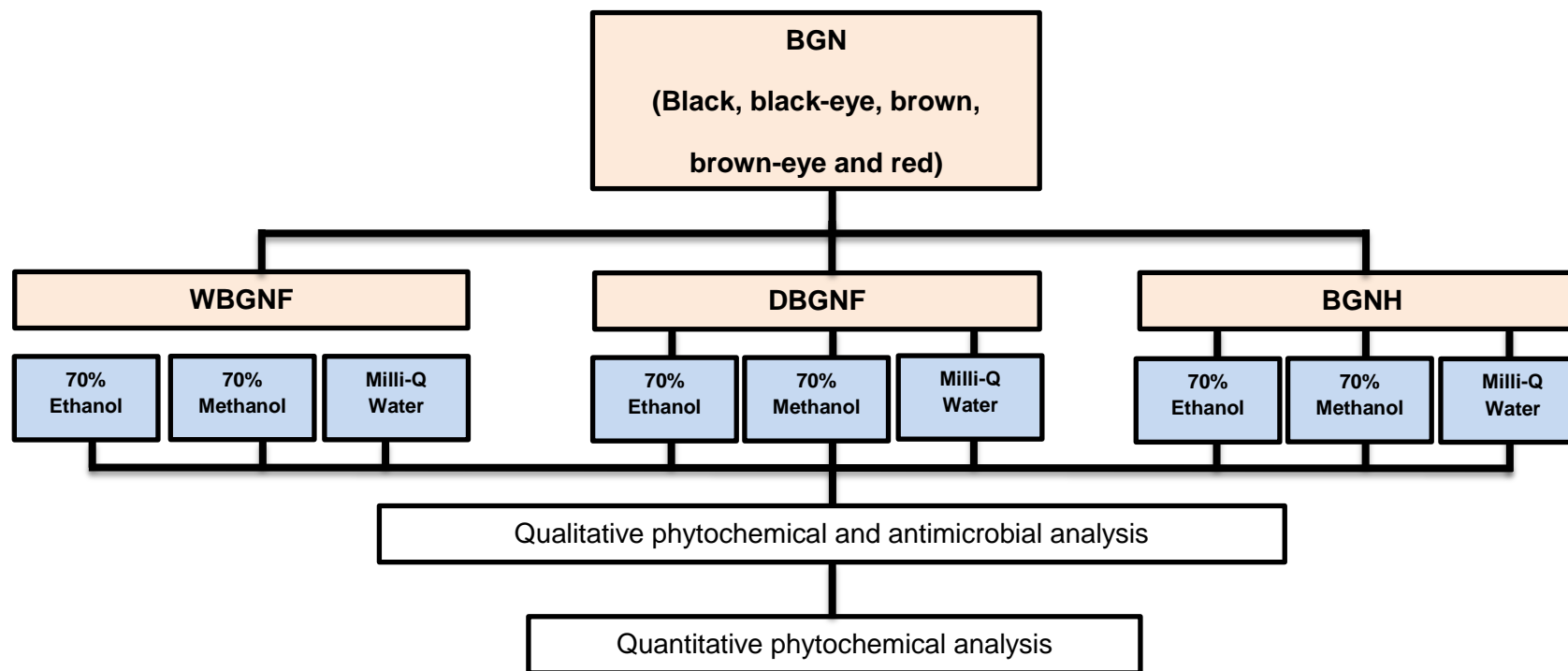


Figure 3.2 Whole Bambara groundnut flour (WBGNF), dehulled Bambara groundnut flour (DBGNF) and Bambara groundnut hulls (BGNH) seed extraction process

3.3.2 Qualitative analysis for flavonoids

As described by Bhandary *et al.* (2012) 1 g of WBGN, DBGN and BGNH per seed colour powdered extract was treated with few drops of ferric chloride solution. Blackish red colour indicated the presence of flavonoids.

3.3.3 Qualitative analysis for alkaloids

Mayer's reagent was prepared by dissolving a mixture of 1.35 g mercuric chloride in 60 mL of distilled water and poured into a solution of 5 g potassium iodide in 10 mL of distilled water. The solution was standardized to 100 mL with distilled water in a volumetric flask (Anon, 2015). WBGN, DBGN and BGNH individual powdered extracts (0.5 g) were stirred with 5 mL of 1% (v/v) HCl in a water bath. The solution was filtered and 1 mL of filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100 mL with distilled water. Turbidity of the extract filtrate with the addition of Mayer's reagent serves as an indicator for the presence of alkaloids (Santosh *et al.*, 2013).

3.4 Quantitative Phytochemical Analysis

Following the qualitative confirmation of phytochemicals, further analysis was performed on WBGN, DBGN and BGNH extracts for each BGN seed colour to quantify the various concentrations present for each phytochemical.

3.4.1 Flavonoid analysis on BGN seed extracts

Analytical standards and preparation of calibration standards

Four flavonoid standards were used as external standards, namely quercetin (Sigma Aldrich), kaempferol (Sigma Aldrich), rutin (Sigma Aldrich) and myricetin (Sigma Aldrich). The standard solutions were prepared as described by Kaliyaperumal *et al.* (2013). All standards were stored at -20°C for a maximum of 2 weeks. The stock solutions of the four flavonoids standards were prepared by dissolving 1 mg into 10 mL HPLC grade methanol (Merck).

HPLC analyses of flavonoids

The HPLC separations of the standards and samples were performed using Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany). The HPLC system consists of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment set at 30°C , an autosampler and a G1315C Diode Array Detector (DAD) set at 190 nm to 600 nm. The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) integrated peak areas was used to record and store data.

The analytical column used was a reversed phase column (ZORBAX SB-C₁₈ 3.5 μm , 4.6 x 150 mm, Agilent, USA) with a reversed-phase C₁₈ guard column at 40°C . The WBGN,

DBGN, BGNH (0.015 g) extracts were reconstituted in methanol (1 mL) following syringe filtration (0.45 μm) and individually injected at a volume of 20 $\mu\text{g}\cdot\text{mL}^{-1}$. The guard cartridge was replaced after every 150 injections. The mobile phase was isocratic at 40:20:39:1 (v/v/v/v) methanol:acetonitrile:water:acetic acid with a flow rate of 0.8 $\text{mL}\cdot\text{min}^{-1}$, the peaks were simultaneously identified using UV absorbance at 350 nm for kaempferol and 254 nm for rutin, myricetin and quercetin (Kaliyaperumal *et al.*, 2013).

The chromatographic peaks of the extracts were confirmed by comparing their retention time and UV spectra with those of the pure standards. The utilisation of the calibration function of the Agilent Chemstation software to interpret the calibration curve and quantification of the flavonoid levels of samples were performed by the external standard method. In terms of quantification, a standard solution, diluted from 20 $\mu\text{g}\cdot\text{mL}^{-1}$ to 0.01 $\mu\text{g}\cdot\text{mL}^{-1}$ was analysed in triplicate at the start of each working day. The software used the resultant peak area to calculate the actual concentration of flavonoids in individual extracts of WBGN, DBGN and BGNH. The column was restored for further use between injections by means of a post-run flushing with 100% acetonitrile (5 min), followed by 100% milli-Q water (5 min), and then recycled to initial conditions (5 min).

3.4.2 Thiamine (vitamin B₁) and riboflavin (vitamin B₂) analysis on BGN seed extracts

Analytical standards and preparation of calibration standards

Two vitamin standards were used as external standards, namely riboflavin and thiamine (Sigma-Aldrich, Germany). The standard solutions were prepared as described by Otemuyiwa & Adewusi (2013). All standards were stored at -20°C for a maximum of 2 weeks. Sodium mono-hydrogen phosphate was purchased from Sigma-Aldrich (Germany). The mobile phases consisted of mobile phase A and mobile phase B at a ratio of 50:50 v/v. Mobile phase A was made up with 20 mM di-potassium hydrogen orthophosphate anhydrous (Unilab) in 500 mL of milli-Q water and adjusted to a pH of 6 using ortho-phosphoric acid 85% (Merck), once pH was adjusted the remaining volume was made up to 1 L. Mobile phase B consisted of milli-Q water only. Both mobile phases were sonicated for 30 min before usage.

Stock solutions of vitamins B₁ and B₂

The stock standard solutions of thiamine and riboflavin were prepared as described by Otemuyiwa & Adewusi (2013). The working standards were prepared from the stock standard solutions by taking 0.1, 0.2, 0.3, 0.4 and 0.5 $\text{mg}\cdot\text{mL}^{-1}$ of the stock into 10 mL standard flask and made up to mark with appropriate solvent. The solutions were sonicated for 30 min and 20 μL of various concentrations of vitamin standards used for HPLC.

HPLC analyses of vitamins B₁ and B₂

The HPLC separations of the standards and samples were performed using an Agilent 1100 HPLC system. The HPLC system consisted of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment, an autosampler, a G1315C Diode Array Detector (DAD), and a Fluorescence Detector (FLD). The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) integrated peak areas were used to record and store data.

The chromatographic separation column consisted of a Phenomenex Luna C₁₈ column (4.6 x 150 mm) 3 µm and column temperature set at 30°C. The HPLC DAD detector was set at wavelengths 360 nm for riboflavin (vitamin B₁) and 254 nm for thiamine (vitamin B₂) to monitor the elution. The WBG, DBG, BGNH extract samples were individually injected at a volume of 20 µg.mL⁻¹. The elution was isocratic with mobile phases of 50:50 v/v ratio (milli-Q water and 20 mM phosphate buffer at pH = 6) and a flow rate 0.60 mL.min⁻¹ (Otemuyiwa & Adewusi, 2013).

3.4.3 Phenolic analysis on BGN seed extracts

Analytical standards

Five phenolic compounds were used as external standards, namely gallic acid (Sigma Aldrich), catechin (Sigma Aldrich), methyl gallate (Industrial analytics), chlorogenic acid (Industrial analytics) and ellagic acid (Sigma Aldrich). The standard solutions were prepared as described by Møller *et al.* (2009).

Stock solutions of phenols

The stock solution of the five phenolic compounds was prepared to a concentration of 0.5 mg.mL⁻¹ in a solvent consisting of 95% methanol and 5% water. From the stock solution five calibration standards was prepared by diluting with 50% (v/v) methanol-water with the following dilutions 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 mg.mL⁻¹.

HPLC-MS analyses of phenols

HPLC was performed using the equipment previously described. As described by Møller *et al.* (2009), the chromatographic separation with a Phenomenex Luna C18 column (250 x 4.6 mm i.d.; 5 µm) was used. The DAD detector was set a wavelength from 245nm -360nm. The HPLC-DAD/MS system used a programmed gradient mobile phase A [methanol, water, and formic acid (5:95:0.1) (v/v/v)] and mobile phase B [methanol and formic acid (100:0.1) (v/v)] in the following gradient sequence: 0 min, 5% B; 7 min, 18% B; 11 min, 18% B; 15 min, 25% B; 20 min, 50% B, 25 min, 75% B, 26 min, 100% B; 29 min, 100% B; and 30 min, 5% B. The WBG, DBG, BGNH extract samples were individually injected at a volume of 20 µg.mL⁻¹. The flow rate was 1.0 mL.min⁻¹ and column temperature was maintained at 40°C.

3.4.4 Linearity, limit of detection (LOD) and limit of quantification (LOQ) for all HPLC analyses (flavonoid, phenols, riboflavin and thiamine)

A linearity curve was prepared using standard solutions at 0.01, 0.03, 0.10, 0.25, 0.50, 1.0, 1.50, 2.0, 2.50, 5.0, 10.0 and 20.0 $\mu\text{g.mL}^{-1}$. Twelve samples per concentration ($n = 12$) was analysed and the multiple correlation coefficient (R^2) and regression coefficient (R) was used to determine whether the peak area plotted was linear over the concentration range. The linearity curve was assessed to find the suitable linear region that was used to quantify flavonoids, riboflavin, thiamine, and tannins in the BGN seed extract samples.

The LOD and LOQ was calculated based on signal (S) to noise (N) ratios of 3 and 10 ($S/N = 3$ and 10), respectively. The standard deviation (SD) of the response and the slope (SP) of the calibration curve was used to calculate the LOD according to the formula: $\text{LOD} = 3 (\text{SD}/\text{S})$ (Anon, 2002).

The LOQ was determined using the response SD and the slope of the calibration curve according to the formula: $\text{LOQ} = 10(\text{SD}/\text{SP})$. The standard deviation of the response was determined based on the standard deviation of y-intercepts of the regression line. The values of SD and slope was obtained from the LINEST function, when creating the calibration curve in SigmaPlot® (Systat Software, San Jose, USA). The SD of the y-intercept was the standard used for LOD and LOQ calculation.

3.4.5 GC-MS alkaloid analysis on BGN seed extracts

Extracts for gas chromatography mass spectrophotometry (GC-MS) were prepared by weighing 15 mg freeze dried extract and dissolving it in 1 mL (99%) methanol solvent in amber vials. The GC-MS Agilent 6890 N gas chromatography instrument with an Agilent 7683 autosampler, electronic pressure control, split-split- less injector and Agilent 5973 MSD mass selective detector with electronic impact was used for alkaloid analyses. GC operating conditions were as follows: capillary column Agilent DB – 5MS (30 m, 0.25 i.d., 0.25 μm film thickness); oven temperature was increased at a rate of 40°C per minute from 50°C to 200°C; 20°C per minute from 200°C to 280°C, and was maintained for the duration of 12.25 minutes; the injector temperature was set at 250°C; the flow rate of carrier gas (helium) was 1 mL.min^{-1} ; the injection was performed in splitless mode and the purge off time was 0.5 min (Djurendic-Brenesel *et al.*, 2012). The WBGN, DBGN, BGNH extract samples were individually injected at a volume of 20 $\mu\text{g.mL}^{-1}$.

3.5 Data Analysis

Multivariate analysis of variance was used to establish mean difference between treatments. Results expressed as mean \pm standard deviation of triplicate measurements. Duncan multiple range test was used to separate means where difference existed (IBM - SPSS, 2015). Principal Component Analysis (PCA) was performed on aggregated mean centred

phytochemical data using Singular Value Decomposition (SVD) algorithm and random cross validation method with 18 segments to determine potential groupings using Unscrambler X version 10.4.

3.6 Results and Discussion

3.6.1 Qualitative phytochemical screening

Phytochemical compounds, flavonoids, phenols and alkaloids were all positively screened for all BGN seed extracts, and were detected in the BGN extracts. Therefore, the BGN extracts were subjected to quantitative phytochemical analysis.

3.6.2 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity curve parameters for phenols, flavonoids, vitamins B₁ and B₂ are detailed in Table 3.1. For phenols, the linearity curves of the calibration mixture consisting of five phenolic compounds interpreted over a concentration range between 0.01 and 50 µg.mL⁻¹ (Table 3.1). The multiple correlation coefficients (R²) of the five phenolic compounds ranged between 0.997 and 0.999 and the correlation coefficients (R) ranged between 0.998 and 0.999. The LOD for gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid were between 0.04 and 0.49 µg.mL⁻¹, while the LOQ was between 0.13 and 1.64 µg.mL⁻¹ (Table 3.1).

For flavonoids, the linearity curves of the calibration mixture consisting of four flavonoid compounds interpreted over a concentration range between 0.50 and 50 µg.mL⁻¹ (Table 3.1). The multiple correlation coefficients (R²) of the four flavonoid compounds ranged between 0.996 and 0.997 and the correlation coefficients (R) ranged between 0.99639 and 0.99893. The LOD for rutin, myricetin, quercetin and kaempferol were between 0.05 and 0.65 µg.mL⁻¹, while the LOQ was between 0.19 and 2.17 µg.mL⁻¹ (Table 3.1).

For vitamins B₁ and B₂ the linearity curves of the calibration mixture were interpreted over a concentration range between 2 and 20 µg.mL⁻¹ (Table 3.1). The multiple correlation coefficients (R²) of the vitamins ranged between 0.98531 and 0.99998 and the correlation coefficients (R) ranged between 0.992 and 0.999. The LOD for thiamine and riboflavin were between 0.0003 and 0.058 µg.mL⁻¹, while the LOQ was between 0.001 and 0.195 µg.mL⁻¹ (Table 3.1).

The calibration procedures for phenols, flavonoids and both vitamins were executed according to the AOAC method (Anon., 2002). High multiple correlation coefficients (R²) and correlation coefficients (R) of > 0.98 were evidence of a good linear fit (Anon., 2002). The correlation coefficient and multiple correlation coefficients for phenols, flavonoids and vitamin B₁ and vitamin B₂ ranged between 0.98 and 0.99, indicating a noteworthy linear relationship between the concentration of the analytical standards and the response, while the proximity

to one of the regression coefficients shows that the regression line fits the data. The HPLC method was, therefore, adequately sensitive and can be used as an analytical tool.

Table 3.1 Limits of detection (LOD), limits of quantification (LOQ) and linear range of all standard solutions

Phytochemicals	LOD ($\mu\text{g.mL}^{-1}$)	LOQ ($\mu\text{g.mL}^{-1}$)	Liner range ($\mu\text{g.mL}^{-1}$)
Phenols			
Gallic acid	0.11	0.37	0.10 – 50.00
Catechin	0.14	0.49	0.10 – 50.00
Methyl gallate	0.04	0.13	0.10 – 50.00
Chlorogenic acid	0.18	0.60	0.10 – 50.00
Ellagic acid	0.49	1.64	0.10 – 50.00
Flavonoids			
Rutin	0.33	1.12	0.50 – 50.00
Myricetin	0.65	2.17	0.50 – 50.00
Quercetin	0.05	0.19	0.50 – 50.00
Kaempferol	0.11	0.39	0.50 – 50.00
Vitamins B₁ & B₂			
Thiamine	0.00	0.00	2.00 – 20.00
Riboflavin	0.05	0.19	2.00 – 20.00

Peak identification and peak purity

The identification of phenols, flavonoids, and vitamin B₁ and B₂ were achieved by means of the retention time (R_t) and the quantification of the compounds and vitamins were performed by measuring the peak area of the samples relative to that of the standards (Anon, 2002). The retention times for all compounds were as follows; gallic acid (6.19 min), catechin (12.42 min), methyl gallate (14.25 min), chlorogenic acid (15.55 min) and ellagic acid (24.50 min) (Figure 3.3); rutin (1.96 min), myricetin (2.23 min), quercetin (2.68 min) and kaempferol

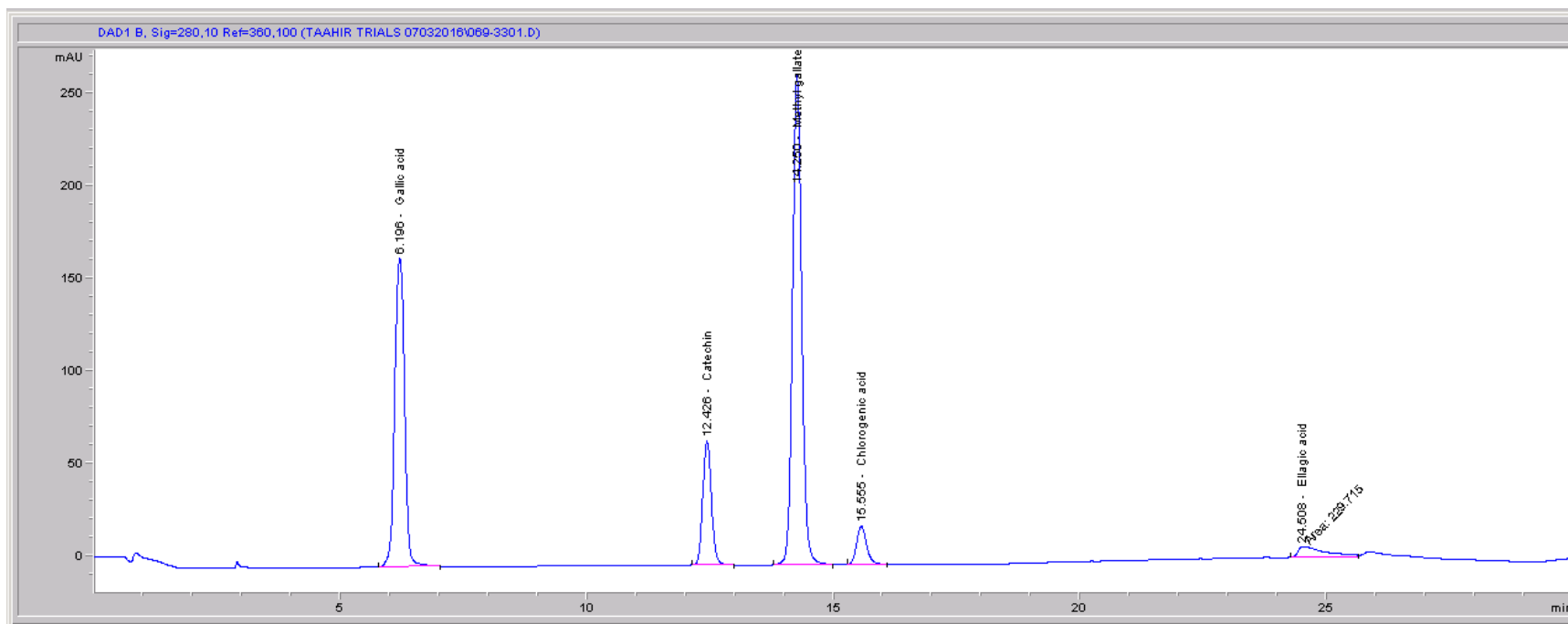


Figure 3.3 Chromatogram of a standard mixture containing five phenols at their respective retention times (R_t), namely gallic acid (6.196), catechin (12.426), methyl gallate (14.250), chlorogenic acid (15.555) and ellagic acid (24.508)

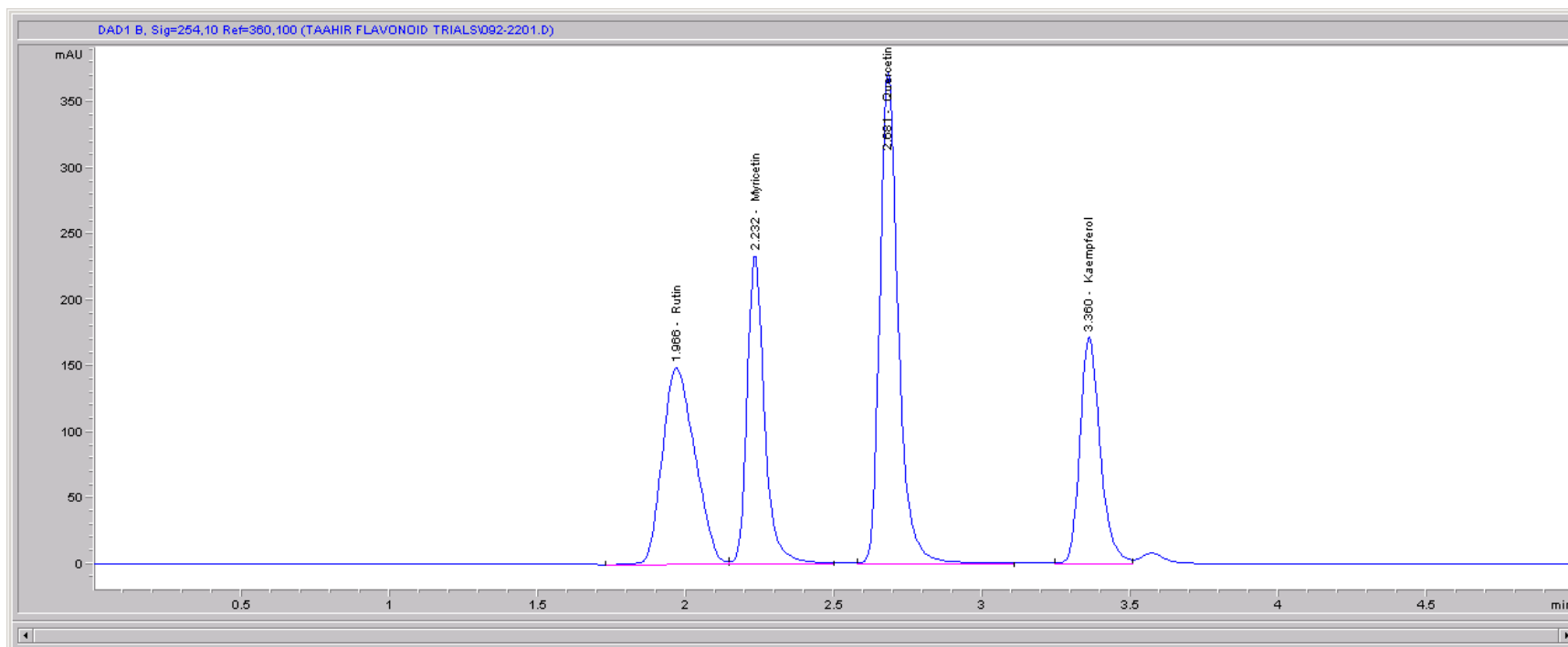


Figure 3.4 Chromatogram of a standard mixture containing four flavonoid at their respective retention times (R_t), namely rutin (1.966), myricetin (2.232), quercetin (2.681) and kaempferol (3.360)

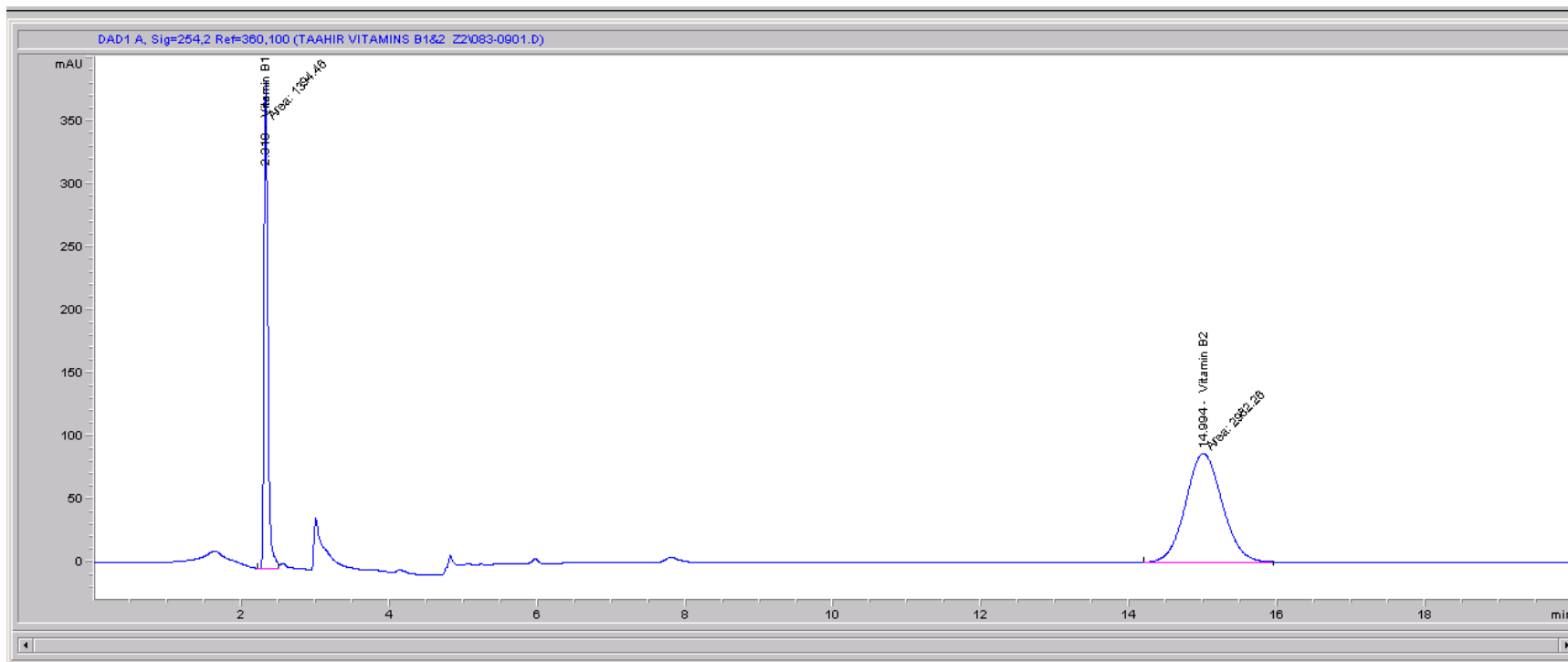


Figure 3.5 Chromatogram of a standard mixture containing two B-vitamins at their respective retention times (R_t), namely vitamin B1 – thiamine (2.318) and vitamin B2 – riboflavin (14.994)

(3.36 min) (Figure 3.4), and for the vitamins: thiamine (2.31 min) and riboflavin (14.99 min) (Figure 3.5). In addition, purity tests were performed using the “Check purity” option of the Chemstation system. This option was used to determine the purity factor of peaks which were within the threshold limit and therefore can be used to calculate the tannin, flavonoid and vitamin B₁ and vitamin B₂ concentrations. The purity factors were > 990, which indicated an excellent peak.

3.6.3 Flavonoid characteristics of BGN extracts based on BGN form

Flavonoid compounds from whole BGN are summarized in Table 3.2. Quercetin ranged between 0.005 and 0.018 mg.g⁻¹. Extraction of BGN with 70% ethanol resulted in an extract with quercetin levels significantly ($p \leq 0.05$) higher than an extract prepared with 70% methanol. Levels of quercetin in milli-Q water did not differ significantly ($p \geq 0.05$) compared to the 70% methanol and 70% ethanol extracts. Kaempferol ranged between 0.052 and 0.138 mg.g⁻¹. Extraction of BGN with milli-Q water resulted in kaempferol levels significantly ($p \leq 0.05$) higher than the extract prepared with 70% methanol. Rutin ranged between 0.352 and 2.599 mg.g⁻¹. Extraction of BGN with 70% ethanol resulted in rutin levels significantly ($p \leq 0.05$) higher than extracts prepared with 70% methanol and milli-Q water, however, there was no significant ($p \geq 0.05$) difference between extracts of BGN prepared with 70% methanol and milli-Q water. Myricetin ranged between 0.022 and 0.135 mg.g⁻¹. Extraction of BGN with 70% ethanol resulted in an extract with myricetin levels significantly ($p \leq 0.05$) higher than extracts prepared with milli-Q water and for 70% methanol.

Ethanol yielded the highest in quercetin, rutin and myricetin amongst all BGN samples. Milli-Q water was the best solvent for the extraction of kaempferol. However, 70% ethanol also resulted in an extract with a relatively high kaempferol concentration. Overall it appeared that ethanol as a solvent yielded the best extraction of the flavonoid compounds for whole BGN. The improved action of the ethanol based extracts as compared to the aqueous extract can be linked to the presence of higher amounts of polyphenols as compared to aqueous extracts. Indicating that they are more efficient in cell walls and seeds degradation which have non-polar characteristics and therefore causing polyphenols to be released from cells (Tiwari *et al.*, 2011). Thus, the type of solvent to optimize better extraction yield depends on which flavonoid to be extracted. Furthermore, other factors that influence the yield of extraction include the polarity of the solvent, pH, temperature, extraction time, and composition of the sample (Do *et al.*, 2014; Dhanani *et al.*, 2017). It should also be noted that quercetin and kaempferol are common flavonoids present in nearly 70% of plants (Doughari, 2012).

Flavonoids of dehulled BGN are summarized in Table 3.3. Quercetin ranged between 0.004 and 0.050 mg.g⁻¹. For quercetin levels, there were no significant ($p \geq 0.05$) differences amongst extracts prepared from BGN.

Table 3.2 Flavonoid characteristics of whole BGN^{1, 2}

BGN sample & solvent	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
Brown				
70% methanol	n.d.	0.052 ± 0.070 ^a	0.645 ± 0.013 ^a	0.062 ± 0.008 ^{ab}
70% ethanol	0.016 ± 0.009 ^a	0.078 ± 0.039 ^{ab}	0.829 ± 0.781 ^b	0.098 ± 0.085 ^a
Milli-Q water	n.d.	0.085 ± 0.074 ^b	0.352 ± 0.305 ^a	0.022 ± 0.038 ^b
Black				
70% methanol	0.006 ± 0.010 ^b	0.131 ± 0.034 ^a	1.427 ± 0.458 ^a	0.123 ± 0.062 ^{ab}
70% ethanol	0.011 ± 0.002 ^a	0.091 ± 0.008 ^{ab}	2.599 ± 0.126 ^b	0.127 ± 0.008 ^a
Milli-Q water	0.009 ± 0.008 ^{ab}	0.104 ± 0.028 ^b	0.849 ± 0.136 ^a	0.079 ± 0.017 ^b
Brown-eye				
70% methanol	0.005 ± 0.008 ^b	0.105 ± 0.013 ^a	0.417 ± 0.337 ^a	0.040 ± 0.038 ^{ab}
70% ethanol	0.016 ± 0.009 ^a	0.104 ± 0.012 ^{ab}	1.773 ± 0.127 ^b	0.094 ± 0.010 ^a
Milli-Q water	0.013 ± 0.003 ^{ab}	0.118 ± 0.015 ^b	0.630 ± 0.234 ^a	0.088 ± 0.065 ^b
Black-eye				
70% methanol	n.d.	n.d.	0.544 ± 0.097 ^a	0.064 ± 0.006 ^{ab}
70% ethanol	0.018 ± 0.007 ^a	0.118 ± 0.011 ^{ab}	1.725 ± 0.370 ^b	0.094 ± 0.012 ^a
Milli-Q water	0.008 ± 0.007 ^{ab}	0.138 ± 0.012 ^b	0.667 ± 0.282 ^a	0.048 ± 0.017 ^b
Red				
70% methanol	0.007 ± 0.012 ^b	0.096 ± 0.085 ^a	0.878 ± 0.858 ^a	0.135 ± 0.151 ^{ab}
70% ethanol	0.009 ± 0.009 ^a	0.113 ± 0.012 ^{ab}	1.148 ± 0.019 ^b	0.162 ± 0.038 ^a
Milli-Q water	0.013 ± 0.022 ^{ab}	0.108 ± 0.013 ^b	0.658 ± 0.119 ^a	0.044 ± 0.052 ^b

¹Results are mean ± standard deviation. Means with different superscripts within each BGN sample column are significantly different ($p \leq 0.05$). ²n.d. = not detected

Table 3.3 Flavonoid characteristics of dehulled BGN^{1, 2}

BGN sample & solvent	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
Brown				
70% methanol	0.021 ± 0.026 ^a	0.129 ± 0.007 ^a	0.881 ± 0.251 ^a	0.070 ± 0.017 ^a
70% ethanol	0.050 ± 0.053 ^a	0.105 ± 0.011 ^a	1.611 ± 0.364 ^b	0.090 ± 0.033 ^b
Milli-Q water	0.004 ± 0.007 ^a	0.125 ± 0.016 ^a	0.566 ± 0.311 ^a	0.060 ± 0.009 ^a
Brown-eye				
70% methanol	0.007 ± 0.011 ^a	0.168 ± 0.062 ^a	0.837 ± 0.190 ^a	0.065 ± 0.008 ^a
70% ethanol	0.010 ± 0.009 ^a	0.129 ± 0.006 ^a	1.146 ± 0.513 ^b	0.062 ± 0.021 ^b
Milli-Q water	0.004 ± 0.007 ^a	0.082 ± 0.071 ^a	0.369 ± 0.432 ^a	0.034 ± 0.030 ^a
Black-eye				
70% methanol	0.004 ± 0.007 ^a	0.077 ± 0.067 ^a	0.476 ± 0.430 ^a	0.042 ± 0.036 ^a
70% ethanol	0.010 ± 0.009 ^a	0.088 ± 0.059 ^a	1.262 ± 0.456 ^b	0.073 ± 0.004 ^b
Milli-Q water	0.010 ± 0.009 ^a	0.122 ± 0.015 ^a	0.460 ± 0.136 ^a	0.054 ± 0.009 ^a
Red				
70% methanol	0.004 ± 0.007 ^a	0.119 ± 0.007 ^a	0.729 ± 0.149 ^a	0.061 ± 0.010 ^a
70% ethanol	n.d.	0.040 ± 0.070 ^a	0.465 ± 0.481 ^b	0.047 ± 0.051 ^b
Milli-Q water	0.005 ± 0.008 ^a	0.119 ± 0.024 ^a	0.561 ± 0.059 ^a	0.013 ± 0.023 ^a

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN solvent in each BGN sample column are significantly different ($p \leq 0.05$). ²n.d. = not detected

Kaempferol ranged between 0.040 and 0.168 mg.g⁻¹. For kaempferol levels, there were no significant ($p \geq 0.05$) differences amongst extracts prepared from BGN. Rutin ranged between 0.369 and 1.611 mg.g⁻¹. Extraction of BGN with 70% ethanol resulted in an extract with rutin levels significantly ($p \leq 0.05$) higher than extracts prepared with 70% methanol and milli-Q water. Myricetin ranged between 0.013 and 0.090 mg.g⁻¹.

Extraction of BGN with 70% ethanol resulted in an extract with myricetin levels significantly ($p \leq 0.05$) higher than extracts prepared with 70% methanol and milli-Q water.

For quercetin and kaempferol the solvents did not differ in their yield, this may possibly be due to BGN being dehulled, and therefore most flavonoids were not situated in the endosperm of the groundnut, but rather in the hulls. As for rutin and myricetin, the extracts of BGN with 70% ethanol seemed superior extracting the highest yields regardless of BGN form.

Flavonoids from BGN hulls are summarized in Table 3.4. Quercetin ranged between 0.011 and 0.077 mg.g^{-1} . For quercetin levels, there were no significant ($p \geq 0.05$) differences amongst BGN extracts. Kaempferol ranged between 0.017 and 0.391 mg.g^{-1} . For kaempferol levels, there were no significant ($p \geq 0.05$) differences amongst BGN extracts. Rutin ranged between 3.977 and 24.458 mg.g^{-1} . Extraction of BGN with 70% ethanol resulted in rutin levels that were significantly ($p \leq 0.05$) lower than extracts prepared with 70% methanol and milli-Q water. For rutin levels, there were no significant ($p \geq 0.05$) differences amongst BGN extracts prepared with 70% methanol and milli-Q water. Myricetin ranged between 0.594 and 1.800 mg.g^{-1} . For myricetin, there were no significant ($p \geq 0.05$) differences amongst BGN extracts.

No significant trend was observed for quercetin, kaempferol and myricetin. However, for rutin, water seemed to not be the best extraction solvent. Furthermore, the BGN hulls contain higher concentration of all flavonoid compounds than whole and dehulled, indicating that the flavonoids are more concentrated in the hulls than in the whole and dehulled. Salawu (2016) reported results of whole BGN (Western States, Sudan) for flavonoid compounds, namely rutin $1.120 \pm 0.010 \text{ mg.g}^{-1}$, quercetin $6.390 \pm 0.010 \text{ mg.g}^{-1}$ and kaempferol $2.180 \pm 0.020 \text{ mg.g}^{-1}$. The rutin level reported by Salawu (2016) was comparable to the present study, values for quercetin and kaempferol were higher compared to those of the present study. This could be due to the source of BGN or the extraction process followed by Salawu (2016). The extraction duration of BGN extracts performed by Salawu (2016) was 24 h, whilst the duration of extraction of extracts for the present study was 30 min. Furthermore, Salawu (2016) used no extraction catalyst (e.g. ultrasound bath), as the methanolic extracts were left to stand for 24 h whereas in the present study, an ultrasound bath was used to aid extraction of phytochemicals.

3.6.4 Flavonoid characteristics of BGN extracts based on BGN colour

Flavonoids of brown BGN are summarized in Table 3.5. Quercetin ranged between 0.004 and 0.077 mg.g^{-1} . For quercetin levels, there were no significant ($p \geq 0.05$) differences amongst extracts. Kaempferol ranged between 0.052 and 0.391 mg.g^{-1} . The hulls were significantly ($p \leq 0.05$) higher in kaempferol level than whole and dehulled. Rutin ranged between 0.352 and 24.458 mg.g^{-1} .

Table 3.4 Flavonoid characteristics of hull BGN form^{1,2}

BGN sample and solvent	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
Brown				
70% methanol	0.077 ± 0.123 ^a	0.297 ± 0.094 ^a	24.458 ± 0.234 ^a	0.932 ± 0.550 ^a
70% ethanol	0.011 ± 0.010 ^a	0.391 ± 0.161 ^a	14.500 ± 6.767 ^a	0.594 ± 0.557 ^a
Milli-Q water	0.059 ± 0.090 ^a	0.287 ± 0.091 ^a	8.374 ± 4.468 ^b	0.875 ± 0.465 ^a
Brown-eye				
70% methanol	0.062 ± 0.041 ^a	0.219 ± 0.090 ^a	3.977 ± 0.447 ^a	0.622 ± 0.279 ^a
70% ethanol	0.047 ± 0.039 ^a	0.257 ± 0.097 ^a	5.942 ± 0.253 ^a	0.844 ± 0.256 ^a
Milli-Q water	0.065 ± 0.040 ^a	0.190 ± 0.178 ^a	4.882 ± 1.347 ^b	0.662 ± 0.380 ^a
Black-eye				
70% methanol	0.057 ± 0.054 ^a	0.224 ± 0.008 ^a	6.233 ± 1.251 ^a	0.986 ± 0.432 ^a
70% ethanol	0.059 ± 0.060 ^a	0.065 ± 0.062 ^a	8.221 ± 1.290 ^a	0.887 ± 0.507 ^a
Milli-Q water	0.042 ± 0.034 ^a	0.017 ± 0.018 ^a	4.821 ± 0.388 ^b	0.617 ± 0.258 ^a
Red				
70% methanol	0.070 ± 0.043 ^a	0.159 ± 0.129 ^a	7.236 ± 4.101 ^a	1.800 ± 0.771 ^a
70% ethanol	0.042 ± 0.053 ^a	0.057 ± 0.098 ^a	8.319 ± 7.669 ^a	0.969 ± 1.010 ^a
Milli-Q water	0.052 ± 0.030 ^a	0.313 ± 0.057 ^a	6.655 ± 2.119 ^b	1.686 ± 0.558 ^a

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN solvent within each BGN sample column are significantly different ($p \leq 0.05$). ²n.d. = not detected

Rutin levels were significantly ($p \leq 0.05$) higher in hulls than whole and dehulled, furthermore there were no significant ($p \geq 0.05$) differences between whole and dehulled. Myricetin ranged between 0.022 and 0.932 mg.g⁻¹. Myricetin levels were significantly ($p \leq 0.05$) higher in hulls than whole and dehulled. Kaempferol, rutin and myricetin were significantly higher in brown hulls regardless of which solvent was used, signifying that most of these flavonoids are concentrated in the hulls.

Table 3.5 Flavonoid characteristics of brown BGN^{1,2}

BGN solvent and form	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
70% Methanol				
Whole	n.d.	0.052 ± 0.070 ^a	0.645 ± 0.013 ^a	0.062 ± 0.008 ^a
Dehulled	0.021 ± 0.026 ^a	0.129 ± 0.007 ^a	0.881 ± 0.251 ^a	0.070 ± 0.017 ^a
Hull	0.077 ± 0.123 ^a	0.297 ± 0.094 ^b	24.458 ± 0.234 ^b	0.932 ± 0.550 ^b
70% Ethanol				
Whole	0.016 ± 0.009 ^a	0.078 ± 0.039 ^a	0.829 ± 0.781 ^a	0.098 ± 0.085 ^a
Dehulled	0.050 ± 0.053 ^a	0.105 ± 0.011 ^a	1.611 ± 0.364 ^a	0.090 ± 0.033 ^a
Hull	0.011 ± 0.010 ^a	0.391 ± 0.161 ^b	14.500 ± 6.767 ^b	0.594 ± 0.557 ^b
Milli-Q water				
Whole	n.d.	0.085 ± 0.074 ^a	0.352 ± 0.305 ^a	0.022 ± 0.038 ^a
Dehulled	0.004 ± 0.007 ^a	0.125 ± 0.016 ^a	0.566 ± 0.311 ^a	0.060 ± 0.009 ^a
Hull	0.059 ± 0.090 ^a	0.287 ± 0.091 ^b	8.374 ± 4.468 ^b	0.875 ± 0.465 ^b

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN form within each BGN solvent column are significantly different ($p \leq 0.05$). ²n.d. = not detected.

With regards to quercetin, there was no difference between solvents used for the extraction of BGN or which form the BGN. Flavonoid compounds of black BGN were summarized in Table 3.6. Due to insufficient raw material to prepare dehulled and hull BGN flour, only the whole form of black BGN could be analysed, therefore there were no comparisons on various forms of BGN to represent significant differences. Nonetheless for black whole BGN, quercetin ranged between 0.006 and 0.011 mg.g⁻¹. Kaempferol ranged between 0.091 and 0.131 mg.g⁻¹. Rutin ranged between 0.849 and 2.599 mg.g⁻¹. Myricetin ranged between 0.079 and 0.127 mg.g⁻¹. Although no significant differences could be identified it can still be noted that ethanol solvent yielded higher extraction than methanol and water solvents.

Flavonoid compounds of brown-eye BGN were summarized in Table 3.7. Quercetin ranged between 0.004 and 0.065 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in quercetin than whole and dehulled. There was no significant ($p \geq 0.05$) difference between

whole and dehulled for quercetin. Kaempferol ranged between 0.082 and 0.257 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in kaempferol than whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for kaempferol. Rutin ranged between 0.369 and 5.942 mg.g⁻¹. Hulls were significantly ($p \leq 0.05$) higher in rutin than whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for rutin. Myricetin ranged between 0.034 and 0.844 mg.g⁻¹. Hulls of BGN were significantly ($p \leq 0.05$) higher in myricetin than whole and dehulled BGN. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for myricetin. The brown-eye hulls were highly concentrated in flavonoid compounds when compared to the whole and dehulled.

Flavonoid compounds of black-eye BGN were summarized in Table 3.8. Quercetin ranged between 0.004 and 0.059 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in quercetin than whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for quercetin. Kaempferol ranged between 0.077 and 0.224 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst all forms of BGN in kaempferol. Rutin ranged between 0.460 and 8.221 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in rutin than whole and dehulled, furthermore there was no significant ($p \geq 0.05$) difference between whole and dehulled forms for rutin. Myricetin ranged between 0.042 and 0.986 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in myricetin than whole and dehulled BGN. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for myricetin.

Table 3.6 Flavonoid characteristics of black BGN^{1,2}

BGN solvent	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
70% Methanol	0.006 ± 0.010	0.131 ± 0.034	1.427 ± 0.458	0.123 ± 0.062
70% Ethanol	0.011 ± 0.002	0.091 ± 0.008	2.599 ± 0.126	0.127 ± 0.008
Milli-Q water	0.009 ± 0.008	0.104 ± 0.028	0.849 ± 0.136	0.079 ± 0.017

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN form within each BGN solvent column are significantly different ($p \leq 0.05$). ²n.d. = not detected

Table 3.7 Flavonoid characteristics of brown-eye BGN^{1, 2}

BGN solvent and form	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
70% Methanol				
Whole	0.005 ± 0.008 ^a	0.105 ± 0.013 ^a	0.417 ± 0.337 ^a	0.040 ± 0.038 ^a
Dehulled	0.007 ± 0.011 ^a	0.168 ± 0.062 ^a	0.837 ± 0.190 ^a	0.065 ± 0.008 ^a
Hull	0.062 ± 0.041 ^b	0.219 ± 0.090 ^b	3.977 ± 0.447 ^b	0.622 ± 0.279 ^b
70% Ethanol				
Whole	0.016 ± 0.009 ^a	0.104 ± 0.012 ^a	1.773 ± 0.127 ^a	0.094 ± 0.010 ^a
Dehulled	0.010 ± 0.009 ^a	0.129 ± 0.006 ^a	1.146 ± 0.513 ^a	0.062 ± 0.021 ^a
Hull	0.047 ± 0.039 ^b	0.257 ± 0.097 ^b	5.942 ± 0.253 ^b	0.844 ± 0.256 ^b
Milli-Q water				
Whole	0.013 ± 0.003 ^a	0.118 ± 0.015 ^a	0.630 ± 0.234 ^a	0.088 ± 0.065 ^a
Dehulled	0.004 ± 0.007 ^a	0.082 ± 0.071 ^a	0.369 ± 0.432 ^a	0.034 ± 0.030 ^a
Hull	0.065 ± 0.040 ^b	0.190 ± 0.178 ^b	4.882 ± 1.347 ^b	0.662 ± 0.380 ^b

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN form within each BGN solvent column are significantly different ($p \leq 0.05$). ²n.d. = not detected

Flavonoids quercetin, rutin and myricetin were significantly higher in the black-eye hulls than whole and dehulled. As for kaempferol it appeared that the form of BGN had no effect on results for black-eye BGN.

Flavonoids of red BGN (Table 3.9) quercetin ranged between 0.004 and 0.070 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in quercetin than whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for quercetin. Kaempferol ranged between 0.040 and 0.313 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in kaempferol than whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for kaempferol. Rutin ranged between 0.465 and 8.319 mg.g⁻¹. The hulls were significantly ($p \leq 0.05$) higher in rutin than whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for rutin.

Myricetin ranged between 0.047 and 1.800 mg.g⁻¹. Hulls were significantly ($p \leq 0.05$) higher in myricetin than in whole and dehulled. There was no significant ($p \geq 0.05$) difference between whole and dehulled forms for myricetin. The red hulls were highly concentrated in flavonoids when compared to the whole and dehulled.

Table 3.8 Flavonoid characteristics of black-eye BGN^{1, 2}

BGN solvent and form	Flavonoids (mg.g ⁻¹)			
	Quercetin	Kaempferol	Rutin	Myricetin
70% Methanol				
Whole	n.d.	n.d.	0.544 ± 0.097 ^a	0.064 ± 0.006 ^a
Dehulled	0.004 ± 0.007 ^a	0.077 ± 0.067 ^a	0.476 ± 0.430 ^a	0.042 ± 0.036 ^a
Hull	0.057 ± 0.054 ^b	0.224 ± 0.008 ^a	6.233 ± 1.251 ^b	0.986 ± 0.432 ^b
70% Ethanol				
Whole	0.018 ± 0.007 ^a	0.118 ± 0.011 ^a	1.725 ± 0.370 ^a	0.094 ± 0.012 ^a
Dehulled	0.010 ± 0.009 ^a	0.088 ± 0.059 ^a	1.262 ± 0.456 ^a	0.073 ± 0.004 ^a
Hull	0.059 ± 0.060 ^b	0.065 ± 0.062 ^a	8.221 ± 1.290 ^b	0.887 ± 0.507 ^b
Milli-Q water				
Whole	0.008 ± 0.007 ^a	0.138 ± 0.012 ^a	0.667 ± 0.282 ^a	0.048 ± 0.017 ^a
Dehulled	0.010 ± 0.009 ^a	0.122 ± 0.015 ^a	0.460 ± 0.136 ^a	0.054 ± 0.009 ^a
Hull	0.042 ± 0.034 ^b	0.017 ± 0.018 ^a	4.821 ± 0.388 ^b	0.617 ± 0.258 ^b

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN form within each BGN solvent column are significantly different ($p \leq 0.05$). ²n.d. = not detected

3.6.5 Flavonoid characteristics of BGN extracts based on extraction solvent

Flavonoids of BGN extracted with 70% methanol solvent summarized in Table 3.10. Quercetin ranged between 0.004 and 0.077 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences for quercetin amongst samples. Kaempferol ranged between 0.052 and 0.297 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences for kaempferol amongst all samples. Rutin ranged between 0.417 and 24.458 mg.g⁻¹. Brown BGN was significantly

($p \leq 0.05$) higher in rutin than black, brown-eye, black-eye and red. There were no significant differences ($p \geq 0.05$) between black, brown-eye, black-eye and red. Myricetin ranged between 0.040 and 1.800 mg.g^{-1} . Brown-eye and black were significantly ($p \leq 0.05$) lower in myricetin, whereas red was significantly ($p \leq 0.05$) higher in myricetin for all samples. There was no significant ($p \geq 0.05$) difference between brown and black-eye.

The different forms of BGN (hulls, whole, dehulled) did not differ in their quercetin and kaempferol content. Rutin was better extracted with 70% methanol from black, brown-eye, black-eye and red BGN. The red BGN had the highest concentration of myricetin when extracted with 70% methanol.

Flavonoids of BGN extracted with 70% ethanol are summarized in Table 3.11. Quercetin ranged between 0.009 and 0.059 mg.g^{-1} . There were no significant ($p \geq 0.05$) differences with regards to quercetin amongst all BGN samples. Kaempferol ranged between 0.057 and 0.391 mg.g^{-1} .

Table 3.9 Flavonoid characteristics of red BGN^{1, 2}

BGN solvent	BGN solvent and form	Flavonoids (mg.g^{-1})			
		Quercetin	Kaempferol	Rutin	Myricetin
70% Methanol	Whole	0.007 ± 0.012 ^a	0.096 ± 0.085 ^a	0.878 ± 0.858 ^a	0.135 ± 0.151 ^a
	Dehulled	0.004 ± 0.007 ^a	0.119 ± 0.007 ^a	0.729 ± 0.149 ^a	0.061 ± 0.010 ^a
	Hull	0.070 ± 0.043 ^b	0.159 ± 0.129 ^b	7.236 ± 4.101 ^b	1.800 ± 0.771 ^b
70% Ethanol	Whole	0.009 ± 0.009 ^a	0.113 ± 0.012 ^a	1.148 ± 0.019 ^a	0.162 ± 0.038 ^a
	Dehulled	n.d.	0.040 ± 0.070 ^a	0.465 ± 0.481 ^a	0.047 ± 0.051 ^a
	Hull	0.042 ± 0.053 ^b	0.057 ± 0.098 ^b	8.319 ± 7.669 ^b	0.969 ± 1.010 ^b
Milli-Q water	Whole	0.013 ± 0.022 ^a	0.108 ± 0.013 ^a	0.658 ± 0.119 ^a	0.044 ± 0.052 ^a
	Dehulled	0.005 ± 0.008 ^a	0.119 ± 0.024 ^a	0.561 ± 0.059 ^a	0.013 ± 0.023 ^a
	Hull	0.052 ± 0.030 ^b	0.313 ± 0.057 ^b	6.655 ± 2.119 ^b	1.686 ± 0.558 ^b

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN form within each BGN solvent column are significantly different ($p \leq 0.05$). ²n.d. = not detected

Table 3.10 Flavonoid characteristics of BGN extracted with 70% methanol solvent^{1,2}

BGN form	BGN sample	Flavonoids (mg.g ⁻¹)			
		Quercetin	Kaempferol	Rutin	Myricetin
Whole	Brown	n.d	0.052 ± 0.070 ^a	0.645 ± 0.013 ^a	0.062 ± 0.008 ^{ab}
	Black	0.006 ± 0.010 ^a	0.131 ± 0.034 ^a	1.427 ± 0.458 ^b	0.123 ± 0.062 ^a
	Brown-eye	0.005 ± 0.008 ^a	0.105 ± 0.013 ^a	0.417 ± 0.337 ^b	0.040 ± 0.038 ^a
	Black-eye	n.d	n.d	0.544 ± 0.097 ^b	0.064 ± 0.006 ^{ab}
	Red	0.007 ± 0.012 ^a	0.096 ± 0.085 ^a	0.878 ± 0.858 ^b	0.135 ± 0.151 ^b
Dehulled	Brown	0.021 ± 0.026 ^a	0.129 ± 0.007 ^a	0.881 ± 0.251 ^a	0.070 ± 0.017 ^{ab}
	Brown-eye	0.007 ± 0.011 ^a	0.168 ± 0.062 ^a	0.837 ± 0.190 ^b	0.065 ± 0.008 ^a
	Black-eye	0.004 ± 0.007 ^a	0.077 ± 0.067 ^a	0.476 ± 0.430 ^b	0.042 ± 0.036 ^{ab}
	Red	0.004 ± 0.007 ^a	0.119 ± 0.007 ^a	0.729 ± 0.149 ^b	0.061 ± 0.010 ^b
Hull	Brown	0.077 ± 0.123 ^a	0.297 ± 0.094 ^a	24.458 ± 0.234 ^a	0.932 ± 0.550 ^{ab}
	Brown-eye	0.062 ± 0.041 ^a	0.219 ± 0.090 ^a	3.977 ± 0.447 ^b	0.622 ± 0.279 ^a
	Black-eye	0.057 ± 0.054 ^a	0.224 ± 0.008 ^a	6.233 ± 1.251 ^b	0.986 ± 0.432 ^{ab}
	Red	0.070 ± 0.043 ^a	0.159 ± 0.129 ^a	7.236 ± 4.101 ^b	1.800 ± 0.771 ^b

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN sample within each BGN form column are significantly different ($p \leq 0.05$).

²n.d. = not detected.

Table 3.11 Flavonoid characteristics of BGN extracted with 70% ethanol solvent^{1, 2}

BGN form	BGN sample	Flavonoids (mg.g ⁻¹)			
		Quercetin	Kaempferol	Rutin	Myricetin
Whole	Brown	0.016 ± 0.009 ^a	0.078 ± 0.039 ^a	0.829 ± 0.781 ^a	0.098 ± 0.085 ^a
	Black	0.011 ± 0.002 ^a	0.091 ± 0.008 ^{bc}	2.599 ± 0.126 ^a	0.127 ± 0.008 ^a
	Brown-eye	0.016 ± 0.009 ^a	0.104 ± 0.012 ^{ab}	1.773 ± 0.127 ^a	0.094 ± 0.010 ^a
	Black-eye	0.018 ± 0.007 ^a	0.118 ± 0.011 ^{bc}	1.725 ± 0.370 ^a	0.094 ± 0.012 ^a
	Red	0.009 ± 0.009 ^a	0.113 ± 0.012 ^c	1.148 ± 0.019 ^a	0.162 ± 0.038 ^a
Dehulled	Brown	0.050 ± 0.053 ^a	0.105 ± 0.011 ^a	1.611 ± 0.364 ^a	0.090 ± 0.033 ^a
	Brown-eye	0.010 ± 0.009 ^a	0.129 ± 0.006 ^{ab}	1.146 ± 0.513 ^a	0.062 ± 0.021 ^a
	Black-eye	0.010 ± 0.009 ^a	0.088 ± 0.059 ^{bc}	1.262 ± 0.456 ^a	0.073 ± 0.004 ^a
	Red	n.d.	0.040 ± 0.070 ^c	0.465 ± 0.481 ^a	0.047 ± 0.051 ^a
Hull	Brown	0.011 ± 0.010 ^a	0.391 ± 0.161 ^a	14.500 ± 6.767 ^a	0.594 ± 0.557 ^a
	Brown-eye	0.047 ± 0.039 ^a	0.257 ± 0.097 ^{ab}	5.942 ± 0.253 ^a	0.844 ± 0.256 ^a
	Black-eye	0.059 ± 0.060 ^a	0.065 ± 0.062 ^{bc}	8.221 ± 1.290 ^a	0.887 ± 0.507 ^a
	Red	0.042 ± 0.053 ^a	0.057 ± 0.098 ^c	8.319 ± 7.669 ^a	0.969 ± 1.010 ^a

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN sample within each BGN form column are significantly different (p ≤ 0.05).

²n.d. = not detected.

Brown was significantly ($p \leq 0.05$) higher in kaempferol compared to black, black-eye and red. Red was significantly ($p \leq 0.05$) lower in kaempferol compared to brown and brown-eye. There were no significant ($p \geq 0.05$) differences in kaempferol between black, brown-eye and black-eye. Rutin ranged between 0.465 and 14.500 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst BGN samples. Myricetin ranged between 0.062 and 0.969 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst BGN samples. Extraction solvent 70% ethanol had no significant effect on flavonoids quercetin, rutin and myricetin. Kaempferol had the highest yield for brown hull BGN. By adding water to the pure ethanol up to 30% for preparing ethanol 70% the polarity of solvent was increased. Thus, polar compounds like flavonoids could be isolated better from sample matrix (Bimakr *et al.*, 2011).

Flavonoids of BGN extracted with milli-Q water solvent are summarized in Table 3.12. Quercetin ranged between 0.004 and 0.065 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst BGN samples in quercetin. Kaempferol ranged between 0.082 and 0.313 mg.g⁻¹. Black-eye was significantly ($p \leq 0.05$) different to red in kaempferol concentration. As for brown, black and brown-eye BGN there were no significant ($p \geq 0.05$) differences amongst them for kaempferol. Rutin ranged between 0.352 and 8.374 mg.g⁻¹. Brown was significantly ($p \leq 0.05$) different to black in rutin concentration. As for brown-eye, black-eye and red BGN there were no significant ($p \geq 0.05$) differences amongst them for rutin. Myricetin ranged between 0.013 and 1.686 mg.g⁻¹. Red was significantly ($p \leq 0.05$) different to black, brown-eye and black-eye. Kaempferol, rutin and myricetin were higher in red and brown hull BGN, suggesting that perhaps the colour is attributing to the higher yields of these flavonoid compounds.

It appeared that ethanol as a solvent yielded the best extraction of the flavonoid compounds. The improved action of the ethanol based extracts as compared to the aqueous extract can be linked to the presence of higher amounts of polyphenols as compared to aqueous extracts. Indigenous people who consume BGN regularly, before consumption would remove the seed coat of BGN as it was regarded as waste. However, the seed coats have been previously reported by Klompong & Benjakul (2015) to possess plenty of phenolic compounds. The largest naturally occurring group of phenolic compounds are flavonoids, which occurs in different plant parts both in free state and as glycosides (Ibrahim *et al.*, 2011).

General discussion on flavonoid content of BGN

When comparing the three forms of BGN for utilization, preference should go to whole BGN, as it contains both the endosperm and the hulls. Reason being, the endosperm or dehulled BGN contained the protein of BGN and the hulls includes the high flavonoid concentration providing overall nutritional benefits.

The pigments that colour most flowers, fruits, and seeds are flavonoids. Flavonoids are also responsible for the display of fall colour in many plants, which may protect leaf cells from photo-oxidative damage, enhancing the efficiency of nutrient retrieval. An example of flavonoids and colour correlation can be seen in plant species as mentioned by Ferreyra *et al.* (2012). The unique structure and combination of different flavonoids in each plant species produce yellow pollen with a range of visible and UV reflection spectra that can be detected by the targeted insects and larger animals, facilitating successful pollination. However, the existence of “white pollen” has been reported in species as diverse as bristle cone pine and morning glory. The correlation between pollen richness and flavonoids was first established in wind pollinated maize, with its numerous and well-characterized anthocyanin mutants. Flavonoid-deficient mutants lacking chalcone synthase were generated in maize and petunia to explain the roles of flavonoids in pollen. These mutants were deficient in flavonoids. This deficiency could be reversed by adding the flavanol kaempferol at pollination. (Ferreyra *et al.*, 2012)

Hence the indication of colour in the yellow pollen displayed the presence of flavonoids at a higher concentration than “white pollen”. Comparable results can be observed with BGN hulls as the darker colours in BGN hulls namely, red and brown have higher concentration of flavonoids than the black-eye and brown-eye counterparts. In summary, the brown and red hulls had the highest concentration of flavonoids compared to whole and dehulled, with the highest flavonoid concentration being rutin at 24.458 mg.g⁻¹ found in brown hulls and myricetin at 1.800 mg.g⁻¹ found in red hulls. Rutin main health role is to help the body utilize vitamin C more efficiently, as well as help produce collagen (skin), whereas myricetin is an antioxidant regulating blood sugar levels, cancer cells and general skin health.

The seed coat (hull) of pulses is often indigestible and may have a bitter taste. Thus, dehulling is one of the most important operations in post-harvest handling of legumes. Dehulling has also been reported to improve the palatability and more importantly reducing the cooking time of pulses by removing its impermeable seed coat that hinders water absorption during cooking (Kanatt *et al.*, 2011).

However, an alternative could be to mill the whole BGN into flour and develop products from the flour instead of cooking the whole BGN to avoid high energy inputs. Consuming BGN with the hulls will therefore also include the phytochemicals namely, flavonoids with many useful properties as seen with rutin and myricetin, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity antiallergic activity, antioxidant activity, vascular activity and cytotoxic anti-tumour activity (Harbone & Williams, 2000; Cushnie & Lamb, 2005).

Therefore, when comparing whole and dehulled it is clear that whole BGN should be considered rather than dehulled when preparing meals or utilized as a medicine, as most of

Table 3.12 Flavonoid characteristics of BGN extracted with milli-Q water solvent^{1, 2}

BGN form	BGN seeds	Flavonoids (mg.g ⁻¹)			
		Quercetin	Kaempferol	Rutin	Myricetin
Whole	Brown	n.d.	0.085 ± 0.074 ^{ab}	0.352 ± 0.305 ^a	0.022 ± 0.038 ^{ab}
	Black	0.009 ± 0.008 ^a	0.104 ± 0.028 ^{ab}	0.849 ± 0.136 ^b	0.079 ± 0.017 ^a
	Brown-eye	0.013 ± 0.003 ^a	0.118 ± 0.015 ^{ab}	0.630 ± 0.234 ^{ab}	0.088 ± 0.065 ^a
	Black-eye	0.008 ± 0.007 ^a	0.138 ± 0.012 ^a	0.667 ± 0.282 ^{ab}	0.048 ± 0.017 ^a
	Red	0.013 ± 0.022 ^a	0.108 ± 0.013 ^b	0.658 ± 0.119 ^{ab}	0.044 ± 0.052 ^b
Dehulled	Brown	0.004 ± 0.007 ^a	0.125 ± 0.016 ^{ab}	0.566 ± 0.311 ^a	0.060 ± 0.009 ^{ab}
	Brown-eye	0.004 ± 0.007 ^a	0.082 ± 0.071 ^{ab}	0.369 ± 0.432 ^{ab}	0.034 ± 0.030 ^a
	Black-eye	0.010 ± 0.009 ^a	0.122 ± 0.015 ^a	0.460 ± 0.136 ^{ab}	0.054 ± 0.009 ^a
	Red	0.005 ± 0.008 ^a	0.119 ± 0.024 ^b	0.561 ± 0.059 ^{ab}	0.013 ± 0.023 ^b
Hull	Brown	0.059 ± 0.090 ^a	0.287 ± 0.091 ^{ab}	8.374 ± 4.468 ^a	0.875 ± 0.465 ^{ab}
	Brown-eye	0.065 ± 0.040 ^a	0.190 ± 0.178 ^{ab}	4.882 ± 1.347 ^{ab}	0.662 ± 0.380 ^a
	Black-eye	0.042 ± 0.034 ^a	0.017 ± 0.018 ^a	4.821 ± 0.388 ^{ab}	0.617 ± 0.258 ^a
	Red	0.052 ± 0.030 ^a	0.313 ± 0.057 ^b	6.655 ± 2.119 ^{ab}	1.686 ± 0.558 ^b

¹Results are mean ± standard deviation. Means with different letter superscripts representing BGN sample within each BGN form column are significantly different ($p \leq 0.05$).

²n.d. = not detected.

the flavonoid compounds are situated in the hulls instead of the endosperm. The intake of flavonoid-rich foods potentially has beneficial effects on health, with different classes of flavonoids having different positive effects on disease risk (Harbone & Williams, 2000).

3.6.6 Phenol characteristics of BGN extracts based on BGN form

Phenols of whole BGN seed extracts were summarized in Figure 3.6. Gallic acid ranged between 0.002 and 0.007 mg.g⁻¹. Extraction of BGN with milli-Q water resulted in gallic acid levels significantly ($p \leq 0.05$) higher than that of 70% methanol and 70% ethanol. Furthermore, there was no significant difference for gallic acid between 70% methanol and 70% ethanol. Catechin ranged between 0.001 and 0.008 mg.g⁻¹. Methyl gallate ranged between 0.001 and 0.008 mg.g⁻¹. Chlorogenic acid ranged from 0.002 mg.g⁻¹ to 0.518 mg.g⁻¹. Ellagic acid ranged between 0.003 and 0.014 mg.g⁻¹.

Solvent milli-Q water was significantly ($p \leq 0.05$) higher in ellagic acid than 70% methanol for all samples. There were no significant differences between milli-Q water with 70% ethanol, and 70% methanol compared to 70% ethanol. Milli-Q water yielded higher in total phenols than other solvents for whole BGN, perhaps due to the variance in solvent properties e.g. polarity, pH etc. (Tiwari *et al.*, 2011).

Phenols of dehulled BGN were summarized in Figure 3.7. Gallic acid was 0.002 mg.g⁻¹, catechin was 0.001 mg.g⁻¹ and methyl gallate was 0.001 mg.g⁻¹ for all extraction solvents. Chlorogenic acid was not detected for all the extraction solvents for BGN form and BGN seeds. Ellagic acid ranged between 0.004 and 0.016 mg.g⁻¹. Extraction solvents 70% methanol and milli-Q water were significantly ($p \leq 0.05$) higher in ellagic acid than 70% ethanol. There were no significant differences between 70% methanol and milli-Q water for ellagic acid.

As seen in Figure 3.7 most phenols compounds gallic acid, catechin, methyl gallate and chlorogenic acid were not detected apart from ellagic acid. With regards to ellagic acid extraction solvents 70% methanol and milli-Q water yielded slightly higher in ellagic acid levels than 70% ethanol, suggesting that sample properties are important for optimized extraction.

Phenols of BGN hull form were summarized in Figure 3.8. Gallic acid ranged between 0.002 and 0.009 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences for gallic acid amongst all extraction solvents. Catechin ranged from 0.001 to 0.026 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences for catechin amongst all extraction solvents. Methyl gallate ranged between 0.002 and 0.005 mg.g⁻¹. There were no significant differences for catechin amongst all extraction solvents. Chlorogenic acid ranged from 0.012 to 0.115 mg.g⁻¹. There were no significant differences for chlorogenic acid amongst solvents. Ellagic acid ranged between 0.003 and 0.105 mg.g⁻¹.

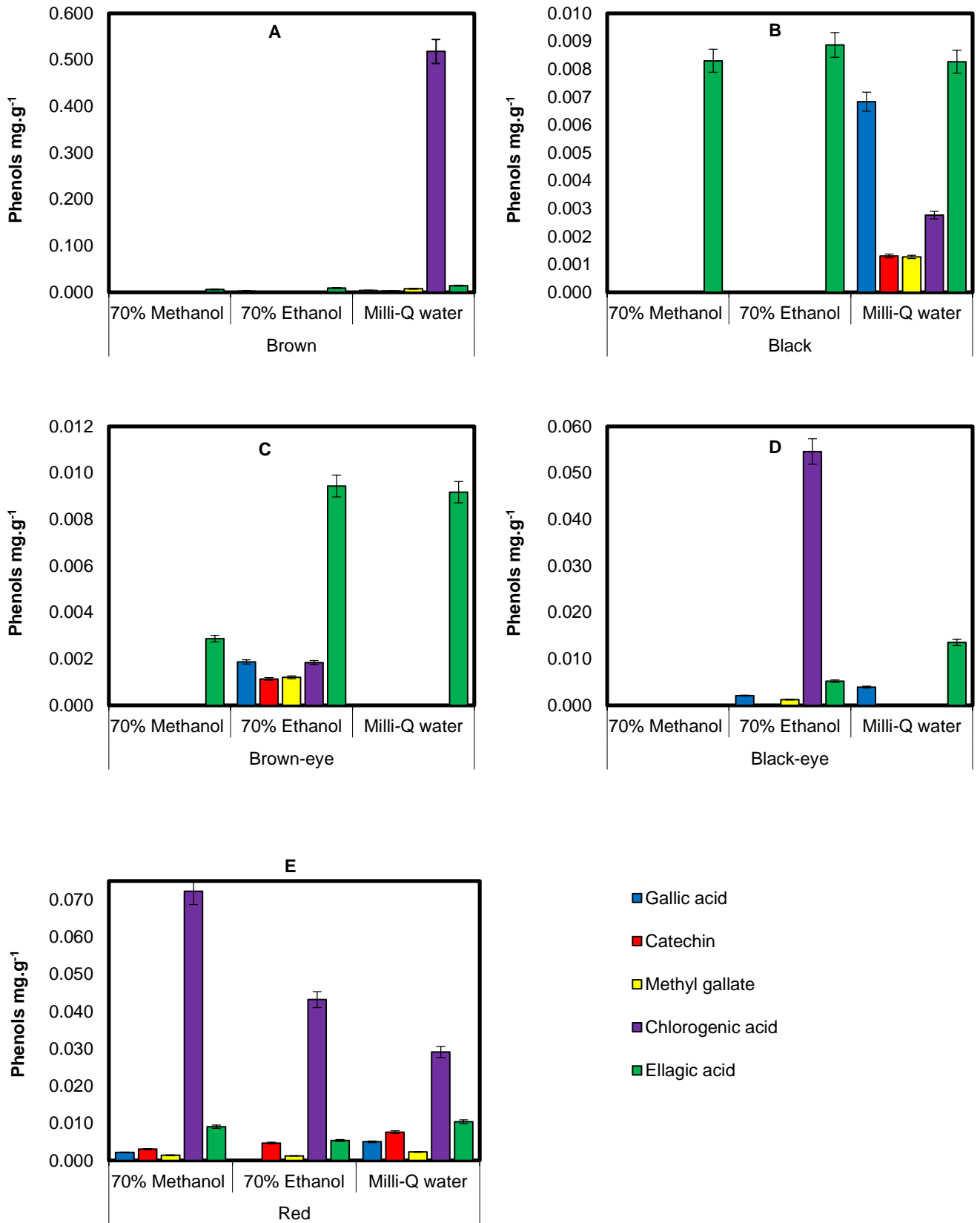


Figure 3.6 Phenols of whole BGN from A = Brown, B = Black, C = Brown-eye, D = Black-eye, E = Red

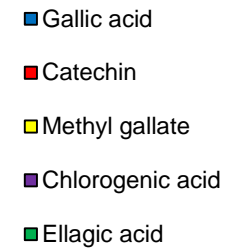
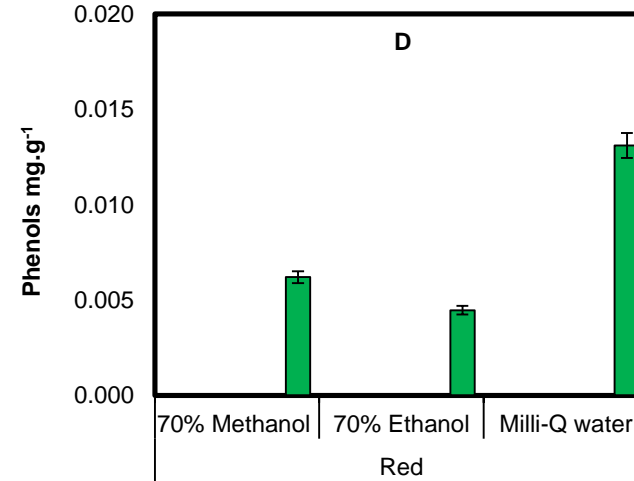
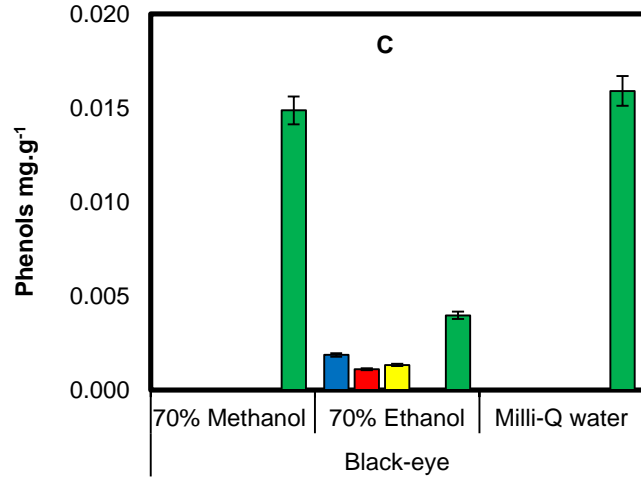
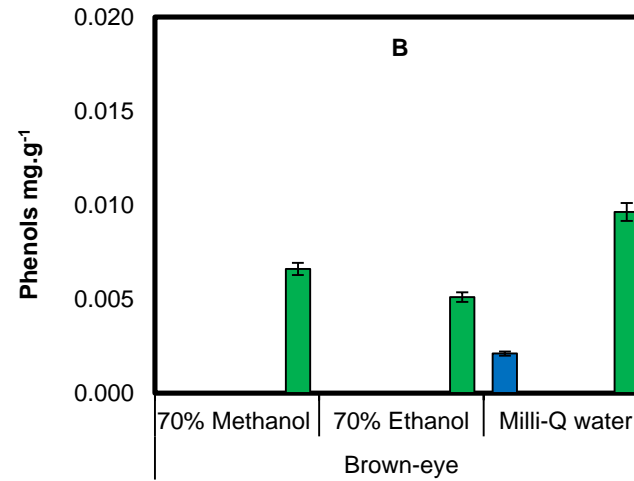
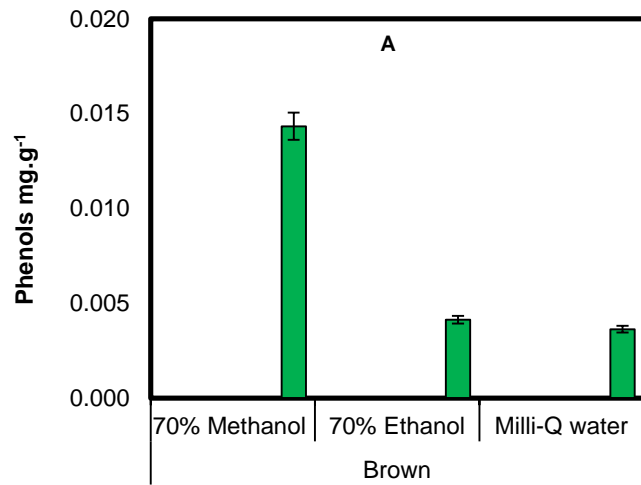


Figure 3.7 Phenols of dehulled BGN from A = Brown, B = Brown-eye, C = Black-eye, D = Red

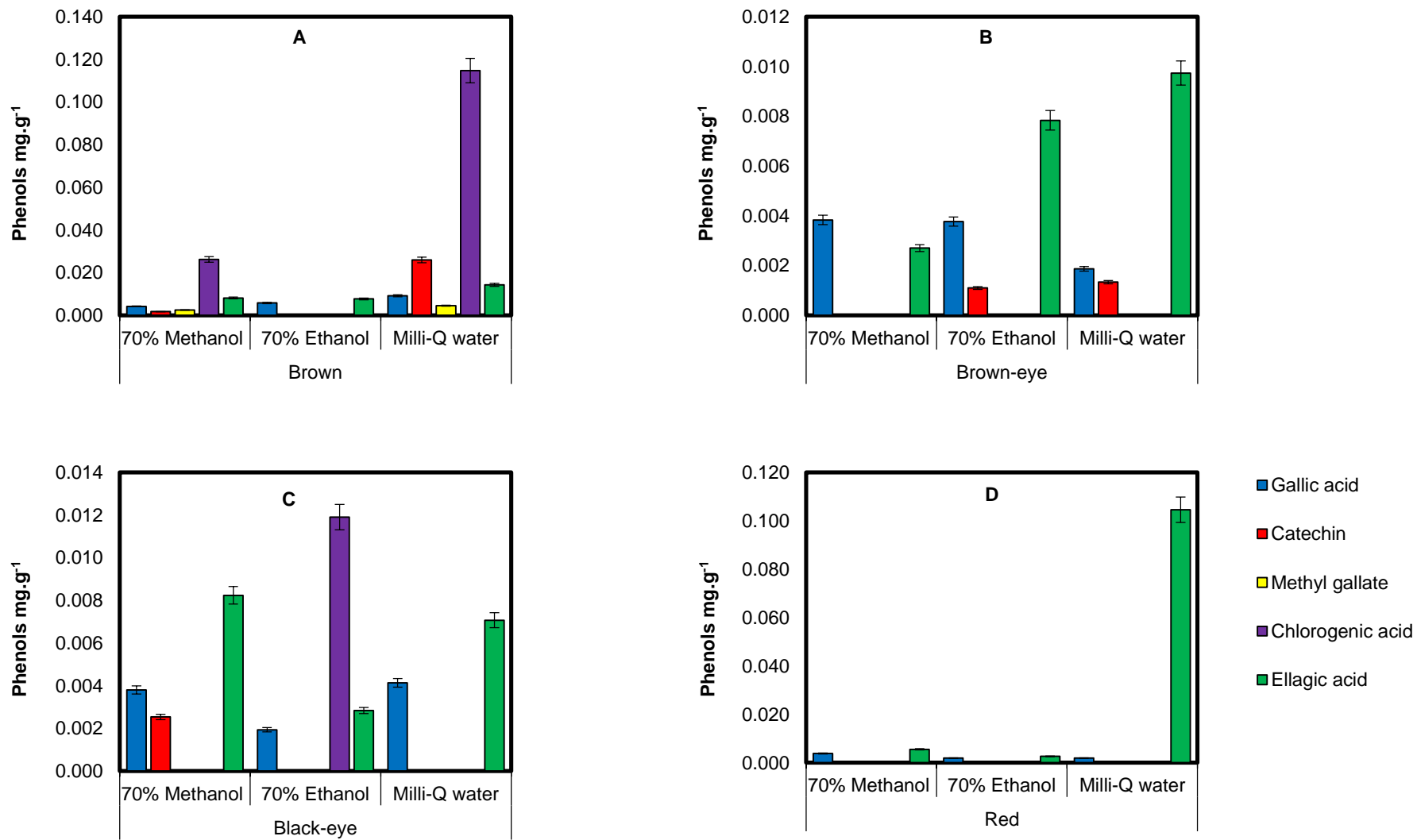


Figure 3.8 Phenols of BGN hulls from A = Brown, B = Brown-eye, C = Black-eye, D = Red

Extraction solvent milli-Q water was significantly ($p \leq 0.05$) higher in ellagic acid than 70% methanol and 70% ethanol. There was no significant ($p \geq 0.05$) difference between 70% methanol and 70% ethanol for ellagic acid. Ellagic acid yielded higher with solvent milli-Q water for all hull BGN samples.

3.6.7 Phenols characteristics of BGN extracts based on BGN colour

The descriptive statistics for phenols compounds of brown BGN were summarized in Figure 3.9. Gallic acid ranged between 0.003 and 0.009 mg.g^{-1} . Amongst all extraction solvents, hulls were significantly ($p \leq 0.05$) higher than whole in gallic acid. Catechin ranged between 0.001 and 0.026 mg.g^{-1} . Methyl gallate ranged between 0.001 and 0.008 mg.g^{-1} . Chlorogenic acid ranged between 0.026 and 0.518 mg.g^{-1} . Ellagic acid between 0.004 mg.g^{-1} and 0.014 mg.g^{-1} . There were no significant differences for catechin, methyl gallate, chlorogenic acid and ellagic acid amongst BGN forms and extraction solvents. The data represented in Figure 3.8 exhibited that the amount of phenol compounds present in brown BGN was lower.

Due to insufficient raw material only black whole BGN could be analysed, therefore there were no comparisons on various forms of BGN (Figure 3.10). Gallic acid was 0.007 mg.g^{-1} for all extraction solvents. Methyl gallate and catechin was 0.001 mg.g^{-1} for all extraction solvents. Chlorogenic acid was 0.003 mg.g^{-1} for all extraction solvents. Ellagic acid ranged between 0.008 and 0.009 mg.g^{-1} .

Phenolic compounds of brown-eye BGN were summarized in Figure 3.11. Gallic acid ranged between 0.002 and 0.004 mg.g^{-1} . Catechin was 0.001 mg.g^{-1} and chlorogenic acid was 0.002 mg.g^{-1} for all BGN extraction solvents. Ellagic acid ranged between 0.003 and 0.010 mg.g^{-1} . There were no significant differences for gallic acid, catechin, chlorogenic acid and ellagic acid amongst BGN forms and extraction solvents.

The data represented in Figure 3.11 showed that the amount of phenols is low for brown-eye BGN in comparison to the others. The phenols concentration correlated with intense seed colour. It has been observed that black and red seeds contain the highest level of phenols whilst the lowest level was found in cream-coloured seeds (Baidoo *et al.*, 2015).

Phenols of black-eye BGN were summarized in Figure 3.12. Gallic acid ranged between 0.002 and 0.004 mg.g^{-1} . Catechin ranged between 0.001 and 0.003 mg.g^{-1} . Methyl gallate was 0.001 mg.g^{-1} for all extraction solvents. Chlorogenic acid ranged between 0.012 and 0.055 mg.g^{-1} . Ellagic acid ranged between 0.003 and 0.016 mg.g^{-1} . There were no significant differences for gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid amongst BGN forms and extraction solvents.

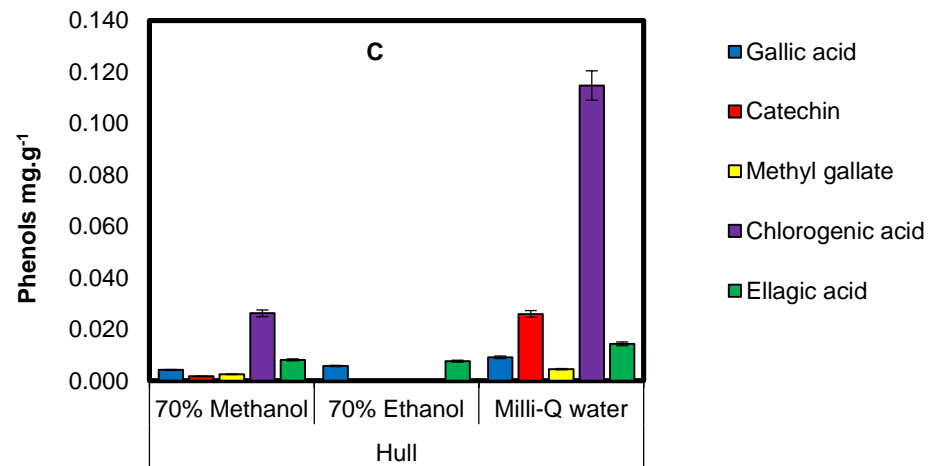
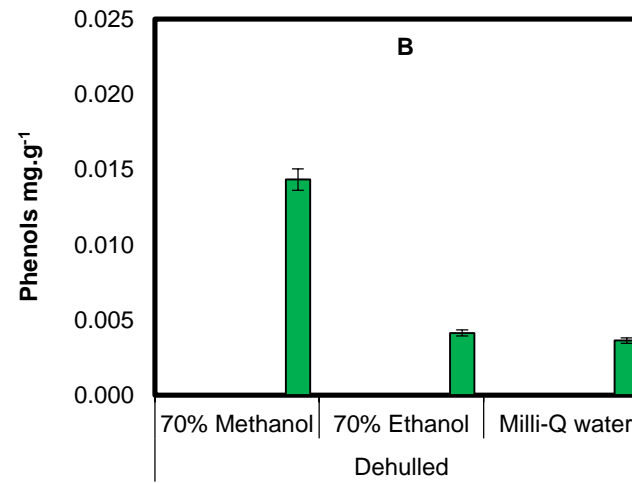
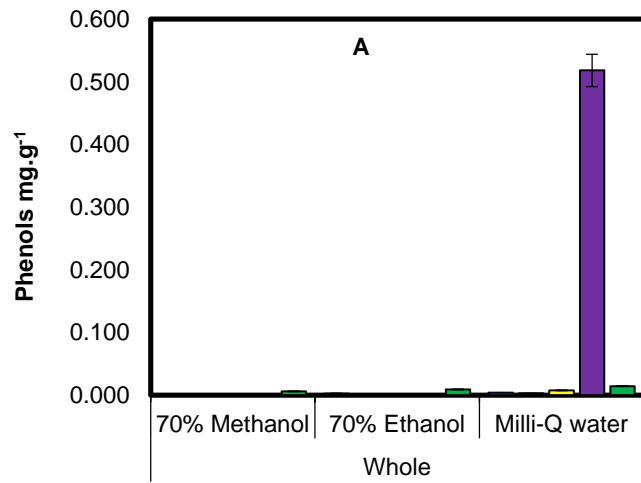


Figure 3.9 Phenols of brown BGN A = Whole, B = Dehulled, C = Hull

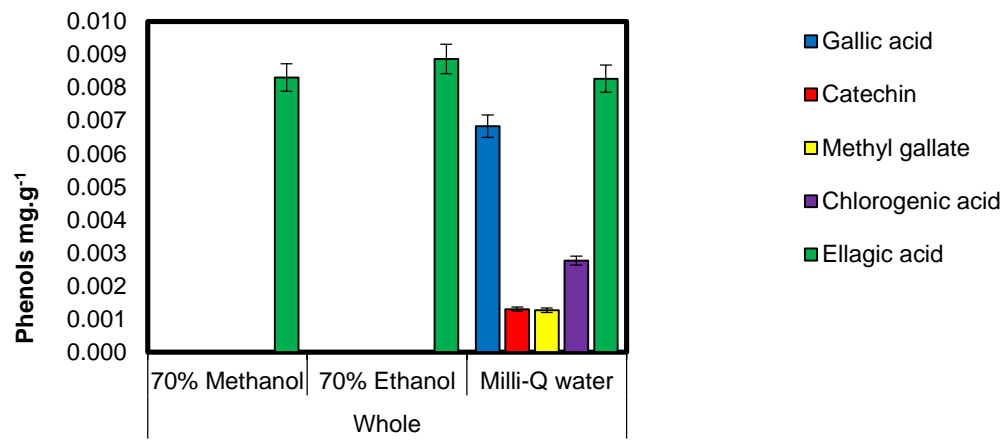


Figure 3.10 Phenols of black whole BGN

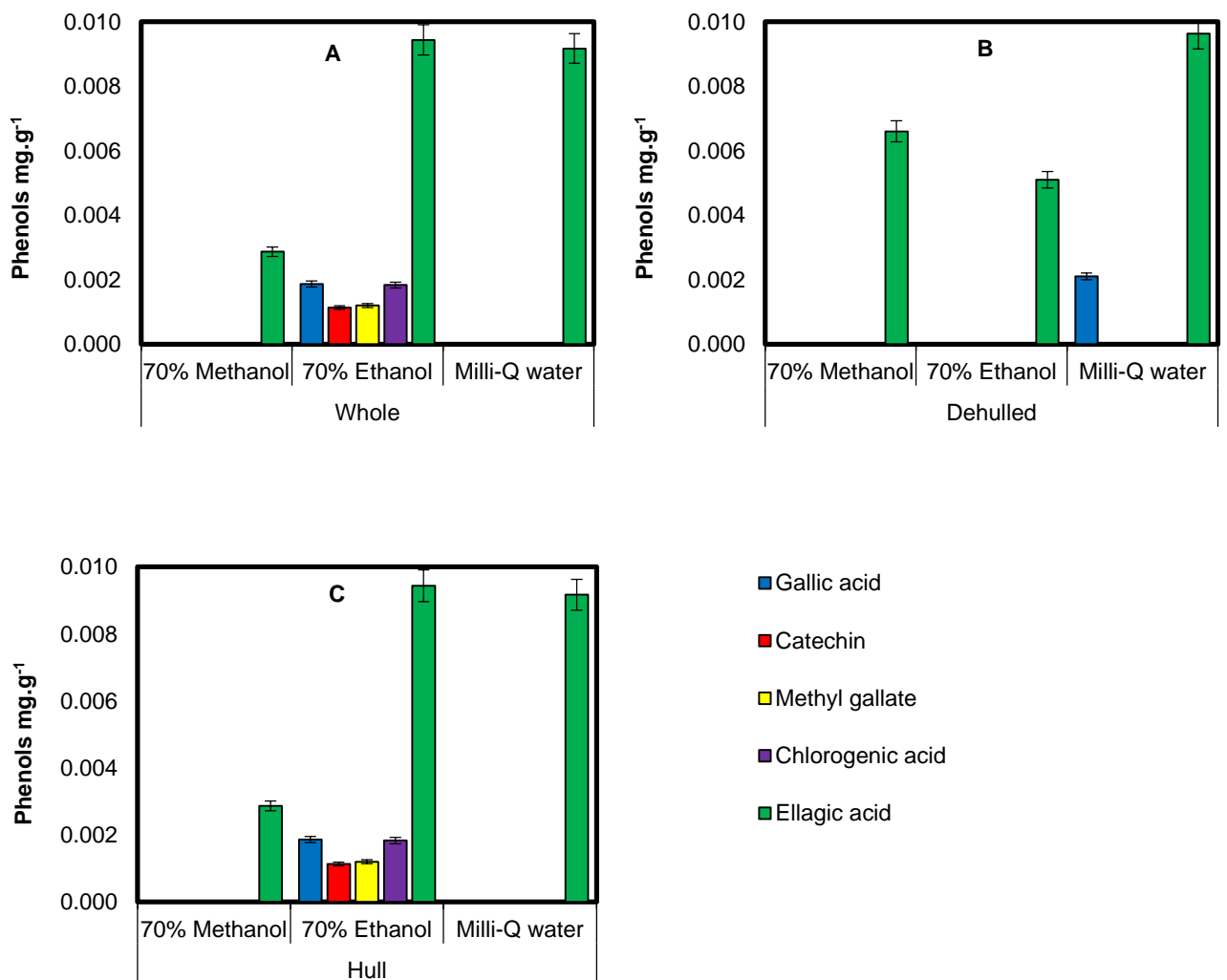


Figure 3.11 Phenols of brown-eye BGN from A = Whole, B = Dehulled & C = Hull

Phenols of red BGN are summarized in Figure 3.13. There were no significant differences for gallic acid (0.002-0.005 mg.g⁻¹), catechin (0.003-0.008 mg.g⁻¹), methyl gallate (0.001-0.002 mg.g⁻¹) and chlorogenic acid (0.029-0.072 mg.g⁻¹) amongst BGN forms and extraction solvents. Ellagic acid ranged between 0.003 and 0.105 mg.g⁻¹, with BGN hull significantly ($p \leq 0.05$) different to whole and dehulled BGN. There was no significant difference between whole and dehulled BGN in ellagic acid.

The data represented in Figure 3.12 displays that the amount of phenols is low for black-eye BGN. As stated by Baidoo *et al.* (2015) phenols are situated primarily in the seed coat. Phenols contribute colour to seeds and their concentrations are correlated with seed colour as seen in Figure 3.13. It has been observed that black and red seeds contain the highest level of phenols whilst the lowest level was found in cream-coloured BGN seeds.

3.6.8 Phenol characteristics of BGN extracts based on extraction solvents

Phenols of BGN extracted with 70% methanol solvent summarized in Figure 3.14. Gallic acid ranged between 0.002 and 0.004 mg.g⁻¹, with no significant differences amongst BGN samples. Catechin (0.002–0.003 mg.g⁻¹) did not differ significantly amongst BGN seeds. Methyl gallate ranged between 0.001 and 0.002 mg.g⁻¹, with no significant differences amongst BGN samples. Chlorogenic acid ranged between 0.026 and 0.072 mg.g⁻¹. There were no significant differences amongst BGN samples for chlorogenic acid.

Ellagic acid ranged between 0.003 and 0.015 mg.g⁻¹ for all BGN samples. There were no significant differences amongst BGN samples for ellagic acid. Phenolic compounds namely gallic acid, catechin, methyl gallate and chlorogenic acid could not be detected in BGN samples, possibly due to limitations of instrument i.e. limit of detection (LOD) and limit of quantification (LOQ) for all extraction solvents.

The phenolics of BGN extracted with 70% ethanol solvent were summarized in Figure 3.15. Gallic acid ranged between 0.002 and 0.004 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst BGN samples for gallic acid. Catechin ranged between 0.001 and 0.005 mg.g⁻¹. There were no significant differences amongst BGN samples for catechin. Methyl gallate was 0.001 mg.g⁻¹ for all BGN forms. Chlorogenic acid (0.002 and 0.055 mg.g⁻¹) did not differ significantly amongst BGM samples. Ellagic acid ranged between 0.003 and 0.009 mg.g⁻¹. The extraction with 70% ethanol exhibited no noticeable effect on phenol compounds. Phenol compounds namely gallic acid, catechin, methyl gallate and chlorogenic acid was not detected for BGN samples, perhaps due to limitations of instrument or low concentration of compounds.

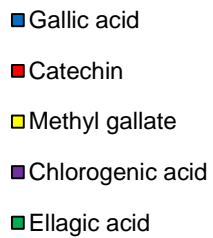
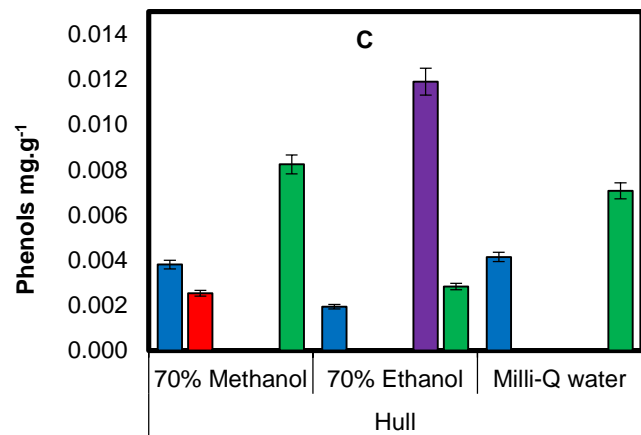
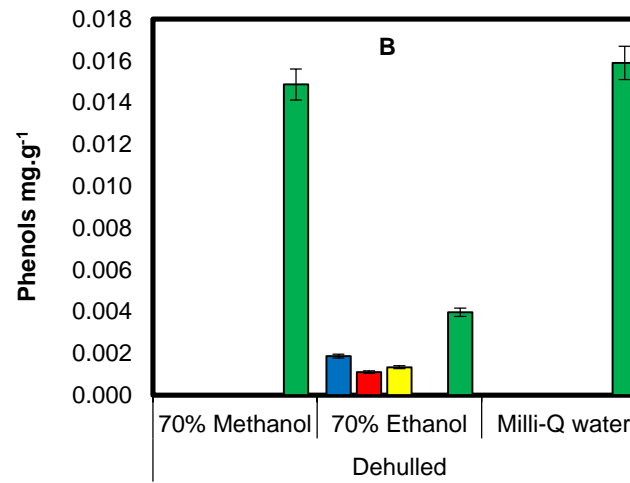
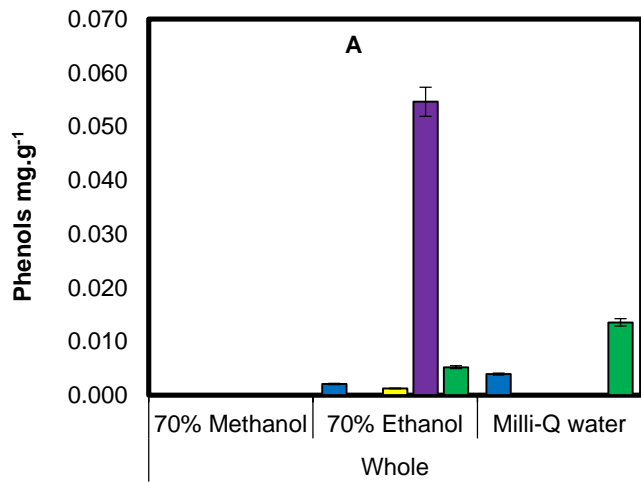


Figure 3.12 Phenols of black-eye BGN from A = Whole, B = Dehulled & C = Hull

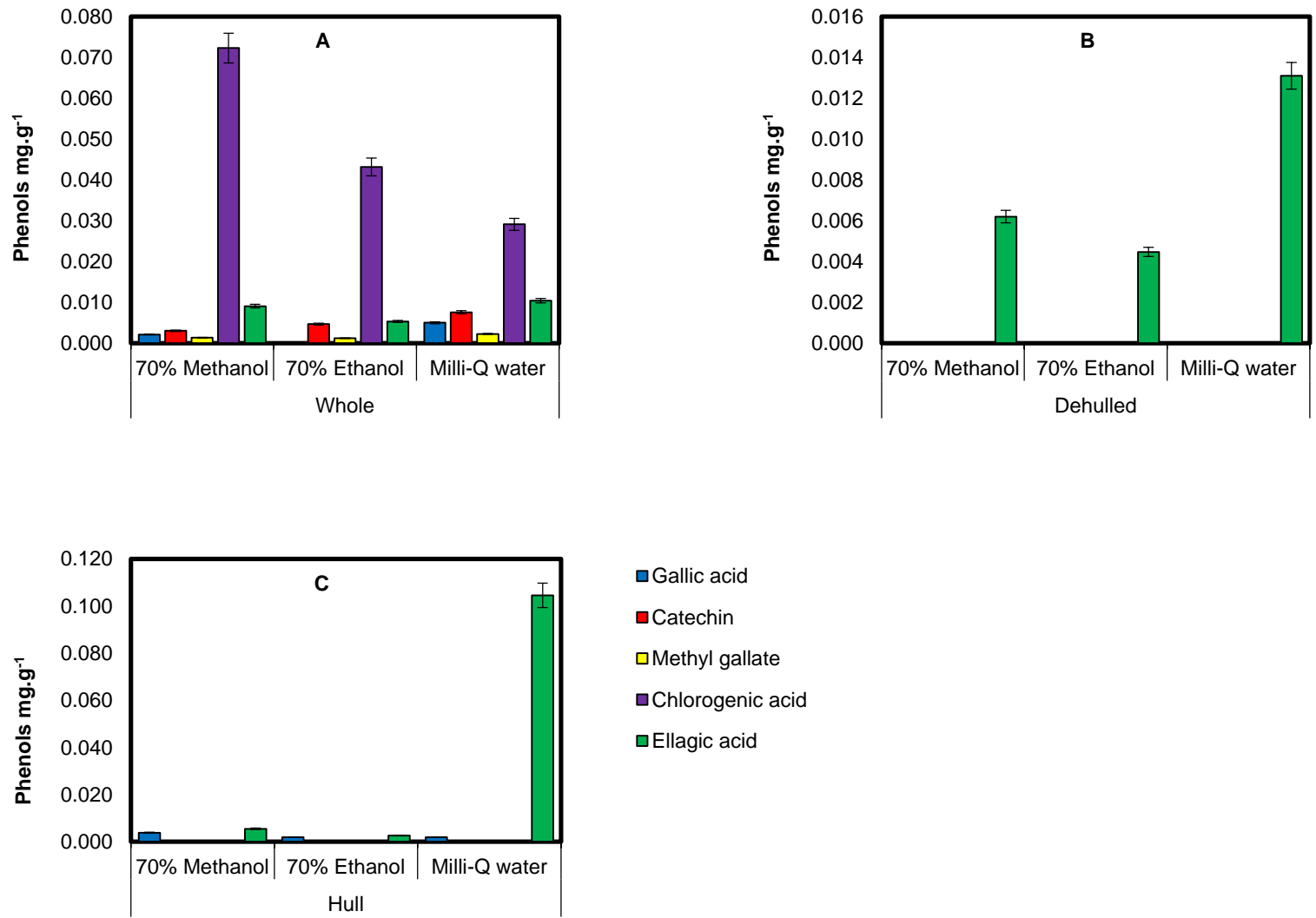


Figure 3.13 Phenols of red BGN for A = Whole, B = Dehulled & C = Hull

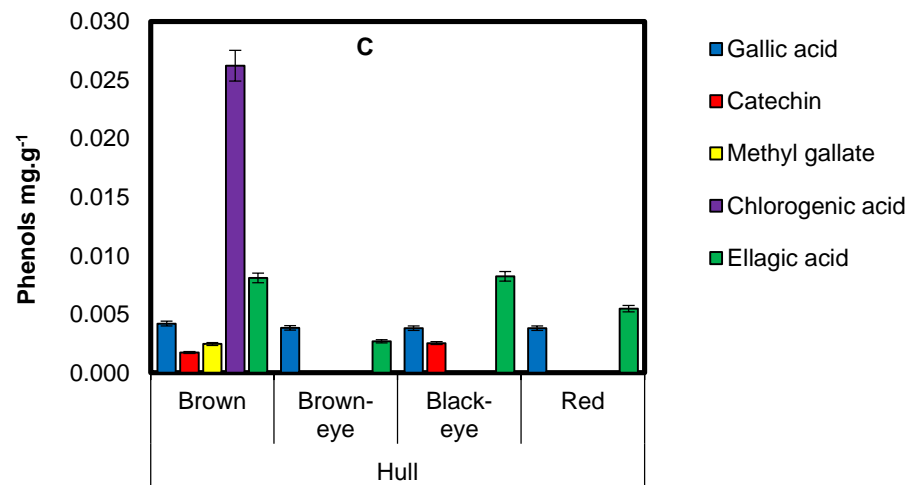
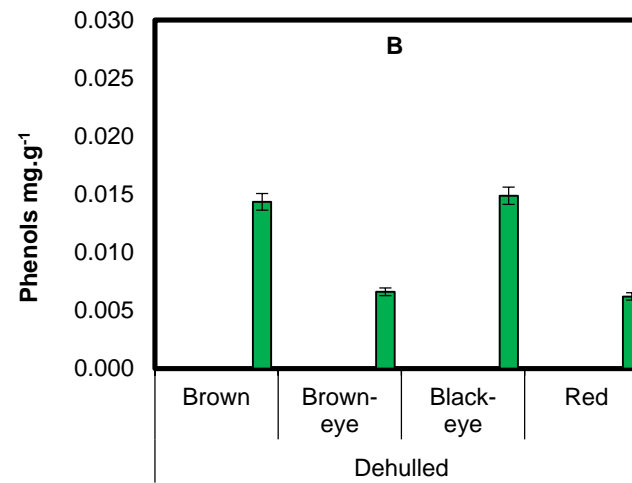
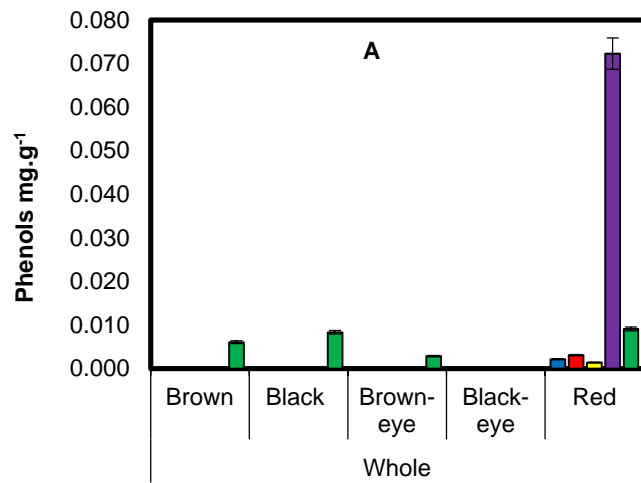


Figure 3.14 Phenols of BGN extracted with 70% methanol solvent from A = Whole, B = Dehulled & C = Hull

Phenols of BGN extracted with milli-Q water solvent were summarized in Figure 3.16. Gallic acid ($0.002 - 0.009 \text{ mg.g}^{-1}$) with no significant differences amongst BGN samples. Catechin ranged between 0.001 and 0.026 mg.g^{-1} and did not differ significant amongst BGN samples. Methyl gallate ranged between 0.001 and 0.008 mg.g^{-1} . There were no significant differences amongst BGN samples for methyl gallate. Chlorogenic acid ranged between 0.003 and 0.518 mg.g^{-1} . There were no significant differences amongst BGM samples for catechin. Ellagic acid ranged between 0.004 and 0.105 mg.g^{-1} . Red BGN was significantly ($p \leq 0.05$) higher in ellagic acid especially within the hulls when compared to other BGN samples i.e. brown, black, brown-eye and black-eye. Phenols reported by Salawu (2016) for whole BGN were, gallic acid ($1.030 \pm 0.010 \text{ mg.g}^{-1}$), catechin ($2.340 \pm 0.030 \text{ mg.g}^{-1}$), chlorogenic acid ($2.370 \pm 0.020 \text{ mg.g}^{-1}$) and ellagic acid ($1.090 \pm 0.020 \text{ mg.g}^{-1}$). These results reported by Salawu (2016) are significantly higher than the present study. This could be due to lower pH of solvent used by Salawu (2016) as it was acidified methanol thus changing the characteristic of the solvent and potentially leading to better extraction of phenolic compounds whereas the present study used unacidified 70% methanol and 70% ethanol.

Furthermore, the extraction time used in Salawu (2016) method was 24 h using plant tissue extraction procedure, whereas in present study the extraction time was only 30 min using sonication according to the method described by Nyau *et al.* (2014). This also could be a contributing factor as the phytochemicals have a longer period to interact with solvent, thereby possibly yielding better extraction and resulting in higher amounts of phenolic compounds (Tiwari *et al.*, 2011).

General discussion on BGN phenolics

Tannins are widely dispersed in the plant kingdom. It is claimed that these polyphenolics have beneficial effects on health as antioxidant, antimicrobial, anti-inflammatory, antiviral and as anti-carcinogen agent (Gudej & Tomczyk, 2004). Tannins are known to have healing properties due to their ability to promote protein precipitation, which gives rise to a protective layer that can improve the regenerative process (De Souza *et al.*, 2008). Hydrolysable tannins comprise of simple phenolic acids such as gallic acid esterified to polyols, typically glucose, whereas condensed tannins are polymers of flavonoid units (Riedl & Hagerman, 2001).

Despite the greater abundance of condensed tannins than hydrolysable tannins in foodstuffs, more nutritional studies have been done with hydrolysable tannins than with condensed tannins. This is probably because hydrolysable tannins such as tannic acid have been more readily available in purified forms suitable for feeding trials than have condensed tannins.

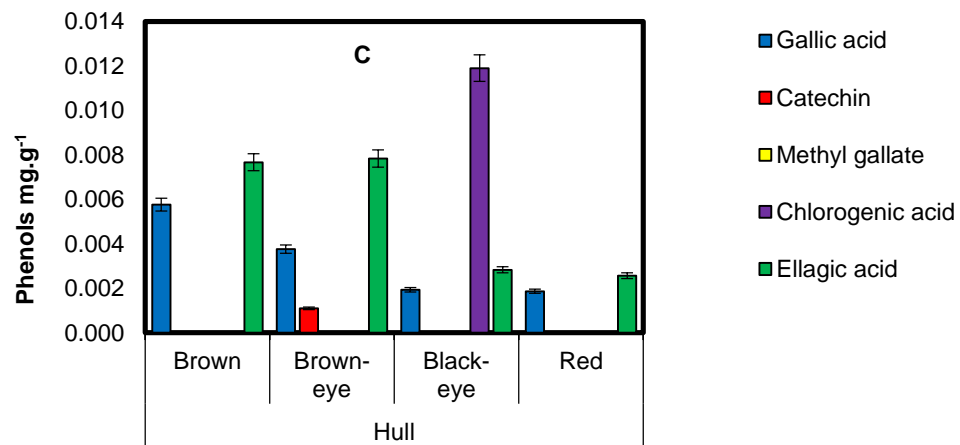
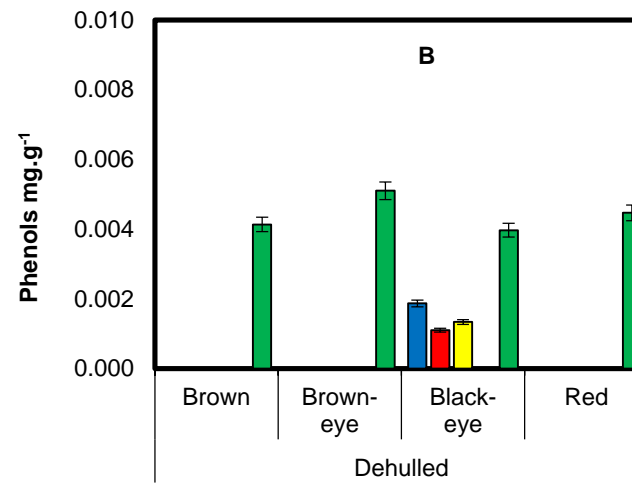
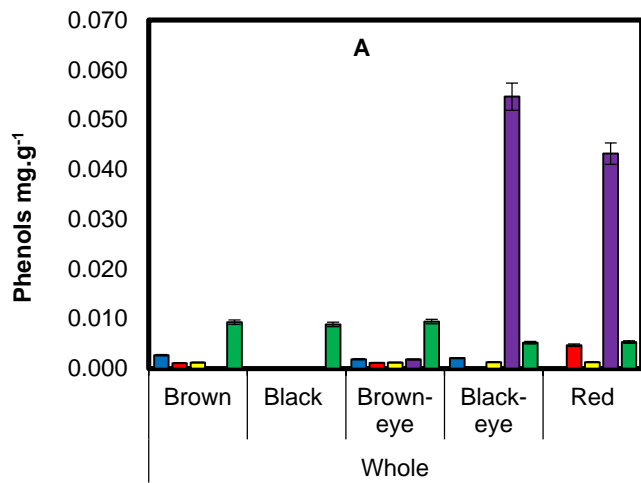


Figure 3.15 Phenols of BGN extracted with 70% ethanol solvent from A = Whole, B = Dehulled & C = Hull

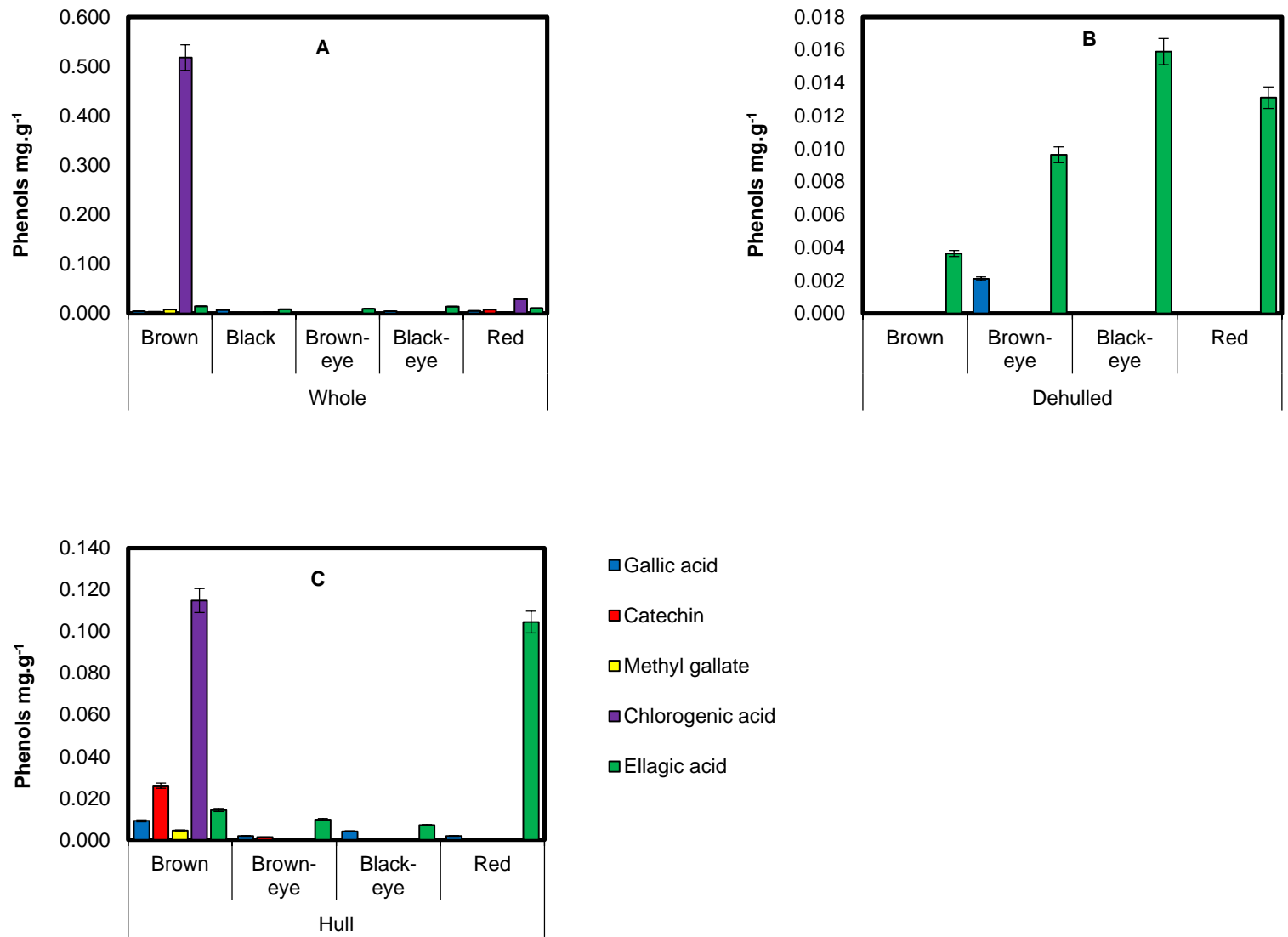


Figure 3.16 Phenols of BGN extracted with milli-Q water solvent from A = Whole, B = Dehulled & C = Hull

Unlike condensed tannins, hydrolysable tannins are subject to breakdown by hydrolysis due to esterolytic 'tannase' enzymes in the digestive tract. The resulting products include gallic acid, which is readily absorbed and excreted in the urine. Absorbed gallate may cause anti-nutritional effects. Despite major structural differences, hydrolyzable and condensed tannins often produce similar anti-nutritional effects. The most common effects are diminished weight gains and lowered efficiency of nutrient utilization. The major biochemical basis for these effects appears not to be inhibition of dietary protein digestion but rather a systemic inhibition of the metabolism of digested and absorbed nutrients, particularly protein (Butler, 1992).

Condensed tannins have been reported to be responsible for decreases in feed intake, growth rate, feed efficiency, net metabolizable energy, and protein digestibility in experimental animals. Consequently, foods rich in tannins are considered to be of low nutritional value (Chung *et al.*, 1998). Additionally, condensed tannins are polyphenolic substances widely distributed in plants, especially in legumes (BGN) and due to their large structure are known to inhibit protein digestibility. It does this by forming irreversible complexes with proteins, thereby reducing the bioavailability of amino acids (Tibe *et al.*, 2007). The highest levels of phenolics in BGN were 0.115 mg.g⁻¹ of chlorogenic acid found in brown hulls and 0.105 mg.g⁻¹ of ellagic acid found in red hulls. Farah *et al.* (2008) stated that recently a series of health benefits have been associated with the consumption of chlorogenic acid, namely a reduction of the relative risk of cardiovascular disease, type 2 diabetes, Alzheimer's disease, antibacterial and anti-inflammatory activities. As for ellagic acid, it is a natural phenol antioxidant.

In the recommendations made by companies selling various nutritional supplements rich in polyphenols, some recommend the consumption of 50 mg per dose for isoflavones or 100-300 mg per dose grape seed extracts rich in proanthocyanidins (condensed tannins) (Mennen *et al.*, 2005). Recent research has also indicated that condensed tannins in low concentrations have beneficial effects in animal and human nutrition and health (Tibe *et al.*, 2007).

3.6.9 Vitamin B₁ and B₂ of BGN extracts based on BGN form

Vitamin B₁ (thiamine) and vitamin B₂ (riboflavin) of whole BGN (Figure 3.17) thiamine ranged between 0.004 and 0.047 mg.g⁻¹. There were no significant differences between solvents for thiamine. Riboflavin was 0.002 mg.g⁻¹ for all solvents. There were no significant differences for riboflavin amongst solvents.

Vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of dehulled BGN form was summarized in Figure 3.18. Thiamine ranged between 0.001 and 0.043 mg.g⁻¹. Solvent milli-Q water was significantly higher than 70% methanol and 70% ethanol. There was no significant difference between 70% methanol and 70% ethanol. Riboflavin ranged between

0.001 and 0.002 mg.g⁻¹. There were no significant differences amongst all solvents for riboflavin. Milli-Q water yielded better extraction of thiamine than that of 70% methanol and 70% ethanol, suggesting that the vitamins were more soluble than in methanol and ethanol. Vitamin B₁ (thiamine) and vitamin B₂ (riboflavin) of hull BGN (Figure 3.19) thiamine ranged between 0.016 and 0.072 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst all solvents for thiamine. Riboflavin was not detected for all BGN solvents. BGN hulls appeared to be similar to whole and dehulled BGN in riboflavin and, milli-Q water extracted higher yields. Furthermore, it should be noted that these vitamins are water soluble therefore extracting better with an aqueous solvent (Sami *et al.*, 2014).

3.6.10 Vitamin B₁ and B₂ characteristics of BGN extracts based on BGN colour

Vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of brown BGN (Figure 3.20) thiamine ranged between 0.001 and 0.047 mg.g⁻¹. Whole BGN was found to be significantly ($p \leq 0.05$) lower in thiamine compared to hull BGN. BGN hulls yielded higher thiamine than dehulled and whole, signifying that thiamine is located primarily in the hulls of BGN. Due to insufficient raw material only, black whole BGN could be analysed. Thiamine and riboflavin was not detected in whole black BGN.

Vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of brown-eye BGN summarized in Figure 3.21. Thiamine ranged between 0.002 and 0.071 mg.g⁻¹. Hull BGN was significantly ($p \leq 0.05$) higher in thiamine than whole and dehulled BGN. There was no significant difference between whole and dehulled. Riboflavin was 0.001 mg.g⁻¹ for all BGN samples. According to the data it can be observed that BGN hulls yielded higher thiamine than dehulled and whole BGN, once more suggesting that thiamine is located primarily in the hulls of BGN.

Vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of black-eye BGN was summarized in Figure 3.22. Thiamine ranged from between 0.002 and 0.072 mg.g⁻¹. Hull was significantly higher in thiamine than whole and dehulled. There was no significant difference between whole and dehulled. Riboflavin was 0.002 mg.g⁻¹ for all black-eye samples. Vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of red BGN thiamine ranged between 0.011 and 0.016 mg.g⁻¹. According to the data a trend was observed whereby suggesting that thiamine is located primarily in the hulls of BGN.

3.6.11 Vitamin B₁ and B₂ characteristics on BGN extracts based on extraction solvents

The descriptive statistics for vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of BGN extracted with 70% methanol solvent thiamine ranged between 0.001 and 0.007 mg.g⁻¹. Brown BGN was significantly ($p \leq 0.05$) lower in thiamine compared to brown-eye, whereas black-eye was not significant ($p \geq 0.05$) to either brown or brown-eye. Riboflavin was not detected. For vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of BGN extracted with 70%

ethanol solvent thiamine was 0.002 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst BGN samples for thiamine. Vitamins B₁ (thiamine) and vitamin B₂ (riboflavin) of BGN extracted with milli-Q water solvent was summarized in Figure 3.23. Thiamine ranged between 0.004 – 0.072 mg.g⁻¹. Samples brown and red were not significant ($p \geq 0.05$), however when compared to brown-eye and black-eye they were significant ($p \leq 0.05$). Sample brown-eye was significant ($p \leq 0.05$) to black-eye, brown and red. Riboflavin ranged between 0.001– 0.002 mg.g⁻¹. There were no significant ($p \geq 0.05$) differences amongst BGN samples for riboflavin. Thiamine and riboflavin reported by Okudu & Ojinnaka (2017) for whole BGN were as follows respectively, 0.004 and 0.001 mg.g⁻¹. Thus, resulting in higher yields for milli-Q water extracts than those of 70% methanol and 70% ethanol.

3.6.12 Classification of BGN phytochemicals based on form, seed, colour and solvents

Principal component analysis (PCA) was performed to summarise the phytochemicals. The variation in the data can be explained by two principal components (PC1 and PC2) as indicated in Figure 3.23 explain 31.0% of the total variance and component PC2, 17.0% with the total cumulative variance of 48.0%.

The BGN hulls (Figure 3.24a) could be associated with flavonoid compounds (Figure 3.24d) namely, quercetin, rutin and myricetin. Whole BGN was associated with methyl gallate which is a phenolic compound, suggesting that phenolic compounds are concentrated in BGN endosperm than the hulls. Extraction solvents (Figure 3.24b) 70% ethanol and 70% methanol were associated with phenols (catechin, gallic acid) and flavonoids (quercetin, rutin and myricetin) whereas 70% ethanol and milli-Q water was associated with phenols (methyl gallate). In Figure 3.24c BGN colours (black-eye and brown-eye) were associated with flavonoids and phenols, for the rest of the colours there were too many outliers to have a clear distinction regarding association of BGN colour and phytochemicals extracted.

3.6.13 Probable compounds in BGN extracts

GC-MS analysis showed the presence of major peaks (Table 3.13) for BGN samples extracted with 70% methanol, 70% ethanol and milli-Q water respectively. The probable compound names, retention times and molecular weights are shown in Table 3.13. Amongst the compounds listed in Table 3.13 some are antioxidants, antimicrobials agents or other phytochemicals specifically alkaloids.

The following compounds occurred more often than others namely 9-Octadecenamide, (Z)-, ethanol 1-methoxy- benzoate, 1-Monolinoleoylglycerol trimethylsilyl and ether butylated hydroxytoluene. According to Oh *et al.* (2010) 9-Octadecenamide, (Z)- or oleamide display anti-inflammatory properties.

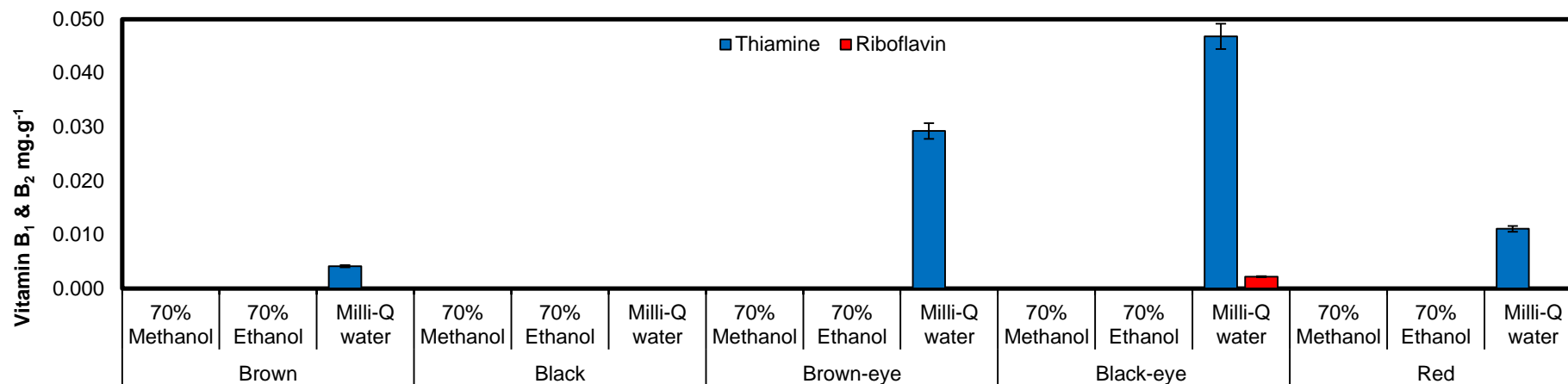


Figure 3.17 Vitamin B₁ (thiamine) and B₂ (riboflavin) of whole BGN

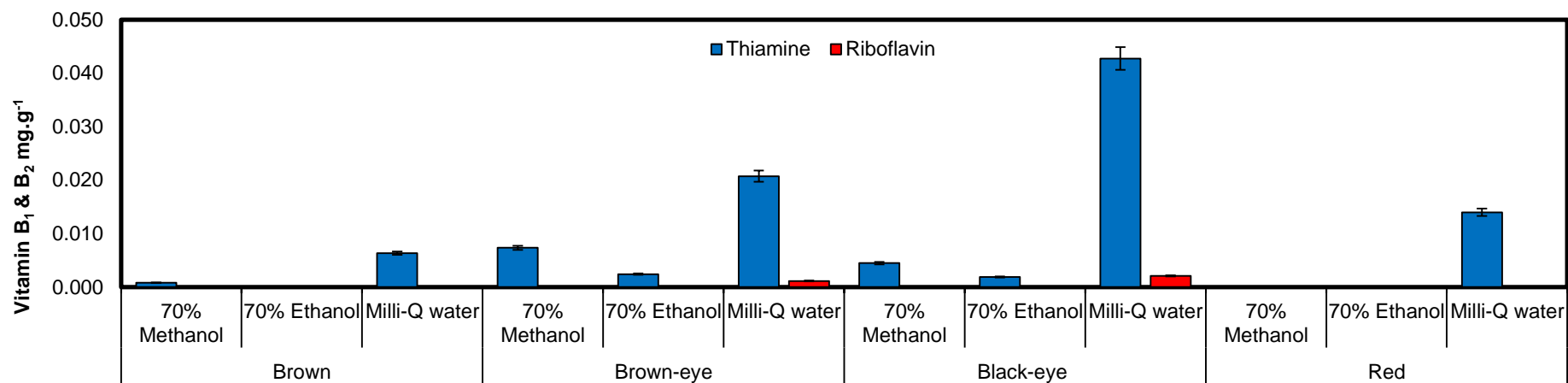


Figure 3.18 Vitamins B₁ (thiamine) and B₂ (riboflavin) of dehulled BGN

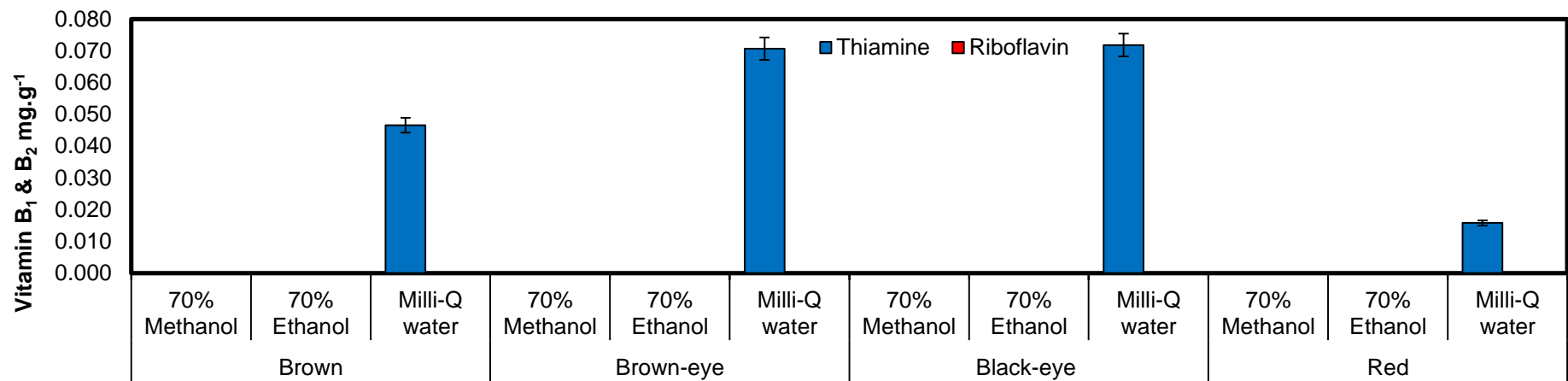


Figure 3.19 Vitamin B₁ (thiamine) and B₂ (riboflavin) of BGN hull

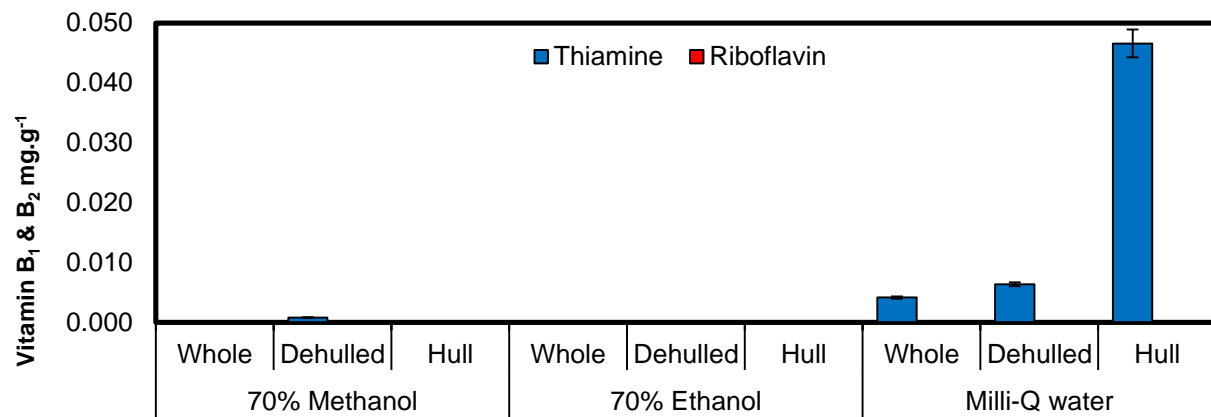


Figure 3.20 Vitamin B₁ (thiamine) and B₂ (riboflavin) of brown BGN

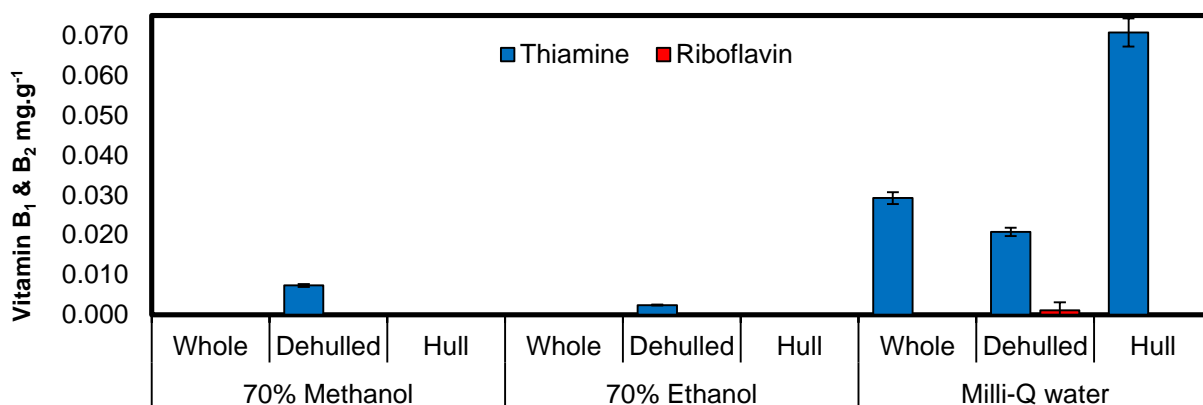


Figure 3.21 Vitamin B₁ (thiamine) and B₂ (riboflavin) of brown-eye BGN

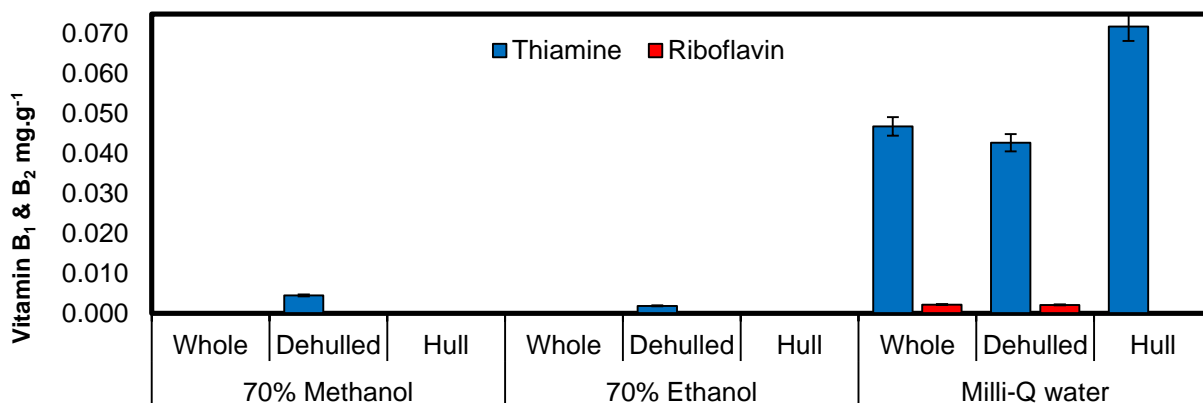


Figure 3.22 Vitamin B₁ (thiamine) and B₂ (riboflavin) of black-eye BGN

Ethanol 1-methoxy- benzoate or ethyl benzoate, is a volatile ester found in organic compounds. It's a colourless liquid that's insoluble in water but miscible with most organic solvents. Linoleic acid or 1-Monolinoleoylglycerol trimethylsilyl (vitamin F) is a polyunsaturated omega-6 fatty acid. Gas chromatography coupled to mass spectrometry is the best for identification of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids and nitrogen compounds (Wesolowska *et al.*, 2016).

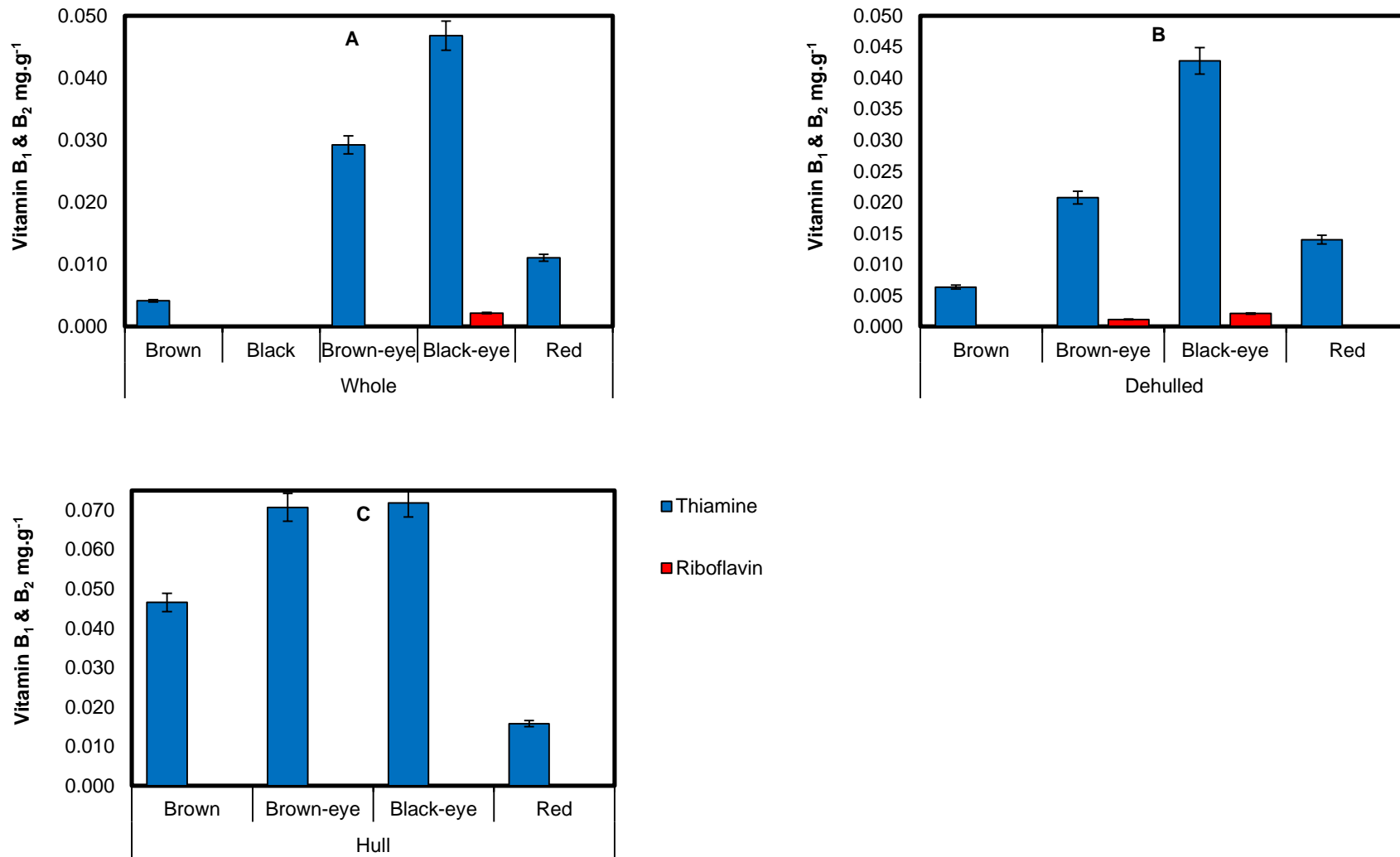


Figure 3.23 Vitamin B₁ (thiamine) and B₂ (riboflavin) of BGN extracted with milli-Q water solvent from A = Whole, B = Dehulled & C = Hull

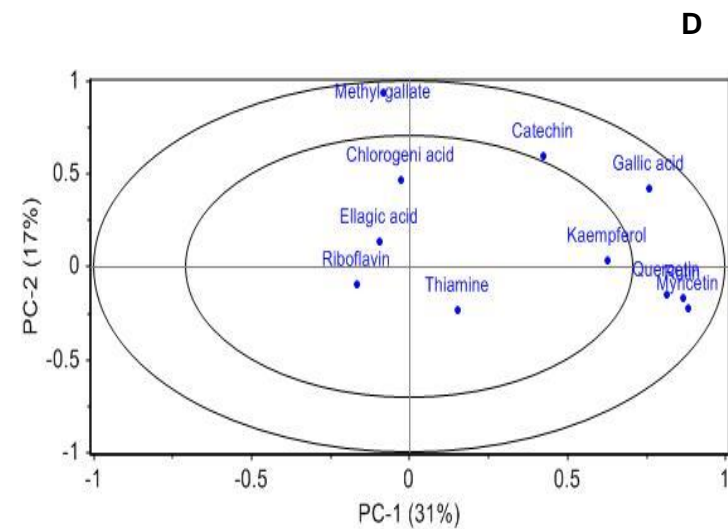
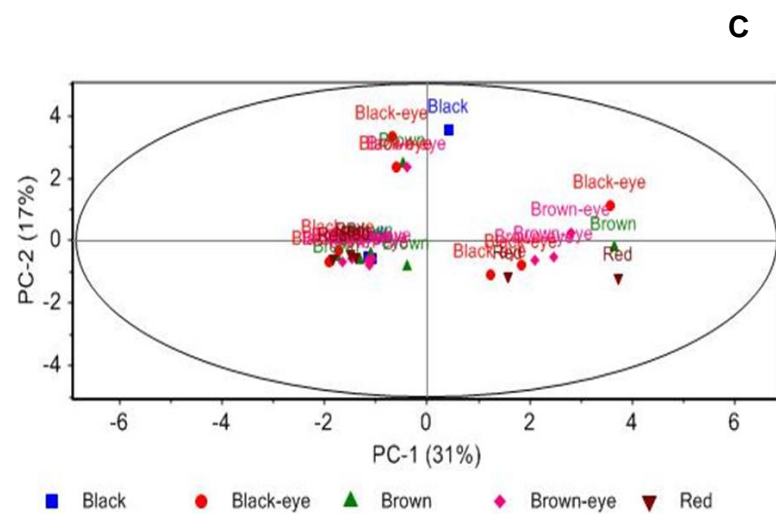
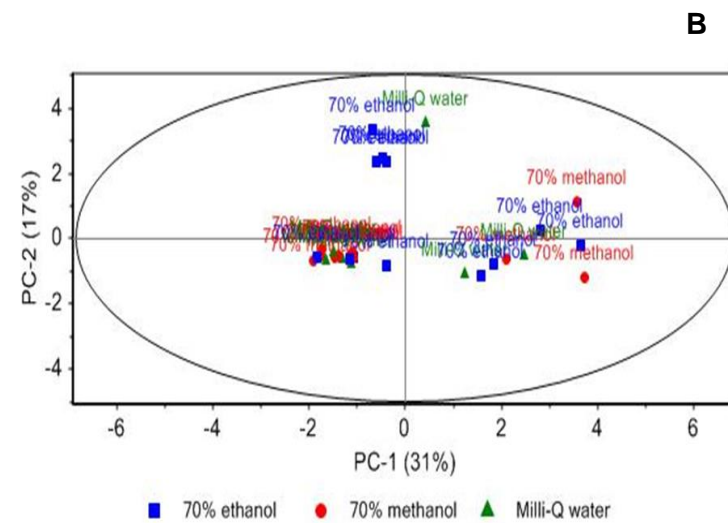
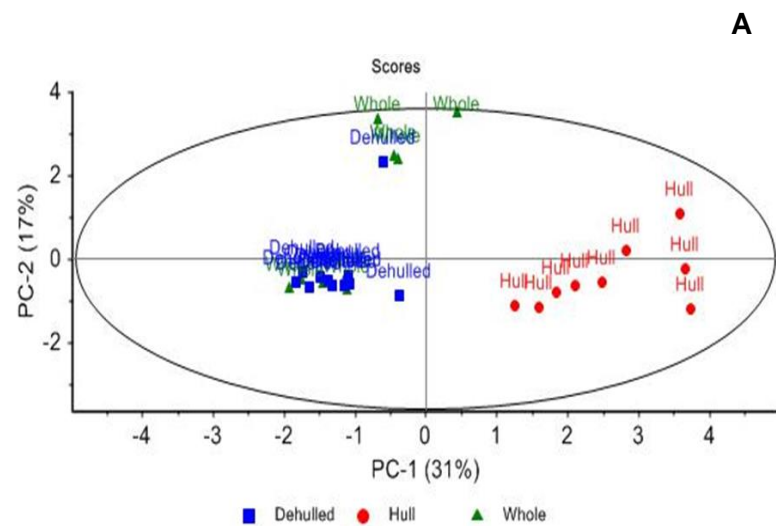


Figure 3.24 PCA plots of independent (scores) and dependent (loadings) variables for BGN phytochemicals

Table 3.13 Probable compounds in BGN extracts

BGN Sample	Solvent	Retention time (mins)	Compound	Molecular weight (g.mol ⁻¹)	Probability (%)	Match	R.Match
Red hull	Milli-Q water	3.728	1,2-Benzenediol	110.110	74.50	850	889
Red hull	Methanol	7.350	1-Monolinoleoylglycerol trimethylsilyl ether	308.640	53.90	622	643
Red hull	Milli-Q water	8.099	1-Monolinoleoylglycerol trimethylsilyl ether	308.640	51.90	644	673
Red hull	Methanol	4.226	2-Methoxy-4-vinylphenol	150.180	52.80	852	892
Red hull	Methanol	3.596	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	144.126	80.30	794	886
Brown-E whole	Ethanol	8.554	9-Octadecenamide, (Z)-	281.484	58.00	700	735
Brown dehulled	Ethanol	8.557	9-Octadecenamide, (Z)-	281.484	87.60	855	855
Brown hull	Ethanol	8.568	9-Octadecenamide, (Z)-	281.484	91.20	872	872
Brown-E dehulled	Methanol	8.568	9-Octadecenamide, (Z)-	281.484	87.70	836	842
Black-E hull	Methanol	7.641	9-Octadecenamide, (Z)-	281.484	81.10	839	839
Red dehulled	Methanol	6.829	9-Octadecenamide, (Z)-	281.484	66.90	779	780
Brown-E dehulled	Ethanol	6.823	9-Octadecenamide, (Z)-	281.484	63.00	724	739
Brown-E-dehulled	Milli-Q water	7.624	9-Octadecenamide, (Z)-	281.484	57.90	657	699
Red whole	Milli-Q water	3.276	Acetic acid, 6-morpholin-4-yl-9-oxobicyclo[3.3.1]non-3-yl ester	281.347	55.90	777	787
Red dehulled	Methanol	4.946	Butylated Hydroxytoluene	220.370	58.30	900	907
Brown dehulled	Methanol	4.952	Butylated Hydroxytoluene	220.371	51.90	854	871
Red hull	Milli-Q water	4.128	Ethanol, 1-methoxy-, benzoate	180.201	64.50	740	825
Brown dehulled	Milli-Q water	4.128	Ethanol, 1-methoxy-, benzoate	180.201	58.30	741	817
Brown hull	Ethanol	4.128	Ethanol, 1-methoxy-, benzoate	180.201	58.20	743	804
Red dehulled	Milli-Q water	4.128	Ethanol, 1-methoxy-, benzoate	180.201	57.60	741	807
Brown dehulled	Ethanol	6.606	Hexadecanoic acid, methyl ester	270.457	84.00	932	934
Brown dehulled	Methanol	3.464	Maltol	126.110	76.70	847	878
Red hull	Methanol	3.464	Maltol	126.110	65.90	795	856
Brown dehulled	Ethanol	5.753	Methyl tetradecanoate	242.403	54.60	773	804
Brown dehulled	Ethanol	6.612	Pentadecanoic acid, 14-methyl-, methyl ester	270.451	59.80	856	893
Black-E hull	Methanol	6.823	Tetradecanamide	227.392	67.00	847	874

However, to identify a compound in an unknown sample with sufficient confidence, the need to verify the mass spectrum as well as the retention time ought to be performed. Therefore, a known standard should be purchased of the selected compound under investigation and be used in GC-FID to determine whether it is present in the BGN samples by comparing their retention times.

3.7 Conclusion

The form of the BGN, solvent used to extract phytochemicals and colour of the BGN were all important factors, to optimize the best extraction yield of phytochemicals. Overall the hulls of the BGN were the optimum source of flavonoids and phenols, however the solvents varied as each phytochemical extraction depended on other characteristics, namely polarity, extraction time and solvent type. In summary, the brown and red hulls had the highest concentration of flavonoids compared to whole and dehulled, with the highest flavonoid concentration being rutin at 24.458 mg.g⁻¹ found in brown hulls and myricetin at 1.800 mg.g⁻¹ found in red hulls. Rutin main health role is to help the body utilize vitamin C more efficiently, as well as help produce collagen (skin), whereas myricetin is an antioxidant regulating blood sugar levels, cancer cells and general skin health. Recent research has also indicated that condensed polyphenols in low concentrations have beneficial effects in animal and human nutrition and health (Tibe *et al.*, 2007). The black-eye BGN hull had the highest concentration for thiamine (vitamin B₁) and riboflavin (vitamin B₂) consisting of 0.072 mg.g⁻¹ (extracted with milli-Q water) and 0.002 mg.g⁻¹, however, the Recommended Dietary Allowances (RDAs) for adults aged 14 and higher are thiamine at a concentration of 1.2 mg and 1.3 mg for riboflavin. Therefore, in order to meet the required RDA's for thiamine, consumption of 100 g black-eye BGN hulls would be required. As for the phytochemicals extraction of BGN extracts the colour, form, solvent used for extraction and concentration of sample extract contributed to the antimicrobial activity. Lastly the GC-MS detected several compounds per extract and a few were alkaloids, antimicrobial agents and antioxidant compounds.

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CHAPTER 4

BAMBARA GROUNDNUT (*VIGNA SUBTERRANEAN*) FROM MPUMALANGA PROVINCE OF SOUTH AFRICA: PHYTOCHEMICAL PROPERTIES OF PRODUCT EXTRACTS

Abstract

Recently, Bambara groundnut (BGN) an indigenous legume has been the focus of scientific research due to having high protein content and indigenous knowledge regarding medicinal value. Therefore, it was necessary to determine if these medicinal properties were present in products (milk and yoghurt) produced from BGN. Qualitative and quantitative phytochemical properties of BGN was reported. Extracts produced from whole and dehulled BGN milk and yoghurt with 70% methanol, 70% ethanol and milli-Q water were screened for the presence of certain phytochemicals, specifically alkaloids, flavonoids, phenols, riboflavin and thiamine using analytical laboratory methods for basic screening, and high-performance liquid chromatography (HPLC) and gas chromatography (GC) for quantification. Flavonoids that were detected at the highest concentrations were rutin (5.694 mg.g⁻¹, whole BGN milk, milli-Q water), quercetin (0.703 mg.g⁻¹, whole BGN yoghurt, milli-Q water) and myricetin (0.987 mg.g⁻¹, whole BGN yoghurt, 70% ethanol). Additionally, highest concentrations of phenols were gallic acid (0.009 mg.g⁻¹, dehulled BGN yoghurt, 70% methanol) and ellagic acid (0.353 mg.g⁻¹, whole BGN yoghurt, 70% methanol). Thiamine and riboflavin was not detected for either BGN milk or BGN yoghurt. The outcome of this research showed that Bambara groundnut products (milk and yoghurt) indeed have phytochemicals.

4.1 Introduction

Functional foods also called nutraceuticals are foods or food components that are scientifically recognized as having physiological benefits beyond those of basic nutrition (Hözer & Kirmaci, 2010). Today, consumers are looking for ways to promote their own health and to enhance their life span. In recent years, rising medical costs have forced people to find cheaper and effective means of protecting their health, and as a result, interest in functional food products has increased (Granato *et al.*, 2010; Hözer & Kirmaci, 2010).

The interest in plant-based nutraceutical products has grown considerably in the last decade due to the large number of beneficial properties. Phytochemicals are non-nutritive compounds with disease-preventive properties that are found in plants. As a result, consumers are increasing their consumption of plant-based nutraceuticals because of their perceived health benefits (López-Gutiérrez *et al.*, 2015).

Furthermore, diseases have led to higher mortality rates due to increased resistance of pathogenic microbes to antimicrobial agents/drugs and the indiscriminate use of synthetic antibiotics. This creates a continued search for new antimicrobials, especially of natural origin. Medicinal plants possess varieties of bioactive compounds (phytochemicals and antimicrobials) having therapeutic potential. These bioactive compounds can be used in the pharmaceutical sector as well as in the food sector which at present contain a variety of food products and functional food used for therapeutic benefit (Vats *et al.*, 2012).

A legume that is becoming more apparent to scientific research is Bambara groundnut (BGN). It is a legume of African origin and used locally as a vegetable milk and can be consumed at any stages of maturation (Atiku *et al.*, 2004; Okonkwo & Opara, 2010). It is reported by Boateng *et al.* (2013) that the trend of solubility observed for the protein isolate of BGN promotes their potential use in beverages namely vegetable milk for lactose intolerant individuals. Furthermore, shelf stable patented products were previously developed by Murevanhema & Jideani (2013) and Hardy & Jideani (2016) from BGN, namely milk and probiotic beverage, demonstrating that BGN is more than suitable for producing lactose intolerant or vegetarian substitutes for dairy milk and yoghurt. Studies on the nutraceutical properties of BGN are essential to establish their potential for use in functional foods and nutraceutical industry.

One way of creating a functional food is through the addition of ingredients such as probiotics and prebiotics to levels that enable the consumer to obtain optimal health benefits (Nyanzi & Jooste, 2012). Probiotics are defined as live microorganisms, which upon ingestion in adequate numbers impart health benefits to the host animal beyond inherent basic nutrition (Nyanzi & Jooste, 2012). Probiotic products are typically marketed in the form of fermented milk and yoghurt. However, with an increase in the consumer vegetarianism throughout developed countries, there is also a demand for vegetarian probiotic products (Rivera-Espinoza & Gallardo-Navarro, 2010; Murevanhema & Jideani 2013; Vasudha &

Mishra, 2013). Non-dairy probiotic beverages may be made from a variety of raw materials, such as cereals, millets, legumes, fruits and vegetables (Murevanhema & Jideani, 2013; Vasudha & Mishra, 2013).

Whilst the nutritional value of BGN and developed products by Murevanhema & Jideani (2013) is generally well known, their potential as nutraceuticals has not been fully exploited. The bioactive compounds present in BGN can potentially assist at promoting consumption of BGN milk and probiotic beverages which could be used as a vehicle to ingest possible phytochemicals for human consumption (Murevanhema & Jideani 2013; Nyau *et al.*, 2015). Investigating the phytochemical properties of BGN milk and probiotic beverage will help to increase the awareness of BGN and its product development capabilities in addition to its potential therapeutic properties. Therefore, the aim of this study was to characterize the phytochemicals in BGN milk and BGN probiotic beverage.

4.2 Materials and Methods

4.2.1 Source of BGN, chemical reagents and equipment

Bambara groundnuts were purchased from Mpumalanga Province, South Africa. The chemicals used in this study were of HPLC and analytical grade and chemical reagents were prepared according to standard analytical procedures: Methanol Chromosolv® for HPLC ≥ 99.9% (Sigma-Aldrich), Acetonitrile LiChrosolv® LC-MS ≥ 99.9% (Merck), Acetic acid (glacial) ≥ 99% (Merck) and Ethanol 99% AR (Merck). Milli-Q water (18.2 MΩ.cm⁻¹), purified using the milli-Q water purification system (Millipore, Microsep, South Africa) were used for making dilutions and solutions. All prepared reagents were stored under conditions (18-20°C in dark environment) that will prevent deterioration or contamination.

All equipment used was from the Department of Food Science and Technology based at Cape Peninsula University of Technology, South Africa.

4.2.2 Production of whole and dehulled Bambara groundnut flour

The mixture of whole BGN (WBGN) varieties was milled into whole BGN flour (WBGNF) using a hammer mill (Bauermeister Inc., Vernon Hills) with a sieve size 250 μm (No. 60), packed and sealed in clear plastic bags and stored in a refrigerator at 4-6°C until further usage.

Whole Bambara groundnut (10 Kg) seeds were dehulled, involving the process of drying the groundnuts in a cabinet dryer (Geiger & Klotzbücher, South Africa) at 40°C for 24 h and subsequently removing the seed coat using a Corona® hand mill, to produce dehulled BGN (DBGN). The DBGN was milled into dehulled BGN flour (DBGNF) using a hammer mill (Bauermeister Inc., Vernon Hills) with a sieve size 250 μm (No. 60), packed and sealed in clear plastic bags and stored in a refrigerator at 4-6°C until further usage.

4.2.3 Production of whole and dehulled Bambara groundnut milk

The production of whole BGN milk (WBGNM) and dehulled BGN milk (DBGNM) comprised of using either the WBGNF or the DBGNF follow the methods described by Hardy & Jideani (2013).

4.2.4 Production of whole and dehulled Bambara groundnut yoghurt

As performed by Murevanhema & Jideani (2013), the WBGNM/DBGNM (100 mL) in 250 mL Schott bottles was warmed to 45°C in a water bath followed by inoculation with 3% (w/v) of yoghurt culture and incubated in a Memmert incubator at 35°C for 24 h. The fermented whole/dehulled BGN yoghurt (WBGNY/DBGNY) was cooled in an ice bath and refrigerated at $2 \pm 4^\circ\text{C}$.

4.2.5 Production of whole/dehulled Bambara groundnut milk and yoghurt extracts

The 70% methanol, 70% ethanol and milli-Q water extracts were prepared using Ultrasound-Assisted Extraction (UAE) on the different BGN products (WBGNM, DBGNM, WBGNY and DBGNY). Approximately, 15 g of sample (WBGNM, DBGNM, WBGNY and DBGNY) in 150 mL of the three different solvents (70% methanol, 70% ethanol and milli-Q water) was sonicated for 30 minutes at 25°C using the Lasec SA 2510 Branson ultrasound bath 42 kHz $\pm 6\%$, USA. After extraction, the mixture was centrifuged at a speed of 15316 g for 15 min at 4°C in a Beckman rotar JA-14 in Beckman Coulter Avanti J-E centrifuge, USA. The resulting supernatant from the 70% ethanol and 70% methanol solvent each was concentrated to ± 30 mL by evaporation under pressure in a rotary evaporator (Buchi RE 011 model, Switzerland) at 40°C to remove residual ethanol and methanol (Nyau *et al.*, 2014). The supernatant of the milli-Q water solvent mixture was syringe filtered directly into 5 mL freeze drier vials without evaporation with rotary evaporator. The extracts of WBGNM, DBGNM, WBGNY and DBGNY per solvent (70% methanol, 70% ethanol and milli-Q water) was frozen at -80°C and freeze dried to obtain a powdered extract using the BenchTop Pro with Omnitronics freeze dryer, United Scientific, Germany. The freeze-dried extracts were stored at -4°C and subjected to qualitative and quantitative phytochemical analyses.

4.3 Qualitative Phytochemical Analysis

Extracts produced from BGN milk (WBGNM and DBGNM), as well as the extracts produced from BGN probiotic beverages (WBGNY and DBGNY) underwent preliminary phytochemical analyses outlined in sections 4.3.1 to 4.3.3 to determine whether specific phytochemicals were present.

4.3.1 Qualitative analysis for tannins/polyphenols

The method consisted of adding 1 mL of freshly prepared 10% (m.v⁻¹) KOH solution to 1 g of individual extracts of WBGNM, DBGNM, WBGNY and DBGNY. If an opaque precipitate appeared, it indicated the presence of tannins (Santosh *et al.*, 2013).

4.3.2 Qualitative analysis for flavonoids

As described by Bhandary *et al.* (2012), 1 g of WBGNM, DBGNM, WBGNY or DBGNY extract was treated with few drops of ferric chloride solution. Formation of blackish red colour indicated the presence of flavonoids.

4.3.3 Qualitative analysis for alkaloids

Mayer's reagent was prepared by dissolving a mixture of 1.35 g mercuric chloride in 60 mL of distilled water and poured into a solution of 5 g potassium iodide in 10 mL of distilled water. The following solution was standardized to 100 mL with distilled water in a volumetric flask (Anon, 2015). WBGNM, DBGNM, WBGNY and DBGNY individual extract (0.5 g) was stirred with 5 mL of 1% (v/v) HCl in a water bath. The solution was filtered and 1 mL of filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100 mL with distilled water. Turbidity of the extract filtrate with the addition of Mayer's reagent was evident for the presence of alkaloids in the extract (Santosh *et al.*, 2013).

4.4 Quantitative Phytochemical Analysis

Following the qualitative confirmation of phytochemicals, further analyses were performed on WBGNM, DBGNM, WBGNY and DBGNY extracts to quantify the various concentrations present for each phytochemical.

4.4.1 Flavonoid analysis on BGN product extracts

Preparation of calibration standards and stock solutions of flavonoids

Four flavonoid standards were used as external standards, namely quercetin (Sigma Aldrich), kaempferol (Sigma Aldrich), rutin (Sigma Aldrich) and myricetin (Sigma Aldrich). The standard solutions were prepared as described by Kaliyaperumal *et al.* (2013). The flavonoid standards used for the calibration were namely quercetin, kaempferol, rutin and myricetin. All standards were stored at -20°C for a maximum of 2 weeks. The stock solutions of the four flavonoids standards were prepared by dissolving 1 mg into 10 mL HPLC grade methanol (Merck).

HPLC analyses of flavonoids

The HPLC separations of the standards and samples were performed using Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany). The HPLC system consisted of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment set at 30°C, an autosampler and a G1315C Diode Array Detector (DAD) set at 190 nm to 600 nm. The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) was used to integrate peak areas as well as record and store data.

The analytical column was a reversed phase column (ZORBAX SB-C18 3.5 μm , 4.6 x 150 mm, Agilent, USA) with a reversed-phase C18 guard column at 40°C. The BGN extract samples at this point were injected at a volume of 20 $\mu\text{g}\cdot\text{mL}^{-1}$. The guard cartridge was replaced after every 150 injections. The mobile phase was isocratic at 40:20:39:1 (v/v/v/v) methanol: acetonitrile: water: acetic acid with a flow rate of 0.8 $\text{mL}\cdot\text{min}^{-1}$, the peaks were simultaneously identified using UV absorbance at 350 nm for kaempferol and 254 nm for rutin, myricetin and quercetin (Kaliyaperumal *et al.*, 2013).

The chromatographic peaks of the extracts were confirmed by comparing their retention time and UV spectra with those of the pure standards. The utilisation of the calibration function of the Agilent Chemstation software to interpret the calibration curve and quantification of the flavonoid levels of samples were performed by the external standard method. In terms of quantification, a standard solution, diluted from 20 $\mu\text{g}\cdot\text{mL}^{-1}$ to 0.01 $\mu\text{g}\cdot\text{mL}^{-1}$ was analysed in triplicate at the start of each working day. The software used the resultant peak area to calculate the actual concentration of flavonoids in individual extracts of WBGNM, DBGNM, WBGNY and DBGNY. The column was restored for further use between injections by means of a post-run flushing with 100% acetonitrile (5 min), followed by 100% milli-Q water (5 min), and then recycled to initial conditions (5 min).

4.4.2 Thiamine (vitamin B₁) and riboflavin (vitamin B₂) analysis on BGN product extracts

Analytical standards and preparation of calibration standards

Two vitamin standards were used as external standards, namely riboflavin and thiamine (Sigma-Aldrich, Germany). The standard solutions were prepared as described by Otemuyiwa & Adewusi (2013). All standards were stored at -20°C for a maximum of 2 weeks. Sodium mono-hydrogen phosphate was purchased from Sigma-Aldrich (Germany). The mobile phases consisted of mobile phase A and mobile phase B at a ratio of 50:50 v/v. Mobile phase A was made up with 20 mM di-potassium hydrogen orthophosphate anhydrous (Unilab) in 500 mL of milli-Q water and adjusted to a pH of 6 using ortho-phosphoric acid 85% (Merck), once pH was adjusted the remaining volume was made up to 1 L.

Mobile phase B consisted of milli-Q water only. Both mobile phases were sonicated for 30 min before usage.

Stock solutions of vitamins B1 and B2

The stock standard solutions of thiamine and riboflavin were prepared as described by Otemuyiwa & Adewusi (2013). The working standards were prepared from the stock standard solutions by taking 0.1, 0.2, 0.3, 0.4 and 0.5 mg.mL⁻¹ of the stock into 10 mL standard flask and making it up to mark with appropriate solvent. The solutions were sonicated for 30 min and 20 µL of various concentrations of vitamin standards used for HPLC.

HPLC analyses of vitamin B1 and B2

The HPLC separations of the standards and samples were performed using an Agilent 1100 HPLC system. The HPLC system consists of a G1322A vacuum degassing unit, a G1311A quaternary pump, a thermostat column compartment, an autosampler, a G1315C Diode Array Detector (DAD), and a Fluorescence Detector (FLD). The Agilent Chemstation software (Agilent Technologies, Waldbron, Germany) was used to integrate peak areas as well as to record and store data.

The chromatographic separation column consists of a Phenomenex Luna C18 column (4.6 x 150 mm) 3 µm and column temperature set at 30°C. The HPLC DAD detector was set at wavelengths 360 nm for riboflavin (vitamin B1) and 254 nm for thiamine (vitamin B2) to monitor the elution. The BGN extract samples at this point were injected at a volume of 20 µg.mL⁻¹. The elution was isocratic with mobile phases of 50:50 v/v ratio (milli-Q water and 20 mM phosphate buffer at pH = 6) and a flow rate 0.60 mL.min⁻¹ (Otemuyiwa & Adewusi, 2013).

The chromatographic peaks of the extracts were confirmed by comparing their retention time and UV spectra with those of the pure standards. The utilisation of the calibration function of the Agilent Chemstation software to interpret the calibration curve and quantification of the flavonoid levels of samples were performed by the external standard method. In terms of quantification, a standard solution, diluted from 20 µg.mL⁻¹ to 0.01 µg.mL⁻¹ was analysed in triplicate at the start of each working day. The software used the resultant peak area to calculate the actual concentration of flavonoids in individual extracts of WBGNM, DBGNM, WBGNY and DBGNY. The column was restored for further use between injections by means of a post-run flushing with 100% acetonitrile (5 min), followed by 100% milli-Q water (5 min), and then recycled to initial conditions (5 min).

4.4.3 Phenolic analysis on BGN product extracts

Analytical standards

Five phenolic compounds were used as external standards, namely gallic acid (Sigma Aldrich), catechin (Sigma Aldrich), methyl gallate (Industrial analytics), chlorogenic acid (Industrial analytics) and ellagic acid (Sigma Aldrich). The standard solutions were prepared as described by Møller *et al.* (2009).

Stock solutions of tannins and mobile phases

The stock solution of the five phenolic compounds was prepared to a concentration of 0.5 mg.mL⁻¹ in a solvent consisting of 95% methanol and 5% water. From the stock solution five calibration standards was prepared by diluting with 50% (v/v) methanol-water with the following dilutions 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 mg.mL⁻¹. Mobile phase A consisted of methanol, water, and formic acid (5:95:0.1) (v/v/v) and mobile phase B consisted of methanol and formic acid (100:0.1) (v/v).

HPLC-MS analyses of phenols

HPLC was performed using the equipment previously described. As described by Møller *et al.* (2009), the chromatographic separation with a Phenomenex Luna C18 column (250 x 4.6 mm i.d.; 5 µm) was used. The DAD detector was set a wavelength from 245 – 360 nm. The HPLC-DAD/MS system used a programmed gradient mobile phase A and mobile phase B in the following gradient sequence: 0 min, 5% B; 7 min, 18% B; 11 min, 18% B; 15 min, 25% B; 20 min, 50% B, 25 min, 75% B, 26 min, 100% B; 29 min, 100% B; and 30 min, 5% B. The flow rate was 1.0 mL.min⁻¹ and column temperature was maintained at 40°C. The BGN extract samples at this point were injected at a volume of 20 µg.mL⁻¹.

The chromatographic peaks of the extracts were confirmed by comparing their retention time and UV spectra with those of the pure standards. The utilisation of the calibration function of the Agilent Chemstation software to interpret the calibration curve and quantification of the phenolic levels of samples were performed by the external standard method. In terms of quantification, a standard solution, diluted from 20 µg.mL⁻¹ to 0.01 µg.mL⁻¹ was analysed in triplicate at the start of each working day. The software used the resultant peak area to calculate the actual concentration of phenols in individual extracts of WBGNM, DBGNM, WBGNY and DBGNY. The column was restored for further use between injections by means of a post-run flushing with 100% acetonitrile (5 min), followed by 100% milli-Q water (5 min), and then recycled to initial conditions (5 min).

4.4.4 Linearity, Limit of detection (LOD) and Limit of quantification (LOQ) for all HPLC analyses (flavonoid, riboflavin, thiamine and tannins)

A linearity curve was prepared using standard solutions at 0.01, 0.03, 0.10, 0.25, 0.50, 1.0, 1.50, 2.0, 2.50, 5.0, 10.0 and 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$. Twelve samples per concentration ($n = 12$) were analysed and the multiple correlation coefficient (R^2) and regression coefficient (R) was used to determine whether the peak area was linear over the concentration range. The linearity curve was assessed to find the suitable linear region that was used to quantify flavonoids, riboflavin, thiamine, and phenols in BGN products (WBGNM, DBGNM, WBGNY and DBGNY).

The LOD and LOQ was calculated based on signal (S) to noise (N) ratios of 3 and 10, respectively $S/N = 3/10$. The standard deviation (SD) of the response and the slope (SP) of the calibration curve was used to calculate the LOD according to the formula: $\text{LOD} = 3 (\text{SD}/S)$ (Anon, 2002).

The LOQ was determined using the response SD and the slope of the calibration curve according to the formula: $\text{LOQ} = 10(\text{SD}/\text{SP})$. The standard deviation of the response was determined based on the standard deviation of y-intercepts of the regression line. The values of SD and slope was obtained from the LINEST function, when creating the calibration curve in SigmaPlot® (Systat Software, San Jose, USA). The SD of the y-intercept was the standard used for LOD and LOQ calculation.

4.4.5 GC-MS analyses of alkaloids

Extracts for GC-MS were prepared by weighing out freeze dried extract and dissolving it in 99% methanol solvent. The GC-MS Agilent 6890 N gas chromatography instrument with an Agilent 7683 autosampler, electronic pressure control, split-split- less injector and Agilent 5973 MSD mass selective detector with electronic impact was used for alkaloid analyses. GC operating conditions included: capillary column Agilent DB – 5MS (30 m, 0.25 i.d., 0.25 μm film thickness); oven temperature was increased at a rate of 40°C per minute from 50°C to 200°C; 20°C per minute from 200°C to 280°C, and was maintained for the duration of 12.25 minutes; the injector temperature was set at 250°C; the flow rate of carrier gas (helium) was 1 $\text{mL}\cdot\text{min}^{-1}$; the injection was performed in splitless mode and the purge off time was 0.5 min (Djurendic-Brenesel *et al.*, 2012).

4.5 Data analysis

Results expressed as mean \pm standard deviation of triplicate means. Multivariate analysis of variance was used to establish mean difference between treatments. Duncans multiple range tests were used to separate means where difference existed (IBM - SPSS, 2015).

4.6 Results and Discussion

4.6.1 Qualitative phytochemical screening

Phytochemical compounds, flavonoids and phenols were positively screened for BGN product (milk and yoghurt) extracts, and were detected in the BGN product extracts. Therefore, the BGN product extracts were subjected to quantitative phytochemical analysis.

4.6.2 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

To determine the response variation in concentrations of tannins, flavonoids, vitamins B₁ and B₂ linearity curves generated were interpreted. As described in Table 4.1 the linearity curves of the calibration mixture consisting of five phenols compounds interpreted over a concentration range between 0.01 and 50 µg.mL⁻¹. The multiple correlation coefficients (R²) of the five phenols compounds ranged between 0.997 and 0.999 and the correlation coefficients (R) ranged between 0.998 – 0.999. The LOD for gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid were between 0.04 and 0.49 µg.mL⁻¹, while the LOQ was between 0.13 and 1.64 µg.mL⁻¹ (Table 4.1).

For flavonoids, the linearity curves of the calibration mixture consisting of four flavonoid compounds interpreted over a concentration range between 0.50 µg.mL⁻¹ and 50 µg.mL⁻¹ detailed in Table 4.1. The multiple correlation coefficients (R²) of the four flavonoid compounds ranged between 0.997 and 0.999 and the correlation coefficients (R) ranged between 0.99639 and 0.99893. The LOD for rutin, myricetin, quercetin and kaempferol were between 0.05 µg.mL⁻¹ and 0.65 µg.mL⁻¹, while the LOQ was between 0.19 µg.mL⁻¹ and 2.17 µg.mL⁻¹ (Table 4.1).

For vitamins B₁ and B₂ the linearity curves of the calibration mixture were interpreted over a concentration range between 2 µg.mL⁻¹ and 20 µg.mL⁻¹ (Table 4.1). The multiple correlation coefficients (R²) of the vitamins ranged between 0.98531 and 0.99998 and the correlation coefficients (R) ranged between 0.992 and 0.999. The LOD for thiamine and riboflavin were between 0.0003 µg.mL⁻¹ and 0.058 µg.mL⁻¹, while the LOQ was between 0.001 µg.mL⁻¹ and 0.195 µg.mL⁻¹ (Table 4.1).

High multiple correlation coefficients (R²) and correlation coefficients (R) of > 0.98 were evidence of a good linear fit (Anon., 2002). The correlation coefficient and multiple correlation coefficients for tannins, flavonoids and vitamin B₁ and vitamin B₂ ranged between 0.98 and 0.99, indicating a noteworthy linear relationship between the concentration of the analytical standards and the response, while the proximity to one of the regression coefficients shows that the regression line fits the data. The HPLC method was, therefore, adequately sensitive to be used as an analytical tool.

Table 4.1 Limits of detection (LOD), limits of quantification (LOQ) and linear range of all standard solutions

Phytochemical	Compound	LOD ($\mu\text{g.mL}^{-1}$)	LOQ ($\mu\text{g.mL}^{-1}$)	Linear range ($\mu\text{g.mL}^{-1}$)
Phenols	Gallic acid	0.11	0.37	0.10 – 50.00
	Catechin	0.14	0.49	0.10 – 50.00
	Methyl gallate	0.04	0.13	0.10 – 50.00
	Chlorogenic acid	0.18	0.60	0.10 – 50.00
	Ellagic acid	0.49	1.64	0.10 – 50.00
Flavonoid	Rutin	0.33	1.12	0.50 – 50.00
	Myricetin	0.65	2.17	0.50 – 50.00
	Quercetin	0.05	0.19	0.50 – 50.00
	Kaempferol	0.11	0.39	0.50 – 50.00
B- Vitamins	Thiamine	0.00	0.00	2.00 – 20.00
	Riboflavin	0.05	0.19	2.00 – 20.00

Peak identification and peak purity

The identification of tannins, flavonoids, and vitamin B₁ and B₂ were achieved by means of the retention time (R_t) and the quantification of the compounds and vitamins were performed by measuring the peak area of the samples relative to that of the standards (Anon, 2002). The retention times for all compounds were as follows; gallic acid (6.19 min), catechin (12.42 min), methyl gallate (14.25 min), chlorogenic acid (15.55 min) and ellagic acid (24.50 min) (Figure 4.1); rutin (1.96 min), myricetin (2.23 min), quercetin (2.68 min) and kaempferol (3.36 min) (Figure 4.2), and for the vitamins: thiamine (2.31 min) and riboflavin (14.99 min) (Figure 4.3). In addition, purity tests were performed using the “Check purity” option of the Chemstation system. The purity factors of peaks were within the threshold limit and therefore can be used to calculate the phenols, flavonoid and vitamin B₁ and vitamin B₂ concentrations. The purity factors were > 990, which indicated an excellent peak.

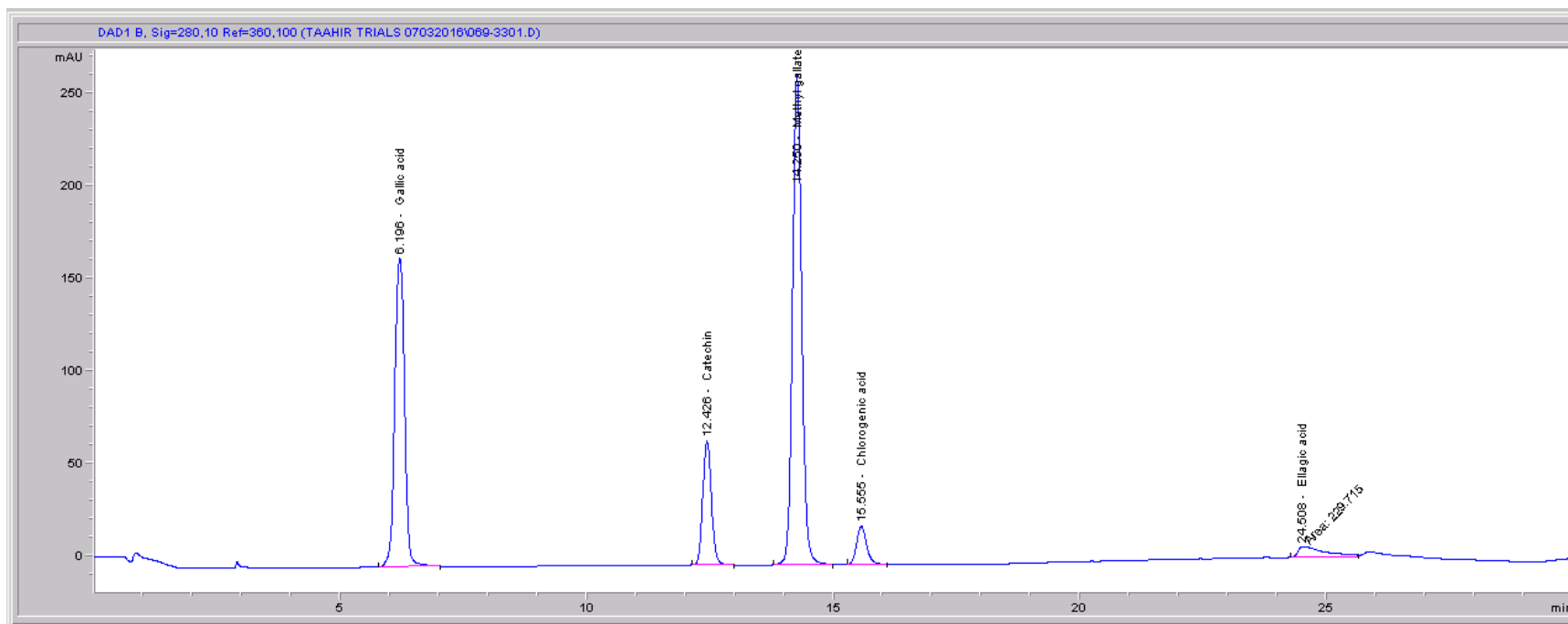


Figure 4.1 Chromatogram of a standard mixture containing five phenolic compounds at their respective retention times (R_t), namely gallic acid (6.196), catechin (12.426), methyl gallate (14.250), chlorogenic acid (15.555) and ellagic acid (24.508)

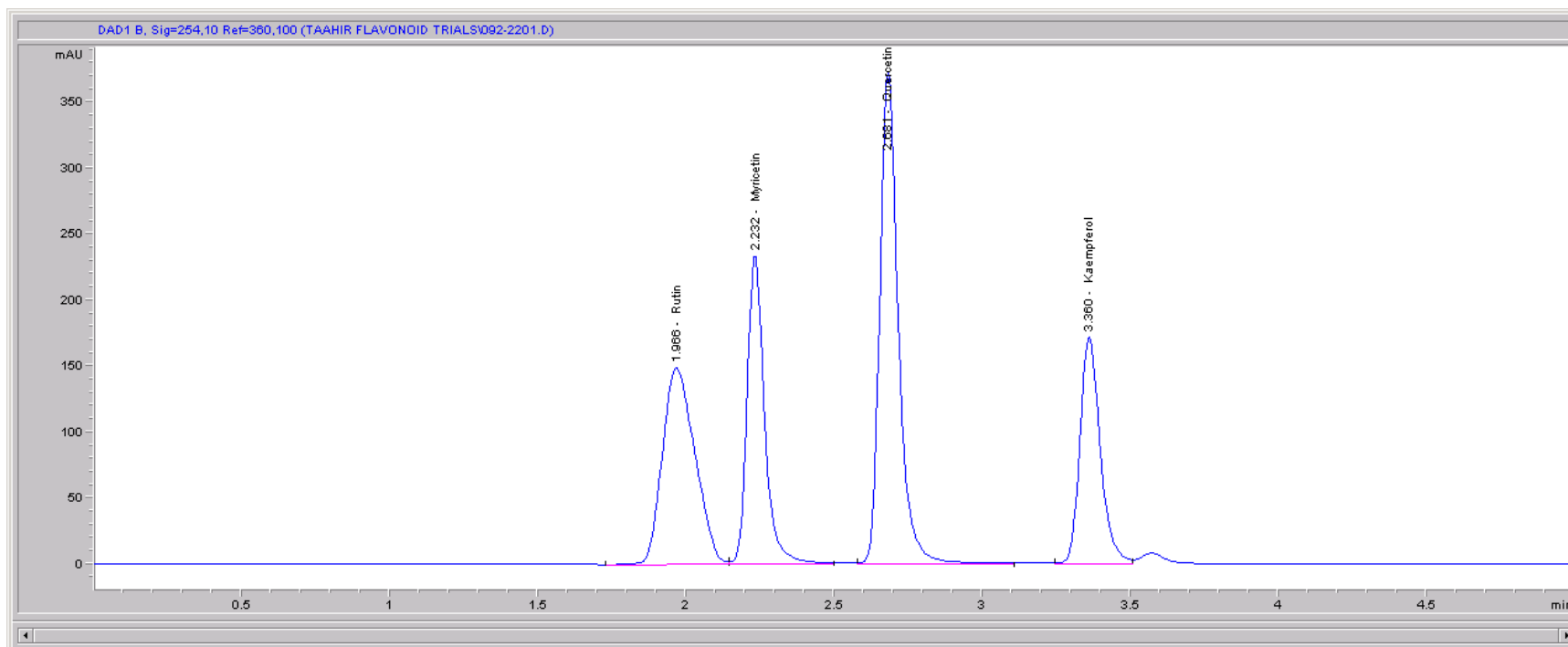


Figure 4.2 Chromatogram of a standard mixture containing four flavonoid compounds at their respective retention times (R_t), namely rutin (1.966), myricetin (2.232), quercetin (2.681) and kaempferol (3.360)

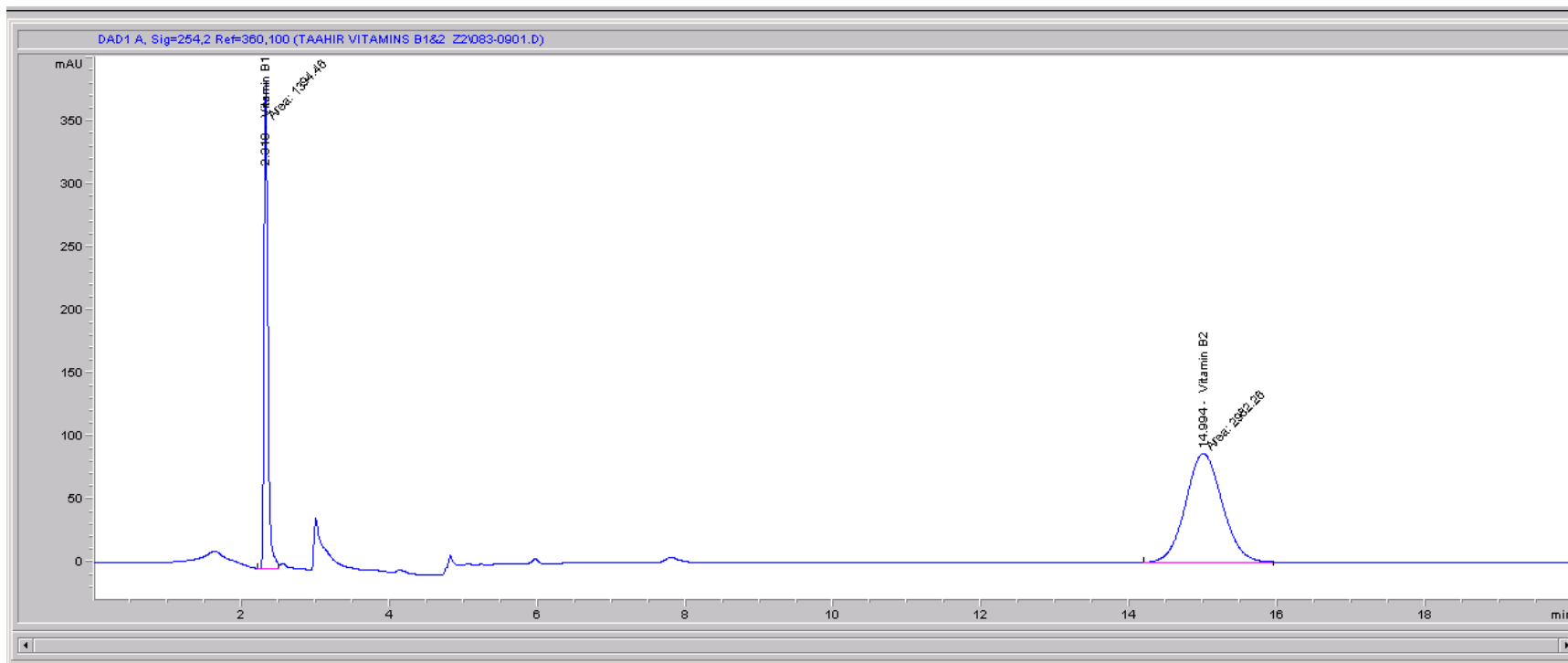


Figure 4.3 Chromatogram of a standard mixture containing two B-vitamins compounds at their respective retention times (R_t), namely vitamin B1 – thiamine (2.318) and vitamin B2 – riboflavin (14.994)

4.6.3 Flavonoid characteristics of whole and dehulled BGN products (milk and yoghurt) extracted with various solvents (70% methanol, 70% ethanol and milli-Q water)

Flavonoid compounds in extracts from whole and dehulled BGN products (milk and yoghurt) are summarized in Figures 4.4 to 4.6. Whole and dehulled BGN milk quercetin levels ranged between 0.035 (70% methanol, dehulled) and 0.693 mg.g⁻¹ (milli-Q water, dehulled). Quercetin levels from whole and dehulled BGN milk extracted with 70% ethanol and milli-Q water were significantly ($p \leq 0.05$) higher than whole and dehulled BGN milk extracted with 70% methanol. Furthermore, as seen in Figure 4.5, the quercetin levels were significantly higher ($p \leq 0.05$) in whole BGN milk extracted with 70% methanol than dehulled BGN milk extracted with 70% methanol. As for the rest of the extraction solvents for whole and dehulled BGN milk observed in Figure 4.6, there were no significant ($p > 0.05$) differences for quercetin levels between 70% ethanol and milli-Q water. Therefore, 70% ethanol and milli-Q water were superior solvents for extracting quercetin from whole and dehulled BGN milk. However, quercetin levels for whole BGN milk extracted with 70% methanol was comparable to 70% ethanol and milli-Q water, thus it was only the dehulled BGN milk that had low quercetin levels for 70% methanol.

Whole and dehulled BGN yoghurt quercetin levels ranged between 0.013 (milli-Q water, dehulled) and 0.703 mg.g⁻¹ (milli-Q water, whole). Quercetin levels from whole and dehulled BGN yoghurt extracted with 70% methanol and 70% ethanol were significantly ($p \leq 0.05$) higher than whole and dehulled yoghurt extracted with milli-Q water (Figure 4.4). Furthermore, the quercetin levels were significantly higher ($p \leq 0.05$) in whole BGN yoghurt extracted with milli-Q water than dehulled BGN yoghurt extracted with the same solvent (Figure 4.6). For whole and dehulled BGN yoghurt, there were no significant ($p > 0.05$) differences for quercetin levels between 70% methanol and 70% ethanol (Figures 4.5 – 4.6). Overall 70% methanol and 70% ethanol were superior solvents when compared to milli-Q water for extracting quercetin from whole and dehulled BGN yoghurt. However, quercetin levels for whole BGN yoghurt extracted with milli-Q water was comparable to 70% methanol and 70% ethanol, thus it was only the dehulled milk that had low quercetin levels for 70% methanol.

Similarities can be observed with whole and dehulled BGN milk and yoghurt for quercetin, only difference is their solvents. With BGN milk 70% methanol was the inferior solvent whereas with BGN yoghurt milli-Q water was less effective solvent. Though, the form of BGN did play a role in quercetin concentration whereby it was always lower in the dehulled BGN products for those specific solvents, suggesting that quercetin is more concentrated in the hulls of BGN than the endosperm.

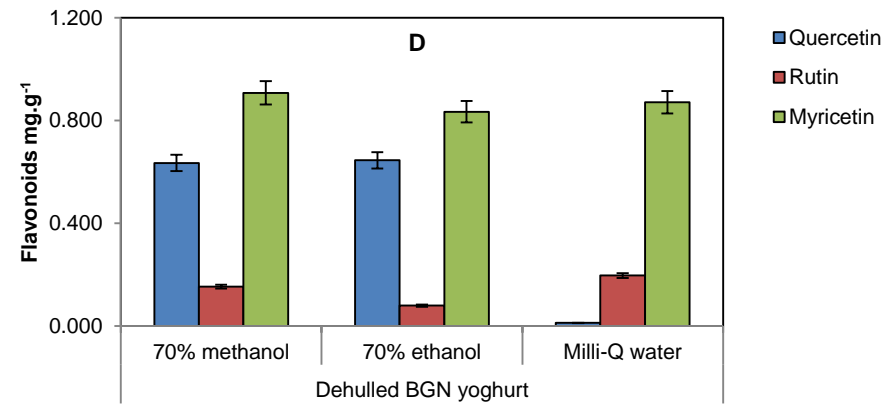
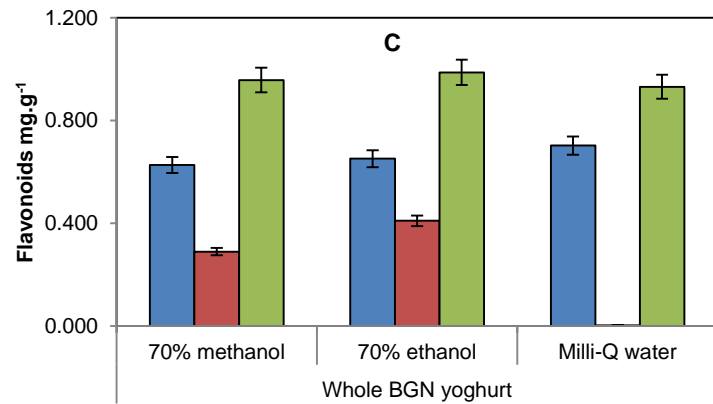
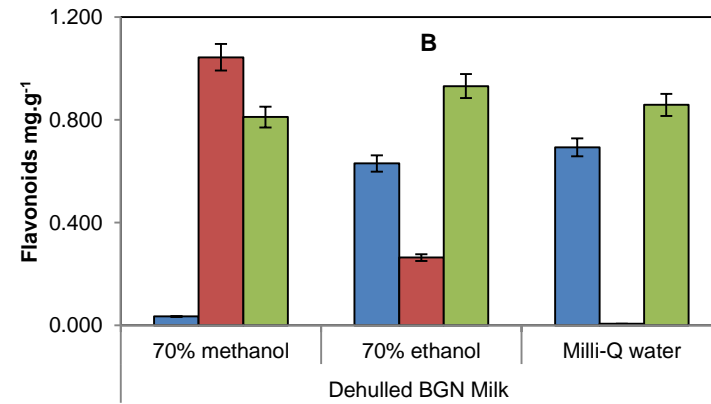
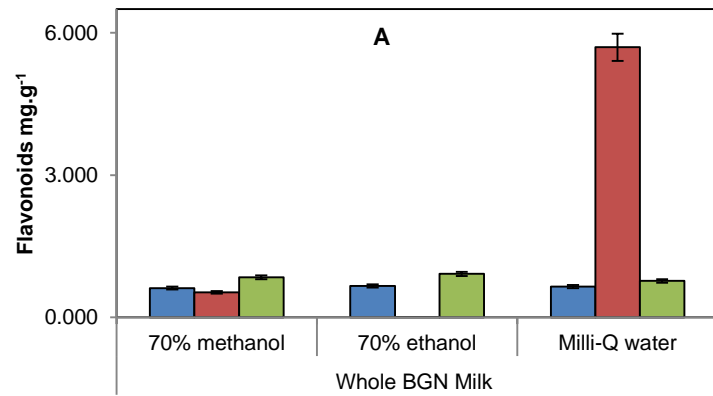


Figure 4.4 Flavonoids dehulled BGN milk and yoghurt from A = whole BGN milk, B = dehulled BGN milk, C = whole BGN yoghurt, D = dehulled BGN yoghurt

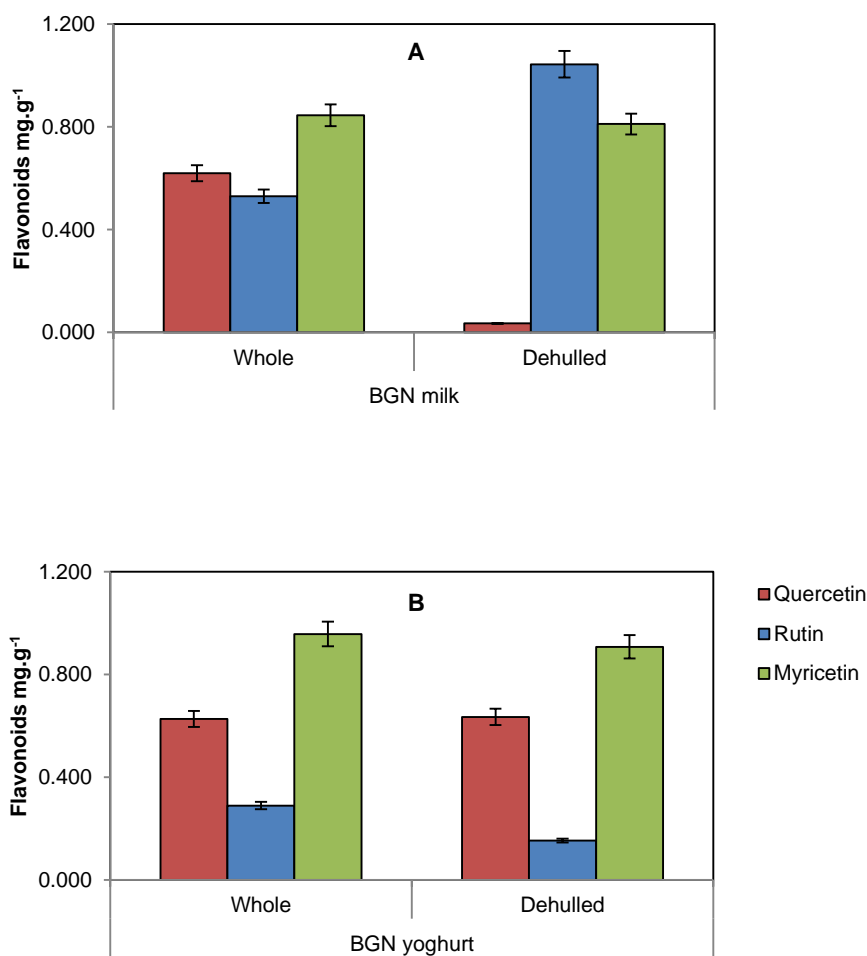


Figure 4.5 Flavonoids of BGN milk (A) and yoghurt (B) extracted with 70% methanol

Rutin levels for whole and dehulled BGN milk ranged between 0.004 (70% ethanol, whole) and 5.694 mg.g⁻¹ (milli-Q water, whole). Whole and dehulled BGN milk extracted with milli-Q water was significantly ($p \leq 0.05$) higher in rutin than 70% methanol, and 70% methanol was significantly ($p \leq 0.05$) higher than 70% ethanol (Figure 4.4). Rutin was significantly higher in dehulled BGN milk than whole BGN milk extracted with 70% methanol and 70% ethanol, whereas with milli-Q water rutin was significantly higher in whole BGN milk than dehulled BGN milk (Figure 4.5 and 4.6). It could be concluded that milli-Q water was the best solvent for extracting rutin when compared to 70% methanol and 70% ethanol. Additionally, whole BGN milk had higher rutin levels than dehulled BGN milk, indicating that the form played a role, and as observed with quercetin, the flavonoid rutin is more present in the hulls than the endosperm.

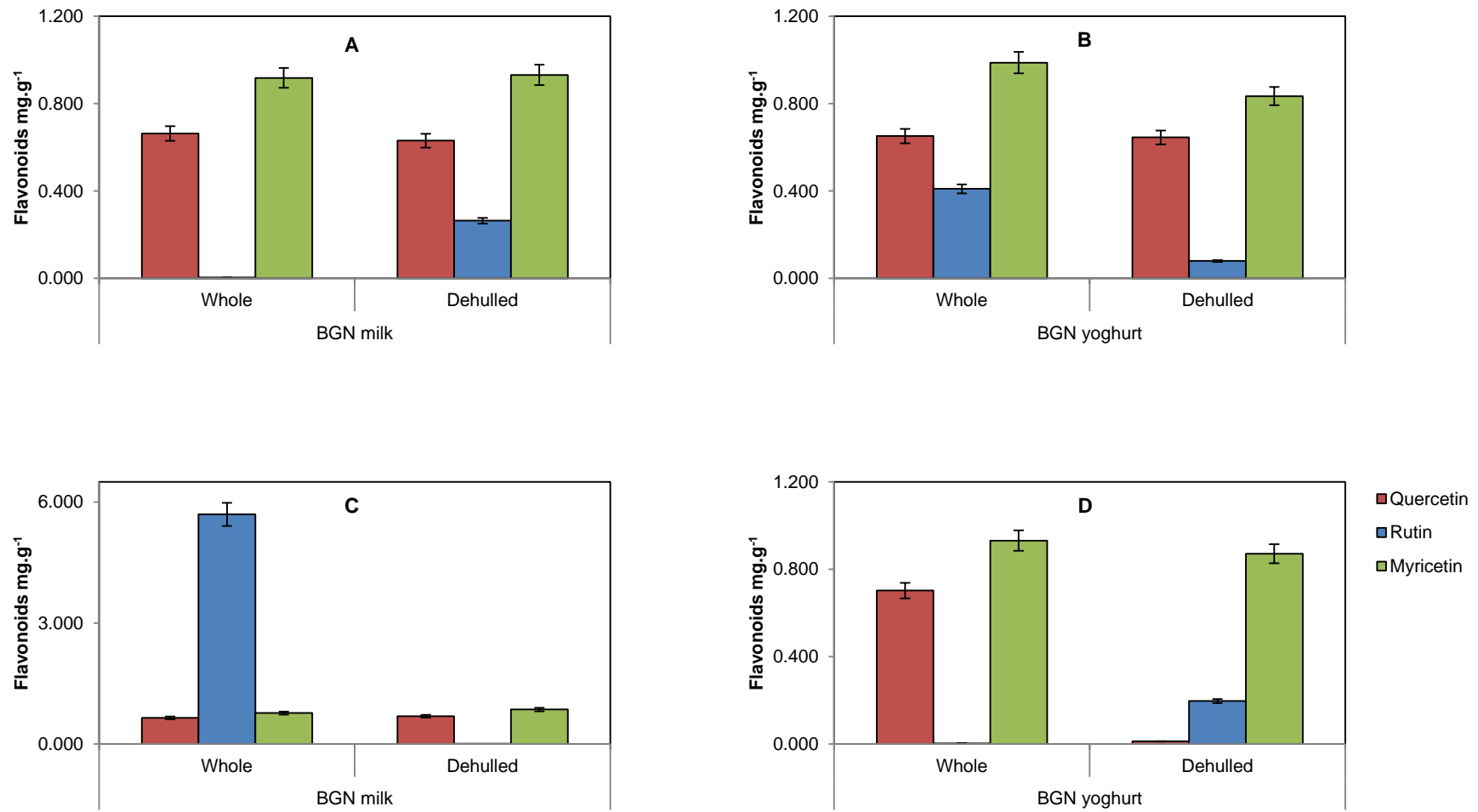


Figure 4.6 Flavonoids of BGN milk and yoghurt from A = BGN milk extracted with 70% ethanol, B = BGN yoghurt extracted with 70% ethanol, C = BGN milk extracted with milli-Q water, D = BGN yoghurt extracted with milli-Q water

Whole and dehulled BGN yoghurt rutin levels ranged between 0.004 (milli-Q water, whole) and 0.410 mg.g⁻¹ (70% ethanol, whole). Rutin extracted with 70% ethanol for whole and dehulled BGN yoghurt was significantly ($p \leq 0.05$) higher than 70% methanol, and 70% methanol was significantly ($p \leq 0.05$) higher than milli-Q water (Figure 4.4). Rutin was significantly higher in whole BGN yoghurt than dehulled BGN yoghurt extracted with 70% methanol and 70% ethanol, whereas with milli-Q water rutin was significantly higher in dehulled BGN yoghurt than whole BGN yoghurt (Figure 4.5 and 4.6). With regards to rutin extraction solvents 70% ethanol was the superior solvent when compared to 70% methanol and milli-Q water. Moreover, the inverse was observed, whereby dehulled BGN yoghurt extracted with milli-Q water had the highest rutin concentration.

Myricetin levels for whole and dehulled BGN milk ranged between 0.770 (milli-Q water, whole) and 0.931 mg.g⁻¹ (70% ethanol, dehulled). Whole and dehulled BGN milk extracted with 70% ethanol was significantly higher than 70% methanol and milli-Q water. Furthermore, there was no significant difference between 70% methanol and milli-Q water. Additionally, myricetin was significantly higher in dehulled BGN milk than whole BGN milk for all three solvents, indicating that myricetin is primarily concentrated in the endosperm of BGN more so than the hulls (Figure 4.5 and 4.6).

Whole and dehulled BGN yoghurt myricetin levels ranged between 0.834 (70% ethanol, dehulled) and 0.987 mg.g⁻¹ (70% ethanol, whole). There was no significant difference amongst the solvents for whole and dehulled BGN yoghurt (Figure 4.4). However, myricetin for all solvents was significantly ($p \leq 0.05$) higher in whole BGN yoghurt than dehulled BGN yoghurt.

Extraction of whole BGN milk with milli-Q water had the highest concentration of rutin, whereas whole BGN yoghurt extracted with milli-Q water, had the lowest concentration of rutin. Quercetin and myricetin levels were higher in the whole BGN yoghurt than whole BGN milk, suggesting that possibly these phenolic compounds increased during the fermentation process. Additionally, plant foods are a natural source of functional phytochemicals. Yet, most of the phytochemicals that exist naturally in plant foods are bound and are less bioavailable than the free form. These phytochemicals and their antioxidant properties could be altered by processing, such as fermentation. As a result, the microorganisms used in fermentation are capable of modifying the bioavailability of phytochemicals in plant foods (Yeo & Ewe, 2015). Rutin (quercetin-3-o-rutinoside) is the glycoside combining the flavonol quercetin and the disaccharide rutinose (α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranose) (Wang *et al.*, 2011). The sugar rutinose is comprised of glucose substituted in the 6-position with an O- α -L-rhamnopyranosyl grouping. The action of β -glucosidases splits off the rutinose resulting in quercetin. The action of α -L-rhamnosidases yields isoquercetin. β -glucosidase activity is widespread amongst lactic acid bacteria (Michlmayr & Kneifel, 2014).

α -L-rhamnosidase activity is present in *Lactobacillus plantarum* and *Lactobacillus acidophilus* (Yadav *et al.*, 2010). The enzymatic conversion of rutin to quercetin by *Lactobacillus bulgaricus* used in this study could account for the decrease in rutin and increase in quercetin contents. As for myricetin, it was significantly ($p \leq 0.05$) higher in BGN yoghurt than BGN milk.

4.6.4 Phenol characteristics of whole and dehulled BGN products (milk and yoghurt) extracted with various solvents (70% methanol, 70% ethanol and milli-Q water)

Phenols of whole BGN milk and yoghurt are summarized in Figures 4.7 – 4.9. Gallic acid for whole and dehulled milk ranged between 0.000 (70% methanol, whole) and 0.006 mg.g⁻¹ (70% ethanol, dehulled). There was no significant ($p > 0.05$) difference amongst all solvents for both whole and dehulled BGN milk. According to Figure 4.7 there was no significant ($p > 0.05$) difference between whole and dehulled BGN milk, however the concentration of gallic acid was slightly higher in dehulled BGN than whole BGN.

Whole and dehulled BGN yoghurt gallic acid levels ranged between 0.006 (milli-Q water, whole) and 0.009 mg.g⁻¹ (70% methanol, dehulled). Gallic acid extracted with 70% methanol was significantly ($p \leq 0.05$) higher than 70% ethanol and milli-Q water, and 70% ethanol was significantly ($p \leq 0.05$) higher than milli-Q water (Figure 4.7). There were no significant ($p > 0.05$) differences between whole and dehulled BGN yoghurt for gallic acid levels. Gallic acid according to Figures 4.7 – 4.9 have very low levels in BGN when compared to ellagic acid. The form and products made from BGN had no impact on levels. However, for the yoghurt there was a significant difference between solvents (Figures 4.7 and 4.9).

Ellagic acid for whole and dehulled milk ranged between 0.113 (70% methanol, whole) and 0.353 mg.g⁻¹ (milli-Q water, whole). There were no significant differences amongst solvents (70% methanol, 70% ethanol and milli-Q water) for ellagic acid levels in whole and dehulled BGN milk. However, whole BGN milk was significantly higher in ellagic acid levels than dehulled BGN milk, indicating that ellagic acid is more concentrated in the hulls than the endosperm of BGN.

Whole and dehulled BGN yoghurt ellagic acid levels ranged between 0.112 (70% methanol, dehulled) and 0.347 mg.g⁻¹ (70% methanol, whole). Ellagic acid levels for whole and dehulled BGN yoghurt extracted with 70% ethanol were significantly ($p \leq 0.05$) higher than whole and dehulled BGN yoghurt extracted with 70% methanol and milli-Q water, additionally, 70% methanol was significantly ($p \leq 0.05$) higher than milli-Q water in ellagic acid (Figure 4.7). Furthermore, whole BGN yoghurt was significantly higher ($p \leq 0.05$) than dehulled BGN yoghurt, indicating yet again as seen with the milk, that ellagic acid is more concentrated in the hulls than the endosperm of BGN.

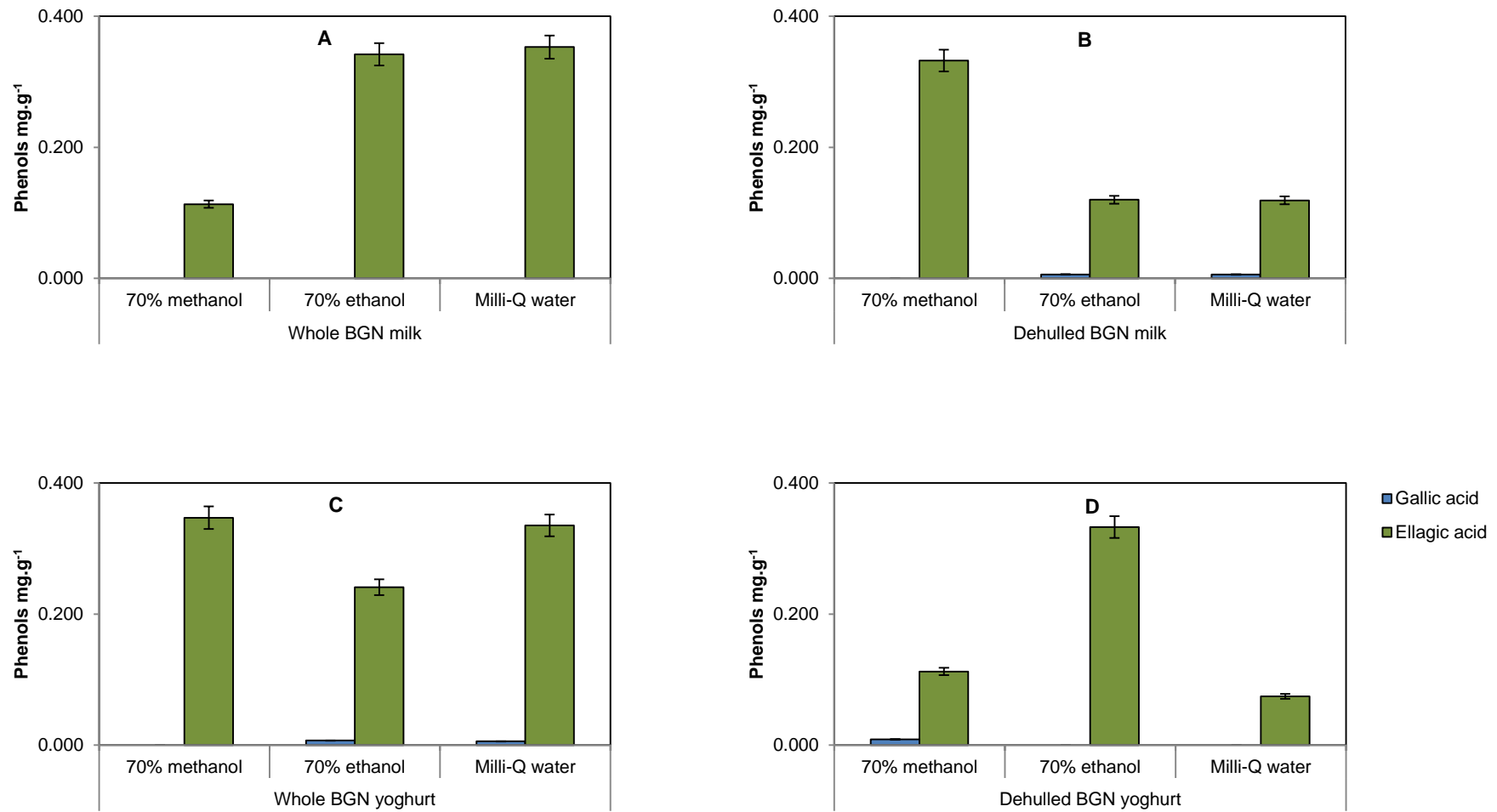


Figure 4.7 Phenols of whole and dehulled BGN milk and yoghurt based on solvents from A = whole BGN milk, B = dehulled BGN milk, C = whole BGN yoghurt, D = dehulled BGN yoghurt

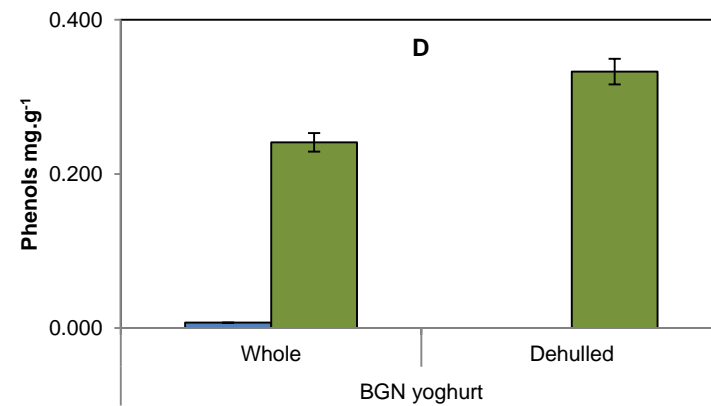
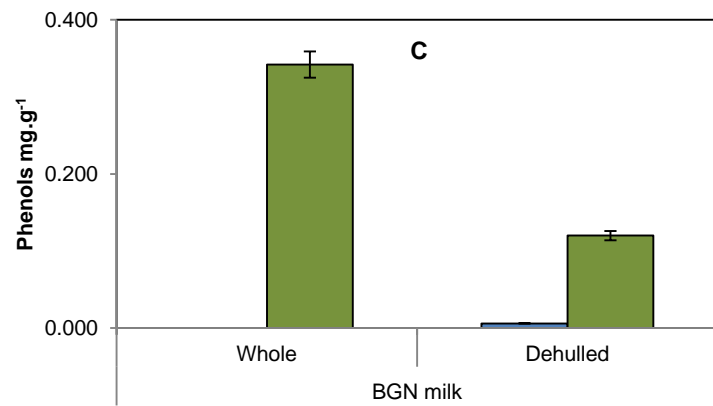
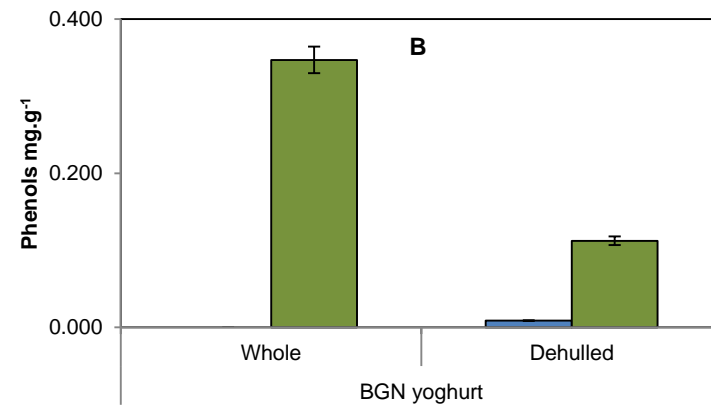
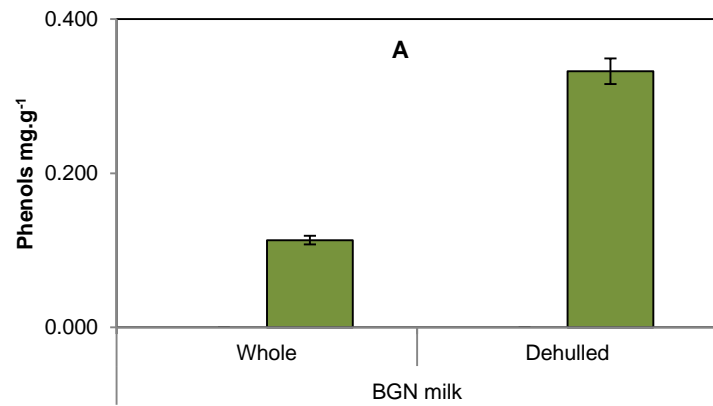


Figure 4.8 Phenols of BGN milk and yoghurt extracted with 70% methanol and 70% ethanol; A = BGN milk extracted with 70% methanol, B = BGN yoghurt extracted with 70% methanol, C = BGN milk extracted with 70% ethanol, D = BGN yoghurt extracted with 70% ethanol

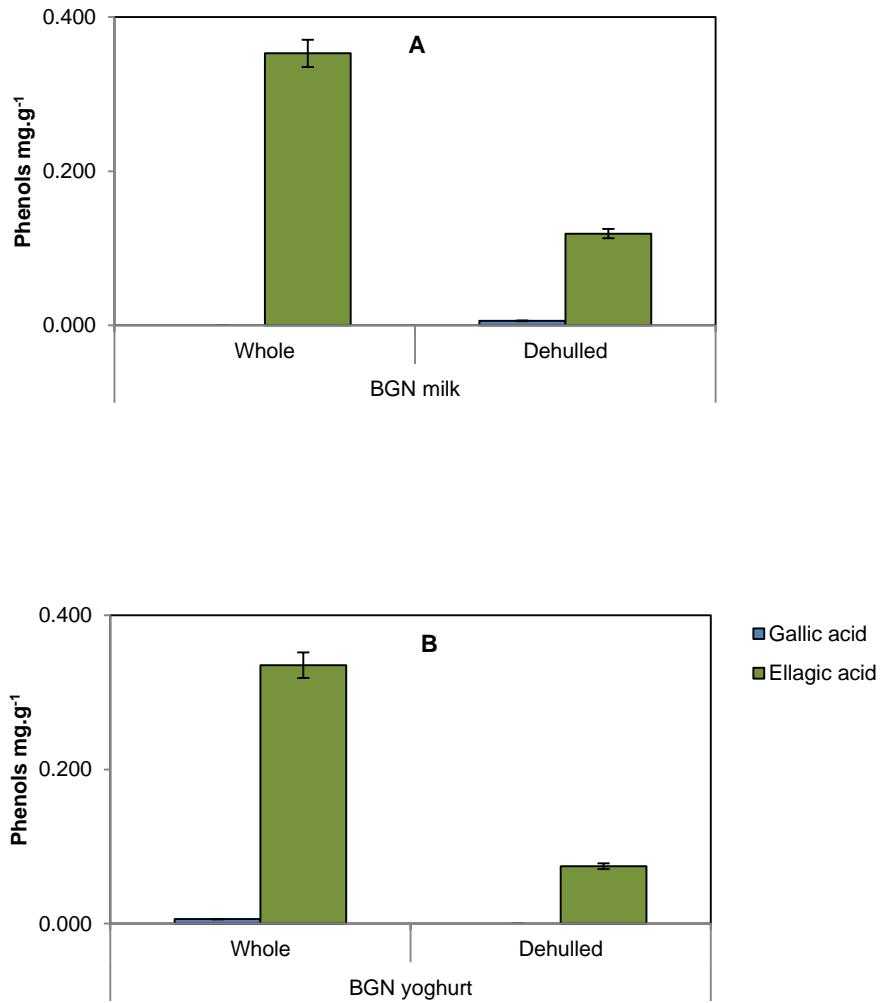


Figure 4.9 Phenols of BGN milk (A) and yoghurt (B) extracted with milli-Q water

Gallic acid was only present in dehulled BGN yoghurt. As reported by Sreeramulu & Raghunath (2011) gallic acid levels in BGN milk and yoghurt is lower than gallic acid found in coconut milk (0.310 mg.g⁻¹) and dairy milk (0.034 mg.g⁻¹). Similarly, ellagic acid was found to be significantly ($p \leq 0.05$) different between BGN yoghurt and BGN milk. Ellagic acid levels were found to be significantly ($p \leq 0.05$) different between dehulled BGN milk and yoghurt. Additionally, different solvents had different yields with different products, suggesting that either the phenolic compounds were lost during the fermentation process or it's due to the nature of each product and solvent properties.

4.7 Conclusion

The study showed that phytochemicals were present in whole and dehulled BGN milk and yoghurt. The phytochemicals specifically flavonoids and phenols found in the BGN milk and yoghurt all have antimicrobial characteristics. Furthermore, these phytochemicals were mostly concentrated in the whole BGN products than dehulled BGN products, suggesting that the phytochemicals are highly concentrated in BGN hulls as it forms part of the whole BGN products. Additionally, the form of BGN and the solvents used to extract were indeed important factors to consider. Formulating products with higher concentration of BGN hulls will result in a product with higher phytochemical content and also potentially increasing therapeutic value of the products.

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CHAPTER 5

BAMBARA GROUNDNUT (VIGNA SUBTERRANEAN) FROM MPUMALANGA PROVINCE OF SOUTH AFRICA: ANTIMICROBIAL PROPERTIES OF SEEDS AND PRODUCT EXTRACTS

Abstract

Bambara groundnut an indigenous legume cultivated in Sub-Saharan Africa has been proclaimed to have medicinal properties from communities in tribes and in rural areas. However, there is not enough scientific information to validate these claims. This study aimed to identify the antimicrobial properties of Bambara groundnut (BGN), using extracts from whole, dehulled and hulls of BGN as well as extracts from BGN milk and yoghurt. The antimicrobial activity methodology involved direct bioautography and minimum inhibitory concentration (MIC) against six antibiotic-resistant microorganisms, *Acinetobacter baumannii* ATCC 19606^T, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. *aureus* ATCC 33591 and *Candida albicans* ATCC 24433. Red and brown hull extracts from organic solvents (70% ethanol and 70% methanol) showed the highest antimicrobial activity, whereas the whole, dehulled and hulls (black-eye and brown-eye) extracts had no antimicrobial activity. Antimicrobial activity of extracts for *C. albicans* ATCC 24433 displayed the highest cell reduction of 55.8% (brown hull, 70% methanol) at 100 $\mu\text{g.mL}^{-1}$, *P. aeruginosa* ATCC 27853 cell reduction of 43.9% (red hull, 70% ethanol) at 30 $\mu\text{g.mL}^{-1}$, *E. faecalis* ATCC 29212 cell reduction of 42.9% (brown hull, 70% ethanol) at 50 $\mu\text{g.mL}^{-1}$, *S. aureus* subsp. *aureus* ATCC 33591 cell reduction of 22.7% (red hull, 70% ethanol) at 5 $\mu\text{g.mL}^{-1}$ while *K. pneumoniae* subsp. *pneumoniae* ATCC 700603 a cell reduction of 30.8% (red hull, 70% ethanol) at 20 $\mu\text{g.mL}^{-1}$. No activity was observed against *A. baumannii* ATCC 19606^T under the tested conditions. As for antimicrobial activity of extracts from BGN products, no activity was observed against the selected microorganism strains under the conditions tested. The outcome of this research showed that Bambara groundnut products though high in phytochemicals, had no antimicrobial activity detected against the experimental microorganisms.

5.1 Introduction

The continuous evolution of bacteria resistant to currently available antibiotics has motivated the search for novel and more effective antibacterial compounds, thus, plant based medicine have become the focus, as their historical use particularly in developing countries, rely on plants for the treatment of infectious and non-infectious diseases (Yagi *et al.*, 2013). These plants are ingested as elixirs, teas or juice preparations to treat respiratory infections (Rojas *et al.*, 2006). These plants remain a great source of natural antioxidants, antimicrobials and possibly anticancer agents that may serve as leads for the development of new potent antibiotics to which pathogenic strains are not resistant (Abu-Shanab *et al.*, 2004; Rojas *et al.*, 2006; Patra *et al.*, 2011; Iwu *et al.*, 2014). Furthermore, the traditional healers state that patients of these villages and communities have reduced risk to infectious diseases from resistant pathogens than patients from urban areas treated with synthetic antibiotics. Subsequently patients treated in hospital environment have a higher risk of contracting a nosocomial infection (Rojas *et al.*, 2006; Mireya *et al.*, 2007).

Bambara groundnut (*Vigna subteranea*) an indigenous African legume is grown across the continent from Senegal to Kenya and from the Sahara to South Africa (Mpotokwane *et al.*, 2008; Eltayeb *et al.*, 2011; Jideani & Diedericks, 2014; Nyau *et al.*, 2014). The colour of the seed varies from black, brown or red and may be mottled with various colours (Onimawo *et al.*, 1998; Jideani & Diedericks, 2014). Before consumption, the hulls are removed and generally regarded as waste. However, BGN hulls have been reported to have quantities of phenolic compounds as well as confirmed in chapter 3. In addition, the hulls are an inexpensive source of nutraceuticals and functional ingredients (Klompong & Benjakul, 2015). Additionally, phenolic compounds (a group of secondary metabolites) have shown to possess antimicrobial properties (Maddox *et al.*, 2010).

Legumes have historically been utilised mainly as whole seeds but in recent years, interest has grown in the utilisation of legumes in other forms (e.g. flour, concentrate and isolate) other than the whole seeds. (Sitohy & Osman, 2010). The medicinal use of BGN is based on information obtained from communities in several parts of Africa, where this legume is reportedly used for the treatment of various ailments. The water boiled from the maize and pulse mixture is ingested to treat diarrhoea. Anon (2011) reported that raw BGN can be chewed and swallowed to treat nausea suffered by pregnant women, while in Kenya, the Luo tribe use BGN to cure diarrhoea (Mkandawire, 2007).

The traditional utilization of BGN to treat several ailments is notable and therefore creates an opportunity for detailed scientific study on the pharmaceutical value of the crop (Mølgaard *et al.*, 2011). According to Mbagwu *et al.* (2011) the phytochemical screening carried out on whole BGN (sourced from Idemili in Anambara State, Nigeria) showed the presence of valuable phytochemicals and contained a high percentage of alkaloids compared to other legumes. Furthermore, Nyau *et al.* (2015) reported that BGN possessed

antioxidant activities. These findings point toward Bambara groundnuts having the potential as a functional food, with medicinal properties. However, there is limited research with regards to antimicrobial properties of whole, dehulled (endosperm) and hulls of Bambara groundnut for all seed colours (black, black-eye, brown, brown-eye and red) as well as the products produced from BGN, namely milk and yoghurt (Hardy & Jideani, 2013; Murevanhema & Jideani, 2013). Therefore, in the present study the aim was to evaluate the antimicrobial properties of BGN (seeds from Mpumalanga province of South Africa) and products (milk and yoghurt).

5.2 Materials and Methods

5.2.1 Source of BGN, chemical reagents and equipment

Bambara groundnuts were purchased from Mpumalanga Province, South Africa. The BGN seeds were sorted into their respective colours according to different testa and hilum colours as black, black-eye, brown, brown-eye and red.

The chemicals used in this study were of HPLC and analytical grade. Reagents were prepared according to standard analytical procedures: Methanol Chromosolv® for HPLC \geq 99.9% (Sigma-Aldrich), Acetonitrile LiChrosolv® LC-MS \geq 99.9% (Merck), Acetic acid (glacial) \geq 99% (Merck) and Ethanol 99% AR (Merck). Milli-Q water ($18.2 \text{ M}\Omega\cdot\text{cm}^{-1}$), purified using the Milli-Q water purification system (Millipore, Microsep, South Africa) were used for making dilutions and solutions. All prepared reagents were stored at 18 – 20°C in dark environment to prevent deterioration or contamination. All equipment utilized was from the Department of Food Science and Technology based at the Cape Peninsula University of Technology, South Africa.

5.2.2 Production of whole, dehulled and hull Bambara groundnut flour

Upon receiving the BGN, it was sorted into its respective colours. The black, black-eye, brown, brown-eye and red colours of whole BGN (WBGN) were milled separately into flour with a sieve size of 250 μm using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) and packed and sealed in clear plastic bags. The whole Bambara groundnut flour (WBGNF) of each variety was stored in a refrigerator at 4 – 6°C until further analysis.

The WBGN seeds were dried at 40°C in an industrial oven dryer (Geiger and Klotzebücher, Cape Town, South Africa) for 48 h. A Corona® manual grain was used to mill BGN to allow easier manual removal of hulls to produce dehulled Bambara groundnut (DBGN). The black, black-eye, brown, brown-eye and red DBGN and their respective hulls were milled into flour using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) with 250 μm sieve size, packed and sealed in clear plastic bags. The dehulled Bambara

groundnut flour (DBGNF) of each BGN seed colour was stored in a refrigerator at 4-6°C until further analysis.

The black, black-eye, brown, brown-eye and red BGN hulls were milled into flour to form Bambara groundnut hull flour (BGNHF) using a hammer mill (Perten Mill, Perten Instruments AB, Sweden) sieved with 250 µm sieve size, packed in clear plastic bags and stored in a refrigerator at 4-6°C until further analysis.

5.2.3 Production of whole and dehulled Bambara groundnut milk

The production of whole and dehulled BGN (WBGNM and DBGNM) comprised of using either the WBGNF or the DBGNF were prepared according to methods described by Murevanhema & Jideani (2013) and Hardy & Jideani (2013).

5.2.4 Production of whole and dehulled Bambara groundnut yoghurt

As performed by Murevanhema & Jideani (2013), the WBGNM/DBGNM (100 mL) in 250 mL Schott bottles was warmed to 45°C in a water bath following by inoculation with 3% (w/v) of yoghurt culture and incubated in a Memmert incubator at 35°C for 24 h. The fermented whole and dehulled BGN yoghurt (WBGNY and DBGNY) was cooled in an ice bath and refrigerated at 2 ± 4°C.

5.2.5 Production of whole, dehulled and hull Bambara groundnut seeds, milk and yoghurt extracts

The 70% methanol, 70% ethanol and milli-Q water extracts were prepared using Ultrasound-Assisted Extraction (UAE) on the different BGN seed flours and products (WBGNF, DBGNF, BGNHF, WBGNM, DBGNM, WBGNY and DBGNY). Approximately 15 g of sample (WBGNF, DBGNF, BGNHF, WBGNM, DBGNM, WBGNY and DBGNY) in 150 mL each of the three different solvents (70% methanol, 70% ethanol and milli-Q water) was sonicated for 30 minutes at 25°C using the Lasec SA 2510 Branson ultrasound bath 42 kHz ± 6%, USA. After extraction, the mixture was centrifuged at a speed of 15316 G for 15 min at 4°C in a Beckman rotar JA-14 in Beckman Coulter Avanti J-E centrifuge, USA. The resulting supernatant from the 70% ethanol and 70% methanol solvent each was concentrated to ± 30 mL by evaporation under the pressure in a rotary evaporator (Buchi RE 011 model, Switzerland) at 40°C to remove residual ethanol and methanol (Nyau *et al.*, 2014). The supernatant of the Milli-Q water solvent mixture was syringe filtered directly into 5 mL freeze dry vials without evaporation with rotary evaporator. The extracts of WBGNF, DBGNF, BGNHF, WBGNM, DBGNM, WBGNY and DBGNY per solvent (70% methanol, 70% ethanol and milli-Q water) was frozen at -80°C and freeze dried to obtain a powdered extract using the BenchTop Pro with Omnitronics freeze dryer, United Scientific, Germany.

The freeze-dried extracts were stored at -4°C and subjected to phytochemical screening and antimicrobial activity.

5.2.6 Antimicrobial activity of BGN seed, milk and yoghurt extracts

Sample preparation

Six bacteria strains were used in this study, namely *Acinetobacter baumannii* ATCC 19606^T, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. *aureus* ATCC 33591 and yeast, *Candida albicans* ATCC 24433. The test microorganisms were grown in broth media brain heart infusion broth (BHI; *E. faecalis*), nutrient broth (NB; *A. baumannii*, *K. pneumoniae* subsp. *pneumoniae*, *S. aureus* subsp. *aureus*, *C. albicans*) and trypticase soy broth (TSB; *P. aeruginosa*) and incubated at 37°C with agitation (160 rpm) overnight.

After confirmation of the purity of the cultures through standard Gram staining, the optical densities (OD) of the test bacterial cultures were determined at 600 nm on a spectrophotometer (SpectraMax M2, USA) using the SoftMax Pro 6.4.2 software and adjusted to 0.50 OD with sterile liquid broth.

Direct bioautography assay of BGN seed extracts, milk and yoghurt

Concentrated extracts (5 µL volumes) were spotted on thin layer chromatography (TLC) plates and clearly labelled. Once the spots have dried to completeness, the plates were used in bioautography (Dewanjee *et al.*, 2014).

The test strains were applied to the surface of the TLC plates containing the extracts with sterile absorbent cotton wool, placed in plastic sealable containers lined with moist paper towel and incubated at 37°C overnight. Thiazolyl blue (MTT) (Sigma Aldrich, South Africa) dissolved in phosphate buffered saline (4.26 g sodium monohydrogen phosphate heptahydrate, 2.27 g potassium dihydrogen phosphate, 8 g sodium chloride per litre, pH to 7.0) at a final concentration of 0.25%, w.v⁻¹, was applied to the plates using a sterile liquid spray bottle and the plates incubated at 37°C for 2 to 3 hours. Colour changes were monitored: if the plate turned purple, it indicated the presence of living cells; white/cream areas on the plate indicated the areas where the test microorganisms were killed, therefore indicating antimicrobial activity (Betina *et al.*, 1973; Dewanjee *et al.*, 2014).

Minimum inhibitory concentration (MIC) of BGN seed extracts, milk and yoghurt

To measure the MIC, a stock solution of 400 µg.mL⁻¹ was prepared for each extract sample and was further serially diluted into a sterile 96-well plate so that the final concentration of the extracts came to 5 µg.mL⁻¹, 10 µg.mL⁻¹, 20 µg.mL⁻¹, 30 µg.mL⁻¹, 50 µg.mL⁻¹ and 100 µg.mL⁻¹.

Microbial suspension of 135 μL at an OD of 0.80 and 45 μL of sample extract was added to the wells of a sterile 96-well microplate. The negative controls were 135 μL culture and 45 μL sterilized distilled water totalling to a volume of 180 μL and a pure control made of 180 μL culture. Positive control involved 135 μL culture and 45 μL antibiotic. Lastly, sterile control included 135 μL sterile broth and 45 μL sterile distilled water. The microplates were incubated at 37°C for 24 h. After 24 h 20 μL of 0.25% MTT was added to the wells and incubated for a further 3 h to observe colour change. Subsequently, 100 μL of DMSO (dimethyl sulfoxide) was added to kill all viable organisms remaining. Following the addition of DMSO, the microplates were analysed with a spectrophotometer (SpectraMax M2, USA) using the SoftMax Pro 6.4.2 software to determine OD readings of each well (Vats *et al.*, 2012).

5.3 Results and Discussion

5.3.1 Antimicrobial properties of whole, dehulled and hull BGN seed extracts

Antimicrobial activity was determined for whole, dehulled and hull BGN using the bioautography assay to determine antimicrobial activity. Whole and dehulled BGN had no activity against any of the strains. Red and Brown BGN hulls displayed antimicrobial activity against most of the selected strains, based on the silica plates (Figure 5.1 – Figure 5.6). On the prominent clear cream/white zones were the position of extract pipetted. As seen in Figure 5.1 – Figure 5.6 certain areas are highlighted on the silica plate with red circles indicating zones of growth inhibition of the selected strains due to extracts, while the yellow circles indicating the zones of growth inhibition due to controls and antibiotics.

Klebsiella pneumoniae subsp. *pneumoniae* ATCC 700603 (Gram-negative, Figure 5.1), *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative, Figure 5.2) and *Staphylococcus aureus* subsp. *aureus* ATCC 33591 (Gram-positive, Figure 5.6) were inhibited by BGN extracts from red and brown BGN hulls. No antimicrobial activity was observed from any of the BGN extracts for *Acinetobacter baumannii* ATCC 19606^T (Gram-negative, Figure 5.3) and *Candida albicans* ATCC 24433 (yeast, Figure 5.4). Lastly, for the strain *Enterococcus faecalis* ATCC 29212 (Gram-positive, Figure 5.5) growth inhibition by the extracts of brown BGN hulls was observed.

Branter *et al.* (1996) and Silver *et al.* (1997) reported that Gram-negative bacteria were not susceptible to plant extracts when compared to Gram-positive bacteria. The resistance of Gram-negative bacteria towards antibacterial substances is related to lipopolysaccharides in their outer membrane (Gao *et al.*, 1999). This is in contrast with results reported by Ajiboye & Oyejobi (2017) since most of the extracts showed prominent activity against Gram-negative bacteria. This contrast is also observed in this present study as most extracts had better activity against Gram-negative than Gram-positive bacteria. The

yellow circles on the silica plates on Figures 5.1 to Figure 5.6 indicated the zones of inhibition/clearance for the positive controls. These positive controls consisted of different antibiotics, namely penicillin, ampicillin, chloromycetin, cycloserine and vancomycin. Starting from Figure 5.1 the antibiotics ampicillin, chloromycetin, cycloserine and vancomycin were effective at inhibiting the growth of *K. pneumoniae* subsp. *pneumoniae* ATCC 700603. Figure 5.2 representing the strain *Pseudomonas aeruginosa* ATCC 27853 showed that the strain was inhibited by chloromycetin and vancomycin. For Figure 5.3 representing *A. baumannii* ATCC 19606^T only vancomycin was effective at resisting this strain. Figure 5.4 which had the strain *C. albicans* ATCC 24433 showed clear zones for positive controls chloromycetin and vancomycin. Figure 5.5 which had *E. faecalis* ATCC 29212, all experimental antibiotics (penicillin, ampicillin, chloromycetin, cycloserine and vancomycin) were effective at resisting this strain. Lastly, Figure 5.6 which had the strain *S. aureus* subsp. *aureus* ATCC 33591 exhibited clear zones around antibiotics chloromycetin and vancomycin.

It appeared that there was no noticeable difference for antimicrobial activity regardless of which solvent was used for the extraction of the brown and red BGN hulls. It seemed however, that each individual colour of BGN hulls had an important characteristic that contributed towards exhibiting antimicrobial activity, as it could either be that multiple colours (i.e. brown and red), singular colours (i.e. brown or red) or neither colours were effective at exhibiting antimicrobial activity. Figures 5.1, 5.2 and 5.6 showed that both brown and red BGN hulls were effective against the selected strains and displayed some degree of antimicrobial activity. In Figure 5.5 only brown BGN hull had showed activity and for Figures 5.3 and 5.4 neither brown nor red had showed any activity.

Brown and red BGN hulls correlated well with high flavonoid and tannin content of BGN as observed in chapter 3, therefore the higher the phenolic content, the greater the chances for antimicrobial activity of these extracts. The antimicrobial activity of these extracts was predominantly attributable to their phenolic compounds (Celis *et al.*, 2011; Carraturo *et al.*, 2014).

It should be noted that the zone of clearance of the red circles representing the pipetted extracts was smaller in size than that of the yellow circles representing the positive controls i.e. antibiotics. Subsequently, this suggests that even though the concentrations ($20 \mu\text{g.mL}^{-1}$) of extracts and antibiotics were the same for the direct bioautography assay the BGN extract at that specific concentration ($20 \mu\text{g.mL}^{-1}$) wasn't as effective as the traditional antibiotics commonly prescribed. However, this was taken further in the next process to determine the optimum concentration level of each extract and the minimum inhibitory concentration (MIC) of each extract for all selected strains. Therefore, a MIC assay was conducted using the different BGN extracts.

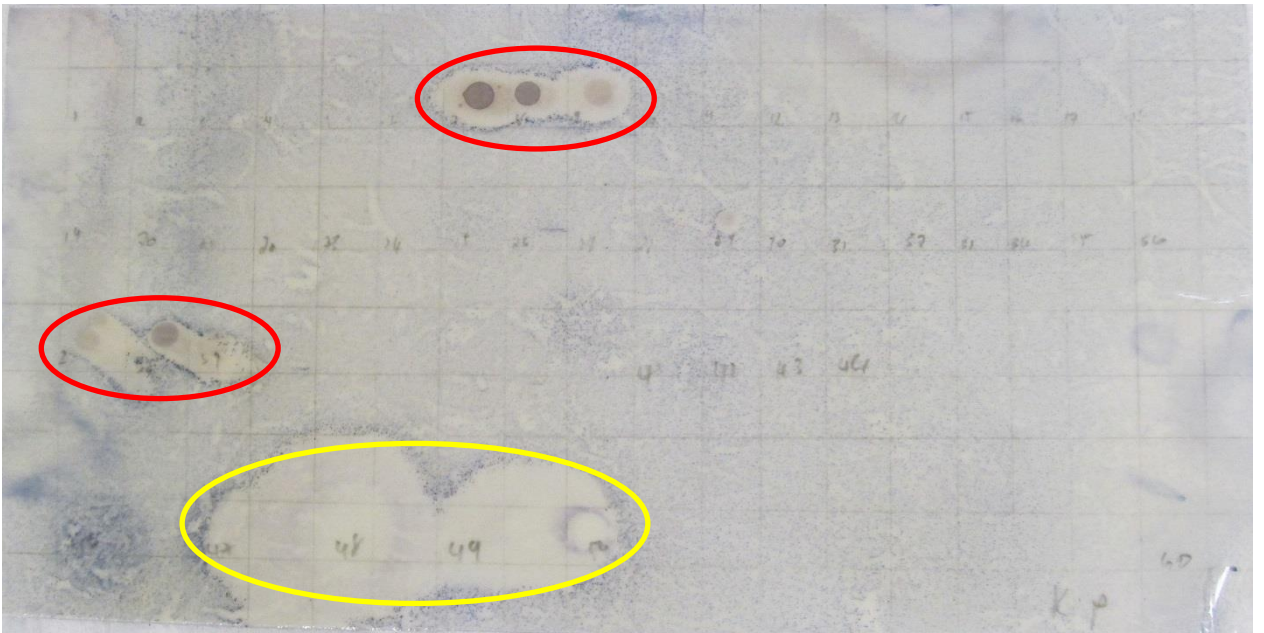


Figure 5.1 *Klebsiella pneumoniae* (subsp. *Pneumoniae*) ATCC 700603 bioautography thin layer chromatography plate

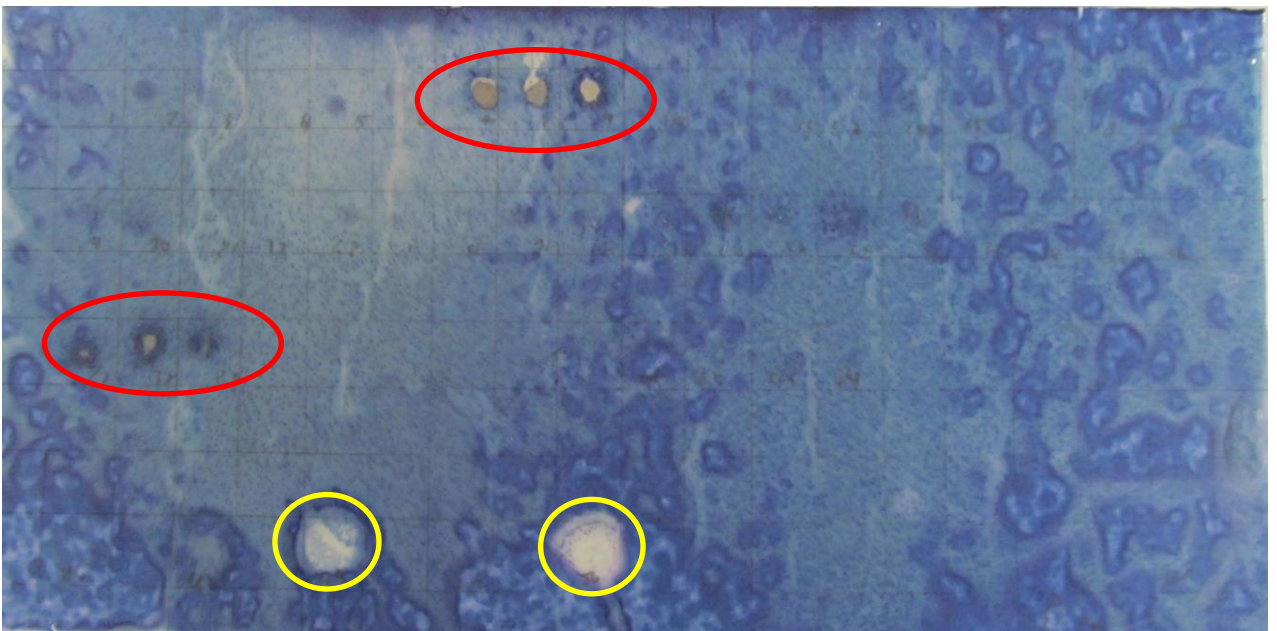


Figure 5.2 *Pseudomonas aeruginosa* ATCC 27853 bioautography thin layer chromatography plate

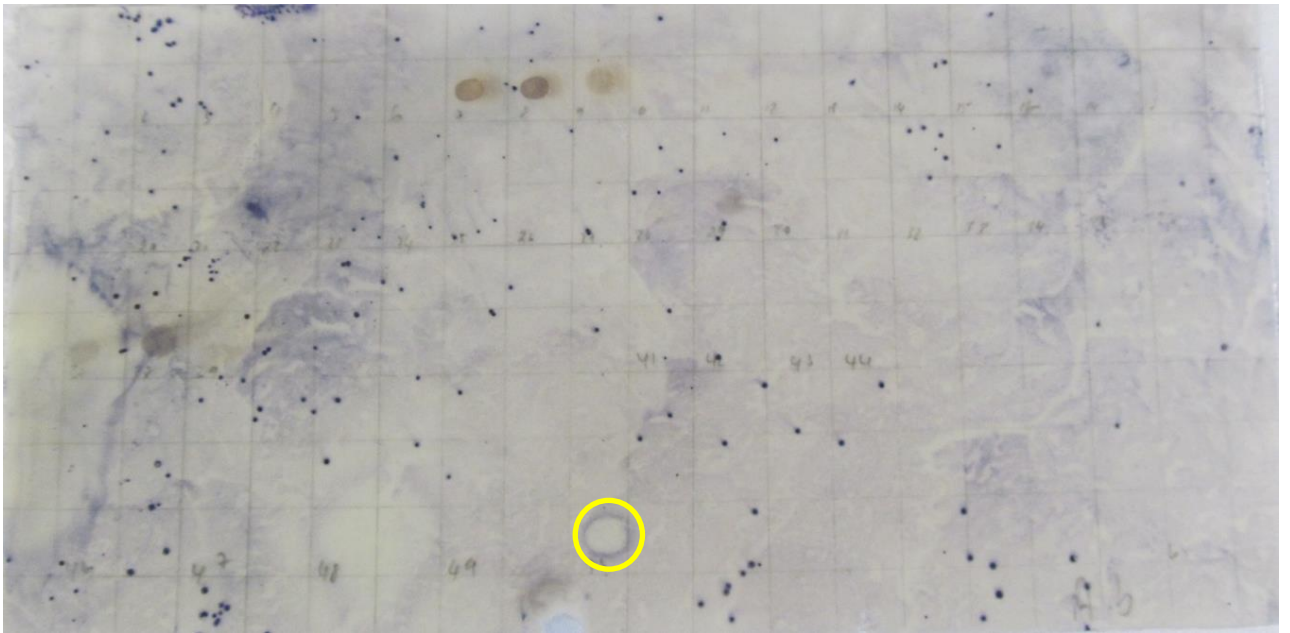


Figure 5.3 *Acinetobacter baumannii* ATCC 19606^T bioautography thin layer chromatography plate

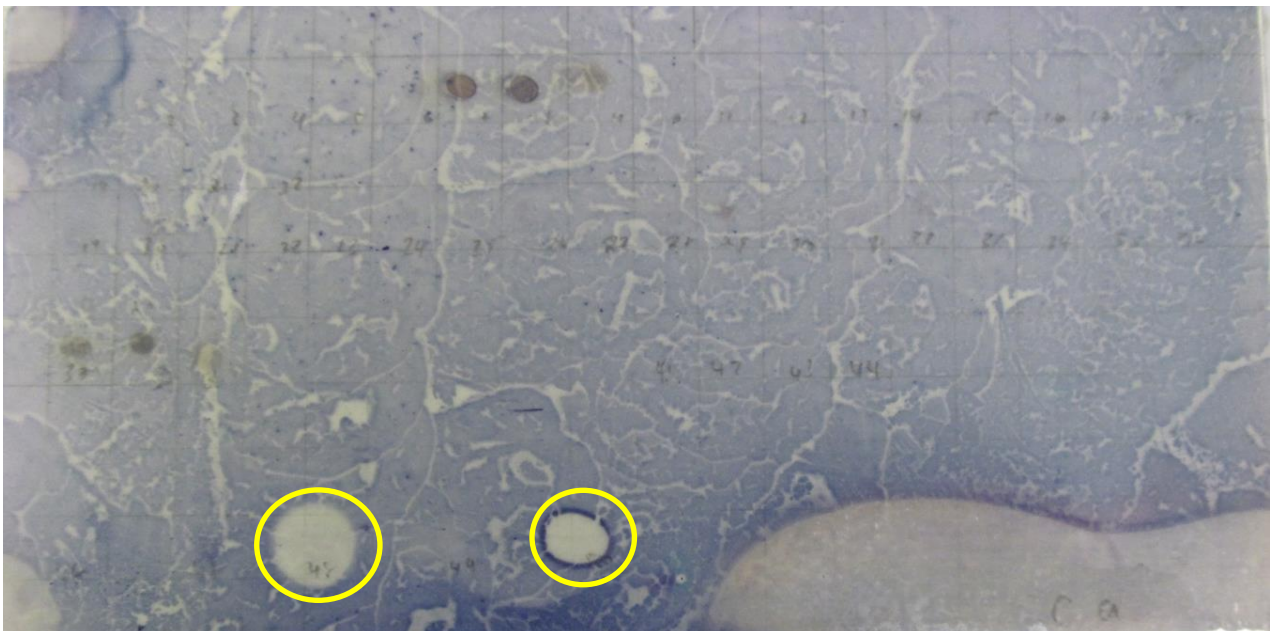


Figure 5.4 *Candida albicans* ATCC 24433 bioautography thin layer chromatography plate

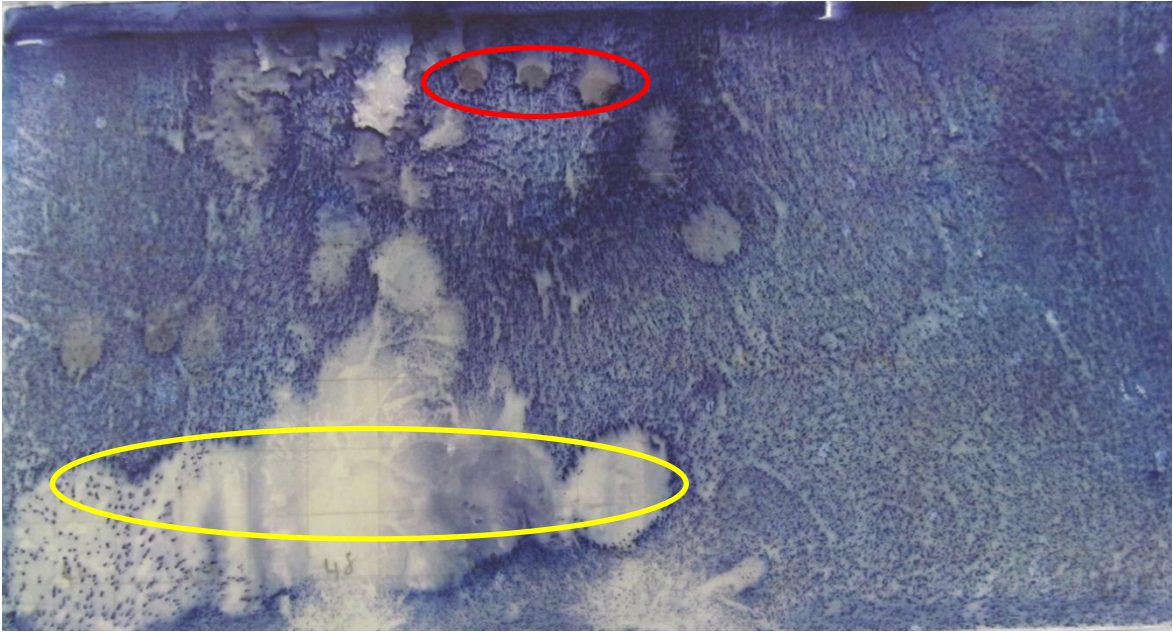


Figure 5.5 *Enterococcus faecalis* ATCC 29212 bioautography thin layer chromatography plate

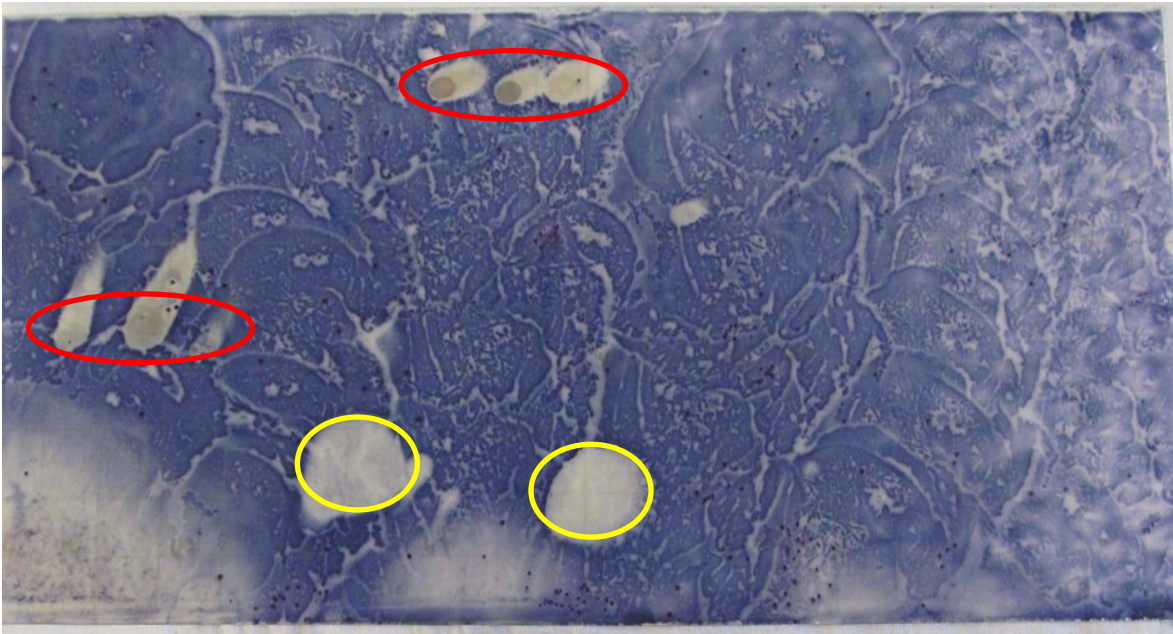


Figure 5.6 *Staphylococcus aureus* (subsp. *aureus*) ATCC 33591 bioautography thin layer chromatography plate

5.3.2 Minimum inhibition concentration (MIC) properties of whole, dehulled and hull BGNseed extracts

An MIC assay was conducted on BGN extracts that displayed positive activity via direct bioautography against all selected strains. The direct bioautography was completed first to eliminate the process of doing MIC on all extracts and their different extraction solvents. The BGN extracts that displayed antimicrobial activity against most strains was brown and red BGN hulls (Figures 5.1 to 5.6) each extracted with respective solvents, namely 70% methanol, 70% ethanol and milli-Q water.

The data was represented using optical density (OD) as an indicator for microbial growth (increased OD = increased growth), whereby comparisons were made from the control bacteria and bacteria inoculated with sample extract (Figures 5.1 – 5.6). Figures 5.7 and 5.8 represented the visual depiction of the minimum inhibition concentration plates used for OD reading. The purple colour (after the addition of MTT solution) indicated microbial growth within each well. However, most wells were purple which suggested an indication of growth but not to the degree of effective growth. Henceforth the OD reading was determined as an indicator for microbial growth within each well.

From Figure 5.9, *S. aureus* ATCC 33591 sample 6 (red BGN hull, milli-Q water) did not grow effectively hence it appeared that sample 6 was the most effective in reducing the growth of *S. aureus* ATCC 33591. The inconsistency of the results across the range could suggest a pipetting error. The red BGN hull extracted with 70% ethanol at 5 $\mu\text{g.mL}^{-1}$ exhibited the best reduction with an OD of 2.45 for *Staphylococcus aureus* ATCC 33591 (Figure 5.9) as well as a low standard deviation. Brown BGN hull extracted with 70% ethanol at 50 $\mu\text{g.mL}^{-1}$ was the second best result with an OD of 2.67 however the standard deviation varied substantially. These results compared favourably as the control OD for *S. aureus* ATCC 33591 was 3.17. Therefore, resulting in a standard deviation of $\pm 15.8\%$ (brown BGN hull, ethanol) to 22.7% (red BGN hull, ethanol) reduction of OD for *Staphylococcus aureus* ATCC 33591.

K. pneumoniae subsp. *pneumoniae* ATCC 700603 (Figure 5.10) control OD was 3.93. It was observed that sample 5 (red BGN hull, ethanol) had the lowest OD of 2.72 at 20 $\mu\text{g.mL}^{-1}$, resulting in a reduction of 30.8% of cells. However, similar results were not observed with concentrations higher than 20 $\mu\text{g.mL}^{-1}$ i.e. 50 $\mu\text{g.mL}^{-1}$ and 100 $\mu\text{g.mL}^{-1}$, suggesting that results aren't conclusive with an exception that a decrease in OD indicated that there was a certain degree of activity. Samples 1 – 5 (brown hull extracted 70% methanol, 70% ethanol, brown 70% and milli-Q water, and red hull extracted with 70% methanol and 70% ethanol) all had antimicrobial activity against *K. pneumoniae*

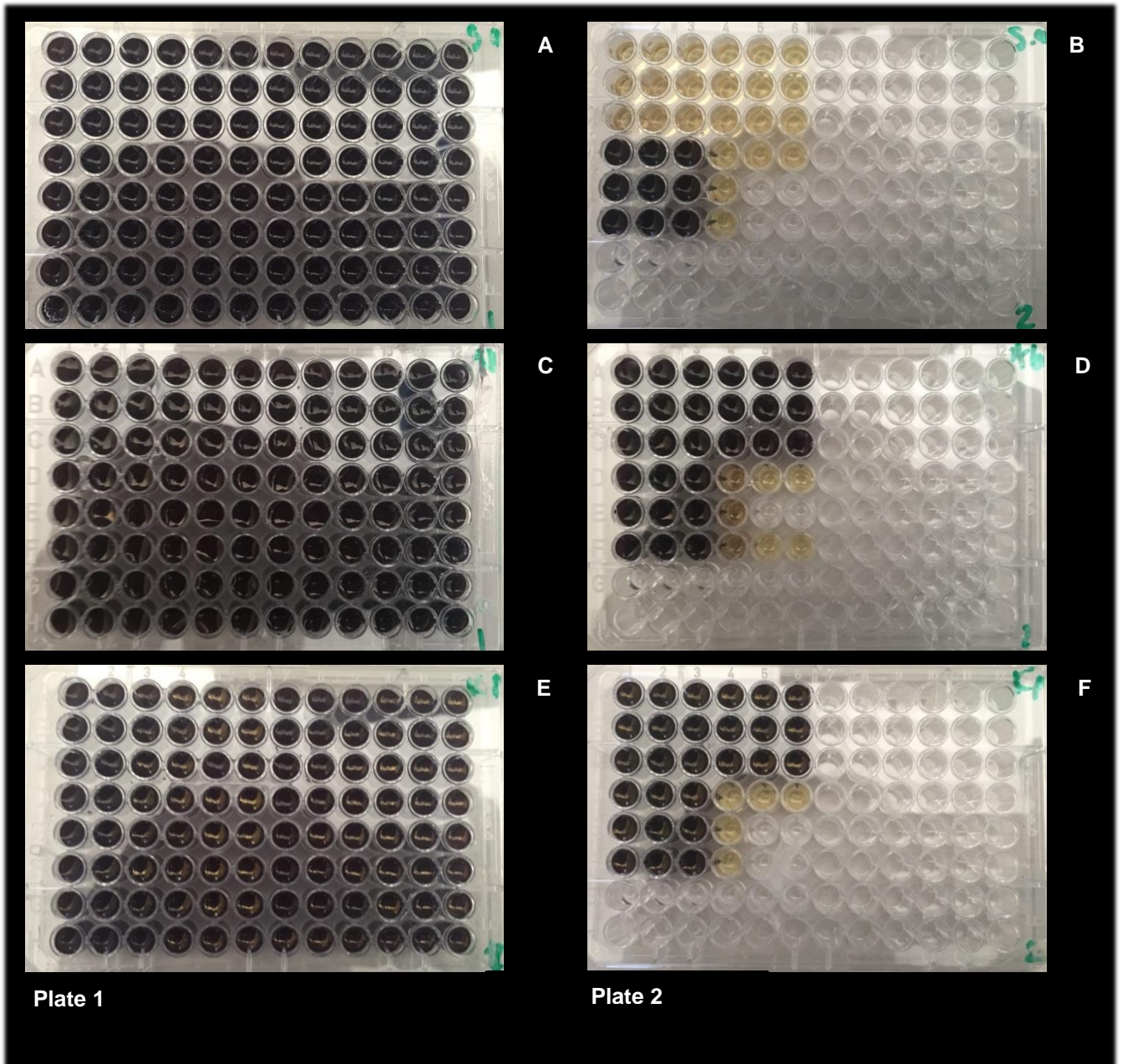


Figure 5.7 Minimum inhibition concentration (MIC) assay on BGN extracts for 3 strains. (A) *Staphylococcus aureus* (subsp. *aureus*) ATCC 33591 plate 1, (B) *Staphylococcus aureus* (subsp. *aureus*) ATCC 33591 plate 2, (C) *Acinetobacter baumannii* ATCC 19606^T plate 1, (D) *Acinetobacter baumannii* ATCC 19606^T plate 2, (E) *Klebsiella pneumoniae* (subsp. *Pneumoniae*) ATCC 700603 plate 1 & (F) *Klebsiella pneumoniae* (subsp. *Pneumoniae*) ATCC 700603 plate 2

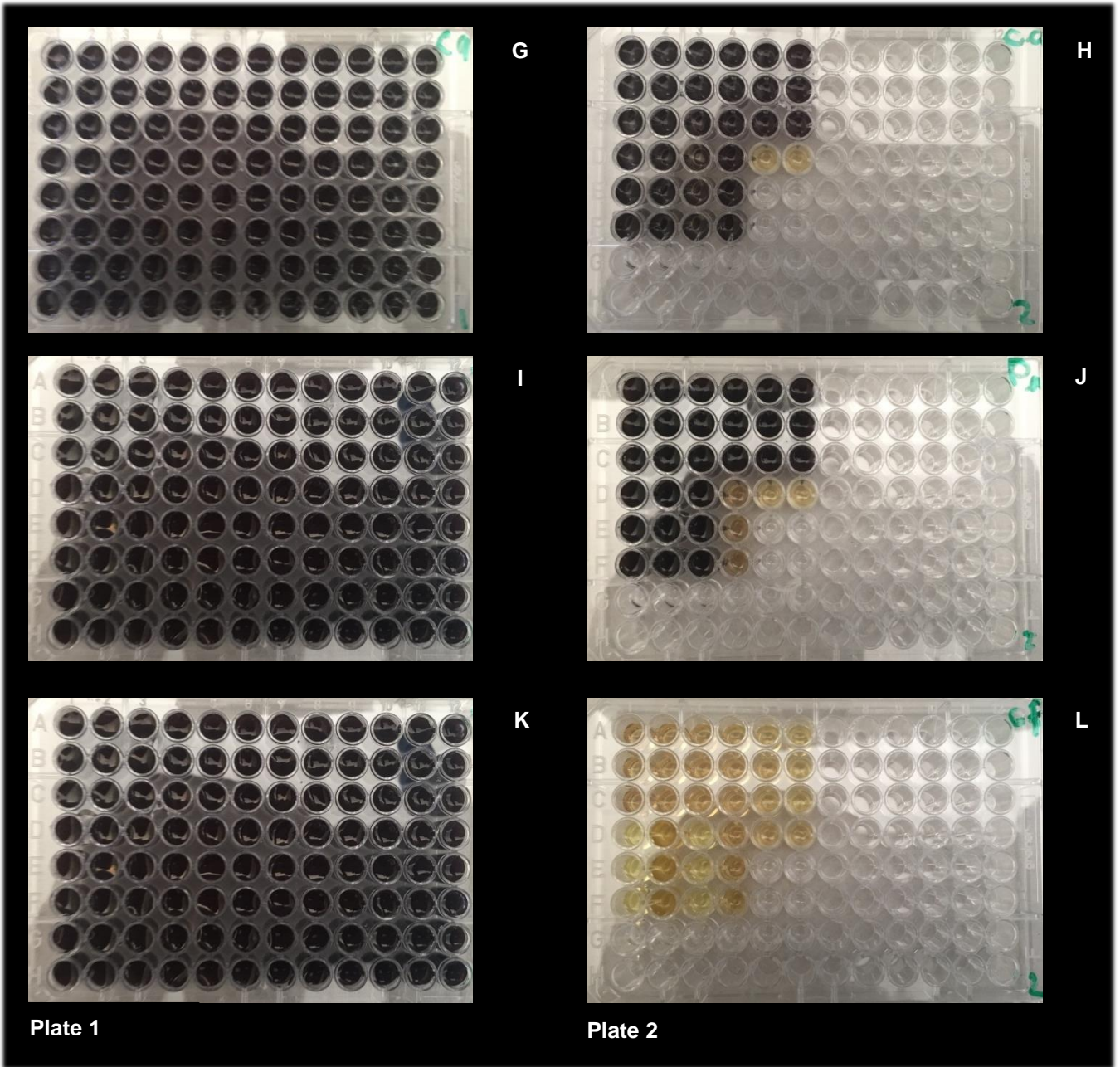


Figure 5.8 Minimum inhibition concentration (MIC) assay on BGN extracts for 3 strains. (G) *Candida albicans* ATCC 24433 plate 1, (H) *Candida albicans* ATCC 24433 plate 2, (I) *Pseudomonas aeruginosa* ATCC 27853 plate 1, (J) *Pseudomonas aeruginosa* ATCC 27853 plate 2, (K) *Enterococcus faecalis* ATCC 29212 plate 1 & *Enterococcus faecalis* ATCC 29212 plate 2

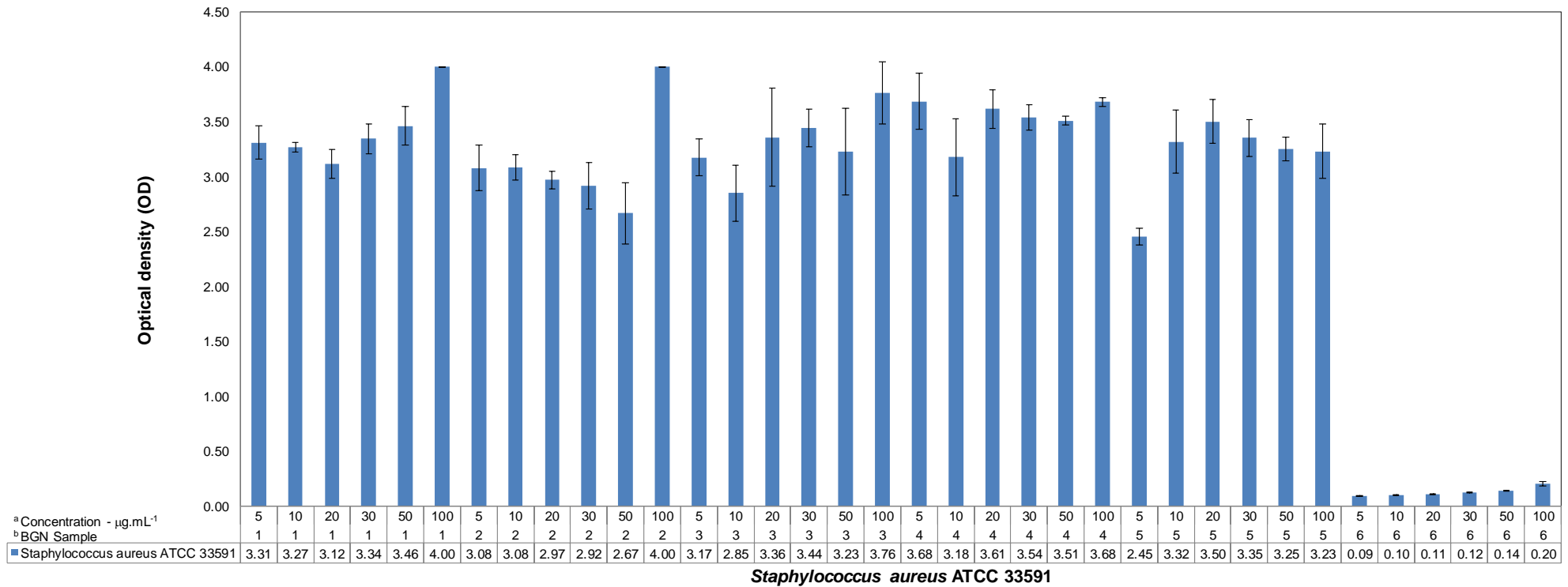


Figure 5.9 *Staphylococcus aureus* ATCC 33591 median optical density (OD) for all BGN sample extracts at various concentrations. ^aConcentration = µg.mL⁻¹ , ^bBGN sample 1 = brown hull methanol; 2 = brown hull ethanol; 3 = brown hull milli-Q water; 4 = red hull methanol; 5 = red hull ethanol; 6 = red hull milli-Q water

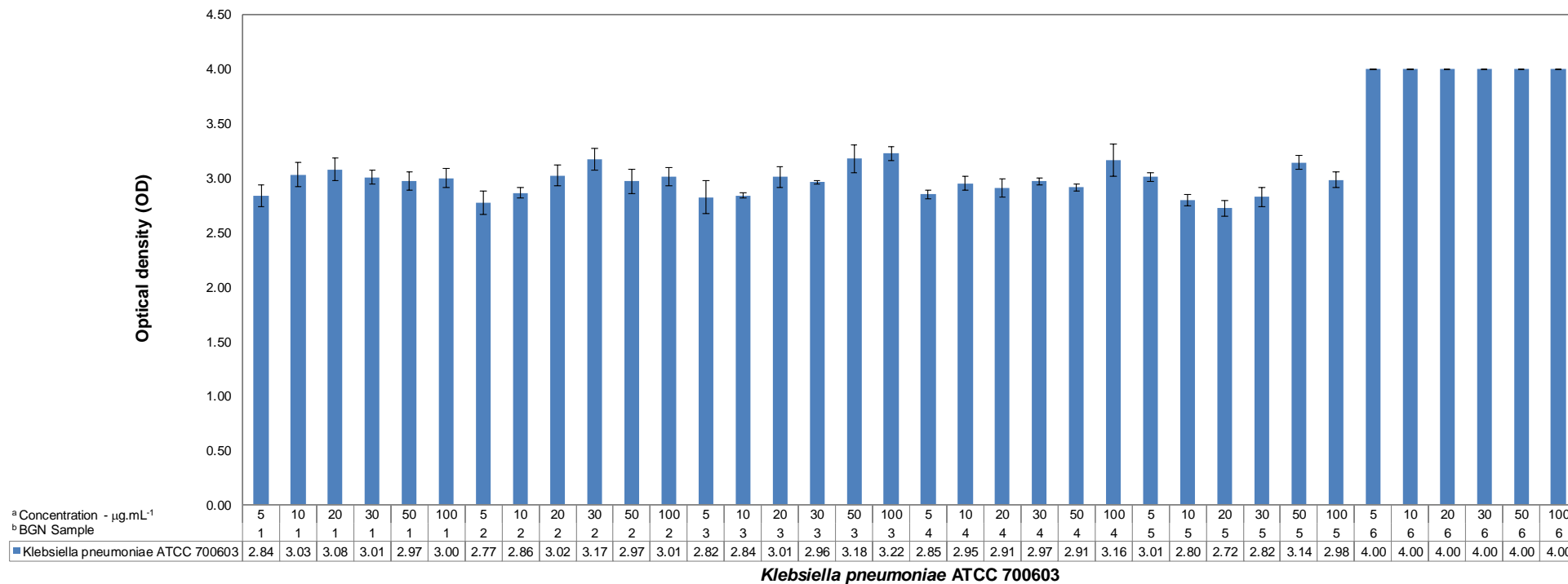


Figure 5.10 *Klebsiella pneumoniae* ATCC 700603 median optical density (OD) for all BGN sample extracts at various concentrations. ^aConcentration = µg.mL⁻¹ , ^bBGN sample 1 = brown hull methanol; 2 = brown hull ethanol; 3 = brown hull milli-Q water; 4 = red hull methanol; 5 = red hull ethanol; 6 = red hull milli-Q water

ATCC 700603 with all results being similar and having low standard deviations. As for sample 6 it appeared that it had no effect on *K. pneumoniae* ATCC 700603.

C. albicans ATCC 24433 (Figure 5.11) control OD was 2.26, with the maximum reading of 3.57 on Figure 3.11, indicating that the sample being tested has a growth enhancing effect on the microbial strain, this growth enhancing effect drastically decreased the OD of *C. albicans* ATCC 24433 by 50.0 – 55.8%. Sample 1 (brown BGN hull, methanol) had the lowest OD of 1.00 at 100 $\mu\text{g.mL}^{-1}$ and sample 2 (brown BGN hull, ethanol) an OD of 1.13 also at 100 $\mu\text{g.mL}^{-1}$. It was also noted that the higher the concentration of extracts the lower the OD, indicating reduction in cell growth and better antimicrobial activity. For *A. baumannii* ATCC 19606^T (Figure 5.12) there appeared to be no effect from either of the extracts as all results had an OD of 4.00 which also resembled the control OD of *A. baumannii* ATCC 19606^T.

P. aeruginosa ATCC 27853 (Figure 5.13) control OD was 3.92. Sample 5 (red BGN hull, ethanol) had the lowest OD of 2.20 at 30 $\mu\text{g.mL}^{-1}$, with a total OD reduction of 43.88%, however similar results were not observed for higher concentrations, therefore results aren't conclusive with the exception that a decrease in OD suggested that there was a certain degree of activity. As for sample 6 (red BGN hull, milli-Q water), similar to *S. aureus* ATCC 33591 it was noted that the bacterium did not grow effectively hence, it appeared that sample 6 was effective at reducing OD. However, this was not the case.

E. faecalis ATCC 29212 (Figure 5.14) had a growth problem therefore the control was 0.45 OD, indicating poor growth. Nonetheless, OD of samples of BGN extracts were compared to control OD. The bacterium did not grow effectively with sample 6 (red BGN hull, milli-Q water) hence, it again appeared that sample 6 was effective at reducing OD. However, this was not the case. Sample 2 (brown BGN hull, ethanol) had an OD of 0.26 and 0.32, respectively at 50 $\mu\text{g.mL}^{-1}$ and 100 $\mu\text{g.mL}^{-1}$, resulting in OD reduction of 28.9 – 42.2%.

As observed the common trend across all bacterium indicated that organic solvents extract of BGN hulls yielded the best reduction in microbial growth. These results and trend correlated well with phytochemical results, as the hulls had the highest concentration of phytochemicals, therefore contributing the best to antimicrobial activity (Kannan *et al.*, 2009; Weerakkody *et al.*, 2010; Celis *et al.*, 2011; Carraturo *et al.*, 2014; Klompong & Benjakul, 2015).

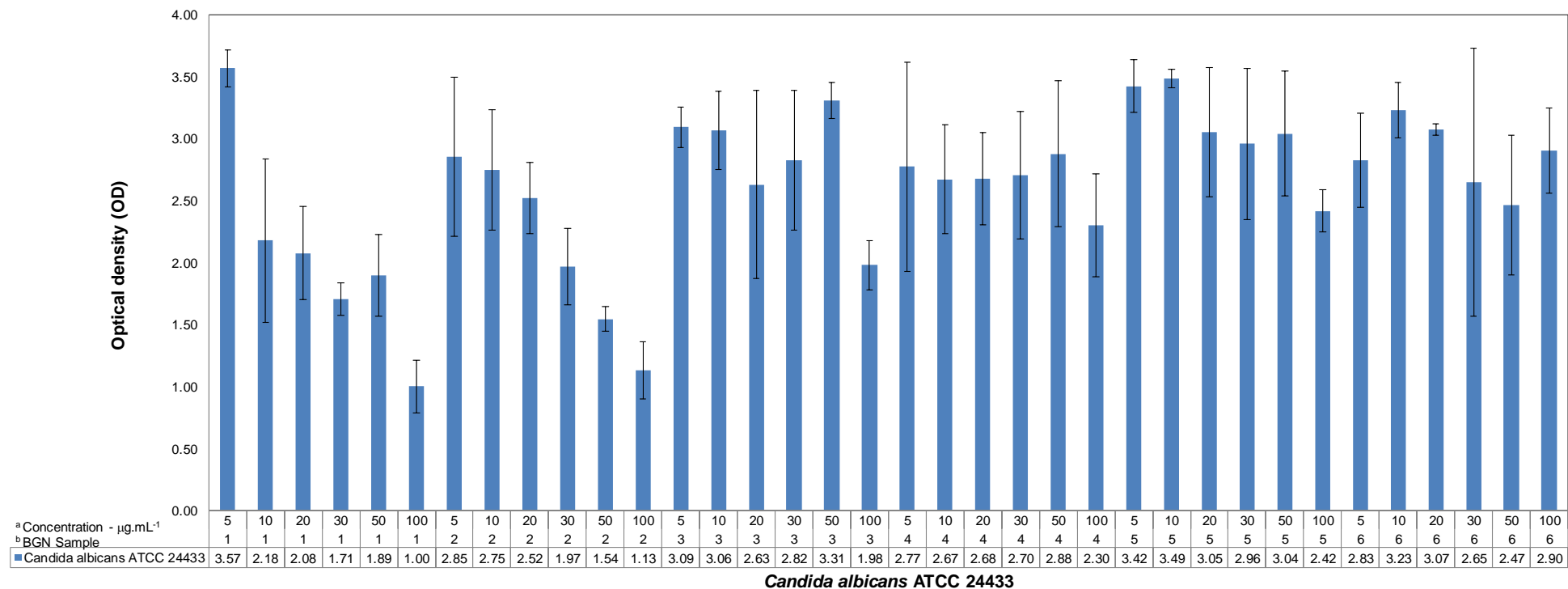


Figure 5.11 *Candida albicans* ATCC 24433 median optical density (OD) reading for all BGN sample extracts at various concentrations. ^aConcentration = $\mu\text{g.mL}^{-1}$, ^bBGN sample 1 = brown hull methanol; 2 = brown hull ethanol; 3 = brown hull milli-Q water; 4 = red hull methanol; 5 = red hull ethanol; 6 = red hull milli-Q water

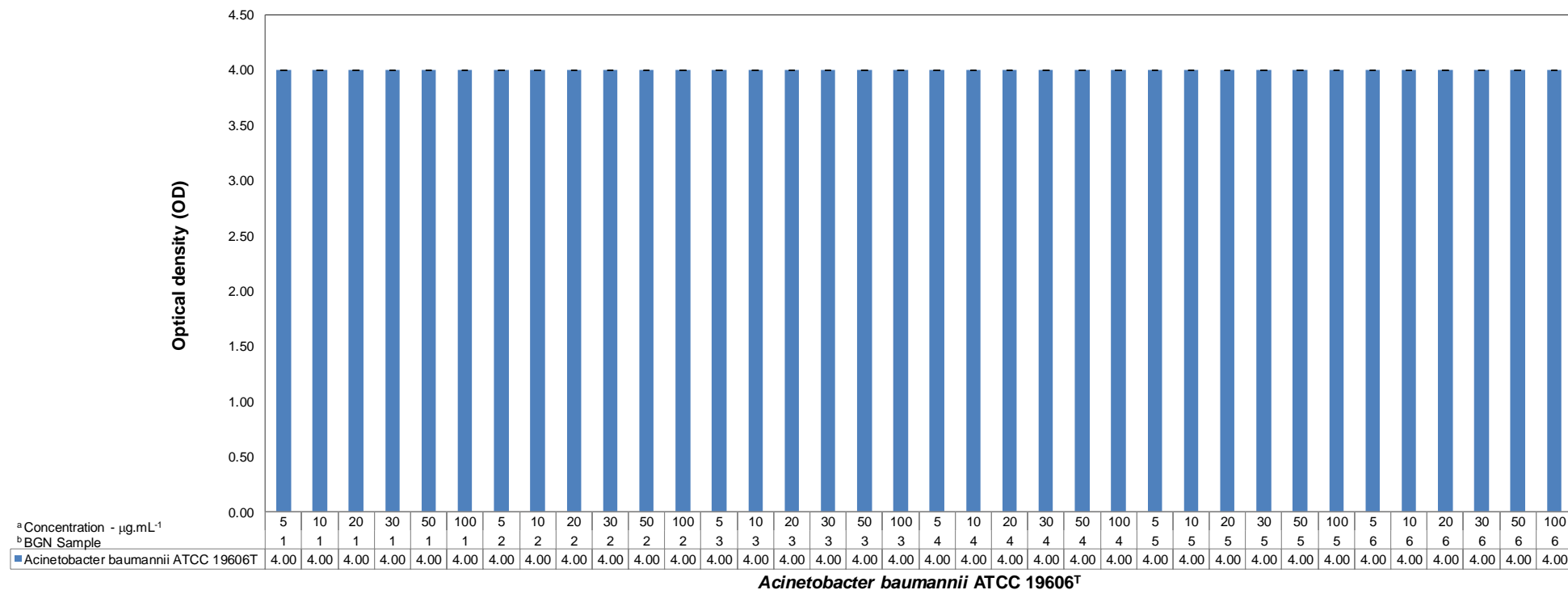


Figure 5.12 *Acinetobacter baumannii* ATCC 19606^T median optical density (OD) reading for all BGN sample extracts at various concentrations. ^aConcentration = $\mu\text{g.mL}^{-1}$, ^bBGN sample 1 = brown hull methanol; 2 = brown hull ethanol; 3 = brown hull milli-Q water; 4 = red hull methanol; 5 = red hull ethanol; 6 = red hull milli-Q water

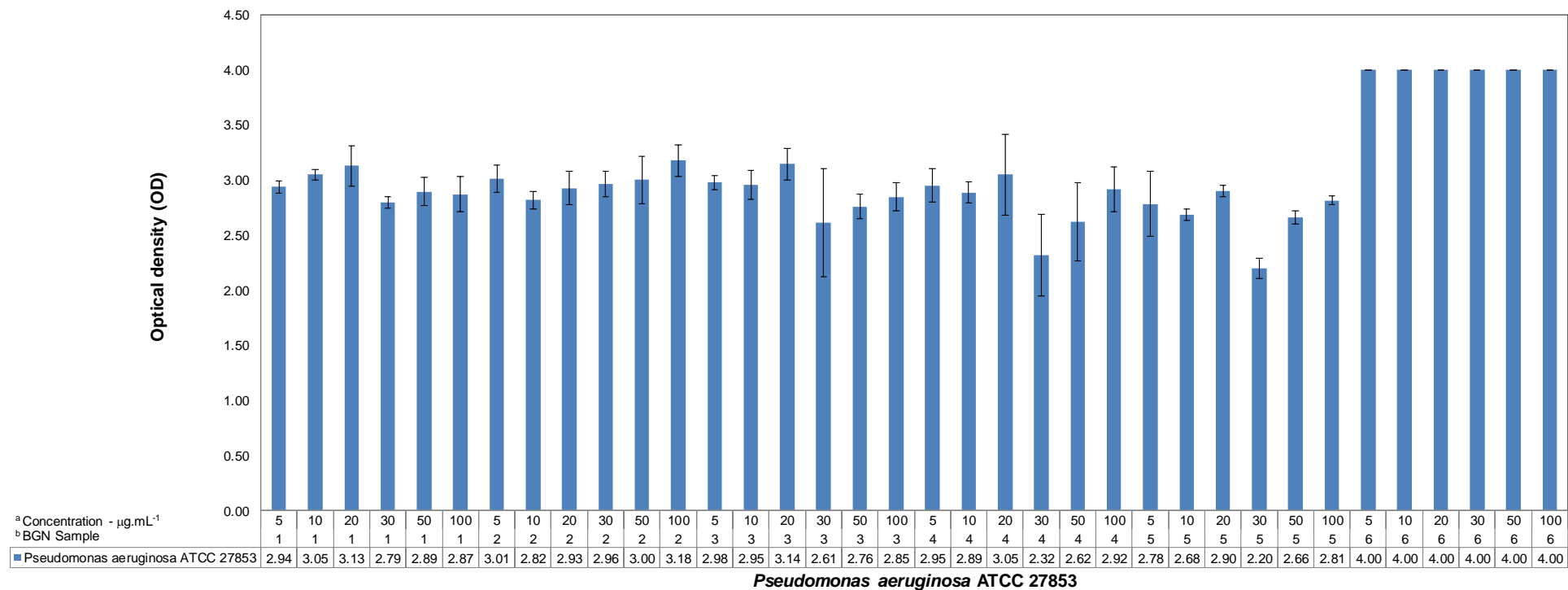


Figure 5.13 *Pseudomonas aeruginosa* ATCC 27853 median optical density (OD) reading for all BGN sample extracts at various concentrations. ^aConcentration = $\mu\text{g.mL}^{-1}$, ^bBGN sample 1 = brown hull methanol; 2 = brown hull ethanol; 3 = brown hull milli-Q water; 4 = red hull methanol; 5 = red hull ethanol; 6 = red hull milli-Q water

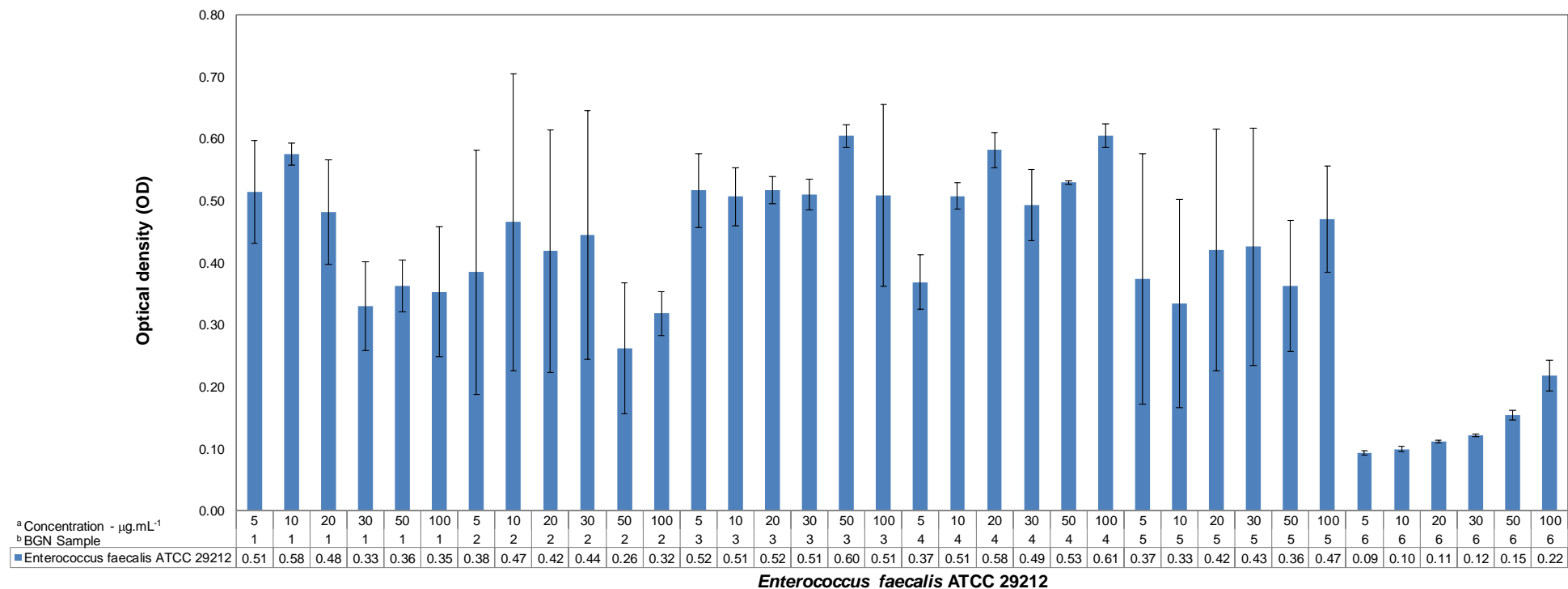


Figure 5.14 *Enterococcus faecalis* ATCC 29212 median optical density (OD) reading for all BGN sample extracts at different concentrations. ^aConcentration = µg.mL⁻¹, ^bBGN sample 1 = brown hull methanol; 2 = brown hull ethanol; 3 = brown hull milli-Q water; 4 = red hull methanol; 5 = red hull ethanol; 6 = red hull milli-Q water

5.3.3 Antimicrobial properties of whole and dehulled BGN products (milk and yoghurt) extracts

From Figures 5.15 – 5.20 certain areas are highlighted on the silica plate with red circles, displaying the area where extracts produced from BGN products were pipetted, while the yellow circles representing the zones of growth inhibition due to controls and antibiotics. There were no zones of inhibition around the red circles, indicating no antimicrobial activity of the extracts produced from BGN milk and yoghurt at the tested concentrations. Hence, it could be concluded that both whole and dehulled BGN products (milk and yoghurt) had no activity against any of the strains under the specific test conditions (*Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606^T, *Candida albicans* ATCC 24433, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* subsp. *aureus* ATCC 33591).

The phytochemicals found in whole and dehulled BGN products as seen from chapter 4, namely quercetin, rutin, myricetin, gallic acid and ellagic acid all have antimicrobial characteristics reported. A study by Hirai *et al.* (2010) investigated the antibacterial properties of quercetin, a flavonoid present in vegetables and fruits. Quercetin was tested against gram-positive and gram-negative bacteria and was reported to have antibacterial activity against *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), and *Staphylococcus epidermidis*. Some clinical MRSA strains showed remarkable susceptibility to quercetin. In combination with antibiotics, such as oxacillin, ampicillin, vancomycin, gentamicin, and erythromycin, quercetin showed markedly enhanced antibacterial activity against MRSA. Another study performed by Singh *et al.* (2008) showed that rutin from *Pteris vittata* L. exhibited potent activity against *B. cereus*, *P. aeruginosa* and *K. pneumoniae* with the MIC values of 0.03 mg/ml. Puupponen-Pimiä *et al.* (2001) reported that myricetin showed strong inhibitory effects on the growth of all LAB strains of human origin. *Lactobacillus plantarum*, a beer isolate, was resistant to myricetin at all test concentrations, as well as to all other pure phenolic compounds. Also, growth of *E. coli* strains was inhibited by myricetin. Myricetin seemed to retard the growth of both *E. faecalis* and *B. lactis*, although no clear-cut inhibition zones in the agar diffusion assay were detected (Puupponen-Pimiä *et al.*, 2001).

Furthermore, it was reported by Fu *et al.* (2016) that gallic acid could restrain the growth of many bacteria, including methicillin-sensitive *S. aureus*, MRSA, *E. coli*, *P. aeruginosa*, and *Salmonella typhi*. Lastly, ellagic acid showed antimicrobial activity against *Staphylococcus epidermatis*, *Bacillus cereus*, *Klebsiella pneumonia* and *Salmonella typhi* (Ghudhaib *et al.*, 2010).

Therefore, the phytochemical compounds present in the whole and dehulled BGN milk and yoghurt indeed have antimicrobial activity properties. However, according to results seen in Figures 5.15 – 5.20 with the methodology of direct bioautography it is not the case. It

could therefore be argued that, either the known phytochemical concentration present in the whole and dehulled BGN milk and yoghurt is not sufficient to exhibit antimicrobial properties or the antimicrobial activity methodology itself may possibly be hindering the results. Potential reason for this is that direct bioautography standardizes the viable microbial cells to a cell count of 0.5 OD (optical density) using spectrophotometry, which equates to $\pm 10^8$ CFU/mL (McFarland standard). Whereas disc diffusion method either uses 10^6 CFU/mL or 10^8 CFU/mL. Considering the lower CFU/mL used in disc diffusion method, that if microbial cells were less, would permit better potential for the whole and dehulled BGN milk and yoghurt extracts to inhibit the microorganisms.

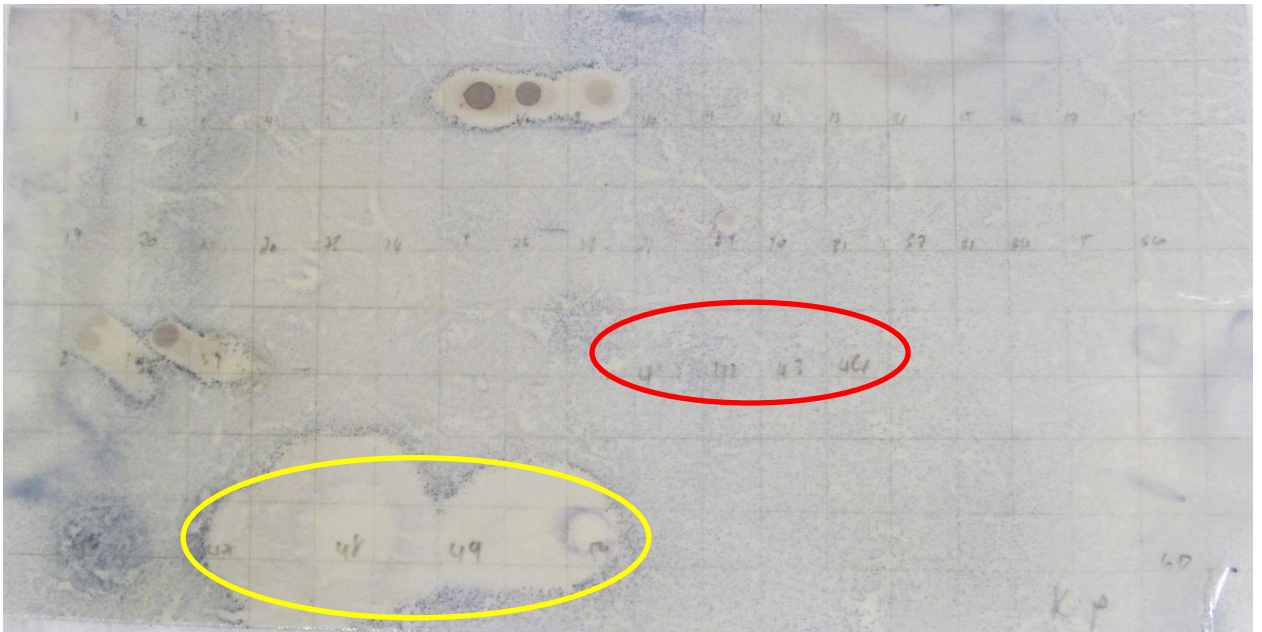


Figure 5.15 *Klebsiella pneumoniae* subsp. *Pneumoniae* ATCC 700603 bioautography thin layer chromatography plate

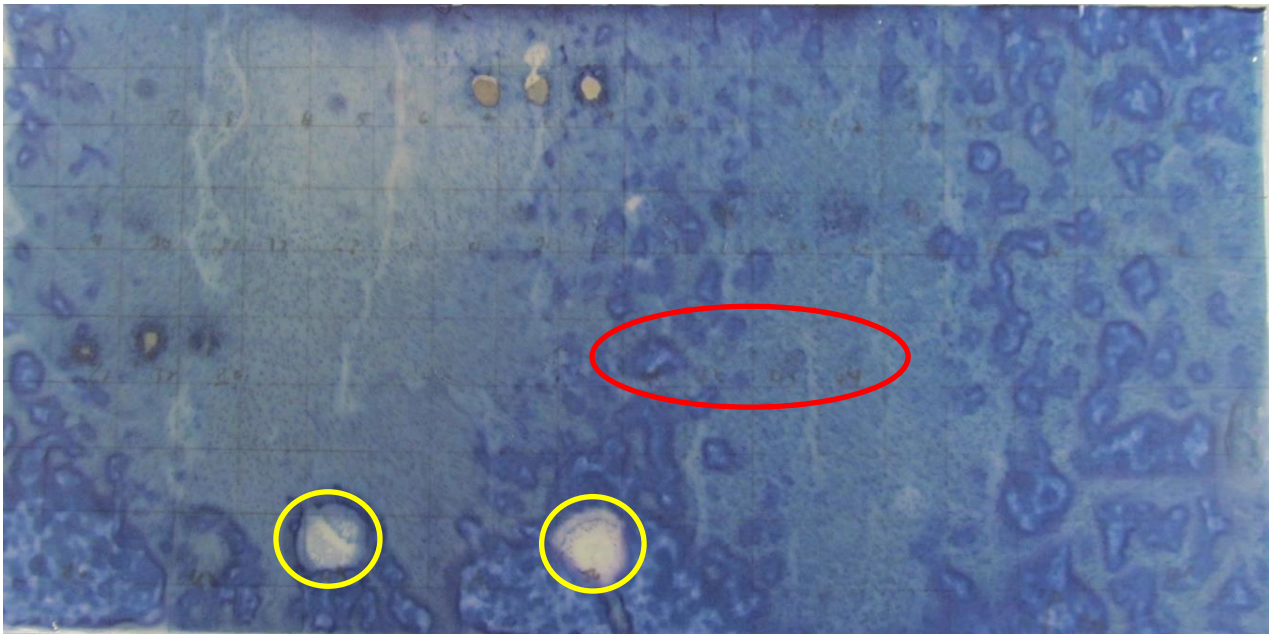


Figure 5.16 *Pseudomonas aeruginosa* ATCC 27853 bioautography thin layer chromatography plate

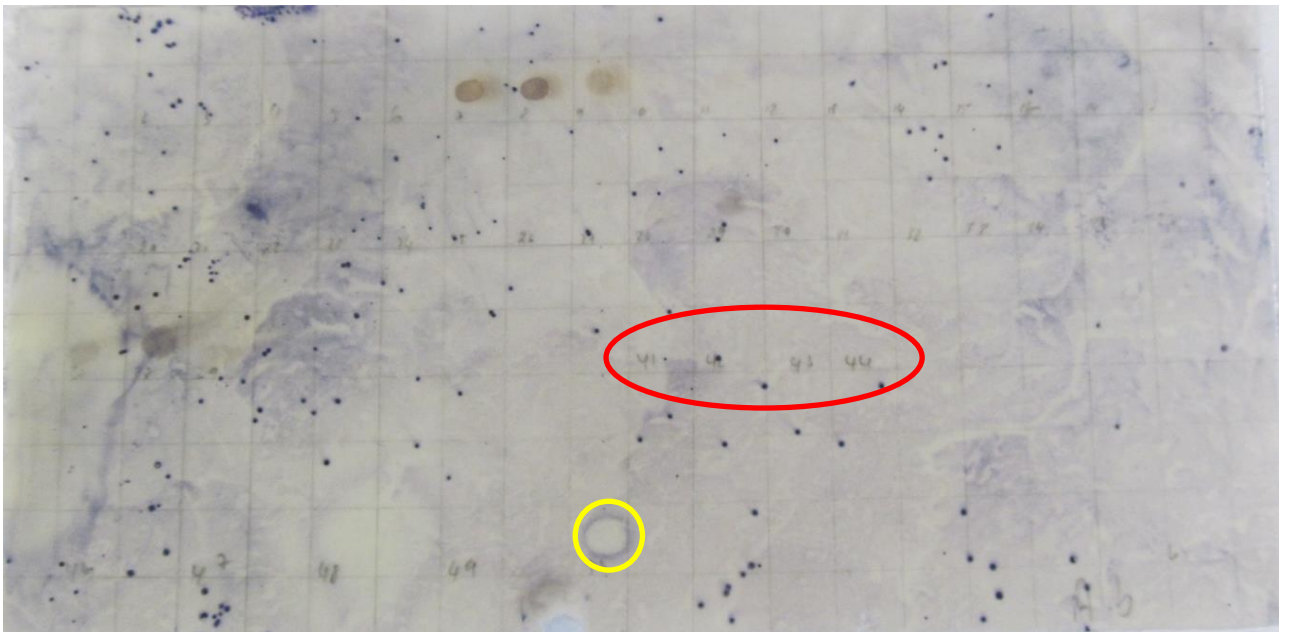


Figure 5.17 *Acinetobacter baumannii* ATCC 19606^T bioautography thin layer chromatography plate

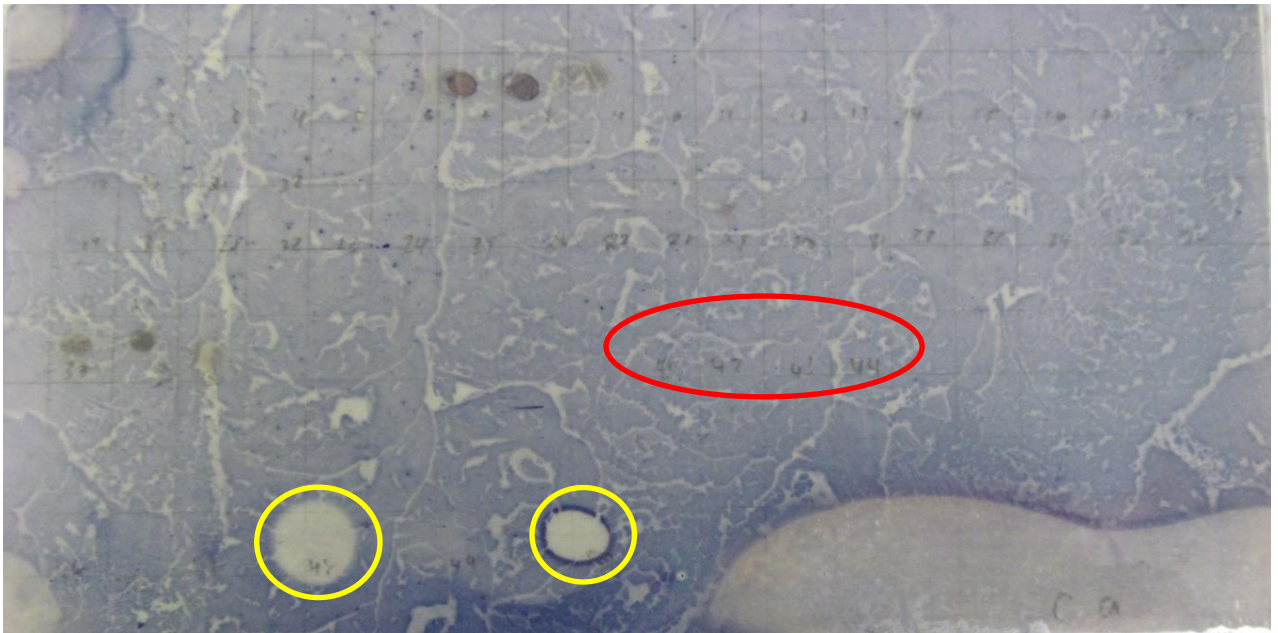


Figure 5.18 *Candida albicans* ATCC 24433 bioautography thin layer chromatography plate

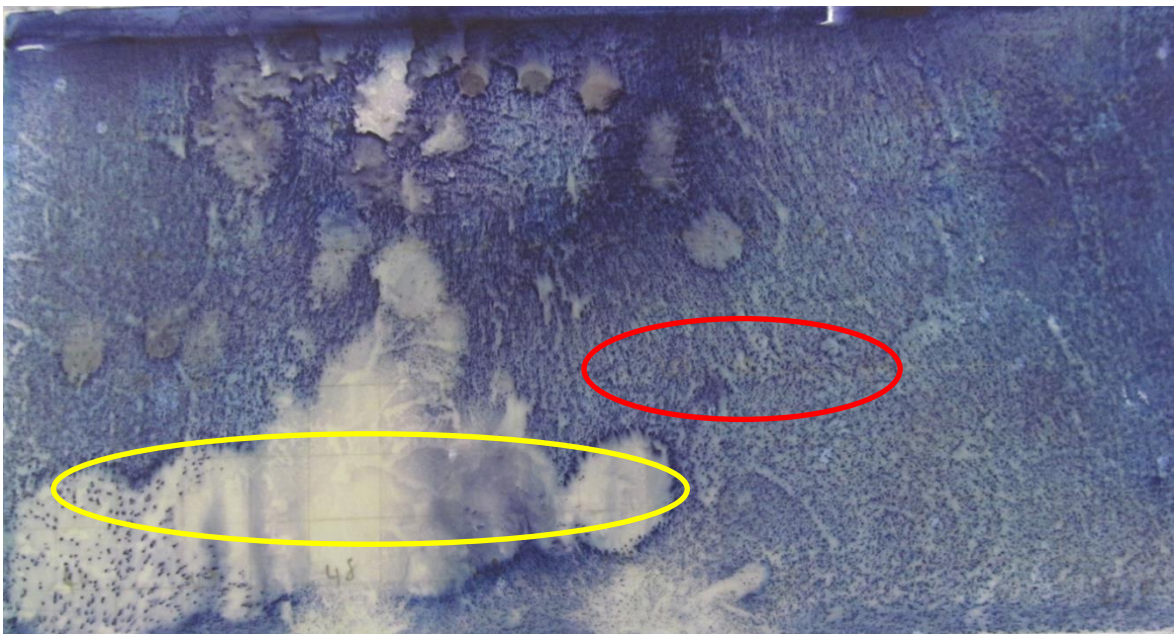


Figure 5.19 *Enterococcus faecalis* ATCC 29212 bioautography thin layer chromatography plate

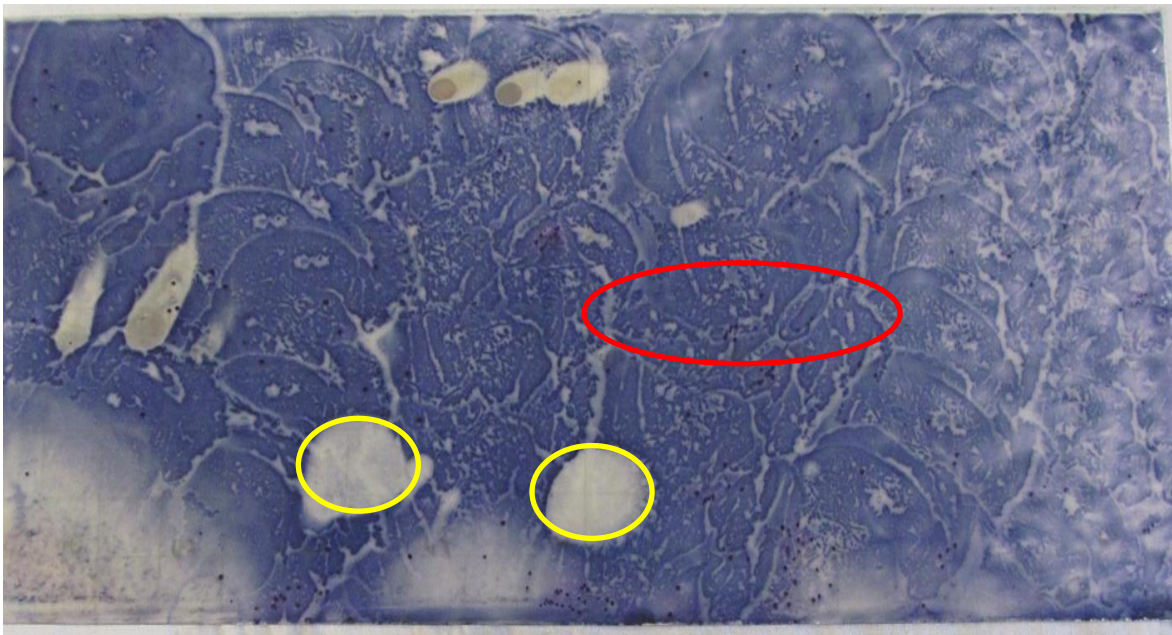


Figure 5.20 *Staphylococcus aureus* subsp. *aureus* ATCC 33591 bioautography thin layer chromatography plate

5.4 Conclusion

The phytochemicals in the BGN seeds, milk and yoghurt extracts as seen in Chapter 3 and 4 all have antimicrobial characteristics. The extracts with the highest cell reduction of microorganisms were found in the BGN hulls, as it decreased the cell count for *Candida albicans* ATCC 24433 by 55.8% (brown hull, 70% methanol) at $100 \mu\text{g.mL}^{-1}$, *P. aeruginosa* ATCC 27853 cell reduction of 43.9% (red hull, 70% ethanol) at $30 \mu\text{g.mL}^{-1}$, *E. faecalis* ATCC 29212 cell reduction of 42.9% (brown hull, 70% ethanol) at $50 \mu\text{g.mL}^{-1}$, *S. aureus* subsp. *aureus* ATCC 33591 cell reduction of 22.7% (red hull, 70% ethanol) at $5 \mu\text{g.mL}^{-1}$ while *K. pneumoniae* subsp. *pneumoniae* ATCC 700603 a cell reduction of 30.8% (red hull, 70% ethanol) at $20 \mu\text{g.mL}^{-1}$. No activity was observed against *A. baumannii* ATCC 19606^T under the tested conditions. However, antimicrobial activity was not exhibited in extracts from BGN milk and yoghurt, potentially suggesting that the concentration of phytochemicals was lower than the raw BGN. The high concentration of phytochemicals in the BGN hulls correlates well with the direct bioautography results, as the hulls were the only extracts sufficient to reduce microorganism cells. It can be concluded that formulating products with high concentration of BGN hulls in the product will result with high phytochemical content and antimicrobial resistance.

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CHAPTER 6

GENERAL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 General Summary and Conclusion

The aim of this study was to characterize the phytochemicals and antimicrobial characteristics of BGN (Mpumalanga province, South Africa) seed extracts and its products (BGN milk and BGN yoghurt). This was achieved by characterising the phytochemical properties of each BGN seed extracts and BGN product extracts and determine whether the phytochemicals present in the extracts had antimicrobial properties.

The first objective of the study was to identify some phytochemicals in five BGN seeds (black, black-eye, brown, brown-eye and red) based on respective extraction solvent (70% ethanol, 70% methanol and milli-Q water). For the extracts produced from BGN flour, the hulls were the optimum source of flavonoids and phenols. However, the solvents varied as each phytochemical extraction depended on other characteristics such as BGN colour, form of BGN, solvent polarity, extraction time and solvent type. Flavonoids and phenols were highly concentrated in the red and brown hulls of BGN compared to whole and dehulled BGN. Organic solvents (70% ethanol and 70% methanol) in comparison to water yielded the highest concentration of flavonoids. It was observed that the form of the BGN (whole, dehulled and hull), solvent (70% methanol, 70% ethanol and milli-Q water) and colour of the BGN were all important factors, to optimize the best extraction yield of phytochemicals. GC-MS analysis of BGN seeds showed the presence of alkaloid compounds with potential to act as antimicrobial agents and antioxidants.

The first hypothesis predicted that BGN seeds, milk and yoghurt extracts will contain some phytochemicals and will exhibit anti-microbial activity. This hypothesis was only partially met or true, as both BGN seed and products (milk and yoghurt) extracts contained some phytochemicals, however, only BGN seed extracts exhibited antimicrobial properties. This could be linked to thermal degradation of certain phytochemicals with antimicrobial properties caused by pasteurization of BGN milk, as the methodology of producing BGN milk required heat processing of BGN seeds. The importance of pasteurizing BGN milk ensured that the product will be safe for human consumption. The BGN yoghurt therefore used this milk as a base and is inoculated with yoghurt cultures to produce BGN yoghurt.

The second objective was to identify some phytochemicals in BGN milk and yoghurt based on extraction solvent. Certain flavonoids (rutin and quercetin) in BGN milk would yield higher in the yoghurt and the inverse would be for BGN yoghurt. This was linked to enzymatic conversion of flavonoids rutin to quercetin by *Lactobacillus bulgaricus* as it could account for the decrease in rutin and increase in quercetin within BGN milk and yoghurt. To ensure higher concentration of vitamin B₁ and B₂ in products, higher concentration of black-

eye BGN flour should be considered for formulating or re-formulating BGN derived products, as black-eye BGN had the highest concentration of vitamins B₁ and B₂. As for BGN milk and yoghurt, further optimization is required on product formulation to produce a product with the best phenolic compound yield, whereby possibly looking at using whole BGN flour in the products instead of dehulled BGN flour as it contains the hulls, which as observed, have the highest source of phytochemicals.

The second hypothesis predicted that the different solvents will differ in the phytochemicals extracted and their antimicrobial activity. This was observed as organic solvents (70% ethanol and 70% methanol) extracts had the highest flavonoid concentration, whereas milli-Q water extracts had the highest concentration of phenols and vitamin B₁ and B₂. With regards to antimicrobial activity, organic solvent extracts of BGN seeds had antimicrobial activity while milli-Q water extracts had no activity. Hence, this hypothesis was accepted according to objectives, and thus entirely met.

The third objective was to determine the antimicrobial potential of each BGN seed (black, black-eye, brown, brown-eye and red) based on extraction solvent. Red and brown BGN extracts from organic solvents (70% ethanol and 70% methanol) showed the highest antimicrobial activity due to the large number of phenolic compounds being extracted, as seen with flavonoids and phenols. Antimicrobial activity of these extracts produced from BGN hulls had a cell reduction ranging between 0.00 – 55.75%. The extracts with the highest cell reduction of microorganisms were found in the BGN hulls, as it decreased the cell count for *Candida albicans* ATCC 24433 by 55.8% (brown hull, 70% methanol) at 100 µg.mL⁻¹, *P. aeruginosa* ATCC 27853 cell reduction of 43.9% (red hull, 70% ethanol) at 30 µg.mL⁻¹, *E. faecalis* ATCC 29212 cell reduction of 42.9% (brown hull, 70% ethanol) at 50 µg.mL⁻¹, *S. aureus* subsp. *aureus* ATCC 33591 cell reduction of 22.7% (red hull, 70% ethanol) at 5 µg.mL⁻¹ while *K. pneumoniae* subsp. *pneumoniae* ATCC 700603 a cell reduction of 30.8% (red hull, 70% ethanol) at 20 µg.mL⁻¹.

The fourth and final objective was to determine the antimicrobial potential of BGN milk and BGN yoghurt. As for antimicrobial activity of extracts from BGN products, no activity was observed against the selected microorganism strains under the conditions tested. This was possibly due to the high concentration of colony forming units (CFU/mL) of the tested microorganism, as the bioautography test conditions requires an optical density (OD) of 0.5 which is approximately 1.5 x 10⁸ CFU/mL according to McFarland Standards (Sutton, 2011; Balouiri, 2016).

The final hypothesis predicted that BGN seeds will differ in their phytochemicals and antimicrobial activity. Red and brown BGN colours had the highest concentration of phytochemicals and were the only BGN seeds that exhibited antimicrobial properties. Therefore, this hypothesis was accepted.

To conclude, all objectives and most hypotheses of the study were met, as there were indeed phytochemicals present in BGN seeds, milk and yoghurt from Mpumalanga origin that displayed antimicrobial activity. The following conclusions can thus be made from this study:

1. Flavonoid (quercetin, kaempferol, rutin and myricetin) and phenols (gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid) compounds detected in BGN and BGN products have beneficial properties that could contribute to human health.
2. From all BGN colours, black-eye BGN variant had the highest thiamine and riboflavin content.
3. Red and brown BGN hulls had the highest antimicrobial activity for the tested microorganism strains (*Candida albicans* ATCC 24433, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *S. aureus* subsp. *aureus* ATCC 33591, *K. pneumoniae* subsp. *pneumoniae* ATCC 700603).
4. BGN has the potential to not only improve food security in many rural areas as a low-cost protein source but also provide health benefits, therefore making it a functional food.

6.2 Recommendations

It is recommended that further work be done to optimize extraction methodology of phytochemicals on BGN flour and BGN products. During the preparation of extracts considerations should be given with regards to increasing extraction time and increasing extraction temperature (Klompong, 2015). Finally, for BGN products currently developed or potential future BGN products, it is advised to consider retaining the hulls in the formulations as it has the highest concentration of phenolic compounds compared to dehulled BGN as seen in the present study, thereby insuring health benefits of these phytochemicals in BGN products.

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