DEVELOPMENT AND CHARACTERIZATION OF ANTIOXIDANT-RICH HASKAP-BASED (*LONICERA CAERULEA* L.) FUNCTIONAL BEVERAGES

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Memorial University of Newfoundland, Grenfell Campus

Corner Brook, Newfoundland, Canada

by

Beverley Reid

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ABSTRACT

Haskap (*Lonicera caerulea* L.) exhibits tremendous potential as a functional dietary constituent, owing to its robust phytochemical composition. The current investigation was carried out to develop a novel technique using sous vide for optimal extraction of phytochemicals from haskap, and subsequent incorporation of these antioxidant-rich extracts into four functional beverages. The beverages were formulated using different combinations of haskap, sorrel (*Hibiscus sabdariffa*), and ginger (*Zingiber officinale*) root extracts. A sensory analysis was conducted on the beverages, which were then characterized for their bioactive compounds, and their total antioxidant activities were measured using ferric reducing antioxidant power (FRAP) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. The sous vide approach showed optimal yield at 40 °C for 60 min using ethanol: water (1:1) solvent. This technique yielded significantly ($p < 0.05$) more anthocyanin and showed higher antioxidant activity than the conventional extraction. Furthermore, haskap with sorrel and ginger showed the highest antioxidant activity of the four beverages, with 369.41 ± 1.70 μmol TE 100 mL^{-1}, and 298.96 ± 1.21 μmol TE 100 mL^{-1} for ABTS and FRAP respectively. Haskap with sorrel and haskap with sorrel and ginger beverages had the highest consumer preference, with bioactive components comparable to marketed functional beverages. These products demonstrate great potential as commercially viable functional beverages. Additionally, the utilization of the sous vide technology is an excellent method for extracting bioactive compounds from berries.

Keywords: Phytochemicals, Sous vide, Conventional extraction, Bioactive compounds, Antioxidant activity

GENERAL SUMMARY

Haskap (*Lonicera caerulea* L.) contains phytochemicals comparable to superfruits such as blueberries (*Vaccinium angustifolium* Ait) and blackberries (*Rubus fruticosus*). In this experiment, a novel sous vide technique was developed for the extraction of antioxidant-rich compounds from haskap berries. This technique showed a higher yield of bioactive compounds compared to extracts from the cold extraction method. The haskap extract created using sous vide, was further incorporated with sorrel and ginger extracts in the development of four antioxidant-rich functional beverages. A survey was then conducted to determine consumers' preferences regarding their sensory attributes, and the beverages analyzed to determine polyphenolic content and antioxidant activity. The haskap with sorrel was preferred for its appearance while haskap with sorrel and ginger, highly ranked due to its aroma and taste, had superior antioxidant activity and total flavonoid content. Haskap with sorrel and haskap with sorrel and ginger, prepared using sous vide extraction, show great potential as marketable functional beverages.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percentage(s)
°C	Degree Celsius
AGE	Advanced glycation end products
Ca	Calcium
CAT	Catalase
cv.	Cultivar
SOD1	Superoxide dismutase
GPX1	Glutathione peroxidase 1
GSS	Glutathione synthetase
HMOX1	Heme oxygenase 1
CPEM	Cold plasma-assisted enzyme method
dH_2O	Deionized water
DPPH	2,2-diphenyl-1-picryl-hydrazl-hydrate
Fe	Iron
FW	Fresh weight
LAA	Lipophilic antioxidant activity
HAA	Hydrophilic antioxidant activity
TAA	Total antioxidant activity
g	Grams
hr	Hour
HUVEC	Human umbilical vein endothelial cells
K	Potassium
L	Liters
m	Meters
Mg	Magnesium
$scCO_2$	Supercritical carbon dioxide
mg L^{-1}	Milligram per liter

min	Minute
mL	Milliliters
mm	Millimeters
MS	Mass spectrometer
ORAC	Oxygen radical absorbance capacity
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
FRAP	Ferric reducing antioxidant potential
QE	Quercetin
PCA	Protocatechuic acid
PGA	Phloroglucinaldehyde
TE	Trolox equivalent
GAE	Gallic acid equivalent
µl	Microliter

CHAPTER 1
INTRODUCTION

1.0 THESIS OVERVIEW

The role of oxidative stress in the pathogenesis and progression of various diseases inside the human body is of notable significance (Dalle-Donne et al., 2006). Conversely, fruits and vegetables are abundant in natural antioxidants which possess the ability to prevent and alleviate the adverse effects of oxidative stress on the human body (Hooda et al., 2023).

Haskap (*Lonicera caerulea* L.), commonly known as the Japanese blue honeysuckle, is a member of the Caprifoliaceae family, within the Dipsacales order (Gołba et al., 2020; Wu and Hou, 2020). The medicinal benefits of haskap have been explored by the people of Russia and Northeastern China for centuries and is considered "the elixir of life" by the Japanese Ainu aboriginal people (Plekhanova, 1999; Thompson, 2006; Rupasinghe et al., 2018). Haskap berries contain substantial amounts of physiologically active phytochemicals, including flavonoids, and other phenolic compounds that can help in the prevention of chronic diseases such as diabetes, heart disease and cancer (Rupasinghe et al., 2012).

The polyphenolic content of some haskap cultivars has been found to surpass that of known superfruits cultivated in Canada like strawberry (*Fragaria* × *ananassa*), blackberry *(Rubus fruticosus*), and blueberry (*Vaccinium angustifolium* Ait.) (Khattab et al., 2016). One remarkable attribute of haskap is its high anthocyanin content, measuring five times the anthocyanin content recorded for black raspberry (*Rubus occidentalis*), which is known for its high anthocyanin content (Bakowska-Barczak et al., 2007). Cyanidin-3-glucoside (C3G) is the most prevalent class of anthocyanin present in haskap (Khattab et al., 2016). Numerous *in vivo* studies have been conducted on C3G and have found that it has great potential as a neuroprotectant, anti-inflammatory, and antioxidative agent (Sukprasansap et al., 2020; Jung et al., 2014). Additionally,

haskap has been shown to exhibit the most potent antioxidant activity of berries such as golden currant (*Ribes aureum* Pursh), chokecherries (*Prunus virginiana*), lingonberries (*Vaccinium vitis-idaea*), and crowberries (*Empetrum nigrum* L.) (Bakowska-Barczak et al., 2007). Several research have demonstrated the biological properties in haskap, including anti-inflammatory, antiproliferative, antimicrobial, antidiabetic, and antioxidant effects (Jin et al., 2006; Wang et al., 2017a; Amararathna et al., 2020; De Silva, 2020; Yemiş et al., 2022).

Haskap is presently commercially promoted in various forms such as jam, jelly, wine, candies, gelatin, and puffed snacks (Liu et al., 2009). Due to the high antioxidant content of haskap, it has often been recommended for use as a functional food ingredient (Celli et al., 2014; Grobelna et al., 2020). Functional foods are defined as food items that possess the potential to confer health benefits beyond their basic nutritional value (Corbo et al., 2014). They aid in promoting optimal health conditions and are significant for fighting and alleviating lifestyle-related diseases and disorders (Granato et al., 2017; Khalaf et al., 2021). The fundamental components that confer these health benefits are ascribed to their bioactive compounds, such as their phytochemicals (Maqsood et al., 2020). These compounds may be naturally occurring, produced during processing, or acquired from external sources and integrated into the food item (Butnariu and Sarac, 2019).

The quality of the extract obtained from the fruit is usually a factor of the extraction method used. According to Castro-López et al. (2017), the composition and efficacy of an extract are based on the extraction method used. Additionally, the yield and quantity of polyphenols in the extracts obtained are based on various factors, which include the extract type, temperature, and time duration of the extraction (Da Silva et al., 2016). This is because the processing of some bioactive substances such as anthocyanin and vitamin C can cause chemical oxidation and thermal degradation thus altering the composition, function, and bioavailability of these antioxidants

(Nicoli et al., 1999; Rawson et al., 2011). Sous vide technology, however, utilizes sealed plastic bags at a precisely regulated temperature that facilitates efficient transfer of heat from the water to the food (Singh et al., 2023). Thus, the technique provides a greater degree of control which helps to prevent nutrient loss by oxidation and thermal processing compared to conventional cooking techniques (Pandita et al., 2023).

Increasing awareness of the correlation between dietary patterns and various health conditions has led to a notable surge in consumer demand for functional foods and nutraceuticals (Saxena et al., 2013). Methods used for the extraction of the bioactive compounds from fruits however are often time-consuming, use high energy consumption, and elevated temperatures, which can lead to loss of thermolabile bioactive compounds (Ameer et al., 2017; Soquetta et al., 2018). Therefore, it is necessary to develop methods for efficient and reliable extraction of phytochemicals from fruits to enhance the functionality of such products (Bennett et al., 2011).

1.1 Research gap

The incorporation of haskap berries as a novel functional food ingredient could have a positive impact on the health and well-being of consumers considering its potent bioactive properties. Haskap-based functional foods are a promising addition to the thriving functional food and nutraceutical market. There is however no published work to date, of haskap berry being integrated into a functional food formula and whether haskap can retain its bioactive and physiochemical composition when processed into a functional beverage. The sensory attributes and consumer acceptability of haskap-based products are lacking in the literature but are very important and useful information that is required in the development of novel products.

Additionally, several methods have been used for preparing haskap extracts solely for analysis of their bioactive compounds, using solvents that are not safe for consumption (Khattab et al., 2016; De Silva and Rupasinghe, 2021). High extraction temperatures are also often used, decreasing the potential yield of thermolabile antioxidants (Khattab et al., 2017; MacLean et al., 2021). Sous vide technology can preserve thermolabile compounds through the regulation of its temperature. This cooking method has demonstrated the ability to enhance the preservation of the nutritional quality of meat, compared to conventional cooking (Ayub and Ahmad, 2019; Gómez et al., 2020). Furthermore, carrots prepared using sous vide cooking, yielded higher anthocyanin content than carrots prepared by traditional cooking (Iborra-Bernad et al., 2015). Therefore, incorporating sous vide for the extraction of phytochemicals from berries has the potential to produce antioxidant-rich extracts, that could be valuable for both household and industrial applications. The sous vide technology, however, is yet to be used for the extraction of antioxidants from berries such as haskap.

1.2 Research hypothesis

Based on the principles of sous vide, it is hypothesized that sous vide can be used to improve the extraction yield of antioxidant properties in berry extracts, compared to conventional methods such as cold extraction.

Secondly, it is hypothesized that haskap berries are a good novel plant-based source for the development of antioxidant-rich functional food products.

1.3 Overall project objective

The research was conducted with three specific objectives, which were:

1. To optimize the sous vide extraction technique using different times (30 min and 60 min), temperatures (30 °C - 50 °C), and solvents (pure ethanol, pure water, and ethanol: water (1:1)), with the aim of enhancing the extraction efficiency of antioxidant-rich bioactive compounds from haskap berry.
2. To compare the antioxidant properties of extracts obtained from optimized sous vide extraction and the conventional cold extraction method.
3. To formulate and develop functional beverages with high antioxidant content, using haskap as the main ingredient.

1.4 Thesis organization

The thesis is organized in a manuscript format and consist of five chapters. Chapter one gives an introduction of the focus of the thesis, the gap in research and major objectives of the study. Chapter two provides an overview of the existing literature on the origin and history of haskap as well as its bioactive properties. It comprises a brief overview of the phytochemical constituents of haskap, their physiological effects, and potential impact on chronic conditions associated with oxidative stress. Chapter three focuses on the optimization of the sous vide technique for haskap berry extraction, which is used in subsequent chapters. The formulation and development of antioxidant-rich haskap-based beverages, along with the consumers' ratings of each flavour are highlighted in chapter four. Chapter five then provides a general conclusion, giving implications of the study, limitations, and future research recommendations.

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CHAPTER 2
LITERATURE REVIEW

2.0 INTRODUCTION

The latest edition of the comprehensive World Health Statistics has revealed that non-communicable diseases like cancer, diabetes, heart disease, and neurological diseases, account for seven of the top ten causes of death worldwide (WHO, 2020). According to Dalle-Donne et al. (2006), oxidative stress has a significant impact on the onset and progression of such diseases. Oxidative stress in humans refers to a state of imbalance in the levels of pro-oxidants and the body's natural antioxidant capacity (Li et al., 2016). These agents can originate from exogenous sources, such as pathogens and environmental factors, or from endogenous sources, such as the leakage of free radicals and the generation of reactive oxygen species (ROS).

The term "ROS" encompasses a group of oxygen-centered radicals, including superoxide (O_2 –), hydroxyl (·OH), and non-radical reactive derivatives of oxygen, which include singlet oxygen ($1\Delta g O_2$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (McDowell et al., 2007). These species result in deleterious effects and structural alterations on various biomolecules such as deoxyribonucleic acid **(**DNA), lipids, proteins and thereby, instigating pathophysiological occurrences (Araujo and Martins, 2016). Additionally, the activation of various transcription factors can be induced by oxidative stress, leading to the differential expression of multiple genes that participate in inflammatory pathways (Rahman and Adcock, 2006). Numerous conditions including cardiovascular disease, inflammatory bowel disease, and pulmonary diseases are believed to result from chronic inflammation that is initiated by oxidative stress and oxidative damage (He et al., 2015; Hussain et al., 2016). In typical circumstances, the intracellular levels of ROS are regulated to remain low through the involvement of a range of defensive mechanisms. These mechanisms contribute to the maintenance of redox homeostasis *in vivo* and play a vital role

in mitigating oxidative stress induced by diverse abiotic stressors (Rahal et al., 2014; Sisein, 2014; Nath et al., 2017). Some of these natural defenses include antioxidant enzymes, such as superoxide dismutase (SOD), catalases (CAT), glutathione peroxidases (GPx), ascorbate peroxidase (APX), glutathione reductase (GR), as well as non-enzymatic antioxidants such as ascorbic acid (vitamin C), α-tocopherols (vitamin E), carotenoids and phenolic compounds (Hamid et al., 2010; Ighodaro and Akinloye, 2018). Oxidative stress ensues when the generation of prooxidants surpasses the ability of antioxidant defense mechanisms to counteract them (McDowell et al., 2007).

Antioxidants can be natural or synthetic. It has been reported that synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) pose potential risks to human health (Sindhi et al., 2013). Conversely, the consensus is that natural antioxidants are safer and more efficient than synthetic ones (Tavasalkar et al., 2012). Fruits such as berries, mango (*Mangiferaindica*), pears (*Pyrus*), and vegetables such as broccoli (*Brassica oleracea var. italica)* and radish (*Raphanussativus*) are rich sources of natural antioxidants (Ravimannan and Nisansala, 2017). They have the capacity to prevent or delay the oxidation process by scavenging harmful free radicals and reducing the impact of oxidative stress. Consequently, they provide a certain level of protection against oxidative damage (Hooda et al., 2023). According to Einbond et al. (2004), an inverse correlation exists between the regular consumption of diets rich in fruits and vegetables and the occurrence and progression of certain diseases such as coronary heart disease and cancers.

Over the last three decades, haskap berries *(Lonicera caerulea* L.) have been gaining popularity among superfruits such as blueberry (*Vaccinium angustifolium* Ait.), strawberry (*Fragaria* × *ananassa)* and blackberry *(Rubus fruticosus)*. There is mounting evidence that haskap berries possess anticarcinogenic, antioxidant, antibacterial, anti-inflammatory, and glucose metabolism-modifying properties (Rupasinghe et al., 2018). According to Rupasinghe et al. (2012), these

berries contain physiologically active phytochemicals, including flavonoids and phenolic acids, that can contribute to preventing a number of chronic diseases like cancer, cardiovascular and neurodegenerative diseases. The primary aim of this study is to present an overview of the existing literature on haskap berries to substantiate a more extensive utilization of this fruit in health-related applications. It will provide a comprehensive analysis of the phytochemicals present in haskap berries in contrast to other berries, elucidate their biological effects, and explore their potential therapeutic applications in the management of chronic health disorders associated with oxidative stress.

2.1 The origin, legends, and history of haskap

The name haskap was coined by the indigenous people of Hokkaido, Japan, and means "many fruits on branches" (Lefol, 2007; Ochmian et al., 2012; **Figure 1**). Haskap was an important part of the Ainu diet and was acknowledged for its ability to enhance physiological and cognitive functions (Gołba et al., 2020). It was commonly employed in the treatment of gastrointestinal disorders, tonsillitis, as well as served as a preventive measure against other metabolic diseases (Jurikova et al., 2011; Minami et al., 2019; Liu et al., 2021). The berries were often consumed fresh or preserved with sugar and salt, particularly during periods when the availability of the fruit was limited, such as in the winter season (Thompson, 2006). Among the Ainu people haskap berries were utilized in the production of spirits and served as a traditional food preservative (Svarcovaa et al., 2007). Furthermore, its products have been marketed on Hokkaido Island as a valuable solution for achieving perpetual youthfulness and longevity (Lefol, 2007).

Haskap breeding programs were started in Japan and Russia after several notable characteristics of the berry were observed (Thompson and Barney, 2007; Celli et al., 2014). These included early

maturation, a unique taste, exceptional frost tolerance in both its plants and flowers, and the significant presence of bioactive compounds in its fruit (Plekhanova, 1999). It was soon recognized beyond the borders of Russia and Japan, which led to an increase in demand and production in Europe and other countries (Hummer, 2006). Its presence in North America was recorded in the early 2000s, when haskap was first introduced to Maxine Thompson by a contact from Oregon who had received a single plant (Thompson, 2006). Owing to growing interest in this fruit, haskap breeding programs were started at the Oregon State University, USA and the University of Saskatchewan, Canada (Bors, 2009).

Figure 1. Haskap and two of the popular cultivars. A. Haskap plant with fruits. B. Tundra. C. Indigo Gem. Adapted from Bors et al. (2012).

2.2 Classifications of haskap

2.2.1 Taxonomic classification of haskap

Haskap also referred to as Japanese blue honeysuckle and honeyberry, is an antioxidant-rich superfruit that has seen an increase in production over recent years. It typically ranges in size from half an inch to two inches and exhibits a spherical, oblong, or irregular shape, depending on the specific cultivar (Lauritzen et al., 2015). Haskap belongs to the Caprifoliaceae family of the order Dipsacales (Wu and Hou 2021; **Figure 2).** *Lonicera* is the largest genus in the Caprifoliaceae

family, comprising over two hundred distinct species that are indigenous to Northeastern Asia, Japan, and Siberia (Naugžemys et al., 2007). *Lonicera caerulea* is a well-known species in the family and consist of several varieties including *Lonicera caerule*a var. *emphyllocalyx* from Hokkaido, Japan (Thompson and Barney, 2007). Researchers have employed physiological, chemical, morphological, and DNA analyses to identify four distinct species of haskap native to Russia (Plekhanova, 1999; Jurikova et al., 2011). They are the most utilized species and include: *Lonicera kamtchatica, Lonicera edulis*, *Lonicera boczkarnikovae*, and *Lonicera altaica*.

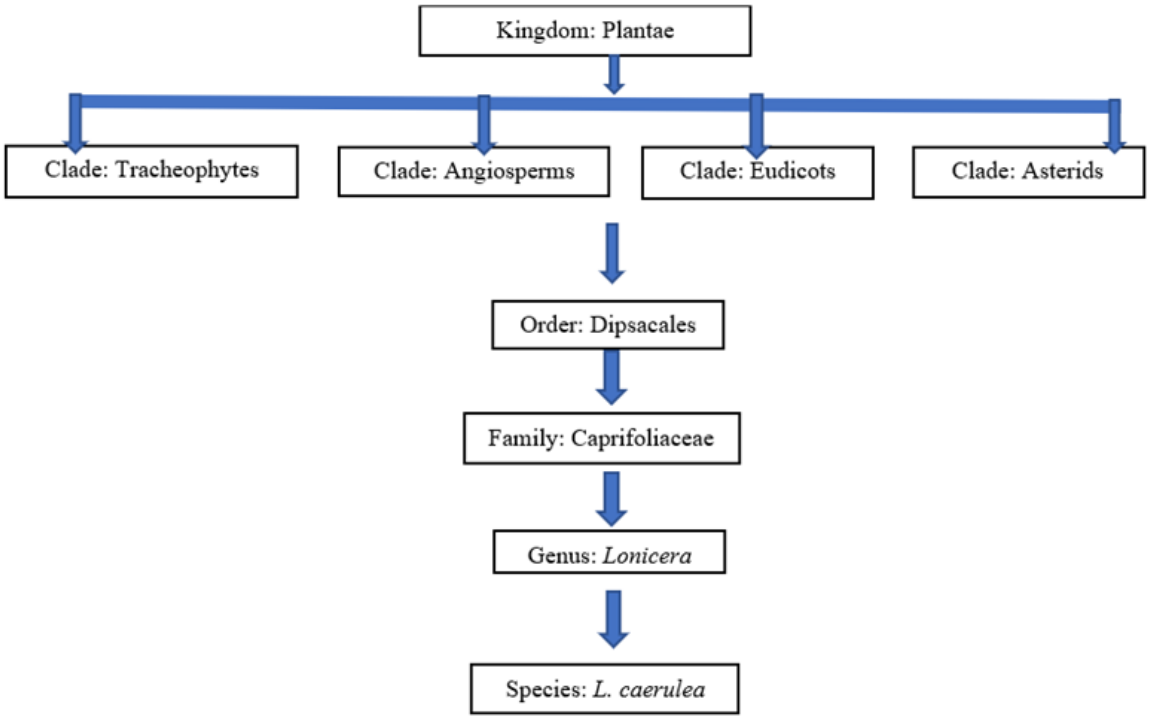


Figure 2. Scientific classification of haskap (*Lonicera caerulea* L.).

2.2.2 Classification of haskap based on the place of origin.

The term "haskap" typically suggests that varieties are a form of Japanese Blue honeysuckle of the *emphyllocalyx* subspecies. The term "honeyberry," on the other hand, is commercially used to refer to Russian and Kuril varieties of blue honeysuckle of the *kamtschatica and edulis* subspecies

(Lauritzen et al., 2015). Haskap varieties that differ based on location may have different nutritional content and tend to respond differently to the climate (De Silva and Rupasinghe, 2021). For example, Japanese varieties show more growth in higher temperatures, while Russian varieties are better adapted to colder, higher-elevation areas (Lauritzen et al., 2015).

There are numerous species of edible honeysuckle, but only a select few possess the desirable qualities of sweet, aromatic berries (Wojdyło et al., 2013). Specifically, crops derived from *Lonicera edulis* and *Lonicera kamtschatica* are recommended due to their superior taste (Pokorná-Juríková and Matuškovič, 2007). Furthermore, these species contain a wide range of valuable biologically active substances such as high contents of ascorbic acid, potassium, and polyphenolic compounds (Jurikova et al., 2010; Jurikova et al., 2012). Some species such as *Lonicera altaica* and *Lonicera pallasii* may exhibit a bitter taste due to the presence of esters of malic and citric acids (Vereshchagin et al., 1989). Popular *Lonicera caerulea* cultivars that are currently undergoing widespread cultivation in Canada due to their exceptional fruit properties are Borealis, Indigo Gem, and Tundra (Rupasinghe et al., 2012).

2.3 Phytochemical composition of haskap berries

Fruits are known to contain high amounts of phenolic compounds, terpenoids, pigments and other natural antioxidants (Bellucci et al., 2022). These are phytochemicals, which represent a class of non-nutritive bioactive compounds that have been shown to have health-promoting properties (Septembre-Malaterre et al., 2018). These compounds are associated with health benefits, including protection against conditions such as heart disease, cancer, diabetes, and hypertension (Prakash, 2020). Haskap is no exception, containing a variety of bioactive compounds with nutritional and medicinal properties, thus earning them recognition as superfruits. (Molina et al., 2019; Bieniek et al., 2021). They are noted for their high concentration of active ingredients, which

include polyphenols, vitamins, carotenoids, and minerals (Celli et al., 2014). These phytochemicals have excellent antioxidant properties, which render haskap fruits as an excellent source of natural dietary antioxidants (Khattab et al., 2020).

This section focuses on the bioactive compounds identified in haskap berries and their mechanism of action. It presents the difference in polyphenolic content in various cultivars and provides a chemical comparison between haskap and other super fruits such as blueberry and blackberry.

2.3.1 Polyphenols

Polyphenols represent the major secondary metabolites found extensively across the plant kingdom (Liu et al., 2017). They are characterized by the presence of phenyl-ring compounds that are carbon-based and aromatic in nature (Mohankumar et al., 2018). Polyphenolic compounds possess the unique property of being readily oxidized to quinones by ROS, which contributes to their ability to scavenge free radicals (Sreejayan and Rao, 1996). The redox properties of phenolic compounds facilitate their function as hydrogen donors thus impeding the activity of free radicals through the transfer of a hydrogen atom from the hydroxyl group. They act as reducing agents and quenchers of singlet oxygen (Ghasemzadeh and Ghasemzadeh, 2011). Phenolic compounds also chelate metal ions, specifically iron and copper, thereby hindering the oxidation of low-density lipoproteins (Santos-Sánchez et al., 2019).

The predominant phenolics identified in haskap fruit extracts are phenolic acids and flavonoids, particularly anthocyanins (Becker and Szakiel, 2019; Amararathna et al., 2020; Ponder et al., 2022).

2.3.1.1 Phenolic acids

Phenolic acids refer to phenolic compounds that possess a single carboxylic acid group and are an important subset of dietary polyphenols (Kumar and Goel, 2019). These compounds have been found to exhibit higher *in vitro* antioxidant activity compared to widely recognized antioxidant vitamins (Tsao and Deng, 2004). The class and concentration of phenolic acids that are found in haskap berries may vary significantly (Orsavová et al., 2022; **Table 1**). The variation may result from genetic differences, place of origin, or the method used for extraction of these bioactive compounds (Ali et al., 2018b; Oreopoulou et al., 2019).

Table 1. Phenolic acid content in haskap berries from different places of origin.

Phenolic acid	Place of origin	Phenolic content	Reference
Chlorogenic acid	Czech Republic	2123.1 - 4770.8 [a]	(Orsavová et al., 2022)
	Lithuania	897.2 - 944.4 [a]	(Raudonė et al., 2021)
	Poland	766.3 - 2940.1 [a]	(Wojdyło et al., 2013)
	Canada	207.0 - 327.0 [b]	(Rupasinghe et al., 2015)
Caffeic acid	Czech Republic	22.3 - 226.4 [a]	(Orsavová et al., 2022)
	Lithuania	9.5 - 25.1 [a]	(Raudonė et al., 2021)
	Poland	598.2 [a]	(Zadernowski et al., 2005)
	Canada	1.0 - 2.0 [b]	(Rupasinghe et al., 2015)
p-Coumaric acid	Czech Republic	71.8- 770.1 [a]	(Orsavová et al., 2022)
	Lithuania	67.76 - 143.2 [a]	(Raudonė et al., 2021)
	Poland	987.1 [a]	(Zadernowski et al., 2005)

[a]Dry weight, [b]Fresh weight phenolic acid in mg kg^{-1} .

One phenolic acid that has been found in all haskap extracts irrespective of species or their geographical location is chlorogenic acid (CGA). The concentration of CGA in haskap extracts varies widely, with levels ranging from 2.85 mg kg^{-1} in Morena berries to 62,400 mg kg^{-1} in

certain Chinese cultivars (Auzanneau et al., 2018; Wang et al., 2018). The compound CGA has notable biological activity, exhibiting various therapeutic functions including anti-inflammatory, anti-microbial, antioxidative, hepatoprotective, antipyretic, cardioprotective, neuroprotective and anti-obesity properties (Naveed et al., 2018). Other phenolic acids commonly found in haskap berries are *p*-coumaric acid (*p*-CA) and caffeic acid (CA). In one study that compared haskap varieties from different locations, all the samples exhibited a prevalence of *p*-CA and CA as the second and third most abundant phenolic acids, respectively, following CGA (Orsavová et al., 2022). Both *p*-CA and CA exhibit antioxidant action (Pei et al., 2016). Previous research has demonstrated that pre-treatment with *p*-CA effectively reduces the production of intracellular ROS generated by 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH). Furthermore, *p*-CA has been shown to upregulate the expression of antioxidant genes *in vivo* (Shen et al., 2019). Gülçin (2006) also showed that CA has effective free radical scavenging ability as measured by *in vitro* antioxidant assays such as 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, and 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging. The presence of these compounds in haskap thus contributes to its biological properties (Jurikova et al., 2011).

2.3.1.2 Flavonoids

Flavonoids are a subclass of polyphenols (Clifford, 2001), and can be further categorized into six distinct subclasses based on the type of heterocycle present (Harborne and Williams, 2001). These subclasses include flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols. The category of subclass is based on the specific carbon of the C ring to which the B ring is connected, as well as the level of unsaturation and oxidation exhibited by the C ring (Panche et al.,2016; **Figure 3**). Plant flavonoids have significant biological activities that support human health while lowering the risk of illness. These biological properties are determined by their

structural classification, diverse substitutions and conjugations, level of hydroxylation, and polymerization (Heim et al., 2002). The antioxidant effects of flavonoids are attributed to the presence of functional hydroxyl groups, which facilitate the elimination of free radicals and the chelation of metal ions (Kumar and Pandey, 2013). The hydroxyl configuration of the B ring can donate both hydrogen and an electron to radicals of hydroxyl, peroxyl, and peroxynitrite, leading to their stabilization and the formation of a relatively stable flavonoid radical (Heim et al., 2002; Panche et al., 2016).

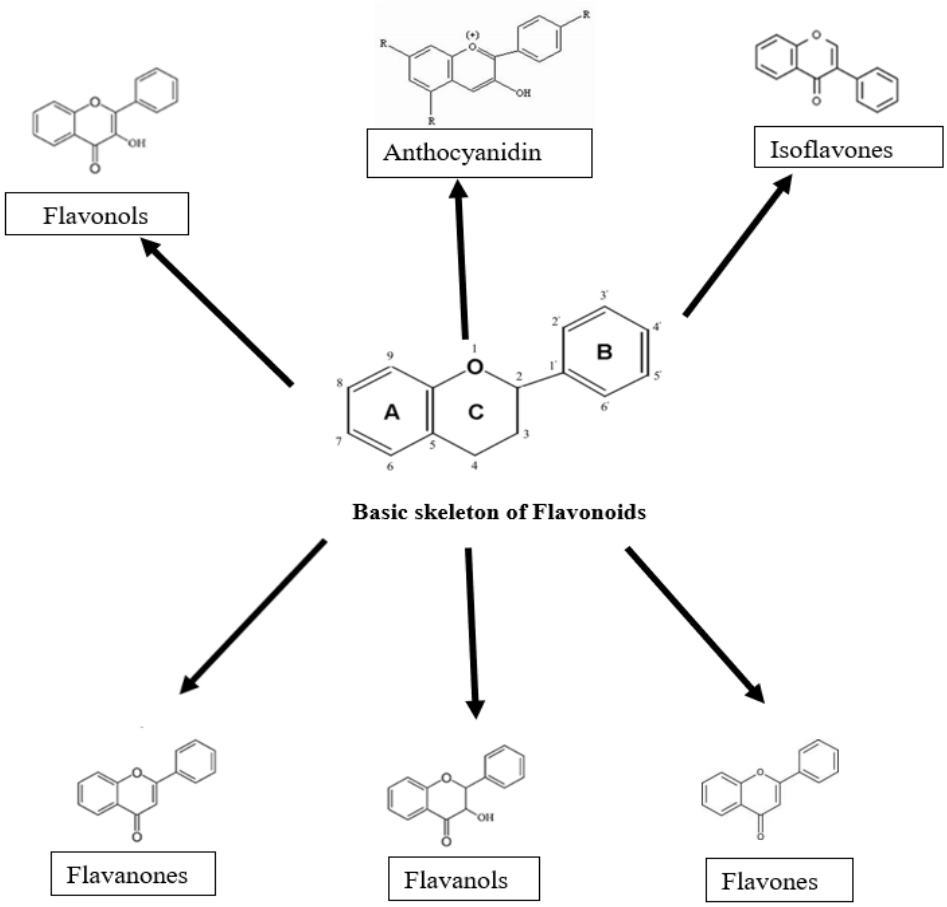


Figure 3. Main skeleton of flavonoids and their sub-classes.

Flavonoids have also been found to inhibit arachidonic acid (AA) metabolizing enzymes, like cyclo-oxygenase (COX) and lipoxygenase (Nworu and Akah, 2015). The metabolites derived from

these enzymes could elicit inflammation and free radicals' generation and appear to be significant causative factor to oxidative stress (Wu et al., 2011; Wang et al., 2020).

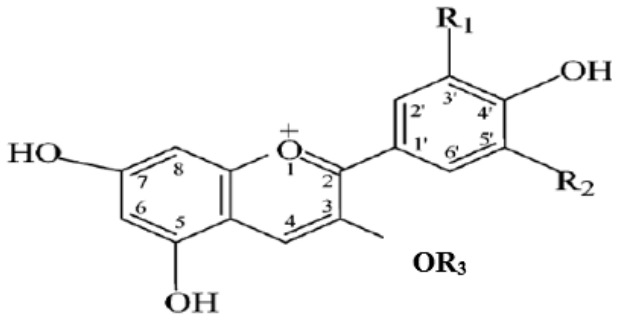
Additionally, flavonoids prevent low density lipoprotein (LDL) cholesterol from being oxidized to dangerous cholesterol oxides (Craig, 1997; El-Tantawy, 2015). This is crucial as cholesterol oxidation products have been linked to adverse health consequences, including inflammation, atherogenesis, carcinogenesis, and the onset of degenerative conditions such as Alzheimer's, Parkinson's, Huntington's, and other chronic ailments (de Oliveira et al., 2018). Flavonoids also have anti-inflammatory and anticancer properties and work synergistically with vitamin C to increase their effectiveness (Craig, 1997; Saini et al., 2017).

Flavonoid compounds that are found in haskap berry include flavanols such as catechin and epicatechin, and flavonols like quercetin-3-β-D-glucoside and quercetin-3-rutinoside (Rupasinghe et al., 2015; Khattab et al., 2016). According to Azeem et al. (2023), quercetin is the most potent antioxidant flavonoid found in nature that protects the human body against free radicals.

2.3.1.2.1 Anthocyanin

The most studied flavonoid subclass in haskap is anthocyanin (Fujita et al., 2020; Zhou et al., 2023). Anthocyanins are anthocyanidins linked with one or more sugar moieties (Zhang et al., 2004). The six most common anthocyanidins in nature are peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt), pelargonidin (Pg), and delphinidin (Dp) (Auzanneau et al., 2018; Raudonė et al., 2021). More than 700 derivatives of anthocyanin have been identified and these differ based the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule (Kong et al., 2003; Nassour et al., 2020; **Figure 4)**. Anthocyanins (*Anthos*-flower, *kyanos* -blue) are polyphenolic pigments and account for a range of colours, varying from

red-orange to blue-violet, that are observed in different fruits (Wallace and Giusti, 2015). These compounds are known to act as free radical scavengers, and thereby reducing oxidative stress (Tena et al., 2020).



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3
Sugar		
R_3	glucose, galactose or arabinose	

Figure 4. Basic anthocyanin structure. Adapted from Thomasset et al. (2009).

The most prevalent and potent anthocyanin is cyanidin 3-glucoside (C3G). Numerous *in vivo* studies have been conducted on C3G and it shows great potential as a neuroprotectant (Sukprasansap et al., 2020). Its anti-inflammatory and antioxidative effects have been shown to potentially reduce H_2O_2-induced cytotoxicity (Jung et al., 2014).

Five anthocyanins (cyanidin-3-glucoside, cyanidin 3,5-di-glucoside, cyanidin-3-rutinoside, peonidin-3-*o*-glucoside, and pelargonidin-3-glucoside) were identified in three haskap varieties, with C3G having the highest concentration. C3G was up to six times greater than other classes of anthocyanin and represented 92.0%, 82.8%, and 89.4% of the total anthocyanin content in Tundra, Berry blue, and Indigo Gem respectively (Khattab et al., 2016). Furthermore, haskap berries exhibit a notably higher concentration of C3G in comparison to local North American berries such

as Saskatoon berries (*Amelanchier alnifolia* Nutt.), alpine bearberries (*Arctostaphylos alpina* L. Spreng.), chokeberries (*Aronia melanocarpa* (Michx.) Elliott), and lowbush blueberries (*Vaccinium angustifolium* Aiton) (Dudonné et al., 2015; Rupasinghe et al., 2018). When haskap was compared to other Western Canadian berries, the total anthocyanin content was found to be highest in the honeysuckle berries, measuring 1080.9 ± 24.9 mg C3G 100 g^{-1} FW, which was four times higher than that which was measured in Saskatoon berry and five times the anthocyanin content recorded for black raspberry (*Rubus leucodermis* Dougl.) (Bakowska-Barczak et al., 2007).

Furthermore, total flavonoid content of various blue honeysuckle cultivars in the literature exhibited a range of 594.4 to 1582.8 mg QE 100 g^{-1} FW (Rupasinghe et al., 2012; Rupasinghe et al., 2015; Gawroński et al., 2020). The variation in flavonoid content could be due to the cultivar (genotype) differences, method of extraction across studies, as well as other environmental conditions such as soil quality and fertilization (Gawroński et al., 2020).

2.3.1.3 Total polyphenolic content

Overall, the total polyphenolic content of haskap berries surpasses that of other berries considered as superfruits that are cultivated in Canada, including blackberry, and blueberry (Khattab et al., 2016). In research conducted by Rupasinghe et al. (2012) haskap varieties including Borealis, Indigo gem and Tundra had the highest phenolic content measured among six other fruits. The total phenolic content (TPC) ranged from 428.2 to 622.5 mg GAE 100 g^{-1} FW among the haskap cultivars and 166.8 to 429.8 mg GAE 100 g^{-1} FW for other fruits such as blackberry (*Rubus fruticosus*), partridgeberry (*Vaccinium vitis-idaea* L.), blueberry (*Vaccinium angustifolium* Ait.), strawberry (*Fragaria* × *ananassa*) cv. Chandler, red raspberry (*Rubus idaeus* L.), and red table grape (*Vitis vinifera* L.). Another analysis of berries from Western Canada revealed that

honeysuckle fruits (1111.2 ± 68.5 mg 100 g^{-1} FW) exhibited the highest total polyphenolic content of all the fruits examined. The other berries included Saskatoon berries (*Amelanchier alnifolia* Nutt.) (620.2 ± 23.4 mg 100 g^{-1} FW), purple raspberries (*Rubus neglectus* Peck) (638.1 ± 33.2 mg 100 g^{-1} FW), crowberries (*Empetrum nigrum* L.) (689.6 ± 40.7 mg 100 g^{-1} FW), golden currant (*Ribes aureum* Pursh) (833.2 ± 2.7 mg 100 g^{-1} FW) and bilberries (*Vaccinum myrtilloides* Michx.) (778.5 ± 32.1 mg 100 g^{-1} FW) (Bakowska-Barczak et al., 2007). Haskap berries often exhibit a greater concentration of antioxidants when compared to other berries that are recognized for their advantageous effects on human health (Celli et al., 2014; **Table 2**).

Table 2. Phytochemicals in haskap berry compared to other superfruits.

Polyphenol	Haskap	Strawberry	Blackberry	Blueberry	Reference
Phenolic content	428.1 - 622.5	201.8	429.8	166.8	(Rupasinghe et al., 2012)
Flavonoid	594.4 - 699.3	63.5	171.4	343.0	(Rupasinghe et al., 2012)
Anthocyanin	10810.0* [b]	169.2	1055.7	4069.0	(Jakobek et al., 2007) *(Bakowska-Barczak et al., 2007)

Phenolic content in mg GAE 100 g^{-1}, Flavonoid in mg QE 100 g^{-1}, Anthocyanin in mg C3G kg^{-1}. [b]converted to C3G kg^{-1} from 1081 mg C3G 100 g^{-1} for comparison. * Reference. All samples were analyzed per fresh mass. GAE = gallic acid equivalence, QE = quercetin, C3G = cyanidin-3-glucoside.

2.3.2 Carotenoids in berries

In addition to polyphenols, another class of non-phenolic compounds in haskap berries responsible for antioxidant activity are carotenoids. Carotenoids are a group of phytochemicals that are responsible for different colours in food and are present in almost all plant species (Rao and Rao, 2007). These substances exhibit high efficacy as antioxidants and can scavenge both singlet molecular oxygen and peroxyl radicals (Stahl and Sies, 2003). The structural base fragment of these compounds, which comprises a conjugated polyunsaturated chain, endows them with

exceptional peroxyl radical scavenging capabilities (Santos-Sánchez et al., 2019a; **Figure 5**). Carotenoids can also function as chemical quenchers by undergoing an irreversible process of oxygenation (Fiedor and Burda, 2014).

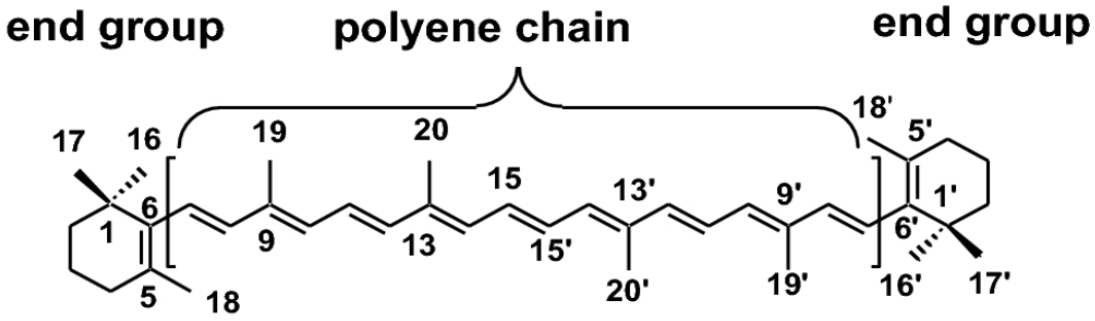


Figure 5. Basic structure of carotenoids. Adapted from Maoka (2020).

The effectiveness of carotenoids in their role as antioxidants is contingent upon their interaction with other co-antioxidants, as it is more potent in synergistic interactions with other antioxidants, particularly vitamins E and C (Young and Lowe, 2001). There is limited research documenting the presence of carotenoids in haskap, however, the four carotenoids that have been identified are *α*-carotene, β-carotene, lutein, and β-cryptoxanthin (Fujita et al., 2023; Mech-Nowak et al., 2014). β-carotene was found to be the most predominant carotenoid in haskap and ranged from 250 to 350 µg 100 g^{-1} FW (Fujita et al., 2023). This is significantly higher ($p \leq 0.05$) than *β*- carotene that was measured by Heinonen et al. (1989) in other berries such as black currant (*Ribes nigrum*) (99 µg 100 g^{-1} FW), bilberry (*Vaccinium myrtillus*) (47 µg 100 g^{-1} FW), strawberry (*Fragaria* × *ananassa*) (8.9 µg 100 g^{-1} FW, cranberry (*Vaccinium macrocarpon*) (22 µg 100 g^{-1} FW), and raspberry (*Rubus idaeus*) (6.4 µg 100 g^{-1} FW), but lower than other non-berry fruits such as tomato (*Solanum lycopersicum*) (390-580 µg 100 g^{-1} FW) and vegetables such as lettuce (*Lactuca sativa*) (730-790 µg 100 g^{-1} FW) and carrot (*Daucus carota*) (2600-5500 µg 100 g^{-1} FW).

Furthermore, the α-carotene Fujita et al. (2023) recorded for haskap (2 to 7 µg 100 g^{-1} FW) is significantly lower than that recorded in raspberry (*Rubus ideaus* L.) (23.7 ± 1.9 µg 100 g^{-1} FW, $p \leq 0.05$) by Marinova and Ribarova (2007). These differences in concentration of carotene could be due to genetic, environmental, or developmental factors among the varying species and may also be influenced by the varying extraction methods used among the different studies (Fanciullino et al., 2014).

2.3.3 Minerals

Numerous microminerals are crucial for antioxidant functions in the human body (Goff, 2018). These compounds serve as cofactors for enzymes that regulate free radicals in the body, maintaining redox homeostasis (Zhao et al., 2015b; Marreiro et al., 2017). These minerals include iron (Fe) which activates catalase (CAT); copper (Cu), manganese (Mn), and zinc (Zn) which serve as activators of superoxide dismutase (SOD); as well as selenium (Se) which functions as a component of glutathione peroxidase (GPX) (Liu et al., 2019). Each of these enzymes can convert peroxides in cells to less toxic forms (i.e., water and alcohols) (Styskal et al., 2012).

Minerals also play a pivotal role in the stabilization of protein and enzyme structures, enabling them to facilitate essential biological activities. Additionally, these trace elements can modify the shape of the membrane, thereby impeding the entry of specific molecules into the cell (Islam et al., 2023). Calcium, for example, plays a crucial role in various physiological processes such as vascular contraction, vasodilation, nerve transmission, intracellular signaling, and hormone secretion (Beto, 2015). Potassium on the other hand serves as the predominant cation in the intracellular fluid, maintaining homeostatic osmolarity (Udensi and Tchounwou, 2017). A select group of enzymes such as pyruvate kinase and aldehyde dehydrogenase, which are involved in

carbon metabolism, rely on the presence of potassium to facilitate their catalytic activity (Pereira et al., 2018; Danchin and Nikel, 2019).

The most predominant mineral found in haskap is potassium (K)(10,175 to 14,764 mg kg^{-1}), followed by phosphorus (P) (1675 to 2,775 mg kg^{-1}), Ca (426 to 1,675 mg kg^{-1}), magnesium (Mg) (469 to 952 mg kg^{-1}), and sodium (Na) (37 to 140 mg kg^{-1}) (Pokorná-Juríková and Matuskovic, 2007). The variation observed was based on the difference in species and mode of irrigation. Rupasinghe et al. (2012) measured the mineral content in berries including but not limited to, three haskap varieties (Borealis, Indigo Gem, and Tundra), as well as strawberry, blueberry, and blackberry. The findings show that strawberries contained the most minerals of all the berries. Compared to blueberry and blackberry however, haskap had an overall higher mineral content except for manganese, copper, and zinc. Additionally, phosphorus and calcium were significantly higher ($p \leq 0.05$) in haskap than in blueberry and blackberry. It is noteworthy that haskap cv. Tundra had the overall highest calcium content, which was twice that of mineral-rich strawberries. The mineral constitution is based on growth circumstances, including soil and climatic conditions, and not just the type of cultivar (Wojdyło et al., 2013).

2.3.4 Vitamins in haskap

Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are two potent vitamins that serve as antioxidant agents. *In vivo*, vitamins C and E are the most significant hydrophilic and lipophilic antioxidants, respectively (Heller et al., 1998). Ascorbic acid is the most significant antioxidant situated in the aqueous compartment of cells, where it potentially safeguards biological membranes by eliminating peroxyl radicals before they initiate peroxidation. It is recognized as a potent antioxidant due to its ability to readily donate electrons and thereby confer stability to ROS (McDowell et al., 2007). Vitamin E on the other hand, functions as a tissue-based antioxidant and

works synergistically with vitamin C by conferring stability to the reactive species by donation of an electron (Traber and Stevens, 2011).

The antioxidant activity exhibited by haskap has been attributed to its vitamin C content. Orsavová et al. (2022) identified a strong positive correlation (r=0.8, $p \leq 0.05$) between vitamin C content and antioxidant activity (DPPH) in haskap extracts. This indicates that vitamin C is a contributor to the antioxidant capacity of haskap. Vitamin C in haskap ranged from 18.4 ± 0.6 to 28.6 ± 0.1 g kg^{-1} and vitamin E ranged from 1.6 ± 0.0 to 3.70 ± 0.0 mg kg^{-1} in various haskap cultivars from two different locations (Orsavová et al., 2022).

2.4 Role of antioxidants in haskap

The presence of ROS is a characteristic of aerobic organisms in their normal state (Lee et al., 2017). An excess of these species however, can have debilitating health implications (Sepand et al., 2023). The endogenous antioxidant defenses have been found to be insufficient to provide complete protection against damage caused by ROS (Li et al., 2014). Hence, the consumption of antioxidants through dietary sources plays a crucial role in preserving overall health (Halliwell, 1996). Polyphenols such as flavonoids, isoflavones, anthocyanins, and catechins are thought to possess the strongest antioxidant capabilities while carotenoids, and vitamins C and E have weaker antioxidant effects (Biel et al., 2020). Furthermore, phenolics have emerged as potent antioxidants *in vitro*, surpassing the antioxidant capabilities of both carotenoids and vitamins (Rice-evans et al., 1995; Rice-Evans et al., 1996) . This section primarily examines the main mechanisms by which antioxidants effectively counteract the detrimental effects of oxidative stress.

2.4.1 Mechanism of action of antioxidants

Antioxidants inhibit ROS and quench free radicals such as hydroxyl (HO•), alkoxyl (RO•), and hydrogen peroxide (ROO•) that may initiate oxidation (**Figure 6**). Antioxidants can be classified into primary and secondary antioxidants based on their respective modes of action (Makris and Boskou, 2014). Primary antioxidants such as vitamin E or C function as scavengers of free radicals by providing a hydrogen atom, thereby actively preventing, or delaying the different stages of autooxidation (Oresajo et al., 2012). The effectiveness of secondary antioxidants such as phenolics are attributed to their ability to inhibit oxidation indirectly (Craft et al., 2012). Secondary antioxidants can chelate prooxidants, scavenge oxygen, and inhibit various enzymes that contribute to oxidative stress (Makris and Boskou, 2014; Santos-Sánchez et al., 2019; Prakash, 2020). It is believed that the effectiveness of antioxidants can be enhanced and the potential for toxicity reduced when agents with varying modes of action are combined (Fraga, 2009).

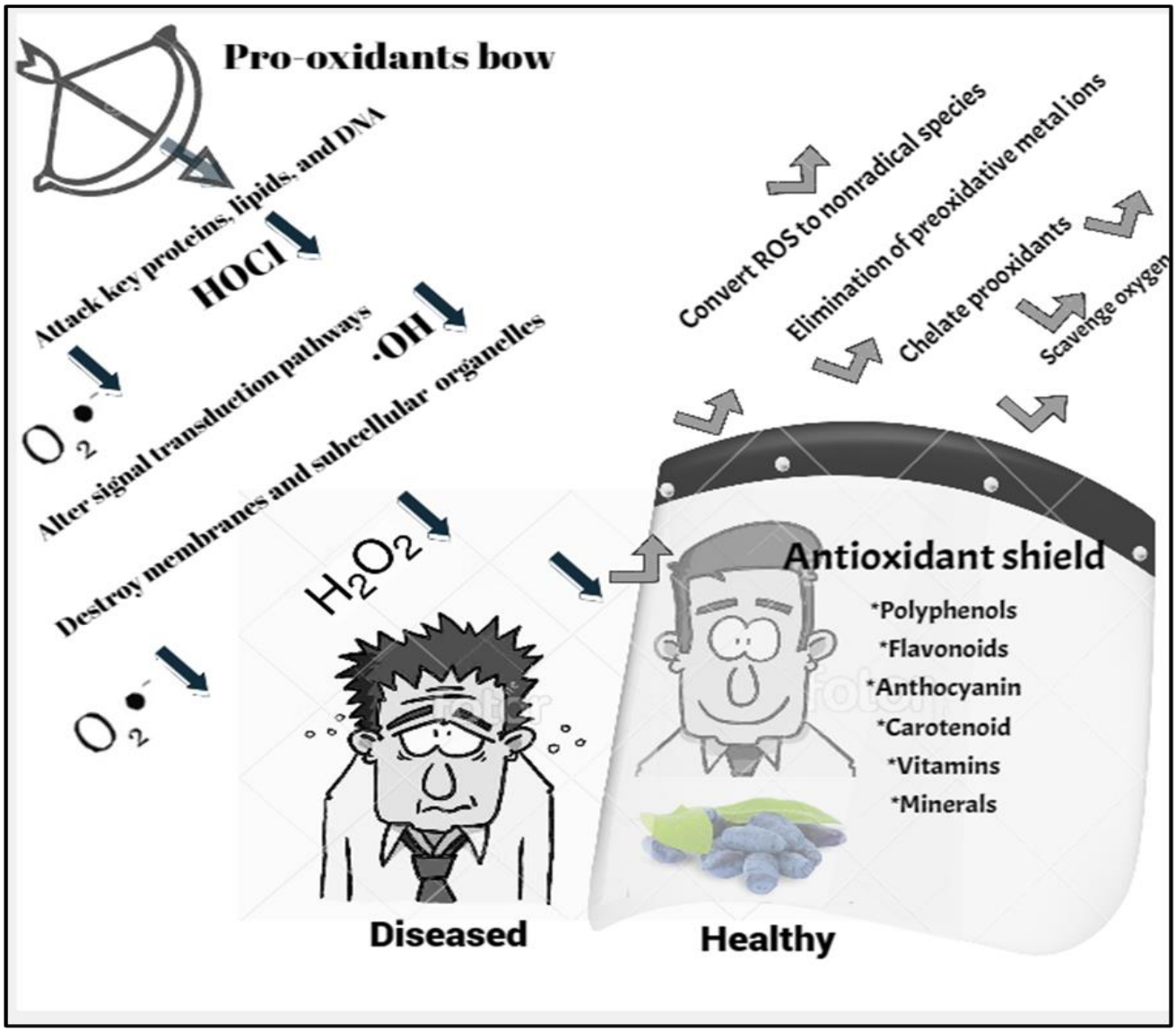


Figure 6. Graphic representation of the potentially protective effects of antioxidant-rich haskap berries against ROS. Graphic modified from https://www.dreamstime.com/stock-illustration-ill-man-healthy-man-looking-both-image43858227.

2.4.2 Antioxidant activity assessment of haskap

The assessment of antioxidant activity cannot be limited to a single methodology owing to the varied modes of action exhibited by antioxidants (Romulo, 2020). Therefore, various assays have been utilized to obtain extensive information on the antioxidant capacities in haskap fruit including

2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC), 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic (ABTS) and ferric reducing antioxidant power (FRAP) assay.

DPPH scavenging activity assay is the most widely used method for evaluating the antioxidant potential of food and biological samples *in vitro* (Nicklisch and Waite, 2014). Its fundamental principle is based on the ability of antioxidants to scavenge DPPH free radicals. Antioxidants react with DPPH and donate hydrogen, thus changing the solution from purple to pale yellow. DPPH exhibits an active absorption band at maximum 515–517 nm in its radical state (Romulo, 2020). The findings can be expressed as the percentage of DPPH decolourization exhibited by the sample or as the EC_{50} value, which represents the concentration necessary to achieve a 50% antioxidant capacity (Locatelli et al., 2009). The lower the effective concentration (EC_{50}) value the more powerful the antioxidant capacity (Chintong et al., 2019). DPPH scavenging activity of haskap ranges from 66.5 to 90.4% in the literature when different cultivars, as well as extraction and processing conditions (time, temperature, pressure) were utilized (Khattab et al., 2016; Jiao, 2018; Li et al., 2019a). In another study haskap (86%) also demonstrated higher scavenging activity when compared to other plants such as tomato (*Lycopersicon esculentum* Mill.) (37%), rhubarb (*Rheum rhaponticum* L.) root (21%), European blueberry *(Vaccinium myrtillus* L.) (37%) and standard ascorbic acid (25%) (Raudsepp et al., 2013). In terms of the EC_{50} value, haskap measured the lowest with a median value of 30 ± 2 µg ml^{-1} compared to western blueberry (*Vaccinium uliginosum* Linn.) with a value of 35 ± 2 µg ml^{-1} and raspberry *(Rubus idaeus*) with an EC_{50} value of 50 ± 3 µg ml^{-1} (Zhao et al., 2011).

ORAC assay utilizes AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride], which when heated in the presence of enough oxygen forms peroxyl radicals (Schaich et al., 2015). These radicals can

quench the fluorescence of a probe, reducing the intensity of the solution and thus the absorbance measured. The extent of reduction is contingent upon the efficacy of the antioxidant (Borlinghaus et al., 2020).

The ABTS test measures the ability of antioxidants to neutralize the ABTS stable radical cation, which is a chromophore exhibiting a blue-green coloration (Singh and Singh, 2008). The presence of antioxidants leads to a reduction in the intensity of this chromophore. The extent of discoloration, measured by a decrease in absorbance at 734 nm, is influenced by the time of the reaction, the potency of the antioxidant involved, and the concentration of the sample (Munteanu and Apetrei, 2021).

Another antioxidant assay regularly used is FRAP. The principle of this assay is founded on the capacity of antioxidants to donate electrons, thus reducing iron from the ferric (Fe^{3+}) to the ferrous (Fe^{3+}) state (Benzie and Devaki, 2018). This changes the solution to a violet-blue colour which is measured at 593 nm. Therefore, the concentration of ferrous ions is proportional to the electron-donating ability of antioxidants (Chen et al., 2010).

Rupasinghe et al. (2012) used ORAC and FRAP to compare the antioxidant activity of berries, including nutrient-packed berries like blueberry, red table grape, blackberry, partridgeberry, strawberry, and raspberry. The berries' ORAC values ranged from 6.2 to 226.2 mmol Trolox equivalent (TE) 100 g^{-1} FW, whereas their FRAP values ranged from 0.8 to 4.7 mmol TE 100 g^{-1} FW. The FRAP values of the haskap cvs Borealis and Indigo Gem were found to be approximately six times higher than those of red table grape, raspberry, and strawberry, and thrice as much as partridgeberry, blueberry, and blackberry. The haskap cultivar, Tundra, exhibited an ORAC value that was twice as high as that of partridgeberry, blueberry, blackberry, and red table grape, and four times greater than that of strawberry and raspberry.

Another study quantified the polyphenols of some Western Canadian berries. In this study, haskap berries had the highest contents of polyphenols such as anthocyanins, as well exhibited the most potent antioxidant capacity (9.6 ± 0.6 mmol TE 100 g^{-1} FW) of all berries. The antioxidant activity of golden currant, chokecherries, lingonberries, and crowberries was found to be comparatively lower and ranged between 4.60 and 5.08 mmol TE 100 g^{-1} FW (Bakowska-Barczak et al., 2007). The variation in antioxidant capacity among the berries could be due to the difference in polyphenolic composition of each berry type, the capacity of the plant to synthesize polyphenols as well as differences in environmental and growth conditions (Debnath and Goyali, 2020).

Furthermore, the variations in total antioxidant activity among the assays may be attributed to the fact that the principle for each test differ significantly (Thaipong et al., 2006; Dudonne et al., 2009; Biskup et al., 2013). Studies comparing various antioxidant assays have concluded that the FRAP assay showed high reproducibility, while the ORAC assay may differ among runs (Thaipong et al., 2006; Shah and Modi, 2015). The ORAC technique has been reported to underestimate of the antioxidant capacity of complex food as compared to other antioxidant assays. This is believed to occur due to inadequate scavenging during the reaction period (Tian and Schaich, 2013). Moreover, compounds that possess the ability to absorb light at a specific wavelength of 734 nanometers have the potential to impede the accuracy of the findings (Apak et al., 2013).

The findings of a correlation study between different antioxidant activity parameters and polyphenols of haskap berries demonstrated a significant positive correlation (r= 0.94, $p \leq 0.05$) between the phenolic and the anthocyanin content. A positive correlation was also observed between the total polyphenolic content and antioxidant activity parameters DPPH (r=0.99, $p \leq 0.05$) and FRAP (r= 0.9, $p \leq 0.05$) (Zhao et al., 2015a). A similar result was obtained by Ponder et al. (2022) in 2019, where the antioxidant activity was measured using ABTS. Strong positive

correlations (r= 0.9, r=0.8, $p \leq 0.05$) were observed between the antioxidant activity and total phenolic content in organic haskap berries and conventional berries respectively. This suggests that anthocyanin may ultimately act as the main polyphenol responsible for haskap's antioxidant ability (Negreanu-Pirjol et al., 2023).

2.4.3 Varying antioxidant content among species

Significant differences have been observed in phytochemical content among different haskap cultivars and will be briefly discussed in this section.

Rupasinghe et al. (2012) quantified the total phenolic content among three haskap varieties and determined that Borealis (622.5 ± 81.5 mg GAE 100 g^{-1} FW) had significantly ($p \leq 0.05$) higher total phenolic content than Indigo Gem (500.8 ± 48.5 mg GAE 100 g^{-1} FW) and Tundra (428.1 ± 9.1 mg GAE 100 g^{-1} FW). Khattab et al. (2016) also had a similar finding in phenolic content between Tundra (8.1 mg GAE g^{-1} FW) and Indigo Gem (8.4 mg GAE g^{-1} FW), which were significantly higher than the Berry Blue (6.17 mg GAE g^{-1} FW) cultivar.

The antioxidant activities were analysed for five Saskatchewan (Canada) bred haskap berries in another study (Zehfus et al., 2021a). The Tundra variety had the highest value for total antioxidant activity as measured by ABTS scavenging antioxidant assay, which was 225.9 ± 5.8 mM TE 100 mg^{-1} FW, higher than the values recorded for Aurora, Blizzard, Honeybee, Indigo Gem cultivars which ranged from 131.4 to 207.0 mM TE 100 mg^{-1} FW.

Polyphenolic compounds also vary amongst the different haskap varieties. For instance, Tundra exhibited the greatest phenolic content at 727.0 ± 3.6 mg 100 g^{-1} FW, while Indigo Gem had a higher content of anthocyanin at 447.8 ± 5.9 mg 100 g^{-1} FW (Zehfus et al., 2021a).

Genotypic differences and extraction conditions might be the reason for the variations in polyphenols between studies. These results could also be influenced by harvesting time (De Silva, 2020) or even origin of cultivars (Orsavová et al., 2022).

2.5 Biological activities of haskap

The therapeutic properties of haskap, has rendered it the title “elixir of life” by Japanese Ainu aborigines. For centuries, haskap has been used for glaucoma, as a hypotensive agent, and for gastrointestinal and cardiovascular diseases (Celli et al., 2014). Additionally, it was used to treat malaria and certain blood disorders, slow down aging, as well as for the promotion of skin elasticity (Thompson, 2006). Haskap berries' biological functions are primarily attributed to their phenolic compounds (Wu and Hou, 2020). Several studies have shown evidence for the potential health benefits of polyphenol-rich haskap berry. Experiments carried out *in vitro* and *in vivo* also suggest the possible effectiveness of haskap in the prevention of chronic health conditions. These benefits, which include haskap’s antioxidant, anti-microbial, anti-carcinogenic, and anti-diabetic effects, will be discussed in this section.

2.5.1 *In vitro* studies

Several experiments have been conducted using animal and human cell lines to determine the efficacy of haskap extracts. According to Palikova et al. (2009), the use of *in vitro* subcellular and cellular model systems is irreplaceable for the determination of the biological activity, toxic properties, and metabolic mechanisms of xenobiotics. Pace et al. (2018) investigated the antiproliferative characteristics of C3G obtained from haskap, and its primary degradation products (protocatechuic acid (PCA) and phloroglucinaldehyde (PGA)), in relation to hepatocellular carcinoma (HepG2) and breast adenocarcinoma (MDA-MB-231) cells. The researchers found that the C3G-rich haskap berry fraction demonstrated a dose-dependent

inhibitory effect on HepG2 cell metabolic activity. This study also showed that PGA demonstrated cytotoxicity against both MDA-MB-231 and HepG2 cell lines. These findings suggest that haskap may have the ability to exhibit anti-carcinogenic effects in cells.

With diabetes being among the top ten non-communicative death-causing diseases worldwide, haskap is acknowledged for its anti-diabetic properties. Haskap berry extracts derived from four distinct cultivars, namely Aurora, Rebecca, Larissa, and Evie, were utilized to assess the potential of haskap berry polyphenols to inhibit carbohydrate-hydrolyzing enzymes and the formation of advanced glycation end products (AGE) *in vitro* (De Silva, 2020). The findings indicate that haskap exhibits an inhibitory effect on amylase and glucosidase in a dose-dependent manner. These enzymes are responsible for regulating the rate at which glucose is released into the bloodstream. The ability to inhibit AGE was observed in all cultivars' extracts. The findings of this investigation suggest that haskap extracts warrant further exploration as a potential therapeutic agent for diabetes.

Determining the effects of antioxidant-rich haskap extracts on ROS and by extension oxidative stress, has attracted the interest of many researchers. A recent study utilized rat microsomes, primary cultures of rat hepatocytes, and human umbilical vein endothelial cells (HUVEC) to evaluate the effects of the phenolic fraction of blue honeysuckle on cell survival and its effectiveness against oxidative damage in low density lipoproteins (oxLDL) (Palikova et al., 2009). The findings revealed that the phenolic fraction of haskap inhibited rat liver microsome peroxidation and that the phenolic fraction exhibited a protective effect against cellular damage caused by oxLDL in HUVEC and *tert*-butyl hydroperoxide (*t*BH) in both HUVEC and hepatocytes. The inhibition of microsome peroxidation and LDL oxidation presents a promising avenue for preventing certain diseases associated with oxidative stress.

Another study analysed the effect of phenolic extracts and fractions of haskap on the production of five intracellular radical scavenging enzymes, namely: catalase (CAT), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1), glutathione synthetase (GSS), and heme oxygenase-1 (HMOX1). The results indicate that the application of haskap phenolic treatments resulted in a reduction of transcripts for all radical scavenging enzymes that were assessed except for HMOX1 (Zehfus et al., 2021b). Similarly, another antioxidative study aimed to evaluate the effectiveness of anthocyanin-rich haskap extracts in preventing DNA damage caused by 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone (NNKOAc), a precursor of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in human lung epithelial BEAS-2B cells was conducted. They found that the utilization of haskap extracts as a pre-treatment technique resulted in a notable reduction in NNKOAc-induced DNA damage, DNA fragmentation, and intracellular ROS. Furthermore, the ataxia-telangiectasia mutated (ATM) dependent DNA damage repair pathway was upregulated in contrast to untreated BEAS-2B cells (Amararathna et al., 2020).

The impact of haskap on bacterial cells has also been studied. Yemiş et al. (2022) investigated the antibacterial efficacy of methanolic haskap extracts and tannic acid against *Cronobacter* spp. These pathogenic bacteria pose a significant risk of life-threatening illnesses including meningitis and sepsis in infant formula. The haskap extract demonstrated pH–dependent bacteriostatic and bactericidal activity against all tested *Cronobacter* spp. Additionally, the supplementation of haskap extracts resulted in a reduction of thermal resistance in *C. sakazakii*. Therefore, the utilization of haskap berry extract has the potential to enhance the safety of powdered infant formula.

2.5.2 *In vivo* studies

Although limited, *in vivo* studies on haskap berries have been conducted in both rodents and humans.

Animal model

Wang et al. (2017a) carried out an investigation in which eight-week-old mice were subjected to a seven-day administration of Yichun blue honeysuckle (YBHS) extract, followed by exposure to carbon tetrachloride (CCl_4) and subsequent sacrifice. Liver samples were obtained and the levels of thiobarbituric acid reactive substances (TBARS) and glutathione, as well as the activities of endogenous antioxidant enzymes, were assessed. The findings indicated that YBHS administration inhibits lipid peroxidation, increases glutathione levels, and elicits the activation of endogenous antioxidant enzymes such as SOD, CAT, GPX1, GSS. These suggest that YBHS may scavenge ROS by increasing the activity of the endogenous antioxidant defense system.

Human model

One human study utilized 20 adults between the ages of 62 and 81 years to investigate the cognitive effects of different dosages of anthocyanin-rich haskap berry extract (Bell and Williams, 2019). Additionally, blood pressure and heart rate measurements were collected and analyzed. The study found that the consumption of haskap berry extract resulted in a notable enhancement of postprandial episodic memory when compared to a control group with similar characteristics. However, the extract had no apparent impact on working memory and executive function. The results of the study also indicated a notable reduction in diastolic blood pressure and heart rate compared to the control group. This suggests that haskap may exhibit vasodilatory mechanisms and should be further analyzed.

Table 3. Summary of the activities of haskap berries based on their biological properties.

Properties	Activity	Reference
Anti-inflammatory effects	Blue honeysuckle extract treatment significantly reduced inflammatory cell infiltration.	(Jin et al., 2006)
Anti-proliferative/ anti-cancer effects	C3G-rich haskap extract exhibited a time and dose-dependent inhibition of HepG2 cell proliferation.	(Pace et al., 2018)
	The utilization of haskap extracts as a pre-treatment technique resulted in a reduction in NNKOAc-induced DNA damage, and DNA fragmentation.	(Amararathna et al., 2020)
Anti-microbial effects	Haskap extracts exhibited both bacteriostatic and bactericidal properties against *Cronobacter* spp.	(Yemiş et al., 2022)
	Haskap demonstrated strong antibacterial activity against foodborne pathogens but not probiotic microorganisms.	(Yemiş et al., 2022)
Anti-neurodegenerative effects	Supplementation with haskap berry extract improved episodic memory.	(Bell and Williams, 2019)
Anti-diabetic effects	Haskap berry extracts exhibit an inhibitory effect on AGE formation and carbohydrate hydrolyzing	(De Silva, 2020)

	enzymes (alpha-amylase & alpha-glucosidase) in a dose-dependent manner.	
Antioxidant effect	Haskap phenolic extract inhibited liver microsome peroxidation in rats.	(Palikova et al., 2009)
	Yichun blue honeysuckle inhibited lipid peroxidation and elicited the activation of endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase.	(Wang et al., 2017a)
	The utilization of haskap extracts as a pre-treatment technique resulted in a reduction of intracellular reactive oxygen species.	(Amararathna et al., 2020).
	The application of haskap phenolic treatments resulted in a reduction of radical scavenging enzymes (CAT, SOD1, GPX1, GSS)	(Zehfus et al., 2021b)

2.6 Applications of haskap berry

Haskap berries have versatile potential applications in the food industry, as well as in the production of health products, cosmetics, medicine, and packaging materials. Haskap is presently promoted in various forms such as jam, jelly, wine, candies, gelatin, and puffed snacks (Liu et al.,

2009). According to Senica et al. (2019), there are additional food options that include honeysuckle liqueur and smoothie. Its potential as a viable source of natural antioxidants and natural colourants should be explored, given the ongoing consumer demand for healthier plant-based alternatives (Grobelna et al., 2020). Owing to the reasons stated above, the utilization of haskap as a functional food source shows potential for future development. According to the study conducted by Yemiş et al. (2022), haskap berry infusions exhibit potent antimicrobial properties while having no adverse effects on probiotics. Consequently, the food industry can use haskap berry infusions as functional ingredients, including antioxidants, antimicrobials, and preservatives during processing.

Numerous studies have shown that haskap has a wide spectrum of biological properties and can be incorporated into pharmaceuticals (Shang, Pan, Li, Miao, & Ding, 2011). One of the most prevalent phenolic acids in haskap is CGA and it has current applications in the pharmaceutical, food processing, and cosmetic chemical industries (Xiang and Ning, 2008). Thus, CGA extracted from haskap can be utilized for its free radical scavenging and antioxidant activities.

The potential utilization of anthocyanin as a functional constituent for health enhancement is restricted due to its chemical instability during the extraction process. The incorporation of encapsulation can potentially increase the stability of anthocyanin (Yousuf et al., 2016). Various novel delivery systems such as encapsulation of drugs into nanoparticles have been proposed to regulate the pharmacokinetics of existing drugs, thus improving the delivery of bioactive agents to specific target sites (Aqil et al., 2013). Additionally, the utilization of proanthocyanins and pure natural anthocyanins found in haskap presents a viable option for formulating mild, nontoxic skin care creams and naturally coloured lipsticks in the cosmetics industry (Łyko et al., 2022).

In a spectrophotometric investigation, it was observed that the leaves of haskap contain compounds that exhibit a diverse range of pharmacological effects. Thus, it is possible to utilize haskap leaf extracts in various applications such as food additives, cosmetic formulations, and pharmaceutical and perfumery products (Krotova et al., 2020).

2.7 Extraction of bioactive compounds using sous vide technology

Bioactive thermolabile compounds in fruits can be lost through food processing methods such as blanching, cooking, pasteurization, dehydration and freezing (Ellong et al., 2015; Mieszczakowska-Frąc et al., 2021). The sous vide technique involves cooking food in a vacuum-sealed plastic bag at a precisely regulated temperature that facilitates efficient and consistent transfer of heat from the water to the food product (Singh et al., 2023). The utilization of precise temperature in cooking allows for almost-perfect reproducibility (Carr et al., 2015). It also provides a greater degree of control which helps to prevent nutrient loss by oxidation and thermal processing compared to conventional cooking techniques (Pandita et al., 2023). Iborra-Bernad et al. (2014) prepared red cabbage by both traditional cooking and sous vide technique and analysed the anthocyanin content of red cabbage from both techniques. The loss of anthocyanin in traditionally cooked cabbage was found to be twice as high as the sous vide samples. Furthermore, Rondanelli et al. (2017) analysed the mineral content of legumes prepared using sous vide technology at 65 °C for 10 hr and 74 °C for 4 hr, versus traditional cooking for 1 hr. They found that magnesium, iron, zinc, and copper, were all significantly higher in the sous vide prepared beans, than the traditionally cooked beans. Cooking in heat-stable and vacuum-sealed bags increase the shelf life of products as lipid oxidation is inhibited due to the absence of oxygen (Bhuyan et al., 2022). Additionally, it limits the loss of flavour volatiles through evaporation, as well as inhibits off flavours from oxidation, while improving nutritional value (Baldwin, 2012). Yang et al. (2020) utilized both sous vide and

traditional cooking in the preparation of beef meat that was then analysed by panelist in a sensory analysis. The beef prepared using sous vide had greater likability among consumers, with regards to colour and taste compared to traditional cooking. Thus, sous vide technology shows potential as a viable approach for preparing antioxidant rich extracts from haskap berry.

2.8 CONCLUSION

Oxidative stress within the human body exerts a significant influence on the initiation and progression of diseases. Fruits such as berries, which are abundant in antioxidants, exhibit the potential to protect against oxidative damage. Haskap is known to possess physiologically active phytochemicals such as phenolic acids, flavonoids, minerals, and vitamins. The antioxidant capacity of haskap may be attributed to its flavonoid content, which could be considered the primary polyphenol responsible for these properties. Polyphenols in haskap possess the ability to eliminate free radicals, regulate pro-oxidant and antioxidant mechanisms, and modulate inflammatory status. As a result, they can effectively inhibit ROS and prevent or mitigate the activity of specific enzymes and pathways that may lead to chronic illnesses. Compared to other berries that are acknowledged for their beneficial impact on human health, haskap demonstrates a higher concentration of several antioxidant compounds. The therapeutic properties of haskap, has earned it the title “elixir of life” by Japanese Ainu aborigines. It has been shown to possess antioxidant, anti-microbial, anticarcinogenic, and anti-diabetic properties in various *in vitro* and *in vivo* studies. These properties have rendered haskap a potentially versatile ingredient in the food industry and a potential component in health products, including functional foods, natural cosmetics, and plant-based drugs.

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CHAPTER 3

DEVELOPMENT OF NOVEL GREEN TECHNOLOGY FOOD-BASED EXTRACTION USING SOUS VIDE TECHNIQUE

3.0 ABSTRACT

Haskap (*Lonicera caerulea* L.) is an antioxidant-rich berry that can be used as a functional food ingredient. Conventional methods for extraction of antioxidants from haskap berries are however time-consuming and often require large quantities of toxic organic solvents. The high extraction temperatures typically used consume a lot of energy and tend to destroy its thermolabile compounds. There is the need to develop a novel food-based technique to obtain the best conditions for extracting antioxidant-rich bioactive compounds from haskap berries. An investigation into the effects of extraction method (conventional and sous vide), extraction solvent (pure ethanol, pure water, and ethanol: water (1:1)), extraction time (30 min and 60 min), and extraction temperature (30 °C - 50 °C) on the phenolics and antioxidant content of haskap cultivars Indigo Gem and Wojtek was conducted using double-factorial experiments. The Folin-Ciocalteu assay was used to evaluate the total phenolics content (TPC) while the total antioxidant activities (TAA) of haskap extracts were measured using ferric reducing antioxidant power (FRAP) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. The results showed that extraction conditions had a significant ($p < 0.05$) effect on the extraction of phenolic compounds and the antioxidants from haskap. The optimized conditions using the sous vide method were ethanol: water (1:1) for 60 min at 40 °C, with FRAP values of 175. 4 ± 0.4 µmol TE g^{-1} FW, while the conventional method yielded 51.5 ± 0.2 µmol TE g^{-1} FW. The ABTS values were 21658.1 ± 0.4 µmol TE 100 g^{-1} FW and 9344. 6 ± 0.1 µmol TE 100 g^{-1} FW for sous vide and conventional methods respectively. Indigo Gem had the highest phenolic content and antioxidant activity of the two cultivars analyzed using the sous vide method. The TPC was significantly correlated with total

anthocyanin content (TAC) (r = 0.937, $p < 0.001$) and TAC showed a strong positive correlation to FRAP (r= 0.844, $p < 0.001$) and ABTS ($r = 0.908$, $p < 0.001$) in extracts that were prepared using the sous vide technique. These results indicate that haskap is a good potential source of dietary antioxidants, and the sous vide technique can be utilized for obtaining antioxidant-rich extracts from berries.

Keywords: Indigo Gem, sous vide, antioxidant, extraction, phenolics, haskap berries.

3.1 INTRODUCTION

Phytonutrients are non-essential plant-derived chemical compounds that are known to provide health benefits to humans and include polyphenols, carotenoids, flavonoids, and anthocyanins (Gibbs, 2007; Salter et al., 2012). They possess various beneficial properties, such as antioxidant, antibacterial, anti-inflammatory, chemopreventive, hepatoprotective, hypolipidemic, neuroprotective, and hypotensive activities (Prakash and Gupta, 2009). Phytonutrients also act as preventive measures against various diseases including diabetes, cancer, and heart disease (Soquetta et al., 2018). In addition, these compounds have been found to induce apoptosis in cancer cells, act as diuretics and central nervous system stimulants, and modulate the immune system (Prakash and Gupta, 2009).

The concentration of polyphenolic compounds in fruit extracts depends on various factors such as variety of the fruit and processing technique used (Munzuroglu et al., 2003). Prior research has demonstrated a correlation between the composition and efficacy of plant extracts and the specific extraction methods used in their preparation (Castro-López et al., 2017). The method of extraction has a substantial influence on the extraction efficiency of antioxidants from plants (Zhang et al., 2015).

The methods used for extraction of polyphenolic compounds can be categorized as either conventional or non-conventional. Many of the conventional methods used for extraction of phytochemicals from haskap use solid-liquid extraction techniques such as hot water bath, maceration, and percolation, requiring large amount of solvents such as formic acid, acetone, and methanol (Rupasinghe et al., 2012; Celli et al., 2015; Khattab et al., 2016; Zehfus et al., 2021a). Extracts prepared using these solvents are only used for phytochemical analysis and are not consumable as these solvents are toxic to the human body (Ashurst and Nappe, 2018) . Additionally, these methods are often time-consuming and use elevated temperatures which can denature bioactive compounds that are beneficial to human health (Khattab et al., 2017; MacLean et al., 2021). Conversely, green technologies often require less time, reduced energy consumption and organic solvents, thus maintaining low environmental impact and health implications, while increasing efficiency of the extraction process (Rodríguez-Pérez et al., 2015; Cannavacciuolo et al., 2022).

There are two non-conventional methods that have been found in the literature to date for extraction of polyphenols from haskap fruit. The first is extraction using supercritical carbon dioxide ($scCO_2$) and water as co-solvent (Jiao, 2018). This is reported to have superior efficiency of anthocyanin extraction and increased antioxidant activity when compared to traditional extraction methods (Jiao, 2018; Silva et al., 2019). While this method is safe for food grade extraction and easily removable from the product, the high pressure required, maintenance costs for equipment, and its low ability to dissolve polar species has limited the industrial applications (Jesus and Meireles, 2014; Bitencourt et al., 2016; Chemat et al., 2019). Cold plasma-assisted enzyme method (CPEM) is another green extraction method that has been used (Zhou et al., 2023). In comparison to alternative extraction methodologies, CPEM offers the

benefits of decreased extraction time, reduced solvent use, and increased active constituents and antioxidant potential (Heydari et al., 2023; Zhou et al., 2023). There is no distillation of the solvent, hence higher efficiency extraction of phytochemicals is simpler and faster, but the use of enzymes is costly for large-scale industrial applications (Rodríguez-Pérez et al., 2015; Chemat et al., 2019).

The antioxidant potential of polyphenolic compounds in extracts is affected by chemical and enzymatic reactions that occur during processing or storage (López-Vidaña et al., 2017). Piepiórka-Stepuk et al. (2023) demonstrated that the loss of bioactive compounds such as vitamin C and anthocyanin in pumpkins (*Cucurbita moschata* and *Cucurbita maxima*) were affected by treatment preparations such as freezing at -18 °C, blanching at 80 - 90 °C or boiling at 100 °C. Boiling resulted in a loss of 23 - 45% in phenolic compounds and 31 - 51% in flavonoid compounds, depending on the variety of pumpkin. Bustos et al. (2018) also showed that preparation methods using high temperatures such as 130° C resulted in higher degradation of polyphenols in berries such as raspberry (*Rubus idaeus*), redcurrants (*Ribes rubrum*) and blackcurrants (*Ribes nigrum*), and boysenberry (*Rubus ursinus x idaeus*), compared to drying at 65 °C. This occurs because the stability of many polyphenolic compounds is affected by temperature (Alara et al., 2021).

It is thus important to consider the influence of temperature on the stability of analytes when formulating analytical methodologies as the degradation of thermolabile analytes during extraction is often an inevitable occurrence (Mediani et al., 2013; Mokrani and Madani, 2016; Muhammad et al., 2019). Sous vide technology provides a solution to this problem by mitigating loss of thermolabile compounds during the cooking process as its temperature can be precisely regulated (Kathuria et al., 2022).

The food industry is actively searching for environmentally sustainable sources of valuable compounds that can be used as potential food ingredients. This is in response to consumers

desirous of plant-based nutrient packed food with increased health benefits (Carpentieri et al., 2021). This creates a demand for green technology method for the extraction of antioxidant rich compounds from fruits that are simple, safe for consumption, and cost effective. The aim of this study therefore is to optimize sous vide technology, for the extraction of antioxidant rich bioactive compounds from haskap berry.

3.2 MATERIALS AND METHODS

3.2.1 Chemical Reagents and standards

Pure ethanol (HPLC grade) was supplied by VWR International (Ontario, Canada) and ultrapure water used where applicable. Quercetin, Folin-Ciocalteu reagent, sodium phosphate monobasic, sodium phosphate dibasic anhydrous, sodium acetate, glacial acetic acid, 2,4,6-tripyridyl-*s*-triazine, hydrochloric acid (HCl 36.5% v/v), iron (III) chloride hexahydrate, trolox, ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], horse radish peroxidase (HRP), aluminum chloride, potassium chloride were purchased from Sigma Aldrich (Ontario, Canada).

3.2.2 Plant Material

Haskap (*Lonicera caerulea* L.) cvs. Indigo gem and Wojtek were obtained from North 45 Orchards, Stewiacke, Nova Scotia, Canada (latitude 45.18˚, longitude - 63.22˚). The haskap plants were cultivated in alluvium, loam to silty clay, and loam soil types, during the 2021 and 2022 growing seasons. Samples were packaged in paper bags and frozen at -20˚C for 24 hrs and subsequently shipped by courier on ice to Memorial University of Newfoundland, Grenfell Campus. Samples were then stored in a refrigerator (-20 ˚C) for two weeks, until sample preparation.

3.2.3 Extraction procedure

Haskap berries were separated into three (3) replicates and freeze-dried at -80 ˚C, until constant dry weight. Aluminum foil was used to cover glass containers to reduce light exposure to samples. The dried berries were ground into powder using a coffee grinder and subsequently stored in airtight amber glass containers at -20 °C to protect from moisture, excess air, and light.

3.2.3.1 Sous vide extraction

For sous vide extraction, 375 mg haskap powder was mixed with 15 mL of extraction solvent which included pure ethanol, ultrapure water, and ethanol: water (1:1) solution. These ethanol-water (EtOH: H_2O) mixtures (100:0, 0:100, and 1:1 v/v) were tested to investigate the effect of solvent type on the phenolic content and antioxidant activity of haskap extract. The mixtures were then transferred to 8″ x 12 ″ BPA and dioxin-free food grade vacuum sealing bags and vacuum sealed using VacMaster VP215 (Ary Inc., Kansas City, MO, USA) vacuum sealer with a sealing time of 21 secs. The bags were then placed in Sous vide Supreme 10LS (Eades Appliance Technology, Dallas, TX 75220, USA) water oven, after the set temperature (30 ˚C, 40 ˚C, or 50 ˚C) was reached and extracted for 30 min or 60 min. The resulting extracts were centrifuged at 5000 g for 15 min at 22 ˚C. The supernatant was collected and stored at -80 ˚C in amber-coloured containers until time for analysis. Sous vide extraction of dried berries was performed in triplicates (n=3).

3.2.3.2 Conventional extraction using cold extraction

The conventional extraction of haskap was performed using the method described by Deng et al. (2014) with modifications. For extraction of the lipophilic phase, 20 mg of powdered haskap was homogenized with 1 mL pure ethanol and the mixture incubated at room temperature for 10 min. Following incubation, the resulting mixture was centrifuged at 14000 g for 10 min at 22 ˚C. The

supernatant (lipophilic extract) was then collected. The remaining residue was used to extract the hydrophilic phase, by adding 1 mL of pure water to each centrifuge tube. The hydrophilic extracts were prepared as previously described for the lipophilic phase. Both lipophilic and hydrophilic extracts were stored at -80˚C until further analysis.

3.2.4 Experimental design for sous vide extraction optimization

In the present study, double-factorial experiments were used to determine the optimal conditions for extracting antioxidants and phenolic compounds from haskap berries. Therefore, multiple extraction parameters were manipulated simultaneously, while the remaining parameters were held constant. A total of five parameters including cultivar (Indigo Gem and Wojtek), extraction solvent (pure ethanol, pure water; and equimolar ethanol: water mixture), extraction time (30 min and 60 min), extraction temperature (30 °C – 50 °C) and method of extraction (sous vide and conventional method) were studied. In this experiment, the time, temperature, and cultivar were initially simultaneously manipulated while the solvent remained constant. The optimal extracting conditions were selected based on the FRAP and ABTS measurements.

The antioxidant activity and phenolic content of extracts prepared using the conventional extraction, was compared to the results of the extracts prepared using the optimized sous vide extraction technique. Furthermore, the total phenolic content, total anthocyanin content, and total flavonoid content of sous vide-based extracts were analyzed to determine the composition of the extracts.

3.2.5 Analysis of haskap extract using Cytation Image microplate reader

The total phenolic, flavonoid and anthocyanin content, as well as antioxidant activity of haskap extract were analyzed by ultraviolet–visible spectrophotometry using Cytation image microplate

reader (BioTek, Vermont, USA). For comparison with the literature, the results of these analyses were represented in fresh weight using an equation adapted from Nielsen (2010) :

$$FW = [DW] \times \frac{(100 - \%MC_{WB})}{100}$$

Where; $\%MC_{WB} = \frac{\text{wt } H_2O}{(\text{wt } H_2O + \text{Dry solid})} \text{ X } 100$

Where FW = fresh weight of sample; DW = dry weight of sample; $\%MC_{WB}$ = percent moisture content in wet basis; wt H_2O=Initial weight of fresh fruit - weight of dried fruit; and wt H_2O = weight of the water

3.2.5.1 Total Phenolic Content

The total phenolic content (TPC) of haskap berries was estimated using the Folin-Ciocalteu assay described by (Rajakaruna et al., 2022). Briefly, 125 µL of 10-fold diluted Folin-Ciocalteu reagent was added to 25 µL of haskap extract in 96 well microplate. Subsequently, 50 µL of water was added for hydrophilic extracts whereas 50 µL pure ethanol was added to lipophilic extracts. This mixture was then incubated for 30 mins and the absorbance measured at 755 nm on the microplate reader. Quercetin standard curves (0-1 mg/mL, $R^2 \geq 0.987$) were used to quantify the phenolic contents and the results expressed as milligrams quercetin equivalents per gram fresh weight fruit (mg QE g^{-1} FW).

3.2.5.2 Total antioxidant activity (TAA) Analysis- FRAP

Antioxidant activity was measured using ferric reducing antioxidant power (FRAP) method from Thomas et al. (2010) with modifications. Here, 20 µL of extract was mixed with 180 µL of FRAP working solution. The resulting mixture was incubated in the dark for 30 minutes, then the absorbance was measured at 593 nm on the microplate reader. The concentration of antioxidants

in haskap was determined based on trolox standard curves (0-25 µM, $R^2 \geq 0.99$) and the TAA, expressed in micromoles equivalents of trolox per gram of fresh weight fruit (µmol TE g^{-1} FW).

3.2.5.3 Total Antioxidant activity (TAA) Analysis - ABTS

The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) antioxidant assay described by Manful et al. (2020) was modified and utilized to validate the TAA results obtained from the FRAP assay. For working solutions, 160 µL of 25 mM ABTS, followed by 480 µL of 125 µM H_2O_2, 200 µL of 0.2 mg mL^{-1} HRP and 1160 µL of water were mixed in this order consecutively for the hydrophilic (HAA) working solution whereas 160 µL of 25 mM ABTS, 480 µL of 125 μM H_2O_2, 1000 µL of 0.2 mg/mL HRP, and 360 µL pure ethanol were combined consecutively to prepare the lipophilic (LAA) working solution. For analysis, 200 µL of either LAA or HAA working solution, depending on whether the extract to be added was lipophilic or hydrophilic was first added to a 96-well microplate and the absorbance read at 730 nm on the microplate reader. Subsequently, 20 µL of haskap extract was added to the working solution and the absorbance of the resulting mixture measured again following incubation in darkness for 10 mins. The antioxidant activity was determined using trolox standard curves (0-25 µM, $R^2 \geq 0.98$) for LAA and HAA respectively. The TAA was calculated by adding both the HAA and LAA result for a specific sample. The results were expressed in micromoles equivalents of trolox per gram of fresh weight fruit µmol TE g^{-1} FW.

3.2.5.4 Total Flavonoid Content

The flavonoid content of haskap berries was analyzed by the aluminum chloride colourimetric method as done by Do et al. (2014) with slight modification in volume of reagents to extract used. Specifically, 10 µL of 10 % w/w $AlCl_3$ (aq) was added to 96 well microplate followed by 150 µL of either water or pure ethanol depending on whether hydrophilic or lipophilic extracts were being

analyzed. After mixing, 10 µL of 1 M CH_3COONa (aq) and 50 µL of haskap extract were added and resulting mixture homogenized. The plates were then incubated for 20 mins in the dark and absorbance measured at 510 nm on the microplate reader. The total flavonoid content was determined using quercetin standard curves (0-1 mg/mL, $R^2 \geq 0.97$) and results expressed as milligrams quercetin equivalents per gram fresh weight fruit (mg QE g^{-1} FW).

3.2.5.5 Total Anthocyanin Content

The anthocyanin content of haskap berries was measured using the differential pH method using buffer solutions of pH 1.0 and pH 4.5 as described by (Chen et al., 2014) with modifications in the ratio of extract to reagent. For pH 1.0, 40 µL of haskap extract was mixed with 160 µL of KCl (0.025 M, pH 1.0). The plate was then incubated at room temperature for 10 min. Afterward, the absorbance was measured at 510 nm and 710 nm on the microplate reader. The same steps were used for pH 4.5 by mixing 40 µL of haskap extract with 160 µL of CH_3COONa buffer (0.4 M, pH 4.5), and the absorbance was measured at 510 nm and 710 nm on the microplate reader. The anthocyanin content was calculated using the equation:

$$\text{Anthocyanin content (mg C3G L}^{-1}) = \frac{A}{\varepsilon \times l} \times MW \times DF \times \frac{V}{W} \times 1000$$

Where; $A = (A_{510nm} - A_{710nm})_{pH1.0} - (A_{510nm} - A_{710nm})_{pH4.5}$; MW (molecular weight) = 449.2 g.mol^{-1} for cyanidin-3-glucoside (C3G); DF = dilution factor; W = sample weight (mg); l = path length in cm; V= volume (L), and ε = 26,900 M extinction coefficient in L mol^{-1} cm^{-1} for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

Results were expressed as milligrams of cyanidin-3-glucoside equivalents per gram fresh weight fruit (mg C3G g^{-1} FW).

3.2.6 Statistical approach

XLSTAT (Premium 2017, Version 19.5, Addinsoft, New York, USA) was used for statistical analysis. All measurements were performed in quadruplicates (n=4), and results were expressed as mean ± standard error (SE). Significant differences ($p \leq 0.05$) between means were determined by Tukey (HSD), one-way analysis of variance (ANOVA). Three-way ANOVA was used to determine statistical differences for factors that doubly interacted. Pearson correlation was used to analyze the relationship between antioxidants and polyphenol compounds.

3.3 RESULTS AND DISCUSSION

3.3.1 Phenolic content and antioxidant activity of haskap cvs. Indigo Gem and Wojtek

One approach to evaluating the bioactive potential of edible haskap berries is to measure their antioxidant activity and polyphenolic compound content (Gazdik et al., 2008). One study also noted that the impact of phenolic compounds should be considered when assessing the bioactive value of food nutraceuticals (de Camargo et al., 2019). In this study, haskap cvs Indigo Gem and Wojtek were analyzed at different times (30- 60 mins) and temperatures (30, 40, 50˚C), to compare the phenolic content and antioxidant activity of extracts prepared using the sous vide technique. This was done to determine which cultivar possessed superior phytonutrient content and would potentially have the most biological benefits on the human body if it were to be incorporated for use as a functional food ingredient.

Table 1 gives a comparison of the impact of haskap cultivar on the phenolic content as measured by Folin-Ciocalteu method. The TPC of the two haskap cultivars using sous vide extraction ranged from 3.6 to 8.1 mg QE g^{-1} FW and 3.9 to 8.3 mg QE g^{-1} FW for Indigo Gem and Wojtek respectively. The greatest yield was observed at 60 min and 40 °C for both cultivars and is consistent with previous reports of high phenolic content in the literature for Indigo Gem, using

total phenolic chromatographic index (659.2 ± 10.5 mg 100 g^{-1} FW) (Zehfus et al., 2021a) as well as phenolic assay (8.4 ± 0.0 mg GAE g^{-1} FW (Khattab et al., 2016). Conversely, Wojtek cultivar showed a lower content of phenolics (149.3 - 183.7 mg 100 g^{-1}) in a previous report than observed in the present study (Ochmian et al., 2012). Under the optimal conditions however, no significant difference ($p \geq 0.05$) was observed in the total phenolic content between Indigo Gem and Wojtek (**Table 1)**.

Table 1. Phenolic content of haskap (*L. caerulea*) cultivars extracted by green sous vide method.

Haskap variety	Time (min)	Temperature (°C)	TPC
Indigo Gem	30	30	4.8 ± 0.2c
		40	3.8 ± 0.1b
		50	3.6 ± 0.1d
	60	30	5.7 ± 0.2b
		40	8.1 ± 0.2a
		50	4.6 ± 0.2b
Wojtek	30	30	4.4 ± 0.1c
		40	5.0 ± 0.3b
		50	4.5 ± 0.2c
	60	30	3.9 ± 0.1c
		40	8.3 ± 0.1a
		50	6.0 ± 0.3b

TPC values represent means ± standard error (n = 4). Means within the same column followed by different letters (a-c) are significantly different ($p \leq 0.05$). TPC = total phenolic content in mg QE g^{-1} FW. The lipophilic fraction was extracted using pure ethanol and deionized water was used for the extraction of the hydrophilic fraction.

This is contrary to the findings of Raudonė et al. (2021), who qualitatively and quantitatively profiled different classes of polyphenols such as flavonoids, including anthocyanin, and phenolic acids in Indigo Gem and Wojtek. He found that while phenolic acids such as quercetin, ferulic

acid, neochlorogenic acid and caffeoylquinic acid content did not differ significantly between the cultivars, anthocyanin and flavonoids were significantly higher in Indigo gem. This resulted in Indigo Gem having a higher total phenolic content than Wojtek.

While both a measure of the antioxidant activity and polyphenols represent its bioactive contents, if one component were to be chosen to determine the best cultivar, based on phytochemical content, it would be the antioxidant activity. The evaluation of antioxidant activity of Wojtek and Indigo Gem were assessed using two *in vitro* methods, namely ABTS and FRAP assays. The highest antioxidant activity was retained by sous-vide extraction of 60 min, 40°C for both Indigo Gem and Wojtek (**Table 2)**.

Table 2. Antioxidant capacity of haskap (*L. caerulea*) cultivars extracted by sous vide method.

Haskap variety	Time (min)	Temperature (˚C)	FRAP-TAA	ABTS-TAA
Indigo Gem	30	30	136.2 ± 0.5c	79.8 ± 0.7c
		40	135.5 ± 0.8c	92.1 ± 0.6b
		50	110.4 ± 0.7c	63.8 ± 0.4d
	60	30	171.9 ± 0.2b	80.6 ± 0.7c
		40	228.3 ± 0.5 a	107.4 ± 1.3 a
		50	178.5 ± 0.5b	74.6 ± 1.0c
Wojtek	30	30	160.5 ± 1.0b	67.9 ± 1.0d
		40	167.3 ± 0.8b	109.8 ± 0.4a
		50	195.0 ± 0.6a	109.3 ± 0.8a
	60	30	160.6 ± 1.2b	72.0 ± 0.4c
		40	182.8 ± 1.2b	104.5 ± 0.4a
		50	165.8 ± 0.7b	99.2 ± 0.8b

Antioxidant capacity values represent mean ± standard error (n = 4). Means within the same column followed by different letters (a-c) are significantly different ($p \leq 0.05$). FRAP = ferric reducing antioxidant power antioxidant assay; ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) antioxidant assay. TAA = Total antioxidant activity in µmol TE g^{-1} FW. The lipophilic fraction was extracted using pure ethanol and deionized water was used for the extraction of the hydrophilic fraction.

The haskap cultivar Indigo Gem had significantly ($p < 0.05$) higher antioxidant activity than Wojtek at 60 min and a temperature of 40 °C when the FRAP assay was utilized, whereas the ABTS does not demonstrate any significant ($p > 0.05$) differences between antioxidant activity of Indigo Gem at 60 min, 30°C, and Wojtek at 30 min, 40°C or 50°C (**Table 2)**.

The FRAP value for Wojtek in one study was 35.5 ± 2.8 µmol TE g^{-1} FW (Kucharska et al., 2017), and Rupasinghe et al. (2012) reported Indigo Gem FRAP values as 46.9 ± 3.0 µmol TE g^{-1} FW, both of which are lower than the values obtained in this study. The observed variation in antioxidant activities of haskap cultivars among the different studies can be attributed to various factors such as climatic conditions and agricultural practices used in the cultivation or different extraction conditions (Gawroński et al., 2020; Gołba et al., 2020; Zehfus et al., 2021a). In the case of the present study however, Indigo Gem and Wojtek were cultivated in the same location and under similar growing practices, and the same method of extraction was used. The findings thus suggest that the primary source of variation in antioxidant activity between the two cultivars is genetic differences.

The general trend of the results from this experiment agrees with others reported by Raudonė et al. (2021), who compared the scavenging ability of Indigo Gem, Wojtek, and other cultivars using a few antioxidant assays, including ABTS and FRAP. It was found that Indigo Gem had significantly higher free radical scavenging ability than Wojtek. He also reported similar results for FRAP assays. In his study, the FRAP values were 707.6 ± 23.8 µmol TE g^{-1} and 153.3 ± 27.7 µmol TE g^{-1} for Indigo Gem and Wojtek cultivars respectively. These results are consistent with those observed in the present study that Indigo gem FRAP-TAA is higher than Wojtek under optimal extraction conditions. Furthermore, while two antioxidant assays were used as a means of validating the results obtained, the methods have different response parameters (Li et al., 2019b).

The results obtained for the same substance using different antioxidant assays thus vary (Müller et al., 2011). Interestingly FRAP assay has demonstrated high reproducibility, while ABTS radicals are not very stable, resulting in lower reproducibility and the assay being less reliable (Shah and Modi, 2015).

Indigo Gem had the highest antioxidant activity at the optimal extraction conditions based on the interaction of cultivar, time, and temperature (**Table 2**). Hence it was the cultivar of choice for subsequent analyses. Additionally, the superior quality of Indigo Gem among other cultivars has been widely noted. It is considered one of the most popular and easiest to grow cultivars (Lauritzen et al., 2015). Indigo Gem is commonly grown in Nova Scotia, Canada, and has often been used as a reference for comparing all the other cultivars (Iheshiulo et al., 2022). It has a higher sugar content in comparison to other analyzed cultivars which may serve to mask the typical astringent taste commonly associated with haskap berries (Auzanneau et al., 2018). Indigo Gem was also found to have the highest anthocyanin content among other varieties such as Aurora, Blizzard, Honey Bee, and Tundra (Zehfus et al., 2021a).

3.3.2 Effect of time and temperature on extraction yield

The efficiency of the method of extraction and the recovery of antioxidant compounds from natural sources are influenced by the extraction time and temperature (Zhang et al., 2007; Wang et al., 2008; Mokrani and Madani, 2016). In this study, the antioxidant activity was measured for Indigo Gem extracts prepared using the sous vide technique at three different temperatures; 30 °C, 40 °C and 50 °C, for a duration of 30 min and 60 min respectively to evaluate the effect of extraction time on the antioxidant activity. The results showed that the radical scavenging activity of Indigo gem increased ($p \leq 0.5$) with extraction time (**Table 2)**. In particular, ABTS-TAA values increased from 92.0 µmol TE g^{-1} FW to 107.4 µmol TE $g^{-}1$ FW when the extraction time was extended from

30 – 60 min **(Table 2)**. A similar trend was observed for TAA-FRAP values which increased from 135.5 µmol TE g^{-1} FW to 228.3 µmol TE $g^{-}1$ FW over the same extraction duration **(Table 2).** The findings revealed that the optimal time for the highest extraction of antioxidants in haskap berries under sous vide conditions was 60 mins at an extraction temperature of 40 °C. These results, taken together, are in good accordance with Hismath et al. (2011) who noted that an extraction time of 59.3 mins at a temperature of 40.88 °C were optimal conditions for the extraction of polyphenols from neem (*Azadirachta indica*) leaves.

Other studies have also shown that polyphenolic content and antioxidant activity in plant extracts increased when extraction time was increased (Zhang et al., 2011; Mokrani and Madani, 2016). However, this is up to a certain time after which increasing extraction time does not improve recovery. This phenomenon may be elucidated by invoking Fick's second law of diffusion, which postulates that a state of equilibrium will eventually be reached between the concentrations of solute present in the solid matrix and those present in the solvent (Cussler, 2013). Mokrani and Madani (2016) noticed an increase in antioxidant activity from 30 min up to 180 min, after which it drastically declined. Considering that this experiment stopped at 60 min, there is still room for optimizing the time that could potentially yield more antioxidants before yield declines. Many studies have used times ranging from a few min to several hours for extraction (Mokrani and Madani, 2016; Ozturk et al., 2018; Tan et al., 2013; Xu et al., 2017). The purpose of optimizing this technique, however, is to establish a method that produces antioxidant rich extracts in a time efficient manner. This is because from an industrial production perspective, an extended extraction time results in reduced efficiency of equipment utilization (Shi et al., 2003). Additionally, compared to these long extraction times, a short extraction time could reduce thermal degradation (MacLean et al., 2021). Moreover, it is important to note that time of contact is sometimes not the

main variables affecting the extraction efficiency. Other research has shown that total phenolic content and thus antioxidant activity did not significantly change when extraction time was increased (Prasad et al., 2009; Belwal et al., 2016; Chen et al., 2018). Furthermore, prolonged extraction periods increase the probability of phenolics oxidation (Naczk and Shahidi, 2006). This may be influenced by other interactions such as temperature and solvent concentration and thus a combination of these factors could result in changes in extraction yield. At a higher temperature, the antioxidants yield may be greater with shorter extraction time, whereas at a low temperature, the yield may be higher with an increase in time as well as an increase in concentration of solvent could also cause higher yield at a lower extraction time (Silva et al., 2007; Quispe-Fuentes et al., 2017).

Temperature is an important parameter to consider as it affects the diffusion of solutes from plant matrices into extraction solvents. As such, it can have a significant impact on the quality and quantity of solutes including antioxidants extracted under sous vide conditions. The impact of extraction temperature on the extraction of antioxidants from Indigo Gem under sous vide conditions was investigated in the range from 30 °C to 50 °C. This low temperature range was selected to mitigate degradation of thermolabile compounds in the berry extract. The results showed that antioxidant activity of the berries increased with increasing sous vide extraction temperature **(Table 2).** For instance, the antioxidant content increased from 80.6 µmol TE g^{-1} FW to 107.4 µmol TE g^{-1} FW with increasing extraction temperature from 30 °C to 40 °C based on ABTS assay while a similar increase from 171.9 µmol TE g^{-1} FW to 228.3 µmol TE g^{-1} FW was observed when antioxidant content was measured by the FRAP assay under the same conditions **(Table 2)**. It is important to note that higher extraction temperatures had a detrimental effect on the extraction of antioxidants from Indigo gem under sous vide extraction conditions. The

antioxidant content was significantly lower for ABTS-TAA (70.1 μmol TE g^{-1} FW) and FRAP-TAA (149.1 μmol TE g^{-1} FW) when sous vide extraction was performed above 40 °C, corresponding to a gradual increase in antioxidant activity with time, up to 40 °C, and the decline with further increases in temperature.

This finding is similar to a study that analysed the impact of different extraction conditions on the antioxidant capacity of grape stem extracts, the highest antioxidant activity of the extracts was obtained at a temperature of 40 °C with 50% ethanol (Jiménez-Moreno et al., 2019). These suggest that an optimal extraction temperature of 40 °C is adequate to sufficiently extract antioxidants from plant materials. The antioxidant activity may have increased due to an increase in thermal energy, enhancing phenolic yield and antioxidative potential of the resultant extracts (Benmeziane et al., 2014). An increase in temperature accomplishes this by weakening the intermolecular bonds, thus softening plant tissues, and leading to a faster diffusivity of polyphenolic compounds into the solvent (Plaza and Turner, 2015). An increase in extraction temperature can also reduce the interfacial tension between the solvent, solute, and sample, thus decreasing the viscosity of the solvent while increasing solubility of the analytes, leading to an increase in the desired compounds (Wijngaard et al., 2012). In contrast, the decrease in antioxidant activity after 40 °C with further increase in temperature was noteworthy **(Table 2)**.

While increased extraction time may enhance the efficacy of extraction, the rate of degradation of thermolabile polyphenolic compounds may also increase with prolong extraction time (González-Montelongo et al., 2010; Carrera et al., 2012; Maran and Priya, 2014).

Anthocyanin is highly concentrated in haskap berry, contributing to its high antioxidant activity. It has been shown that anthocyanins have increased susceptibility to elevated temperatures and to oxidation (Cacace and Mazza, 2003), therefore elevating temperatures beyond the range of 30-

35°C can lead to the degradation of anthocyanins (Celli et al., 2015). This is particularly evident in the findings of Liu et al. (2018) who determined that the degradation rate of blueberry anthocyanin content; was 36 times more at an extraction temperature of 40 °C than it was at an extraction of 80°C and attributed this to the destruction of glycosidic bonds by high temperature.

The highest antioxidant activity was observed at the temperature of 40 °C and was thus used for extracting phenolic compounds from haskap berry extracts.

3.3.3 Evaluation of extraction solvent

The efficacy of the extraction of phenolic compounds and the resulting antioxidant capacity of the extract can be impacted by various factors, including the solvent type and composition, utilized during the process (Mokrani and Madani, 2016). Antioxidants have been extracted from plant sources using various combinations of solvents such as water, ethanol, methanol, acetone, and their aqueous solutions, with or without the presence of acid (Bunea et al., 2012; Boeing et al., 2014). In this study, pure ethanol, pure water, and ethanol: water (1:1) were the three solvents used for the extraction of antioxidants from Indigo Gem extracts at the optimal conditions described above (60 min at 40 °C). The results showed that the ethanol: water (1:1) solvent had a significantly higher ($p \leq 0.05$) yield in antioxidants compared to pure water or pure ethanol extraction solvents **(Table 3)**. For the FRAP assay, the antioxidant activity using the ethanol to water solvent was more than twice the yield when either water solvent or pure ethanol was used. A similar result was observed in ABTS antioxidant assay as the ethanol: water was twice, and up to four times greater than the yield of pure ethanol and pure water respectively (**Table 3)**.

Table 3. Phenolic content and antioxidant activity of haskap cultivar Indigo gem using different solvents in sous vide extraction.

Solvent	TPC	FRAP-TAA	ABTS-TAA
EtOH	3.1 ± 0.1c	102.7 ± 0.2b	100.5 ± 0.2b
H_2O	4.8 ± 0.1b	57.6 ± 0.1c	59.4 ± 0.3c
EtOH: H_2O (1:1)	6.9 ± 0.2a	175.2 ± 0.5a	216.6 ± 0.4a

Values represent means ± standard error (n = 4). Means within the same column followed by different letters (a-c) are significantly different ($p \leq 0.05$). TPC = total phenolic content in mg QE g^{-1} FW. FRAP-ferric reducing antioxidant power, ABTS- 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). TAA = Total antioxidant activity in µmol TE g^{-1} FW. FW-fresh weight, QE- quercetin equivalent, TE = Trolox equivalents. EtOH = ethanol, H_2O = water.

These observations are in accordance with prior findings that indicated the superiority of a binary solvent system over a mono solvent system in relation to the relative polarity of the extracted phenolic chemicals. Wong et al. (2014) analysed the impact of solvent and temperature on the extraction of polyphenols and antioxidants of red grape and found that ethanol: water (1:1) solvent is the most efficient solvent for polyphenol extraction. Another study also showed that the extraction yield of polyphenols was almost four-fold higher in ethanol: water (1:1) in comparison to water extracts (Baron et al., 2021).

The solubility of phenolic compounds is determined by the influence of both the chemical composition of the plant tissue and the polarity of the solvent system (Rahman et al., 2013). The observed differences can be elucidated by the variances in the polarities of the solvents, which preferentially isolate distinct hydrophobic or hydrophilic phenolic compounds present in the extract (Ngo et al., 2017). The water solvent had the lowest antioxidant activity in the present study (**Table 3**), although water is an effective solvent for hydrophilic compounds such as hydrophilic procyanidins, tannins and terpenoids (Selvamuthukumaran and Shi, 2017). The challenge occurs with lipophilic compounds, such as anthocyanins, of which haskap is known to contain significant amounts. Pure water may not have been the best solvent for the absorption of these lipophilic

compounds, thus there was decreased antioxidant activity in extracts for which water was used as the solvent.

The decrease in antioxidant activity observed in pure ethanol extracts may have been impacted by the inadequacy of pure ethanol to extract phenolic compounds which have a hydrophilic nature and are more soluble in water. Water is necessary for the liberation of hydrophilic antioxidants in the extract (Trabelsi et al., 2010; Wong et al., 2014). Many studies have demonstrated that organic solvent-water mixtures are more efficient in extracting antioxidant compounds than their respective pure solvents (Thoo et al., 2010; Rahman et al., 2013; Boeing et al., 2014; Jacotet-Navarro et al., 2018). As a result, the extract obtained through the binary solvent exhibited significantly greater ($p \leq 0.05$) activity compared to the extract obtained through pure water or pure ethanol extraction (Wong et al., 2014).

3.3.4 The impact of method of extraction on antioxidant activity

The method of extraction used is one primary factor in the yield of phytonutrients from a source. The best solvent (ethanol: water (1:1)), extraction time (60 min), and temperature (40°C), as well as the material that showed the highest yield (Indigo Gem) under these conditions, were applied using sous vide technique. The resulting antioxidant activity from sous vide method was compared to the values of antioxidant activity obtained when the conventional method was used. This was done to determine the method of extraction that was best for obtaining an antioxidant-rich extract. The results revealed higher antioxidant activity in sous vide extracts compared to those prepared using conventional extraction. Specifically, for FRAP- TAA, sous vide extracts (175.24 ± 0.47 µmol TE 100 g^{-1} FW) were three times greater than those obtained using the conventional method (51.58 ± 0.20 µmol TE 100 g^{-1} FW). A similar trend was observed for ABTS-TAA in which sous vide extracts were twice as high as those obtained using the conventional method (**Table 4)**.

Table 4. Comparison of the total polyphenols and antioxidant activity of haskap berries in this study with those previously recorded in the literature.

Antioxidant	Sous vide	Conventional	Literature	Reference
TPC	723.1 ± 0.2	715.4 ± 0.0	784.5 ± 0.3	(Česonienė et al., 2021)
			8.42 ± 0.0	(Khattab et al., 2016)
	-	-	1111*	(Bakowska-Barczak et al., 2007)
	-	-	500.8 ± 48.5	(Rupasinghe et al., 2012)
	-	-	427 - 1142*	(Thompson and Chaovanalikit, 2002)
FRAP-TAA	175.2 ± 0.5	51.6 ± 0.2	46.9 ± 3.0	(Rupasinghe et al., 2012)
	-	-	37- 113	(Thompson and Chaovanalikit, 2002)
ABTS-TAA	21658.2 ± 0.4	9345. 0 ± 0.1	9.6**	(Celli et al. 2014)
Anthocyanin	2010.50 ± 0.0	-	4.5–7.0***	(Khattab et al., 2016)
		-	1081	(Bakowska-Barczak et al., 2007)
	-	-	116 - 339	(Thompson and Chaovanalikit, 2002)
	-	-	343.9 ± 3.7	(Česonienė et al., 2021)

Sous vide and conventional values represent means ± standard error (n = 4). TPC = total phenolic content in mg QE 100 g^{-1} FW. FRAP; ferric reducing antioxidant power, ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). TAA = Total antioxidant activity in µmol TE 100 g^{-1} FW. Anthocyanin measured in mg QE 100 g^{-1} FW. FW- fresh weight, QE - quercetin equivalent, TE = Trolox equivalents. * mg GAE 100 g^{-1}, ** mmol TE 100 g^{-1} FW, *** mg QE g^{-1} FW. Solvent used for conventional method was pure ethanol for lipophilic portion, followed by ultrapure water for hydrophilic portion at 22 °C.

The sous vide results for FRAP and ABTS were also higher than those reported in the literature for antioxidant activity, using the same antioxidant assay (Thompson and Chaovanalikit, 2002; Rupasinghe et al., 2012; Celli et al., 2014; **Table 4**). The variance between results obtained from the present study and those previously reported in the literature could be due to various factors

such as the haskap cultivar used for the study, the extraction conditions, the climatic conditions (e.g., temperature, rainfall, sunlight) as well as agricultural practices (e.g., fertilization, watering techniques) used in their respective cultivation (Zehfus et al., 2021a)

The total phenolic content was also measured for extracts using the conventional and sous vide methods. The TPC measured in both extraction methods in this study were comparable to the total phenolic content of haskap berry measured in the literature which range from 500.9 - 842 mg 100 g^{-1}(Rupasinghe et al., 2012; Khattab et al., 2016; Česonienė et al., 2021).

The anthocyanin content was analysed using extracts prepared using the best parameters of the sous vide technique. It was found that extract prepared using sous vide method showed higher yield of anthocyanins than those previously recorded in the literature. The total anthocyanin yield for the sous vide extracts was 2010.5 mg 100 QE g^{-1} (**Table 4**). This was lower than the studies previously reported in the literature using different methods of extraction and haskap cultivars, ranging from 116- 1081 mg 100 g^{-1} FW (Thompson and Chaovanalikit, 2002; Bakowska-Barczak et al., 2007; Khattab et al., 2016; Česonienė et al., 2021). The high anthocyanin yield using the novel sous vide technique could be the reason for the high antioxidant activity that was measured using the sous vide method, as opposed to the other methods of extraction. The antioxidant activity gives a more inclusive measurement of phytonutrients and their biological impact, as it can be influenced by a wide range of bioactive compounds that have the ability to scavenge free radicals (Shahidi and Ambigaipalan, 2015).

Haskap is an anthocyanin-rich fruit. However, anthocyanin is thermolabile and has been found to degrade at temperatures greater than 35-40°C (Celli et al., 2015). The sous vide extraction was conducted at 40 °C, which may have helped in preventing the loss of anthocyanin by thermal processing (Pandita et al., 2023). These conditions could have facilitated the recovery of

anthocyanin compounds which further contribute to the increased antioxidant activity in sous vide extracts compared to the conventional method as well as other methods that have been used in the literature.

Pearson correlation was used to determine the relationship between polyphenols and antioxidant activity as a function of FRAP and ABTS, measured in this study. There is a strong positive relationship ($r = 0.937$, $p < 0.001$) between the TPC and total anthocyanin content (TAC) (**Table 5**). This signifies that anthocyanin is one of the main compounds contributing to the total polyphenols in haskap fruit. Another strong relationship was observed between antioxidant activity measured using FRAP and total flavonoid content (TFC) ($r = 0.963$, $p < 0.001$). A similar finding was observed when antioxidant activity was measured by ABTS and showed a strong positive correlation with TFC ($r = 0.952$, $p < 0.001$), which served as a validation of the results (**Table 5**). This suggests that the flavonoids are contributing heavily to antioxidant activity.

Table 5. Pearson correlation of haskap extracts prepared using the sous vide extraction.

Variables	TPC	FRAP	TAC	TFC	ABTS
TPC	1	-	-	-	-
FRAP	0.655**	1	-	-	-
TAC	0.937***	0.844***	1	-	-
TFC	0.619**	0.963***	0.808***	1	-
ABTS	0.746***	0.991***	0.908***	0.952***	1

Coefficient values without (*) are not significant. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. TPC = total phenolic content, FRAP = ferric reducing antioxidant power, ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), TAC = total anthocyanin content, TFC = total flavonoid content.

Additionally, the total flavonoid content shows a positive relation to anthocyanin (r = 0.808, $p < 0.001$) which indicates that anthocyanin is the main compound contributing to the total flavonoid content and thus the total antioxidant activity of haskap extract. Furthermore, a few studies have demonstrated a strong positive association between the total phenolic content and flavonoid content, suggesting that the flavonoid content may serve as the primary polyphenolic component responsible for the antioxidant capacity of these fruits (Rupasinghe et al., 2012; Orsavová et al., 2022). Bakowska-Barczak et al. (2007) also noticed that anthocyanins are a significant contributor to the antioxidant activity of berries. Hence it has been seen that the high anthocyanin yield using the novel sous vide technique preserves anthocyanin, contributing to the antioxidant activity of haskap. Thus sous vide extracts have higher antioxidant activity than the extracts prepared using the conventional techniques as well as those mentioned in the literature.

3.4 Conclusion

It can be concluded that the efficiency of the method of extraction and the recovery of antioxidant compounds from natural sources were indeed influenced by several factors including material used, duration of extraction, extraction temperature, the solvent that is used as well as the method of extraction. Increased extraction time and temperature enhanced antioxidant yield. There was however a sharp decline in antioxidant activity with an increase in temperature beyond 40 °C. This could be due to the thermolabile compounds present in haskap such as anthocyanin which would degrade above 35-40°C. The ethanol: water (1:1) solvent was determined to be more efficient in extracting antioxidant compounds than pure ethanol or pure water solvents. Extracts prepared using sous-vide were found to have higher anthocyanin content and antioxidant activity than the extracts prepared using the conventional method.

The results of the present study indicate that the binary (ethanol: water (1:1)) extract of haskap showed notable antioxidant activity in different assays *in vitro*. Ethanol: water (1:1) extract is rich in polyphenolics, particularly in anthocyanins. Therefore, the ethanol: water (1:1) extract of haskap could be explored as a natural antioxidant for application in functional food and nutraceuticals formulations. Sous vide technique can also be utilized as a simple but effective method for preparing antioxidant rich extraction of berries' polyphenols. This method can be further expanded and used on the wider scale in industries involved in natural extract preparation of fruits.

3.5 REFERENCE

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CHAPTER 4

FORMULATION AND DEVELOPMENT OF THE ANTIOXIDANT-RICH FUNCTIONAL BEVERAGE USING HASKAP (*LONICERA CAERULEA* L.) AS A BASIC INGREDIENT

4.0 ABSTRACT

Consumers are increasingly becoming aware of the significant impact of beverages and foods on the overall health of consumers. As a result, there is a heightened demand for functional beverages that can aid in the prevention or inhibition of degenerative diseases caused by oxidative stress. Haskap (*Lonicera caerulea* L.) is a rich source of bioactive compounds and possess various nutritional and health-promoting properties. In this study, haskap extracts and iceberg water were combined in a ratio of 1:5 v/v. Three additional beverages were formulated using different combinations of haskap, sorrel (*Hibiscus sabdariffa*), and ginger (*Zingiber officinale*) root extracts.150 untrained panelists were recruited for a sensory evaluation in determining consumers' preference and acceptability of these novel functional drinks. The vitamins, total phenolics, total flavonoid, anthocyanin, and carotenoid contents were analyzed for each of the formulations and the antioxidant activity was measured using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging abilities and ferric reducing antioxidant potential (FRAP). Haskap with sorrel and ginger was desired for the taste and aroma, but haskap with sorrel showed higher acceptability among consumers in terms of appearance. Haskap with sorrel and ginger had significantly ($p < 0.05$) higher flavonoid (21.1 ± 0.2 mg QE 100 mL^{-1}) and carotenoid content and showed stronger antioxidant activity than the other formulations, with 369.4 ± 1.7 µmol TE 100 mL^{-1}, and 299.0 ± 1.2 µmol TE 100 mL^{-1} for ABTS and FRAP respectively. Conversely, haskap with sorrel had significantly ($p < 0.05$) higher contents of total phenolics (311 ± 1.7 mg QE 100 mL^{-1}), total anthocyanin (61.4 ± 0.1 mg 100 mL^{-1}), and vitamins B2 (28.6 ± 0.1 mg 100 mL^{-1}),

B3 (16.1± 3.7 mg 100 mL^{-1}), B6 (173.8 ± 4.8 mg100 mL^{-1}) and B9 (23.0 ± 0.8 mg 100 mL^{-1}) than the other flavoured beverages. Haskap beverage (79.2 ± 1.0 mg100 mL^{-1}) had the highest vitamin C content of all the formulations. The formulations incorporating haskap with sorrel and haskap with sorrel and ginger demonstrated significant potential as commercially viable functional food products, considering factors such as consumer acceptability and bioactivity.

Keywords: Oxidative Stress, Degenerative diseases, Carotenoid, Functional Food, Antioxidant.

4.1 INTRODUCTION

The Hippocratic era was characterized by a medical approach that predominantly relied on dietary interventions, and medicine was employed solely in instances deemed essential (Joly, 1960; Cardenas, 2013). Several works of literature have mentioned a quotation from Hippocrates, the father of medicine, that states, “Let food be thy medicine and medicine be thy food” (Grabowska et al., 2019; Patel, 2021; Hall, 2022). Hippocrates is said to have made this statement centuries ago, but it is now universally acknowledged that one's diet has a substantial impact on their health (Conlon and Bird, 2014; Funtikova et al., 2015).

The global market has observed that the focus on food has transitioned from its traditional role of providing vital nutrients for supporting life and development, to its potential in preventing or treating diverse forms of illnesses (Betoret et al., 2011). Furthermore, there is a growing trend among consumers to prioritize their health and place greater emphasis on their lifestyle choices and dietary habits (Szakály et al., 2012). The increased awareness of the impact of diet on health has resulted in a significant increase in the market's desire for functional foods and nutraceuticals (Saxena et al., 2013). In 2021, functional food had an estimated global market of $258 billion including functional drinks and beverages and was projected to grow to $530 billion by 2028 (Egea

et al., 2022). Tadesse and Emire (2020) primarily attribute this rapid surge in demand for functional foods to the rise in non-communicable diseases and technological advancements.

Functional foods are defined as food items that possess the potential to confer health benefits beyond their basic nutritional value. They aid in promoting optimal health conditions and are significant for fighting and alleviating lifestyle-related diseases and disorders (Granato et al., 2017; Khalaf et al., 2021). The fundamental components that confer these health benefits are ascribed to their bioactive compounds, such as phytochemicals. These compounds may be naturally occurring, produced during processing, or acquired from external sources and integrated into the food item (Butnariu and Sarac, 2019). Bioactive compounds are considered the basic building blocks of functional foods, originating from diverse sources such as plants, mushrooms, and animals (Martirosyan et al., 2022). They include flavonoids, anthocyanin, vitamins, and miscellaneous phenolics (Wang and Li, 2014). These compounds have the potential to mitigate inflammation through the inhibition of oxidative damage and modulation of immune system communication (Maqsood et al., 2020). Moreover, they possess various biological properties, including antioxidant and anti-microbial activity, regulation of enzyme detoxification, modulation of the immune system, reduction of platelet aggregation, metabolism of hormones, and anti-cancer activities (Khalaf et al., 2021).

As the global food market expands, producers are increasingly drawn towards developing innovative products that offer health benefits, making them more attractive to potential consumers (Granato et al., 2010). An increasing number of consumers are seeking plant-based products that provide enhanced nutritional benefits and reduce adverse health consequences (Tso and Forde, 2021). This trend has driven the advancement of substitute food alternatives that possess the ability to rival conventional offerings in the marketplace (Choudhury et al., 2020). In the functional food

market, there has been increasing research into products targeting health and mental well-being (Vicentini et al., 2016). Some nutraceuticals and food ingredients that have been tested for their functional food potential due to their phytochemical properties include Goji berry (*Lycium barbarum)* (Vidović et al., 2022), red onion (*Allium cepa* L.) (Chadorshabi et al., 2022), and elderberry (*Sambucus ebulus)* (Kiselova-Kaneva et al., 2022). The bioactive compounds from ingredients used to create functional food can be obtained through physical, chemical, or enzymatic processes (Deepak and Jayadeep, 2022).

The consumption of berry fruits is a common practice globally, owing to their abundance in bioactive compounds that possess significant potential in promoting human health (de Souza et al., 2014). Berries are known to possess phytochemicals, which exhibit various health-promoting effects, such as antioxidant and anti-inflammatory properties. Berries are a promising pharmacological treatment option for various diseases due to their high concentration of polyphenols (Kelly et al., 2017). These compounds have the potential to mitigate oxidative stress and inflammation, which are frequently implicated in the pathogenesis of conditions such as diabetes, neurological disorders, cardiovascular diseases, and cancer (Golovinskaia and Wang, 2021).

Haskap is a berry that has great potential as a functional food ingredient due to its high antioxidant activity (Celli et al., 2014; Grobelna et al., 2020). Furthermore, scientific evidence supports the notion that functional foods, which incorporate plant parts containing bioactive substances, can have a positive impact on human health (Gong et al., 2020; Verma and Thakur, 2021). According to Rupasinghe et al. (2018), haskap berries are characterized by a significant concentration of vitamin C, comparable to, or exceeding the levels present in fruits such as oranges, strawberries, raspberries, and blackberries, widely acknowledged as primary sources of this nutrient. Haskap

has a remarkably high content of anthocyanins (Becker and Szakiel, 2019). It also contains high amounts of phenolics, carotenoids, and flavonoids, which exhibit potent antioxidant properties that safeguard against the onset of various ailments resulting from heightened oxidative stress within the organism (Rauf et al., 2019). Phenolic acids and flavonoids have attracted considerable interest from nutritionists and food technologists as essential constituents in a diverse range of nutraceutical, pharmaceutical, and medicinal implementations (Yadav, 2021). This is due to its anti-oxidative, anti-inflammatory, and anti-carcinogenic properties (Panche et al., 2016). Additionally, the persistent intake of anthocyanins and foods that are rich in anthocyanins has been hypothesized to confer a range of health advantages, such as safeguarding cardiovascular health, preserving neurological function, enhancing vision, as well as exhibiting antidiabetic and anticancer benefits (Pojer et al., 2013; Wallace and Giusti, 2015).

In addition to its antioxidant properties, haskap extract was found to have anti-inflammatory properties (Rupasinghe et al., 2015). Haskap was also shown to exhibit enhanced glucose tolerance as well as antimicrobial activity against bacteria such as *Cronobacter* species (De Silva, 2020; Yemiş et al., 2022).

Across various historical periods, ginger (*Zingiber officinale*) root has been recognized not only as a culinary spice for enhancing taste, promoting nutrition, and preserving food, but also as a medicinal agent for mitigating a range of ailments such as the flu, asthma, dyspepsia, and gastrointestinal disorders (Srinivasan, 2017). Ginger has been reported in many studies for its anti-oxidative ability (Chakraborty et al., 2012; Ali et al., 2018a), anti-inflammatory activity (Farombi et al., 2020; Liu et al., 2020), anti-microbial potential (Wang et al., 2010; Tang et al., 2020), anti-obesity (Wang et al., 2019), anti-diabetic (Roufogalis, 2014) and anti-neurodegenerative properties (Choi et al., 2018).

Another well-known therapeutic plant is sorrel *(Hibiscus sabdariffa* L.). Several studies have documented the medicinal properties of sorrel plants in the treatment of various ailments, including cutaneous diseases, jaundice, sore throat, and warts (Lee et al., 2005; Guarrera and Savo, 2013). Notably, sorrel has been found to possess therapeutic properties for the treatment of fever, diarrhea, lack of appetite, and worms, and has been traditionally used as a blood cleanser (Kucekova et al., 2011; Guarrera and Savo, 2016). According to Khalifa et al. (2022), the medicinal value of sorrel can be attributed to its chemical makeup, which includes a variety of bioactive compounds like flavonoids, vitamins, proteins, carbohydrates, reducing sugars, phenols, tannins, and organic acids. Sorrel also has relatively high carotenoid content (Molnár et al., 2005; Ślesak et al., 2014). Carotenoids aid in the promotion of human health primarily through their antioxidant effects but are not synthesized endogenously and must be consumed through dietary intake or supplements (Eggersdorfer and Wyss, 2018).

Water is another compound that is essential for life. It aids the body in thermoregulation, increases physical and cognitive performance, and improves the skin (Popkin et al., 2010). Notwithstanding the extensively recorded health advantages linked to habitual water consumption, the overall public's daily water intake remains below the recommended level (Papies et al., 2021). Interestingly however, the United States is currently experiencing a significant expansion in the functional water market (Department, 2022). This is a non-alcoholic beverage and is usually enhanced through the addition of supplementary components, such as vitamins, minerals, herbs, raw fruits, or vegetables. Furthermore, approximately 60% of respondents in a survey express a preference for their beverage to possess a substantial quantity of antioxidants (Department, 2022).

There is an increasing need for both fresh and processed functional food items that offer health benefits beyond basic nutrition when consumed as part of a regular diet (Manful et al., 2023).

The development of functional foods however is a complex process that involves collaboration among various stakeholders. The growth and sustainability of the functional food sector depend on the cooperative efforts of its partners throughout the entire process, from conceptualization to successful marketing (Jones and Jew, 2007; Siro et al., 2008). The process starts with a novel idea which is then developed into the product. This is followed by an evaluation of the physicochemical, chemical, and sensory attributes of the newly developed product. Efficacy testing in cell and human models should then be done to ascertain the toxicological implications and therapeutic dosages (Granato et al., 2017). Then, there needs to be substantiating evidence of its functionality, regulatory review, consumers' sensitization, and perception before it can officially be introduced in the market as a functional food (Jones and Jew, 2007).

The evolving patterns of consumption pose novel challenges for the food industry (Sala et al., 2017). Specifically, food companies are required to create and introduce fresh products that can prevent or slow the spread of communicable ailments by virtue of their health-preserving properties while still appealing to the sensual desires of consumers. Greater water consumption must also be considered for its several health benefits and in the mitigation of some disease conditions. Water should be formulated to appeal to the appetites and desires of consumers. This desire may be brought about by different flavours as well as a knowledge of the increased health benefits of its consumption. The objective of this research then, is to formulate and develop novel antioxidant-rich haskap-based flavoured waters, which can be further tested for its functional properties before being introduced to the market as a functional beverage.

4.2 MATERIALS AND METHODS

4.2.1 Chemical Reagents and standards

Pure ethanol (HPLC grade) was supplied by VWR International (Ontario, Canada) and ultrapure water used where applicable. Quercetin, Folin-Ciocalteu reagent, sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), sodium phosphate dibasic anhydrous (Na_2HPO_4), anhydrous sodium acetate, glacial acetic acid (HPLC grade), TPTZ (2,4,6-tripyridyl-*s*-triazine), 36.5% hydrochloric acid (HCl), $FeCl_3 \bullet 6H_2O$, Trolox, ABTS, horse radish peroxidase (HRP), β-carotene, $AlCl_3$ (aq), 1 M CH_3COONa (aq), potassium chloride, sodium acetate, thiamine tydrochloride (vitamin B1), riboflavin (vitamin B2), niacinamide (vitamin B3), calcium d-pantothenate (vitamin B5), pyridoxine hydrochloride (vitamin B6) , pyridoxamine dihydrochloride (vitamin B6 amine), pyridoxal hydrochloride (Vitamin B6-aldehyde), d-biotin (vitamin B7), folic acid (vitamin B9), p-aminobenzoic Acid (vitamin B10), DL-6,8-thioctic Acid (DL-α-Lipoic acid) (vitamin Bx), L-ascorbic acid (vitamin C) were purchased from Sigma Aldrich (Ontario, Canada) . Mineral standards (IV-ICPMS-71A) were purchased from Inorganic Ventures, Virginia, USA.

4.2.2 Plant Material

Haskap cultivar, Indigo gem was obtained from North 45 Orchards, Stewiacke, Nova Scotia, Canada (latitude 45.18˚, longitude - 63.22˚). The haskap plants were cultivated in alluvium, loam to silty clay, and loam soil types, during the 2021 and 2022 growing seasons. Samples were packaged in paper bags and frozen at -20˚C for 24 hrs and subsequently shipped by courier on ice to Memorial University of Newfoundland, Grenfell Campus. Samples were then stored in a refrigerator (-20 ˚C) until extract preparation. Ginger root and sorrel were purchased from Dominion supermarket, Corner Brook.

4.2.3 Preparation of extracts using sous vide technique

10 g of haskap berries, 82 g of ginger and 10 g of sorrel were puréed separately using a blender. Three separate solutions were then prepared using these ingredients, by adding 250 mL of water to the haskap as well as the ginger purée and 50 mL of water to the sorrel purée. The mixtures were then poured into 8" ×10" food grade vacuum seal bags and vacuum sealed using VacMaster VP215 (Ary Inc., Kansas City, MO, USA) vacuum sealer. The bags were placed in Sous vide Supreme 10LS (Eades Appliance Technology, Dallas, TX 75220, USA) water oven and cooked at 40 ˚C for 60 min. After cooking, the haskap, ginger, and sorrel extracts were individually strained using a flour sieve then a cheese cloth to remove any residual particles from the solutions.

4.2.4 Analysis of extract using Cytation Image microplate reader

All the extracts were analyzed in replicates of four using ultraviolet–visible spectroscopy on the Cytation Image microplate reader (BioTek, Vermont, USA). The assays performed were used to quantify the antioxidant capacity and includes TPC, ABTS, FRAP, flavonoid, anthocyanin, carotenoid, chlorophyll a, chlorophyll b. All the results were expressed on fresh weight basis.

4.2.4.1 Total Phenolic Content (TPC)

TPC of haskap was estimated using the analysis as described by (Rajakaruna et al., 2022) with modification. 125 µL of 10-fold diluted Folin-Ciocalteu reagent was added to a 96-well microplate along with 25 µL of each sample in replicates of four. 50 µL of water was then added for hydrophilic extracts followed by the addition of 95% v/v ethanol was added to lipophilic antioxidant extracts after mixing. This solution was then incubated for 30 mins and the absorbance measured at 755 nm on the microplate reader. Quercetin standard curve (0-1 mg/mL, R2 = 0.987) and (0-1 mg/mL, R2= 0.982) was used to determine the phenolic content at both hydrophilic and

lipophilic phases respectively. For calculation of total phenolic content, results from the lipophilic and hydrophilic mixtures were combined for each sample and expressed as mg quercetin equivalents per gram fresh weight fruit (mg QE g^{-1}FW).

4.2.4.2 Total antioxidant activity (TAA) - FRAP

Total antioxidant activity was determined using two methods. Antioxidant activity was measured using FRAP method from (Thomas et al., 2010) with modification. 20 µL of the haskap extract was mixed with 180 µL of working solution. This solution was then mixed and incubated in the dark for 30 minutes. After which, the absorbance was measured on the microplate reader at 593 nm. The concentrations of Trolox equivalent antioxidants in the samples were determined by using the standard curve (0-25 µM, R^2 = 0.992) and (0-25 µM, R^2 = 0.999) for hydrophilic anthocyanin activity (HAA) and lipophilic anthocyanin activity (LAA) respectively. The results for LAA and HAA for each sample were added to give the TAA, expressed in µmol Trolox equivalent antioxidant capacity per gram fresh weight (µmol TE g^{-1} FW) of haskap.

4.2.4.3 Total antioxidant activity (TAA) - ABTS

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) procedure as conducted by Manful et al. (2020) with modifications to the ratio of ABTS reagent to extract used. This method was utilized in validating the TAA results obtained from the FRAP assay. 160 µL of 25 mM ABTS, followed by 480 µL of 125 µM hydrogen peroxide (H_2O_2), 200 µL of 0.2 mg mL^{-1} HRP and 1160 µL of water were mixed in this order consecutively for the hydrophilic reaction mixture while, 160 µL of 25 mM ABTS, followed by 480 µL of 125 µM H_2O_2, 1000 µL of 0.2 mg mL^{-1} HRP and 360 µL ethanol (95% v/v) were combined consecutively for the lipophilic reaction mixture.

200 µL of either LAA or HAA reaction mixture, depending on whether the extract to be added was lipophilic or hydrophilic was first added to a 96-well microplate and the absorbance read at

730 nm using the Cytation Image reader. 20 µL of sample was then added to the reaction mixture and after 10 minutes the absorbance again measured. The amount of antioxidant present in the sample is reflected by the difference in these absorbances. A Trolox standard curve (0-25 µM, R^2 =0.973) and (0-25 µM, R^2 = 0.988) for LAA and HAA respectively were used to determine the antioxidant activity. The TAA was calculated by adding both the HAA and LAA result for a specific sample. The results were expressed as micromoles Trolox equivalents antioxidant capacity per gram fresh weight fruit (µmol TE g^{-1} FW).

4.2.4.4 Flavonoid

Flavonoid content was analyzed using the aluminum chloride colourimetry method as done by Do et al. (2014) with slight modification in volume of reagents to extract used. 10 µL of 10 %w/w $AlCl_3$(aq) was added to 96 well microplate followed by 150 µL of either water or 95% v/v ethanol depending on if the samples were hydrophilic or lipophilic antioxidant extracts. These were then gently rotated to mix and 10 µL of 1 M CH_3COONa (aq) added and the contents again mixed to get a homogenous solution. 50 µL of haskap extract was added last and the solution mixed carefully. The plates were then incubated for 20 mins in the dark and absorbance measured at 510 nm in the Cytation Image reader. The concentration of flavonoid in the haskap extract was determined by the Trolox standard curve (0-1 mg mL^{-1}, R^2 = 0.976) and (0-1 mg mL^{-1}, R^2 = 0.97) for hydrophilic and lipophilic extracts respectively. Results were expressed as milligrams quercetin equivalents per gram fresh weight fruit (mg QE g^{-1} FW).

4.2.4.5 Anthocyanin

The Anthocyanin content was measured using the differential method as described by (Chen et al., 2014) with modifications. Two microwell plates were selected for solutions at pH of 1.0 and 4.5 respectfully for lipophilic extracts. For pH 1.0, 20 µL of each sample was added to microplate

wells followed by 180 µL of potassium chloride (0.025 M, pH 1.0). The plate was then incubated at room temperature for 10 min. The absorbance was then measured at 510 nm and 710 nm in the Cytation Image reader (BioTek, Vermont, USA). The microwell plate for pH 4.5 was then filled with 20 µL of each sample, followed by 180 µL of sodium acetate buffer (0.4 M, pH 4.5). This plate was incubated in the dark at room temperature for 10 min and the absorbance measured at 510 nm and 710 nm in the Cytation Image reader. The same steps were repeated for hydrophilic extracts. The following equation was then used to calculate the Anthocyanin content in lipophilic and hydrophilic extracts and the results for each sample combined to determine the total anthocyanin content.

$$\text{Total anthocyanin content} = \frac{A}{\varepsilon \times l} \times MW \times DF \times \frac{V}{W} \times 1000$$

where, $A = (A_{510nm} - A_{710nm})_{pH1.0} - (A_{510nm} - A_{710nm})_{pH4.5}$

MW (molecular weight) = 449.2 $g.mol^{-1}$ for cyanidin-3-glucoside (cyd-3-glu)

DF = dilution factor

W = sample weight (mg)

l = path length in cm

V= volume (L)

ε = 26,900 M extinction coefficient in L mol^{-1} cm^{-1} for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

4.2.4.6. Total carotenoid and chlorophyll analysis

Carotenoid and Chlorophyll content were analyzed as described by Rainha et al. (2011) with modification. 200 µL of the haskap extract was pipetted in the microwell plate and the absorbance measured at 470 nm, 645 nm and 662 nm on a Cytation Imaging microplate reader. The content

of carotenoid, chlorophyll a and chlorophyll b for both hydrophilic and lipophilic extracts were calculated using the formulae:

Chl a (µg/mL) = (13.36 × A662) - (5.19 × A645)

Chl b (µg/mL) = (27.43 × A645) - (8.12 × A662)

Chl t (µg/mL) = (5.24 × A662) + (22.24 × A645)

Car (µg/mL) = (1000 × A470) - (2.13× Chl a) – (97.64× Chl b)

Where Chl a= chlorophyll a; Chl b= chlorophyll b; Car= carotenoid content

A662, A645, A470= Absorbance at 662, 645 and 470 respectively

The results from lipophilic and hydrophilic extracts were added to give the total carotenoid as well as total chlorophyll a and b. The results were expressed as micrograms per gram fresh weight fruit (µg g^{-1} FW) basis.

4.2.4.7 Vitamin analysis of haskap flavoured water

For extraction of water-soluble vitamins, 0.2 mL of each of the haskap flavoured water samples was diluted to 2 mL using distilled water. These were then filtered using 2mL filtration vials and applied to UHPLC-HRAMS analysis for vitamin C and B complex vitamins (B1, B2, B3, B5, B6, B7, B9, and B12).

Water soluble vitamins were resolved on RP-C18 column (Polar Acclaim II 4.6 × 150 mm, 5 μm) coupled to a dionex ultimate 3000 ultra-high performance liquid chromatography system (**Table 1**) (Thermo Fisher Scientific, Ontario, Canada) and LTQ Orbitrap high-resolution accurate mass spectrometer (Thermo Fisher Scientific, Ontario, Canada).

Table 1. LC Parameters for water soluble vitamins

Solvent A	10 mM aqueous ammonium acetate
Solvent B	Pure acetonitrile
The mobile phase gradient had the following settings	
0 – 5 mins	0 – 20 % B
5 – 10 min	20 – 50 % B
10 – 15 min	50 – 80 % B
15 – 17 min	80 – 90 % B
17 – 18 min	90 % B
18 – 19 min	0 % B
19 – 22 min	0 % B
Column temperature	35 °C
mobile phase flow rate (mL/min)	0.4

In all runs, 10 µL of the sample or standards was injected into the instrument.

The mass spectrometer (MS) was operated under positive electrospray ionization (ESI) conditions in selected ion monitoring (SIM) mode for vitamin C (m/z = 175), vitamin B1 (m/z = 265), vitamin B2 (m/z = 377), vitamin B3 (m/z = 123), vitamin B5 (m/z = 220), vitamin B6 (m/z = 170), vitamin B7 (m/z = 245), vitamin B9 (m/z = 442), vitamin B12 (m/z = 678).

Quantification of vitamins in berries was based on individual standard curves generated from vitamin standards, and concentrations expressed as milligram per milliliter (mg mL^{-1}) of water.

4.2.6 Sensory Analysis

A sensory evaluation was conducted using four flavours of haskap flavoured water: haskap, haskap with sorrel, haskap with ginger, and haskap with sorrel and ginger. The procedure using human subjects for the panel was approved by Memorial University of Newfoundland (MUN), Grenfell Campus Research Ethics Board as seen in document 1 of the appendix. A total of 150 individuals representing various age groups and ethnic backgrounds, attended the sensory and willingly participated in this survey. The participants were selected randomly from the employees, students, and community members of Corner Brook, NL. Prior to their involvement, all participants provided written consent. Each panelist was provided with four samples of haskap flavoured water, along with unsalted biscuits and water to cleanse their palate between samples. Participants were given explicit instructions to complete an anonymous questionnaire in a genuine manner. The questionnaire required them to express their preferences about the appearance, aroma, and flavour of the offered samples. Additionally, participants were asked to evaluate each sample and indicate their overall preference in terms of appearance, taste, and likelihood of purchase. The experiment was conducted using the SIMS Software, version 6, at the Functional Food Laboratory at Memorial University, Grenfell Campus.

4.2.7 Statistical approach

XLSTAT (Premium 2017, Version 19.5, Addinsoft, New York, USA) was used to conduct all statistical analysis. All measurements were performed in quadruplicate, and results were expressed as mean ± standard deviation (SD). Significant differences between means were determined by One-way analysis of variance (ANOVA). For the sensory analysis, to understand the overall association between the samples and each attribute tested for, principal component analysis (PCA) was done. Pearson correlation was used to analyze the relationship between compounds.

4.3 RESULTS AND DISCUSSION

4.3.1 The active ingredients used in the formulation of haskap water and their bioactive compounds.

In the formulation of the functional beverages developed in this experiment, extracts from haskap, sorrel and ginger were prepared using a novel sous vide extraction technique for optimal yield of antioxidants. The total phenolic, flavonoid, anthocyanin, carotenoid, and chlorophyll content, as well as the antioxidant activity (ABTS and FRAP) of the extracts, were then analyzed. Haskap extracts contained significantly ($p < 0.05$) higher total anthocyanin and flavonoid content and showed higher antioxidant activity than both the sorrel and ginger extracts (**Table 2)**. Of the three ingredients analyzed, haskap was chosen as the main material for all the products, due to the high content of bioactive compounds in the extract.

Table 2. Bioactive compounds in the ingredients used in the formulation of flavoured water.

Ingredient	TPC	Anthocyanin	Flavonoid	ABTS-TAA	FRAP-TAA	Carotenoids	Total chlorophyll
Haskap	6.9 ± 0.2b	20.1 ± 0.0a	2.3 ± 0.1a	216.6 ± 0.4a	175.2 ± 0.5a	36.6 ± 0.2c	2.0 ± 0.1c
Sorrel	10.2 ± 0.1a	1.5 ± 0.0b	1.7 ± 0.0b	160.5 ± 0.2b	133.1 ± 0.2b	91.6 ± 2.7a	31.1 ± 0.4b
Ginger	4.9 ± 0.0c	0.0 ± 0.0c	1.2 ± 0.0c	37.1 ± 0.1c	25.7 ± 0.1c	49.1 ± 0.1b	140.6 ± 0.4a

TPC = total phenolic content in mg QE g^{-1} FW. FRAP; ferric reducing antioxidant power, ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). TAA = Total antioxidant activity in µmol TE g^{-1} FW. Anthocyanin measured in mg QE g^{-1} FW. Flavonoid in µg QE g^{-1} . Total chlorophyll and carotenoids in µg $100\ g^{-1}$. Values represent means ± standard error (n=8). Significant ($p < 0.05$) differences are represented by different letters (a-c) within the same column. All results are presented in fresh weight.

The anthocyanin content was twice as high as that which was previously reported by Bakowska-Barczak et al. (2007) (1081 mg QE 100 g^{-1} FW/ 10.8 mg QE g^{-1}) and four times that of Zehfus et al. (2021a) (447.8 mg QE 100 g^{-1} FW/ 4.5 mg QE g^{-1}). The difference in values is possibly due to the extraction method and conditions or varying cultivars among the studies. The anthocyanin content of haskap extracts in this study is higher than the anthocyanin content of ripe blueberry (0.9- 145.2 mg100 g^{-1}) reported by Hwang et al. (2020). Other studies have shown anthocyanin content in blueberry to range from 0.7 to 4.3 mg g^{-1} (Shibata et al., 2021; Wang et al., 2017b) which is lower than the anthocyanin content of the haskap extract in this study. This is noteworthy as blueberries have been recognized and utilized as a functional food ingredient (Patel, 2014; Estupiñan-Amaya et al., 2020; Gonçalves et al., 2022).

The antioxidant activity was also significantly higher ($p < 0.05$) in the haskap extracts (**Table 2)**, than that which was reported by Rupasinghe et al. (2012) (46.9 µmol TE g^{-1} FW) and Celli et al. (2014) (9.6 mmol TE 100 g^{-1} /95.5 µmol TE g^{-1} FW) for FRAP and ABTS respectively. Negreanu-Pirjol et al. (2023) noted that haskap berries exhibit significantly greater antioxidant activity, ranging from three to five times higher than that of more commonly consumed berries, such as blackberries or strawberries. The high antioxidant activity may be explained by the high anthocyanin content observed in the haskap extract. The findings of our study revealed a strong positive correlation between haskap, and anthocyanin content, flavonoid content, and *in vitro* antioxidant activity (ABTS and FRAP) (**Table 3)**. These findings indicate that the potent antioxidant and radical-scavenging properties of haskap extracts may be ascribed to the elevated concentrations of the antioxidant compounds (total flavonoids, and anthocyanidin contents) contained in these extracts, which offer protection against reactive oxygen radicals-induced damage (Zhou et al., 2023).

Table 3. Pearson correlation matrix for bioactive compounds in haskap, ginger, and sorrel extracts

	Ginger	Haskap	Sorrel	TPC	FRAP	TAC	TFC	ABTS	Carotenoids	Total Chlorophyll
Ginger	1	-	-	-	-	-	-	-	-	-
Haskap	-0.533	1	-	-	-	-	-	-	-	-
Sorrel	-0.483	-0.483	1	-	-	-	-	-	-	-
TPC	-0.775	-0.107	0.914***	1	-	-	-	-	-	-
FRAP	-0.964	0.739**	0.233	0.585*	1	-	-	-	-	-
TAC	-0.588	0.998***	-0.424	-0.042	0.781**	1		-	-	-
TFC	-0.794	0.852***	-0.060	0.299	0.899***	0.877***	1	-	-	-
ABTS	-0.955	0.760**	0.202	0.559*	0.999***	0.801***	0.906***	1		-
Carotenoids	-0.269	-0.657	0.958***	0.789**	0.008	-0.607	-0.263	-0.023	1	-
Total Chlorophyll	0.981***	-0.686	-0.306	-0.642	-0.997	-0.733	-0.877	-0.994	-0.082	1

Coefficient values without (*) are not significant. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. TPC = total phenolic content, FRAP = ferric reducing antioxidant power; ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), TAC = total anthocyanin content, TFC = total flavonoid content.

A closer look at the relationship between antioxidant activity and anthocyanin in **Supplemental Fig. 1c** suggests that anthocyanin is the major contributor to the antioxidant activity of haskap. This is in line with various researchers who have demonstrated that anthocyanins make a significant contribution to the antioxidant activity and health-promoting effects that are exhibited by fruits and their associated products (Rivero-Pérez et al., 2008; D'Archivio et al., 2010). A few studies have suggested further exploration of haskap preparations as a potential functional food due to its high polyphenolic content, especially anthocyanin (Celli et al., 2015; Rupasinghe et al., 2018; Cheng et al., 2022).

While haskap had the highest anthocyanin content, the sorrel extract contained significantly ($p < 0.05$) higher total phenolic content and carotenoids than the haskap or ginger extracts (**Table 2)**. Sorrel was highly correlated with phenolic content and carotenoids as indicated (**Table 3)**. This suggests that polyphenolics and carotenoids contributed most to the bioactive compounds in sorrel. Although showing potential, there are not many studies on the yield and nutraceutical composition of sorrel (Ceccanti et al., 2020). The phenolic content in this study is lower than the range of the reported values (32.9 ± 0.3 g GAE kg^{-1}, 41.7 ± 2.0 mg GAE g^{-1}) despite variations in the reported unit value (Cisse et al., 2009; Dias et al., 2013; Riaz et al., 2021). These variations are primarily caused by the species used, environmental factors, the extraction process, or the extraction solvent (Liu et al., 2006; Puro et al., 2017). Isbilir and Sagiroglu (2013) noticed that there is a positive correlation between phenolic content and antioxidant assays in sorrel. This is comparable to the results of the present study, which also observed a strong positive correlation between antioxidant activity (FRAP and ABTS) and total phenolic content as illustrated in **Supplementary Figure 1b**.

Ginger extracts yielded the lowest phenolic content in this experiment (**Table 2)**. The phenolic content of ginger in this study is lower than the reported range of phenolic content (5.2 - 35.2 mg QE g^{-1}) in ginger extracts using different solvents (Tung et al., 2017). This could be due to the use of more potent solvents in the extraction process, the extraction method used, or even the cultivar of ginger that was utilized in the previous study. A similar occurrence was observed in one study that compared ginger extracts that were prepared using different solvents. They found that total phenolic content was twice as high in ethanol than it was when water was used as the solvent (Tanweer et al., 2020). Ginger, however, contains significant amounts of phytochemicals. Compounds such as 6-gingerol, 6-shogaol, and zingerone, as well as various phenolics and flavonoids, have been found to be primarily responsible for its pharmacological activities (Ha et al., 2012)

The first component that was considered in the formulation was the bioactive component of the ingredients. This is because in making functional food, the ingredients should contain a variety of bioactive substances that have positive effects on the health of the body (Koraqi, 2022). Furthermore, having a knowledge of the bioactive compounds in the extracts helps in determining the potential benefit of these extracts on the body as these compounds are the substances that will render functionality to the beverage. The analysis revealed that haskap extracts exhibit a high concentration of flavonoids and anthocyanins and display notable antioxidant properties. Additionally, sorrel and ginger extracts exhibit a high concentration of phenolic compounds and carotenoids. The above benefits underline the importance and relevance of having these compounds in a functional beverage. Thus, these bioactive compounds found in the sorrel, haskap and ginger extracts were used to enhance the haskap-flavoured water.

4.2.3 Sensory evaluation of haskap-based functional beverages.

The success of food and beverage products in the marketplace is significantly influenced by human perception and preference (Finlayson et al., 2007). The selection of a food product is also contingent on the multisensory experience it provides (Mielby et al., 2018). A sensory evaluation was conducted to determine consumer preference for haskap-flavoured beverages. **Supplementary Table 1** shows of the parameters that were assessed, only a few had statistically significant ($p < 0.05$) findings. These included the colour intensity, turbidity, turbidity preference, aroma intensity, sweetness preference, flavour, and overall liking. A report of the average preference of participants in ranking the four flavours based on sensory attributes are presented in **Figure 1.**

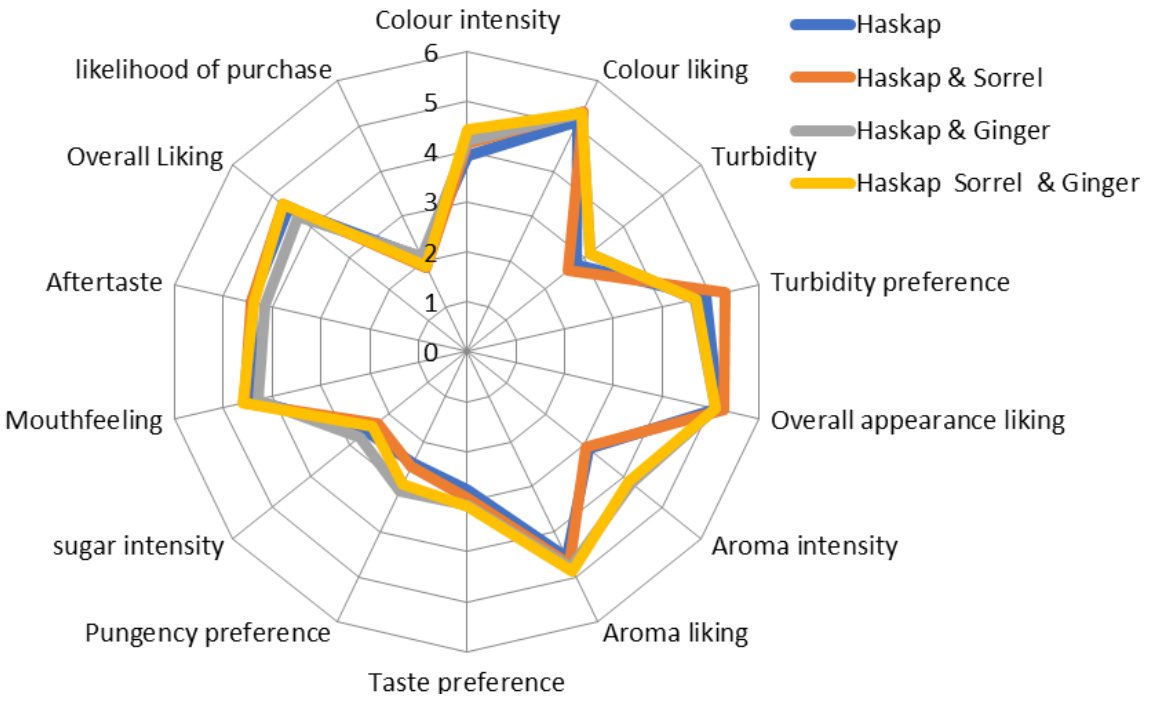

Figure 1. Sensory profile of haskap-based functional beverages.

The panelist noted a difference in the colour intensity pointing out that 'haskap with sorrel and ginger' was the beverage that had the most intense colour. This is possibly due to the presence of the three extracts, two of which had rich red colours, that were combined and could have added to this intense colour. There are several studies that points out the association between colour and liking preference (Spence, 2015, 2019; Spence and Piqueras-Fiszman, 2016). This was not observed in this study as the four beverages were red, although the intensity of red colour varied, which was noticed by participants. To analyze the relation between colour and taste preference, the colour of the beverages should have had more variation.

The haskap with sorrel and ginger beverage was noted as being significantly ($p < 0.05$) more turbid than the other beverages that were tested. The ginger extract would have introduced a level of cloudiness to the beverage that resulted in this increased turbidity to the flavours with ginger. Similarly, Arifan et al. (2023) noticed that increasing the quantity of ginger powder in robusta coffee (*Coffea canephora)* leaf tea resulted in an increase in the yellow hue of the green-coloured tea. The participants however preferred the appearance of haskap with sorrel which they observed as being the least turbid. The participants also had an overall preference for the appearance of haskap with sorrel **(Figure 1)**. This is possibly due to the low turbidity which would suggest that in terms of selecting a beverage for water, the clarity is more important than the colour. This offers insightful knowledge as it indicates that consumers would prefer to have a clear drink for their flavoured water, as opposed to a turbid beverage. The literature shows a trend in consumers' preference for clarity in some of their beverages. In one study, the darker and more turbid beers despite being fruity and more nutritious, had a low preference among consumers. da Costa Jardim et al. (2018) analyzed the sensory profile and consumer preference of Craft beers from Brazil and the findings showed that the more turbid beers had low preference among consumers. Another

study revealed that clear apple juice was more preferred than cloudy apple juice (Włodarska et al., 2016). Additionally, according to the research conducted by Zellner and Durlach, it was observed that clear beverages were perceived to be more refreshing (Zellner, 2003).

Increased aroma intensity was perceived by participants in the flavours that contained ginger. There was a strong liking for the aroma of the haskap with ginger, and haskap with ginger and sorrel flavours. This indicates that participants prefer to have the spiciness of aroma that ginger provides. This is similar to a study that showed that the essential oils present in ginger gave rise to a characteristic aroma that was well-received by the panelists. The researchers therefore concluded that adding ginger powder to robusta coffee leaf tea significantly increased the preference for the aroma of the product (Arifan et al., 2023). The preference for ginger flavours could be influenced by familiarity with this spice or a knowledge of its perceived health benefits. Gaikwad et al. (2013) noted that the increasing recognition of the health advantages associated with ginger-based beverages has resulted in significant interest in these products. A study was conducted to determine the knowledge of individuals on the health benefits and use of ginger and majority of the respondents reported that they consume fresh ginger. Some take ginger as a spice, while others use it for medicinal purposes or both. The respondents were also interested in food that had incorporated ginger as a spice (Amoah et al., 2022).

Haskap with ginger was perceived as being the sweetest of the four beverages as displayed in **Figure 1**. Nevertheless, all three flavours contained approximately the same amount of sugar. The sorrel and haskap concentrates added to the other beverages may have resulted in the inclusion of sour and bitter compounds respectively. The name sorrel seems to be a synonymous of its taste. According to Muhammad and Umar (2007), sorrel refers to any shrub belonging to a vast genus of diminutive, fleshy plants that have leaves containing acidic sap, which gives a sour flavour. The

general belief, however, is that sweetness and sourness suppress each other, and bitterness can supress sweetness (Melis and Tomassini Barbarossa, 2017; Mielby et al., 2018). The original haskap flavour was considered bland by many participants, but the presence of sorrel could have potentially led to the masking of the sweetness in the other beverages. Interestingly, the phenomenon of taste interactions arises when two or more tastants are presented concurrently, leading to a mutual influence on each other's perception. This was possibly what happened when haskap and sorrel were combined in the beverage. The phenomenon of taste interactions can have either a positive or negative effect on the overall taste experience, based on the quality of taste, the specific tastants involved, and their respective concentrations (Wilkie and Phillips, 2014). Apart from the sweetness, participants had a taste preference for the ginger flavoured beverages, which may again be due to the health benefits that ginger is known to offer.

The overall liking based on the hedonic rating analysis suggest that haskap with sorrel and ginger was the favourite of the four beverages. This may be due to the mixture of extracts in this beverage, each of which adds a distinctive flavour in addition to the spicy ginger aroma. Additionally, with this combination, there is an increased probability that participants know at least one ingredient. It is important to note that the likelihood of purchase of these beverages is low based on the responses from the survey (**Figure 1).** Prior sensitization to the ingredient could have potentially improved the acceptability of these products by panelist, as there is a tendency of consumers to be reluctant to accepting new food (Lusk et al., 2014).

The results from the sensory were used to run a principal component analysis (PCA) to visualize and better determine how the attributes clustered and thus the possibly related to each of the formulated beverage. The output is shown in Figure 6. The upper right quadrant of the PCA in Figure 6 indicates that haskap with sorrel and ginger shows a relation to the attributes related to

colour liking, pungency, and taste. Conversely, haskap with sorrel is clustered with the overall appearance and liking.

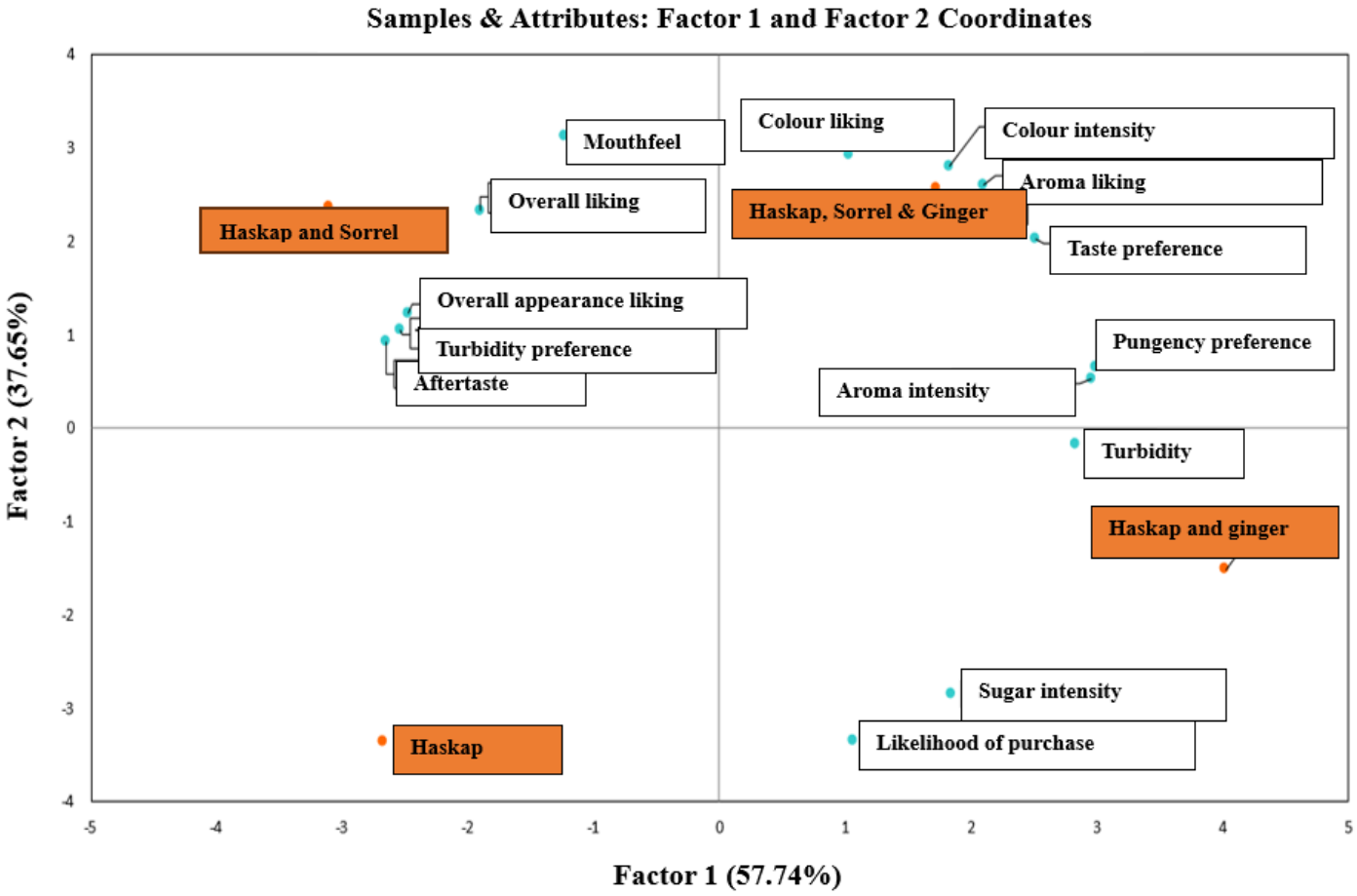

Figure 2. Scatter plots of principal component analysis (PCA) scores for specific sensory attributes of four haskap- flavoured functional beverages.

This may suggest that while there is a preference for haskap with sorrel and ginger in terms of aroma and taste, the visual attribute, specifically the clarity takes precedence when deciding on one's overall preferred flavour. This confirms the hypothesis that appearance is one of the contributing factors to consumer choice to the products. In the lower left quadrant, haskap is clustered with none of the attributes. This may suggest that the haskap flavour is not preferred by

itself but should be combined with other flavours to improve the potential of it being liked by consumers.

The participants of the sensory analysis were asked to give an overall ranking of the four flavours. Rank one was the overall favourite beverage and four was the least favourite overall. **Figure 3** establishes that haskap with sorrel was ranked as the preferred flavour, followed by haskap with sorrel and ginger. The original haskap flavoured water was ranked number four which suggest that it was the least preferred among the flavours. This is in line with the representations of the PCA in **Figure 2**.

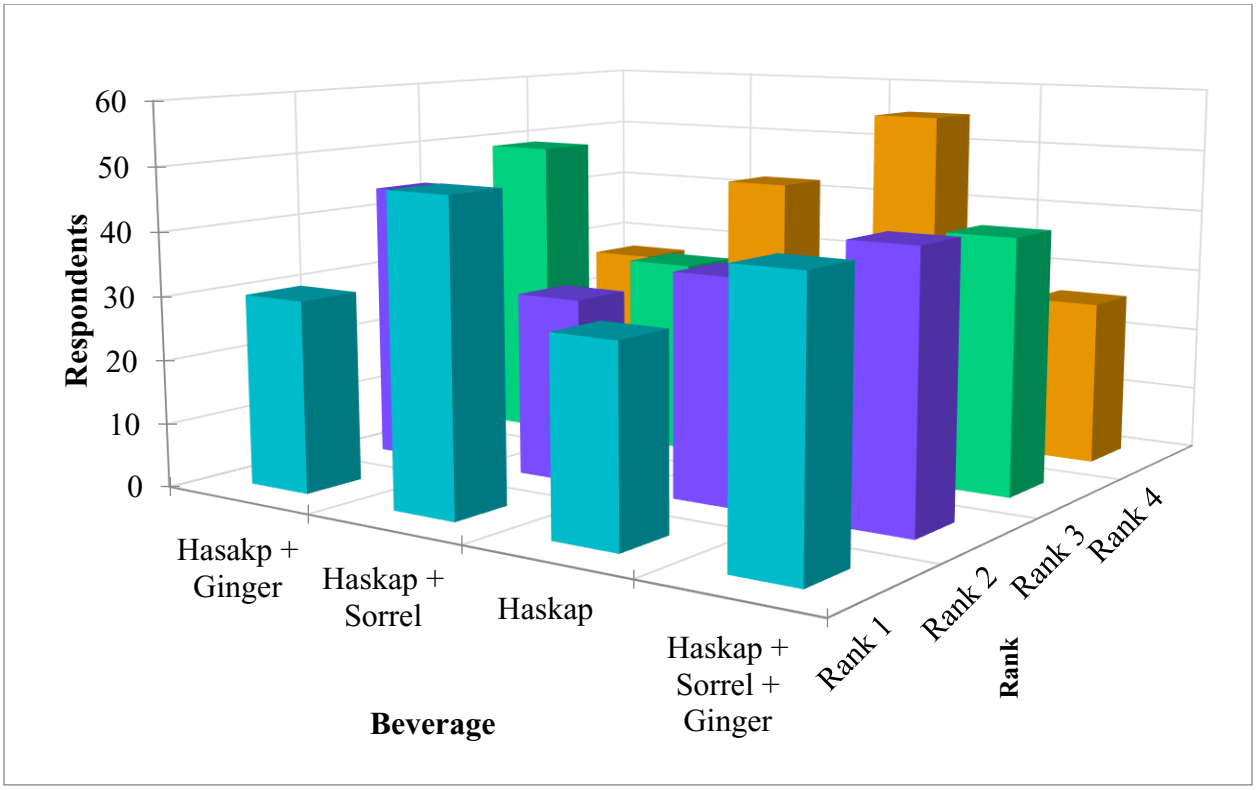

Figure 3. Contingency ranking of each of the four functional beverages based on the mean score of the participants.

Haskap with sorrel and ginger is the preferred beverage in terms of taste but the perceived cloudiness associated with it may be the cause of it being ranked second. The clarity therefore

needs to be improved to potentially increase consumers' acceptance of the beverage. Haskap can therefore be eliminated from options of beverages and more attention focused on developing the other three flavours that seem to be preferred by the participants.

4.3.4 Characterization of functional beverages

Haskap flavoured water created in a 1:5 ratio of haskap extract to Iceberg water v/v, was selected as the best blend that retained the refreshing iceberg taste after adding the extract.

The formulation for haskap with sorrel, and haskap with ginger was 1:1:8 and 3:1:16 v/v respectively (haskap extract: sorrel/ ginger extract: Iceberg water). The haskap with sorrel and ginger beverage was combined in a ratio of 2:1:1:16 v/v (haskap extract: sorrel extract: ginger extract: iceberg water). Samples of these beverages were then characterized to quantify their bioactive compounds.

4.3.4.1 Bioactive composition of haskap- based beverages

The four formulated products were characterized for their phenolic content, anthocyanin, flavonoid, carotenoid, and antioxidant activity. **Table 4** gives an outline of the compounds that were present in each beverage. Haskap with sorrel had significantly ($p < 0.05$) higher phenolic and anthocyanin content than the other three flavours. Both the haskap and sorrel extracts had high anthocyanin and phenolic content respectively which could have contributed to this finding.

Table 4. Bioactive compounds in haskap flavoured beverages

Beverage	TPC	TAC	TFC	ABTS-TAA	FRAP-TAA	Carotenoid
Haskap+ Sorrel+ Ginger	245.5 ± 1.17b	54.69 ± 0.04c	21.07 ± 0.16a	369.41 ± 1.70a	298.96 ± 1.21a	1976.92 ± 28.83a
Haskap + Sorrel	311 ± 1.73a	61.40 ± 0.09a	19.19 ± 0.16b	344.06 ± 0.81b	261.17 ± 0.56b	1781.42 ± 69.93b
Haskap+ Ginger	218.50 ± 3.45c	51.95 ± 0.08d	16.38 ± 0.14c	324.76 ± 1.03c	223.53 ± 1.44c	1233.03 ± 27.26c
Haskap	184.06 ± 1.65d	56.76 ± 0.07b	16.57 ± 0.18c	280.20 ± 1.56d	216.11 ± 0.87d	1277.40 ± 37.62c

TPC = total phenolic content in mg 100 QE mL^{-1}. F*RAP*= ferric reducing antioxidant power, ABTS= 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). TAA = Total antioxidant activity in µmol TE 100 mL^{-1} . TAC = total anthocyanin content measured in mg 100 mL^{-1}. TFC= total flavonoid content in mg QE 100 mL^{-1} . Carotenoid measured µg per 100 mL of sample. Values represent means ± standard error (n=8). Significant ($p < 0.05$) differences are represented by different letters (a-d) within the same column.

The presence of anthocyanin has been noted as a significant contributing factor to the overall phenolic composition of haskap (Rupasinghe et al., 2018). Furthermore, the antioxidant properties of sorrel are primarily attributed to anthocyanins, which constitute the major phenol content (Borrás-Linares et al., 2015; Peredo Pozos et al., 2020). One cup of the sorrel drink or dried powdered roselle extract twice daily has been recommended for some disease conditions, as it provides 250 mg anthocyanin per day (Salami and Afolayan, 2020). Additionally, the higher ratio, and thus the concentration of sorrel to haskap extracts in the haskap with sorrel beverage may have caused the significant difference between phenolic and anthocyanin content between the haskap with sorrel and the haskap with sorrel and ginger samples, as both beverages contain sorrel and haskap.

Haskap with sorrel and ginger had significantly ($p < 0.05$). higher flavonoid and carotenoid content and showed greater antioxidant activity than the other three beverages. Both sorrel and haskap naturally have high carotenoid content (Wu et al., 2021; Cheng et al., 2022), however, this increase was possibly due to the addition of ginger extract with its antioxidant compounds. The result in the present study is comparable to the findings of another study in which gum arabic and Aloe vera *(Aloe barbadensis Miller)* gel-based edible coatings in combination with ginger, garlic (*Allium sativum)*, and aloe vera extracts were compared. Total carotenoids were higher in the ginger extract and gum arabic combination than the other combinations without ginger (Anjum et al., 2020). Ginger is rich in carotenoids and has high antioxidant activity. The results of the correlation analyses conducted on the biochemical composition of ginger rhizomes indicated a strong positive correlation between total phenolics and antioxidant activity ($r = 0.973$, $p < 0.001$). Additionally, carotenoids strongly correlated with total phenolic content ($r= 0.915$, $p < 0.05$) in another study (Ghafoor et al., 2020). This suggests that carotenoid contributes to the antioxidant activity in ginger. Thus, the bioactive compounds in the ginger extract could have boosted the antioxidant activity of the haskap with ginger and sorrel beverage. The potent antioxidant activity of ginger can be seen in a study conducted by Attia et al. (2013) in which rats were treated with lead acetate for the first 50 days, and then treated with ginger solution for the next 50 days. The study showed an increase in lipid peroxidation and a decrease in glutathione (GSH) levels in the control rats that did not receive the ginger solution. There was however an increase in the superoxide dismutase and glutathione peroxidase activities in rats that were treated with the ginger solution. The upregulation of protective enzymes is just one means by which ginger defends the body against oxidative damage.

Most phenolic assays of functional drinks in the literature were measured using gallic acid while in this study, quercetin was used. The highest TPC in this study was 311 mg QE 100 mL^{-1} for haskap with sorrel (**Table 4)**. The TPC in other plant-based functional drinks was 15.2 mg GAE 100 mL^{-1} in cocoa and red tea (Kittibunchakul et al., 2021), 158.9 mg GAE 100 mL^{-1} in an orange peel enriched functional drink (Selahvarzi et al., 2022) 18.7 to 54.2 mg GAE 100 mg GAE 100 mL^{-1} in a ready to drink orange juice and nectar (Stella et al., 2011), 1602-195.0 mg GAE 100 mL^{-1} in a novel functional fruit beverage consisting of Cornelian Cherry (*Cornus mas*) Juice (Mantzourani et al., 2018), and 14.8 and 33.0 mg GAE 100 mL^{-1} for hibiscus *(Hibiscus sabdariffa L.)* and green tea *(Camellia sinensis L)* (Preciado-Saldaña et al., 2019) respectively.

The total flavonoid content in the haskap with sorrel and ginger (21.1 mg QE 100 mL^{-1}) represented in **Table 4**, was lower than TFC in other plant-based products such as 15% orange peel enriched functional drink (83.4 mg QE 100 mL^{-1}) (Selahvarzi et al., 2022) but higher than flavonoid in seaweed infused coffee (1.5 mg QE 100 mL^{-1}) (Kumar et al., 2019).

Furthermore, the total antioxidant activity in haskap with sorrel and ginger (ABTS- 369.41 ± 1.70, FRAP- 299.0 ± 1.21 µmol TE 100 mL^{-1}) was within close range of ready-to-drink orange juice and nectar functional beverages (57.9 to 349.3 µmol TE 100 mL^{-1}) in the literature (Stella et al., 2011). The findings of this investigation are consistent with a previous study that reported on the antioxidant properties of sorrel beverage as 335.1 ± 4.6 µmol TE 100 mL^{-} and 312 ± 7.8 µmol TE 100 mL^{-1} for ABTS and FRAP respectively (Sáyago-Ayerdi et al., 2007).

4.3.4.2 Vitamin composition of haskap-based functional beverages.

A vitamin analysis was done on the four haskap-based samples for water-soluble vitamins. The results are illustrated in **Figure 4**. All beverages were found to contain Vitamins B2 (riboflavin), B3(niacin), B5(pantothenic acid), B6 (pyridoxine), B9 (folic acid), B10 (Para-aminobenzoic acid), and vitamin C (ascorbic acid) in varying concentrations. Haskap (79.2 ± 1.0 mg 100 mL^{-1}) beverage contained statistically higher vitamin C followed by haskap and sorrel (75.9 ± 1.0 mg 100 mL^{-1}). This is expected due to the high concentrations of vitamin C in haskap extracts.

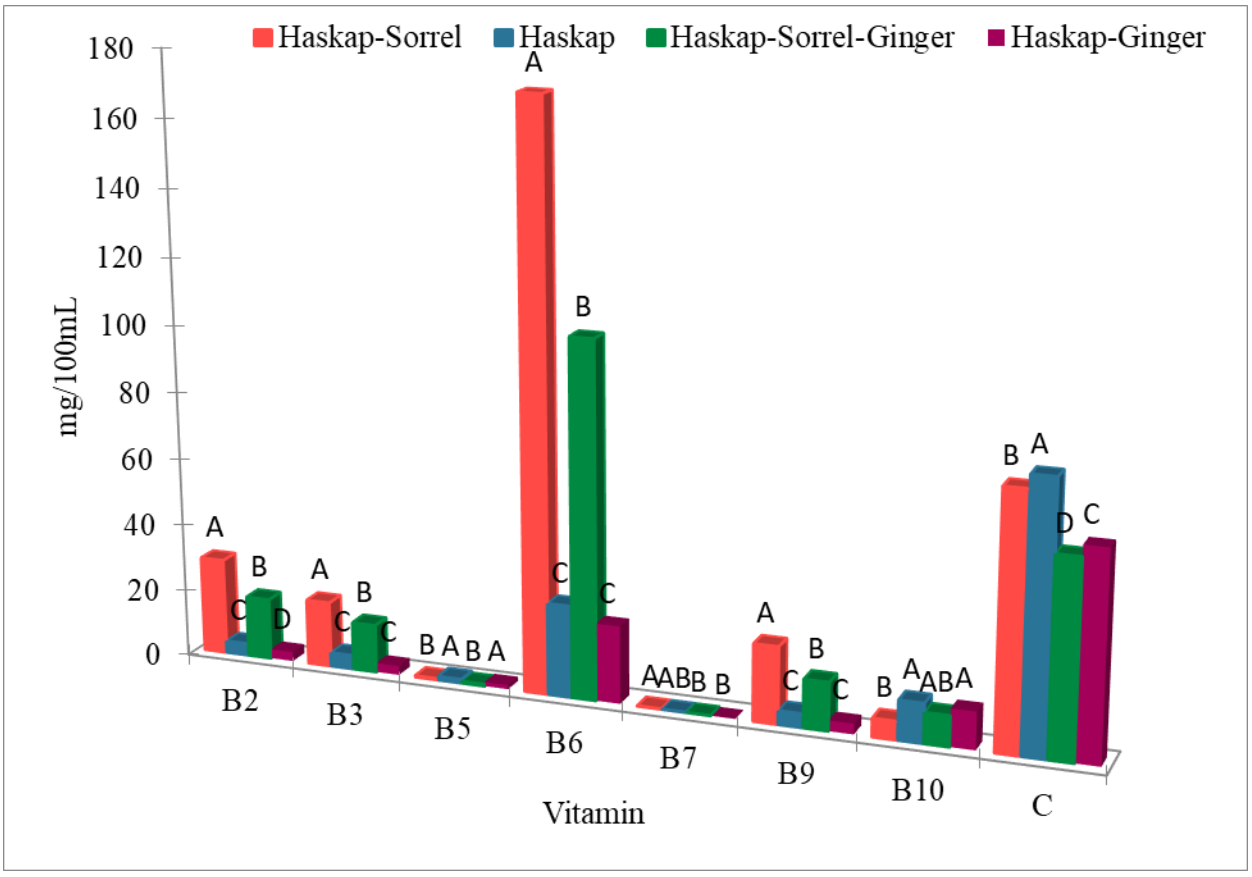

Figure 4. 3-D clustered column showing water soluble vitamins in the haskap based- beverages. Vitamins B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxin), B7 (Biotin), B9 (folic acid), B10 (Para-aminobenzoic acid), and vitamin C (ascorbic acid). The letters a-*d* for the same vitamin complex represent significant differences ($p < 0.05$).

Haskap is known to contain higher vitamin C content than other vitamin C-rich sources like orange and strawberry (Grobelna et al., 2020; Rupasinghe et al., 2018). Furthermore, studies have revealed that the calyx of sorrel plants is rich in vitamin C and Riboflavin (Babalola, 2000; Muhammad and Umar, 2007; Mohammed et al., 2017; Riaz et al., 2021). The samples containing ginger were significantly lower in vitamin C than the other beverages without ginger. This is because ginger does not have high concentrations of vitamin C. According to Latona et al. (2012), ginger contains 1.0 % vitamin C. The ginger diluted the concentration of the haskap and sorrel extracts in the sample which resulted in lower levels of the individual vitamin complexes in the haskap with sorrel and ginger extract. This is similar to a study in which adding orange or pineapple juice to sorrel extract decreased the concentration of mineral content in the sorrel extract (Oboh and Elusiyan, 2004).

The recommended dietary allowance (RDA) for vitamin C differs across countries and is based on one's gender, age, and health status (i.e., pregnancy, smoker) (Carr and Lykkesfeldt, 2023). In North America, the current guidelines suggest that women should consume 75 mg vitamin C daily, while men should consume 90 mg/day (Ihara et al., 2004). Thus, drinking one cup (250 mL) of haskap flavoured water or 'haskap with sorrel' would supply more than 200% RDA of vitamin C.

Additionally, haskap with sorrel was found to contain significantly higher amounts of vitamins B2 (28.6 ± 0.1 mg 100 mL^{-1}), B3 (16.1 ± 3.7 mg 100 mL^{-1}), B6 (173.8 ± 4.8 mg 100 mL^{-1}) and B9 (23.0 ± 0.8 mg 100 mL^{-1}) than the other beverages. In addition to vitamin C, sorrel extracts have been found to contain vitamins B1(thiamine), B2, and B6 (Mukhtar, 2008). This high content of vitamins in sorrel contributed to the increased concentration in the overall vitamin content in the beverage containing sorrel. Vitamin B1 however was not found in the beverages in this study.

4.4 CONCLUSION

In this study, four different formulations of functional beverages were developed by incorporating haskap as a potential antioxidant agent into iceberg water. To provide a more desirable product, sorrel and ginger were also added into the formulation, which further improved the physicochemical and bioactive properties. A sensory evaluation revealed that the panelist preferred haskap with sorrel and haskap with sorrel and ginger-flavoured waters. A combination of flavours can increase the willingness of consumers to try these products as it has been suggested to facilitate the introduction of novel food into the market The beverages were evaluated for their bioactive compounds and haskap with sorrel was found to contain significantly higher phenolic contents and anthocyanin, while haskap with sorrel and ginger contained higher flavonoids and carotenoids showed significantly greater antioxidant. Interestingly, it was found that haskap contained the highest vitamin C content overall. The results suggest that this novel formulation of functional drink holds great potential as a natural, refreshing, and nutritious source of antioxidants and vitamins. Our study also demonstrates the great potential of future scale-up production and commercialization of this functional beverage. It substantiates the applicability of haskap-flavoured water as a functional beverage. Further experimentation is recommended to validate the *in vivo* antioxidant characteristics and other bioactive effects, with an animal model or human trial.

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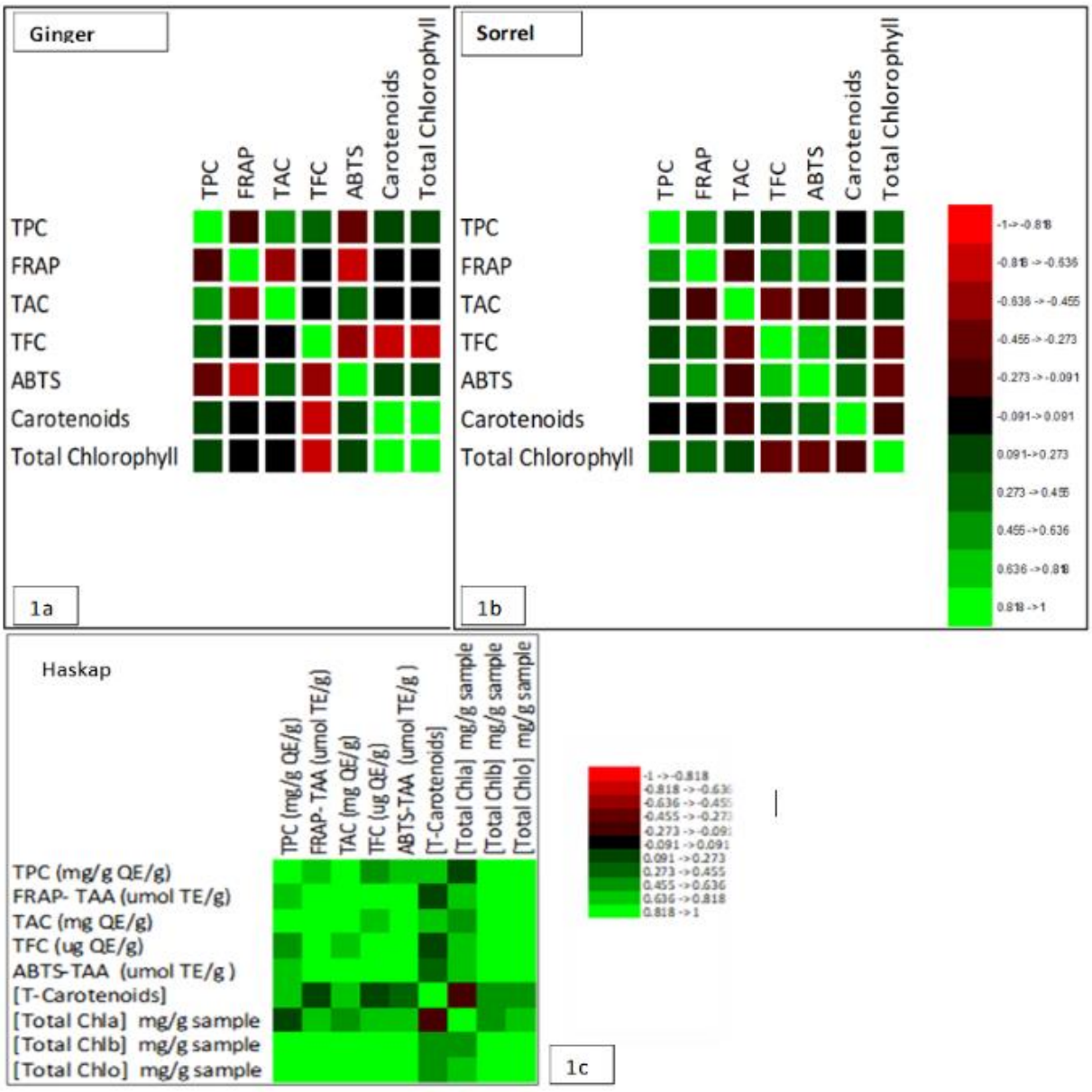
4.6 SUPPLEMENTARY DATA



Supplementary Figure 1a-c. Correlation map shows the relation between bioactive compounds in ginger (1a), sorrel (1b), and haskap (1c) extracts respectively.

Summary of MeanScores, P-Values, and Significance
Test Result Code: BEV
Test Description: HASKAP BERRY

This test was performed on 150 panelists.

Attribute	Haskap	Haskap & Sorrel	Haskap & Ginger	Haskap, Sorrel, & Ginger	P-Value	Sig
Colourintensity	3.91	4.16	4.21	4.44	0.0001	***
Colour liking	5.12	5.33	5.29	5.29	0.3226	NS
Turbidity	2.79	2.59	3.13	3.13	0.0001	***
Turbidity preference	4.90	5.29	4.67	4.69	0.0001	***
Overall appearance liking	5.15	5.27	5.09	5.10	0.4057	NS
Aroma intensity	3.12	3.05	4.21	4.15	0.0001	***
Aroma Liking	4.57	4.73	4.79	4.87	0.2063	NS
Taste preference - Sweet	0.15	0.31	0.12	0.25	0.0001	***
Taste preference - Salty	0.08	0.05	0.07	0.08	0.6267	NS
Taste preference - Sour	0.21	0.19	0.30	0.25	0.1401	NS
Taste preference - Bitter	0.15	0.12	0.20	0.15	0.2990	NS
Taste preference - Bland	0.26	0.21	0.24	0.17	0.2842	NS
Flavor intensity	2.77	2.93	3.07	3.09	0.0024	**
Pungency preference	2.47	2.56	3.10	2.95	0.0001	***
sugar intensity	2.58	2.29	2.73	2.41	0.0001	***
Mouthfeeling	ong4.39	4.58	4.30	4.59	0.1129	NS
Aftertaste	4.44	4.44	4.15	4.39	0.1667	NS
Overall Liking	4.58	4.71	4.32	4.72	0.0205	*
Likelihood to purchase	2.07	1.87	2.10	1.89	0.1909	NS

Supplementary Table 1. ANOVA result showing the attributes that were selected by the 150 participants for each of the four for formulated beverages in the sensory analysis. Significant factors are highlighted.

CHAPTER 5
GENERAL CONCLUSION

Berries are recognized as significant natural sources of antioxidants, which have the potential to safeguard the body against oxidative stress. The findings of the current investigation suggest that the extract derived from haskap exhibited significant antioxidant activity *in vitro*. The extraction parameters, particularly the utilization of a binary solvent pure ethanol: water (1:1), at a temperature of 40°C for a duration of 60 minutes in sous vide, exhibited a significant influence on the extraction of polyphenolic compounds and the antioxidant activity of the extracts specifically anthocyanins. Thus, the binary extract derived from haskap berries has the potential to be investigated as a natural antioxidant suitable for incorporation into functional foods or nutraceuticals.

Under the optimized conditions, haskap cultivar Indigo Gem exhibited a significantly ($p< 0.05$) higher level of bioactive potential in comparison to Wojtek. This suggests that Indigo Gem may possess noteworthy biological properties. Additional research is required to comprehensively evaluate the biological characteristics of this cultivar of haskap. Furthermore, the utilization of the sous vide method in the extraction of bioactive compounds, particularly heat-labile ones like anthocyanin and vitamin C from fruits, can result in a more efficient and time-effective approach on a broader scope.

The method of sous vide extraction was employed to generate antioxidant rich extracts, which were subsequently utilized in the development of haskap-based functional beverages. The sensory evaluation of the four beverage formulations revealed that the panellists displayed a preference for haskap with sorrel in terms of appearance, while haskap with sorrel and ginger was favoured for aroma and flavour. The bioactive compounds and antioxidant activity of the haskap and haskap

with ginger beverages were found to be lower than those of the two aforementioned beverages. Among the beverages analzyed, the haskap-flavoured water exhibited the highest concentration of vitamin C. The participants ranked the haskap with sorrel as the top choice overall. This implies that while sensory attributes such as aroma and flavour may impact consumer preference, the visual appearance of the product was the primary determinant of consumer selection for these beverages.

In conclusion, it is crucial to acknowledge that while the antioxidant capacity serves as the primary factor in determining the advantageous characteristics of such beverages as functional food, the sensory approval of these commodities by consumers holds equal significance. Hence, it is recommended that emphasis be placed on the visual appeal of the beverage in the continued advancement of these functional products and other beverages. The opaqueness resulting from the presence of ginger in beverages needs to be eliminated, while ensuring that the characteristic scent and taste of ginger are preserved, in order to enhance the desirability of such drinks. The intrinsic desirability of haskap as a lone flavour appears to be limited, suggesting that its palatability is enhanced when it is integrated with other complementary flavours, thereby increasing consumer acceptability. It is imperative to consider these factors when formulating and developing alternative products utilizing haskap. Due to the significant presence of vitamin C and polyphenols in haskap, a greater proportion of haskap extract to other flavours is recommended when formulating functional food, to achieve higher concentrations of these compounds.

A constraint of this investigation pertains to the restricted duration (30 and 60 minutes) and temperature (30, 40, 60) variables employed in formulating the extraction technique. In order to ascertain the precise moment at which the heat-labile compounds began to diminish, it would have been advisable to conduct more frequent time-point measurements during the experiment. By

comparing the results obtained at these intervals, it would have been possible to pinpoint the exact temperature range between 30 and 40°C at which the yield began to decrease. The objective of the study was to ascertain the minimum duration required to attain maximum compound production. However, it is recommended that a broader time frame be incorporated to identify the inflection point at which the yield begins to decrease. It is plausible that an increase in temperature and duration of the process may have resulted in a higher yield of bioactive compounds. Therefore, additional experimentation may be conducted to enhance the extraction protocol and achieve an optimized yield of antioxidants from fruits and vegetables. Additionally, to enhance the sensory analysis, incorporating a greater range of colours in the beverages could have facilitated an assessment of the potential impact of colour on consumer preference, thereby contributing to the advancement of functional beverage development.

As the functional food market seeks novel nutritious products, our study demonstrates the great potential of future scale-up production and commercialization of haskap with sorrel and haskap with sorrel and ginger as functional beverages. It substantiates the antioxidant potential and applicability of haskap-flavoured water as a functional beverage. Further experimentation is recommended however, to validate the *in vivo* antioxidant characteristics and other bioactive effects of these haskap-based products, using an animal model and human trial. After this, more sensitization of the health benefits of the product could also improve the commercialization potential and future advancements of these beverages.

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APPENDIX

Document # 1- Approval for sensory analysis

GRENFELL CAMPUS | MEMORIAL UNIVERSITY
Research Ethics Board
University Drive, Corner Brook, NL Canada A2H 5G4
Tel: 709-639-2399 Fax: (709) 637-2885 http://www.grenfell.mun.ca/research-ethics-board

June 2, 2022

Reference number: **20160641**

Dear Dwayne Keough, Dr. Thomas, Dr. Stewart

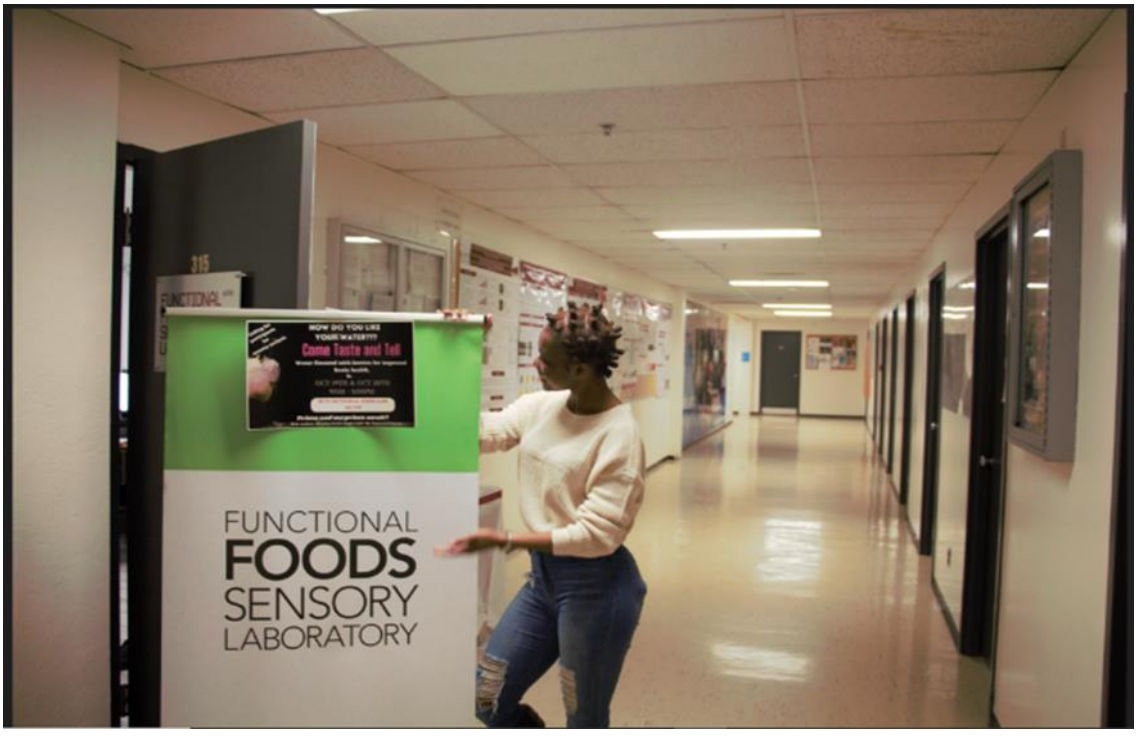
Thank you for your application for the extension of ethical clearance for your proposals *Taste Perception Influenced by Marinade Type and Time? A Multisensory Investigation*. The Grenfell CampusResearch Ethics Board (GC-REB) has reviewed your application and finds the proposal in ethical compliance with the Tri-Council Guidelines. Your approval for this extension expires on June 2, 2023. To remain in compliance with Article 6.14 (Continuing Research Ethics Review) of the Tri-Council Policy Statement on Ethics in Human Research (TCPS2), should your project continue past that date, you are required to renew your ethics approval in a timely manner. As well, please note that any changesto the proposed study will need to be cleared by the GC-REB first.

The Board wishes you success with your research.

Best wishes,

John Bodner, Ph.D., Chair

Important Notice regarding COVID-19: Memorial University, including Grenfell Campus, currently has a ban on all in-person data collection. Although projects involving face -to-face contact may be ethically approved, they cannot recommence until Memorial changes the policy.You can follow information on the current status of policy here: https://www.mun.ca/research/.



Collage of participants (top) and M.Sc. student Beverley Reid (bottom) at the sensory analysis conducted at Grenfell campus.

Haskap flavoured water- samples of the final packaged functional product