Bioactivity of anthocyanins from *Hibiscus sabdariffa*

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A thesis submitted in accordance with the requirements for the degree

Doctor of Philosophy

University of Leeds

School of Food Science and Nutrition

October 2013

The candidate confirms that the work submitted is her own work except where work which has formed part of jointly- authored publications has been included. The contribution of the candidate and other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given with in the thesis where reference has been made to the work of others.

Articles ready for submission:

- Comparative Chemical and Biochemical Analysis of Extracts of *Hibiscus sabdariffa*. Heba A. Sindi, Lisa J. Marshall* and Michael R. A. Morgan.
- Comparison study of antioxidant capacity and bioactive compounds between pure aqueous extract of dried *Hibiscus sabdariffa* and selected commercially available products contain *Hibiscus sabdariffa.*
  Heba A. Sindi, Lisa J. Marshall* and Michael R. A. Morgan

Abstracts:

- Systematic study of the polyphenol: Composition of extracts of *Hibiscus sabdariffa*. 241th American Chemical Society National Meeting, Anaheim, Los Angeles, USA, March 27-31, 2011.
- Determination of Octanol- Water partition coefficients of anthocyanin standards and dried calyces of *Hibiscus sabdariffa*. EuroFoodChem XVII, Istanbul, Turkey, May 7-10, 2013.

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Acknowledgements

I would like to thank my supervisors Professor Michael Morgan and Doctor Lisa Marshall for all their support, effort, guidance and excellent supervision through my study. Your advice, experience and professional discussions help me a lot to resolve many problems through my study.

Thanks to King Abdul-Aziz University give me the chance to complete my study abroad by giving me scholarship and support me financially and Saudi cultural bureau for all their help.

Also, I would like to thank James Zijun Luo for his collaboration with identifying the compounds using LC-MS as part of the thesis.

Am grateful to simply Habit Company, who is supplied the human study with *H. sabdariffa* drink.

Thank you my entire friends and colleagues inside the school and outside the school for all the support and kindness. Special thanks for my best friends Sarah Al hamli and Merfat Almagrabi we have been through a lot together wish you the best in your life and study as well; you are enriching my days in and outside the department. Thank you Wessal, Pattanan, Maha, David , Idolo, Wejdan, Nesreen, Bayan, Hawazen, Rawiah, Mashaeal, Shahad and Mona you are really good friends.

Finally I would like to dedicate my thesis as reward to my mother who has shown me that hard work the first step of success, and to my only and lovely sister Hala and to all my brothers your support help me a lot during my study. Ahmed you teach me that I cannot achieve my PhD without management and organisation also you gave me all the encouragements to complete my study, Mohammed, Taher and Khalid I'm so proud of you thank you all for your beliefs in me, thank you for always encouraging me to have

belief in myself. I won’t be in this position without your encouragement. Big hug and thanks to all my nephews and nieces

Abstract

A systematic study on extraction of *Hibiscus sabdariffa* was carried out for the first time using different solvents (water, methanol, ethyl acetate and hexane) in the presence and absence of formic acid, using different extraction times and temperatures. The extracts were analysed for total phenol content, antioxidant capacity using DPPH, FRAP and TEAC assays, and total monomeric anthocyanin content. In addition, specific anthocyanins were determined using HPLC and LC-MS. The results showed the highest antioxidant capacities were obtained by extracting using water, with or without formic acid, for 10 min at 100 °C. These extracts provided the highest concentrations of cyanidin 3-sambubioside and delphinidin 3-sambubioside.

Commercially available herbal teas containing *H. sabdariffa* were analysed. The study found that contents of total phenols, anthocyanins and antioxidant capacity were higher when using the optimal extraction procedure, suggesting that putative health benefits could be increased by altering processing methods.

The partition coefficients (log *p*) of anthocyanins found in *H. sabdariffa*, were measured showed that aglycone and glucoside forms of hibiscus anthocyanins behave differently when in the presence of cell wall material. Such behaviour could, *in vivo,* affect the absorption and bioactivity of these anthocyanins, and therefore, their efficacy.

A human crossover study investigated the effect of daily consumption for 8 weeks of a *H. sabdariffa* juice for 8 weeks on the blood pressure of healthy subjects (n= 29). Cranberry juice was used as the control. A significant reduction was found in systolic blood pressure (but not diastolic) compared to the baseline. No significant effect on blood pressure was seen with cranberry juice. The study suggests that regular consumption of extracts of *H. sabdariffa* may reduce the risk of cardiovascular disease,

on the other hand people with low blood pressure should consumed it very carefully due to hypotensive effect of the extract.

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List of abbreviation

ACE _ Angiotensin Converting Enzyme

ANP _Atrial Natriuretic Peptide

Apo B _ Apolipoprotein B

ALT_ Alanine aminotransferase or Alanine transaminase

AST _ Aspartate aminotransferase or Aspartate transaminase

ASE _ Accelerated Solvent Extraction

Bcl-2_ B-cell lymphoma 2

BHA _Butylated Hydroxyanisole

BNP _Brain Natriuretic Peptide

BP_ Blood Pressure

BRAI _ Briggs–Rauscher Antioxidant Index

CAT _ Catalase

cGMP_ Cyclic Guanosine MonoPhosphate

CIS_ Cisplatin it is chemotherapy drug

DBP _ Diastolic Blood Pressure

DNA_ Deoxyribonucleic acid

DPPH _ 2, 2-Diphenyl-1-Picrylhydrazyl

eNO _ Endothelial Nitric Oxide

eNOS_ Endothelial Nitric Oxide Synthase enzyme

FasL_ Fas ligand (type-II transmembrane protein that belongs to the tumour necrosis factor)

FMD_ flow-mediated endothelium-dependent dilation

FRAP _ Ferric Reducing Antioxidant Potential

GIT _ Gastrointestinal tract

GLUT1_Glucose transporter 1

GLUT2_Glucose transporter 2

GPx_ Plutathione Peroxidase

GSH_ Glutathione

H. sabdariffa _ *Hibiscus sabdariffa*

HPLC_ High-Performance Liquid Chromatography

HTN_ Hypertension

iNOS_ Inducible Nitric Oxide Synthase enzyme

IL-10_ Interleukin-10 known as human cytokine synthesis inhibitory

LDL_ Low Density Lipoprotein

LOD_ Limits of detection

LOQ_ Limits of quantification

Log *P*_ Partition Coefficient

miRNAs _ Micro Ribonucleic Acid

mRNA_ Messenger Ribonucleic acid

MKN-28_ Adenocarcinoma stomach cells as a gastric barrier

NADPH_ Nicotinamide Adenine Dinucleotide Phosphate

NHS_ National Health Service

NO_ Nitric Oxide

1-NP _ 1- Nitropyrene

OD _Optical Density

oxLDL_ Oxidized Low Density Lipoprotein

P 53_ Tumor protein 53

PCA_ Protocatechuic Acid

PDA_ Photodiode array

PKa_ the negative base-10 logarithm of the acid dissociation constant of a solution

PLE _ Pressurised Liquid Extraction

PPAR _ Peroxisome Proliferator-Activated Receptors

REM_ Relative Electrophoretic Mobility

ROS_ Reactive Oxygen Species

SBP_ Systolic Blood Pressure

SFE_ Supercritical Fluid Extraction

SOD_ Superoxide Dismutases

ST _ Sulfotransferase

TBARS _ Thiobarbituric Acid and Reactive Substances

TEAC _Total Equivalent Antioxidant Capacity

TNF- α _ Tumour Necrosis Factor Alpha

UGT_ Uridine Diphospho-glucuronosyltransferase

USDA_ United States Department of Agriculture

Terminology

- Vasoconstriction: Tight in blood vessels as a result of muscle contraction on the vessel wall.
- Vasodilation: Expansion occurs in blood vessels due to relaxation of smooth muscles cells.
- Natriuretic: is a peptide responsible for secretion large quantities of sodium in urine. and causes naturesis,
- Endothelial cells: the internal cells of blood and lymph vessels which in contact with blood.
- Tachycardia: irregularity in heartbeat rate.
- Ambulatory blood pressure: measured blood pressure over 24 hours which affected by fear of the white coat of the doctors or nurses.
- Glycosylation: it is a process forming glucoside form as a result of the hydroxyl group in an aglycone molecule links with one or more sugars.
- miR122: type of genes responsible for control the bile acid, fatty acid oxidation and cholesterol.
- Cardiac output: the volume of blood pumped by the heart.
- Bilitranslocase: an organic anion carrier involved in bilirubin and phthalein uptake by the liver.
- Kimbap food: rice rolled in dried laver seaweed.

Chapter 1 Introduction

In the last few decades many people have changed the way they treat themselves from classic medication to herbal medication, due to lack of major side effects with natural medicine and to cut the cost of the treatment especially with chronic diseases. This is known as alternative medicine. Therefore several studies have been done on natural plants to estimate the potential antioxidant activity when consuming these plants as food or drugs to reduce the side effect of usual synthetic medication (Komes *et al.*, 2011). There has been more attention from food science experts and the food industry in last few years in the area of polyphenols owning to their ability as antioxidants which play major role in reducing the risk of cancer, cardiovascular and neurodegenerative diseases (Belscak *et al.*, 2011).

1.1 *Hibiscus sabdariffa*

There has been much focus in recent years on phytochemicals from plants which are believed to have bioactive properties that may prevent chronic diseases such as diabetes, cardiovascular disease and some types of cancer (Ali *et al.*, 2005; Maganha *et al.*, 2010). Consuming fruits and vegetables rich in anthocyanins can help to reduce the amount of reactive oxygen which can damage the cells and lead to certain type of disease such as cancer and cardiovascular disease (Gonzalez-Mendoza *et al.*, 2010). *Hibiscus sabdariffa* is an example of a plant that contains large amounts of phytochemicals including anthocyanins and phenols. An effect of anthocyanins on cardiovascular disease and hypertension has been reported (Ali *et al.*, 2005; Maganha *et al.*, 2010).

H. sabdariffa is one of many plants containing anthocyanins (Prenesti *et al.*, 2007). *H. sabdariffa* is a plant from the *Malvaceae* family, with flowers which are traditionally used as a hot or cold drinks, or in jam.

Mohamed *et al.* (2007) described *H. sabdariffa* as ‘’an annual, erect, bushy plant, with smooth, cylindrical, red stems. Its leaves are green with reddish veins and petioles. Flowers, borne singly in the leaf axils, are yellow or buff with a rose or maroon eye, and turn pink as they wither. The calyx, which encloses the capsule, consists of 5 large sepals with a collar of pointed bracts around the base. The capsule is green when immature, 5-valved, with each valve containing 3 to 4 kidney-shaped seeds. The capsule turns brown and splits open when mature and dry. Calyx, stems, and leaves are acidic and closely resemble the cranberry (*Vaccinium* spp.) in flavour’’ (Mohamed *et al*., 2007).

H. sabdariffa has different names in different countries, such as karkad'e in Arabic regions and Africa, red or sour tea or Roselle in the UK. In Thailand, it is known as karchiap daeng, and in Spanish it is called Jamaica. In France, *H. sabdariffa* is famous as l’oiselle. The *Hibiscus* family contains more than 220 genera, of which 15 have been studied more extensively (Maganha *et al*., 2010).

H. sabdariffa is rich source of polyphenols, anthocyanins, and has antioxidative effects. The antioxidant effect of these types of bioactive compounds perhaps is not because of their antioxidant capacity, but other mechanisms might be involved (Piljac-Zegarac *et al.*, 2010). Therefore, the aim of this chapter is to study the composition of *H. sabdariffa*, extraction, stability, bioavailability and the potential pharmacological effects and the possible health benefits of *H. sabdariffa.* Figure 1-1 shows example of dried *H. sabdariffa* cycles and extract.

Figure 1-1. Dried *H. sabdariffa* calyces and extract

1.2 Polyphenols and flavonoids

Polyphenols can be defined as complex compounds with two or more aromatic rings attached together (Strack, 1997), and are found in different parts of all plants such as fruits, vegetables, nuts and flowers. Polyphenols can be classified, depending on their chemical structures, into 10 categories. The polyphenol family found in plants include phenolic acids, flavonoids, stilbenes, lignans, though 60% of flavonoids and 30% of phenolic acids contribute to total dietary polyphenols (Strack, 1997).

Nichenametla *et al.* (2006) reported the *in vitro* antioxidant effects of polyphenol compounds. Antioxidant compounds may protect the body from the risks of chronic diseases. Moreover, plants rich in polyphenols have been stated that they work as antimutagenic and anticarcinogenic which could reduce mutagenic and carcinogenic compounds, and an association between consumption of polyphenols and enhanced health has been demonstrated in epidemiological studies (Chong *et al.*, 2010; De Pascual-Teresa *et al.*, 2010; Cassidy *et al.*, 2011; Chanet *et al.*, 2012; Del Rio *et al.*, 2013). In addition, polyphenols have been reported to have potential pharmacological effects on many types of diseases (Nichenametla *et al.*, 2006).

According to Nichenametla *et al.* (2006), five main categories of polyphenols are particularly important: phenolic acids, flavonoids, iso-flavones, anthocyanins and

proanthocyanidins. The common phenolic acids in plants come from either hydroxybenzoic acids, such as gallic acid, vanillic acid, procatechuic acid or syringic acid, or from hydroxycinnamic acids, which consist of p-coumaric acid, caffeic acid and ferulic acid. Flavonoids may also be sub-classified into the following: flavonols, which are also known as (flavan-3-ol), flavanones and flavanonols. Figure 1-2 shows the basic skeleton structure for flavonoids, which contains two benzene rings attached to heterocyclic carbon ring.

The difference between flavones and flavonols is that flavonols have a hydroxyl group at position 3. Similarly, flavanonols vary from flavanones by their addition of a hydroxyl group in the C3-position. The differences in the structures of common flavonoids are reviewed in Table 1-1 (Nichenametla *et al*., 2006).

Figure 1-2. Flavonoid structure

Flavonoid class	Carbons involved in double bonds in the B-ring	Functional group in B-ring
flavanols	______	3-hydroxy, 3-*o*- gallate
flavonols	2 and 3	3-hydroxy, 4-oxo
flavones	2 and 3	4-oxo
flavanones	______	4-oxo
anthocyanidin	1 and 2, 3and 4	3-hydroxy

Table 1-1. Structural differences between flavonoid classes

1.3 Anthocyanins

Anthocyanins are one of the most important types of flavonoids. The name anthocyanin is a Greek word (antho), meaning flower and (kaynos) meaning red or blue (Schwartz *et al.*, 2008). Anthocyanins are secondary plant metabolites found in many fruits and vegetables, and are one of the subclasses of polyphenols (Castaneda-Ovando *et al.*, 2009).

Researchers nowadays are very interested in anthocyanins as natural antioxidants, because of their supposed health benefits in fighting several diseases, such as cardiovascular disease and cancer. According to Prior (2004) and Takeoka and Dao (2008), anthocyanins can be defined as red, purple or blue water-soluble pigments that occur in fruits and vegetables and particularly in flowers.

Cyanidin: R=OH, R_2=H
Malvidin: R_1=R_2= OCH_3
Pelargonidin: R_1=R_2=H
Pednodin: R_1= OCH_3,R_2= H
Petunidin: R_1= OCH_3, R_2=OH
Delphinidin: R_1=R_2=OH

Figure 1-3. Anthocyanidin structure

1.3.1 Anthocyanin structures

Figure 1-3 shows the general chemical structure of anthocyanins. The chemical structure of anthocyanin contains three rings C6, C3, C6. The benzene ring is important in anthocyanin structure. The main anthocyanins structure exists in the form of flavylium cations (Jackman and Smith, 1996; Schwartz *et* al., 2008).

Anthocyanins exist in the aglycone form (anthocyanidin) and the glucoside form. Anthocyanidin is the basic structure of anthocyanins (Prior, 2004; Schwartz *et al.*, 2008; Takeoka and Dao, 2008).

Six common aglycones are present in nature. Cyanidin is the main aglycone of the anthocyanins. Table 1-2 shows the names and structures of the most common anthocyanidins. The glucosides are normally formed by conjugating sugar molecules to

the aglycone forms, most of the time the glycosylation occurs at carbon 3. Glucose is the predominant sugar attached, while arabinose, galactose, rhamnose and xylose mostly are attached with glucose to form the di-glycoside (Jackman and Smith, 1996; Schwartz *et al.*, 2008; Takeoka and Dao, 2008). There are three important features in anthocyanins, the aglycone structure, sugar moiety and acylation groups. The structures and types of anthocyanins may vary due to the position of the hydroxyl and methoxyl group, the number and location of sugars and the extent of the acylating sugar. Methoxyl groups can donate electrons easily compared with hydroxyl groups. The methoxyl group is responsible for the red colour and the hydroxyl group responsible for the blue colour (Prior, 2004; Jackman and Smith, 1996; Brat *et al.*, 2008; Schwartz *et al.*, 2008). Anthocyanins can appear as monomers or dimers (Prior, 2004; Jackman and Smith, 1996; Brat et al., 2008; Schwartz et al., 2008). In addition, acylated anthocyanins result from esterifying sugars with aliphatic or aromatic acids. Most of the sugars attach to carbon number 3, but others can bind with any of the hydroxyl groups at carbon numbers 5, 7, 3', 4' or 5'. Anthocyanins and other flavonoids differ in that flavonoids are colourless or have a yellow colour, and they do not have the ability to react by proton transfers in polar solution (Takeoka and Dao, 2008).

In addition, anthocyanins are characterized as powerful antioxidants. Cyanidin glycosides have a high antioxidant capacity compared with peonidin- or malvidin-glycosides because of the free hydroxyl groups found on positions 3 and 4 of the B-ring of cyanidin (Prior, 2004; Brat *et al*., 2008).

Dietary anthocyanins are absorbed quickly in to the blood within 15-30 minutes. The anthocyanins are hydrolysed into aglycone and sugar before absorption (Amorini *et al.*, 2001). It was also found that the concentration of anthocyanins in the skin was much higher—five-fold the level found in plasma after 4 hours (Lietti and Forni, 1976). The high water solubility and relatively high molecular weight of anthocyanins and

proanthocyanidins make them incapable of passing through the small intestinal wall without prior hydrolysis (Deprez *et al.*, 1999).

Name	Substitution pattern	Aglycone structure
Delphinidin	3, 5,7, 3', 4', 5'-OH	
Cyanidin	3, 5,7, 3', 4'-OH	
Pelargonidin	3, 5,7, 4'-OH	
Petunidin	3, 5,7, 4', 5'-OH; 3'-OMe	
Peonidin	3, 5,7, 4'-OH; 3'-OMe	
Malvidin	3, 5,7, 4'-OH; 3, 5'-OMe	

Table 1-2. Common anthocyanin names, substitution patterns and structures

1.3.2 Extraction of anthocyanins from plants

According to Giusti and Jing (2008), extraction of anthocyanins usually depend on the complexity of the sample matrices and in which tissue the pigment is located. Anthocyanins pigments are located in the Vacuole near the surface. The first step of the

extraction is started by grinding and crushing the samples to powder to reduce the particle size (Giusti and Jing, 2008). Liquid nitrogen has been used to maximise the stability of anthocyanins by lowering the temperature, which inactivates the enzymes responsible for anthocyanin deterioration and provides a very fine powder which increases the surface area (Giusti and Jing, 2008).

1.3.2.1 Extraction methods

In general, there is no one type of procedure that can be used to extract all types of anthocyanins from plants (Santos-Buelga and Williamson, 2003). Wrolstad (2005) reported that choosing an appropriate method to extract the anthocyanins should be based on the method that gives high recovery and reduces the degradation in the samples. Jackman and Smith (1996) reported that if extracted anthocyanins are to be quantified then the method for extraction should extract the anthocyanin pigments in their natural forms. The procedure for extraction and purification needs to be simple, cheap and not time-consuming as much as possible (Jackman and Smith, 1996). Methods used for the extraction of polyphenols are solvent extraction, solid phase extraction, supercritical extraction and pressurised liquid extraction (Santos-Buelga and Williamson, 2003).

The pressurized liquid method is used to extract samples under high pressure and temperatures between 50-100 °C, generally methanol is used as a solvent to increase the contact between the solvent and the sample and to protect the sample from degradation and oxidation. Another method used is supercritical fluid extraction, which can also be classified as a type of solvent extraction, although an advanced method. In this method, gas and liquid are used to minimize extraction time and degradation in the samples under low temperatures. Commonly, carbon dioxide is used as a polar solvent to extract the polar compounds, and then a solvent such as methanol, ethanol or ethyl acetate is

used, depending on the type of polyphenol, to extract the phenol compounds in the cell walls; a C18 cartridge is then used to trap the phenols (Santos-Buelga and Williamson, 2003).

Giusti and Jing (2008) reported using supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and pressurised liquid extraction (PLE) to extract anthocyanins. The latter two methods are commonly used in extracting plant, animal and biological samples. However, PLE method results in anthocyanin deterioration when temperature reached above 70 °C. SFE method in general used to extract nonpolar compounds such as seeds oil, the SFE method is not preferable for extraction of anthocyanins due to the effect of the method on hydrophilic properties.

1.3.2.2 Factors affect the extraction of anthocyanins

According to Giusti and Jing (2008), there are three factors that must be considered before performing any extraction: the reason for extraction, the character of the anthocyanins being extracted and material resources.

Santos-Buelga and Williamson (2003) reported that an abundance of factors could affect the efficiency of extraction, such as type of solvent, pH, temperature, number of extraction steps, volume of solvent and finally, particle size and shape. Grinding of plants releases the phenol in the cells; therefore, when solvent is added to the sample, phenol is dissolved inside the particles and diffused form inside the cells to the outside.

1.3.2.3 Extraction solvents

In general, reducing the ratio between solid and solvent is recommended to avoid the concentration step that may lead to anthocyanin degradation (Giusti and Jing, 2008). Anthocyanins have interesting polarities that can show solubilities in different types of solvents (Wrolstad, 2005). Santos-Buelga and Williamson (2003) defined solvent

extraction as “a process designed to separate soluble phenolic compounds by diffusion from a solid matrix (plant tissue) using a liquid matrix (solvent).”

According to Jackman and Smith (1996), Santos-Buelga and Williamson (2003) and Giusti and Jing (2008), the most common solvents used in anthocyanin extraction are methanol, acetone, ethanol or aqueous solvents, because of the polar nature of these solvents and anthocyanins. Most of the anthocyanins in the glycoside form are compounds which are highly soluble in water. Non-polar aglycones can be extracted with non-aqueous solvents, whereas glycosides can be extracted using aqueous solvents.

Jackman and Smith (1996) and Giusti and Jing (2008) reported that several studies have used different solvents, like 60% methanol, *n*-butanol, cold acetone, mixtures of acetone, methanol and water, or water alone which extracted anthocyanins close to their natural form. However, Giusti and Jing (2008) and Castaoeda-Ovando *et al.* (2009) found that methanol was more efficient than the other solvents. Methanol is widely used as a solvent due to its low boiling point though the sample may need further processing to purify the sample because the extract may be contaminated with low polarity components (Wrolstad, 2005). When extraction with methanol, ethanol and water were compared, it appeared that methanol was 20% more efficient than ethanol and 73% more efficient than water at extracting anthocyanins from grapes (Castaoeda-Ovando *et al.*, 2009). Wrolstad (2005) also indicated that acetone increase the recovery by 30% efficiency of anthocyanins in 20 extracted samples of cranberries and elderberries in liquid and dry forms compared to chloroform and methanol in the presence or absence of acid. Jackman and Smith (1996) have reported that ethanol is not a preferred solvent due to the low yield of extracted anthocyanins and the high boiling point of the solvent which make it very hard to dry the extract. Acetone and chloroform were used for anthocyanin extraction, acetone to extract the anthocyanins and chloroform for separating anthocyanin from non-polar compounds such as lipids, immiscible organic

solvent, chlorophyll and carotenoids (Wrolstad, 2005). Similarly, Giusti and Jing (2008) indicated that using acetone-chloroform as a solvent for extraction, gave the advantage that it separated liquids, chlorophyll and insoluble water materials from anthocyanins. Castaoeda-Ovando *et al.* (2009) reported a group of researchers comparing acetone-chloroform solvent extraction with acidified methanol and found that acid minimised the deterioration of pigments and was more efficient than the acetone-chloroform solvent extraction. However, acid is useful to stabilise the pigments and flavylium cations, and it would also induce hydrolysis of the acyl and sugar and fracture the binding with metals or co-pigments. Usually, anthocyanin extracts are diluted due to the use of large quantities of solvents. Therefore, extracts should be concentrated and the evaporation process should be done under vacuum at 30 °C or by using a freeze dryer (Jackman and Smith, 1996) and (Wrolstad, 2005). In general, reducing the ratio between solid and solvent is recommended to avoid the concentration step that may lead to anthocyanin degradation (Giusti and Jing, 2008).

1.3.2.4 The effect of using acid in extraction

According to Wrolstad (2005), the aim of adding acid to the extraction solvent is because most of the anthocyanins are located near the cell wall or at the surface. Therefore, by adding acid the membrane of the cell wall will be disrupted and pigment will be more readily released. Acid will also break down the co-pigments or any association with metals, acid also stabilises the anthocyanins pigment the flavylium cation. Figure 1-4 shows the effect of low pH on flavylium structure.

To reduce degradation, weak acids and volatile organic acids such as formic, acetic, citric or tartaric acids were used in ranges from 0.01% to 3%, or HCl from 0.01% to 0.05% (Jackman and Smith, 1996; Santos-Buelga and Williamson, 2003; Giusti and Jing, 2008). Weak acids were used to extract the flavylium cation form. Conversely,

acid may result in hydrolysis of the acylated anthocyanins to colourless products or insoluble compounds. Therefore, understanding the concentration and type of acid is essential to knowing if an extract will be effective (Santos-Buelga and Williamson, 2003; Giusti and Jing, 2008).

1.3.2.5 Anthocyanin purification

Santos-Buelga and Williamson (2003) reported that solid phase extraction was often used for purification after extraction, to decrease the amount of solvent used in extraction and also as a method to purify and fractionate the bioactive compounds. On the other hand, one of the disadvantages of this method is that it could under-estimate the phenolic compound. The purification starts by removing the polar non-phenolics, and then separated the anthocyanins by washing with water to remove the sugar then acid eluted with ethanol aqueous with 0.01% HCl to acquire anthocyanins. Another column used Toyopear gel to separate anthocyanins and anthocyanin-derived pigments, but generally the reversed phase method is recommended for better results with HPLC. In addition, the solid phase can also be used to fractionate the acid and neutralise it using a C18 cartridge washed with water, then HCl, and then adding the phenol sample and adjusting the pH to 2 with HCl after that, the column is washed with 0.01% HCl and water.

1.4 Extraction of *H. sabdariffa*

Different methods have been used to extract *H. sabdariffa* using different solvents and different amounts of *H. sabdariffa*; this could be one of the reasons why each study has different results compared to other. Santos-Buelga and Williamson (2003) and Castaoeda-Ovando *et al.* (2009) reported that the most frequently used method for the extraction of anthocyanins was solvent extraction by adding a ground, freeze-dried or dried sample to the solvent, an ordinary aqueous mixture of solvents such as methanol,

ethanol and acetone. However, this method requires further purification of the extract due to the sugar, organic acid and proteins extracted with the anthocyanins. In addition, acidified solvents have been used for extraction by using weak acid such as 0.1% of hydrochloric acid, 1-3% formic acid or acetic acid, which has been reported as extracting increased amounts and different kinds of anthocyanins.

The Castaoeda-Ovando *et al.* (2009) review reported that acidified methanol is more efficient than ethanol or acetone, but there was one disadvantage to using the acidified method: if a strong acid was used, it could hydrolyse the acylated anthocyanins and damage the glycoside bond. However, with this kind of method, it cannot be guaranteed that the aglycones in the extract are hydrolysed. The types of solvent and the concentration of flavylium salts affect extract colour; therefore, using a protic solvent or a high concentration of flavylium salt revealed a red colour, which matches with the monomer, while aprotic solvent or a low concentration of flavylium showed a yellow colour due to the dimer.

Agoreyo *et al.* (2008) produced two extracts of *H. sabdariffa,* the first using 100 g of hibiscus with 200 ml of water, and the other using 400 ml of water brought to a boil for 15 minutes, to measure the effects of the *H. sabdariffa* extracts on blood cholesterol and glucose levels. However, the report reviewed about 26 studies on *H. sabdariffa* and the methods used for extraction. It could thus conclude that there were no reliable results and many different concentrations, time-frames, temperature, solvents and different solid to solvent ratio were used in extractions, which are all summarised in table 1-3. In addition, several studies did not declare the amount of sample used or even the time or temperature of the extraction

Solvent type	Solvent amount (ml)	Temperature (◦C)	Time (min)	Other conditions	Reference
Mixture of water and 95 % ethanol (1:1)+ 0.1M HCl	5		30	_____	(Duangmal *et al.*, 2008)
Hexane Water	2 2 to 4	Room temperature	60	Extract in the dark frozen under liquid nitrogen	(Mohamed et al., 2007)
Mixture water/+methanol +HCl (50:50:2) Water	125 125	_____	30	Using sonication	(Juliani *et al.*, 2009)
Water solid-to-solvent ratio 1:5 and 1:10	5 and 10	25 to 90	10 to 600	_____	(Cisse *et al.*, 2012a)
Water	10	_____	5	_____	(Aurelio *et al.*, 2008)
	5	60 90	180, 30 30	_____	(Mounigan and Badrie, 2007)
Water ratio of calyx to water of 1:15	15	30 100	240 30	pasteurisation	(Cisse *et al.*, 2012b)
Water	4 40	22 98	240, 16	_____	(Ramirez-Rodrigues *et al.*, 2011a)
	4	25 60 90	360, 720 and 1080 120, 240 and 360 60, 120 and 180	_____	(Ramirez-Rodrigues *et al.*, 2011b)

Solvent type	Solvent amount (ml)	Temperature (◦C)	Time (min)	Other conditions	Reference
Acidified ethanol of (1.5 mol/L HCl)	50	_____	_____	_____	(Tsai and Huang, 2004)
Water	20	90	20	_____	(Olawale, 2011)
	100	100	10	Filtered with Whatman No1	(Aoshima *et al.*, 2007)
	50	25	60	Filtered with Whatman No1	(Wong *et al.*, 2006)
	10	25	600	Stainless steel sieve (1mm)	(Cisse *et al.*, 2009)
	100	100	5	Centrifuge 2000 rpm for 10 min	(Oboh and Rocha, 2008)
	40	27, 30,50,60, 70,90,100	30,120,165, 210 and 300	_____	(Wong *et al.*, 2003)
	50	100 cold	3 5,30,180,540 and 930	Buchner funnel	(Amin *et al.*, 2008)
	100	_____	3	Buchner funnel	(Tsai *et al.*, 2002)
	2.5	25 or 100	720 or 10	_____	(Olvera-Garcia *et al.*, 2008)
	2	_____	_____	Filtered with Whatman No1	(Yin and Chao, 2008)

Solvent type	Solvent amount (ml)	Temperature (◦C)	Time (min)	Other conditions	Reference
Water	5	100	10	2 h with N2 at 40 C 60 mL/min into a stainless steel tube packed with 200 mg of Tenax TA	(Chen *et al.*, 2005)
	40	100	120	lyophilised	(Chen *et al.*, 2004; Lin *et al.*, 2007)
	33.3	100	240	End volume 11 litre	(Fakeye *et al.*, 2007)
Water Methanol 80% (v/v)	1000	25	120	Filtered with (Whatman no. 4)	(Mohd-Esa *et al.*, 2010)
Methanol + 3% formic acid	_____	4	1440	evaporate solvent below 40 c Residue dissolve in water+3% of formic acid	(Gradinaru *et al.*, 2003)
Methanol/water (60 : 40, v/v) + 0.3% HCl	50	_____	_____	_____	(Farombi and Fakoya, 2005b)
Methanol/water (50:50 v/v) Acetone/water (70:30 v/v)	50 50	_____	60	Centrifuged for 15 min at 25 c in 3000 g and the two extracts combined	(Sayago-Ayerdi *et al.*, 2007)
Ethanol 70%	10	Microwave High power	2	Filtered and evaporated under vacuum at 40 c	(Amin et al., 2008)

Solvent type	Solvent amount (ml)	Temperature (◦C)	Time (min)	Other conditions	Reference
Acidified ethanol (1.5 mol/l HCl)	50	_____	_____	_____	(Tsai and Huang, 2004)
Water water/ethanol(50:50) ethanol	10 10 10	100	240	Filtered with (Whatman paper)	(Fakeye *et al.*, 2008)
Water	10	50	60	re-dissolved in 4000 ml water and filtered	(Alarcon-Aguilar *et al.*, 2007)
Ethanol with 0.1% HCl	15	25	1440	evaporated at 30 c then 5 ml of diethyl ether added incubate 12 h	(Wang *et al.*, 2000)
Water	99	_____	5	_____	(Ali *et al.*, 2003)
	2 4	100	15	Filtered	(Agoreyo et al., 2008)
	192	100	6	_____	(McKay *et al.*, 2010)
ethanol 70%	_____	_____	480	Concentrated and re-dissolved in water	(Essa and Subramanian, 2007)

Table 1-3. Different conditions used to extract 1 g of *H. sabdariffa*. (h) Extraction time by hours, (__) Information not available

1.5 Chemical composition of anthocyanins bioactive compounds in *H. sabdariffa*

Maganha *et al.* (2010) showed that *H. sabdariffa* contains many chemical substances, such as ascorbic acid, β-carotene, anisaldehyde, arachidic acid, citric acid, malic acid, tartaric acid glycinebetaine, trigonnelline and anthocyanins (cyanidin-3-rutinoside, delphinidin and delphinidin-3-glucoside, the most important anthocyanin in *H. sabdariffa*). *H. sabdariffa* also contains many polyphenolic acids. Figure 1-6 show the structures of delphinidin-3-glucoside, delphinidin-3-sambubioside, cyanidin-3-glucoside and cyanidin-3-sambubioside; figure 1-7 shows the structure of protocatechuic acid. The content of the seed oil of *H. sabdariffa* includes campasterol, stigmasterol, ergosterol, β-sitosterol and α-spinasterol (Maganha *et al*., 2010). Prenesti *et al*. (2007) determined the total polyphenol content of *H. sabdariffa* and showed that extraction at 100 °C for 3 minutes with boiling water gave the highest yield of total polyphenols, 39.1 mg/ml of gallic acid equivalents, and that *H. sabdariffa* is rich in anthocyanins and ascorbic acid.

Hsieh *et al.* (2008) identified the most important anthocyanin pigments in *H. sabdariffa,* utilizing HPLC and LC/MS, to be delphinidin-3-sambubioside, delphinidin-3-glucoside and cyanidin-3-sambubioside. In addition, it was found that delphinidin-3-sambubioside and delphinidin-3-glucoside are important antioxidants. Cyanidin-3-sambubioside had a lower antioxidant activity compared to delphinidin-3-sambubioside and delphinidin-3-glucoside (Hsieh *et al.*, 2008).

Sayago-Ayerdi et al. (2007) demonstrated that the flowers of *H. sabdariffa* contain 33.9% dietary fibre and 6.13% phenolic compounds, while Olvera-Garcia *et al.* (2008) detected

phenolic compounds in hot and cold aqueous extracts, and in chloroform and ethyl acetate extracts of *H. sabdariffa,* and found that hot aqueous extracts contained 22.27 mg of protocatechuic acid per gram of lyophilized dried extract. In contrast, ethyl acetate extracts contained the lowest amounts of protocatechuic acid, 7.60 mg of protocatechuic acid per gram (Olvera-Garcia et al., 2008). Ali *et al*. (2005) reported in their review that *H. sabdariffa* contained malic, citric, oxalic and tartaric acids; in addition, ascorbic acid was found in the aqueous extract. Certain types of anthocyanins were detected in an extract of *H. sabdariffa,* such as cyanidin-3-glucoside, delphinidin-pentoside-glucoside, delphinidin-3-sambubioside, small quantities of delphinidin-3-monoglucoside, cyanidin-3-monoglucoside, delphinidin and cyanidin-3-monoglucoside. In addition, protocatechuic acid, eugenol, β-sitosterol, ergosterol and quercetin were also been reported. In contrast, flavonol glycoside (hibiscetin) and hydroxyflavone (sabdaretin) were isolated via acidified solvents. However, the concentration of flavonol glycoside in the calyces was very small compared with the concentration in the flower petals (Ali *et al*.,2005).

Cyanidin 3-sambubioside **Delphinidin 3-sambubioside**

Cyanidin 3-glucoside

Delphinidin 3-glucoside

Figure 1-4. Chemical structures for some anthocyanin compounds found in dried *H. sabdariffa* calyces

Protocatechuic acid (PCA)

Figure 1-5. Structure of protocatechuic acid

1.6 Antioxidant activity and the scavenging effects of *H. sabdariffa* extracts

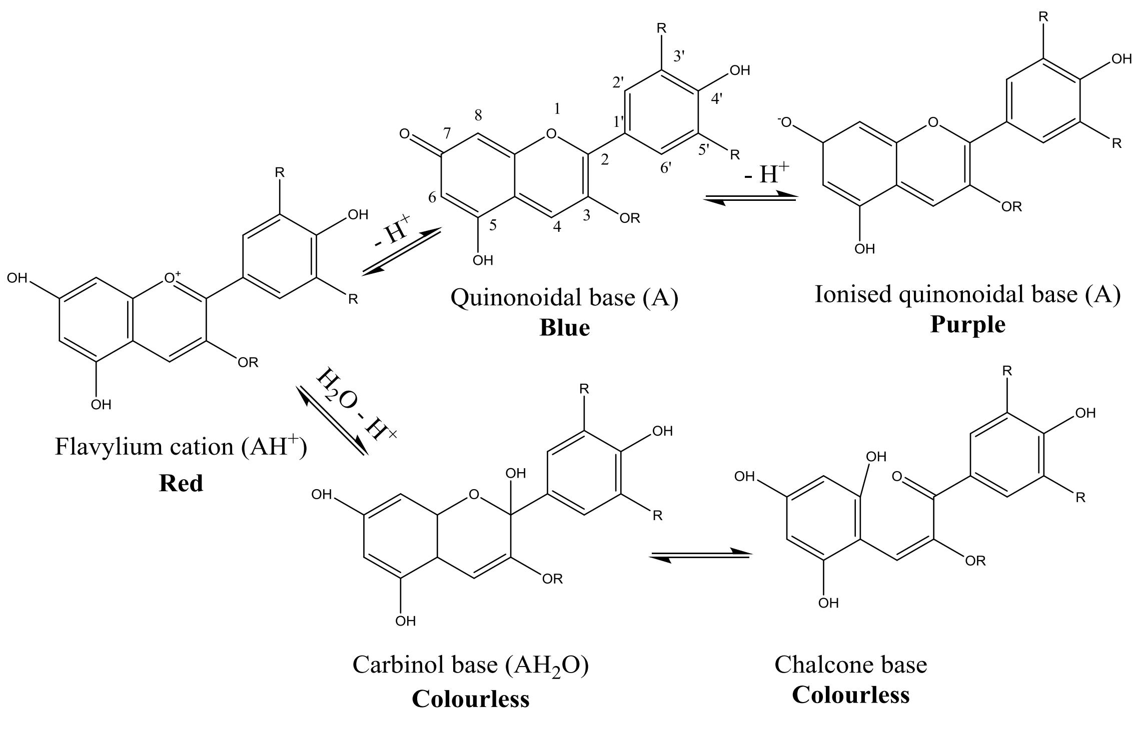
Prenesti *et al*. (2007) determined the antioxidant power of *H. sabdariffa* using the Briggs–Rauscher antioxidant index (BRAI), and they found that *H. sabdariffa* extracted with boiling water for 3 minutes at 25 °C and 37 °C had the highest antioxidant power

compared with other extracts at the same temperatures (320, 665 mg/100ml gallic acid equivalents, respectively; (Prenesti et al., 2007). Sayago *et al*. (2007) measured the antioxidant activity of *H. sabdariffa,* and found that it was 335 µmol Trolox equivalents/100 ml. Chang *et al*. (2006) examined the antioxidant activity of *H. sabdariffa,* and the effect of its extracts on LDL oxidation and anti-apoptotic abilities *in vitro*. The results showed that *H. sabdariffa* extracts*,* at doses of 2mg/ml, reduced the amount of oxidised low-density lipoproteins (oxLDL) by 50%. In addition, fragmentation of apo-protein B (Apo B) was reduced by 61%. Moreover, it was shown that adding more than 0.1 mg/ml extract could scavenge approximately 95% of free radicals. The extract that reduced the LDL oxidation was made using copper, so *H. sabdariffa* reduced oxLDL and LDL oxidation (Chang *et al.*, 2006). Kao *et al.* (2009) supported the findings of Chang *et al.* (2006) by examining the effects of *H. sabdariffa* on foam cell formation and gene expression of scavenger receptors. Lipid accretion in oxLDL-treated cells was significantly elevated, and treatment with *H. sabdariffa* extracts at 0.05-0.2mg/ml avoided lipid accretion and reduced mRNA and PPAR levels (Kao *et al.*, 2009).

1.7 Stability of anthocyanins

Anthocyanins may deteriorate during extraction, processing and storage. There are several factors that could affect the stability of anthocyanins. These factors can be categorised as factors with major and minor effects. The factors with major effects are pH, temperature and oxygen. Ascorbic acid, sulfur dioxide, metal ions, sugar and co-pigmentation are the factors with minor effects (Schwartz et al., 2008; Castaneda-Ovando et al., 2009). Regarding stability of anthocyanins Castaneda-Ovando et al. (2009) reported that anthocyanins are fairly labile therefore, solvents and the presence of other flavonoids and proteins can affect the stability of extracted anthocyanins.

Jackman and Smith (1996), Brat *et al*. (2008),Schwartz *et al*. (2008) and Mercadante and Florinda (2008) have studied anthocyanin stability extensively the following sections about the factors affect the anthocyanin stability are summaries for their findings.



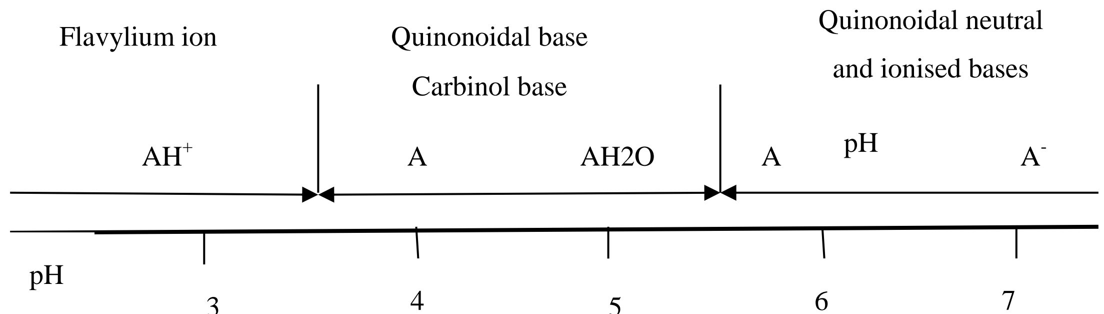


Figure 1-6. The effect of pH on anthocyanins chemical structure adapted from (Linden and Lorient, 1999)

1.7.1.1 Anthocyanins and pH

In general, acid increases the stability of anthocyanins. If the number of hydroxyl groups increase then anthocyanin degradation will increase. In contrast, increased numbers of methoxy groups will increase the stability of anthocyanins. For example, malvidin and petunidin are more stable aglycones compared to others due to the blocking the active hydroxyl group. In addition, increasing the glycosylation increased the stability of anthocyanins, so that a di-glucoside is more stable than a mono-

glucoside. A 3, 5 di-glucoside is more stable than a 3-glucoside. Adding aromatic acid increased the stability. Figure 1-4 shows, the anthocyanin structures formed due to the effect of pH. The flavylium structure appears at low pH from 1 to 2, whereas, the colourless carbinol structure is found at pH from 4 to 6. Chalcone is formed when pH value is 4 or above, and yellow compounds appear. The anthocyanin extract become colourless when pH value reaches 6. Therefore, increasing the pH above 4.5 reduces the stability (Jackman and Smith, 1996; Brat *et al*., 2008; Schwartz *et al*., 2008).

1.7.1.2 Temperature

In general increasing the temperature above 100 °C leads to break the linkage between sugar and aglycone and deteriorate the anthocyanins. Therefore, high temperatures and short times are recommended for optimal extraction of anthocyanins due to minimising changes in anthocyanin structure (Jackman and Smith, 1996). The degree of deterioration depends on the type of anthocyanins and the temperature. The thermal stability is also related to anthocyanin structure. Therefore, anthocyanins containing more methoxy groups are highly stable at high temperatures. Moreover, increasing temperature increased the time for anthocyanins in the chalcone form to reach equilibrium. The thermal degradation of anthocyanins follows one of these reactions; either, the flavylium structure converts to quinonoidal structure that goes through several reactions which produce intermediate products and finally produce coumarin with brown colour, or the flavylium structure converts to colourless carbinol which transformed to chalcone and produces brown degradation products, or the flavylium structure converts to colourless carbinol which is transformed to chalcone (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz *et al*., 2008).

1.7.1.3 Oxygen and light

Juices rich with anthocyanins contain ascorbic acid. In the presence of oxygen, ascorbic acid become oxidised and with increased time of storage the amount of ascorbic acid degrades until eventually, it vanishes. Oxidation of ascorbic acid in juices rich with anthocyanins produces hydrogen peroxide which is responsible for anthocyanins degradation. The degradation is increased if copper is available. Hydrogen peroxide (H_2O_2) attacks the pyrylium ring in the carbon at position 2 and produces a colourless ester with coumarin, that may lead to further degradation which leads to brown material precipitating (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz et al., 2008). In addition, light deteriorates acylated and methylated anthocyanins. Di-glucosides are found to be more stable than mono-glucosides (Schwartz *et al*., 2008).

1.7.1.4 Sugar

High concentration of sugar increased the stability of anthocyanins due to the effect of sugar on lowering the water activity $a_{w.}$ The presence of fructose, sucrose, arabinose, lactose and sorbose deteriorated anthocyanins considerably than other sugar did. According to Mercadante and Florinda (2008), increasing the amount of sucrose by 20 % in anthocyanin products reduced the thermo-stability of anthocyanins. However, pigment stability increased when adding 40% sucrose. Increasing the concentration of fructose was found to reduce stability due to the production of furfural products as components of anthocyanin degradation. Anthocyanin sugars are transformed to furfural compounds due to Maillard reaction giving rise to a brown product. The reaction happens in the presence of oxygen and at high temperature (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz *et al*., 2008).

1.7.1.5 Metal

Anthocyanins with two adjacent functional hydroxyl groups can chelete metals. Consequently, aluminium chloride can distinguish different aglycones. Malvidin for example, does not have two adjacent hydroxyl groups so it will not react with Al^{3+}. The availability of metal ions such as Ca^{++}, Fe^{+++}, Al^{+++} and Sn in juices rich in anthocyanins increase the stability, although, blue or brown compounds are formed in the presence of tannin which leads to deterioration in product colour (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz *et al*., 2008).

1.7.1.6 Sulfur dioxide

Using high concentrations of SO_2 in the bleaching step when processing anthocyanin juices results in a colourless product, because of the presence of SO_2 attached to carbon in position 4 of the anthocyanin structure which is block the joining with double bond. However, some anthocyanins are not affected by SO_2 possibly due to the blockage of the carbon at position 4, or dimer formation through carbon at position 4 (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz *et al*., 2008).

1.7.1.7 Co-pigmentation

Co-pigmentation happens when anthocyanin extracts are concentrated alone or in the presence of other organic compounds (Schwartz et al., 2008). Co-pigmentation could occur when weak acid is added to increase stability (Jackman and Smith, 1996). Co-pigmentation means that anthocyanins react with proteins, tannins or other type of polyphenols or polysaccharides to produce a complex product. These types of compounds are colourless. Co-pigmentation increases the light absorbed and results in greater stability during processing and storage.

Co-pigmentation of anthocyanin with itself results in more stability at different pH because the linkage between the anthocyanins occurs at position 4, meaning that discolouration by SO_2 cannot occur. Other co-pigmentations lead to colourless products (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz et al., 2008).

1.7.1.8 Enzymes

There are two main types of enzymes involved with anthocyanin degradation. The glycosidase enzymes which break the link between sugar and the aglycone, leading to loss of colour due to the poor solubility of the aglycone form. The other main enzyme group are the polyphenol oxidases, which work in presence of the substrate (di-phenols) and oxygen. The reaction produces benzo-quinones which react with anthocyanins and give rise to oxidised anthocyanin products. Blanched fruits rich with anthocyanins heated to 90 °C to 100 ° C for 45-60 seconds inhibit the enzyme (Jackman and Smith, 1996; Mercadante and Florinda, 2008; Schwartz *et al.*, 2008).

1.8 Bio-accessibility and bioavailability of anthocyanins

1.8.1 Factors interfering with absorption and bioavailability of anthocyanins

It is very difficult to study the absorption and metabolism of polyphenols in humans due to the number of factors that could interfere with the research (Rothwell *et al.*, 2005). Each polyphenol has a unique chemical structure, causing each compound to behave differently (Rothwell *et al.*, 2005). One type of polyphenol can interact with another type of polyphenol or nutrient (Rothwell *et al.*, 2005). Until now, the interaction between different polyphenols has not been well-studied. For example, it is very difficult to say that all flavonoids show the same behaviour or bioactivity after consumption, digestion and absorption (Rothwell *et al.*, 2005). Luo *et al.* (2011)

reported that for a better understanding of flavonoids, absorption and metabolism, bioavailability, physicochemical characteristics and bioactivity must all be understood.

Bioavailability of polyphenols depends on several factors solubility, penetrability, metabolism, secretion, target tissue uptake and distribution. For example, phenolic glucosides are hydrophilic compounds characterized by being relatively large molecules and having high polarity. These characteristics reduce the potential to penetrate the intestinal membrane (Hu, 2007). However, enzymes in the gut microflora may transform glucosides to aglycones by removing the attached sugars (Hu, 2007). The aglycone formed may be absorbed better than the glucoside form, but poor solubility of aglycone in water may in contrast lower the absorption and bioavailability (Hu, 2007). Glycosylation and acylation affect anthocyanin bioavailability. The glycosidase enzyme in the gastrointestinal tract hydrolyses the glucoside forms to aglycones that reduce the stability of aglycones (Wallace, 2011).

The chemical structure of anthocyanins and the type of sugar conjugated with the aglycone can affect the absorption and excretion of anthocyanins, (McGhie *et al.*, 2003). The type of sugar had an effect on the bioavailability of anthocyanins from cranberry juice. Glucose attached to cyanidin or peonidin increased the bioavailability compared with the presence of a galctose or arabinose (Wallace, 2011). The sugar moiety attached to the aglycone had an effect on the absorption and transport of the anthocyanin (Del Rio *et al*., 2013). It has been found that anthocyanins hydroxlyated in position 3 in pelargonidin 3-glucoside metabolites meant that they could be absorbed better than anthocyanins hydroxlyated in position 3 and 4 of pelargonidin 3 -glucoside (Del Rio *et al*., 2013). Therefore, hydroxyl or methoxy groups found in position 3 and 4 reduced the absorption (Del Rio *et al*., 2013). According to Yang et *al.* (2011), anthocyanins with glucoside sugars attached are broken down by the bacteria in the colon and in the aglycone form are transformed into phenolic acids or other types of

metabolites. Yang *et al.* (2011) indicated that protocatehuic acid is one of the anthocyanin metabolites that have high antioxidant activity and bioavailability.

The plant matrix has been shown to effect anthocyanin absorption and bioavailability. A long time is needed to release anthocyanins from the food matrix, delaying the rate of absorption (Charron *et al.*, 2009). Bioavailability of anthocyanins is reduced if there are free hydroxyl groups in front of methoxyl groups in the aglycone form (Charron *et al.*, 2009).

The effect of food matrix was further examined, showing that consuming strawberry with high fat content food affected the absorption of anthocyanins. Pelargonidin-O-glucuronide concentration in strawberry did not change when healthy subjects consumed 200g strawberries, with or without 100 ml double cream. The cream delayed the anthocyanin metabolites to reach the maximum concentration on plasma due to the gastric emptying effect. Amounts of pelargonidin-O-glucuronide in urine were less after strawberry was consumed with cream between 0-2 hours (Mullen *et al.*, 2008).

In general, when polyphenols are extracted from plants by using organic solvents or a mixture of organic and aqueous solvents such as water, methanol, and aqueous acetone, they can be divided into two groups: organic solvent extractable polyphenols, which have relatively low and medium molecular weights and which may be absorbed through the intestinal wall and have a systemic effect; and polyphenols which have a larger molecular weight and can be identified in the residue of water-organic solvent extracts (Sayago-Ayerdi et al., 2007). Each type of polyphenol is processed differently in the gastrointestinal tract. Therefore, one of the matrix factors that could affect the absorption and bioavailability of anthocyanins is dietary fibre because non-extractable polyphenols could adsorb to soluble and insoluble dietary fibre. Therefore, polyphenols with a large molecular weight or that are conjugated with other compounds are not bio-accessible in the small intestine and they might degraded by the microflora of the large

intestine (Sayago-Ayerdi *et al*., 2007). It has been found that phytic acid or food rich with phytic acid, such as nuts, seeds and grains raised the anthocyanin concentration in plasma and urine of rats and humans. Phytic acid lowers the movement of the gastrointestinal tract (GIT) and that allows anthocyanin to stay for a longer time in the stomach, duodenum and jejunum, consequently leading to increased absorption of anthocyanins (Lucioli, 2010).

Bioavailability of anthocyanins in plasma was studied in 10 healthy subjects who had consumed 50, 150 and 250 ml of purple carrot juice for 8 hours (Charron *et al.*, 2009). The juice was used to avoid the possible effect of the intact plant matrix. The juice contained 76% of acylated anthocyanins. High concentrations of anthocyanins were found in plasma from 0 to 2 hours, while after 8 hours anthocyanins were still detectable (Charron *et al*., 2009). The study revealed that the bioavailability of non-acylated anthocyanins was significantly higher than the bioavailability of acylated anthocyanins. Absorption of anthocyanins reduced with increasing dose of anthocyanins. The study concluded that variation in bioavailability of acylated and non-acylated anthocyanins was not mainly due the interactions with the plant matrix but due to chemical structure (Charron *et al*., 2009).

1.8.2 Transporters and enzymes involved absorption and bioavailability

The activity of bioactive compounds begins when the compounds reach their target organs or specific tissues (McGhie *et al*., 2003). If compounds are bioavailable, then they can be absorbed and transported through the bloodstream (McGhie *et al*., 2003). Anthocyanins in fruits can be transported into cells even in low concentrations in humans and in rats through different mechanisms, using the glucose transport protein or

a translocater such as bilitranslocase (an organic anion carrier involved in bilirubin and phthalein uptake by the liver) for transportation (McGhie *et al.*, 2003).

Flavonoids can be absorbed and distributed in tissues via the systematic circulation, but if the appropriate transporter is lacking, then bioactive compounds absorbed from the gut or entering the systematic circulation through the intestinal membrane by the process of diffusion (Rothwell *et al*., 2005). The sodium-glucose transporter could transfer the glucoside to the mucosa of the small intestine where the glucoside could be hydrolysed by a β-glucosidase enzyme. Consequently, glucosides are absorbed slowly because they need transporters or enzymes to hydrolyse the glucose prior to absorption, compared with aglycones, which absorb much faster (Wiczkowski *et al.*, 2008).

Charron *et al.* (2009) indicated that anthocyanins are transported either by the sodium-dependent glucose transporter or the organic anion membrane carrier bilitranslocase. It has been found that anthocyanins with glucose attached had high rate of transport and bioavailability compared with anthocyanins attached to galactose in Caco-2 human intestinal cell monolayers (Charron *et al*., 2009). As Yang *et al.* (2011) reported in their review, anthocyanin glucosides need a transporter such as GLUT 2 (glucose transporter 2) or SGLT1 (sodium glucose co-transporter) to be absorbed into the epithelial cells. However, Yang *et al.* (2011) reported that anthocyanins transported from the intestines and endothelial cells could affect the bioavailability in plasma. Fernandes *et al.* (2012) reported that the identity of the transporter and the mechanism of absorption in the stomach are still unknown.

1.8.3 Explanation of poor absorption and bioavailability

Lucioli (2010) reported that anthocyanins are rapidly absorbed and secreted from plasma and urine within 1.5 and 2.5 hours respectively. Only 0.004% to 0.1% of the dose of anthocyanin from dietary intakes are absorbed intact (Lucioli, 2010). Therefore, anthocyanins are poorly absorbed (Lucioli, 2010). Poor absorption could be explained in that most anthocyanins are acidified before analysis therefore anthocyanins are converted to the stable flavylium form. But other forms (Quinonoidal, carbinol or chalcone) appeared in neutral pH in the plasma or urine (Lucioli, 2010). Metabolites of anthocyanins were found in urine within 24 hours, though less than 0.1% of anthocyanin intake could be determined in urine, an indication that only small amounts of anthocyanins are absorbed from the small intestine, and that ingested anthocyanins continue to the large intestine (Lucioli, 2010). Bacteria in the large intestine metabolise anthocyanins very fast. The bacteria hydrolyse the anthocyanin glucosides and therefore large amounts of anthocyanins could be found in GIT (Lucioli, 2010). Unstable aglycones formed at neutral pH are transformed to monomeric phenolic acids and aldehydes.

Del Rio *et al*. (2013) reported that pH- induced changes in anthocyanin structure causes poor absorption of anthocyanins. The predominant anthocyanin structure in the stomach at pH 1–3 is the red flavylium cation, but when the anthocyanins cross the small intestine the red flavylium form changes to the colourless carbinol pseudobase form at pH 4-7, with minor amounts of chalcone pseudobase and a blue quinoidal base (Del Rio *et al*. (2013).

It is very difficult to determine anthocyanin bioavailability due to difficulties of maintaining the equilibrium stability of anthocyanins, because the effect of the pH on shifting anthocyanin structure. Therefore, less than 1% of the dose of anthocyanins were absorbed in the intestine (Fernandes *et al*., 2012).

The stomach is one of the metabolizing organs along with intestines, liver and kidneys (Fernandes *et al*., 2012). The role of the stomach in absorption and hydrolysis of polyphenols was unnoticed by researchers for many years for several reasons. These include the rapid absorption of some polyphenols in the stomach and the failure to detect the small amounts involved. In addition, there is poor absorption of some polyphenols, such as anthocyanins, leading to difficulties in detecting the small amounts of polyphenols such as anthocyanins in urine and plasma. It is difficult to work with isolated gastric cells which require expense and effort. Finally, because many types of cells appear in the stomach there are difficulties in analysing the transport roles played by one single type of stomach cell (Fernandes *et al*., 2012). All of these reasons have limited the research in this area, and few studies have examined the gastric absorption of anthocyanins from food (Fernandes *et al*., 2012).

Anthocyanins are probably absorbed in the upper Gastrointestinal tract (GIT) because they appear very quickly within the bloodstream (Fernandes *et al*., 2012). Yang *et al.* (2011) reported that some studies on humans and rats have claimed that anthocyanin glucosides are absorbed and released without any transformation. Anthocyanin antioxidant activity and bioavailability depends on the chemical structure of the anthocyanins, the number of hydroxyl groups and catechol rings, and oxonium ions in rings B and C of the anthocyanin structure (Yang *et al.* 2011).

Borges *et al.* (2007) studied the bioavailability of raspberry juice anthocyanins in rats. After consumption, amounts of anthocyanins were measured in plasma, the stomach, small intestinal and urine. The study reported that anthocyanins were poorly absorbed and that the main absorption sites for anthocyanins were the stomach and the jejunum of rats (Borges *et al*., 2007). The majority of the anthocyanins pass to the large intestine and they are then fermented and broken down by bacteria. The anthocyanin concentration in plasma reduced after two hours and after four hours was undetectable,

while in urine, only 1.2% of the dose was found after 24 hours. Accumulation of red colour in the stomach and small intestine tissues through to gastrointestinal tissue was observed after intake of acidic extract of anthocyanins (Borges *et al*. (2007). Anthocyanins have been found attached to an unknown protein, possibly a transporter protein, which would make the anthocyanins difficult to detect by HPLC. The low concentration of anthocyanins in the blood stream could be because anthocyanins were not transported from the gastrointestinal tract to the blood stream (Wallace, 2011).

Anthocyanin derivatives (methylated, glucuronidated and sulfoconjugated) were found in the blood stream and urine very quickly due to the fast absorption from the stomach and small intestine. Anthocyanin residues have been found in rat organs such as stomach, small intestine, liver, kidney and brain after consuming anthocyanins for 15 days (Lucioli, 2010). High concentrations of the glucoside forms and their glucuronide derivatives were found in blood from 0-5 hours after oral consumption of anthocyanins. After 5 hours the concentration of methylated forms increased up to 24 hours (Lucioli, 2010). Further transformation of anthocyanin metabolites occurs with time. The unstable aglycone form under neutral pH is transformed to monomeric phenolic acids and aldehydes (Lucioli, 2010).

A study carried out on three different anthocyanins (delphinidin 3-glucoside, cyanidin 3-glucoside and malvidin 3-glucoside), examined the absorption of the compounds in the stomach at pH 3 and pH 5 incubated with adenocarcinoma stomach cells (MKN-28) as a gastric barrier (Fernandes *et al*., 2012). After testing the capability of the cells to survive at these pHs it was found that anthocyanins need precise active transport mechanisms (Fernandes *et al.*, 2012). The results from samples taken from the basolateral side at pH 7.4 showed that there was no red colour present, additional peak was detected in HPLC LC-MS analysis at 345 nm and it was identified as chalcone the was 511 *m/z*, which fragmented to produce(MS^2 349 *m/z* -162 *m/z* loss of glucose) and

(MS^3 223 *m/z* -162 *m/z* loss of glucose) (Fernandes *et al*., 2012). The authors suggested that the peak could be the result of formation of a hemiketal or chalcone occurring at pH 7.4 (Fernandes *et al*., 2012). The difficulties explained that non-acidified antocyanins have unstable flavlyium form that converted to hemiketal or chalcone form which could not be detected due to HPLC conditions (gradient, time and solvents) therefore, that led to underestimation of anthocyanins (Fernandes *et al*., 2012). The study found that absorption rate was increased at pH 3 but that there were no significant differences between pH 3 and 5 in efficiency of the transport (Fernandes *et al.*, 2012). Anthocyanins penetrate the cells through a saturated transport mechanism, perhaps via bilitranslocase, a plasma membrane organic anion carrier (Fernandes *et al*., 2012). The study found that at pH 3, anthocyanins in the cationic form could not passively diffuse through the cells. After three hours of being incubated, anthocyanins in the cell membrane were analysed by HPLC and LC-MS and only parental anthocyanins could be identified, and there was no detection of any metabolites (Fernandes *et al*., 2012). Consequently, the study concluded that the cells could not metabolise the anthocyanins and suggested the reason why the amount of anthocyanins was low was due to the ability of HPLC to only measure the anthocyanins in the red flavylium form (Fernandes *et al.*, 2012).

The metabolites of anthocyanins (ellagic acid and ellagitannins) were studied in healthy subjects and in ileostomy subjects after consuming 300 g of raspberries (González-Barrio *et al.*, 2010). Urine and plasma were collected and analysed by HPLC. No anthocyanins or their metabolites were found in the plasma of healthy subjects (González-Barrio *et al.*, 2010).

However, three major types of anthocyanins were found in the urine of both healthy and ileostomy subjects 7 hours after intake of the raspberries (González-Barrio *et al*., 2010). In the ileostomy subjects, 40% of anthocyanins and 23% of the ellagitannins were found

in ileal fluid 4 hours after intake of raspberries, indicating that hydrolysis of the ellagitannins occurred in the stomach and/or the small intestine and that there was no absorption in the small intestine, while in healthy subjects degradation by the colonic microflora in the large intestine occurred.

However, there was variation in ellagitannin degradation due to colonic microflora (González-Barrio *et al*., 2010). In conclusion, the study revealed low absorption of anthocyanins after consumption of raspberries in humans, although the recovery of individual compounds varied greatly ranging from 5.9% for cyanidin-3-O-glucoside to 93% for cyanidin- 3-O-(2-O-xylosyl) rutinoside (González-Barrio *et al*., 2010).

Phenolic metabolites were identified by Iswaldi *et al.* (2013) in subjects consuming cranberry syrup. Urine was analysed by HPLC and LC-MS after using solid phase extraction of the metabolites. The study found 6 types of anthocyanins 0-6 hours after ingestion. Peonidin 3-O-galactoside was the predominant anthocyanin after consuming the cranberry juice. The majority of the free polyphenols were detected between 2 and 4 hours after cranberry-syrup intake. 32 metabolites were detected including methylated and glucuronide conjugated forms (Iswaldi *et al*., 2013).

Compounds become more hydrophilic due to oxidation, reduction, and hydrolysis reactions. The majority of the free polyphenols were detected between 2 and 4 h after cranberry-syrup intake (Iswaldi *et al*., 2013).

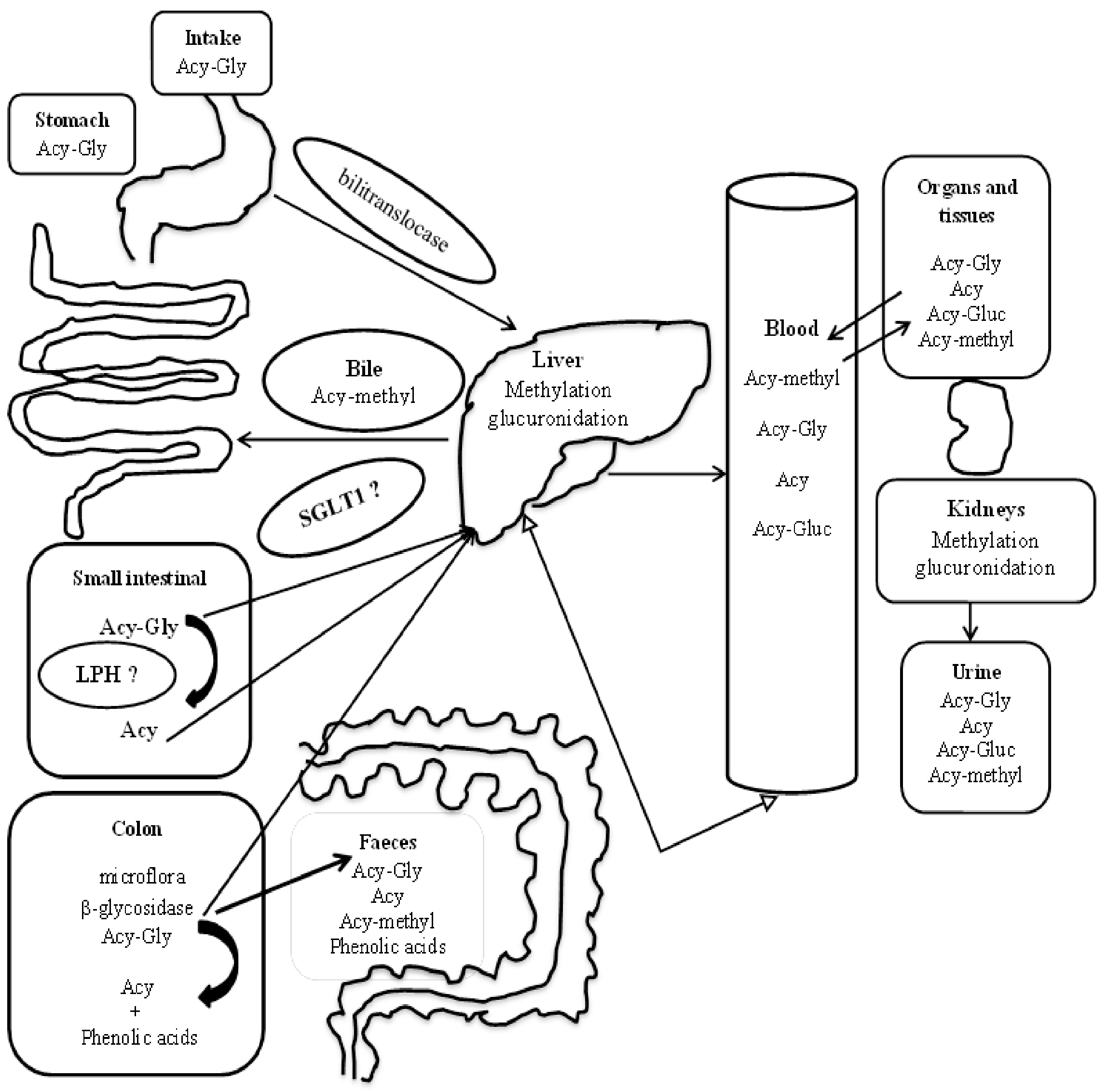


Figure 1-7. Absorption and bioavailability pathways of anthocyanins in the human body. Anthocyanin glucosides (Acy-gly), anthocyanin aglycones (Acy) and their metabolites [methylated (Acy-methyl) or glucuronidated (Acy-Gluc)] and the effect of the sodium dependent glucose transporter (SGLT1) and lactase phloridzin hydrolase (LPH). Adapted from (McGhie and Walton, 2007)

During digestion and absorption, anthocyanins pass through different pH conditions that may change the structure from the flavylium form to other structures such as quinonoidal, chalcone and hemiketal and that transformations are not reversible (McGhie and Walton, 2007). Therefore the bioactivity of these forms will be different,

though the concentration of the red flavylium form is low compared with other forms due to the neutral pH following absorption. The colourless anthocyanin form cannot always be determined by HPLC because only the flavylium form can be detected (McGhie and Walton, 2007).

According to McGhie and Walton (2007) and as figure 1-5 shows, anthocyanins absorbed very fast in the stomach and passing into the liver enter the blood stream by help from bilitranslocase. Metabolites formed from anthocyanins after methylation and glucuronidaton process pass to the intestine in bile. Anthocyanin glucoside that not absorbed in the stomach level pass through to the small intestine where the pH is neutral therefore the structure will change to hemiketal, chalcone, and quinonoidal forms (McGhie and Walton, 2007). The part of the absorption that happens in the jejunum occurs by unknown mechanism. some anthocyanins may be also metabolised in the liver before the anthocyanins pass through the liver to reach the blood stream (McGhie and Walton, 2007). Once the anthocyanins enter the colon, the microflora hydrolyses the remaining anthocyanins to sugar and phenols, which go under further degradation to phenolic acids. More information is still needed about the absorption and how other forms of anthocyanins can contribute to health (McGhie and Walton, 2007).

Basu *et al.* (2013) reviewed all findings about the bioavailability of anthocyanins from strawberry. From these findings it was clear that until now the mechanism of anthocyanin absorption was not fully understood and needed more investigation. It was proposed that bilitranslocase could have a crucial role in anthocyanins bioavailability. Some anthocyanins were absorbed as glycosides. The review revealed that gastric juice transformed pelargonidin-3-glucoside to pelargonidin glucuronide in the stomach, before further transformation in the small intestine and by the microflora in the colon converted the pelargonidin glucuronide to 4-hydrobenzoic acid. Pelargonidin-3-O-glucoside, one of the major anthocyanins found in strawberry, gives rise to metabolites

(such as pelargonidin mono-glucoronides and sulfoconjugates) found in human urine 24 hours from consuming of strawberry (Basu *et al*., 2013). Excretion of strawberry anthocyanins and their metabolites increased with increasing the intake of strawberry, it was recommended to consume fresh or pureed strawberry for better absorption of anthocyanins (Basu *et al*., 2013).

Borges *et al.* (2013) examined the effect of consuming 350 ml of polyphenol juice drink contains (green tea, apples, grapes and citrus fruit) on six healthy ileostomy subjects. The drink contained significant amounts of peonidin- 3, 5-O-diglucoside and malvidin-3, 5-O-diglucoside. The ileal fluid and urine was collected for 24 hours after consuming the juice and analysed by HPLC-MS. The data was compared with 10 healthy subjects who had consumed the same juice. The study indicated that in healthy subjects when absorption took place in the small intestine considerable amounts of the ingested compounds still went through to the large intestine to be metabolised by the microflora to phenolic acid (Borges *et al*., 2013). Anthocyanins were found in ileal fluid unchanged. The study did not find any of the anthocyanins or their metabolites in urine but if a high dose was consumed low concentrations could be detected (Borges *et al.*, 2013). The study concluded that there was no inactivation or competition in the sites responsible for transporting and metabolising polyphenols from the GIT to the blood. In addition, the amount of absorption and excretion of several flavonoids were found to be the same to the amount in the polyphenol juice drink or individual drink consumed (Borges *et al*., 2013).

1.9 Pharmacological effects of *H. sabdariffa*

This section will emphasize the pharmacological effects of *H. sabdariffa* and review selected important studies. Maganha *et al*. (2010) argued that *H. sabdariffa* can be utilized to treat several kinds of diseases, such as kidney disease and bladder stones; in addition, it can be used as a laxative and diuretic, and it may possess uricosuric, anti-hypertensive, anti-inflammatory, anti-mutagenic, anti-tumour, anti-bacterial and anti-fungal properties. Prenesti *et al*. (2007) claimed that *H. sabdariffa* has potential anti-hypertensive effects, and may also protect individuals from cardiovascular disease, hepatic disease and hyperlipidemia.

1.9.1 Role of anthocyanins in reducing blood pressure

Anthocyanins are considered a very important type of flavonoid today due to their ability to help prevent cardiovascular disease (Wallace, 2011). This section briefly discusses some important research on anthocyanins and their effect on cardiovascular disease.

Aviram and Dornfeld (2001) studied the effect of consuming 50 ml of pomegranate juice for two weeks on BP in seven non-smoking hypertensive participants aged between 62 and 77 years. They studied ACE and BP before the consumption of the juice and two weeks later. They found a 36% reduction in ACE serum activity in parallel with a reduction in SBP of 5%, which was low but significant $p<0.05$. Moreover, the study revealed that there was no significant relation between the reduction in serum ACE and the reduction in BP, but there was a significant correlation between ACE in serum and lipid peroxidation.

The influence of consuming pomegranate juice for two weeks was examined in 21 subjects with high BP who were aged between 30 and 67 years. The subjects were separated into two groups. The first group consumed 150 ml of fresh juice (containing 8.7 mg anthocyanins) per day between lunch and dinner time. The second group consumed 150 ml of water per day. The results revealed that pomegranate juice reduced the subjects' SBP (from 130.91 ± 13 to 124.55 ± 15.72 mmHg) and DBP (from 80 ± 8.49 to 76.36 ± 6.74 mmHg) and that the endothelial function was improved with consumption of the juice. The findings may be due to the bioactive compounds ellagitannins and anthocyanins (Asgary *et al.*, 2013).

The study by Aviram and Dornfeld (2001) was criticised because there was no information about where the pomegranate juice used in the study was obtained and whether it was commercially available or extracted. There was also no information on the extraction method (if the juice was extracted), on the instruction given to the participants about how often to consume the juice or on how the BP was measured. Moreover, the study did not have a control group or a placebo group for comparison. In addition, the duration of the study (two weeks) was not long enough to monitor or detect significant changes in BP due to the effect of the juice (Asgary *et al*., 2013). Finally, the number of participants was insufficient to judge the effect of the juice.

The effect of freeze-dried chokeberry juice *in vitro* and in *vivo* on the inhibition of the ACE enzyme and BP has also been examined. *In vivo* hypertensive rats were separated into four groups. The control group was fed 1% potato starch, the positive control was fed enalapril (2 mg/kg of body weight/day), the third group was fed potato starch and polyphenol extract (50 mg/kg), and the fourth group consumed chokeberry juice (50 mg/kg/day) with potato starch for 10 days. The BP was measured after the animals had rested for 10 min in a warm room using the tail-cuff

method. An average of four readings was taken per day before the meal and after the meal at one, three and six hours. The results showed that 4.5 mg dry material/ml of chokeberry juice and 1.5 to 2.5 mg of chokeberry polyphenols had a weak effect on the inactivation of the ACE enzyme. All bioactive compounds that were isolated from the juice inhibited the activity of ACE. The maximum reduction in SBP (20 ± 8 to 15 ± 7 mmHg) and DBP (23 ± 6 to 13 ± 2 mmHg) occurred three hours after consuming the 50 mg/kg/day chokeberry polyphenols due to the juice enhancing the activation of the eNO endothelial enzyme and the secretion of NO. The results revealed that polyphenols increased endothelial nitric oxide synthases (eNOS), showing that it is not necessary to inhibit ACE to produce a vasorelaxation effect. The study concluded that the polyphenols in the chokeberry juice affected the vascular function and BP (Hellström *et al.*, 2010).

Jennings *et al.* (2012) tested the relationship between the intake of flavonoids and BP, atherosclerosis and arterial stiffness in women aged between 18 and 75 years, using information obtained from a cross-sectional study done in 1989 in the U.K. The BP of 728 women was measured twice on a flat bed. The study also incorporated data from a food questionnaire administered in 1996 and 2007. The total flavonoids and individual types of flavonoids, especially anthocyanins, were calculated using the UDSA database and polyphenol explorer database. The results revealed that a high consumption of anthocyanins correlated with a reduction of SBP and DBP, but there was no correlation between reduction of SBP and DBP with total flavonoids or other types of flavonoids consumed by individuals. The study suggested that consuming one or two portions of berries equal to 44 mg of anthocyanins could prevent or reduce cardiovascular disease.

A double-blind crossover study examined the effect of grape juice using 64 healthy pre-hypertensive subjects with an average BP of 138/82 mmHg. The subjects were asked to drink 7 ml/kg/day of either grape juice or a placebo for eight weeks with four weeks' rest between each phase. Their BP was measured three times manually after 10 min rest, with 3 min intervals between each reading. An auto-BP monitor also recorded the subjects' BP every 15 min/hour during the day and every 30 min/hour during the night. The recordings were obtained twice before consuming the drink and 24 hours after the eight-week period had ended. The study found that grape juice had no effect on BP monitored continuously (ambulatory BP) (Dohadwala *et al.*, 2010).

Finkel *et al.* (2013) studied the influence of 300 mg of purple corn extract in capsules/day (each 100 mg extract contained 6 mg anthocyanins) for three weeks on the BP of 30 pre-hypertensive subjects aged 21–70 years in a double-blind crossover study. BP was taken three times in sitting and flat positions. The results showed a reduction in the BP compared with a placebo after three weeks. The subjects who had high BP values at the start of the study showed substantial reductions in their BP.

The peel of purple passion fruit extract was examined to determine the effect of the extract on reducing BP in hypertensive humans and rats after eight weeks. The fruit was extracted with hot water at 60°C for 5 min by solid phase extraction, and the extract was then freeze dried. The study used 24 six-week-old female rats, which were divided into three groups. The control group consumed a basic diet, the second group consumed 10 mg of extract /kg of body weight, and the third group consumed 50 mg of extract /kg / body weight and a basic diet. The study found a significant reduction in SBP (by 12.3 mmHg) after the consumption of 50 mg/kg of the extract associated with a 65% decline in levels of NO in rats. In the human part of the study,

30 participants aged between 40 and 75 years were divided randomly into two groups. A washout period of one week was used to eliminate the effect of antihypertensive medication. Their BP was taken three times after resting for 10 min every week for eight weeks. In the second visit, the subjects took 200 mg of extracted pills twice a day or a placebo. The results revealed a reduction in the SBP and the DBP of the subjects who consumed 400 mg of the extract per day (Zibadi *et al.*, 2007).

Hassellund *et al.* (2012) studied the effect of pure anthocyanins on reducing BP in 27 healthy subjects aged between 35 and 51 years in a crossover study. The subjects consumed 320 mg twice a day for one month, with four weeks rest between the two phases. Pure anthocyanins were extracted from bilberry and blackcurrant in capsule form. Each capsule contained 80 mg of 17 different types of anthocyanins. The main compounds had a high concentration of cyanidin 3-glucoside and delphinidin 3-glucoside. BP was monitored once a week with an ambulatory BP recorder 24 hours after the second and fourth visit. The BP was measured in two positions, sitting and resting positions, to determine whether the position had an effect on the subjects' BP. The results showed that pure anthocyanins had no effect on reducing BP and that the position had no effect on the BP readings.

The influence of blueberry consumption on the BP of rats consuming a high-fat, high-cholesterol diet for 10 weeks was examined in normal and hypertensive rats. Thirty-two rats were separated into four groups. The control group was fed a normal diet, the second group consumed a normal diet plus 2% blueberry (w/w), the third group was fed a high-fat diet consisting of 10% lard and 0.5% cholesterol, and the last group consumed a high-fat diet and 2% of blueberry extract. The BP was measured from the tail of the rat in a warm room, after a 10 min rest, with an

average of three readings, at weeks 1, 2, 4, 6, 8 and 20. The results revealed that the consumption of the 2% blueberry and a high-fat diet reduced the SBP of the rats at week eight and week 10. The blueberry also had an effect on aorta relaxation, showing that the blueberry improved endothelial function. A low level of serum NO is associated with cardiovascular disease. Therefore, the absorption and the circulation of flavonoid metabolites in the blood increased the bioavailability of NO due to the stimulation by the flavonoids of excreted eNOS. (Rodriguez-Mateos *et al.*, 2013).

Shaughnessy *et al.* (2009) studied the influence of freeze-dried blueberry extract on reducing BP in 12 male rats aged eight weeks that were divided into two groups, The groups were fed either a basic diet or a diet consisting of 3% of blueberry extract for eight weeks. The BP was measured from the tail three times after resting for 10 min in a warm room. The results showed that blueberry extract lowered the BP of the rats by 19% at week four and by 30% at week six. The authors attributed the effect of the blueberry to the accumulation of the extract over time during the study. The consumption of the blueberry extract also relaxed the vascular smooth muscle through the endothelium-dependent pathway. The study by Shaughnessy *et al.* (2009) was criticised because it did not describe the extraction process, which could affect the antioxidant activity and the bioactive compounds in the extracts and the amount of blueberry in each pill.

Several studies on *H. sabdariffa* noted that the compounds delphinidin 3-sambubioside and cyanidin 3-sambubioside are known to decrease BP due to the predominance of these two compounds in a water extract (Herrera-Arellano *et al.*, 2004; Herrera-Arellano *et al.*, 2007; Gurrola-Díaz *et al.*, 2010; McKay et al., 2010). In addition, hibiscus acid is known to reduce BP, but how it exerts its effect is not

known (Carvajal-Zarrabal *et al.*, 2005). Due to the large quantity of anthocyanins in *H. sabdariffa*, Hopkins *et al.* (2013) suggested that anthocyanins functions as an antihypertensive compounds. The results of animal trials showed a reduction in blood pressure with an increase in the dose of *H. sabdariffa.* In hypertensive and nonhypertensive rats, doses between 1 mg and 1000 mg/kg were effective in reducing BP, particularly the lower doses (Onyenekwe *et al.*, 1999; Mojiminiyi *et al.*, 2007; Inuwa *et al.*, 2012). In human studies, the consumption of *H. sabdariffa* tea or extract lowered the SBP and DBP of patients diagnosed with diabetes or HTN. Most of the studies involved patients with early-stage HTN. In general, Hopkins *et al*. (2013) reported that most of the human study used *H. sabdariffa* as a treatment for HTN varied in the quantity used for extraction *H. sabdariffa* between 1.5 g to 10 g, usually low concentration was consumed three times/day and a high concentration once/day also there were variation in the period of the study ranged from 4 weeks to 6 weeks (Hopkins *et al*., 2013). The extract of *H. sabdariffa* had a diuretic effect, without affecting the electrolytes level in the body. Comparison between the effect *H. sabdariffa* extract and a antihypertensive drug called captopril was done 90 patients from 30 to 80 years old divided into two groups, the control group was taken 25 mg of captopril twice a day for one month, the second group consumed infusion of *H. sabdariffa* extract of (10g/0.5l) by adding boiling water and infused for 10 min once a day for one month. The study found that *H. sabdariffa* extract lowered SBP from 139.05 to 123.73mm Hg $P = 0.03$ and DBP from 90.81 to 79.52mm Hg $P = 0.06$. The results found there were no significant differences between *H. sabdariffa* extract and captopril $P = 0.25$, therefore, the study concludes that *H. sabdariffa* lowered BP as the antihypertensive drug captopril (Herrera-Arellano *et a*l., 2004).

The review of the research on the effect of anthocyanins on BP points to significant evidence for an effect on BP, with anthocyanins reducing the risk of cardiovascular disease in *vivo* and *vitro* in most of the studies. Only two of ten of the reviewed studies reported no effect on BP: the study by Hassellund *et al*. (2012) on pure anthocyanins and that of Dohadwala *et al.* (2010) on the effect of grape juice. With respect to the effect of anthocyanins on reducing BP and the risk of cardiovascular disease, the previous research points to the following possibilities: they may inhibit ACE, their bioactive compounds may have antioxidant effects on oxidative stress and lipid peroxidation, and they may increase the release of NO by endothelial cells. Anthocyanins also could work as a vasodilator, improve the endothelial function and relax the aorta, the smooth muscles cells and vascular cells. From a scientific point of view, some of the previous studies lacked accuracy or information, which may have affected the results. The problems included unclear or unmentioned extraction procedures, a short duration of the study, a small sample size and the absence of an appropriate placebo or a control group. Importantly, all the previous animal and human studies involved hypertensive or pre-hypertensive cases and not healthy models with a normal BP.

1.9.2 Effects of *H. sabdariffa* on reducing blood pressure

Haji Faraji and Haji Tarkhani (1999) evaluated the effect of *H. sabdariffa* on hypertension in 54 participants with an average age of 52.6 and moderate high blood pressure, divided into two groups, the control group and the experimental group, for 15 days. The results showed a reduction in systolic and diastolic blood pressure by 11.2% and 10.7%, respectively, in the experimental group after 12 days of consuming two spoonful of *H. sabdariffa* in one glass of water boiled for 20-30 min. However, within three days of stopping the intervention, systolic and diastolic blood

pressure rose by 7.9% and 5.6%, respectively, corresponding to the antihypertensive or the diuretic effect or the components of the *H. sabdariffa.*

In addition, Chen *et al*. (2004) examined the effect of *H. sabdariffa* as an anti-atherosclerotic agent on white New Zealand rabbits. Rabbits were fed normal diets containing 1.3% cholesterol and 3% lard oil adding or excluding 0.5 % or 1% H. *sabdariffa* for a 10 week period. The results showed a decrease in the serum triglycerides, cholesterol and LDL values of the rabbits fed with *H. sabdariffa*. The study suggested that feeding the rabbits with 0.5 % or 1% *H. sabdariffa* extensively decreased atherosclerosis in the aorta due to the inhibition of smooth muscle cell migration and cell formation.

Ali *et al*. (2005) demonstrated the effect of *H. sabdariffa* on hypertension in animal experiments. *H. sabdariffa* extract inhibited angiotensin I converting enzyme (ACE). So that angiotensin II was not generated and aldosterone secretions from the adrenal gland were prevented, which are causing an eventual decline in vascular resistance.

Haji Faraji and Haji Tarkhani (1999) and Ali *et al*. (2005) reported that *H. sabdariffa* extract may responsible for relaxing the smooth muscles, which is likely related to endothelium-independent mechanisms present or because of calcium channels influence (Ali *et al.*, 2005).

Wahabi *et al.* (2010) reviewed the data regarding the effectiveness and safety of *H. sabdariffa* in the treatment of hypertension by looking at a number of studies. The results showed that *H. sabdariffa* reduced blood pressure compared with black tea, but to a lesser degree than ACE inhibitors. They also mentioned that there were poor experimental designs and a lack of consistent data in the studies, and all but one lacked a standardised dose or standardised conditions for preparing the *H. sabdariffa* extracts. The systematic review discussed in the Haji Faraji and Haji Tarkhani (1999)

study reported incomplete data with unbalanced dropout from the groups; moreover, there was no report of undesirable outcomes or a baseline for the study, and the study did not report how the randomized sample was selected.

McKay *et al*. (2010) studied the role of *H. sabdariffa* as an anti-hypertensive substance in 65 persons, 30-70 years of age, who were diagnosed as pre- and mildly hypertensive, but were not taking any medications. The trial provided the participants with three servings/d (720 mL/d) of *H. sabdariffa* tea, hot or cold; in addition, volunteers were given information on how to prepare the beverages by infusing the bag provided (containing 1.25 g of *H. sabdariffa*) with 240 mL of boiled water for 6 min, before removing the bag, or adding 16–18 drops (1.2 mL) of an artificial hibiscus flavour concentrate to 240 mL water and stirring, thus obtaining a placebo beverage. The trial lasted for six weeks. After six weeks, they found that the hibiscus drink dramatically decreased systolic blood pressure relative to the placebo. In addition, diastolic pressure was low; this affect could be the results of anthocyanin substances, such as delphinidin-3-sambubioside, cyanidin-3-sambubioside or other bioactive components may also give this effect. Therefore, the study recommended consuming *H. sabdariffa* as a daily beverage to reduce blood pressure. The study also pointed out that in spite of age, gender or other dietary supplements used, there was a significant effect of *H. sabdariffa* toward reducing blood pressure, and thus it may also be helpful in lowering the risk of heart and cardiovascular disease by raising endothelial function and inhibiting platelet accumulation.

1.9.3 Reducing cholesterol and blood sugar

A six-week study by Agoreyo *et al.* (2008) investigated the effect of *H. sabdariffa* and ginger on lowering plasma cholesterol and blood sugar levels in 30 albino rats

weighing between 225-270 g, divided into six groups, with each group containing five rats. Groups 1 and 2 were the control groups; one was fed a 100% normal diet and the other a 99% normal diet with 1% cholesterol. Group 3 were fed 0.8 ml/kg of *H. sabdariffa* extract, while group 4 was treated with 0.2 ml of ginger extract/kg of body weight, group 5 was fed 1 ml of a *H. sabdariffa* and ginger mixture (17.2 mg/400 ml + 8.6 mg/200 ml) and rats in group 6 were given a high concentration of the mixture (17.2 mg/200 ml + 8.6 mg/100 ml). Results indicated that groups fed the *H. sabdariffa* extract and ginger extract, or a mixture of the two, lowered plasma cholesterol and glucose dramatically, especially group 6, who received a high dose of the hibiscus and ginger mixture compared to the control (Agoreyo *et al*., 2008).

1.9.4 The effects of *H. sabdariffa* on lipid oxidation and hyperlipidaemia

Oboh and Rocha (2008) evaluated the capacity of aqueous extracts of *H. sabdariffa* and green tea (1 g/ 100 ml of hot water) to prevent lipid peroxidation by inhibiting some pro-oxidants, such as Fe, sodium nitroprusside and quinolinic acid in rat brains. The study indicated that there was a dramatic reduction in lipid peroxidation and pro-oxidant compounds when using *H. sabdariffa* and green tea, presumably due to the high concentrations of phenols in *H. sabdariffa* and green tea (13.3 and 24.5 mg/g, respectively).

Hainida *et al.* (2008) examined the effects of *H. sabdariffa* on lipid oxidation and hypercholesterolemia in samples of 23 and 20 male rats. The rats were fed a normal diet, hypercholesterol and different doses of *H. sabdariffa* seed powder (10, 20, 50 or 150 g kg^{-1} for 5 weeks). The study revealed that feeding 50 g kg^{-1} and 150 g kg^{-1} of *H. sabdariffa* seeds powder reduced serum total cholesterol 5.69 ± 1.24 mmol L^{-1},

4.44 ± 0.58 mmol L^{-1} respectively compared with hypercholesterol rats 8.93 ± 2.51 mmol L^{-1} and LDL levels 5.19 ± 1.27 mmol L^{-1}, 3.96 ± 0.60 mmol L^{-1} respectively compared with hypercholesterol rats 8.44 ± 2.49 mmol L^{-1}.

Hirunpanich *et al.* (2006) reported similer findings when examining the effects of *H. sabdariffa* on lipid oxidation in rats for 6 weeks using dried extracts in the following amounts: 250, 500 and 1000 mg/kg. *H. sabdariffa* dramatically reduced serum cholesterol levels by 22% and 26%, respectively; serum triglyceride levels by 33% and 28%, respectively and serum LDL by 22% and 32%, respectively. In addition, thiobarbitutic acid-reactive substances (TBARS) formation considerably decreased after 6 weeks, and the oxidation of LDL formed by $CuSO_4$ slightly reduced.

Carvajal-Zarrabal et al. (2005) studied the effects of different doses of *H. sabdariffa* (5%, 10% and 15%, extracted with ethanol). The rats were fed with normal diets containing high cholesterol or *H. sabdariffa* extracts for 4 weeks. The results indicated that weight, triacyglycerol and LDL levels significantly decreased at all over *H. sabdariffa* doses compared with controls, whereas total lipids dramatically declined at a dose of 5% compared to the 10% and 15% doses. Overall, the 5% dose was the most effective dose for lowering LDL levels due to its inhibition of the production of triacylglycerol (Carvajal-Zarrabal *et al.,* 2005).

In a separate study, Chen *et al*. (2004) examined the antioxidant activity of *H. sabdariffa* extract on LDL oxidation using 150 g of hibiscus extracted with 6 L of water at 100°C for two hours, then filtered and lyophilized to the equivalent of 75 g of hibiscus. They also measured the relative electrophoretic mobility (REM) and TBARS of *H. sabdariffa* extract in the animal treatments. The experiment used male Sprague-Dawley rats (5 weeks old), and 15 g/day of diet was given to each rat. In the first model, 24 rats were separated into four sets: the first set was fed a basal diet;

the second, a basal diet plus 300 g/kg fructose; the third, 300 g of fructose and10 g/kg of hibiscus extract and the fourth set was fed 300g of fructose with 20 g/kg *H. sabdariffa* extract.

In the second experiment, rats were split into four groups: the first group was fed a basal diet; the second, a basal diet with 30 g/kg lard oil and 13 g/kg cholesterol; the third group, the same as the second group plus 10 g/kg of *H. sabdariffa* extract and the last group, 30 g/kg lard oil and 13 g/kg cholesterol with 20 g/kg *H. sabdariffa*. The duration of the experiment was 12 weeks. The results showed that *H. sabdariffa* extract significantly decreased REM by 18.29%, 29.67% and 52.85% with treatment with 0.5, 1.0 or 2.0 mg/ml of *H. sabdariffa,* respectively, and also decreased TBARS by 16.30%, 44.56% and 94.47% with treatment of 0.5, 1.0 and 2.0 mg/ml of *H. sabdariffa,* respectively. Overall, hibiscus decreased the production of LDL oxidation, catalysed by copper. There was also a reduction in the serum triglycerides of the rats fed with the high fructose diet in the rats fed 10 and 20 g/kg *H. sabdariffa*, and serum cholesterol declined significantly in rats fed the high cholesterol diet. In addition, the proportion of LDL to HDL decreased when utilizing hibiscus extract in both models. The authors also concluded that *H. sabdariffa* not only avoided peroxidation, but also reduced ApoB fragmentation and cholesterol degradation in LDL *in vitro*. Therefore, the study recommend that *H. sabdariffa* extract might be used to reduce the risk of hyperlipidaemia in both models, and moreover, to reduce the distribution levels of triglycerides and LDL-C (Chen *et al.*, 2004).

1.9.5 The effect of *H. sabdariffa* on the immune system and the effect of *H. sabdariffa* on mutagenic compounds and tumours

Fakeye *et al*. (2008) investigated whether water, ethanol and water/ethanol (50:50) extract of dried *H. sabdariffa* in mice could reduce or increase the production of two tumours' necrosis factor-alpha (TNF- α) and interleukin-10 (IL-10). The study showed that, to some degree, the extracts, and to a large degree, the two fractions were capable of encouraging the immune system *in vivo*, especially with the water/ethanol and absolute ethanol extracts. The interaction between the production of interleukin 10 and the reduction of tumour necrosis TNF- α resulted from the activity of the extracts. Results found that antibody production depends on TNF- α and B-cells. The study revealed that *H. sabdariffa* extract could be used as therapy in immunosuppressant diseases.

Anthocyanins play a major role as anti-cancer and anti-estrogenic substances because of their capability of binding with oestrogen receptors and inducing estrogenic reactions. Moreover, anthocyanins specifically have the ability to quench free radical singlet oxygen by either providing hydrogen or regulate enzyme activity or protein kinases. In addition, anthocyanins are capable of preventing the formation of the free radicals that can initiate cancer in cells and can possibly protect DNA from damage by inhibiting tertiary butyl hydroperoxide-induced cytotoxicity. In addition, the study indicated that there are several mechanistic roles for anthocyanins that may be important in preventing cancer. The first is to reduce matrix metalloproteinase secretions, which are critical in tumour initiation, the second is to block nitrogen from activation, the third is to reduce tumorigenesis and the activity of epidermal growth factor receptors and the fourth function is to minimise further cell proliferation (Nichenametla *et al*., 2006).

Nichenametla *et al.* (2006) also speculated that anthocyanins may have an effect on colon carcinogenesis, decreasing the development of tumours in the intestines of rats. This is significant, as a decline in the propagation of human colon cells with anthocyanin supplements could reduce tumour size remarkably, occurrence and multiplication by 20-95% compared with the control.

Olvera-Garcia *et al.* (2008) investigated the effects of different extracts (aqueous, ethyl acetate) from *H. sabdariffa* on mutagenic compounds and antiproliferative agents. The results showed reductions in cell proliferation and DNA fragmentation of human cells. In addition, the extracts reduced the mutagenicity of 1-nitropyrene (1 NP). The study also reported that the extract did not encourage DNA fragmentation (Olvera-Garcia et al., 2008)

Another study (Lin *et al.*, 2005) examined the effects and the mechanisms of hibiscus polyphenol extracts as anticancer agents. The study revealed that the hibiscus extract (100g of dried *H. sabdariffa* extracted using 300 ml of methanol, then 200 mL hexane and finally 180 mL ethyl acetate and lyophilized) encouraged cell death of eight types of cancer cells, but among all these types of cells, the human gastric carcinoma cells were the most vulnerable. Meanwhile, the results indicated that 0.95 mg/ml of *H. sabdariffa* extract reduced the growth of cells by 50%, and after 24 hours, *H. sabdariffa* extract at a concentration of 2 mg/ml was introduced, increasing the hypodiploid phase, at which point, the gastric cells showed massive changes in DNA fragmentation. In addition, the study pointed out that *H. sabdariffa* extract is able to encourage cell apoptosis through two mechanisms; the first was through activated p38 and increased phosphorylation of c-jun in the gastric carcinoma cells treated with *H. sabdariffa* extract, which may have led to activated FasL (an AP-1 target gene) and the release of FasL. Releasing FasL

initiates apoptosis throughout Fas death receptors, which become caspase 8 activated and Bid involved. The other is by making p53 stable, while at the same time increasing the release of Bax and cytochrome c to activate caspase 3, which provokes apoptosis. The results signified that hibiscus, when encouraging apoptosis, is also linked to inducing the protein levels of p53 and phosphorylation and changing the appearance of the Bcl-2 family. The study concluded that the effect of *H. sabdariffa* extract in inducing apoptosis was due to the antioxidant activity of hibiscus combating oxidative stress (Lin *et al.*, 2005).

Liu *et al.* (2006) investigated the effects of *H. sabdariffa* on liver fibroses in male rats fed normal diets with different doses of *H. sabdariffa*, from 1-5%, for 9 weeks, and the results revealed that *H. sabdariffa* inhibited liver damage, containing steatosis and fibrosis. Plasma aminotransferase (AST) and alanine aminotransferase (ALT) were also reduced. The extracts also reduced the production of lipid peroxide and decreased the activation of hepatic cells (Ajay *et al.*, 2007).

1.9.6 Antibacterial and antimicrobial effects of *H. sabdariffa*

Chao and Yin (2009) studied the effect of *H. sabdariffa* and protocatechuic acid in both aqueous and ethanol extracts on different bacteria such as *Salmonella typhimurium*, *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus*, and *Bacillus cereus* in apple juice and ground beef. The study found that after 3 days of storage at 25°C, both *H. sabdariffa* and protocatechuic acid inhibited the growth of bacteria. The ethanol extracts showed better results as antibacterial agents than the aqueous extracts (Chao and Yin, 2009). Olaleye (2007) confirmed and supported Chao and Yin (2009) by investigating the antimicrobial effects of aqueous-methanolic extracts of *H. sabdariffa* on brine shrimps, and notcing that there was inhibiton growth of bacteria such as *Bacillus stearothermophilus, Micrococcus*

luteus, Serratia mascences, Clostridium sporogenes and *Klebsiella pneumoniae* (Olaleye, 2007). Further research examined the antimicrobial effects of different extracts of *H. sabdariffa* (70% ethanol, then hexane,cloroform, ethyl acetate, n-butanol and water). The research emphasized that the ethanol extract of *H. sabdariffa* had the highest antimicrobial effect compared to the other extracts in kimbap (rice rolled in dried laver) when useing the paper disc method, which slowed down the development of in spoilage bacteria kimbap. Ethyl acetate extracts were capable of inhibiting *Bacillus cereus* and *Clostridium perfringens* (Kang *et al.*, 2006).

1.9.7 Increased reproductive function

Amin *et al*. (2008) assessed the effects of *H. sabdariffa* and ginger extracted via a microwave method to protect the plants from degradation (100 g of dried material mixed with 1L of 70% ethanol for 2 minutes). In a rat model, 1g/kg/day *of H. sabdariffa* and ginger were provided orally to male albino rats weighing between (150-200 g) for 26 days pre-experiment, before the rats were injected with cisplatin drug chemotherapy (CIS) (10 mg/kg) according to body weight. The results showed positive protection of reproductive function, by decreasing CIS reproductive toxicity and maintaining the normal testes morphology; in addition, a reduction in testicular lipid peroxidation was enhanced by the *H. sabdariffa* and ginger. The study concluded that hibiscus and ginger showed defensive effects against testicular damage and oxidative stress induced by cisplatin (CIS) when 1g/kg/day of ethanol extracts of *H. sabdariffa* and ginger were utilized.

1.9.8 The anti-hyper-ammonemic effect

Essa and Subramanian (2007) examined the effect of *H. sabdariffa* extract on hyper-ammonemia, which is defined as unevenness in the oxidation conditions of nervous tissue in addition to the occurrence of free radical damage. In the study, eight rats were separated into four groups; the first group was the control, the second one was treated with 250 mg/kg of *H. sabdariffa*, the third group was given 100 mg/kg, while the final group was treated with both 100 mg ammonium chloride and 250 mg/kg *H. sabdariffa* extract. These treatments were kept up for eight weeks, and blood samples were taken to measure circulating ammonia, urea, uric acid, creatinine and non-nitrogenised protein. Results showed remarkably increased levels in ammonium chloride in treated rats, while these levels returned to an approximately normal level in the *H. sabdariffa* extract-treated group, possibly because of the effects of phenols and flavonoids (such as anthocyanins, glycosides, PCA and hydroxycitric acid) in eliminating the excessive amount of ammonia, urea, uric acid and creatinine throughout hyper-ammonemic and nephrotoxic conditions. In addition, the activity of thiobarbituric acid, hydroperoxides and glutathione peroxides were measured in the brain tissues. The results of TBARS and HP were extremely high compared with SOD, CAT, GSH and GPx, which are considerably reduced in the brains of ammonium chloride-treated rats. In contrast, these levels were brought back to normality in the *H. sabdariffa* extract group due to the effect of *H. sabdariffa* extract as free radical scavenger and nitric oxide scavenger which may reduce the products of lipid peroxidation among the hyper-ammonemia rats. Moreover, the enzyme antioxidants and non-enzyme antioxidants were reduced in the brains of the rats, whereas the group treated with hibiscus went back to near normal; thus, it is assumed that phenolic antioxidants could both inhibit and slow down production

free radicals. The study concluded that the *H. sabdariffa* alcohol extract considerably depressed the amounts of lipid peroxidation in the brain. In addition to increasing the level of antioxidants, it also decreased glutathione in the brain tissues of rats; in fact, *H. sabdariffa* had potential in encouraging anti-hyper-ammonemic and antioxidant activity.

From research point of view, after reviewing numerous studies on *H. sabdariffa* pharmacological effects *H. sabdariffa* is not a magical medication that can treat or relieve many types of diseases, the effect also may varies due to usage of different type of solvents which could not consumed by human. As with any other type of plant, it may have side-effects; even if it is an antioxidant or has beneficial pharmacological effects, it does not mean it is necessarily 100% safe, and adding to this possibility is a shortage of studies on its toxic effects *in vivo*. According to Aoshima *et al*. (2007), a number of polyphenols formed toxic compounds such as H_2O_2 and caused cell death. Therefore, the shortage of the toxic studies are probably because a small proportion of dietary polyphenols go through the intestines, and thus oxygen radicals and hydrogen peroxide are generated, but the cells have defence mechanisms to protect the body and the epithelial cells can regenerate in the stomach and intestines. In addition, Ali *et al.* (2005) reported that *H. sabdariffa* extract is non-toxic in doses between 500-1,000 mg/kg.

Aims and objectives

More attention has focused recently on increasing the knowledge about the *H. sabdariffa* plant as a source of functional food. *H. sabdariffa* is a rich source of anthocyanins and other bioactive compounds and has high potential antioxidant activity that could be responsible for protecting against several types of disease, as used in folk medicine. After reviwing several articles on the types of extraction and the concentrations used for extracts of *H. sabdariffa,* it was found that there was no consistency in the results or methods for determining the best extract. There have been no previous systematic studies of *H. sabdariffa* extraction. Moreover it is hard to confirm the pharmalogical effects of the plant may be due to different solvents used for extraction. Therefore, the first aim of the study was to characterise *H. sabdariffa* extracts by conducting a systematic study with different solvents particularly with regard to polyphenol contents and antioxidant activity. Secondly, commercially-available products containing *H. sabdariffa* were analysed. Thirdly, log *P* values for particular hibiscus anthocyanins will be determined in the presence and absence of hibiscus cell wall material. Fourthly, a human study looking at hibiscus bioactivity was carried out.

1.10 Objectives

- To identify the best solvent and condition for extracting anthocyanins from *H. sabdariffa*.
- To measure the antioxidant capacity of *H. sabdariffa* extracts and conclude the best method to measure antioxidant capacity.
- To identify and quantify anthocyanins in *H. sabdariffa* extracts by HPLC and LC-MS.

- To apply the optimal conditions for extraction to extracts commercially-available products of *H. sabdariffa* and compare with typical infusion methods.
- To determine the effect of different matrices on log *P* values of particular hibiscus anthocyanins.
- To investigate the effect of *H. sabdariffa* drink on blood pressure by an intervention study with healthy human subjects.

Chapter 2 Materials and methods

In this chapter general analytical methods, including antioxidant assays and HPLC method, and preparation of buffers are described. Other methods are described in individual chapters.

2.1 Chemicals

6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Iron(III) chloride hexahydrate ($FeCl_3.6\ H_2O$), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), L-ascorbic acid, butylated hydroxy anisole (BHA), Tris-HCl, Tris base, gallic acid, Folin Ciocalteu's phenol reagent (2N), sodium carbonate (Na_2CO_3), acetic acid glacial,1-octanol (HPLC grade), sodium acetate (tri-hydrate; $CH_3CO_2Na.3\ H_2O$), potassium chloride (KCl) and potassium persulphate ($K_2S_2O_8$), sodium chloride (NaCl) and formic acid (HCOOH) were supplied by the Sigma Aldrich Chemical Company Ltd. (Poole, Dorset, UK). 2, 2-Diphenyl-1-picrylhydrazyl free radical (DPPH) and hydrochloric acid were from Merck Chemicals Ltd. (Darmstadt, Germany). All analytical grade solvents (methanol, ethyl acetate and hexane) and HPLC grade solvents (acetonitrile, methanol and water) were obtained from Fisher Scientific Ltd (Loughborough, UK). Water for general laboratory use was obtained from a Milli-Q purification system. Sodium dihydrogen phosphate anhydrous (NaH_2PO_4) was supplied from BDH Laboratory Supplies (Poole, UK).

2.2 Herbal teas, juices and plant materials

Dried flowers of *H. sabdariffa* were obtained from a local market in Jeddah, Saudi Arabia. Two types of herbal tea, pomegranate tea (manufactured by the Health and Heather Herb Specialists Company working with the National Institute for Medical Herbalists, NIMH, UK) and Yogi Tao Rose tea manufactured by Golden Temple Natural Products, Hamburg, Germany) both containing *H. sabdariffa* as the main ingredient, were bought from Holland and Barrett, Leeds. Ocean Spray Concentrated Cranberry Juice Drink was obtained from local supermarkets. Concentrated *H. sabdariffa* juice drinks (Simply Hibi, Hibiscus Elixir) was obtained from lbis Organic Ltd, Towa, UK.

2.3 Anthocyanins

Anthocyanin standards (cyanidin, cyanidin 3-glucoside, cyanidin 3-sambubioside, delphinidin, delphinidin 3-glucoside and delphinidin 3-sambubioside) were purchased from PhytoLab GmbH & Co. KG (Dutendorfer, Germany). Apigeninidin was obtained from Extrasynthese (Genay Cedex, France). All standards were stored at – 20 °C. Standards were dissolved in methanol + 1% (v/v) HCl when it was required.

2.4 Preparation of buffers and solutions

2.4.1 Potassium chloride buffer (0.025 M, pH 1)

KCl (1.86 g) was dissolved in 980 ml of water. The pH was measured and adjusted to pH 1 with concentrated HCl. The buffer was then transferred to a volumetric flask and water was added to make up to 1 litre. The buffer was stored at room temperature and the pH was checked each time before use.

2.4.2 Sodium acetate buffer (0.4 M, pH 4.5)

Sodium acetate (tri-hydrate) $CH_3CO_2Na.3H_2O$ (54.43 g) was mixed with 960 ml of water. The pH was measured and adjusted to pH 4.5 with concentrated HCl. The buffer was then transferred to a volumetric flask and water was added to make up to 1 litre. The buffer was stored at room temperature and the pH was checked each time before use.

2.4.3 Acetic buffer (0.1 M, pH 5.5)

Acetic acid (0.1 M): concentrated acetic acid (5.8 ml) was added to water in a volumetric flask and made up to 1 litre.

Sodium acetate (0.1 M): sodium acetate (tri-hydrate) $CH_3CO_2Na.3H_2O$ (13.6. g) was dissolved in water (1 litre). The solution was stored at room temperature.

0.1 M Acetic acid (4.5 ml) was mixed with 0.1M sodium acetate (57.7 ml) and the solution was diluted with water to 100 ml to make up the buffer. The solution was stored at room temperature.

2.4.4 Sodium acetate buffer (300 mM, pH 3.6)

($CH_3COONa.3H_2O$; 3.10 g) was dissolved in glacial acetic acid (16 ml) and then made up with water to 1 litre. The pH was adjusted to pH 3.6 by adding concentrated acetic acid.

2.4.5 Phosphate buffered saline (PBS)

5mM Na_2HPO_4 was prepared by dissolving 0.710 g in water (100 ml). 5mM NaH_2PO_4 was prepared by dissolving 0.599 g in water (100 ml).

5mM Na_2HPO_4 (81ml) and 5 mM NaH_2PO_4 (19 ml) were mixed with NaCl (0.9 g) to achieve (pH 7.4).

2.4.6 Tris-HCl buffer (50 mM, pH 7.4)

Tris-HCl (6.61 g) and Tris base (0.97 g) were dissolved in water (800 ml). The pH was adjusted to pH 7.4 with HCl and the volume made up to 1 litre with water.

2.4.6.1 HCl (40 mM)

40 mM HCl was prepared by adding 1N HCl (4 ml) to water in a volumetric flask and making up to 100 ml with water.

2.4.6.2 Sodium carbonate solution (20%, w/v)

Anhydrous sodium carbonate (200g) was dissolved in water (800 ml). The solution was heated to boiling and left to cool. A few extra crystals of sodium carbonate were added to ensure the solution remained saturated. After 24 hours the solution was filtered using Whatman filter paper No 1 and the volume adjusted to 1 litre.

2.5 Equipment

- The following laboratory equipment was used throughout
- Spectrophotometer (CE 3021), capable of scanning wavelengths between 190 nm to 650 nm, Cecil Instruments Limited (Cambridge, UK).
- Quartise Cuvettes of 3 ml volume were obtained from the Sigma Aldrich Chemical Company Ltd. (Poole, Dorset., UK).
- Eppendorf IEC desktop micro-centrifuge (maximum centrifugal force 17000g) Thermo, Electron Corporation (Germany).
- Genevac EZ-2 series, Genevac Ltd. (Ipswich, UK).

- Mettler Toledo Xs 104 Analytical Balance (maximum weight 120 g d= 0.1 mg), Metter-Toledo Ltd, (Beaumont Leys Leicester, UK).
- Vortex Mixer FB 15013 Top Mix 230v 50 Hz ZX Wizard Fisherbrand, Fisher Scientific, (Loughborough, UK).
- Grant OLS 200 Ultrasonic Water Bath and Grant TxF 200 Series Heated Circulating Bath with maximum temperature 100˚C, Grant Instruments, (Cambridge, UK).
- Hanna Basic pH Bench Top Meter HI 2210 calibrated with buffer solutions at pH 4 and 7, Hanna Instruments (USA).
- Kenwood Chef Classic KM336, 4.6 Litre Kitchen Grinder Machine, 800 Watt, with Major AT320B Mini Chopper/Mill Attachment with 3 Additional Glass Jars, Kenwood Limited (Havant, UK).
- Omron Blood Pressure Monitor, Omron Health Care Manufacturing, Vietnam Co., Ltd (Binh Duong Province, Vietnam).
- Shimadzu LC-20AD Liquid Chromatograph, with SIL-20AC autosampler, RF-10AXL fluorescence detector and SPD-20A UV/VIS detector, Shimadzu Corporation (Kyoto, Japan).
- A Gemini C18 column (5µm, 250 mm×4.6 mm), (Phenomenex: P/NO.00G-4435-E0, desc. Gemini 5µ C18 110A, S/NO540974-22), Phenomenex (Macclesfield, Cheshire, UK).
- Shim-pack-ODS (2.2µm, 100 mm×4.6 mm), (Shimadzu: P/N:228-41607-94, S/NO 70644748), Shimadzu Corporation (Kyoto, Japan).

- Agilent 6410 Triple Quadrupole LC /MS 6410, Agilent Technologies 1200 Series, from Agilent Technologies Inc. (Santa Clara, California, USA).

2.6 Methods

2.6.1 Extraction of samples

2.6.1.1 Extraction of *H. sabdariffa* calyces

Dried *H. sabdariffa* powder (100 mg) was added to water, methanol, hexane or ethyl acetate solvents (10 ml), with and without the addition of 1% (v/v) formic acid solution. Different temperatures (25 °C, 50 °C and solvent boiling point) were used for each of the eight solvent mixtures. The times of extraction used at all temperatures were 3, 5 and 10 minutes. At the conclusion of each extraction, the whole mixture was filtered through Whatman No. 1 filter paper.

2.6.1.2 Extraction of herbal teas

Two methods were used to infuse the herbal tea, firstly by following the directions on the package by adding 200 ml of boiled water to the tea bag and leaving it to infuse for 5 minutes (infusion method). The second method used the optimal extraction conditions as determined in chapter 3 using the method in previous section (2.6.1.1) (boiling water at 100 °C for 10 min), with the herbal tea (100 mg) being extracted using 10 ml of water (decoction method). The final extract was obtained by filtering through Whatman No. 1 filter paper. Freshly prepared extracts were used each time for analysis without further treatment or storage.

2.6.2 Determination of total monomeric anthocyanins by pH differential.

Total anthocyanins were measured by applying the method of Wrolstad (2005). All *H. sabdariffa* extractions were diluted until the absorbances of the samples were within the range of the spectrophotometer. A first dilution was made with 0.025 M potassium chloride buffer (0.025 M, pH 1) and the second dilution with 0.4 M sodium acetate buffer (0.4 M, pH 4.5). The samples were incubated for 15 min until they reached the equilibrium phase. The absorbance was measured in all diluted samples at wavelength 520 nm (maximum absorbance) and at 700 nm to correct the haze against water as blank. Red flavylium cation was the predominant form of anthocyanins at pH 1while, at pH 4.5, the flavylium cation was transformed to the colourless carbinol form. The absorbance of samples at pH 4.5 was subtracted from the absorbance at pH 1 to obtain the free anthocyanin content.

The absorbance of the diluted sample was calculated using the following equation.

$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5.}$ The concentration of total monomeric anthocyanins in the original samples was calculated using the following formula:

Monomeric anthocyanins pigment (mg/l) = $(A \times MW \times DF \times 1000)/(\varepsilon \times 1)$ where MW was the molecular weight of cyanidin 3-glucoside= 449.2, DF was the dilution factor and ε was the molar absorptivity of cyaniding 3-glucoside= 26900.

2.6.3 Determination of total phenols using the Folin assay

Total polyphenol content was evaluated using the Folin assay according to Singleton *et al.* (1999). The method utilises the change of the yellow colour of the Folin reagent to the blue colour due to sample oxidation. Stock solution of gallic acid was

made by dissolving 0.05 g in ethanol (10 ml), and completed up to 100 ml with water to produce a standard curve from 50 to 500 mg/L.

H. sabdariffa extracts (0.1ml) were added to water (7.9 ml), and then mixed with Folin–Ciocalteu's reagent (0.5 ml) for 1 minute. Sodium carbonate solution (20%; w/v; 1.5 ml) was added to the reaction mixture. The reaction solution was protected from the light and kept for 2 hours at room temperature, and then the absorbance was measured at a wavelength of 765 nm against a water blank The optical density (OD) was compared to a standard curve prepared with 50 to 500 mg/L gallic acid and results are expressed as (dried weight) as mg of gallic acid equivalents (GAE)/g. Samples were analysed in triplicate. Concentration (mg/g) = Concentration (mg/ml) $\times$FV $\times$ DF /Sample weight, where FV = final volume and DF = dilution factor.

2.6.4 Determination of ascorbic acid and other compounds that interfere with the estimation of total phenols

Interference by ascorbic acid and other oxidizing agents was determined according to Perla *et al.* (2012) by following the same procedure used to determine total phenols (2.6.3) with minor modifications in the method. Sodium carbonate solution was substituted with the same amount of water in this procedure. Therefore, *H. sabdariffa* extracts (0.1ml) were added to water (7.9 ml), and then mixed with Folin–Ciocalteu's reagent (0.5 ml) for 1 minute. Water (1.5 ml) was added to the reaction mixture. The reaction solution was protected from the light and kept for 2 hours at room temperature, and then the absorbance was measured at a wavelength of 765 nm against a water blank The OD was compared to a standard curve prepared with 50 to 500 mg/L gallic acid and results were expressed as (dried weight) as mg of gallic acid equivalents (GAE)/g.

2.6.5 Free radical scavenging assay

The 2, 2-Diphenyl-1-picrylhydrazyl free radical (DPPH) assay was used to measure the antioxidant capacity of the *H. sabdariffa* extracts. A modified of the method of Aoshima *et al.* (2007) was used. An acetic acid buffer (pH 5.5, 0.5 ml) was mixed with DPPH (0.2 mM, 1 ml), but instead of dissolving the DPPH in the ethanol, the study was modified by using methanol to match with dilution solution. The *H. sabdariffa* extract (1.5 ml) was diluted 50 times using methanol/water (50/50; v/v), and L-ascorbic acid and butylated hydroxy anisole (BHA; 50 mM) were used as external antioxidants. The standards were also dissolved in methanol/water (50/50; v/v). The mixtures were vortexed, and then kept in the dark at room temperature for 30 minutes, and the absorbance was read at a wavelength of 517 nm. The radical-scavenging activity (%) was measured by applying the following equation:

(%) = 100× (absorbance of the control - absorbance of the sample)/absorbance of the control.

2.6.6 Ferric reducing antioxidant potential assay (FRAP)

The ability to reduce ferric ions (FRAP) was measured by using a modified version of the method described by Benzie and Strain (1996). Extracts (diluted 10 fold; 200 µl) were added to 3 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution prepared by dissolving the TPTZ in 40 mM HCl, and 1 part of 20 mM, FeCl3 6H2O solution) and the reaction mixtures were incubated for 10 minutes. A dark blue colour was achieved due to the ability of the samples to oxidise the substrate. The absorbance was measured at 593 nm after 30 min. OD was compared to a Trolox standard curve (0-1000 µM) was prepared by dissolving 5 mg Trolox in a small amount of methanol (2 ml) then making up with

water to (20 ml). Results are expressed as (dried weight) as mg of Trolox acid equivalents (GAE)/g. Samples were analysed in triplicate.

2.6.7 Total equivalent antioxidant capacity (TEAC)

According to Huang *et al*. (2005), stock solution of Trolox standard at a concentration of 100 µM was prepared by dissolving 5 mg of Trolox in a small amount of ethanol (5 ml) to dissolve completely, then making up with phosphate buffered saline (pH 7.4) to 50 ml.

All samples were diluted with water. Before the samples were measured, the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was diluted with phosphate buffered saline to reach an OD of 0.700. Reagent blank was made by mixing ABTS (2 ml) with phosphate buffered saline (40 µl). ABTS (2 ml) was added to all the standards and diluted samples (40 µl) and mixed well. The OD at 734 nm was recorded after one minute for the reagent blank, standard and samples. Blue colour was formed due to the reaction between ABTS cation and peroxidase and hydrogen peroxide. The results were expressed as (dried weight) of mg/ g Trolox equivalents.

2.6.8 High performance liquid chromatography

2.6.8.1 HPLC method

Reversed-phase (RP) high performance liquid chromatograph (HPLC) was used to quantify compounds in individual extracts, using a Shimadzu LC-20AD liquid chromatography, with SIL-20AC autosampler and SPD-20A UV/VIS detector. A Gemini C18 column (5µm, 250 mm×4.6 mm) was used (Phenomenex: P/NO.00G-4435-E0, desc. Gemini 5µ C18 110A, S/NO540974-22) and the wavelength was set

at 520 nm. The column was incubated at 40 ° C and the autosampler cooled to 4 °C. The method used was described by Juliani *et al.* (2009) with minor modifications in the gradient. The elution system was binary. The mobile phase was (A) 0.2% (v/v) formic acid in water (B) acetonitrile. The starting mobile phase was 10% (B). Subsequently, solvent B was increased to 20% (0-10 min) and to 70% (10-11 min). The conditions were held at 70% B for 4 min prior to returning to 10% B (15-16 min) with a final isocratic run of 10% B (16-25 min). The flow rate was 1 ml/min and the injection volume was 10 µl. Peaks were provisionally identified according to the retention time of anthocyanin standards. Recovery was calculated only for the major peaks for cyanidin and delphinidin 3-sambubioside. After running the samples spiked with 100 µl internal standard (20µM apigeninidin) samples were re-run by adding 50 µl of 0.01 mM of cyanidin and delphinidin 3-sambubioside. A mixture of water: acetonitrile in the ratio (50:50, v: v) was pumped through the system to remove residues from the system and the column. For cyanidin 3-glucoside the limits of detection (LOD) was 0.01 mM and the limits of quantification (LOQ) 0.04 mM; for delphinidin 3-glucoside the LOD was 0.06 mM and the LOQ 0.18 mM; for cyanidin 3-sambubioside the LOD was 0.06 mM and the LOQ 0.18 mM and for delphinidin 3-sambubioside the LOD was 0.05 mM and the LOQ 0.15 mM.

2.6.8.2 Standard curves

Anthocyanin standards were used to plot standard curves, ranging from 0.01 mM to 0.5mM over 5 concentrations. Apigeninidin (20µM) was used as the internal standard because it is type of anthocyanin and it was chosen due to absence of this compound in the extracts. The curves were calculated as the ratio of the peak areas of internal standard and anthocyanin standards of three triplicate injections and plotted with standard deviations.

2.6.9 Mass-spectrometric analysis

LC-MS was used to confirm the identity of the bioactive compounds in the extracts, using an Agilent Technologies 1200 series LC with triple quad MS 6410. Analysis was carried out using positive ion mode for the detection of anthocyanin compounds in *H. sabdariffa* samples. Full scan mode was used for the accurate determination of the parent ion and MS2 mode used to obtain fragmentation data. The most significant ion in the scan was then collected in the ion trap, fragmented and the spectrum of the fragments recorded. The mass spectral information was used to identify the compounds. In addition, use of a PDA detector also provided absorbance spectra, which gives useful information in recognising the presence of anthocyanins (λ_{max} 520 nm). Co-chromatography was done with standards to identify and confirm the presence in the extracts.

The same column, mobile phases and monitoring wavelength were used as for HPLC (section 2.6.8). The starting mobile phase was 10% B; subsequently, solvent B was increased to 50% (0-8 min) and to 70% (8-15min). The conditions were held at 70% B for 0.30 min prior to returning to the final isocratic run of 10% B (15.30-25 min). The flow rate was 0.3 ml/min and the injection volume was 1 µl. Nitrogen gas temperature was 35 °C, gas flow 11 L/min, Nebulizer 60 psi. The standard and the sample extracts were run in positive mode (+).

2.6.10 Determination of log *p* values

2.6.10.1 Theoretical calculations

Theoretical determinations of log *p* values for anthocyanins were calculated via online chemical software (Marvin and calculator plugin demo) after chemical structures was converted to smile form using the web site: http://www.chemaxon.com/marvin/sketch/index.php (accessed July, 2012).

2.6.10.2 Experimental determinations - preparation of standards

Each anthocyanin standard was prepared by dissolving in methanol acidified with (0.1% HCl to make up solution of 0.5 mM. Each standard (200 µl) was transferred into a 2 ml Eppendorf tube and dried down in a Genevac for 30 minutes in a low-pressure mode and with the lamp turned off. Octanol (400 µl) was added and vortexed for 1 minute. Tris-HCl buffer (400 µl; pH 7.4) was then added and vortexed for a further minute. The samples were centrifuged using a microcentrifuge at 17,000 g for 5 minutes. After centrifugation, each phase was taken by syringe and filtered using syringe filters with pore size 0.02 µm, before transfer into a micro amber HPLC vial. Octanol and buffer phases were analysed using reverse-phase HPLC.

$$\text{Log } P = \frac{\text{peak area of the compound in octanol phase}}{\text{Peak area of the compound in buffer phase}}$$

2.6.10.3 Calculation of log *p* using *H. sabdariffa* water extract

A water extract of *H. sabdariffa* was prepared to see how the extract affected the phase behaviour of the anthocyanin standards. Ground powder of dried *H. sabdariffa* calyces (0.1 g) was added to boiling water (10 ml) and left at 100 °C for 10 minutes. The sample was then immediately filtered (Whatman No 1). *H.*

sabdariffa extract (200 µl) with 0.5 mM of the different anthocyanins (200 µl) were added to Eppendorf tubes (capacity 3 ml). The samples were dried in the Genevac using the HPLC fraction mode with the lamp off. Each sample took approximately 30 minutes to dry. Octanol (400 µl) was added and vortexed for 1 minute. Tris-HCl buffer (400 µl; pH 7.4) was then added and was vortexed for a further minute. All samples were centrifuged at 17,000 g for 10 minutes and phases were separated using a syringe and filtered using syringe filters with pore size 0.02 µm.

2.6.10.4 Calculation of log *p* using a dried powder from *H. sabdariffa*

Anthocyanin standards were prepared as described previously (section 2.6.10.2). Octanol (400 µl) was added and vortexed for 1 minute. Prior to addition of Tris-HCl buffer, 0.08 g of ground *H. sabdariffa* powder was added and vortexed for 3 minutes. Tris-HCl buffer (400 µl; pH 7.4) was then added and vortexed for 1 minute. All samples were centrifuged at a force of 17,000g for 10 minutes and the phases were separated using a syringe and filtered using syringe filters with pore size 0.02 µ.

All the samples were analysed by HPLC using a Shim-pack-ODS (2.2µm, 100 mm×4.6 mm), (Shimadzu: P/N: 228-41607-94, S/NO 70644748) column. Samples were analysed by using the same conditions and gradients that were used to identify and quantify the anthocyanins by HPLC (section 2.6.8).

2.6.10.5 Calculation of recovery of standards

Each standard (0.5 mM, 200 µL) was dissolved in methanol with 0.1 % (v/v) HCl and then dried down using the Genevac. Standards were re-dissolved in methanol and treated as the previous samples (section 2.6.10.2) by adding octanol and buffer and vortexing. Samples of octanol and buffer phases of anthocyanin standards were analysed using reverse-phase HPLC.

To determine recovery, the following equation was applied:

$$\% \text{ recovery} = \frac{(\text{Peak area of octanol + peak area of buffer})}{\text{Peak area of standard}} * 100$$

2.6.10.6 Calculation of recovery for *H. sabdariffa* water extract and powder

Water extract alone without adding the standard was dried down in the Genevac using the same conditions mentioned previously (section 2.3.13). After dryning samples were treated as described in section 2.3.13. In the case of ground *H. sabdariffa* powder, the same amount (0.08 g) without adding the standard was added after the addition of octanol and buffer and was then vortexed and centrifuged. All samples of octanol and buffer phases were analysed using reverse-phase HPLC. To check recovery, the following equation was applied:

% recovery = [(peak area of octanol standard + peak area of buffer standard)-(peak area of standard in octanol water extract + peak area of standard in buffer water extract)]*100/peak area of standard.

2.6.11 Human study

A human study was carried out assess the ability efficacy of anthocyanin- rich *H. sabdariffa* to reduce blood pressure (BP).

2.6.11.1 Ethical approval

The human study was performed to test whether a *H. sabdariffa* drink rich in anthocyanins, could reduce BP in healthy human volunteers. The study protocol was approved by the Mathematics and Physical Sciences (MaPS) and Engineering Research Ethics Committee (MEEC), University of Leeds, United Kingdom (Ethics reference number, MEEC 11-009). The approved protocol is presented in appendix.

2.6.11.2 Study design

A randomised controlled cross-over design study (each participant was their own control), was performed with 30 healthy or pre-hypertensive, 4 male and 26 female participants. One of the female volunteers withdrew from the study after completion of the first phase. Another participant was recruited to replace the missing volunteer. Another participant dropped out at the beginning of the study, so the total number of female participants was 25. Participants were recruited from students and staff of the University of Leeds, and from the general population. Volunteers were aged between 20 - 48 years and not taking medication that interfered with the study. Written informed consent was obtained from each participant. All participants followed their usual diet throughout the intervention which means that participants were not required to avoid any foods or beverages. A standardized protocol was followed for each BP measurement. The same arm (left) and cuff was used for all measurements. For each measurement, the subject was sat in a quiet environment in a comfortable chair, with their feet on the floor. BP was measured with the arm at heart level. A value for 3 determinations of systolic blood pressure (SBP) and Diastolic Blood Pressure (DBP) was subsequently averaged. Before the intervention, BP was measured on two separate occasions to get the mean for the baseline (rested state, no test drink). BP was measured 3 times at 5 min intervals. The second visit was one week after the first visit were the participants completed the medical questionnaire (Appendice).

Volunteers were randomised into two groups of 15 volunteers; the *H. sabdariffa* drink group and the control group (cranberry drink). Participants in both groups were asked to consume 35 ml of concentrated drink and diluted up to 250 ml once every day for 8 weeks. On the first week of the study participants were asked to

complete the first week-study questionnaire. BP was taken every week at the same day and time (three times, 5 minutes between each measurement). In addition, subjects were asked to complete a 4 day dietary recall before, in the middle, and at the end of the 8 week study period in order to determine if results were due to the intervention or a change in dietary pattern. The questionnaire involved the volunteer being asked what he/she ate in all meals and snacks for 4 days (one of which was at the weekend). The food record was not further analysed and kept only to explain any strange or odd results. Volunteers had a 1 month break after completion of the first 8 weeks of the study. They continued the study by switching drinks and their BP was measured again every week for 8 weeks to the end of the study. The total time for the study was 16 weeks plus the one month break which was not included in the study time.

2.6.11.3 Criteria

Inclusion criteria were being a healthy adult, 19 years of age and above, with ability to consent, being non-pregnant or breast feeding, and should not be suffering from any chronic diseases especially cardiovascular diseases. Mean blood pressure had to be between the range for SBP of 90-130 mm hg and for DBP of 60-80 mm Hg. Body mass index was between 19-27 kg/M^2.

2.7 Statistical analysis

Data are presented as mean values ± standard deviation (SD), n=3. Each sample was analysed in triplicate and calibrated against relevant standards where appropriate. Multivariate correlation analysis of experimental data was performed according to Pearson's Correlation (Chapter 3). Calculations quantified the relationship between two sets of experimental variables. The correlation coefficient r quantified the

direction and magnitude of the correlation and ranged from -1 to +1. Independent 2-sample paired t-tests were used to assess differences between groups (Chapter 5). Statistical analysis of variance (ANOVA) was conducted one-way, and two-way ANOVA was used to determine the differences in antioxidant capacity between samples (Chapter 3 and 4). Values at $P<0.05$ were considered statistically significant. Post-hoc tests (Tukey's) were carried out to determine the sources of variation. Analysis was carried out using IBM SPSS Statistics software version 20.

Chapter 3 Comparative Chemical and Biochemical Analysis of Extracts of *H. sabdariffa*

Summary

- Water and methanol extracts were efficient and extracted the highest amounts of phenols compared with other types of solvents.
- Adding acid to the solvents did not enhance the yield of the extracts or changed their antioxidant activity.
- There was a significant correlation between phenols and antioxidant activity.
- The highest antioxidant capacities were obtained by extracting using water both with and without formic acid for 10 min at 100 °C.
- Water extracts with and without formic acid for 10 min at 100 °C had the highest concentrations of cyanidin 3-sambubioside and delphinidin 3-sambubioside.
- Hexane and ethyl acetate extracts had low antioxidant capacities.
- Cyanidin 3-sambubioside and delphinidin 3-sambubioside were not found in hexane and ethyl acetate extracts.

3.1 Introduction

The current interest in natural antioxidants from plant sources has become overwhelming, particularly in bioactive antioxidants such as polyphenols and flavonoids. Anthocyanins are a subgroup of flavonoids appearing in plants mostly in the glycoside form as anthocyanins. Anthocyanins differ in the number and position of hydroxyl and methoxy groups (Nollet, 2000) and have been associated with anticancer, antihypertensive and anti-inflammatory effects (Vasapollo *et al.*, 2007).

3.2 Importance of the study

Several researchers have examined the antioxidant capacity of *H. sabdariffa* extracts using different solvents to extract and different assays for analysis, but none have carried out a comparative study of extraction or analytical protocols. At present, it is difficult to compare different studies because of the different doses and different extraction protocols used. Furthermore, a wide range of extraction conditions have been used (Table 3-1). Each of these studies reported different results for *in vitro* antioxidant capacity and reported different levels of bioactivity *in vivo*. Rodriguez-Mateos et al. (2013) commented on the difficulty of comparing studies due to the variations in extraction conditions.

Solvent	Solvent volume (ml)	Time (min)	Temperature (°C)	Study
water	50	60	25	(Wong *et al.*, 2006)
	100	5	100	(Oboh and Rocha, 2008)
	10	600	25	(Cisse *et al.*, 2009)
acidified methanol	50	180	64	(Farombi and Fakoya, 2005a)
80% methanol	1000	120	25	(Mohd-Esa *et al.*, 2010)
70% ethanol	10	2	Heated in microwave on 150 W	(Amin *et al.*, 2008)

Table 3-1. Examples of extraction conditions used in different reported studies on *H. sabdariffa* (1 g) the main table in chapter 1 section 1.5

3.3 Aim of the study

The aim of the present study was to investigate the optimal extraction conditions for extracting *H. sabdariffa* by using different solvents (water, methanol, ethyl acetate and hexane), times (3 min, 5 min and 10 min) and temperatures (25 °C, 50 °C and solvent boiling point). Types of solvents were used based the most solvents commonly used in extraction polyphenols and because each type of solvent could extract different type of polyphenols. Time and temperature were selected based on preliminary study, the 10 min was the maximum after that the bioactive compounds degraded and solvent boiling point was the maximum temperature to reach without deterioration of the compounds in various times. Each extract was evaluated for its antioxidant activity, and the anthocyanin compounds of each extract were identified and quantified using different chemical and biochemical analyses. The hypothesises were the 10 min and solvent boiling point for all solvent have the highest value in all measurements and acidified solvents have the highest value in all measurements compared with non-acidified solvents.

3.4 Material and methods

All the materials and methods used in extraction, determination of antioxidant activity and identification and quantification of anthocyanin bioactive compounds by RP-HPLC and LC-MS in this part of the study are described in detail in chapter 2 section (2.6.2 to 2.6.9).

3.5 Results

Figure 3-1 shows the colour of *H. sabdariffa* extracts using different solvents. Water extract had red colour, while the colour of methanol extract was red-purple colour, ethyl acetate extract characterised by the yellow colour compared with colourless hexane extract.

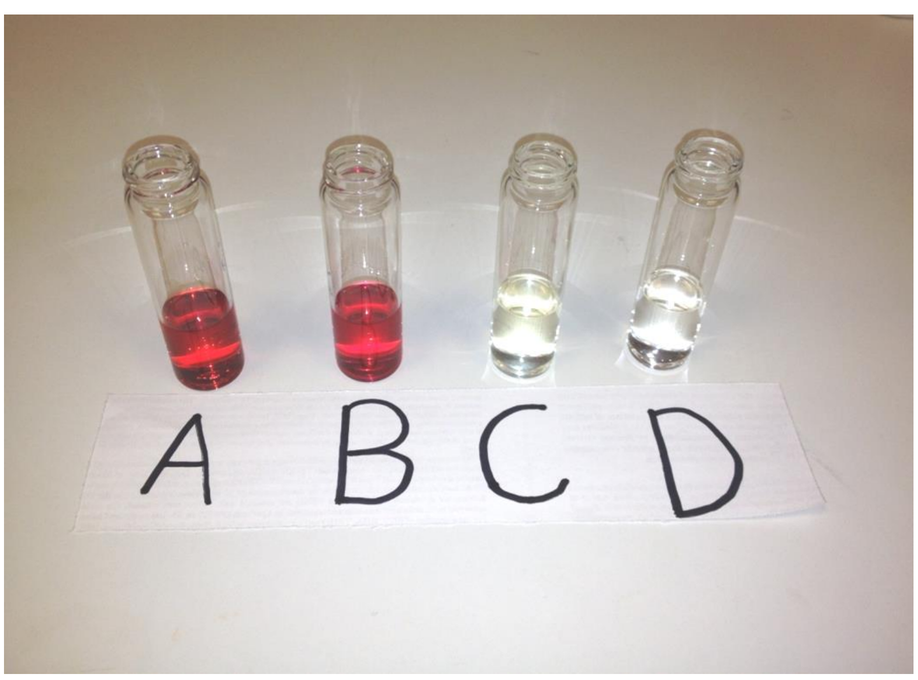


Figure 3-1. Image of dried *H. sabdariffa* extracts extracted by using different solvents, (A) water extract, (B) methanol extract, (C) ethyl acetate extract (D) hexane extract

3.5.1 Time of extraction

The study examined the effect of extraction time (3, 5 and 10 min) on the total polyphenol content, the amount of antioxidant capacity and the total monomeric anthocyanin content.

The results revealed from Table 3-2, shows that time of extraction had no significant differences between extracts analysed by TEAC assay and for the total anthocyanins determination ($P > 0.05$). For total polyphenol content, a significant difference was found between 3 min and 10 min of extraction ($P = 0.026$), but there was no significant difference between 5 min and 10 min of extraction ($P = 0.872$). Extraction for 10 min was the optimum time. For FRAP assay, there was no significant difference between 3 min and 5 min ($P = 0.916$) but there was a significant difference between both 3 min and 10 min and 5 min and 10 min extraction ($P = 0.014$ and $P = 0.004$ respectively). Both 3 and 5 min extraction time gave optimal results. Finally, DPPH showed no significant difference between 5 and 10 min extraction ($P = 0.870$) but did show a significance between both 3 and 5 min and 3 and 10 min extraction ($P < 0.001$ and $P = 0.002$ respectively). 3 min extraction time was optimum.

Assays	3-5 min	3min-10min	5-10 min
Total phenols	0.091	0.026	0.872
DPPH	< 0.001	0.002	0.870
FRAP	0.916	0.014	0.004
TEAC	0.990	0.999	0.983
Total anthocaynins	0.791	0.829	0.933

Table 3-2. *P* value results of effect of time extraction on different antioxidant assays by using (ANOVA) statistical analysis, significant different determined by using (Tukey's test, p<0.05)

3.5.2 Extraction temperature

Temperature had an effect on all extractions measuring total polyphenol content, DPPH, FRAP and total anthocyanin content as shown in table 3-3. Increasing the extraction temperature increased the extractable bioactive compounds and result in increased the assays values. There was no significant differences between 50 °C and solvent boiling point ($P > 0.05$) in all assays except in DPPH and FRAP assays ($P < 0.05$). However, temperature did not have any effect on TEAC values ($P > 0.05$). For DPPH and FRAP assays the results showed significant differences between extraction at 25 °C and solvent boiling point ($P = 0.000$ for both DPPH and FRAP assays) with solvent boiling point being optimal. For total monomeric anthocyanins significant differences ($P = 0.011$, $P < 0.001$ and $P = 0.02$) were found for yields between all the temperatures tested (25 °C, 50 °C and solvent boiling point) with maximum yield at the solvent boiling point. Depending on both the solvent used for

extraction and the assay, there were variations in significance. However, for some solvents and determinations the optimum yield was seen at 50 °C.

Assays	25-50 ° C	25 °C and solvent boiling point	50 °C and solvent boiling point
Total phenols	0.136	0.057	0.919
DPPH	< 0.001	< 0.001	0.803
FRAP	0.806	< 0.001	< 0.001
TEAC	0.793	0.071	0.264
Total anthocaynins	0.011	< 0.001	0.002

Table 3-3. *P* value results of effect of extraction temperature on different antioxidant assays by using (ANOVA) statistical analysis, significant different determined by using (Tukey's test, $p<0.05$)

3.5.3 Effect of solvent on the total phenol content

Figure 3-2 shows the standard curve for gallic acid, which was used to calculate the equivalent amount of total phenols in the extracts.

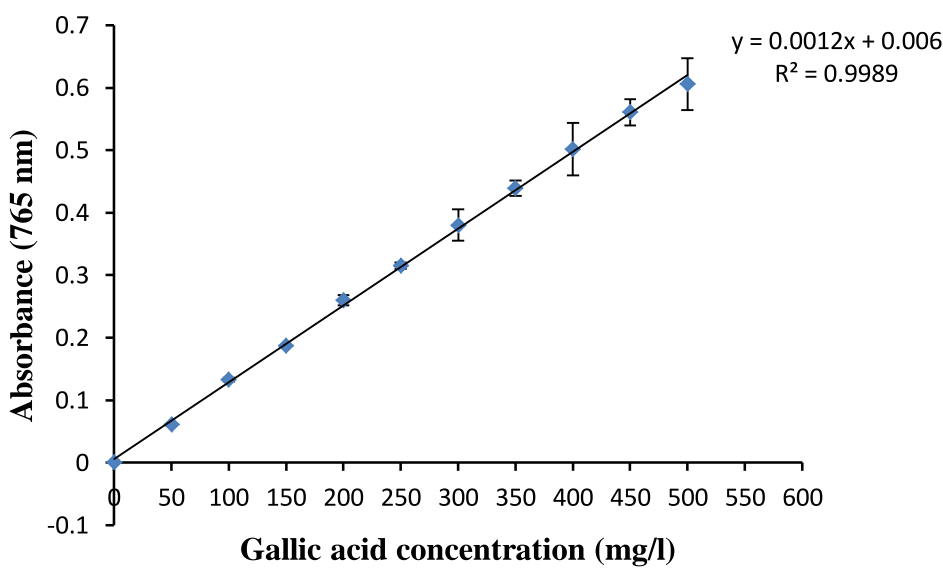


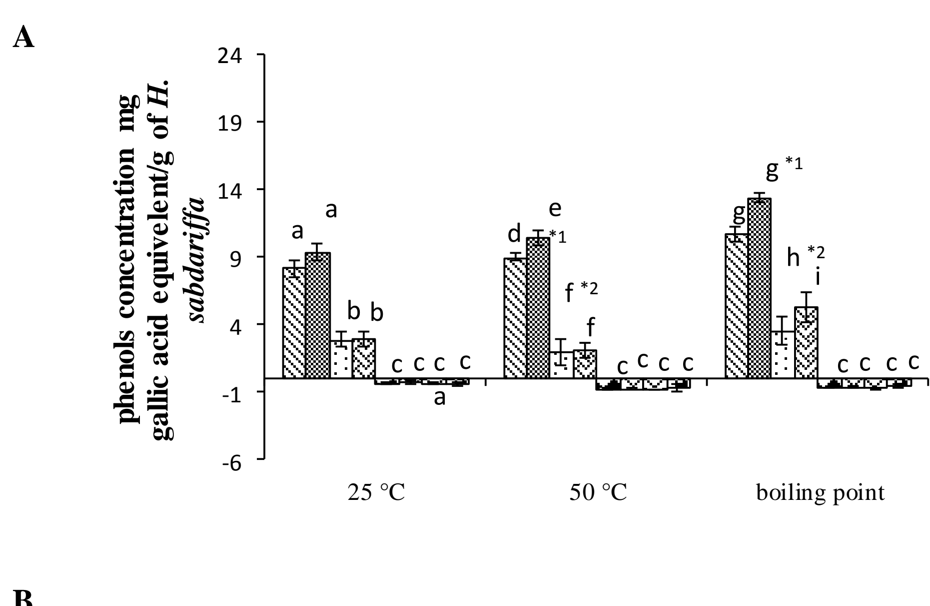
Figure 3-2. Standard curve for gallic acid from (0-500 mg/l), using folin assay

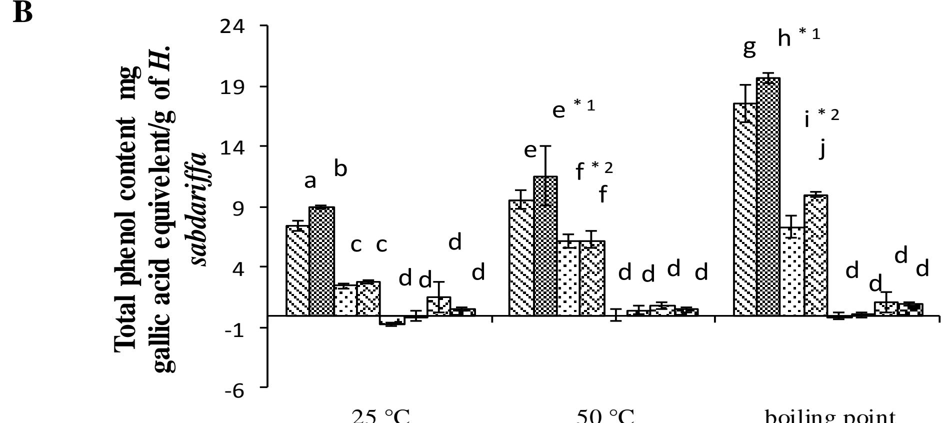
From Figures 3-3 (A), 3-3 (B) and 3-3 (C), results show the amount of total phenol in mg as gallic acid equivalent per (1) g of dried *H. sabdariffa* in different solvent extracts extracted at different time and temperature. The highest amount of total phenol obtained in extracts extracted at solvent boiling point for 5 min and at 50 °C for 10 min respectively.

Total phenol content in extracts extracted at 3 min and 5 min reduced following ascending patterns. In general, for the 10 min extraction, extracts reach the maximum amount of total phenol at 50 °C, after that the amount of total phenol for all types of extracts, decreased sharply when solvent boiling point used in extraction.

In terms of solvent extracts, at temperatures of 25 °C, 50 °C and solvent boiling point, water and methanol acidified and non-acidified extracts have the highest concentrations of phenols compared with hexane and ethyl acetate, with or without formic acid, which had the lowest amounts of phenols. There was a significant difference between water extracts and other solvents (P <0.001) at all conditions.

At the 3 min extraction figure 3-3 (A), water and water with formic acid extracts have the highest amounts of phenols at boiling point (10.7 ± 0.58 mg/g, 13.4 ± 0.33 mg/g gallic acid equivalent respectively). At 50 °C and boiling point temperatures the differences between the acidified and non-acidified aqueous extracts were significant (P = 0.027, P = 0.001) but at 25 °C there was no significant difference between them (P = 0.064). Whereas, methanol and methanol with formic acid extracts have the same amount of total phenols at 25 °C (2.9 ± 0.55 mg/g and 2.9 ± 0.59 mg/g gallic acid equivalent) and 50 °C (1.9 ± 0.96 mg/g and 2.1 ± 0.59 mg/g gallic acid equivalent respectively), with no significant differences between them (P = 1). The total phenol content of non-acidified and acidified methanol at the solvent boiling point were (3.6 ± 1.1 mg/g and 5.3 ± 1.1 mg/g gallic acid equivalent) respectively. Significant differences were found between methanol with and without formic acid (P = 0.037).





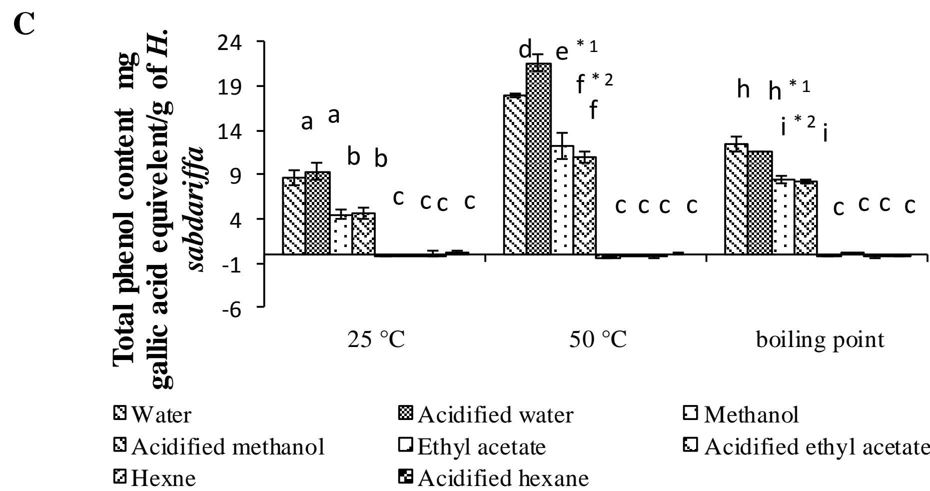


Figure 3-3. Total phenol content of dried *H. sabdariffa* extracts, (A) at 3 min extraction time, (B) at 5 min extraction time and (C) at 10 min extraction time, extracted by different solvents at different temperatures 25 °C, 50 °C and solvent boiling point. Values with different letters or numbers in each chart are significantly different (Tukey's test, $p<0.05$). Results are triplicates (± SD)

Total phenols were higher at the 5 min extraction time figure 3 (B), than at the 3 min extraction figure 3-3 (A).

Using solvent boiling point temperature for extraction for 5 min increased the total phenols in non-acidified water and acidified water extracts to 17.6 ± 1.5 mg/g and 19.7 ± 0.42 mg/g gallic acid equivalent respectively. Methanol with and without formic acid extracts were ranked second after the water extracts (10.02 ± 0.17 mg/g and 7.3 ± 0.87 mg/g gallic acid equivalent with and without formic acid). Significant differences between acidified and non-acidified water extracts were found at 25 °C and solvent boiling point for 5 min extraction figure 3-3 (B) clearly showing the significant differences (P = 0.033, P = 0.034 respectively). Also, at boiling point temperature the differences between methanol extracts with and without formic acid, were significant (P = 0.005).

Total polyphenol content was measured for each extract (10 min extraction times). Figure 3-3 (C) shows that the water extracts with and without formic acid, gave the highest yields of total polyphenols (21.7 ± 0.93 and 17.9 ± 0.29 mg/g gallic acid equivalent, respectively). In contrast, the ethyl acetate and hexane extracts with, and without formic acid, resulted in the lowest amounts of polyphenols. At 25 °C and boiling point temperatures the differences between the acidified and non-acidified aqueous extracts were not significant (P = 0.790, P = 0.088) but at 50 °C there was a significant difference between them (P <0.001). Methanol extracts had lower amounts of total polyphenols in comparison with water extracts by approximately 1.3 fold. Ethyl acetate and hexane extracts resulted in approximately 75% less total polyphenols at all temperatures, compared with aqueous extracts. There was a

significant difference between water extracts and other solvents ($P < 0.05$) at all conditions.

Total phenol content (mg/g) at 3 min extraction									
	25 °C			**50 °C**			**Solvent boiling point**		
samples	**before**	**after**	**differences**	**before**	**after**	**differences**	**before**	**after**	**differences**
Water	15.5	12.3	3.2	18.5	14.9	3.6	23.1	18.1	5.1
Acidified water	15.9	12.5	3.4	19.6	16	3.6	23.9	19.1	4.8
Methanol	5.7	4.6	1.1	11.4	9.2	2.2	12.4	9.9	2.4
Acidified methanol	5.9	4.9	1	13.6	11.4	2.2	13.9	11.2	2.8
Total phenol content (mg/g) at 5 min extraction									
	25 °C			**50 °C**			**Solvent boiling point**		
samples	**before**	**after**	**differences**	**before**	**after**	**differences**	**before**	**after**	**differences**
Water	15.2	11.9	3.3	20.2	16.2	4	25.3	20.0	5.3
Acidified water	15.4	12.0	3.4	21.8	17.7	4.1	25.3	20	5.4
Methanol	7.7	6.3	1.4	13.2	10.8	2.4	12.5	10.1	2.4
Acidified methanol	6.9	5.8	1.1	14.1	11.5	2.6	14.7	11.6	3.0

Table 3-4. Average total phenol content (mg/g gallic acid equivalent) before and after correction of vitamin C interference, for acidified and non- acidified water and methanol extracts of *H. sabdariffa* extracted at 25 ° C, 50 ° C and solvent boiling point for 3min, 5 and 10 min (n= 3)

Total phenol content (mg/g) at 10min extraction									
	25 °C			50 °C			Solvent boiling point		
samples	**before**	**after**	**differences**	**before**	**after**	**differences**	**before**	**after**	**differences**
Water	17.1	13.8	3.3	18.9	15.1	3.8	25.7	20.1	5.6
Acidified water	14.8	11.8	3.1	17.7	14.3	3.4	24.3	19.0	5.3
Methanol	9.5	7.7	1.8	13.9	11.3	2.6	14.6	11.8	2.9
Acidified methanol	9.1	7.4	1.7	12.7	10.4	2.3	16. 8	13.4	3.4

Table 3-5. Average total phenol content (mg/g gallic acid equivalent) before and after correction of vitamin C interference, for acidified and non- acidified water and methanol extracts of *H. sabdariffa* extracted at 25 ° C, 50 ° C and solvent boiling point for 10 min (n= 3)

3.5.4 Total phenols after correction of the interference of vitamin C

Table 3-4 and 3-5 show the amount of total phenols mg/g gallic acid equivalent for water and methanol extracts in the presence and absence of formic acid at 25 °C, 50 °C and solvent boiling point for 3min, 5 and 10 min. It is clear from the table that vitamin C had an interference effect on the amount of total phenols. Increasing the temperature increased the vitamin C amount. Statistical analysis was done using a paird student t-test to see if there was a significant difference before and after the correction of vitamin C for acidified and non-acidified water and methanol extracts of *H. sabdariffa*. The analysis found that there was a significant difference between the amount of total phenols before and after the correction for both for acidified and non-acidified water and methanol extracts of *H. sabdariffa* (P <0.001).

3.5.5 Effect of solvent on the DPPH assay

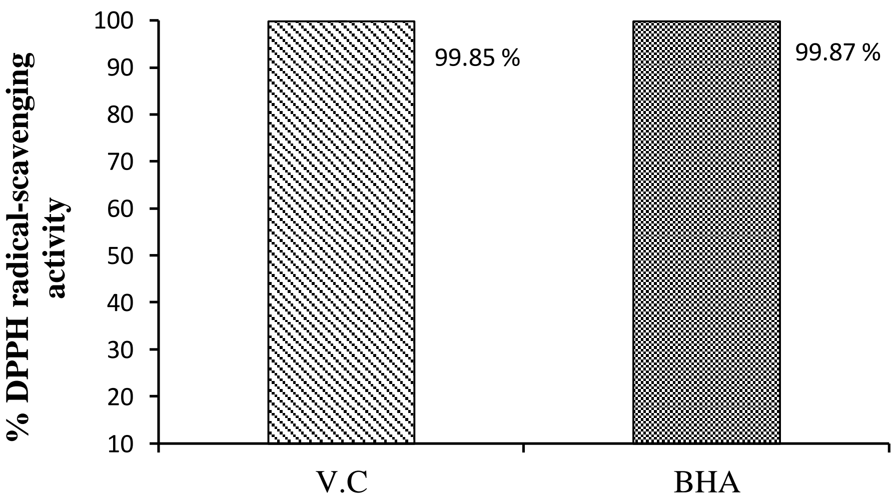


Figure 3-4. The percentage of DPPH radical scavenging activity for antioxidant standards vitamin C (V.C) and butylated hydroxyanisole (BHA)

Figure 3-4 illustrates the percentage of DPPH radical scavenging activity for the antioxidant standards vitamin C and butylated hydroxyanisole (BHA), which show a stable % of DPPH.

Figures 3-5 (A), 3-5 (B) and 3-5 (C) show DPPH percentage radical scavenging activity in 3, 5 and 10 min extractions using different solvents at different temperatures (25 °C, 50 °C and solvent boiling point). The percentage of DPPH radical scavenging activity in the extracts was (in descending order) water, acidified water, methanol, acidified methanol, acidified ethyl acetate, ethyl acetate, hexane and acidified hexane. The percentage of DPPH radical scavenging activity is high in the *H. sabdariffa* water extracts, made with and without formic acid, while it is low in hexane and ethyl with and without formic acid extracts. A significant difference was found between the antioxidant capacity of the water extracts and all other solvents when measured by the DPPH assay ($P < 0.001$).

The highest antioxidant capacity of water with formic acid, water, methanol and methanol with formic acid extracts occur when extracted for 10 minutes at boiling point temperature with 72, 70, 35 and 34% DPPH radical scavenging activity respectively. Over all, the radical scavenging activity decreased with an increase in the time of extraction in all solvent extracted at 25 °C and 50 °C. While, the percentage remained high in solvent boiling point temperature.

Formic acid does not have any significant effect on radical scavenging activity in all extraction temperatures, except between non-acidified and acidified water extracts at both solvent boiling point for 5 min extraction time and at 50 °C temperature for 10 min extraction time ($P = 0.030$ $P < 0.05$, $P < 0.001$) respectively.

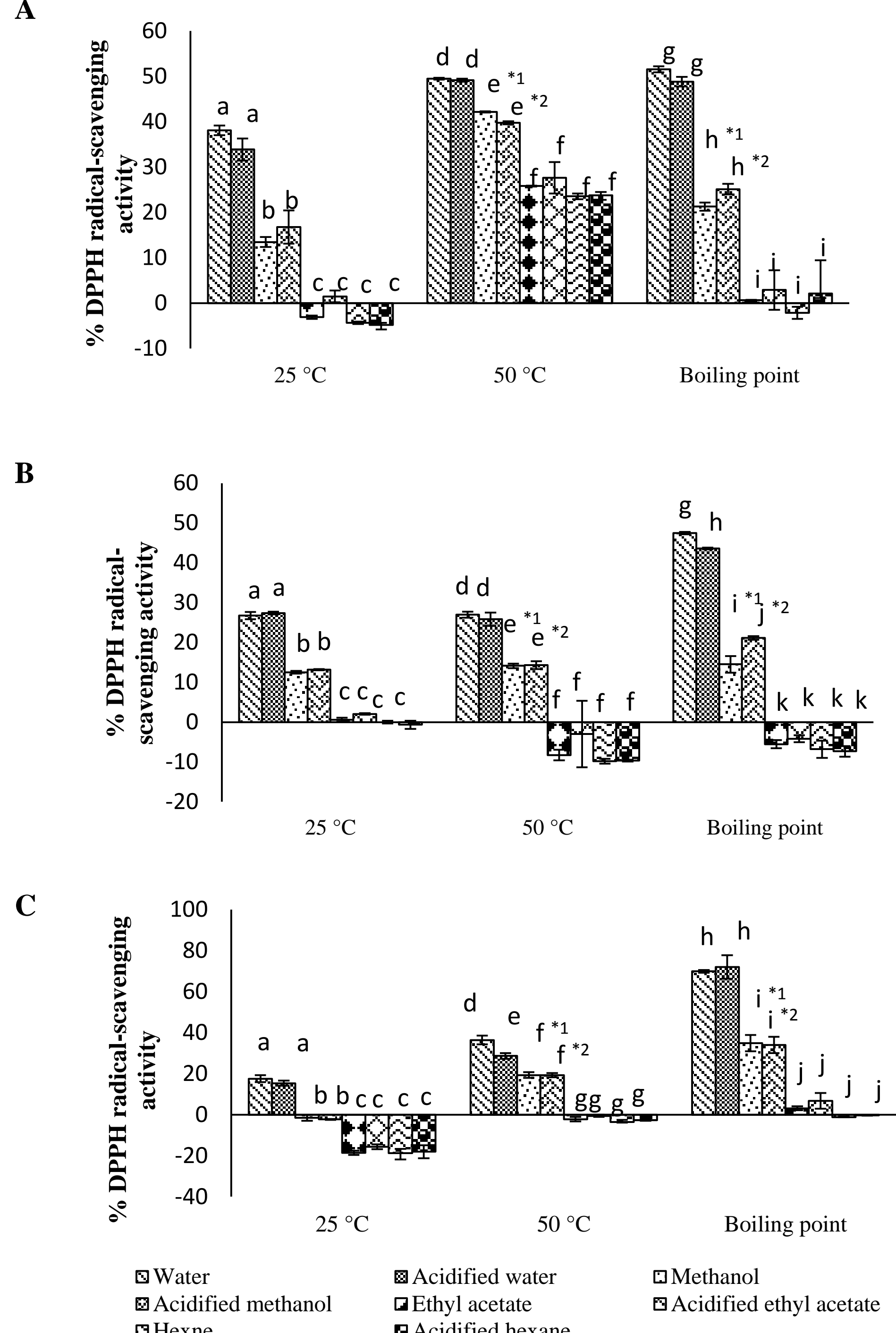


Figure 3-5. Percentage of DPPH radical scavenging activity of dried *H. sabdariffa* extracts, (A) at 3 min extraction time, (B) at 5 min extraction time and (C) at 10 min extraction time, extracted by different solvents at different temperatures 25 °C, 50 °C and solvent boiling point. Values with different letters and numbers in each chart are significantly different (Tukey's test, $p<0.05$). Results are triplicates (± SD)

3.5.6 Effect of solvent on the FRAP assay

Figure 3-6 shows the standard curve for Trolox, which was used to calculate the antioxidant activity in the extracts.

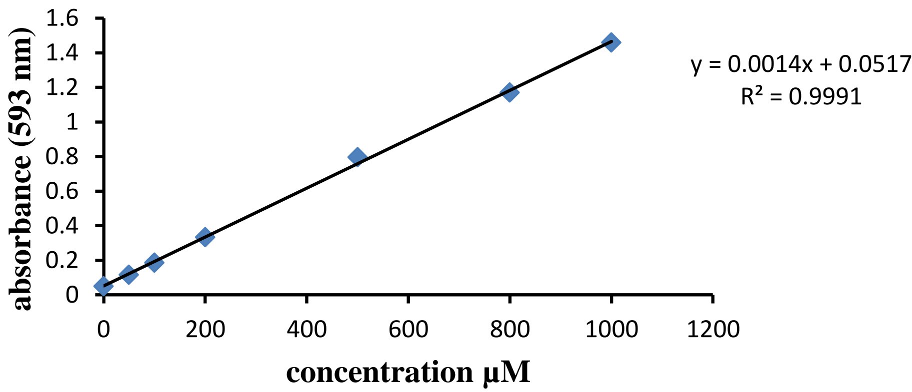


Figure 3-6. Trolox standard curve for FRAP assay (0-1000 µM)

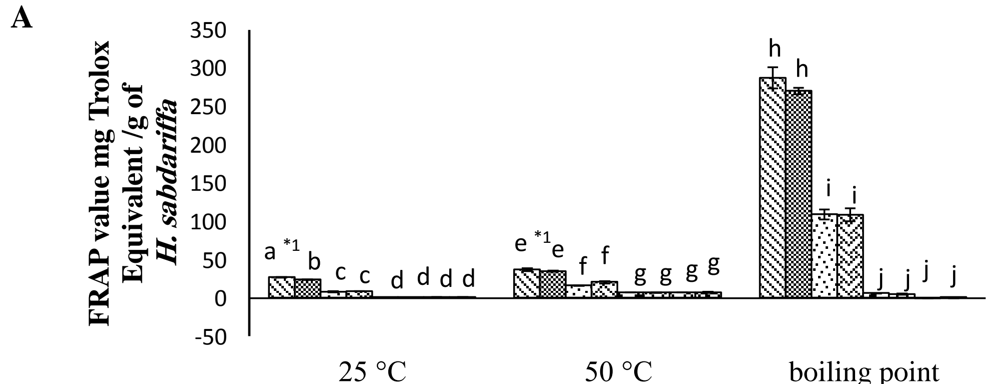
Figure 3-7 (A), 3-7 (B) and 3-7 (C) shows the FRAP assay result as Trolox equivalents for 3 min, 5 min and 10 min extraction time at 25 °C, 50 °C solvent boiling point temperatures using different solvents for extraction. From figure 3-7 (A) and 3-7 (B), it is appears that the results are similar for extraction temperatures 25 °C and 50 °C. The highest FRAP values were obtained at solvent boiling point for water and methanol with and without formic acid at 3 min 270 ± 4.2 mg/g, 287.7 ± 13.8 mg/g Trolox equivalent, and 108.8 ± 8.3 mg/g, 109.3 ± 6.4 mg/g Trolox equivalent respectively and at 5 min extraction were 271 ± 5.1 mg/g, 306.3 ± 13.8 mg/g Trolox equivalent, 142 ± 4.6 mg/g, 119.5 ± 3 mg/g Trolox equivalent respectively.

At 10 min extraction time figure 3-7 (C) FRAP values increased with increasing extraction temperature, but the values of FRAP for acidified and non-acidified water and methanol extracts at solvent boiling point lower than both 3 min and 5 min extraction time at the same temperature. With 10 min extraction time, acidified and non-acidified aqueous extracts made at boiling point had the highest FRAP values (38.18 ± 0.64 and 39.14 ± 1.69 mg/g Trolox equivalents, respectively), whereas acidified and non-acidified hexane and ethyl acetate extracts made at boiling point had the lowest values (0.03 ± 0.37 and 0.15 ± 0.47 mg/g Trolox equivalent, respectively). There was no significant difference between water with or without formic acid at 50 °C and boiling point (P = 0.932, P = 0.795) and methanol extracts with or without formic acid at 25 °C and 50 °C (P = 0.998, P = 0.986) respectively. FRAP results showed a significant difference between the antioxidant levels of water extract with and without formic acid compared to ethyl acetate and hexane with and without formic acid (P <0.001).

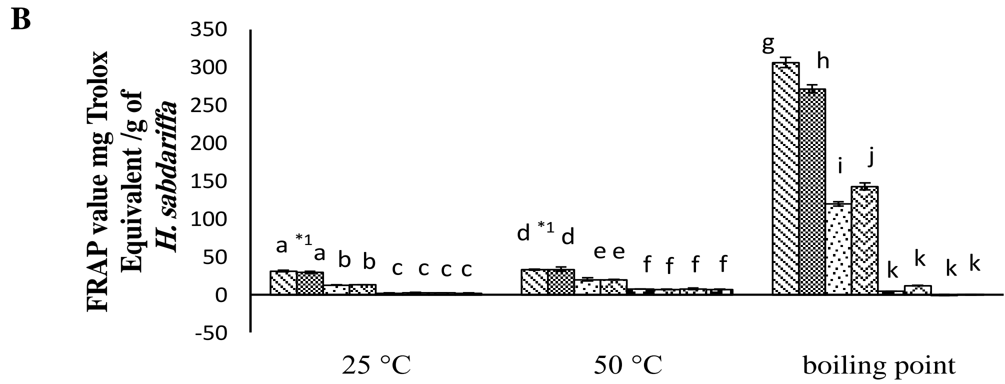
3.5.7 Effect of solvent on the TEAC assay

Figure 3-8 shows the standard curve for Trolox, which was used to calculate the concentration of TEAC in the extracts. Figure 3-9 (C) illustrates that extracts at solvent boiling point temperature has the highest concentration of antioxidant activity in TEAC assay.

A



B



C

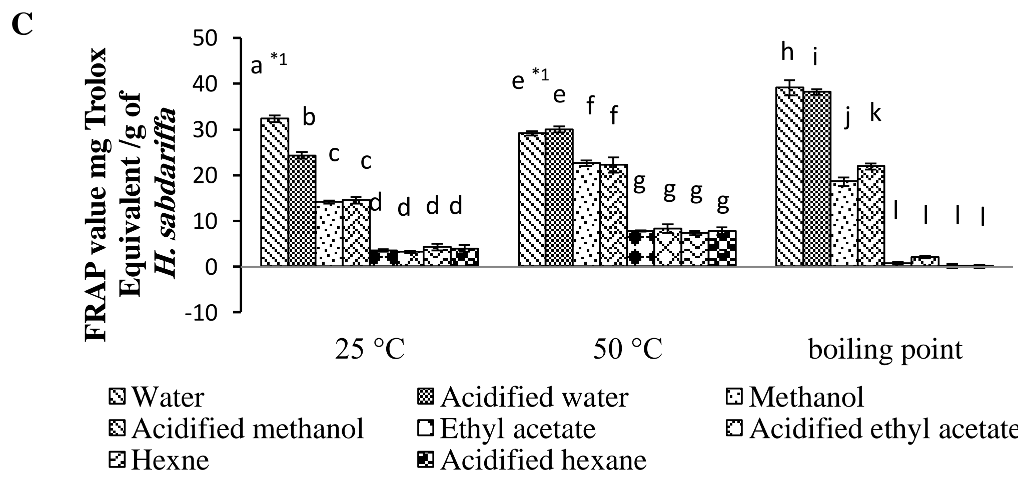


Figure 3-7. FRAP values of dried *H. sabdariffa* extracts, (A) at 3 min extraction time, (B) at 5 min extraction time and (C) at 10 min extraction time, extracted by different solvents at different temperatures 25 °C, 50 °C and solvent boiling point. Values with different letters or numbers in each chart are significantly different (Tukey's test, p<0.05). Results are triplicates (± SD)

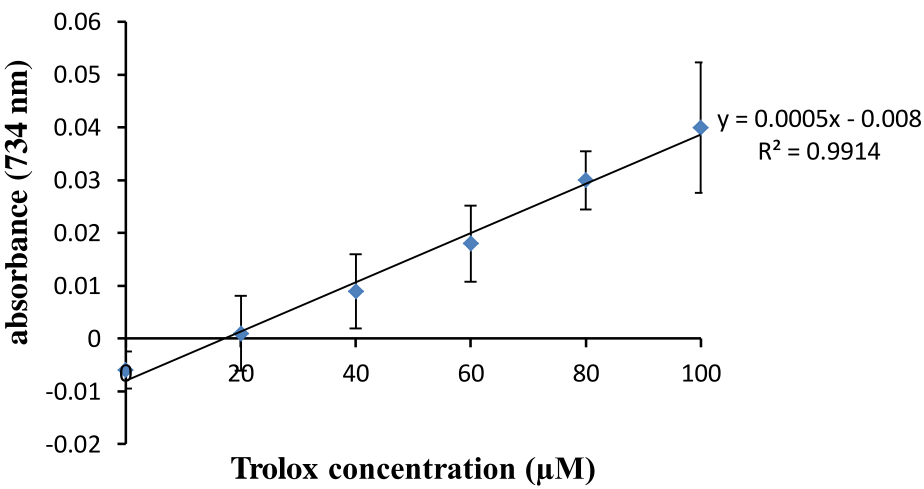
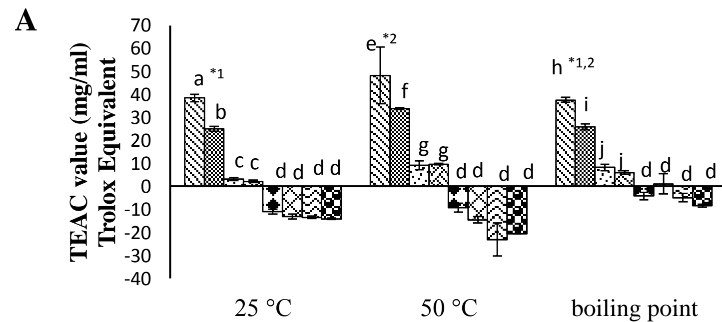


Figure 3-8. Trolox standard curve for TEAC assay (0-100 µM)

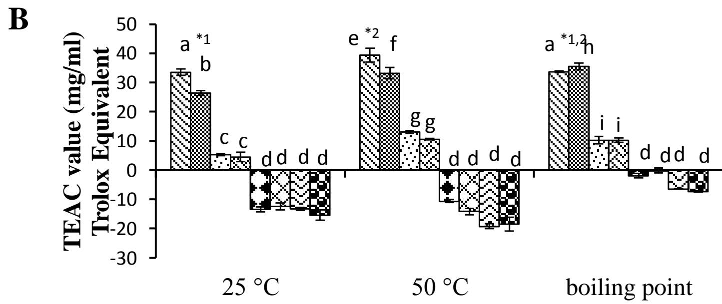
Figure 3-9 (A) shows the effect 3 min extraction time has on the antioxidant activity of TEAC at 25 °C, 50 °C and solvent boiling point. The highest antioxidant activity was found for acidified and non-acidified aqueous extracts (33.9 ± 0.30 mg/g, 48.3 ± 12.3 mg/g Trolox equivalent respectively) at 50 °C compared with the TEAC activity at 25 °C and solvent boiling point. There was a significant difference at 3 min extraction between acidified and non-acidified aqueous extracts and all other solvent extracts ($P < 0.001$). The TEAC antioxidant capacity values of extracts of *H. sabdariffa* extracted at 5 and 10 min are shown in figure 3-9 (B) and 3-9 (C). There was a significant difference between the TEAC values for aqueous extracts with and without formic acid at 25 °C and 50 °C ($P < 0.001$) and between the water extracts and all other solvents ($P < 0.001$). At 3 min and 5 min extraction time, formic acid has significant effect on water extracts for 25 °C and 50 °C ($P < 0.001$), were formic acid lowered the antioxidant capacity at 25 °C and 50 °C. In addition, at all

extraction times, formic acid lowered the antioxidant capacity of water extract at 50 °C. The TEAC antioxidant capacity of acidified water and methanol extracts remain the same as non-acidified water extract at solvent boiling point for 5 min and 10 min extraction time. At 5 min and 10 min extraction, the TEAC antioxidant capacity of ethyl acetate and hexane extracts was not significantly different from the values obtained in the presence of formic acid at all extraction temperatures. Also, there was no significant differences between acidified and non-acidified methanol extracts at 25 °C and solvent boiling point ($P = 0.058$, $P = 0.052$). In addition, there was no significant differences between acidified and non-acidified methanol extracts at 5 min extraction time for 25 °C, 50 °C and solvent boiling point ($P = 0.825$, $P = 0.201$, $P = 1$), but for 10 min extraction time there was a significant differences between acidified and non-acidified methanol extracts at 50 °C ($P = 0.023$), were formic acid reduced the antioxidant capacity. The TEAC antioxidant capacity of acidified water and methanol extracts was the same as non-acidified water extract at solvent boiling point for 5 min and 10 min extraction time. In terms of acidified and non-acidified water extracts, no significant differences were found between TEAC antioxidant activity at 5 min and 10 min extraction time for solvent boiling point ($P = 0.110$, $P = 0.986$ respectively). Acidified and non-acidified ethyl and hexane extracts have poor antioxidant activity, however the TEAC values increase with increasing temperature of extraction.

A



B



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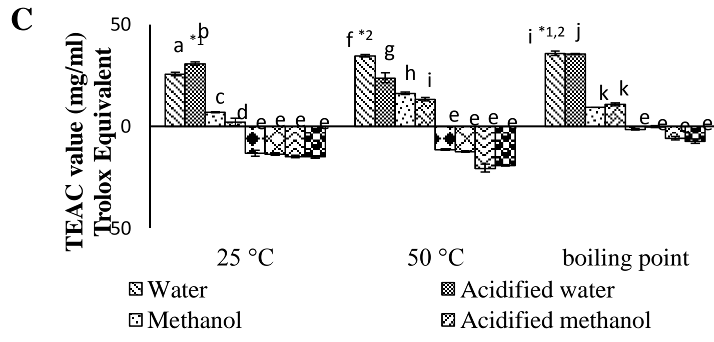


Figure 3-9. TEAC values of dried *H. sabdariffa* extracts, (A) at 3 min extraction time, (B) at 5 min extraction time and (C) at 10 min extraction time, extracted by different solvents at different temperatures 25 °C, 50 °C and solvent boiling point. Values with different letters and numbers in each chart are significantly different (Tukey's test, $p<0.05$). Results are triplicates (± SD)

3.5.8 Correlation of antioxidant assays and total phenol content

Statistical results from the present study in table 3-6 shows that total phenol content had moderate correlation with TEAC values (r^2 = 0.622) with FRAP (r^2 = 0.450) and DPPH (r^2 = 0.448). In addition, TEAC values had a positive strong correlation with DPPH (r^2 = 0.649). While, there was a weak correlation between FRAP and DPPH (r^2 = 0.360) and between TEAC and FRAP values (r^2 = 0.280).

Assays	**Total phenols**	**DPPH**	**FRAP**	**TEAC**
Total phenols	1	0.448*	0.450*	0.622*
DPPH	0.448*	1	0.360*	0.649*
FRAP	0.450*	0.360*	1	0.280**
TEAC	0.622*	0. 649*	0.280*	1

Table 3-6. Linear correlation coefficients (r^2) among total phenols, DPPH, FRAP and TEAC of different solvent extracts of dried *H. sabdariffa*.
***Correlation is significant at the 0.01 level (2-tailed)**

3.5.9 Effect of solvent on the total monomeric anthocyanin content

Figure 3-10. Aqueous and methanol extract of dried *H. sabdariffa* extracted at solvent boiling point for 10 min

Figure 3-10 represents images of water and methanol extracts of dried *H. sabdariffa* extracted at solvent boiling point for 10 min, it is clear from figure 3-10 that water extract darker than methanol extract.

Figure 3-11 shows the total monomeric anthocyanin content (extracted as cyanidin 3-glucoside equivalent) for acidified and non-acidified aqueous and methanol extracts. These are the only extracts that could be determined. Anthocyanins were undetectable in acidified and non-acidified ethyl acetate and hexane extracts due to difficulties in solubility. Figure 3-11 shows that the total monomeric anthocyanin content increases with increasing the temperature of extraction. Acidified and non-acidified water extracts yield higher amounts of anthocyanins compared to methanol extracts ($P < 0.001$).

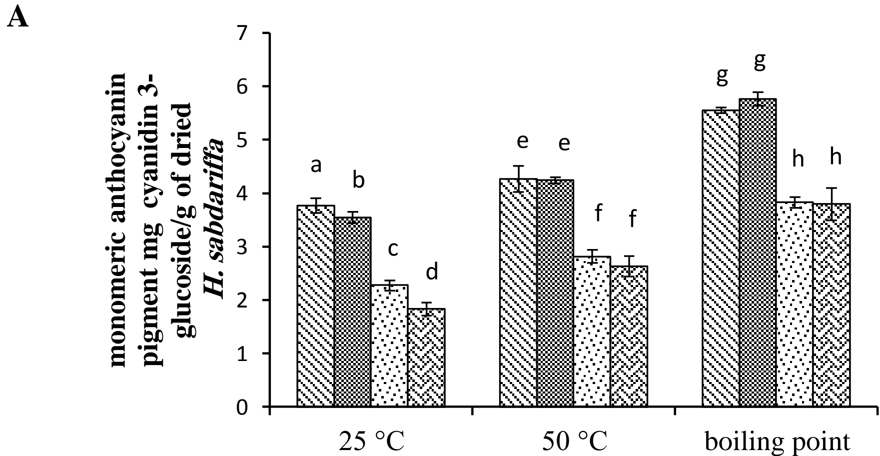
Figure 3-11 (A) shows the total monomeric anthocyanin content 3 min at extraction time. Total anthocyanin content in water extracts with and without formic acid was higher than methanol extracts at the same conditions. At 25 °C, significant difference were found between water and methanol extracts with and without formic acid ($P < 0.001$, $P = 0.005$, $P < 0.05$). Addition of formic acid to the water and methanol extracts lowered the amount of extracted anthocyanins. However, no significant difference was found in total anthocyanin content of acidified and non-acidified water and methanol extracts at 50 °C and solvent boiling point for 3 min ($P = 0.997$, $P = 0.500$ for water $P > 0.05$; $P = 0.570$, $P = 0.966$ for methanol $P > 0.05$).

Figure 3-11 (B) demonstrates the amount of monomeric anthocyanins at 5 min extraction time. It is appears that acidified and non-acidified aqueous extracts have the maximum yield of anthocyanins at solvent boiling point (5.8 ± 0.26 mg/g, 6.2 ± 0.10 mg/g cyanidin 3-glucoside equivalent) compared with acidified and non-

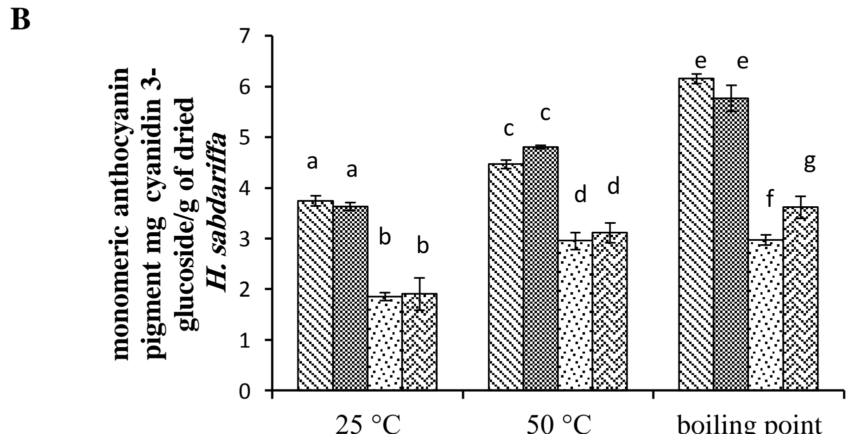
acidified methanol extracts (3.6± 0.22 mg/g, 3 ± 0.10 mg/g cyanidin 3-glucoside equivalent). There were no significant differences between acidified and non-acidified water extracts at all extraction temperatures 25 °C, 50 °C and solvent boiling point ($P = 0.984$, $P = 0.064$ and $P = 0.116$, $P > 0.05$ respectively) at 5 min extraction. For acidified and non-acidified methanol extracts at 25 °C and 50 °C, adding formic acid had no effect on the amount of anthocyanins ($P = 0.984$, $P = 0.512$, $P > 0.05$ respectively).

At solvent boiling point, a significant difference was found between acidified and non-acidified methanol extracts at 5 min extraction time ($P = 0.011$, $P < 0.05$). Formic acid increased the amount of extracted anthocyanins.

A



B



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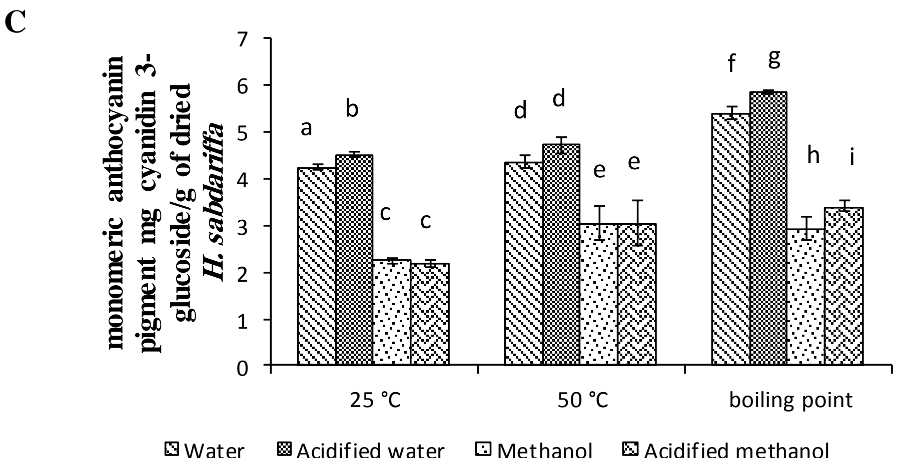


Figure 3-11. Total monomeric anthocyanins content expressed as cyanidin 3-glucoside equivalents (mg/g of *H. sabdariffa*) of extracts of *H. sabdariffa* for 3 min (A), 5 min (B) and 10 min (C) extraction time. Values with different letters in each chart are significantly different (Tukey's test, p<0.05). Results are triplicates (± SD)

Figure 3-11 (C) illustrates the effects of extraction on the total monomeric anthocyanin content (expressed as cyanidin 3-glucoside equivalents) for water and methanol extracts with and without formic acid. Water extracts with and without formic acid had the highest amount of anthocyanin (5.860 ± 0.48, 5.408 ± 1.38 mg/g, respectively), compared with methanol extracts There is no significant difference between acidified and non-acidified solvents at 50 °C (P = 0.586 for water, P =1 for methanol; P >0.05, respectively). However, there was a significant difference between water with and without formic acid at 25 °C and solvent boiling point (P <0.001), and a significant difference between methanol with and without formic acid at solvent boiling point (P = 0.033, P <0.05). Formic acid increased the anthocyanin content.

3.5.10 Correlation of total anthocyanin content with antioxidant assays and total phenol content

Linear correlation coefficients (r^2) were determined between total anthocyanin content, total phenol content and antioxidant assays for acidified and non-acidified aqueous and methanol extracts. Statistical results from the present study showed that total anthocyanin content had strong correlation with other assays; for TEAC values (r^2 = 0.781), total phenol (r^2 = 0.756), DPPH (r^2 = 0.718) and moderate correlation with FRAP (r^2 = 0.630).

Assays	Total phenols	DPPH	FRAP	TEAC	Total anthocyanins
Total phenols	1	0.448*	0.450*	0.622*	0.756*
DPPH	0.448*	1	0.360*	0.649*	0. 718*
FRAP	0.450*	0.360*	1	0.280**	0.630*
TEAC	0.622*	0. 649*	0.280*	1	0. 781*
Total anthocyanins	0.756*	0. 718*	0.630*	0. 781*	1

Table 3-7. Linear correlation coefficients (r^2) among total phenols, total anthocyanins, DPPH, FRAP and TEAC of different solvent extracts of dried *H. sabdariffa*. * Correlation is significant at the 0.01 level (2-tailed)

3.5.11 Identification of anthocyanin compounds in *H. sabdariffa* extracts by LC-MS

Table 3-6 summarise the identification of anthocyanin compounds in acidified and non-acidified aqueous and methanol extracts extracted at solvent boiling point temperature for 10 min, as well as using standards to identify the peaks eluted using HPLC, *H. sabdariffa* extracts were also analysed by LC-MS to provide confirmation. Peak 1 detected at average retention time 4.5 min was identified as delphinidin 3-sambubioside. It had a $[M]^+$ at *m/z* 597 which fragmented to produce MS^2 a minor ion at *m/z* 465 ($[M]^+$ -132, loss of a xylosyl group) and a major fragment *m/z* 303 ($[M]^+$ -132 -162, quench of xylosyl and glucosyl units). Peak 2 separated at retention time 5.4 min, also had a $[M]^+$ at *m/z* 465, which yielded a MS^2 fragment at *m/z* 303 (delphinidin, $[M]^+$ -162, loss of a glucose). This peak was identified as delphinidin 3-glucoside and this was confirmed by standards. Peak 3 detected at retention time 5.8 min, was identified as cyanidin 3-sambubioside.

It had a $[M]^+$ at *m/z* 581 which splited to produce MS^2 a small ion at *m/z* 449 ($[M]^+$ -132, loss of a xylosyl group) and a main fragment *m/z* 287 ($[M]^+$ -132 -162, split of xylosyl and glucosyl units). Peak 4 eluted at retention time 6.8 min, produced a MS containing a ($[M]^+$ at *m/z* 449, which fragmented on MS^2 to produce a cyanidin ion at *m/z* 287 ($[M]^+$ -162, loss of a glucose). This peak is cyaniding 3-glucoside and the identification was confirmed by co-chromatography with a cyanidin-3-glucoside standard.

peak	**t_R[a]**	**Compound**	**Molecular ion M^+ (*m/z*)**	**Fragment ion (*m/z*)**
1	4.5	Delphinidin 3-sambubioside	597*	303 delphinidin (M^+- glucose-xylose)
2	5.4	Delphinidin 3-glucoside	465*	303 delphinidin (M^+- glucose)
3	5.8	Cyanidin 3-sambubioside	581*	287 cyanidin (M^+- glucose-xylose)
4	6.8	Cyanidin 3- glucoside	449*	287 cyanidin (M^+- glucose)

Table 3-8. anthocyanin compounds identified in acidified and non-acidified water and methanol extracts by HPLC and LC/MS at λ_{max} (nm) 520, t_R retention time of anthocyanins detected in HPLC, peaks number and retention time refer to HPLC result in figure 14 and 15, * positive ionisation molecular ion $[M]^+$

3.5.12 Identification and quantification of anthocyanin compounds in *H. sabdariffa* extracts by HPLC

Figure 3-12 shows the maximum absorbance of anthocyanins at 520 nm spectrum scan using photodiode array (PDA) detector.

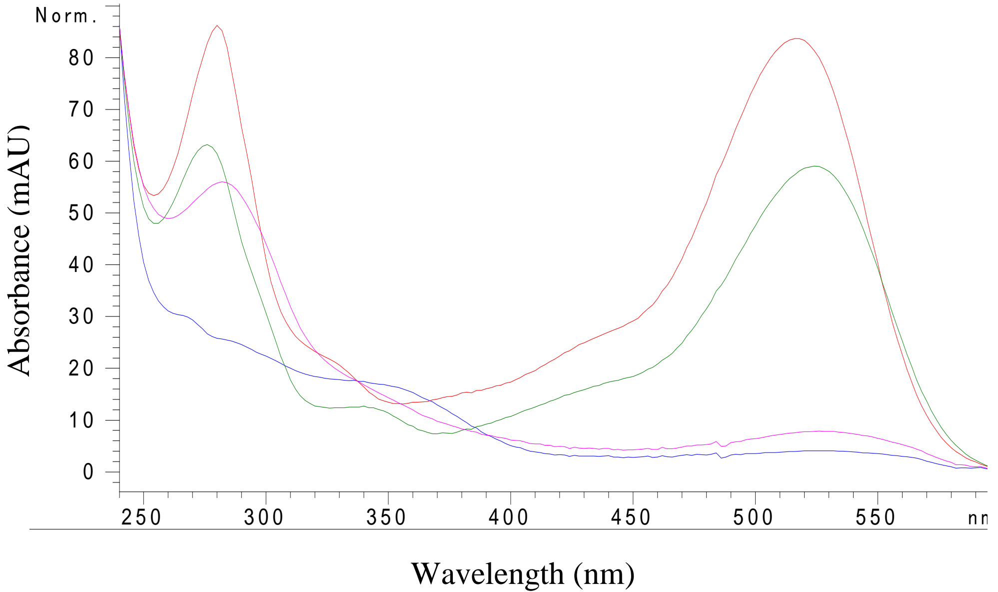


Figure 3-12. UV-Visible spectrum scan of aqueous extract of *H. sabdariffa* at wavelength 520 nm using photodiode array detector

Figures 3-13 (A), (B), (C) and (D) illustrate the standard curves of 4 common anthocyanins found in *H. sabdariffa*. Apigeninidin (20µM) was used as an internal standard. The elution order of the anthocyanins standards and retention times of the compounds were as follows: delphinidin 3-sambubioside (t_R =4.5min), delphinidin 3-glucoside (t_R =5.4 min), cyanidin 3-sambubioside (t_R =5.8 min) and cyanidin 3-glucoside (t_R =6.8 min).

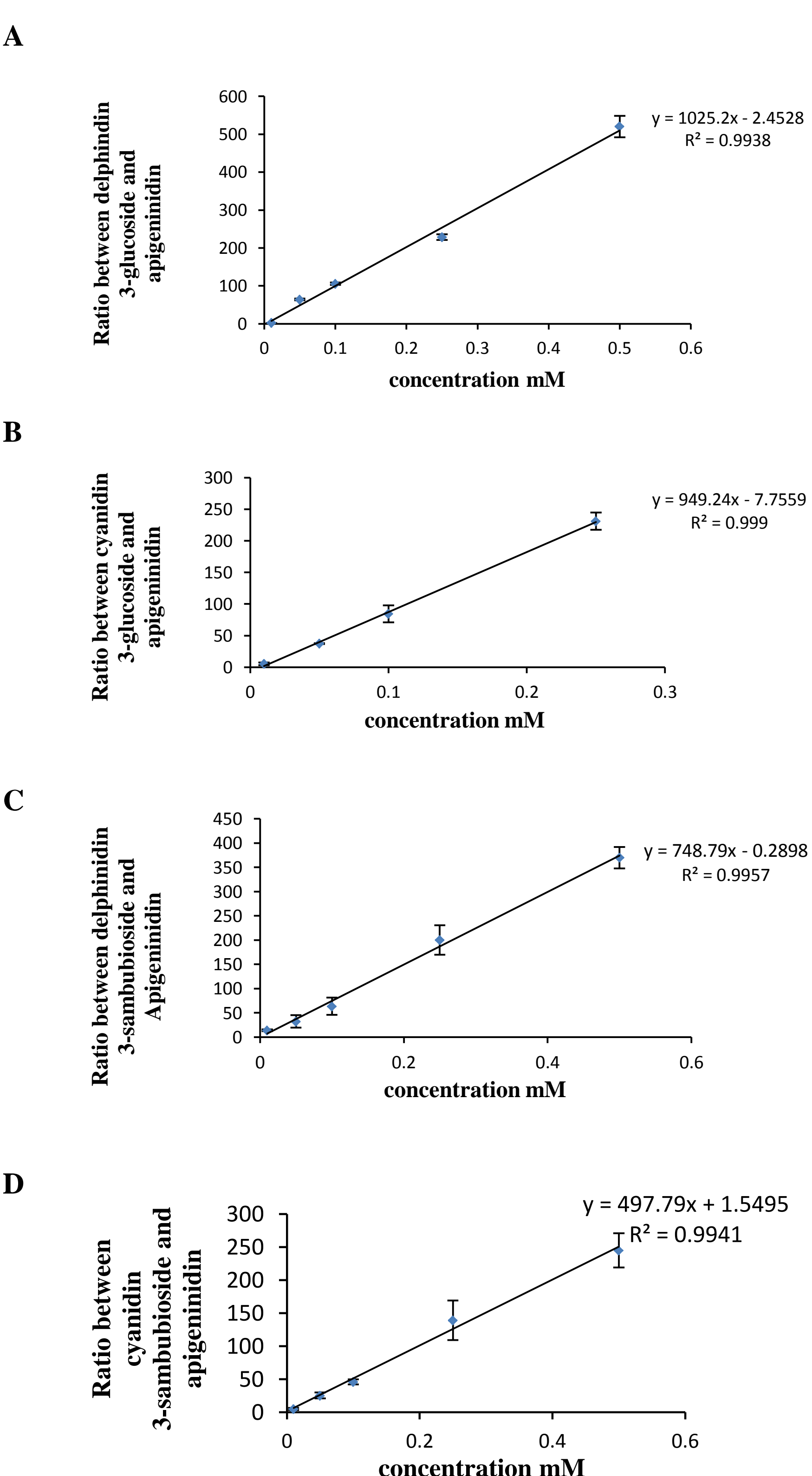


Figure 3-13. Anthocyanin standard curves (A) delphinidin 3-glucoside, (B) cyanidin 3-glucoside, delphinidin 3-sambubioside (C) and cyanidin 3-sambubioside (D) (range from 0.01 mM to 0.5mM over 5 concentrations) n=3 ± SD range, apigeninidin (20µM) was used as an internal standard

Figures 3-14, 3-15 and 3-16 show the concentrations of the four anthocyanins in the different aqueous and methanolic extracts. Results show that water with and without formic acid extracted the highest amount of the individual anthocyanins. This was significantly greater than the methanol extracts with or without formic acid ($P < 0.001$). Time had an effect on the concentration of anthocyanins. 3 min compared to 5 min does not have any significant effect on the anthocyanins concentration ($P = 0.724$, $P > 0.05$). But, significant difference was found between 5 min and 10 min extraction time ($P = 0.049$, $P > 0.05$).

From figure 3-14 it is appears that the concentration of delphinidin 3-glucoside and cyanidin 3-glucoside are the same in all solvent extracts, where as there are differences in the concentration of delphinidin 3-sambubioside and cyanidin 3-sambubioside. Water extracts in the presence and absence of formic acid have the highest concentration of delphinidin 3-sambubioside and cyanidin 3-sambubioside. In the 3 min extracts, concentration of delphinidin 3-sambubioside and cyanidin 3-sambubioside increased in 25 °C then the concentration decreased gradually with increasing the temperature. Addition of formic acid to the water and methanol extracts does not has any significant effect on the concentration of delphinidin 3-sambubioside, cyanidin 3-sambubioside, delphinidin 3-glucoside and cyanidin 3-glucoside ($P > 0.05$).

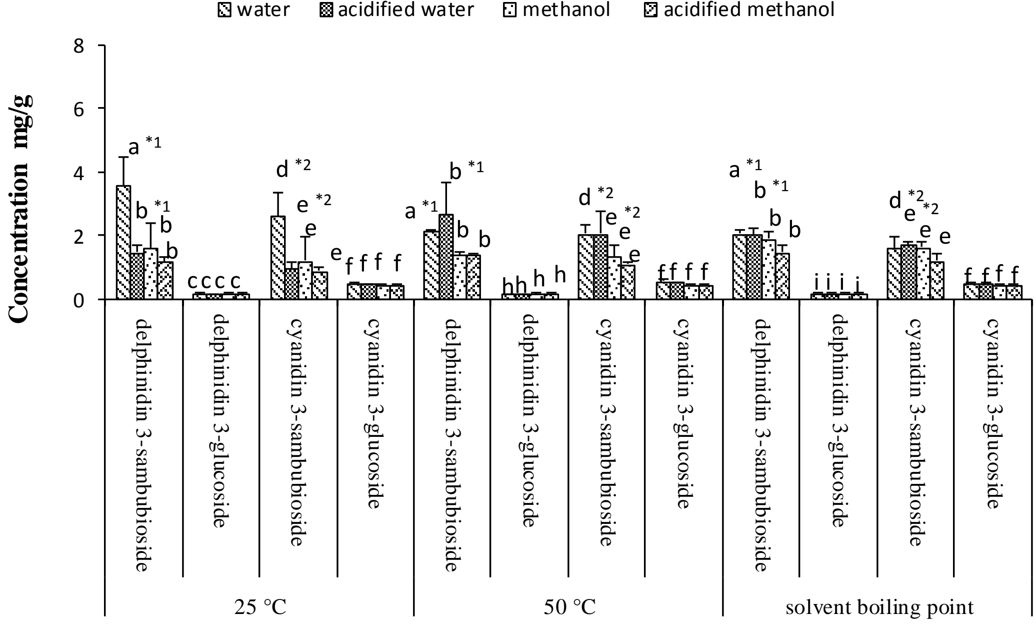


Figure 3-14. Concentration of anthocyanins (mg/g) identified and quantified by HPLC in acidified and non-acidified aqueous and methanolic extracts of *H. sabdariffa* at 3 min extraction time for 25 °C, 50 °C and solvent boiling point. Values with different letters numbers in each chart are significantly different (Tukey's test, p<0.05). Results are mean of triplicates (± SD)

At 5 min extraction time (figure 3-15), it is clear that increasing extraction temperature has an effect on increasing the anthocyanin concentration of delphinidin 3-sambubioside and cyanidin 3-sambubioside. Acidified and non-acidified aqueous extracts have the highest concentration of anthocyanins at solvent boiling point. Delphinidin 3-sambubioside was the greatest in the acidified water extract (3.4 ±0.43 mg/g) followed by water extract (2.8 ± 0.67 mg/g). The concentration of cyanidin 3-sambubioside for water extract was 2.5 ± 0.82 mg/g and for acidified water extract was 2.9 ± 0.15 mg/g. No significant differences were found between water and methanol extracts in the presence or absence of formic acid ($P< 0.05$), the concentration of delphinidin 3-glucoside and cyanidin 3-glucoside does not change with increasing extraction temperature. There were significant diffrences between 25 °C and 50 °C and between 25 °C and solvent boiling point ($P = 0.03$ $P <0.05$, P

<0.001) but no significant differences was found between 50 °C and solvent boiling point (*P* = 0.230, *P* >0.05) for delphinidin 3-sambubioside. Also, there were significant differences between 25 °C and solvent boiling point also between 50 °C and solvent boiling point (*P* <0.001) but no significant differences found between 25 °C and 50 °C (*P* = 0.098, *P* >0.05) for cyanidin 3-sambubioside. Extraction temperature does not has a significant effect on the concentration of delphinidin 3-glucoside and cyanidin 3- glucoside (*P*, <0.05) for all solvent extracts.

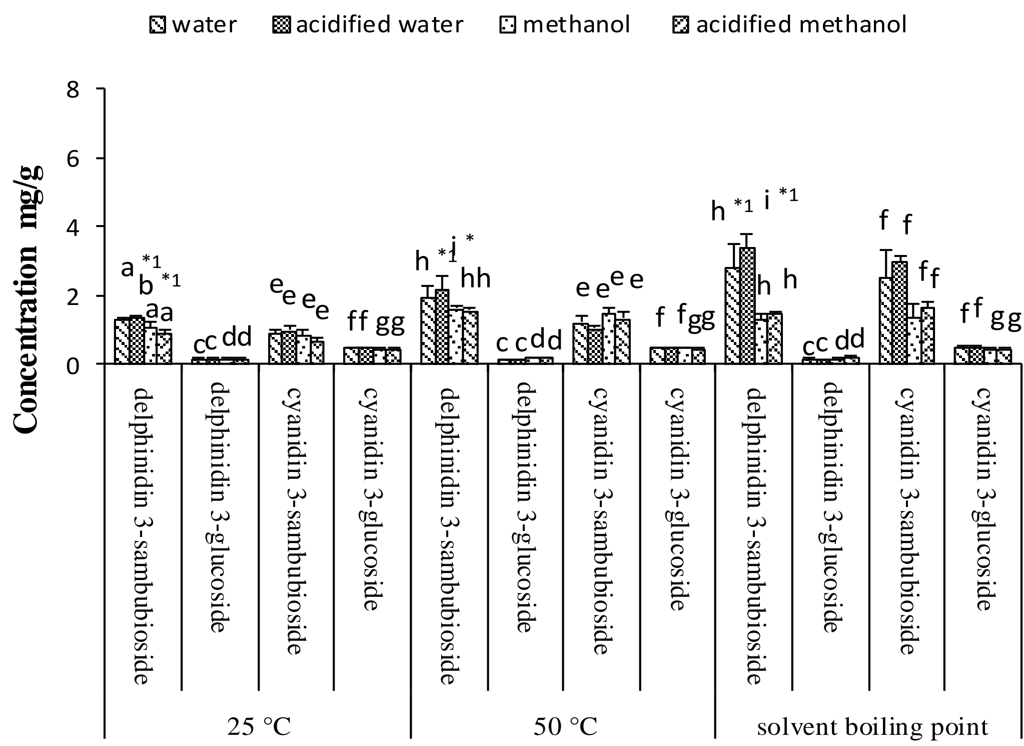


Figure 3-15. Concentration of anthocyanins (mg/g) identified and quantified by HPLC in acidified and non-acidified aqueous and methanolic extracts of *H. sabdariffa* at 5 min extraction time for 25 °C, 50 °C and solvent boiling point. Values with different letters numbers in each chart are significantly different (Tukey's test, p<0.05). Results are mean of triplicates (± SD)

At 5 min extraction time, adding acid to the solvent does not have any effect on the concentration of delphinidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-sambubioside and cyanidin 3-sambubioside extracted.

Figure 3-16 shows the concentration of anthocyanins in water and methanolic extracts with and without formic acid at 10 min. Figure 3-16, the highest amount of delphinidin 3-sambubioside was seen at solvent boiling point in aqueous extract without formic acid (5.1 ± 0.49 mg/g) and in aqueous extract with formic acid (4.9 ± 0.78 mg/g), compared with methanolic extract without formic acid (2.6 ± 0.69 mg/g) and in methanolic extract with formic acid (2.4 ± 0.09 mg/g). The highest concentration of cyanidin 3-sambubioside was found also at solvent boiling point in aqueous extract without formic acid (3.8 ± 1.21 mg/g) and in the aqueous extract with formic acid (3 ± 0.21 mg/g), compared with methanolic extract without formic acid (1.7 ± 0.44 mg/g) and in methanolic extract with formic acid (2.1 ± 0.02 mg/g). No significant differences were found between water and methanol extracts with or without formic acid. Temperature does not have any significant effect on the concentration of delphinidin 3-glucoside and cyanidin 3-glucoside. Whereas, a significant difference was found between 25 °C and solvent boiling point also, between 50 °C and solvent boiling point (P <0.001) for delphinidin 3-sambubioside and cyanidin 3-sambubioside. Pearson correlation was done between total monomeric anthocyanins and total anthocyanins detected by HPLC to see whether there is any relationship. The result shows moderate correlation between total monomeric anthocyanins and total anthocyanins detected by HPLC (r^2= 0.622, P <0.01).

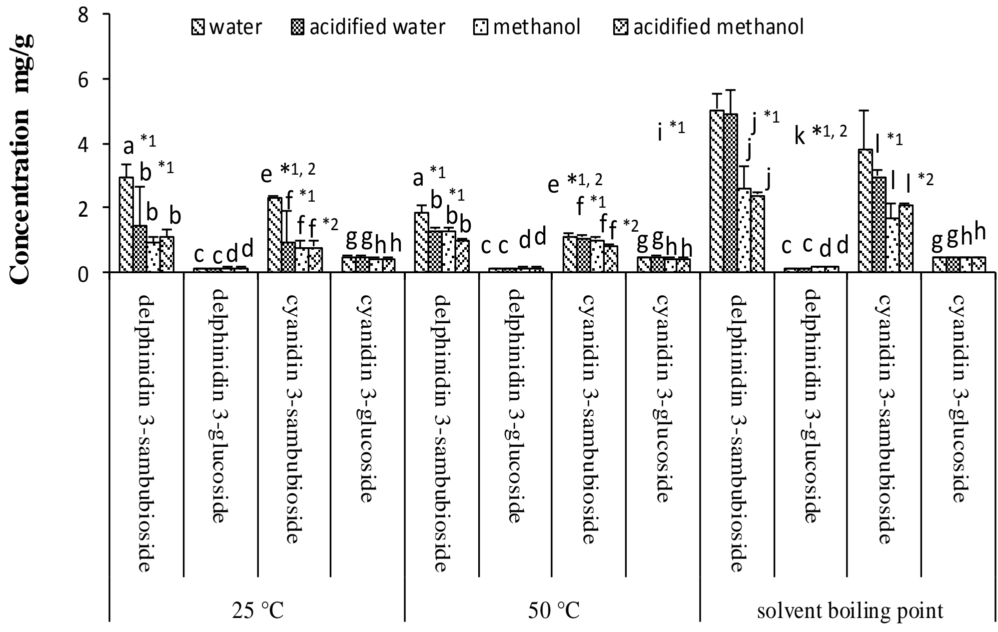


Figure 3-16. Concentration of anthocyanins (mg/g) identified and quantified by HPLC in acidified and non-acidified aqueous and methanolic extracts of *H. sabdariffa* at 10 min extraction time for 25 °C, 50 °C and solvent boiling point. Values with different letters numbers in each chart are significantly different (Tukey's test, p<0.05). Results are mean of triplicates (± SD)

Delphinidin 3-sambubioside, delphinidin 3-glucoside, cyanidin 3-sambubioside and cyanidin 3-glucoside were identified by HPLC (confirmed by LC-MS) in all acidified and non-acidified water and methanol extracts of *H. sabdariffa*. Figure 3-19 shows that none of the anthocyanins analysed were detected in ethyl acetate or in hexane with or without formic acid**.**

Figures 3-17 and 3-18 show chromatograms of the anthocyanins detected in acidified and non-acidified aqueous and methanolic extracts of *H. sabdariffa* made at solvent boiling point temperature for 10 min. It is clear from figures 3-17 and 3-18, that the anthocyanin compounds are similar for water and methanol extracts the only different between the two extract was the concentration of the anthocyanins which was higher in water extract than in methanol extracts. Adding formic acid to

the water and methanol extracts does not have any significant effect on peaks height. Peak 1 is delphinidin 3-sambubioside which is the highest peak, peak 3 is cyanidin 3-sambubioside and it is the second highest peak in acidified and non-acidified water extracts. Peaks 2 and 4 are for delphinidin 3-glucoside and cyanidin 3-glucoside. The concentrations of the latter two compounds are very low especially in acidified and non-acidified methanol extracts.

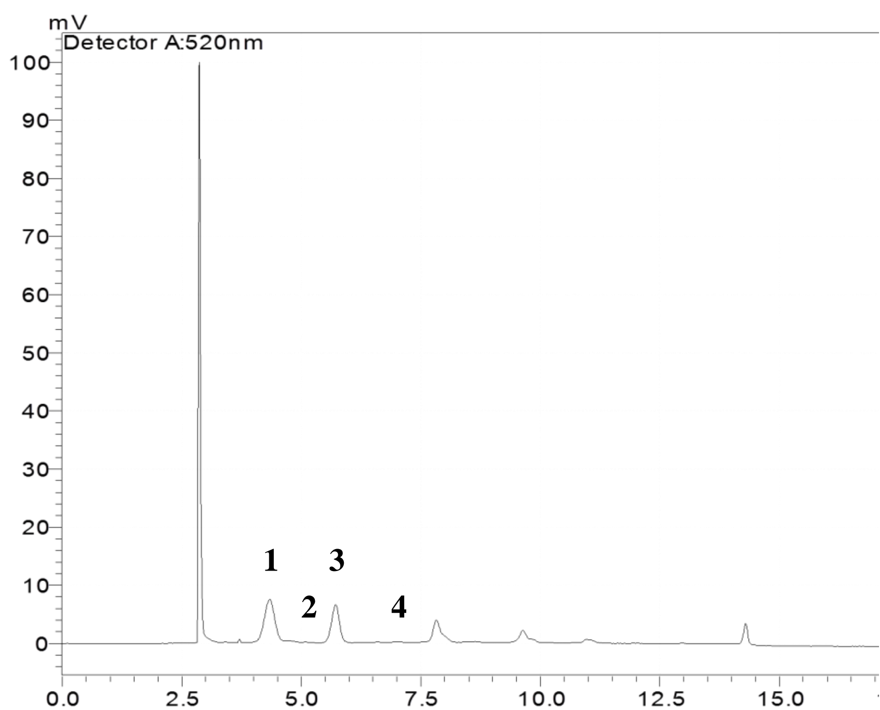


Figure 3-17. Typical chromatogram of compounds in acidified and non-acidified methanol extracts of dried *H. sabdariffa* for 10 min extraction time at solvent boiling point. Peak (1) delphinidin 3-sambubioside (t_R = 4.5min), peak (2) delphinidin 3-glucoside (t_R = 5.4 min), peak (3) cyanidin 3-sambubioside (t_R = 5.8 min) and peak (4) cyanidin 3-glucoside (t_R = 6.8 min)

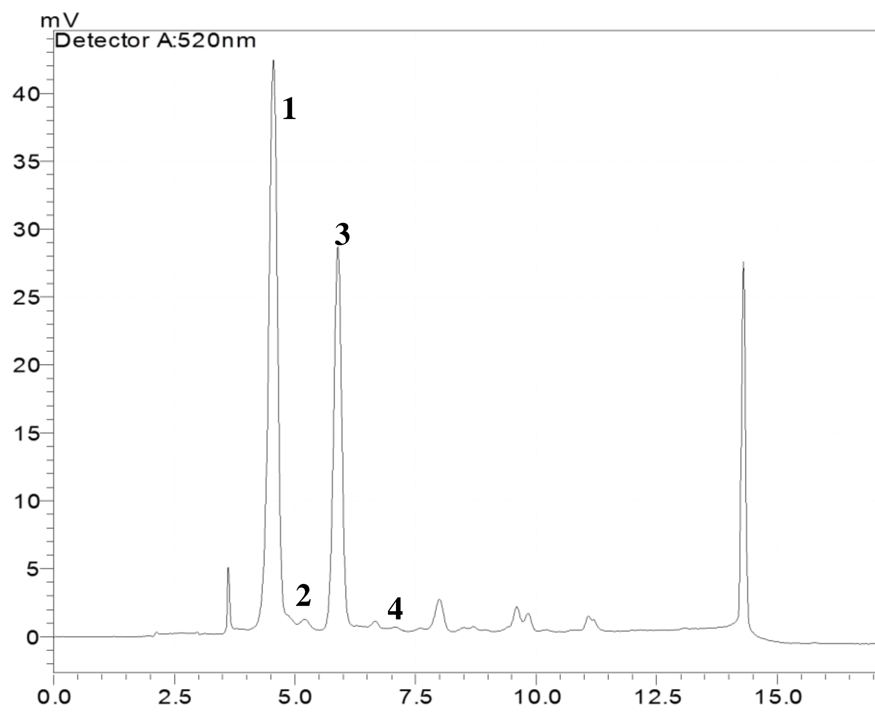


Figure 3-18. Typical chromatogram of compounds in acidified and non-acidified water extracts of dried *H. sabdariffa* for 10 min extraction time at solvent boiling point. (1) Delphinidin 3-sambubioside (t_R = 4.5min), (2) delphinidin 3-glucoside (t_R = 5.4 min), (3) cyanidin 3-sambubioside (t_R = 5.8 min) and (4) cyanidin 3-glucoside (t_R = 6.8 min)

Table 3-7 and 3-8 show the percentage recovery for delphinidin 3-sambubioside and cyanidin 3-sambubioside in acidified and non-acidified water and methanol extracts of *H. sabdariffa*. The highest percentage recovery for both major compounds delphinidin 3-sambubioside and cyanidin 3-sambubioside were achieved in water extracts with or without formic acid. The percentage recovery of anthocyanins in water extracts were between 75.5 % and 100 % , while, in methanol extracts the percentage recovery of anthocyanins were range from 59.5 % to 83.7 %. But most of the time 80 % of major anthocyanins were recovered in water extracts compared with 60 % up to 70 % of major anthocyanins were recovered in methanol extracts.

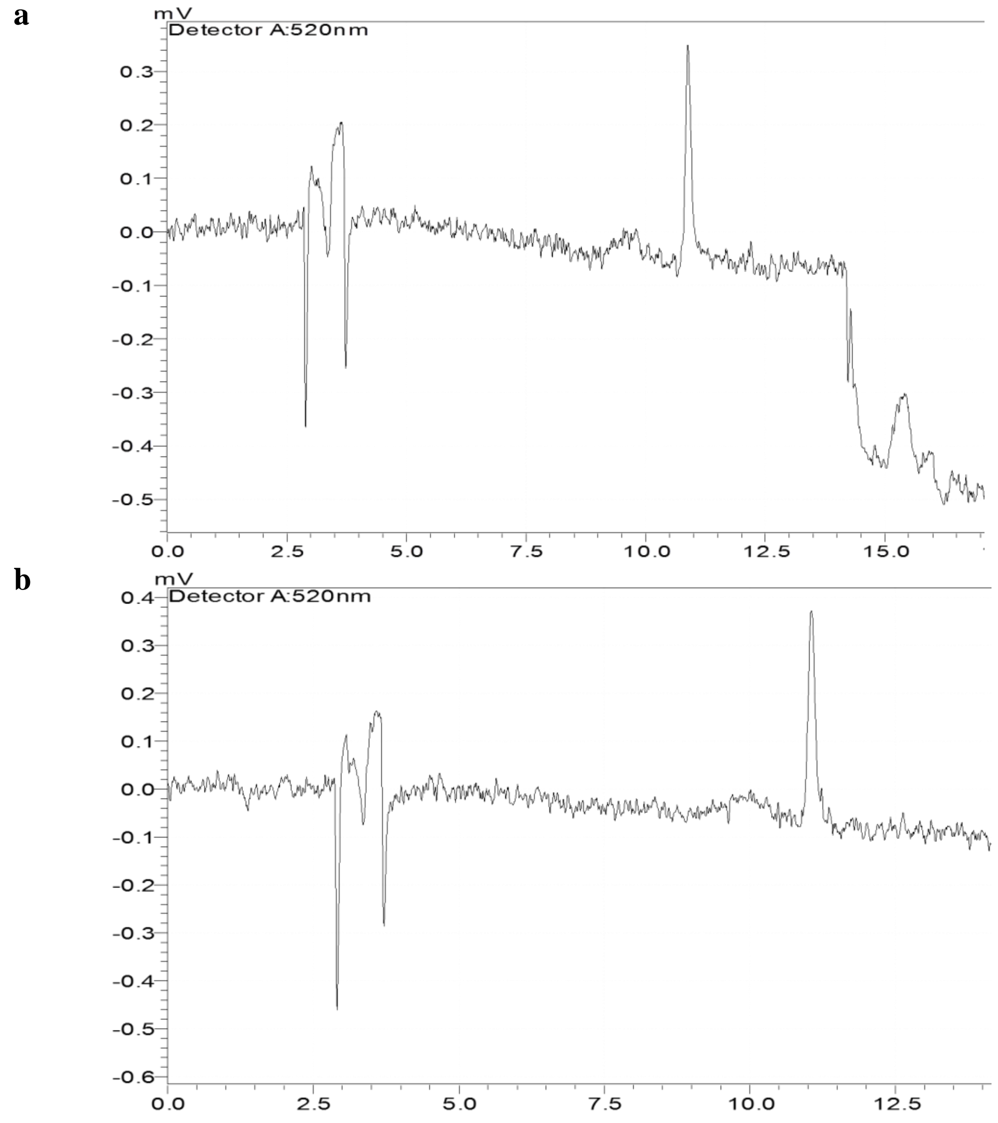


Figure 3-19. Example of chromatogram for ethyl acetate extract (a) and hexane extract (b) of dried *H. sabdariffa* for 10 min extraction time, at solvent boiling point. No anthocyanin standards were detected (n=3)

% of anthocyanins recovery at 3 min extraction time						
Extracts	**25 °C**		**50 °C**		**Solvent boiling point**	
	% del 3-sam [1]	**% Cy 3-sam [2]**	**% del 3-sam [1]**	**% Cy 3-sam [2]**	**% del 3-sam [1]**	**% Cy 3-sam [2]**
Water	81.7	83.6	78.5	88.3	83.9	100.2
Acidified water	83	83.8	84.9	90.5	83.9	96.8
Methanol	72	70.6	73.8	73.2	73.6	68.9
Acidified methanol	100	86.8	74.6	74.7	87	94.2
% of anthocyanins recovery at 5 min extraction time						
Extracts	**25 °C**		**50 °C**		**Solvent boiling point**	
	% del 3-sam [1]	**% cy 3-sam [2]**	**% del 3-sam [1]**	**% Cy 3-sam [2]**	**% del 3-sam [1]**	**% Cy 3-sam [2]**
Water	79.8	75.7	85.8	94.4	81.4	87.5
Acidified water	85.6	85.7	90.2	99	86.1	101.9
Methanol	64.6	62	72.9	70.3	68.7	70
Acidified methanol	64.9	65.8	68.7	68.2	73.6	81.8

Table 3-9. Percentage recovery of the major Anthocyanin compounds ([1] del 3-sam = delphinidin 3-sambubioside and [2] cy 3-sam = cyanidin 3-sambubioside) in acidified and non-acidified aqueous and methanol extracts of dried *H. sabdariffa* extracted at 3 min and 5 min using different temperature (25 °C, 50 °C and solvent boiling point) were analysed by reversed phase HPLC. Results are average of triplicates

Extracts	25 °C		50 °C		Solvent boiling point	
% of anthocyanins recovery at 10 min extraction time						
	% del 3-sam [1]	% cy 3-sam [2]	% del 3-sam [1]	% Cy 3-sam [2]	% del 3-sam [1]	% Cy 3-sam [2]
Water	82.6	78.6	80.8	87.5	85.9	125.5
Acidified water	84	85.7	82.1	78.6	84.1	126.9
Methanol	65.5	59.5	80	86	77.9	83.7
Acidified methanol	67.4	67.1	71.7	71.6	74.7	81.7

Table 3-10. Percentage recovery of the major Anthocyanin compounds ([1] del 3-sam = delphinidin 3-sambubioside and [2] cy 3-sam = cyanidin 3-sambubioside) in acidified and non-acidified aqueous and methanol extracts of dried *H. sabdariffa* extracted at 10 min using different temperature (25 °C, 50 °C and solvent boiling point) were analysed by reversed phase HPLC. Results are average of triplicates

3.6 Discussion

Despite significant interest in the bioactive properties of *H. sabdariffa* there has been no systematic study of the characterisation and efficiency of different extraction procedures.

The total phenol content and antioxidant capacity of extracts of *H. sabdariffa*, were determined using four solvents (water, methanol, ethyl acetate and hexane with and without formic acid) at different temperatures (25 °C, 50 °C and solvent boiling point) and different extraction times (3, 5 and 10 min). The total phenol content was determined by the Folin-assay and three different assays were used to determine the antioxidant capacity (DPPH, FRAP and TEAC). Total monomeric anthocyanins were determined and anthocyanin contents were identified and quantified by HPLC and LC-MS.

3.6.1 Time and temperature of extraction

The study by Ramirez-Rodrigues *et al*. (2011b) concluded that increasing extraction time led to a significant increase in total polyphenol content, antioxidant capacity and amounts of anthocyanins. The present study is in partial agreement with this, however, the antioxidant capacity assays show varying results. Tsai *et al*. (2002) found that antioxidant capacity as measured by the FRAP assay plateaued at extraction times of 3 and 5 min for extracts of *H. sabdariffa* petals. The present results also show the FRAP assay reaches its maximum at 3 min. According to the study done by Hsieh *et al.* (2008) which was evaluating the antioxidant capacity of *H sabdariffa* using different solvent extraction solutions (50% acetonitrile, 50% methanol, 50% ethanol, boiling water and water at room temperature). 10 min extraction time was enough to extract all important materials and bioactive

compounds from 3 g of *H. sabdariffa.* Prenesti *et al.* (2007) and Ramirez-Rodrigues *et al*. (2011b) also showed an increased yield of polyphenols at a higher temperature coupled with minimal losses of antioxidant compounds. That in agreement with current study which found significant difference between 25 °C and solvent boiling point in all antioxidant assays and total anthocyanin content except TEAC which the temperature had no effect on the activity.

Taking all assays into account, we suggest a 10 min extraction time for *H. sabdariffa* for optimal extraction of bioactive compounds, which provides a safety margin and certainty for all assays.

3.6.2 Total phenols assay

The results of the total phenols assay concluded that the amount of phenols varies in the extracts, probably due to the usage of different solvents in the extraction of *H. sabdariffa,* and also due to the antioxidant activity of the extracts. Mohd-Esa *et al*. (2010) stated this in their study, and explained that variations in extracted samples occurred because several samples may have had water soluble phenolic compounds and some did not. They also mention that solvents could change the polarity of these compounds; therefore, solvents with a high polarity have a higher yield of polyphenols in the extracts.

The minimum content of phenols was found in the ethyl acetate extract, after 3 minutes at 25 °C and 50 °C, might be due to the kinds of phenols in the ethyl acetate extraction degrading after 3 minutes. According to Wrolstad (2005) ethyl acetate solvent used in the purification steps of anthocyanins to remove another flavonoids and phenolic acids. Also,Jakobek and Seruga (2012) reported that quercetin, kaempferol, caffeic acid, *p*-coumaric acid and ellagic acid were detected at

wavelength (320 and 360 nm) in ethyl acetate fraction of red berry, blackberry, sour cherry, strawberry, chockeberry, elderberry and blueberry using HPLC. In general, the decrease in total phenols that emerged for the ethyl acetate and ethyl acetate/formic acid extracts compared with other solvents differed from Ramakrishna *et al.* (2008), who reported that ethyl acetate has approximately same amount of total phenol content as a methanol extract.

Another study by Aoshima *et al.* (2007) agreed that there was strong correlation between the % of DPPH and the phenol content, due to the occurrence of the anthocyanin pigments in the extracts, and they explain that *H. sabdariffa* decreased the hydrogen peroxide (H_2O_2) in several extracts due to the phenol content, which participated as auto-oxidised hydrogen donation and reduced the creation of H_2O_2.

Therefore, the results show that a 3 min extraction might not provide enough time to release the phenols from the dried hibiscus. In most cases, after three minutes, ethyl acetate extracts contained small amounts of phenols, and it also appears from the results that hexane has a lower quantity of phenols when extraction temperature increased above room temperature or if the time of extraction exceeded five minutes.

3.6.3 Correction of the interference of vitamin C

According to Singleton *et al.* (1999) the presence of ascorbic in samples could increase the amount of total phenol determined by producing blue colour before adding the sodium carbonate. Singleton *et al*. (1999) explained that the remaining amount of ascorbic acid in extracted grape juice oxidised to de-hydroascorbic acid and reduced the quinone form of polyphenol oxidase, therefore it is recommend to determine ascorbic acid and subtracted it from the amount of total phenols. Also, Perla *et al.* (2012) study found high level of ascorbic acid in the red and purple

potatoes. The current study found it was interesting to see whether vitamin C had an effect on *H. sabdariffa.* Therefore, the correction of the interference of vitamin C was done separately. The results of total phenol content after correction of ascorbic acid may vary compared with the original result done earlier due to variation in preparation of the reagents. Interference of vitamin C was not determined in ethyl acetate and hexane extracts due the low amount of total phenol and antioxidant activity compared with acidified and non-acidified aqueous and methanol extracts.

The present study was found significant differences between total phenol content before and after the calculation of vitamin C interference. However, after subtraction the ascorbic acid effect the results of total phenol content remained high therefore, the study suggest that ascorbic acid had no effect on total phenol content because with high temperature the phenol oxidase should be inhibited also the amount of vitamin C in dried *H. sabdariffa* was low. According to Prenesti *et al*. (2007) study they related the antioxidant activity of *H. sabdariffa* extract to the total phenol content and reported that the amount of vitamin C in the *H. sabdariffa* was insignificant between 100 and 140 mg of ascorbic acid /100g of dried material therefore, it could not be responsible for the high antioxidant activity of the extract. From Prenesti *et al.* (2007) it can be concluded that the high antioxidant activity of the extract of *H. sabdariffa* is not related to vitamin C even if it is interferes with total phenol determination.

3.6.4 Effect of solvent on the DPPH assay

Ramakrishna *et al*. (2008) measured the antioxidant capacity of ethyl acetate, acetone and methanol extracts of both the calyces and fruit of *H. sabdariffa* using the DPPH assay. The study found that the methanol extract gave the highest DPPH value in comparison with ethyl acetate and acetone. This is in agreement with the

findings from this study, as the methanol extract of *H. sabdariffa* had a higher percentage of DPPH scavenging activity (34.88% ± 3.94) than ethyl acetate extract (3.10% ± 0.94).

The high percentage of scavenging activity for the water and methanol with and without formic acid extracts could be explained by the positive correlation between the amount of total phenols and the antioxidant activity, as indicated in the results. According to Mohd-Esa *et al*. (2010) study which examined the fresh calyces, stem, seeds and leaves of *H. sabdariffa* extracts extracted by adding (1000 ml) of water or 80 % methanol to (1 g) of sample at room temperature for 2 hours , the DPPH amounts of extracts had different patterns in each extract; however, the DPPH value for the methanol extract was higher than for the water extract, and it was correlated to the amount of polyphenols, which may have contributed to high antioxidant activity, also to the strong relationship between total phenol content and DPPH (R^2 = 0.701).

The current study found that water extract had high DPPH percentage compared to methanol extract this might due to the use of dried *H. sabdariffa* and the increase of extraction temperature which help to released more phenols compounds compared with Mohd-Esa *et al*. (2010) study, in addition the present study found that high amounts of total phenol content or anthocyanins in specific due to strong correlation between DPPH assay and total anthocyanins could be the reason in water extract had high DPPH percentage.

From the results of the study, it appears that increasing temperature does not reduce antioxidant activity, possibly because high temperatures released the bioactive compounds in the *H. sabdariffa,* especially the polyphenols. The decline in DPPH value in the samples after 50 °C for the 3 minute extractions may be due to

increasing temperatures degrading the bioactive compounds in the hibiscus extracts. The increases in DPPH value in both water and methanol with 1% of formic acid or without the formic acid compared with the ethyl acetate and hexane extracts is in agreement with Ramakrishna *et al*. (2008), who indicated in their study that the methanol extract showed high antioxidant activity compared with the ethyl acetate and acetone extracts. However, our study disagreed with the percentage of DPPH, which was higher than our maximum obtained results by 49 % and 37 % for methanol and ethyl acetate extracts, respectively.

It was noticed in this experiment that there were variations in the DPPH data, perhaps because of the different types of solvents used for hibiscus extraction, and it could be assumed that different types of solvents extract different types of polyphenols. However, Ramakrishna *et al*. (2008) also mentioned that the methanol extract had higher antioxidant activity, and as a result, the variety of compounds extracted by methanol differed from the other compounds extracted by other types of solvents. Ramakrishna *et al*. (2008) also clarified that there were differences between extracts on amount of total phenols and/or the composition

3.6.5 Effect of solvent on the FRAP assay and TEAC assay

Antioxidant capacity of aqueous extract for *H. sabdariffa* extracted at boiling point using FRAP assay had a high value (Segura-Carretero *et al.*, 2008). The present study found that FRAP value increased with increasing extraction temperature which is in agreement with what Segura-Carretero *et al*. (2008) found.

Total phenol content is thought to be responsible for increasing the antioxidant capacity of in FRAP assay (Wong *et al*., 2006). On the other hand, Tsai *et al*. (2002) related 51 % of *H. sabdariffa* the FRAP antioxidant activity to the delphinidin 3-

sambubioside and cyanidin 3-sambubioside. Kruawan and Kangsadalampai (2006) also related high antioxidant activity of FRAP assay to the high amount of total phenol in the extracts.The present study observed that the TEAC values are lower compared with the rest of the antioxidant activity assays and this is in agreement with Yang *et al.* (2006) who reported low values of 127 edible plant species when determined by TEAC assay compared with other methods used.

3.6.6 Correlation between antioxidant assays and total phenol content.

Amin and Mukhrizah (2006) reported that antioxidant activity determined in plant extracts by one assay is correlated well with other assays. Statistical results from the present study showed that total phenol content had strong correlation with other assays TEAC, FRAP and DPPH. In addition, TEAC values had a positive correlation with both DPPH and with FRAP. Also, there was a significant correlation between FRAP and DPPH. However, DPPH, FRAP and TEAC assays do not give the same results due to their different mechanisms' (Fukumoto and Mazza, 2000; Pellegrini *et al.*, 2003). Pearson correlation coefficient indicates there is a significant correlation between total phenol content and antioxidant assay with high positive correlation.

The present study found that strong correlation between total phenols and total anthocyanins and between total anthocyanins and antioxidant activity which was indicating that increasing the amount of extractable anthocyanins in extract increased the total phenols and the antioxidant activity as well. The hydroxyl group in the B ring of anthocyanins usually attached to the free radical and donate the hydrogen to stabilise the radicals.

3.6.7 Effect of solvent on the total monomeric anthocyanin content

In the current study, the high amount of total monomeric anthocyanin content was achieved by using boiling point temperature for 10 min and solid to solvent ratio 1:10. This is in agreement with Cisse *et al*. (2012a) study which examined the effect of particle size and the ratio between solid-solvent extraction, time and temperature on the efficiency of *H. sabdariffa* extraction. The study revealed that anthocyanins could be extracted within 10 min or less. Reducing the particles size and increasing solvent ratio by 1:10 or 1:15, allowed the solvent to reach the centre of the anthocyanin molecules very fast and reached equilibrium quickly due to increasing the speed to exchange the mass between the solid and the solvent which increased the amount of extractable anthocyanins. High temperature is required to achieve good extraction of anthocyanins. In addition, according to (Cisse *et al*., 2012b) determined the effect of using cold water at 30 °C or hot water at 100 °C to extract *H. sabdariffa* by using a solid: solvent ratio of 1:15. The result showed that using hot water for extraction increased the total anthocyanins by 30 % but the rate of degradation increased during storage for 6 month when hot water extraction was used followed by pasteurisation compared with the juice extracted by hot water without pasteurisation. Tsai *et al*. (2002) reported that anthocyanins may be the main compounds responsible to the antioxidant activity in the *H. sabdariffa* extract and related 85% of total anthocyanins content to delphinidin 3-sambubioside and cyanidin 3-sambubioside.

The present study, found strong correlation between TEAC assay and the total anthocyanin content (R^2= 0.781) and weak correlation between FRAP assay and anthocyanins content but (R^2= 0.360). Bahorun *et al.* (2003) also found positive correlation between FRAP and TEAC antioxidant results with total anthocyanin

contents and Tsai *et al*. (2002) found strong correlation (R^2= 0.837) between FRAP assay and anthocyanins content.

3.6.8 Identification of anthocyanins compounds by LC-MS

Four peaks were identified by LC-MS in acidified and non-acidified aqueous and methanol extracts, two peaks were major anthocyanin compounds in both extracts which are delphinidin 3-sambubioside and cyanidin 3-sambubioside and two peaks were minor anthocyanin compounds, delphinidin 3-glucoside and cyanidin 3-glucoside.

The results of the mass spectrum were in agreement with Giusti *et al.* (1999); Rodriguez-Medina *et al.* (2009); Juliani *et al*. (2009); Amor and Allaf (2009) and Ramirez-Rodrigues *et al*. (2011a) studies, their identified two anthocyanin compounds delphinidin 3-sambubioside and cyanidin 3-sambubioside by the mass of the molecular ion (597 and 581 respectively) in the positive mode in aqueous extracts of dried *H. sabdariffa*. Also, Mourtzinos *et al.* (2008) identified the anthocyanin compounds delphinidin 3-sambubioside and cyanidin 3-sambubioside in an aqueous extract of dried *H. sabdariffa* at 60 °C and related 64 % of the total monomeric anthocyanins to delphinidin 3-sambubioside and 25 % corresponding to cyanidin 3-sambubioside.

In addition, recent study used 3 different methods for extracting *H. sabdariffa*. Two methods used acidified methanol with HCl (99:1, v/v) for extraction one gram of *H. sabdariffa,* one sample extracted at room temperature for 4 hours and the other used sonication for 30 min, while the third method used acidified water with 15% acetic acid as solvent for extraction. In each of the three methods, delphinidin 3-sambubioside and cyanidin 3-sambubioside were identified. However, delphinidin

3-glucoside was only found and identified in acidified water extracts (Segura-Carretero *et al*., 2008). The current study identified the major compounds delphinidin 3-sambubioside and cyanidin 3-sambubioside in acidified methanol and water extracts in Segura-Carretero *et al*. (2008) only delphinidin 3-glucoside was detected and identified water extract but the current study was identified delphinidin 3-glucoside in both in acidified methanol and water extracts.

Cyanidin 3-glucoside was identified by the mass of fragment ion 287 m/z from the parent ion 449 m/z in brain extracts of European birds fed with blackberry for two weeks (Mullen *et al.*, 2010). This study identified the same mass of fragment ion from the parent ion. Nayak *et al.* (2010) found the ion peak at 581 m/z and at 449 m/z which were identified as cyanidin 3-sambubioside and cyanidin 3-glucoside respectively, in fresh *Kokum* fruit extract extracted by acidified water with 0.1% HCl.

According to the study done by Hsieh *et al*. (2008) which was evaluating the antioxidant capacity of *H. sabdariffa* using different solvent extraction solutions (50% acetonitrile, 50% methanol, 50% ethanol, boiling water and water at room temperature). The study revealed that two superior delphinidin-3-sambubioside and cyanidin-3-sambubioside and one minor delphinidin-3-glucoside compounds were identified by LC-MS in 3g of *H. sabdariffa* extracted for 10 min.

3.6.9 Determination of anthocyanins in extracts by HPLC.

Delphinidin and cyanidin 3-sambubioside were the major compounds present and delphinidin and cyanidin 3-glucoside were minor compounds present in dried *H. sabdariffa* , which is in agreement with Segura-Carretero *et al*. (2008) and Ali *et al.* (2005) reviewed several studies isolating bioactive compounds from several parts of *H. sabdariffa* using different types of Hibiscus species, and also found the most predominant compounds from these extracts were delphinidin and cyanidin 3-sambubioside and delphinidin and cyanidin 3-glucoside. Amor and Allaf (2009) determined the anthocyanins in *H. sabdariffa* water extract and found delpinidin 3-sambubioside (6.2 mg/g) and cyanidin 3-sambubioside (2.4 mg/g). The present study is in agreement with Amor and Allaf (2009) as the concentration of delpinidin 3-sambubioside (5.1 ± 0.49 mg/g) was higher than cyanidin 3-sambubioside (3.8 ± 1.21 mg/g). The current study found moderate correlation between total monomeric anthocyanins and anthocyanins measured by HPLC (r^2= 0.622). The result is in agreement with Juliani et al. (2009) who also found a strong relationship (R^2= 0.820). Christian *et al.* (2006) reported the presence of delpinidin 3-sambubioside and cyanidin 3-sambubioside in the acidified methanol extract (0.1 % HCl) of early bearing *H. sabdariffa* samples. According to the study done by (Hsieh et al., 2008) which was evaluating the antioxidant capacity of *H. sabdariffa* using different solvent extraction solutions (50 % acetonitrile, 50 % methanol, 50 ethanol, boiling water and water at room temperature), the study revealed that 61 % of antioxidant of *Hibiscus sabdariffa* due to anthocyanins (51 % from delphinidin compounds and 10% from cyanidin compounds). Salazar-Gonzalez *et al.* (2012) determined the anthocyanins of four different types of solvent extraction of *H. sabdariffa* water: ethanol (50:50 and 70:30 v: v), water, acidified ethanol with HCl (1.5 N) and 96%

ethanol by using HPLC. The study detected three anthocyanin compounds which were delphinidin 3- sambubioside, delphinidin 3- glucoside and cyanidin 3- sambubioside in all extracts, but water: ethanol (50:50 v:v) extracts was the best for extracting the anthocyanin compounds then water: ethanol (70:30 v:v) extracts. In contrast, water extract alone or ethanol with and without acid had the lowest concentration of anthocyanins. Also, ethanol: water mixture (50:50 v: v) had approximately the same total monomeric anthocyanins (2.09 mg/g) as the total concentration of the three anthocyanin compounds detected by HPLC (2.2 mg/g).

3.6.10 Comparison of results to other researches

Due to inconsistencies in methodology used in previous studies measuring the antioxidant capacity of *H. sabdariffa,* it is difficult to compare the results of the present study with other work as each study has used a different method for extraction. Wrolstad *et al.* (2002) attributed the variation in the results of different studies to differences in extraction methods, temperatures or other factors, which could affect the enzyme activity and oxidation. Kruawan and Kangsadalampai (2006) reported that extraction with water at boiling point could give different results for antioxidant activity because of minimizing enzyme activity and oxidation. Additionally, when studying *H. sabdariffa*, different cultivars, and different locations of cultivation, different climatic conditions and condition of storage will all contribute significantly to the variation in results. Light can also influence the amount of antioxidant activity and the total anthocyanin content (Lajolo *et al.*, 2005).

3.6.11 Optimal extraction conditions

Using the optimal extraction time of 10 min and the optimal extraction temperature of solvent boiling point, it can been seen that water either with or without formic

acid is the best solvent for extraction to maximise the total polyphenols, total anthocyanins and antioxidants extracted from *H. sabdariffa*. Acidified and non-acidified water extracts gave the highest values in all antioxidant capacity assays, which is in concurrence with Fukumoto and Mazza (2000). However, there was no significant difference between acidified and non-acidified solvents. These findings are in disagreement with Nollet (2000) who reported that adding acid helps anthocyanins to form the flavylium cation and eliminates degradation, which increases the amount of extracted compounds. Nollet (2000) and Ramakrishna *et al*. (2008) related the strong antioxidant capacity of *H. sabdariffa* extracts to the total phenol content. They explained the variance between the antioxidant capacities of extracts as due to the hydrogen-donating ability of the compounds in the extract as different solvents extracted different types of phenol compounds. Fukumoto and Mazza (2000) reported that antioxidant activity of extracts increased when compounds with 2 to 3 hydroxyl groups in the B ring, increased, since the level of antioxidant capacity is dependent on the number of hydroxyl groups in the compound. Any loss of these groups will mean a reduction in the antioxidant capacity. The HPLC results in this study show the presence of delphinidin 3-glucoside and delphindin 3-sambubioside, which have 3 hydroxyl groups in the B ring, and cyanidin 3-glucoside and cyanidin 3-sambubioside, which both have 2 hydroxyl groups in the B ring in acidified and non-acidified water and methanol extracts. We did not detect any of these compounds in acidified and non-acidified ethyl acetate or hexane extracts which would explain the low antioxidant values obtained from these extracts. The present report is the only study to have compared different solvents when determining the antioxidant capacity and anthocyanins present in *H. sabdariffa*. We used different solvents to identify which solvent would give the maximum antioxidant activity. However, it should be noted that different

solvents extract different compounds, which is why antioxidant values vary so using one type of solvent for extraction can underestimate total antioxidant activity (Pellegrini *et al*., 2003) and no single solvent can optimally extract all antioxidants (Yang *et al*., 2006). Pellegrini *et* al. (2003) suggested that solvent extracts with low antioxidant activity may contain other types of compounds such as those with bioactive properties and therefore, they recommend using at least two different solvents for extraction. A study by Orhan *et al.* (2009) found that some solvents were unable to extract flavonoids from edible plants and exhibited low antioxidant activity. In the present study, ethyl acetate and hexane extracts had both a low total phenol content and antioxidant capacity in comparison with water and methanol extracts and did not extract any anthocyanins. However, the lower values could also be due to the polarity of these solvents compared with water and methanol. Some of the assays used are hydrophilic, so the ethyl acetate and hexane when mixed with high polarity solvents will be immiscible, which would affect the mechanism of the assay. This is in agreement with findings from Amin and Mukhrizah (2006). Mohd-Esa *et al*. (2010) also explains that if the polarity of the solvent increased it would increase the extractable compounds and extraction yields. This theory justifies the results of this study as the polarity of solvents used in this study in descending order were water>methanol>ethyl acetate>hexane. HPLC results found that *H. sabdariffa* is an important source of anthocyanins, which may play an important role in the prevention of several diseases. The results of the antioxidant capacity *in vitro* cannot be related to the in *vivo* results, due to insufficient information about the bioavailability, absorption and metabolism of anthocyanins. Therefore, further study must be done *in vivo.*

Chapter 4 A comparison between a aqueous extract of dried *H. sabdariffa* and selected commercially available products containing *H. sabdariffa*

Summary

- The effect of decoction method for extraction pure and two types of herbal teas (pomegranate tea and rose tea) compared with the ordinary infusion method on total phenol content, antioxidant activity and anthocyanin content.
- Decoction method was found to be optimal for extraction; the herbal teas compared with infusion method.
- Herbal teas extracted by decoction method exhibited higher total phenol content, total monomeric anthocyanins and antioxidant activity compared with infusion method.
- Strong correlation was found between total phenols, FRAP, TEAC and DPPH.
- In pomegranate tea both infusion and decoction had highest amount of delphinidin 3-sambubioside and cyanidin 3-sambubioside compared with pure aqueous extract of *Hibiscus sabdariffa*.
- Concentration of delphinidin 3- sambubioside in all samples was larger than cyanidin 3-sambubioside except for the diffusion method for pomegranate tea.

4.1 Introduction

Fruit and herbal tea infusions are rich sources of polyphenol antioxidants and have high antioxidant properties (Belscak *et al*., 2011). In addition, the bioactive compounds may vary or differ from one herbal tea to another due to the type of plant, the different extraction methods and different measurements (Komes *et al*., 2011). Each country has her traditional way to consume fruit and herbal tea; tea usually made by soaking the dried tea leaves in hot water (Komes *et al*., 2011). It can be easily prepared, and is preferable among another types of traditional tea such as green tea, oolong tea and black tea (Belscak *et al*., 2011).

Consumption of herbal and fruit teas around the world has increased dramatically in the last few years due to the health effect of those teas on prevention of diseases (Belscak *et al*., 2011). Research has found that consumption of tea ranks second after water consumption, which shows the increase in the consumption of tea around the world (Piljac-Zegarac *et al*., 2010).

Currently, most of Arab countries such as Egypt use plants to cure diseases (AbouZid and Mohamed, 2011). Twenty three percent (23 %) of Egypt's population have been using plants as alternative medicine. The most important plants and spices used by Egyptians are used as medication to treat diseases, such as cardiovascular disease. *H. sabdariffa* is also used to treat this type of disease and specifically hypertension in comparison with other type of plants (AbouZid and Mohamed, 2011).

H. sabdariffa extract and fruit tea infusion have high portion of pigments specially anthocyanins. Bioactive compound content of wine, fruit juice and coffee were already known and have been studied extensively, in particular flavonoids are the

most widely studied in classic hot beverages' such as oolong, green, black and white teas. These classic teas contain high amount of bioactive compounds in parallel with a high antioxidant activity, which help to reduce free radicals. Consuming tea has been linked with lower the risks of cardiovascular disease, cancer, arthritis, viral and bacterial infections and skin damage due to the flavonoid effect on UV rays, therefore, fruit tea or herbal tea should be studied further (Piljac-Zegarac *et al*., 2010).

There are limited number of articles and data that investigate the phenol compounds and antioxidant activity in herbal and fruit tea infusions or beverages. According to Belscak *et al*. (2011) most studies determined the phenol content of green, black and white teas, with only a slight focus on bioactive compounds of fruit and herbal tea. There are some studies which monitor the stability of anthocyanins, bioactive compounds and antioxidant capacity, especially on *H. sabdariffa*, but few studies done in the area of commercial products available in the market, which contain *H. sabdariffa* as the main ingredient.

This study found that there is no pure *H. sabdariffa* tea in UK market. It is usually mixed with other type of fruits or herbs but *H. sabdariffa* is usually the main ingredient in these fruit or herbal teas. Table 4-1 and Figure 4-1 show pictures and ingredients in two types of herbal tea used in this study. Therefore, the antioxidant activity will vary in each product depending on the percentage of *H. sabdariffa* in the fruit or herbal teas and this could affect the power of *H. sabdariffa* to reduce blood pressure or cardiovascular disease and other types of chronic diseases.

Table 4-1. Ingredients of two herbal teas products (Pomegranate tea and Rose tea) used in this study

Name of the herbal tea	Content
Pomegranate tea	Hibiscus, Apple pomace, Blackberry, Natural pomegranate flavouring and juice 4.5%, Natural apple flavouring and juice 3.5%, Malic acid, Liquorice root, Natural vanilla flavouring, Orange peel
Rose tea	Hibiscus, Chamomile flower, Elderflower, Rose petal 5%, Cinnamon, Lavender flowers, Yarrow flower, Ginger, Black pepper, Fennel, Turmeric root, Sunflower petals, Mullein, Alfalfa, Buckhorn, Cardamom, Cloves, Dried kmobucha drink

4.2 Aim of the study

The aim of this study is to determine the antioxidant activity, total phenol content and total anthocyanins, using the optimal extraction conditions obtained in chapter 3, for pure water extracts of dried *H. sabdariffa* and two herbal teas compared with using the infusion method instructions on the tea pack.

A

B

Figure 4-1. Pictures of two herbal teas products (A) Pomegranate tea and (B) Rose tea used in this study

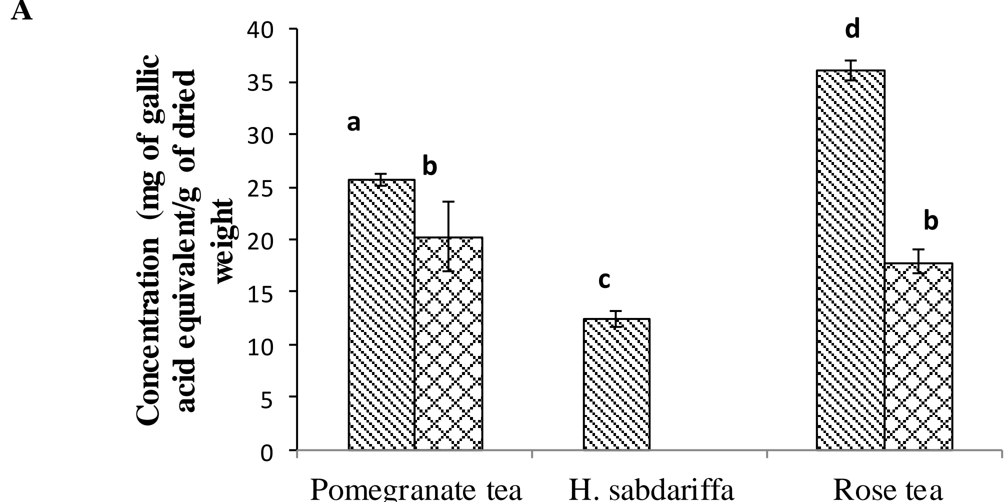
4.3 Material and method

The decoction method (10 min, boiling point) and the infusion method (as stated on the tea packet) used in the extraction herbal teas and pure *H. sabdariffa* were fully described in chapter 2 (section 2.6.1.2). The methods used to determine total phenols, total anthocyanins and antioxidant activity are described in section 2.6.2 to section 2.6.8.

4.4 Results

Figure 2 shows the total anthocyanins, phenolic content and antioxidant capacity of dried *H. sabdariffa* products commercially available in the market. The study found that applying the optimal condition (decoction for 10 min at boiling point temperature) increased the antioxidant capacity. The decoction samples of herbal tea had a larger antioxidant value compared with the infusion method.

A



B

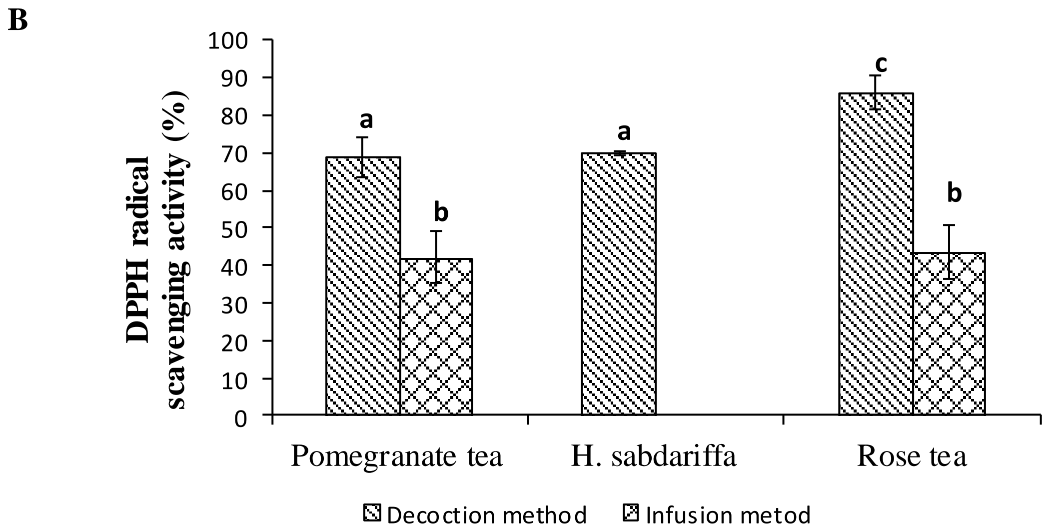


Figure 4-2. Total phenol content and antioxidant capacity assays of infusion and decoction method of different *H. sabdariffa* products commercially available in the market (A) Total phenol content expressed as gallic acid equivalents (mg/g of dried weight), (B) % DPPH radical scavenging activity. Values with different letters in each chart are significantly different (Tukey's test, $p<0.05$). Results are mean of triplicates (± SD)

4.4.1 Total phenol content

Figure 4-2 (A) shows the total phenol content of *H. sabdariffa* water extract extracted by using the decoction method and herbal teas extracted by both infusion and decoction methods. Rose tea extracted by the decoction method for 10 min has the maximum amount of total phenols (36.02± 0.92 mg of gallic acid equivalents /g of dried weight) compared with pomegranate tea and *H. sabdariffa* water extract extracted by the same method. In the rose tea the optimal extraction condition (decoction method) increased the amount of total phenols by 2 fold in comparison with the infusion method. No significant difference was found between pomegranate tea and rose tea extracted by infusion method for 5 min (P = 0.377, P>0.05).

4.4.2 Total phenol content after correction for of vitamin C interference

		Total phenol content (mg/g)		
Extracts	**Method of extraction**	**before**	**after**	**differences**
Water extract	Decoction[1]	25.7	20.1	5.6
Pomegranate tea	Infusion[2]	28.3	22.0	6.3
Rose tea	Infusion[2]	28.6	23.0	5.5
Pomegranate tea	Decoction[1]	30.3	23.3	7
Rose tea	Decoction[1]	40.9	32.8	8.2

Table 4-2. Total phenol content (mg/g gallic acid equivalent) before and after correction of vitamin C interference, for a water extract of *H. sabdariffa* and herbal teas extracted either by decoction method for 10 min[1] or by infusion method for 5 min[2] (n= 3).

Table 4-2 shows the amount of total phenols (mg/g gallic acid equivalent) for water extract of *H. sabdariffa* and herbal teas extracted either by infusion method for 5 min or by decoction method for 10 min before and after the correction of vitamin C

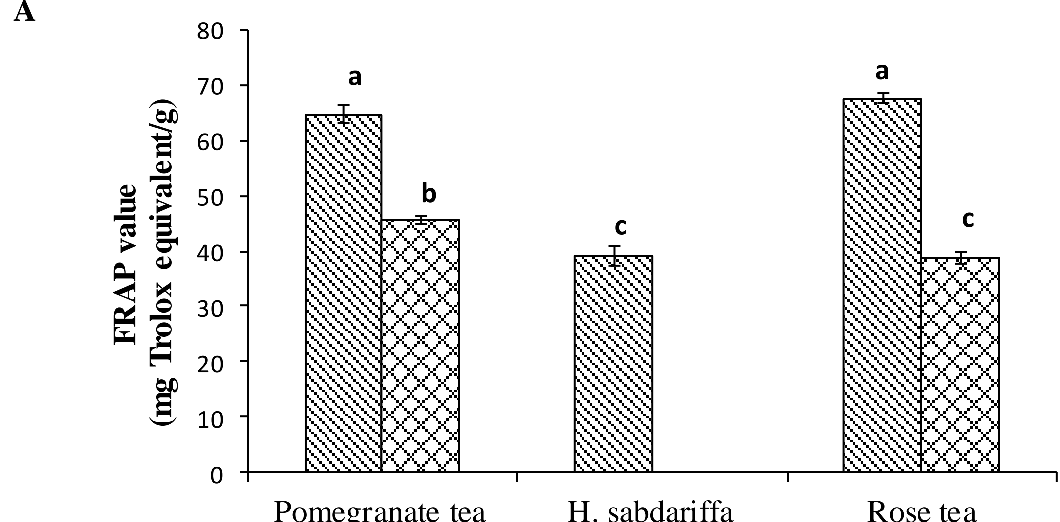
interference. It is clear from the table that interference of vitamin C increased with increasing the temperature. However, the amount of total phenol content still remained high even after the correction of ascorbic acid interference. Samples were not normally distributed due to the small number of samples. Therefore, statistical analysis was done using Wilcoxon non- parametric test to see if there were significant differences before and after the correction of vitamin C. The analysis found that there were significant differences between the amount of total phenols before and after the correction of vitamin C ($P = 0.043$, $P<0.05$).

4.4.3 DPPH assay

Figure 4-2 (B) illustrates the percentage of radical scavenging activity using the DPPH assay for *H. sabdariffa* water extract and herbal teas. Rose tea extracted at boiling point for 10 min had the highest percentage of radical scavenging activity of DPPH at 86.12 % ± 4.48, whereas the minimum percentage of DPPH was found in pomegranate tea by the infusion method 41.99% ± 6.91 and that maximum percentage of DPPH was in decoction rose tea for 10 minutes 86.12% ± 4.48 followed by water *H. sabdariffa* extract 69.88 ± 0.66 which was surprising result.

The ANOVA shows no significant difference between pomegranate tea and *H. sabdariffa* water extracted by the decoction method ($P = 0.950$, $P>0.05$). An insignificant difference was found between pomegranate tea and rose tea extracted by the infusion method for 5 min ($P = 0.944$, $P>0.05$). In rose tea the optimal extraction condition (decoction method) increased the amount of DPPH by 2 folds in comparison with the infusion method.

A



B

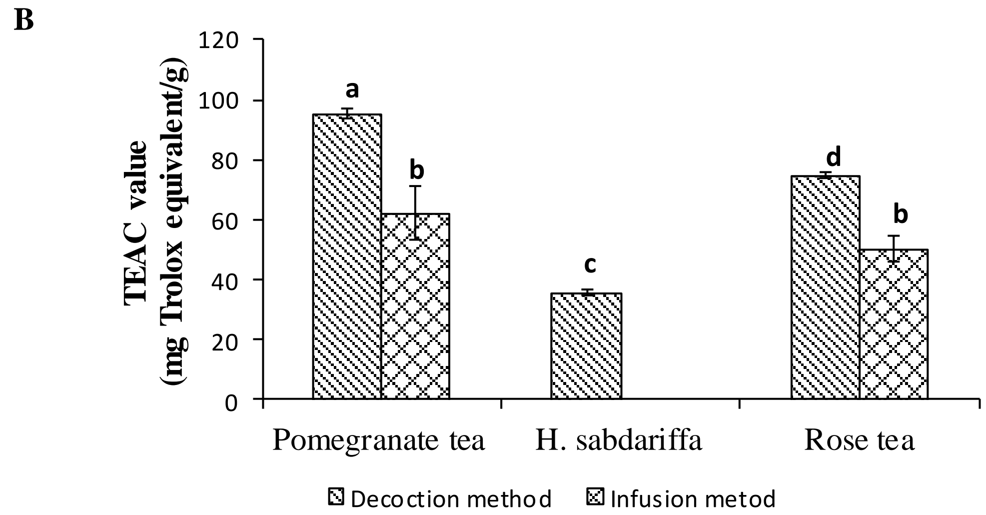


Figure 4-3. Antioxidant capacity assays of infusion and decoction and infusion method of different *H. sabdariffa* products commercially available in the market (A) FRAP antioxidant capacity as Trolox equivalents (mg/g of dried weight) and (B) TEAC antioxidant capacity as Trolox equivalents (mg/g of dried weight).Values with different letters in each chart are significantly different (Tukey's test, p<0.05). Results are mean of triplicates (± SD)

4.4.4 FRAP assay

From figure 4-3 (A) it is clear that rose tea and pomegranate tea extracted at boiling point for 10 min have the highest values. An insignificant difference ($P = 0.130$, $P > 0.05$) was found between rose tea and pomegranate tea extracted by decoction

method. In the rose tea the optimal extraction condition (decoction method) increased the amount of FRAP by 1.8 fold in comparison with the infusion method.

An insignificant differences (P=0.917, P >0.05) was found for FRAP between *H. sabdariffa* water extract and rose tea extracted by infusion method. In addition, with the infusion method, pomegranate tea has a higher value of FRAP than rose tea (45.70 ± 0.77 mg; 38.73 ± 1.08 mg of Trolox equivalent / g of dried weight respectively).

4.4.5 TEAC assay

From figure 4-3 (B) it is appears that pomegranate tea extracted by decoction method at boiling point for 10 min has the highest TEAC value of 95.34 ± 0.8 mg of Trolox equivalents / g of dried weight, While, water *H. sabdariffa* extract has the lowest TEAC value of 35.86 ± 1.11 mg of Trolox equivalent / g of dried weight. The TEAC value for of *H. sabdariffa* water extract extracted by decoction method is less by 2.7 fold than pomegranate tea extracted by same method.

Applying the optimal extraction condition (decoction method) increases TEAC value in the pomegranate tea by 1.5 fold in comparison with the infusion method. There was no significant difference found between rose tea and pomegranate tea extracted by the infusion method (P=0.098, P >0.05)

4.4.6 Total monomeric anthocyanin content

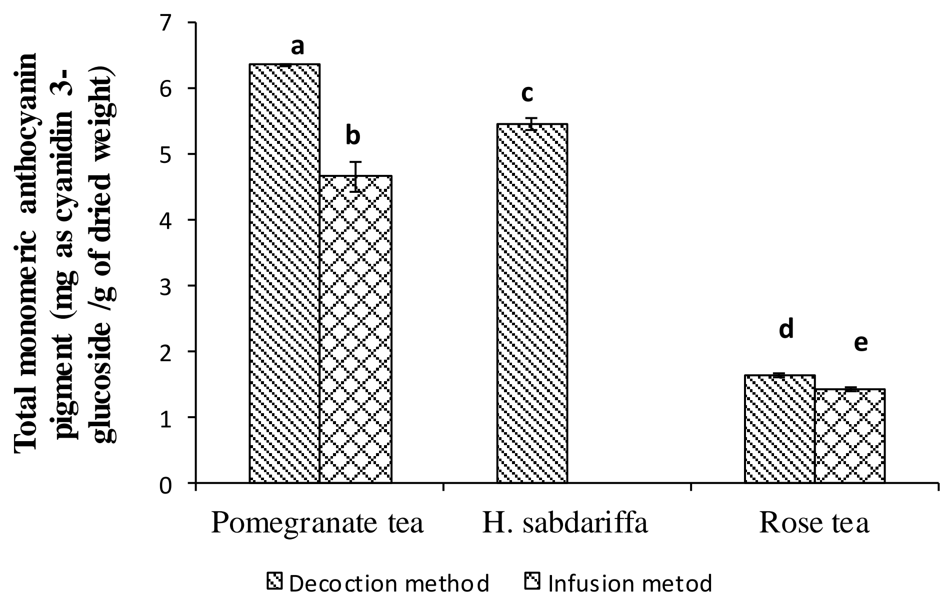


Figure 4-4. Total monomeric anthocyanin content expressed as cyanidin 3-glucoside equivalents (mg/g of dried weight) for 5 min infusion and 10 min decoction for herbal teas and *H. sabdariffa* water extract. Values with different letters are significantly different (Tukey's test, p< 0.05). Results are triplicates.± SD

Figure 4-4 shows that pomegranate tea extracted by decoction method at boiling point for 10 min has the highest amount of total anthocyanins (6.34 ± 0.02 mg of cyanidin 3-glucoside/g of dried weight), while, rose tea extracted by the same method had a much lower amount of anthocyanins (1.63 ± 0.04 mg/g of cyanidin 3-glucoside/g of dried weight). The anthocyanins concentration in the water extract of *H. sabdariffa* is less than the pomegranate tea by 1.2 fold.

Applying the optimal extraction condition (decoction method) increases the amount of total anthocyanins in the pomegranate tea by 1.8 fold in comparison with the infusion method. A significant difference between total anthocyanins of decoction and infusion of rose tea was found (p<0.001).

4.4.7 Correlation between total phenol content, total monomeric anthocyanin and antioxidant assays

Table 4-3 illustrates the linear correlation between total anthocyanins, total phenol content and antioxidant capacity of different assays. Strong correlations were found between total phenols and the FRAP assay ($r^2 = 0.898$ $p > 0.01$), total phenols and the TEAC assay ($r^2 = 0.698$ $p > 0.01$) and DPPH. No relationship was found between total anthocyanin content and total phenol content or antioxidant assays.

Assays	Total phenols	DPPH	FRAP	TEAC	Total anthocyanins
Total phenols	1	0.533*	0.898**	0.687**	-0.320
DPPH	0.533*	1	0.654**	0.308	0.169
FRAP	0.898**	0.654**	1	0.860**	0.091
TEAC	0.687**	0.308	0.860**	1	0.206
Total anthocyanins	-0.320	0.169	0.091	0.206	1

Table 4-3. Linear correlation coefficients (r^2) among total anthocyanins, total phenols and 3 different antioxidant assays of dried *H. sabdariffa* products were commercially available in the market) for 5 min infusion and 10 min decoction. ** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed)

4.4.8 Identification and quantification of anthocyanins by HPLC

Extracts	del 3-sam [3] mg/g	% recovery del 3-sam [3]	cy 3-sam [4] mg/g	%recovery cy 3-sam [4]
H. sabdariffa water extract [1]	5.1 ± 0.5 [a]	85.3	3.8 ± 1.2 [a]	89.3
Pomegranate tea [2]	7.5 ± 0.1[c]	82.8	9 ± 0.2 [d]	79.3
Pomegranate tea [1]	6 ± 0.6 [a]	85.6	3.5 ± 0.3 [a]	88.8
Rose tea [2]	1.8 ± 0.5 [e]	72.3	0.93 ± 0.2 [h]	99.8
Rose tea [1]	2.3 ± 1.7 [g]	75.9	1.2 ± 1.0 [a]	83.5

Table 4-4. Anthocyanin concentration expressed as mg/g of dried weight and percentage recovery of [3] delphinidin 3-sambubioside and [4] cyanidin 3-sambubioside in different herbal teas products commercially available in the market and *H. sabdariffa* water extract were analysed by reversed phase HPLC. [1]decoction method of extraction for 10 min, [2] infusion method of extraction for 5 min. Values with different letters in a column are significantly different (Tukey's test, $p < 0.05$). Results are mean of triplicates.± SD

Table 4-4 shows delphinidin 3-sambubioside and cyanidin 3-sambubioside concentration and percentage of recovery of these compounds in a *H. sabdariffa* water extract and herbal teas extracts, extracted either by decoction or infusion methods, which were identified and quantified using reverse phase HPLC. Pomegranate tea has the highest amount of delphinidin 3-sambubioside and cyaniding 3-sambubioside when extracted by the infusion method. In all *H. sabdariffa* products delphinidin 3-sambubioside has the highest concentration compared with cyanidin 3-sambubioside.

The percentage of recovery for delphinidin 3-sambubioside varied from 82.8 - 85.6 % in pomegranate tea and water extract, while lower recovery was seen in rose tea.

Cyanidin 3-sambubioside has the highest percentage of recovery in the infusion extraction method (99.8 %) compare to the other samples recovery range between 79 to 89.3%. Overall cyanidin 3-sambubioside had a better recovery compare with delphinidin 3-sambubioside.

4.5 Discussion

The herbal teas used in this study, are traditionally consumed as an infusion in the UK. The present study, determined the total phenol content, total anthocyanin content and the antioxidant capacity of herbal tea compare to the *H. sabdariffa* water extract using both the decoction and the infusion methods for extraction in order to choose an optimal method for extraction of the herbal teas and to compare the activity of *H. sabdariffa* in pure and mixed herbal tea extracts. In general, samples extracted by the decoction method had the highest amount of total phenol content, total anthocyanin content and antioxidant activity.

4.5.1 Total phenol content

The result of the total phenol content in the current study of pure water extract of dried *H. sabdariffa* extracted by decoction method (10 min) is greater than what was determined in a study by Prenesti *et al*. (2007) which looked at decoction extract of *H. sabdariffa* (0.1 g with 5 ml water) for 3 min (total phenol content 19.6 mg/g of gallic acid equivalents/g of dried *H. sabdariffa*). The increase in the amount of total phenol content in the present study may be due to the effect of increasing the time of extraction to 10 min in decoction methods which allowed more anthocyanins and other phenols to release from the Vacuole. Also, in Prenesti *et al.* (2007) study the volume of water used for extraction was lower than this study by the half which is not enough to extract all the phenols. On the other hand, the samples were analysed

with three days of preparation and that could affect the stability and increase the degradation of phenols, compared with the present study which made the extracts freshly every time and keep them in dark place even during the experiment. Finally, the *H. sabdariffa* obtained from Egypt which also my responsible for lowering the amount of total phenol content.

Total phenol content was measured in 12 selected pasteurized and sterilized healthy beverages commonly used in Thailand (including a *H. sabdariffa* beverage). The results indicated that the *H. sabdariffa* drink had the highest value of antioxidant capacity and total phenols among other beverages (Abdullakasim *et al.*, 2007).

Furthermore, total phenol content was also determined in different types of herbal teas including *H. sabdariffa* by an infusion method (0.1g of herbal tea in 10 ml of water 100 ° C for 10 min). The study revealed that total phenol content in the *H. sabdariffa* infusion was 0.073 mg of gallic acid equivalent / g of dried weight (Aoshima *et al*., 2007). The condition that Aoshima *et al*. (2007) used is similar to the current study, but there are two main differences can be summarised as responsible for the variations in total phenol content compare with the current study. Firstly, the uses of the infusion method not the decoction in the extraction, which is found in the current study that the decoction method extract more phenols. Secondly, Aoshima *et al*. (2007) study did not mention if the samples were kept in dark place during storage and analysis or not which also may affect.

Kruawan and Kangsadalampai (2006) studied the total phenol content of ten herbal extracts extracted by using boiling water (0.1 g in 15 ml) for 10 min. The results indicated that total phenols in *H. sabdariffa* extract were 210.72 ± 14.62 mg/g of Gallic acid equivalents of dried *H. sabdariffa*. The result of the total phenols in the current study of pure water extract of dried *H. sabdariffa,* pomegranate tea and rose

tea extracted by decoction method are much greater than what found by Kruawan and Kangsadalampai (2006), Abdullakasim *et al.* (2007), Prenesti *et al*. (2007) and Aoshima *et al*. (2007).

In Prenesti *et al*. (2007), Aoshima *et al*. (2007) and Kruawan and Kangsadalampai (2006) they used dried *H. sabdariffa* from different places Egypt, Japan and Thailand individually while the present study from obtained the dried *H. sabdariffa* from local market in Saudi Arabia which is usually imported from Sudan or Nigeria therefore, the plant original and cultivar may affect in the total phenol content.

Overall the variation in extraction and experiment condition as explained above study that could be related to low amount of total phenols in previous studies and high amount in current study.

4.5.2 Total phenol content after correction for of vitamin C interference

The present study found that the amount of total phenol content was remained high even after the correction of ascorbic acid interference as shown in chapter 3. According to Abdullakasim *et al*. (2007) study which indicated that high concentration of total phenols in samples and low antioxidant activity could be explained by non-phenolic compounds like, ascorbic acid, sugars, sulfur dioxide and protein could interfered the phenolic compounds and increase the total phenols but cannot react or effect in the antioxidant activity. The study suggests that the interference of vitamin C did not responsible for high amount of the total phenol. As Prenesti *et al*. (2007) reported that the amount of vitamin C in in dried *H. sabdariffa* was ranged between 1 to 1.4 mg/g which is a small amount. The high value of total phenol content could be explained due to the strongly correlation of antioxidant

activity of the extract of *H. sabdariffa* with total phenols, so if the interference of vitamin C increased the total phenol that should be affect the antioxidant activity and reduced the antioxidant activity of herbal teas and pure *H. sabdariffa* extract as Abdullakasim *et al*. (2007) found and that could be confirmed by the high antioxidant activity and high total phenol content even after correction of the interference. In addition, the current study suggests that increasing the temperature for extraction to 100 °C in both the decoction and the infusion methods allowed to free more phenols during extraction in parallel with releasing more vitamin C (which is not a large amount), and inhibit the polyphenols oxidase that is responsible to oxidise the ascorbic acid and reduce the forming of complex compounds that increase the values of total phenols content (Singleton *et al*., 1999).

4.5.3 DPPH and FRAP assays

The DPPH for pure water extract of dried *H. sabdariffa,* pomegranate tea and rose tea was highest when extracted by decoction method (69.88 ± 0.66 %, 68.88 ± 5.32 %, 86.12 ± 4.4 8% respectively). In addition, pomegranate tea and rose tea extracted by decoction method had the highest FRAP values.

Kruawan and Kangsadalampai (2006) studied the radical scavenging activity (DPPH) of ten herbal extracts extracted by using boiling water for 10 min. The results indicated that the percentage of radical scavenging capacity of dried *H. sabdariffa* extract was 93.12 % (Kruawan and Kangsadalampai, 2006). The high percentage of DPPH in the study done by Kruawan and Kangsadalampai (2006) compared to this study could be explained due to the differences in the volume of water added to 0.1g was 15 ml compared with this study 10 ml increasing the water may increase the soluble compounds and increased the DPPH.

In contrast, radical scavenging activity DPPH was determined in different types of herbal teas including *H. sabdariffa* by infusion method (0.1g of herbal tea in 10 ml of water 100 °C for 10 min). The study revealed that radical scavenging capacity for *H. sabdariffa* was 11.7% (Aoshima *et al*., 2007). The lower percentage of radical scavenging activity DPPH may be because (Aoshima *et al*., 2007) study used the infusion method for extraction as explained in section 4.5.1

The results of the DPPH revealed that rose tea extracted by decoction method had the highest percentage of radical scavenging activity that may be due to the effect of other phytochemical ingredients in the rose tea which released more of the compounds by the decoction method, also the insignificance between both pure extract and pomegranate tea can be explained as the amount of *H. sabdariffa* added to the pomegranate nearly the same or the combined of blackberry with pomegranate and *H. sabdariffa* reduce the effect of the antioxidant compared with activity of each extract alone.

The increase of the DPPH percentages and FRAP values could be linked to the strong relationship between total phenol content and both FRAP and DPPH. That is in agreement with Kruawan and Kangsadalampai (2006) study which found strong correlation between total phenol content and both FRAP and DPPH. The strong correlation could explained that high total phenol content responsible for high percentage of DPPH and FRAP values, while the radical scavenging percentage in Aoshima *et al*. (2007) is below our results of infusion and decoction method may be related also due to the low phenol content of *H. sabdariffa* sample as mentioned in section 4.51. Moreover, Tsai *et al*. (2002) reported that 51% of the FRAP activity belong to the delphinidin 3-sambubioside and cyanidin 3-sambubioside. The results

study of Tsai *et al*. (2002) linked the high values of FRAP to the major anthocyanins in *H. sabdariffa* which may explain the high value of FRAP in the present study.

4.5.4 TEAC assay

The TEAC value of pure water extract of dried *H. sabdariffa* in the current study was 35.86 ± 1.69 mg of Trolox equivalents /g of dried *H. sabdariffa*.

According to Yang et al. (2006), TEAC antioxidant capacity was evaluated in 150 types of edible plants including *H. sabdariffa*. The results found the value of TEAC was 0.038 mg of Trolox equivalents per gram of *H. sabdariffa*. This value is much lower than the results for all determinations in the current study. The low value of TEAC in Yang *et al*. (2006) could be due to the usage of fresh sample and low extraction temperature which not enough to extract all soluble compounds, further more Yang *et al.* (2006) used methanol and water for extraction, therefore solvents could affect TEAC values.

4.5.5 Total monomeric anthocyanins

In general, the total monomeric anthocyanin content was high in pure *H. sabdariffa* extract and herbal teas extracted by the decoction method compared with the infusion method. Among the extracts extracted by decoction method pomegranate tea had the highest total anthocyanin content. The results could be correlated to the high temperature of extraction for 10 min extracted more anthocyanin compounds than the infusion method, and the high amount of anthocyanins in pomegranate tea compared with pure extract of *H. sabdariffa* could be linked to the presence of pomegranate, blackberry combined with *H. sabdariffa* which may responsible for increasing the total anthocyanin content.

The influence of different processing methods (hot water extraction, hot water blending, cold water blending and screw press) on the total anthocyanin content was investigated. The results showed that the anthocyanin content was 0.043 mg of delphinidin 3-glucoside /ml of *H. sabdariffa* juice extracted by hot water extraction method (Wong *et al*., 2003). The present study found that the total anthocyanin content in the pure water extract of dried *H. sabdariffa* was 5.5 mg of delphinidin 3-glucoside /ml which means that our determination of anthocyanins was above the amount of anthocyanins in the study by Wong *et al*. (2003). The difference is possibly due to the use fresh *H. sabdariffa* samples in the study by Wong *et al*. (2003) compared to dried as we used. Tsai *et al*. (2002) related 85% of total anthocyanin content to the presence of delphinidin 3-sambubioside and that might explain the high total anthocyanin content of pure *H. sabdariffa* extract and pomegranate tea in the current study.

4.5.6 Correlation between total phenol content, total anthocyanin content and antioxidant assays

The study found strong correlation between total phenols and all antioxidant assays.

Fu *et al.* (2011) also found strong association between total phenols and antioxidant capacity for TEAC and FRAP in 51 different types of tea and herbal tea infusions in China. Also Belscak *et al*. (2011) found a strong relationship between total phenols and FRAP assay when studying the effect of vitamin C and honey on total phenols and antioxidant capacity in 10 fruit tea infusions. These studies are in agreement with our study results. In addition, Deetae *et al.* (2012), found strong correlation between total phenol and both TEAC and FRAP assays (r^2 = 0.954, r^2 = 0.885), and a strong relationship between TEAC and FRAP assays (r^2 = 0.946) when 18

different types of Thai herbal tea infusions were measured. Piljac-Zegarac *et al*. (2010) also found a strong relationship between FRAP, TEAC and DPPH as well as between total phenols and the antioxidant assays when examining the antioxidant activity of 2 g of 10 different fruit tea infusion extracted by adding 100 ml of boiling water (95 °C) for 8 min (Piljac-Zegarac *et al*., 2010).

Phenols and other bioactive compounds had an effect on increasing the antioxidant capacity in different herbal teas. These compounds can decrease oxidants and quench free radicals which can protect the body from oxidative stress diseases (Fu *et al*., 2011). The high antioxidant activity in the infusions is explained due to the ability of the components in the infusion to donate hydrogen and oxygen atoms which inhibit the creation of hydroxyl radicals (Deetae *et al*., 2012).

The current study found no relationship between total anthocyanin content and total phenol content or antioxidant assays. This finding could be explain by that anthocyanins content are not responsible or the increasing of total phenols and antioxidant activity, other type of polyphenols could have the effect and responsible on the increasing of total phenols and antioxidant activity. In addition, the fruit herbal teas that used in the present study include other ingredients which may had the effect on total phenols and antioxidant activity. However, the *H. sabdariffa* was the first main ingredients but the proportion of *H. sabdariffa* in herbal tea was minor compared with the proportion of other ingredients.

4.5.7 Identification and quantification of anthocyanins by HPLC

The current study found the highest concentration of delphinidin 3-sambubioside and cyanidin 3-sambubioside were found in pomegranate tea extracted by infusion method. When the amount was compared with the current study pure *H. sabdariffa*

extract extracted by the decoction method the result revealed that 100 mg of dried *H. sabdariffa* contained 0.506 mg of delphinidin 3-sambubioside and 0.381 of cyanidin 3-sambubioside , therefore the increase of the pomegranate tea extracted by infusion could assumed to be related that the amount of *H. sabdariffa* in pomegranate tea is more than 100 mg assumed between (135 mg to 150 mg) in contrast, the low amount of major anthocyanin in rose tea due to the amount of *H. sabdariffa* is less than 100 mg assuming in the range of (30 to 50 mg). Due to the lack of the studies on the product of *H. sabdariffa,* the results could not explain or compared. Also, the low concentration of both delphinidin 3-sambubioside and cyanidin 3-sambubioside in pomegranate tea extracted by decoction method due deterioration of the anthocyanins with high temperature which may other ingredients such as blackberry and pomegranate anthocyanins increased the instability of *H. sabdariffa* anthocyanins.

4.5.8 Effect of infusion and decoction methods

The current study indicates that using the decoction method for 10 min for extraction herbal teas gives the highest amount of total phenols and antioxidant activity compared with the infusion method for 5 min, possibly due to the decoction for time being longer which could extract more antioxidant and phenols than the infusion method. Prior and Cao (1999), reported that amount of antioxidant activity of tea was affected by the infusion conditions. 84% of the antioxidant activity was achieved at 5 min and 13% more antioxidant activity was achieved at 10 min. Komes *et al*. (2011) also studied the effect of extraction time on the total phenol content and antioxidant activity was measured by TEAC and FRAP assays of 6 medical plants. Their study found that extraction for 15 min was the optimal time to extract bioactive compounds. In addition, Goncalves *et al.* (2013) were examined

the total phenol content and the antioxidant activity of 10 Mediterranean medicinal plants by using cold and hot extraction methods. By using the ratio of solid to water (1:20) cold extraction was done at room temperature for 2 hours while the hot extraction done at 90 °C for 5 min. The study showed that there was antioxidant activity for all infusions, with high antioxidant activity and total phenols revealed in tea infusions extracted by hot water. Duh and Yen (1997) found that *H. sabdariffa* extracted with boiling water for 10 min had high total phenols and antioxidant capacity due to the ability of antioxidant compounds to react with free radicals and convert it to stable forms by hydrogen donation. The present study agree with these studies, that increasing time of extraction to 10 min increases the activity of antioxidants and the amount of total phenol content. Another explanation for the high antioxidant activity and total phenol content of herbal teas could be the presence of other ingredients in herbal teas.

Deetae *et al*. (2012), measured the total phenol content and the antioxidant capacity using TEAC and FRAP assays in 18 different types of Thai herbal tea infusions. Overall, most of the herbal teas had a low amount of total phenols except a few samples; the study related the variation in the results of total phenols to the method, time and temperature of extraction and the dose of herbal tea and part used for infusion. The study concludes that the antioxidant capacity depends on the type and the amount of phenols available in the tea infusions.

4.5.9 Comparison between pure extract and mixed

Bekhit *et al.* (2011) examined the influence of green tea and *H. sabdariffa* on total phenols and DPPH in pure green tea and *H. sabdariffa* and in five types of grape skin tea infusions (two type of grape skin wine waste alone, grape skin with 50% of extracted green tea and 50% of grape skin with 25% of extracted green tea and 25%

H. sabdariffa). The study found that tea infusions with 25% of *H. sabdariffa* had a high amount of total phenols and antioxidant activity. The total phenol content of *H. sabdariffa*, green tea alone were (1.1 ± 0.1 mg, 6 ± 0.3 mg of gallic acid equivalents /cup, respectively), while the total phenol content of 25% *H. sabdariffa* mixed with grape skin and green tea was 2.6 ± 0.1 mg compared with grape skin mixed with 50% green tea 5.4 ± 0.3 mg of gallic acid equivalents /cup (Bekhit *et al*., 2011). Adding green tea and *H. sabdariffa* to the grape skin wine waste were responsible for increasing the total phenol content.

Piljac-Zegarac *et al*. (2010) indicated that the high antioxidant capacity of fruit tea infusions studies related to the presence of a high percentage of *H. sabdariffa* in the fruit tea infusions. In addition, Costa *et al.* (2012) showed that tea and juice containing *H. sabdariffa* in the ingredient list is high in total phenols, ascorbic acid and has a high radical scavenging activity compared with other products.

In the current study, the results found the herbal teas which contained *H. sabdariffa* as the major ingredient had higher total phenols and antioxidant capacity than that of pure *H. sabdariffa* extracted by decoction method. This is in agreement withPiljac-Zegarac *et al*. (2010), Bekhit *et al*. (2011) and Costa *et al*. (2012). The study suggests that other ingredients mixed with *H. sabdariffa* could increase the antioxidant capacity.

Overall, the study found that extract herbal teas by the decoction method was the optimal method due to high antioxidant activity, total phenol content and total anthocyanins content may be due to the presence of *H. sabdariffa*. Therefore, the present study suggests using decoction method for more health benefit and trying to send that message to the manufacture to change the instructions of preparation on pack of herbal teas.

Chapter 5 Determination of the log *P* of anthocyanins in different conditions

Summary

- Log *P* values measure the distribution of compounds between octanol and water, and can be determined experimentally or theoretically to give information about transport across biological membranes.
- There have been no previous studies for measuring log *P* values for anthocyanins from *H. sabdariffa*.
- There was no significant difference between log *P* values done experimentally and theoretically for most anthocyanins tested but there were differences for the glucosides for both methods. Sambubioside-derived anthocyanins were significantly more water-soluble than glucosides.
- Addition of water extracts from *H. sabdariffa* to the octanol-water system significantly changed determined log *P* values; in all cases, lower log *P* values were observed.
- Log *P* values determined in the presence of *H. sabdariffa* powder showed that all anthocyanins tested had negative log *P* results; even hydrophobic aglycones appeared to demonstrate apparent water solubility.
- Hydrophobic anthocyanins would be expected to be readily absorbed across lipid bilayers. However, the presence of plant extracts (with significant availability of hydrophobic binding capacity) acts to reduce log *P* values and may act to reduce the potential for intestinal absorption.

5.1 Introduction

5.1.1 Transportation through a biological membrane barrier

For any food component to be absorbed from the intestine it must pass across the biological barrier provided by cell membranes at the site of administration. Much of our knowledge about the processes involved comes from pharmacology rather than food source or nutrition. Drugs and food-derived chemical compounds are transported either transcellularly (through the cells) or paracelluarly (between the cells) (Pandit, 2007).

The Transcellular Method, in which, compounds move from the outside of the cell, through the cell and outside again occurs through four different mechanisms; passive diffusion, facilitated diffusion, active transport and transcytosis.

The first mechanism of transcellular transport is through passive diffusion. In this way compounds moves from a highly concentrated site outside the cell through to inside the cell where there is a low-concentration site until both sides reach equilibrium. Only un-ionised compounds can travel across membranes by passive diffusion. A weak base with the logarithmic measure of the acid dissociation constant (pK_a) of 10 or higher and a weak acid with a pK_a of less than 3 are poorly absorbed from the intestine because they exist in the ionised form at relevant pH condition. Therefore, if the concentration is relatively high, the amount of un-ionised compounds is enough for them to diffuse down the concentration gradient. Compounds can be diffuse through protein (or hydrophilic) channels or through the hydrophobic lipid bilayer. The protein channel is responsible for carrying small molecules and ions through the channel by force of diffusion from high concentration to low concentration until both sides are balanced. In the lipid bilayer,

un-ionised and lipophilic compounds can travel through the hydrophobic lipid bilayer. Only un-ionised hydrophilic compounds and un-ionised parts of weak acids or bases can be transported via the lipid bilayer (Pandit, 2007).

Carrier-Mediated transporters are used to transfer ions and nutrients such as amino acids, sugars, minerals and vitamins from the intestines. This can be achieved by combining different transporters to reach the epithelial membrane. Using transporters can resolve the difficulty of hydrophilic compounds passively crossing the lipid bilayer by allowing attachment to transporters and transferring from one side of the membrane to another. Each transporter is specialised to transport specific molecules or substrates (Pandit, 2007).

The second mechanism of transcellular transport is facilitated diffusion, this mechanism happens only by the effect of (uniporters) transporter which transport one compound or molecule each time, and the transporter can only transport compounds form high concentration to the low concentration and this mechanism does not need energy, such as demonstrated by the glucose transporter 1 (GLUT1 receptor). Transporters can bind to other compounds such as inhibitors, which reduce the amount of compound absorbed (Pandit, 2007).

The third mechanism of transcellular transport is active transport is responsible for carrying substrates from low concentration to high concentration, which requires energy to complete the process. Active method transporters carry two compounds in one way (symporters) and other transporters (antiporters) carry two compounds in two ways ; from outside the cell to inside the cell, and at the same time from inside the cell to outside the cell. Hydrophobic compounds can return back to the site of administration more than hydrophilic compounds due to the effect of the efflux protein transporter, which attach to the compound when it cross the lipid bilayer and

pumped the compound out of the cells which leads to maintain the concentration of the compound low in the intracellular (Pandit, 2007).

The fourth mechanism of transcellular transport is transcytosis. In this mechanism, transport of large molecules occurs by endocytosis, passing across the epithelial and the endothelial cells by the creation of sacs that capture and then release the compounds into the cell. Exocytosis transfers molecules in the other direction (Pandit, 2007).

The second method for transportation of compounds is Paracelluar Transport. In this method, small hydrophilic or hydrophobic molecules and ions with a molecular weight no greater than 200 Da are transported through epithelial membranes. With paracelluar diffusion un-ionised compounds are more rapidly absorbed than ionised compounds (Pandit, 2007).

To conclude, some lipophilic compounds can be transported by three different processes. They may pass the endothelial tissues by paracelluar transport, and then attach to a transporter in a capillary membrane, then they pass through the capillary by passive transcelluar diffusion.

5.1.2 The importance of partition coefficient

The partition coefficient (Log P) is very important as a parameter in determining the transport behaviour of compounds across a membrane. It is also important in understanding the defence mechanisms of the cells towards xenobiotic and to understand the compounds' relative solubility in water and the interaction between molecules (Rothwell *et al.*, 2005).

In determination of log P, molecules are usually mixed with a water phase and an octanol phase until they reach equilibrium. Some compounds, including ionised

compounds, may locate at the surface of the two phases (Luo *et al.*, 2011). The effect of pH on ionisation can affect the log *P* value for that compound. Therefore, a weak base may be highly soluble at a low pH and a weak acid may be highly soluble in a high pH, but the salt form of a weak acid or base does not change its solubility (Pandit, 2007).

Only neutral forms and un-ionised forms can partition from the aqueous phase to the octanol phase. If the concentration of a compound is high in the octanol phase, that means it is highly soluble in lipids. Also, if the log *P* value of the compound is below zero (too hydrophilic) or above 3.5 (too lipophilic). It is impossible for the compound to cross lipophilic biological membranes. Log *P* is usually measured experimentally, the result giving an idea of the absorption behaviour of the compound in the body (Pandit, 2007).

5.1.3 Lipophilicity and the partition coefficients

Lipophilicity is defined for any chemical compound as the solubility of a compound in oils or non-polar solvents (Pandit, 2007), and it is a very important measurement in medicinal chemistry and the pharmaceutical field because it is a helpful measure in understanding how compounds might behave in non-polar surroundings such as those present in cell membranes. Consequently the log *P* can help understand absorption from intestine.

The solubility of a compound is affected by its molecular weight, whether it is small or large, and whether the molecule is branched or straight. In general, the smaller the molecular weight, the more it is absorbed. Branched molecules are absorbed better as well. Most compounds with large molecular weights are hydrophobic and less volatile, while most polar compounds are hydrophilic. Also, if a compound contains

oxygen or nitrogen in its structure, this means that the compounds are more likely to be hydrophilic. In contrast, a large number of hydroxyl groups increases hydrophilicity (Tehrany *et al.*, 2004).

Determination of the partition coefficient is the main method used to measure lipophilicity. Therefore, log *P* is usually used as an indicator of the hydrophobicity of drugs, pharmaceuticals and dietary compounds to understand the absorption and distribution of these compounds in the human body, and also to study the relationship between structure and activity (Muller *et al.*, 2005).

5.1.3.1 Log *P* determination methods

There are two types of method used to determine the log *P* value the first one is the theoretical method which is based on mathematical equations calculation.

5.1.3.1.1 Theoretical method

Log *p* can be calculated using different calculation methods such as methods based on substituents to calculate log *P* as additive- constitutive free energy based on the differences in log *P* between specific substituent and hydrogen atom which can exchanged (Fujita *et al.*, 1964). This method is applicable only if a swap group happened on aromatic ring or the hydrogen is exchanged with hydrocarbon (Fujita *et al*., 1964). Leo *et al.* (1971) and Leo (1993) reported three methods for calculating log *P* value which are fragments method, method established using the charge densities of the atoms and atomic contributions method. The fragments method based on constants of hydrophobic fragment, the fragment table was developed by adding some parameters such as chain length, ring size, branching, ring joining, H polar to achieve more accurate results. The method based on atomic contributions

categories depend on individual chemicals and include the experiment data of log *P* values.

for example Tehrany et al. (2004) used prediction model to calculate log *P* with account all the variable such as Polarity, total energy, boiling point, binding energy, mass percent of hydrogen and oxygen and molecular weight. The prediction model of Tehrany *et al*. (2004) has given better results compared with classic calculations. An alternative a quantitative method has been used to determine log *P* with a linear correlation between the log *P* of octanol-water (Ibrahim *et al.*, 2008).

Currently, none of the calculation methods are used widely due to the complexity of the equations and the multi-steps to get the value, therefore many software programmes has been built based on those method mentioned earlier.

5.1.3.1.2 Experimental methods

The usefulness of the partition coefficient to determine solubility is based on the simplicity of the experimental procedure (Ibrahim *et al.*, 2008). The distribution of the compound between the octanol phase or the aqueous phase is measured by shake flask method then analysed by chromatographic or spectrophotometric techniques (Ibrahim *et al*., 2008). According to Cairns (2008) the shake flask method which is based on using separation funnel and two types of immiscible solvents then the compound added and shake in the funnel for approximately an hour and allow to stand for a while to let the two phase separate well. Usually the concentration of polar phase is analyses by titration or spectrophotometer. The concentration of nonpolar phase calculated by differences between the original amounts of compound added in the beginning of the experiment and the concentration in the polar phase (Cairns, 2008).

However, the shake flask method has some drawbacks, including the need for a pure compound, the time taken to complete the procedure and that can be difficult to control the temperature (Ibrahim *et al*., 2008). Also, Liu *et al.* (2005) stated that the shake-flask method is not preferred in determining log *P* due to the effect of extended times and inconsistencies in the results. Muller *et al.* (2005) mentioned that stability of compounds affects the determination of log *P*, so it was very difficult to measure the concentration of compounds in the two phases.

According to Cairns (2008) log *P* value can be determine by thin layer chromatography. The method based on using thin layer plate coated with organic solvent then drops from the compound added to the plate and the whole plate inserted in the mobile phase which usually water or mixture of water with organic solvent, the plate left to let the mobile phase move upward (Cairns, 2008). At the end the plate is visualised using UV lamp or iodine vapour, the log *P* value calculated by dividing the movement distance of the compound to the movement distance of the solvent (Cairns, 2008). The drawbacks of the method that it takes long time to finish due to the large amount of water in the mobile phase which makes the movement very slow, but the advantage of the method that only small amount is needed for the experimental and several sample can be run in one plate (Cairns, 2008).

Another method to determine log *P* is the RP-HPLC method. However, the majority of the studies used RP-HPLC as an alternative method because this method is not required to use pure compounds, due to previous preparation of samples depend on the complexity of the sample such as solid phase extraction or filtration by using filters with small pore size and eliminates degradation and impure compounds as much as possible in the products, impurity could limiting the log *P* range (Liu *et al.*,

2005). Moreover, only a small amount of sample is needed and the temperature was also controlled quickly for analysis (Ibrahim et al., 2008). RP-HPLC had the same concept of thin layer chromatography , the retention time for the compound to elute from the column is measured (Cairns, 2008).

The RP- HPLC method still has drawbacks due to the limit range of hydrophobicity and also because the border between the solvents, mixed or partially mixed, is not enough to act as a model of bio-membranes due to inflexibility of the structure, size limitation and the proportion between polar and non-polar compounds (Muller *et al.*, 2005). Compounds may take a long time to elute from the column also if UV could not be used then the machine should be attached to an electrochemical detector or refractive index detector, the advantages of the method are that small amount of sample are used, high purity of compounds are not required and low cost of solvent of the mobile phase (Cairns, 2008).

On the other hand, an advanced method, used recently is called Micellar Electrokinetic Chromatography (MEKC) (Muller et al., 2005). This method is based on the partitioning of the solute between the two mobile phases to determine the partition between liposomes, cells or between cells and liposomes is closer to bio-membranes in the human body (Muller *et al.*, 2005). The advantage of this method is that the mechanisms are close to the real compound–membrane interaction, but also has disadvantage such as inconsistency in the results (Muller *et al.*, 2005).

After reviewing some of the theoretical methods, until now the methods had errors and not accurate which may mislead the behaviour of compounds distribution and absorption in biological membranes. Also usually the ionised form calculated as constant which is not exactly the total amount. The calculation for log *P* is very complicated and now there are several softwares that can do the calculation and that

reduced the errors. Therefore, to confirm the log *P* values, experimental methods are recommended to mimic the biological membranes and also to calculate the ionised part correctly.

The experimental methods that were usually used to determine most log *P* values, it can be concluded that shake flask method is still used in micro tubes combined with reverse-phase HPLC to analysis the peaks is the most preferred method used due to the simplicity and availability of the equipment.

5.1.4 Absorption and delivery

The way that our bodies absorb compounds depends on the structure of those compounds, their molecular size and the site of absorption. Usually there is no one single organ or site for compounds to be absorbed, and they can be absorbed by different sites or organs, or by only one. Compounds move from the site of administration to the site of absorption and cross the epithelial tissues either by transporters or by passive diffusion, or both. It is difficult for the compounds to move to the endothelium by diffusion and finally to reach the bloodstream by force of blood flow. Sometimes the rate of absorption is slow due to the rate of operation of the efflux pump in the epithelial membrane, so it returns back to the starting point, which explains the poor absorption of some compounds and drugs figure 5-1 explain the process (Pandit, 2007).

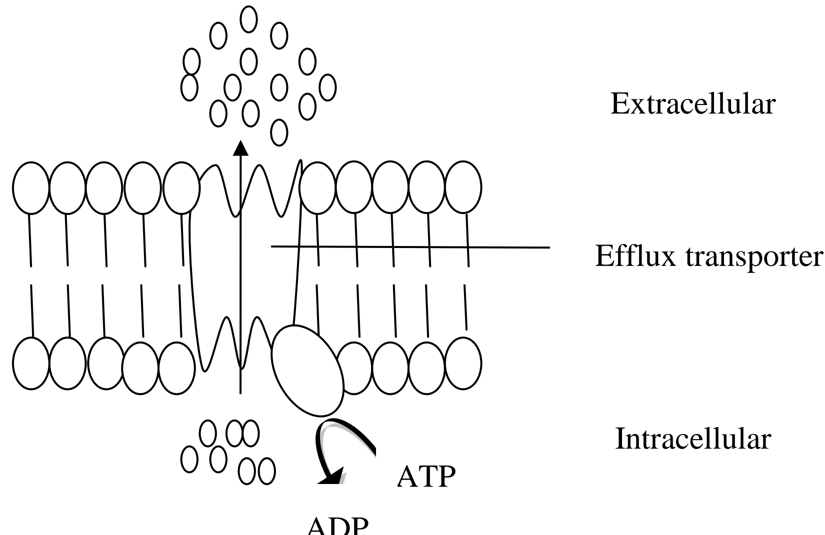


Figure 5-1. Diagrammatic representation of the efflux pump. Efflux protein transporter attach to the compound when it cross the lipid bilayer and pump the compound out of the cells which leads to maintain the concentration of the compound low in the intracellular, the process needs energy to be done. adapted from (Pandit, 2007)

Compounds travel from the mouth to the oesophagus to the GI tract. All parts of the GI tract have different surface areas, different enzymes and are subject to different pH values. All of these factors affect the absorption of compounds. In the stomach, the pH ranges between 1 and 3.5. Weak bases are ionised in this range and weak acids are incompletely ionised due to lack of solubility in the gastric medium. The ionisation of compounds increases the solubility and decreases the partition coefficient during the movement of the compounds from the stomach to the small intestine. If the compounds are highly soluble in water, they will be absorbed in the stomach. A small amount of the compounds may be absorbed transcellularly by passive diffusion, but the chance of this is very low due to the short stay in the stomach and the relatively small surface area. The compounds move to the small intestine to be absorbed by crossing the epithelial tissue transcellularly using passive diffusion for transportation dependent on the partition coefficient. The pH varies in

each part of the small intestine. Lipophilic compounds are the most likely to be absorbed and move to the capillary endothelium either through the bloodstream or though lymphatic circulation. Other, hydrophilic compounds without active transport are not absorbed and pass from the small intestine to the large intestine. Compounds can be absorbed in the colon and rectum by crossing the epithelial membrane using passive transcelluar diffusion (Pandit, 2007).

5.2 Aim of the study

Several studies have examined the log *P* of flavonoids and their relation to absorption and metabolism (Murota *et al.*, 2000; McGhie et al., 2003; Rothwell et al., 2005; Wiczkowski et al., 2008); however, the partition coefficients of anthocyanins from *H. sabdariffa* have not been documented, and few researchers have looked at anthocyanins log *P* due to the relatively poor absorption of this type of flavonoid. In general, the presence of anthocyanins in the polar phase (aqueous phase), and their concentration, depends on the pH range. If it is between 1 and 3, it means that the anthocyanins are stable in the red flavylium form. However, if it is above 4, this means that the anthocyanins formed a chalcone or other type of structure (Qiu *et al.*, 2009).

Determination of log *P* could be useful for a better understanding of anthocyanin bioavailability, absorption and metabolism. The results of the log *P* determinations are based on using anthocyanin standards alone. The behaviour of the standards was compared to their behaviour in association with aqueous extracts or a powder of *H. sabdariffa*.

5.3 Materials and methods

5.3.1 Materials

1-Octanol (HPLC grade ≥ 99.8%), methanol (HPLC grade ≥ 99.8%) and acetonitrile were obtained from Fisher Scientific, Loughborough, UK. Formic acid, tris-HCL and tris-base were obtained from Sigma Aldrich (Gillingham, UK). Seven anthocyanin standards were used, three in the form of aglycones (cyanidin, delphinidin and malvidin) and four were in the form of glucosides (cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-sambubioside and delphinidin 3-sambubioside). The standards were purchased from Phytolab and Extrasynthese (Germany, France), respectively. Water was purified by treatment with a Milli-Q apparatus (Millipore, Bedford, UK).

5.3.2 Methods

5.3.2.1 Theoretical method of determination of log *P* values

Theoretically determined log *P* predicted values were obtained via java based online chemical software (Marvin and calculator plugin demo) from the web site: http://www.chemaxon.com/demosite/marvin/index.html (accessed June 2012)

5.3.2.2 Experimental method of determination of log *P* values

The experimental method described in chapter 2 sections (2.6.10).

5.4 Results

Log *P* values for seven anthocyanins were determined with recovery percentage using standards, a water extract of *H. sabdariffa* and dried *H. sabdariffa* powder.

5.4.1 Comparison between theoretical and experimental log *P* of anthocyanin standards

Table 5-1, show that there is no significant difference between theoretical and experimental log *P* anthocyanin standards *p* value (0.148), $p > 0.05$, but the experimental log *P* gave a higher value than the theoretical log *P*, except for the aglycone cyanidin, which was lower than the theoretical results. The standard deviation of the samples was small and ranged between 0.01 and 0.05.

Anthocyanins	Theoretical log *P*	Experimental log *P*	% recovery for standards in the experimental log *P*
Cyanidin	3.05	2.86 ± 0.03	42.5
Delphinidin	2.77	3.20 ± 0.01	34.1
Malvidin	2.83	3.16 ± 0.05	27
Cyanidin 3-glucoside	0.39	-0.83 ± 0.01	67.1
Delphinidin 3-glucoside	0.10	-1.13 ± 0.01	75.2
Cyanidin 3-sambubiside	-1.06	-1.41 ± 0.01	52.7
Delphinidin 3-sambubioside	-1.5	-2.24 ± 0.05	79

Table 5-1. Theoretical log P values calculated using Marvin and calculator plugin demo and experimental log P value calculated using HPLC analysis of octanol and buffer phase for replicates (n=3)of anthocyanin standards.

Log *P* values of aglycones in the experiment were between 2.86 and 3.20. Delphinidin had the highest value followed by malvidin, at 3.16. Cyanidin had the lowest value of 2.86, which means that delphinidin was the most lipophilic anthocyanin aglycone. Delphinidin 3-glucoside and sambubioside gave the lowest values of log *P* at -1.13 ± 0.01 and -2.24 ± 0.05, respectively, compared with the cyanidin3-glucoside and the 3-sambubioside values, which were -0.83 ± 0.01 and -1.41 ± 0.01, respectively. Anthocyanins attached with mono-glucoside had lower log *P* values than aglycones, but anthocyanins conjugated with sambubioside were the most hydrophilic compounds.

The high values of log *P* for the aglycones show that the aglycones prefer the (non-polar) octanol phase. Anthocyanin glucoside forms were found in both phases with a minority of the molecules (the un-ionised form) appeared in the non-polar phase

while the majority of the molecules (the ionised forms) appeared in the polar phase. The anthocyanins, which contain two types of sugars (the sambubioside group) were found mainly in the polar phase. Table 1 also illustrates the percentage recovery for the standards in the experimental log *P* .The highest recovery for the standard alone was for delphinidin 3-sambubioside at 79%, and the lowest percentage recovery was for malvidin at 27%.

5.4.2 Effect of adding a water extract of *H. sabdariffa* and a *H. sabdariffa* powder to anthocyanin standards on log *P* determination

Log *P* values of seven types of anthocyanins were determined with recovery percentage in presence of a water extract of *H. sabdariffa* and a dried *H. sabdariffa* powder.

From figure 5-2, it can be observed that there was a decrease in log *P* values in all the standards when the water extract was added, and the value decreased dramatically more with the *H. sabdariffa* powder compared to the log *P* value of the standards alone. The anthocyanidins appear to become more hydrophilic. log *P* value of aglycone standards (cyanidin, delphinidin and malvidin) with water extract were 1.2, 1.2 and 2.5 while with *H. sabdariffa* powder were – 0.2, – 0.8 and – 0.6 respectively. In contrast, log *P* value of glucoside standards (cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-sambubioside and delphinidin 3-sambubioside) with water extract were – 1.1, – 1.5, – 1.9 and – 2.4 while with *H. sabdariffa* powder were – 2.7, – 3.4, – 3.4 and – 3.3 respectively.

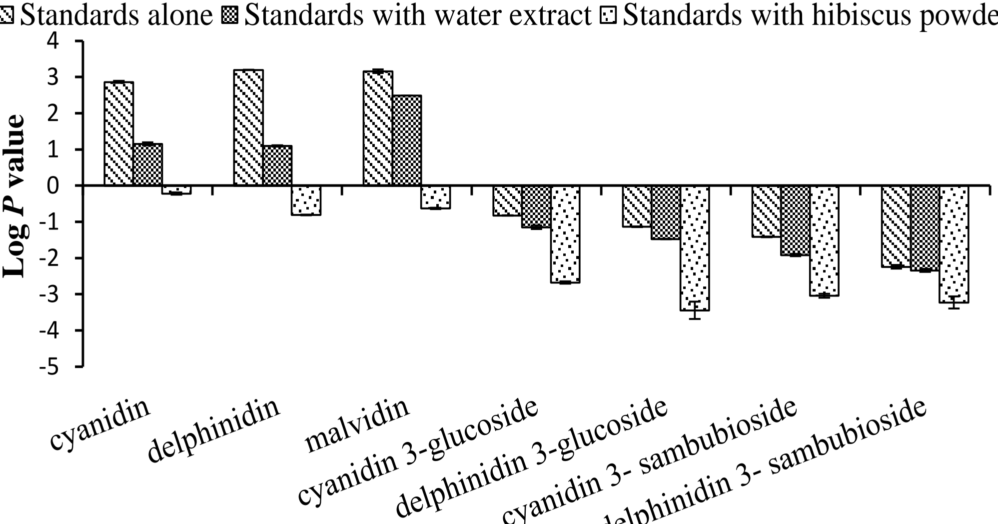


Figure 5-2. Log *P* values calculated for anthocyanin standards alone and in the presence of a *H. sabdariffa* water extract and in the presence of a *H. sabdariffa* powder based on the ratio between the octanol and buffer phase were analysed by HPLC (n=3)

Figures 5-3, 5-4 and 5-5 show examples of chromatographs of three types of anthocyanin standards (aglycone form, mono-glucoside and sambubioside) of delphinidin in the octanol phase and the buffer phase using water extract and hibiscus powder. The retention time for the aglycone form (delphinidin) was 5.5 minutes, 2.5 minutes for delphinidin 3-mono-glucoside and 2.4 minutes for delphinidin 3-sambubioside. The amounts of delphinidin 3-sambubioside and delphinidin 3-glucoside in the buffer phase were much higher than the octanol phase compared with the aglycone form, which dissolved in the octanol phase much more than it did in the buffer phase. Also from the experiment, it was found that the number of the sugar moieties affected the dissolved standard; therefore, standards containing a 3-glucoside moiety dissolved more in the octanol phase compared with sambubioside, which dissolved less in the octanol and more in the buffer phase.

Also, cyanidin dissolved more than delphinidin. For example, cyanidin 3-glucoside dissolved more in the buffer phase but delphinidin 3-glucoside dissolved less.

Anthocyanins	**Retention time (t_{R})**	**% recovery for standards with water extract**	**% recovery for standards with hibiscus powder**
Cyanidin	7.37	11.99	26.37
Delphinidin	5.5	9.04	46.93
Malvidin	10.4	167.74	28.17
Cyanidin3-glucoside	3.67	106.77	77.59
Delphinidin 3-glucoside	2.5	71.61	63.92
Cyanidin 3-sambubioside	3.60	102.60	43.80
Delphinidin 3- sambubioside	2.4	110.78	70.323

Table 5-2. Percentage recovery of the anthocyanin standards alone and in the presence of water extract or *H. sabdariffa* powder (n=3).

Table 5-2 illustrates the percentage recovery for the standards in the presence of water extract or *H. sabdariffa* powder. The recovery for the standards with *H. sabdariffa* powder had lower percentages than the recovery for the standards with water extract. The data in the table illustrates that the highest percentages of recovery for the standard with water extract, the highest percentages of recovery were for malvidin (167.74%), followed by delphinidin 3-sambubioside (110.8 %) and the lowest percentages were for delphinidin (9%), followed by cyanidin (12%). While, percentages of recovery for the *H. sabdariffa* powder with the standard, cyanidin (26.4%) and malvidin (28.2%) had the lowest recovery. Comparing with cyanidin 3-glucoside (77.6%) and delphinidin 3-sambubioside (70.3 %) had the

highest rates of recovery. The recovery percentages of anthocyanin aglycone standards were reduced in the presence of the water extract compared to the anthocyanins with 3-sambubioside.

In these, the percentage recovery increased. The percentage of recovery for standards with hibiscus powder were reduced for cyanidin and delphinidin 3-sambubioside, delphinidin 3-glucoside and cyanidin compared to the standard alone, while the percentage was increased for malvidin, delphinidin and cyanidin 3-glucoside.

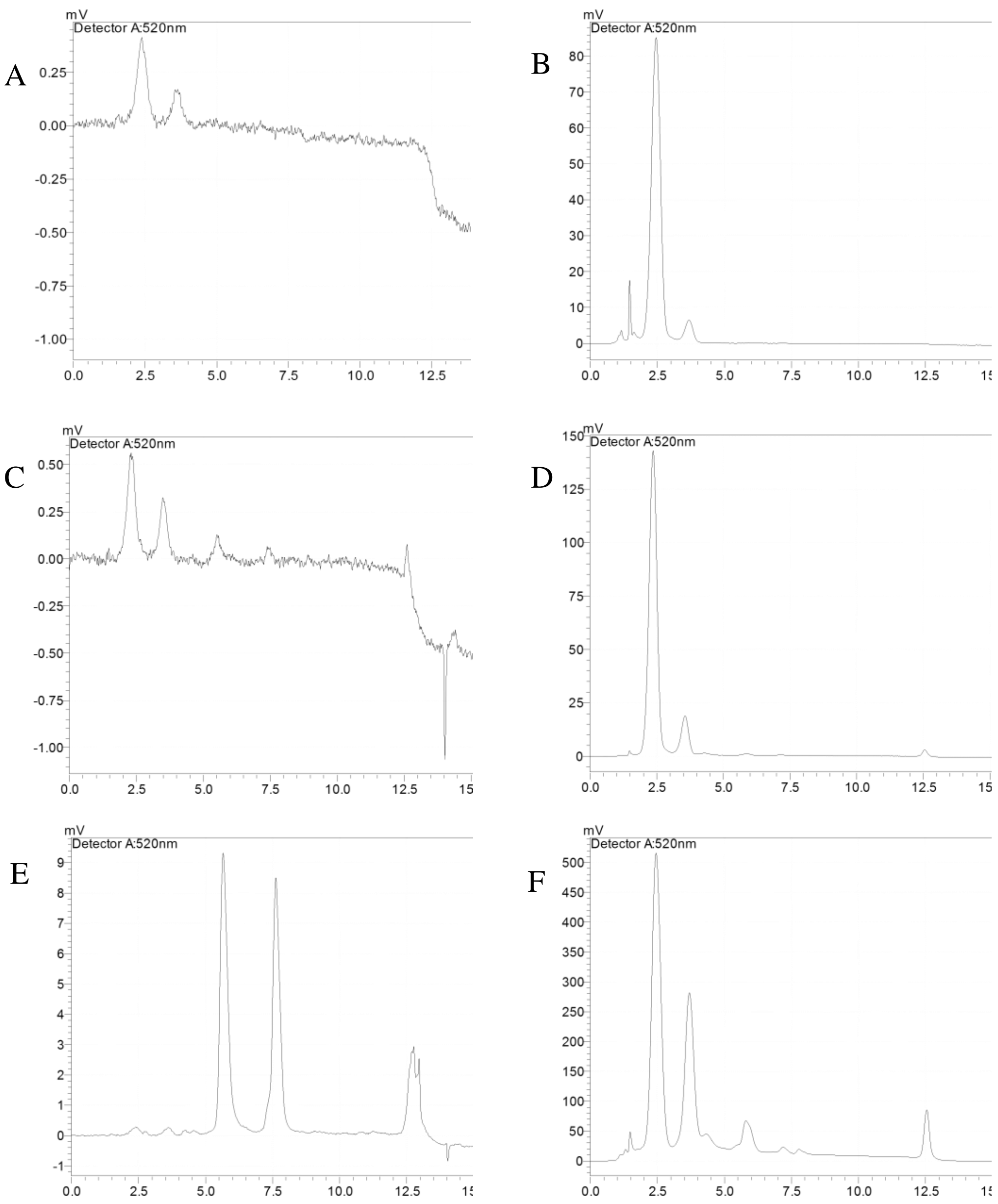


Figure 5-3. Determination of log *P* value of delphinidin 3-sambubioside in standard log *P* =-2.3 (A, B), water extract log *P* = -2.4 (C, D) and powder of *H. sabdariffa* log *P* = -3.3 (E, F), chromatograms showing HPLC peaks of the octanol phase and buffer phase of delphinidin 3-sambubioside in standard (A,B), water extract (C, D)and powder of *H. sabdariffa* (E, F) respectively. Peaks appear in (t_R = 2.4), (N=3) of 10 µl injection volume of each sample.

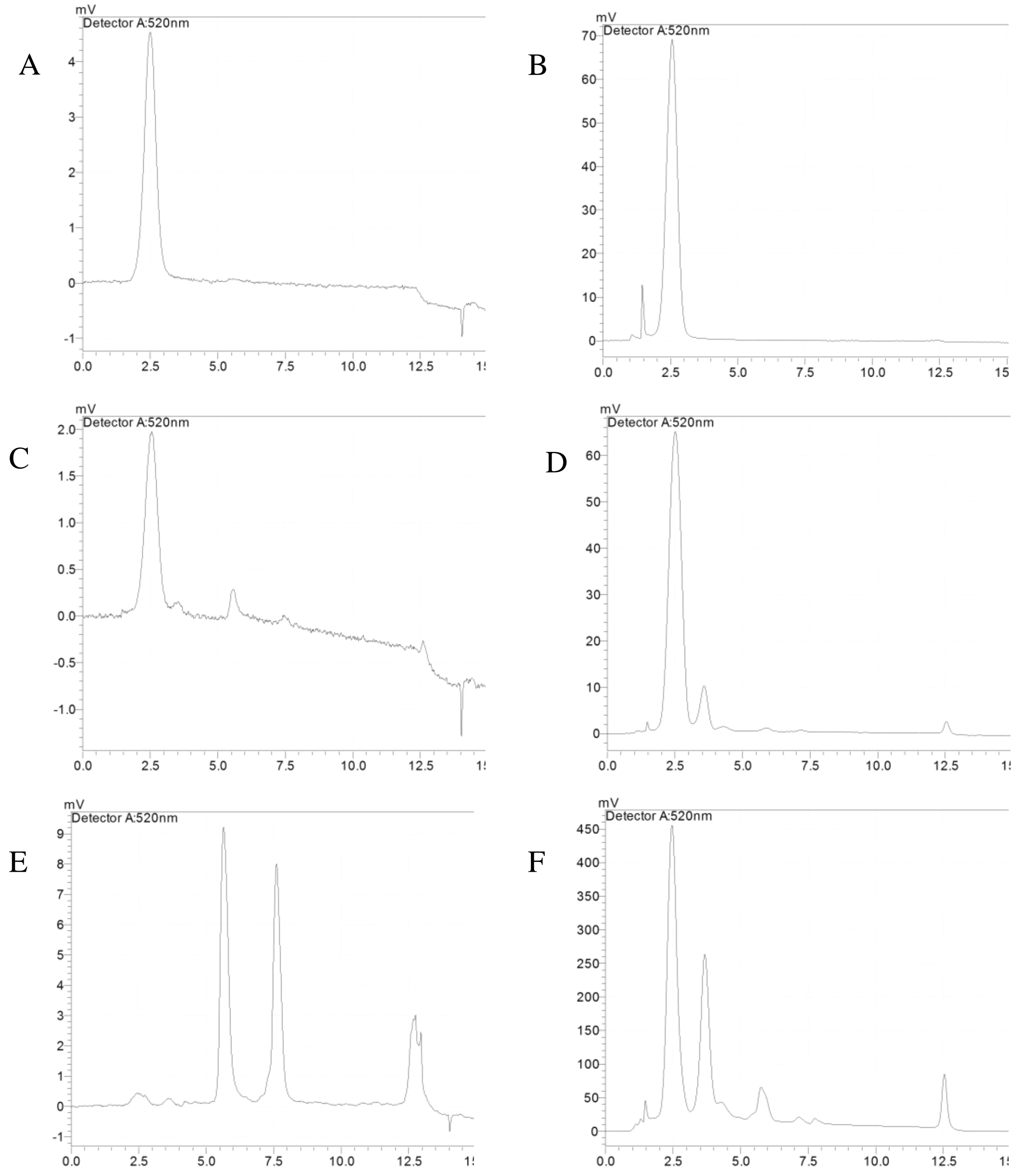


Figure 5-4. Determination of log *P* value of delphinidin 3-glucoside in standard log *P* =0.1 (A, B), water extract log *P* = -1.5 (C, D) and powder of *H. sabdariffa* log *P* = -3.4 (E, F), chromatograms showing HPLC peaks of the octanol phase and buffer phase of delphinidin 3-glucoside in standard (A,B), water extract (C, D)and powder of *H. sabdariffa* (E, F) respectively. Peaks appear in (t_R = 2.5), (N=3) of 10 µl injection volume of each sample.

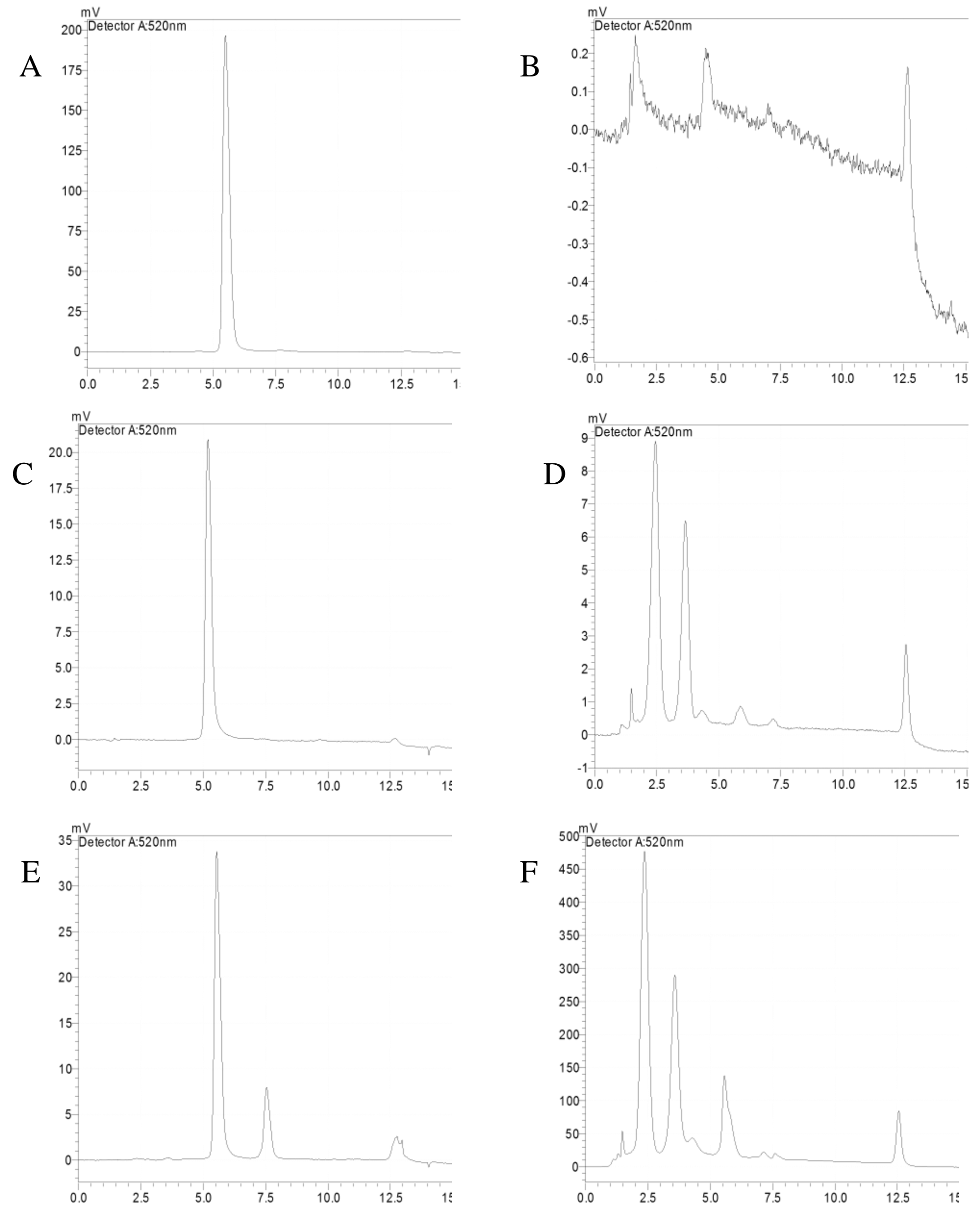


Figure 5-5. Determination of log *P* value of delphinidin in standard log *P* = 2.7 (A, B), water extract log *P* = 1.2 (C, D) and powder of *H. sabdariffa* log *P* = -0.8 (E, F), chromatograms showing HPLC peaks of the octanol phase and buffer phase of delphinidin 3-sambubioside in standard (A,B), water extract (C, D)and powder of *H. sabdariffa* (E, F) respectively. Peaks appear in (t_R = 5.5), (N=3) of 10 µl injection volume of each sample.

5.5 Discussion

From experimental observation, peaks were not always found in one or both of the phases. Especially for aglycone form cyanidin and malvidin, did not appear in the buffer phase some times. Therefore, it was difficult to identify and quantify peaks (in the octanol and buffer phases) it could be that anthocyanins deteriorated when they were kept in the refrigerator for 24 hours or more. Therefore, samples had to be determined as soon as possible in the same day of preparation.

The reason why peaks did not always appear or the anthocyanin standards deteriorated if kept in the refrigerator might due to the lack of stability of the anthocyanins. Felgines *et al.* (2005) observed a similar phenomenon, and degradation was observed in the chromatograms of frozen samples of urine and plasma whereas extra peaks were found in samples prepared and determined directly (Felgines *et al*., 2005).

According to Fleschhut *et al.* (2006) increasing the hydroxyl or methoxyl groups on the structure of anthocyanindin will reduced the stability of the aglycone, while if the anthocyanidin attached to the sugar to form anthocyanins it will increase the stability of the anthocyanins especially at neutral pH. In the current study, the anthocyanidin that were used were less stable due to the exist of two hydroxyl group in carbon at position 3' and 4' in cyanidin and three hydroxyl group in carbon at position 3', 4'and 5' in delphinidin also the hydroxyl group in carbon at position 4' and methoxyl group in carbon at position 5' in malvidin and that also explain the low recovery of the aglycones standards. In contrast, the recovery and the stability of anthocyanins glucosides were increased compared with aglycones due to the

effect of glucose in cyanidin 3-glucoside and delphinidin 3-glucoside and the glucose and xylose in cyanidin 3-sambubioside and delphinidin 3-sambubioside.

According to Fleschhut et al. (2006) study that examined the stability of five types of anthocyanidins and 4 types of anthocyanins in potassium buffer (0.05 M, PH 7.4) and in cell culture at 37 °C for 5 hours and analysed by HPLC. The study found that pH affect the stability of anthocyanidins and the aglycones vanished after one hour and new peaks appeared and analysed by LC-MS and the molecular ions were 605, 587 and 589 *m/z*. the study related the molecular ions to the dimer products and the quinoid base of anthocyanidins. On the other hand anthocyanins attached to mono-glucoside or di-glucoside were more stable in pH 7.4 due to the prevent effect of sugar in the deterioration of quinoid base and phenolic acids. Increasing the pH reduced the positive charge of anthocyanins and transformed the flavylium cation to quinoidal base or pseudobase carbinol, which is reduced the amount of the falvylium and reduced the bioavailability of anthocyanins in general.

The pH of the buffer phase is the most valid reason that could also explain the observation in the present study, a pH used to mimic the small intestinal medium and the pH of the blood stream around 7.4. For the anthocyanins to be stable they should be at pH around 2–3, in which a more acidic environment. The increase of the pH changed the equilibrium of the anthocyanins to another type of chalcone. Therefore, pH could affect the peaks in HPLC due to the machine will just determine the anthocyanins in the red flavylium form. This clarification is in agreement with Fleschhut *et al.* (2006) study. The pH 7.4 deteriorated cyanidin, delphinidin and malvidin and that were very clear from the low recovery of aglycones in the study, on the other hand both glucose and xylose attached with the aglycone to form the sambubioside or the glucose alone to form the glucoside

increased the stability of anthocyanins and that in the same time increased the recovery of the compounds.

McGhie and Walton (2007) also, found that for anthocyanins absorbed through the stomach and the intestines, pH affected their stability due to the acid medium of the gastric juice of the stomach. Anthocyanin is found in a stable form of red flavylium in stomach but when it reaches the small intestine, the pH increased and transformed the flavylium to carbinol pseudobase which is low absorbed in small intestine site (Crozier *et al.*, 2009).

Log *P* values for anthocyanin standards were consistent, with low standard deviation. The results of the current study found that no significant differences between theoretical and experimental log *P* values of anthocyanin standards, but there was a variation between the theoretical and the experimental results. Usually experimental method gives the correct values of log *P* because it is measured exactly the ionised and non-ionised part of anthocyanins. Whereas, the calculated log *P* values not always correct because in theoretical the ionised part not calculated or they use constant value for calculation. According to Ruiz-Ángel *et al.* (2011), log *P* values can be used as measurements of the hydrophobicity of molecules. Theoretically, log *P* only measures the distribution constant (using a constant value for the AH molecular form, and a constant value of the A^- anionic form in calculations for ionisable compounds), while experimental log *P* measures the distribution ratio (expressed by the ratio of AH in all forms in the octanol phase over the ratio of AH in all forms in the aqueous phase in calculation of ionisable compounds). Therefore, the study prefer to use the experimental method because when water extracts of *H. sabdariffa* or *H. sabdariffa* powder was added to anthocyanin standards, the

standards behaved differently and different log P values found due to the effect of matrix which is difficult to know if theoretical method was used.

From this study it was observed that the sugar moiety in the anthocyanins affected the solubility and increased the hydrophilicity of the anthocyanins. While in the absent of the sugar the aglycone forms became more lipophilic. Also it was found that the number of the sugar moieties affected the dissolved standard.

In terms of the reasons why the aglycones were more lipophilic and the anthocyanin glucosides were more hydrophilic, Valls *et al.* (2009) reported that anthocyanins become more (polar) soluble in water, ethanol and methanol if the aglycone is attached with one or more sugars and acylated by organic acid, or if there is more than one hydroxyl group in the B ring with the occurrence of one or more sugar moieties. Also, the nature of the sugars and their sites affect the polarity of anthocyanins, therefore the presence of two sugar moieties in different positions increases the polarity more than one sugar in one position.

Polyphenols are separated between the polar phase (Tris-HCL buffer pH 7.4) and the non-polar phase (octanol which contains an alkyl chain and a hydroxyl group) to mimic the lipid membranes in living tissues (Rothwell *et al*., 2005). According to the studies by McGhie and Walton (2007) and (Vather, 2006), the partition coefficients of anthocyanins with two sugar moieties of glucoside were larger (-0.71) than the for anthocyanins attached to two rutinoside sugars (-1.56), and affected the ability of anthocyanins to pass through the cell membranes (McGhie and Walton, 2007). Moreover, in general, Murota *et al.* (2000) found that number of glucoside groups may affect the lipophilicity, and by adding the glucoside group to quercetin, the lipophilicity is reduced. Therefore, a minor amount of quercetin glucoside is absorbed by the small intestine due to the hydrophilicity of the glucoside, which

makes them difficult to be soluble in micelles. This may also explain why anthocyanin glucosides show negligible amounts of absorption in the body.

The current study in agreement with previous studies, that number of sugars reduced the lipophilicity and that could reduce the amount of compounds absorbed in the small intestinal. The cyanidin 3-sambubioside and the delphinidin 3-sambubioside had lowest log *P* values due to the glucose and xylose moieties attached to the cyanidin and delphinidin.

Luo *et al.* (2011) found that the chemical structure could have a strong effect on solubility in the body. For example, the number of hydroxyl groups will increase the hydrophilicity of the compounds, whereas the number of rings in the structure will increase the hydrophobicity of the compounds. The present study in agreement with Luo *et al.* (2011) that the increase number of hydroxyl group increase the hydrophilicity, the log *P* of delphindin 3- glucoside and delphinidin 3-sambubioside alone were very low that's mean they were more hydrophilic (-1.13 and -2.24 respectively) compared with the log *P* of cyanidin 3- glucoside and cyanidin 3-sambubioside alone were(-0.83 and – 1.41 respectively) because there were three hydroxyl group in position 3', 4' and 5' in the structure of delphinidin 3- glucoside and delphinidin 3-sambubioside compared with cyanidin 3- glucoside and cyanidin 3-sambubioside which have only two hydroxyl group in position 3' and 4'.

The dramatic decrease in log *P* values was observed in all the standards when the water extract or *H. sabdariffa* powder were added, compared to the log *P* value of the standards alone, may reduce the bioavailability. the finding was in agreement with the study by Rothwell *et al*. (2005) in which they concluded that if the compound was too hydrophilic, it would be difficult to enter the cells. If it is too hydrophobic, it will not be able to interact with the water phase. Therefore, if

polyphenols are too lipophilic or hydrophilic, they will have a reduced ability to inhibit lipid peroxidation (Rothwell *et al*., 2005). As Pandit (2007) reported that if the log *P* close to zero means that large amount of ionised part of the compounds in the aqueous fluids of the body which is make it difficult for the compound to pass through the lipid membrane.

The present study in agreement with Rothwell *et al*. (2005) and Pandit (2007) because adding the water extracts and *H. sabdariffa* powder to the standards all the log *P* values were below the zero and became more hydrophilic and it might reduce the bioavailability due the difficulties to penetrate the cell membrane and that also in agreement with Fernandes *et al.* (2012) which reported that anthocyanins are weak acids due to the effect of ionisable hydroxyl groups which are less capable to penetrate the lipid membranes by passive method because the positive charge may remained.

When water extracts of *H. sabdariffa* or *H. sabdariffa* powder was added to anthocyanin standards, the standards behaved differently and different log *P* values found due to the effect of matrix. Adding water extract extracted at 100 °C for 10 min, the effect of temperature increase the extracted anthocyanins by releasing and de-attaching them from the cell wall of the *H. sabdariffa*. Therefore, adding water extract to the standard increases the amount of polar anthocyanin compounds, and this enhances the solubility of anthocyanins in the buffer phase specially the ionised part compared with low amount of anthocyanins in the octanol phase.

While adding *H. sabdariffa* powder part of anthocyanins still attached to the cell wall which could have an effect in different ways, non-extractable or soluble fibre could bind with some anthocyanin compounds and that could possibly increase the

bioavailability of *H. sabdariffa* anthocyanins and reach the gut hydrolysis by the microflora.

Padayachee *et al.* (2013) studied the effect of plant cell wall materials on anthocyanins and phenolic acids in two different system the cellulose-pectin and the puree of black carrot and also examined that in *vitro* digestion. The results indicated that approximately 60-70% of anthocyanins and phenolic acids attached to the plant cell wall released after the cell wall destroyed in the black carrot puree system and using the acidified organic solvent only released third of the amount (20% of anthocyanins and 30% of phenolic acids). While in *vitro* digestion of gastric and small intestinal less than 2% of attached polyphenols only released this was could be possible because the phenolic acid formed complex products with anthocyanins and polysaccharides. The study found that anthocyanins attached to the cell walls could be reach the colon and released due to the effect of gut microflora (Padayachee *et al*., 2013).

According to Sayago-Ayerdi *et al.* (2007), in the *H. sabdariffa* beverage (which is rich in anthocyanins), approximately 85% of dietary fibre is insoluble, while only 15% is soluble. The study indicated that soluble dietary fibre is used as a substrate to the microflora and small fatty acids, which are stimulated in the blood flow, fluids and electrolytes in the colon which may have various effects on digestion process, absorption, gastrointestinal content and the large intestine. The Sayago-Ayerdi *et al.* (2007) study reports that 30% of polyphenols in the *H. sabdariffa* beverage were conjugated with soluble dietary fibre, which could reach the colon and have an effect on the microflora and metabolism (Sayago-Ayerdi *et al.*, 2007). The previous study explains why adding *H. sabdariffa* water extract or powders increased hydrophilicity. It also explains why reducing the absorption and accessibility of

hydrophilic compounds such as cyanidin and delphinidin 3-sambubioside and delphinidin 3-glucoside by increasing hydrophilicity increases the number of ionised compounds, making it harder for them to pass through the cell membrane. Wiczkowski *et al*. (2008) reported that quercetin 3-glucoside is better absorbed than quercetin aglycone which is opposite with what found the study results according to the log *P* values. The study explains that the quercetin aglycone cannot be compared with quercetin glucoside from a dietary source due to the food matrix, which may affect absorption and could behave differently; also because pure quercetin is hard to dissolve in the digestive tract (Wiczkowski *et al*., 2008).

The chemical structure may have an effect on the partition coefficient as well. Galvano *et al.* (2009) addressed the reasons for the poor bioavailability of anthocyanins. First, because anthocyanins are unstable chemicals, changes in temperature or pH could affect their absorption; therefore, only four forms could be absorbed into the GI tract: flavylium, hemiketals, quinoid base and chalcones. Second, glucuronidation and/or sulfation could reduce the stability of the compounds (Galvano et al., 2009). In addition, using HPLC can only determine one form of anthocyanin, which is the red flavlyium (McGhie and Walton, 2007; Carkeet *et al.*, 2008). Another factor may be that anthocyanins are transformed into metabolites such as protocatechuic acids or hydroxyl benzoic acids, and these compounds are not considered to be anthocyanin derivatives (McGhie and Walton, 2007).

Ruiz-Ángel *et al*. (2011) found that log *P* values for ionisable molecules were inconsistent due to the pH of the aqueous phase. For aqueous phase compounds that have ions in their chemical structure, this depends on the pH, and that could affect the log *P* value.

All anthocyanins standards were salt of weak acids according to Pandit (2007) the salt form did not change the pk_a therefore the solubility of weak acids and their salts behaved the same as they did not fully ionised in aqueous solution. The unionised part of weak acid with pk_a around 6 and at pH 7 diffused through the lipid bilayer due to the driving force for diffusion and once some weak acid reached the receive side of membrane it will ionised .Unionised part and the diffusion will continue across membrane in slow rate until reach the equilibrium, so the concentration of un ionised the same in both side. If the pk_a different with pH the equilibrium will take longer time to achieve. In the current study, the pk_a of cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-sambubioside and delphinidin 3-sambubioside were 6.01, 6.37, 6.39 and 6.37 respectively. Therefore, the unionised part was diffused through the lipid bilayer and minority of the compounds ionised. Unionised part and the diffusion continued across membrane in slow rate due to the differences between the pk_a and pH.

According to Sangster (1989) phenols were weak acids and minor part of the compounds ionised in water at pH 7. Also reported that the effect of temperature on log P value were insignificant less than 0.01. The log P value decreased with increasing the temperature.

Borrirukwisitsak *et al.* (2012) examined the hazard of Bisphenol A and toxicity in the environment by using octanol-water partition coefficient in different conditions (salinity, different pH and temperature) using shake flask method. The results indicated that increasing the pH was increased the log P value, for example changed the pH from 6 to 8 increased log P value from 3.39 to 3.47 the change was 0.08. In addition log P was decreased with increasing the temperature between 25 °C and 45 °C the value decreased from 3.42 to 3.18 the changed was 0.24.

The current study was determined log *P* value of anthocyanins at 25 °C (room temperature) not 37 °C which is the temperature of human body fluids and according to the previous two studies they found that increasing temperature increased the log *P* value but the increased was insignificant.

Anthocyanins are very soluble in water due to their ionised form. The separation and concentration of the compound between the aqueous phase (hydrophilic) and lipids (hydrophobic) phase is very important to know if the compound has the ability to cross by passive diffusion or by transporter (Luo *et al.* 2011). Also, increasing hydrophilicity increases the amount of ionised part of the compound, which cannot pass the cell membrane by passive diffusion.

From the retention times in table 2 and figures 3 and 4 delpinidin 3-sambubioside elutes first, then the 3-mono-glucoside and finally the aglycone form. Valls *et al.* (2009) reported that B ring of anthocyanins had a major effect on the separation of anthocyanins and depends on the level, nature and number of groups of replaced on the B ring. The polarity of the sugar might have an effect on the separation of anthocyanins, with three groups of hydroxyl, which are likely to separate first (delphinidin, petunidin and malvidin), followed by anthocyanins with two hydroxyl groups (peonidin, cyanidin) (Valls *et al*., 2009). According to Qiu *et al.* (2009), polar compounds separated first due to low similarity with the stationary phase. Sometimes, the stationary phase of the column could reduce the amount of separated polar anthocyanin compounds due to the high polarity of anthocyanins.

From an observational point of view, the percentage recovery of the anthocyanins varied. Escribano-Bailon *et al.* (2004) found that anthocyanins became more stable if the number of methoxy groups increased in the B ring and if the pH in an acid range that could increase the stability. Therefore, in the current study cyanidin was

more stable and had high percentage of recovery than delphinidin in anthocyanin standards alone or with water extract added but not *H. sabdariffa* powder. Diglucoside was also more stable compared with monoglucoside. Anthocyanin structures, number of hydroxyl groups, number and position of sugars and the aliphatic or aromatic carboxylates attached to the sugar all had an effect on stability, equilibrium and antioxidant activity (Valls *et al*., 2009). In addition, pH could affect recovery because HPLC will just determine the anthocyanins in the red flavylium form.

During the experiment, it was found that after drying the anthocyanin standard in the Genevac and adding the octanol and buffer, some of the standard did not dissolve, which could have affected the recovery, especially for delphinidin and malvidin.

The observation could be explained as Bridle and Timberlake (1997) reported, in the interaction between anthocyanin products and metabolites in the small intestine, produce (insoluble complex) could be bound to the bile site and lower the amount of anthocyanins absorbed. Therefore, *in vitro*, the same thing could happen in that a part of the anthocyanins are hydrolysed and yield complex compounds that cannot be dissolved in buffer or octanol phases, which lowers the amount of compound that can be detected in each phase. In general, the recovery for anthocyanins could be varied due to part of the anthocyanin not dissolving or being located in the interface between the octanol and the buffer phase. As , Luo *et al.* (2011) reported that some compounds can be absorbed at the interface between both polar and nonpolar phases, which means that they can be used as stabilising agents in emulsions. They also found that tiliroside, which has a glucoside sugar moiety, accumulates in interface and that is why it could be used as a stabiliser-due to the effect that it has on stabilising emulsions (o/w).

Another explanation for low standard recovery with *H. sabdariffa* powder could be due to adding *H. sabdariffa* powder increase the anthocyanins distributed in the octanol phase due to the presence of fibre, which acts as a carrier, or that the amount of nonpolar compounds was high, which is why the recovery increased for delphinidin, malvidin and cyanidin 3-glucoside. Or non-extractable fibre could bond with some anthocyanin compounds and reduced the recovery or still part of the anthocyanins attached to the cell wall.

Chapter 6 Effect of *H. sabdariffa* on reducing blood pressure

Summary

- This study revealed that the consumption of *H. sabdariffa* drink had a significant effect on the reduction in systolic blood pressure (SBP) in healthy adults.
- The reduction in SBP was small compared with the baseline, but still significant, possibly suggesting that the extract could have the ability to reduce blood pressure (BP) in hypertensive patients and contribute to a reduction in cardiovascular disease.
- Consumption of cranberry juice had an insignificant effect on SBP and diastolic blood pressure (DBP).
- The mechanism of *H. sabdariffa* on reducing BP is unclear. It may be due to one or several mechanisms.
- The unique anthocyanin bioactive compounds cyanidin 3-sambubioside and delphinidin 3-sambubioside may be responsible for reducing BP.

6.1 Introduction

6.1.1 Definition of hypertension

Lim (2007) and Beevers *et al.* (2007) defined hypertension (HTN) as a high level of BP, which is controlled with antihypertensive drugs. Using population-level data, Lim (2007) defined HTN as a continuous increase in SBP in the range of 140 and 159 mmHg or as a continuous increase in DBP in the range between 90 to 99 mmHg. The WHO (2013) defined HTN as increased BP, a condition in which the blood vessels have insistently elevated pressure. When blood pressure is increased in blood vessels it is very hard for the heart to pump blood. WHO defined HTN as a SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg.

Mackenzie *et al.* (2005) defined HTN as a polygenic disorder involving environmental and genetic factors. Mackenzie et al. (2005) stated that practitioners define HTN as "a level of BP that is associated with a significantly increased risk of cardiovascular disease" (Mackenzie *et al.*, 2005, pp. 4-5). The current study adopted the population definition of HTN used by Lim (2007) because it is more precise than other definitions.

6.1.2 Types of hypertension

According to Lim (2007), HTN can be classified into two types: essential (primary) and secondary. Essential HTN is responsible for 95% of cases and may be due to interactions between genetic and environmental factors or one of these factors. Secondary HTN affects about 5% of patients and is usually the result of kidney disease, heart disease or endocrine system disease (Beevers *et al.*, 2007; Lim, 2007).

It may also be due to the intake of long-term medications (Beevers et al., 2007; Lim, 2007).

6.1.3 Prevalence of hypertension

According to a WHO (2013) report, cardiovascular disease is responsible for one-third of deaths every year. HTN is the main cause of death in 45% of those with heart disease and 51% of those with stroke. The WHO, HTN organisations and heart organisations have focused on HTN recently due to the elevation in the number of untreated individuals, individuals with uncontrolled BP and individuals not being diagnosed (Lim, 2007; WHO, 2013). HTN increases the risk of cardiovascular disease and Lim (2007) reported that DBP can be used to predict death and illness from cardiovascular disease.

The management of HTN can reduce the risk of cardiovascular disease and stroke. Lim (2007) and WHO (2013) stated that a decline in the DBP between 5 and 6 mmHg reduces heart disease-related mortality by 20–25% and stroke-related mortality by 40% per year. Beevers *et al*. (2007) mentioned that maintaining SBP at 140 mmHg reduced deaths from stroke by 28–44% and deaths from heart disease by 20–35% per year.

Geographic region influence HTN, with the highest percentage (46%) of HTN found in the African region and the lowest in the U.S (35%). Moreover, high-income countries tend to have the lowest incidence of HTN (35%) compared with low-income countries, which have the highest incidence (40%) (WHO, 2013). Ethnic origin also has an effect on HTN. Beevers et al. (2007) reported a high BP among African people living in cities compared with Caucasians who had a lower BP. The high BP may be related to high consumption of salt, with Beevers *et al.* (2007)

finding that Africans in the U.K. and the U.S. consumed a large amount of salt compared with Africans in Europe. In addition, epidemiological studies found that African-Americans had a low amount of renin and angiotensin in their plasma compared with Africans in European countries. According to Mackenzie *et al*. (2005), in Western countries the presence of HTN increased the risk of stroke seven-fold.

Gender also affects BP before the age of 50. Beevers *et al*. (2007) found that women younger than 55 years old have a low risk of cardiovascular disease compared with men of the same age. In addition, an increase in high BP is associated with men and with increase of age. Before the age of 50 men tend to have a higher BP than women, but after the age of 50 women and men had the same level of BP. Beevers *et al*. (2007) also found that in western societies if a person's BP was high at baseline, their BP increased at a faster rate over the years compared with normal person. Moreover, age affected the relationship between essential HTN and an increase in DBP and SBP in people younger than 50 years (Mackenzie *et al*., 2005). Beevers *et al.* (2007) stated that with increasing age, BP and complications from HTN increased. Lim (2007) also confirmed that gender and ethnic origin contributed to HTN.

Usually, individuals with HTN also suffer from hyperlipidaemia, diabetes and decreased glucose intolerance (Beevers *et al*., 2007). The risk of cardiovascular disease increased in smokers with HTN (Beevers *et al*., 2007). Beevers *et al*. (2007) concluded that HTN is caused by multiple factors and that treating one of these will not reduce the risk of stroke and heart disease because the other factors also influence BP.

6.1.4 Causes of hypertension

The WHO (2013) listed several factors that could contribute to essential HTN, such as an increase in the population, which reduces the chances of good living conditions, and the ability to find a job, which increase stress, the consumption of large amounts of alcohol, a lack of physical activity, an increase in body weight, stress, ageing, individual behaviour and life style, high cholesterol in the diet and plasma, diabetes mellitus and salt intake. Lim (2007) noted that obesity contributes to HTN. Figure 6-1 illustrates the causes of HTN.

Factors such as weight, salt, alcohol intake and exercise should be managed carefully to help prevent increased BP (Mackenzie *et al*., 2005).

Beevers *et al*. (2007) declared that salt sensitivity affected BP, with a positive correlation found between sodium excreted from urine and BP. The same study found that a high intake of potassium was associated with reduced BP. Mackenzie *et al*. (2005) reported a positive correlation between sodium excretion and SBP, and the correlation was strong in older subjects with high a proportion between aldosterone and renin. Mackenzie *et al*. (2005) reported that a reduction in BP was associated with a decrease in salt intake. Salt sensitivity varies between humans due to genetic effects. A restricted salt diet decreased SBP in hypertensive patients by 6 mmHg and DBP by 2 mmHg compared with pre-hypertensive patients where SBP reduced by only 1 mmHg (Mackenzie *et al*., 2005).

Weight was correlated positively with increasing BP in overweight people. Weight reduction and weight management decreased the SBP and DBP of healthy individuals by 2.5 mmHg compared with hypertensive patients by 5.2 mmHg (Mackenzie *et al*., 2005). Each kilogram of body weight lost reduced the BP by 1 mmHg (Beevers *et al*., 2007). In overweight individuals, vasoconstriction and renin

formation increased in the kidneys due to influence from sympathetic nervous system (Mackenzie *et al*., 2005).

In terms of alcohol consumption, a positive relationship was found between BP and alcohol consumption. This relationship may be due to the excitation of the sympathetic nervous system or an increase in adrenocorticoid hormones excreted from the adrenal gland (Beevers *et al*., 2007). Decreasing the consumption of alcohol lowered the SBP (5 to 8 mmHg) and DBP (2 to 3 mmHg) of hypertensive patients. Although the reduction was minor, it was equal to the effect of hypertensive drugs. Weight loss is associated with reduced consumption of alcohol. Therefore, people are advised to maintain their level of alcohol intake to two units/day (Mackenzie et al., 2005).

According to Mackenzie *et al*. (2005) moderate exercise also had a long-term effect on SBP and DBP, reducing it by 8 mmHg and 4 mmHg, respectively. To maintain these benefits, people are advised to undertake physical activity three to four times a week for 45–60 min (Mackenzie *et al*., 2005).

6.1.5 Pathophysiology of hypertension

Lim (2007) stated that the pathophysiology of HTN is very complicated because HTN is affected by many variables. There is no one clear reason or factor that can explain the 95% incidence of essential HTN. Only a minority of people (2–5%) with certain types of diseases suffer from secondary HTN. Many physiological mechanisms are involved in the development of HTN. According to Beevers *et al*. (2007), there are three important factors that affect HTN: the balance between the cardiac output and peripheral resistance, the renin-angiotensin-aldosterone system and the autonomic nervous system.

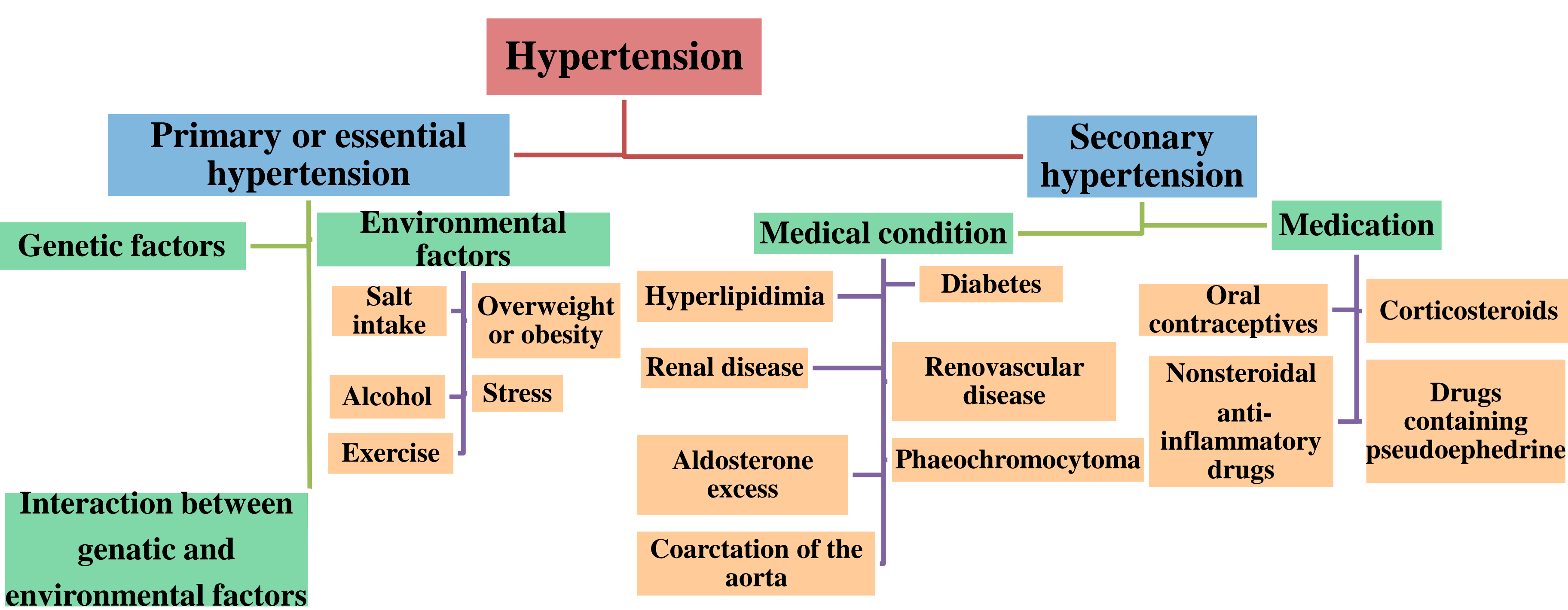


Figure 6-1. Causes of hypertension Environmental and genetic factors and the interaction between factors lead to primary hypertension. Chronic diseases and medication contribute to secondary hypertension adapted from (Beevers *et a*l., 2007; Lim, 2007).

6.1.5.1 Balance between cardiac output and peripheral resistance

People suffering from essential HTN usually have a normal level of cardiac output and an increase in peripheral resistance (which is the force against blood flow). An increase in peripheral resistance increases vasoconstriction, which would decrease blood flow (Beevers *et al*., 2007; Lim, 2007). The increase in the peripheral resistance in patients with essential HTN is because of the abnormal activity of the sympathetic nervous system (Beevers *et al.*, 2007; Lim, 2007). The cardiac output and the peripheral resistance should be balanced to maintain normal tissue perfusion (Beevers *et al*., 2007; Lim, 2007). Usually, peripheral resistance is measured by the small arterioles. When contracted smooth muscle cells located in the arterioles have an increased calcium level in the intracellular cells, this leads to an increase in the wall thickness, narrowing of the vessels, and an increase in BP (Beevers *et al*., 2007; Lim, 2007).

6.1.5.2 Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system plays a crucial role because of its effect on three important factors: kidney function, BP and the balance between water and salts (Mackenzie *et al*., 2005). Indirectly, pathophysiological effects of the renin-angiotensin-aldosterone system increase BP (Mackenzie *et al*., 2005).

Renin may be excreted by the kidney or the sympathetic nervous system, or it may be excreted as a result of a deficiency in sodium. Renin substrate (angiotensinogen) is converted to angiotensin I by renin, the angiotensin-activating enzyme (ACE) then converts angiotensin I to angiotensin II, which has a vasoconstriction effect (Mackenzie *et al*., 2005; Beevers *et al*., 2007; Lim, 2007). This results in an increase in the BP and causes organ damage. Also any increase in renin or aldosterone levels

may lead to the development of HTN (Mackenzie *et al*., 2005; Beevers *et al*., 2007; Lim, 2007). In addition, angiotensin II causes aldosterone to be secreted from the adrenal gland. Aldosterone increases the accumulation of sodium and fluid in the body, which leads to an increase in the BP and the development of HTN (Mackenzie *et al*., 2005; Beevers *et al*., 2007; Lim, 2007). On the other hand, this system can also reduce a high BP via one of two routes. The first involves inhibition of ACE, and the second involves blocking the angiotensin receptors (Mackenzie *et al*., 2005; Beevers *et al*., 2007; Lim, 2007). Figure 6-2 shows the role of the renin-angiotensin-aldosterone system in the development of HTN.

Aviram and Dornfeld (2001) mentioned that ACE inhibitors reduce the percentage of cardiovascular disease by decreasing BP and preventing the accumulation of platelets and lipid peroxidation. The role of ACE inhibitors is to prevent the conversion of angiotensin I to angiotensin II by inactivation of the ACE enzyme (Aviram and Dornfeld, 2001). In addition, ACE controls nitric oxide (NO) and rennin-angiotensin in endothelial cells and inactivate the vasodilation (relax the smooth muscles cells which lead to widening blood vessels), inactivation of ACE via inhibitors, such as polyphenols, reduces BP (Hellström et al., 2010).

6.1.5.3 Autonomic nervous system

The autonomic nervous system plays an important role in maintaining BP in a normal range in case of stress or physical activity. Any motivation in the system causes arteriolar contraction and dilation, depending on the type of receptors (i.e. inhibitory or excitatory). Systolic dysfunction activates the system, which has an important role in heart failure (Beevers *et al*., 2007; Lim, 2007).

6.1.5.4 Auto-regulation in hypertension

Auto-regulation prevents arterial and arteriolar vasodilatation, Unsuccessful auto-regulation occurs when the BP is above 160/100 mmHg, which leads to an elevation in arterial pressure and vascular damage (Lim, 2007). Peripheral vasoconstriction in peripheral organs leads to ischaemia of the brain, the activation of neurohormones and the section of cytokines (Lim, 2007). Endothelial function and natriuretic peptides affect auto -regulation in HNT (Lim, 2007).

6.1.5.5 Endothelial function

Endothelial function plays a critical role in heart disease, and it is usually measured as an indicator of organ damage and heart disease (Beevers *et al*., 2007; Lim, 2007). Endothelial cells secrete important substances, such as the vasoconstrictor peptide endothelin (confers salt sensitivity and increases BP) and bradykinin (works as a vasodilator, which is inhibited by ACE) (Beevers *et al.*, 2007; Lim, 2007). NO, which is released from endothelial cells, affects blood flow, vascular tone (the degree of blood vessel constriction to achieve maximum dilated state) and BP (Beevers et al., 2007; Lim, 2007). NO enters the smooth muscles cell through the vessels and leads to vasodilatation (Beevers et al., 2007; Lim, 2007). In essential HTN, imbalance between the vasodilators and vasoconstrictors permanently damaged the endothelial function and changed the vascular tone of the cells (Beevers *et al*., 2007; Lim, 2007).

Zibadi et al. (2007) stated that endothelial nitric oxide (eNO) is very important to maintain vascular homeostasis, which is the balance between injured and repaired vasculature. They further noted that NO from the inducible NO synthase enzyme (iNOS) can increase BP and the (iNOS) enzyme in some organs, such as the aorta and kidney, decrease levels of endothelial synthase (eNOS) and increase levels of

these enzymes in plasma (Zibadi *et al*., 2007). Shaughnessy et al. (2009) described the role of oxidative stress in increased BP following the production of reactive oxygen species (ROS) in vascular endothelial cells and kidney cells by NADPH oxidases. This leads to inflammation in endothelial cells, destruction of the endothelium, lowered NO bioavailability or inflammation in kidney cells. Subsequently, there is an imbalance between O_2 and NO formation in the kidney, leading to salt-sensitive HTN (Shaughnessy *et al*., 2009).

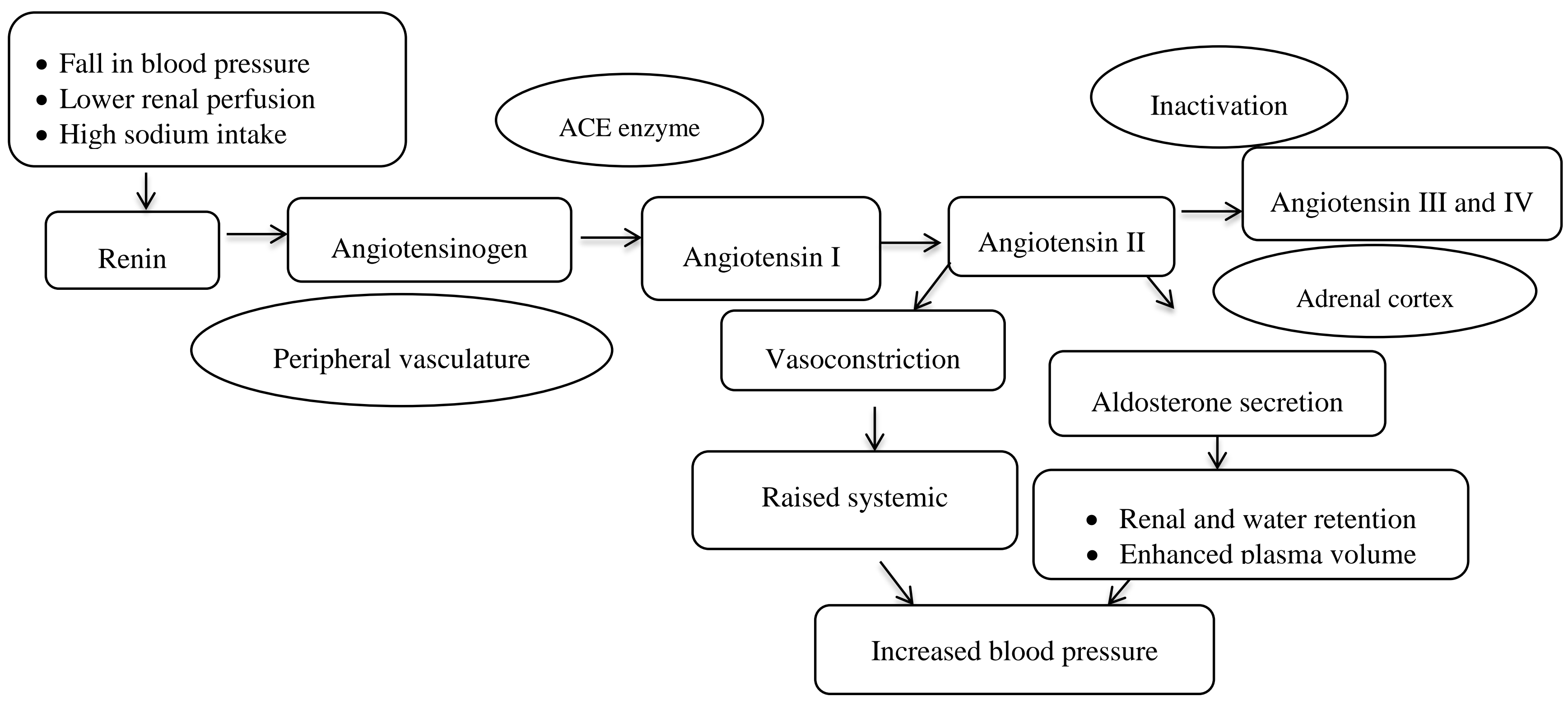


Figure 6-2. The mechanism of renin-angiotensin-aldosterone system, that leads to increase BP. An increase of salt intake, low perfusion and low BP stimulates the kidneys or sympathetic nervous system to secrete renin, which convert to angiotensin substrate and activates the ACE enzyme, which converts angiotensin I to II. This leads to the secretion of vasoconstrictor substances, which raise the vascular resistance and increase the BP, leading to the release of aldosterone. Subsequently, there is an increase in the salt inside the cells, causing water retention in the kidney and an increase in BP adapted from (Mackenzie *et al*., 2005; Lim, 2007).

6.1.5.6 Natriuretic peptides

Natriuretic peptides cause naturesis, which is the excretion of an abnormal amount of sodium in the urine (Alarcón-Alonso *et al.*, 2012)

The atria of the heart are release important hormones, atrial natriuretic peptide (ANP), and secrete brain natriuretic peptide (BNP) from the left ventricle. BNP is an indicator of ventricular systolic dysfunction (Beevers *et al*., 2007; Lim, 2007). An elevation in ANP levels increases the secretion of sodium and fluid from the kidney (Beevers *et al*., 2007; Lim, 2007). Fluctuations in the ANP hormone cause accumulation in fluid in the extracellular and HTN, and any increase in BNP reduces the systolic dysfunction. The gene responsible for induced HTN is not known (Beevers *et al*., 2007; Lim, 2007).

6.2 Aim of the study

The study aimed to examine the effect of cranberry juice and *H. sabdariffa* drink that contain anthocyanins on reducing BP in healthy subjects after eight weeks.

6.3 Importance of the study

To the best of our knowledge, this is the first study to investigate the effect of cranberry juice and *H. sabdariffa* drink contain anthocyanins on reducing BP in healthy subjects after a long period (eight weeks). Numerous studies have investigated different types of anthocyanins but only in pre-hypertensive or hypertensive volunteers (Haji Faraji and Haji Tarkhani, 1999; Herrera-Arellano *et al*., 2004; Mozaffari-Khosravi *et al.*, 2009b; McKay *et al.*, 2010). The results of this study could be distributed in the community and suggest the drink for healthy people as a precaution to reduce cardiovascular disease.

6.4 Sample size

Theoretical calculations were made to determine the sample size and the required number of individuals needed to obtain a representative sample and maintain a realistic approach in accordance with the statistical probabilities of the study. The sample size was calculated using a confidence level of 95%, corresponding to a z level of 1.96. The sampling error was set at 0.05. The sample size was selected to detect a clinically relevant difference in SBP between the intervention and the placebo groups. A previous study reported that the consumption of a beverage prepared with *H. sabdariffa* reduced SBP by 17.66 ± 6.8 mm Hg (mean ± SD) in hypertensive patients (n=54) (McKay *et al*., 2010).

On the basis of the McKay *et al.* (2010) study, a sample size of 30 participants/group is sufficient to detect a 5 mm Hg change in SBP, with 80% power and $P = 0.05$.

6.5 Materials and method

Prior to commencement, the study was approved by the MaPS and Engineering Ethics Committee (MEEC) (11-009) committee. The total phenols, total anthocyanins and antioxidant capacity of Ocean Spray cranberry juice and Simply Hibi drink which is a water extract of dried *H. sabdariffa* were analysed using DPPH, FRAP and TEAC. Further details about the procedure are available in Sections 2.6.2 to 2.6.8 in Chapter 2 of the Materials and methods. The amount of major and minor anthocyanins (cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-sambubioside and delphinidin 3-sambubioside) in both juices was determined with the HPLC method described in Section 2.6.8 of Chapter 2 in the Materials and methods.

6.6 Calculation of the amount of juice consumed in the study

Concentrated cranberry Juice and *H. sabdariffa* drink (35 ml) were used in the study. This amount was calculated based on the study of McKay *et al.* (2010) on *H. sabdariffa* tea. The same total amount of anthocyanins as was used in the study by McKay *et al*. (2010) in *H. sabdariffa* tea (21.12 mg/3 serving), was used to calculate the amount of juice of *H. sabdariffa* should consumed in the current study.

6.7 Selection of the placebo

Cranberry juice was used as the placebo in the current study for several reasons. First, it is very difficult to prepare a unique placebo because of health and safety concerns. The subject will consume the placebo for a long period (eight weeks), and it is hard to pasteurise a placebo to ensure it remains free of pathogens or fungi during the full eight weeks of the study. Second, cranberries have a red colour and a similar sour taste to that of *H. sabdariffa*, although cranberries are light red and *H. sabdariffa* are dark red. Both are also contain anthocyanins. Therefore, the participants will not be able to distinguish the juices upon examination. Third, Dohadwala *et al.* (2011) found that cranberry juice has no effect on BP.

6.8 Study design

The study was a randomised controlled crossover study with 29 healthy subjects. The study was started after the subjects signed consent forms. The design and the criteria of the study are described in Chapter 2 in the materials and methods sections (2.6.11.2 and 2.6.11.3). Also figure (6-3) shows a brief summary of the study designed.

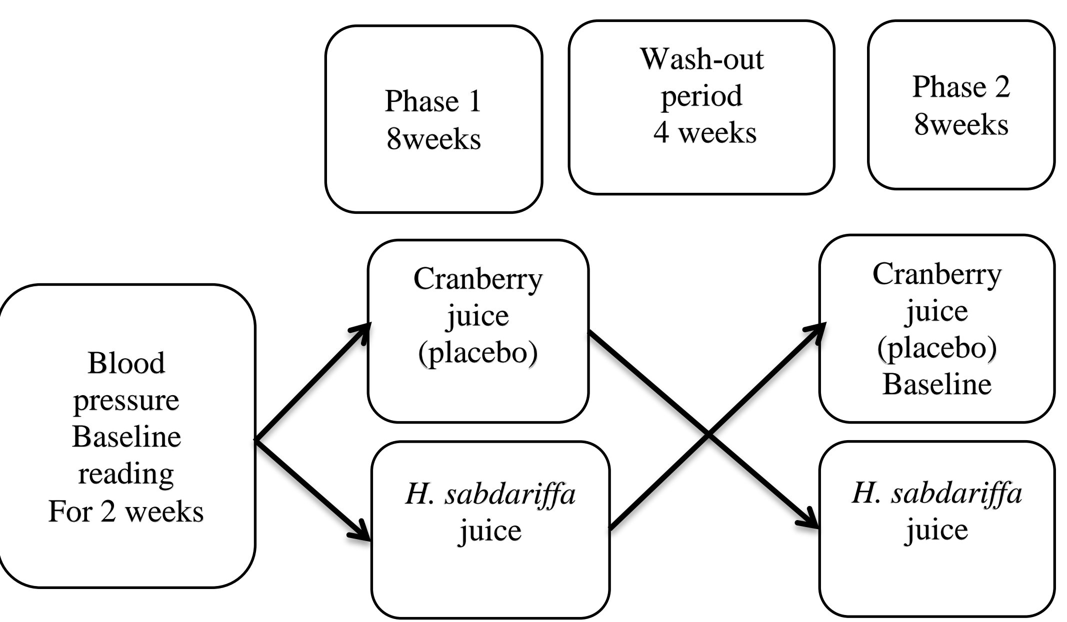


Figure 6-3. Study design

Subjects (29) were randomly assigned in the study. They consumed 35 ml of cranberry juice diluted up to 250 ml water/day for 8 weeks. BP reading was measured every week. After 8 weeks, there were rest period 4-weeks, and then subjects crossed over to consume 35 ml of *H. sabdariffa* drink diluted up to 250 ml water/day for 8 weeks or via versa.

6.9 Compliance

Only 29 of 30 subjects completed the study. Two participants dropped out during the study.

6.10 Results

6.10.1 Analysis of the cranberry and *H. sabdariffa* juices used in the study

Table 6-1 shows the total anthocyanins, total phenol content and antioxidant capacity of the juices studied. The amount of anthocyanins and the antioxidant capacity values in the FRAP and TEAC assays were higher in the Simply Hibi drink than in the Ocean Spray cranberry juice by 24.5-, 5- and 2.5-fold, respectively. The phenol content and the DPPH scavenging activity were 2.5-fold and 1-fold greater, respectively, in the Ocean Spray cranberry juice than in the Simply Hibi drink.

Juice	[1]Total anthocyanin mg/g	Total phenol mg/g	DPPH %	FRAP mg/g	TEAC mg/g
Ocean Spray cranberry	0.02 ± 0.002	85.36 ± 2.05	98.47 ± 0.01	2.15 ± 0.01	3.77 ± 0.22
Simply Hibi *H. sabdariffa*	0.49 ± 0.01	34 ± 0.24	89.90 ± 0.07	10.97 ± 0.21	9.42 ± 0.38

Table 6-1. Total anthocyanins, total phenols and antioxidant capacity of the juices were used in the human study. [1]Cyanidin glucoside equivalents. (*n*=3, ± SD)

The concentration of major anthocyanins in the cranberry and the *H. sabdariffa* juices was analysed using reverse phase HPLC. Table 6-2 shows the type of major anthocyanins differed in both juices, but when the result of total anthocyanins was compared with HPLC anthocyanins after combined the compounds together the amount of total anthocyanins in general was similar to the anthocyanins identified by HPLC. Obviously Simply Hibi had a high concentration of anthocyanins. The cranberry juice contained more cyanidin 3-glucoside (0.13 mg/ml) than delphinidin 3-gulcoside, and Simply Hibi had a greater concentration of cyanidin 3-sambubioside (0.34 mg/ml) than delphinidin 3-sambubioside.

Juice	Cy 3-sam [1] mg/ml	Del 3-sam [2] mg/ml	Cy 3-glu [3] mg/ml	Del3-glu [4] mg/ml
Ocean Spray cranberry	ND	ND	0.13 ± 0.008	0.05 ± 0.002
Simply Hibi ***H. sabdariffa***	0.34 ± 0.009	0.19 ± 0.005	ND	ND

Table 6-2. Concentration of major anthocyanins identified by HPLC in juices used in the human study, [1] cy 3-sam = cyanidin 3-sambubioside, [2] del 3-sam = delphinidin 3-sambubioside, [3] cy 3-glu = cyanidin 3-glucoside and [4] del-3glu = delphindin 3-glucoside (ND= not detected). (n=3, ± SD)

6.10.2 Demographic and medical data of study participants

The participants completed a short medical questionnaire before starting the study and signed a consent form. Table 6-3 illustrates the percentage and the frequency of participant's health status results from the questionnaire. The vast majority of the volunteers were female (87%). All the subjects were in good health. Only a minority of the subjects (six subjects) were taking routine medication (oral contraceptives) or suffered from allergies. Approximately 3% of the participants smoked 10 cigarettes per day or had a family history of heart disease. A high percentage (93%) of the volunteers had normal BP (normal BP level between 90-120 mmHg for the SBP and between 60-80 mmHg for the DBP), and 3.2% had low BP previously but not anymore (low BP level between 70-90 mmHg for the SBP and 40-60 mmHg for the DBP) BP (hypotensive), or they did not remember whether the doctor said it was normal. A high percentage (64.5%) of the subjects had checked their BP before, whereas 35.5% had not checked.

Table 6-4 shows the characteristics of the study participants. They were between 19 and 48 years old. The maximum height was 190 cm, and the minimum height was

152 cm. The average body weight and body mass index (BMI) was 64 kg and 22.3 respectively. According to the statistical data, the vast majority of the participants were of normal weight.

Categories	Frequency (n)	Percentage %
Male	4	12.9
Female	27	87.1
Health status (good)	31	100.0
Taking medications routinely	6	19.4
Not taking medications routinely	25	80.6
Abnormal BP before	1	3.2
Normal BP	29	93.5
Do not know the BP	1	3.2
BP checked	20	64.5
BP not checked	11	35.5
History of disease	1	3.2
No history of disease	30	96.8
Smoking	1	3.2
Non-smoking	30	96.8
Allergy	6	19.4
No allergy	25	80.6

Table 6-3. Frequency (n= number) and percentage (%) of health status in the short medical questionnaire

Statistic	Age	Height (Cm)	Weight (Kg)	BMI (kg/M^2)
Minimum	19	152	45	17
Maximum	48	190	91	31
Mean	30	165	63	23
Median	28	163	64	22
Mode	22	163	60	20

Table 6-4. Characteristics of the study participants

6.10.3 Effect of cranberry and *H. sabdariffa* juices on blood pressure

Tables 6-5 and 6-6 show the *p* values of the normality tests for the SBP and the DBP before consumption of the cranberry and *H. sabdariffa* juices (p = 0.863, 0. 680, p >0.05) (p = 0.045, 0.628, p >0.05) respectively, and after consumption of the cranberry and *H. sabdariffa* juices (p = 0.869, 0.188, p >0.05) (p = 0.692, 0.827, p >0.05) respectively. The results show that the SBP and DPB data for both juices before and after the intervention were normally distributed. The effect of cranberry juice on the SBP of the participants at the end of the study was non-significant when compared with baseline (103.9 ± 8.7 mmHg and 102.9 ± 9.7 mmHg, respectively; p = 0.422) with the change in the SBP - 0.96 ± 6.3 mmHg. There was also no significant effect on the DBP of the subjects before and after consuming the cranberry juice (70.9 ± 6.6 mmHg and 70 ± 7.4 mmHg, respectively; p = 0.378) at the end of the study, with the change in DBP -0.93 ± 5.6 mmHg.

As shown in Table 6-6, consumption of *H. sabdariffa* juice significantly reduced the SBP. When the SBP before the intervention (103.3 ± 10.8 mmHg) was compared with the SBP after the intervention (100.8 ± 7.2 mmHg), the change was significant (p = 0.014). The change in the SBP was – 2.58 ± 5.2 at the end of the study. There was no significant reduction in the DBP. When the DBP before consuming the juice

was compared with the DBP at the end of the study (70.9 ± 7.5 mmHg and 69.5 ± 7.2 mmHg, respectively; $p = 0.128$), the change in the DBP was - 1.4 ± 4.7. Figure 6-4 shows the changes in the SBP and the DBP for both the placebo (cranberry) juice and the *H. sabdariffa* juice.

The large differences between the SBP at the baseline with the SBP at the end of study (- 2.85 ± 5.2 mmHg) show that the H. *sabdariffa* juice reduced the SBP in comparison with the cranberry juice (- 0.96 ± 6.3 mmHg).

Cranberry	**Normality test sig.**	**Mean (mmHg) (± SD)**	**Change (mmHg)**	**Paired *T*-test sig. (2-tailed)**
Pre-intervention SBP	0.863	103.9 ± 8.7	- 0.96 ± 6.3	0.422
Post-intervention SBP	0.680	102.9 ± 9.7		
Pre-intervention DBP	0.869	70.9 ± 6.6	-0.93 ± 5.6	0.378
Post-intervention DBP	0.188	70 ± 7.4		

Table 6-5. Paired *T*-test and normality test of SBP and DBP for participants before and after consuming cranberry juice

H. sabdariffa	**Normality test sig.**	**Mean (mmHg) (± SD)**	**Change (mmHg)**	**Paired *T*-test sig. (2-tailed)**
Pre-intervention SBP	0.045	103.3 ± 10.8	- 2.58 ± 5.2	0.014
Post-intervention SBP	0.628	100.8 ± 7.2		
Pre-intervention DBP	0.692	70.9 ± 7.5	- 1.40 ± 4.7	0.077
Post-intervention DBP	0.827	69.5 ± 7.2		

Table 6-6. Paired *T*-test and normality test of SBP and DBP for participants before and after consuming *H. sabdariffa* juice

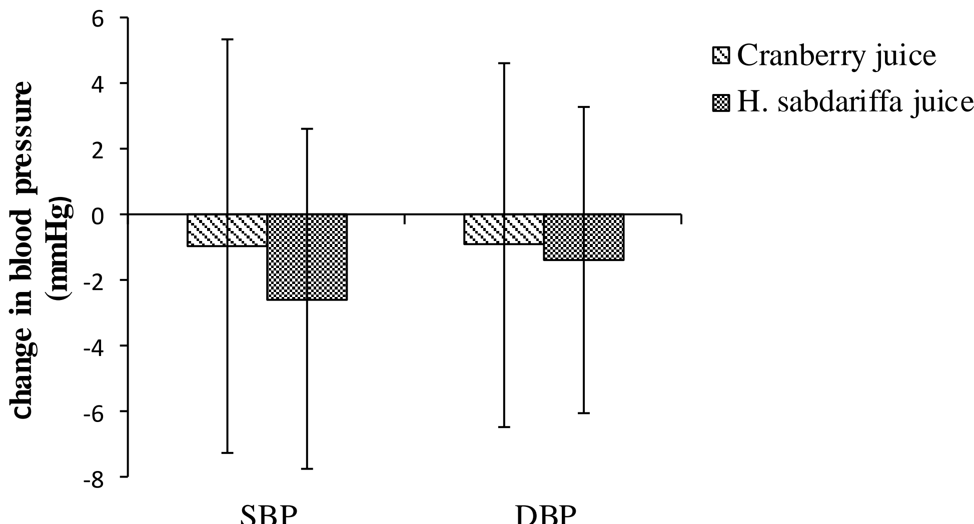


Figure 6-4. Effect of *H. sabdariffa* extract on SBP and DBP in healthy subjects. The BP at the baseline was compared with the BP after consuming the placebo juice (cranberry) and the *H. sabdariffa* juice for eight weeks. The results demonstrated that the *H. sabdariffa* juice reduced the SPB compared with the placebo. Values are means ± standard error (n=29).

Figure 6-5 shows the SBP and DBP for subjects consuming cranberry juice and *H. sabdariffa* juice pre-intervention and post-intervention. It is clear from figure 6-5 (a) that SBP pre-intervention and post-intervention was the same, while there was a slight significant reduction in SBP post-intervention compared with pre-intervention when *H. sabdariffa* juice was consumed. On the other hand, there was no significant differences in DBP readings during the 8 weeks when DBP was compared between the pre- intervention and post-intervention for both cranberry and *H. sabdariffa* juices at the end of the study figure 6-5 (b). In addition, figure 6-6 illustrates the accumulation effect and the changes in SBP and DBP for participants during the whole 8 weeks consuming either cranberry or *H. sabdariffa* juice. From Figure 6-6 (a) it is clear that the decline in SBP following consumption of *H. sabdariffa* juice started from week 2 until week 5, then a slightly increased SBP was observed in week 6, but the SBP fell again at the end of week 8. The opposite effect on SBP in participants consuming cranberry juice was observed. SBP increased considerably

from week 2 until week 5 then declined at week 6 then increased again until the end of the study. Figure 6-6 (b) shows the DBP for both cranberry and *H. sabdariffa* juice during the 8 weeks. Participants who consumed *H. sabdariffa* juice showed a sharp decline in DBP until week 2 then it increased in week 3 and after week 3 the DBP was reduced slightly and maintained until the end of the study. In cranberry juice SBP decreased sharply during the first week, then DBP rose gradually until week 4. After week 4 the DBP declined until week 6 and increased after that to the end of the study.

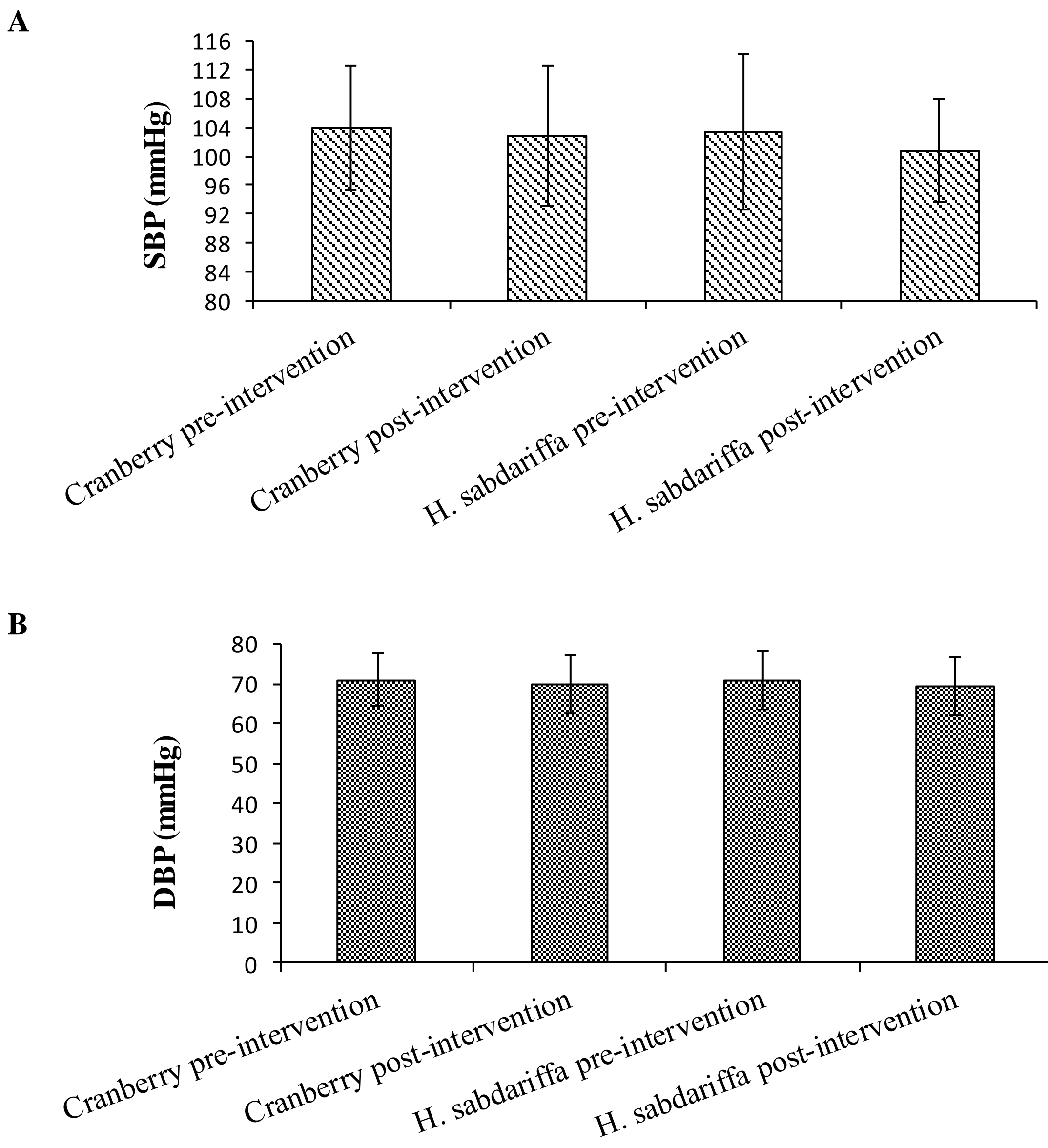
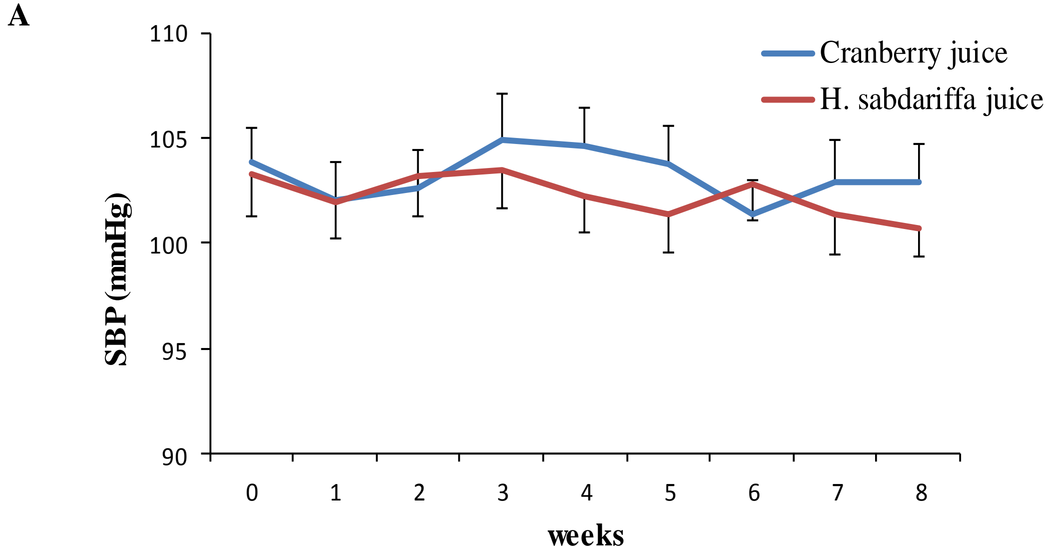


Figure 6-5. (A) Systolic blood pressure (SBP) (mmHg) and (B) Diastolic blood pressure (DBP) (mmHg) of healthy volunteers before and after consumig cranberry or *H. sabdariffa* juices for 8 weeks. Values are means (n=29) ± SD

A



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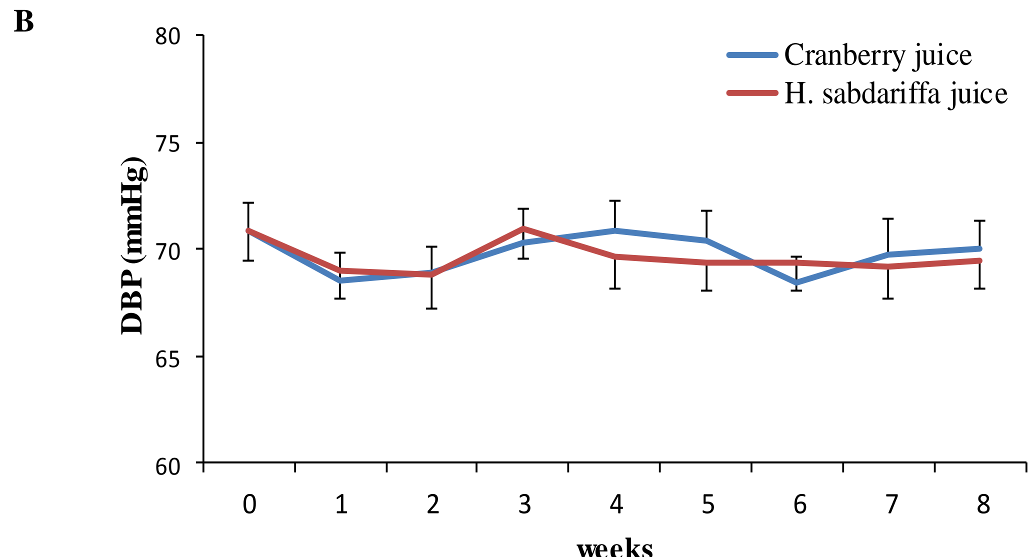
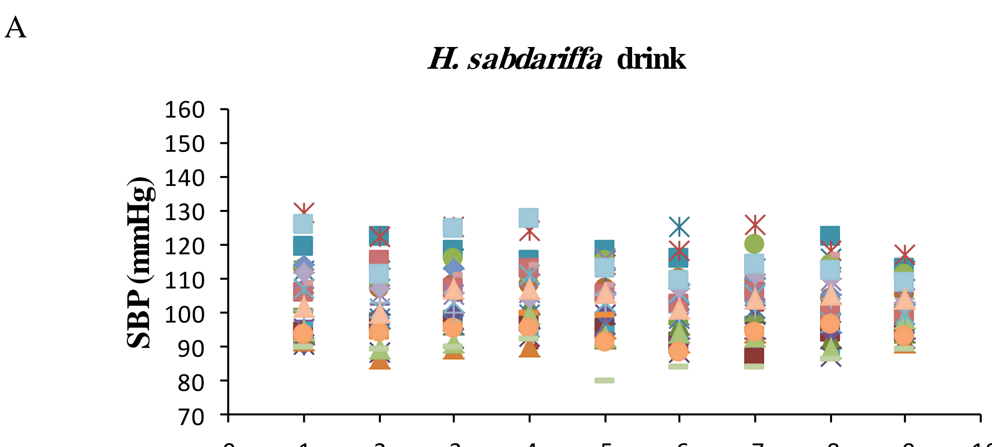


Figure 6-6. Effect of cranberry and *H. sabdariffa* juices for 8 weeks on BP of participants (n=29) showing the mean of triplicate BP readings ± SEM. (A) systolic blood pressure, SBP, (B) diastolic blood pressure, DBP.

A



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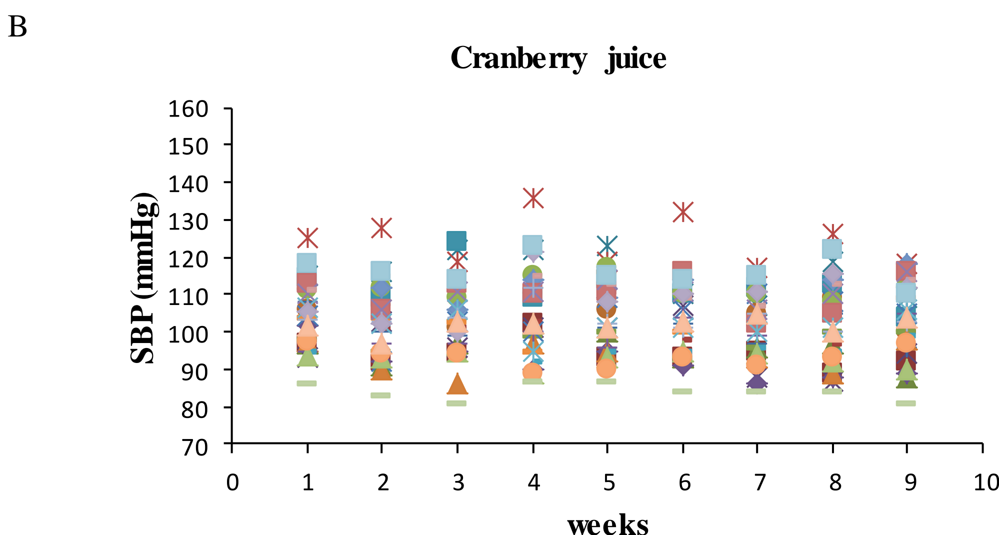
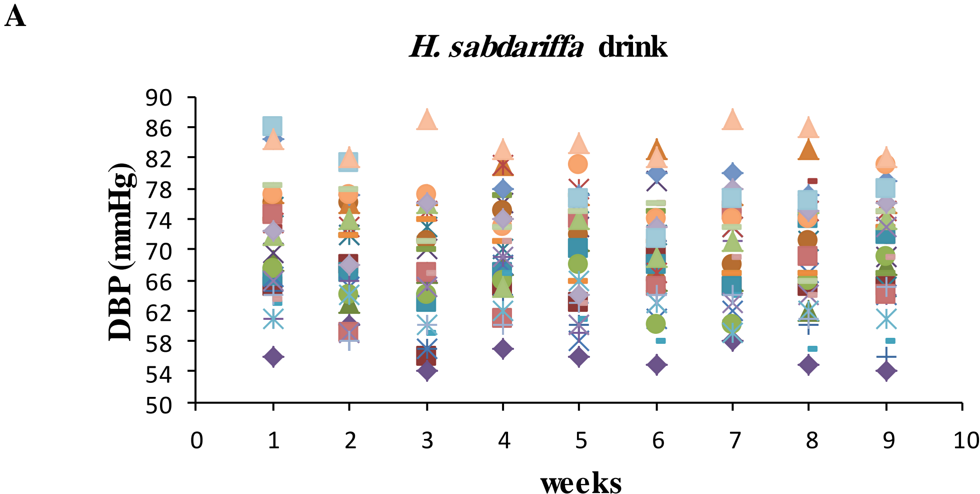


Figure 6-7. (A) Systolic blood pressure (SBP) (mmHg) of participants consumed *H. sabdariffa* drink , (B) Systolic blood pressure (SBP) (mmHg) of participants consumed cranberry juice for 8 weeks. The readings of SBP are average of triplicates reading each week (n=29)

A



B

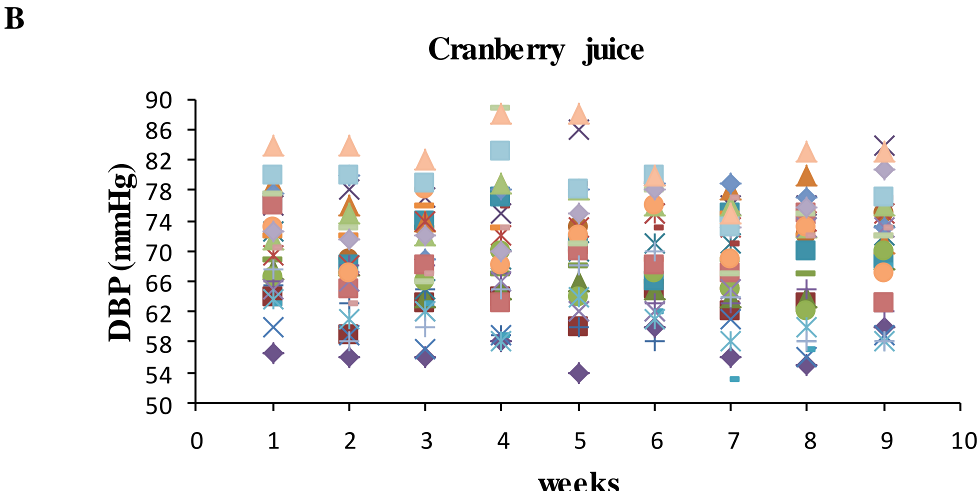


Figure 6-8. (A) Diastolic blood pressure (DBP) (mmHg) of participants consumed *H. sabdariffa* drink , (B) Diastolic blood pressure (DBP) (mmHg) of participants consumed cranberry juice for 8 weeks. The readings of DBP are average of triplicates reading each week (n=29)

Figure 6-7 and figure 6-8 show the mean of individuals reanding of SBP and DBP in the baseline and during the 8 weeks of consumption of *H. sabdariffa* drink and cranberry juice. For participants consumed *H. sabdariffa* drink the were differences in SBP reading at the end of 8 weeks compared with baseline, the SPB decreased in some indivuals. While in individuals consumed cranberry juice there were no differences between the baseline and the end of the 8 weeks. Moreover, there was variation in the SPB of individuals, some of the participants after consuming the *H. sabdariffa* drink SPB remaied the same and others had hypotesive effect on them, also cranberry reduced the SBP in some participants and increased it in others. In contrast, the DBP readings for participants consumed *H. sabdariffa* drink slightly decresed at the end of the study compared with the baseline, while remained the same in participants who consumed cranberry juice.

6.11 Discussion

The current study found that cranberry juice had no significant effect on the SBP or the DBP. This finding is in agreement with that of Dohadwala *et al*. (2011) who examined the effect of cranberry juice on vascular function in a crossover study including 44 participants diagnosed with coronary artery disease. There was a washout period of one week before commencing the study, and cranberry juice was the only type of juice allowed during the study. All the subjects consumed 480 ml of cranberry juice with different concentration (45%, 27% of cranberry juice), and the results were compared with a placebo group who consumed a drink similar in taste and colour to cranberry but that did not contain polyphenols. Each group consumed the drinks for four weeks, with two weeks rest between the two drinks. The BP was measured before the drink was consumed and two and four hours after consuming the drink. The c study found that cranberry juice had no effect on the BP and no

vasodilator effect on endothelial function. But, some of the participants the SBP reduced after consumed cranberry juice this could be due to the amount of anthocyanins in the juice could reduce the SBP, also the bioavailability of anthocyanins in participants could be varied and was more effect on reduce SBP in some participants than others.

The important finding of the current study was that *H. sabdariffa* juice had an accumulation effect and reduced the SBP after eight weeks compared with the cranberry juice.

Kalt *et al.* (2008) found that dietary anthocyanins accumulated in pig tissues after feeding pigs anthocyanins for long periods of time, remaining longer in tissues than in the bloodstream. The place of accumulation of anthocyanins still not clear if it was in cardiac or in vascular tissues, nevertheless, anthocyanins had effect on vascular reactivity (Kalea *et al.*, 2009).

In addition, Mckay *et al.* (2010) investigated the influence of consuming *H. sabdariffa* tea 3 times/day on BP compare with placebo. In 65 pre-hypertensive and mild hypertensive subjects aged from 30-70 years, BP reading was taken every week. The study was found a decline in SBP (-7.2 ± 11.7 mmHg) compared with placebo (-1.3 ± 10.0 mmHg) after 6 weeks. There was no difference in DBP between *H. sabdariffa* tea and placebo. Mckay *et al.* (2010) related the effect of the *H. sabdariffa* tea to the large amount of the major anthocyanins cyanidin 3-sambubioside and delphinidin 3-sambubioside in tea, and their effects as vasodilators, on inhibition of ACE, on controlling calcium channels or on a diuretic effect.

In the present study, *H. sabdariffa* drink characterised by high antioxidant activity and high amount of anthocyanins compared with cranberry juice and that could be

responsible for the reducing the SPB. Acquaviva *et al.* (2003) and Ramirez-Tortosa *et al.* (2001) reported that anthocyanin working as anti-inflammatory and as antioxidants which could reduce the oxidative stress by hunting the reactive oxygen species and free radicals thus protecting the DNA from damage that effect could reduce the BP and the incidence of heart disease.

The effect of the hibiscus drink could be due to one of several mechanisms. Aviram and Dornfeld (2001) discussed, in relation to the effect of pomegranate juice on reducing BP, that anthocyanin antioxidants in pomegranate juice may affect endothelial dysfunction and reduce BP. Shaughnessy *et al*. (2009) attributed the reduction in BP following consumption of blueberry extract to the antioxidant activity of blueberries, reducing oxidative stress in the kidneys and vascular structures by hunting O_2 species and reducing peripheral resistance, usually by increasing vasoconstriction. Vasoconstriction occurs because of the increase in the NO concentration, leading to increased SBP.

Zibadi *et al.* (2007) stated that bioactive compounds, such as querecetin 3-glucoside and cyanidin 3-glucoside, in passion fruit peel extract acted as antioxidants and reduced iNOS. They also noted that the passion fruit peel extract prevented the formation of NO at concentrations from 10 to 100 µmol/L, hunting the NO and transforming the vascular tone and the peripheral resistance.

(Ajay *et al*., 2007) examined the vasodilator effect of a methanol extract of *H. sabdariffa* on relaxing smooth muscles. Vascular smooth muscle cells were isolated from the aortas of hypertensive rats, the aorta cut in rings and soaked in Krebs solution for 45 min, and the solution changed every 15 min interval with adjustment of tension to 1 g. The aorta rings were tested before use by adding KCl solution and the response recorded isometrically through the force displacement. The extract (10

ng/ml up to 1 mg/ml) was bought into contact with the aorta rings. The response was recorded and further extract concentrations were added at 3 min intervals. The study revealed that *H. sabdariffa* extracts had a vasodilator effect on the muscles. The vasodilation reduced the endothelium-denuded aortic rings by activating the eNO relaxing pathway and preventing calcium from entering to the vascular smooth muscles. A high concentration of the extract blocked the drug inhibitor cGMP-specific phosphodiesterase (cGMP), which resulted in the contraction of aorta. The blockage stopped the calcium from entering smooth muscle cells and relaxed the aorta (Ajay *et al*., 2007). Moreover, Zibadi *et al*. (2007) stated that anthocyanins in passion fruit peel extract enhanced eNOS, resulting in increased bioavailable of vascular NO. The NO is working as a vasodilator to protect vascular homeostasis and reduce blood pressure. Beevers *et al*. (2007) also reported that compounds that block the calcium channel have vasodilation effects and decrease BP.

As noted by Ojeda *et al.* (2010), *H. sabdariffa* may act as an ACE inhibitor. They suggested that bioactive compounds isolated from *H. sabdariffa* water extract (cyanidin 3-sambubioside and delphinidin 3-sambubioside) by RP-HPLC were responsible for ACE inhibition. The *H. sabdariffa* extract was obtained by dissolving 2 g/ml in water at 60°C for two hours and freeze-dried. Their results revealed that 84.5 µg/ml of delphinidin 3-sambubioside and 68.4 µg/ml cyanidin 3-sambubioside competed with the substrate at the active site, suggesting that they are competitive inhibitors of ACE. The ACE active site consists of three parts: carboxylate binding functionality, a pocket providing a host for the hydrophobic side chains of the amino acid residue and zinc ions. The study suggested that polyphenol forms chelate complexes with the zinc atoms in the active site or creates hydrogen bridges between the inhibitor and the amino acids near the active site due to the

straight structure of cyanidin 3-sambubioside and delphinidin 3-sambubioside. These compounds contain two hydroxyl groups on an aromatic ring, and hydroxylation of both compounds plays a role in the inhibition of metalo-peptidases. Hellström et al. (2010) concluded that chokeberry juice had a long-term effect on reducing BP and that it acts as an ACE inhibitor.

As shown by Inuwa *et al*. (2012) the reduction of BP by *H. sabdariffa* could be due to a combination of two effects: the inhibition of ACE, which improves angiogenesis in ischemic myocardium, and a decrease in the distance of diffusion between the capillaries and the myocytes, leading to the creation of new vessels. They studied the influence of water extracts of *H. sabdariffa* on hypertensive rats. The *H. sabdariffa* was prepared by soaking dried *H. sabdariffa* in water (5, 10 and 20 g in 995, 990 and 980 ml of water, respectively) for 24 hours at room temperature. The male hypertensive rats were divided into eight groups. A control group consumed water and the other three groups were given *H. sabdariffa* extract as the only drink (5, 10 and 20%). The other four groups were divided into a control group, which consumed saline, and three groups that consumed *H. sabdariffa* anthocyanins (50, 100, and 200 mg/kg) for 5 days. The BP of the animals was measured from the tail cuff once a week. The results showed that a high dose of *H. sabdariffa* extract and of *H. sabdariffa* anthocyanins reduced the BP. The surface and the length of the capillaries were increased by 15–20% after consumption of the *H. sabdariffa*. However, the extract had no effect on heart rate.

According to Alarcón-Alonso *et al*. (2012), another explanation for the effect of *H. sabdariffa* on reducing BP is that the extract works as a diuretic. They examined the diuretic effect of freeze-dried water extract of *H. sabdariffa* (extracted at 55°C for two hours) in vivo in a model system. 7.5 ml/100 g of saline solution was given

through gastroesophageal probe, the 45 min later all the treatments were given by mouth. The rats were separated into seven groups. The negative control group consumed 7.5 ml water/kg, and the positive control groups consumed a diuretic drug (Furosemid, 13 mg/kg). The remainder of the groups consumed extract dissolved in water (500 mg, 100 mg, 1500 mg, 2000 mg and 2500 mg/kg, respectively). The renal filtration rate was examined in isolated kidneys from the rats. Urine was collected for five hours, and the urine volume/hour, and the sodium, potassium and chloride were determined. The results revealed an increase in the kidney filtration rate of 48% with the water extract of *H. sabdariffa.* The authors attributed the increase in the kidney filtration rate to the effect of the anthocyanins on the vascular endothelium and the release of NO, which increased renal vasorelaxation. Increasing the dose of the extract increased the production of urine, demonstrating that the extract functioned as a diuretic. The excretion of sodium and chloride also increased with an increased dose of the extract.

As shown in Figure 6-7, the ability of a antihypertensive drug (warfarin) to reduce BP may be because it has a structure that similar to that of anthocyanins (Fakhree and Nazemiyeh, 2011). Fakhree and Nazemiyeh (2011) found that anthocyanins from black mulberry reduced BP and anti-platelet activity, the first study that related the effect of a antihypertensive drug on reducing BP to the structure of anthocyanins.

Figure 6-9. Structure of warfarin, an antihypertensive drug, which has a very similar structure to anthocyanins

The effect of *H. sabdariffa* on the reduction of BP could also be related to gene regulation. Although miRNAs are involved in the regulation of BP their exact role is not known (Bladé *et al.*, 2013). For example, miR-126 controls vascular endothelial pathogenesis in hypertension and acts as an anti-angiogenic factor (Urbich *et al.*, 2008). miR-155 plays a role in the control of BP by regulating the target renin angiotensin-aldosterone system, and by affecting smooth muscles cells (Martin *et al.*, 2006) miR-143 and miR-144 may reduce BP by affecting smooth muscles cells (Ella *et al.*, 2009; Batkai and Thum, 2012).

Mozaffari-Khosravi *et al.* (2009a) concluded that the mechanism of *H. sabdariffa* in reducing BP is not clear yet. They proposed that it could be due to the effect of *H. sabdariffa* on vascular muscles, the calcium channels in the sympathetic nervous system, cholinergic and histaminic mechanisms or the renin-angiotensin system. They suggested that the antioxidant and diuretic effects of *H. sabdariffa* are the most important mechanisms. They studied 60 subjects that were not taking any medications and diagnosed with type 2 diabetes and moderate hypertension. The subjects consumed *H. sabdariffa* tea (a 2 g sachet containing 5 g of sugar) and black

tea. The tea (240 ml) was brewed for 20–30 min. The subjects consumed the two types of tea twice/day for four weeks. Their BP was measured at the beginning, middle and the end of the study three times (0, 15, 30 min after consumption of the tea). The study found that consumption of the *H. sabdariffa* tea reduced the subjects' SBP and pulse by the end of the study compared with black tea, which increased their SBP and pulse in comparison with the baseline. Neither the black tea nor the *H. sabdariffa* tea affected the subjects' DBP. This was also the case in the present study.

Ojeda *et al*. (2010) stated that the reduction in BP following the consumption of *H. sabdariffa* may be due to the activity of *H. sabdariffa* as a vascular relaxant. They also suggested that it could be due to *H. sabdariffa* preventing angiotensin 1 converting ACE or to *H. sabdariffa* acting as a vasodilator or a diuretic (an aldosterone antagonist). Alternatively, the anthocyanins may act to inhibit ACE (Ojeda *et al*., 2010).

In the present study, the reduction in SBP was small but significant, but the change in each participant was more than 5 mmHg. Therefore, *H. sabdariffa* is likely to have a major effect on hypertension or reduce the incidence of cardiovascular disease. The *H. sabdariffa* drink had hypotensive effect on some of participants that could be due to the diuretic or vasodilation effect of the juice or that participants already characterised by low blood pressure the minimum reading for DBP was 54 mmHg or other factors such as sickness or fasting for long period. The *H. sabdariffa* drink had no effect on other these variation could be related to the absorption of anthocyanins in each individual was different or other factors.

According to Lim (2007), the management of BP in hypertensive patients and the maintenance of the BP at a certain level by reducing (a few mmHg) of mean BP can reduce the incidence of stroke and heart disease. Lim (2007) also stated (BP as a

continuous variable from the low to the high, distributed normally most of the people located in the middle of the curve, few located at the end which had high BP and high risk of heart disease), Therefore, minor reductions in BP (in entire populations will reduce the incidence of stroke and heart disease better than large reductions in BP pressure in patients with severe HTN which had less impact on the incidence of stroke and heart disease because only minor individual suffered from severe HTN and most the incidence of cardiovascular disease happened in high or normal BP. The study suggests that regular consumption of extracts of *H. sabdariffa* may reduce the risk of cardiovascular disease, on the other hand people with low blood pressure should consumed it very carefully due to hypotensive effect of the drink. So the study concludes that healthy individuals and pre-hypertensive need more care and education on who to reduce the risk of cardiovascular disease to protect them in the future.

6.11.1 Observation during the study

During the study some of interesting observations were highlighted; that blood pressure elevate when participant talk during the measurement or if they did physical activity 1 hour before taking the reading, also due to large proportion of the volunteers recruited from the school it was observed that also blood pressure increased due to the stress which self-reported from the volunteer in addition, high heart rate and low diastolic pressured was observed when participants reported that they did not eat anything until the time of the measurement or if they have flu. Moreover, interesting observation was found that blood pressure readings overall females are lower than males. Last observation, when participants asked if they have problem with drinking the juice some of them reported bitter taste after consuming the *H. sabdariffa* juice and some of them complain from the sweetness of the juice

and they diluted more than the recommended amount to drink it therefore, they prefer to take it as shot instead of diluting it. Which raise a question if the concentration may have any effect. The investigator could not control that due to the long duration of the study and the important thing to the researcher that they consume the whole amount of the juice to sure they have they take the specific amount of anthocyanins other than these observation most of the subjects quite like the *H. sabdariffa* juice and they do not bother to keep consuming as daily drink, it was being habit for them to drink it and ask about the availability of the juice in the UK market. The study will suggest for the manufacture that provide the study by juice to try to reduce the amount of sugar or develop new product free sugar suitable for diabetic people.

Chapter 7 General discussion

Recently *H. sabdariffa* has had more attention from researchers due to its high antioxidant and high anthocyanin content. *H. sabdariffa* is used in folk medicine because of the health benefits of the aqueous extract on reducing the risk of cardiovascular disease, hypertension, diabetes, cancer and liver disease.

This thesis on the bioactivity of anthocyanins from *H. sabdariffa* has covered four key areas in order to characterise *H. sabdariffa* confirming its properties and health benefits. Firstly, the total phenols, total anthocyanins and antioxidant capacity of different extracts of *H. sabdariffa* extracted with different solvents, was determined and the major anthocyanins were identified and quantified by HPLC and LC-MS. This is the first systematic study of *H. sabdariffa*. The second study evaluated two commercially available herbal teas, which contained *H. sabdariffa*. The optimal extraction method was compared to the infusion method recommended on the packaging. The third study measured the partition coefficients of anthocyanin standards in the presence of a *H. sabdariffa* water extract or *H. sabdariffa* powder. This is the first study to measure partition coefficients of anthocyanins from *H. sabdariffa*. The final part of this thesis was very important in advancing the research knowledge on the health benefits of consumption of *H. sabdariffa*. In the first study of its kind, the effect of a commercial *H. sabdariffa* juice on the blood pressure of healthy subjects was investigated.

7.1 Systematic study of the extraction of *H. sabdariffa*

Different types solvent (water, methanol, ethyl acetate and hexane) with and without addition of formic acid were used to extract *H. sabdariffa* for various times and

temperatures for extraction. In order to choose the optimal method, the study found that using water at 100 °C for 10 min to extract *H. sabdariffa* gave the highest total phenol content, total anthocyanin content and boiling water gave the highest antioxidant activity. Optimal extraction time varied depending on the antioxidant assay but the optimal time was selected as 10 min as there was a strong positive correlation between total phenol content and the antioxidant assays. Adding formic acid had no significant effect. Methanol is a good alternative solvent to use. Ethyl acetate had some antioxidant activity, which was possibly due to flavonoid bioactive compounds as ethyl acetate is used in purification methods to remove of flavonoids and phenolic acids (Wrolstad, 2005). However, no anthocyanins were detected with this solvent. Although, Mohamed *et al*. (2007) found the hexane extract of leaf, seed, stem and sepal of *H. sabdariffa* contained α- tocopherol it did not have any significance in the assays carried out in this study. The presence of vitamin C in *H. sabdariffa* had indirect effect on the total phenol content. However, along with Tsai *et al*. (2002) anthocyanins are thought to be the major component responsible for the antioxidant activity and total phenol content in *H. sabdariffa* water extracts compared with ascorbic acid, because the results for the total phenol content are still high even after correction of vitamin C. It is difficult to compare the finding of this study with other studies due to different experimental conditions, number of extractions per each sample, the part used from the *H. sabdariffa* plant, its location, cultivar and climate. In addition, Goncalves *et al.* (2013) indicated difficulties in comparing antioxidant results with other studies due to the use of different assays, experimental conditions or because samples are processed differently. This study was systematic in nature and therefore the results obtained were comparable. Using water at 100 °C for 10 min is the optimal method when using dried *H. sabdariffa*.

7.2 Commercially available herbal teas containing *H. sabdariffa*

Two types of herbal teas (pomegranate tea and rose tea) were analysed using two different methods of extraction. The first method was the optimal method (water at 100 °C for 10 min) and the second method was an infusion method, typically used for infusing tea at home by following the direction on the tea package (water at 100 °C for 5 min). The optimal method (decoction) exhibited more total anthocyanins, total phenols and antioxidant activity compared with the infusion method. This suggests that changing the method of making a cup of tea could allow for a greater consumption of potential health benefiting compounds. Pomegranate tea had a higher propotion of anthocyanins and higher antioxidant activity compare with a pure *H. sabdariffa* water extract. This may be due to the presence of other ingredients but also suggests a high proportion of the tea is *H. sabdariffa*. And therefore many commercially available herbal teas containing *H. sabdariffa* could be of benefit to health.

7.3 Identification and quantification of anthocyanins

LC-MS identified delphinidin 3-sambubioside, cyanidin 3-sambubioside, delphinidin 3-glucoside and cyanidin 3-glucoside in acidified and non-acidified aqueous and methanol extracts of *H. sabdariffa*.

These four anthocyanins were quantified using HPLC and LC-MS. Delphinidin 3-sambubioside and cyanidin 3-sambubioside were the major compounds and delphinidin 3-glucoside and cyanidin 3-glucoside were the minor compounds According to Maganha *et al*. (2010) delphinidin 3-sambubioside, cyanidin 3-

sambubioside, delphinidin 3-glucoside and cyanidin 3-glucoside also have bioactivity in biological models and therefore these compounds may be of significance to the pharmacological effects of *H. sabdariffa* extracts.

7.4 Partition coefficients of anthocyanins from *H. sabdariffa*

There have been many reports about the poor absorption and bioavailability of anthocyanins. To give an idea about the absorption of anthocyanin compounds in both their aglycone and glucoside form, the partition coefficients (log P) of the four anthocyanins identified in *H. sabdariffa*, (delphinidin 3-sambubioside, cyanidin 3-sambubioside, delphinidin 3-glucoside and cyanidin 3-glucoside) were determined. How these anthocyanins behave in presence of a *H. sabdariffa* water extract or *H. sabdariffa* powder was also determined, before the human study recruitment, to give an idea about the effect of the food matrix. Experimental values of log P were compared with theoretical values. The results showed that the glucoside and sambubioside forms of anthocyanins were more hydrophilic (log $P < 0$) due to the effect of xylose and glucose sugars attached to carbon in position number 3. According to Hu (2007) phenolic glucosides are hydrophilic compounds, with large molecules and have a high polarity. These characteristics reduce the potential of these compounds to penetrate the intestinal membrane. However, though those anthocyanins glucoside may penetrate the cell membrane by efflux pump even in low concentration and may became bioavailable.

The aglycone form of the anthocyanins had a large positive log P value so it is more hydrophobic. Therefore, a small concentration of these anthocyanins may be able to pass through the lipid bilayer of the intestine and absorbed. This was confirmed by

other researchers who found traces of the anthocyanins in the brain tissues of animals. Adding a water extract of *H. sabdariffa* further reduced the value of the log *P* for anthocyanin glucosides. Whereas adding *H. sabdariffa* powder reduced the log *P* values for both anthocyanin glucosides and aglycones so they become more hydrophilic. This is likely to be due to the effect of fibre, which it attached to the aglycone and glucoside forms of anthocyanin and would help them to be in the water phase, thereby affecting their absorption. However, McGhie and Walton (2007) reported that the neutral pH in the small intestine had an effect on anthocyanins and changed the flavylium structure to another structure such as quinonoidal , chalcone or hemiketal and that transformation was not reversible. This could therefore have an effect on the bioavailability in *vivo*.

7.5 Effect of *H. sabdariffa* juice on the blood pressure of healthy subjects

The final part of the current research was a human cross-over study, which investigated the effect of drinking (35 ml of concentrated juice diluted up to 250 ml) cranberry and *H. sabdariffa* juice on the blood pressure of healthy volunteers aged (between 19 and 45 years) for 8 weeks. The human study showed a significant reduction in systolic blood pressure after 8 weeks compared with the baseline, after consumption of *H. sabdariffa* juice. There are was no effect on diastolic blood pressure. The results are same as what McKay *et al*. (2010) found when studying the effect of *H. sabdariffa* tea consumption on pre-hypertensive patients. In contrast, no significant difference was found in systolic or diastolic blood pressure after 8 weeks from consumption of cranberry juice when compared with the baseline. It is therefore suggested that the reduction in blood pressure may be due to the

contribution of the two major compounds delphinidin 3-sambubioside and cyanidin 3-sambubioside in *H. sabdariffa*. The results obtained from the analysis of the commercial juice that used in the human study were compared with the pure water extract of *H. sabdariffa* analysed in this study. Pure water extract of *H. sabdariffa* was higher in total phenol content, total anthocyanin content, FRAP and TEAC than in commercial water extract of *H. sabdariffa* juice. DPPH radical scavenging activity was the only antioxidant assay was higher in commercial juice compared with pure water extract of *H. sabdariffa*. The low values obtained when commercial juice analysed could be explained due to the effect of processing on anthocyanins compounds or the high content of sugar which may affect the anthocyanins stability and increased the deterioration. However, the commercial *H. sabdariffa* drink had significant effect on reducing systolic blood pressure. Reducing blood pressure may help to protect from cardiovascular disease.

7.5.1 Compliance and limitation

Compliance in study was good; 29 of 30 subjects completed the study. Two participants dropped out during the study. Check table were provided for volunteers in each phase to make sure that they consumed the amount of juice every day. Certain limitations in the design of the human study have to be addressed. It was hard to take ethical approval for hypertensive patients and it was required another approval from the National Health Service (NHS). Therefore, participants in the human study were all healthy, thought that if the study could approved that the drink have effect on the healthy subjects blood pressure it is also approved that it could have the same effect on pre-hypertensive or hypertensive subjects. Another limitation that placebo juice was not controlled. It was chosen in the study due to the long duration of the study and it was and it is hard to pasteurise a placebo to ensure

it remains free of pathogens or fungi during the full eight weeks of the study. The result of the human study showed reduction in BP of some participants when the consumed cranberry juice as placebo drink even that some study such as Dohadwala *et al.* (2011) found that cranberry juice has no effect on BP. In future, controlled placebo should be used to avoid any effect on BP. The study found that the check list not enough to guarantee that subjects consumed the daily amount of juice, therefore, in future blood test should be included to check the plasma level of anthocyanins. Variation in the subjects' blood pressure increased the need to analyses the 24 hour recall which was not analysed due to the short of the time and test another variables effect such as ethnicity, physical activity, the time of taking the juice which could have effect on BP. The current study did not check the bioavailability of the *H. sabdariffa* drink. In future, urine sample should be taken and metabolites compounds should be analysed.

7.6 Conclusion

Water extraction at solvent boiling point for 10 min was the optimal extraction for *H. sabdariffa* due to the extract having high antioxidant activity, total phenol content, total anthocyanin content and anthocyanin bioactive compounds. Similarly, applying the optimal extraction condition to extract commercially-available herbal teas containing *H. sabdariffa* also increased the antioxidant activity, total phenol content, total anthocyanins content and anthocyanin bioactive compounds. The study recommends not using hexane or ethyl acetate for anthocyanin extraction. Anthocyanin glucosides become more hydrophilic when *H. sabdariffa* powder and water extract are added, and anthocyanin aglycones become more hydrophilic when *H. sabdariffa* powder was added. However, the anthocyanin glucosides may penetrate the cell membrane even in low concentration and may become

bioavailable. More attention is needed to study the effect of food matrix and fibre on absorption and bioavailability of anthocyanins because the behaviour of anthocyanin may change by effecting of food matrices. From the finding of the human study, it is recommended that *H. sabdariffa* should be consumed as part of the daily diet due to its effect on lowering the systolic blood pressure. However, due to participants comment about the sweetness of the *H. sabdariffa* juice the amount of sugar should be reduced, a new sugar free product should be developed. *H. sabdariffa* products need more marketing especially in the UK because to our knowledge the Simply Hibi is the only company who produces hibiscus drink and therefore Hibiscus is not widely known about. Although hibiscus is present as the major ingredient in many herbal teas, they are not sold nor consumed due to the presence of Hibiscus. As this study has proven, Hibiscus can have an effect on even healthy subjects and therefore its consumption could help the health of the nation. From this research there are still some questions which need to be answered, such as which have more effect on reducing blood pressure, a pure extract of *H. sabdariffa* or a herbal tea containing *H. sabdariffa*. Also, if a small quantity of anthocyanin glucoside is absorbed is it bioavailable and can the metabolites be detected?

7.7 Impact of the research on the community

The key finding of this research is that long term consumption of *H. sabdariffa* juice may help to protect from cardiovascular disease. It is thought that the effect is due to the high content of phenols and high antioxidant activity, which could be due to the presence of delphinidin 3-sambubioside and cyanidin 3-sambubioside. This study recommends drinking 35 ml of concentrated *H. sabdariffa* juice or 250 ml of diluted *H. sabdariffa* juice everyday as part of daily diet protect from developing high blood pressure and therefore protect from the cardiovascular disease. On the other hand

people with low blood pressure should consume it very carefully due to hypotensive effect of the drink. Due to the effect of the juice on reducing blood pressure in healthy people and its high antioxidant activity, it can also be suggested that pre-hypertensive and hypertensive patients consume the juice as a nutraceutical product to help lowering their blood pressure. The media could emphasise the health benefits of *H. sabdariffa* which would educate society about the natural food and juices which may help to lower blood pressure. Due to the findings of the study, it can also be recommended that manufactures pay more attention to *H. sabdariffa* by producing supplements or alternative products such as salad dressing or seasoning, confectionery or soft drinks to increase the amount of *H. sabdariffa* that is currently consumed by the population, thereby helping towards a healthier nation.

7.8 Future work

There is more to investigate in order to answer questions about *H. sabdariffa,* which have arisen due to this research. Relation to antioxidant activity, there is a need to determine the antioxidant activity in the human body using *in vivo* assays after consumption of the *H. sabdariffa*, to determine if there is an increase in the plasma antioxidant activity. This will give a clear vision as to whether *H. sabdariffa* has any likely health benefits *in vivo*. Also it is important to study both Hexane and ethyl acetate extracts of *H. sabdariffa* further in order to identify if they contain another flavonoids and tocopherol.

Protocatechuic acid is thought to be present in *H. sabdariffa* and many researches have related the health benefit of *H. sabdariffa to* this compound (Tseng *et al.*, 2000; Ali et al., 2005; Hsieh *et al.*, 2006; Lin *et al.*, 2011). Therefore, there is need to identify whether protocatechuic acid is present in the water extract of *H. sabdariffa*.

Following this, if protocatechuic acid is present, we need to confirm if the effect of *H. sabdariffa* on systolic blood pressure is related to this compound or anthocyanins present that could be done by using *in vitro* experiment or *in vivo* animal study.

To detect other anthocyanin structures formed in neutral pH, methods needs to be developed. These anthocyanins can then be used for absorption experiments using cell culture and possibly a human study using ileostomy patients. This would resolve the mystery surrounding anthocyanin absorption and bioavailability by focusing the analysis on the faecal matter of the volunteers instead of the urine because many studies reviewed in the section of anthocyanins bioavailability (section 1.3.4) reported the importance of the micro-flora in the gut in hydrolysing the anthocyanins.

Due to limitation of time the study did not analyse the 24 hour recall data of the participants in the human study. Therefore, the 24 recall should be analysed and correlated with the blood pressure readings, as it is possible an interesting result could be seen. Following on from the human study there is a need to investigate the effect of the *H. sabdariffa* water extract on the flow-mediated endothelium-dependent dilation (FMD) to confirm the effect on cardiovascular disease.

It will be interesting to compare the results of the human study with a sample of participants from Saudi Arabia to see the effect of stress, ethnicity, gender and physical activity on large scale in Saudi Arabia.

Finally, investigate the effect of the *H. sabdariffa* water extract in the presence of sugars, fibre and protein on the blood pressure, is important because these nutrients may reduce or enhance the anthocyanins bioactive compounds in *H. sabdariffa* which may affect the role of *H. sabdariffa* on reducing blood pressure.

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Appendices