Identification of Polyphenolic Compounds within Fruit Extracts and Their Role in the Treatment of Glioma

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common and malignant primary brain tumour in adults. Despite current treatment options, including surgery followed by radiation and chemotherapy with temozolomide (TMZ) and cisplatin, the median survival rate remains below 16 months. Epidemiological studies have shown a positive correlation between consumption of fruits and vegetables in the reduced risk and prevention of cancers, resulting in improved mortality rates. This is due to various fruits encompassing differing antioxidant abilities, which are derived from their phytochemical components. Research has emphasised the importance of antioxidants and their ability to neutralise reactive oxygen species (ROS) which cause oxidative damage to lipids, proteins and nucleic acids. This study examined the presence of polyphenolic compounds within five fruits: cranberry, strawberry, goji berry, maqui berry and acai berry, and their effect on cell viability. Through High Performance Liquid Chromatography, it was possible to identify the potential of all fruits to contain antioxidants; specifically, gallic acid, punicalagin, cyanidin-3-glucoside and malvidin. Subsequently, flavonoid and phenolic assays quantified the levels of antioxidants present within the fruits whereby, cranberry had the highest total flavonoid content (670.93 ±45.30 µg/CE/serving) and strawberry had the highest total phenolic content (2835.11± 26.48 µg/GAE/serving). Also, the DPPH· radical scavenging assay quantified antioxidant activity of fruits and phytochemical compounds showing punicalagin to have the highest antioxidant activity (6522.74 ± 59.30 µM TROLOX equivalents). The results presented a significant difference in each assay, between all fruits samples ($p<0.001$). This identified cranberry to contain the highest antioxidant activity when compared to the other fruits. This study examined the effect of the fruit extracts and antioxidant compounds in comparison to cisplatin and determined effects on cell viability.

Cyanidin-3-glucoside and maqui berry significantly reduced the viability of U87-MG cells. These results suggest a potential for antioxidants as chemotherapeutic agents in the treatment of glioblastoma multiforme.

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ABBREVIATIONS

AC	Antioxidant capacity
ANOVA	Analysis of variance
ATR	Ataxia telangiectasia
CDDP	Cisplatin
CE/ml	Catechin equivalents per millilitre
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EBRT	External beam radiotherapy
EMEM	Eagles Minimum Essential Medium
F: P	Flavonoid to phenolic ratio
FRAP	Ferric reducing antioxidant power
GAE/ml	Gallic acid equivalents per millilitre
GBM	Glioblastoma Multiforme
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory concentration 50%
LOD	Limit of detection
MAPK	Mitogen-activated protein kinase
mAU	milliArbitary units
MGMT	Methylguanine methyltransferase
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

NEAA	Non-essential amino acids
NHS	National Health Service
ORAC	Oxygen radical absorbing capacity
PBS	Phosphate Buffer Saline
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RT	Radiation therapy
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TE/ml	TROLOX equivalents per millilitre
TFC	Total flavonoid content
TMZ	Temozolomide
TNF	Tumour necrosis factor
TPC	Total phenolic content
TROLOX	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
WHO	World Health Organisation

Chapter 1

INTRODUCTION

1.1 Brain Tumours

Cancers are a huge research topic as 292,680 invasive malignant cancer diagnoses were registered in 2013, in England (Office for National Statistics, 2015; Cancer Registration Statistics, England, 2013). Of these, 133,000 deaths occurred, in England in 2013, from a total of 162,000 in the UK (Macmillan Cancer Support). Specifically, little significance is given to brain tumours in comparison to other cancers such as breast cancer. Brain tumour incidences within the UK range from 2,398 in males and 1,803 in females (Figure 1.1) (Office or National Statistics, 2016).

Like other cancer, the cause of brain tumours is unknown. However, several risk factors have been established such as age, medical radiation, recurrence from previous cancers and other medical conditions such as HIV or AIDS (cancer Research, UK). This report emphasises the challenges that remain in the diagnosis and treatment of brain cancers and understands the importance of developing new treatments.

1.2. Glioblastoma Multiforme

Glioma can be astrocytic, oligodentritic and ependymal in origin and accounts for 70% of brain tumours; of which the most frequent (65%) is glioblastoma (Ohgaki et al., 2005). Current classification is based on the widely accepted World Health Organisation (WHO) grading system. As shown in Table 1.1, glioma are identified by the type of cell affected: divided into astrocytoma, oligodendroglioma, mixed oligoastrocytomas and glioblastoma. They are also classified by grade (II-IV), based on abnormal nuclear structure, cellular proliferation, microvascular proliferation and necrosis. Tumours can be classified as grade I which are usually solid and non-infiltrative (Vigneswaran et al., 2015; Louis et al., 2016).

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	9400/3
Gemistocytic astrocytoma, IDH-mutant	9411/3
Diffuse astrocytoma, IDH-wildtype	*9400/3*
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH-mutant	9401/3
Anaplastic astrocytoma, IDH-wildtype	*9401/3*
Anaplastic astrocytoma, NOS	9401/3
Glioblastoma, IDH-wildtype	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epithelioid glioblastoma	*9440/3*
Glioblastoma, IDH-mutant	9445/3*
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M–mutant	9385/3*
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9451/3
Anaplastic oligodendroglioma, NOS	*9451/3*
Oligoastrocytoma, NOS	*9382/3*
Anaplastic oligoastrocytoma, NOS	*9382/3*
Other astrocytic tumours	
Pilocytic astrocytoma	9421/1
Pilomyxoid astrocytoma	9425/3
Subependymal giant cell astrocytoma	9384/1
Pleomorphic xanthoastrocytoma	9424/3
Anaplastic pleomorphic xanthoastrocytoma	9424/3
Ependymal tumours	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Papillary ependymoma	9393/3
Clear cell ependymoma	9391/3
Tanycytic ependymoma	9391/3
Ependymoma, *RELA* fusion–positive	9396/3*
Anaplastic ependymoma	9392/3
Other gliomas	
Chordoid glioma of the third ventricle	9444/1
Angiocentric glioma	9431/1
Astroblastoma	9430/3
Choroid plexus tumours	
Choroid plexus papilloma	9390/0
Atypical choroid plexus papilloma	9390/1
Choroid plexus carcinoma	9390/3
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	9413/0
Gangliocytoma	9492/0
Ganglioglioma	9505/1
Anaplastic ganglioglioma	9505/3
Dysplastic cerebellar gangliocytoma (Lhermitte–Duclos disease)	9493/0
Desmoplastic infantile astrocytoma and ganglioglioma	9412/1
Papillary glioneuronal tumour	9509/1
Rosette-forming glioneuronal tumour	9509/1
Diffuse leptomeningeal glioneuronal tumour	
Central neurocytoma	9506/1
Extraventricular neurocytoma	9506/1
Cerebellar liponeurocytoma	9506/1
Paraganglioma	8693/1
Tumours of the pineal region	
Pineocytoma	9361/1
Pineal parenchymal tumour of intermediate differentiation	9362/3
Pineoblastoma	9362/3
Papillary tumour of the pineal region	9395/3
Embryonal tumours	
Medulloblastomas, genetically defined	
Medulloblastoma, WNT-activated	9475/3*
Medulloblastoma, SHH-activated and *TP53*-mutant	9476/3*
Medulloblastoma, SHH-activated and *TP53*-wildtype	9471/3
Medulloblastoma, non-WNT/non-SHH	9477/3*
Medulloblastoma, group 3	
Medulloblastoma, group 4	
Medulloblastomas, histologically defined	
Medulloblastoma, classic	9470/3
Medulloblastoma, desmoplastic/nodular	9471/3
Medulloblastoma with extensive nodularity	9471/3
Medulloblastoma, large cell / anaplastic	9474/3
Medulloblastoma, NOS	9470/3
Embryonal tumour with multilayered rosettes, C19MC-altered	9478/3*
Embryonal tumour with multilayered rosettes, NOS	*9478/3*
Medulloepithelioma	9501/3
CNS neuroblastoma	9500/3
CNS ganglioneuroblastoma	9490/3
CNS embryonal tumour, NOS	9473/3
Atypical teratoid/rhabdoid tumour	9508/3
CNS embryonal tumour with rhabdoid features	*9508/3*
Tumours of the cranial and paraspinal nerves	
Schwannoma	9560/0
Cellular schwannoma	9560/0
Plexiform schwannoma	9560/0

Table 1.1: World Health Organisation examples of classification of gliomas. This is in accordance with type, grade, and survival rate alongside a description of the cancer (Louis et al., 2016).

1.3 Treatment of Glioblastoma Multiforme

Despite medical advances in cancer, GBM has limited success. Current treatment consists of surgery followed by radiation therapy (RT) in the form of fractionated external beam radiotherapy (EBRT) and chemotherapy with temozolomide (TMZ) (Stupp et al., 2005).

Initial treatment begins with surgery to obtain a histological diagnosis and safely remove as much tumour as possible. Resection can be problematic due to the invasiveness, increased vascularisation or position such as proximity to the motor cortex. In such cases, the tumour is partially removed if safe to do so. Full or partial removal of the tumour can be advantageous by decreasing symptoms thus reducing the need for additional radiation and chemotherapies, reducing side effects resulting in improved quality of life (Watts et al., 2014). However, risks are associated with surgery as it is difficult to clearly differentiate between tumorous and healthy tissue which has potential for error (Watts et al., 2014).

RT is assumed to be effective at delivering a high dose, localised treatment with minimal invasion to surrounding tissue. Studies explored the success rate of RT, however Souhami et al. (2004) found RT to have no significant difference within the survival rates. Although there have been steady advances in treatment to improve survival rates whilst improving the quality of life amongst patients (Barani and Larson., 2015).

Medical therapy in the form of alkylating agents such as TMZ have been found advantageous due to their rapid and complete absorption upon oral administration and their excellent penetration into body tissues including the brain. TMZ is able to select a methylation site upon DNA, specifically N^7 and O_6 on guanine and O_3 on adenine resulting

in a cascade of events leading to repetitive futility in base pair mismatch repair causing chronic strand breaks, triggering an apoptotic response (Stupp et al., 2001). The exploration into the combination of TMZ and RT were found to increase cytotoxicity which established a success in the relationship and was suggested to be used as a combination therapy (Stupp et al., 2001).

Another medical therapy consists of cisplatin or Cis-diamminedichloroplatinum (CDDP), considered one of the most potent and widely used drugs for cancer treatment. Cisplatin is a metallic- platinum coordination compound with a square planar structure which is composed of a doubly charged platinum ion surrounded by four ligands. Within this structure, the amine ligands form stronger bonds with the platinum ion and the chloride ions forming leaving groups allowing the platinum ion to bond with DNA bases (Dasari et.al., 2014). The mode of action of its cytotoxicity is mediated by its interaction with DNA, activating several pathways including those with the involvement of ATR, p53, p73 and MAPK, all which cumulate in activation of apoptosis as summarised in Figure 1.2. Although TMZ is the current drug treatment for GBM, cisplatin therapy is used for recurrent childhood brain tumours. However, due to cisplatin's interaction with DNA it is known to have cytotoxic effects including nephrotoxicity, hepatotoxicity and cardiotoxicity (Yousef et.al.,2009)

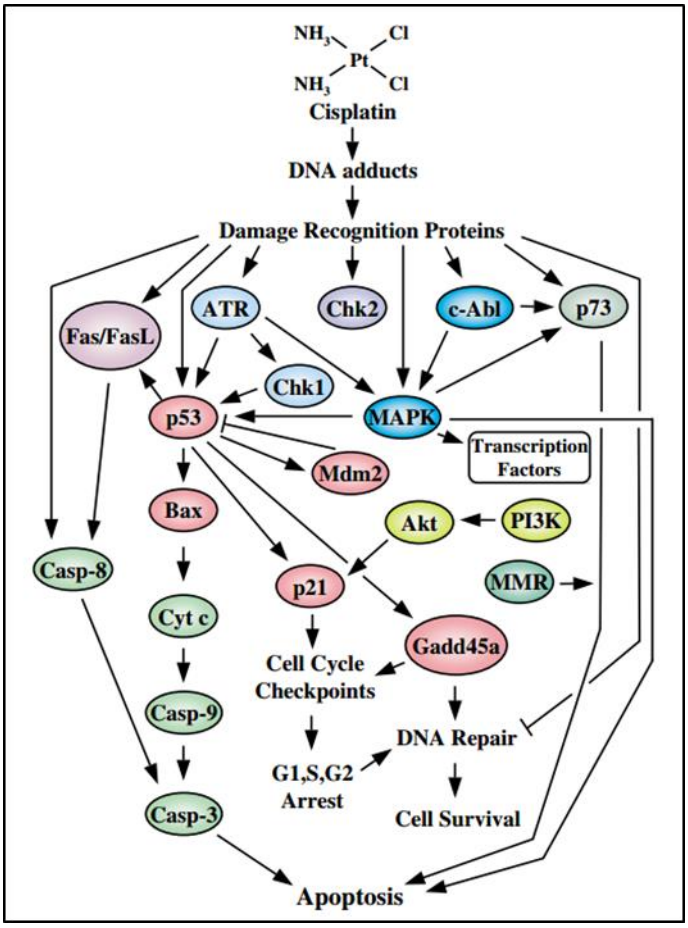


Figure 1.2: shows the pathways through which cisplatin leads to apoptosis dependent upon crosstalk between pathways and the intensity of signal67tnming (Siddik, 2003).

Despite these combinations, as well as advances in each treatment option individually, prognosis is still poor. This could be due to the GBM cell resistance to TMZ and cisplatin possibly due to the role of a specific microRNA (Clarke et al., 2010). In TMZ, methylguanine methyltransferase (MGMT), a DNA repair protein which is able to bind to damaged substrate DNA, resulting in a conformational change of the DNA, detaching and then allowing the DNA to go through the degradation system. MGMT is able to protect cells against carcinogens however, it can also protect cancer cells from chemotherapeutic agents such as TMZ (Zhang et al., 2012) (Figure 1.3). As well as investigations into intensity modulated radiotherapy and other therapies, there are still few approved treatment

alternatives. Recurrence of the disease has been found problematic, where the same treatment is administered and fails to show the same success; as well as the difficulty of drugs crossing the blood brain barrier (Clarke et al., 2010).

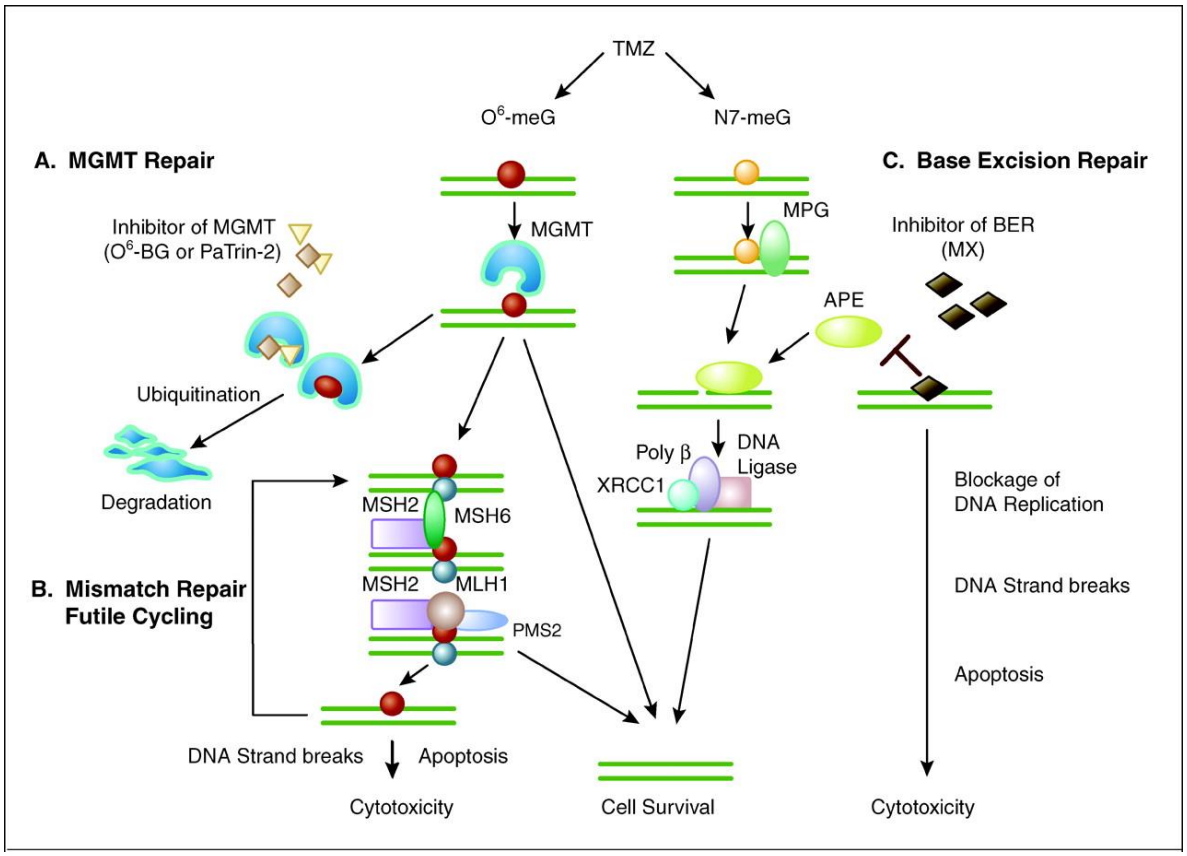


Figure 1.3: Shows the pathway through which TMZ interacts with MGMT and other DNA repair mechanisms leading to cytotoxicity within cells (Liu, 2006).

1.4 Reactive Oxygen Species and Antioxidants

Reactive oxygen species (ROS) are chemically reactive by-products of oxygen metabolism and can be generated in response to xenobiotics, cytokines and bacterial invasion; and external exposure such as air pollutants, X-rays and industrial chemicals (Lobo, 2010). Under normal cellular conditions, ROS can be obtained through enzymatic and non-

enzymatic reactions. Where enzymatic reactions are a source of ROS through involvement in defence mechanisms such as phagocytosis and redox signalling; non-enzymatic reactions form ROS through oxygen and organic compound reactions having similar beneficial effects (Lobo, 2010; Halliwell, 2007).

In high concentrations ROS can have damaging effects upon cellular components including DNA, proteins and lipids; contributing to the development of diseases such as cancer, atherosclerosis, cardiovascular disease, diabetes mellitus and neurodegenerative disease (Gordon 1996; Gey, 1990; Scalbert et al., 2005). Free radicals known to create such damage consist of the hydroxyl (·OH), superoxide anion and hydrogen peroxide. For example, the reduction of molecular O_2 produces superoxide ($\cdot O_2^-$) which can be further converted to hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH).

Cell damage can occur due to protein and lipid peroxidation leading to apoptosis. In proteins, as a result of oxidative stress, physical and chemical changes can occur causing biological consequences including disease and aging. Specifically, oxidative modifications of proteins take place at the side chains of amino acids. Cysteine and methionine are especially susceptible to oxidation due to the presence of reactive sulphur atoms which is rich is electrons and can be removed. ROS can directly attack the backbone of proteins to cause conformational changes in the secondary and tertiary structures (Zhang et al., 2013).

Lipid peroxidation takes place due to an antioxidant deficiency (α-tocopherol). The process consists of the formation and propagation of lipid radicals, uptake of oxygen, and a rearrangement of the double bonds in unsaturated lipids, specifically within polyunsaturated fatty acids (PUFA). This causes an increase in radical formation by

hydrogen abstraction due to the susceptibility of a weak C-H bond within the methylene bridge resulting in the formation of a peroxyl radical (ROO·) and hydroperoxides. This leads to a chain reaction, where the ROO· alongside a yield of alcoxyl (RO.) or hydroxyl (·OH) radicals is able to start the procedure of forming and propagating lipid radicals again. Under high levels of lipid peroxidation, the membranes under attack are overwhelmed by oxidative damage such that they are unable to put repair mechanisms in place, inducing apoptosis or necrosis. This leads to the eventual destruction of PUFA and therefore lipid membranes, with breakdown products including: alcohols, ketones, alkanes, aldehydes and ethers (Dianzani et al., 2008; Repetto, 2012; Ayala et al., 2014).

To defend against such damage, oxidative mechanisms are put in place with the aid of antioxidants which can prevent radical formation, remove radicals before they can cause damage to key components and repair oxidative damage. However, it is imperative for an exacting amount of ROS to remain, to carry out necessary processes and so the aim of these mechanisms is not to remove all ROS but to maintain the levels to prevent such damage from occurring (Ames et al., 1993; Gordon, 1996).

According to Lobo et al., (2010), proposed defence mechanisms consist of the donation of an electron from the antioxidant, neutralising the free radical; removal of ROS by quenching the chain initiating catalyst; metal ion chelation; co-antioxidants or gene expression regulation. These antioxidants can be generated naturally or obtained via the diet.

Reduction of molecular O_2 produces superoxide ($\cdot O_2^-$) which can be further converted to hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). Naturally occurring enzymatic antioxidants found within cells, have the ability to convert ROS in to less toxic products. The antioxidant superoxide dismutase (SOD) enzyme catalyses the dismutation (alternately adding or removing an electron from the superoxide molecules it encounters) of the $\cdot O_2^-$ radical to produce the less harmful products H_2O_2 and O_2 (Figure 1.3). Alongside SOD, another enzymatic antioxidant catalase, prevents the increased production of H_2O_2, also a harmful by product; decomposing it to O_2 and H_2O. Other antioxidants include, ascorbic acid (vitamin C), α-tocopherol (vitamin E), uric acid and the glutathione (GSH) (all non-enzymatic) which also target H_2O_2.

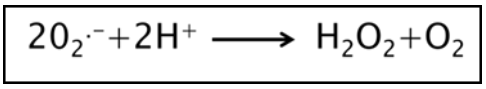


Figure 1.4: Equation showing the breakdown of $\cdot O_2^-$ (adapted from Gordon, 1996).

1.5 Phytochemicals

Phytochemicals are bioactive non-nutrient plant compounds which have particular roles within plants such as contributing to reproduction and growth, defence mechanisms against pathogens and parasites, as well as colour of the plant (Liu, 2004; Lampe et al., 2007). Upon human consumption of these phytochemicals, studies have indicated a potential for them to have chemotherapeutic advantages (Ames, 1983; Gey, 1990). Phytochemicals can be classified into five categories; phenolics, carotenoids, nitrogen-containing compounds, organosulfur compounds and alkaloids, of which carotenoids and phenolics are the most

studied and phenolics are the most common and widely distributed (Figure 1.4) (Tsao, 2010).

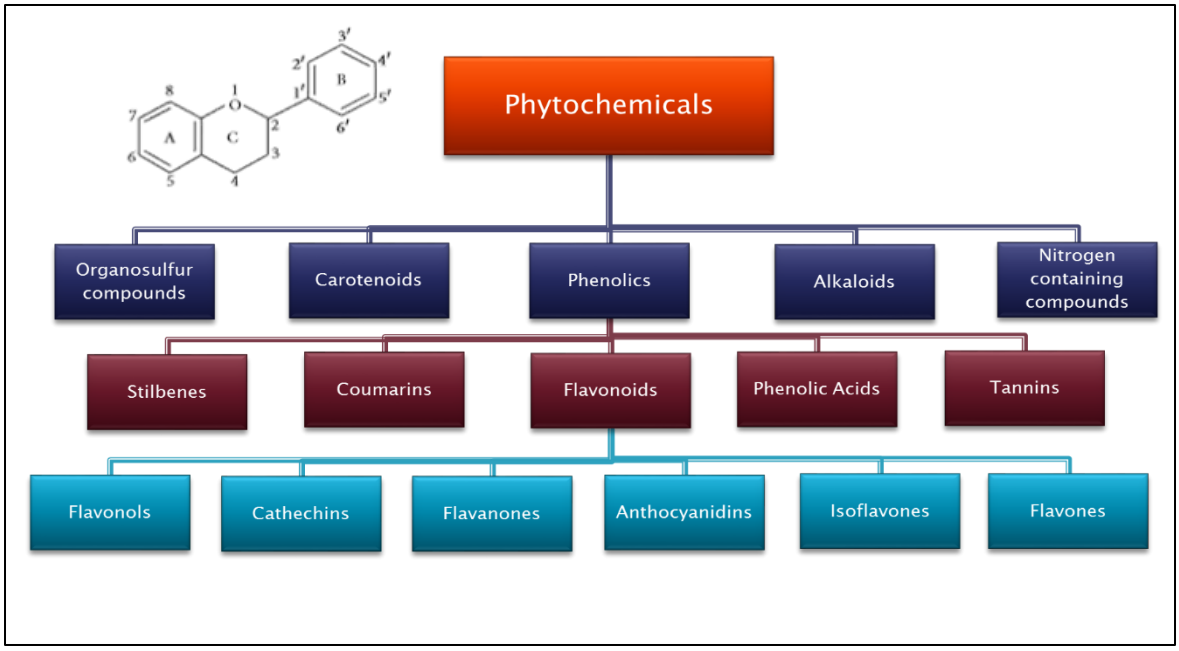


Figure 1.5: Classification of phytochemicals recognising those which have been widely researched (Adapted from Liu, 2004)

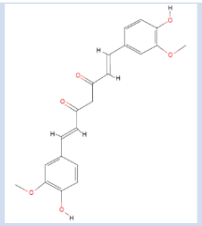
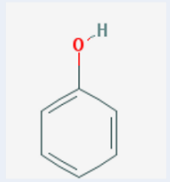
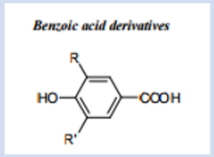
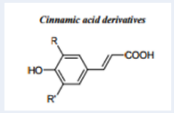
Carotenoids and phenolics from the same basic chemical structure and are differentiated by the addition of functional groups which also differentiate the phytochemicals' roles within plants (Table 1.2). It is their roles within plants that have been identified to aid in antioxidant defence mechanisms due to their ability to quench ROS and prevent lipid peroxidation (Gordon 1996).

1.5.1 Flavonoids

Flavonoids are the most widely studied of the phenolics with over 4000 discovered to date. They are typically found in glycosylated or esterified forms, all leading to variation in role, and can be further divided into subgroups: flavonols, catechins, flavones, flavanones, anthocyanins and isoflavones (Liu, 2004; Manach, 2004). The general structure of flavonoids contains a C6-C3-C6 backbone, of which rings A and B are phenolic in nature (Table 1.3). Ring C is the part of the structure which further sub-divides the flavonoids where variations can occur due to hydroxylation patterns, and positioning of the connection between rings B and C, which can result in the flavonoids existing as glycosides (Tsao, 2010).

Like other phytochemicals, the variants of flavonoids have antioxidant capabilities as they are hydroxylated phenolic substances. The sub-divisions of flavonoids are structure dependent which in turn arranges their chemical nature through their degree of hydroxylation polymerisation and substitutions. The variant consists of the functional hydroxyl group which determines the flavonoids antioxidant capability which allows the scavenging of free radicals (Kumar et al., 2013).

Table 1.2: Classification of phytochemicals explained according to subtype, chemical structure, examples, and sources alongside additional information to understand each type (continued on page 29).

Phytochemical Classification	Subtypes	Chemical Structure	Description		Examples	Sources:	References
Carotenoid			• < 600 have been identified • Have an important role in photosynthesis and photoprotection • Scavenging properties also reflected in humans		α-Carotene, β-Carotene, β-Cryptoxanthin Lutein.	Carrots Broccoli Spinach Zucchini	Raduly et al., 2015 Liu, 2004
Phenolics			• Secondary metabolite				National Centre for Biotechnology Information Liu, 2004
	Phenolic Acids		• Found in few plants within human diet	• Antioxidant activity depends on amount of hydroxyl groups present. • Alongside chemical structure	Hydroxybenzoic acid (e.g. vanillic acid; gallic acid)	Raspberries Strawberries Blackberries	Seabra et aql., 2006 Tsao, 2010 Tomas-Barberan et al., 2000 Manach et al., 2004 Liu, 2004 Rice-Evans et al., 1995 Zambonin et al., 2012
			• More common within human diet		Hydroxycinnamic acid (e.g. caffeic acid; ferulic acid)	Seeds Plant leaves Outer part of ripe fruit	

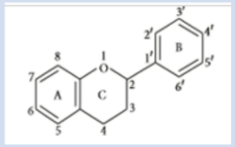
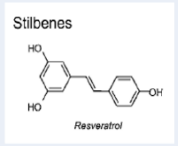
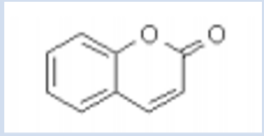
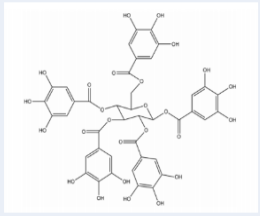
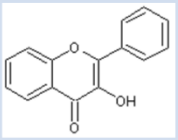
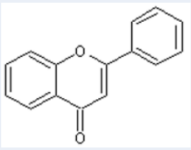
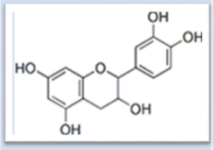
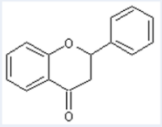
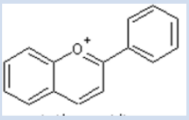
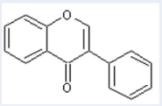
	Flavonoids		• Most abundant phenolic • <4000 have been disocvered	flavonols, flavones, catechins, flavanones, anthocyanidines isoflavones.		Liu, 2004
	Stilbenes		• Present in small quantities in consumables • Thus, present in low quantities in human diet • Protective properties less likely to be seen	Resveratrol	Wine	Manach et al., 2004
	Coumarins		• <300 have been identified • Compex coumarins aid in chronic infections, cancer treatment, blood coagulation and inflammation • Effective antioxidant properties dependant upon the positioning of the hydroxyl group within structure • Belong to the benzopyrone group which is similar to flavonoids • However, coumarin has low bioavailability as it acts as a prodrug	Umbelliferone esculetin scopoletin	Essential oils Bilberry Cloudberry Fruits Roots Stems Leaves	Bhatnagar et al., 2010 Jain et al., 2012 Hoult et al., 1996
	Tannins		• Water soluble polyphenols • Are similar to flavonoids as, hydrolysable tannins undergo hydrolysis to obtain the flavonoid structure • Show *in vitro* health benfits • Not enough information to suggest health benefits occur in humans	condensed tannins, hydrolysable tannins, phlorotannins and complex tannins	Grains Legumes Blackberries Bananas Apples Red wine cocoa	Chung et al., 1998 Serrano et al., 2009

Table 1.3: Classification of flavonoids, divided into their subtypes, chemical structure, examples and structure.

Phytochemical Classification	Subtypes	Chemical Structure	Examples	Sources:	References
Flavonoid	Flavonols		Kaempferol Quercetin Myricetin Tamarixetin	Onions Berries Grapefruit Broccoli	Liu, 2004 Tsao, 2010 Kumar, 2013 Manach, 2004
	Flavones		Luteolin Apigenin Chrysin Rutin	Parsley Celery Fruit skins Red pepper Tomato skins Red wine	
	Catechin		Epicatechin Epigallocatechin	Tea leaves Chocolate Grapes Apple and Blueberry skins	
	Flavanones		Naringin Naringenin Taxifolin Herperidin	Citrus fruits Grapefruits	
	Anthocyanadines		Cyanidin Pelargonidin Delphinidin Peonidin Malvidin	Gives colour and pigment in flower petals, fruit vegetables and grains (black rice)	
	Isoflavones		Genistein Daidzein Glycitein Formonoetin	Soya bean (leguminous family of plants)	

RATIONALE

The aim of this project was to measure the phytochemical and antioxidant levels of a variety of fruits: strawberry, cranberry, acai berry, maqui berry and goji berry, to determine their chemotherapeutic effects alone and in combination with current therapies within human cell lines - human brain glioblastoma, astrocytoma; classified as grade IV compared to human foetal astroglia as a control for the treatment of glioma. These five particular fruits were chosen due to emerging claims promoting their health benefits being advertised to the public due to their high antioxidant levels and their current frequency in the regular diet in freeze dried form.

Initial studies focussed on determining the presence of particular polyphenolic compounds present with the fruit samples via the conduction of High Performance Liquid Chromatography (HPLC). The abundance of phytochemicals within each fruit extract were identified with the use of previously optimised flavonoid and phenolic assays. Further characterisation of the antioxidant effects were measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Following this, the mechanism of action of cell death in glioma cells was measured by cell viability, cell cycle, proliferation, apoptosis and metabolic assays in increasing concentrations.

Cell culture studies were performed alone and compared with current chemotherapeutic agents used in the treatment of glioma, namely cisplatin in cell viability, cell cycle, proliferation and apoptosis assays.

HYPOTHESIS AND AIMS

Working Hypothesis: Can polyphenolic compounds found in cranberry, strawberry, goji berry, maqui berry and acai berry become a potential chemotherapeutic agent in *in vitro* models of glioma?

Main Aim: The main aim of this study was to analyse fruit extracts, determine the composition of antioxidants and study their effects on glioma cell lines.

Specific Aims:

1. To determine the composition of antioxidants present within freeze-dried cranberry, strawberry, goji berry, maqui berry and acai berry.

2. To determine the total flavonoid, total phenolic and total antioxidant capacity of the five fruit extracts.

3. To investigate the time course and dose dependant effects of cisplatin, antioxidants and the five fruit extracts on glioma cell lines.

Chapter 2

METHODS AND MATERIALS

2.1 Chemicals and Plastics

All chemicals used were of the highest quality available.

Scientific Laboraestory Supplies (Nottingham, UK)

Sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), 2N Folin-Ciocalteu's reagent, sodium carbonate (Na_2CO_3), gallic acid, (+) catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX)

Fisher Scientific, (Loughborough, UK)

HPLC Grade methanol, 96 well plates, PBS tablets, 25 and 75 cm^2 filter cap, angled neck tissue culture flasks, serological pipettes, 50 and 15 ml centrifuge tubes, multi-well plates and Foetal Bovine Serum, 20ml syringes

BMG LABTECH (Offenburg, Germany)

FLUOstar OPTIMA plate reader

Sigma Aldrich (Poole, UK)

SUPELCO Analytical LC18, 5µm, 25cm x 4.6mm column, (+) catechin hydrate, gallic acid, myricetin, elagic acid, kaempferol, quercetin, green tea catechin mix, punicalagin and 2 ml vials with screw tops, Trypan blue, cisplatin and DMSO

Greyhound Chromatography (Merseyside, UK)

Cyanidin chloride, malvidin chloride, deplphinidin chloride and pelargonidin chloride

Chemfaces (Hubei, Wuhan, China)

Cyanidin-3-O-glucoside chloride

Agilent Technologies (Waldbronn, Germany)

Agilent Technologies Series 1200 system: G1322A degasser, G11311A quaternary pump, G1329A standard auto sampler, G1316A thermostatted column compartment, G1315D diode array detector and multi wavelength detector and G1159A six position column selector valve.

Life Technologies (Paisley, UK)

PrestoBlue® Cell Viability Reagent

Lonza (Slough, UK)

Eagle's MEM w/o L-Glut, NEAA (100x), L-Glutamine (2mM), Trypsin (10x) and sodium pyruvate (100mM)

ECACC (Porton Down, UK)

U-87 MG Grade IV Human Glioblastoma cell line

ATCC (Masassas, VA, USA)

SVG-P12 human foetal astroglia cell line

www.healthysupplies.co.uk: Healthy Supplies & Sussex Wholefoods (Lancing, West Sussex, UK)

Freeze-Dried Strawberry Powder (100g), Freeze-Dried Cranberry Powder (100g), Goji Juice Powder - organic (150g), Freeze Dried Maqui Berry Powder - organic (100g), Freeze-Dried Acai Berry Powder- Organic (100g)

GE Healthcare Life Sciences (Buckinghamshire, UK)

Puradisc™ 25mm Sterile and Endotoxin Free 0.2µm PES Filter Media

2.2. Sample Preparation

The fruit samples used within experimentation were prepared with the mind – set of the consumer. This was defined by interpreting the serving suggestions given by the manufacturer:

Table 2.1: description of fruit sample preparation. Each fruit had serving suggestions which were applied to the sample preparation in accordance to the experiments conducted in order to obtain results as per these serving suggestions.

Fruit	Serving Suggestions	Fruit sample (g)	Solvent (ml)
Cranberry	1:5 ratio of powder to liquid	1.29	25
Strawberry	1:5 ratio of powder to liquid	1.48	25
Goji berry	2 tsp in 250 ml	2.69	250
Maqui berry	2 tsp in 250 ml	4.11	250
Acai berry	2 tsp in 250 ml	5.39	250

2.3. High Performance Liquid Chromatography (HPLC)

The fruit samples, cranberry, strawberry, goji berry, maqui berry and acai berry were weighed out in accordance to their serving suggestions and dissolved in HPLC grade methanol and filtered using a 0.2 µm filter (Table 2.1). These were compared against the 12 standards consisting of catechin, quercetin, myricetin, kaempferol, punicalagin, ellagic acid, gallic acid, cyanidin, cyanidin-3-glucoside, delphinidin, malvidin, pelargonidin. All standards were prepared as 1mg/ml and were stored at -20°C. Two HPLC methods were preliminarily explored with the use of an Agilent Technologies Series 1200 system consisting of a degasser, quaternary pump, standard auto sampler, thermostatted column compartment, diode array detector and multi wavelength detector and a six position column selector valve.

2.3.1 HPLC: Method 1

All standards and samples were analysed following a gradient elution programme (modified from Huang et al., 2012). This required solution A, 0.1% formic acid, and solution B, 100% methanol. This was used to follow a graduated method consisting of: 0-10 min, where solution B was increased from 0%–10%; at 10–25 min, solution B was increased from 10%–20%; at 25–35 min, solution B increased from 20%–23%; at 35–45 min, solution B increased from 23%–28%; at 45–60 min, solution B increased from 28%–35%; at 60–75 min, solution B increased from 35%–50%; at 75–80 min, solution B increased from 50%–55%; at 80–85 min, solution B increased from 55%–75%; at 85–90 min, solution B increased from 75%. The flow rate was 0.8 ml/min and the injection volume was 20 µl. Detection was monitored at 280 nm.

2.3.2 HPLC: Method 2

All standards and samples were analysed following a method modified from Brauch et al., 2016. Elution solvent A consisted of water/methanol/formic acid (77/13/10), while solvent B was a mixture of water/methanol/formic acid (30/60/10). Separation was achieved using: 0-15 min, 6% (solvent B), increased to 30% (solvent B), 15-20 min, 30% - 36% (solvent B), 20-25 min, 36%- 100% (solvent B), 25-27 min, 2 min isocratic period, 27-30 min, 100% - 6% (solvent B), 30-35 min, re-equilibration at 6% (solvent B). Total run time was 35 min at a flow rate of 0.8 mL/min. The injection volume was 10µl. Samples were monitored at 520 nm.

2.3.3 Identification of Phytochemicals in Fruit Extracts

Running the HPLC methods gave an indication of what could be present within the fruit extracts. To seek confirmation of what was observed, the process of spiking was undertaken. Spiking was the process in which a standard antioxidant was added to a fruit sample to determine the presence of that antioxidant within the fruit extract. This was expressed as a larger peak on the resulting chromatograms. Fruit extracts and standards were prepared in a 1:1 ratio and subjected to HPLC using a modified version of method 1.

2.3.4 Quantification of Phytochemicals in Fruit Extracts

The determination of the presence of antioxidants within the fruit samples led to the progression of quantifying this. This was achieved by creating a standard curve per relevant antioxidant against which each fruit sample could be quantified. This was accomplished by serial, 1:2 dilutions of the antioxidant standard from 1mg/ml to 0.0009 mg/ml. From this, it was possible to interpolate the amount of antioxidant present within the fruit sample.

2.4. Determination of Phytochemical Content and Antioxidant Capacity of Fruit Extracts

The fruit samples, cranberry strawberry, goji berry, maqui berry and acai berry were prepared as recommended by the serving suggestions (Table 2.1) and dissolved in dH_2O.

2.4.1 Measurement of Total Phenolic Content

The total phenolic content (TPC) was determined using a modified Folin-Ciocalteu method (Yang et al., 2012). Samples consisted of undiluted and diluted, in dH2O, at 1:10, 1:100 and 1:1000 using serial dilutions and 12.5µl was added in triplicate to the wells, incubated with 62.5µl of a master mix containing 50µl dH2O and 12.5µl of 1N Folin- Ciocalteu reagent for 5 minutes at room temperature. 125µl of 7% Na2CO3 was added per well and samples incubated for 30 minutes at room temperature. Absorbance was measured at 750nm. TPC was quantified using a standard calibration curve, prepared using 0.5mg/ml gallic acid in dH20, and expressed as mg of gallic acid equivalents (GAE)/ml of sample (Appendix 2.2).

2.4.2 Measurement of Total Flavonoid Content

The total flavonoid content (TFC) of the samples was determined using a modified $AlCl_3$ method (Marinova et al., 2005). Samples consisted of undiluted and diluted, in dH_2O, at 1:10, 1:100 and 1:1000 using serial dilutions and 20µl added in triplicate to the

wells. 6µl of 5% NaNO2 and 80µl of dH_2O added to each well and incubated for 5 minutes, after which 94µl of a master mix containing 6µl 10% $AlCl_3$, 40µl 1M NaOH and 48µl of dH2O was added and the absorbance was measured at 510nm. The TFC of the samples was quantified using a standard calibration curve, prepared using 1mg/ml catechin in dH_2O, and expressed as mg of catechin equivalents (CE)/ml of sample (Appendix 2.1).

2.4.3 DDPH Radical-Scavenging Capacity

The antioxidant capacity (AC) was determined by the DPPH· radical scavenging method modified from Kodama et al., (2012) and Thaipong et al., (2006). Samples consisted of undiluted and diluted, in MeOH on ice, at 1:10, 1:100 and 1:1000 using serial dilutions. 50µl of sample was incubated with 250µl of 0.5mM DPPH per well for 20 minutes at room temperature. Absorbance was measured at 515nm. AC was quantified using a standard calibration curve, prepared using 1mM TROLOX in MeOH and expressed as µM TROLOX equivalents (TE)/ ml of sample (Appendix 2.3.1). Percentage scavenging was also calculated using a standard calibration curve (Appendix 2.3.2).

2.5 Cell Culture

Fruit samples, cranberry, strawberry, goji berry, maqui berry and acai berry were prepared in accordance to the serving suggestions and dissolving in media, in sterile conditions in a Labcaire laminar flow hood.

2.5.1 Cell Maintenance

U-87 MG and SVG-p12 cells were maintained in EMEM supplemented with, Foetal Bovine Serum, L-Glutamine (2mM), NEAA (1%), and sodium pyruvate (1mM) in an environment of 37oC and 5% CO2 atmosphere within an IncuSafe incubator. Upon confluency of cells at approximately 80%, cell monolayers were washed with 3ml PBS solution, replaced with 2ml trypsin and the flask was returned to the incubator to allow the cells to detach. After detachment, 4ml of the supplemented EMEM (media) was added to the flask to neutralise the trypsin. The cells were gently pipetted to ensure a single cell suspension was obtained before passaging into suitable ratios or seeded into plates for experimental analysis.

2.5.2 Growth Curves

In order to determine the growth rate of the cell lines, performed over seven days. 6 well plates were seeded with 20,000 cells/ml and topped with 2ml media. Cells were trypsinised and the cell number was determined by manually counting the cells with the use of a haemocytometer on 2-5 days, with an interval of 2 days and then read on the 7th day. This process was repeated in triplicate.

2.5.3 Presto Blue® Cell Viability Assay

Concentration dependent effects of each treatment on cell viability were measured by the addition of PrestoBlue®, resulting in a resazurin reduction by metabolically active

cells, changing in colour from blue to red (absorbance 570 nm) and becoming highly fluorescent (excitation 535 nm/ emission 612 nm), over a 7 day period and repeated in triplicate. Thus, allowing it to be possible to quantify changes. This was accomplished by expressing viability via linearity of fluorescence versus cell number. Cells were seeded at 0, 500, 1000, 2000, 2500, 5000 and 10,000 cells/ well per 100µl of media in 96-well plates. These plates were then left to incubate over a period of 7 days within normoxic and hypoxic conditions.

After 24, 48, 72, 96 and 120 hrs of incubation, 10 µl of PrestoBlue® was added to each well and the plate was returned to incubate for one hour after which the fluorescence was obtained by the Tecan GENius PRO plate reader.

2.5.4 Concentration Response Curves

The concentration response assay was conducted to determine the IC_{50} values for cisplatin, punicalagin, cyanidin-3-glucoside, cranberry, strawberry, maqui berry and acai berry fruit samples in SVG-p12 and U-87 MG cells in normoxic conditions.

This was accomplished by seeding cells at 1000 cells/ well in 100 µl media and incubated for 24 hours before (11 µl) drug treatments were added (ranging from 1µM-300µM) in order to determine the dose response. This was left to incubate for another 24 hours before 10µl of PrestoBlue® was added for the fluorescence to be measured one hour later at the same wavelength as the cell viability assay.

2.6. Statistical Analysis

Statistical analyses were performed using Microsoft Excel 2013, GraphPad Prism 5 and IBM SPSS Statistics Version 22. Fruit samples were compared by performing one-way and two-way ANOVA tests with Tukey's and Gabriel's *post- hoc* analysis on SPSS and GraphPad Prism 5, with $p<0.05$ considered as indicating a statistically significant difference. Results were expressed as an average ± the Standard Deviation or ± the Standard Error of Mean (SEM) where appropriate. HPLC used ChemStation to aid in the analysis of chromatograms.

Chapter 3

RESULTS

3.1 High Performance Liquid Chromatography

3.1.1 Comparison of HPLC Methods for Sample Separation

Based on previously published protocols, investigations into the phytochemical composition of the five fruit samples were undertaken using two different graduated HPLC methods. Method 1 comprised of a 90 minute run time with a mobile phase of methanol and 0.1% formic acid and read at 280 nm. Method 2 consisted of a 30 minute graduated method with a mobile phase of two solvent mixtures containing water: methanol: formic acid in the ratios of 77:13:10 (Solvent A) and 30:60:10 (Solvent B). Peaks in method 2 were detected at 520nm. Method 2 had been demonstrated previously to be optimal for detecting anthocyanidins in fruit samples and so was of particular interest for this study.

Initial investigations using HPLC Method 1 showed an elution time for the fruit samples of between 3.38 ± 0.28 - 3.59 ± 0.06 mins (Figure. 3.1 and Table 3.1). All of the standards used were eluted from the column within 3.00 ± 0.90 – 84.27 ± 2.04 mins except catechin, quercetin, myricetin and kaempferol, which did not show discernible peaks using this method (Table 3.1). Comparison of the traces and peak elution times suggested that the fruit samples may contain punicalagin (3.46 ± 0.45 mins), gallic acid (3.35 ± 0.11 mins), cyanidin-3-glucoside (3.33 ± 0.15 mins) and malvidin (3.47 ± 0.04 mins) (Table 3.1).

Method 2 showed elution times between 4.04 ± 0.00 mins (maqui berry) and 4.66 ± 0.03 mins (strawberry) for the fruit extracts (Figure. 3.2). The standards showed a more wide-ranging elution rate ranging from 4.23 ± 0.01mins (cyanidin-3-glucoside) to 27.75 ± 2.7 mins (malvidin) (Table 3.2). Based on these results, and as this method was

known to be favourable for separating anthocyanidins, it was suggested that the samples contained only cyanidin-3-glucoside which had shown a retention time of 4.23 ± 0.01mins (Table. 3.2).

3.1.2 Identification of Phytochemicals Present in Fruit Extracts

Having identified a number of antioxidants which may have been present in the fruit extracts, further investigation was undertaken. Fruit extracts and standards were prepared in a 1:1 ratio and subjected to HPLC using Method 1. This process of 'spiking' the samples with the addition of a known reference material can help to confirm the identity of the sample component peaks.

Each fruit extract was mixed with punicalagin, gallic acid, cyanidin-3-glucoside or malvidin. As the retention times for the extracts and standards was within the first 5 minutes, the run time for Method 1 was decreased to 20 mins.

Analysis of the spiked samples showed an increase in the height of the peaks detected on the chromatograms with no additional peaks observed (Figure. 3.3). Retention times for the spiked samples were comparable to those obtained from the fruit extracts and standards alone (Table 3.3) indicating that punicalagin, gallic acid, cyanidin-3-glucoside and malvidin could be present in the fruits investigated. This demonstrated that there is tentative evidence that punicalagin, cyanidin-3-glugoside, and malvidin could be present within the fruit extracts.

3.1.3 Quantification of Phytochemical Components used with the HPLC system and comparison of Fruit Extracts between HPLC, TFC and TPC

Having determined the presence of 4 antioxidant compounds within each of the fruit extracts, a standard curve was generated in order to measure the amount of each compound in each extract. Decreasing concentrations of the standards (1 – 0.0009 mg/ml) were run through HPLC using Method 1. The minimum amount of standard that could be reliably detected was determined (limit of detection; LOD) and plotted as concentration vs average peak area (Figure 3.4) thus expressing the validity of the system used.

From this, we were able to use the peak areas from the fruit extracts obtained from the HPLC method 1 and compare them to the results obtained from the TFC and TPC assays. This showed similarities where by the

3.2 Determination of the Phytochemical Content and Antioxidant Capacity of Fruit Extracts

Phenolics are products of secondary metabolism in plants and provide essential functions for the maturation and reproduction. They are compounds which have one or more aromatic benzene rings, with one or more hydroxyl groups attached to it. The phenolics are further divided into 5 subgroups according to the number of phenol rings that they contain and the structural elements that bind these rings to one another. Flavonoids, the largest sub-group of phenolics, have been shown to possess antioxidant, anti-proliferative and anti-inflammatory activity.

The therapeutic potential of plants can be measured by a variety of parameters including the ratio of flavonoid to phenolic compounds present in the extract, and the overall antioxidant activity observed. The ratio of flavonoids to overall phenolic content in plant extracts, in combination with the measurement of the antioxidant capacity of these

extracts can be used as an indicator of the therapeutic potential of these compounds. Having identified the presence of antioxidant compounds within the fruit extracts, total phenolic, total flavonoid and overall antioxidant capacity was measured.

3.2.1 Total Phenolic and Total Flavonoid Content

To determine the total phenolic content (TPC) of the fruit extracts, a modified Folin-Ciocalteu assay was used (Section 2.4.1). The average TPC ranged from 281.55 ± 6.97 (goji berry) to 2835.11 ± 26.48 (strawberry) mg/ml GAE as calculated for a 'serving' as specified by the manufacturer (Table 3.5; Figure 3.6; Section 2.4). Within these samples, a significant difference was found ($p<0.001$) in the range of TPC values (Table 3.6). A Tukey's post hoc analysis revealed a significant difference in the mean TPC between: cranberry and strawberry and goji berry ($p<0.05$); strawberry and all fruit extracts ($p<0.05$ cranberry; $P<0.001$) as shown in table 3.7.

Using a modified AlCl3 method (Section 2.4.2), total flavonoid content (TFC) of each extract was quantified. The TFC of the extracts was found to range from 166.37 ± 33.38 (goji berry) to 670.93 ± 45.30 µg/ml CE (cranberry) per serving (Table. 3.5; Figure 3.5; Section 2.2). Significant differences ($p<0.001$) in the TFC were found between all fruit extracts (Table 3.6). Further analysis identified the most significant differences in the mean TFC between: cranberry and goji berry ($p<0.05$); cranberry and acai berry ($p<0.05$) was observed (Table 3.7).

3.2.2 Flavonoid to Phenolic Ratio

Although flavonoids are the most abundant phenolic, there are four other sub-groups present within this classification. Therefore, the flavonoid to phenolic ratio expresses the TFC as a proportion of the TPC. The flavonoid to phenolic ratio states the proportion of flavonoids that make up the phenolic content within the fruit extracts. The flavonoid: phenolic ratio ranged from 0.14 in strawberry to 0.59 in goji berry. This ratio is expressed for all the fruits extracts as shown is table 3.5.

3.2.3 Total Antioxidant Capacity

Following determination of a high ratio of flavonoid to phenolic compounds in a number of the fruit extracts, the antioxidant capacity (AC) of each fruit was measured. The AC of the compounds previously identified by HPLC as being present in the fruit extracts were also measured in parallel (gallic acid, punicalagin, cyanodin-3-glucoside and malvidin). Using the DPPH· radical scavenging method (Section 2.4.3), the average AC as shown in table 3.8 was found to range from 2239.07 ± 1021.27 (cranberry) to 16.84 ± 82.60 mM TE (goji berry) per serving ($p<0.001$) (Figure 3.7; Table 3.9). Table 3.9 shows that the Gabriel's post hoc test for the DPPH assay revealed a significant difference in the mean AC between: cranberry and strawberry, goji berry, and punicalagin ($p<0.05$, excluding maqui berry, acai berry, gallic acid, cyanidin-3-glucoside and malvidin, $p<0.001$).

3.3. Cell Culture

3.3.1 Growth Curves

Proliferation and the conditions in which it occurs are factors influencing cell growth. This affects any lag periods or plateaus in growth following prolonged incubation of SVG-p12 and U87-MG cells. In this case, growth curves were performed in conditions of normoxia and hypoxia over a period of 7 days. Both cell lines expressed an exponential growth over a period of seven days, displaying signs of a lag phase over the first 48 hours (Figure 3.8). A significant difference was found between the incubation conditions of normoxia and hypoxia within both cell lines at 168 hours ($p<0.001$).

3.3.2 Cell Viability

The relationship between fluorescence and cell number as measured by the PrestoBlue® viability assay was determined by an assay using increasing numbers of cells (Section 2.5.4)). SVG-p12 and U87-MG cell lines displayed a linear relationship between 0-24 hours between 0-10,000 cells. However, following this at 48 hours, fluorescence was linear to 2,500 cells and 72 - 168 hours a linearity is only present between 0-1000 cells (Figure 3.9).

3.3.3 Dose Response Curves

The results obtained from the TAC, indicated punicalagin and cyandin-3-glucoside to contain the highest AC as measured by the radical scavenging activity. Conversely, goji berry consistently showed low levels of potential antioxidants throughout the study. Therefore, punicalagin and cyandin-3-glucoside were taken forward for in vitro analysis of their activity. Of the fruits, goji berry was not analysed further. Concentration response assays were undertaken in SVGp12 and U87-MG cell lines to establish the IC_{50} values for the fruit extracts, the previously identified standards punicalagin and cyanodin-3-glucoside and the standard chemotherapeutic agent, cisplatin.

Following treatment with cisplatin, a reduction in cell viability was observed in SVG-p12 and U87-MG cell lines at 24hrs (37.04 µM vs10.82 µM), 48hrs (10.11 µM vs 7.13 µM) and 72hrs (9.42 µM vs 1.94 µM). Punicalagin showed SVG-p12 and U87-MG IC_{50} values at; 24 hrs (85.42 µM vs 149.90 µM), 48 hrs (48.56 µM vs 45.55 µM) and 72 hrs (46.50 µM vs 57.11 µM). Cyanidin-3-glugoside displayed a decrease in cell viability in both cell lines at 24hrs (103.60 µM vs 101.30 µM), 48hrs (109.40 µM vs 46.24 µM) and 72hrs (36.65 µM vs 31.43 µM). Further analysis found there to be a significant difference between cyanidin-3-glucoside and cisplatin and punicalagin ($p<0.001$) in SVGg-p12 cells (Figure 3.10 and Table 3.10).

In addition, concentration response assays were conducted with fruit extracts prepared at 1mg/ml in EMEM (Section 2.2). IC_{50} values for cranberry showed a decrease in cell viability at 24hrs (3.88 mg/ml). In the U87-MG cell line, IC_{50} values at 48hrs (0.13 mg/ml) and 72 hrs (0.01 mg/ml). Strawberry at 48 hours in SVG-p12 resulted in an IC_{50}

value of 0.11 mg/ml. U87-MG showed IC_{50} values at 24 hrs (0.24 mg/ml), 48 hrs (3.43 mg/ml) and 72 hrs (0.01 mg/ml) when strawberry was added to the cell line. Maqui berry had an IC_{50} value of 0.001 mg/ml at 72 hrs in SVG-p12 cells (Figure 3.11 and Table 3.11). Further analysis from this expressed a significant difference between maqui and cranberry ($p<0.001$ at 24, 48 and 72 hrs); maqui and strawberry ($p<0.001$ at 24 and 48 hrs); and maqui and acai berry ($p<0.001$ at 24 and 48 hrs; $p<0.05$ at 72 hrs) in the SVG-p12 and U87-MG cell lines.

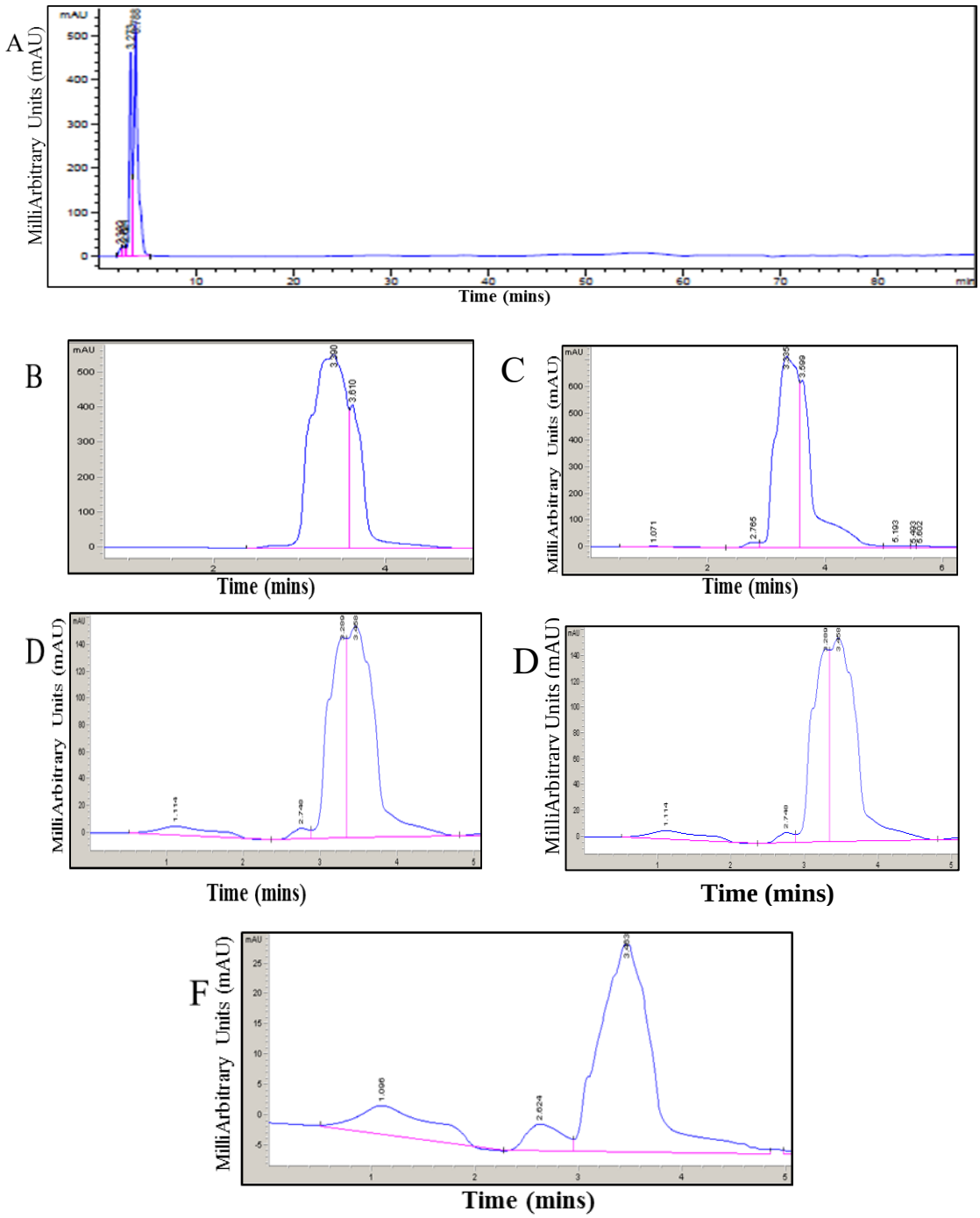


Figure 3.1 (A-F): Example of Chromatograms of fruit samples obtained from method 1. Fruit samples obtained from Method 1; A. cranberry- entire chromatogram; B. cranberry; C. strawberry; D. goji; E. maqui; F. acai. These samples were run on a 90 minute graduated method alongside 12 standards. Results shown were obtained from one independent experiment.

Table 3.1 (A & B): Average retention times, peak areas and percentage peak areas for all standards (A) and samples (B) obtained using Method 1. Average retention

times, peak areas and percentage peak areas were obtained from two independent experiments (A). The averages for the fruit samples were obtained from the biggest peak found from two independent experiments. This information led to the possibility of punicalagin, gallic acid, cyanidin-3-glucoside and malvidin to be present within the fruit samples.

Sample	Average Retention Time (mins)	Average Peak Area (mAU*s)
Catechin	-	-
Quercetin	-	-
Myricetin	-	-
Kaempferol	-	-
Punicalagin	3.46 ± 0.45	17392.15 ± 301.02
Elagic Acid	3.00 ± 0.90	2094.53 ± 1886.3
Gallic Acid	3.35 ± 0.11	30839.75 ± 29997.36
Cyanidin	84.27 ± 2.04	46945.95 ± 446.11
Cyandin 3-glucoside	3.33 ± 0.15	1027.37 ± 602.65
Delphinidin	77.73 ± 2.39	28622.65 ± 383.46
Malvidin	3.47 ± 0.04	1269.96 ± 945.73
Pelargonidin	44.57 ± 62.08	14422.43 ± 14548.96
Cranberry	3.59 ± 0.06	15145.05 ± 427.45
Strawberry	3.38 ± 0.28	20876.90 ± 3319.44
Gojiberry	3.43 ± 0.03	4615.724 ± 869.16
Maqui berry	3.43 ± 0.01	9818.40 ± 518.87
Acai berry	3.43 ± 0.05	857.86 ± 279.11

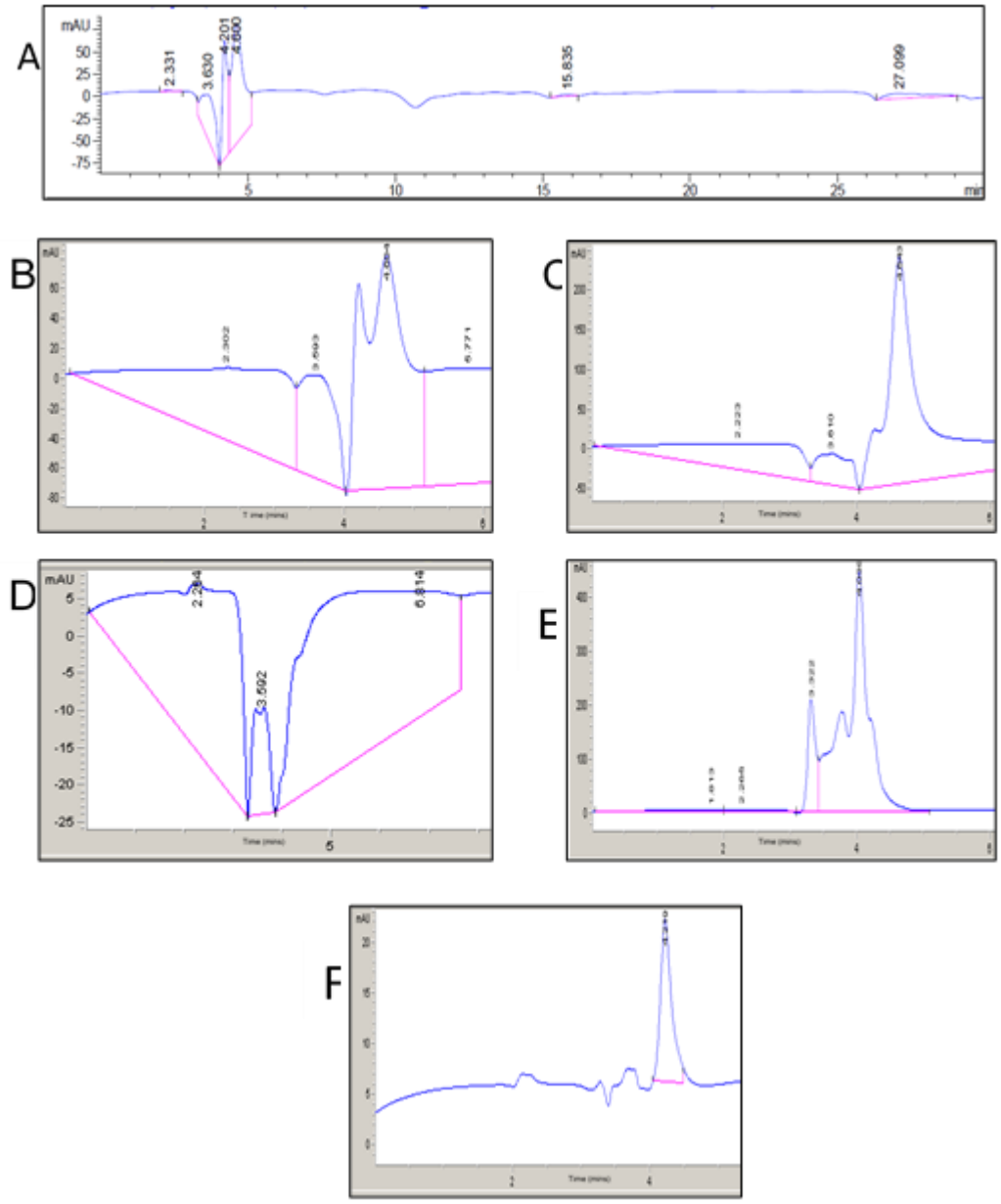


Figure 3.2 (A-E): Zoomed chromatograms of fruit samples obtained from Method 2. Fruit samples obtained from method 1; A. cranberry – entire chromatogram; B. cranberry; C. strawberry; D. goji; E. maqui; F. acai. These samples were run on a 30 minute graduated method alongside 12 standards. Results shown were obtained from one independent experiment.

Table 3.2 (A & B): Average retention times, peak areas and percentage peak areas for all standards (A) and samples (B) obtained from Method 2. Average retention times, peak areas and percentage peak areas were obtained from two independent experiments (A). The averages for the fruit samples were obtained from the biggest peak obtained from two independent experiments. This information led to the possibility cyanidin-3-glucoside to be present within the fruit samples.

Sample	Average Retention Time (mins)	Average Peak Area (mAU*s)	Average Peak Area (%)
Catechin	-	-	-
Quercetin	-	-	-
Myricetin	-	-	-
Kaempferol	-	-	-
Punicalagin	-	-	-
Elagic Acid	-	-	-
Gallic Acid	-	-	-
Cyanidin	15.77 ± 0.21	57104.65 ± 2842.78	38.03
Cyandin 3-glucoside	4.23 ± 0.01	19849.85 ± 1000.63	34.15
Delphinidin	9.37 ± 0.16	42544.35 ± 1823.23	35.64
Malvidin	27.75 ± 2.7	19646.15 ± 8599.62	43.74
Pelargonidin	21.98 ± 0.18	44731.95 ± 2050.91	40.59
Cranberry	4.62 ± 0.03	3651.97 ± 331.70	14.66
Strawberry	4.66 ± 0.03	7332.82 ± 355.91	26.31
Gojiberry	-	-	-
Maqui berry	4.04 ± 0.00	7220.45 ± 454.62	20.38
Acai berry	4.23 ± 0.01	258.43 ± 43.57	28.13

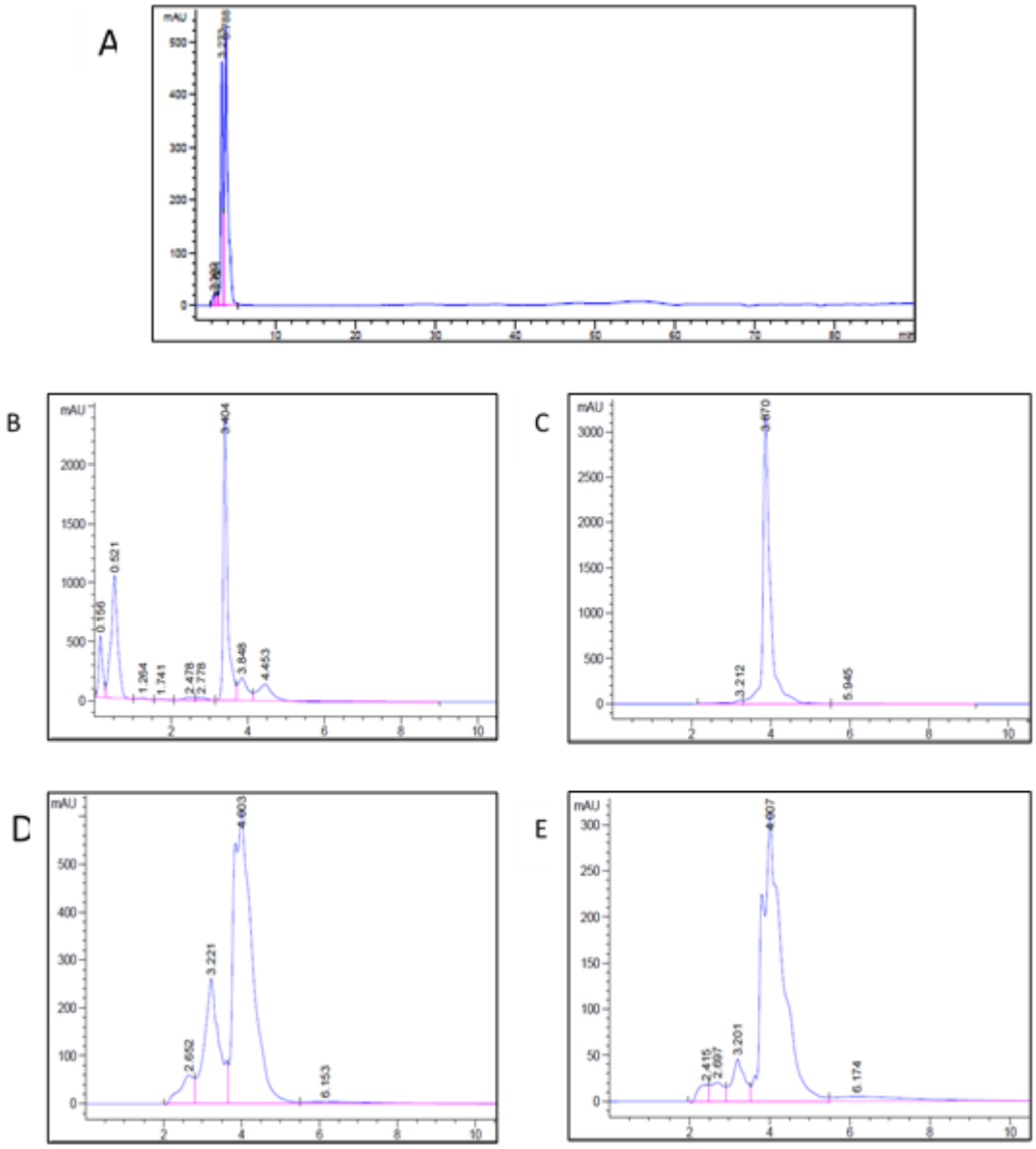


Figure 3.3 (A-D): Zoomed chromatograms of spiked samples obtained from method 1 as compared to non-spiked sample. Example of fruits spiked with compounds obtained from method 1; A. Cranberry; B. Cranberry + punicalagin; C. Cranberry + gallic acid; D. Cranberry + cyanidin-3-glucoside; E. Cranberry + malvidin.

Table 3.3: Average retention times spiked samples obtained from Method 1. Average retention times and peak areas were obtained from two independent experiments. Percentage presence represents the percentage increase in the peak areas of the fruit sample compared to the corresponding standard, once the samples were spiked.

	Punicalagin (Mins)			Gallic Acid (Mins)			Cyandin 3-glucoside (Mins)			Malvidin (Mins)		
Fruit	Standard Retntion Time	Fruit Sample Retention Time	Spiked Sample Rention Time	Standard Retntion Time	Fruit Sample Retention Time	Spiked Sample Rention Time	Standard Retntion Time	Fruit Sample Retention Time	Spiked Sample Rention Time	Compound Retntion Time	Fruit Sample Retention Time	Spiked Sample Rention Time
Cranberry	3.46 ± 0.45	3.59 ± 0.06	3.64 ± 0.34	3.35 ± 0.11	3.59 ± 0.06	3.87 ± 0.01	3.33 ± 0.15	3.59 ± 0.06	4.01 ± 0.01	3.47 ± 0.04	3.59 ± 0.06	4.01 ± 0.00
Strawberry	3.46 ± 0.46	3.38 ± 0.28	3.82 ± 0.00	3.35 ± 0.12	3.38 ± 0.28	3.86 ± 0.01	3.33 ± 0.16	3.38 ± 0.28	3.84 ± 0.02	3.47 ± 0.05	3.38 ± 0.28	3.81 ± 0.00
Goji berry	3.46 ± 0.47	3.43 ± 0.03	3.79 ± 0.02	3.35 ± 0.13	3.43 ± 0.03	3.86 ± 0.00	3.33 ± 0.17	3.43 ± 0.03	3.88 ± 0.06	3.47 ± 0.06	3.43 ± 0.03	3.96 ± 0.02
Maqui berry	3.46 ± 0.48	3.43 ± 0.01	3.80 ± 0.00	3.35 ± 0.14	3.43 ± 0.01	3.86 ± 0.00	3.33 ± 0.18	3.43 ± 0.01	3.90 ± 0.01	3.47 ± 0.07	3.43 ± 0.01	3.92 ± 0.02
Acai berry	3.46 ± 0.49	3.43 ± 0.05	3.79 ± 0.01	3.35 ± 0.15	3.43 ± 0.05	3.86 ± 0.00	3.33 ± 0.19	3.43 ± 0.05	3.88 ± 0.08	3.47 ± 0.08	3.43 ± 0.05	4.44 ± 0.04

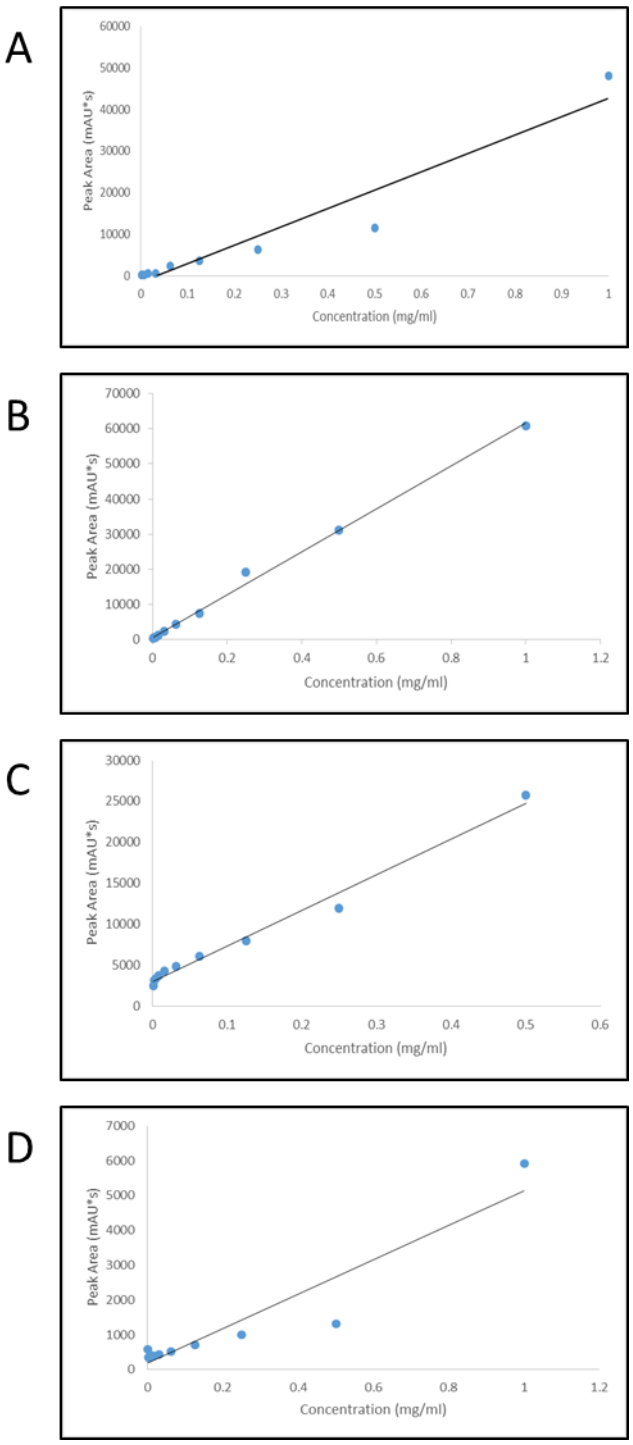


Figure 3.4 (A-D): Standard curves of compounds obtained via LOD from Method 1. Standard curve used to quantify the concentration of the compounds; A. Punicalagin (a positive correlation was observed between peak area and concentration at r2=0.93); B. Gallic Acid; (a positive correlation was observed between peak area and concentration at r2= 0.99) C. Cyanidin-3-glucoside (a positive correlation was observed between peak area and concentration at r2= 0.99); D. Malvidin (a positive correlation was observed between peak area and concentration at r2= 0.89) within the fruit samples expressed as mg/ml.

Table 3.4: Comparison between the average peak areas of the fruit extracts and the TFC and TPC. The HPLC values were obtained from n=2 of HPLC method 1; TFC and TPC values were expressed as µg/serving Catechin and µg/serving Gallic acid from n=3 independent experiments. The TFC and TPC values are also expressed individually in figures 3.5 and 3.6.

Fruit	Fruit Sample Average Peak area	Total Flavonoid Content	Total Phenolic Content
	(mAU)	Avg µg/serving Catechin	Avg µg/serving Gallic acid
Cranberry	15145.05 ± 427.45	670.93 ± 45.30	1439.16 ± 7.59
Strawberry	20876.90 ± 3319.44	394.72 ± 39.09	2835.11 ± 26.484
Goji berry	4615.724 ± 869.16	166.37 ± 33.38	281.55 ± 6.97
Maqui berry	9818.40 ± 518.87	427.83 ± 151.46	1104.52 ± 23.32
Acai berry	857.86 ± 279.11	251.57 ± 52.45	851.60 ± 18.17

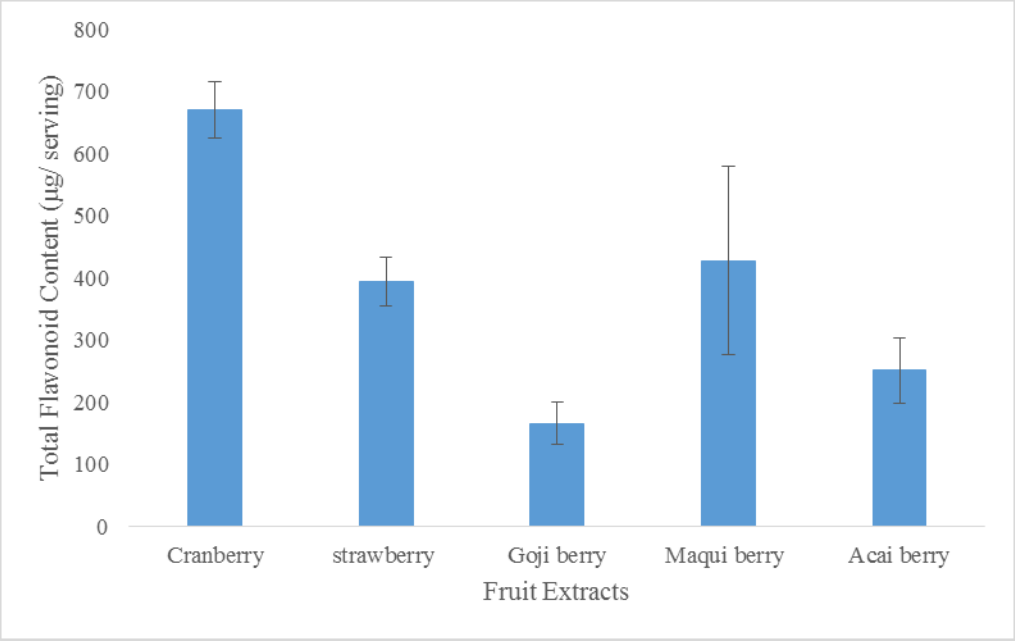


Figure 3.5: Shows the total flavonoid content results obtained for each fruit extract

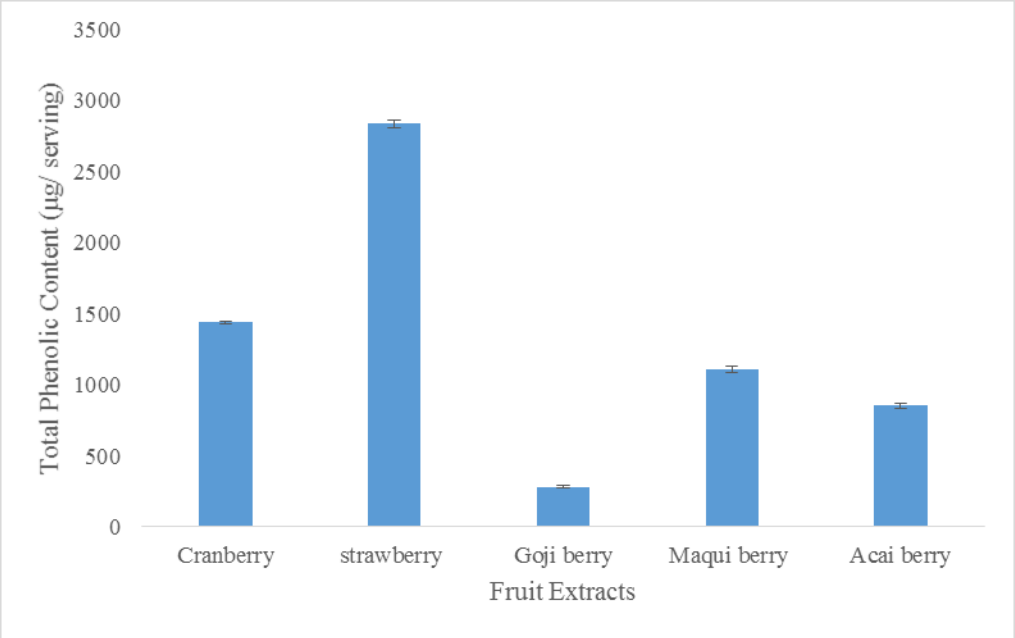


Figure 3.6: Shows the total phenolic content results obtained for each fruit extract

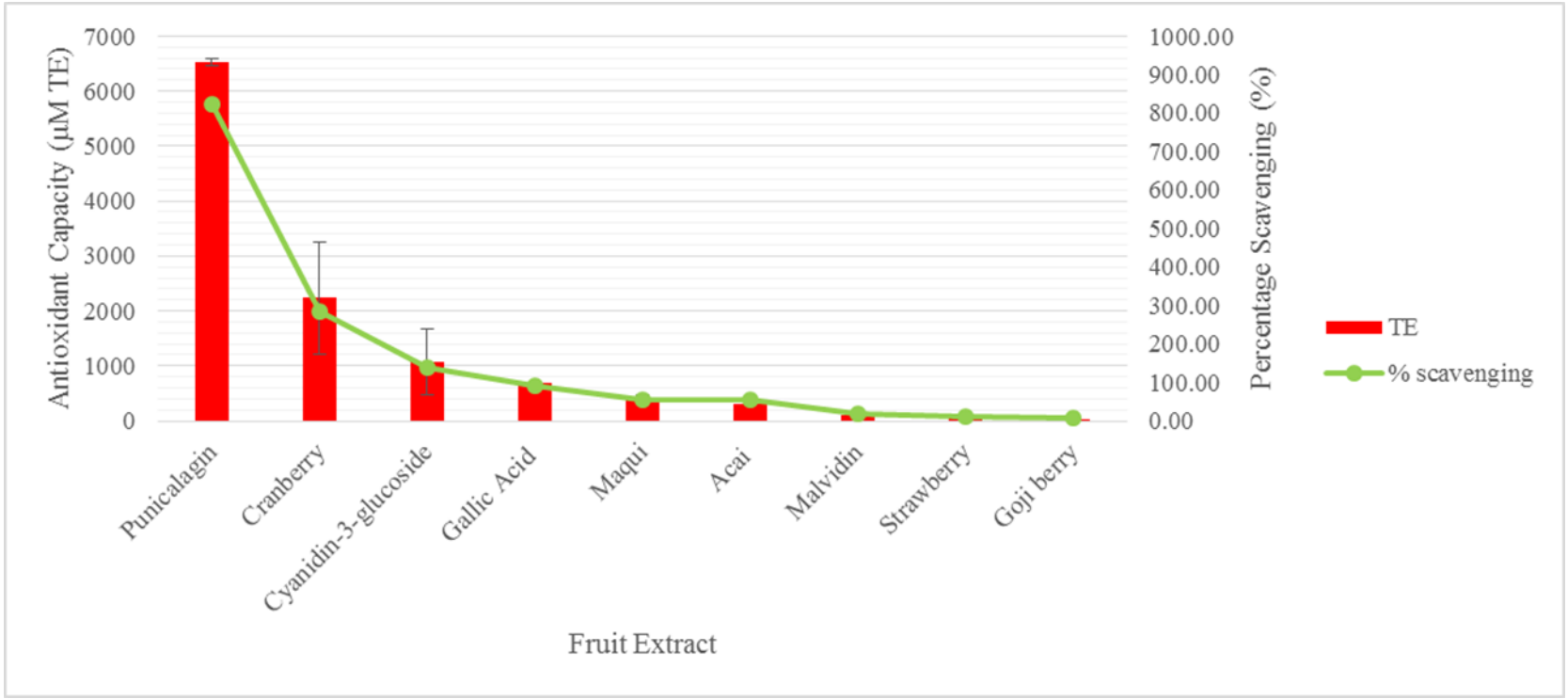


Figure 3.7: Shows the antioxidant capacity and the percentage scavenging results obtained via the DPPH assay for each fruit type within each fruit form in ascending order.

T.E = TROLOX equivalence

Table 3.5: Total Flavonoid Content, Total Phenolic Content, Flavonoid to Phenolic Ratio and Total Antioxidant Capacity: TFC per fruit extract expressed as µg/serving catechin equivalence; TPC per fruit extract expressed as µg/serving gallic acid equivalence; flavonoid to phenolic ratio as obtained from n=3 independent experiments.

Fruit	Total Flavonoid Content	Total Phenolic Content	
	Avg µg/serving Catechin	Avg µg/serving Gallic acid	Flavonoid: Phenolic Ratio
Cranberry	670.93 ± 45.30	1439.16 ± 7.59	0.47
Strawberry	394.72 ± 39.09	2835.11 ± 26.484	0.14
Goji berry	166.37 ± 33.38	281.55 ± 6.97	0.59
Maqui berry	427.83 ± 151.46	1104.52 ± 23.32	0.39
Acai berry	251.57 ± 52.45	851.60 ± 18.17	0.30

Table 3.6: One- way ANOVA for antioxidant activity: Showing the degrees of freedom (df), mean square and p values within each antioxidant assay; total flavonoid content, total phenolic content and DPPH.

Assay		df	Mean Square	*P*
Total Flavonoid Content	Between Groups	4	111937.996	<0.001
	Within Groups	10	18231.067	-
	Error	14	-	-
Total Phenolic Content	Between Groups	4	2739308.764	<0.001
	Within Groups	10	100870.852	-
	Error	14	-	-
DPPH	Between Groups	8	14928052.92	<0.001
	Within Groups	33	1029608.886	-
	Error	41	-	-

Table 3.7: Post hoc analysis: A Tukey's post hoc analysis was conducted to show the difference between the fruit extracts within total flavonoid and total phenolic content.

n.s. = No Significant Difference. a = TFC, b = TPC

Fruit Extract	Cranberry	Strawberry	Goji berry	Maqui berry	Acai berry
Cranberry		n.s.[a] P<0.05 [b]	P<0.05 [a] P<0.05 [b]	n.s.[a] n.s.[b]	n.s.[a] n.s.[b]
Strawberry	n.s.[a] P<0.05 [b]		n.s.[a] P<0.01 [b]	n.s.[a] P<0.01 [b]	n.s.[a] P<0.01 [b]
Goji berry	P<0.05 [a] P<0.05 [b]	n.s.[a] P<0.01 [b]		n.s.[a] n.s.[b]	n.s.[a] n.s.[b]
Maqui berry	n.s.[a] n.s.[b]	n.s.[a] P<0.01 [b]	n.s.[a] n.s.[b]		n.s.[a] n.s.[b]
Acai berry	n.s.[a] n.s.[b]	n.s.[a] P<0.01 [b]	n.s.[a] n.s.[b]	n.s.[a] n.s.[b]	

Table 3.8: Total antioxidant capacity of fruit extracts and antioxidants. Total antioxidant capacity for each sample was expressed as µM/serving TROLOX equivalence as obtained from n=6 independent experiments.

Sample	Antioxidant Capacity	
	Avg µM/serving TROLOX	Percentage Scavenging (%)
Cranberry	2239.07 ± 1021.27	284.83
Strawberry	63.35 ± 52.60	12.00
Goji berry	16.84 ±82.60	6.16
Maqui berry	407.05 ±76.59	55.09
Acai berry	308.34 ± 32.65	42.72
Gallic Acid	697.25 ± 2.17	91.49
Punicalagin	6522.74 ± 59.30	822.00
Cyanidin-3-glucoside	1067.87 ± 592.15	137.96
malvidin	101.22 ± 105.41	16.74

Table 3.9: Post hoc analysis for DPPH assay. A Gabriel's post hoc analysis was conducted to show the difference between the fruits within the total antioxidant capacity assay.

n.s. = No significant difference

Sample	Cranberry	Strawberry	Goji berry	Maqui berry	Acai berry
Cranberry		n.s.	n.s.	n.s.	n.s.
Strawberry	n.s.		n.s.	n.s.	n.s.
Goji berry	n.s.	n.s.		n.s.	n.s.
Maqui berry	n.s.	n.s.	n.s.		n.s.
Acai berry	n.s.	n.s.	n.s.	n.s.	
Gallic Acid	n.s.	n.s.	n.s.	n.s.	n.s.
Punicalagin	n.s.	n.s.	n.s.	n.s.	n.s.
Cyanidin-3-glucoside	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Malvidin	n.s.	n.s.	n.s.	n.s.	n.s.

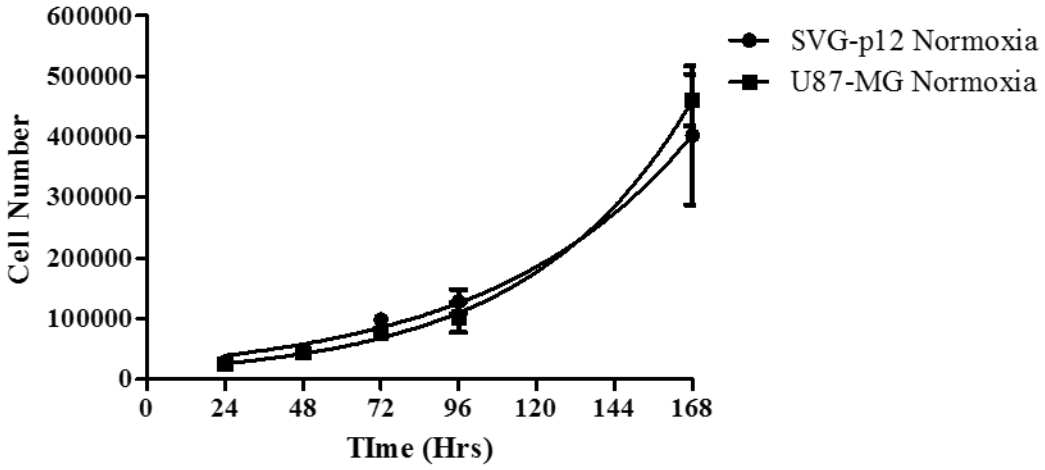
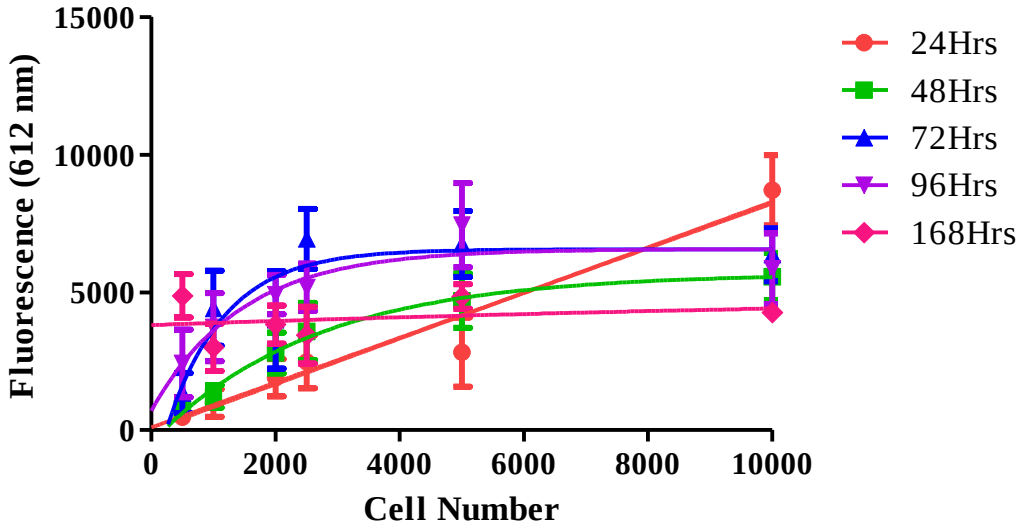


Figure 3.8: Growth rate for SVG –p12 and U87 MG cells. Upon initial seeding of 20,000 cells per well, proliferation was determined by a manual cell count of a seven day period.SVG-p12 demonstrated a doubling time of approximately 30-72 hours. U87-MG demonstrated a doubling time of 30-42 hours. The data illustrates mean cell number ± SEM generated from n=3.



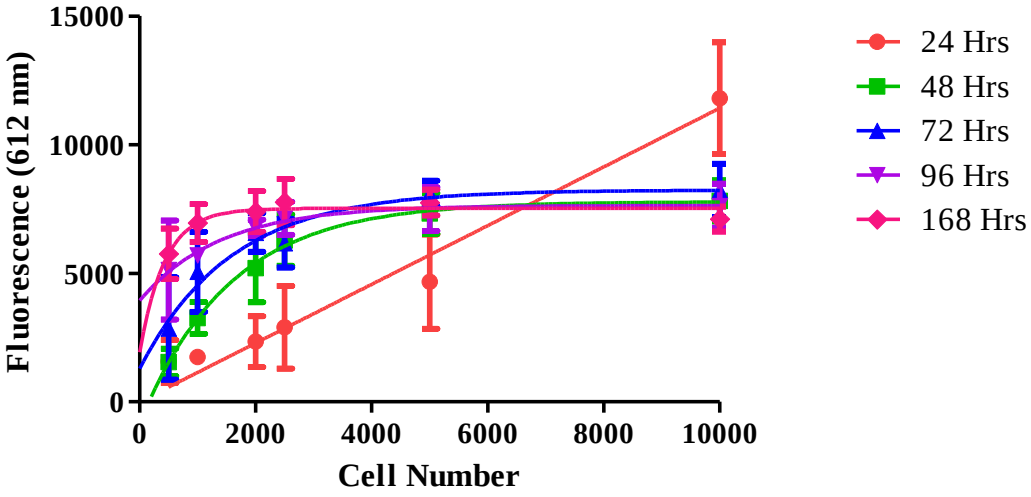


Figure 3.9: The relationship between fluorescence and increasing cell number for SVG –p12 (A) and U87 MG (B) cells. The data illustrates ± SEM generated from three independent experiments per cell line.

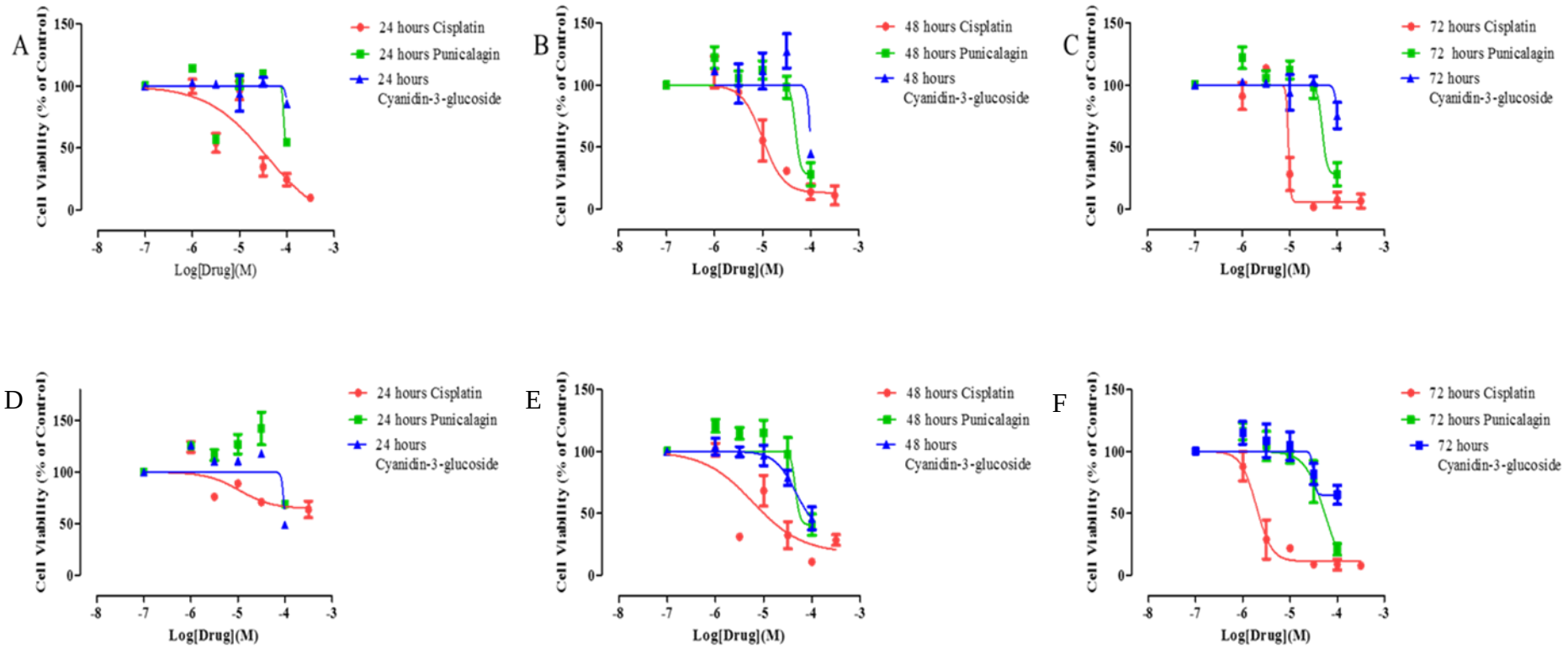


Figure 3.10 (A-F): Graphs illustrating the effect of dose-dependent treatment on of cisplatin, punicalagin, and cyanidin-3-glucoside on SVG-p12 and U87-MG cell lines. A-C display the dose – dependent effect of the three compounds on the SVG-p12 cell line over 24, 48 and 72 hours respectively. D-F display the dose – dependent effect of the three compounds on the U87-MG cell line over 24, 48 and 72 hours respectively. The data illustrates ± SEM generated from n=3 experiments per cell line.

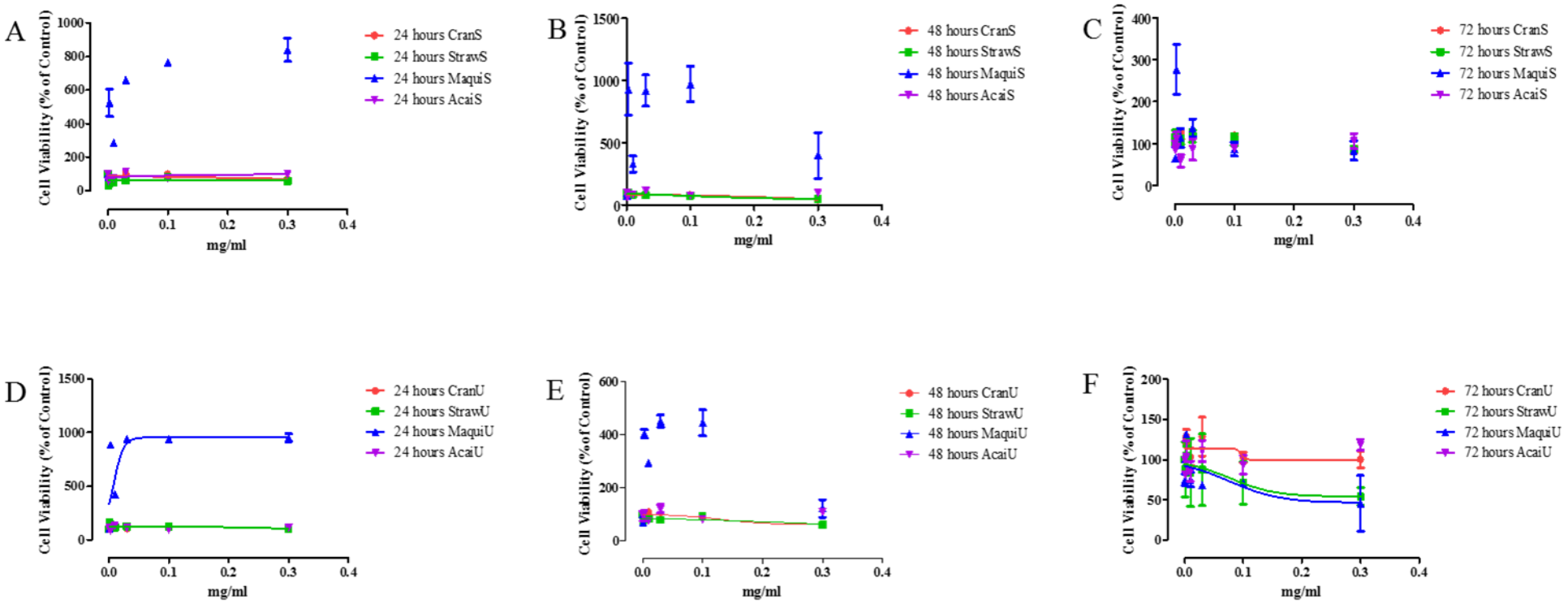


Figure 3.11 (A-F): Graphs illustrating the effect of dose-dependent treatment on of cranberry, strawberry, maqui berry and acai berry on SVG-p12 and U87-MG cell lines. A-C display the dose – dependent effect of the three compounds on the SVG-p12 cell line over 24, 48 and 72 hours respectively. D-F display the dose – dependent effect of the three compounds on the U87-MG cell line over 24, 48 and 72 hours respectively. . The data illustrates ± SEM generated from n=3 experiments per cell line.

Table 3.10 (A & B): IC_{50} values for SVG-p12 cell line (A) and IC_{50} values for U87-MG cell line (B) following 24, 48 and 72 hours incubation with cisplatin, punicalagin and cyanidin-3-glucoside.

A

SVG-p12	IC_{50} values (µM)		
	24 hours	48 hours	72 hours
Cisplatin	37.04	10.11	9.42
Punicalagin	85.42	48.56	46.50
Cyanidin-3-glucoside	103.60	109.40	36.65

B

U87-MG	IC_{50} values (µM)		
	24 hours	48 hours	72 hours
Cisplatin	10.82	7.13	1.94
Punicalagin	149.90	45.45	57.11
Cyanidin-3-glucoside	101.30	46.24	31.43

Table 3.11: IC_{50} values for SVG-p12 cell line and U87-MG cell line following 24, 48 and 72 hours incubation with cranberry, strawberry, maqui berry and acai berry fruit extracts.

A

SVG-p12	IC_{50} values (mg/ml)		
	24 hours	48 hours	72 hours
Cranberry	3.88	1.21	-
Strawberry	-	0.11	-
Maqui berry	-	-	-
Acai berry	-	-	-

B

U87-MG	IC_{50} values (mg/ml)		
	24 hours	48 hours	72 hours
Cranberry	-	0.13	0.01
Strawberry	0.24	3.43	0.01
Maqui berry	0.01	-	0.01
Acai berry	-	-	-

Chapter 4

DISCUSSION

4.1 Discussion

Due to increasing media influences surrounding healthy lifestyle choices and the claims that berries such as maqui and goji contain high levels of antioxidants, this study investigated the levels of potential antioxidant compounds within freeze-dried extracts of strawberry, cranberry, maqui, goji and acai berries; and their potential to be used as alternative therapies for the treatment of glioma.

These five fruits were of particular interest for this study due to the increasing level of media coverage they have received over the past 2-3 years. The fruits themselves, as well as products containing these fruits are heavily promoted via blogs and websites stating information regarding how these berries benefit health (www.purehealingfoods.com; www.globalhealingcentre.com). In addition to this, the NHS, a trusted public resource within the UK, provides information regarding superfoods, and in particular, goji berries (NHS Choices, 2015). Products such as powder mixes are also advertised to the general public which are aimed at those who take an interest in healthy living, claiming to 'contribute to normal immune and nervous system function' (www.myprotein.com). Due to the unavailability of the fresh fruit in the UK, the uncommon fruits such as maqui and acai berries are readily available online and particularly, within freeze dried forms. This form is claimed to retain the benefits of fresh fruit, particularly when referring to the presence of antioxidants (www.HealthySupplies.co.uk; www.Lio-Licious.com; www.MamasHealth.com).

The aim of this study was therefore to investigate the claims made within public forums suggesting that these berries contain higher levels of antioxidants than other fruits, and therefore bestow greater health benefits to the consumer. To maintain similarity to the

end user, the freeze-dried powders used within the study were prepared according to the serving suggestions advertised to the consumer, accounting for the requirements of the experiment being conducted.

4.2 HPLC

HPLC analysis provided an indication of the presence of antioxidants within the chosen fruit extracts. Due to the preliminary nature of this analysis, the presence of antioxidants was identified by comparing the fruit extracts to a range of the most abundant phytochemicals within the phenolic subtype in particular, phenolic acids, flavonols and anthocyanidins.

Initial HPLC studies were performed using a protocol optimised for the separation of strawberry samples. The strawberry extract was found to contain a number of phytochemicals as previously reported, confirming the suitability of this method for sample separation within our system (Huang et al., 2012). Ideally the protocol for separation of each berry would have been optimised however, as this was not the sole purpose of this study, and the fact that not all fruits had an associated published protocol, each fruit sample was analysed using the strawberry protocol. The results concluded that the strawberry extract contained the highest number of antioxidants as compared to all other fruit extracts measured however, this may have been influenced by the fact that the method used was optimised for this particular berry.

In addition, as the berries chosen for this study are known to contain high levels of anthocyanidins, contributors to the bright red, blue and purple colours of fruits and vegetables; a protocol optimised for this phenolic subgroup was also investigated (Wang and Stoner, 2008; Aabay et al., 2007; Brauch et al., 2016). On comparison of the

separation of the samples using the two protocols, it was found that there was no enhanced separation of anthocyanidins and confirmed that strawberry extract contained the highest amount of antioxidants; suggesting that the strawberry protocol was suitable for this study. Furthermore, the fruit extracts tested, displayed similar results to previously published studies showing the presence of gallic acid, punicalagin and cyanidin-3-glucoside (Thermo Scientific., 2012).

It was evident from comparison to the literature that a varying level of sensitivity exists within HPLC. Antioxidants such as cyanidin can exist with attached moieties for example, glucose or galactose, which contribute to the antioxidant capacity of the compound. These small differences in the structure of the compound can be detected by altering the HPLC conditions such as solvent, solvent ratios, column type and temperature. Upon further optimisation of the protocol, there may have been potential to distinguish these structural differences.

In an attempt to confirm the peaks seen were those of the antioxidant standards, samples were spiked. A single, higher peak at the same retention time suggests that the sample contains the compound of interest. In order to absolutely define the compound present within the sample, further experimentation could be performed. A previous study used gas chromatography-mass spectrometry (GC-MS) to identify polyphenolic compounds within a root extract (Ajayi et al., 2011). This process consisted of using GC to separate compounds to a greater degree of resolution however, this alone does not achieve an identification of the compound. In combination with MS, which has the ability to identify compounds through their molecular weight, detailed structural information can be obtained, which has the potential to identify a compound.

4.3 Flavonoid: Phenolic Ratio and Antioxidant Capacity

Following the identification and quantification of the polyphenolic compounds within the fruit extracts, it was important to recognise the type of antioxidants that were contributing to this activity. The flavonoid to phenolic ratio (F: P) was calculated to quantify the amount of flavonoid present in the total phenolic content of each fruit extract. As flavonoids are a subgroup of phenolics, the F: P ratio determines how much the flavonoid subgroup contributes to the overall phenolic content (Marinova et al., 2005). It was hypothesised that this result would be comparable with the concentrations of antioxidants as measured by HPLC. Unexpectedly, these two results were not analogous as goji berry showed the largest F: P with strawberry displaying the lowest. One factor which may have influenced these results is that the HPLC method was optimised for strawberry as previously stated, therefore the full potential of the other fruits was not exhibited. However, this may not be a true representation of the full potential of the fruit extracts, as previous studies have found cyanidin-3-glucoside to be the main antioxidant contributor for these berries (Del Pozo-Insfran et al., 2006; Aaby et al., 2007; Brito et al., 2014). Further optimisation of the TFC assay could investigate the use of a cyanidin compound as the measurement standard.

4.4 Antioxidant Capacity (AC)

The DPPH assay is an end point assay which measures the AC through the ability of neutralisation of DPPH molecules by TROLOX, resulting in discolouration which can

expressed as total antioxidant capacity (TAC) and percentage scavenging. Percentage scavenging is defined as the percentage at which the DPPH free radicals accept hydrogen atoms available from the hydrogen donor; this was obtained from the fruit or antioxidant tested with a given TROLOX equivalent.

DPPH· radical scavenging assay is known as a gold standard assay for measuring the AC. This method is defined as the standardised method to use when calculating the AC and is known to be of superior quality in comparison to other available methods of AC calculation. The values yielded from this are utilised as reference points for the AC value in other fruits (Naik et al., 2015).

Upon comparison of the fruits to their F: P ratio goji berry would have been expected to contain the highest AC, followed by cranberry>maqui berry>acai berry>strawberry. However, goji berry displayed the lowest antioxidant capacity and was surpassed by maqui an acai berry. In conjunction with this, the percentage scavenging was displayed to be above 100% for punicalagin, cranberry and cyanidin-3-glucoside vs TROLOX. This display of the percentage scavenging being above 100% is due to it being expressed as a TROLOX equivalence which may introduce the possibility to compare these fruits to a different antioxidant with a higher scavenging ability. A lack of knowledge on how the fruits were freeze dried makes it difficult to establish the reasoning behind this. A study by Asami et al., (2003) compared freeze-dried strawberry to other available forms, showing the freeze-dried form to display the highest AC values. It could be possible that this process contributed to enhance the antioxidant availability, particularly within the cranberry sample.

Freeze drying is the method by which water is removed via the sublimation of ice crystals. There have been many known advantages to this form of food preservation along with obvious explanations such as efficient food storage of heat sensitive biological material, the retention of morphological, biochemical and immunological properties as well as high viability or activity levels. The process of freeze drying can instigate these attributes from the food sample which may be an explanation of the high cranberry result. In addition to this, pre-treatment of the fruit may have occurred for the company to save in production costs (Ciurzyńska and Lenart., 2011).

Other factors contributing to this result may include the combination of antioxidants present within the sample. Although the results display four antioxidants it may be possible that the AC is contributed to by these in combination with other antioxidants not detected at this time. Although this study was able to determine four antioxidants to be present within the fruit samples, in comparison to other studies it is conceivable to suggest that these compounds are not the only antioxidants present within the fruit extracts. For example, goji berries were found to contain a variety of antioxidants particularly vitamin C with maqui berry found to contain anthocyandins and proanthocyanidins contributing to their AC (Ionică et al., 2012; Fredes et al., 2012). Sources have found acai berry to contain a multitude of antioxidants consisting of anthocyanidins and proanthocyanidins as well as catechin, and phenolic acids (Schauss et al., 2006; Pacheco-Palencia et al., 2008).

Although the DPPH assay is of gold standard, this is not bound by its comparisons to TROLOX. It may be possible to utilise another antioxidant that could prove to be a better comparison of antioxidant activity within the fruit samples e.g. ascorbic acid (Floegel et al., 2016). The comparison between TROLOX and the samples may have

been a limitation for expressing the percentage scavenging as there were clear indications of punicalagin having a higher scavenging capability.

Also, despite the reputation of this assay, there are other assays available that express the AC of samples. The oxygen radical absorbing capacity (ORAC) assay expresses AC by the use of a free radical generator mixed with a fluorescein probe. Upon heating of the generator, the fluorescein is damaged resulting in a loss of fluorescence. When antioxidants interact with the free radicals, the loss in fluorescence is delayed and this is used to calculate the AC (Floegel et al., 2016; FLUOstar OPTIMA; Bmglabtech.com; 2014). The ferric reducing antioxidant power (FRAP) assay follows a single electron transfer mechanism where the antioxidants are oxidised by oxidants such as Fe (III). This is measured by obtaining an absorbance value to quantify the reducing capabilities of the antioxidant (Ou et al., 2002).

The antioxidant content of the fruits is not the only factor to consider when exploring antioxidant capacity. It is also conceivable that although there is a low amount of a particular antioxidant present within the fruit, the antioxidant itself, regardless of amount may have the ability to exceed the capabilities of the other antioxidants which are more highly present.

Another factor to consider when exploring the results obtained from this experiment consist of the DPPH assay being and in vitro assay in which there is no consideration of the living organism (the consumer) and the effect this fruit would have in an in vivo environment. This concerns the bioavailability of the fruit within the consumer. The question is raised as to how this fruit affects the body. This is due to the assumption of

the entire fruit being utilised within the assay whereas, due to bioavailability it may be possible that the fruit portions contributing the antioxidant activity may not impact the consumer. Miller et al., (2013) showed that absorption of vitamin E, C, carotenoids and catechins are well established and that diets high in fruits and vegetables increased blood serum antioxidant activity by 10%. An avenue to explore could be of bioavailability as conducted by Lila et al., (2013) in which the anthocyandins from maqui berry were tested within a model of the human gastrointestinal tract which showed that less than 10% of anthocyandin is bioaccessible. This is imperative to the exploration of the administration of antioxidants as a potential chemotherapeutic agent due to it the antioxidants being primarily present within the fibrous aspects of the fruits which are generally not consumed or are excreted without being digested.

4.5 Growth Curves and Cell Viability

The correct use of cell lines is an imperative factor when conducting assays of this kind. As expected from the SVG-p12 and U87-MG cell lines, it was apparent that the cancerous cell line had a higher rate of proliferation. This would be expected due to the hallmark of cancer which causes a disruption of the negative feedback mechanisms in place to decrease proliferative signalling. Defects within this feedback mechanism are what are capable of enhancing this proliferative effect (Hanahan and Weinberg., 2011).

The U87-MG cell line doubled in cell number over 48 hours and continued to grow exponentially over a period of seven days. In comparison, the SVG-p12 cell line doubled over 72 hours and continued to grow exponentially over a seven day period. Both cell lines expressed an initial lag phase in growth which is to be expected in order for the cells to become acclimatised to their new environment post- seeding. The

PrestoBlue® used the reducing cell environment as an indicator of viability. However, upon conduction of the assay it became evident that a large impacting factor is cell number when measuring the response of cell. The linearity assay established that fluorescence was proportional to cell density over 24 hours. In combination with the doubling time of the cells, the results recognised that the appropriate time for subsequent testing would be after a 24 hour incubation period with an initial seeding density of 1000 cells per well.

4.6 Dose Response

To be able to explore the result of antioxidants within a diseased state, the antioxidant compounds showing the highest AC were carried into dose dependant experimentation in U87-MG and SVG-p12 cell lines. This was compared to the chemotherapeutic agent cisplatin. Cisplatin demonstrated cytotoxic effects by reducing the cell viability of both U87-MG and SVG-p12 cell lines in a concentration dependant manner. In comparison to this, punicalagin and cyanidin-3- glucoside demonstrated a decrease in viability over the time period in both cell lines but at a higher concentration.

Cisplatin is a common chemotherapeutic treatment due to its ability to induce cytotoxicity through a variety of mechanisms. This could consist of the characteristics of the platinum anticancer drug by inhibiting molecular pathways necessary for cellular division. Also, platinum compounds have the ability to damage tumours by apoptosis in cancer cells where signalling networks that regulate proliferation and survival are altered. This may occur by the activation of various signal transduction pathways due to interactions with ROS, DNA, tumour necrosis factor (TNF), mitochondria, p53, calcium

signalling and caspases. This may be due to the inhibition of DNA synthesis and repair resulting in cell cycle arrest at the G1, S or G2-M phase – thus inducing apoptosis (Florea and Büsselburg., 2011).

However, cisplatin is known for its side effects in normal tissue and so novel compounds to work alone or in combination with this are constantly being sought. The aim of identifying antioxidants as a potential chemotherapeutic agent is due to their interaction with ROS. ROS have the ability to contribute to cancer initiation, progression and metastasis. Cancerous cells have increased ROS as compared to non-cancerous cells which may contribute to oncogenic activation. Although this relationship remains unclear, oxidative DNA damage has been known to play a role within carcinogenesis and malignant transformation. ROS could mediate signalling cascades relating to survival, proliferation and resistance to apoptosis. This could occur within cancer cells due to the ischemic environment and increased rates of metabolism (Wang and Yi., 2016).

Clerkin et al., 2008, explained how anti-oxidative therapy could aid in the retardation of angiogenesis, rendering them to be a promising antiangiogenic strategy in cancer therapy. This may suggest antioxidants to be a suitable anti-cancer therapy due to their nutraceutical properties which could result in reduced side effects upon administration. Another avenue that could be explored may consist of a combination of therapies as suggested previously.

As well as adding the antioxidant compounds to the cells, the fruit samples were also directly added to the cells. The results seem to address previous statements of how the

antioxidant capacity does not reflect the ability of the compound. In this instance, it is possible to suggest that strawberry and maqui berry have an effect upon the cell viability of U87-MG cells due to their ability to reduce the viability of the cells by 50%. To determine the effectivity of the assay, it could be possible to compare the method with the MTT assay ((3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) which has been used in studies where antioxidants have been the focus (Ghalli et al., 2013). The commonality of the assay suggests extraction of the antioxidant from the fruit sample to be applied to the suitable cell line. Fruits used in this study were highly pigmented in their nature, this suggested that this interfered with the PrestoBlue® assay viability assay and so would not detect the true level of viability on the cells.

4.7 Future Work

In order to achieve a logical progression it would be advantageous to determine the full antioxidant profile of each fruit. From this it would be beneficial to identify the antioxidant activity of the compounds present, which could indicate the relevant antioxidants contributing to the AC of the fruit extract. To ensure this is accomplished it could be beneficial to prepare the fruit sample rather than purchasing from a company. This would ensure the ability of the processing to be factored in to the results obtained. Previous studies form this lab attempted to process the fruit extracts from the fresh form and at the time of expermentation however, this did not have a desirable outcome. Fresh fruits were processed with the aim of use within experimentation, yet the fruits were difficult to solubilise and utilise whilst maintaining them in their purest form. This was overcome within the study by diluting the fruits to suit the relevant experimentation (Patel, Raani and Salim; unpublished data).

As previously stated, the standard used in the antioxidant assays can contribute to the results obtained. Upon determination of the optimal standard to use to compare the results against, it may be ideal to explore the effect of the combination of antioxidants within the sample. This could enable further understanding into how the antioxidants interact with each other and to identify combinations that would have a higher AC thus, a better effect within the cell lines. Previous research has extracted the antioxidants from the fruit samples prior to addition to cell culture. This would be ideal to determine the amount of antioxidants found in a serving size or choosing to explore a particular section of the fruit, for instance the skin, flesh, pith, seeds or the entire fruit which could solve the complication of the interference pigmentation. Having determined an AC of the fruit and the effect on cells, it would be beneficial to measure the bioavailability of the fruit. Experimentation for this could consist of the use of a model of the human gastrointestinal tract to quantify the bioavailability of the compound (Lila et al., 2013).

The antioxidants explored within this study suggest an effect upon cell viability however, this does not determine cell death. Cell death could be explored with regards to these antioxidants through apoptosis assays. Leading on from this it could be beneficial to identify the interaction of the antioxidant within the cell cycle by considering cell cycle analysis and proliferation assays. Another avenue to explore could be the potential to use the antioxidant as a targeted drug therapy. In this case, exploration into the blood brain barrier could be beneficial.

4.8 Conclusions

Results from this study have demonstrated that berries do contain antioxidants. Due to their pigmentation the flavonoid subgroup of anthocyanidins is favoured in this fruit type. However, this may not be the sole contributor to the antioxidant capacity. This study provides information about the interaction between antioxidants and glioblastoma multiforme cell line U87-MG. This opens the potential to explore antioxidants as a chemotherapeutic agent alone, or in combination with a platinum based chemotherapeutic agent.

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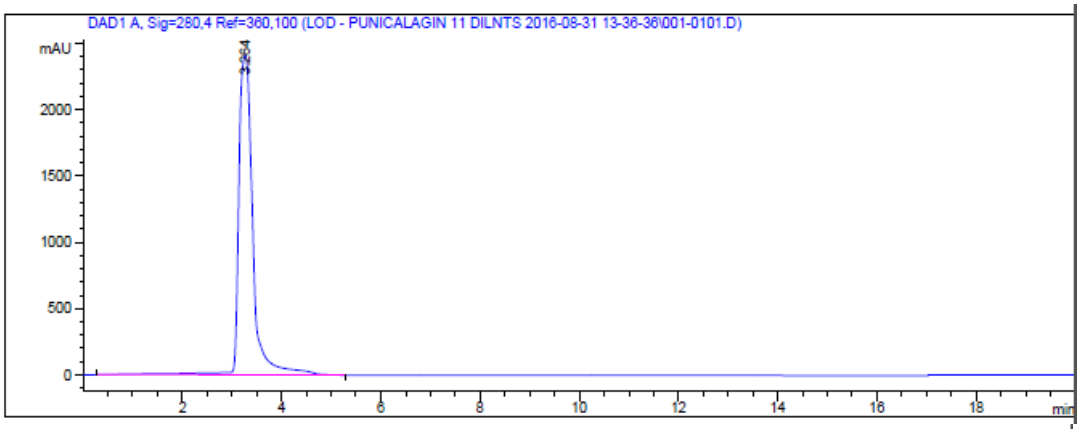
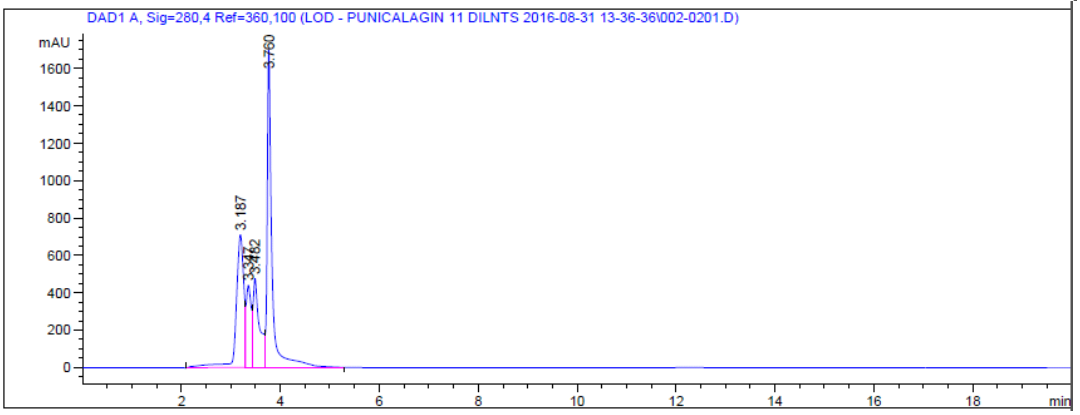
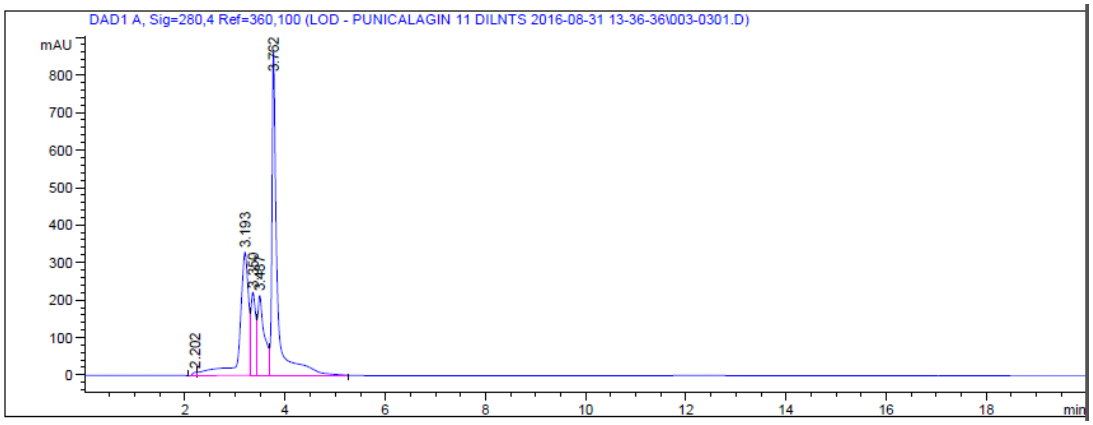
APPENDIX

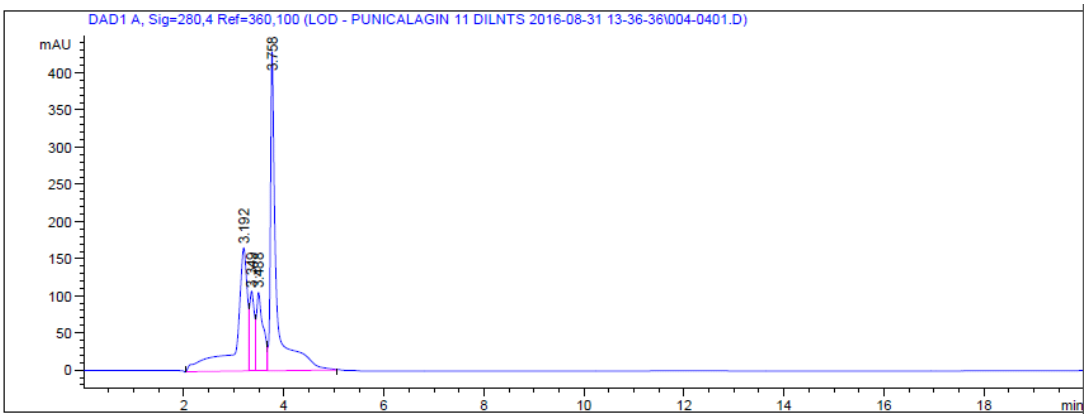
Appendix 1: Limit of Detection

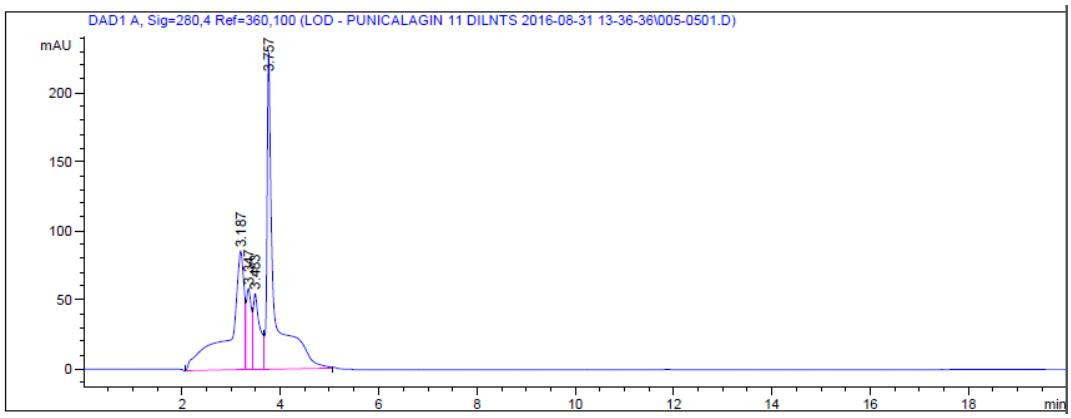
Appendix 1.1: Limit of Detection of Punicalagin using HPLC method 1.

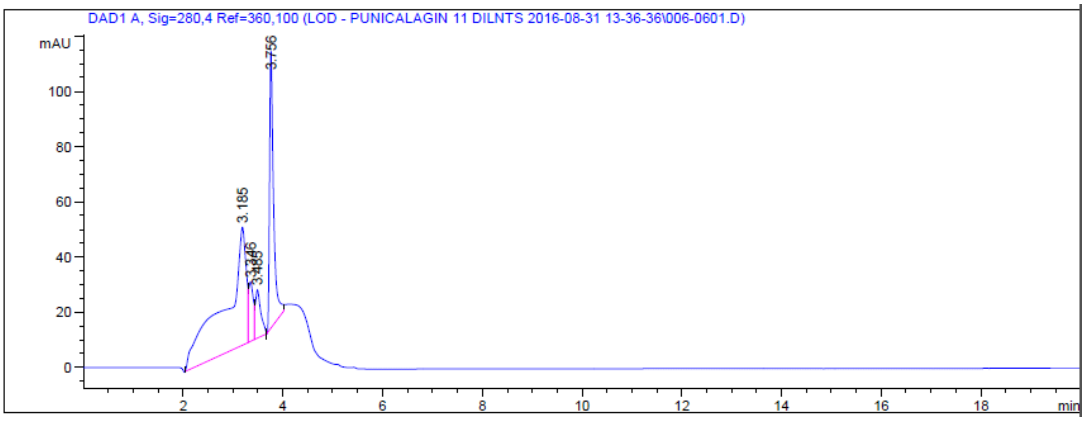


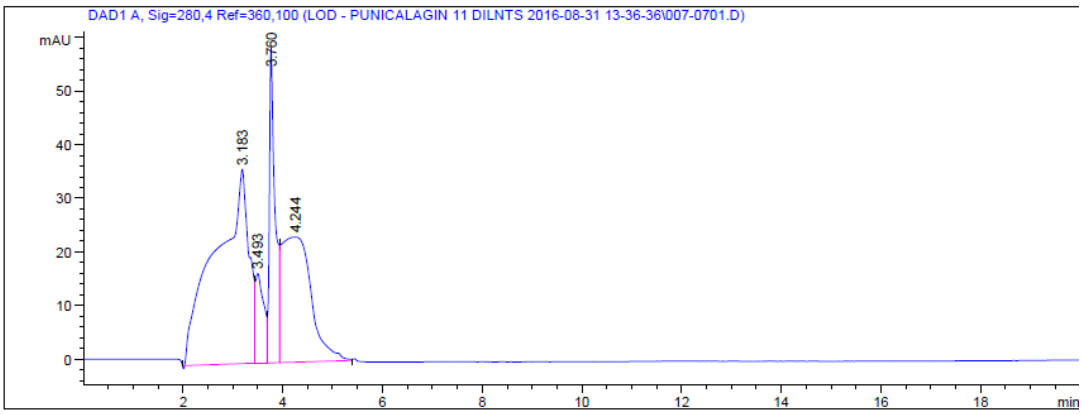


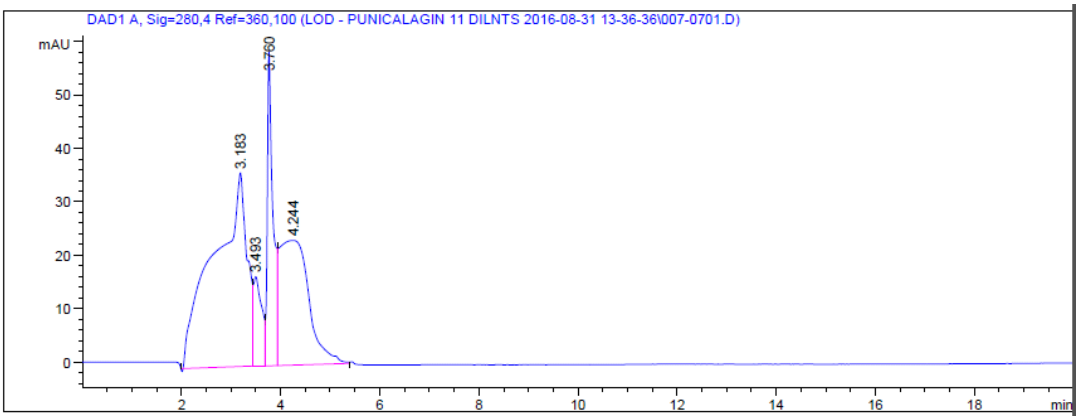


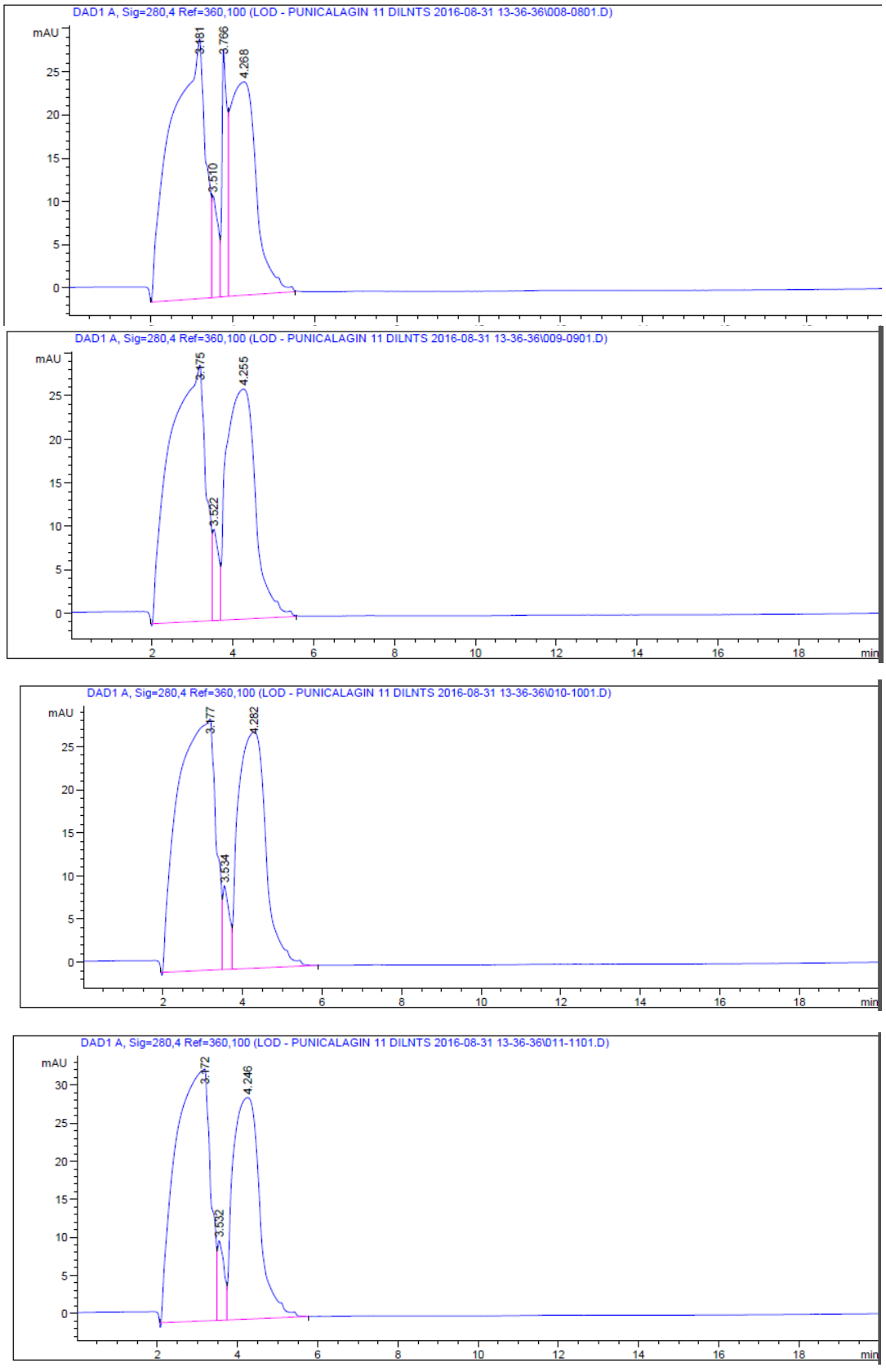



DAD1 A, Sig=280,4 Ref=360,100 (LOD - PUNICALAGIN 11 DILNTS 2016-08-31 13-36-36\005-0501.D)
mAU
3.757
3.187
3.493
3.244
min

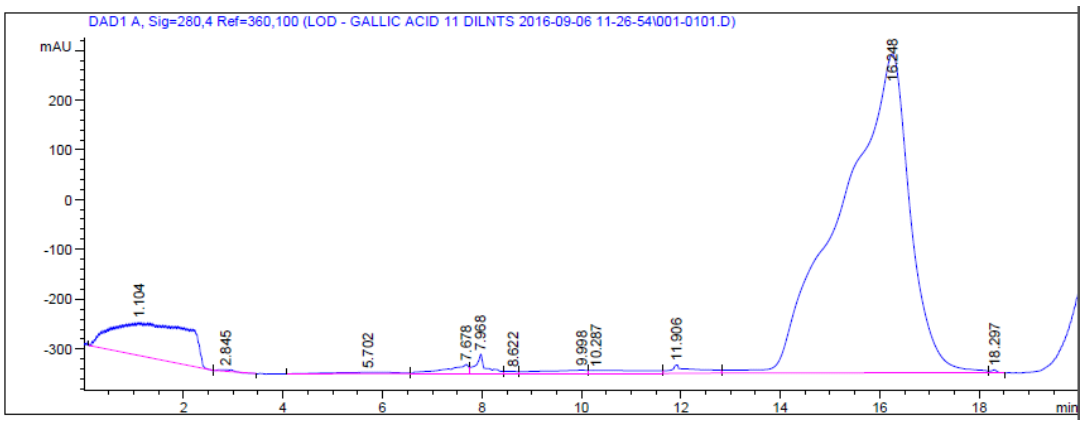

DAD1 A, Sig=280,4 Ref=360,100 (LOD - PUNICALAGIN 11 DILNTS 2016-08-31 13-36-36\006-0601.D)
mAU
3.756
3.185
3.493
3.244
min

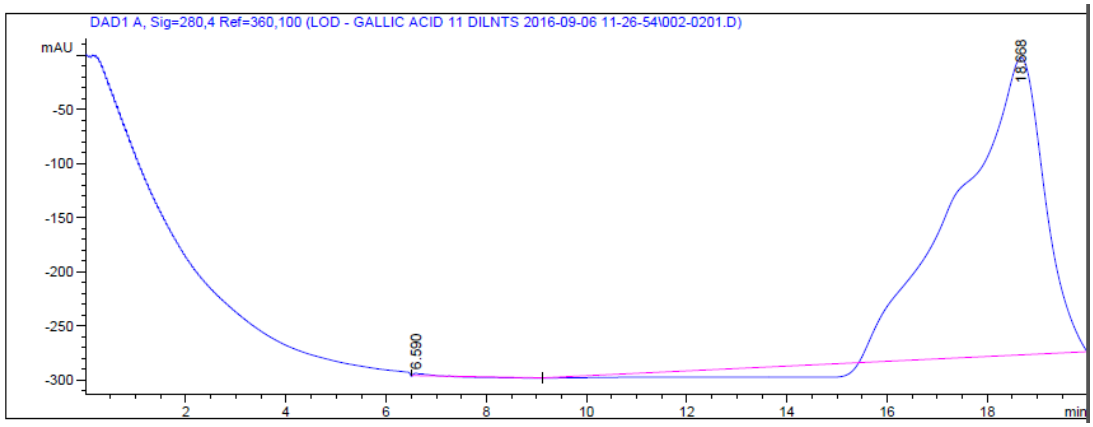

DAD1 A, Sig=280,4 Ref=360,100 (LOD - PUNICALAGIN 11 DILNTS 2016-08-31 13-36-36\007-0701.D)
mAU
3.760
3.183
3.493
4.244
min

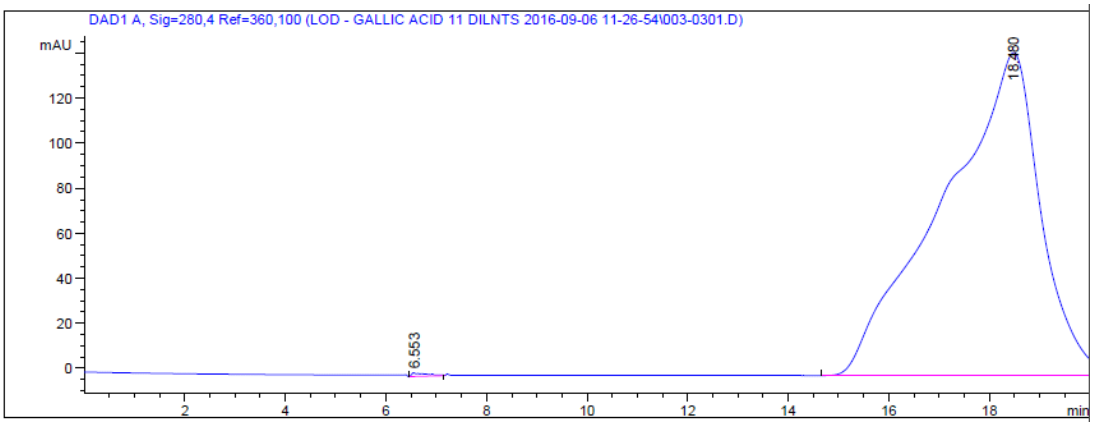

DAD1 A, Sig=280,4 Ref=360,100 (LOD - PUNICALAGIN 11 DILNTS 2016-08-31 13-36-36\007-0701.D)
mAU
3.760
3.183
3.493
4.244
min

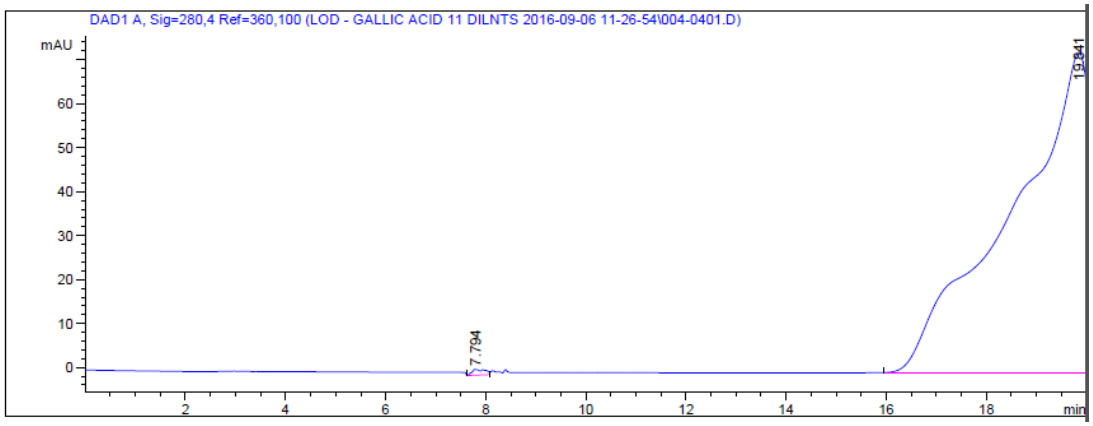


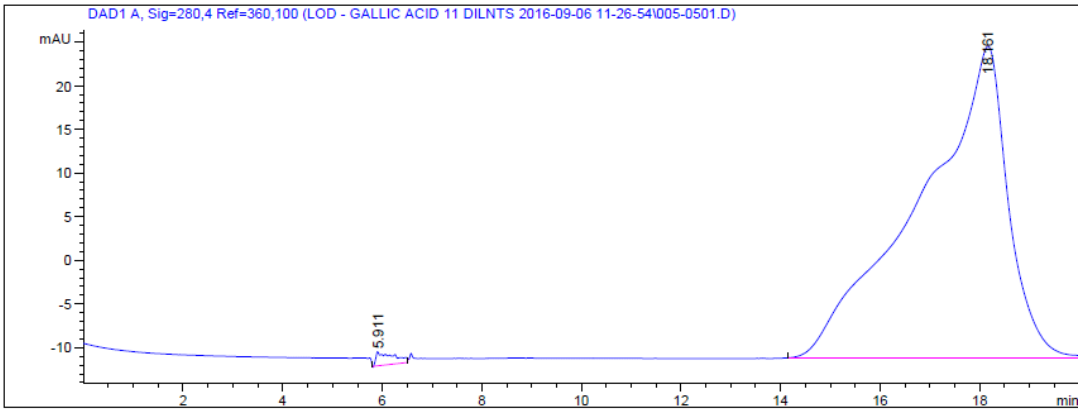
Appendix 1.2: Limit of Detection of Gallic acid using HPLC method 1

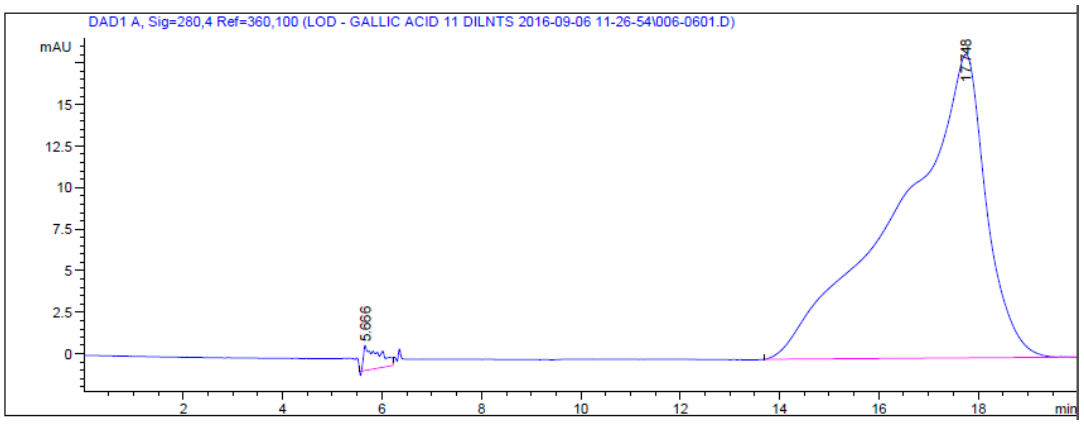

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\001-0101.D)
mAU
1.104
2.845
5.702
7.678
7.968
8.622
9.998
10.287
11.906
16.248
18.297
min

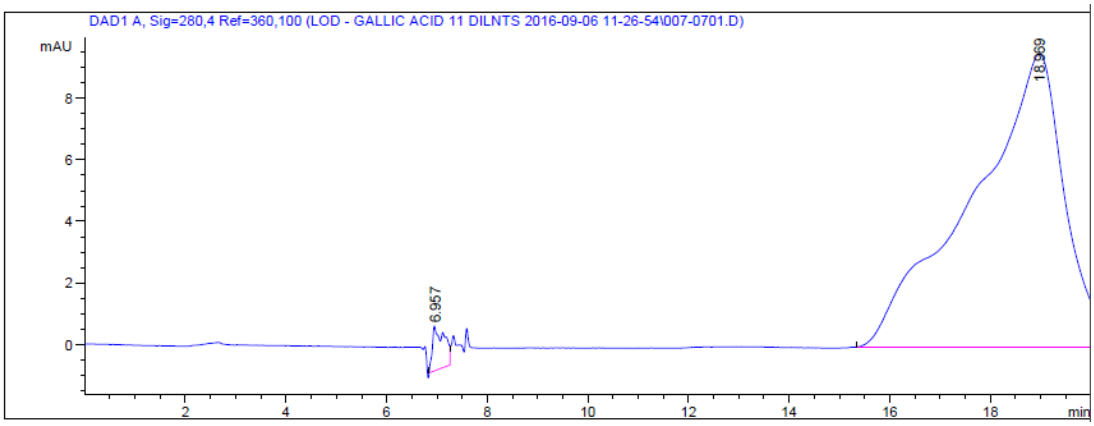

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\002-0201.D)
mAU
6.590
18.668
min

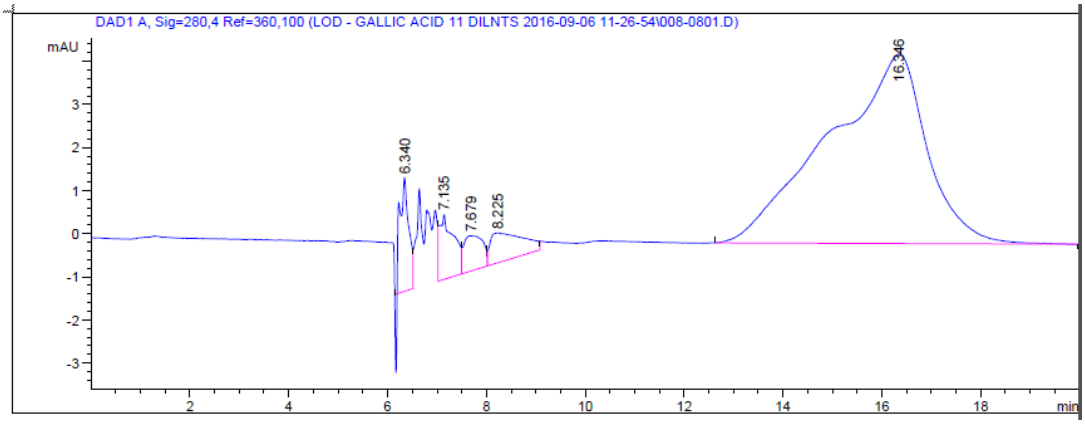

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\003-0301.D)
mAU
6.553
18.480
min

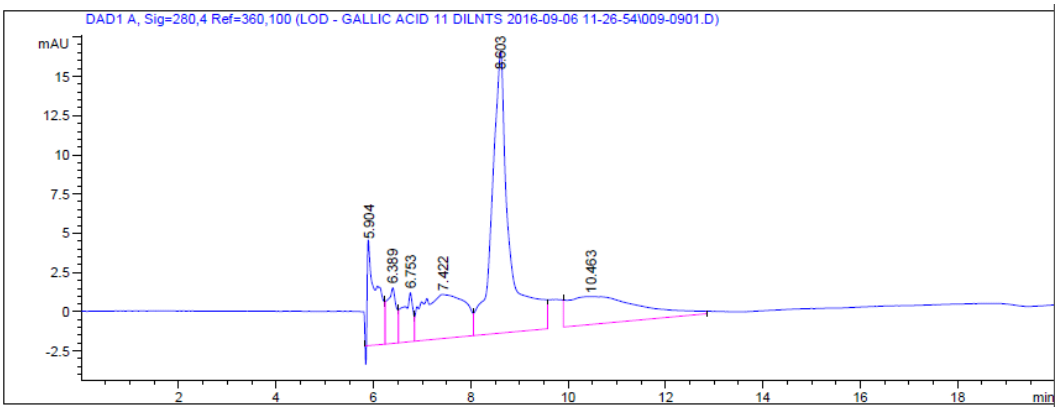

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\004-0401.D)
mAU
7.794
19.841
min

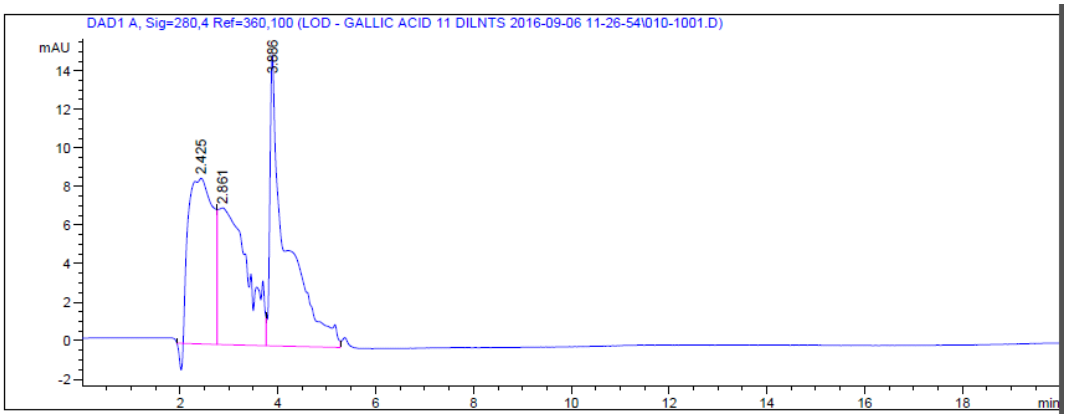

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\005-0501.D)
mAU
18.161
5.911
min

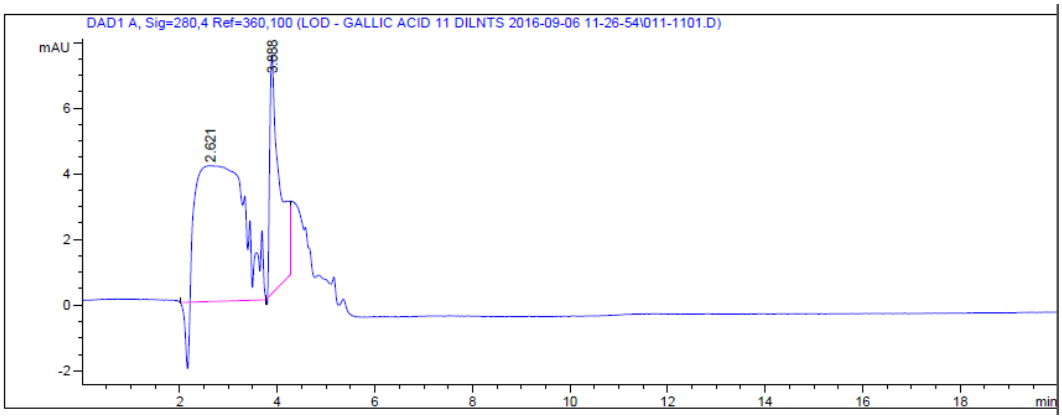

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\006-0601.D)
mAU
17.748
5.666
min


DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\007-0701.D)
mAU
18.969
6.957
min

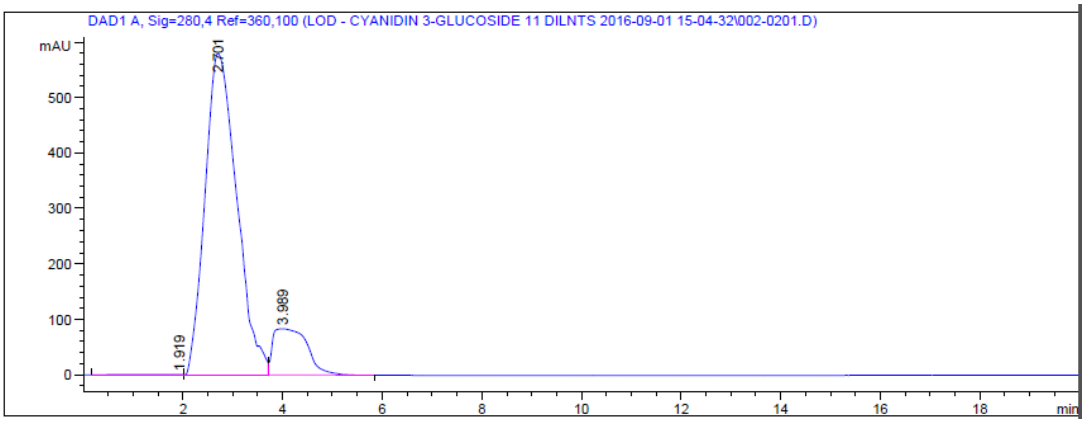

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\008-0801.D)
mAU
16.346
6.340
7.135
7.679
8.225
min

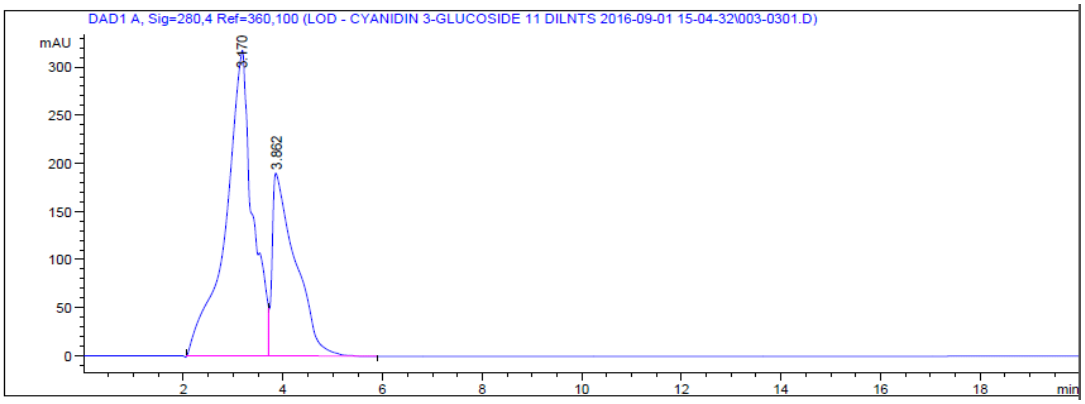

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\009-0901.D)
mAU
15
12.5
10
7.5
5
2.5
0
-2.5
5.904
6.389
6.753
7.422
8.603
10.463
2
4
6
8
10
12
14
16
18
min

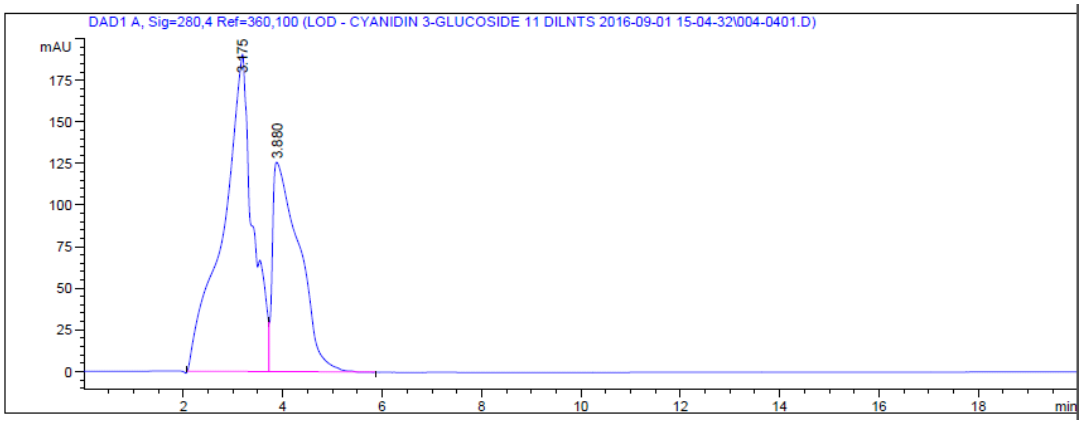

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\010-1001.D)
mAU
14
12
10
8
6
4
2
0
-2
2.425
2.861
3.886
2
4
6
8
10
12
14
16
18
min

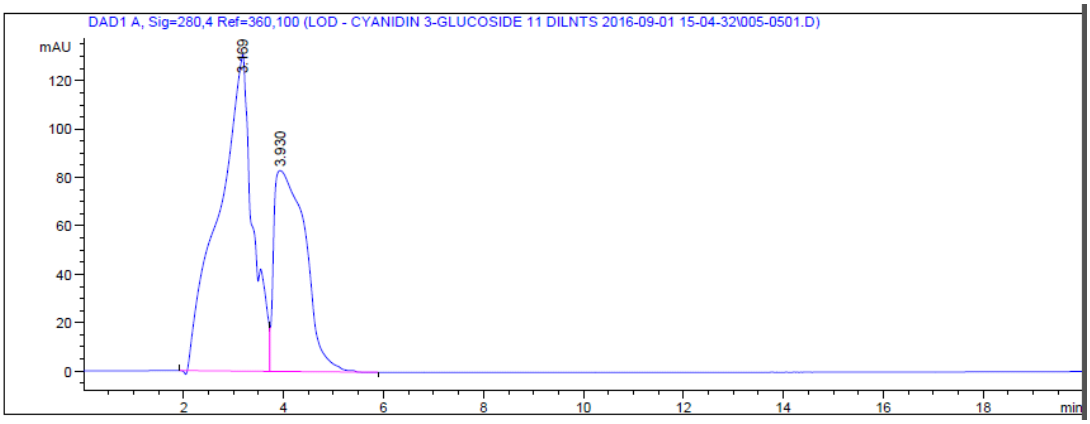

DAD1 A, Sig=280,4 Ref=360,100 (LOD - GALLIC ACID 11 DILNTS 2016-09-06 11-26-54\011-1101.D)
mAU
6
4
2
0
-2
2.621
3.888
2
4
6
8
10
12
14
16
18
min

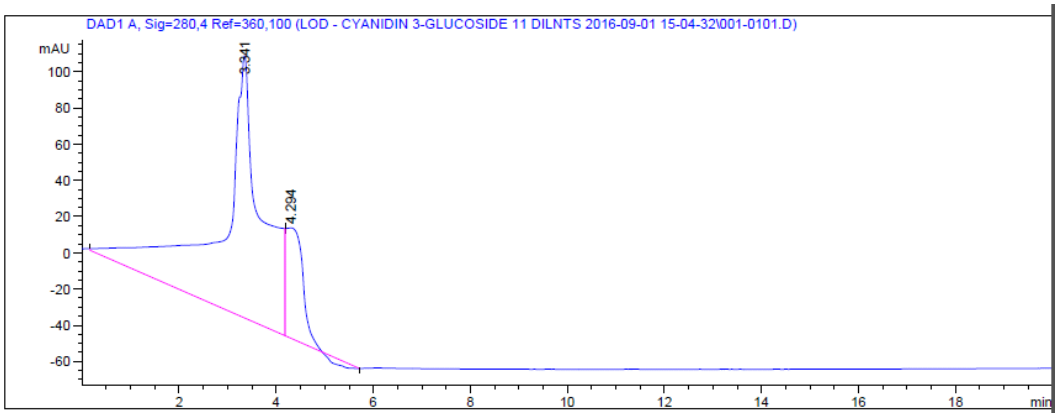
Appendix 1.3: Limit of Detection of cyanidin-3-glucoside using HPLC method 1.

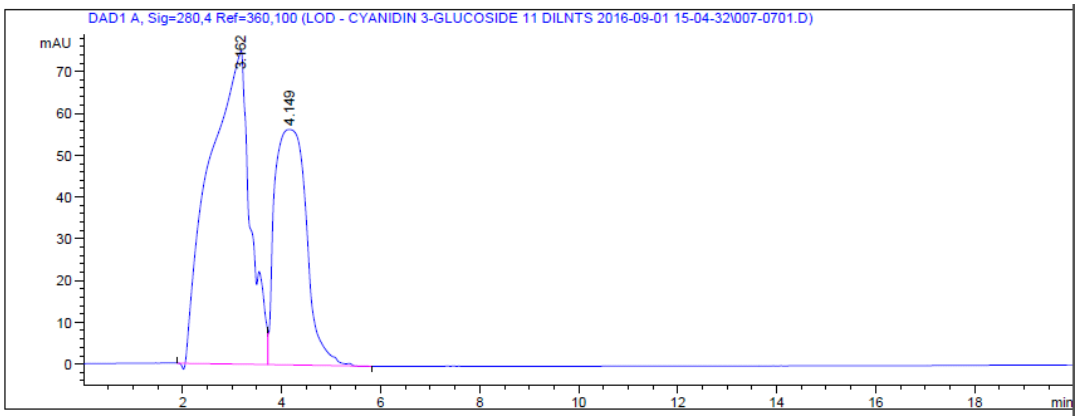


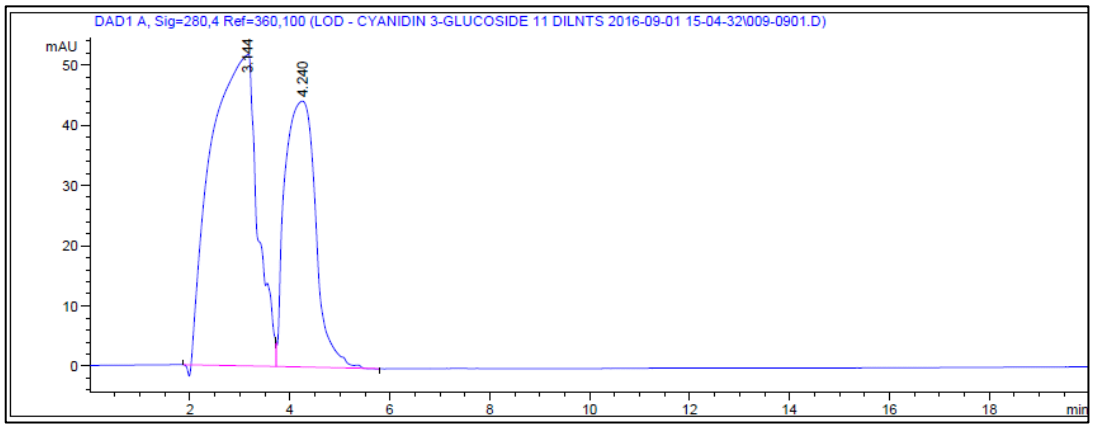


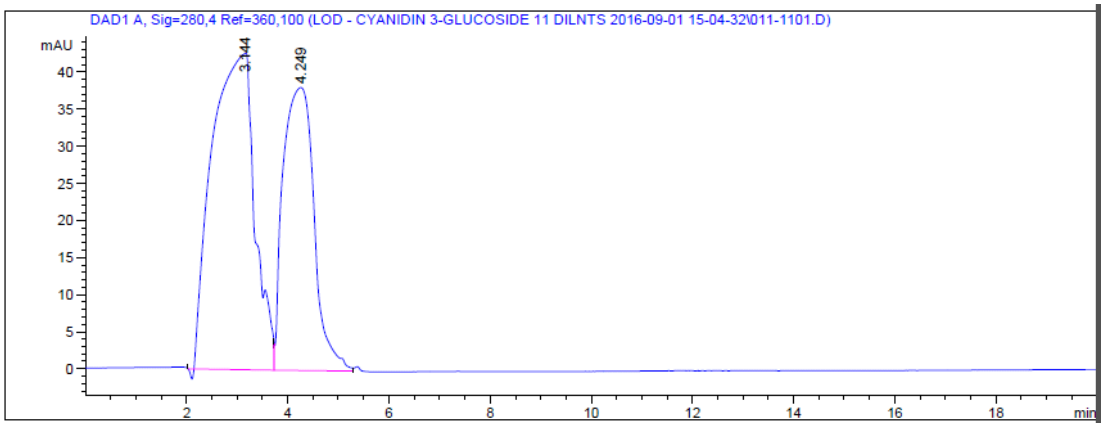


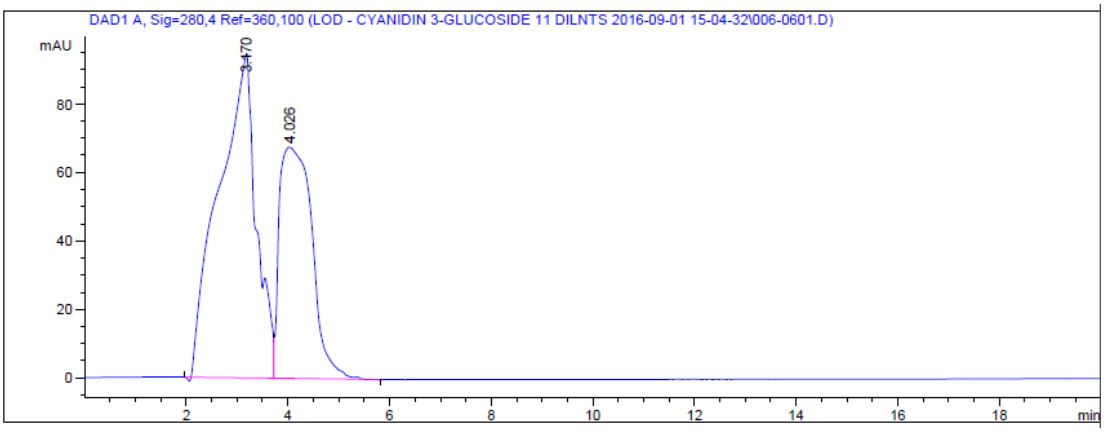


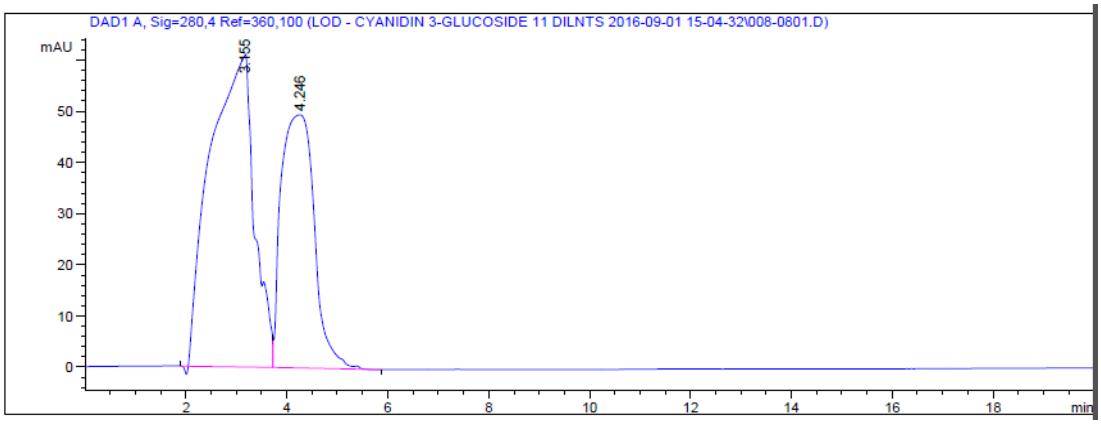

DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\001-0101.D)
mAU
3.341
4.294
min

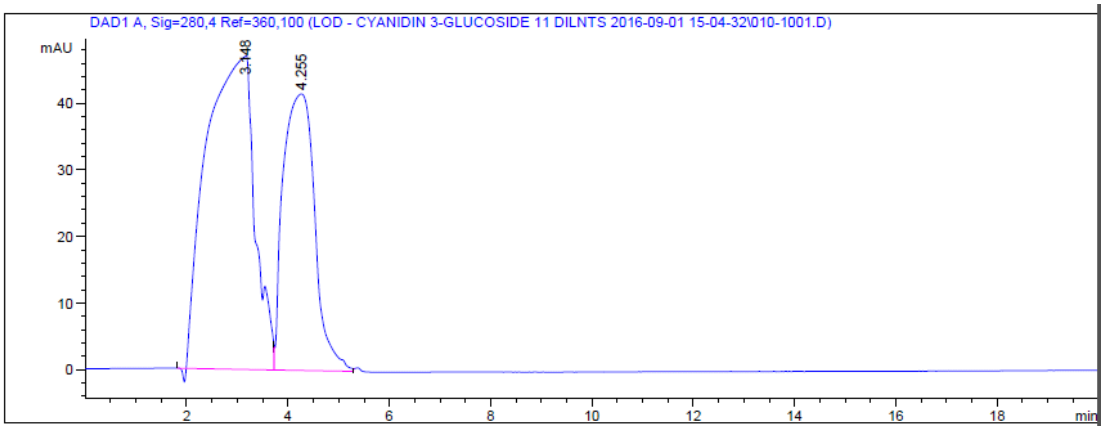

DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\007-0701.D)
mAU
3.162
4.149
min


DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\009-0901.D)
mAU
3.144
4.240
min

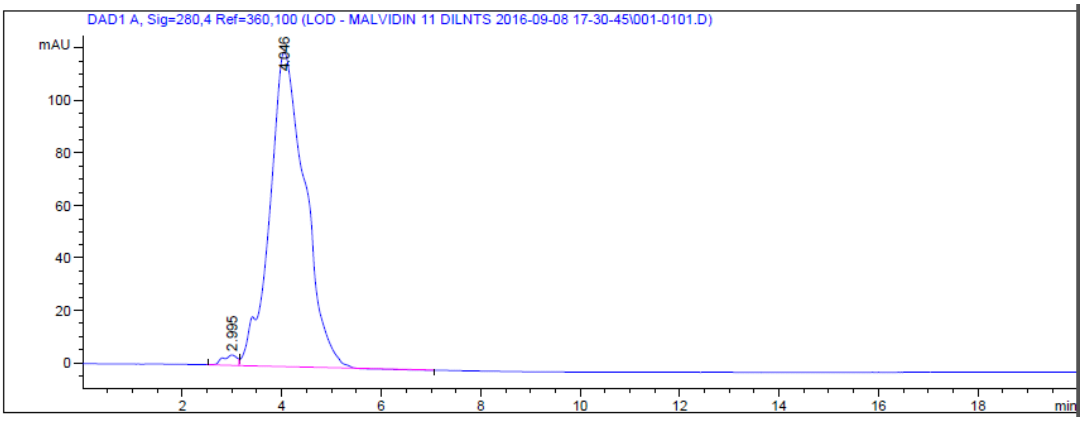

DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\011-1101.D)
mAU
3.144
4.249
min

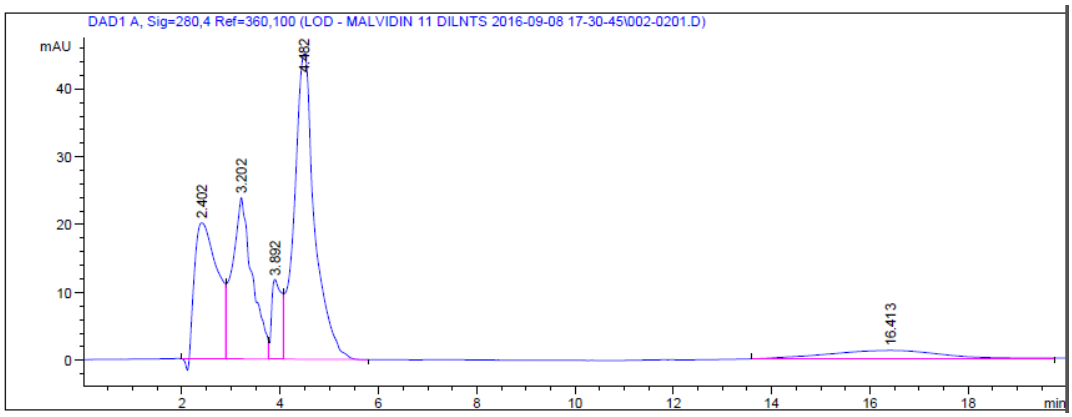

DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\006-0601.D)
mAU
3.170
4.026
min

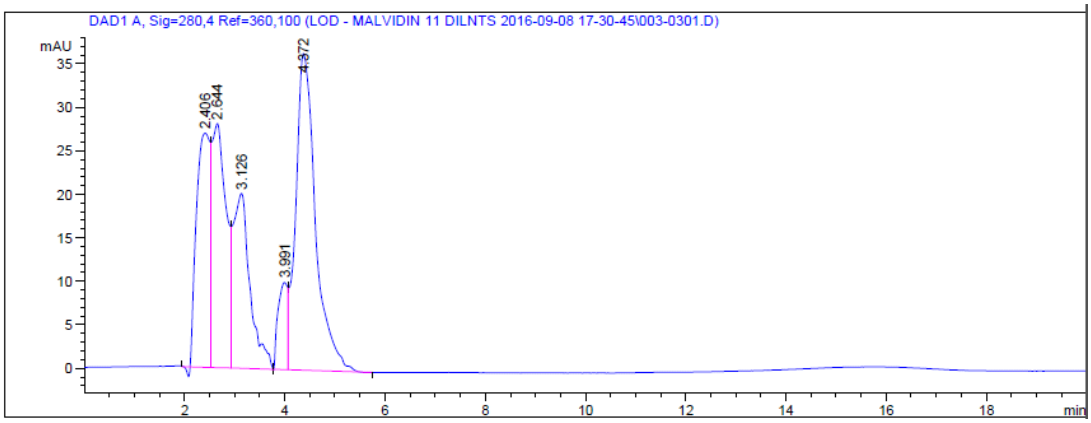

DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\008-0801.D)
mAU
3.155
4.246
min

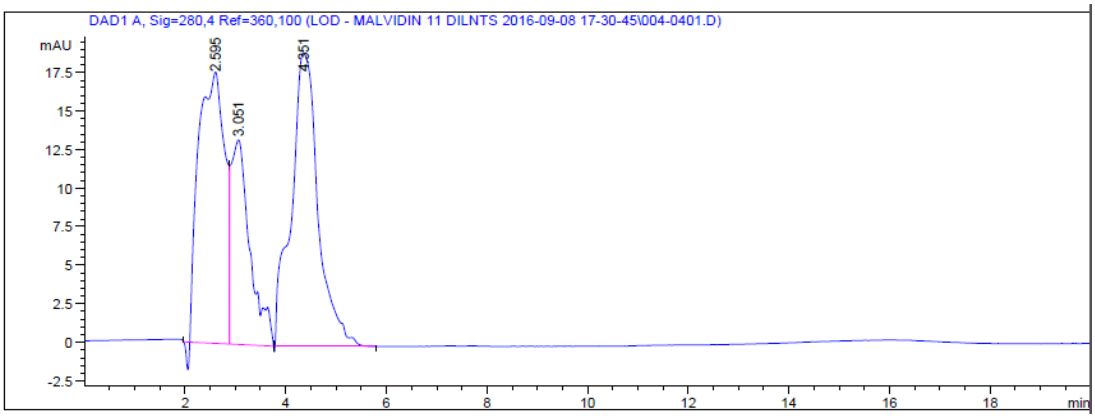

DAD1 A, Sig=280,4 Ref=360,100 (LOD - CYANIDIN 3-GLUCOSIDE 11 DILNTS 2016-09-01 15-04-32\010-1001.D)
mAU
3.148
4.255
min

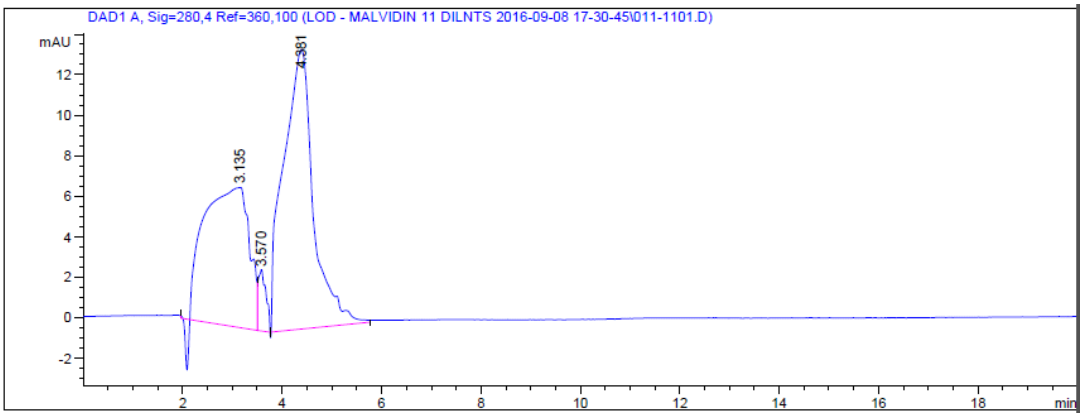
Appendix 1.2: Limit of Detection of Malvidin using HPLC method 1.

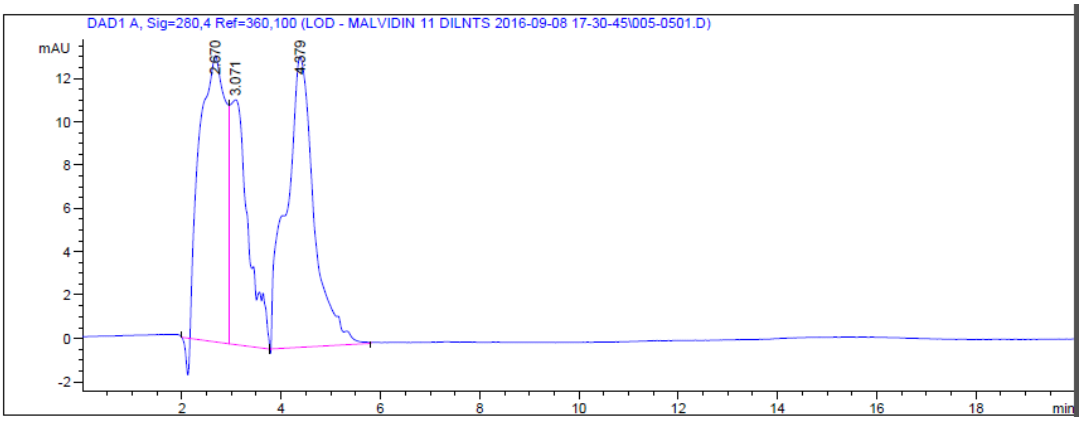


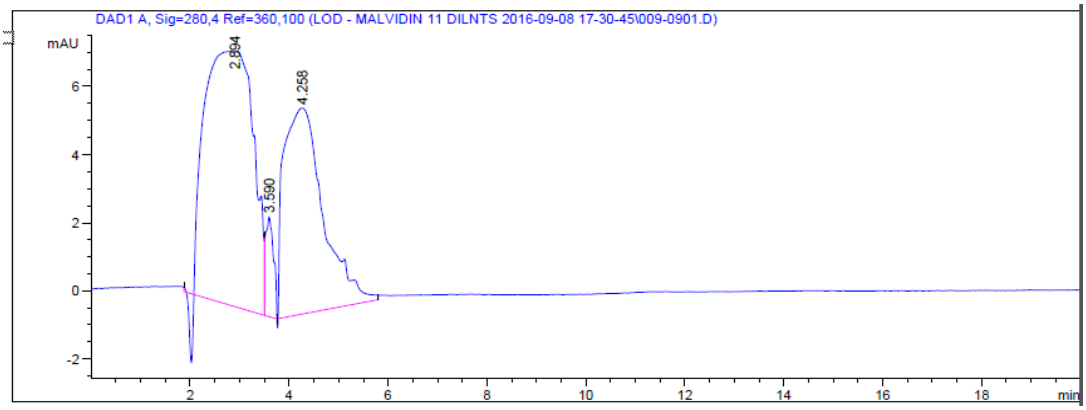


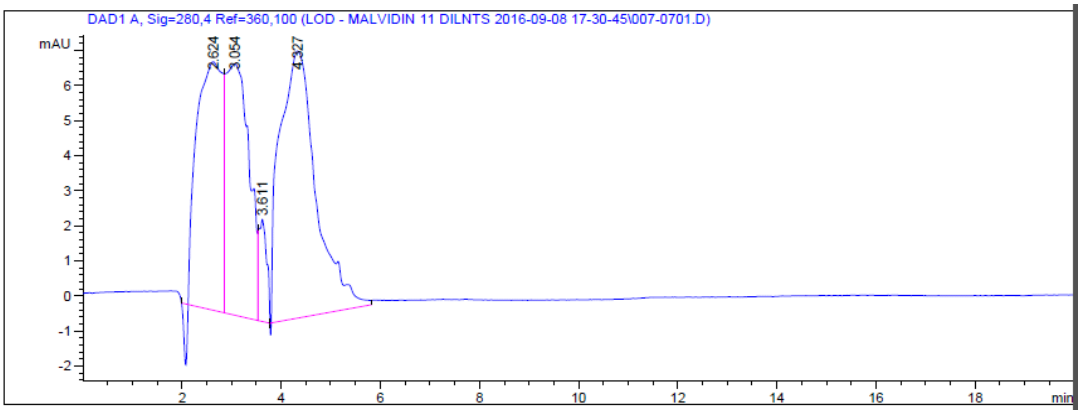


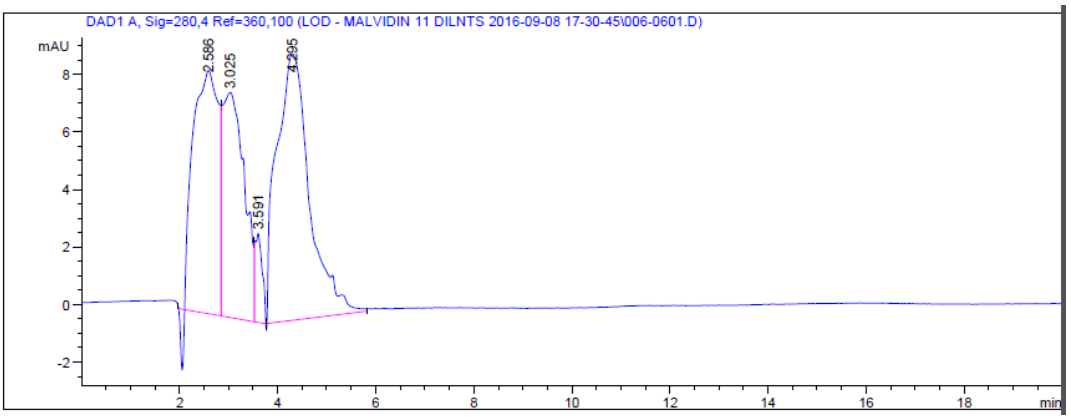


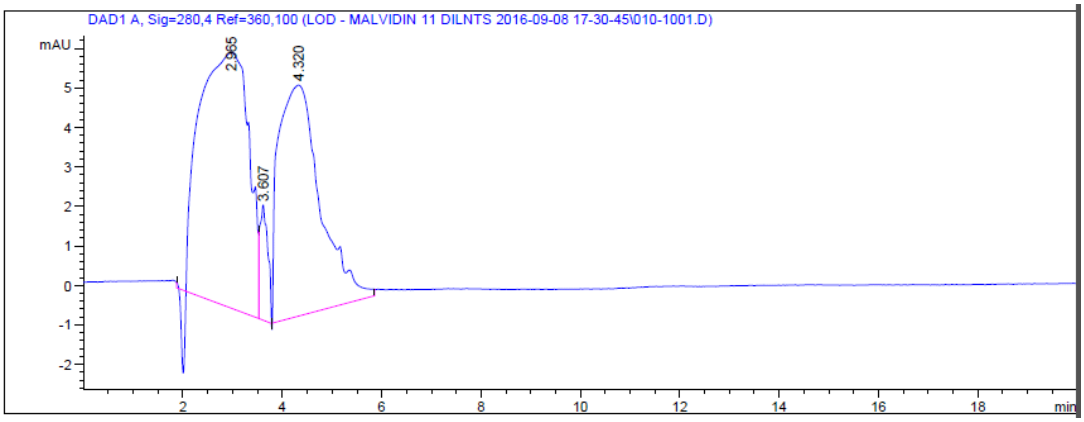

DAD1 A, Sig=280,4 Ref=360,100 (LOD - MALVIDIN 11 DILNTS 2016-09-08 17-30-45\011-1101.D)
mAU
3.135
3.570
4.381
min


DAD1 A, Sig=280,4 Ref=360,100 (LOD - MALVIDIN 11 DILNTS 2016-09-08 17-30-45\005-0501.D)
mAU
2.670
3.071
4.379
min


DAD1 A, Sig=280,4 Ref=360,100 (LOD - MALVIDIN 11 DILNTS 2016-09-08 17-30-45\009-0901.D)
mAU
2.894
3.590
4.258
min

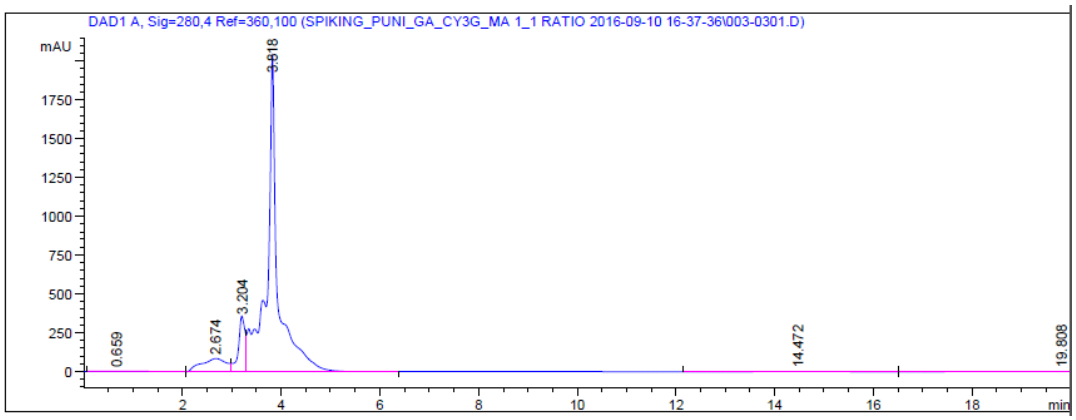

DAD1 A, Sig=280,4 Ref=360,100 (LOD - MALVIDIN 11 DILNTS 2016-09-08 17-30-45\007-0701.D)
mAU
2.624
3.054
3.611
4.327
min

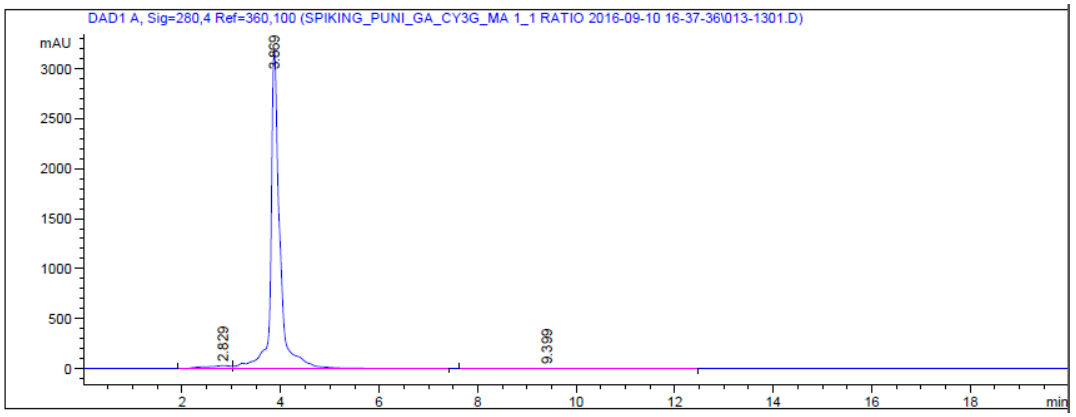

DAD1 A, Sig=280,4 Ref=360,100 (LOD - MALVIDIN 11 DILNTS 2016-09-08 17-30-45\006-0601.D)
mAU
2.586
3.025
3.591
4.295
min

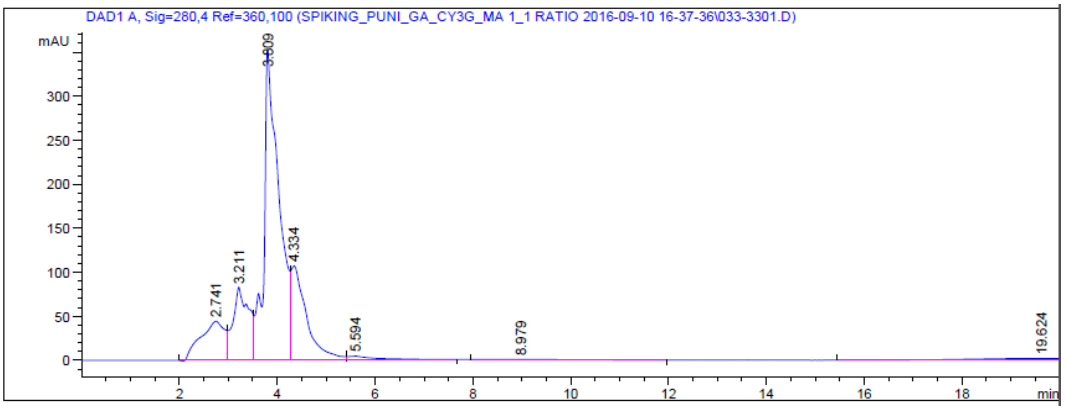

DAD1 A, Sig=280,4 Ref=360,100 (LOD - MALVIDIN 11 DILNTS 2016-09-08 17-30-45\010-1001.D)
mAU
2.965
3.607
4.320
min

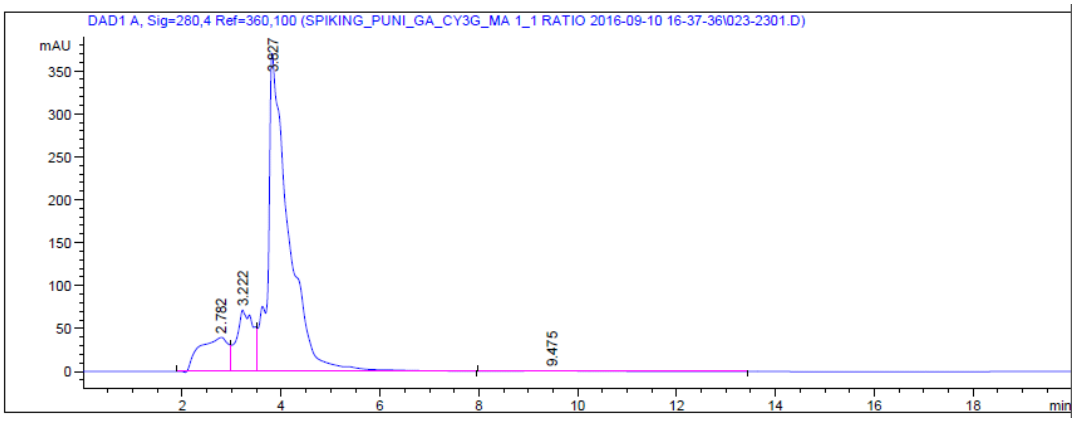
Appendix 2: Spiking of Fruit Extracts

Appendix 2.1: Strawberry extract spiked with punicalagin, gallic acid, cyanidin-3-glucoside and malvidin.

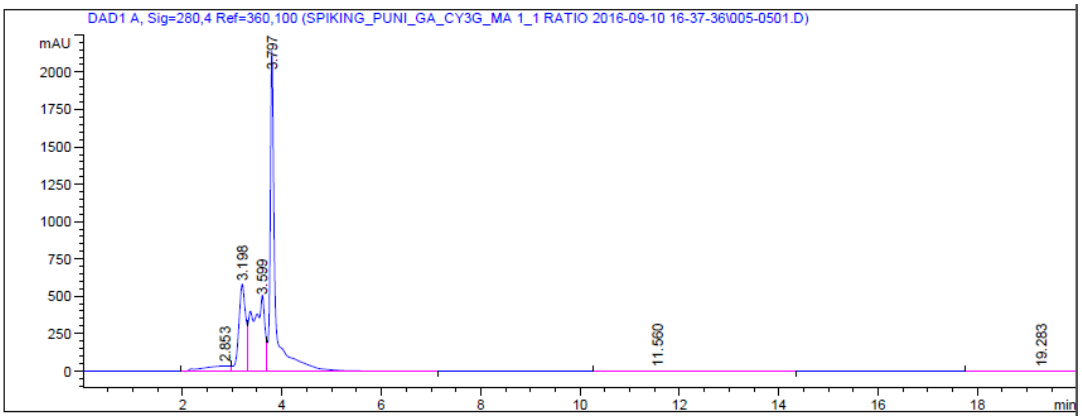


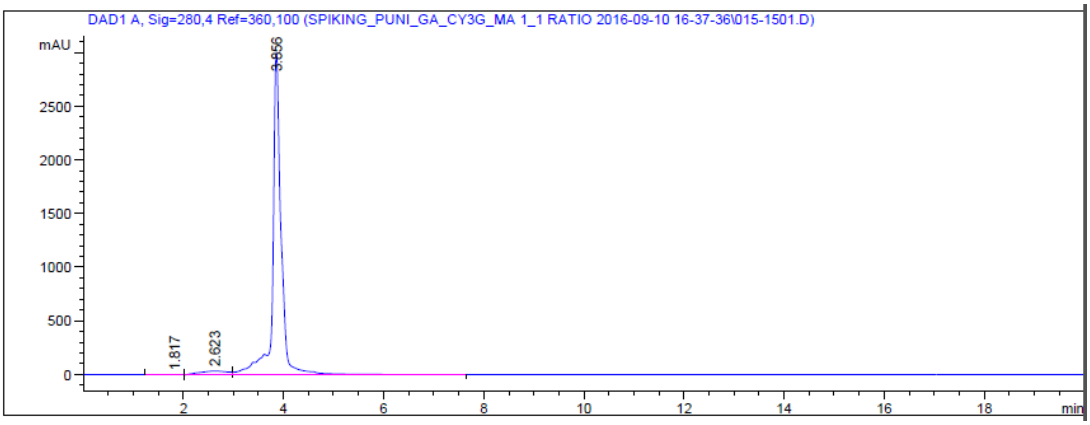


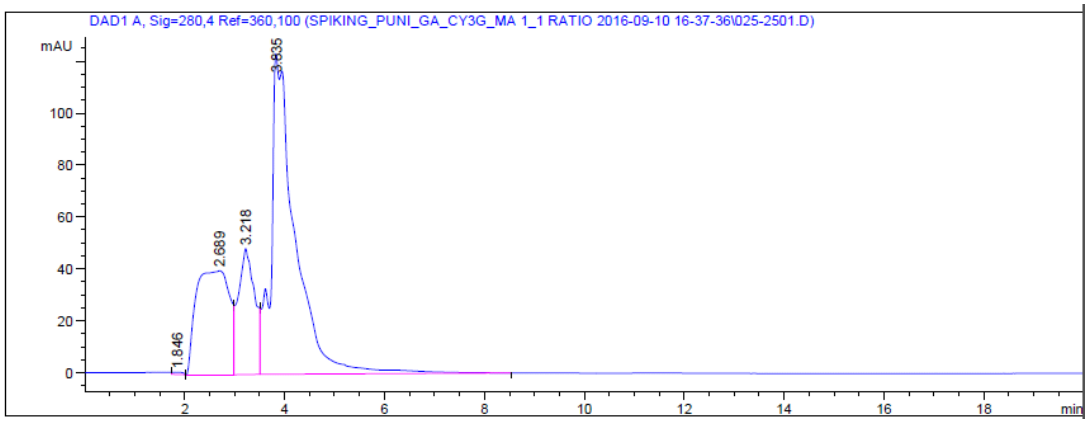


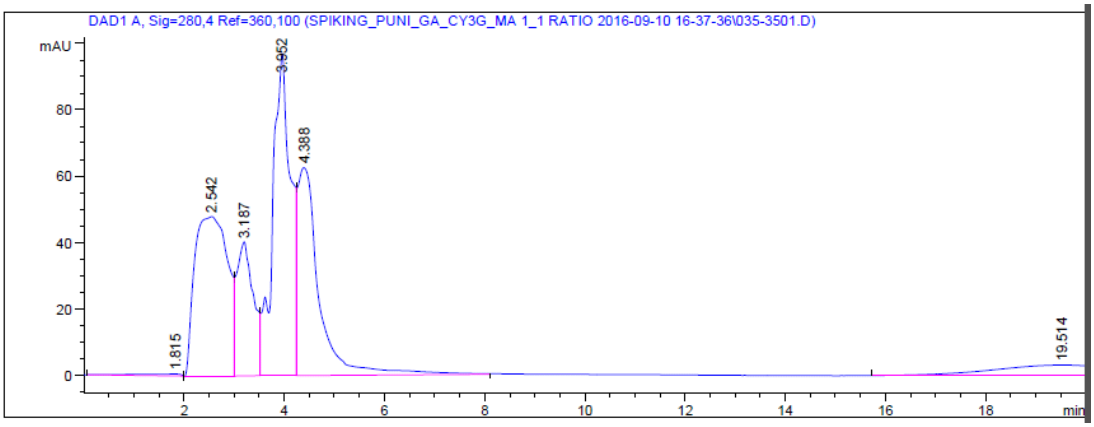


Appendix 2.2: Goji berry extract spiked with Punicalagin, gallic acid, cyanidin-3-glucoside and malvidin.

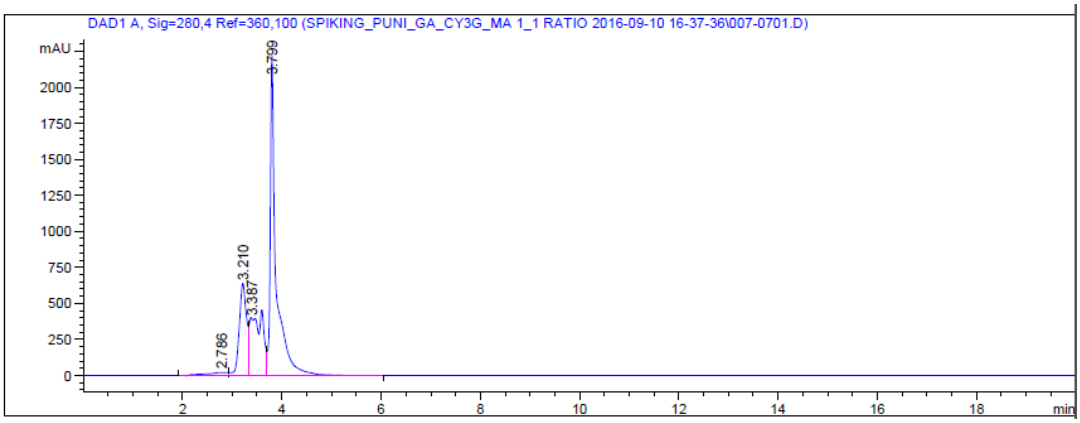


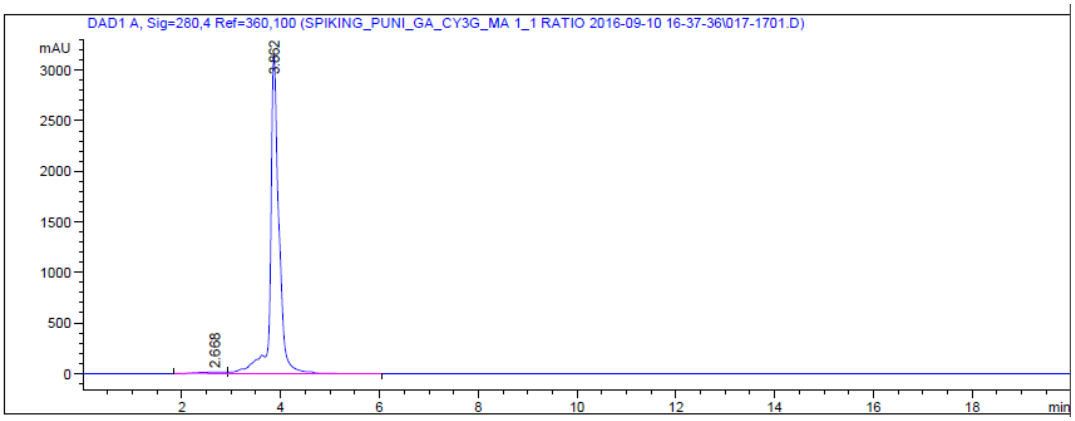


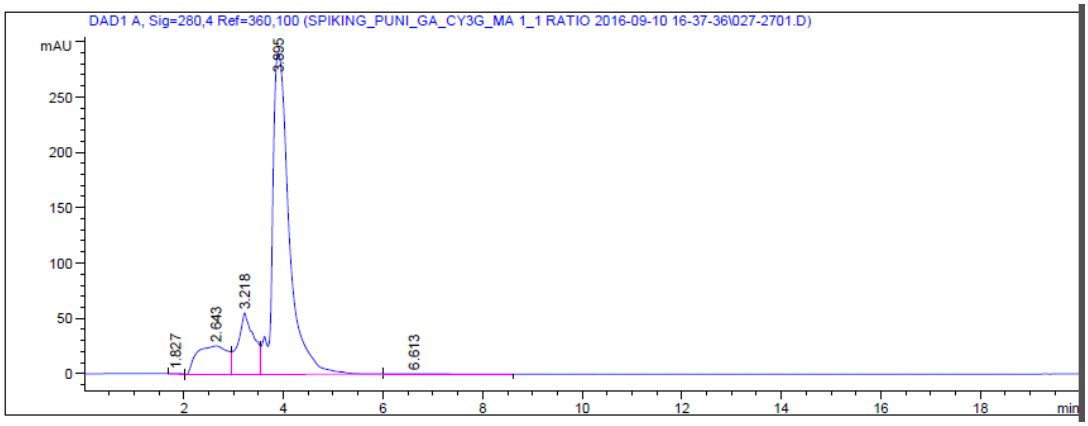


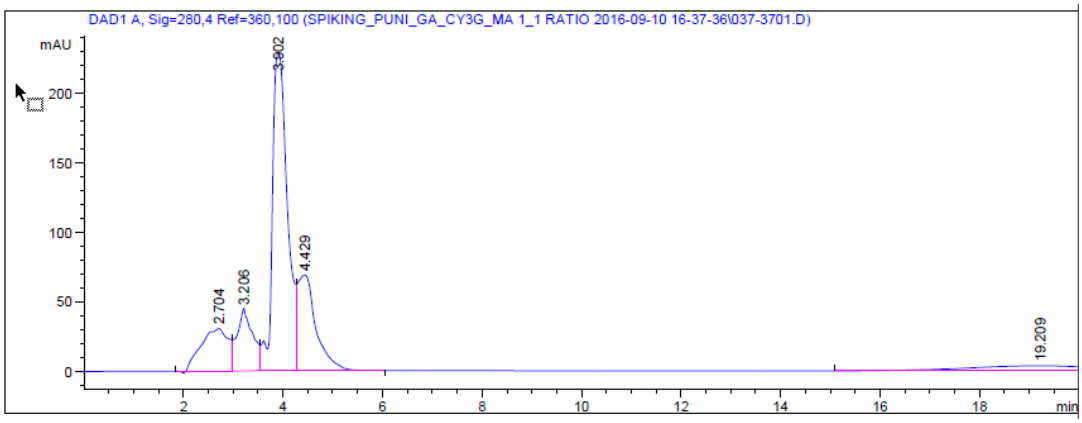


Appendix 2.3: Maqui berry extract spiked with Punicalagin, gallic acid, cyanidin-3-glucoside and malvidin.

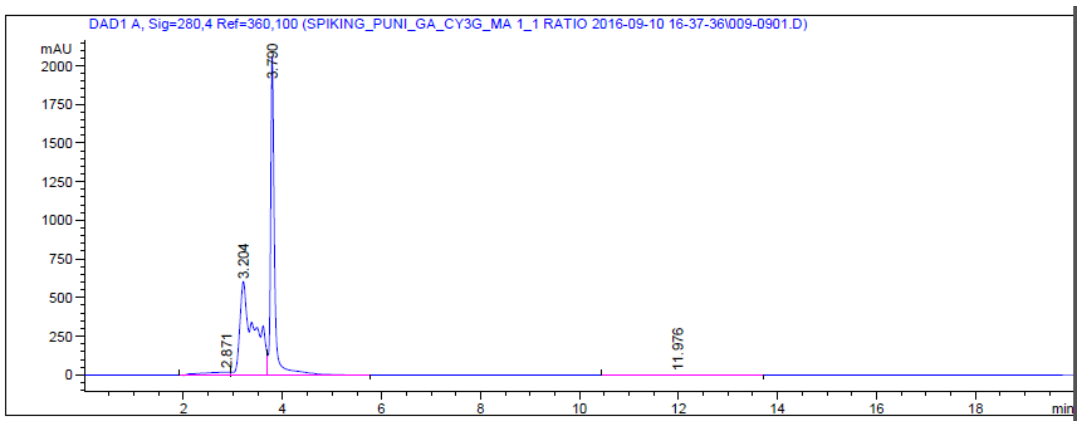


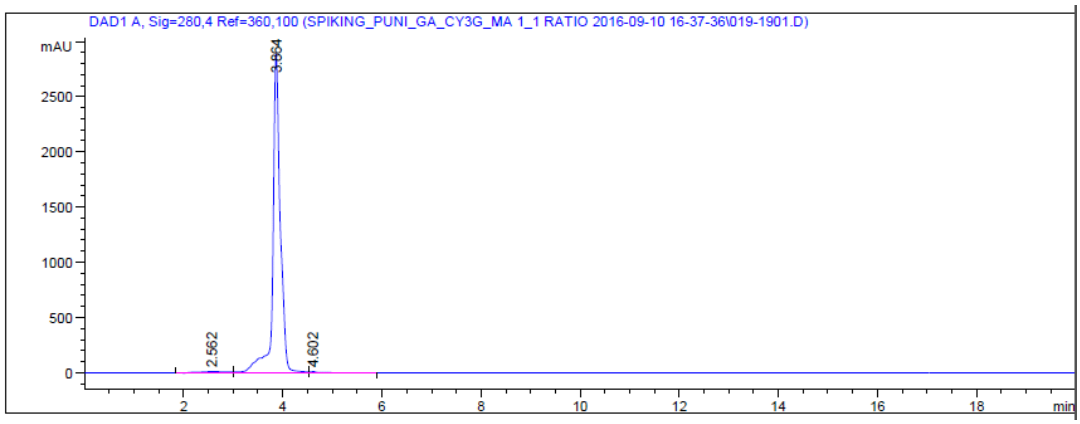


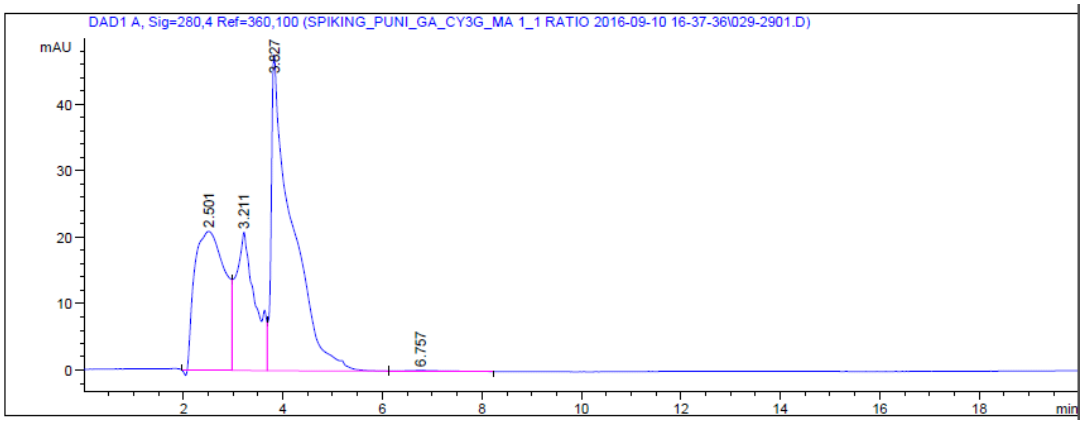


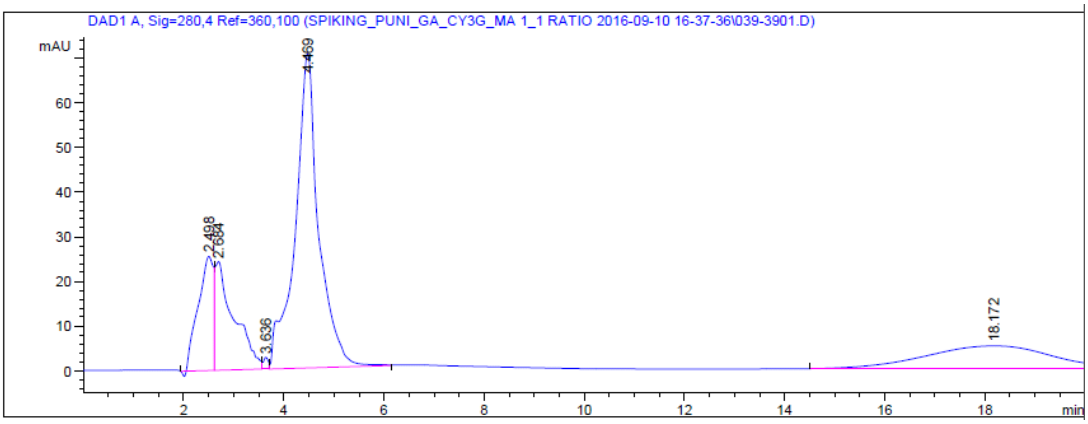


Appendix 2.3: Acai berry extract spiked with Punicalagin, gallic acid, cyanidin-3-glucoside and malvidin

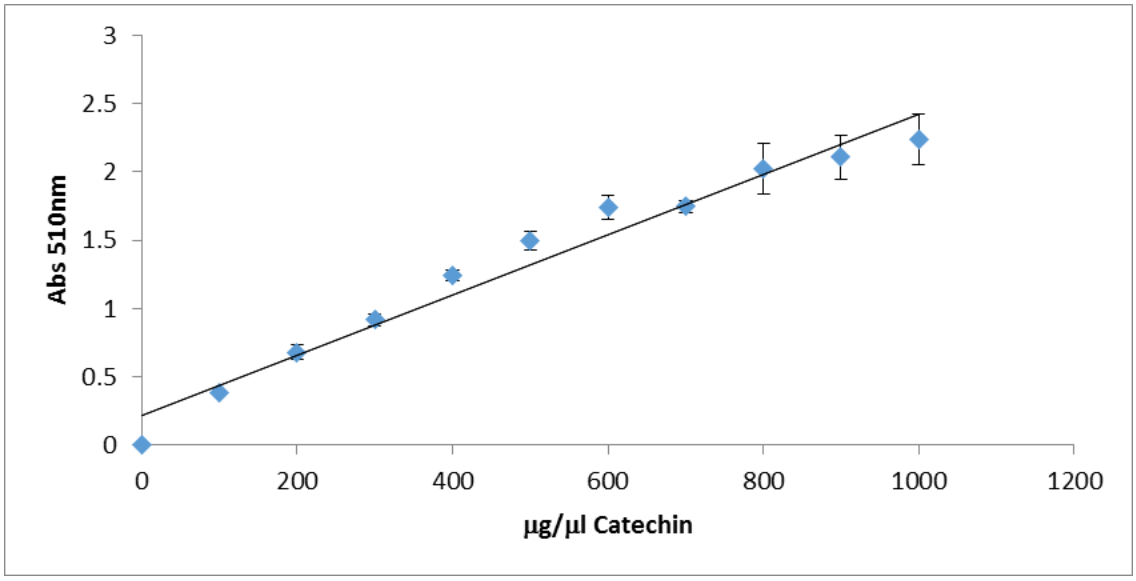




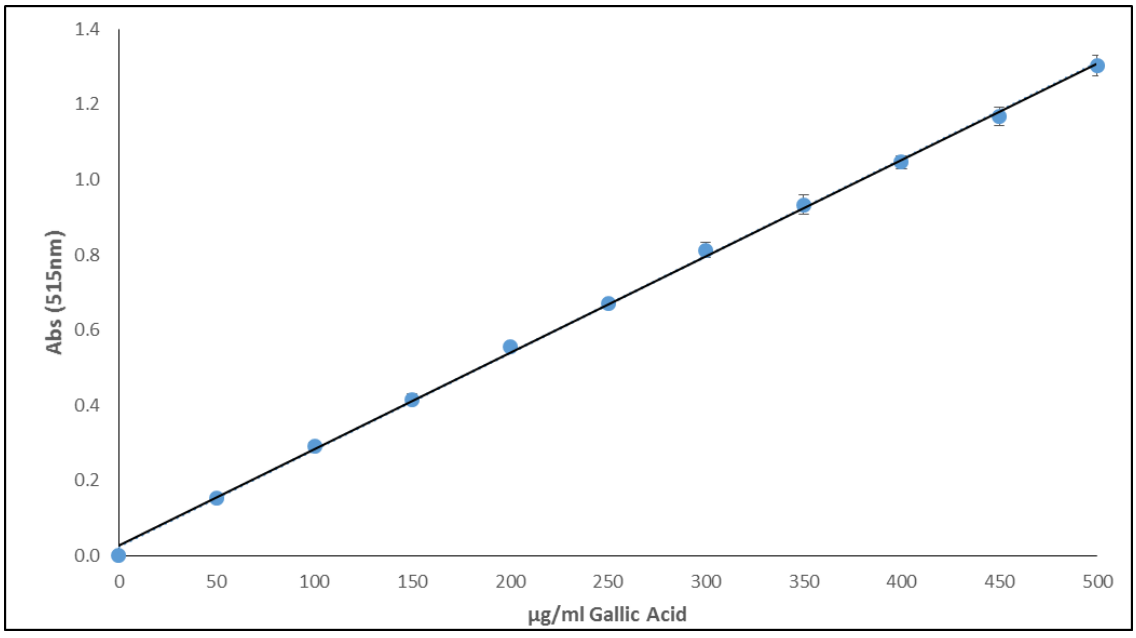




Appendix 3: Standard Curves & Graphs

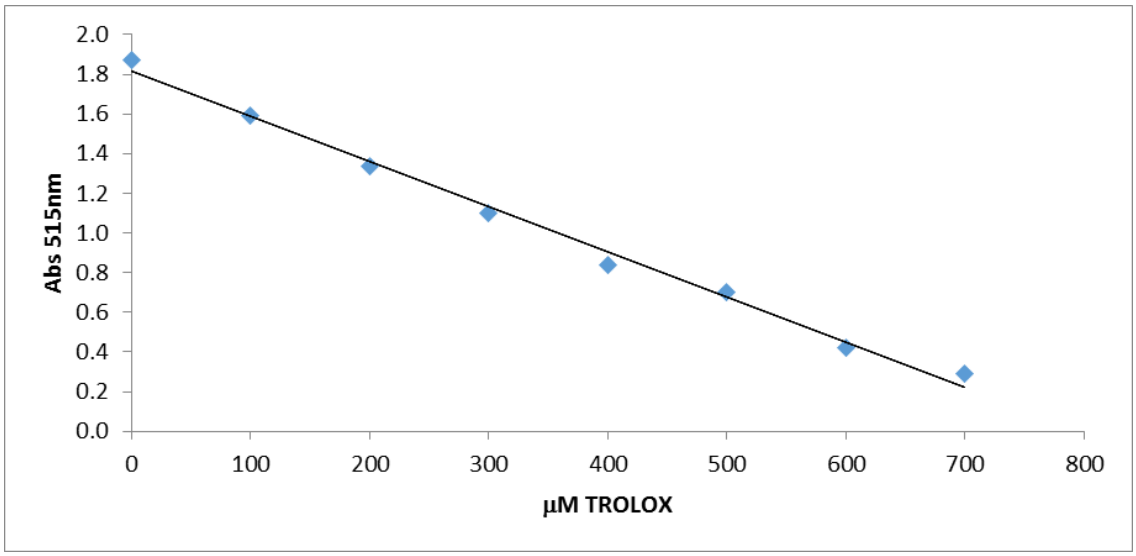


Appendix 3.1: Standard curve constructed from the Total Flavonoid Content. Standard curve used to quantify the TFC for samples expressed as µg of catechin equivalents (CE)/ml. A positive correlation was observed between absorbance and catechin concentration at r^2=0.97.

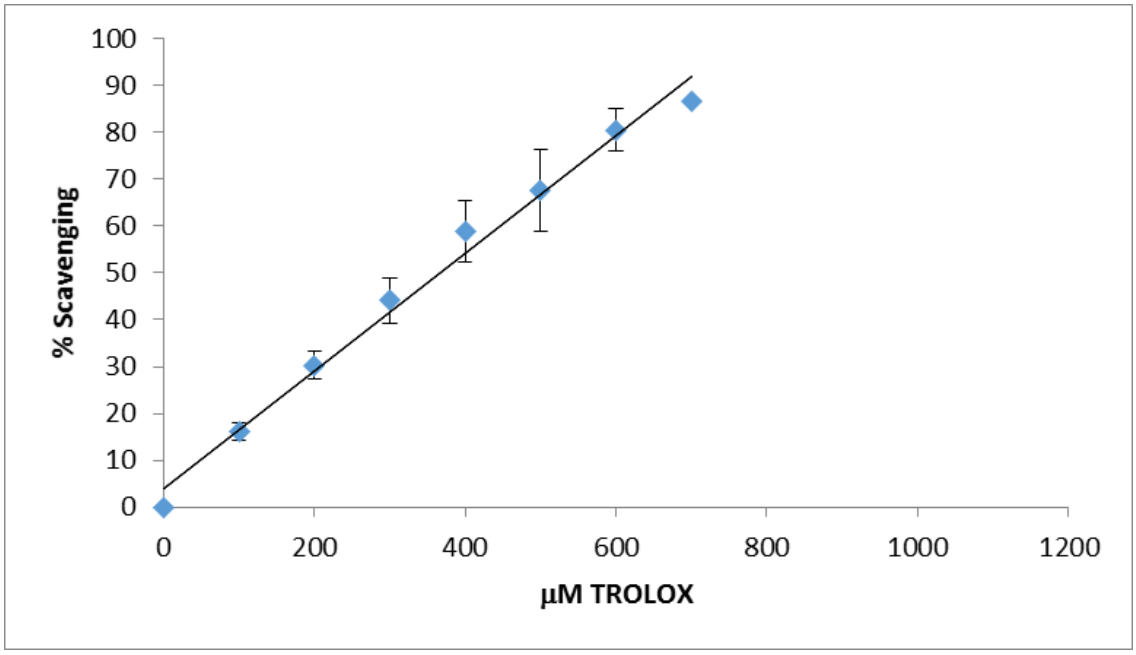


Appendix 3.2: Standard curve constructed from the Total Phenolic Content. Standard curve used to quantify the TPC for samples expressed as µg of gallic acid equivalents (GAE)/ml. A positive correlation was observed between absorbance and gallic acid concentration at r^2 = 0.99.

Appendix 3.3: Graphs from the DPPH assay



Appendix 3.3.1: Standard curve constructed from the DPPH assay. Standard curve used to quantify the antioxidant capacity for samples expressed as µM of TROLOX equivalents (TE). A negative correlation was seen between absorbance and concentration of TROLOX at r^2=0.99.



Appendix 3.3.2: Percentage scavenging graph for the DPPH assay. Percentage scavenging curve, used to quantify the percentage at which the AAPH molecules were quenched by the antioxidant, for samples expressed as µM of TROLOX equivalents (TE). A positive correlation was found between percentage scavenging and TROLOX concentration at r^2 = 0.85.