I declare that the work reputed in this thesis is entirely my own and has been carried out at Kingston University, UK

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Magali Barbara Nadine Chohan

Abstract

Culinary herbs, the use of which has increased significantly in the last decade, are known to possess health promoting properties, which are attributed mainly to their polyphenols. However, despite the fact that such herbs undergo some form of cooking prior to consumption, there is a paucity of data concerning the effects of cooking on their properties. Furthermore, little is known of the disposition of these polyphenols following digestion and absorption, and thus it is not known whether these herbs have the potential to be significant contributors of dietary polyphenols at amounts used domestically. Thus the aim of this study was to investigate: the impact of cooking processes on the polyphenolic antioxidant activity (AA) of parsley, rosemary, sage and thyme; the impact of digestion *in vitro* on the AA of the herbs post cooking; the role of the gut on the AA of these herbs using the Caco-2 model of intestinal transport; and whether the AA of these herbs, contributes to their anti-inflammatory and antimicrobial activities .

Results showed that cooking significantly increased the AA of these herbs. AA was further enhanced following *in vitro* digestion with rosmarinic acid identified as the predominant polyphenol. Polyphenolic activity was detected post absorption *in vitro* but, possibly due to the dilute nature of the samples used, individual polyphenols were not identified. Anti-inflammatory activity of the herbs investigated was significantly associated with their AA. However, there was no association between AA and the anti-microbial activity of aqueous extracts of herbs. Some oil extracts possessed anti-microbial activity, which was enhanced by cooking. In conclusion, this study suggests that the culinary herbs investigated have the potential to contribute to dietary polyphenol intake and thus their health promoting properties. However, the biological significance of this contribution *in vivo* is yet to be established.

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List of abbreviations

AA	Antioxidant Activity
ABTS	2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulfonic acid)
A.M.U	Atomic Mass Unit
AOC	Antioxidant Capacity
FC	Folin-Ciocalteu's phenol reagent
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
GAE	Gallic Acid Equivalent
^{1}H NMR	Proton Nuclear Magnetic Resonance
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) 2-(N-Morpholino) ethanesulphonic acid hydrate
HPLC	High Pressure Liquid Chromatography
IL-8	Interleukin 8
MAPK	Mitogen-activated protein kinase
MES	4-Morpholineethanesulphonic acid
NEAA	Non-essential amino acids
NF-κB	Nuclear Factor kappa B
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
SBF	Simulated Buccal Fluid
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOD	Superoxide Dismutase
SODm	Superoxide Dismutase mimetic
TEAC	Trolox Equivalent Antioxidant Capacity
TEER	Transepithelial Electrical Resistance
TLC	Thin Layer Chromatography
TNFα	Tumor Necrosis Factor alfa
TPC	Total Phenolic Content

Chapter 1 Introduction

1.0 An Introduction to Culinary Herbs

Culinary herbs, as defined by the Oxford English Dictionary (2002), are "any plants, with leaves, seeds or flowers used for flavouring in the kitchen". Culinary herbs are grown and used with food as they have attractive aromatic scents due to the presence of essential oils and oleoresins. Culinary herbs have also traditionally been used as cosmetic ingredients and medicinal agents in many forms such as strong teas, inhalants, oils macerations, decoctions, tinctures and poultices. In fact most western medicines in use today have originated from plant extracts, and around 40 % of the world's prescription drugs contain herbs (Walker, 1996). The World Health Organisation (WHO, 2009) estimates that 80% of the global population rely on traditional medicine, including herbal extracts, for minor ailments.

1.1 Uses of Culinary Herbs (a brief history).

The use of herbs and plants for nutrition, as cosmetics and for medicinal purposes by indigenous populations has been an integral part of human history. The first documented record of a medicinal plant was found in Iraq, in the tomb of a Neanderthal man and dates back to 60,000 BC (Leroi-Gourhan,1975 in Williamson, 2003). Ancient Sumerian tablets thought to date back to 4500 BC were found in a Bronze Age excavation. These tablets listed around 250 herbs, including thyme, and suggest that herbs were important in the traditions of prehistoric times (Atha, 2001).

The first record of the use of plants as herbal medicine was in China, and dates back to Sheng Nong, "The Divine Husbandman" (2838–2698 BC) and Huang Di, "The Yellow Emperor's Classic of Internal Medicine" (2698–2598 BC), (Tapsell *et al.*, 2006).

The Egyptians and the Greeks also have a long tradition of using herbs for medicinal purposes: the Egyptians used herbal medicines for various religious rituals, including embalment. Papyri from 1555 BC record the use of coriander, fennel, juniper, cumin, garlic and thyme (Tapsell *et al.*, 2006). Hippocrates (460–377 BC) had compiled a list of 300 herbal remedies such as locally grown rosemary, which he used as memory enhancers (Bellamy and Pfister, 1992).

"Plant Researches" and "Plant Aetiology" were written by Theophrastus, a pupil of Aristotle, around 300 BC. Furthermore, the Greek physician, Pedanius Dioscorides published the first plant record in the first century, which included 600 herbs entitled "*De Materia Medica"* in which he describes how to choose the herbs, store them and use them for health benefits. Some of his knowledge surrounding the health benefits of herbs and spices was acquired by Arabic physicians and that knowledge made its way to Europe in the 11th century, enhanced by the spice trade between Europe, Africa and Asia which continued throughout the 13th century. Dioscoride's *"De Materia Medica*" was still used in Europe until the 17th century as a reference for herbal medicine (Tapsell *et al.*, 2006).

Herbal medicine is still a subject of much interest today. A search in the ISI Web of Knowledge for "Culinary Herbs" resulted in 84 records, a majority of which were original articles published between 1991 and 2010 with an increase in publication numbers from 1996 (Web of Knowledge, 2010)

1.2 Culinary herbs: Current trends

In the UK, for the past 30 years, the trend has moved from the traditional 'meat and two veg' staple of the everyday meal, towards rice and pasta based dishes. Income and social status are thought to influence this change in food choice, and it is usually the better-off socio-economic groups, influenced by via the media, travel, and eating out, that are more likely to be exposed to, and purchase, a wider variety of foods.

A UK survey showed that the majority of people spend between 16 and 45 minutes preparing the weekday evening meal (Mintel, 2009). This growth in the interest of cooking 'from scratch' as opposed to readymade meals, has led to an increased interest in the use of herbs in the preparation of food, an interest that is said to be fuelled mainly by the media (Tapsell *et al.*, 2006). This increased interest has been reflected in the strong growth in the culinary herb (dried and fresh) market which grew by 15% between 2002 and 2007 and increased by 9.5 % between 2007 and 2009 (Mintel, 2009).The interest in culinary herbs, and also spices, may also be enhanced by the reported health benefits of consuming a diet low in salt: the Food Standards Agency, UK, recommends using culinary herbs and spices instead of salt in an attempt to reduce salt intake, the high consumption of which is linked to high blood pressure and its cardiovascular consequences, including stroke and coronary heart disease (SCAN, 2003).

1.3 Culinary herbs: the basis for their reported health benefits

The increase in interest in culinary herbs has occurred in conjunction with the emergence of research into these foodstuffs. However, in terms of nutrition, the contribution of culinary herbs is thought to be negligible in comparison to other plant-derived foods such as fruits and vegetables, as they are not rich sources of micronutrients and macronutrients (Garrow *et al.* 2002). However, recent research has shown that culinary herbs possess bioactive compounds that are reported to have health benefits (Halvorsen *et al.*, 2002; Dragland *et al.*, 2003; Tapsell *et al.*, 2006) and have been termed as 'functional foods'. A major group of compounds, which are believed to be significant contributors to the putative health benefits of culinary herbs, are collectively called the polyphenols (Tapsell *et al.*, 2006).

1.4 Polyphenols

Polyphenols are organic secondary metabolite compounds ubiquitous in plants and plant derived foods including culinary herbs (Bravo, 1998; Cheynier, 2005). Polyphenols are thought to have developed at an early stage in terrestrial plant evolution to protect the plants from UV light (Pietta, 1999). There is evidence to support the hypothesis that these polyphenols combine with carbohydrates to form esters, glycosides or polymers, and are produced to protect the plant from UV light, pathogens, parasites and environmental stresses such as harsh temperatures (Raven, 1998; Pietta, 1999; Regnault-Roger *et al.*, 2004). Wolski *et al.* (2010) suggested that plants increase their polyphenol content once they have been under stress, and that these polyphenols were most concentrated in the proximity of the sites of the insult/damage. The major classes of polyphenols in plant foods are the flavonoids and non-flavonoids, The flavonoids include the anthocyanins, flavonols, flavones, flavanones, isoflavones, monomeric and polymeric flavonols and the proanthocynadins. The non-flavonoid phenolic compounds include the phenolic acids, lignins and stilbenes (Singh *et al., 2008*).

1.4.1 Biological activity of polyphenols

Epidemiological evidence suggests that polyphenols are beneficial to health (Kris-Etherton *et al.*, 2002). The protective effects of flavonoids such as luteolin and apigenin, found in parsley and thyme, have been demonstrated against many cancers including, oral, breast, thyroid, lung, prostate, colon and leukaemia, and have been described as "very impressive" (Kris-Etherton *et al.*, 2002). In addition, these flavonoids are thought to show promise as anti-cancer agents, especially in

terms of prevention, and as important tools in the protection of non-cancerous cells during chemotherapy (Ren *et al.*, 2003).

The antimicrobial activity of polyphenols found in culinary herbs has also been investigated. Shan *et al.* (2007) showed a close correlation between the antioxidant activity, total phenolic content and antibacterial activity of freeze dried methanol extracts of culinary herbs. In addition, Moreno *et al.* (2006) showed a correlation between the antioxidant and antimicrobial activity of rosemary extracts which was due to their polyphenol composition, and more precisely to the phenolic acids, carnosic acid and rosmarinic acid. Furthermore, Khan *et al.* (2005) conducted a systematic review of evidence concerning herbs used for medicinal purposes. The review noted that some herbs possessed anti-viral activity that may be due to polyphenol compounds including caffeic acid, chlorogenic acid, ferulic acid, and p-coumaric acid.

Anti-inflammatory activity of culinary herbs has also been attributed to polyphenols. Yoon and Baek (2005) discuss the various ways in which polyphenols found in the diet demonstrate anti-inflammatory properties. They suggest that polyphenol compounds inhibit several enzymes involved in inflammation, namely the cyclooxygenase and lipoxygenase enzymes polyphospholipase A_2 and nitric oxide synthase.

The main property associated with the polyphenols, however, is their antioxidant activity. Polyphenols appear to be significant contributors to the antioxidant activity of culinary herbs (Rice-Evans *et al.*, 1997). Zheng and Wang (2001) observed that, in 27 culinary herbs, there was a positive correlation between the polyphenols identified in these herbs and their antioxidant activity, and subsequent studies concur with these (Pellegrini *et al.*, 2003; Matsingou *et al.*, 2003; Javanmardi *et al.*, 2003; Wu *et al.*, 2004; Shan *et al.*, 2006). Some suggest that polyphenols protect against oxidative damage to a greater degree than other dietary antioxidants such as vitamin A, C, E, and carotenoids (Craig, 1999). Furthermore, the antioxidant activity of some culinary herbs are reported to exceed those of fruits and vegetables (Halvorsen *et al.*, 2002 and 2006).

Craig (1999) performed a systematic review on the health promoting properties of common herbs, and found evidence to support the role of polyphenols in protecting against the oxidation of LDL cholesterol. However, whether or not their

role as antioxidants contributes, or forms part of, their other biological properties remain unclear.

1.5 The importance of looking at the effects of food processing on biological activities of culinary herbs.

Polyphenols are reported to be the most abundant antioxidants in the diet and their intake could be as high as 1g/day which is 10 times more than vitamin C intake and 100 times more than vitamin E and carotenoid intakes (Scalbert *et al.*, 2005). However, despite major studies on the antioxidant activity possessed by culinary herbs (Craig, 1999; Zheng and Wang 2001; Halvorsen *et al.*, 2002; Pellegrini *et al.*, 2003; Matsingou *et al.*, 2003; Javanmardi *et al.*, 2003; Wu *et al.*, 2004; Shan *et al.*, 2006; Hinneburg *et al.*, 2005; Pellegrini *et al.*, 2006; Halvorsen *et al.*, 2006; Carlsen *et al.*, 2010), few have taken into consideration the impact of cooking methods which are commonly used prior to consumption of herbs on their biological activity, specifically their polyphenol antioxidant activity.

Price *et al.* (1997) looked at the effects of various cooking methods on the stability of flavonoid glycosides of quercetin in onions using HPLC analysis. They concluded that there were no changes in this particular polyphenol caused by cooking, although they observed a mean loss of polyphenols of 25%. Furthermore, Chohan *et al.* (2008) showed that cooking either increased or decreased the polyphenol antioxidant capacity of several culinary herbs, depending on the method used.

Another question that has yet to be addressed is whether these herbs, when combined, increase polyphenol antioxidant activity synergistically, as has been seen with foods such as broccoli and tomato in relation to anti-cancer activity (Canene-Adams *et al.*, 2007). Furthermore, although the bioavailability of polyphenols has been subject to much research (Garrett *et al.*, 2000; Williamson *et al.*, 2000; Scalbert and Williamson, 2000; Rechner *et al.*, 2002; Manach *et al.*, 2004; Nurmi *et al.*, 2006; Gladine *et al.*, 2007; Baba *et al.*, 2005; Singh *et al.*, 2008) there is a paucity of literature on the consequences of digestion and absorption on the polyphenol activity of culinary herbs. Such information is essential in determining if culinary herbs have the potential to be significant contributors of dietary polyphenols and thus their reported healthy benefits.

1.6 Aims of the study

When considering the health effects of dietary constituents, many factors come into play, including the synergistic activities of bioactive compounds and the impact of the food matrix. However, since antioxidants are deemed to be beneficial to health, the first aim of this study was to investigate the overall contribution to negating oxidative stress by a selection of culinary herbs (individually and in combination). This aim was investigated by assessing the impact of cooking methods commonly used in the preparation of culinary herbs on their biological activity, specifically their polyphenol antioxidant activity and polyphenolic profile.

The second aim of this study was to investigate the role of the gut on the polyphenol antioxidant activity and polyphenolic profile of the same selection of cooked culinary herbs. The second aim was investigated by setting up, validating and using *in vitro* models of digestion and absorption.

The final aim was to determine if the polyphenol antioxidant activity of these herbs, pre- and post-cooking and post–absorption, contributed to the presence of any anti-microbial and anti-inflammatory activity possessed by the selected herbs.

Achieving these aims was essential for the evaluation of the polyphenol contribution of culinary herbs to the diet.

Chapter 2 Selection of culinary herbs.

2.0 Introduction

There is a wide range of culinary herbs available in the UK which are sold in various forms such as "fresh still in pots" or freshly cut, air dried, freeze dried, frozen, as well as "in oil as paste". Therefore for the purposes of this investigation it was important to set up and apply criteria for the selection of herbs to be used. Thus the aim of this chapter is to explain the basis for the criteria and to demonstrate how each criterion was used for the selection of the culinary herbs used.

2.1 Selection criteria

For each selection criterion the basis is stated first and then the selection criterion is highlighted in bold.

- One of the aims of the study was to assess the role of culinary herbs as significant contributors to dietary polyphenol antioxidant intake. Thus, it was necessary to consider culinary herbs that are commonly consumed and that are freely available in the UK (Mintel, 2009a). **The first selection criterion for this study was that the herb is commonly used for the purposes of cooking.**

- The form of the culinary herb also needed to be considered. Fresh herbs are becoming more popular, affordable and available as a result of leading UK supermarkets investing in the advertising of fresh herbs (Mintel, 2009a). However, dried herbs are cheaper and more convenient as they have a long shelf life; they do not require refrigeration and are available for cooking all year long. **Thus, dried culinary herbs were used in this study.**

- Poor quality, especially microbial contamination, could affect the results of biological assays that the selected herbs would undergo and thus limit the usefulness of data regarding their activity. **Therefore, it was essential that the herbs selected be assessed for, and shown to be free of, microbial contamination.**

- It is well established that the content of bioactive compounds in plant derived foods including culinary herbs are affected by seasonal variations (Luis and Johnson, 2005; Dragland *et al.*, 2003) and the place of provenance (Cuvelier et al., 1996; Halvorsen *et al.*, 2002) as well as pre-sale processing and storage (Stafford *et al.*, 2005). **Thus, it was necessary that batch numbers, place of provenance and processing details of the culinary herbs selected were readily available.**

- Many culinary herbs have medicinal properties, and are part of the *materia medica, or* classic herb medicine as stated in Chapter 1. The medicinal effects of culinary herbs are mainly attributed to bioactive compounds especially the polyphenols, (Craig, 1999; Pietta., 1999; Kris-Etherton *et al.*, 2002; Williamson, 2003). In addition, some of the health benefits of polyphenols have been attributed to their high antioxidant activity (Zheng and Wang, 2001; Williamson, 2003; Matsingou *et al.*, 2003; Dragland *et al.*, 2003; Shan *et al.,* 2006 and 2007). **Thus, for the purposes of this investigation, culinary herbs were chosen that, from the literature were identified as having a track record for use for medicinal purposes and possessing high polyphenol content and antioxidant activity**

- All herb products retailed in the UK for culinary and medicinal purposes are required to have either a traditional herb registration (THR) or marketing authorisation (MA) in order to be deemed safe for public health consumption (MHRA.gov.uk, 2009). **Thus, herbs used in this study had to have either a THR or a MA.**

2.2 The Culinary Herbs Selected

Based on the criteria stated above, parsley, rosemary, sage and thyme were selected. All four herbs used came from the same supplier "The Organic Herb Trading Company", UK. The herbs were grown in different countries but were dried in warm air, and processed by the supplier, as stated in the summary table 2.1.

Herbs were stored at ambient temperature, with free air flow. The herbs were then packaged in a sealed brown paper bag with a blue polythene liner and distributed by "Neal's Yard remedies" Richmond, Surrey, and UK, in clear polythene bags with labelled batch numbers (Table 2.1).

The cut off for microbiological contamination was expressed in colony forming units per gram for each dried herb sample. The microbiological evaluation was performed by the supplier using the maximum acceptale limits (MAL) set by the British retail consortium (BRC certified, Table 2.2)

The summary of evidence for the presence of polyphenol compounds and antioxidant activity for each herb is tabulated below. All four herbs are commonly used for cooking and authorised for the herb medicine by the MHRA summarised in Table 2.3.

Table 2.1 Summary of culinary herbs selected based on the selection criteria.

Herbs	**Parsley**	**Rosemary**	**Sage**	**Thyme**
Botanical name	*Petroselinum crispum*	*Rosmarinus officinalis*	*Salvia Officinalis*	*Thymus vulgaris*
Plant family	Umbelliferae	Labiatae	Labiatae	Labiatae
Country of origin	Egypt	Spain	Croatia	France
Pre-sale processing	Dried in warm air Coarse cut*	Dried in warm air Uncut	Dried in warm air Uncut	Dried in warm air Coarse cut *
Storage	Ambient temperature, light and humidity with free air flow	Ambient temperature, light and humidity with free air flow	Ambient temperature, light and humidity with free air flow	Ambient temperature, light and humidity with free air flow
Packaging	Sealed brown paper bag with blue polythene liner	Sealed brown paper bag with blue polythene liner	Sealed brown paper bag with blue polythene liner	Sealed brown paper bag with blue polythene liner

* Blade cut size was 6 millimetres for Coarse cut herbs.

Table 2.2 Summary of microbiological evaluation for parsley, rosemary, sage and thyme.

Isolates	**For parsley, rosemary, sage and thyme.**
Escherichia coli cfu/g dried herb	Below the MAL set as: $1x10^2$
Salmonella cfu/g dried herb	Negative in 25g
Yeast cfu/g dried herb	Below the MAL set as: $1x10^5$
Mould (generic) cfu/g dried herb	Below the MAL set as: $1x10^5$
Staphylococcus aureus cfu/g dried herb	<20
Enterobactriacae cfu/g dried herb	Below the MAL set as: $1x10^5$

Maximum acceptable limit (MAL) of colony forming units per gram of each dried (Cfu/g herb) set by the British retail consortium.

Table 2.3 Literature demonstrating the presence of polyphenol compounds and antioxidant activity.

Herbs	**Parsley**	**Rosemary**	**Sage**	**Thyme**
Summary of evidence for High Polyphenol content	Zheng and Wang (2001) Wu *et al.* (2003) Hinneburg *et al.* (2005) Shan *et al.* (2006)	Zheng and Wang (2001) Proestos *et al.* (2005) Proestos *et al.* (2006) Shan *et al.* (2006) Hossain *et al.* (2010)	Zheng and Wang (2001) Proestos *et al.* (2005) Shan *et al.* (2006) Hossain *et al.* (2010)	Zheng and Wang (2001) Proestos *et al.* (2005) Shan *et al.* (2006) Hossain *et al.* (2010)
Summary of evidence for the High Antioxidant Activity [Type of assay]	Zheng and Wang (2001) [ORAC] Halvorsen *et al.* (2002)[FRAP] Dragland *et al.* (2003) [FRAP] Wu *et al.* (2004) [ORAC] Shan *et al.* (2006) [ABTS] Shan *et al.* (2007 [ABTS] Pellegrini *et al.* (2006) [ABTS, FRAP, TRAP]	Mantle *et al.* (2000) [ABTS] Zheng and Wang (2001) [ORAC], Dragland *et al.* (2003) [FRAP] Shan *et al.* (2006) [ABTS] Shan *et al.* (2007) [ABTS] Pellegrini *et al.* (2006) [ABTS, FRAP, TRAP]	Mantle *et al.* (2000) [ABTS] Zheng and Wang, (2001) [ORAC], Dragland *et al.* (2003) [FRAP] Shan *et al.* (2006) [ABTS] Shan *et al.* (2007) [ABTS] Pellegrini *et al.* (2006) [ABTS, FRAP, TRAP]	Mantle *et al.* (2000) [ABTS] Zheng and Wang (2001) [ORAC] Dragland *et al.* (2003)[FRAP] Shan *et al.* (2006) [ABTS] Shan *et al.* (2007) [ABTS] Pellegrini *et al.* (2006) [ABTS, FRAP, TRAP]

ORAC: Oxygen Radical Absorption Capacity assay; FRAP: Ferric Reducing Antioxidant Power assay; TRAP: Total Radical-Trapping Antioxidant Potential assay. ABTS: (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) assay

2.3 Discussion

2.3.1 Parsley, (*Petroselinum crispum*)

Parsley (Figure 2.1) is often used fresh to garnish meat dishes or as a dried herb added to salads and soups (Food Standards Agency, 2002). Parsley originates from the eastern Mediterranean and is cultivated worldwide. In herb medicine parsley has been used as a diuretic, carminative, spasmolytic and a uterine tonic due to the properties of its polyphenols (Williamson, 2003). In addition, parsley has been shown to possess polyphenolic constituents such as the flavonoid apigenin, that have free radical scavenging and anti-inflammatory properties (Williamson, 2003).

Figure 2.1 Parsley dried leaves

This picture was captured using a Zeiss Stemi 2000C stereomicroscope (X 10). The picture compares well with the description provided by the supplier: "cut olive and green leaves".

2.3.2 Labiatae: Rosemary (*Rosmarinus officinalis*), Sage (*Salvia officinalis*) and Thyme (*Thymus Vulgaris*).

Unlike parsley, rosemary, sage and thyme are from the plant family Labiatae and all originate from the Mediterranean. All are known to have a high content of volatile oils and are primarily used for their palatability as culinary herbs to flavour dishes. Several aromatherapy studies have reported psychological effects and mental health benefits of the essential oils from these herbs for example the

aromas of these oils have been shown to modulate mood and memory (Diego *et al*. 1998; Aburjai and Natesheh, 2003).

2.3.2.1 Rosemary, (Rosmarinus officinalis)

Rosemary (Figure 2.2) is cultivated worldwide and is a classic component of many dishes and sauces. It is extensively used in tonics and cosmetic products. In herb medicine it is mainly used as an anti-bacterial, antiviral and anti-inflammatory (Petersen, 2002). Rosemary possesses antioxidant, anti-inflammatory, anti-tumour, antispasmodic, anticonvulsant, astringent, tonic, antiseptic, antimutagen activity and is also used as a nervine (Williamson, 2003). In addition, rosemary oil has been found to have antiviral and antitumor activity (Williamson, 2003).

The bioactive compounds found in rosemary include a number of polyphenol compounds including the flavonoids, luteonin and apigenin, and the phenolic acids caffeic acid and rosmarinic acid (Hossain *et al*. 2010). Rosmarinic acid has been found to suppress the inflammatory response, as well as inhibit genotoxicity and tumour growth (Williamson, 2003).

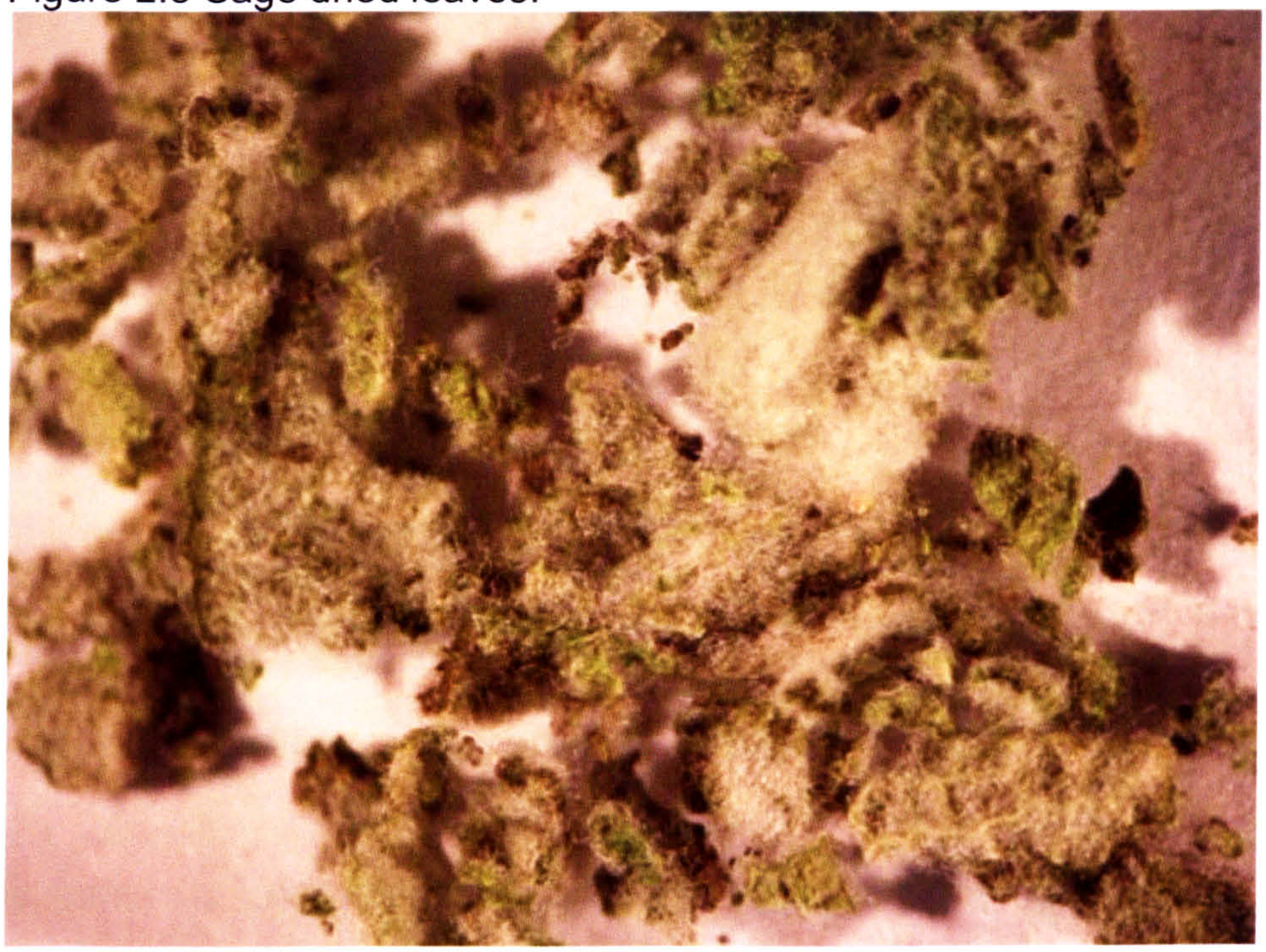
Figure 2.2 Rosemary dried leaves

This picture was captured using a Zeiss Stemi 2000C stereomicroscope (X 10). The picture compares well with the description provided by the supplier: "Light and dark green linear leaves" although some leaves could be described as brown.

2.3.2.2 Sage, (Salvia officinalis)

Sage (Figure 2.3), as with rosemary, is grown worldwide. In addition to being a very popular culinary herb it is widely used as an astringent, as well as an antiseptic; it is classically used to treat mouth lesions and throat infections. In addition, sage is used as a memory enhancer, to reduce perspiration and to manage symptoms associated with the menopause (Williamson, 2003). Sage is reported to possess high antioxidant activity properties and both rosmarinic acid and caffeic acid have been shown to be major contributors to this activity (Cuvelier *et al.,* 1996; Mantle *et al*., 2000). Furthermore, the high levels of rosmarinic acid in sage have been associated with the anti-inflammatory and antimicrobial properties of this herb (Williamson, 2003).

Figure 2.3 Sage dried leaves.



This picture captured using a Zeiss Stemi 2000C stereomicroscope (X 10). The picture compares well with the description provided by the supplier: "Green and green/grey furry leaves".

2.3.2.3 Thyme, (Thymus Vulgaris).

Thyme (Figure 2.4) is widely used as a culinary herb in stews and roasted meats. It is mainly used as an expectorant and antiseptic in herb medicine and, like sage, thyme is used in mouth rinses and toothpaste preparations. Studies have found that thyme's high antioxidant activity is mainly due to the presence of phenolic

acids such as rosmarinic acid and caffeic acid. Some studies have reported the presence of tannins in thyme (Williamson, 2003). Tannins are large polyphenols (MW: 500-20,000 A.M.U) and are thus potentially important biological antioxidants in this herb (Hagerman *et al.* 1998).

Figure 2.4 Thyme dried leaves

This picture was captured using a Zeiss Stemi 2000C stereomicroscope (X 10). The picture compares well with the description provided by the supplier: "Rough angular pieces with green and brown leaf", although there is evidence of flowering tops and twigs in the sample which was not mentioned in the Organic Herb Trading Company OHTC product specification sheet. The presence of twigs could explain why some studies have found traces of tannins in thyme samples (Williamson, 2003).

In summary, four commonly used culinary herbs, parsley, rosemary, sage and thyme were selected in a dried form and the batches used were certified safe to use by the MHRA. Herbs were purchased from a reputable source where they could be traced; these were processed, stored and packaged well and had low microbiological contamination. Although all the herbs selected are established as containing high levels of polyphenols and high antioxidant activity this study focussed further on their role as dietary sources of polyphenols, specifically with regards to their antioxidant properties.

Chapter 3. The impact of cooking on the antioxidant activity of parsley, rosemary, sage and thyme.

3.0 Introduction

Parsley, rosemary, sage and thyme are known to possess high antioxidant activity (AA) (Zeng and Wang, 2001; Williamson, 2003; Matsingou *et al.*, 2003; Dragland *et al.*, 2003, Shan *et al.*, 2006 and 2007; Craig, 1999; Pietta, 1999, Zeng and Wang, 2001; Kris-Etherton *et al.*, 2002; Williamson, 2003; Proestos *et al.*, 2005). Several studies on culinary herbs that have assessed their AA concluded that the main contributors of this AA are the polyphenols (Zeng and Wang, 2001; Pellegrini *et al.*, 2003; Shan *et al.*, 2005). However, most studies on the AA of culinary herbs have focussed on the uncooked form; however, although many herbs undergo cooking prior to consumption, there is a paucity of data on the impact of cooking on their AA.

Some studies have looked at the effects of cooking on the antioxidant capacity/polyphenolic content of non herb plant foods. Miglio *et al.* (2008) looked at carrots, courgettes and broccoli individually cooked for lengths of times that were determined to meet specified conditions of tenderness, palatability and taste (cooking times were not specified). The vegetables were boiled, steamed, or fried with peanut oil at 170°C. The study showed a consistent increase in antioxidant capacity of all cooked carrots, courgettes and broccoli compared to their uncooked counterparts. Kim *et al.* (2005) observed an increase in the antioxidant capacity of heated grape seed extracts (roasted at 50, 100, 150 and 200 ° C) compared to non-heated grape seed extract. In addition, Choi *et al.* (2005) studied the effects of heat on Shiitaki mushrooms (which were heated for 15 and 30 minutes at 100°C and 121° C) and reported an increase in antioxidant capacity which appeared to be due to an increase in polyphenolic content. However, in contrast, Mulinacci *et al.*, (2009) showed that heat treatment on potatoes (boiling) for up to 2 hours did not change their polyphenol, specifically phenolic acid, content. These studies indicate that cooking techniques involving heat increase the antioxidant capacity of some plant derived foods and this increase may be due to an increase in polyphenolic content although the latter has yet to be fully established. With regard to the impact of cooking on culinary herbs, a preliminary study by Chohan *et a.* (2008) reported that microwaving (10min), simmering (30min), stewing (1 hour) significantly increased the herbs' polyphenolic antioxidant capacity. However,

grilling sage (5 min) and frying rosemary (5 min) resulted in a decrease in this capacity. These results suggest that the type of cooking technique used influences antioxidant capacity, but how is not entirely clear, and the one measure that might have shed some light on how, phenolic content, was not determined. Thus, further investigation of the effect of cooking was required

3.1 Methods used to quantify antioxidant activity.

Several methods are available to determine the AA of food stuff. The most commonly used are the hydrogen atom transfer assays (HAT), that measure the ability of an antioxidant to quench free radicals by hydrogen donation, and single electron transfer assays (SET) that detect the ability of potential antioxidants to transfer one electron to not only reduce radicals but also metals and carbonyls. HAT assays include the oxygen radical absorption capacity assay (ORAC) and the total radical-trapping antioxidant potential assay (TRAP). SET assays assume that the antioxidant capacity of a compound or food is equal to their reducing capacity and these include the Trolox equivalent antioxidant capacity (TEAC) assay, the total phenolic content assay (TPC) (Singleton, 1974), also known as the gallic acid equivalent assay (GAE), the ferric reducing antioxidant power assay (FRAP), and the 2,2-diphenil-1-picrylhydrazyl (DPPH) assay. Prior *et al.* (2005) reviewed these methods as well as the copper reduction assay (CUPRAC), the total oxidant scavenging capacity (TOSC), both SET assays, LDL oxidation (HAT) and photochemiluminescence (PHOTOCHEM) which is based on the photochemical generation of superoxide free radicals combined with a chemically induced light detector. All these assays have advantages and disadvantages based on biological relevance, ease of use, speed of assay, and instrumentation requirement. From their review Prior *et al.* (2005) proposed that the ORAC, TEAC and the TPC assays are adequate candidates to be used to determine AA and should be considered for the standardizations of AA assays for foods to enable comparison between studies. In the current study, TEAC and the TPC assay were used to quantify the AA of a selection of herbs. The TEAC assay, also called the $ABTS^{\bullet+}$ assay, is based on the ability of the antioxidant to scavenge the blue-green coloured $ABTS^{\bullet+}$ radical cation relative to the scavenging ability of Trolox, the water soluble alpha-tocopherol (vitamin E) analogue. Upon addition of a sample, for example a food sample, the amount of $ABTS^{\bullet+}$ scavenged results in a loss of chromogenic activity, and this loss of colour can be measured spectrophotometrically and compared to Trolox, giving a Trolox equivalent

antioxidant capacity (TEAC). The TEAC assay is simple to set up, fast and has been shown to be adequate in measuring the AA of a range of foods and beverages; it has been used widely in other studies (Mantle *et al.*, 2000 ; Shan *et al.*, 2006 and 2007; Pellegrini *et al.*, 2006) and therefore enables comparison with published work. The TPC assay uses the Folin- Ciocalteu (FC) reagent. Phenolic compounds react with this reagent at pH 10, to generate a blue product, which can be measured spectrophotometrically and compared to gallic acid used as a standard, giving a gallic acid equivalent (GAE). It is a widely used method to measure the AA of foods due to it being simple, speedy, cheap and robust and enables comparison with other studies (Prior *et al.*, 2005).

Culinary herbs, as stated above, are normally cooked prior to consumption. The most common cooking techniques are steaming, stewing, microwaving, stir-frying and grilling. Thus, the aim of this investigation was to determine the impact of such cooking techniques on the AA of quantities of parsley, rosemary, sage and thyme, likely to be used in a domestic kitchen, using the TEAC and GAE assays. This investigation was carried out to shed some light on the potential of these culinary herbs as dietary sources of polyphenols once cooked.

3.2 Materials and Methods

3.2.1Materials

Reagents

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) in diamonium salt form, (ABTS), Folin-Ciocalteu's phenol reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, 97% (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), ethanol 99.6%, potassium persulphate ($K_2S_2O_8$), were all purchased from Sigma Aldrich, Poole, UK.

Culinary herbs

Parsley, rosemary, sage and thyme were selected and purchased from "Neal's Yard Remedies" Richmond, Surrey, UK, as stated in Chapter 2.

3.2.2 Methods of herb preparation

The amount of herbs added in cooking is subjective and varies between the herbs, individuals, recipies, and the quantity of food prepared. For the purpose of this

study 1g of each dried herb were used for each cooking technique, repeated in triplicate experiments.

Preparation of cold extractions

1 g of each dried herb was lightly crushed using a pestle and mortar with distilled water (25ml). Extracts were then covered with a lid in a dark glass bottle (to protect the antioxidants from light oxidation) and left to infuse for 10 minutes.

Preparation of hot extractions

Distilled water (25ml) was boiled, added to each dried herb (1g) (in a glass bottle). The mixture was stirred gently, covered with a lid in a dark glass bottle and left to infuse for 10 minutes.

Preparation of microwaved herb extracts ('soup')

Culinary herbs are often added to soup recipes; therefore the production of "soup" equivalent to one portion (200ml) of a can of soup (Crawley, 1988) was simulated under laboratory conditions by microwaving the herbs in water (using a standard kitchen microwave). Herbs (1g) were added to distilled water (200ml) in a Pyrex beaker. The solutions were covered with cling film and microwaved (Panasonic, 1000 W) at the highest level for 4 minutes. The total remaining solution was cooled quickly in an ice bath.

Preparation of stewed herb extracts

Casseroles and stews take an average of 1 to 2 hours on a low heat at 180° C/mark 4 in an oven, whilst the average time for hob stewing is between 35-90 minutes on low heat (Good House Keeping Cook Book).Therefore the production of a "stew" was simulated under laboratory conditions by stewing herbs in water (one portion 200ml), on a hot plate for various lengths of time (30, 60 and 120 min). Each herb (1g) was placed in distilled water (200ml) in a Pyrex beaker covered with foil, put on a hot plate (Stuart CB162, Sterilin Ltd, UK), boiled for 10 minutes (300˚C), covered and left to stew for 30, 60, and 120 minutes (at 50˚C).

Preparation of herbs heated in a Teflon® stir frying pan.

Stir frying takes an average of 10 minutes in a wok or a Teflon® pan in little or no oil. It is a very popular and modern way of cooking as it is quick. Thus stir frying was simulated under laboratory conditions by heating the herbs (dry) in Teflon® stir frying pan for various lengths of time (5 to 20 minutes) to compare the effects of heat over time on the antioxidant capacity of culinary herbs.

Herbs (1g) were added to a Teflon® stir frying pan without oil as vegetable oil is known to possess antioxidant activity (Pellegrini *et al.*, 2003). The pan was placed on a hot plate (300˚C) for 5, 10, 15 and 20 minutes, the herbs were stirred regularly, and then were transferred to a beaker. Boiled water (25ml) was added to the beaker that contained the herb and the mix was stirred gently. The mix was then transferred to a dark glass bottle where it was left to infuse for 10 minutes.

Following the initial method of preparation, hot solutions were left to cool. The entire preparation was then drained using a conical flask, a funnel and a Whatman filter #1. Extracts were filter-sterilised using a 0.22µm filter membrane, aliquoted into micro-centrifuge tubes, labelled and frozen at -80 º C until needed.

3.3 Evaluation of evaporation rates.

Cold, hot and heated preparations were considered to have negligible evaporation rates, and calculations of antioxidant activity were performed with the initial volume. Evaporation rates were calculated for stewing (see appendix 1 for details on evaporation rates for stewing) and microwaving (9%) and were performed by running triplicate blank experiments (water only) and then subtracting the final weight of water after cooking from the initial weight of water before cooking. The values obtained were used to adjust the volumes used for the calculation of the AA assays.

3.4 Determination of antioxidant activities of herb extracts.

3.4.1 Trolox equivalent antioxidant capacity assay (TEAC)

The TEAC/ABTS•+ assay was adapted from the method by Re *et al.* (1999). Briefly, ABTS (7 mM) was dissolved in 10 ml of phosphate buffer saline (PBS). Potassium persulphate (24.5 mM) was dissolved in 10 ml PBS. The addition of 9 ml ABTS stock to 1 ml of potassium persulphate stock solution resulted in a 10 ml dark green solution which was kept in foil in a dark cupboard at room temperature for at least 18 hours. The radical obtained ($ABTS^{\bullet+}$) was stable when stored in the dark for over 48 hours and the colour was measured, as an absorbance reading, at 734 nm. The absorbance of $ABTS^{\bullet+}$ was checked; the optimum working absorbance at 734nm of 0.70 (+/- 0.2) was obtained generally at a ratio of 10µl $ABTS^{\bullet+}$ to 990µl PBS. Absorbance was obtained using the Beer-Lambert's equation $A = \varepsilon cl$, the extinction coefficient for ABTS was $\varepsilon = 1.5 \times 10^4\ mol^{-1}L\ cm^{-1}$ .As Trolox is not soluble in PBS, it was prepared in ethanol at a concentration of

20mM (0.05g Trolox in 10ml ethanol). This solution was then stored in 0.5ml aliquots at -80° C until needed. For each standard curve, an aliquot of Trolox solution (0.5ml) was diluted with PBS up to 5ml to obtain a final concentration of 2mM. A new aliquot of Trolox was used each time a new standard curve was set up. Extracts were diluted appropriately and absorbance values were read after 60 seconds. Trolox was used as a standard (0-20µM) to draw a standard curve (see Figure A1.2a in Appendix 1 for assays standard curve). The assay's repeatability was very good, r = 0.987, a percentage inhibition curve was drawn with an equally good correlation coefficient, r = 0.983 (Please see Figure A1.2b in Appendix 1 for percentage inhibition curve).

3.4.2 Total phenolic content/gallic acid equivalent assay (GAE)

Extracts diluted in distilled water (100µl) were plated in 12 well Nunc plates in triplicates, after which 200 µl Folin Ciocalteu reagent was added, followed by 2 ml distilled water and finally 1 ml of Na_2CO_3 (15%) . Solutions were then incubated for 2 hours at 25°C and absorbance read on a Cary Microplate reader (Varian, UK) at 765 nm. Concentrations of gallic acid solution were prepared, ranging from 0.05mg/ml to 0.5mg/ml, and a standard curve was generated for each assay in order to determine the gallic acid equivalents in mg/ml. Each herb extract was diluted appropriately. The assay's repeatability was very good, r = 0.993 (please see Figure A1.3 in Appendix 1 for standard curve).

3.5 Expression of data and statistical analysis

Extracts were prepared for each herb in triplicate (n=3). Each extract was tested three times to ensure the precision of the measurements. Data are presented as the mean of the samples taken from each extract, unless otherwise stated, in µmoles of Trolox Equivalent Antioxidant Capacity (TEAC) per gram of herb and in mg Gallic acid equivalent (GAE) per gram of herb. Statistical analysis was done using SPSS for windows. Independent sample T tests were used to compare hot and cold extracts. ANOVA was used to compare herbs stir fried for 5, 10, 15 and 20 minutes and herbs stewed for 30, 60 and 90 minutes. Post hoc tests (Tukey) were carried out to determine where the significant differences lay. P values equal to or less than 0.05 indicate statistically significant differences between samples tested. Correlation coefficients were calculated using Pearson's correlation coefficient analysis for both standard and inhibition response curves, as well as for

correlations between TEAC and GAE assays (** statistically significant at 0.001 level, 2-tailed).

3.6 Results

3.6.1 TEAC and GAE for cold and hot control of parsley, rosemary, sage and thyme

Hot extracts of parsley and sage had a significantly higher AA than their cold extract counterparts (Table 4, $p<0.001$ for both assays) and rosemary cold extract was significantly higher than its hot extract counterpart ($p<0.001$ for both assays). There were no statistical differences between the antioxidant activities of cold and hot extracts of thyme: TEAC: $p=0.964$, and GAE: $p=0.212$ (Table 3.1).

3.6.2 TEAC and GAE for microwaved, parsley, rosemary, sage and thyme.

All microwaved extracts had significantly higher AA than cold and hot extracts ($p \leq 0.005$; Table 3.1). The percentage of water loss to evaporation during microwaving was determined: 9 ±1 % (n=3), this was taken into consideration in the calculations.

Table 3.1. TEAC and GAE for cold and hot control and standardised parsley, rosemary, sage and thyme.

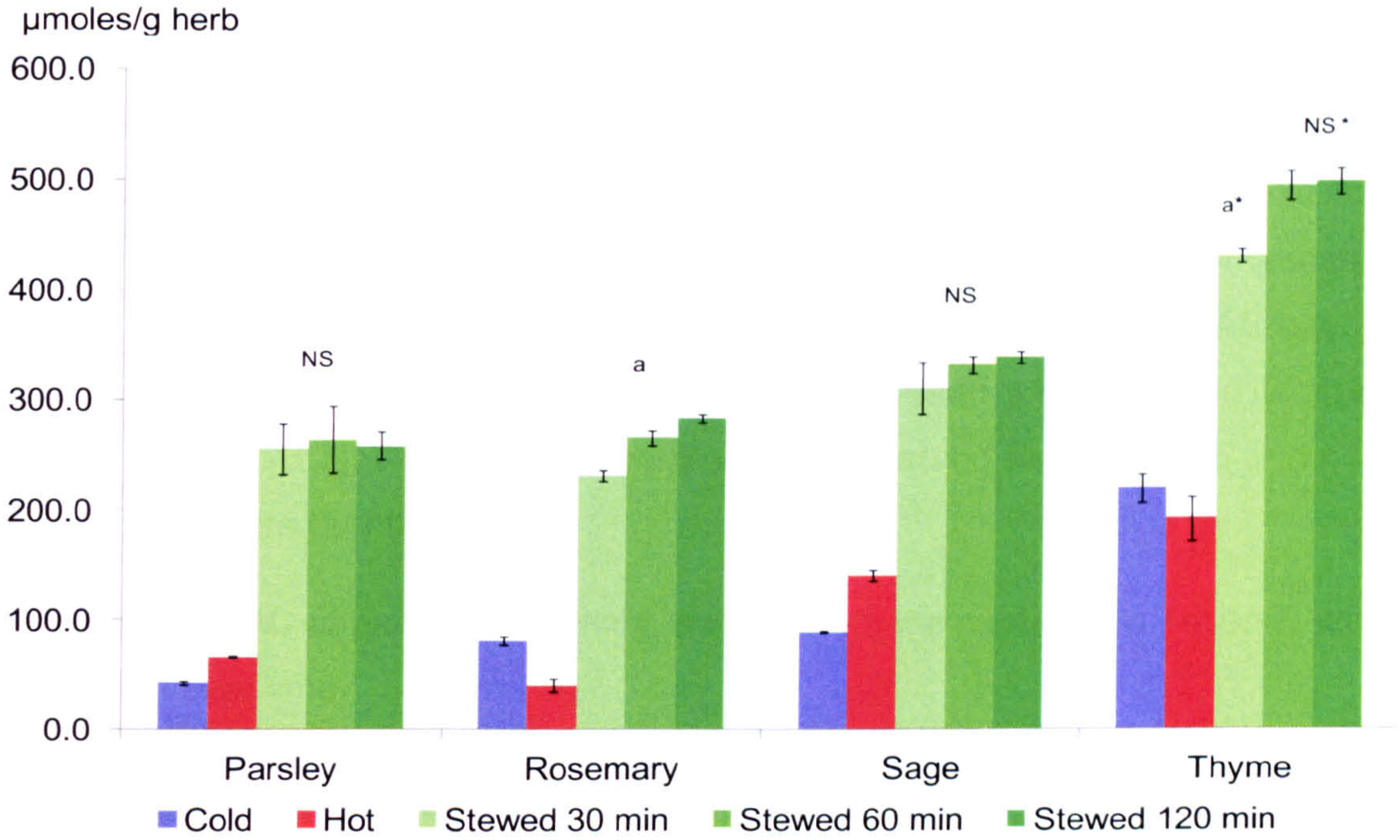
Samples	TEAC (µmoles/g)	GAE (mgGAE/g)
Cold extract		
Parsley	42.3±1.52	12.12±0.17
Rosemary	82±3.6	16.6±0.17
Sage	89.3±10.4	18± 0.35
Thyme	220±12.6	32.5±0.7
Hot extract		
Parsley	66±0.73[a]	15.5±0.35[a]
Rosemary	40.6±6.2[a]	1.82±0.03[a]
Sage	140.3±5[a]	22±0.02[a]
Thyme	192±20.1[c]	31±0.35[d]
Microwaved extracts		
Parsley	126±4.8	19.8±1.8
Rosemary	98.3±5.7	8.9±0.3
Sage	209.3±7.3	67.7± 0.1
Thyme	357.6±24.3	76.7±0.1

Data are presented as mean ±SD (n=3). Independent T tests between cold and hot extract: [a] P≤0.001, [b] p=0.964, [c]p=0.212. ANOVA between microwaved, hot and cold extracts all: P ≤0.002.

3.6.3 TEAC and GAE of parsley, rosemary, sage and thyme stewed over time.

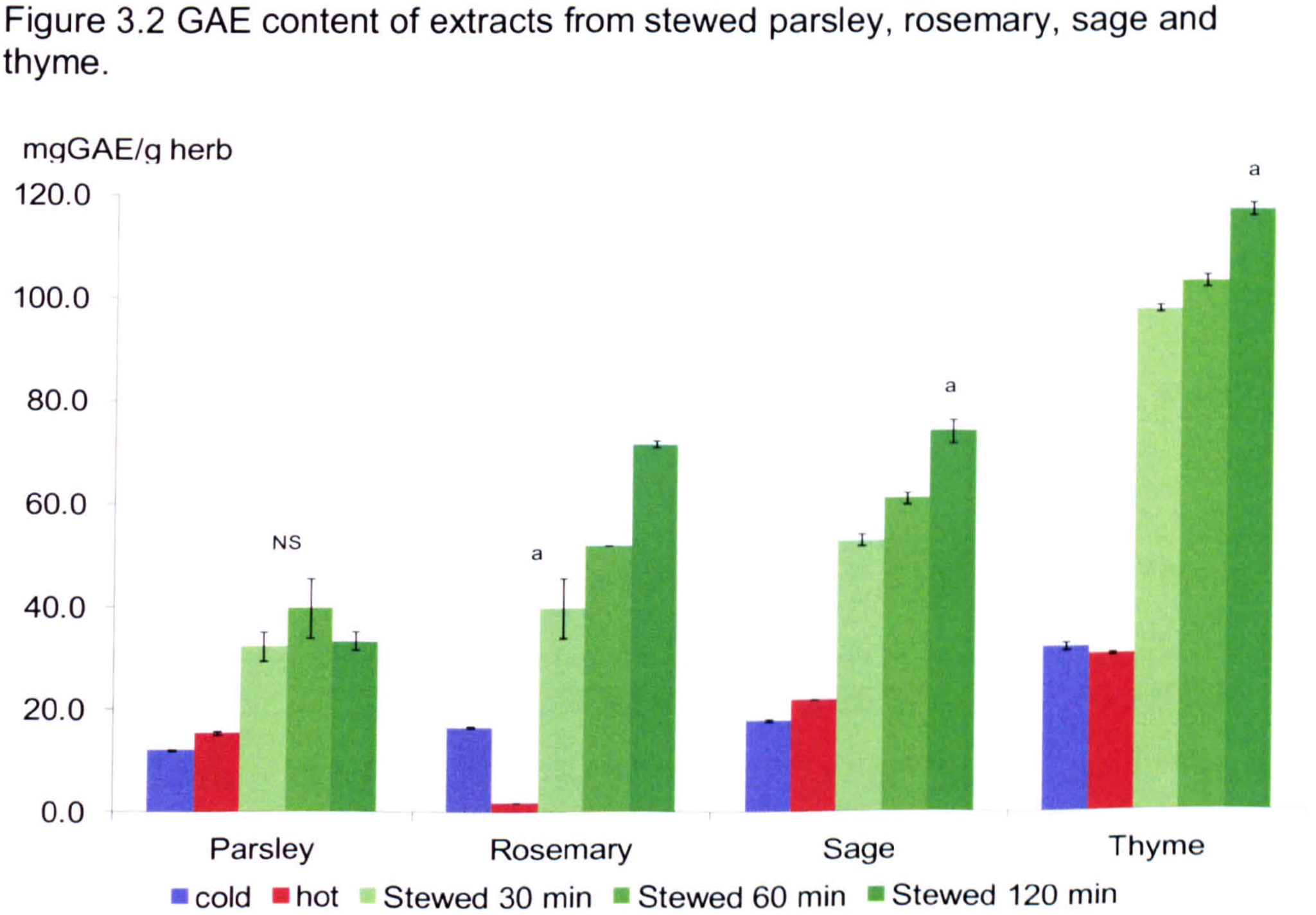
The water losses over the cooking time were determined as a percentage evaporation loss of 13-22% (n=3); this was taken into consideration when calculating values. Results were significantly different than both cold and hot extracts (all p≤ 0.003 for both assays). Rosemary and thyme GAE and TEAC were significantly increased over time of stewing ($p<0.001$). Sage TEAC increased up to 60 minutes stewing and then reached a plateau. Parsley's TEAC and GAE increased slightly from 30 to 60 minutes stewing and then decreased; however, there were no significant differences between TEAC for parsley (p=0.916), and sage (p=0.165) and GAE for parsley (p=0.065) at the three time points (Figures 3.1 and 3.2).

Figure 3 1 TEAC content of extracts from stewed parsley, rosemary, sage and thyme.



Data are presented as mean ±SD (n=3). NS: Non significant differences between stewed times (ANOVA), a, P≤0.001 between cooking times, a* P≤0.001 between 30 min and 60 as well as 120 minutes, NS*: Non significant difference between 60 and 120 min.

Figure 3.2 GAE content of extracts from stewed parsley, rosemary, sage and thyme.



Data are presented as mean ±SD (n=3). NS: Non significant differences between stewed times (ANOVA), [a] P≤0.001 between the three cooking times.

3.6.4 TEAC and GAE of parsley, rosemary, sage and thyme heated in a Teflon ® stir frying pan over time

Results were significantly different than both cold and hot extracts except TEAC for rosemary heated 5 minutes and rosemary hot extract: p=0.077 and TEAC for rosemary heated for 15 minutes and rosemary cold extract: p=0.066 (Table 3.2). There were no significant differences for herbs heated for 5 and 10 minutes in either assay but there were significant increases in both TEAC and GAE beyond 10 minutes heating for all herbs over time, with a mean 1.8 ± 0.3 fold increase (Tables 3.2 and 3.3). A degree of browning was observed for each herb and none of the herbs were burnt.

Table 3.2. TEAC of parsley, rosemary, sage and thyme heated in a Teflon ® stir frying pan over time.

µmoles TEAC/ g herb	Parsley	Rosemary	Sage	Thyme
Heated 5 min	139±5	47*±5.8	239±6.4	303±16.8
Heated 10 min	126^{NS}±3.6	49.1^{NS}±4.3	207.5^{NS}±11.2	307^{NS}±6.6
Heated 15 min	202±0.95	73**±6.7	314.3±4	370±15.5
Heated 20 min	273±1	110.6±2.8	370.6±15.5	403.6±28.5

Data are presented as mean ±SD (n=3). NS: no significant differences between herbs heated for 5 min and 10 min. All heated extracts:p≤ 0.033 when compared to cold and hot extracts, except rosemary 5 min and rosemary hot extract: *p=0.077, and rosemary 15 min and rosemary cold extract:** p=0.066.

Table 3.3 GAE of parsley, rosemary, sage and thyme heated in a Teflon ® stir frying pan over time.

mg GAE/ g herb	Parsley	Rosemary	Sage	Thyme
Heated 5 min	11.3±0.1	5.75±1.06	26.87±0.44	45.2±0.35
Heated 10 min	11.55^{NS}±0.17	6.06^{NS}±0.08	27.87^{NS}±0.88	54.37^{NS}±0.88
Heated 15 min	16.37±0.7	7.08±0.05	31.6±0.53	70.25±0.35
Heated 20 min	24.48±1.19	7.4±0.44	53.37±4.06	81.5±1.06

Data are presented as mean ±SD (n=3). NS: no significant differences between herbs heated for 5 min and 10 min. All p≤ 0.016 when compared to cold and hot extracts.

3.6.5 Correlation between TEAC and GAE

There were good correlations between TEAC and GAE values for cold, hot, microwaved, stewed and stir fired herb extracts of parlsey, rosemary, sage and thyme (Table 3.4).

Table 3.4. TEAC versus GAE correlation data.

Extracts	Correlation coefficients
Hot extracts	0.938
Cold extracts	0.977
Microwaved extracts	0.916
Stewed extracts	0.932
Stir fried extracts	0.920

All statistically significant at 0.001 level (2-tailed).

3.7 Discussion

This study was designed to provide an insight into how cooking affects the polyphenolic antioxidant activity of culinary herbs in order to understand the potential of cooked culinary herbs as dietary sources of polyphenols.

There was a good repeatability for each assay. The level of association between TEAC and GAE agreed well with those reported in the literature (Zeng and Wang, 2001; Pellegrini *et al.*, 2003; Shan *et al.*, 2005).The high correlation between the TEAC and the GAE for all cold, hot, microwaved, stewed and stir fried herbs is expected as the chemistry behind the TEAC and the GAE assay is based on similar redox reactions via electron donation (Huang *et al.* 2005). As discussed in Chapter 1 and 2 this AA is thought to be mainly attributed to the polyphenols present in the culinary herbs (Rice-Evans *et al.*, 1997; Lu and Foo, 2000; Zheng and Wang, 2001; Halvorsen *et al.*, 2002; Dragland *et al.*, 2003; Pellegrini *et al.*, 2003; Matsingou *et al.*, 2003; Javanmardi *et al.*, 2003; Wu *et al.*, 2004; Shan *et al.*, 2006; Tapsell *et al.*, 2006).

There were variations in AA between herbs and cooking methods. The ranking for both assays for standardised, cold, and stewed herbs in descending order was thyme, sage, rosemary, parsley; for microwaved, hot and stir fired herbs the ranking in descending order was thyme, sage, parsley, rosemary. These rankings correlate quite well with previous studies with a degree of variation that is to be

expected in natural products and with different extraction techniques for uncooked herbs. Mantle *et al.* (2000) ranked the following herbs in descending order: thyme, sage, rosemary. Dragland *et al.* (2003) ranked the following herbs in descending order: rosemary, thyme, parsley. Shan *et al.* (2007) ranked the following herbs in descending order: sage, rosemary, thyme, parsley. Chohan *et al.* (2008) ranked the following herbs in descending order: sage, thyme rosemary, and parsley. Carlsen *et al.* (2010) ranked the following herbs in descending order: thyme, rosemary, sage.

The differences in AA encountered with cold and hot extractions methods for each herb indicate that the domestic processing of herbs can alter their AA, as was shown by Chohan *et al.* (2008). The current study also showed that different herbs react differently to the same processing technique; it was noticed that the thyme leaves, and more markedly the rosemary leaves, were coarse and hard, while parsley and sage had much softer leaves. For the herbs with softer leaves, an increase in heat caused an increase in AA which agrees with the literature (Kim *et al.*,2005; Choi *et al.*, 2005; Chohan *et al.*, 2008). Polyphenols are thought to be most concentrated in the vacuoles of the paraveinal and parenchymal cells below the epidermis (Raven, 1998). If these cells become more exposed after the softening effects of hot water, the release of the polyphenols is higher and hence an increase in AA of the extracts. It is possible that due to its coarseness the hot water had less of a softening effect on the hard rosemary leaves, hence the low AA and total phenolic content observed. However, the light crushing used for the cold extraction method may have enabled a greater release of polyphenols, and hence a higher AA, compared to their release following hot water extraction. There were no significant differences in AA for cold and hot thyme extracts. It may be that the light crushing involved in the cold extraction method may have had an effect equal to the softening impact of the hot water extraction method in liberating polyphenolic compounds from thyme leaves.

3.7.3 TEAC and GAE for microwaved, parsley, rosemary, sage and thyme.

The antioxidant capacity and total phenolic content of microwaved extracts were significantly higher than those of both hot and cold extracts for all herbs studied. This observation agrees with those of Chohan *et al.* (2008) who showed a significant increase in TEAC for parsley and thyme microwaved against controls (hot extracts). These results also correlated well with Miglio *et al.* (2008) who

looked at the effects of cooking on the physiochemical characteristics and antioxidant capacity of vegetables, and suggested that the increase in AA observed after boiling, steaming and frying was due to the softening of the plant matrix, which ultimately caused an increase in the release of polyphenols.The increased liberation of polyphenols due to the softening of the plant cell wall as a consequence of exposure to heat during microwave cooking seem a logical explanation for the effects observed in the present study.

3.7.4 TEAC and GAE of parsley, rosemary, sage and thyme stewed over time.

Results of stewed herbs were significantly higher than both cold and hot extracts, and showed that for rosemary and thyme an increase in processing time correlated with a linear increase in AA. There was no more increase in AA for parsley and sage beyond 60 minutes of stewing and there was a decrease in AA from 60 minutes for parsley. It is not known whether these trends would continue or not but it ought to be noted that the aim of this experiment was to mimic an average domestic stewing time, which is approximately 30 to 120 minutes on a hob. The increase in antioxidant activity observed in this study upon stewing rosemary and thyme over time is in agreement with the literature (Miglio *et al.*, 2008; Chohan *et al.*, 2008). The AA plateau effect observed for the softer leaves of parsley and sage beyond 60 minutes could be explained by the breakdown of their leaves through cooking triggering the leaching out of all the available polyphenols in these herbs into the surrounding cooking water. The cooking water of the stew containing all these food matrix-free polyphenols is likely to be ingested directly or via the absorption of water by other food stuff during domestic cooking.

3.7.5 TEAC and GAE of parsley, rosemary, sage and thyme heated in a Teflon ® stir frying pan over time.

The results for herbs heated dry in a Teflon® frying pan were significantly different from both cold and hot extracts, and showed an increase in TEAC and GAE values that is directly proportional to the time of exposure; there was a 1.8 fold average increase in AA at 20 minutes compared to 5 minutes. Rosemary heated for 5 minutes and rosemary hot extracts, which gave similar TEAC values, suggesting that heating the coarse leaves of rosemary in a Teflon pan for less than 10 minutes makes little difference in the amount of TEAC made available, although this was not mirrored by their GAE values as these, (whilst similar) remained statistically different. Heating rosemary for 15 minutes was statistically

similar to the TEAC of rosemary cold extract, suggesting than the effect of light crushing used in the cold extraction method had a similar impact on the TEAC of rosemary as heating in a Teflon pan for 15 minutes. In addition, the herbs underwent some degree of browning. These results differ from those of Chohan *et al.* (2008) in which herbs that were heated in a non- Teflon® pan, were heated until burnt and the AA was reduced greatly. However, the Teflon® coating of the stir frying pan used in the current study protected the herbs from burning. These results correlated well with Choi *et al.* (2005), who observed an increased antioxidant capacity for Shiitake mushrooms under the influence of heat, and suggested that this may be due to both the breakdown of the insoluble section of the mushrooms' cell walls and also may be due to the Maillard reaction. The Maillard reaction is a series of chemical reactions initiated by amino acids and reducing sugars, and results in the formation of a range of products, known as Maillard reaction products (MRPs) that influence the taste, aroma and colour of food. The MRPs that are responsible for browning are the melanoidins and these are reported to possess antioxidant capacity (Kim *et al.*, 2005; Tuohy *et al.*, 2006). In the current study, as with the impact of the other heating techniques, the liberation of polyphenols by the breakdown of the plant cell wall as a consequence of exposure to heat remains a plausible explanation. However, the Maillard reaction, specifically the melanoidins, could have also contributed to the increase in AA observed. It is not known whether this increase in AA would have continued beyond 20 minutes. However, the average time for stir frying is approximately 20 minutes; thus, from a cooking perspective, stir-frying for longer would not have provided additional insight and may have resulted in burning.

3.8 Conclusions

This study indicates that culinary herbs parsley, rosemary, sage and thyme possess AA which is thought to be attributed to their polyphenol content. Both the cooking method and duration of cooking had an impact on the AA. The physical softening/breakdown of the plant cell wall as a consequence of exposure to heat or mechanical crushing, appear to be a logical explanation for the AA increases. However, the physiological characteristics of the plant leaves may also be a factor in the liberation of polyphenols. The production of antioxidant metabolites as a result of the Maillard reaction is also a plausible explanation for the increase AA of herbs heated dry in a Teflon® frying pan. Cooking appears to have a major effect on the availability of polyphenol antioxidants in herbs. However, to fully establish

the significance of these observations, the impact of digestion and absorption on these antioxidants needs to be investigated.

Chapter 4. An investigation on the antioxidant activity of combined herbs.

4.0 Introduction

Although a number of studies have investigated the antioxidant properties of culinary herbs individually (Zeng and Wang, 2001; Pellegrini *et al.*, 2003; Shan *et al.*, 2006; Chohan *et al.*, 2008), it is not uncommon for these herbs to be used combined for the purpose of cooking. A question that has yet to be addressed, therefore, is whether these herbs, when combined, have an additive or synergistic effect on antioxidant activity.

The synergistic properties of bioactive compounds, including polyphenols, in plant-derived foods is not a novel concept. So *et al.* (1996) demonstrated that flavonoids tested in pairs (genistein, baicalein, hesperetin, narengenin and quercetin) synergistically inhibited the growth of MDA-MB 435 breast cancer cells. Furthermore, in a review on synergies and interactions in phytomedicine, Williamson (2001) discussed reported evidence of synergy within single herbs (willow bark, hypericum, ginger) explaining that synergy can occur in a single plant that is more than the sum of its parts, that is that synergy exists through the interaction of a plant's constituents. For example, the essential oils found in sage may interact with other compounds present in the plant, including polyphenols, resulting in increases in antioxidant activity and cholinesterase inhibitions (Perry *et al.*, 2000).

Williamson (2001) also discussed a more classical interpretation of synergy; that synergy can occur in a combination of plants that are significantly more than the sum of either alone (examples include nettles and pygeum bark, valerian and kavakava, ginseng and ginkgo). The synergies in these combinations are thought to be due to the interactions between their bioactive constituents (Berenbaum, 1989). In light of this evidence Williamson (2001) suggested that further testing is required to prove the "synergistic phenomenon" in order to use these herbs combined as therapeutic tools, and additional studies have been carried out.

Peyrat-Maillard *et al.* (2003) investigated the antioxidant activity of polyphenolic compounds combined in pairs, including the phenolic acids *p*-coumaric acid, ferulic acid, caffeic acid, rosmarinic acid, chlorogenic acid, the flavonoids (+)-catechin, (-)-epichatechin, (-)-epigallocatechin, quercetin, rutin as well as α-

tocopherol using an induced oxidation method. The authors found synergistic antioxidant effects between rosmarinic acid and quercetin, and between rosmarinic acid and caffeic acid, and found antagonistic effects between (+)-catechin and caffeic acid, between caffeic acid and quercetin, between α-tocopherol and rosmarinic acid, and between α-tocopherol and caffeic acid. Thus this study highlighted that additive and synergistic effects cannot be assumed when polyphenols are combined.

More recently, Canene-Adams *et al.* (2007) reported synergetic effects for broccoli and tomato in relation to anti-tumour activity on R3327-H prostate adenocarcinomas, and phytochemicals, including polyphenols, are suggested to be one of the group of bioactive compounds that may be responsible for this effect. Most studies have taken a reductive approach and focussed on isolated compounds, yet it is the plant derived foods that are the major dietary contributors of the polyphenols. Furthermore, and as stated above, little is known about a possible synergy occurring between polyphenols in culinary herbs. Therefore to provide further insight into their potential to contribute to dietary polyphenols (and to identify factors that impact this potential) the potential synergetic effects in antioxidant activity of hot and cold extracts of parsley, rosemary, sage and thyme used in combination was investigated.

4.1 Material and methods

4.1.1Material

Reagents

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulphonic acid) in diamonium salt form, (ABTS), Folin-Ciocalteu's phenol reagent, Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, 97% (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), potassium persulphate ($K_2S_2O_8$), all purchased from Sigma Aldrich, Poole, UK.

Culinary herbs

The culinary herbs parsley, rosemary, sage and thyme were selected as stated in Chapter 2, were and purchased from "Neal's Yard remedies" Richmond, Surrey, UK.

4.1.2 Methods

Single herb extractions

Cold and hot extractions of single herbs were prepared as described in Chapter 3, (section 3.2.2).

Combined herbs extractions

The methods used for extracting the combined herbs in hot and cold water were designed to keep the ratio of 1g herb to 25ml water, as used in Chapter 3, (section 3.2.2). The combinations are listed and described below.

Parsley (1g) + Rosemary (1g) + 50 ml distilled water (PR)

Parsley (1g) + Sage (1g) + 50 ml distilled water (PS)

Parsley (1g) + Thyme (1g) + 50 ml distilled water (PT)

Rosemary (1g) + Sage (1g) + 50 ml distilled water (RS)

Rosemary (1g) + Thyme (1g) + 50 ml distilled water (RT)

Sage (1g) +Thyme (1g) +50 ml distilled water (ST)

Parsley (1g) + Rosemary (1g) + Sage (1g) + 75 ml distilled water (PRS)

Parsley (1g) + Rosemary (1g) + Thyme (1g) + 75 ml distilled water (PRT)

Rosemary (1g) + Sage (1g) +Thyme (1g) + 75 ml distilled water (RST)

Parsley (1g) + Rosemary (1g) + Sage (1g) +Thyme (1g) + 100 ml distilled water (PRST)

The combination PST was omitted. All herb extracts were drained using a conical flask, a funnel and a Whatman filter #1. Extracts were then filter sterilised using a 0.22µm filter membrane, aliquoted into micro-centrifuge tubes, labelled and frozen at -80 ° C until needed.

4.2 Determination of antioxidant activity of herb extracts.

Trolox equivalent antioxidant capacity assay (TEAC) and Total phenolic content/ gallic acid equivalent assay (GAE) were performed as described in chapter 3, (section 3.4.1 and 3.4.2 respectively).

4.2.1 Expression of data

Data are presented as the mean ± SD of triplicate samples of triplicate preparations (preparations A, B and C; n=3) in µmoles of Trolox Equivalent Antioxidant Capacity (TEAC) per gram of herb for antioxidant capacity assay, and in mg Gallic acid equivalent (GAE) per gram of herb for total phenolic content assay. TEAC and GAE values obtained from the extracts of individual herbs were added up and the sum was then divided by the total amount (g) of herbs. For example [parsley (TEAC/GAE) g + Rosemary (TEAC/GAE)g] /2. These values were them termed 'mean expected values'. The TEAC and GAE values of the combined herbs (g) were termed the 'mean combined value'. Statistical analyses were performed to determine whether the expected values were statistically different from the combined values. Since the single herbs and combined herbs were extracted and then tested for TEAC and GAE on three different occasions (A, B and C) the analysis was performed as follows for each occasion: [Combined value herbs (TEAC/GAE) g] – [expected value herbs (TEAC/GAE) g]. The obtained values were entered in SPSS 17 for windows and data were checked for normal distribution. A 1-sample t test was carried out, using 0 as the expected value (that is, 0 represents an additive, and thus no synergistic, effect). P values ≤ 0.05 indicated that there was a significant difference between samples, showing either a significant synergistic effect (combined values significantly higher than expected values) or significant antagonistic effect (combined values significantly lower than expected values). To quantify the level of synergy or antagonism, the percentage increase/decrease of the mean combined value compared to the mean expected value was compared. The calculation is as follows: [(Mean combined value (TEAC/GAE)) ÷ mean expected value (TEAC/GAE)] x 100.

4.3 Results

4.3.1 TEAC of cold extracts of combined herb compared to expected values.

Parsley and rosemary had a mean combined value of 70.6 µmoles/g which was 114.2% of the expected value (61.8µmoles/g; Table 4.1), representing a significant synergistic effect for their antioxidant capacity (p=0.004). Parsley and sage had a mean combined value of 105.7 µmoles/g which was 161.1% of the expected value (65.6 µmoles/g), (Table 4.1) and had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined (p<0.001). The mean combined value was 61.1% higher than the mean expected value.

Parsley and thyme had a mean combined value of 155.6 µmoles/g, which was 118.8% of the expected value (131.2 µmoles/g), (Table 4.1) and had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p<0.001$). The mean combined value was 18.8% higher than the mean expected value. Rosemary and sage had a mean combined value of 125.4 µmoles/g, which was 146.8% of the expected value (85.4 µmoles/g), (Table 4.1) and had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p<0.001$). The mean combined value was 46.8% higher than the mean expected value.

Rosemary and thyme had a mean combined value of 157.1 µmoles/g, which was 104% of the expected value (151µmoles/g), (Table 4.1) and had no significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p=0.067$). Sage and thyme had a mean combined value of 190% µmoles/g, which was 122.8% of the expected value (154.7 µmoles/g), (Table4.1). This combination had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p<0.001$). The mean combined value was 23% higher than the mean expected value.

Parsley, rosemary and sage had a mean combined value of 119 µmoles/g, which was 167.7% of the expected value (70.9 µmoles/g), (Table4.1). This combination had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p<0.001$). The mean combined value was 67.7% higher than the mean expected value. Parsley, rosemary and thyme had a mean combined value of 130 µmoles/g, which was 113.4% of the expected value (114.7 µmoles/g), (Table4.1). This combination had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p=0.003$). The mean combined value was 14.7% higher than the mean expected value. Rosemary, sage and thyme had a mean combined value of 166.6 µmoles/g, which was 127.7% of the expected value (130.4 µmoles/g), (Table4.1). This combination had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p=0.001$). The mean combined value as 27.7% higher than the mean expected value.

Table 4.1 TEAC of cold and hot extracts of combined herb compared to expected TEAC values.

Herb combination	µmoles/g TEAC of cold extracts				µmoles/g TEAC of hot extracts			
	Mean combined value	Mean expected value	% of expected value	statistical significance	Mean combined value	Mean expected value	% of expected value	statistical significance
PR	70.6±2.5	61.8	114.2	p=0.004	52.7±1.7	51.8	101.7	NS
PS	105.7±1.4	65.6	161.1	p<0.001	110.0±5.6	92.2	119.3	p=0.037
PT	155.8±1.7	131.2	118.8	p<0.001	146.2±6.4	141.3	103.5	NS
RS	125.4±.3	85.4	146.8	p<0.001	78.8±1.5	81.0	97.2	NS
RT	157.1±1.7	151.0	104.0	NS	156.5±3.9	130.1	120.3	p=0.014
ST	190.0±5.4	154.7	122.8	p<0.001	191.9±17.2	170.5	112.5	p=0.023
PRS	119.0±3.6	70.9	167.7	p<0.001	120.5±2.1	75.0	160.5	p=0.001
PRT	130.0±3.9	114.7	113.4	p=0.003	160.4±27.5	107.7	148.9	p=0.002
RST	166.5±2.7	130.4	127.7	p<0.001	153.8±5.5	127.2	120.9	p<0.001
PRST	124.6±1.7	108.3	115.0	p<0.001	118.7±9.8	111.2	106.7	NS

Data are presented as mean ±SD (n=3). PR: parsley + rosemary, PS: parsley +sage, PT: parsley +thyme, RS: rosemary + sage, RT: rosemary + thyme, ST: sage + thyme, PRS: parsley + rosemary + sage, PRT: parsley + rosemary + thyme, RST: rosemary + sage + thyme, PRST: parsley + rosemary + sage + thyme. The mean expected value was calculated by adding individual assay values for each herb and divided by the amount of herbs. NS Non significant ($p \geq 0.05$) % of expected values: [(Mean combined value)÷(mean expected value)]x100.

Table 4.2. GAE of cold and hot extracts of combined herb compared to expected GAE values.

Herb combination	mg GAE/g of cold extracts				mg GAE/g of hot extracts			
	Mean combined value	Mean expected value	% of expected value	statistical significance	Mean combined value	Mean expected value	% of expected value	statistical significance
PR	15.4± 3.6	16.6	93.0	NS	14.3±1.2	12.4	115.2	NS
PS	16.3±4	17.5	93.3	NS	22.7±0.1	16.6	136.3	P<0.001
PT	21.6±4.1	25.1	86.3	NS	22.4±.4	25.3	88.7	NS
RS	20.1±6	18.9	106.1	NS	16.3±0.6	15.4	106.4	NS
RT	27.3±10.5	26.5	102.8	NS	17.2±1.3	24.0	71.5	NS
ST	32.6±1	27.4	118.7	NS	29.9±0.3	28.2	106.1	NS
PRS	16.4±4.9	17.7	92.8	NS	20.4±1.7	14.8	137.9	p<0.001
PRT	22.2±5.2	22.7	97.6	NS	23.2±0.1	20.6	112.6	NS
RST	23.4±3.6	24.3	96.1	NS	23.1±.4	22.5	102.5	NS
PRST	18.3±2.5	22.0	83.0	NS	20.4±1.1	20.3	100.2	NS

Data are presented as mean ±SD (n=3). PR: parsley + rosemary, PS: parsley +sage, PT: parsley +thyme, RS: rosemary + sage, RT: rosemary + thyme, ST: sage + thyme, PRS: parsley + rosemary + sage, PRT: parsley + rosemary + thyme, RST: rosemary + sage + thyme, PRST: parsley + rosemary + sage + thyme. The mean expected value was calculated by adding individual assay values for each herb and divided by the amount of herbs. NS: Non significant ($p \geq 0.05$) . % of expected values: [(Mean combined value)÷(mean expected value)]x100.

All 4 herbs combined had a mean combined value of 124.6 µmoles/g, which was 115% of the expected value (108.3 µmoles/g), (Table4.1). This combination had a significant synergistic effect for the antioxidant capacity of cold extracts of the herbs combined ($p<0.001$). The mean combined value as 15% higher than the mean expected value.

4.3.2 TEAC of hot extracts combined herbs compared to expected TEAC values.

Parsley and rosemary had a mean combined value of 52.7 µmoles/g, which was 101.7% of the expected value (51.8 µmoles/g, Table 4.1). This combination had no significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.610$). Parsley and sage had a mean combined value of 110 µmoles/g, which was 119.3% of the expected value (92.2µmoles/g, Table 4.1). This combination had a significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.037$). The mean combined value was 19.3% higher than the mean expected value. Parsley and thyme had a mean combined value of 146.2 µmoles/g wich was 103.5% of the expected value (141.3 µmoles/g, Table 4.1). This combination had no significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.292$). Rosemary and sage had a mean combined value of 78.8 µmoles/g, which was 97.2% of the expected value (81 µmoles/g, Table 4.1). This combination had no significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.557$).

Rosemary and thyme had a mean combined value of 156.5 µmoles/g, which was 120.3% of the expected value (130.1 µmoles/g, Table 4.1). This combination had a significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.014$). The mean combined value was 20.3 % higher than the mean expected value. Sage and thyme had a mean combined value of 191.9 µmoles/g, which was 112.5% of the expected value (170.5 µmoles/g, Table 4.1). This combination had a significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.023$). The mean combined value was 12.5% higher than the mean expected value. Parsley, rosemary and sage had a mean combined value of 120.5 µmoles/g, which was 160.5% of the expected value (75µmoles/g, Table 4.1). This combination had a significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined ($p=0.001$). The

mean combined value was 60.5% higher than the mean expected value. Parsley, rosemary and thyme had a mean combined value of 160.4 µmoles/g, which was 148.9% of the expected value (107.7 µmoles/g, Table 4.1). This combination had a significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined (p=0.002). The mean combined value was 48.9% higher than the mean expected value. Rosemary, sage and thyme had a mean combined value of 153.8 µmoles/g, which was 120.9% of the expected value (127.2 µmoles/g, Table 4.1). This combination had a significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined (p< 0.001). The mean combined value was 20.9% higher than the mean expected value. All 4 herbs combined had a mean combined value of 118.7 µmoles/g which was 106.7% of the expected value (111.2 µmoles/g, Table 4.1). This combination had no significant synergistic effect for the antioxidant capacity of hot extracts of the herbs combined (p=0.196).

4.3.3 GAE of cold extracts of combined herb expressed in mg GAE/g compared to expected GAE values.

Parsley and rosemary had a mean combined value of 15.4 mg GAE/g, which was 93% of the expected value (16.6 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.134). Parsley and sage had a mean combined value of 16.3 mg GAE/g, which was 93.3% of the expected value (17.5 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.203). Parsley and thyme had a mean combined value of 21.6 mg GAE/g, which was 86.3% of the expected value (25.1 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.160).

Rosemary and sage had a mean combined value of 20.1 mg GAE/g, which was 106.1% of the expected value (18.9 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p =0.522). Rosemary and thyme had a mean combined value of 27.3 mg GAE/g, which was 102.8% of the expected value (26.5 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.715). Sage and thyme had a mean combined value of 32.6 mg GAE/g, which was 118.7% of the expected value (27.4 mg GAE/g), (Table 4.2). This combination had no significant

synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.116).

Parsley, rosemary and sage had a mean combined value of 16.4 mg GAE/g which was 92.8% of the expected value (17.7 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.665). Parsley, rosemary and thyme had a mean combined value of 22.2 mg GAE/g, which was 97.6% of the expected value (22.7 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.832).

Rosemary, sage and thyme had a mean combined value of 23.4 mg GAE/g, which was 96.1% of the expected value (24.3 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.062). All 4 herbs combined had a mean combined value of 18.3 mg GAE/g, which was 83% of the expected value (22 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of cold extracts of the herbs combined (p=0.066).

4.3.4 GAE of hot extracts of combined herb expressed in mg GAE/g compared to expected GAE values.

Parsley and rosemary had a mean combined value of 14.3 mg GAE/g, which was 115.2% of the expected value (12.4 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.178). Parsley and sage had a mean combined value of 22.7 mg/g, which was 136.3% of the expected value (16.6 mg GAE/g), (Table 4.2). This combination had a significant synergistic effect for the total phenolic content of hot extracts of parsley and rosemary combined (p= 0.001). The mean combined value was 36.3% higher than the mean expected value. Parsley and thyme had a mean combined value of 22.4 mg GAE/g, which was 88.7% of the expected value (25.3 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.135).

Rosemary and sage had a mean combined value of 16.3 mg GAE/g, which was 106.4% of the expected value (15.4 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.553). Rosemary and thyme combined had a mean combined

value of 17.2 mg GAE/g, which was 71.5% of the expected value (24 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.106). Sage and thyme had a mean combined value of 29.9 mg GAE/g, which was 106.1% of the expected value (28.2 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.081). Parsley, rosemary and sage had a mean combined value of 20.4 mg/g, which was 137.9% of the expected value (14.8 mg GAE/g), (Table 4.2). This combination had a significant synergistic effect for the total phenolic content of hot extracts of parsley and rosemary combined (p< 0.001). The mean combined value was 37.9% higher than the mean expected value.

Parsley, rosemary and thyme had a mean combined value of 23.2 mg GAE/g, which was 112.6% of the expected value (20.6 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.259). Rosemary, sage and thyme had a mean combined value of 23.1 mg GAE/g, which was 102.5% of the expected value (22.5 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.778). All 4 herbs combined had a mean combined value of 20.4 mg GAE/g, which was 100.2% of the expected value (20.3 mg GAE/g), (Table 4.2). This combination had no significant synergistic effect for the total phenolic content of hot extracts of the herbs combined (p=0.465).

4.4. Discussion

The aim of this study was to provide further insight into the potential synergistic effect in antioxidant activity for hot and cold extracts of parsley, rosemary, sage and thyme used in combination.

GAE results for the cold extracts showed no significant synergistic effects with any combinations, whilst TEAC results for combined cold extracts all showed significant synergy with the exception of RT. GAE results for hot extracts only showed statistically significant synergy for PS and PRS, whilst TEAC results for combined hot extracts showed statistically significant synergy for most combinations except for PR, PT, RS and PRST. The results show that both PS and PRS were the only combinations that demonstrated synergistic effects for antioxidant capacity and total phenolic content (hot extracts for the latter). At the

other end of the spectrum the combination RS did not show significant synergistic effects with the exception of the TEAC for the hot extracts.

The results for PS suggest that the combination of polyphenols present in parsley and sage interact synergistically and, in conjunction with those of PRS and RS, suggest that for PRS the synergistic interaction originated primarily from the combination of polyphenols present in parsley and sage, and that this effect was diminished by the addition of the polyphenols present in rosemary.

Peyrat-Maillard *et al.* (2003) found evidence of synergism in antioxidant activity of the paired polyphenols of quercetin and rosmarinic acid and of rosmarinic acid with caffeic acid. The synergy observed between rosmarinic acid and caffeic acid was low whilst that of quercetin and rosmarinic acid synergy was high. The mechanisms responsible for these effects, although not entirely clear, may be explained by the structure of the molecules and their ability to form intermolecular complexes that are stable. The authors suggested that the high synergy observed between quercetin and rosmarinic acid was due to the less antioxidant efficient molecule (quercetin) being able to regenerate the more antioxidant efficient molecule (rosmarinic acid), forming a more stable intermolecular complex.

Parsley is part of the *Umbelliferae* plant family, and contains the flavonoids apiin, apigenin and quercetin and apigenin-7-glucoside (Yoshikawa *et al.,* 2000; Williamson, 2003; Kaefer and Milner, 2008), whilst sage is part of the *Labiatae* plant family and reported to possess the phenolic acids rosmarinic acid and caffeic acid (Cuvelier *et al.,* 1996; Mantle, 2000); and the flavonoids luteolin and salvigenin (Williamson, 2003). Therefore, it may be possible to hypothesise that there are similar interactions between the predominant flavonoid in parlsey (apigenin-7-glucoside) and the predominat phenolic acid in sage (rosmarinic acid) as that proposed for rosmarinic acid with quercetin by Peyrat-Maillard *et al.* (2003). However, no evidence was found in the literature to explain the interactions between these two compounds (apigenin-7-glucoside and rosmarinic acid) and thus these two polyphenols in combination warrant further investigation.

Possible potential synergistic interactions were suggested between polyphenols and essential oils within individual *Labiatiea* herbs and between different herbs of this family by Williamson (2001); however, in the current study, where aqueous methods of extraction were employed, it is unlikely that volatile oils were present in

the extracts, and therefore it is more likely that the synergistic effects observed were due to polyphenols which are mainly hydrophilic compounds whilst volatile oils are essentially hydrophobic.

Rosemary is part of the plant family *Labiatae,* as is sage, and is thought to contain significant amounts of rosmarinic acid as well as caffeic acid, and the flavonoids luteonin and apigenin (Yoshikawa *et al.,* 2000). It is possible that a complex interaction between phenolic acids and flavonoids present in parsley and sage worked synergistically with the phenolic acids and flavonoids present in rosemary, which was not possible with sage and rosemary only. For example, if apigenin in parsley forms a complex with rosmarinic acid in sage, it may be that the apigenin in rosemary and parlsey also forms a complex with the rosmarinic acid in rosemary and sage, and that these interactions contribute to the stability of their intermolecular complexes and thus to an increase in antioxidant activity observed in the combination PRS. However, it is possible that these interactions are highly complex and, as the number of herbs increases, so does the complexity of interactions.

In the current study, no synergy occurred in GAE for combinations of hot extracts of rosemary, sage and thyme (RS, RT, ST, RST). However, the reasons for this are not entirely clear. Peyrat-Maillard *et al.* (2003) reported antagonistic antioxidant activity between the flavonoids (+)-catechin and quercetin but no explanation as to why was put forward. So *et al.* (1996) showed that paired flavonoids, genistein, baicalein, hesperetin, narengenin and quercetin synergistically inhibited the growth of MDA-MB 435 breast cancer cell line with the exception of the pairing of arengenin with hesperetin. The authors suggested that the lack of synergy between these two compounds was due to the similarity of their chemical structure which prevented the formation of a stable intermolecular complex. If compounds that are too similar in structure do not give rise to synergy when combined and sometimes have antagonistic effects (as was the case for (+)-catechin and quercetin in the study by Peyrat-Maillard *et al.* (2003)). It may explain why the combinations of herbs of the same plant family with the same predominant phenolic acid (rosmarinic acid, in R, S and T; Shan *et al.*, 2006; Zheng and Wang, 2001) or structurally similar polyphenols (caffeic acid, coumaric acid, ferulic acid), had no synergistic antioxidant effects.

Regarding the GAE for the cold extracts in combination, it is unclear as to why the GAE results for cold extracts, and for most of the hot extracts, showed no significant synergistic effects, but the PS and PRS combinations for the hot extracts appear to give rise to synergy. The chemistry behind the TEAC and the GAE assay is similar, these are both electron transfer assays that measure the capacity of an antioxidant in reducing an oxidant. A possible advantage of the GAE assay is that the longer wavelength (765nm) absorption of the chromophore compared to the TEAC assays (734nm) may marginally minimise interferences from the colour of the herb samples (Huang, Ou and Prior, 2004). Thus, further work is required to determine if the effects reported are truly synergistic.

4.5. Conclusions

This study showed that, when combined, some culinary herbs appeared to increase the polyphenolic antioxidant activity of these herbs synergistically, and this was especially true for the combination of parsley with sage and parsley with sage and rosemary. However, this synergy was not consistent despite evidence that suggests that synergy exists between polyphenols, specifically flavonoids and phenolic acids. Certainly using combinations of culinary herbs would provide a variety of polyphenols in the diet, but clearly how polyphenol variety, as a result of herb combination, impacts on the antioxidant activity of a combination remains to be answered in full. Further research on the actual compounds present in the herbs studied and research into the chemical interactions between flavonoids and phenolic acids should shed some light on how polyphenols interact. In addition, to help address the question of the biological significance of synergy, the effect of combinations of herbs in "biologically relevant" systems such as cell and tissue based models, is required.

Chapter 5. The impact of *in vitro* digestion on the antioxidant activity of cooked parsley, rosemary, sage and thyme.

5.0 Introduction

A number of *in vitro* studies have looked at the effects of digestion on the polyphenolic content and antioxidant activities of plant foods. Record & Lane (2001) investigated the effects of *in vitro* digestion of green and black tea catechin levels and their antioxidant activity. Their results showed that the simulated stomach acid with HCl at pH 2 for 1 hour incubation had little effect on individual catechin concentration or their antioxidant activity. The pH of the solution was then brought up to 7.5 with sodium bicarbonate for an additional 1 hour in order to simulate the pH of the intestinal environment. The authors did observe a reduction in antioxidant activity and an even greater reduction in catechin concentration. From these observations, the authors suggested that the formation of dimers of green and black tea catechin with antioxidant activity may be responsible.

Bermudez-Soto *et al.* (2007) studied the stability of polyphenols in chokeberry juice which is known to have high levels of polyphenols (anthocyanins, flavan-3-ols, procyanidins, flavonols and cinnamic acids). The juice was subjected to *in vitro* gastric and intestinal digestion. The authors found that simulated gastric digestion with HCl at pH 2 with pepsin for 2 hours had no significant effect on the major polyphenols found in chokeberry. A simulated intestinal digestion at pH 7.5 with pancreatin and bile salts for an additional 2 hours incubation resulted in a 26% decrease in flavonoids, a 19% decrease in flavan-3-ols, a 28% decrease in neochlorogenic acids, and a 23.9% increase in chlorogenic acid. Coates *et al.* (2007), investigated the anti-cancer effects of raspberry polyphenols post digestion (*in vitro*). Gastric digestion was simulated by acidifying homogenised raspberry extracts to pH 1.7 with HCl and pepsin followed by a 2 hour incubation. Intestinal digestion was simulated with the addition of pancreatin and bile salts with incubation in $NaHCO_3$ in cellulose dialysis tubing for a further 2 hours until a neutral pH was reached. Reductions of total phenolic content and total anthocyanin content of 30% and 50% respectively occurred. In addition, the authors reported that new polyphenolic compounds formed during the digestive process. Cilla *et al.* (2008) subjected a selection of fruit beverages to an *in vitro* digestion process in order to assess the bioavailability of polyphenols present in these juices. They used a simulated gastric digestion with HCl and pepsin at pH 2 for a 2 hour

incubation and simulated intestinal digestion with pancreatin and bile salts at pH 6.5 for a further 2 hour incubation. The authors observed a reductions in derivatives of hydroxycinnamic acid (37%), neochlorogenic acid (68%) and chlorogenic acid (53%).

The above mentioned studies seem to indicate that the major polyphenols studied in these plant materials decreases with *in vitro* digestion, particularly with a more alkaline environment, whilst the formation of new polyphenolic compounds may also occur. However many of the *in vitro* digestion models mentioned are incomplete and thus do not mimic closely digestion *in vivo*; for example none of the studies included an *in vitro* buccal simulation. The combination of saliva and mastication that occurs in the mouth is an established part of the physiological and biological processes that form digestion in the upper parts of the gastrointestinal tract (Pederson *et al.*, 2002). Therefore the effects of the buccal phase need to form part of any investigation into the effects of digestion on the polyphenolic content and activity of plant foods. Furthermore, the studies above clearly indicate that little work has been done to understand the impact of digestion on culinary herbs; and knowledge of how digestive processes influence the polyphenolic content and activity of this group of foods is essential to understanding their dietary significance . Therefore, the aim of this study was to investigate the impact of digestion *in vitro* on the antioxidant capacity and total phenolic content profile of cooked (heated) parsley rosemary, sage and thyme.

5.1 Material and methods

5.1.1 Materials

Reagents

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulphonic acid) in diamonium salt form, (ABTS), Folin-Ciocalteu's phenol reagent, Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, 97% (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), and potassium persulphate ($K_2S_2O_8$), Sodium Hydroxyde (NaOH), Sodium Bicarbonate (Na HCO_3), hydrochloric acid (HCl), PH Hydrion Brilliant dip sticks were all purchased from Sigma Aldrich, Poole, UK.

Culinary herbs

Four culinary herbs, parsley, rosemary, sage and thyme were selected as stated in Chapter 2 and purchased "Neal's Yard Remedies" Richmond, Surrey, UK.

5.1.2 Methods

5.1.2.1 Preparation of control (undigested heated) herb extracts (H) and rosmarinic acid.

Herbs (1g) were stir-fried (heated) under laboratory conditions by adding them to a Teflon® stir frying pan without oil, as vegetable oil has been shown to possess antioxidant activity (Pellegrini e*t al.,* 2003). The pan was placed on a very hot plate (300˚C) for 10 minutes during which the herbs were stirred before being placed in a beaker to which heated (37°C) distilled water (25ml) was added. This mixture was then stirred gently, transferred to a tinted glass bottle, which was covered with a lid. The herbs were then left to infuse for 10 minutes. The entire preparation was then drained using a conical flask, a funnel and a Whatman filter #1. Extracts were then filtered sterilised using a 0.22µm filter membrane, aliquoted into micro-centrifuge tubes, labelled and frozen at -80°C until needed.

To gain a better understanding of the impact of the food matrix on the digestion of polyphenols in these culinary herbs, the effect of *in vitro* digestion on rosmarinic acid was carried out. Rosmarinic acid was chosen as it has been determined from the literature to be a major polyphenol in rosemary, sage and thyme (Wang *et al.,* (2004), Kivilompolo *et al*., (2007 a&b), Shan *et al.,* (2006), Yoshikawa *et al., (*2000) Lu and Foo, (2001)).

Rosmarinic acid 10mg in 1ml solution (100µl ethanol 900µl distilled water) was digested in triplicate experiments using the same *in vitro* process as the herbs except it was not heated. Rosmarinic acid controls 10mg/ml (non-digested) were prepared by adding the same amount of distilled water equivalent to the amounts of digestive fluids used throughout the *in vitro* digestion process described below.

5.1.2.2 In vitro digestion of herb extracts

In vitro digestion of the herbs was carried out in three stages, stage 1 the buccal (mouth) phase, phase 2 the gastric phase and stage 3 the intestinal phase. Preparation of these stages is described below.

Stage 1: Simulated Buccal fluid

"Simulated buccal fluid" was prepared by adding heated herbs (1 g) (prepared as described above) to a pre-warmed (37°C) glass mortar. In order to simulate physical mastication in the mouth the leaves were crushed with a glass pestle minutes to break them into large fragments. Salivary α-Amylase (1.4 units/ml, pH 7; 1 unit corresponds to the amount of enzyme that liberates 1µmol of maltose per minute at pH 6.9 at 25°C (Sigma)) and 14ml of distilled water pre- warmed to 37° C were then added to the herbs in the glass mortar for 2 minutes. Buccal fluid controls were made up the same way without α-Amylase.

Stage 2: Simulated Gastric Fluid

The mixture (the buccal digest) was then processed as per Garrett *et al.* (2000) and Glahn *et al.* (1995) with minor adjustments. The mixture was acidified to pH 2, with HCl (4 ml, 0.1 mol/L) (to mimic the gastric environment) followed by the addition a solution (1ml) made up of pepsin (0.4g) (800-2.500 units/mg of protein; 1 unit gives a ΔA_{280} of 0.001 per min at pH2.0 at 37 °C (Sigma), in HCl (10 ml, 0.1 mol/L)). The mixture was then poured into a dark glass bottle with a screw cap and placed in a shaking water bath (Grant, SS40-2, Fisher, UK) for 1 hour at (37° C) which operated at 190 strokes per minute to mimic both the gastric environment and contractions. The pH of the mixture was checked every 15 minutes and an average of 1ml HCl (0.1M) was added drop by drop to keep the pH of the solution at 2. Gastric fluid controls were made up the same way without pepsin.

Stage 3: Simulated intestinal fluid

The mixture, (the gastric digest) pH was brought to 4.5-5 using sodium hydroxide (NaOH, 1M) added drop by drop. Sodium bicarbonate ($NaHCO_3$), (5ml, 0.1 M) containing pancreatin (2mg/ml) (The National Formulary (N.F.) and the U.S. Pharmacopeia (USP) x4 specification) and bile salts (12mg/ml) was then added (pH 7.5-8). Partially digested mixtures were then incubated at 37°C for 2 hours in the shaking bath, 190 strokes per minute to simulate the environment of the small intestine. Sodium hydroxide (NaOH, 1M) was added drop by drop to maintain the

pH (7.5-8) when required, which was checked every 15 minutes. Intestinal fluid controls were made up the same way without pancreatin and bile salts.

Following stage 3, the final volume totalled, conveniently, 25 ml which enabled a direct comparison with control preparations (see section 5.1.2). The preparations of extracts of the control (H) and digested (H&D) [where H stands for heated and D for digested] herbs were filtered using a Whatman grade #1, and filter sterilised (pore size 0.22 μm, Sartorius from Sigma).

In addition to the final digest, samples were taken for assaying (antioxidant capacity and total phenolic content) at each stage of digestion, with blanks for each simulated fluid (no herbs) and controls (without enzymes). Non-digested and digested rosmarinic acid (simulated intestinal fluid) was also assayed for antioxidant capacity.

5.1.2.3 Determination of antioxidant activities of herb extracts.

Trolox equivalent antioxidant capacity assay (TEAC) and Total phenolic content/ gallic acid equivalent assay (GAE) were performed as described in chapter 3, (section 3.4.1 and 3.4.2 respectively).

5.1.2.4 Scanning Electron Microscopy (SEM) of uncooked, heated and heated and digested herbs

In order to gain further insight as to the effects of heat and *in vitro* digestion on culinary herbs, the histology of rosemary and thyme was investigated using SEM (it was not possible to mount parsley and sage leaves on the SEM after they had been through the digestion *in vitro* due to the soft/crumbled structure of their leaves post digestion; therefore, a comparison could not be made between these herbs uncooked, heated and heated and digested via SEM). Dried herb leaves (non-heated), heated herbs, and heated and digested herbs (dried), were fractured across their long axis, carefully mounted on aluminium stubs and then sputter coated with gold palladium alloy. The samples were examined in the secondary mode using a Zeiss EVO 50 SEM (Carl Zeiss, Hertfordshire, UK) at an accelerating voltage of 20 kV, after ascertaining that no damage to the samples was induced by this voltage.

Investigation of the Stability of Rosmarinic Acid with treatment at various pH values that occur in the Digestive Tract using ^{1}H NMR spectroscopy

Nuclear magnetic resonance (^{1}H NMR) spectroscopic experiments were performed in order to assess the stability of rosmarinic acid exposed to the various pH values encountered in the digestive tract and thus ascertain how this major polyphenol (rosmarinic acid), when part of a herb matrix, may be affected by the digestive process. First, rosmarinic acid (5mg) was dissolved in acetonitrile (50µl) and then deuterium oxide (D_2O) (100µl) and water (850µl) were added. The solution, at pH7, was incubated at 37 °C for 30 minutes to mimic rosmarinic acid in buccal fluid. Second, rosmarinic acid (5mg) was prepared as described above but with the pH adjusted to 2 using HCl. The solution was then, to mimic rosmarinic acid in gastric fluid, incubated at 37 °C for 1 hour. Third, rosmarinic acid (5mg) was dissolved in acetonitrile (50µl) and then D_2O (100µl) and phosphate buffer (850µl) at pH 8 were added. This third solution was then, to mimic rosmarinic acid in intestinal fluid, incubated at 37 °C for 2 hours and measured every 30 minutes for two hours at 37 °C +/- 0.5 °C ^{1}H NMR performed using a JEOL Eclipse+ 400 FT-NMR spectrometer using Delta version 4.3.6 control and processing software (JEOL, Hertfordshire, UK). Each solution (approximately 0.6ml) was placed in a 5 mm OD Wilmad 527-PP-8 tube containing 3-(Trimethylsilyl) propionic acid-d_4 sodium salt (1-2mg) as the internal reference (Gottlieb *et al.*, 1997). The samples were heated to the required temperature +/- 0.5 °C allowing 5 minutes thermal equilibration before the first measurement. For each temperature and each replicate the position of the water signal was determined using a survey protin spectrum measured in automation mode (16 accumulations, 32K points, 6.0 KHz sweep width, centred at 5 ppm). A pre-saturation experiement (Hoult, 1976), (a single pulse homogated experiment) was run to selectively remove the water peak from the spectrum (water/on-resonance signal = 4.788 ppm, attenuation = 35 dB, relaxation delay = 3s, 16 accumulations, 32K points, 6.0 KHz sweep width, centred at 5 ppm). Raw data were processed using single exponential windowing function (line width = 0.2Hz).

5.2 Expression of data and statistical analysis

The antioxidant activity (AA) of heated and digested culinary herbs was measured by determining their total equivalent antioxidant capacity (TEAC) in µmoles of Trolox per gram of herb and their total phenol content in mg Gallic acid equivalent (GAE) per gram of herb. Antioxidant capacity and total phenol content data are

presented as the mean of triplicate samples of triplicate preparations (n=3). TEAC and GAE values for blanks (tests and controls) were subtracted at each stage of digestion for each herb. Statistical analysis was conducted using SPSS for windows. ANOVA and a *post hoc* test using Tukey's test, if $p \leq 0.05$, were carried out to compare herb samples at each stage of digestion for each herb (H and H&D). An independent T test was done to compare TEAC values for rosmarinic acid non-digested to rosmarinic acid digested. P values equal to or less than 0.05 indicate statistically significant differences between samples tested.

5.3 Results

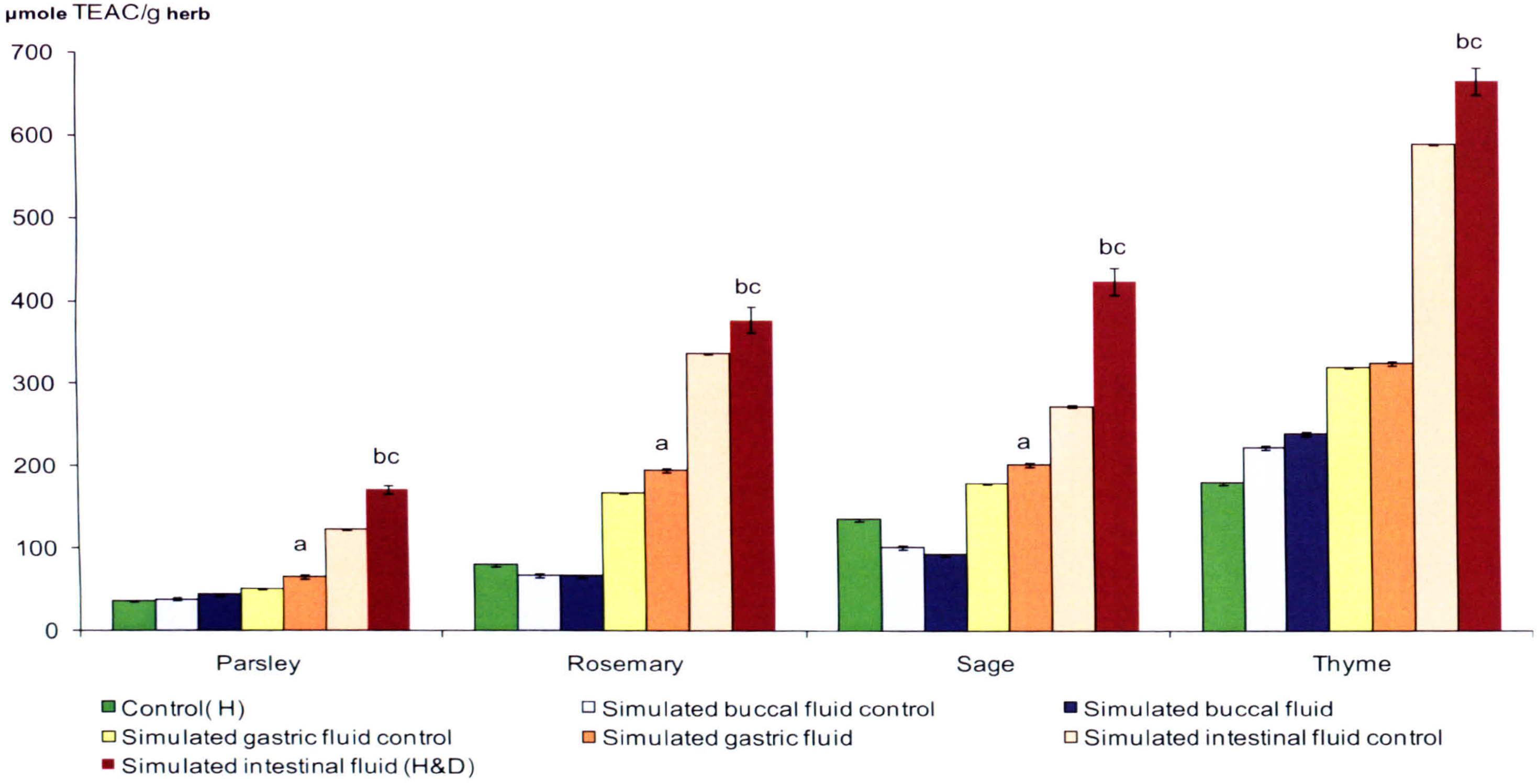
5.3.1 Effects of *in vitro* digestion on TEAC and GAE of culinary herbs

The TEAC for parsley H&D (172.2±4.9 µmoles TEAC/g herb) was significantly higher than that for parsley H (36±3.4 µmoles TEAC/g herb), ($p<0.001$). The TEAC for parsley in simulated buccal fluid (43.6±1.9 µmoles TEAC/g herb) was not significantly different from that of parsley in the simulated buccal fluid control (38.2±3.4 µmoles TEAC/g herb), ($p=0.504$). The TEAC for parsley in simulated gastric fluid (65.5±1 µmoles TEAC/g herb) was significantly higher than that for parsley in the simulated gastric fluid control (51±5.6 µmoles TEAC/g herb), ($p=0.004$). The TEAC for parsley in simulated intestinal fluid (172.2±4.9 µmoles TEAC/g herb) was significantly higher than that for parsley in the simulated intestinal fluid control (122.4±2.3 µmoles TEAC/g herb), ($p<0.001$). The TEAC for parsley in simulated buccal fluid stage was significantly lower than that for parsley in simulated gastric fluid stage ($p<0.001$) and the later was significantly lower than that for parsley in simulated intestinal fluid stage ($p<0.001$) (Figure 5.1).

The GAE for parsley H&D (21.4±1.7 mg GAE/g herb) was significantly higher than that for parsley H (3.7±1.9 mg GAE/g herb), ($p<0.001$). The GAE for parsley in simulated buccal fluid stage (4.6±0.4 mg GAE/g herb) was not significantly different from that for parsley in the simulated buccal fluid control (4.1±0.4 mg GAE/g herb), ($p=0.999$). The GAE for parsley in simulated gastric fluid stage (11.9±0.4 mg GAE/g herb) was not significantly different than that for parsley in the simulated gastric fluid control (11.2±0.7 mg GAE/g herb), ($p=0.106$). The GAE for parsley in simulated intestinal fluid stage (21.4±1.7 mg GAE/g herb) was significantly higher than that for parsley in the simulated intestinal fluid control (12.6±0.2 mg GAE/g herb), ($p=0.001$). The GAE for parsley in simulated buccal

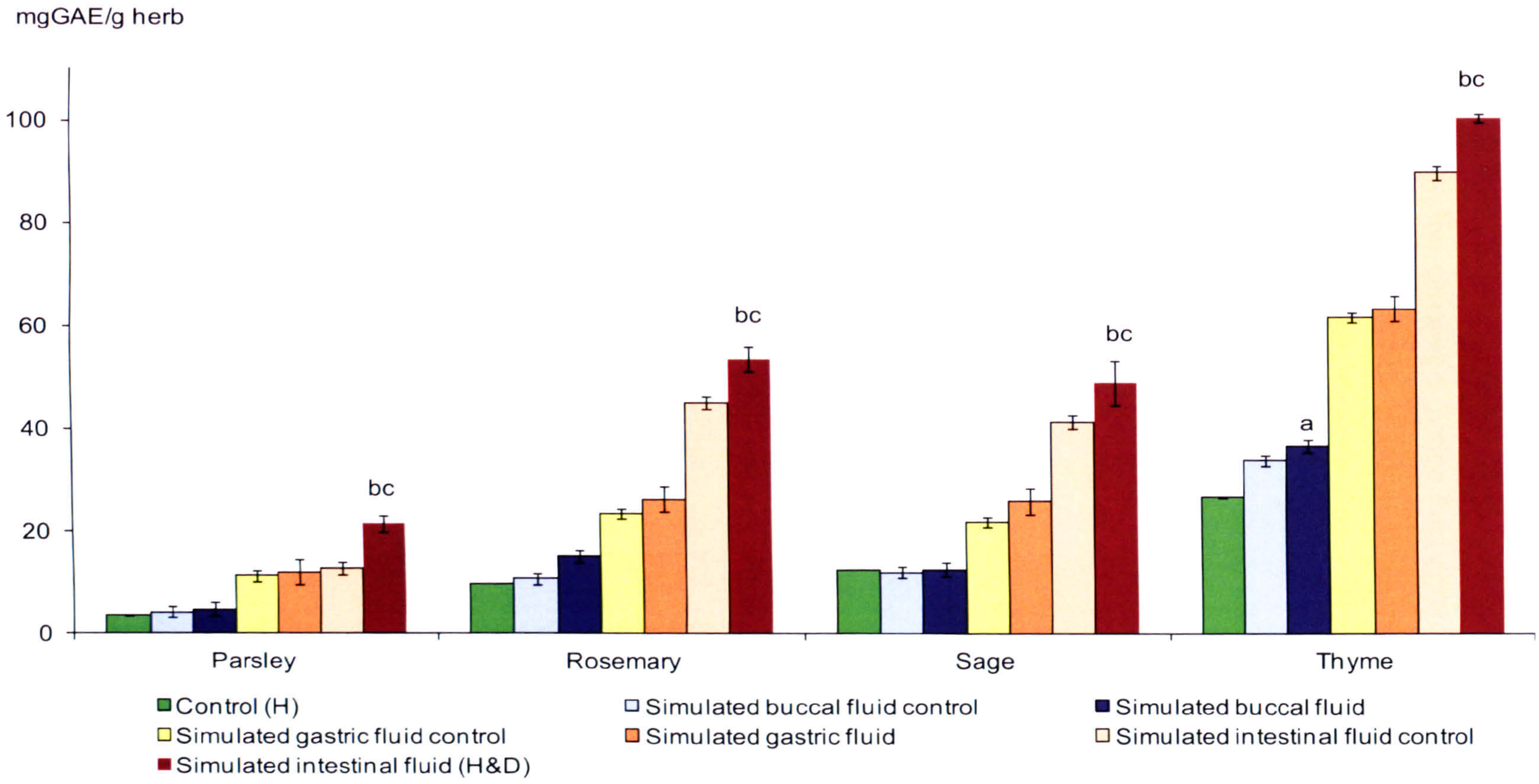
fluid stage was significantly lower than that for parsley in simulated gastric fluid stage ($p<0.001$) and the latter was significantly lower than that for parsley in simulated intestinal fluid stage ($p<0.001$) (Figure 5.2).

Figure 5.1 Antioxidant capacity of herbs (TEAC µmoles /g) at various stages of digestion compared with controls.



[a]Significant difference between simulated gastric fluid and simulated gastric fluid control $p<0.05$, (n=3).
[b]Significant difference between simulated intestinal fluid (H&D) and simulated intestinal fluid control $p<0.05$, (n=3).
[c]Significant difference between controls (H) and simulated gastric fluid (H&D) $p<0.05$, (n=3)

Figure 5.2 Total phenolic content of herbs (mg GAE/g) at various stages of digestion compared with controls.



[a]Significant difference between simulated gastric fluid and simulated gastric fluid control $p<0.05$, (n=3).
[b]Significant difference between simulated intestinal fluid (H&D) and simulated intestinal fluid control $p<0.05$, (n=3).
[b]Significant difference between controls (H) and simulated gastric fluid (H&D) $p<0.05$, (n=3)

The TEAC for rosemary H&D (377.5±15.5 µmoles TEAC/g herb) was significantly higher than that for rosemary H (79.8±4.8 µmoles TEAC/g herb), ($p<0.001$). The TEAC for rosemary in simulated buccal fluid (66.5±1.4 µmoles TEAC/g herb) was not significantly different from that for rosemary in the simulated buccal fluid control (67.4.2±4.2 µmoles TEAC/g herb), ($p=1.000$). The TEAC for rosemary in simulated gastric fluid (195±4.5 µmoles TEAC/g herb) was significantly higher than that for rosemary in the simulated gastric fluid control (168±4.5 µmoles TEAC/g herb), ($p=0.042$). The TEAC for rosemary in simulated intestinal fluid (377.5±15.5 µmoles TEAC/g herb) was significantly higher than that for rosemary in the simulated intestinal fluid control (337.1±2.3 µmoles TEAC/g herb), ($p<0.001$). The TEAC for rosemary in simulated buccal fluid stage was significantly lower than that for rosemary in simulated gastric fluid stage ($p<0.001$) and the later was significantly lower than that for rosemary in simulated intestinal fluid stage ($p<0.001$) (Figure 5.1).

The GAE for rosemary H&D (53.5±2.5 mg GAE/g herb) was significantly higher than that for rosemary H control (9.8±0.8 mg GAE/g herb), ($p<0.001$). The GAE for rosemary in simulated buccal fluid stage (15.1±6.7 mg GAE/g herb) was not significantly different from that for rosemary in the simulated buccal fluid control (10.7±2.6mg GAE/g herb), ($p=0.288$). The GAE for rosemary in simulated gastric fluid stage (26.2±1 mg GAE/g herb) was not significantly different from that for rosemary in the simulated gastric fluid control (23.5±1.7 mg GAE/g herb), ($p=0.772$). The GAE for rosemary in simulated intestinal fluid stage (53.5±2.5 mg GAE/g herb) was significantly higher than that for rosemary in the simulated intestinal fluid control (45.1±3.2 mg GAE/g herb), ($p=0.010$). The GAE for rosemary in simulated buccal fluid stage was significantly lower than that for rosemary in simulated gastric fluid stage ($p<0.001$) and the later was significantly lower than that for rosemary in simulated intestinal fluid stage ($p<0.001$) (Figure 5.2).

The TEAC for sage H&D (425±16.4 µmoles TEAC/g herb) was significantly higher than that for sage H (135.8±14 µmoles TEAC/g herb), ($p<0.001$). The TEAC for sage in simulated buccal fluid (92.7±1.6 µmoles TEAC/g herb) was not significantly different from that of sage in the simulated buccal fluid control

(101.8±3.7 µmoles TEAC/g herb), (p=0.873). The TEAC for sage in simulated gastric fluid (202.6±5 µmoles TEAC/g herb) was significantly higher than for sage in the simulated gastric fluid control (178.5±5.4), (p<0.001). The TEAC for sage in simulated intestinal fluid (425±16.4 µmoles TEAC/g herb) was significantly higher than that for sage in the simulated intestinal fluid control (273.4±4.2 µmoles TEAC/g herb), (p<0.001). The TEAC for sage in simulated buccal fluid stage was significantly lower than that for sage in simulated gastric fluid stage (p<0.001) and the latter was significantly lower than that of sage in simulated intestinal fluid stage (p<0.001) (Figure 5.1).

The GAE for sage H&D (48±4.4 mg GAE/g herb) was significantly higher than that for sage H (12.5±0.3 mg GAE/g herb), (p<0.001). The GAE for sage in simulated buccal fluid (12.4±1.9 mg GAE/g herb) was not significantly different from that for sage in the simulated buccal fluid control (11.9±2.2 mg GAE/g herb), (p=1.000). The GAE for sage in simulated gastric fluid (25.9±2.3 mg GAE/g herb) was not significantly different than that for sage in the simulated gastric fluid control (21.9±0.9 mg GAE/g herb), (p=0.192). The GAE for sage in simulated intestinal fluid (49±4.4 mg GAE/g herb) was significantly higher than that for sage in the simulated intestinal fluid control (41.3±1.6 mg GAE/g herb), (p=0.005). The GAE for sage in simulated buccal fluid stage was significantly lower than that for sage in simulated gastric fluid stage (p=0.001) and the latter was significantly lower than that for sage in simulated intestinal fluid stage (p<0.001) (Figure 5.2).

The TEAC for thyme H&D (667.8±12.6 µmoles TEAC/g herb) was significantly higher than for thyme H (179.9±34 µmoles TEAC/g herb), (p<0.000). The TEAC for thyme in simulated buccal fluid stage (240.1±12.6 µmoles TEAC/g herb) was not significantly different from that of thyme in the simulated buccal fluid control (223.3±23.1 µmoles TEAC/g herb), (p=0.997). The TEAC for thyme in simulated gastric fluid stage (320±1.7 µmoles TEAC/g herb) was not significantly different from that of thyme in the simulated gastric fluid control (326±4.5 µmoles TEAC/g herb), (p=1.000). The TEAC for thyme in the simulated intestinal fluid stage (667.8±12.6 µmoles TEAC/g herb) was significantly higher than that for thyme in the simulated intestinal fluid control (591.7±34.2 µmoles TEAC/g herb), (p=0.003). The TEAC for thyme in simulated buccal fluid stage was significantly lower than that for thyme in simulated gastric fluid stage (p=0.001) and the later was

significantly lower than that for thyme in simulated intestinal fluid stage ($p<0.001$) (Figure 5.1).

The GAE for thyme H&D (100.8±2.3 mg GAE/g herb) was significantly higher than that for thyme H (26.7±1.3 mg GAE/g herb), ($p<0.001$). The GAE for thyme in simulated buccal fluid (36.6±0.7 mg GAE/g herb) was significantly higher from that for thyme in the simulated buccal fluid control (33.9±0.5 mg GAE/g herb), ($p=0.013$). The GAE for thyme in simulated gastric fluid (63.4±0.6 mg GAE/g herb) was not significantly different from that for thyme in the simulated gastric fluid control (61.8±1 mg GAE/g herb), ($p=0.275$). The GAE for thyme in simulated intestinal fluid (100.8±2.3 mg GAE/g herb) was significantly higher than that for thyme in the simulated intestinal fluid control (90±1.6 mg GAE/g herb), ($p<0.001$). The GAE for thyme in simulated buccal fluid stage was significantly lower than that for thyme in simulated gastric fluid stage ($p=0.001$) and the latter was significantly lower than that for thyme in simulated intestinal fluid stage ($p<0.001$) (Figure 5.2).

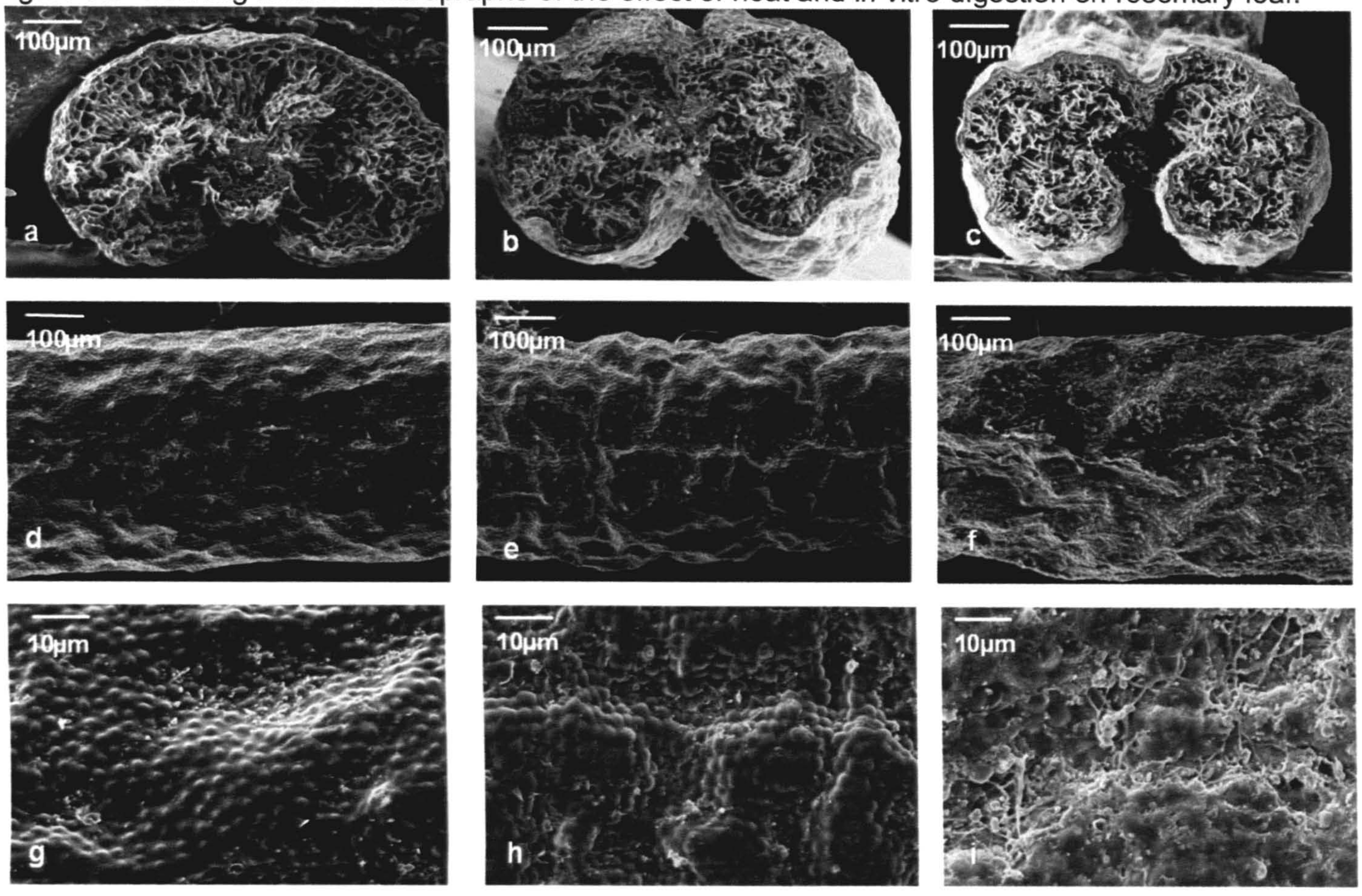
5.3.2 Effects of *in vitro* digestion on TEAC of rosmarinic acid

There were no statistical differences between the TEAC for non-digested rosmarinic acid (2901± 149.1 µmoles TEAC/g) and that for digested rosmarinic acid (2973.1 ± 140.6 µmoles TEAC/g), ($p=0.692$).

5.3.4 Scanning Electron Microscopy of controls (heated) and heated and digested rosemary and thyme leaves

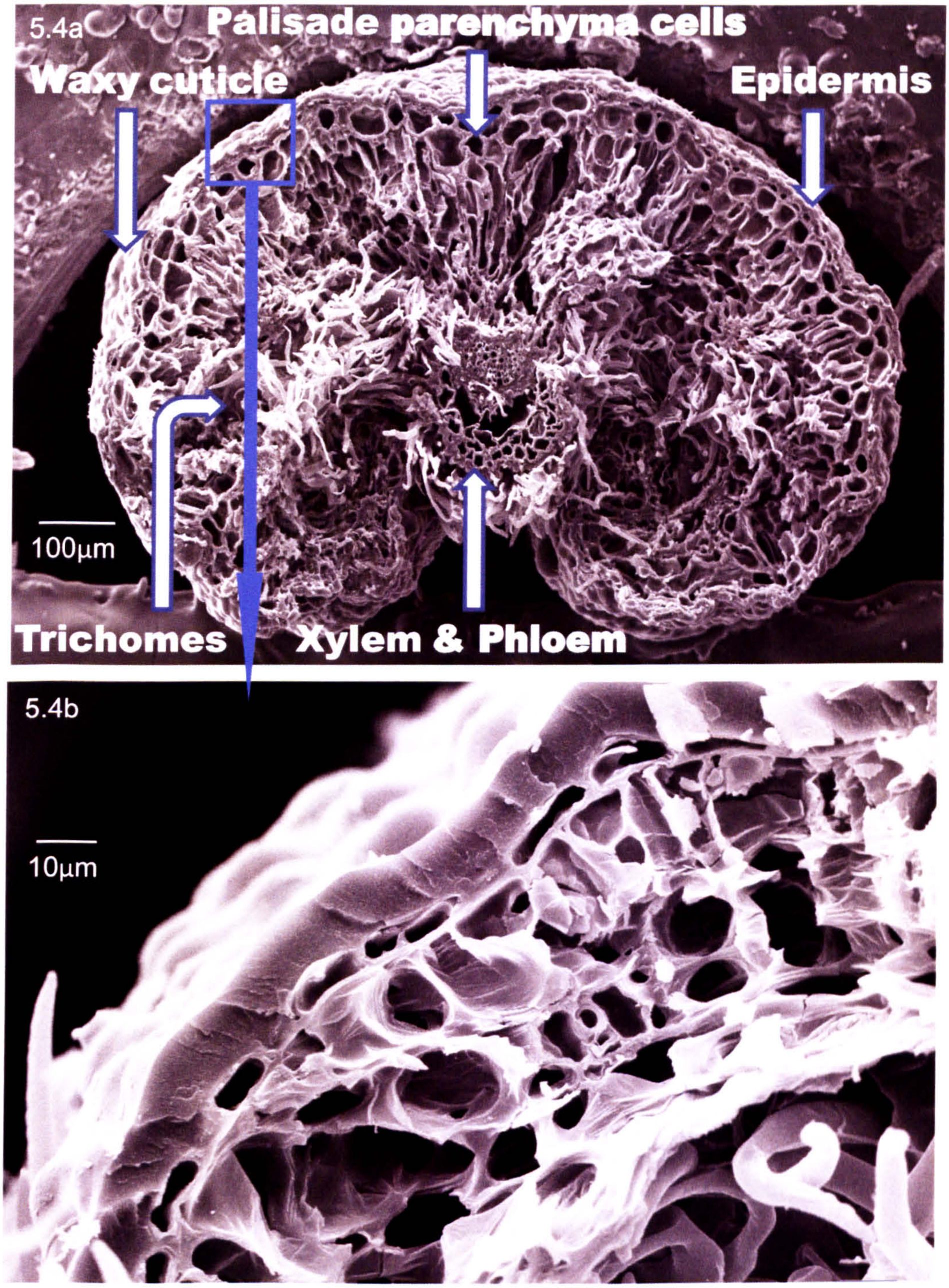
Scanning electron microscopy (SEM) showed that there was a difference in structure between the non-heated, heated and heated digested herb samples. On the surface of the heated samples, the SEMs show that the waxy cuticles of these herbs are shrunken and wrinkled and the epidermis is shrunken (Figures 5.3 (b, e, h) and 5.5 b). For the digested herbs the surface not only appears shrunken but there is also evidence of damage to the cuticle (5.3 (c, f, i) and 5.5 c).The cross sectional images show the presence of the epidermis, the waxy cuticle, on the upper part of the leaf, xylem and phloem which provide water and nutrient exchange to the leaf at the centre of the leaf as well as palisade parenchymal cells below the epidermis and trichomes towards the bottom part of the leaf (Figure 5.4a and 5.4b).

Figure 5.3 Scaning electron micropraphs of the effect of heat and *in vitro* digestion on rosemary leaf.



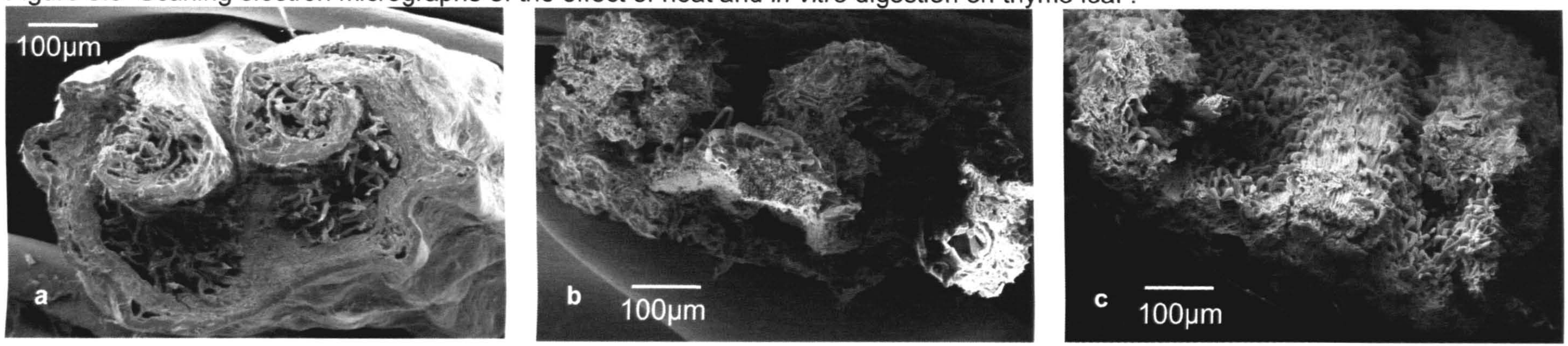
a: Cross section of the leaf uncooked, b: cross section of the leaf heated, c: cross section of the leaf heated and digested, d and g: surface of the leaf uncooked, e and h: surface of the leaf heated, f and i: surface of the leaf heated and digested.

Figure 5.4a and 5.4b Scaning electron micrographs close up for rosemary uncooked leaf.



SEM of cross section of rosemary leaf uncooked with labels (5.4a).
SEM of cross section of rosemary leaf uncooked close up (5.4b).

Figure 5.5 Scaning electron micrographs of the effect of heat and *in vitro* digestion on thyme leaf .

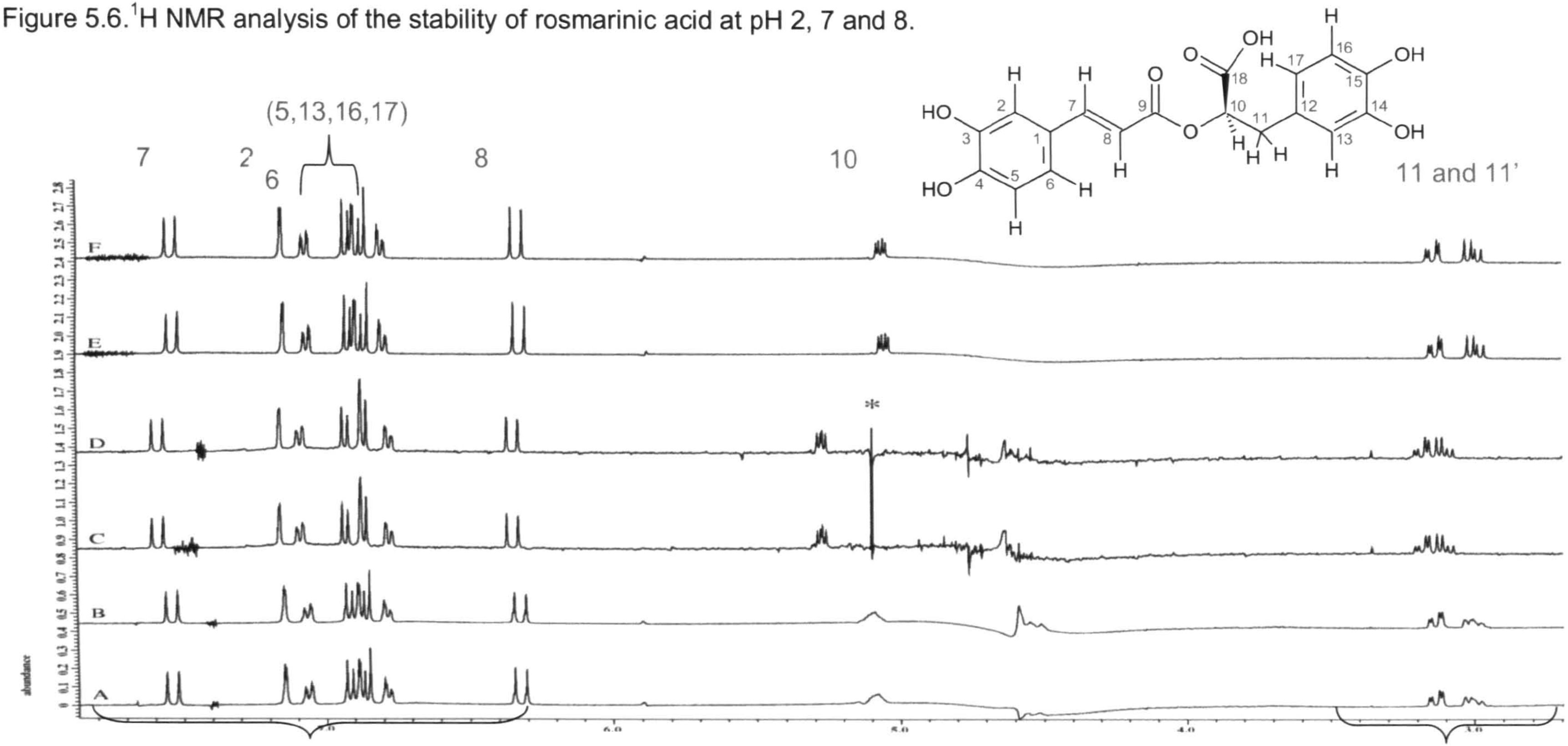


a: Cross section of the leaf uncooked, b: cross section of the leaf heated, c: cross section of the leaf heated and digested.

5.3.5 ^{1}H NMR analysis of rosmarinic acid exposed to different digestion pH values

^{1}H NMR determination and quantification of rosmarinic acid expressed as: [x(1H ,d , *j*=$y_{(a,b)}$Hz, z] where x is the peak in ppm integrating for 1 proton (relative to the total number of protons found), d= the peak is a doublet with a coupling constant J (between proton a and b) equal to 2 hertz, and the peak is that of proton z (Figure 5.4) (400 MHz in H_2O/D_2O (9:1) at pH 8.0): 7.53 (1H, d, *J*=$16_{(7,8)}$Hz, H-7), 7.14 (1H, d, *J*=$2.0_{(2,6)}$Hz, H-2), 7.06 (1H, dd, *J*=$8.2_{(6,5)}$Hz, *J*=$2.0_{(6,2)}$Hz, H-6), 6.92 (1H, d, *J*=$8.2_{(5,6)}$Hz, H-5), 6.89 (1H, d, *J*=$2.0_{(13,17)}$Hz, H-13), 6.86 (1H, d, *J*=$8.1_{(16,17)}$Hz, H-16), 6.79 (1H, dd, *J*=$8.1_{(17,16)}$Hz, *J*=$2.0_{(17,13)}$Hz, H-17), 6.32 (1H, d, *J*=$16_{(8,7)}$Hz, H-8), 5.05 (1H, dd, *J*=$9.0_{(10,11)}$Hz, *J*=$3.9_{(10,11')}$Hz, H-10), 3.13 (1H, dd, *J*=$14.4_{(11',11)}$Hz, *J*=$3.9_{(11',10)}$Hz, H-11'), 2.99(1H, dd, *J*=$14.4_{(11,11')}$Hz, *J*=$9.0_{(11,10)}$Hz, H-11). There were no qualitative or quantitative changes in rosmarinic acid at the various pH values of 2, 7 and 8 over time (Figure 5.6).

Figure 5.6.[1]H NMR analysis of the stability of rosmarinic acid at pH 2, 7 and 8.



The above spectra represent the attempt to replicate the pH of the three stages of digestion. Rosmarinic acid was studied at pH 7 for 30 minutes (A= time 0, B= 30 min); at pH 2 for 1 hour (C= time 0, D = 1 hour); and at pH 8 for 2 hours (E= time 0, F= 2 hours). Numbers in figures above represent carbon positions.* solvent suppression artefact.

5.4 Discussion

In this study the AA of culinary herbs in the upper part of the gastrointestinal digestion (excluding the colon) was investigated using an *in vitro* model of digestion. This investigation showed that significant increases in AA were observed between herbs heated and undigested (H) and heated and digested (H&D). Increases in AA at the simulated intestinal fluid stage were highly significant, suggesting that the enzymes have a key role to play in the effects observed. The plant cell walls of culinary herbs are made up of proteins and dietary fibre including lignin, cellulose, hemicelluloses and pectins. By definition non starch polysaccharides (NSPs) are resistant to hydrolysis by enzymes in humans (Trowel, 1972), however, the simulated intestinal fluid contained proteases which may have facilitated the breakdown of the proteins present in the cell walls of culinary herbs and allowed for cracks in the epidermis of the leaves. Also, unlike cellulose, lignin, and most hemicellusoles, pectins can swell in aqueous solutions, and may have helped to stretch the epidermis, the leaves and plant cell walls. Furthermore the presence of amylases contained in the pancreatin may have also contributed to the cleavage of α 1- linkages between D-galacturonic acid units of pectin (Groff and Gropper, 1989) and caused further damage to both the epidermis of leaves and the plant cell walls. These results taken together with those from the previous chapters of this study suggest that the increase in available polyphenols may be due to the gradual breakdown of plant cell walls and this increase results in the increase in AA following digestion. This suggestion is further supported by the SEM results, which showed that the waxy cuticles and epidermis of heated and heated and digested rosemary are shrunken and damaged compared to the unprocessed leaf, more so in the digested leaf (Fig 5.3a and 5.3b). Furthermore, the cracks in the cuticle and epidermis favour the exposure of inner cells. Polyphenols are thought to be most concentrated in the vacuoles of paraveinal and parenchymial cells below the epidermis (Raven, 1998). If these cells become more exposed after heating and digestion, it (exposure of these cells) could explain the increase in antioxidant activity observed post digestion compared to uncooked and heated herb.

Concerning the buccal phase of digestion, non significant differences in AA were found between tests and controls at the first stage of digestion "simulated buccal fluid". The short period of time spent in this phase (2 min) in the presence of α-

amylase may not have allowed for significant plant cell wall degradation to promote the release of polyphenols. Since α-amylase is rapidly inactivated by gastric acids, this phase may therefore be considered to be of minor significance in the digestion of culinary herbs and other plants due to their polysaccharide make up (Peterson *et al.*, 2002). However, it is possible that in this phase minor physical changes may occur that may influence the entire digestive process (Hoebler *et al.*, 1998).

Both significant and non significant differences in AA were found at the simulated gastric fluid stage between tests and controls. It is unlikely that the significant effects observed were caused by acid hydrolysis of flavonoid glycosides as the conditions required for the hydrolysis of red spring onions and spinach leaves is reported to be 80 °C for 2 h with 1.2 M HCl in 50% aqueous methanol (Nuutila *et al.*, 2001). In addition, most flavonoids are thought to resist human gastric digestion and arrive intact in the duodenum (Manach *et al.*, 2004). It is likely that the plant cells walls of culinary herbs remained mostly undamaged and somehow "protected" some of the polyphenols within the leaf from being released, that some (tests and controls) became damaged via the swelling of pectins as mentioned above, and that some became damaged via the action of pepsin on the proteins present in the cell membranes, all of which may explain the inconsistencies at the simulated gastric fluid stage. However the first two stages (simulated buccal fluid and simulated gastric fluid) of the *in vitro* digestion may have contributed to the overall final increase in AA observed at the simulated intestinal fluid stage by weakening the plant cell walls.

Overall, the results of this study do not correlate with those of other *in vitro* digestion studies that indicated that the major polyphenols decrease with *in vitro* digestion, particularly when digested in a more alkaline environment such as the simulated intestinal fluid (Record and Lane, 2001; Bermudez-Soto *et al.*, 2007; Coates *et al.*, 2007; Cilla *et al.*, 2008). These observations may be due to differences in the types of plant material extracted and differences in the methods used for preparing the extracts as well as differences in the *in vitro* digestion model used, specifically the use of a buccal phase. Although this phase does not appear to contribute to the increase in AA as stated above it could be that structural changes occurred to facilitate an increase in the release of polyphenols

at intestinal phase. The culinary herbs in the current study were gently crushed to mimic mastication at the buccal simulation fluid stage and leaves were digested in order to reflect the fate of culinary herbs *in vivo*.

In addition, in some studies fruit concentrates were used (Bermudez-Soto *et al.*, 2007,Cilla *et al.*, 2008,) as opposed to whole fruits. Coates *et al.*, (2007) obtained their raspberries extracts by homogenising ripe berries with glacial acetic acid and acetonitrile. Clearly the types of sample that was used and the extractions that were carried out are very different from those used in the present study. In addition, the fruits listed above have much less rigid plant cell walls that those of culinary herbs.

The rosmarinic acid ^{1}H NMR spectra was in agreement with Lu and Foo, (2000) and Mehrabani *et al.* (2005) except that acidic protons were not visible in the current study due to differences in solvent. The current study shows that rosmarinic acid was stable when run through the *in vitro* digestion system, as no significant decrease in TEAC was observed compared to non digested rosmarinic acid. This result correlates well with the ^{1}H NMR results were rosmarinic acid was found to be stable at pH 8 as well as pH 2 and pH 7. Other studies ((Record and Lane, 2001; Bermudez-Soto *et al.*, 2007; Coates *et al.*, 2007; Cilla *et al.*, 2008) reported that polyphenols decreased in an alkaline environment, but this was not the case in the current study. This observation also correlates well with that of Baba *et al.*, (2005) who investigated the fate of orally administered rosmarinic acid in 6 healthy males volunteers, in the form of *Perilla frutescens* leaf extract tablets (200mg rosmarinic acid). The authors found that rosmarinic acid was mostly recovered in plasma within 30 minutes of administration as rosmarinic acid and its metabolites, suggesting that rosmarinic acid was absorbed and metabolised. Nurmi *et al.* (2006) also reported that rosmarinic acid metabolites were found in human plasma and urine after ingestion of oregano herb extracts, and demonstrated that these were rapidly excreted mainly as *p*- hydroxybenzoic acid and to a lesser extent as ferulic acid with levels of *p*- hydroxybenzoic acid peaking in urine 2- 6 hours after ingestion. These results indicate that rosmarinic acid, on its own, is not destroyed through digestion. This observation may apply to rosmarinic acid contained in the herbs investigated and also to other polyphenols in these herbs. Whether parent polyphenols such as rosmarinic acid remain intact

after the digestion of their host culinary herbs or whether they undergo some metabolism requires identification and quantification of polyphenols before and after digestion

5.5 Conclusions

In conclusion, the results of the present study suggest that heating the culinary herbs followed by *in vitro* digestion significantly increases polyphenol antioxidant activity of these herbs, which is thought to be due to the physical breakdown of the plant cell wall, promoting an increase exposure of inner cells containing the polyphenols. These results give further insight as to the impact of the upper gastrointestinal system on the availability of polyphenols in these culinary herbs. However, it is unclear from this study how much of the polyphenols released are made available post absorption. Thus, in order to ascertain the significance of these findings in terms of the antioxidant contribution of culinary herbs to the diet, studies on the impact of absorption on the antioxidant activity and action of processed culinary herbs are required.

Chapter 6 Bioavailability study of heated digested culinary herbs

6.0 Introduction

Polyphenols are secondary metabolites widely distributed in plant food. They are diverse and vary in types and can be classified into different groups based on their number of phenolic rings. Due to the different classes of polyphenols, their bioavailability is thought to vary (Manach *et al.*, 2004). Most polyphenols are usually present in foods as esters, glycosides or polymers and not readily absorbed from the small intestine. They first need to be hydrolysed to bio available aglycones via intestinal enzymes or the colonic microflora (Manach *et al.*, 2004). Aglycones are then absorbed by intestinal enterocytes and conjugated by glucuronidation via UDP-glucuronyl transferase during their transport from the gut to the portal vein. In the liver, remaining aglycones are conjugated by glucuronidation or sulphation, and methylated polyphenols are demethylated, some are then excreted in urine and eliminated or excreted in bile into the duodenum (entheropathic recycling) where they can be broken down further via bacterial enzymes (β-glucuronidases), (Singh *et al.*, 2008).

Garrett *et al.* (2000) estimated the bioavailability of carotenoid compounds from fresh stir fried vegetables using an *in vitro* model of digestion and the Caco-2 cell culture model. They found that 29% of lutein, 3.2% of lycopene 14.7% of α-carotene and 16% of β-carotene were transferred from the meal to the Caco-2 micellar fraction. Rechner *et al.* (2002) investigated the fate of polyphenols in 20 healthy human male and female subjects using a 3 day flavonoid free diet followed by a 5 day controlled flavonoid rich meal. The study focussed on the metabolites formed by colonic microflora degradation and their reabsorption. Results showed that glucoronides of naringenin, quercetin and hydroxycinnamic acids were detected in plasma and urine. In a study by Baba *et al.* (2005) the fate of orally administered rosmarinic acid in 6 healthy males volunteers, in the form of *Perilla frutescence* leaf extract tablets (200mg Rosmarinic acid) was investigated. It was found that rosmarinic acid reached its highest concentration in plasma within 30 minutes of administration (1.15μM) as rosmarinic acid and metabolites (conjugated form) with 75% of total rosmarinic metabolites being excreted in the urine within 6 Hours. Similar findings were observed by Nurmi *et al.* (2006) who investigated the fate of phenolic compounds from oregano extracts in the form of encapsulated

oregano leaf extracts (3.75g, single dose) in healthy human subjects (4 female, 2 male). It was found that p-hydroxybenzoic acid was the main metabolite of rosmarinic acid and it was rapidly excreted via the urine (2-6 hours after ingestion) in all 6 subjects. This study suggested that rosmarinic acid was rapidly absorbed in the upper part of the gastrointestinal system, metabolised and excreted.

To ascertain the significance of the antioxidant contribution of culinary herbs to the diet, studies on the impact of gut bioavailability on the antioxidant capacity and action of processed culinary herbs are required.

A model commonly used to investigate the bioavailability of the constituents of food is the Caco-2 cell epithelial transport model. This model of bioavailability has been validated and used for the past 25 years because of its morphological and biochemical similarities to enterocytes. The Caco-2 cell line, which is derived from a human colon adenocarcinoma, when differentiated, possesses key structural characteristics of human enterocytes, specifically apically directed microvilli and tight junctions (Pinto *et al*, 1983). Although *in vitro* studies may never replace the accuracy of human trials, the main advantage of using Caco-2 cells as a model of bioavailability is the low cost compared to animal and human studies and also its impressive high degree of agreement with human studies, (Mc Clement and Decker, 2009) therefore this model was deemed appropriate for the purpose of the current study.

Thus, the aim of this study was to investigate the impact of epithelial transport on the bioavailability of polyphenols, including their total phenolic content and total antioxidant capacity, post cooking and digestion of the culinary herbs under investigation.

6.1 Material and methods

6.1.1 Material

Reagents

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulphonic acid) in diamonium salt form, (ABTS), Folin-Ciocalteu's phenol reagent, Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, 97% (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), potassium persulphate ($K_2S_2O_8$), Transwell 6 well plates and Coning inserts, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 2-(N-Morpholino) ethanesulphonic acid (MES) all purchased from Sigma Aldrich, Poole, UK. Dulbecco's Modified Eagle Medium (DMEM) with Glucose (4.5g/L), 1 % non-essential amino acids (NEAA), L-glutamine and heat inactivated fetal bovine serum were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Caco-2 cells were purchased from the European Collection of Cell Culture (ECACC), Health Protection Agency, Salisbury, UK.

Culinary herbs

Four culinary herbs, parsley, rosemary, sage and thyme were selected as stated in Chapter 2 and purchased from "Neal's Yard remedies" Richmond, Surrey, UK.

Preparation of herb extracts

Extracts of herbs heated and digested *in vitro* were prepared as previously described, (please see Chapter 5 for details).

Following the extractions, hot solutions were cooled quickly using cold water, entire solutions were first filtered using a Whatman #1, then filter sterilised using a 0.22 µm filter membrane (Millipore), aliquoted into microcentrifuge tubes (1.5 ml) labelled and frozen at -80 ° C until needed.

6.1.2 Methods

6.1.2.1 Determination of antioxidant activities of herb extracts

Trolox equivalent antioxidant capacity assay (TEAC) and Total phenolic content/ gallic acid equivalent assay (GAE) were performed as described in chapter 3, (section 3.4.1 and 3.4.2 respectively).

6.1.2.2 Cell culture

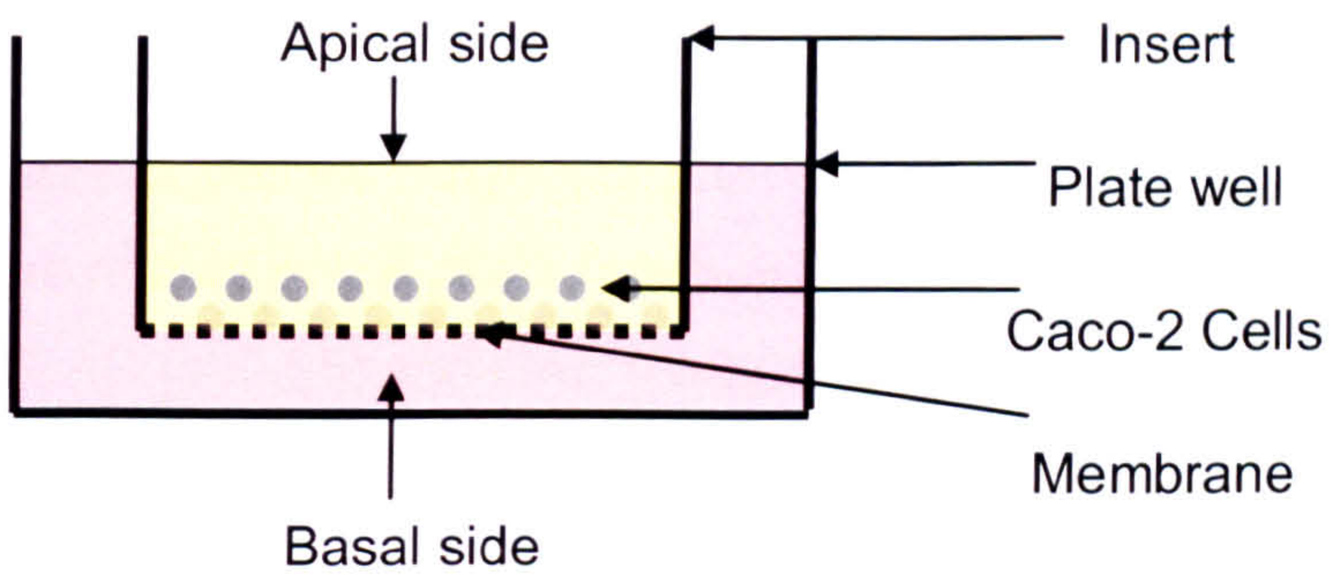
Caco-2 cells (ECACC) were established and maintained by serial passage in a sterile tissue culture environment. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with glucose (4.5g/L), 1 % non-essential amino acids (NEAA), 1% L-glutamine and heat inactivated fetal bovine serum (10%). Resuscitation of Caco-2 cell, passage 43, was done as per the manufacturer's recommendations. Briefly, vials were left at room temperature for one minute, and then thawed in a water bath (37°C) for another minute. The entire content of the vial was then transferred to a T-25 culture flask containing culture medium (10ml) and then placed in an incubator (37°C, 5% CO_2). Medium was replaced every 3-4 days until the cells reached 70-80% confluency (Behrens and Kissel, 2003). For details of cells growth curves please see Appendix 2.

6.1.2.3 Bioavailability experiments

6.1.2.3.1 The setting up of the cells

Caco-2 cells were seeded at 2 $x10^5/cm^2$ on Transwell Coning inserts (4.7cm^2 surface area and 0.4µm pore size) inside 6 well plates, and incubated for 21 days in DMEM supplemented with 1 % non-essential amino acids (NEAA), 1% L-glutamine 100U/ml penicillin,100µg/ml streptomycin, and 10% heat inactivated foetal bovine serum. Culture medium was replaced every 3-4 days (Beherns & Kissel 2003). The integrity of the monolayer was determined using transepithelial electrical resistance (TEER) as a measure of the integrity of the monolayer. After 21 days, TEER was checked with a Millicell-ERS unit (Millippore) and cells with TEER above and below 300-350 Ω were discarded as this range indicates that the integrity of the monolayer has not been compromised (Li *et al.*, 2005). For details of how TEER was measured and the model was validated, please see Appendix 3.

Figure 6.1 Caco-2 epithelial transport set up illustration .



6.1.2.3.2 Transport buffers

Transport buffers were prepared under sterile conditions: Hanks' balance salt solution (HBSS) was prepared (1 L) with 4.7 ml sodium carbonate (1M) pH 7.6. This was filtered sterilised using a 0.22µm filter membrane (Millipore), and aliquoted into two 500 ml bottles. The first bottle contained buffer (A) which was adjusted to pH 7.4 with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (25 mM). HEPES buffer A (1.2 ml) was used throughout the experiment for equilibration on the basal side of the insert (Figure 6.1). The second bottle contained buffer (B) which was adjusted to pH 6 with 2-(N-Morpholino) ethanesulphonic acid hydrate, 4-Morpholineethanesulphonic acid (MES) (25 mM); this buffer (B) (0.4 ml) was used on the apical side for equilibration (Figure 6.0). The pH gradient across the monolayer was maintained. Extracts were diluted 1 in 10 with transport buffer pH 6 (the dilution did not affect the pH of the solution (pH 6) on the apical side and transportation was carried out for 60 and 120 minutes in triplicate as determined using the TEER validation tool, please see Appendix 3, figure 3.A.2 for details on the TEER validation tool. Appropriate blank samples were set up. Positive controls (no monolayer) were also run in order to calculate a percentage passage across the monolayer with the positive control assumed to be 100% passage. At 60 and 120 minutes, samples from the basal side were carefully removed. After each sampling, fresh buffer A was added to replenish the basal side, samples stored at -80°C.

6.1.2.3.4 Impact of extracts of digested herbs on Caco-2 monolayer.

Preliminary cytotoxicity tests showed that the digest caused the membrane to detach from the inserts. This cytotoxic effect was reduced by pouring the digest in glass test tubes placed in boiling water boiled 5 minutes. The cytotoxic effects on

the Caco-2 cells and the % viability of cells did not fall below 97.5% for all three concentrations (25, 50 and 75%) over time (30 to 240 min), please see appendix 4 for details on viability data. Therefore all heated and digested (H&D) herb extracts were boiled for 5 minutes prior to carrying out the transport experiments. Boiling the heated and digested herb extracts for 5 minutes did not significantly reduce the TEAC ($p \geq 0.05$) and GAE ($p \geq 0.05$) of H&D herb extracts please see appendix 5 for details on AA of herbs H&D compared to H&D boiled.

6.1.2.3.5 Determination of dilution of herb extracts heated, digested and boiled for transport study.

Growth curves of Caco-2 cells were calculated to determine correct dilutions of heated and digested herb extracts to use to set up the monolayers. Cells were seeded 2x 10^5 cells per ml in 24 well plates and grown for 7 days. Dilutions of 1 in 2, 1 in 5, 1 in 10 and 1 in 100 were set for each extract of heated and digested herb in duplicate with blanks (sterile water) and counted daily for three consecutive days using the trypan blue exclusion assay, please see appendix 6 for details on growth curves.

6.1.2.3.6 Scanning Electron Microscopy on Caco-2 monolayer.

Scanning electron microscopy (SEM) using a Zeiss EVO50 Scanning electron microscope (Carl Zeiss, Hertfordshire, UK) was used in this study to provide physical evidence of the presence of microvilli in the Caco-2 cells. Samples of membranes on inserts (n=4) were washed twice with cold PBS and prepared for SEM. Primary fixation of the membranes was done using 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for 1 hour, then washed four times with PB for 15 minutes. Secondary fixation was done in 1% osmium tetroxide in PB for 1 hour and aspirated. Membranes were then dehydrated on both the apical and basal sides in six stages by applying ethanol mixed with de-ionised water (50, 70, 80, 90, 95 %) each for 20 minutes and final 6th stage with 100% ethanol three times for 20 minutes. Membranes were then immersed in hexamethyldisilazane (HMDS) for 5 minutes and air dried for 24 hours. Membranes were separated from the plastic culture inserts using a scalpel and tweezers and carefully mounted on double sided, adhesive carbon tabs which were fixed on aluminium stubs. They were then sputter coated with gold palladium. The samples were examined in secondary mode using a Zeiss EVO 50 SEM at an accelerating voltage of 20 kV, after ascertaining that no damage to the samples was induced by this voltage.

6.2 Expression of data and statistical analysis

The bioavailability of polyphenols from extracts of heated and digested herbs are expressed as the final TEAC/GAE on the basolateral side – the TEAC/GAE for the blank) /TEAC/GAE of extracts of heated and digested herb in 400µl*100). Statistical analysis was done using SPSS for windows. The independent sample T test was used to compare H&D herb extracts non-boiled to H&D herb extracts boiled. The independent sample T test was also used to compare % bioavailability at 60 min to that at 120 min incubation for each herb and to compare % bioavailability between TEAC and GAE assays for each herb at each incubation time.

6.3 Results.

6.3.1 Determination of dilution of herb extracts heated, digested and boiled for transport study.

Extracts diluted 1 in 2 and 1 in 5 showed clear signs of causing growth curve inhibition of the Caco-2 cell line at 48 and 72 hours (Figure A6.1 and A6.2 in Appendix 6), suggesting these may be too concentrated for the experiment and were affecting the natural growth of the cells. Extracts diluted 1 in 10 and 1 in 100 dilutions showed growth rates similar to those for the blanks suggesting that these concentrations did not affect the cells growth (Figure A6.3 and A6.4 in Appendix 6). In order to maximise the sensitivity of the experiments, the higher concentration (extracts diluted 1 in 10) was used for the transport experiments.

6.3.2 Scanning Electron Microscopy on Caco-2 monolayer.

SEM showed that the Caco-2 monolayer was successfully established. Pores and microvilli, which appear to be interlocked or fused, are clearly visible (Figure 6.2a&b).

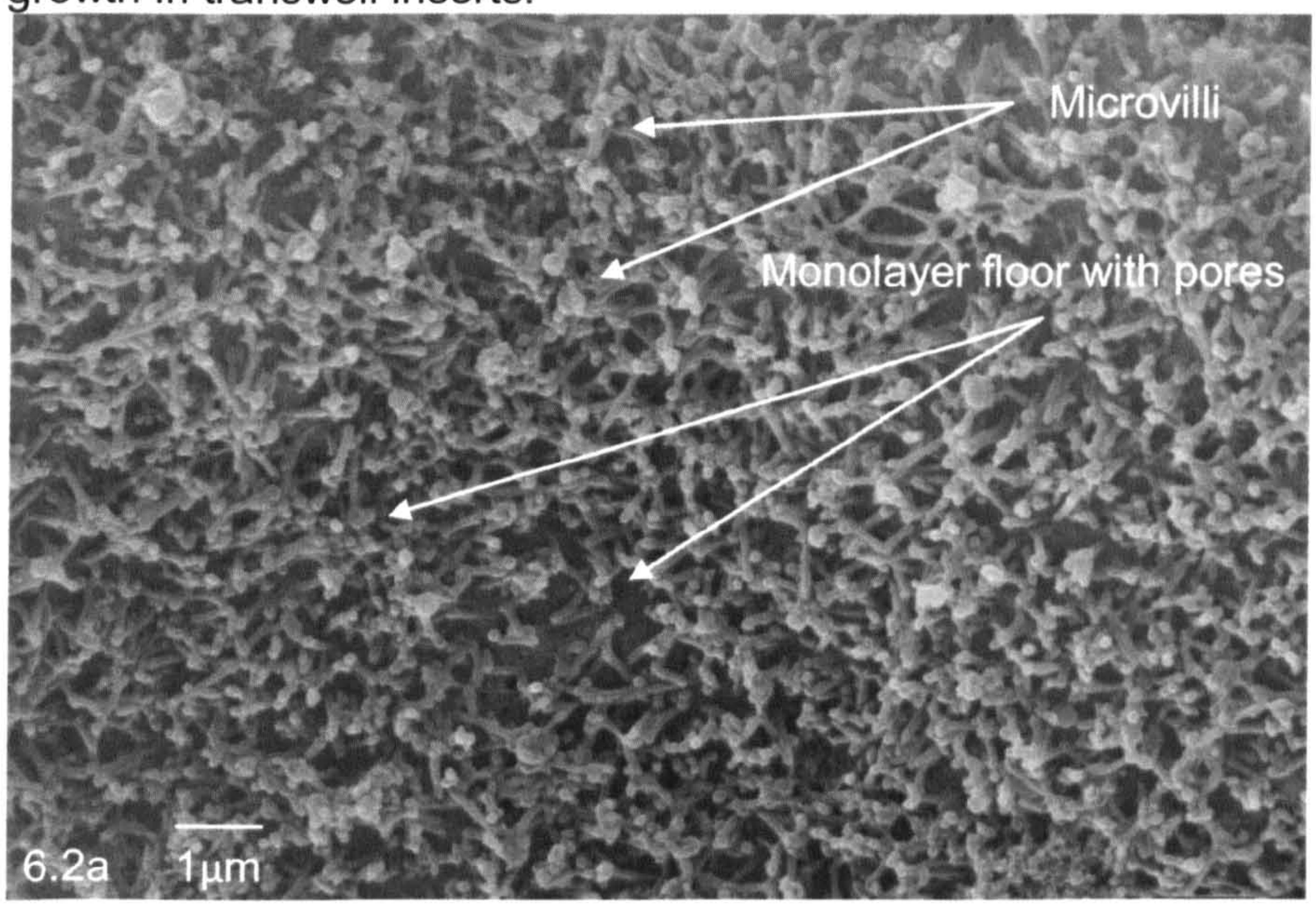
6.3.3 Bioavailability of polyphenols from extracts of heated and digested herb.

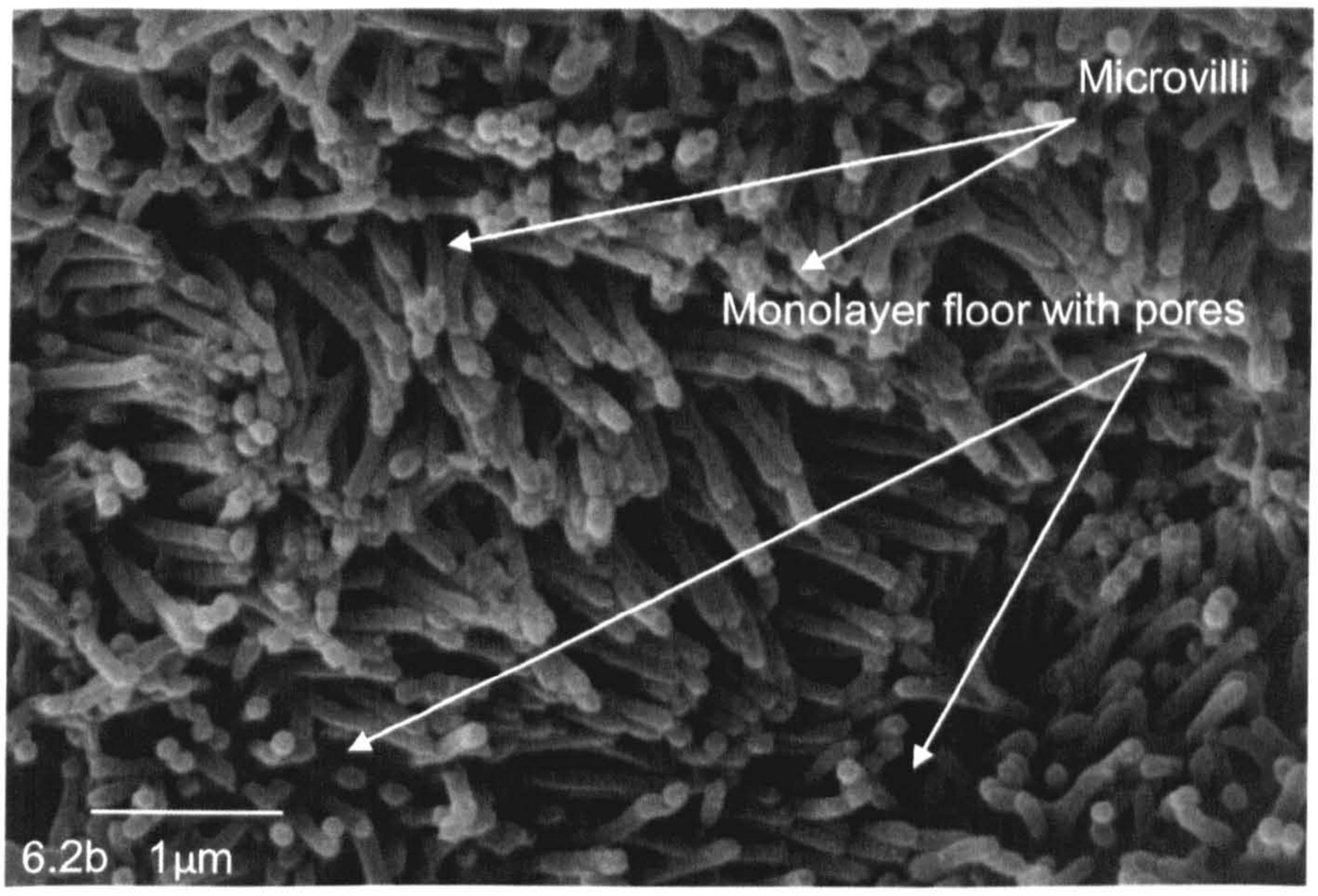
Percentage bioavailability expressed as TEAC was between 8.5% ± 1.5 and 15 % ± 2.1 and expressed as GAE was between 8.33% ± 2.7 and 14.8% ± 6.5 (Table 6.1).

There were no statistical differences between herb extracts % bioavailability at 60 min and 120 min for TEAC assay ($p>0.05$) and no statistical differences between

herb extracts % bioavailability at 60 min and 120 min for GAE assay ($p>0.05$). There were no significant differences in % bioavailability between TEAC and GAE assays for each herb at each incubation time ($p>0.05$) with the exception of rosemary 60 min incubation (TEAC: 12.9±1.3% and GAE: 9.5±3.1%) ($p\leq0.05$) and rosemary at 120 min incubation (TEAC: 15±2.1% and GAE: 10±2.9%) ($p\leq0.05$).

Figure 6.2a and 6.2b Scaning electron micrographs of Caco-2 cells after 21 days growth in transwell inserts.





(6.2a) X 20,000, (6.2b) X 42,000

Table 6.1 Percentage bioavailability of antioxidant activity in digested and boiled stir fried herbs.

Samples	**TEAC % Bioavailability mean ±SD**	**GAE % Bioavailability Mean ±SD**
Parsley digested and boiled post absorption (60 minutes)	12.6 ± 1.4	13.3 ± 6.2
Parsley digested and boiled post absorption (120 minutes)	13.9 ± 2.6	14.8 ± 6.5
Rosemary digested and boiled post absorption (60 minutes)	12.9 ± 1.3	9.5 ± 3.1*
Rosemary digested and boiled post absorption (120 minutes)	15 ± 2.1	10 ± 2.9*
Sage digested and boiled post absorption (60 minutes)	11.1 ± 1.5	8.33 ± 2.7
Sage digested and boiled post absorption (120 minutes)	12.1± 2.1	10.6 ± 1.2
Thyme digested and boiled post absorption (60 minutes)	8.5 ± 1.5	8.83 ± 2.7
Thyme digested and boiled post absorption (120 minutes)	11.9 ± 4.5	10.5 ± 1.3

Percentages are expressed as the final TEAC on the basolateral side – the TEAC for the blank) /Total TEAC of herb stir fried digested in 400µl*100) and as as the final GAE on the basolateral side – the GAE for the blank) /Total GAE of herb stir fried digested in 400µl*100) with standard deviations. Significant differences in % bioavailability between TEAC and GAE assays: *$p<0.05$, n=3.

6.4 Discussion.

The aim of this study was to investigate the role of absorption on the antioxidant activity (AA) of a selection of culinary herbs. The SEM images obtained demonstrate the integrity of the monolayer grown on collagen coated inserts for 21 days used as an *in vitro* model of transport and bioavailability in this study. The presence of a tight monolayer indicates that there was little to no leaking of compounds from the apical to the basal side of the membrane thus indicating that the model was appropriate for investigating bioavailability.

The polyphenols' flux was measured by assessing the AA on either side of the membrane at 60 and 120 minutes of incubation and their bioavailability was on average 11.5% with a good agreement between assays, and supports the hypothesis that polyphenolic antioxidants can cross through the epithelial membrane of the gut into the blood. These results suggest that only a small portion may reach the systemic circulation and therefore the rest of the body and logically that most of the polyphenols in these herbs may be acting as "local antioxidants" in the gut. Establishing whether or not this AA post absorption had a threshold (a point above which there was no further increase in AA) would have helped to shed further light as to whether or not most of the polyphenols act locally but bioavailabilty beyond 120 minutes was not investigated.

Concerning the above suggestion, the predominant polyphenol in rosemary sage and thyme has been shown to be the hydroxicinammic acid, rosmarinic acid (Wang *et al.*, 2004; Kivilompolo *et al.*, 2007 a & b; Shan *et al.*, 2006; Yoshikawa *et al.*, 2000; Lu and Foo, 2001). Konishi and Kobayashy, (2005) investigated the bioavailability of this polyphenol using the Caco-2 cell model and HPLC. They reported that rosmarinic acid (as well as gallic acid) has a low affinity for the monocarboxylic acid transporters (MCT) (possibly due to the ester groups) and cross the Caco-2 cell monolayer mainly via paracellular diffusion. From these findings they concluded that the intestinal absorption efficiency of pure rosmarinic acid was not only low (99.88% remained on the apical side) but that rosmarinic acid was resistant to metabolism by Caco-2 cell mucosa esterase hydrolysis. However, in relation to the current study, as the most abundant polyphenol present in the herbs, it is possible that rosmarinic acid contributed to the AA measured on the basal side of the Caco-2 membrane.

Human intervention studies have shown that rosmarinic acid obtained from plant extracts can be absorbed in the upper part of the gastrointestinal system (Baba *et al.*, 2005; Nurmi *et al.*, 2006). Baba *et al.* (2005) detected rosmarinic acid (methylated), and conjugated forms of caffeic acid, ferulic acid and m-coumaric acid, with 75% of total rosmarinic acid metabolites (free and conjugated) being excreted in the urine within 6 hours of ingestion (these represented 6.3%± 2.2 of the total initial dose of rosmarinic acid).

Concerning other hydroxycinnamic acids, it is possible that caffeic acid, ferulic acid and p-coumaric acid (all known to be present in the culinary herbs investigated) may have contributed to the AA on the basal side. Kern *et al.* (2003) showed that the Caco-2 *in vitro* model was able to metabolise such polyphenols, specifically the dietary hydroxycinnamates and diferulate esters, by various phase I (de-esterification) and phase II (glucuronidation, sulfation, and O-methylation) reactions, suggesting that the epithelium of the small intestine is involved in the metabolism of these phenolic compounds. Other studies have reported that caffeic acid is absorbed from the upper part of the gastrointestinal tract (GI) (Konishi *et al.*, 2006; Lafay *et al*, 2006). Olthof *et al.* (2001) reported that 11% of an orally administered dose of pure caffeic acid (2.8mM) was absorbed in the upper part of the GI tract, metabolised and rapidly excreted in humans. A recent review on the bioavailability of hydroxycinnamates (pure compounds) suggests that their relative bioavailability is in the following order, rosmarinic acid<caffeic acid<ferulic acid<p-coumaric acid involving passive diffusion, MCT transport and possibly facilitated diffusion (Zhao and Moghadasian, 2010). However, it is possible that food matrices may affect this order. For instance, Konishi and Shimizu, (2003) demonstrated that a significant percentage of pure ferulic acid (30.5% at 50 min incubation) was able to cross the Caco-2 membrane via monocarboxylic acid (MCT) mediated transport and paracellular diffusion. This percentage is much higher than that obtained in the current study (11.5%) and this difference may be due to the food matrix and/or the array of polyphenols present in the herb extracts which vary in their ability to cross the Caco-2 membrane.

Concerning the biological relevance of the bioavailability of polyphenols from food matrices such as the culinary herbs, Nurmi *et al.* (2006) in their study on encapsulated oregano leaf extracts in human subjects, reported that most ingested rosmarinic acid is quickly absorbed in the upper part of the

gastrointestinal tract, metabolised and excreted as p-hydroxybenzoic acid (possibly from the metabolism of p-coumaric acid) ferulic acid and p-coumaric acid, with a peak urine concentration at 2-6 hours after ingestion. In the context of the current study, the study by Nurmi et al (2006) suggests that some rosmarinic acid present in culinary herbs may be quickly metabolised and excreted. If this is true its relevance as biologically active molecules (antioxidants and anti-inflammatory) may be questionable bearing in mind that rosmarinic acid is excreted within 6 hours after ingestion according to Baba *et al.*, (2005) and Nurmi *et al.*, (2006); rosmarinic acid would have to act quickly to be biologically relevant. Future studies would need to involve determining the biological activity of rosmarinic acid and the other hydroxycinnamic acids (as part of a food matrix) over a set period of time following ingestion and mapping that activity to plasma and urine levels.

In addition to the upper part of the GI tract it is suggested that the colon, specifically its microflora influence the bioavailability of pure polyphenols (Konishi and Kobayashy 2005). However, colonic microflora may also play an important role in the bioavailaibility of polyphenols from a food matrix (Konishi and Kobayashy, 2005). If one considers the food matrices of the culinary herbs investigated most of the hemicelluloses and celluloses in plant food form the bulk of human faeces which helps with colonic transit, however some naturally occurring gut bacteria are able to break these linkages down in the human gut via fermentation. Over 400 species of gram positive and gram negative bacteria strains have been reported in the human gut, including *Bifidobacteria, Bacterioids, Lactobacteria, Clostridia, Coliforms, Eubacteria, Streptococci* (Groff and Gropper, 1998). Thus the non starch polysaccharide constituents of culinary herbs, could be broken down further by gut flora as suggested by Kroon *et al.* (1997); Scalbert *et al.* (2000); Manach *et al.* (2004); Tuohy *et al.* (2006). Thus, it is possible that the polyphenols in these herbs can, via their exposure to the colonic microflora, following the breakdown of cellulose, be metabolised and absorbed in the colon. The work of Jenner *et al.* (2005) goes some way to support this possibility as they showed that the phenolic acid, phenylacetic acid and its metabolites 3-phenylpropionic acid and 3-(4-hydroxy)-phenylpropionic acid were found in high amounts in human faecal water.

Regarding the rosmarinic acid that reaches the colon, studies suggest that it is metabolized and degraded by colonic microflora to m-coumaric acid and caffeic acid which are subsequently absorbed, conjugated and methylated in tissues and then excreted in urine over time (up to 48 hours) (Nurmi *et al.*, 2006). Thus rosmarinic acid (from plant derived foods such as culinary herbs) may be more relevant as a local antioxidant and anti-inflammatory agent, for chronic gastro-intestinal diseases as, based on the literature discussed above, it appears to have a longer window of opportunity in the gut and thus, possibly, a more clinically significant effect.

Concerning the other hydroxycinnamic acids thought to contribute to AA post absorption, if portions of these compounds in culinary herbs, when ingested, never reach the colon and are quickly metabolised and excreted their potential as biologically significant molecules (antioxidants and anti-inflammatory) may also be questionable. However, for those that do reach the colon "protected" within the plant cells, they may be metabolised (Kern *et al.*, 2003) and/or further degraded by colonic bacteria, absorbed and transported to the systemic circulation by both paracellular diffusion and MCT transporter molecules. Thus their biological and clinical significance/potential may be greater. Furthermore, smaller hydroxycinnamic acids such as ferulic acid and p-coumaric acid may be absorbed and transported to the systemic circulation more efficiently than larger molecules such as rosmarinic acid which first has to be metabolized and degraded by colonic microflora to m-coumaric acid and caffeic acid as stated above (Konishi and Kobayashy 2005). However, in the context of the current study, the amounts of these compounds present in the herbs extracts remains to be detected and quantified in order to attempt to determine their biological relevance.

6.5 Conclusions.

This study showed that on average 11.5% of the initial AA present on the apical side was made available on the basal side after 60 and 120 minutes thus indicating that some polyphenols from the culinary herbs would be made available to the systemic circulation. Whether or not there is a threshold to the passage of polyphenols remains unknown and warrants further research to help determine the roles of the polyphenols as locally (in the gut), or systemically, acting compounds. In addition, the identification and quantification of those polyphenols that are made

available post absorption would be required to further understand the significance of these culinary herbs as dietary contributors of polyphenols.

Chapter 7 Biological activity of processed Labiatae: An investigation of the anti-inflammatory properties of rosemary, sage and thyme.

7.0 Introduction

There is a body of evidence that indicates that polyphenols possess anti-inflammatory activity (Singh and Aggarwal 1995; Baba *et al.*, 2004; Yoon and Baek, 2005; Tapsell *et al.*, 2006; Peng *et al.*, 2007; Dhandapani *et al.*, 2007; Lin and Lin 2008; Singh *et al.*, 2008; Kaefer and Milner 2008; Romier *et al.*, 2008; Romier-Crouzet *et al.*, 2009). As well as their antioxidant activity (AA) by single electron transfer to reduce radicals (TEAC and GAE assays), there are several possible mechanisms of action by which polyphenols may help to combat inflammation. Yoon and Baek, (2005) suggest that polyphenolic compounds such as the phenolic acid gallic acid and the flavonoid apigenin have effects on one or possibly several cellular pathways that are involved in the inflammatory process. These pathways include the arachidonic acid independant pathways, which includes peroxisome proliferator activated receptors (PPARs), Nitric oxide synthase (NOS), nuclear transcription factor kappa B (NF-κB), and NAG-1, as well as the non-steroidal anti-inflammatory drug (NSAID) activated gene. NF-κB is a transcription factor, part of an inactive complex in the cytoplasm that migrates to the nucleus upon activation by free radicals, inflammatory mediators or other stimuli. NF-κB induces the expression of over 200 genes that affect inflammation as well as cell apoptosis, proliferation and metastasis, all of which are potential activators of cancer (Yoon and Baek, 2005).

Culinary herbs have been reported to possess anti-inflammatory activity and polyphenols are thought to be responsible for this anti-inflammatory activity (Singh and Aggarwal 1995; Dhandapani *et al.*, 2007; Lin and Lin 2008; Romier *et al.*, 2008) however, as indicated above, the mechanism of action is not clear. A study by Lu and Foo (2001) showed that polyphenols present in sage extracts possess superoxide dismutase (SOD) mimetic activity (SODm). SOD is one of the most important cellular antioxidant enzymes in biological systems. It catalyses the removal of superoxide radicals generated by cells during aerobic respiration, enzymatic reactions and drug metabolism (the superoxide radicals are produced by oxidising enzymes including xanthine oxidase) by dismutation into hydrogen

peroxide (H_2O_2) and oxygen, the H_2O_2 is then converted to water by catalase (Garrow *et al.* 2002). Superoxide is also thought to be a messenger of inflammation by controlling cellular enzyme activity (Ullrich *et al.*, 2003).Therefore, the reduction of superoxide radicals either via inhibition of xanthine oxidase, or by SODm activity is a possible mechanism of reducing inflammation, meaning that compounds with SODm activity are potential therapeutic agents in inflammatory conditions (Lu and Fu, 2001; Fisher *et al.*, 2003 and 2004; Samai *et al.*, 2008).

Several studies demonstrated that the polyphenol curcumin, present in turmeric and curry leaves, was able to interrupt NF-κB signalling by inhibiting I-KB kinase (Singh and Aggarwal, 1995; Dhandapani *et al.*, 2007; Lin and Lin, 2008) and Lin and Lin, (2008) suggested that, as such, curcumin has anti-inflammatory properties. Romier *et al.* (2008) demonstrated that the polyphenol chrysin can block NF-κB activation by inhibiting I-KB-alfa phosphorylation and also reduced release of the pro-inflammatory cytokine IL8 by Caco 2 cells (Son *et al.*, 2008).

Studies that have looked in to the anti-inflammatory activities of culinary herbs have used various amounts of herbs and various methods of extractions that do not necessarily relate well to human nutrition. Therefore, little is known about the impact of cooking and digestion on anti-inflammatory activity (if any) of culinary herbs used in a quantity commonly used in domestic kitchens. Furthermore, it is unclear whether this activity is linked to the antioxidant activity of polyphenols in these herbs. Thus, the aims of this part of the study were to determine whether aqueous extracts of rosemary, sage and thyme possess anti-inflammatory activities; what effect cooking and digestion *in vitro* have on such anti-inflammatory properties; and whether these activities are linked to polyphenolic mediated antioxidant capacity. To investigate anti-inflammatory activity two approaches were used: firstly determining if the herbs possessed SODm activity, this approach has been used in previous studies (Lu and Foo, 2000 and 2001; Fisher *et al.*, 2003; Thring *et al.*, 2009; Hague, 2010) to assess the SOD activity of herbs and isolated compounds, secondly, to investigate the effect of the herbs on the release of IL8 from peripheral blood lymphocytes and Caco 2 cells.

7.1 Materials and methods

7.1.1 Materials

Reagents

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulphonic acid) in diammonium salt form (ABTS), Folin-Ciocalteu's phenol reagent, Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (97%) (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), potassium persulphate ($K_2S_2O_8$), nitroblue tetrazolium (NBT), xanthine, xanthine oxidase (XO) (Grade 4 from bovine milk), hydrogen peroxide (H_2O_2), tumor necrosis factor alfa (TNFα), and Citranox® were all purchased from Sigma Aldrich, Poole, UK. Dulbecco's Modified Eagle Medium (DMEM) with Glucose (4.5g/L), 1 % non-essential amino acids (NEAA), L-glutamine, RPMI 1640 medium, 100U/ml penicillin/100µg/ml streptomycin and heat inactivated fetal bovine serum, 24 well plates (Coning), 15ml flasks with vented caps and 25 ml flasks with vented caps (Coning) were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Caco 2 cells passage 44 were purchased from the European Collection of Cell Culture (ECACC), Health Protection Agency, Salisbury, UK. The Quantikine Elisa kit assay D8000C was purchased from R&D systems, (Europe) Limited, (www.R&Dsystems.com).

Culinary herbs

Rosemary, sage and thyme were used (please see Chapter 2 for details).

Preparation of herb extracts

Extracts of uncooked (U), heated (H) (1g, in 25 ml water (37°C)), heated and digested "boiled" (H&D), blank digests (as previously described in Chapter 5).

Preparation of standardised extracts.

Precision is essential when comparing biological activities of plants, and therefore herb standards were prepared. This was carried out by drying preparations of hot extract controls for each herb to prepare aqueous standardised herb extracts at 30 mg of plant material per ml solution.

Standardised extracts were made by placing the entire hot aqueous herb extract solution in a dark glass vial at room temperature. These were left to evaporate under a fan over night until no moisture was visible. The resulting dry material was

weighed and re-suspended with sterile distilled water at 30mg/plant material per ml and stored at -80° C until needed.

7.1.2 Methods

7.1.2.1 Determination of antioxidant activities of herb extracts.

Trolox equivalent antioxidant capacity assay (TEAC) and Total phenolic content/ gallic acid equivalent assay (GAE) were performed as described in chapter 3, (section 3.4.1 and 3.4.2 respectively).

7.1.2.2 Quantification of Metal Ions by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES).

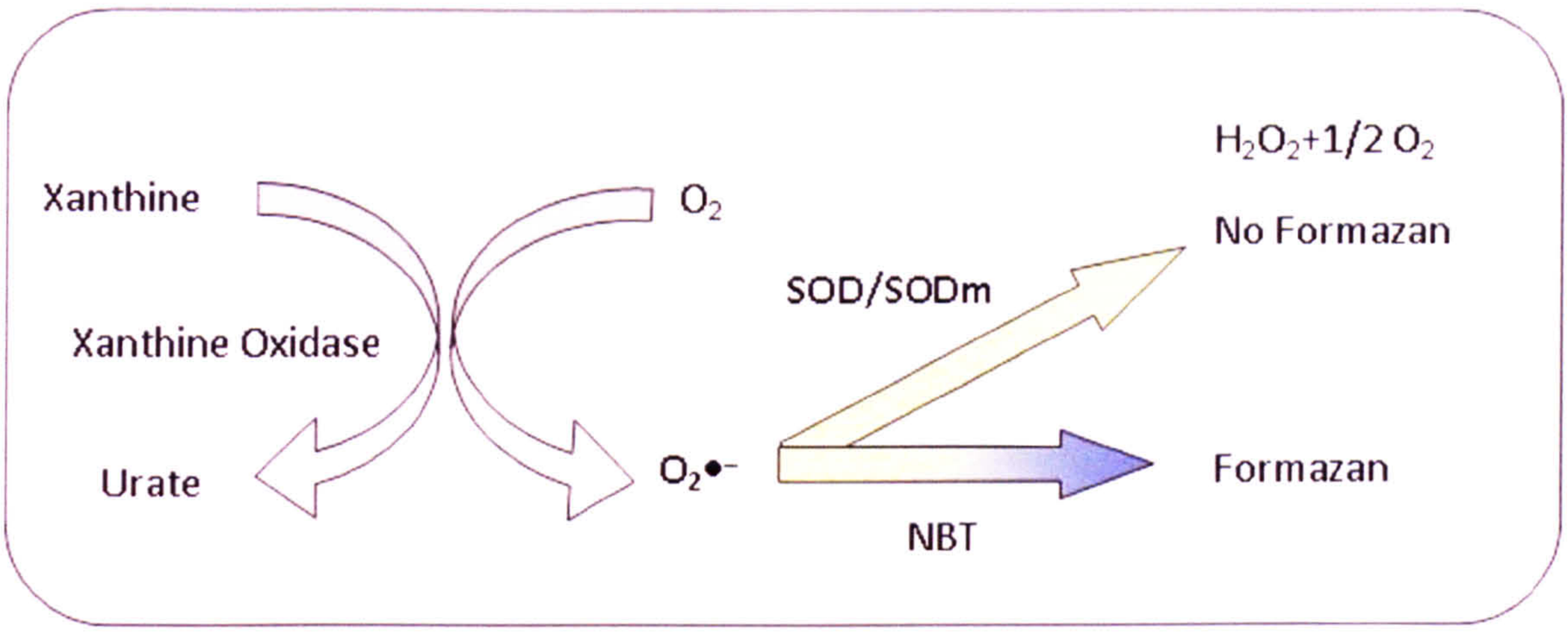
The metal ion content of each herb extract U, H and U&D was determined, using ICP-AES spectrometry using an ULTIMA 2C, Jobin Yvon, France. ICP-AES is an efficient way to determine the transition metal ions content of the herb extracts prior to carrying out the SODm assay as some metal ions could interfere with the assay by electron donation (Hague, 2010).

Glassware used to carry out herb extractions was acid washed with Citranox® acid solution and rinsed three times with deionised water to remove possible metal ion contamination of the samples. Metal ion content was expressed in mg/litre. Herb extracts (10ml) were analysed and data are expressed as mean of duplicates for each sample.

7.1.2.3 Determination of Superoxide Dismutase Mimic (SODm) Activity.

Herb extracts uncooked (U), heated (H), heated and digested (H&D) and standardised (STD, uncooked 30 mg/ml), were assessed for SOD mimetic activity using the nitroblue tetrazolium (NBT) assay (Fisher *et al.*, 2003). The modified NBT assay monitors SODm activity indirectly by converting superoxide ($O_2^{\bullet-}$) and NBT to formazan. The enzymatic reaction between xanthine and xanthine oxidase (XO) produces a solution containing $O_2^{\bullet-}$ and urate. Urate can be measured spectrophotometrically at 290 nm, and in the presence of NBT, $O_2^{\bullet-}$ is converted to a blue solution containing formazan that can be measured at 550nm (Figure 7.1). In the presence of SODm activity $O_2^{\bullet-}$ is converted to hydrogen peroxide (H_2O_2) and oxygen (1/2 O_2). Therefore there is an inverse linear relationship between absorbance at 550 nm (blue formazan) and SOD mimetic activity of the tested samples.

Figure 7.1 The NBT assay.



Phosphate buffer (PBS), (pH 7.4, 50 mM) was degassed under nitrogen to prepare the NBT working solution (50 µM xanthine, 100 µM NBT) which was kept on ice in the dark. The enzyme concentration (XO) was adjusted with PBS to produce a XO stock solution with a rate of 0.025 absorbance units over 3 minutes (550nm) to insure a constant output of $O_2^{\bullet-}$ in the catalytic reaction. All herb extracts were diluted by 1 in 10 in PBS prior to the assay. To assess SODm activity of the herbs, diluted extracts (10 µL) in distilled water (20 µL) and the controls (30 µl distilled water) were equilibrated for 5 minutes with NBT solution (3ml) in a thermostatically controlled Cary 300 UV-Visible spectrophotometer (25°C), then XO stock solution (20 µL) was added and the rate of formazan production was measured at 550 nm over 3 minutes. Superoxide dismutase (3.33 units from bovine erythrocytes) was used as the positive control for assessing superoxide dismutase mimetic (SODm) activity. Tests were performed in triplicate and were also done without XO to ensure extracts alone did not reduce NBT by producing $O_2^{\bullet-}$ (550 nm) as well as without NBT to ascertain whether the extracts inhibited the formation of uric acid from xanthine by xanthine oxidase (measured spectrophotometrically at 290nm).

7.1.2.4 Determination of anti-inflammatory activity.

7.1.2.4.1 Preparation of peripheral blood lymphocytes (PBLs).

Ethical approval was obtained from the Ethics Committee of the Faculty of Science, Kingston University. Subjects were recruited once they had read the participant information sheet (Appendix 7) and had given informed consent (Appendix 7). Blood collections and storage procedures were carried out in accordance with the Human Tissue Act (HTAct). PBLs were isolated from

heparinised venous whole blood (15 ml) using Ficoll-Paque Plus, and then cultured in RPMI media supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100U/ml penicillin/100µg/ml streptomycin, placed in 15 ml flasks with vented caps (Coning) to incubate standing at 37° C in a 5% atmosphere for 24 hours.

7.1.2.4.2 Preparation of Caco 2 cells.

Caco 2 cell lines were established and maintained by serial passage in a sterile tissue culture environment. Cells were cultured using a complete medium containing Dulbecco's Modified Eagle Medium (DMEM). DMEM (500ml) with Glucose (4.5g/L), 1 % non-essential amino acids (NEAA) was used, supplemented with 1% L-glutamine and heat inactivated fetal bovine serum (10%). Cells were then placed in 25 ml culture flasks with vented caps (Coning) containing 10 ml of pre-warmed complete medium (37°C), the flasks were then placed in an incubator (37°C) and 5% CO_2 and grown for 14 days. Media was replaced by new media every 3-4 days until the cells reached 70-80% confluence (Beherns and Kissel, 2003).

7.1.2.4.3 Cytotoxicity tests on PBLs and Caco 2 cells exposed to herb extracts.

PBLs cells were seeded at a concentration of $1x10^5$ cells/ml and incubated with herb extracts (diluted 1:10 in FBS free RPMI media supplemented with 100U/ml penicillin/100µg/ml streptomycin). Caco 2 cells seeded at $2x10^5$ in 24 well plates (Coning) were incubated with herb extracts (diluted 1:10 in FBS free Dulbecco's Modified Eagle Medium (DMEM) with Glucose (4.5g/L), 1 % non-essential amino acids (NEAA), supplemented with 1% L-glutamine. Both cell lines were placed in an incubator (37°C) and 5% CO_2 for 24 hours with herb extracts and assessed for cytotoxicity using the trypan blue assay.

7.1.2.4.4 Investigation of anti-inflammatory effects of herb extracts on PBLs and Caco 2 cells.

The anti-inflammatory activities of the herb extracts were assessed in two ways, first cells were co-incubated with U, H, H&D or standardised (STD, uncooked 30 mg/ml), diluted 1:10 in medium, and hydrogen peroxide H_2O_2 (2mM) or TNFα (100 µg/ml) for 24 hours. Second, cells were pre-incubated for 3 hours with H&D extracts diluted 1:10 in medium and then exposed to hydrogen peroxide (H_2O_2) (2mM) or TNFα (100 ng/ml) for 24 hours. Controls were also set up with H_2O_2 or TNFα only. Spontaneous release of IL8 was assessed by incubating cells in media

only (in the absence of herbs, H_2O_2 or TNFα). Media was then collected and the release of IL8 was determined using the quantitative sandwich enzyme immunoassay ELISA Interleukin 8.

7.1.2.4.4.1 Determination of IL-8

Il-8 was determined using the Quantikine ELISA kit assay, which is based on antibodies raised against the 72 amino acid variant of human IL8 derived from *E. coli.* The kit contained a 96 well microplate pre-coated with monoclonal antibody specific for IL8. A diluent was added to each well (100µl). Standards and samples (50µl) were pipetted into the wells and incubated for two hours at room temperature. The samples/standards were then removed from the wells and the wells were washed four times using the wash buffer. An enzyme-linked polyclonal antibody specific for IL8 was then added to the wells and incubated for an hour at room temperature and then washed again four times to remove any unbound antibody-enzyme reagent. A substrate solution (H_2O_2 with tetramethylbenzidine) (200µl) was added and a pink colour developed proportionally to the amount of IL8 bound. A colour stop solution (50µl) was added to each well in order to stop the development and the intensity of the colour which was measured at 450nm, with a correction at 570nm for more accuracy, using a multiplate Carry 50 Spectrophotometer, Varian, UK.

8.1.3 Expression of data and statistical analysis

Data are expressed as means of triplicate analysis ±SD unless otherwise stated. Statistical analysis and standard deviations were calculated using SPSS for windows. ANOVA with post hoc Tukey were used to compare AA of herbs uncooked (U), heated (H) , heated and digested (H&D- blank digest values) and standardised (STD uncooked 30 mg/ml) and to compare the ICP levels of metal ions (µg/g herb) for U, H and H&D extracts and blank digests (n=2). Results of the NBT assay are presented as a percentage inhibition of formazan production by herb extracts, calculated using a straight line equation, where the maximum rate equals 0% inhibition and where no absorbance change equals 100% inhibition. ANOVA with post hoc Tukey tests were used to compare SOD mimetic activities expressed in % inhibition of formazan production for herbs U, H, H&D and STD. The percentage inhibition of IL8 release from PBLs or Caco 2 cells is presented as means ±SEM (n=3): " % inhibition IL8 =[(IL8 release from respective control cells (H_2O_2 or TNFα)– IL8 release from cells co-incubated or pre-incubated with herb

extracts and H_2O_2 or TNFα])/ IL8 release from respective control cells (H_2O_2 or TNFα)] x100". ANOVA with post hoc Tukey tests were also used to compare the percentage inhibition by herbs preparations U, H, H&D, STD and H&D pre-incubated with H_2O_2 or TNFα against their respective controls. As percentages are bound data the percentages for SODm activity and IL8 inhibition were checked for normality using the Shapiro-Wilk test prior to running the ANOVA tests. Pearson's correlation coefficients (r) with level of significance (p) (2-tailed) were used to compare all herb extracts irrespective of treatments for the following: TEAC and GAE assay results, the AAs (TEAC and GAE) with the SODm activities, and to compare AAs (TEAC and GAE) and percentage inhibition of TNFα or H_2O_2 stimulated IL8 release by PBLs or Caco 2 cells.

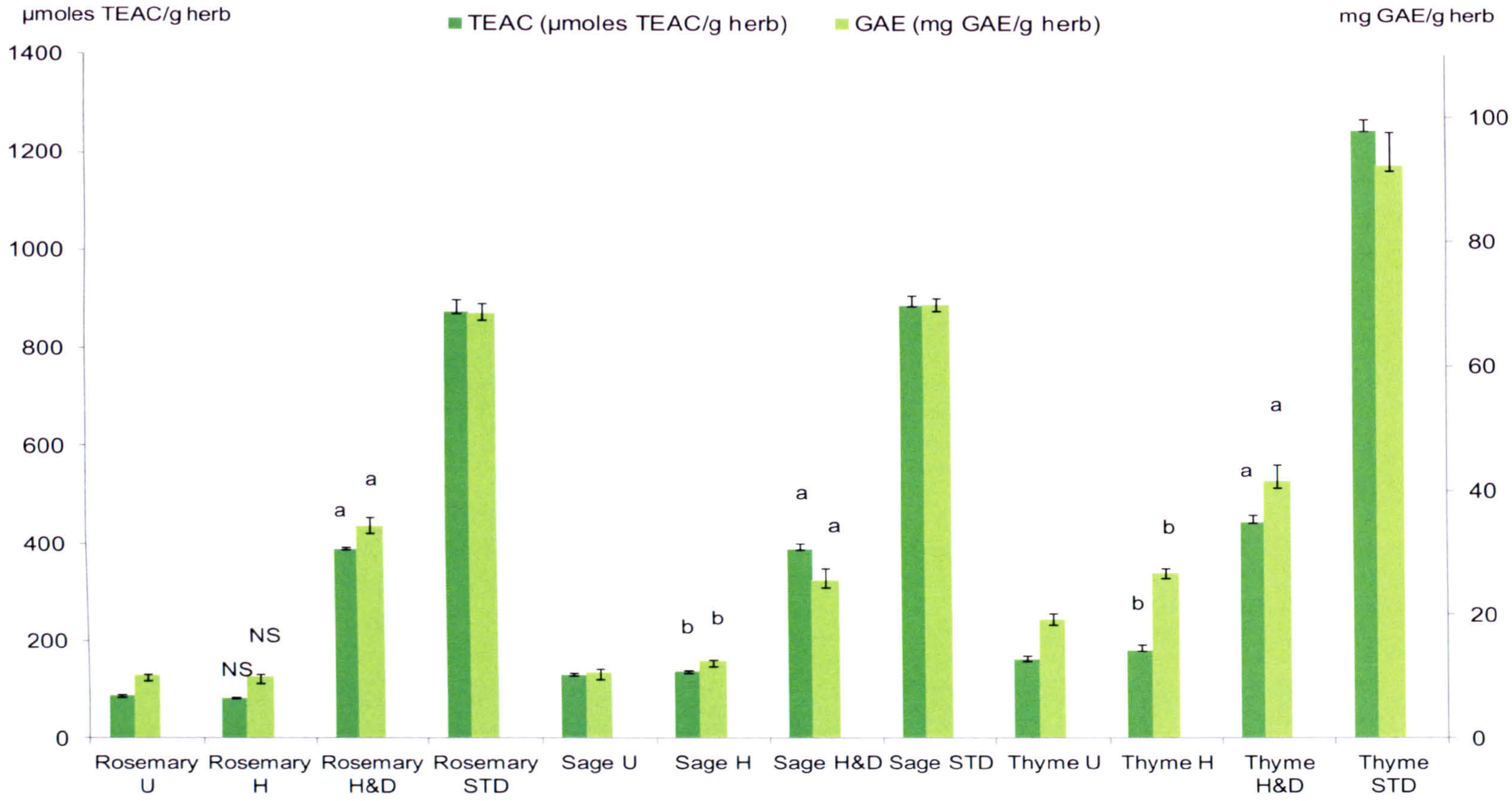
7.2 Results.

7.2.1 Antioxidant activity of herb extract.

The H&D extracts assayed for antioxidant capacity (rosemary 387.1±5.6 µmoles TEAC/g, sage 387.8±12.8 µmoles TEAC/g and thyme 443.5±16.1µmoles TEAC/g) and total phenolic content (rosemary 34.2±1.4 mg GAE/g, sage 25.4±2 mg GAE/g and thyme 41.5±2.7 mg GAE/g) were significantly higher than their heated (H) counterparts (rosemary 82±1.5µmoles TEAC/g, sage 135.7±5 µmoles TEAC/g and thyme 179.8±12.1 µmoles TEAC/g) and (rosemary 9.9±0.4 mg GAE/g, sage 12.5±0.1mg GAE/g and thyme 26.7 ±0.7 mg GAE/g) ($P \leq 0.001$) which were significantly higher than uncooked (U) extracts (rosemary 85.1±4.1 µmoles TEAC/g, sage 129.6±5.4 µmoles TEAC/g and thyme 159.7±9.2 µmoles TEAC/g) and total phenolic content (rosemary 10.2±0.1 mg GAE/g, sage 10.6±0.5 mg GAE/g and thyme 19.2±1 mg GAE/g) ($P \leq 0.05$) except for rosemary ($p \geq 0.05$for both TEAC and GAE assays) (Figure 7.2).

The STD extracts assayed for antioxidant capacity (rosemary 873±27 µmoles TEAC/g, sage 887 ±21 µmoles TEAC/g and thyme 1246.5±23 µmoles TEAC/g) and total phenolic content (rosemary 68.4±1.6 mg GAE/g, sage 69.9±1 mg GAE/g and thyme 92.3±5.4mg GAE/g) were significantly higher than their U, H and H&D counterparts ($P \leq 0.001$).

Figure 7.2 Antioxidant capacity (TEAC) and total phenolic content (GAE) of herb extracts



Data are presented as the mean ±SD (n=3). For standardised extracts, hot herb extracts (1g, 25 ml water) were air dried, then re-suspended at 30mg/ml in sterile water. NS: no significant differences between TEAC rosemary U and rosemary H ($p \geq 0.05$.) and no significant differences between GAE rosemary U and rosemary H ($p \geq 0.05$.). Herbs H&D significantly different from their H counterparts a $p \leq 0.001$, b:≤ 0.05.

7.2.2 ICP-AES analysis of herb extracts.

Data are presented as means of duplicate (n=2) (Table 7.1). Transition metal ions concentration for U and H extracts were not significantly different unless otherwise stated. H&D herb extracts were blanked for metal ions using the blank digest.

Copper (Cu) ion levels for rosemary (4.27±0.80 µg/g), and thyme (3.09±0.75 µg/g) H&D were significantly different from their U and H counterparts ($p \leq 0.05$). Cu ion levels for sage H&D (2.36±0.02 µg/g) was not significantly higher than sage U (0.92±0.03 µg/g, $p=0.080$) and than sage H (1.29±0.03 µg/g, $p=0.269$).

Iron (Fe) ion levels for rosemary (1.00±0.61 µg/g) and sage (5.61±0.04 µg/g) H&D were significantly different from their respective U and H counterparts Fe ion levels for and thyme H&D (3.41±0.66 µg/g) was significantly higher than thyme U (2.02±<0.01, $p=0.042$) but not significantly different from thyme H (2.68±0.46 µg/g, $p=0.499$).

Manganese (Mn) ions levels for rosemary (10.39±1.72 µg/g) and sage (12.32±0.14 µg/g) H&D were significantly different from their respective U and H counterparts ($p<0.05$). The Mn level for thyme H&D (26.28±1.77 µg/g) was significantly different from thyme U (18.2±0.04 µg/g, $p=0.020$) but similar to thyme H (24.4±4.59 µg/g, $p=0.967$). Thyme U and H Mn levels were also significantly different ($p<0.05$).

Nickel (Ni) ion levels for rosemary (0.56±0.14 µg/g), sage (0.01±≤0.01 µg/g) and thyme (0.11±0.04 µg/g) H&D were not significantly different from their U or H counterparts ($p>0.05$).

Vanadium (V) ions levels for rosemary (4.43±0.74 µg/g), and thyme (6.78±0.40 µg/g) H&D were significantly different from their respective U and H counterparts ($p<0.05$). V ions levels for sage H&D (4.82±2.10 µg/g) were not significantly different from its U (3.58±0.01 µg/g) and H (3.58±0.12 µg/g) counterpart ($p>0.05$).

Zinc (Zn) ion levels for rosemary (1.51±0.91 µg/g), sage (7.29±0.13 µg/g) and thyme (8.29±0.99 µg/g) H&D were not significantly different than their U and H counterparts ($p>0.05$).

Table 7.1 ICP-AES data for uncooked (U), heated (H) and heated and digested (H&D) herb extracts

Sample	Cu (µg/g)	Fe (µg/g)	Mn (µg/g)	Ni (µg/g)	V (µg/g)	Zn (µg/g)
Rosemary U	0.69±0.05	0.77±0.06	2.96±0.03	0.36±0.08	1.13±0.04	1.93±0.01
Rosemary H	0.36±0.01	0.57±0.03	2.58±0.03	0.31±0.11	1.03±0.04	1.71±0.04
Rosemary H&D	4.27±0.80**	1.00±0.61**	10.39±1.72**	0.56±0.14	4.43±0.74**	1.51±0.91
Sage U	0.92±0.03	1.33±0.04	6.40±0.01	0.11±0.15	3.58±0.01	7.11±0.09
Sage H	1.29±0.03	1.22±0.03	6.40±0.33	0.15±0.06	3.58±0.12	8.22±0.20
Sage H&D	2.36±0.02	5.61±0.04**	12.32±0.14**	0.01±0.00	4.82±2.10	7.29±0.13
Thyme U	1.30±0.02	2.02±0.00	18.2±0.04	0.27±0.15	3.18±0.0	6.88±0.06
Thyme H	1.86±0.44	2.68±0.46	24.4±4.59	0.40±0.14	3.82±0.53	9.17±1.72
Thyme H&D	3.09±0.75**	3.41±0.66*	26.28±1.77*	0.11±0.04	6.78±0.40**	8.29±0.99

Data are presented as the means ±SD (n=2) at 2decimal place for uncooked (U), heated (H) and heated and digested (H&D), H&D were blanked using the blank digest. *significantly higher than U counterpart, **significantly higher than both U and H counterparts ($p<0.05$)

7.2.3 SODm activity of herb extracts.

All extracts possessed SODm activity (Figure 7.3) and SODm activity data expressed in % inhibition formazan production were normally distributed ($p \geq 0.177$).

The SODm activities of rosemary (24.4±0.9%), and sage (19.5±1.7%) U were significantly lower than those for herbs H (rosemary 32.9±0.2%, sage 27.3±3.1%, $p \leq 0.001$); Rosemary and sage H had significantly lower SODm activities than rosemary and sage H&D (rosemary 38.7±2.4%, sage 32.9±0.3%; $p<0.05$). There was no significant difference between the SODm for thyme U, H and H&D (ANOVA, $p=0.095$).

STD herbs possessed the highest SODm activities (rosemary 65.6±6.1%, sage 58.3±3.1% thyme 70.6±4.1%) and all were significantly higher than those of their respective U, H and H&D counterparts ($p \leq 0.001$)

7.2.4 PBLs and Caco 2 cells' cytotoxicity tests

The viability of both PBLs and Caco 2 cells exposed to herb extracts U, H and H&D for 2 hours were not significantly different from that of the controls ($p=0.715$).

7.2.5 Spontaneous release of IL8 from PBLs and Caco 2 cells compared to TNFα and H_2O_2 stimulated PBL and Caco 2 cells controls

Both PBLs and Caco 2 cells secreted IL8 spontaneously (60.4 ± 1.1pg/ml and 855.5 ± 10.7pg/ml respectively, Table 7.2). Cells exposed to TNFα (100ng/ml) significantly increased IL8 secretions (1011± 63.9pg/ml for PBLs and183 ± 14.5pg/ml for Caco 2 cells) compared to non-stimulated cells ($p \leq 0.05$) and cells exposed to H_2O_2 (2mM) significantly increased in IL8 secretions (1144.6±47.5pg/ml for PBLs and 91.8 ± 8.2pg/ml for Caco 2 cells compared to non-stimulated cells) ($p \leq 0.05$, Table 7.2).

7.2.6 Investigation of anti-inflammatory effects of herb extracts on PBLs.

Percentage inhibition of IL8 data for PBLs was normally distributed for each treatment for each herb ($p \geq 0.101$).

7.2.6.1: Co-incubating PBLs of herb extracts with pro-inflammatory agents.

For herbs U decreases in IL8 release were observed in PBLs but these decreases were only significant for PBLs exposed to H_2O_2 (rosemary 41±8.3%, sage 38.2±1.5% and thyme 36.9±8.6%) ($P \leq 0.05$) and were not significant for PBLs exposed to TNFα (rosemary 25.1±10.9%, sage 24.1±11.8% and thyme 33.8±9.6%) ($p \geq 0.05$).

For herbs H, decreases in IL8 release were observed in PBLs but these decreases were only significant for PBLs exposed to H_2O_2 (sage 39.7±6.5%, and thyme 49.7±8%) with the exception of rosemary (35.2±7.9%). There were no significant decreases for PBLs exposed to TNFα (rosemary 16.4±10.2%, sage 27.7±9.3% and thyme 31.8±8.1%), ($p \geq 0.05$).

For herbs H&D, decreases in IL8 release were observed but these decreases were only significant for PBLs exposed to H_2O_2 (rosemary 41±8.3%, sage 38.2±1.5% and thyme 36.9±8.6%) ($p \leq 0.05$) and were not significant for PBLs exposed to TNFα (rosemary 37.4±8.9%, 26.3±1.7% and thyme 34.7±8.1%) ($p \geq 0.05$).

Extracts of STD herbs (3mg/ml) significantly inhibited the release of IL8 from PBLs exposed to H_2O_2 (rosemary 98.7±0.1%, sage 99.3±0.1% and thyme 99.6±0.3%) and PBLs exposed to TNFα (rosemary 96.1±1.4%, sage 92.5±3.1%, and thyme 99.2±0.3%) ($p \leq 0.001$, Figure 7.4).

7.2.6.2: Pre-incubating PBLs with herb extracts prior to adding the pro-inflammatory agents.

Pre-incubating PBLs with extracts of H&D herbs caused a significant inhibition in IL8 release with both PBLs exposed to H_2O_2 (rosemary 40.4±0.5%, sage 30.5±1% and thyme 46.9±0.5%) and PBLs exposed to TNFα (rosemary 21.6±5.3%, sage 14.7±5.8% and thyme 35.6±3.8%) ($p \leq 0.001$).

7.2.7 Investigation of anti-inflammatory effects of herb extracts on Caco 2 cells.

Percentages inhibition of IL8 data for Caco 2 cells was normally distributed for each treatment for each herb ($p \geq 0.517$).

7.2.7.1 Co-incubating Caco 2 cells with herb extracts and pro-inflammatory agents.

Extracts of H&D herb extracts significantly inhibited the release of IL8 from Caco 2 exposed to TNFα (rosemary 20.9±4.5%, sage 22.6±5% and thyme 25±5.5%) ($p \leq 0.001$, Table 1). No effect was observed for herbs U and H when compared with controls (Figure 7.5).

Extracts of STD herbs (3mg/ml) significantly inhibited the release of IL8 from Caco 2 cells exposed to TNFα (rosemary 47.4±4.2%, sage 41.9±6.4%, and thyme 47.7±5.1%) ($p \leq 0.001$, Figure 7.5).

Herb extracts U, H, H&D and STD had no effects on IL8 release from Caco 2 exposed to H_2O_2 (data not shown).

7.2.7.2 Pre-incubating Caco 2 cells with herb extracts prior to adding the pro-inflammatory agents

Pre-incubating the Caco 2cells with H&D extracts had no effects on IL8 release from Caco 2 exposed to H_2O_2 or TNFα.

7.2.8. Correlations between antioxidant capacity and total phenolic content, SODm and total phenolic content and SODm and antioxidant capacity of herb extracts irrespective of treatments

There was a strong correlation between TEAC and GAE ($r = 0.989$, $p \leq 0.01$), (Figure 8.2). SODm activity and GAE assay ($r = 0.931$) and between the SODm activity and the TEAC assay ($r = 0.929$, $p \leq 0.01$, Table 7.3).

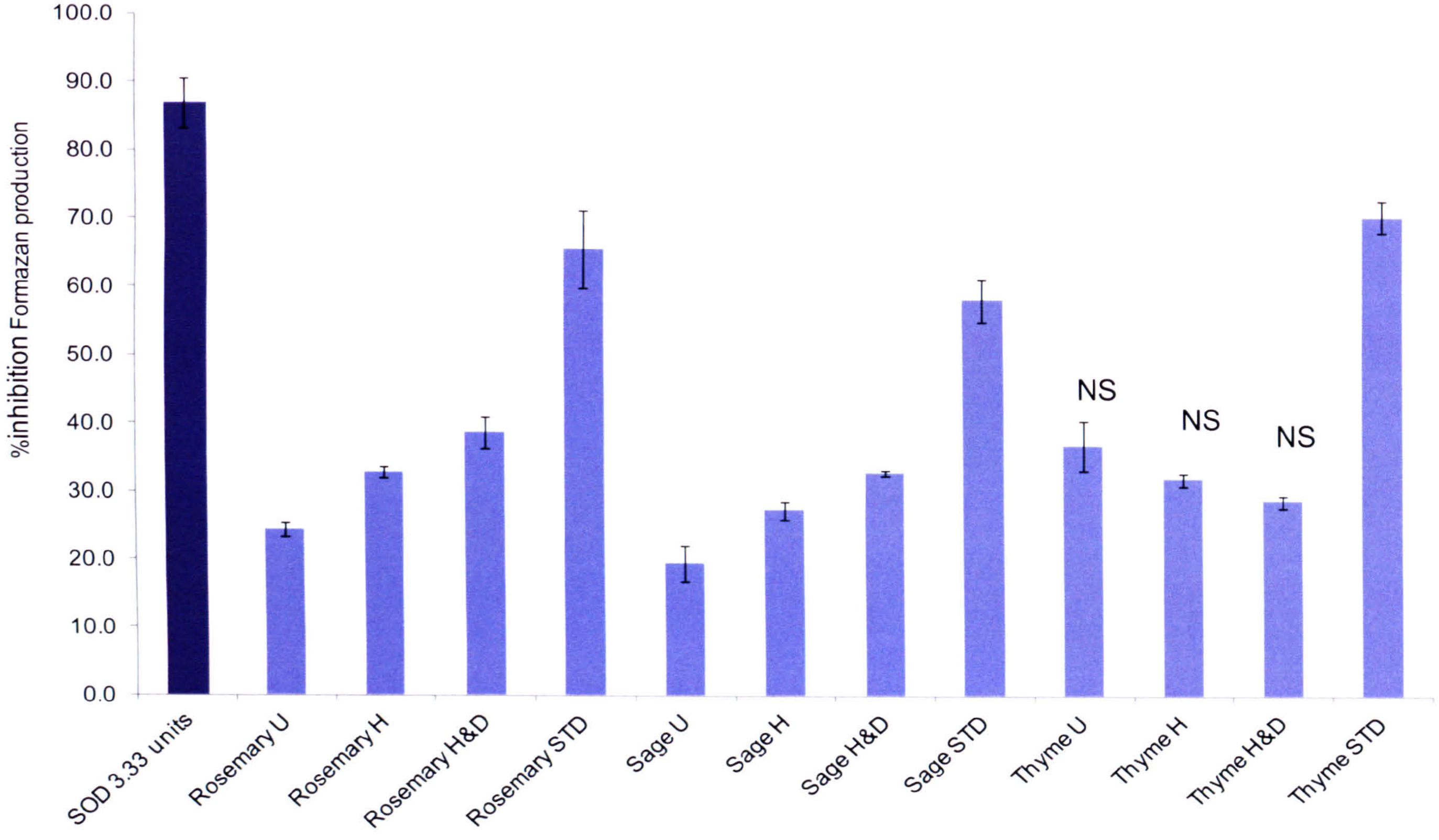
7.2.9 Correlations between, the anti-inflammatory action of herb extracts on PBLsand their total phenolic content and antioxidant capacity irrespective of treatments

There was a strong correlation between the anti-inflammatory action of the herb extracts (U,H, H&D and STD) and their total phenolic content ($r = 0.914$ for action against TNFα, $r = 0.922$ for action against H_2O_2) and between the anti-inflammatory actions of herb extracts (U,H, H&D and STD) and their antioxidant capacity ($r = 0.901$ for action against TNFα, $r = 0.905$ for action against H_2O_2), ($p \leq 0.01$, Table 7.3).

7.2.10. Correlation between the anti-inflammatory action of herb extracts on Caco 2 cells, their total phenolic content and antioxidant activity irrespective of treatments

The % inhibition of the anti-inflammatory action of these herb extracts (U, H, H&D and STD) against TNFα appeared to correlate with their total phenolic content (r= 0.876, p≤0.001), and their antioxidant capacity (r= 0.892, p≤0.01, Table 7.3).

Figure 7.3.SOD mimetic activities of culinary herb extracts U (uncooked), H (heated) H&D (heated and digested),



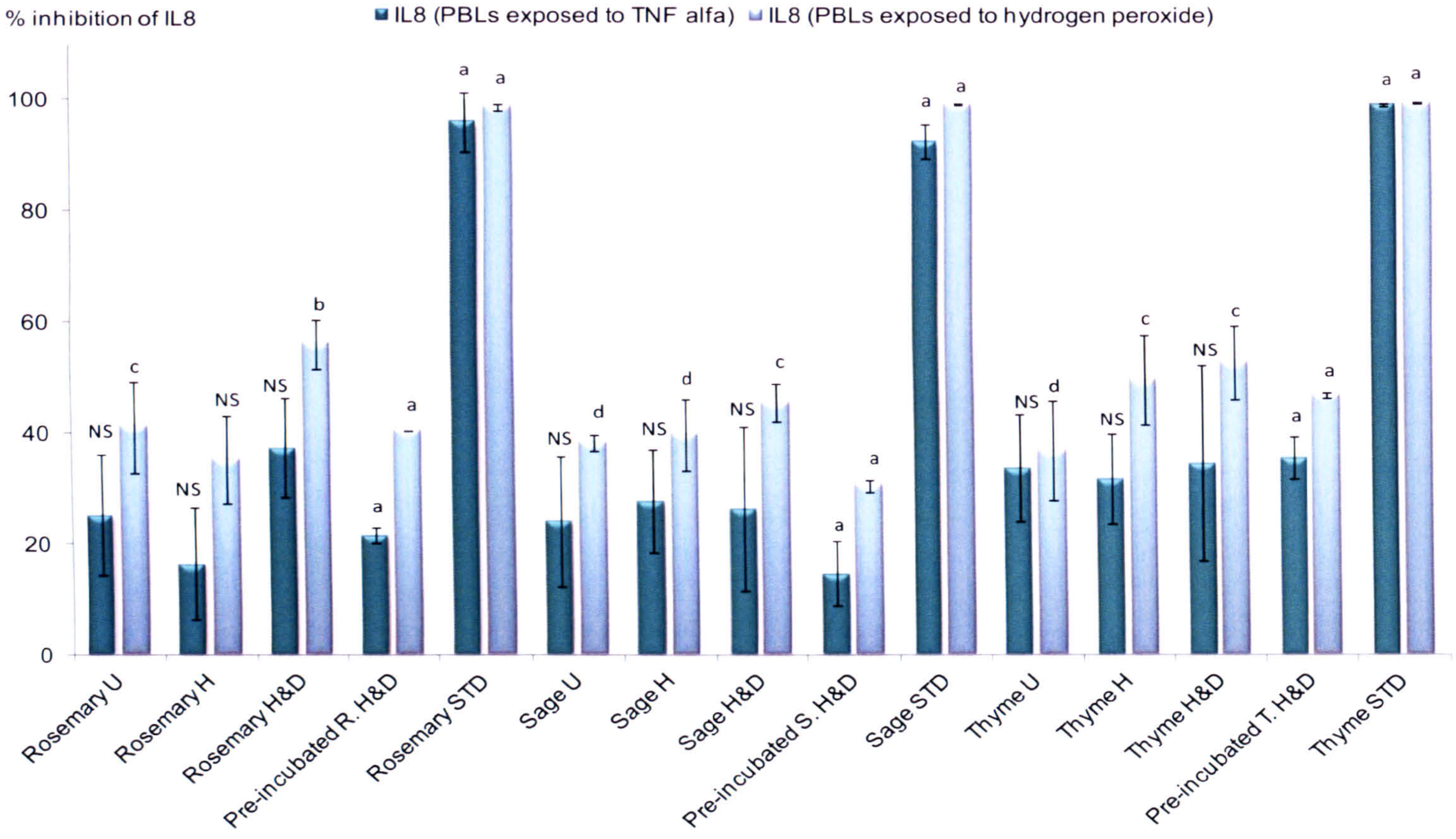
3.33 units/ml SOD = the positive control. Data are presented as the mean ±SD (n=3). Blank digest was subtracted from herbs H&D. NS: no significant differences between thyme U, H and H&D (ANOVA, p=0.095). STD: Standardised extract 30µg in final assay volume.

Table 7.2 Spontaneous secretion of IL8 by PBLs and Caco 2 cells compared to TNFα and H_2O_2 stimulated PBL and Caco 2 cells

	IL8 pg/ml Non-stimulated	**H_2O_2 (2mM)**	**TNFα (100ng/ml)**
PBLs	855.5 ± 10.7 (n=8)	1144.6± 47.5*(n=6)	1011± 63.9*(n=6)
Caco 2 cells	60.4 ± 1.1 (n=6)	91.8 ± 8.2*(n=6)	183 ± 14.5* (n=6)

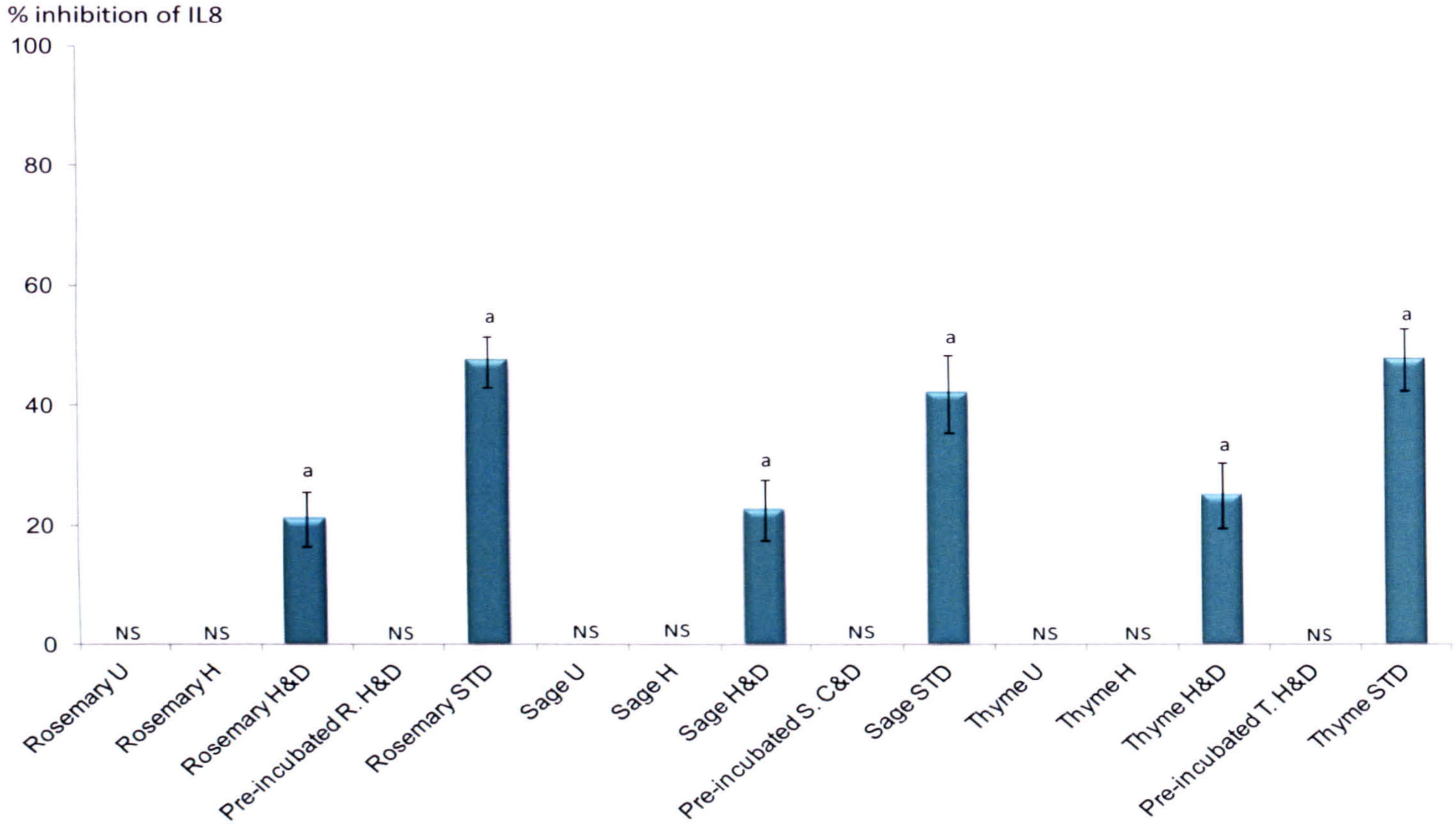
* p≤0.05 for IL8 release from TNFα and H2O2 stimulated cells vs. non stimulated controls.

Figure 7.4 Percentage inhibition of IL8 release by H_2O_2 or TNFα exposed PBLs pre-incubated or co-incubated with herb extracts



Data are presented as the means ±SEM (n=3). % inhibition of IL8 =[(IL8 release from control PBLs(H_2O_2 or TNFα)– IL8 release from PBLS co-incubated/pre-incubated with herb extracts)/ IL8 release from control PBLs(H_2O_2 or TNFα)–] x100,. Significant differences between IL8 released from control PBLs and PBLs incubated/pre-incubated with herb extracts, a P≤0.0001, b P≤0.001, c P≤0.01, d P≤0.05 n=3 . NS: non-significant difference between IL8 release for control PBLS and that of PBLs co-incubated with herb extracts and TNFα or H_20_2, or pre-incubated with herb extracts.

Figure 7.5 .Percentage inhibition of IL8 release by TNFα exposed Caco 2 cells pre-incubated or co-incubated with herb extracts



Data are presented as the Means ±SEM (n=3). % inhibition of IL8 =[(IL8 release from control Caco 2 cells (TNFα)– IL8 release from Caco 2 co-incubated/pre-incubated with herb extracts)/ IL8 release from control Caco 2 cells (TNFα)] x100]. Significant differences between IL8 released from control PBLs and PBLs incubated/pre-incubated with herb extracts, a P≤0.0001, (n=3). NS: non-significant difference between IL8 release for Caco 2 cells control (TNFα) and that of Caco 2 cells co-incubated with herb extracts and TNFα, or pre-incubated with herb extracts.

Table 7.3 Correlations between antioxidant capacities, Total phenolic contents, formazan inhibition and anti-inflammatory activities of culinary herbs, irrespective of treatments STD, U, H, H&D and Pre incubated H&D (if applicable).

Comparisons between assays	**r**	**p**
GAE *vs* TEAC	0.989	≤0.01
% inhibition of formazan production *vs* GAE	0.931	≤0.01
% inhibition of formazan production *vs* TEAC	0.929	≤0.01
% inhibition of IL8 for PBLs exposed to TNFα *vs* GAE	0.914	≤0.01
% inhibition of IL8 for PBLs exposed to TNFα *vs* TEAC	0.901	≤0.01
% inhibition of IL8 for PBLs exposed to H_2O_2 *vs* GAE	0.922	≤0.01
% inhibition of IL8 for PBLs exposed to H_2O_2 *vs* TEAC	0.905	≤0.01
% inhibition of IL8 for Caco 2 exposed to TNFα *vs* GAE	0.876	≤0.01
% inhibition of IL8 for Caco 2 exposed to TNFα *vs* TEAC	0.892	≤0.01
% inhibition of IL8 for Caco 2 exposed to H_2O_2 *vs* GAE	NA	NA
% inhibition of IL8 for Caco 2 exposed to H_2O_2s *vs* GAE	NA	NA

NA= Not applicable, as the herb extracts incubated with Caco 2 cells did not show inhibition of IL8 when exposed to H_2O_2.

7.4 Discussion

The aim of this study was to investigate the effectiveness of aqueous herb extracts of rosemary, sage and thyme at reducing inflammation, what effect cooking and digestion *in vitro* have on such anti-inflammatory properties, and whether these activities are linked to polyphenolic antioxidant activity.

None of the herb extracts interfered with the NBT assay when tested without XO at 550nm and none of the tested extracts inhibited the formation of uric acid by XO in the presence of xanthine at 290nm which is in agreement with Thring *et al.* (2009), therefore the extracts mimicked SOD activity (SODm) by inhibiting formazan production directly. In the current study all extracts possessed SODm activity ranging from 19.5% to 70.6% (inhibition of formazan production) for sage U and STD thyme respectively. All three STD herb extracts (30µg in assay) exhibited high SODm activities and high AA efficacies suggesting that there is an association between SODm properties of these herbs and their polyphenolic antioxidant activity.

Although there was a trend to an increase in SODm activity with H herbs compared to U, the current results do not show definitively that heating increases SODm activity because there was no SODm increase for thyme (H). The same applies to H&D herbs where SODm activities were significantly higher than for U and H herbs for rosemary and sage but not for thyme. It is unclear as to why U, H and H&D extracts for thyme had similar SODm activities. H&D extracts had higher amounts of Cu, Fe, Mn and V than their U and H counterparts not all of which were significant, and this may be due to the acid digestion of the herbs at the simulated gastric fluid stage causing the release of these metal ions. Transition metal ions are known to enhance oxidative damage by Fenton and Haber-Weiss reactions (Fisher and Naughton, 2005). Phenolic acids with catechol groups (benzene ring with 2 hydroxyl groups (-OH)) such as caffeic acid and rosmarinic acid, have been shown to chelate metal ions and reduce cell membrane damage on metal ion intoxicated rat hepatocytes (Psotova *et al.*, 2003). However, in the presence of O_2, and transition metal ions, the redox cycling of these phenolic acids can lead to the formation of ROS, therefore the pro/antioxidant activity of these complexes depends on the metal reducing properties and chelating abilities of the phenolic acids (Sakihama, 2002). It is possible that, as the metal ions increased

via the gastric acid digestion the phenolic acids present in the mixture also increased. It may be that these phenolic acids were present in insufficient quantities to reduce/chelate the metal ions and thus kept the balance of the mixture in favour of antioxidants in the free radical/antioxidant balance. However in the case of thyme H&D which had the highest levels of Mn, V and Zn metal ions, compared to rosemary and sage H&D, the balance in favour of antioxidants may have been compromised by a possible pro-oxidant activity in the presence of superoxide in the NBT assay. This may explain why the SODm activity of thyme H&D was not higher in proportion to its uncooked and heated counterpart.

Despite the results for thyme, there were high correlations between the AA assays and SODm activity irrespective of treatments, suggesting that the SODm activities were due to a dose dependant polyphenolic antioxidant activity of the herb extracts. This observation agrees well with a study by Thring *et al.* (2009) who showed good correlations between SOD activity and AA for nine herb extracts. Lu and Foo (2001) also showed that the antioxidant activity of sage correlated well with its SOD activity and suggested that this correlation was due to the polyphenols, specifically rosmarinic acid, and its derivatives; caffeic acid, salvianolic acid, sagecoumarin, sagerinic acid, more so than the flavonoid glycosides. The catechol structures present in hydroxycinnamic acids (caffeic acids, rosmarinic acid, ferulic acid, coumaric acid and derivatives) are thought to readily form a resonance stabilised phenoxy radical responsible for their high antioxidant activity and SOD activity. Rosmarinic acid is the predominant polyphenol in rosemary sage and thyme (Yoshikawa *et al.*, 2000; Lu and Foo, 2001; Wang *et al.*, 2004; Kivilompolo *et al.*, 2007 a & b; Shan *et al.*, 2006), therefore it is possible that rosmarinic acid and/or its derivatives were responsible for the SODm activities observed for all the samples studied.

The potential anti-inflammatory action of herbs was also investigated by measuring IL8 secretions upon exposure to herb preparations and H_2O_2 or TNFα. There were no significant differences between the number of PBLs or Caco 2 cells incubated for 24 hours in sterile water or herb extracts therefore it was assumed that the herb extracts diluted 1:10 were not cytotoxic to either PBLs or Caco 2 cells. Standardised extracts of rosemary, sage and thyme (3mg/ml in final cell assay volume) significantly inhibited the release of IL8 from PBLs incubated with

H_2O_2 or TNFα suggesting that high amounts of herb have some anti-inflammatory activity. Regarding the Caco 2 cells, STD extracts significantly inhibited the release of IL8 when cells were exposed to TNFα but to a lesser extent than with PBLs. However, there were no decreases in IL8 release with Caco 2 cells incubated with STD herbs and H_2O_2 .Caco 2 cells are human colonic adenocarnicoma (cancer cells) and the PBLs were healthy cells, their differences in nature may explain their differences in inflammatory responses observed. The results of this study suggests that rosemary, sage and thyme (standardised, uncooked, heated and heated and digested) may reduce inflammation by reducing oxidative stress and consequently reducing the inflammatory signalling cascade responsible for chronic inflammation.

Extracts of rosemary, sage and thyme (H&D) caused a decrease in IL8 release from PBLs exposed to H_2O_2 and TNFα but these decreases were only statistically significant for PBLs exposed to H_2O_2. Interestingly, the opposite effects were observed for Caco 2 cells where H&D herbs significantly decreased the IL8 release from Caco 2 cells exposed to TNFα with no effects on cells exposed to H_2O_2. Shin *et al.* (2010) found that Caco 2 cells exposed to H_2O_2 and TNFα promoted higher levels of IL8 secretions compared to non-stimulated cells, and that it was the NF-κB signaling pathway (transcriptional activity and p65 nuclear translocation) that was affected. Furthermore, some polyphenols were shown to reduce IL8 secretions by 1.6 fold in Caco 2 cells by inhibiting IKB-α phosphorylation and blocking NF-κB activation (Romier *et al.*, 2008). Therefore it is possible that the polyphenols present in the culinary herbs studied (shown to reduce IL8 release when exposed to H_2O_2 and TNFα) acted via the NF-κB signalling pathway.

For extracts of herbs (U and H) some decreases in PBL IL8 release were observed but, as for H&D extracts, these decreases were only significant for PBLs exposed to H_2O_2 with the exception of rosemary H. This could be due to the different types and amount of polyphenolic compounds present in the three culinary herbs and requires further investigation via identification of the polyphenolic compounds present in the culinary herbs studied. There were no decreases in IL8 release by either H_2O_2 or TNFα stimulated Caco-2 cells exposed to U and H herbs. TNFα and H_2O_2 may affect different anti-inflammatory pathways

within the cells. The ways in which these factors contribute to inflammation are complex. H_2O_2 or TNFα have pro-inflammatory effects that could be mediated at several levels including transcription, translation and degradation (Son *et al.*, 2005). The reduction/inhibition of IL8 release reduces/inhibits the PKC/ NF-κB pathway resulting in switching off pro-inflammatory genes and therefore putting a stop to the production of pro-inflammatory mediators involved in chronic inflammation (Romier *et al.*, 2008). These factors taken together suggest that Labiatae culinary herbs, in a quantity commonly used in domestic kitchen, contribute to reducing factors involved in chronic inflammation that contribute to the development of chronic diseases and cancer.

Pre-incubating PBLs with extracts of heated and digested rosemary, sage and thyme significantly protected the PBLs from the inflammatory effects of both H_2O_2 and TNFα, this protection was not greater than that of the co-incubated extracts in terms of percentage inhibition, but the results of the latter were not significant, therefore the pre-incubation of PBLs with H&D extracts for 3 hours does appear to offer some protection to PBLs against inflammation. Pre-incubating Caco 2 cells with H&D herbs had no effects on their IL8 release in the presence of either H_2O_2 or TNFα thus negating the pre-incubation protection hypothesis for Caco 2 cells, the reason why this happened is unclear although it is possible to hypothesise as above that the differences in nature of these cells may be responsible for such results and this warrants further investigation.

It remains unclear why none of the herb extracts inhibited IL8 secretions in Caco 2 cells exposed to H_2O_2 (for the pre and co-incubation experiments). Differences in H_2O_2 and TNFα induced secretion of IL8 were also reported by Zhao *et al*, (2008) with caffeic acid, a metabolite of rosmarinic acid. The authors demonstrated that caffeic acid (2.00 mmol/l) was able to block the H_2O_2 or TNFα induced secretion of IL8 in Caco 2 cells, however, whilst caffeic acid inhibited the H_2O_2-induced IL8 secretion and its mRNA expression, it did not suppress the TNFα-induced increase in the IL8 mRNA expression, suggesting that the suppressive mechanisms are different between for TNFα-induced and H_2O_2-induced IL8 production. These results are in disagreement with the current study, where only TNFα-stimulated IL8 release with herbal extracts inhibited IL8 secretions. Whether or not caffeic acid was present in these extracts warrants further investigation via

HPLC analysis. The concentration of caffeic acid (if any) may be a factor in the differences observed between the current study and Zhao *et al.* (2008).

The good correlations between percentage inhibition of IL8 on PBLs and polyphenolic antioxidant activity of the herbs irrespective of treatments, supports the hypothesis that the polyphenols were responsible for the anti-inflammatory responses detected and these responses appear to be dose dependant which correlates well with the literature (Yoon and Baek 2005; Peng *et al.*, 2007; Kaefer and Milner 2008; Zhao *et al.*, 2008). As stated above rosmarinic acid is the predominant polyphenol in rosemary sage and thyme, and Osakabe *et al.*, (2004) demonstrated the effectiveness of rosmarinic acid at reducing inflammation by inhibiting the PKC/ NF-κB pathway in mice, therefore it is possible to speculate that the rosmarinic acid present in the herbs studied may be responsible, at least in part, for the IL8 inhibition observed.

7.5 Conclusions

This study indicates that the culinary herbs rosemary, sage and thyme, in quantities used for cooking, possess anti-inflammatory activity that appears to be due to their high polyphenol content. Heating and *in vitro* digestion increased this activity and this may be the result of increased availability of polyphenols. The literature supports the role of rosmarinic acid and its derivatives in contributing to the anti-inflammatory action of the herbs but further work is required to fully elucidate the polyphenols responsible for this action and their mechanisms of action.

Chapter 8. Anti-microbial activity of heated herb extracts of rosemary, sage thyme.

8.0. Introduction

Although the majority of work concerning the biological properties of culinary herbs has focussed on their antioxidant activity (Crowell, 1999; Mantle *et al.*, 2000; Zheng and Wang, 2001; Manach *et al.*, 2004; Hinnerburg *et al.*, 2005; Halvorsen *et al.*, 2006; Shan *et al.*, 2007; Chohan *et al.*, 2008; Carlsen *et al.*, 2010) which is mainly attributed to polyphenols (Crowell, 1999; Zheng and Wang, 2001), culinary herbs have also been shown to possess anti-microbial activity. Liu and Nakamo, (1996) investigated the antibacterial activity of 17 spices and 5 herbs, and concluded that the effects of herbs and spices varied greatly with gram types and species of bacteria. Whether this property can be attributed fully to the polyphenols in these herbs has yet to be established. However, Moreno *et al.*, (2006) showed that both rosmarinic acid and carnosic acid, extracted from rosemary, possess anti-microbial activity. Furthermore, Shan *et al,* (2007) showed that there is a good correlation between the antioxidant activity and antimicrobial activity of methanolic extracts of the Labiatae rosemary, sage and thyme. From this observation Shan *et al.* (2007) suggest that there is an association between the phenolic constituents of culinary herbs and their anti-microbial activity. To establish if this relationship holds for the Labiatae culinary herbs under investigation and to determine if cooking affects such a relationship, the aim of this study was to determine if the anti-microbial activity of rosemary, sage and thyme is associated with their polyphenolic antioxidant activity and if cooking affected this relationship. The mode of cooking chosen for this investigation was stir frying in a Teflon® pan, as small quantities of both water or oil can be used as mediums to extract the herbs and then compared. In addition, to further investigate the influence of synergy (see Chapter 4) the effect of herbs in combination on their anti-microbial activity was also investigated.

8.1 Materials and Methods

8.1.1Materials

Three Labiatae, rosemary (*Rosmarinus officinalis*), sage (*Salvia Officinalis*) and thyme (*Thymus vulgaris*) were purchased from Neals' Yard Remedies, Covent Garden London, UK. Olive oil was purchased from Tesco's Osterley, UK. All reagents were purchased from Sigma-Aldrich (Poole Dorset, UK) unless otherwise stated.

It was necessary to record the energy content and carbohydrate content of each of the herbs as energy, especially from carbohydrate supports microbial growth. Thus, energy and carbohydrate contents are important factors to consider when investigating anti-microbial activity. Energy and carbohydrate content were obtained from the supplier's product specification sheet (OHTC, UK).

8.1.2 Methods

8.1.2.1 Preparation of herb extracts.

Aqueous Herb extracts

Distilled water (12.5ml heated to 37˚C) was added to the herbs (0.5g) either uncooked (aqueous uncooked; AU) or heated (aqueous heated; AH). Heated herbs were prepared by stir-frying herbs (without oil) for 10 minutes in a Teflon® stir frying pan, covered with a lid in a tinted glass bottle and left to infuse for 10 minutes.

Oil herb extracts

Olive oil was chosen as opposed to an extra virgin olive oil which is known to contains (hydroxythyrosol) polyphenolic antioxidant compounds. Olive oil (12.5ml heated to 37˚ C) was added to herbs (0.5 g) in tinted glass bottles, covered with a lid and left to infuse for 10 minutes (oil uncooked; O.U), or to herbs (0.5g) that were then heated and stirred in a Teflon® stir frying pan for 10 minutes (oil heated; O.H).

Combined herbs

Uncooked and heated rosemary, sage and thyme (0.5g each) were extracted in 37.5 ml of either water or olive oil.

Following the above extractions, hot solutions were cooled quickly on cold water. For the aqueous extracts, entire solutions were filter sterilised using a 0.22 µm filter membrane (Millipore), aliquoted into microcentrifuge tubes (1.5 ml), labelled and frozen at -80 ° C until needed. Entire solutions of oil extracts were aliquoted into (25 ml) centrifuge tubes, spun at 12000 rpm for 5 minutes and the supernatants re-aliquoted into microcentrifuge tubes (1.5 ml). These were, kept at room temperature in the dark to protect antioxidants from light oxidation and tested promptly.

8.1.2.2 Microbiological contamination assessment

Prior to carrying out any sensitivity tests, herb extracts were tested for microbiological contamination. Each herb extract was swabbed evenly on to Nutrient agar (biomeriux, UK) the plates were then incubated at 37°C for 24 hours. Following incubation, the plates were examined for the presence of bacterial growth (the cut off point for microbial contamination was equal to or less than 5 colony forming units; C.F.U).

8.1.2.3 Anti-microbial activity testing of herb extracts

Each herb extract was tested against a panel of potentially pathogenic bacterial isolates *Staphylococcus aureus* (NCTC 6571), *Staphylococcus epidermidis* (NCTC 13360), methicillin resistant *Staphylococcus aureus* (MRSA) (NCTC 12493) all of which are gram positive; and *Escherichia coli* (NCTC 12241), *Pseudomonas aeruginosa* (NCTC 950) and *Proteus mirabilis* (NCTC 7827) all of which are gram negative. Overnight cultures were suspended in Ringer's solution (Oxod, Basingstoke, U.K.) to a turbidity equivalent to 0.5 McFarland (1.5×10^8 CFU/ml) and diluted 1:10 in ringer solutions (*Gutierez et al.*, 2008). Bacterial suspensions were swabbed evenly over the surface of Iso-sensitest plates, which were then left to dry (for no more the 15 minutes). Herb extracts (10 µL) were then spotted on to the plates in triplicate, water for aqueous extracts and olive oil for oil extracts were used as negative controls. Penicillin G (1 unit, Mast Diagnostic, UK) was used as the positive control. Plates were then incubated at 37° C for 24 hours. The presence of microbial growth over the plate and under the spots meant that the extracts had no anti-microbial activity for the isolate tested and was reported as "no inhibition". Microbial growth absent under the spot (5mm in diameter) but present on the rest of the plate, meant that the extract had anti-microbial activity in direct contact with the isolate tested and this was reported as a "contact inhibition".

Microbial growth absent under the spot with a clear zone spread outside of the spot (diameter >5mm), meant that the extracts had a stronger anti-microbial activity than contact inhibition, the zone was measured using a standard ruler and reported as a "zone of inhibition" in millimetres, (a greater diameter meant a greater anti-microbial activity).

8.1.2.4 Investigation of antioxidant activity of herb extracts

Extracts and controls (water for aqueous extracts and olive oil uncooked and heated for oil extracts) (5 µl) were each spotted in duplicate on to silica TLC plates (5 cmx20cm). The plates were then left to dry at room temperature and sprayed with a solution of 0.2% 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) in methanol as described by Mc Gaw *et al.* (2007). The appearance of yellow areas over a light purple background indicated the presence of polyphenolic antioxidant compounds in the extracts.

8.3 Results

8.3.1 Energy and Carbohydrate content of the Labiatae: Rosemary, Sage and Thyme

The energy per 100g herb for rosemary and sage were similar 331Kcal and 315 Kcal respectively and less with thyme (276 Kcal), however the amount of carbohydrates/g for 100 g of herbs was similar for rosemary, sage and thyme, 64g, 61g and 64g respectively (Table 8.1).

Table 8.1 Energy and Carbohydrate content of the Labiatae: Rosemary, Sage and Thyme

Herbs	**Rosemary**	**Sage**	**Thyme**
Botanical name	*Rosemarinus officinalis*	*Salvia officinalis*	*Thymus vulgaris*
Energy (KJ)/100g	1387	1317	1156
Energy (Kcal)/100g	331	315	276
Carbohydrate (g/100g)	64	61	64

8.3.2 Microbiological contamination assessment

No bacterial growth was observed following incubation of the extracts.

8.3.3 Anti-microbial activity of rosemary, sage and thyme – uncooked and cooked (heated)

A zone of inhibition (14 mm) was observed with Penicillin G (positive control) for *S aureus*, and *S epidermidis*. (Table 8.2) .

None of the aqueous herb extracts uncooked, heated, combined or aqueous controls showed any antimicrobial activity on the isolates tested (Table 8.2).
None of the oil controls (uncooked or heated) showed any antimicrobial activity on the isolates tested (Table 8.2).

Contact inhibition (5 mm) was present with the three gram positive isolates *S aureus*, *S epidermidis* and MRSA when exposed to oil extracts of uncooked and heated sage, and heated rosemary (Table 8.2). No effect was observed for uncooked rosemary and uncooked and heated thyme on these isolates (Table 8.2). Contact inhibition (5 mm) was present with the three gram positive isolates with the oil extract of uncooked combined herbs (Table 8.2).

A zone of inhibition (7 mm) was present for oil extracts of the heated combined herbs on the three gram positive isolates. None of the herbs extracted in oil showed any activity against the gram negative bacterias: "*E. coli, Ps.aeruginosa* and *P. Mirabilis*", (Table 8.2).

Table 8.2 Diameter of the zones of inhibition (mm) of the uncooked and heated oil herb extracts at 24 Hour.

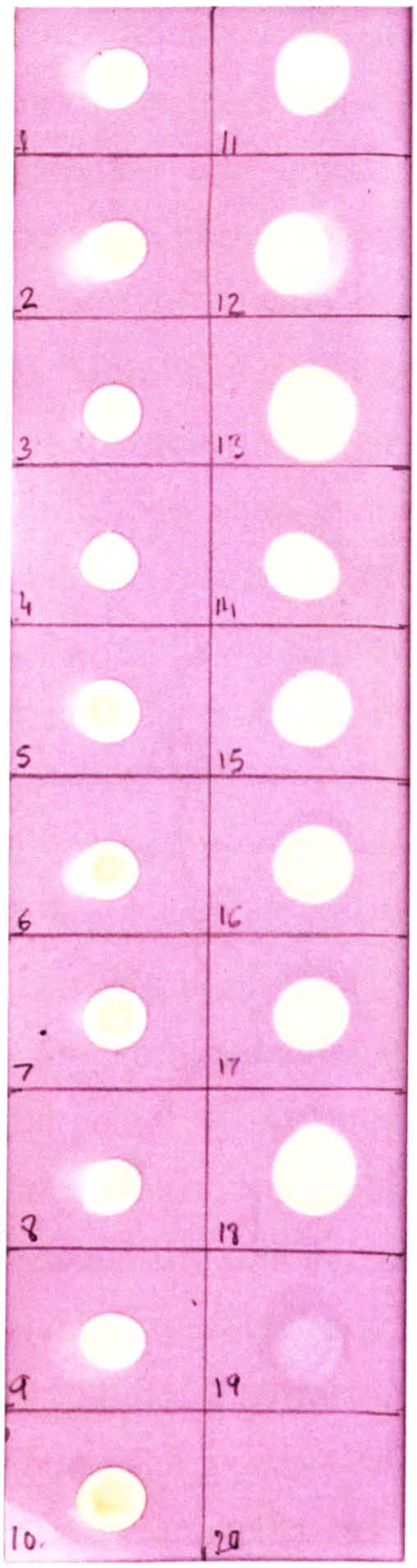
	Gram positive			Gram negative		
	S. aureus	*S. epidermidis*	MRSA	*E.coli*	*Ps. aeruginosa*	*P.mirabilis*
Control: Penicillin G	14mm	14mm	0	0	0	0
Olive oil uncooked	0	0	0	0	0	0
Rosemary (in olive oil; uncooked)	0	0	0	0	0	0
Sage (in olive oil; uncooked)	5mm	5mm	5mm	0	0	0
Thyme (in olive oil; uncooked)	0	0	0	0	0	0
All three herbs (in olive oil; uncooked	5mm	5mm	5mm	0	0	0
Olive oil heated	0	0	0	0	0	0
Rosemary (in olive oil; heated)	5mm	5mm	5mm	0	0	0
Sage (in olive oil; heated)	5mm	5mm	5mm	0	0	0
Thyme (in olive oil; heated)	0	0	0	0	0	0
All three herbs (in olive oil; heated)	7mm	7mm	7mm	0	0	0

Each assay was conducted in triplicate on three different days (n=3). 0 =No inhibition. 5mm =contact inhibition, >5mm =zones of inhibition (mm).

8.3.4 Investigation of antioxidant activity of herb extracts

Each extract (aqueous and oil) possessed some antioxidant activity with the exception of the control 3 (water blank) and control 4 (DPPH only) (Fig 8.2).

Figure 8.1 A thin Layer chromatography plate of antioxidant activity of aqueous and oil extracts (uncooked and cooked) detected using DPPH



1. Rosemary oil uncooked
2. Sage oil uncooked
3. Thyme oil uncooked
4. Control 1: olive oil uncooked
5. Rosemary oil heated
6. Sage oil Heated
7. Thyme oil heated
8. Control 2: olive oil heated
9. R, S & T oils uncooked
10. R, S & T oils heated
11. Rosemary aqueous uncooked
12. Sage aqueous uncooked
13. Thyme aqueous uncooked
14. Rosemary aqueous heated
15. Sage aqueous heated
16. Thyme aqueous heated
17. R, S & T aqueous uncooked
18. R, S & T aqueous heated
19. Control 3: Water blank
20. Control: (DPPH only)

R: Rosemary, S: sage, T: thyme.

8.4 Discussion.

The aim of this study was to investigate whether heating culinary herbs affects their anti-microbial activity and to determine if such activity was associated with their antioxidant activity. The herbs used showed no signs of bacterial contamination. The yellow spots over purple background indicated the presence of

antioxidant activity. The intensity in yellow colouration did vary but was not measured in this study as the purpose of this assay was to determine the presence of antioxidant activity of herb extracts and not to quantify it. Aqueous extracts did possess antioxidant activity which could be due to the presence polyphenols; this would correlate well with previous studies on uncooked and heated extracts of rosemary, sage and thyme (Chohan *et al.*, 2008). However, these aqueous herb extracts had no effect on the microbiological isolates tested, therefore there appeared to be no association between the antioxidant activity detected and anti-microbial activity. The absence of anti-microbial activity of aqueous herb extracts on the isolates tested was contrary to the findings of Shan *et al.* (2007), who studied extracts of rosemary, sage and thyme, and found all three to have anti-microbial activity against *S aureus*. However the differences in the extraction method used by Shan *et al.* (2007) (in methanol with fine grinding) and those used in the present study may account for the different results, the method used by Shan *et al* . (2007) may have increased the availability of phenolic compounds thus providing levels of polyphenols sufficient to provide an anti-microbial response. The results of the present study also contradict that of Moreno *et al.*, (2006) who showed that both rosmarinic acid and carnosic acid, extracted from rosemary, possess anti-microbial activity; both these polyphenols are found in the three Labiatae investigated in the current study; rosemary, sage and thyme (Cuvelier *et al.,* 1996; Shan *et al.*, 2005). However as with Shan *et al.* (2007) the extraction methodology used by Moreno *et al.* (2006) differed (rosemary was extracted in methanol). Thus, in the present study for aqueous extracts, polyphenolic compounds may not have been present in sufficient amounts to display any anti-microbial activity, or at least, the assay used was not sensitive enough to detect any. The study by Liu and Nakano (1996) further supports the hypothesis that bacterial sensitivity is proportional to the concentration of extracts used. The authors showed that at a 0.5% concentration, rosemary, sage and thyme extracts caused a perfect inhibition against S.*aureus*, a perfect inhibition for sage at 0.1% and weak inhibition for rosemary and thyme at 0.2% and a strong inhibition (less than perfect) with sage at 0.1% and no inhibition for rosemary and thyme at 0.1%. An obvious solution to address the possibility that aqueous extraction did not provide polyphenols in high enough amounts to demonstrate anti-microbial activity is to use a higher concentration of herbs, however it is unlikely that amounts larger than the ones used in this study would be used in

domestic kitchen. Nevertheless, water based methods of cooking (microwaving, stewing) involving heat have been shown to increase the antioxidant activity of these herbs, more so than heating the herbs (chapter 3) and this is possibly due to the increase in the amount of polyphenols made available (Chohan *et al.*, 2008). Thus, these methods of cooking may release polyphenols in sufficient amount to have some anti-microbial activity, and this warrant further research.

An additional factor that needs to be considered is volatile oils. Culinary herbs also possess volatile oils, including aromatic compounds and terpenes, and based on their chemical structures most volatile oils can be classified as lipophillic phenolic compounds (Shan *et al.*, 2005); for instance, bourneol, thymol and cineol are present in rosemary, sage and thyme (Williamson, 2003). The anti-microbial properties of volatile oils are well known (Graven *et al.*, 1997; Baratta *et al.*, 1998; Shan et al., 2005; Hinnerburg *et al.*, 2005; Barry-Ryan and Bourke, 2008; Gutierez *et al.*, 2008) and the anti-microbial activity of essential oils has been attributed to their phenolic groups (Shan *et al.*, 2005, Viuda-Martos *et al.*, 2011). Volatile oils are secreted by lysigenous glands and glandular trichome on the waxy cuticle surface of the leaf they are responsible for the distinctive scent of the plants (Manach et al., 2004; Bussell *et al.*, 1995) and can be extracted in fats and oils. Thus, it is possible that the volatile oils are responsible for anti-microbial activity of culinary herbs. Furthermore, as these oils are poorly soluble in water, they would not have been extracted using an aqueous based technique but may have been released by the extraction techniques used by Shan *et al.* (2007) and Moreno *et al.* (2006) which involved methanol. Volatile oils may explain the anti-microbial activities observed for the oil extracts in the present study (see below).

All oil extracts did possess antioxidant activity and so did the blanks (olive oil uncooked and olive oil heated); these results are in agreement with the literature (Pellegrini *et al.*, 2003; Halvorsen *et al.*, 2006).The antioxidant activity of culinary herbs extracted in oils could also be due to both the vitamin E content of olive oil as well as the volatile oils extracted from the culinary herbs. However, the oil controls did not possess anti-microbial activity and only some of the oil extracts did, therefore an association between antioxidant activity and anti-microbial activity was not apparent in this study.

Regarding the combined herbs, the contact inhibition present with the three gram positive isolates with the oil extract of uncooked combined herbs seemed to be due mainly to uncooked sage as the oil extracts of uncooked rosemary and thyme did not possess anti-microbial activity when tested alone. However, this observation may also indicate a possible synergy between all three herbs, as sage only made up 1/3 of the combination and yet the same contact inhibition (5mm) was observed for uncooked sage oil on its own. Regarding the effect of the heated combined herbs on the three gram positive isolates, heated rosemary showed signs of contact inhibition that was not present in uncooked rosemary suggesting that heat promoted an increase in anti-microbial activity. It appears, from the results, that sage is a major contributor to the anti-microbial activity displayed in conjunction with rosemary for the combined herbs, as the oil extract of heated thyme was the only herb that did not show signs of contact inhibition. Again, it is possible that synergy occurred in this combination which, based on the clear zone of inhibition (7mm), denotes a stronger anti-microbial activity than the contact inhibition (5 mm) observed for the individual oil extracts of heated rosemary and sage. The carbohydrate content of each herb was similar and therefore could not account for any differences in microbial sensitivity between the herbs.

As mentioned above the anti-microbial activity observed in the oil extracts could be due to volatile oils and these volatile oils may have acted synergistically to increase the anti-microbial activity of culinary herbs when combined. Shan *et al.* (2007) suggested that the most active anti-microbial compounds in culinary herbs are the volatile oils, which include bourneol and cineol present in rosemary, sage and thyme and carvacrol in thyme. Shan *et al.* (2007) reviewed several studies on the microbiological action of volatile oils and reported that they degrade the cell wall thus disturbing proteins, fatty acids and phospholipids of the cytoplasmic membrane of bacteria causing holes, impairing enzymes, energy production, metabolism, nutrient uptake electron transport, DNA and RNA synthesis and protein translocation (Shan *et al.*,2007).

The absence of anti-microbial activity for both aqueous and oil extracts against the gram negative bacterias *E. coli, Ps.aeruginosa* and *P. mirabilis* (Table 7.2) in the present study was similar to that reported for extracts of rosemary, sage and thyme by Liu and Nakano, (1996) where none of the concentrations of herb

extracts tested (0.5, 0.2 and 0.1 %) had inhibitory effects on *E. coli.* The authors found that in general, gram positive bacteria were more sensitive to herbs than gram negative bacteria and suggested that this was due to the differences in outer membrane in gram positive and gram negative bacteria. The same effects were observed by Shan *et al.* (2007) where neither rosemary, sage nor thyme extracts had inhibitory effects on *E. coli* and again the authors proposed that this effect was due the complex structure of the membrane of gram negative bacteria compared to the thick but simple membrane of gram positive bacteria. A barrier is provided in the form of the hydrophilic nature of the surface of the outer membrane of gram negative bacteria, which is rich in lipopolysaccharide molecules and the periplasmic space below the outer membrane of these bacteria (which is associated with the production of enzymes capable of breaking down invading molecules) (Shan *et al.*, 2007). Therefore in the present study, it is possible that neither the phenolic acids present in aqueous extracts nor the volatile oils present in oil extracts were able to affect the outer membrane of *E. coli, Ps.aeruginosa* and *P. Mirabilis* due to the complexity of their outer membranes.

8.5 Conclusion

In conclusions, no association between the polyphenolic antioxidant activity and anti-microbial activity was observed for the culinary herbs investigated. This lack of association may be due to insufficient levels of polyphenols, an antibacterial assay of low sensitivity or the anti-microbial activity that was detected being due to volatile oils. The results of this study suggest that culinary herbs extracted in olive oil, particularly sage, may be of benefit to the host by inhibiting the gram positive bacterial growth and this effect may be enhanced by heat as was the case for rosemary. In addition, the study supports the possible role of synergy between the herbs although it is not clear which constituents may be responsible for this effect. The possibility of synergy agrees with that of Gutierez *et al.* (2008) who suggested that although some food compounds have, individually, low anti-microbial activity their effects may be greater if used in combination or as part of a food matrix. Furthermore, the polyphenolic compounds present in the herbs studied were not identified and each phenolic compound in each herb extract may have mechanisms of action against bacteria that are complex. If so, then one can assume that as the number of herbs in combination increases so will the complexity of the mechanisms of action. It would therefore be necessary to identify

the chemical profile of the herbs studied in order to give further insight as to their antibacterial activities. Based on these findings and those reported in Chapter 4, the possible influence of synergy requires further investigation.

Chapter 9. Identification of polyphenolic compounds found in the Labiatae: rosemary, sage and thyme.

9.0 Introduction

The molecules of interest in this study, the polyphenols, are secondary metabolites that occur in a cascade of biochemical pathways within the plant (Craig, 1999) and as is clear from the literature, these compounds are ubiquitous in plants and plant derived foods including culinary herbs (Cheynier, 2005; Bravo, 1998). Focussing on the Labiatae herbs (rosemary, sage and thyme), a search of the literature indicated that the hydroxycinnamic acid rosmarinic acid is the major phenolic acid in rosemary, sage and thyme. A summary of the concentrations of polyphenols in the culinary herbs investigated was obtained from the Phenol-explorer website (Table 9.1) for rosemary, sage and thyme (dried). The hydroxycinnamic acids caffeic acid, ferulic acid and p-coumaric acid are also present alongside rosmarinic acid in all of the three herbs studied (Table 9.1). The hydroxybenzoic acids, gallic acid, syringic acid and vanillic acid have also been found in all three herbs. Rosemary, sage and thyme are also known to possess luteolin and apigenin flavonoid glycosides however several studies suggest that the antioxidant activity and anti-inflammatory activity of these flavonoid glycosides are weaker than their phenolic acids, (Cuvelier *et al.*, 1996; Lu and Foo, 2001).

These polyphenol profiles of the Labiatae herbs suggests that a number of polyphenols could be responsible for the biological activities investigated in this study, however the group that predominates are the phenolic acids and in particular rosmarinic acid. It is possible that volatile oils may contribute to the biological properties of the herbs under investigation. It is well established that rosemary sage and thyme also possess volatile oils and that these oils possess antioxidant activity and antimicrobial activity (Graven *et al.*, 1997; Baratta *et al*, 1998; Shan *et al.*, 2005; Hinneburg *et al.*, 2005; Barry-Ryan and Bourke, 2008; Gutierez *et al.*, 2008). However for the antioxidant and anti-inflammatory studies reported here, aqueous extracts were used and thus the contribution of volatile oils to these activities, in the current study, is unlikely.

Table 9.1 Summary of polyphenols in rosemary, sage and thyme (Polyphenol content obtained from www.phenol-explorer)

Data are presented as mean ±SD µg/g derived from various studies. NA: papers in which polyphenols were detected but concentrations not reported.

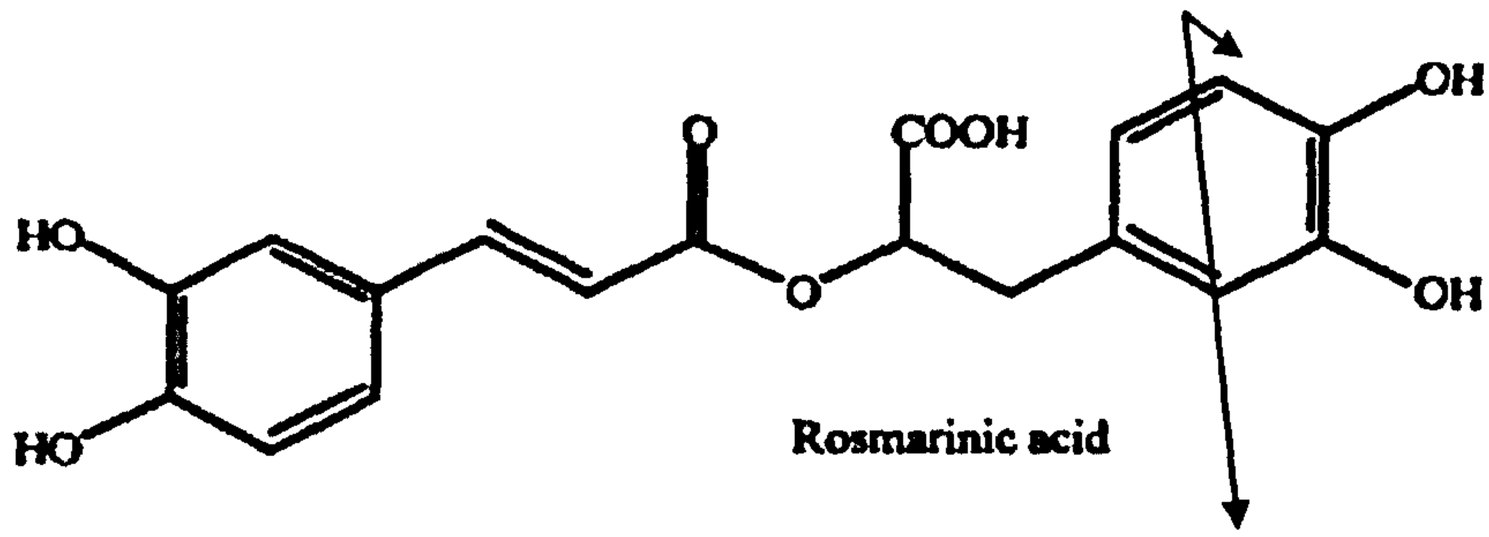
Polyphenols	**Rosemary**	**Sage**	**Thyme**
Gallic acid	Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Hossain *et al*. (2010)
	NA	52.5±74.2µg/g	NA
Syringic acid	Proestos *et al*. (2005) Kivilompolo et al. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)
	10.5±17.9µg/g	33.5±47.4µg/g	117.0±4.2 µg/g
Vanillic acid	Proestos *et al*. (2005) Kivilompolo et al. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)
	30.7±53.2µg/g	58.5±82.7µg/g	61.0±86.3 µg/g
5-Caffeoylquinic acid	Kivilompolo *et al*. (2007 a & b)	Kivilompolo *et al*. (2007 a & b)	
	60.0±84.9µg/g	198.5±44.5µg/g	

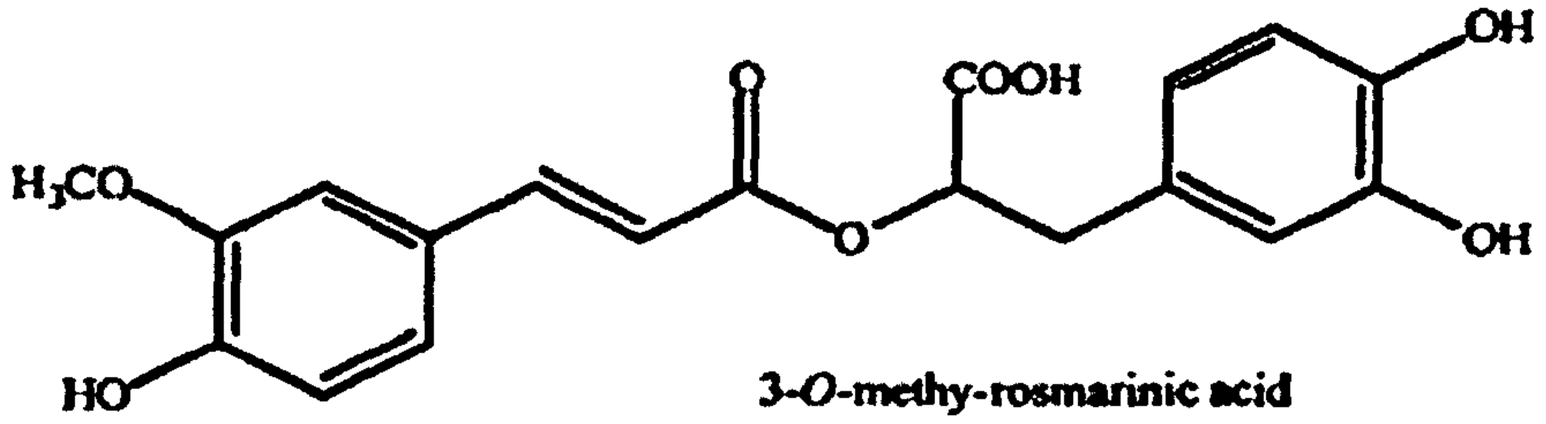
Polyphenols	Rosemary	Sage	Thyme
Caffeic acid	Cuvelier *et al*. (1996) Wang *et al*.(2004) Proestos *et al*. (2005) Shan *et al*. (2006) Kivilompolo *et al*. (2007 a & b) Yoshikawa *et al*. (2000) Hossain *et al*. (2010)	Cuvelier *et al*. (1996) Wang *et al*. (2004) Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Wang *et al*. (2004) Kivilompolo *et al*. (2007 a & b) Shan *et al*. (2006) Hossain *et al*. (2010)
	96.7±56.9µg/g	264.0±109.2µg/g	212.8±98.3 µg/g
Ferulic acid	Proestos *et al*. (2005) Kivilompolo et al. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)
	20.0±34.6µg/g	58.0±82.0µg/g	56.5±79.9 µg/g
p-Coumaric acid	Proestos *et al*. (2005) Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)	Kivilompolo *et al*. (2007 a & b) Hossain *et al*. (2010)
	36.7±63.5µg/g	49.5±70.0µg/g	49.5±70.0 µg/g

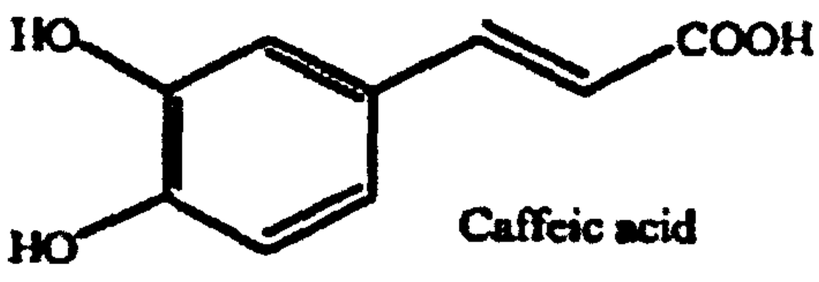
Polyphenols	Rosemary	Sage	Thyme
Rosmarinic acid	Cuvelier *et al*. (1996) Wang *et al*. (2004) Kivilompolo *et al*. (2007 a & b) Shan *et al*. (2006) Yoshikawa *et al. (*2000) Lu and Foo (1999 and 2001) Regnault-Roger *et al. (*2004) Hossain *et al*., (2010)	Cuvelier *et al*. (1996) Wang *et al*.,(2004) Baskan *et al*. (2007) Kivilompolo *et al*. (2007 a & b) Shan *et al*. (2006) Lu and Foo (1999 and 2001) Hossain *et al*. (2010)	Wang *et al*. (2004) Kivilompolo *et al*. (2007 a & b) Shan *et al*., (2006) Lu and Foo (1999 and 2001) Hossain *et al*. (2010)
	9872.±752.3µg/g	6102.5±3933.1µg/g	8290.0±2597.8 µg/g
Carnosic acid	Cuvelier *et al*. (1996) Hossain *et al*. (2010)	Cuvelier *et al*., (1996) Kivilompolo *et al*. (2007 a & b) Shan *et al*. (2006)	
	NA	5258.6±1628.8µg/g	
(+)- Catechin	Proestos *et al*. (2005)		
	27.0±00.0µg/g		
Luteolin	Proestos *et al*. (2005) Yoshikawa *et al*. (2000) Hossain *et al*. (2010)	Hossain *et al*. (2010)	Hossain *et al*. (2010)
	30.0±00.0µg/g	NA	NA

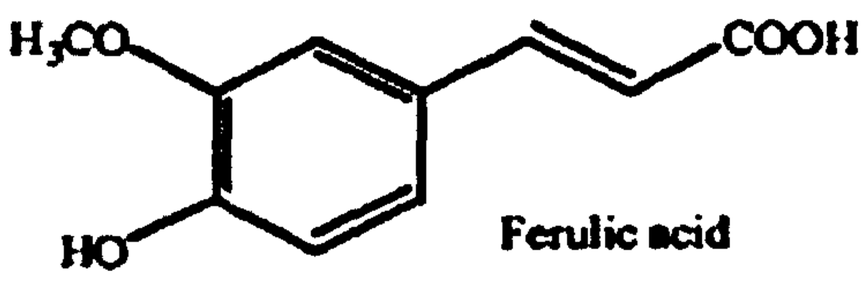
Figure 9.1 Rosmarinic acid metabolites

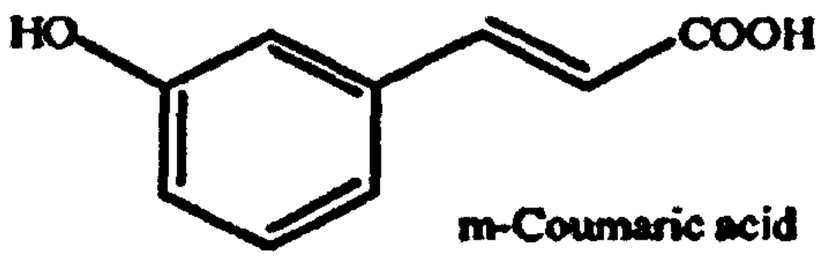
Catechol group: Benzene ring with 2 Hydroxyl groups (-OH)











Adapted from Baba *et al.*, (2004).

As stated in Chapter 3 and 5, there is a paucity of data concerning the effects of cooking and digestion on these polyphenols and their concentration in culinary herbs. The bioavailability of culinary herbs heated and digested *in vitro* showed an average of 11.5% transport of polyphenols antioxidant activity to the basal side of the membrane. Since, based on the literature review summarised above, rosmarinic acid is the major polyphenol found in the Labiatae, it could be hypothesised that this antioxidant activity (AA) was due at least in part to the presence of rosmarinic acid. Therefore the aim of this study was to quantify the rosmarinic acid and its metabolites (caffeic acid, ferulic acid and m-coumaric acid (Baba *et al.*, 2004) as well as gallic acid and vanillic acid in uncooked, heated, heated and digested extracts of rosemary sage and thyme and to investigate the presence of these phenolic acids post *in vitro* bioavailability for herbs heated and digested.

9.1 Material and methods.

9.1.1 Materials

9.1.1.1 Reagents

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), in diamonium salt form, Folin-Ciocalteu's phenol reagent, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic Acid, 97% (Trolox), phosphate buffered saline 5 mM at pH 7.4 (PBS), potassium persulphate ($K_2S_2O_8$), Transwell 6 well plates and Coning inserts, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 2-(N-Morpholino) ethanesulphonic acid hydrate, 4-Morpholineethanesulphonic acid (MES), rosmarinic acid (MW:360.31), caffeic acid (MW:180.16) ferulic acid (MW:194.18), Gallic acid (MW:170.12), m-coumaric acid (MW:164.16), vanillic acid (MW:168.15), all purchased from Sigma Aldrich, Poole, UK. Dulbecco's Modified Eagle Medium (DMEM) with Glucose (4.5g/L), 1 % non-essential amino acids (NEAA), L-glutamine and heat inactivated fetal bovine serum were purchased from Fisher Scientific, Loughborough, Leicestershire, UK. Caco-2 cells were purchased from the European Collection of Cell Culture (ECACC), Health Protection Agency, Salisbury, UK.

9.1.1.2 Culinary herbs

Three Labiatae culinary herbs rosemary, sage and thyme were selected as stated in Chapter 2 and purchased from "Neal's Yard Remedies" Richmond, Surrey, UK.

9.1.1.3 Preparation of herb extracts

Herb extracts uncooked (U), heated (H) and heated and digested *in vitro* (H&D) were prepared as previously described (see Chapter 5 and 7 for details). Herbs U and H were analysed via ^{1}H NMR. Herbs U, H and H& D were investigated for the presence of tannins and analysed via TLC and HPLC.

9.1.2 Methods.

9.1.2.1 Determination of antioxidant activities of herb extracts.

The Trolox Equivalent Antioxidant Capacity (TEAC) and the total phenolic content (TPC) expressed in GAE were used to determine the antioxidant capacity and total phenolic content respectively (please see Chapter 3 for details).

9.1.2.2 Cell culture and bioavailability study.

The Caco-2 cell epithelial transport model of bioavailability and transport buffers were prepared as previously described (please see Chapters 6 for details).

9.1.2.3 Tannin assay Method.

From literature reports it is not clear whether tannins were present in rosemary, sage and thyme (furthermore, rosmarinic acid was often referred to as tannin in older textbooks). Tannins are large polyphenols with a molecular weight range of 500 - 20,000 A.M.U. They are potentially important biological antioxidants (Hagerman *et al.*, 1998) and may interact with the antioxidant assays used throughout this study (TEAC and GAE). Therefore, to determine if tannins contribute to the antioxidant activity of the culinary herbs under investigation a tannin precipitation assay was performed as described by Duncan *et al.* (1999) as it was a simple and reliable method. To each herb extract (1ml) a 10% NaCl solution (50µl) was added to "salt out" the non-tannin compounds. The solution was then filtered using a Whatman grade # 1 paper and black tea was used as the positive control. The herb solutions were tested in triplicate, aliquoted (40µl) into a 48 well plate placed over a white piece of paper (to visualise precipitation and coloration). In the first well no reagents were added to the herb extract (control). In a second well 1% gelatine solution (20 µl) was added to observe for the formation of precipitate, in a third well a 1% gelatine solution and 10% NaCl solution (20 µl) was added to the herb extracts to observe the formation of a precipitate (gelatine-salt block test). Finally a 10% ferric chloride solution (20 µl) was added to the fourth well to observe colour changes. Providing there was precipitation after the gelatine-salt block test, a greenish/black colour after the addition of ferric chloride indicated the presence of tannins. No precipitation on the gelatine-salt block test meant that there are no tannins present; the colouration to greenish brown with ferric chloride solution is due to other phenolic plant constituents. No reaction with ferric chloride solution indicates that there are no phenolic compounds present in the sample.

9.1.2.4 Thin Layer chromatography method.

Thin layer chromatography (TLC) is an easy way to investigate and identify the presence of phenolic acids and flavonoid glycosides present in plants (Wagner and Bladt, 1996) and can also be used to highlight the presence of antioxidant activity of these compounds using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Herb extracts U, H and H&D (5µL, pH6), and rosmarinic acid (0.5mg/mL, 1.4 mM) (5µL, pH6), caffeic acid, ferulic acid, gallic acid, m-coumaric acid, vanillic acid (1mg/mL, 5µL), and the herb extracts H&D post-bioavailability (5µL, pH6.8±0.2) were spotted on to silica TLC plates (10cmx10cm) and air dried for 15 minutes. The optimal solvent system was made up of 1,2 ethyl acetate, formic acid, acetic acid and water (100:11:11:26) (Wagner and Bladt, 1996). The silica TLC plates were run using the solvent system detailed above and air dried, sprayed with natural product reagent (NP) and polyethylene glycol (PEG) to determine the polyphenols present, control plates were prepared and sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) 0.2 % in methanol to highlight areas with antioxidant activity.

9.1.2.5 Nuclear magnetic resonance (^{1}H NMR) spectroscopy.

^{1}H NMR spectroscopy was used for this study to give a ^{1}H NMR spectroscopy "fingerprint" of the herbs before and after heating in order to assess any differences that have occurred in the polyphenols via the heating treatment.

^{1}H NMR was carried out on a JEOL Eclipse$^+$ 400 FT-NMR spectrometer using Delta version 4.3.6 control and processing software (JEOL, Hertfordshire, UK). For each replicate, a survey proton spectrum was measured in automation mode (16 accumulations, 32K points, 6.0 KHz sweep width, centred at 5ppm) from this the water signal was determined and a single pulse homogated experiment was run to selectively remove the water peak from the spectrum (water/on-resonance signal = 4.788 ppm, attenuation = 35 dB, relaxation delay = 3s, 16 accumulations, 32K points, 6.0 KHz sweep width, centred at 5 ppm). Raw data were processed using single exponential windowing function (line width = 0.2Hz). Each herb extract U and H for rosemary, sage and thyme (approximately 0.6ml) was placed in a 5 mm OD Wilmad 527-PP-8 tube containing 3-(trimethylsilyl)propionic acid-d_4 sodium salt (1-2mg) as the internal reference (δ = 0ppm). After an initial room temperature ^{1}H NMR experiment, used as (t_0), the samples were heated to 37 °C +/- 0.5 °C.

9.1.2.6 High-performance liquid chromatography (HLPC) method.

High performance Liquid chromatography (HPLC) is a widely used method to qualify and quantify polyphenols present in herb extracts (Cuvelier *at al.*, 1996; Zheng and Wang 2001; Wang *et al.*, 2004; Shan *et al.*, 2005; Proestos *et al.*, 2005). HPLC can give further insight as to the effect of heating, *in vitro* digestion and post bioavailability (120min) of rosemary, sage and thyme by providing values of phenolic acid content that enables comparison between treatments.

HPLC analyses were conducted to identify rosmarinic acid and rosmarinic acid metabolites caffeic acid, m-coumaric acid, ferulic acid as well as two hydroxybenzoic acids; gallic acid and vanillic acid. The calibration data were obtained using standards (65.5mg/L to 1000mg/L) dissolved in methanol. Peak areas for the standards were plotted and the data were extrapolated from the line of best fit (Please refer to Appendix 8 for HPLC calibration details and sample of HPLC Chromatograms). Analyses of standards and herb extracts (U, H and H&D) were performed with a Perkin Elmer series 200 HPLC with UV detection (Perkin Elmer, Cambridgeshire, UK) at 278 nm and equipped with a 46mm x 250mm 5 µm Phenomenex PFP analytical column (Phenomenex, Cheshire, UK). Elution was achieved with a two way gradient, water (containing 1% formic acid) for mobile phase A and methanol (containing 1% formic acid) for mobile phase B. Mobile phase B was set to pre-run at 20% for 8 minutes and then maintained at 100% for 30 minutes, and back to 20% for 2 minutes; the flow rate was 1 ml/minute with a 20µl injection volume.

9.2 Statistical Analysis.

All data are presented as the mean ± standard deviations (SD) with n=3 analysed in triplicates unless otherwise stated. For TLC, the Retention Factor (Rf) value was calculated by dividing the distance travelled by the compound by the distance travelled by the solvent front. Results of HPLC are expressed in µg/g of plant material. ANOVA (SPSS for windows version 17) and a *post hoc* tests "Tukey" were carried out to compare the amount of polyphenols in each extract obtained using HPLC for U, H and H&D extracts.

9.3 Results

9.3.1 Tannin precipitation assay: Uncooked and Heated herb extracts

The tests for tannins were negative for all extracts of culinary herbs. There was no precipitation on the gelatine-salt block test however a greenish-brown colour for all extracts of rosemary, sage and thyme indicated the presence of other phenolic compounds.

9.3.2 Thin Layer Chromatography of Herbs: Uncooked, Heated and Heated and *in vitro* Digested

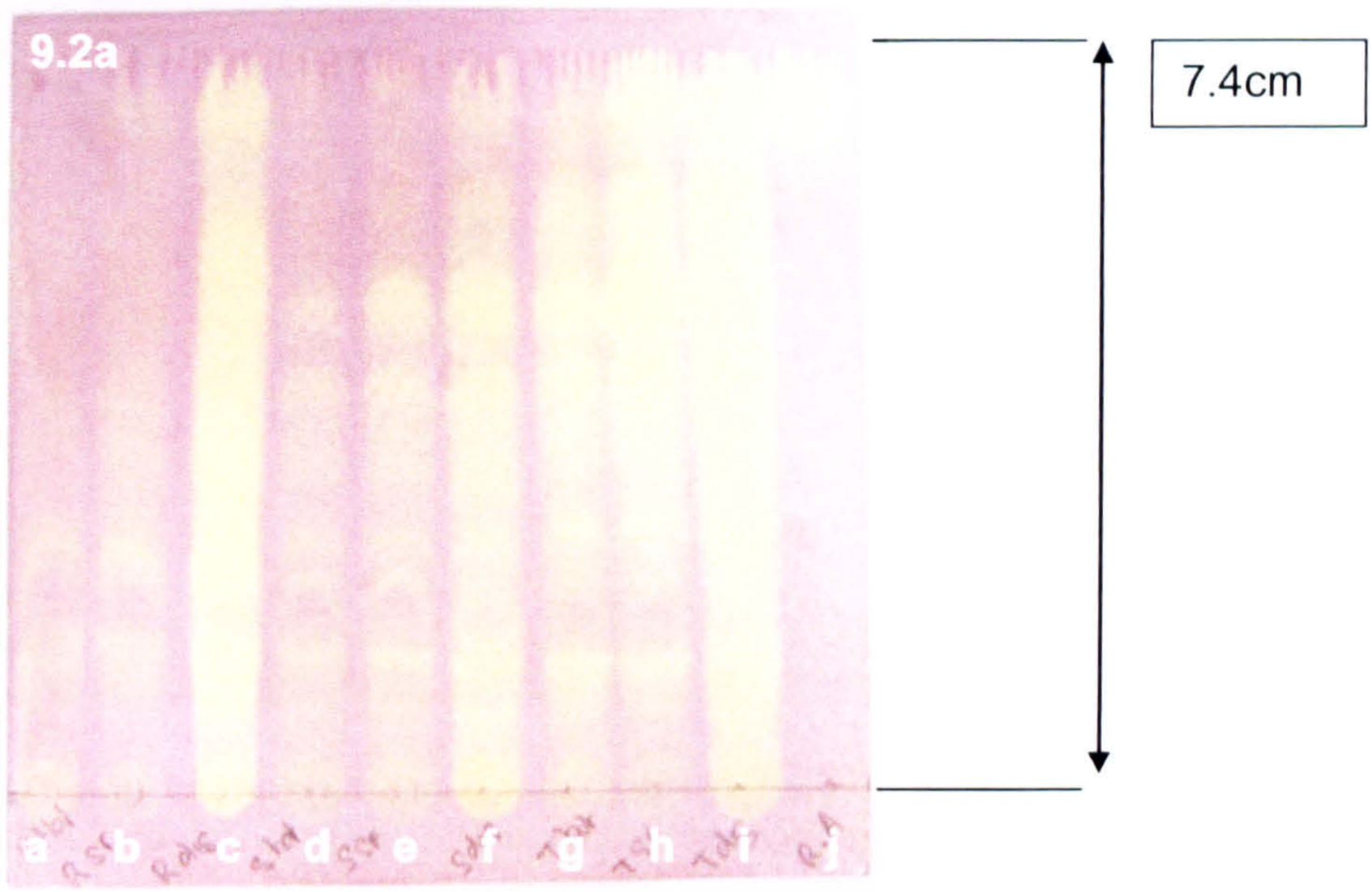
The solvent front for the TLC plates was 7.4 cm. For the TLC plate spotted with extracts of herb samples (5µl) and rosmarinic acid in methanol (0.5mg/mL), caffeic acid, ferulic acid, gallic acid, m-coumaric acid and vanillic acid in methanol (1mg/mL) and sprayed with DPPH (0.2%) the yellow areas against purple background correspond to antioxidant activity (Figure 9.1a and 9.2a). The H&D extracts provided a deeper yellow colour compared to their undigested counterparts, indicating a higher antioxidant activity (Figure 9.1a). m-coumaric acid and vanillic acid showed little not no yellow colouration compared to rosmarinic, caffeic, ferulic and gallic acid (Figure 9.2a). There were no yellow areas over the purple backgroup for the post-bioavalibility herb extracts (Data not shown).

For the TLC plate spotted with extracts of herb samples (5µl) and rosmarinic acid in methanol (0.5mg/mL), caffeic acid, ferulic acid, gallic acid, m-coumaric acid and vanillic acid in methanol (1mg/mL) and sprayed with natural product reagent and PEG, phenolic acids appear in shades of blue towards the upper part of the plate (Figure 9.2b) (rosmarinic acid R_f 0.93, Figure 9.1b) and various spots in shades of green and yellow thought to be flavonoid glycosides were visible with coresponding antioxidant activity on the control plate (Figure 9.1b) The colours of phenolic acids were more intense for the H&D herb samples compared to their undigested counterparts indicating higher levels of phenolic compounds. For rosemary, sage and thyme U, there were no obvious differences in colour intensity when compared with their H counterparts (Figure 9.1b). Rosmarinic acid was less visible in sage H&D than the other two herbs H&D and instead, a green compound was visible at (R_f 0.95) for sage U, H and H&D).

Caffeic, ferulic and gallic acid (Figure 9.2b) had various shades of blue towards the upper part of the plate, m-coumaric acid had a faint blue colour and vanillic

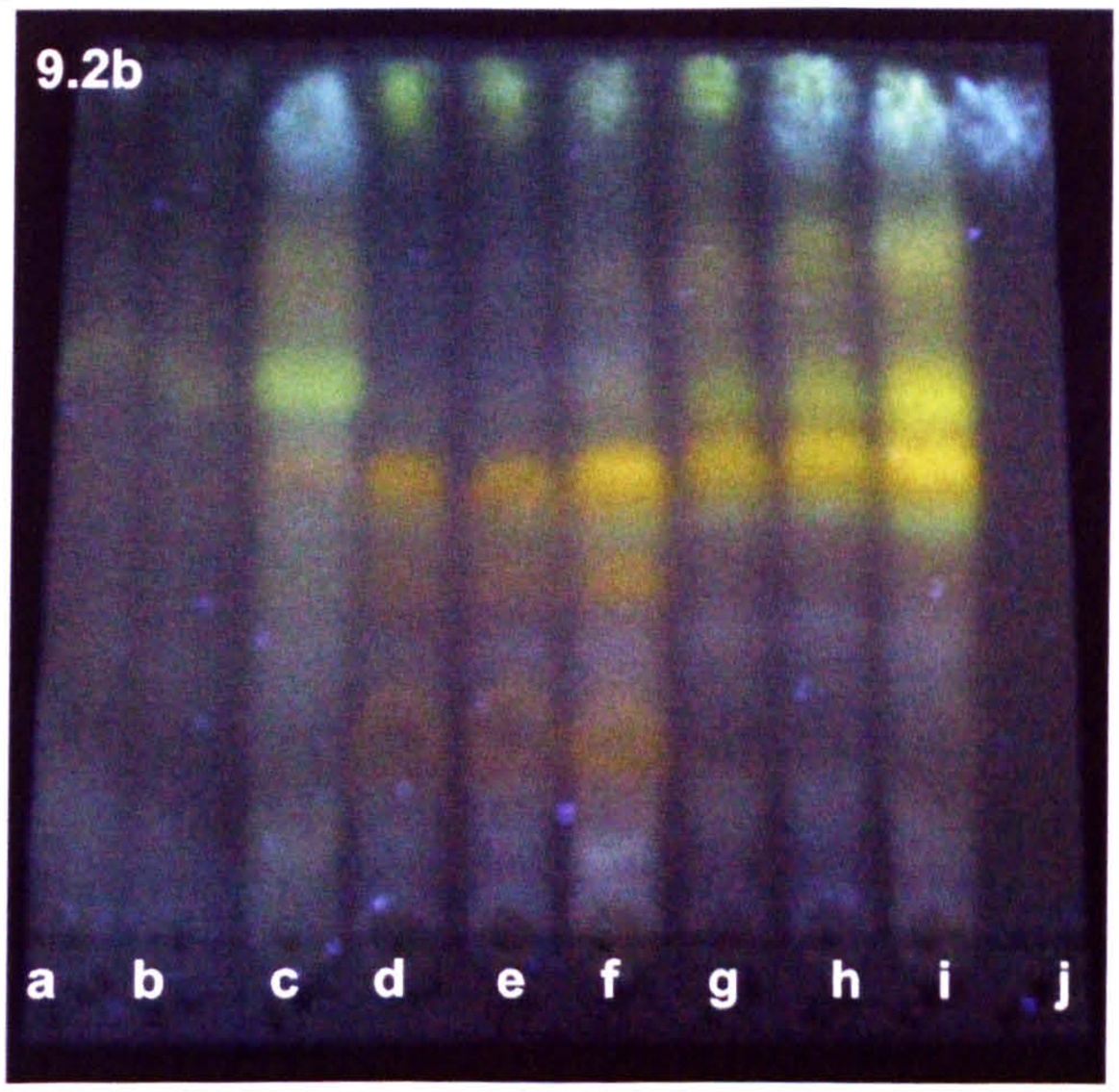
acid had no colouration (Figure 9.2b). There were no visible colours for the post-bioavalibility herb extracts (Data not shown).

Figure 9.2.a TLC of herb extracts and rosmarinic acid sprayed with DPPH (0.2%)



TLC plate spoted with extracts of herb samples (5µl) and rosmarinic acid (0.5mg/ml) and sprayed with DPPH (0.2%) the yellow areas correspond to antioxidant activity:

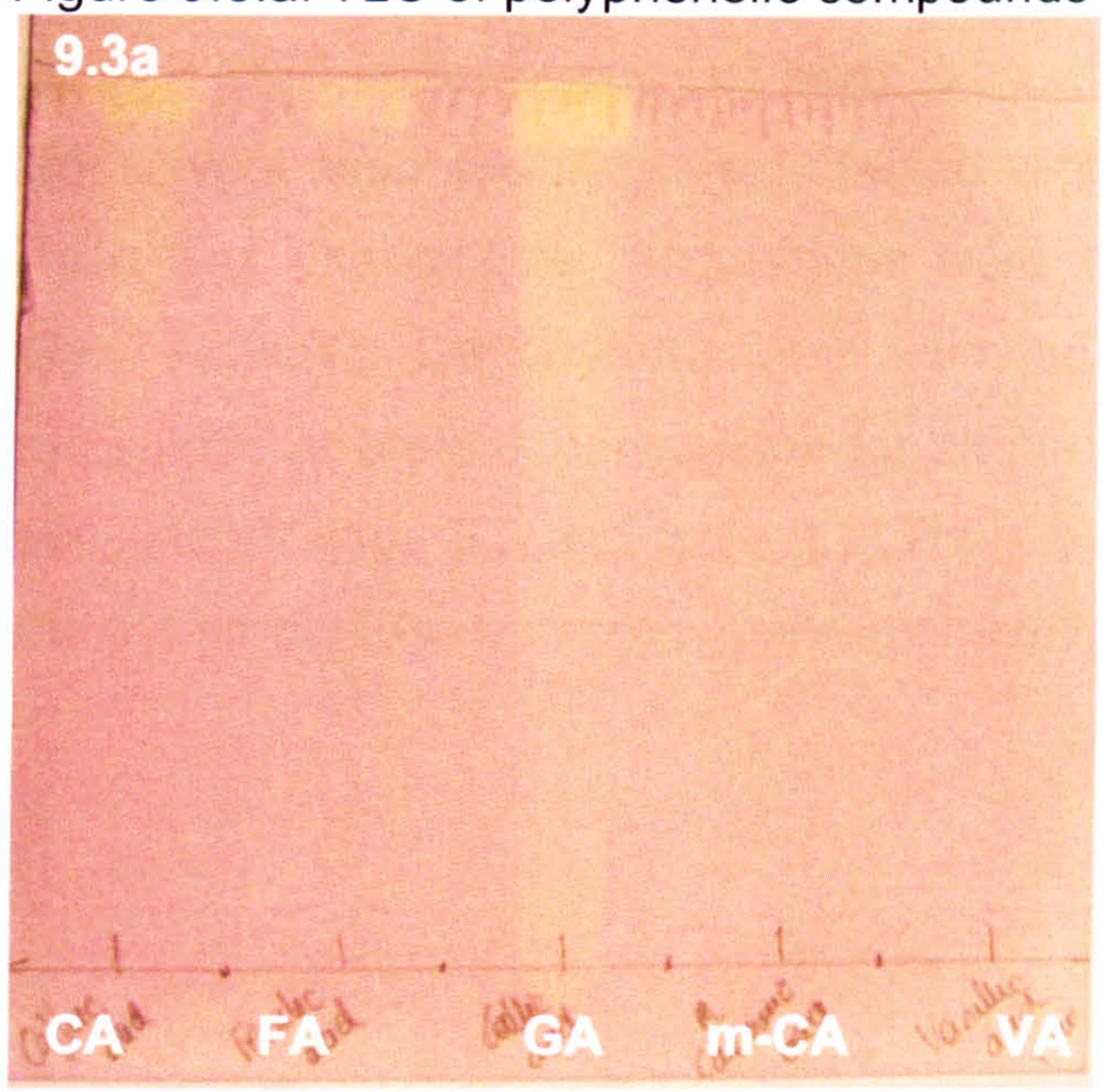
Figure 9.2.b TLC of herb extracts and rosmarinic acid sprayed with natural product and PEG.



TLC plate spoted with extracts of herb samples (5µl) and rosmarinic acid (0.5mg/ml) and sprayed with natural product and PEG.

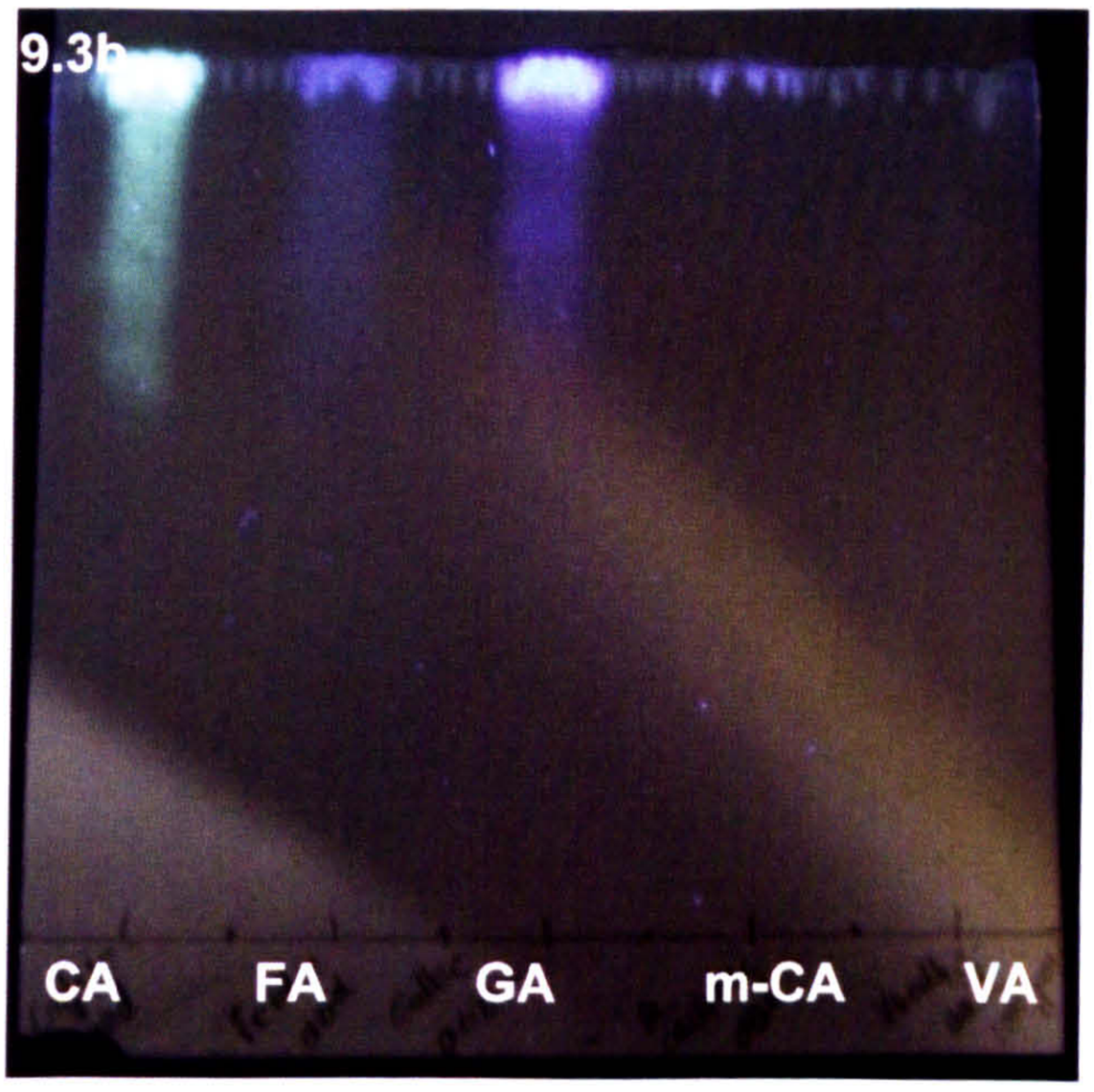
For both 9.1.a and 9.1.b: **a:** Rosemary uncooked (U), **b:** Rosemary heated (H), **c:** Rosemary heated and digested *in vitro* (H & D) , **d:** Sage uncooked (U), **e:** Sage heated (H) , **f:** Sage heated and digested *in vitro* (H & D), **g**:Thyme U, **h:** Thyme H, **i:**Thyme heated and digested *in vitro* (H & D), **j:** Rosmarinic acid (R_f 0.93), solvent front:7.4cm.

Figure 9.3.a. TLC of polyphenolic compounds sprayed with DPPH (0.2%).



TLC plate spoted with standards (5µl, 1mg/ml in methanol) and sprayed with DPPH (0.2%) the yellow areas correspond to antioxidant activity

Figure 9.3.b TLC of polyphenolic compounds sprayed with natural product and PEG.



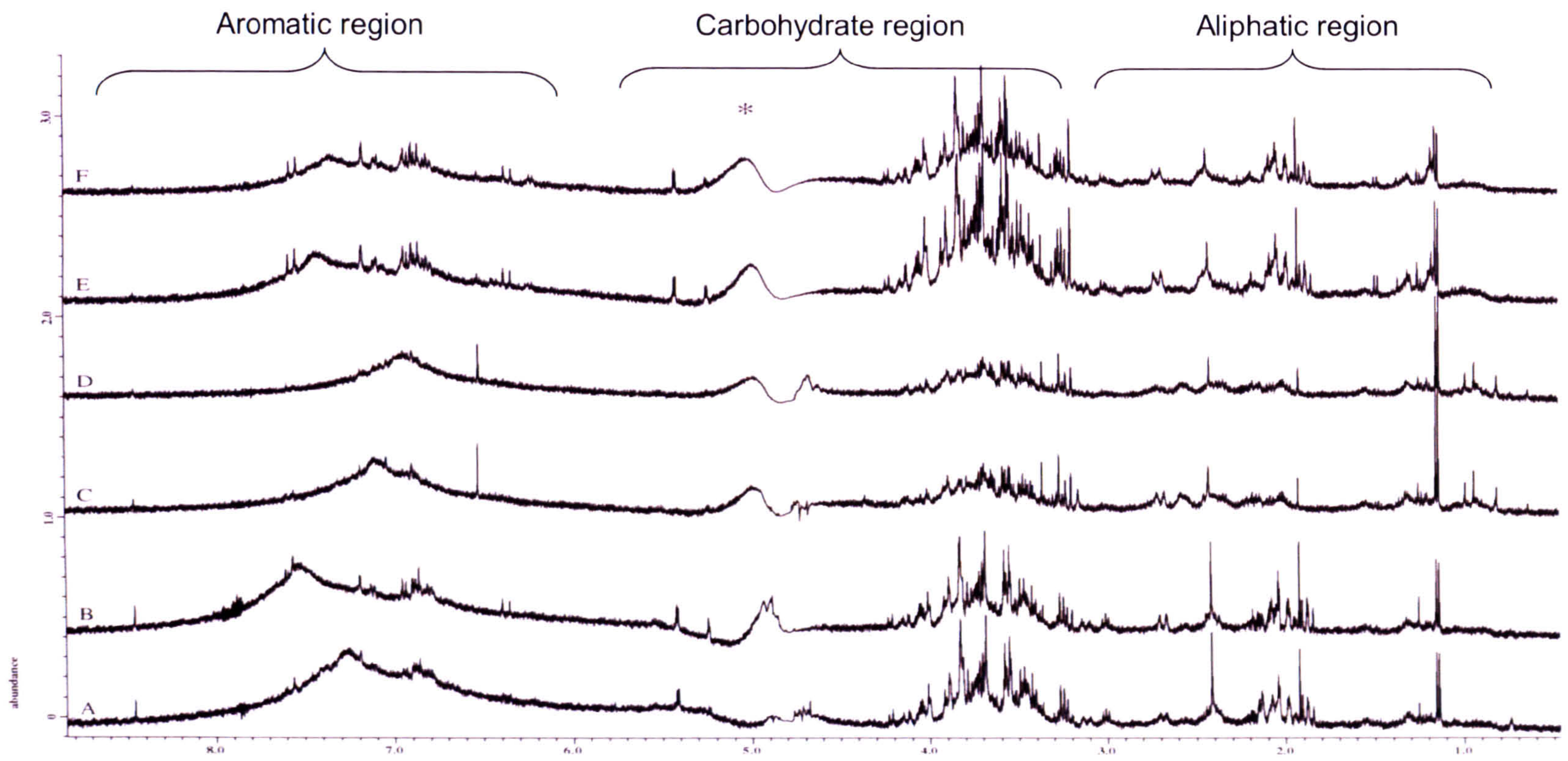
TLC plate spoted with with standards (5µl, 1mg/ml in methanol) and sprayed with natural product and PEG.

For 9.2.a and 9.2.b: **CA**: Caffeic acid, **FA**: Ferulic acid, **GA**: Gallic acid, **m-CA**: m-Coumaric acid, **VA**: Vanillic acid

9.3.3 ^{1}H NMR spectroscopy of herb extracts.

The solvent suppression (water) created slight artefacts by skewing the baseline which was unavoidable. Acquisitions were performed once on 2 to 5 different days. Aliphatic, carbohydrate and aromatic regions were highlighted and the percentage of protons per regions for all three herbs uncooked and heated were calculated (Figure 9.3 and table 9.1).There were little changes in the finger prints and the proportions of protons per regions of herb extracts U compared to H.

Figure 9.4. H^1 NMR spectrum of herbs Uncooked versus their Heated counterpart.



(*) solvent suppression artefact, (A) Rosemary U (B), Rosemary Heated (C), Sage U, (D) Sage H, (E) Thyme U, (F) Thyme H.

Table 9.2 Percentages of proton peaks per H^1 NMR spectrum regions of herbs Uncooked versus their Heated counterpart.

Sample	**% protons in Aromatic Region (8.5-6.0ppm)**	**% protons in Carbohydrate Region (5.5-3.0ppm)**	**% protons in Aliphatic Region (3.0-0.5ppm)**
Rosemary U (A)	46	37	17
Rosemary H (B)	42	37.5	20.5
Sage U (C)	48.5	23.5	28
Sage H (D)	49.5	23	27.5
Thyme U (E)	42	26.5	15.5
Thyme H (F)	46	27.5	17.5

9.3.4 HPLC analysis of herb extracts

There were significant differences between treatments for each herbs ($p \leq 0.05$) except for gallic acid in sage, Tukey *post hoc* tests showed differences in between treatments (Table 9.2) described below for individual compounds.

Caffeic acid

Caffeic acid was undetectable in rosemary (U, H and H&D). Caffeic acid was also undetectable in sage H however levels were detected in sage U and H&D with sage U having significantly higher levels than sage H&D ($p \leq 0.05$). Caffeic acid was statistically significantly increase with treatments for thyme U<H<H&D ($p \leq 0.05$, table 9.1).

m-Coumaric acid

m-Coumaric acid was not detected in any of the samples tested.

Ferulic acid

Ferulic acid significantly increased with treatments (U<H<H&D) for thyme ($p \leq 0.05$); this was not the case for rosemary and sage. Ferulic acid was undetectable in rosemary U and was significantly lower in rosemary H&D compared to rosemary H ($p \leq 0.05$). For sage H&D there was a significantly higher level of ferulic acid compared to sage U and H ($p \leq 0.05$, Table 9.1).

Gallic acid

Gallic acid significantly increased with treatments for rosemary (U<H<H&D) ($p \leq 0.05$) but this was not the case for sage and thyme. For sage U and H&D the levels of gallic acid were similar and although these levels were approximately twice as high as that of sage H, these differences were not significant. For thyme U and H&D the levels of gallic acid were similar and were significantly lower than that of thyme H ($p \leq 0.05$, Table 9.1).

Rosmarinic acid

Rosemary and sage H&D contained significantly higher levels of rosmarinic acid than rosemary and sage U and H respectively ($p<0.05$). Thyme H and H&D had similar levels of rosmarinic acid, which were significantly higher than that of thyme U ($P<0.05$, Table 9.1).

Vanillic acid

For vanillic acid rosemary U and H&D had significantly higher levels than rosemary H ($p \leq 0.05$) (Table 2). However, there were no differences between

vanillic acid in rosemary U and rosemary H&D. There was no difference in the levels of vanillic acid between sage H and H&D but they had significantly higher levels of vanillic acid compared to sage U ($p \leq 0.05$). Thyme H had significantly higher levels of vanillic acid than that of thyme H&D ($p \leq 0.05$); thyme U and H&D had similar levels (Table 9.1)

9.3.5 HPLC analysis of herb extracts heated and digested post bioavailability.

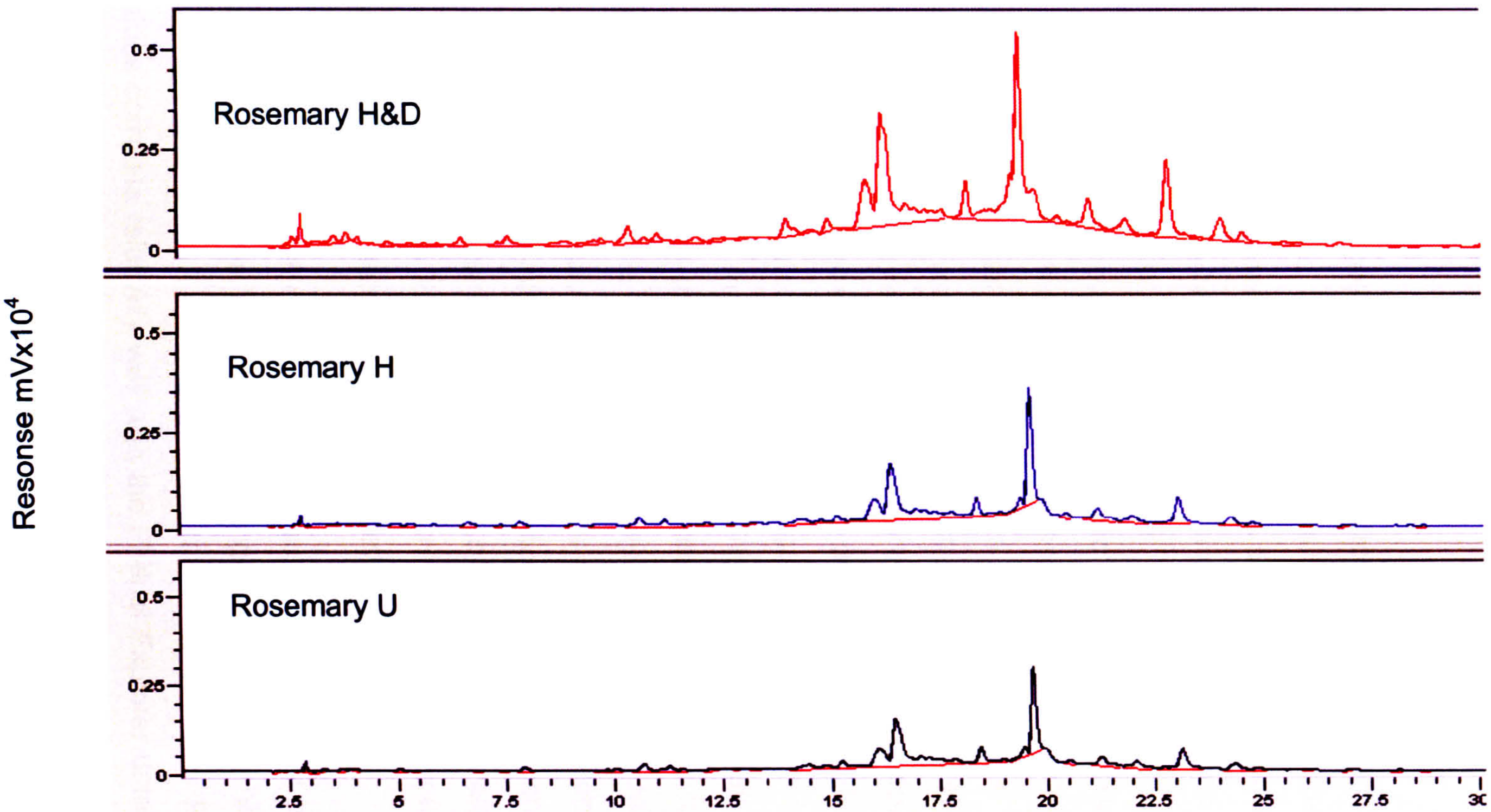
There were no visible traces of peaks on the HPLC chromatograms for the post bioavailability samples of herbs H&D.

Table 9.3 HPLC analysis of herb extracts expressed in µg/g herb.

Samples	Caffeic acid	Ferulic acid	Gallic acid	Rosmarinic acid	Vanillic acid
Rosemary U	UD	UD	12.6 ± 2 [a]	1154.2 ± 56.7	56.9 ± 4.8 [a]
Rosemary H	UD	55.5 ± 1.9 [b]	27 ± 2.4 [b]	1808.1 ± 26.7 [b]	16.5 ± 1.8 [b]
Rosemary H&D	UD	19.3 ± 0.6 [c]	39.5 ± 3.8 [c]	6004.6 ± 579.1 [c]	50.8 ± 6.4
Sage U	301.8 ± 77.5	48.6 ± 48.6	25.9 ± 8	1067.6 ± 116.5	156.4 ± 10.3 [a]
Sage H	UD	34.5 ± 34.5 [b]	12.1 ± 5.8	1566.8 ± 110 [b]	376.8 ± 12
Sage H&D	118.3 ± 51.7 [c]	530.5 ± 68.1 [c]	21.3 ± 17.8	2341.4 ± 314.8 [c]	427.7 ± 12.3 [c]
Thyme U	41.2 ± 3.7	852.5 ± 27.9 [a]	12.3 ± 1.3 [a]	3871.5 ± 83.7 [a]	243.2 ± 12.4
Thyme H	72.2 ± 3.2 [b]	1130.7 ± 80.1 [b]	26.7 ± 0.2 [b]	6530.9 ± 448.4	273.1 ± 14.8 [b]
Thyme H&D	151.4 ± 11.4 [c]	1689.9 ± 68.78 [c]	16.9 ± 0.5	7211.2 ± 371.6 [c]	189 ± 0.7

[a]p≤0.05: significant difference between U and H; [b]p≤0.05: significant difference between H and H&D; [c]p≤ 0.05 significant difference between U and H&D; UD: undetectable, Data expressed as mean ±SD, (n=3).

Figure 9.5 HPLC example chromatograms for herbs U, H and H&D.



9.4 DISCUSSION

The aim of this study was to identify the phenolic acids shown to be prominent in Labiatae, rosemary sage and thyme, specifically rosmarinic acid and its metabolites and give further insight as to the effect of heating, digestion and absorption on these compounds.

The results of the tannin precipitation assay showed that tannins were absent from all the herb samples and thus had no impact on the AA of these herbs. TLC analysis confirmed the presence of rosmarinic acid and other phenolic compounds with antioxidant activity in herb extracts U, H and H&D with a good correlation with results reported by Wagner and Bladt, (1996). The compounds on the upper part of the TLC plates are thought to be phenolic acids and the compounds in the centre of the plates are thought to be flavonoid glycosides (Wagner and Bladt, 1996). No TLC work has been reported in the literature on comparing the polyphenolic profiles of rosemary, sage and thyme post cooking and *in vitro* digestion and the current results clearly show that there were visible increases in colour intensity of bands at the same retention times for H & D extracts compare to their uncooked and heated counterpart, suggesting a higher concentration of polyphenolic compounds in H&D extracts. This observation was supported by a visible increase in AA on the control plate sprayed with DPPH for H&D extracts and these results correlate well with AA assay results (please see Chapter 8 for TEAC and GAE assay values for the extracts analysed). The lack of colouration for the herb extracts post-bioavailability does not necessarily mean the absence of polyphenols, and suggests a weak AA which correlates with results from Chapter 6 (average 11.5% of the initial loaded amount) with a polyphenol concentration which was too low to be detected using this method,.

HPLC results showed that rosmarinic acid was the predominant phenolic acid for rosemary, sage and thyme U, H and H&D, ranging from a mean of 1067.6 ± 116.5 µg/g for sage U to 7211.2 ± 371.6 µg/g for thyme H&D (Table 9.1). Rosmarinic acid values correlate relatively well with the Phenol Explorer database, rosemary (9872.±752.3µg/g), sage (6102.5±3933.1µg/g) and thyme (8290.0±2597.8 µg/g, (Table 9.0). Rosemary H&D contained over 3 times more rosmarinic acid than rosemary uncooked or heated. Sage and thyme H&D had twice as much rosmarinic acid as sage and thyme U respectively which correlates with both TLC

results and significant increases in TEAC and GAE (Chapter 8) suggesting that rosmarinic acid is a major contributor to the AA of the Labiatae.

Caffeic acid was not detected in rosemary which differs from the literature (Phenol-Explorer, rosemary: 96.7±56.9µg/g, Table 9.0). Variations in chemical composition in natural products are common and this is illustrated in the large error bars obtained from the Phenol Explorer database. Herb chemical compositions are known to differ with seasons, provenance and abiotic factors (Luis and Johnson, 2005; Dragland *et al.*, 2003). The caffeic acid levels were significantly increased with treatments (U<H<H&D) for thyme but not sage. Thyme has coarser leaves than sage and as discussed in Chapter 3 and 5, the *in vitro* digestion may have facilitated the release of caffeic acid from thyme. Sage U had the highest amount of caffeic acid (301.8 ± 77.5 µg/g, Table 9.1) which correlated well with the Phenol-Explorer data (264.0±109.2µg/g, Table 9.0). No caffeic acid was detected in sage H, it is a possibility that caffeic acid is sensitive to degradation by heat, and this could be due to the physiology of sage leaves (soft and thin) compared to the coarser leaves of thyme. Regarding sage H&D about a third of the initial amount of caffeic acid in U sage was present, this could indicate some degree of caffeic acid degradation from heat treatment as stated above, followed by an increase in caffeic acid from sage leaves via *in vitro* digestion. Similar patterns were observed for levels of gallic acid and ferulic acid in sage, but not for rosmarinic acid or vanillic acid in sage. Therefore different polyphenolic compounds may have various thresholds to heat degradation. Furthermore this highlights the importance of food matrices in the availability of polyphenols, coarser leaves may offer some "protection" against heat degradation to the polyphenols.

Gallic acid, vanillic acid and ferulic acid, were present in all three herbs in all three treatments but in fairly small amounts compared to rosmarinic acid, and some with very large standard deviations, which did not translate into any real statistically significant pattern when comparing U, H and H&D extracts. There was a trend towards an increase in compounds for H&D herbs thus suggesting that these polyphenols may contribute to the overall increase AA from U to H to H&D herbs. Rosemary H&D had the highest amount of gallic acid (39.5 ± 3.8 µg/g) compared to its U and H counterparts and sage and thyme (U, H and H&D). Although gallic acid was detected by Hossain *et al.* (2010) in rosemary, it was not detected or quantified in the Phenol Explorer website. In the current study, gallic acid was

quantified in rosemary U (12.6 ± 2 µg/g). Sage H&D had the highest amount of vanillic acid (427.7 ± 12.3 µg/g), and the vanillic acid value for sage U (156.4 ± 10.3 µg/g, Table 9.1) in the current study was higher but not dissimilar to the value reported in to the Phenol Explorer website (58.5±82.7 µg/g, maximum level detected was 117 µg/g, Table 9.0). Thyme H&D had the highest amount of ferulic acid (1689.9 ± 68.78 µg/g, Table 9.1). The ferulic acid value for thyme U (852.5 ± 27.9 µg/g, Table 9.1) was a lot higher than the values reported in the Phenol-Explorer database (56.5±79.9 µg/g, Table 9.0). As stated above, chemical variations are common in natural products.

m-Coumaric acid is a metabolite of rosmarinic acid as a result of metabolism and was not detected in any herb samples. Rosmarinic acid is the predominant polyphenol in the herbs studied therefore the absence of *m*-coumaric acid provides further evidence to support the hypothesis that rosmarinic acid remains stable through heating and *in vitro* digestion as discussed in Chapter 5.

TLC results suggest that there was no evidence of new compounds forming as a result of heating the herbs and this correlated well with the ^{1}H NMR spectroscopic investigation where the same fingerprint of compounds was visible in both uncooked and heated extracts for all three herbs with little changes after heating. Although HPLC showed that some compounds were reduced or increased in some heated herbs compared to their uncooked counterparts there was no real pattern that provides evidence to suggest that heating affects all these compounds equally, and it was more likely to be due of either natural variations or individual behaviour of the polyphenols to heat. For *in vitro* digestion the TLC analysis did not appear to show that new compounds formed either but only showed an increase in the existing compounds which correlates well with both AA results and HPLC results. The absence of new compounds would require further investigation with a more sensitive tool such as LC-MS linked to a comprehensive library of compounds.

These results taken together suggest that it is the increase in polyphenols, specifically rosmarinic acid, which is responsible for the observed increase in AA following *in vitro* digestion. However, it is possible that another compound with high AA was present in sage alongside rosmarinic acid, as the rosmarinic acid content of sage H&D (HPLC) was less than half that of the other two herbs H&D. An unknown compound was visible at (R_f 0.95) on the TLC for sage U, H and

H&D. The fact that this spot was located towards the upper part of the plate suggests that it may be a phenolic acid (Wagner and Bladt, 1996) however these are usually in shades of blue (Figure 9.2a) therefore further investigation into the identification would be required to confirm the identity of this compound.

The lack of polyphenolic compounds detected post bioavailability may be due to a lack of sensitivity of the HPLC technique and not necessarily an absence of polyphenolic compounds as the results of both TEAC and GAE assays (Chapter 6) showed that an average of 11.5% of the antioxidant activity of the herb extract, post bioavailability, was detected. These herb extracts (H&D) were diluted 1:10 before transport and further diluted with the basolateral transport medium .LC-MS which can detect smaller amounts of polyphenols and could have been used instead to identify and quantify the polyphenols present and this would require further investigation.

It also needs to be born in mind that the focus on this study was on the phenolic acid, specifically rosmarinic acid and its metabolites, however, complex mixtures of other compounds such as other phenolic acids and flavonoid glycosides are present in herbs and may also contribute to the biological activities of the culinary herbs studied.

9.5 Conclusion

Results of this study suggest that the phenolic acids are contributors to the antioxidant capacity of the herbs, with rosmarinic acid as the predominant phenolic acid, prior to and following heating and *in vitro* digestion. There was no evidence of tannins in any of the extracts and no evidence of new compounds forming as a result of heating and *in vitro* digestion, but instead there was an increase in the compounds already present. This study provides yet further evidence to support the hypothesis that the physical breakdown of the plant cell wall promotes an increased exposure of inner cells containing the polyphenolic compounds and that the food matrix may be an important factor in the availability of these compounds.

Chapter 10 General Discussion and future work.

10.0 Introduction

Culinary herbs are functional foods with health benefits that are attributed mainly to their polyphenols (Tapsell *et al.*, 2006). However, the literature often refers to the antioxidant activity, anti-inflammatory activity, and antimicrobial activity of culinary herbs which were ground or powdered, and extracted in solvents, such as acetone, ethanol and methanol, rather than water, which is used in the preparation of culinary herbs for domestic purposes (Peng *et al.,* 2007; Shan *et al.*, 2007). In addition, culinary herbs undergo some form of heating/cooking prior to consumption and then undergo digestion followed by the absorption of some of their constituents. However there is a paucity of data concerning the impact of such processes on the polyphenolic properties of culinary herbs but understanding their impact is key to assessing and evaluating the dietary polyphenol contribution of culinary herbs and ultimately the biological significance of this contribution.

10.1 Limitations of the study

One of the ongoing challenges of carrying out such an investigation was the standardising of the methods used to investigate the polyphenolic properties of the culinary herbs selected. Batch variation was a major issue: Batch variation is a well known and common limitation when studying the biological properties and chemical profiles of plant derived natural products and highlights the importance of looking at abiotic factors (pH, soil nutrient and metal ion content, altitude, water, temperature and exposure to sunlight) when addressing the nutritional quality of such foods. Dragland *et al.* (2003) reported that seasonal variations affect the antioxidant activity of culinary herbs and are thus important when regarding their contribution to total dietary antioxidant intake in the form of bioactive compounds. Therefore such factors will have influenced the bioactive compound profile and thus the biological activities of the culinary herbs used in the current study. Storage may also have influenced the findings of the current study as it can affect quality and biological activities (Stafford *et al.*, 2005). There is little that can be done to address the natural variations due to abiotic factors; however, it is important to be aware of these variations. Information on the commercially available culinary herbs used in the current study including genus, species, place of provenance and pre-sale processing and storage helped to maintain some degree of standardisation. Additional information consisting of genus and species,

and where relevant, sub-species, variety and cultivar name, batch number, and advice on storage procedure post-sale, would certainly help to maximize standardisation for future studies.

10.2 The role of cooking on polyphenols and antioxidant activity (AA) from culinary herbs and the possible role of synergy in the provision of polyphenols and AA from culinary herbs.

The first objective of this study was to investigate the overall contribution of the culinary herbs selected (individually and in combination) to negating oxidative stress. This objective was investigated by assessing the impact of cooking methods, commonly used in the preparation of culinary herbs, on their antioxidant activity. Four commonly used culinary herbs, parsley, rosemary, sage and thyme (dried) were selected. The results of this part of the investigation suggest that the quantity of culinary herbs used in a domestic kitchen contributes to dietary antioxidant intake with cooking (microwaving, stewing, heated in a Teflon ® stir frying pan) significantly increasing both gallic acid equivalent (GAE) and Trolox equivalent antioxidant capacity (TEAC) (collectively termed antioxidant activity – AA). Furthermore, both the cooking method and the length of time of cooking impacted on the AA. Stewing for 30 and 60 minutes caused an increase in AA for all herbs compared to cold and hot extracts. For herbs heated in a Teflon ® stir frying pan, there was an increase in AA with an increase time of exposure (10, 15 and 20 minutes). There is a paucity of studies on the impact of cooking on the antioxidant activity of culinary herbs, as stated above, however, results that are available suggest that such cooking methods could increase AA via the physical breakdown of the plant cell wall, thereby promoting an increased exposure of inner cells containing the polyphenolic compounds (Choi *et al.*, 2005; Kim *et al.*, 2005).

If there were polyphenol losses during cooking via leaching, heat damage or oxidation in the current study, these losses were outweighed by the liberation of more compounds from within the plant cell walls translating in higher AA. However, when considering the role of these herbs as significant dietary contributors of polyphenols further studies are needed involving complex food mixtures. One study looked at the changes in AA on spice-based marinating sauces after marinating and cooking with chicken and observed losses in AA (from polyphenols and other dietary antioxidants, these were non-identified), possibly due to the complexity in the pro/anti-oxidant balance within the food

mixtures (Thomas *et al.*, 2010). Thus this area requires further investigation; the contribution of dietary polyphenols of culinary herbs cooked with other foods would make an interesting study.

The individual AA and polyphenol profiles of culinary herbs are, as stated above, well established and synergistic and antagonistic biological effects have been reported between culinary herbs (Perry *et al.*, 2000; Williamson, 2001) and between polyphenols (So *et al.*, 1996; Peyrat-Maillard *et al.*, 2003). It is not uncommon for these herbs to be used combined for the purpose of cooking therefore to shed some light on the polyphenol contribution of culinary herbs to the diet, the AA of combined herbs was investigated.

Combining culinary herbs appeared to increase the polyphenolic antioxidant capacity of these herbs although there was no consistently statistically significant synergy observed. This does not necessarily mean that synergistic effects may not occur *in vivo* where culinary herbs are ingested as part of a meal in a complex food matrix, and this area (the impact of a complex food matrix) needs to be investigated further as suggested above. It must also be borne in mind that three out of the four herbs investigated were Labiatae (rosemary, sage and thyme) and these are known to have similar polyphenols (predominantly rosmarinic acid) thus it may be that a selection of culinary herbs from different families may generate synergistic interactions when combined due to their different polyphenol profiles. For instance, Peyrat-Maillard *et al.* (2003) found evidence of synergism in antioxidant activity of the paired polyphenols quercetin (flavonoid found in the *Umbelliferae* parsley) and rosmarinic acid (a phenolic acid fond in Labiatae such as rosemary, sage and thyme).The advantage of using a selection of culinary herbs in the diet from different plant families is the exposure of the host to a wider variety of polyphenols with an array of biological activity. When considering the role of these herbs as significant dietary contributors of polyphenols further work is needed, involving combining culinary herbs from different plant families as well as assessing the overall AA and polyphenol profile of recipes involving culinary herbs as part of complex food mixtures.

10.3 The role of digestion and absorption on the provision of polyphenols and AA from culinary herbs.

The second objective of this study was to investigate the role of the gut on the AA and polyphenolic profile of the same selection of cooked culinary herbs (H) using

validated *in vitro* models of digestion and absorption. No work has been reported in the literature on the polyphenolic profiles of culinary herbs post cooking and post cooking followed by *in vitro* digestion. However, as stated above such work is essential in determining the dietary contribution of culinary herbs to polyphenol intake. This study demonstrated that the AA of culinary herbs is enhanced via in *vitro* digestion. As with the effects of cooking, the effect of digestion *in vitro* was thought to be due to the physical breakdown of the plant cell wall, thereby promoting an increased exposure of inner cells containing the polyphenolic compounds. The scanning electron microscope (SEM) analysis of rosemary and thyme leaves uncooked (U) compared to heated (H) and heated and digested (H&D) provided further evidence to support this hypothesis by showing visual damage to the leaf epidermis post heating with further damage occurring post *in vitro* digestion. Thin layer chromatography (TLC) analysis demonstrated that rosmarinic acid and other phenolic acids as well as flavonoid glycosides were present in rosemary, sage and thyme (U), (H) and (H&D). Furthermore, high performance liquid chromatography (HPLC) analysis of (U), (H) and (H&D) herb extracts confirmed the presence of gallic acid, ferulic acid, vanillic acid and rosmarinic acid; the latter being the predominant polyphenol in all three herbs which is in accordance with the literature (Wang *et al.*, 2004; Kivilompolo *et al.*, 2007 a & b; Hossain *et al.*, 2010).

No work was reported in the literature on the bioavailability of cooked and digested culinary herbs and it appears, based on limited studies, that the bioavailability of pure hydroxycinnamates polyphenols such as rosmarinic acid, caffeic acid or ferulic acid, known to present in rosemary sage and thyme, varies greatly (Zhao and Moghadasian, 2010).The successful set up of the *in vitro* Caco-2 model of transport (Chapter 6) and bioavailability results suggested that on average 11.5% of the initial AA present in herb extracts placed on the apical side of the model was made available on the basal side after 60 and 120 minutes, thus indicating that these herbs are providers of dietary polyphenols and antioxidant activity post absorption and indicating that such herbs provide polyphenols to the systemic circulation *in vivo* although the bioavailability threshold for these compounds is unknown. In addition, it needs to be borne in mind that some of the polyphenols in these herbs may remain in the small intestine for a longer period and therefore may not be made available after 60 – 120 minutes and thus the *in vivo* significance of these observations remains unclear. Identification of polyphenolic compounds is

required to understand fully the impact of digestion and absorption on the antioxidant capacity of these herbs however the polyphenols present in the post-bioavailability herb extracts appeared to be too dilute for HPLC detection. However, the small starting herb sample was necessary as it was important to mimic as closely as possible the ingestion and digestion of the culinary herbs used domestically so as to further establish their role as dietary providers of polyphenols; liquid chromatography-mass spectrometry (LC-MS) may have been a better method for detecting small amounts of polyphenolic compounds, and, used in conjunction with a comprehensive library of compounds, identifying them.

Again the food matrix needs to be considered when investigating the bioavailability of polyphenols from the culinary herbs investigated. The leaves of such herbs are often ingested whole (fresh/dried) and whilst some of the non-starch polysaccharides (NSPs) in these leaves may be broken by food processing, some of these NSPs may still be retained. The results of the current study suggest that the physiology of culinary herbs (thick epidermis and cell walls made up of NSPs) especially for coarse leaves such as those of rosemary, may have the advantage of "protecting" the polyphenols from digestion in the upper part of the gastro-intestinal tract. It is feasible, therefore, that, *in vivo*, when partially digested cooked herbs reach the colon, the NSP constituents, mainly cellulose, of these herbs are metabolised by colonic microflora (Kroon *et al.*, 1997, Tuohy *et al.*, 2006). Therefore, the polyphenols in these herbs could, via their exposure to the same microflora following the breakdown of cellulose, be metabolised in the colon. The work of Jenner *et al.*, (2005) goes some way to support this theory as they showed that the phenolic acid, phenylacetic acid and its metabolites 3-phenylpropionic acid and 3-(4-hydroxy)-phenylpropionic acid were found in high amounts in human faecal water.

Based on studies on the absorption and metabolism of polyphenols present in the culinary herbs investigated (Chapter 6), the local (gut based) biological activities of these polyphenols could last quite some time, varying with colonic transit time. Therefore, it could be hypothesised that with regular ingestion of culinary herbs such as the Labiatae, the host could be provided with a regular supply of polyphenols providing significant local antioxidant, anti-inflammatory activities (especially from rosmarinic acid) and antimicrobial activities (volatile oils). Thus, by acting directly on sites of chronic inflammation in GI disorders (Crohn's disease,

celiac disease and IBS) one could speculate that these polyphenols may be key in maintaining long term gut health.

In contrast to the possible role of the colon on the provision of polyphenols from culinary herbs, the significant breakage of the leaf structure (epidermis and cell walls) by the grinding/powdering of these herbs, may mean that most of the phytochemicals in these herbs are released at once when ingested and will go through the upper part of the digestive system "unprotected". ^{1}H NMR demonstrated the stability of rosmarinic acid at the different pHs (2, 7 and 8) along the digestive track. However, this does not necessarily mean that all the rosmarinic acid ingested would arrive intact in the colon, as human intervention studies have demonstrated that some rosmarinic acid is absorbed in the upper part of the digestive tract and excreted rapidly (Baba *et al.*, 2005; Nurmi *et al.*, 2006). It could be that the body identifies polyphenols as unwanted compounds and thus metabolises and disposes of these polyphenols as quickly as possible (Baba *et al.*, 2005; Nurmi *et al.*, 2006). This not only gives a small window of opportunity for these polyphenols to have any biological activities in the upper gastrointestinal tract and beyond (i.e. post absorption), but it also reduces the possibility of these polyphenols reaching the large intestine. Thus, the biological effects of polyphenolic compounds at the site of gastro-intestinal (GI) inflammation would then become questionable. Furthermore, contrary to large rosmarinic acid molecules which are thought to be poorly absorbed in the colon, according to Konishi and Shimizu (2003) a significant percentage of smaller hydroxycinnamates molecules such as ferulic acid could be quickly absorbed from the colon without requiring prior metabolism which adds to the complexity of this area of research. In light of all the questions generated in the current study concerning the bioavailability of polyphenols, it would be interesting to further investigate the differences between the ingestion of whole leaves (fresh or dried) compared to ground/powdered leaves, looking at plasma, blood and faecal levels of polyphenols and their metabolites in human studies to determine if there are any differences, due to differences in the structures of food matrices, to confirm this hypothesis. Furthermore, studies are also needed on the effects of fermentation of the culinary herbs cell walls by gut micro flora, especially by species of the genera lactobacillus and bifidobacteria the consumption of which has increased due to an impressive media campaign in support of the use of probiotics in the past decade

(Mintel, 2010). Maybe it is the profile of the flora that is key in influencing the biological significance of polyphenols in the gut and beyond.

10.4 The role of cooking and digestion on the biological activities of culinary herbs.

Little is known about the impact of cooking and digestion on the anti-inflammatory activity of culinary herbs or whether this activity is linked to the antioxidant activity of polyphenols in these herbs. Studies that have investigated the anti-inflammatory activities of culinary herbs have used various amounts of herbs and various methods of extractions that do not necessarily relate well to human nutrition. The final objective of this study was to determine if the polyphenols of these herbs, U, H, H&D and post absorption contributed to the presence of any anti-inflammatory and anti-microbial activity and no work has been reported in the literature on the biological activities of rosemary, sage and thyme U, H and H&D. Results on anti-inflammatory activity of culinary herbs, determined by measuring SOD mimetic (SODm) activity and the inhibition of release of IL-8 from peripheral blood mononuclear cells exposed to H_2O_2 or TNF α, in this study showed that aqueous herb extracts of rosemary sage and thyme U, H and H&D had no cytotoxic effects on the cells studied but did possess SODm activity as well as IL8 inhibition activities. These activities correlated positively with their AA. This investigation also suggested that rosmarinic acid, as the predominant polyphenol in rosemary sage and thyme, may be responsible, for this anti-inflammatory activity. The results of the current study are supported by the work of Osakabe *et al.* (2004) who demonstrated the effectiveness of rosmarinic acid at reducing inflammation by inhibiting the PKC/ NF-KB pathway in mice. However, further studies using molecular biology techniques such as ELISA, Western Blotting and/or Microarray are required to ascertain if the anti-inflammatory activity reported in the current study is due to the polyphenol content of the herbs investigated and their antioxidant activity and further understand these mechanisms of action.

Shan *et al.* (2007) showed that there is a good correlation between the antioxidant activity and antimicrobial activity of methanolic extracts of the Labiatae rosemary, sage and thyme and suggested that there was an association between the phenolic constituents of culinary herbs and their anti-microbial activity. However, it was important to establish if this relationship was true for the quantity of Labiatae culinary herbs used in the current study (i.e. levels used for domestic purposes)

and to further investigate the influence of synergy (see Chapter 4) between the herbs when combined, on their anti-microbial activity. No association was established between the AA of the herbs and any anti-microbial activity in their aqueous extracts either alone or combined as they appeared not to possess any anti-microbial activity. Liu and Nakano (1996) hypothesised that bacterial sensitivity is proportional to the concentration of extracts used. It is a possibility that the aqueous extraction did not provide polyphenols in high enough amounts to demonstrate anti-microbial effects. However, increasing the amount of herbs was not an option in the current study, as it is unlikely that larger amounts of culinary herbs would be used in cooking. Nevertheless, water based methods of cooking (microwaving, stewing) involving heat and longer cooking times may release polyphenol compounds in sufficient amounts to have some anti-microbial activity, and thus warrant further research.

In contrast to the aqueous extracts, oil extracts of sage and sage in combination with rosemary and thyme appeared to support the anti-microbial potential of volatile oils, and this effect was enhanced further by heating the herbs in a Teflon ® stir frying pan for 10 minutes. The anti-microbial effects of volatile oils are supported by the literature (Graven *et al*., 1997; Baratta *et al*, 1998; Shan et al., 2005; Hinnerburg *et al*., 2005; Barry Ryan and Bourke, 2008; Gutierez *et al*., 2008). However, this investigation did not go further by investigating the compounds responsible. The investigation of the chemical profile of the oil herb extracts via HLPC/GC-MS/LC-MS may provide a clearer picture as to the antimicrobial synergistic effects of volatile oils. In addition, a comprehensive identification and quantification of the polyphenol compounds present in the aqueous herb extract would be also be required, as the literature suggests that polyphenols such as rosmarinic acid possess anti-microbial activity (Moreno *et al*., 2006). Furthermore, it would be useful to establish the threshold of anti-microbial activity of these herbs, alone and combined and polyphenols alone and combined in order to further understand the biological significance of dietary polyphenols.

10.5 Conclusion

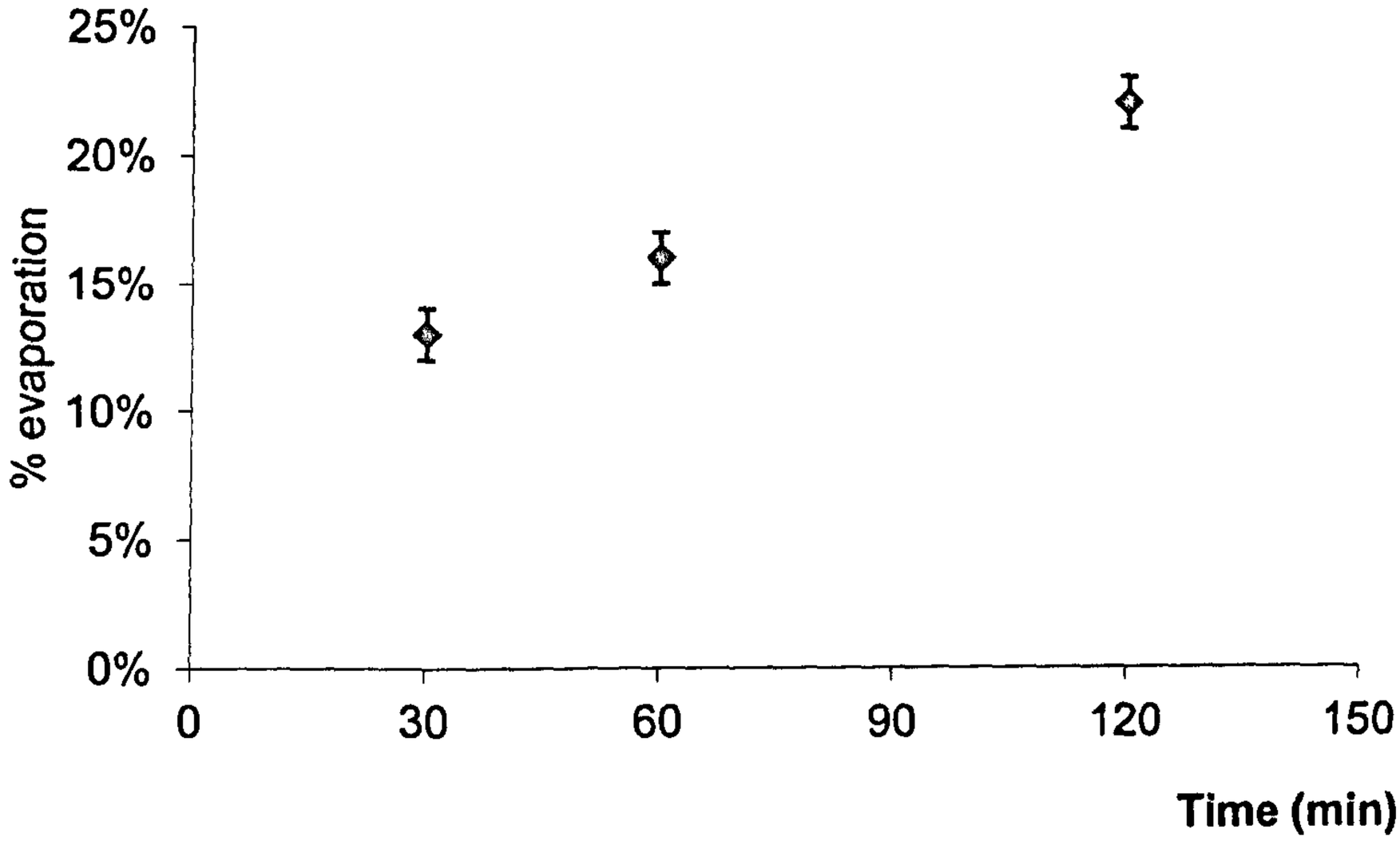
This study provides further knowledge of the potential dietary contribution of polyphenols by culinary herbs and evidence of biological properties that appear to extend beyond the role of these herbs as antioxidant providers. However the key question is do these culinary herbs have real potential as significant dietary

sources of polyphenols? The potential health benefits of polyphenols are widely known and recent studies have started looking at ways to optimise the delivery of polyphenols in humans (Yaakob, Wilde and Kroon, 2010). For example, the flavonoid quercetin, which is thought to have a poor bioavailability, was extracted from plants, purified, processed and blended with an oil-water emulsion that facilitates its delivery and absorption in the human digestive system. The end product is seven times more bio-available than quercetin in aqueous solution. Looking at the vitamin and mineral supplements market (which was worth £396 million in UK, 2009; Mintel, 2009b), with a clever marketing strategy, polyphenols formulated to increase their bioavailability may generate significant profits for food companies and multinational pharmaceutical companies. However, studies on conventional antioxidant supplements, including β carotene, vitamin A and vitamin E, and the prevention of chronic diseases, such as cancer, have shown them to be ineffective (Bleys et al., 2006; Park *et al.*, 2011) or detrimental (Bjelakovic et al., 2007; WCRF/AICR, 2007). Thus, it is possible that polyphenol products with a high absorption potential, such as the one mentioned above, have negative effects on human health similar to those associated with some conventional antioxidant supplements. Overall, the literature concerning nutritional supplements vs. nutrients/bioactive compounds in foods supports the role of the latter in conferring health benefits. With regards to polyphenols, the literature reports that, although effective in small quantities (Yoon and Baek, 2005; Romier et al., 2008) more than adequate amounts, can be attained with a diet rich in plant foods (Kris-Etherton et al., 2002; Ren et al., 2003, Scalbert et al 2005). Furthermore, based on the findings of the current study, the consumption of culinary herbs, in small quantities, appears to be associated with the health benefits of polyphenolic compounds post cooking and post digestion and absorption *in vitro*. Thus, further studies concerning culinary herbs (as single herbs, in combination and as part of a meal) and the biological significance of their polyphenols *in vivo*, both locally, for example in the gut, and systemically, would significantly increase knowledge and understanding of the dietary potential of culinary herbs with regards to polyphenol intake.

Appendix 1 (Chapter 3)

Evaluation of percentage of water lost to evaporation during stewing

Figure A1.1 Evaluation of percentage of water lost to evaporation during stewing



Data are presented as mean ±SD (n=3).

Standard curves for antioxidant assays.

Figure A1.2a The standard curve for Trolox in the $ABTS^{\bullet+}$ assay.

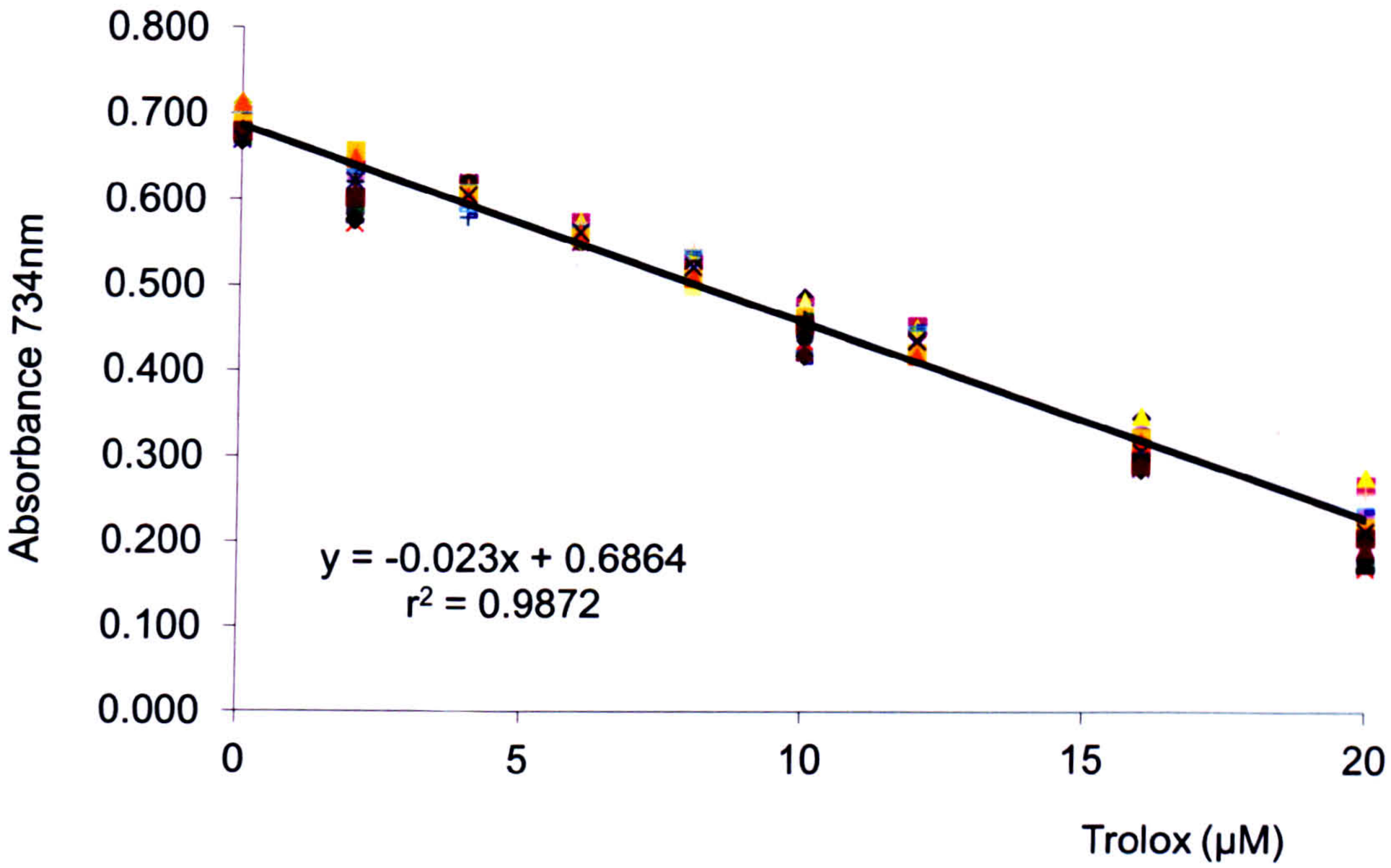


Figure A1.2b The Percentage inhibition curve for Trolox in the $ABTS^{\bullet+}$ assay.

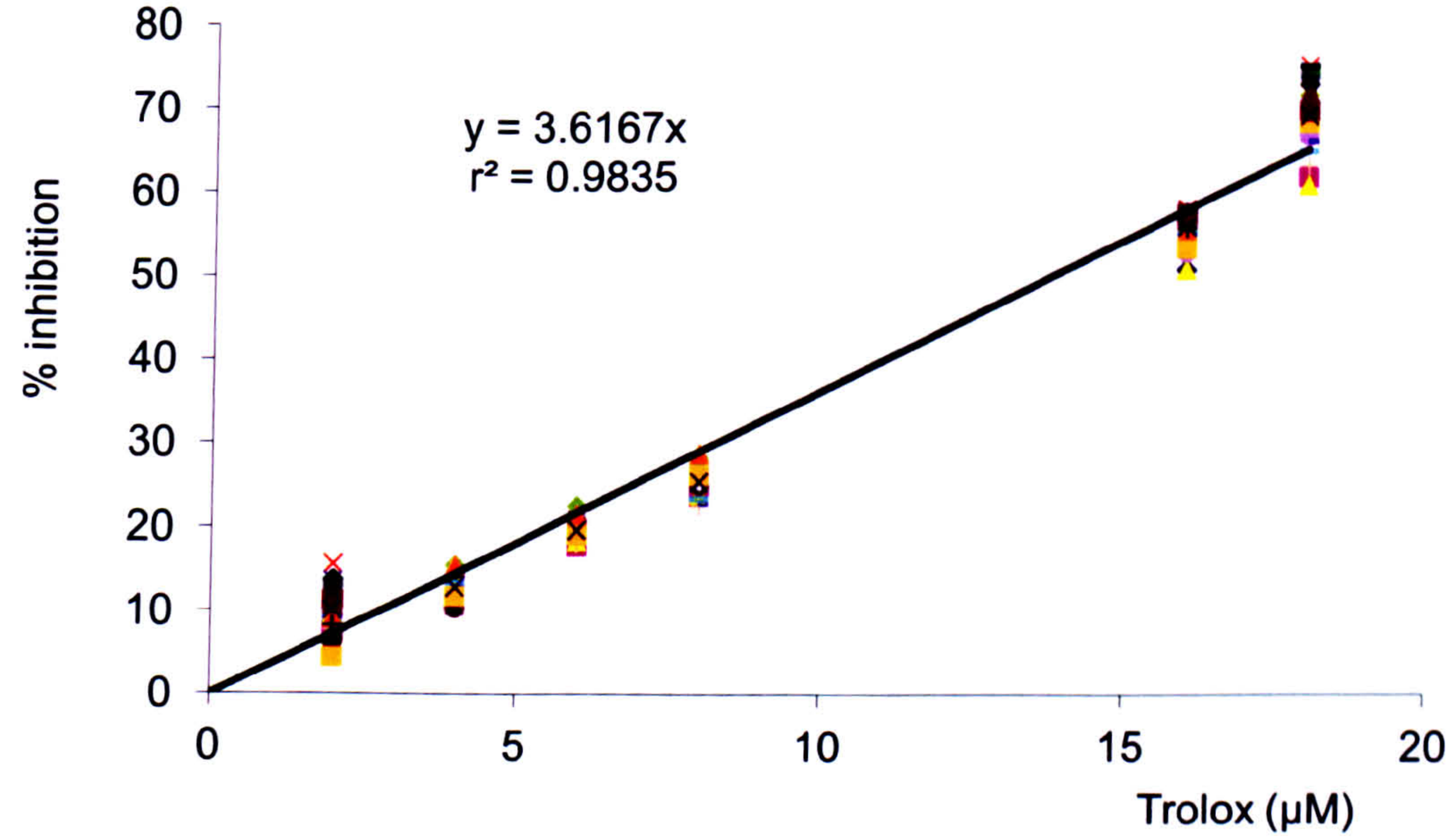
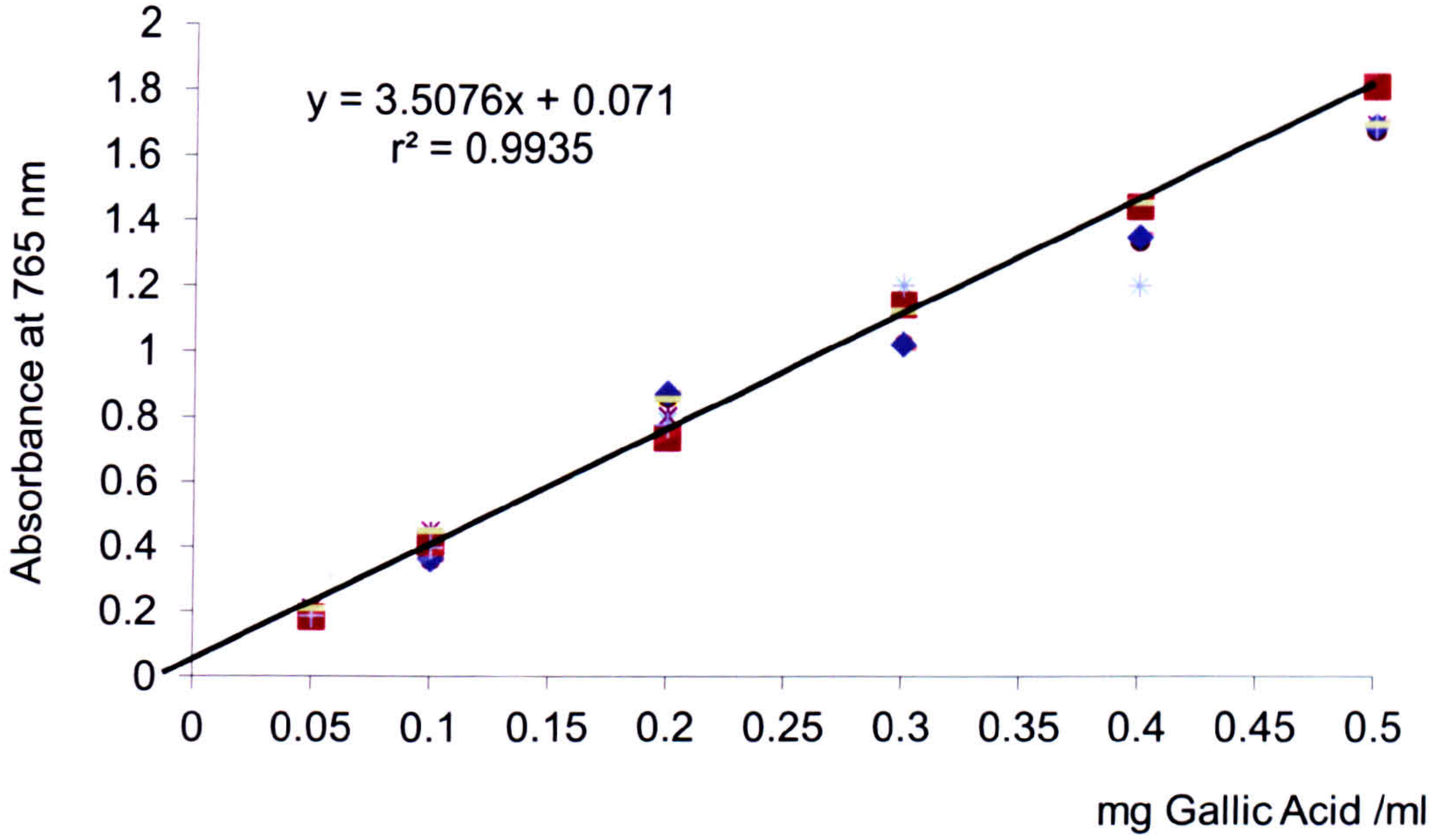


Figure A1.3 The standard curve for Gallic acid in the Total phenolic acid assay.

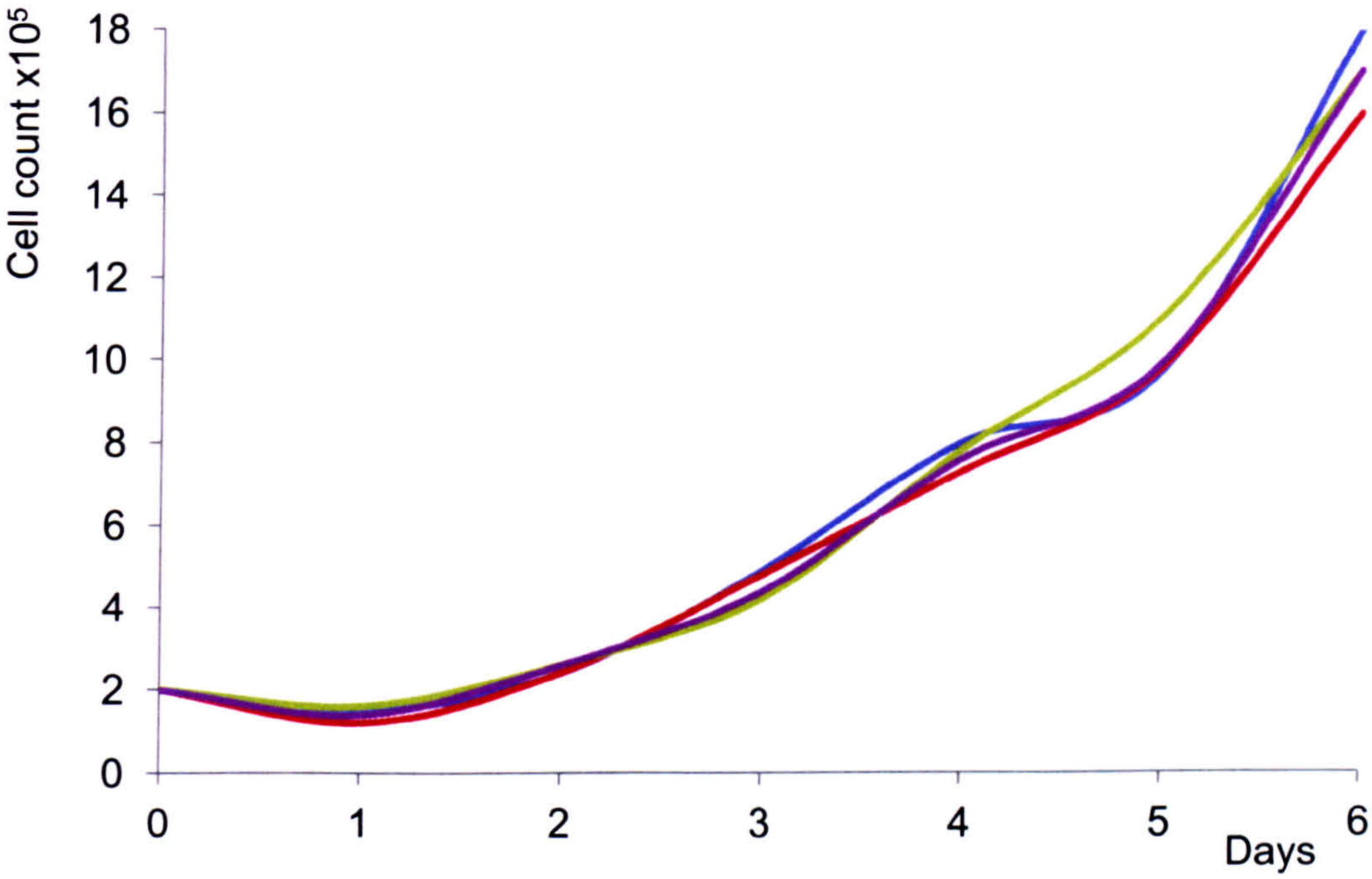


Appendix 2 (Chapter 6)

Caco-2 growth curves.

Caco-2 cells were seeded 2x 10^5 cells per ml (Li *et al.*, 2002), (1ml) in 24 well plates in triplicates and counted daily for six consecutive days using the trypan blue exclusion assay (figure A2.1).

Figure A2.1 Growth curve for Caco-2 cell line.



Growth curves Correlation p=0.986, statistically significant at 0.001 level, (2-tailed), (n=4).

Summary

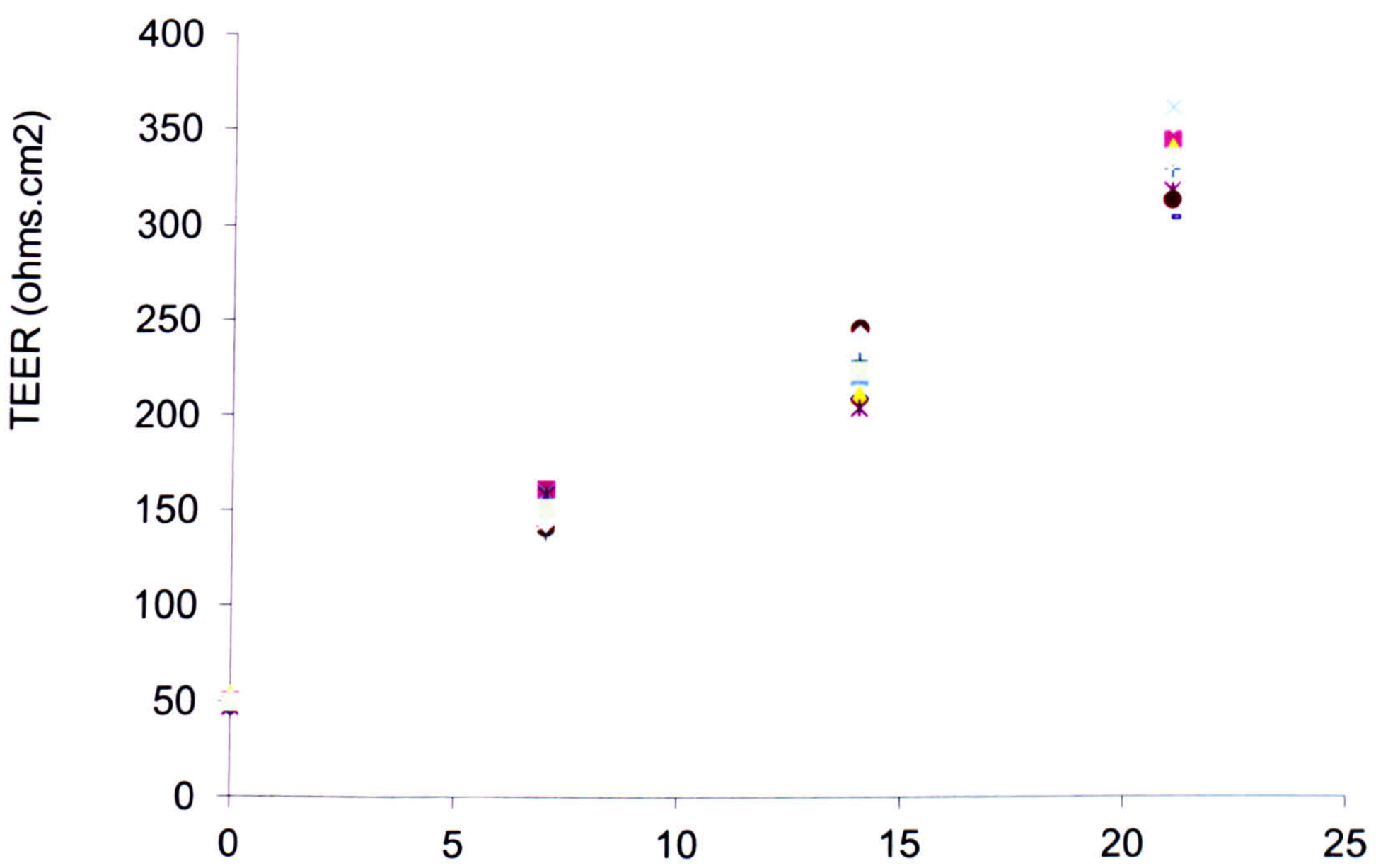
Growth curves (N=4), consistently showed similar results, with significant correlation p=0.986 (p=0.001, Figure A2.1). The log phase was reached at day 5 (70-80% confluent). In order to maintain the cell line, the cells were split every 4 to 5 days with fresh culture medium. Caco-2 cells growth curves over a seven day period were done three times and showed a constant good correlation with previously published data (Paniagitou, 2006) suggesting that a healthy cell line was established.

Appendix 3 (Chapter 6)

Transepithelial electrical resistance (TEER).

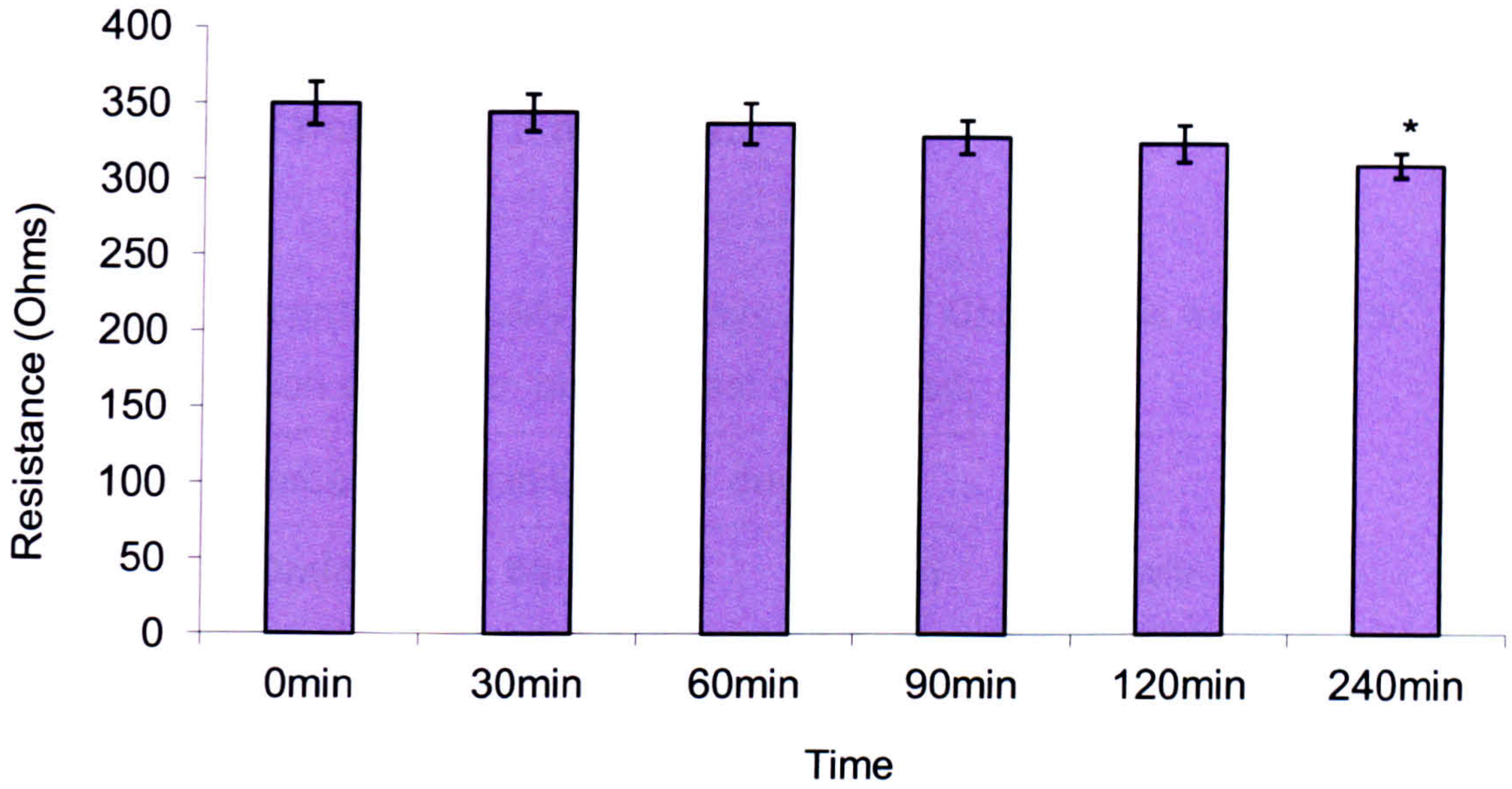
Prior to running the transport experiments, 10 inserts were prepared to assess the TEER of the Caco-2 monolayers over the 21 day. These readings measure the resistance of the electrical membrane potential across the epithelial cell monolayer; and provide valuable information on the integrity of the monolayer by assessing the degree of confluence and cell junction tightness (Millipore, 2009). Readings were taken using cell medium only to assess the growth rate of the monolayer and were taken at day 0, 7, 14 and 21 (Figure 3A.1). TEER readings were also taken using the transport buffers to determine the integrity of the fully grown monolayer and this was done at various time of incubation (Figure 3A.2). Prior to the transport studies, monolayers with TEER values outside the range of 300-350Ω cm^2 were discarded (Li *et al.*, 2005).

Figure A3.1 TEER readings for 21 days Caco-2 monolayer in relations to the growth period.



The Pearson correlation coefficient r= 0.993, (p=0.001, 2-tailed) indicates that the data obtained was consistent, (n=10).

Figure A3.2 TEER readings of fully grown Caco-2 monolayer over various transport incubation times.



At 240 min TEER were significantly different from time 0, 30, 60 and 90 min *($p \leq 0.05$), (n=10).

Summary

The Pearson correlation coefficient r= 0.993 was significant at p=0.001 2-tailed and indicates that the data obtained from TEER readings for 21 days incubation period of Caco-2 monolayers in relations to the growth period for the ten inserts was consistent and repeatable (Figure A.3.1). The overall analysis of TEER values showed significant differences between incubation times (ANOVA p=0.000). TEER at time 0 and 30 min incubation were significantly different from times 90, 120 and 240 min (Tukey post hoc, $p \leq 0.05$). TEER at 60 and 90 minutes incubation were significantly different from 240 min (Tukey post hoc p=0.01). TEER at 120min incubation were similar to 240 min (Tukey post hoc p=0.241). (Figure A3.2).

The integrity of fully grown Caco-2 monolayer over various incubation times used in the transport experiments was maintained. Significantly lower TEER results at 240 minutes, suggested that the membrane was slightly leakier beyond 120 min incubation, the values remained within the suitable experimental ranges (300-350 Ω), and the low error bars suggested that transport results were consistent with a good repeatability. Based on these results it was decided that 60 min and 120 min incubation time would be used for the transport experiment.

Appendix 4 (Chapter 6)

Percentage viability of 21 days cultured Caco-2 cells exposed for various incubation times with blank digest non-boiled compared to blank digests boiled for 5 minutes.

Table A4.1 Percentage viability of 21 days cultured Caco-2 cells exposed for various incubation times with blank digest non-boiled.

% extract in insert n=3	Percentage viability at 21 days			
	30min	**60min**	**120min**	**240min**
25%	98.8 ± 2.4	91.7 ± 7.1	61.9 ± 3.9	36.9 ± 18
50%	96.4 ± 4.6	59.5 ± 2.7	48.8 ± 9.8	28.6 ± 8.7
75%	55.9 ± 2.4	46.4 ± 13.1	42.9 ± 3.9	19. ± 5.5

Table A4.2 Percentage viability of 21 days cultured Caco-2 cells exposed for various incubation times with blank digest boiled for 5 minutes.

% extract in insert n=3	Percentage viability at 21 days			
	30min	**60min**	**120min**	**240min**
25%	99.59 ± 6.7	99.48 ± 5.5	99.48 ± 4.3	99.48 ± 4.3
50%	99.48 ± 4.3	98.53 ± 4.7	98.53 ± 3.3	98.64 ± 6.1
75%	99.48 ± 6.4	97.57 ± 4.8	97.68 ± 5.2	98.53 ± 4.7

Summary

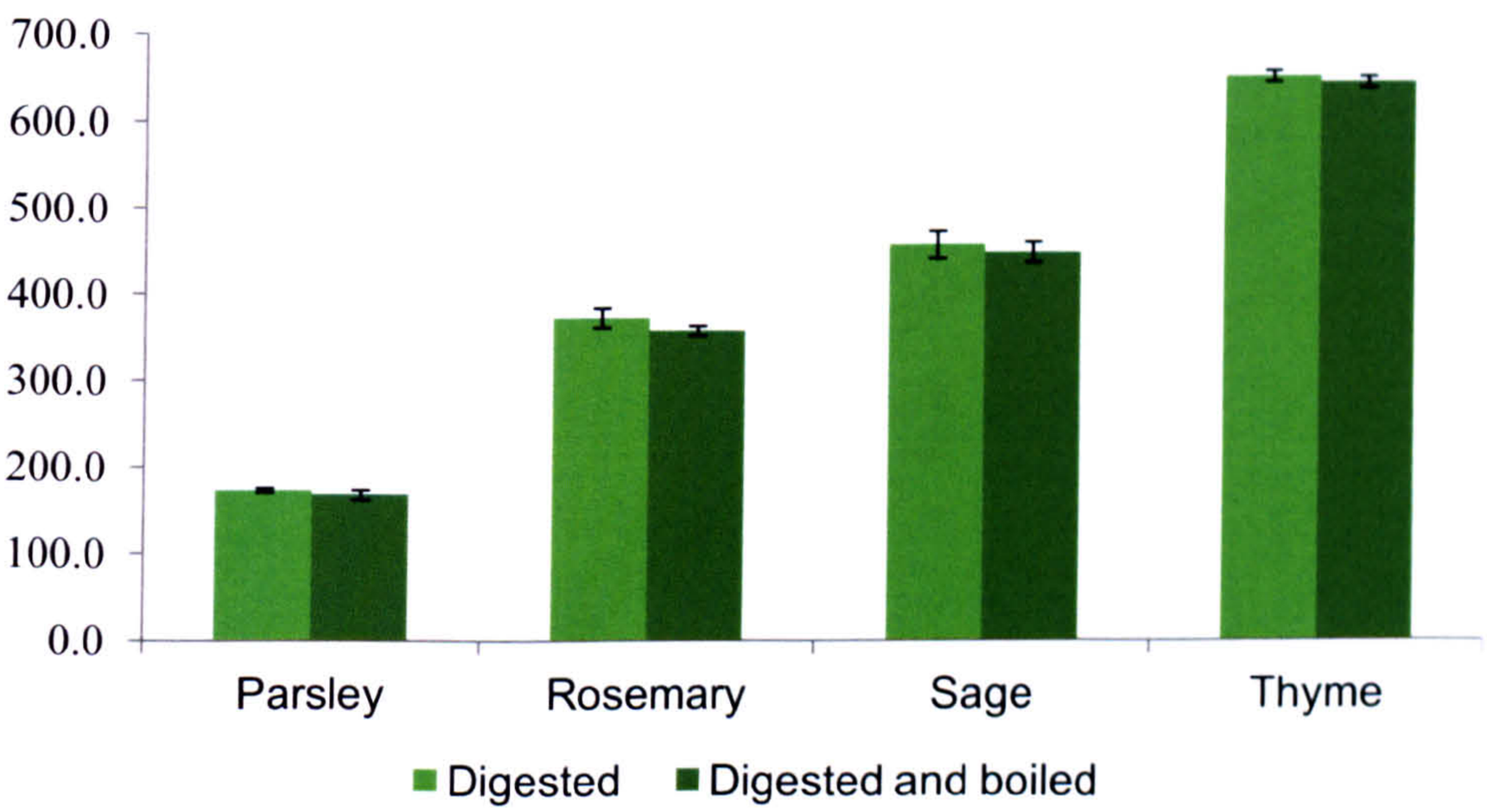
Boiling the H&D extracts was successful at reducing the cytotoxic effects of remaining enzyme activity in the blank digest (cell viability: 97.5- 99.5%). It can therefore be assumed that any growth inhibition that would be observed with the herb extracts heated digested boiled, would be due to the effects of the herb extracts and not the digest.

Appendix 5 (Chapter 6)

Comparison between herb extracts H&D and H&D boiled

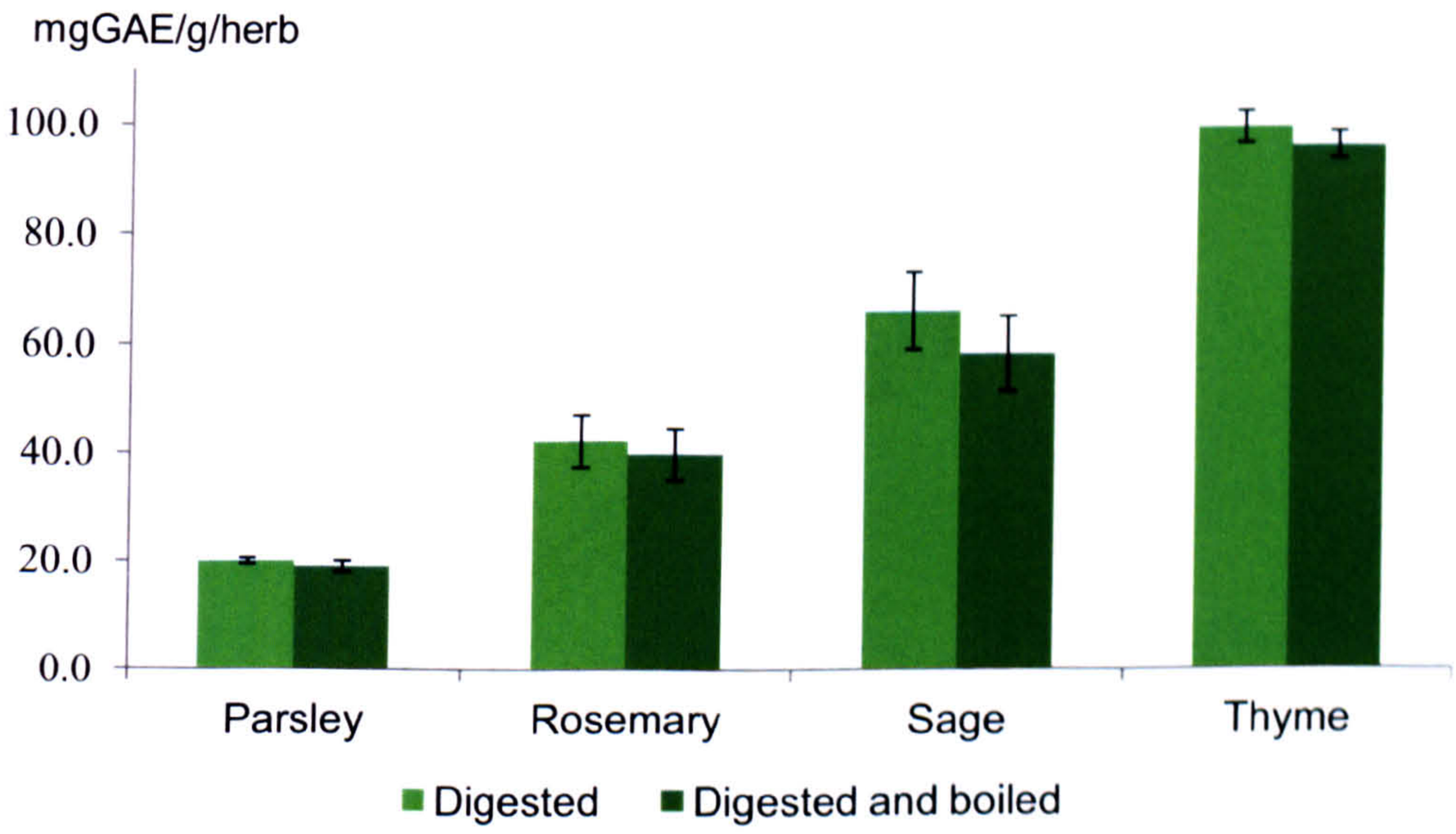
Extracts were poured in glass test tubes placed in boiling water for 5 minutes to denature digestive enzymes.

Figure A5.1 Comparison between TEAC of herbs H&D and herbs H&D boiled



Data are presented as mean ±SD, comparison between digested and digested and boiled for each herb extract p >0.05 (T tests), (n=3).

Figure A5.2 Comparison between GAE of herbs H&D and herbs H&D boiled



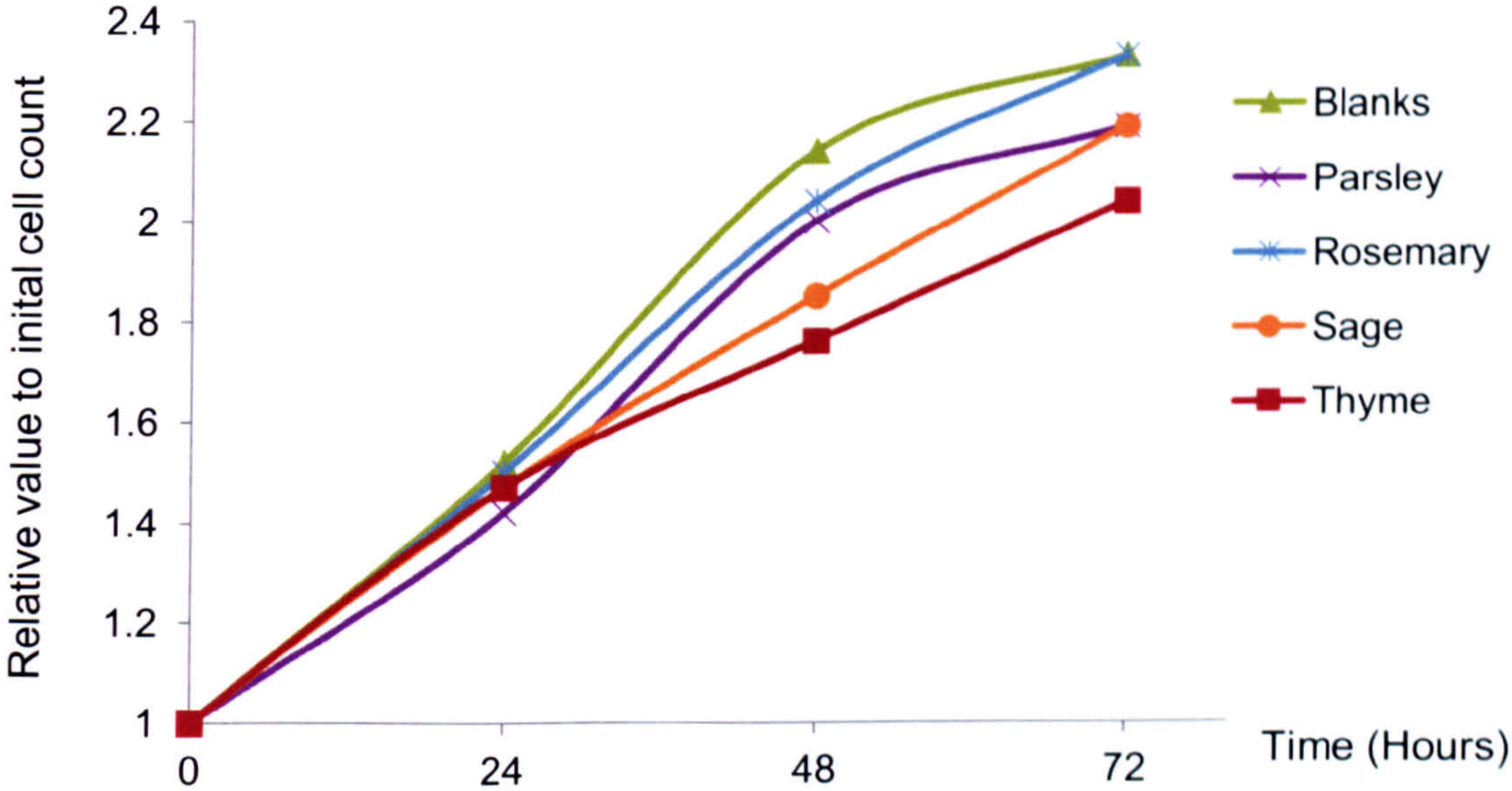
Data are presented as mean ±SD, comparison between digested and digested and boiled for each herb extract p >0.05 (T tests), (n=3).

Appendix 6 (Chapter 6)

Determination of dilution of herb extracts heated, digested and boiled for the transport study.

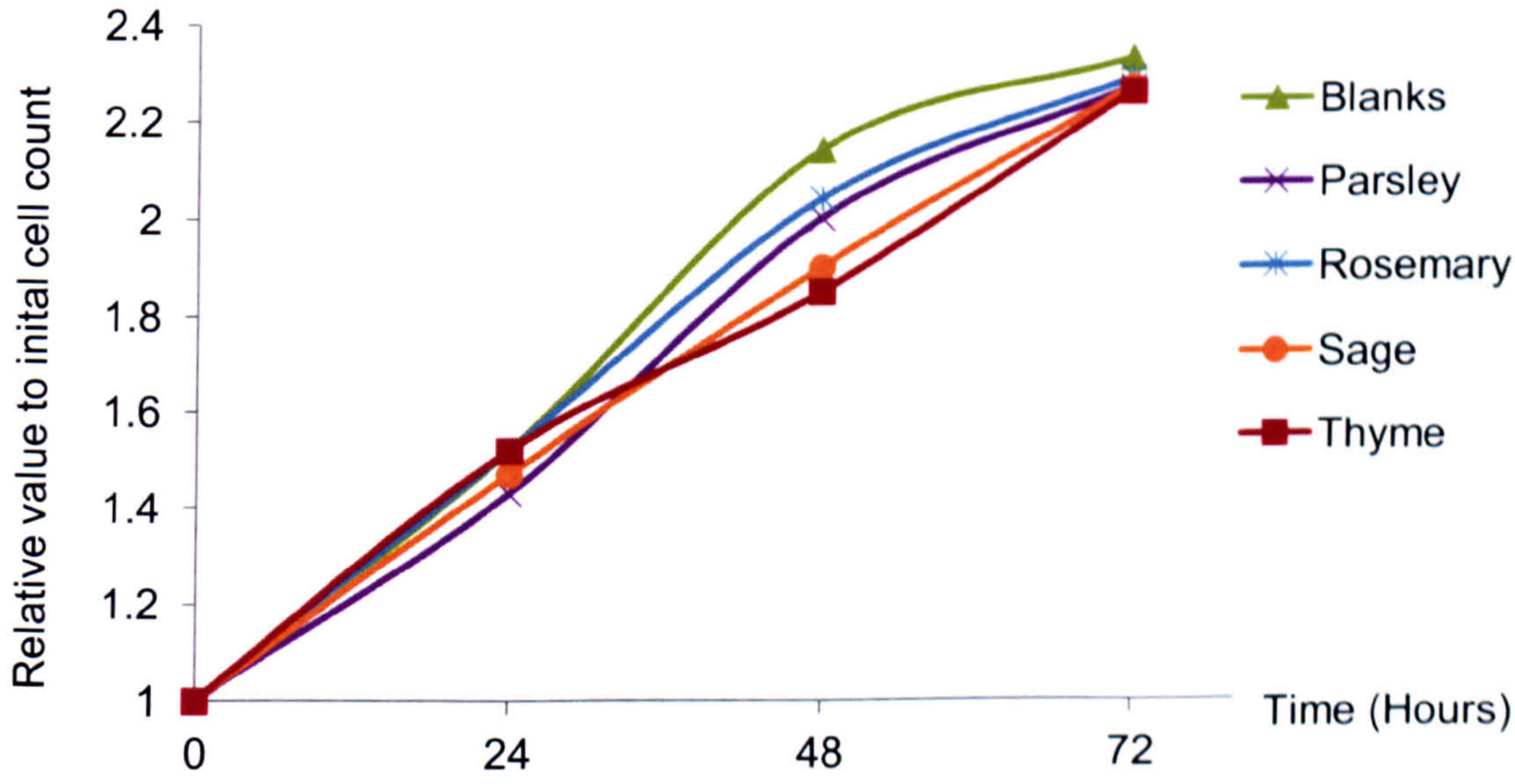
Relative values were calculated by dividing the cell count for each time point by the intitial cell count.

Figure A6.1a Caco-2 growth curves with heated digested boiled extracts 1 in 2 in media.



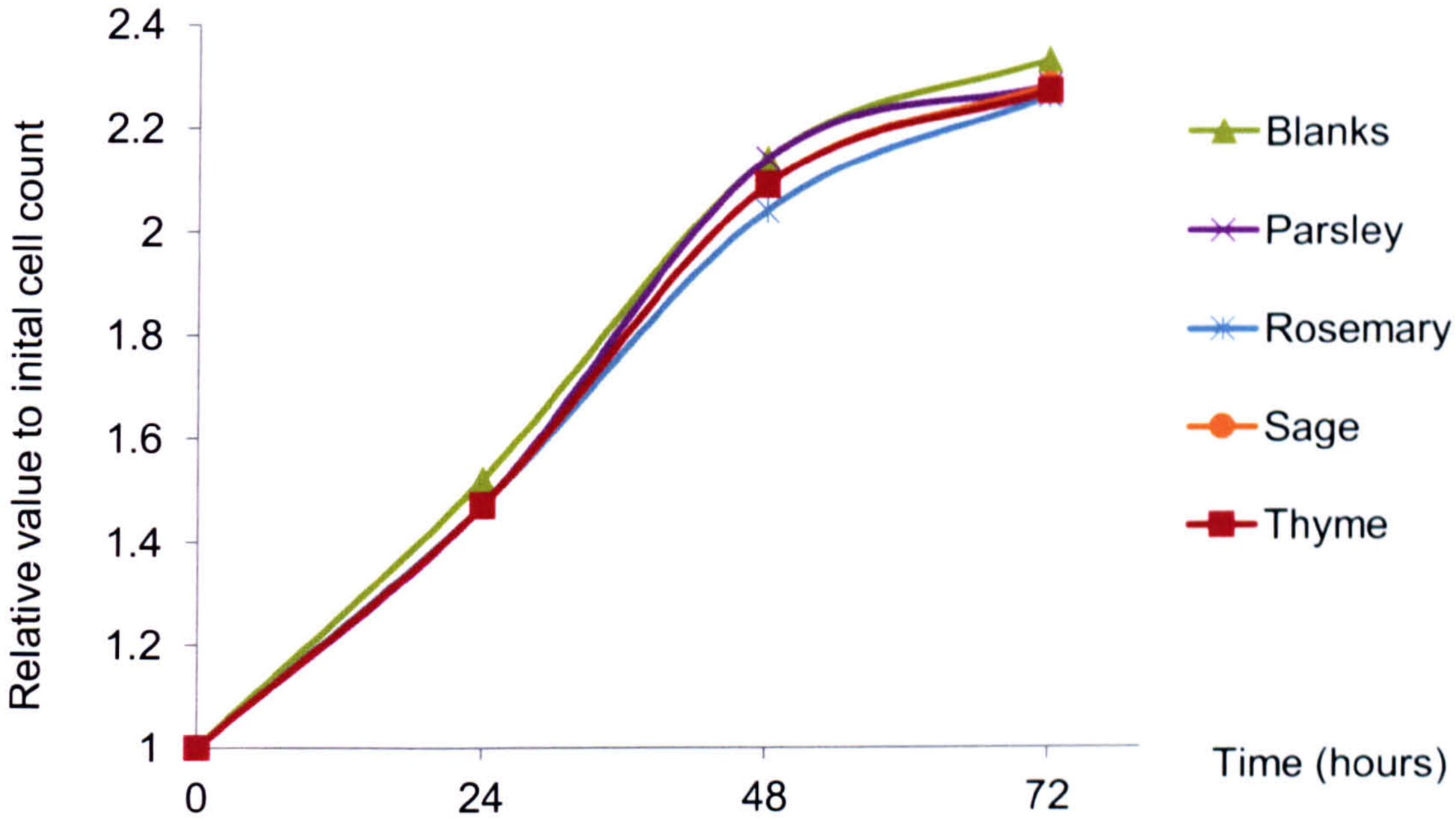
Blanks were made of sterile water diluted 1 in 2 in media (n=2).

Figure A6.1b Caco-2 growth curves with heated and digested boiled extracts 1 in 5 in media.



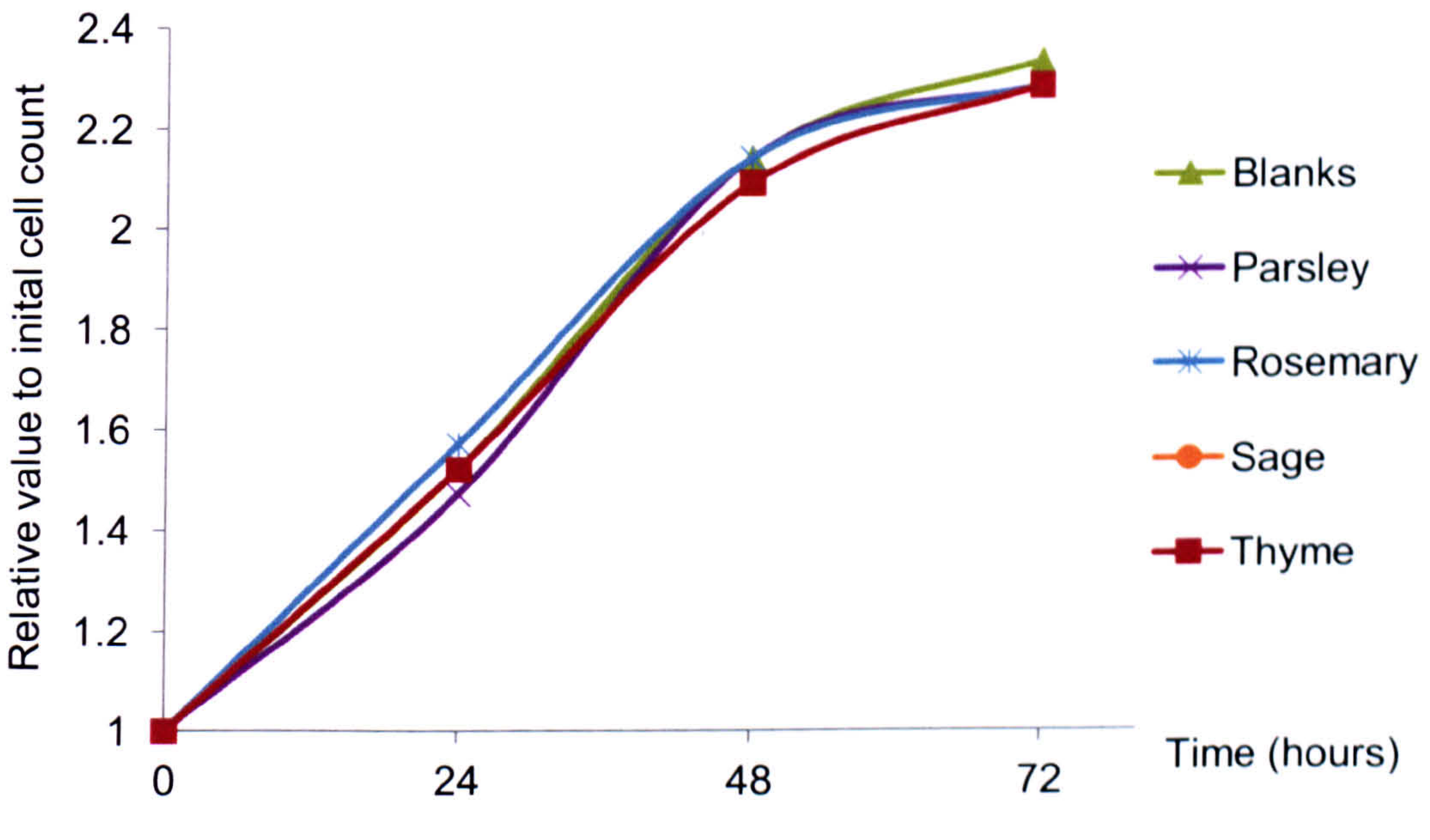
Blanks were made of sterile water diluted 1 in 5 in media (n=2).

Figure A6.1c Caco-2 growth curves with heated and digested boiled extracts 1 in 10 in media.



Blanks were made of sterile water diluted 1 in 10 in media (n=2).

Figure A6.1d Caco-2 growth rates with heated and digested boiled extracts 1 in 100 in media.



Blanks were made of sterile water diluted 1 in 100 in media; (n=2).

Appendix 7 (Chapter 7)

Participant Information Sheet (PSI).

'The impact of gut bioavailability on the antioxidant capacity and action of cooked culinary herbs'

You are asked to kindly assist a PhD research project at Kingston University by donating a small amount of blood. The research project is being carried out by Magali Chohan (a Nutrition PhD student) investigating the impact of gut bioavailability on the antioxidant capacity and action of cooked culinary herbs. Before you decide to take part please read the information carefully so that you understand why the research is being done and what it involves before you agree to take part in the study. Please read the following information.

What is the aim of the study?

Magali Chohan will be investigating the potential anti-inflammatory effects of cooked culinary herbs (parsley, rosemary, sage and thyme) *in vitro*, using human blood. This study may help to give further insight as to the anti-inflammatory effect of culinary herbs, following the novel approach to using foods (culinary herbs) in preventative medicine. This study is expected to be completed in six weeks in laboratory and it is part of a three year PhD project. Here is a brief description of the rationale of the study. The literature indicates that culinary herbs are becoming a major source of dietary antioxidants. This is thought to be due to polyphenolic compounds. A recent study has shown that cooking can significantly increase or decrease the antioxidant capacities of a selection of culinary herbs and that digestion further increased this antioxidant capacity even further. Additional studies have shown that once absorbed by the gut some of the antioxidant capacity is retained. A recent study has shown that these culinary herbs procure an inflammatory activity when cancer cells of the colon are used. Therefore this experiment is to be repeated on healthy lymphocytes (white blood cells) and healthy volunteers are needed to donate some blood.

Why have I been invited to participate?

You have been invited to help because you are a healthy human volunteer. One volunteer and one blood sample should suffice for the study; however another

volunteer may be required for another sample if the experiment needs to be repeated.

Do I have to take part?

You do not have to take part if you choose not to. Your participation is entirely voluntary. If you decide to take part you will be given a consent form to sign. You are free to refuse or withdraw at any time without giving a reason.

What will happen if I do take part?

A single small blood sample is needed, this is done via venopuncture. Venopuncture which involves entering a vein with a needle, is one of the most commonly performed invasive procedures routinely performed by qualified phlebotomist usually a doctors or a nurse. Venopuncture is usually performed on the median cephalic vein (inside your arm) because it is close to the surface therefore easy to find and the skin less sensitive. Magali Chohan, PhD student will introduce you to the phlebotomist (who is also a qualified medical doctor) who will draw a small blood sample from you, 15ml (3 teaspoons) once. The lymphocytes (white blood cells) obtained from the blood sample will be used to study the potential anti-inflammatory effects of culinary herbs using a commonly used laboratory based assay.

What are the potential risks and harm in participating?

Venopuncture is a commonly used, easy and quick clinical procedure however there are potential risks. These include excessive bleeding, feeling light-headed or fainting, hematoma (blood accumulating under the skin), infection (a slight risk any time the skin is broken) and in rare cases, nerve damage which may result in the loss of use of the hand. In order to reduce these risks the qualified phlebotomist has received training and is required to adhere to good hand hygiene, the use of sterile blood collection equipment, gauze and dressing, the wearing of protective clothing, and the safe disposal of needles as well as insuring the procedure is performed in a clean environment. Venopuncture must not be performed on bruised skin or damaged veins. You may feel a prick sensation during puncture. You may experience slight bruising at the site of puncture after the blood is taken and the phlebotomists will advise you on how to minimise this risk.

What are the potential benefits of taking part? Your contribution to this study by donating a small sample of blood may help to give further insight as to the beneficial role of processed culinary herbs in preventative medicine.

Confidentiality. One blood sample will be collected for this study, however another volunteer may be required for another sample if the experiment needs to be repeated.The origin of the blood sample will be kept confidential. No personal information will be obtained from you.

What should I do if I want to take part?

You should contact Magali Chohan (k0225713@kingston.ac.uk) and tell her you wish to take part. Magali will meet with you to answer any additional questions you may have. If you are satisfied with her responses then a consent form will be given to you to sign.

Who is organising and funding the research?

The project is organised and funded by the Biomedical and Pharmaceutical Sciences Research Group at Kingston University.

Contact for further information:

Magali Chohan, PhD student of Kingston University,
Life science research office, Room: EM131, Kingston University, School of Life Sciences, Faculty of Science, Penryhn Road, Kingston upon Thames, KT1 2EE, Kingston.
Tel: 020 8547 2000 ext 62476
Email: k0225713@kingston.ac.uk

Dr Elizabeth Opara, Director of Studies and Principal Supervisor of Magali Chohan,
Principal Lecturer in Nutrition, Nutrition Field Leader, Kingston University, Kingston University, School of Life Sciences , Faculty of Science , Penryhn Road , Kingston upon Thames , KT1 2EE , Kingston.
Tel: 020 8547 2000 ext 62047
Email: e.opara@kingston.ac.uk

Thank you for taking time to read the information sheet. 25th May 2009

CONSENT FORM

'The impact of gut bioavailability on the antioxidant capacity and action of cooked culinary herbs'

Magali Chohan, student of Kingston University,
Life science research office, Room: EM131, Kingston University, School of Life Sciences, Faculty of Science, Penryhn Road , Kingston upon Thames , KT1 2EE , Kingston.
Tel: 020 8547 2000 ext 62476
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Dr Elizabeth Opara, Principal supervisor of Magali Chohan, Principal Lecturer in Nutrition, Nutrition Field Leader, Kingston University
Kingston University, School of Life Sciences , Faculty of Science , Penryhn Road , Kingston upon Thames , KT1 2EE , Kingston.
Tel: 020 8547 2000 ext 62047
Email: e.opara@kingston.ac.uk

I confirm that I have understood all the information provided in the Participant information sheet (PSI) and have had the opportunity to ask any questions relating to the study.	☐
I understand that my participation in this study is voluntary and that I am able to withdraw at any time, without giving a reason.	☐
I agree to take part in this study	☐

_____	_____	_____
Name of Participant (PRINT)	Signature	Date
_____	_____	_____
Name of Researcher (PRINT)	Signature	Date

Appendix 8 (Chapter 9)

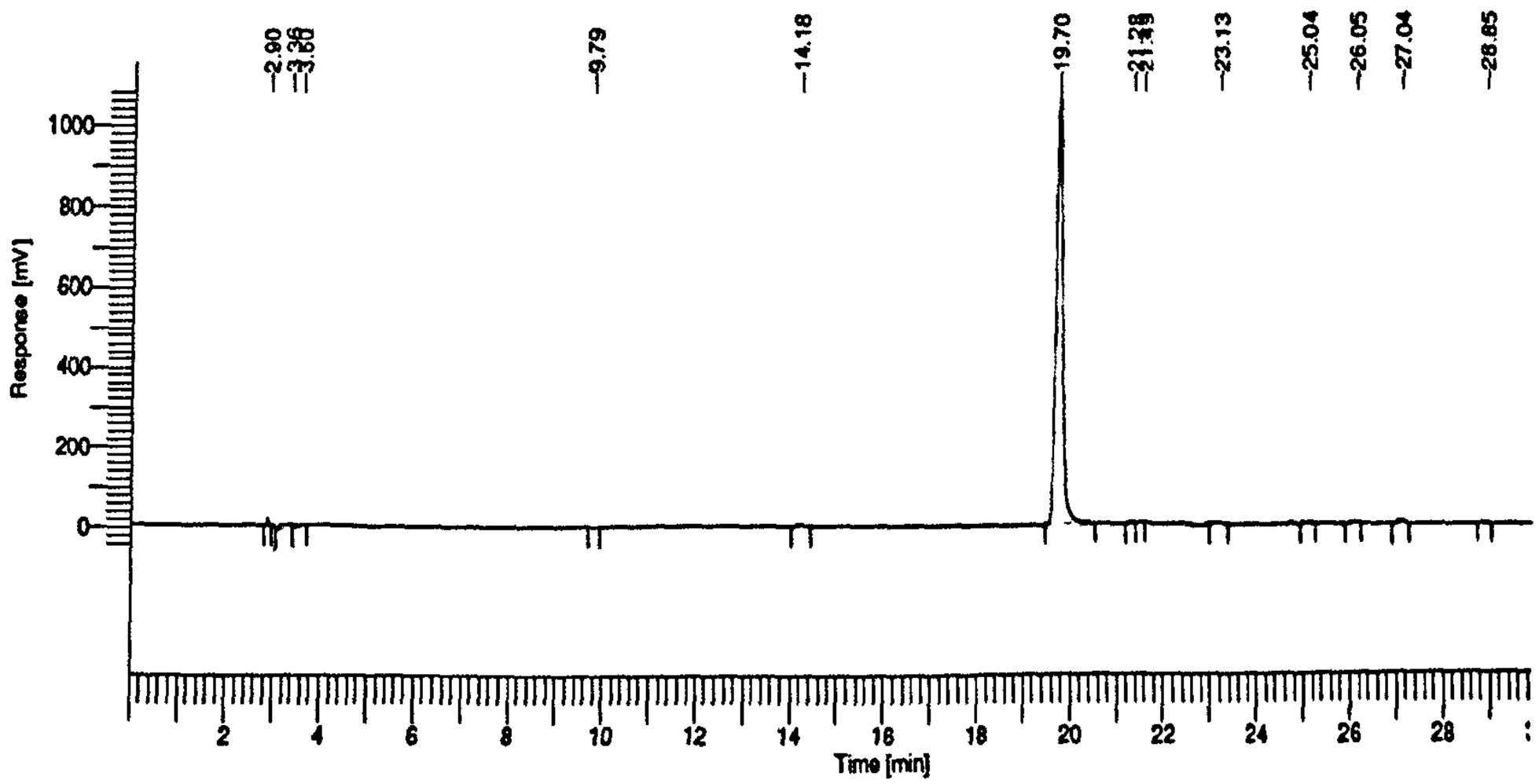
HPLC Calibration

The HPLC calibration data were obtained using standards (65.5mg/L to 1000mg/L) dissolved in methanol. Peak areas for the standards were plotted and the data were extrapolated from the line of best fit (regression line), the "goodness of fit" (r^2) was calculated using EXEL for Windows.

Table A8.1. HPLC calibration data.

Standard	Regression line	r^2
Caffeic acid	y= 71747x + 3E+06	0.9965
Ferulic acid	y = 40489x + 917452	0.9997
Gallic acid	y= 73129x-411247	0.9944
Rosmarinic acid	y = 23325x - 114031	0.9995
Vanillic acid	y = 40138x - 30943	0.9981
m-coumaric acid	y = 73888x + 1E+07	0.9272

Figure A8.1 Rosmarinic acid HPLC chromatogram



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