

Elucidation of mechanisms by which culinary herbs and spices exert their inhibitory action on the growth of CRC cells *in vitro*

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Abstract

Colorectal cancer (CRC) is one of the most commonly diagnosed types of cancer in the developed countries and the incidence is rising in the developing regions. Chronic inflammation, which is propagated by overexpression of cyclooxygenase-2 (COX-2) and its major product prostaglandin E2 (PGE2), plays a key role in the development of CRC. Culinary herbs and spices (CHS) are rich in polyphenols, have a high anti-oxidant capacity and possess anti-inflammatory activity. It has been shown that CHS inhibit the growth of CRC cells, however, their anti-carcinogenic mechanisms are mainly unknown. Hence, the aim of this study was to identify the CHS that were most potent inhibiting the growth of CRC cells, and subsequently to elucidate their anti-carcinogenic mechanisms, in particular, focusing on COX-2, the Wnt/ β -catenin signalling pathway, and proteins involved in apoptosis. Another goal was to investigate whether combining the CHS would result in synergistic effects on the above. This study demonstrated that CHS extracted in water/or ethanol and their combinations inhibited CRC cell growth. This study also revealed that the most potent CHS extracted in ethanol (turmeric (TE), bay leaf (BLE) and ginger (GE)) and combinations downregulated the expression of COX-2 and suppressed COX-2 activity by reducing PGE2 release; their effect was comparable to that of the selective COX-2 inhibitor Celecoxib (50 μ M). These CHS also induced apoptosis in CRC cells by targeting several key proteins: p53, caspase-3, and PAPR. However, the CHS did not have an effect on Wnt signalling pathway, which partially could be due to insufficient treatment time. In conclusion, this study demonstrated that CHS and their combinations inhibited CRC cell growth, inhibited COX-2 expression and activity, and modulated several key molecules involved in the development of CRC. Based on these findings, CHS have the potential to be utilized for CRC chemoprevention and possibly be used as a complimentary treatment. However, in vivo studies are needed to establish the true potential of these foods.

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List of abbreviations:

AA – Arachidonic acid

APC - Adenomatous polyposis coli

BLA – Bay leaf aqueous

BLE – Bay leaf ethanol

BLSE – Bay leaf sage ethanol

BLTE – Bay leaf and turmeric ethanol

CA – Carnosic acid

CL - Carnosol

COX-2 – Cyclooxygenase-2

CRC – Colorectal cancer

FAP - Familial adenomatous polyposis

FC - Folin-Ciocalteu's reagent

DMEM - Dulbecco's Modified Eagle Medium

GAE - Gallic acid equivalent

GE – Ginger ethanol

GSE – Ginger and sage ethanol

MAPK/ERK – Mitogen-activated protein kinase/extracellular signal regulated kinase

NF-kB - Nuclear Factor kappa B

NSAIDS - Non-steroidal anti-inflammatory drugs

PGE2 – Prostaglandin E2

RA – Rosemary aqueous

RE – Rosemary ethanol

RTE – Rosemary and turmeric ethanol

SA – Sage aqueous

SE – Sage ethanol

SRB - Sulforhodamine B

TCF – T-cell factor

TE – Turmeric ethanol

TPC - Total phenolic content

Chapter 1 General introduction

1.1 Colorectal cancer: general overview

Colorectal cancer (CRC) is defined as any malignant neoplasm arising from the inner lining of the colonic or rectal epithelium (Suwannalert et al. 2016; Hamza et al. 2017). About 85% of CRC develops from untreated/unremoved adenomatous polyps (Roy & Majumdar 2012; Dulai et al. 2016). CRC is one of the most common cancers in high income countries, including the UK (Ferlay et al. 2010; Jemal et al. 2010; Siegel et al. 2014). In 2015, over 34 000 new CRC cases were diagnosed in the UK. The occurrence was 84.5 (in males) and 56.8 (in females) cases per 100,000 people (Office for National Statistics 2017). In US, in 2017 there were estimated 134 000 new CRC cases and 50 000 deaths from this condition. It was also estimated that in 2013 there were over 1.3 million people in the US living with CRC (National Institute of Cancer 2017). Furthermore, CRC cases are increasing in low and medium income countries, hence world-wide CRC cases are projected to increase by 60% by 2030 with 2.2 million new cases per year and 1.1 million deaths (Arnold et al. 2016).

Early symptoms of CRC are blood in stool and/or changes in bowel habits indicating possible presence of the tumour (WCRF 2011; National Cancer Institute 2017). CRC diagnosis involves performing a colonoscopy, and is then confirmed by biopsy and histological analysis (NICE guidelines). If diagnosed early the 5-year survival rate is 90%, however, over 50% of CRC patients are diagnosed when the tumour is locally advanced or metastasised to other organs, and in these cases the survival rates are 70% and 13% respectively (Siegel 2014).

Conventional treatment for CRC is surgery, and if required, is followed by chemotherapy and/or radiation. For advanced and metastasised CRC cases, a combination of chemotherapy is offered: FOLFOX (folinic acid plus fluorouracil plus oxaliplatin) as the first line of treatment then a single agent, irinotecan, or FOLFOX with FOLFIRI (folinic acid plus fluorouracil plus irinotecan) as the second-line of treatment. Alternatively, XELOX (capecitabine plus oxaliplatin) as the first-line treatment then FOLFIRI as second-line treatment is used (NICE Guidelines 2012).

1.2 Causes and Risk Factors of CRC

Only 10% of CRCs are caused by inherited mutations such as those of the adenomatous polyposis coli (APC), which involves disruption of the tumour suppressor gene adenomatous polyposis coli (APC), and hereditary non-polyposis colorectal cancer

(HNPCC) (Fajardo & Piazza 2015; Patel, et al. 2010). Another 20% of CRC cases are diagnosed in people that have family history of CRC (WCRF 2011). However, the majority of CRC cases are sporadic, which does not occur due to inherited pro-cancerous genes, and mutations of the genes develops during the life of the person due to various environmental, non-genetic factors that are linked to the development of CRC. Age is a big risk factor with the majority of CRC cases are diagnosed in patients over 65 years old. However, the increased prevalence of CRC is still evident when age is taken in to consideration thus highlighting the role of other factors in its development (Fearhead et al. 2002; Hagggar et al. 2009). However, it is lifestyle factors including diet and physical activity that have been the focus of most attention in relation to this and other cancers primarily as a consequence of epidemiological studies. Such studies have shown that CRC cancer risk is increased greatly when people migrate from countries with low CRC incidence to countries with high incidence of CRC and adopt their dietary and other lifestyle habits (Hagggar et al. 2009). For example, CRC risk for Japanese migrant's children in the US is 3-4 times higher in comparison to Japanese people living in Japan (WCRF 2007). Recognised lifestyle risk factors for CRC include smoking, heavy alcohol consumption, physical inactivity, obesity, the high intake of red and processed meat and/or saturated fat, and insufficient consumption of fibre, fruits and vegetables (Hagggar et al. 2009; Gingras & Béliveau 2011; Tantamango et al. 2011; Hou et al. 2013; Grosso et al. 2015; WCRF 2011). It is estimated that at least half of CRC cases in the UK are attributed to these factors, and at least half of these cases could be prevented by changing them (Parkin & Boyd 2011). In US, according to American institute for cancer research up to 45% of CRC cases could be avoided by eating more fibre, less meat, limiting alcohol consumption and maintaining a healthy body weight (AICR 2012). Therefore, CRC is a particularly good type of cancer for chemoprevention and various dietary and lifestyle interventions (Arber 2008; Shemesh & Arber 2014).

1.3 Development of CRC

It takes 20-40 years for CRC to develop, and it occurs through several stages: a healthy colonic mucosa develops into a benign adenoma, and then into an adenocarcinoma (Fearon 2011; Fajardo & Piazza 2015). The development of CRC can involve a number of different pathways that based on the literature may be interlinked. A disruption of tumour suppressor genes such as p53 and APC, and activation of oncogenes including KRas are very common in CRC tumours (Tachibana et al. 2004; Markowitz, S.D. 2009; Fearon

2011). KRas mutations, which are found in ~50% of CRC patients) activate EGFR (epidermal growth factor receptor) signalling causing increased cell proliferation (Fearon 2011; Douillard et al. 2013; Dinu et al. 2014). p53 is a tumour suppressor protein, which is involved in cell cycle arrest and apoptosis, hence its mutations can lead to the development of CRC. Approximately half of sporadic CRC patients have mutated p53 gene, and p53 mutations linked to poor treatment outcome (Li et al. 2015). The mutation of APC, which is a common feature of sporadic CRC, disrupts the Wnt/ β -catenin signalling pathway causing aberrant crypts and early adenomas (Kauh & Umbreit 2004). In fact, analysis of patients' tumour biopsy samples (276 patients) revealed that the majority (94%) had mutations in the Wnt/ β -catenin signalling pathway (Muzny et al. 2012).

1.4 The role of the Wnt signalling pathway in the aetiology of CRC

As stated above the mutations in the Wnt signalling pathway are very common in CRC cells, hence it is a good molecular target for prevention and treating this disease (Amado et al. 2011; Novellademunt et al. 2015). There are at least three different Wnt signalling pathways. The Wnt/ β -catenin (also known as canonical) (Figure 1.1), which activates target genes in the nucleus, is the most studied and is involved in the development of CRC (Giles et al. 2003; Clevers 2006; Mohammed et al. 2016). There are other important molecules in the canonical pathway including dishevelled (Dsh), APC, glycogen synthase kinase 3 β (GSK-3 β), casein kinase 1 (CK1), axin and TNIK however, the protein β -catenin is a central mechanism for this particular pathway (Willert & Nusse 1998; Kuhl et al. 2000; Giles et al. 2003; Sawa et al. 2015).

When Wnt signalling is not activated, β -catenin is phosphorylated by GSK-3 β , CK1 and serine/threonine kinase complex at Thr41, Ser37 and Ser33. This phosphorylation process is stabilized by axin and APC, both of which bind to β -catenin making it easier to phosphorylate. Phosphorylated β -catenin is degraded by the E3 ubiquitin complex, and therefore, does not enter the nucleus (Pennisi 1998; Liu et al. 2002; Clevers 2006; Badalà et al. 2007; Valkenburg et al. 2011). To activate the canonical Wnt pathway, extracellular ligands have to bind the frizzled receptor on the cell surface and, in addition co-receptors, low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6, have to be occupied, which then disrupts GSK-3 β thus preventing β -catenin phosphorylation and subsequent degradation (Badalà et al. 2007; Papatriantafyllou 2012).

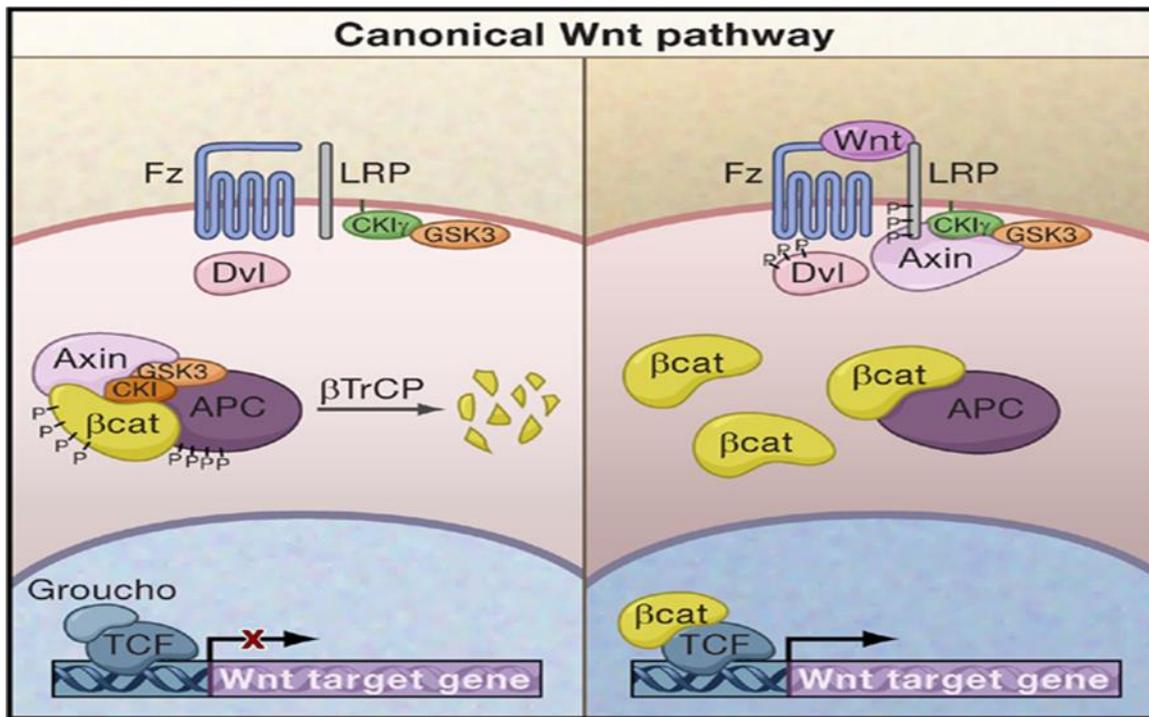


Figure 1.1 Canonical Wnt/ β -catenin pathway

On the left the Wnt signalling is switched off, thus β -catenin is phosphorylated and then degraded and does not enter the nucleus. On the right, the extracellular Wnt ligand is bound to the frizzle receptor and Wnt signalling is activated, as a result β -catenin phosphorylation is disrupted. Unphosphorylated β -catenin entered the nucleus and triggers cell proliferation. Taken from Clevers 2006.

Wnt signalling and β -catenin are tightly controlled by numerous intracellular and extracellular mechanisms (Eo et al. 2016), because a higher than normal level of unphosphorylated β -catenin in the nucleus leads to uncontrolled cell division and, ultimately, cancer (Willert & Nusse 1998; Clevers 2006). However, extracellular Wnt signalling is often up-regulated in CRC cells and some cancer cells can even secrete Wnt activating ligands (Najdi et al. 2011; Voloshanenko et al. 2013). As a result, Dsh becomes activated, which inactivates the GSK-3 β . Consequently, the β -catenin phosphorylation process is interrupted, and β -catenin is not phosphorylated, therefore, preventing its degradation. Unphosphorylated β -catenin is then translocated from the cytoplasm into the nucleus and interacts with transcription coactivator p300 triggering T-cell factor (Tcf)-dependent transcription, which leads to colon crypt proliferation and, therefore, a higher risk of CRC (Pennisi 1998; Karim et al. 2004; Clevers 2006; Valkenburg et al. 2011; Williams 2012; Mohammed et al. 2016; Morris & Huang 2016). In addition, in the nucleus β -catenin can be acetylated, which leads to the up-regulation of the oncogene c-Mys, thus further triggering carcinogenesis (Wolf et al. 2002). In the cytoplasm unphosphorylated β -

catenin can also be held by E-cadherin, therefore preventing unphosphorylated β -catenin from entering the nucleus and triggering increased cell proliferation (Pennisi 1998).

β -catenin phosphorylation and its subsequent degradation, can also be affected by mutations or loss of function of the proteins involved in this pathway, for example, mutations in β -catenin itself and the absence or mutation of the APC gene which leads to the synthesis of mutated APC lacking binding sites for β -catenin. As a result β -catenin cannot be phosphorylated and degraded (Sieber et al. 2000; Sansom et al. 2004; Clevers 2006; Kroboth et al. 2007). In fact, the APC gene is absent or inactive in 85% of sporadic CRCs (Pennisi 1998; Sparks et al. 1998). β -catenin regulates the expression of at least 66 genes including pro-oncogenes MYC and CCND1 (Herbst et al. 2014). Furthermore, the tumour suppressor p53 regulates β -catenin degradation hence mutations in p53 can result in increased level of nuclear unphosphorylated β -catenin (Matsuzawa & Reed 2001). Nuclear β -catenin over-expression in lymph nodes is associated with liver metastasis and poor prognosis (Cheng et al. 2011). In addition, WNT/ β -catenin signalling is also involved in regulation of the expression of COX-2, which plays a key role in the inflammatory process (Carlson 2003).

1.5 Inflammation and CRC

Chronic inflammation creates a tumour favourable microenvironment, which leads to the development of CRC (Aggarwal et al. 2006; Wang & DuBois 2011; Derry et al. 2013). It has been shown that patients with inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease have a much greater risk of developing CRC than people not affected by these conditions (Eaden et al. 2001; Freeman 2008; Haggard et al. 2009), which proves that prolonged inflammation is a big risk factor for CRC. Moreover, people with elevated blood inflammatory markers including C-reactive protein, erythrocyte sedimentation rate (ESR), tumour necrosis factor- α (TNF α), interleukin 6 (IL-6), have a higher risk of CRC (Ananthakrishnan et al. 2014).

The cyclo-oxygenase-2 (COX-2) enzyme plays a central role in the inflammatory process (Brown & DuBois 2005; Greenhough et al. 2009; Telliez et al. 2006). COX-2 is a bifunctional enzyme. First, cyclooxygenase converts arachidonic acid to prostaglandin G₂, which then is converted to prostaglandin H₂ (PGH₂) by peroxidase enzyme, and then specific enzymes convert PGH₂ to various prostaglandins including prostaglandin E₂ (PGE₂) (Chandrasekharan & Simmons 2004; Ricciotti & Fitzgerald 2011), which further mediate pro-inflammatory processes and carcinogenesis (Brown & DuBois 2005; Yarla et

al. 2016). COX-2 is usually not expressed in healthy colonic mucosa and only induced in response to inflammatory stimuli. It is induced in the early stages of cancer by various growth factors and tumour promoters, including pro-inflammatory cytokines, the epidermal growth factor (EGF), insulin-like growth factor (IGF) (Wang & DuBois 2011). Dietary factors can also contribute to the increased COX-2 expression: high intake of saturated fatty acids and a high n-6 to n-3 polyunsaturated fatty acid ratio (Singh et al. 1997; Hwang 2001; Wang et al. 2007). It has been shown that COX-2 promotes carcinogenesis via its product –PGE₂, which activates numerous oncogenic pathways including the Wnt/ β -catenin signalling pathway. PGE₂ also suppresses apoptosis and cell-mediated immunity, and stimulates angiogenesis (Figure 1.2) (Brown & DuBois 2005; Castellone et al. 2006; Telliez et al. 2006; Wang et al. 2007; Mohammed et al. 2016). DuBois et al. (1994) demonstrated that the mRNA of COX-2 becomes over-expressed after the administration of carcinogens (Eberhart et al. 1994). Astring et al. (2011) reported that, numerous genes regulating COX-2 expression are altered in CRC patients' tumours (Astring et al. 2011). The expression of COX-2 in CRC tissues increases with tumour progression and metastases (Tsuji et al. 1997; Zhang & Sun 2002). Biopsy samples obtained from CRC patients revealed that COX-2 is over-expressed in 40% of human adenomas and 71-90% of adenocarcinomas (Eberhart et al. 1994; Soslow et al. 2000; Wiese et al. 2003; Sinicrope & Gill 2004). Moreover, over-expression of COX-2 is linked to poor prognosis among CRC patients (Sheehan et al. 1999; Ogino et al. 2008; Zhang & Sun 2002).

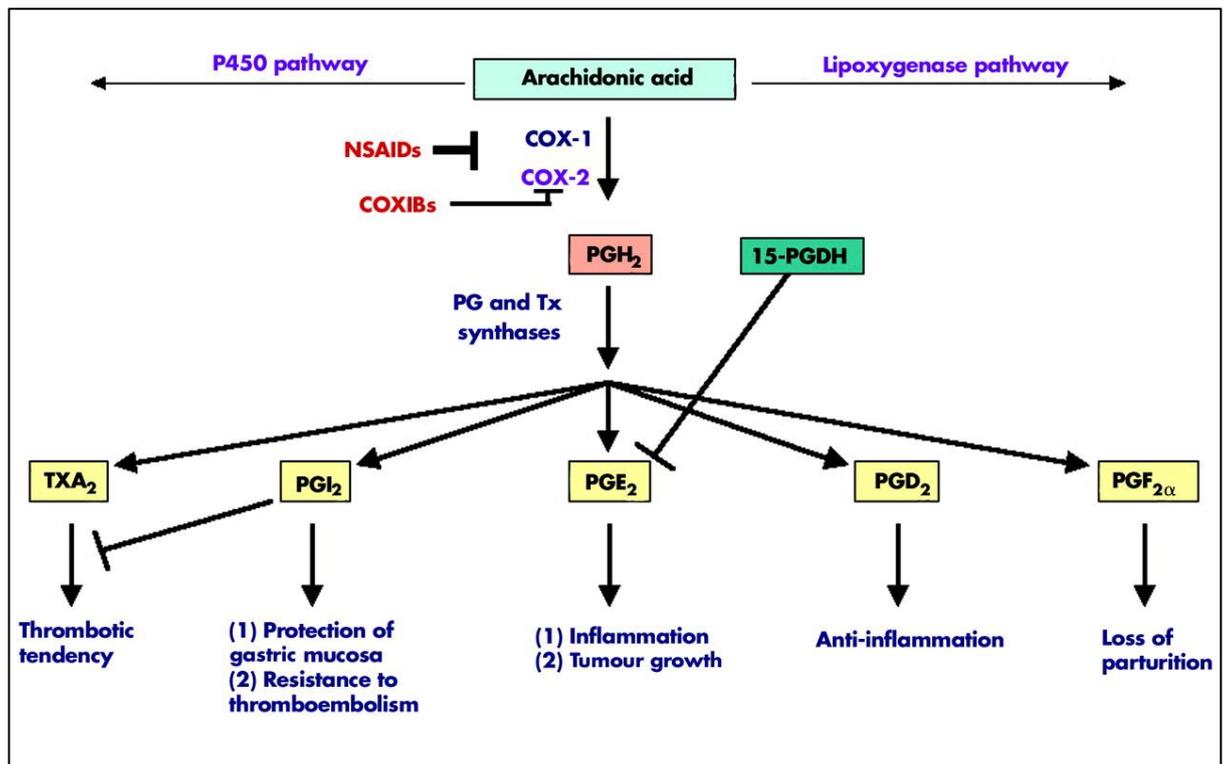


Figure 1.2 COX-2 role in prostaglandin production and development of CRC

Taken from Wang et al. 2006.

Bearing in mind the significant role that COX-2 plays in CRC, anti-inflammatory agents should be included in chemoprevention interventions and even treatment of CRC by combining anti-inflammatory agents with chemotherapy (Rayburn et al. 2009; Barbosa Vendramini-Costa et al. 2016; Dulai et al. 2016; Urbanska et al. 2015). COX-2 inhibiting drugs have been used in several clinical trials, and a number epidemiological studies and their meta-analyses (Cole et al. 2009; Rothwell 2011; Thun et al. 2012; Friis et al. 2015) revealed that non-steroidal anti-inflammatory drugs (NSAIDs), which target COX-2, and selective COX-2 inhibitors, reduced the risk of, and mortality from CRC; the latter by 50% (Tsuji et al. 1997; Temraz et al. 2013). Moreover, the anti-inflammatory drugs that inhibit COX-2 such as aspirin, Sulindac and Celocobix, reduced the size and number of adenomas in subjects with FAP (Giardiello et al. 1993). However, they have numerous side effects such as stomach ulcers and intestinal bleeding (Shemesh & Arber 2014). Hence, alternative solutions are needed. Research indicates that natural agents are available which have the potential to be used for the prevention and treatment of CRC (Aggarwal et al. 2006; Aravindaram & Yang 2010). These include dietary polyphenols such as curcumin, genistein. Both possess anti-COX-2 activity and do not have the stated above side effects (Mutoh et al. 2000; Goel et al. 2001; Aggarwal et al. 2003).

1.6 Polyphenols: general overview

Polyphenols are a large group of natural compounds that possess phenolic structure containing at least one benzene ring with at least one hydroxyl group. Polyphenols are found in various plant foods, for example, curcumin found in turmeric and gingerols found in ginger (Figure 1.3) (Scalbert et al. 2005; Tsao 2010; Little et al. 2015). So far over 8000 polyphenols have been identified and they can be subdivided into the following groups: phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavones, flavonols, flavanones, flavan-3-ols anthocyanindis and isoflavones), stilbenes and lignans (Manach et al. 2004; Pandey & Rizvi 2009). Over the last two decades, due to their versatile properties, polyphenols have attracted a lot of interest (Manach et al. 2004; Crozier et al. 2009; Pereira et al. 2009; Tsao 2010; Tomás-Barberán & Andrés-Lacueva 2012), and as a consequence polyphenol research and the polyphenol market are on the rise, according to Grand View Research: the global polyphenol market (covering cancer and cardiovascular disease prevention and weight management) is forecast to reach \$1.3 billion by 2024 (Grand View Research 2016).

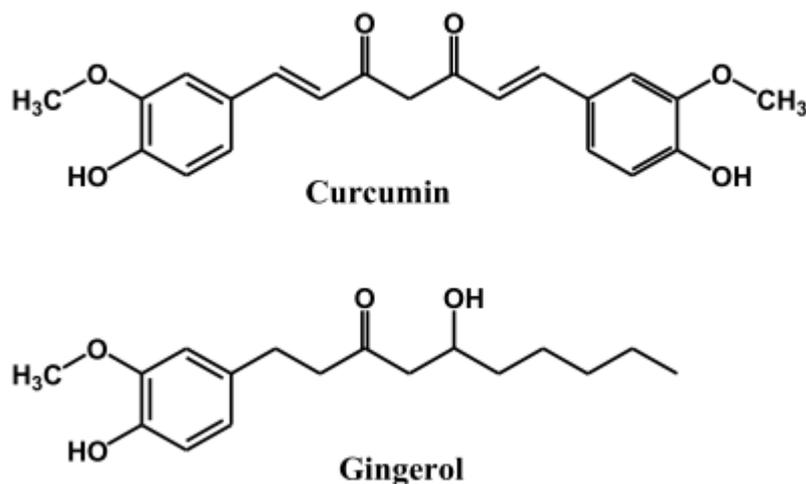


Figure 1.3 Chemical structure of curcumin. Take from Tsao et al. (Tsao 2010)

1.7 Polyphenols and CRC

Various polyphenols including carnosic acid, rosmarinic acid, curcumin and gingerols have been shown to inhibit the proliferation of various CRC cancer cell lines in vitro, including HT-29, Caco-2, SW480 and HCT116 (Ramos 2007; Sa & Das 2008; Jeong et al. 2009; Yesil-Celiktas et al. 2010; Lim et al. 2014). However, as polyphenols are pluripotent in relation to their mechanisms of action they thus have the potential to modulate various pathways and molecules involved in carcinogenesis including:

- Epidermal growth factor receptor (EGFR), Phosphoinositide 3-kinase (PI3K)/Akt (curcumin, genistein, EGCG) (Aggarwal & Shishodia 2006).
- Mitogen-activated protein kinases (MAPKs) (curcumin, resveratrol, EGCG) (Aggarwal & Shishodia 2006).
- The cell cycle protein cyclin D1 (curcumin, urosilic acid, quercetin) (Aggarwal & Shishodia 2006) .
- The anti-apoptotic proteins: Bcl 2, survivin, caspases 2,3,6,8,9 (curcumin, resveratrol, quercetin) (Aggarwal & Shishodia 2006; Shemesh & Arber 2014)
- Growth factor pathways including TNF, insulin growth factor (IGF) (curcumin, resveratrol, apigenin, EGCG) (Aggarwal & Shishodia 2006; Macdonald & Wagner 2012).
- Transcription factors including nuclear factor-kappaB (NF-κB) (curcumin, resveratrol, epigallocatechin gallate (EGCG), genistein, urosilic acid, quercetin) (Surh 1999; Aggarwal & Shishodia 2006; Macdonald & Wagner 2012)
- Signal transduction and transcription (STAT), β-catenin (curcumin, resveratrol) (Aggarwal & Shishodia 2006; Tarapore et al. 2012).

Furthermore, polyphenols (curcumin, resveratrol, EGCG) have been shown to modulate COX-2 activity and expression, and the Wnt signalling pathway (Aggarwal & Shishodia 2006; Macdonald & Wagner 2012; Tarapore et al. 2012), all of which as stated above, play an important role in the development of CRC. Moreover, natural compounds present in CHS such as curcumin, gingerols and shagaols selectively target COX-2, whilst have no effect on COX-1 (Goel et al. 2001; van Breemen et al. 2011), which is mainly expressed in normal tissue, thus avoiding negative side effects caused by many synthetic cyclooxygenase inhibitors (Rayburn et al. 2009).

The current emphasis regarding dietary polyphenols has been on the isolated constituents alone, so far a lot of focus in scientific community has been on isolated constituents from CHS such as curcumin (found in turmeric), gingerols (found in ginger), rosmarinic acid, carnosic acid, carnosol (found in rosemary, sage) (Ravindran et al. 2009; Johnson et al. 2011; Bauer et al. 2012; Simão da Silva et al. 2012; Mileo & Micedei 2015). However, it is well known that isolated phytochemicals do not always produce the same effects that are generated by the food within which they are found (Jacobs et al. 2009). One possible reason for this is that the isolated constituents are no longer in an environment where interactions between them are possible. Interactions between

phytochemicals found in plants foods, have been shown to be synergistic in nature, (Liu 2004). For example, Majumdar et al. (2009) reported that a combination of curcumin, and resveratrol were more effective at inhibiting the growth of HCT116 cell lines in vitro and in vivo (HCT116 cells were implanted immune-deficient mice) than either of individual compounds (Majumdar et al. 2009). Del Follo-Martinez et al. (2013) also demonstrated that the IC₅₀ values (HT-29) of resveratrol/quercetin combination were about a third lower compared to the IC₅₀s resveratrol or curcumin alone (Del Follo-Martinez et al. 2013). Mazue et al. (2014) also demonstrated that red wine polyphenols resveratrol and quercetin had a synergistic anti-proliferative effect on the SW480 cell line (Mazué et al. 2014). Such effects bring to mind the possibility that interactions between constituent polyphenols within whole foods could also give rise to synergistic responses; evidence suggests that the whole food should be the focus for understanding the true benefits of dietary and food constituents when considering disease prevention, and there is growing evidence of the whole food being more efficacious than its constituents (Liu 2004; Jakobs & Tapsell 2007). With regards to polyphenols and the inhibition of mechanisms involved in the development of CRC, candidates for such foods would be those that are known to be rich sources of polyphenols and known to possess anti-inflammatory properties, and one such group of foods are culinary herbs and spices (CHS) (Jungbauer & Medjakovic 2012; Rubió et al. 2013; Bhagat & Chaturvedi 2016; Zheng et al. 2016). Furthermore, polyphenols have limited bioavailability suggesting that a significant part of their action may be limited to the gut (Manach et al. 2004; Opara & Chohan 2014).

1.8 Culinary herbs and spices (CHS)

In general, a culinary herb is defined as a leaf of the plant that is used in cooking, whilst a culinary spice comes from other parts of the plant, such as bud, bark, root and seed, they often used in dried form (Tapsell et al. 2006). Culinary herbs and spices (CHS) have been used for centuries to enhance the flavour and aroma of foods and also for medicinal purposes such as digestive issues, viral and bacterial infections (Tapsell et al. 2006; Bhagat & Chaturvedi 2016). In developing countries populations continue to use CHS in herbal medicine for a range of ailments (Ekor 2014). One reason for this focus on these foods is their relatively high polyphenol content, for example the total phenolic content (TPC) of the foods that are listed among the foods with the highest phenolic content, dried herb and spice TPC ranges 20-158 mg of gallic acid equivalents (GAE) per 1 gram of dry weight (DW), whilst in comparison some fruits/berries and vegetables contain

0.08 - 14 mg and 0.06 - 3 mg of GAE per 1g respectively, however, fruits and vegetables consumed in higher quantities than CHS (Pérez-Jiménez & Torres 2011). Nevertheless, although CHS are consumed in relatively small quantities, they still can provide a substantial amount of polyphenols and thus may provide certain health benefits that are linked to their polyphenolic constituents which include anti-oxidant, anti-inflammatory and anti-carcinogenic activity (Tapsell et al. 2006; Jungbauer & Medjakovic 2012; Rubió et al. 2013; Bhagat & Chaturvedi 2016; Zheng et al. 2016). Furthermore, polyphenols found in CHS and other foods have limited bioavailability suggesting that a significant part of their action may be limited to the gut (Manach et al. 2004; Opara & Chohan 2014). Despite the fact that they are consumed in relatively low amounts, due to the beneficial properties discussed above, CHS may be considered by some as a functional food – although it must be borne in mind that they are not used to meet basic nutritional need (Tapsell et al. 2006).

As it was already stated above, numerous foods and natural compounds possess anti-inflammatory activity, thus they have a potential to be used for cancer prevention and treatment (Yoon & Baek 2005; Aggarwal et al. 2006; Lu & Yen 2015). Concerning anti-inflammatory activity of CHS, it was found that CHS reduced COX-2 expression and lowered other pro-inflammatory markers, IL-6, IL-10 and TNF-alpha in macrophages (Mueller et al. 2010). Several studies have also revealed that CHS possess anti-proliferative activity against various cancer cell lines (Kwon et al. 2010; Yesil-Celiktas et al. 2010; Dilas et al. 2012; Dimas et al. 2015; González-Vallinas et al. 2015). Yi & Wetzstein (2011) investigated the anti-proliferative effect of five culinary herbs (thyme, rosemary, sage, spearmint and peppermint) against SW480 CRC cells (Yi & Wetzstein 2011). Individually all the investigated herbs showed anti-proliferative activity with the strongest one being sage (IC₅₀ (inhibitory concentration at which growth inhibition is 50% compared to the control with no treatment)) - 35.9 µg/ml (Yi & Wetzstein 2011). Kogiannou et al. (2013) have shown that several herbal infusions (herb extracts prepared using boiling water and then dried residue used for experiments), which included oregano, showed anti-proliferative and anti-inflammatory effects against HT-29 cells by targeting NF-κB and IL-8 (Kogiannou et al. 2013). Another study demonstrated that an infusion of rosemary, suppressed the growth of HT-29 cells, and also reduced the level of IL-8, which plays an important role in promoting inflammation (Kaliora et al. 2014). It also has been shown that whole ginger extract inhibited the proliferation of CRC cells (HCT116 and HT-29) *in vitro* (Abdullah et al. 2010). Regarding the effects of CHS in comparison to their constituent polyphenols, evidence suggests that synergy is occurs in the whole food.

Arranze et al. (2015) reported that rosemary extract produced a stronger anti-inflammatory effect than its major polyphenol - carnosic acid (Arranz et al. 2015). Furthermore, there is evidence that CHS are more effective/potent than their individual constituents: Kim et al. (2012) found that whole turmeric was better at inhibiting the growth of CRC cell growth than the equivalent amount of curcumin, indicating that other phytochemicals present in turmeric also contributed to its anti-proliferative activity (Kim et al. 2012). Combining CHS also results in synergistic effects: Yi & Wetzstein (2011) found the combination of sage and peppermint produced a synergistic anti-proliferative effect compared to the sum of the effects of the individual herbs. Interestingly, the effects of whole foods known to be high in polyphenols have been shown to have the opposite effect to combinations of their isolated constituents: Durak et al (2015) reported that whereas coffee and ginger increased anti-inflammatory activity, mixtures of the isolated active components of these two foods resulted into an antagonistic effect, which disappeared after the in vitro digestion process removed one compound (Durak et al. 2015). The examples above illustrate the importance of interactions of constituents within food matrices namely those of CHS thus indicating that the foods themselves should be the focus of further investigation regarding their chemopreventative/therapeutic potential.

1.9 The chemopreventative/therapeutic potential of CHS with regards to CRC: previous research

Recent work by Jaksevicius (2012) and Baker (2012) has shown that a number of CHS (bay leaf, rosemary, sage, thyme, ginger, clove, turmeric, cinnamon) exerted inhibitory effects on the growth of CRC cells (HT-29 and HC116) in vitro. The most potent CHS extracts in relation to CRC cell growth were bay leaf (*Laurus nobilis*), ginger (*Zingiber officinale*), rosemary (*Rosemarinus officinalis*), sage (*Salvia officinalis*) and turmeric (*Curcuma longa*). Some of the CHS extracts also showed some potential in reducing COX-2 expression. However, in this study the effect of CHS on COX-2 was not fully established and one of the reasons why was that the expression of this molecule in HT-29 cell line was very low. Thus, it was accepted that to study the effect on COX-2 expression other CRC cell lines that express COX-2 at high levels needed to be identified. The main candidate is the HCA-7 cell line, which according to the literature, has the highest expression of COX-2 (Shao et al. 2000).

The effect of these CHS on the Wnt signalling pathway was also investigated with a focus on the central molecule β -catenin. However, their effect on unphosphorylated β -catenin was, as with COX-2, not fully established. It was suggested at the time that longer

or shorter exposure times might be needed and/or molecular targets further downstream needed to be investigated. Another suggestion was to look at the CHS effect on nuclear β -catenin, where it produces the major effect in carcinogenesis process.

The inconclusive results concerning the effect of these CHS on COX-2 and β -catenin expression also gave rise to a number of questions that need to be addressed to allow for further understanding of the chemo-preventative/chemotherapeutic potential of the CHS under investigation. Is the CHS' anti-proliferative activity on CRC cells *in vitro* due to their effect on COX-2 alone or does it also involve Wnt signalling pathway, and/or do they target other pro and anti-carcinogenic mechanisms such as caspase signalling?

1.10 Aims of the study

The up-regulation of COX-2 expression and the mutation of the Wnt/ β -catenin signalling pathway play key roles in development of CRC thus in light of the questions and suggestions that were made based on the previous research summarised above the aim of this project was to further investigate the effect of the most potent CHS (bay leaf, ginger, rosemary, sage and turmeric) on COX-2 and β -catenin expression in CRC cells whose growth has been inhibited by these CHS.

Research (Yi & Wetzstein 2010) suggests that combining CHS may result in greater effects than those of the sum of the constituent therefore another aim of this study was to investigate the effect of combining two herbs/spices to see whether they can produce stronger anti-carcinogenic effect than the sum of the effect of individual herbs/spices.

Cancer cells develop the ability to avoid apoptosis (Fernald et al. 2013). Hence, another aim of this study was to evaluate the effect of CHS on ability to induce apoptosis in CRC cells, and they do what key markers of apoptosis are affected, and to attempt to determine if this effect was linked to their effect on COX-2 (if any is identified).

1.11 Hypothesis

As it was stated above CHS and polyphenols present in CHS possess anti-inflammatory activity, whilst chronic inflammation plays a key role in the development of CRC. Hence, it was hypothesised that due to their anti-inflammatory activity CHS could inhibit the growth of CRC cells by targeting COX-2 enzyme, which is overexpressed in CRC. Another hypothesis was that if CHS can inhibit CRC cancer cell growth, they possibly could be able to induce apoptosis, which is a key outcome in preventing and curing CRC (Hu & Kavanagh 2003).

Chapter 2 Growth inhibitory and cytotoxic activity of CHS against CRC cells

2.1 Introduction

Colorectal cancer (CRC) is one of the most common cancers diagnosed in UK and other developed countries (Siegel et al. 2014). Dietary factors play an important role in the development and prevention of this disease (Hagggar et al. 2009; Gingras & Béliveau 2011) with epidemiological studies linking the consumption of foods rich in polyphenols with reduced risk of CRC (Arts & Hollman 2005; Johnson & Mukhtar 2007; Wang et al. 2013; Afrin et al. 2016). For example, consumption of berries, coffee and green tea, all of which contain high amounts of polyphenols, has been found to be associated with reduced incidence of CRC (Li et al. 2013; Nechuta et al. 2012; Sinha et al. 2012; Afrin et al. 2016).

Polyphenols are a large number of phytochemicals possessing a variety of health properties. It is well established that polyphenols possess growth inhibitory activity against cancer cells including CRC cells (Araújo et al. 2011; Núñez-sánchez et al. 2015; O’Keefe 2016). For example, carnosol and carnosic acid found in rosemary and other *Lamiaceae* family herbs, inhibited the growth of several cancer cell lines: NCI-H82 (lung cancer), MCF-7 (breast cancer), DU-145 (prostate cancer), Hep-3B (liver cancer) and K-562 (leukaemia) (Yesil-Celiktas et al. 2010). Curcumin, a major polyphenol of turmeric, also inhibited the growth of various cancer cell lines including CRC cells (Kim et al. 2012). However, although individual polyphenols can be effective at inhibiting cancer cell growth (Aggarwal & Shishodia 2006; Ibáñez et al. 2012; Catchpole et al. 2015), there are numerous examples in the literature that show that combining polyphenols can be more effective than using them individually (Jacobs & Tapsell 2013; Alshatwi et al. 2016). For example, a combination of carnosic acid (CA) and carnosol (CL) inhibited the growth of HT-29 cells better than these two polyphenols individually (Valdes et al. 2014). Majumdar et al. (2009) found that a combination of curcumin and resveratrol was more effective at inhibiting CRC cell growth than these polyphenols used individually. Another study found that a combination of resveratrol and quercetin had an IC₅₀ that was approximately 30% lower than those of the individual polyphenols indicating that the combination was more potent (Del Follo-Martinez et al. 2013). Gingerol, found in ginger, combined with γ -tocotrienol showed synergistic anti-proliferative activity against two CRC cell lines – HT-29 and SW837 (Yusof et al. 2015).

The evidence above also shows that the growth inhibitory action of their combinations on CRC cells is more potent. However, these polyphenols are part of complex plant food matrices such as CHS, so it is important to consider the growth inhibitory action of their polyphenols with this matrix, especially as studies concerning the combinations of polyphenols show an increased potency compared to the individual constituents (Williamson 2001; Liu 2004; Wagner 2010; Jacobs & Tapsell 2013). CHS are known to be able to inhibit the growth of CRC cells. For example, melissa ethanol extract and its major polyphenol – rosmarinic acid reduced growth of HCT116 cell line (Encalada et al. 2011). Ginger reduced the growth of CRC cells - HT-29 (Tahir et al. 2015). Yi and Wetzstein (2010) found that thyme, rosemary, sage, spearmint, and peppermint extracts inhibited the growth of SW480 cells. Valdés et al. (2013) reported that several types of rosemary extract inhibited the growth of SW480 and HT-29 cells (Valdés et al. 2013). Moreover, there is evidence that the growth inhibitory effect of whole CHS extracts is stronger than their major active constituents used individually. For example, Kim et al. (2012) found that whole turmeric extract was more effective at inhibiting CRC cell growth (HCT116 and HT-29) than its major active polyphenol – curcumin. Hence, the literature indicates that it could be more effective to use whole herb/spice extract rather than their isolated active constituent polyphenols. Thus, the main aims of this study was to determine and investigate CHS extracts can inhibit the growth of several CRC cells and to identify the most potent extracts for further studies concerning the elucidation of their growth inhibitory mechanisms related to CRC.

2.2 Materials and methods

2.2.1 Selection of culinary herbs and spices

Five CHS were used in this study: bay leaf (*Laurus nobilis*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*). The selection of the CHS was based on CRC cell growth inhibition data from previous work (Baker 2012; Jaksevicius 2012), and the most potent CHS were selected for this project, for more details see Appendix 1. CHS were purchased from Neal's Yard Remedies.

2.2.2 Preparation of CHS extracts

Aqueous and ethanol extracts of bay leaf, rosemary, and sage were prepared. Based on previous work (Baker 2012), only ethanol extracts were prepared for ginger and turmeric. The extraction method used was adapted from Huang et al. (2009) with some modifications (Huang et al. 2009). Briefly, herbs were ground up using a pestle and mortar

and 0.45 g of ground herb/spice was added to a glass bottle, and extracted in 27 ml of solvent (ultra-pure water or 42% ethanol (v/v)). The solid to solvent ratio was 1:60 as determined to be the best ratio by Huang et al (2009). The bottles were then wrapped in aluminium foil and placed on an orbital shaker (OS71, Fisher Scientific) for 2.5 hours. Thereafter, the bottles were placed in a sonicator (Fisherbrand™ S-Series Ultrasonic Cleaners, FB15015) and sonicated for 70 min. at 35 kHz frequency. After the sonication, the CHS extracts were filtered using a two-stage filtration process: at stage 1, the extracts were filtered using Whatman No1 filter and at stage 2 a Whatman No 6 filter paper was used. The filtered extracts were then aliquoted into Eppendorf tubes (1 ml per Eppendorf tube) and then stored at -80°C.

Prior to using the CHS for the tissue culture experiments, the extracts were filter sterilised using a 0.22 µm filter (Millipore express PES membrane, Millipore Ireland Ltd) and then transferred to a bijou. For the growth inhibition studies combination of two extracts were used with ratio 1:1 by the TPC (10 µg GAE/ml of each). Based on preliminary potency studies, the following combinations were used: rosemary aqueous and rosemary ethanol (RAE), sage aqueous and sage ethanol (SAE), bay leaf and turmeric ethanol (BLTE), sage and ginger ethanol (SGE), bay leaf and sage ethanol (BLSE) and rosemary and turmeric ethanol (RTE).

2.1.1 Total phenolic content assay

The total phenolic content (TPC) for each herb extract was determined using the Folin-Ciocalteu (F-C) colorimetric method (Singleton et al. 1999), modified by (Tang et al. 2004). Samples of the herb extracts (100 µl) were diluted with distilled water and then added to the 12-well plates followed by 200 µl F-C reagent, 2 ml of deionised water and 1 ml of sodium carbonate. The standard curve was generated using gallic acid standards (0.05 mg/ml to 0.5 mg/ml). Samples were left for 2 hours, after which absorbance was measured at 765 nm using an Epoch microplate reader (Biotek, UK).

2.1.2 Cell culture

Three CRC cell lines were used in the present study: HCT116, CCL235 and HCA-7, which were originally purchased from European Collection of Cell Cultures (ECACC All cell lines listed above were grown in DMEM supplemented with 10% FBS and antibiotics: penicillin (50 units per ml), streptomycin (0.05 mg/ml) and neomycin (0.1 mg/ml), at 37°C, 5% CO₂ atmosphere. A new batch of cells was replaced every 2-3 months. In order to determine whether CHS inhibit cell growth by targeting COX-2 enzyme, COX-2 negative (HCT116) and COX-2 positive cell lines were chosen (Shao et al. 2000).

Confluent cells (80-100% of confluency) were trypsinised and placed into a new flask, placed into the incubator for continuous growth. Usually cell culture media was not replaced between trypsinisation. To investigate the effect of CHS (BLE and TE only) on normal, control, cells and to determine if any effect on the CRC cells may be specific, human fibroblast cells (HFF-2) were used. The cells were donated by Dr. M.Chioni, Kingston University London, and grown under the same conditions as CRC cell lines.

2.1.3 Growth inhibition studies

The growth inhibition studies were performed on the CRC cells using the sulforhodamine B (SRB) assay, and the protocol was adapted from Khelwatty et al. (2011). In brief, confluent cells (80-100% of confluency) were trypsinised and re-suspended in DMEM with 10% FBS and then seeded on to 96-well plates (5000 cells suspended in 100 µl of DMEM per well) and placed into an incubator for four hours. Based on previous work (Baker 2012; Jaksevicius 2012) the following CHS extracts and their combinations were used: rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA), bay leaf and turmeric ethanol (BLTE), sage and bay leaf ethanol (SBLE), rosemary aqueous ethanol (RAE), sage aqueous and ethanol (SAE) and sage and ginger ethanol (SGE). CHS extracts were then prepared using a doubling dilution technique; the starting concentration for each extract was 20 µg GAE/ml. Following the 4-hour incubation period, the herb extracts (100 µl) at various concentrations were added to the wells. Cells were treated with extracts for 5-7 days and when the control wells (no herb extract, just cells in DMEM containing 10% FBS) reached confluence. Thereafter, the plate was fixed for 1 hour with 10% TCA, then washed with tap water, dried and stained with SRB (0.4 % (w/v) in acetic acid (1%, v/v) (100 µl per well) for 1 hour. Thereafter, SRB was removed and the stain was re-solubilised by adding 100 µl Tris-base (10mM) into each well, and absorbance was then read at 564 using an Epoch microplate reader (Biotek, UK). Subsequently, the SRB assay was also performed on the normal cells (HFF-2) using the same concentrations (starting concentration was 20 µg GAE/ml with eight double dilutions) that were applied to the cancer cells. Only BLE and TE, which were two of most potent extracts with regards to their effect on the growth of the CRC cells, were tested.

2.1.4 The effect of CHS extracts on CRC cell viability using MTT

The effect on cell viability of the most potent CHS extracts and combinations based on SRB assay) was further investigated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The following CHS extracts and their combinations

used were TE, GE, BLE, BLA, BLTE and SGE. Cells were trypsinised and seeded on 96-well plate and left for 24 hours, thereafter the CHS extracts were added at concentrations based on the SRB investigation: 20, 10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µg GAE/ml. Following the treatment periods (24, 48 and 72 hours), the media containing the CHS extracts were removed and MTT (0.5 mg/ml) added. After 4 hours, media containing MTT was removed and DMSO was added to solubilize the cells. Absorbance was then read at 570 nm (Epoch microplate reader, Biotek, UK) and cell viability was determined.

A further experiment was performed to evaluate what would happen if CHS extracts were removed after 24 hours and replaced with fresh media and left for another 48 hours. It was hypothesised that if the IC₅₀ values after the removal of the extracts were similar to the ones that received the whole 72-hour treatment, the action of CHS extracts could be cytotoxic. To confirm this, the lactate dehydrogenase (LDH) cytotoxic assay was performed (Promega, UK) using two of the most potent extracts (BLE and TE) and their combination (BLTE). The concentrations of the extracts were the same as for SRB and MTT assays and the treatment period was the longest period used for the MTT assays described above - 72 hours. The assay procedure was followed using the manufacture's protocol.

2.1.5 Monitoring cell growth with IncuCyte

Cells (HCT116, HCA-7 and the normal control cells HFF-2) were set up and exposed as described above for the MTT assay. For BLE the concentrations were 15, 7.5, 3.75, 1.88 and 0.94 µg GAE/ml. The starting concentration for bay leaf was different in order to match the concentration used in western blot experiment. For TE the concentrations were 20, 10, 5, 2.5, 1.25, 0.625 µg GAE/ml. The cell growth was monitored using IncuCyte camera for 3-5 days. The effect on HFF-2 cell growth was compared to the effect on CRC cells (HCT116 and HCA-7) at the same concentrations.

2.1.6 Data expression and statistical analysis

All experiments were done in triplicate (n=3), which represents three separate experiments and data are expressed as mean and standard deviation (\pm SD) unless otherwise stated. The TPC data are expressed as milligrams (mg) of gallic acid equivalents (GAE) per gram of dry herb/spice weight. Growth inhibition data (SRB and MTT) are presented as 50% inhibitory concentration (IC₅₀), at which 50% of cells growth is inhibited compared to the no treatment group (the control for which cell growth is 100%). The IC₅₀ concentration was determined for each CHS (individual and in combination) extract (unless IC₅₀ was not achieved) using Gen5 (Biotek, UK) software and expressed as µg

GAE/ml and DW equivalents $\mu\text{g/ml}$ in order to show the importance of polyphenols found in the CHS extracts.

To determine if synergy occurred as a result of the CHS combinations, the interaction factor was calculated for each combination using the analysis described by Gawlik-Dziki (2011). $IF = \frac{\text{IC}_{50} \text{ value for combination}}{(\text{IC}_{50} \text{ value for herb1}/2 + (\text{IC}_{50} \text{ value for herb2}/2))}$ IF values of <1 indicate synergy, IF values >1 indicate antagonism, and IF values =1.

Statistical analysis was performed using PASW 18 software package. Independent T sample test was used to compare the TPC of non-filter-sterilised and filter-sterilised extracts. One-way ANOVA with Tukey's post-hoc test was performed to assess whether the in the IC₅₀ values between three different cell lines (SRB assay). A statistically significant difference was set at $p < 0.05$.

2.2 Results

2.2.1 Total phenolic content of CHS

In order to determine the dose for growth inhibition studies, the total phenolic content (TPC) was established for each extract (for non-filter-sterilized – NF – and filter sterilized - F) (Table 1), and extracts were ranked by their TPC in the following order: SE > RE > RA > SA > BLE > TE > BLA > GE. Filter-sterilisation (F) reduced TPC, and with the exception of ginger, there was a statistically significant difference ($p < 0.05$) between NF and F extracts. Filter-sterilised (F) extracts ranked in the following order: SE > RE > RA > BLE > SA > TE > GE > BLA. Three ethanol CHS extracts had a higher phenolic content in comparison to their aqueous counterparts: bay leaf – 48.5 vs 23 GAE mg/g DW; rosemary 62.2 vs 61.4 GAE mg/g DW, sage 80.2 vs 50.9 GAE mg/g DW (Table 1).

Table 2.1 Total phenolic content of CHS extracts: non-filtered (NF) vs filter-sterilised (F)

CHS	NF (GAE mg/g of DW), SD (\pm)	F (GAE mg/g of DW), SD(\pm)
SE	80.2 (\pm 0.3)*	74.2 (\pm 1.2)
RE	62.2 (\pm 1.3)*	59.8 (\pm 0.2)
RA	61.4 (\pm 0.4)*	55.3 (\pm 0.2)
BLE	48.5 (\pm 0.4)*	44.7 (\pm 0.5)
SA	50.9 (\pm 1.1)*	41.0 (\pm 0.2)
TE	26.1 (\pm 0.3)*	23.2 (\pm 0.2)
GE	16.2 (\pm 0.1)	15.6 (\pm 0.3)
BLA	23.0 (\pm 0.7)*	13.3 (\pm 0.5)

Total phenolic content was determined using total phenolic content assay (TPC), and data presented as gallic acid equivalent (GAE) per 1g of dry weight (DW) of the herb/spice. Data expressed as mean (n=3), and \pm SD. *Statistically significant difference between non-filter-sterilised and filter-sterilised extracts (P<0.05). Rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA).

2.2.2 The effect of activity of CHS and their combinations CRC cell growth using the SRB assay

The results show that all investigated CHS extracts, individual and in combination, inhibited the growth of HCT116, CCL235 and HCA-7 CRC cells in a dose-dependent manner (Table 2). There was a statistically significant difference ($p < 0.05$) between BLAE for HCT116 cells, and RAE for both cell lines (Tables 2). Individually TE was the most potent extract with IC₅₀ values of 1.4 μ g GAE/ml (HCT116), 2.3 μ g GAE/ml (CCL235) and 3.0 μ g GAE/ml (HCA-7). For SRB assay the IC₅₀ values for HFF-2 cells were several times higher in comparison to the IC₅₀ values CRC cancer cell lines: for TE – 7.1, and for BLE also 7.1 μ g/ml GAE (Table 2). The IC₅₀ values were also expressed in dry weight equivalent of the herb/spice (Table 3). Based on dry weight equivalent, the most potent CHS for HCT116 cell line were RE (71 μ g/ml), SE (78 μ g/ml) and BLE (102 μ g/ml); for CCL235 – RE (66 μ g/ml), SE (100 μ g/ml) and SA (122 μ g/ml) and for HCA-7 – BLE (117 μ g/ml), BLA (200 μ g/ml) and TE (300 μ g/ml). For the CHS were the most potent for the combination when the IC₅₀ was expressed in dry weight equivalent: for HCT116 – RAE (77 μ g/ml) and SAE (92 μ g/ml); for CCL235 – RAE (83 μ g/ml) and SAE (87 μ g/ml) and for HCA-7 – SBLE (180 μ g/ml) and BLTE (227 μ g/ml) (Table 2).

Table 2.2 IC50 values of CHS extracts for HCT116, CCL235 and HCA-7 cell lines

CHS and combinations	HCT116	CCL235	HCA-7	HFF-2
	IC 50 (µg GAE/ml) SD (±)			
TE	1.4 (±0.2)	2.3 (±0.6)	3.0 (±0.4)**	7.1 (±1.6)
GE	2.5 (±0.2)	3.2 (±0.5) ^{\$}	5.5 (±0.5)**	
RE	2.6 (±0.4)	2.4 (±1.0) ^{\$}	15.9 (±0.7)**	
BLE	2.7(±0.1)	3.4 (±0.7) ^{\$}	4.7 (±0.4)**	7.1 (±1.6)
SE	2.8 (±0.1)	3.6 (±1.1) ^{\$}	12.5 (±1.6)**	
BLA	3.1 (±0.4)	4.9 (±0.6)	4.0 (±0.8)	
SA	4.8 (±0.9)	5.0 (±0.8)	>20	
RA	5.6 (±0.5)	5.4 (±0.3) ^{\$}	17.1 (±0.2)**	
RTE	1.7 (±0.1)	2.5 (±0.6) ^{\$}	6.0 (±0.6)**	
BLTE	1.8 (±0.4)	3.5 (±0.7)	3.3 (±1.2)	
RAE	2.9 (±0.4)	3.1 (±0.7) ^{\$}	16.2 (±0.8)**	
SBLE	3.1 (±0.4)	4.0 (±1.2)	5.5 (±0.5)	
SGE	3.3 (±0.7)	2.7 (±0.3) ^{\$}	6.8 (±0.1)**	
SAE	3.5 (±1.1)	3.3 (±0.9) ^{\$}	15.7(± 1.0)**	

IC50 values were established using SRB assay. Cells were treated with a range of concentrations of CHS. Data expressed as mean (n=3), and ±SD.

*Statistically significant difference between IC50 dose for CCL235 and HCT116, for the same extract, p<0.05

** Statistically significant difference between IC50 dose for HCA-7 and HCT116 for the same extract, p<0.05

\$ Statistically significant difference between IC50 dose for HCA-7 and CCL235 for the same extract, p<0.05

Rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA), bay leaf and turmeric ethanol (BLTE), sage and bay leaf ethanol (SBLE), rosemary aqueous ethanol (RAE), sage aqueous and ethanol (SAE) and sage and ginger ethanol (SGE).

Table 2.3 IC50 values of CHS extracts for HCT116, CCL235 and HCA-7 cell lines expressed in dry weight equivalent

CHS and combinations	HCT116	CCL235	HCA-7	HFF-2
	IC 50 (µg/ml DW)			
TE	140	230	300	710
GE	189	242	417	
RE	71	66	347	
BLE	102	128	117	268
SE	78	100	347	
BLA	117	185	200	
SA	117	122	>442	
RA	145	140	442	
RTE	108	159	382	
BLTE	124	241	227	
RAE	77	83	432	
SBLE	102	131	180	
SGE	171	140	352	
SAE	92	87	414	

Data presented as dry weight equivalent. Data expressed as mean (n=3), and \pm SD. DW – dry weight of the herb/spice. Rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA), bay leaf and turmeric ethanol (BLTE), sage and bay leaf ethanol (SBLE), rosemary aqueous ethanol (RAE), sage aqueous and ethanol (SAE) and sage and ginger ethanol (SGE).

Regarding synergy/antagonism for the combinations according to the IF index the strongest synergistic effect was by RAE on HCT116 cell line, SAE on CCL235, and SGE on CCL235, HCA-7; SGE on HCA-7 and BLTE on HCA-7 cell lines (Table 3). Several other combinations had IFs just below 1 including RAE (CCL235/ HCA-7), RTE (HCT116), and BLTE (HCT116). SGE (HCT116), SGE (both cell lines), BLTE (CCL235), and RTE (HCA-7) had IF index above 1 (Table 4).

Table 2.4 IF index for CHS extract combinations based on SRB assay

Combinations	HCT116	CCL235	HCA-7
RAE	0.83	0.93	0.98
SAE	0.99	0.80	n/a
RTE	0.92	1.01	1.20
BLTE	0.95	1.26	0.90
SGE	1.27	0.80	0.67
SBLE	1.11	1.16	0.80

Bay leaf ethanol (BLE), bay leaf aqueous (BLA), bay leaf and turmeric ethanol (BLTE), sage and bay leaf ethanol (SBLE), rosemary aqueous ethanol (RAE), sage aqueous and ethanol (SAE) and sage and ginger ethanol (SGE). IF index <1 indicates synergy, IF index >1 indicates antagonism.

2.2.3 The effect of CHS extracts on CRC cell viability using MTT assay

The effect of the most potent CHS extracts and their combinations on CRC cell viability was also tested using the MTT assay. Turmeric ethanol (TE) had the lowest IC₅₀ values across all three time points – 24, 48 and 72 hours - 6 ± 0.1 , 2.1 ± 0.5 , 2.5 ± 0.3 μg GAE/ml (Table 5.). At the 24-hour time point, for all extracts and combinations, the IC₅₀ values were higher than those for the 48 and 72-hour treatments. Bay leaf aqueous (BLA) extract was the least potent and did not achieve an IC₅₀ value (for 24 and 72 hours). The combination of BLTE had a strong effect on reducing cell viability of both cell lines, and its IC₅₀ values for 48 and 72h were second lowest after TE (Tables 5 and 6).

A further experiment was performed to investigate what would happen if the treatment was removed from the CRC cells. Results revealed that the removal of extracts after 24 hours did not have a significant effect on the IC₅₀ values, in comparison to the whole 72-hour treatment, with exception for SGE on HCT116 cell line. For the other tested extracts and their combinations, the difference between the IC₅₀ values was not statistically significant (Table 5 and 6). Thereafter LDH cytotoxic assay was conducted and at the higher concentrations (5, 10 and 20 μg GAE/ml) TE, BLE and BTLE each produced a cytotoxic effect (Figure 2.1 and 2.2).

Table 2.5 Effect of CHS and their combinations on HCA-7 cell viability (MTT assay)

Herbs/spices	24hours	48hours	72hours	Extracts removed from media*
	IC50 (μg GAE/ml) ($\pm\text{SD}$)			
TE	6 (± 0.1)	2.1 (± 0.5)	2.5 (± 0.3)	2.5 (± 0.7)**
GE	10 (± 0.8)	6.1 (± 1.1)	5.8 (± 0.3)	7.8 (± 1.5)**
BLE	10.5 (± 0.5)	6 (± 0.9)	9.2 (± 0.4)	8.4 (± 0.4)**
B A	>20 (n/a)	17.3 (± 2.5)	>20 (n/a)	>20 (n/a)
BLTE	11.1 (± 1.6)	4.9 (± 0.8)	3.6 (± 1.1)	3.7 (± 1.0)**
SGE	11.1 (± 1.3)	10.7 (± 0.8)	10.9 (± 1.4)	11.3 (± 1.5)**

Data expressed as mean (n=3), and $\pm\text{SD}$.

*Extracts removed after 24hrs and replaced with fresh media and left for another 48hours

**Difference between the IC50 values were statistically insignificant compared to 72-hour treatment $p > 0.05$; n=3. IC50 – the concentration of the extract at which cell growth is reduced by 50% in comparison to control. GAE – gallic acid equivalent, which is used to express the total polyphenol content (TPC). Rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA), bay leaf and turmeric ethanol (BLTE), sage and bay leaf ethanol (SBLE), rosemary aqueous ethanol (RAE), sage aqueous and ethanol (SAE) and sage and ginger ethanol (SGE).

Table 2.6 Effect of CHS and their combinations on HCT116 cell viability (MTT assay)

Herbs/spices	24 hours	48 hours	72 hours	Extracts removed from media*
	IC50 (μg GAE/ml) ($\pm\text{SD}$)			
TE	2.5 (± 0.5)	1.3 (± 0.3)	1.5 (± 0.4)	1.7 (± 0.2)
GE	11.9 (± 0.8)	6.1 (± 2.0)	6.6 (± 1.4)	6.6 (± 1.4)
BLE	7.0 (± 2.0)	5.0 (± 1.6)	5.4 (± 1.6)	5.3 (± 1.2)
B A	>20	14.7 (± 3.5)	12.8 (± 3.8)	13.4 (± 0.9)
BLTE	3.9 (± 0.8)	2.8 (± 0.3)	2.3 (± 0.3)	2.7 (± 0.4)
SGE	9.4 (± 0.2)	6.2 (± 1.4)	3.2 (± 1.3)	6.1 (± 0.7)**

Data expressed as mean (n=3), and $\pm\text{SD}$.

*Extracts removed after 24hrs and replaced with fresh media and left for another 48hours

** Statistically significant difference between the IC50 values when treatment was removed after 24 hours compared to 72-hour treatment $p > 0.05$; n=3. IC50 – the concentration of the extract at which cell growth is reduced by 50% in comparison to control. GAE – gallic acid equivalent, which is used to express the total polyphenol content. Rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA), bay leaf and turmeric ethanol (BLTE), sage and bay leaf ethanol (SBLE), rosemary aqueous ethanol (RAE), sage aqueous and ethanol (SAE) and sage and ginger ethanol (SGE).

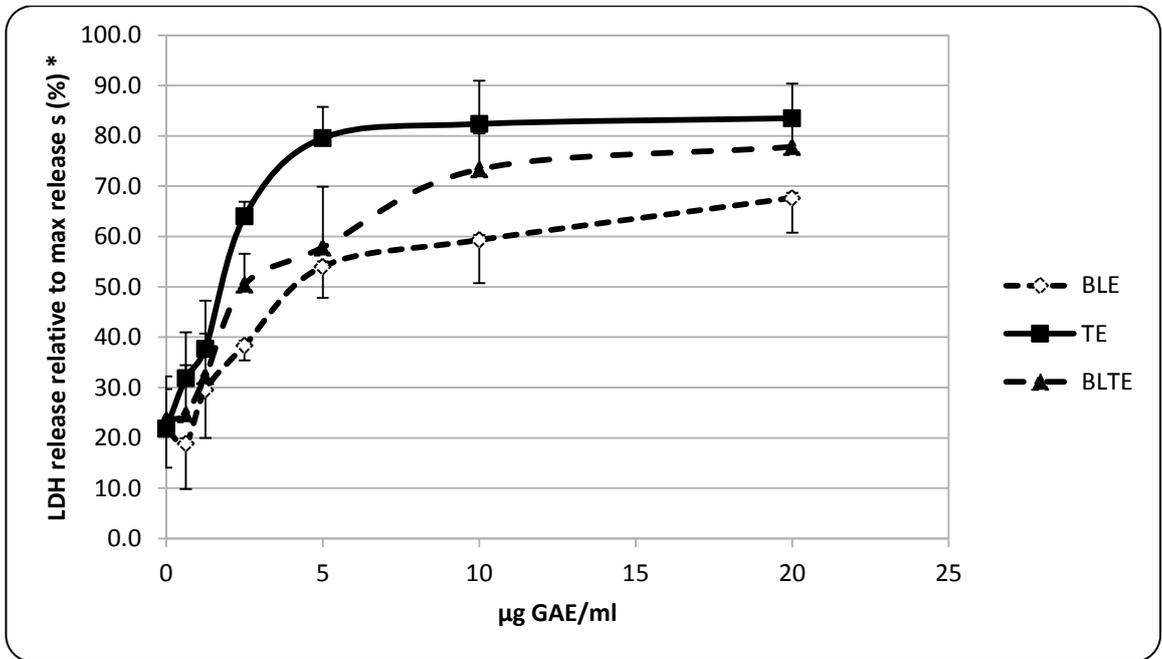


Figure 2.1 Cytotoxic effect of CHS against HCA-7 cells using LDH assay.

Cells were treated for 72 hours with bay leaf ethanol (BLE), turmeric ethanol (TE) extracts, and combination of bay leaf and turmeric ethanol (BLTE). *Data expressed as a percentage of maximum release of lactate dehydrogenase (LDH), which was achieved using lysis buffer provided by the kit manufacturer, n=3, and ±SD.

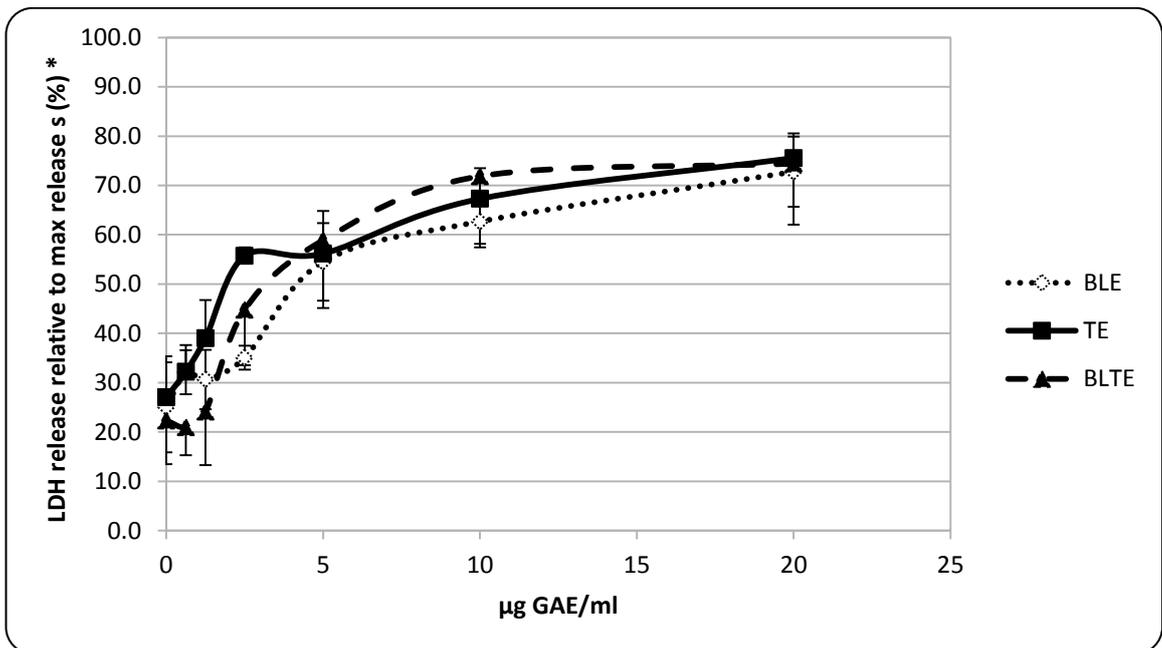


Figure 2.2 Cytotoxic effect of CHS against HCT116 cells using LDH assay.

Cells were treated for 72 hours with bay leaf ethanol (BLE), turmeric ethanol (TE) extracts, and combination of bay leaf and turmeric ethanol (BLTE). *Data expressed as a percentage of maximum release of lactate dehydrogenase (LDH), which was achieved using lysis buffer provided by the kit manufacturer, n=3, and ±SD. Bay leaf ethanol (BLE), turmeric ethanol (TE), bay leaf and turmeric ethanol (BLTE).

2.2.4 The effect of CHS on growth healthy cells - human fibroblasts (HFF-2)

The IncuCyte data revealed that for TE 20 and 10 μg GAE/ml stopped HFF-2 cell growth, whilst for BLE two highest concentration 15 and 7.5 μg GAE/ml also inhibited HFF-2 cell growth (Figure 2.5). The doses used to inhibit the growth of CRC cells were lower (Figures 2.3 and 2.4). According the IncuCyte data the lowest BLE dose that inhibited CRC cell dose was 3.75 μg GAE/ml (HCA-7 and HCT116) and for TE 5 μg GAE/ml (HCA-7) and 2.5 μg GAE/ml (HCT116) (Figures 2.3 and 2.4).

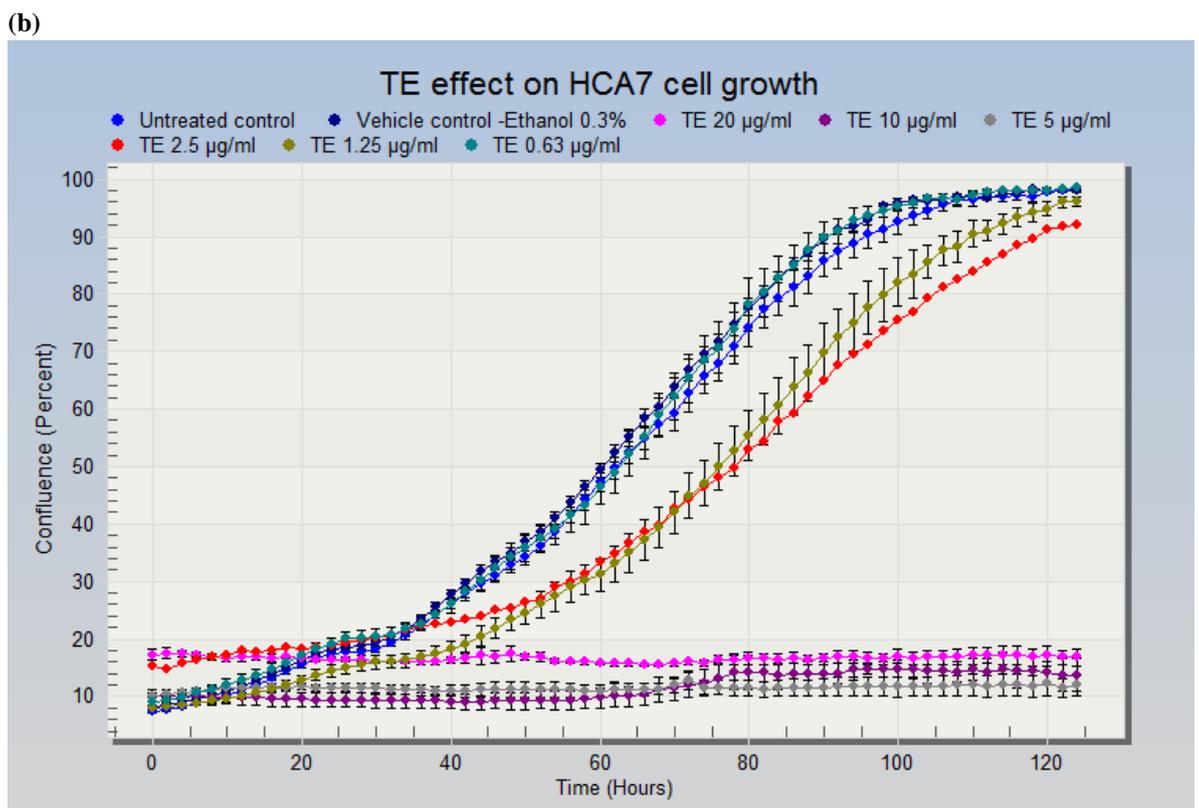
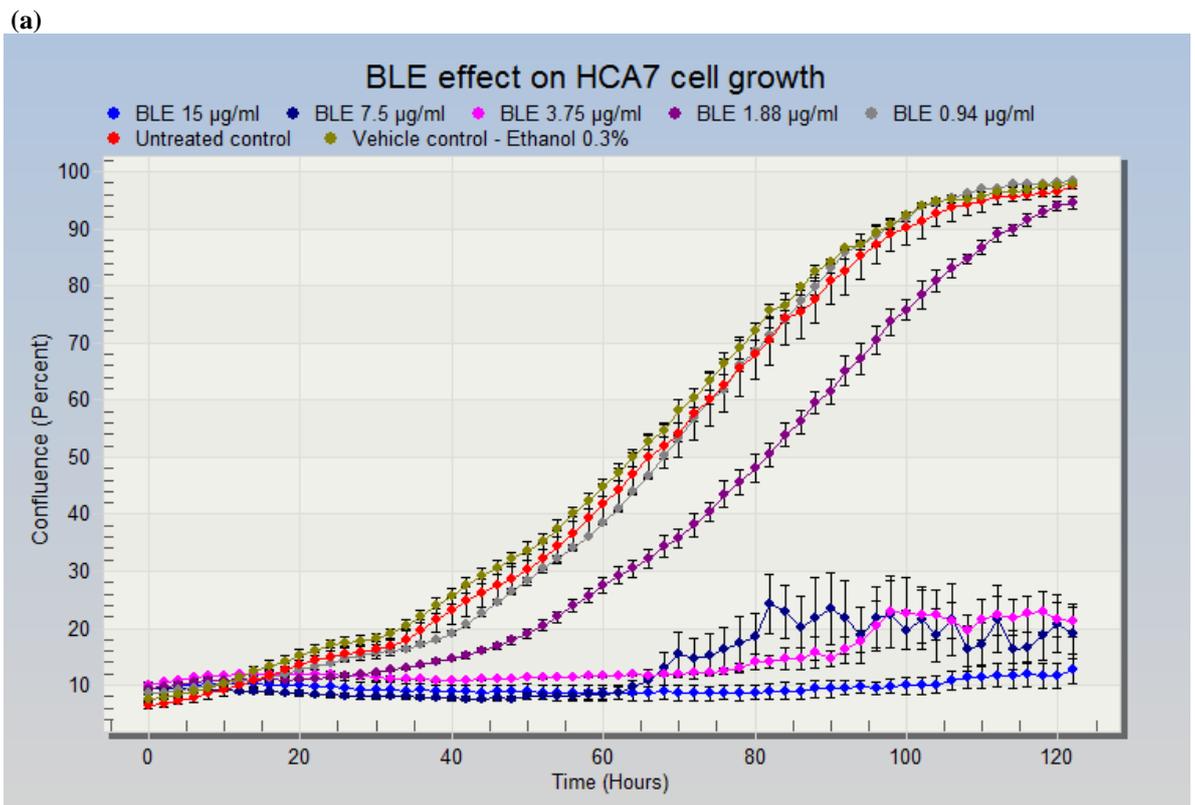
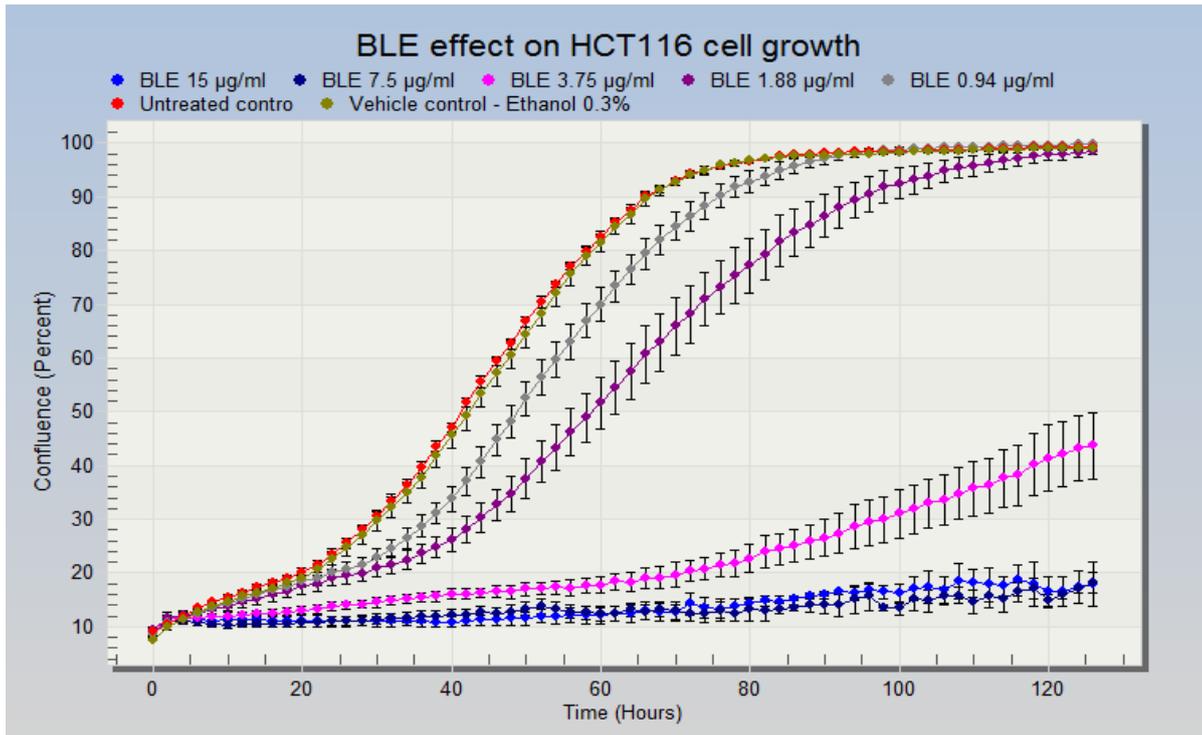


Figure 2.3 (a) BLE effect on HCA-7 cell growth. (b) TE effect on HCA-7 cell growth. Cells were treated with a range of concentrations of turmeric ethanol extract (TE) (0.63 – 20 µg GAE/ml), and bay leaf ethanol (BLE) (0.94 – 15 µg GAE/ml), and the growth was monitored using IncuCyte camera. Data was analysed and the graph was generated using IncuCyte software (Essen Bioscience, UK).

(a)



(b)

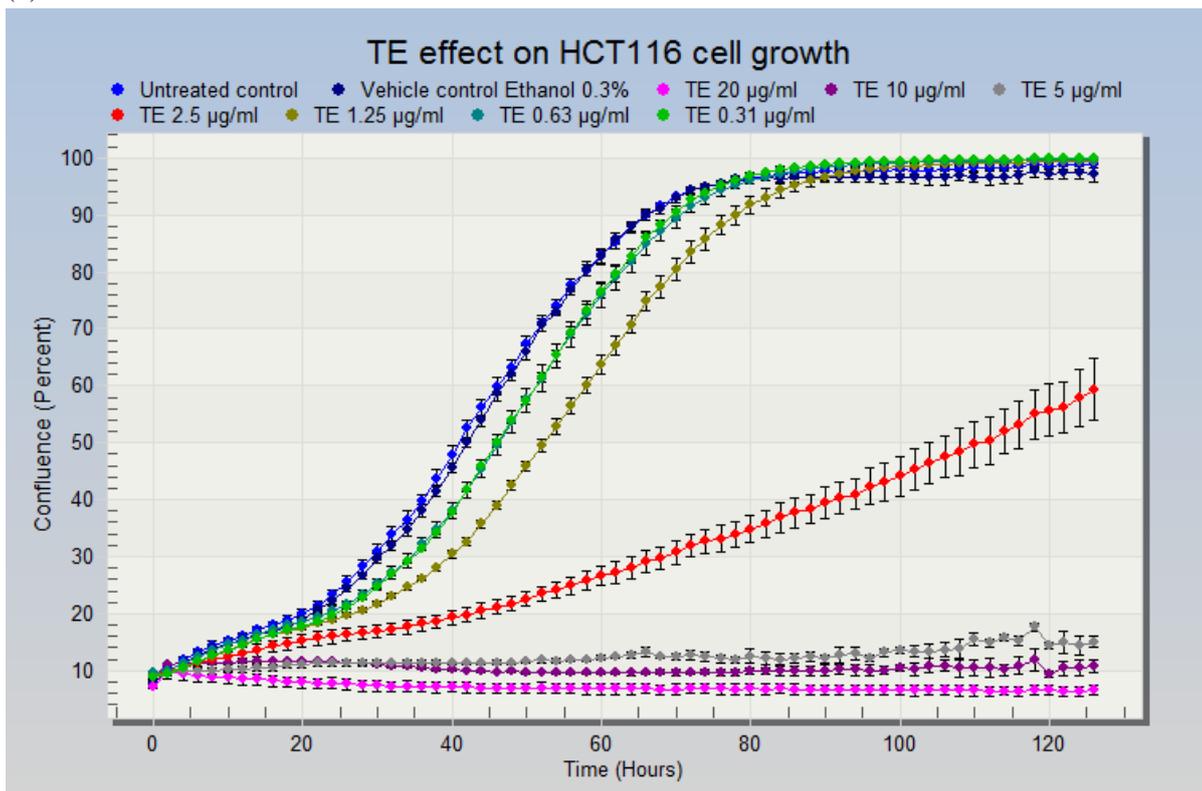


Figure 2.4 (a) BLE effect on HCT116 cell growth. (b) TE effect on HCT116 cell growth.

Cells were treated with a range of concentrations of turmeric ethanol extract (TE) (0.63 – 20 µg GAE/ml) and bay leaf ethanol (BLE) (0.94 – 15 µg GAE/ml), and the growth was monitored using IncuCyte camera. Data was analysed and the graph was generated using IncuCyte software (Essen Bioscience, UK).

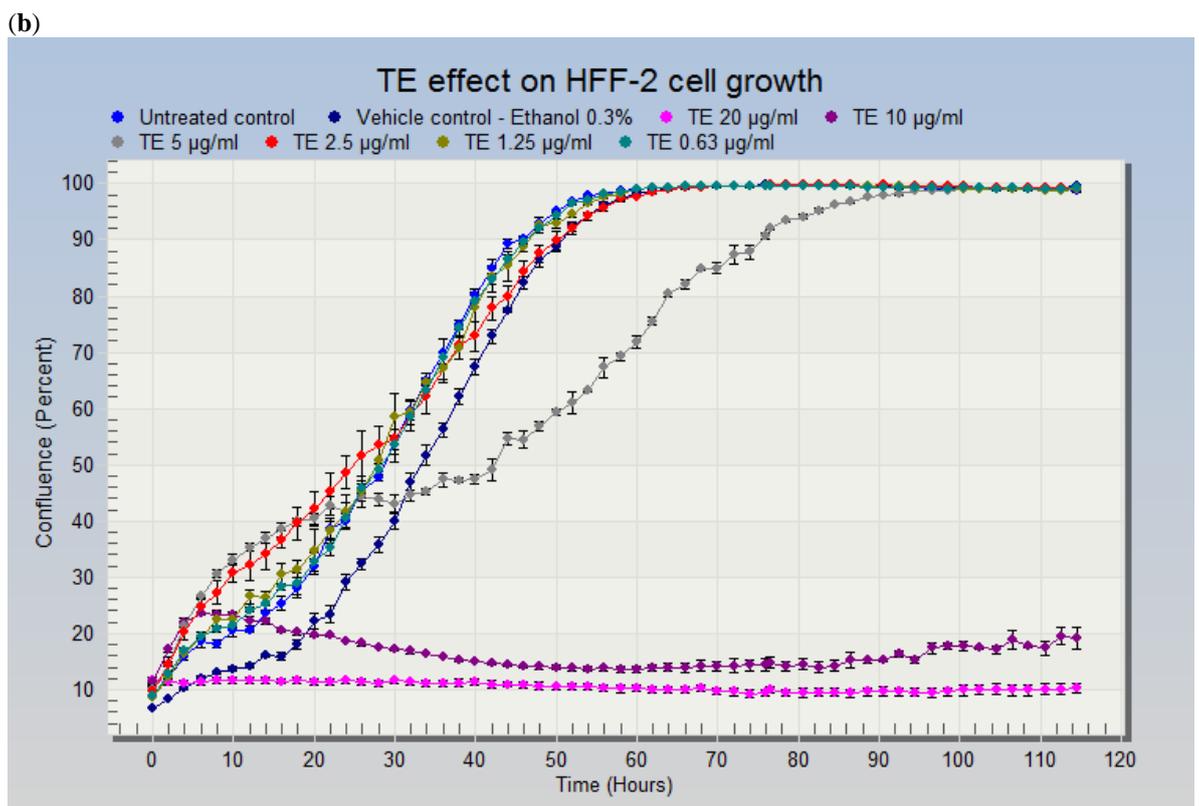
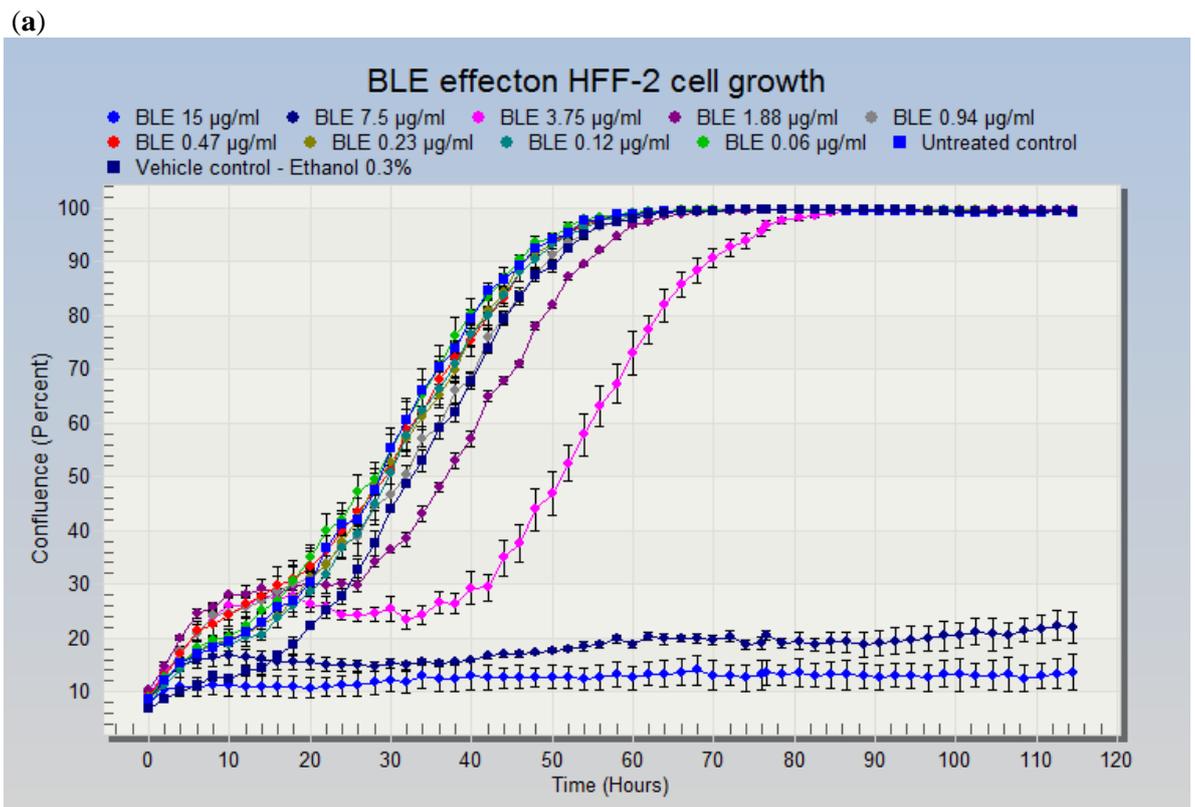


Figure 2.5 (a) BLE effect on HFF-2 cell growth. (b) TE effect on HFF-2 cell growth. Cells were treated with a range of concentrations of turmeric ethanol extract (TE) (0.63 – 20 μg GAE/ml) and bay leaf ethanol (BLE) (0.94 – 15 μg GAE/ml), and the growth was monitored using IncuCyte camera. Data was analysed and the graph was generated using IncuCyte software (Essen Bioscience, UK).

2.3 Discussion

The main aim of this study was to evaluate the growth inhibitory effect activity of CHS extracts against CRC cells and identify the most potent extracts and their combinations. The TPC was established for each extract and then was used to calculate the dose for the subsequent growth inhibition studies and other experiments. This study confirmed the findings of previous studies that CHS have a high phenolic content (Shan et al. 2005; Conforti et al. 2006; Kivilompolo & Hyötyläinen 2007; Chohan et al. 2012). Sage, rosemary and bay leaf had higher TPCs in comparison to ginger and turmeric extracts which could be explained by the different phenolic compositions of these herbs and spices (Puangsombat & Smith 2010; An et al. 2016; Chinedum et al. 2015). According to Phenol-Explorer.eu data base (Neveu et al. 2010), major polyphenols in rosemary and sage are carnosic acid and rosmarinic acid. Ginger's major polyphenols are gingerols, but it also contains shagoals, paradols and some other compounds (Sajid et al. 2012; Švarc-gajić et al. 2016). Curcumin is the major polyphenol in turmeric, however, it also contains at least another 235 phenolic and non-phenolic compounds (Aggarwal et al. 2013). Whilst for the bay leaf, phenol-explorer.eu does not provide phenolic composition. In general, the polyphenols were better extracted in ethanol than water, as most ethanol extracts had a higher TPC in comparison to aqueous. Several studies reported that mixture of ethanol and water was the most effective method to extract polyphenols from CHS (Wang et al. 2004; Dvorackova et al. 2015). Ethanol can mix with water but it also has a hydrophobic region, in addition, in this study, 42% ethanol was used to extract some hydrophilic polyphenols such as rosmarinic acid, which is one of the most abundant polyphenols in rosemary (Puangsombat & Smith 2010; Kowalczyk et al. 2013). Hence, ethanol is more effective when it comes to extracting both hydrophilic and lipophilic polyphenols from CHS and as a result most ethanol extracts had a higher TPC than aqueous. Filter-sterilisation reduced the TPC to some extent, which can be explained by the fact that some polyphenols or their polymers attached to the filter (Ogunrinola 1996).

When the IC₅₀ values were expressed per dry weight equivalent of the CHS, the potency order of the extracts differed from the order expressed per GAE. This can be explained by the fact that the TPC of the most potent CHS (TE, BLE, BLA, GE) were much lower in comparison to less potent CHS (RE, SE, RA and RE). In addition, this pattern also strongly suggests that specific polyphenols present in CHS are responsible for their growth inhibition rather than TPC.

The results from SBR, MTT and InCuCyte clearly have shown that most CHS extracts and their combinations inhibited the growth and/or reduced cell viability of the three CRC cell lines (HCT116, CCL235 and HCA-7). There were no statistically significant correlations between TPC and IC50 values (SRB data) suggesting that specific polyphenols are more likely to be responsible for anti-proliferative activity of CHS. For example, TE had had one of the lowest TPC among investigated extracts, but it had the lowest IC50 values. Similar results were found by Yi and Wetzstein (2011) who also reported only a weak correlation between TPC and IC50 values. The differences in the IC50 values between MTT and SRB assays could be explained by the different mechanisms upon which these assays are based: MTT utilises NAD(P)H-dependent cellular oxidoreductase enzymes that convert colourless tetra-zolium to the purple-coloured formazan dye, whilst SRB measures cell mass and does not distinguish between dead and live cells (Vichai et al. 2006). In addition, it must also be noted that for the MTT experiments the exposure times were shorter than that for the SRB assay. Nevertheless, the order of potency of CHS extracts and their combinations was similar across all assays/methods. It seems that HCT116 was more sensitive to CHS treatments than CCL235 and especially than HCA-7, which is in line with our previous work (Baker 2012; Jaksevicius 2012) in which HCT116 was also more sensitive than another CRC cells line, HT-29. Differences in sensitivity could be explained by the different mutations in the cell lines (Xavier et al. 2009), for example, HCA-7 is a COX-2 positive cell line and it could be the major factor that the IC50 values for this cell line were higher in comparison to HCT116, which is COX-2 negative cell line (Shao et al. 2000). Moreover, the literature indicates that COX-2 positive tumours are more difficult to treat and are associated with lower survival rate (Ogino et al. 2008) thus suggesting that the expression of COX-2 could be a major factor influencing the potency of the CHS. However, Lev-Ari et al. (2006) found that COX-2 expressing HT-29 cells (these cells have a low level of COX-2 expression) were more sensitive to treatment with curcumin (IC50 – 15 μ M) than the COX-2 negative CRC cell line SW480 (IC50 - 50 μ M) (Lev-Ari et al. 2006). However, these cell lines were different from the CRC cells used in present study, which may explain the opposite findings.

Concerning the effect of the extracts on normal cells, high concentrations of BLE and TE inhibited the growth of healthy cells (human fibroblasts). However, the concentrations are higher in comparison to the ones needed to inhibit the growth of cancer cells, which indicates that BLE and TE are selective towards cancer cells, in this case CRC cells.

Although it is not fully understood through which mechanisms phytochemicals differentiate between health and cancerous cells, it is thought that they target the molecules that are more highly expressed (for example NF- κ B) in cancer cells (Ravindran et al. 2009). In the literature, there are numerous examples of where phytochemicals or food extracts are non-toxic to healthy cells. For example, curcumin at 50-100 μ M had no effect on hepatocytes, whilst it induced apoptosis in liver cancer cells (Syng-ai et al. 2004; Santel et al. 2008). This differential effect is possibly due to the pro-oxidant effect of polyphenols at high doses (50-100 μ M). Healthy liver cells have a capacity to neutralise superoxide generation caused by a high dose of curcumin, whilst cancer cells do not have such a mechanism and thus a high level of superoxide triggers apoptosis (León-González et al. 2015). Cinnamon extract (up to 50 μ g/ml) was not harmful to healthy kidney cells (Elkady & Ramadan 2016). Srivastava and Gupta (2007) found that aqueous and methanol extracts of chamomile inhibited the growth and induced apoptosis of various cancer cell lines including CRC (RKO), but at the same concentrations (with concentrations as high as 4000 μ g/mL for aqueous extract and 400 μ g/ml for methanol extract) were not toxic to human prostate epithelial PZ-HPV-7 cells (Srivastava & Gupta 2007). Hong et al. (2016) reported that mango ginger (*Curcuma mangga* from *Gingiberaceae* family) extracts (hexane and ethyl acetate) was more toxic to HT-29 cells than to human normal colon cell lines (CCD-18Co) with IC₅₀ values ranging from 18-16 μ g/ml for cancer cells and 46-47 μ g/ml for CCD-18Co (Hong et al. 2016). Another study found that [6]-gingerol at the dose twice as high as that of the IC₅₀ for CRC cells (HCT116 (285 μ M) and SW480 (205 μ M)) only reduced viability of normal intestinal epithelial cells (IECs) by 10-15% (Radhakrishnan et al. 2014). [6]-, [8]- and [10]-gingerols up to 100 μ M, which was enough to inhibit the growth of breast cancer cells, were non-toxic to human fibroblast, whilst 500 and 1000 μ M caused ~50% inhibition (da Silva et al. 2012).

The results of the present study showed that most ethanol extracts were more potent on CRC cell growth in comparison to the aqueous extracts which again suggest that active components are better extracted in ethanol rather than water. Srivastava et al. (2007) reported that the IC₅₀ values of chamomile aqueous extract against several cancer cell lines were ~10 times higher in comparison to methanol extracts. MTT assay gave indication that the effect of BLE, TE (two the most potent extracts) and BLTE produced cytotoxic rather than cytostatic effect, as after the removal of treatment (24h treatment), cells did not start growing, and the IC₅₀ values were very similar to whole 72-hour treatment. The cytotoxic action of these extracts (BLE, TE and BLTE) was confirmed with

cytotoxic LDH assay. In the present study TE was the most potent extract as it produced the lowest IC₅₀ values for all treated cell lines.

Regarding the active components of the extracts, it is widely believed that curcumin is the major active component of turmeric and it kills cancer cells in numerous ways (Ravindran et al. 2009). Guo et al. (2014) also reported that curcumin inhibited the growth of HT-29 cells (10 and 25 μM) (Guo et al. 2014). Another study also found that curcumin (10 and 50 μM) inhibited the growth of HCT116, HT-29 and SW620 cells; the latter is also a CRC cell line (Kunnumakkara et al. 2009). However, there is evidence in the literature that some other phytochemicals present in turmeric could also produce anti-proliferative effects against cancer cells (Aggarwal et al. 2013). Kim et al. (2012) found that turmeric was more potent at inhibiting growth of six cancer cell lines (two of them were CRC, HCT116 and HT-29) than the same amount of curcumin (5 μg/ml) present in the extract. This is not surprising as turmeric contains at least 235 other phytochemicals, some of which individually or synergistically can produce anti-carcinogenic effects (Aggarwal et al. 2013). For example, the turmeric isolated novel non-phenolic compound β-sesquiphellandrene (5-50 μM) reduced HCT116 cell viability at the similar level to curcumin (Tyagi et al. 2015). Another study reported that turmerones (lipophilic non-phenolic compounds found in turmeric) inhibited non-CRC cell (liver and breast cancer) growth at the similar rate to curcuminoids (Yue et al. 2010). These examples above suggest that apart from curcumin, turmeric contains other compounds that can contribute to its anti-proliferative activity.

Ginger belongs to the same family (Zingiberaceae) as turmeric (Surh 1999), and the results of this study show that it possesses a strong anti-proliferative activity, although it was not as potent as turmeric. Gingerols and shagols are the most abundant active compounds in ginger (Sang et al. 2009). Sang et al. (2009) found that both shagols and gingerols inhibited the growth of HCT116 cells; however, shagols were more potent than gingerols. Radhakrishnan et al. (2014) reported that [6]-gingerol inhibited that growth of HCT116 and SW480 cells with IC₅₀ values 283 and 205 μM, respectively. Another study also reported that [6]-gingerol and two of its metabolites (150 and 200 μM) inhibited the growth of two CRC cell lines (HCT116 and HT-29), but this study did not test the whole ginger extract (Lv et al. 2012). Furthermore, the concentrations of the individual polyphenols are far higher than could be found in whole ginger extracts.

Rosemary and sage belong to *Lamiaceae* family and in the present study these two herbs also inhibited the growth of CRC cells with exception for sage aqueous extract

(HCA-7 cell line). Several studies reported growth inhibitory activity of these herbs against various cancer cell lines including CRC. González-Vallinas et al. (2013) demonstrated that rosemary extract inhibited the growth of two CRC cell lines - SW620 and DLD-1, with the IC50 values of 36 and 34 $\mu\text{g/ml}$, respectively. Yi and Wetzstein (2011) also found that sage (IC50 - 35.9 $\mu\text{g/ml}$) and rosemary inhibited (IC50 - 72 $\mu\text{g/ml}$) the growth of several cancer cell lines including CRC (SW480). The major active components of these herbs are carnosic acid (CA), carnosol (CL), and rosmarinic acid, but the ratios vary depending on what solvents are used for extract preparations, the growing conditions and harvesting time (Puangsombat & Smith 2010; Generalić et al. 2012) for the herbs. Yesil-Celiktas et al. (2010) found that several rosemary extracts reduced the growth of various cancer cell lines (none were CRC) and the most potent extracts were the ones containing the highest amount of CA, which suggest that CA was the most likely polyphenol responsible for anti-proliferative activity of rosemary extract. Dilas et al. (2012) reported that rosemary extracts, enriched with CA and CL, at IC50s greater than 62.5 $\mu\text{g/ml}$ and rosmarinic acid, at an IC50 of 190 $\mu\text{g/ml}$, alone reduced the growth of HT-29 cells. Barni et al. (2012) showed that CA inhibited the growth of three CRC cell lines (HT-29, Caco and LoVo) with IC50 values ranging from 24 to 96 μM . Rosmarinic acid also inhibited the growth of another CRC cell line HCT116 at concentrations of 5-1000 $\mu\text{g/ml}$ (Encalada et al. (2011). In relation to the present study's IC50 value for rosemary, it was not possible to compare with values in the literature due to the different way in which the values are expressed.

Bay leaf is a less researched herb, nevertheless, the data of this study showed that it was one of the most potent extracts (BLE) at inhibiting CRC cell growth. It was reported that bay leaf methanol extract produced anti-proliferative activity against HT-29 cells (Konczak et al. 2012). Another study found that bay leaf extract (oil and phenolic fraction) inhibited the growth of several CRC cell lines: HT-29, HCT-116, Caco-2, and SW-480 with inhibitory doses ranging from 200 to 1000 $\mu\text{g/ml}$ (Bennett et al. 2013). Whole bay leaf extract and its several fractions reduced viability of brain tumour cells (SK-N- BE(2)-C, SH-SY5Y) with IC50 values ranging from 15 to 153 $\mu\text{g/ml}$ (Pacifico et al. 2013). Regarding the active constituents, it is not clear which constituents are responsible for bay leaf anti-proliferative activity. Some potential phytochemicals (not all phenolic) that may contribute to the anti-proliferative activity of bay leaf are dehydrocostus lacton, limonene, β -sitosterol, eugenol, p-Coumaric acid, ferrulic and eremanthin. However, their presence and amount can vary depending on the solvent used for the purposes of extraction (Pacifico et al. 2013; Vallverdú-Queralt et al. 2014).

In the present study, although the polyphenol content in CHS extracts is lower in comparison to the studies cited above that used individual polyphenols, it is clear that there are enough polyphenols in most of these extracts to inhibit CRC cell growth, which not only suggests that they possess anti-carcinogenic potential but that this potential may be due in part, or whole, to synergistic interactions occurring within their matrices. The enhanced effect of synergistic interactions found in foods could be partially explained by the fact that they target multiple molecular targets and thus lower concentrations are sufficient to produce a desirable effect (Parasramka & Gupta 2012). Continuing with the discussion on possible synergistic effects, some of the combinations of CHS used in the present study showed potential at inhibiting growth of CRC cells, however, the IC₅₀ values for even the most potent combinations were still higher in comparison to the most potent extract –TE. In addition, some combinations produced an antagonistic effect. The data clearly indicate that some phytochemical interactions in the investigated CHS may be beneficial in the context of their effect on CRC cells, whilst some may interfere with each other's action thus reducing overall effect. Similar findings regarding synergistic and antagonistic effects of food extracts were reported by Wang et al. (2013) on breast cancer cells (MCF-7) in which they found that combining onion and grape resulted in a synergistic anti-proliferative activity, but grapes and adzuki bean showed antagonistic interaction (Wang et al. 2013). There are a few other studies that looked into anti-carcinogenic effect at combining several foods. Combining garlic with tomatoes better prevented formation of intestinal precancerous lesions in rats than when these two foods were used individually (Sengupta et al. 2004). Another animal study has shown that combining tomato with broccoli was more effective at slowing the growth of prostate tumour than feeding just a single vegetable (Canene-Adams et al. 2007). Regarding CHS, Yi and Wetzstein (2010) found that some herb combinations were more effective than individual herb extracts, for example, sage and spearmint combination was the most potent (SW480 CRC cells), even though individually spearmint was the least potent extract. There is little in the literature to help explain the effects of the combinations used in the present study, but interactions between constituents likely play a role. Furthermore, it is possible that varying combinations may alter such combinations. In the present study only one ratio (1:1) was used for the combinations and from it the interaction factor (IF) was calculated. Although this factor is a quick method for identifying synergistic, antagonistic and additive effects of the combinations used, to obtain a fuller picture of the nature of these

interactions the effect of different ratios of the same combinations need to be investigated and an isobologram plotted (Gawlik-Dziki 2011; Durak et al. 2015).

Finally, in attempting to ascertain the bioactive compounds responsible for the anti-inflammatory properties reported in the present study, it must be borne in mind that the phenolic composition of these CHS vary depending on solvents used and other factors including the region where the CHS were grown and how they were stored (Puangsombat & Smith 2010; Generalić et al. 2012; Dvorackova et al. 2015; Anandaraj et al. 2014), thus it is possible that for some the main bioactive compound(s) responsible as well as the impact of their effect(s) will vary.

2.4 Conclusion

The results of this study show that most CHS extracts inhibited the growth of several CRC cell lines with TE being the most potent extract. Some CHS combinations produced a synergistic effect, however, the IC₅₀ were lower in comparison to TE alone. The concentrations of CHS extracts needed to inhibit the growth of CRC cells were non-toxic to normal cells. It seems that reduced CRC cell growth and viability by CHS was not directly related to the total phenolic content of the extracts, which suggests that specific polyphenols were most likely responsible of the growth inhibition of CRC cells. Future studies are needed to elucidate the anti-proliferative mechanisms of the most potent CHS extracts, and to identify the most active components in bay leaf. A more in-depth study of the synergistic effect of combining several CHS extracts is needed as a number of the combinations were shown to be effective at inhibiting CRC cell growth.

Chapter 3 Effects of polyphenol CHS on COX-2 expression, and activity

3.1 Introduction

Inflammation is one of the hallmarks in the development of cancer including colorectal (CRC) (Lasry et al. 2016). Cyclooxygenase 2 (COX-2) is a key enzyme involved in the process of inflammation, and as detailed in Chapter 1 is known to play an important role in the development of CRC. In contrast to COX-1, which is constitutive, COX-2 is induced by pro-inflammatory agents, hormones and growth factors (Ricciotti & Fitzgerald 2011). COX-2 is a bifunctional enzyme with cyclo-oxygenase and peroxidase activities, as explained in detailed in Chapter 1. It is well known that overexpression of COX-2 and subsequent increase in PGE2 promote carcinogenesis (Subbaramaiah & Dannenberg 2003; Greenhough et al. 2009). Moreover, it has been found that patient histological samples of CRC tumours have overexpressed COX-2 (Sinicrope FA et al. 2004; Zhang & Sun 2002). Furthermore, when this enzyme is targeted using non-steroidal anti-inflammatory drugs (NSAIDs), the risk of CRC has been shown to be reduced (Thun et al. 2012; Friis et al. 2015; Jacobs et al. 2012), and thus could be used for CRC prevention (Chun & Surh 2004). However, these drugs have adverse side effects, and hence safer alternatives are required (Subbaramaiah & Dannenberg 2003; Saloheimo et al. 2006; Aggarwal & Shishodia 2006; Murakami & Ohigashi 2007).

There are numerous foods and food constituents that have been shown to have anti-inflammatory effects (Aggarwal & Shishodia 2006; Aravindaram & Yang 2010) and culinary herbs and spices (CHS) are among them with many being shown to inhibit a number of inflammatory mediators (Jungbauer & Medjakovic 2012; Peng et al. 2007; Baker et al. 2013) (See Chapter 1, section 1.3.6). Although CHS are consumed in small amounts, these foods possess high levels of phytochemicals especially polyphenols, which have limited bioavailability suggesting that a significant part of their action may be limited to the gut (Manach et al. 2004; Opara & Chohan 2014). However, with the exception of a study by Bennett et al. (2013) who looked at the effects of bay leaf on COX-2 activity in HT-29 cells, there is a paucity of information on the effects of CHS on COX-2 activity and expression in CRC cells in vitro. Most of the current work in this area is on the effect of their polyphenolic constituents especially curcumin (Zhang et al. 1999; Goel et al. 2001) and as detailed in Chapter 1, the effect of the whole CHS may be greater than that of these constituents. Furthermore, the need to know and understand more fully the potential beneficial effects of whole foods rich in phytochemicals has ignited a growing interest in

the bioactivity of foods in combination (Opara & Chohan 2014; see Chapter 1). It has been established in this investigation that the CHS, individually and in combination, are cytotoxic to CRC cells. Furthermore, some of the CHS combinations namely sage and ginger ethanol (SGE), bay leaf and turmeric ethanol (BLTE) and sage and bay leaf ethanol (SBLE)) had a synergistic effect on their growth (see Chapter 2). One of the CRC cell lines used was the COX-2 expressing HT-29 and based on the knowledge that polyphenols are anti-inflammatory two questions were asked: 1) could the effect of the CHS, individually and in combination, on CRC growth be associated with a decrease in COX-2 expression and PGE2 synthesis in this cell line? 2) is the magnitude of the cytotoxic effect of the CHS, individually and in combination, mirrored in their effect on COX-2 expression and PGE2 synthesis? For example, the combinations named above had a synergistic effect on HCA-7 cell growth so does this mean that they also have a synergistic effect on its COX-2 expression and PGE2 synthesis? Thus, the aim of this study was to investigate the effect of the most potent CHS identified in Chapter 2 (individually and in combination) on COX-2 expression and activity in HCA-7 CRC cells within the same time frame used to investigate their effect on cell viability and within which they were shown to be cytotoxic.

3.2 Materials and methods

3.2.1 Preparation of CHS extracts and TPC

This part of the investigation involved determining the effect of the CHS on COX-2 expression and thus western blotting was used so to reduce the amount of solvent and increase the phenolic content of the extracts, more concentrated extracts (with higher phenolic content per volume of solvent) were prepared by increasing the dry herb/spice content: 2 g of herb/spice into 27 ml of solvent (ultra-pure water or 42% ethanol (v/v)), with exception for sage, for which 1g was used, as it was impossible to fit 2 g of this very light-weight herb into the bottle with 27 ml of solvent. The remaining extract preparation was the same as described in Chapter 2. The total phenolic content (TPC) was established as described in Chapter 2. The TPC was used to calculate the dose for the experiments conducted in this study.

3.2.2 The effect of culinary herb and spice extracts on COX-2 expression in HCA-7 CRC cells

Based on the growth inhibition studies, described in Chapter 2, the most potent extracts and combinations were: rosemary ethanol (RE), sage ethanol (SE), bay leaf ethanol (BLE), ginger ethanol (GE), turmeric ethanol (TE), rosemary and turmeric ethanol (RTE), BLSE, SGE and BLTE were chosen to study their effect on COX-2 expression in

HCA-7 CRC cells. In addition, dose response experiments on COX-2 expression were also performed for the most potent CHS (TE, GE, BLE and BLA). HCA-7 cells were seeded into 6-well plates (Nunclon delta, Fisher, UK) with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK, D5796 500 ml) in 10% foetal bovine serum (FBS) (Sigma-Aldrich, UK F7524) and incubated at 37 °C and 5% CO₂. After 48 hours, when the cells were almost 80% confluent, the CHS extracts were added and left for another 24 hours. The concentrations of the CHS used were based on their highest tolerated concentrations. Controls were also set up and these were: 'no treatment' (HCA-7 cells in cell culture media only); ethanol control (HCA-7 cells exposed to the equivalent volume of ethanol contained by extracts, i.e. 0.2% v/v); and a positive control - HCA-7 cells exposed to a selective COX-2 inhibitor, Celecoxib (Sigma-Aldrich,UK) (50 µM), (Greenhough et al. 2009; Lev-Ari 2005) and a non-selective COX-2 inhibitor – salicylic acid (Aspirin) (Sigma-Aldrich, UK) (1 mM) These drugs were used at the highest concentrations that could be tolerated by the cells without killing them. A positive control was used to gain some idea of the therapeutic potential of the CHS as Celecoxib has been shown to reduce adenomas in humans (Arber et al. 2006). Celecoxib were made up in DMSO. Celecoxib was used as the positive control for the RE, SE, BLE, GE, TE, RTE, BLSE, SGE and BLTE experiments and salicylic acid was used as the positive control for the dose-response experiments. After incubation with the CHS or the control cells were lysed using LDS NUPAGE lysis buffer (4x) (Fisher, UK 10718414). Prior using lysis buffer was diluted with deionized water, added protease inhibitors (104 mM AEBSF, 80 µM aprotien, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A and 1.4 mM E-64 (Sigma-Aldrich, UK)) (10 µl for 1 ml of diluted lysis buffer), then lysis buffer was heated for 10 min at 75°C and put onto the cells for lysing. Lysates were collected with 1 ml pipette, transferred into Eppendorf tube, placed on ice, and syringe was used to reduce viscosity, and then stored at -80°C freezer. Western blotting was performed using equal amounts of sample (lysed cells) based on protein content, which was 30 µg. Prior loading onto the 4–12% Bis-Tris gel (Invitrogen, UK), samples were mixed with reducing agent (Invitrogen UK) (10 µl + 90 µl of the lysate), left for at least 10 min on ice, and then heated for 10 min at 75°C. Following electrophoresis, the separated proteins were transferred on to Immobilon® PVDF membranes (IPFL 00010; Merck Millipore, UK). Thereafter, the membrane was placed in blocking solution for at least one hour and then primary antibodies were applied: COX-2(D5H5) XP® Rabbit mAb #12282 (Cell Signalling), (dilution 1:1000) and β-actin

(1:1000; Cell Signalling) which was used as an internal control to show that equal amounts of protein were loaded. After incubating with the primary antibody, the membranes were washed with wash solution (3 times each for 5 minutes) and incubated with IRDye 689 Rd, donkey anti-Rabbit secondary antibody (Licor, UK). The signal was detected and quantified using Licor Image studio (Licor, UK).

3.2.3 The effect of culinary herb and spice extracts on COX-2 activity in HCA-7 CRC cells

The same CHS used in the COX-2 expression experiments were also used to investigate their effect on COX-2 activity in HCA-7 cells which was determined by measuring their release of PGE₂. From the western blot experiments cell culture media was collected and stored at -20°C. Prior to carrying out the PGE₂ assay, samples were defrosted, centrifuged at 1000 rpm for 4 min and then assayed using a PGE₂ ELISA kit according to the manufacturer's instructions (RND Systems, UK KGE004B). To further investigate the effect of the CHS on HCA-7 COX-2 activity the effect of the two most potent COX-2 CHS inhibitors, BE and TE, on this cell line's COX-2 enzyme activity and PGE₂ synthesis was investigated using a COX-2 Inhibitor Screening Assay Kit (CAY560131-96; Cayman).

3.2.4 Data expression and statistical analysis

All experiments were done in triplicate (n=3), which represents three separate experiments and data are expressed as mean and standard deviation (\pm SD) unless otherwise stated. Western blot band intensity was analysed using Odyssey Image Studio software (Licor, UK), the data were normalised against β -actin and reduction in band intensity was expressed as a percentage in comparison with the intensity of the 'no treatment' band (HCA-7 cells in cell culture media only) which represented 100% expression.

COX-2 activity was determined based on PGE₂ release data, which are expressed as % reduction) in comparison to the control (HCA-7 cell in cell culture media only), which represented 100% activity. One-way ANOVA with Tukey's post-hoc test was performed to assess whether the differences in effect of the extracts were statistically significant. Pearson's correlation coefficient (r) (2-tailed) was used to determine correlations between COX-2 expression and PGE₂ production. For all statistical tests SPSS software was used and p<0.05 was considered statistically significant.

3.3 Results

3.3.1 Total phenolic content of CHS

The total phenolic content (TPC) was established for each extract. The ranking order of the extracts was in the following order - (NF): SE > RA > RE > BLE > S > TE > GE > BLA, and F reduced the TPC: Sage E > RA > RE > BLE > SA > BLA > TE > GE (Table 1.).

Table 3.1 Total phenolic content of CHS extracts: non-filtered (NF) vs filter-sterilised (F)

CHS	NF (GAE mg/g of DW) SD (\pm)	F (GAE mg/g of DW) SD (\pm)
SE	94.7 (\pm 1.3)*	85.2 (\pm 0.6)
RE	46.7 (\pm 0.7)	44.3 (\pm 1.7)
RA	49.4 (\pm 0.3)	47.1 (\pm 1.2)
BLE	42.4 (\pm 0.3)*	37.8 (\pm 0.3)
SA	37.0 (\pm 0.5)*	31.8 (\pm 0.5)
TE	16.5 (\pm 0.2)	14.8 (\pm 0.2)
GE	15.9 (\pm 1.2)*	14.2 (\pm 1.0)
BLA	15.3 (\pm 0.2)	14.8 (\pm 0.4)

Total phenolic content was determined using total phenolic content assay (TPC), and data presented as gallic acid equivalent (GAE) per 1g of dry weight (DW) of the herb/spice. Data expressed as mean (n=3), and \pm SD. *Statistically significant difference between non-filter-sterilised and filter-sterilised extracts (P<0.05). Rosemary ethanol (RE), rosemary aqueous (RA), sage ethanol (SE), sage aqueous (SA), turmeric ethanol (TE), ginger ethanol (GE), bay leaf ethanol (BLE), bay leaf aqueous (BLA).

3.3.2 The effect of culinary herb and spice extracts on COX-2 expression in HCA-7 CRC cells

Based on growth inhibition data (see Chapter 2) dose-response experiments were performed using the most potent CHS (TE, BLE, BLA and GE). The results showed that the highest doses (15 μ g/ml GAE for BLE, BLA and GE; 10 μ g GAE/ml for TE) were the most effective at down-regulating COX-2 expression (15-40% reduction) in HCA-7 cell and the effect was similar or better than non-selective COX-2 inhibitor – salicylic acid (Aspirin) (1 mM), (see Figures 3.1; 3.2; 3.3; and 3.4). The quality of BLE blots was not the best, hence the effect was not as good as for the combination experiments when only the highest dose was used (Figure 3.5).

Additional experiments on COX-2 expression were also conducted using the highest doses of individual CHS and their combination. Tested extracts, RE, SE, BLE, GE and TE reduced COX-2 expression in HCA-7 cells (Figure 3.5). The effect was slightly better than for the dose-response experiment. BLE and TE extracts reduced COX-2 expression by 59 % and 57% respectively (Figure 3.5). All four tested combinations: rosemary and turmeric ethanol (RTE), bay leaf and turmeric ethanol (BLTE), sage and ginger ethanol (SGE) and

sage and bay leaf ethanol (SBLE) reduced COX-2 expression by 53%, 60%, 58% and 62% respectively. The effects of the most potent of the CHS extracts and combinations were slightly less, but of the same magnitude as that of the selective COX-2 inhibitor Celecoxib (50 μ M), which reduced COX-2 expression by 70% (Figure 3.5).

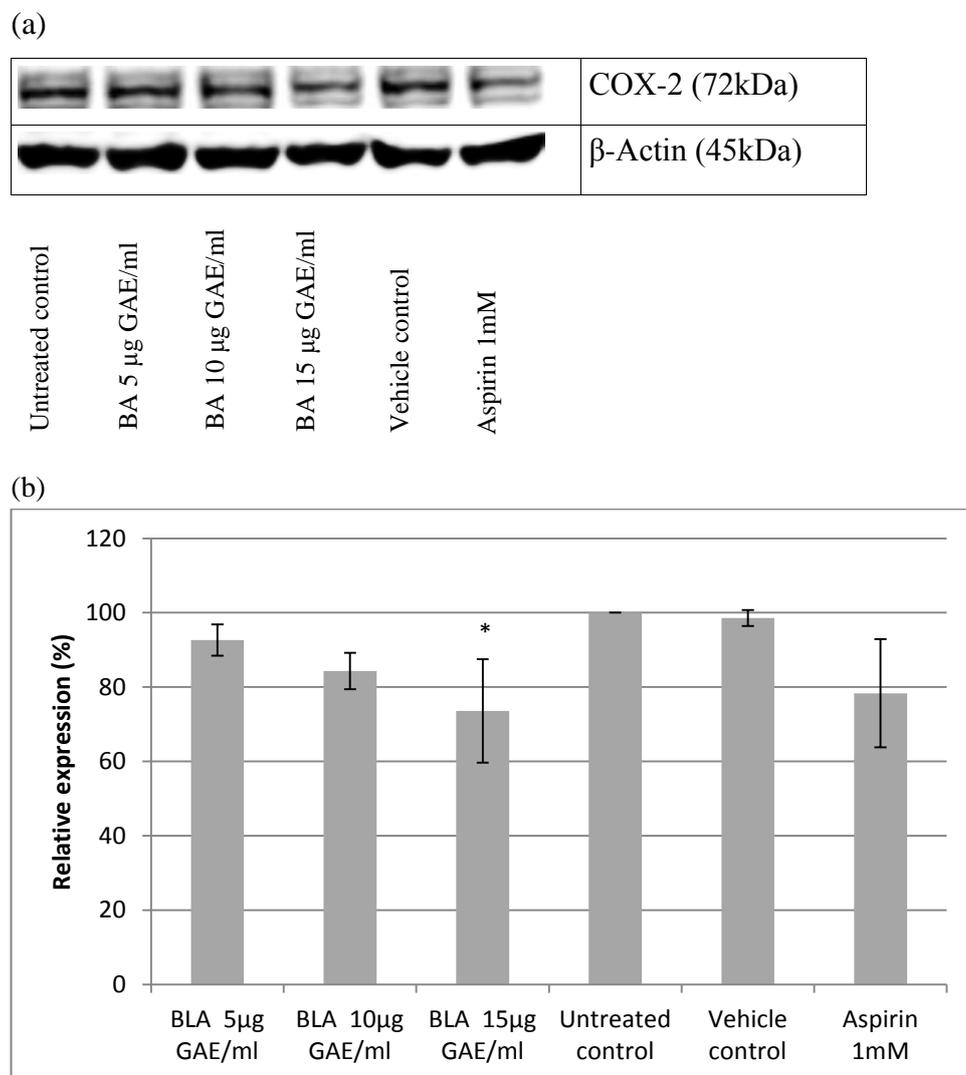
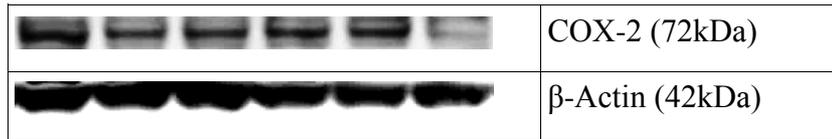


Figure 3.1 Dose response effect of BLA (bay leaf in water) extract on COX-2 expression in HCA-7 cells.

(a) Western blot; Cells were treated with bay leaf aqueous (BLA) extract for 24h with concentrations ranging from 5 to 15 μ g GAE/ml. Untreated control contained just DMEM with 10% FBS, vehicle control – water (1.3% v/v), the highest amount found in the extracts. (b) Quantitative analysis of COX-2 bands. Data are expressed in comparison to control (100%) after the signal was normalized against β -actin, mean (n=3), \pm SD. *Statistically significant difference from control ($p < 0.05$).

(a)



Control/No treatment
TE 10 µg GAE/ml
TE 7.5 µg GAE/ml
TE 5 µg GAE/ml
TE E 2.5 µg
Aspirin 1mM

(b)

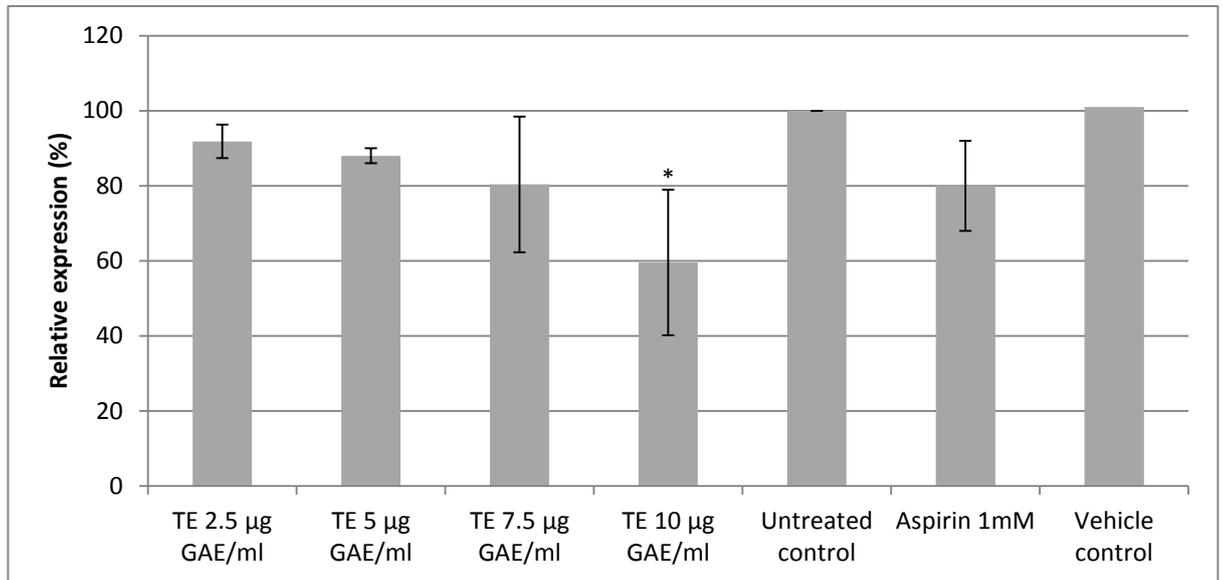
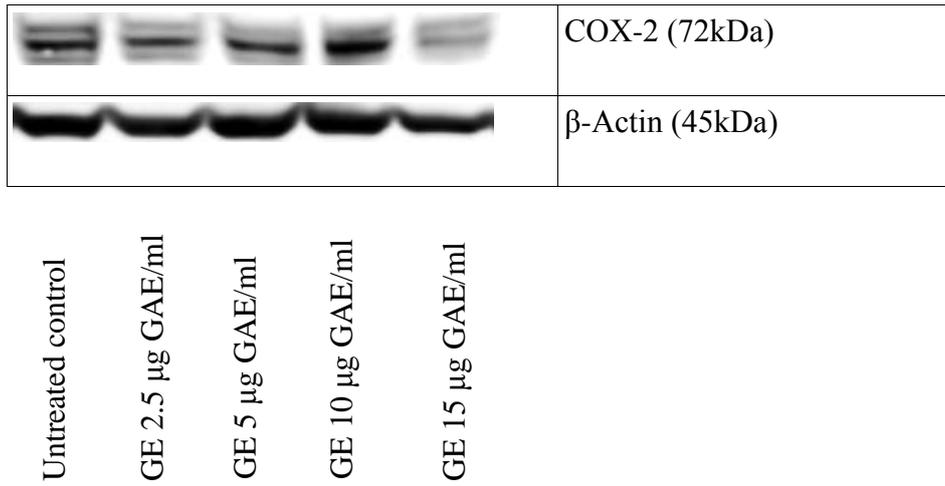


Figure 3.2 Dose response effect of TE extract on COX-2 expression in HCA-7 cells.

(a) Western blot; Cells were treated with turmeric ethanol (TE) extract for 24h with concentrations ranging from 5 to 10 µg GAE/ml. Untreated control contained just DMEM with 10% FBS, vehicle control – water (1.3% v/v), the highest amount found in the extracts. (b) Quantitative analysis of COX-2 bands; Data are expressed in comparison to control (100%) after the signal was normalized against β-actin, mean (n=3), ±SD. *Statistically significant difference from control (p<0.05).

(a)



(b)

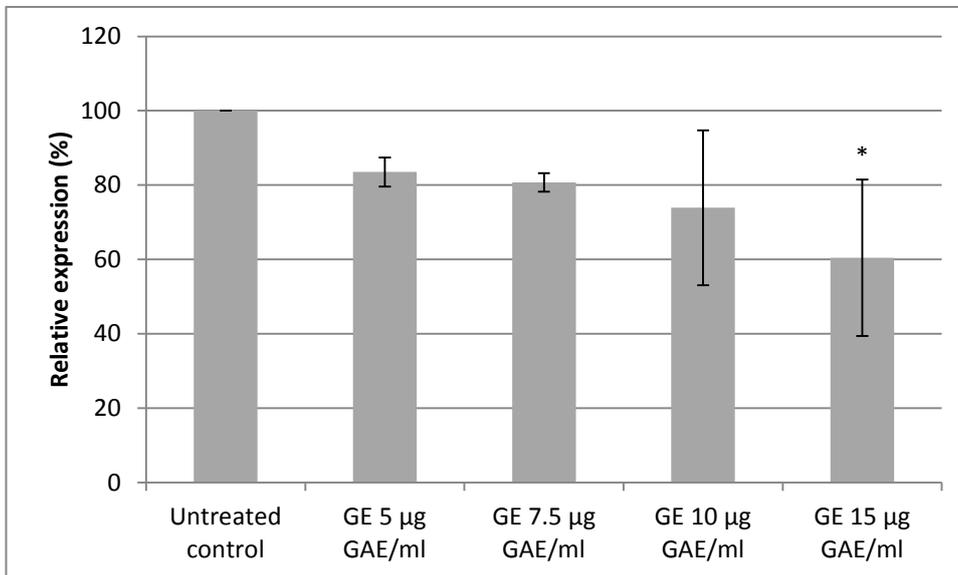
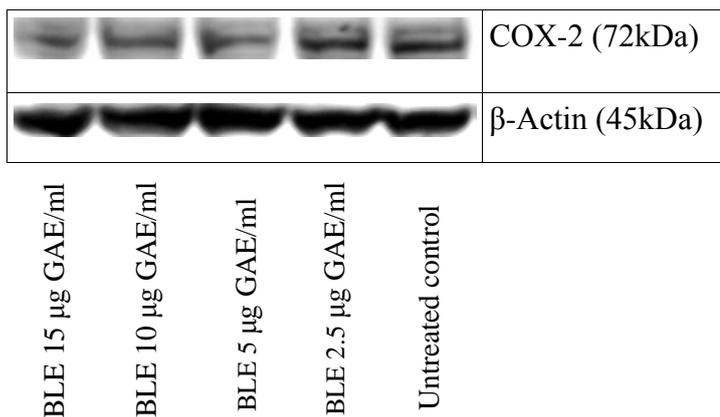


Figure 3.3 Dose response effect of GE extract on COX-2 expression in HCA-7 cells.

(a) Western blot; Cells were treated with ginger ethanol (GE) extract for 24h with concentrations ranging from 5 to 15 μ g GAE/ml. Untreated control contained just DMEM with 10% FBS, vehicle control – water (1.3% v/v), the highest amount found in the extracts. Data are expressed in comparison to control (100%) after the signal was normalized against β -actin, mean (n=3), \pm SD; (b) Quantitative analysis of COX-2 bands.

*Statistically significant difference from control ($p < 0.05$).

(a)



(b)

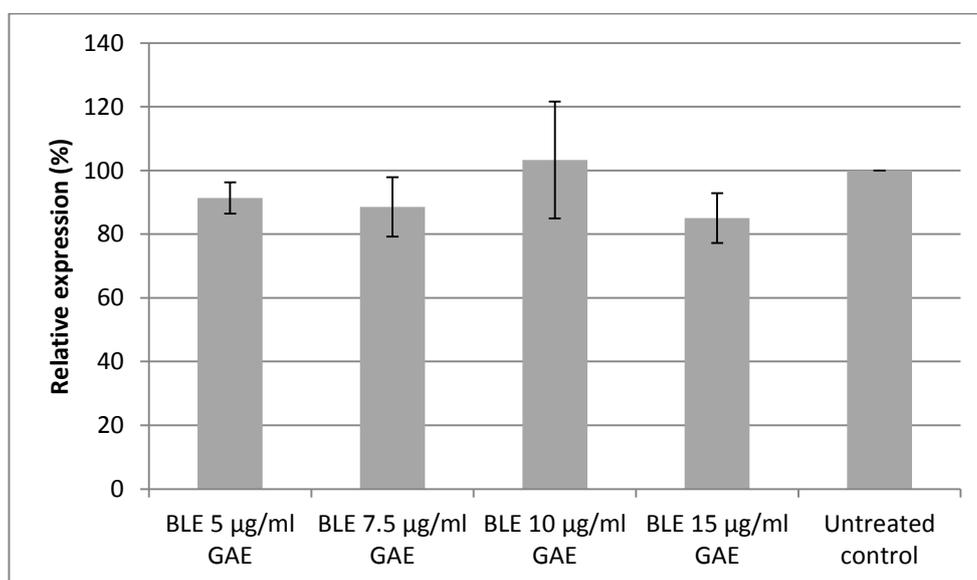


Figure 3.4 Dose response effect of BLE extract on COX-2 expression in HCA-7 cells.

(a) Western blot; Cells were treated with bay leaf ethanol (BLE) extract for 24h with concentrations ranging from 5 to 15 µg GAE/ml. Untreated control contained just DMEM with 10% FBS, vehicle control – water (1.3% v/v), the highest amount found in the extracts. (b): Quantitative analysis of COX-2 bands. Data are expressed in comparison to control (100%) after the signal was normalized against β-actin, mean (n=3), ±SD. *Statistically significant difference from control (p<0.05).

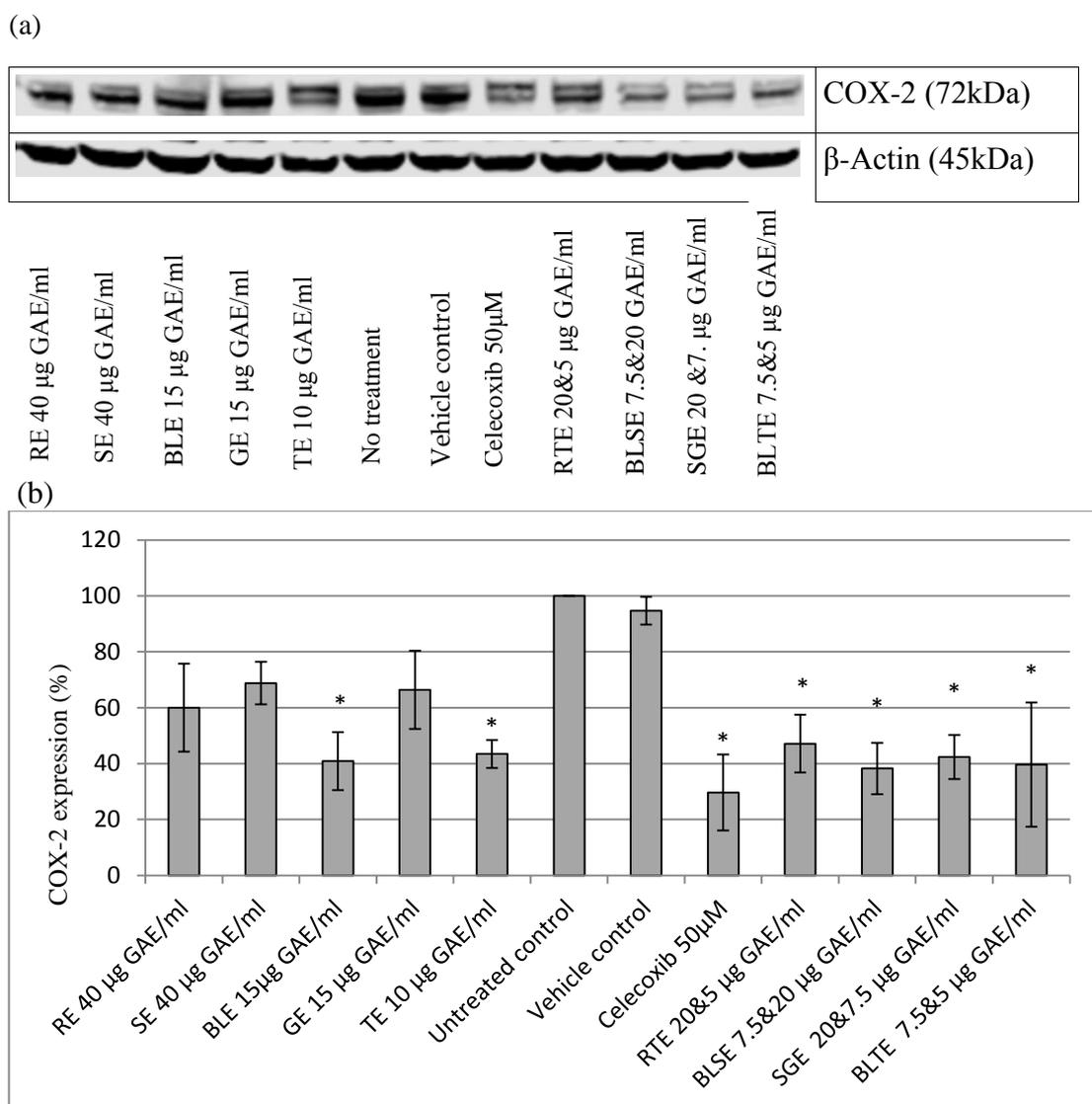


Figure 3.5 Effect of culinary herb and spice extracts on COX-2 expression.

(a) Western blot; HCA-7 cells were treated with following CHS and their combinations: rosemary ethanol (RE); sage ethanol (SE); bay leaf ethanol (BLE), ginger ethanol (GE) and turmeric ethanol (TE) and their combinations (rosemary and turmeric ethanol (RTE), bay leaf and sage ethanol (BLSE), sage and ginger ethanol (SGE). Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v), the highest amount found in the extracts. (b) Quantitative analysis of COX-2 bands. Data are expressed in comparison to control (100%) after the signal was normalized against β -actin, mean (n=3), \pm SD.. *Statistically significant different from control ($p < 0.05$).

3.3.3 The effect of culinary herb and spice extracts on COX-2 activity in HCA-7 CRC cells

The CHS extracts TE, BLE and GE almost completely inhibited PGE₂ production and for the combinations, BLTE and RTE had the strongest inhibitory effect (Figure 3.6). The pattern of inhibition of PGE₂ release by these CHS was similar to that of COX-2 expression: the most potent CHS and their combinations significantly reduced PGE₂ production: TE (92%), BLE (91%), GE (88%), BLTE (92%), RTE (91%), SGE (87%) and BLSE (80%). In addition, as with PGE₂ release, the effects of the most potent extracts

were slightly less than that of Celecoxib (50 μ M), which produced a 97% reduction (Figure 3.6). Furthermore, most extracts produced a stronger reduction in PGE2 than COX-2 expression. There was a strong ($r=0.78$) statistically significant correlation between PGE2 production and COX-2 expression.

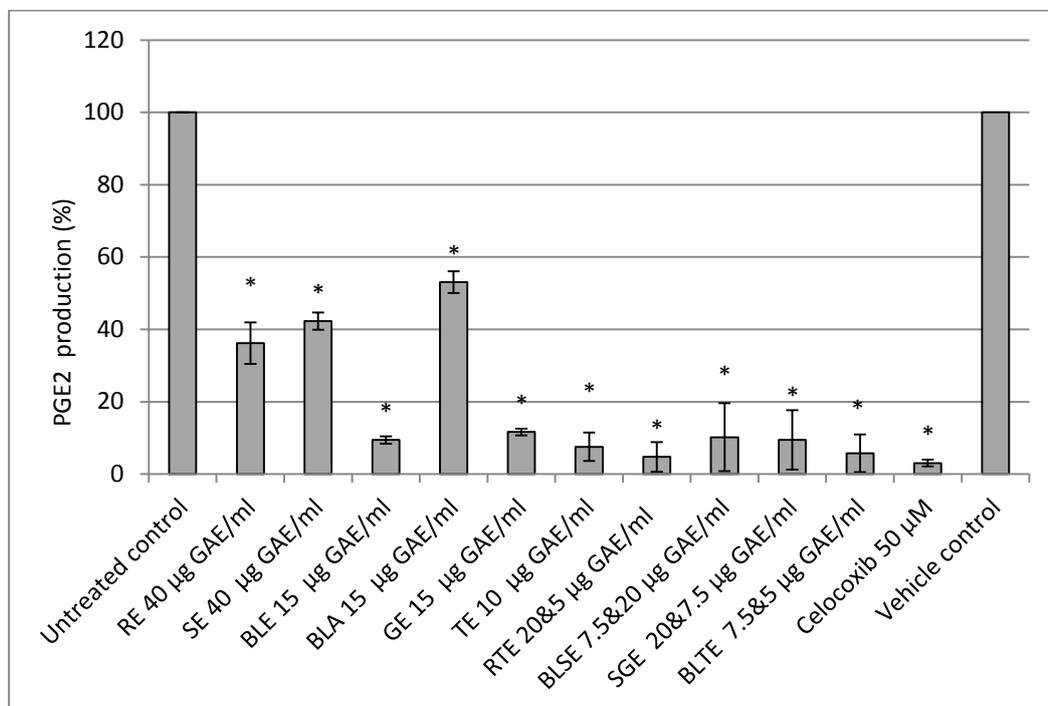


Figure 3.6 Effect of CHS (RE, SE, BLE, GE and TE) and their combinations (RTE, BLSE, SGE and BLTE) on PGE2 release from HCA-7 cells.

HCA-7 cells were treated with the following CHS and their combinations: rosemary ethanol (RE), sage ethanol (SE), bay leaf ethanol (BLE), ginger ethanol (GE), turmeric ethanol (TE), and rosemary and turmeric ethanol (RTE), bay leaf and sage ethanol (BLSE), sage and ginger ethanol (SGE), bay leaf and turmeric ethanol (BLTE). Untreated control contained just DMEM with 10% FBS, vehicle control (ethanol) – 0.4% (v/v), the highest amount found in the extracts. *Statistically significant difference from control ($p<0.05$), $n=3$, \pm SD.

To confirm that BLE and TE directly targets COX-2 activity, rather than just purely reducing its expression, and that the effect observed in vitro were not due to the inhibition of HCA-7 cell growth by the CHS, an in vitro COX-2 inhibition screening assay was performed. The assay revealed that BLE and TE reduced PGE2 production by 53% and 25% respectively (Figure 3.7).

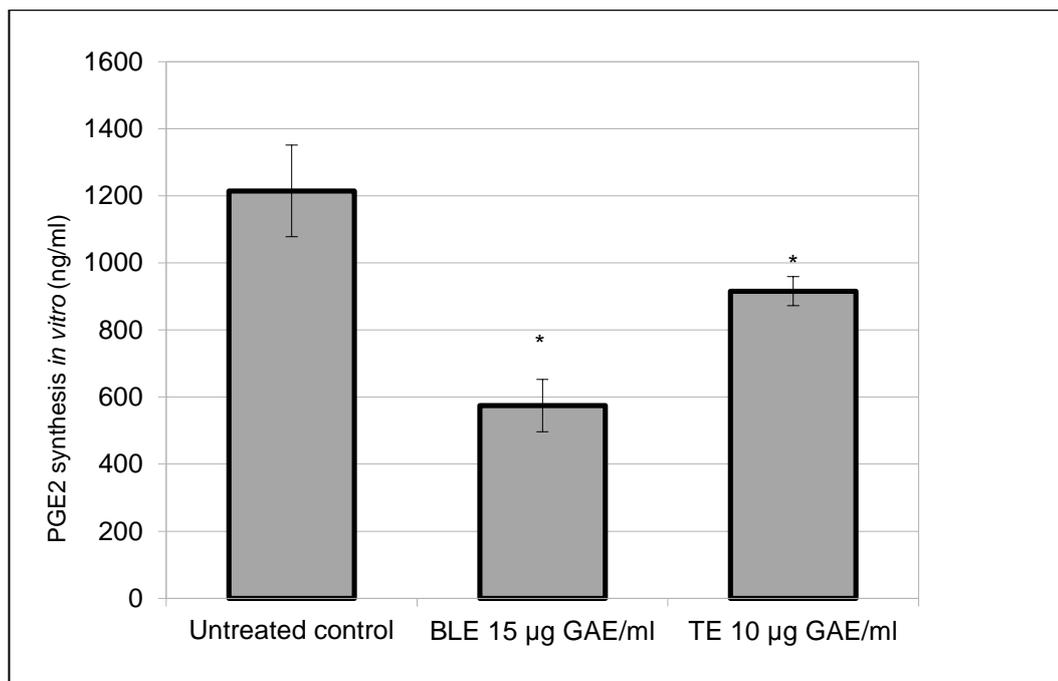


Figure 3.7 7 BLE and TE effect on COX-2 activity and PGE2 production.

COX-2 enzyme activity and PGE2 synthesis was investigated using *in vitro* enzyme reaction: COX-2 enzyme was pipetted into an Eppendorf tube, and then haeme, arachidonic acid (to initiate the reaction) and TE (turmeric ethanol) or BLE (bay leaf ethanol) extracts or vehicle control (ethanol) were added. *Statistically significant difference from control ($p < 0.05$), data expressed as mean ($n=3$), and \pm SD.

3.4 Discussion

The main aim of this study was to investigate the effect of a the potent CHS (individually and in combination) on COX-2 expression and activity in HCA-7 CRC cells, which expresses COX-2 in high levels, *in vitro*, within the same time frame used to investigate their effect on cell viability and within which they were shown to be cytotoxic. The results show that the CHS (individually and in combination) inhibited COX-2 expression and activity, and PGE2 synthesis and that for TE, BLE, GE, RE, SE, RTE, SGE, BLTE and BLSE. Furthermore, the dose response experiments demonstrated the reduction of COX-2 expression was dose dependent. These effects happened within the same timeframe that they were shown to cytotoxic. The individual CHS that proved to be the most potent in inhibiting COX-2 expression and activity (PGE2 release and synthesis) were TE and BLE. TE extract was also the most potent extract in the growth inhibition, cell viability and cytotoxicity studies, which suggests a possible link between downregulation of COX-2 expression and growth inhibition reported in Chapter 2. Such an association is supported by the work of Levi-Ari et al., who found that the growth inhibitory IC50 values of curcumin were lower for a COX-2 positive cell line (HT-29; 15 μ M) than SW480 (40 μ M), which does not expresses COX-2 (Lev-Ari et al. 2006),

although their work is focused on a major constituent of turmeric – curcumin. One of the striking observations of this part of the present investigation was the effect of a number of the CHS (individual and in combination) on COX-2 expression and activity (specifically PGE2 release) in comparison to that of the COX-2 specific inhibitor Celecoxib (50 μ M), which is an established treatment for a number of conditions caused by chronic inflammation (Chen et al. 2014). The effect of these CHS was comparable to that of Celecoxib supporting their not inconsiderable potency which is likely due primarily to their polyphenol content. This is a key point to note in light of literature suggesting that in comparison to anti-inflammatory drugs including Celecoxib, food polyphenols are of limited biological relevance regarding their effect on COX-2 activity (Willenberg et al. 2015). Moreover, there is evidence in the literature, that conversely to anti-inflammatory drugs, CHS selectively targets COX-2, and have much lower inhibitory effect on its isoform COX-1, which is expressed in most healthy tissues, thus avoiding side effects. Indeed, Yi and Wetzstein demonstrated that several CHS including rosemary and sage inhibited COX-2 activity, and the selectivity for COX-2 vs COX-1 was the highest at low concentrations (1mg of dw per ml) (Yi & Wetzstein 2010).

Although Willenberg et al (2015) investigated the effects of food polyphenols that are not major constituents of the CHS used in the present study, their focus on the individual constituents rather than their food sources may explain the lack of potency. The work of the present study reinforces the need to consider these constituents within their food matrices, in which interactions may likely influence the biological potency of the whole food.

Regarding the effect of the most potent of the CHS, research has clearly established the anti-inflammatory effects of turmeric, although not in CRC cells, primarily because of the action of its major bioactive polyphenolic constituent curcumin (Goel et al. 2001). Zhang et al. (1999) demonstrated that curcumin (10–20 μ M) blocked the induction of COX-2 expression by bile and the phorbol ester - phorbol-12-myristate-13-acetate (PMA) in HCA-7 cells and other gastrointestinal cancer cell lines. In addition, Goel et al. (2001) found that curcumin (5-75 μ M) reduced COX-2 expression in HT-29 cells (CRC). It is unclear how curcumin acts to inhibit COX-2 activity. One possible way in which this polyphenolic constituent present in turmeric affects COX-2 expression is by targeting the transcription factor NF- κ B, which is involved in regulating COX-2 expression (Surh et al. 2001; Romier et al. 2008). There are additional routes via which curcumin inhibits COX-2

and thus PGE2 synthesis in HT-29 cells. It has been shown to target AP-1, which is a downstream transcription factor that regulates COX-2 expression (Zhang et al. 1999) and it has been reported that curcumin (10 μ M) inhibited the release of arachidonic acid, which is a substrate of COX-2, in HT-29 cells (Hong et al. 2004). Furthermore, the bifunctional property of COX-2 may also be a target as curcumin inhibits both cyclo-oxygenase, and peroxidase activities (Zhang et al. 1999). This additional route via which curcumin, and therefore turmeric, can decrease the level of PGE2 means that this CHS could potentially be more advantageous in chemoprevention than non-steroidal anti-inflammatory drugs that only target cyclo-oxygenase, but have no effect on peroxidase (Zhang et al. 1999).

Curcumin (~1 μ M) has also been shown to decrease PGE2 synthesis by inhibiting microsomal PGE2 synthase-1 activity, which is functionally linked to COX-2 and is induced by pro-inflammatory stimuli, and often overexpressed in various cancers (Koeberle et al. 2009; Ricciotti & Fitzgerald 2011). PGE2 synthase-1 is required to convert PGH2 into PGE2 (Koeberle et al. 2009). The same study (Koeberle et al. 2009) also tested other polyphenols that are structurally similar to curcumin and also present in CHS, namely rosmarinic acid (up to 10 μ M), which is found in sage and rosemary, and [6]-gingerol (up to 10 μ M), which is present in ginger. However, neither showed inhibitory activity against microsomal PGE2 synthase-1, and was suggested by the authors of the study that a very specific structure of a polyphenol was needed to target this enzyme. Interestingly, other studies showing that curcumin reduced COX-2 activity required a higher dose in comparison to the dose needed to inhibit PGE2 synthase-1 activity (1 μ M vs 5-16 μ M) (Zhang et al. 1999; Gafner et al. 2004).

Curcumin, however, may not be the only bioactive compound in turmeric that is responsible for the effects the authors observed in the present study as other constituents (turmerones, elemes, furanodiene, cyclocurcumin, bisacurone, germacrone) of this spice have recently been identified as possessing anti-inflammatory activity and targeting various pro-inflammatory molecules including COX-2, PGE2 and NF- κ B (Aggarwal et al. 2013), so the effect of turmeric on COX-2 and PGE2 may also be due to the combined effect of a number of its phytochemical constituents .

Regarding ginger and bay leaf, it would not be unreasonable to assume that their polyphenolic constituents also contributed to their COX-2/PGE2 inhibitory action. In the present study, bay leaf, specifically BLE, proved to be almost as potent as that of TE. Bay leaf is a less studied herb, a small number of studies have reported its ability to decrease

COX-2 expression (in macrophages) (Mueller et al. 2010; Guo et al. 2014) and also to moderately inhibit COX-2 activity (Bennett et al. 2013), however in the latter study processed bay leaf (cooked and enzymatically treated) was used and the inhibition of cellular COX-2 expression and activity was not investigated. In the present study, BLE was far more effective at reducing PGE2 release than BLA, which suggest that constituents targeting COX-2 activity are better extracted in ethanol than water. Willenberg et al. (2015) found that naringenin and apigenin, which are present in this herb reduced COX-2 expression and activity in HCA-7 cells (Willenberg et al. 2015). However, these polyphenols are only present in bay leaf in trace amounts (Haghighi et al. 2016), and hence are unlikely to be the main polyphenols responsible for the significant reduction in COX-2 expression by BLE. Other potential constituents (not all phenolic) that may contribute to the anti-inflammatory activity of bay leaf are dehydrocostus lacton, limonene, β -sitosterol, eugenol, p-Coumaric acid, ferrulic and eremanthin. However, their presence and amount can vary depending on the solvent used for the purposes of extraction (Pacifico et al. 2011; Vallverdú-Queralt et al. 2014). Ginger (GE) also reduced COX-2 expression in the present study, and its main active phenolic constituents of ginger - gingerols, shogaol, and paradols have been shown to possess anti-inflammatory properties (Shukla & Singh 2007; Dugasani et al. 2010; Mashhadi et al. 2013). Ginger extract has been shown to reduce COX-2 gene expression in another COX-2 expressing CRC cell line – HT-29 (Dufour et al. 2014). Moreover, one clinical trial demonstrated that ginger (2 g/d) reduced PGE2 in subjects at normal risk of developing CRC (Zick et al. 2011). The results of the present study also showed that its effect on activity (based on PGE2 release) was greater than on expression. van Breemen et al (2011) who showed that ginger constituents, gingerols, and shagols (at 32 μ M, 17.5 μ M and 7.5 μ M) inhibited COX-2 activity by selectively binding to this enzyme with high affinity, so the marked effect on COX-2 activity in the present study could be due to the presence of these polyphenols. The greater effect on activity (based on PGE2 release) compared to expression was not limited to ginger. A number of other CHS (individually and in combination) had a similar effect suggesting that they too contain polyphenolic constituents that may have a high affinity for COX-2 and thus be potent inhibitors.

The effect of combinations of the CHS on COX-2 expression and activity suggests that some additive, and possibly synergistic, effects came into play as some of the combinations, specifically SGE, BLTE and BLSE produced slightly stronger effects than

those of their constituent individual CHS extracts. Interestingly, these three combinations also had a synergistic effect on growth inhibition for the same cell line and as stated above suggest that interactions within this combination of food matrices influences their biological activity. Indeed, there is evidence in the literature that combining several foods can result in synergistic effects suggesting that some combinations are more beneficial than the constituent single food (de Kok et al. 2008). However, synergistic interactions of plant phytochemicals are complex, and the literature also reports that food combinations not always produce a stronger effect than individual foods (van Breda et al. 2005), although this was not the case in the present study, thus highlighting the complexity of the ‘within matrix’ interactions. It appears that some polyphenols might interfere with each other’s activity, hence their effect becomes antagonistic. For example, antagonistic anti-inflammatory effect of compounds from ginger and coffee disappeared when one particular compound found in coffee was removed by the process of digestion (Durak et al. 2015). It is clear that as with the growth inhibition results discussed in Chapter 2, the effect of such combinations requires further investigation as the mechanisms of these synergistic effects of CHS remain unknown.

3.5 Conclusion

As stated above, the results of the present study clearly show that a selection of CHS (individually and in combination) inhibits the growth of the HCA-7 cell line and its COX-2 expression and activity within the same time frame as their effect on cell viability and cytotoxic action, and at levels similar to those achieved by Celecoxib, which is a strong selective COX-2 inhibitor, highlighting the greater therapeutic potential of these foods over their respective polyphenolic constituents. These results suggest an association as it is well established that COX-2 and PGE2 play an important role in the development of CRC. However, how these two actions of the CHS are linked is unclear, plus their inhibitory effect on growth also occurs in CRC cells that do not express COX-2 namely the HCT116 cell line (Chapter 2) indicating that the CHS also target mechanisms that are not COX-2 dependent (Issa et al. 2006).

Chapter 4 The effect of culinary herbs and spices on Wnt/ β -catenin signalling in CRC cells

4.1 Introduction

The Wnt signalling pathway was first discovered as a regulator of tissue morphogenesis and regeneration however it was later revealed that dysregulations and mutations in this pathway lead to the development of various cancers including CRC (Giles et al. 2003; Valkenburg et al. 2011; Muzny et al. 2012; Basu et al. 2016). There are two other Wnt signalling pathways, but in this chapter the focus will be on the canonical pathway, which is also known as Wnt/ β -catenin pathway, and in particular, on its key molecule β -catenin (Clevers 2006; S. Basu et al. 2016). Mutations in the Wnt/ β -catenin signalling pathway are very common in CRC (Munzy et al. 2012). Hence, it is a potential molecular target for the prevention and treatment of this disease (Teiten et al. 2012; Sawa et al. 2015; Novellademunt et al. 2015).

As stated in Chapter 1 under normal circumstances β -catenin is phosphorylated and subsequently degraded in the cytosol. If the phosphorylation process is disrupted, which is common in most CRC cell lines, unphosphorylated (active) β -catenin accumulates in the cytosol and then enters the nucleus where it binds to T-cell factor (Tcf), forming the β -catenin/TCF/lymphoid enhancer-binding factor (LEF) complex which together with coactivators triggers transcription cyclin D1 and c-Myc, which are cancer-promoting genes (Novellademunt et al. 2015).

There is some evidence in the literature that certain polyphenol-rich foods and their constituents are able to target Wnt signalling and β -catenin phosphorylation and degradation (Tarapore et al. 2013; Afrin et al. 2016). A study with human subjects revealed that consumption of a large amount of red seedless grapes (0.15-0.45kg), which are rich in polyphenols, reduced Wnt signalling in colon mucosal cells (Holcombe et al. 2015). In addition, consumption of black raspberries for two weeks reduced β -catenin expression in adenomas in CRC patients (Wang et al. 2011). Animal studies also indicate that such foods rich in polyphenols are able to modulate the Wnt signalling pathway: feeding mice with white currants, which are rich in polyphenols, reduced nuclear β -catenin level and a number of adenomas (Rajakangas et al. 2008). Another study with mice also showed that consuming cloudberries caused reduced nuclear β -catenin level and also reduced one its target gene - cyclin D1 expression in adenomas of the animals (Mutanen et al. 2008). Lupeol, a dietary triterpene found in some fruits, modulated Wnt signalling in melanoma

cells, by increasing β -catenin in the cytosol and reducing it in the nucleus, which suggests that this compound blocked β -catenin translocation into the nucleus (Tarapore et al. 2010). Some individual polyphenols may also target the Wnt/ β -catenin signalling pathways. For example, (-)-epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea, increased β -catenin phosphorylation (Sangtaek, Jungsug Gwak, Seoyoung Park 2011). Paul et al. (2010) found that pterostilbene (present in blueberries) reduced β -catenin level (in the whole cell lysates) and its translocation into the nucleus in HT-29 cells thus suppressing cyclin D1 and c-Myc gene transcription and cell proliferation (Paul et al. 2010). Treating prostate cancer cells with genistein, a polyphenol found in soy, resulted in increased β -catenin phosphorylation and degradation (Li et al. 2010). Furthermore, treating HT-29 cells (CRC) with fisetin, a polyphenol found in various fruits and vegetables, downregulated β -catenin and Tcf and cyclin D1 expression (Suh et al. 2009). Other dietary polyphenols have been reported to affect this pathway: silibinin found in milk thistle, reduced nuclear and cytosolic β -catenin levels and reduced the transcription of cancer-related genes in SW480 CRC cells (Kaur et al. 2010). In addition, silymarin, also found in milk thistle, increased β -catenin phosphorylation and downregulated the signalling of the Wnt pathway (Eo et al. 2016). Quercetin, a flavonoid found in many fruits and vegetables, lowered the expression of the β -catenin/Tcf complex thus reducing activation of precancerous gene in CRC cells (Park et al. 2005). Black raspberry anthocyanins suppressed CRC cell (HCT-116, Caco-2, and SW480) growth and induced apoptosis by modulating β -catenin (Wang et al. 2013). Thus, the evidence above clearly indicates that dietary polyphenols and polyphenol-rich foods are able to target Wnt/ β -catenin signalling. However, as yet there is no information about the effect of culinary herbs and spices, known to be rich in polyphenols, on the Wnt/ β -catenin in CRC cells.

Culinary herbs and spices (CHS) contain high amounts of polyphenols, in fact, they contain amongst the highest amounts of polyphenols based on dry weight (Pérez-Jiménez & Torres 2011). Our research has shown that CHS inhibited the growth of CRC cells (HCT116, HCA-7) and their effect was cytotoxic (see Chapter 2). Thus, the first aim of this study was to determine if the CHS used could modulate this pathway, specifically β -catenin levels in CRC cells. Moreover, these CHS also possess anti-inflammatory activity by decreasing COX-2 expression and activity, and PGE2 in HCA-7 cells (see Chapter 3). Literature suggests that there is a link between COX-2/PGE2 and Wnt/ β -catenin, and PGE2 has been shown to activate the Wnt signalling pathway and that this relationship may play a role in cancer development (Castellone et al. 2005, Suh et al 2009). However, it

is not known whether the CHS, specifically those used in the present study, act by targeting Wnt/ β -catenin signalling pathway. Thus, the second aim of this study was to investigate whether CHS extracts can modulate Wnt/ β -catenin pathway in CRC cells within the same time period that their inhibition of COX-2 in HCA-7 cells occurred. This part of the study focused on, in particular, the CHS' effect on β -catenin phosphorylation/degradation, because one of the ways to reduce the level of unphosphorylated β -catenin and thus prevent its translocation into the nucleus is to stimulate β -catenin phosphorylation.

4.2 Materials and methods

4.2.1 Chemicals/reagents/drugs

The following chemicals were used in this study: ethanol (Sigma-Aldrich UK), foetal bovine serum (FBS) ((Sigma-Aldrich UK), antibiotics: penicillin, streptomycin and neomycin (Sigma-Aldrich UK), Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich UK), trypsin (Sigma-Aldrich UK), Dulbecco's Phosphate Buffered Saline (DPBS) (Fisher Scientific UK), NuPage LDS sample buffer 4x (Invitrogen, UK), NuPage reducing agent 10x (Invitrogen, UK), NuPage sharp prestained protein standard (Invitrogen, UK), NuPage antioxidant (Invitrogen, UK), protease inhibitors: 104 mM AEBSF, 80 μ M aprotien, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A and 1.4 mM E-64. (Sigma-Aldrich, UK), reducing agent (Invitrogen UK), 4–12% Bis-Tris gels (Invitrogen, UK), NuPage SDS transfer buffer 20x (Invitrogen, UK), NuPage MOPS SDS running buffer 20x (Invitrogen, UK).

4.2.2 Preparation of culinary herb and spice extracts

For this study two of the most potent extracts were investigated: bay leaf ethanol (BLE) and turmeric ethanol (TE). The extract preparation was the same as described in Chapter 3.

4.2.3 Cell culture

HCA-7 and HCT116 cell lines were used in this study and were. To see whether the effect of CHS on the Wnt/signalling pathway is dependent on COX-2 expression, a COX-2 positive (HCA-7) and COX-2 negative cell lines were chosen. Cell culture procedure were as described in Chapter 2.

4.2.4 The effect of BLE and TE on β -catenin in whole cell extracts

Initially cells (HCT116 and HCA-7) were treated for 24 hours with the two most potent extracts (TE and BLE) based on the COX-2 data (Chapter 3) and growth inhibition studies (Chapter 2). The treatment protocol was the same as described in Chapter 3, and then any changes in the level of unphosphorylated β -catenin (active), specifically its

decreased, were looked for in the whole cells lysates using the same protocol as for COX-2 western blot analysis in Chapter 3. However, as the CHS appeared to have no effect it was decided to decrease the treatment time to three hours; this decision was based on work done by Rooney et al. (Rooney et al. 2011). For the 3-hour experiments unphosphorylated and total β -catenin (which includes phosphorylated and unphosphorylated β -catenin) were measured in whole cell lysates. Total β -catenin was looked at because phosphorylated β -catenin is degraded so it is possible that if the CHS increased phosphorylation it would subsequently be degraded hence total β -catenin would also be decreased. The concentrations used were 15 μ g GAE/ml for BLE and 10 μ g GAE/ml for TE for 24-hour and 3-hour treatments. At these doses cell growth was inhibited and the reduction of COX-2 expression occurred in HCA-7 cells (see Chapter 3).

4.2.5 The effect of BLE and TE extracts on nuclear β -catenin in HCT116 cells

In order to see whether the effect of BLE and TE extracts on nuclear β -catenin in HCT116 cells could reduce the level of unphosphorylated β -catenin (active) in the nucleus, where initiation of the transcription of several precancerous genes occurs, cell fractionation involving the separation and isolation of the nucleus was carried out. For this purpose, the NE-PER™ Nuclear and Cytoplasmic Extraction kit (78833) (Thermo Fisher Scientific, UK) was used (Choi et al. 2010). Following 24-hour treatment with BLE (15 μ g GAE/ml) and TE (15 μ g GAE/ml), cells were trypsinised and transferred to Eppendorf tubes. Cells were then centrifuged at 500 x g for 5min. and then 1×10^6 cells were transferred to a new Eppendorf tube and centrifuged for 3min. at 500 x g. The supernatant was then removed and ice-cold CER I (200 μ l) was added to the cell pellet, which was immediately re-suspended, vortexed for 15s and incubated on ice for 10min. Thereafter ice-cold CER II was added into the tube, vortexed for 5s and placed on ice again for 1min. and vortexed for another 5s. Then cells were centrifuged at 16000 x g for 5min. The supernatant (cytoplasmic extract) was then transferred to a clean pre-chilled tube and placed on ice and later stored at -80°C until it was used for western blotting. The remaining pellet was suspended in ice-cold NER, vortexed for 15s and placed on ice for 40min., whilst vortexing every 10min. Thereafter, it was centrifuged at 16000 x g for 10min. The supernatant was then immediately transferred to pre-chilled tubes and stored at -80°C until it was used for western blotting. Then electrophoresis was performed on both cytosol and nuclear fractions following the protocol below.

4.2.6 Western blot procedure

The expression of total (unphosphorylated and phosphorylated) β -catenin and unphosphorylated β -catenin (active) in treated and controls cells (whole cell lysates) was determined by Western blotting. Sample preparation and western blot procedure were as described in Chapter 3. The following primary antibodies were applied: β -catenin (D10A8) XP® Rabbit mAb #8480 (for total β -catenin, which includes phosphorylated and unphosphorylated β -catenin) (1:1000); non-phospho (active) β -catenin (Ser33/37/Thr41) (D13A1) rabbit mAb #8814 (1:500), which was used to detect unphosphorylated form of β -catenin and β -Actin (13E5) rabbit mAb #4970 (1:1000) (all from Cell Signalling).

4.2.7 Data expression and analysis

The β -catenin experiments were carried out at least three times ($n=3$) for the 3 hour treatment, whilst for the 24 hour experiments they were performed twice (whole cell lysates) ($n=2$). Western blot band intensity was analysed using LICOR Image studio software (Licor, UK) and the data were normalised against β -actin and any decrease/increase in band intensity are expressed as a percentage in comparison with the intensity of the 'no treatment' band (cells in cell culture media (DMEM) with 10% FBS,) which represented 100% expression. Data are expressed as mean ($n=3$) \pm standard deviation (SD) for 3-hour treatment. For the 24-hour treatment data are expressed as mean ($n=2$) \pm standard deviation (SD) for the HCA-7 cell line. It was not possible to determine the mean for the HCT116 cell line for the 24-hour treatment as an $n=1$ was carried out for HCT116 (whole cell lysates) and also for the HCT116 cytosol vs nuclear extracts.

4.3 Results

4.3.1 The effect of BLE and TE on β -catenin in whole cell extracts

The results of the experiments showed that the 24-hour treatment CRC cells (HCT116 and HCA-7) with BLE and TE did not have an effect on unphosphorylated β -catenin (active) in whole cell lysates (Figures 4.1, 4.2). A shorter treatment time with the same extracts (BLE and TE) also did not change unphosphorylated and total β -catenin during a shorter treatment time (3h) (Figures 4.3 and 4.4).

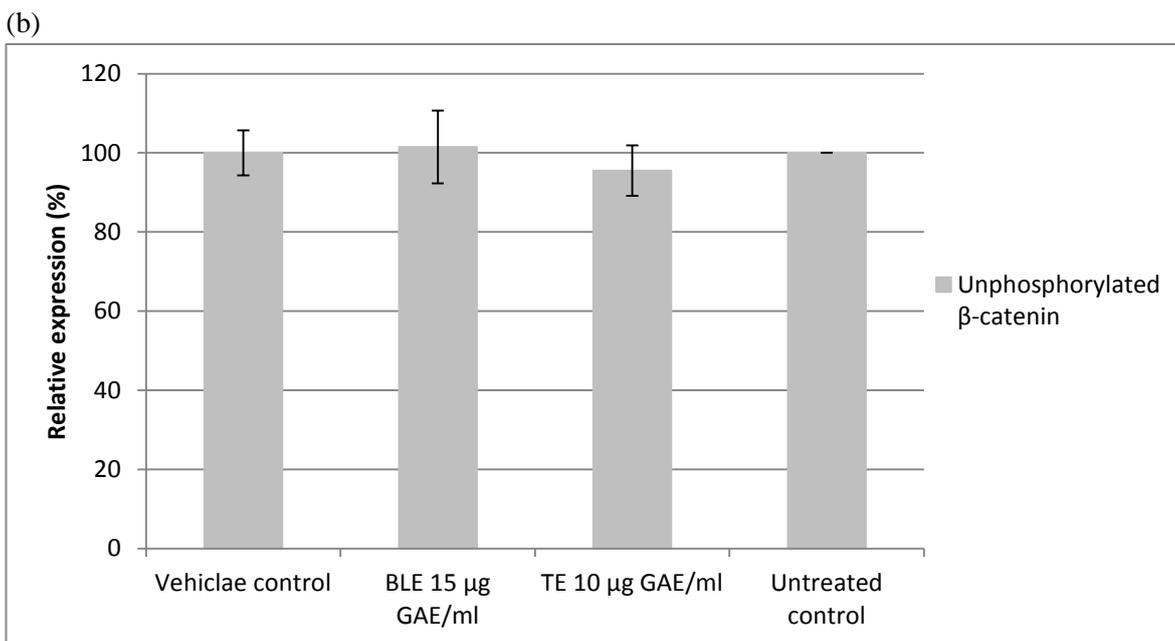
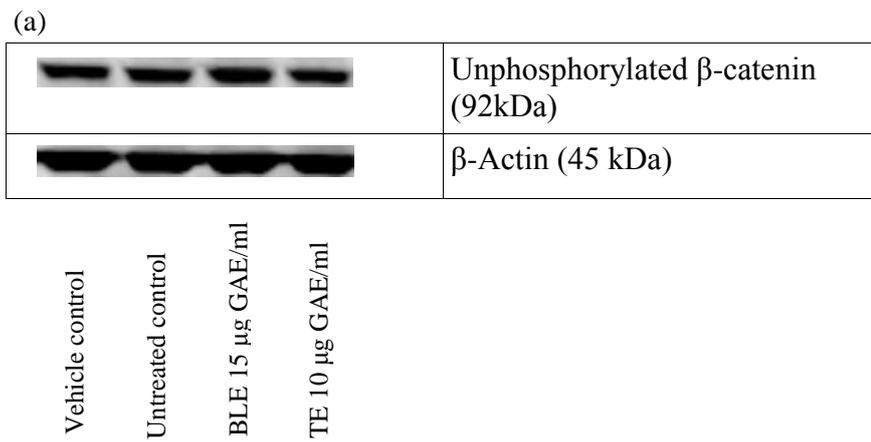


Figure 4.1 BLE and TE effect on unphosphorylated (active) β -catenin in HCA-7 cell line (whole cell lysates).

(a) Western blotting; Cells were treated for 24 hours with bay leaf (BLE 15 μ g GAE/ml), turmeric (TE 10 μ g GAE/ml), then lysed using the procedure explain in the method section. Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v). (b) Quantitative analysis of β -catenin bands. Data are expressed in comparison to control (100%) after the signal was normalized against β -actin, mean (n=2), \pm SD.

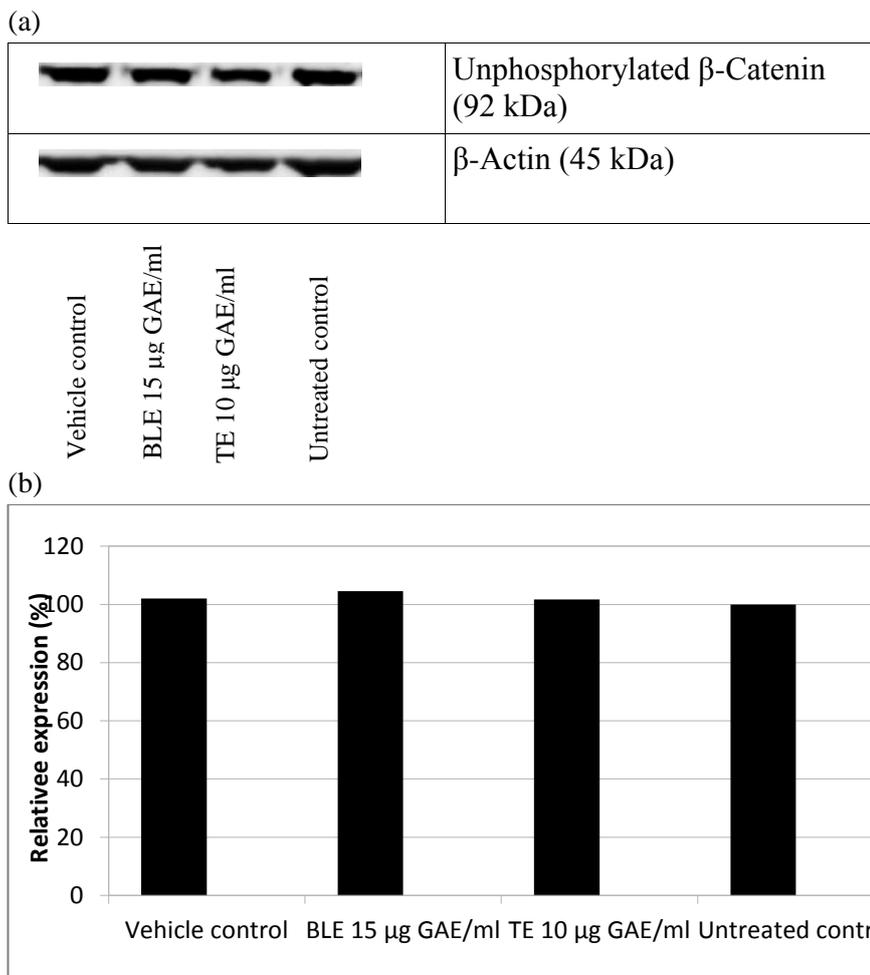
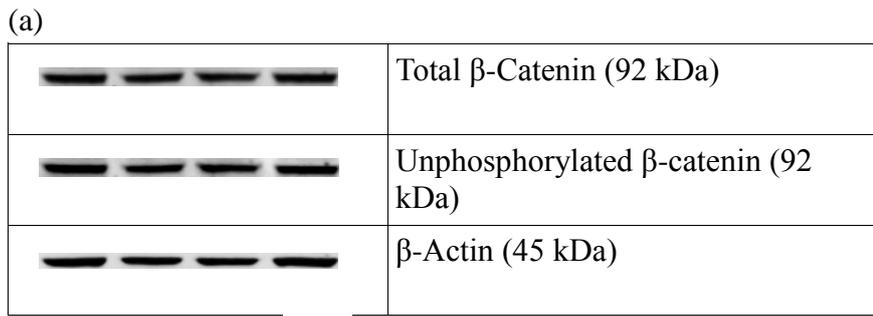


Figure 4.2 2 BLE and TE effect on unphosphorylated β -catenin in HCT116 cell line (whole cell lysate).

(a) Western blot; Cells were treated for 24 hours with bay leaf (BLE 15 μ g GAE/ml) and turmeric (TE 10 μ g GAE/ml), then lysed using the procedure explain in the method section. Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v). (b) Quantitative analysis of β -catenin bands Data are expressed in comparison to control (100%) after the signal was normalized against β -actin n=1.



Vehicle control
BLE 15 µg/ml
TE 10 µg/ml
Untreated control

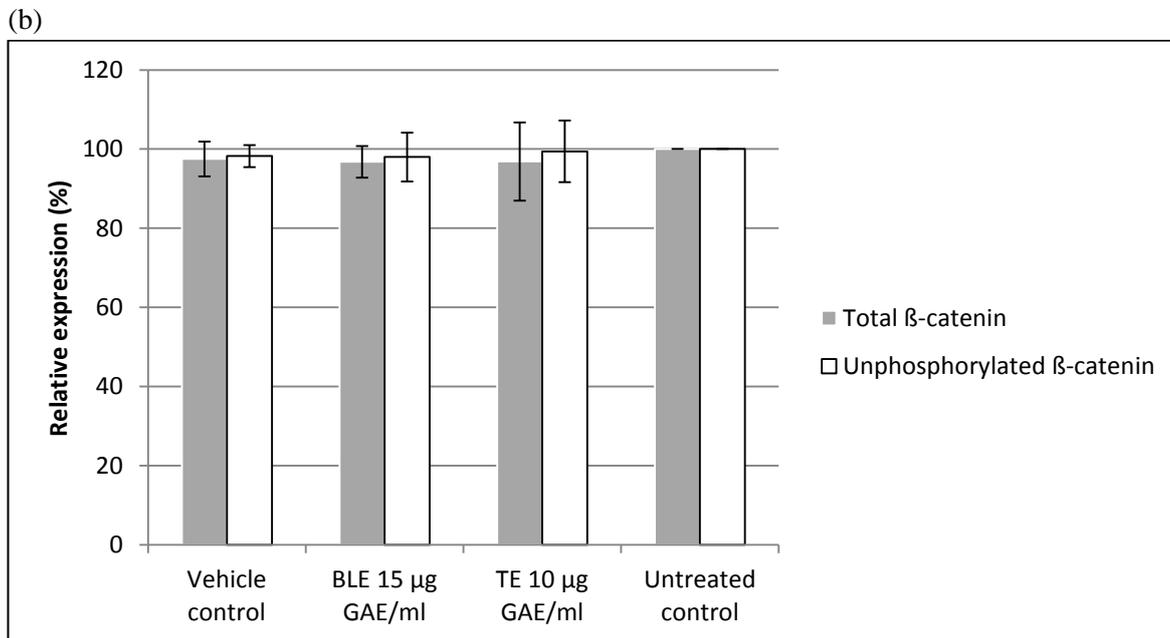


Figure 4.3 The effect of BLE and TE extracts on unphosphorylated and total β-catenin in HCA-7 cells, whole cell lysates.

(a) Western blot; Cells were treated for 3 hours with bay leaf (BLE 15 µg GAE/ml) and turmeric (TE 10 µg GAE/ml). Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v), the highest amount of ethanol found in the extracts. (b) Quantitative analysis of β-catenin bands. Data expressed in comparison to control (100%) after the signal was normalized against β-actin, mean (n=3), ±SD.

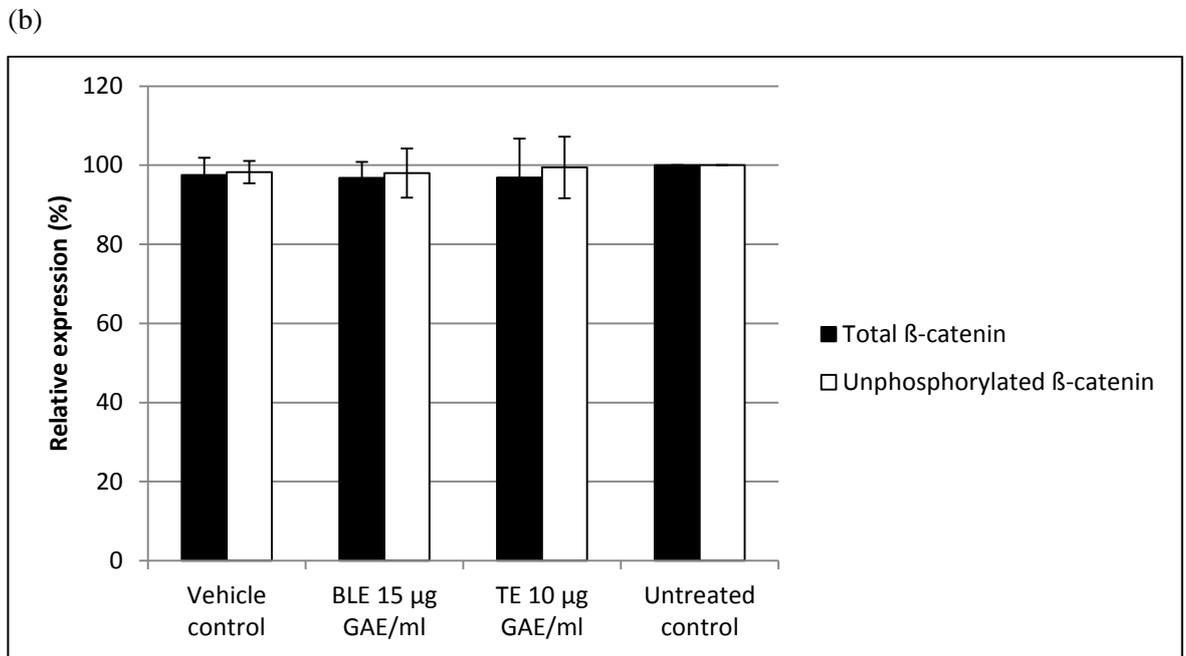
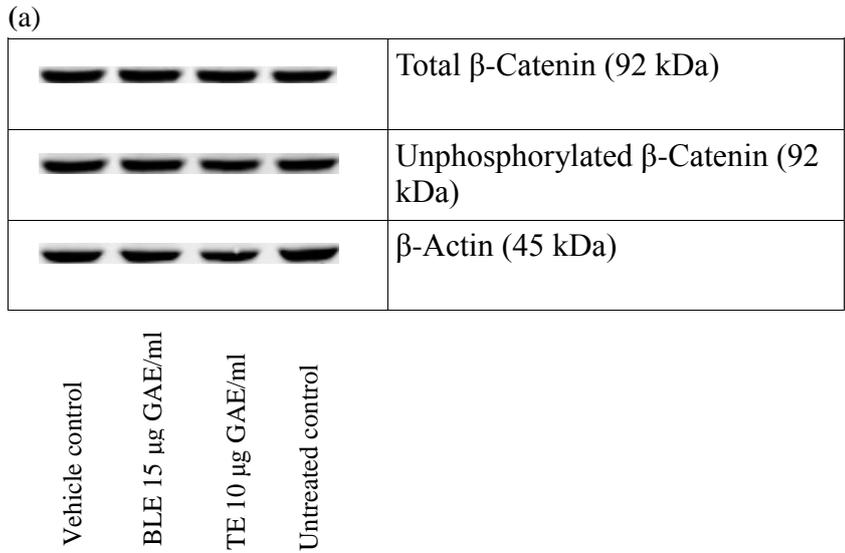


Figure 4.4 The effect of BLE and TE extracts on unphosphorylated and total β -catenin in HCT116 cells.

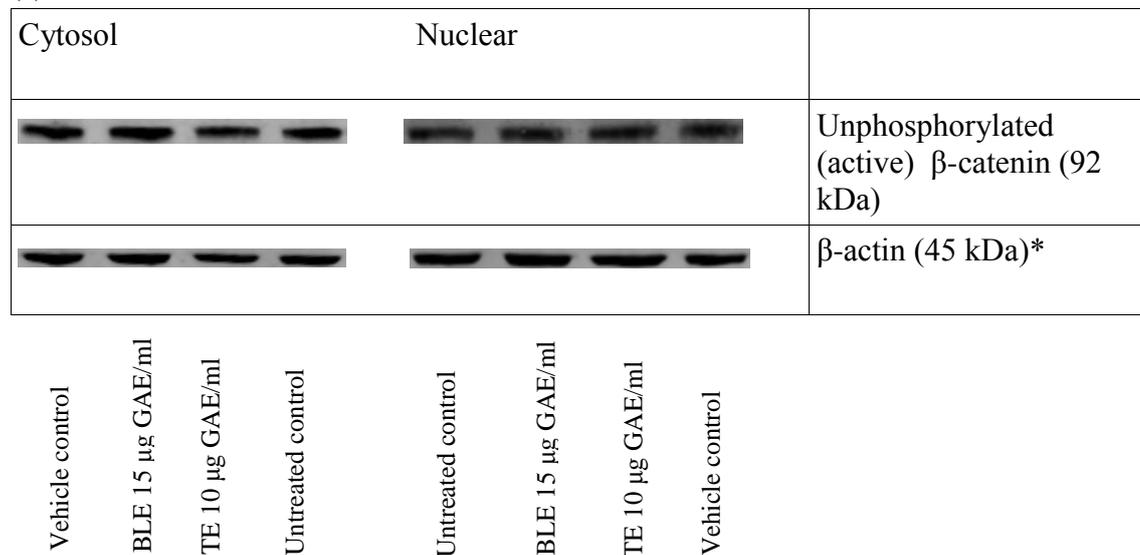
(a) Western blot; Cells were treated for 3 hours with bay leaf (BLE 15 μ g GAE/ml) and turmeric (TE 10 μ g GAE/ml). Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v), the highest amount of ethanol found in the extracts. (b) Quantitative analysis of β -catenin bands. Data are expressed in comparison to control (100%) after the signal was normalized against β -actin, mean (n=3), \pm SD.

4.3.2 The effect of BLE and TE extracts on nuclear β -catenin in HCT116 cells

As treating cells for 24 hours with BLE and TE did not have effect on unphosphorylated β -catenin (active) in whole cell lysates, it was decided to investigate their effect on unphosphorylated β -catenin, which moves into the nucleus hence the preparation of the nuclear fraction. Their effect on unphosphorylated β -catenin in the

cytosol was also investigated. The results showed that neither BLE nor TE had an effect on unphosphorylated β -catenin in the nucleus or in cytosol fraction (Figure 4.5).

(a)



(b)

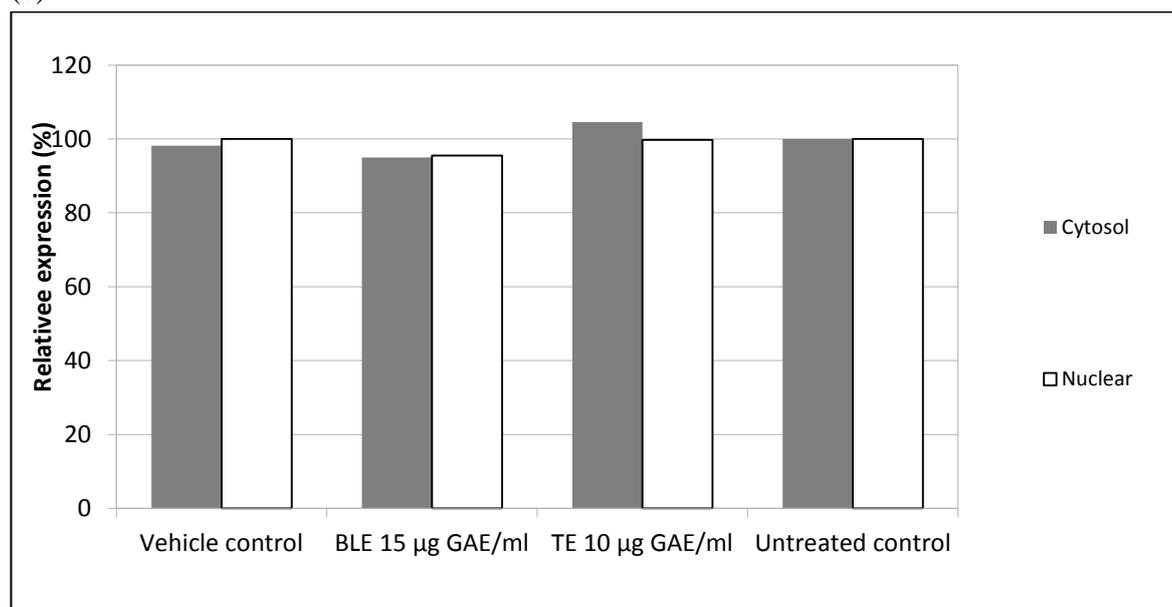


Figure 4.5 The effect of CHS extracts on unphosphorylated β -catenin in the nucleus, in HCT116 cells.

(a) Western blot, Cells were treated for 24 hours with bay leaf (BLE 15 μ g GAE/ml) and turmeric (TE 10 μ g GAE/ml), then lysed using the procedure explain in the method section. Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v). (b) Quantitative analysis of β -Catenin bands. Data are expressed in comparison to control (100%) after the signal was normalized against β -actin, n=1.

*Nuclear fraction has β -Actin band which suggests that nuclear fraction is not clean and it contains some cytosolic fraction.

4.4 Discussion

Wnt/ β -catenin signalling plays a key role in the development of CRC hence there have been numerous attempts to find compounds or foods that could target this pathway (Novellademunt et al. 2015; Afrin et al. 2016). The main aim of this study was to determine whether CHS, specifically bay leaf (BLE) and turmeric (TE), can target the Wnt/ β -catenin signalling pathway with a particular focusing on β -catenin phosphorylation and degradation as phosphorylation of β -catenin signals its degradation thus preventing translocation into the nucleus (Clevers 2006). However, the results revealed that the extracts (TE and BLE) did not have an effect on β -catenin phosphorylation/degradation, as non-phospho (unphosphorylated) β -catenin level remained unchanged after 3 and 24-hour treatments in whole cell lysates. Total β -catenin (phosphorylated and unphosphorylated) also was not affected after the 3-hour treatment. These studies suggesting that the effect of curcumin on β -catenin phosphorylation and degradation could be time-specific. It is possible that a longer 30-48h treatment period would have resulted in an effect as was reported in studies by Jaiswal et al. (2002); Xiang et al. (2006) and Xie et al. (2012). Jaiswal et al. (2002) found that curcumin (20 μ M) increased β -catenin degradation after a 30h treatment on HCT116 cells, whilst no effect on β -catenin degradation was observed during a shorter treatment period (Jaiswal et al. 2002). The same study also found that curcumin prevented β -catenin/Tcf-Lef binding resulting in reduced transcription of the β -catenin target gene – c-Myc, which is associated with increased cell proliferation. Park et al. (2005) found that 20 μ M of curcumin was enough to reduced upphosphorylated β -catenin level in the nucleus (HCT116 cells), but 40 μ M produced the stronger effect (Park et al. 2005). Xiang et al. (2006) using caffeic acid phenethyl ester (a polyphenol) found that it only reduced 48h treatment reduced β -catenin expression (HCT116 and SW480 cells), whilst no effect was observed after 24h (Xiang et al. 2006) . However, in contrast Prasad et al. (2009) found that curcumin (20 μ M) reduced β -catenin expression during a shorter treatment period of 12h, but this study used breast cancer cells (Prasad et al. 2009), which suggests that the effect may be cell-specific. Ryu et al. (2008) found that 20 μ M of curcumin, did not have effect on either nuclear or cytosolic β -catenin level in embryonic kidney cells, where Wnt signalling was stimulated using Wnt ligands (15h treatment), however, 40 μ M reduced both nuclear and cytosolic β -catenin (Ryu et al. 2008), which suggest that the effect on β -catenin is dose-dependent.

The results of this study may suggest that these CHS only target β -catenin phosphorylation in CRC cells with specific mutations. For example, Kaur et al. (2010) found that silibinin reduced cytosolic and nuclear β -catenin only in SW480 cells that possess a mutated APC gene and also wild-type β -catenin (Kaur et al. 2010). However, they reported no effect on the HCT116 cell line, which was also used in the present study. The HCT116 cell line has a wild-type APC but mutated β -catenin. However, Park et al. (2005) found that curcumin (20-40 μ M, 4-24h treatment) reduced unphosphorylated β -catenin in the nucleus of HCT116 cells without affecting its level in the cytosol, which suggests that β -catenin level can be targeted in this cell line (Park et al. 2005).

After not seeing an effect on β -catenin phosphorylation/degradation in the whole cell lysates, the effect of CHS on unphosphorylated β -catenin in the nucleus was investigated as it is possible that CHS could decrease unphosphorylated β -catenin levels in the nucleus only. However, the effect of CHS on nuclear β -catenin level could not be fully established in the present study. Our results suggest that BLE and TE do not affect nuclear unphosphorylated β -catenin level. However, it is likely that the nucleus separation from the cytosol was incomplete, as β -actin band was still present in the nuclear fraction. Most studies use other loading markers such as lamin for nuclear fraction and β -actin should not appear in the nuclear fraction (Yang et al. 2006; Leow et al. 2014; Hwang et al. 2016), however, Ryu et al. (2008) and Lee et al. (2017) had β -actin band present in the nuclear fraction samples without giving explanation about it. Lee et al. (2017) use another marker - U1 snRNP70 (Lee et al. 2017). Literature indicates that β -actin can be present in the nucleus (Olave et al. 2002; McDonald et al. 2006), so without using another marker for nuclear fraction it is impossible to determine whether the nuclear fraction samples used in the present study were contaminated or not.

It is possible that BLE and TE target some downstream molecules in Wnt/ β -catenin signalling pathway. There is evidence in the literature that some polyphenols have an effect on this pathway at various levels, although again as stated above the concentrations of isolated polyphenols used in the studies are manifold higher than those found in the extracts used in the present study. For example, curcumin analogues (demethoxycurcumin [DMC] and bisdemethoxycurcumin [BDMC]), and the curcumin metabolite (tetrahydrocurcumin) (15 μ M) inhibited β -catenin/Tcf transcription signalling by downregulating coactivator p300 expression in the CRC cells, whilst nuclear β -catenin levels remained unchanged (Ryu et al. 2008), which suggest that lower concentrations are more likely to act on downstream molecular targets in the Wnt/ β -catenin pathway. Park et al. (2005)

found that flavanone polyphenols (50-100 μM) did not change β -catenin levels and distribution between cytosol and nucleus (24h treatment) but still managed to inactivate β -catenin/Tcf transcription and reduce cyclin D1 expression (Park et al. 2005). Another polyphenol – resveratrol (20 μM ; 16h treatment) also prevented β -catenin binding to Tcf and also cyclin D1 expression, and as a result stopped the proliferation of HCT116 cells without altering β -catenin levels (Chen et al. 2012). Curcumin (20 μM) also increased GSK3 β expression, which facilitates β -catenin phosphorylation and subsequent degradation (Prasad et al. 2009). Moreover, the same study also found that curcumin reduced cyclin D1 expression, whose transcription is regulated by β -catenin.

Targeting downstream molecules in Wnt pathway could be more effective than targeting β -catenin phosphorylation and degradation or/and translocation into the nucleus. For example, it was found that knocking out Tcf gene, produced a stronger anti-proliferative, pro-apoptotic effect and enhancement of chemo-sensitivity than knocking out the β -catenin gene (Xie et al. 2012). Thus, future work with BLE, TE and other CHS should focus on other targets in Wnt/ β -catenin signalling pathway rather than β -catenin phosphorylation. It could also be worth further assessing the effect of CHS extracts on β -catenin phosphorylation and degradation using a longer treatment time (30-48 hours), which was observed by Jaiswal et al. (2002), and also their effect on nuclear β -catenin. However, one has to bear in mind that concentrations of individual polyphenols used in the studies cited above cannot be achieved in the whole CHS extracts. For example, in order to achieve micro-molar concentrations of curcumin in a TE extract, the extract would have to be approximately thousand times more concentrated than the extract used in the present study, which is not achievable with such a food source.

4.5 Conclusion

The results of this study revealed that CHS, specifically TE and BLE did not affect β -catenin phosphorylation at 3 and 24 hours. A longer treatment time (30-48h) could result in a positive result based on the literature. Although literature suggests that there is a link between COX-2/PGE2 and Wnt/ β -catenin signalling, it appears that there was no association between the anti-inflammatory activity of CHS and β -catenin phosphorylation/degradation in the present study. Thus, the anti-proliferative and cytotoxic effects of these CHS are unlikely to occur through regulating β -catenin phosphorylation/degradation. Future studies need to investigate the effect of CHS on downstream molecules in Wnt/ β -catenin signalling pathway.

Chapter 5 CHS ability to induce apoptosis in CRC cells

5.1 Introduction

Apoptosis is one of the main processes through which cell proliferation is controlled and understanding its underlying mechanism, and how it can be affected by external, including dietary factors is important because of the crucial role it plays in the pathogenesis of cancer (Ghobrial et al. 2005). Apoptosis is described as ‘a set of morphologic changes including chromatin condensation, nuclear fragmentation, membrane blebbing and cell shrinkage’ (Khan et al. 2007). In contrast to normal cells, cancer cells through various pathways and mechanisms develop the ability to suppress apoptosis (Gupta et al. 2010; Hanahan & Weinberg 2011) so its induction is a desirable outcome in cancer prevention and therapy (Hu & Kavanagh 2003). There are two major pathways of apoptosis: the extrinsic pathway, which occurs through the death receptor located on the cell surface, and the intrinsic pathway, which is mediated via mitochondria; both of these pathways can be interlinked (Khan et al. 2007). Apoptosis is executed by caspases, which are a group of protease enzymes that play an important role in the process of apoptosis. There are initiator caspases and executor caspases (McIlwain et al. 2013; Huai et al. 2010). One way to induce apoptosis is by activating executor caspases (3 and 7), which can be activated through both extrinsic and intrinsic pathways leading to cleavage of poly(ADP-ribose) polymerase (PARP) and results in irreversible cell death (Fernald 2013). Another way to induce apoptosis is through p53, which is a tumour suppressor protein that plays a key role in the regulation of apoptosis. Many cancer cells have p53 mutations, and it is one of the most common mutations in cancer (Fernald 2013; Li et al. 2015). Moreover, mutations and loss of function of p53 is mediated by inflammation (Lasry et al. 2016), so activation of executor caspases and upregulating p53 expression are good targets for natural anti-carcinogenic compound and foods like culinary herbs and spices (CHS).

Another protein of interest is cyclin D1, which plays an important role in cell cycle regulation and apoptosis (Tashiro et al. 2007). It belongs to the cyclin family of proteins, and by binding to cyclin-dependent kinases (CDK4 and CDK6) form active complexes, which promote cell cycle progression and cell proliferation (Aggarwal et al. 2003; Alao 2007; Ravindran et al. 2009). It is a proto-onco gene and many cancer cells including those that are CRC cells over-express this protein and the result is uncontrolled cell division (Joyce et al. 2001; Ravindran et al. 2009; Shishodia 2013). The dietary polyphenol curcumin, which as stated previously is a major constituent of the spice turmeric, has been

shown to target cyclin D1 expression in leukaemia and neck cancer cells (Aggarwal et al. 2006; Shishodia 2013) so as with the executor caspases and p53 it is another good target for chemoprevention and therapy by the CHS under investigation. ,

It has been demonstrated that CHS extracts inhibit CRC cell growth and possess cytotoxic activity (see Chapter 2). Moreover, within the time frame of their cytotoxic activity these extracts also target COX-2 and its main product – PGE2 (see Chapter 3), both of which are involved in apoptosis (Greenhough et al. 2009). Thus, it is hypothesised that CHS extracts can induce apoptosis in these CRC cells however the mechanism by which this is achieved is unknown. Hence, the aim of this study was to investigate whether CHS extracts can induce apoptosis in CRC cells, and elucidate the mechanism(s) by which this is achieved.

5.2 Materials and methods

5.2.1 Preparation of culinary herb and spice extracts

The extract preparation was the same as described in Chapter 3.

5.2.2 Cell culture

For this study two CRC cell lines were used: HCT116 and HCA-7, both were grown as described in Chapter 2. These two cell lines were chosen to see if there could be a link between induction of apoptosis and COX-2, hence COX-2 positive (HCA-7) and COX-2 negative (HCT116) cell lines were used.

5.2.3 The effect of CHS on the cell cycle and apoptosis in HCA-7 and HCT116 CRC cells

Based on the results of the SRB growth inhibition data (Chapter 2), the most potent CHS extracts and their combinations were tested for their effect on cell cycle distribution. The CHS investigated were rosemary ethanol (RE), sage ethanol (SE), bay leaf ethanol (BLE), bay leaf aqueous (BLA), ginger ethanol (GE), turmeric ethanol (TE), rosemary and turmeric ethanol (RTE), bay leaf and sage ethanol (BLSE), sage and ginger (SGE) and bay leaf and turmeric ethanol (BLTE). The CHS extracts were screened for their ability to modulate the cell cycle and induce apoptosis using FACS analysis. Trypsinised cells (1×10^6) were seeded into a flask containing 10ml of media and CHS extract. The doses used for the cell cycle analysis were based on the SRB growth inhibition study, and were slightly higher than their IC₅₀ values (approximate IC₇₀) so that an effect could be observed without the CHS killing a large proportion of the cells. Following the exposure periods of 24 or 48 hours, the supernatant of floating (dead) cells and trypsinised cells were pooled together. Then cells were washed three times by centrifugation (at 1000rpm for

4min) and re-suspended in cold (4°C) PBS. After the final wash, cells were re-suspended in 200 µl of cold PBS and fixed by adding 1ml of ice cold 70% ethanol (in PBS). Cells were then kept overnight at 4°C, and then washed 3 times as above. Thereafter, cells were incubated with 0.5ml of propidium iodide (PI) buffer (BD, UK) for 30min at room temperature and analysed using a FL3 detector (PI detector, 620 nm) (FACS calibur, BD). At least 10,000 events were counted. Cells present in the sub G1 phase were considered to be apoptotic (Wlodkowic et al. 2009; Dimas et al. 2015). HCA-7 cells were also treated with selective COX-2 inhibitor - Celecoxib (50 µM) to compare its effect with that of the CHS extracts. But this experiment was done only once for 24-hour treatment.

5.2.4 Activation of caspase-3/7 by BLE in HCA-7 and HCT116 CRC cells

To confirm that apoptosis had occurred, a caspase-3/7 assay was performed using IncuCyte live-cell imaging. The manufacturer's instructions were followed (EssenBioscience, UK). Briefly, cells were seeded on 96-well plates and placed into an incubator for 24h, then one of the most potent extracts - BLE was added at their approximate IC70 (for the reasons stated above) – 6 µg GAE/ml with caspase-3/7 reagent. Etoposide was used as a positive control for caspase-3/7 activation, and a caspase-3/7 inhibitor (MMPSI, Caspase-3/7 Inhibitor I (ab145046)) was used as a negative control. Another negative control – (media without caspase-3/7 reagent) was also used to make sure cell culture media was not generating a fluorescence signal. An untreated control containing solely cell culture media and caspase-3/7 reagent was also included. On caspase-3/7 activation the probe emits a green fluorescent light which is detected by the IncuCyte camera. Cells were treated with the CHS extracts for 48h and constantly monitored (images were taken every 2 hours). The data were analysed using IncuCyte ZOOM® software (EssenBioscience, UK). It was intended that TE extract would also be used as it was the most potent CHS extract but it could not be used for this experiment as its yellow colour caused fluorescence and affected the data readings.

5.2.5 The effect of CHS on proteins involved in apoptosis in HCA-7 and HCT116 CRC cells

To further investigate the effect of the CHS, specifically BLE and TE, on apoptosis, their effect on key protein markers of apoptosis, cleaved caspase-3, p53 and cleaved PARP and cyclin D1 was determined. Etoposide (25 µM) was used as a positive control for caspase-3 activation. The western blot procedure and treatment was the same as described in Chapter 3, for the COX-2 experiments. All antibodies were purchased from Cell Signalling: p53 (#9282 Cell Signalling), p53 (1C12) mouse mAb #2524; cleaved PARP

(Asp214) (D64E10) XP® Rabbit mAb #5625 (dilution 1:1000); cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb #9664; cyclin D1 (92G2) #2978 rabbit mAb #2978 and β -actin (1:1000; Cell Signalling), which was used as an internal control to show that equal amounts of protein were loaded.

5.2.6 Data expression and statistical analysis

All experiments were done in triplicate (n=3) (unless stated otherwise), which represents three separate experiments and data are expressed as mean and standard deviation (\pm SD) unless otherwise stated. Western blot band intensity was analysed using Odyssey software (Licor, UK), the data were normalised against β -actin and any reduction in band intensity was expressed as a percentage in comparison with the intensity of the 'no treatment' band (HCA-7 cells in cell culture media only), which represented 100% expression. Cell cycle distribution was expressed as a percentage of cells in each cycle/phase (sub G1, G1, S and G2). To determine if there was a statistically significant difference between treated (exposed to CHS) and untreated cells for the sub G1 phase one-way ANOVA with Tukey's post-hoc test was performed. For all statistical tests SPSS software was used and $p < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 The effect of CHS on the cell cycle distribution in HCA-7 and HCT116 CRC cells

Twenty four hour treatment resulted in an increased number of cells in the sub G1 phase for both HCA-7 and HCT116 cell lines (Table 5.1). For HCA-7 cells BLE and GE were the most potent extracts causing 28% and 27%, respectively, of the cells to accumulate in the sub G1 phase. Other CHS extracts were slightly less potent: TE -23%, BLA - 21%, SE - 16% and RE - 14% of cells were in the sub G1 phase. The BLTE combination caused 33% of cells to accumulate in the sub G1 phase, which was higher than for its individual herb constituents. The combinations were less potent than at least one of their individual CHS constituents: SGE - 23%, BLSE - 19% and RTE - 16%. After the 48-hour treatment, the extracts also caused cells to accumulate in the sub G1 phase with TE (49%) and GE (49%) being the most potent, followed by BLE (43%), BLA (35%), SE (30%) and RE (17%) (Table 5.1); the numbers in this phase were greater than those for the 24hr treatments. Treatment with BLTE and RTE combinations resulted into 35% and 33% of cells accumulating in this phase, whilst BLSE and SGE produced slightly lower figures: 26% and 22%, respectively.

For HCT116 cell line (Table 5.2), during the 24-hour treatment the most potent extracts were TE, and BLE and the BLTE combination; all caused 15% of cells to accumulate in the sub G1 phase, followed by RTE (14%), BLSE (13%), SE (13%), RE (12%), SGE (8%), GE (8%) and BLA (8%). During the 48-hour treatment TE and BLE extracts were the most potent, causing 36% and 23% of cells to accumulate in the sub G1 phase. The most potent combination was BLTE (26% cells in sub G1 phase), followed by BLSE (22%), SGE (17%) and RTE (14%).

Table 5.1 The Effect of CHS on the cell cycle in HCA-7 cells over 24 and 48 hours

Herbs/spices	Sub G1 (%) (\pm SD)		G1 (%) (\pm SD)		S (%) (\pm SD)		G2 (%) (\pm SD)	
	24h	48h	24h	48h	24h	48h	24h	48h
Untreated control	10 (\pm 2.3)	7 (\pm 2.5)	40 (\pm 2.6)	46 (\pm 1.7)	26 (\pm 2.1)	25 (\pm 4.0)	23 (\pm 2.0)	20 (\pm 5.0)
Vehicle control (ethanol)	9 (\pm 2.1)	4 (\pm 1.2)	41 (\pm 2.8)	47 (\pm 2.3)	27 (\pm 2.1)	24 (\pm 2.0)	23 (\pm 0.7)	23 (\pm 0.6)
Vehicle control (H ₂ O)	10 (\pm 1.7)	4 (\pm 1.7)	39 (\pm 1.5)	45 (\pm 1.0)	25 (\pm 3.2)	25 (\pm 0.6)	24 (\pm 2.0)	23 (\pm 0.6)
TE (2 μ g GAE/ml)	23 (\pm 7.0)*	49 (\pm 5.3)*	41 (\pm 5.5)	21 (\pm 8.1)	20 (\pm 0.6)	19 (\pm 2.1)	15 (\pm 1.5)	9 (\pm 1.0)
GE (8 μ g GAE/ml)	27 (\pm 6.7)*	49 (\pm 5.3)*	41 (\pm 4.0)	25 (\pm 5.0)	17 (\pm 0.6)	15 (\pm 2.1)	12 (\pm 1.0)	8 (\pm 1.2)
BLE (6 μ g GAE/ml)	28 (\pm 5.5)*	43 (\pm 4.3)*	38 (\pm 5.9)	28 (\pm 2.6)	20 (\pm 3.2)	15 (\pm 2.1)	13 (\pm 3.2)	12 (\pm 1.7)
BLA (6 μ g GAE/ml)	21 (4.5)*	35 (\pm 11.6)*	37 (\pm 1.5)	28 (\pm 7.9)	23 (\pm 4.0)	20 (\pm 8.7)	18 (\pm 3.2)	14 (\pm 4.0)
SE 16 (μ g GAE/ml)	16 (\pm 4)	30 (\pm 2.3)*	42 (\pm 0.6)	31 (\pm 7.2)	23 (\pm 2.0)	21 (\pm 6.0)	18 (\pm 2.6)	17 (\pm 0.6)
RE (20 μ g GAE/ml)	14 (\pm 0.6)	17 (\pm 7.4)	42 (\pm 1.5)	41 (\pm 4.2)	31 (\pm 1.2)	20 (\pm 0.7)	10 (\pm 0.6)	20 (\pm 2.8)
BLTE (3 μ g GAE/ml BLE & 1 μ g GAE/ml TE)	33 (\pm 1.5)*	33 (\pm 1.0)*	34 (\pm 0.6)	35 (\pm 2.3)	19 (\pm 1.0)	16 (\pm 1.2)	11 (\pm 1.2)	14 (\pm 2.6)
BLSE (3 μ g GAE/ml BLE & 8 μ g/ml GAE SE)	19 (\pm 1.4)*	26 (\pm 3.1)*	41 (\pm 1.4)	42 (\pm 5.5)	18 (\pm 2.8)	18 (\pm 2.9)	12 (\pm 0.7)	14 (\pm 1.7)
RTE (10 μ g GAE/ml GAE RE & 1 μ g GAE/ml TE)	16 (\pm 1.5)*	35 (\pm 0.6)*	42 (\pm 2.6)	32 (\pm 4.4)	26 (\pm 4.0)	17 (\pm 2.1)	18 (\pm 0.6)	12 (\pm 0.6)
SGE (8 μ g GAE/ml SE & 4 μ g GAE/ml GE)	23 (\pm 0.6)*	22 (\pm 2.1)	37 (\pm 1.2)	45 (\pm 1.2)	27 (\pm 0.6)	20 (\pm 1.0)	11 (\pm 1.0)	12 (\pm 1.7)
Celecoxib (50 μ M)**	23	-	45	-	18	-	13	-

Cells were treated for 24 or 48 hours with the following CHS and their combinations: rosemary ethanol (RE), sage ethanol (SE), bay leaf ethanol (BLE), ginger ethanol (GE), turmeric ethanol (TE), and rosemary and turmeric ethanol (RTE), bay leaf and sage ethanol (BLSE), sage and ginger ethanol (SGE), bay leaf and turmeric ethanol (BLTE). Data are expressed as a percentage of cells in each phase, mean (n=3), \pm SD. *Statistically significant difference in comparison to control (p<0.05). Vehicle control (ethanol) – 0.2% (v/v), the highest volume found in the extracts. Vehicle control (filter-sterilised distilled H₂O) 0.7% (v/v), the highest volume found in the extracts. **Celecoxib had only one experiment (n=1).

Table 5.2 The Effect of CHS on the cell cycle in HCT116 cells over 24 and 48 hours

Herbs/spices	Sub G1 (%) (\pm SD)		G1 (%) (\pm SD)		S (%) (\pm SD)		G2 (%) (\pm SD)	
	24h	48h	24h	48h	24h	48h	24h	48h
Untreated control	4 (\pm 0.6)	4 (\pm 2.3)	44 (\pm 0.6)	54 (\pm 7.6)	29 (\pm 1.0)	23 (\pm 7.6)	22 (\pm 1.2)	18 (\pm 1.2)
Vehicle control (ethanol)	4 (\pm 0.6)	4 (\pm 3.1)	45 (\pm 3.2)	50 (\pm 4.5)	26 (\pm 6.1)	23 (\pm 6.6)	24 (\pm 3.1)	17 (\pm 2.1)
Vehicle control (H ₂ O)	6 (\pm 1.5)	6 (\pm 0.6)	47 (\pm 2.3)	49 (\pm 2.1)	26 (\pm 2.3)	26 (\pm 2.1)	24 (\pm 1.7)	18 (\pm 0.6)
TE (2 μ g GAE/ml)	15 (\pm 0.6)*	36 (\pm 10.1)*	37 (\pm 2.5)	26 (\pm 5.5)	25 (\pm 1.7)	17 (\pm 5.3)	20 (\pm 1.7)	19 (\pm 9.1)
GE (8 μ g GAE/ml)	8 (\pm 1.0)*	12 (\pm 1.5)*	52 (\pm 1.0)	50 (\pm 1.7)	21 (\pm 1.0)	21 (\pm 1.7)	16 (\pm 1.2)	14 (\pm 1.2)
BLE (6 μ g GAE/ml)	15 (\pm 0.6)*	23 (\pm 9.1)*	37 (\pm 0.6)	40 (\pm 6.8)	24 (\pm 1.0)	20 (\pm 4.2)	22 (\pm 1.5)	15 (\pm 1.0)
BLA (6 μ g GAE/ml)	8 (\pm 0.6)*	4 (\pm 0.6)	40 (\pm 0.6)	52 (\pm 0.6)	29 (\pm 2.5)	25 (\pm 1.2)	22 (\pm 2.1)	18 (\pm 1.2)
SE 16 (μ g GAE/ml)	13 (\pm 0.6)*	18 (\pm 3.0)*	37 (\pm 2.0)	47 (\pm 3.6)	28 (\pm 6.9)	19 (\pm 1.5)	24 (\pm 1.0)	12 (\pm 2.1)
RE (20 μ g GAE/ml)	12 (\pm 0.6)*	17 (\pm 1.0)*	38 (\pm 0.6)	45 (\pm 3.0)	27 (\pm 1.2)	23 (\pm 0.6)	23 (\pm 0.6)	18 (\pm 1.7)
BLTE (3 μ g GAE/ml BLE & 1 μ g GAE/ml TE)	15 (\pm 2.9)*	22 (\pm 1.5)*	39 (\pm 1.5)	41 (\pm 1.7)	24 (\pm 1.7)	18 (\pm 1.2)	22 (\pm 0.6)	17 (\pm 1.0)
BLSE (3 μ g GAE/ml BLE & 8 μ g/ml GAE SE)	13 (\pm 1.0)*	26 (\pm 3.1)*	40 (\pm 0.6)	41 (\pm 3.5)	25 (\pm 1.0)	17 (\pm 3.1)	20 (\pm 1.7)	15 (\pm 2.1)
RTE (10 μ g GAE/ml GAE RE & 1 μ g GAE/ml TE)	14 (\pm 2.1)*	14 (\pm 2.0)*	45 (\pm 6.1)	48 (\pm 1.5)	21 (\pm 3.1)	18 (\pm 0.6)	18 (\pm 5.3)	19 (\pm 1.2)
SGE (8 μ g GAE/ml SE & 4 μ g GAE/ml GE)	9 (\pm 0.6)*	17 (\pm 1.5) *	51 (\pm 7.8)	46 (\pm 1.2)	24 (\pm 5.8)	19 (\pm 1.7)	17 (\pm 1.2)	17 (\pm 0.6)

Cells were treated for 24 or 48 hours with the following CHS and their combinations: rosemary ethanol (RE), sage ethanol (SE), bay leaf ethanol (BLE), ginger ethanol (GE), turmeric ethanol (TE), and rosemary and turmeric ethanol (RTE), bay leaf and sage ethanol (BLSE), sage and ginger ethanol (SGE), bay leaf and turmeric ethanol (BLTE). Data are expressed as a percentage of cells in each phase, mean (n=3), \pm SD. *Statistically significant difference in comparison to control (p<0.05). Vehicle control (ethanol) – 0.2% (v/v), the highest volume found in the extracts. Vehicle control (filter-sterilised distilled H₂O) 0.7% (v/v), the highest volume found in the extracts.

5.3.2 Effect of CHS on caspase3/7 activation in HCA-7 and HCT116 cells

The results showed that BLE extract (6 µg GAE/ml) activated caspase-3/7 in HCA-7 cells (Figures 5.1 and 5.2). The activation of caspase-3/7 by BLE was not inhibited by the presence of the caspase 3/7 inhibitor. In HCT116 BLE (6 µg GAE/ml) also activated caspase-3/7, however, the data were a bit anomalous as after 30 hours untreated control and lower doses of BLE exceeded caspase-3/7 activation.

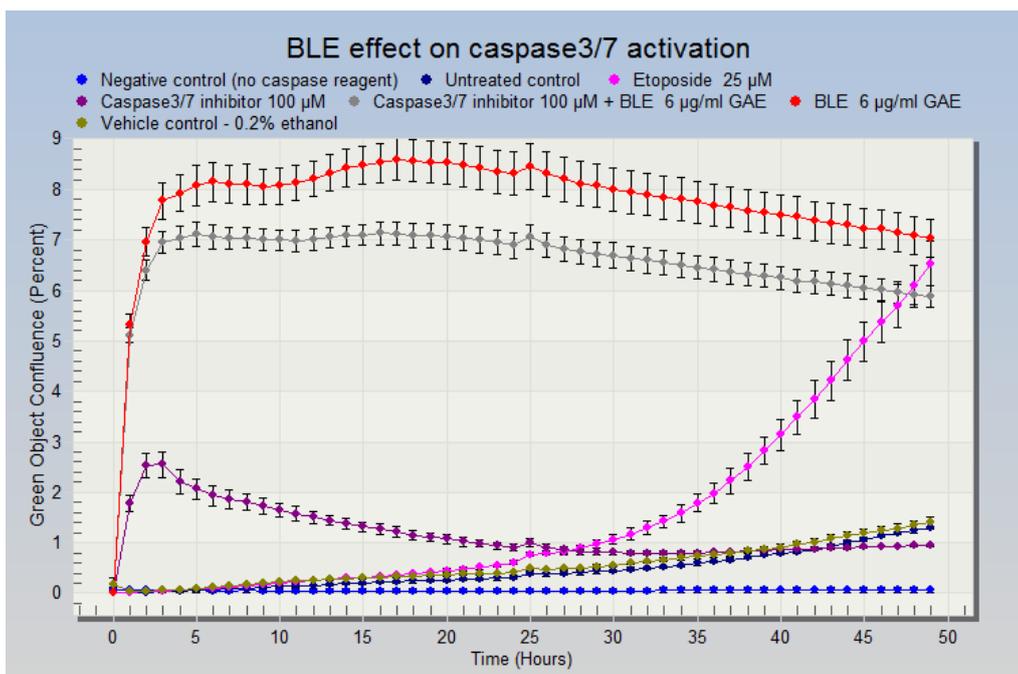


Figure 5.1 BLE effect on cell death and caspase-3/7 activation in HCA-7 cells.

HCA-7 cells were treated with BLE (bay leaf ethanol) (6 µg GAE/ml); Etoposide (25µM) was used as a positive control for caspase-3 activation; caspase-3/7 inhibitor (100 µM) was used as a negative control. Another negative control – (media without caspase-3/7 reagent) was used to ensure the cell culture media does not generate fluorescence signal. Vehicle control – 0.2% ethanol (v/v). Before the first scan was performed by the IncuCyte ZOOM®, cells were exposed to the treatment for ~30min so time 0 is approximately 30 minutes are cells were exposed.

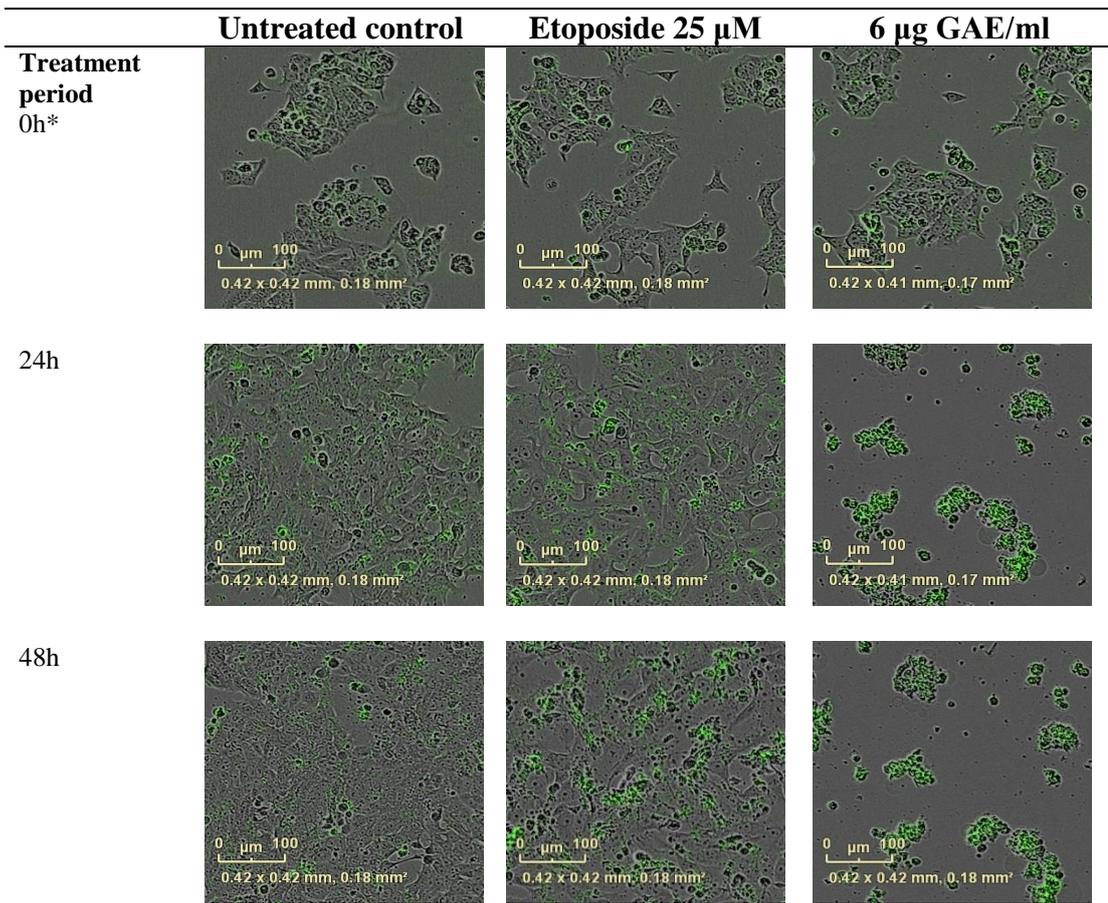


Figure 5.2 BLE effect on caspase-3/7 activation in HCA-7 cells.

Images recorded using the IncuCyte ZOOM® camera (x10 zoom). HCA-7 cells were treated with bay leaf ethanol (BLE) at 6 μ g GAE/ml concentration; *The first scan taken by the IncuCyte ZOOM®; before the first scan was performed cells were exposed to the treatment for ~30min. On caspase-3/7 activation reagent turns green and is recorded by IncuCyte ZOOM® camera.

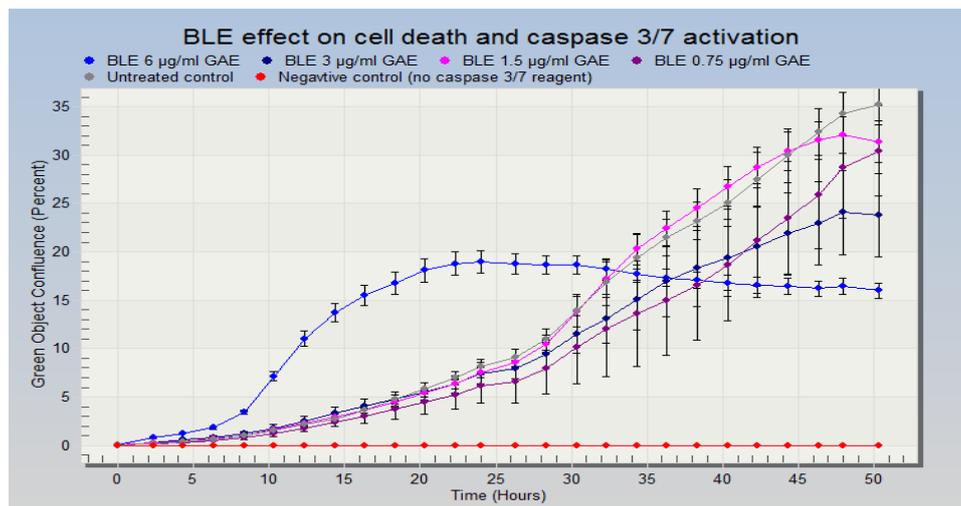


Figure 5.3 BLE effect on cell death and caspase-3/7 activation in HCT116 cells.

HCT116 cells were treated with several concentrations of BLE (bay leaf ethanol) (6; 3; 1.5 and 0.75 μ g GAE/ml); Negative control – (media without caspase-3/7 reagent) was used to ensure the cell culture media does not generate fluorescence signal. Before the first scan was performed by the IncuCyte ZOOM®, cells were exposed to the treatment for ~30min so time 0 is approximately 30 minutes are cells were exposed.

5.3.3 The effect of BLE and TE on proteins involved in apoptosis process

In the HCA-7 cell line, both extracts increased the expression of cleaved caspase-3 and cleaved PARP and the increase was comparable to that of the caspase-3 activating drug - Etoposide. Concerning p53, BLE did not affect its expression whilst TE reduced it slightly (Figures 5.4a and 5.4b). In addition, TE also slightly reduced the expression of cyclin D1; BLE no consistent data could be obtained (Figure 5.5a and 5.5b).

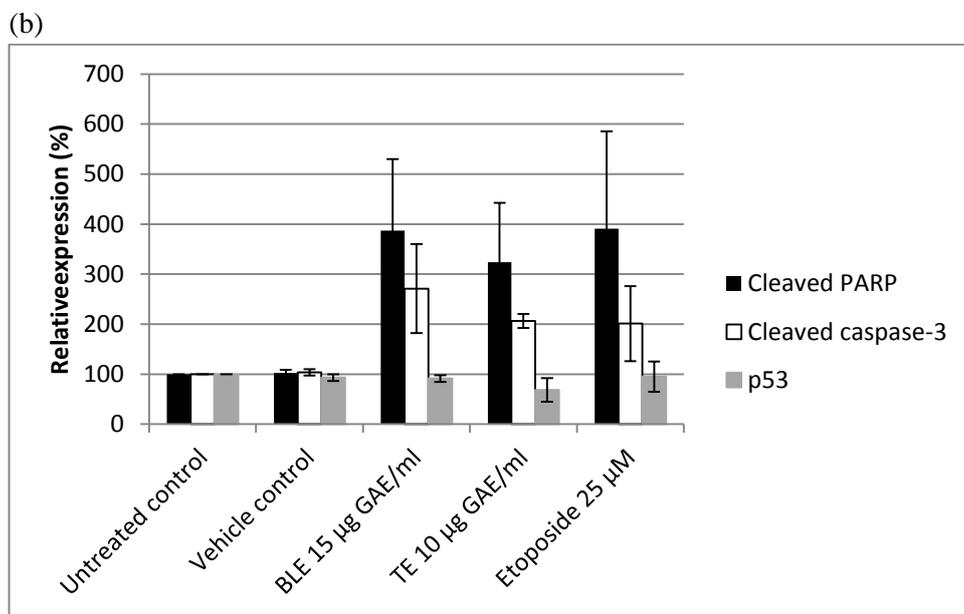
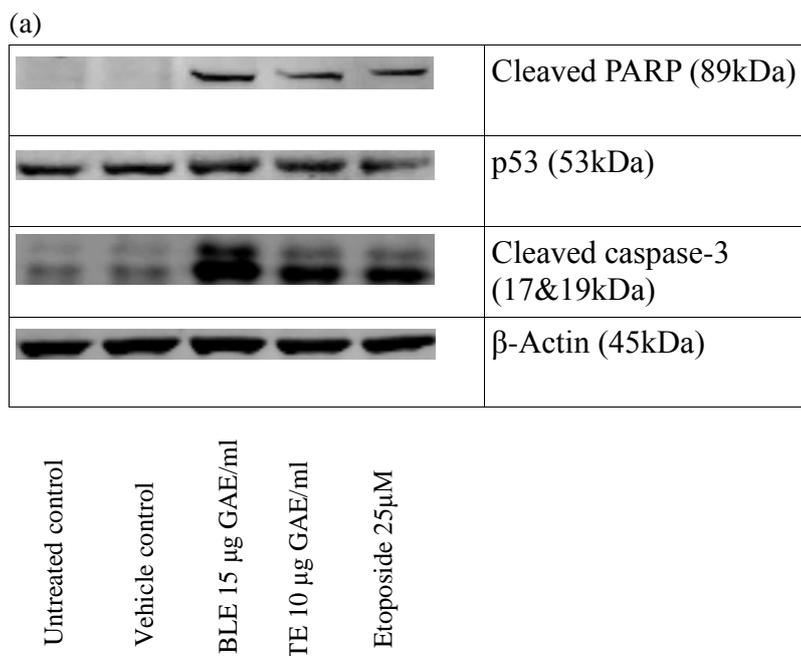


Figure 5.4 BLE and TE effect on proteins markers for apoptosis in HCA-7 cell line.

(a) Western blot; (b) Quantitative analysis of western blot bands. Cells were treated for 24 hours with bay leaf (BLE 15 μ g GAE/ml), turmeric (TE 10 μ g GAE/ml) and Etoposide 25 μ M, which was used as a positive control for caspase-3 activation. Protein expression was normalised against β -Actin and expressed relative to untreated control, where control is 100%. Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v), the highest amount found in the extracts. Data expressed as mean (n=3), \pm SD.

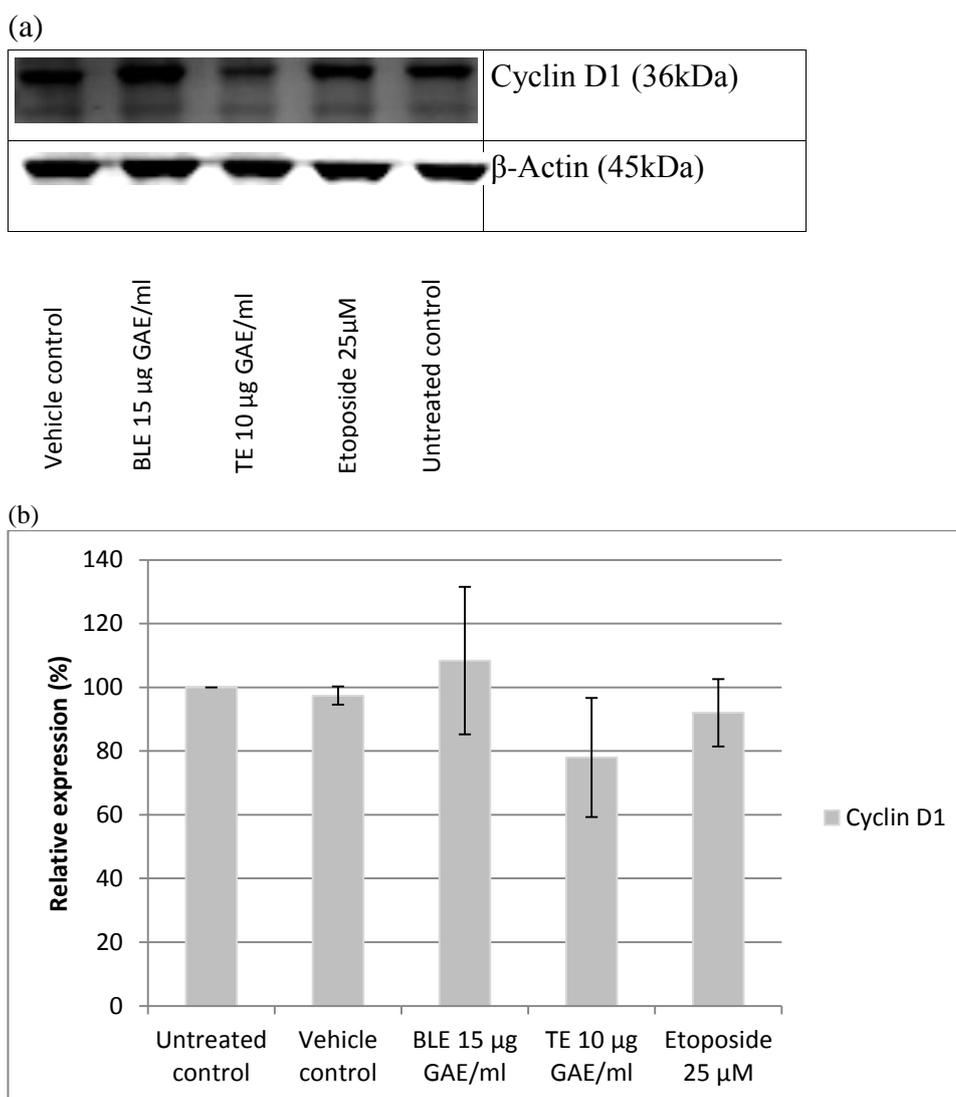
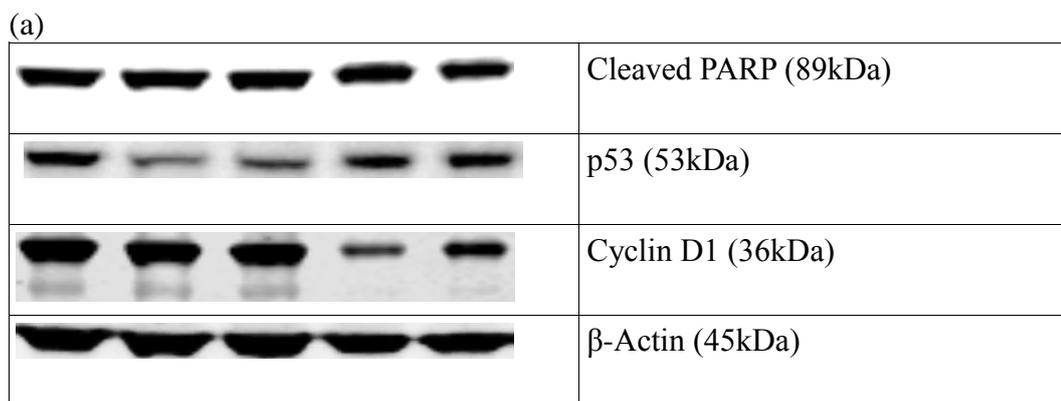


Figure 5.5 BLE and TE effect on cyclin D1 expression in HCA-7 cell line.

(a) Western blot; Cells were treated for 24 hours with bay leaf (BLE 15 μ g GAE/ml), turmeric (TE 10 μ g GAE/ml) and Etoposide 25 μ M. Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v), the highest amount found in the extracts. (b) Quantitative analysis of western blot bands. Protein expression was normalised against β -Actin and expressed relative to untreated control, where control is 100%. Data expressed as mean (n=3), \pm SD.

For the HCT116 cell line TE and BLE upregulated the expression of p53, and reduced the expression of cyclin D1. The results for cleaved PARP and cleaved caspase-3 were, respectively, anomalous and inconsistent with the former being detected in both control and experiments (Figure 5.6).



Etoposide 25 μ M
 Untreated control
 Vehicle control
 BLE 15 μ g GAE/ml
 TE 10 μ g GAE/ml

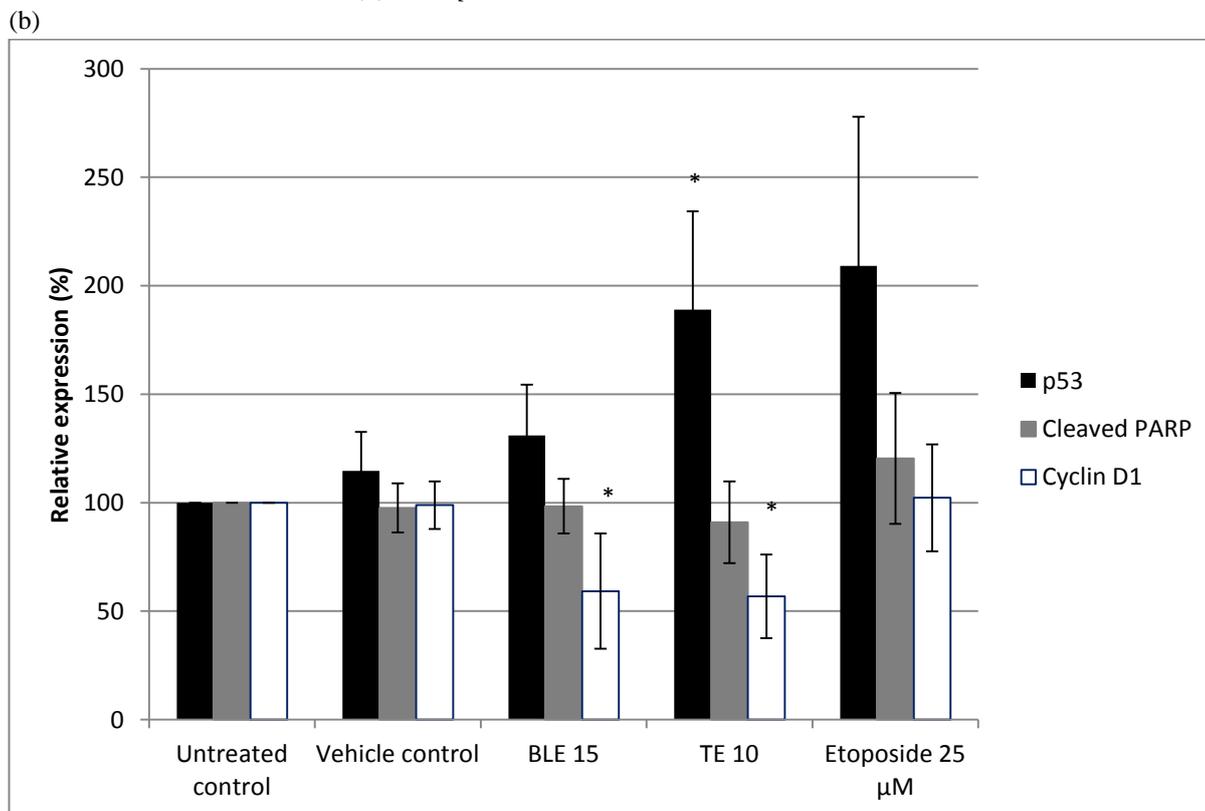


Figure 5.6 BLE and TE effect on proteins markers for apoptosis in HCT116 cell line.

(a) Western blot; Cells were treated for 24 hours with bay leaf (BLE 15 μ g GAE/ml), turmeric (TE 10 μ g GAE/ml) and Etoposide 25 μ M, which was used as a positive control for caspase-3 activation. Untreated control contained just DMEM with 10% FBS, vehicle control - ethanol (0.4% v/v), the highest amount found in the extracts. (b) Quantitative analysis of western blot bands. Protein expression was normalised against β -Actin and expressed relative to untreated control, where control is 100%. Data expressed as mean (n=3), \pm SD. *Statistically significant difference between untreated control and treatment with BLE or TE.

5.4 Discussion

The main aim of this study was to determine if the CHS extracts were able to induce apoptosis and to elucidate their mechanisms. This study revealed that treating CRC cells, HCA-7 and HCT116, with CHS extracts resulted in an increased number of cells in sub G1 phase, which indicated that apoptosis was induced (Hong et al. 2014; T. Basu et al. 2016). The results of the present study are in line with Dimas et al. (2015) who also reported that whole turmeric ethanol extract increased the number of HCT116 cells in sub G1 phase, whilst there were no significant changes in other cell cycle phases (Dimas et al. 2015). Abdullah et al. (2010) found that ginger extract induced apoptosis in HT-29 and HCT116 cells and also caused cell cycle arrest at G0/G1 checkpoint (Abdullah et al. 2010). *Curcuma manga*, commonly referred to as mango ginger, which belongs to the same family as turmeric and ginger, has been shown to induce apoptosis in HT-29 cells (Hong et al. 2016).

One clear observation from the present study is that the potency of the CHS varied depending on the cell line with the effect on the HCA-7 cells (COX-2 expressing cells) being greater than on the HCT116 cells (COX-2 negative cells). In addition, more apoptotic cells were detected after a longer treatment period of 48 hours compared to 24 hours, which is in line with cell viability data where the IC50 values were lower after 48-hour treatment in comparison to 24h (see Chapter 2). Interestingly, two CHS (GE, BLE) and a combination of BLTE produced a stronger apoptotic effect than the selective COX-2 inhibitor drug – Celecoxib (50 μ M), whilst TE and SGE were equally as potent the drug, which again highlights their therapeutic potential as identified in previous chapters. The combinations (BLTE, 24h, HCA-7; and BLSE, 48h, HCT116) appeared to be synergistic as they produced stronger apoptotic effects than their individual CHS constituents. A synergistic effect of BLTE and SGE was also observed during growth inhibition for HCA-7 cell line. The fact that for the other combinations no synergy was evident again highlights how interactions between constituents impacts on the biological potency of the CHS in different ways.

Studies on the polyphenol constituents of these CHS and their ability to induce apoptosis in CRC cells provide some insight into how whole extracts work to suppress CRC cell growth. One study by Rajitha et al (2016) found that curcumin (20 μ M) and two of its synthetic analogues (EF31 and UBS109) caused G0/G1 arrest, which was achieved by downregulating cyclin D1 expression in HT-29 and HCT116 cells. Major active

components of ginger are also capable of inducing apoptosis in cancer cells. Ryu and Chung (2015) reported that [6]-gingerol (30 μM), one of the major polyphenols found in ginger, induced apoptosis in HCT116 cell causing cells to accumulate in sub G1 phase, and these findings are similar to the effect of whole ginger extract on cell cycle distribution in the present study. Another study also found that [6]-gingerol induced apoptosis and caused cell cycle arrest at G1 phase in several CRC cells (SW480, LoVo and HCT116) (Lee et al. 2008).

Following the treatment with CHS and their combinations no increase in the G2 phase, which is associated with cell cycle arrest (G2/M arrest) was observed (Tang et al. 2013), however, Hanif et al. (1997) reported that treatment with curcumin (10-75 μM) resulted in increased number of cells in G2/M phase in dose and time-dependent manner (HT-29 and HCT15, both CRC) (Hanif et al. 1997). This could be explained by the different cell lines, the amount of curcumin used, and the fact that the whole turmeric extract was used in the present study thus dealing with a complex matrix of constituents.

To further investigate, and elucidate, the effect of CHS on the cell cycle and apoptosis in HCA-7 cells, the effect of BLE and TE on the expression of key protein markers of the cell cycle and apoptosis was investigated. The pro-apoptotic action of these CHS was further confirmed by activation of executor caspases-3 and 7 by BLE and the increase in cleaved caspase-3 and cleaved PARP by TE and BLE in HCA-7 cells. The effect on the former indicates irreversible apoptosis (Elmore 2007) and was comparable to that of the caspase activating drug Etoposide (25 μM), a chemotherapy drug (Rezonja et al. 2013) again reinforcing the biological significance of the whole CHS. PARP protein is considered a hallmark of the apoptosis process, and it is targeted by caspase-3 (Poirier et al. 2002; Chaitanya et al. 2010; Hassen et al. 2012). The results of the present study further strengthen the evidence that these extracts can target the pathways involving caspase-3 and cleaved PARP, however, the results for the HCT116 could not be confirmed because the PARP western blot data were anomalous as cleaved PARP was detected in the control cells suggesting that there were cells undergoing apoptosis as the activation of PARP by its cleave only occurs when this process is induced (O'Brien et al. 2001). This also coincided with caspase-3/7 activation (IncuCyte data) for HCT116 cell line, where after 30 hours of treatment untreated control exceeded caspase activation of the treatment (BLE 6 μg GAE/ml), again suggesting that when confluency is achieved some cells start dying. Another possible explanation is the difference in cell number, as untreated controls

continued growing whilst BLE (6 µg GAE/ml) inhibits cell growth. So the effects of CHS on caspase-3 activation and PARP in HCT116 cell line need to be further analysing for both cleaved and intact PARP protein. Several treatment time frames could be tested too, as for example, one study found that cleaved PARP in HCT116 cell line only became apparent after 48 and 72-hour treatment with mastic gum extract (Balan et al. 2005). However, it has been reported that [10]-gingerol (30 µM, 24h treatment), which is found in ginger, induced apoptosis in HCT116 cells by activating caspase signalling, which resulted in increased cleaved PARP (Ryu & Chung 2014). Polyphenol-rich Chinese olive fruit extract also induced cleaved PARP in HCT116 cells (24h treatment) (Hsieh et al. 2016). Polyphenol-rich longan seed extract induced cleavage of PARP in three CRC cell lines - Colo 320DM, SW480 and HT-29 (48h treatment) (Chung et al. 2010). So these studies demonstrate that PARP can be cleaved in HCT116 cells using 24h treatment.

There are very few studies that have investigated the pro-apoptotic mechanisms involving caspase-3 and cleavage of PARP of whole CHS extracts in CRC cells. Rodd et al. (2015) reported that unfractionated and low molecular mass fractions of bay leaf were pro-apoptotic in HT-29 CRC cells (based on caspase-3/7 activity). There are however a number of studies showing that phenolic constituents of the CHS under investigation, such as curcumin, are capable of inducing apoptosis via the activation of caspase-3. Song et al. (2005) reported that curcumin induced apoptosis in HT-29 cells by activating caspase-3 and another pro-apoptotic protein – Bax; it also downregulated anti-apoptotic protein - Bcl-2. Radhakrishnan et al. (2014) reported that [6]-gingerol (50-200 µM), one of major active constituents in ginger, induced apoptosis in SW480 CRC cells by activating caspases (including caspase-3) and cleaving PARP, whilst leaving healthy colon cells unharmed (up to 500 µM) (Radhakrishnan et al. 2014).

Regarding the effect of the CHs on another pro-apoptotic protein p53, there was a clear difference between the HCA-7 and HCT116 cells. In the HCT116 cell line, TE and BLE upregulated p53 expression, which was not the case for HCA-7 cells. p53 is a tumour suppressor protein involved in apoptosis, and cancer cells often have mutations in this protein in order to avoid apoptosis (Fernald 2013). Loss of p53 function is also linked to chemo-resistance. Several studies have looked at the effect of CHS and food polyphenols on p53 expression in CRC cells. Song et al. (2005) reported that curcumin (50 µM) upregulated p53 expression in HT-29 cells (Song et al. 2005). Another study found that [6]-gingerol upregulated p53 in LoVo CRC cells (Lin et al. 2012). Baek et al. (2004)

reported that EGCG, a polyphenol found in tea, upregulated p53 in HCT116 cells (Baek et al. 2004). Hong et al. (2016) found that *Curcuma mangga* caused upregulation of p53 in HT-29 cells. Polyphenol-rich olive oil extract also upregulated p53 expression in HCT116 and RKO (Fini et al. 2008) Furthermore, in a human study, administration of curcumin was shown to increase p53 expression in CRC tissues (He et al. 2011).

The lack of an effect on p53 could be explained by the fact that HCA-7 has partially mutated/dysfunctional p53 so, with regards to TE, the slight reduction observed in the present study could be a reduction in the mutated p53, which can result in sensitisation of chemo resistance cell to undergo apoptosis thus circumventing chemo resistance (Thongrakard et al. 2014). Indeed, it has been shown that turmeric and curcumin are able to degrade mutated p53 in skin cancer cells (Thongrakard et al. 2014). Another study demonstrated that curcumin reduced levels of p53 expression in the CRC cell line HCT15, which like HCA-7, also possesses mutated p53 (Shehzad et al. 2013).

p53 controls the check point that allows cell progression from G1 to S phase, and upregulation of p53 is linked to a decrease in cyclin D1, which causes cell cycle arrest and halts cell cycle progression until the DNA is repaired or the cell undergoes apoptosis (Meeran & Katiyar 2008). In the present study upregulation of p53 by TE and BLE coincided with downregulation of cyclin D1 in the HCT116 cell line so it is possible that upregulation of p53 by TE and BLE resulted in downregulation of cyclin D1 in HCT116 cells. Cyclin D1 is another important target in chemo prevention and treatment (Alao 2007). In the HCA-7 cell line, only TE caused the downregulation of cyclin D1; the source of this effect is unclear in light of the effect of this CHS on p53 in this cell line although one could speculate that as the reduction in mutated p53 is a pro-apoptotic action it could lead to the downregulation of cyclin D1. Targeting cyclin D1 expression could be another of the ways by which these extracts control cell division and trigger apoptosis. There are studies conducted on the effect of CHS on cyclin D1 expression in CRC cells. Lee et al. (2008) demonstrated that [6]-gingerol (200 μ M), one of the polyphenols found in ginger, induced apoptosis in HCT116, SW480 and Caco-2 cells by reducing the expression of cyclin D1 (Lee et al. 2008). Kunnumakkara et al. (2009) study reported that curcumin (10 and 50 μ M) reduced cyclin D1 expression in HCT116 cells. Whilst Elkady et al. (2014) found that ginger extract decreased cyclin D1 and upregulated p53 in HCT116 cells, which is similar to the effect of TE and BLE observed in the present study (Elkady et al. 2014). Whilst polyphenol-rich longan seed extract also downregulated cyclin D1 expression in

two CRC cell lines - Colo 320DM and SW480 (Chung et al. 2010). These studies support our findings that CHS and polyphenols present in CHS under investigation can target cyclin D1 expression in cancer cells.

Based on these results and those of Chapter 3 for the HCA-7 cells, it is possible, in light of the findings reported in Chapter 3, that the CHS induction of apoptosis occurs via the downregulation of COX-2 expression and inhibition of its activity. However, the results for the HCT116 cell line, which is COX-2 negative, show clearly that downregulation/inhibition of COX-2 by the CHs is not necessary to induce apoptosis. Furthermore, in the literature there are reports that the selective COX-2 inhibitor - Celecoxib can induce apoptosis in COX-2 negative CRC cells (He et al. 2008). To determine definitively that COX-2 is essential in the induction of apoptosis, its expression in the HCA-7 cell line would need to be blocked. Moreover, it is also unclear whether p53 upregulation is necessary for the induction of apoptosis by CHS, because these CHS induced apoptosis in HCA-7 cells that have mutated p53, but on other hand, they also upregulated p53, inhibited the growth and induced apoptosis in HCT116 cells, which possess wild type and not mutated p53.

5.5 Conclusion

The results of this study show that CHS extracts and their combinations can induce apoptosis in CRC cells and suggest that the effect of the CHS on particular molecular targets involved in apoptosis is cell line dependent. Bearing in mind that the polyphenols in the CHS are known to have pleotropic effects and may thus target multiples molecules (Aggarwal et al. 2003; Ravindran et al. 2009; Shehzad et al. 2010), it is very likely that the CHS investigated also modulate apoptosis through other molecular targets that have not been investigated in the present study.

Chapter 6 General discussion

6.1 Introduction

Culinary herbs and spices (CHS) have a very high anti-oxidant capacity per dry weight, and also possess anti-inflammatory activity. These properties are related to beneficial health properties, such as protection against cardiovascular diseases, neurodegenerative diseases, type II diabetes and cancer. Hence the interest in studying these properties of CHS has grown over the last two decades (Manach et al. 2004; Opara & Chohan 2014; Vallverdú-Queralt et al. 2014; Mueller et al. 2010; Imran et al. 2017). Polyphenols are the major active constituents in CHS and so far in the scientific community the major focus has been on isolated dietary phenolic compounds such as curcumin (a major polyphenol found in turmeric), gingerol (found in ginger), carnosic acid and rosmarinic acid (both found in sage and rosemary) (Surh 1999; Prasad et al. 2014; Tsai et al. 2011; Kunnumakkara et al. 2016). For example curcumin, which is one of the most studied polyphenols has had since 2003 over 5600 citations (Aggarwal et al. 2013) showing that it possesses various health beneficial properties especially its ability to target a variety of anti-carcinogenic mechanisms including those involved in the development of CRC one of the most common cancers in high income countries (Aggarwal et al. 2003; Aggarwal & Harikumar 2009; Cruz-Correa et al. 2006; Aggarwal et al. 2011). However, the literature shows that isolated compounds, including curcumin, do not always produce the same/best results as their host food matrices (Williamson 2001; Liu 2004; Aggarwal et al. 2013). For example, it has been shown that whole turmeric is more effective than curcumin at inhibiting growth of various cancer cells including CRC (HCT116 and HT-29 cell lines) (Kim et al. 2012). Furthermore, it has been shown that curcumin-free turmeric extract possess anti-inflammatory activity (Yue et al. 2016). These examples show that whole food extracts such as CHS have the potential for be a functional food with additional health properties beyond basic nutrition (Liu 2003). It is this potential that provided, in part, the foundation for the present study as very few studies have investigated the effect of CHS extracts on cancer so knowledge in, and understanding of the anti-carcinogenic mechanisms of CHS are mainly unknown. Finally, again with specific reference to CRC, although over the last decade the survival of patients with this cancer has improved, conventional cancer therapies are still very toxic and produce numerous side effects (Palumbo et al. 2013; Siegel et al. 2014). Therefore, there is a need to explore the safer alternatives therapies, and the identification of natural compounds and foods which could

prevent this disease in the first place (Johnson & Mukhtar 2007; Russo 2007). Hence, the aim of this study was to establish the most potent CHS at inhibiting CRC cell growth *in vitro*, and elucidate their potential anti-carcinogenic mechanisms, specifically focusing on two targets associated with the development of CRC, COX-2 and β -catenin as well as key proteins of apoptosis, namely p53, caspase-3, cyclin D1 and PARP. To further understand the chemo-preventative and therapeutic potential of CHS, their action in combination was also investigated.

6.2 CHS effect on CRC cell growth and viability

The investigation into the effects of the CHS, both individually and in combination, showed that the majority inhibited CRC cell growth, reduced their viability and also had a cytotoxic effect. The latter was shown to be the case for two of the most potent CHS - turmeric in ethanol (TE) and bay leaf in ethanol (BLE) (Chapter 2). There were some differences in the IC₅₀ values between the different cell lines used. HCT116 was the most sensitive to the CHS with IC₅₀ values ranging from 1.4 to 5.6 $\mu\text{g GAE/ml}$, whilst HCA-7 was more resistant with the IC₅₀ values from 3 to 17.1 $\mu\text{g GAE/ml}$ (for more details see the Chapter 2, table 2). A similar pattern was observed in previous work (Baker 2012; Jaksevicius 2012), where HCT116 cells were more sensitive to CHS extracts in comparison to another CRC cell line - HT-29, another CRC cell line that expresses COX-2, but at the lower level than HCA-7 (Shao et al. 2000). The HCA-7 cell line is not commonly used in the published studies, compared to more commonly used cell lines like HT-29, HCT116 or SW480, and there are no data in the literature about the growth inhibitory effect of CHS on this cell line. The IC₅₀ values for CCL235 cell line ranged from 2.3 to 5.4 $\mu\text{g/ml GAE}$ and these findings clearly indicates that the majority of tested extracts inhibited the growth of all three tested CRC cell lines used in the present study (HCT116, CCL235 and HCA-7; see Chapter 2), underlining their potency. TE was the most potent extract in most of the growth inhibition and cell viability experiments which is not surprising as its major phenolic constituent – curcumin is well-known for its anti-carcinogenic properties (Kunnumakkara et al. 2009; Uzzan & Benamouzig 2016; Qadir et al. 2016). However, one has to bear in mind that turmeric possesses many another bioactive compounds such as turmerones, elemene, cyclocurcumin, some of which also possess anti-carcinogenic properties which were also likely to have influenced the effect of TE on CRC cell growth and viability reported in Chapter 2 through interactions within the food matrix (Yue et al. 2010; Aggarwal et al. 2013; Yue et al. 2016). Although TE (IC₅₀

values ranging from 1.4 to 3.0 μg GAE/ml) was the most potent, some other CHS (BLE, GE) had the IC₅₀ values (2.5 - 5.5 μg GAE/ml for GE and 2.7 – 4.7 μg GAE/ml for BLE) very close to TE, and the actions of other CHS extracts clearly indicate that the interaction of their bioactive compounds also needs to be further investigated. Such interactions are likely to be key as the CHS investigated produced significant inhibitory and cytotoxicity effects despite their approximate concentration of the major individual polyphenols present in CHS being at the lower range (according to approximate calculations, in a low micro molar range (2 to 22.5 μM), see Appendix 2) in comparison to the concentrations of individual dietary polyphenols such as curcumin gingerols, rosmarinic acid and carnosic acid, which have been used in numerous studies at the concentrations ranging from 1 to 200 μM (Lev-Ari et al. 2005; Shakibaei et al. 2013; Moore et al. 2016; Lim et al. 2014; Lv et al. 2012).

In theory, the IC₅₀ value of some CHS used in this study could be achieved in the gut, for example, the IC₅₀ value of bay leaf aqueous extract was 200 $\mu\text{g}/\text{ml}$ of dry weight, which would be 200 mg/l, suggesting that such concentration can be easily achieved in a cooked meal with bay leaf by adding 200 mg or more of dry bay leaf into the meal that has a volume of approximately 1L. However, it is unlikely that the same amount of active components of bay leaf that were extracted, using sonicator, will be extracted in the gut when it is mixed with other foods and passed the digestion. Nevertheless, there is evidence that polyphenols interact with micronutrients (fat, proteins and carbohydrates) in the intestinal tract and some of these interactions can enhance their absorption (Jakobek 2014). Thus, to shed light on the bioavailability of the bioactive compounds in the CHS, an *in vivo* study could be performed to replicate the *in vitro* findings of the present study using CHS at the concentrations used in the present study.

6.3 CHS effect on COX-2 activity and expression

CRC are promoted and stimulated by chronic inflammation, whilst COX-2 and its product prostaglandin E₂ (PGE₂) play a major role in this process (for more detail on CRC and inflammation see Chapter 3). The present study demonstrated that CHS extracts downregulated COX-2 expression, and also inhibited COX-2 activity by reducing PGE₂ release (see Chapter 3). Inhibition of COX-2 activity was confirmed with a COX-2 inhibition assay, which showed that TE and BLE, two of the most potent CHS, inhibited COX-2 activity *in vitro*. These results prove that PGE₂ reduction was not purely due to a reduction in COX-2 expression, and that the CHS target both COX-2 activity and

expression. The effect of the most potent CHS was very close to that of the selective COX-2 inhibitor – Celecoxib (50 μ M) which demonstrates that these CHS are of potential in the prevention of CRC and could also be used as a complementary treatment (Garcea et al. 2005; Hatcher et al. 2008; Carroll et al. 2011). Currently, curcumin is being investigated for its complementary potential as it is being tested in combination with a chemotherapy drug used commonly to treat cancer, 5-fluorouracil (5FU), in patients who have 5FU-resistant metastatic colon cancer (ClinicalTrials.gov 2016). Thus, light of the results of the present study the efficacy of CHS, rich in this and other polyphenols, in combination with established chemotherapeutic drugs should be investigated.

Celecoxib has severe cardiovascular side effects, and hence, is not very suitable for individuals with normal risk of CRC (Arber 2008). Other anti-inflammatory drugs such as aspirin, and non-steroidal anti-inflammatory drugs that have been used for purpose of prevention of CRC also have severe side effects such as internal bleeding (Dulai et al. 2016), so natural compounds and food extracts including CHS that possess anti-inflammatory activity and inhibit COX-2 activity could be a safe alternative to these anti-inflammatory drugs. One of the reasons for absence of side effects by natural COX-2 inhibitors are that CHS are more selective towards COX-2, which is only induced to the inflammatory stimulus, and have little effect on COX-1, which is expressed in most tissues including healthy intestinal tract cells (Arber 2008; Yi & Wetzstein 2010). Another reason could be their pleotropic action, which is discussed below. Although the present study did not test whether CHS inhibit COX-1 activity, Yi and Wetzstein (2009) showed that several CHS including rosemary and sage selectively inhibited COX-2 activity, especially at a lower concentration of 1 mg of dry weight per ml in comparison to 10 mg/ml (Yi & Wetzstein 2009). The Yi and Wetzstein (2009) study did not use a control drug like Celecoxib but their results support the findings of the present study, which further strengthens the chemopreventative/therapeutic potential of the CHS identified in the present study. To further confirm the effects of CHS on COX-2 an animal study could be performed to determine whether these positive effects on COX-2 can be replicated *in vivo*.

Regarding the mechanism of action by which the CHS inhibit COX-2, literature, and the present study, indicate that their action is pleotropic. In the present study, the CHS targeted both COX-2 activity and expression possibly via more than one molecular target/mechanism. Turmeric has been shown to affect COX-2 expression by targeting the transcription factor NF- κ B, which is involved in regulating COX-2 expression, and it is

believed to be another factor that minimises side effects, as NF- κ B is highly overexpressed in CRC cells (Surh et al. 2001; Romier et al. 2009). In addition, it has been shown that curcumin, which is a major active polyphenol in turmeric, inhibits PGE2 synthase-activity, which is required to convert PGH2 into PGE2 (Koeberle et al. 2009) thus reinforcing the pleiotropic action of turmeric. Another possible COX-2 inhibition mechanism is through preventing the release of arachidonic acid, which is a substrate for COX-2 (Hong et al. 2004). It has been shown that curcumin, a major polyphenol in turmeric, prevented the release of arachidonic acid (Hong et al. 2004).

6.4 CHS effect on Wnt signalling

The Wnt/ β -catenin signalling pathway plays an important role in the development of CRC, and over half of CRC tumours has mutations in this pathway (Sparks et al. 1998). As a result the phosphorylation of β -catenin, which is a central molecule in this pathway, is disrupted and unphosphorylated β -catenin is then translocated into the nucleus and triggers the transcription of precancerous genes (for more details about this pathway see Chapter 4) (MacDonald et al. 2009; Giles et al. 2003; Barker & Clevers 2006). Hence, β -catenin and its phosphorylation are a good target for cancer prevention and possibly treatment (Teiten et al. 2012). Results of the present study showed that two of the most potent CHS – TE and BLE did not have an effect on Wnt signalling pathway based on the effect on β -catenin phosphorylation and degradation (see Chapter 4). However, it is possible that the exposure times (3h and 24h) were not long enough as there are studies showing that the effect on β -catenin phosphorylation was only observed after the treatment for 32-48 hours (Jaiswal et al. 2002). The choice of cell line could be another reason why CHS did not have an effect on this pathway. HCT116 has a mutated β -catenin, which cannot be phosphorylated, thus it could be the reason why β -catenin phosphorylation remained unchanged following the treatment with CHS. Although, there was no effect observed in HCA-7 cell line, in the future it is to gain more insight into whether this pathway plays a role in the effect of CHS on other CRC cell lines.

The present study attempted to do a cell fractionation to see whether CHS have an effect on nuclear β -catenin as in the nucleus this compound binds to transcription factors and initiates transcription of genes involved in the development of CRC (for more details see Chapter 4). However, the effect on nuclear β -catenin could not be fully established due to what appeared to be contamination of the nuclear fraction as β -actin was present in the nuclear fraction. For future studies, the use of another marker such as lamin (which is only

present in the nucleus) would be needed for this experiment to ensure that the nuclear fraction is well-separated.

The absence of an effect of CHS on β -catenin could suggest that the CHS have an effect on Wnt/ β -catenin pathway by targeting some other downstream molecules in this pathway, such as p300 and T-cell factor-4 (TCF-4) as the literature provides evidence in their involvement in the effects of polyphenols found in CHS on the Wnt pathway. For example, it has been shown that curcumin suppressed CRC cell growth by targeting p300 and TCF-4, whilst cytosol and nuclear β -catenin remained unchanged (Ryu et al. 2008). Finally, it may be that another method is needed to evaluate the effect on Wnt/ β -catenin pathway. One such alternative is the luciferase reporter assay, which can be used to study this pathway (Zhang 2017). It has been shown that Reishi mushroom extract inhibited Wnt/ β -catenin signalling in breast cancer cells using the method above (Zhang 2017).

6.5 CHS effect on apoptosis and molecular targets involved in apoptosis

Apoptosis is a key process through which cells regulate their proliferation and death, and the induction of apoptosis by external agents including drugs, natural compounds and foods is a very desirable outcome in cancer treatment (Ghobrial et al. 2005). The results of the present study have shown that most tested CHS extracts and their combinations are capable of inducing apoptosis in CRC cells as there was a significant increase in number of cells in sub G1 phase. The effect was stronger after a longer exposure (48h vs 24h) (see Chapter 5). This study also looked at some molecular targets involved in apoptosis. It seems that CHS, specifically BLE target caspase-3/7 activation, whilst BLE and TE increased cleaved caspase-3 and cleaved PARP (in HCA-7 cells only), which indicates irreversible apoptosis, and is a much desired outcome in cancer treatment and prevention (Chaitanya et al. 2010; Hassen et al. 2012; Fernald 2013). In fact, the effect was similar to control drug – Etoposide which further supports the therapeutic potential of these CHS in CRC prevention and possibly treatment. In addition, TE, the most potent extract, also upregulated another important molecule p53 (in the HCT116 cell line only), which plays a key role in cell cycle progression and triggering apoptosis. Moreover, this effect coincided with downregulation of cyclin D1 a molecule responsible for cell progression through the cell cycle thus TE slowed down cell proliferation via its effect on this protein. Whilst for HCA-7, which has a mutated p53, TE reduced its expression, once again indicating the pleiotropic effect of this plant-based extracts. Furthermore, in the present study bay leaf (BLE) appeared to have little effect on the expression of p53 in HCA-7 cells suggesting

that its apoptotic action does not involve this protein in this COX-2 positive cell line, although further studies are required to confirm this observation.

The cleaved PARP data for the HCT116 cell line was anomalous as cleaved PARP was also detected in untreated control CRC cells. This result could partially be explained by the fact that there were some dying/apoptotic cells in the control sample as by the end of the treatment control cells reached full confluence, and it is possible that some of these cells were dying at the time when they were lysed. In addition, to confirm the effect of CHS on cleaved PARP by CHS, it would be a good idea to detect both cleaved and intact PARP which would confirm whether the cleaving of PARP was caused by the treatment with CHS.

6.6 Synergy

The use of the extracts in combination in the present study further highlighted the possible role and importance of interactions between bioactive compounds in a food matrix. None of the combinations had a lower IC50 value than the most potent individual CHS extract – TE, however, the interaction factor (IF) index suggests that several combinations had a synergistic effect (see Chapter 2). However, the synergistic effect was not consistent enough to draw firm conclusions and the effect varied across the experiments and cell lines. The only consistency was that the combinations (SGE; BLTE; SBLE) with the best IF index for HCA-7 cell line were also the most potent at reducing COX-2 activity and expression (see Chapters 2 and 3). It is clear therefore that the synergistic interactions and potential benefits of combining several CHS need to be studied further using different ratios of the same combination thus establishing the best possible combinations and ratios using isobolographic analysis (Williamson 2001; Gawlik-Dziki 2011). In addition, a further study could be done combining more than two CHS or combining CHS with other foods with which they are normally combined for culinary purposes - vegetables and meats. A larger variety of polyphenols available in the mixture from different herbs and spices may enhance their synergy further than any synergy occurring between polyphenols from two CHS. For example, it was found that combining four fruits enhanced their total antioxidant activity (Liu 2004). Another study found that for optimal synergistic activity four tea polyphenols were required in comparison to ECGC alone, which is a major active polyphenol found in tea, however, the synergic effect was not consistent across all the experiment performed in the study (Williams et al. 2003). This inconsistent synergistic effect across different experiments was also observed in the

present study, and once again highlights the complexity of synergistic interactions found in CHS.

With regards to the interactions between individual constituents present in CHS, it is very likely that a major phenolic constituent – curcumin, present in turmeric, interacts with other compounds such as turmerin, turmerone, elemene, furanodiene, curdione, bisacurone, cyclocurcumin, calebin A, and germacrone (Aggarwal et al. 2013). For example, Kim et al. (2012) demonstrated that turmeric was more effective at inhibiting growth of breast cancer cells in comparison to the same amount of curcumin present in whole turmeric extract. Another study demonstrated that whole turmeric ethanol extract had a stronger growth inhibitory effect against CRC cells (HT-29 and HCT116) in comparison to curcumin alone (Yue et al. 2016). The authors of the study attributed the enhanced growth inhibitory effect of the turmeric extract to the presence of turmerones as a combination of curcumin and turmerones produced a stronger inhibition in comparison to curcumin alone, whilst turmerones alone (at the same concentration present in the whole turmeric extract) did not inhibit CRC cell growth at all. These two examples once again demonstrates the potential synergistic interactions between compounds present in whole turmeric extract, and hence provide a strong argument for the use whole extracts of CHS instead of isolated polyphenolic compounds .

6.7 Limitations

The present study has shed some light on the chemopreventative and chemotherapeutic potential of CHS in relation to CRC. However, there are questions that still need to be answered to understand more fully what the capabilities of these foods are. One area that needs to be addressed is bioavailability and metabolism. It has been shown that many of the polyphenols present in CHS are poorly absorbed into the systemic circulation and even when absorbed they are quickly metabolised (conjugated) and then excreted (Manach et al. 2004; D'Archivio et al. 2007; Percival et al. 2012). However, to be beneficial in CRC prevention and treatment, CHS and their active constituents need to get into contact with precancerous and cancer cells in the gut without being absorbed into the blood. In fact, it had been shown that curcumin can be absorbed by cancer cells/tumours *in vivo* (Carroll et al. 2011) suggesting that polyphenols present in CHS could be absorbed by tumour cells and be beneficial in prevention and treatment of CRC. Polyphenols in the intestinal tract can also be metabolised by gut bacteria which could lower the amount of the active parent polyphenols present in CHC (Hidalgo et al. 2012). For example, it has

been shown that curcumin is converted by gut bacteria into tetrahydro-curcumin (M1), hexahydro-curcumin (M2) and octahydro-curcumin (M3) (Li et al. 2017). According to another study, curcumin also can be converted to desmethoxycurcumin, demethylcurcumin, demethyldemethoxycurcumin and bisdemethylcurcumin (Burapan et al. 2017), and some of these metabolites possess similar anti-inflammatory and anti-proliferative activity to their parent compound curcumin (Sandur et al. 2007).

The present study used only three CRC cell lines so to further confirm the anti-carcinogenic effects of CHS discovered in this study, CHS could be tested on a wider panel of CRC cell lines with different mutations, such as SW480, CCL-221; CCL-228, LoVo DiFi (derived from the patient with familial adenomatous polyposis) (Khelwatty et al. 2010; Shakoori et al. 2005).

Finally, little is known about the stability of polyphenols present in CHS in cell culture media. It has been shown that polyphenols are more stable in the plasma in comparison to cell culture media (DMEM) (Xiao & Högger 2015). In the present study, the total phenolic content (TPC) could not be measured because the red colour of cell culture media interfered with absorption readings however other methods such as HPLC could be used to assess for how long the active components of CHS are stable/remain undegraded in cell culture media

6.8 Future work

The present study demonstrated that CHS are potent inhibitors of CRC cell growth and also are capable of modulating several key molecular targets in CRC such as COX-2, PPAR and p53. Moreover, their efficacy was similar to drugs such as celecoxib and etoposide which demonstrates the therapeutic potential of these CHS hence they should be further investigated both *in vitro* and *in vivo*. The latter could involve the use of CRC animal models or human subjects, who have normal or high risk of CRC, to assess if consumption of CHS can prevent or reduce the risk of CRC. The human study could follow a research design similar to that of Carrol et al. who showed that 4g/day of curcumin reduced the number of aberrant crypt foci, which can form colorectal polyps, in human subjects, and later develop into cancerous lesions (Carroll et al. 2011). Another human study could involve instructing subjects to consume CHS for several weeks/months to see whether their blood could have an enhanced ability to fight cancer cell growth *ex vivo*, One human study demonstrated consuming various spices (including ginger, turmeric and rosemary) reduced several blood anti-inflammatory markers (IL-6, TNF-a, and IL-1a),

ex vivo (Percival et al. 2012) and thus demonstrated that bioactive components from CHS were available, post consumption, digestion and absorption, to generate an anti-inflammatory effect.

CHS are often consumed as a part of a cooked meal and it has been shown that cooking methods and *in vitro* digestion do not lower the phenolic content of CHS (Chohan et al. 2012). Moreover, these cooked CHS also maintained anti-inflammatory activity and in some cases this activity was increased (Chohan et al. 2012; Baker et al. 2013). However, there are no studies showing whether cooked and digested CHS mixed with other foods can inhibit cancer cell growth and retain other anti-carcinogenic properties studied in the present study so this area needs to be studied further.

Chemo-resistance is a big issue in cancer therapies and there is evidence in the literature that plant phytochemicals including polyphenols can re-sensitise or even kill chemo-resistant cancer cells (Wang et al. 2015). For example, it has been reported that curcumin in combination with dasatinib, a cancer drug used to treat leukaemia and some other cancers, eliminated chemo-resistance in CRC cells (Nautiyal et al. 2011). Thus, the effect of CHS on cancer stem and chemo-resistant cells is another area which should be investigated.

Cancer stem cells are a relatively new topic in cancer research and it is believed they are the key factors for chemo-resistance and re-occurrence of cancer (Li et al. 2011; Roy & Majumdar 2012; Butler et al. 2017). It has been shown that curcumin can target cancer stem cells (Li & Zhang 2014; Buhrmann et al. 2014; Huang et al. 2016), however, there are no studies showing if any of CHS could target cancer stem cells. Hence, future studies are needed to investigate the effect of CHS on cancer stem cells.

Although the present study focused on CHS extracts rather than isolated polyphenols, it would be interesting to determine the phenolic composition of these extracts, identify the most active components, and then make a direct comparison between the activity of the isolated polyphenol and the extract.

Over recent years three-dimensional (3D) cell culture systems have gained increasing interest in studying cancer. Unlike 2D tissue culture, which is limited to a cell monolayer, a 3D cell culture system creates a microenvironment that allows cells, in this case cancer cells, to grow in all directions and interact with stromal cells. Hence, the results of 3D studies are more to indicate what is happening *in vivo*. (Edmondson et al. 2014). 3D tissue culture has been used to study the effect of individual polyphenols, specifically curcumin,

on CRC viability (Shakibaei et al. 2013) thus the effect of CHS could be tested using a 3D cell culture model to see whether the results of 2D system obtained in the present study can be replicated in a 3D environment.

Evidence is emerging that microbiome composition is an important factor in the development of CRC and one of the ways to prevent CRC is to maintain or modulate gut microbiome (Bongers et al. 2014; Manzat-Saplacan et al. 2015). It has been shown that polyphenols can have a positive effect on gut flora composition, and potentially reduce the risk of CRC (Laparra & Sanz 2010; Cardona et al. 2013; Dueñas et al. 2015). In animal study, curcumin changed colonic microbiota thus reducing inflammation and reducing or eliminating tumour formation (Ramalingam et al. 2016). Moreover, it has been shown that some spices including turmeric, ginger and rosemary possess prebiotic-like activity promoting the growth of beneficial bacteria whilst at the same time suppressing the growth of harmful bacteria (Lu et al. 2017). Thus, this area could be further explored by investigating if this gut flora modulation by CHS can prevent or reverse CRC.

6.9 Conclusion

The present study demonstrated that CHS and their combinations inhibited CRC cell growth. These CHS and combinations target several key molecular targets in CRC. CHS inhibited COX-2 activity and expression and the effect of the most potent CHS (TE, BLE and GE) were very close to that of the COX-2 inhibitor Celecoxib. CHS also induced apoptosis via activation of caspase-3 and PARP, and upregulated p53 expression and the effect was comparable to another drug – Etoposide. Wnt/ β -catenin signalling seems to be unaffected by CHS, however, the effect by CHS of this pathway could be studied further. Although combining several CHS showed some synergistic effect, in most experiments their effect was outperformed by the most potent individual CHS – TE. This study has therefore shed some light on the chemopreventive and chemotherapeutic potential of CHS however, it is clear that more work is required to establish definitively if their significant potential can become a reality.

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Appendix 1 Selection of culinary herbs and spices

Culinary herbs and spices (CHS) for the present study was selected based on previous work (Table 1.) (Baker 2012; Jaksevicius 2012). Based on the growth inhibition studies the most potent CHS were selected. For the MSc project, selection of CHS was based on the phenolic content and antioxidant activity, which was measured for the BSc projects. Presence of well-studied anti-carcinogenic compounds such as curcumin and gingerol was also considered. Clover was not selected as it had many times higher phenolic content in comparison to other CHS.

Table 1: IC50 of selected culinary herbs and spices for HT-29 and HCT116 cell lines

Culinary Herbs&spices	HT-29 (Ethanollic) IC50 (µg GAE/ml; n=3) ± SEM	HCT116(Ethanollic) IC50 (µg GAE/ml; n=3) ± SEM	HT-29(Aqueous) IC50 (µg GAE/ml; n=3) ± SEM	HCT116(Aqueous) IC50 (µg GAE/ml; n=3) ± SEM
Sage	4.0 (±0.4)	0.8 (±0.1)	2.4 (±1.1)	0.9 (±0.5)
Bay Leave	4.4 (±2.3)	1.8 (±1.1)	1.2 (±0.6)	0.5 (±0.3)
Rosemary	7.1 (±0.3)	1.1 (±0.2)	2.5 (±0.8)	2.3 (±0.2)
Thyme	7.4 (±1.3)	1.4 (±0.2)	2.5 (±0.3)	0.8 (±0.3)
Parsley	16.4 (±0.2)	7.6 (±0.4)	n/a	8.5 (±0)
Turmeric	4.7 (±0.2)	4.6 (±0.3)	12.2(±2.4)	14.8 (±2.5)
Ginger	6.4 (±0.2)	5.4 (±0.2)	6.5(±0.2)	18.1 (±0.2)
Nutmeg	5.5 (±0.7)	7.3 (±1.5)	11.8(±2.1)	n/a
Cinnamon	n/a	8.6 (±0.5)	7.0 (±0.4)	18.4 (±0.7)
Clove	n/a	8.9 (±0.7)	3.5 (±0.1)	9.0 (±0.02)

n/a – the IC50 was not achieved at highest tested concentration (20 µg GAE/ml)

Appendix 2 Estimation of polyphenol content in CHS extracts

According to a study that used methanol as a solvent, turmeric contains approximately 3% of curcumin by weight (Tayyem et al. 2006). Some studies that used more sophisticated extractions techniques found it to be higher, for example, Kim et al. (2012) found that turmeric extract prepared for supplement sale contained 7.9% of curcumin. However, for the present study 3% was used to make an approximate calculation of the amount of curcumin in the turmeric extract.

Based on 3% figure, 1g of turmeric contains ~30 mg of curcumin, 1mg of curcumin contains ~30 µg of curcumin, 1 µg of turmeric = ~30 ng of curcumin. The IC₅₀ value in dry weight for turmeric for HCA-7 cell line was 300 µg/ml DW. So, 3% of 300 µg/ml equals 9 µg/ml of curcumin. $9 \mu\text{g/ml} = 9 \times 10^{-3} \text{g/l}$.

Molecular weight of curcumin is 368g/L.

Mass (g) = Concentration (mol/L) * Volume (L) * Molecular Weight (g/mol)

Concentration (mol/L) = Mass (g)/ (Volume (L) * Molecular Weight (g/mol))

$C \text{ (mol/L)} = 9 \times 10^{-3} \text{g} / (11 \times 368 \text{g/mol})$

$C \text{ (mol/L)} = 24.5 \mu\text{mol/L} = 24.5 \mu\text{M}$.

[6]-gingerol is a major active component in ginger. Dried ginger contains 18.8 mg [6]-gingerol per 1g of dry weight (Puengphian & Sirichote 2008). 18.8 mg/g equals approximately 2% of dry weight

The IC₅₀ value for HCA-7 for ginger was 415 µg/ml DW. [6]-gingerol molecular weight is 294 g/mol

2% of 415 µg/ml equals 8.3 µg/ml of [6]-gingerol

$C \text{ (mol/L)} = 8.3 \times 10^{-3} \text{g} / (11 \times 294 \text{g/mol})$

$C \text{ (mol/L)} = 22.2 \mu\text{mol/L} = 22.2 \mu\text{M}$ of [6]-gingerol.

According to (Puangsombat & Smith 2010) study, 40% ethanol extract of rosemary contains 1.7 mg/g (~0.2%) DW of rosmarinic acid (RA), 5.9 mg/g (~0.6%) DW of carnosic acid (CA) and 115 5.9 mg/g (11.5%) DW carnosol (CL).

The IC₅₀ value for HCA-7 for rosemary ethanol extract was 347 µg/ml DW. RA molecular weight is 360 g/mol; CA molecular weight is 332.4 g/mol; CL molecular weight is 330.4 g/mol

0.2% of 347 µg/ml equals 0.7 µg/ml of RA.

$C \text{ (mol/L)} = 0.7 \times 10^{-3} \text{g} / (11 \times 332.4 \text{g/mol})$

$C \text{ (mol/L)} = 2 \mu\text{mol/L} = 2 \mu\text{M}$ of RA.

0.6% of 347 $\mu\text{g/ml}$ equals 2 $\mu\text{g/ml}$ of CA.

$$C (\text{mol/L}) = 2 \cdot 10^{-3} \text{ g} / (11 \cdot 332.4 \text{ g/mol})$$

$$C (\text{mol/L}) = 6 \mu\text{mol/L} = 6 \mu\text{M of CA.}$$

11.5% of 347 $\mu\text{g/ml}$ equals 3.8 $\mu\text{g/ml}$ of CL.

$$C (\text{mol/L}) = 3.8 \cdot 10^{-3} \text{ g} / (11 \cdot 330.4 \text{ g/mol})$$

$$C (\text{mol/L}) = 11.4 \mu\text{mol/L} = 11.4 \mu\text{M of CL.}$$

However, as it was stated in the Chapter 2 the phenolic content of the CHS can be influenced by numerous factors such as growing conditions, harvesting time, extraction method (Puangsombat & Smith 2010; Generalić et al. 2012; Dvorackova et al. 2015; Anandaraj et al. 2014), hence the phenolic composition and the amounts of the major polyphenols in the CHS extracts used in the present study could be different from the estimated amounts above.

Appendix 3 Publication

Andrius Jaksevicius, Mark Carew, Calli Mistry, Helmout Modjtahedi and Elizabeth I. Opara (2017) Inhibitory Effects of Culinary Herbs and Spices on the Growth of HCA-7 Colorectal Cancer Cells and Their COX-2 Expression, *Nutrients*, 9(10), 1051; doi:10.3390/nu9101051.

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