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PORTULACA OLERACEA EXTRACT CAN INHIBIT NODULE FORMATION OF COLON CANCER STEM CELLS BY REGULATING GENE EXPRESSION OF NOTCH SIGNAL TRANSDUCTION PATHWAY

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PORTULACA OLERACEA EXTRACT CAN INHIBIT NODULE FORMATION OF COLON CANCER STEM CELLS BY REGULATING GENE EXPRESSION OF NOTCH SIGNAL TRANSDUCTION PATHWAY

By

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Preface

All the chapters of this dissertation were organized from a manuscript which is already submitted to peer-reviewed journals and book publishers for publication. The author has main contribution to the manuscripts. Other than Introduction and summary and future perspective section, this dissertation is organized into three main chapters.

Chapter 1 and chapter 2 is on differences between HT29 cancer cells and HT29 stem cells derived from the submitted manuscript. The author of this dissertation was the first author of the manuscript, who was responsible for most of the out experiments, data processing, manuscript writing, organizing the content, leading the writing context, and drawing the illustrations. Chao Deng did the image quantification analysis and data organization.

Chapter 3 is on the effects of *P. oleracea* extracts on HT-29 stem cells and HT-29 cells also derived from the submitted manuscript. The author of this dissertation was a joint first author and was responsible for organizing the idea, manuscript writing, experiment designing, imaging, data collection and analysis. Dr. Heiying Jin provided support on funding and guidance. Dr. Xiaofeng Wang helped with part of the experiments.

Chapter 4 is on the effects of *P. oleracea* extracts on the expression of Notch1, Notch2, and Beta-Catenin mRNA in HT-29 cells and HT-29 stem cells also derived from the submitted manuscript. The author of this dissertation was responsible for carrying out experiments, data processing, analysis and manuscript writing. Dr. Heiying Jin is the co-first author on this submitted manuscript. Dr. Heiying Jin also provided organization and revision of this submitted manuscript.

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Abstract

Purpose: The main aim of this study was to investigate the effects of *Portulaca oleracea* extract on tumor formation in colon cancer stem cells and chemotherapy sensitivity. In addition, this study analyzed the genetic changes within the Notch signal transduction pathway associated with the effects of the extracts.

Methods: Serum-free cultures of colon cancer cells (HT-29) and HT-29 stem cells were treated with the chemotherapeutic drug 5-FU to assess sensitivity. Injections of the stem cells were also given to BALB/c mice to confirm tumor growth and characteristics. In addition, the effect of different concentrations of *P. oleracea* extract was tested on the growth of HT-29 and HT-29 stem cells, which was determined by the MTT method. The effects of *P. oleracea* extract on the expression of β-catenin, Notch1, and Notch2 in HT-29 cells were detected by RT-PCR and Western blotting.

Results: The tumor volume of HT29 cells was two times larger than that of HT29 stem cells. Treatment with *P. oleracea* extracts inhibited the proliferation of both HT-29 and HT-29 stem cells in doses from 0.07 - 2.25 μg/mL. Apoptosis of HT-29 and HT-29 stem cells, as assessed by flow cytometry, was enhanced by the addition of *P. oleracea* extract. Finally, treatment with *P. oleracea* extracts significantly down-regulated the expression of the Notch1 and β-catenin genes in both cell types.

Conclusion: This study shows that *P. oleracea* extract inhibits the growth of colon cancer stem cells in a dose-dependent manner. Furthermore, it inhibits the expression of the Notch1 and β-catenin genes. Taken together, this suggests that it may elicit its effects through regulatory and target genes that mediate the Notch signal transduction pathway.

Key Words: Colon Cancer Stem Cells; *Portulaca oleracea* Extract; Notch Signal Transduction Pathway

1.0 Introduction and Study Background[1]

1.1 Abstract

Cancer stem cells are cells which have been reported to have the ability to self-differentiate and self-renew. In colon cancer, the cells have been shown to play a significant role in tumorigenesis, abnormal differentiation, recurrence, and metastasis of colon cancer. Additionally, research has shown that the cancer stem cells are insensitive to chemotherapy and immunotherapy, hence the need for an alternative therapy. The use of herbal medicines is seen as a viable alternative with reports indicating that *P. oleracea* extracts have the ability inhibit a number of different cancer cell types. However, the effectiveness of *P. oleracea* extracts in inhibiting colon cancer stem cells has not been investigated. Also, although it is known that dysregulations of the Notch signal transduction pathway play a significant role in the development of colon cancer stem cells, no study has been conducted to investigate the effects of *P. oleracea* extracts on Notch signal transduction pathway. Therefore, the purpose of this study was to determine if *P. oleracea* extracts can inhibit colon cancer stem cells, and if the effects observed are mediated by the Notch signal transduction pathway.

[1] The material contained in this chapter was already submitted to *Tumor Biology*.

1.2 Introduction

Colon cancer is a significant public health problem worldwide ranked as the fourth leading cause of cancer-related mortalities worldwide (S. Liu et al., 2015). The development, recurrence, and metastasis of colon cancer cells are mediated by colon cancer stem cells, a small proportion of tumor cells that can self-renew, infinitely proliferate, and have the potential for multi-directional differentiation. The stem cells must be inhibited in the initiating cells associated with carcinogenesis to maintain the growth of the tumor (Kucia et al., 2005). It was once believed that tumor stem cells only existed in hematological and lymphocytic malignancies; however, it is now known that tumor stem cells heavily influence all solid tumors (Patrawala et al., 2005; Setoguchi et al., 2004).

Some cells present in human colon crypts can self-renew and differentiate. Mediated by a series of genes that maintain a dynamic balance in the colon, these stem cells play an important role in the regeneration and restoration of the organ (Dhawan, Ahmad, Srivastava, & Singh, 2011). External forces, such as mutations, can have long-term effects when they impact colon stem cells. During a prolonged period of time, oncogene mutations can accumulate thereby promoting the transformation of stem cells and the asymmetric division of stem cells and ultimately leading to the

production of colon cancer stem cells (Dhawan et al., 2011). Colon cancer stem cells have the same self-differentiation and self-renewal characteristics of normal colon stem cells; however, cancer stem cells play a role in tumorigenesis and abnormal differentiation (L Ricci-Vitiani, Pagliuca, Palio, Zeuner, & De Maria, 2008). In clinical practice, stem cells do not invade the tumor but rather are present during the earliest stages of colon cancer. Additionally, cancer stem cells are present only at the primary tumor site and do not appear to be involved in distant metastases. However, recent studies have indicated that colon cancer stem cells are important to the recurrence and metastasis of colon cancer (Dhawan et al., 2011; L Ricci-Vitiani et al., 2008).

Currently, many molecular markers can be used to identify and isolate colon cancer stem cells. The most commonly used molecular markers include CD133 and CD44, as well as CD24, epithelial-specific antigen (ESA), CD166, CD29, and leucine-rich repeat containing G protein-coupled receptor (Lgr) (Dalerba, Dylla, et al., 2007; Kemper et al., 2012; O'Brien, Pollett, Gallinger, & Dick, 2007; Lucia Ricci-Vitiani et al., 2007; Todaro et al., 2007; Vermeulen et al., 2008). Most colon cancer stem cells have been reported to be insensitive to chemotherapy and immunotherapy, and these cells are thought to be involved in the development of resistance to chemotherapy (Dhawan et al., 2011). Taken together with their

important roles in many cancer processes, these cells may be an important target for the prevention and treatment of colon cancer (Dhawan et al., 2011).

At present, there are no effective methods for the suppression of colon cancer stem cells. Previous studies have found that the foods used in traditional Chinese medicine can prevent and treat colon cancer; for example, extract from the edible vegetable *P. oleracea* has been shown to inhibit a number of different cancer cell types (H.-Y. Jin, Zhang, Wang, Xu, & Ding, 2012). However, at present no study has yet determined the effect of *P. oleracea* extract on colon cancer stem cells. Additionally, the Notch signal transduction pathway has been shown to be important for the development of colon cancer stem cells (H.-Y. Jin et al., 2012; Qiao & Wong, 2009). Therefore, the purpose of this study was to determine if *P. oleracea* can inhibit colon cancer stem cells, and if the effects observed were mediated by the Notch signal transduction pathway.

1.3 Study Background

1.3.1 Colon Cancer: Epidemiology

Colon cancer, also known as colorectal cancer, accounts for more than 9% of all the cases of cancers in the world (Haggar & Boushey, 2009) and the third most common type of cancer worldwide (Fitzmaurice et al., 2015). Epidemiological data have also shown that colon cancer is the fourth leading cause of cancer-related

mortalities worldwide (S. Liu et al., 2015). The cancer is common in both men and women with reports indicating that the cancer accounts for 9.4% of all cancer incidences in men and 10.1% in women (Haggar & Boushey, 2009). Therefore, colon cancer is a significant public health problem worldwide requiring appropriate and effective therapy.

1.3.2 Colon Cancer: Causes and Risk Factors

There are several factors which have been shown to be risk factors and/or causes of colon cancer. The factors are grouped into environmental, other health conditions and genetic factors as well as interactions between the genetic and environmental factors. Environmentally, obesity (Bardou, Barkun, & Martel, 2013), sedentary lifestyle (Durko & Malecka-Panas, 2014; Shen et al., 2014) and unhealthy dietary (Béliveau & Gingras, 2007; Myles, 2014) habits have been shown to increase the risk of colon cancer in both women and men.

According to Bardou, Barkun, and Martel (2013), obesity leads to metabolic syndromes, insulin resistance and dysregulation of adipocytokines levels which results in increased risk of colon cancer. Through these mechanisms, obesity is also associated with poor cancer outcomes including recurrence ad metastasis of the cancers as well as increased mortalities (Bardou et al., 2013). Indeed, it has also been reported that hyperinsulinemia, due to obesity-associated type 2 diabetes mellitus,

plays a significant role in the pathogenesis and spread of colon cancer (Frezza, 2006). A systematic literature review of studies by Ma et al. (2013) also found that obesity is associated with up to 60% greater risk of colon cancer when compared to individuals with normal body mass index. Therefore, the available research evidence indicates that obesity does not only increase the risk of developing colon cancer by up to 60%, but is also associated with poor clinical outcomes of the cancer.

Sedentary lifestyle is another environmental factor which has been associated with the risk of colon cancer. According to Shen et al. (2014), a sedentary lifestyle characterized by low levels of physical activity is associated with obesity and poor weight management which in turn increases the risk of colon cancer and results in poor clinical outcomes among patients with the cancer. Boyle, Fritschi, Heyworth and Bull (2011) also found that people who spent 10 or more years in sedentary work had twice the risk of colon cancer when compared to the people who did not spend any time in sedentary work. The Boyle et al. (2011) study also found that the association between sedentary lifestyle and colon cancer was independent of recreational physical activity. The findings demonstrate the significant role played by sedentary lifestyle in increasing the risk of developing colon cancer. The association can occur via obesity, where the sedentary lifestyle increases the body mass index of a person, or directly where the sedentary lifestyle results in colon cancer independent of body

weight and physical activity.

Like sedentary lifestyle, unhealthy eating habits such as consumption of high-calorie food and food rich in fats can result in obesity which in turn increases the risk of developing colon cancer and/or complicates the clinical outcomes of the cancer (Myles, 2014). Research has also shown that high-fat contents in food disrupt the composition of the gut normal flora which in turn increase the risk of colon cancer (Zackular et al., 2013). Indeed, the Zackular et al. (2013) study established that patients with colon cancer have altered gut flora when compared to healthy patients. The study also found that the manipulation of gut flora in mice with antibiotics increased the number and size of the gut tumours. The findings suggest that disruption of the gut microbiota increases the risk and pathogenesis of colon cancer. Therefore, consumption of high-fat content foods which alters the gut microbiota increases the risk of colon cancer and may be associated with the pathogenesis, invasion, and metastasis of the cancer.

In addition to these environmental factors, other factors such as alcohol consumption and tobacco smoking have also been reported to be significantly associated with colon cancer (Cho et al., 2015). It is known that consumption of alcohol and smoking cigarette are major risk factors for gastrointestinal cancers including colon cancer (Cho et al., 2015). Alcohol is a carcinogen (group 1

carcinogen) which has been shown to be a significant contributor to the risk of colon cancer (Tsong et al., 2007). Indeed, a study cited by Tsong et al. (2007) found that there is 30% higher risk of colon cancer in people who consume two alcoholic drinks per day compared to non-drinkers. Similarly, cigarette is a carcinogen which has been associated with the risk of colon cancer at a relative risk of 1.19 at 95% confidence interval (Limsui et al., 2010). Botteri et al. (2008) also found that ever-smokers are 18% more likely to develop colon cancer compared to non-smokers. Research has also shown that the metabolites of cigarette such as cotinine, O-cresol sulfate, and hydroxycotinine are the carcinogens that induce colon cancer in cigarette smokers (Cross et al., 2014).

In addition, certain health conditions such as gastrointestinal inflammation and high blood levels of insulin have also been associated with colon cancer. According to Terzić, Grivennikov, Karin and Karin (2010), the association between gastrointestinal inflammations such as inflammatory bowel disease and colon cancer is well-established. During inflammation, activated inflammatory cells produce reactive oxygen species (ROS) and reactive nitrogenous metabolites which cause DNA damage and mutations resulting in uncontrolled cell proliferation and cancer (Terzić et al., 2010). Therefore, gastrointestinal inflammation results in the production of ROS and reactive nitrogenous intermediates which induce DNA mutations in the

colon leading to colon cancer. High levels of insulin in blood has also been shown to increase the risk of colon cancer. According to Johnson and Gale (2010), insulin is a mitogenic hormone and overexpresses their receptors on the surface of cancer cells if present in high levels in the blood. Therefore, high level of insulin in blood acts as tumour growth factors which promote the development and spread of cancer cells. Increased levels of insulin in the blood, like the case in type 2 diabetes mellitus due to insulin resistance can, therefore, promote the development and spread of colon cancer cells.

Genetically, research has shown that up to 10% of all the cases of colon cancer are hereditary conditions (Jackson-Thompson, Ahmed, German, Lai, & Friedman, 2006). The mist common hereditary colon cancers are hereditary nonpolyposis colon cancer and familial adenomatous polyposis (Haggar & Boushey, 2009). Hereditary nonpolyposis colon cancer is caused by is due to mutations in genes that regulate DNA repair such as the MLH1 and MSH2 genes. Mutations in these genes (MLH1 and MSH2 genes) results in hereditary nonpolyposis colon cancer, which accounts for approximately 2% to 6% of all the forms of colon cancer (Haggar & Boushey, 2009). Familial adenomatous polyposis is caused by mutations in the APC gene (a tumour suppressor gene) which occur in an autosomal dominant manner (Haggar & Boushey, 2009).

Interactions between genetic and environmental factors also contribute to the risk of developing colon cancer. For instance, increased physical activity has been shown to interact with certain genes to influence the clinical outcome of the cancer (Marley & Nan, 2016). Indeed, it has been shown that the level of physical activity with colon cancer survival in patients diagnosed to be prostaglandin-endoperoxide synthase 2-positive (PTGS2-positive) (Yamauchi et al., 2013). Similarly, Meyerhardt et al. (2009) found that physically active colon cancer patients with P27 expression had a 68% lower mortality rates from the cancer compared to colon cancer patients who were physically inactive. In addition, further research has shown that the interaction between environmental factors such as alcohol consumption, obesity, intake of vitamin D and intake of polyunsaturated fatty acids have also been shown to influence the risk of colon cancer (Marley & Nan, 2016; Morikawa et al., 2013).

1.3.3 Colon Cancer Stem Cells and Colon Cancer

1.3.3.1 Differences between Colon Cancer Stem Cells and Colon Cancer Cells

In addition to environmental and genetic factors, colon cancer stem cells also play a significant role in the development, recurrence and metastasis of colon cancer (Dhawan et al., 2011; L Ricci-Vitiani et al., 2008), hence the need to identify the stem cells for diagnosis and treatment. Cancer stem cells are a small sub-population of tumour cells with the ability to efficiently propagate cancer development and

pathogenesis (Puglisi, Tesori, Lattanzi, Gasbarrini, & Gasbarrini, 2013). Therefore, cancer stem cells can be defined as a small proportion of tumor cells, making 0.1to 10% of tumour cells (Deonarain, Kousparou, & Epenetos, 2009), that can self-renew, infinitely proliferate, and have the potential for multi-directional differentiation.

Cancer cells are abnormal cells capable of differentiating and proliferating indefinitely and can invade surrounding tissues and metastasize to distant organs (Sitas et al., 2006). The cancer cells can either be benign or malignant. Benign cancer cells are tumours that remain confined to the original site of location without invading the surrounding tissues or metastasizing to distant organs. Malignant tumours are cancer cells with abnormal and unregulated proliferation and differentiation with the ability to invade surrounding tissues and metastasize to distant organs (Sitas et al., 2006). On the other hand, cancer stem cells are cells within benign or malignant tumours with the same characteristics as normal stem cells (can give rise to new cells) (Magee, Piskounova, & Morrison, 2012).

Since cancer stem cells form a small sub-population of tumour cells, their identification can be challenging. However, comparison of other cancer stem cells and cancer cells have revealed some significant differences which can be used to identify and isolate colon cancer stem cells. For instance, it has been reported that cancer cells and cancer stem cells can be distinguished based on their sensitivity to

chemotherapeutic agents where cancer stem cells have been found to be more insensitive to the agents than cancer cells (Vinogradov & Wei, 2012). The expression of CD133 and CD44 has also been utilised as a basis of differentiating cancer cells from cancer stem cells. For instance, it has been shown that cancer stem cells have higher expression of CD133 and CD44 compared to cancer cells (Guo, 2012; Nagata et al., 2011). Other reports have also established that cancer cells take up more Hoechst33342 stain and have a higher growth rate compared to cancer cells (Gao, Geng, Kvalheim, Gaudernack, & Suo, 2009; Toloudi et al., 2014).

1.3.3.2 Role of Colon Cancer Stem Cells in Cancer Development, Recurrence and Metastasis

There is research evidence to show that colon cancer stem cells are associated with development (Abdul Khalek, Gallicano, & Mishra, 2010), recurrence (Ramasamy, Ayob, Myint, Thiagarajah, & Amini, 2015) and metastasis (Basu, Haase, & Ben-Ze'ev, 2016) of colon cancers. When compared to normal stem cells, cancer stem cells has no restricted or regulated rate of proliferation and have a slow rate of cycling which plays a significant role in tumour recurrence and initiating resistance to chemotherapy and radiotherapy (Moore & Lyle, 2011; Pannuti et al., 2010). In addition, cancer stem cells have the ability to initiate new tumours hence plays a significant role in metastatic colorizations (Hanahan & Weinberg, 2011).

1.3.3.3 Properties of Colon Cancer Stem Cells

The main properties of colon cancer stem cells include self-renewal potential, ability to differentiate into diverse cell populations and ability to balance and modulate the processes of self-renewal and differentiation (Garza-Treviño, Said-Fernández, & Martínez-Rodríguez, 2015). Self-renewal refers to the ability of the colon cancer stem cells synthesize new cells with the same ability to proliferate, expand and differentiate as the parental cells thereby maintaining a pool of the stem cell (Garza-Treviño et al., 2015). The mechanisms utilised for the self-renewal of the colon cancer stem cells, such as the Wnt/β-catenin and Notch pathways, makes use of proto-oncogenes. In addition, the self-renewal pathway of colon cancer stem cells is regulated by the sonic hedgehog (Hh) signaling pathway, a pathway commonly reported in multiple myeloma (Varnat et al., 2009). Research has shown that the self-renewal of colon cancer stem cells is promoted by the Wnt pathway and the prevention of the β-catenin-dependent transcription (Ong, Vega, & Houchen, 2014).

Additionally, the Notch receptors pathway have also been reported to promote the self-renewal of colon cancer stem cells (Ong et al., 2014). In the Notch pathway, the DLL4 genes stimulate the Notch receptors on adjacent cells and in combination with the effects of β-catenin promotes the self-renewal of the cancer stem cells by directing an immature transcription profile (Garza-Treviño et al., 2015). However, the

self-renewal pathways are inhibited by BMP4 genes which bind BMP receptors leading to interference with the Wnt pathway and thus promote cell differentiation. The process of differentiation has also been reported to be inhibited by hepatocyte growth factor (HGF) leading to the maintenance of the cells in a stem-cell state (Ong et al., 2014).

Cancer stem cells also have the ability into differentiate to diverse cell populations (Garza-Treviño et al., 2015). That is, the stem cells have the ability to differentiate into a heterogeneous progeny of cells that can undergo progressive diversification and specialization following self-renewal. The cells of this differentiation are specialized to carry out certain unique physiological functions (Dalerba, Cho, & Clarke, 2007). However, histopathological studies of colon cancers have indicated that the cells are relatively undifferentiated containing higher proportions of colon cancer stem cells than more differentiated cancer cells (Ashley, Yeung, & Bodmer, 2013; Merlos-Suárez et al., 2011). The undifferentiated colon cancer cells have worse clinical prognosis compared to differentiated cancer cells (Merlos-Suárez et al., 2011).

Homeostatic control where the cells have the ability to balance and modulate the processes of self-renewal and differentiation is also a key property of cancer stem cells (Garza-Treviño et al., 2015). Through research, it has been established that

differentiated intestinal epithelial cells reside in the intestinal crypts as repeats of stem cells containing leucine and B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1) or G protein-coupled receptor 5 (Lgr5) (Tian et al., 2011). The Bmi-1 AND Lgr5 are used to maintain the capacity of tissues to regenerate (Tian et al., 2011). The intestinal cells that express Lgr5 undergo active proliferation and very sensitive to stimulatory effects of Wnt and Dkk1-mediated Wnt inhibition (Tian et al., 2011). On the other hand, intestinal cells that express Bmi-1 are less sensitive to environmental stressors and are, therefore, not affected by the Wnt modulation. The Bmi-1-expressing intestinal cells are normally quiescent, only giving rise to cell progeny under certain conditions (Yan et al., 2012). The Wnt proteins and the Notch pathway has been reported to play a significant role in maintaining cancer stem cells hemostasis by providing signals required for the maintenance of the phenotype of colon cancer stem cells in the tumour mass (Garza-Treviño et al., 2015).

1.3.3.4 Markers of Colon Cancer Stem Cells

Colon surface antigens on the surface of the stem cells have been identified as the markers for colon cancer stem cells. According to Deonarain et al. (2009), these antigens are not identified based on their overexpression on typical tumour cells, but on their presence in a population of cells with stem cell-like properties. However, the

identification of the cancer stem cell antigen has been challenging due to their variable expression on cancer stem cells and frequent co-expression in normal stem cells (Deonarain et al., 2009). Currently, many molecular markers have been discovered for the identification and isolation of colon cancer stem cells.

The most commonly used molecular markers include CD133 and CD44, as well as CD24, epithelial-specific antigen (ESA), CD166, CD29, and Lgr (Dalerba, Dylla, et al., 2007; Kemper et al., 2012; O'Brien et al., 2007; Lucia Ricci-Vitiani et al., 2007; Todaro et al., 2007; Vermeulen et al., 2008). Most colon cancer stem cells have been reported to be insensitive to chemotherapy and immunotherapy, and these cells are thought to be involved in the development of resistance to chemotherapy (Dhawan et al., 2011). Taken together with their important roles in many cancer processes, these cells may be an important target for the prevention and treatment of colon cancer (Dhawan et al., 2011).

1.3.4 Colon Carcinogenesis

The transformation of normal cells to cancer cells requires six hallmarks; self-sufficiency in signals required for growth, the potential for unlimited replication, insensitivity to snit-growth signals, ability to evade apoptosis, tissue invasion and sustained angiogenesis (Garza-Treviño et al., 2015). Initially, there must be genetic defects within the cell that result in DNA instability and then inactivation of tumour

suppressor genes. This results in uncontrolled cell division, differentiation, and self-renewal leading to cancer development (Garza-Treviño et al., 2015).

In colon cancer, the transformation of normal intestinal cells to colon cancer cells begins with an increase in the intracellular β-catenin in normal colon epithelial tissue. This results in prolonged activation of the Wnt pathway, stabilization of β-catenin, and activation of the C-terminal binding protein 1 (CtBP1) (Garza-Treviño et al., 2015). Additionally, there is inactivation of the APC gene which initiates adenoma while the nuclear localization and activation of β-catenin promote the activation of the formed adenoma to carcinoma (Phelps et al., 2009). The progression of colon tumours is driven by mutations in phosphatidylinositol 3-kinase (PIK3CA) and transforming growth factor-beta receptor (TGFBR) genes (Armaghany, Wilson, Chu, & Mills, 2012). In addition to these mechanisms, research has also established that normal stem cells, instead of progenitor and differentiated cells, are also involved in the initiation of colon cancers of cellular origin (Armaghany et al., 2012).

1.3.5 Colon Cancer Stem Cells in Colon Carcinogenesis

Cancer stem cells are transformed to more malignant phenotypes during the process of primary cancer progression via three main pathways; microsatellite instability (defects in DNA mismatch repair), chromosomal instability, and CpG island methylator phenotype pathways (Pino & Chung, 2010). These carcinogenic

pathways cooperate leading to the activation of different downstream signaling effectors such as mitogen-activated protein kinases (MAPKs), PI3K/Akt/molecular target of rapamycin (mTOR) and nuclear factor-kappaB (NF-kB) conferring high-level self-renewal ability to the cells (Birnie et al., 2008; Chiba et al., 2008; S. Ma, Lee, Zheng, Chan, & Guan, 2008; Mimeault, 2012; Shiras et al., 2007; Venugopal et al., 2012; Wang et al., 2012). In addition to transforming themselves to more malignant phenotypes, colon cancer stem cells also initiate the development, recurrence, and metastasis of colon cancers (Dhawan et al., 2011; L Ricci-Vitiani et al., 2008).

1.3.6 Chemotherapy and Chemoresistance

Colon cancer is primarily managed with chemotherapy regiments of "5 fluorouracil (5FU), oxaliplatin and/or leucovorin or 5-FU, leucovorin and irinotecan (FOLFIRI)" (Garza-Treviño et al., 2015, p.2). The drugs display anti-cancer properties by inducing oxidative damage of the cancer cells. However, the chemotherapy regimen has been reported to be ineffective leading to treatment failures in 90% of metastatic colon cancer and this has been attributed to chemoresistance (Zhao, Butler, & Tan, 2013). The oxidative damage of the cancer cells increases the glycolysis of the cells resulting in high levels of the antioxidant NADPH, which can be associated with chemoresistance (Wu, Calcagno, & Ambudkar,

2008).

Colon cancer stems also displays chemoresistance by expressing HIF-1α which decreases the pro-apoptotic signaling. The expression of HIF-1α induces the expression of decoy receptors like DcR2 which competes for the pro-apoptotic signaling factors such as the tumor necrosis factor-related apoptosis inducing ligand. This reduces the signaling through the apoptosis-inducing receptors and, therefore, attenuates apoptosis thereby allowing cells to tolerate high levels of chemotherapeutic agents (Chen et al., 2009; Flamant, Notte, Ninane, Raes, & Michiels, 2010; Sullivan & Graham, 2009; Wen et al., 2010). Indeed, there is research evidence to show that most colon cancer stem cells are insensitive to chemotherapy and immunotherapy, leading to the development of resistance to chemotherapy (Dhawan et al., 2011).

1.3.7 Treatment Implications of Colon Cancer Stem Cells

Colon cancer stem cells play a significant role in the development, recurrence, and metastasis of colon cancer and can, therefore, be an important target for the prevention and treatment of colon cancer (Dhawan et al., 2011). The cancer stem cells are insensitive to chemotherapy hence the need for alternative therapy. Research has shown that immunotherapy with antibodies can be utilised as an alternative therapy. For instance, studies have shown that colon cancer stem cells that are insensitive to 5FU can be made sensitive by the use of interleukin-4 blocking antibody (Todaro et

al., 2007; Todaro, Perez Alea, Scopelliti, Medema, & Stassi, 2008). Similarly, it has been demonstrated that the use of anti- epiregulin, epidermal growth factor family (anti-EREG) antibodies is an effective immunotherapy against colon cancer metastasis (Kobayashi et al., 2012). Therefore, there is research evidence to demonstrate the effectiveness of immunotherapy against colon cancer stem cells. However, immunotherapy is an expensive alternative to chemotherapy due to the high cost of producing, storing and transporting the antibodies (Lynch & Murphy, 2016).

Alternatively, nanotechnology can also be utilised to suppress colon cancer stem cells which are resistant to chemotherapy. For instance, siRNA-mediated knockdown of the drug efflux protein MDR1 (the protein is overexpressed in colon cancer stem cells) has been described as a nanotechnology method of overcoming chemoresistance by the colon cancer stem cells (Liu et al., 2009). However, nanotechnology is also an expensive therapy which cannot be afforded by most colon cancer patients (Kim, Buddolla, & Lee, 2016). Therefore, a more affordable and effective alternative is necessary to overcome the chemoresistance of colon cancer stem cells in the treatment of colon cancers.

1.3.8 *Portulaca oleracea* Extracts for the Inhibition of Colon Cancer Stem Cells

As discussed earlier, the chemoresistance of colon cancer stem cells, despite their significant role in the development, recurrence, and metastasis of colon cancers, can

be overcome by immunotherapy (Kobayashi et al., 2012) and nanotechnology (Liu et al., 2009). However, these therapies are expensive and may not be affordable to many colon cancer patients(Kim et al., 2016; Lynch & Murphy, 2016). Therefore, a more affordable but effective alternative is necessary to overcome the chemoresistance. The use of medicinal plants has been utilised as an affordable alternative for cancer therapy and can also be utilised to overcome the chemoresistance of colon cancer stem cells. For instance, a study by Min, Lim, Kim, Kim, and Kim (2015) found that the leaves of *Sasa quelpaertensis* can inhibit colon cancer stem cells and influence gene expression related to stem cell development. In another study, Kumar, Kumar, Raina, Agarwal and Agarwal (2014) found that grape seed extract can inhibit the effect of colon cancer stem cells. *P. oleracea* extracts, a traditional Chinese medicine can prevent and treat colon cancer. Extracts of the plant have been to inhibit a number of different cancer cell types (H.-Y. Jin et al., 2012). However, at present no study has yet determined the effect of *P. oleracea* extract on colon cancer stem cells. Therefore, the purpose of this study was to determine if *P. oleracea* can inhibit colon cancer stem cells, and if the effects observed were mediated by the Notch signal transduction pathway.

1.4 Aims of the Study

Colon cancer stem cells have the ability to self-differentiate and self-renew and

play a significant role in tumorigenesis and abnormal differentiation (L Ricci-Vitiani et al., 2008). Although the stem cells do not invade tumors, they are present during the early stages of colon cancer development and have been shown to play a significant role in the recurrence and metastasis of colon cancer (Dhawan et al., 2011; L Ricci-Vitiani et al., 2008). In addition, research has shown that most cancer stem cells are insensitive to chemotherapy and immunotherapy and contributes to the development of resistance to chemotherapy (Dhawan et al., 2011). Despite the significant role played by the stem cells in the pathogenesis of cancers and resistance to chemotherapy, there are no effective methods for the suppression of colon cancer stem cells currently.

However, previous studies have found that the foods used in traditional Chinese medicine can prevent and treat colon cancer; for example, extract from the edible vegetable *P. oleracea* has been shown to inhibit a number of different cancer cell types (H.-Y. Jin et al., 2012). The effectiveness of the *P. oleracea* vegetable in inhibiting colon cancer stem cells has, however, not been investigated. In addition, research has shown that Notch signal transduction pathway plays a significant role in the development of colon cancer stem cells (H.-Y. Jin et al., 2012; Qiao & Wong, 2009). Therefore, dysregulation of the Notch signal transduction pathway by this vegetable can it the development of colon cancer stem cells. As a result, the main

purpose of this study was to determine if *P. oleracea* extracts can inhibit colon cancer stem cells, and if the effects observed were mediated by the Notch signal transduction pathway. This was accomplished by meeting the following general and four specific objectives.

1.4.1 General Objective

To investigate the role and mechanism of *P. oleracea* extracts in inhibiting the proliferation of colon cancer stem cells.

1.2.2 Specific Objectives

- To investigate the differences between colon cancer cells (HT29 cells) and colon cancer stem cells (HT29 stem cells)
- To determine if *P. oleracea* extracts can inhibit colon cancer stem cells
- To investigate the dysregulations of the Notch signal transduction pathway in *P. oleracea* extracts-mediated inhibition of colon cancer stem cells

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2.0 Chapter 1: Differences between HT29 Cancer Cells and HT29 Stem Cells

2.1 Abstract

Identification of the unique features of cancer stem cells that can be used to differentiate them from cancer cells is significant to the management of cancers due to their association with cancer development, recurrence, and metastasis. This study used HT29 colon cancer cells and HT29 colon cancer stem cells to compare the characteristics of the two cell lines. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and then flow cytometric analysis of cd133 and cd44, chemotherapy drug sensitivity assay, Hoechst 33342 staining, proliferation assessment and in vivo tumorigenesis assay carried out. The results showed that the HT29 colon cancer stem cells have a significantly higher Hoechst33342 staining, significantly increased expression of CD133 and CD44 markers, significantly faster growth and have a lower sensitivity to chemotherapeutic drug 5-FUdrug compared to the HT29 colon cancer cells. These characteristics can be used in clinical practice to differentiate colon cancer stem cells from colon cancer cells for diagnostic purposes. All institutional and national guidelines from Chinese Association for Laboratory Animal Science for care and use of laboratory animals were followed.[2]

[2] The material contained in this chapter was already submitted to *Tumor Biology*.

2.2 Introduction

There is research evidence to show that colon cancer stem cells are associated with the development (Abdul Khalek et al., 2010), recurrence (Ramasamy et al., 2015) and metastasis (Basu et al., 2016) of colon cancers. These cells form a small sub-population of tumour cells with the ability to efficiently propagate cancer development and pathogenesis (Puglisi et al., 2013). Therefore, cancer stem cells can be defined as a small proportion of tumor cells, making 0.1to 10% of tumour cells (Deonarain et al., 2009), that can self-renew, infinitely proliferate, and have the potential for multi-directional differentiation.

On the other hand, cancer cells are abnormal cells capable of differentiating and proliferating indefinitely and can invade surrounding tissues and metastasize to distant organs (Sitas et al., 2006). The cancer cells can either be benign or malignant. Benign cancer cells are tumours that remain confined to the original site of location without invading the surrounding tissues or metastasizing to distant organs. Malignant tumours are cancer cells with abnormal and unregulated proliferation and differentiation with the ability to invade surrounding tissues and metastasize to distant organs (Sitas et al., 2006). On the other hand, cancer stem cells are cells within benign or malignant tumours with the same characteristics as normal stem cells (can

give rise to new cells) (Magee et al., 2012).

Since cancer stem cells form a small sub-population of tumour cells, their identification can be challenging. However, comparison of other cancer stem cells and cancer cells have revealed some significant differences which can be used to identify and isolate colon cancer stem cells. For instance, it has been reported that cancer cells and cancer stem cells can be distinguished based on their sensitivity to chemotherapeutic agents where cancer stem cells have been found to be more insensitive to the agents than cancer cells (Vinogradov & Wei, 2012). The expression of CD133 and CD44 has also been utilised as a basis for differentiating cancer cells from cancer stem cells. For instance, it has been shown that cancer stem cells have higher expression of CD133 and CD44 compared to cancer cells (Guo, 2012; Nagata et al., 2011). Other reports have also established that cancer cells take up more Hoechst33342 stain and have a higher growth rate compared to cancer cells (Gao et al., 2009; Toloudi et al., 2014).

Currently, many molecular markers can be used to identify and isolate colon cancer stem cells. The most commonly used molecular markers include CD133 and CD44, as well as CD24, epithelial-specific antigen (ESA), CD166, CD29, and leucine-rich repeat containing G protein-coupled receptor (Lgr) (Dalerba, Dylla, et al., 2007; Kemper et al., 2012; O'Brien et al., 2007; Lucia Ricci-Vitiani et al., 2007;

Todaro et al., 2007; Vermeulen et al., 2008). Most colon cancer stem cells have been reported to be insensitive to chemotherapy and immunotherapy, and these cells are thought to be involved in the development of resistance to chemotherapy r (Dhawan et al., 2011). Taken together with their important roles in many cancer processes, these cells may be an important target for the prevention and treatment of colon cancer (Dhawan et al., 2011).

Indeed, the identification of cancer stem cells has been shown to be critical in the management of cancers because inhibiting such stem cells will reduce the risk of cancer development and recurrence (Huang, Cheng, Guryanova, Wu, & Bao, 2010). Therefore, it is important to understand the differences between colon cancer cells and colon cancer stem cells for the identification of the later. An example of cancer cell lines is the HT29 cancer cells which are derived from human colon carcinoma cells (Schreier et al., 2014). The HT29 stem cells are examples of cancer stem cells derived from human colon carcinoma cells (Yeung, Gandhi, Wilding, Muschel, & Bodmer, 2010). In this study, the HT29 cancer cells and HT29 stem cells were used to investigate the differences in the characteristics of cancer cells and cancer stem cells.

2.3 Materials and Methods

2.3.1 Cell Culture

The colon cancer cells and colon cancer stem cells were cultured and separated

as previously described (Todaro et al., 2007; Vermeulen et al., 2008). Briefly, the cells of the HT29 human colon cancer cell line were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) until they reached 90% confluence, at which point the cells were collected. Next, the cells were centrifuged and the supernatant was removed. The cells were re-suspended in serum-free DMEM/F12 medium (containing 20 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 5 μg/mL insulin, 0.4% bovine serum albumin, and 2% B27), counted, and then cultured in 6-pore suspension culture plates at a concentration of 1×10^4 cells/mL at 37°C and 5% CO_2. After culturing for 3-7 days, the growth of the glomus cells could be visualized. After 30 minutes at 37°C, the cells precipitated, and the supernatant was removed (containing a small amount of individual cells and cell debris). Serum-free DMEM/F12 was added for an expanded culture. After centrifuging the glomus cells, digestion and passage, as well as cultivation, was performed for several generations to purify the cells. Finally, the cells were collected for drug sensitivity testing, proliferation assays, flow cytometry, and Hoechst 33342 staining, as indicated.

2.3.2 Flow Cytometric Analysis of CD133 and CD44

HT-29 cells and HT-29 stem cells were first scattered and digested with EDTA-containing trypsin, before quickly diluting and mixing with DMEM/F12

medium. After centrifuging at 1500 rpm for 5 min, the supernatants were removed and the cells were washed twice with PBS. Cells were re-suspended in 300 µL of PBS and a homogenous mixture was obtained before each cell suspension was divided into three separate suspensions, each consisting of 100 µL, which received different treatments. The first was used as a blank/control. The second was treated with 10 µL of anti-CD44-APC in the dark for 15 minutes at 4°C. The third was treated with 10 µL of Fcr Block for 15 minutes at 4°C, and then received 10 µL of anti-CD133-APC for 20 minutes at 4°C. The third was treated with 10 µL of Fcr Block for 15 minutes at 4°C, and then received 10 µL of both anti-CD133-APC and anti-CD44-APC for 20 minutes at 4°C (all procedures were carried out in the dark), before washing with PBS. All cells were re-suspended in 400 µL of PBS and then used for flow cytometry experiments to detect expression of the surface markers CD44 and CD133.

2.3.3 Chemotherapy Drug Sensitivity Assay

HT-29 cells and HT-29 stem cells were plated in 96-well cell culture plates at a concentration of 5000 cells/well. After cell adherence was noted, the chemotherapeutic drug 5-Fu (Fluorouracil) was added at different concentrations for 72 hours (each concentration was tested in triplicate) and 20 mL of MTT solution was added. The medium was discarded after four hours and 150 µL of DMSO was added

and mixed with an oscillating movement. An optical density (OD) of 570 nm wavelength was used as a quick approximation of cell abundance in cell culture. And the IC50 values (the half maximal inhibitory concentration) were calculated to determine drug efficacy.

2.3.4 Hoechst 33342 Staining

HT-29 cells and HT-29 stem cells were first scattered and digested with EDTA-containing trypsin, before quickly diluting and mixing with DMEM/F12 medium. After washing twice with PBS, the cells were re-suspended with PBS, mixed and incubated at 37°C for 2-3 hours. 500μl of Hoechst 33342 was added to the cultivated cells at a final concentration of 2 μg/mL and incubated at 37°C and for 10 min. Cells were washed 3 times with PBS followed by 400 mesh screen filtration and analysis by flow cytometry. Heochst33342 dye excites with a krypton laser: the wavelength of excitation used was 352 nm with an expected emission wavelength was 400-500 nm (blue).

2.3.5 Proliferation Assessment of HT-29 Colon Cancer Stem Cells and HT-29 Colon Cancer Cells

HT-29 cells and HT-29 stem cells were scattered and digested with EDTA-containing trypsin into single cell suspensions, and 1640 medium containing 10% serum was quickly added to stop the digestion. After washing twice with PBS, the cells were re-suspended with PBS, mixed, and pipetted into 6-well plates. Cells

were cultured for 96 hours, cell growth was recorded, and photographs were taken with Zeiss inverted microscope.

2.3.6 In Vivo Tumorigenesis Assay of HT-29 Colon Cancer Stem Cells and HT-29 Colon Cancer Cells

Bilateral stem cell injections were performed in the subscapularis of BALB/c mice, and the inguinal and axillary nodal status of HT29 stem cells and HT29 cells were noted with respect to tumorigenesis. A total of 20 average body weight of 20g, female BALB/c nude mice were used for each cell line. The first group was set as negative control. The second group was treated with Xeloda as a positive control. The third, the fourth and the fifth group were enhanced with resveratrol in the dose of 50mg, 100mg, and 200mg respectively. The BALB/c mice' physical conditions were observed after injecting the stem cells and the experiment was ended by death or by the presence of a tumor with a diameter over 20 mm. Autopsies were performed to determine tumorigenesis in specific locations of the animal's body and photographs were taken. Tumor incidence was calculated at each location of interest. Calculations of tumor volume and the growth rate were performed according to the following formulas:

Tumor volume (mm^3) = $1 / 2ab^2$ (where a = tumor diameter and b is the tumor minor diameter)

Tumor growth rate = (measurement of tumor size at the time point – starting tumor

size) / starting tumor size.

2.3.7 Statistical Analysis

All the experimental data were analyzed using SPSS statistical software version 13.0 (IBM, Chicago, IL). A confidence interval of 95% was used and, therefore p-values of less than 0.05 were considered statistically significant.

2.4 Results

After serum-free cultivation, HT-29 colon cancer cells formed spindle-shaped cells, while HT-29 colon cancer stem cells showed spherical growth(Figure 1.1). After staining with Hoechst33342, the nuclei of HT29 colon cancer cells showed significantly less staining compared to the HT-29 colon cancer stem cells (Figure 1.2). The positive expression rate of CD133 and CD44 in HT-29 cells was 44.6% and 0.6%, respectively (Figure 1.3A). Conversely, the expression rate of CD133 and CD44 in cultured and purified HT-29 stem cells was 92.6% and 97.8%, respectively, and was significantly increased (p <0.001) compared to HT-29 colon cancer cells (Figure 1.3B). Additionally, results of the *in vivo* experiments showed that HT-29 stem cells grew significantly faster than the HT-29 cells. Moreover, the volume of tumors formed by HT-29 cells was twice as large as those formed by HT-29 stem cells (Figure 1.4; p <0.05). In contrast, the growth rate of HT-29 cells was slow (Figure 1.4), and a continuous measurement data comparisons analysis revealed that

the difference was statistically significant ($p < 0.001$). Finally, the results of a drug sensitivity assay for the chemotherapeutic drug 5-FU showed that the IC50 of HT-29 cells was 1.394 µg/mL, compared to 13.087 µg/mL in HT-29 stem cells (Figure 1.5).

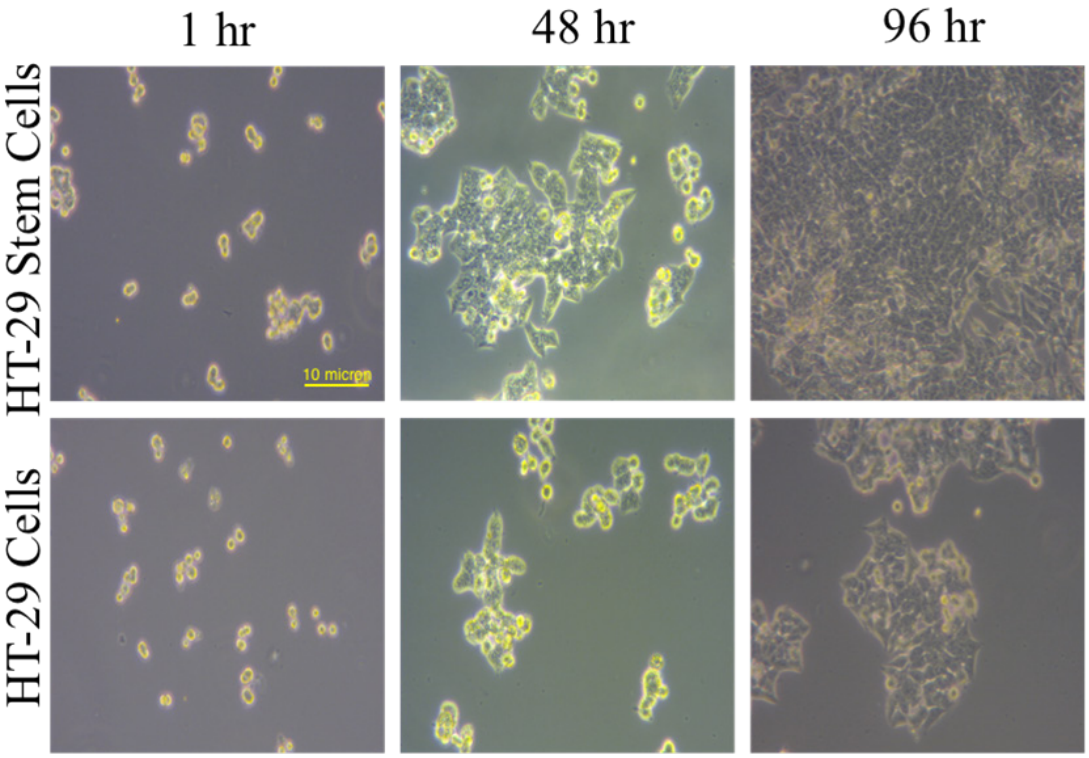

Figure 2.1: Proliferation Assessment of HT-29 Colon Cancer Stem Cells and HT-29 Colon Cancer Cell. In vitro Proliferation Assessment showed HT-29 stem cell's proliferative activity dramatically increased after 48 hours. Magnification 100X Scale bar 10 micron.

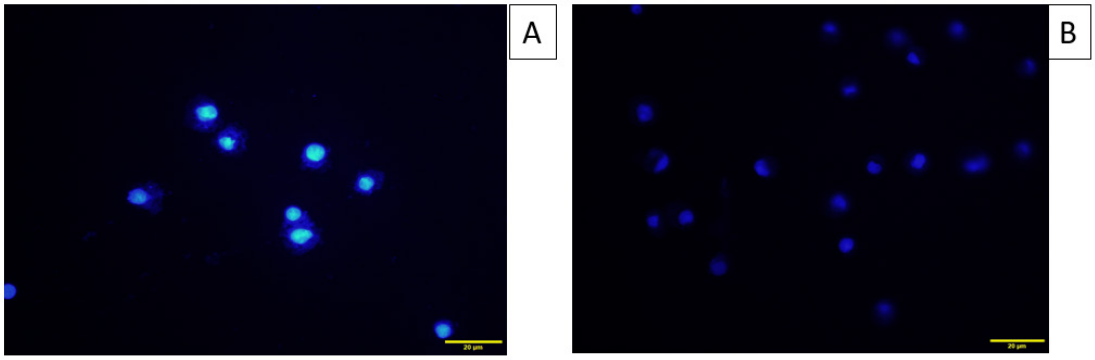

Figure 2.2: Hoechst33342 staining for HT-29 cells (A) and HT-29 stem cells (B). Representative images are shown of Hoechst33342 staining for HT-29 cells (A) and HT-29 stem cells (B). Magnification was 400x for both, and scale bar = 20 µm.

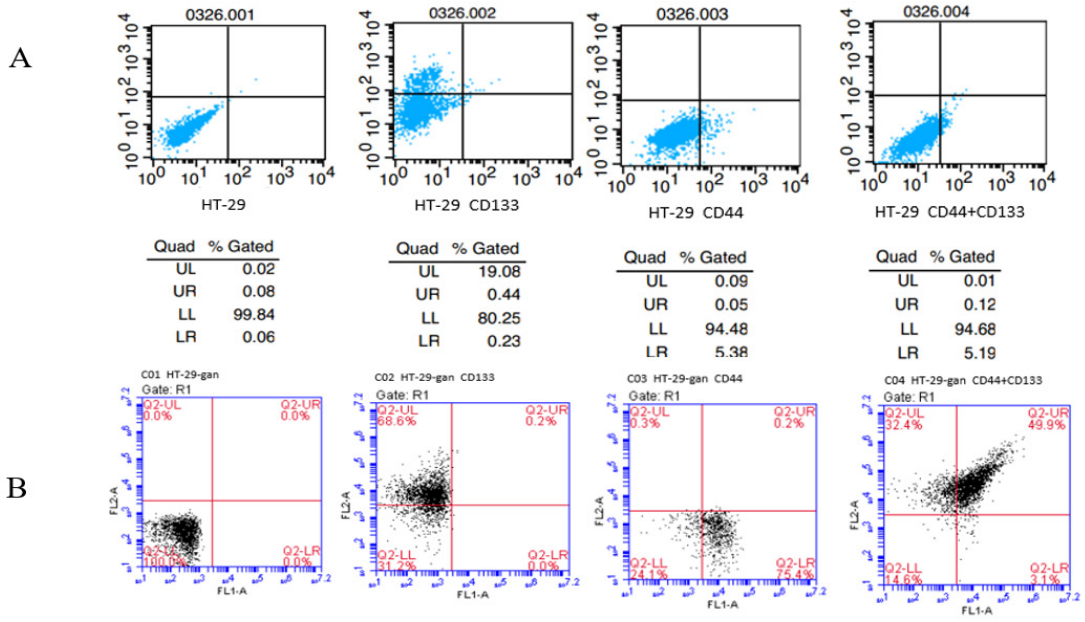

Figure 2.3: Flow Cytometric Analysis of CD133 and CD44 in HT-29 cells (A) and HT-29 stem cells (B).

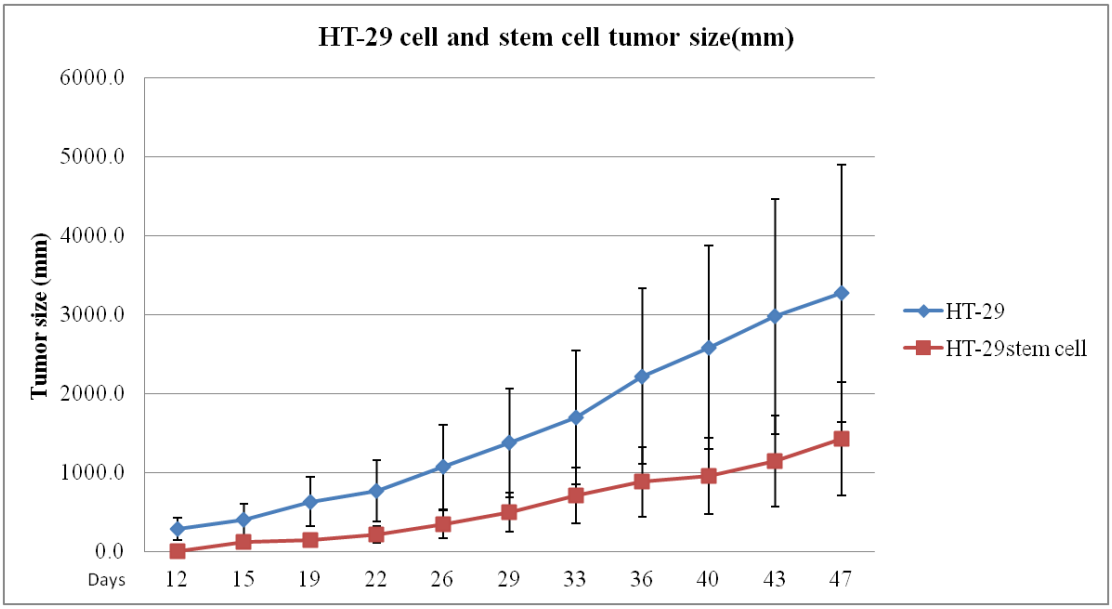

Figure 2.4: In Vivo Tumorigenesis Assay of HT-29 Colon Cancer Stem Cells and HT-29 Colon Cancer Cells

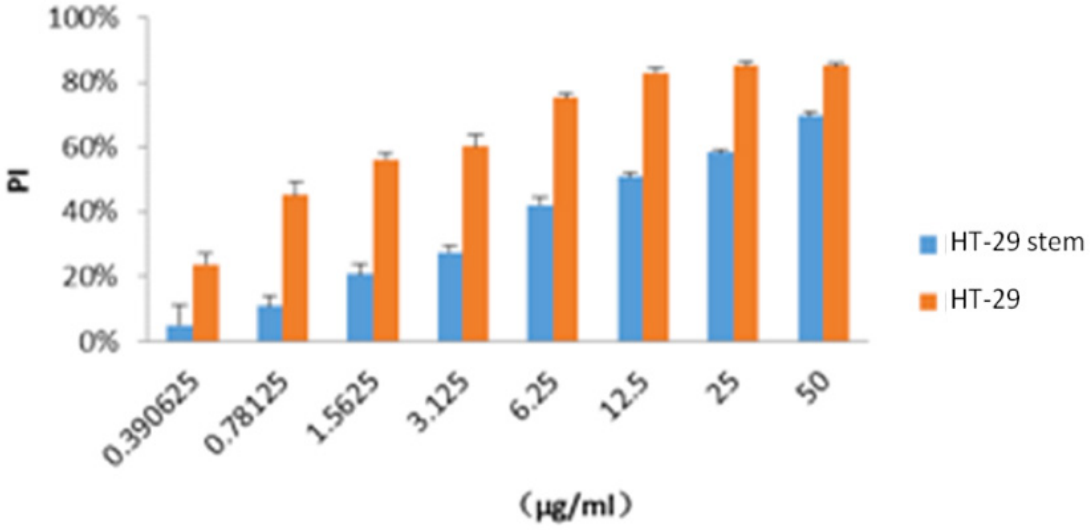

Figure 2.5: 5-FU Drug Sensitivity Assay for HT-29 Colon Cancer Stem Cells and HT-29 Colon Cancer Cells

2.5 Discussion

In this study, stem cells were isolated from tumor cells by culturing them in serum-free medium. Tumor cells cannot grow in these conditions, whereas colon cancer stem cells have the ability to auto-synthesize (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Kondo, Setoguchi, & Taga, 2004). The results showed spherical growth of the isolated HT-29 stem cells and staining with Hochest33342 revealed a phenomenon in which dye was excluded from the nuclear compartments. In addition, it was observed that there is a positive expression of CD133 and CD44, at 92.6% and 97.8% respectively, suggesting that these were correctly identified as colon cancer stem cells. Importantly, HT-29 stem cells were much less sensitive to 5-FU in this study than HT-29 cells: the IC50 of HT-29 cells was 1.394 μg/mL, compared to 13.087 μg/mL in HT-29 stem cells. These findings suggest that at conventional doses, colon cancer stem cells are resistant to chemotherapeutic drugs. Similar results were previously reported by Reya, Morrison, Clarke and Weissman (2001). Taken together, the results suggest that isolated HT-29 stem cells had the general characteristics of colon cancer stem cells.

Previously, it had been shown that colon cancer stem cells play an important role in the occurrence, development, recurrence, and metastasis of colon cancer, leading to the hypothesis that they may be an important target for cancer prevention and

treatment (Kanwar, 2012). Unfortunately, colon cancer stem cells are insensitive to chemotherapy drugs, such as 5-FU (Dhawan et al., 2011), and the toxicity associated with the chemotherapeutic treatment of these cells is high (Han, Shi, Gong, Zhang, & Sun, 2013; Vinogradov & Wei, 2012). Therefore, it is of great importance to explore new methods to inhibit colon cancer stem cells. This study explored the use of *P. oleracea* extracts, as a new method to inhibit colon cancer stem cells.

2.6 Conclusion

In conclusion, this study established that there are significant differences between HT29 colon cancer stem cells and HT29 colon cancer cells. The HT29 colon cancer stem cells have a significantly higher Hoechst33342 staining, significantly increased expression of CD133 and CD44 markers, significantly faster growth and have a lower sensitivity to chemotherapeutic drug 5-FUdrug compared to the HT29 colon cancer cells. These characteristics can be used in clinical practice to differentiate colon cancer stem cells from colon cancer cells for diagnostic purposes.

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3.0 Chapter 2: The Effects of *P. oleracea* Extracts on HT-29 Stem Cells and HT-29 Cells

3.1 Abstract

Novel cancer therapies that target cancer stem cells, which has a low insensitivity to chemotherapeutics, so as to inhibit their proliferation or induce their apoptosis is significant to the management of cancer development, invasion, recurrence, and metastasis. *P. oleracea* extract is of potential value in inhibiting the proliferation of cancer stem cells because studies have shown that to inhibit cancer cells. However, no study has investigated the effect of *P. oleracea* extracts on the proliferation and apoptosis of colon cancer stem cells. This study investigated the effect of ethanol extracts of *P. oleracea* on the proliferation and apoptosis of HT-29 cells and HT-29 stem cells. Ethanol extracts of the medicinal plant were added to the cultured cells and the cell proliferation and apoptosis were assessed. The study found that *P. oleracea* extracts can inhibit the proliferation and increases the apoptosis of colon cancer stem cells at the cellular level in a dose-dependent manner. These results provide a new basis for the prevention and treatment of colon cancer at the level of colon cancer stem cells using *P. oleracea* extracts.[3]

[3] The material contained in this chapter was already submitted to *Tumor Biology*.

3.2 Introduction

Colon cancer, also known as colorectal cancer, is a significant public health problem and has been reported to account for more than 9% of all the cases of cancers in the world (Haggar & Boushey, 2009) and the third most common type of cancer worldwide (Fitzmaurice et al., 2015). Epidemiological data have also shown that colon cancer is the fourth leading cause of cancer-related mortalities worldwide (S. Liu et al., 2015). The cancer is common in both men and women with reports indicating that the cancer accounts for 9.4% of all cancer incidences in men and 10.1% in women (Haggar & Boushey, 2009).

Several risk factors including environmental factors such as obesity (Bardou et al., 2013), sedentary lifestyle (Durko & Malecka-Panas, 2014; Shen et al., 2014) and unhealthy dietary habits (Béliveau & Gingras, 2007; Myles, 2014) as well as other health conditions Terzić, Grivennikov, Karin and Karin (2010) and genetic factors (Jackson-Thompson et al., 2006) have been shown to increase the risk of colon cancer in both women and men. In addition to environmental and genetic factors, colon cancer stem cells also play a significant role in the development, recurrence and metastasis of colon cancer (Dhawan et al., 2011; L Ricci-Vitiani et al., 2008), hence the need to inhibit the stem cells for effective colon cancer therapy.

Currently, colon cancer is primarily managed with chemotherapy regiments of "5 fluorouracil (5FU), oxaliplatin and/or leucovorin or 5-FU, leucovorin and irinotecan (FOLFIRI)" (Garza-Treviño et al., 2015, p.2). The drugs display anti-cancer properties by inducing oxidative damage of the cancer cells. However, the chemotherapy regimen has been reported to be ineffective leading to treatment failures in 90% of metastatic colon cancer and this has been attributed to chemoresistance (Zhao, Butler, & Tan, 2013). The oxidative damage of the cancer cells increases the glycolysis of the cells resulting in high levels of the antioxidant NADPH, which can be associated with chemoresistance (Wu et al., 2008).

Colon cancer stems also displays chemoresistance by expressing HIF-1α which decreases the pro-apoptotic signaling. The expression of HIF-1α induces the expression of decoy receptors like DcR2 which competes for the pro-apoptotic signaling factors such as the tumor necrosis factor-related apoptosis inducing ligand. This reduces the signaling through the apoptosis-inducing receptors and, therefore, attenuates apoptosis thereby allowing cells to tolerate high levels of chemotherapeutic agents (Chen et al., 2009; Flamant et al., 2010; Sullivan & Graham, 2009; Wen et al., 2010). Indeed, there is research evidence to show that most colon cancer stem cells are insensitive to chemotherapy and immunotherapy, leading to the development of resistance to chemotherapy (Dhawan et al., 2011).

The cancer stem cells are insensitive to chemotherapy hence the need for alternative therapy. Research has shown that immunotherapy with antibodies can be utilised as an alternative therapy. For instance, studies have shown that colon cancer stem cells that are insensitive to 5FU can be made sensitive by the use of interleukin-4 blocking antibody (Todaro et al., 2007, 2008). Similarly, it has been demonstrated that the use of anti- epiregulin, epidermal growth factor family (anti-EREG) antibodies is an effective immunotherapy against colon cancer metastasis (Kobayashi et al., 2012). Therefore, there is research evidence to demonstrate the effectiveness of immunotherapy against colon cancer stem cells. However, immunotherapy is an expensive alternative to chemotherapy due to the high cost of producing, storing and transporting the antibodies (Lynch & Murphy, 2016).

Alternatively, nanotechnology can also be utilised to suppress colon cancer stem cells which are resistant to chemotherapy. For instance, siRNA-mediated knockdown of the drug efflux protein MDR1 (the protein is overexpressed in colon cancer stem cells) has been described as a nanotechnology method of overcoming chemoresistance by the colon cancer stem cells (Liu et al., 2009). However, nanotechnology is also an expensive therapy which cannot be afforded by most colon cancer patients (Kim et al., 2016). Therefore, a more affordable and effective alternative is necessary to overcome the chemoresistance of colon cancer stem cells

in the treatment of colon cancers.

The use of medicinal plants has been utilised as an affordable alternative for cancer therapy and can also be utilised to overcome the chemoresistance of colon cancer stem cells. For instance, a study by Min, Lim, Kim, Kim, and Kim (2015) found that the leaves of *Sasa quelpaertensis* can inhibit colon cancer stem cells and influence gene expression related to stem cell development. In another study, Kumar, Kumar, Raina, Agarwal and Agarwal (2014) found that grape seed extract can inhibit the effect of colon cancer stem cells. *P. oleracea* extracts, a traditional Chinese medicine can prevent and treat colon cancer. Extracts of the plant have been to inhibit a number of different cancer cell types (H.-Y. Jin et al., 2012). However, at present no study has yet determined the effect of *P. oleracea* extract on colon cancer stem cells. Therefore, this study investigated the effects of *P. oleracea* extracts on HT-29 stem cells and HT-29 cells.

3.3 Materials and Methods

3.3.1 *P. oleracea* Extract Identification and Concentration Determination

The compound was extracted according to manufacturer's protocol, which is summarized as follows: 50 g of decocted *P. oleracea* were pulverized for approximately three minutes, boiled, and refluxed for 1.5 hours with 10 times the volume of 50% ethyl alcohol. *P. oleracea* was filtered by 200 mesh sieve filtrations

and the residue was extracted twice in the manner described above. The filtrates were combined three times to reach a final volume of 100 mL and incubated in a heated water bath (temperature 95°C). Vacuum distillation was performed to transform the concentrated solution into oil, which was dissolved in 50 mL of ultrapure water and centrifuged at 12000 x g for 20 min. The supernatant was retained and the sample solution was read at 510 nm.

3.3.2 High-performance liquid chromatography-Mass Spectrometry Analysis of *P. oleracea* Extracts

High-performance liquid chromatography (HPLC) was used to identify, quantify and purify the individual components of the compounds present in the plant extract. The *P. oleracea* extract was dissolved in methanol and water prior to mass spectrometry with a method previously described by De Vos, Schipper and Hall (2011). The analysis was carried out using Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA). The column used was ODS column Hedera ODS-2 column measuring 250mm × 4.6 mm at a temperature of 25^{O}C. The mobile phase was 0.1% (v/v) formic acid in acetic acid acetonitrile. The flow rate was 1 ml/min. The UV detector was operated at a wavelength of 358 nm.

3.3.3 Proliferation Assessment of HT-29 cells and HT-29 Stem Cells

HT-29 colon cancer stem cells and HT-29 stem cells were seeded in 21 wells of a

96-well plate at a concentration of 5×10^3; 100 μL of medium was added to each well that did not contain cells. The *P. oleracea* ethyl alcohol extract was dissolved in the complete medium after adding DMSO (concentration of less than 0.01%). After 24 hr and normal cell culture conditions, cells from each of the 21 wells were divided into seven treatment groups. Each group was cultured in triplicate and *P. oleracea*-containing medium was added at 200 μL to a final concentration that was dependent on the treatment group, as follows: 0 (control group), 0.07 μg/mL, 0.14 μg/mL, 0.28 μg/mL, 0.56 μg/mL, 1.12 μg/mL, and 2.25 μg/mL (experimental group). Cells were then placed in an incubator at 37°C and 5% CO_2. After incubation for 24 or 72 hours, MTT reaction liquid was added to the wells at a ratio of 100 μL:10 μL MTT. Following another 1 hour period in the 37°C incubator, the absorbency at 450 nm (A value) was determined with a microplate reader. The following equation was used: inhibition rate = [1 - (A value/drug adding group - A value/blank control group)/(A value /drug group - A value /blank control group)]×100%.

3.3.4 Flow Cytometry Assay for Apoptosis after Treatment of Cells with *P. oleracea*

HT-29 cells and HT-29 stem cells were scattered and digested with EDTA-containing trypsin into single cell suspensions. Cells were stained with trypan blue, the number of live cells was counted, and the living cell concentration was adjusted to 1×10^4 cells/ ml. Cells were seeded into 6-well plates at 1×10^5 cells/well.

After conventional culturing for 24 hours, the cell medium was changed and the complete solution with *P. oleracea* ethyl alcohol was added at 2.25 μg/ml. No drugs were added to the control group: only culture medium was present. After 72 hours of reaction, the old solution was discarded and the cells were washed once with PBS, before adding 1 mL of a digestive solution containing EDTA and trypsin, which was incubated for 1-2 min at 37°C. Cells were observed carefully, and at the point when they exhibited around appearance serum-containing medium was added to stop the digestion. Using a sucker device to blow and suck several times, the cells detached to form a monoplast suspension, and were moved to centrifuge tubes and incubated for 1 hours at 37°C. The cells were centrifuged at 2000 rpm for 5mins at room temperature and re-suspended with 500μl of annexin binding buffer. In the dark, 5.0 μL of Annexin V-FITC(AV) and propidium iodide (PI) dye was added and allowed to mix for 15 min at 4°C, followed by analysis by flow cytometry. PI dye excites with an argon ion laser: the excitation light wavelength is 488 nm with an emission wavelength of over 630 nm (red). The subsequent analysis included blue to red fluorescence scatterplots or topographic maps.

3.3.5 Statistical Analysis

All the experimental data were analyzed using SPSS statistical software version 13.0 (IBM, Chicago, IL). A confidence interval of 95% was used and,

therefore p-values of less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 Compounds Present in *P. oleracea* Extracts, Identified by HPLC-MS

The mass spectrometry analysis of the ethanol extracts of *P. oleracea* showed that the plant contains five flavonoid components; quercetin, kaempferol, myricetin, apigenin, and luteolin (Figure 2.1). The HPLC analysis also identified non-flavonoid components of the extract(Figure 2.2); cyclopentanecarboxylic acid, furan, 9H-Fluorene, benzeneacetic acid, andazaheneicosanoic acid (Table 2.1).

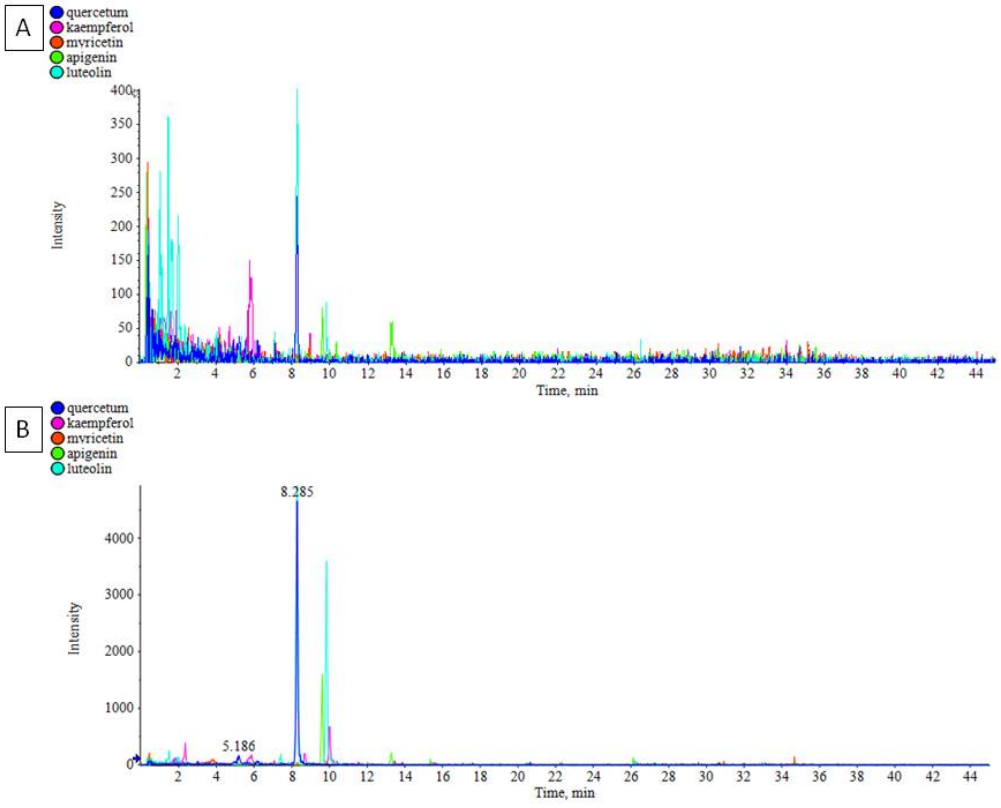


Figure 3.1: Flavonoid Components XIC Manager screening of Ethanol *P.*

***oleracea* Extracts with water(A) and extract with ethanol(B)**.

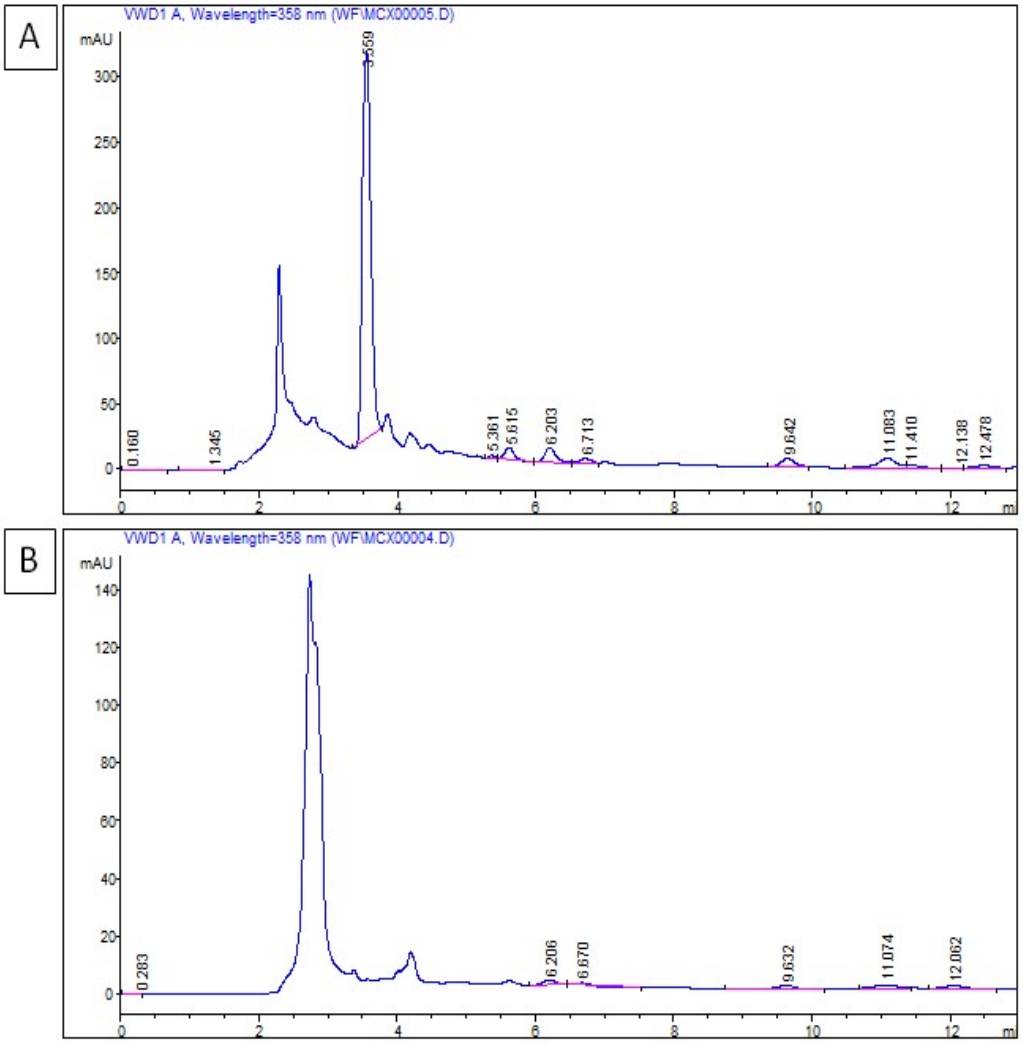
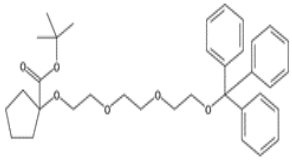
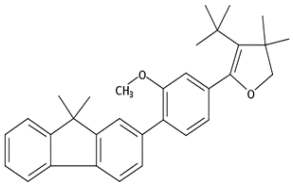
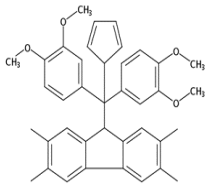
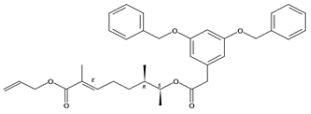
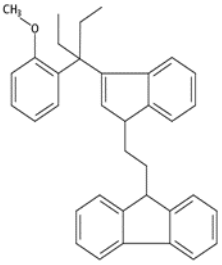
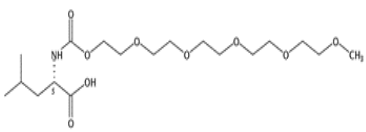


Figure 3.2 HPLC chromatogram of *P.oleracea* extract with water(A) and extract with ethanol(B) The components were identified by mass spectrometry analysis at a mobile phase of 0.1% (v/v) formic acid in acetic acid acetonitrile. The UV detector was operated at a wavelength of 358 nm and separation performed on an ODS column Hedera ODS-2 column (250mm × 4.6mm) at a temperature of 25^{O}C.

Table 3.1

Non-flavonoid Components of Ethanol Extracts of *P. oleracea*

Peak NO	Molecular formula	Identification	Structure
1	$C_{35}H_{44}O_6$	Cyclopentanecarboxylic acid, 1-[2-[2-[2-(triphenylmethoxy) ethoxy] ethoxy]ethoxy]-, 1,1-dimethylethyl ester	
2	$C_{32}H_{36}O_2$	Furan, 4-(1,1-dimethylethyl)-5-[4-(9,9-dimethyl-9*H*-fluoren-2-yl)-3-methoxyphenyl]-2,3-dihydro-3,3-dimethyl-	
3	$C_{39}H_{40}O_4$	9H-Fluorene, 9-[2,4-cyclopentadien-1-ylbis(3,4-dimethoxyphenyl)methyl]-2, 3,6,7-tetramethyl-	

4	$C_{35}H_{40}O_6$	Benzeneacetic acid, 3,5-bis(phenylmethoxy)-, (1R, 2S,5E)-1,2,6-trimethyl-7-oxo-7-(2-propen-1-yloxy)-5-hepten-1-yl ester, rel-	
5	$C_{36}H_{36}O$	9H-Fluorene, 9-[2-[3-[1-ethyl-1-(2-methoxyphenyl) propyl]-1H-inden-1-yl]ethyl] -	
6	$C_{18}H_{35}NO_9$	5,8,11,14,17,20-Hexaoxa-3-azaheneicosanoic acid, 2-(2-methylpropyl)-4-oxo-, (2*S*)-	

3.4.2 Effect of *P. oleracea* extracts on the Proliferation of HT-29 Cells and HT-29 Stem Cells

The results of a proliferation assay showed that HT-29 cells proliferated more slowly than HT-29 stem cells (Figure 2.2). *P. oleracea* extracts exerted an inhibitory effect on the proliferation of HT-29 cells and HT-29 stem cells after 72 hours at concentrations ranging from 0.07 - 2.25 μg/mL, and the level of inhibition was found to increase with dosage (Figure 2.2). Interestingly, *P. oleracea* extracts had a significantly more potent effect on HT-29 colon cancer cells than on HT-29 colon cancer stem cells, based on a repeated measurement data comparisons analysis of

different treatment concentrations ($p < 0.001$). However, for both cell types, as the concentration increased, the difference in the rate of proliferation inhibition was reduced.

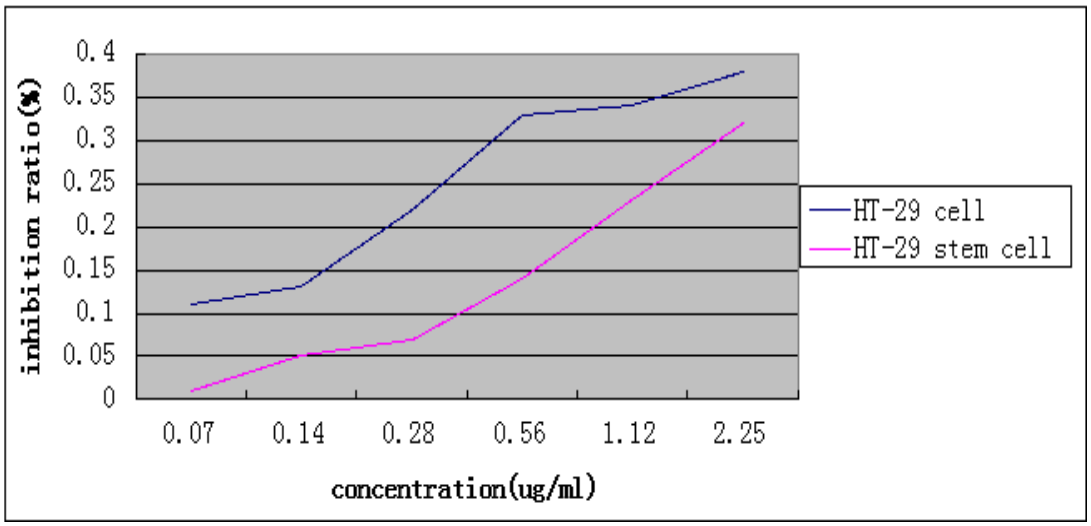


Figure 3.3: Proliferation Assay for HT-29 Cells and HT-29 Stem Cells and the Effect of *P. oleracea* Extract. Stem cells were treated with varying concentrations of *P. oleracea* extract, as indicated, and the effect on proliferation of both cell types was measured after 72 hours of culture. The inhibition rate was determined by an MTT assay, and the ratio was calculated as indicated in the methods. An increased inhibition ratio is associated with a significantly higher inhibition rate of proliferation (p-value < 0.001).

3.4.3 The effect of *P. oleracea* Extracts on Apoptosis, as Measured by Flow Cytometry.

Treatment with *P. oleracea* extract (2.25 µg/mL) resulted in a significant increase in apoptosis in both HT-29 stem cells and HT-29 cells (Figure 2.3; $p < 0.05$ and $p < 0.025$, respectively) compared to untreated controls. Taken together with the previous section, this result suggests that *P. oleracea* extract can inhibit the proliferation of cancer stem cells by inducing apoptosis.

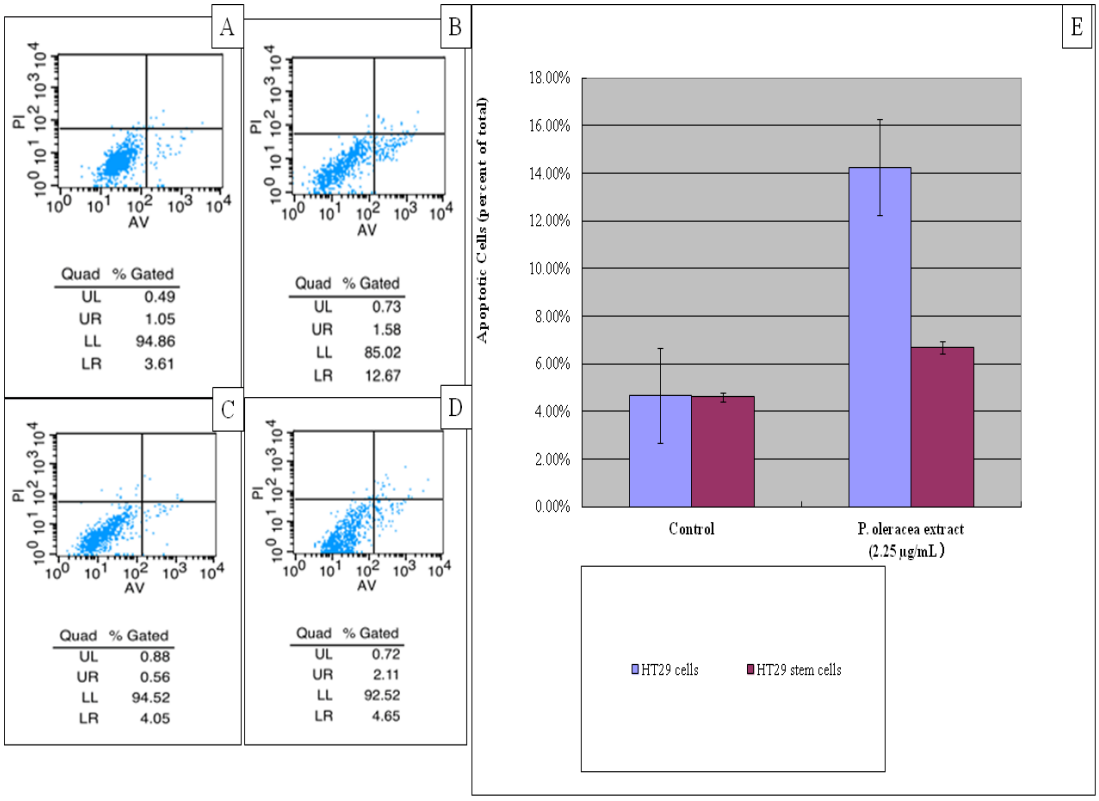


Figure 3.4: The Effects of *P. oleracea* Extracts on Apoptosis of HT-29 Cells and HT-29 Stem Cells. Cells were stained for markers of cell viability: propidium iodine

(PI) and Annexin V (AV). Viable cells are AV and PI negative. Increased uptake of AV is correlated with apoptosis. Double staining with AV and PI is consistent with late apoptosis or dead cells. (A) Untreated HT-29 cells (B) HT-29 cells treated with 2.25 µg/mL *P. oleracea* (C) Untreated HT-29 stem cells (D) HT-29 stem cells treated with 2.25 µg/mL *P. oleracea*. (E) Quantification of flow cytometry data shown in A-D. AV positive cells were considered apoptotic (upper and lower right quadrants).

3.5 Discussion

The results of this study showed that *P. oleracea* extracts can inhibit the proliferation of both colon cancer cells and colon cancer stem cells in a dose-dependent manner. When treated with lower concentrations of *P. oleracea*, the inhibition rate was higher in colon cancer cells than in colon cancer stem cells by more than a two-fold difference. Additionally, *P. oleracea* extracts induced apoptosis in both cell types. The dosage used in this study was a comparatively low dose of *P. oleracea* extract. A study by Al-Sheddi et al. (2015) utilized doses of 250-1000 µg/mL to inhibit the liver cancer cell line HepG2 and lung cancer cell line A-549, the lowest dose being 100 times higher than the highest dose in this study.

Similarly, many previous studies have found beneficial effects of *P. oleracea* on cancer cells. For instance, a study by Ji et al. (2015) showed that *P. oleracea* extract inhibits the proliferation, invasion, and metastasis of the liver cancer cell line

HCCLM3. Another study by Gu et al. (2015) found that both fresh and dry *P. oleracea* extract inhibit liver cancer cell proliferation and have anti-oxidant effects. In addition, Farshori et al. (2014) reported that *P. oleracea* extract can inhibit the influence of liver cancer cells. Finally, a study by Zhao et al. (2013) found that *P. oleracea* extracts can inhibit cervical cancer cells. However, this is the first study to show that *P. oleracea* has a similar influence on colon cancer and can inhibit colon cancer stem cells.

3.6 Conclusion

In conclusion, the results of this study show that *P. oleracea* extracts can inhibit the proliferation and increases the apoptosis of colon cancer stem cells at the cellular level in a dose-dependent manner. These results provide a new basis for the prevention and treatment of colon cancer at the level of colon cancer stem cells using *P. oleracea* extracts.

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4.0 Chapter 3: The Effects of *P. oleracea* Extracts on the Expression of Notch1, Notch2, and Beta-Catenin mRNA in HT-29 Cells and HT-29 Stem Cells

4.1 Abstract

The Notch signal transduction pathway has been shown to be important for the development of colon cancer stem cells (H.-Y. Jin et al., 2012; Qiao & Wong, 2009). After translation, the Notch pathway has also been shown to regulate the Beta-Catenin mRNA in stem cells (Kwon et al., 2011). This study investigated the effect of *P. oleracea* extracts on the expression of Notch1, Notch2, and Beta-Catenin mRNA in HT-29 cells and HT-29 stem cells. It was found that *P. oleracea* extracts significantly down-regulate the expression of Notch1 and β-catenin in both HT-29 cells and HT-29 stem cells. However, the treatment of HT-29 cells and HT-29 stem cells with *P. oleracea* extract caused a slight increase in expression of Notch2, although this difference was not statistically significant. This suggests that alterations in the Notch signal transduction pathway underlie the inhibitory effects of *P. oleracea* on proliferation, as well as the increase in apoptosis, of colon cancer stem cells.[4]

[4] The material contained in this chapter was already submitted to *Tumor Biology*.

4.2 Introduction

The main properties of colon cancer stem cells (which plays a significant role in the development, recurrence, and metastasis of colon cancers) include self-renewal potential, ability to differentiate into diverse cell populations and ability to balance and modulate the processes of self-renewal and differentiation (Garza-Treviño et al., 2015). Self-renewal refers to the ability of the colon cancer stem cells synthesize new cells with the same ability to proliferate, expand and differentiate as the parental cells thereby maintaining a pool of the stem cell (Garza-Treviño et al., 2015). The mechanisms utilised for the self-renewal of the colon cancer stem cells, such as the Wnt/β-catenin and Notch pathways, makes use of proto-oncogenes. In addition, the self-renewal pathway of colon cancer stem cells is regulated by the sonic hedgehog (Hh) signaling pathway, a pathway commonly reported in multiple myeloma (Varnat et al., 2009). Research has shown that the self-renewal of colon cancer stem cells is promoted by the Wnt pathway and the prevention of the β-catenin-dependent transcription (Ong et al., 2014).

Additionally, the Notch receptors pathway has also been reported to promote the self-renewal of colon cancer stem cells (Ong et al., 2014). In the Notch pathway, the DLL4 genes stimulate the Notch receptors on adjacent cells and in combination with

the effects of β-catenin promotes the self-renewal of the cancer stem cells by directing an immature transcription profile (Garza-Treviño et al., 2015). However, the self-renewal pathways are inhibited by BMP4 genes which bind BMP receptors leading to interference with the Wnt pathway and thus promote cell differentiation. The process of differentiation has also been reported to be inhibited by hepatocyte growth factor (HGF) leading to the maintenance of the cells in a stem-cell state (Ong et al., 2014).

Cancer stem cells also have the ability into differentiate into diverse cell populations (Garza-Treviño et al., 2015). That is, the stem cells have the ability to differentiate into a heterogeneous progeny of cells that can undergo progressive diversification and specialization following self-renewal. The cells of this differentiation are specialized to carry out certain unique physiological functions (Dalerba, Cho, et al., 2007). However, histopathological studies of colon cancers have indicated that the cells are relatively undifferentiated containing higher proportions of colon cancer stem cells than more differentiated cancer cells (Ashley et al., 2013; Merlos-Suárez et al., 2011). The undifferentiated colon cancer cells have worse clinical prognosis compared to differentiated cancer cells (Merlos-Suárez et al., 2011).

Homeostatic control where the cells have the ability to balance and modulate the

processes of self-renewal and differentiation is also a key property of cancer stem cells (Garza-Treviño et al., 2015). Through research, it has been established that differentiated intestinal epithelial cells reside in the intestinal crypts as repeats of stem cells containing leucine and B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1) or G protein-coupled receptor 5 (Lgr5) (Tian et al., 2011). The Bmi-1 AND Lgr5 are used to maintain the capacity of tissues to regenerate (Tian et al., 2011). The intestinal cells that express Lgr5 undergo active proliferation and very sensitive to stimulatory effects of Wnt and Dkk1-mediated Wnt inhibition (Tian et al., 2011). On the other hand, intestinal cells that express Bmi-1 are less sensitive to environmental stressors and are, therefore, not affected by the Wnt modulation. The Bmi-1-expressing intestinal cells are normally quiescent, only giving rise to cell progeny under certain conditions (Yan et al., 2012). The Wnt proteins and the Notch pathway has been reported to play a significant role in maintaining cancer stem cells hemostasis by providing signals required for the maintenance of the phenotype of colon cancer stem cells in the tumour mass (Garza-Treviño et al., 2015).

Based on these three properties of colon cancer stem cells, it can be deduced that the dysregulation of the Notch pathway and Beta-Catenin mRNA plays a role in the development of cancer. Therefore, the ability of *P. oleracea* extracts to inhibit the proliferation but promote the apoptosis of colon cancer stem cells, in case of any,

may suggest that the extracts dysregulate the Notch pathway and Beta-Catenin mRNA. However, there is no study that has investigated the effect of *P. oleracea* extracts on the expression of Notch1, Notch2, and Beta-Catenin mRNA in HT-29 cells and HT-29 stem cells. Therefore, this study investigated the effect of *P. oleracea* extracts on the expression of Notch1, Notch2, and Beta-Catenin mRNA in HT-29 cells and HT-29 stem cells.

4.3 Materials and Methods

4.3.1 Real-time Fluorescence Quantitative PCR (FQ-PCR) Assay for the Expression of Notch1, Notch2, and β-catenin mRNA

HT29 cells were cultured in McCoy's 5A medium with 10% FBS, while HT29 stem cells were cultured in RPMI-1640 medium with 10% FBS. Both cell strains were seeded at a concentration of 5×10^5 cells/mL in complete medium containing 10 cm dishes, with two dishes for each strain. After 24 hours under normal conditions, a complete medium that contained the following concentrations of *P. oleracea* extract was added: 0 μg/mL (control group) or 2.25 μg/mL (experiment group). After 72 hours in an incubator at 37° and 5% $C0_2$, the cells were washed twice with pre-cooled PBS. Total RNA was isolated with Trizol kit. RNA was reverse-transcribed to cDNA (Real Time PCR) using the primers shown in Table 3.1. Ribosome 18S (18S rRNA) was used as an internal reference.

Table 4.1
Primers used for the Quantitative RT-PCR Assay

Gene	Primer	Primer sequence	Length
Notch1	Notch1 (+)	CCGCAGCACTATTGAGAACA	213 bp
	Notch1 (-)	ATCCATGTGTAGCCGTAGCC	
Notch2	Notch2 (+)	TAATACGACTCACTATGAGA	229 bp
	Notch2 (-)	ATTTAGGTGACACTATCTGT	
β-catenin	β-catenin (+)	AAGTTCTTGGCTATTACGCA	203 bp
	β-catenin (-)	ACAGCACCTTCAGCACTATG	
18s rRNA	18s rRNA (+)	TTTGTTGGTTTTCGGAACTGA	198 bp
	18s rRNA (–)	CGTTTATGGTCGGAACTACGA	

4.3.2 Western Blot Assays for the Expression of ß-catenin, Notch1, and Notch2 Proteins

HT-29 cells and HT-29 stem cells were treated with 2.25 μg/mL of *P. oleracea* extract, a cell scraper was used to lift cells in the primary culture solution, total protein was extracted, and a Western blot assay was prepared, according to the manufacturer. The total grey value in the resulting Western blots was measured with Band Scan Analysis software, which allowed for a quantitative comparative analysis. Measurements were normalized to β-actin.

4.3.3 Statistical Analysis

All the experimental data were analyzed using SPSS statistical software version 13.0 (IBM, Chicago, IL). A confidence interval of 95% was used and, therefore

p-values of less than 0.05 were considered statistically significant.

4.4 Results

4.4.1 Expression of Notch1, Notch2, And B-Catenin mRNA in HT-29 Colon Cancer Cells And HT-29 Colon Cancer Stem Cells with and Without Treatment with *P. Oleracea* Extracts.

At baseline, the expression of Notch1, Notch2, and β-catenin mRNA was lower in HT-29 colon cancer cell lines than in HT-29 colon cancer stem cells. While the differences in the expression of Notch1 and β-catenin were statistically significant, the differences in Notch2 were not significant (Table 3.2). After treatment with *P. oleracea* extract (2.25 μg/mL), the expression of Notch1 and β-catenin in both HT-29 cells and HT-29 stem cells were significantly down-regulated. Conversely, treatment with *P. oleracea* extract caused a slight increase in expression of Notch2, although this difference was not statistically significant (Table 3.3).

Table 4.2
Expression of Notch1, Notch2, and β-catenin mRNA in HT-29 cells and HT-29 stem cells

	HT-29 cells (mean±SD)	HT-29 stem cells(mean±SD)	*p-value*

β-catenin	1.16 ± 0.17	2.34 ± 0.28	0.003
Notch1	1.01 ± 0.01	1.66 ± 0.03	<0.001
Notch2	1.11 ± 0.11	1.16 ± 0.10	0.584

Table 4.3
Expression of Notch1, Notch2, and β-catenin mRNA in HT-29 cells and HT-29 stem cells after treatment with P. oleracea (mean±SD)

	β-catenin	Notch1	Notch2
HT-29 cells	1.16 ± 0.17	1.01 ± 0.01	1.11 ± 0.11
+ *P. oleracea*	0.003 ± 0.0004	0.004 ± 0.001	1.93 ± 0.30
p-value	0.000	0.000	0.32
HT-29 stem cells	2.34 ± 0.28	1.66 ± 0.03	1.16 ± 0.10
+ *P. oleracea*	0.003 ± 0.001	0.003 ± 0.0003	1.37 ± 0.067
p-value	<0.001	<0.001	0.053

4.4.2 Results of Western Blot Analyses for β-catenin, Notch1, and Notch2

Treatment with *P. oleracea* extracts decreased the expression of Notch1, Notch2, and β-catenin protein in HT-29 cells and HT-29 stem cells (Table 3.4, Figure 3.1).

Table 4.4
Relative expression of Notch1, Notch2, and β-catenin proteins in HT-29 cells and HT-29 stem cells after treatment with *P. oleracea* compared to β-actin

	β-catenin	Notch1	Notch2
HT-29 stem cells	1.64	1.26	0.69
HT-29 stem cells + *P. oleracea*	0.16	0.41	0.03
p-value	0.027	0.032	0.052
HT-29 cells	0.70	0.45	0.17
HT-29 cells + *P. oleracea*	0.55	0.32	0.05
p-value	0.002	0.011	0.355

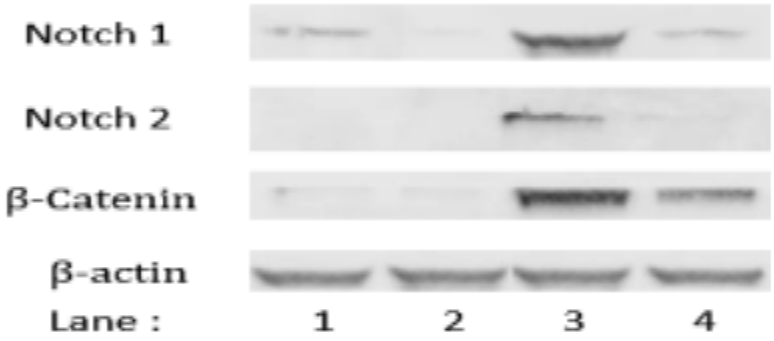

Figure 4.1: Representative Western blots for the expression of proteins in the Notch signal transduction pathway after treatment with *P. oleracea.* Lane 1: HT-29 stem cells + *P. oleracea*; Lane 2: HT-29 cells + *P. oleracea*; Lane 3: HT-29 stem cells; Lane 4: HT-29 cells.

4.5 Discussion

The Notch signal transduction pathway is an important regulator gene for the development of colon cancer stem cells (H.-Y. Jin et al., 2012; Qiao & Wong, 2009). Preliminary results have shown that tea polyphenol can inhibit and regulate the genetic effects of the Notch signal transduction pathway (H. Jin, Tan, Liu, & Ding, 2010). The target genes of the Notch signal transduction pathway are closely related to colon cancer stem cells and include Notch1, Notch2, and β-catenin. In this study, it was found that the expression of Notch1 and β-catenin mRNA was significantly higher in colon cancer stem cells than in colon cancer cell lines. This suggests that Notch1 and β-catenin gene expression decreases when colon cancer stem cells mature. However, the reason for this is unclear and requires further study. Importantly, this study established that treatment with *P. oleracea* extracts inhibited the expression of Notch1 and β-catenin mRNA by close to 10-fold. Taken together, it is possible that alterations in the Notch signal transduction pathway underlie the inhibitory effects of *P. oleracea* on proliferation, as well as the increase in apoptosis, of colon cancer stem cells. At present, there are only a limited number of studies on *P. oleracea* extract;

therefore, next study will continue to explore the possible mechanisms underlying these suppressive effects on colon cancer stem cells.

4.6 Conclusion

In conclusion, this study established that *P. oleracea* extracts significantly inhibit the expression of Notch1 and β-catenin mRNA by close to 10-fold, suggesting that alterations in the Notch signal transduction pathway underlie the inhibitory effects of *P. oleracea* on proliferation, as well as the increase in apoptosis, of colon cancer stem cells.

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5.0 Summary and Future Perspective

Given the importance of preventing and treating colon cancer at the level of the cell, a number of difference avenues have been explored for inhibiting colon cancer stem cells. Aside from immunological therapy, chemotherapy, and the other standard treatments, some investigators have reported on the merits of using natural drugs or foods to prevent and treat this disease. For example, a study by Min, Lim, Kim, Kim, and Kim (2015) found that the leaves of *Sasa quelpaertensis* can inhibit colon cancer stem cells and influence gene expression related to stem cell development. In another study, Kumar, Kumar, Raina, Agarwal and Agarwal (2014) found that grape seed extract can inhibit the effect of colon cancer stem cells. Similarly, in this study, alcohol was used to extract the flavone content of *P. oleracea*, and this extract was found to inhibit colon cancer and colon cancer stem cells. These findings suggest that *P. oleracea* extract could represent a potential option in the prevention and treatment of colon cancer, as well as for adjuvant therapy.

The results of this study demonstrated that *P. oleracea* extracts can inhibit colon cancer stem cells at the cellular level in a dose-dependent manner. This result provides a new basis for the prevention and treatment of colon cancer at the level of colon cancer stem cells using *P. oleracea* extracts. This study also established that *P. oleracea* extracts significantly inhibit the expression of Notch1 and β-catenin mRNA

by close to 10-fold, suggesting that alterations in the Notch signal transduction pathway underlie the inhibitory effects of *P. oleracea* on proliferation, as well as the increase in apoptosis, of colon cancer stem cells. However, further studies should be conducted to explore the possible mechanisms underlying these suppressive effects on colon cancer stem cells considering the limited number of studies on *P. oleracea* extracts in this area currently.

5.1 References

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