

LEBANESE AMERICAN UNIVERSITY

A Comparative Study of the Antiproliferative Effect of Kohlrabi and Green Cabbage on Colorectal Cancer Cell Lines

By
Valia M. Zarzour

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Molecular Biology

School of Arts and Sciences

September 2012

Thesis Proposal Form (Annex I)

Name of Student: Valia Zarzour I.D.#: 200503272

Department: Natural Sciences

On (dd/mm/yy) 19-05-2010, has presented a Thesis proposal entitled

A Comparative Study of the Antiproliferative Effect of Kohlrabi and Green Cabbage on Colorectal Cancer Cell Lines

In the presence of the Committee members and Thesis Advisor:

Dr. Sandra Rizk 
(Name and Signature of the Thesis Advisor)

Proposal Approved on (dd/mm/yy): 14-07-2010

Comments/Remarks/Conditions to Proposal:

Student must follow the proposal suggested, taking into consideration the committee's suggestions

Date: 24/09/2012

Acknowledged by 
(Dean, School of Arts and Sciences)

cc: Chair
Advisor
Student
File Graduate Studies

Thesis Defense Result Form (Annex II)

Name of Student: Valia Zarzour I.D.#: 200503272

On (dd/mm/yy) 21-09-2012, has defended a Thesis entitled:

A Comparative Study of the Antiproliferative Effect of Kohlrabi and Green Cabbage on Colorectal Cancer Cell Lines

In the presence of the following Committee members:

Advisor Dr. Sandra Rizk
(Name and Signature)

Committee Member Dr. Roy Khalaf
(Name and Signature)

Committee Member Dr. Sima Tokajian
(Name and Signature)

Committee Member _____
(Name and Signature)

The student has passed/~~not passed~~ the Thesis defense in partial Fulfillment of the requirements of the degree of MS in Molecular Biology

Comments/Required Changes to Thesis due on (dd/mm/yy):

Sep. 24th, 2012

Advisor's report on completion of above Thesis conditions:

She fulfilled the requirements.

Changes Approved by Thesis Advisor: SANDRA RIZK Signature: _____ 24/09/2012

Date: 24/09/2012 Acknowledged by _____
(Dean, School of Arts and Sciences)

cc: Registrar
Advisor
File Graduate Studies

Date of the Thesis defense public announcement (dd/mm/yy) 05/09/2012

Thesis approval Form (Annex III)

Student Name: **Valia Zarzour** I.D. #: 200503272

Thesis Title : **A Comparative Study of the Antiproliferative Effect of Kohlrabi and Green Cabbage on Colorectal Cancer Cell Lines**

Program : M.S. in Molecular Biology

Division/Dept : Natural Sciences Department

School : **School of Arts and Sciences**

Approved by:

Thesis Advisor: Dr. Sandra Rizk  _____

Member : Dr. Roy Khalaf  _____

Member : Dr. Sima Tokajia  _____

Date: September 24, 2012

THESIS/PROJECT COPYRIGHT RELEASE FORM

LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants to Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic format and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Valia Zarzour

Signature: 

Date: 24 Sep
2012

PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that I have read and understood LAU's Plagiarism Policy. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.

This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Valia Zarzour

Signature: 

Date: 24 Sep - 2012

Dedication

First and foremost, I am genuinely thankful for my supervisor Dr. Sandra Rizk-Jamati. Throughout all my years at the Lebanese American University, she has shown me continuous support and encouragement academically and socially. My senior project with Dr. Rizk and her field of research were the motives that encouraged me to enroll in the masters' program. I am truly grateful to have had worked under her supervision, she guided me and provided me with all her expertise, and mostly she was considerate, devoted, committed to the project and patient. By all means, Dr. Rizk is a role model as a professor, mentor and mother.

I would also like to convey my appreciation to the Dean of Arts and Sciences Dr. Philippe Frossard, the Chairperson of the Biology department Dr. Costantine Daher, the faculty members of the Biology department Dr. Mirvat El-Sibai and Dr. Ralph Abi-Habib for their technical support and insightful comments, Dr. Pierre Zalloua, Dr. Brigitte Wex and to the committee members Dr. Sima Tokajian and Dr. Roy Khalaf.

I sincerely thank Mrs. Sawsan Jabi for her technical support and more importantly for her moral soothing support without which I couldn't have overcome the disappointments of some experiments and the long h in the lab. I would also like to thank Dr. Katia Maalouf who taught me most of the cell culture techniques, Dr. Ghinwa Barakat who taught critical lab techniques and was a great friend more than a mentor, Ms. Maya Farah and Mrs. Helena Bou Farah for their technical support, and Omayr for his lab assistance.

Finally special thanks go to my husband, mom and dad for their unconditional encouragement in pursuing my education, and I thank my dear friends Angelique Salloum and Sonia Youhana for their follow up and care during the master's program and thesis project.

A Comparative Study of the Antiproliferative Effect of Kohlrabi and Green Cabbage on Colorectal Cancer Cell Lines

Valia Zarzour

Abstract

Experimental studies showed that phytochemicals from Brassicaceae Cruciferae vegetables possess anticarcinogenic properties. This association is attributed to glucosinolates molecules, mainly isothiocyanates and indoles released upon consumption of these vegetables. Two members of this family, namely cabbage (*Brassica oleracea*, group *Capitata*) and kohlrabi (*Brassica oleracea*, group *Gongylodes*) seem to share similar anticarcinogenic characteristics. The aim of this study is to compare the effect of the ethanolic extracts of the edible parts of these two plants on the proliferation and apoptosis of HT-29 and Caco-2 colorectal cancer cell lines. The cytotoxicity of the extracts was assayed using the Trypan Blue Exclusion method and the IC50 was determined to be higher in kohlrabi compared to cabbage. Consequently, WST-1 assay was used to assess the effect of these plant extracts on the proliferation of colorectal cancer cell lines: both extracts exhibited a sharp anti-proliferative effect in a dose and time dependent manner, even though the cell viability recorded upon kohlrabi treatment was slightly higher. Analysis of cell DNA content by flow cytometry revealed the ability of both kohlrabi and cabbage extracts to induce cell cycle arrest in HT-29 cells at the G phase and S phase in similar patterns. Furthermore both extracts promoted apoptosis in colorectal cancer cell lines, as revealed by Cell Death ELISA Assay, as well as Annexin/PI staining followed by flow cytometry analysis. The apoptotic pathway seems to involve the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2: the expression of these apoptotic regulatory proteins was altered upon cabbage and kohlrabi treatments in a dose-dependent manner, with a slightly higher increase in the Bax/Bcl-2 ratio upon treatment with cabbage extract. As a conclusion, kohlrabi should be considered, like cabbage, among the potent anti-carcinogenic cruciferous vegetables.

Keywords: Kohlrabi, Colorectal cancer, Proliferation, Apoptosis, DIM, SFN, Erucin, AITC, Bax, Bcl-2.

Table of Contents

List of tables.....	xii
List of figures.....	xiii
Glossary.....	xvi
1. Literature Review.....	1
1.1. Cancer.....	1
1.1.1. Introduction.....	1
1.1.2. Colorectal Cancer (CRC).....	1
1.1.2.1. Introduction.....	1
1.1.2.2. Prevalence of Colorectal cancer.....	2
1.1.2.3. Polymorphisms associated to CRC	3
1.1.2.4. The EGFR pathway and CRC.....	3
1.1.2.5. The role of VEGF in CRC.....	4
1.1.2.6. Treatment of colorectal cancer.....	4
1.1.2.7. CRC and Diet.....	5
1.2. Brassica Oleracea	6
1.2.1. Brassica oleracea background and history	6
1.2.2. Cabbage Brassica oleracea Capitata.....	6
1.2.2.1. Health benefits of cabbage.....	7
1.2.2.2. Anti-nutritional effects of cabbage and constituents	13
1.2.3. Kohlrabi <i>Brassica oleracea</i> var. <i>gongylodes</i>.....	13
1.3. Purpose of the study	17
2. Materials and Methods	18
2.1 Cell lines and cell culture.....	18
2.2 Preparation of kohlrabi and cabbage ethanolic extract	18
2.3 Antibodies and reagents	18
2.4 Cytotoxicity: Trypan Exclusion Method.....	19
2.5 Proliferation: Cell proliferation reagent (WST-1).....	19
2.6 Cell cycle Analysis: Flow cytometry.....	20
2.7 Apoptosis.....	20
2.7.1 Cell Death ELISA Plus Kit.....	20
2.7.2 Flow Cytometry: Annexin V-FITC/PI double staining method.....	21

3. Results	24
3.1. Cytotoxicity effect of cabbage and kohlrabi on Ht-29 and Caco-2 cell lines	24
3.2. Effect of cabbage and kohlrabi on the proliferation of HT-29 and Caco-2 cell lines	29
3.3. Cell cycle arrest induction of HT-29 cells by kohlrabi and cabbage	35
3.4. Proapoptotic effect of kohlrabi and cabbage on HT-29 and Caco-2 cell lines. ...	37
3.4.2. Apoptosis by Flow Cytometry:	41
3.5. Effect of Kohlrabi and Cabbage on Bax and Bcl-2 in HT-29 cells	43
4. Discussion	45
5. Bibliography	52

List of Tables

Table 1: Major glucosinolates found in Brassicae Oleracea	8
Table 2:GLS Content (Milligrams per 100 g) of Cabbage and kohlrabi grown in 1994.....	16

List of Figures

Figure 1: Schematic pathways of the antitumor activity of ITCs	20
Figure 2A: Cytotoxicity of Kohlrabi on two cell lines HT-29 and Caco-2 after 24h of treatment.....	25
Figure 2B: Cytotoxicity of Kohlrabi on two cell lines HT-29 and Caco-2 after 48h of treatment.....	25
Figure 2C: The cytotoxicity of cabbage on two cell lines HT-29 and Caco-2 after 24h.....	26
Figure 2D: Cytotoxicity of cabbage on two cell lines HT-29 and Caco-2 after 48h of treatment.....	26
Figure 2E: Cytotoxicity of kohlrabi versus cabbage extracts after treating HT-29 cells with different concentrations for 24h.....	27
Figure 2F: Cytotoxicity of kohlrabi versus cabbage extracts after treating Ht-29 cells with different concentrations for 48h.....	28
Figure 2G: Cytotoxicity of kohlrabi versus cabbage extracts after treating Caco-2 cells with different concentrations for 24h.....	28
Figure 2H: Cytotoxicity of kohlrabi versus cabbage extracts after treating Caco-2 cells with different concentrations for 48h.....	29
Figure3A: Proliferation of Caco-2 cells and HT-29 cells after 24h of treatment with different concentrations of kohlrabi.....	30
Figure 3B: Proliferation of Caco-2 cells and HT-29 cells after 48h of treatment with different concentrations of kohlrabi.....	30
Figure3C: Proliferation of Caco-2 cells and HT-29 cells after 24 h of treatment with different concentrations of cabbage.....	31
Figure3D: Proliferation of Caco-2 cells and HT-29 cells after 48 h of treatment with different concentrations of cabbage.....	31
Figure 3E: Antiproliferative effect of kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 24h.....	33
Figure 3F: Antiproliferative effect of kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 48h.....	33
Figure 3G: Antiproliferative effect of kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 24h.....	34

Figure 3H: Antiproliferative effect of kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 48h.....	34
Figure 4: Cell cycle analysis by flow cytometry.....	36
Figure 5A: Induction of apoptosis in HT-29 and Caco-2 cells 24h after kohlrabi treatment.....	37
Figure 5B: Induction of apoptosis in HT-29 and Caco-2 cells after 48h of kohlrabi treatment.....	38
Figure 5C: Induction of apoptosis in HT-29 and Caco-2 cells 24h after cabbage treatment.....	38
Figure 5D: Induction of apoptosis in HT-29 and Caco-2 cells 48h after cabbage treatment.....	39
Figure 5E: Apoptosis induction of Kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 24h.....	40
Figure 5F: Apoptosis induction of Kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 48h.....	40
Figure 5G: Apoptosis induction of Kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 24h.....	41
Figure 5H: Apoptosis induction of Kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 48h.....	41
Figure 6: Analysis of apoptosis induced by cabbage and kohlrabi in HT-29 cells by flow cytometry using annexinV-FITC and PI.....	43
Figure 7: Western Blot analysis for Bax and Bcl-2.....	44

Glossary

CRC: Colorectal cancer

mCRC: metastatic colorectal cancer

WCRF/AICR: World Cancer Research Fund and the American Institute of Cancer
Research

ITC: Isothiocyanates

AITC: Allyl-isothiocyanate

PEITC: Phenethyl isothiocyanate

PITC: Phenyl isothiocyanate

BITC: Benzyl-isothiocyanate

SFN: Sulfuraphane

ER: Erucin

DIM: 3, 3'-diindolylmethane

I3C: Indole-3-carbinol

FOBT: Fecal occult blood test

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

mRNA: messenger ribonucleic acid

TNF- α : Tumor necrosis factor-alpha

ROS: reactive oxygen species

MCF-7: Michigan Cancer Foundation-7

VEGF: Vascular endothelial growth factor

GSTP-1: Glutathione S-transferase P 1

EGFR: Epidermal growth factor receptor

EGF: Epidermal Growth Factor

TGF- α : Transforming growth factor alpha

Ras: Rat sarcoma

K-Ras: Kirsten rat sarcoma

PI3K: Phosphatidylinositol 3-kinase

PI3KCA: Phosphoinositide-3-kinase, catalytic, alpha polypeptide

PTEN: Phosphatase and tensin homolog

PKC: Protein kinase C

VEGFR: Vascular endothelial growth factor receptor

CYP: Cytochrome P450
GST: Glutathione S-transferase
JNK: c-Jun N-terminal kinase
HDAC: Histone deacetylase
MAPK: Mitogen-activated protein kinase
Cdk: Cyclin dependent kinase
CDI: Cyclin dependent inhibitor
NFκB: nuclear factor kappa B
CYP DME: Cytochrome P450 drug-metabolizing enzymes
FBS: Fetal Bovine Serum
Bax: Bcl-2-associated X protein
Bcl-2: B-cell lymphoma 2
PI: Propidium Iodide
PBS: Phosphate Buffered Saline
G0: Gap 0
G1: Gap 1
S: Synthesis
M: Mitosis
ELISA: Enzyme-linked immunosorbent assay
ABTS: 2, 2'-azino-di(3-ethylbenzthiazolin-sulfonate)
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
PVDF: Polyvinylidene fluoride
ECL: Enhanced Chemiluminescence
IC50: Inhibitory concentration 50
p53: protein 53
PARP: Poly (ADP-ribose) polymerase
UGT1A1: UDP glucuronosyltransferase 1 family, polypeptide A1
XPD: xeroderma pigmentosum group D
XRCC-1: X-ray repair complementing defective repair in Chinese hamster cells 1
MGMT: Methylated-DNA-protein-cysteine methyltransferase
Bcl-2: B-cell lymphoma 2
HRP: horseradish peroxidase
RPMI: Roswell Park Memorial Institute
PC: Prostate cancer

IgG: immunoglobulin
PBS: phosphate buffered saline
Rb: Retinoblastoma
COX2: cyclooxygenase-2
ERK: extracellular signal-regulated protein kinase
APAF1: apoptotic-protease-activating factor 1
GST: glutathione S-transferase
NQO-1: quinone oxidoreductase
UGT: UDP-glucuronosyltransferase
Akt: Ak mouse strain thymoma
RTK: transmembrane tyrosine kinase
FC- γ -RIIa: Low affinity immunoglobulin gamma Fc region receptor II-a
ALA: alpha-linolenic acid
EPA: eicosapentaenoic acid
DHA: docosahexaenoic acid

Chapter I

Literature Review

1.1. Cancer

1.1.1. Introduction

The World Cancer Research Fund and the American Institute of Cancer Research (WCRF/AICR) projects that in 2030, 22 million people will be diagnosed with cancer (excluding skin cancers) and about 14 million people may be recorded as dying from cancer. This exponential increment in cancer rates is overlapping the increase in global population and is including all income classes: high, middle and low-income countries.

The scientific community considers that the sole genetic vulnerability to cancer is limited to a few cases whereas the majority of most adult cancers account for the epigenetic intervention mainly by environmental factors (Vargas & Thompson, 2012). For instance, one major cause of cancer is smoking or exposure to tobacco, solar radiation, and dietary factors that affect directly or indirectly proteins such as caspases, protein cycle inhibitors, cyclin dependent kinases etc, metabolites and gene expression. Therefore, one maybe theoretically convinced that most cancers are preventable by avoiding these factors and by modifying one's nutritional state since they are collectively and individually significant in cancer predisposition.

1.1.2. Colorectal Cancer (CRC)

1.1.2.1. Introduction

Colorectal cancer is the cancer of the colon, the last section of the intestinal tract that prolongs from the caecum to the rectum. CRC can originate in any section of the colon: the caecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon and the rectum. However, studies have shown that most common CRCs arise in the colon regions and few in the rectum (Giovannucci, 2002).

Estimates show that around 95% of CRCs are adenocarcinomas, while other forms of cancer also are mucinous carcinomas and adenosquamous carcinomas (Kufe et al. 2003).

Studies list some facts show that around 5–10 % of CRC is genetically inherited. The other CRC incidences engage an accumulation of endogenous and exogenous factors that damage and alter the genetic material. The exogenous (environmental) factors include nutrition and physical activity. The risk factors of developing colorectal cancer are smoking, excessive alcohol consumption and a diet rich in animal fat and low on vegetables and fruits intake (Ambrosone & Tang, 2009).

The increase in colorectal incidence was also related to age, gender and individuals with a medical history of colorectal cancer, endometrial, ovarian or breast cancer, colorectal polyps and chronic ulcerative colitis or Crohn's disease (Aiello et al. 2011).

1.1.2.2. Prevalence of Colorectal cancer

The World Cancer Research Fund International revealed that CRC is the third most widespread type of cancer in men and women. However, it is important to highlight the variability in the worldwide occurrence and mortality rates. Industrialized and high income countries such as Western Europe, North America, New Zealand and Australia have elevated rates for colorectal neoplasms, and most developing nations are on the verge of an increase in colorectal cancer incidence (Center, Jemal, Smith, & Ward, 2009).

Johns Hopkins colon cancer center revealed that colorectal cancer marks 21% of all cancers in the United States, and ranks second in mortality in males and females to lung cancer. In the United States, CRC is the 3rd most common type of cancer in both men and women. 75,590 cases of CRC in men and 71,380 cases in women were diagnosed in 2009 in the United States (Jemal et al. 2009). The Singapore Cancer Registry revealed that the epidemiological data for the years 2004 to 2008 show that colorectal cancer was the most prevalent cancer in men (17.7%) and the second most common in females 14.6% after breast cancer (29%). Further and for both genders, Chinese followed by Malays and Indians prevail the highest occurrence of CRC for the period of 2004 to 2008, in addition to the substantial increase of CRC incidence

with age above 50 years. The CRC rates in these nations are increasing due to the modification of their dietary habits towards the “western type” (Seow et al. 2002). Nevertheless, the risk of developing colorectal cancer can be reduced by following a screening routine for the prevention and early diagnosis of CRCs. These checkups decreased the mortality rates from colon cancer over the last 20 years as stated by Johns Hopkins colon cancer center.

1.1.2.3. Polymorphisms associated to CRC

Colorectal cancer is determined when normal mucosa show an unusual cell replication and proliferation, in addition to the appearance of large polyps with proliferative, biomolecular and biochemical irregularities (Srivastava, Lippman, Hong & Mulshine, 1994; Ponz de Leon, 2002; Bostick et al. 1997). Transforming growth factor- α (TGF- α) belonging to the epidermal growth factor family (EGFR), is associated with proliferating cells within colon crypts and has shown its expression to be 51% higher in CRC cases compared with controls ($P = 0.05$). Therefore, detection of TGF- α expression level is an early modifiable biomarker of risk for colorectal cancer and crucial for the prevention of colorectal neoplasms (Daniel et al. 2009).

Further, specific mutations and some polymorphic genes in colorectal cells have been recognized to be directly linked to colorectal carcinogenesis. These genes and mutations were predictive biomarkers in finding targeted treatments against CRC. Few examples comprise mutation of K-Ras, BRAF mutation, PI3K-PIK3CA mutations and expression of PTEN and p-AKT (Krasinskas A. 2011), XPD (Lys751Gln), XRCC1 Arg399Gln, MGMT 2535G>T, VEGF C936T (Aiello et al. 2011)

1.1.2.4. The EGFR pathway and CRC

The epidermal growth factor receptor (EGFR) EGFR is a 170-kDa receptor belonging to the transmembrane tyrosine kinase (RTK) family. Ligand activation, mainly TGF α and EGF, of EGFR induces the activation of downstream signaling cascades (Krasinskas, 2011). These pathways comprise the RAS-RAF-MAP kinase pathway (Alroy & Yarden, 1997), the phosphatidyl inositol 3-kinase (PI3K), and the

Akt pathway (Chan, Rittenhouse & Tsichlis, 1999). Stimulation of these pathways was shown to intervene in cell cycle arrest and activation, proliferation and angiogenesis (Petit et al. 1997), in the promotion of tumor cell motility, adhesion, and metastasis (Engebraaten, Bjerkvig, Pedersen & Laerum, 1993). These signaling cascades were of high interest to researchers in finding targeted therapies by inhibiting their corresponding pathways (Aboud-Pirak, Hurwitz, Pirak, Bellot, Schlessinger, & Sela, 1988). For instance, the anti-EGFR monoclonal antibodies the panitumumab (Vectibix, Amgen) and cetuximab (Erbix, Bristol Myers Squibb and Merck KGaA) are the fruit of such research and are currently permitted as a treatment of metastatic colorectal cancer (Gibson, Ranganathan & Grothey, 2006; Jonker et al. 2007).

1.1.2.5. The role of VEGF in CRC

Vascular endothelial growth factor (VEGF) is a protein that stimulates angiogenesis in colorectal cancer cells when overexpressed. The intracellular portion of VEGF receptor hosts a tyrosine kinase domain, upon VEGF binding to its receptor, the downstream effectors lead to endothelial cell proliferation, migration, survival and vascular permeability. All these factors will hence promote angiogenesis (Wang et al. 2004). 50% of CRC cases were reported to show an upregulation of VEGFR, this upregulation is a predictive marker for worse prognosis and rapid tumor progression (El Zouhairi, Charabaty & Pishvaian, 2011). Since VEGF promotes angiogenesis which is mandatory for tumor growth and metastasis, researchers considered the VEGF receptor/ ligand system as a targeted therapy to reducing tumor growth and extending survival (Ellis, Takahashi, Liu & Shaheen, 2000).

1.1.2.6. Treatment of colorectal cancer

For over 50 years, fluoropyrimidine- based (5-fluorouracil) drug was a main treatment of colorectal cancer. Yet lately, the treatment of mCRC has been modified to combine chemotherapeutic drugs such as irinotecan and oxaliplatin. These combinations increased the overall survival in mCRC patients (Iqbal & Lenz, 2004). Chemical agents were found to inhibit the EGFR or bind to VEGF in clinical activity when combined with chemotherapy in phase II and phase III clinical trials. One of the prominent agents is cetuximab. Cetuximab is an EGFR antagonists that obstructs

the binding of TGF- α and EGF to EGFR (Laszlo, 2010). The other promising agent bevacizumab is an anti-VEGF monoclonal antibody. Bevacizumab and in combination with chemotherapy has been tested in patients with stage III and IV colon cancer. This combination was considered due to bevacizumab antiangiogenic result and to its alteration of tumor vasculature and reduction of the elevated interstitial pressure needed in chemotherapy (Aiello et al. 2011) . The post treatment response for these therapeutic agents varies according to the underlying polymorphism. For instance, patients with BRAF and/or K-Ras mutations show resistance to anti-EGFR therapy (Loupakis et al. 2009; de Roock et al. 2010). The percentage of patients who show positive effects is mostly, low (Saltz et al. 2004, Aiello et al. 2011; Laszlo, 2010) (Table4). Consequently, ongoing researches about the use of multiple targeted therapies (i.e. bevacizumab combined with cetuximab) with chemical agents other than irinotecan is being assessed. These different combinations will be evaluated while underlying the side effects such as gastrointestinal perforation, hypertension and myocardial infarction (El Zouhairi et al. 2011; Laszlo, 2010).

1.1.2.7. CRC and Diet

In November 2007, the WCRF/AICR Second Expert Report “Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global Perspective” presented in vitro and in vivo experimental evidences that phytochemicals from vegetables, fruits, grains, nuts, herbs and spices, are associated with cancer predisposition, recurrence and therapy. Phytochemicals intervened in several pathways of cancer such as cell cycle control, apoptosis, angiogenesis, differentiation, DNA repair, carcinogen metabolism and inflammation. Examples of phytochemicals that have been shown to influence colorectal cancer pathways in experimental models include isothiocyanates and indole-3-carbinol from cruciferous vegetables (Hecht, 1999). However, studies and trials about the correlation of CRC and these vegetables are still to be delineated; hence the purpose of our study.

1.2. Brassica Oleracea

1.2.1. Brassica oleracea background and history

Brassicaceae Cruciferae, one of the economically top 10 plant families worldwide, comprises a wide group of consumed vegetables.

One specie of brassica crucifera, *Brassica oleracea* comprises different varieties *Brassica oleracea* Acephala Group (kale and collard greens), *Brassica oleracea* Alboglabra Group (Chinese broccoli), *Brassica oleracea* Botrytis Group (cauliflower, Romanesco broccoli and broccoflower), *Brassica oleracea* Capitata Group (cabbage), *Brassica oleracea* Gemmifera Group (brussels sprouts), *Brassica oleracea* Gongylodes Group (kohlrabi) and *Brassica oleracea* Italica Group (broccoli). All these varieties of *Brassica oleracea* are thought by most scholars to have developed from the wild cabbage, “the head of the family tree” (Fahey, 2001). The parent wild cabbage originated in northern Europe and the Mediterranean region, and led to the descent of some 400 varieties like cabbages, kohlrabi, Brussels sprouts, oilseed rape, broccoli and cauliflower etc. Neither Hebrews, nor Egyptians consumed cabbages, while Greeks and ancient Romans were accustomed to cabbages and cauliflower, where they considered cabbage the “first of all vegetables” and its consumption during a banquet will avoid one from alcohol intoxication. Ancient figures Cato the Elder an ancient Roman ancestor of Nero, Diogenes of Sinope founder of the Greek Cynic philosophy, and Pythagoras the Greek mathematician included cabbage in their writings (Katz & Weaver, 2003).

1.2.2. Cabbage Brassica oleracea Capitata

Cabbage is a vegetable that belongs to the *Brassica* genus and the Brassicaceae family. Cabbage are biennial plants that are usually cultivated in well drained soil of pH between 6.0 and 6.8, and in locations fully exposed to sun with varying temperatures between 4 and 24 °C (Bradley, Ellis & Martin, 2009). Cabbage heads generally range in between 0.45 to 3.6 kg (Delahaut & Newenhouse, 1997). The color of these heads varies from white, to green and purple, while their shape may differ from round to oblate and pointy; as for the leaves they are thick with curvy to dissected margins. Cabbage with all its forms is an edible vegetable that is used in many cuisines as raw, steamed, stewed, sautéed and pickled (Ingram, 2000).

1.2.2.1. Health benefits of cabbage

Scientists investigated the composition of cabbage and reported that cabbage comprises a rich supply of vitamin K and vitamin C, a fair source vitamin B6, vitamin B1 (thiamin), potassium, calcium, and molybdenum. Further, cabbage constitutes a decent source of fiber, folate and manganese. In addition, 70g of cabbage comprises 38.5mg of alpha-linolenic acid, or ALA (a kind of omega-3 fatty acid which can be converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which is the omega-3 in fish oil (Egert et al. 2007). Many of the cabbage constituents have health benefits. For instance, due to its high content in vitamin C sailors used cabbage as a remedy for scurvy and as dressings against gangrene in the 1700s (Fahey, 2003). Further, the high concentration in fiber results in decreasing the level of cholesterol and hence the incidence of cardiovascular events (Kahlon, Chiu & Chapman, 2008).

Cabbage health benefit against Cancer:

The exceptional health benefit provided by cabbage is its high involvement in cancer prevention. This feature is assured by the presence of anti-inflammatory compounds such as anthocyanins, antioxidant molecules and glucosinolates in cabbage. Researchers ranked polyphenols as a primary phytonutrient antioxidant in cabbage. The antioxidant intake will reduce oxidative stress. Chronic oxidative stress is considered among the main participants in the prevalence of cancer and cardiovascular disease (Ames, Gold & Willett, 1995). These antioxidants are involved in preventing the initiative stages of cancer and helping in reducing proliferation at the progressive stages (Manson, 2003; Surh, 2003).

On the other hand, glucosinolates, besides being responsible for the pungent flavor and odor of brassica vegetables, are considered to be the “trump card” against cancer. The chemopreventive efficiency of glucosinolates is basically due to its hydrolysis into isothiocyanates and indoles (Fahey, 2001; Arikawa & Gallaher, 2008). The latter molecules have shown remarkable evidence in preventing different forms of cancer such as stomach cancer (Hansson et al. 1993) breast cancer (Fowke et al. 2003; Ambrosone et al. 2004), prostate cancer (Cohen, Kristal & Stanford, 2000; Giovannucci, Rimm, Liu, Stampfer & Willett, 2003; Kirsh et al. 2007), lung cancer

(London et al. 2000; Spitz et al. 2000; Zhao et al. 2001; Wang et al. 2004; Lam et al. 2009), bladder cancer (Zhao et al. 2007) and colorectal cancer (Ambrosone & Tang, 2009.) However, it is to note that the plant's age (Fahey et al. 2001), soil fertility (Booth & Walker, 1992; Fahey & Stephenson, 1999), and plant's growth limiting factors determine the quantitative and qualitative levels of glucosinolates and its distribution within the plant's organs (Bodnaryk, 1994).

A-Glucosinolates and Cancer:

The predominant glucosinolates found in cabbage are glucoiberin, progoitrin, sinigrin of the aliphatic group of glucosinolates and majorly glucobrassicin of the indoles (Cartea & Velasco, 2008) as well as other glucosinolates and indoles as indicated in Table 1. Upon consumption or tissue rupturing, the glucosinolates in the vacuole will be in contact with myrosinase enzyme localized in the cytoplasm and will be hydrolyzed into indole-3-carbinol I3C and isothiocyanates such as SFN (sulforaphane), allyl-isothiocyanate (AITC) and BITC (benzyl-isothiocyanate), PEITC (phenethyl isothiocyanate), PITC (phenyl isothiocyanate) and many others (Fahey et al. 2001).

Table 1: Major glucosinolates found in Brassicae Oleracea (Cartea & Velasco, 2007).

GIB: glucoiberin (3-methylsulfinylpropyl); PRO: progoitrin (2-hydroxy-3-butenyl); SIN: sinigrin (2-propenyl); GAL: glucoalysiin (5-methylsulphinylpentyl); GRA: glucoraphanin (4-methylsulphinylbutyl); GNA: gluconapin (3-butenyl); GBN: glucobrassicinapin (4-pentenyl); GIV: Glucoiberin (3-methylthiopropyl); GER: glucoerucin (4-methylthiobutyl); GNL: gluconapoleiferin (2-hydroxy-4-pentenyl); GBS: glucobrassicin (3-indolylmethyl); NGBS: neoglucobrassicin (1-methoxy-3-indolylmethyl); 4HGBS: 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl); 4MGBS: 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl), GST: Gluconasturtiin (2-phenylethyl).

Principal glucosinolates identified in leaves of <i>Brassica</i> vegetable crops														
Crop	Aliphatic glucosinolates									Indole glucosinolates				Aromatic
	GIB	PRO	SIN	GAL	GRA	GNA	GBN	GIV	GER	GNL	GBS	NGBS	4HGBS	4MGBS
<i>Brassica oleracea</i>														
White cabbage	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Savoy cabbage	+	+	+	-	+	+	+	+	-	-	+	+	-	+
Red cabbage	+	+	+	-	+	+	-	+	-	-	+	+	-	-
Kohlrabi	+	+	+	+	+	+	-	+	-	-	+	+	+	-

A1- ITCs induce apoptosis:

Apoptosis follows 2 pathways: extrinsic and intrinsic. The extrinsic pathway revolves around the activation of surface death receptors the tumor necrosis factor (TNF) and Fas and their interaction with their appropriate ligands. Both factors result in the activation of procaspase 8 and the activation of its downstream effectors leading to apoptosis (Alberts et al. 2002). However, the intrinsic pathway consists of releasing cytochrome c from the mitochondria and allowing it to bind to the adaptor protein named apoptotic-protease-activating factor 1 (APAF1). Once activated, APAF1 cleaves procaspase-9 molecules and triggers the caspase cascade leading to apoptosis. Upstream regulator of the intrinsic pathway involves the Bcl-2 family, the protein modulators of programmed cell death. This family encloses the proapoptotic members Bax, Bak, Bik, Bad, and Bid and antiapoptotic members Bcl-2 and Bcl-xL. Their property will either suppress or promote the discharge of cytochrome *c* (Gross, McDonnell & Korsmeyer, 1999). ITCs were shown to induce apoptosis via the release of cytochrome *c* from the mitochondria, Bcl-2 family regulation, MAPK signaling and continuous activation of caspases (Traka & Mithen, 2009).

SFN studies showed the induction of the proapoptotic protein BAX in HT-29 colon cells which was due to the release of cytochrome *c* from the mitochondria and to the occurrence of a proteolytic fragment of the caspase-3 substrate poly(ADP-ribose)polymerase (PARP) (Gamet-Payraastre et al. 2000). However, it is important to note that the expression of tumor suppressor gene p53 remained unchanged and the antiapoptotic Bcl-2 protein was not perceived in any conditions. These results suggested that SFN stimulates apoptosis in a p53 independent and Bax dependent pathway (Gamet-Payraastre et al. 2000). Studies on SFN in PC-3 cells reported the activation AP-1 which necessitates the activation of the MAPKs c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) thus regulating apoptosis (Xu et al. 2006). Further, studies on 40-16 colon cancer cells showed that 15 μ M of SFN led to programmed cell death by increasing the Bak to Bcl-xL ratio 2 folds and Bax to Bcl-xL ratio 1.5 fold; these data suggested the involvement of Bcl-2 family in inducing apoptosis by SFN against 40-16 colon cancer cells (Pappa, Lichtenberg, Iori, Barillari, Bartsch & Gerh"ausser, 2006).

PEITC played role in inducing apoptosis in HT-29 cells by activating caspase 3 and 9 followed by nucleus condensation and DNA disintegration, and three MAPKs, c-Jun N-terminal kinase(JNK), extracellular signal-regulated protein kinase (ERK) and p38 kinase post treatment, and releasing cytochrome *c* from the mitochondria in a

time and dose dependant manner. This study showed the importance of JNK activation in treating HT-29 since it marked JNK as a significant mediator of PEITC-mediated apoptosis. JNK inhibitor SP60012 inhibited PEITC-induced apoptosis suggesting JNK is upstream signaling mediator of caspases in PEITC induced apoptosis (Keum, Jeonga & Konga, 2004).

PEITC also showed a minor increase in the Bax:Bcl-xL and the Bak:Bcl-xL ratios in 40-16 colon cancer cells but an efficient and rapid PARP cleavage only after 10h of 10 μ M PEITC treatment. These results implied that apoptosis induction by PEITC in 40-16 colon cancer cells slightly relies on Bcl-2 protein (Pappa et al. 2006). In A549 non-small cell lung human carcinoma, PEITC and AITC inhibited cell growth by activating caspase-3 and caspase-8 to induce apoptosis (Wu, Zhou & Xu, 2009).

BITC induced apoptosis in Hela Cells 60 and rat hepatocytes via JNK-dependent Bcl-2 phosphorylation pathway. This induction was due to the phosphorylation of Bcl-2 decreasing its interaction to bind to Bax, Therefore, Bax expression increases to enhance the propensity of cells to program cell death. However, when HT-29 were treated with BITC the expression of Bax was not increased (Fimognari et al. 2002) suggesting apoptosis induction to be cell specific (Nakamura & Miyoshi, 2006).

A2- ITCs induce cell cycle arrest:

Hyperproliferation, the hallmark of all cancers, is caused by the loss of the cell cycle regulators cyclin dependent kinases (CDKs), cyclins, and CDK inhibitors (CDI). Cyclin proteins govern G1, S, G2 and M phases in a cell cycle. They bind and activate cyclin-dependent kinases (CDKs) which maybe modulated by CDK inhibitors (CDIs) such as p21/WAF-1, p16/INK41 and p27/Kip-1. The cyclin/CDK complexes endorse cell cycle sequence whereas the CDK inhibitors induce cell cycle arrest (Keum et al. 2004).

In 1993, ITCs were first studied in inducing cell cycle arrest in Hela cells. Results showed that 10 μ M AITC, 2.5 μ M BITC, or PEITC arrested the cells at G2/M phase (Hasegawa, Nishino & Iwashima, 1993). Ten years later, a research by Zhang, Tang, and Gonzalez (2003) showed that PEITC, AITC and BITC only needed 3 h to inhibit cell growth apart from the origin of cancer cells and in cells showing an overexpression of multidrug resistance-P-glycoprotein-1 or associated protein-1 (Zhang et al. 2003); however SFN was remarkably time dependant. Whereas for

Human promyelocytic leukemic cells (HL-60 cells), AITC arrested them at the G1 phase, while BITC caused the cell cycle arrest to be the G1 and the G2/M phases (Zhang, Yao & Li 2006). AITC arrested prostate cancer PC-3 cell at the G2/M phase of their cell cycle as it decreased the levels of cyclin B1, cdk1, cell division cycle 25B (cdc25B), and cdc25C (Xiao, Johnson, Trump & Singh, 2004). 12mM of AITC arrested HT-29 cells in G2/M phase after 24 h of treatment (Smith, Lund, Clarke, Bennet & Johnson, 2004).

As of SFN in inducing cell cycle arrest in HT-29 cells, SFN adopts a two phase pattern in CRCs. For up to 6h of SFN exposure, HT-29 stopped growing and were arrested in reversible G2/M phase, while a constant 12h exposure led to an irreversible arrest in G2/M phase and consequently to apoptosis (Pappa et al. 2007). The stop led to a low percentage of cells in the S phase and to a high expression of cyclinA and B1 when HT-29 are treated with 15mM of SFN (Gamet-Payrastre et al. 2000).

PEITC on the other hand induces a G1 cell cycle arrest in HT-29 cells by downregulating cyclins A, D, and E (Cheung & Kong, 2010). Cyclin D1 is known to phosphorylate Rb and launch E2F transcription factors which permit the transition of the cell cycle phases and mainly the start of DNA synthesis (Keum et al. 2004). Thus its downregulation will promote cell cycle arrest. As of PC-3 cells, PEITC resulted in G2/M phase cell cycle arrest by reducing more than 80% Cdk1 and Cdc25C levels. This decrease was suggested to be through the increase of proteasome mediated breakdown since lactacystin, a proteasome inhibitor, blocked the cell cycle in pretreated PC-3 cells (Xiao et al. 2004).

In Caco2 cells, 5.1 $\mu\text{mol/L}$ of BITC and 2.4 $\mu\text{mol/L}$ of PEITC stopped DNA synthesis and arrested the cells in the G2/M phase which was maintained by the upregulation of p21. This arrest was considered due to the activation of Chk2 and its phosphorylation (Visanji, Duthie, Pirie, Thompson, & Padfield, 2004). However, with a dose of 20 μM SFN, Caco-2 cells were arrested at G2/M phase, but accumulated at Sub-G1 with concentrations $>20 \mu\text{M}$ (Jakubikova, Sedlak, Mithen & Bao, 2005).

Studies have shown that other mechanisms in inducing cell cycle arrest are through inhibiting histone deacetylase (HDACs). High expression of HDACs was noticed to be common in several cancer malignancies, where it represses transcription and silences tumor suppressor gene p21 by deacetylation resulting in cell cycle arrest

(Clarke, Dashwood & Hoa, 2008). In HT-29 cells, HDAC2 was knocked down upon treatment with SFN and cell cycle arrest was induced (Zhu et al. 2004).

Treatment of human prostate cancer cells with PEITC and SFN showed inhibition of HDACs which was correlated to the upregulation of p21. As a result the cell cycle was arrested and CDIs expression, p21 and p27 were upregulated in a p53 independent pathway (Wang et al. 2008). Similarly, SFN increased acetylated histones binding to p21 promoter and inhibited HDAC suggesting a mode of action in arresting cell cycle (Myzack, Karplus, Chung & Dashwood, 2004).

A3- Indoles and DIM induce apoptosis and cell cycle arrest:

It is noteworthy that indoles and their derivatives play role in inducing apoptosis and cell cycle arrest. The glucosinolate derivative I3C self dimerizes and oligomerizes to result in a stable compound 3, 3'-diindolylmethane (DIM). DIM induced cell cycle arrest at the G1 phase, and promoted apoptosis in leukemic HSB2 and CEM cells (Shorey et al. 2012). Moreover, DIM reduced tumorigenesis in C57BL/6 mice injected with a mouse prostate cell line (TRAMP-C2) (Choi, Lim & Park, 2004); it also reduced induced mammary tumors (DMBA) in rats (Wattenberg & Loub, 1978), benzo [a]pyrene-induced stomach cancers in mice, and the growth of transplantable human breast carcinoma and angiogenesis in mice. It was also shown that DIM leads to the inhibition of the proliferation and cell survival of prostate cancer cells by inactivating Akt and NFκB (Sarkar & Li, 2004), while breast cancer cells proliferation will be inhibited by cell cycle arrest at the G1 phase and activation of CDIs p21 (WAF1/CIP1) (Choi et al. 2004).

I3C and DIM induced apoptosis in HT-29 cells by stimulating PARP degradation in the wild cell line as in the mutated form where p53 was knocked out. These results suggested apoptosis induction via a p53 independent mechanism (Pappa et al. 2006).

DIM (30 μmol/L) inhibited HT-29 growth in a time dependent manner. It induced apoptosis and cell cycle arrest at the G1 and G2/M phases by inhibiting the activity of Cdc2, Cdk2, Cdc25c and cyclin B1 (Choi et al. 2009). At 2h of treatment, Cdk2 activity was reduced, while at 6h, the levels of p21CIP1/WAF1 and p27KIP1 the CDK inhibitors proteins and their corresponding mRNA remarkably increased, and at 12h Rb was hypophosphorylated and the levels of the E2F-1 protein were reduced. All these changes suggest the arrest at the G1 phase. However, the arrest at the G2/M

phase in HT-29 cells was regulated by the decrease of Cyclin B1 and Cdc25c, and Cdc2 kinase activity (Figure1) (Choi et al. 2009).

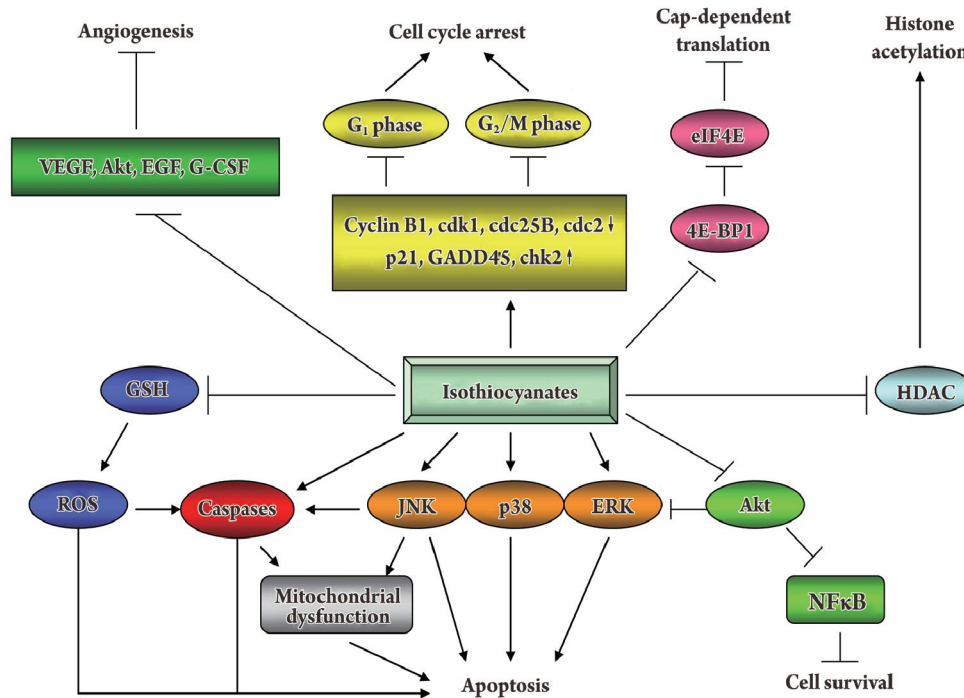


Figure 1: Schematic pathways of the antitumor activity of ITCs (Wu et al. 2009).

1.2.2.2. Anti-nutritional effects of cabbage and constituents

In contrast to the previously remarkable health benefits, glucosinolates and several ITCs are considered to be toxic in given concentrations. In high concentrations 10–50mg/kg body weight in rats, oxazolidine-2-thione, deriving from progoitrin glucosinolate, and nitriles may be mutagenic and cytotoxic (Cartea & Velasco, 2008). Progoitrin was a cause of goiter, delayed growth, liver damage and poor egg production in animals, however, Mithen, Verkerk, Rabot, and Johnson (2000) showed the absence of evidence of any goitrogenic consequence on humans after excessive consumption of Brassica.

1.2.3. Kohlrabi *Brassica oleracea* var. *gongylodes*

Kohlrabi member of the brassica family is recognized as turnip cabbage. Five centuries ago, Kohlrabi was developed in northern Europe as a perennial vegetable.

In color, kohlrabi may be white, green or purple on the outside but the inner edible portion is always pale white. The turnip-like structure of kohlrabi is the engorged bottom of the stem holding leaves and this tuber may reach 13 to 15cm in diameter (Macleod G. & Macleod J. 1990).

As the other member of the brassica family, kohlrabi is rich in polyphenols and phytonutrients mainly Vitamin C where it supplies 62g per 100g weight which 102% of recommended daily allowance, carotenes, vitamin A, vitamin K, minerals, and B-complex group of vitamins. Therefore, kohlrabi is considered to be a rich source of antioxidants. In addition, kohlrabi is a source of phytochemicals mainly glucosinolates and indoles (Mas et al. 2007). The most important ITCs found in kohlrabi flesh are methylthiobutyl ITC or erucin, and SFN, where the latter compound was previously described to be a promising anticarcinogen (Holst & Williamson, 2004).

Erucin (ER), a major ITC in kohlrabi flesh and seed (You, Wu, Mao, Zou & Liu, 2008), is the hydrolysis product of glucoerucin isothiocyanate and is also obtained when SFN is reduced in vivo. Erucin is hence considered an analogue of SFN and is of interest as being an anticarcinogenic agent similar to SFN (Fimognari et al. 2004). Researchers showed that erucin arrested the cell cycle of Jurkat T-leukemia cells (You et al. 2008). Erucin also induced phase II enzymes, upregulated phase III detoxifying system and modulated the cell proliferation in Caco-2 cells. In fact Caco-2 cells treated with 20 μ M of erucin, were arrested in the G2/M phase (Jakubikova et al. 2005), and decreased their G1 phase compared to the high percentage of cells found in G0/G1 and S phase of untreated cells. When the concentration of erucin reached 50 μ M, the percentage of Caco-2 cells in the sub-G1 stage increased significantly showing a raise in apoptosis (Jakubikova et al. 2005). Additionally, ER-induced phase II enzymes were inhibited in Caco-2 cells when PI3K/Akt and Raf/MEK/ERK were blocked by their inhibitors. This factor reflects how apoptosis induction by erucin in Caco-2 cells maybe PI3K/Akt and Raf/MEK/ERK dependant (Melchini & Traka, 2010). When comparing erucin to its analogue SFN, erucin increased the mRNA of multidrug resistant protein MRP2 in Caco-2 cells with a better potency than SFN; however, SFN induced the increase of both MRP1 and MRP2 in Caco-2 cells and A549 lung cells (Jakubikova et al. 2005). Fimognari et al. (2004) showed in experiments that erucin inhibits the proliferation of human leukemia cells but not in non-transformed human peripheral T lymphocytes, whereas

SFN exhibited antiproliferation in both transformed and non-transformed human peripheral T lymphocytes (Fimognari et al. 2004). These findings may indicate that SFN and its analogue erucin might be cell specific with respect to their mode of action. Studies have shown that kohlrabi seeds have lower erucin concentration compared to cabbage (You et al. 2008), while the leaves of kohlrabi show an absence of erucin compared to its presence in cabbage leaves (Cartea & Velasco, 2008). As of the flesh, kohlrabi was investigated to carry erucin predominantly (Gawęda & Nizioł-Lukaszewska, 2011). SFN on the other hand is largely found in kohlrabi seeds and few cabbage seeds cultivars show SFN similar presence while in other cultivars it is absent (You et al. 2008), while for the leaves and flesh of cabbage and kohlrabi, SFN is found in abundance in kohlrabi 8.73mg/100g compared to cabbage 0.26mg/100g (Ciska, Martyniak-Przybyszewska & Kozłowska, 2000; Farag & Abdel Motaal, 2009). Further it was found that kohlrabi is a less carrier of indoles particularly glucobrassicin, the precursor of indole 3 carbinol, and sinigrin precursor of AITC, whereas cabbages carry the indole glucobrassicin in a high percentage followed by isothiocyanates glucoiberin and sinigrin (Sarıkamı, Balkaya & Yanmaz, 2010) (Table 2).

These differences and similarities in ITCs and indoles contents in between 2 members of the same Brassica oleracea family raise the question to whether kohlrabi reveals similar health benefits and anti-nutritional effect as cabbage and more importantly similar anticarcinogenic properties. Further, despite the presence of major ITCs in kohlrabi such as PEITC, SFN and iberberin, most studies describing the potency of these compounds excluded kohlrabi as a rich source of these ITCs and focused on other members such as broccoli, brussels sprouts, cabbage and cauliflower (Smith et al. 2004; Fahey et al. 1997; Zhang et al. 2006; Wu et al. 2010, Cheung & Kong, 2010, Clark et al. 2008; Gamet-Payraastre et al. 2000). This observation led us to question whether these predominant ITCs in kohlrabi and of known anticarcinogenic properties are insignificant or have a minimal potency in inducing anticarcinogenic properties as compared to its family member cabbage. Further, no studies showed the exact glucosinolates concentrations differences and similarities in between the edible parts of cabbage leaves and kohlrabi flesh, or their comparative effect on specific cell lines (You et al. 2008; Melchini & Traka, 2010; Gawęda & Nizioł-Lukaszewska Z. 2011). Therefore, we aimed in this study to investigate kohlrabi since it is a minor crop in Lebanon and not well known, and more

importantly it belongs to a major Brassica family and possesses phytochemicals known for their inhibitory attributes against cancer. Consequently, our search was designed to check for the potency of kohlrabi flesh on colorectal cancer cell lines and compare its effect to the leaves of its family member cabbage.

Table 2:GLS Content (Milligrams per 100 g) of Cabbage and kohlrabi grown in 1994 (Ciska, Martyniak-Przybyszewska & Kozłowska, 2000).

	glucoiberin	progoitrin	sinigrin	gluco-raphanin	gluco-raphenin
white cabbage	12.38 ± 6.14	3.73 ± 1.60	22.73 ± 11.32	0.26 ± 0.13	–
kohlrabi	2.95 ± 1.31	2.05 ± 1.04	0.43 ± 0.57	8.73 ± 3.64	1.33 ± 0.34

4-methylthio-but-3-enyl GLS	aralkyl		4-hydroxy-glucobrassicin	glucobrassicin	4-methoxy-glucobrassicin	neoglucobrassicin
	gluconastrutiin					
–	0.42 ± 0.18	1.07 ± 0.37	35.84 ± 9.82	9.74 ± 5.98	0.53 ± 0.25	
–	0.12 ± 0.16	1.11 ± 0.76	4.81 ± 1.91	0.18 ± 0.15	1.09 ± 0.68	

aliphatic						
glucoraphenin	glucoapoleiferin	glucoalyssin	glucunapin	glucoibervirin	glucobrassicinapin	glucercucin
–	–	–	0.56 ± 0.37	1.24 ± 0.74	–	na
1.33 ± 0.34	–	–	tr	1.18 ± 0.81	–	4.48 ± 2.39

1.3. Purpose of the study

Cabbage and kohlrabi, members of the cruciferous family, are rich in phytochemicals that influence colorectal cancer. The purpose of this study is to establish a comparative study that investigates the effect of the ethanolic extract of the edible parts of cabbage leaves and kohlrabi flesh on human colorectal adenocarcinoma cells in vitro.

The main objectives reflect three tasks:

- Identifying and comparing the effect of the ethanolic extracts of cabbage and kohlrabi on the cytotoxicity of Caco-2 and HT-29 cells.
- Determining and comparing the effect of the ethanolic extracts of cabbage and kohlrabi on proliferation, cell cycle progression and apoptosis in Caco-2 and HT-29 cells.
- Elucidating and comparing the expression of proapoptotic and anti-apoptotic proteins in HT-29 cells when treated with the ethanolic extracts of cabbage and kohlrabi.

Chapter II

Materials and Methods

2.1 Cell lines and cell culture

Caco-2 and HT-29, two human colorectal adenocarcinoma cell lines, were cultured in RPMI 1640 medium with 25 mM Hepes and Lglutamine, and supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml of Penicillin Streptomycin and grown in a humidified incubator at 37°C and 5% CO₂ (Smith et al. 2004).

2.2 Preparation of kohlrabi and cabbage ethanolic extract

Kohlrabi and Cabbage plants were cultivated in the mountains of Zahleh. The cabbage leaves and the flesh of the kohlrabi were each blended and incubated in 90% ethanol for a week in a shaker.

The extract was strained and then placed in a rotoevaporator. The suspension filtrate was then filtrated through a 0.22µm millipore filter, transferred to a weighed eppendorf tube then concentrated in a speed vacuum (eppendorf centrivap concentrator). The mass of the pellet and the percentage yield were determined. The percentage yield of cabbage was 1.57% and that of kohlrabi was 1.43%. The pellet was then resuspended in RPMI 1640 media to a final concentration of 0.5mg/µl. The filtrates were then stored in the deep freeze until required.

On the day of treatment, the suspended extract was applied on the seeded cells in different volumes to establish the required final concentrations of 10µg/µl, 20µg/µl, 40µg/µl or 60µg/µl.

2.3 Antibodies and reagents

Rabbit polyclonal IgG anti-β-Actin, anti-Bax, and anti-Bcl2 antibodies were provided by Santa Cruz Biotechnology.

Anti-rabbit IgG HRP-conjugated secondary antibody was obtained from Santa Cruz biotechnology.

Propidium Iodide (PI)/ Annexin V was obtained from Sigma LifeScience.

2.4 Cytotoxicity: Trypan Exclusion Method

Trypan blue exclusion method determines the viability percentage of cells in suspension. Trypan blue dye will penetrate the cell membrane of damaged dead cells and will stain their cytoplasm in blue, while live cells will exclude the dye and will appear bright. The viability of cells is assessed by counting the blue dead cells and the bright live ones on a hemocytometer.

HT-29 and Caco-2 cells were seeded in 24 well plates at a density of 1×10^6 cells/ml. 24 h later and after forming a monolayer, control wells were left untreated, while the cells in other wells were treated independently with cabbage and kohlrabi filtrates at the following concentrations $10 \mu\text{g}/\mu\text{l}$, $20 \mu\text{g}/\mu\text{l}$ and $40 \mu\text{g}/\mu\text{l}$. The treatment of each concentration and extract was performed in triplicates. Cytotoxicity was assayed 24 and 48h after treatment. The supernatant of each well was collected in a labeled eppendorf tube, and the adhered cells were washed with PBS. The latter washes were then pipetted to their corresponding collected supernatants. Adhered cells were then detached by trypsin then resuspended in media. $10 \mu\text{l}$ of each collect was mixed with $10 \mu\text{l}$ of Trypan Blue. $10 \mu\text{l}$ of this mix was placed in a hemocytometer and the blue and bright cells were counted. The percentage of viable cells was calculated applying the following formula:

Live cell count/ Total cell count * 100.

2.5 Proliferation: Cell proliferation reagent (WST-1)

WST-1 (water soluble tetrazolium salt) is a tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases enzymes present in metabolically active cells. Colorimetric quantification of formazan at 450 nm will assess cell proliferation using BIO TEK EKX808 ELISA Reader.

HT-29 and Caco-2 cells were plated in 96 well plates at a concentration of 1×10^6 cells/ml. After 24h of seeding, triplicates of cells were treated independently with kohlrabi and cabbage at the following concentrations $10 \mu\text{g}/\mu\text{l}$, $20 \mu\text{g}/\mu\text{l}$ and $40 \mu\text{g}/\mu\text{l}$. Control wells were left untreated, and blank wells containing media alone, and other

wells with the volumes of treatment were marked. After 24 and 48h of treatment, 10µl of the reconstituted WST-1 reagent (Roche, Germany) was mixed to each well according to manufacturer's instructions. The plates were placed in a humidified incubator at 37°C and 5% CO₂ for 4h. The optical density was then recorded at 450nm using the ELISA reader machine and the percent of proliferation was calculated.

2.6 Cell cycle Analysis: Flow cytometry

Flow cytometry is a tool used to measure the properties of cells including the cells' size, granularity or inner complexity. This tool indicates the cell's phase cycle according to its DNA content; G0/G1 phase is 2n, S-phase cells is >2n but <4n, while G2/M cells is 4n. The percentage of cells in the different phases was measured to reflect the progression of cell cycle.

HT-29 cells were seeded in 6 well plates at a concentration of 1×10^6 cells/ml. 24 h later, control cells were left untreated while others were treated independently with kohlrabi and cabbage extracts at the following concentrations 10µg/µl, 20µg/µl and 40 µg/µl. 24 h later, the cells were detached using trypsin, resuspended in media with 10%FBS, then centrifuged at 1000rpm at 4°C for five min. The supernatant was discarded and the pelleted cells were washed and resuspended in 1 ml of ice-cold 1 × phosphate-buffered saline (PBS). The suspension was centrifuged at 1500rpm at 4°C for five min and the supernatant discarded. The cells were then fixed using ethanol to a final concentration of 70% with ice cold 1X PBS. Ethanol fixation was overnight at -20°C. 24h later, the cells were centrifuged under the previous conditions and the supernatant discarded. The pellet was resuspended in 500µl PI solution constituted of 6ml 1X PBS, 10µl of RNase A and 300µl of Propidium iodide.

The samples were incubated for 40 min at 37°C then analyzed using an Accuri C6 flow cytometer (Ann Arbor, MI USA). The data was collected in list mode on 10,000 events and analyzed using CFlow[®] Software (Maalouf, Baydoun & Rizk, 2011).

2.7 Apoptosis

2.7.1 Cell Death ELISA Plus Kit

In order to assess the apoptotic effect of kohlrabi and cabbage on colorectal cancer cells, the Cell Death ELISA kit (Roche, Mannheim, Germany) was utilized. This

assay is based on the quantification of histone-complexed DNA fragments into the cytoplasm which are a hallmark of apoptosis.

HT-29 and Caco-2 cells were seeded in sterile eppendorf tubes at a concentration of 1×10^5 cells/ml. After 24h, the cells were treated independently with cabbage and kohlrabi extracts at the following concentrations $10 \mu\text{g}/\mu\text{l}$, $20 \mu\text{g}/\mu\text{l}$ and $40 \mu\text{g}/\mu\text{l}$. Control tubes were left untreated. 24 and 48 h after treatment, the cells were centrifuged and the supernatant discarded. The pelleted cells were lysed with the supplied lysis buffer according to the manufacturer's instructions. The lysate was then centrifuged for 10 min at $200 \times g$ and $20 \mu\text{l}$ of each supernatant was transferred to a precoated streptavidin microtiter plate. Biotin-labeled anti-histone and peroxidase-conjugated anti-DNA antibodies were added to each well. The plates were incubated at room temperature for 2h, and then washed three times with the provided incubation buffer. ABTS (2,2'-azino-di(3-ethylbenzthiazolin-sulfonate)) substrate solution of peroxidase enzyme was added to each well and was incubated for 15min at room temperature. The absorbances were read at 405nm using the ELISA reader machine and the enrichment factor was calculated (Maalouf et al. 2011).

2.7.2 Flow Cytometry: Annexin V-FITC/PI double staining method

Annexin V-FITC/PI double staining method was followed. The cells were seeded in 6 well plates at a concentration of 1×10^6 cells/ml. 24h later, control cells were left untreated while others were treated independently with kohlrabi and cabbage extracts at the following concentrations $10 \mu\text{g}/\mu\text{l}$, $20 \mu\text{g}/\mu\text{l}$ and $40 \mu\text{g}/\mu\text{l}$. 24h later, the cells were detached using trypsin, resuspended in media with 10%FBS, then centrifuged at 1000 rpm at 4°C for 5min. The supernatant was discarded and the pelleted cells were washed and resuspended in Annexin binding buffer, $5 \mu\text{l}$ of Annexin is added followed by $5 \mu\text{l}$ of Propidium Iodide (PI). The cells were then incubated for 5 min at room temperature in the dark. The samples were then analyzed using an Accuri C6 flow cytometer (Ann Arbor, MI USA). The data was collected in list mode on 10,000 events and analyzed using CFlow[®] Software (Smith et al. 2004).

2.8 Western Blot Analysis

Western Blot is a technique used to identify and quantify the presence of a specific protein in a given sample.

HT-29 cells were cultured in 6 well plates at a concentration of 1×10^6 cells/ml. 24h later and after forming a monolayer, the cells were treated with cabbage and kohlrabi extracts at the following concentrations $10 \mu\text{g}/\mu\text{l}$, $20 \mu\text{g}/\mu\text{l}$ and $40 \mu\text{g}/\mu\text{l}$. After 24h, protein extraction was performed using Cell lytic MEM protein extraction kit (Sigma). The medium was aspirated, $500 \mu\text{l}$ of 1xPBS was added to the cells that were scraped and transferred to labeled eppendorf tubes. The cells were then centrifuged at 300rpm for 5min, the supernatant was discarded and the pellet was stored on ice. $6 \mu\text{l}$ of protease inhibitor and $600 \mu\text{l}$ of ice cold lysis and separation solution were added to each pellet. The vortexed suspension was then incubated on ice for 10min then centrifuged in a precooled centrifuge at 12000rpm, 4°C for 5min. The lysate was stored at -20°C until used. The proteins were quantified using Quick start Bradford all protein Assay according to the manufacturer's instruction.

Lamelli sample buffer was added to the protein samples in a 1:1 ratio and then boiled for 5min prior to loading. The samples were resolved on a 12 % SDS polyacrylamide gel and transferred to PVDF membranes at 100V for 1hour using wet transfer system. The membranes were then blocked with 5% non-fat dry milk in 1xPBS containing 0.1% Tween-20 overnight at 4°C . The second day, the membranes were incubated with primary Rabbit polyclonal IgG anti- β -Actin antibody at a concentration of 1:400 for 2h at 25°C on a shaker, then washed four times with 0.5% Tween-20 in 1xPBS for one hour on a shaker. Afterwards, the membranes were incubated with Anti-rabbit IgG HRP-conjugated secondary antibody at a concentration of 1:1000 for 1h at 25°C with shaking then were washed as previously described. The membranes were then treated with the ECL chemiluminescent reagent for western blots (Amersham ECL Advance Western Blotting Detection Kit GE Healthcare) and visualized on Bio Rad Moleuclar Imager chemi doc XRS^t imaging system (abcam protocol).

The same procedure was followed using Rabbit polyclonal IgG anti-Bax, and anti-Bcl-2 primary antibodies.

2.9 Statistical Analysis

All experiments were carried out in triplicate. Results were reported as the mean value \pm standard deviation. Data were analyzed by one-way analysis of variance (ANOVA). The differences between the means of treated and control groups were tested for significance using Fisher's least significant differences at $P \leq 0.05$ (Fisher PLSD). An effect was considered significant when the value (\pm) of mean difference between groups exceeded Fisher PLSD in the one-factor ANOVA test.

Chapter III

Results

3.1. Cytotoxicity effect of cabbage and kohlrabi on Ht-29 and Caco-2 cell lines

The cytotoxicity of cabbage and kohlrabi was assessed on 2 colorectal cancer cell lines HT-29 and Caco-2 cells for 24 and 48h post treatment using Trypan Blue exclusion method.

Cytotoxicity at 24h was almost insignificant for HT-29 and Caco-2 cells when treated with 60µg/µl of kohlrabi; the cytotoxicity percentage recorded 15% and 9% respectively (Figure 2A, 2B). However, the percentage increased to 45% for HT-29 and 12.5% for Caco-2 cells upon treatment with 80µg/µl of kohlrabi (Data not shown). On the contrary, cytotoxicity with 60µg/µl of cabbage 24h post treatment recorded 55% for HT-29 and 30% for Caco-2 cells, while the cytotoxicity percentage with 80µg/µl was calculated to be 80% for HT-29 and 68% for Caco-2 cells (Data not shown).

The maximal cytotoxic values reported 48h after treatment were as follows: 34% for HT-29 cells, 30% for Caco-2 cells when treated respectively with 60µg/µl of kohlrabi, while 77% for HT-29 cells and 83% for Caco-2 cells when treated respectively with 60µg/µl of cabbage. No significant cytotoxicity was accounted for the values of the control and the 60µg/µl of kohlrabi; however a remarkable cytotoxicity was reported for the 60µg/µl values of cabbage. The IC₅₀ for the latter treatment was shown to be at 50µg/µl beyond which the viability of both cell lines decreased significantly ($p \leq 0.05$) (Figures 2C, 2D).

These results were calculated as the percent of viable cells to the total number of dead and live cells.

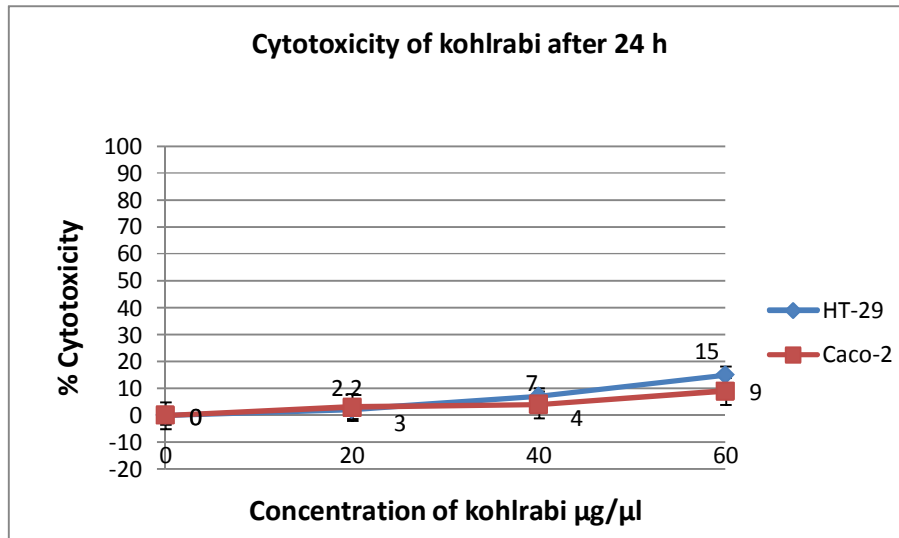


Figure 2A: Cytotoxicity of Kohlrabi on two cell lines HT-29 and Caco-2 after 24h of treatment. HT-29 and Caco-2 cells were treated with different concentrations of kohlrabi for 24h. Cell viability was then determined using the Trypan Blue Exclusion method. The results were expressed as percent of total number of cells. Data is the mean \pm SD.

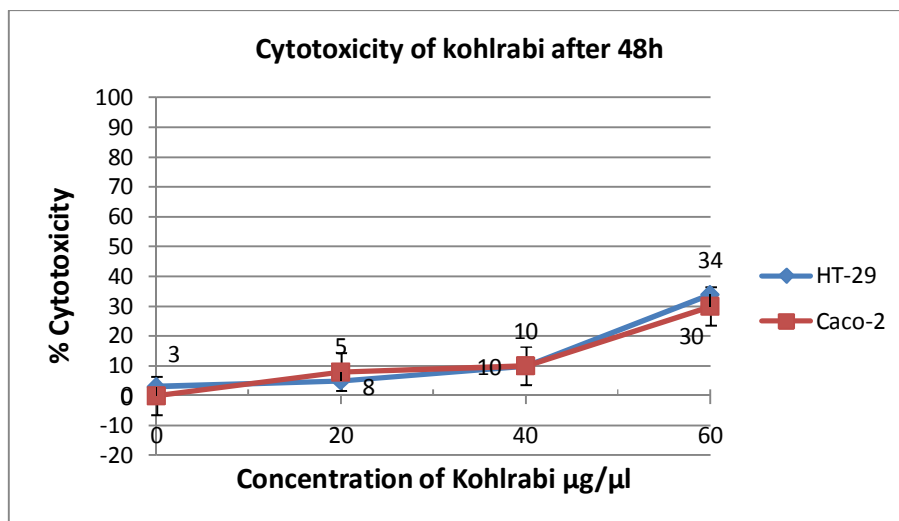


Figure 2B: Cytotoxicity of Kohlrabi on two cell lines HT-29 and Caco-2 after 48h. HT-29 and Caco-2 cells were treated with different concentrations of kohlrabi for 48h. Cell viability was then determined using the Trypan Blue Exclusion method. The results were expressed as percent of total number of cells. Data is the mean \pm SD.

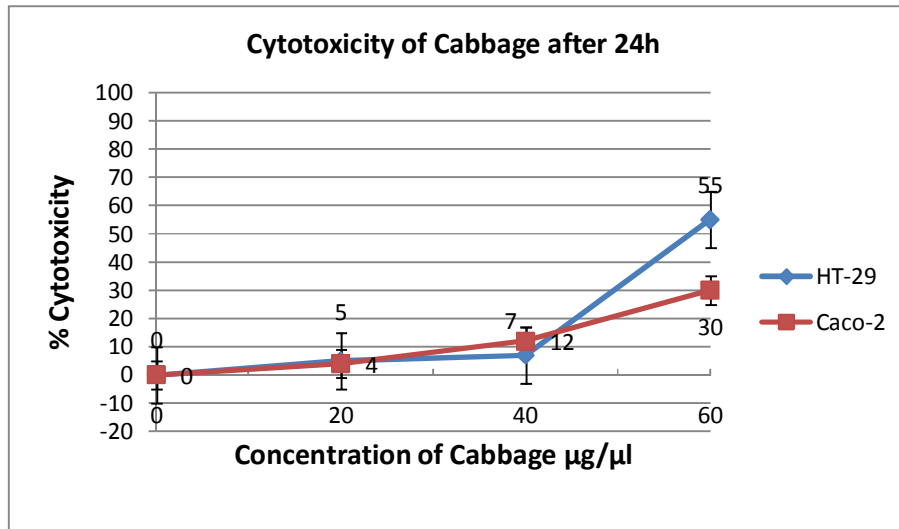


Figure 2C: The cytotoxicity of cabbage on two cell lines HT-29 and Caco-2 after 24h. HT-29 and Caco-2 cells were treated with different concentrations of cabbage for 24h. Cell viability was then determined using the Trypan Blue Exclusion method. The results were expressed as percent of total number of cells. Data is the mean \pm SD.

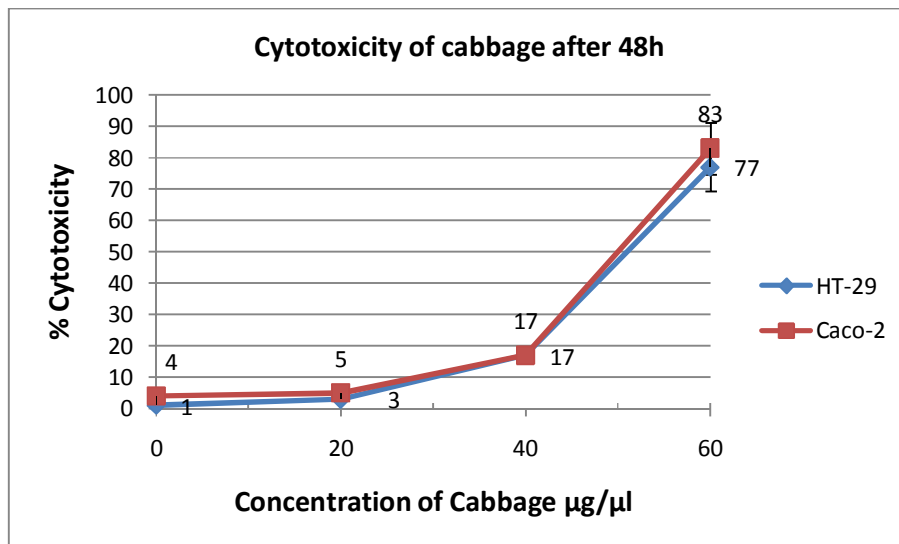


Figure 2D: Cytotoxicity of cabbage on two cell lines HT-29 and Caco-2 after 48h of treatment. HT-29 and Caco-2 cells were treated with different concentrations of cabbage for 48h. Cell viability was then determined using the Trypan Blue Exclusion method. The results were expressed as percent of total number of cells. Data is the mean \pm SD.

Therefore, to properly compare the effect of these two extracts, the cytotoxicity of kohlrabi versus cabbage after 24 and 48h of treatment on each cell line was established. The results were as follows: after 24h, and with 60µg/µl of treatment in

HT-29 cells, kohlrabi marked 15% of cytotoxicity while cabbage marked 55% (Figure 2E). While after 48h of treatment and for the same concentration and cell line, the cytotoxicity of kohlrabi only increased to 34% while that of cabbage reached 77%. These results show that cabbage is about two times more cytotoxic than kohlrabi on HT-29 cells (Figure 2F).

On the other hand, Caco-2 cells treated with 60 $\mu\text{g}/\mu\text{l}$ of kohlrabi for 24h reported 9% of cytotoxicity while the same concentration marked 30% for cabbage (Figure 2G). While after 48h of treatment for the same concentration and cell line, the cytotoxicity of kohlrabi slightly increased to 30% while that of cabbage increased to reach 83% (Figure 2H). Similarly, cabbage is shown to be 2 times more cytotoxic than kohlrabi on Caco-2 cells. As result, we can state that cabbage is more cytotoxic than kohlrabi on both colorectal cell lines.

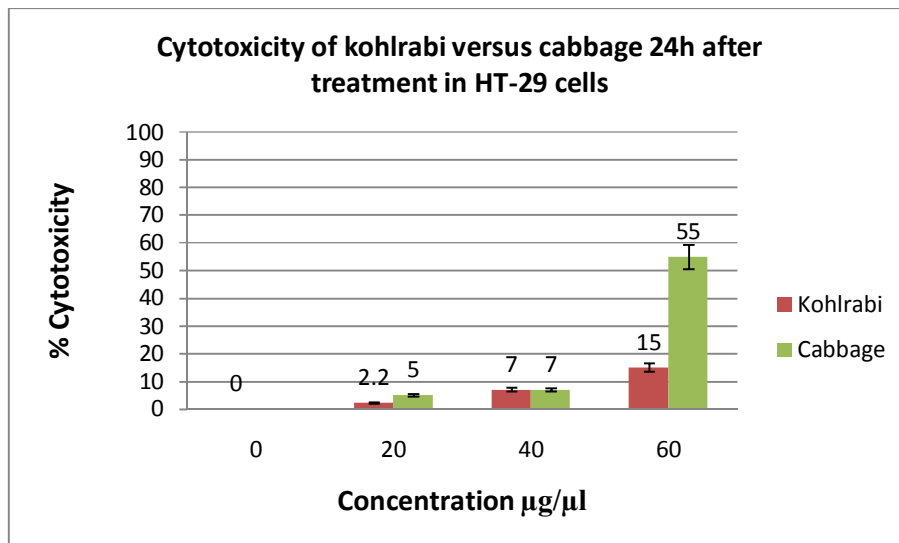


Figure 2E: Cytotoxicity of kohlrabi versus cabbage extracts after treating HT-29 cells with different concentrations for 24h.

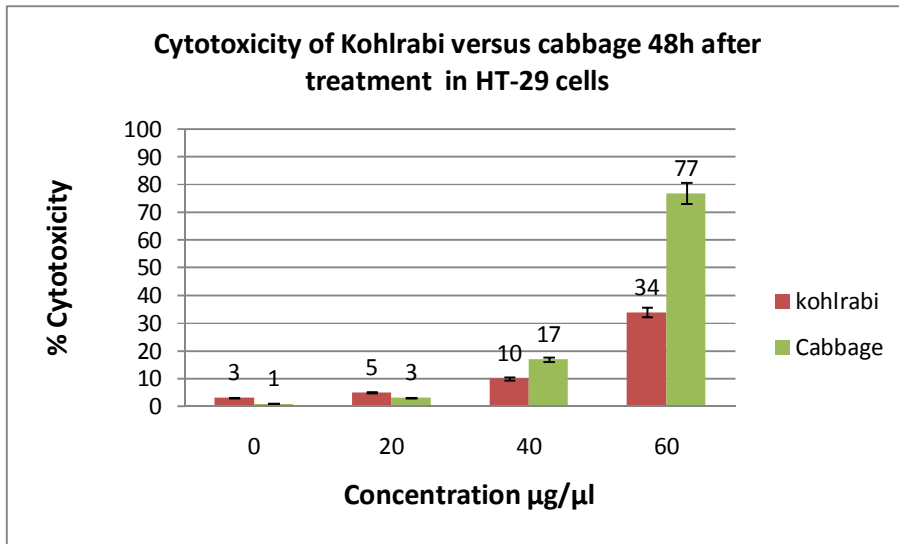


Figure 2F: Cytotoxicity of kohlrabi versus cabbage extracts after treating HT-29 cells with different concentrations for 48h.

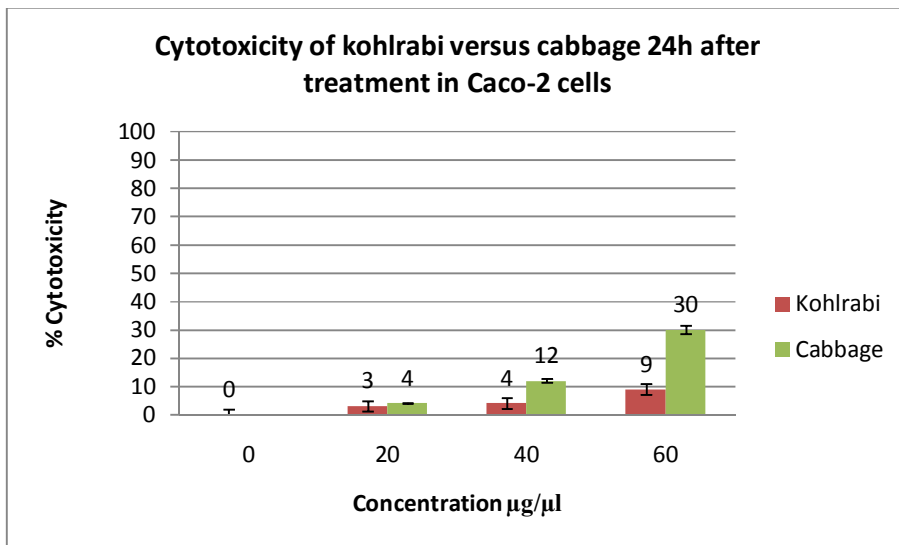


Figure 2G: Cytotoxicity of kohlrabi versus cabbage extracts after treating Caco-2 cells with different concentrations for 24h.

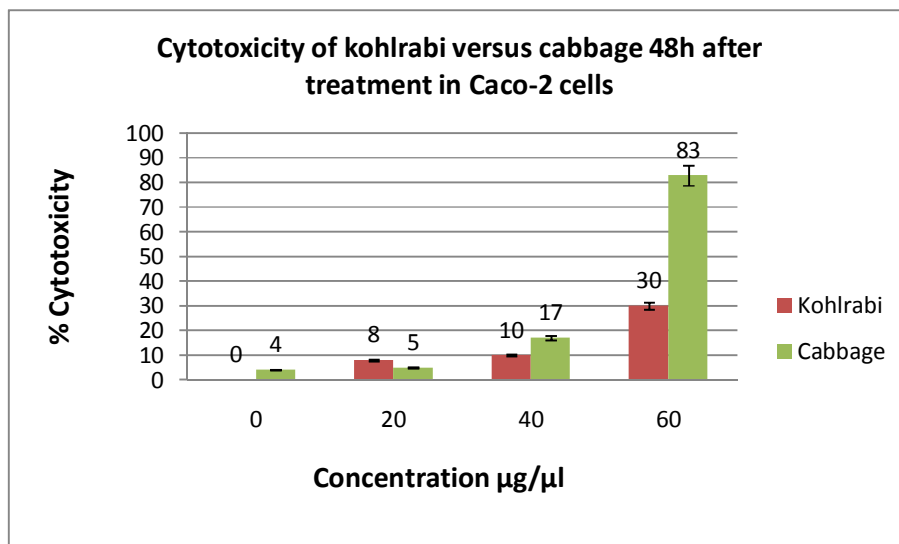


Figure 2H: Cytotoxicity of kohlrabi versus cabbage extracts after treating Caco-2 cells with different concentrations for 48h.

3.2. Effect of cabbage and kohlrabi on the proliferation of HT-29 and Caco-2 cell lines

HT-29 and Caco-2 cells were seeded in 96-well plate and were treated with Kohlrabi and cabbage independently at the following concentrations 10µg/µl, 20µg/µl, 40µg/µl and 60µg/µl for 24 and 48h.

The proliferation of these two cell lines treated with these extracts was assessed using WST-1 which tetrazolium salt is cleaved to formazan by mitochondrial dehydrogenases enzymes present in live cells. Kohlrabi and cabbage extracts were found to show a remarkable decrease in the proliferation of HT-29 and Caco-2 cells in a dose dependent manner. 24h post treatment and at the concentration of 40µg/µl of kohlrabi (slightly below the IC50), the proliferation of HT-29 was reduced to 10.5% while that of Caco-2 cells decreased to 11%. However, when treated with 40µg/µl of cabbage extract, HT-29 cells proliferation percentage decreased to 1.8% while that of Caco-2 cells reached 2% (Figure 3A, 3C). Additionally, 48h post treatment and at a concentration of 40µg/µl of kohlrabi, the proliferation of HT-29 was inhibited to reach 8% while that of Caco-2 cells the proliferation was 8%. On the other hand and for the same concentration of cabbage extracts, the proliferation percentage decreased to reach 1.5% in HT-29 cells and 1% in Caco-2 cells (Figure 3B, 3D). These results showed a significant antiproliferative effect of both extracts

on HT-29 and Caco-2 cells, yet it is noteworthy the proximity of the experimental values obtained in each cell line suggesting a similar extent of action of kohlrabi or of cabbage on these two colorectal cancer cell lines.

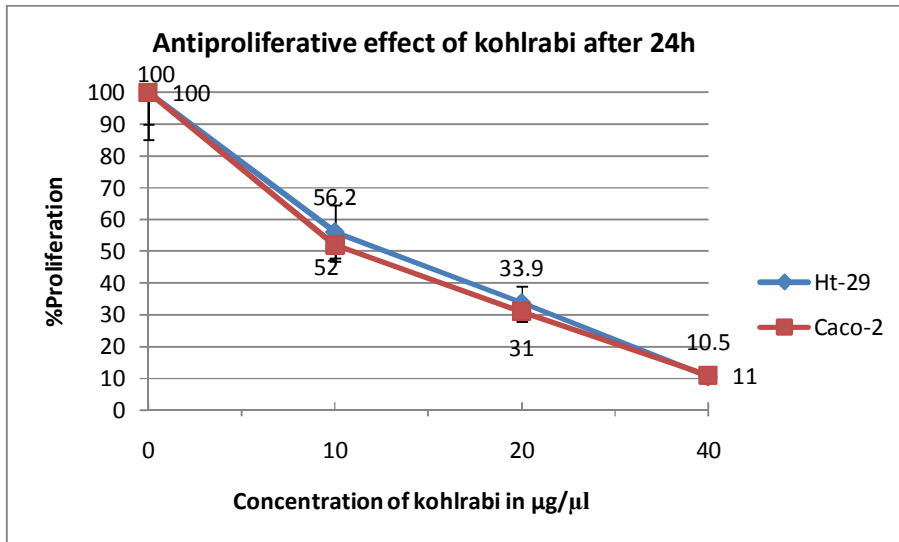


Figure3A: Proliferation of Caco-2 cells and HT-29 cells after 24h of treatment with different concentrations of kohlrabi. Cells were treated with different concentrations of kohlrabi. Absorbance was measured at 450 nm after 4h of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. Data is the mean \pm SD.

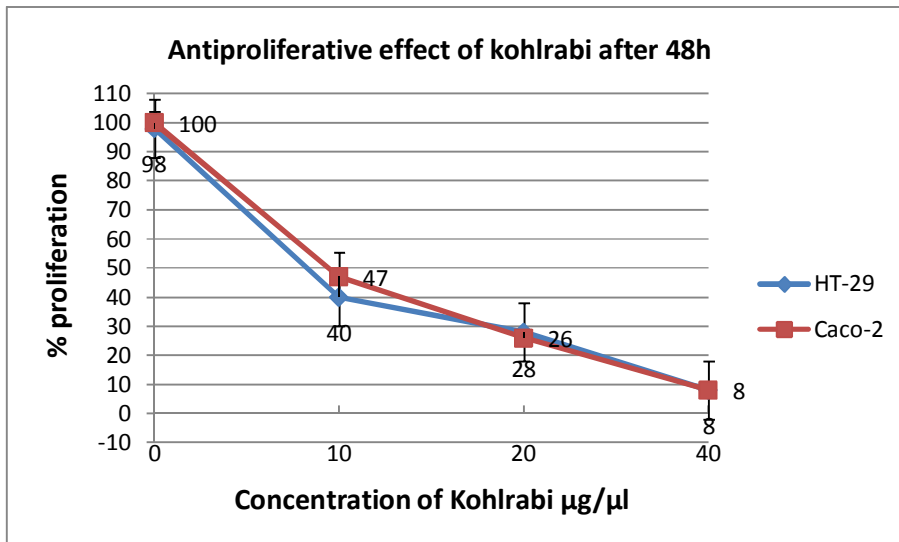


Figure 3B: Proliferation of Caco-2 cells and HT-29 cells after 48h of treatment with

different concentrations of kohlrabi. Cells were treated with different concentrations of kohlrabi. Absorbance was measured at 450 nm after 4h of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. Data is the mean +/- SD.

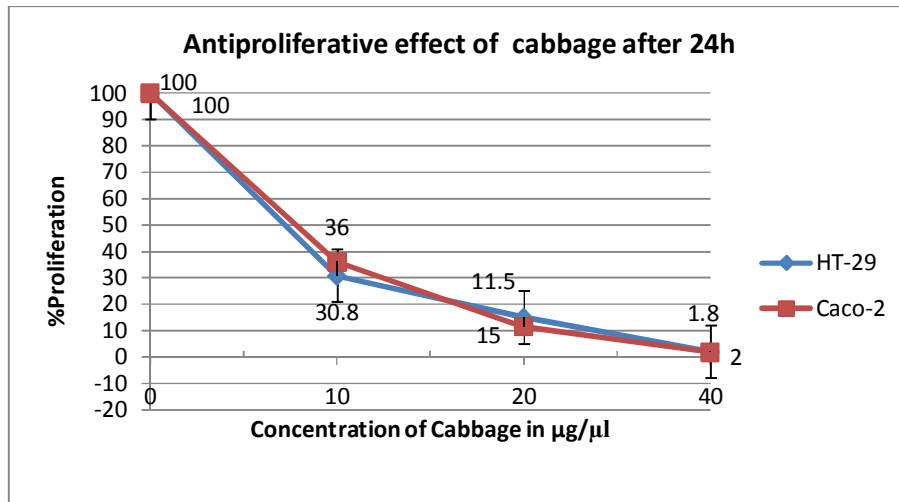


Figure 3C: Proliferation of Caco-2 cells and HT-29 cells after 24h of treatment with different concentrations of cabbage. Cells were treated with different concentrations of cabbage. Absorbance was measured at 450 nm after 4h of incubation with WST-1 cell proliferation reagent. Results were normalized to the untreated cells. Data is the mean +/- SD.

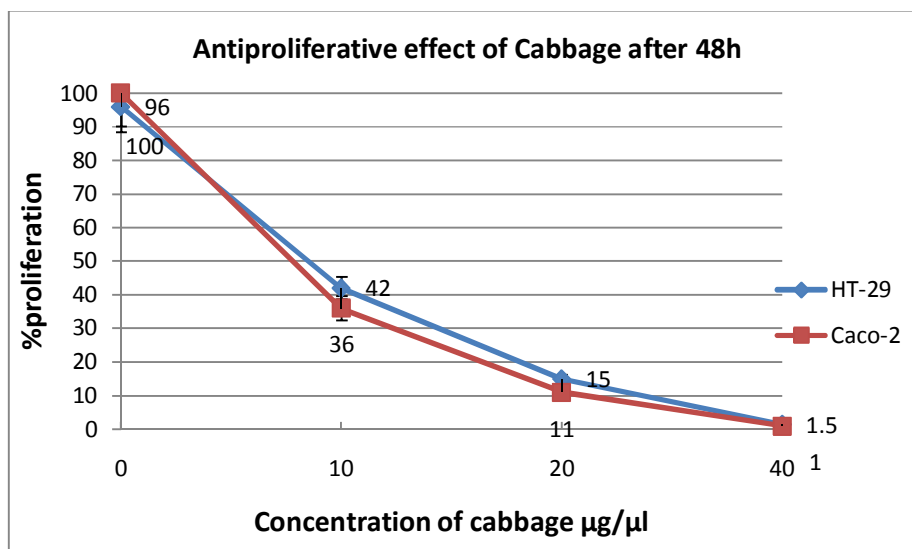


Figure 3D: Proliferation of Caco-2 cells and HT-29 cells after 48h of treatment with different concentrations of cabbage. Cells were treated with different concentrations of cabbage. Absorbance was measured at 450 nm after 4h of incubation with WST-1 cell

proliferation reagent. Results were normalized to the untreated cells. Data is the mean \pm SD.

These experiments were also assessed to establish a comparison in between kohlrabi and cabbage extract and their antiproliferative effect on colorectal cell lines. This comparison illustrates that the proliferation percentage of HT-29 cells treated with $40\mu\text{g}/\mu\text{l}$ after 24h is 10.5% while with the cabbage treatment and for the same concentration the proliferation is reduced to 2% (Figure 3E); while after 48h $40\mu\text{g}/\mu\text{l}$ of the kohlrabi extract further reduced the proliferation percentage of HT-29 to 8% while the proliferation of HT-29 cells treated with cabbage reached 1.5% (Figure 3F).

On the other hand, Caco-2 cells treated with $40\mu\text{g}/\mu\text{l}$ of kohlrabi for 24h showed 11% of proliferation while cabbage recorded for the same concentration and cell line 1.8%. 48h later, the proliferation of Caco-2 treated cells with kohlrabi decreased to 8% while the cells treated with $40\mu\text{g}/\mu\text{l}$ of cabbage were reduced to 1% (Figure 3G, 3H). At $60\mu\text{g}/\mu\text{l}$ 24 h post treatment, the decrease in the proliferation of HT-29 with kohlrabi and cabbage reached 5% and 2% respectively, while for Caco-2 cells the inhibition reached 5% and 2.9% correspondingly (data not shown). However, 48h post treatment, and at $60\mu\text{g}/\mu\text{l}$ of kohlrabi and cabbage, the proliferation decreased to 4% and 0.5% respectively, while in Caco-2 cells proliferation was reduced to 5% when treated with kohlrabi and 0.4% when treated with cabbage (data not shown). Accordingly, we can conclude that the antiproliferative effect of kohlrabi on Caco2 cells is 3.5 times weaker than cabbage. Therefore, kohlrabi has a weaker antiproliferative effect on HT-29 and caco-2 cells compared to cabbage.

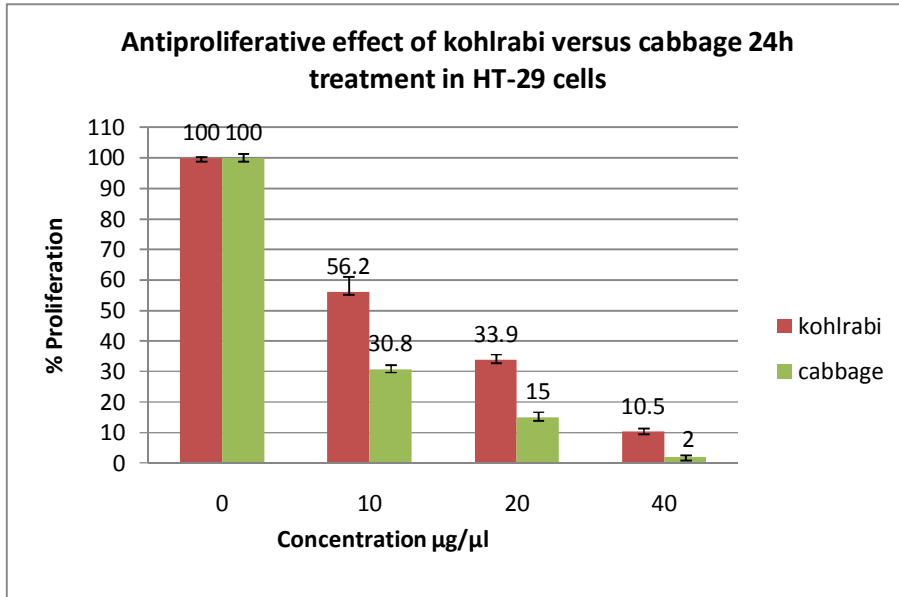


Figure 3E: Antiproliferative effect of kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 24h.

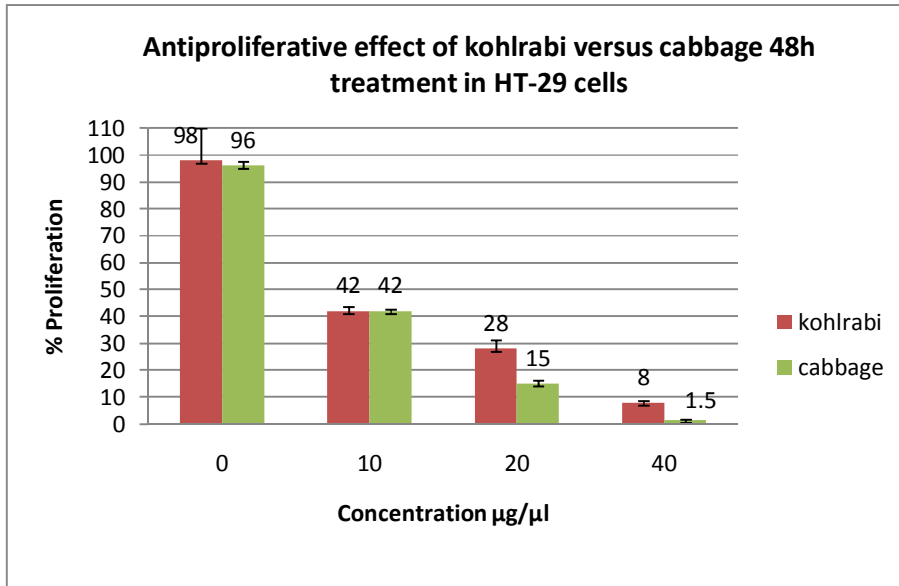


Figure 3F: Antiproliferative effect of kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 48h.

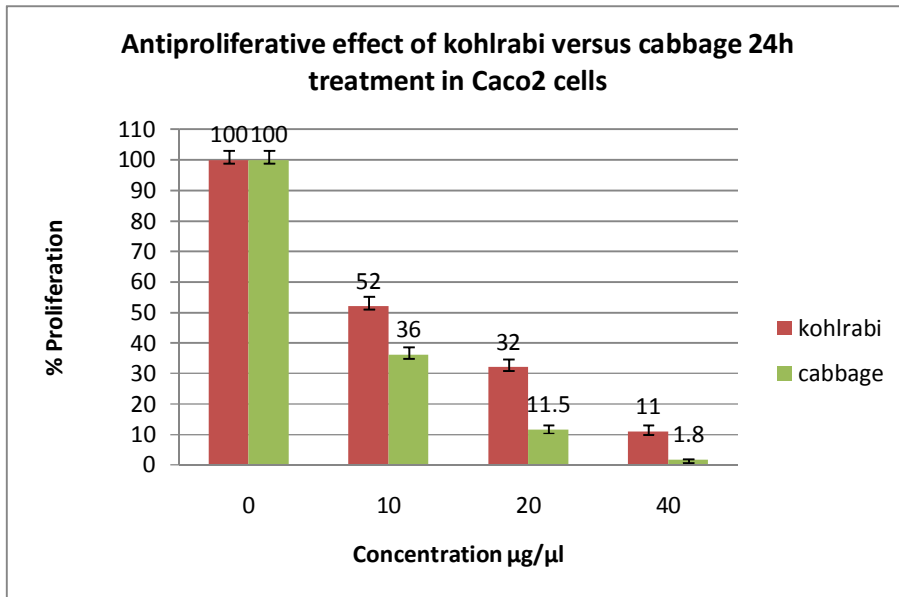


Figure 3G: Antiproliferative effect of kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 24h.

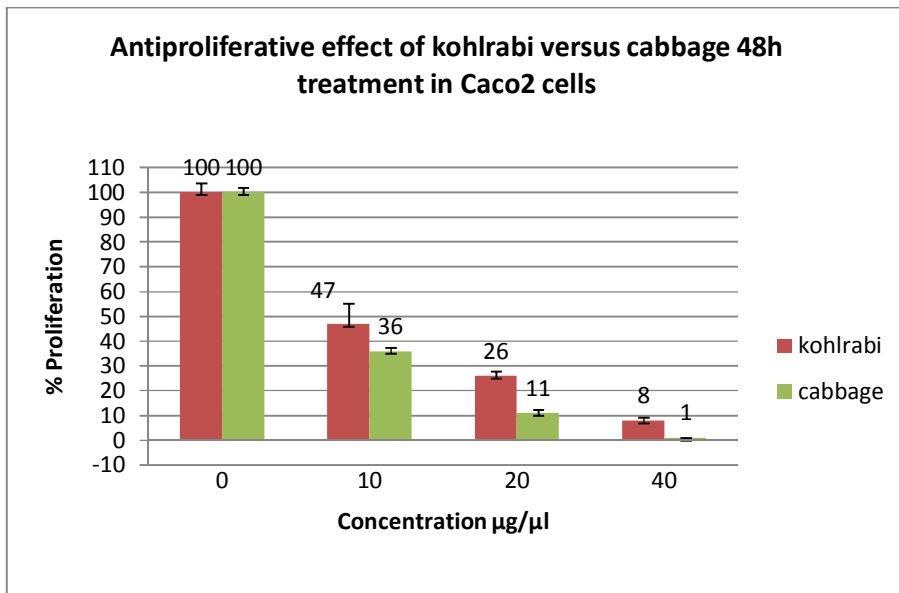


Figure 3H: Antiproliferative effect of kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 48h.

3.3. Cell cycle arrest induction of HT-29 cells by kohlrabi and cabbage

After determining the antiproliferative effect of kohlrabi and cabbage on HT-29 cells and verifying the effect of kohlrabi in inhibiting cell progression to be 2.5 folds lower than cabbage, we aimed to assess whether this result is through cell cycle arrest using flow cytometry analysis. HT-29 cells were treated with 10 μ g/ μ l; 20 μ g/ μ l and 40 μ g/ μ l of cabbage extract and kohlrabi extract separately for 24h and then were stained with PI. The cell cycle comprises the following phases according to its DNA content. Sub-G0/G1 cells are < 2n, G0/G1 cells are equal to 2n, S phase cells are >2n but < 4n, and G2/M phase cells are 4n. Accordingly, the G0 and G1 DNA content of cells are alike, and so is the content of the G2 and phase. Therefore, the cell cycle is frequently illustrated by the G0/G1, S, and G2/M phases (Kues WA. et al. 2000).

After treating the cells with the different increasing concentrations of cabbage and kohlrabi extracts, we notice a change in the phases' distribution particularly a shift from the M phase to the G phase. Both kohlrabi and cabbage extracts induced an increase in the G phase after exhibiting a decrease in the M phase after treatment. The nontreated cells show a 42.8% in the G phase, 13.9% in the S phase where DNA is replicating and 28.1% in the M phase where cells prepare for division and enter mitosis for a new cell cycle (Figure 4A). However, when comparing the cells treated with 10 μ g/ μ l, 20 μ g/ μ l and 40 μ g/ μ l of cabbage to the cycles of the control cells we observe that the nontreated cells show a lower G peak and a higher M peak in a dose dependent manner. The high peak of the M phase indicates the readiness of the daughter cells to exit the mitotic cycle. While when the peak of M is lowered in favor to the increase of G and S phases, the cells decreased their proliferation level to arrest it at the G phase and at the S/M transition checkpoint (Figures 4D, 4G). Figures 4B, 4C, 4E and 4F show an accumulation in the S phase suggesting the augmentation of S to be due to a hindrance in movement of cells from the S phase to the G2/M phase upon increasing the concentrations of the treatments. This suggests a checkpoint at the S/M phase. Further, when comparing the analysis of the treated cell cycles of cabbage to those of kohlrabi, we notice that cabbage induction to cell cycle arrest at the G phase displays higher peaks as we increased the concentrations. However, it is noteworthy to mention that the two extracts seem to follow a similar pattern in inducing cell cycle arrest at the G phase in HT-29 cells treated with

40 $\mu\text{g}/\mu\text{l}$ for 24h. Consequently, this outcome asserts a lower impact of antiproliferation of kohlrabi compared to cabbage as previously assessed by WST-1 assay.

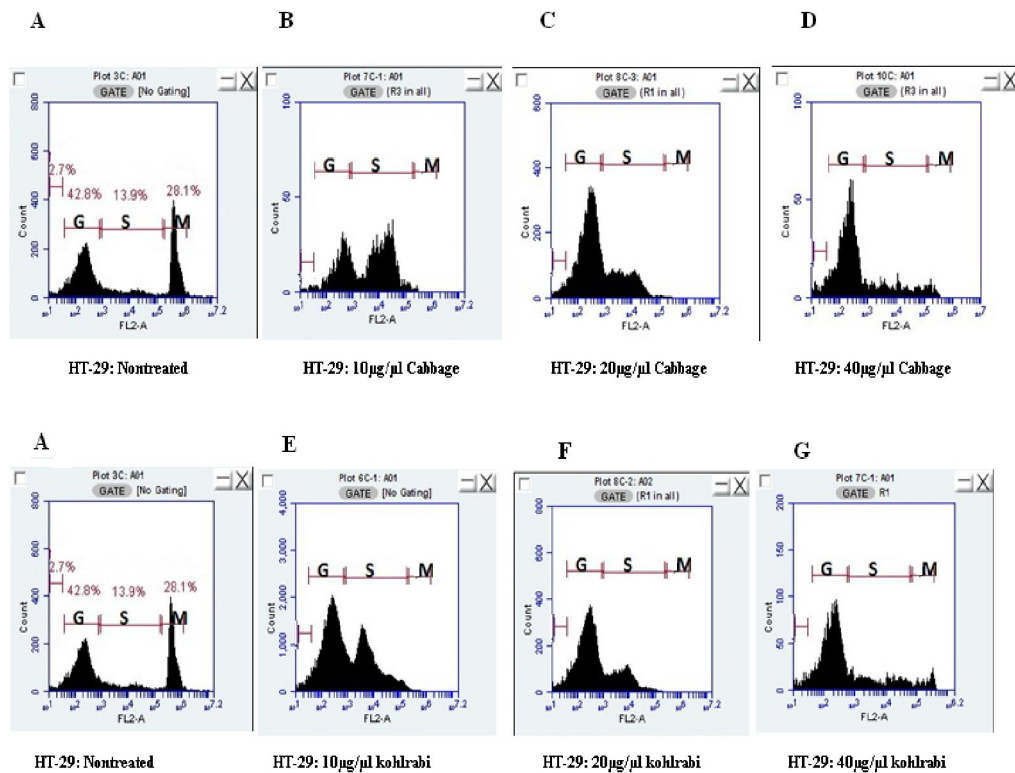


Figure 4: Cell cycle analysis by flow cytometry. HT-29 treated with different concentrations of cabbage (upper panels) and kohlrabi (lower panels) for 24 h. Control cells were left untreated. After overnight fixation, cells were incubated with Propidium Iodide (PI). Cells were analyzed using a C6 flow cytometer indicating the distribution of the cells based on their DNA content into their respective cell cycle phases: Sub-G0/G1 cells were less than 2n, G0/G1 cells were 2n, S/M phase cells were >2n. A: Stained control; B: 10 $\mu\text{g}/\mu\text{l}$ cabbage, C: 20 $\mu\text{g}/\mu\text{l}$ cabbage, D: 40 $\mu\text{g}/\mu\text{l}$ cabbage, E: 10 $\mu\text{g}/\mu\text{l}$ kohlrabi, F: 20 $\mu\text{g}/\mu\text{l}$ kohlrabi, G: 40 $\mu\text{g}/\mu\text{l}$ kohlrabi.

3.4. Proapoptotic effect of kohlrabi and cabbage on HT-29 and Caco-2 cell lines.

3.4.1. Apoptotic effect by Cell death ELISA:

To confirm the effect of kohlrabi and cabbage extracts on the induction of apoptosis, HT-29 and Caco-2 cells were seeded, treated and assayed for apoptosis using Cell Death ELISA kit.

This assay showed an increase of DNA/histone fragments in a dose-dependent manner after treatment with both extracts. The enrichment factor was calculated based on the presence of degraded nucleosomes sandwiched in streptavidin and anti-histone-biotin anti-DNA-peroxidase antibodies. These results were normalized to untreated cells.

After treating HT-29 and Caco-2 cells with 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi extracts for 24h, HT-29 showed a 2.2 fold increase in apoptosis while Caco-2 cells exhibited one fold increase (Figure 5A). While for cabbage and for the same dose and time, both HT-29 and Caco-2 cells expressed one fold increase (Figure 5C). While after 48h, the apoptotic fold increased of HT-29 remained the same when treated with kohlrabi while that of Caco 1.7 fold (Figure 5B). While after 48h of treating HT-29 with 40 $\mu\text{g}/\mu\text{l}$ of cabbage, the apoptotic rate increased to reach 2 folds while that of Caco increased to reach 1.7 fold (Figure 5D). Consequently, we can deduce that HT-29 cells will undergo apoptosis at a higher rate than Caco-2 when treated with kohlrabi and cabbage extracts.

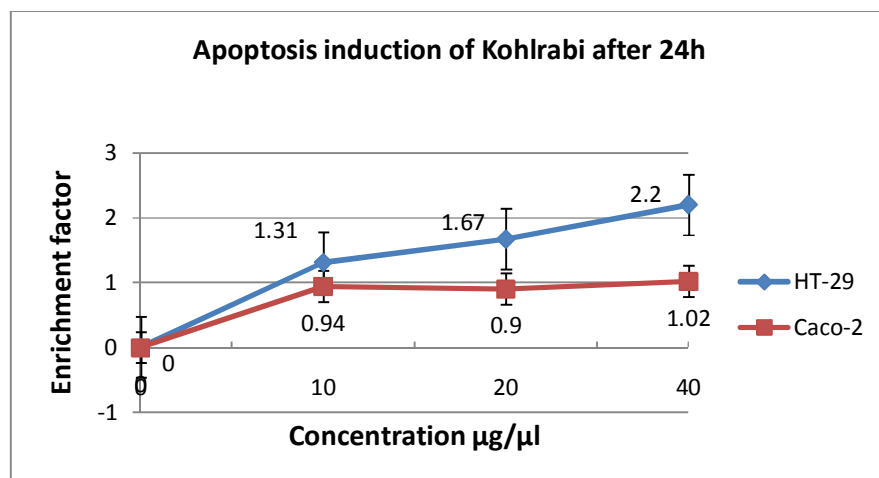


Figure 5A: Induction of apoptosis in HT-29 and Caco-2 cells 24h after kohlrabi treatment. Cells were treated with different concentrations of kohlrabi. Absorbance was

read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control).

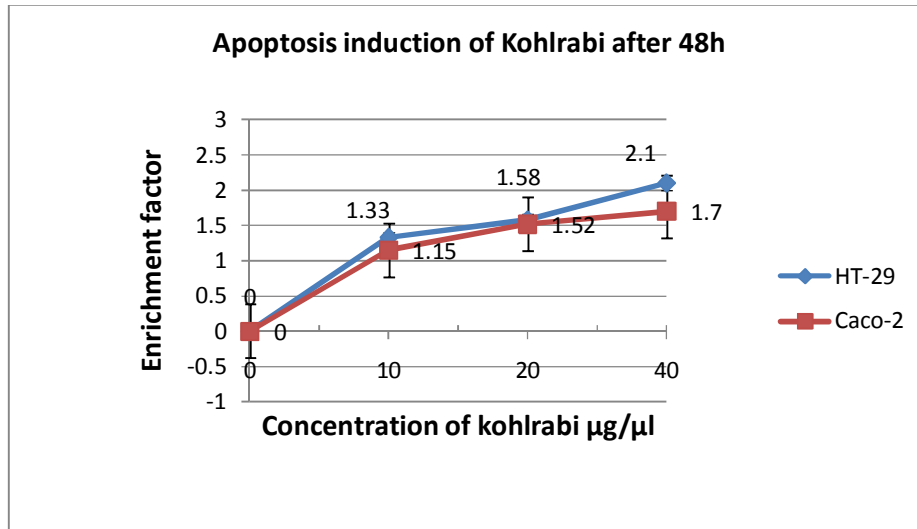


Figure 5B: Induction of apoptosis in HT-29 and Caco-2 cells after 48h of kohlrabi treatment. Cells were treated with different concentrations of kohlrabi. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control).

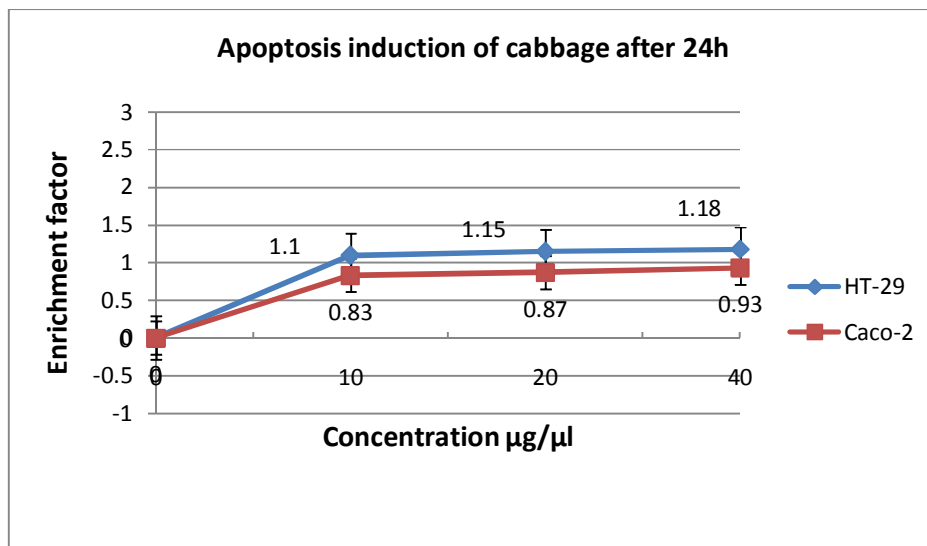


Figure 5C: Induction of apoptosis in HT-29 and Caco-2 cells after 24h of cabbage treatment. Cells were treated with different concentrations of cabbage. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control).

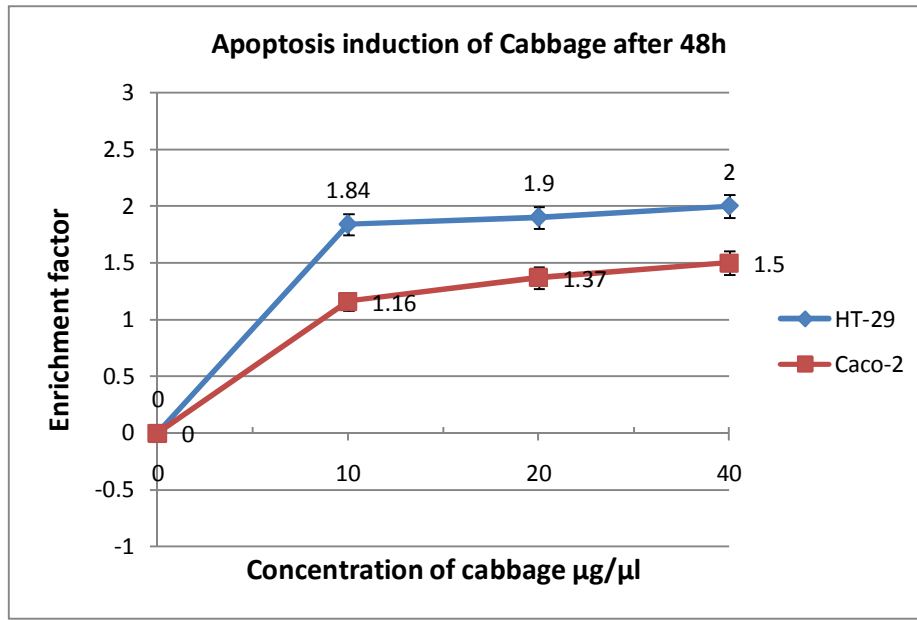


Figure 5D: Induction of apoptosis in HT-29 and Caco-2 cells after 48h of cabbage treatment. Cells were treated with different concentrations of cabbage. Absorbance was read at 405 nm after the addition of ABTS substrate. Results are shown as the enrichment factor (absorbance of sample divided by the absorbance of untreated control).

Kohlrabi and cabbage extracts belonging to same family were apoptotically assessed and compared over the same cell lines. HT-29 showed a 2.2 fold increase in apoptotic cells when treated independently for 24h with 40µg/µl of kohlrabi and 1fold increase with 40µg/µl of cabbage extracts (Figure 5E). Further, HT-29 almost maintained their 2 fold increase constant when treated for 48h with 40µg/µl of kohlrabi and increased their apoptotic rate to 2 fold when treated with 40µg/µl of cabbage extracts for 48h (Figure 5F). While, Caco-2 cells barely showed an increase of 1 fold in apoptotic cells when treated independently with 40µg/µl of kohlrabi and cabbage extracts for 24h (Figure 5G). Whereas, Caco-2 cells exhibited 1.7 fold increase when treated with 40µg/µl of kohlrabi and 1.5 fold increase with 40µg/µl of cabbage for 48h (Figure 5H). These results showed that kohlrabi induced apoptosis in HT-29 at 24h and maintained it constant for 48h, while cabbage needed 48h for a 2 fold increase in apoptosis. Consequently, kohlrabi appears to induce apoptosis faster in HT-29 cells compared to cabbage. However, in Caco-2 cells, kohlrabi and cabbage reveal a similar apoptotic pattern in a time and dose dependant manner. Both extracts required a concentration of 40µg/µl for 48h to initiate a noticeable apoptotic rate of 2 folds.

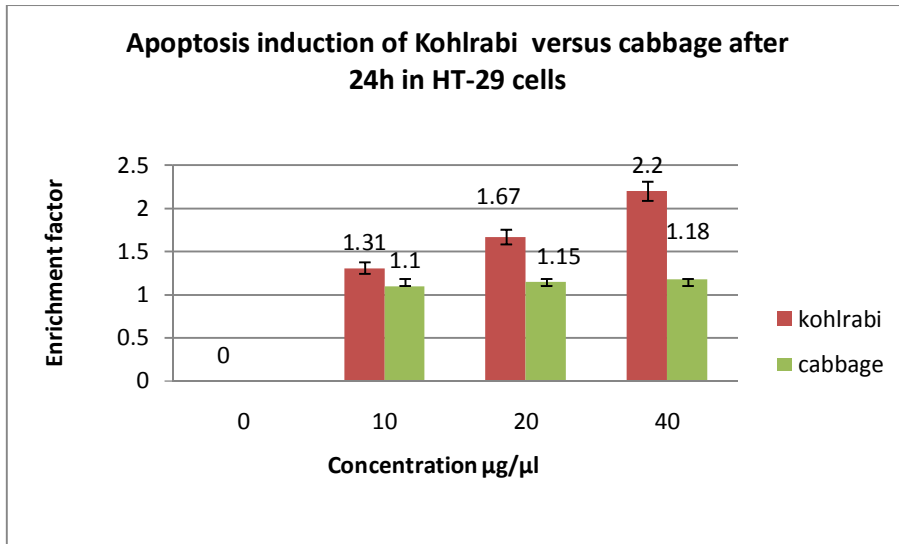


Figure 5E: Apoptosis induction of Kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 24h.

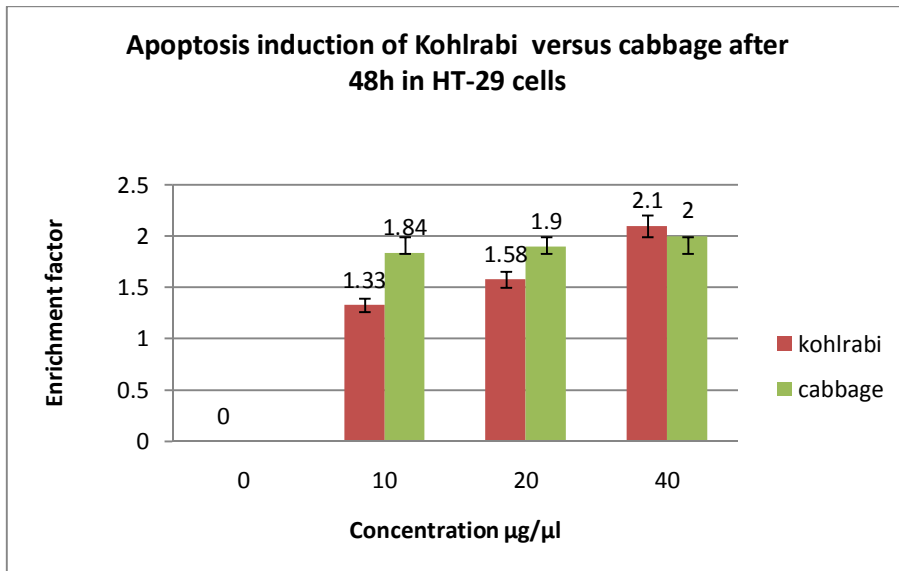


Figure 5F: Apoptosis induction of Kohlrabi versus cabbage after treating HT-29 cells with different concentrations for 48h.

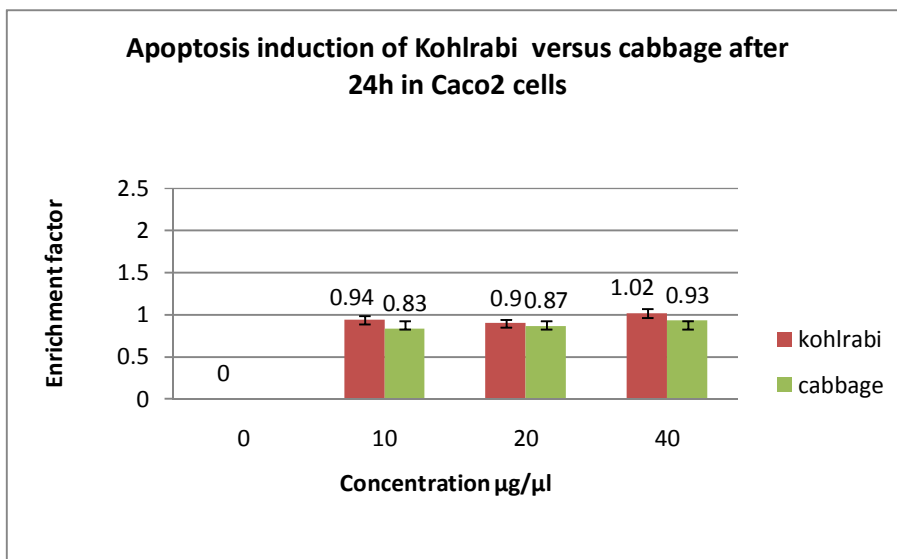


Figure 5G: Apoptosis induction of Kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 24h.

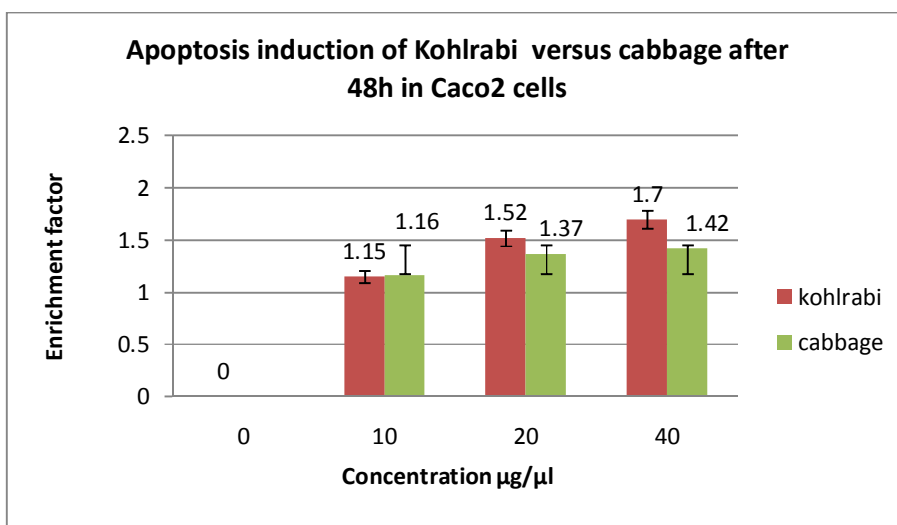


Figure 5H: Apoptosis induction of Kohlrabi versus cabbage after treating Caco-2 cells with different concentrations for 48h.

3.4.2. Apoptosis by Flow Cytometry:

In order to further evaluate apoptosis, a more specific Annexin V-FITC/PI double staining method flow cytometry assay was used. When undergoing apoptosis, phosphatidylserine (PS) residues get exposed to the extracellular side of the cell membrane. These revealed PS residues possess a high binding affinity to the natural ligand annexin V. Propidium iodide (PI) fluorochrome on the other hand binds to fragmented DNA (Mukherjee et al. 2010).

The quadrant that indicates the presence of early apoptosis in cells is the bottom right quadrant where the cells are negative for PI staining while positive for annexin staining. The quadrant in the upper right corner displays cells of late apoptotic stages where they are positive for both PI and Annexin V staining. As of the lower left quadrant, healthy cells are displayed and necrotic cells are found in the upper left quadrant. As figure 6 shows we can compare apoptosis induction in HT-29 by cabbage and kohlrabi. The assay used showed that nontreated HT-29 cells had 4.1% of early apoptotic cells and 6.9% of late apoptotic cells, while 88% of healthy cells (Figure 6A). Upon treatment with 10 μ g/ μ l of cabbage extract for 24h the early apoptotic rate increased to 12.6% and the late apoptosis increased to 22.9%, while the healthy cells percentage decreased to 63.1% (Figure 6B); with 20 μ g/ μ l of cabbage early apoptosis further augmented to 17.9% and late apoptosis to 33.4%, while healthy cells proliferation decreased to 48.1% (Figure 6C). Finally, at 40 μ g/ μ l of cabbage led to 22.8% of early apoptotic cells while 54.9% of late apoptosis and a further reduction in healthy cells to 21.9% (Figure 6D). As for 10 μ g/ μ l of kohlrabi extract treatment for 24h, the early apoptotic rate increased to 9.7% while the late apoptotic rate reached 15.9% and the percentage of healthy cells decreased to 74.3% (Figure 6E); with 20 μ g/ μ l of kohlrabi early apoptosis slightly increased to 13.3% as late apoptosis was amplified to 24.2% and healthy cells' percentage further decreased to 62.1% (Figure 6F); and with 40 μ g/ μ l of kohlrabi extract early apoptosis induction attained 15.7% while late apoptosis reached 45% and healthy cells were reduced to 38.7% (Figure 6G). Consequently, we can deduce that for the concentration of 10 μ g/ μ l, cabbage showed the early apoptotic rate to be 12.6% versus 9.7% for kohlrabi, and late apoptosis to be 22.9% versus 15.5% respectively and healthy cells in cabbage to 63.1% versus 74.0% in treated cells with kohlrabi; while for 20 μ g/ μ l, cabbage recorded 17.9% versus 13.3% for kohlrabi for early apoptosis, 33.4% versus 24.2% for late apoptosis and 48.1% versus 62.1% for healthy cells in cabbage and kohlrabi respectively. As for the highest concentration of 40 μ g/ μ l, cabbage early apoptotic rate reached 22.8% while that of kohlrabi only indicated 15.7%, 54.9% versus 45% for late apoptosis and 21.9% versus 38.6% for living cells in cabbage and kohlrabi consecutively. Accordingly, one can conclude that apoptosis induction in HT-29 cells treated with kohlrabi for 24h is 1.3 fold less than cabbage induction.

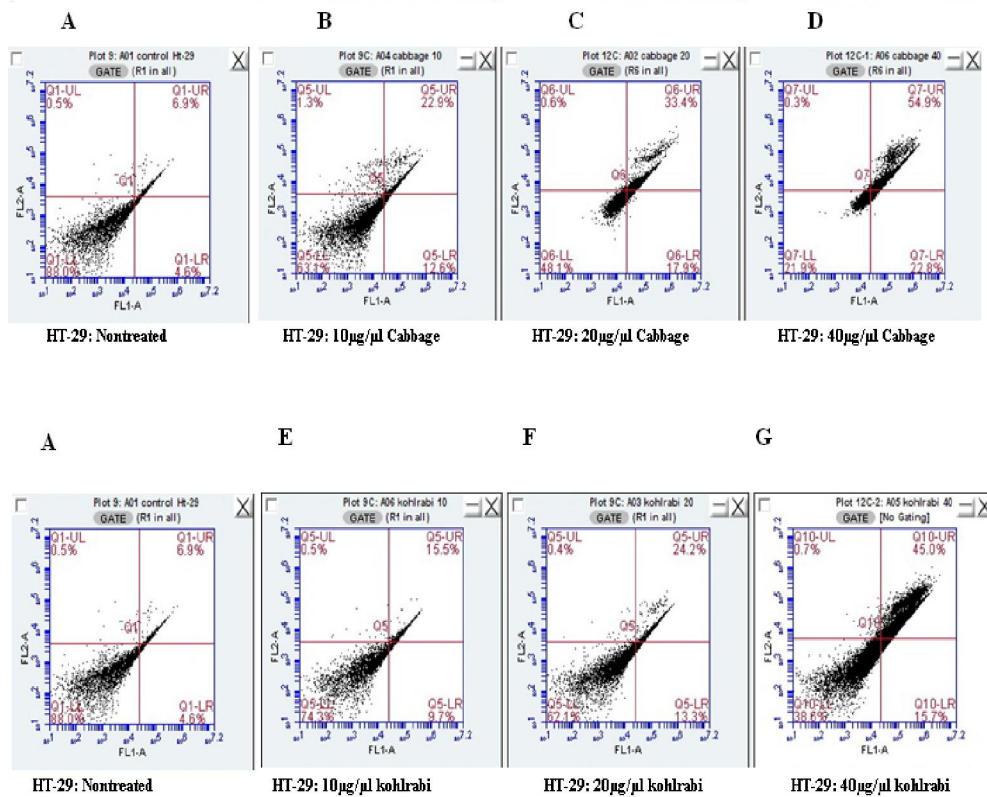


Figure 6: Analysis of apoptosis induced by cabbage and kohlrabi in HT-29 cells by flow cytometry using annexinV-FITC and PI. Quadrant analysis of fluorescence intensity of gated cells in FL-1 (annexin V-FITC) and FL-2 (PI) channels was from 10,000 events. A: Stained control; B: 10µg/µl cabbage, C: 20µg/µl cabbage, D: 40µg/µl cabbage, E: 10µg/µl kohlrabi, F: 20µg/µl kohlrabi, G: 40µg/µl kohlrabi.

3.5. Effect of Kohlrabi and Cabbage on Bax and Bcl-2 in HT-29 cells

The programmed cell death, apoptosis, had to be further assessed molecularly. Therefore, Bcl-2 and Bcl-2-associated X protein, or Bax levels were quantified using Western Blot analysis. The treatments used in this experiment were control, 10µg/µl, 20µg/µl, and 40µg/µl, for 24h and it showed the regulations of Bcl-2 and Bax in a dose dependent manner.

A control protein, β-Actin, was loaded to confirm equal concentration among the different samples. The kohlrabi and cabbage extracts promoted the down regulation

of the anti apoptotic Bcl-2 protein, while exerted the upregulation of the pro apoptotic Bax protein. Results are shown in the figure below.

The Bax/Bcl-2 ratio was calculated using Image J program. The Bax/Bcl-2 ratio was calculated to be 1.38 in the control sample. This ratio increased to become 1.6 when the HT-29 cells were treated with 10 μ g/ μ l of kohlrabi extract. The ratio further increased to 1.8 with 20 μ g/ μ l treatment and to 2.12 with 40 μ g/ μ l of kohlrabi extract. Whereas for the cabbage extract, 20 μ g/ μ l increased the Bax/Bcl-2 ratio to 2.03 and the 40 μ g/ μ l further increased the ratio to 2.16. As a consequence one may suggest that the apoptotic pathway of kohlrabi and cabbage extract on HT-29 cells is Bax/Bcl-2 dependant.

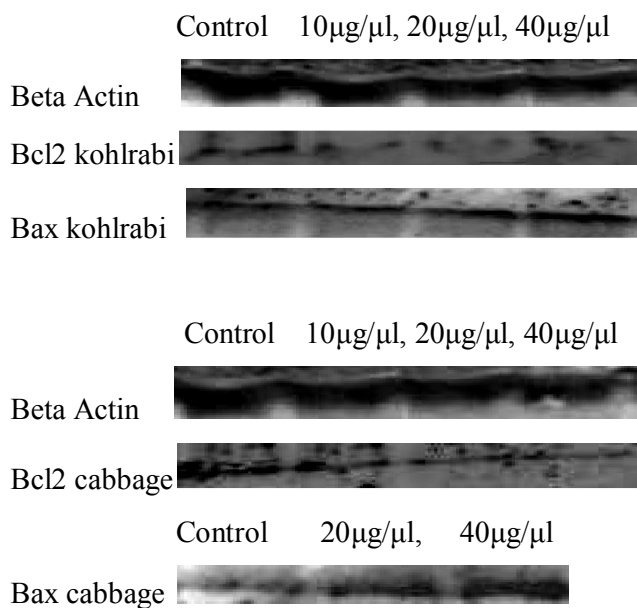


Figure 7: Western Blot analysis for Bax and Bcl-2. HT-29 cells were treated with 10 μ g/ μ l, 20 μ g/ μ l, and 40 μ g/ μ l of kohlrabi and cabbage extracts. Control cells were left untreated. At the end of the treatment period, cells were lysed and blotted for β -Actin (upper panel) as internal loading control, Bcl-2 (middle panel) and Bax (lower panel).

Chapter IV

Discussion

Colorectal cancer is the third most frequent cancer in the world. Epidemiological prospective and retrospective studies investigated and confirmed an inverse relationship between the prevalence of colon cancer and the consumption of *Brassica* vegetables such as cabbage and kohlrabi (Van Poppel, Verhoeven, Verhagen & Goldbohm, 1999). Isothiocyanates and indoles extracted from these plants showed a decrease in the proliferation and cell survival of different forms of cancer particularly colorectal cancer (Ambrosone & Tang, 2009). Most studies tackled HT-29 and Caco-2 cells treatment using defined ITCs such as AITC, SFN, BITC, PEITC and erucin, and indoles such as I3C derived from *Brassica oleracea* plants, however, very few studies included kohlrabi (You et al. 2008; Ciska et al. 2000). Thus, the aim of this study was to investigate and compare the antiproliferative and pro-apoptotic effect of kohlrabi versus cabbage on colorectal cancer cell lines in vitro. Kohlrabi flesh and cabbage leaves were blended in ethanol and incubated for a week, and then the filtrate was concentrated in a speed vacuum. The tissue damage of the plants caused by blending exposed the enzyme myrosinase to be in contact with glucosinolates molecules. The glucosinolates, glucobrassicin, sinigrin, gluconapin, gluconasturtiin, glucoerucin and glucoraphanin, identified to be present in both cabbage and kohlrabi (Cartea & Velasco, 2007; Melchini & Traka, 2010) were most likely hydrolyzed into I3C, AITC, BITC, PEITC, erucin and SFN respectively upon blending the tissues of the plants. However, the exact ITCs and indoles were not identified nor quantified in our extracts.

The attained results of cabbage extract were in accordance with preceding studies where the constituents of cabbage, ITCs and indoles, induced apoptosis and cell cycle arrest in HT-29 and Caco-2 cells (Zhu et al. 2004, Cartea & Velasco, 2007; Pappa et al. 2007; Mas et al. 2007; Choi et al. 2009); on the other hand, the results obtained after treatment with kohlrabi ethanolic extracts seem quite promising. We first determined the cytotoxicity of kohlrabi and cabbage extracts on HT-29 and caco-2 cell lines. HT-29 cells treated with 60µg/µl of kohlrabi for 24h exhibited a 15% decrease in viability, while with cabbage it reached 55%. On the other hand,

60 $\mu\text{g}/\mu\text{l}$ of kohlrabi caused a cytotoxicity of 9% in Caco-2 cells while cabbage reported 30%. Cytotoxicity levels were time and dose –dependent where higher concentrations and a longer exposure of both extracts led to a decrease in cell viability. Treating HT-29 cells with 60 $\mu\text{g}/\mu\text{l}$ of kohlrabi for 48h lead to 34% of cytoxicity while cabbage treatment reached 77%. As for Caco-2 cells, 60 $\mu\text{g}/\mu\text{l}$ of kohlrabi caused 30% decrease in cell viability while cabbage caused 83% decrease. The results obtained show that kohlrabi flesh extract is less cytotoxic than cabbage leaves extract when treating colorectal cancer lines H-T29 and Caco-2 cells. Consequently, the IC-50 of both kohlrabi and cabbage ethanolic extracts were determined. The IC-50 is the concentration of an inhibitor at which 50% of the cells remain viable. In HT-29 cells the IC-50 of cabbage was determined to be at 50 $\mu\text{g}/\mu\text{l}$ compared to 70 $\mu\text{g}/\mu\text{l}$ in Caco-2 cells. On the other hand, the IC-50 for Kohlrabi was calculated to be 80 $\mu\text{g}/\mu\text{l}$ for HT-29 cells and much higher for Caco-2 cells. Accordingly, we used the maximal concentration of 40 $\mu\text{g}/\mu\text{l}$ throughout the remaining experiments in order to examine and compare the extracts, while ensuring the presence of a considerable number of viable cells post-treatment. Additionally, cell proliferation was assessed using WST-1 assay in order to evaluate the effect of the high concentrations of these extracts on the survival of metabolically active cells. After treating HT-29 cells with 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi for 24h, the proliferation of HT-29 cells decreased to 10.5% compared to non-treated cells, while upon treatment with cabbage, only 1.8% of the cells remained metabolically active. However, in Caco-2 cells, the proliferation decreased to 11% when treating with 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi while it was reduced to 2% with the same concentration of cabbage. Similarly, the proliferation further decreased after treating HT-29 cells for 48h with both extracts reaching 8% with kohlrabi and 1.5% with cabbage, while the proliferation of Caco-2 cells decreased to 8% with kohlrabi and to 1% with cabbage. Consequently, we observe that kohlrabi extract is less antiproliferative than cabbage when treating HT-29 and Caco-2 cells. A possible explanation for this observed difference would be the different bioactive compounds present in these extracts. Ciska et al. (2000) reported that glucobrassicin, glucoiberin, and sinigrin were the dominant glucosinolates in all *B. oleracea* species, except for kohlrabi, while kohlrabi was discernible by an elevated content of glucoerucin and glucoraphanin. Glucobrassicin is a bioactive compound found in significant amounts in cabbage at a concentration of 35.84mg/100g (Sarkamı et al. 2010; Ciska et al. 2000), compared

to the concentration of 4.83mg/100g found in kohlrabi (Ciska et al. 2000; Gawęda & Nizioł-Lukaszewska, 2011). Glucobrassicin is hydrolyzed to I3C which in its turn is converted to DIM. DIM showed a dose dependent cytotoxicity effect on HT-29 cells where 10 – 30 $\mu\text{mol/L}$ were reported to decrease the proliferation of HT-29 and HCT-116 human colon cancer cells without affecting the viability of normal small intestinal epithelial cells (IEC-6 cells) (Gamet- Payraastre et al. 1998). Further, based on Cartea et al. (2000) and Sarıkamı et al. (2010), glucosinolate sinigrin, precursor of AITC, is found at a lower concentration in kohlrabi (0.77mg/100g) compared to cabbage (22.7mg/100g) (Ciska et al. 2000). AITC treatment caused HT-29 cells to round-up 7 h post-treatment and consequently to detach from the bottom of the flask. This morphological feature and detachment indicate a decrease in cell proliferation (Verhoeven, Goldbohm, Van Poppel, Verhagen & Van den Brandt, 1996). Thus these differences in the concentrations of DIM and sinigrin may be the reason behind the lower cytotoxic and anti-proliferative effect of kohlrabi compared to cabbage on colorectal cancer cell lines.

The decreases observed in the rate of proliferation of the cells upon treatment with different extracts could be due to either an increase in cell death/apoptosis, or an induction of cell cycle arrest at different point intervals of the cell cycle, or both. Thus we aimed to check for cell cycle arrest induction by analyzing DNA content, where the G0/G1 phase is 2n, the S-phase cells is greater than 2n but less than 4n, while M phase cells is 4n. The percentage of cells in the different phases reflects the progression of the cell cycle. DNA content analysis of nontreated HT-29 cells revealed the presence of 42.8% of the cells in the G0/G1 phase, 13.9% in the S phase and 28.1% in the M phase where cells are undergoing mitosis.

When HT-29 cells were treated with 10 $\mu\text{g}/\mu\text{l}$ of cabbage for 24h, we observed an increase in the S phase and a decrease in the M phase compared to the nontreated cells, the result implies an S/M checkpoint and an arrest at the S phase. Upon treatment with 20 $\mu\text{g}/\mu\text{l}$ of cabbage, we detected a remarkable increase at the G phase, a slight increase at the S phase and a significant decrease at the M phase. This result suggests arrests at the G phase and S phase. On the other hand, when HT-29 cells were treated with 40 $\mu\text{g}/\mu\text{l}$ of cabbage, we observed a significant increase at the G phase and a considerable decrease at the M phase. The highest concentration used seemed to induce an arrest at the G phase. However, when HT-29 cells were treated with 10 $\mu\text{g}/\mu\text{l}$ of kohlrabi, an increase in the G and S phases, and a major decrease in

the M phase were observed. This result entails a G arrest and an S/M checkpoint. Whereas, when HT-29 cells were treated with 20 $\mu\text{g}/\mu\text{l}$ and 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi extracts, we noticed similar patterns of cell cycles to those of cells treated with cabbage, yet with lower peaks. The 20 $\mu\text{g}/\mu\text{l}$ concentration of kohlrabi induced an arrest at the G and S phases, while the 40 $\mu\text{g}/\mu\text{l}$ concentration of kohlrabi induced an arrest at G phase. The cell cycle analysis of HT-29 cells treated with kohlrabi and cabbage extracts seems to be dose dependant in inducing cell cycle arrest. The low concentrations of 10 $\mu\text{g}/\mu\text{l}$ and 20 $\mu\text{g}/\mu\text{l}$ provoke an arrest at the S phase and S/M checkpoint while the high concentration of 40 $\mu\text{g}/\mu\text{l}$ induces an arrest at the G phase. The results obtained after treating the cells with kohlrabi extracts are in accordance with the abundance of the bioactive compounds, SFN and erucin, found in that plant. Studies of SFN and erucin showed their induction of cell cycle arrests in HT-29 and Caco-2 cells. SFN found in kohlrabi at a concentration of 8.73mg/100mg compared to cabbage 0.26mg/100mg (Ciska et al. 2000) induced cell arrest at the G1 phase in HT-29 cells causing an increment in p21^{CIP1}, and a reduction in cyclin D1, cyclin A, and c-myc (Shen et al. 2006). While in Caco-2 cells, SFN induced cycle arrest at G2/M phase at a concentration of 20 μM ; when the concentration increased to >20 μM , an accumulation at sub-G1 was observed accompanied with loss of the mitochondrial membrane, elucidating the pro-apoptotic effect of SFN in Caco-2 cells (Jakubikova et al. 2005). Further, SFN induces the expression of p21 protein cycle inhibitor in HT-29 and Caco-2 cells (Parnaud et al. 2004). Additionally, erucin found in kohlrabi at a concentration of 4.48mg/100g compared to traces in cabbage increased the percentage of the subG1 phase in Caco-2 cells (Jakubikova et al. 2005). On the other hand, the induction of cell cycle arrest by cabbage seems to be due to the presence of the high concentrations of AITC, DIM and PEITC bioactive compounds. Studies have shown that 12mM of AITC arrested HT-29 cells in G2/M phase after 3h of treatment (Smith et al. 2004). DIM was found to induce G1 and G2/M phase cell cycle arrest in HT-29 cells, and this arrest was correlated to the reduced activity of cyclin A, cyclin D1 cyclin B1, CDK4, CDK2 and the upregulation of protein cycle inhibitors p21^{CIP1/WAF1} and p27^{KIP1} (Choi et al. 2009). Further, gluconastrutin precursor of PEITC found at a concentration of 0.42mg/100g in cabbage compared to 0.12mg/100mg in kohlrabi (Ciska et al. 2000) caused cell cycle arrest at the G1 phase in HT-29 cells through the downregulation of cyclins A, D, and E (Cheung et al. 2008). The findings about the concentrations of

glucosinolates, precursors of AITC, BITC, PEITC, SFN, DIM and erucin, in cabbage is 89.85mg/100g compared to the concentration of 37.2mg/100g of total glucosinolates in kohlrabi (Ciska et al. 2000). These differences may explain why cabbage appears to provoke cell cycle arrest at G0/G1 phase and S/M phase with higher peaks than those displayed by kohlrabi. However, to confirm those results and elucidate the mechanisms involved, it is important to check for the expressions, regulations and ratios of the protein cycle inhibitors p21 and p27, cyclins A, B1, D and E, and CDK4 and CDK2 in treated cells with both extracts.

In addition, we needed to verify that the dead cells observed using Trypan Blue exclusion method died of apoptosis and not necrosis. Therefore, we tested the treated cells using Cell Death ELISA Kit which is based on the quantification of the hallmark of apoptosis, histone-complexed DNA fragments. The results obtained showed that HT-29 cells treated with 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi for 24 and 48h only had 2 fold increase in apoptosis, and 1 fold and 2 fold increase when treating cells with 40 $\mu\text{g}/\mu\text{l}$ of cabbage for 24 and 48h respectively. Results obtained upon treatment of Caco-2 cells with the different extract showed less pro-apoptotic effect; 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi and cabbage separately only induced 1 fold increase 24h post-treatment and 1.7 fold increase after 48h. These results were not as expected since several epidemiological studies continue to show that brassicas vegetables rich in ITCs such as cabbage report apoptosis induction in cancer cells in vitro (Fimognari et al. 2004). Therefore, we recurred to a more reliable method than the colorimetric assay of Cell death ELISA, and used flow cytometry Annexin V-FITC/PI double staining method to evaluate the pro-apoptotic effect of kohlrabi and cabbage extracts. In this technique, apoptotic cells expose their PS residues at an early stage in apoptosis and bind to the ligand annexin, while necrotic cells reveal their PS residues and lose the integrity of their membrane concurrently and right after cell damage. This damage allows PI to bind to fragmented DNA (Mukherjee et al. 2010). This double staining technique reveals the percentage of cells in early and late apoptosis. The results obtained show that 40 $\mu\text{g}/\mu\text{l}$ of kohlrabi induced a lower early apoptotic rate of 15.7% compared to 22.8% induced by the same concentration of cabbage 24 h after treating HT-29 cells. The literature demonstrates the ability of ITCs to induce apoptosis mainly through the intrinsic pathway whereby cytochrome c released from the mitochondria activates APAF1 and cleaves procaspase 9 molecules triggering the caspase cascade. Apoptosis induction is also accomplished by the regulation of the

MAPK signaling and continuous activation of caspases, Bcl-2 family and PARP cleavage (Traka & Mithen, 2009). Results obtained in this study indicate that apoptosis induction upon cabbage treatment is higher compared to kohlrabi: this may be due to AITC, PEITC, DIM, SFN and erucin collectively found in higher concentrations in cabbage than in kohlrabi. AITC induces apoptosis in HT-29 cells by activating caspase-3 and caspase-8 (Choi et al. 2009), and 82% of nonadherent HT-29 treated cells are left unstained when using Annexin V-FITC/PI double staining method suggesting they are undergoing apoptosis (Smith et al. 2004). PEITC molecules activated caspase 3 and 9 followed by nuclear condensation and DNA disintegration, and three MAPKs, JNK, ERK and p38 kinase post treatment in HT29 cells (Keum et al. 2004). Additionally, PEITC displayed a minor increase in the Bax:Bcl-xL and the Bak:Bcl-xL ratios in 40-16 colon cancer (Pappa et al. 2006). On the other hand, SFN high concentration in kohlrabi may have induced apoptosis in HT-29 cells. HT-29 cells treated with SFN released cytochrome c and increased PARP cleavage, and activated MAPKs, JNK, ERK and p38 kinase post treatment (Shen et al. 2006). SFN also showed the induction of the proapoptotic protein Bax in HT-29 colon cells (Gamet-Payraastre et al. 2000). Further, studies on 40-16 colon cancer cells confirmed that 15 μ M of SFN led to programmed cell death by increasing the Bax to Bcl-xL and the Bak to Bcl-xL ratios (Gamet-Payraastre et al. 2000). Additionally, erucin was found to induce apoptosis in Caco-2 cells through diverse kinases, such as MAPKs and PI3K (Jakubikova et al. 2005). Therefore, based on the previous findings of ITCs and apoptosis, we aimed to evaluate the expression levels of Bax and Bcl-2 proteins involved in the apoptotic pathway. The western blot analysis of HT-29 cells treated with cabbage and kohlrabi for 24h showed an upregulation of the proapoptotic protein Bax and a downregulation of the antiapoptotic protein Bcl-2 in all of the concentrations used. The ratio of Bax/Bcl-2 was calculated and showed an increase as the concentrations of both cabbage and kohlrabi extracts increased; it also showed the ratio of Bax/Bcl-2 upon cabbage treatment to be slightly higher than the ratio induced by kohlrabi. These outcomes were in agreement with the results of flow cytometry. However, further confirmation and comparison of apoptosis could be done by evaluating the expression levels of caspases 3, 8 and 9, cytochrome c and PARP cleavage in treated cells. These results may be due to the high concentrations of AITC and PEITC in cabbage compared to their concentration in kohlrabi. A study performed on HT-29 cells has

shown that the potency of AITC is greater than that of PEITC which are even greater than SFN in reducing the cell number of adherent cells (Lund et al. 2001).

Therefore, to further compare the antiproliferative and pro-apoptotic effect of kohlrabi and green cabbage, we suggest in the future work to subfractionate the extracts and determine the nature and exact concentrations of the found glucosinolates. In addition, the antiproliferative mechanism of each extract may be elucidated after evaluating the expression levels of p21 and p27, cyclins A, B1, D and E, and CDK4 and CDK2 in treated cells, whereas the proapoptotic pathway may be clarified after checking the expression levels of caspases 3, 8 and 9, cytochrome c and PARP cleavage.

In conclusion, all of our results show that kohlrabi extracts is antiproliferative and apoptotic in HT-29 cells, however, cabbage is more potent in reducing proliferation and inducing apoptosis in colorectal cancer cell lines. Nevertheless, kohlrabi should be considered, like cabbage, among the potent anti-carcinogenic cruciferous vegetables.

Bibliography

- Aboud-Pirak, E., Hurwitz, E., Pirak, M.E., Bellot, F., Schlessinger, J., & Sela, M. (1988). Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma in vitro and in nude mice. *J Natl Cancer Inst*, 80(20), 1605-1611.
- Aiello, M., Vella, N., Cannavo, C., Scalisi, A., Spandidos, D. A., Toffoli, G., ... Stivala, F. (2011). Role of genetic polymorphisms and mutations in colorectal cancer therapy (Review). *Mol Med Report*, 4(2), 203-208.
- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). The cell cycle and programmed cell death. In A. Johnson & M. Raff (Eds.), *Molecular biology of the cell*, (pp. 212-214). New York: Garland Science.
- Alroy, I., & Yarden, Y. (1997). The ErbB signaling network in embryogenesis and oncogenesis: Signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett*, 410(1), 83-86.
- Ambrosone, C.B., McCann, S.E., Freudenheim, J.L., Marshall, J.R., Zhang, Y., & Shields, P.G. (2004). Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. *J Nutr*, 134, 1134–1138.
- Ambrosone, C.B., & Tang, L. (2009). Cruciferous vegetable intake and cancer prevention: role of nutrigenetics. *Cancer Prev Res (Phila Pa)*, 2(4), 298-300.
- Ames, B.N., Gold, L.S., & Willett, W.C. (1995). The causes and prevention of cancer. *Proceedings of the National Academy of Sciences of USA*, 92, 5265–5268.

- Arikawa, Y., & Gallaher, D. (2008). Cruciferous vegetables reduce morphological markers of colon cancer risk in dimethylhydrazine-treated rats. *J. Nutr.* *138*, 526–532.
- Bodnaryk, R.P. (1994). Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry*, *35*, 301-305.
- Boivin, D., Lamy, S., Lord-Dufour, S., Jackson, J., Beaulieu, E., Côté, M., ... Béliveau, R. (2009). Antiproliferative and antioxidant activities of common vegetables: A comparative study. *Food Chemistry*, *112*, 374–380.
- Booth, E.J., & Walker, K.C. (1992). The effect of site and foliar sulfur on oilseed rape: Comparison of sulfur responsive and non-responsive seasons. *Phyton*, *32*, 9-13.
- Bostick, R.M., Fosdick, L., Grandits, G.A., Lillemoe, T.J., Wood, J.R., Grambsch, P., ... Potter, J.D. (1997). Colorectal epithelial cell proliferative kinetics and risk factors for colon cancer in sporadic adenoma patients. *Cancer Epidemiol Biomarkers Prev*, *6*, 1011–1019.
- Bradley, F.M., Ellis, B.W., & Martin, D.L. (Eds.). (2009). *The organic gardener's handbook of natural pest and disease control*. N.p.: Rodale Inc.
- Center, M.M., Jemal, A., Smith, R.A., & Ward, E. (2009). Worldwide variations in colorectal cancer. *CA Cancer J Clin*, *59*(6), 366-378.
- Cartea, M.E., & Velasco, P. (2008). Glucosinolates in Brassica foods: Bioavailability in food and significance for human health. *Phytochem Rev*, *7*, 213–229.
- Chan, T.O., Rittenhouse, S.E., & Tschlis, P.N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: Kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem*, *68*, 965-1014.

- Chen, C., & Kong, A.N. (2005). Dietary cancer-chemopreventive compounds: From signaling and gene expression to pharmacological effects. *Trends Pharmacol Sc*, 26(6), 318–26.
- Cheung, K.L., & Kong, A. (2010). Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. *The AAPS Journal*, 12(1), 87-97.
- Choi, H.J., Lim, D.Y., & Park, J.H.Y. (2009). Induction of G1 and G2/M cell cycle arrests by the dietary compound 3,3'-diindolylmethane in HT-29 human colon cancer cells. *BMC Gastroenterology*, 9, 39.
- Clarke, J.D., Dashwood, R.H., & Hoa, E. (2008). Multi-targeted prevention of cancer by sulforaphane. *Cancer Lett*, 269(2), 291–304.
- Daniel, C.R., Bostick, R.M., Flanders, W.D., Long, Q., Fedirko, V., Sidelnikov, E., & Seabrook, M.E. (2009). TGF- α expression as a potential biomarker of risk within the normal-appearing colorectal mucosa of patients with and without incident sporadic adenoma. *Cancer Epidemiol Biomarkers Prev*. 18(1), 65–73.
- Delahaut, K.A. & Newenhouse, A.C. (1997). *Growing broccoli, cauliflower, cabbage and other cole crops in Wisconsin*. Wisconsin: University of Wisconsin.
- De Roock, W., Claes, B., Bernasconi, D., De Schutter, J., Biesmans, B., Fountzilas, G., ... Tejpar, S. (2010). Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: A retrospective consortium analysis. *Lancet Oncology*, 11, 753–762.
- Egert, S., Somoza, V., Kannenberg, F., Fobker, M., Krome, K., Erbersdobler, H.F., & Wahrburg, U. (2007). Influence of three rapeseed oil-rich diets, fortified with alpha-linolenic acid, eicosapentaenoic acid or docosahexaenoic acid on the composition and oxidizability of low-density lipoproteins: Results of a controlled study in healthy volunteers. *Eur J Clin Nutr*, 61(3), 314-325.

- Ellis, M.L., Takahashi, Y., Liu, W., & Shaheen, R.M. (2000). Vascular endothelial growth factor in human colon cancer: Biology and therapeutic implications. *The Oncologist*, 5(1), 11-15.
- Engebraaten, O., Bjerkvig, R., Pedersen, P.H., & Laerum, O.D. Effects of EGF, bFGF, NGF and PDGF(bb) on cell proliferative, migratory and invasive capacities of human brain-tumour biopsies in vitro. *Int J Cancer*, 53(2), 209-214.
- Fahey, J.W., & Stephenson, K.K. (1999). Cancer chemoprotective effects of cruciferous vegetables. *HortScience*, 34, 4-8.
- Fahey, J.W., Zalcmann, A.T., & Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*. 56, 5–51.
- Farag, M.A., & Abdel Motaal, A. (2010). Sulforaphane composition, cytotoxic and antioxidant activity of crucifer vegetables. *Journal of Advanced Research*, 1, 65–70.
- Fimognari, C., Nusse, M., Berti, F., Iori, R., Cantelli-Forti, G., & Hrelia, P. (2004). Isothiocyanates as novel cytotoxic and cytostatic agents: Molecular pathway on human transformed and nontransformed cells. *Biochem. Pharmacol*, 68, 1133-1138.
- Fowke, J.H., Chung, F.L., Jin, F., Qi, D., Cai, Q., Conaway, C., ... Zheng, W. (2003). Urinary isothiocyanate levels, brassica, and human breast cancer. *Cancer Res*, 63, 3980–3986.
- Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M.A., Chevolleau, S., ... Terce, F. (2000). Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res*, 60, 1426–1433.
- Gamet- Payrastre, L., Lumeau, S., Gasc, N., Cassar, G., Rollin, P., & Tulliez J. (1998). Selective cytostatic and cytotoxic effects of glucosinolates hydrolysis

products on human colon cancer cells in vitro. *Anti-cancer drugs*, 9(21), 41-148.

Gawęda, M., & Nizioł-Łukaszewska, Z. (2011). Quality of kohlrabi stems (*Brassica oleracea* var. *gongylodes* L.) kept in cold storage. *Folia Horticulturae*, 23(2), 107-110.

Gibson, T.B., Ranganathan, A., & Grothey, A. (2006). Randomized phase III trial results of panitumumab, a fully human anti-epidermal growth factor receptor monoclonal antibody, in metastatic colorectal cancer. *Clin Colorectal Cancer*, 6(1), 29-31.

Giovannucci, E. (2002). Modifiable risk factors for colon cancer. *Gastroenterol Clin North Am*, 31(4), 925-943.

Giovannucci, E., Rimm, E.B., Liu, Y., Stampfer, M.J., & Willett, W.C. (2003). A prospective study of cruciferous vegetables and prostate cancer. *Cancer Epidemiol Biomarkers Prev*, 12, 1403-1409.

Gross, A., McDonnell, J.M., & Korsmeyer, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*, 1, 1899-1911.

Hansson, L.E., Nyren, O., Bergstrom, R., Wolk, A., Lindgren, A., Baron, J., & Adami, H.O. (1993). Diet and risk of gastric cancer. A population-based case-control study in Sweden. *Int J Cancer*, 55, 181-189.

Hecht, S.S. (1999). Chemoprevention of cancer by isothiocyanates, modifiers of carcinogen metabolism. *J Nutr*, 129, 768s-774s.

Holst, B., & Williamson, G. (2004). A critical review of the bioavailability of glucosinolates and related compounds. *Nat. Prod. Rep*, 21, 425-447.

Hong, C., Kim, H.A., Firestone, G.L., & Bjeldanes, L.F. (2002). 3, 3'-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast

cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. *Carcinogenesis*, 23(8), 1297-1305.

- Ingram, C. (2000). *The cook's guide to vegetables*. New York: Hermes House.
- Iqbal, S., & Lenz, H. (2004). Integration of novel agents in the treatment of colorectal cancer. *Cancer Chemother Pharmacol*, 54(1), s32-s39.
- Jakubikova, J., Sedlak, J., Mithen, R., & Bao, Y. (2005). Role of pi3k/akt and mek/erk signaling pathways in sulforaphane- and erucin-induced phase ii enzymes and mrp2 transcription, g2/m arrest and cell death in caco-2 cells. *Biochem Pharmacol*, 69, 1543–1552.
- Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., & Thun, M. J. (2009). Cancer statistics, 2009. *CA Cancer J Clin*, 59(4), 225-249.
- Jeong, W.S., Kim, I.W., Hu, R., & Kong, A.N. (2004). Modulation of AP-1 by natural chemopreventive compounds in human colon HT-29 cancer cell line. *Pharm. Res*, 21, 649–660.
- Jonker, D.J., O'Callaghan, C.J., Karapetis, C.S., Zalberg, .JR., Tu, D., Au, H.J., ... & Moore, M.J. (2007), Cetuximab for the treatment of colorectal cancer. *N Engl J Med*, 357(20), 2040-2048.
- Katz, S.H., & Weaver, W.W. (2003). *Encyclopedia of food and culture*. New York: Scribner.
- Kahlon, T.S., Chiu, M.C., & Chapman, M.H. (2008). Steam cooking significantly improves in vitro bile acid binding of collard greens, kale, mustard greens, broccoli, green bell pepper, and cabbage. *Nutr Res*, 28(6), 351-357.
- Keum, Y-S., Jeonga, W-S., & Konga, A.N.T. (2004). Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. *Mutation Research*, 555, 191–202.

- Kirsh, V.A., Peters, U., Mayne, S.T., Subar, A.S., Chatterjee, N., Johnson, C.C., & Haye, R.R. (2007). Prospective study of fruit and vegetable intake and risk of prostate cancer. *J Natl Cancer Inst*, 99, 1200–1209.
- Krasinskas, A. (2011). EGFR signaling in colorectal carcinoma. *Pathology Research International*, 2011. Doi:10.4061/2011/932932.
- Kufe, D., Pollock, R., Weichselbaum, R., Bast, R., Gansler, T., Hollad, J., & Frej, E. (2003). *Holland frei cancer medicine* (6th ed.). Hamilton, Ontario: BC Decker.
- Lam, T., Gallicchio, L., Lindsley, K., Shiels, M., Hammond, E., Tao, X., ... Alberg, A.J. (2009). Cruciferous vegetable consumption and lung cancer risk: A systematic review. *Cancer Epidemiol Biomarkers Prev*, 8(1), 184–95.
- Laszlo, L. (2010). Predictive and prognostic factors in the complex treatment of patients with colorectal cancer. *Magy Onkol*, 54(4), 383-394.
- London, S.J., Yuan, J., Chung, F., Gao, T., Coetzee, G.A., Ross, R.K., & Yu, M.C. (2000). Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: A prospective study of men in Shanghai, China. *Lancet*, 356, 724–729.
- Loupakis, F., Ruzzo, A., Cremolini, C., Vincenzi, B., Salvatore, L., Santini, D., ... Graziano, F. (2009). KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. *British Journal of Cancer*, 101(4), 715–721.
- Lund, E.K., Smith, T.K., Clarke, R.G., & Johnson, I.T. (2001). Cell death in the colorectal cancer cell line HT-29 in response to glucosinolate metabolites. *Journal of the Science of Food and Agriculture*, 81(9), 959–961.
- Maalouf, K., Baydoun, E., & Rizk, S. (2011). Kefir induces cell-cycle arrest and apoptosis in HTLV-1-negative malignant T-lymphocytes. *Cancer Manag Res*, 3, 39-47.
- Macleod, G., & Macleod, A. (1990). The glucosinolates and aroma of volatiles of green kohlrabi. *Phytochemistry*, 29(4), 1183-1187.

- Mahéo, K., Morel, F., Langouët, S., Kramer, H., Le Ferrec, E., Ketterer B, & Guillouzo, A. (1997). Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. *Cancer Res*, 57, 3649–3652.
- Manson, M. M. (2003). Cancer prevention – the potential for diet to modulate molecular signalling. *Trends Molecular Medicine*, 9, 11–18.
- Melchini, A., & Traka, M.H. (2010). Biological profile of erucin: A new promising anticancer agent from cruciferous vegetables. *Toxins*, 2, 593-612.
- Mukherjee, A., Dutta, S., Shanmugavel, M., Mondhe, D.M., Sharma, P.R, Singh, S.K., ... Sanya, U. (2010). 6-Nitro-2-(3-hydroxypropyl)-1H-benz[de]isoquinoline- 1,3-dione, a potent antitumor agent, induces cell cycle arrest and apoptosis. *Journal of Experimental & Clinical Cancer Research*, 29, 175.
- Nakamura, Y., & Miyoshi, N. (2006). Cell death induction by isothiocyanates and their underlying molecular mechanisms. *BioFactors*, 26, 123–134.
- Pappa, G., Lichtenberg, M., Iori, R., Barillari, J., Bartsch, H., & Gerhäuser, C. (2007). Comparison of growth inhibition profiles and mechanisms of apoptosis induction in human colon cancer cell lines by isothiocyanates and indoles from *Brassicaceae*. *Mutation Research*, 599, 76–87.
- Parnaud, G., Li, P., Cassar, G., Rouimi, P., Tulliez, J., Combaret, L., & Gamet-Payrastre, L. (2004). Mechanism of sulforaphane-induced cell cycle arrest and apoptosis in human colon cancer cells. *Nutr Cancer*, 48, 198–206.
- Petit, A.M., Rak, J., Hung, M.C., Rockwell, P., Goldstein, N., Fendly, B., & Kerbel, R.S. (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: Angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol*, 151(6), 1523-1530.
- Ponz de Leon, M. (2002). *Colorectal cancer*. New York: Springer-Verlag Berlin Heidelberg.

- Saltz, L.B., Meropol, N.J., Loehrer, P.J.Sr., Needle, M.N., Kopit, J., & Mayer, R.J. (2004). Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol*, *22*, 1201.
- Sarıkamı, G., Balkaya, A., & Yanmaz, R. (2009). Glucosinolates within a collection of white head cabbages (*Brassica oleracea* var. *capitata* sub.var. *alba*) from Turkey. *African Journal of Biotechnology*, *8*(19), 5046-5052.
- Seow, A., Quah, S.R., Nyam, D., Straughan, P., Chua, T., & Aw, T.C. (2002). Food groups and the risk of colorectal carcinoma in an Asian population. *Cancer*, *95*(11), 2390-2396.
- Shen, G., Xu, C., Chen, C., Hebbar, V., & Kong, A.N. (2006). P53-independent G1 cell cycle arrest of human colon carcinoma cells ht-29 by sulforaphane is associated with induction of p21cip1 and inhibition of expression of cyclin d1. *Cancer Chemotherapy and Pharmacology*, *57*, 317–327.
- Shorey, L.E., Hagman, A.M., Williams, D.E., Ho, E., Dashwood, R.H, & Benninghoff, D. (2012). 3'-Diindolylmethane induces G₁ arrest and apoptosis in human acute T-cell lymphoblastic leukemia cells. *PLoS ONE*, *7*(4). Doi:10.1371/journal.pone.0034975.
- Smith, T.K., Lund, K.L., Clarke, R.G., Bennet, R.G., & Johnson, I.T. (2004). Effects of brussels sprout juice on the cell cycle and adhesion of human colorectal carcinoma cells (HT-29) in vitro. *J. Agric. Food Chem*, *53*, 3895-3901.
- Spitz, M.R., Duphorne, C.M., Detry, M.A., Pillow, P.C., Amos, C.A., Lei, L., ... Wu, X. (2000) Dietary intake of isothiocyanates: Evidence of a joint effect with glutathione S-transferase polymorphisms in lung cancer risk. *Cancer Epidemiol Biomarkers Prev*, *9*, 1017–1020.
- Srivastava, S., Lippman, S.M., Hong, W.K., & Mulshine, J.L. (1994). *Early detection of cancer: Molecular markers*. Armonk (NY): Futura Publishing Company, Inc.
- Surh, Y.J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer*, *3*, 768–780.

- Traka, M., & Mithen R. (2009). Glucosinolates, isothiocyanates and human health. *Phytochem Rev*, 8, 269–282.
- Uauy, R., & Solomons N. (2005). Diet, nutrition, and the life-course approach to cancer prevention. *J Nutr*, 135, 2934S-45S.
- Van Breda, S.G.J., de Kok, T.M.C.M., & Van Delft, J.H.M. (2008). Mechanisms of colorectal and lung cancer prevention by vegetables: A genomic approach. *Journal of Nutritional Biochemistry*, 19, 139–157.
- Van Poppel, G., Verhoeven, D.T., Verhagen, H., & Goldbohm, R.A. (1999). Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Adv Exp Med Biol*, 472, 159–168.
- Vargas, A.J., & Thompson, P.A. (2012). Diet and nutrient factors in colorectal cancer risk. *Nutr Clin Pract*, 27(5), 613-623.
- Verhoeven, D.T.H., Goldbohm, A., Van Poppel, G., Verhagen, G., & Van den Brandt, P. (1996). Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiology, Biomarkers & Prevention*, 5, 733-748.
- Wang, L.I., Giovannucci, E.L., Hunter, D., Neuberg, D., Su, L., & Christiani D.C. (2004). Dietary intake of cruciferous vegetables, glutathione S-transferase (GST) polymorphisms and lung cancer risk in a Caucasian population. *Cancer Causes Control*, 15, 977–985.
- Wattenberg, L.W., & Loub, W.D. (1978). Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res*, 38(5), 10-1413.
- Wu, X., Zhou, Q-H., & Xu, K. (2009). Are isothiocyanates potential anti-cancer drugs? *Acta Pharmacol Sin*, 30(5), 501–512.
- Wu, X., Zhu, Y., Yan, H., Liu, B., Li, Y., Zhou, Q., & Xu, K. (2010). Isothiocyanates induce oxidative stress and suppress the metastasis potential of human non-small cell lung cancer cells. *BMC Cancer*, 10, 269.

- Xiao, D., Johnson, C.S., Trump, D.L., & Singh, S.V. (2004). Proteasome-mediated degradation of cell division cycle 25C and cyclin-dependent kinase 1 in phenethyl isothiocyanate-induced G2-M-phase cell cycle arrest in PC-3 human prostate cancer cells. *Mol Cancer Ther*, 3, 567–75.
- Xu, C., Yuan, X., Pan, Z., Shen, G., Kim, J.H., Yu, S., ... Kong, A.N. (2006). Mechanism of action of isothiocyanates: The induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. *Mol Cancer Ther*, 5, 1918–1926.
- You, Y., Wu, Y., Mao, J., Zou, L., & Liu, S. (2008). Screening of Chinese brassica species for anti-cancer sulforaphane and erucin. *African Journal of Biotechnology*, 7(2), 147-152.
- Zhao, H., Lin, J., Grossman, H.B., Hernandez, L.M., Dinney, C.P., & Wu, X. (2007). Dietary isothiocyanates, GSTM1, GSTT1, NAT2 polymorphisms and bladder cancer risk. *Int J Cancer*, 120, 2208–2213
- Zhang, Y., Tang, L., & Gonzalez, V. (2003). Selected isothiocyanates rapidly induce growth inhibition of cancer cells. *Mol Cancer Ther*, 2, 1045–52.
- Zhang, Y., Yao, S., & Li, J. (2006). Vegetable-derived isothiocyanates: Anti-proliferative activity and mechanism of action. *Proceedings of the Nutrition Society*, 65, 68–75.
- Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K.P., & Gottlicher, M. (2004). Induction of hdac2 expression upon loss of apc in colorectal tumorigenesis. *Cancer Cell*, 5, 455–463.