INVESTIGATION ON THE EFFECT OF FIG AND DATE VINEGAR ON HT-29 HUMAN COLON CANCER CELLS

by

RANA AHMED MAHMOUD EL KADI

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LIST OF SYMBOLS AND ABBREVIATIONS

% – percentage

°C – degree celcius

µl – microliter

ANOVA – analysis of variance

cm^3 – cubic centimeter

CO_2 – carbon dioxide

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

FBS – fetal bovine serum

g – gram

h – hour

HeLa – human cervical adenocarcinoma cell line

HPLC – high performance liquid chromatography

HPTLC – high performance thin layer chromatography

HT-29 – human colorectal adenocarcinoma cell line

IC_{50} – half maximal inhibitory concentration

ml – milliliter

mM – milimolar

mm – milimeter

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

nm – nanometer

OD – optical density

PBS – phosphate buffer saline

PCR – polymerase chain reaction

SD – standard deviation

SPSS – Statistical Package for the Social Sciences

SRB – sulforhodamine B

TCA – trichloroacetic acid

USA – United States of America

KAJIAN TENTANG KESAN CUKA ARA DAN KURMA KE ATAS HT-29 SEL BARAH KOLON MANUSIA

ABSTRAK

Kanser kolon disenaraikan sebagai punca kedua kematian selepas kanser payudara. Walaupun rawatan canggih dengan teknologi terkini telah digunakan untuk merawat pesakit kanser kolon, kadar insiden dan kematian masih meningkat dengan kadar yang membimbangkan. Oleh itu, produk semula jadi dilihat sebagai pendekatan baru untuk membantu dalam rawatan atau sekurang-kurangnya pencegahan penyakit ini. Kajian terdahulu telah mengenal pasti sifat-sifat anti-kanser daripada ara, kurma dan juga cuka. Dalam kajian ini, kami bertujuan untuk melihat kesan sitotoksik cuka ara dan cuka kurma ke atas sel barah kolon sel. Ujian SRB dilakukan untuk mengira nilai IC_{50} dan menilai kesan sitotoksik cuka-cuka tersebut ke atas sel HT-29. Analisis statistik dilakukan dengan menggunakan ujian ANOVA satu hala. Rawatan cuka ara dan cuka kurma tarikh (ditapis dan tidak ditapis) selama 24, 48 dan 72 jam pada sel HT-29 menyebabkan perencatan pertumbuhan sel yang signifikan dengan nilai IC_{50} yang pelbagai bermula pada 0.013% kepada 0.125% keasidan. Cuka kurma ditapis yang dirawat selama 24 jam mempunyai aktiviti sitotoksik yang terbaik diikuti dengan cuka kurma tidak ditapis dan akhirnya cuka ara. Pada 48 jam inkubasi, cuka kurma ditapis mempunyai aktiviti sitotoksik tertinggi diikuti dengan cuka ara dan cuka kurma tidak ditapis. Lebih 72 jam tempoh inkubasi, cuka kurma tidak ditapis adalah yang paling

berkesan berbanding daripada cuka ara dan cuka kurma ditapis. Kesimpulannya, cuka ara dan cuka kurma memang mempunyai aktiviti sitotoksik terhadap sel HT-29.

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ABSTRACT

Colon cancer is listed as the second cause of death after breast cancer globally. Although advanced treatment with the latest technology has been applied to treat colon cancer patients, the incidence and mortality rate is still increasing alarmingly. Therefore, natural product is seen as a new approach to help in treatment or at least prevention of this disease. Previous studies have identified the anticancer properties of fig, date and also vinegar. In this study, we intended to look at the cytotoxic effect of commercial fig and date vinegar on colon cancer cell line. SRB assay was performed to calculate the IC_{50} value and evaluate the cytotoxicity effect of the above-mentioned vinegars on HT-29 cells. Statistical analysis was performed by using one way ANOVA test. 24, 48 and 72 hours treatment of fig and date (filtered and unfiltered) vinegars on HT-29 cells resulted in significant cell growth inhibition with diverse IC_{50} value ranging from 0.013% to 0.125% acidity. 24 h filtered date vinegar has the best cytotoxic activity followed by unfiltered date vinegar and finally fig vinegar. At 48 h incubation, filtered date vinegar has the highest cytotoxic activity followed by fig vinegar and unfiltered date vinegar. Over 72 h incubation period, unfiltered date vinegar is the most effective than fig vinegar and filtered date vinegar. In conclusion, fig and dates vinegars do have cytotoxic activity against HT-29 cells.

CHAPTER I

INTRODUCTION

1.1 Research Background

Colorectal cancer (CRC) can be considered as an international burden as the fatal consequences of this malignant disease is by far among the most common cause of death these days. The rate of incidence and mortality of CRC are increasing in a scary ways in advanced countries particularly US, European and part of Asian countries including Malaysia. In Malaysia, CRC is the commonest cancer diagnosed among men and the third most common cancer among women (Samat & Abd Shattar, 2014).

Currently, treatment for this cancer includes surgery and a series of chemotherapy. However, chemotherapeutic drugs can induce secondary malignancies (Vega-Stromberg, 2003) and have additional adverse effects due to their inability to distinguish between rapidly proliferating cancer cells and healthy dividing cells.

Due to the potential treatment complications, the researchers are actively looking for natural products. Over the recent years, there has been growing interest in naturally occurring phytochemical compounds with anticancer potential, as they are believed to be safe and effective (Kuppusamy *et al*., 2014).

Fig and date vinegars are commonly consumed especially in Middle Eastern countries. The anticancer properties of fig, date and vinegar have been assessed separately but to

date, there are no study on the effect of fig vinegar and date vinegar on colon cancer cells.

1.2 HYPOTHESIS

Fig and date vinegars potentially have active compounds that could halt the proliferation of HT-29 colon cancer cells.

1.3 OBJECTIVES OF STUDY

To evaluate the growth inhibitory effect of commercial fig vinegar, unfiltered date vinegar and filtered date vinegar on HT-29 cancer cells.

CHAPTER II

LITERATURE REVIEW

2.1 Colorectal Cancer (CRC)

2.1.1 Prevalence

Cancer has been known as one of the most imminent health problems all over the world (Tárraga López *et al.*, 2014; Prabhu *et al.*, 2009). Developing countries are characterised by higher incidence of stomach, cervix, oropharynx and liver cancer, while developed countries have higher incidence of colorectal, breast and prostate cancer (Wiseman, 2008). Colorectal cancer (CRC) is the third leading cause of death in both genders in USA (Rebecca *et al.,* 2014). There is an alarming rate that half of colon cancer patients will die because of this malignant disease (Nagel *et al.*, 2012).

It has a strong relationship with age and gender; 90% of CRC cases occur among people aged fifty and older (Rajamanickam & Agarwal, 2008). Men are more susceptible to have CRC (Tárraga López *et al.*, 2014). In Malaysia, CRC affects 14.2% males, making it the commonest cancer among men. On the other hand, 10.1% females are diagnosed with CRC hence it is declared that CRC is the third most common cancer among women (Samat & Abd Shattar, 2014).

2.1.2 Development of CRC and the risk factors

CRC arises from a clone of cells that escaped normal growth pattern, differentiation and interaction among themselves (Wiseman, 2008). Development of CRC consists of many stages; from normal colonic epithelium to polyps development and finally invasion (Peddareddigari *et al.*, 2010). The polyps may be present as early as ten years before the malignant tissue formation (Cooper *et al.*, 2010). The most dangerous event of CRC is during the invasive stage where it can reach and harm liver, ovary, lung and other gastrointestinal parts (Kuppusamy *et al.*, 2014).

One of the key predictor of colorectal cancer is the inflammatory activity of the stroma (Peddareddigari *et al.*, 2010). Inflammation is considered as a contributor to cancer (Cappellani *et al.*, 2013) hence assessment of the environment of the stroma is vital in understanding the initial stage of tumorigenic effect of CRC (Mclean *et al.*, 2011). Figure 2.1 illustrates the relationship between inflammation, intestinal dysbiosis and colon cancer formation.

CRC is believed to be more predominant in the developed countries than the developing countries, mainly due to modern life style (O'Keefe *et al.*, 2009). There are also many other factors that contribute to the increasing number of CRC incidence such as inherited cancer predisposition, hereditary non-polyps colon cancer, obesity, laziness, alcohol intake and smoking (Ashktorab *et al.*, 2014; Yarnall *et al.*, 2013; Rajamanickam & Agarwal, 2008). There is also a link between CRC and diabetes, high dietary glycemic load, high sugar-sweetened beverage, and obesity. These factors are believed

to have a contribution in the increase of CRC incidence (Fuchs *et al.*, 2014; Huxley *et al.*, 2009; Gunter and Leitzmann, 2006).

Interestingly, approximately 90-95% of CRC case is caused by food intake and life style and little is resulted from genetic cause (Paul *et al.*, 2010). This is because food is interacting directly with alimentary tract thus the colon cancer could highly associate with dietary components that had been taken in (Woo *et al.*, 2013). Dietary components play a crucial role in CRC tumorigenicity, therefore food components is considered as modifiable risk factors (Westergaard *et al.*, 2014; Zhang *et al.*, 2011).

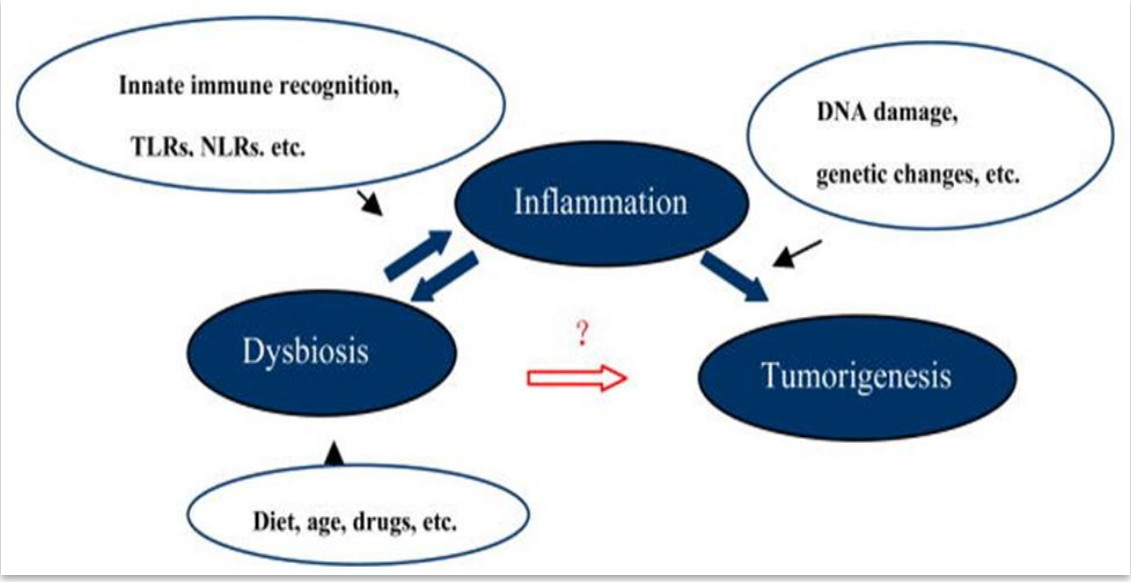

Figure 2.1: Relationship between inflammation occurrence, intestinal dysbiosis and colon cancer formation (Huxley *et al.*, 2009).

2.1.3 Treatment and prevention

Conventional treatments such as chemotherapy, surgery and radiotherapy remain to be the most common approach to treat cancer patients (Kuppusamy *et al.*, 2014). However, despite having the ability to prolong survival among cancer patients, the synthetic chemical anticancer drugs often have off-target effects and many adverse consequences such as nausea, hair loss and vomiting (Kuppusamy *et al.*, 2014).

Primary prevention to absolutely avoid CRC till now remains quite challenging (Qasim & O'Morain, 2010). Thus, secondary and tertiary prevention is very critical in limiting the spread of CRC (Rajamanickam & Agarwal, 2008). Figure 2.2 shows the different preventive strategies applied in poor countries against CRC incidence.

Dietary food intake has the ability to control cancer progression, recurrence occurrence and overall survival of cancer patients (Rock *et al.*, 2012). Based on this, many natural products have been investigated for colon cancer therapeutics for it is believed to be more safe and effective (Kuppusamy *et al.*, 2014).

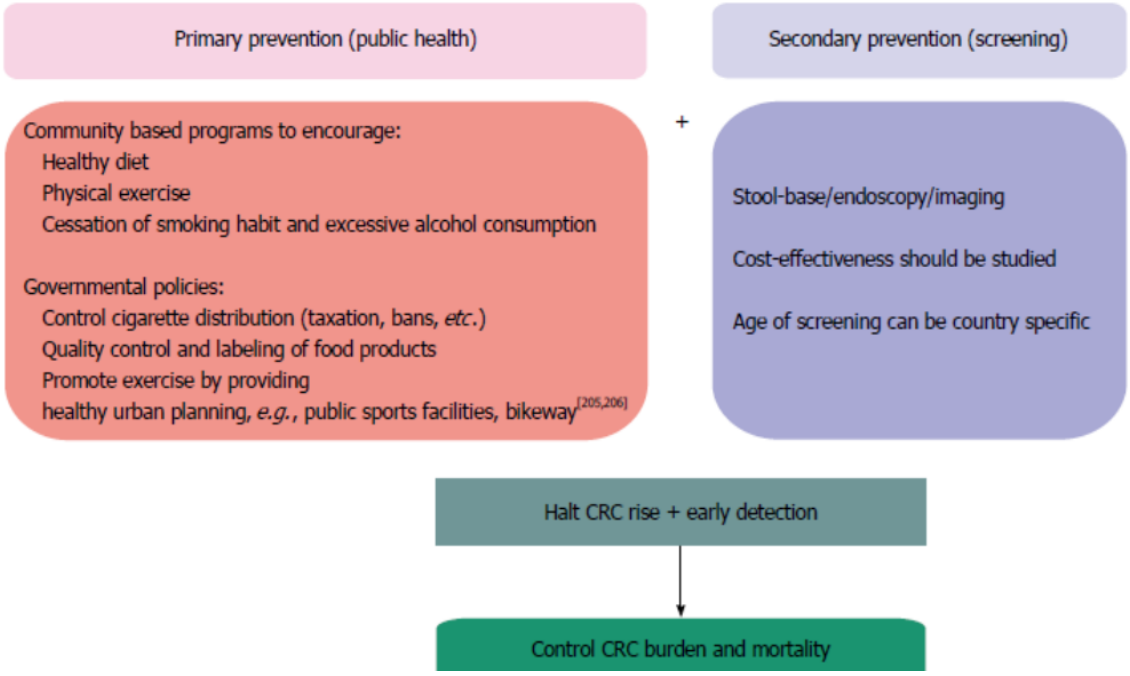

Figure 2.2: The different preventive strategies applied in poor countries against CRC incidence (Bishehsari *et al.*, 2014)

2.1.3.1 Dietary as CRC prevention

Food is involved in the occurrence and prevention of CRC by several biological mechanisms (Williams *et al.*, 2010). Monogenetic study showed that around four out of ten CRC case could be reduced with increasing fiber intake such as whole grains, vegetables and fruits (Kuppusamy *et al.*, 2014; Galas *et al.*, 2013; Kyrø *et al.*, 2013; Aune *et al.*, 2011a; Bravi *et al.*, 2009; Haas *et al.*, 2009). Dietary fiber may increase the bulk of the stool, dilute carcinogens in the lumen of the gastrointestinal tract, and reduce transit time and fermentation of fibers by gut microorganisms (Aune *et al.*, 2011b). Meanwhile, fermentable dietary fibers contribute to healthier and more balanced macronutrient foods (Paturi *et al.*, 2012).

Apart from its potential to stop cancer progression, fruits and vegetable also promote healthy weight management. There are strong recommendations for taking at least 1-2 cups of fruits every day (Rock *et al.*, 2012). Fruits and vegetables also help in modification of inflammatory pathways and carcinogen metabolism (Cappellani *et al.*, 2013). Some natural fruits even have the ability to modify human genes for example the increase expression of certain anti-oxidant genes by apple juice (Soyalan *et al.*, 2011). Apple can also reduce DNA damage in the pre-neoplastic liver cells (Poulsen *et al.*, 2011).

There are many other food constituents that can help to reduce the incidence of CRC such as low fat and calorie intake, high selenium and high calcium content (Tárraga López *et al.*, 2014). Calcium and vitamin D can help against CRC by many mechanisms including induction of apoptosis, limiting proliferation and stimulating cell

differentiation (Zhang *et al.*, 2011; Mizoue *et al.*, 2008; Ishihara *et al.*, 2008; Larsson *et al.*, 2006).

There are also plant-derived polysaccharides that has protective role in the development of colon lesions (Kuppusamy *et al*., 2014). Flavonoid containing diet is also a useful component in decreasing CRC disease (Zamora-Ros *et al.*, 2013; Theodoratou *et al.*, 2007).

Recently, scientists have discovered that the interactions between food and gut microbiota in determining the risk of CRC (Akin & Tözün, 2014) (Figure 2.3). All gut microorganisms; approximately 100 trillion of them in adult gut; are called microbiota (Davis *et al.*, 2009). The microbiota forms a symbiotic relationship with external and internal factors including diet (Zhu *et al.*, 2011). They produce bioactive compounds from the component of food consumed (Davis *et al.*, 2009). They also have the capability to generate new compounds that have both beneficial and harmful effect (Davis *et al.*, 2009). Moreover, they also regulate the proliferation of colonic mucosa, hence, they indirectly becoming the source of CRC protection. The interactions between microbiota, food and the host are basic for the homeostasis of the body system, which any defect of the interaction between those elements can lead to a pathological state (Bernalier-Donadille, 2010).

Microbiota manipulation is becoming the latest strategy to fight CRC (H. Shmuely *et al.*, 2012). Since the microbial composition is influenced by nutrition (Zhu *et al.*, 2011), the type and the numbers of microbiota can easily be modulated through eating behaviors (Davis *et al.*, 2009).

The other aspect to look at is the ability of certain food constituents to act as mutagens, or interfere with external mutagens by two ways; either directly or by eliminating the effect of the mutagen (Wiseman, 2008). Foods may change the cell internal environment by modifying hormonal axes thus it will have impact on the growth and proliferation of specific cell population (Wiseman, 2008). In summary, modification to healthier diet is a crucial step in solving many problems contributes to CRC occurrence (Grosso *et al.*, 2013).

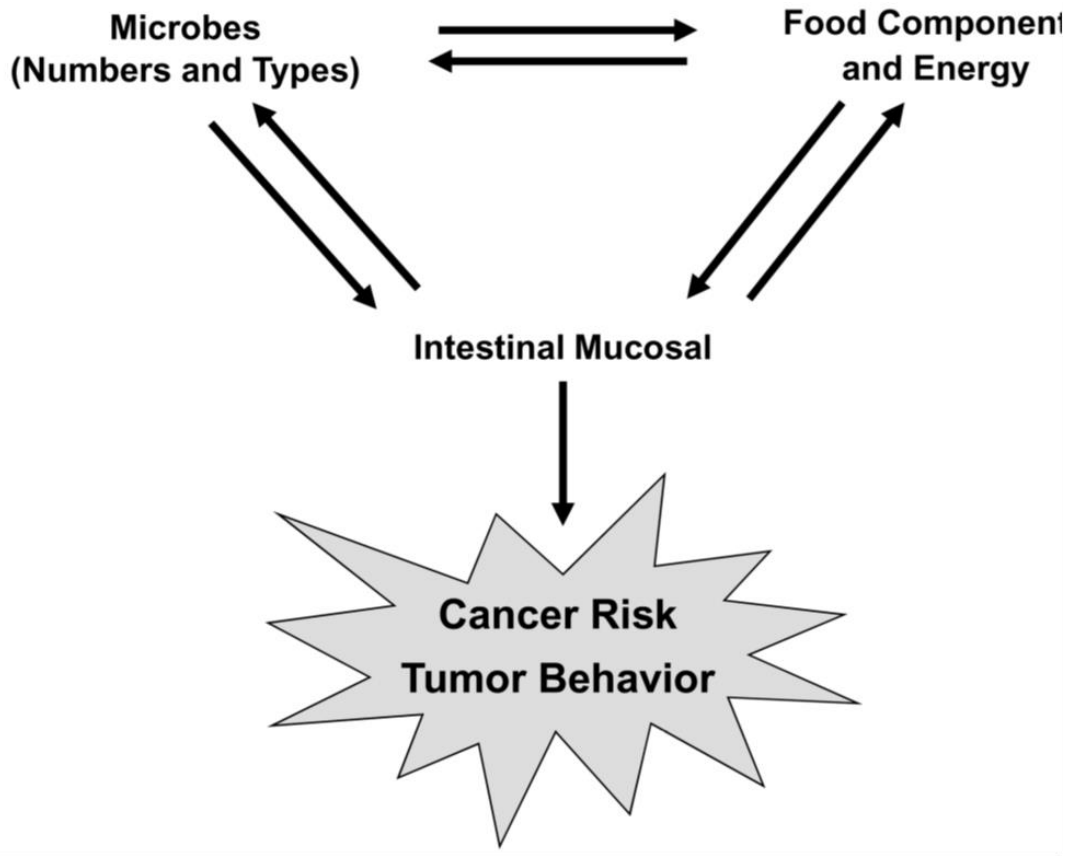

Figure 2.3: The dynamic link between food component, gastrointestinal microorganisms and cells of intestinal mucosa. Variation in type and number of gut microorganisms and food constituents could influences the incidence and the behavior of CRC. (Davis *et al.*, 2009).

2.2 PLANT-DERIVED NUTRACEUTICALS

Natural compounds exhibit extensively wide biological activity spectrum. These natural compounds have the ability to suppress both early and late stages of carcinogenesis (Rajamanickam & Agarwal, 2008).

Among many types of natural products, plant-derived nutraceuticals are notably advantageous for CRC treatment with additional benefit of improving overall health (Figure 2.4). Also, those nutritional compounds could provide better treatment with fewer undesirable side effects (Kuppusamy *et al*., 2014). In addition to that, natural antioxidants derived from fruits and vegetables can treat and control damage caused by free radical (Kuppusamy *et al*., 2014).

Furthermore, plant-based chemo-preventive and their phytochemicals components have the ability to control initiation and progression of colon cancer by many molecular pathways (Rajamanickam & Agarwal, 2008). They could influence overall parts of the regulation of colon cells, establish complex relationship with the microorganisms present in colon and afford protection against colon cancer (Macdonald & Wagner, 2012). Their consumption may also encounter many mechanisms like controlling DNA damage and regulating transcription of DNA in cancer patients (Kuppusamy *et al.*, 2014).

There are many examples that prove the effectiveness of natural plants. For instance, the polyphenolic compounds present in green tea has showed a convincing anticancer effect (Sukhthankar *et al.*, 2008; Hao, 2007). Onion is also well known to be

able to limit proliferation of colon cancer cells by inducing apoptosis. (Kuppusamy *et al.*, 2014)

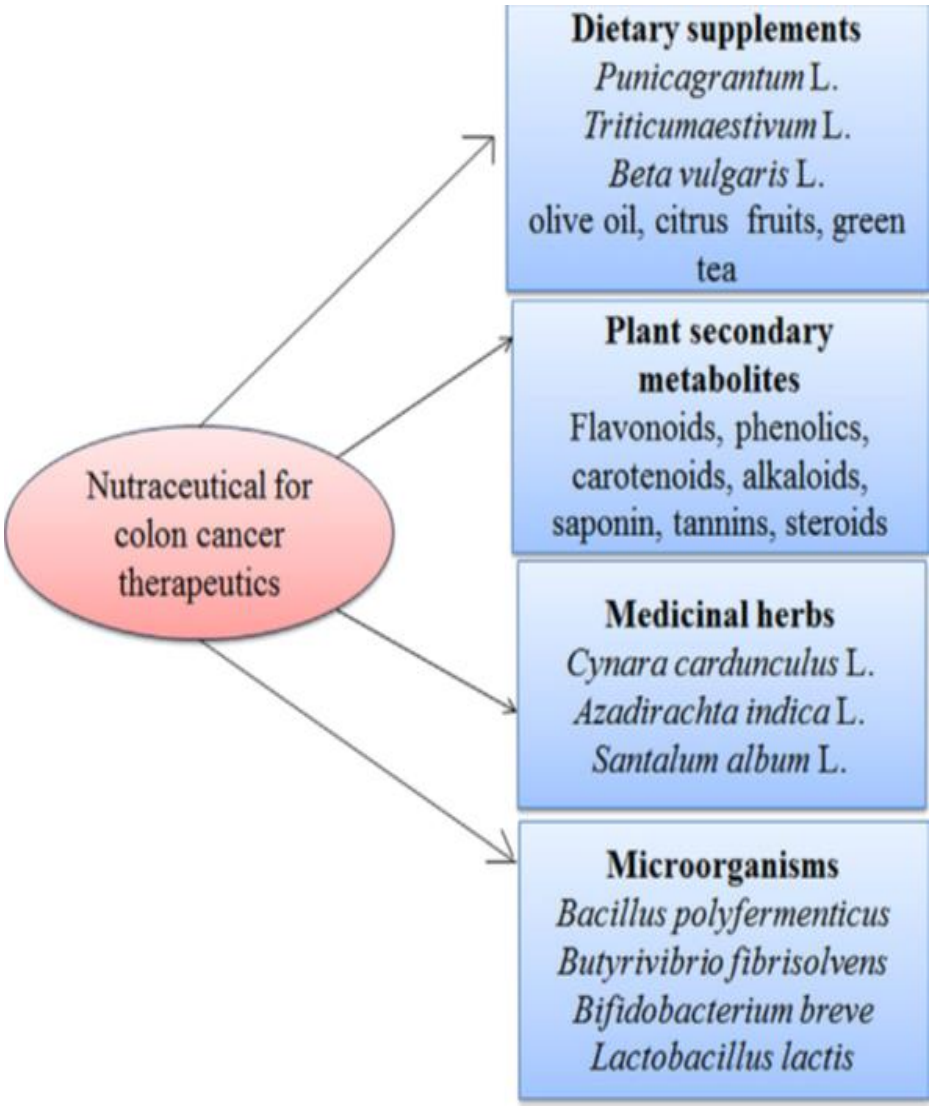

Figure 2.4: Various types of natural products used in colon cancer therapeutics (Kuppusamy *et al.*, 2014).

2.2.1 Fig and its medicinal properties

Fig has a reputable historical background over many centuries concerning its nutritional and medicinal value (Barolo *et al.*, 2014). It is a rich nourishing food and used in industry as an industrial product. It contains many beneficial constituents like valine, leucine, arginine, lysine, some minerals and vitamin (Joseph & Raj, 2010). All plants in ficus genus contain active constituent with medicinal value. Those active compounds contain mostly secondary metabolites like phenols, steroids, tannins and alkaloidal compounds (Joseph & Raj, 2010).

Dried fig contain high amount of crude fibers compared to other common fruits (Solomon *et al.*, 2006). Figs are totally safe for hypertensive patients, as it does not contain sodium salts. It is also useful in cardiovascular diseases and weight loss management as it is cholesterol free (Solomon *et al.*, 2006). Some species of fig also show high anti-oxidant activity (Tamuly *et al.*, 2014; Abdel-Hameed, .2009).

Some fig fruits show an anticancer activity due to the presence of various bioactive compounds (Joseph & Raj, 2010; Lansky *et al.*, 2008; Wang & Ma, 2005). Fig can also modulate immunity and has anti-inflammatory effects due to its antidiabetic property (Kirana *et al.*, 2011).

Fig fruit latex has prominent cytotoxic effect in cancerous cells compared to normal cells. It is recorded that fig fruit latex induces the mechanisms of apoptosis, inhibition DNA synthesis and cell cycle arrest (Wang *et al.*, 2008). It also has significant amount of polyphenolic compounds with anti-oxidant activity (Wang *et al.*, 2008).

Ficus racemosa, known as cluster fig is considered as one of the bona fide medicinal plants and is used in diabetes treatment (Vijayagiri & Mamidala, 2013; Paraakh, 2009). *Ficus fistulosa* has anti-proliferative mechanisms on cancer cells due to its alkaloidal contents (Vijayagiri & Mamidala, 2013).

Ficus religiosa is another example of ficus genus that has medicinal and religious importance and used as anticancer, anti-inflammatory, antibacterial, antidiabetic and antiulcer. It also can enhance immunity to act against various types of diseases (Eshwarappa *et al.*, 2015)**.** *Ficus shispida* and *Ficus schwarzii* are used in treatment of adenocarcinoma of colon in HT-29 colon cell line by anti-proliferative effect. (Abubakar *et al.*, 2014)

Ficus glomerata has significant gastroprotective mechanism and this could be due to the presence of certain gastric defense factors and some phenolic compounds (Rao *et al.*, 2008). It also have antioxidant activity contributed by the high content of phenolic compounds and presence of some acids includes ellagic, gallic and chlorogenic acids (Verma *et al.*, 2010). Its alcoholic extract has more antioxidant activity than aqueous extract as it contain more phenolic and flavonoid compounds (Eshwarappa *et al.*, 2015).

Ficus microcarpa is one of the traditional medicinal plants that used as a food additive in Japan. It has strong antioxidant and antibacterial properties with significant amount of phenolic components (Ao *et al.*, 2008).

Ficus species extracted with ethanol show better effect than standard drugs in treatment of renal carcinoma, worms and microbes (Joseph & Raj, 2010).

2.2.2 Date and its medicinal properties

Date is considered as one of the historical plant with impressive 7000-year-old history. Asia including China and India is the largest importer and consumer of date (Ashraf & Hamidi-Esfahani, 2011). Date is imported in many forms including vinegar and yeast-fermented products, syrup and jam (Saafi-Ben Salah *et al.*, 2012; Ashraf & Hamidi-Esfahani, 2011).

Date is a major supply of energy. Each kilogram of dry date contains 3000 calories, while fresh dates contain 1570 calories per kilogram (Ashraf & Hamidi-Esfahani, 2011). Seeds of date are considered an important source of valuable nutrition with interesting function (Saafi-Ben Salah *et al.*, 2012).

The main constituents of date include water, polyphenols, sugar, protein, fat, pectin, minerals, vitamins, crude fiber, and ash, (Ashraf & Hamidi-Esfahani, 2011). The texture of date is depending on the amount of methylated pectin; the lower the amount of methylated pectin the softer the date (Ashraf & Hamidi-Esfahani, 2011). The odour of date palm seeds were characterised by the presence of various constituents which responsible for specific odour for each variety *e.g.* alcohols, aldehydes and ketones (Saafi-Ben Salah *et al.*, 2012).

Date fruit is reasonably important source of many micro and macro elements (Al-Hooti *et al.*, 1997). Approximately, it contains 15 different minerals including a large quantity of potassium, magnesium, sodium, phosphorous, iron, calcium, selenium and manganese (Ashraf & Hamidi-Esfahani, 2011).

Date is a versatile product. Beside nutritional value, it has potential medicinal value due to variety of biological actions including antioxidant, anti-inflammatory and antiviral (Saafi-Ben Salah *et al.*, 2012). The antioxidant activity of date increase during storage (Ashraf & Hamidi-Esfahani, 2011; F. Biglari *et al.*, 2009).

Date seed extract has the ability to restore normal function against liver toxicity (Saafi-Ben Salah *et al.*, 2012). Meanwhile, date palm can reduce the risk of cancer and atherosclerosis activity, this may be because of individualised constituent or a mixture of constituents (Vayalil, 2002).

2.2.3 Medicinal properties of vinegar

Like fig and date, vinegar is also a common product known from ancient time and used in both food and medicine (Dogaru *et al.*, 2008). Commercial vinegar was sold since 5000 years ago (Budak *et al.*, 2014). Hippocrates (c. 420 BC) used vinegar to control wound infections. Sung Tse, creator of forensic medicine in the 10th century, used vinegar to prevent infection during autopsies (Johnston & Gaas, 2006). Ancient Babylonians used commercial vinegar with many flavors like malt, fruits and honey (Budak *et al.*, 2014).

Vinegar has beneficial characteristics. It is available with affordable prices and possesses safety profile. It is very useful in developing countries (Cortesia *et al.*, 2014). It is used as a source of food condiments and in the preparation of mustard, salad dressings and mayonnaise (Budak *et al.*, 2014).

Any natural product obtained from vinegar will be linked with the decrease overall perishability. Vinegar contains many constituents including acetic acids, mineral salts, amino acids, non-volatile organic acids, polyphenolic compounds and vitamins (Poiană *et al.*, 2007). Acetic acid is one of the main components of vinegar and forms in over-ripe fruits and vegetables (Johnston, 2008).

The polyphenolic compounds from vinegar are responsible for antioxidant activity and reduction of cancer incidence (Poiană *et al.*, 2007). Vinegar shows anticancer effect *in vivo* and *in vitro* (Johnston, 2008). Oral consumption of vinegar has prominent effect against esophageal cancer (Poiană *et al.*, 2007). Vinegar also has both bactericidal and tuberculocidal activity (Cortesia *et al.*, 2014).

Traditional Japanese black vinegar has *in vivo* and *in vitro* anti-colitis and anti-carcinogenic effects (Shizuma & Fukuyama, 2013; Shizuma *et al.*, 2011). The traditional Japanese vinegar is found to have anticancer effect at 4.2% acidity concentration (Baba *et al.*, 2013). Phenolic compounds from the vinegar of distal residues of Japanese liquor shochu also have anticancer and antioxidant effect (Seki *et al.*, 2008).

In addition, vinegar from black soybean also has apoptotic effect (Inagaki *et al.*, 2014). Fermented brown rice also exhibits strong anti-carcinogenic role (Kuno *et al.*,

2006; Kuno *et al.*, 2004). It protect against urinary bladder cancer by antagonistic effect of chemicals that induce urinary bladder formation through anti-proliferative mechanisms (Kuno *et al.*, 2006). Rice vinegar added to drinking water reduces colon cancer occurrence in rats (Johnston, 2008).

Meanwhile, sugarcane vinegar has apoptotic property against leukemia (Johnston, 2008). It shows high physiological function with anticancer activity by several mechanisms such as anti-proliferation, stimulation of natural killer cells and induction of apoptosis (Yoshimoto *et al.*, 2008).

Furthermore, vinegar is very helpful in diabetes as it can improve insulin resistance (Wu *et al.*, 2013; Johnston & Gaas 2006; Ostman *et al.*, 2005). Vinegar with different fruits like sherry, grape fruit, orange, lemon, strawberry and lime are found to have high superoxide anion scavenger property (Cejudo *et al.*, 2010).

Vinegar can also be used as a diagnostic tool. It can help in prediction of early gastric cancer and gastric intestinal metaplasia (Liang & Yang, 2010).

2.3 SRB assay

The sulforhodamine B (SRB) assay is created by Skehan and colleagues and initially is used by anticancer drug discovery programme launched by the National Cancer Institute (NCI) in 1985 (Voigt, 2005).

SRB principle is based on binding ability of sulforhodamine B dye to basic amino acid residues of cells that is fixed by trichloroacetic acid under mild acidic conditions. Under weak bases, for example Tris base, it is solubilised, quantitatively extracted from cells and is measured by optical density (OD) (Voigt, 2005).

SRB assay is inexpensive, rapid and sensitive for drug-induced cytotoxicity. SRB assay's results were linear at density ranging from 1 to 200% of confluence of cells in the 96-well plate with advantages of being stable, non-destructive and colorimetric products (Voigt, 2005).

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Cell lines

Human colon cancer cell lines, HT-29 was provided by Professor Ishak Mat (Advanced Medical & Dental Institute, USM). The cell lines were maintained in a humidified atmosphere at 5% CO_2 and 37°C. The HT-29 cell lines were grown in DMEM (Dulbecco's Modified Eagle Medium) containing 0.1 mg/ml fetal bovine serum (FBS) to final concentration of 10% and 5 ml of warmed penicillin-streptomycin antibiotic. All reagents and chemicals were purchased from Life Technologies, Gibco, USA.

3.1.2 Fig and date vinegars

Fig vinegar was commercially obtained from VOM FASS (Malaysia) Sdn. Bhd. The product was made from concentrated fig juice and the final acidity concentration is 3%. Meanwhile, date vinegar was purchased from Takchin Factory in Iran.

3.2 METHODS

3.2.1 Cell culture methods

3.2.1.1 Preparing complete medium

FBS was first thawed in 56°C water bath for 30 minutes. Then 10 % of warmed FBS and 5 % penicillin-streptomycin antibiotic were added to 500 ml pre-warmed DMEM medium.

3.2.1.2 Resurrection of frozen HT-29 cell Line

It was very essential to thaw the cryovial containing cells quickly because the cryopreservative agent of the frozen cells contain DMSO that is toxic above 4°C. Once taken out from liquid nitrogen tank, the vial was rubbed using hand until half thawed. The half thawed cells were immediately transferred into falcon tube containing 5 ml fresh medium. Then the suspension was centrifuged at 500 x g for 5 minutes and the supernatant containing DMSO was removed. The cells were resuspended in 1 ml fresh growth medium. The cells were transferred to 25 cm^2 flask with additional 4 ml medium to make the final volume of 5 ml. They were incubated in the CO_2 incubator with 5% CO_2 at 37°C. Monitoring of cell attachment was continued until sub culture of cell lines.

3.2.1.3 Trypsination of cells

Cells were washed with PBS and then incubated for 5 minutes in CO_2 incubator with trypsin; 1 ml trypsin for 25 cm^2 flask and 4 ml trypsin in 75 cm^2 flask, respectively. The flask was gently tapped to detach cells from the wall and the cells were observed under microscope to evaluate the detachment process. The detached cells were transferred to a 15 ml falcon tube with additional 1 ml (or 4 ml) medium. The cells were mixed well before spinning them at 500 x g for 5 to 6 minutes. Subsequently, the supernatant was discarded and fresh medium was added.

3.2.1.4 Sub culturing of HT-29 cells

1 ml (for 25 cm^2 flask) or 2 ml (for 75 cm^2 flask) fresh complete medium was added into the cell pellet from the trypsinisation procedure (3.2.2). The cell pellets were re-suspended and transferred into two separate flasks and then new medium were added adequately to a final volume of 5 ml for 25 cm^2 flask and 10 ml for 75 cm^2 flask. Then the cells were incubated at 37°C in a CO_2 incubator until the cell confluence is about 90% for each flask. The cell medium was changed every 3-4 days (the confluent of cells and color of medium were monitored).