CYTOTOXIC AND APOPTOTIC EFFECTS OF CURCUMIN AND THYMOQUINONE ON HSC-2 CELL LINE

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CYTOTOXIC AND APOPTOTIC EFFECTS OF CURCUMIN AND THYMOQUINONE ON HSC-2 CELL LINE

by

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LIST OF ABBREVIATIONS

ACF	Aberrant crypt foci
ALA	Alpha linolenic acid
ALL	Acute lymphoblastic leukemia
AR	Androgen receptor
ASR	Age standardized rate
BDMC	Bis-desmthoxy curcumin
BDNF	Brain derived neurotrophic factor
CSC	Cancer stem cells
CTL	Cytotoxic T lymphocytes
CV	Cell viability
DHA	Docosahexaenoic acid
DISC	Death inducing signaling complex
DMC	Desmethoxy curcumin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent-activated cell sorter
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum

FITC	Fluorescein isothiocyanate
GLOBOCAN	Global cancer incidence, mortality and prevalence
HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IAP	Inhibitors of apoptosis proteins
IC_{50}	50% inhibitory concentration
IL	Interleukin
LOX	Lipoxygenase
MEM	Minimal essential medium
MRSA	Methicillin-resistant Staphylococcus aureus
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National cancer institute
OSCC	Oral squamous cell carcinoma
PBS	Phosphate buffer saline
PCD	Programmed cell death
PenStrep	Penicillin-Streptomycin
PI	Propidium Iodide
PMS	Phenazine methosulphate
PS	Phosphatidylserine

PUMA	p-53 modulator of apoptosis
R^2	R-squared
ROS	Reactive oxidative stress
SD	Standard deviation
SPSS	Statistical package of social sciences software
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TQ	Thymoquinone
TRADD	Tumor necrosis factor receptor associated death domain
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
USM	Universiti Sains Malaysia
VEGF	Vascular endothelial growth factor
WHO	World health organization
XTT	(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2H-tetrazolium)

KESAN APOPTOSIS DAN KESITOTOKSIKAN "CURCUMIN" DAN "THYMOQUINONE" KE ATAS BARISAN SEL HSC-2

ABSTRAK

Kanser adalah salah satu penyebab kematian dan morbiditi yang paling biasa di kalangan manusia. Kanser mulut adalah barah ke-11 yang paling biasa . Karsinoma sel skuamosa oral menyumbang 90% daripada semua barah mulut. Pilihan-pilihan rawatan yang biasa dilakukan bagi kanser mulut adalah pembedahan, radiasi dan kemoterapi. Semua ini adalah pilihan rawatan yang sangat mahal dan agresif yang gagal membasmi barah sepenuhnya dan mempunyai banyak kesan yang melemahkan. Oleh itu, terdapat keperluan yang tinggi untuk pilihan rawatan yang lebih baik dan selamat. Salah satu pilihan tersebut adalah penggunaan sebatian semula jadi yang mempunyai sifat sitotoksik dan anti-barah. "Curcumin" dan "thymoquinone" adalah dua sebatian tersebut. Mereka berdua adalah bahan kimia yang berasal dari tumbuhan (fitokimia) yang merupakan unsur aktif "*Curcuma longa"* dan "*Nigella sativa"* masing-masing. Kedua-dua bahan kimia ini telah digunakan selama berabad-abad untuk membantu merawat pelbagai penyakit. Peranan mereka sebagai agen sitotoksik dan antikanser telah dikaji secara meluas. Sifat sitotoksik dinilai menggunakan ujian "3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide" (MTT) pada pelbagai kepekatan (7.8µM-250µM) selama 24, 48 dan 72 jam. Hasil daripada ujian MTT menunjukkan penurunan ketara dalam daya hidup sel HSC-2 pada 24 jam dan seterusnya, kepekatan perencatan 50% (IC50) dikira pada masa ini dan didapati 54.47µM dan 32.70µM bagi "curcumin" dan "thymoquinone" masing-masing. Kepekatan 50% perencatan (IC50) telah dikirakan dan didapati adalah

54.47µM dan 32.70µM bagi “curcumin” dan “thymoquinone” pada jam ke-24 masing-masing. Sifat yang mendorong apoptosis mereka disahkan melalui sitometri aliran menggunakan perkakas pengesanan apoptosis Annexin-V. Hasil kajian menunjukkan peratusan sel apoptosis awal yang signifikan untuk “curcumin” (min = 9%) dan “thymoquinone” (min = 8%) pada 24 jam pada kepekatan 62.5µM. Hasil yang diperoleh daripada eksperimen ini menyokong sifat sitotoksik dan anti-proliferatif “curcumin” dan “thymoquinone” yang telah dibuktikan dan menyokong hasil daripada kajian-kajian yang serupa .

CYTOTOXIC AND APOPTOTIC EFFECTS OF CURCUMIN AND THYMOQUINONE ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

ABSTRACT

Cancer is one of the most prevalent causes of mortality and morbidity amongst humans. Oral cancer is the 11th most common cancer. Oral squamous cell carcinoma accounts for 90% of all oral malignancies. The current commonly-practiced treatment options for oral cancer are surgery, radiation and chemotherapy. These are extremely expensive and aggressive treatment options that fail to completely eradicate the tumor and have multiple debilitating outcomes. There is a thus a strong need for better and safer treatment options. One such option is the use of naturally-occurring compounds that have cytotoxic and anti-cancer properties. Curcumin and thymoquinone are two such compounds. They are both plant-derived chemicals (phytochemicals) which are the active constituents of *Curcuma longa* and *Nigella sativa* respectively. Both these chemicals have been used for centuries to help treat various diseases. Their roles as cytotoxic and anticancer agents have been extensively studied. In this study, we test their cytotoxic and apoptotic effect on HSC-2 cell line, a type of oral squamous cell carcinoma. The cytotoxic properties were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at various concentrations (7.8µM-250µM) for 24, 48 and 72h. The results from MTT assay showed significant decrease in cell viability of the HSC-2 cells at 24h and so, 50% inhibitory concentration (IC_{50}) was calculated at this time and was found out to be 54.47µM and 32.70µM for curcumin and thymoquinone respectively. Their apoptosis inducing property was confirmed via flow cytometry using the Annexin-

V apoptosis detection kit. The results showed a significant percentage of early apoptotic cells for curcumin (mean= 9%) and thymoquinone (mean= 8%) at 24h at the concentration of 62.5µM. The results obtained from these experiments support the established cytotoxic and anti-proliferative properties of curcumin and thymoquinone and support results from similar studies.

CHAPTER 1

INTRODUCTION

1.1 Background

Oral cancer is the eleventh (11^{th}) most common cancer in the world (García-Martín *et al.*, 2019). Squamous cell carcinoma encompasses 90% of all oral malignancies (*Oral Cancer*, 2010) while the remaining 10% comprise of rare malignancies such as minor salivary gland tumors, lymphomas and benign tumorous growths for example lipomas and papillomas (Genden *et al.*, 2010). According to GLOBOCAN 2018, which is an online database providing estimates of incidence and mortality and is part of International Agency for Research on Cancer (IARC) Global cancer observatory, 354,864 new cases of cancers of lip and oral cavity were reported. Out of these 177,384 died (Bray *et al.*, 2018).

The increased prevalence and poor prognosis of OSCC results from its late diagnosis. There is a wide scope of probable preventive approaches that can prevent or delay the development of cancer. Various cancer prevention strategies such as behavioral modification, surgical management and chemoprevention have evolved with remarkable research efforts (Lippman and Hawk, 2009).

The current treatment modalities for cancer include surgery, chemotherapy, radiotherapy, immunotherapy (Day *et al.*, 2003). Despite the practice of such aggressive methods, the overall 5-year survival rate has not been able to improve which is around 50% (Silverman Jr, 2001). Several investigations have proven that healthy lifestyles, regular exercise, balanced diet, reduced intake of alcohol, smoking cessation, stress management and

weight control are beneficial for decreasing risk of cancer and can never be overstated (Brennan *et al.*, 1995; Calle *et al.*, 2003; Pollack *et al.*, 1984).

M.B. Sporn, coined the term chemoprevention, first in 1976. He defined it as a preventive modality in which synthetic or natural agents can be employed to stop, slow, reverse or prevent the development of cancer (Sporn, 1976). Researchers have investigated numerable agents for this purpose since then with a few successes. Medicinal herbs and their phytochemical derivatives are being rapidly recognized as valuable and useful complimentary treatments for cancer. A great volume of laboratory and clinical studies have reported the beneficial effects of herbal medicine, when used alone or in combination with conventional therapies, on the survival, quality of life and immune modulation of cancer patients (Yin *et al.*, 2013). Two such commonly used herbs are turmeric (Curcumin) and black seed (*Nigella sativa* (*N. sativa*)).

Turmeric (*Curcuma longa*) is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae. It is native to the Subcontinent and Southeast Asia. Curcumin is a hydrophobic polyphenol derived from turmeric. Chemically, it is a bis-α, β-unsaturated β-diketone (commonly called diferuloylmethane). Commercial curcumin is a mixture of curcuminoids, comprising of approximately 77% diferuloylmethane, 18% demethoxycurcumin, and 5% bisdemethoxycurcumin (Aggarwal *et al.*, 2007). Conventionally, turmeric and other curcuminoids have been used in therapeutic preparations for several ailments in various parts of the world. Modern scientific research has confirmed multiple therapeutic effects of turmeric/curcumin (Anand *et al.*, 2008). It has an anti-inflammatory, anti-oxidant, anti-cancer and an anti-microbial effect (Chainani-Wu, 2003; Gunnars, 2017).

Nigella sativa is an annual herbaceous plant native to (and cultivated in) Mediterranean countries, India and Pakistan (Gali-Muhtasib *et al.*, 2006b). Its seeds are commonly known as black cumin. Latest research reports conducted in Muslim countries have demonstrated that N. sativa is very commonly used as a dietary supplement, by cancer patients, along with chemotherapy (Jazieh *et al.*, 2012; Mayadagli *et al.*, 2011). N. sativa seed extract and oil have shown a broad spectrum of favorable biological activities, most prominent being, anti-inflammatory (El-Dakhakhny *et al.*, 2002), anti-oxidant (Ashraf *et al.*, 2011), anti-bacterial (Bakathir and Abbas, 2011), anti-mutagenic (Bourgou *et al.*, 2008), hepatoprotective (Michel *et al.*, 2011), and antitumor activities (Aikemu *et al.*, 2013; Majdalawieh *et al.*, 2010). These beneficial properties of *Nigella sativa* are primarily attributed to it's essential oil component which is pre-dominantly (30-48%) thymoquinone (Hajhashemi *et al.*, 2004).

Curcumin and TQ have been extensively used as a medicine in Ayurvedic and Chinese medicine for centuries. Their beneficial properties are now being proven scientifically by testing and proving their role as anti-inflammatory, anti-angiogenic, anti-oxidant and anti-cancer agents. Studies have also shown that they possess cytotoxic and apoptotic effects against a wide variety of cell lines (Woo *et al.*, 2012). TQ can prevent cancer through the activation or inactivation of molecular cell signaling pathways (Rahmani *et al.*, 2014). In addition, recent studies have shown that these compounds, either alone or in combination with other anticancer agents, can efficiently induce apoptosis of tumor cells (Duarte *et al.*, 2010; Jafri *et al.*, 2010). This is supported by their inhibitory effects on the growth of numerous tumor cell lines both *in vitro* and *in vivo*, including melanoma, mantle cell lymphoma, glioblastoma, hepatic, prostatic, ovarian, breast and pancreatic

carcinomas. Apoptosis is a tightly-regulated process of programmed cell death, including the activation of various molecules for initiating cell death. Specific activation of apoptosis in tumor cells offers a promising approach for cancer therapy. Along with these promising antitumor effects, several studies have reported the safety of TQ and curcumin (Al-Ali *et al.*, 2008; Chainani-Wu, 2003).

1.2 Problem statement

Oral cancer is one of the most common malignancies that affect humans worldwide (Warnakulasuriya, 2009a). Despite the ease of access to the oral cavity, early diagnosis is often almost missed due to the asymptomatic nature of the disease. This leads to a delayed diagnosis which further results in an unsuccessful/incomplete treatment. The current available treatment methods are extremely aggressive and expensive. Furthermore, the postoperative complications of surgery, radiotherapy and chemotherapy are mostly longstanding and painful (de Melo *et al.*, 2001; Sciubba and Goldenberg, 2006; YAJIMA *et al.*, 2000). Moreover, even with these invasive and high-cost treatments, survival rates have not been able to improve (Baykul *et al.*, 2010). Therefore, there is a pressing need for better and safer treatment options. A great volume of laboratory and clinical studies have reported the beneficial effects of herbal medicine on the survival, quality of life and immune modulation of cancer patients (Ouyang *et al.*, 2014).

1.3 Justification of study

Phytochemicals are the biologically active compounds found in plants that possess substantial anticarcinogenic and antimutagenic properties (Surh, 2003). A number of studies have been conducted that prove many beneficial properties exhibited by numerous

herbal extracts and natural products which can help in our battle against cancer (Nobili *et al.,* 2009). Positive results from such studies advocate the need for similar studies to be conducted. Curcumin and TQ are two such plant extracts that have exhibited strong anti-cancer properties in various models and have been proven to be non-toxic (Chainani-Wu, 2003; Tavakkoli *et al.*, 2017). To the best of our knowledge, no similar data is available explaining the cytotoxic and apoptotic effects of curcumin and TQ on HSC-2 cell line.

1.4 Objectives

1.4.1 General

To study the cytotoxic and apoptotic effects of curcumin and TQ individually on HSC-2 cell line.

1.4.2 Specific

1. To determine the cytotoxic effect of curcumin and TQ on HSC-2 cell line by using Methyl Thiazole Tetrazolium (MTT) assay.

2. To determine the maximal inhibitory concentration (IC_{50}) for curcumin and TQ against HSC-2 cell line at 24h.

3. To evaluate the pro-apoptotic effect of curcumin and TQ on HSC-2 cell line by flow cytometry.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Development of cancer is caused by abnormal growth of cells in a particular part of the body. Some types of cancerous cells, travel via blood or lymph to other parts of the body (metastasis), where they begin to grow. Cancer cells, generally, develop from normal cells due to damage of DNA and /or DNA related proteins (Collins *et al.*, 1997; Szent-Györgyi, 1965). Gain-of-function mutations was the first genetic alteration described to contribute in development of cancer (Aaronson, 1991). Loss of function mutations in cancer development was also discovered (Weinberg, 1991). One can also inherit mutated DNA from their parents, which accounts for inherited cancers (Collins *et al.*, 1997). DNA damage can commonly be caused by various environmental factors like smoking, radiation, etc. (Sudhakar, 2009). The correlation between the pathological processes of infection, inflammation and cancer is now being actively studied. Studies show that approximately 15% of human deaths resulting from cancer are associated with longstanding viral or bacterial infections (Karin and Greten, 2005; Lu *et al.*, 2006).

2.2 Oral cancer

Oral cancer can be defined as a malignant neoplasia which develops on the lip or oral cavity (Rivera, 2015). There are several types of oral cancers but, approximately 90% are squamous cell carcinomas which is seen typically on the lateral border of the tongue, floor of the mouth and oral mucosa (Lingen *et al.*, 2008; Van Zyl and Marnewick, 2012). They may appear as a red lesion (erythroplakia), white lesion (leukoplakia), or a mix of the two (erythroleukoplakia) with an ulcer. The mortality rate particularly in these types

of cancers is significantly high and they possess different levels of differentiation and affinity for lymph node metastasis (Barnes *et al.*, 2005).

2.2.1 Etiology

Oral cancer is a multi-factorial disease. Tobacco and Alcohol are the two major and most common contributing factors for the development of oral cancer, causing up to 75% of oral cancers. These are the most important risk factors for squamous cell carcinoma and they have a synergistic effect (Feller *et al.*, 2013; Marttila *et al.*, 2013). Infections and nutrition can also play an important role in cancer development (Jerjes *et al.*, 2012; Scully, 2011). Several microorganisms can produce carcinogenic acetaldehyde from alcohol (Scully, 2011). Poly-microbial supra-gingival plaque, contains both oral streptococci and Neisseria which can have mutagenic reactions with saliva and may produce acetaldehyde from alcohol (Scully and Bagan, 2009). Candida can and may also convert ethanol into carcinogenic acetaldehyde. Candidal leukoplakias can sometimes develop into carcinomas due to nitrosamines that are produced by candida. They can or may activate specific proto-oncogenes (Scully, 2011). Human papilloma virus (HPV) has been linked to oral cancer as well (Chaturvedi *et al.*, 2011; Syrjänen *et al.*, 2011). Another important etiological factor is betel quid chewing, with or without the addition of tobacco (IARC, 2004). The prevalence of alcohol drinking (2 billion people), smoking (1.25 billion people), and betel quid chewing (up to 1.2 billion people) worldwide, risk numerous lives (IARC, 2007). Other contributing factors include chronic irritation, ionizing radiation, chronic exposure to sunlight, which is the leading cause of lip cancers and diet (Scully, 2011). A consistent finding across multiple cultures and ethnicities is the protective effect of fruits and vegetables against oral cancer (Scully and Bagan, 2009).

Anti-oxidants and folate present in these foods may possibly protect against cancer (Pelucchi *et al.*, 2003; Suzuki *et al.*, 2006). Studies have shown that a high intake of fruits and vegetables can potentially prevent the development of squamous cell cancers even in the presence of tobacco and alcohol consumption (Boccia *et al.*, 2008; Pavia *et al.*, 2006). Genetic events within signal transduction pathways that govern normal cellular physiology, if altered, can initiate carcinogenesis (Vogelstein and Kinzler, 1993). Basic cellular functions like cell division, differentiation, senescence and adhesion are governed by tightly controlled excitatory and inhibitory pathways (Bishop, 1991). It has been estimated that around 3-6 somatic mutations are required for the transformation of a normal cell into a malignant cell (Vogelstein and Kinzler, 1993). The accumulation of these changes causes the cell to function independently from the surrounding normal tissue. The abnormal tissue has increased ability to stimulate vascularization. It may grow locally or metastasize to distant sites (Weiner and Cance, 1994). More than 63 karyotypes have been described for oral cancer. Recurrent loss of chromosome 9, 13, 18 and Y deletions are more commonly reported than others (Jurel *et al.*, 2014). A study conducted on patients with OSCC to establish the importance of the alterations in chromosome 8, revealed that amplifications most commonly happened in the 8q region whereas losses were observed in 8p (Yong *et al.*, 2014). Approximately two-third of all head and neck cancer cells contain a deleted region located in chromosome 9p21-22 (Ah-See *et al.*, 1994) which appears in dysplastic and carcinoma-*in-situ* lesions, thereby suggesting that gene in this region is knocked out early in carcinogenesis (Van Der Riet *et al.*, 1994). A study recognized a four genetic marker signature useful for the prognosis of OSCC. According to this study, the co-alteration of 7p, 8q, 9p, and 11q is associated with poor

prognosis (Vincent-Chong *et al.*, 2017). Chromosomal region in 3p and 13q also contain frequently deleted regions (Califano *et al.*, 1996). Numerous oncogenes have been found responsible for oral carcinogenesis (Rowley *et al.*, 1995). Growth factors are also deregulated through increased production in oral carcinogenesis. Transforming growth factor-alpha (TGF-alpha) is over expressed early in oral carcinogenesis (Todd *et al.*, 1991). Epithelial growth factor receptor (EGFR) is commonly found to be over expressed in human oral cancer. 5 to 50 times more EGFR is found in malignant oral keratinocytes (Christensen *et al.*, 1993). Transcriptional factors that regulate the expression of other genes are altered in oral cancer. c-myc, a transcription factor, is commonly over expressed in oral cancer (Rowley *et al.*, 1995). Tumor suppressor genes (TSGs) are lost due to chromosomal alterations. This functional loss of TSGs is considered to be a major cause leading to the development of malignancies (Yokota and Sugimura, 1993) (Lee, 1993). The TSG p53, in normal cell biology, acts as DNA regulator. It helps in blocking cell division of damaged DNA and initiates DNA repair. It also activates apoptotic pathways (Hartwell and Kastan, 1994). Alteration of p53 gene occurs as either point mutations or deletions. In oral cancers, not only has p53 been found to be functionally inactive but its restoration of function has shown reversion in malignancies, in animal models (Schantz, 1995). Human papilloma virus (HPV) has been shown to interact and degrade the p53 protein (Scheffner *et al.*, 1990; Werness *et al.*, 1990). Smoking and tobacco use has been associated with the mutation of the p53 gene in squamous cell carcinoma of the head and neck (Langdon and Partridge, 1992).

2.2.2 Epidemiology

Oral cancer is one of the most common malignancies and its prevalence varies from region to region (Gupta *et al.*, 2016). Asia is home to more than half of oral cancers in the world (Cheong *et al.*, 2017). Of these, 11% belong to South East Asia (SEA) (Warnakulasuriya, 2009a). In these regions, oral cancer can be as much common in females as in males (Ferlay *et al.*, 2015; Vatanasapt *et al.*, 2011). Mortality rates are also highest in this region (Ng *et al.*, 2015). Malaysia is one of the five countries in this region (total = 11) that has an active population based cancer registry (Cheong *et al.*, 2017). The incidence of oral cancer in Malaysia is ethnicity-dependent with Indians being the most affected (Ghani *et al.*, 2011). The Indian sub-continent accounts for one-third of oral cancers globally (Khan *et al.*, 2015). Cancers of lip and oral cavity rank first in Bangladesh and second in Pakistan, India and Nepal (Fitzmaurice *et al.*, 2015). Recent studies indicate the increased rate of people below the age of 45 being affected by it (Majchrzak *et al.*, 2014).

Oral cancer is most common in men than women and this has been attributed to a stronger indulgence and association with risk factors (Dhanuthai *et al.*, 2018). A low 5-year survival rate of 50% is noted among patients with oral cancer (Greenlee *et al.*, 2001; Gupta *et al.*, 2016)

2.2.3 Prognosis and Treatment

Oral cancer is considered a major health concern in most countries with poor survival rate but they are preventable (Dissanayaka *et al.*, 2012). Most cases of oral cancer get reported at very late stages thus making treatment very difficult (Warnakulasuriya, 2009b). Thus, in order to improve survival and cure rates for oral cancer, early diagnosis is extremely

necessary (Seoane *et al.*, 2016). The survival rate of OSCC is highly dependent on the stage of the tumor at the time of diagnosis, the earlier, the better (Barboza *et al.*, 2008). A tumor identified in its early stage gives an 80% chance of survival whereas at an advanced stage, the prognosis falls down to 30-50% (Koch *et al.*, 2011) .One of the most important factors in late diagnosis is the unawareness and lack of public knowledge about oral cancer (Elango *et al.*, 2011). A study showed that there was general unawareness amongst smokers about the role of alcohol in oral cancer. They also lacked awareness about the early signs and symptoms of oral cancer (Zulkiffli and Kallarakkal, 2017). Oral cancer is a big problem worldwide and is associated with extremely expensive treatment, high mortality and permanent impairment (Rao *et al.*, 2013). Surgery, chemotherapy and radiotherapy are the primary treatment options available for oral cancer and all of them are associated with adverse side effects and low response rates (Silva *et al.*, 2012). Surgery can result in functional abnormalities and physical disfiguration (Furness *et al.*, 2011). Hair loss, bone marrow suppression, gastro-intestinal lesions, drug resistance, neurological dysfunction and cardiac toxicity are some of the major side effects of chemotherapy (Nussbaumer *et al.*, 2011).

Despite these aggressive interventions, patients continue to suffer and the survival rate fails to improve and thus it is imperative that new, safer anticancer agents be discovered and introduced. Naturally-occurring chemicals have proven to be useful against various diseases including cancer (Hosseini and Ghorbani, 2015). This has been experimentally proven for numerous herbal products as well (Teiten *et al.*, 2013). Furthermore, a number of phytochemical compounds, extracted from plants, have demonstrated a decrease in cell proliferation, induced apoptosis and inhibit metastasis (Sadeghnia *et al.*, 2014).

2.3 Curcumin

2.3.1 Introduction

Curcumin is a polyphenol and the active ingredient of the spice turmeric (*Curcuma longa).* It is extracted from its plant and purified. It constitutes approximately 3.14% (on average) of powdered turmeric (Tayyem *et al.*, 2006). Turmeric has been used in Asia for around 5000 years and is an integral part of Ayurvedic and traditional Chinese medicine. Vogel and Pierre Joseph Pelletier isolated it first in 1815, reporting the isolation of a "yellow coloring matter", from the rhizomes of turmeric and named it curcumin (Vogel and Pelletier, 1815). In addition to curcumin, there are 2 other curcumoids present known as desmethoxycurcumin (DMC) and bis-desmethoxycurcumin (BDMC). Furthermore, turmeric contains numerous volatile oils, for example; atlantone, tumorone, zingiberone, sugars proteins and resins. It is important to note that besides curcumin, none of the constituents of turmeric possess any anti-inflammatory and anti-proliferative effect (Sandur *et al.*, 2007). Curcumin is pharmacologically safe (Ammon and Wahl, 1991). The US Food and Drug administration classified it safe (US Food and Drug Administration) and is commonly used as a spice in multiple cuisines with no side effects.

2.3.2 Chemical structure and properties

Its chemical structure was accurately mapped in 1910 (Miłobędzka and Lampe, 1910).

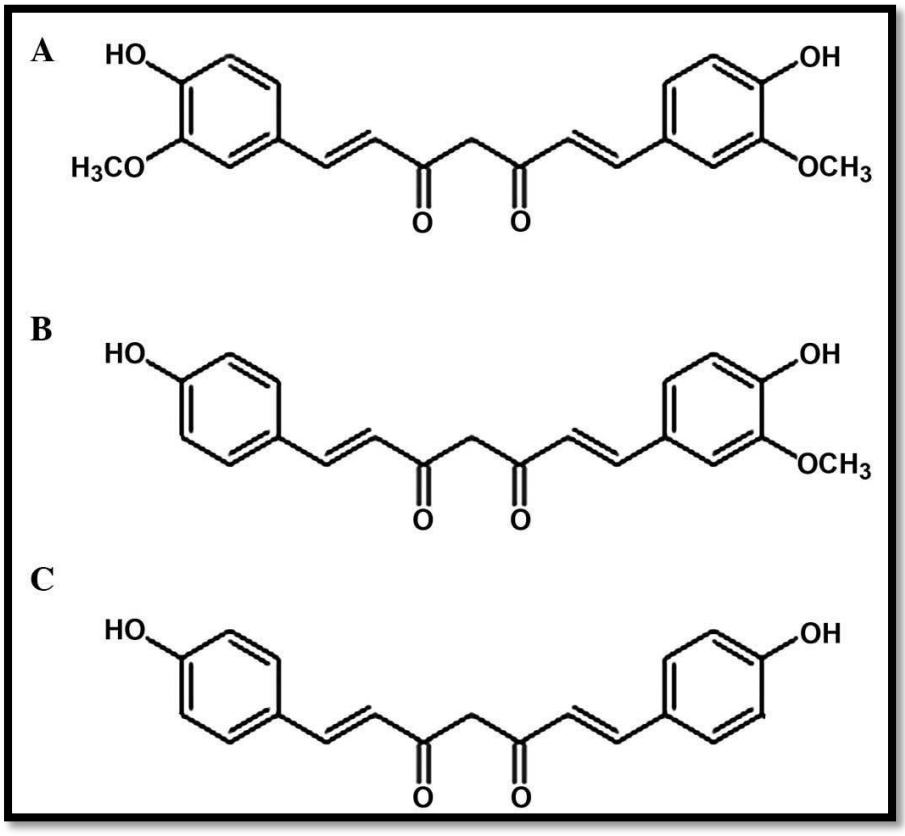

Figure 2.1: Primary extracts of the Curcuma longa plant. (A) Curcumin (diferuloloymethane), (B) demethoxycurcumin and (C) bisdemethyoxycurcumin (Perrone *et al.*, 2015).

Curcumin has a molecular weight of 368.38g/mole (Sharma *et al.*, 2005). Curcumoids comprise of two methoxylated phenols joined by two α, β-unsaturated carbonyl groups. Terpene derivatives, mainly tumerone and zingibrene are predominant in Curcumin (Aggarwal *et al.*, 2015). The rhizome comprises of 3-5% curminoids and 2-7% essential oils (Wang *et al.*, 1997). Curcumin is not readily soluble in water or aqueous solutions but dissolves in organic solvents for example, dimethyl sulfoxide (DMSO), ethanol or acetone. Its melting point is 183°C (Anderson *et al.*, 2000).

2.3.3 Uses

Research on curcumin has been vast and it has proven to have innumerable beneficial health effects. The use of curcumin as a folk remedy continues even today. As part of Ayurveda, ancient Indian medical system, a poultice of turmeric paste is used to dress wounds, treat bites, acne, burns, common eye infections and several skin diseases (Thakur *et al.,* 1989). It is applied as a paste to the perineum to help in the healing of any lacerations in the birth canal during child birth in North India. Women are also given milk with fresh turmeric paste with powdered ginger and honey to drink post childbirth (Pandeya, 2005) and roasted turmeric is one of the ingredients used as an anti-dysenteric for children (Thakur *et al.,* 1989). Cough and related respiratory ailments are cured by taking powdered turmeric with a glass of warm milk (Thakur *et al.,* 1989). Turmeric Band-Aids™ are made for the Indian market by the company Johnson & Johnson (MacGregor, 2006). Dental diseases, digestive disorders such as acidity, indigestion, dyspepsia, ulcers are also treated by this ancient remedy, as well as to alleviate the hallucinatory effects of psychotropic drugs like hashish (Tilak *et al.,* 2004). In food and manufacturing, curcumin is currently used as an approved food additive to flavor different types of mustards and curries. It is also being used in perfumes as a natural yellow coloring agent (Tilak *et al.,* 2004) (Shishodia *et al.,* 2005).

Recent importance on the use of natural and complimentary medicines in Western medicine has caused the scientific community to pay attention to this ancient remedy. It has been revealed through research that curcumin has a wide range of beneficial properties; antioxidant, anti-inflammatory, chemotherapeutic and chemopreventive activity. These activities have been demonstrated in cultured cells and animal models.

One major advantage of curcumin is its safety not only in comparison with normally used chemotherapeutic drugs (fluorouracil, cisplatin etc.) but, also, with other natural agents (resveratrol, lycopene etc.). It does not cause any significant complications even at high concentrations (Gupta *et al.*, 2013).

2.3.4 Medicinal properties of curcumin

Curcumin fights against autoimmune diseases. These diseases can be defined as a condition in which tissue injury is caused by T-cell or antibody reactivity to self. This immune reaction may be initiated by a pathogen but it persists even in its absence (Davidson and Diamond, 2001). Multiple sclerosis (MS), rheumatoid arthritis, inflammatory bowel disease (IBD), type 1 diabetes, thyroiditis, psoriasis, Sjögren disease and myasthenia gravis are examples of autoimmune diseases (Cooper and Stroehla, 2003). Curcumin has proven effective against multiple autoimmune diseases. It has been shown to ameliorate multiple sclerosis, psoriasis and inflammatory bowel disease (Bright, 2007). A study suggested that low doses of curcumin taken daily can reduce the incidence and severity of autoimmune inflammation in MS, however, controlled systematic studies in patients are still required (Natarajan and Bright, 2002). Type 1 diabetes is an autoimmune disease of the pancreas that leads to insulin deficiency and thus increased blood sugar levels (Devendra *et al.*, 2004). Dietary curcumin has shown to decrease blood sugar levels in animal models and patients (Babu and Srinivasan, 1995; Srinivasan, 1972). Moreover, it has also found to be helpful in preventing and inhibiting diabetes related complications; delayed wound healing, cataract etc. (Kumar *et al.*, 2005; Sidhu *et al.*, 1999; Suryanarayana *et al.*, 2005). In a mice study it lowered blood sugar, reduced urine sugar, improved insulin sensitivity and in some mice, reversed diabetes (Zhang *et al.*,

2013). Moreover, it helped improve muscular insulin resistance in rats and prevented insulin resistance and obesity (Shao *et al.,* 2012). It lowers blood sugar by stimulating insulin secretion from pancreatic cells and also helps in pancreatic regeneration (Abdel Aziz *et al.*, 2010). It alleviates diabetic cardiomyopathy in rats (Yu *et al.*, 2012). Rheumatoid arthritis (RA) is characterized by synovitis within diarthroidal joints. Activated macrophages and dendritic cells cause accumulation of inflammatory cells and synthesis of cytokines, matrix metalloproteinases (MMPs) and other inflammatory mediators. This ongoing inflammation potentially produces autoantibodies in RA (Edwards and Cooper, 2006; Firestein, 2005). Curcumin has shown antirheumatic activity in humans (Aggarwal and Shishodia, 2004; Dcodhar *et al.,* 2013; Liacini *et al.,* 2002). It blocks the signaling pathways thus inhibiting inflammatory cytokines (Liacini *et al.,* 2003). In patients with rheumatoid arthritis, dosing at 500mg curcumin + diclofenac sodium was found to be effective (Chandran and Goel, 2012). Inflammatory bowel disease (IBD) is a common health problem (Shi *et al.,* 2006a). Several cytokines are upregulated in IBD and they perpetuate tissue damage (Ricart *et al.*, 2004). Beneficial effects of curcumin have been seen in murine models of IBD (Salh *et al.,* 2003; Sugimoto *et al.*, 2002). Curcumin is a potent inhibitor of inflammation in autoimmune diseases (Nirmala and Puvanakrishnan, 1996) and studies have that that it inhibits myocardial inflammation associated with ischemia, thereby, preventing and protecting against myocarditis (Yeh *et al.,* 2005). Dry eyes are one of the most common symptom of Sjögren syndrome. Curcumin may also have therapeutic potential for dry eye (Chen *et al.,* 2010).

Curcumin reduces the expression of COX-2 and PGE2 synthase 1 which play a key role in the formation of PGE2 and prostaglandin. They play a vital role in tumor development

and inflammation (Zhang *et al.*, 1999). Moreover, it markedly inhibits the production of IL-8, a pro-inflammatory cytokine, that causes serious inflammatory reactions (Yadav *et al.*, 2015). Inflammation is one of the symptoms of osteoarthritis (Belcaro *et al.*, 2010). A study conducted on rats suggested that orally administered curcumin diminishes the neutrophil inflammatory response against arthritis induced by zymosan (Funk *et al.*, 2006). Furthermore, curcuminoid has been argued as a safe and effective treatment against osteoarthritis (Panahi *et al.*, 2014). It also inhibited obesity induced inflammation (Aggarwal *et al.*, 2013).

Curcumin protects against various pathogens. It showed anti-viral activity against influenza, adenovirus, coxasckievirus, HIV and reduces hepatitis C gene expression (Kim *et al.*, 2010). It also has antifungal properties against Candida albicans by inhibiting its hyphae development (Sharma *et al.*, 2010) (Neelofar *et al.*, 2011) (Kumar *et al.*, 2014a). Moreover, it protects against septicemia in mice exposed to the pathogenic bacteria responsible for cholera and reduces mortality rates (Na *et al.*, 2011). Curcumin, when used alongside antibiotic, helps decrease lung inflammation in pneumonia (Bansal and Chhibber, 2010).

Curcumin also helps brain function in several ways. It elevates levels of enzymes involved in the synthesis of DHA (Docosahexaenoic acid) from ALA (alpha-Linolenic acid) in both liver and brain tissues (Wu *et al.*, 2015). This is significant because even DHA supplements and fish oil often do not increase DHA in the brain. Bioavailable curcumin used in brain hemorrhaging improves neurological function and reduces brain water content (Zhang *et al.*, 2017). It is also protective against cell death in brain injuries caused by rapid blood return (Li *et al.*, 2016). It inhibited cell death and neuron loss in

spinal cord injury and markedly improved neurologic deficit seven days after injury (Lin *et al.*, 2011b). In an animal model of dementia, curcumin prevented memory loss (Agrawal *et al.*, 2010). Curcumin improved working memory, sustained attention and mood in a healthy older population significantly (Cox *et al.*, 2015). Curcumin was found effective in reducing amyloid plaque (Begum *et al.*, 2008). It can stimulate adult and developmental hippocampal neurogenesis, neural plasticity and repair (Kim *et al.*, 2008). Similarly, to the prescription antidepressant imipramine, curcumin promotes the development of new cells in the hippocampus in chronically stressed rats, while simultaneously protecting BDNF (brain derived neurotrophic factor) stores (Xu *et al.*, 2007). Curcumin protects against metal toxicity and fluoride. It decreases inflammatory markers in copper-overloaded rats and reduces aluminum induced inflammatory responses in rat brains (Aggarwal *et al.*, 2013). It reduces tissue mercury concentrations (Agarwal *et al.*, 2010). Curcumin is protective against selenium toxicity in the liver and kidneys (Manikandan *et al.*, 2010b). A marked decrease in iron accumulation in liver and spleen was noted after being treated with curcumin (Badria *et al.*, 2015). Curcumin has also been found to ameliorate the neurodegenerative effects of fluoride on the brain (Sharma *et al.*, 2014).

Curcumin is also therapeutic for muscle tissue generation and accelerated healing after injury (Thaloor *et al.*, 1999). Curcumin is also said to be anti-aging. Being a powerful anti-oxidant and anti-inflammatory agent, it inhibits the release of cytokines (IL-1, IL-6. TNF-alpha) that are responsible for the inflammation. Aging slows when inflammation is reduced (Sikora *et al.*, 2010).

Curcumin also has protective effect on kidneys. A turmeric-based diet protected against kidney injury in rats (Aggarwal *et al.,* 2013). Moreover, it prevents Tylenol overdose-induced damage to kidney cells (Kheradpezhouh *et al.,* 2010). Renal ischemia followed by reperfusion can cause renal failure. A study conducted on rats concluded that curcumin protects the kidneys against this ischemia/reperfusion (I/R) injury via its anti-oxidant effects (Bayrak *et al.,* 2008).

Some miscellaneous effects include its ability to prevent cataracts in animal models (Manikandan *et al.,* 2010a). Curcumin augments erectile function in diabetic male rats (Aziz *et al.,* 2012). Curcumin has also shown to be beneficial in reducing the severity of Premenstrual syndrome (Khayat *et al.,* 2015). Curcumin may regenerate cartilage and in a study conducted, was preferred by patients, over analgesic and anti-inflammatory drugs (Appelboom *et al.,* 2014).

Curcumin is an oxygen radical scavenger. It acts as an anti-oxidant by increasing glutathione levels and as an anti-inflammatory agent through the inhibition of IL-8 (in lung cells) (Biswas *et al.,* 2005). It binds to iron, which is one mechanism by which it combats free radicals. Additionally it inhibits the enzymes responsible for mediating inflammation (Menon and Sudheer, 2007). It is a more potent anti-inflammatory than aspirin and ibuprofen (Takada *et al.,* 2004). Furthermore, it prevents alcohol-induced oxidative stress by reducing inflammation and lipid peroxidation and increasing antioxidant enzymes and it also prevents liver disease (Varatharajalu *et al.,* 2016). Likewise, it protects the liver from aflatoxin (El-Agamy, 2010).

Another extremely significant property of curcumin is that it is anti-cancer. Curcumin not only reduces the growth of new blood vessels in tumors (Ravindran *et al.,* 2009), but also,

induces programmed cell death in human malignant brain cancer cells (Dhandapani *et al.*, 2007), melanoma cancer cells (Lu *et al.*, 2010) and T-cell lymphoma cells (Zhang *et al.*, 2010).

2.3.5 Curcumin vs Cancer

The National Cancer Institute (NCI) has tested over a 1000 different potential chemopreventive agents since 1987. Forty promising agents amongst them moved onto clinical trials. One such agent, which is currently under clinical investigation for cancer prevention is, curcumin. It is the most abundant and potent polyphenol present in turmeric (*Curcuma longa*) (Amin *et al.*, 2009). Traditionally, curcumin has been used as a remedy against many illnesses but, it is only in the past few years that its effects against cancer and complications resulting from cancer therapy have emerged. Anti-cancer properties of curcumin were first clinically reported from Kuttan and his coworkers who used a 1% curcumin ointment on skin cancer lesions and observed a decrease in the size of the lesion and pain in 10% patients (Kuttan *et al.*, 1987). The chemopreventive activity was observed in a study of esophageal cancer prevention in curcumin fed F344 rats (Ushida *et al.*, 2000). Furthermore, a significant decline was noted in the initiation of carcinoma in an experimental model of 7,12-dimethylbenz-[a]-anthracene (DMBA) induced mammary cancer in female rats. These experimental models were infused with curcumin intraperitoneally four days before being administered with DMBA (Singletary *et al.*, 1996). Additionally, an oral curcumin diet prevented the development of adenomas in mice proposing the chemopreventive role of curcumin in colorectal cancer (Perkins *et al.*, 2002). Studies have shown that curcumin aids in hepatic cancer prevention as well (Sreepriya and Bali, 2006). Various studies on cancer prevention have proven this

chemopreventive and multi-targeted anti-cancer properties of curcumin, thus, making it an extremely favorable chemopreventive agent.

Chemopreventive agents can be classified into three groups based on their mode of action; anti-oxidants, antiproliferative and/or carcinogen-blocking agents. Given its multiple mechanisms of action, curcumin falls in all three groups. Curcumin mainly exhibits its anticancer effects by regulating multiple biochemical mechanisms responsible for programmed cell death and survival signals. Growth factors, transcription factors, inflammatory cytokines, receptors and enzymes are targeted by curcumin in signaling pathways. The various actions by which curcumin exhibits its anticancer properties in several kinds of cancer include anti-inflammatory action, survival signal reduction, scavenging reactive oxygen species and pro-apoptotic promotion to varying degrees. Curcumin's effects on the signaling pathways are complicated and its chemopreventive, chemosensitizing and radiosensitizing properties are being actively and vigorously studied now.

2.4.5(a) Curcumin and host factors:

Curcumin has poor bioavailability and it is thus almost impossible to achieve the *in vitro* effective dose of curcumin in modulating biomarkers and inhibiting tumor growth *in vivo*. This suggests that there are certain host factors, for example, metabolic system and the host immune system that affect the actions of curcumin. A decrease in T-cell derived cytokines like interferon-γ and functional T-cells stimulates spontaneous as well as carcinogen-induced tumorigenesis. Cytotoxic T lymphocytes (CTL) and CD8 are responsible for antigen-specific tumor destruction and CD4(+) T cells are vital for assisting this CD8(+) T–cell-dependent tumor obliteration. A study showed that curcumin

prevents and weakens the tumor-induced inhibition of T-cell proliferation and reverses the type 2 immune bias. It also inhibited the synthesis of immunosuppressive cytokines like IL-10 and TGF-B (Bhattacharyya *et al.,* 2010). Curcumin improved the production of INF-γ and CD8+ T cells in another study (Luo *et al.*, 2011). There is also a host effect on the metabolism of curcumin in which curcumin is either transformed to hexahydrocurcumin or is modified to glucuronide–sulfate forms (Belkacemi *et al.*, 2011). Hydrocurcumins are more effective anti-oxidants, both *in vitro* and *in vivo,* as suggested by several studies (Murugan and Pari, 2006; Somparn *et al.*, 2007). These anti-oxidant effects of curcumin were proven to be critical for its chemopreventive potential.

2.4.5(b) Trophic signals: (Growth factors and Cytokines)

Various types of trophic factors can aid the growth signals in cancer cells. Curcumin impedes epidermal growth factor receptor (EGFR) kinase phosphorylation which in turn inhibits cancer growth (Tikhomirov and Carpenter, 2003). Curcumin targets VEGF and EGFR to inhibit cell growth (Lin *et al.*, 2009a). Moreover, in an estrogen receptor-negative breast cancer cell line, curcumin inhibited basic fibroblast growth factor along with VEGF at the transcription level (Shao *et al.*, 2002). In another study, curcumin inhibited phosphorylation of Akt (protein kinase B) within the MAPK/PI3K pathway, leading to apoptosis (Squires *et al.*, 2003). It has also shown to inhibit the expression of pro-inflammatory cytokines and growth inhibitory effects via inhibition of MAPK and NF-Kb pathways (Cho *et al.*, 2007).

2.4.5(c) Survival signals- nuclear factor-κB:

The downstream cascades of nuclear factor-κB (NF-κB) and Akt can cause the upregulation of anti-apoptotic proteins. These signals can be modulated by curcumin by

inhibiting the NF-κB pathways at various levels (Shishodia *et al.*, 2007) (Notarbartolo *et al.*, 2005). In nude mice with xenograft tumors of squamous cell carcinoma of head and neck, curcumin markedly inhibited the growth of the carcinoma (Duarte *et al.*, 2010). Similarly, curcumin blocked NF-κB and reduced radioresistance in a colon cancer cell line (Sandur *et al.*, 2009).

2.4.5(d) Role of reactive oxidative stress:

It has divergent effects on cancers; it can cause an insult leading to DNA mutations in carcinogenesis as well as drive mitochondrial apoptosis. It is important to scavenge reactive oxidative stress (ROS), thereby minimizing DNA damage. It is important for cancer prevention. Curcumin was shown to induce many important ROS-scavenging enzymes in laboratory (Balogun *et al.*, 2003) and animal studies (Iqbal *et al.*, 2003). Conversely, curcumin also kills cancer cells, by using ROS. Curcumin generated ROS induced apoptosis in human renal Caki cell by downregulating the inhibitors of apoptosis proteins (IAP) (Woo *et al.*, 2003). ROS generated by curcumin, activated extracellular signal regulated kinase in cervical cancer cell lines, thereby modifying radiosensitivity (Javvadi *et al.*, 2008). Curcumin possess anti-cancer activity regardless of its paradoxical roles in generating and scavenging ROS.

2.4.5(e) Inflammation:

Leaky vessels and loss of adhesion are linked to cancer development and invasiveness. Curcumin has an antagonistic effect to these. The association between pro-inflammatory enzymes, lipoxygenase (LOX) and COX-2, with the possible development of lung, breast and colorectal cancers has been examined (Rao *et al.*, 1999). Curcumin markedly inhibited colonic ACF (aberrant crypt foci) formation, by functioning as a nonspecific

iNOS inhibitor, in F344 rats (Rao *et al.*, 1999).In a breast cancer cell line, curcumin, via NF-κB inhibition downregulated CXCL-1 and -2 and also downregulated CXCR4, the metastasis promoting gene (Bachmeier *et al.*, 2007). Curcumin also inhibited MMP-9 in an invasive hepatocellular cancer cell line resulting in diminished invasiveness (Lin *et al.*, 1998).

2.4.5(f) Cancer stem cells and miRNA:

Cancer stem cells are a rare population of cells within the tumor exhibiting cell-renewal properties and are supposed to be responsible for tumor initiation and treatment failures. Targeting these stems cells can decrease cancer relapse and recurrence and treatment failure. Curcumin and its analogs can target cancer stem cells. Curcumin, along with its analog, decreased cancer stem cell markers in prostate cancer (Johnson *et al.*, 1998). In colon cancer cells, the curcumin analog, GO-Y030 suppressed CSC growth (Lin *et al.*, 2011a).

miRNAs exhibit the ability to target oncogenes and tumor suppressors. Owing to these properties, they play a pivotal role in anti-cancer drug development and tumorigenesis. Curcumin also targets miRNA, enhancing its chemopreventive potential. By targeting transcription factors, curcumin and its analogs (piperidine and cyclohexanone), inhibited various colon cancers (Gandhy *et al.*, 2012). Furthermore, curcumin targets miR-203 in bladder cancers (Saini *et al.*, 2011).

2.4.5(g) Apoptotic signals (intrinsic and extrinsic):

Curcumin induces apoptosis (programmed cell death), both, via intrinsic and extrinsic pathways. Several cell stresses like loss of growth factors, defective cell cycle, irreversible DNA damage can produce death signals which are ultimately passed to