Individual and synergetic effects of Umbelliferone with Vitamin C .......................................................... an in vivo and in vitro study

1. INTRODUCTION

Cancer is a group of diseases in higher multicellular organisms and is characterized by alterations in multiple gene expression, leading to dysregulation in normal cellular program of cell division and cell differentiation. This results in an imbalance between cell replication and cell death promotes tumour population. To invade nearby cells, to extend to regional lymph nodes, and to metastasize to distant organs are the characteristics that delineate a malignant cancer from a benign tumour (Corner, 2001). In cancerous growth progresses, genetic glide in the cell population produces cell heterogeneity, such as cell antigenicity, invasiveness, metastatic potential, cellular proliferation, differentiation and response to chemotherapeutic agents (Corner, 2001; Yarbro, et al., 2005).

Multistep nature of carcinogenesis

Carcinogenesis is multistep process that involves sequence of genetic and epigenic alterations, such as activation of dominantly acting oncogenes and activation of tumour suppressor genes. The theory of multi-stage carcinogenesis was first proposed by Berenblum and Shubik in 1948. The progression of cancer, through the stages of initiation, promotion, and progression has been generally accepted, and mutation and growth of mutated cell clones appear to be the key factors in carcinogenesis (Gutiérrez and Salsamendi, 2001; Trosko, 2001).

Initiation

This is considered to be the first step in carcinogenesis. It involves one or more stable cellular changes arising spontaneously or induced by exposure to a carcinogenic agent, creating the potential for neoplastic development (UNSCEAR,
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1993; Cox, 1994). It predisposes the affected cell and its progeny to subsequent
neoplastic transformation. The human DNA sequences responsible for
transformation are called oncogenes. Although the activation of more than one
oncogene appears to be necessary for neoplastic transformation, the studies imply
that initiation may be induced with one hit kinetics (Bishop, 1987).

Fig.1. Multi-step of carcinogenesis [Adapted from Mary Helen Barcellos-Hoff,
et al., 2013]

Tumour promotion

The intermediate stage of promotion, which does not involve structural
alterations in the genome, may also occur ‘spontaneously’ as a result of endogenous or
exogenous promoting agents (Pitot, 1993). The initiated or transformed cells can
remain harmless, unless they are stimulated to undergo further proliferation,
disrupting the cellular balance. The subsequent changes of an initiated cell, which lead
to neoplastic transformation, may involve more than one step and require repeated and
prolonged exposures to promoting stimuli (Upton, et al., 1986). However, the
continued action of a promoting agent on spontaneously initiated cells can result in the
development of preneoplastic lesions (Schulte-Hermann, et al., 1983).
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Progression

The progression is the final stage of carcinogenesis, which is usually accompanied by more rapid growth, invasiveness, metastasis and increased genetic instability, and changes in the metabolical and morphological characteristics of cells (Gutiérrez and Salsamendi, 2001; Dixon and Kopras, 2004). Angiogenesis is crucial for neoplastic progression and the development of characteristics that contribute to malignancy is preceded by acquisition of an angiogenic phenotype, and its inhibition delays neoplastic development (Hawighorst, et al., 2001).

Structure of liver

The liver is a significant vital organ present in vertebrates and some other animals. It is positioned in the right upper quadrant part of the abdominal cavity, below the diaphragm. It is a reddish brown organ with four lobes of unequal size and shape (Cotran, et al., 2005). It has a wide range of biologically significant functions, such as detoxification, production of biochemicals, that are necessary for digestion and protein synthesis. It produces an alkaline bile compound that is involved in digestion of lipids via emulsification of lipids (Maton, et al., 1993).

Fig.2. Structure of liver (Adapted from Robin Smithuis, 2006)
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Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy. The annual number of new cases of HCC worldwide is over one million, making it the fifth most common type of cancer worldwide and the third leading cause of cancer-related deaths, preceded by the lung and stomach cancers (NCI, 2011; Dhanasekaran, et al., 2012). The global distribution varies by region due to factors at the origin of the disease. This increase is related to the spread of chronic infections due to hepatitis B or hepatitis C (Kew, 2010).

Epidemiology of liver cancer

Because of the aging population and growth of the world population with an increasing adoption of cancer-causing behaviours, particularly smoking, the global burden of cancer continues to increase largely (Jemal, et al., 2011).

International status

Hepatocellular carcinoma affects around 700,000 patients every year worldwide (Villannueva and Josep, 2014). According to the estimates of the American Cancer Society for primary liver cancer and intrahepatic bile duct cancer in the USA for 2014 about 33,190 new cases (24,600 in men and 8,590 in women) will be diagnosed and about 23,000 people (15,870 men and 7,130 women) will die of these cancers (American Cancer Society, 2014). In Asian countries, cases of liver cancer account for 77%, which is highest of all new male cases worldwide. Although Mongolia has the highest incidence of liver cancer among men and women (99 and 57 per 100,000 men and women, respectively) in the 15 Asian countries, it translates to less than 700 new cases in men and 500
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National status

According to the results of a study carried out on 140 patients, HCC was found to be the third most common cause of cancer death in India; hepatitis B was the most common cause of liver cancer affecting as many as 56 (39%) patients, followed by alcohol, which affected 31 (22%) patients (Times of India, 27 August 2013). In India, 2.5 million people are living with cancer and more than 550,000 people die every year from cancer, with 70% in patients aged between 30 and 69 years (Kelly, 2013). Among the cases reported; an estimated 1.1 million new cases are added per year and 0.5 million people die annually because of liver cancer (National Cancer Institute, India (NCI), 2013). According to the available data, the incidence rate of HCC in India ranges from 0.7 to 7.5 and from 0.2 to 2.2 per 100,000 persons/year in men and women, respectively. The male/female ratio of HCC incidence in India is 4:1. According to another study, the incidence of HCC in patients with cirrhosis in India is 1.6% per year (Acharya, 2014).
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RISK FACTORS

Recently, different trends related with incidence of HCC have been observed in various regions, but the reasons were not fully understood. Some risk factors that might have played an important role hepatocarcinogenesis are given below in the following sections.

Hepatitis B virus infection

Hepatitis B virus (HBV) is not directly hepatotoxic but its interaction with the host immune system creates opportunity for HBV and DNA integration into the host genome (But, et al., 2008). Its integration and protein expression alter cell proliferation cycle and apoptosis process. Many other factors, including viral-induced alterations in p53 and telomerase, HBV genotypes, co-infection with hepatitis C virus (HCV), may be involved in this process. Necro-inflammation, cellular proliferation and fibrosis processes facilitate the initial carcinogenic development of HCC (Leung, 2005).

Hepatitis C virus infection

The mechanism of HCC causing HCV remains unsolved. Unlike HBV, it does not integrate into human genome, and does not seem to encode a transforming protein. Continuous inflammation and hepatocyte regeneration in the setting of chronic hepatitis and subsequent progression to cirrhosis is believed to cause chromosomal damage and possibly to initiate hepatic carcinogenesis (Gomaa, et al., 2008).

Co-infections of hepatitis B and hepatitis C

Epidemiological studies have shown that co-infection of HBV with HCV is associated with a higher risk of HCC development than either infection alone. The
Individual and synergetic effects of Umbelliferone with Vitamin C during an in vivo and in vitro study cumulative risk of developing HCC was found to be 10%, 21%, and 23% after 5 years and 16%, 28%, and 45% after 10 years (Chiaramonte, et al., 1999). A meta-analysis indicated that co-infection of HBV with HCV was associated with an odds ratio of 136 for HCC development compared with 20.4 and 23.6 for HBV or HCV infection alone (Donato, et al., 1998).

![Diagram of liver disease progression](image)

Fig. 3. Risk factors of hepatocellular carcinoma (adapted from Paraskevi, et al., 2006)

**Aflatoxin intoxication**

Aflatoxin B1 (AFB1) frequently induces G:C to T:A transversions at the third base of codon 249 of TP53 (Yuan, et al., 2004). AFB1 is classified as group 1 carcinogen by International Agency for Research on Cancer (IARC, 1987). A carcinogenic effect of aflatoxins, particularly of AFB1, has found to be independent from (or to interact) with that exerted by HBV infection (London, 2006).

**Drinking water contamination**

Several carcinogens have been isolated from the surface water, including blue-green algae toxins, organochlorine pesticides, nitrite, and some microelements. Among
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these, microcystins (MCs) are the most convincing carcinogen. They are able to induce
proliferation at low doses, severe liver necrosis, and intrahepatic haemorrhages; and
may have possible synergistic effect with aflatoxins (Yu, et al., 2001; Clark, et al.,
2008). There is a wide spectrum of cyanotoxins of hepatotoxins. They are cyclic
peptides, predominantly MCs, nodularins, and cylindrospermopsin. Therefore, it was
defined by IARC as possibly carcinogenic to humans (Cogliano, et al., 2008).

Alcohol drinking

Alcohol consumption was found to be associated with both HCC incidence
and mortality in ecological studies carried out in the mid 1980s. In areas with low
prevalence of HBV and HCV infection, alcohol is an important risk factor of HCC
and has been defined as a causal relationship by IARC. In high incidence areas,
alcohol may exacerbate viral liver damage and promote tumour development (Gao,
2012). Furthermore, synergistic interactions have been observed between alcohol
intake and risk factors such as hepatitis virus, diabetes, obesity, and smoking
(Singal, et al., 2007; Chuang, et al., 2009).

Tobacco

Tobacco smoking is found to be causally associated with liver cancer (IARC,
2004). The synergistic interactions have been reported between tobacco smoking and
other existing risk factors including HBV, HCV, alcohol, obesity, and diabetes
(Marrero, et al., 2005; Hassan, et al., 2008), but the relationships are still inconclusive.

Oral contraceptives

Oral contraceptives (OCs) were first associated with benign liver tumours
such as hepatic adenoma and focal nodular hyperplasia (Kenya, et al., 1990; Korula,
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et al., 1991). Transition from benign hepatic adenoma to HCC was reported
(Gyorffy, et al., 1989; Korula, et al., 1991) and an association between OCs and
HCC was indicated by cumulative evidence.

Obesity

Obesity may cause HCC via development of non-alcoholic fatty liver
disease, accumulation of fat in the liver to non-alcoholic steatohepatitis (NASH),
cirrhosis, and liver cancer. A recent study has shown obesity to be a genuine
promoter of HCC in a mice model, depending on enhanced production of the
tumour-promoting cytokines such as interleukin 6 (IL-6) and tumour necrosis factor
(TNF), which induce hepatic inflammation and activation of the oncogenic
transcription factor like STAT3 (Park, et al., 2010).

Diabetes mellitus

Most epidemiological studies of diabetes mellitus (DM) and HCC have
reported a positive association so far. A possible explanation for the association is
diabetes is part of the metabolic syndrome, which increases the risk of NASH and
then leads to liver cancer (El-Serag, et al., 2007). Despite the possibility of a causal
association, several arguments have been raised in the study of DM and HCC. One
is that HCV infection might be a consequence of the relation between diabetes and
HCC (White, et al., 2008).

Iron overload

Most commonly, iron overload led to HCC through cirrhosis, but there was
evidence that it could cause HCC independently (Blumberg, et al., 1988). Moreover,
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it may interact with HBV, HCV, alcohol and many other known HCC risk factors and act as a co-factor in the pathogenesis of HCC (Kew, et al., 2009).

Pathogenesis of liver cancer

Hepatocellular carcinoma is a multistep process in which external stimuli induce the genetic alteration in mature hepatocytes, which leads to cellular proliferation and cell death (Thomas, et al., 2005). HCC tumour cells are characterized by loss heterozygosity, which includes multiple chromosomes; in addition, mutations are found in several important genes, such as p57, p53, Rb, APC, DLC-1 (deleted in liver cancer), p16, Smad2 and Smad4, b-catenin, c-myc, and cyclin D1, PTEN, IGF-2BRCA2, and SOC-1 (Fujimori, et al., 1991; Tsuda, et al., 1992). The expression of epidermal growth factor (EGF), transforming growth factor-α and heparin-binding EGF, EGF receptor, has been described in several cell lines and in HCC tissue (Thomas, et al., 2005). Angiogenic factor such as vascular endothelial growth factor (VEGF), platelet-derived growth factor receptor, and fibroblast growth factor are released by the tumour; moreover, inflammatory cells and tumour stromal cells participate in HCC neovascularization (Poon, et al., 2001).

HCC is characterized by an imbalance in growth-promoting and regulation signals, and the mitogen-activated protein kinase (MAPK) cascade is a signalling pathway that has undergone more extensive characterization in this type of cancer (Thomas, et al., 2005). This signalling pathway is activated when a growth factor binds to specific receptors located on the membrane of target cells. Next, there is autophosphorylation of the receptor, and the cytosolic Ras protein relays the signal through the activation of other components of the MAPK pathway, including the
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kinases Raf, mitogen-activated protein kinase kinase (MEK) and extracellular-signal
regulated kinase (ERK). Ras and Raf are important molecular signal transducers,
and MEK performs the intermediate signalling by phosphorylating and activating
ERK1 and ERK2, the effector molecules involved in this signalling process
(Chaparro, et al., 2008). The tumour angiogenesis is important in the development
of HCC and also increases the number of receptors required for MAPK cascade
signalling, thereby helping proliferation, differentiation, and tumour cell survival
(Llovet, et al., 2008). Other signalling pathways involved in the development of
HCC include PI3K/Akt/mTOR and Wnt/b-catenin (Keating, et al., 2009).

ROS and oxidative stress

Oxidative stress is a disturbance in the oxidant–antioxidant balance, leading
to potential cell damage. The imbalance between oxidant–antioxidant can result
from a lack of antioxidant capacity due to production and distribution of reactive
oxygen species (ROS) (Leung and Nieto, 2013). Oxidative stress found to be a key
contributor in the development and progression of many pathological conditions,
including liver carcinogenesis. Any disturbances in normal cell redox state can exert
toxic effects through the production of peroxides and free radicals that damage all
components of the cell, including proteins, lipids, and DNA (Noda and Wakasugi,
2000).

Lipid peroxidation

Lipid peroxidation has been defined as oxidative degradation of lipids, in this
process the free radicals “steal” electrons from membranes lipids, resulting in
cellular damage (Knight, 1995; Ahsan, et al., 2003). Lipid peroxidation and the
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breakage of lipids with the formation of reactive compounds can cause changes in
the permeability and fluidity of the membrane lipid bilayer and can significantly
alter cell integrity (Dix, et al., 1993). The reactive metabolites, which are produced
by oxidative stress, can alter the membrane bilayers and cause lipid peroxidation of
polyunsaturated fatty acids (PUFA), which contain multiple double bonds in
methylene bridges (–CH2–) that possess reactive hydrogens. Therefore, PUFA are
mostly affected by free radicals, resulting lipid peroxidation are involved in various
and numerous pathological states including cancer (Gueraud, et al., 2010).

Oxidative stress and HCC

Oxidative stress resulting from excess ROS production act as potential
carcinogens and has an important function in mutagenesis, tumour promotion, and
progression (Droge, 2003). This excess ROS modulate different signalling
pathways, which may alter the gene expression, cell metabolism to induce oxidative
DNA damage, which in turn increases the chromosomal aberrations with cell
transformation (Choi and Ou, 2006). ROS production may also activate cellular
signal pathways, such as those mediated by nuclear factor-κB (NF-κB), MAPK,
phosphatidylinositol 3-kinase (PI3K), p53, β-catenin/Wnt (Martindale and
Holbrook, 2002; Kuo and Savaraj, 2006; Czaja, 2007). Importantly, HBx stimulates
MAPK, NF-κB, PI3K activities, and β-catenin contributes to the development of
HCC.

Antioxidants

Antioxidants are natural or artificial substances that may inhibit or delay the
cell damage by oxidation of free radicals. They are found in many food sources,
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including fruits and vegetables, and also available as dietary supplements such as, Vitamin A, Vitamin C, and Vitamin E, and β-carotene. An antioxidant is a molecule that inhibits the oxidation process of other molecules (Augusti, et al., 2012). There are evidence that eating a diet with lots of vegetables and fruits is healthy and lowers risks of certain chronic diseases such as osteoporosis, diabetes, and cardiovascular diseases. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S- transferase (GST) and some minerals such as selenium, magnesium, copper, and zinc come under antioxidants. Mainly SOD and CAT are the first line of defence against free radical-induced oxidative stress. The SOD enzyme destroys the superoxide radical; however, as a result of that it creates hydrogen peroxide, which is also highly toxic (Kusvuran, 2012). It has been reported as one of the most important antioxidant defence enzyme that scavenge superoxide anion by converting to hydrogen peroxide, thus diminish the toxic effect caused by this radical (Dincer, et al., 2006). CAT is responsible for the catalytic decomposition of hydrogen peroxide to molecular oxygen and water (Scandalios, 1987). GPx are substantially more efficient on a molar basis than other enzymes (Michiels, et al., 1994; Gomathi, et al., 2012). It acts as a radical scavenger, membrane stabilizer (Pric, et al., 1990), and precursor of heavy metal binding peptides (Ruegesgger, et al., 1990). Glutathione S-transferase is a family of cytosolic enzymes that catalyzes conjugation of glutathione with various reactive electrophilic compounds by neutralizing their active electrophilic sites and subsequently making the parent compound more water soluble. GR is a homodimeric flavoprotein that catalyzes the reduction of oxidized glutathione (GSSG) to the reduced form (GSH) in the presence of nicotinamide adenine
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dinucleotide phosphate-oxidase is a reducing cofactor (Kim, et al., 2010). GR has a
diverse function in cellular process, including the defensive response against free
radicals and ROS, as well as protein and DNA biosyntheses via maintenance of a
high ratio of GSH/GSSG (Estrela, et al., 2006)

![Diagram showing oxidative damage and antioxidants](image)

**Fig.4.** Oxidative damage and antioxidants [adapted from Rao and Rao, 2013]

Vitamin C, Vitamin E, and GSH are well-known non-enzymatic antioxidant
defence system. Vitamin E is fat soluble and is the most powerful membrane bound
antioxidant used by the cell (Hensley, et al., 2004). It protects biological membranes
from lipid peroxidation (Pryor, 2000), and it is also found that α-tocopherol and
ascorbic acid work together in a cyclic-type of process. In antioxidant reaction,
α-tocopherol is converted to an α-tocopherol radical by labile hydrogen to lipid
peroxyl radical donation, and the α-tocopherol radical is reduced to the original
α-tocopherol form by ascorbic acid (Kojo, 2004). Vitamin C is considered as the
first-line natural antioxidant defence in plasma and a powerful inhibitor of lipid
peroxidation (Maxwell, 1995). It has been found in the chloroplast, cytosol, and
vacuole of plant cells and functions as a reluctant for many free radicals (Kumar and
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Hemalatha, 2011). GSH is a tripeptide produced by the liver and neutralizes the oxygen radicals before they cause cell damage. It is found in two forms: free or bound to proteins. Free form is present mainly in its reduced form (GSH), is converted to the oxidized form (GSSG) during oxidative stress, and reverted to the reduced form by the action of the enzyme GR (Ames, 1989; Ames, et al., 1993).

Antioxidants and cancer

Antioxidants are important role reducing free radical reaction that may cause cell damage and DNA mutations and alter enzymatic activity and lipid peroxidation of cellular membranes, including DNA (Gaby, 1991). Inhibition of malignant transformation by antioxidants may be due to inhibition or neutralization of ROS, preventing the metabolic activation of procarcinogens, inactivating carcinogens, inhibiting carcinogen binding to DNA, enhancing DNA repair mechanisms, inhibiting chromosomal aberrations, decreasing the expression of proto-oncogenes, and reducing the expression of transcription factors and nuclear binding proteins involved in tumour progression (Abdi and Ali, 1999).

Liver marker enzymes and HCC

Liver damage generally shows instability of liver cell metabolism, which leads to distinctive changes in hepato-specific enzymes such as α-fetoprotein (AFP), carcinoembryonic antigen (CEA), transaminases, phosphates and lactate dehydrogenase (LDH), and these enzymes leak from the damaged tissues into the body fluids due to their tissue specificity and catalytic activity (Whittby, et al., 1984). As these enzymes are representative of liver function so they are considered to be the most sensitive and dramatic indicators of hepatic injury and loss of
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functional integrity of the membrane (Rajesh and Latha, 2004). α-Fetoprotein (αFP) is a protein of foetal component produced during the embryonic period by the visceral endoderm of the gestational sac. Several studies have shown that the presence of elevated levels of αFP in patients with liver cancer is a risk factor for the development of HCC (Peng, et al., 2004).

CEA is a glycoprotein involved in the process of cell adhesion and produced in gastrointestinal tissue during the foetal development. In general, CEA is usually present only at very low levels in the blood of healthy adults because its stops before birth. It is elevated more in tumours with lymph node and distant metastasis than in tumours confined to an organ. The tumours causing obstruction and liver dysfunction also enhance CEA levels (Zimmer, et al., 2001). Transaminases (AST and ALT), the first marker enzymes of the liver, are reliable and are used in diagnostic enzymology. ALP is another key marker enzyme located in the bile canalicular lipid membrane, so any interference with the bile flow (whether extra- or intrahepatic) alters these enzymes (Whittby, et al., 1984; Iqbal, et al., 2004). LDH is a fairly sensitive marker of solid neoplasm (Lipport, et al., 1981).

Lipids

Dysregulated lipid metabolism is an established characteristic of cancer. Lipids are a diverse group of water-insoluble molecules that play many roles in maintaining cellular structure, forming membrane microdomains for functional scaffolding of protein complexes, serving as fat storage depots, and acting as signalling molecules (Nomura and Cravatt, 2013). Fatty acids are the building blocks for the synthesis of triacylglycerides, which are mainly used for energy
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storage. Cholesterol is required to build and maintain membranes. It modulates
membrane fluidity over the range of physiological temperatures. Phospholipids are a
class of lipids that are a major component of all cell membranes as they can form
lipid bilayers. It is observed that cancer cells show specific changes in different
aspects of lipid metabolism, which can affect numerous cellular processes, such as
cell growth, proliferation, differentiation, and cell motility (Das, et al., 1998;
Bartsch, et al., 1999).

Tumour suppressor genes

Tumour suppressor genes are normal genes that regulate cell division and
repair DNA mistakes. If the DNA mistakes are not repairable, cells undergo the
process known as apoptosis or programmed cell death. When tumour suppressor
genes fail to work properly, cells can grow out of control, leading to cancer.
Different type of tumour suppressor genes have been identified, including TP53
(p53), BRCA1, BRCA2, APC, and RB1. p53 mutations are found in several tumours
and so contribute to the complex network of molecular events leading to tumour
promotion (El-Deiry, et al., 1991). The p53 inactivation is essential for the
development of the majority of human tumours; therefore it is a uniquely valuable
target for applied research (Vogelstein, et al., 2010).

Inflammation and cancer

Chronic inflammation has also been associated with various steps involved
in carcinogenesis and cellular transformation, promotion, survival, proliferation,
invasion, angiogenesis, and metastasis (Coussens and Werb, 2002; Mantovani,
2005). Inflammation is a process by which the body’s white blood cells and
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chemicals they produce protect us from infection with foreign substances, such as bacteria and viruses (Philip, et al., 2004). Tumour invasion and metastasis are a multi-stepped and complex process consisting of cell division and proliferation, proteolytic digestion of extracellular matrix (ECM), cell migration through the basement membranes to reach the circulation systems, and the remigration and growth of tumours at metastatic sites (Grivennikov, et al., 2010). HCC is a prominent example for inflammation-associated cancer, thus it is a model to gain insight about the role of matrix metalloproteinases (MMPs), TNF-α, and NF-κB in the carcinogenesis (Maeda, et al., 2005).

Matrix metalloproteinases

Matrix metalloproteinases (MMP-2 and MMP-9) are a kind of highly conserved proteolytic enzymes (Orlichenko and Radisky, 2008) and are important in the invasion and metastasis of carcinomas. Invasion and metastasis of malignant tumours is closely correlated with the tumour angiogenesis (Saharinen, 2011). Several studies have shown that MMPs can stimulate VEGF secretion besides degrading ECM components, therefore gradually leading to tumour angiogenesis (Van Hinsbergh, et al., 2006; Anne, et al., 2011). Overexpression of MMP-9 and VEGF is related to the recurrence and metastasis of HCC. MMP-9 was predominantly involved in the capsular infiltration and portal vein invasion of HCC (Man, et al., 2010; Hou, et al., 2007). Activation of MMP-2 gene by NF-κB enhances the expression of MMP-2. It has been found to be associated with the progression and invasion of tumours (Shieh, et al., 2010).
Mast cells

Mast cells (MC) are a long-lived, bone-marrow-derived, heterogeneous cellular population that function as both positive and negative regulator of immune responses. They are commonly observed in various tumours and have been attributed alternatively with tumour rejection or tumour promotion. They can not only directly influence tumour cell proliferation and invasion but also help tumours indirectly by organizing the microenvironment and modulating immune responses to tumour cells. In HCC, higher peritumoural MC density is found to be associated with worse clinical outcomes and shorter recurrence-free survival, whereas higher density has been related to increased probability of early recurrence (Khazaie, et al., 2011).

Tumour necrosis factor

Tumour necrosis factor (TNF-α) super family is a group of cytokines that plays an important role in immunity, inflammation, control of cell proliferation, cell differentiation and apoptosis. TNF-α inhibits tumour-induced vascularization of major blood vessel formation by damaging the tumour-associated vasculature. It blocks blood flow and causes ischaemia of the tumour cells (Watanabe, et al., 1988a). Furthermore, TNF-α can exert a direct effect on tumour cells by increasing lysozymal enzymes and hydroxyl radicals, and inducing cytochrome c release from the mitochondria and apoptosis (Watanabe, et al., 1988b). Although high doses of TNF-α have anti-tumour activity, data suggest that endogenous TNF-α acts as a tumour promoter. Both in vivo mouse model studies and data from cancer patients suggest a significant role for TNF-α in tumour promotion. Endogenous TNF-α
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production by cancer cells positively correlates with increased expression of cytokines and chemokines such as CXCL12, CCL8, VEGF, MMPs, and TNF-α itself, leading to neovascularisation, angiogenesis and metastasis (Balkwill, 2004; Nabors, et al., 2003). TNF-α production via the NF-κB pathway in tumour cells is also central to the induction of cell survival and proliferation of malignant cells (Pikarsky, et al., 2004).

Nuclear Factor-κB

Nuclear factor-κB is a collective term referring to dimeric transcription factors of the Rel family. It is activated by inflammatory stimuli and suspected to be a critical promoter facilitating the development from inflammation into cancer (Karin, et al., 2000). It has an important function in the regulation of different biological processes such as immune responses, cell growth, and development. Deregulated NF-κB is associated with various human diseases, particularly cancers (Karin, 2009; Perkins, 2007; Staudt, 2010). As a transcription factor, NF-κB is involved in all stages of tumourigenesis, from initiation all the way to metastasis by regulation of expression of various tumour-related genes. Like tumour itself, however, the role of NF-κB in tumourigenesis is complex and dynamic. During tumour initiation, NF-κB is activated to induce expression of chemokines and cytokines, leading to activate immune cells, particularly myeloid cells. The activated immune cells in turn produce a large amount of pro-inflammatory cytokines/chemokines and growth factors, such as IL-1, IL-6, TNF, and EGF, which is also through NF-κB activation within the cells (Karin, 2009). The NF-κB-mediated inflammation contributes to DNA damage and induction of oncogenic
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mutations in pre-malignant cells through both NF-κB-dependent and -independent mechanisms (Matsumoto, et al., 2007; Takai, et al., 2009; Tergaonkar, et al., 2002; Ak and Levine, 2010), facilitating tumour initiation and progression. Furthermore, NF-κB, which is activated by NF-κB-induced cytokines and growth factors as well as inflammation-induced ROS/RNS and DNA damage, regulates the transcription of genes involved in cell survival, proliferation, angiogenesis, invasion and metastasis, promoting tumour growth and progression. Thus, NF-κB participates in tumourigenesis in both extrinsic (inflammatory cells) and intrinsic (tumour cells) ways.

Fig.5. NF-κB and carcinogenesis [Adapted from Tom Luedde and Robert, 2011]

Cyclooxygenase 2

Prostaglandin (PG) metabolism controlled by cyclooxygenase 2 (COX-2), a protein known as PG synthase, has recently been implicated in the pathogenesis of HCC (Okuda, et al., 1993), and abnormal COX-2 expression has an important role in HCC. It induces the following advantageous properties for tumourigenesis: the angiogenic property through accelerated production of both VEGF and
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prostaglandins (Murono, et al., 2001; Cianchi, et al., 2001); the anti-apoptotic
property mediated by Bcl-2 and protein kinase B (Akt/PKB) signalling (Sheng, et
al., 2001; Nzeako, et al., 2002), and the highly invasive property via activation of
MMPs (Abiru, et al., 2002). Several transcription factors (e.g., NF-κB, NF-IL-6)
have the potential to regulate COX-2 expression (Lim, et al., 2001), suggesting
involvement of inflammation-mediated induction of COX-2 in inflammation-
mediated carcinogenesis.

Proliferation and cancer

The cell proliferation is the process whereby cells reproduce themselves by
growing and then dividing into two equal copies. The normal cells divide only a
finite number of times before they enter into a permanent state of growth arrest or
simply die, cancer cells never cease to proliferate (Andreeff, et al., 2000). Proliferating cell nuclear antigen is a protein, commonly known as PCNA, that acts
as a processivity factor for DNA polymerase δ, which is synthesized between late G1
and S phases. It has been recognized as a useful index of cell proliferation. It is
important in nucleic acid metabolism as a part of the replication and repair
machinery (Isozaki, et al., 1994). This toroidal-shaped protein encircles the DNA
and can slide bidirectionally along the duplex. Interphase argyrophilic nucleolar
proteins (AgNORs) are structural–functional units of the nucleolus and their number
is strictly related to rRNA transcriptional activity and, present in continuously
proliferating cells, to the rapidity of cell proliferation (Derenzini, et al., 1990).

One of interesting and promising methods for determining the rate at which
cells do mitosis is the assessment of the number of AgNORs, regulating the activity
Individual and synergetic effects of Umbelliferone with Vitamin C ……………… an in vivo and in vitro study of ribosomal genes (Sirri, et al., 1995). Generally AgNOR expression depends on the cell-cycle phase, with minimal expression in the G₀ phase and the highest expression in the S and G₂ phases, and the degree of this expression depends mainly on the tumour growth rate and grade. The structure and functions of interphase AgNOR are qualitative tool of measuring the cell proliferation rate (Derenzini, 2000). The abnormal cellular proliferation is one of the important mechanisms in carcinogenesis (Cohen and Ellwein, 1990).

Apoptosis and carcinogenesis

Apoptosis, or programmed cell death, is a major control process by which cells die if DNA damage is not repaired (Lowe and Lin, 2000). This is important in controlling cell number and proliferation as part of normal development. Cancer can be viewed as the outcome of a succession of genetic changes during which a normal cell is transformed into a malignant cell whereas evasion of cell death is one of the essential changes in a cell that cause this malignant transformation (Hanahan, et al., 2000). Apoptosis, characterized by a set of morphological changes, can occur in mammalian cells by the extrinsic or intrinsic pathways (Khan, et al., 2007). The first, referred to as extrinsic pathway or cytoplasmic pathway, is triggered by Fas death receptor, a member of the TNF receptor superfamily (Zapata, et al., 2001).

The second pathway is the intrinsic or mitochondrial pathway that, when stimulated, leads to the release of cytochrome c from the mitochondria and activation of the death signal (Hockenbery, et al., 1990). Both pathways converge to a final common pathway involving the activation of proteases called caspases that cleave regulatory and structural molecules, resulting in cell death. This caspase
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activation has a central role in the offset of apoptosis. The activation of caspase-3 is
a common event in two major pathways, death receptor and mitochondrial pathways
(Sarada, et al., 2008; Hengartner, 2000; Paris, et al., 2007). Caspase-9 is the most
thoroughly characterized initiator caspase and its activation is mediated by the
apoptosome, a multimeric complex involving Apaf-1, cytochrome c, and the
cofactor dATP/ATP.

The overexpression of Bcl-2 in the intrinsic pathway may lead to the
inhibition of extrinsic-mediated apoptosis (Scaffidi, et al., 1998). Conversely,
TNF-α may increase the expression of NF-κB and stimulate anti-apoptotic members
of the Bcl-2 family proteins. Bcl-2 and Bax proteins are members of a large family
of proteins called the Bcl-2 family and both are important regulators of apoptosis
(Ramakrishnan, et al., 2009). Bcl-2 overexpression does not promote cell
proliferation but inhibits cell death (Vaux, et al., 1988) but renders tumour cells
refractory to diverse therapeutic drugs and radiation both in vivo and in vitro.
Selection for drug resistance in cancer cells is often accompanied by up-regulation
of Bcl-2 in vitro (Cory and Adams, 2002). Bcl-2 protein can prolong cell survival by
suppressing apoptosis whereas Bax protein can enhance apoptosis. Thus, the ratio
between Bcl-2 and Bax might be one of the critical factors of a cell’s threshold for
undergoing apoptosis (Cory and Adams, 2005).
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Fig.6. Apoptotic pathways [Adapted from Favaloro, et al., 2012]

Another important protein is the transcription factor p53 (tumour protein 53), which regulates cell cycle and apoptosis. If the DNA is damaged, p53 arrests cell cycle, allowing time for cells to repair DNA. If the damage cannot be successfully repaired, p53 acts as proapoptotic protein signal. It down-regulates several anti-apoptotic genes and/or can directly activate apoptotic pathways (Baptiste, et al., 2002).

In vitro model and HCC
Human hepatoma cell line

Human hepatoma cell line (HepG2) is a human liver carcinoma cell line (ATCC no. HB-8065) regarded as a model system for hepatocytes on widely used in vitro studies (Ihrke, et al., 1993). Human hepatocytes are the most suitable in vitro model for biotransformation in human liver and are important for toxicological and pharmaceutical studies. The cells secrete major plasma proteins such as albumin and transferrin, and the acute-phase proteins such as 2-macroglobulin, α-1-antitrypsin,
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and transferrin. The human hepatocytes have been grown successfully in large-scale
cultivation systems. The HepG2 cell line is easy to handle and provides a
reproducible in human system. However, they are less suitable to predict metabolism
in adult human liver cells, because their expression of drug-metabolizing enzymes is
distinct from the in vivo situation (Knasmuller, et al., 2004). To estimate the risk of
specific compounds that cause cancer in humans, several studies have been
conducted in animal models. However, due to the species differences, there is a need
for a reliable human test system. Therefore, human cell culture models have been
established for toxicity assays to reduce the use of animals. The preferred human in
vitro models are primary hepatocytes and HepG2, because they retain most of the
liver-specific proteins, metabolic enzymes, and functions of human hepatocytes
(Kanazawa, et al., 2006).

In vivo model and HCC

N-Nitrosodiethylamine (DEN), also known as diethylnitrosamine, is widely
used as a liver carcinogen in experimental animal models. It is an N-nitroso alkyl
compound, producing reproducible liver tumours after repeated administration
through drinking water (Bansal, et al., 2005; Ghosh, et al., 2012). Investigations
have proved that N-nitrosamines cause a wide range of tumours in all animal species
and they have widespread industrial uses. Exposure of humans to them occurs
through foodstuffs, cooked meat, in certain occupational settings and as a result of
the use of tobacco products, cosmetics, pharmacological products and agricultural
chemicals (Hecht, 1997). DEN and its metabolites are frequently used to study liver
cancer in animal models because they induce liver tumours with a similar histology,
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morphology, and anatomy to human liver neoplasms (Feo, et al., 2000; Bansal, et al., 2005; Ramakrishnan, et al., 2006; Sivaramkrishnan, et al., 2008).

Mechanism of DEN

DEN was used to induce liver tumours in rats as DEN -induced liver cancer is considered as one of the most accepted experimental models (Bansal, et al., 2005; Ghosh, et al., 2012). It is activated by cytochrome P450, which causes the formation of procarcinogenic, adducts that could lead to DNA damage (Archer, 1989). It is well known to cause perturbations in the nuclear enzymes involved in DNA repair\replication and is normally used as a carcinogen to induce liver cancer in animal models (Bhosale, et al., 2002; Aiub, et al., 2004). The metabolic activation of DEN produces promutagenic adducts, O\(^6\)-ethyl deoxyguanosine and O\(^4\)- and O\(^6\)-ethyl deoxy thymidine that can cause DNA damage and miscode sequence, which are responsible for its carcinogenic effects (Verna, et al., 1996). It is hydroxylated by cytochrome P-450 isozymes present in liver, by an alkylation mechanism, to become bioactivated. Subsequently, DEN reacts with DNA, causing ethylation of the bases. The ethyl DNA adducts can interrupt base pairing, resulting in mutations and the activation of proto-oncogenes, which would lead to carcinogenesis (Li, et al., 2005; Archer, 1989). Furthermore, oxidative stress caused by DEN can lead to hepatocarcinogenesis (Kolaja and Klaunig, 1997; Qi, et al. 2008).

![Chemical structure of N-Nitrosodiethylamine](image)

Fig.7. Chemical structure of N-Nitrosodiethylamine
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Chemoprevention

The increasing number of cancer cases, and the failure of conventional chemotherapy for advanced invasive diseases, indicate that new approaches are urgently required (Sporn, et al., 2000). Chemoprevention is defined as the use of natural, synthetic, or biological chemical agents to reverse, suppress, or prevent carcinogenesis (Anne, et al., 2004). A synergy of natural active principles will bring about the maximum of therapeutic efficacy compared with using single active principle. Recent development in synergy research has opened new avenues for generation of new pharmaceutical drugs.

Flavonoids and cancer

Flavonoids are polyphenolic compounds that are found ubiquitously in plants. They have been shown to possess various biological activities at non-toxic concentrations in organisms (Ren, et al., 2003). These polyphenolic compounds might be able to influence processes that are dysregulated during cancer development. These include antioxidant, anti-allergic, anti-inflammatory, anti-mutagenic, anti-carcinogenic, and modulation of enzymatic activities (Craig, et al., 1999; Middleton, et al., 1994; Galati, et al., 2000).

Umbelliferone

UMB (UMB; aka 7-hydroxycoumarin (7-OH), hydrangine, skimmetine, and ß-umbelliferone) is a natural antioxidant. It has the ability to absorb ultraviolet light at several wavelengths. This chemical is used in sunscreens despite several indications it is photomutagenic. It is a yellowish-white crystalline solid that has a slight solubility in hot water, but high solubility in ethanol. Its
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structure includes two 6-member rings, one of which incorporates an oxygen atom.
UMB has a hydroxyl group and adds a molecular fragment at a carbon adjacent to it.
These two side fragments then close by joining their other ends to form a second
ring, the one with the oxygen atom. During this cyclization, called Pechmann
condensation, a molecule of water is released.

Fig.8. Structure of Umbelliferone (C₉H₈O₃)

A UMB molecule has one of the longer sides polar and the other apolar,
which is the result of locating a hydroxyl group next to the seventh carbon atom, and
it is assumed to have the amphiphilic character “across” the molecule. It is hard for
such a molecule to incorporate itself into the lipid membrane and if it does, this
occurs very close to polar heads almost without interaction with lipid acyl chains
and thus only slightly affecting phase transition parameters (Raj, et al., 1998).

Natural occurrences

UMB is present in many familiar plants from the Apiaceae (Umbelliferone)
family such as carrot, coriander, and garden angelica, plants from other families such as
the mouse-ear hawkweed; and also in the edible fruits such as golden apple (Aegle
marmelos Correa) (Wu and Sheu, 1992) and bitter orange (Citrus aurantium)
(Bouwmeester, et al., 1995), Apium graveolens and Carum carvi (Halim, et al., 1989),
Pituranthos triradiatus (Cheung, et al., 2008), Prunella vulgaris (Khalil, et al., 2003),
Hydrangea chinensis (Eliašová, et al., 2004), Dystaenia takeshimana (Suleimanov,
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2004), Carthamus tinctorius (Rajbir, et al., 2010), Acacia nilotica (L.) (Abyshev, et al.,
2007), Betula pubescens (Jeong, et al., 2001), Dictamnus albus (Parkhomenko, et al.,
2006), Sophora flavescens (Schaufelberger and Mccloud, 1991), Edgeworthia
chrysantha Lindl (Jeong and Lachance, 2001), Ficus carica Linn (Naser, et al., 2005),
Thuja occidentalis (Murray, et al., 1982), Peucedanum palustre, and Angelica
archangelica L. (Flores, et al., 2004).

Pharmacological activities

UMB has been reported to possess antioxidant properties (Ramesh and
Pugalendi, 2006a). 4-Methylcoumarins, having one hydroxyl or two acetoxy groups
in the benzoid ring at positions ortho to each other, have shown very strong
antioxidant and radical scavenging properties better than those of α-tocopherol
(Ramesh and Pugalendi, 2006a). UMB has one hydroxyl and one acetoxy group in
the benzoid ring, which may be responsible for the antioxidant and radical
scavenging properties. A previous report has shown that UMB has alkylperoxy
radical scavenging property (Ohnishi, et al., 1982), and we have also reported that
UMB has antioxidant properties and regulates glycoprotein components (Ramesh
and Pugalendi, 2007; O’Kennedy and Thornes, 1997) in STZ-diabetic rats. It is also
used in sunscreen lotion as an antioxidant and has a minimal toxicity (Ramesh and
Pugalendi, 2006a). It is also reported to have anti-hyperlipidemic, anti-diabetic, and
anti-hyperglycaemic properties (Ramesh and Pugalendi, 2006b; Lacy and
O’Kennedy, 2004). A recent study has shown that UMB inhibits the release of
cyclin D1, which is overexpressed in many types of cancer (Lopez-Gonzalez, et al.,
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2004). Its most important properties include spasmyloytic, anti-tumour and anti-
diabetic (Ramesh and Pugalendi, 2005a; Fort, et al., 1998) properties.

Anti-cancer activity

UMB isolated from Glycyrrhiza glabra L ameliorates oxidative stress
induced by oxidative mutagens and inhibits COX-2, proving it to have strong anti-
cancer effects (Kaur, et al., 2012). Another study reported that UMB has shown anti-
tumour activity in human cancer cell lines, such as A549 (lung), ACHN (renal),
H727 (lung), MCF-7 (breast), and HL-60 (leukaemia) (Stanchev, et al., 2008;
Thornes, et al., 1994; Mohler, et al., 1992). Recently, a study reported that the
percentage of cells expressing cyclin D1 in the lung adenocarcinoma cell line A-427
is reduced by exposure to UMB (Lopez-Gonzalez, et al., 2004). Maucher and Van
Angerer (1996) reported the anti-tumour activity of coumarin and UMB against 7,
12-dimethylbenz[a]anthracene-induced mammary carcinomas in rats.

Anti-inflammatory activity

Islam et al., (2012) reported the anti-inflammatory activity of UMB
6-carboxylic acid isolated from Angelica decursiva. The study confirmed that UMB
6-carboxylic acid showed anti-inflammatory effects, via suppression of NF-κB
activation in RAW 264.7 cells, which suppressed the release of pro-inflammatory
mediators NO, PGE2, iNOS, COX-2, and TNF-α. Kim, et al. (2006) found that
UMB and other coumarins present in the hexane and EtOAc fractions of the MeOH
extract from the root of D. Takeshima [Nakai] Kitagawa [umbelliferae] showed
COX-2 and 5-lipoxygenase (5-LOX) dual inhibitory activity by assessing their
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effects on the production of prostaglandin D2 and leukotriene C4 in mouse bone marrow-derived MCs.

Anti-venom activity

Toyama et al., (2009) reported that UMB is a potential anti-inflammatory drug, and its action involves the selective inhibition of sPLA2 and possibly other enzymes in the inflammation cascade. It interacts with sPLA2 and causes some structural modifications that lead to a sharp decrease or inhibition of the edematogenic and myotoxic activities of this enzyme, indicating its potential use to suppress inflammation induced by sPLA2 from the snake venom. Toyama, et al., (2009) also found that UMB induces changes in the structure and pharmacological activities of Bn IV, a phospholipase A2 isoform isolated from Bothrops neuwiedi. UMB can modify pharmacological ability of sPLA2 by chemical modification of amino acid residues, specifically serines. This sPLA2 also has a highly conserved C-terminal amino acid sequence, which has been shown as important for the pharmacological activities of Lys49 sPLA2. Sequencing of Bn IV previously treated with UMB revealed modification of S(1) and S(20).

Anti-diabetic activity

Ramesh and Pugalendi (2005b and 2006a) reported that UMB inhibits diabetes in streptozotocin-induced diabetic rats. Ramesh, et al., (2005b) stated that UMB protects the membranous fatty acid composition in streptozotocin-induced diabetic rats, which indicates that UMB has minimized the risk of diabetic complications. Kato, et al., 2008) showed that daily consumption of chamomile tea (which contains UMB and other components) with meals could prevent the progress of hyperglycaemia and diabetic complications.Suppressive effect of chamomile on
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blood glucose level was independent of the inhibition of intestinal R-glycosidases
but depended on the inhibition of hepatic GP.

Radioprotective activity

Kanimozhi, et al., (2012) reported that administration of UMB before
radiation exposure prevented crypt cell damage and hepatocyte degeneration in
Swiss albino mice. Pre-treatment with UMB maintained jejunum villus height and
crypt cells architecture in irradiated mice. The protection of intestinal epithelium by
UMB might have a role in the prevention of radiation-induced nutritional mal
absorption. Hence, UMB can be used as an adjuvant in the radiotherapy to protect
normal tissues from deleterious effects of γ-radiation.

Bioavailability of UMB

The compound and its phase II metabolite 7-OHC glucuronide are rapidly
excreted via the kidneys (Rautio, et al., 1992). Matrix calibrations of analyze-free
blood plasma and urine, spiked with the internal standard to a final concentration of
1000 ng/mL and 7-OHC to give final concentrations from 0 to 3000 ng/mL, were
carried out daily before the measurement of experimental samples. The 7-OHC
content was calculated on the basis of the 7-OHC-d5 internal standard. Results of
single samples are averages of duplicate analyses. The analytical method was
validated in-house assessing repeatability (VC 53.6%), linearity, limit of detection
(approximately 3.4 ng/mL), and limit of quantification (approximately 11 ng/mL).
Within each analysis series, a quality assurance sample (pooled urine and pooled
plasma) was analyzed in duplicate; the values of these measurements were
monitored with a Shewhart control chart. Plasma levels of 7-OHC within the first
105 minutes after application are shown next. The 7-OHC concentrations were
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observed below the limit of detection at 15 minutes; mean maximum levels of
190±46 ng/mL were reached after 75 minutes. At the end of the observation period
(105 minutes), mean 7-OHC levels were 151±46 ng/mL. The amount of urinary
excretion of 7-OHC was evaluated for the four 2-hours periods and calculated as a
percentage of coumarin dose administered.

Vitamins and cancer

Vitamins are a class of organic compounds essential for an adequate diet and
are required by various biochemical and physiological processes. They are essential
nutrients for human metabolism, playing as coenzymes or enzymes in many
enzymatic vital processes for the normal functioning of the human body (Young,
et al., 1981). Recently, several studies have shown relationship between the
Vitamins and diseases. Several studies state that Vitamins have an important role in
the prevention and treatment of some cancer types (Mamede, et al., 2011).

Ascorbic acid (Vitamin C)

Vitamin C is a water-soluble antioxidant and enzyme cofactor in some vital
process present in plants and animals. Unlike most mammals, humans do not
synthesize this nutrient endogenously; therefore, they must obtain it through diet. It
is an unstable, gets easily oxidized, and can be destroyed by oxygen, alkali, and high
temperature. Vitamin C has two chemical forms: one is the reduced form (ascorbic
acid; AA) and other one is the oxidized form (dehydroascorbic acid, DHA). The
reduced form of DHA is the more predominant chemical structure in human body,
as an essential micronutrient involved in biochemical and biological functions
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Fig.9. Chemical structure of Vitamin C

History

Vitamin C was first isolated by the Hungarian biochemist and Nobel Prize winner Albert Szent-Györgyi in 1928. In the mid-18th century James Lind showed that the juice of citrus fruits cures scurvy. The active agent, the enolic form of 3-keto-L-gulofuranlactone, christened ascorbic acid, was isolated in the late 1920s by Szent-Györgyi (Szent-Györgyi, 1942). In the mid 1930s, it was available at low cost and in non-toxic form at any dosage.

Biological role in mammals

In humans, Vitamin C is essential to a healthy diet as well as being a highly effective antioxidant, acting to lessen oxidative stress (Higdon, 2007) and an enzyme cofactor for the biosynthesis of many important biochemicals. It acts as an electron donor for certain important enzymes. Vitamin C or Ascorbic acid performs numerous biophysiological functions in the human body including the synthesis of collagen, synthesis and catabolism of tyrosine, and metabolism of microsome and carnitine, and act as neurotransmitter (Gropper, et al., 2005). During biosynthesis of ascorbate, it acts as a reducing agent, donating electrons and preventing oxidation to keep iron and copper atoms in their reduced states (Levine, et al., 1996).

Antioxidant

Vitamin C is well known for its antioxidant activity, functioning as a reducing agent to reverse or inhibit the oxidation in liquids. A large number of
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studies show that antioxidants, including Vitamin C supplementation, may reduce the oxidative stress-mediated diseases including cancer (Bjelakovic, et al., 2007). Vitamin C may contribute to decreased risk of cardiovascular disease and strokes through a small reduction in systolic blood pressure (Fotherby, et al., 2000). However, there has been no consensus that Vitamin C intake has an impact on cardiovascular risks in general, and some studies found negative results (Mayer-Davis, et al., 1997).

Pro-oxidant

Vitamin C also acts as a pro-oxidant (McGregor et al., 2006) and shown to reduce transition metals, cupric ions (Cu$^{2+}$) to cuprous (Cu$^{1+}$) and ferric ions (Fe$^{3+}$) to ferrous (Fe$^{2+}$), during conversion from ascorbate to dehydroascorbate in vitro (Satoh, et al., 1997). This reaction can generate superoxide radicals and other free radicals. However, free transition elements are unlikely to be present while iron and copper are bound to diverse proteins (McGregor, et al., 2006). Thus, ascorbate as a pro-oxidant is unlikely to convert metals to create free radicals in in vivo. However, Vitamin C supplementation has been associated with increased DNA damage in the lymphocytes of healthy volunteers in one study (Podmore, et al., 1998) that has been criticized on methodological grounds (Carr and Frei, 1999).

Immune system

Vitamin C is found in high concentrations in immune cells, and is consumed quickly during infections. It has been shown to modulate the activities of phagocytes, production of cytokines, lymphocytes, and cell adhesion molecules in monocytes (Preedy, et al., 2010).
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Antihistamine

Vitamin C is one of the natural anti-histamines. It prevents histamine release and increases the detoxification of histamine. A study by Johnston et al., (1992) found that taking 2 g Vitamin C per day lowered the blood histamine levels 38% in healthy adults in just 1 week. Low concentrations of serum Vitamin C have been correlated with increased serum histamine levels (Johnston, et al., 1996; Clemetson, 1980).

Therapeutic use

Vitamin C is mostly used for the treatment and prevention of scurvy (WHO, 2001; Olmedo, et al., 2006; Shenkin, 2006; Woodside, et al., 2005; Stanner, et al., 2004). It may be useful in lowering serum uric acid levels, resulting in a correspondingly lower incidence of gout (Stamp, et al., 2013). Neither prophylactic nor therapeutic use is supported in the prevention or treatment of pneumonia (Hemilla and Louhiala, 2013). The highest levels of ascorbic acid in bloodstream are shown to significantly reduce the risk of stroke, and low ascorbic acid has been suggested as a way of identifying high risk of stroke (Myint, et al., 2008). The effect of Vitamin C on common cold has been extensively researched but could not be substantiated, except in limited circumstances (Douglas, et al., 2007; Heimer, et al., 2009).

Vitamin C and cancer

Vitamin C has a controversial history in cancer therapy (Padayatty, et al., 2003). Several observational reports described ascorbate, given in pharmacologic doses of 10 g/day, as effective in treating some cancers and improving patient health and well-being (Cameron and Pauling, 1973; Cameron and Pauling, 1974). Vitamin C acts as antioxidant and prooxidant, promoting the formation of ROS, such as hydrogen peroxide, hydroxyl radicals, and many other radicals. ROS,
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generated in response to high concentration of Vitamin C, interacts with critical
cellular molecules and organelles and results in oxidative degradation and damage of
these cellular compounds in tumour mass, impairing their viability. It acts
selectively on tumour cells because they show decreased levels of several
antioxidant enzymes compared to normal cells, so there is an increased production
of ROS when exposed to Vitamin C. The presence of transition metals in Vitamin C
is due to the increased oxidation of AA to DHA; this selective cytotoxic effect is
enhanced in tumour cells (Hoffer, et al., 2008).

Bioavailability of Vitamin C

Vitamin C is mainly present in fruits and vegetables, and fruit sources rich in
Vitamin C include cantaloupe, grapefruit, honeydew, kiwi, mango, orange, papaya,
strawberries, watermelon tangelo, and tangerine (Carr and Vissers, 2013; Carr, et al.,
2013). Fruit juices also containing Vitamin C include grapefruit and orange juices.
Several fruit juices, such as apple, cranberry and grape juices, are fortified with Vitamin
C. Vitamin C-rich vegetable sources include asparagus, broccoli, brussels sprouts,
cabbage, cauliflower, kale, mustard greens, pepper (red or green), plantains, potatoes,
sweet potatoes, tomatoes, tomato juices, and snow peas (Platt, et al., 1963). As a
supplement, the Vitamin C is available in tablet and powder forms in different doses. In
addition, it is formulated as many multi-Vitamin tablets. It is commonly combined with
other selected Vitamins and the resulting complex is collectively sold as an
“antioxidant” supplements (Elwood and Mccluskey, 1985). It is mostly present in
human breast milk, but only in limited quantity is present in raw cow milk (Clark,
2007). It is most present in the liver and least amount of Vitamin C is present in the
muscle.