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2. SCOPE OF THE THESIS

Hepatocellular carcinoma (HCC) is a life-threatening form of human cancer with more than one million fatalities occurring annually. It is the fifth most widespread cancer and the third leading cause of cancer death worldwide. The chronic hepatitis virus B and C infections are the key threat for HCC. Other factors that contribute to the development of HCC include exposure to ecological carcinogens, iron overload, fatty liver disease, and alcohol abuse. N-Nitrosodiethylamine (DEN) is one of the most significant ecological carcinogens and is present in tobacco smoke, cosmetics, gasoline, and various processed foods such as milk and meat products. Moreover, DEN is frequently used to induce lesions in rats that imitate different types of benign and malignant tumors occurring in humans. The success of several recent clinical trials in preventing cancer in high-risk populations suggests that chemoprevention is a rational and appealing strategy.

Chemoprevention refers to the use of agents to inhibit, reverse, or retard tumorigenesis. Numerous phytochemicals derived from edible plants have been reported to interfere with a specific stage of the carcinogenic process and to improve endogenous mechanisms against diverse stages of cancer development. Recently, a lot of attention has been given on exploring the chemopreventive properties of natural herbs and plants. Flavonoids are the universally occurring secondary metabolites in plants. These are the most common set of polyphenolic compounds consumed as dietary constituents by humans. This polyphenolic compounds show an outstanding spectrum of biological activities including antioxidant, antiviral,
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an in vivo and in vitro study antiallergic, anti-inflammatory, antimutagenic, anticarcinogenic, antineoplastic, and modulation of enzymatic activities.

Hydroxycoumarin, also known as umbelliferone (UMB), is a major biotransformed product of coumarin (1,2-benzopyrone), which is found in a few of the plants belonging to Umbellifera family, but the chief source of these compounds is a plant named Ferula galbaniflua, which is found in a vast area in Damavand and Alborz in Iran. It is also present in lots of familiar plants from the Apiaceae (Umbelliferone) family such as carrot, coriander, mouse-ear hawkweed, and garden angelica, and in the edible fruits such as golden apple (Aegle marmelos Correa) and bitter orange (Citrus aurantium), Apium graveolens, Carum carvi, Pituranthos triradiatus, Prunella vulgaris, Hydrangea chinensis, Matricaria chamomilla, Dystaenia takeshimana, and Carthamus tinctorius. A study discovered that UMB as an antioxidant possesses antidiabetic, antihyperglycemic, anti-inflammatory and antihyperlipidemic properties.

UMB has other biological effects including scavenging of reactive oxygen species (ROS); antibacterial, antithrombotic, and vasodilatory; lipoxygenase and cyclooxygenase inhibition; and antimutagenic and antitumorigenic effects. It has most significant properties including spasmylytic and antitumor properties. It also has UV–protective properties, which are used by skin to guard against radiation. In addition, UMB is well known for its anticoagulant and analgesic properties and used in the synthesis of drugs, especially for anticancer drugs. UMB has shown antitumor activity in human cancer cell lines, such as A549 (lung), ACHN (renal), H727 (lung), MCF-7 (breast), and HL-60 (leukemia). A recent study has shown that UMB
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inhibits the release of cyclin D1, which is overexpressed in numerous types of
cancer.

Vitamin C is broadly found in plants, such as citrus fruits, tomatoes, green
peppers, red peppers, strawberries, broccoli, turnip and other leafy vegetables. Smaller
amounts of Vitamin C are present in fish and milk. All known physiological and
biochemical actions of Vitamin C are due to its action as an electron donor. It is can be
obtained in reduced (L-ascorbic acid) and oxidized (L-dehydroascorbic acid) forms.
Vitamin C is a potent reducing agent (antioxidant) that efficiently quenches potentially
harmful free radicals produced through biological processes in numerous extracellular
and intracellular reactions. It also acts as a prooxidant, promoting the formation of ROS
such as hydrogen peroxide, hydroxyl radicals, and several other ROS, which, generated
in response to high concentration of Vitamin C, interact with hazardous cellular
molecules and organelles and result in the oxidative degradation of these compounds in
cancer cells, impairing their feasibility. It also acts selectively on tumor cells because the
tumor cells show diminished levels of several antioxidant enzymes compared to normal
cells. Owing to the presence of transition metals and due to the increased oxidation of
AA to DHA, selective cytotoxic effect of Vitamin C is enhanced in some tumor cells.

Many in vitro studies showed the selectivity of Vitamin C: it killed a few
cancer cells but not normal cells. Chen, et al., revealed that AA in pharmacologic
concentrations could act as a prodrug, which is important to the formation of
ascorbate radical (Asc−) and hydrogen peroxide radicals in the extracellular region.
Later, an in vivo study showed that DHA can act as an antitumor agent and it reacts
with homocysteine thiolactone, which is a compound present in normal cells in large
quantity. Homocysteine thiolactone is converted to a toxic compound,
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3-mercaptopropionaldehyde, in tumor population. The same effect has been observed in leukemia and lymphoma tumor cell lines.

A single active principle alone may not be sufficient in the treatment of most active cancers. Vitamin C appears to improve the life quality and extend the survival, it should be considered as a part of the treatment procedure for all patients with cancer, whether they have selected as primarily orthodox or complementary approach. Therefore, nowadays scientists are showing interest in the synergy of the natural active principles, which will bring the maximum of therapeutic efficacy compared with using the single active principle. Recent development in synergy research has opened highly interesting perspectives for a new generation of pharmaceutical drugs. Till date, the exact mechanism of the anticancer effect of UMB+Vitamin C is not clear. This study used a well-described model of HCC to study the mechanism of the anticancer action of UMB+Vitamin C by evaluating its antioxidant, anti-inflammatory, antilipid peroxidative, antilipidemic, proapoptotic, and antiproliferative effects.

Specific objectives

In vitro study

1. To carry out MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and to determine the IC₅₀ concentration for the UMB, Vitamin C, and UMB+Vitamin C in human hepatic carcinoma (HepG2) cell line models

2. To study the morphological changes in human hepatic carcinoma (HepG2) cell lines by dual staining with ethidium bromide/Acridine Orange
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3. To study the morphological changes in human hepatic carcinoma (HepG2) cell lines by fluorescence microscopic examination of propidium iodide staining

4. To study the DNA fragmentation induced by UMB, Vitamin C, and UMB+Vitamin C in human hepatic carcinoma (HepG2) cell lines by agarose gel electrophoresis

5. To assess the apoptotic efficacy of UMB, Vitamin C, and UMB+Vitamin C by flow cytometry analysis of the cell cycle

6. To study the proapoptotic and apoptotic proteins such as Bax, Bcl-2, caspase-3, and caspase-9 in human hepatic carcinoma (HepG2) cell lines by Western blotting

In vivo study

Experiment : I

1. The effect of UMB on liver weight and body weight control and experimental rats were observed.

2. Morphological changes in liver of control and experimental rats were observed.

3. Histological evaluation was performed on a portion of 10% formalin-fixed rat liver. Sections (5 μm thick) were cut, stained with hematoxylin and eosin, and viewed under a light microscope for histological changes in the control and experimental groups.

4. Plasma and tissue lipid peroxidation by-products (TBARS (thiobarbituric acid reactive substances), MDA (malondialdehyde) and CD (conjugated dienes) levels were measured in the control and experimental groups.
5. The activities of plasma and tissue antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione transferase (GST), glutathione reductase (GR), and non-enzymatic antioxidants, reduced glutathione (GSH), Vitamin C and Vitamin E were measured in the control and experimental groups.

6. The activities of serum and tissue liver-specific marker enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were measured in the control and experimental groups.

Experiment: II

1. Histological evaluation was performed on a portion of 10% formalin-fixed rat liver. Sections (5 μm thick) were cut, stained with hematoxylin and eosin, and viewed under a light microscope for observing histological changes in the control and experimental groups.

2. Ultrastructural changes in liver tissues were studied using transmission electron microscopy to scrutiny cellular changes initiated by UMB and Vitamin C in the control and experimental groups.

3. Quantitative estimation of tumor markers α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) was done by ELISA in the control and experimental groups.

4. The activities of serum and tissue lipid profiles such as total cholesterol (TC), triglycerides (TG), phospholipids (PL), and free fatty acids (FFA) and total protein, albumin, and bilirubin, were measured in the control and experimental groups.
5. Histochemical analysis of mast cells (MCs) by toluidine blue staining was performed in the control and experimental groups.

6. The expression of matrix metalloproteinases (MMP-2 and MMP-9) in the control and experimental groups was determined by ELISA.

7. Expression of NF-κBp65, TNF-α, and COX-2 in the control and experimental groups was determined by RT-PCR.

8. The expression of NF-κBp65 and TNF-α in the control and experimental groups was determined by Western blot analysis.

9. The expression of cell proliferation marker such as Argyrophili nucleolar organizer regions (AgNORs) and proliferating cell nuclear antigen (PCNA) were determined in liver tissue of the control and experimental groups.

10. The levels of apoptotic proteins such as Bax, Bcl-2, caspase-9 and caspase-3, and p53 were measured in the control and experimental groups by Western blotting.

11. DNA fragmentation pattern induced by UMB and Vitamin C in the experimental groups was determined by agarose gel electrophoresis and in situ TdT-mediated dUTP nick end labeling (TUNEL assay).