4. SUMMARY AND CONCLUSION

Hepatocellular carcinoma (HCC) is one of the most fatal cancers having universal prevalence. Most of the cases have been reported in the developing countries of Asia and Africa, but there is an alarming increase in the HCC cases in Western Europe as well as the USA. The major risk factors involved in the development of HCC include chronic liver diseases, viral infections, and dietary carcinogens such as aflatoxins and nitrosamines. The surgical resection and liver transplantation offered limited treatment options. Accordingly, there exists a critical need to investigate and evaluate possible alternative chemopreventive and therapeutic strategies that may be effective in the control of liver cancer. Flavonoids are endowed with potent antioxidant as well as anti-inflammatory, antitumor, antiviral properties, offered an appropriate choice for alleviation of HCC.

Umbelliferone (UMB), a derivative of coumarin, is a benzopyrone and most extensively present in golden apple (Aegle marmelos Correa) and bitter orange (Citrus aurantium). Several studies have shown that UMB exerts potent antioxidant, antidiabetic, and antitumor effects against lung cancer cell line. A study revealed that UMB also exerts a protective effect against oxidative stress in the heart and brain. It is also used in sunscreen lotion as an antioxidant and has minimal toxicity. Several studies reported that UMB has antihyperglycemic, antihyperlipidemic, anticoagulant, anti-inflammatory, and analgesic properties.

Vitamin C is widely distributed in plants, such as citrus fruits, tomatoes, green peppers, red peppers, strawberries, broccoli, turnip, and other leafy vegetables. There are substantial epidemiological data pointing to the benefits of Vitamin C in the prevention of a numerous cancer types. A number of studies show an inverse
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relationship between Vitamin C status in the treatment of cancers, including breast cancer, cervical cancer, esophageal cancer, bladder cancer, colorectal cancer, pancreatic cancer, reticulum cell sarcoma, salivary cancer, and stomach and gastrointestinal cancer.

Therefore, this study was designed to elucidate the effects of UMB alone and along with Vitamin C in preclinical in vitro and in vivo models of HCC with particular emphasis to its antioxidant, anti-inflammatory, antilipid peroxidative, antilipdemic effects as well as involvement in various molecular signaling mechanisms. The results of the in vitro and in vivo studies are summarized as follows:

In vitro study
1. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed in HepG2 cells to determine the IC$_{50}$ concentrations for UMB, Vitamin C, and UMB+Vitamin C. UMB, Vitamin C, and combined effect of UMB+Vitamin C treated with HepG2 cells resulted in concentration-dependent cytotoxicities, especially at 25.47, 10.95, and 7.55mM for 24 hours. UMB and the synergistic effect of both exhibited significant attenuation of cell proliferation.
2. UMB, Vitamin C, and UMB+Vitamin C treatment at 25.47, 10.95, and 7.55mM for 24 hours resulted in significant morphological changes, nuclear cleavage, and apoptosis, as revealed by dual staining with ethidium bromide/Acridine Orange staining on HepG2 cells.
3. UMB, Vitamin C, and UMB+Vitamin C treatment at 25.47, 10.95, and 7.55mM for 24 hours resulted in significant morphological changes, nuclear cleavage, and apoptosis, as revealed by propidium iodide staining on HepG2cells.
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4. UMB, Vitamin C, and UMB+Vitamin C treatment at 25.47, 10.95, and 7.55mM for 24 hours resulted in DNA fragmentation in HepG2 cells, as evident from DNA ladder-like pattern determined by agarose gel electrophoresis.

4. Cell cycle analysis was determined by administering UMB, Vitamin C, and UMB+Vitamin C on HepG2 cells. This treatment could arrest the HepG2 cells at G₀/G₁ phase more effectively than the S and G₂/M phase.

5. Immunoblot analysis of apoptotic proteins of HepG2 cells treated with UMB, Vitamin C, and UMB+Vitamin C confirmed the apoptosis by upregulation of proapoptotic proteins such as Bax and caspase-3 and downregulation of antiapoptotic protein Bcl-2.

In vivo study

Experiment I

1. N-Nitrosodiethylamine (DEN)-induced rats showed significant increase in body weight for the first 8 weeks, later it was observed that the body weight was declined sequentially and there was a sharp loss in body weight. On UMB treatment, the body weight was significantly increased when compared to DEN-induced rats.

2. Morphological studies of DEN-induced rats showed enlarged liver, and the nodule incidence of DEN group was 100%. The maximum diameter of nodules was about 10 mm and clear necrosis regions were observed in DEN induced rats. Interestingly, a significant reduction in liver enlargement, nodule incidence, and the nodule numbers was observed in DEN with UMB-treated rats than in DEN group.

3. Histopathological studies of DEN-induced rat liver showed loss of normal liver hepatocyte architecture due to HCC, and examination of UMB-treated rat liver revealed near-normal architecture of the liver hepatocytes.
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4. A significantly increased activity of serum and tissue lipid peroxidation end
products such as TBARS, MDA, and CD was seen in DEN-induced rats, whereas
treatment with UMB resulted in a marked decrease in the levels of these substances
to near-normal levels, indicating the protective antioxidant effect of UMB.

5. A significantly decreased activity of serum and tissue enzymatic and non-
 enzymatic antioxidants such as SOD, CAT, GR, GST, GPx, and GSH, Vitamin C
and Vitamin E was seen in DEN-induced rats, on treatment with UMB could reverse
the above-said antioxidants to near-normal level, suggesting the anticancer potential
action of UMB.

6. Liver-specific serum markers such as AST, ALT, ALP, and ACP were
significantly increased in DEN-induced rats, whereas near-normal levels were
observed after treatment with UMB.

Experiment II

1. Histopathological studies of DEN-induced rat liver showed loss of normal liver
hepatocytes architecture due to HCC, and examination of UMB and UMB+Vitamin
C-treated rat liver revealed near-normal architecture of the liver hepatocytes.

2. Ultrastructural changes in DEN-induced rat liver showed multiple irregularly
shaped nuclei close to each other with irregular cytoplasm, but treatment with UMB
and UMB+Vitamin C showed liver cells with shrunken nucleus and condensed
chromatin undergoing apoptosis.

3. Lipid profiles such as TC,TG, FFA, PL, total protein and albumin were
significantly increased and bilirubin was decreased in DEN-induced rats, whereas
near-normal levels of these lipid profiles and protein were noted after treatment with
UMB and UMB+Vitamin C.
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4. Liver cancer-specific serum markers such as α-fetoprotein (AFP) and
carcinoembryonic antigen were found to be significantly increased in DEN-induced
rats, whereas near-normal levels of these serum markers were observed after
treatment with UMB and UMB+Vitamin C.

5. The expressions of inflammatory markers such as MMP-2 and MMP-9 were
found to be increased in DEN-induced rats, whereas those in UMB+Vitamin C-
treated rats greatly reduced to near-normal level.

6. The expressions of mast cells by using toluidine blue staining were found to be
increased in DEN-induced rats, whereas those in UMB and UMB+Vitamin C treated
rats greatly reduced to near-normal level.

7. Reverse transcriptase-PCR (RT-PCR) was performed for NF-κB65, COX-2 and
TNF-α, and the levels of these mRNA genes were increased in DEN induced rats,
whereas treatment with UMB alone for different regimens and with Vitamin C
significantly suppressed the levels of these genes to near-normal in DEN-induced
experimental rats.

8. The expression of NF-κBp65 and TNF-α in control and experimental groups was
determined by Western blot analysis. Increased expressions of NF-κBp65 and TNF-α
were observed in DEN-induced rats, whereas treatment with UMB alone for different
regimens and with Vitamin C significantly suppressed the levels of these genes to near
normal in DEN induced experimental rats.

9. Increased expression of argyrophili nucleolar organizer regions (AgNORs) and
proliferating cell nuclear antigen (PCNA) (markers for cell proliferation) were noted
in DEN-induced liver cancer-bearing rats whereas those in UMB and UMB+Vitamin
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Created rats were observed to be near normal in DEN-induced rats, proving the individual and synergistic anticancer effects UMB and Vitamin C have.

10. The expression levels of apoptotic proteins such as Bax, caspase-9, and caspase-3 were found to be decreased and antiapoptotic protein Bcl-2 was significantly increased in DEN-induced rats whereas treatment with UMB, UMB+Vitamin C found to reverse the apoptotic and antiapoptotic proteins to near-normal levels in DEN-induced experimental groups.

11. DNA fragmentation pattern induced by UMB and Vitamin C treatment in experimental groups was determined by agarose gel electrophoresis and in situ TdT-mediated dUTP nick end labeling (TUNEL) staining. DEN-induced rats showed no DNA fragmentation whereas treatment with UMB+Vitamin C in DEN-induced rats (groups 4, 5, and 6) showed a DNA fragmentation and induction of cell death in the form of apoptosis.

CONCLUSION

Our study clearly demonstrated that, the combined treatment of UMB+Vitamin C combination appreciably attenuates the reversible alterations in the DEN induced hepatocellular carcinoma with no harmful adverse effects. This provides an experimental proof on the therapeutic impact of UMB+Vitamin C against hepatocellular carcinoma. This will be provided the awareness about natural dietary compounds and their combination has significant impact in treating cancer to avoid toxicity and further studies needed to prove the molecular mechanisms implicated in the efficacy of UMB+Vitamin C as anticancer agents.
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