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Saffron-Based Crocin Prevents Early Lesions of Liver Cancer: *In vivo*, *In vitro* and *Network Analyses*

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Abstract: Background: The angiogenesis inhibitor, sorafenib, remains the only available therapy of hepatocellular carcinoma (HCC). Only recently patents of VEGF receptors-3 inhibitors are developed. Thus, a novel approach against HCC is essential for a better therapeutic outcome. **Objective:** The aims of this study were to examine the chemopreventive action of saffron's main biomolecule, crocin, against chemically-induced liver cancer in rats, and to explore the mechanisms by which crocin employs its anti-tumor effects. **Method:** We investigated the anti-cancer effect of crocin on an experimental carcinogenesis model of liver cancer by studying the anti-oxidant, anti-inflammatory, anti-proliferation, pro-apoptotic activities of crocin *in vivo*. In addition, we provided a network analysis of differentially expressed genes in tissues of animals pre-treated with crocin in comparison to induced-HCC animals' tissues. To further support our results, *in vitro* analysis was carried out. We assessed the effects of crocin on HepG2 cells viability by treating them with various concentrations of crocin; in addition, effects of crocin on cell cycle distribution of HepG2 cells were investigated. **Results:** Findings reported herein demonstrated the anti-proliferative and pro-apoptotic properties of crocin when administrated in induced-HCC model. Crocin exhibited anti-inflammatory properties where NF-$_k$B, among other inflammatory markers, was inhibited. *In vitro* analysis confirmed crocin's effect in HepG2 by arresting the cell cycle at S and G2/M phases, inducing apoptosis and down regulating inflammation. Network analysis identified NF-$_k$B as a potential regulatory hub, and therefore, a candidate therapeutic drug target. **Conclusion:** Taken together, our findings introduce crocin as a candidate chemopreventive agent against HCC.

Keywords: Apoptosis, crocin, inflammation, liver cancer, network analysis, NF-κB.

INTRODUCTION

Despite the global concerted efforts, cancer continues to be a major global health burden. Hepatocellular carcinoma (HCC) remains among the leading causes of cancer-related death worldwide [1]. Viral liver infections with hepatitis (B and C) often advance to HCC. In fact, HBV and HCV account for 70% - 90% of HCC patients' cases [2]. Other factors that contribute to the formation of HCC include fatty liver disease, iron overload [3], alcoholism and exposure to environmental carcinogens [4] such as diethylnitrosamine (DEN) [5].

Sorafenib has been reported beneficial at early stages of HCC, yet its effectiveness as an angiogenesis inhibitor has been modest [6]. As vascular endothelial growth factor (VEGF) is highly expressed in HCC liver [7, 8], Phase III study is currently underway to evaluate the effect of regorafenib, a derivative of sorafenib that inhibits angiogenic kinases like VEGFR-1/3 and its efficacy and safety in HCC patients [9]. VEGF receptors-3 has been shown to be differentially upregulated on tumor vasculature supporting the majority of solid cancers including HCC. Such upregulation introduces a significant antiangiogenic effect for VEGFR-3 - targeted therapy [10]. Multiple VEGFR-3 inhibitors for treating HCC have been recently developed in patent US20150183780 [11].

Thanks to the genetic stability of endothelial cells, angiogenesis inhibitors are emerging as single-agent and/or combination therapies particularly for the hypervascular HCC. VEGF regulates the proliferation and survival of sinusoidal endothelial cells as well as their ability to form fenestrations. Unlike other capillaries, endothelial cells of liver sinusoids have open fenestrations and lack basal lamina. The plasmalemma vesicle-associated protein (PLVAP) is the only protein that has been identified as a critically required com-

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ponent of endothelial diaphragms [12]. Defenestration in HCC involves PLVAP expression [6]. PLVAP protein was reported to be specifically expressed only in vascular endothelial cells of HCC livers. A recombinant monoclonal anti-PLVAP Fab fragment that co-expresses a water-soluble extracellular domain of human tissue factor (anti-PLVAP Fab-TF) was developed and shown effective to treat HCC in Hep3B xenograft model [6]. Anti-PLVAP Fab-TF has been developed in patents KR20150056489, TW201519906, TW201510218 and may soon become a viable therapy for patients with advanced disease and compromised liver function [13-15].

In addition of being expressed on the surface of breast cancer cells [16], new antibody against cytoplasmic- and proliferation-associated protein 1 (CAPRIN-1) has been developed, patent US2015218285, specifically against this protein in HCC liver [17]. The majority of liver cancer started with hepatitis C virus infection. Thus, effective agent for inhibiting the development or progression of liver cancer in hepatitis C virus-positive human cirrhosis patients is desired. In a recent patent, KR20150055100, a composition comprised of isoleucine, leucine and valine as active ingredients that has an inhibitory action on the development and/or progression of liver cancer in hepatitis C virus-positive human cirrhosis patients has been developed [18].

Aminothiazoles are a group of compounds that have been reported to possess various biological activities [19]. Substituted aminothiazoles as inhibitors of HCC progression and of hepatitis virus replication is a recent patent HK1200361 that targets the cell types that have been shown to aid viral infection in the liver. This patent is particularly effective in treating patients infected with hepatitis viruses A, B, C, D virus, and E virus [20]. Similarly, a novel pyridazine derivative has been developed in patent KR20150079942 and introduced recently as a potent treatment of HCC [21].

Cancer chemoprevention, particularly with natural products, represents a promising strategy against cancer development and metastasis. Thus, natural compounds with robust antioxidative, hepatoprotective and anti-inflammatory properties are normally evaluated for their abilities to hinder tumors' growth [22]. One particular plant product that has gained much attention is saffron. This golden spice is the flower's dried stigma of *Crocus sativus* and is known for its medicinal benefits against a host of health disorders [23]. Saffron and its active constituents exhibited antioxidant, anti-inflammation, and antitumor activities [24]. The major biological components of saffron are safranal, crocin, and picrocrocin; which attribute the saffron aroma, color, and bitter taste, respectively [25]. Crocin was found to have anti-proliferation and pro- apoptotic effects against HepG2 [26] and gastric adenocarcinoma cells [27]. In our laboratory, we have shown pro-apoptotic, anti-inflammatory, anti- oxidative stress of saffron extract against colorectal cancer [28] and against HCC both *in vitro* and *in vivo* [29]. Similarly, both the method of extraction and the extract's effects of *Graptopetalum paraguayense*, known to possess many beneficial effects including alleviating hepatic disorders, have been recently patented in US2015182572 for treating both liver fibrosis and cancer [30].

Thus, well-designed mechanism-based preclinical studies in animal models are needed to introduce crocin as effective chemopreventive agent against HCC. In experimental carcinogenesis, pre-neoplastic foci of altered hepatocytes (FAH) are considered the earliest indicative morphological changes prior to the appearance of HCC [31]. Similar progression has been described in human hepatocarcinogenesis [32].

Figure **1** illustrates the experimental scheme, where animals were randomly divided into different groups; namely control, crocin-alone, DEN-induced and crocin-protected groups. Moreover, real-time gene expression profiling was performed to identify the molecular basis for phenotypic differences and select the gene expression targets for in-depth study. Gene expression profiling provided valuable insight into the role of differential gene expression in normal biological and disease conditions.

MATERIALS AND METHODS

Additional methods are described in the Supplementary Information.

Animals

Adult male albino Wistar rats (150g-200g) were provided with free access to standard pellet diet and tap water, *ad libitum*. Rats were housed in polycarbonate cages lined with wood chip bedding, with a light/dark cycle of 12 hours at room temperature (22°C- 24°C). Prior to the experimental procedure, rats were acclimatized to the environment for two weeks. Rats were acquired from UAE University Animal House. All experiments involving animals were performed with the approval of the Animal Research Ethics Committee of UAE University.

Hepatocarcinogenesis Model

A protocol by Espandiari, *et al*. [33] was modified for our purposes, to initiate and promote hepatocarcinogenesis. DEN (Sigma Chemical Co., Missouri, USA) was used to initiate hepatocellular carcinoma, and 2-AAF (Sigma Chemical Co., Missouri, USA) was used to promote it. In this model, hepatocarcinogenesis was initiated by a single intraperitoneal injection of DEN dose (200 mg/kg b.wt.) dissolved in saline. After initiation, all rats underwent one period of 5-days fasting followed by one day re-feeding, as mitotic proliferative stimuli. Two weeks after DEN treatment, rats received 6 daily intragastric doses of 2-AAF then one dose every week for 4 weeks (30mg/kg in 1% Tween 80), for promoting hepatocarcinogenesis.

Experimental Design

Crocin (17304 SIGMA; Sigma Chemical Co., Missouri, USA) at doses 100 (Low Dose; LD) and 200 (High Dose; HD) mg/kg were administrated orally to rats. Those doses have been reported to suppress chemically induced oxidative damage in rats [34]. A total of 40 adult male albino Wistar rats were randomly divided into 5 groups (n = 8) and were subjected to different treatments. In control group (Group 1), animals were administered with distilled water (5ml/kg b.wt.) and were subjected to a single dose saline injection throughout the experimental period. Group 2 animals were

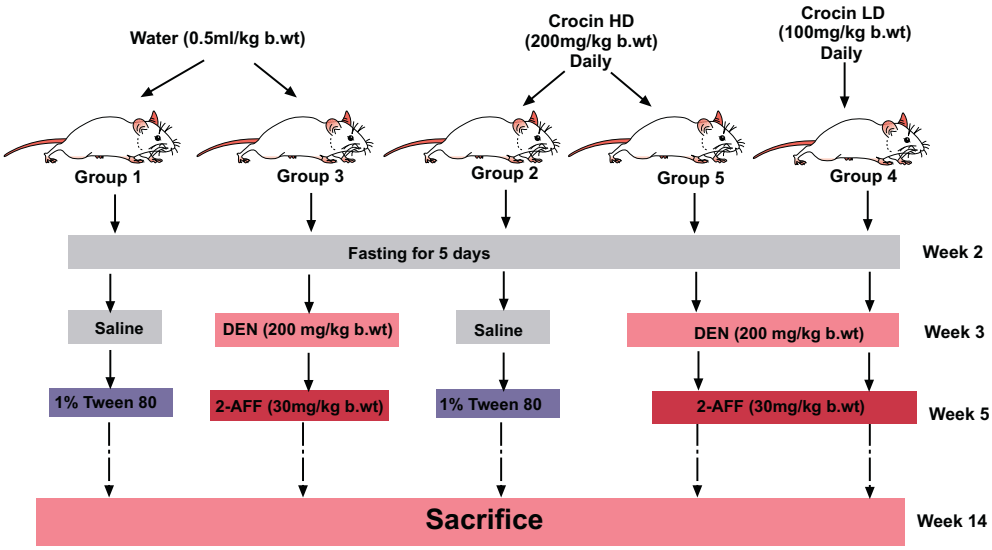


Fig. (1). Schematic representation of the experimental setup followed.

subjected to a high crocin dose (200mg/kg) through oral administration daily for the duration of the experimental period. In HCC-induced animals (Group 3), hepatocarcinogenesis was induced by DEN and promoted by 2-AAF, as reported previously. Rats of protected groups (Groups 4 and 5) were administered with LD and HD of crocin suspensions, respectively, at the beginning of promotion periods and continued daily for 12 weeks. For Group 4, animals were orally-fed with crocin at 100mg/kg; for Group 2 and 5, animals were orally fed with crocin at 200mg/kg. The experimental design is illustrated in Fig. (**1**). Fourteen weeks post DEN administration and 24 hrs after last treatment; all rats were anesthetized by diethylether 24 hrs post last treatment. Blood samples were collected through retro orbital puncture, and animals were then sacrificed.

Morphology and Histopathology

For histopathological examination, random samples of right, left, caudate lobes were obtained and immediately fixed in 10% buffered formalin. After fixation, tissues were processed and then embedded in paraffin. Five-micrometer sections were mounted onto slides for examination under light microscope (Olympus DP71; Olympus Tokyo, Japan), after performing routine staining by Hematoxylin and Eosin. The remaining liver samples were flash-frozen in liquid nitrogen and stored at 80°C.

Sample Preparation

Liver homogenates were prepared by homogenization of frozen liver samples in ice-cold 150mM Tris-HCl buffer (pH 7.4) of 1:10 wt/v ratio. Aliquots were prepared for the purpose of biochemical markers determination. Collected blood samples via retro-orbital puncture were centrifuged at 3000 rpm for 20 minutes at 4°C to obtain serum.

Immunohistochemical Staining

To examine apoptotic cells, four-micrometer liver sections were prepared. They were subjected to de-paraffinization and subsequent gradual hydration prior to probing with M30 CytoDeath monoclonal antibody (Enzo life Science, Lausen, Switzerland) to confirm cell death by detecting the caspase-cleaved fragment of cytokeratin-18.

For the purposes of immunohistochemistry assay, mounted sections were immersed in sodium citrate buffer (0.1 M, pH 6) and placed in a water bath for 15 minutes to unmask antigen epitopes. To prevent nonspecific binding to endogenous peroxidase, sections were incubated with 0.3% H_2O_2 (Sigma Chemical Co., Missouri, USA) in methanol. Rabbit anti-rat primary antibodies, anti-COX-2 (Clone SP 21; Thermo Fisher Scientific, Massachusetts, USA), anti-iNOS (Ab-1; Thermo Fisher Scientific, Massachusetts, USA), anti-NF-kB-P65 (Rel A, Ab-1; Thermo Fisher Scientific, Massachusetts, USA), and anti-Ki-67 (Thermo Fisher Scientific, Massachusetts, USA) were incubated with the slides overnight (4°C); in addition to M30 CytoDeath, monoclonal ED-2 anti-rat antibody (Santa Cruz, CA, USA), and polyclonal anti-rabbit antibodies, anti- GST-p (Medical and Biological laboratories Co., Tokyo, Japan) and anti- p-TNFR (Santa Cruz, CA, USA). Afterwards, slides were washed with PBS and incubated with secondary antibody, polyvalent biotinylated goat-anti-rabbit antibody for 10 minutes at room temperature (1:200 dilution). Universal LSAB kit and DAB plus substrate kit were both used to perform a standard staining protocol and an additional counter-staining was performed using hematoxylin. Slides were mounted and observed under an optical microscope (Olympus DP71; Olympus, Tokyo, Japan), and tissue images were obtained. In individual samples, ten fields were randomly selected to quantify positive cells (400x). Color image processor was used to count GST-p foci more than 15 cells.

Statistical and Computational Analysis

One-way analysis of variance (ANOVA) of our data was carried out using SPSS statistical program version 18 (SPSS Inc., Chicago, IL, USA). Upon detection of significant differences by ANOVA, Dunnett's t test was performed to analyze the differences between means of the treated and control groups.

Network and gene association analyses were done using Search Tool for The Retrieval of Interacting Genes (STRING) version 9.1 (http://string-db.org/) [35]. STRING is an online gene association database curated from literature, high-throughput experimental data, and predicted associations from systemic genome comparisons. Users can query the database using single or multiple name(s) and protein sequence(s) for a specific species. For our analyses, *Rattus norvegicus* databases were queried to generate association network for genes that show significant fold change in expression. STRING was also used to carry out GO term enrichment analyses of the set of genes interrogated in this study; Hypergeometric test was used to calculate P-value and Benjamini-Hochberg False Discovery rate with the cut-off value after correction < 0.05 was used for multiple testing correction. We considered the enrichment of terms with p <0.05 (after FDR correction) to be significantly overrepresented.

RESULTS

Crocin Decreases the Expression of Tumor Biomarkers and Increases Apoptotic Proteins in HCC

Histological examination of Group 3 liver sections showed pale-colored hepatocellular nodules of large, irregular morphology and a larger nuclear/cytoplasmic ratio. This is consistent with FAH's features that represent the first alteration in hepatocellular morphology prior to the emergence of HCC. However, such altered morphology was neither observed in sections taken from animals of Group 2 nor Group 1. Nevertheless, these hepatocellular nodules were morphologically less apparent in sections taken from animals of Groups 4 and 5, respectively Fig (**2a**).

Placental glutathione S-transferase (GST-p) is considered a reliable tumor-biomarker owing to its expression specificity through the extent of hepatocarcinogenesis [36]. Upon histological examination, liver sections of group 3 showed a significant increase in GST-p positive foci; which is consistent with hepatocarcinogenesis. However, GST-p positive foci were neither observed in sections taken from animals of Group 2 nor Group 1 liver sections. Color image processor was used to measure GST-p positive foci larger than 15 cells. Calculations of the number and areas of foci/cm^2 in liver sections were carried out. Upon crocin pre-treatment, the number of GST-p positive foci was significantly reduced in animals pretreated with either the low or the high dose of crocin Fig. (**2a**) & Supplementary Fig. (**1**).

Dividing cells normally have a high level of Ki-67 expression. Overexpression of this protein is usually associated

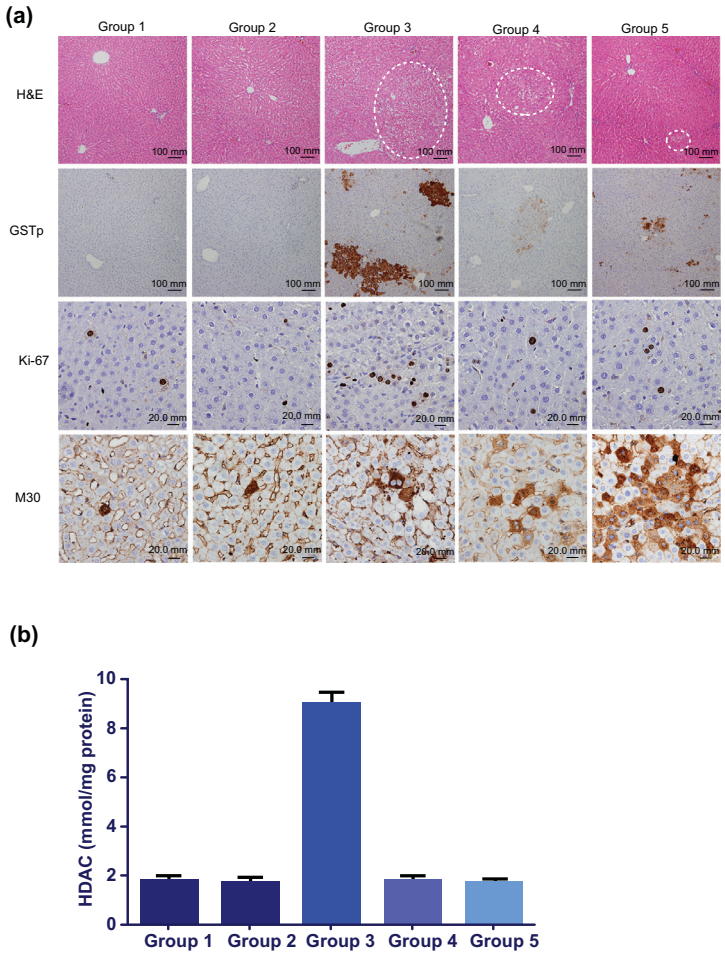


Fig. (2). Crocin decreases the expression of cancer markers and apoptotic proteins. (**a**) Representative Images of hematoxylin and eosin-stained sections (encircled parts are one representative areas of FAH) and immunohistochemical stained liver sections with GST-p, Ki-67, and M30 of all five groups studied. (**b**) Crocin inhibited HDAC activity. Concentration is expressed in mmol/ mg protein; n = 8.

with tumorgenesis as well; as seen in liver sections of group 3. The number of Ki-67-expressing hypatocytes of cancer-induced animals increased dramatically, compared to group 1. Expectedly, compared to group 1, no significance was noted for the number of Ki-67 positive cells in liver sections from animals of Group 2. Nevertheless, the numbers of Ki-67-expressing hypatocytes in liver sections from DEN-2-AAF-treated animals were significantly reduced once protected with low/high doses of crocin Fig. (**2a**) & Supplementary Fig. (**1**).

Early apoptosis was assessed by M30 CytoDeath antibody. This antibody is an apoptotic marker that specifically detects an epitope caused by caspase-cleaved keratin 18. In liver sections of Group 3, the number of M30 CytoDeath-positive cells was significantly decreased in comparison to group 1. Nevertheless, up-regulation of apoptosis was evident in liver sections of animals treated with low/high doses of crocin after DEN-2-AAF treatment. Upon histological examination, these sections displayed a dramatic elevation in M30 CytoDeath-positive cells, compared to Group 3 Fig. (**2a**) & Supplementary Fig. (**1**).

The effect of crocin, on the activity levels of Histone deacetylase (HDAC) was evaluated *in vivo*. Livers of Group 2 animals exhibited no significant change in HDAC activity, compared to Group 1. Nonetheless, chemical induction of cancer caused a significant increase in HDAC activity, in comparison to Group 1. Pretreatment with crocin restored normal expression levels of HDAC in animals treated with low and high doses of crocin prior to HCC induction Fig. (**2b**).

Crocin Inhibition of HCC is Mediated Through Inflammation

p-TNF-R1 positive cells exhibited an increase in number in liver sections of group 3. Nevertheless, the number p-TNF-R1 positive cells in liver sections from DEN-2-AAF-treated animals were significantly reduced in animals pretreated with low and high doses of crocin, in comparison to that of Group 3. Treatment with crocin alone had no significant effect on the number of p-TNF-R1 positive cells in comparison to the group 1 animals Fig. (**3a**) & Supplementary Fig. (**1**). TNF-α expression levels were assessed using ELISA Fig. (**3a**). A significant increase in the expression of TNF-α was demonstrated in Group 3, compared to group 1. However, the difference in the activity of TNF-α in animals treated with crocin alone was of no significance, compared to control animals. Nonetheless, TNF-α expression was dramatically reduced in animals treated with DEN-2AAF and pretreated with crocin.

Levels of COX-2, and iNOS, were estimated in animals treated with DEN-2-AAF. They demonstrated highly elevated levels of COX-2 and iNOS. Those levels were significantly decreased upon pre-treatment with low and high doses of crocin compared to Group 1 Fig. (**3a**) & Supplementary Fig. (**1**).

To assess macrophages' level of activity, ED-1 was used as a cellular marker. In liver sections of Group 1, a dramatic overexpression of macrophages' ED-1 is evident in Fig. (**3a**) & Supplementary Fig. (**1**). Such up-regulation of macrophages activity was significantly inhibited in Groups 4 and 5. No significant difference was detected in liver sections of animals in Group 1. To assess the activity of Kupffer cells resident macrophages, ED-2 was used as a cellular marker. In Group 3, a significant increase in the expression of Kupffer cell marker was displayed in obtained liver sections. Treatment with crocin at low and high doses after DEN-2-AAF exposure, dramatically reduced the number of ED2-expressing Kupffer cells; compared to Group 1. However, liver sections of Group 2 animals exhibited a difference of no significance compared to animals in Group 1 Fig. (**3a**) & Supplementary Fig. (**1**).

DEN-2-AAF exposure significantly increased the number of NF-κB-p65-positive cells which were expressed mostly in hepatocytes around the central vein and in Kupffer's cells, this increase in NF-κB-p65-positive cell numbers was significantly inhibited in Groups 4 and 5 compared to rats in group 3 Fig. (**3a**) & Supplementary Fig. (**1**). As parallel with NF-κB-p65 expression, crocin treatment resulted in decreased level of NF-κB-p65 in nuclear extracts indicating that crocin is able to inhibit NF-κB translocation to the nucleus Fig. (**3c**). Thus in HCC model, the crocin's anti-inflammatory effect might be mediated by NF-κB signaling pathway.

Crocin Arrests Cell Cycle and Inhibits Inflammation of HepG2 Cells

Human hepatoma cells, HepG2 were used to assess crocin's anticancer effects *in vitro*. HepG2 cells were treated with various concentrations of crocin (0.01, 0.03, 0.1, 0.3, 1mM) for 24 and 48hrs. Using CellTiter-Glo kit, crocin caused a significant dose-dependent reduction of cell viability of HepG2 cells. Crocin at a concentration of 1mM was able to reduce cell viability by almost 40% Fig. (**4a**). Effect of crocin was then investigated on cell cycle distribution using flow cytometry. Crocin induced S and G2/M phase accumulation in HepG2 cells. S Phase accumulation occurred from 24 to 72 h whereas cells accumulated at G2/M phase at 72h Fig. (**4d & 4e**). A dramatic decrease of interleukin-8 (IL-8) secretion by HepG2 cells was noted as early as 6 hrs post crocin treatment Fig. (**4b**). In support of the previous *in vivo* results, protein levels of TNF receptor 1 (TNFR1) were reduced after treatment with crocin starting at 12hours, but most notably at 48 hours Fig. (**4c**).

Network Analysis Showed NFκB and CCl20 as Key Genes of Crocin's Action in HCC

Based on the analysis of genes differentially expressed in tissues of animals in Groups 4 and 5 in comparison to their expression in Group 3 animals' tissues, 29 out of 160 genes have shown an observable fold change which were selected for further network analysis Fig. (**5a**).

The set of genes interrogated in this study consisted of genes implicated in apoptosis and inflammation. A network was created based on multiple association criteria, i.e., neighborhood, gene fusion, co-occurrence, co-expression experiments, databases, text-mining and homology, by plotting the corresponding proteins of the selected genes with obtained connections from published literature. Highly

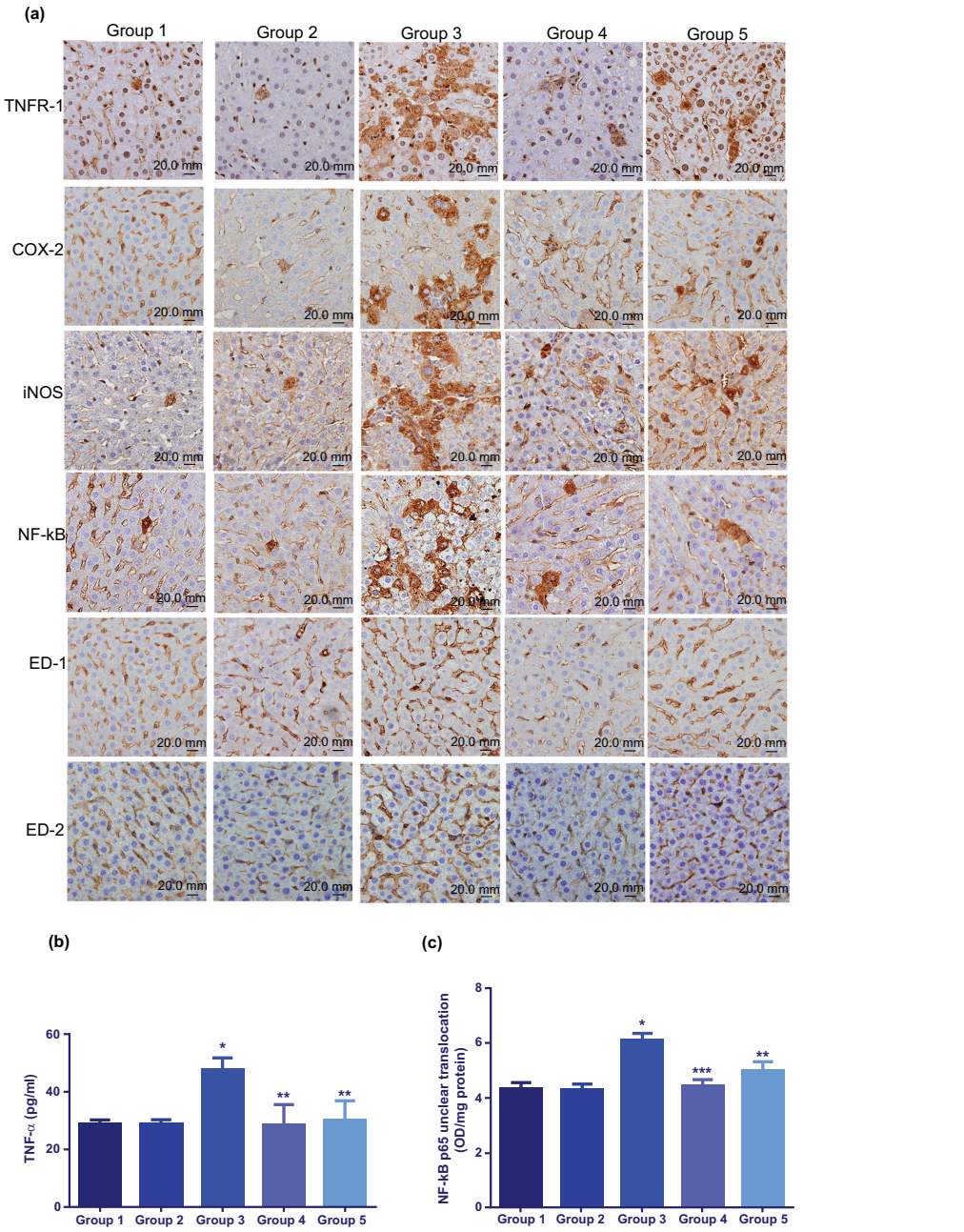


Fig. (3). Crocin treatment ameliorates the inflammatory markers and core-signaling molecule NF-κB. (**a**) Representative images of immunohistochemical staining with p-TNF-R1, COX-2, iNOS, NFκB- p65, ED-1, ED-2 in the liver sections from all the groups studied. (**b**) Crocin affected TNF-α content in serum. Concentration is expressed in pg/ml. (**c**) Quantitative analysis of NFκB- p65 nuclear localization. NFκB- p65 nuclear localization represented as fold change (relative to control group).

connected nodes or hubs in networks often carry important or essential functions. Hubs were identified in the context of the generated network based on the highest number of connections to other target proteins Fig. (**5a**). One particular hub of interest is NF-κB1 which was expressed in both inflammatory and apoptotic pathways and exhibited an observable fold change. Notably, CCL20 was ranked first among the 29 genes with significant observable fold changes of -4.9143. Consistently, CCL20 receptor, CCR6 exhibited and observable fold change with a value of -1.6211. Furthermore, NF-κB interacts with DNA fragmentation complex through DFFB Fig. (**5a**), an interaction that can be DFFB Fig. (**5a**), an interaction that can be implicated with the observed apoptotic effect of crocin.

Gene Set Enrichment Analysis Identifies Immune Response and Cell Death as Key Processes Affected by the Treatment

To define the over-representation of functions and biological processes, as defined by the Gene Ontology Consortium (or "GO") [37], we queried the STRING database (http://string-db.org/) for enrichment of "Biological Process" and "Molecular Function" terms Fig. (**5b & 5c**) and Supple-

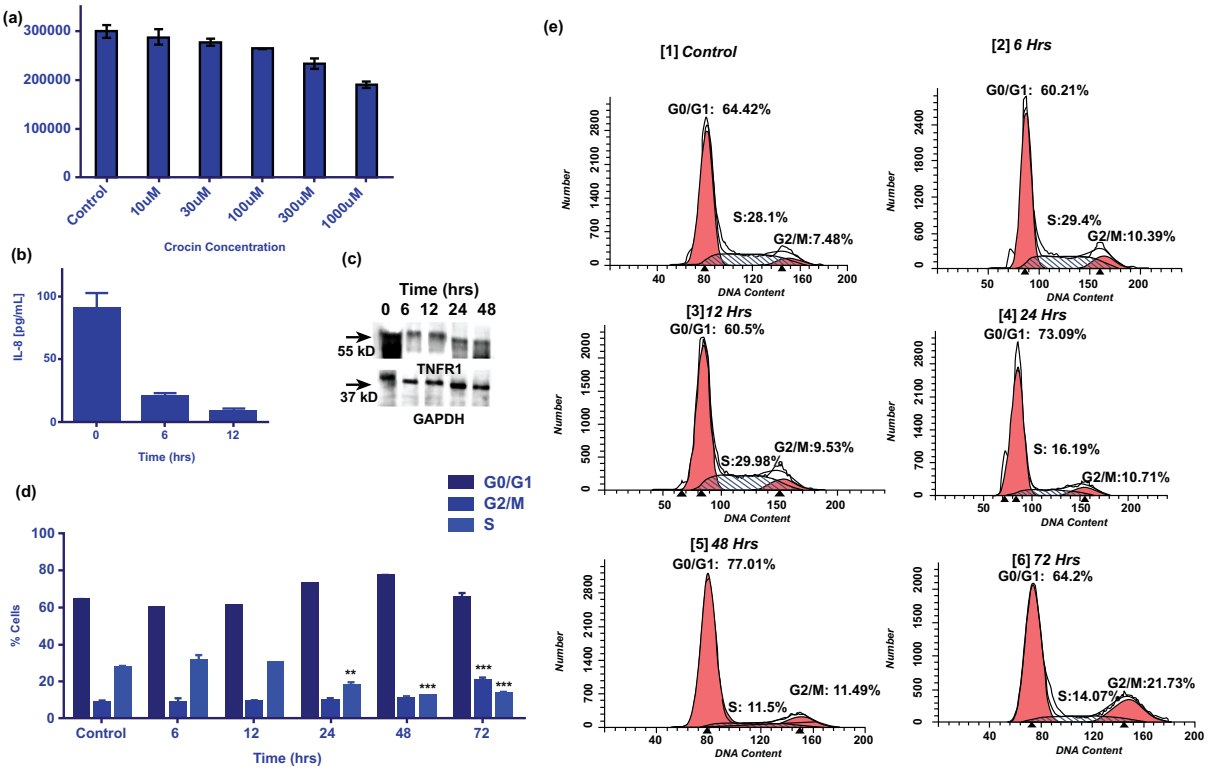


Fig. (4). The effect of crocin on liver cancer cell line HepG2. (**a**) Viability assessment after crocin treatment showing the HepG2 cells untreated (control) or treated with different concentrations of crocin (10-1000μM) for 24 hours. Data shown are performed in triplicate (*$P <$ 0.001). (**b**) IL-8 secretion after (3mM) crocin treatment. HepG2 cells were treated with 3mM for 6 and 12hrs, and subsequently, the supernatants were analyzed by IL-8 ELISA. (**c**) Cell lysates prepared from HepG2 cells treated with (3mM) crocin for 6, 12, 24, and 48 hrs, were analyzed by anti-TNFR1 and anti-GAPDH western blotting. GAPDH served as an internal control for equal loading. (**d**) Cell cycle distribution after crocin treatment for 6, 12, 24, 48, 72 hrs. Values were expressed as mean ± SEM and significance was determined by one-way analysis of variance followed by Dunnett's t test. ** $P < 0.005$ vs. Control, *** $P < 0.001$ vs. Control (**e**) Effect of crocin on cell cycle progression. Cells were untreated (control) or treated with (3 mM) crocin for 6, 12, 24, and 48 hours, harvested, and DNA was stained with PI for flow cytometric analysis of DNA content with BD FACSCanto II.

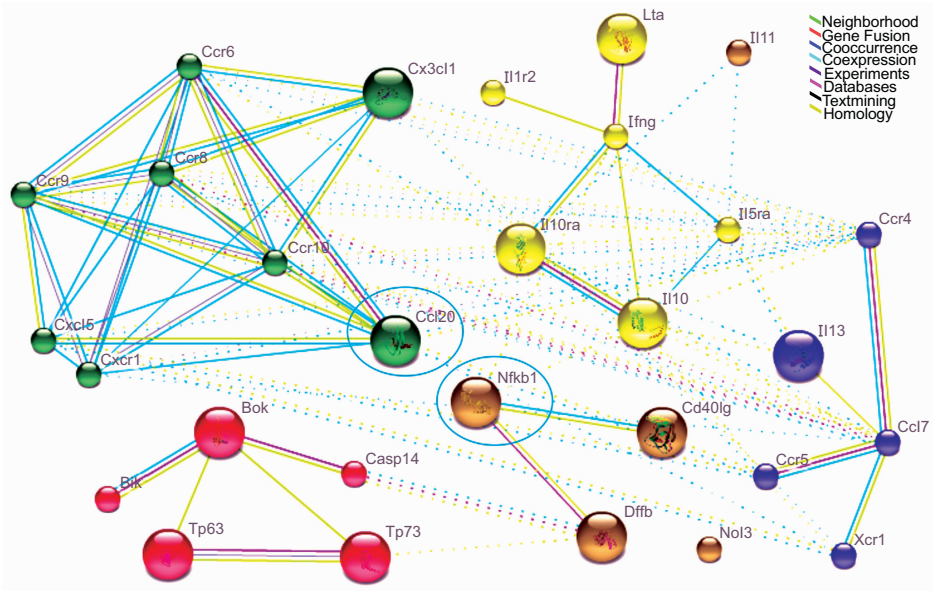
mentary Fig. (**3**). We selected the results that returned *p* values of < 0.05, after false discovery rate (FDR) correction. Our analyses indicated over representation of a large number of biological processes terms (931 terms with $p < 0.05$) in the full gene set Supplementary Fig. (**3a**); however, only 46 terms were overrepresented among the 29 DEGs. Among these, "immune response" has the highest number of genes (10 associated genes), followed by "response to stress" and "immune system process (9 and 7 genes respectively) Fig. (**5b**) and Supplementary Fig. (**2**). Enrichment analysis for Molecular Function ontology indicated enrichment of "cytokine receptor activity" (8 genes), "cytokine activity", "G-protein coupled peptide receptor activity", and "peptide receptor activity" (5 genes), "cytokine binding" and "C-C chemokine receptor activity" (3 and 2 genes respectively) Fig. (**5C**) and Supplementary Fig. (**3**). The present results indicate a highly specific response for crocin in relation to DEN-induced cancer treatment.

DISCUSSION

Crocin's anti-inflammatory, anti-proliferative, and pro-apoptotic effects are well-documented here both *in vitro* and *in vivo*. The present results introduce crocin as a potential natural-based chemopreventive agent against early hepatic pre-neoplastic events.

GST-P overexpression is associated with carcinogenesis and drug resistant cancers [38, 39], and is commonly used as a reliable marker of hepatocarcinogenesis [36]. In this study, the initiation-promotion DEN-induced cancer model used mimiced the early events of the latent period of human carcinogenesis. The initiation stage of cancer development can be produced by the administration of single dose of DEN, a carcinogen that causes DNA methylation and mutagenesis. DEN has been used to study different types of benign and malignant tumors in humans by inducing resembling lesions in rats [33]. Initiation is followed by a growth stimulus (Fasting and re-feeding) during treatment with promoting agent such as 2-acetylaminofluorene (2-AAF) that induces selective proliferation of the initiated cell population over non-initiated cells in the target tissue [40]. In this study, administration of crocin to DEN-induced HCC model caused a dramatic decrease in the number of GST-P positive foci that was consistent with a visible reduction in FAH formation that represents the initial morphological change in HCC development.

Key alterations in the physiology of cancer cells that are essential for tumor progression include a persistent increase in cellular proliferation and disrupted apoptosis machinery. To that end, this investigation reported a significant antiproliferative activity of crocin that was evident in the dramatic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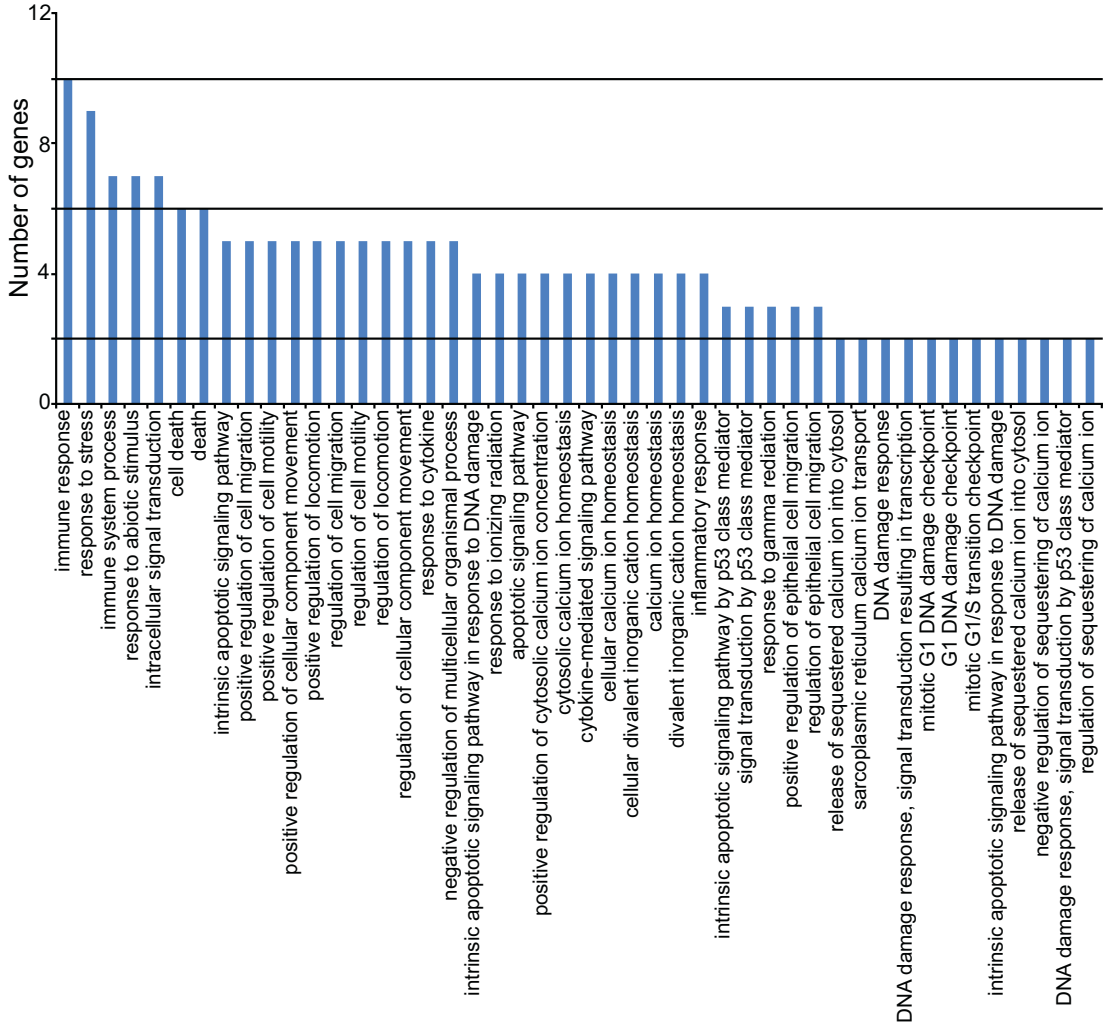
Number of genes
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immune response
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immune system process
response to abiotic stimulus
intracellular signal transduction
cell death
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intrinsic apoptotic signaling pathway
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mitotic G1/S transition checkpoint
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B

Fig. (5) contd….

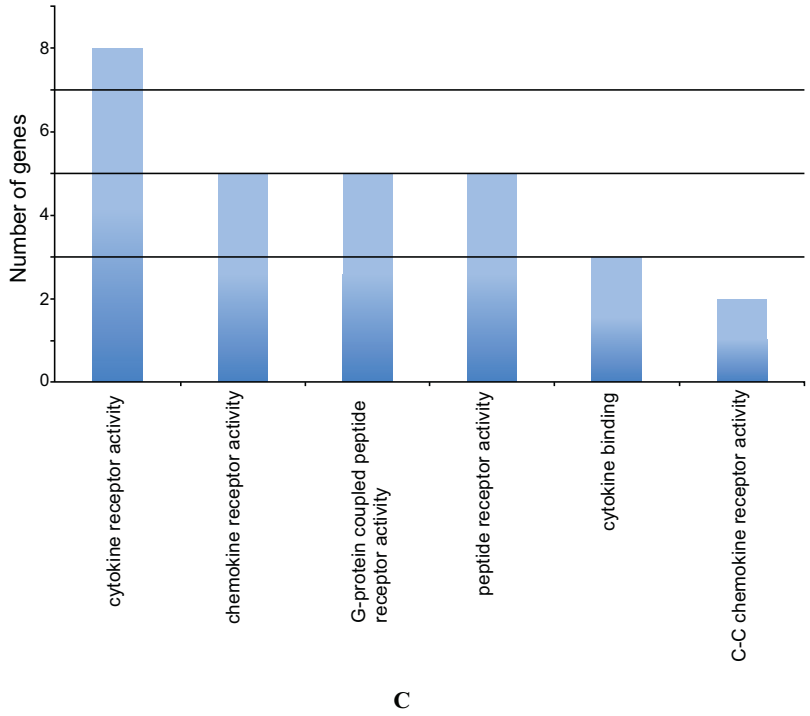

Fig. (5) Network Analysis shows NFκB and CCl20 as main mediators of crocin action in HCC. (**a**) Network of the functional relationship of the 29 genes and corresponding proteins that exhibited statistically significant fold changes. Differentially colored lines and nodes indicate the different association type and corresponding protein complexes, respectively. The purple cluster of chemokines, CCL20, CCR6, CX3CL1, CXCL6, are involved in cancer cell proliferation, migration, leukocytes recruitment and angiogenesis, respectively. Members of this cluster are under either in-direct or direct control of NFκB. (**b**, **c**) GO term enrichment analyses for Biological Process and Molecular Function. The set of differentially expressed genes (DEG's) was queried for overrepresentation through the STRING database. Only those with enrichment probability value of $p < 0.05$ (after FDR correction) are reported; (**b**), enrichment for Biological Process (**c**), enrichment for Molecular Function. The number of genes per term is indicated in the Y-axis.

reduction of Ki-67 expression, which is strictly associated with proliferation, in HCC-induced rats. The antiproliferative activity of crocin was also associated with the induction of apoptosis as evidenced by increasing the number of and M30 CytoDeath-positive cells. A similar trend was evident *in vitro* where a significant decrease in cell viability was observed in crocin-treated HepG2 cells, particularly at 300uM dose. Crocin also caused significant cell cycle arrest at S and G2/M phases which was consistent with the crocin-induced antiproliferative effect *in vivo*. In addition, crocin treatment caused a significant increase in the percentage of apoptotic HepG2 cells as early as 6 hours. These results are in agreement with previous investigations describing the anti-proliferative and pro-apoptotic effects of crocin in different cancer cell lines [26, 27]. These results indicate that the inhibition of hepatic neoplasia in rats was associated with an up-regulation of apoptosis and a down-regulation of cellular proliferation.

Chronic inflammation elicits early alterations associated with the development of cancer through the activation of macrophages and Kupffer cells as well as the production of pro-inflammatory mediators such as nitric oxide, TNF-α, and transcription factors such as NF-κB [41]. Crocin exhibited great efficacy in eliminating inflammation in HCC-induced rats in the present work. This is evident in restoring hepatic MPO levels Supplementary Fig. (**3**), a marker of neutrophil infiltration [42]. Moreover, administration of crocin significantly decreased the number of hepatic ED1- and ED2- positive macrophages and, the number of p-TNF-R1 positive cells and the content of TNF-α. TNF-α is a major mediator of proliferations and inflammations and its tumor-promoting action involves TNFR1 activation. Such results were supported by *in vitro* analyses where crocin-treated HepG2 cells demonstrated a reduction in TNF-R1 protein level, in a time dependent manner. Elevated levels of COX-2 and iNOS enzymes have been shown to play a key role in promoting cell growth and inhibiting cell death in many malignant tumors [43]. Hence, the inhibition of these enzymes can inhibit tumor growth. In the present work, both COX-2 and iNOS levels were dramatically decreased in groups 4 and 5 animals. Several studies described NF-κB as procarcinogenic regulator in DEN-induced HCC [44]. NF-κB transduces oxidative stimuli to nucleus to modulate the expression of many genes involved in inflammatory responses, the up-regulation of COX-2 and iNOS, the cell proliferation, and the cell death [45]. In addition, IL-8 is a chemokine with CXC-motif (CXCL8) associated with inflammatory responses. Tumorigenic and proangiogenic properties of IL-8 have been reported where increased expression of IL-8 is detected in many solid tumors including prostate [46], and intestine [47]. Moreover, overexpression of IL-8 has been described in infiltrating neutrophils and tumor-associated macrophages. It has also been reported to increase proliferation and survival

of endothelial cells, which suggest a role that IL-8 may play in regulating the tumor microenvironment [48]. In this report, administration of crocin to HCC-induced rats reversed DEN-induced up-regulation and nuclear translocation of NF-κB-p65 subunit. Taken together, these results suggest that crocin's anti-cancer properties could be mediated through its anti-inflammatory activity by down-regulating NF-κB, IL-8, COX-2 and iNOS expression levels and reducing the expression of both TNF-α and its receptor.

HDAC is a class of enzymes that facilitates the removal of acetyl groups from ε-amino groups from lysine amino acids on histones tails which allow DNA to be more tightly packed around histones; and their role in modulating gene expression has been shown [49]. A change in HDAC activity has been described in several diseases, including many types of cancer [50]. Moreover, higher levels of HDAC expression were reported in HCC that correlated with higher incidence of cancer invasion, and considered as an independent prognostic factor after liver resection in HCC patients [51]. HDAC inhibitors (HDACis) have been reported to trigger apoptosis, in transcription-dependent and -independent manners, in both hematological and solid cancers [52]. Moreover, HDACis have been implicated in causing DNA damage [53], in arrested cells, and in inducing pro-apoptotic genes expression [54]. In this study, crocin demonstrated a significant efficacy in abolishing the elevated HDAC activities in HCC-induced rats, where HDAC activity was restored to normal levels when compared to group 1 and 2. Together, these results suggest that crocin's anti-proliferative and pro-apoptotic effects could be attributed, at least in part, to their ability to inhibit HDAC activity in cancerous cells. Indeed, it has been suggested that HDACis mechanism of lethality partially depends on the anti-inflammatory mode of action through the reduction of inflammatory mediators such as NF-κB [55]. For instance, HDACis are reported to induce cell cycle arrest in colorectal cancer, and differentiation of colon cancer cell lines through suppressing proteasome activity mediating TNF-α-induced NF-κB activation [56]. Similar results were reported in non–small cell lung cancer, *in vivo* and *in vitro*, where HDACis reduced NF-κB activation through TNF-α stimulation [57].

According to our network analysis, we were able to identify 160 genes that were differentially expressed in rats in Group 5 from those in group 3; 29 genes of which exhibited a statistically significant fold-change, including NF-$_k$B, CX3CL1, CCL20 and its receptor CCR6. The functional relationship of the 29 corresponding proteins has led to the identification of NF-$_k$B and CCL20 as regulatory hubs based on their high number of connections to other components in the system. For example, NF-$_k$B was associated with CCL20 according to their co-expression in other species. Indeed, CCL20 is reported to be under direct control of NF-$_k$B [58] through TNF-α stimulation; which has been blocked in HCC-induced rats in response to crocin treatment. In addition, Inhibitor of NF-$_k$B, I-$_k$B, was reported to inhibit such stimulation in murine liver injury model; illustrating that CCL20 expression is highly influenced by TNF-induced NF-$_k$B activation. Moreover, a significant increase in CCL20 mRNA level was triggered due to overexpression of NF-$_k$B-p-65 *in vitro* [59]. CCL20 has been shown to play a paramount role in cancer cell proliferation and migration *in vitro* [60], and consistently, overexpression of CCR6 was observed in colorectal cancer [61]. Additionally, CCL20/CCR6 has been reported to have a significant role in HCC progression, *in vitro* and *in vivo*. Similar results were reported in HepG2 cell line, where CCL20/CCR6 was found to be up-regulated [62]. Such up-regulation of both CCL20 and its receptor CCR6 has been reported in HCC tissue as well, where levels of expression and differentiation degree were highly associated [63]. According to differential gene expression analysis in our study, NF-$_k$B expression has shown a fold decrease in groups 4 and 5 when compared to its expression in group 3 animals. Similarly, CCL20 expression has shown the highest fold change with four-fold decrease; which could be attributed to crocin effects against NF-$_k$B.

Moreover, CX3CL1 expression is partially coordinated by NF-$_k$B in vascular endothelial cells [64]. Many studies have reported the involvement of CX3CL1 in several inflammatory diseases and malignancies through its recruitment of leukocytes [65]. For instance, increased expression of CX3CL1 has been linked to inflammatory bowel disease due to its significant role in leukocyte mediation, adhesion, and chemo-attraction [66]. Additionally, higher expression of CX3CL1 has been reported to induce cellular migration in human RCC cell lines, and to promote metastasis of clear cell renal cell carcinoma *in vivo* [66]. Moreover, up-regulation of CX3CL1 has been observed during liver injury in humans, which can also be attributed to the ability of CX3CL1 to recruit leukocytes [67]. CX3CL1 up-regulation has been reported in HepG2 cell lines as well [62]. According to our differential gene expression analysis, CX3CL1 showed a two-fold decrease in groups 4 and 5 animals when compared to group 3 animals; which could also be attributed to crocin effects against NF-$_k$B. Taken together, all of the previous results illustrated the significant role of NF-κB in HCC tumorigenesis; which could identify NF-κB as a regulatory hub and a candidate therapeutic drug target.

In summary, this study demonstrated the effects of crocin in chemically-induced HCC animal model and promoting crocin as a potential HCC drug. Crocin inhibited the formation of FAH in DEN-induced HCC models, which was accompanied with reduced oxidative stress and restored levels of antioxidants. Moreover, treatment with crocin decreased the activity of inflammatory markers, COX-2, iNOS, NF-$_k$B, TNF-α and its receptor p-TNF-R1; in addition to a reduction in the number of Kupffer cells and macrophages. Crocin exhibited anti-proliferative and pro-apoptotic properties when administrated to DEN-treated rats. Similar results were shown *in vitro* where crocin caused G2 cell cycle arrest and thus blocked proliferation of HepG2 cells. Pro-apoptotic and anti-inflammatory effects of crocin have also been shown. The evident anticancer properties of the naturally-occurring crocin make it not only a significant chemopreventive agent but also a potent adjuvant therapy as well.

CURRENT & FUTURE DEVELOPMENTS

Liver cancer continues to make the list of handful most lethal types of cancer worldwide. The poor diagnosis and limited therapeutic options of liver cancer mandate more concerted efforts to overcome the tumor genetic heterogeneity that often leads to an inevitable drug resistance. In fact,

numerous cytotoxic chemotherapies, molecular target drugs, immuno- and hormonal therapies against different types of cancers have been developed and patented as detailed earlier. It is becoming increasingly evident however that most cancers cannot be successfully addressed solely with single-target therapies. Developing such a broad-spectrum approach [22] necessitates more preclinical models for testing of combinatorial therapies of agents comprising multiple phytochemicals. The preclinical investigations presented here clearly demonstrated the crocin's anti-inflammatory, proapoptotic and antiproliferative effects against liver cancer. Further studies are currently underway in our lab to assess the effects of crocin (along with other natural occurring ingredients) against liver cancer in combinations with other commonly used chemotherapeutic drugs.

LIST OF ABBREVIATIONS

2-AAF	=	2-Acetyl Aminofluorene
DEN	=	Diethylnitrosamine
FDR	=	False Discovery Rate
FAH	=	Foci of Altered Hepatocytes
GO	=	Gene Ontology Consortium
GST-p	=	Glutathione S-Transferase-Placental Form
HDACis	=	HDAC Inhibitors
HCC	=	Hepatocellular Carcinoma
HDAC	=	Histone Deacetylase
IL-8	=	Interleukin-8
HD	=	High Dose
LD	=	Low Dose
TNFR1	=	TNF receptor 1

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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