IN VIVO ASSESSMENT OF SAFRANAL'S NOVEL THERAPEUTIC EFFECTS ON CHEMICALLY INDUCED HEPATIC NEOPLASIA

Ameera Ali M. AlMansoori

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IN VIVO ASSESSMENT OF SAFRANAL’S NOVEL THERAPEUTIC EFFECTS ON CHEMICALLY INDUCED HEPATIC NEOPLASIA

Ameera Ali Mohammed AlMansoori

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Professor Amr Amin

November 2018
Declaration of Original Work

I, Ameera Ali Mohammed AlMansoori, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “In Vivo Assessment of Safranal’s Novel Therapeutic Effects on Chemically Induced Hepatic Neoplasia”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Amr Amin, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Chronic liver insult leads to fibrosis, which often ends up causing cirrhosis and most of the time that progresses into hepatic neoplasms (early Hepatocellular carcinoma; HCC). HCC is the fifth most common cancer, and the third cause of cancer-related deaths. Chemotherapy is the most common treatment of cancer patients. HCC is however, chemo-resistant, and the side-effects of chemotherapy are typically exhausting to the patient. Sorafenib is the only anti-HCC drug approved by the U.S Food and Drug Administration. It is a multikinase inhibitor that blocks tumor cells proliferation and angiogenesis. Although sorafenib is successful treating early and mid HCC lesions, it is not efficient in advanced HCC cases. Safranal, a major biomolecule of saffron “stigmas of the flowers of Crocus sativus L.”, is known for its anti-oxidant, proapoptotic and anti-inflammatory effects against different cancer types. Compared with monotherapy, combination therapy (safranal + sorafenib) targeting multiple signaling pathways offered a better treatment alternative potentially abolishing resistance, feedback activation, and compensatory activation of survival pathways. This study investigated the therapeutic effect of safranal on DEN-induced hepatic neoplasms, in vivo, using male Wistar rats. Safranal was found to be involved in cell cycle arrest particularly at G2/M phase, and to induce the intrinsic, mitochondrial, apoptotic pathway leading to cell death. This study highlights safranal’s therapeutic potential against HCC and introduces it as a novel natural therapeutic and adjuvant agent against HCC.

Keywords: Hepatocellular carcinoma, safranal, sorafenib, apoptosis, cell cycle arrest.
تقييم الآثار العلاجية للسافرانال ضد سرطان الكبد المستحث كيميائياً في الجرذان

الملخص

تؤدي إصابة الكبد المزمنة إلى تليفه، والذي غالباً ما يؤدي إلى تشمع الكبد، و في معظم الوقت يتطور إلى أورام كبدية (سرطان الكبد). سرطان الكبد هو خامس نوع من السرطانات الأكثر شيوعاً، والسبب الثالث للوفيات المرتبطة بالسرطان. العلاج الكيميائي هو الأكثر شيوعاً لعلاج مرضى السرطان، ومع ذلك، فإن سرطان الخلايا الكبدية يقاوم العلاج الكيميائي، بالإضافة إلى أن الآثار الجانبية للعلاج الكيميائي عادة ما تكون مرهمة للمريض. سورافينيب هو الدواء الوحيد المرخص من قبل إدارة الغذاء والدواء الأمريكية المستخدم في علاج سرطان الكبد. وهو يمنع انتشار الخلايا الورمية وتولد الأوعية الدموية الجديدة. على الرغم من نجاح دواء السورافينيب في علاج الأعاب المبكرة والمتوسطة من سرطان الخلايا الكبدية، إلا أنه غير فعال في حالات سرطان الكبد المتقدمة. سافرانال، هو جزيء حيوي رئيسي من نبات الزعفران معروف باثاره المضادة للأكسدة والمضادة للالتهاب ضد أنواع مختلفة من السرطان. مقارنة بالعلاج الأحادي، العلاج المركب (سورافينيب + سافرانال) الذي يستهدف المسارات المتعددة للسرطان قد خيارًا أفضل للعلاج عن طريق توفير طريقة لإبطال المقاومة، وتفعيل ردود الفعل من الجسم. هذه الدراسة بحثت تأثير السافرانال العلاجي على سرطان الكبد الناتج عن حقن مركب Diethylnitrosamine في الجرذان من نوع Wistar. نتائج البحث تشير أن السافرانال يؤثر في دورة الخلية المزمنة Diethylnitrosamine في اعتقال دورة الخلية خصوصاً في مرحلة G2/M (طور النمو الثاني وطور الانقسام)، وأيضاً يبحث على موت الخلية المبرمج في المسار الداخلي، مما يؤدي إلى موت الخلايا السرطانية. هذه الدراسة تسلط الضوء على أهمية السافرانال ضد سرطان الكبد كعامل علاجي طبيعي ومساعد.

مفاهيم البحث الرئيسية: سرطان الكبد، سافرانال، سورافينيب، موت الخلية المبرمج، اعتقال دورة خلايا الخلية.
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Dedication

To my beloved parents, Ali & Rana, who always believe in me
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<th>Description</th>
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<tbody>
<tr>
<td>$\times g$</td>
<td>Centrifugal Force</td>
</tr>
<tr>
<td>$^\circ C$</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>Microgram</td>
</tr>
<tr>
<td>$\mu l$</td>
<td>Microliter</td>
</tr>
<tr>
<td>$\mu m$</td>
<td>Micrometer</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>Apaf1</td>
<td>Apoptotic Protease Activating Factor-1</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2-associated X Protein</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid Assay</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell Lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAK</td>
<td>Cdk-activating Kinase</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-aspartic Protease</td>
</tr>
<tr>
<td>Cdc25B</td>
<td>Cell Division Cycle 25B</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent Kinase</td>
</tr>
<tr>
<td>DEN</td>
<td>Diethylnitrosamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>gm</td>
<td>Gram</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatoma G2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal Injection</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram Per Kilogram</td>
</tr>
<tr>
<td>mU/ml</td>
<td>Milliunits Per Milliliter</td>
</tr>
<tr>
<td>PARP</td>
<td>PolyADP-ribose Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay Buffer</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SB</td>
<td>Sorafenib</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SF</td>
<td>Safranal</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single Stranded DNA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline with Tween</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Cancer

Cancer starts with a single defected cell that grows and multiplies into a group of cancerous cells. This group of cells, tumor, needs to go through several important hallmarks to survive and spread. The normal cell goes through complex mechanisms of reproduction, growth, and death with the help of signals and enzymes to maintain a normal tissue or organ size. Cancer cells, on the other hand, keep proliferation and replication signals on, skip growth suppressors and cell death, invade neighboring tissue and make new blood vessels to survive (Figure 1; Hanahan & Weinberg, 2011). There are several factors that could lead to cancer development such as smoking, radiation, and viruses, thus, a healthy lifestyle is highly recommended especially for those with a family history of cancer (Martin & McFerran, 2017). The International Agency for Research on Cancer reported 14.1 million cases of cancer and 8.2 million deaths worldwide in 2012 (Ferlay et al., 2015). The number of deaths increased to 8.8 million in 2015 advancing cancer to be the second cause of death worldwide (World Health Organization, 2018). In the United Arab Emirates (UAE), cancer is the third cause of death, and according to the UAE Ministry of Health – Abu Dhabi, there are around 500 cancer deaths every year (Tadmouri & Al-Sharhan, 2004).
1.2 The Liver

The liver is the largest internal organ of the body, which lies in the top right abdominal cavity and weighs around 1500 gm. It secretes bile; which helps digesting fat and neutralizing stomach acid. It is also, where carbohydrates, proteins, and fats are metabolized. Liver also helps maintaining blood sugar and producing blood proteins essential for the detoxification process. Different types of cells make up the liver tissue, with hepatocytes being the most represented cell type where they occupy 80% of the liver (Kmieć, 2001). Hepatocytes form sheets that face the blood veins in the liver, they synthesize, degrade and store many substances, including glycogen. Important vitamins are stored in the liver such as vitamins A, D, K, and B₁₂ (Cammack et al., 2006c, 2006b, 2006a; Concise Medical Dictionary, 2010).
1.2.1 Hepatocellular Carcinoma

There are different types of liver cancer; intrahepatic cholangiocarcinoma and hepatoblastoma, but the most common type is hepatocellular carcinoma (HCC). Repeated liver injury or disease causes acute inflammation in the liver, which is usually reversible, then if not treated progresses to fibrosis and cirrhosis (irreversible), and finally hepatic neoplasms (Ramakrishna et al., 2013). HCC is the fifth most common cancer type and the third cause of cancer-related deaths worldwide (Zhang et al., 2014). In the UAE, liver cancer is the fourth cause of death related to cancer in both men and women (Figure 2; Department of Health - Abu Dhabi, 2016). The mortality rate has increased with an average of only 5-year survival. Among known HCC risk factors, hepatitis C and B viral infections and alcohol consumption are the most common. However, 15 – 50% of HCC cases emerge without known causes (Zhou et al., 2016). Different chemotherapeutic drugs are used to treat HCC including fluorouracil, cisplatin, doxorubicin, paclitaxel and mitomycin. Side effects, caused by the non-selective cytotoxicity of these drugs, remain a major challenge (Subramaniam et al., 2013). Increasing numbers of patients opt for surgical intervention. Removing the tumor and liver transplant are most common in early stages of HCC with 50% survival chances, however, most of later-staged patients have limited treatment options (Pang & Lam, 2015).
Figure 2: Top four main causes of death related to cancer in the United Arab Emirates – Abu Dhabi in men and women (Department of Health - Abu Dhabi, 2016)

1.3 Sorafenib

Sorafenib, a multikinase inhibitor, is known to block tumor proliferation, and inhibit angiogenesis. It is also, the first U.S. Food and Drug Administration (FDA) approved HCC therapy (Finn et al., 2013; Subramaniam et al., 2013). However, survival rates with sorafenib are barely extended by 5 – 10 months compared to placebo (El-Serag, 2017). The common side effects of sorafenib are skin toxicity, diarrhea, hypertension, and bleeding (Crissien & Frenette, 2014). Therefore, exploring other treatment options is well justified in an attempt to provide an efficient treatment or adjuvant anti-HCC drug with minimum side effects.

1.4 Plants in Medicine

Plants have always been the first source of treatment throughout history. As a main source of human food, plants are generally considered to be safe. Thus, plants have traditionally been used for health promoting purposes (Prinsloo et al., 2018). Around 50,000 plant species have been exploited as a source of different drugs. Aspirin from willow bark, quinine from cinchona bark and morphine from the opium poppy are a few examples.
Thanks to their broad spectrum benefits that are associated with minimal side-effects, people are increasingly turning to plants for treatment despite all recent developments in the field of drug discovery (Rana & Rana, 2014). Nearly 50% of approved anti-cancer drugs are plant in origins (Veeresham, 2012; Greenwell & Rahman, 2015).

1.4.1 Plants and Cancer Therapy

Recent studies have shown the beneficial effects of plant extracts in cancer therapy due to their low side effects, and anti-tumor activities. Modern therapy is also shifting to plants as boosters for current medicines of chemotherapy, or even for novel replacements. Investigations showed that plants stimulate the immune system, and exhibit antibacterial, anti-inflammatory, antioxidant, and anti-cancer effects (Ahmad et al., 2017).

1.4.1.1 Saffron

*Crocus sativus* L. from the family Iridaceae has a purple flower with three red stigmas, called saffron, which is a natural spice and colorant. Saffron is mainly grown in Iran and Spain. Due to the extreme delicacy needed in harvesting and handling saffron, it is considered the most expensive “golden” spice. To yield 500 gm of pure saffron, 70,000 flowers must be handpicked. Saffron contains more than 150 bioactive ingredients. Crocin, picrocrocin, and safranal are the most common among all those ingredients. They are responsible for saffron color, taste and odor, respectively. Saffron has traditionally been used as antidepressant, antispasmodic, decongestant, and a sedative. It was also used to treat fever, smallpox, and colds (Gohari et al., 2013; Rezaee & Hosseinzadeh, 2013; Srivastava et al., 2010).
1.4.1.1 Safranal

Safranal, the oily compound of saffron, is the main source for saffron odor. Safranal is known as an antioxidant and it protects against seizure and has a cytotoxic effect against cancer cells (Rezaee & Hosseinzadeh, 2013). In neuroblastoma cells, safranal has been reported to inhibit the growth of malignant cells by inducing apoptosis (Samarghandian et al., 2014).

1.5 Cancer in Research

Research has helped to understand the molecular mechanisms of cancer and therefore offering ways to control it. In the lab, *in vivo* and *in vitro* methods are commonly used to investigate and assess different treatment strategies. *In vitro* setting is based on assessing different drugs in isolated human cancer cell lines; outside the living body (Hine & Martin, 2015). The main disadvantage of such approach is that cultured cancer cells do not mimic the natural environment of a tumor in a living body. *In vivo* setting research induces and hence studies tumors in living organisms rather than in isolated cells (Lackie, 2010). Whole organisms, like rats, mice, and rabbits, are often used *in vivo* where natural tumor environment is better assessed. There are several ways to induce cancer in animals by using chemicals, viruses, gene editing, or a transplant (Figure 3; Santos et al., 2017).
1.5.1 Diethylnitrosamine

Diethylnitrosamine (DEN) is a widely used chemical for inducing cancer. N-nitroso compounds are known for their mutagenic and carcinogenic properties. In 1956, investigators reported liver carcinogenesis in albino rats fed with a diet containing dimethylnitrosamine. Then few years later, a sheep died of liver toxicity after feeding from a meal that was preserved with nitrite. Thus, DEN effect was heavily studied and was shown to initiate liver injury, and lead to liver fibrosis, cirrhosis, and finally HCC. DEN can also induce tumor in respiratory tract, kidney, upper digestive tract, and hematopoietic system (Santos et al., 2017).
1.6 Objectives of the Study

The aim of this study is to examine safranal’s therapeutic effects against chemically induced hepatic neoplasms and that involves:

2. Biochemical assessment of liver function.
3. Histopathological examination of liver tissues.
4. Western blotting examination of representative markers.

1.7 Hypothesis

We hypothesize that safranal will have a suppressive effect on DEN-induced hepatic neoplasms *in vivo*. 
Chapter 2: Methodology

2.1 In vivo Study

2.1.1 Animals

Male Wistar rats, weighing around 160 gm, were used in this study. Rats were provided by the animal research facility at the College of Medicine and Health Sciences, UAEU. Rats were housed under a 12-hour light/dark cycle at 24 – 26°C. They were maintained on a standard laboratory animal diet with food and water ad libitum. Animal ethics form has been submitted and approved by the Animal Research Ethics Committee of the College of Medicine and Health Sciences, UAEU (Approval No. A8-15).

2.1.2 Experimental Design

A modified version of the protocol described by DePeralta et al. (2016) and Schiffer et al. (2005) was used here to establish the hepatocarcinogenesis model. Animals were divided into five groups, each group has eight animals labelled as follows: control phosphate buffered saline (PBS), HCC, HCC + sorafenib, HCC + safranal, and HCC + safranal + sorafenib (Figure 4).

On the first 15 weeks, control PBS group was treated with 1x PBS, whereas the experimental groups were given an intraperitoneal injection (IP) of 50 mg/kg DEN (Sigma Aldrich), once a week. DEN was diluted with 1x PBS. Followed by a one-week break (week 16), the next three weeks (weeks 17 to 19) treatment started. All drugs were administrated by oral gavage. All doses were chosen according to literature (Alsaied et al., 2014; Karafakioğlu et al., 2017). For HCC + sorafenib group, the drug (Carbosynth Limited) was administered at a dose of 10 mg/kg, five days a week.
For HCC + safranal group, the drug (Sigma Aldrich) was administered at a dose of 200 mg/kg, five days a week. For HCC + safranal + sorafenib group, the drugs were administered at a dose of 200 mg/kg safranal + 10 mg/kg sorafenib, five days a week. Both safranal and sorafenib were diluted with 1x PBS and drops of Tween 80. Oral LD$_{50}$ of safranal is 5.53 mL/kg in male rats (Hosseinzadeh et al., 2013).

After 24-hours from last treatment, rats were euthanized by mild diethyl ether and dissected in equal conditions. Blood and whole liver were collected.

Figure 4: Experimental design
2.2 Sample Preparation

2.2.1 Blood Samples

Rats were euthanized then blood was collected by decapitation and processed for later investigation. The blood was collected in collection tubes (BD Vacutainer) and serum was separated by centrifugation at 1200 × g for 10 minutes. Serums was collected and flash frozen immediately then stored at -80 °C for further analysis.

2.2.1.1 Biochemical Analysis

Alanine Transaminase (ALT), and Aspartate Aminotransferase (AST) assays were performed using commercial kits (Abcam), according to the protocol provided. ALT and AST activities were measured spectrophotometrically using Epoch by BioTek.

2.2.2 Liver Samples

Part of the liver was immediately flash frozen in liquid nitrogen then stored at -80°C for further analysis. The other part was kept in 10% neutral buffered formalin at room temperature for histology.

2.2.2.1 Histopathological Examination

Liver sample specimens were fixed in 10% neutral buffered formalin, dehydrated in a series of graded ethanol, embedded in paraffin blocks, and cut into 3 μm-thick sections. To detect histopathological changes, sections were stained with hematoxylin and eosin (H&E), and reticulin stain kit according to the protocol provided (Abcam), then examined under light microscope (Ozkececi et al., 2016). Blinded examination of tissue samples was done by a pathologist from Tawam Hospital – UAE.
2.2.2.2 Western Blotting

One hundredth gm (10 mg) liver was homogenized using 200 µl RIPA buffer (Sigma Aldrich) mixed with 2 µl protease inhibitor and 2 µl phosphatase inhibitor (Sigma Aldrich), and centrifuged at 4°C, 15,000 rpm for 15 minutes. Whole cell lysate was taken and stored at -80°C. Protein concentration was measured by Pierce BCA Protein Assay Kit with Promega GloMax Discover. A total of 35 µg of protein was loaded on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The gel was then transferred to polyvinylidene difluoride membrane. The membrane was then blocked with 5% BSA in TBST for one hour at room temperature. Membranes were incubated with anti-Proliferating Cell Nuclear Antigen (PCNA), anti-PolyADP-ribose Polymerase (PARP), anti-caspase-3 (Cell Signaling Technology Inc.), anti-caspase-9 (Novus Biologicals), anti-Bax, anti-Bcl-2 (Santa Cruz), anti-Cdk1, anti-Cyclin B1, anti-Cdc25B (Cell Signaling Technology Inc.) over night at 4°C, then with HRP conjugated secondary, anti-mouse or anti-rabbit, antibody (Cell Signaling Technology Inc.) for one hour at room temperature. All primary and secondary antibodies were diluted in 5% BSA in TBST. Blots were incubated in WesternSure PREMIUM Chemiluminescent Substrate for antibodies’ detection. Signal was visualized using Bio-Rad ChemiDoc XRS+ System. Band density was measured using ImageJ (Amin et al., 2011). Total protein was used as a loading control and stained using SYPRO Ruby protein gel stain according to the protocol provided (Thermo Fisher Scientific) (Aldridge et al., 2008; Hu et al., 2016).
2.2.2.2.1 Total Protein as a Loading Control

Due to technical reasons, total protein was used in this study as the loading control instead of the other common markers like GAPDH, β-tubulin, and β-actin. A study published in 2003 used liver samples from normal, cirrhotic, and HCC tissues to inspect the housekeeping genes. Ten internal controls were used, and their expressions were determined using RT-PCR. Results showed that all internal control genes varied more than a 2-fold, and the commonly used genes like GAPDH and β-actin varied from 7- to 23-fold, precisely in tumor tissue (Kim & Kim, 2003). Following studies then tried to find an alternative way for this issue. Total protein, depending on the amount of total protein rather than a single protein, served as a better control for colorectal cancer and HCC compared with different common housekeeping proteins. Also, testing the signal’s linearity with the loading amounts was preserved in total protein, while in the other housekeeping proteins it was lost (Aldridge et al., 2008; Hu et al., 2016). Due to technical problems with all common internal controls, I followed a protocol that was mentioned by Aldridge et al. (2008) and Hu et al. (2016).
Chapter 3: Results

Several enzymes are released from hepatocytes into the blood and are measured in the blood serum to test the efficiency of liver function, ALT and AST are the most common enzymes for testing liver function. The more severe the liver is damaged, the higher their serum levels get. Together, they are considered the best markers for liver injury (Liu et al., 2012). In addition to serum, the whole liver tissues were collected and properly stored for further histological and immunoblotting analyses. In histological examination, liver tissues were processed and stained for final imaging using the microscope (Martin, 2015). To detect markers of specific pathways, selected proteins were targeted using western blotting.

3.1 Biochemical Analysis

As shown in (Table 1), ALT ($P < 0.01$) and AST levels were elevated in HCC group as compared to control group, thus indicating liver damage. Treatment with safranal and with both safranal + sorafenib significantly ($P < 0.01$) decreased ALT levels in the treated groups as compared to HCC group. Safranal and the combination therapy caused a significance decrease ($P < 0.05$) as compared to sorafenib alone (HCC + sorafenib).

<table>
<thead>
<tr>
<th></th>
<th>Control PBS</th>
<th>HCC</th>
<th>HCC + sorafenib</th>
<th>HCC + safranal</th>
<th>HCC + safranal + sorafenib</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>7.44 ± 0.67</td>
<td>14.10 ± 0.15$^a$</td>
<td>13.42 ± 1.12</td>
<td>10.93 ± 0.29$^b$</td>
<td>9.22 ± 1.92$^c$</td>
</tr>
<tr>
<td>AST</td>
<td>9.00 ± 0.39</td>
<td>10.18 ± 1.69</td>
<td>11.14 ± 0.62</td>
<td>7.80 ± 2.02</td>
<td>10.98 ± 0.50</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of six rats per group ($n = 6$). Activity is expressed as mU/ml for ALT and AST. Significance was determined using Microsoft Excel Data Analysis Tool Pack, $t$-test: two-sample assuming equal variances (a versus PBS, b versus HCC, c versus HCC + Sorafenib; $^aP < 0.05$, $^{**}P < 0.01$)
3.2 Anti-tumorigenic and Anti-proliferative Activities of Safranal on DEN Induced Rat Liver Tumors

3.2.1 Liver Gross

Control PBS liver shows normal liver structure and color with no macroscopic lesions. Treatments showed “lesser levels of damaged livers” compared to livers from HCC group (Figure 5). DEN caused lesions and rough liver surface and caused abnormality in liver color in HCC animals. Drug treatments of HCC rats restored to variable degrees the normal liver architecture where lesions were evidently less in drug-treated groups. Treatments with safranal (HCC + safranal) and with both safranal and sorafenib (HCC + safranal + sorafenib) reduced lesions comparing to HCC animals, safranal also dramatically decreased lesions comparing to treatment with sorafenib alone (HCC + sorafenib) (Figure 6).

Figure 5: Representative images of livers on week 20 to demonstrate the anti-tumorigenic effect of safranal (n = 6). Whole liver excised from control rats (PBS), DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC SB), safranal (HCC SF) individually or combined (HCC SF SB)
Figure 6: Quantitative analysis of number of liver nodules from DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC + SB), safranal (HCC + SF) individually or combined (HCC + SF + SB). Statistical significance was determined using Microsoft Excel Data Analysis Tool Pack, $t$-test: two-sample assuming equal variances (b versus HCC; $^*P < 0.05$, $^{**}P < 0.01$)

3.2.2 Histology

Light Microscope images of liver sections (Figure 7). The structure of tissues and cells need to be stained in order to be visible. Cellular components are normally stained with a different color for proper distinction and analysis. Hematoxylin stains nucleic acids (nucleus) with blue color. Eosin stains proteins (cytoplasm) with pink color. The stain reveals plentiful structural and functional information (Fischer et al., 2008). Normal structure and histology of liver as seen in the control group where the liver is organized into hexagonally shaped lobules with the central vein at lobular centers. Hepatocytes are arranged in single-cell thick plates that radiate out from the central vein. In the animal model that has been developed in this study, macroscopic nodules were observed in the livers of mainly DEN-induced groups (Figure 5).
However, microscopic histological examination of livers of rats in DEN-induced group showed clear neoplastic changes such as altered hepatocellular foci (AHF). In the present study, AHF are generally distinguished as delineated areas of hepatocytes with altered staining properties. Safranal alone or in combination with sorafenib seem to enhance \( P < 0.001 \) the restoration of the normal architecture of the liver in DEN-treated groups (Figure 8).

Figure 7: Representative images of hematoxylin and eosin-stained sections (arrows point to representative areas of AHF), n = 6. Sections from control rats (PBS), DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC SB), safranal (HCC SF) individually or combined (HCC SF SB)
Figure 8: Quantitative analysis of area of neoplastic foci for histology from DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC + SB), safranal (HCC + SF) individually or combined (HCC + SF + SB). Statistical significance was determined using Microsoft Excel Data Analysis Tool Pack, t-test: two-sample assuming equal variances (b versus HCC; **P < 0.01, ***P < 0.001)

3.2.3 Reticulin Staining

Light Microscope images of liver sections from all groups (Figure 9). Control PBS liver shows normal liver morphology and defined reticular fibers. Liver sections from HCC animals show that DEN has caused reticular fiber breakage indicating hepatic neoplasia diagnosis. Treatment with safranal (HCC + safranal) and with both safranal and sorafenib (HCC + safranal + sorafenib) reduced reticular fibers’ breakage and restored their morphology comparing to HCC group, with a higher improvement comparing to treatment with sorafenib alone (HCC + sorafenib).
Figure 9: Representative images of reticulin-stained sections (arrows point to reticulin fibers), n = 6. Sections from control rats (PBS), DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC SB), safranal (HCC SF) individually or combined (HCC SF SB)
3.2.4 Anti-proliferative Effect of Safranal

Figure 10: Safranal inhibits proliferation of induced hepatic neoplasia. (a) Western blot analysis of the proliferation-related protein (PCNA) on DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC SB), safranal (HCC SF) individually or combined (HCC SF + SB) (b) Each band intensity was quantified using ImageJ, normalized in relative to the total protein from the liver.

Results are expressed as mean ± S.D for n = 4 animals in each group. Statistical significance was determined using Microsoft Excel Data Analysis Tool Pack, t-test: two-sample assuming equal variances

Western blot results and quantification for PCNA (Figure 10) showed that PCNA was significantly ($P < 0.01$) increased in DEN induced livers comparing to controls, while treatment with safranal ($P < 0.001$) and with both safranal and sorafenib ($P < 0.05$) significantly downregulated PCNA. Interestingly, the effect of safranal was more evident when applied alone compared to its combined administration with sorafenib, as well as compared to the effect of sorafenib alone.
3.3 Western Blotting

3.3.1 Effect of Safranal on Cell Cycle Progression

Figure 11: Safranal causes G2/M cell cycle arrest of induced hepatic neoplasia. Western blot analysis of the cell cycle-related proteins (Cdk1, Cyclin B1, Cdc25B) on DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC SB), safranal (HCC SF) individually or combined (HCC SF + SB).

![Western Blot Images]

Figure 12: Quantification of proteins of G2/M cell cycle arrest of induced hepatic neoplasia. Each band intensity was quantified using ImageJ, normalized in relative to the total protein from the liver. Results are expressed as mean ± S.D for n = 4 animals in each group. Statistical significance was determined using Microsoft Excel Data Analysis Tool Pack, t-test: two-sample assuming equal variances.
To study the pathway responsible for safranal mediated cell cycle effect in DEN-induced rat liver neoplasia, the expression levels of cell cycle-related proteins were examined. Cdk1, cyclin B1, Cdc25B western blot results (Figure 11) showed that they are significantly \((P < 0.01, P < 0.01, P < 0.05, \text{respectively})\) increased in HCC animals as compared to control animals. Treatment in (HCC + safranal) and (HCC + safranal + sorafenib) groups significantly decreased their levels \((P < 0.001)\) comparing to HCC animals. Treatment with safranal (HCC + safranal) and the combination drug (HCC + safranal + sorafenib) showed a greater decrease than treatment with sorafenib alone (HCC + sorafenib) (Figure 12). It is possible then that safranal may sensitize hepatic cells to sorafenib’s effect by further decreasing the expression of cell cycle-related proteins in the co-treated group. These results suggest that safranal causes \(G_2/M\) cell cycle arrest of drug-treated hepatic cells.

### 3.3.2 Effect of Safranal on Apoptosis

Figure 13: Safranal induces intrinsic apoptosis of induced hepatic neoplasia. Western blot analysis of the intrinsic apoptosis-related proteins (Bcl-2, Bax, Pro-Caspase-9, Pro-Caspase-3, PARP) on DEN-induced hepatic neoplasia in rats untreated (HCC group) or treated with sorafenib (HCC SB), safranal (HCC SF) individually or combined (HCC SF + SB).
Figure 14: Quantification of Bax, Bcl-2, Bax/Bcl ratio. Each band intensity was quantified using ImageJ, normalized in relative to the total protein from the liver. Results are expressed as mean ± S.D for n = 4 animals in each group. Statistical significance was determined using Microsoft Excel Data Analysis Tool Pack, *t*-test: two-sample assuming equal variances

b versus HCC; *$P < 0.05$

a versus PBS, b versus HCC; *$P < 0.05$, **$P < 0.001$

a versus PBS, b versus HCC; *$P < 0.05$
Figure 15: Quantification of Pro-Caspase-9, Pro-Caspase-3, PARP. Each band intensity was quantified using ImageJ, normalized in relative to the total protein from the liver. Results are expressed as mean ± S.D for n = 4 animals in each group. Statistical significance was determined using Microsoft Excel Data Analysis Tool Pack, t-test: two-sample assuming equal variances.
To study the pathway responsible for safranal mediated apoptosis in DEN-induced rat liver tumor cells, the expression levels of apoptosis-related proteins were examined. The results showed that safranal treatment significantly ($P < 0.05$) increased the expression of the pro-apoptotic protein Bax and significantly ($P < 0.05$) decreased the expression of the anti-apoptotic protein Bcl-2 compared to HCC group. The Bax/Bcl-2 ratio favored the apoptotic effect of safranal ($P < 0.05$) in DEN-induced rat liver tumors (Figure 14). Interestingly, the apoptotic effect of safranal was more evident when administered alone compared to where both safranal and sorafenib, were administered or when sorafenib alone was used. To further investigate the apoptotic effect of safranal, western blot analysis showed that pro-caspase-9, pro-caspase-3, and PARP results confirmed caspase cascade activation and PARP cleavage, where the expression of pro-caspases-9 & 3 and whole PARP were significantly decreased compared to HCC group after treatments with safranal ($P < 0.01, P < 0.001, P < 0.001$, respectively) and with both safranal + sorafenib ($P < 0.01, P < 0.01, P < 0.001$, respectively) (Figure 15). These results further support the pro-apoptotic effect of safranal on drug-induced neoplasia.
Chapter 4: Discussion

4.1 Safranal Restores Liver Function by Reducing ALT and AST

Three main biological processes are carried out in the liver; biochemical metabolism, bilirubin transport, and immune defense. Aminotransferases like ALT and AST are enzymes that are responsible for amino group transfer. AST is found in the cytoplasm and mitochondria of the liver cells, also in cardiac and skeletal muscles. ALT on the other hand is specifically found in the cytoplasm of liver cells. Any injury in the liver leads to an elevation of these aminotransferases (Lui, 2018).

Saffron has been shown to restore normal levels of liver enzymes (ALT) thus restoring liver function (Amin et al., 2011). Similar data has been reported with sorafenib in DEN-induced liver cancer in Sprague Dawley rats. Sorafenib decreased ALT an AST levels in the treated livers and retained normal functions of the liver (Lv et al., 2017). In the present study, serum activities of ALT ($P < 0.01$), and AST were increased in HCC group comparing to the control group, which indicates liver damage. As the results show in (Table 1); treatment with safranal decreased the elevation of ALT comparing to HCC group ($P < 0.01$) with a higher efficiency of safranal alone and the synergic group (HCC + safranal + sorafenib) over sorafenib alone ($P < 0.05$). This highlights the efficiency of safranal and its potential in combination therapy. Since ALT and AST increase in liver injury, the results show that safranal significantly restores liver function. The insignificant change of AST can be explained as ALT is a more diagnostic enzyme found exclusively in the liver, while AST is not just found in the liver, but also in the muscles. AST is also cleared from the liver twice as fast as ALT, therefore ALT level is increased more than AST after liver injury (Lui, 2018).
4.2 Safranal has Anti-tumor and Anti-proliferative Activities on DEN Induced Rat Liver Tumors

4.2.1 Histology

The biopsy taken from the patients’ livers for examination is important for diagnosis of liver diseases. It often helps to unravel the cause of a demonstrated damage, or abnormality that have not been reported with other examinations. Any changes in the normal morphology of the liver found by clinical examination could be an indication of serious liver lesion (Hübscher, 2015). Histological examination of DEN-induced (HCC group) showed loss of hexagonal lobular architecture, abnormal proliferation, loss of the single cell plate arrangement, and nodular neoplastic transformation that indicate the formation of hepatic neoplasia. similar nuclear pleomorphism and hyperchromatism were reported elsewhere (Hamza et al., 2018). Treatment with safranal alone or in combination with sorafenib seem to enhance the restoration of the normal architecture of the liver in DEN-treated groups.

4.2.2 Reticulin Staining

Connective tissue is a group of cells surrounded by extracellular substances. These extracellular substances contain fibers; reticular fibers are a network of thin and delicate type III collagen. In liver tissue, hepatocytes are surrounded by reticular fibers that provide support and exchange of substances between the cells and blood vessels (Slomianka, 2009). As reticular fibers network is important for hepatocytes support, attention was drawn to reticulin staining to investigate changes in reticular fibers in the liver to identify liver damage. Thioacetamide was used to induce liver fibrosis in rats and degraded reticular fibers suggesting that breakage of hepatic reticular fibers is associated with liver damage (Wen et al., 2016).
Another study investigated metastasis of oral squamous cell carcinoma to lymph nodes by examining reticular fibers, where in tumor metastasis they formed thinner and separate strands while in the control they formed thick and bundled reticular fibers (Yinti et al., 2015). Reticulin staining in human HCC also shows abnormal reticular structure that surrounds cancerous hepatic cells. Previous reports revealed that reticulin staining is the most accurate marker for HCC, and the degradation of reticular fibers supports the diagnosis of HCC (Singhi et al., 2012; Yao et al., 2013).

Consistent with those studies, present microscopic analysis (Figure 9) showed that liver tissue of control group showed a uniformed network of reticular fibers surrounding the hepatic cells. In the experimental groups, DEN-treated livers showed abnormal, broken reticular fibers that surrounded the cancerous cells indicating that DEN caused HCC. Whereas in the drug treated groups, the reticulin fibers have retained their normal morphology being uniformed with less breakage and smaller lesions comparing to HCC animals. Treatments with safranal (HCC + safranal) and with both safranal and sorafenib (HCC + safranal + sorafenib) showed more intact bundles of reticulin fibers, and smaller lesions compared to their counterparts in livers of animals treated with sorafenib alone (HCC + sorafenib).

4.2.3 Safranal has Anti-proliferative Effect on DEN Induced Rat Liver Tumors

PCNA is highly expressed in dividing cells in the S phase. PCNA plays an important role in DNA replication, DNA repair and in controlling the cell cycle. Its structure is best described as a sliding clamp, that stabilizes polymerases during DNA synthesis. It acts as a signal for proliferation, and if absent, DNA replication is inhibited, and cells opt for apoptosis.
Studies also suggest that in case of DNA damage, PCNA recruits special proteins such as polymerase δ and ligase to the site of damage, and resynthesizes DNA (Juríková et al., 2016). In cancer research, the first markers checked are proliferation markers where they increase dramatically in tumors. In a study of non-small cell lung cancer, results showed elevation in PCNA, and it was related to tumor metastasis as well, and its elevation inhibited apoptosis (Wang et al., 2018). In another study, zebrafishes were exposed to DEN which caused liver abnormalities, and PCNA expression was also elevated (Machado et al., 2014). In this study, western blotting analysis (Figure 10) showed that PCNA was significantly increased in DEN induced livers comparing to controls ($P < 0.01$), while treatments with safranal and with both safranal and sorafenib downregulated PCNA.

4.3 Western Blotting

4.3.1 Safranal Induces G₂/M Cell Cycle Arrest

Living organisms grow and reproduce to survive and maintain their own characteristics; the same is true for cells. Cells grow and divide into two daughter cells that are identical to their mother cells. For cells to divide, they must go through the cell cycle that includes four main phases; G₁, S, G₂, and M. During these phases the cell grows and DNA replicates in preparation for ultimate cell division. Checkpoints are present to ensure that all the cellular materials and DNA are readily available before division can start. For everything to go smoothly, the cell cycle has its own control system. This control system is present at different phases, G₁/S, G₂/M, and at the M phase. G₁/S checkpoint checks that the cell is ready to go for DNA replication, G₂/M checkpoint prepares the cell to go to cell division, and the M phase checkpoint insures that sister chromatids are ready to separate at the anaphase.
If checkpoints sense any defect, whether within the cell or outside the cell, they will halt the next step until such defect is repaired. The main components in the control system of the cell cycle are the cyclin-dependent kinases (Cdks) and cyclins. Cdks are kinases that transfer phosphate to regulate the activity of their substrate proteins. Cyclins, on the other hand, are proteins that are needed to activate Cdks and form a cyclin-cyclin dependent kinase complex. Cyclin-Cdks are the cell cycle checkpoints. Cdks are present throughout the cell cycle in the same concentration, whereas cyclins’ concentration varies depending on which phase is the cell at, and each phase has its own cyclins (Nair & van Staden, 2018).

Cyclin B and Cdk1 are involved in the last step which the cell goes through, cell division. Cyclin B attaches to Cdk1, yet it is still inactive. Two kinases are involved in the next step, Wee1, which adds an inhibitory phosphate, and CAK (Cdk-activating kinase), which adds an activating phosphate. To have an active cyclin B-Cdk1 complex, another enzyme, Cdc25, a phosphatase, removes the inhibitory phosphate that was added by Wee1, and the resulting complex of cyclin B-Cdk1 is active, and the cell is ready to divide (Figure 16) (Gardino & Yaffe, 2011).

![Diagram of Cyclin B1-Cdk1 activation]

Figure 16: Cyclin B1-Cdk1 activation
The important role of Cdks and cyclins in cell cycle made them imperative targets for cancer therapy, especially that their weak control of the cell cycle is linked to cancer. Liu and colleagues (2007) suggested that nitrogen-containing flavonoids are good candidates for inhibiting CDK1/Cyclin B and consequently has led to the inhibition of cancer cells in vitro (Liu et al., 2007). Plant-derived compounds from *Appendicula reflexa* have been also reported to lead to CDK1/Cyclin B inhibition (Apel et al., 2012). In addition to CDKs and cyclins and thanks to the important role of Cdc25B in the cell cycle as well by activating the cyclin-Cdk complex, a recent study showed that microRNA-152 inhibited Cdc25B in human endometrial cancer cells which induced G2/M phase arrest (Xie et al., 2018). Cdc25B was also reported to be inhibited by using thiadiazole amides and their derivatives which helped in halting the cell cycle (Li et al., 2014).

In the current study, western blot results for Cdk1, cyclin B1, Cdc25B (Figure 11), and their quantification (Figure 12), showed significant upregulation of all assessed markers in HCC animals comparing to control animals (*P* < 0.01, *P* < 0.01, *P* < 0.05, respectively). Livers of animal groups treated with safranal alone and with both safranal and sorafenib showed significant (*P* < 0.001) downregulations as compared to HCC animals. These results show that safranal targets the cell cycle arrest at the G2/M phase, blocks cell division suggesting a great potential of future utilization of safranal as a novel combination/therapy against HCC. Similar results have recently been reported where safranal caused G2/M cell cycle arrest in HepG2 cells (Al-Hrout et al., 2018).
4.3.2 Safranal Induces Apoptosis

Life and death are the normal cycle of life in order to keep balance. In the body, a group of cells forming a tissue needs to die and reproduce in a matter that keeps the tissue size uniformed. The growth of tissue should be balanced by the forming of new cells and the removal of old cells. Cells also die if they sense a harmful invasion, or DNA damage. Certain cellular mechanisms control this balance between cell death and cell proliferation to avoid any tumor formation. Programmed cell death (apoptosis) is mainly triggered by caspases, which are typically inactive in healthy cells, and are activated upon their cleavage during apoptosis. There are two classes of caspases: initiators and executioners. When the cell undergoes apoptosis, initiators get activated first, activating executioners in return, which eventually activates proteases and nucleases to undergo apoptosis. Two pathways are used by the cell to undergo apoptosis: intrinsic and extrinsic (Bi et al., 2018). This study will focus on the intrinsic pathway.

Any cellular stress such as DNA damage, triggers the intrinsic apoptotic pathway which starts in mitochondria. The released cytochrome c from the mitochondria binds to a protein called apoptotic protease activating factor-1 (Apaf1) forming an apoptosome. This apoptosome activates the initiator caspase-9, which activates the executioner caspase-3, the main apoptosis protein. Cytochrome c release must be regulated to ensure that cells do not undergo apoptosis all the time, this regulation occurs by Bcl-2 and Bax proteins. Bcl-2 and Bax bind together in normal cases, but when an apoptotic signal is received, Bcl-2 breaks loose from Bax which leads to forming holes in the mitochondria allowing cytochrome c to be released (Figure 17) (Li et al., 2013).
PARP is also an important protein that its inhibition regulates apoptosis. PARP regulates DNA repair and stability. During DNA replication, some errors could occur breaking the single strand of the growing DNA. The cell senses this error and sends PARP to seal this ssDNA breakage. In apoptosis, the activation of caspase-3 cleaves PARP leading to a broken DNA then the whole DNA collapses and the cell goes to apoptosis (Chaitanya et al., 2010).

Cancer however, usually finds its way to escape apoptosis to survive and even spread. Studies have focused on the caspase cascade for cancer therapy. Saffron has been shown to induce apoptosis in lung cancer through the caspase-3 cascade in a dose and time dependent manner (Liu et al., 2014). DEN induced HCC in rats treated with melatonin showed a significant increase in active caspsases and cleaved PARP, along with Bax/Bcl-2 ratio in treated rats which resulted in tumor shrinkage via apoptosis (Moreira et al., 2015). In addition, HCC rats treated with cape gooseberry juice led to an increase in apoptosis via Bax/Bcl-2 and cleaved caspase-3 upregulation (Hassan et al., 2017).
The role of caspase-9 & 3 and bcl-2 was also studied in eosinophil apoptosis in humans, where the activation of CD30 lead to apoptosis via caspase-3 activation and bcl-2 inhibition (Lee et al., 2017). Another study examined PARP by inducing apoptosis via death receptor, CD95. Results showed that activating caspase-3 resulted in PARP cleavage and mediated apoptotic cell death (Los et al., 2002).

In the present study, western blot analysis (Figure 13, Figure 14, Figure 15), showed that the intrinsic apoptotic pathway is activated by safranal via activating Bcl-2/Bax and caspase-9 & 3 followed by PARP cleavage. The first focus was on the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. The present results showed that safranal treatment significantly increased the expression of Bax \( (P < 0.05) \) and decreased the expression of Bcl-2 \( (P < 0.05) \) as compared to HCC animals. Subsequently, the Bax/Bcl-2 ratio favored the apoptotic effect of safranal in DEN-induced rat liver tumors \( (P < 0.05) \). Interestingly, the apoptotic effect of safranal was stronger when treated alone compared to the combined treatment and was stronger than sorafenib alone. The apoptotic effect of safranal was further confirmed by determining the expression of apoptotic markers, caspase-9, caspase-3, and PARP. Western blot analysis showed that the proportions of pro-caspase-9 and pro-caspase-3 were significantly \( (P < 0.01, P < 0.001, \text{respectively}) \) decreased as a result of caspase cascade activation and similarly there was a significant decrease in whole PARP \( (P < 0.001) \) compared to HCC group and compared to treatment with sorafenib alone \( (P < 0.05) \). These results mark the apoptotic fate of tumor cells. Interestingly, safranal seemed to help sorafenib-induced PARP cleavage in animal group treated with both safranal and sorafenib.
Chapter 5: Conclusion

The novelty of this research stems from the use of a bioactive compound of a natural source that has been traditionally used as a popular spice and showing how it may carry the potential to be a novel therapy of liver cancer.

*In vivo* HCC model was successfully induced in male Wistar rats, then treated with sorafenib alone, safranal alone, and with both safranal + sorafenib. Data analysis showed the efficiency of safranal as a drug and an adjuvant in restoring liver function. Presented results also showed safranal’s inhibitory role of cell cycle, and its pro-apoptotic capacity suggesting safranal’s high potential as a novel anti-cancer drug.

Further investigations are underway to unravel the molecular mechanisms of safranal, particularly, in ER stress, UPR activation and DNA repair pathways. At the present stage, this study could soon be translated to benefit cancer patients as it convincingly argues for the advantage of safely using safranal/saffron as an adjuvant therapy.
References


