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IN-VITRO ANALYSIS OF POTENTIAL AUTOPHAGY MEDIATED SAFRANAL-SORAFENIB COMBINED EFFECT ON HEPATOCELLULAR CARCINOMA

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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 Dr. Mohammed Ayoub

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Declaration of Original Work

I, Aaminah Farrukh, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*In-vitro analysis of potential autophagy mediated Safranal-Sorafenib combined effect on Hepatocellular Carcinoma*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Mohammed Ayoub, 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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

Hepatocellular carcinoma (HCC) is one of the most commonly occurring cancers worldwide. The risk factors include obesity, hepatitis, alcoholism, smoking etc. One of the biggest associated challenges is the current treatment strategies. The therapeutic options are limited with economical and accessibility challenges, especially with increased HCC incidence. Sorafenib, a multikinase inhibitor, is one of the first FDA approved drugs for HCC. But drug resistance and cancer relapse are a common drawback for this treatment. The need for better treatment is crucial now more than ever. The use of saffron as a potential natural therapeutic option is not a novel strategy. It has been long used as an analgesic, anti-inflammatory and anti-spasmodic in folk medicine. Recently, saffron ant its bioactive constituents have shown anti-cancer properties through different mechanisms. In this study, Safranal was tested against the hepatocellular carcinoma cell line (HepG2) for its effectiveness on cell viability and autophagy. The cells exhibited a decreased survival on treatment with Safranal which was associated with induced autophagy. This was confirmed by SDS-PAGE and western blot showing an increase in the expression of the major autophagic protein markers such as Beclin-1 and Atg 12. These effects were also supported by microscopic analysis where phagosome formation was evident. Moreover, the combination of Safranal with Sorafenib showed antagonistic effect on Safranal inhibiting cell viability. the effect of Safranal and Sorafenib alone and their combination on the canonical kinase, ERK1/2, controlling the survival pathway, was also investigated. Safranal had no effect on AKT expression level but inhibited the phosphorylated form of AKT which is consistent with the inhibition of cell survival and the induction of autophagy. These results have provided with a possibility of Safranal being an effective chemotherapeutic against HCC. The combination treatment led to more complex observations that require further investigation in the future. Also, Safranal's effect on other cancerous cell lines and the other possible mechanisms by which Safranal affects HCC can be potential areas to broach in the future.

Keywords: Hepatocellular carcinoma, Safranal, Sorafenib, Autophagy, Combination therapy, Combination index

Title and Abstract (in Arabic)

دراسة خلوية للتأثير المحتمل للسافرانال وسورافينيب على البلعمة الذاتية في خلايا سرطان الكبد

الملخص

سرطان الكبد هو أحد أكثر أنواع السرطانات شيوعًا في جميع أنحاء العالم. تعتبر السمنة والتهاب الكبد وإدمان الكحول والتدخين من العوامل المؤدية للإصابة. خيارات العلاج الحالية محدودة وليست اقتصادية ولا يمكن الوصول إليها بسهولة في جميع أنحاء العالم، خاصة في الحالات التي يزيد فيها معدل الإصابة بسرطان الكبد. يعد سورافينيب ,مثبط العديد من الانزيمات , أحد الأدوية الأولى المعتمدة من قِبل إدارة الأغذية والعقاقير لعلاج سرطان الكبد. الا أن مقاومه هذا العقار وعوده المرض من أخطر سلبيات هذا العقار. مما جعل الحاجة الى علاج أفضل أمر بالغ الأهمية الآن أكثر من أي وقت مضى. استخدام الزعفران كمركب علاجي ليس بالمفهوم جديد فلطالما استخدم كمسكن ومضاد للالتهابات ومضاد للتشنج في الطب الشعبي. وفي الآونة الأخيرة أظهرت مكونات الزعفران خصائص مضادة لأنواع مختلفة من السرطان من خلال آليات جزيئيه مختلفة. في هذه الدراسة، تمت دراسة فاعليه السافرانال في حيوية والالتهام الذاتي لخلايا سرطان الخلايا الكبدية. أظهرت الخلايا انخفاضًا في معدل البقاء على قيد الحياة عند العلاج باستخدام السافرانال الذي تسبب في الالتهام الذاتي. تم تأكيد ذلك من خلال تجارب معمليه دقيقه التي اظهرت زيادة في انتاج بروتينات البلعمة الرئيسية مثل Beclin-1 و Atg 12 ، وكذلك من خلال التحليل المجهري لتشكيل البلعمة. علاوة على ذلك، فإن الجمع بين السافرانال وسورافينيب أظهر تأثيرًا معاكسًا على حيوية الخلايا المثبطة لـ السافرانال .لقد بحثنا أيضًا في تأثير السافرانال و سورافينيب ومزيجهما على انزيمات ERK1/2 ، والتحكم في مسارات التكاثر والبقاء ، على التوالي. لم يكن للسافرانال أي تأثير على مستوى تعبير AKT ولكنه أعاق بشكل كبير الشكل الفسفوري لـ AKT والذي يتوافق مع تثبيط بقاء الخلية وتحريض الالتهام الذاتي. وقد أتاحت هذه النتائج إمكانية أن يكون السافرانال علاجًا كيميائيًا فعالًا ضد سرطان الكبد. أدى العلاج المركب إلى ملاحظات أكثر تعقيدًا تتطلب مزيدًا من التحقيق في المستقبل. كما يمكن أن يكون تأثير السافرانال على خطوط الخلايا السرطانية الأخرى والآليات المحتملة الأخرى التي يؤثر بها السافرانال على سرطان الكبد مجالات بحثيه محتملة للتطرق إليها في المستقبل.

مفاهيم البحث الرئيسية: خلايا سرطان الكبد, لسافرانال ,سورافينيب ,الذاتية , الجمع بين العلاج, مؤشر الجمع

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Dedication

To my beloved parents; my backbone, my strength and my most loyal and faithful well-wishers.

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List of Abbreviations

ATG	Autophagy Related Genes
CI	Combination Index
DMSO	Dimethyl Sulfoxide
FDA	Food and Drug Authority
HCC	Hepatocellular Carcinoma
IC	Inhibitory Concentration
MTT	Methylthiazolyldiphenyl-tetrazolium bromide

Chapter 1: Introduction

1.1. Overview

Hepatocellular Carcinoma (HCC) continues to be one of the mostly commonly occurring cancers worldwide, with very high mortality rate. Recent medical advancements have enhanced the knowledge and understanding of the diseases and its pathophysiology. In some countries advancements in early screening for susceptible patients has made it possible to improve the treatment plan. The treatment for HCC is a complex procedure that involves taking into consideration multiple factors, like liver damage, tumor metastasis and patient comorbidity. Unfortunately, the overall survival rate has not been considerably improved owing to different environmental and economic factors. The availability of resources and competency varies among different parts of the world and this disparity is the reason for increased HCC cases in some parts of the world, like Asia and Africa [1, 2] .

1.2. Research Problem

The increase in HCC incidence calls for potent and alternative curative treatments. The limited treatment options demand the pursuit of treatment plans that are formulated in a way to achieve maximum effectiveness, with improved survival rate, and minimal side effects.

1.3. Literature Review

HCC is the most commonly occurring primary liver malignancy. It is typically caused by excessive alcohol consumption, nonalcoholic fatty liver disease, obesity, aflatoxin B1 exposure, autoimmune hepatitis or infection caused by Hepatitis B and/or C viruses. HCC is also common in patients with liver cirrhosis, occurring in at least one-third of cirrhotic patients. HCC has high mortality rate with most patients being diagnosed at advanced tumor stages. The current treatment options include; liver resection, ablation and transplantation. However, these treatment options are mostly viable for patients in early stages of HCC, which is only 30-40% of patients diagnosed with HCC. For advanced stages, most often systematic drugs are used as HCC is also resistant to conventional chemotherapeutics [3–6].

High recurrence rate is the main drawback of resection and ablation. Besides being uneconomical, liver transplantation, is only feasible if the patients meet certain strict requirements. HCC treatment protocol often involves intricate planning taking into consideration HCC stage, patients health status, underlying/pre-existing conditions and extent of metastasis, if any [7].

Apart from the surgical and non-invasive therapies, certain systemic medical therapies are also used in the treatment of HCC. The First-line targeted agents for HCC include Sorafenib and Lenvatinib. Since, resistance to these first line agents became a commonly occurring problem, Second-line targeted agents such as Regorafenib, Cabozantinib and Ramucirumab have been introduced [8].

Sorafenib is a tyrosine kinase inhibitor that was the first FDA approved drug for the treatment of HCC. It has shown antiangiogenic properties by targeting platelet-derived growth factor receptor, vascular endothelial growth factor receptor and other

proteins involved in tumor angiogenesis. The anti-proliferative nature of Sorafenib is owing to its ability to inhibit Raf-1, B-Raf, and kinase activity in the Ras/Raf/MEK/ERK signaling pathways. Typically patients develop resistance to Sorafenib within six months and other concerning side effects may arise including hand and foot skin reactions, diarrhea, and extreme weight loss [9, 10].

As current HCC treatment options deem to be inadequate and ineffective, health of patients in advanced stages continues to deteriorate. Natural compounds have now recently momentum and are being explored as therapeutics for different diseases including HCC. Many natural compounds such as; Solamargine (derivative of Chinese herb), Capsaicin (a spice), Curcumin (a spice), Resveratrol (a plant derived polyphenol), Silibinin (a flavonoid) etc. have shown therapeutic effects in HCC *in-vitro*. Their mechanisms of action include, but not limited to; cell cycle arrest, induction of apoptosis, and anti-angiogenesis [11–13].

Saffron, the dried stigma of *Crocus sativus* flower is one of those promising compounds derived from natural resources. Saffron has long been used as a spice and food coloring agent by Asian and Middle Eastern nations. It has also been used as a traditional medicine acting mostly as anti-inflammatory and analgesic. Lately, Saffron and its derivatives; Safranal, Crocin and Crocetin have shown *in-vitro* and *in-vivo* anticancer, antioxidant and anti-inflammatory properties. These derivatives have been tested across different cancer cell lines, including Hela (human cervical epithelioid carcinoma), A549 (human lung cancer), N2A (neuroblastoma), colon cancer (HCT116) and PC-3 (human prostate cancer) cells. These compounds have reported to inhibit cell proliferation and induce apoptosis across all tested cell lines [14–21].

Safranal is one of the main components of Saffron's essential volatile oil, giving it it's characteristic odor and aroma. As a therapeutic agent, Safranal has proven

to act as an anti-oxidant, anticonvulsant, antidepressant and hypotensive [22–25]. A study conducted by Al-Hrout *et al.* [19] signified Safranal's potent ability as an anticancer agent. The results of the study, that was conducted on HepG2 cells, revealed that Safranal inhibits growth, survival and cellular proliferation *in-vitro*. Safranal affected key cell cycle regulators and arrested cell cycle, induced DNA damage and breakage and apoptosis [19].

The use of combination drugs for cancer treatment is an idea that is being explored to a great extent. The benefits of using a combination therapy as compared to the standard mono-therapies, are numerous. The toxicity is much less in combination therapy as compared to single-drug treatment, the chances of developing drug-resistance is reduced and in-fact, the possibility of introducing cancer-stem-cell inhibitor has been shown to reduce the incidence of a relapse [26]. Previously, Safranal has not been used in combination with other drugs for cancer studies. However, two combination studies include; an *in-vivo* study by Erfanparast *et al.* [27] where Safranal was used alone or in combination with crocin, morphine, diclofenac and naloxone to investigate the effect on orofacial pain in rats. The second Safranal combination study, conducted by Delkhosh-Kasmaie *et al.* [28], was also an *in-vivo* study where the rats were examined for the effects of Safranal and metformin on learning and memory abilities. Sorafenib has been tested, on different cancer cell lines, in combination with other drugs, natural compounds and specific inhibitors [29–32]. The use of Sorafenib in combination with other anti-cancer agents, preferably natural compounds, can change the course of HCC treatment regimen. HCC is a complex biological process of genetic and epigenetic alterations. These alterations range from mutations in promoter regions to irregularities in DNA methylation. Recent advancements have aided in understanding of the molecular complexity of HCC, oncogenic and cell signaling

pathways and tumor suppressor pathways involved in the progression of HCC. These findings serve as an opportunity for the development of therapeutic agents that can successfully target these pathways and their critical checkpoints [5, 6].

Autophagy is an evolutionary conserved multistep process. It is a catabolic pathway essential for maintaining cellular homeostasis and cytoplasmic quality by facilitating the removal of misfolded and/or long-lived proteins, damaged organelles and protein aggregates. Physiologically autophagy is active at basal levels in all cells. But under certain stressful conditions, such as oxidative stress, hypoxia, nutrient starvation, growth factors deficiency and anticancer agents, autophagy can be up-regulated. A number of liver diseases, including alcoholic liver disease, non-alcoholic fatty liver disease, hepatomegaly and HCC have exhibited abnormal autophagy [33–35]. Hence, drugs targeting autophagy can prove to be a beneficial treatment approach for HCC.

The aim of this study was to investigate the anti-survival and the pro-autophagic effects of Safranal and the effects on cellular and molecular levels in HepG2 cells. Moreover, a combination of Sorafenib, known as a kinase inhibitor, and Safranal was also investigated.

Chapter 2: Materials and Methods

2.1 Cell Culture

The cell line used for this study is HepG2, purchased from ATCC. The cells were cultured in RPMI 1640 Media (HyClone), supplemented with 10% Fetal Bovine Serum (Sigma Aldrich (USA)) and containing 1% of 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich) at 37°C in a humidified 5% CO_2 atmosphere. Cells were sub-cultured every 3-5 days using Trypsin 0.25% (HyClone).

2.2 Drugs Preparation

Safranal was obtained from Sigma Aldrich and was prepared to a stock concentration of 10 mM by dissolving in Dimethyl Sulfoxide (DMSO) and RPMI media. Sorafenib was obtained from Sigma Aldrich and prepared to a stock concentration of 38 mM by dissolving in DMSO. The working concentrations for both drugs were then prepared, from their respective stocks, accordingly.

2.3 Cell Viability Assay

The Cell viability of the HepG2 on treatment was determined by using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma Aldrich). All cells were seeded at a density of 5000 cells/well in 96-well plate in 100 µl of complete growth medium. Cells were allowed to attach for 24 – 36 hr, before being treated with different concentrations of the drugs, alone or in combination, for 24 hr. Cells were then treated with MTT and incubated for 3 hr at 37°C in a humidified 5% CO_2 atmosphere. The

formed formazan crystals were dissolved using DMSO and the absorbance of the resulting product was measured at 570 nm using GloMax Microplate Reader (Promega). The experiment was carried out in triplicates. The Inhibitory Concentrations (IC) were measured using Fit Spline/LOWESS analysis of GraphPad Prism software. The Combination Index (CI) was calculated using CompuSyn software. The percentage of dissolving agent was maintained at < 0.1%.

2.4 Autophagic Vacuoles Detection

The formation of autophagic vacuoles was assessed using Autophagy Assay Kit (ab139484). The assay was performed following manufacturer's protocol. Briefly, the cells were seeded at a density of 5000 cells/well in 96-well clear bottom plate in 100 µl of complete growth medium. Cells were allowed to attach before being treated with Safranal IC25 and IC50, for 24 hr. The cells were then washed with 1X assay buffer, supplied with the kit. Next, detection reagent was added 100 µl/well and incubated in dark for 45 min at 37°C. The cells were then once again washed with 1X assay buffer and then visualized using IX53 microscope (Olympus).

2.5 Detecting Lysosomes using Lysosomal Staining Reagent

The formation of lysosomes was monitored using Abcam's Lysosomal Staining Reagent - Orange | Cytopainter (ab176827). The assay was performed following manufacturer's protocol. Briefly, the cells were seeded at a density of 20,000 cells/well in 2 mL media of 6 well plate and incubated. The cells were then treated with Safranal IC25 and Safranal IC50 concentrations and incubated for 24 hr. The control was

untreated cells. The cells were then washed, twice, and the dye-working solution was then added for 30 min. The cells were incubated with the reagent at 37°C in a humidified 5% CO_2 atmosphere. The cells were then washed again and visualized using IX53 microscope (Olympus).

2.6 Protein Extraction and Quantification

HepG2 cells were seeded at a density of $1x10^6$ cells/90 mm plates and allowed to attach. The cells were then treated with Safranal IC25 and IC50 for 24 hr. The proteins were then extracted using and following the protocol of RIPA Lysis and Extraction Buffer (Sigma). The protein quantification was performed using Bradford Reagent (Sigma) and absorbance was measured using GloMax Microplate Reader (Promega).

2.7 SDS-PAGE and Western Blot

Proteins were separated using 5-15% SDS polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membranes prior to incubation with various primary antibodies; LC3B, p.AKT, AKT, pERK1/2, Beclin1 and Atg12. GAPDH was used as loading control. Appropriate HRP conjugated secondary antibodies were used. Protein bands were detected using WesternSure Chemiluminescent Substrate (LI-COR) and detected using ChemiDoc XRS+ Gel Imaging System (Biorad). The images were then analyzed using Image Lab and ImageJ software.

Chapter 3: Results

3.1 Safranal and Sorafenib Inhibit HepG2 cell proliferation

In order to examine the effect of Safranal and Sorafenib on HepG2 cells, the cells were treated with different concentrations of either Safranal (100-1000 µM) or Sorafenib (1-100 µM) for 24 hours and the cell proliferation was analyzed using MTT assay. Both treatments resulted in a significant dose-dependent decrease of cell viability with Sorafenib being more potent that Safranal (Figure 1 and Figure 2). Indeed, the IC25 and IC50 values were calculated to be 56 µM and 195 µM, respectively, for Safranal, and 13 µM and 22 µM, respectively, for Sorafenib.

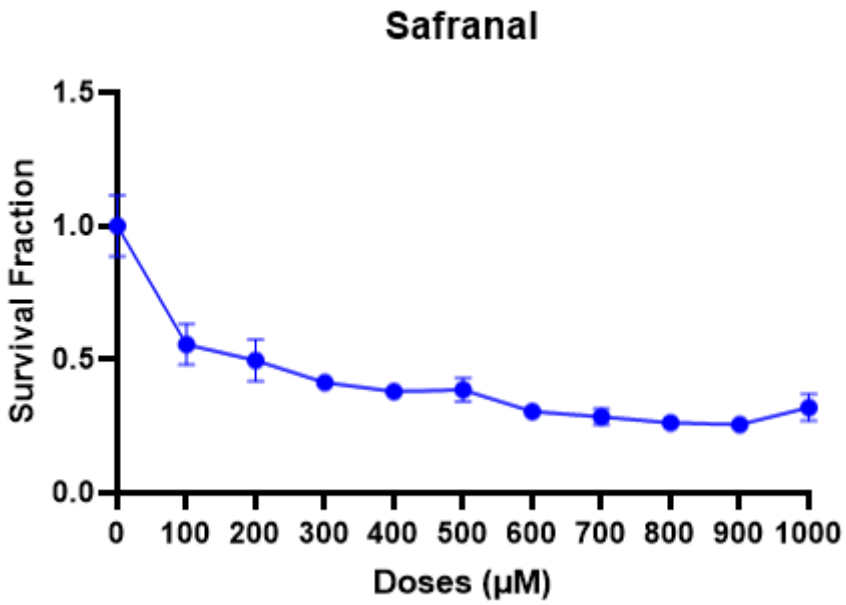


Figure 1: Survival fraction of HepG2 cells upon treatment with increasing doses of Safranal.

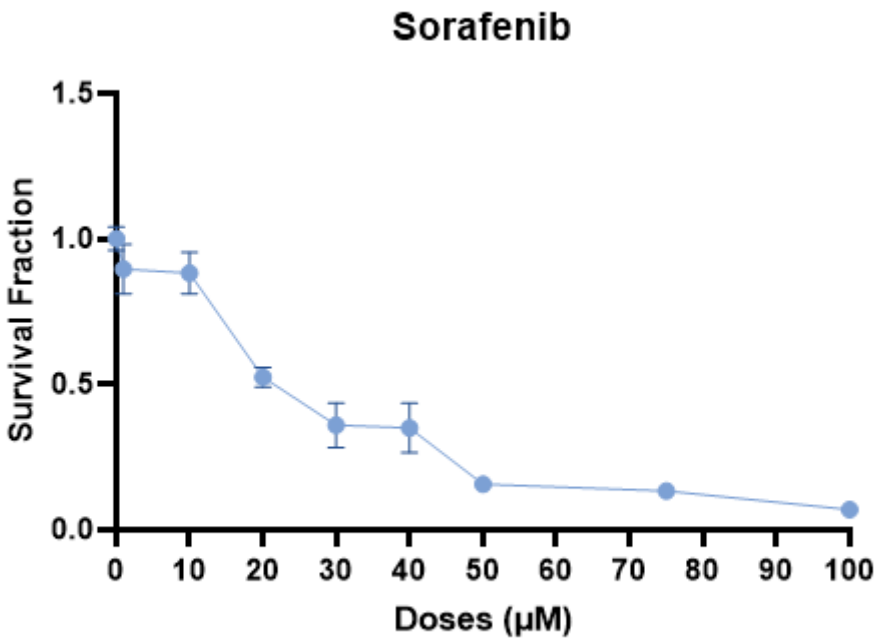


Figure 2: Survival fraction of HepG2 cells upon treatment with increasing doses of Sorafenib.

3.2 Safranal and Sorafenib combination showed an antagonistic effect

The cells were then treated with the combination of both drugs. First, we used constant does of Sorafenib at IC25 and/or IC50 in combination with increasing doses of Safranal (100 – 1000 μM) as shown below in Figure 3. Such a treatment showed antagonistic action of Sorafenib on Safranal-induced inhibition of HepG2 viability.

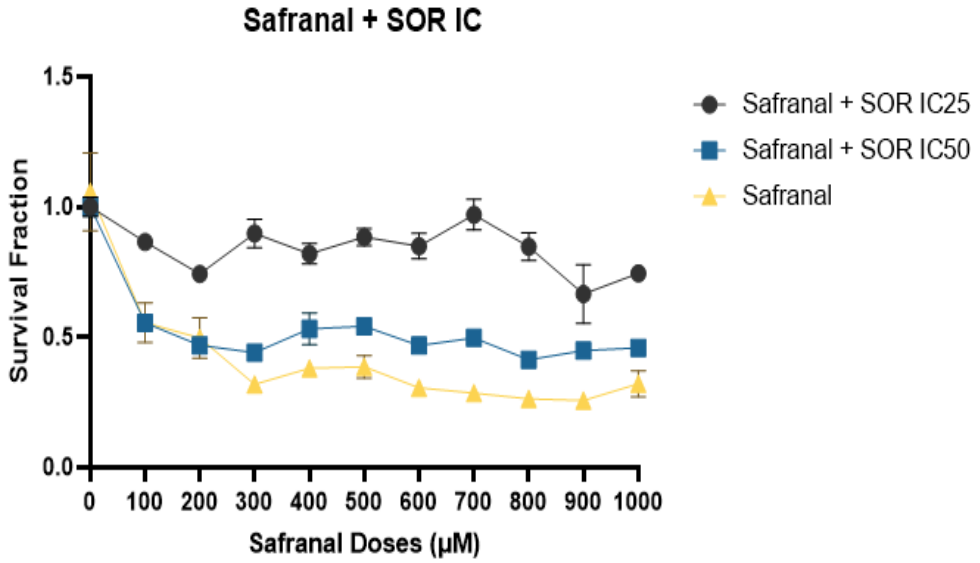


Figure 3: The effect of the combination treatment with a constant dose of Sorafenib with different doses of Safranal.

Next, cells were treated with different doses of Sorafenib (1-100 µM) and IC25 and/or IC50 of Safranal (Figure 4). Here, the combined treatment did not show any significant difference as compared to the single treatment with Sorafenib. This may be due to the high potency and efficacy of Sorafenib on the inhibition of HepG2 cell viability.

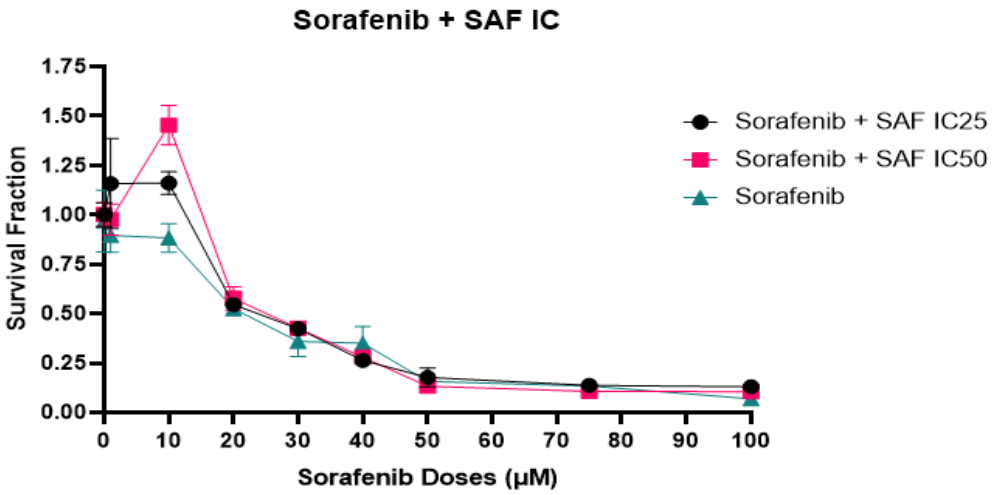
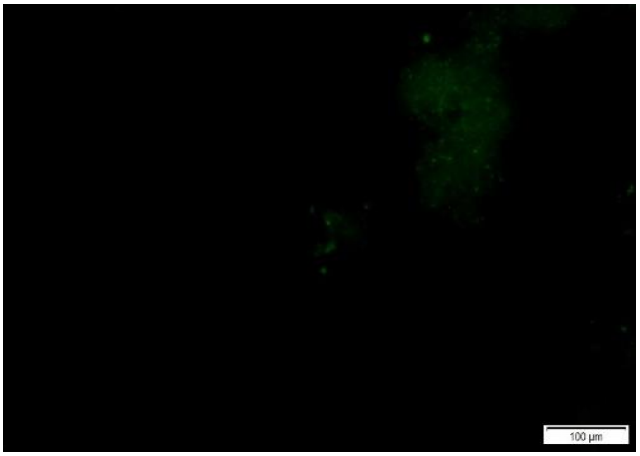


Figure 4: The effect of the combination treatment with a constant dose of Safranal with different doses of Sorafenib.

3.3 Autophagic vacuoles are formed upon treatment with Safranal

In order to determine the effect of Safranal on autophagy, the cells were treated with IC25 and IC50 doses of Safranal and autophagic vacuoles were visualized using an autophagy assay kit, as shown in Figure 5. The data showed the formation of autophagic vacuoles which were more visible with the treatment of HepG2 cells with Safranal at its IC50 value (Figure 5C).



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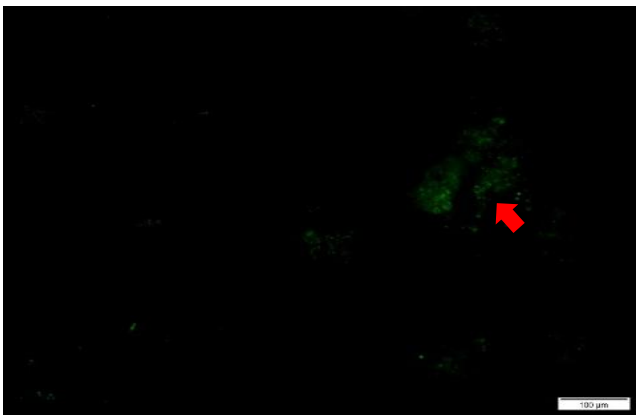

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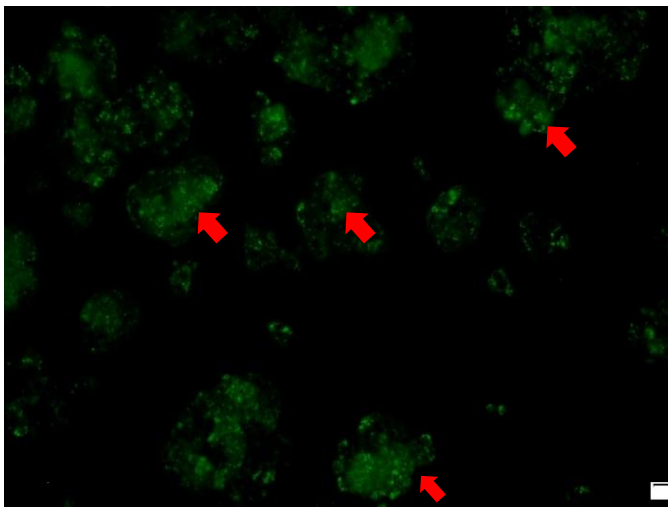
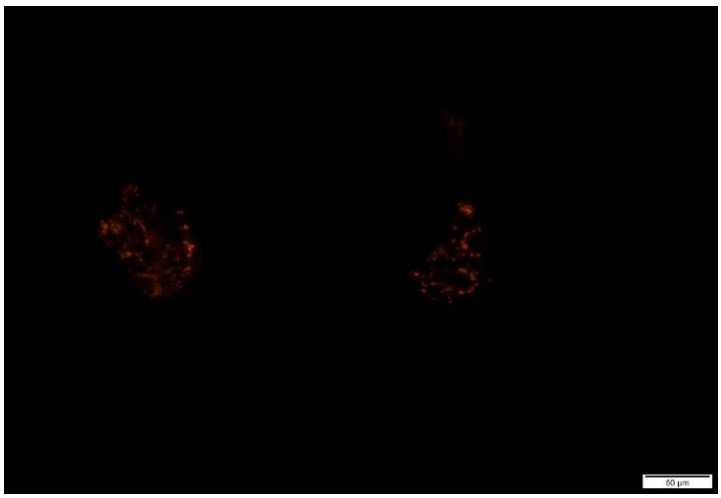


C

Figure 5**:** Autophagic vacuoles visualized at 10X magnification after treating with Safranal IC25 and IC50. (A-Control, B-Safranal at IC25, C-Safranal at IC50)

3.4 Treatment with Safranal results in formation of autolysosomes

The formation of autophagosome was followed by the formation of autolysosomes. The cells were treated with Safranal IC25 and IC50 and autolysosomes were visualized using a fluorescence microscope (Figure 6). The data showed the formation of autolysosomes upon the treatment of HepG2 cells with Safranal (Figure 6B and C).



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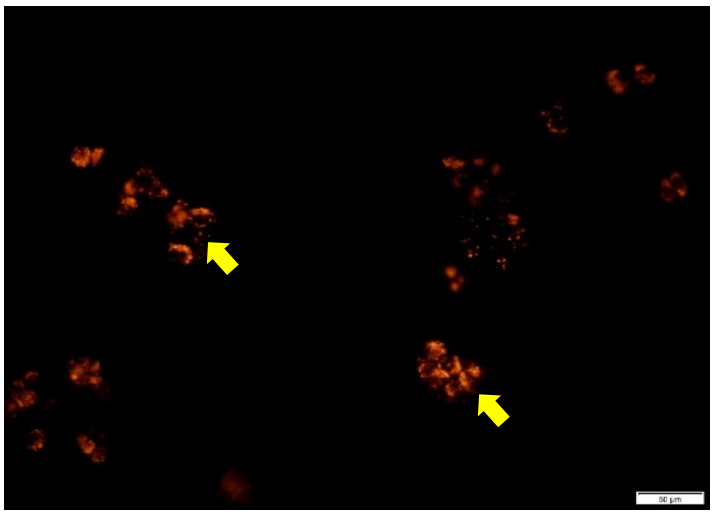

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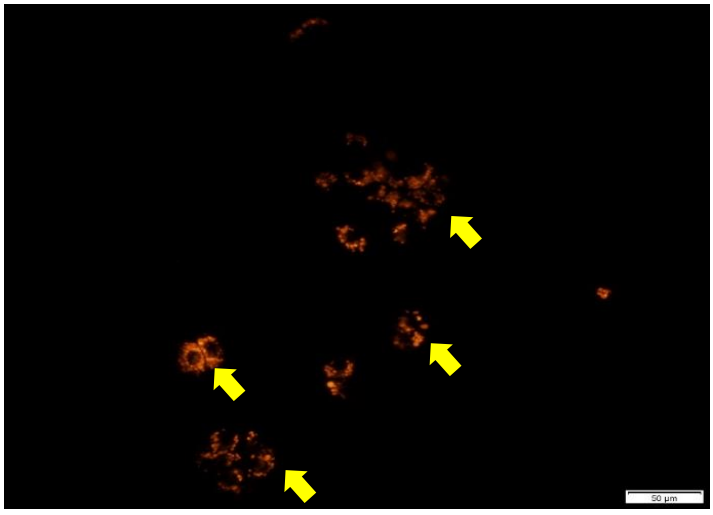

C

Figure 6**:** Autolysosomes visualized at 20X magnification after treating with Safranal IC25 and IC50. (A-Control, B-Safranal at IC25, C-Safranal at IC50)

3.5 Effect of Safranal on autophagic proteins expression

In order to validate the previous results on autophagy, the expression of the major proteins involved in autophagy was analyzed in HepG2 cells. For this, cells were treated with Safranal at IC25 or IC50 and proteins were extracted and analyzed by SDS-PAGE followed by western blot using specific antibodies (Figure 7 and Figure 8).

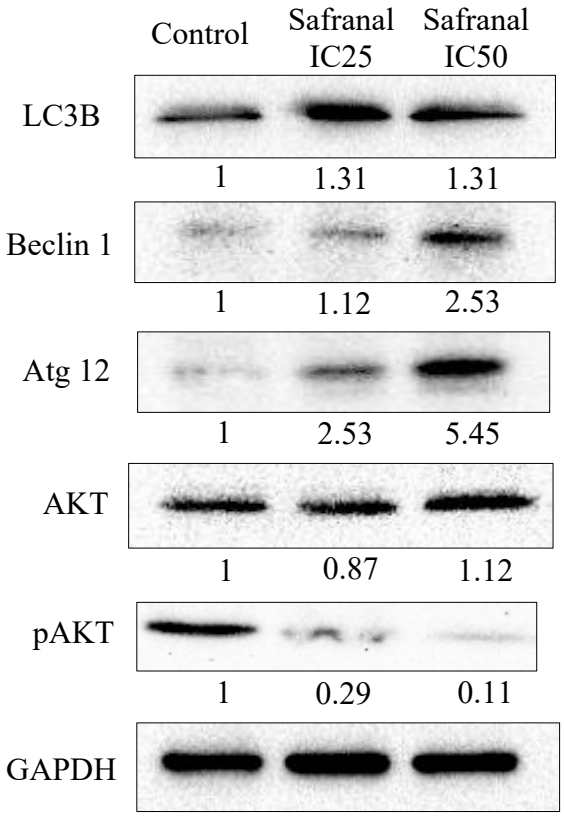

Figure 7**:** Effect of Safranal on the expression of the key proteins known as autophagic markers.

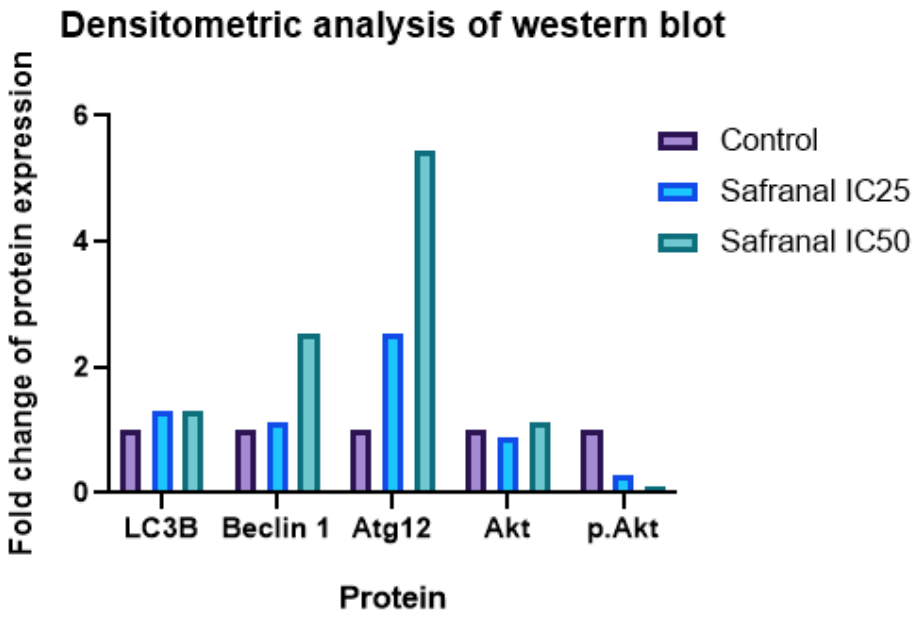

Figure 8: Densitometric Analysis of Western Blot.

All the pro-autophagic markers (LC3B, Beclin 1, Atg 12) tested showed an increased expression in a dose-dependent manner with the treatment with Safranal as compared to control. This is very consistent with the observations on cell viability and autophagy induced by Safranal treatment.

Interestingly, Safranal treatment significantly reduced the phosphorylation level of the kinase AKT (pAKT), known to control the survival pathway (Figure 7). However, Safranal had no effect on the total AKT expressed in HepG2 cells (Figure 7). This is consistent with the previous data showing Safranal inhibiting cell viability and inducing autophagy. All the variations can be considered specific as no change was observed on GAPDH, used as a loading control (Figure 7).

Chapter 4: Discussion

Hepatocellular Carcinoma has become a global burden since it's the most commonly occurring primary liver malignancy with high mortality rates. It is a multi-stage disease and treatment approach and subsequently the treatment outcome depends on liver cancer stage at the time of diagnosis. The available treatment options such as surgery, loco-regional therapies and systematic therapies are proving to be discouraging due to their varying limitations [36]. This study aims at evaluating the effect of Safranal, which is obtained from Saffron, treatment on HCC *in-vitro*. A combination treatment with Sorafenib, the first FDA approved drug for HCC, was also analyzed.

The concept of using natural products as chemotherapeutics is an idea that is gaining attention compared to the use of modern chemotherapeutics and anti-cancer therapies. One such promising agent is safranal, which is a volatile oil of Saffron. Safranal has shown positive therapeutic effects on nervous system, respiratory tract and as an antimicrobial agent [37]. In this study, Safranal resulted in a dose-dependent inhibition of cell survival (Figure 1). The cells were treated with varying concentrations and cell survival was analyzed. Safranal had exhibited apoptotic effects in oral squamous cell carcinoma cell line (KB) and anti-proliferative effects were observed in alveolar lung epithelial cancerous cell line (A549), colon colorectal cell line (HCT-116), breast epithelial cancerous cell line (T47D) prostrate cancerous cell line (PC-3) and hepatocellular carcinoma cells (HepG2). The IC50 values that were calculated, across different studies, are summarized in Table 1 [19, 38, 39].

Table 1: IC50 values of different cell lines on treatment with Safranal.

Cell Line	**IC50 values (µM)**
KB	300
A549	330
HCT-116	170
T47D	420
PC-3	360
HepG2	500

The IC50 value that was calculated in this study is 195 µM, which is in accordance with the previously published values, as shown in Table 1. The IC25 was calculated to be 55 µM.

Sorafenib being the first FDA approved and most widely used drug, for HCC treatment, is studied extensively. In this study, Sorafenib was very potent by inhibiting the cell viability of HepG2 cells in a dose-dependent manner (Figure 2). The IC50 was calculated to be 22 µM. This IC50 value is closer to the published values. The published IC50 values for Sorafenib include; 7.42 µM and 3.4µM in HepG2 cells [32, 40], and 4.4±0.18 µM in A549 cells [31].

The use of combination therapy for the treatment of different diseases is a revolutionary concept. The use of multiple drugs that target the same pathway increase the efficacy of drugs, and in case of drugs that target different pathways, the chances of developing drug resistance are reduced [26].

In this study, Sorafenib and Safranal were combined to investigate their effect on HepG2 cells (Figure 3 and Figure 4**).** As seen from the results, the combination tends to produce an antagonistic action while a synergistic or additive effects were expected. This analysis was also confirmed by calculating the CI, as shown in Figure 9 and Figure 10.

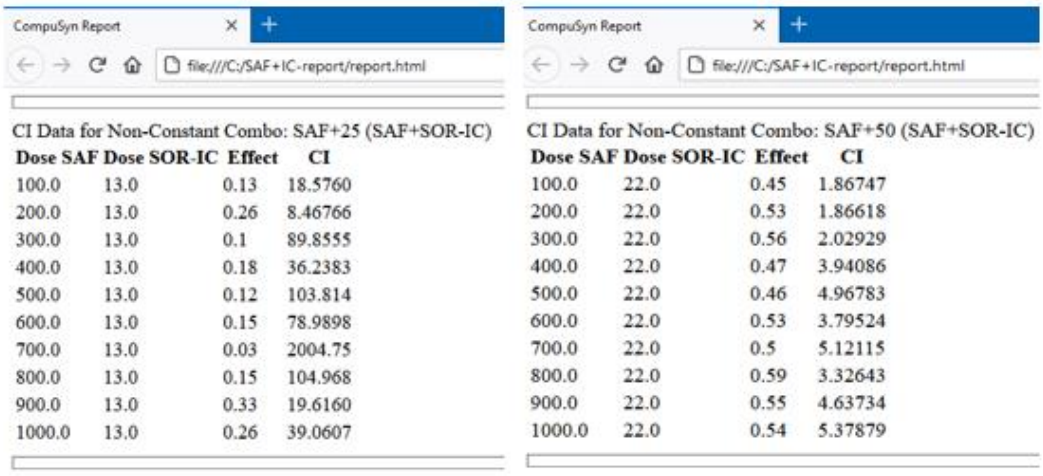

Figure 9: Combination Index values analyzed using CompuSyn Software for treatment with various does of Safranal combined with Sorafenib at IC25 and IC50.

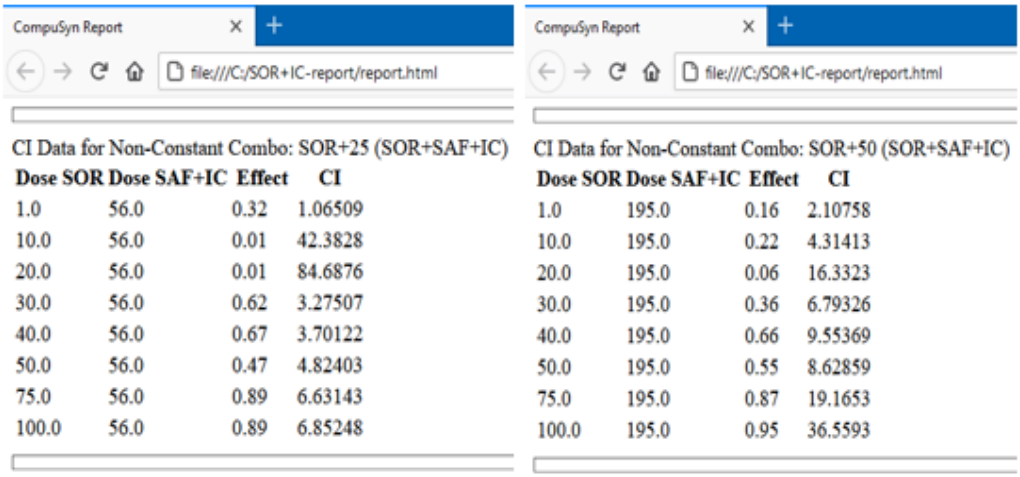

Figure 10: Combination Index values analyzed using CompuSyn Software for treatment with various does of Sorafenib combined with Safranal at IC25 and IC50.

Combination Index are used to quantitatively represent the synergistic (CI<1), additive (CI=1) and antagonist (CI >1) effect of the drugs in combination. The analysis in this study was conducted using CompuSyn software designed by Chou T-C (2010) [41]. Since the combination did not have a synergistic or additive effect, the study was continued using Safranal only.

Autophagy, as mentioned earlier, is a metabolic process responsible for maintaining cellular homeostasis. The role of autophagy in liver under diseased conditions such as HCC itself is of profound significance, since liver is a major metabolic organ. In case of any damage to the liver such as inflammation, autophagy can function as a tumor suppressor mechanism and prevent any malfunction to liver physiology and hence homeostasis [42]. Across different studies autophagy has acted as a tumor suppresser mechanism [33–36, 42] and hence the objective of this study was to determine the effect of safranal treatment on autophagy in HepG2cells.

In other words, autophagy's mechanism of action is the transport of damaged or unwanted substances from different parts of the cell to lysosome for degradation or recycling. In the first step of autophagy, a small isolation membrane called phagophore is formed, this step is called as nucleation. The formation of phagophore is a de-novo process. This phagophore then begins to elongate and form a mature spherical membrane around targeted molecules. These molecules can be damaged or unwanted proteins, cellular debris, dead or dying cells or lipid droplets. The spherical membrane formed from phagophore is a double membraned structure called autophagosome. The size of the autophagosome varies across organisms and also depends on cargo size. For instance, the autophagosome of yeast cells range from ~0.4 μm – 0.9 μm while in mammals the diameter can be 0.5 to 1.5 μm [34, 43]. The autophagosome along with its cargo, then fuses with lysosomes and forms autolysosomes. The lysosomes then

undergo the process of either recycling the cargo, to provide with energy as happens in nutrient starvation or degradation in the case of aggregated proteins etc. These functions of lysosomes are carried out by lysosomal enzymes [44].

The maturation of phagophore forms spherical autophagosome or autophagic vacuoles. Treatment with Safranal IC25 and IC50 of HepG2 cells, lead to the increase of these autophagic vacuoles within the cell. This was visualized using an assay, where the autophagic vacuoles were fluorescently labelled. As seen in Figure 5, there was an increase in autophagic vacuoles on treatment as compared to control. There was an increased vacuoles formation on treatment with Safranal IC50 as compared to IC25 (Figure 5B and Figure 5C).

The next step after the autophagosome formation is the fusion of these vacuoles with lysosomes to form autolysosomes. These autolysosomes were visualized after treatment with Safranal IC25 and IC50. The autolysosomes were fluorescently labelled using a kit. As was seen previously in autophagosome formation, here also there was an increase in autolysosomes in treated cells as compared to control (Figure 6).

The role of autophagy is controversial in HCC. Inflammation is one of the hallmarks of HCC, and autophagy has acted as a tumor suppressor mechanism by suppressing this inflammation. The potential of autophagy to inhibit inflammation was first discovered after an autophagic inhibited mice had increased inflammation. Autophagy also acts as a tumor suppressor mechanism by downregulating certain tumor-promoting miRNA's. Autophagy can also act as a tumor promoter mechanism. As mentioned earlier, hypoxia induces autophagy and due to their constant proliferation and growth, cancerous cells often undergo hypoxic stress. Under such stressful conditions, autophagy is initiated and acts as a tumor promoting mechanism [35].

In this study, autophagy has acted as a tumor suppressing mechanism as is evident from decreased cell viability (Figure 1). The different protein expressions, favorable for autophagy suggest that Safranal induces autophagy.

At the molecular levels and to confirm the previous analyses of induced autophagy, expression of the key autophagic protein markers was analyzed using western blot. LC3 is one of the most abundant protein present in autophagic vacuoles (autophagosome), hence it is also one of the most commonly used marker for autophagy and autophagic activities. LC3 is present abundantly in the cell, within nucleus as well as cytosol. During stressful conditions, such as starvation or hypoxia, LC3 binds with certain autophagy related proteins in the cytosol and initiates the formation of autophagosome [45, 46]. As seen in Figure 7, on treatment with Safranal the expression of LC3 has increased as compared to control. This increase in expression suggest an induction of autophagy within cells.

Beclin 1 belongs to a class of genes called as autophagy related genes (ATG). Beclin 1 is sometimes also referred to as ATG6. The role of Beclin 1 is complex in autophagy. It acts by allosterically modulating class III phosphatidylinositol 3-kinase (PI3KC3) complexes, PI3KC3-C1 and PI3KC3-C2. The PI3KC3-C1 complex is involved in the formation of autophagosome and the PI3KC3-C2 complex is involved in the maturation of autolysosomes. Both of these complexes contain Beclin 1 along with certain other proteins [47]. On treatment with safranal, Beclin 1 expression had increased compared to control (Figure 7). Bcl-2 is an anti-autophagic protein that binds to Beclin 1 and makes binding with PI3KC3 complexes difficult [47]. A study conducted by Al-Hrout *et al.* [19] showed that on treatment with Safranal Bcl-2 protein expression is decreased. The increased expression of Beclin 1, in this study correlates well with this already published data.

Just like some other proteins mentioned earlier, Atg12 is another protein that is involved in the formation of autophagosome. Atg 12 is a part of small complex made of Atg12-Atg5-Atg16, which are first recruited at phagophore assembly site and then through a series of complicated steps leads to the formation of autophagosome [48]. In this study Atg 12 expression was elevated on treatment with Safranal.

AKT is a serine/threonine kinase that plays a crucial role in cell survival, proliferation, growth and metabolism. Increased AKT activity is reported in many cancers, and hence acts as an interesting drug target. AKT also activates another kinase called as TOR (target of rapamycin), which is involved in cell survival and inhibits autophagy. Autophagy can be induced directly either by inhibiting TOR or indirectly via AKT pathway [49]. In this study, after treatment with Safranal the AKT levels remained same, whereas the phosphorylated form of AKT (p.AKT) had decreased, suggesting inhibition of cell survival and thereby an induction of autophagy. This result is very consistent with the data on cell viability and autophagy.

ERK (Extracellular signal-regulated kinase 1/2) belongs to the mitogen activated protein kinase family, and is responsible for cellular proliferation, differentiation and stress response. Abnormalities of this pathway is observed in many cancers and increased phosphorylated ERK (p.ERK) levels are consistent with development of tumors [50]. Numerous studies have shown elevated ERK levels in ovarian cancer and HCC and is responsible for their metastasis and recurrence. And hence ERK/MAPK has become one of the targets for cancer treatment [50, 51]. In order to determine the effect of Sorafenib-Safranal combination on this pathway, since sorafenib is a RAF/MEK/ERK kinase inhibitor, cells were treated with the IC50 of Safranal or Sorafenib alone. Or Safranal IC50 combined with Sorafenib maximum dose (50 μM) and Sorafenib IC50 with Safranal maximum dose (200 μM) (Figure 11).

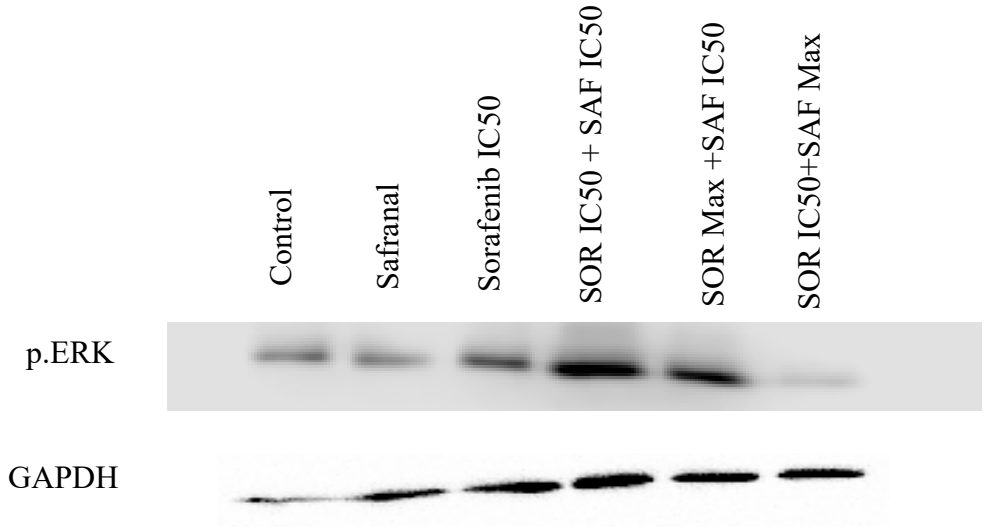

Figure 11: Expression of p.ERK on treatment with Safranal, Sorafenib alone and in combination.

As seen in Figure 11the combination of Saf max+SorIC50 has decreased the p.ERK expression. But a contradictory effect is seen for the other two combinations.

Physiological induction of ERK pathway depends on RAS pathway. When RAS is activated, B-RAF and C-RAF (two distinctive kinases of RAF family) form homo (BRAF-BRAF) or hetero (BRAF-CRAF) dimers. The binding of ATP molecule to both these dimers then leads to phosphorylation of MEK, and hence leads to the activation of the ERK pathway. For ERK pathway to be activated, ATP has to bind to both the catalytic domains of the dimer. In case of mutant cell lines, binding of ATP to anyone of the domains does not trigger a reaction since RAS is inhibited. But in wild type cell lines, binding of ATP to one of the catalytic domain causes transactivation of the second catalytic domain. Since sorafenib is ATP competitive, overtime as drug concentration and ATP increases, ATP occupies both the catalytic domains and causes the phosphorylation cascade to continue [52–54].

It can be hypothesized that the conflicting results in this study with this combination can be explained due to the increased ATP concentration at higher doses. The response of ERK pathway on treatment with Safranal-Sorafenib combination depends on the drug concentrations, as is evident from the varying response in Figure 11. This hypothesis needs to be further experimented on and clarified.

Chatter 5: Conclusion

Hepatocellular Carcinoma is becoming a global burden due to its high mortality rate and increasing incidences. Different factors play crucial role in the treatment regimen for different patients. Even with the scientific advancements, the survival rate is low and the recurrence rate is high. Such disturbing statistics calls for urgent effective alternatives. Natural compounds are one such alternative that are being researched immensely for different diseases including HCC. In this study, Safranal which is a component of Saffron is tested in HepG2 cells for its therapeutic efficacy and ability to induce autophagy. Safranal had successfully inhibited cellular proliferation which was the result of an induced autophagy. This was confirmed by visualizing autophagic vacuoles and autophagosome formation using microscopic analysis. For further investigations, protein expression of different key autophagic markers was also analyzed. LC3B, Beclin 1 and Atg12 are involved in the formation of autophagosome and/or autolysosomes. The expression of these proteins was elevated on treatment with Safranal, as compared to control. AKT which is a key cell survival protein was also investigated for safranal treatment. The expression of active AKT (p.AKT) was decreased on treatment, suggesting a decrease in overall cell survival. Sorafenib which is a multikinase inhibitor and a commonly used drug for HCC was also tested in combination with Safranal against HCC. Interestingly, the combination revealed antagonistic nature of the drugs. The effect of this combination was examined on the expression of an important proliferative pathway, ERK. The treatment seemed to increase the proliferative capacity of the cells, except for in one combination. This suggests that the response of ERK depends on the careful selection of doses.

It would be interesting to see the other pathways that safranal triggers apart from previously reported pathways and autophagy, reported in this study. The use of safranal in other cancerous cell lines can also be investigated. For the combination therapy, it is necessary to further understand the pharmacological aspects and then proceed to understand the underlying antagonistic nature of Safranal and Sorafenib combination.

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