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# Antitumor Effect of Esculetin on Sorafenib -Treated Human Hepatocellular Carcinoma Cell Lines Via Suppression of VEGF-EGFR/RAS/ERK/NF-κB Pathway and Modulation of pI3K/ P38 Axis Crosstalk

**Islam Adel [1,2], Mohamed A. Tantawy [3,4], Ahmad Salahuddin [1], Maha E. Houssen [1 *]**

[1] *Biochemistry Department, Faculty of Pharmacy, Damanhour University,22511, Egypt.*

[2]*The Egyptian Drug Authority, Cairo, Egypt.*

[3]*Hormones Department, Medical Research, and Clinical Studies Institute, National Research Centre, Cairo, Egypt*

[4]*Stem Cells Lab, Center of Excellence for Advanced Sciences, National Research Centre, Dokki, Cairo, Egypt.*

**Abstract**

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Sorafenib is a multi-tyrosine kinase inhibitor used to treat HCC. However, it has many side effects and induces resistance. Esculetin is a natural compound with reported anticancer activity. This study was designed to assess the chemotherapeutic effects of the sorafenib and esculetin combination on human HCC cells. Stock solutions from sorafenib and esculetin in Dimethyl sulfoxide (DMSO) were prepared and then diluted by Dulbecco's Modified Eagle Medium (DMEM) to obtain the required working concentrations. HepG2 and Huh-7 cells have been selected as in vitro model of HCC. Cytotoxicity was evaluated using an MTT assay. Flow cytometry analysis and scratch assay were performed to assess apoptosis and migration. Finally, vascular endothelial growth factor (VEGF), VEGFR-2, EGFR, NF-κB, and Ki-67, PI3K, p38MAPK levels were quantified through ELISA, while HRAS and ERK2 gene expression were detected through RT-PCR. The sorafenib and esculetin combination exerted a potent synergistic anti-tumor effect by modulating the EGFR and VEGF-RAS/ERK/PI3K/NF-κB axes associated with significant upregulation of the apoptotic p38MAPK/caspase-3 axis, modulation of pI3k/p38MAPK crosstalk and inhibition of the proliferation marker Ki67. This study paves the way for using esculetin as good adjuvant to sorafenib as a promising future treatment for hepatocellular carcinoma.

*Keywords*: Hepatocellular carcinoma, Sorafenib, Esculetin, HepG2, Huh-7, Synergism, Ki67, VEGF, NF-κB.

## 1. Introduction

Hepatocellular carcinoma (HCC) is a particularly deadly type of cancer [1]. It is the second leading cause of cancer-related mortality worldwide [2]. Besides, it is the fourth most common cancer type in Egypt [3]. HCC is characterized by a major dysregulation of several oncogenes and tumor suppressor genes, along with abnormalities in diverse critical cell signaling pathways contributing to cancer progression, such as the RAS/RAF/MEK/ERK [4], the PI3K/Akt/mTOR [5], NF-κB [6], Ki-67 [7], EGFR [8], and angiogenic pathways [9].

Angiogenesis is a crucial biological process mediating HCC progression. The vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR-2) signaling pathway plays a key role in tumor angiogenesis, which promotes tumor growth by allowing oxygen and nutrient delivery [10]. VEGF activates VEGFR-2, causing a rapid proliferative response that promotes the destruction of the extracellular matrix and basement membrane by matrix metalloproteinase-2 (MMP-2). Besides, VEGFR-2 promotes endothelial cell migration and invasion, leading to the formation of new blood vessels [11]. Thus, targeting these pathways should provide new treatments for advanced HCC patients.

Sorafenib (BAY 43-9006) is a multi-tyrosine kinase inhibitor and an FDA-approved HCC treatment [12]. Since 2007, it has been the standard of care for patients with advanced unresectable HCC

*Corresponding author e-mail: mahahoussen@pharm.dmu.edu.eg, mahahoussen@yahoo.com.

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[13]. It inhibits multiple tyrosine kinases, reducing cancer cell growth and increasing apoptosis by inhibiting the RAS/RAF/MEK/ERK [12] and PI3K/Akt pathways [14]. Despite the chemotherapeutic efficacy of sorafenib, many patients develop acquired resistance to sorafenib, suppressing their responsiveness to the drug [15]. As a result, many researchers have attempted to ameliorate the chemotherapeutic efficacy of sorafenib and counteract its resistance by combining it with herbal extracts or natural active products as adjuvant chemotherapeutic agents [15].

Esculetin (also called cichorigenin or 6,7-dihydroxycoumarin) is a coumarin derivative extracted from many natural plants, such as *Artemisia capillaries* (capillary wormwood), *Artemisia scoparia* (redstem wormwood) and *Citrus limonia* (mandarin lime) leaves [16]. Esculetin inhibits VEGF-induced proliferation and DNA synthesis and downregulates MMP-9 expression in human umbilical vein endothelial cells [17]. Furthermore, esculetin reduces VEGFR-2 phosphorylation and inhibits the ERK1/2 and eNOS/Akt signaling pathways, showing its essential role in inhibiting cancer development [18].

Thus, the present study assessed the chemotherapeutic effect of sorafenib combined with esculetin against HCC *in vitro* using HepG2 and Huh-7 cells and investigated the underlying molecular mechanisms.

## 2. Material and methods

### *2.1. Chemicals*

Sorafenib tosylate (CAS number 475207-59-1) and esculetin (6,7-dihydroxycoumarin) (CAS number 305-01-1) were purchased from Sigma Aldrich (St Louis, MO, USA). High glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were obtained from Biowest Co (Nuaillé, France). Penicillin/streptomycin (Pen/Strep) and trypsin were ordered from Lonza (Basel, Switzerland). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany).

### *2.2 Cancer cell lines*

The protocols complied with the rules of the ethical committee of the Faculty of Pharmacy, Damanhour University (ref no 321PB21). The experiments were performed on two human HCC cell lines: HepG2 and Huh-7, procured from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were cultured in DMEM containing 10% heat-inactivated FBS and 1% penicillin/streptomycin and incubated at 37°C under 5% $CO_2$ atmosphere at 95% humidity. Cells were passaged around 5–6 times after reaching 90% confluence.

Stock solutions from sorafenib and esculetin in DMSO were prepared and diluted using DMEM to obtain the required working concentrations. The vehicle solution for controls contained only DMEM and DMSO, in proportions similar to the experimental solutions.

### *2.3 Cell viability assay (MTT assay)*

The cytotoxic effect of sorafenib, esculetin, and their combination on human HCC cells was investigated through the MTT assay. The cells were cultured in 96-well plates ($10^4$ cells/well) and incubated them at 37°C overnight. After discarding the old media, we added 100 µl of media containing sorafenib at 0, 1.25, 2.5, 5, 10, 20, or 40 µM and esculetin 0, 25, 50, 100, 200, 400, or 800 µM. We then incubated the cells for 48 h, added the MTT solutions to each well, and incubated again for 4 h. The supernatant was discarded and the formazan crystals were dissolved in 150 µl of DMSO.

Cell viability was determined by reading the absorbance at 492 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Then, we calculated the cell viability percentage using the formula:

(OD of treated sample/control OD) × 100

The assay was performed three times and median inhibitory concentrations ($IC_{50}$) was calculated using CompuSyn software version 1 (CompuSyn, Inc., Paramus, NJ, USA) [19].

### *2.4 Cytotoxicity analysis*

Cell viability assays and data analysis with CompuSyn software were performed to assess the synergistic anti-tumor efficacy of sorafenib and esculetin on HepG2 and Huh-7 cells. The combination index (CI) and dose reduction index (DRI) were calculated using the percentage of cell viability as reported by Chou 2010 [19].

### *2.5 Flow cytometry analysis*

Annexin V-FITC/propidium iodide (PI) staining was performed as described previously [20] to determine the effect of the tested agents on apoptosis extent. HepG2 cells were treated with sorafenib (9.6 µM), esculetin (225 µM), or both for 48 h, and Huh-7 cells with sorafenib (4.5 µM), esculetin (350 µM), or both for 48 h. The doses used in this assay correspond to each compound's $IC_{50}$ for each cell type. After trypsinization, the cells were collected and washed twice with ice-cold PBS (pH = 7.4), then added 500 µl of Annexin V-FITC/PI solution. At this stage, the cells were kept in the dark for 30 min at room temperature. The cells were injected into a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, Indiana, USA) and recorded FITC and PI fluorescence signals. Finally, the obtained data were analyzed using CytExpert Software.

### *2.6 Scratch assay (wound healing assay)*

We evaluated migration through a wound scratch assay. For both cell lines, we seeded $5 \times 10^5$ cells/well in a 6-well plate and incubated them overnight at 37°C under a 5 % $CO_2$ atmosphere. We

removed the medium, scratched the adherent sheets of cells with a sterile 10 µl pipette tip, and washed them with PBS. Finally, we added 3 ml of treatment low serum media (1% FBS DMEM) and treated the cells with sorafenib, esculetin, or both, at doses corresponding to each compound's $IC_{50}$ for each cell type. We recorded cell migration into the wound space using an inverted microscope and optical camera (ZEISS ZEN microscope software blue edition) 0 and 48 h after treatment.

*2.7 Experimental cell design and cell lysate preparation*

Cells of each line were cultured in 12 T-75 flasks ($2 \times 10^5$ cells/flask) [21] and incubated them overnight. Then, we kept untreated cells as controls, treated the rest with sorafenib, esculetin, or both, and incubated them for 48 h. For each compound, we used a dose equal to its $IC_{50}$ on each cell type. Next, we collected the cells and stored cell supernatants and pellets at −80°C until further investigation. We prepared cell lysates using a RIPA buffer (Thermo Fisher Scientific, MA, USA) containing 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. Following the manufacturer's instructions, we added 1 ml of RIPA lysis buffer to HepG2and Huh-7 cell pellets, shook gently for 15 min on ice, then centrifuged at 14,000×g for 15 min to remove the cell debris. Next, we stored the lysates at −20°C until further investigations.

*2.8 Biomarker analysis using the sandwich ELISA technique*

Several tumor markers in HepG2 and Huh-7 cells were quantified using the reliable, highly specific, and sensitive sandwich ELISA technique following the manufacturer's instructions [22]. These biomarkers include NF-κB (Catalog # MBS450580, MyBioSource, CA, USA), Ki-67 (Catalog # ab253221, Abcam, Cambridge, UK), VEGF (Catalog # DVE00, R&D Systems, MN, USA), VEGFR-2 (Catalog # ab213476, Abcam, Cambridge, UK), EGFR (Catalog# I6176, Glory Science, Jiangsu, China), PI3K (Catalog # MBS703766,MyBioSource, CA, USA), and p38MAPK(Catalog # KHO2261, Thermo Fisher Scientific Inc. MA, USA).The results represent the mean ± SEM of three separate experiments, each carried out in triplicate.

*2.9 Caspase-3 activity assay*

Active caspase-3 levels were quantified using a colorimetric kit (Catalog # ab39401, Abcam). Briefly, active caspase-3 hydrolyzes the peptide substrate (Ac-DEVD-pNA) in cell lysates and releases a *p*-nitroaniline moiety quantifiable colorimetrically at 405 nm. We determined the cleaved *p*-nitroaniline concentration using a calibration curve as described by Nicholson *et al*. [23]. Data were expressed as the mean ± SEM of three separate experiments, each carried out in triplicate.

*2.10 Quantitative real-time polymerase chain reaction RT-PCR*

Total RNA was extracted using an RNeasy RNA Extraction Mini Kit (Cat. No 74104, Qiagen, Hilden, Germany). We measured the purity and concentration of extracted RNA using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc. MA, USA). Next, we reverse-transcribed the RNA equivalent concentrations of isolated RNA using a RevertAid First Strand cDNA Synthesis Kit (Cat. No. k1622, Thermo Fisher Scientific, MA, USA). We quantified HRAS and ERK2 gene expression levels on a RT-PCR system (Dtlite Real-Time system) using Rotor-Gene SYBR Green RT-PCR Kit (Cat. No. 204174, Qiagen, Hilden, Germany) with the following primer pairs: HRAS, 5'-GAT GCC TTC TAC ACG TTG GT-3'/5'-AGC TTG TGC TGC GTC A-3'; ERK2, 5'-CCG AAG CAC CAT TCA AGT TC-3'/5'-AGA AGA ACA CCG ATG TCT GA-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CCG CAT CTT CTT TTG CGT C-3'/5'-CCC AAT ACG ACC AAA TCC GT-3'. We determined the fold changes in HRAS and ERK2 gene expression using GAPDH as a housekeeping gene, as published by Livak and Schmittgen [24]. We evaluated each sample three times to ensure accuracy.

*2.11 Statistical analysis*

Data were presented as the mean ± SEM. We performed multiple comparisons using one-way analysis of variance followed by Tukey's post hoc multiple comparison test and considered $p < 0.05$ as significant. We used Graph Pad Prism® software package version 8 (GraphPad Software Inc., CA, USA) for all statistical analyses and plots.

## 3. Results

*3.1. Cytotoxicity of sorafenib and esculetin on HCC cell lines*

Both drugs showed a concentration-dependent cytotoxic effect. Indeed, treating cells with sorafenib inhibited the viability of HepG2 and Huh-7 cells with $IC_{50}$ values of 9.6 and 4.5 µM, respectively. Meanwhile, esculetin inhibited cell growth with an $IC_{50}$ of 225 µM for HepG2 cells and 350 µM for Huh-7 cells. Furthermore, combining sorafenib and esculetin at the same concentration ranges inhibited cell viability with a combined $IC_{50}$ of 100.7 (3.7 µM for sorafenib + 97 µM for esculetin) for HepG2 cells, and of 41.96 µM (1.96 µM for sorafenib + 40 µM for esculetin) for Huh-7 cells (Fig. 1).

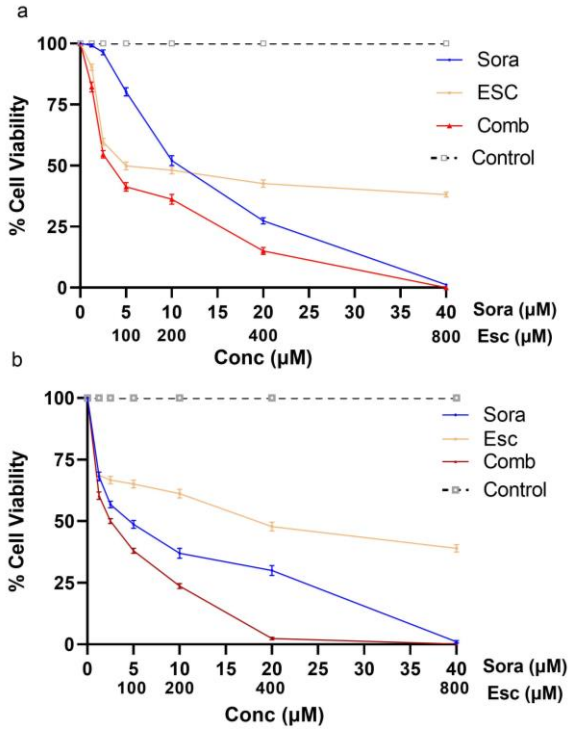


**Fig. 1.**Cytotoxicity of sorafenib (Sora, 1.25–40 μM), esculetin (Esc, 25–800 μM), and both. a) HepG2 cells, b) Huh-7 cells. The dotted black line represents the viability of untreated cells.

*3.2 Synergy analysis of sorafenib and esculetin*

We assessed the synergy of sorafenib and esculetin on HCC cells using CompuSyn software. Table 1 shows the CompuSyn software-calculated combination indices from HepG2 and Huh-7 cells treated with the sorafenib and esculetin combination (at doses equal to each compound's $IC_{50}$). The CI was 0.93 in HepG2 cells and 0.53 in Huh-7 cells. These values are markedly lower than 1, revealing a synergy in the sorafenib and esculetin combination. A CI equal to 1 would indicate an additive effect, while a value higher than 1 would indicate an antagonism. Furthermore, the DRI values indicated that the combination allowed a dose decrease to obtain the same effect as the individual compounds [19].

In addition, Table 1 shows that in HepG2 cells, sorafenib and esculetin had individual $IC_{50}$ values of 9.6 μM and 225 μM, respectively. Meanwhile, combining the drug decreased the $IC_{50}$ values of sorafenib and esculetin to 3.7 μM and 97 μM, respectively. The corresponding DRI values were 2.59 for sorafenib and 2.32 for esculetin. Regarding Huh-7 cells, sorafenib and esculetin had individual $IC_{50}$ values of 4.5 and 350 μM, respectively. Meanwhile, combining the drugs decreased the $IC_{50}$ values of sorafenib and esculetin to 1.96 μM and 40 μM, respectively. The corresponding DRI values were 2.3 for sorafenib and 8.75 for esculetin. These findings indicate that esculetin improves the effectiveness of sorafenib.

*3.3 Apoptosis analysis*

Phosphatidylserine flipping from the inner membrane leaflet to the outer membrane surface after cellular damage indicates apoptosis. This phenomenon can be monitored by double-staining cells with annexin-FITC and PI [25]. we treated HepG2 cells with sorafenib (9.6 μM, 48 h), esculetin (225 μM, 48 h), or both. Besides, we treated Huh-7 cells with sorafenib (4.5 μM, 48 h), esculetin (350 μM, 48 h), or both, then compared them with untreated cells. The doses correspond to each compound's cytotoxicity $IC_{50}$ on each cell type. Next, we performed flow cytometric analyses, including Annexin-V-FITC/PI differential apoptosis/necrosis assessment.

In HepG2 cells, sorafenib induced apoptosis by 111.19-fold (35.58 % vs. 0.32 % for control, $P \leq 0.05$), while esculetin induced it by 125.19-fold (40.06 % vs. 0.32 % for control, $P \leq 0.05$). In comparison, the combination of sorafenib plus esculetin increased apoptosis by 182.8-fold (58.5% vs 0.32 % for control, $P \leq 0.05$).

Esculetin yielded a 1.13-fold higher apoptosis increase than sorafenib (40.06 % and 35.58 %, respectively). Meanwhile, the sorafenib and esculetin combination yielded a 1.64-fold and 1.46-fold higher apoptosis induction than sorafenib and esculetin alone, respectively (Figs. 2a and 2c).

In Huh-7 cells, sorafenib induced apoptosis by 38.96-fold (60.39 % vs. 1.55 % for control, $P \leq 0.05$), while esculetin induced it by 23.59-fold (36.56 % vs. 1.55 % for control, $P \leq 0.05$), while the sorafenib plus esculetin combination increased it by 49.86-fold (77.28% vs. 1.55 % for control, $P \leq 0.05$).

Sorafenib yielded a 1.65-fold higher apoptosis increase than esculetin (60.39 % and 36.56 %, respectively). Meanwhile, the sorafenib plus esculetin combination yielded a 1.28-fold and 2.11-fold higher apoptosis increase than sorafenib and esculetin alone, respectively (Figs. 2b and 2d).

Table 1. Combination indices and dose reduction indices of sorafenib and esculetin on HepG2 and Huh-7 cells.

| Cell line | CI | Individual $IC_{50}$ | | $IC_{50}$ of each drug in combination | | DRI of Sorafenib | DRI of Esculetin |
|---|---|---|---|---|---|---|---|
| | | Sorafenib (μM) | Esculetin (μM) | Sorafenib (μM) | Esculetin (μM) | | |
| HepG-2 | 0.93 | 9.6 | 225 | 3.7 | 97 | 2.59 | 2.32 |
| Huh-7 | 0.53 | 4.5 | 350 | 1.96 | 40 | 2.3 | 8.75 |

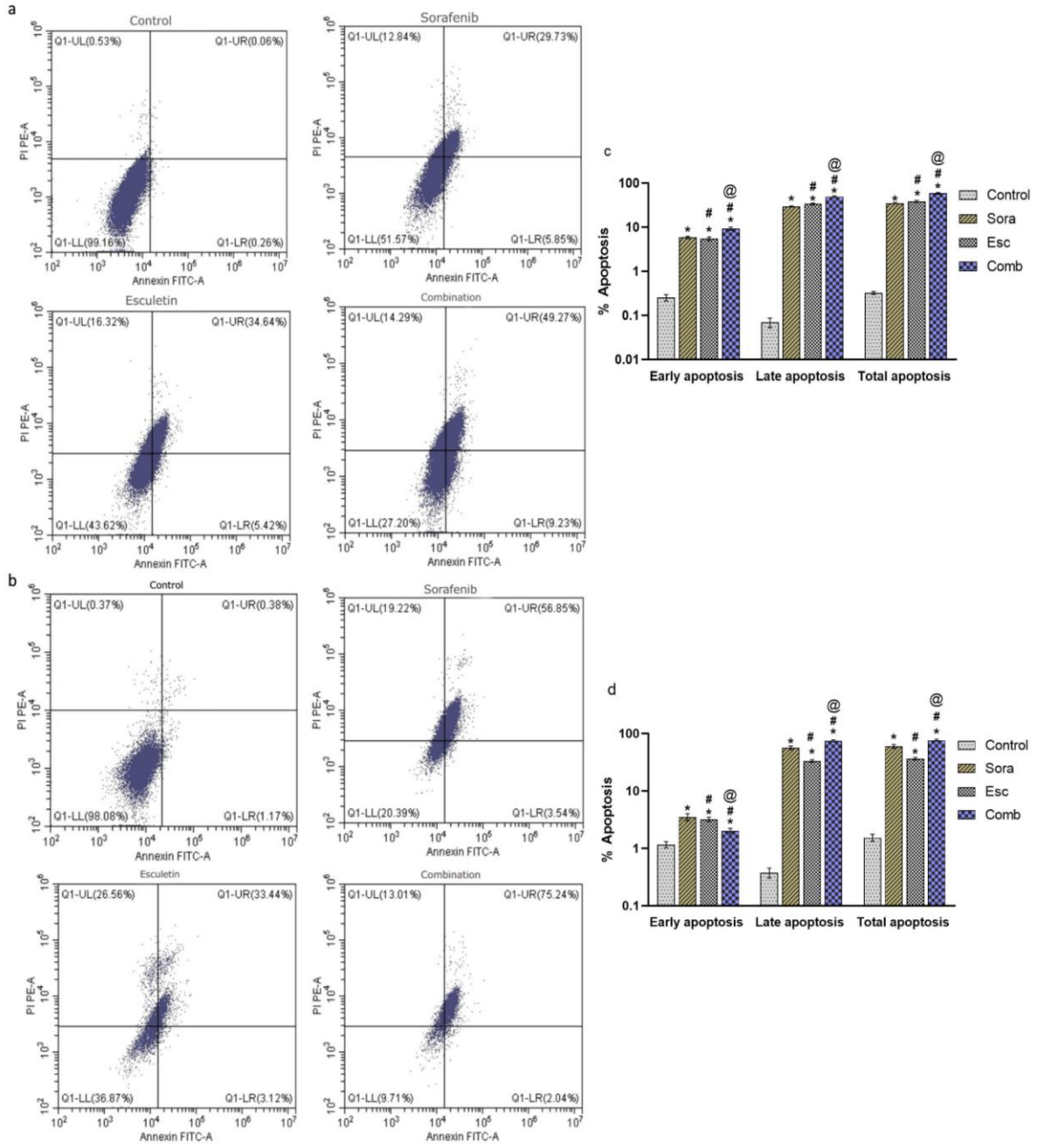


**Fig. 2.** Cytograms showing annexin-V/PI-stained cells: (a) HepG2 cells. (b) Huh-7 cells. Quadrant charts show Q1-UL (necrotic cells, AV-/PI+), Q1-UR (late apoptotic cells, AV+/PI+), Q1-LL (normal cells, AV-/PI-), and Q1-LR (early apoptotic cells, AV+/PI-). (c) Bar chart of apoptosis percentage in HepG2 cells and (d) Bar chart of the apoptosis percentage in Huh-7 cells treated with sorafenib, esculetin, or both. Data are represented as mean ± SEM.*P ≤ 0.05, compared with control cells. #P ≤ 0.05, compared with sorafenib-treated cells. @P ≤ 0.05, compared with esculetin-treated cells.

*3.4 Scratch assay (wound healing assay)*

Treating hepatocellular carcinoma cells with sorafenib, esculetin, or both for 48 h inhibited cell migration. Moreover, when combined, sorafenib and esculetin had a synergistic effect (Fig. 3).

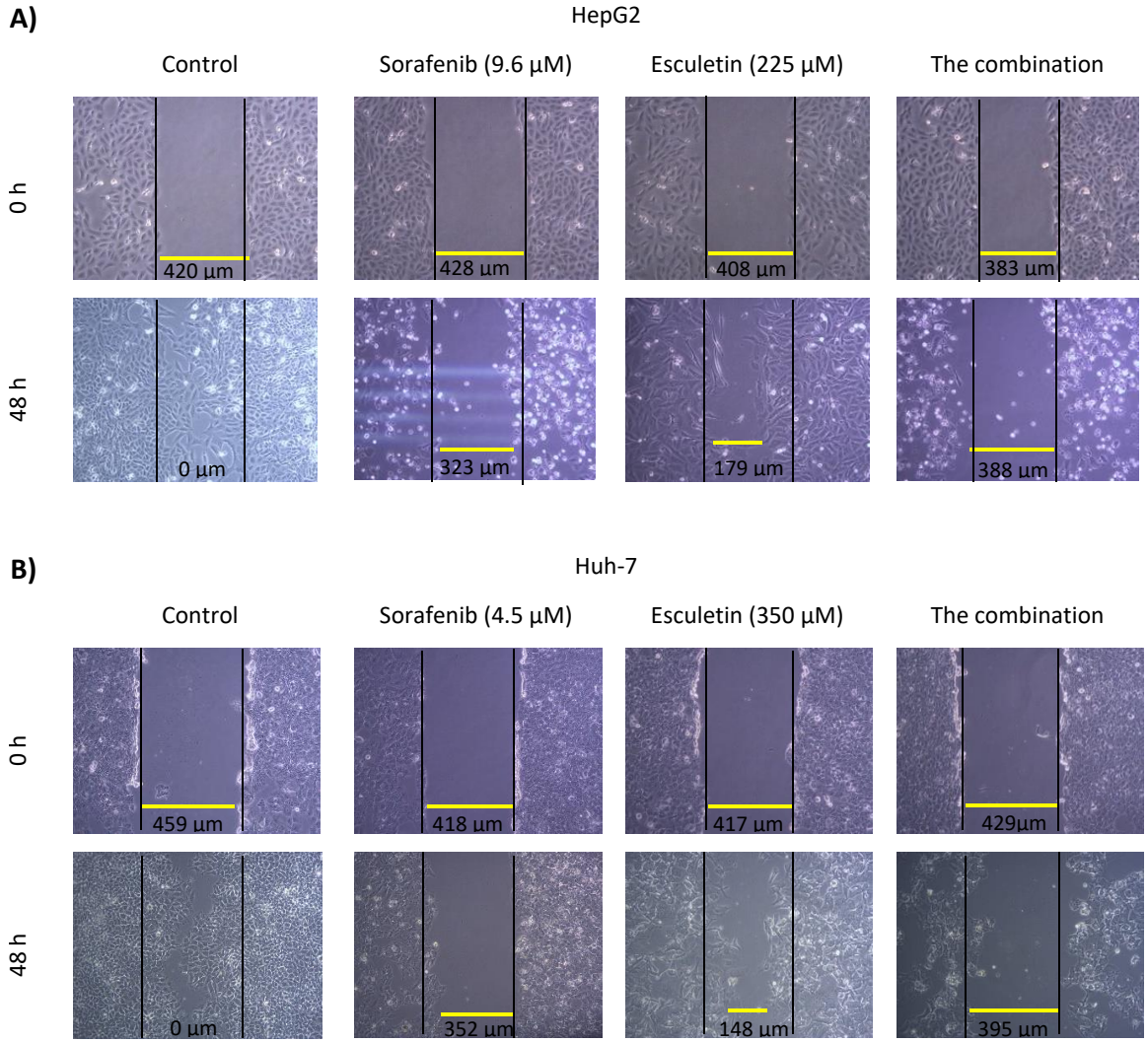


**Fig. 3.** Cell migration inhibition by sorafenib, esculetin, and their combination. (A) HepG2 cells treated with 9.6 µM sorafenib, 225 µM esculetin, or both, and untreated. (B) Huh-7 cells treated with 4.5 µM sorafenib, 350 µM esculetin, or both, and untreated.

*3.5 Sorafenib and/or esculetin induced apoptosis and activated caspase-3*

Further on, and in addition to the flow cytometry, we estimated apoptosis induction through caspase-3 activity. The basal expression of caspase-3 in untreated cells were for HepG2 was 0.42 ± 0.16 µM pNA/min/ml, and for Huh-7 was 0.67 ± 0.013 µM pNA/min/ml. HepG2 cells, sorafenib, esculetin, and the combination increased apoptosis by 219.8% (1.34 ± 0.017 µM pNA/min/ml), 122.1% (0.933 ± 0.02 µMpNA/min/ml), and 301.7% (1.69 ± 0.017 µMpNA/min/ml), respectively. In Huh-7 cells, sorafenib increased apoptosis by 40.44% (0.667 ± 0.012 µMpNA/min/ml), esculetin by 23.43% (0.937 ± 0.012 µMpNA/min/ml), and the combination by 98.9% (1.33 ± 0.013µM pNA/min/ml) (Fig. 4a).

*3.6 Sorafenib and/or esculetin reduced NF-κB levels*

Compared with the control HepG2 cells, sorafenib-, esculetin-, and combination-treated cells had NF-κB levels reduced by 27.31% (6.3 ± 0.163 ng/ml), 21.19 % (6.83 ± 0.124 ng/ml), and 39.66 % (5.23 ± 0.125 ng/ml), respectively. Meanwhile, in Huh-7 cells, sorafenib, esculetin, and the combination reduced NF-κB levels by 24.85 % (8.47 ± 0.124 ng/ml), 23.07 % (8.67 ± 0.126 ng/ml), and 44.37 % (6.23 ± 0.12 ng/ml), respectively (Fig. 4b).

*3.7 Sorafenib and/or esculetin reduced Ki-67 levels*

Compared with control HepG2 cells, sorafenib-, esculetin-, and combination-treated cells had Ki-67 levels reduced by 28.3% (391 ± 2.44 pg/ml), 13.8% (474 ± 2.16 pg/ml), and 34.6 % (356.6 ± 3.39 pg/ml), respectively. Meanwhile, in Huh-7 cells, sorafenib, esculetin, and the combination reduced Ki-67 levels by 13.56 % (709.7 ± 3.85 pg/ml), 3.45 % (792.7 ± 5.31 pg/ml), and 25.01 % (615.6 ± 4.1 pg/ml), respectively (Fig. 4c).

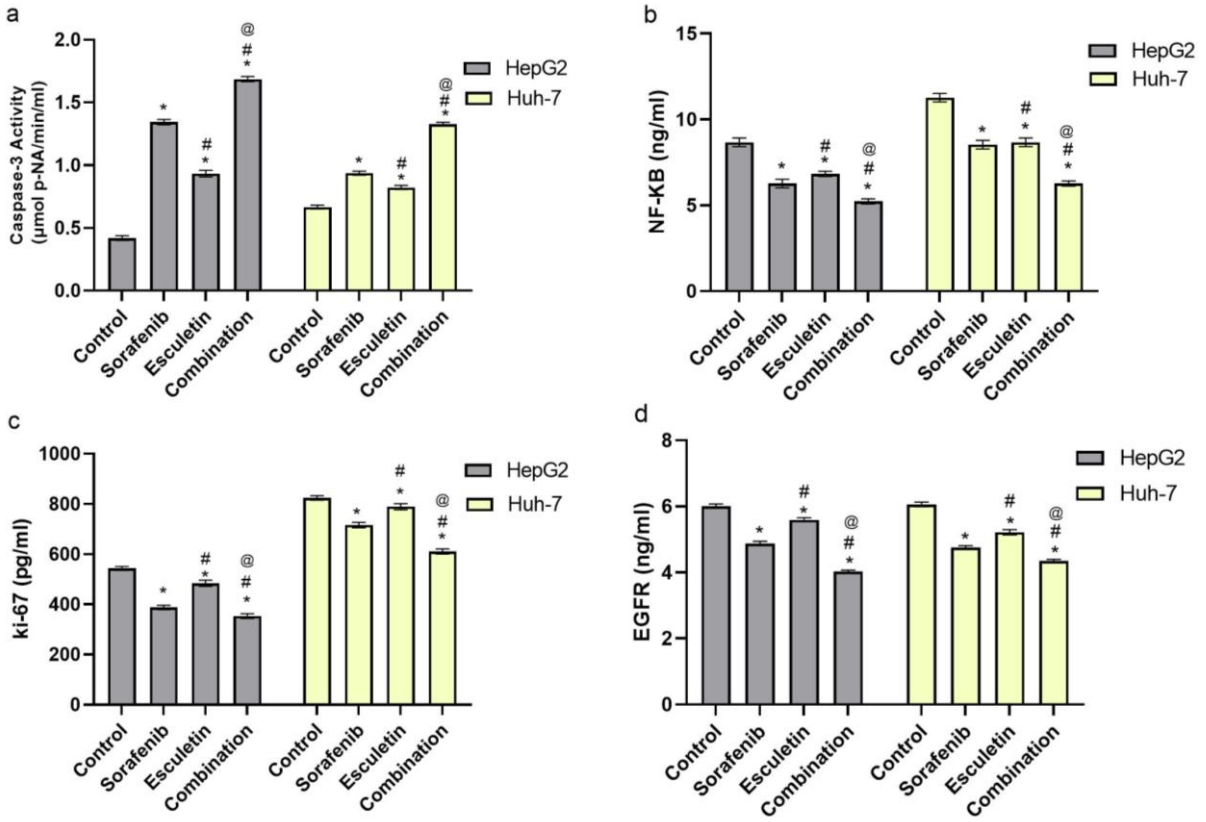


**Fig. 4.** Effects of sorafenib (9.6 µM), esculetin (225 µM), and their combination in HepG2 cells and sorafenib (4.5 µM), esculetin (350 µM), and their combination in Huh-7 cells on proliferation and apoptosis markers. The levels of caspase-3 activity (a), NF-κB (b), Ki-67 (c), and EGFR (d) were measured using ELISA or colorimetry. Data are expressed as the mean ± SEM of three experiments, each conducted in triplicate. *P< 0.05 vs. control cells, #P< 0.05 vs. sorafenib-treated cells, and @P< 0.05 vs. esculetin-treated cells.

*3.8 Sorafenib and/or esculetin reduced EGFR levels*

Compared with control HepG2 cells (6.01 ± 0.057 ng/ml), EGFR expression was reduced 18.76% (4.88 ± 0.049 ng/ml), 6.94% (5.59 ± 0.053 ng/ml), and 33.02% (4.02 ± 0.04 ng/ml) in sorafenib, esculetin-, and combination-treated cells, respectively. In Huh-7 cells, sorafenib, esculetin, and the combination reduced EGFR expression by 21.5% (4.75 ± 0.042 ng/ml), 13.86% (5.22 ± 0.065 ng/ml), and 27.5% (4.36 ± 0.016 ng/ml), respectively (Fig. 4d).

*3.9 Sorafenib and/or esculetin decreased VEGF expression*

Compared withHepG2 control cells, sorafenib-, esculetin-, and combination-treated cells had VEGF protein expression levels reduced by 39.03% (684.6 ± 5.31 pg/ml), 26.8% (822 ± 6.68pg/ml), and 57.01% (482.6 ± 6.54pg/ml), respectively. In Huh-7 cells, sorafenib, esculetin, and the combination reduced VEGF expression by 48.57% (663.7 ± 3.39 pg/ml), 42.74% (739 ± 2.16 pg/ml), and 54.88% (582.3 ± 3.29 pg/ml), respectively (Fig. 5a).

*3.10 Sorafenib and/or esculetin decreased VEGFR-2 expression*

In HepG2 cells, control cells had the highest VEGFR-2 levels. Sorafenib-, esculetin-, and combination-treated cells decreased VEGFR-2 levels by 40.28% (1717 ± 6.16 pg/ml), 33.66% (1907.3 ± 3.29 pg/ml), and 61.22% (1114.6 ± 6.79 pg/ml), respectively. Meanwhile, in Huh-7 cells, sorafenib, esculetin, and the combination reduced VEGFR-2 levels by 34.06% (2122.3 ± 8.65 pg/ml), 32.96% (2156.7 ± 2.49 pg/ml), and 63.91% (1161.3 ± 7.04 pg/ml), respectively (Fig. 5b).

*3.11 Sorafenib and/or esculetin decreased PI3K levels*

Compared with the control HepG2 cells, sorafenib-, esculetin-, and combination-treated cells had PI3K levels reduced by 44.11 % (2.67 ± 0.287 ng/ml), 30.67 % (3.3 ± 0.327 ng/ml), and 60.08 % (1.9 ± 0.33ng/ml), respectively. Meanwhile, in Huh-7 cells, sorafenib, esculetin, and the combination reduced PI3K levels by 36.7 % (4.53 ± 0.29 ng/ml), 29.74 % (5.03 ± 0.249 ng/ml), and 69.83 % (2.16 ± 0.205 ng/ml), respectively (Fig. 5c).

*3.12 Effect of sorafenib and/or esculetin on p38MAPK levels*

Compared with control HepG2 cells, sorafenib-, esculetin-, and combination-treated cells had p38MAPK levels increased by 14.5 % (134.6 ± 3.68 pg/ml), 6.5 % (125.3 ± 4.49 pg/ml), and 23.89 % (145.6 ± 4.5pg/ml), respectively. Meanwhile, in Huh-7 cells, sorafenib, esculetin, and the combination increased p38MAPK levels by 10.54 % (171.7 ± 3.29pg/ml), 7.34 % (166.6 ± 4.49pg/ml), and 17.39 % (182.3 ± 4.92pg/ml), respectively (Fig. 5d).

*3.13 Effect of sorafenib and/or esculetin on HRAS and ERK2 gene expression levels*

To assess the effect of sorafenib, esculetin, and their combination on the MAPK cascade, we measured HRAS and ERK2 gene expression.

First, in HepG2 cells, sorafenib and esculetin reduced the relative HRAS gene expression by 31.85% (0.682 ± 0.012) and 24.38% (0.756 ± 0.01), respectively. Interestingly, the combination downregulated the HRAS gene expression significantly: combination-treated cells had levels 39.7% (0.603 ± 0.012) lower than control cells. In Huh-7 cells, combination-treated cells also had the lowest HRAS gene expression levels, reaching 48.83% (0.512 ± 0.007) inhibition. Meanwhile, sorafenib and esculetin induced a 36.85% (0.631 ± 0.008) and 17% (0.83 ± 0.011) decrease in HRAS gene expression, respectively, compared with control cells (Fig. 5e).

In HepG2 cells, sorafenib, esculetin and the combination reduced ERK2 gene expression by 28.13% (0.719 ±0.017), 16.48% (0.835 ± 0.012), and 47.27% (0.527 ± 0.007), respectively, compared with control cells. In Huh-7 cells, sorafenib, esculetin, and the combination reduced ERK2 gene expression by 29.93% (0.701 ± 0.015), 14.73% (0.853 ± 0.013), and 36.56% (0.634 ± 0.01), respectively (Fig. 5f).

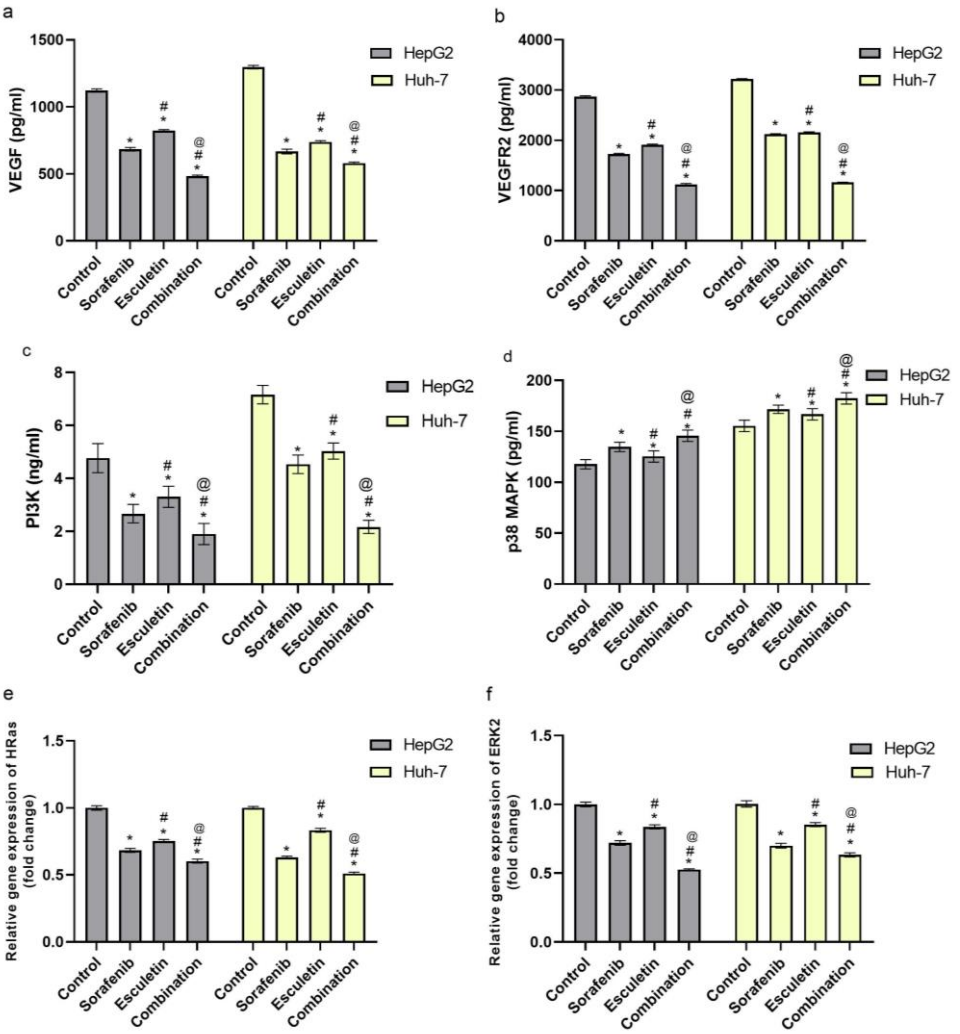


**Fig. 5.** Effects of sorafenib (9.6 μM), esculetin (225 μM), and their combination in HepG2 cells and sorafenib (4.5 μM), esculetin (350 μM), and their combination in Huh-7 cells on (a) VEGF, (b) VEGFR-2 (a) and VEGFR-2, (c) PI3K and (d) p38MAPK levels measured through ELISA after treating cells with sorafenib and/or esculetin for 48 h. And on (e) HRAS and (f) ERK2 gene expression measured using RT-PCR. Data are expressed as the mean ± SEM of three experiments, each conducted in triplicate. *P< 0.05 vs. control, #P< 0.05 vs. sorafenib-treated cells and @P< 0.05 vs. esculetin-treated cells.

## 4. Discussion

HCC is one of the most common cancers with a high mortality rate [2]. Sorafenib is first-line therapy for advanced HCC and improves patient survival. However, despite positive initial clinical reactions, long-term sorafenib usage can have several side effects and induce drug resistance [26]. Combination therapies can improve the efficacy, reduce the dosage, and reduce the toxicity of a single medicine while also effectively decreasing drug resistance [27]. Herein, we used HepG2 and Huh-7 cells to investigate the new powerful sorafenib and esculetin combination and its chemotherapeutic effects mediating HCC progression through multiple signaling pathways.

To the best of our knowledge, the current study is the first to demonstrate the anti-tumor effects of the sorafenib and esculetin combination. In line with previous studies, sorafenib and esculetin reduced cell growth [28,29]. Moreover, the combination significantly decreased the cell viability compared with the single drugs, and the CI indicated a synergistic effect [19].

In both cell lines, untreated cells had higher HRAS and ERK relative gene expression levels, higher EGFR, VEGF, VEGFR-2, Ki-67, PI3K and NF-κB levels, and lower caspase-3 activity than treated cells. These results are coherent with an up-regulation of these oncogenic signaling pathways in HCC [30].

We showed that, in both HepG2 and Huh-7 cells, the esculetin and sorafenib combination effectively reduced VEGF levels. This was in agreement with a study from Park *et al*. in 2016 [17] showing that esculetin inhibited VEGF-induced angiogenesis in human umbilical vein endothelial cells. This effect was ascribed to the antiangiogenic effect of esculetin described by Kimura *et al*. in 2015 [31], indicating that esculetin exerts antiangiogenic actions by inhibiting MMP-2 expression, transforming growth factor (TGF)-β1generation, and VEGF secretion at the tumor site.

Additionally, we observed that esculetin markedly inhibited the ERK1/2 pathway by a dual effect on HRAS and ERK2 gene expression. This result is consistent with previous findings by Kuo *et al*. in 2006 [32] showing that esculetin inhibited ERK, leading to cell cycle arrest in HepG2 cells. Besides, this pathway mediates cell growth, survival, differentiation, and migration [33]. Oncogenic Ras can increase VEGF gene transcription through the HIF-binding region in the VEGF promoter [34]. EGFR and VEGFR are upstream of the ERK cascade and are involved in the progression of HCC [8]. EGFR regulates several biological processes, including proliferation, angiogenesis, differentiation, and apoptosis [35]. One of the important mechanisms of sorafenib resistance is abnormal activation of the PI3K/AKT/mTOR pathway [36]. We also observed that esculetin markedly decreased EGFR and PI3K levels, which is in line with the study by Jeon *et al*. in 2016 [37] showing that esculetin inhibited oral squamous cell carcinoma proliferation through inhibition of the EGFR/PI3K/Akt survival signaling pathways.p38 MAPK is an important regulator of cell proliferation and death [38]. The sorafenib-esculetin combination group showed a significant increase in p38MAPK levels compared to the control group in the current investigation. These findings contrast prior research that found a considerable reduction of p38MAPK activity in cancer cell lines treated with sorafenib [39]. The genetic variability of distinct human cancer cell lines and the type of kinetic activation can contribute to this paradoxical conclusion. It could be attributed to the sorafenib-esculetin combination's effect on PI3K inhibition, which resulted in Raf phosphorylation inhibition at serine residue and subsequent MAPK activation [40].

Esculetin also notably decreased NF-κB levels, inhibiting invasion and angiogenesis by suppressing MMP-9 and VEGF expressions in HCC [41]. This result agreed with those from Zhu *et al*. in 2018 [42], who reported that esculetin suppressed NF-κB expression in murine Lewis lung carcinoma cells.

Regarding cell proliferation, we observed that esculetin significantly reduced Ki-67 levels in both HepG2 and Huh-7 cells. This result was in agreement with Wang *et al*. in 2017 [7], who found that esculetin decreased Ki-67 expression in a xenograft gastric cancer model. We also found that esculetin markedly enhanced apoptosis by restoring caspase-3 activity, which is in line with a previous study suggesting that esculetin induces apoptosis and inhibits cell proliferation in a xenograft gastric cancer model [7] and SMMC-7721 cells [29].

## 5- Conclusion

In conclusion, we demonstrated that esculetin potentiates the chemotherapeutic effects of sorafenib by modulating the angiogenic VEGF and EGFR/RAS/ERK/NF-κB pathways, promoting apoptosis and inhibiting proliferation, as indicated by the observed caspase 3 activation and Ki-67 suppression, respectively. Thus, esculetin is a promising therapeutic agent against HCC in combination with and future studies should investigate further molecular mechanisms mediating its chemotherapeutic effects.

## 6- Conflicts of Interest

The authors declare no conflict of interest.

## 7-Acknowledgements

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## 8-References

[1] J. Balogh, D.V. Iii, E.H. Asham, S.G. Burroughs, M. Boktour, A. Saharia, X. Li, R.M. Ghobrial, H.P.M. Jr, Hepatocellular

carcinoma: a review, J. Hepatocell. Carcinoma. 3 (2016) 41–53. https://doi.org/10.2147/JHC.S61146.
[2] K. Schulze, J.-C. Nault, A. Villanueva, Genetic profiling of hepatocellular carcinoma using next-generation sequencing, J. Hepatol. 65 (2016) 1031–1042. https://doi.org/10.1016/j.jhep.2016.05.035.
[3] O.B.S. Osman, E.F. Mohammed, Z.G. Mahran, A.O. Bakr, D.A. Mohareb, E.N.A.-E. Hakim Nasr, Evaluation of serum Midkine as a novel biomarker for the diagnosis of hepatocellular carcinoma, J. Curr. Med. Res. Pract. 3 (2018) 154. https://doi.org/10.4103/JCMRP.JCMRP_153_18.
[4] H. Huynh, T.T.T. Nguyen, K.-H.K.-P. Chow, P.H. Tan, K.C. Soo, E. Tran, Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: Its role in tumor progression and apoptosis, BMC Gastroenterol. 3 (2003) 19. https://doi.org/10.1186/1471-230X-3-19.
[5] Q. Zhou, V.W. Lui, W. Yeo, Targeting the PI3K/Akt/mTOR pathway in hepatocellular carcinoma, Future Oncol. 7 (2011) 1149–1167. https://doi.org/10.2217/fon.11.95.
[6] T. Luedde, R.F. Schwabe, NF-κB in the liver—linking injury, fibrosis and hepatocellular carcinoma, Nat. Rev. Gastroenterol. Hepatol. 8 (2011) 108–118. https://doi.org/10.1038/nrgastro.2010.213.
[7] G. Wang, M. Lu, Y. Yao, J. Wang, J. Li, Esculetin exerts antitumor effect on human gastric cancer cells through IGF-1/PI3K/Akt signaling pathway, Eur. J. Pharmacol. 814 (2017) 207–215. https://doi.org/10.1016/j.ejphar.2017.08.025.
[8] L. Zhong, D. Liao, M. Zhang, C. Zeng, X. Li, R. Zhang, H. Ma, T. Kang, YTHDF2 suppresses cell proliferation and growth via destabilizing the EGFR mRNA in hepatocellular carcinoma, Cancer Lett. 442 (2019) 252–261. https://doi.org/10.1016/j.canlet.2018.11.006.
[9] M.A. Morse, W. Sun, R. Kim, A.R. He, P.B. Abada, M. Mynderse, R.S. Finn, The Role of Angiogenesis in Hepatocellular Carcinoma, Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 25 (2019) 912–920. https://doi.org/10.1158/1078-0432.CCR-18-1254.
[10] J. Li, W. Gu, X. Bi, H. Li, C. Liao, C. Liu, W. Huang, H. Qian, Design, synthesis, and biological evaluation of thieno[2,3-d]pyrimidine derivatives as novel dual c-Met and VEGFR-2 kinase inhibitors, Bioorg. Med. Chem. 25 (2017) 6674–6679. https://doi.org/10.1016/j.bmc.2017.11.010.
[11] L. Lamalice, F. Le Boeu, J. Huot, Endothelial Cell Migration During Angiogenesis, Circ. Res. 100 (2007) 782–794. https://doi.org/10.1161/01.
[12] T.-C. Kuo, H.-P. Lu, C.C.-K. Chao, The tyrosine kinase inhibitor sorafenib sensitizes hepatocellular carcinoma cells to taxol by suppressing the HURP protein, Biochem. Pharmacol. 82 (2011) 184–194. https://doi.org/10.1016/j.bcp.2011.04.008.
[13] G. Marisi, A. Cucchetti, P. Ulivi, M. Canale, G. Cabibbo, L. Solaini, F.G. Foschi, S.D. Matteis, G. Ercolani, M. Valgiusti, G.L. Frassineti, M. Scartozzi, A.C. Gardini, Ten years of sorafenib in hepatocellular carcinoma: Are there any predictive and/or prognostic markers?, World J. Gastroenterol. 24 (2018) 4152–4163. https://doi.org/10.3748/wjg.v24.i36.4152.
[14] H. Chai, A.Z. Luo, P. Weerasinghe, R.E. Brown, Sorafenib downregulates ERK/Akt and STAT3 survival pathways and induces apoptosis in a human neuroblastoma cell line, (2010) 8.
[15] Y.H. Habiba, G.A. Omran, M.W. Helmy, M.E. Houssen, Antitumor effects of rhamnazinon sorafenib-treated human hepatocellular carcinoma cell lines via modulation of VEGF signaling and PI3K/NF-κB p38/caspase-3 axes cross talk, Life Sci. 297 (2022) 120443. https://doi.org/10.1016/j.lfs.2022.120443.
[16] G. Wang, M. Lu, Y. Yao, J. Wang, J. Li, Esculetin exerts antitumor effect on human gastric cancer cells through IGF-1/PI3K/Akt signaling pathway, Eur. J. Pharmacol. 814 (2017) 207–215. https://doi.org/10.1016/j.ejphar.2017.08.025.
[17] S.L. Park, S.Y. Won, J.-H. Song, S.-Y. Lee, W.-J. Kim, S.-K. Moon, Esculetin Inhibits VEGF-Induced Angiogenesis Both *In Vitro* and *In Vivo*, Am. J. Chin. Med. 44 (2016) 61–76. https://doi.org/10.1142/S0192415X1650004X.
[18] A. Parveen, L. Subedi, H. Kim, Z. Khan, Z. Zahra, M. Farooqi, S. Kim, Phytochemicals Targeting VEGF and VEGF-Related Multifactors as Anticancer Therapy, J. Clin. Med. 8 (2019) 350. https://doi.org/10.3390/jcm8030350.
[19] T.-C. Chou, Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method, Cancer Res. 70 (2010) 440–446. https://doi.org/10.1158/0008-5472.CAN-09-1947.
[20] S.W. Kattan, M.S. Nafie, G.A. Elmgeed, W. Alelwani, M. Badar, M.A. Tantawy, Molecular docking, anti-proliferative activity and induction of apoptosis in human liver cancer cells treated with androstane derivatives: Implication of PI3K/AKT/mTOR pathway, J. Steroid Biochem. Mol. Biol. 198 (2020) 105604. https://doi.org/10.1016/j.jsbmb.2020.105604.

[21] T.K. Motawi, H.A. Darwish, I. Diab, M.W. Helmy, M.H. Noureldin, Combinatorial strategy of epigenetic and hormonal therapies: A novel promising approach for treating advanced prostate cancer, Life Sci. 198 (2018) 71–78. https://doi.org/10.1016/j.lfs.2018.02.019.

[22] M.W. Helmy, A.I. Ghoneim, M.A. Katary, R.K. Elmahdy, The synergistic anti-proliferative effect of the combination of diosmin and BEZ-235 (dactolisib) on the HCT-116 colorectal cancer cell line occurs through inhibition of the PI3K/Akt/mTOR/NF-κB axis, Mol. Biol. Rep. 47 (2020) 2217–2230. https://doi.org/10.1007/s11033-020-05327-4.

[23] D.W. Nicholson, A. Ali, N.A. Thornberry, J.P. Vaillancourt, C.K. Ding, M. Gallant, Y. Gareau, P.R. Griffin, M. Labelle, Y.A. Lazebnik, N.A. Munday, S.M. Raju, M.E. Smulson, T.-T. Yamin, V.L. Yu, D.K. Miller, Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis, Nature. 376 (1995) 37–43. https://doi.org/10.1038/376037a0.

[24] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method, Methods. 25 (2001) 402–408. https://doi.org/10.1006/meth.2001.1262.

[25] L. Beckerbauer, J.J. Tepe, R. AEastman, P.F. Mixter, R.M. Williams, R. Reeves, Differential Effects of FR900482 and FK317 on Apoptosis, IL-2 Gene Expression, and Induction of Vascular Leak Syndrome, Chem. Biol. 9 (2002) 427–441. https://doi.org/10.1016/S1074-5521(02)00122-9.

[26] O. Abdel-Rahman, A. Lamarca, Development of sorafenib-related side effects in patients diagnosed with advanced hepatocellular carcinoma treated with sorafenib: a systematic-review and meta-analysis of the impact on survival, Expert Rev. Gastroenterol. Hepatol. 11 (2017) 75–83. https://doi.org/10.1080/17474124.2017.1264874.

[27] R. Zhang, Z. Chen, S.-S. Wu, J. Xu, L.-C. Kong, P. Wei, Celastrol Enhances the Anti-Liver Cancer Activity of Sorafenib, Med. Sci. Monit. 25 (2019) 4068–4075. https://doi.org/10.12659/MSM.914060.

[28] N. Dai, R. Ye, Q. He, P. Guo, H. Chen, Q. Zhang, Capsaicin and sorafenib combination treatment exerts synergistic anti-hepatocellular carcinoma activity by suppressing EGFR and PI3K/Akt/mTOR signaling, Oncol. Rep. 40. (n.d.) 3235–3248. https://doi.org/10.3892/or.2018.6754.

[29] J. Wang, M.L. Lu, H.L. Dai, S.P. Zhang, H.X. Wang, N. Wei, Esculetin, a coumarin derivative, exerts in vitro and in vivo antiproliferative activity against hepatocellular carcinoma by initiating a mitochondrial-dependent apoptosis pathway, Braz. J. Med. Biol. Res. 48 (2015) 245–253. https://doi.org/10.1590/1414-431x20144074.

[30] S.M. Akula, S.L. Abrams, L.S. Steelman, M.R. Emma, G. Augello, A. Cusimano, A. Azzolina, G. Montalto, M. Cervello, J.A. McCubrey, RAS/RAF/MEK/ERK, PI3K/PTEN/AKT/mTORC1 and TP53 pathways and regulatory miRs as therapeutic targets in hepatocellular carcinoma, Expert Opin. Ther. Targets. 23 (2019) 915–929. https://doi.org/10.1080/14728222.2019.1685501.

[31] Y. Kimura, M. Sumiyoshi, Antitumor and antimetastatic actions of dihydroxycoumarins (esculetin or fraxetin) through the inhibition of M2 macrophage differentiation in tumor-associated macrophages and/or G1 arrest in tumor cells, Eur. J. Pharmacol. 746 (2015) 115–125. https://doi.org/10.1016/j.ejphar.2014.10.048.

[32] H.-C. Kuo, H.-J. Lee, C.-C. Hu, H.-I. Shun, T.-H. Tseng, Enhancement of esculetin on Taxol-induced apoptosis in human hepatoma HepG2 cells, Toxicol. Appl. Pharmacol. 210 (2006) 55–62. https://doi.org/10.1016/j.taap.2005.06.020.

[33] K. Rajalingam, R. Schreck, U.R. Rapp, Š. Albert, Ras oncogenes and their downstream targets, Biochim. Biophys. Acta BBA - Mol. Cell Res. 1773 (2007) 1177–1195. https://doi.org/10.1016/j.bbamcr.2007.01.012.

[34] O. Kranenburg, M.F.B.G. Gebbink, E.E. Voest, Stimulation of angiogenesis by Ras proteins, Biochim. Biophys. Acta BBA - Rev. Cancer. 1654 (2004) 23–37. https://doi.org/10.1016/j.bbcan.2003.09.004.

[35] Y.-J. Jeon, J.H. Cho, S.-Y. Lee, Y.H. Choi, H. Park, S. Jung, J.-H. Shim, J.-I. Chae, Esculetin Induces Apoptosis Through EGFR/PI3K/Akt Signaling Pathway and Nucleophosmin Relocalization: E SCULETIN I NDUCES A POPTOSIS IN O RAL S QUAMOUS C ELL C ARCINOMA, J. Cell. Biochem. 117 (2016) 1210–1221. https://doi.org/10.1002/jcb.25404.

[36] X. Zhou, T.-M. Li, J.-Z. Luo, C.-L. Lan, Z.-L. Wei, T.-H. Fu, X.-W. Liao, G.-Z. Zhu, X.-P. Ye, T. Peng, CYP2C8 Suppress Proliferation, Migration, Invasion and Sorafenib Resistance of Hepatocellular Carcinoma via PI3K/Akt/p27kip1 Axis, J. Hepatocell. Carcinoma. 8 (2021) 1323–1338. https://doi.org/10.2147/JHC.S335425.

[37] Y.-J. Jeon, J.H. Cho, S.-Y. Lee, Y.H. Choi, H. Park, S. Jung, J.-H. Shim, J.-I. Chae, Esculetin Induces Apoptosis Through EGFR/PI3K/Akt Signaling Pathway and Nucleophosmin Relocalization, J. Cell. Biochem. 117 (2016) 1210–1221. https://doi.org/10.1002/jcb.25404.

[38] W. Wang, J. Wu, Q. Zhang, X. Li, X. Zhu, Q. Wang, S. Cao, L. Du, Mitochondria-mediated

apoptosis was induced by oleuropein in H1299 cells involving activation of p38 MAP kinase, J. Cell. Biochem. 120 (2019) 5480–5494. https://doi.org/10.1002/jcb.27827.

[39] D. Kang, Z. Han, G.-H. Oh, Y. Joo, H.J. Choi, J.J. Song, Down-Regulation of TGF-β Expression Sensitizes the Resistance of Hepatocellular Carcinoma Cells to Sorafenib, Yonsei Med. J. 58 (2017) 899–909. https://doi.org/10.3349/ymj.2017.58.5.899.

[40] Y. Cui, P. Lu, G. Song, Q. Liu, D. Zhu, X. Liu, Involvement of PI3K/Akt, ERK and p38 signaling pathways in emodin-mediated extrinsic and intrinsic human hepatoblastoma cell apoptosis, Food Chem. Toxicol. 92 (2016) 26–37. https://doi.org/10.1016/j.fct.2016.03.013.

[41] J.-M. Wu, H. Sheng, R. Saxena, N.J. Skill, P. Bhat-Nakshatri, M. Yu, H. Nakshatri, M.A. Maluccio, NF-κB inhibition in human hepatocellular carcinoma and its potential as adjunct to sorafenib based therapy, Cancer Lett. 278 (2009) 145–155. https://doi.org/10.1016/j.canlet.2008.12.031.

[42] X. Zhu, J. Gu, H. Qian, Esculetin Attenuates the Growth of Lung Cancer by Downregulating Wnt Targeted Genes and Suppressing NF-κB, Arch. Bronconeumol. 54 (2018) 128–133. https://doi.org/10.1016/j.arbres.2017.09.005.