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Research Article

Phytochemical Modulation of Fibrosis-Linked Oncogenic Pathways in Hepatocellular Carcinoma Cells

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Abstract

Background: Liver fibrosis is a progressive pathological process that is the super-accumulation of extracellular matrix (ECM) due to chronic liver damage. Dysregulated signaling pathways involving interleukin-6 (IL-6), epidermal growth factor receptor (EGFR), and protein kinase B (AKT1), are implicated in hepatic stellate cell (HSC) activation, fibrogenesis, and progression toward cirrhosis and hepatocellular carcinoma (HCC). Despite advances in understanding these mechanisms, effective pharmacological interventions with favorable safety profiles remain limited. **Methods:** The current research examined the effects of two naturally occurring flavonoids, Kaempferol and Coumestrol on fibrosis-linked oncogenic signaling using an integrated *in silico* and *in vitro* study. Molecular docking studies were conducted to assess the binding affinities and the interaction profiles of both compounds with IL-6, EGFR, AKT1, and Caspase-3 (*CASP3*). Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) profiling was used to predict pharmacokinetic and toxicity properties to evaluate drug-likeness and safety parameters. *In vitro* experiments were conducted in HepG2 and Huh7 hepatocellular carcinoma cell lines to evaluate cytotoxicity, cell viability, migration, invasion, colony formation, and expression of IL-6, EGFR, AKT1, and Caspase-3. IC_{50} values were derived from dose-response curves generated from three independent technical experimental replicates. **Results:** Docking analysis showed stable binding interactions of both compounds at the active sites of IL-6, EGFR, AKT1, and CASP3, supported by hydrogen bonding and hydrophobic interactions, and ADMET predictions indicated favorable absorption with manageable toxicity profiles. In HepG2 and Huh7 cells, Kaempferol and Coumestrol showed dose-dependent reductions in cell viability, migration, invasion, and colony formation, along with downregulation of EGFR, AKT1, and IL-6 and upregulation of Caspase-3 at the transcript level. **Conclusion:** These findings suggest that Kaempferol and Coumestrol modulate IL-6/EGFR/AKT1-associated oncogenic signaling in hepatocellular carcinoma cells *in vitro*, indicating possible relevance to fibrosis-to-HCC progression. However, this study did not directly assess hepatic stellate cell activation or classical fibrogenic markers, and further studies using HSC models and *in vivo* validation are needed to confirm anti-fibrotic potential.

Keywords: liver fibrosis; IL-6; EGFR; AKT1; Kaempferol; Coumestrol; molecular docking; ADMET; hepatic stellate cells; fibrogenesis

1. Introduction

Liver fibrosis is a dynamic and progressive condition that develops in response to chronic liver injury caused by viral infections, alcohol abuse, metabolic disorders, autoimmune diseases, and toxic exposures [1,2]. It is characterized by abnormal accumulation of extracellular matrix (ECM) proteins, especially collagen types I and III, leading to structural reorganization of the liver tissue [3]. If untreated, fibrosis can advance to cirrhosis and eventually hepatocellular carcinoma (HCC), emphasizing the fibrosis-HCC continuum as a major global health concern [4].

Hepatic stellate cells (HSCs) are the key cells in fibrogenesis. When the liver is injured, HSCs remain in the dormant state and differentiate into myofibroblast-like cells that produce ECM and pro-inflammatory cytokines, a process directly induced by IL-6 through MAPK and JAK/STAT signaling [5].

One of them is interleukin-6 (IL-6), a pleiotropic cytokine involved in chronic inflammation and fibrogenesis via the Janus kinase/signal transducer (JAK/STAT) pathway [5]. Continuous IL-6 signaling induces the survival and growth of inflamed HSCs and has been shown to lead to the development of fibrosis into HCC [6]. High levels of IL-6 are associated with disease severity in chronic liver diseases [7]. Another important growth factor is the epidermal growth factor receptor (EGFR) that regulates hepatic regeneration and fibrosis. EGFR activation triggers Phosphoinositide 3-kinase/Ak strain transforming (PI3K/AKT) and Mitogen-Activated Protein Kinase (MAPK) activation and cascades of downstream signaling that induce cellular survival and proliferation [8]. Unchecked EGFR signaling has been linked to increased fibrosis remodeling and tumorigenesis [9].

AKT1 is a PI3K/AKT pathway central node that controls cell survival, metabolism, and proliferation [10]. The abnormal AKT1 activation increases the fibrogenic reactions and promotes the pathological remodelling of tissues [11]. Since IL-6, EGFR, and AKT1 are related to each other, inhibiting this pathway could be a logical treatment

approach to prevent fibrogenic stimulation and possibly lessen the risk of HCC [12].

The natural bioactive compounds have received significant interest owing to their multi-targeted property and good safety profiles [13]. Kaempferol is a flavonol that is found abundantly in fruits and vegetables and has anti-inflammatory, anti-oxidant, and anti-proliferative effects [14,15]. It has been shown to be able to modulate fibrosis and cancer-related signaling pathways in the past [16].

Coumestrol is a phytoestrogen, which is an antioxidant and anti-inflammatory plant compound in legumes [17]. The new evidence indicates that it may be useful in the regulation of the kinase-mediated pathways and cytokine signaling [17,18]. Although there are reports of biological activities, the mechanisms of action of Kaempferol and Coumestrol on IL-6/EGFR/AKT1-regulated fibrogenic signaling have not been fully assessed using computational and experimental methods.

The current research was intended to perform molecular docking of Kaempferol and Coumestrol with IL-6, EGFR, AKT1, and CASP3; evaluate the pharmacokinetic and toxicity profiles using Absorption, Distribution, Metabolism, Excretion, Toxicity (ADMET) prediction; and determine the *in vitro* anti-fibrotic action in activated hepatic cell models. IC_{50} values were calculated using reproducible experimental data, and the findings were reviewed in light of fibrosis progression and the risk of HCC. This integrated strategy provides mechanistic insight while maintaining a cautious interpretation of therapeutic potential, given the frequent gap between preclinical promise and clinical translation for plant-derived compounds [19].

2. Materials & Methodology

2.1 Selection and Collection of Plant Material

The study used medicinal plants obtained from the local market in Lahore, Pakistan. Prof. Dr. Muhammad Iqbal from the Department of Botany, Government College University, Faisalabad, Pakistan, taxonomically identified the plant material used in this study. A voucher specimen of the plant material has been deposited in the herbarium of Government College University, Faisalabad, Pakistan, where it is available for reference under the voucher specimen numbers GBM-347/25 and GBM-348/25. Extracts were made using *Euphorbia hirta* (whole plant) and *Lepidium sativum* (seeds) plants.

Chemicals and Compounds

Kaempferol and Coumestrol used in this study were purchased as analytically grade purified (>98% purity, as tested by the supplier) from Sigma Aldrich (St. Louis, MO, USA). Chromatographic analysis was used to determine the purity of the two compounds. The stock solutions were made in dimethyl sulfoxide (DMSO) and then stored at –20 °C until required. Experimental procedures involved preparing fresh working concentrations before the experiment, regardless of stability or reproducibility. All reagents used for cell culture and biochemical assays were of molecular biology grade.

2.2 In silico *Study of Medicinal Plants*

2.2.1 Retrieval of Bioactive Compounds From Plant Target

69 bioactive phytochemicals from *Euphorbia hirta* and *Lepidium sativum* were retrieved from Pharmacology of Traditional Chinese Medicinal Systems (TCMSP), Indian Medicinal Plants, Phytochemistry and Therapeutics (IMPPAT), and PubChem databases. Their 3D conformers were saved in SDF format for analysis [20,21,22].

2.2.2 Protein Preparation

The Protein Data Bank (PDB) was used to find three-dimensional crystal structures of IL-6, EGFR, and AKT1. Docking studies were performed on structures with good resolution and completeness. Protein preparation was performed by removing water molecules, eliminating ligand co-crystallization, adding polar hydrogens, and minimizing energy to ease steric clashes. The prepared protein structures were stored in SDF formats compatible with docking [23].

2.2.3 Selection of Molecular Targets

The molecular targets were IL-6, EGFR, and AKT1 because they are known to play a role in fibrogenesis and are connected via signaling in inflammatory and proliferative pathways. IL-6 was selected because it induces chronic inflammatory stimulation of hepatic stellate cells and contributes to the fibrosis-HCC transition. EGFR was selected for its regulatory effects on cellular growth and its downstream fibrogenic effects. AKT1, a central signaling kinase in the PI3K/AKT pathway, which mediates cell survival and ECM production, was also selected. The combination of these three targets leads to evaluation of multi-pathway modulation not a single-node inhibition [23].

2.2.4 Ligand Preparation

The 3D structures of phytochemicals were obtained from PDB and prepared for docking by optimizing protein structures using Discovery Studio (Software 2021, Pakistan). Phytochemical structures from PubChem were energy-minimized using PyRx (Virtual Screening software), following conversion to PDBQT format, target molecules and ligands were produced and prepared for additional docking studies [24].

2.2.5 Molecular Docking Analysis

Molecular docking identified candidates for the treatment of hepatic fibrosis. Molecular docking analysis was performed using the validated docking software platform, PyRx. The binding sites of IL-6, EGFR, AKT1, and CASP-3 were targeted to grid boxes using previously published ligand-binding residues. PyRx enabled docking, and the

Discovery Studio. The binding affinity was calculated referring to binding energy (kcal/mol). The interaction analysis involved identifying hydrogen bonds, hydrophobic interactions, π–π stacking interactions, and electrostatic interactions. Molecular visualization software, Discovery Studio was used to visualize receptor-compound interactions, revealing optimal binding conformation at the active site of the receptors [25].

2.2.6 ADMET and Drug-Likeness Prediction

In silico ADMET prediction tools were used to assess pharmacokinetic properties. Parameters that were evaluated were: Lipinski's Rule of Five, gastrointestinal absorption, blood-brain barrier, hepatotoxicity prediction, carcinogenicity risk, and acute toxicity prediction. These parameters were considered to assess an initial safety and drug-likeness profile [26,27,28,29].

2.2.7 *In Vitro* Experimental Design

2.2.7.1 Cell Culture. Hepatic cell lines with fibrogenic conditions were activated and cultured in a suitable growth medium supplemented with fetal bovine serum and antibiotics. The cells were kept at 37 °C in a humidified incubator at 5% CO_2. All cell lines were validated by Short Tandem Repeat (STR) profiling and tested negative for mycoplasma.

2.2.7.2 Treatment Protocol. Kaempferol and Coumestrol were added to cells in different concentrations. The control groups were the untreated cells and the vehicle-treated cells (DMSO control). The amount of time spent on treatment was standardized among the experiment groups.

2.2.8 Cytotoxic Study of Selected Phytochemicals: Cell Viability

Cell viability was determined by the Cell Count Kit-8 (CCK-8) assay. HepG2 and Huh7 cells were purchased from Sigma-Aldrich under catalog number 85011430 for HepG2 and 1042712 for Huh7 cell line. Both the cell lines were cultured and treated with various concentrations of Kaempferol and Coumestrol, and incubated. After adding 50 µL of CCK-8 solution, absorbance was taken at 450 nm via a microplate reader. Cell viability was computed with a higher absorbance value indicating greater cell viability. This procedure was performed according to the established methodology [30].

2.2.9 Colony Formation Assay

HepG2 and Huh7 cells were seeded into 6-well plates, treated with varying concentrations of Kaempferol and Coumestrol, and cultured for 10–14 days to enable colony formation. Every two to three days, the medium was changed to provide sufficient nutrients and prevent contamination. After fixation with four percent paraformaldehyde and dyed with 0.1% crystal violet, the colonies were allowed to air dry. Imaging software was used to count clusters with at least 50 cells. The number of colonies created was divided by the number of cells that were first seeded and multiplied by 100% to determine the colony formation efficiency [31].

2.2.10 Wound Healing Assay

A wound healing assay assessed the migratory ability of HepG2 and Huh7 cells treated with purified peptides. Cells were seeded into 6-well plates and cultured to confluency and then scratched. Detached cells were washed off, and migration was observed using Kaempferol and Coumestrol in a serum-free medium. Images of the wound area were captured at 0 h and 24 h post-scratch using imaging software to quantify wound closure. Migration was quantified by a reduction in the wound width using imaging software. The assay was performed in triplicate, and the results were analyzed to determine the impact of the peptides on cell migration [32].

2.2.11 Transwell Invasion Assay

The transwell invasion assay analyzed the invasive potential of HepG2 and Huh7 cells. Cells were seeded in Matrigel-coated transwell inserts with an 8 µm pore size. Their invasion ability through the extracellular matrix, toward a chemoattractant, was quantified after Matrigel solidification. HepG2 and Huh7 cells were harvested, suspended, and seeded into the upper chamber. The upper chamber was filled with different concentrations of kaempferol and Coumestrol. A chemoattractant was poured into the lower compartment. After incubating the plates, the non-invading cells were carefully extracted. 0.1% crystal violet was used to stain the invasive cells that had moved to the underside after they had been fixed with four percent paraformaldehyde. Stained cells were visualized, counted in random fields, and quantified as the average or percentage of invaded cells from triplicated assays relative to the control group [31].

2.2.12 LDH Assay

Lactate dehydrogenase (LDH) release analyzed cell membrane integrity and cytotoxicity in HepG2 and Huh7 cells. HepG2 and Huh7 cells were incubated to attach, then treated with Kaempferol and Coumestrol, and supernatants were carefully collected to avoid disrupting the cell monolayer. LDH levels in the supernatants were measured using an LDH assay kit. Supernatants were incubated, and absorbance was measured at 490 nm. Cytotoxicity was expressed as a percentage with experiments performed in triplicate, for statistical analysis of peptide effects on cell membrane integrity [33].

2.2.13 Gene Expression

Quantitative real-time PCR (qRT-PCR) assessed inflammatory and necrotic markers. Gene expression was

Table 1. Primer sequences for qRT-PCR.

Sr. No.	Gene name	F	R
1	*EGFR*	AGGCACGAGTAACAAGCTCAC	ATGAGGACATAACAGCCAC
2	*AKT1*	CACAAACGAGGGGAGTACATC	GCCATCATTCTTGAGGAGGAAGT
3	*IL-6*	TTCCATCCAGTTGCCTTGTTGG	TTCTCATTTCCACGATTTCCCAG
4	*Caspase 3*	TTAATAAAGGTATCCATGGAGAACACT	TTAGTGATAAAAATAGAGTTCTTTTGTGAG

qRT-PCR, quantitative real-time PCR; *EGFR*, epidermal growth factor receptor; *AKT1*, AKT serine/threonine kinase 1; *IL-6*, interleukin-6.

normalized to 18S using the ΔCt method. RNA was isolated with Trizol, normalized to 18S levels, and validating purity through spectrophotometry and ensuring integrity using 1% agarose gel electrophoresis. Analysis of *EGFR, AKT1, IL-6, Caspase 3* and 18S gene expression levels occurred through qRT-PCR testing using the StepOne real-time PCR system. The reverse transcription and Syrian Blue Yellow Radiant (SYBR) Green amplification step used 20 ng of RNA. The system recorded fluorescence signals to measure gene expression levels using the ΔCt method, with triplicate measurements to verify accuracy [34]. Primer sequences used for the qRT-PCR reactions are listed in Table 1.

2.2.14 Determination of IC_{50}

Dose-response curves were constructed to determine the values of IC_{50} with the help of nonlinear regression analysis. The values were presented in the form of mean ± Standard Error of Mean (SEM), where the data were taken from three independent experiments.

2.2.15 Statistical Analysis

Data was presented as mean ± Standard Error of Mean (SEM). To compare differences between groups, a one-way analysis of variance (ANOVA) was used. Dunnett's test was used for post-hoc multiple comparisons, and $p < 0.05$ was used as the threshold for statistical significance. The Kruskal-Wallis H test was used to analyze group differences for non-parametric data, and the Mann-Whitney U test was used to assess meaningful pairwise comparisons. For all statistical analysis, SPSS software (Version 26.0, SPSS Inc., Chicago, IL, USA) was used, while graphical illustrations and data visualizations were created with GraphPad Prism (Version 9.0, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Molecular Docking Analysis

Initially, sixty-nine phytochemicals were retrieved from *Euphorbia hirta* and *Lepidium sativum*, and seven phytochemicals were selected for further study based on Lipinski's rule of five and blood-brain barrier (BBB) crossing. PyRx Virtual Screening software was used to generate ligands, and Discovery Studio software 2021 was used to visualize associations between key active substances and receptor proteins. Two of the seven phytochemicals, Kaempferol and Coumestrol, were selected based on high binding energy (Table 2) and maximum interaction with the target proteins at the binding site (Figs. 1,2,3,4). Coumestrol exhibited higher binding affinities for three of the target proteins, AKT1 (–6.4 kcal/mol), EGFR (–7.1 kcal/mol), and CASP3 (Caspase 3) (–7.1 kcal/mol).

3.1.1 Binding Affinity With IL-6

Kaempferol showed constant binding to the active site of IL-6, and the binding energy is favorable. Interaction analysis showed hydrogen bonding with important residues that were associated with cytokine receptor interaction. The complex was also more stable with the aid of hydrophobic stabilization. Coumestrol was also found to exhibit high binding affinity with hydrogen bonding and hydrophobic interactions in the IL-6 binding pocket. The results indicate the possibility of changing the IL-6-mediated inflammatory signaling.

3.1.2 Interaction With EGFR

The binding of Kaempferol was found to be strong in the Adenosine Triphosphate (ATP)-binding domain of EGFR by docking results. Stability was facilitated by the formation of hydrogen bonds. Coumestrol also displayed a stable docking orientation in the kinase domain. Inhibition of EGFR kinase activity may attenuate downstream proliferative and fibrogenic signaling.

3.1.3 Interaction With AKT1

Both compounds were found to bind in the catalytic site of AKT1. Hydrogen bonds were observed with critical active-site residues, and hence may have interfered with kinase activation. Altogether, the docking results suggest multi-target activity in the IL-6/EGFR/AKT1 signal transduction pathway.

3.2 ADMET Analysis

Kaempferol and Coumestrol both satisfied the Lipinski Rule of Five, supporting their drug-likeness. Both the phytochemicals were predicted to have acceptable gastrointestinal absorption profiles. However, *in silico* analysis identifies inhibitory activity against multiple cytochrome P450 enzymes, including CYP3A4, CYP2C9, and CYP2D6 for both compounds, and CYP2C19 for Coumestrol. Kaempferol was tested positive for AMES

Table 2. Binding energies of phytochemicals with target proteins as shown by molecular docking studies.

Sr. No.	Target protein	PDB ID	Phytochemical	Binding energy (kcal/mol)
1	IL-6	1alu	Kaempferol	–6.3
			Coumestrol	–6.3
2	AKT1	2uvm	Kaempferol	–6
			Coumestrol	–6.4
3	EGFR	5gty	Kaempferol	–6.7
			Coumestrol	–7.1
4	CASP3	2cdr	Kaempferol	–6.4
			Coumestrol	–7.1

CASP3, Caspase 3.

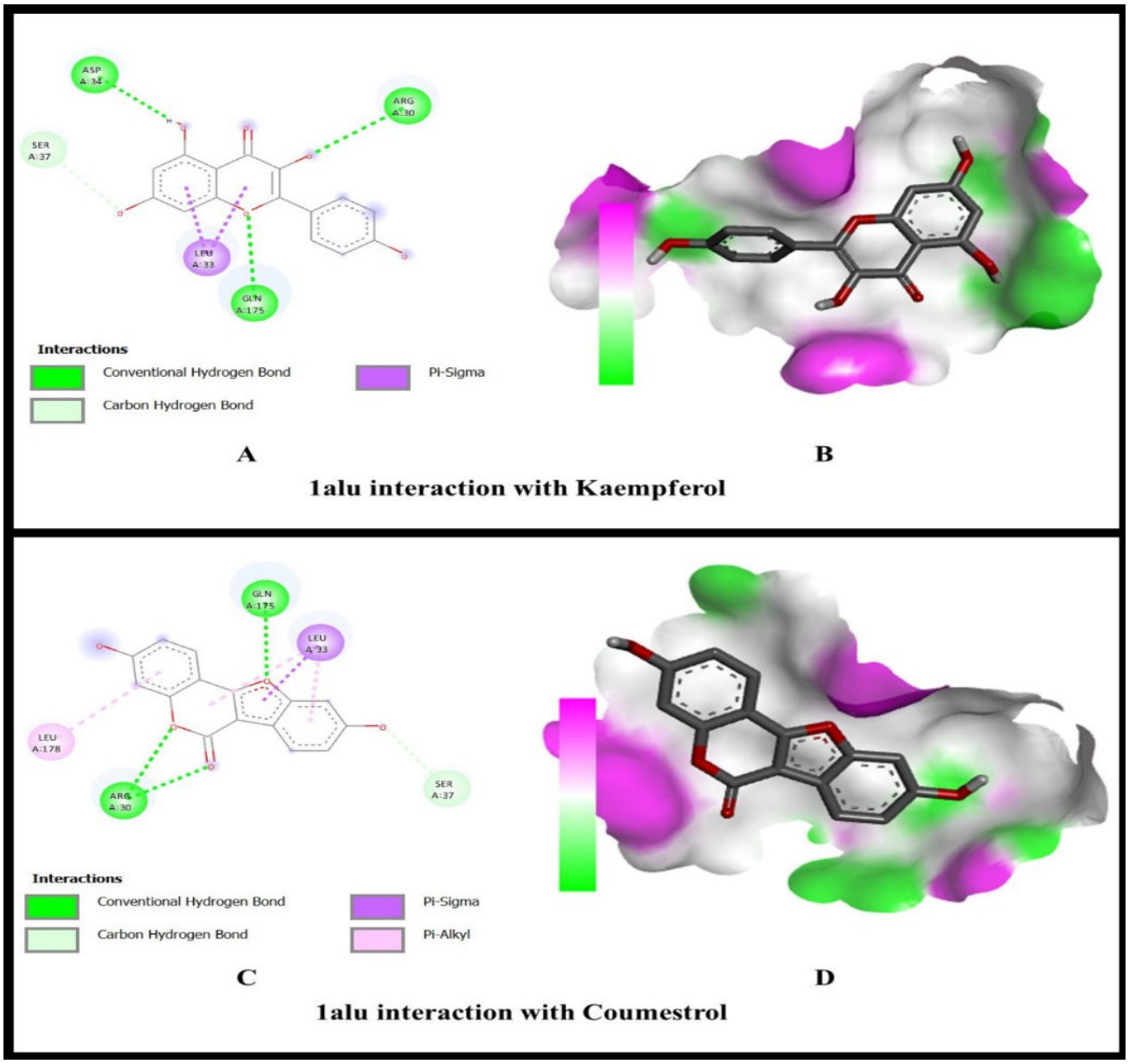

Fig. 1. Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein IL-6, and interaction (C) and binding sequence (D) of Coumestrol with functional domains of protein IL-6.

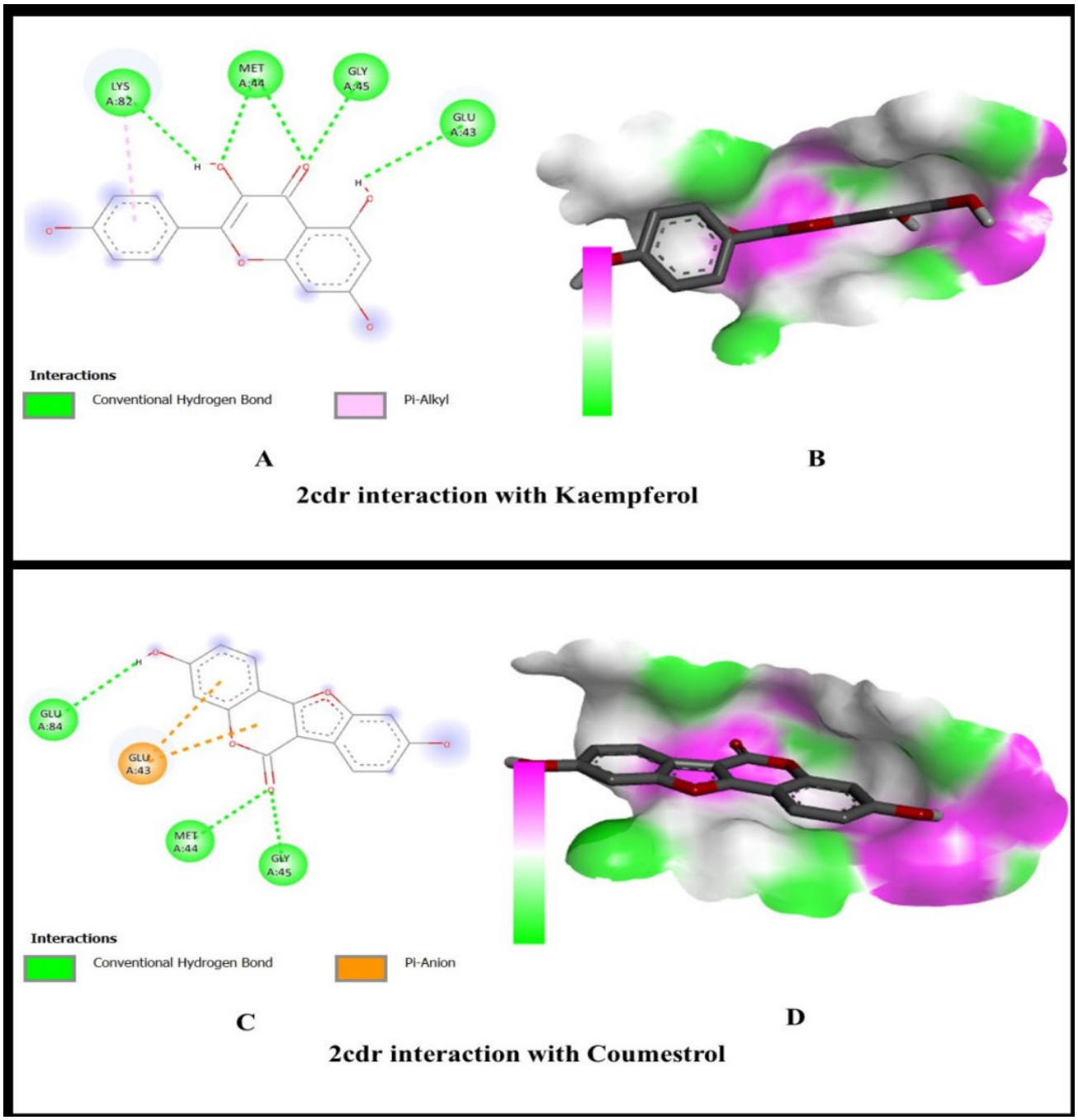

Fig. 2. Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein CASP3, and interaction (C) and binding sequence (D) of Coumestrol with functional domains of protein CASP3.

toxicity *in silico*, which indicates a mutagenic potential that needs to be confirmed experimentally. Coumestrol exhibited some toxicity, which represents a safety concern that must be verified through *in vitro* and *in vivo* testing before any pharmacological application. Premedication regarding hepatotoxicity did not show high toxicity. It is to be noted that these ADMET predictions reported are *in silico* estimated that are generated using different computational tools and need further experimental pharmacokinetic and toxicological validation. *In silico* ADMET predictions are, however, early predictions that have to be verified through testing. ADMET Profiling parameters are presented in Table 3.

3.3 Cell Viability of HepG2 and Huh7 cell lines based on Higher Absorbance Values

Following treatment with increasing concentrations of Kaempferol and Coumestrol, cell viability was assessed 24 hours post-treatment. All concentrations showed variable results, as summarized in Fig. 5. The results showed that

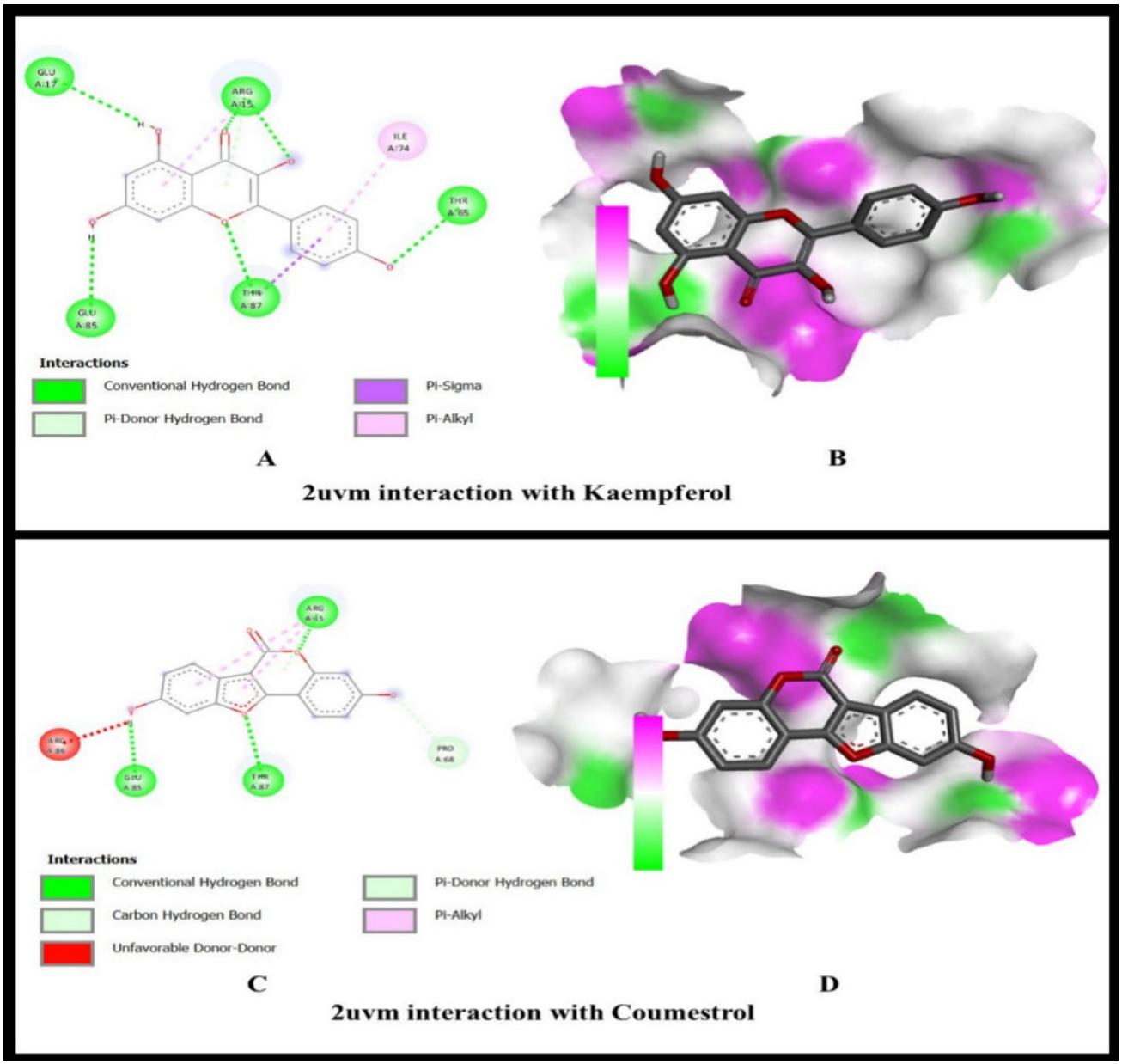

Fig. 3. Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein AKT1, and interaction (C) and binding sequence (D) of Coumestrol with functional domains of protein AKT1.

Kaempferol exhibited dose-dependent reduction in cell viability with a corrected IC_{50} value of 1.41 µM for HepG2 and 9.04 µM for Huh7 cell lines. Coumestrol demonstrated a comparatively moderate reduction in cell viability, with an IC_{50} of 7.07 µM for the HepG2 cell line and 19.58 µM for the Huh7 cell line after the treatment (Fig. 5). The findings suggest that Kaempferol exhibits greater cancer cell inhibition than Coumestrol.

3.4 Assessment of Cytotoxicity in HepG2 and Huh7 Cells by LDH Release Assay

LDH release analyzed membrane integrity and cytotoxicity in HepG2 and Huh7 cells. In HepG2 cells, LDH release increased in a dose-dependent manner with increasing concentrations of the drug candidates relative to the negative control, indicating partial cytotoxicity without complete membrane disruption. Kaempferol induced a higher release of LDH than Coumestrol in the HepG2 cells and thus showed significant cytotoxic activity. In Huh7 cells, the release of LDH increased with increasing the concentration of the drug candidates as compared to the NC, resultantly increasing cytotoxicity (Fig. 6). Both Kaempferol and Coumestrol induced a comparable release of LDH in the Huh7 cells, and thus showed comparable cytotoxic activity.

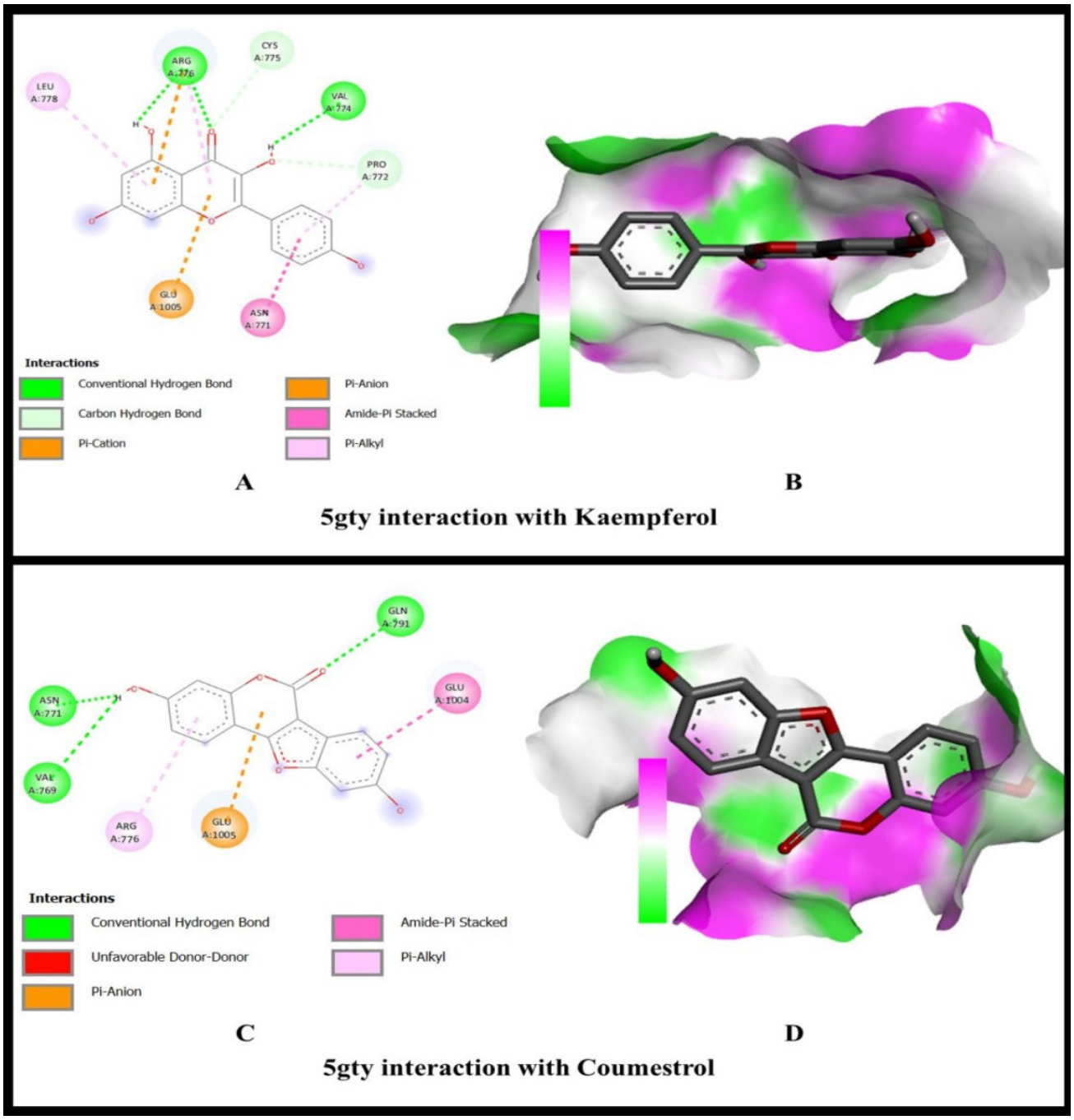

Fig. 4. Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein EGFR, and interaction (C) and binding sequence (D) of Coumestrol with functional domains of protein EGFR.

3.5 Cell Migration Effect of the Drug Candidates Using Wound Healing Assay

In HepG2 cells, treatment with Kaempferol significantly reduced cell migration at 24 h post-incubation at 37 °C compared with the negative control. In the same cell line, Coumestrol showed a compataively smaller reduction in migration. In the Huh-7 cell line, both the drug candidates showed comparable inhibitory effects on wound healing at 30 µM (Fig. 7).

3.6 Effect of the Drug Candidates on the Invasion Potential of HepG2 and Huh7 Cells

The transwell assay showed that Kaempferol visibly reduced the invasive potential of HepG2 cells at 30 µM as compared to the NC. Coumestrol showed comparatively lesser reduction in the invasive potential of HepG2 cells at the same concentration. In the Huh7 cell line, Coumestrol suppressed the invasive potential of cancer cells to a greater extent as compared to Kaempferol at the same concentration of 30 µM (Fig. 8). Qualitative assessment of

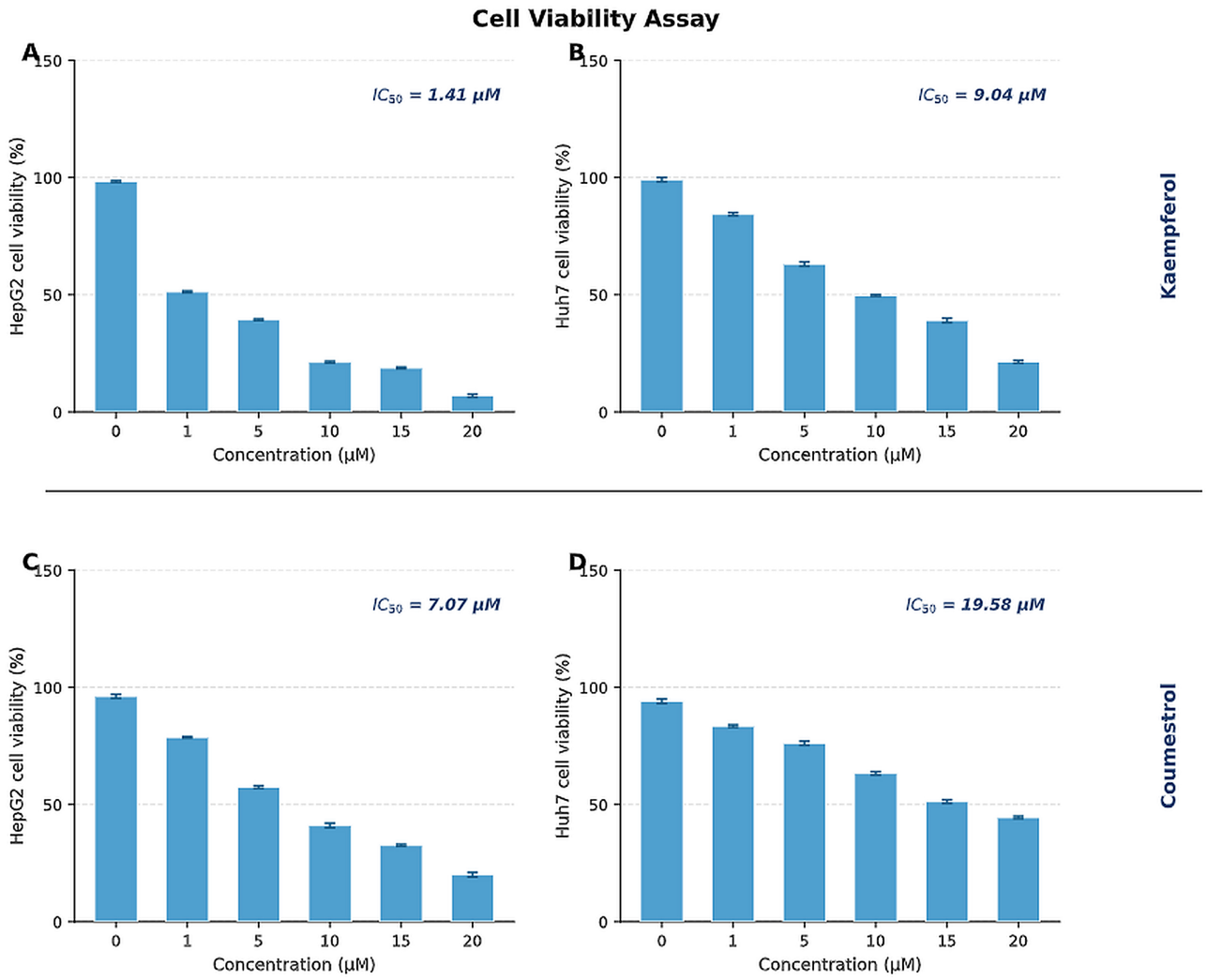

Fig. 5. Cell viability of Kaempferol and Coumestrol on HepG2 and Huh-7 cell lines. The cell viability was evaluated using the Cell Count Kit-8 (CCK-8) test 24 hours post-treatment. (A) Effect of Kaempferol on HepG2 cells, showing decrease in cell viability with increasing concentration of Kaempferol. (B) Effect of Kaempferol on Huh7 cells, showing decrease in cell viability with increasing concentration of Kaempferol. (C) Effect of Coumestrol on HepG2 cells, showing decrease in cell viability with increasing concentration of Coumestrol. (D) Effect of Coumestrol on Huh7 cells, showing decrease in cell viability with increasing concentration of Coumestrol.

crystal violet stained cells, the results suggested that both Kaempferol and Coumestrol effectively impede cancer cell invasiveness, with Coumestrol appearing more potent in Huh7 cells. However, these observations are qualitative in nature, as formal quantitative cell counting and statistical analysis were not performed.

3.7 Inhibition of Clonogenesis of HepG2 and Huh7 Cells by the Drug Candidates

The colony formation assay revealed that clonogenesis of HepG2 and Huh7cells was significantly suppressed following the treatment with the drug candidates compared to the control group. The quantity of colonies produced by HepG2 cells after being treated with Kaempferol was far less than those of the control group, and the quantity of colonies reduced even more in the group treated with Coumestrol than NC. The inhibition of clonogenesis was more prominent in Huh7 cells, where Kaempferol reduced colony formation at 30 μM concentration, and Coumestrol also reduced the quantity of colonies formed as compared to NC (all $\#p < 0.0001$). Coumestrol exhibited a more marked inhibition of clonogenesis than Kaempferol (Fig. 9).

3.8 Gene Expression Analysis of EGFR, AKT1, IL-6, and Caspase 3 Following Treatment With Kaempferol and Coumestrol

In the HepG2 cells, on treatment with Kaempferol, the results showed the downregulation of *EGFR* ($\#p < 0.0001$), *AKT1* ($\#p < 0.0001$), and *IL-6* ($\#p < 0.01$) as compared to the NC. Meanwhile, in the same cell line, *CASP3* ($\#p < 0.0001$) showed significant upregulation following the treatment with Kaempferol. On treatment with Coumestrol,

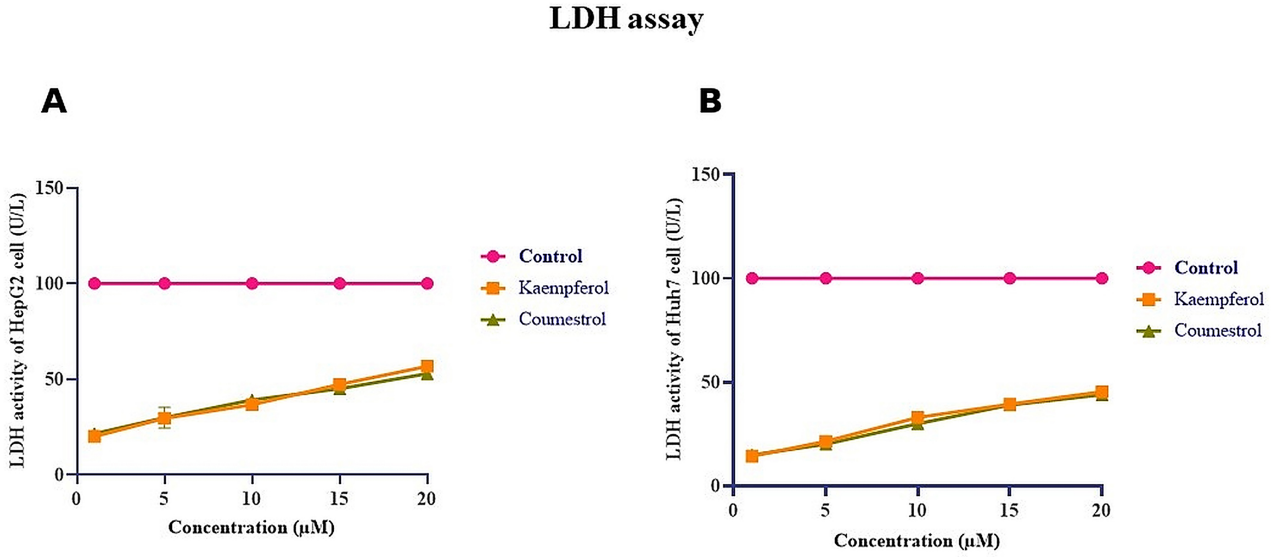

Fig. 6. Lactate dehydrogenase (LDH) assay to evaluate cytotoxicity of Kaempferol and Coumestrol. Release of LDH and the resulting cytotoxicity measured by the LDH assay. (A) LDH activity in HepG2 cells showing a dose-dependent increase after treatment with Kaempferol and Coumestrol to the negative control. (B) LDH activity in Huh7 cells showing a dose-dependent increase after treatment with Kaempferol and Coumestrol relative to the negative control. This indicates comparable partial cytotoxicity for both compounds.

Table 3. ADMET Profiling parameters of phytochemicals.

ADMET parameters	Phytochemicals	
	Kaempferol	Coumestrol
Absorption and distribution		
BBB	No	No
HIA		
Caco-2 Permeability	–4.974	–4.889
Metabolism		
CYP3A4 substrate	No	No
CYP2D6 substrate	No	Yes
CYP3A4 inhibition	Yes	Yes
CYP2C9 inhibition	Yes	Yes
CYP2C19 inhibition	No	Yes
CYP2D6 inhibition	Yes	Yes
Excretion		
Total clearance	6.868	8.085
Toxicity		
AMES toxicity	Yes	No
Hepatotoxicity	No	No
Carcinogenicity	No	Yes
Skin sensitization	Yes	Yes

BBB, blood brain barrier; HIA, human intestinal absorption; ADMET, absorption, distribution, metabolism, excretion, and toxicity; AMES, bacterial reverse mutation assay.

the results showed a more considerable downregulation of *EGFR* (#$p < 0.0001$), *AKT1* (#$p < 0.0001$), and *IL-6* (#$p < 0.001$) as compared to the NC. Meanwhile, in the same cell line, *CASP3* (#$p < 0.0001$) showed a more significant upregulation following the treatment than the treatment group of Kaempferol (Fig. 10). In the Huh7 cells, on treatment with Kaempferol, the results showed a remarkable downregulation of *EGFR* (#$p < 0.0001$), *AKT1* (#$p < 0.0001$), and *IL-6* (#$p < 0.0001$) as compared to the NC. Meanwhile, in the same cell line, *CASP3* (#$p < 0.0001$) showed a more significant upregulation following the treatment with the Kaempferol. On treatment with Coumestrol, the results showed a more substantial downregulation of *EGFR* (#$p < 0.0001$) as compared with the NC, and *AKT1* (#$p < 0.0001$) and *IL-6* (#$p < 0.0001$) showed lesser downregulation than Kaempferol treatment group. Meanwhile, in the same cell line, *CASP3* (#$p < 0.0001$) showed a more significant upregulation following the treatment with Coumestrol than the treatment group of Kaempferol (Fig. 11).

4. Discussion

Liver fibrosis, produced by collagen and ECM accumulation that replaces hepatic parenchyma, was thought to be incurable and might lead to liver failure. Although traditionally considered irreversible, technological advances have made it possible that, the progression of fibrosis can be halted or even reversed. Novel therapeutic strategies targeting anti-fibrotic strategies, such as HSC downregulation, removal of damaging stimuli, and matrix degradation, have been enabled by improved understanding of the mechanisms underlying liver fibrosis [5,14,15,35]. However, limited diagnostic facilities and adverse drug effects continue to challenge effective clinical management [36,37].

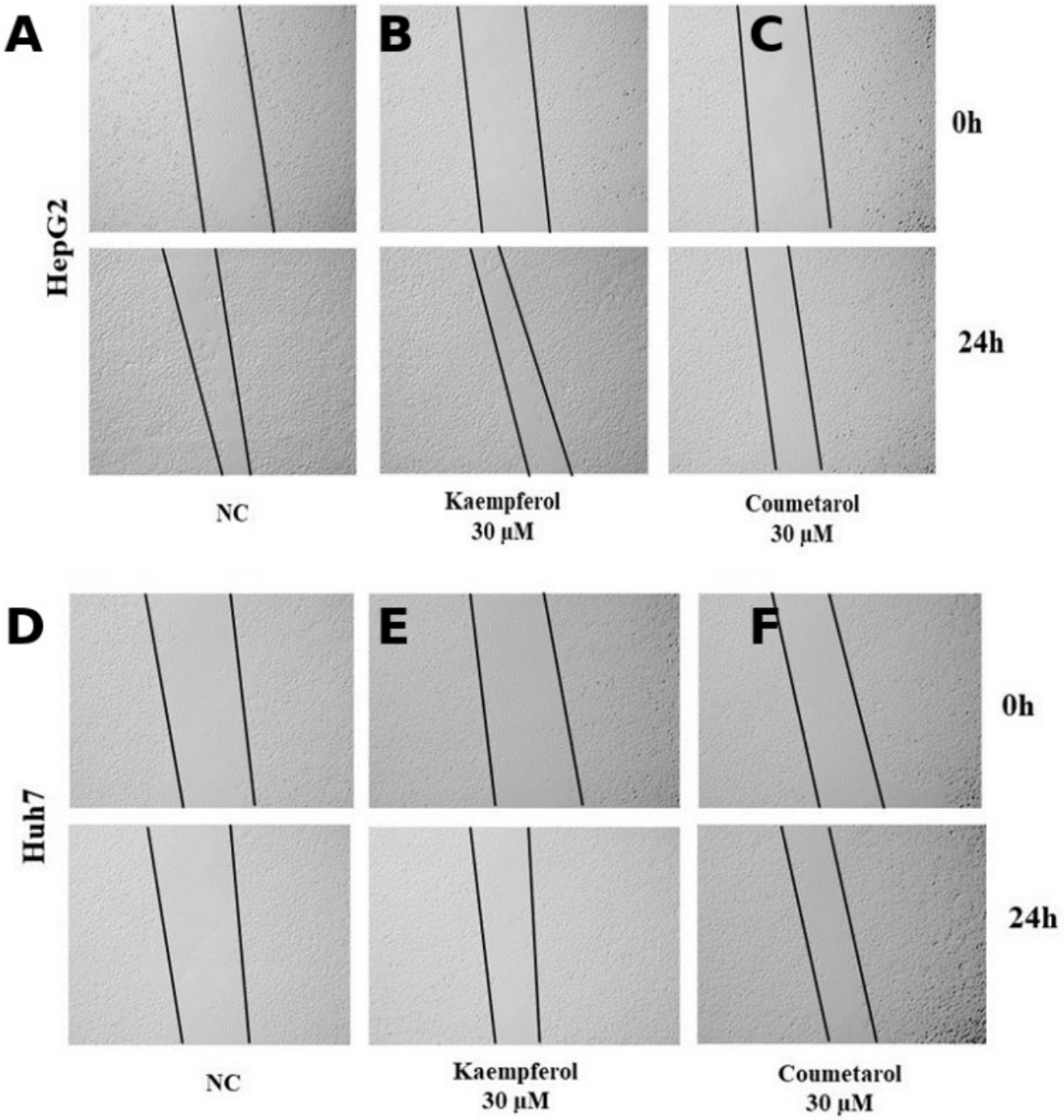

Fig. 7. Wound healing assay of HepG2 and Huh7 cell lines. Wound closure in HepG2 cells at 0 h and 24 h after treatment with the Kaempferol and Coumestrol at 30 µM concentration. (A) Negative control (NC) showing normal cell migration and partial wound closure over time. (B) HepG2 cells treated with Kaempferol at 30 µM concentration showing reduced wound closure and inhibition of cell migration compared with negative control. (C) HepG2 cells treated with Coumestrol at 30 µM concentration showing reduced wound closure and inhibition of cell migration compared with negative control. (D) NC showing normal cell migration and partial wound closure over time. (E) Huh7 cells treated with Kaempferol at 30 µM concentration showing reduced wound closure and inhibition of cell migration compared with negative control. (F) Huh7 cells treated with Coumestrol at 30 µM concentration showing reduced wound closure and inhibition of cell migration compared with negative control. Note: Scale bars are not provided because the original image-acquisition parameters required for spatial calibration were not recorded and could not be retrieved; estimated scale bars were not added to avoid misrepresentation.

Euphorbia hirta and *Lepidium sativum* are highly recognized for their medicinal efficacy, fewer side effects, and relative safety when compared to synthetic pharmaceuticals. This study identified Kaempferol and Coumestrol as

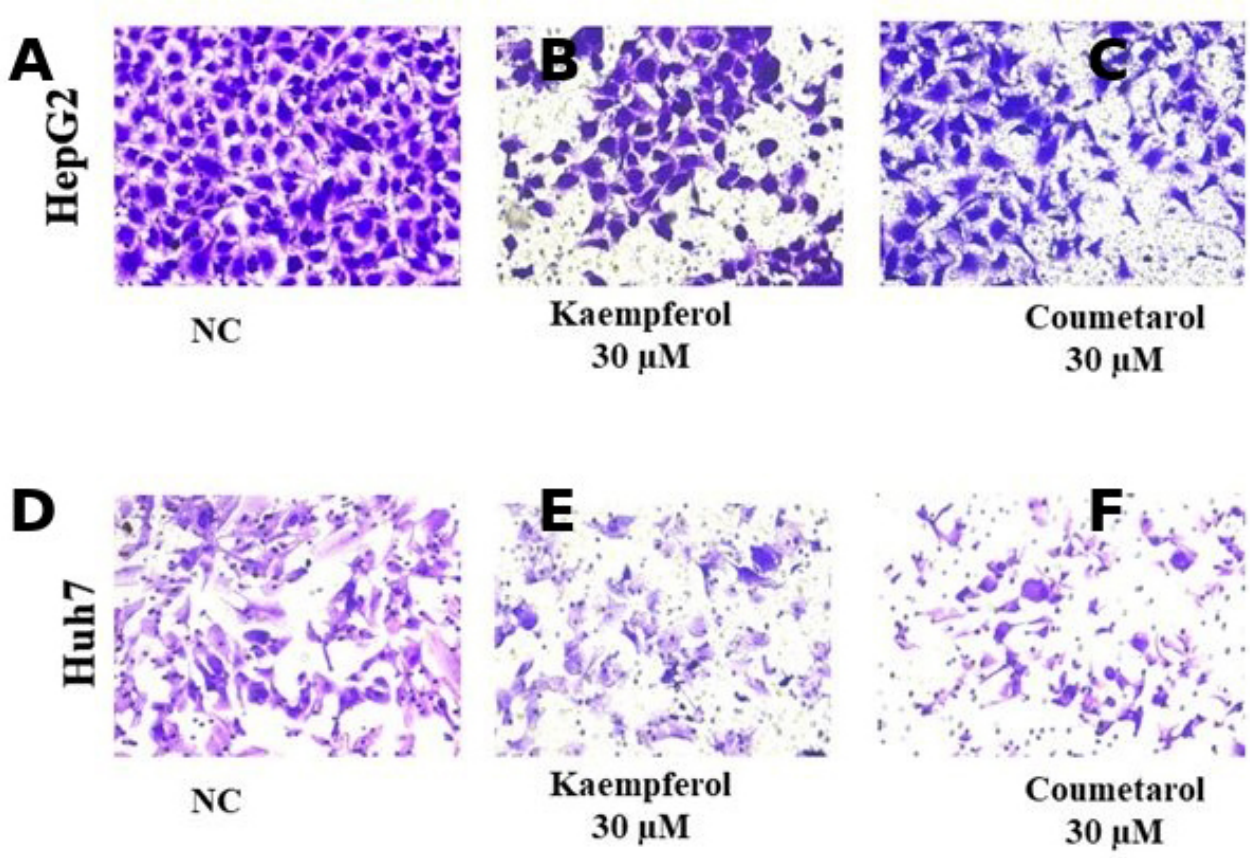

Fig. 8. Transwell invasion assay. Invasion propensity of HepG2 and Huh7 cells evaluated. (A) Invasiveness of HepG2 cells in NC. (B) Invasion propensity of HepG2 cells after treatment with Kaempferol at 30 μM. (C) Invasion propensity of HepG2 cells after treatment with Coumestrol at 30 μM. (D) Invasiveness of Huh7 cells in NC. (E) Invasion propensity of Huh7 cells after treatment with Kaempferol at 30 μM. (F) Invasion propensity of Huh7 cells after treatment with Coumestrol at 30 μM. Note: Scale bars are not provided because the original image-acquisition parameters required for spatial calibration were not recorded and could not be retrieved; estimated scale bars were not added to avoid misrepresentation.

leading phytochemicals using molecular docking, and were further evaluated experimentally. The selected targets, IL-6, AKT1, EGFR, and Caspase-3 are involved in signaling pathways associated with inflammation, apoptosis, and cell survival, which are relevant to both progression of fibrosis and HCC. It has explored the anti-fibrotic effects of Kaempferol and Coumestrol based on a combined computational and experimental analysis. We discuss our results that two compounds affect key signaling molecules in fibrogenesis, such as IL-6, EGFR, Caspase-3, and AKT1.

Previous studies have demonstrated the anti-inflammatory and anti-proliferative effects of Kaempferol [16]. In the present study, docking results suggested favorable interactions of both compounds with IL-6, EGFR, and AKT1, indicating potential multi-target activity. However, these docking findings should be interpreted as indicative of possible interactions and require further validation.

Kaempferol has been reported in previous studies to exhibit anti-inflammatory and anti- proliferative effects [16]. The docking results suggested favorable interactions of both compounds with IL-6, EGFR, and AKT1, indicating potential multi-target activity, which are consistent with these observations. Similarly, the literature has shown that EGFR inhibition reduces activation of hepatic stellate cells [9]. Such effects have a potential mechanistic explanation by the binding affinity that we observed in the study. However, these docking results should be indicators of possible interactions and require further validation.

In vitro experiments using HepG2 and Huh7 cell lines showed that Kaempferol and Coumestrol significantly suppressed cell viability, migration, clonogenesis, and invasion in the HepG2 and Huh7 cell lines. Cell viability evaluated using CCK-8 assay showed that Kaempferol and Coumestrol exhibited cytotoxic effects on both cell lines, where Kaempferol exhibited greater inhibitory potential (38.8%) than Coumestrol (53.6%). The IC_{50} values of Kaempferol were 1.41 μM and 9.04 μM in HepG2 and Hhuh7 cell lines, respectively. The IC_{50} values of Coumestrol were 7.07 μM and 19.58 μM in HepG2 and Huh7 cell lines, respectively. This suggests that Kaempferol can be more potent in reduc-

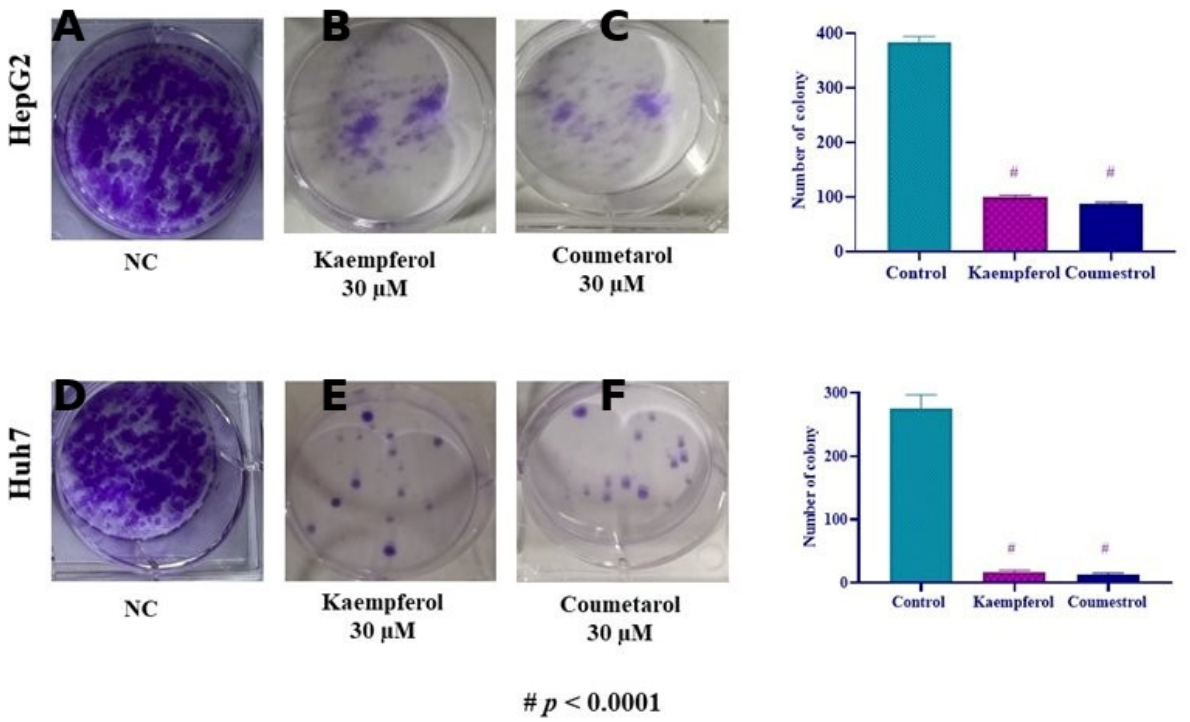

Fig. 9. Inhibition of clonogenesis of HepG2 and Huh7 cells by Kaempferol and Coumestrol. Inhibition of colony formation of HepG2 and Huh7 cells evaluated. (A) Inhibition of clonogenesis of HepG2 cells in NC. (B) Inhibition of clonogenesis of HepG2 cells after treatment with Kaempferol at 30 µM. (C) Inhibition of clonogenesis of HepG2 cells after treatment with Coumestrol at 30 µM. (D) Inhibition of clonogenesis of Huh7 cells in NC. (E) Inhibition of clonogenesis of Huh7 cells after treatment with Kaempferol at 30 µM. (F) Inhibition of clonogenesis of Huh7 cells after treatment with Coumestrol at 30 µM.

ing the proliferation of cancerous liver cells. These findings are consistent with previous studies highlighting the anticancer properties of these compounds.

A 2021 study revealed that Kaempferol exhibits an inhibitory effect on HepG2 cancer cells and cell viability was found to be higher in the group treated with Kaempferol. It also showed that Kaempferol inhibits the clonogenesis of liver cancer cells [31]. Another study, conducted in 2023, that correlates with the current study's results, showed that Kaempferol significantly inhibits HepG2 cell proliferation. With the increase in drug concentration, the rate of cell proliferation increased as well. Therefore, Kaempferol-induced cell inhibition of HepG2 is found to be dose-dependent [30].

LDH activity was evaluated cytotoxicity of phytochemicals after the release of LDH. Kaempferol induced a higher release of LDH than Coumestrol in the HepG2 cell line, signifying greater toxicity. This implies that Kaempferol may be more effective in limiting metastasis of HCC. Abid et al. [37], reported in 2022 that the LDH assay showed more LDH release in the groups treated with the plant drugs. The invasive potential of the cell lines was determined using a transwell assay, and this reinforced the potential of both the phytochemicals as anti-metastatic agents in HCC. Coumestrol showed a stronger inhibitory effect than Kaempferol. This suggests that Coumestrol can be more potent for fighting cancer cells. A study conducted in 2021 assessed the level of apoptotic potential of Kaempferol using transwell assay on the SK-Hep-1 cell line. The results showed that Kaempferol alone restricted the invasive ability of liver cancer cells and decreased the number of invasive cells [31].

Gene expression analysis using qRT-PCR demonstrated downregulation of EGFR, AKT1, and IL-6, along with upregulation of Caspase-3, which corresponds to cytotoxic impacts and metastasis suppression through AKT1 inhibition. This positively correlates with a study conducted in 2016 that showed significant upregulation of AKT1 in different liver cancer cell lines. Clonogenesis and cell viability were also suppressed in AKT-knockout liver cancer cells [38]. A more recent experimental study conducted in 2020 showed that an alkaloid, Cyclovirobuxine (CVB-D), suppressed the invasion, migration, and proliferation of liver cancer cell lines by inhibiting the EGFR pathway [39]. These results suggest that Kaempferol and Coumestrol may influence inflammation and apoptosis pathways at the transcriptional level. However, these findings are based on mRNA expression data and do not confirm direct modulation of signaling pathways at the protein level.

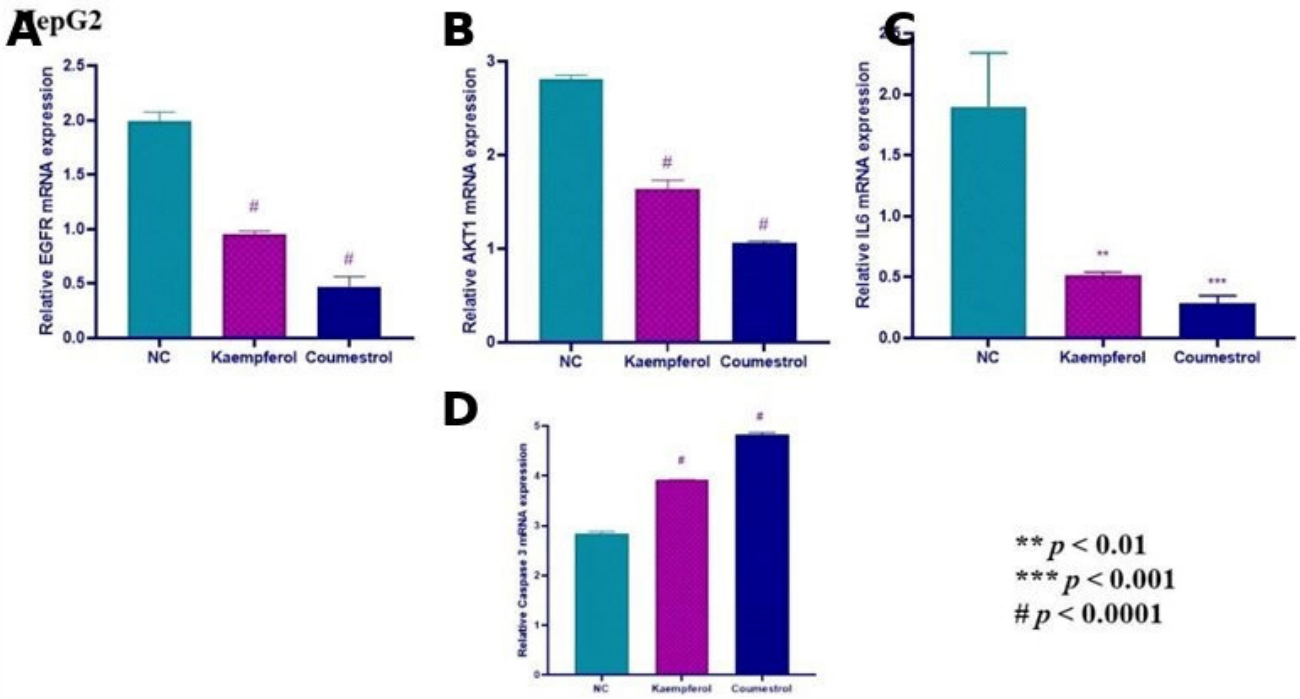

Fig. 10. EGFR, AKT1, IL-6, and Caspase 3 expression levels in HepG2 cell line. (A) Downregulation of EGFR on treatment with the drug candidates as compared to the NC. (B) Downregulation of AKT1 on treatment with the drug candidates as compared to the NC. (C) Downregulation of IL-6 on treatment with the drug candidates as compared to the NC. (D) Upregulation of Caspase 3 on treatment with the drug candidates as compared to the NC.

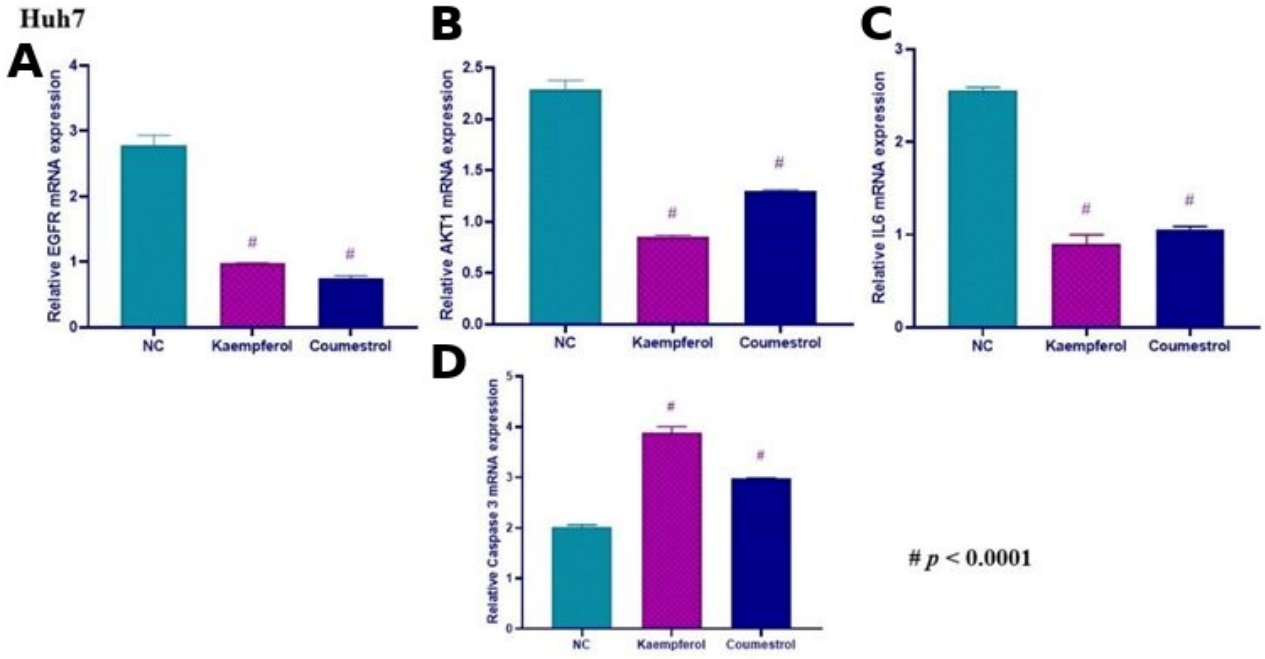

Fig. 11. EGFR, AKT1, IL-6, and Caspase 3 expression levels in Huh7 cell line. (A) Downregulation of EGFR on treatment with the drug candidates as compared to the NC. (B) Downregulation of AKT1 on treatment with the drug candidates as compared to the NC. (C) Downregulation of IL-6 on treatment with the drug candidates as compared to the NC. (D) Upregulation of Caspase 3 on treatment with the drug candidates as compared to the NC.

The use of HepG2 and Huh7 cell lines provides insight into fibrosis-associated signaling in hepatocytes; however, the absence of hepatic stellate cell models limits direct assessment of fibrogenesis. This study provides a comparative evaluation of Kaempferol and Coumestrol using an integrated *in silico* and *in vitro* approach, highlighting their potential effects on fibrosis-associated signaling pathways in the hepatocellular carcinoma model. Overall, these combined approaches provide preliminary evidence supporting the therapeutic activity of Kaempferol and Coumestrol. Further studies, including protein-level interactions and *in vivo* models, are necessary to understand the therapeutic relevance of the compounds.

5. Limitations

In this study, *in vivo* validation was not included, and ADMET predictions were not supported by further experimental validation. Gene-specific pathways and protein-expression analyses were not extensively discussed, and aspects of long-term toxicity and pharmacodynamics remain to be investigated.

6. Conclusion

Liver fibrosis remains a serious health issue that can progress to incurable hepatocellular carcinoma. In this study, Kaempferol and Coumestrol exhibited stable binding interactions with IL-6, EGFR, and AKT1 in molecular docking, produced significant reductions in viability, migration, invasion, and colony formation in HepG2 and Huh7 hepatocellular carcinoma cell lines, accompanied by downregulation of EGFR, AKT1, and IL-6 and upregulation of Caspase-3. These combined *in silico* and *in vitro* results indicate that both compounds modulate oncogenic signaling pathways relevant to the fibrosis-HCC continuum, though the present study evaluated these effects in cancer cell models rather than hepatic stellate cells, and did not assess classical fibrogenic markers directly. However, the given results are encouraging; more mechanistic and *in vivo* studies are required to support the therapeutic applicability. Future studies incorporating HSC-based models, protein-level validation, and *in vivo* testing are needed to establish the anti-fibrotic relevance of these compounds and their therapeutic applicability.

Availability of Data and Materials

All data generated or analyzed during the study are included in this article.

Author Contributions

AI: Conceptualization, methodology, data collection, writing, and original draft preparation. SMAS: Data collection, formal analysis, supervision, and review. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of GC University, with approval number GCUF/1040/23 dated 21/10/2024 (No human- or animal-derived materials, data or samples were used in this study, IRB approval was solely obtained for institutional purposes).

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Conflicts of Interest

The authors declare no conflicts of interest.

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