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RESEARCH ARTICLE
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DISRUPTING FIBROGENIC PATHWAYS: INSILICO AND INVITRO EVALUATION OF KAEMPFEROL AND COUMESTROL AS POTENTIAL ANTI-FIBROTIC AGENTS

Areeba Imtiaz [1*], Syed Muhammad Ali Shah[2], Sultan Ayaz[3]

[1*]PhD Scholar, Department of Eastern Medicine Government College University Faisalabad
Email: areeba.dahda247@gmail.com Phone no: +923361166096
[2]Assistant Professor, Department of Eastern Medicine Government College University Faisalabad
Email: smalishah@hotmail.com
[3]Associate Professor & Chairperson Department of Eastern Medicine Government College University Faisalabad Email: ayaz_libra@hotmail.com

***Corresponding Author:** Areeba Imtiaz
*PhD Scholar, Department of Eastern Medicine Government College University Faisalabad
Email: areeba.dahda247@gmail.com

Abstract

Background: Liver fibrosis is caused by chronic liver injury which is a reversible process of excessive extracellular matrix deposition. The continuous activation of hepatic stellate cells progresses to fibrosis, resulting in cirrhosis and hepatocellular carcinoma. Conventional treatment focuses only on symptoms, but natural phytochemicals as antifibrotic drugs offer a prospective option for novel therapeutic techniques that target fibrogenic pathways.

Objective: To explore the hepatoprotective potential of selected phytochemicals using computational and experimental approaches involving fibrogenic pathways.

Methodology: Bioactive compounds of medicinal plants *Euphorbia hirta* (whole plant) and *Lepidium sativum* (seeds) were studied. The 69 phytochemicals were molecular docked against IL6, AKT1, EGFR, and CASP3 proteins. Safety was assessed by ADMET profiling, while cytotoxicity, migration, invasion, and gene expression effects of Kaempferol and Coumestrol were assessed by in vitro assays. Statistical analysis was conducted using SPSS software.

Results: Kaempferol and Coumestrol were chosen from 69 phytochemicals based on high binding energies and atomic interactions with proteins. Coumestrol showed superior anti-invasive properties, and higher binding affinities with AKT1 (-6.4 kcal/mol), EGFR (-7.1 kcal/mol), and CASP3 (-7.1 kcal/mol) when compared to Kaempferol, plus no AMES toxicity and exceeded total clearance (8.085) of Kaempferol (6.868). Kaempferol exhibited greater cytotoxicity, colony-building inhibition, and stronger inhibition of cancer cell viability (38.8%) compared to Coumestrol (53.6%). Both bioactive compounds downregulated EGFR, AKT1, and IL6 while upregulating Caspase-3 expression, to efficiently target fibrogenic pathways.

Conclusion: The study concluded that *Euphorbia hirta* (whole plant) and *Lepidium sativum* (seeds) have the potential for hepatoprotection for liver fibrosis.

Keywords: Coumestrol, *Euphorbia hirta*, Fibrogenic Pathways, Kaempferol , *Lepidium sativum*, Liver Fibrosis

Introduction:

The liver is a vital organ with multifaceted physiological functions critical to metabolism, detoxification, and homeostasis. It stores glycogen, decomposes red blood cells, detoxifies harmful substances, and synthesizes essential plasma proteins and hormones. (1) (2) (3). The liver is highly prone to pathological conditions because of its persistent exposure to both exogenous and endogenous toxins which results in fibrosis. This medical condition is now among the biggest causes of morbidity and mortality worldwide. The characteristic feature of liver fibrosis involves increased matrix component accumulation within the extracellular space, which diminishes normal hepatic tissue (4). The advancement of medical science now suggests that controlled therapeutic treatment can reverse liver fibrosis despite its previous status as an irreversible condition that leads to cirrhosis and hepatocellular carcinoma (HCC). The development of fibrosis progresses from various causative factors which include viral hepatitis (HBV, HCV), autoimmune hepatitis (AIH), alcoholic liver disease (ALD), drug-induced liver injury (DILI), and metabolic disorders such as non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disorder (NAFLD). Research shows that liver diseases impact more than 10% of the worldwide population thus emphasizing the need for efficient therapeutic solutions (5) (6) (7) (8) (9). The dynamic process of liver fibrosis produces persistent injury that dysregulates hepatic stellate cells (HSCs) which acts as a central mechanism in fibrogenesis (10). HSCs react to liver injury by converting into active myofibroblast cells while losing their lipid storage functions and starting to express profibrotic properties (11). Transforming growth factor-beta (TGF-β) together with platelet-derived growth factor (PDGF) serve as the main mediators of ECM production and hepatic architectural disruption during this process. Chronic inflammation causes fibrosis progression through persistent cytokine activity together with oxidative stress which results in cirrhosis that leads to organ failure without treatment. The therapeutic potential of HSC activation therapies coupled with the management of profibrotic signaling cascade offers substantial promise. Additionally, a better understanding of epigenetic mechanisms and gene regulatory sequences in fibrosis progression presents new therapeutic opportunities (12) (13) (14). Current therapy techniques mostly aim at targeting the underlying cause rather than directly reversing fibrosis, and there are no FDA-approved antifibrotic medications available, despite substantial research in the field (15) (16). Antiviral treatments for hepatitis viruses, alcohol withdrawal for alcohol-induced liver disease, and behavioral changes for obesity-related fibrosis are all part of the management strategy. Novel antifibrotic therapies, such as those that block HSC activation, modulate immunological responses, and enhance ECM breakdown, have been developed as a result of the increasing awareness of the fibrosis pathophysiology (17). However, the prognosis for liver fibrosis remains poor due to diagnostic limitations and the side effects associated with current pharmacological therapies. This necessitates the investigation of alternative therapy approaches with higher efficacy and safety profiles. (18) (19) (20). Natural compounds receive substantial research interest for hepatoprotection because of their bioactive characteristics and lower harmful potential compared to synthetic medications (21). The historical use of plants for liver disease treatment is restricted by three major barriers including standardization difficulties, poor bioavailability, and toxicity testing requirements. The identification of antiviral anti-inflammatory and antifibrotic plant compounds through biomolecular sciences research provides a promising approach to treating fibrosis (22) (23) (24). However, a systematic evaluation, including molecular docking and in vivo validation, is essential to establish their therapeutic potential. The present study investigates the hepatoprotective potential of selected phytochemicals through computational and experimental approaches to identify novel bioactive compounds for liver fibrosis treatment. By targeting key fibrogenic pathways, this study contributes to developing plant-derived antifibrotic agents with translational potential for clinical applications (25).

Materials and Methods:

Selection and Collection of Plant Material: The study utilized medicinal plants that were obtained from the local market of Lahore, Pakistan. Their taxonomy was verified by the Department of Botany, Government College University, Faisalabad, Pakistan. Extracts were made using *Euphorbia hirta* (whole plant) and *Lepidium sativum* (seeds) plants.

***In silico* Study of Medicinal Plants: Retrieval of bioactive compounds from plant target:** 69 bioactive phytochemicals from *Euphorbia hirta* and *Lepidium sativum* were retrieved from TCMSP (Pharmacology of Traditional Chinese Medicinal Systems), IMPPAT (Indian Medicinal Plants, Phytochemistry and Therapeutics), and PubChem databases. Their 3D conformers were saved in SDF format for analysis (26) (27, 28). **Retrieval and preparation of receptor proteins:** Proteins involved in hepatic fibrosis, including IL6 (PDB ID: 1alu), AKT1 (PDB ID: 2uvm), EGFR (PDB ID: 5gty), and CASP3 (PDB ID: 2cdr), were retrieved from the literature and downloaded in PDB format (29). **Ligands and Receptors Preparation:** The 3D structure of Phytochemicals was accessed from PDB and prepared for docking by optimizing protein structures using the Discovery Studio tool. Phytochemicals chemical structures from the PubChem were energy minimized using the PyRx (Virtual Screening software), Following conversion into PDBQT format, protein molecules and ligands were produced and prepared for additional docking study analysis (30). **Molecular docking**: Molecular docking identified candidates for treating hepatic fibrosis. PyRx enabled docking, and the Discovery Studio visualized receptor-compound interactions, revealing optimal binding conformation at the active site of the receptors (31). **ADMET Profiling:** The ADMET parameters—toxicity, excretion, metabolism, distribution, and absorption—were assessed using SwissADME (32) and pkCSM (33), free online tools were used to evaluate the ADMET parameters of the drug candidates (34). (Azeem et al., 2015)**. Drug Scan:** Lipinski's five rules—a molecular weight of 500 Daltons, a refractivity index of 40–130, a log P ≤5, ≤5 hydrogen bond donors, and ≤10 hydrogen bond acceptors—were used to conduct the drug scan of the candidates for drugs (35). These parameters were checked through SwissADME, and the drug-likeliness of the drug candidates was evaluated.

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 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 (36). **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 being fixated 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 (37). **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 scratched. Detached cells were washed off, migration was observed using Kaempferol and Coumestrol in a serum-free medium. Images of the wound area were monitored via image at intervals (e.g., 12, 24, or 48 hours). Migration was quantified by measuring the wound width reduction using imaging software. The assay was performed in triplicate, and the results were analyzed to determine the impact of the peptides on cell migration (38).**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 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 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. (37). **LDH Assay:** Lactate dehydrogenase (LDH) release analyzed cell membrane integrity and cytotoxicity in HepG2 and Huh7 cells. HepG2 and Huh7 cells were incubated for attachment, 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 (39). **Gene Expression:** Quantitative real-time PCR (qRT-PCR) assessed inflammatory and necrotic markers. Gene expression was 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, IL6, Caspase 3 and 18S gene expression levels occurred through qRT-PCR testing using the StepOne real-time PCR system. The reverse transcription and 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 (40). Primer sequences used for the qRT-PCR reactions are listed in Table 1.

Table I: Primer sequences for qRT-PCR

Gene Expression

Sr. No	Gene name	F	R
1	EGFR	AGGCACGAGTAACAAGCTCAC	ATGAGGACATAACCAGCCAC
2	AKT1	CACAAACGAGGGGAGTACATC	GCCATCATTCTTGAGGAGGAAGT
3	IL6	TTCCATCCAGTTGCCTTCTTGG	TTCTCATTTCCACGATTTCCCAG
4	Caspase 3	TTAATAAAGGTATCCATGGAGAACACT	TTAGTGATAAAAATAGAGTTCTTTTGTGAG

Statistical Analysis: Data was presented as 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 further assess meaningful pairwise comparisons. For all statistical studies, SPSS software was used, while graphical illustrations and data visualizations were created with GraphPad Prism.

Results:

Molecular Docking: Initially, sixty-nine phytochemicals were retrieved from *Euphorbia hirta* and *Lepidium sativum,* and seven phytochemicals were selected for further study based on Lipnski's rule of five and Blood blood-brain (BBB) crossing. PyRx Virtual Screening software was used for ligand formation, and the 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 (Figures 1-8). Coumestrol exhibited higher binding affinities for three of the target proteins, AKT1 (-6.4 kcal/mol), EGFR (-7.1 kcal/mol), and CASP3 (-7.1 kcal/mol).

Table II: 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

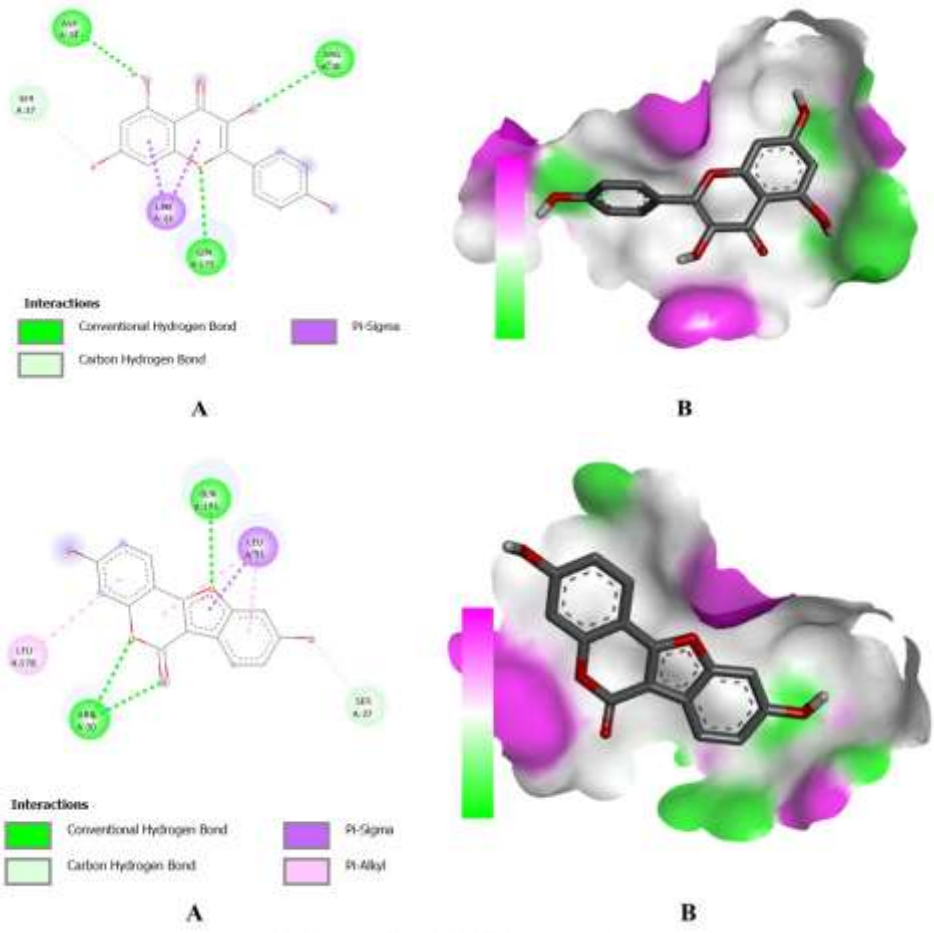


Fig. 1: Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein IL-6

Fig. 2: Interaction (A) and binding sequence (B) of Coumestrol with functional domains of protein IL-6

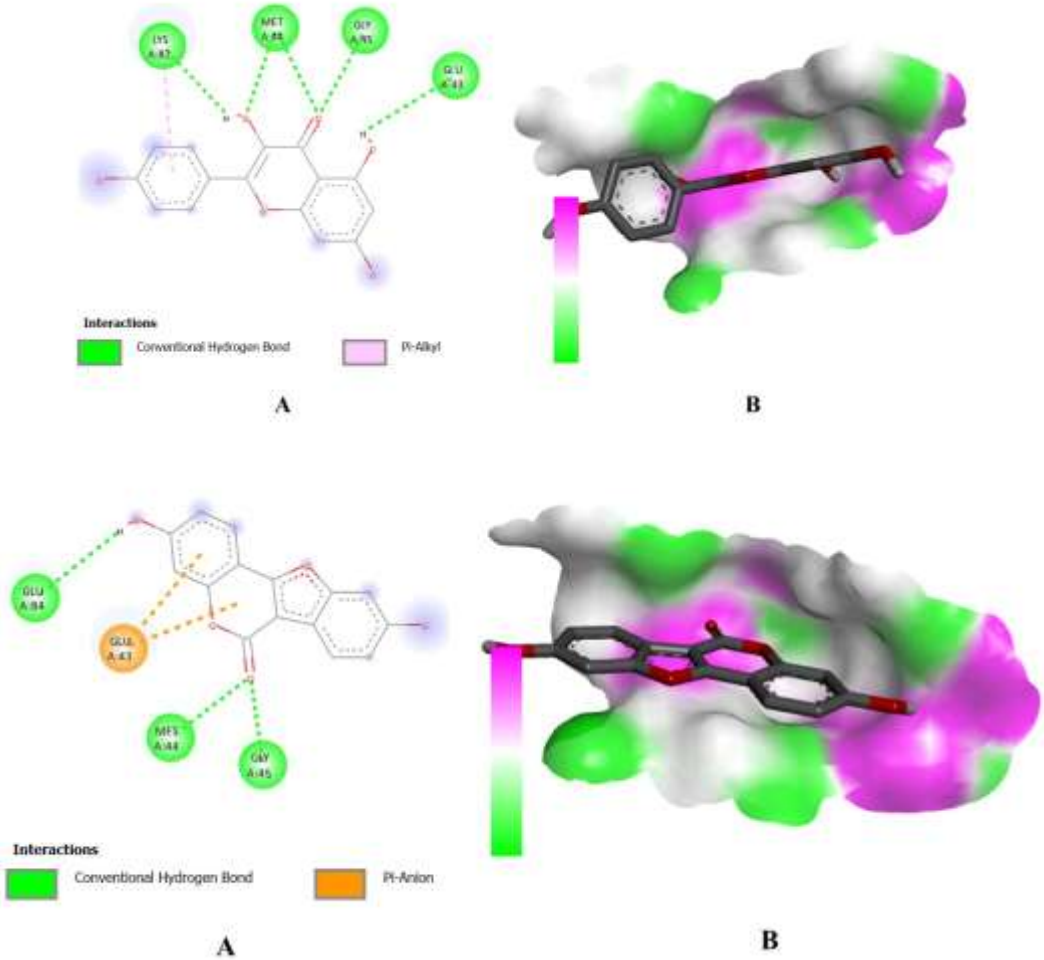
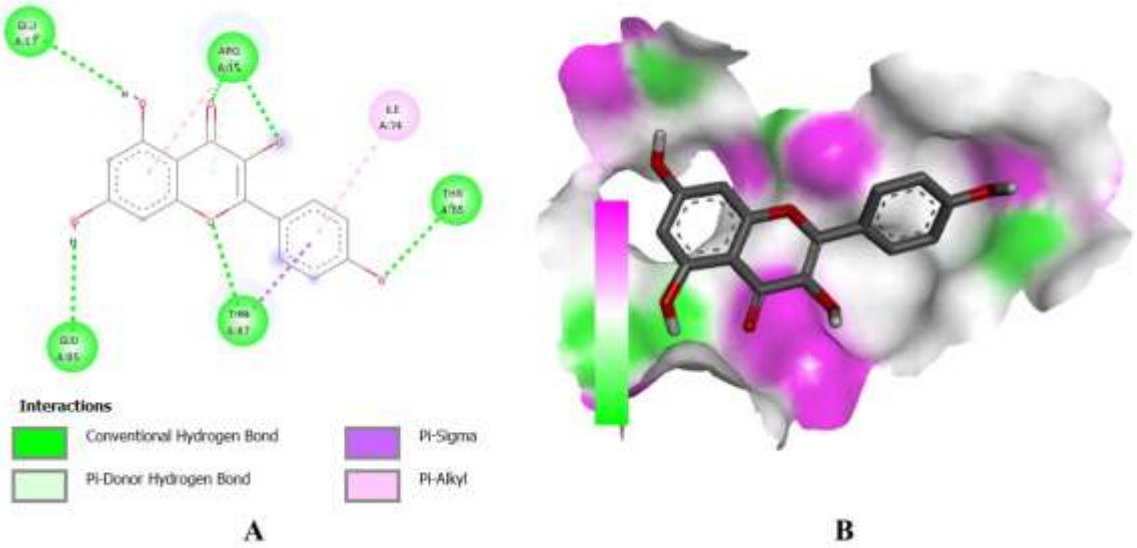


Fig. 3: Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein CASP3

Fig. 4: Interaction (A) and binding sequence (B) of Coumestrol with functional domains of protein CASP3



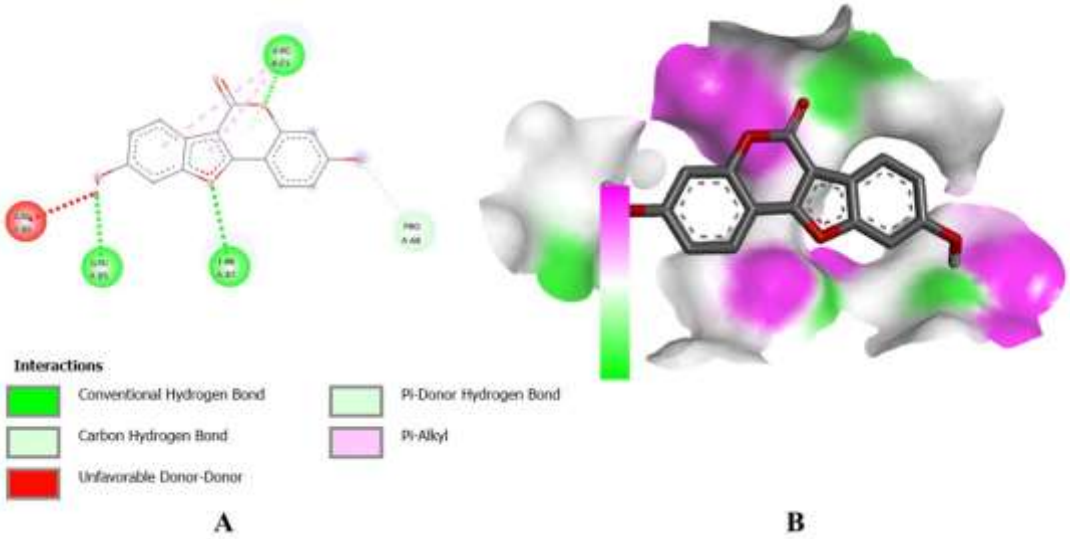


Fig. 5: Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein AKT1

Fig. 6: Interaction (A) and binding sequence (B) of Coumestrol with functional domains of protein AKT1

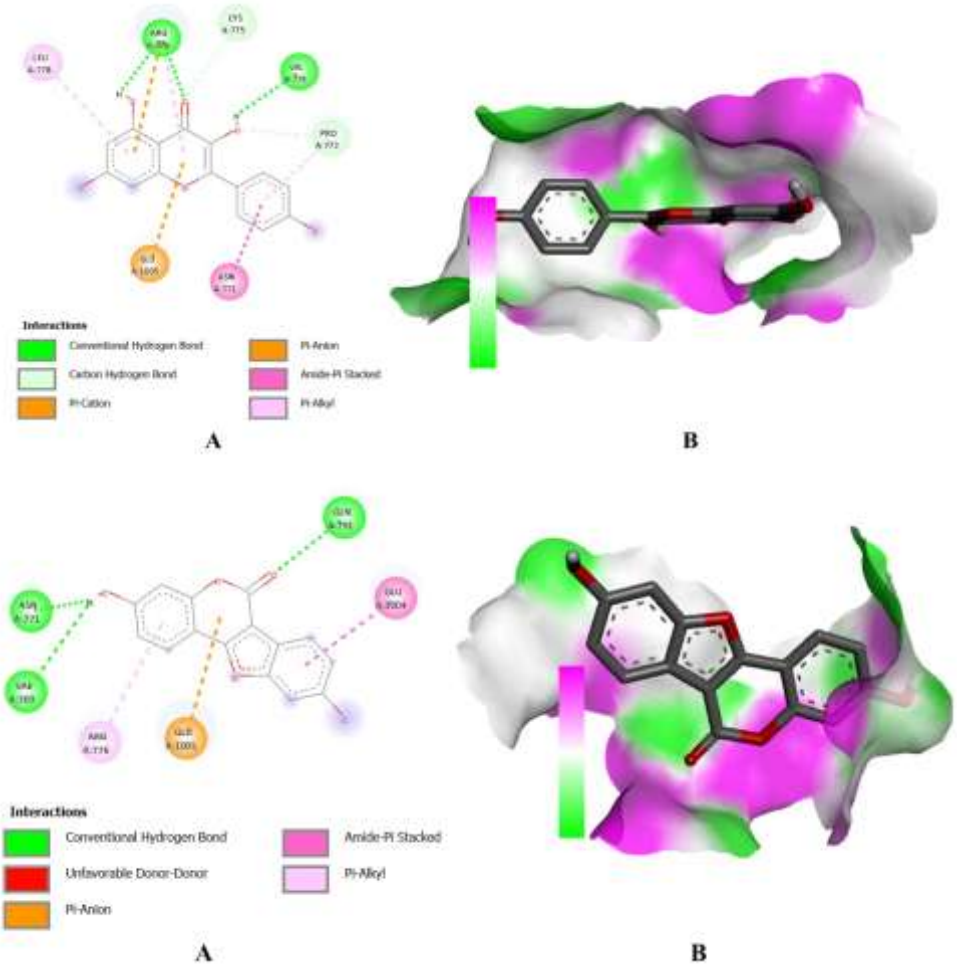


Fig. 7: Interaction (A) and binding sequence (B) of Kaempferol with functional domains of protein EGFR

Fig. 8: Interaction (A) and binding sequence (B) of Coumestrol with functional domains of protein EGFR

ADMET Profiling: ADMET Profiling parameters are presented in Table III

Table III: ADMET Profiling parameters of phytochemicals

ADMET Parameters	Phytochemicals	
	Kaempferol	**Coumestrol**
Absorption and distribution		
BBB	No	No
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; PGS: P-glycoprotein substrate; PGI: P-glycoprotein inhibitor

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 the concentrations exhibited variable results, as summarized in Fig. 9. The results show that Kaempferol exhibited 38.8% cell viability, and Coumestrol demonstrated 53.6%, after the treatment. The findings suggest that Kaempferol has a higher cancer cell inhibition activity than Coumestrol.

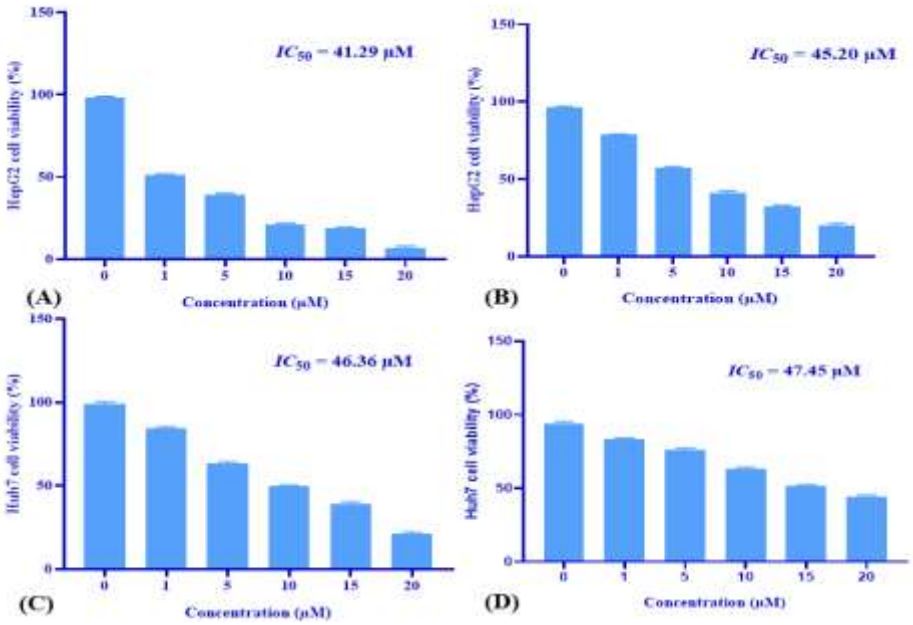


Fig. 9: Cell viability of Kaempferol and Coumestrol on HepG2 and Huh-7 cell lines.

The cell viability was evaluated using the CCk-8 test 24 hours post-treatment. (A) HepG2 cells treated with Kaempferol (B) HepG2 cells treated with Coumestrol (C) Huh7 cell treated with Kaempferol (D) Huh7 cells treated with Coumestrol.

Assessment of Cytotoxicity in HepG2 and Huh7 Cells by LDH Release Assay: Lactate dehydrogenase (LDH) release analyzed membrane integrity and cytotoxicity in HepG2 and Huh7 cells. In HepG2 cells, the release of LDH increased with increasing the concentration of the drug candidates as compared to the NC, resultantly increasing cytotoxicity. 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. Both Kaempferol and Coumestrol induced a comparable release of LDH in the Huh7 cells, and thus showed comparable cytotoxic activity.

Cell Migration Effect of the Drug Candidates Using Wound Healing Assay: In HepG2 cells, the treatment group of Kaempferol showed significant cell migration ability at 12, 24, and 48 hours after incubation at 37ºC. In the same cell line, the treatment group of Coumestrol showed lesser cell migration ability at the different time intervals after incubation at 37ºC. (Fig. 10a) In the Huh-7 cell line, both the drug candidates showed comparable wound-healing effects at the same concentration of 30µM after incubation at 37ºC. (Fig. 10b)

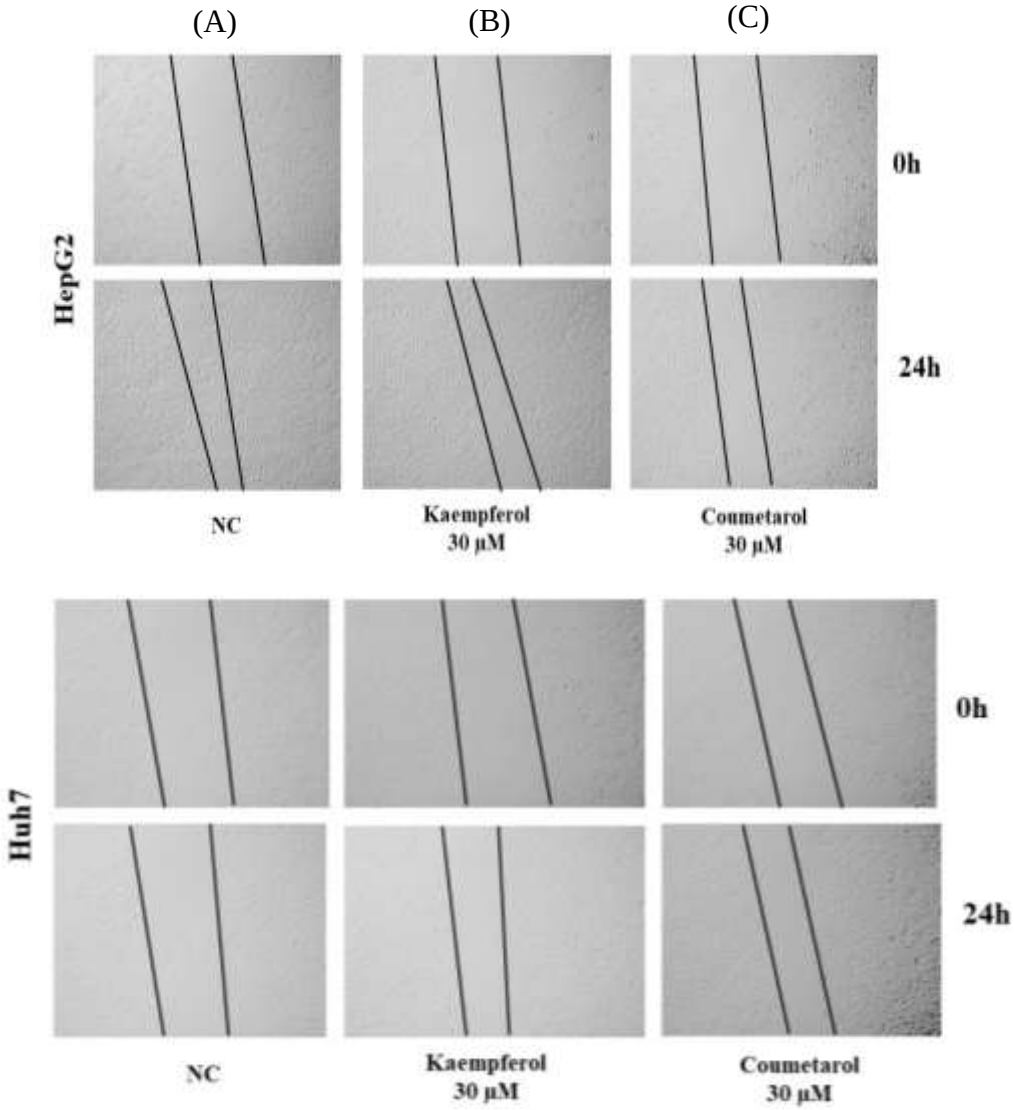
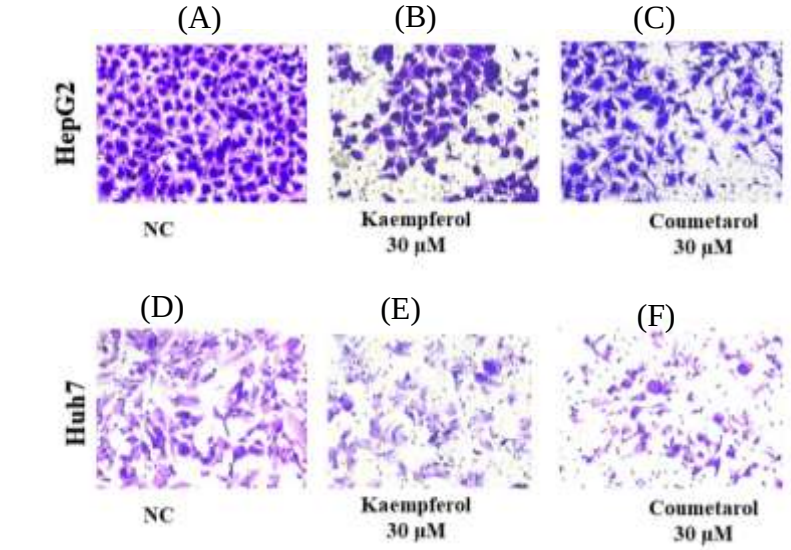


Fig. 10a: Wound Healing Assay of HepG2 cell line.
Fig. 10b: Wound Healing Assay of Heuh7 cell line.

Effect of the Drug Candidates on the Invasion Potential of HepG2 and Huh7 Cells: The transwell assay exhibited that Kaempferol inhibited the invasive potential of HepG2 cells at 30µM at a significant rate as compared to the NC. Coumestrol showed lesser inhibition of the invasive potential of HepG2 cells at the same concentration. In the Huh7 cell line, Coumestrol significantly suppressed the invasive potential of cancer cells as compared to Kaempferol at the same concentration of 30µM. Quantified by counting stained cells, the results suggested that Kaempferol and Coumestrol effectively impede cancer cell invasiveness, with Coumestrol being more potent.



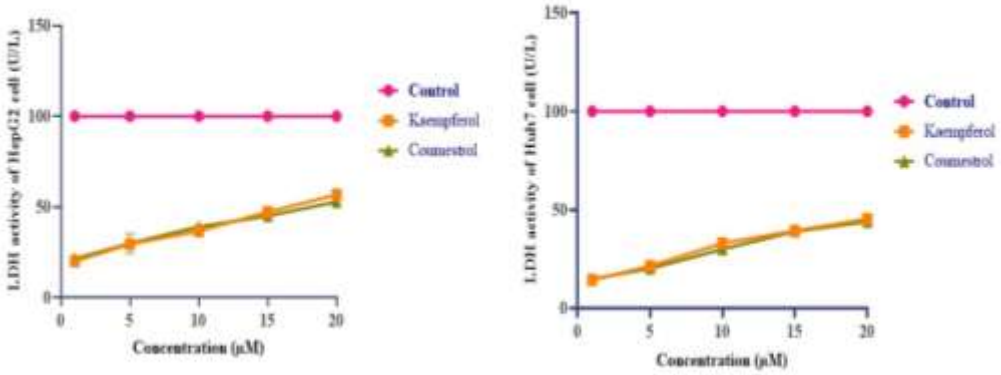


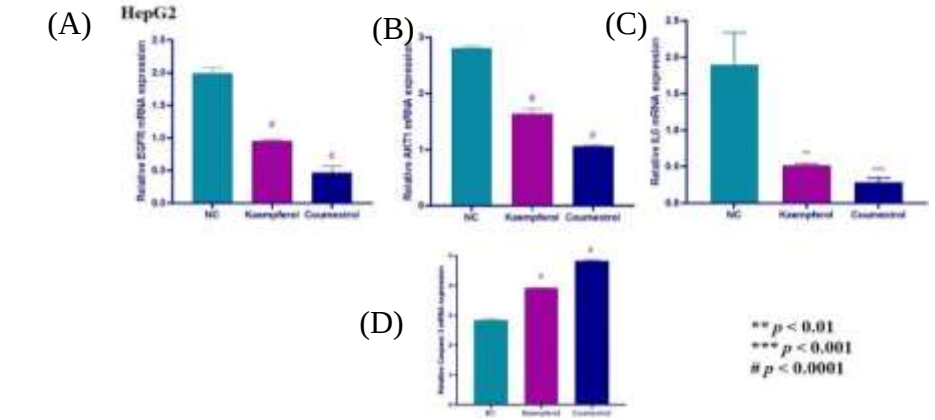
Fig. 11: Transwell invasion assay.
Fig. 12: LDH assay to evaluate cytotoxicity of Kaempferol and Coumestrol.

Fig. 11: 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 30µM (C) Invasion propensity of HepG2 cells after treatment with Coumestrol at 30µM (D) Invasiveness of Huh7 cells in NC (E) Invasion propensity of Huh7 cells after treatment with Kaempferol at 30µM (F) Invasion propensity of Huh7 cells after treatment with Coumestrol at 30µM. Fig. 12: Release of LDH and the resulting cytotoxicity measured by the LDH assay. (A) LDH activity of Kaempferol and Coumestrol in HepG2 cells (B) LDH activity of Kaempferol and Coumestrol in HepG2 cells

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 30μM concentration, and Coumestrol also reduced the quantity of colonies formed as compared to NC. (all #p<0.0001) The inhibitory effect of Kaempferol and Coumestrol on colony formation of HepG2 and Huh7 cells was positively correlated with the dose. Coumestrol exhibited a more marked inhibition of clonogenesis than Kaempferol.

Gene Expression Analysis of EGFR, AKT1, IL6, 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 IL6 (*#p* <0.01) as compared to the NC. Meanwhile, in the same cell line, Caspase3 (*#p* <0.0001) showed significant upregulation following the treatment with Kaempferol. On treatment with Coumestrol, the results showed a more considerable downregulation of EGFR (*#p* <0.0001), AKT1 (*#p* <0.0001), and IL6 (*#p* <0.001) as compared to the NC. Meanwhile, in the same cell line, Caspase3 (*#p* <0.0001) showed a more significant upregulation following the treatment than the treatment group of Kaempferol. (Fig. 13a) In the Huh7 cells, on treatment with Kaempferol, the results showed a remarkable downregulation of EGFR (*#p* <0.0001), AKT1 (*#p* <0.0001), and IL6 (*#p* <0.0001) as compared to the NC. Meanwhile, in the same cell line, Caspase3 (*#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 IL6 (*#p* <0.0001) showed lesser downregulation than Kampferol treatment group. Meanwhile, in the same cell line, Caspase3 (*#p* <0.0001) showed a more significant upregulation following the treatment with Coumestrol than the treatment group of Kaempferol. (Fig. 13b)



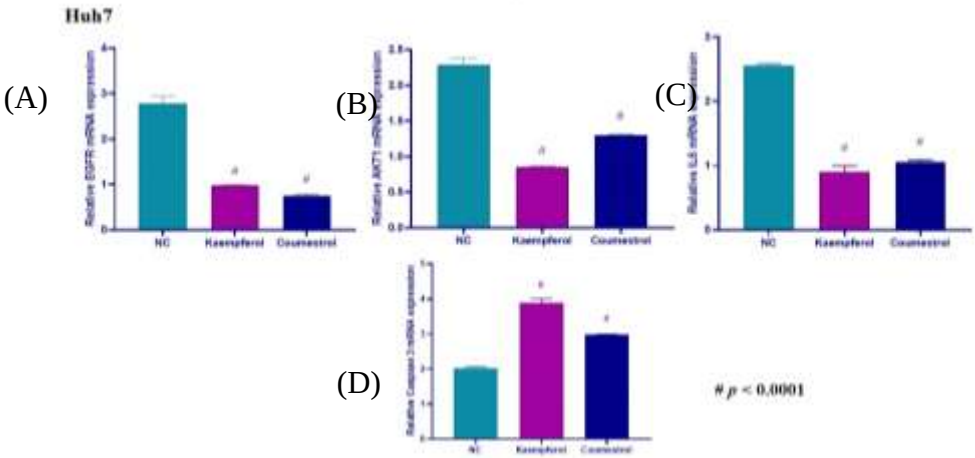


Fig. 13a: EGFR, AKT1, IL6, and Caspase 3 expression levels in HepG2 cell line.
Fig. 13b: EGFR, AKT1, IL6, and Caspase 3 expression levels in Huh7 cell line.

Fig. 15a: (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 IL6 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. 15b: (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 IL6 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.

Discussion:

Liver fibrosis, produced by collagen and ECM accumulation that replaces hepatic parenchyma, was formerly thought to be incurable and might lead to liver failure. While irreversible, technological advances have made it bearable by stopping progression. Novel treatments that focus on anti-fibrotic strategies such as HSC downregulation, the removal of damaging stimuli, and matrix disintegration have been made possible by a better understanding of the mechanisms underlying liver fibrosis (7) (8) (18) (19) (20). Despite the lack of adequate diagnostic facilities and the poor prognosis caused by drug side effects, medical innovations and botanical treatments have improved results (41) (42). *Euphorbia hirta* and *Lepidium sativum*, are highly esteemed for their medicinal efficacy, lack of side effects, and relative safety when compared to synthetic pharmaceuticals. This study focused on the genes EGFR, AKT1, Caspase-3, and IL-6 that are implicated in liver fibrosis. Kaempferol and Coumestrol were found to be the most effective phytochemicals by molecular docking utilizing PyRx and Discovery Studio software, exhibiting strong receptor contacts and high binding energy. In terms of binding affinities, Coumestrol was found to have a stronger ability to block cancer-promoting pathways, with values of -6.4 kcal/mol for AKT1, -7.1 kcal/mol for EGFR, and -7.1 kcal/mol for CASP3. The anti-cancer qualities of both substances were demonstrated by in vitro tests such as LDH analysis, wound healing, clonogenesis inhibition, and cell viability. The cytotoxic and anti-migration actions were supported by qRT-PCR, which also verified the overexpression of Caspase-3 and the downregulation of EGFR, AKT1, and IL-6. Phytochemicals like quercetin and oroxylin-A and others have also been demonstrated in earlier studies to be useful against fibrosis (43) (44) (45). The findings highlight the potential benefits of kaempferol and coumestrol in slowing the evolution of liver fibrosis and lowering the rate of metastases in hepatocellular carcinoma.

This study found that both the phytochemicals, Kaempferol and Coumestrol significantly suppressed cell viability, migration, clonogenesis, and invasion in the HepG2 and Huh7 cell lines. HepG2 and Huh7 cell lines of HCC were used to perform these *in vitro* activities. 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 41.29μM and 46.36μM in HepG2 and Hhuh7 cell lines, respectively. The IC_{50} values of Coumestrol were 45.20μM and 47.45μM in HepG2 and Huh7 cell lines, respectively. This suggests that Kaempferol can be more potent in reducing the proliferation of cancerous liver cells. A study conducted in 2021 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 (37). A 2007 study shows that Kaempferol suppresses the proinflammatory cytokines IL-6 and IL-8 and inhibits cell migration in a ROS-dependent manner (46). 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 (36).

LDH activity was performed to evaluate the 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., reported in 2022 that the LDH assay showed more LDH release in the groups treated with

the plant drugs constituting *Acacia modesta* and *Opuntia monocantha*. (42). The invasive potential of the cell lines was determined with the help of 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. The same experimental study validated the results of the transwell assay by measuring the expression levels of MMP-2, MMP-9, P13K, AKT, and S6K (37). qRT-PCR demonstrated downregulation of EGFR, AKT1, and IL6, as well as overexpression 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. It further revealed that AKT-knockout liver cancer cells displayed lower levels of AKT1. Clonogenesis and cell viability were also suppressed in AKT-knockout liver cancer cells (47). Another study conducted in 2017 showed that a phytochemical, ilexgenin A, exerted antitumor activity in the HepG2 cell line, by inhibiting the production of VEGFR (Vascular Endothelial Growth Factor) and the process of transcription. It also inhibited different signaling pathways and suppressed the inflammatory cytokines, IL-6 and TNF-β (48). 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 (49).

With the help of *in silico* and *in vitro* studies, the main targets were evaluated as potential targets for Kaempferol and Coumestrol to treat hepatic fibrosis, leading to HCC. The molecular docking studies confirmed the potential of Coumestrol as the leading drug candidate. The minimum energy score of Coumestrol (Table 2) shows its higher binding affinity as a ligand for the target proteins. The findings of this research highlight the potential therapeutic properties of Kaempferol and Coumestrol in the treatment of HCC. The ability of both the phytochemicals to regulate key oncogenic pathways suggests that these may prove to be promising drug candidates for further clinical evaluations.

Conclusion:

Liver fibrosis, a serious health issue, can lead to incurable liver cancer. Researchers looked at the anti-inflammatory and antioxidant capabilities of plant-based medications including Euphorbia hirta and Lepidium sativum. After applying Lipinski's rule to seven phytochemicals, two of them—Kaempferol and Coumestrol—were found to have substantial interactions with proteins involved in hepatic fibrosis. The results of the in vitro tests showed that Kaempferol had a greater cytotoxic effect, whereas Coumestrol had a stronger inhibitory effect on colony development and invasion. In LDH experiments, both phytochemicals showed similar cytotoxicity and wound-healing properties. The results of the qRT-PCR showed that EGFR, AKT1, and IL6 were downregulated, whereas Caspase 3 was markedly upregulated, especially with Coumestrol which indicates their therapeutic potential.

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