Int. J. Morphol.,
42(6):1746-1754, 2024.

Potential Anticancer Activity of *Alcea rosea* and Molecular Docking of Selected Target Proteins in Liver Cancer Cells

Actividad Anticancerígena Potencial de la Alcea rosea y Acoplamiento Molecular de Proteínas Diana Seleccionadas en Células de Cáncer de Hígado

Nael Abutaha; Mohamed A Wadaan; Raed Ahmad Alghamdi & Fahd A. Al-Mekhlafi

ABUTAHA, N.; WADAAN, M.A.; ALGHAMDI, R.A. & AL-MEKHLAFI, F.A. Potential anticancer activity of *Alcea rosea* and molecular docking of selected target proteins in liver cancer cells. *Int. J. Morphol., 42(6)*:1746-1754, 2024.

SUMMARY: The incidence of cancer is rising globally. In this study, the cytotoxic effects of *Alcea rosea* L., hexane seed (ARHS) extract were examined against liver (HuH-7) and breast (MDA-MB-231) human cancer cell lines, as well as non-cancerous HUVEC cell line. The ARHS extract displayed dose-dependent cytotoxic effects on HuH-7 and MDA-MB-231 cells, with respective IC_{50} values of 2.39 and 5.67 μg/mL. Additionally, the ARHS extract impeded the migration of HuH-7 cells in a time-dependent fashion. Morphological assessments showed characteristics indicative of apoptosis in the treated cells. ARHS extract induced ROS production in HuH-7 cells. This study revealed that the *A. rosea* hexane extract effectively inhibited HuH-7 cell growth by inducing apoptosis, as evidenced by the changes in cell and nuclear morphology observed using DAPI and AO/EBr staining. Heptadeca-8,11-dien-1-yl demonstrated the highest docking score, forming three hydrogen bonds with the amino acids ARG-842A, ALA-1050A, and ASP-1052A in the 4ASD receptor. Although it has potential as an anticancer agent, additional research is needed to explore the *in vivo* anticancer efficacy.

KEY WORDS: *Alcea rosea*; cytotoxicity; apoptosis; molecular docking.

INTRODUCTION

Globally, the prevalence of liver cancer is increasing, posing an ongoing challenge to global health (Balogh *et al*., 2016; Villanueva, 2019). Annual liver cancer cases are predicted to reach over 1 million by 2025 (Llovet *et al*., 2021). Liver cancer ranked ninth in cancer incidence among Saudi females and sixth among Saudi males (Saudi Cancer Registry, 2014). Furthermore, compared to other nations in the Arabian Gulf region, Saudi Arabia and Kuwait exhibited a slightly elevated rate of liver cancer (Alghamdi & Alghamdi, 2020).

Although chemotherapeutic medicines are successful treatments for hepatocellular carcinoma, their side effects, development of drug resistance, and high toxicity result in poor clinical outcomes and a high mortality rate (Anwanwan *et al*., 2020). Therefore, Alternative treatment options are required. Numerous published reports indicate the potential of using plant extracts as a treatment option for hepatocellular carcinoma. Utilizing natural compounds treatment may reduce toxicity and minimize side effects (Aung *et al*., 2017; Lin *et al*., 2020).

Alcea rosea L. (Malvaceae) is widely distributed from the east Mediterranean region to Central Asia. Sometimes found in waste places and along roadsides (Fahamiya *et al*., 2016; Singh & Panda, 2005). The medicinal use of *A. rosea* involves the utilization of its roots, leaves, seeds, and flowers. The fruits and leaves of the plant contain various compounds, including mannose, glucose, sucrose, phellandrene, b-sitosterol, limonene, cyclohexanol, and primary alcohols. Furthermore, seed oil primarily comprises linoleic acid, which is its major constituent (Fahamiya *et al*., 2016). Several pharmacological activities of the plants have been reported, such as analgesic, anti-inflammatory, diuretic, and antibacterial (Wang *et al*., 1989; Seyyednejad *et al*., 2010; Tuba *et al*., 2010; Ahmadi *et al*., 2012). The plant is also used for cough, bronchitis, asthma, inflammatory conditions, arthritis, kidney disorders, renal calculi, peptic ulceration, chest pain, skin cuts, ulcers, burns, boils, abscesses, gastrointestinal disorders and many more (Fahamiya *et al*., 2016). The root of *A. rosea* has been used in traditional medicine for a wide range of ailments, including bronchitis, diarrhea, constipation, inflammation, severe coughs and angina (Ahmadi *et al*., 2012).

Department of Zoology, College of Science, King Saud University, Riyadh , Saudi Arabia.
FUNDED. Researchers Supporting Project number (RSP2024R112), King Saud University, Riyadh, Saudi Arabia.

Received: 2024-05-28 Acccepted: 2024-07-26

Gas chromatography-mass spectrometry (GC–MS) has been commonly used to identify bioactive secondary metabolites in plants (Satapute *et al*., 2019). Advanced computer-based tools have revolutionized drug discovery by allowing for the screening of bioactive secondary metabolites from medicinal plants (Sliwoski *et al*., 2014). Computational prediction models predict pharmacological, pharmacokinetic, and toxicological properties, aiding in selecting promising candidates for further research and accelerating pharmaceutical advancements (Loza-Mejía *et al*., 2018). In silico (computer-based) techniques are quick and affordable for developing and testing pharmaceuticals. This approach generates insights into the drug-receptor interactions, enabling predictions of how the drug model will interact with target proteins (Bharathi *et al*., 2014; Lee & Kim, 2019).

For an anticancer drug to be considered effective, it should be able to eliminate cancer cells while leaving normal cells unharmed selectively. The key to achieving this is by restoring the apoptosis machinery in cancer cells, making apoptosis modulation a promising approach in cancer prevention and therapy (Motadi *et al*., 2007). Natural products have shown the potential to provide such templates for inducing apoptosis. Consequently, screening plant extracts for apoptotic inducers is crucial, either in their crude form or as isolated compounds (Yang *et al*., 1998; Choene & Motadi, 2016).

Thus, this study aimed to investigate cytotoxicity of *A. rosea* seed extract against liver (HepG2) and breast cancer (MDA-MB-231) cells lines, apoptosis, cell migration, molecular docking, and chemical constituent of *A. rosea* seed extract.

MATERIAL AND METHOD

Extraction procedure. *Alcea rosea* seeds were purchased from a herbal store in Riyadh, Saudi Arabia, and subsequently stored in the herbarium under the voucher BRS-KSU-082. The seeds were washed in distilled water before being dried at 50 °C in an oven. A commercial mill was used to grind the plant material into a coarse powder. The powdered substance (34 g) was macerated for five days at 25 °C with periodic shaking in 300 mL of 85 % ethanol. The resultant mixture was centrifuged at 3000 rpm, and the solvent was concentrated using a rotary evaporator at 45 °C (Sadiq *et al*., 2014).

Fractionation. The evaporated extract was suspended in distilled water (500 mL) and was poured into a separating funnel, and fractionated by liquid-liquid extraction using hexane (fraction 1), chloroform (fraction 2), and ethyl acetate (fraction 3). The remaining aqueous extract was evaporated until completely dried and then suspended in methanol. The soluble methanol extract was filtered (fraction 4) using Whatman filter paper 1. After separation, each fraction was concentrated using a rotary evaporator at 40 ˚C under reduced pressure.

Culture of Cell Lines. HuH-7 cells, representing human hepatocarcinoma; MDA-MB-231 cells, representing triple-negative human breast adenocarcinoma; and non-cancerous HUVEC cells derived from human umbilical vein endothelium, were sourced from the German cell culture collection. Subsequently, these cell lines were cultured in a 5 % CO_2 humidified atmosphere at 37 °C, using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal calf serum from Gibco (USA).

Cytotoxicity Assays. This study investigated the cytotoxic effects of extracts on cancer cell lines using the colorimetric MTT assay. For the MTT assay, 24-well culture plates were used, with each well initially seeded with 5×10^4 cells and left for 24 h. The wells were treated with varying concentrations (0, 10, 5.2, 2.5, 1.25, and 0.625 µg/mL) of different seed extract fractions (Fraction 1, 2, 3, and 4) and then incubated for 48 h. Control cells were treated with 0.01 % methanol. In each well, 100 µL of MTT solution was added and incubated for 2 h afterwards, the medium was removed, and 100 mL of 0.01 % hydrochloric acid in isopropanol was added to solubilize the formazan crystals. The optical density of the plates was measured at a wavelength of 550 nm using a plate reader (ChroMate, England). The MTT assay procedure followed a similar protocol as described by Abutaha (Abutaha *et al*., 2023).

Scratch Wound Healing Assay. Hexane extract of *A. rosea* (ARHS) was used to assess plant extracts effects on the cell migration using HuH-7 cells. Six well-plates with 5×10^4 cells/mL were seeded and given time to reach 80 % confluence. PBS was used to wash the cells, and a 10 µL tip was used to make a scratch. The cells were washed with PBS, and the unattached cells were removed. 1 µg/mL of ARHS extract was added to the cells. Negative control cells were treated with 0.1 % methanol. An inverted microscope with a digital camera was used to capture the images. The tests were carried out three times. The scratch width was measured at different time intervals (24 and 48 hours) using the Image J software. The percentage of migration was calculated using the following formula:

Percentage of migration = (Distance at 0 h-Distance at 48 h)/(Distance at 0 h)×100

Assessment of Cell Morphology. Cells were cultivated on 24-well plates and exposed to ARHS extract at 0 to 10 µg/mL concentrations for 24 h, either with or without treatment. Morphological alternations were photographed with the help of a digital camera (Leica, Germany).

Fluorescence Microscopy

DAPI staining. HuH-7 cells (5×10^4 cells/mL) were seeded in a 24-well plate and incubated at 37 °C for 24 h. The cells were treated with 1 µg/mL of ARHS extract and incubated for 24 h. Treated and vehicle control cells (0.1 % methanol) were rinsed with PBS and fixed using ice-cold ethanol. 4′, 6′-diamidino-2-phenylindole (DAPI, 1 µgml-1) was used to look for nuclear damage or chromatin condensation. The fluorescent images were captured using a fluorescence microscope (EVOS, USA).

Dual Acridine Orange (AO)/Ethidium Bromide Staining (EB). HuH-7 cells (5×10^4 cells/mL) were seeded in a 24-well plate and incubated at 37 °C for 24 h. The cells were treated with 1µg/mL of ARHS extract and left for 24 h. The cells were stained with AO/EB solution (1µg/mL) for 2 min in the dark. Similar treatment was used for vehicle control cells (0.1 % methanol). The fluorescent images were captured using a fluorescence microscope (EVOS, USA).

DCFH-DA ROS Assay. To evaluate the production of reactive oxygen species (ROS), a dichlorofluorescein diacetate (DCFH-DA) assay was conducted. The oxidation of DCFH in cells results in the production of dichlorofluorescein. Huh-7 cells were incubated with ARHS extract in a 24-well plate for 24 h, as reported in the previous sections. Post-treatment period, cells were incubated with DCFH-DA for 30 min at 37 °C. Similar treatment was used for vehicle control cells (0.1 % methanol). Cells were resuspended in 500 µL of PBS and imaged with a fluorescent microscope (EVOS, USA).

Gas chromatography-mass spectrometry (GC–MS) analysis. The ARHS extract from seeds was analyzed using the Perkin-Elmer Clarus 680 system (Perkin-Elmer, Inc., USA). This system was equipped with an Elite-5MS capillary column made of fused silica. The dimensions of the column were 30 m in length, 250 µm in diameter, and 0.25 µm in thickness. Pure helium (99.99 %) served as the carrier gas, with a continuous flow rate of 1 mL per min. The spectral detection in the GC-MS analysis used an electron ionization method with high ionization energy of 70 electron volts (eV), a scan time of 0.2 s, and fragments in the range of 40 to 600 m/z. The injected volume was 1 µL, with a split ratio of 10:1, and the injector temperature was consistently maintained at 250 °C. The column oven temperature was initially set at 50 °C for 3 min, and then gradually increased at a rate of 10 °C per min until it reached 280 °C. Finally, the temperature was ramped up to 300 °C and held for 10 minutes. The phytochemical constituents were identified by comparing them with the spectral database of verified compounds stored in the National Institute of Standards and Technology (NIST) library.

Molecular docking. The possible interaction between GC-MS detected compounds and the protein targets, including 1CP3, 2O2F, 1M17, 4ASD, 4QVF,1SVC, 4XCU, 4C612, BMC, 4XI3, 5KIR, 2Q7K, 2OH4, 4LQM, 2AR9 (Table I). Each protein target was retrieved from the Protein Data Bank (*www.rcsb.org/pages/policies*). To predict the interaction between each ligand and its corresponding protein target, virtual screening tools such as AutoDock Vina 1.1.2 were employed. PyMOL 2.5 was utilized to construct the interactions between the ligands and target proteins. Additionally, the investigation of the three-dimensional (3D) and two-dimensional (2D) visualization of the ligand and its corresponding protein target interactions was carried out using PLIP (Protein–Ligand Interaction Profiler).

Statistical Analysis. The obtained data are displayed as the mean ± SD, the experiments were carried out in triplicate and Student's t-test to assess significance between groups. Results were considered significant when $p < 0.05$.

Table I. Apoptotic proteins with PDB ID.

S.NO	PDB ID	Target
1	2BMC	Aurora-2
2	4C61	Jak2 Kinase
3	4XCU	Fibroblast growth factor receptor 4 (VEGFR-4)
4	1SVC	Nuclear factor kappa-B (NF-KB)
5	4QVF	Bcl-2-like protein 1
6	4ASD	Vascular endothelial growth factor receptor 2 (VEGFR-2)
7	1M17	epidermal growth factor receptor
8	2O2F	Apoptosis regulator Bcl-2
9	1CP3	Apopain
10	4XI3	homodimer Estrogen receptor
11	5KIR	human COX-2 protein
12	2Q7K	prostate cancer receptor
13	2OH4	vascular endothelial growth factor 2 (VEGFR-2)
14	4LQM	Epidermal Growth Factor Receptor
15	2AR9	caspase 9

RESULTS

The yield of the crude extract. Upon extraction 34 grams of *A. rosea* seed powder with 85 % ethanol, the resulting extract yielded the following fractions: 2.5 mg from hexane (Fraction 1), 700 mg from chloroform (Fraction 2), 4.5 mg from ethyl acetate (Fraction 3), and 637 mg from methanol (Fraction 4).

Cytotoxicity of hexane extract on cancer cell lines. All extracts and control were assessed for their cytotoxicity on Huh-7 (human hepatoma) and MDA-MB-231 (breast adenocarcinoma) cells, and HUVEC (human umbilical vein endothelial cell). Fraction 1 was the only potent extract, and its IC50 values on Huh-7, MDA-MB 231, and HUVEC were 2.39, 3.44, and 5.67µg/mL, respectively. However, the hexane extract showed a less cytotoxic effect on non-cancer cell lines HUVEC. After 24 h of incubation, the cell lines tested exhibited dose-dependent mortality in response to the extract's varying concentrations (0-10 µg/mL) (Fig. 1).

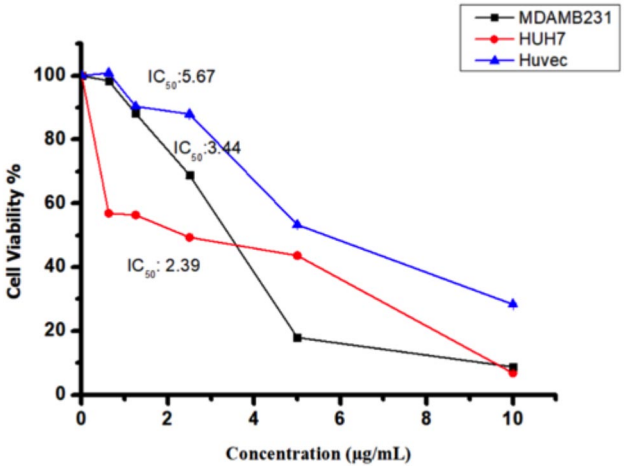

Fig. 1. The results of viability assessments conducted on three different cell lines: Huh-7 and MDA-MB-231 (both cancer cell lines) and HUVEC cells (a non-cancerous cell line). The viability of these cells was examined followaing treatment with the fraction 1 (*A. rosea* seed).

Fraction 1 induces apoptosis. Most anticancer medications in clinical oncology exploit the apoptotic mechanism to trigger cancer cell death (Reed, 2006). Huh-7 cells were used to study the role of apoptosis in hexane-induced cytotoxicity since hexane extract was the only active extract. The Light microscopic study of cells treated with 1 µg/mL of hexane extract for 24 h shown characteristics of apoptotic changes like rounding and detachment of cells, unlike the control (0.01 % methanol). DAPI, a nuclear stain, emits blue fluorescence when viewed under a fluorescence microscope. In our current

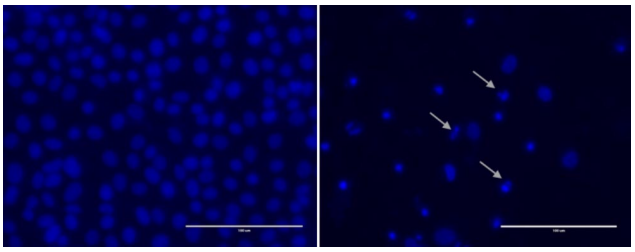

Fig. 2. The nuclear changes seen in cells after treatment with fraction 1 of *A. rosea*. HuH-7 cells treated with ARHS extract of *A. rosea* seeds for 24 h were seen under a fluorescence microscope following nuclear staining with DAPI.

study, we used DAPI staining to highlight the changes related to Apoptosis in Huh-7 cells treated with the hexane extract of *A. rosea* seeds, as shown in Figure 2. Upon treatment, noticeable morphological alterations associated with apoptosis, including margination of the nucleus, nuclear fragmentation, and chromatin condensation Figure 2 (indicated by white arrows), were seen in the Huh-7 cells.

AO/EtBr staining can be used to detect changes in the nucleus of apoptotic cells. This technique is commonly used to explore apoptosis mechanisms and evaluate the efficacy of anticancer drugs that induce apoptosis in cancer cells. The AO dye can permeate living cells and stain their nuclei green, while EB can only penetrate dead cells and stains their red nuclei. Therefore, viable cells display intact green nuclei, while necrotic cells exhibit bright orange round nuclei. Early apoptotic cells present with bright green fragmented and condensed nuclei, whereas late apoptotic cells show red fragmented and condensed nuclei. In this study, AO/EB staining was performed, and it revealed intact green nuclei in control cells, while treated cells displayed red fragmented and condensed nuclei after 24 hours of treatment (Fig. 3). Thus, the microscopic analysis suggests that the hexane extract-induced cell death in Huh-7 cells was primarily due to apoptosis.

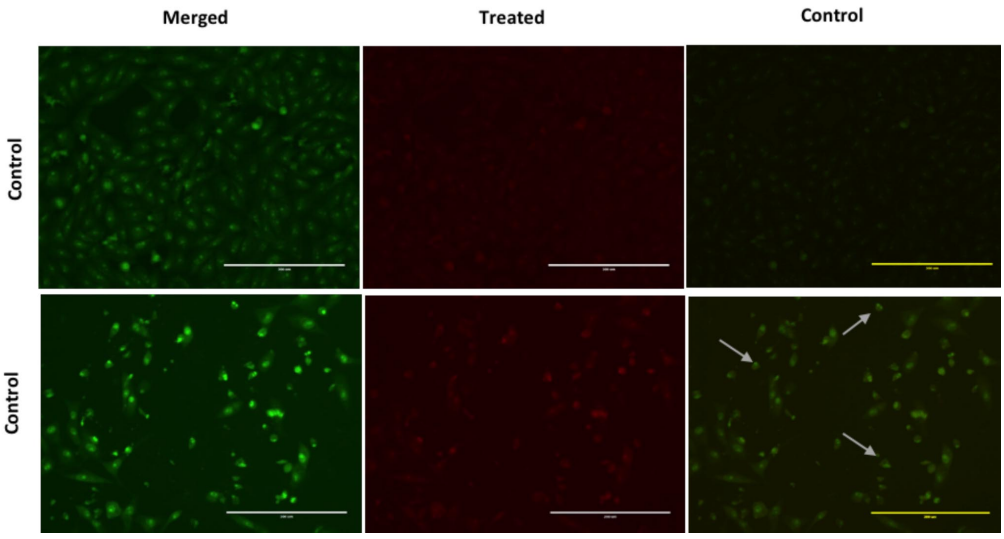

Fig. 3. illustrates the results of AO/EB staining performed on Huh-7 cells to detect apoptosis induced by treatment with fraction 1 from *A. rosea* at a 1 µg/mL concentration. In the vehicle control group, the cells exhibit uniform green fluorescence. However, the apoptotic cells display distinct characteristics in the experimental group (treated with the extract). The nuclei of these apoptotic cells are stained with a yellow-green fluorescence and appear concentrated in a crescent or granular pattern on one side of the cells.

Fraction 1 increases the production of ROS in Huh-7 cells. To examine the influence of ARHS extract on reactive oxygen species (ROS), we employed DCFH-DA staining. Huh-7 cells were subjected to DCFH-DA staining to assess changes in intracellular ROS levels following 24 h of treatment with fraction 1. The fluorescent micrographs revealed that treatment with fraction 1 resulted in a pronounced increase in DCF-fluorescence intensity within Huh-7 cancer cells, indicating an enhanced generation of intracellular ROS triggered by the ARHS extract (Fig. 4).

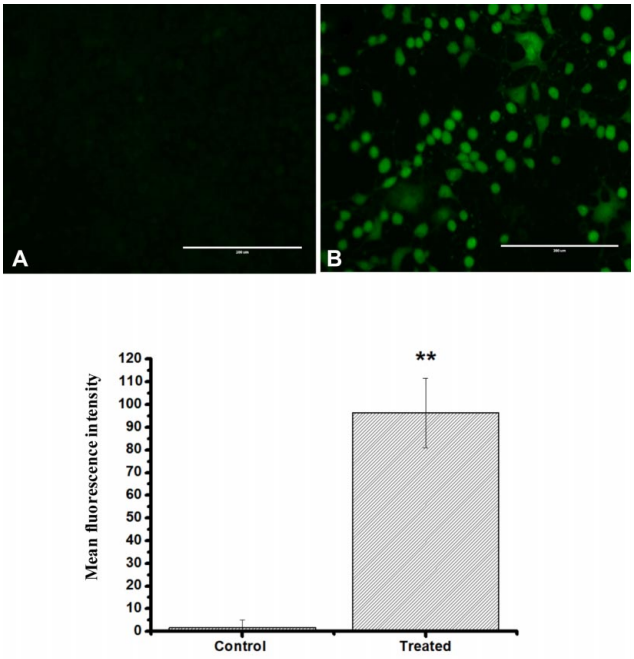

Fig. 4. Representative images of DCFH-DA assay showing ROS generation in Huh-7 cells after exposure to fraction 1 of *A. rosea* seed (A) Control, (B) Treated. B: Measurement of fluorescence intensity of ROS in fraction 1-treated cells. Values were expressed in mean±SD of three independent experiments. The asterisk (*) (P 0.05) shows a statistically significant difference.

Scratch assay. In the control wells, the cells migrated and completely covered the scratched area within 48 h, achieving full confluence. However, upon treating the cells with fraction 1 (Fig. 5), the migration of cells was significantly inhibited. The inhibitory effect on Huh-7 cell migration by fraction 1 was analyzed at a concentration of 1 µg/mL, and the results were reported as a percentage of cell migration. Treatment with fraction 1 led to a time-dependent reduction in the migration of Huh-7 cells. The percentage of cell migration was significantly lower in the presence of the ARHS extract compared to the negative control ($p<0.05$) after 24 and 48 h of treatment. These findings suggest that fraction 1possesses antimetastatic potential, likely attributed to its cytotoxic activity.

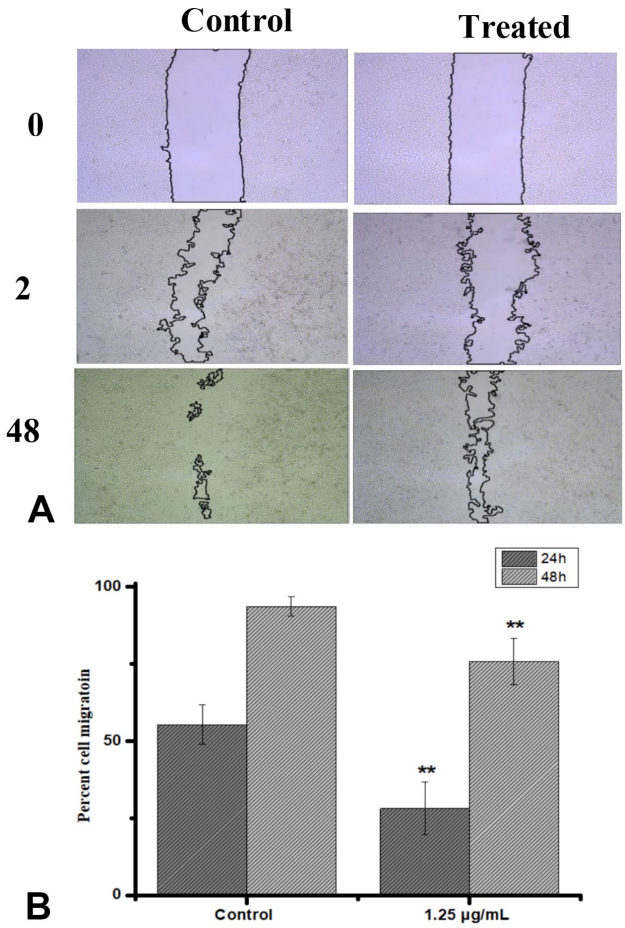

Fig. 5. Effect of fraction 1 of *A. rosea* seeds on the migration of Huh-7 cells. (A): The wound closure potential of Huh-7 cells was evaluated by creating a scratch in both treated and control wells. Images of the fraction 1-treated Huh-7 cell monolayers were captured using an inverted light microscope at 24-hour and 48-hour intervals. (B): Quantitative analysis of Huh-7 cell migration after treatment with 1 µg/mL of fraction 1. The percentage of cell migration used to depict the results. Values were expressed in mean±SD of three independent experiments. The asterisk (**) (P 0.05) shows a statistically significant difference.

GC–MS analysis. To identify the biologically active compounds, present in fraction 1 of *A. rosea*, gas chromatography-mass spectrometry (GC-MS) analysis was performed. A total of 11 compounds were detected (Table II). The major compounds were nonanoic acid (18.4 %), 9,12-octadecadienoic acid (17.6 %), 1,3,5-trimethyl benzene (14.6 %), 9,12-octadecadien-1-ol (14 %) and 2-hydroxy-1 hexadecanoic acid (12.07 %). The presence of these compounds in the ARHS extract may contribute to its demonstrated anticancer activity.

Molecular docking: This study employed a molecular docking approach to predict the potential target proteins for the twelve compounds identified by GC-MS analysis (Figs. 6 and 7). The

liver proteins available in the Protein Data Bank (PDB) were used for this purpose. Molecular docking is a computational technique that uses computational methods to predict the complex structure formed between a ligand and a receptor. We conducted molecular docking simulations to gain insights into the interactions between the proteins 1SVC, 1CP3, 1M17, 2AR9, 2BMC, 2OH4, 2O2F, 2Q7K, 4ASD, 4C61, 4LQM, 4QVF, 4XI3, 4XCU, and 5KIR with different ligands. Table I displays the binding energies (kcal/mol) of all the compounds detected. The best Molecular docking scores recorded for 4ASD, 2OH4, and 4ASD were -8.1 kcal/mol (4ASD) and -7.8 kcal/mol for 2OH4, and 4ASD, respectively. Heptadeca-8,11-dien-1-yl shows three hydrogen bonds with ARG- 842A, ALA-1050A, and ASP- 1052A amino acids with 4ASD receptor. Also, the formation of 15 hydrophobic interactions with ALU- 840A, VAL-840A, ALA- 866A, LYS- 868A, VAL-899A, VAL- 916A ASN- 923A, LEU- 1035A, PHE- 1047A, and ARG- 1051A. Similarly, the docking results of Z-1-phenyl-7-methyl-1,6-octadiene to 2OH4 resulted in eleven hydrophobic interactions (LEU- 838A VAL- 846A, ALA-864A, LYS- 866A, VAL- 914A, LEU- 1033A, PHE- 1045A) with binding energies of -7.8 kcal/mol. The 3D and 2D docking images of 4ASD are represented in Figures 6 and 7.

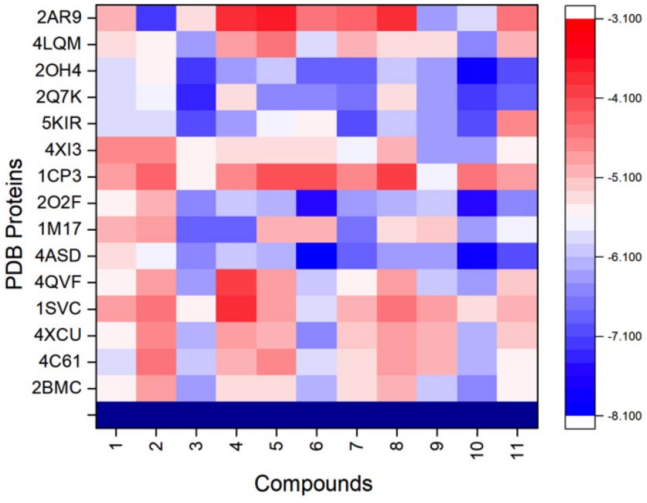

Fig. 6. Heatmap of molecular docking scores of ligands and target proteins. 1: 1,3,5-trimethyl benzene, 2: Nonanoic Acid, 3: 2,4-bis (1,1-dimethyl ethyl)-phenol,4: 5: Palmitic acid, 5: Ethyl ester-hexadecanoic acid, 6: Heptadeca-8,11-dien-1-yl, 9,12-7: octadecadien-1-ol, 8: 9-Octadecenal,9: 2-hydroxy-1 hexadecanoic acid,10: Z-1-phenyl-7-methyl-1,6-octadiene,11: 9,12-Octadecadienoic acid.

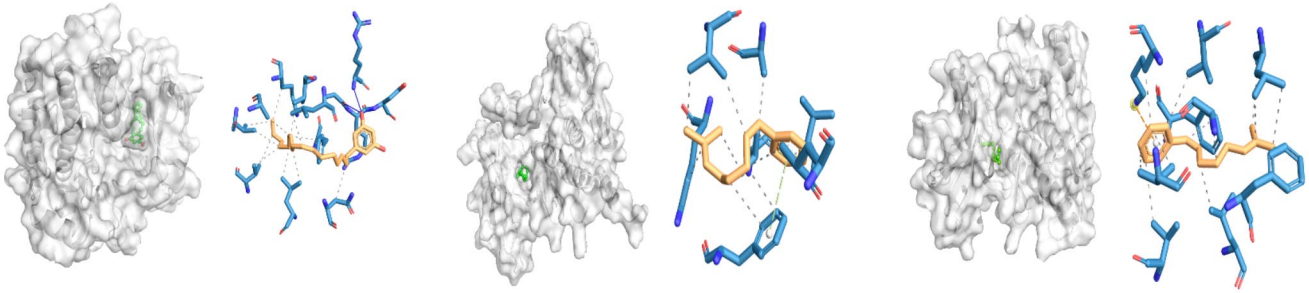

Fig. 7. In silico molecular docking of heptadeca-8,11-dien-1-yl and z-1-phenyl-7-methyl-1,6-octadiene compounds with the liver cancer target protein (4ASD and 2OH4) based on the binding energy generated by the AutoDock Vina 1.1.2. (A) A close-up view of the surface structure of 4ASD and 2OH4 with heptadeca-8,11-dien-1-yl and z-1-phenyl-7-methyl-1,6-octadiene binding at the active site. (B) 2D structure of heptadeca-8,11-dien-1-yl and z-1-phenyl-7-methyl-1,6-octadiene interacting with 4ASD and 2OH4 active site residues. - - - - - Hydrophobic interaction ▬ Hydrogen bond.

Table II. Lipinski's properties for compounds identified from fraction 1 of *A. rosea* using GC-MS and assessed by ADMIT web tool.

Sl. No	Compound name	RT	Area %	Molecular weight (<500kD)	Log P (<5)	H-bond donor (<5)	H-bond acceptor (<10)	Lipinski Rule
1	1,3,5-trimethyl benzene	5.84	14.6	120.090	3.587	0	0	Accepted
2	Nonanoic acid	8.79	18.4	158.130	3.366	2	1	Accepted
3	2,4-bis(1,1-dimethylethyl)-phenol	11.48	3.8	206.170	4.832	1	1	Accepted
4	Palmitic acid	15.22	9.2	256.240	6.732	1	2	Accepted
5	Ethyl ester-hexadecanoic acid	15.37	3.8	284.270	7.448	0	2	Accepted
6	Heptadeca-8,11-dien-1-yl	16.06	2.3	344.270	5.988	2	2	Accepted
7	9,12-octadecadien-1-ol	16.38	14.0	266.260	6.848	1	1	Accepted
8	9-octadecenal	17.00	3.2	266.260	6.665	0	1	Accepted
9	2-hydroxy-1 hexadecanoic acid	18.63	12.07	345.320	4.880	4	4	Accepted
10	Z-1-phenyl-7-methyl-1,6-octadiene	19.48	1.03	200.320	5.064	0	0	Accepted
11	9,12-octadecadienoic acid	19.67	17.6	280.240	6.652	1	2	Accepted

DISCUSSION

Despite significant advancements in basic research and clinical investigations, cancer remains the leading cause of death globally (Singh & Panda, 2005). The incidence of malignancies can be reduced through early detection and chemoprevention. Safe and effective chemotherapeutics must be developed because the side effects of existing chemotherapeutics lower the quality of patient life. Even though research has been done on natural therapies for cancer treatment, a complete and effective chemotherapeutic agent has yet to be discovered (Pucci *et al*., 2019; Sedighi *et al*., 2019).

The importance of plant extracts in cancer treatment lies in their potential to offer alternative and complementary therapeutic options. Many plant extracts have been found to contain bioactive compounds with anticancer properties, including the ability to inhibit cell proliferation, induce apoptosis, and prevent metastasis. These natural compounds may provide new treatment strategies with fewer side effects than conventional chemotherapy drugs while enhancing the efficacy of existing treatments (Lin *et al.*, 2020). Additionally, plant extracts may help overcome drug resistance, a significant challenge in cancer therapy, by targeting multiple signalling pathways and cellular processes (Ullah, 2008). This study investigated the effects of *A. rosea* extracts on cell cytotoxicity using MTT assay and cell and nuclear morphology. Cell morphology changes were studied to examine the mechanism of cell death brought on by fraction 1. Observing morphological changes in apoptotic cells is crucial for determining Apoptosis (Stewart, 1994). Fraction 1-treated Huh-7 cells revealed classic characteristics of apoptosis, including rounding of cells, shrinkage of cells, and condensation of cytoplasm, as demonstrated through microscopic study. These morphological changes in apoptotic cells have previously been documented (Wyllie *et al*., 1981; Ezhilarasan *et al*., 2019). Fluorescence microscopy also showed apoptotic effects of extracts, which were consistent with the morphological abnormalities. Hexane extract-treated cells revealed altered cellular morphology of Huh-7 cells, which was also observed by DAPI staining. Apoptotic cells have smaller nuclear areas and increased DAPI brightness due to chromatin condensation. A dual AO/EB staining assay further confirmed the apoptosis induction.

The metastasis of cancer cells to vital organs remains a critical obstacle in cancer treatment and is accountable for over 90 % of cancer-related deaths (Weinberg & Hanahan, 2000; Steeg, 2006). The successful targeting of highly metastatic cancer cells is required to develop chemotherapeutic anticancer agents. The investigation findings showed that the ARHS extract successfully prevented the migration of Huh-7 cells. This indicates that the inhibitory properties of ARHS extract on Huh-7 cell migration were likely attributed to its cytotoxicity. To validate the occurrence of oxidative stress induced by the ARHS fraction, we examined the production of reactive oxygen species (ROS) in Huh-7 cells. This is significant because apoptotic effects often correlate with oxidative stress (Martindale & Holbrook, 2002).

As shown in Figure 4, fraction 1 increased the intracellular ROS production in Huh-7 after 30 min of incubation with DCFH-DA. The findings of this study are consistent with earlier research that indicated that botanical extract treatment elevated the production of ROS in cancer cells. (Abhyankar *et al*., 2010; Isa *et al*., 2013; Kazberuk *et al*., 2022). According to several reports, evidence suggests that elevated levels of reactive oxygen species (ROS) can lead to apoptosis and increase the sensitivity of cancer cells to chemotherapy treatments (Shao *et al*., 2023).

Molecular docking is a robust computational technique that analyses interactions and binding energies between a therapeutic molecule and its target. This approach provides valuable insights into discovering new drugs and their potential efficacy (Tufail *et al*., 2021). Combining this in-silico method with in-vitro analysis offers a practical base for drug testing in experimental animals (Jan *et al*., 2020). The binding efficiency of different compounds with most liver cancer proteins investigated was promising. However, the current study reveals that among various liver cancer proteins, two compounds, heptadeca-8,11-dien-1-yl, and Z-1-phenyl-7-methyl-1,6-octadiene, demonstrated the best interaction with 2OH4 and 4ASD, with binding energies of -7.8 kcal/mol for 2OH4 and -8.1 kcal/mol for 4ASD, respectively. Protein-ligand interactions are crucial in identifying the potential mechanisms by which a ligand binds to its target and exerts its pharmacological effects (Thavamani *et al*., 2016). Heptadeca-8,11-dien-1-yl was found to bind with 4ASD with the lowest free energy compared to other liver cancer proteins. This suggests it may stimulate apoptotic proteins in liver cancer cells, acting as an effective anticancer agent. However, additional research is needed to clarify the interaction mechanisms between the compounds, the target proteins and the practicality of using these inhibitors *in vivo* and *in vitro*.

Adverse effects resulting from drug toxicity often contribute to the failure of drugs during the clinical trial stage (Sun *et al*., 2022). The compounds in the preparation had a good investigational new drug following Lipinski's rule of five. Lipinski's Rule of Five is a set of guidelines used in drug discovery to predict a compound's oral bioavailability. The criteria include molecular weight, hydrogen bond donors

and acceptors, and the octanol-water partition coefficient (Log P). These rules help researchers assess a molecule's potential to become a successful orally administered drug. If a ligand fails to fulfill these parameters, it will cause problems if ingested (Pollastri, 2010) .

CONCLUSIONS

The present study demonstrated that fraction 1 of *A. rosea* seeds induced apoptosis and inhibited cell migration in Huh-7 cells against liver cancer cell lines. Heptadeca-8,11-dien-1-yl and z-1-phenyl-7-methyl-1,6-octadiene displayed the highest docking scores for target proteins 4ASD and 2OH4. Our findings indicate that this plant may contribute to developing effective cancer therapies. Nevertheless, additional research is needed to evaluate the bioactivity of these compounds and conduct clinical trials to assess their effectiveness. These steps are crucial in advancing the development of new drug formulations.

ACKNOWLEDGMENTS. Researchers Supporting Project number (RSP2024R112), King Saud University, Riyadh, Saudi Arabia.

ABUTAHA, N.; WADAAN, M.A.; ALGHAMDI, R.A. y AL-MEKHLAFI, F.A. Actividad anticancerígena potencial de la *Alcea rosea* y acoplamiento molecular de proteínas diana seleccionadas en células de cáncer de hígado. *Int. J. Morphol., 42(6)*:1746-1754, 2024.

RESUMEN: La incidencia del cáncer está aumentando a nivel mundial. En este estudio, se examinaron los efectos citotóxicos del extracto de semilla de hexano (ARHS) de *Alcea rosea* L. contra las líneas celulares de cáncer humano de hígado (HuH-7) y mama (MDA-MB-231), así como contra la línea celular HUVEC no cancerosa. El extracto de ARHS mostró efectos citotóxicos dependientes de la dosis en las células HuH-7 y MDA-MB-231, con valores de CI50 respectivos de 2,39 y 5,67 mg/ml. Además, el extracto de ARHS impidió la migración de células HuH-7 de manera dependiente del tiempo. Las evaluaciones morfológicas mostraron características indicativas de apoptosis en las células tratadas. El extracto de ARHS indujo la producción de ROS en células HuH-7. Este estudio reveló que el extracto de hexano de *A. rosea* inhibió eficazmente el crecimiento de células HuH-7 al inducir la apoptosis, como lo evidencian los cambios en la morfología celular y nuclear observados mediante tinción con DAPI y AO/EBr. Heptadeca-8,11-dien-1-yl demostró el puntaje de acoplamiento más alto, formando tres enlaces de hidrógeno con los aminoácidos ARG-842A, ALA-1050A y ASP-1052A en el receptor 4ASD. A pesar de mostrar potencial como agente anticancerígeno, se requieren estudios adicionales de investigación para explorar la eficacia anticancerígena *in vivo*.

PALABRAS CLAVE: *Alcea rosea*; Citotoxicidad; Apoptosis; Acoplamiento molecular.

REFERENCES

Abhyankar, G.; Suprasanna, P.; Pandey, B.; Mishra, K.; Rao, K. & Reddy, V. Hairy root extract of *Phyllanthus amarus* induces apoptotic cell death in human breast cancer cells. *Innov. Food Sci. Emerg. Technol., 11(3)*:526-32, 2010.

Ahmadi, M.; Rad, A. K.; Rajaei, Z.; Mohammadian, N. & Tabasi, N. S. *Alcea rosea* root extract as a preventive and curative agent in ethylene glycol-induced urolithiasis in rats. *Indian J. Pharmacol., 44(3)*:304, 2012.

Alghamdi, I. G. & Alghamdi, M. S. The incidence rate of liver cancer in Saudi Arabia: an observational descriptive epidemiological analysis of data from the Saudi Cancer Registry (2004–2014). *Cancer Manag. Res., 12*:1101-11, 2020.

Anwanwan, D.; Singh, S. K.; Singh, S.; Saikam, V. & Singh, R. Challenges in liver cancer and possible treatment approaches. *Biochim. Biophys. Acta Rev. Cancer, 1873(1)*:188314, 2020.

Aung, T. N.; Qu, Z.; Kortschak, R. D. & Adelson, D. L. Understanding the effectiveness of natural compound mixtures in cancer through their molecular mode of action. *Int. J. Mol. Sci., 18(3)*:656, 2017.

Balogh, J.; Victor 3rd, D.; Asham, E. H.; Burroughs, S. G.; Boktour, M.; Saharia, A.; Li, X.; Ghobrial, R. M. & Monsour Jr., H. P. Hepatocellular carcinoma: a review. *J. Hepatocell. Carcinoma, 3*:41-53, 2016.

Bharathi, A.; Roopan, S. M.; Vasavi, C. S.; Munusami, P.; Gayathri, G. A. & Gayathri, M. In silico molecular docking and *in vitro* antidiabetic studies of dihydropyrimido[4,5-a]acridin-2-amines. *Biomed Res. Int., 2014*:971569, 2014.

Choene, M. & Motadi, L. Validation of the antiproliferative effects of *Euphorbia tirucalli* extracts in breast cancer cell lines. *Mol. Biol. (Mosk.), 50(1)*:98-110, 2016.

Ezhilarasan, D.; Apoorva, V. S. & Ashok Vardhan, N. *Syzygium cumini* extract induced reactive oxygen species-mediated apoptosis in human oral squamous carcinoma cells. *J. Oral Pathol. Med., 48(2)*:115-21, 2019.

Fahamiya, N.; Shiffa, M. & Aslam, M. A comprehensive review on *Althaea rosea* Linn. *J. Pharm. Res., 6(11)*:6888-94. 2016.

Isa, N. M.; Abdul, A. B.; Abdelwahab, S. I.; Abdullah, R.; Sukari, M. A.; Kamalidehghan, B.; Hadi, A. H. A. & Mohan, S. Boesenbergin A, a chalcone from *Boesenbergia rotunda* induces apoptosis via mitochondrial dysregulation and cytochrome c release in A549 cells *in vitro*: Involvement of HSP70 and Bcl2/Bax signalling pathways. *J. Funct. Foods, 5(1)*:87-97, 2013.

Jan, M. S.; Ahmad, S.; Hussain, F.; Ahmad, A.; Mahmood, F.; Rashid, U.; Abid, O. U.; Ullah, F.; Ayaz, M. & Sadiq, A. Design, synthesis, in-vitro, *in-vivo* and *in-silico* studies of pyrrolidine-2,5-dione derivatives as multitarget anti-inflammatory agents. *Eur. J. Med. Chem., 186*:111863, 2020.

Kazberuk, A.; Chalecka, M.; Palka, J. & Surazynski, A. Nonsteroidal anti-inflammatory drugs as PPARg agonists can induce PRODH/POX-dependent apoptosis in breast cancer cells: new alternative pathway in NSAID-induced apoptosis. *Int. J. Mol. Sci., 23(3)*:1510, 2022.

Lee, K. & Kim, D. *In-silico* molecular binding prediction for human drug targets using deep neural multi-task learning. Genes, 10(11):906, 2019.

Lin, S. R.; Chang, C. H.; Hsu, C. F.; Tsai, M. J.; Cheng, H.; Leong, M. K.; Sung, P. J.; Chen, J. C. & Weng, C. F. Natural compounds as potential adjuvants to cancer therapy: Preclinical evidence. *Br. J. Pharmacol., 177(6)*:1409-23, 2020.

Llovet, J. M.; Kelley, R. K.; Villanueva, A.; Singal, A. G.; Pikarsky, E.; Roayaie, S.; Lencioni, R.; Koike, K.; Zucman-Rossi, J. & Finn, R. S. Hepatocellular carcinoma. *Nat. Rev. Dis. Primers, 7(1)*:6, 2021.

Loza-Mejía, M. A.; Salazar, J. R. & Sánchez-Tejeda, J. F. *In Silico* studies on compounds derived from Calceolaria: Phenylethanoid glycosides as potential multitarget inhibitors for the development of pesticides. *Biomolecules, 8(4)*:121, 2018.

Martindale, J. L. & Holbrook, N. J. Cellular response to oxidative stress: signaling for suicide and survival. *J. Cell. Physiol., 192(1)*:1-15, 2002.

Motadi, L.; Misso, N.; Dlamini, Z. & Bhoola, K. Molecular genetics and mechanisms of apoptosis in carcinomas of the lung and pleura: therapeutic targets. *Int. Immunopharmacol., 7(14)*:1934-47, 2007.

Pollastri, M. P. Overview on the Rule of Five. *Curr. Protoc. Pharmacol., Chapter 9*:Unit 9.12, 2010.

Pucci, C.; Martinelli, C. & Ciofani, G. Innovative approaches for cancer treatment: current perspectives and new challenges. *Ecancermedicalscience, 13*:961, 2019.

Reed, J. C. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat. Clin. Pract. Oncol., 3(7)*:388-98, 2006.

Sadiq, A Z. A.; Ullah, F. & Ayaz, S. A. M. Phytochemical and toxicological investigations of crude methanolic extracts, subsequent fractions and crude saponins of *Isodon rugosus*. *Biol. Res., 47(1)*:1-6, 2014.

Satapute, P.; Paidi, M. K.; Kurjogi, M. & Jogaiah, S. Physiological adaptation and spectral annotation of Arsenic and Cadmium heavy metal-resistant and susceptible strain *Pseudomonas taiwanensis*. *Environ. Pollut., 251*:555-63, 2019.

Sedighi, M.; Zahedi Bialvaei, A.; Hamblin, M. R.; Ohadi, E.; Asadi, A.; Halajzadeh, M.; Lohrasbi, V.; Mohammadzadeh, N.; Amiriani, T.; Krutova, M.; *et al*. Therapeutic bacteria to combat cancer; current advances, challenges, and opportunities. *Cancer Med., 8(6)*:3167-81, 2019.

Seyyednejad, S. M.; Koochak, H.; Darabpour, E. & Motamedi, H. A survey on Hibiscus rosa—sinensis, *Alcea rosea* L. and *Malva neglecta* Wallr as antibacterial agents. *Asian Pac. J. Trop. Med., 3(5)*:351-5, 2010.

Shao, W.; Wang, X.; Liu, Z.; Song, X.; Wang, F.; Liu, X. & Yu, Z. Cyperotundone combined with adriamycin induces apoptosis in MCF-7 and MCF-7/ADR cancer cells by ROS generation and NRF2/ARE signaling pathway. *Sci. Rep., 13(1)*:1384, 2023.

Singh, M. P. & Panda, H. *Medicinal Herbs with their Formulations.* Delhi, Daya Publishing House, 2005. pp.97-408.

Sliwoski, G.; Kothiwale, S.; Meiler, J. & Lowe, E. W. Computational methods in drug discovery. *Pharmacol. Rev., 66(1)*:334-95, 2014.

Steeg, P. S. Tumor metastasis: mechanistic insights and clinical challenges. *Nat. Med. 12(8)*:895-904, 2006.

Stewart, B. W. Mechanisms of apoptosis: integration of genetic, biochemical, and cellular indicators. *J. Natl. Cancer Inst., 86(17)*:1286-96, 1994.

Sun, D.; Gao, W.; Hu, H. & Zhou, S. Why 90 % of clinical drug development fails and how to improve it? *Acta Pharm. Sin. B, 12(7)*:3049-3062, 2022.

Thavamani, B. S.; Mathew, M. & Dhanabal, S. P. *Cocculus hirsutus*: Molecular docking to identify suitable targets for hepatocellular carcinoma by in silico technique. *Pharmacogn. Mag., 12(Suppl. 3)*:S350-2, 2016.

Tuba, M.; Fafal, T.; Kivçak, B. & Öztürk, H. T. Antimicrobial and cytotoxic activities of the extracts obtained from the flowers of *Alcea osea* L. *Hacettepe Univ. J. Fac. Pharm., (1)*:17-24, 2010.

Tufail, M. B.; Javed, M. A.; Ikram, M.; Mahnashi, M. H.; Alyami, B. A.; Alqahtani, Y. S.; Sadiq, A. & Rashid, U. Synthesis, pharmacological evaluation and Molecular modelling studies of pregnenolone derivatives as inhibitors of human dihydrofolate reductase. *Steroids, 168*:108801, 2021.

Ullah, M. F. Cancer multidrug resistance (MDR): a major impediment to effective chemotherapy. *Asian Pac. J. Cancer Prev., 9(1)*:1-6, 2008.

Villanueva, A. Hepatocellular carcinoma. *N. Engl. J. Med., 380(15)*:1450-62, 2019.

Wang, D. F.; Shang, J. Y. & Yu, Q. H. Analgesic and anti-inflammatory effects of the flower of *Althaea rosea* (L.) *Cav. Zhongguo Zhong Yao Za Zhi, 14(1)*:46-8, 64, 1989.

Weinberg, R. & Hanahan, D. The hallmarks of cancer. *Cell, 100(1)*:57-70, 2000.

Wyllie, A.; Beattie, G. & Hargreaves, A. Chromatin changes in apoptosis. *Histochem. J., 13(4)*:681-92, 1981.

Yang, G. Y.; Liao, J.; Kim, K.; Yurkow, E. J. & Yang, C. S. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis, 19(4)*:611-6, 1998

Corresponding author:
Nael Abutaha
Department of Zoology
College of Science
King Saud University
PO Box 2455
Riyadh 11451
SAUDI ARABIA

E-mail: nabutaha@ksu.edu.sa