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INVESTIGATION OF ANTICANCER MECHANISMS OF A CHALCONE/XANTHINE HYBRID IN NON-SMALL CELL LUNG CANCER

Omar Mahmoud Elrehany[1*], Shaymaa Faysal[2], Hesham A. Abou-Zieda[3], Alaa M. Hayallah[4], Mohamed Abdel-Azize[5], Maiiada Hasan Nazmy[1]

[1]*Biochemistry Department, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt*
[2]*Pharmacology Department, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt*
[3]*Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Deraya University, Minia, Egypt, Universities Zone, New Minia City 61111, Egypt*
[4]*Pharmaceutical Organic Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut 71526, Assiut Egypt. Pharmaceutical Chemistry Department Faculty of pharmacy, Sphinx University, New Assiut, Egypt*
[5]*Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt*

Despite major advances in therapy, Lung cancer remains the major cause of death among cancer cases. Chalcone/xanthine Hybrid is a synthetic xanthine derivative that was found to exert an anticancer action in various cancer types. Thus, the current study sought to discover if the chalcone/xanthine hybrid influences the etiology of non-small cell lung cancer (NSCLC). We found that the chalcone/xanthine hybrid inhibited cell cycle progression while inducing apoptosis and causing arrest of the cell cycle in HOP-92 cells. Flow cytometry demonstrated that chalcone/xanthine hybrid strongly induced apoptosis in HOP-92 cells, as well as alterations in apoptosis-related protein expression, including an increase in Bax, caspase3, caspase8, caspase-9, P53 expression, as well as a decrease in Bcl-2 expression.

Moreover, the chalcone/xanthine hybrid slowed cell cycle progression from the G1 phase to the S phase. Furthermore, the ratio of phosphorylated proteins (AKT, MEK, ERK½, and P38) to total protein quantity reduced, indicating regulation of downstream protein signaling pathways governing cell cycle growth. Overall, this chalcone/xanthine hybrid may be considered a therapeutic option for NSCLC via apoptosis induction.

Keywords: *NSCLC (non-small cell lung cancer), HOP-92 (Hopkins-92), MAPK (mitogen-activated protein kinase)*

INTRODUCTION

Cancer of the lung is a common kind of cancer, constituting about 11.6% of all cases in 2018. It's also one of the principal causes of cancer-related deaths, accounting for 18.4% of all lung-related deaths. NSCLC and SCLC are the two most frequent kinds of lung cancer.

NSCLC registered about 85% of lung cancer cases[1,2]. The early discovery of lung cancer is becoming more common each year, while death rates are decreasing, thanks to new and precise detection and diagnosis procedures. However, in less developed nations, lung cancer is often classified as either progressed or locally advanced. In 2020, Asia accounted for more than 50% of lung cancer cases and around 61.9% of deaths, with an age-standardized incidence rate (ASIR) of 22.9 cases and an age-standardized mortality rate (ASMR) of 32.7 cases between 100,000. However, in Europe, cases decreased to 21.6%

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*Corresponding author: Omar Mahmoud Elrehany, E-mail: o_elrehany@yahoo.com

and 21.3% of deaths. The ASIR in Europe is about 29.4 per 100,000 and the ASMR is 22.6 per 100,000. In Africa, ASIR is the lowest (6.2 per 100,000) and ASMR is 5.6 per 100,000[3].

In 2020, there were about 2.2 million diagnosed new lung cancer cases and 1.8 million lung cancer-related deaths. ASIRs ratio differs between nations, In Denmark, it reached 36.8 cases between 100,000 while in Mexico it registered 5.9 cases per 100,000. ASMR levels in Poland showed 32.8 cases/100,000 while in Mexico it reached 4.9 cases/100,000. Both ASIR and ASMR in men are two fold greater than in women.[3] Thus, the development and identification of new candidate agents for managing lung cancer therapy is critical.

Chalcones (1,3-diphenyl-2-propen-1-one) the precursors involved in the production of flavonoids in plants, are naturally occurring chemicals that display a diverse set of biological functions, including anticancer action. Chalcone contains α and β-unsaturated ketones, which have anti-inflammatory, antimalarial, antibacterial, and anti-cancer properties[4-7].

Caffeine and theobromine are examples of methylxanthine derivatives that have been shown to promote doxorubicin-induced cytotoxicity by inducing apoptosis[8]. It is thought that methylxanthine's apoptogenic activity is mediated by a decrease in intracellular levels of the antiapoptotic mediator Bcl-2. As a result, methylxanthine-based compounds provide an appealing foundation for structural alteration in the pursuit of new anticancer medicines with multimodal pharmacodynamics.[9]

The usage of hybrid molecules is one of the useful strategies to increase the efficacy of anticancer drugs and diminish treatment resistance. There are multiple mechanisms proposed for the chemotherapeutic action of chalcone derivatives, including angiogenesis suppression, anti-proliferative activity, and correction of multidrug resistance, antimitotic action, and apoptotic activation in a range of cancer cell lines. Their anti-proliferative action may be achieved by arrest of the cell cycle, stability of p53, or regulation of numerous signaling pathways involved in cell survival or death[10]. Some studies demonstrated that chalcone hybridization with biologically active pharmacophores gives new hybrids that have synergistic biological activity. The purpose of the current study was to assess the antineoplastic mechanism of a new chalcone/xanthine hybrid by evaluating its effect on apoptosis and proliferation of human lung adenocarcinoma[11] Mitogen-activated protein kinases (MAPKs) are examples of serine and threonine protein kinases that are abundant conserved in eukaryotes and are engaged in signal transduction pathways that control physiological and pathological cell responses. There are several processes involved in the reduction of NSCLC cell growth including P38/MAPK and ERK ½ signaling pathways that promote inflammatory responses, oxidative stress, cell aging, apoptosis, and autophagy[12-15].

The present study investigated the effect of the chalcone/xanthine hybrid on certain signaling proteins responsible for apoptosis regulation and cell cycle progression. Furthermore, the mRNA expression of Bcl-2, Bax, P53, and caspases 3, 8, and 9 which play important roles in apoptosis were also evaluated.

MATERIALS AND METHODS

The most applicable approach for the synthesis of chalcone **(fig. 1a)** is the Claisen-Schmidt condensation of substituted ketones with substituted aldehydes in the presence of different condensing agents, such as alkali and acids as shown in **Fig. 2a**[16-18]

Purine is composed of a pyrimidine ring and an imidazole ring[19]**.** There are a number of alkyl xanthine derivatives in nature, including caffeine (1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione) II[20], and theobromine (3,7-dimethyl-1H-purine-2,6(3H,7H)-dione) III[21]**.** The synthesis of certain xanthine derivatives has already been published in the literature from substituted urea **Fig. 2b**[22]**.**

Using the Scheme in **Fig. 2c**, the desired chalcone/xanthine hybrid molecule was synthesized through our previous work[23]**.** The chalcone 3 was produced by a 4-aminoacetophenone Claisen-Schmidt condensation with substituted benzaldehyde derivative 2 catalyzed by a base. Acetylated chalcone 4 was obtained by stirring chalcone 3 in a biphasic layer of potassium carbonate solution in dichloromethane with

bromoacetylbromide. Alkylation of xanthine 1 with acylated chalcone 4 results in a high yield of the desired chalcone/xanthine hybrid **5**.

Reagents and reaction conditions

a) NaOH 60%; b) $BrCH_2COBr$/ CH_2Cl_2; c) TEA/ acetonitrile.

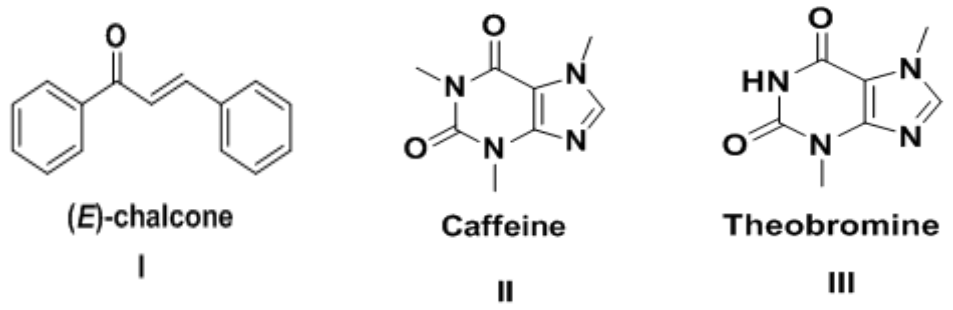

Fig.1: Chemistry of chalcone**.** (a) Structure of chalcone [(E)-1,3-diphenyl-3prop-2-en-1-one], (b) naturally occurring alkyl xanthine derivatives caffeine and (c) theobromine.

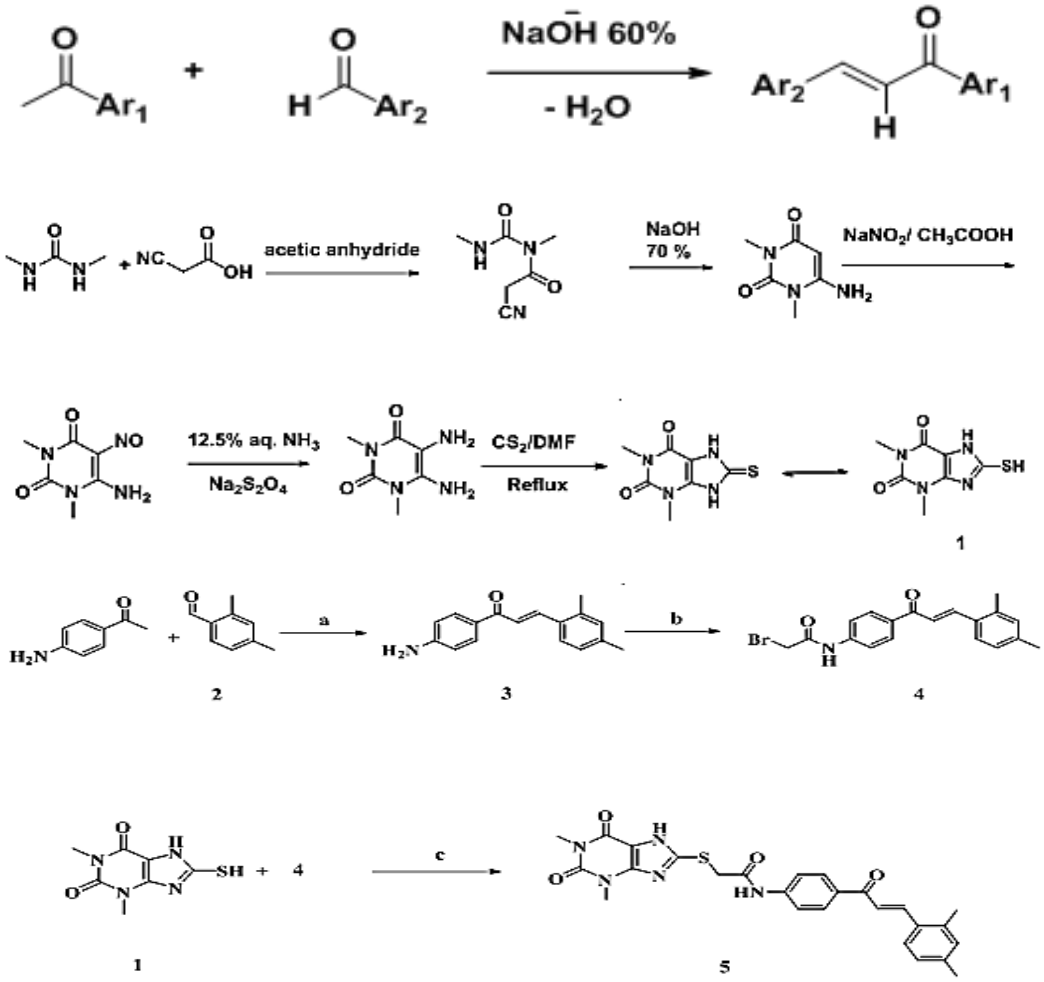

Fig.2: (a) The synthesis of chalcone derivatives under basic condition, (b) synthesis of 1,3,8-trisubstituted xanthine **1** derivatives from substituted urea and (c) synthesis of target chalcone/xanthine hybrid **5.**

Preparation of cell lines for culture

HOP-92 is one of the NCI-60 cell lines that represent several cancer types and is commonly used for drug screening and molecular target discovery. The Hopkins-92 human cell line (HOP-92 cell line) is a Human NSCLC Adenocarcinoma commonly used for drug screening and molecular target identification. The cell line used in the current study was obtained from the American Type Culture Collection. Invitrogen/Life Technologies provided DMEM cells supplemented with 10% FBS (Hyclone), 10 µg/ml insulin (Sigma), and 1% penicillin-streptomycin. Sigma and Invitrogen supplied the remaining chemicals and reagents.

Growth media was removed; cells were washed with warm PBS and aspirated. Adhered cells were detached from the media by the addition of 5 ml 10% trypsin/EDTA and incubated for 2 minutes in the incubator. The suspended cells were then harvested, aspirated into a falcon tube, and pelleted at 2000 rpm for 5 minutes. Cell count was performed after suspending the cells into a fresh media and staining with trypan blue, counted, and used for the preparation of the appropriate number of cells[24].

MTT assay

Sigma Saint Louis, Missouri 63103 USA in vitro toxicity test kit developed for assessing Spectrophotometric analysis related to number of viable cells as a function of mitochondrial activity. Generally, the MTT assay method is used to determine the drug concentration which exhibited 50% cell viability (IC50). For accurate results, the IC50 of the chalcone/xanthine hybrid determined by this method was repeated 3 times and then used in each subsequent experiment along the study. Before the MTT assay, the last detached cells were plated in a multiwell plate at a density of (1×10^5/ml) in a volume of 100 µl complete growth media. Add 100 µl of different concentrations of chalcone/xanthine hybrid compound or the standard control staurosporine (0, 0.4, 1.6, 6.3, 25, and 100 µg/ml) per well. Cells were grown in the multiwell plate for 24 hours and incubated at 37˚C with 5% CO_2[24]. To each well, about 20 µl of MTT solution (5 mg/ml dissolved in PBS) was added and then incubated for two hours. The medium was then aspirated and the crystals of purple formazan were dissolved by the addition of 200 µl of DMSO and then shaking. The OD was measured at λ570 nm using a micromultiplate reader. Any change in viable cell number affects the quantity of formazan produced which reflects the extent of cytotoxicity induced by the test substance[25]. The standard control, staurosporine, used in this experiment is a natural alkaloid isolated from Streptomyces staurosporeus, have a cytotoxic effect against many tumor cell lines[26]. The IC50 of the chalcone/xanthine hybrid determined by this method (6.57 µg/ml) was used along the study for cell line treatment in all experiments.

Analysis of cell cycle

Propidium Iodide Flow Cytometry Kit (ab139418) from Abcam used for cell cycle measurement is developed to analyze the content of DNA in cells by using the nucleic acid stain propidium iodide (PI), prepared for flow cytometry analysis.

Adherent cells were collected after detachment from the media with trypsin. Cell pellets were washed using PBS and then fixed in ice-cold 66% ethanol. The cell pellet was gently suspended in 400 µl ice-cold 1X PBS. Ice-cold 100% ethanol (800 µl) was slowly added, mixed well, and incubated at +4ºC for 2 hours. The fixed cells were stored ready for flow cytometry analysis for at least 4 weeks. The previously prepared cells were washed with PBS, centrifuged, pelleted again, and gently suspended by the addition of 200 µl Propidium Iodide 1x solution and RNase staining solution. Cells were then incubated in the dark at 37ºC for 30 minutes and then stored on ice ready for flow cytometry analysis. Settled cells were gently suspended to remove aggregates and then samples were run on a flow cytometer; set FSC vs. SSC gates to exclude aggregates and cell debris. collect propidium iodide fluorescence in FL2 using laser illumination at 488 nm.

Apoptosis detection

Apoptosis Detection Kit Annexin V-FITC (Catalog #: K101-25), Bio-Vision Research Products, 980 Linda Vista Avenue, Mountain View, CA 94043 USA is based on the finding that Phosphatidylserine (PS) is transported

across the membrane by cells from the plasma membrane's inner face to the cell surface shortly after apoptosis initiation. PS is easily identified on the cell surface by labeling with a fluorescent compound of Annexin V, a protein with a strong affinity for PS. When using both Annexin V-FITC and PI staining, the kit can differentiate between apoptosis and necrosis. Thus, early apoptotic cells possess intact plasma membranes and expose phosphatidylserine (PS) on the cell surface to enable its detection by phagocytes expressing Annexin-V but not PI. Late apoptotic cells express both Annexin-V and PI**.** According to the protocol of the kit, pelleted cells (about $5x10^5$) suspended in about 0.5 ml of 1x binding buffer, then 5 µl of propidium iodide (50 mg/ml) and Annexin V-FITC were added and incubated for 5 minutes in the dark at room temperature. Annexin V-FITC binding was measured using flow cytometry (Ex = 488 nm; Em = 530 nm) with a FITC signal detector (typically FL1) and PI staining with a phycoerythrin emission signal detector (usually FL2).

Western blot analysis

Western Blot Detection Kit (Elabscience Biotechnology Inc. Catalog No. E-IR-R304A) was used to determine the phosphorylation of proteins related to the apoptotic pathway. RIPA lysis solution with a protease inhibitor (E-BC-R327) was used to produce HOP-92 cell lysates. SDS-PAGE electrophoresis technique was used for protein sample separation after the determination of the total protein content of samples using a BCA Protein assay kit (E-BC-K318). Proteins were applied onto a 12% SDS-PAGE gel before being transferred to a PVDF membrane (20-30 µg/lane) (E-BC-R266). The membrane was blocked by soaking with 3% BSA for 1.5 hours at room temperature before washing with tris borate sodium buffer containing 0.1% Tween-20. The membrane was then treated with the appropriate primary antibody separately for each protein overnight at 4°C before rinsing three times with TBST. The main antibodies used were bought from Cell Signaling Technology; AKT (#9272), p-AKT (#9271), MEK (#9122), p-MEK (#9121), ERK (#9102), p-ERK (#9101), P38 (#9212), and p-P38 (#9211). β-actin was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA (#sc-1615). After washing the membrane for 1 hour, it was treated with the goat anti-rabbit IgG-HRP conjugated 2^{ry} antibody (1: 2,000; E-AB-1003). The Excellent Chemiluminescent Substrate Detection Kit (E-BC-R347) was used to detect bands. The bands were quantified using the automated developer (ChemiDocXRS imaging system).

Total RNA extraction and real-time PCR test

Total RNA was extracted following cultivated cell homogenization using the TRIzol total RNA extraction kit (Invitrogen, CA, USA) according to the kit protocol. At A260 nm, the total RNA concentration was calculated, and purity was determined using the A260/A280 ratio. Purity ≥ 1.7 for samples were satisfied for qRT-PCR. DNase I (Invitrogen) was used to get rid of DNA impurities and the cDNA synthesis was prepared from 1 µg of total RNA by using a cDNA Synthesis Kit (Thermo Science Fermentas, St. Leon-Ro, Germany, #K1632). Quantitative Real-time was used to investigate the expression of BAX, caspases (3, 8, 9), P53, and Bcl-2, as well as the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as an internal reference[27]**.**

Table (1) shows the primer sequences collected from the National Center for Biotechnology Information (NCBI). Real-time PCR was used to quantify mRNA in triplicate for each sample. The qPCR reactions were carried out by using SYBR Green (Thermo Scientific Fermentas St. Leon-Ro, Germany-Maxima, #K0251) master mix and then detected by Applied Biosystems StepOne Real-Time PCR Detection System.

After normalization to GAPDH, gene expression levels were evaluated using the housekeeping gene. The comparative cycle threshold (Ct) was calculated for each sample to determine the relative amount of the products, and the average Ct was calculated. The relative expression was calculated using the formula $2^{(-\Delta\Delta Ct)}$. A melting curve analysis was done using the Rotor-Gene 6000 Series Software 1.7 and the SYBR Green fluorescent dye between 60-95 °C at 1 °C intervals to avoid contamination, characterize the resultant amplified combination, and eliminate non-specific compounds[28].

Table 1: Sequences of the primers obtained from NCBI.

Primer	*Accession No.*	*Primer sequence*
BAX	*NM_138761.4*	*Forward 5'-CAGATCATGAAGACAGGGGC-3'* *Reverse 5'-CCCGGAGGAAGTCCAATGTC-3'*
caspase 3	*NM_004346.4*	*Forward 5'-CTAGCGGATGGGTGCTATTGT-3'* *Reverse 5'-AGAATGGGGGAAGAGGCAGG-3'*
Caspase 8	*NM_001228.4*	*Forward 5'-AGCCCTTGAGTTGGTCACTT-3'* *Reverse 5'-CAGAAGTGGAACCTGTAGGCA-3'*
Caspase 9	*NM_001229.5*	*Forward 5'-TCAGGCCCCATATGATCGAG-3'* *Reverse 5'-CAAGAGCACCGACATCACCA-3'*
P53	*NM_001276761.3*	*Forward 5'-AAGTCTAGAGCCACCGTCCA-3'* *Reverse 5'-CTGGCATTCTGGGAGCTTCA-3'*
Bcl-2	*NM_000633.3*	*Forward 5'-AAAAATACAACATCACAGAGGAAGT-3'* *Reverse 5'-TCCCGGTTATCGTACCCTGT-3'*

Table 2: Relative band intensities of different proteins and their phosphorylated forms normalized to β-actin.

	p-AKT	*AKT*	*p-MEK*	*MEK*	*p-ERK1*	*ERK1*	*p-ERK2*	*ERK2*	*p-P38*	*P38*
Untreated	*0.732*	*0.641*	*0.734*	*0.726*	*0.791*	*0.673*	*0.725*	*0.691*	*0.702*	*0.751*
Ratio ph/T	*1.142*		*1.01*		*1.17*		*1.04*		*0.935*	
Treated	*0.314*	*0.392*	*0.19*	*0.24*	*0.231*	*0.356*	*0.261*	*0.313*	*0.175*	*0.225*
Ratio ph/T	*0.80*		*0.789*		*0.64*		*0.8338*		*0.778*	

Statistical analysis

The data was represented by the mean ± SD calculated from experiments that were repeated at least three times independently. A t-test was employed for the detection of any statistically significant differences between the control and treatment groups. A statistically significant difference was considered at *P < 0.05.

RESULTS AND DISCUSSION

Results

Cytotoxicity of Chalcone/xanthine hybrid

The National Cancer Institute (NCI) tested the chalcone/xanthine hybrid molecule against 60 human tumor cell lines *in vitro* to explore its anticancer efficacy. Compounds that inhibited the proliferation of any tested cell line by 32% or less were considered active. Our group tested previously the chalcone/xanthine hybrid effect on cell line growth inhibition. NCI report revealed that the HOP-92 cell line is one of the most affected cell lines, with a -35% growth inhibition[23]**.**

The cytotoxicity of the Chalcone/xanthine hybrid on the HOP-92 cell line was conducted using the MTT test. The *in-vitro* results showed that the IC50 of chalcone/xanthine hybrid was 6.57 µg/ml and the reduced HOP-92 cell growth was dose-dependent **(Fig. 3a)**; whereas the standard control used, staurosporine, demonstrated an IC50 of 2.09 µg/ml **(Fig. 3b).**

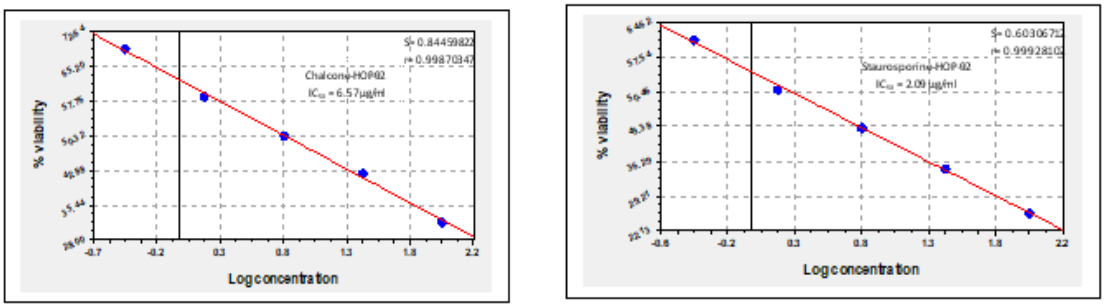

Fig.3: (a) Survival of the lung cancer cell line HOP-92 following treatment with chalcone/xanthine hybrid showed IC50 = 6.57 µg/ml and (b) after treatment with standard control; staurosporine with IC50 = 2.09 µg/ml. Cell survival is given as a proportion of untreated cells.

Effect of chalcone/xanthine hybrid on cell apoptosis

Annexin V-FITC/PI staining was utilized to evaluate the proportion of apoptotic cells. Apoptosis was triggered in HOP-92 cells by Chalcone/xanthine hybrid treatment **(Fig. 4b)**. When compared to untreated cells **(Fig. 4a)**, the percentages of cell apoptosis were elevated significantly ($p < 0.05$) to 9.86 for early apoptosis and 18.45 for late apoptosis **(Fig. 4c)**.

Apoptosis was also studied by evaluating the expression of apoptotic markers with qPCR. Following chalcone/xanthine hybrid treatment, the expression of apoptotic markers BAX, caspases (3, 8, 9), P53, and Bcl-2 were altered **(fig. 5)**. When compared to untreated cells, the expression of BAX, caspases 3, 8, and 9, and P53 increased by 2.83±0.042, 2.4±0.018, 1.9±0.025, 1.88±0.031, and 3.92±0.034 fold. Bcl-2 mRNA expression, on the other hand, was reduced by 0.8±0.022 fold after treatment with the chalcone/xanthine hybrid. Additionally, gene expression analysis revealed that treating HOP-92 cells raised the ratio of BAX/Bcl-2, indicating an increase in cell death.

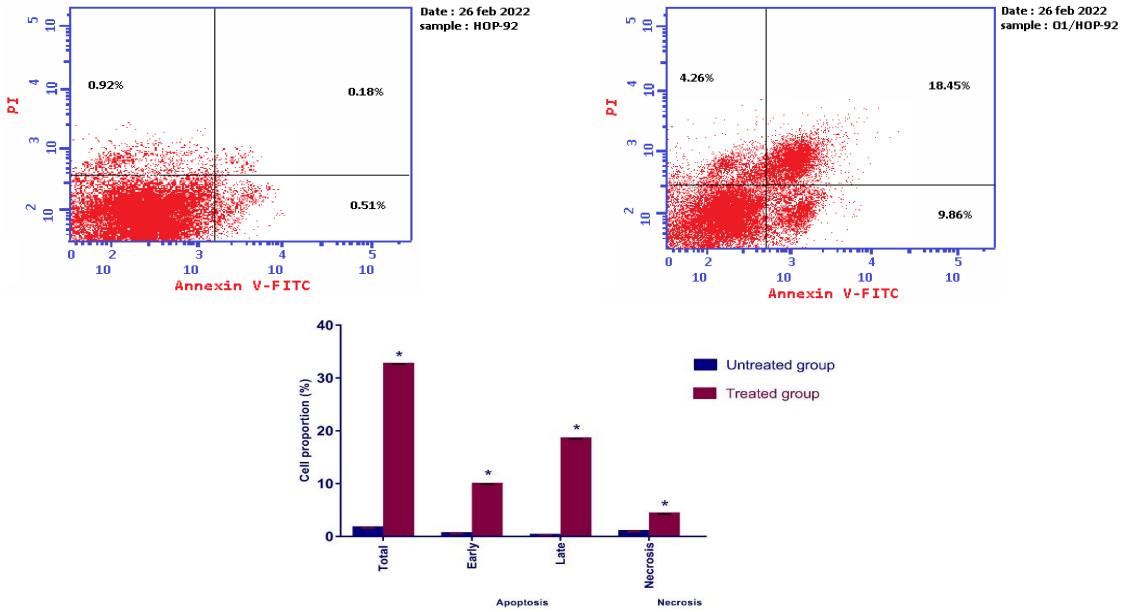

Fig.4: Flow cytometric dot plot of HOP-92. (a) Untreated cells, (b) chalcone/xanthine hybrid-treated cells and (c) a bar graph showing the percentage of early, late apoptotic, and necrotic cells. Low Left (LL) viable cells, Low Right (LR) early apoptotic cells, Upper Left (UL) necrotic cells, and Upper Right (UR) late apoptotic cells. The bars represent the mean ± SD. A t-test was applied to determine statistically significant important differentiation between the treated and untreated groups, with *p value < 0.05 compared to the untreated group.

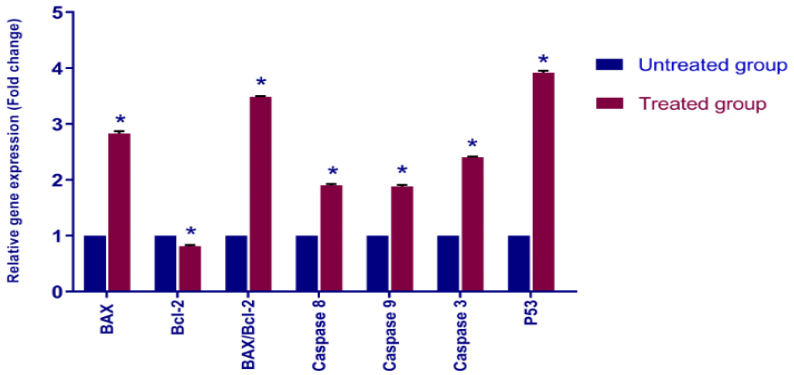

Fig.5: BAX, Bcl-2, and the BAX/Bcl-2 ratio, caspases (8, 9, 3) and P53 gene expression. Normalized relative expression calculated to the internal control GAPDH. The bars represent the mean ± SD. A t-test was applied to identify statistically significant results at * $p < 0.05$ compared to the untreated group.

Cell cycle analysis

To examine cell cycle progression, PI labeling was conducted, followed by flow cytometry in response to malignant lung cancer cells (HOP-92) exposure to 6.57 µg/ml chalcone/xanthine hybrid. According to our findings, the Chalcone/xanthine hybrid can dramatically induce cell cycle arrest by slowing G1/S cell cycle progression **(fig. 6b).** This is clear when comparing the G2/M phase after treatment (3.4%) to the same value before treatment (13.05%). Also, the G1 phase increased after treatment from 53.27% to 56.34% and the S phase from 33.68% **(fig. 6a)** to 39.66% **(fig. 6b)**. Moreover, the bar graph **(fig. 6c)** shows decreased value of the percentage of cell cycle rate in G2/M phase in the treated group. Our findings confirmed the anti-neoplastic action of the Chalcone/xanthine hybrid and offered important information for its role in the regulation of the proliferation of HOP-92 cells.

Western blotting

The MAPK signaling pathway includes the classic circuits ERK½ and p38/MAPK. The effect of the chalcone/xanthine hybrid on protein kinase activation was studied using western blot. Immunoblots of AKT, ERK ½, P38, and MEK, as well as their phosphorylated counterparts, in comparison to β -actin is illustrated **(fig. 7a)**. Untreated cells demonstrated a higher level of phosphorylation of the investigated proteins than treated cells for AKT (0.732±0.01), MEK (0.734±0.01), ERK-1 (0.691±0.011), ERK-2 (0.725±0.01) and P38 (0.702±0.11) as illustrated in **Fig (7b)**. In HOP-92 lung cancer cells, chalcone/xanthine hybrid reduced significantly *(P<0.001)* phosphorylation of AKT (0.314±0.14), MEK (0.19±0.01), ERK-1 (0.331±0.012), ERK-2 (0.261±0.013) and P38 (0.181±0.01) **(Fig. 7b)**.

Furthermore, the relative ratio of phospho-proteins to the total amount of proteins showed decreased values in treated cells **(Fig, 7c)**. P-AKT/Total protein decreased from (1.142±0.01) for untreated cells to (0.82±0.016) after treatment with a chalcone/xanthine hybrid. The other markers demonstrate the same results. MEK, ERK-1, ERK-2 and P38 decreased significantly from (1.01±0.01), (1.175±0.011), (1.03±0.01) and (0.937±0.011) to (0.79±0.01), (0.648±0.02), (0.836±0.018) and (0.778±0.01) after treatment with chalcone/xanthine hybrid *(P<0.001).*

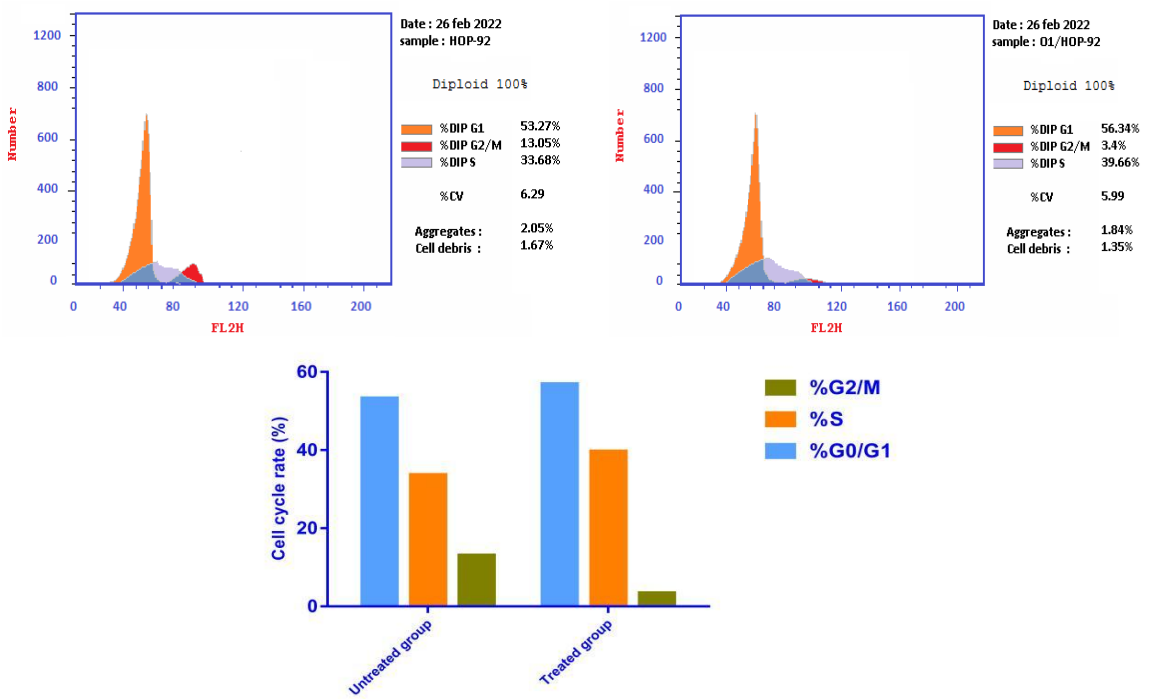

Fig.6: (a) Cell cycle progression study of HOP-92 cells before treatment and (b) following 6.57 µg/ml chalcone/xanthine hybrid treatment, (c) bar graph revealed cell cycle arrest at the G1/S phase. A t-test was used to determine whether there was a statistically significant difference.

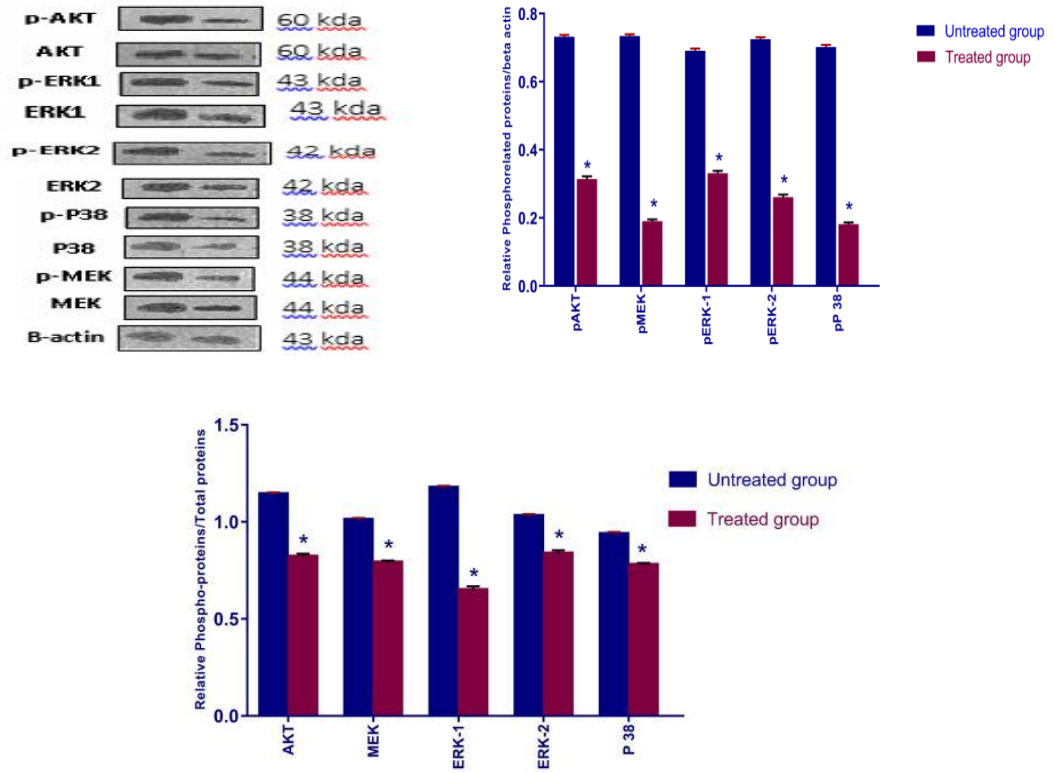

Fig.7: (a) Immunoblots of AKT, ERK ½, P38, and MEK as well as their phosphorylated counterparts, showed in comparison to β-actin. (b) bar graph depicts the relative protein phosphorylation of AKT, MEK, ERK ½, and P38 in HOP-92 cells treated with 6.57 µg/ml chalcone/xanthine hybrid. (c) Ratio of phosphorylated proteins to total proteins. The bars represent the mean ± SD. A t-test was applied to identify whether there is an important differentiation, where * $p < 0.05$ compared to the untreated group.

Discussion

The temporal linkage of cell cycle withdrawal and differentiation is vital for appropriate growth and development and remains so throughout life for tissue homeostasis and cell replenishment. Failure to stop proliferation or loss of differentiation, lead to a number of disorders and is a characteristic of cancer cells. We investigated the molecular pathways that connect cell cycle halt and apoptosis to the antineoplastic effect of the chalcone/xanthine hybrid, with implications for human cancer therapy.

Many investigations have shown that chalcone derivatives have antineoplastic properties. This compound's anti-proliferative effect is due to EGFR inhibition as mentioned in our previous study[23] which created a chalcone/xanthine hybrid as an antiproliferative agent with potential EGFR inhibition. Moreover, the docking molecular modeling technique was used to predict how the chalcone/xanthine hybrid interacts with EGFR. This docking experiment was carried out on the 3D EGFR's crystal structure in conjunction with AQ4999. (PDB ID: 1M17)[29,30]. The docking results of the chalcone/xanthine hybrid against EGFR revealed that it fits precisely inside the protein's ATP-active region. Furthermore, the chalcone moiety forms a hydrogen bonds network with the crucial residue methionine 769 (Met769), which is vital for modifying kinase activity. Interestingly, the xanthine moiety of the hybrid formed an extra hydrogen bond with the His871 residue while being oriented toward the hinge region[23]

The current study revealed that chalcone/xanthine hybrid treatment of cell lines has a discernible influence on early and late apoptosis. The rate of late apoptosis over early apoptosis increased following treatment, validating the cytotoxic test results. Moreover, the increase in the percentage of cells in the G1 phase after treatment and the decreased G2/M

phase indicates cell cycle growth inhibition after chalcone/xanthine hybrid treatment.

A number of tumors overexpress epidermal growth factor receptors (EGFR), which upon interaction with the ligand starts tyrosine kinase receptor signaling and activates downstream numerous signal cascades like RAS/MAPK, PI3K/AKT, and others which results in cancerous cell proliferation and apoptosis inhibition, thereby regulating tumor growth, survival, and angiogenesis. This is accomplished through two processes. The first involves EGFR translocation to the nucleus upon ligand binding to control target gene expression via transcription factor binding. The second includes receptor dimerization, which activates effector proteins and, as a result, phosphorylation of signaling cascades such as RAS/RAF/MEK/ERK and PIK3/AKT/mTOR, which drive cell proliferation and tumor spread[31-33].

In the current study, chalcone/xanthine hybrid induced suppression of AKT phosphorylation in malignant HOP-92 cell lines which is hypothesized to be associated with reduced cancer growth and survival. This can be explained by the fact that when EGFR is active in cancer cells, it activates the downstream signaling mediator AKT, which prevents BAX, a member of the pro-apoptotic Bcl-2 family from activation. Furthermore, inhibiting AKT phosphorylation by chalcone/xanthine hybrid relaxes direct suppression on BAX and activates P53 via suppression of Mdm2 phosphorylation, which inhibits P53 activation and induces apoptosis[34,35].

Chalcone/xanthine hybrid inhibitory impact on P38 phosphorylation as shown in our study predicts its chemotherapeutic action in cell cycle regulation. MAP kinases are involved in transcription factor phosphorylation and activation, translational machinery components, and other proteins that regulate the cytoskeleton or the cell cycle[36-38]. The inhibition of p38 phosphorylation by our drug affects the phosphorylation of transcription factors that regulate cell cycle growth.

The current study highlighted the modulatory impact of the RAS/RAF/MEK/ERK pathway on the HOP-92 cancer cell line following chalcone/xanthine hybrid therapy. ERK½ is activated and translocated to the nucleus in response to the downstream cascade, where it can trigger transcription of target genes, controlling cell proliferation and death. Carcinogenesis is characterized by dysregulation of the RAS/RAF/MEK/ERK signaling cascade, in which numerous components of the cascade are overexpressed. Moreover, multiple studies have discovered that the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways may collaborate to cause cancer[39-41].

Cancerous cells overexpress the anti-apoptotic family member Bcl-2, which suppresses apoptosis and reduces treatment efficiency. Overexpression of Bcl-2 interacts with the pro-apoptotic protein BAX, limiting mitochondrial cytochrome c release. In our study, HOP-92 cells treated with chalcone/xanthine hybrid showed down-regulation of Bcl-2 which relieves the suppression of apoptosis induced by the interaction of Bcl-2 with BAX. Moreover, overexpression results of BAX in our study increase cell death and kill cancer cells[42].

The actions of initiator caspases 8 and 9 were investigated in our study to see if the chalcone/xanthine hybrid influences the extrinsic and/or intrinsic apoptotic pathways. Apoptosis was found to be triggered by two main pathways: the extrinsic pathway mediated by the cell death receptor and the intrinsic pathway mediated by mitochondria, which led to the activation of caspase-8 and caspase-9, respectively, and subsequent activation of the cell death receptor. Intrinsic apoptosis is induced by mitochondrial changes such as the release of mitochondrial cytochrome c and a drop in mitochondrial transmembrane potential. **Fig. 5** shows that treatment of HOP-92 cells by chalcone/xanthine hybrid increased expression of both caspase-8 and caspase-9. Interestingly, the increase in caspase 8, 9, and, as a result, caspase 3 activity in the current study implies that both the intrinsic and extrinsic routes are implicated in causing apoptosis[43].

Conclusion

The anticancer effects of the chalcone/xanthine hybrid on HOP-92 cells investigated in this work. It reduced proliferation, triggered apoptosis, and stalled the G1/S phase. Furthermore, the

chalcone/xanthine hybrid decreased Bcl-2 expression while increasing P53, caspases 3, 8, 9, and the pro-apoptotic production of BAX at the transcriptional level. Furthermore, following treatment, phosphorylation of downstream proteins involved in the cell cycle and apoptosis regulation was suppressed. These findings emphasize the compound's powerful anticancer properties, indicating that it might be used as a novel medication for the treatment of NSCLC.

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دراسة الآليات المضادة للسرطان في هجين الشالكون/الزانثين في سرطان الرئة ذو الخلايا غير الصغيرة

عمر محمود الريحاني[*1] – شيماء فيصل[2] – هشام علاء أبو زيد[3] – علاء عرفات حيالله[4] – محمد عبد العزيز أبو النيل[5] – ميادة حسن نظمي[1]

[1]قسم الكيمياء الحيوية كلية الصيدلة جامعة المنيا

[2]قسم الفارماكولوجي والسموم كلية الصيدلة جامعة المنيا

[3]قسم الكيمياء العضوية الصيدلية كلية الصيدلة جامعة دراية

[4]قسم الكيمياء العضوية الصيدلية كلية الصيدلة جامعة أسيوط، قسم الكيمياء الصيدلية كلية الصيدلة جامعة سفنكس

[5]قسم الكيمياء الصيدلية كلية الصيدلة جامعة المنيا

على الرغم من التقدم الكبير في العلاج، لا يزال سرطان الرئة السبب الرئيسي لوفيات السرطان. هجائن الشالكون هي أحد مشتقات الزانثين الاصطناعية التي وجد أنها تمارس تأثيرًا مضادًا للسرطان في مجموعة متنوعة من أنواع السرطان. وهكذا، سعت الدراسة الحالية إلى اكتشاف ما إذا كان الشالكون الهجين يؤثر على مسببات سرطان الرئة ذو الخلايا غير الصغيرة (NSCLC). لقد وجد أن الشالكون الهجين يمنع تقدم دورة الخلية أثناء إحداث موت الخلايا المبرمج وإيقاف دورة الخلية في HOP-92 cells. أظهر قياس التدفق الخلوي أن الشالكون الهجين يسبب موت الخلايا المبرمج بقوة في خلايا HOP-92 cells ، بالإضافة إلى التغيرات في التعبير البروتيني المرتبط بموت الخلايا المبرمج، بما في ذلك زيادة في تعبير Bax، وcaspase3، وcaspase8، وcaspase-9، وP53، بالإضافة إلى انخفاض في تعبير Bcl-2.

علاوة على ذلك، أدى الشالكون الهجين إلى إبطاء تقدم دورة الخلية من المرحلة G1 إلى المرحلة S. علاوة على ذلك، انخفضت نسبة البروتينات المفسفرة (AKT، وMEK، وERK½، وP38) إلى كمية البروتين الإجمالية، مما يشير إلى تنظيم مسارات إشارات البروتين النهائية التي تحكم نمو دورة الخلية. بشكل عام يمكن اعتبار هذا الشالكون الهجين خيارًا علاجيًا لـ NSCLC عن طريق تحفيز موت الخلايا المبرمج.