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ANTICANCER POTENTIAL OF NOVEL PYRIMIDINE DERIVATIVES AND SCHIFFS' BASES CONTAINING PYRIMIDINE MOIETY AND RELATED APOPTOTIC ALTERATIONS

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ABSTRACT
A number of pyrimidine derivatives and chalconyl pyrimidines were synthesized. Their structures were elucidated by physical measurements such as IR, ^{1}H-NMR and MS, as well as by microanalysis of the elements. They were tested for their cytotoxicity against three cell lines such human liver carcinoma cell line (HEPG2), human breast adenocarcinoma cell line MCF7, and human lung adenocarcinoma cell line A549 and the results were encouraging for their activity on cell cycle arrest and apoptotic genes expression.

KEYWORDS: Pyrimidine derivatives, Chalcones, Anticancer activity and apoptotic alterations.

INTRODUCTION

Many clinically successful anticancer drugs either are natural products or have been developed from naturally occurring lead compounds, such as taxol, topotecan and irinotecan.

Pyrimidine nucleus is a pharmacophoric scaffold and represents a class of heterocyclic compounds with a wide range of biological applications. Many of pyrimidine derivatives are widely used for their antitumor activities[1,2,3]; antimicrobial[4]; and anti-inflammatory.[5] Many compounds containing pyrimidine moiety were reported as widely used anticonvulsants[6] and analgesics.[7] It has been reported that the introduction of an additional pyrimidinyl moiety to the chalcone core tends to exert pronounced influence in conferring novel biological in such compounds.[8]

Chalcones constitute an important group of natural products and serve as precursors for the synthesis of different heterocyclic derivatives namely, pyrazolines, isoxazolines[9]; pyrimidines[10,11]; benzimidazoles[12] as well as triazoles.[13] Chalcone derivatives have received a great deal of attention due to their relatively simple structure and wide variety of pharmacological activities reported for these compounds including antifungal[14,15], antibacterial[16] and antitumor activities.[17,18]

Cancer is a leading cause of mortality worldwide accounting for almost 13% of all death.[19] Among all types of cancer, lung, breast, colorectal, stomach, and prostate are the underlying cause for the majority of cancer death.

Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges. Cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects. The understanding of the molecular mechanisms involved in cancer has opened new ways in the development of new anticancer compounds.[20] This has opened the door for new prospects in chemotherapy to stop or reverse this proliferative process, especially using targeted approaches based on regulation of the cancer cell cycle and regulation of apoptotic pathways.1 Apoptosis is a physiological process for killing cells Signaling for apoptosis occurs through multiple independent pathways. These pathways converge on a common machinery of cell destruction.[21,22,23,24,25]

Pyrimidine derivatives attract great interest due to the wide variety of interesting biological activities, such as anticancer[26,27], antiviral[28], anti-inflammatory[29,30], and antimicrobial activities.[31,32,33] Moreover, Schiffs' bases derived from various heterocyclic compounds possess cytotoxic[34,35], anticonvulsant[36and37], antiproliferative[38and39], antimicrobial[40and41], and anticancer.[42]

Therefore, we have looked for novel pyrimidine derivatives as well as Schiffs' bases containing pyrimidine unit. The study was also extended to predict the mode of action of certain pyrimidine and chalcone derivatives as well as Schiffs' bases.

MATERIALS AND METHODS

1. Chemistry

Synthesis of 4,6-diaryl-2-(1H) pyrimidin-2-one(3a,b); and 4,6-diaryl-2(1H)pyrimidin-2-thione(3c,d)

A mixture of 1,3-diaryl prop-2-en-1-one(1a,b)(0.01mol)[18] and urea(0.01mol)/ or thiourea (0.01mol) in ethanol (30 ml) containing catalytic amount of sodium ethoxide (prepared from 0.2g of sodium metal and 5ml of absolute ethanol) was heated under reflux for 10h. The product that separated was collected by filtration, washed well with water and recrystallized from the proper solvent as **(3a-d)** Scheme1. The physical data are listed Table.1.

Synthesis of 5, 5`-(1, 4-phenylenebis (methaneylylidene)) bis (pyrimidine-2, 4, 6(1H,3H,5H)-trione)

A mixture of terphathaldehyde (0.01mol), barbituric acid (0.02mol) and (0.02mol) of sodium hydroxide was heated in microwave for 90 sec. after cooling , the product was collected, washed well with water and dilute alcohol then recrystallized from ethanol to give 4 as yellow crystals, M.p.230°C in 83% yield . IR(KBr,cm^{-1}) : 3205(NH); 3030-2850(CH aromatic and aliphatic); 1678(C=O); 1593, 1601(C=C).

Table 1: Physical data of Pyrimidine Derivatives (3a-d).

Compounds	M.p. C° Solvent of cryst.	Molecular formula (Mol.wt.)	Analysis Calcd/found C H N S F	I R cm^{-1}	NMRδppm	MS[EI^+]m/z (%)
3a	236 EtOH	$C_{15}H_{10}N_4O_3$ (294)	61.22 3.401 19.04 - - 61.31 3.49 19.13 - -	3330(NH); 1681(C=O); 1626(C=N); 1593(C=C)	4.33(s,1H),CH.pyrimidin, 7.07-7.71(m,8H,Ar-H) 10.71(s,1H,NH) (D_2Oexchangeable)	294.41(11.3%) 293.7(1.3%)
3b	242 EtOH	$C_{15}H_{10}N_4O_2S$ (310)	58.06 3.22 18.061 10.32 - 58.15 3.31 18.15 10.41 -	3334(NH); 2627(SH); 1635(C=N); 1591(C=C); 1241(C=S)	4.31(s,1H,CH.pyrimidin), 7.12-7.38(m,8H,Ar-H) 9.98(s,1H,NH) (D_2Oexchangeable)	311.5(1.1%) 310(12.16%)
3c	210 EtOH	$C_{15}H_{10}N_3OF$ (267)	67.41 3.74 15.73 - 6.88 67.50 3.83 15.82 - 6.97	3300(NH); 1679(C=O); 1622(C=N); 1594(C=C)	4.31(s,1H,CH.pyrimidin), 7.03-7.28(m,8H,Ar-H) 10.71(s,1H,NH) (D_2Oexchangeable)	268.51(7.13%) 267.33(1.65%)
3d	178 EtOH	$C_{15}H_{10}N_3SF$ (263)	63.602 3.53 14.84 11.307 6.71 63.69 3.62 14.93 11.39 6.80	3320(NH); 2622(SH); 1622(C=N); 1592(C=C); 1240(C=S)	4.23(s,1H,CH.pyrimidin), 7.01-7.23(m,8H,Ar-H) 10.71 (s,1H,NH) (D_2Oexchangeable)	284.60(3.71%) 283.10(4.53%)

^{1}H-NMR (DMSO-d_6,300MHz):δ 7.29-7.30 (m,4H,Ar-H); 11.07,11.08,11.19,11.20(4xS,4xH,4xNH) (D_2O exchangeable). ^{13}C-NMR(DMSO.do); δ 119.3, 129.7,132.9,150.8, 154.8,162.9 for CH,C=O. MS:[EI^+] 354.60 (1.3%), 353.4(12.7%)analysis calcd. For $C_{16}H_{10}N_4O_6$ (354). Required: C,54.23; H,2.82; N,15.81. Found: C,54.32; H,2.41; N,15.89%.

Synthesis of 2-amino-4-(4-(2-amino-5,7-dioxo-5,6,7,8-tetrahydro-2H-pyrimido[4,5-d][1,3] oxazine-4-yl)phenyl)-2H-pyrimido[5,4-d] [1,3]oxazine-6,8(5H,7H)-dione (4a) and 2-amino-4-(4-(2-amino-5,7-dioxo-5,6,7,8 tetrahydro-2H-pyrimido[4,5-d][1,3]thiazine-4-yl)phenyl)-2H-pyrimido[5,4-d][1,3]thiazine-6,8(5H,7H)-dione (4b)

A mixture of 5, 5`-(1, 4-phenylenebis (methaneylylidene)) bis (pyrimidine-2, 4, 6(1H,3H,5H)-trione)(0.01 mol) and urea /or thiourea (0.01mol) in 30 ml of ethanol containing 5ml of concentrated hydrochloric acid was refluxed for 8h. After cooling the product was collected by filtration washed well with water and recrystallized from ethanol to give **(4a and 4b)** Scheme 2. The physical data are listed in Table2.

Table 2: IR* and ^{1}H-NMR# spectra of compounds (4a,4b).

Compound No.	IR, cm-1	1H-NMR(DMSO-d_6) δppm	^{13}C-NMR(DMSO-d_6)
4a	**3369(NH_2); 3205(NH);3128,3089,2924,2850 (CH aromatic and aliphatic);1701,1676(C=O); 1649(C=N);1575(C=C).**	**4.9(s,1H,CH);5.01(s,1H,CH); 7.19-7.28(m,4H,Ar-H);8.52, 8.62(2xs,2x2H,2xNH_2) (D_2O exchangeable);9.12,9.23(2xs,2x1H,2xNH); 10.50,11.13(2xs,2x1H,2xNH) (D_2O exchangeable)**	**77.4(CH-NH_2); 101(CH); 104.1(CH); 126.3,129.5,137.7,147.0,175.6,152.4, 157.6, 164.9(C=O)**
4b	**3329(NH_2); 3201(NH);3101,2931,2850,2831 (CH aromatic and aliphatic);1701,1678(C=O); 1635(C=N);1589(C=C).**	**4.92(s,1H,CH);5.03(s,1H,CH); 7.21-7.30(m,4H,Ar-H);8.73, 8.79(2xs,2x2H,2xNH_2) (D_2O exchangeable);9.35,9.36(2xs,2x1H,2xNH); 11.01,11.13(2xs,2x1H,2xNH) (D_2O exchangeable)**	**71(CH-NH_2); 74.2(CH); 126.1(CH); 126.3,128.8,135.3,147.2(C=C),152.4, 147.3,157.1, 162.9,164.9(C=O)**

* Where: IR in RB in pellet and # NMR in DMSO-d_6 at 300MHz.

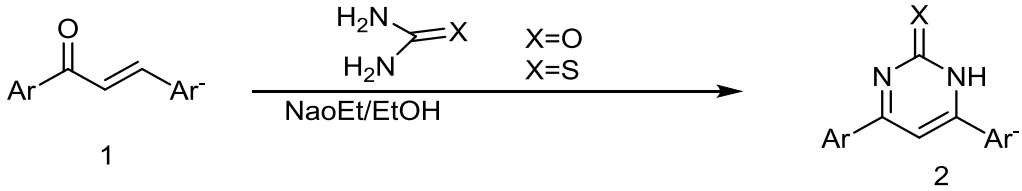
Synthesis of the Schiffs' bases (E)-4-(4-(2-amino-5,7-dioxo-4,4a,5,6,7,8-hexahydro-2H-pyrimido[4,5-d][1,3]oxazin-4-yl)phenyl)-2-((2-chlorobenzylidene)amino)-4a,5-dihydro-2H-pyrimido[5,4-d][1,3]oxazine 6,8(4H,7H)-dione (5a) and (E)-4-(4-(2-amino-5,7-dioxo-4,4a,5,6,7,8-hexahydro-2H-pyrimido[4,5-d][1,3]thiazin-4-yl)phenyl)-2-((2-chlorobenzylidene)amino)-4a,5-dihydro-2H-pyrimido[5,4-d][1,3]thiazine-6,8(4H,7H)-dione (5b)

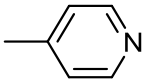
To a solution of 4a and /or 4b (0.01mol) in 20 ml of acetic acid was added 2-chlorobenzaldehyde (0.01mol; 1.4g) and the mixture was reflux for 8 h. after completion, the solvent was evaporated (reduced pressure) and the residue was washed well with diluted alcohol then recrystallized from the proper solvent to give **5a** and/or **5b** as Schiffs' bases. Scheme 2. The physical data are listed in Table3.

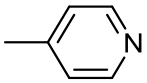
5a: Crystallized from ethanol as dark brown crystals in 65% yield.
5b: Crystallized from ethanol as brown crystals in 67% yield.

Table 3: Physical data of Schiffs'bases (5a,b).

compounds	M.p. C° Solvent of cryst.	Molecular formula (Mol.wt.)	Analysis Calcd/found C H N S O	I R cm^{-1}	NMRδppm	MS[EI^+]m/z (%)
5a	340 EtOH	$C_{25}H_{21}N_8O_6Cl$ (564.5)	53.15 3.75 19.83 - 6.27 53.24 3.84 19.91 - 6.35	3220(NH_2); 3318(NH); 1688(C=O);1620 (C=N); 1589(C=C)	8.82(s,2H,NH_2) (D_2Oexchangeable); 9.91(s,1H,NH); 11.36(s,1H,NH); 4.91(d,1H,O-CH); 3.79(d,1H,CO-CH); 7.31-7.95,m,8H,Ar-H; 8.39(s,1H,N=CH)	564.5 (1.3%); 566.12(3.2%)
5b	315 EtOH	$C_{25}H_{21}N_8O_4$ S2Cl (596.5)	50.29 3.55 18.77 10.74 5.94 50.38 3.64 18.86 10.83 6.03	3228(NH_2); 3368(NH); 1679(C=O); 1635(C=N); 1599(C=C); 1242(C=S)	3.7(s,1H,CH-NH_2); 3.81(d,1H,COCH); 4.31(d,1H,CHS); 7.21-7.79(m,8H,Ar-H); 9.19(s,1H,NH); 11.25(s,1H,NH); 8.69(CH=N); 8.91(s,2H,NH_2) (D_2Oexchangeable)	596.5(2.1%) 599.05(1.1%)



a,Ar=$C_6H_4NO_2$-4 ; Ar^-= 

b,Ar=C_6H_4F-4 ; Ar^-= 

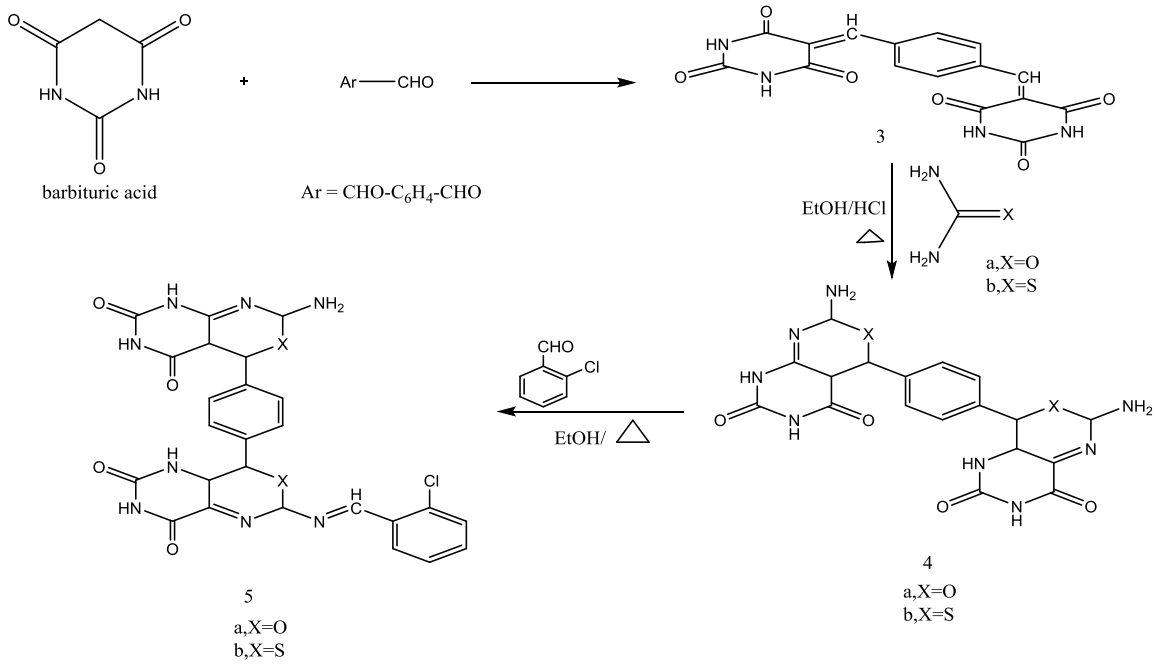
a,Ar =$C_6H_4NO_2$-4 ;Ar^- =C_5H_4-N-4; X=O

b,Ar =$C_6H_4NO_2$-4 ;Ar^- =C_5H_4-N-4; X=S

c,Ar =C_6H_4F-4 ;Ar^- =C_5H_4-N-4; X=O

d,Ar =C_6H_4F-4 ;Ar^- =C_5H_4-N-4; X=S

Scheme 1



Scheme 2

2. Biochemical Study

Cell Lines

Human hepatocellular carcinoma (Hep-G2), human breast cancer (MCF-7) and human lung carcinoma (A549) were provided by the Holding Company for Biological Products and Vaccines (VACSERA), Dokky, Egypt. Cells were cultured in 75 cm2 cell culture flasks (TPP-Swiss) using RPMI 1640 medium supplemented with 10% (v/v)fetal bovine serum (GIBCO, USA) and 1% penicillin–streptomycin (Invitrogen, USA) at 37ºC in a humidified atmosphere containing 5% CO2 (Jouan-France). Confluent cells were detached using 0.25% (w/v) trypsin EDETA (GIBCO-USA).

Cytotoxicity Assay

The cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5- the metabolites were added to pre-cultured confluent cell monolayer. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO2 for a period of 48 h. Three wells were used for each concentration of the test compounds. Negative control cells were considered. At the end of the incubation period, MTT 0.5mg/ml was dispensed to treated plates post decanting the treatment medium. Detached cells were washed-out using sterile phosphate buffer saline (PBS). All experiments were carried out in

triplicate. The percent viability was calculated by the formula:

$$\text{Percent viability} = \frac{MOD\ of\ test\ X100}{MOD\ of\ control}$$

Cell cycle analysis

Flow cytometric analysis was performed in order to investigate the type of cell death induced by the tested compounds. The tested cancer cell lines were treated with 1/2 the IC50 value for 24, 48, and 72h. Detached were pelleted down, by cold centrifugation at 2000 rpm(Jouan-Ki 22, Franc) for 10 min at 4°C, and pellets were washed with (PBS) phosphate buffer saline (ADWIA-Egypt), fixed with cold methanol. Fixed cells were kept at -4°C overnight. Finally the pellets suspended in PBS treated with RNase A enzyme for 30 min at 37°C and stained with propidium iodide 50 µg/ml from stock solution (2.5mg/ml) in 500 µl of PBS solution, incubated in the dark at room temperature for 15 min. The cells were analyzed using flow cytometry (Becton Dickinson, San Jose, CA, USA). Distribution of cell cycle phase of nuclear DNA was determined on fluorescence detector equipped with 488 nm laser light source and 623 nm and pass filter (Hsu et al., 2007).

Molecular biology assay

Total RNA was extracted from control and treated human lung cancer (A549) cell line using Gene JET RNA Purification kit (Fermantus-UK) according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed using nanodrop apparatus. First-strand cDNA was synthesized with 1 µg of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Samples were subsequently frozen at -80°C until use for expression level of P53, Bax and Bcl-2 genes for three time intervals (24, 48, and 72h) using real-time PCR. Real-time PCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and RT-PCR (Reverse Transcriptase Polymerase Chain Reaction). The primer sequences of the oligonucleotides used for PCR (Table 4). Total RNA was extracted using RNeasy® Mini Kit (QIAGEN®) according to manufacturer's instruction. Total RNA (2µg) was applied for the synthesis of cDNA with QuantiTect® Reverse Transcription Kit (QIAGEN®). PCR was performed in a DNA Thermal Cycler (Applied Biosystems) using QuantiTect SYBR Green PCR Master Mix.

Table 4: Primers sequence.

Genes	Primer sequence	Annealing temperature
p53	F: 5`-CCCCTCCTGGCCCCTGTCATCTTC-3` R:5`-GCAGCGCCTCACAACCTCCGTCAT-3`	53°C 51°C
Bax	F: 5`-GTTTCATCCAGGATCGAGCAG -3` R: 5`-CATCTTCTTCCAGATGGTGA-3`	55°C 49°C
Bcl2	F :5`-CCTGTGGATGACTGAGTACC-3` R :5`-GAGACAGCCAGGAGAAATCA-3`	49°C 47°C
β-actin	F: 5`-GTGACATCCACACCCAGAGG-3' R: 5`-ACAGGATGTCAAAACTGCCC-3'	52°C 49°C

F: Forward strand and R: Reverse strand

Oxidative stress markers

The cell lysates of S3, S4 and cisplatin treated and untreated cell for three intervals 24, 48 and 72 hours were assessed for Malondialdehyde (MDA), measured by the thiobarbituric acid assay (BioVision Incorporated, USA) according to (Draper and Hadley, 1990). Also, Glutathione reductase (GR), peroxidase, and hydrogen peroxide levels were assayed calorimetrically according to Ellman (1959) using commercial assay Kit (abcam, US).

Statistical Analysis

Experiments were performed in triplicates and data were presented as mean ± SD. One-way ANOVA was performed using SAS (version 9.1.3, SAS Institute, Cary, Nc). The value of $P \leq 0.05$ was considered statistically significant.

RESULTS

The synthesized compounds and Cisplatin toxicity was evaluated using MTT assay against HepG2, MCF-7and A549. The inhibitory concentrations (IC50) of the tested compounds were listed in (Table 5). Compounds **2b**, **2d**, **5a** and **5b** showed a lower cytotoxic potential on test cell lines. However, compound 2a was effective only against lung cancer (A549) cell line. While, compounds **1b** and **2c** showed the highest cytotoxicity on treated cells, even more potent than Cisplatin on A549 cell line. Treated cells showed detachment, loss of membrane integrity, swelling, and knockdown of metabolic process and release of cellular components to the surrounding environment. Also the IC50 values of different compounds were cell type and concentration dependent.

Table 5: *In Vitro* evaluation of inhibitory concentration (IC50) of synthesized compounds against different cancer cell lines.

Compound	Cell line/IC_{50} value (µg/mL)		
	HepG2	MCF7	A549
2a	903	717	161
2b	949	791	930
1b	164	176	176
2c	360	350	126
2d	230	451	387
5a	458	485	329
5b	711	456	220
Cisplatin	70	75	181

Values are mean ± S.E (n =3).

Cell cycle analysis

Cell cycle analysis was performed targeting human lung cancer (A549) cell line at three time intervals (24, 48 and 72h). Cell cycle analysis showed that, both S3 and S4 induced cell arrest at the G2-M and pre-G1 checkpoints, and relatively to time the cells started to accumulate more in these after phases with concomitant reduction of cells arrest during S-phase relative to time. Also, S3 was significantly enhancer for cell arrest during the G2/M and PreG1 phases than S4 ($P<0.05$). In addition, both S3 and S4 were significantly of arresting potential of treated cells ($P<0.05$) at the G2-M and Pre-G1 checkpoints than untreated cell control (Table 6). Regarding the apoptotic profile of A549 cells **1b** and **2c** treated data recorded revealed that untreated A549 cells possess the least % of apoptotic cell population in the order of 0.24%, in the meantime the IC_{50} values (176 and 126 µg /ml) **1b** and **2c** treated A549 cells for 24, 48, and 72 h resulted in a little increase of the apoptotic populations in the order of 1.94%, 4.28%, and 4.72% for **1b** and 3.32%, 4.27%, and 6.19%, for **2c** respectively. Furthermore, a large population of late apoptotic/necrotic cells (5.71% for **1b** and 6.37% for **2c**) was also observed after 72 h treatment (Fig.1-1a).

Table 6: Cell cycle analysis of 1b and 2c treated A549 cell line expressed by (%) in each phase during different intervals (mean± SD).

TPT	Groups	% G0-G1	% S	% G2-M	% Pre-G1
24h	Cont. A549	67.59±1.02	27.59±0.18	4.15±0.73	0.67±0.10
	S3/A549	63.88±0.63[a]	24.04±0.77[a]	7.32±0.65[a]	4.82±0.69[a]
	S4/A549	61.98±0.66[ab]	22.67±0.49[a]	7.10±0.30[a]	8.26±0.13[ab]
48h	Cont. A549	67.59±1.02	27.59±0.18	4.15±0.73	0.67±0.10
	S3/A549	60.34±1.02[a]	18.99±0.57[a]	10.47±1.10[a]	10.21±0.49[a]
	S4/A549	57.46±4.20[ab]	15.75±0.01[ab]	15.77±3.97[ab]	11.03±0.25[ab]
72h	Cont. A549	67.59±1.02	27.59±0.18	4.15±0.73	0.67±0.10
	S3/A549	55.34±3.15[a]	19.45±0.07[a]	13.71±2.80[a]	11.51±0.29[a]
	S4/A549	55.66±4.14[a]	15.88±4.27[ab]	14.52±0.88[ab]	13.95±0.75[ab]

a: significance vs. control,
b: significance vs. compound 1b.

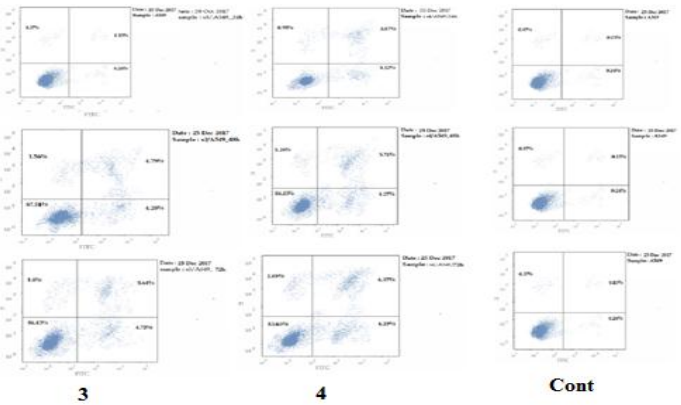


Figure 1: Flow cytometric analysis of A549 cell line either 1b and 2c treated and untreated (Control).

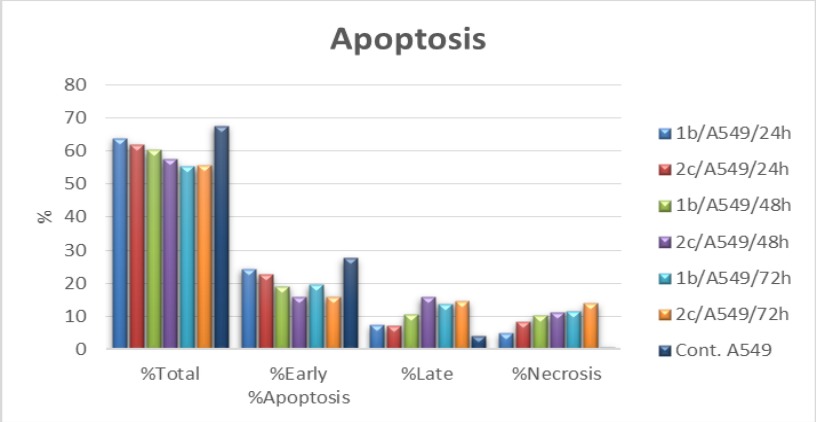


Figure 1a: Percentages of apoptosis and necrosis in A549 cell line exposed to 1b and 2c compared with cell control.

Molecular biology profile

The pro- apoptotic (P53 and Bax) and anti-apoptotic (Bcl-**2**) genes of A549 treated cells treated with both compound; **1b** and **2c** showed a significant up regulation of P53 and Bax genes and down regulation of Bcl-2 compared with those of untreated cell control (P<0.05). Up/down regulation profile was time and compound dependent as it was noticed that the expression of Bax and Bcl-2 was significantly up-regulated/ down regulated in **2c** cells treated cells than in case of using compound**1b** 48 and 72 for Bax and BCl-2 was significantly down regulated 72 hrs. for Bcl-2 post treatment with S4 (P<0.05]. While, P53 expression showed an insignificant changes in case of **1b** and **2c** cell treatment [Fig.3]. As regard to the anti-apoptotic gene Bcl-**2**, it was significantly down-regulated (P<0.05) in cells treated with compounds **1b** and **2c**, during all time intervals, compared to untreated cells.

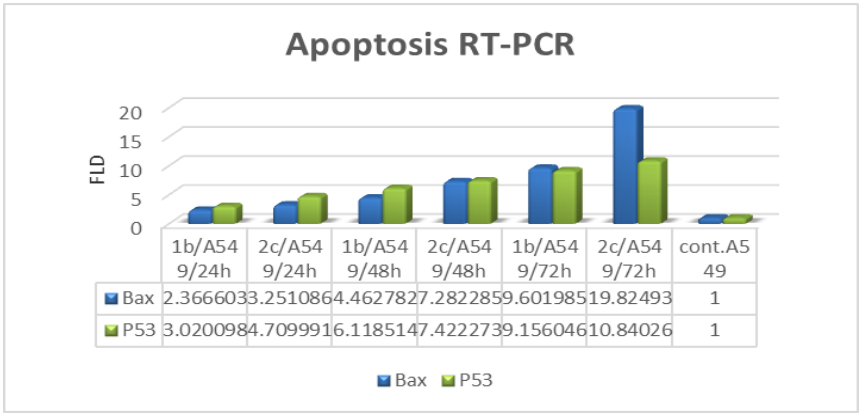


Figure 2: Effect of compounds 1b and 2c on Bax and P53 expression in human lung cancer (A549) cell line at different time intervals as determined by qRT-PCR.

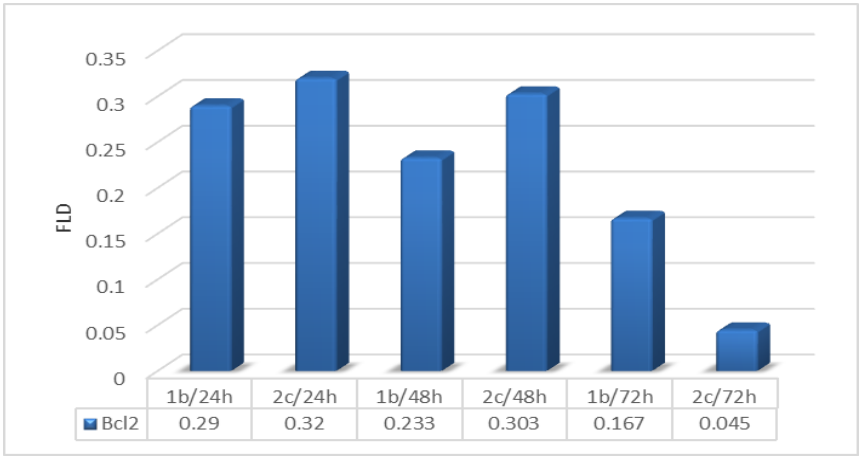


Figure 3: Effect of compounds 1b and 2c on Bcl-2 expression in human lung cancer (A549) cell line relative to time intervals.

Oxidative stress markers

The levels of H_2O_2, MDA and glutathione peroxidase showed a significant elevation relative to time in treated cells compared to untreated cells. Oppositely, glutathione reductase levels were significantly reduced relative to time in treated cells compared with its value in untreated cells (Table 7).

Table 7: Evaluation of antioxidant [H_2O_2, MDA, GPx and GR] in treated and untreated A549 cell Lysates relative to time.

Groups	H2O2	MDA	GPx	GR
24 hours				
Cont.	**27.11±0.90**	**1.20±0.02**	**8.69±0**	**16.05±0.116**
S3/A549	**31.10±0.35a**	**1.53±0.04**	**20.63±0.15a**	**12.75±0.19a**
S4/ A549	**32.33±0.85a**	**1.60±0.03**	**24.55±0.23a**	**13.02±0.10a**
48 hours				
Cont.	**27.11±0.90**	**1.20±0.02**	**8.69±0**	**16.05±0.116**
S3/ A549	**35.65±0.39a**	**1.97±0.05a**	**29±0.39a**	**11.65±0.20a**
S4/ A549	**37.68±0.43a**	**2.19±0.03a**	**29.32±0.36a**	**11±0.07a**
72 hours				
Cont.	**27.11±0.90**	**1.20±0.02**	**8.69±0**	**16.05±0.116**
S3/ A549	**39.46±0.89a**	**3.46±0.06a**	**33.86±0.23a**	**10.38±0.17a**
S4/ A549	**59.50±1.61ab**	**3.46±0.03a**	**34.99±0.31a**	**10.70±0.19a**

a: significance vs control, b: significance vs 1b.

DISCUSSION

Cell culture techniques allow researchers to look *in vitro* on human tumors reactivity to either natural or synthetic compounds, under controlled conditions. Three different cell lines (human breast cancer, hepatocellular carcinoma and lung cancer) were used through the current study for valuation of the anticancer activity of seven newly synthesized compounds in comparison with cisplatin as a positive control drug currently used in cancer therapy. It has been clinically proven that cisplatin acts against various human cancers, and it has been used for the treatment of lung cancer[43and44], carcinoma[45and46] and breast cancer.[47and48] Cisplatin treatment induces cell arrest at the DNA synthesis phases, relatively to time. The cells gradually accumulate at the sub-G1 phase. In addition, it induces cells apoptosis and necrosis. The current data demonstrates that among all tested compounds two compounds 1b, 2c produced the highest growth inhibition percentage especially against human lung cancer cell line (A549). Consequently, these two compounds were furtherly tested against A549 cell line to investigate their mode of action in tumor growth inhibition. Cell cycle analysis was performed on untreated and treated lung carcinoma cell line. The treated cells were arrested at G2-M and pre-G1 phases. The accumulation of treated cells in sub-G1 phase of cell cycle may be due to the cells entering into apoptotic or necrotic phases. So to determine whether apoptosis is the main cause of cell death induced by S3 and S4 derivatives, cells were examined for molecular and morphological markers of apoptosis. Translocation of phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface is a particular sign of apoptosis.[49] Therefore, PS can be detected by staining with a protein that has a high affinity for PS, annexin V, followed by flow cytometry analysis. In parallel, cells are stained with propidium iodide (PI), which can enter the cell only if the plasma membrane is damaged.[50] These results are in consistence with the expressions of the key apoptotic genes. The gene expression results illustrate that the expression of the pro-apoptotic genes (Bax and P53) were upregulated, while the anti-apoptotic gene (Bcl-2) is significantly down-regulated, at all-time intervals. Previous gene expression studies have revealed the existence of more than one pathway regulating growth inhibition and apoptotic processes.[51] This antineoplastic effect can be mediated by activation of different target genes such as p21, caspases, Bax and Bcl-2 that act as cross-point regulators able to induce or inhibit apoptosis.[51] P53 is one of the most important tumor suppressors. It plays a key role in the cellular response to DNA damage. P53 acts primarily at the G1 checkpoint (controlling the G1 to S transition), where it blocks cell cycle progression in response to damaged DNA. It is well known that some chemotherapeutic drugs such as amino benzene sulfonamide may result in the accumulation of reactive oxygen species (ROS), induction of apoptosis, and reduction of reduced glutathione levels in patients.[52] Oxidative stress (OS) is a result of an imbalance between generation of ROS and insufficient antioxidants.[53] The use of antitumor drug with oxidative capacity cannot harm normal cells because such drugs amplify the levels of ROS, however the production of ROS in normal cells can be regulated efficiently by its antioxidant defense system.[54] Also, Yilmaz[53] (2018) exposed human lung cancer cell line (A549) to H_2O_2 in order to generate oxidative stress. Incubation of A549 cells with H_2O_2 resulted in concentration-dependent cell death with reduction of cell number in G2/M phase and increased the number of apoptotic cells. In the current study, we determined the levels of two of the oxidative stress markers (H_2O_2 and MDA) and the antioxidant enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR). A549 cell treatment with S3 and S4 produced a state of OS proved via elevation of H_2O_2 and MDA level

with a concomitant reduced GR that responsible for regeneration of reduced glutathione (GSH). Free radicals, particularly ROS, have been reported to be common mediators for apoptosis.[55] However, due to deregulated redox balance, cancer cells escape programmed cell death regardless of the persistently higher ROS, in a more efficient manner than normal cells, while the higher intracellular levels of GSH promote tumor cell survival. Additionally, anticancer drugs have been shown to exert apoptotic effects based on GSH depletion.[56]

In conclusion, the present results indicate that both S3 and S4 can induce cell cycle arrest followed by cell death due to their apoptotic potential and these effects may be attributed to the predominantly induction oxidative stress.

The structure of compounds tested as well as their in vitro cytotoxicity to tumor cells are given in table 5. The compounds included not only pyrimidines but also compounds related structurally to pyrimidines and Schiffs'bases of barbituric with terphthaldehyde. The pyrimidine derivatives had substitutions with 4-nitro; 4-fluoro and N at the para- positions. The maximum in vitro cytotoxicity was exhibited by **2c** followed **2a** then **1b** in A549 with substitution of F at the para-position of the phenyl ring then the second with NO_2 at para-position of the phenyl ring. The third was chalcone 1b (which had highest activity against A549 and moderate activity against Hepg2 and MCF7) with F at para – position at the phenyl ring while the N was present in all the derivatives as pyridine system. Other compounds showed no cytotoxicity.

The present study is an attempt to investigate whether the substitution of various groups can change or improve the activity of the chalcones as well as the pyrimidines towards all the tested cell lines. The minimum concentration inhibitory needed for the 50% cell death was the highest for the compound **2c** then **2a** then **1b.** All of these compounds contained pyridine ring system attached to either the α, β unsaturated carbonyl compound or the pyrimidine ring system.

The pyrimidinone derivatives **(2c)** and its chalcone **(1b**) with 4- F phenyl ring had the highest activity that may be due to the increased permeability of the pyrimidine and the chalcone into the cell. The presence of pyridine ring system increases the antitumor activity whereas the presence of the nitro group attached to the phenyl ring decreased the antitumor activity on the three-cell lines hepg2, MCF7, and A549 respectively (949,791,930). This may be due to the hydrophobic nature of the NO_2-phenyl and maybe responsible for this activity decrease. The presence of a thioxo-group in the pyrimidine didn't seem to make any significant effect on the three cell lines.

Compound **5b** has shown moderate activity compared to cisplatin when tested against A549 cell line, this might be due to the presence of an azomethine group linking between the chlorophenyl moiety and the pyrimidine moiety in either **5a** and /or **5b** derivatives. The hydrophobic nature of the azomethine group may be responsible for this activity difference.

CONCLUSION

In this study novel chalcone derivatives were synthesized either by conventional method/or by microwave assisted friendly environmental method. Similarly 4-pyrimidine derivatives were synthesized and two Schiffs'bases as well. They were tested against three carcinogenic cell lines and the results of the screening were encouraging.

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