DOI 10.1007/s11094-018-1712-x
Pharmaceutical Chemistry Journal, Vol. 51, No. 10, January, 2018 (Russian Original Vol. 51, No. 10, October, 2017)

IN VITRO ANTITUMOR ACTIVITY OF NEWLY SYNTHESIZED PYRIDAZIN-3(2H)-ONE DERIVATIVES VIA APOPTOSIS INDUCTION

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Original article submitted February 25, 2017.

Systemic toxicity associated with drug resistance continues to be the major obstacle to curative therapy of cancer. Tumor cell resistance to chemotherapeutic drugs often results in coordinate resistance to other structurally and functionally unrelated drugs and the subsequent development of cross resistance phenotype. Therefore, it seems necessary to identify new molecules as anticancer agents. In this process, we synthesized a series of new pyridazin-3(2H)-one derivatives and evaluated their antitumor potential. These cyclic molecules were synthesized and designed as a combination of benzofuran with pyridazinones. All final compounds have been characterized by spectral and elemental analyses to confirm successful synthesis reactions. To evaluate their anticancer activity, all derivatives were assessed against the human breast adenocarcinoma cell line (MCF-7) and the murine mastocytoma cell line (P815) using the methyl tetrazolium Test (MTT assay). The cytotoxic activity was found to be dose-dependent and the IC_{50} values of the synthesized compounds ranged from 14.5 to 40 μM against MCF-7 and from 35 to 82.5 μM against P815. At the same time, no cytotoxic activity was observed against normal cells. In order to investigate the molecular mechanism of the most cytotoxic product (**6f**), apoptosis induction was measured against MCF-7 cells. Using the annexin-V FITC staining technique, we showed that the cytotoxic effect of this product is associated with apoptosis induction.

Keywords: Pyridazin-3(2H)-one derivatives; cytotoxicity; tumor cells; MTT assay; apoptosis.

1. INTRODUCTION

Cancer, also known as a malignant tumor or malignant neoplasm, is a frightful disease. As estimated by the World Health Organization (WHO), more than 21 million new cancer cases and 13 million deaths are expected by 2030 [1]. In particular, breast cancer is now considered the leading cause of cancer-related death among women worldwide [2]. Despite the tremendous success of anticancer drugs such as cisplatin derivatives, these compounds suffer from two main disadvantages: they are encumbered by the development of secondary resistance by cancer cells and they have severe side effects such as nephrotoxicity [3, 4, 5]. Thus, there is a serious need for newly synthesized or natural products with enhanced bioactivity. Pyridazin-3(2H)-one derivatives constitute an interesting class of heterocyclic lead compounds for drug discovery and research into anticancer molecules [6, 7, 8]. Recently, it was reported that some pyridazin-3(2H)-one derivatives specifically interact with and inhibit PIM (potential tumor targets) kinases with low nanomolar potency [9, 10, 11, 12, 13]. In addition, pyridazin-3(2H)-ones are endowed by many biological activities, e.g., antidepressant, antihypertensive, antithrombotic, anticonvulsant, cardiotonic, antibacterial, diuretic, anti-inflammatory, anti-HIV, hepatoprotective, and prostaglandin inhibitor [14, 15, 16]. In this context, the present study was aimed at synthesizing new pyridazin-3(2H)-one derivatives with cytotoxic activity. The obtained molecules were tested for their antitumor activity against two tumor cell lines: MCF-7 (human breast adenocarcinoma) and P815 (murine mastocytoma). The structure-activity relationship has been considered.

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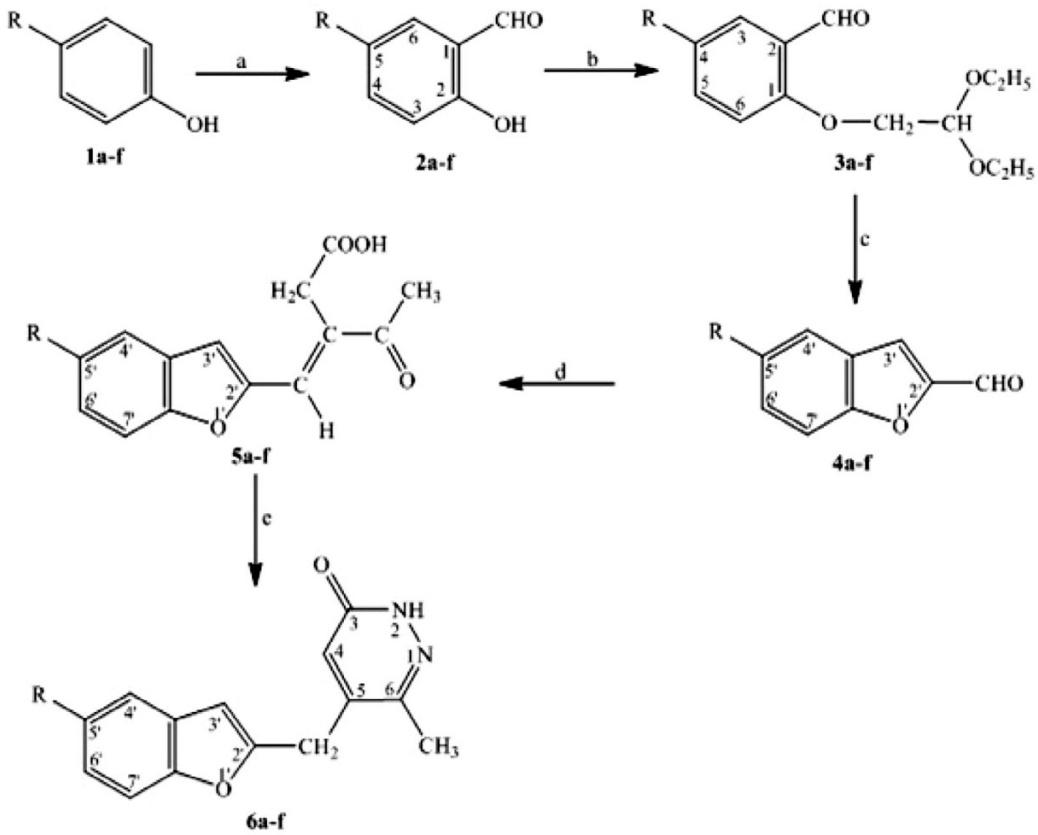

Fig. 1. Synthesis of target compounds **6a - 6f**. Reagents : (a) $CHCl_3$/NaOH 10N, reflux 2h ; (b) $BrCH_2CH(OC_2H_5)_2/K_2CO_3$/DMF, reflux 4h ; (c) CH_3COOH, reflux 24h ; (d) $H_3CCOCH_2CH_2COOH/CH_3COOH$, reflux 24h ; (e) H_2NNH_2/EtOH, reflux 2h.

2. MATERIALS AND METHODS

2.1. Chemicals and Cell Lines

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Gibco BRL, Cergy Pontoise, France. Penicillin G-streptomycin, methotrexate (MTX), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin/ethylenediaminetetraacetic acid (EDTA), phosphate buffer solution (PBS), ficoll-hypaque, annexin-V biotin-streptavidin fluorescein isothiocyanate (FITC) were from Sigma Aldrich, France. Tumor cell lines MCF-7 and P815 were from the stock cultures of the Laboratory of Biological Engineering, Faculty of Science & Technology, Sultan Moulay Slimane University, Morocco.

TABLE 1. Substituents R in Synthesized Compounds

Compound	R
a	H
b	CH_3
c	CH_2CH_3
d	$CH(CH_3)_2$
e	OCH_3
f	$OCH_2CH_2CH_3$

2.2. Synthesis of Drug Molecules

The scheme of synthesis of 5-(benzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2H)-one derivatives is outlined in Fig. 1 and Table 1. Salicylaldehydes **2a - 2f**, which are not commercially available, were synthesized via Reimer - Thieman formylation of the appropriate substituted phenols **1** with $CHCl_3$ and NaOH. Aldehydes **2a - 2f** had been previously synthesized with very low yield. Briefly, reactions of intermediates **2a - 2f** with bromacetaldehyde diethylacetal in the presence of potassium carbonate in DMF (dimethylformamide) yielded compounds **3a - 3f**. Compounds **3** were cyclized to benzo[b]furan-2-ylcarboxaldehydes **4a - 4f** by heating in concentrated acetic acid. Benzo[b]furanaldehydes **4** were prepared according to the method described in literature [17]. Treatment of substituted aldehydes **4** with levulinic acid in the presence of acetic acid gave products **5a - 5f**, which were treated by hydrazine hydrate in ethanol at reflux temperature to afford the target pyridazin-3(2H)-ones **6a - 6f** [18].

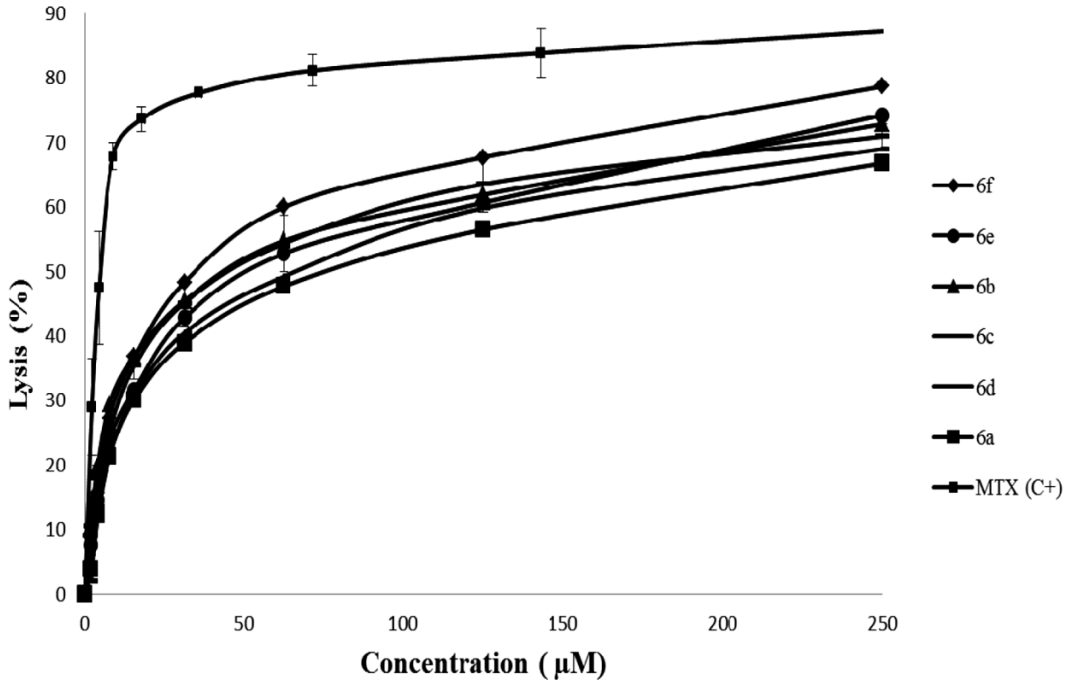

Fig. 2. *In vitro* cytotoxicity of compounds **6a - 6f** against P815 tumor cell line. Cells were treated with increasing concentrations of compounds **6a - 6f**. After 48-h incubation, cytotoxicity was determined as described in Section 2.3.2. Each value represents the mean ± SD of three independent experiments.

2.3. Pharmacological Activity

2.3.1. Cell cultures. MCF-7 (human breast adenocarcinoma) and P815 (murine mastocytoma) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL, CergyPontoise, France), penicillin G-streptomycin (1%), and 0.2% sodium bicarbonate (Sigma). Incubation was performed at 37°C in humidified atmosphere containing 5% CO_2**.**

2.3.2. Cytotoxic activity evaluation. MCF-7 and P815 cell lines were harvested from starting cultures at the exponential growth phase. After PBS wash, adherent cells were harvested from sub-confluent cultures using trypsin/EDTA (0.05%/0.02%) and suspended in DMEM. The harvested cells were poured in flat-bottomed 96-well microtiter plates containing 100 µL of complete medium per well (5×10^4 cells/mL). Three hours later, several concentrations of pyridazin-3(2H)-one derivatives in DMSO completed to 100 µL with complete DMEM were added. Control cells were treated with DMSO alone. In all cases, the final concentration of DMSO never exceeded 2%. After 48 h incubation in humidified atmosphere at 37°C and 5% CO_2, 100 µL of medium was carefully removed from each well and replaced with 20 µL MTT solution (5 mg/mL PBS). After 4 h incubation under the same conditions, the cleavage of MTT to formazan by metabolically active cells was quantified by scanning the plates at 540 nm using a Multiskan EX (Finland) apparatus. Three independent sets of experiments performed in duplicate were evaluated. The relative inhibition of cell proliferation was calculated by the following formula:

$$\% \text{ Inhibition} = 100 \times (1 - A/A_0)$$

where A_0 and A are the absorbances of negative control and test culture, respectively.

The cytotoxic effects of pyridazin-3(2H)-one derivatives against the two cell lines were compared using their IC_{50} values (concentration of tested molecules leading to 50% inhibition of cell viability).

2.3.3. Cytotoxic effect against peripheral blood mononuclear cells (PBMCs). This test was realized in order to evaluate the effect of synthesized pyridazin-3(2H)-one derivatives against normal cells using the MTT colorimetric assay described above. To isolate the human PBMCs, blood samples were collected from healthy donors in heparinized tubes and the PBMCs were isolated using standard Ficoll-hypaque density centrifugation. The interface lymphocytes were washed twice with phosphate buffer solution (PBS). Cells were incubated in 96-well microtiter plates in the presence of various concentrations of synthesized pyridazin-3(2H)-one derivatives.

2.4. Apoptosis Assay

Apoptosis induction by the most cytotoxic compound (**6f**) was evaluated using the annexin-V biotin-streptavidin FITC assay to examine the occurrence of phosphatidylserine

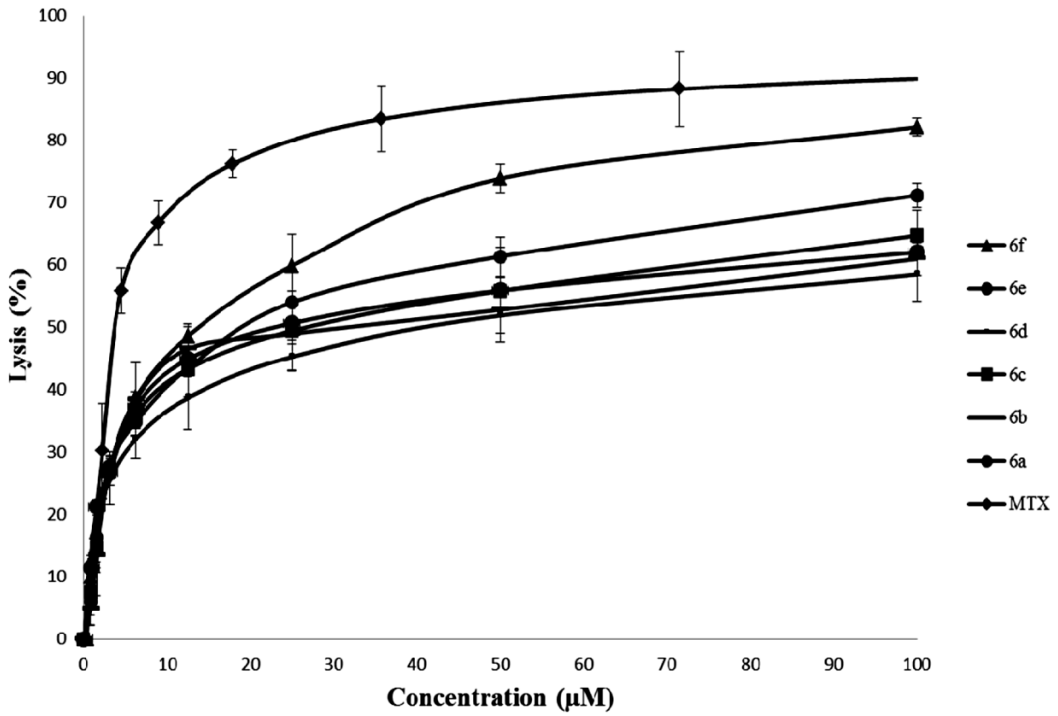

Fig. 3. *In vitro* cytotoxicity of compounds **6a - 6f** against MCF-7 tumor cell line. Cells were treated with increasing concentrations of compounds **6a - 6f**. After 48-h incubation, cytotoxicity was determined as described in Section 2.3.2. Each value represents the mean ± SD of three independent experiments.

externalization and loss of membrane integrity. Briefly, MCF-7 tumor cells (2×10^5 cells/well), treated with IC_{50} of compound **6f**, were incubated at 37°C under 5% CO_2 for 6 h. After incubation in the same culture conditions as above, cell pellets were washed twice with PBS, resuspended in Annexin-V biotin, and treated with streptavidin conjugated to FITC for 30 min. The fluorescence was visualized using an Olympus BX51 microscope equipped with an appropriate fluorescence filter in order to detect apoptosis induction [19, 20].

2.5. Statistical Analysis

Experiments were performed in duplicate and data were expressed as the mean ± SD of three experimental runs. The statistical Student's or ANOVA tests were performed on Graph Pad Prism6 Software and considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Chemistry

3.1.1. Physicochemical analysis. Melting points were determined on a Büchi SMP 20 apparatus and remained uncorrected. Infrared (IR) spectra were recorded with a VERTEX 70 FT-IR (Bruker Optics) spectrometer. The ^1H NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer using tetramethylsilane (TMS) as internal standard and $CDCl_3$ and DMSO-d_6 as solvent. The mass spectra were recorded on an API 3200 LC/MS/MS mass spectrometer using electrospray ionization (ESI) in positive polarity.

3.1.2. General procedure for the synthesis of phenols 2a - 2f (Method A).

A solution of substituted phenol **1** (0.5 mol) in 300 mL of 10 N NaOH (3 mol) was heated to 65°C. Then, 80 mL of $CHCl_3$ was added in three portions over 15 min. The mixture was heated under reflux in chloroform for 2 h. After cooling, the mixture was acidified to pH 1 with 12 N HCl, the organic layer was collected and the aqueous layer was extracted with chloroform. The combined chloroform solution was dried and evaporated to give a crude product which was distilled or recrystallized from an appropriate solvent.

2-Hydroxybenzaldehyde (**2a)**. This compound was obtained as yellowish oil; yield, 20%; bp, 75 – 77°C (0.4 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1655 (C=O); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 7.00 – 8.10 (m, 4H, H_3, H_4, H_5, H_6), 9.85 (s,1H, -CHO), 10.85 (s, 1H, exch D_2O, -OH).

2-Hydroxy-5-methylbenzaldehyde (**2b)**. This compound was obtained as white solid; yield, 95%; mp, 48 – 50°C (petroleum ether); IR (KBr; ν_{max}, cm^{-1}): 1650 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 2.37 (s, 3H, $-CH_3$), 6.90 (d, 1H, J = 8.60 Hz, H_3), 7.25 – 7.50 (m, 2H, H_4, H_6), 9.80 (s, 1H, -CHO), 10.75 (s, 1H, exch D_2O, -OH).

2-Hydroxy-5-ethylbenzaldehyde (2c). This compound was obtained as yellow oil; yield, 36%; bp, 74 – 76°C (0.3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1651 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.25 (t, 3H, J = 7.41 Hz, $-CH_2-C\underline{H}_3$), 2.70 (q, 2H, J = 7.41 Hz, $-C\underline{H}_2-CH_3$), 6.83 (d, 1H, J = 9.50 Hz, H_3), 7.26 – 7.52 (m, 2H, H_4, H_6), 9.87 (s, 1H, -CHO), 10.88 (s, 1H, exch D_2O, -OH).

2-Hydroxy-5-isoprpylbenzaldehyde (2d). This compound was obtained as yellow oil; yield, 32%; bp, 78 – 80°C (0.3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1650 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.25 (d, 6H, J = 6.70 Hz, $\underline{H}_3C-CH-C\underline{H}_3$), 2.95 (h, 1H, J = 6.70 Hz, $H_3C-C\underline{H}-CH_3$), 6.88 – 7.38 (m, 3H, H_3, H_4, H_6), 9.87 (s, 1H, -CHO), 10.88 (s, 1H, exch D_2O, -OH).

2-Hydroxy-5-methoxybenzaldehyde (2e) and 2-hydroxy-5 propoxybenzaldehyde (2f). These compounds are commercially available products.

3.1.3. General procedure for the synthesis of 2-formylphenoxyacetadehyde diethyl acetals 3a - 3f (Method B). To a stirred suspension containing substituted 2-hydroxybenzaldehyde **2** (0.15 mol) and potassium carbonate (0.16 mol) in 100 mL of DMF, bromoacetaldehyde diethyl acetal (0.16 mol) was added dropwise and the mixture was refluxed for 4 h. After cooling, the precipitate was filtered off, the solvent was evaporated under reduced pressure, and the oily residue was distilled.

2-Formylphenoxy acetaldehyde diethylacetal (3a). This compound was obtained as yellowish oil; yield, 85%; bp, 181 – 183°C (5 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; d, ppm): 1.23 (t, 6H, J = 6.42 Hz, $(-OCH_2-C\underline{H}_3)_2$), 3.51 – 4.12 (m, 4H, $(-OC\underline{H}_2-CH_3)_2$), 4.14 (d, 2H, J = 5.13 Hz, $-C\underline{H}_2-CH$), 4.89 (t, 1H, J = 5.13 Hz, $-CH_2-C\underline{H}$), 6.95 – 7.82 (m, 4H, H_3, H_4, H_5, H_6), 10.48 (s, 1H, -CHO).

(2-Formyl-4-methylphenoxy) acetaldehyde diethylacetal (3b). This compound was obtained as yellow oil; yield, 80%; bp, 192 – 194°C (5 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.25 (t, 6H, J = 6.40 Hz, $(-OCH_2-C\underline{H}_3)_2$), 2.30 (s, 3H, $-CH_3$), 3.50 – 4.00 (m, 4H, $(-OC\underline{H}_2-CH_3)_2$), 4.12 (d, 2H, J = 5.10 Hz, $-C\underline{H}_2-CH$), 4.87 (t, 1H, J = 5.10 Hz, $-CH_2-C\underline{H}$), 6.90 (d, 1H, J = 8.20 Hz, H_6), 7.40 (dd, 1H, J_1 = 2.11 Hz, J_2 = 8.20 Hz, H_5), 7.62 (d, 1H, J = 2.11 Hz, H_3), 10.50 (s, 1H, -CHO).

(2-Formyl-4-ethylphenoxy) acetaldehyde diethylacetal (3c). This compound was obtained as yellow oil; yield, 81%; bp, 198 – 200°C (5 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1690 (C=O); 2900 – 3000(C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.00 – 1.38 (m, 9H, $(-OCH_2-C\underline{H}_3)_2$, $-CH_2-C\underline{H}_3$), 2.62 (q, 2H, J = 7.61 Hz, $-C\underline{H}_2-CH_3$), 3.50 – 4.00 (m, 4H, $(-OC\underline{H}_2-CH_3)_2$), 4.08 (d, 2H, J = 4.70 Hz, $-C\underline{H}_2-CH$), 4.88 (t, 1H, J = 4.70 Hz, $-CH_2-C\underline{H}$), 6.88 (d, 1H, J = 8.50 Hz, H_6), 7.57 (dd, 1H, J_1 = 8.50 Hz, J_2 = 3.20 Hz, H_5), 7.64 (d, 1H, J = 3.20 Hz, H_3), 10.50 (s, 1H, -CHO).

(2-Formyl-4-isopropylphenoxy) acetaldehyde diethylacetal (3d).This compound was obtained as yellow oil; yield, 80%; bp, 160 – 162°C (0.3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.00 – 1.30 (m, 12H, $(-OCH_2-C\underline{H}_3)_2$, $\underline{H}_3C-CH-C\underline{H}_3$), 2.92 (h, 1H, J = 6.41Hz, $H_3C-C\underline{H}-CH_3$), 3.50 – 3.90 (m, 4H, $(-OC\underline{H}_2-CH_3)_2$), 4.10 (d, 2H, J = 5.30 Hz, $-C\underline{H}_2-CH$), 4.88 (t, 1H, J = 5.30 Hz, $-CH_2-C\underline{H}$), 6.90 (d, 1H, J = 8.51 Hz, H_6), 7.40 (dd, 1H, J_1 = 2.70, J_2 = 8.50 Hz, H_5), 7.70 (d, 1H, J = 2.70 Hz, H_3), 10.50 (s, 1H, -CHO).

(2-Formyl-4-methoxyphenoxy) acetaldehyde diethylacetal (3e). This compound was obtained as yellow oil; yield, 68%; bp, 154 – 156°C (0.3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.25 (t, 6H, J = 6.40 Hz, $(-OCH_2-C\underline{H}_3)_2$), 2.38 (s, 3H, $-OCH_3$), 3.45 – 4.30 (m, 4H, $(-OC\underline{H}_2-CH_3)_2$), 4.42 (d, 2H, J = 5.10 Hz, $-C\underline{H}_2-CH$), 4.97 (t, 1H, J = 5.10 Hz, $-CH_2-C\underline{H}$), 6.95 (d, 1H, J = 8.21 Hz, H_6), 7.45 (dd, 1H, J_1 = 2.11, J_2 = 8.21 Hz, H_5), 7.65 (d, 1H, J = 2.11 Hz, H_3), 10.56 (s, 1H, -CHO).

(2-Formyl-4-propoxyphenoxy) acetaldehyde diethylacetal (3f). This compound was obtained as yellow oil; yield, 67%; bp, 180 – 183°C (0.5 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.01 (t, 3H, J = 7.21 Hz, $-OCH_2-CH_2-C\underline{H}_3$), 1.24 (t, 6H, J = 6.91 Hz, $(-OCH_2-C\underline{H}_3)_2$), 1.78 (m, 2H, $-OCH_2-C\underline{H}_2-CH_3$), 3.69 (m, 4H, $(-OC\underline{H}_2-CH_3)_2$), 3.90 (t, 2H, J = 6.21 Hz, $-OC\underline{H}_2-CH_2-CH_3$), 4.07 (d, 2H, J = 5.11 Hz, $-C\underline{H}_2-CH$), 4.83 (t, 1H, J = 5.11 Hz, $-CH_2-C\underline{H}$), 6.76 – 7.31 (m, 3H, H_3, H_5, H_6), 10.46 (s, 1H, -CHO).

3.1.4. General procedure for the synthesis of substituted benzo[b]furan-2-yl carboxaldehydes 4a - 4f (Method C). A stirred solution of compound **3** (0.1 mol) in 35 mL of concentrated acetic acid was refluxed for 24 h. After cooling, the solution was evaporated to dryness and the crude product was distilled or recrystallized from an appropriate solvent.

2-Formylbenzo[b]furan (4a). This compound was obtained as yellow oil; yield, 75%; bp, 121 – 123°C (3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1680 (C=O); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 7.26 – 7.74 (m, 5H, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{6'}$, $H_{7'}$), 9.84 (s, 1H, -CHO).

2-Formyl-5-methylbenzo[b]furan (4b).This compound was obtained as yellow solid; yield, 66%; mp, 26 – 27°C (ehanol/water 9/1); IR (KBr; ν_{max}, cm^{-1}): 1680 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 2.38 (s, 3H, $-CH_3$), 7.40 – 7.77 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{6'}$, $H_{7'}$), 9.87 (s, 1H, -CHO).

5-Ethyl-2-formylbenzo[b]furan (4c).This compound was obtained as yellowish oil; yield. 80%; bp, 130 – 132°C

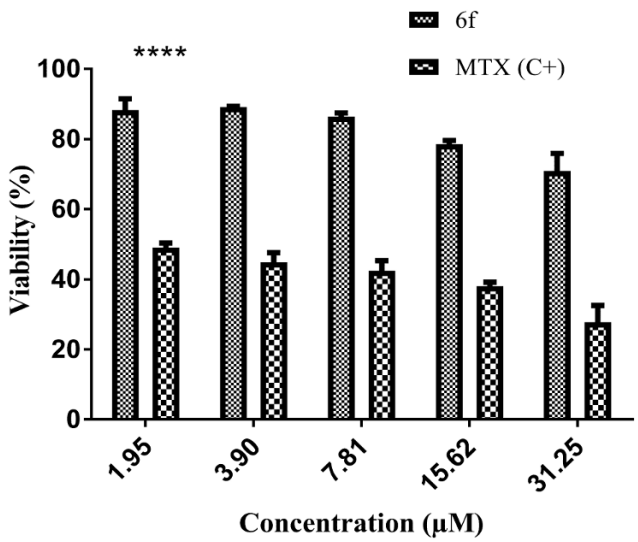

Fig. 4. Cytotoxicity of compound **6f** against PBMCs from healthy donors (****p values < 0.0001 indicate significant difference).

(3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1680 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.30 (t, 3H, J = 7.90 Hz, -CH_2-C$\underline{H}_3$), 2.76 (q, 2H, J = 7.90 Hz, -C$\underline{H}_2$-CH_3), 7.25 – 7.70 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{6'}$, $H_{7'}$), 9.86 (s, 1H, -CHO).

2-Formyl-5-isopropylbenzo[b]furan (4d). This compound was obtained as yellowish oil; yield, 56%; bp, 131 – 133°C (3 mmHg); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 1.28 (d, 6H, J = 6.30 Hz, $\underline{H}_3$C-CH-C$\underline{H}_3$), 3.00 (h, 1H, J = 6.30 Hz, H_3C-C$\underline{H}$-CH_3), 7.48 – 7.63 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{6'}$, $H_{7'}$), 9.85 (s, 1H, -CHO).

2-Formyl-5-methoxybenzo[b]furan (4e). This compound was obtained as yellow solid; yield, 70%; mp, 82 – 84 (diisopropyl ether); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 2.41 (s, 3H, -OCH_3), 7.43 – 7.80 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{6'}$, $H_{7'}$), 9.89 (s, 1H, -CHO).

2-Formyl-5-propoxybenzo[b]furan (4f). This compound was obtained as yellow solid; yield, 85%; mp, 65 – 67°C (diisopropyl ether); IR (KBr; ν_{max}, cm^{-1}): 1700 (C=O), 2900 – 3000 (C-H); ^1H NMR ($CDCl_3$, 400 MHz; δ, ppm): 0.97 (t, 3H, J = 7.20 Hz, -OCH_2-CH_2-C$\underline{H}_3$), 1.73 (m, 2H, -OCH_2-C$\underline{H}_2$-CH_3), 3.94 (t, 2H, J = 6.60, -OC$\underline{H}_2$-CH_2-CH_3), 7.15 (dd, 1H, J_1 = 9.10 Hz, J_2 = 2.70 Hz, $H_{6'}$), 7.31 (d, 1H, J = 2.70 Hz, $H_{4'}$), 7.61 (d, 1H, J = 9.10 Hz, $H_{7'}$), 7.86 (s, 1H, $H_{3'}$), 9.79 (s, 1H, -CHO).

3.1.5. General procedures for the synthesis of substituted 3-benzo[b]furan-2-ylmethylene-levulinic acids 5a - 5f (Method D). A stirred solution of compound **4** (0.1 mol) in 35 mL of concentrated acetic acid was refluxed for 24 h. After cooling, the solution was evaporated to dryness and obtained residue was used crude for continuation of the drug synthesis.

3.1.6. General procedures for the synthesis of substituted 5-(benzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2H)-ones 6a - 6f (Method E). The mixture of acid **5** and hydrazine hydrate solution in ethanol was refluxed for 2h. The precipitate was filtered and recrystallized from an appropriate solvent.

5-(Benzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2 H)-one (6a). This compound was obtained as yellow solid; yield, 69%; mp, 181 – 183°C (ethanol); IR (KBr; ν_{max}, cm^{-1}): 1604 (C=N), 1662 (C=O), 2900 – 3000 (C-H); ^1H NMR ($DMSOd_6$, 400 MHz; δ, ppm): 2.22 (s, 3H, -N=C-CH_3), 4.08 (s, 2H, -CH_2-), 6.97 (s, 1H, H_4), 6.71 (s, 1H, $H_{3'}$), 7.19 – 7.58 (m, 4H, $H_{4'}$, $H_{5'}$, $H_{6'}$, $H_{7'}$), 12.71 (ls, 1H, NH); MS (*m/z*): 241.2 $[M+H]^+$, 238.9 $[M+H]^-$, 263.3 $[M+Na]^+$.

5-(5-Methylbenzo[b]furan-2-ylmethyl)-6-methylpyri dazin-3(2H)-one (6b). This com-pound was obtained as yellow solid: yield, 80%; mp, 183 – 184°C (ethanol); IR (KBr; ν_{max}, cm^{-1}): 1603 (C=N), 1666 (C=O), 2900 – 3000 (C-H); ^1H NMR ($DMSOd_6$, 400 MHz; δ, ppm): 2.23 (s, 3H, -N=C-CH_3), 2.37 (s, 3H, Ar-CH_3), 4.07 (s, 2H, -CH_2-), 6.58

TABLE 2. Cytotoxic Activity of Pyridazin-3(2H)-one Derivative **6f** against P815 Cells

Compound	Lysis (%)								
	Concentration (µM)								
	1,95	3,90	7,81	15,62	31,25	62,50	125	250	IC_{50}
6a	3,80 ± 0,13	12,57 ± 0,86	21,35 ± 1,62	30,12 ± 2,36	38,89 ± 3,12	47,66 ± 3,87	56,43 ± 4,65	66,705 ± 7,49	82,5 µM
6b	10,50 ± 4,91	19,73 ± 3,28	29,43 ± 2,05	36,84 ± 2,19	45,41 ± 3,07	54,72 ± 2,78	61,85 ± 1,29	72,78 ± 3,08	42,5 µM
6c	1,92 ± 2,58	11,45 ± 2,83	21,12 ± 2,89	30,78 ± 2,96	40,45 ± 3,03	49,19 ± 1,78	59,78 ± 3,16	68,96 ± 2,52	66 µM
6d,	10,62 ± 4,60	17,96 ± 2,01	25,17 ± 1,36	35,38 ± 2,14	57,08 ± 13,33	64,84 ± 10,49	63,49 ± 4,28	70,88 ± 1,93	46 µM
6e	7,62 ± 3,246	14,67 ± 3,79	23,84 ± 4,47	31,64 ± 2,77	42,79 ± 3,45	52,76 ± 2,68	60,63 ± 3,02	74,18± 2,905	50 µM
6f	8,82 ± 1,18	15,68 ± 2,12	27,19 ± 2,14	36,73 ± 2,12	48,29 ± 2,16	59,93 ± 1,92	67,63 ± 3,08	80,34 ± 2,44	35 µM
MTX	24,17 ± 0,28	34,46 ± 5,36	44,463 ± 5,36	60,81 ± 1,75	77,69 ± 0,93	78,35 ± 0,93	79,56 ± 1,71	81,23 ± 3,13	7,5 µM

(s, 1H, H_4), 6.64 (s, 1H, $H_{3'}$), 7.07 (dd, 1H, J_1 = 8.41 Hz, J_2 = 1.21 Hz, $H_{6'}$), 7.36 (d, 1H, J = 1.21 Hz, $H_{4'}$), 7.40 (d, 1H, J = 8.41 Hz, $H_{7'}$), 12.74 (ls, 1H, NH); MS (*m/z*): 255.1 $[M+H]^+$, 253.3 $[M+H]^-$, 276.9 $[M+Na]^+$.

5-(5-Ethylbenzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2H)-one (**6c).** This com-pound was obtained as brown solid; yield, 73%; mp, 180 – 181°C (ethanol); IR (KBr; ν_{max}, cm^{-1}): 1605 (C=N), 1661 (C=O), 2900 – 3000 (C-H); ^{1}H NMR ($DMSOd_6$, 400 MHz; δ, ppm): 1.18 (t, 3H, J = 7.50 Hz, $-CH_2-C\underline{H}_3$), 2.21 (s, 3H, $-N=C-CH_3$), 2.65 (q, 2H, J = 7.50 Hz, $-C\underline{H}_2-CH_3$),), 4.05 (s, 2H, $-CH_2-$), 6.56 (s, 1H, H_4), 6.64 (s, 1H, $H_{3'}$), 7.08 (dd, 1H, J_1 = 8.40 Hz, J_2 = 1.80 Hz, $H_{6'}$), 7.37 (d, 1H, J = 1.80 Hz, $H_{4'}$), 7.41 (d, 1H, J = 8.40 Hz, $H_{7'}$), 12.76 (ls, 1H, NH); MS (*m/z*): 269.5 $[M+H]^+$, 267.1 $[M+H]^-$, 291.3 $[M+Na]^+$.

5-(5-Isopropylbenzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2H)-one (**6d).** This compound was obtained as brown solid; yield, 82%: mp, 185 – 186°C (ethanol); IR (KBr; ν_{max}, cm^{-1}): 1605 (C=N), 1682 (C=O), 2900 – 3000 (C-H); ^{1}H NMR ($DMSOd_6$, 400 MHz; δ, ppm): 1.24 (d, 6H, J = 6.90 Hz, $\underline{H}_3C-CH-C\underline{H}_3$), 2.10 (s, 3H, $-N=C-CH_3$), 2.99 (h, 1H, J = 6.90 Hz, $H_3C-C\underline{H}-CH_3$), 3.90 (s, 2H, $-CH_2-$), 7.30 – 7.76 (m, 5H, H_4, $H_{3'}$, $H_{4'}$, $H_{6''}$, $H_{7'}$), 12.32 (ls, 1H, NH); MS (*m/z*): 283.4 $[M+H]^+$, 281.2 $[M+H]^-$, 337.4 $[M+Na]^+$.

5-(5-Methoxybenzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2H)-one (**6e)**. This compound was obtained as yellow solid; yield, 78%; mp, 190 – 192°C (ethanol); IR (KBr; ν_{max}, cm^{-1}): 1650 (C=O), 2950 – 3000 (C-H); ^{1}H NMR ($DMSOd_6$, 400 MHz; δ, ppm): 2.25 (s, 3H, $-N=C-CH_3$), 3.74 (s, 3H, $-OCH_3$), 4.04 (s, 2H, $-CH_2-$), 6.55 (s, 1H, H_4), 6.64 (s, 1H, $H_{3'}$), 6.82 (dd, 1H, J = 8.70 and 2.71 Hz, $H_{6'}$), 7.08 (d, 1H, J = 2.71 Hz, $H_{4'}$), 7.40 (d, 1H, J = 8.70 Hz, $H_{7'}$), 12.75 (ls, 1H, NH); MS (*m/z*): 299.4 $[M+H]^+$, 297.1 $[M+H]^-$, 321.4 $[M+Na]^+$.

5-(5-Propoxybenzo[b]furan-2-ylmethyl)-6-methylpyridazin-3(2H)-one (**6f)**. This compound was obtained as brown solid; yield, 87%; mp, 194 – 196°C (ethanol); IR (KBr; ν_{max}, cm^{-1}): 1652(C=O), 2940 – 3000(C-H); ^{1}H NMR ($DMSOd_6$, 400 MHz; δ, ppm): 0.96 (t, 3H, J = 6.90 Hz, $-OCH_2-CH_2-C\underline{H}_3$), 1.71 (m, 2H, $-OCH_2-C\underline{H}_2-CH_3$), 2.21 (s, 3H, $-N=C-CH_3$), 3.90 (t, 2H, J = 6.90 Hz, $-OC\underline{H}_2-CH_2-CH_3$), 4.04 (s, 2H, $-CH_2-$), 6.55 (s, 1H, H_4), 6.62 (s, 1H, $H_{3'}$), 6.81 (dd, 1H, J = 9.00 and 2.71 Hz, $H_{6'}$), 7.06 (d, 1H, J = 2.71 Hz, $H_{4'}$), 7.38 (d, 1H, J = 9.00 Hz, $H_{7'}$), 12.74 (ls, 1H, NH).

3.2. Cytotoxic Activity

Biological evaluation of pyridazin-3(2H)-one derivatives **6a - 6f** using the MTT assay showed that compounds **6b, 6e** and **6f** exhibited potent cytotoxicity against both tumor cell lines (MCF7 and P815) in the micromolar range, whereas the other three compounds (**6a, 6c**, and **6d)** exhibited moderate cytotoxicity as compared to that of the former compounds and positive control (methotrexate, MTX). Analysis of results suggested that the activity was dose- and cell-dependent. Comparison of data presented in Figs. 2 and 3 allow the six compounds to be classified on the basis of their cytotoxicity levels as **6f > 6e > 6a > 6c > 6b > 6d** against MCF-7 cell line and as **6f > 6b > 6d > 6e > 6c > 6a** against P815 cell line. The obtained IC_{50} values (Tables 2a and 2b) ranged from 14.5 to 40 μM for MCF-7 and from 35 to 82.5 μM for P815. Compound **6f** bearing the propoxy ($OCH_2CH_2CH_3$) group in the radical position showed maximum activity, i.e., the lowest IC_{50} values (14.5 and 35 μM for MCF-7 and P815, respectively).

3.3. Cytotoxicity of Tested Molecules with Respect to Human Normal Cells

The majority of clinically approved anticancer drugs are characterized by a narrow therapeutic window that results mainly from a high systemic toxicity of drugs. Thus, we tested the most important compounds (which were more cytotoxic against tumor cells) with respect to human peripheral blood mononuclear cells (PBMCs) in order to determine their effect against normal cells. The results presented in Fig. 4 show the absence of any cytotoxic effect of com-

TABLE 3. Cytotoxic Activity of Pyridazin-3(2H)-one Derivative **6f** against MCF-7 Cells

Compound	Lysis (%)								
	Concentration (μM)								
	0,78125	1,5625	3,125	6,25	12,5	25	50	100	IC_{50}
6a	11,51 ± 2,33	21,26 ± 6,49	26,71 ± 1,67	37,28 ± 5,91	44,90 ± 3,52	50,65 ± 0,94	55,92 ± 5,19	62,06 ± 2,66	23 μM
6b	4,71 ± 2,56	13,37 ± 3,28	27,29 ± 2,48	38,44 ± 3,29	46,58 ± 2,76	48,9 5 ± 2,53	52,81 ± 1,21	60,94 ± 1,05	31 μM
6c	7,51 ± 2,75	14,62 ± 3,14	26,97 ± 2,32	36,14 ± 2,46	43,30 ± 4,42	49,41 ± 6,42	55,86 ± 6,88	64,73 ± 3,94	28 μM
6d	12,31 ± 1,14	18,90 ± 3,39	25,50 ± 5,82	32,09 ± 3,92	38,69 ± 3,05	45,28 ± 5,19	51,88 ± 2,14	58,47 ± 4,23	40 μM
6e	6,06 ± 3,87	16,36 ± 5,71	27,86 ± 2,20	34,83 ± 2,19	43,44 ± 1,42	54,04 ± 4,80	61,35 ± 3,16	71,17 ± 2	19 μM
6f	9,94 ± 1,28	17,05 ± 4,73	27,61 ± 0,65	38,68 ± 0,93	48,55 ± 1,98	59,84 ± 5,09	73,87 ± 2,24	82,14 ± 1,37	14,5 μM
MTX	19,72 ± 7,63	26,18 ± 3,66	30,18 ± 3,50	61,38 ± 2,20	70,72 ± 5,29	76,183 ± 6,02	80,40 ± 2,92	90,21 ± 5,39	4,9 μM

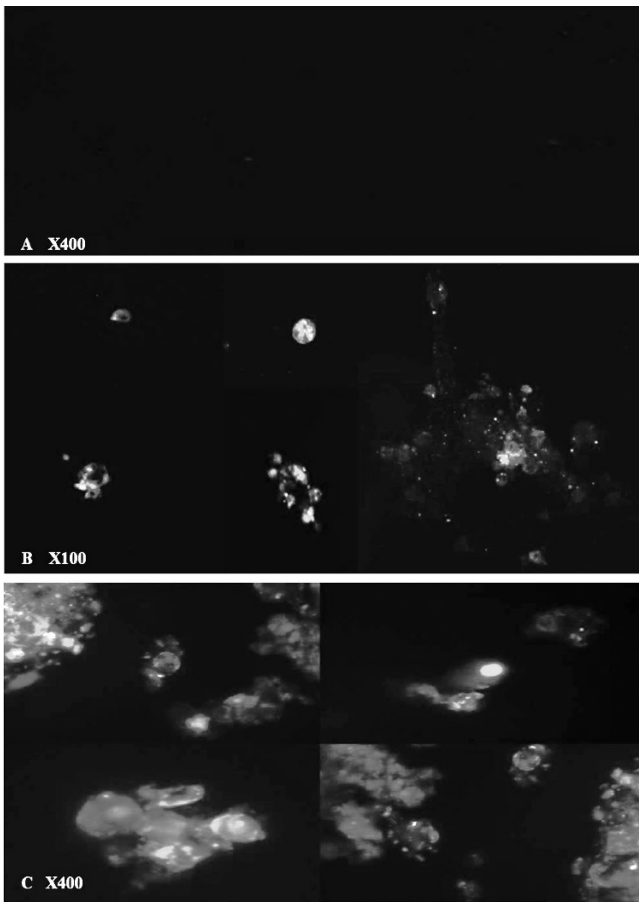

Fig. 5. Apoptosis induction analysis by annexin-V FITC test on MCF7 tumor cells.

pounds tested on normal PBMCs, thus indicating a selective cytotoxicity of these substances.

3.4. Annexin-V Biotin-Streptavidin FITC Analysis for Apoptosis Assay

In order to understand the molecular mechanisms involved in the observed cytotoxic activity of the most cytotoxic compound **(6f)**, apoptosis induction assay was performed using the MCF-7 cell line and analyzed by Annexin-V Biotin-Streptavidin staining as described in materials and methods. The results are reported in Fig. 5. As shown in this figure, the product **6f** induced a significant cell apoptosis.

MCF-7 cells were treated with the IC_{50} of compound **6f** (**C**) after 6-h incubation. The assay was based on the ability of annexin-V (green fluorescence) to bind to phosphatidylserine exposed on the surface of cells undergoing apoptosis. Cells cultured in a medium without serum were used as the positive control (**B**). Cells were treated with DMSO alone as negative control (**A**). The technique was performed as described in materials and methods.

During the last decade, many interesting synthetic compounds such as pyridazin-3(2H)-ones draw the attention of medicinal chemists due to their potential bioactivity. In fact, the handleable functional structure of pharmacophore moieties in pyridazinones makes them an attractive source of new active derivatives [21, 22]. To the best of our knowledge, the combination of benzofuran and pyridazinones is of this kind and has led to good results for the first time. Indeed, our results demonstrated the ability of newly synthesized pyridazin-3(2H)-ones derivatives to induce cytotoxicity against human breast adenocarcinoma (MCF-7) and the murine mastocytoma (P815) tumor cell lines (Figs. 2 and 3) suggesting no specificity of organs. These results corroborate recent findings showing a cytotoxic activity of other pyridazin-3(2H)-one derivatives against lung cancer, central nervous system cancer, leukemia, and pancreatic cell lines [23, 24, 25].

More importantly, the most active compound **6f** did not show any cytotoxic effect against human PBMCs from healthy donors. These PBMCs considered as normal cells were used to evaluate the side effects of this product (Fig. **4**). These results suggest that this compound acts selectively against tumor cells.

Considering the structure–activity relationship, the substitution at C_5 in the benzofuran ring was of interest. In fact, the increase in the aliphatic chain size in radical in the C_5 of benzofuran enhanced the cytotoxic effect of yridazin-3(2H)-one derivatives against both MCF7 and P815 cell lines. Furthermore, as shown in Fig. 2 and 3, compound **6f** was the most active among all tested pyridazinones. The interesting cytotoxic effect of **6f** can probably be attributed to the propoxy radical, which is characterized by its long aliphatic chain associated with oxygen molecule. These results are in agreement with data demonstrating that compounds substituted with this radical (propoxy) are relatively potent as cytotoxic agents [26].

The search for new anti-tumor molecules employs various approaches. The known approaches involving antimitotics, antimetabolites as well as alkylating and intercalating agents are currently classified as classical. However, new developing approaches include the inducers of apoptosis/cellular senescence, molecules that control the cell cycle, those that interfere with cell signaling, and agents controlling cellular resistance, metastasis development, and angiogenesis [27]. Among these new approaches, we are interested in that based on the induction of apoptosis.

Apoptosis is the very tightly programmed cell death with distinct biochemical and genetic pathways that play a critical role in the development and homeostasis in normal tissues [28]. Upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive changes occur in the cell. A family of proteins known as caspases is typically activated in the early stages of apoptosis. These proteins cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair en-

zymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

During the apoptotic process, apoptotic cells display distinctive morphology. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. The apoptotic breakdown of chromatin in the nucleus often leads to nuclear condensation and/or a "horseshoe" like appearance [28]. In order to correlate between the cytotoxic activity of the compound 6f and induction of apoptosis, cells were treated for 6 h with this compound and then studied using the annexin-V/FITC test with fluorescence microscopy. Figure **5** shows a series of changes such as membrane blebbing, cell shrinkage, and apoptotic body formation. These data confirmed that cells treated with compound **6f** were involved in the apoptotic process. Other molecular mechanisms should also be investigated.

4. CONCLUSION

The obtained data showed an interesting cytotoxic activity of the newly synthesized pyridazin-3(2H)-one with propoxy radical substitution. This substitution elongating the aliphatic chain enhances the structure reactivity. Generally, all the obtained compounds exhibited promising cytotoxic effect that was especially pronounced for compound **6f,** which was highly cytotoxic against P815 and MFC-7 cell lines (with IC_{50}=35 and 14,5 μM, respectively). It is important that this compound produces no cytotoxic action against normal PBMCs. The interesting cytotoxic effect of **6f** may probably be attributed to the presence of propoxy radical. Furthermore, the morphological alteration of MCF-7 cells observed using the annexin-V/FITC test indicated that compound **6f** acted in part via apoptosis induction. In addition, further studies should be conducted to explore the molecular mechanisms underneath the cytotoxic activity, in vivo investigation and the interaction effects between this synthetizes compounds with conventional drugs.

ACKNOWLEDGEMENTS

The authors are grateful to the University Mohammed V and the Laboratory of Control of Drugs, Rabat-Morocco, for measuring IR, NMR and mass spectra.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. S.-K. Kim (ed.) in: *Handbook of Anticancer Drugs from Marine Origin,* Springer, Switzerland (2015), (p.) 1.
2. C. E. DeSantis, F. Bray, J. Ferlay, et al., *Cancer Epidemiol., Biomarkers Prev.,* **24**(10), 1495 – 1506 (2015).
3. K. Sato, S. Watanabe, and A. Ohtsubo, *BMC Cancer,* **16**(1), 222 (2016).
4. S. Zhu, N. Pabla, C. Tang, et al., *Arch. Toxicol,* **89**(12), 2197 – 2205 (2015).
5. G. S Oh, H. J. Kim, A. Shen, et al., *Electrolyte Blood Pressure,* **12**(2), 55 – 65 (2014).
6. S. Ovais, K. Javed, S. Yaseen, et al., *Eur. J. Med. Chem,* **67**, 352–358 (2013).
7. M. S. R. Murty, K. R. Ram, R. V. Rao, et al., *Med. Chem. Res,* **20**(5), 576 – 586 (2011).
8. M. B. Boxer, J. K. Jiang, M. G. Vander Heiden, et al., *J. Med. Chem.,* **53**(3), 1048 – 1055 (2010).
9. V. Pogacic, A. N. Bullock, O. Fedorov, et al., *Cancer Res.,* **67**(14), 6916 – 6924 (2007).
10. K. Natarajan, J. Bhullar, S. Shukla, et al., *Biochem. Pharmacol.,* **85**(4), 514 – 524 (2013).
11. L. S. Chen, S. Redkar, P. Taverna, et al., *Blood,* **118**(3), 693 – 702 (2011).
12. J. M. Foulks, K. J. Carpenter, B. Luo, et al., *Neoplasia,* **16**(5), 403 – 412 (2014).
13. L. Brault, S. Knapp, L. Brault, et al., *Br. J. Cancer,* **107**(3), 491 – 500 (2012).
14. M. Asif, *Int. J. Adv. Chem,* **2**(2), 148 – 161 (2014).
15. I. G. Rathish, K. Javed, S. Ahmad, et al., *Eur. J. Med. Chem*., **49**, 304 – 309 (2012).
16. R. Bansal and S. Thota, *Med. Chem. Res.,* **22**(6), 2539 – 2552 (2013).
17. T. Hirota, H. Fujita, K. Sasaki, et al., *J. Heterocycl. Chem.,* **23**(6), 1715 – 1716 (1986).
18. A. Benmoussa, J. El harti, M. Ansar, et al., *Int. J. PharmTech Res*., **4**(4), 1591 – 1594 (2012).
19. M. Tilaoui, H. A. Mouse, A. Jaafari, et al., *Nat. Prod. Bioprospect.*, **4**(3), 189 – 196 (2014).
20. R. Sen, K. Natarajan, J. Bhullar, et al., *Mol. Cancer Ther.,* **11**(9), 2033 – 2044 (2012).
21. M. A. Ibrahim, A. H. Elmenoufy, M. Elagawany, et al., *J. Biosci. Med*., **3**(10), 59 (2015).
22. R. Bansal and S. Thota, *Med. Chem. Res.,* **22**(6), 2539 – 2552 (2013).
23. I. G. Rathish, K. Javed, S. Ahmad, et al., *Eur. J. Med. Chem*., **49**, 304 – 309 (2012).
24. A. Pau, G. Murineddu, B. Asproni, et al., *Molecules,* **14**(9), 3494 – 3508 (2009).
25. W. Malinka, A. Redzicka, and O. Lozach, *Farmaco,* **59**(6), 457 – 462 (2004).
26. B. Wang, Z. W. Miao, J. Wang, et al., *Amino Acids,* **35**(2), 463 – 468 (2008).
27. R. A. Lockshin and C. M. Williams, *J. Insect Physiol*., **11**(2), 123 – 133 (1965).
28. M. Hassan, H. Watari, A. AbuAlmaaty, et al., *BioMed Res. Int*., **2014**, 1 – 23 (2014).