Pharmaceutical Sciences, 2025, 31(2), 179-186
doi:10.34172/PS.025.40811
https://ps.tbzmed.ac.ir/

Research Article

Cytotoxicity and Pro-apoptosis Activity of Synthetic Phenylacetamide Derivatives on Cancer Cells

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Article Info

Article History:
Received: 26 Aug 2024
Accepted: 12 Jan 2025
ePublished: 15 Mar 2025

Keywords:
-Apoptosis
-Cancer cells
-Cytotoxicity
-Phenylacetamide derivatives

Abstract

Background: Despite advancements in cancer treatments, patient survival rates have not significantly improved due to drug resistance and side effects. Developing new anticancer drugs is crucial to providing more options and increasing chances of recovery, ultimately improving patient quality of life and global healthcare. We evaluated the cytotoxicity and pro-apoptosis activity of eleven phenylacetamide derivatives.

Methods: The derivatives were first synthesized and characterized before being tested on MCF7, MDA-MB468, and PC12 cell lines using the MTT assay. We also assessed their potential anticancer effects through the TUNEL assay, caspase 3 activity testing, and real-time PCR.

Results: Phenylacetamide derivatives, specifically **3d** derivative, are highly effective against cancer cells by triggering apoptosis through upregulation of Bcl-2, Bax, FasL RNA expression, and caspase 3 activity. It has shown an IC_{50} value of 0.6±0.08 µM against MDA-MB-468 and PC-12 cells, while **3c** and **3d** also had significant cytotoxic effects against MCF-7 cells with IC_{50} values of 0.7±0.08 µM and 0.7±0.4 µM, respectively.

Conclusion: Notably, phenylacetamide derivatives have shown great promise in controlling the growth and death of cancer cells. These derivatives have been found to possess the remarkable ability to stimulate both internal and external apoptotic pathways, which could greatly benefit conventional cancer treatment methods.

Introduction

Cancer treatment primarily involves chemotherapy, which utilizes synthetic drugs. However, these drugs are associated with severe side effects and are often administered at suboptimal levels. Several approaches have been explored in the quest to discover new compounds with chemotherapeutic properties, including extraction from plants and animals, molecular modeling, and synthetic and combinatorial chemistry.[1] The ability of many chemotherapeutic agents to trigger apoptosis is believed to be crucial to their antitumor effectiveness. New methods to induce apoptosis in cancer cells could pave the way for developing novel anticancer agents. Furthermore, these treatments may also prove effective in overcoming tumor resistance to conventional antitumor agents.[2-9]

Apoptosis is a vital process in the growth and development of multicellular organisms. However, if it malfunctions, it can lead to abnormal development and pathogenesis. The hallmarks of apoptosis include cell contraction, chromosomal DNA segmentation, and chromatin compression. Two primary pathways are involved in apoptosis: extrinsic and intrinsic. In the extrinsic pathway, their ligand activates death receptors like DR3, DR4, TNFαR, and Fas, triggering caspase 8 and executive caspases 3 and 7. In the intrinsic pathway, mitochondrial outer membrane permeability is regulated by Bcl-2 family proteins. Following the release of mitochondrial cytochrome c, activation of caspases such as caspase 9 and executive caspases occurs.[10, 11]

Recent research has uncovered evidence that Phenylacetate (PA) and other related aromatic fatty acids may hold great promise in the fight against various types of cancer. Several studies have demonstrated that PA exhibits in vivo antitumor activity in humans and can induce apoptosis both in vitro and in vivo. In addition, researchers have found that PA exhibits low toxicity at clinically effective doses, making it a potentially safer option for cancer treatment. To build on these promising findings, scientists have synthesized several derivatives of phenylacetic acid that show great potential as effective anticancer agents.[12-14]

Our study aimed to find new cancer treatments using

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phenylacetamide derivatives to induce cytotoxic and pro-apoptotic effects on cancer cell lines.

Methods

Synthesis of phenylacetamide derivatives (3a-3k)

One mmol of the derivative of acetophenone (**1a-1k**) was mixed with *N*-ethyl-*N´*-dimethylaminopropylcarbodiimide (EDC) and hydroxybenzotriazole (HOBt) in a 50 ml flask containing 20 ml of acetonitrile solvent. The resultant mixture was stirred for 30 minutes to synthesize the derivatives (**3a-3k**). After this, one mmol of the amino compound (**2**) was added to the reaction mixture and stirred at room temperature for 24 h. Upon the completion of the reaction, the solvent was removed by rotary. After adding a combination of water and ethyl acetate to the residue, the organic phase was isolated and rinsed twice with a sodium solution. Finally, the isolated organic phase was dehydrated by sodium sulfate, then filtered and dried by rotary. Ultimately, the final products were obtained with high purity and efficiency (Table 1 and Figure 1).

N-Butyl-2-(2-chlorophenyl)acetamide (3a)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.87 (t, 3H, $-CH_3$), 1.29 (m, 2H, $-CH_2-CH_2-\underline{C}H_2-CH_3$), 1.39 (m, 2H, $-CH_2-\underline{C}H_2-CH_2-CH_3$), 3.21 (q, 2H, $-\underline{C}H_2-CH_2-CH_2-CH_3$), 3.67 (s, 2H, $-\underline{C}H_2-CO-$), 5.44 (brs, NH), 7.34 (m, 2H, 2-chlorophenyl), 7.40 (m, 2H, 2-chlorophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.66 (C_a, CH_3), 19.92 (C_b, CH_2), 31.48 (C_C, CH_2), 39.43 (C_e, CH_2), 41.53 (C_d, CH_2), 127.33 (C_5-phenyl), 128.87 (C_4-phenyl), 129.76 (C_2-phenyl), 131.68 (C_3-phenyl), 133.20 (C_6-phenyl), 134.37 (C_1-phenyl), 169.49 ($\underline{C}$=O). IR (KBr, cm^{-1}) ῡ: 3302 (stretch, NH), 3066 (stretch, C-H, aromatic), 2956, 2929, 2870 (stretch, C-H, aliphatic), 1645 (stretch, C=O).

N-Butyl-2-(3-chlorophenyl)acetamide (3b)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.88 (t, 3H, $-CH_3$), 1.28 (m, 2H, $-CH_2-CH_2-\underline{C}H_2-CH_3$), 1.42 (m, 2H, $-CH_2-\underline{C}H_2-CH_2-CH_3$), 3.20 (q, 2H, $-\underline{C}H_2-CH_2-CH_2-CH_3$), 3.51 (s, 2H, $-\underline{C}H_2-CO-$), 5.56 (brs, NH), 7.15 (m, 4H, 3-chlorophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.68 (C_a, CH_3), 19.98 (C_b, CH_2), 31.46 (C_C, CH_2), 39.52 (C_e, CH_2), 43.16 (C_d, CH_2), 127.37 (C_2-phenyl), 127.46 (C_6-phenyl), 129.39 (C_4-phenyl), 130.05 (C_5-phenyl), 134.53 (C_3-phenyl), 137.08 (C_1-phenyl), 170.24 ($\underline{C}$=O). IR (KBr, cm^{-1}) ῡ: 3302 (stretch, NH), 3064 (stretch, C-H, aromatic), 2960, 2931, 2872 (stretch, C-H, aliphatic), 1645 (stretch, C=O).

N-Butyl-2-(4-chlorophenyl)acetamide (3c)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.88 (t, 3H, $-CH_3$), 1.27 (m, 2H, $-CH_2-CH_2-\underline{C}H_2-CH_3$), 1.38 (m, 2H, $-CH_2-\underline{C}H_2-CH_2-CH_3$), 3.20 (q, 2H, $-\underline{C}H_2-CH_2-CH_2-CH_3$), 3.51 (s, 2H, $-\underline{C}H_2-CO-$), 5.56 (brs, NH), 7.19 (d, 2H, J = 8 Hz, $H_{2,6}$-4-chlorophenyl), 7.31 (d, 2H, J = 8 Hz, $H_{3,5}$-4-chlorophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.66 (C_a, CH_3), 19.96 (C_b, CH_2), 31.48 (C_C, CH_2), 39.52 (C_e, CH_2), 42.96 (C_d, CH_2), 129.05 ($C_{2,6}$-phenyl), 130.70 ($C_{3,5}$-phenyl), 133.44 (C_4-phenyl), 170.41 ($\underline{C}$=O). IR (KBr, cm^{-1}) ῡ: 3300 (stretch, NH), 3064 (stretch, C-H, aromatic), 2958, 2931, 2870 (stretch, C-H, aliphatic), 1647 (stretch, C=O). MS (*m/z*): 227 (M^++2, 10), 225 (M^+, 3).

N-Butyl-2-(2-fluorophenyl)acetamide (3d)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.86 (t, 3H, $-CH_3$), 1.26 (m, 2H, $-CH_2-CH_2-\underline{C}H_2-CH_3$), 1.40 (m, 2H, $-CH_2-\underline{C}H_2-CH_2-CH_3$), 3.19 (q, 2H, $-\underline{C}H_2-CH_2-CH_2-CH_3$), 3.51 (s, 2H, $-\underline{C}H_2-CO-$), 5.70 (brs, NH), 7.04-7.15 (m, 2H, J = 8 Hz, $H_{4,6}$-2-fluorophenyl), 7.26-7.33 (m, 2H, J = 8 Hz, $H_{3,5}$-2-fluorophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.66 (C_a, CH_3), 19.91 (C_b, CH_2), 31.50 (C_C, CH_2), 36.93 (C_e, CH_2), 39.46 (C_d, CH_2), 115.39, 115.73 (d, J = 21.39 Hz, C_3-phenyl), 122.17 (C_1-phenyl), 124.56 (C_5-phenyl), 129.27 (C_4-phenyl), 131.68 (C_6-phenyl), 162.86 (C_2-phenyl) 169.70 ($\underline{C}$=O). IR (KBr, cm^{-1}) ῡ: 3298 (stretch, NH), 3086 (stretch, C-H, aromatic), 2953, 2929, 2868 (stretch, C-H, aliphatic), 1645 (stretch, C=O).

N-Butyl-2-(3-fluorophenyl)acetamide (3e)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.88 (t, 3H, $-CH_3$), 1.28 (m, 2H, $-CH_2-CH_2-\underline{C}H_2-CH_3$), 1.42 (m, 2H, $-CH_2-\underline{C}H_2-CH_2-CH_3$), 3.21 (q, 2H, $-\underline{C}H_2-CH_2-CH_2-CH_3$), 3.57 (s, 2H, $-\underline{C}H_2-CO-$), 5.51 (brs, NH), 6.95-7.03 (m, 3H, $H_{4,5,6}$-3-fluorophenyl), 7.25-7.33 (m, 1H, H_2-3-fluorophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.66 (C_a, CH_3), 19.96 (C_b, CH_2), 31.49 (C_C, CH_2), 39.50 (C_e, CH_2), 43.39

Table 1. Physical and chemical properties of the phenylacetamide derivatives.

Compound	R	MW (g/ml)	Chemical Formula	Yield (%)	mp (°C)
3a	2-F	382	$C_{12}H_{16}FNO$	75	51
3b	3-F	398	$C_{12}H_{16}FNO$	72	51
3c	4-F	398	$C_{12}H_{16}FNO$	78	63
3d	2-Cl	393	$C_{12}H_{16}ClNO$	83	66
3e	3-Cl	393	$C_{12}H_{16}ClNO$	85	50
3f	4-Cl	393	$C_{12}H_{16}ClNO$	90	69
3g	2-OCH_3	379	$C_{13}H_{19}NO_2$	86	47
3h	4-OCH_3	379	$C_{13}H_{19}NO_2$	92	95
3i	2-NO_2	379	$C_{12}H_{16}N_2O_3$	76	75
3j	4-NO_2	408	$C_{12}H_{16}N_2O_3$	73	114
3k	4-Br	440	$C_{12}H_{16}BrNO$	80	82

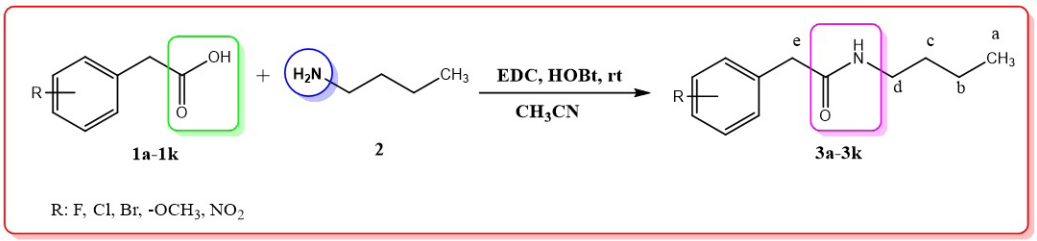

Figure 1. Synthetic pathway of phenylacetamide derivatives **3a-3k**.

(C_d, CH_2), 114.06, 114.39 (d, J = 20.8 Hz, C_4-phenyl), 116.14, 116.48 (d, J = 21.4 Hz, C_2-phenyl), 125.03 (C_6-phenyl), 130.33, 130.46 (d, J = 8.2 Hz, C_5-phenyl), 137.49 (C_1-phenyl), 160.99, 164.93 (d, J = 247.8 Hz, C_3-phenyl), 170.16 (C=O). IR (KBr, cm^{-1}) ῡ: 3302 (stretch, NH), 3082 (stretch, C-H, aromatic), 2958, 2933, 2872 (stretch, C-H, aliphatic), 1633 (stretch, C=O).

N-Butyl-2-(4-fluorophenyl)acetamide (3f)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.87 (t, 3H, $-CH_3$), 1.27 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 1.41 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.20 (q, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.52 (s, 2H, $-CH_2$-CO-), 5.41 (brs, NH), 7.03 (t, 2H, $H_{2,6}$-4-fluorophenyl), 7.23 (m, 1H, $H_{3,5}$-4-fluorophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.67 (C_a, CH_3), 19.95 (C_b, CH_2), 31.51 (C_C, CH_2), 39.45 (C_e, CH_2), 42.86 (C_d, CH_2), 115.61, 115.95 (d, J = 21.39 Hz, $C_{3,5}$-phenyl), 130.85, 130.98 (d, J = Hz, $C_{2,6}$, C_1), 160.09, 164.00 (d, J = 8.2 Hz, C_4), 170.67 (C=O). IR (KBr, cm^{-1}) ῡ: 3244 (stretch, NH), 3066 (stretch, C-H, aromatic), 2962, 2931, 2875 (stretch, C-H, aliphatic), 1656 (stretch, C=O). MS (*m/z*, %): 209 (M^+, 20).

N-Butyl-2-(2-methoxyphenyl)acetamide (3g)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.86 (t, 3H, $-CH_3$), 1.25 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 1.38 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.17 (q, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.55 (s, 2H, $-CH_2$-CO-), 3.84 (s, 3H, $-OCH_3$), 5.67 (brs, NH), 6.88-6.96 (m, 2H, $H_{3,5}$-2-methoxyphenyl), 7.21-7.30 (m, 2H, $H_{4,6}$-2-methoxyphenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.68 (C_a, CH_3), 19.86 (C_b, CH_2), 31.52 (C_C, CH_2), 38.7 (C_e, CH_2), 39.2 (C_d, CH_2), 55.34 ($-OCH_3$), 110.64 (C_3-phenyl), 121.05 (C_5-phenyl), 123.78 (C_1-phenyl), 128.78 (C_4-phenyl), 131.30 (C_5-phenyl), 157.16 (C_2-phenyl), 171.24 (C=O). IR (KBr, cm^{-1}) ῡ: 3296 (stretch, NH), 3074 (stretch, C-H, aromatic), 2953, 2929, 2868 (stretch, C-H, aliphatic), 1647 (stretch, C=O).

N-Butyl-2-(4-methoxyphenyl)acetamide (3h)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.87 (t, 3H, $-CH_3$), 1.26 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 1.39 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.18 (q, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.51 (s, 2H, $-CH_2$-CO-), 3.81 (s, 3H, $-OCH_3$), 5.37 (brs, NH), 6.89 (d, 2H, J = 8 Hz, $H_{3,5}$-4-methoxyphenyl), 7.16 (d, 2H, J = 8 Hz, $H_{2,6}$-4-methoxyphenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.68 (C_a, CH_3), 19.95 (C_b, CH_2), 31.50 (C_C, CH_2), 39.41 (C_e, CH_2), 42.83 (C_d, CH_2), 55.27 ($-OCH_3$), 114.41 ($C_{3,5}$-phenyl), 126.88 (C_1-phenyl), 130.55 ($C_{2,6}$-phenyl), 158.85 (C_4-phenyl), 171.44 (C=O). IR (KBr, cm^{-1}) ῡ: 3263 (stretch, NH), 3076 (stretch, C-H, aromatic), 2960, 2931, 2872 (stretch, C-H, aliphatic), 1643 (stretch, C=O). MS (*m/z*, %): 221 (M^+, 40).

N-Butyl-2-(2-nitrophenyl)acetamide (3i)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.88 (t, 3H, $-CH_3$), 1.31 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 1.45 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.21 (q, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.80 (s, 2H, $-CH_2$-CO-), 5.95 (brs, NH), 7.40-7.49 (m, 2H, $H_{4,6}$-2-nitrophenyl), 7.58 (t, 1H, H_5-2-nitrophenyl), 8.01 (d, 1H, J = 8 Hz, H_3-2-nitrophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.67 (C_a, CH_3), 19.94 (C_b, CH_2), 31.50 (C_C, CH_2), 39.53 (C_e, CH_2), 40.86 (C_d, CH_2), 125.02 (C_3-phenyl), 128.29 (C_4-phenyl), 130.54 (C_1-phenyl), 133.36 (C_5-phenyl), 148.86 (C_2-phenyl), 168.83 (C=O). IR (KBr, cm^{-1}) ῡ: 3288 (stretch, NH), 3089 (stretch, C-H, aromatic), 2954, 2929, 2870 (stretch, C-H, aliphatic), 1645 (stretch, C=O), 1529 (stretch, asymmetric, NO_2), 1346 (stretch, symmetric, NO_2).

N-Butyl-2-(4-nitrophenyl)acetamide (3j)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.89 (t, 3H, $-CH_3$), 1.30 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 1.45 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.24 (q, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.62 (s, 2H, $-CH_2$-CO-), 5.56 (brs, NH), 7.46 (d, 2H, J = 8 Hz, $H_{2,6}$-4-nitrophenyl), 8.19 (d, 1H, J = 8 Hz, $H_{3,5}$-4-nitrophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.67 (C_a, CH_3), 19.98 (C_b, CH_2), 31.53 (C_C, CH_2), 39.65 (C_e, CH_2), 43.36 (C_d, CH_2), 123.95 ($C_{3,5}$-phenyl), 130.18 ($C_{2,6}$-phenyl), 142.51 (C_1-phenyl), 147.18 (C_4-phenyl), 168.96 (C=O). IR (KBr, cm^{-1}) ῡ: 3305 (stretch, NH), 3061 (stretch, C-H, aromatic), 2960, 2931, 2866 (stretch, C-H, aliphatic), 1647 (stretch, C=O), 1537 (stretch, asymmetric, NO_2), 1352 (stretch, symmetric, NO_2). MS (*m/z*, %): 236 (M^+, 10).

2-(4-Bromophenyl)-N-butylacetamide (3k)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 0.88 (t, 3H, $-CH_3$), 1.28 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 1.41 (m, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.21 (q, 2H, $-CH_2-CH_2-CH_2-CH_3$), 3.50 (s, 2H, $-CH_2$-CO-), 5.36 (brs, NH), 7.14 (d, 2H, J = 8 Hz, $H_{2,6}$-4-bromophenyl), 7.48 (d, 1H, J = 8 Hz, $H_{3,5}$-4-bromophenyl). ^{13}CNMR ($CDCl_3$, 62.90 MHz) δ (ppm): 13.67 (C_a, CH_3), 19.96 (C_b, CH_2), 31.51 (C_C, CH_2), 39.51 (C_e, CH_2), 43.12 (C_d, CH_2), 121.32 (C_1-phenyl), 131.08 ($C_{2,6}$-phenyl), 132.04 ($C_{3,5}$-phenyl), 133.97 (C_4-phenyl), 170.19 (C=O). IR (KBr, cm^{-1}) ῡ: 3302 (stretch, NH), 3061 (stretch, C-H, aromatic), 2956, 2931, 2870 (stretch, C-H, aliphatic), 1647 (stretch, C=O). MS (*m/z*): 269 (M^+).

Cell culture

The synthesized derivatives were dissolved in DMSO to create a stock solution with varying concentrations, including 0.125 µM, 0.25 µM, 0.5 µM, and 1 µM. As required, the solution was diluted in DMEM medium (Gibco, DMEM- F /12, USA); the final DMSO concentration was kept below 0.1%.

To culture human cancer cells, MCF7, MDA-MB468, and PC12 cells were grown in DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA). The cells were kept in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Once the cells reached more than 70% confluency, they were washed with PBS and incubated with the trypsin-EDTA solution for 3 min at 37°C to detach them from the flask. The cells were then re-suspended in the culture medium for seeding. The medium was changed every 2-3 days, and the cells were sub-cultured when the population density reached 70-80% confluence.

Cell viability assay

MTT colorimetric assay was performed to determine the effect of phenylacetamide derivatives on MCF7, MDA-MB468, and PC12 cell lines. Cells were cultured in 96 well microplates and incubated for 24 hours at 37°C. Then, fresh media containing phenylacetamide derivatives at varying concentrations (0.125, 0.25, 0.5, and 1 µM) were added and incubated for another 48 hours. After washing with PBS, 20 µL of 5 mg/mL MTT was added, followed by incubation for 3 hours. Formazan crystals were dissolved by adding 100 µL of Dimethyl Sulfoxide (DMSO) and shaking for 10 minutes. Finally, the absorbance was measured at 570 nm by an ELISA plate reader Infinite F500 (Tecan, Autriche).

TUNEL assay

Following the manufacturer's instructions, we detected DNA fragmentation in MCF7, MDA-MB468, and PC12 cell lines using the TUNEL Assay Kit (Titer TACS, R&D System, USA). The cells were treated with IC_{50} concentration of 3d compound for 24 h, labeled with the TdT reaction mix, and the colorimetric reaction was measured at absorbances 450 nm and 630 nm using a spectrophotometer (BioTek Synergy, USA). Negative controls were labeled without the TdT enzyme, and positive controls were created using TACS-Nuclease™ to create DNA breaks.

Caspase 3 activity

The cells (4×10^4 cells) were plated on a 96-well plate and incubated with IC_{50} concentration of 3d derivative for 24 hours. Caspase 3 activity was measured in triplicate using the Caspase-3 Colorimetric Assay Kit (Promega, USA), and the results were recorded at 400-450 nm using a spectrophotometer (BioTek Synergy, USA).

RNA extraction and cDNA synthesis

We extracted total RNA from 1×10^7 cells using a SinaPure™ RNA kit (SinaClon, Iran), dissolved the RNA pellet in 50 µl RNase-free water, and kept it in aliquots at -80°C. RNA integrity was confirmed via agarose gel electrophoresis. Total RNA was used for first-strand cDNA synthesis using the First Strand cDNA Synthesis kit (SinaClon, Iran). The reaction mix included 1 µg RNA, 1 µl oligo(dT), 15-20 pmol reverse transcription primer, 1 µl dNTP mix, 0.5 µl M-MuLV reverse transcriptase RNase H, 2 µl 10X buffer M-MuLV, 0.5 µl RNase inhibitor, and DEPC water up to a total volume of 10 µl. The reverse transcription reaction was incubated at 42°C for 60 minutes and terminated at 85°C for 5 minutes.

Quantitative polymerase chain reaction (qPCR)

The research employed gene-specific primers (Pishgam Biotech Co. Iran) to amplify mRNA transcripts of Bax, Bcl-2, and FasL genes, along with Glyceraldehyde 3-phosphate dehydrogenase (GADPH) as a reference gene. The Bcl-2 specific primers produced a 114 base pair (bp) PCR amplicon, the Bax specific primers generated an amplicon of 99 bp, and the FasL primers resulted in a product of 101 bp (Table 2).

Reverse transcription-qPCR analysis was done using a Corbet Research, UK thermal cycler. The reaction mixture was used consisting of 1 µg cDNA, 1 µl primers, 10 µL qPCR Master Mix, and DEPC water, with a total volume of 20 µl. The reactions were performed in duplicate with specific conditions, which included a polymerase activation step at 95°C for 15 min, 40 cycles of denaturation at 95°C for 45 seconds, and primer annealing, extension, and fluorescence detection at 60°C for 1 min.

Gene expression analysis was conducted using the comparative Ct ($2-\Delta\Delta Ct$) method. The study concluded that Relative Quantification (RQ) units accurately reflected the normalized expression of the target genes.

Statistical analysis

The graphs display mean ± SEM values. Biological triplicates were used for experiments. Statistical analyses used GraphPad PRISM 8.0 software (GraphPad Software, Germany). Two-tailed t-tests or one-sample t-tests were utilized for all statistical analyses. Statistical significance was determined by p values of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Table 2. Specific primer sequences for Real-Time PCR.

Primer	Sequence
Bcl-2 Forward	5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′
Bcl-2 Reverse	5′- GGTGCCGGTTCAGGTACTCAGTCA -3′
Bax Forward	5′- CCTGTGCACCAAGGTGCCGGAACT-3′
Bax Reverse	5′-CCACCCTGGTCTTGGATCCAGCCC-3′
FasL Forward	5′-GCAATGAAACTGGGCTGTACTTT-3′
FasL Reverse	5′-GGAGTTCCTCATGTAGACCTTGT-3′

Results and Discussion

Structure-activity relationship (SAR)

The synthesized phenylacetamide derivatives demonstrated potent cytotoxic effects (Table 3) on all tree cancerous cell lines (MDA-MB468, PC12, MCF7). Various substituents with different electronic impacts were investigated on the phenyl residue. F, Cl, Br, NO_2, and -OCH_3 were utilized on all three possible positions of the phenyl ring. Compound **3j** with *para* nitro group caused a strong cytotoxic effect against MDA-MB468 cells compared to other derivatives (IC_{50} = 0.76±0.09 µM). None of the tested derivatives rendered higher activity than doxorubicin (IC_{50} = 0.38±0.07 µM) toward this cell line. Evaluation of the fluorine atom as a potent electron-withdrawing moiety against MDA-MB468 showed the best activity at position *meta* (compound **3b**, IC_{50} = 1.5±0.12 µM). The best position for the chlorine atom was the *para* position (compound **3f**, IC_{50} = 1±0.13 µM) for cytotoxicity. Examining the methoxy moiety as an electron-donating substituent caused a robust cytotoxic activity at the *ortho* position (compound **3g**, IC_{50} = 1.3±0.03 µM). Substitution of the fluorine and bromine atoms at position *para* (compounds **3c**, **3k**) induced an extremely detrimental effect on the cytotoxic activity. Chlorine atom when substituted at position *meta* (compound **3e**) possessed the highest cytotoxic effect against PC12 cells (IC_{50} = 0.67±0.12 µM). Fortunately, the observed potency was superior to doxorubicin (IC_{50} = 2.6±0.13 µM) on this cell line. Introduction of the potent electronegative moieties like fluorine and nitro at the position *ortho* of the phenyl residue (compounds **3a** and **3i**) afforded remarkable cytotoxic derivatives with higher potency than doxorubicin. This potentiating effect was also seen for *para* methoxylated compound (**3h**, IC_{50} = 1.73±0.13 µM). MCF7 cells as the representative of breast cancer were applied and some of the tested derivatives inhibited their growth and caused the cytotoxic effect higher than doxorubicin. *Meta* fluorinated derivative (**3b**) and methoxylated derivatives (**3g** and **3h**) induced a significant cytotoxicity against MCF7 cells.

Cytotoxic effect of phenylacetamide derivatives

The study aimed to investigate 11 phenylacetamide

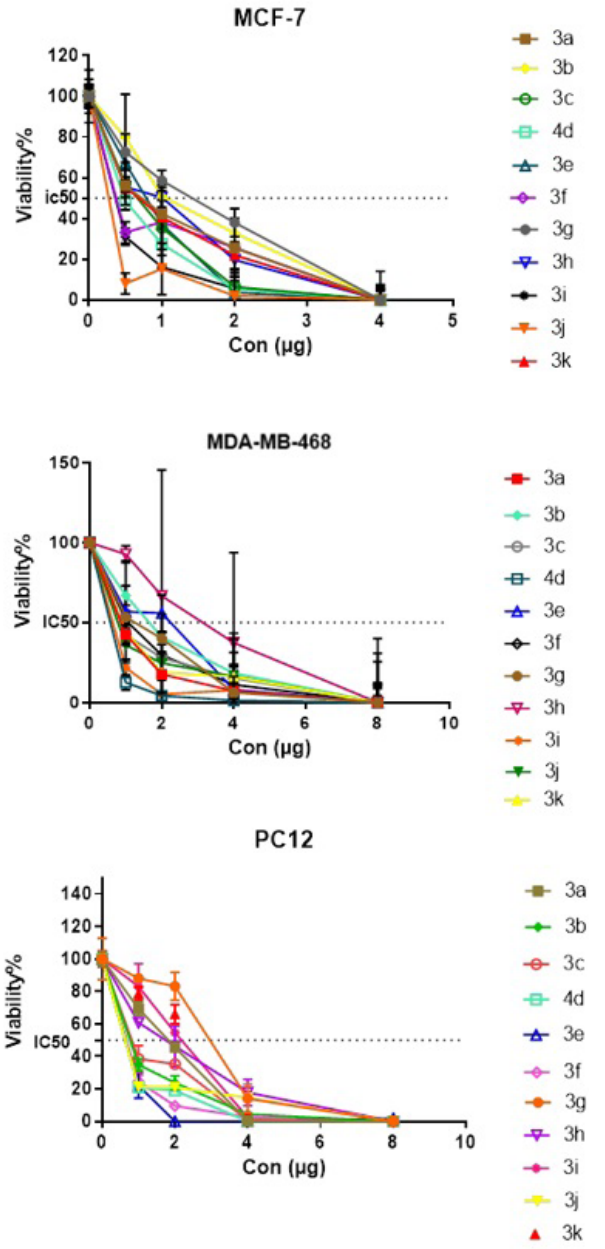

Figure 2. Cytotoxic effect of 11 phenylacetamide derivatives on MCF7, MDA-MB468, and PC12 cell lines by MTT assay. Results were expressed as Mean ± SEM of three independent experiments compared to the control group (DMSO-treated cell lines).

Table 3. IC_{50} (µM) values for the cytotoxic activity of 11 phenylacetamide derivatives on MCF7, MDA-MB468, and PC12 cell lines.

Compound	R	MDA-MB468	PC12	MCF7
3a	2-F	8±0.07	1.83±0.05	9±0.07
3b	3-F	1.5±0.12	77±0.08	1.5±0.06
3c	4-F	87±0.05	8±0.06	7±0.08
3d	2-Cl	6±0.08	6±0.07	7±0.4
3e	3-Cl	2.2±0.07	0.67±0.12	9±0.09
3f	4-Cl	1±0.13	7±0.09	ND
3g	2-OCH_3	1.3±0.03	2.97±0.07	1.53±0.12
3h	4-OCH_3	3.13±0.06	1.73±0.13	1.4±0.12
3i	2-NO_2	6±0.4	2.20±0.43	ND
3j	4-NO_2	0.76±0.09	6±0.4	ND
3k	4-Br	87±0.13	2.50±0.13	85±0.09
Doxorubicin	-	0.38±0.07	2.6±0.13	2.63±0.4

derivatives' effects on MCF7, MDA-MB468, and PC12 cell lines. To determine the IC_{50} value, we conducted an MTT assay using different concentrations of synthesized compounds. The results indicated that the phenylacetamide derivatives inhibited the growth of the cell lines in a dose-dependent manner. Notably, the 3d derivative demonstrated the highest rate of cell death at a lower concentration than the other derivatives (Table 3 and Figure 2).

The effect of phenylacetamide derivative on DNA fragmentation

The TUNEL test showed that exposure to the 3d derivative caused a rise in apoptotic cells in all cancer cell lines compared to the control group. In addition, Figure 3 demonstrated that the compound significantly increased DNA fragmentation in PC12 cells when compared to the negative control group. These results suggest that the compound induced apoptosis in the cells.

The effect of phenylacetamide derivative on caspase 3 activity

After conducting a colorimetric assay to measure caspase 3 activity, it was observed that the 3d derivative (in IC_{50} concentration) caused a significant increase in Caspase 3 activity in three cell lines compared to their control groups after 24 hours (Figure 4).

The effect of phenylacetamide derivative on the expression of Bax, Bcl-2, and FasL genes

After performing Real-Time PCR, the study analyzed mRNA levels of Bax, Bcl-2, and FasL genes. Results in Figure 5 show that the MCF7 cell line had increased expressions of Bax and FasL genes after 3d derivative treatment, while the PC12 cell line had a significaninduction of Baxgene expression. In contrast, the MDA-MB468 cell line increased the expressions of both Bcl-2 and FasL genes, with a significant rise observed in the expression of the FasL gene.

Extensive research has revealed that specific derivatives of phenylacetamide possess remarkable anticancer

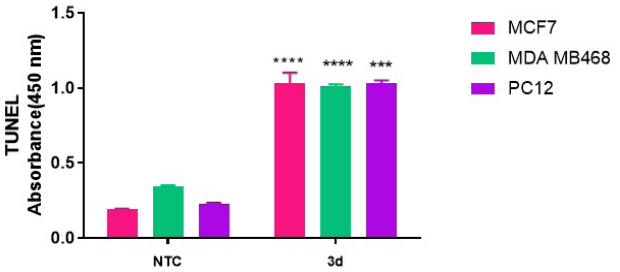

Figure 3. TUNEL assay for cell apoptosis investigation in MCF7, MDA-MB468, and PC12 cell lines after treatment with IC_{50} concentration of 3d compound for 24h. The results are expressed as the average of independent experiments in comparison with the negative control group (NTC, cell line treated with DMSO).
*Indicates Pvalue <0.05, ** Indicates Pvalue <0.01, *** Indicates Pvalue <0.001 and **** Indicates value < 0.0001.

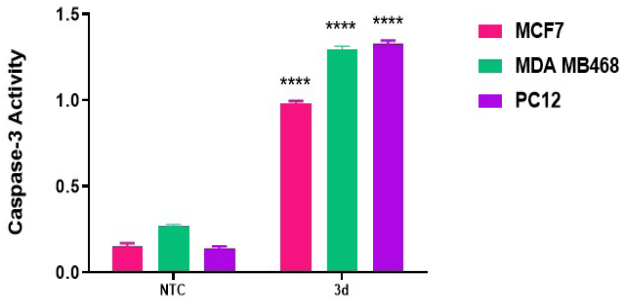

Figure 4. Effect of IC_{50} concentration of 3d derivative for 24h on caspase-3 activity in three cell lines PC12, MDA MB468 and MCF7. The results were expressed as Mean ± SEM of three independent experiments (n = 3) in comparison with the negative control group (NTC, 3 cell lines treated with DMSO).
*Indicates P value <0.05, ** Indicates P value <0.01, *** Indicates P value <0.001 and **** Indicates value < 0.0001

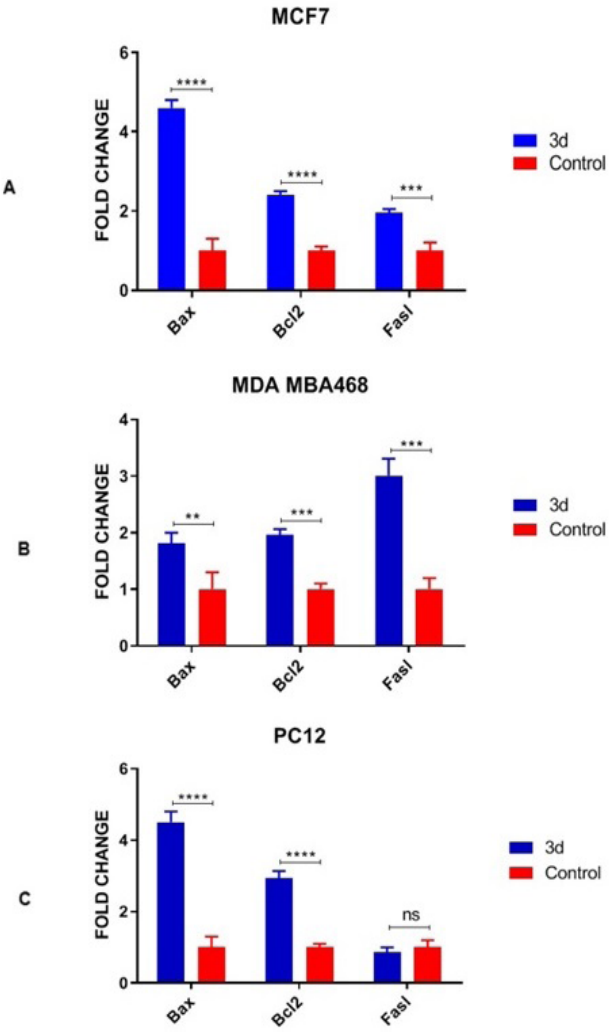

Figure 5. Comparison of the fold changes in RNA expression levels of Bax, Bcl-2 and FasL genes in A) MCF7 B) MDA-MB468 and C) PC12 cell lines after treatment with IC_{50} concentration of 3d compound for 24h. The results were expressed as Mean ± SEM of three independent experiments (n = 3) compared to the negative control group (3 cell lines treated with DMSO).
*Indicates Pvalue <0.05, ** Indicates Pvalue <0.01, *** Indicates Pvalue <0.001 and **** Indicates value < 0.0001

properties.[15-21] Therefore, this study examined the effects of 11 different phenylacetamide derivatives on several cell lines, including MCF7, MDA-MB468, and PC12. The results obtained from this study were impressive, as the phenylacetamide derivatives demonstrated antiproliferative effects on all three cell lines in a dose-dependent manner (Figure 1). Among all the tested compounds, the **3d** derivative exhibited significant efficacy in reducing cell viability compared to other compounds and even the standard drug doxorubicin (Table 1). Notably, this compound contains a remarkable ortho chloro-moiety responsible for its effectiveness on these cell lines.

Apoptosis is an essential process that cytotoxic drugs utilize to destroy tumor cells[22] and is the primary goal of cancer treatment.[23] Apoptosis is a programmed cell death that can be initiated through the extrinsic or intrinsic pathway. Extrinsic pathways, such as Fas/FasL, TNF-α/TNFR1, and TRAIL/D4/D5, can activate effector caspases like caspase-3. Meanwhile, various signals can trigger the intrinsic pathway, including extracellular stimuli that activate caspase-8. BH3-only proteins like Noxa and Puma use anti-apoptotic Bcl-2 family proteins to alleviate inhibition of Bax and Bak, activating them.[24] Bax can stimulate the release of cytochrome c and Smac-diablo, promoting apoptosis. Bcl-2 can prevent cytochrome c release, counteracting Bax-mediated cell death by forming homo-dimers and hetero-dimers.[25,26] The balance between homo- and hetero-dimers of Bcl-2 and Bax proteins impact apoptosis levels. Bax-homodimers promote it, while Bcl-2/Bax hetero-dimers can counteract it.[27]

In this study, we sought to investigate the mRNA expression levels of the Bax, Bcl-2, and FasL genes, which are critical in apoptosis, better to comprehend the effects of phenylacetamide derivatives on cancer cells. The findings of this investigation were particularly compelling, showing that the mRNA levels of these genes increased in all three cell lines after treatment with the 3d derivative for 24 hours. Furthermore, it was discovered that the **3d** derivative effectively increased both Bax and Bcl-2 expressions simultaneously, which is a significant finding (Figure 4).

It has been discovered that caspase 3, an essential executive caspase, plays a pivotal role in the progression of apoptosis through intrinsic and extrinsic pathways.[28] Based on our results, the activity of caspase 3 was increased in all three cell lines, indicating that the 3d derivative induced apoptosis in these cells effectively (Figure 3).

A comparison of the current project with other reported works like phenylacetamides bearing aromatic residue demonstrated that aliphatic derivatives caused higher cytotoxic activity. Former reported phenylacetamides incorporated phenyl or 1,3,4-thiadiazole, did not exhibit a significant cytotoxic effect.[16,17,19] The introduction of an alkyl side chain such as butyl moiety enhanced the cytotoxic effects. Increasing the lipophilicity of the residue on the amide functional group is a favorite parameter for the improvement of the cytotoxic potency. Fortunately, the currently studied compounds activated the caspase enzymes as observed with the 1,3,4-thiadiazole-based phenylacetamides. Certainly, the activation of caspases and induction of apoptosis is one of the likely mechanisms for phenylacetamide derivatives.

Finally, we confirmed DNA fragmentation activation using the TUNEL assay that demonstrated apoptosis induction. Our study found that the 3d derivative induces apoptosis in MCF-7, MDA-MB-468, and PC-12 cancer cell lines through extrinsic and intrinsic apoptotic pathways, with remarkable outcomes (Figure 2).

Conclusion

The study findings indicate that 11 phenylacetamide derivatives have antiproliferative effects on MCF7, MDA-MB468, and PC12 cell lines. The **3d** phenylacetamide derivative has demonstrated notable effectiveness in inhibiting proliferation and inducing apoptosis in these cancer cells. Research also suggests this compound's treatment can activate intrinsic and extrinsic apoptosis signaling pathways in three cancer cell lines. These encouraging results warrant further investigation to assess this compound's potential as a new anticancer agent for tumor treatment.

Author Contribution

Omid Tavallei: Data Curation, Methodology. **Shayesteh Rezazadeh:** Investigation, Writing-Original Draft. **Marzieh Marzbany:** Investigation, Writing - Review & Editing. **Alireza Aliabadi:** Conceptualization, Project Administration, Funding Acquisition.

Acknowledgments

The authors appreciate the research council of Kermanshah University of Medical Sciences for financial support (proposal No. 980373).

Conflict of Interest

The authors report no conflicts of interest.

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