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Research Article

Synthesis, Characterization, and Biological Evaluation of Gabapentinoid Hybrids with Isoindole-1,3(2H)-Dione Moiety as Potential Antioxidant, Antimicrobial, and Anticancer Agents

Mirna Jabbour,[1] **Mohammad Ammar Al-Khayat**,[2] **and Mahmoud Al-Ktaifani**[3]

[1]*Department of Pharmaceutical Chemistry and Quality Control, Faculty of Pharmacy, Damascus University, Damascus, Syria*
[2]*Department of Pharmaceutical Chemistry and Drug Quality Control, Faculty of Pharmacy, Arab International University (AIU), Ghabagheb, Syria*
[3]*Department of Radioisotopes, Head of Organic Synthesis Division, Atomic Energy Commission of Syria, Damascus, Syria*

Correspondence should be addressed to Mirna Jabbour; mirna88_j@yahoo.com

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Purpose. To synthesize new isoindole-1,3(2H)-dione derivatives by molecular hybridization of gabapentin and pregabalin with phthalic anhydride derivatives and to evaluate their biological activity as promising antioxidant, antimicrobial, and anticancer agents. *Method*. Molecular hybridization was successfully achieved by two procedures; synthesized compounds were characterized using analytical and spectral methods. The free radical scavenging properties of synthesized compounds were evaluated using the DPPH method. The antibacterial activity of synthesized compounds and parent compounds was evaluated against two microbial Gram-positive and Gram-negative strains by the well diffusion method. Furthermore, we have studied the effect of compounds on proliferation, cell cycle, and cell death in two human cancer cell lines (Caco-2 and HCT-116). *Results*. Compounds **1**, **3**, and **4** exhibited a good free radical scavenging effect, and compound **3** is the most effective with IC_{50} value of 2.525 μmol/mL. All compounds showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* related to concentration, while parent drugs did not exhibit any antibacterial effect. Compounds **1** and **2** showed a good zone of inhibition against *E. coli* at micromolar concentrations, and they are more effective than Gentamicin Sulfate. Treatment with the studied compounds suppresses proliferation, arrests progress throughout the cell cycle, and induces apoptosis in Caco-2 and HCT-116 cancer cells. Compound **2** is highly effective against Caco-2 cells and more effective than thalidomide, with IC_{50} value less than 1 μmol/L. *Conclusion*. Our results showed that molecular hybridization of gabapentin and pregabalin in the isoindole-1,3(2H)-dione moiety results in promising anticancer and antimicrobial molecules. Results of this preliminary study show that halogenation of the isoindole-1,3(2H)-dione moiety improves antimicrobial and anticancer activity and that tetra-brominated derivatives are comparable to or more effective than related tetra-chlorinated derivatives.

1. Introduction

Research for new, safe, and effective drugs has been a continuous need over the years, especially since there are still challenging diseases with no sufficient treatments, such as cancer, neurodegenerative diseases, autoimmune diseases, and infectious diseases related to resistant microorganisms.

Therapeutic choices for complex, heterogeneous diseases can be very limited, considering that one drug is not sufficient to control the illness in an effective way, which demands the use of combinations of pharmaceuticals with different pharmacotherapeutic profiles. In this context, molecular hybridization has emerged as a new strategy in medicinal chemistry for rational drug design based on the fusion of pharmacophoric units of active parent compounds, leading to a new hybrid architecture containing characteristics of both original active compounds.

The molecular hybridization approach increases the possibility of generating new active molecules with optimized pharmacokinetic and pharmacodynamic properties

compared with parent compounds, taking into consideration the advantage of available data about the bioactive profile, targets, and pharmacokinetic and pharmacodynamic properties of parent compounds due to the high percentage of structure similarity and homology [1–3].

The structural diversity and biological importance of nitrogen-containing heterocycles have made them attractive targets for synthesis over the years. Among heterocyclic scaffolds, isoindole-1,3(2H)-dione is of particular interest in drug discovery and drug development.

This moiety has been described as a multitarget structure with different biological effects, and many compounds of this scaffold are effective antimicrobial [4, 5] and anti-inflammatory agents [6, 7], anticonvulsants [8, 9], antivirals [10], antioxidants [11], xanthine oxidase inhibitors [12], and carbonic anhydrase inhibitors [12]. Many compounds of this moiety are effective for the treatment of Leishmaniasis [13], diabetes [14, 15], hyperlipidemia [14, 16], cancer [5, 11, 17, 18], and Alzheimer's disease [19–21]. Recently, many compounds of this moiety were synthesized and studied as SARS-CoV-2 virus, for the treatment of the COVID-19 pandemic [22, 23].

Furthermore, many isoindole-1,3(2H)-dione drugs have been approved and marketed, like thalidomide, pomalidomide, lenalidomide, and apremilast [24].

Gabapentinoids, gabapentin and pregabalin, are analogues of the inhibitory neurotransmitter γ-amino-butyric acid (GABA); they block $\alpha 2\delta$ subunit of voltage-dependent calcium channels (VDCCs).

They are first-line treatments for the management of neuropathic pain, and they are used to prevent seizures [25].

The aim of this study is to synthesize novel promising drug candidates by molecular hybridization of gabapentinoids and isoindole-1,3(2H)-dione scaffolds (Figure 1) and to evaluate their biological activity. In addition, we report a preliminary study of the structure-activity relationship and mechanism of action of compounds **1**–**5** as anticancer candidates and antimicrobial agents.

Two synthesis methods have been successfully applied: the conventional method and the microwave-assisted method (MAS). The microwave-assisted method has many advantages as an eco-friendly method, including a short reaction time and better yield and purity of products.

Synthesized compounds were screened for their promising biological activity as antioxidants and antibacterial and anticancer agents.

Antimicrobial effect was studied against Gram-negative *E. coli* and Gram-positive *Staphylococcus aureus*. Hybrids of pregabalin and gabapentin show antimicrobial activity against both strains in contrast to their parent compounds.

Hybrids of pregabalin are highly effective against *E. coli* and more effective than the standard drug Gentamicin Sulfate.

Antiproliferative activity was studied against two cancer cell lines, Caco-2 and HCT-116, as models of colorectal adenocarcinoma, in order to study the potential effect of compounds in the treatment of colon cancer.

All hybrid compounds showed significant antiproliferative effects; they arrest progression throughout the cell cycle and effectively induce apoptosis in cancer cell lines.

Compound **2** is the most effective against Caco-2 cells, 137.7 times more effective than thalidomide, with an $IC_{50} = 0.00064\ \mu$mol/mL. Compound 2 inhibits the progression of the cell cycle to the synthesis phase and induces apoptosis.

Compound **4** is the most effective against the HCT-116 cell line; with IC_{50} 0.228 μmol/mL, it inhibits the synthesis of DNA and mitosis in HCT-116 cells and induces cell death by apoptosis and necrosis.

Our results show that molecular hybridization of gabapentin and pregabalin within the isoindole-1,3(2H)-dione moiety results in new promising drug-like molecules, potent as antimicrobial and anticancer agents.

Herein, we report that substitution of aromatic hydrogens of the isoindole-1,3(2H)-dione moiety with bromine or chlorine atoms leads to more potent derivatives, and that bromine derivatives are comparable or more effective than chlorine derivatives; this result is in agreement with previous studies [5].

2. Materials and Methods

2.1. Synthesis and Characterization. Starting materials and solvents were purchased from commercial sources and used without further purification.

Scheme 1 shows synthesis reactions. We used two procedures, procedure A [26] and procedure B [27]. The progress of the reactions was monitored by TLC on silica-gel plates (Merck 60F254).

> Procedure A: 0.010 mole of phthalic anhydride or its derivatives and 0.011 mole of (S) pregabalin [1, 2] or gabapentin [3–5] were dissolved in 5 mL of dimethylformamide (DMF) in a round-bottom flask. The solution was refluxed in the oil path at a temperature of 180°C. The completion of the reaction was determined by TLC. The reaction mixture was poured into ice-cold water, and the crude product was precipitated, filtered out on a Buchner funnel, washed with water, dried, and purified by recrystallization from appropriate solvents.
>
> Procedure B: Equimolar quantities of phthalic anhydride or its derivatives and (S) pregabalin [1, 2] or gabapentin [3–5] were milled together in a mortar for 1 minute, and then the mixture was transferred to a glass beaker containing a magnetic stirrer. A few drops of DMF were added to the beaker, and it was irradiated with microwaves (750 W) with continuous stirring for 5 minutes. The completion of the reactions was determined by TLC.

The mixture was cooled to room temperature, 50 mL of ice-cold water was poured into the flask to precipitate the crude product, and it was filtered off, washed with water, dried, and recrystallized in an appropriate solvent.

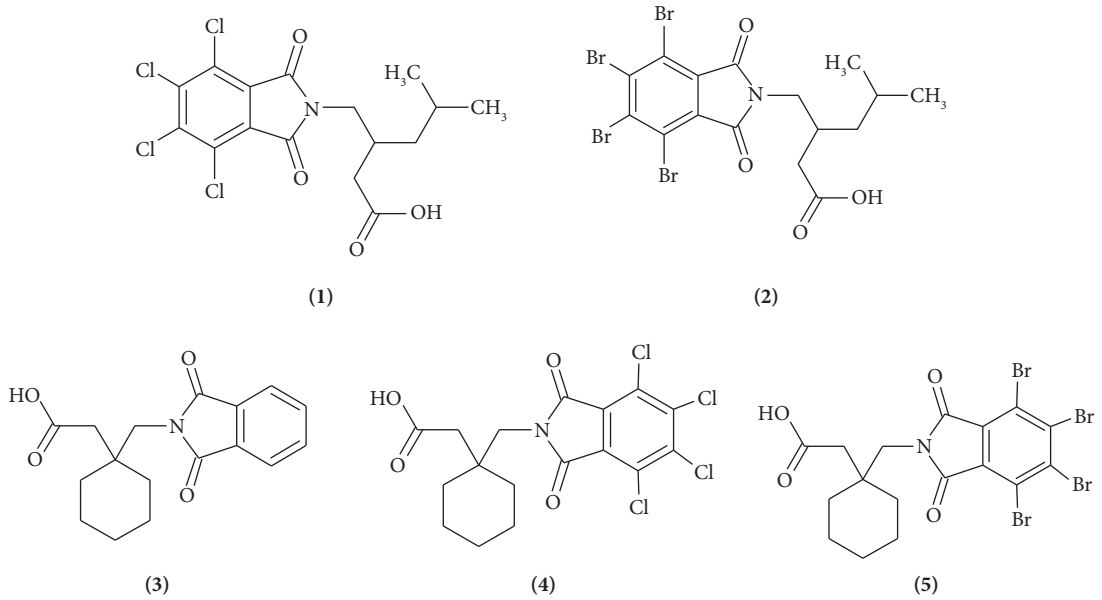
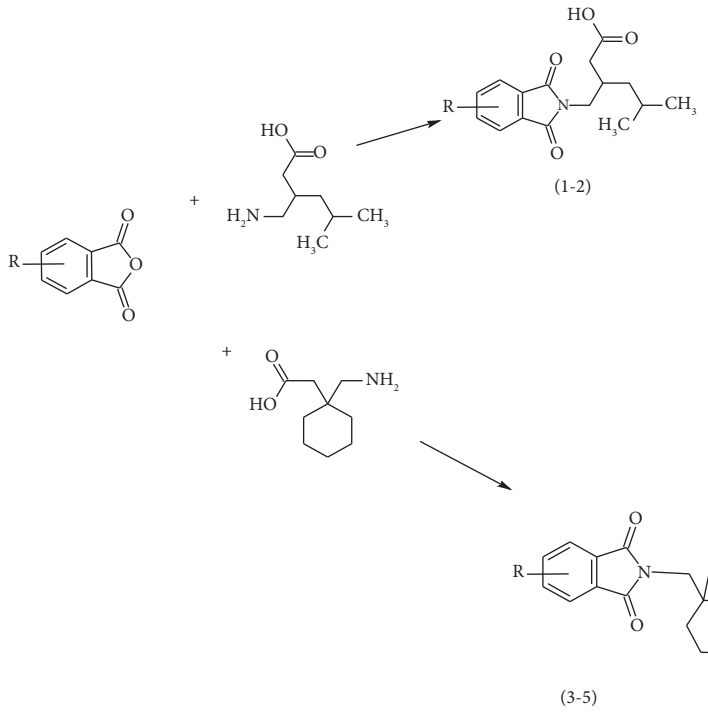


Figure 1: Structures of synthesized compounds.



Compound number	R
Compound **1**	Tetra Cl
Compound **2**	Tetra Br
Compound **3**	H
Compound **4**	Tetra Cl
Compound **5**	Tetra Br

Scheme 1: Synthesis reactions of compounds.

Melting points were determined using the Stuart melting point apparatus SMP30 and used without calibration. High-performance liquid chromatography was performed using the Shimadzu UFLC apparatus A20.

IR spectra were recorded in KBr on the Thermo Nicolet 6700 FT-IR spectrophotometer apparatus. UV spectra were recorded using a Jenway 6850 UV/Vis spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Ultra Shield 400 Instrument (400 MHz for ^{1}H, 100 MHz for ^{13}C).

ESI-MS experiments were performed using an Agilent 6420 Triple Quadrupole LC/MS apparatus equipped with a standard ESI source. The instrument was operated in positive-ion mode. Spectra were recorded for samples dissolved in DMSO: H_2O.

*2.1.1. 5-Methyl-3-[(4,5,6,7-tetrachloro-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) Methyl] Hexanoic Acid N-Tetra Chloro-phthaloyl (S) Pregabalin (**1**).* Pale yellow crystals,

yield 90% (procedure A) and 92% (procedure B), MP 177.6–180 ˚C, UV (methanol, nm): λ_{max} 288, 333.

IR (KBr, cm^{-1}): 3330.2 (OH), 2954.9, 2870 (CH_2 stretching), 1772.4 (CO), 1732.4 (CO), 1702.5 (CO), 1437 (CH_2 bending), 1165 (C-N).

^{1}H NMR (400 MHz, DMSO-d_6, ppm): 12.009 (s, 1H, OH), 3.6–3.42 (m, 2H, N-CH_2), 2.18–2.35 (m, 2H, CH_2-COOH), 2.18–2.048 (m, 1H, N-CH_2-CH), 1.793–1.634 (m, 1H, CH $(CH_3)_2$), 1.0785–1.267 (m, 2H, CH_2-$CH(CH_3)_2$), 0.7587–1.0058 (2d, 6H, $(CH_3)_2$).

^{13}C NMR (100 MHz, DMSO-d_6, ppm): 174.037 (COOH), 164.175 (2CO), 138.474 (2 aromatic carbons), 128.887 (2 aromatic carbons), 128,454 (2 aromatic carbons), 42.637, 41.572, 37.682, 32.157, 25.198, 23.207, 22.677.

DEPT 135 ^{13}C NMR (100 MHz, DMSO-d_6, ppm): 42.592 (N-$\underline{C}H_2$), 41.547 ($\underline{C}H_2$-COOH), 37.659 ($\underline{C}H_2CH$ $(CH_3)_2$), 32.135 (N-CH_2-$\underline{C}H$-), 25.185 ($\underline{C}H$ $(CH_3)_2$), 23.223 ($\underline{C}H_3$), 22.664 ($\underline{C}H_3$).

ESI + MS-MS (M/Z): 101.0, 240.8, 285.9, 287.9, 289.9, 291.9, 307.8, 309.9, 311.9, 362.1.

*2.1.2. 5-Methyl-3-[(4,5,6,7-tetrabromo-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) Methyl] Hexanoic Acid N-Tetra Bromo-phthaloyl (S) Pregabalin (**2**).* White crystals and crystalline powder, yield: 83% (procedure A) and 86% (procedure B), MP 212–212.6 ˚C.

UV λ_{max} (methanol, nm): 288, 338. IR (KBr, cm^{-1}): 3399.5 (OH), 2954.6, 2869.6 (CH_2 stretching), 1769.9 (CO), 1732.3 (CO), 1703.4 (CO), 1610.7 (C=C arom.), 1561.7 (C=C arom.), 1435 (CH_2 bending), 1163.5 (C-N).

^{1}H NMR (DMSO-d6, 400 MHz, ppm): 11.995 (s, 1H, OH), 3.6–3.42 (m, 2H, N-$C\underline{H}_2$), 2.18–2.35 (m, 2H, $C\underline{H}_2$-COOH), 2.18–2.0414 (m, 1H, N-CH_2-$C\underline{H}$), 1.776–1.636 (m, 1H, $CH(CH_3)_2$), 1.059–1.216 (m, 2H, CH_2-$CH(CH_3)_2$), 0.790–0.994 (2d, 6H, $(C\underline{H}_3)_2$).

^{13}C NMR (DMSO-d6, 100 MHz, ppm): 174.074 ($\underline{C}$OOH), 164.493 (2$\underline{C}$O), 136.463 (2 aromatic carbons), 131.614 (2 aromatic carbons), 120.831 (2 aromatic carbons), 42.796 (N-$\underline{C}H_2$), 41.632 ($\underline{C}H_2$COOH), 37.762 (($\underline{C}H_2$-$CH(CH_3)_2$), 32.083 (N-CH_2-$\underline{C}H$), 25.215 ($\underline{C}H(CH_3)_2$), 23.250 ($\underline{C}H_3$), 22.652 ($\underline{C}H_3$).

DEPT 135 ^{13}C NMR: 42.796 (N-$\underline{C}H_2$), 41.632 ($\underline{C}H_2$COOH), 37.762 (($\underline{C}H_2$-$CH(CH_3)_2$), 32.083 (N-CH_2-$\underline{C}H$), 25.215 ($\underline{C}H(CH_3)_2$), 23.250 ($\underline{C}H_3$), 22.652 ($\underline{C}H_3$).

ESI + MS-MS (M/Z): 679.8 $[M + DMSO + H]^+$, 604.7 $[M]^+$, 587.7 $[M - OH]^+$, 565.7, 475.6, 463.7, 396.9.

*2.1.3. {1-[(1,3-Dioxo-1,3-dihydro-2H-isoindole-2-yl) Methyl] Cyclohexyl} Acetic Acid (**3**).* White fine powder, yield: 80% (procedure A) and 84% (procedure B), MP 151–152 ˚C, UV λ_{max} (methanol, nm): 290, 302.

IR (KBr, cm^{-1}): 3434 (OH), 3096 (Ar-H stretch.), 2929, 2869 (CH_2 stretching), 1770 (CO), 1720 (CO), 1710 (CO), 1611 (C=C arom), 1466 (CH_2 bending), 1195 (C-N).

^{1}H NMR (400 MHz, CD_3CN, ppm): 11.99 (s, 1H, OH), 7.91–7.78 (m, Ar- $\underline{4H}$), 3.654 (s, 2H, N-$C\underline{H}_2$), 2.304 (s, 2H, CH_2-COOH), 1.670–1.108 (m, 10H of cyclohexane).

^{13}C NMR (100 MHz, CD_3CN, ppm): 173.287 ($\underline{C}$OOH), 169.243 (2$\underline{C}$O), 134.784 (2 aromatic carbons), 132.131(2 aromatic carbons), 123.454 (2 aromatic carbons), 46.146, 38.098, 33.365, 25.768, 21.551.

DEPT 135 ^{13}C NMR: 134.787 (2 aromatic carbons), 123.459 (2 aromatic carbons), 46.141 ($\underline{C}H_2$-N), 38.098 ($\underline{C}H_2$-COOH), 33.349 (2(CH_2) of cyclohexane), 25.767 (CH_2 of cyclohexane), 21.546 (2(CH_2) of cyclohexane).ESI + MS-MS (M/Z): 339.9 $[M + K]^+$, 323.9 $[M + Na]^+$, 302.0 $[M + H]^+$, 284.0 $[M\text{-}OH]^+$, 256.1 $[M\text{-}COOH]^+$, 242 $[M\text{-}(CH_2\text{-}COOH)]^+$, 160 $[M\text{-}(COOH\text{-}CH_2\text{-}C_6H_{10})]^+$

*2.1.4. {1-[(4,5,6,7-Tetrachloro-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) Methyl] Cyclohexyl} Acetic Acid (**4**).* White crystals yield: 70% (procedure A) and 75% (procedure B), MP 218–218.4 ˚C, UV λ_{max} (methanol, nm): 202, 212, 236, 333.

IR (KBr, cm^1): 3299 (OH), 2862, 2931 (CH_2 stretching), 1772 (CO), 1733 (CO), 1703 (CO), 1434 (CH_2 bending), 1157 (C-N), 736 (C-Cl).

^{1}H NMR (400 MHz, DMSO-d_6, ppm): 12.031 (S, 1H, OH), 3.662 (S, 2H, N-$C\underline{H}_2$), 2.323 (s, 2H, $C\underline{H}_2$-COOH), 1.618–1.139 (m, 10H of cyclohexane).

^{13}C NMR (DMSO-d_6, 100 MHz, ppm): 173.281 ($\underline{C}$OOH), 164.820 (2$\underline{C}$O), 138.412 (2 aromatic carbons), 128.975 (2 aromatic carbons), 128.375 (2 aromatic carbons), 46.954, 38.212, 33.144, 25.668, 21.533.

DEPT 135 (DMSO-d_6, 100 MHz, ppm): 46.948 (CH_2-N), 38.212 (CH_2COOH), 33.144 (2(CH_2) of cyclohexane), 25.690 (CH_2 of cyclohexane), 21.526 (2(CH_2) of cyclohexane).

ESI^+MS-MS (M/Z): 477.9 $[M + K]^+$, 461.9 $[M + Na]^+$, 409.9, 497.8, 387.8.

*2.1.5. {1-[(4,5,6,7-Tetrabromo-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl)methyl]cyclohexylacetic Acid} (**5**).* Pale yellow crystals yield: 77% (procedure A) and 80% (procedure B), MP 241.5–243 ˚C, UV λ_{max} (methanol, nm): 287, 336.

IR (KBr, cm^1): 3299 (OH), 2929, 2861 (CH_2 stretching), 1770 (CO), 1735 (CO), 1702 (CO), 1559 (C=C),1432 (CH_2 bending), 1155 (C-N), 669 (C-Br).

^{1}H NMR (400 MHz, DMSO-d_6, ppm): 12.004 (S, 1H, O$\underline{H}$), 3.645 (S, 2H, N-$C\underline{H}_2$), 2.308 (S, 2H, CH_2-COOH), 1.72–1.07 (m, 10H of cyclohexane).

^{13}C NMR (DMSO-d_6, 100 MHz, ppm): 173.308 (COOH), 165.135 (2CO), 136.588 (2 aromatic carbons), 131.689 (2 aromatic carbons), 120.784 (2 aromatic carbons), 47.112 (N-$C\underline{H}_2$), 38.192 ($\underline{C}H_2$-COOH), 33.171 (2C of cyclohexane), 25.665 (1C of cyclohexane), 21.555 (2C of cyclohexane).

DEPT 135 ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): 47.059 ($\underline{C}H_2$-N), 38.361 ($\underline{C}H_2$-COOH), 33.226 (2($\underline{C}H_2$)), 25.778 ($\underline{C}H_2$), 21.547 (2($\underline{C}H_2$)).

ESI + MS-MS (M/Z): 588.7, 565.7, 475.6.

2.2. Evaluation of Biological Activity

2.2.1. Antioxidant Activity. The antioxidant activity of prepared compounds was determined by measuring free radical scavenging activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the standard method, and ascorbic acid was used as a positive control [28].

We have studied serial concentrations of 250, 500, 750, and 1000 μg/mL of each compound and ascorbic acid in methanol.

1 mL of the studied solution was mixed with 3 mL of DPPH solution and allowed to stand in the dark for 30, 60, 90, and 120 minutes.

Methanol was used to prepare the negative control. Pure methanol was used as a blank. Absorbance was measured at 517 nm after 30, 60, 90, and 120 minutes. All experiments were done in triplicate, and the values are averaged.

DPPH scavenging activity was calculated using the following equation:

$$\text{Scavenging effect \%} = \left(A_{\text{negative control}} - \frac{A_{\text{sample}}}{A_{\text{negative control}}} \right) \text{x } 100. \quad (1)$$

IC_{50} was calculated from the concentration-inhibition curve by plotting the sample concentration versus the corresponding DPPH scavenging activity.

2.2.2. Antimicrobial Activity. Antimicrobial activity of parent compounds (pregabalin and gabapentin) and synthesized compounds was investigated against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria by the well diffusion method reported by Perez et al. [29].

A stock solution of each compound was prepared and diluted to prepare other concentrations through the two-fold dilution method. At least six concentrations were studied for each compound (3200, 1600, 800, 400, 200, and 100 μg/mL).

A microbial suspension was prepared in physiological saline serum and adjusted to the 0.5 McFarland standard.

Culture media were prepared by the pour plate method, in which microbial suspension was added to the Mueller–Hinton agar at a percentage of 1% and then poured into Petri dishes (depth 3-4 mm).

Poured plates were left to cool, and then similar wells (8 mm in diameter) were made in the agar and loaded with 100 μl of the tested compound solution. Inculcated plates were incubated at 37°C for 24 h.

Antimicrobial activity was evaluated by measuring the zone of inhibition (IZ) in mm. Each experiment was carried out in triplicate, and the average zone of inhibition was calculated.

Gentamicin Sulfate was used as the standard drug for antibacterial activity, and the pure solvent (methanol) was used as a negative control.

2.2.3. Anticancer Activity. The effect of compounds was studied on human cancer cell lines Caco-2 and HCT-116 (Sigma-Aldrich).

A stock solution of each compound was prepared in DMSO and diluted with a culture medium to prepare serial concentrations. The final concentration of DMSO in wells is less than or equal to 0.1%.

Viability was measured after 48 hours using the XTT method, cell cycle changes of treated cells were studied by flow cytometry, and an assay of apoptosis and necrosis cell death was performed by the Annexin V/propidium iodide (PI) double staining assay method using flow cytometry.

(1) Cell Cultures. Caco-2 and HCT-116 cells were cultured in sterile flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

(2) XTT Assay. Human Caco-2 and HCT-116 cultures were seeded in 96-well plates (1.5×10^4 cells per well).

Plates were incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2, then the culture medium was removed, and cells were treated with different concentrations of the studied compounds.

At least six concentrations were studied for each compound, and each concentration was studied in triplicate. DMSO alone was added to a set of wells as the solvent control, and culture medium was added to another set of wells as the negative control.

After treatment, plates were incubated for 48 hours at 37°C in a 5% CO_2 atmosphere.

The antiproliferative effect was measured after 48 hours by the XTT assay reported by Skehan et al. [30], according to the instructions of the manufacturing company of the kit (Roche).

Absorbance was measured using an ELISA reader spectrophotometer at 450 nm. All experiments were carried out in triplicate.

Inhibition of cell viability was expressed as a percentage, and it was calculated as follows:

Growth inhibition GI% = [1 − (absorbance of the test compound/absorbance of the negative control)] ∗ 100.

The inhibition ratio was calculated as the mean of the results of all replicates. IC_{50} was calculated from the dose-response curve.

(3) Cell Cycle Analysis. We have studied cell cycle phases in treated cells compared with untreated cells in order to get more information about the mechanism of the anticancer effect of synthesized compounds.

Cells were cultured and treated with the studied compounds for 72 hours, and untreated cells were incubated with culture medium in the same conditions as a negative control.

Cells were harvested by trypsin, samples were centrifuged, and the cellular precipitate was washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640.

Samples were fixed by incubation with methanol in a dark place for 30 minutes at −20°C.

Samples were centrifuged, and fixed cells were rinsed twice with PBS. A staining solution (DNA fluorochrome PI in a solution containing Triton X-100 and RNase) was added, and samples were kept in the dark for 30 minutes at 4–8 °C. Stained cells were analyzed by a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's protocol [31].

The tests were performed in duplicate and repeated at least twice.

(4) Apoptosis Assay. An assay of apoptosis and necrosis cell death was executed by the Annexin V/propidium iodide (PI) double staining assay method in order to know the effect of compounds on cell death.

Cells were treated with compounds for 72 h, and untreated cells were incubated with RPMI 1640 as a negative control.

Cells were harvested by trypsin and precipitated by centrifugation. The cellular precipitate was washed with PBS and resuspended in culture medium.

Cells were fixed by incubation with methanol in a dark and cold place for 30 minutes at −20°C.

Fixed cells were separated by centrifugation and rinsed twice with PBS.

Cells were stained with 5 μL Annexin V, 5 μL PI, and 350 μL Annexin binding buffer and analyzed by a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

The number of cells analyzed for each sample was 10^4 cells [31]. Gating was implemented on the basis of negative-control staining profiles. The tests were performed in duplicate and repeated at least twice.

3. Results and Discussion

3.1. Synthesis and Characterization. Isoindoline-1,3-dione derivatives (**1**–**5**) were synthesized by molecular hybridization of (S) pregabalin or gabapentin with phthalic anhydride derivatives. A one-step synthesis reaction occurs by dehydrative condensation in the presence or absence of solvent, according to Scheme 1.

Synthesis was successfully executed by two methods: the conventional method and the microwave-assisted method.

The reaction is shorter (5–10 minutes) with the microwave-assisted method, while it takes more than 2 hours for the completion of the reaction with the conventional method.

The microwave-assisted method is an eco-friendly and green chemistry method. Short reaction time, good yield, and mild conditions are additional advantages of this method, over the conventional method.

The structures of compounds are confirmed by ^{1}H NMR, ^{13}C NMR, DEPT 135 NMR, LC-MS, FT-IR, and UV-visible spectra.

The analytical and spectral results of all identification tests comply with the structures of compounds.

The molecular data of compounds are tabulated in Table 1.

Compound 3 is known, and published elsewhere. The other four compounds are novel, however similar derivatives are studied as promising drug-like molecules [32].

3.2. Evaluation of Biological Activity

3.2.1. Antimicrobial Activity. Compounds **1**–**5** showed moderate to good zones of inhibition against tested bacterial strains at micromolar concentrations in a concentration-dependent manner, while negative control (solvent) and parent compounds did not show any effect (Tables 2 and 3).

Compounds **1**-**2** are more effective than the standard drug Gentamicin Sulfate against *Escherichia coli*, and they are promising antimicrobial agents.

Isoindole-1,3(2H) dione moiety is well known for antimicrobial effect per previous studies [4, 5, 33, 34].

Though pregabalin and gabapentin do not have an antimicrobial effect, hybridization into an unsubstituted, tetra chlorinated, or tetra brominated isoindoline-1,3(2H)-dione moiety gives new hybrids with moderate to good antimicrobial activity against Gram-positive and Gram-negative microorganisms, and this is in agreement with previous studies that confirm the antimicrobial effect of isoindole-13(2H)-dione moiety [33].

Hybrids of pregabalin are more effective against Gram-negative *E. coli*, while hybrids of gabapentin are more effective against Gram-positive *Staphylococcus aureus*.

Results show that halogenation of isoindole-1,3(2H)-dione derivatives enhances the antimicrobial effect of compounds, and brominated derivatives are more effective than chlorinated ones.

3.2.2. Antioxidant Activity. We have evaluated the free radical scavenging properties of compounds by the DPPH free radical scavenging assay in an attempt to find new sources of antioxidants.

The results of our compounds and antioxidant, ascorbic acid, are shown in Table 4.

Though they are less efficient compared with ascorbic acid, compounds **1**–**5** showed the ability to scavenge free radicals of DPPH in a time- and concentration-related manner. A dose-response curve was plotted to determine IC_{50} where it was applicable.

Compound **3** showed considerable free radical scavenging activity, superior to the other four compounds with IC_{50} value of 2.525 μmol/mL, and can thus ensure protection against oxidative stress caused by free radicals.

Table 1: Molecular and physical properties of synthesized compounds.

Compound no.	Compound name	Molecular formula	MW	Purity (%)	mp	Yield (%)
Compound 1	5-Methyl-3-[(4,5,6,7-tetrachloro-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) methyl] hexanoic acid	$C_{16}H_{15}Cl_4NO_4$	427.11	98.50	177.6–180	A: 90 B: 92
Compound 2	5-Methyl-3-[(4,5,6,7-tetrabromo-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) methyl] hexanoic acid	$C_{16}H_{15}Br_4NO_4$	604.91	99.91	212-212.6	A: 83 B: 86
Compound 3	{1-[(1,3-Dioxo-1,3-dihydro-2H-isoindole-2-yl) methyl] cyclohexyl} acetic acid	$C_{17}H_{19}NO_4$	301.34	99.18	151-152	A: 80 B: 84
Compound 4	{1-[(4,5,6,7-Tetrachloro-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) methyl] cyclohexyl} acetic acid	$C_{17}H_{15}Cl_4NO_4$	439.12	99.99	218–218.4	A: 70 B: 75
Compound 5	{1-[(4,5,6,7-Tetrabromo-1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl) methyl] cyclohexyl} acetic acid	$C_{17}H_{15}Br_4NO_4$	616.92	95.90	241.5–243	A: 77 B: 80

Table 2: In vitro antimicrobial activity of synthesized compounds against *Staphylococcus aureus*.

	Zone of inhibition on *Staphylococcus aureus* (mm)					
Conc. μg/mL	3200 μg/mL	1600 μg/mL	800 μg/mL	400 μg/mL	200 μg/mL	100 μg/mL
Pregabalin	—	—	—	—	—	—
Gabapentin	—	—	—	—	—	—
Compound **1**	20	18.67	18	14.34	12	9.67
Compound **2**	21.67	20	19.67	19.34	18.67	16.34
Compound **3**	16	12	0	0	0	0
Compound **4**	19	17.33	16.5	15.67	14.67	11.67
Compound **5**	20	18.34	17.67	17	16.67	14.67
Gentamicin Sulfate	26	25	24	22.34	21.34	19.34

Table 3: In vitro antimicrobial activity of synthesized compounds against *Escherichia coli*.

	Zone of inhibition on *E. coli* (mm)					
Conc. μg/mL	3200 μg/mL	1600 μg/mL	800 μg/mL	400 μg/mL	200 μg/mL	100 μg/mL
Pregabalin	—	—	—	—	—	—
Gabapentin	—	—	—	—	—	—
Compound **1**	23	20	18	16	14	13
Compound **2**	24	23	22.67	22.34	21.67	20.34
Compound **3**	14.34	13.34	12.37	11.67	11	10.34
Compound **4**	15	13.67	13	11.67	11.34	10.67
Compound **5**	16	15	14	13.67	13.34	12.67
Gentamicin Sulfate	21	20	17	13	0	0

Scavenging activity may be attributed to the hydrogen-donating ability of the compounds.

3.2.3. Anticancer Activity. We have studied the effects of compounds **1**–**4** on two colon cancer cell lines, Caco-2 and HCT-116.

Both cell lines are models of colorectal adenocarcinoma; however, each cell line represents different histological grade of colon cancer. Caco-2 is a model of less metastatic, less invasive, and more differentiated colon cancer cells; however, HCT-116 is known to be a poorly differentiated and highly invasive cell line, and it is one of the higher chemoresistant colon cell lines [35].

We were not able to study the effect of compound **5** on cells due to the inappropriate solubility of the compound in the safety range of DMSO.

(1) Antiproliferative Assay. The antiproliferative effect of compounds was evaluated by an XTT cell viability assay after 48 hours of treatment on two cell lines, Caco-2 and HCT-116. Antiproliferative percentage and IC_{50} values are shown in Tables 5 and 6, respectively.

Results indicate a significant antiproliferative effect of the studied compounds on both cell lines, and they are in good correlation with concentrations.

IC_{50} values of compounds **1, 2**, and **4** against Caco-2 and HCT-116 are less than 1 μmol/mL, assuring a good antiproliferative effect.

Results on Caco-2 show that compound **2** has the most efficient antiproliferative effect against Caco-2 with an IC_{50} value of 0.00064 μmol/mL.

Compound **4** is 14 times more effective than compound **3**. This result shows that chlorination of the isoindole-1,3(2H)-dione moiety increases antiproliferative effect against Caco-2 cells.

Compound **2** is 426 times more effective than compound **1**. This result shows that the halogenation of isoindole-1,3(2H)-dione compounds increases the antiproliferative activity.

The antiproliferative effect of compound **2** ($IC_{50} = 0.64$ μmol/L) against Caco-2 cells is 137.7 times more effective than thalidomide ($IC_{50} = 88$ μmol/L) [36].

Results on the HCT-116 cell line show that compound **4** is the most effective with an IC_{50} value of 0.228 μmol/mL, with compounds **1** and **2** having the same antiproliferative effect against HCT-116 with an IC_{50} value of 0.412 μmol/mL.

IC_{50} of compound **3** is more than 3.39 μmol/mL, and it is not applicable in the studied concentrations.

Compound **3** is less effective than compound **4** and other compounds against Caco-2 and HCT-116 cells; this result indicates that the halogenation of isoindole-1,3(2H)-dione moiety increases the antiproliferative effect against HCT-116 cells, and this result is in agreement with previous studies [5].

Caco-2 cells seem to be more sensitive to our compounds compared with HCT-116 cells, and this result is in agreement with chemosensitivity properties of the two lines [35].

(2) Cell Cycle Analysis. For further understanding of the mechanism of the antiproliferative effect of compounds, we have defined the percentage of cells in each phase of the cell cycle by flow cytometry using the PI staining method.

Table 4: Free radical scavenging activity of studied compounds and ascorbic acid.

Conc. μg/mL	Compound 1				Compound 2				Compound 3				Compound 4				Compound 5				Ascorbic acid			
	Free radical scavenging activity %				Free radical scavenging activity %				Free radical scavenging activity %				Free radical scavenging activity %				Free radical scavenging activity %				Free radical scavenging activity %			
	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min
250	14.82	16.64	39.88	44.02	2.04	9.03	13.71	19.95	28.94	34.47	39.84	42.58	4.2	8.4	10.85	14.85	12.32	15.52	19.35	22.04	95.42	98.02	98.13	98.23
500	18.02	20.28	40.93	44.86	6.54	14.35	18.26	23.33	41.48	44.07	49.55	56.45	27.37	38.37	42.44	44.27	14.98	19.03	20.65	23.25	96.58	98.15	98.22	98.36
750	20.56	22.98	43.05	48.16	7.59	16.26	20.2	25.76	50.4	55.57	60.12	71.45	29.93	58.62	56.86	63.97	21.2	27.84	30.52	33.84	96.65	98.16	98.26	98.41
1000	21.06	26.25	44.26	48.88	8.38	17.29	21.48	26.87	55	58.39	65.34	77.74	32.2	61.3	58.6	67.95	25.6	28.96	32.2	40.16	96.66	98.18	98.3	98.47

Table 5: Antiproliferative effect of studied compounds on the Caco-2 cell line.

Compound number	Antiproliferative effect % on Caco-2 cell line						IC_{50} (μmol/mL)
Compound **1**	Conc. μg/mL	500	250	125	75	25	0.2724
	*GI %	74.58	73.08	65.37	0	0	
Compound **2**	Conc. μg/mL	125	75	35	10	5	0.000639
	GI %	72.9	72.66	69.25	58.12	52.25	
Compound **3**	Conc. μg/mL	1000	500	250	125	75	2.0845
	GI %	69.9	39	19	0	0	
Compound **4**	Conc. μg/mL	100	50	25	12.5	10	0.1450
	GI %	72.67	44.2	8.67	0	0	

*GI%: percentage of growth inhibition.

Table 6: Antiproliferative effect of studied compounds on the HCT-116 cell line.

Compound number	Antiproliferative effect % on HCT-116 cell line						IC_{50} (μmol/mL)
Compound **1**	Conc. μg/mL	500	250	125	75	25	0.412
	GI %	55.774	52.102	49.392	48.308	30.462	
Compound **2**	Conc. μg/mL	1000	500	250	125	75	0.413
	GI %	33.133*	45.384*	50.02	49.855	49.189	
Compound **3**	Conc. μg/mL	1000	500	250	125	75	>3.39
	GI %	48.322	22.858	15.685	7.255	1.683	
Compound **4**	Conc. μg/mL	100	50	25	12.5	10	0.228
	GI %	49.558	47.406	24.692	7.236	0	

Results of studying the cell cycle of Caco-2 and HCT-116 cells treated with compounds for 72 hours compared with untreated cells show that all compounds strongly delay progress throughout the cell cycle, which explains and confirms the already outlined antiproliferative effect.

Results of the cell cycle study of the Caco-2 cellular line after treatment with compounds show that compounds **1** and **2** arrest the cell cycle of treated cells on phase G1, compound **3** arrests the cell cycle on phase S, and compound **4** arrests the cell cycle on phases S and G2/M (Figure 2).

Compounds **1** and **2** arrest the progression of the cell cycle to the synthesis phase, compound **3** inhibits the synthesis of DNA, and compound **4** inhibits the synthesis of DNA and inhibits mitosis as well in treated cells.

Results of the cell cycle study of HCT-116 cells after treatment with compounds show that compounds arrest the cell cycle at phases S and G2/M, which indicates their ability to inhibit the synthesis of DNA and mitosis as well (Figure 3).

Results explain the antiproliferative effect of compounds on the Caco-2 and HCT-116 cell lines, and they are in agreement with our previous results.

(3) Apoptosis Assay. For further understanding of the method of action of synthesized compounds as promising anticancer agents and for further understanding of the mechanism of cell death induced by compounds, Caco-2 and HCT 116 cells were treated with the studied compounds and incubated for 72 hours. Flow cytometry was executed to determine the percentage of live, early apoptotic, apoptotic, and necrotic cells using Annexin V Binding and Propidium Iodide Uptake method.

Results show that treatment of Caco-2 and HCT-116 cell lines with the studied compounds induces cell death by apoptosis. Results show a decrease in the percentage of live cells and an increase in the percentage of apoptotic and/or necrotic cells compared with untreated cells.

Results of the cell death assay in Caco-2 cells confirm that all compounds effectively induce apoptosis in Caco-2 cells compared with untreated cells (Figure 4).

Results of the cell death assay in HCT-116 cells show that all studied compounds effectively induce apoptosis and necrosis in HCT-116 cells compared with untreated cells (Figure 5).

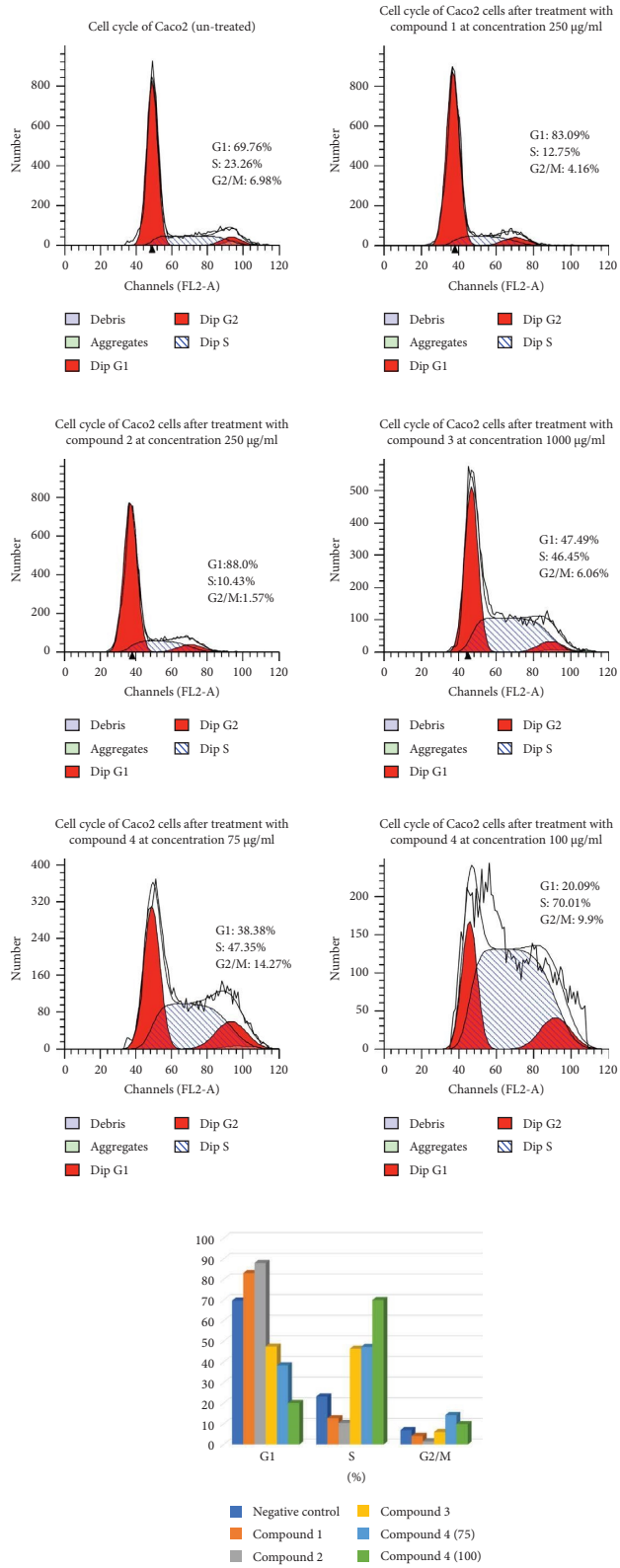


Figure 2: Cell cycle analysis of Caco-2 cells untreated and after treatment with studied compounds using PI staining method by flow cytometry.

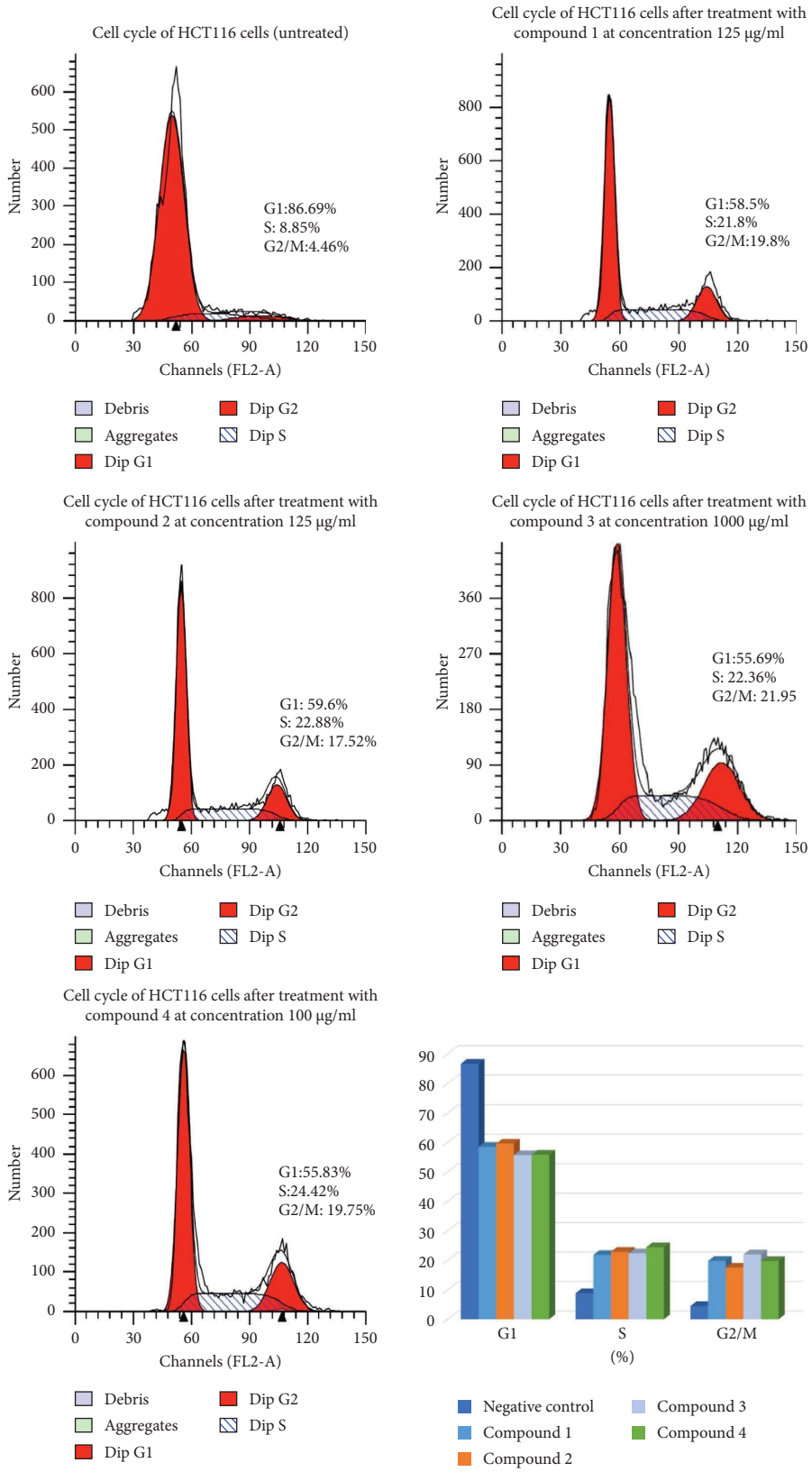


Figure 3: Cell cycle analysis of HCT-116 cells untreated and after treatment with studied compounds using PI staining method by flow cytometry.

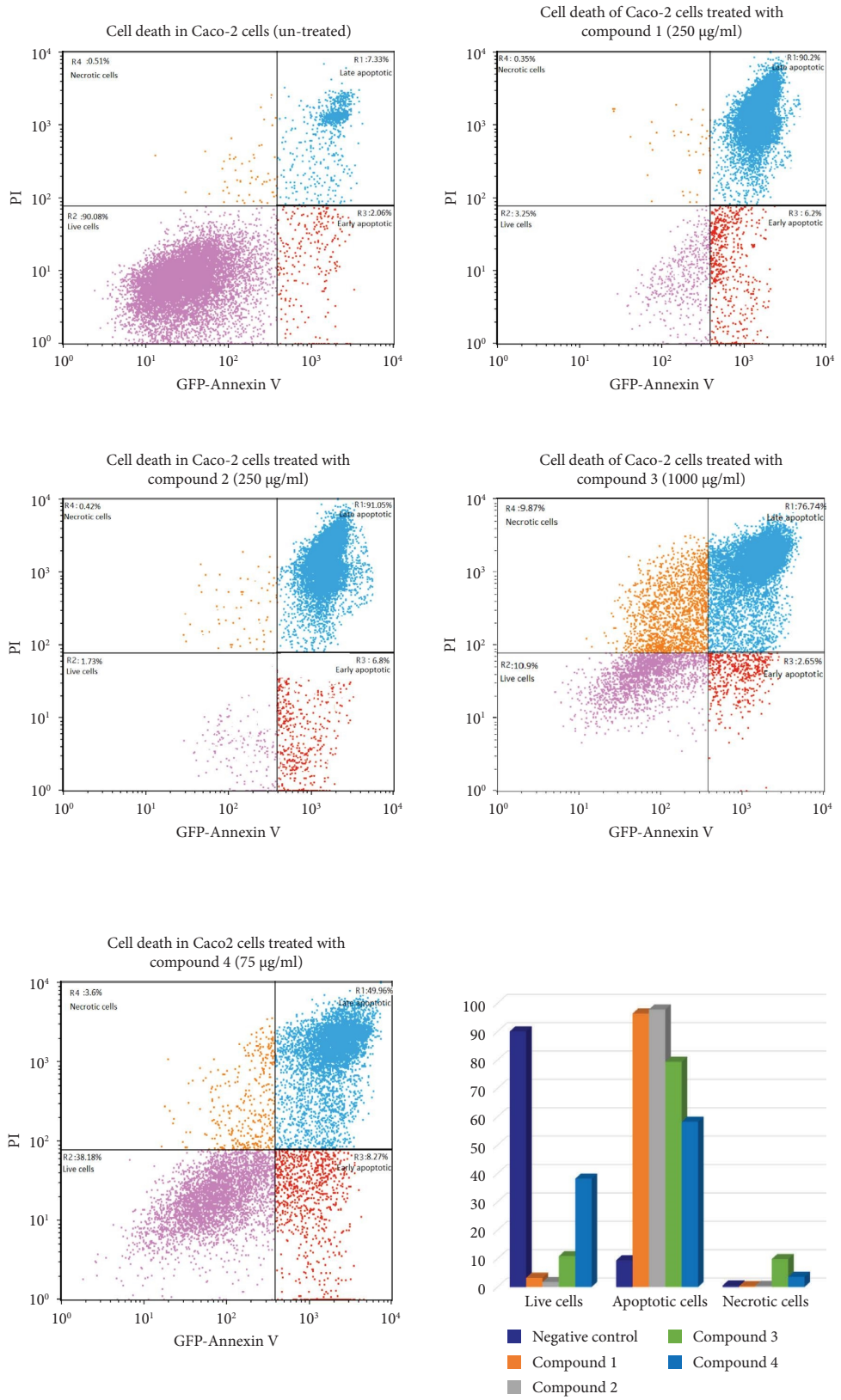


Figure 4: Results of the cell death assay of Caco-2 cells, untreated and treated with studied compounds.

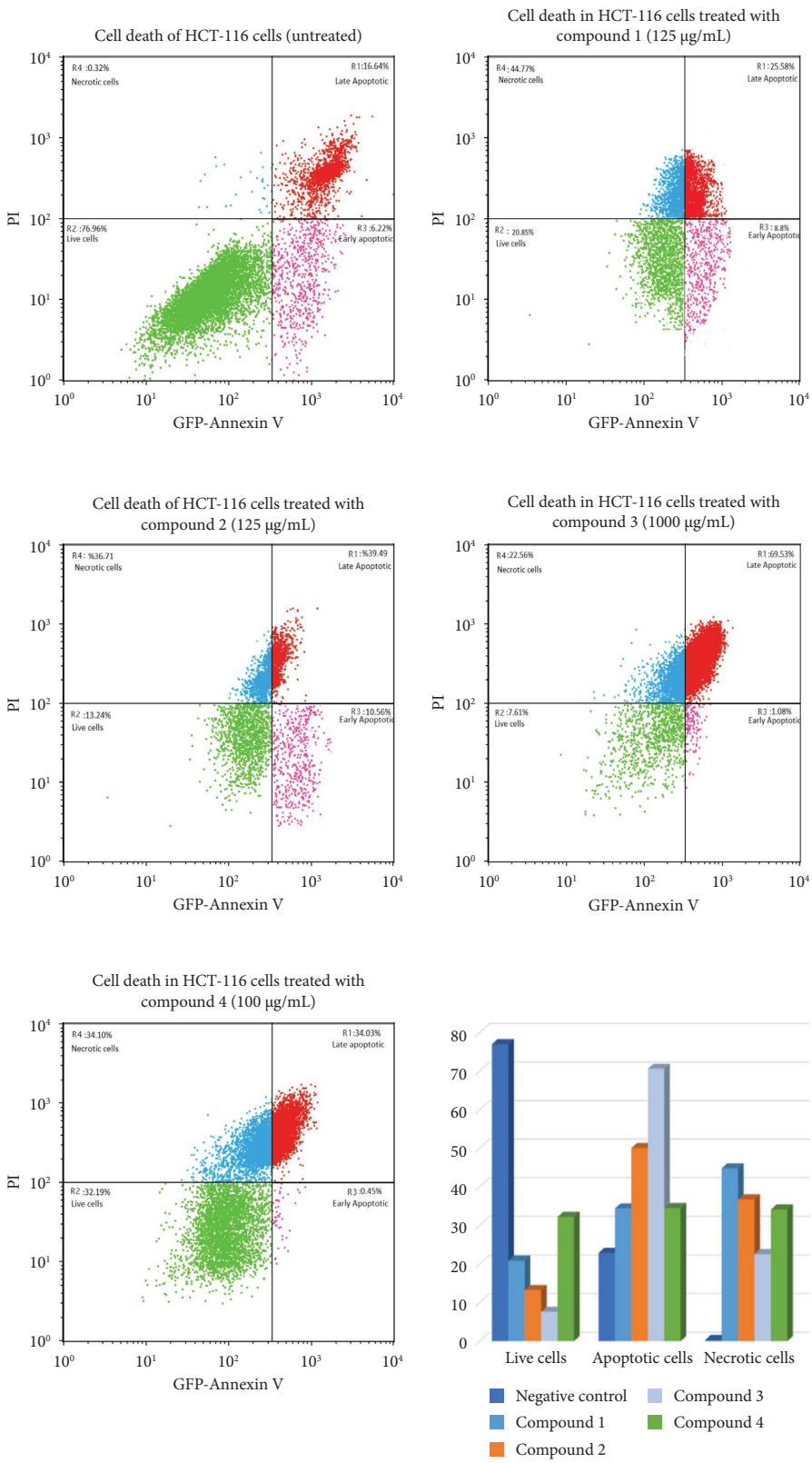


Figure 5: Results of the cell death assay of HCT-116 cells, untreated and treated with the studied compounds.

4. Conclusion

The biological importance of isoindole-1,3(2H)-dione scaffold has promoted the synthesis of some promising multitarget derivatives by molecular hybridization of this moiety with gabapentin and pregabalin.

Five derivatives of gabapentin and pregabalin hybrids with isoindole-1,3(2H)-dione were synthesized. Two synthesis methods have been successfully carried out.

Synthesized compounds were characterized by UV spectra, IR spectra, ^{1}H-NMR, ^{13}C-NMR, DEPT 135 NMR, and ESI-MS. Results showed good agreement with the proposed chemical structures of compounds.

Compounds were screened for antimicrobial activity against two bacterial species, Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*, and for anticancer effects against two cancer cell lines, Caco-2 and HCT-116.

In vitro studies indicated that compounds **1** and **2** are promising antimicrobial agents, and they are more effective than Gentamicin Sulfate against *E. coli*.

Viability studies indicate that tested compounds have a significant antiproliferative effect against Caco-2 and HCT-116 cancer cells. Treatment with compounds arrests progression through the cell cycle and induces cell death in treated cells. Halogenation of the isoindole-1,3(2H)-dione moiety improves antimicrobial and anticancer activity and that tetra-brominated derivatives are comparable to or more effective than related tetra-chlorinated derivatives.

Data Availability

The data used to support the findings of the study are available from the corresponding author upon request.

Disclosure

A preprint has previously been published online [37].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors have read and approved the manuscript.

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Supplementary Materials

The supplementary materials contain IR spectrum of compound 1 (Figure S1), ^{1}H-NMR spectrum of compound 1 (Figure S2), ^{13}C NMR spectrum of compound 1 (Figure S3), DEPT 135 ^{13}C NMR spectrum of compound 1 (Figure S4), IR spectrum of compound 2 (Figure S5), ^{1}H-NMR spectrum of compound 2 (Figure S6), ^{13}C NMR spectrum of compound 2 (Figure S7), DEPT 135 ^{13}C NMR spectrum of compound 2 (Figure S8), IR spectrum of compound 3 (Figure S9), ^{1}H-NMR spectrum of compound 3 (Figure S10), ^{13}C NMR spectrum of compound 3 (Figure S11), DEPT 135 ^{13}C NMR spectrum of compound (Figure S12), IR spectrum of compound 4 (Figure S13), ^{1}H-NMR spectrum of compound 4 (Figure S14), ^{13}C NMR spectrum of compound 4 (Figure S15), DEPT 135 ^{13}C NMR spectrum of compound 4 (Figure S16), IR spectrum of compound 5 (Figure S17), ^{1}H-NMR spectrum of compound 5 (Figure S18), ^{13}C NMR spectrum of compound 5 (Figure S19), and DEPT 135 ^{13}C NMR spectrum of compound 5 (Figure S20). (*Supplementary Materials*)

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