

Research Article

Synthesis, Characterization, and Biological Evaluation of Some Isoindole-1,3-(2H) Dione Derivatives

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In this study, we have established promising drug candidates with approved antimicrobial, antioxidant, antileishmanial, and anticancer activities. We hereby report drug likeliness, ADME prediction, synthesis, characterization, and in vitro biological evaluation of isoindole-1,3-(2H) dione derivatives. Synthesized compounds showed a free radical scavenging effect, with compound **1** being the most effective (IC₅₀ value 1.174 μmol/mL). The antibacterial activity of compounds was studied against two microbial Gram-positive and Gram-negative strains by well diffusion method. The inhibition zone of compound **3** is comparable with the inhibition zone of gentamycin at the same concentration. The compounds are highly effective against *Leishmania tropica* with compound **3** being the most effective one (IC₅₀ 0.0478 μmol/mL). The compounds are highly potential for the treatment of *Leishmania tropica*, and they are more effective than the first-line treatment, Glucantime. Compounds showed good anti-proliferative activity against two human cancer cell lines (Caco-2 and HCT-116). Treatment with studied compounds arrests progress throughout the cell cycle and induces apoptosis in cancer cells. Data from structure-activity relationship (SAR) analysis revealed that lipophilic properties of compounds might enhance their activity as antimicrobial, antileishmanial, and anti-proliferative activity. The halogenation of isoindole-1,3 (2H) dione moiety increases antimicrobial, antileishmanial, and anticancer activities. Tetra-brominated derivatives are more effective than tetra-chlorinated derivatives.

1. Introduction

Many diseases are still incurable and represent a perturbing challenge for the pharmaceutical research such as infectious diseases associated with resistant microorganisms (such as resistant bacteria, Leishmania, and malaria), tropical diseases, autoimmune diseases, and cancer, the fact that demands a continuous effort to the development of new therapeutic molecules: more efficient, selective, and economically accessible.

Based on the broad spectra of thalidomide biological activities, *isoindole-1,3(2H) dione* pharmacophore has been the target of drug discovery and drug development research studies with varying intentions, and this moiety has been described as a privileged scaffold to design new drug

candidates with multitarget effects and different biological activities such as androgen receptor antagonists [1], anti-convulsant [2, 3], antimicrobial [4], hypoglycemic [5, 6], antihyperlipidemic [5, 7], anti-inflammatory [8, 9], antitumor [10–13], antiviral activities [14], anti-Alzheimer [15–17], xanthine oxidase inhibitors, carbonic anhydrase inhibitors [18], antioxidants [13], and antileishmanial effect against *Leishmania amazonensis* and *Leishmania braziliensis* [19].

Recently, many compounds of this moiety were designed and studied as SARS-CoV-2 inhibitors effective against the breakthrough of the COVID-19 pandemic [20, 21].

N-substituted *isoindole-1,3(2H) dione* scaffold possesses a unique architecture with structural features necessary for its biological activity: a hydrophobic aryl ring, a hydrogen

bonding domain, an electron-donor groups, and other structural features depending on substitution on the N atom that may affect the biological activity [22].

Furthermore, many *isoindole-1,3(2H) dione* derivatives have been already approved and marketed as drugs, and Table 1 contains some examples of marketed pharmaceutical products of this moiety, with their indications [23].

From this brief review, *isoindole-1,3(2H) dione* represents a multitarget scaffold, an interesting and promising aim for drug discovery and drug development researches, attracting a growing interest in the recent years the fact that motivated us to perform this study.

Molecular hybridization is a strategy of rational design of new promising ligands based on the recognition of pharmacophoric units in bioactive parent compounds which, through the adequate fusion, lead to the design of new hybrid architectures that maintain preselected pharmacophoric characteristics of the original molecules [24].

Microwave-assisted synthesis (MAS) is a major breakthrough in chemical synthesis methodologies. It is a green chemistry approach since reaction occurs in solid phase, with no need to use toxic solvents, and because microwave irradiation provides a power-saving, and ecofriendly heating source as an alternative to the conventional heating methods [25]. Furthermore, this method has many advantages over conventional method for better yield, short reaction time, and better purity of the products [25].

Microwave-assisted synthesis has revolutionized chemical synthesis the fact that motivated us to use this method in this research.

The aim of this work is to achieve a green and ecofriendly synthesis of *isoindole-1,3(2H) dione* hybrids with phenylalanine amino acid, compounds **1–3** (Figure 1), and to evaluate their biological activity for the first time as potential drug-like molecules. In addition, we report a preliminary study of the structure-activity relationship and preliminary study of the mechanism of action of compounds **1–3** as anticancer candidates.

In this study, the compounds are synthesized following two methods. Microwave-assisted synthesis (MAS) has been successfully applied. Spectacular accelerations, better yields under milder reaction conditions, have been reported.

We investigated, for the first time, the biological activity of compounds **1–3** as an effective antioxidant, antimicrobial, antileishmanial, and anticancer drug-like molecules. All compounds are promising highly effective drugs for the treatment of *Leishmania* as they are more effective against *Leishmania tropica* than the first-line treatment, Glucantime, with IC_{50} in the micromolar range.

Compound **3** is comparable to or more effective than thalidomide for the treatment of Caco-2 colon cancer cells with IC_{50} value $0.080 \mu\text{mol/mL}$.

Herein, we report that the substitution of the four aromatic hydrogen atoms of *isoindole-1,3(2H) dione* analogues with bromine or chlorine atoms leads to more potent antileishmanial, antimicrobial, and anticancer derivatives, and those brominated derivatives are more effective than chlorinated derivatives.

2. Material and Method

2.1. Drug Likelihood and ADME Prediction. Drug likelihood and molecular physicochemical properties were calculated using Molinspiration web service [26]. Pharmacokinetic properties including absorption, distribution, metabolism, and excretion (ADME) characteristics were predicted using PreADMET [27].

2.2. Synthesis and Characterization. Starting materials, solvents, and reagents were purchased from Sigma-Aldrich, and Santa Cruz Biotechnology, Germany, and used without further purification. Reactions were monitored by TLC on silica-gel plates (Merck 60F254). The melting point was determined using Stuart melting point apparatus SMP30 and used without calibration. High-performance liquid chromatography was performed using Shimadzu UFLC apparatus A20.

IR spectra were recorded in KBr on Thermo Nicolet 6700 FT-IR spectrophotometer apparatus. UV spectra were recorded using a JENWAY 6850 UV/Vis spectrophotometer. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra were recorded on a Bruker UltraShield 400 instrument (400 MHz for ^1H , 100 MHz for ^{13}C).

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

2.2.1. General Procedure for the Synthesis of Isoindole-1, 3 (2H) Dione Derivatives. Scheme 1 shows the synthesis reaction, we followed two synthesis methods, method A [28], and method B [29], reactions were monitored by TLC, and crude products were purified by recrystallization from appropriate solvents.

(1) Method A. In a 25 mL round bottom flask, a mixture of 0.010 mole of phthalic anhydride or its derivatives, 0.011 mole of phenylalanine hydrochloride, and 5 mL (0.0648 mole) dimethyl form amide (DMF) was prepared. The mixture was refluxed for 2-3 hours in an oil bath at a temperature of 180°C , and the completion of the reaction was determined by TLC. After cooling to room temperature, 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.

(2) Method B. Equimolar quantities of phthalic anhydride, and phenylalanine hydrochloride, were ground together in a mortar for 1 minute, then the mixture was transferred to a 50 mL glass beaker containing a magnetic stirrer, and the beaker was covered with a water glass and irradiated with microwaves in a microwave oven (700 W) with continuous stirring for 5 minutes. The completion of the reaction was determined by TLC.

TABLE 1: List of some marketed drugs of *isoindole-1,3(2H) dione* derivatives and their analogues.

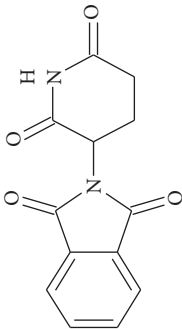
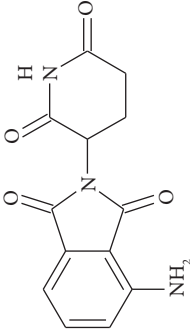
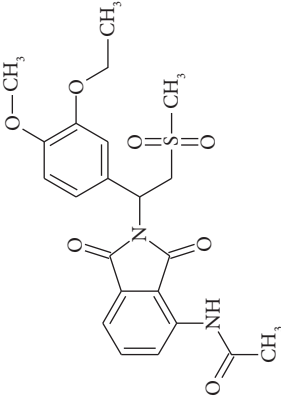
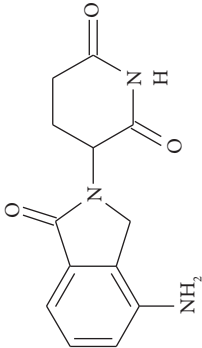
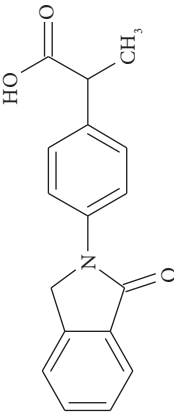
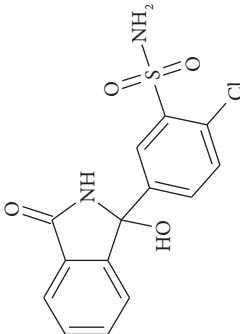
Structure	Name	Use
	Thalidomide (<i>RS</i>)-2-(2,6-Dioxopiperidin-3-yl)-2,3-dihydro-1 <i>H</i> -isoindole-1,3-dione	Antineoplastic, antileptotic, FDA approved for multiple myeloma treatment, erythema nodosum leprosum (ENL)
	Pomalidomide (Actimid®) (<i>RS</i>)-4-Amino-2-(2,6-dioxopiperidin-3-yl) isoindoline-1,3-dione	Angiogenesis inhibitor, immunomodulator, FDA approved for multiple myeloma treatment
	Apremilast (Otezla®) <i>N</i> -(2-[(1 <i>S</i>)-1-(3-Ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-1, 3-dioxo-2,3-dihydro-1 <i>H</i> -isoindol-4-yl) acetamide	Psoriasis and psoriatic arthritis
	Lenalidomide (Revlimid®) (<i>RS</i>)-3-(4-Amino-1-oxo-1, 3-dihydro-2 <i>H</i> -isoindol-2-yl) piperidine-2,6-dione	FDA approved for multiple myeloma
	Indoprofen 2-[4-(1-Oxo-1,3-dihydro-2 <i>H</i> -isoindol-2-yl) phenyl] propanoic acid	Anti-inflammatory (withdrawn)

TABLE 1: Continued.

Structure	Name	Use
	Chlororthalidone <i>(RS)</i> -2-Chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1 <i>H</i> -isoindol-1-yl) benzene-1-sulfonamide	Diuretic

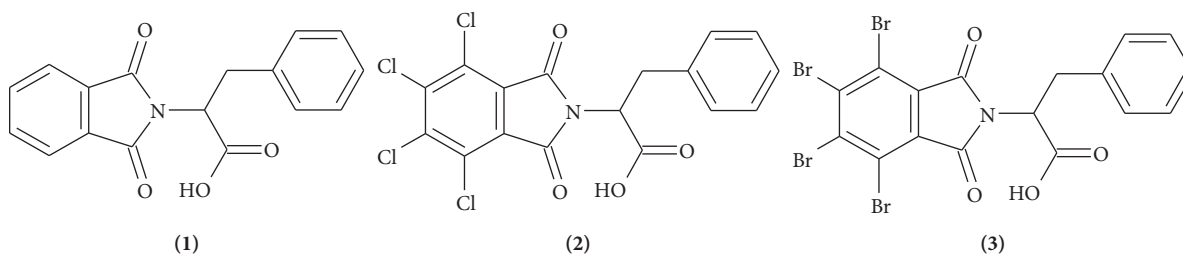
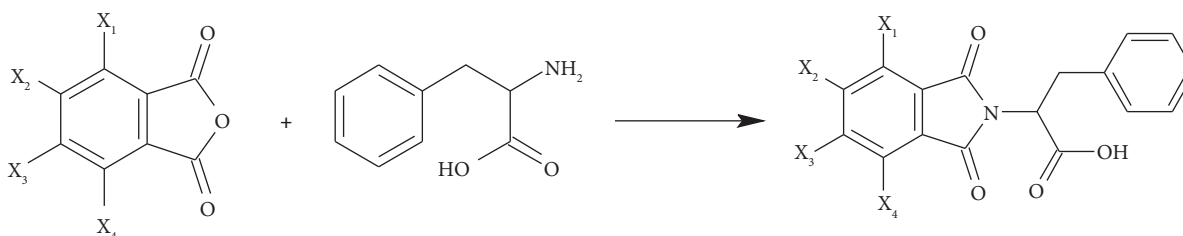


FIGURE 1: Structure of synthesized compounds.



Compound number	X ₁	X ₂	X ₃	X ₄
Compound 1	H	H	H	H
Compound 2	Cl	Cl	Cl	Cl
Compound 3	Br	Br	Br	Br

SCHEME 1: Synthesis reaction of compounds (1–3).

The mixture was cooled to room temperature, and then, 50 mL of ice-cold water was poured into the flask. The crude product was precipitated, filtered off, washed with water, dried, and purified by recrystallization from appropriate solvents.

Structures were characterized by analytical and spectral methods, and furthermore, specific details can be found in the supplementary materials (available here).

2.2.2. 2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-phenylpropanoic Acid (1). White crystals yield: 82%, MP 172–173°C, UV λ_{\max} (methanol, nm): 218, 294. IR (KBr, cm^{-1}): 3267 (OH), 3029, 3065 (Ar-H stretch.), 2923 (CH_2 stretching), 1773 (CO), 1746 (CO), 1697 (CO), 1610 (C=C), 1467 (CH_2 bending). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , ppm): 13.371 (s, 1H, OH), 7.8782–7.776 (m, 4H, Ar-4H in Isoindole moiety), 7.207–7.055 (m, 5H, Ar-5H of benzene), 5.15–5.06 (m, 1H, N-CH), 3.560–3.125 (m, 2H, CH_2 -phenyl). $^{13}\text{C NMR}$ (DMSO-d_6 , 100 MHz, ppm): 170.502 (COOH), 167.559 (2CO), 137.776, 135.404, 131.193, 129.169, 128.769, 127.027, 123.845, 53.399 (N-CH), 34.405 (CH_2 -phenyl). DEPT135 $^{13}\text{C NMR}$ (DMSO-d_6 , 100 MHz, ppm): 135.423, 129.178, 128.778, 127.037, 123.861, 53.387 (N-CH), 34.394 (CH_2 -phenyl). ESI+ MS-MS: 334.2 [M+K]⁺, 318.0 [M+Na]⁺, 295.9 [M+H]⁺, 277.9 [M-OH]⁺, 250.0 [M-COOH]⁺.

2.2.3. 3-Phenyl-2-(4,5,6,7-tetrachloro-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl) Propanoic Acid (2). Pale yellow powder yield: 80%, MP 265.5–266°C, UV λ_{\max} (methanol, nm): 211, 236, 332. IR (KBr, cm^{-1}): 3437 (OH), 3060 (Ar-H stretch.), 2920 (CH_2 stretching), 1784 (CO), 1729 (CO), 1610 (C=C), 1426 (CH_2 bending), 1197 (C-N), 743 (C-Cl). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , ppm): 13.453 (s, 1H, OH), 7.430–6.950 (m, 5H, Ar-5H of benzene), 5.234–5.055 (s, 1H, N-CH), 3.593–3.442 (m, 2H, CH_2). $^{13}\text{C NMR}$ (DMSO-d_6 , 100 MHz, ppm): 169.888 (COOH), 163.066 (2CO), 139.683, 137.585, 129.186, 129.127, 128.900, 127.499, 127.120, 54.221 (N-CH), 34.265 (phenyl- CH_2). DEPT 135 $^{13}\text{C NMR}$ (DMSO-d_6 , 100 MHz, ppm): 129.186 (2C aromatic), 128.900 (2C aromatic), 127.120 (1C aromatic), 54.221 (N-CH), 34.265 (phenyl- CH_2).

2.2.4. 3-Phenyl-2-(4,5,6,7-tetrabromo-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl) Propanoic Acid (3). Yellow powder yield: 60%, MP 300–302°C (degradation), UV λ_{\max} (methanol, nm): 206, 245, 338. IR (KBr, cm^{-1}): 3435.9 (OH), 3028 (Ar-H stretch.), 1775 (CO), 1721 (CO), 1632 (C=C), 1426 (CH_2 bending), 1162 (C-N), 670 (C-Cl). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , ppm): 13.454 (s, 1H, OH), 7.308–7.0318 (m, 5H, Ar-5H of benzene), 5.218–5.046 (s, 1H, N-CH), 3.702–3.431 (m, 2H, Phenyl- CH_2). $^{13}\text{C NMR}$ (DMSO-d_6 , 100 MHz, ppm): 169.934 (COOH), 163.356 (2CO), 137.912, 137.637, 130.300,

TABLE 2: Calculated values for molecular weight (MW), TPSA, logP, H-donor, and H-acceptor for compounds.

	Compound 1	Compound 2	Compound 3
Log P	2.56	4.41	4.93
TPSA (Å ²)	76.38	76.38	76.38
MW (g/mol)	295.29	433.07	610.88
Hydrogen acceptor	5	5	5
Hydrogen donor	1	1	1

TPSA value indicates good absorption after oral administration (TPSA < 140 Å²) and moderate ability to cross the blood-brain barrier (TPSA < 60 Å²) [36].

TABLE 3: Values of ADME properties of the studied compounds obtained using the in silico method.

ADME descriptor	Compound 1	Compound 2	Compound 3
HIA (human intestinal absorption) %	98.92	97.58	98.163
Caco-2 (nm/second)	21.0324	17.203	17.08
BBB (C brain/C blood)	0.456	1.959	1.82
Plasma_Protein_Binding (PPB%)	96.935	95.219	97.33
CYP_2C19_inhibition	Non	Non	Non
CYP_2C9_inhibition	Inhibitor	Inhibitor	Inhibitor
CYP_2D6_inhibition	Non	Non	Non
CYP_2D6_substrate	Non	Non	Non
CYP_3A4_inhibition	Non	Non	Non
CYP_3A4_substrate	Weakly	Weakly	Substrate
Pgp_inhibition	Non	Inhibitor	Inhibitor

129.178, 128.862, 127.084, 121.496, 54.272 (N-CH), 34.272 (CH₂). DEPT135 ¹³C NMR (DMSO-d₆, 100 MHz, ppm): 129.192 (2C aromatic), 128.876 (2C aromatic), 127.098 (1C aromatic), 54.265 (N-CH), 34.268 (phenyl-CH₂).

2.3. Biological Evaluation

2.3.1. Antioxidant Activity. The antioxidant activity of prepared compounds was determined by measuring their free radical scavenging activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) according to an already reported method [30]. The solution of 0.1 mM of DPPH in methanol was prepared, saved in a cool dark place, and used fresh.

The stock solution of each material in methanol was prepared and diluted to prepare serial concentrations (250, 500, 750, and 1000 µg/mL). The stock solution of ascorbic acid in methanol was prepared and diluted to prepare serial concentrations as well. 1 mL of compound solution was mixed with 3 mL of DPPH solution and allowed to stand in dark for 30, 60, 90, and 120 minutes.

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

DPPH scavenging activity was calculated by the following equation:

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

IC₅₀ was calculated from the concentration-inhibition curve by plotting the sample concentration versus the corresponding DPPH scavenging activity.

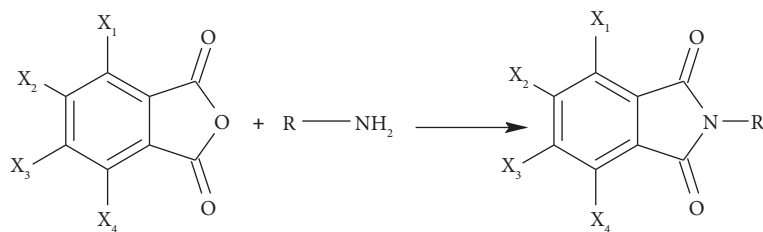
2.3.2. Antimicrobial Activity. The antimicrobial activity of prepared compounds against Gram-positive (*Staphylococcus aureus*, ATCC 6538) and Gram-negative (*Escherichia coli*, ATCC 8739) bacteria was investigated by the well diffusion method as reported by Perez et al. [31]. Bacterial strains are supplied by Thermo Fisher Scientific Inc., USA.

The stock solution of each compound (3200 µg/mL) was prepared in an appropriate solvent and used to prepare other concentrations (1600, 800, 400, 200, and 100 µg/mL) through a twofold dilution method.

Overnight culture of each microorganism was prepared and used in sterile conditions, and few colonies were taken by sterile loop, dispersed in physiological saline serum, and vortexed for few minutes. The process was repeated to adjust microbial concentration to 0.5 McFarland standard.

Culture media were prepared by the pour plate method, in which Mueller-Hinton Agar culture was prepared, sterilized in an autoclave (temperature 121°C for 15 minutes), and then cooled to temperature 45–50°C with stirring, and then, the microbial suspension was added to culture media at percentage 1%, mixed clockwise and counterclockwise, and then poured in Petri dishes (depth 3–4 mm).

Poured plates were left to cool and solidify in sterile conditions; then, similar wells (8 mm in diameter) were made in the agar and loaded with 100 µL of the tested compound solution. Inoculated plates were left at room



Compound number	X ₁	X ₂	X ₃	X ₄	R
Compound 1	H	H	H	H	3-Phenylpropanoic
Compound 2	Cl	Cl	Cl	Cl	3-Phenylpropanoic
Compound 3	Br	Br	Br	Br	3-Phenylpropanoic

SCHEME 2: Synthesis of isoindole-1,3 (2H) dione compounds.

temperature for 15 minutes without movement and then incubated at 37°C for 24 h.

After incubation time, antimicrobial activity was evaluated by measuring the zone of inhibition (IZ) against the test organisms and compared with that of the standard. Antimicrobial activities were expressed as inhibition diameter zones in mm. Each experiment was carried out in triplicate, and the average zone of inhibition was calculated.

Gentamycin sulfate was used as the standard drug for antibacterial activity, and the pure solvent was used as the negative control.

2.3.3. Antileishmanial Activity. Tropical Leishmania cell culture (SYR113) was used for this experimental study, carried out at Leishmaniasis Research Center for Epidemiological and Biological Studies (LCEBS), Damascus University.

The inhibition of Leishmania growth was assessed against the promastigote forms of Tropical Leishmania using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)-based method as reported by Mosmann [32] with modifications [33].

MTT was purchased from Sigma Chemical Co. DMSO was used to dissolve compounds to prepare the stock solution and standard serial concentrations.

Stationary-phase promastigotes of *L. tropica* (5×10^6 cells) were cultured in 12-well plates with synthesized compound solution and incubated for 48 hours at 26°C. Negative control wells contained culture medium with parasite culture. Glucantime was used as a standard drug. Absorbance was measured using an ELISA reader spectrophotometer at 540 nm. All experiments were carried out in triplicate, and the results were expressed in percent of inhibition as follows:

$$\text{inhibition} = \left[100 - \left(\frac{\text{absorbance of test compound}}{\text{absorbance of the negative control}} \right) \right] * 100. \quad (2)$$

2.3.4. Anticancer Activity. The effect of compounds was studied on human Caco-2 and HCT-116 cell lines (Sigma-Aldrich), and passage numbers are 15 and 10 consequently. The density of cells used in tests is 150 cells/ μ L.

Studied compounds were dissolved in DMSO to prepare stock solutions that were diluted with culture medium to prepare serial concentrations of each compound.

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

(1) Cell Cultures. Human Caco-2 and HCT-117 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μ g/mL streptomycin in sterile flasks. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

(2) Antiproliferative Activity Assay. Human Caco-2 and HCT-117 cultures were seeded in 96-well plates (1.5×10^4 cells/well). The final concentration of DMSO in wells is less or equal to 0.1%.

Plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 hours, then the culture medium was removed, and cells were treated with different doses of studied compounds. DMSO alone was added to a set of wells as the solvent control, and culture medium was added to another set of wells as a negative control. Five concentrations (62.5, 125, 250, 500, and 1000 μ g/mL) were

TABLE 4: Physiochemical properties of compounds.

Compound no.	Compound name	Molecular formula	MW	Purity (%)	M.P. (°C)	Yield (%)
1	2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-phenylpropanoic acid	C ₁₇ H ₁₃ NO ₄	295.29	99.50	172-173	82-88
2	3-Phenyl-2-(4,5,6,7-tetrachloro-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoic acid	C ₁₇ H ₉ Cl ₄ NO ₄	433.07	99.55	265.5-269	78-80
3	3-Phenyl-2-(4,5,6,7-tetrabromo-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoic acid	C ₁₇ H ₉ Br ₄ NO ₄	610.87	99.70	** 300-302	60-63

*Melting with degradation.

TABLE 5: In vitro antimicrobial activity of synthesized compounds against *Staphylococcus aureus*.

Conc. ($\mu\text{g/mL}$)	Zone of inhibition on <i>Staphylococcus aureus</i> (mm)					
	100 $\mu\text{g/mL}$	200 $\mu\text{g/mL}$	400 $\mu\text{g/mL}$	800 $\mu\text{g/mL}$	1600 $\mu\text{g/mL}$	3200 $\mu\text{g/mL}$
Compound 1	0	0	0	0	13	16.34
Compound 2	18	19	21	26.67	30	31.34
Compound 3	19	20	22	27.34	30.34	32
Gentamycin sulfate	19.34	21.34	22.34	24	25	26

TABLE 6: In vitro antimicrobial activity of synthesized compounds against *Escherichia coli*.

Conc. ($\mu\text{g/mL}$)	Zone of inhibition on <i>Escherichia coli</i> (mm)					
	100 $\mu\text{g/mL}$	200 $\mu\text{g/mL}$	400 $\mu\text{g/mL}$	800 $\mu\text{g/mL}$	1600 $\mu\text{g/mL}$	3200 $\mu\text{g/mL}$
Compound 1	0	8.34	9	10.34	11.34	13
Compound 2	9.34	11	12.67	14.34	15	17
Compound 3	12	13.34	14	15	17	20
Gentamycin sulfate	0	0	13	17	20	21

studied for each compound, and each concentration was studied in three replicates.

Plates were incubated for 48 hours at a temperature of 37°C and 5% CO₂ atmosphere.

Anticancer activity assay was measured after 48 hours by XTT assay as reported by Skehan et al. [34] according to 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 was calculated as follows:

$$\text{inhibition ratio} = \left[1 - \left(\frac{\text{absorbance of test compound}}{\text{absorbance of the negative control}} \right) \right] * 100. \quad (3)$$

The inhibition ratio was calculated as a mean of the results of all replicates. IC₅₀ of the most effective compounds was calculated from the dose-response curve.

(3) *Cell Cycle Analysis*. In order to get more information about the anticancer activity of studied compounds, we have studied cell cycle phases in treated cells, compared to untreated cells.

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

Cells were trypsinized, harvested, and combined with floating cells by centrifuge, and the cellular precipitate was washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640.

Samples were fixed by adding methanol and incubation in dark for 30 minutes at -20°C.

Samples were centrifuged, suspended liquid was thrown, and fixed cells were rinsed twice by PBS. Then, the DNA fluorochrome PI in a solution containing Triton X-100 and RNase was used as a staining solution, and samples were kept in dark for 30 minutes at 4-8°C. Stained cells were analyzed by BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's protocol. The number of cells analyzed for each sample was 10⁴ cells [35]. The percentage of the cells in each cell cycle phase was determined. The tests were performed in duplicates and repeated at least twice.

(4) *Quantitative and Qualitative Cell Death Assay*. Quantitation assay of apoptosis and necrosis cell death of cells treated with studied compounds for 72 hours was executed by Annexin V/propidium iodide (PI) double staining assay method.

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

Cells were trypsinized, harvested, and combined with floating cells by centrifuge. The cellular precipitate was washed with phosphate-buffered saline (PBS) and resuspended in RPMI1640, and then, samples were fixed by adding methanol and incubating them in dark for 30 minutes at -20°C.

Samples were centrifuged, suspended liquid was thrown, and fixed cells were rinsed twice by PBS and stained with 5 μL Annexin V and 5 μL PI and 350 μL Annexin binding buffer for 10 minutes in the dark at room temperature, and then, samples were analyzed by BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

TABLE 7: Free radical scavenging activity of studied compounds and ascorbic acid.

Conc. ($\mu\text{g}/\text{mL}$)	Free radical scavenging activity %															
	Compound 1			Compound 2			Compound 3			Ascorbic acid						
	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	50.23	57.33	61.48	62.39	10.5	11.99	12.85	16.85	13.84	16.19	20.16	26.35	95.42	98.02	98.13	98.23
500	50.61	57.52	63.15	65.46	36.12	41.05	45.06	46.71	16.26	20.24	25	31.14	96.58	98.15	98.22	98.36
750	55.65	59.58	64.81	71.62	51.87	54.32	60.41	66.76	30.2	36.44	39.92	47.9	96.65	98.16	98.26	98.41
1000	58.66	63.33	68.52	75.32	54.78	60.8	63.98	70.33	40.65	44.53	49.19	55.89	96.66	98.18	98.3	98.47

TABLE 8: Antileishmanial effect of studied compounds (GI%) against *Leishmania tropica*.

Compound name	Antileishmanial effect of studied compounds (GI%)						IC ₅₀ μ mol/mL
	75 μ g/mL	125 μ g/mL	250 μ g/mL	500 μ g/mL	1000 μ g/mL	2000 μ g/mL	
Compound 1	50.2	62.81	64.52	66.74	69.12	76.75	0.25
Compound 2	59.85	67.78	69.18	73.15	77.8	85.75	0.111
Compound 3	64.5	72.86	74.42	79.31	82.65	95.12	0.0478
Glucantime	20.2	22.5	30.2	58.5	70.2	75.25	1.226

Compound 1: 0.001; compound 2: 0.001; compound 3: 0.0001; glucantime: 0.001.

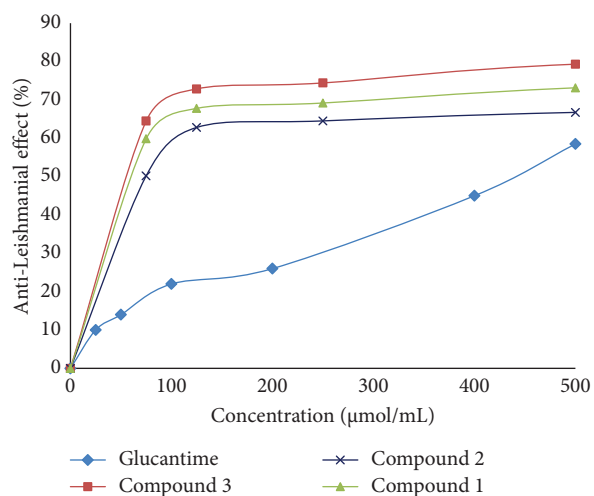


FIGURE 2: Concentration-antileishmanial effect curve of studied compounds and positive control.

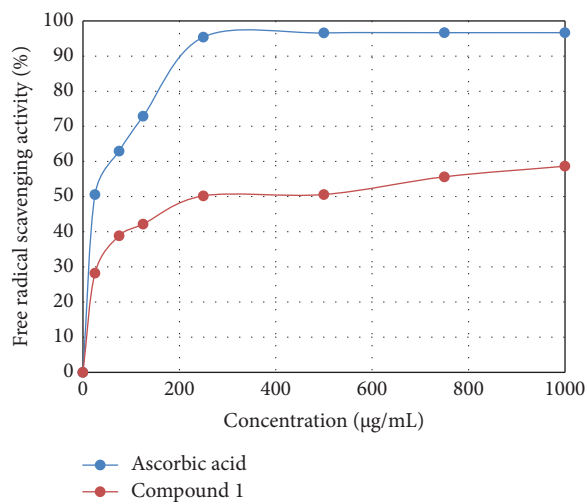


FIGURE 3: DPPH radical scavenging activity of ascorbic acid and compound 1.

TABLE 9: Antiproliferative effect of studied compounds on Caco-2 cell line.

Compound number	Conc. μ g/mL	Antiproliferative effect % on Caco-2 cell line					IC ₅₀ (μ mol/mL)
		62.5	125	250	500	1000	
Compound 1		42.591	42.748	54.932	62.768	68.77	0.556
Compound 2		48.85	52.25	60.85	65.8	70.5	0.215
Compound 3		48.69	64.12	68	73.11	73.26	0.080

Compound 1: 0.001; compound 2: 0.001; compound 3: 0.001.

TABLE 10: Antiproliferative effect of studied compounds on HCT-116 cell line.

Compound number	Conc. ($\mu\text{g/mL}$)	Antiproliferative effect % on HCT-116 cell line					IC_{50} ($\mu\text{mol/mL}$)
		62.5	125	250	500	1000	
Compound 1		0	21.317	28.723	39.546	46.154	>3.39
Compound 2		20.5	35.8	38.2	44.8	50.25	1.941
Compound 3		36.098	48.232	48.126	50.715	54.432	0.651

Compound 1: 0.001; compound 2: 0.001; compound 3: 0.001.

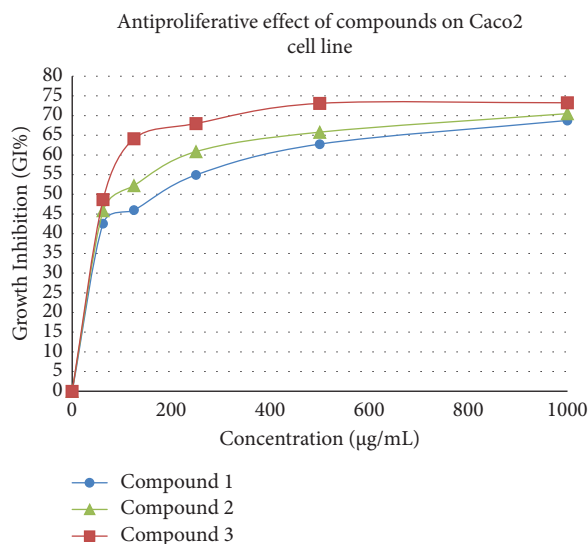


FIGURE 4: Antiproliferative effect of studied compounds on Caco-2 cell line.

The number of cells analyzed for each sample was 10^4 cells [35]. Gating was implemented on the basis of negative-control staining profiles.

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

3. Result and Discussion

3.1. Drug Likelihood and ADME Prediction. Physicochemical and drug-likeness properties of synthesized compounds were calculated using online Molinspiration, and the results are presented in Table 2.

Results ensure that compounds comply with Lipinski's rule of five [37], as hydrogen bond donors are less than 5, hydrogen bond acceptors are less than 10, molecular weight does not exceed 500, and the partition coefficient of compounds in n-octanol/water biphasic system ($\log P$) is less than 5.

ADME properties of compounds were calculated using the online PreADMET webservice, and the results are presented in Table 3.

Results of ADME descriptors indicate that blood-brain barrier penetration is moderate (BBB is within the range 0.1–2) [36, 38], gastrointestinal absorption of compounds is high (HIA is more than 70%) [39], and

results of BBB and HIA are in good agreement with results of TPSA.

The penetration rate of compounds through Caco-2 cells is moderate (within the range of 4–70 nm/sec) [40], and plasma protein binding is high (PPB > 90%).

Compounds are expected to inhibit cytochrome enzymes CYP 2C9 and CYP 3A4. Compounds 2 and 3 are expected to inhibit glycoprotein P.

3.2. Synthesis and Characterization. Isoindoline-1,3-dione derivatives (**1**, **2**, **3**) were synthesized by molecular hybridization of phenylalanine and phthalic anhydride derivatives according to a one-step synthesis reaction indicated in Scheme 2.

Synthesis occurs by dehydrative condensation of phthalic anhydride derivatives and phenylalanine in the presence or absence of solvent. We have followed two methods of synthesis, the conventional method and the microwave-assisted method.

The microwave-assisted method is a solvent-free, fast, mild, and highly efficient method. In addition, it is green chemistry and an eco-friendly method, the reaction time is shorter (a few minutes only), the yield is better, and conditions are milder in the microwave-assisted method, compared to the conventional method.

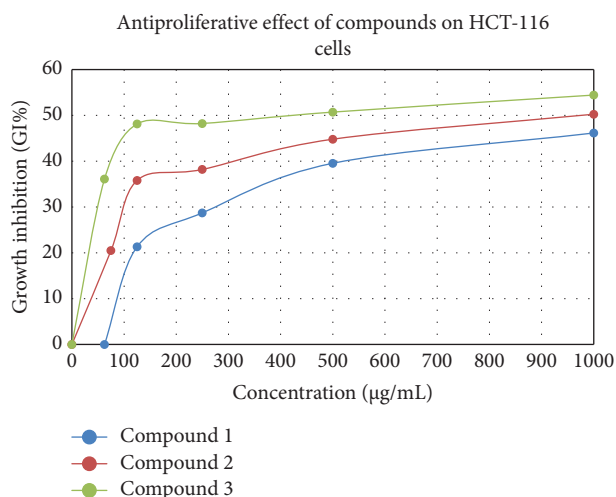


FIGURE 5: Antiproliferative effect of studied compounds on HCT-116 cell line.

TABLE 11: Effect of 72 hours of treatment with studied compounds on the cell cycle of Caco-2 cells.

Compound number	Concentration (µg/mL)	Percentage of Caco-2 cells in each phase			Effect on cell cycle
		G1%	S%	G2/M %	
Negative control	—	69.76	23.26	6.98	—
Compound 1	1000	52.09	37.47	10.44	Arrest on S and G2/M
Compound 2	500	50.8	40.52	8.68	Arrest on S and G2/M
Compound 3	500	48.39	43.13	8.48	Arrest on S and G2/M

TABLE 12: Effect of 72 hours of treatment with studied compounds on the cell cycle of HCT-116 cells.

Compound number	Concentration (µg/mL)	Percentage of HCT-116 cells in each phase			Effect on cell cycle
		G1%	S%	G2/M %	
Negative control	—	86.69	8.85	4.46	—
Compound 1	500	79.32	15.18	5.51	Arrest on S
Compound 2	125	72.5	18.4	9.1	Arrest on S and G2/M
Compound 3	125	65.48	20.22	14.3	Arrest on S and G2/M

Structures of compounds are confirmed by $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, LC-MS, FT-IR, and UV-visible spectra. Analytical and spectral results of all identification tests comply with the structures of compounds.

The physicochemical properties of compounds are tabulated in Table 4.

3.3. Determination of Biological Activity. Synthesized compounds have been evaluated for their *in vitro* antimicrobial, antioxidant, antileishmanial, and antiproliferation activity.

3.3.1. Antimicrobial Activity. The antimicrobial effect of studied compounds against *Staphylococcus aureus* and *Escherichia coli* is indicated in Tables 5 and 6.

Results showed that synthesized compounds are effective against Gram-positive and Gram-negative microorganisms compared to negative control (solvent), with concentrations

in the micromolar range. Results showed that Gram-positive microorganisms are more sensitive to studied compounds than Gram-negative microorganisms.

Compounds 2 and 3 showed a good zone of inhibition against tested bacterial strains, and their results are comparable with those of the standard drug gentamycin sulfate.

Compound 1 showed no to moderate inhibitory activity against tested bacterial strains depending on the studied concentration.

The results show that the halogenation of isoindole-1,3(2H) dione enhances the antimicrobial effect of compounds.

3.3.2. Antioxidant Activity. Antioxidant properties of compounds were evaluated by the DPPH free radical scavenging assay in an attempt to find new sources of antioxidants. This *in vitro* assay provides a method to evaluate the antioxidant activity in a relatively short period of time.

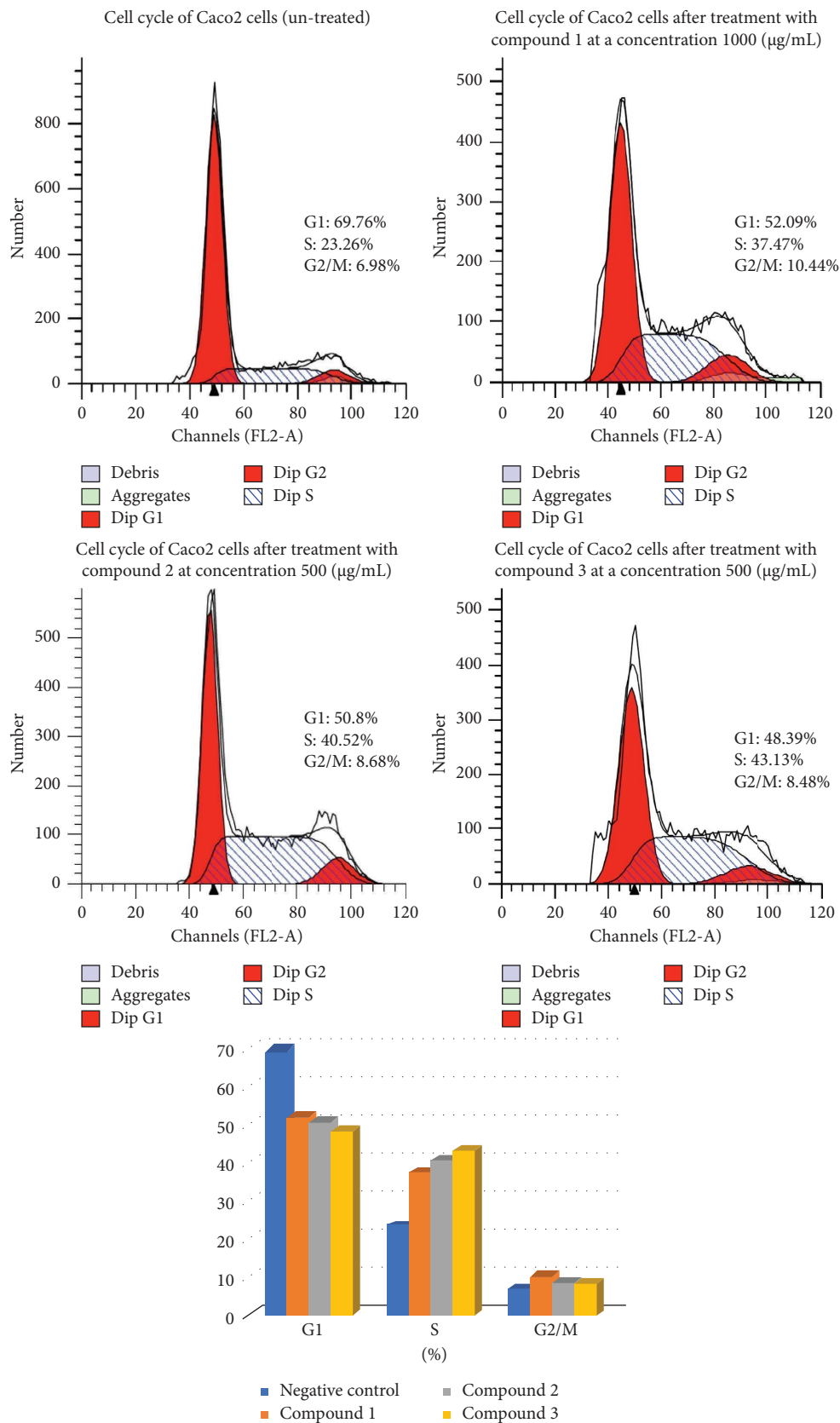


FIGURE 6: Cell cycle analysis of Caco-2 cells untreated and after treatment with studied compounds using PI staining method by flow cytometry.

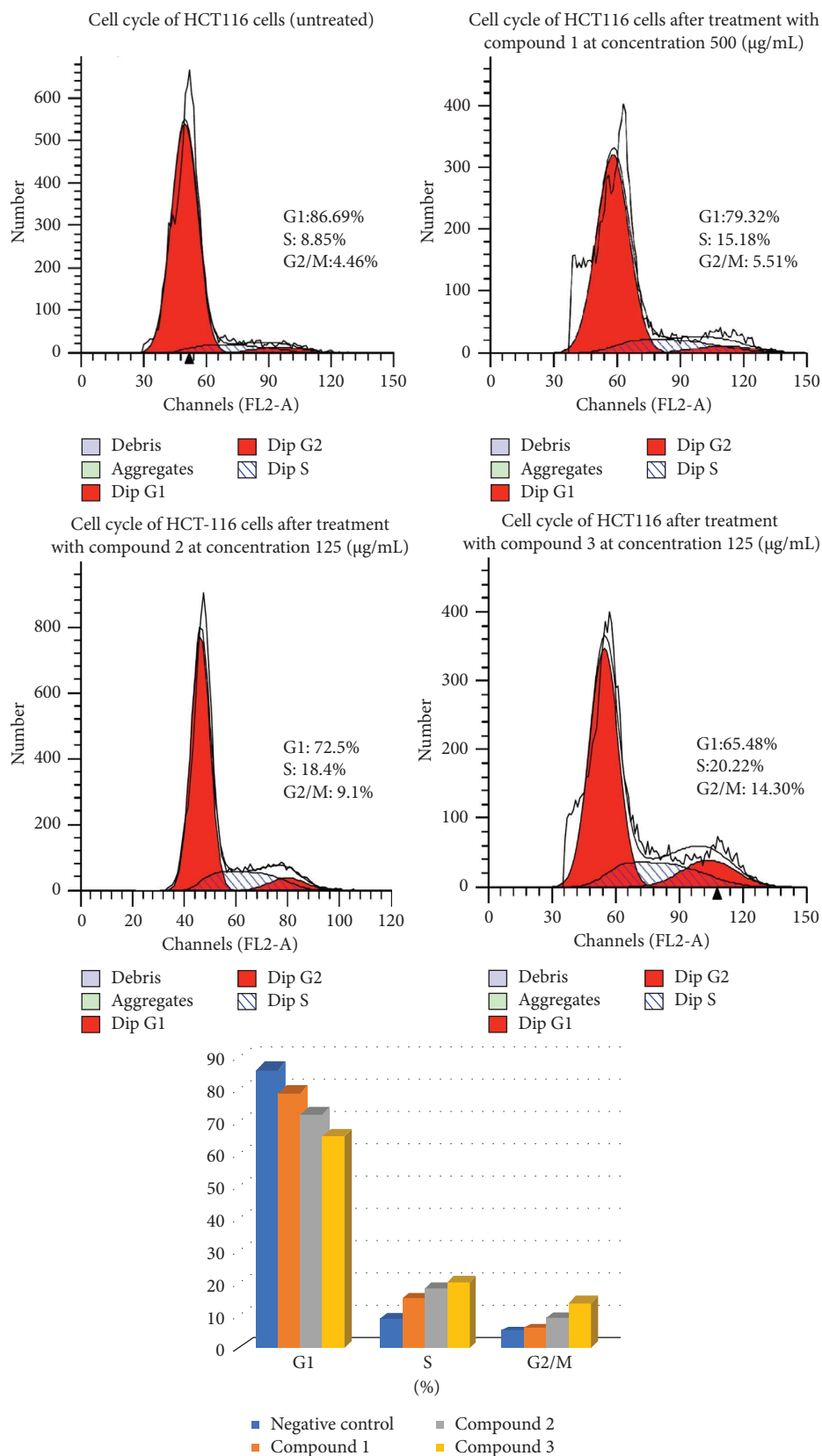


FIGURE 7: Cell cycle analysis of HCT-116 cells untreated and after treatment with studied compounds using PI staining method by flow cytometry.

TABLE 13: Cell death assay of Caco-2 cells untreated and treated with studied compounds.

Compound number	Concentration ($\mu\text{g}/\text{mL}$)	Cell death assay in Caco-2 line			
		R1% late apoptotic cells	R2% live cells	R3% early apoptotic cells	R4% necrotic cells
Negative control	—	7.33	90.08	2.06	0.51
Compound 1	1000	12.94	74.04	12.22	0.95
Compound 2	500	53.52	25.75	20.48	0.25
Compound 3	500	89.04	5.65	5.04	0.29

Results of our compounds are shown in Table 7 compared to results of known antioxidant ascorbic acid.

Studied compounds showed the ability to scavenge DPPH free radicals in a time- and concentration-related manner, although they are less efficient compared to ascorbic acid.

Compounds **1** and **2** showed considerable activity in quenching DPPH free radicals with significant IC_{50} values (1.174 and 1.816 $\mu\text{mol}/\text{mL}$ consequently) and can thus ensure protection against oxidative stress caused by free radicals.

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

3.3.3. Antileishmanial Activity. Six concentrations were studied for each compound, and each concentration was studied in three duplicates. Table 8 displays the antileishmanial effect of compounds and standard drug, Glucantime, against *Leishmania tropica*, along with IC_{50} .

The inhibitory activity demonstrated a dose-dependent pattern (Figure 2). All compounds inhibited the growth of promastigotes effectively and more efficiently than the standard drug, Glucantime. IC_{50} of all compounds is less than 1 $\mu\text{mol}/\text{mL}$, which assures high efficiency.

Results show that compound **3** is the most effective antileishmanial compound, then compound **2**, compound **1**, and Glucantime consequently, with IC_{50} in the micromolar range.

All studied compounds have the potential of Leishmania treatment, and they are more effective than the standard drug Glucantime, which is the first-line treatment of Leishmania; however, compound **3** showed the highest inhibitory effect (Figure 3), with IC_{50} 0.0478 $\mu\text{mol}/\text{mL}$, 25.6 times more effective than Glucantime.

The higher antileishmanial effect of compound **3** may be explained due to its higher $\log P$ value and the higher lipophilicity, which affects the bioavailability of the compound

within parasite cells and may affect the affinity of the compound to the targeted protein as well.

The IC_{50} values of compounds are in good correlation with $\log P$ values, the fact that assures the importance of lipophilicity of compounds for their antileishmanial activity.

Molecular hybridization of phenylalanine with isoindole-1,3(2H) dione moiety gives hybrid compounds with high antileishmanial activity.

The substitution of hydrogens of isoindole moiety by tetra halogen atoms has a positive effect on antileishmanial activity due to increased lipophilicity. Bromide-substituted derivatives are more effective than chloride-substituted derivatives.

3.3.4. Anticancer Activity. We have studied the effect of the compounds on two cancer cell lines, Caco-2 and HCT-116.

(1) Antiproliferative Assay. The anticancer activity/antiproliferative activity of compounds was evaluated by XTT assay (cell viability assay). Five concentrations (62.5, 125, 250, 500, and 1000 $\mu\text{g}/\text{mL}$) were studied for each compound, and each concentration was studied in three triplicates. The antiproliferative effect of compounds was studied on two cell lines, Caco-2 and HCT-116, after 48-hour treatment. Antiproliferative percentage and IC_{50} values are shown in Tables 9 and 10, respectively.

Results indicate significant antiproliferative effect of studied compounds on both cell lines, and results are in good correlation with studied concentrations (Figures 4 and 5).

Compounds have a high antiproliferative effect against Caco-2 with IC_{50} values 0.560, 0.264, and 0.075 $\mu\text{mol}/\text{mL}$, consequently.

Compound **3** is seven times more effective than compound **1**, and compound **2** is twice more effective than compound **1** against Caco-2 cells, and this result improves that the halogenation of isoindole-1,3(2H) dione moiety increases the antiproliferative effect against Caco-2 cells and

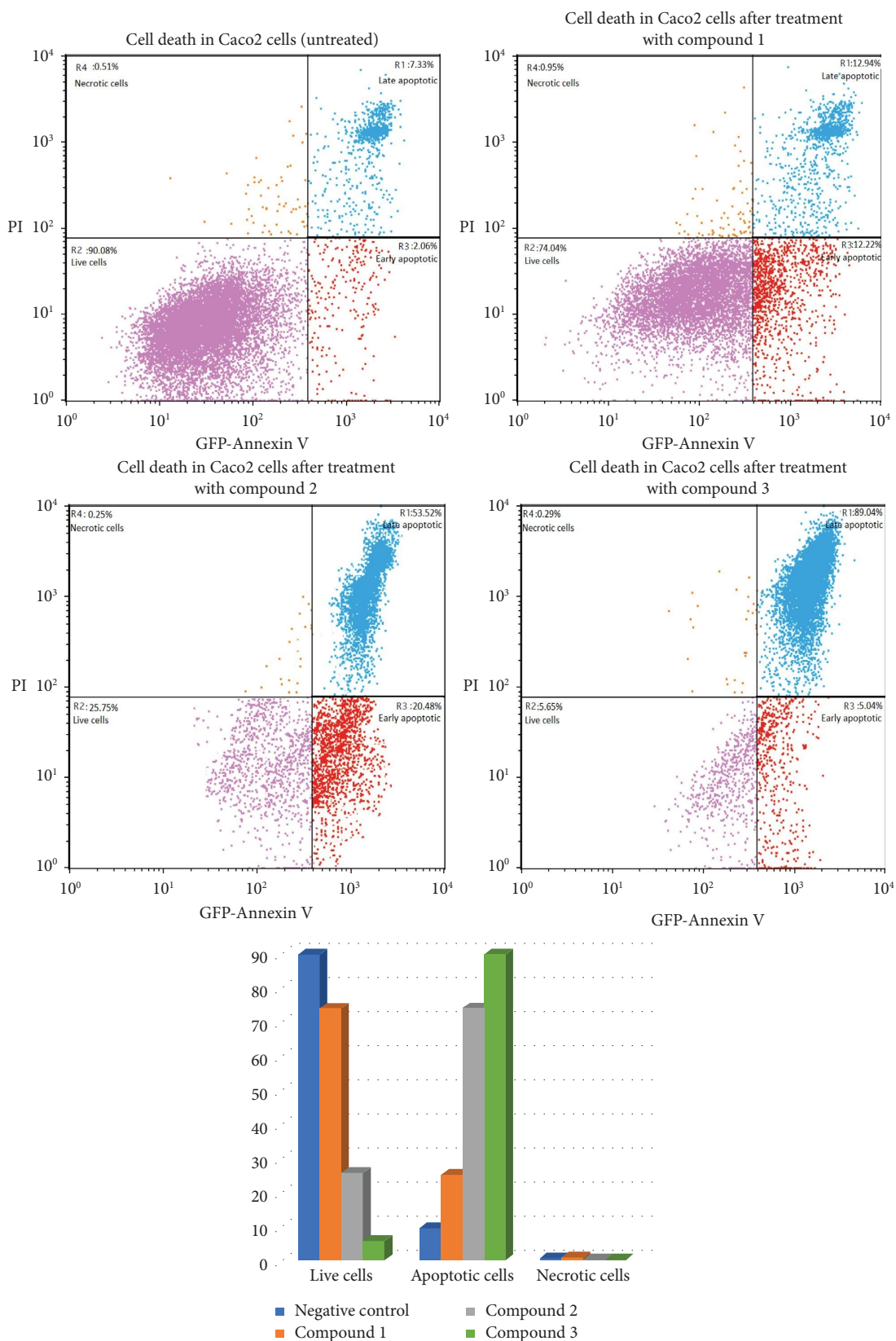


FIGURE 8: Results of cell death assay of Caco-2 cells untreated and treated with studied compounds.

TABLE 14: Cell death assay of HCT-116 cells untreated and treated with studied compounds.

Compound number	Cell death assay in HCT-116 line				
	Concentration ($\mu\text{g/mL}$)	R1% late apoptotic cells	R2% live cells	R3% early apoptotic cells	R4% necrotic cells
Negative control	—	16.64	76.96	6.22	0.32
Compound 1	500	36.95	34.29	3.57	26.28
Compound 2	125	42.5	35.2	10.5	11.8
Compound 3	125	17.14	33.71	0.64	49.52

that brominated derivatives are more effective than chlorinated derivatives, and this result is in agreement with results of previous studies [41].

The antiproliferative effect of compound 3 ($\text{IC}_{50} = 0.080 \mu\text{mol/mL}$) against Caco-2 cells is comparable to or more effective than thalidomide ($\text{IC}_{50} = 0.088 \mu\text{mol/mL}$) [42].

Compounds have an antiproliferative effect against HCT-116 with IC_{50} values 1.941 and $0.651 \mu\text{mol/mL}$ for compounds 2 and 3, consequently. IC_{50} of compound 1 is not applicable in studied concentrations.

Compounds 2 and 3 are more effective than compound 1 against HCT-116 cells (Figure 5), compound 3 is 2.9 times more effective than compound 2, and this result improves that the halogenation of isoindole-1,3-(2H) dione moiety increases antiproliferative effect against HCT-116 cells and that brominated compounds are more effective than chlorinated compounds, and this result is in agreement with results of previous studies [41]. Results on HCT-116 cells are in agreement with results on Caco-2 cells.

(2) *Cell Cycle Analysis.* For further understanding of the mechanism of the antiproliferative effect of compounds, we have treated Caco-2 and HCT-116 cells with studied compounds at concentrations indicated in Tables 11 and 12 for 72 hours, and then, we have defined the percentage of cells in each phase of the cell cycle using PI staining method by flow cytometry.

Results of studying the cell cycle of Caco-2 and HCT-116 cells treated with compounds for 72 hours compared with untreated cells (Tables 11 and 12) show that all compounds strongly delay the progress throughout the cell cycle, the fact that 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 they arrest the cell cycle of treated cells at phases S and G2/M

(Figure 6). This result confirms that compounds inhibit the synthesis of DNA and mitosis in treated cells, the fact that explains their antiproliferative effect, and this result is in agreement with our previous results of antiproliferative effect.

Figure 7 shows the results of the cell cycle study of the HCT-116 cellular line after treatment with compounds.

Compound 1 arrests the cell cycle of treated cells at phase S, which indicates its ability to inhibit the synthesis of DNA in treated cells. Compounds 2 and 3 arrest the cell cycle of treated cells at phases S and G2/M, which indicates their ability to inhibit the synthesis of DNA and mitosis in treated cells.

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

(3) *Quantitative and Qualitative Cell Death Assay.* For further characterization and understanding of the mechanism of cell death induced by treatment with active compounds, we have treated Caco-2 and HCT-116 cells with studied compounds for 72 hours and assayed the percentage of necrotic cells and percentage of cells in different stages of apoptosis using Annexin V binding propidium iodide uptake method by flow cytometry.

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

Results of the cell death assay in Caco-2 cells are indicated in Table 13. Results show that compounds effectively induce apoptosis in Caco-2 cells compared with untreated cells (Figure 8).

This result is in agreement with our previous results.

Results of the cell death assay in HCT-116 cells are indicated in Table 14. Results show that compounds effectively induce apoptosis and necrosis in HCT-116 cells compared with untreated cells (Figure 9).

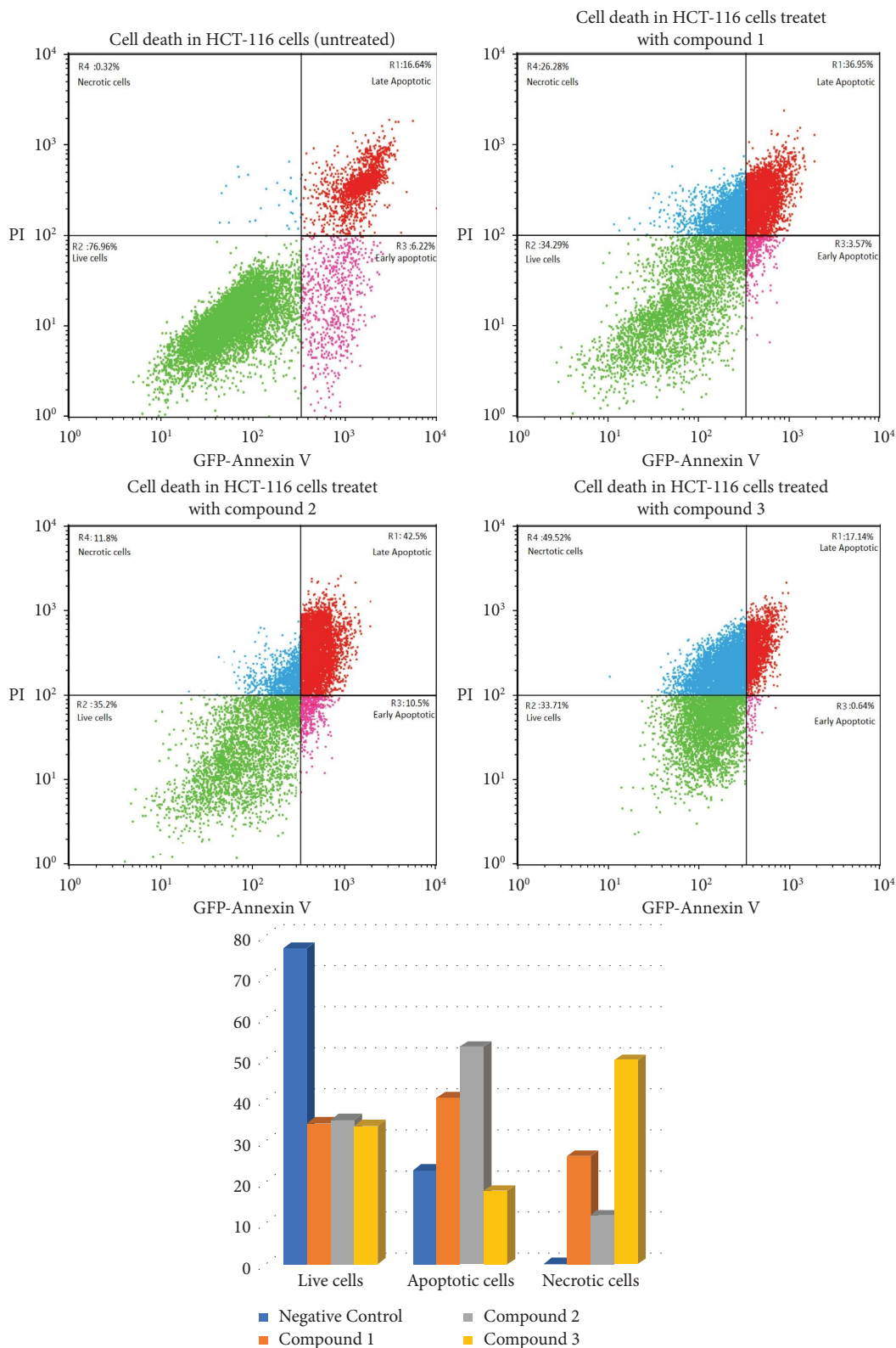


FIGURE 9: Results of cell death assay of HCT-116 cells untreated and treated with studied compounds.

4. Conclusion

The biological importance of *isoindole-1,3(2H) dione* scaffold has promoted us for synthesis and screening biological activity of some promising derivatives with high potency and multitarget affinity by molecular hybridization of this moiety with phenylalanine.

Three derivatives of phenylalanine hybrids with *isoindole-1,3(2H) dione* were synthesized, and two synthesis methods have been successfully carried out.

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

Resulted products were screened for measuring their antimicrobial activity against two bacterial species, Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*, *Leishmania tropica*, and two cancer cell lines.

In vitro studies indicated that compounds have a considerable antileishmanial effect, with IC_{50} 0.0478–0.250 $\mu\text{mol/mL}$, and all compounds are more effective than the first-line treatment, Glucantime.

Viability studies indicate that tested compounds have an antiproliferative effect against Caco-2 and HCT-116 cancer cells, and the efficacy of compound **3** is comparable with the efficacy of thalidomide against Caco-2 cells.

Based on the results of this study, we suggest that compounds **1**, **2**, and **3** might be good potential antileishmanial agents. In addition, compound **3** is a potential anticancer agent for the treatment of Caco-2 cell cancer.

Data Availability

All data used to support the findings are available from the corresponding author upon request.

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), UV spectrum of compound **1** (Figure S2), $^1\text{H-NMR}$ spectrum of compound **1** (Figure S3), ^{13}C NMR spectrum of compound **1** (Figure S4), DEPT135 ^{13}C NMR spectrum of compound **1** (Figure S5), MS spectrum of compound **1** (Figure S6), IR spectrum of

compound **2** (Figure S7), UV spectrum of compound **2** (Figure S8), $^1\text{H-NMR}$ spectrum of compound **2** (Figure S9), ^{13}C NMR spectrum of compound **2** (Figure S10), DEPT135 ^{13}C NMR of compound **2** (Figure S11), IR spectrum of compound **3** (Figure S12), UV spectrum of compound **3** (Figure S13), $^1\text{H-NMR}$ spectrum of compound **3** (Figure S14), ^{13}C NMR spectrum of compound **3** (Figure S15), DEPT135 ^{13}C NMR spectrum of compound **3** (Figure S16). (*Supplementary Materials*)

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