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RESEARCH ARTICLE

Anticancer Potential of Piperidine-Substituted Mannich Bases: Evidence of Cytotoxic Activity and Apoptotic Effects on Various Cancer Cells

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Anticancer Potential of Piperidine-Substituted Mannich Bases: Evidence of Cytotoxic Activity and Apoptotic Effects on Various Cancer Cells

SUMMARY

Cancer remains the second leading cause of death worldwide, accounting for approximately 20% of annual mortalities. Lung, breast, colon, and melanoma are among the most common cancer types, especially in developing countries. Kojic acid and its synthetic derivatives are known for their wide range of biological activities and applications in medicine, cosmetics, food, and agriculture. Mannich base derivatives of kojic acid have been previously reported to exhibit promising cytotoxic potential on various tumor cell lines. In this study, a series of kojic acid–based Mannich bases containing a piperidine ring were synthesized and evaluated for their anticancer effects on several cancer cell lines (SK-MEL, MCF-7, MDA-MB-231, A549, HeLa, Hep3B, HT-29, Neuro2A) and one healthy cell line (Vero). Among the synthesized compounds, Compound 4, containing a piperidinopiperidine moiety, demonstrated the highest and broadest cytotoxic activity, with IC50 values of 5.02 ± 0.09, 5.80 ± 0.06, and 5.92 ± 0.12 μM against MCF-7, Neuro2A, and SK-MEL cells, respectively. Flow cytometric analysis using the Annexin V method suggested that Compound 4 may induce apoptosis in a time-dependent manner, accompanied by G1 phase arrest in MCF-7 cells, although further molecular verification is required.

Keywords: *Kojic acid, piperidine, cytotoxicity, apoptosis.*

Piperidin İçeren Kojik Asit Mannich Bazlarının Sentezi ve Antikanser Değerlendirmesi

ÖZ

Kanser, dünya çapında ikinci önde gelen ölüm nedeni olmaya devam etmekte ve yıllık ölümlerin yaklaşık %20'sini oluşturmaktadır. Akciğer, meme, kolon ve melanom, özellikle gelişmekte olan ülkelerde en yaygın kanser türleri arasındadır. Kojik asit ve sentetik türevleri, tıp, kozmetik, gıda ve tarım alanlarında geniş bir yelpazede biyolojik aktiviteleri ve uygulamalarıyla bilinmektedir. Kojik asidin Mannich bazı yapısındaki türevlerinin, çeşitli tümör hücre hatlarında umut verici sitotoksik potansiyel sergilediği daha önce bildirilmiştir. Bu çalışmada, piperidin halkası içeren bir dizi kojik asit türevi Mannich bazları sentezlenmiş ve çeşitli kanser hücre hatları (SK-MEL, MCF-7, MDA-MB-231, A549, HeLa, Hep3B, HT-29, Neuro2A) ve bir sağlıklı hücre hattı (Vero) üzerinde antikanser etkileri değerlendirilmiştir. Sentezlenen bileşikler arasında, piperidinopiperidin parçası içeren Bileşik 4, MCF-7, Neuro2A ve SK-MEL hücrelerine karşı sırasıyla 5,02 ± 0,09, 5,80 ± 0,06 ve 5,92 ± 0,12 μM IC50 değerleri ile en yüksek ve en geniş sitotoksik aktiviteyi göstermiştir. Annexin V yöntemi kullanılarak yapılan akış sitometrik analiz, daha fazla moleküler doğrulama gerekmekle birlikte, Bileşik 4'ün MCF-7 hücrelerinde G1 fazı durması ile birlikte zamana bağlı bir şekilde apoptozu indükleyebileceğini ortaya koymuştur.

Anahtar Kelimeler: *Kojik asit, piperidin, sitotoksisite, apoptoz.*

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INTRODUCTION

Cancer is a major and growing global health issue, affecting millions of people each year regardless of region or socio-economic background. As a primary cause of death worldwide, it accounted for approximately 10 million deaths in 2020 (Filho et al., 2025). To effectively tackle this critical problem and overcome drug resistance, focused efforts are necessary to develop novel therapeutic agents that will improve both patient outcomes and quality of life (Koirala & DiPaola, 2024).

Kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one, KA), a natural chelating agent, is a versatile starting material for making new anticancer agents due to its known antioxidant, radioprotective, antiproliferative, and anti-inflammatory qualities (Brtko, 2022; Burnett et al., 2010; He et al., 2021; Mohamad et al., 2010; Phasha et al., 2022; Zilles et al., 2022). Its chelating properties, attributed to the hydroxyl group at position 3 and ketonic oxygen at position 4 of the hydroxypyranone ring, are particularly significant for its antimicrobial and antioxidant activities (Emami et al., 2022). Previously, some KA derivatives were tested for their antimelanogenic activity by our research group, and several compounds showed stronger inhibition than the reference drugs, while maintaining low toxicity toward normal cells (Karakaya et al., 2019a, b). Moreover, the structural resemblance of KA to allomaltol (ALM), another pyrone derivative, suggests a shared potential for modulating cellular pathways critical for cancer progression (Ercan et al., 2020) (Figure 1.). Given its structure and established biological activities, KA becomes an attractive scaffold for synthesizing novel Mannich bases with potential anticancer properties. Specifically, using Mannich reactions allows us to strategically attach diverse amine moieties, such as piperazine and piperidine rings. These additions can significantly boost the compound's affinity for biological targets and enhance its pharmacokinetic profiles.

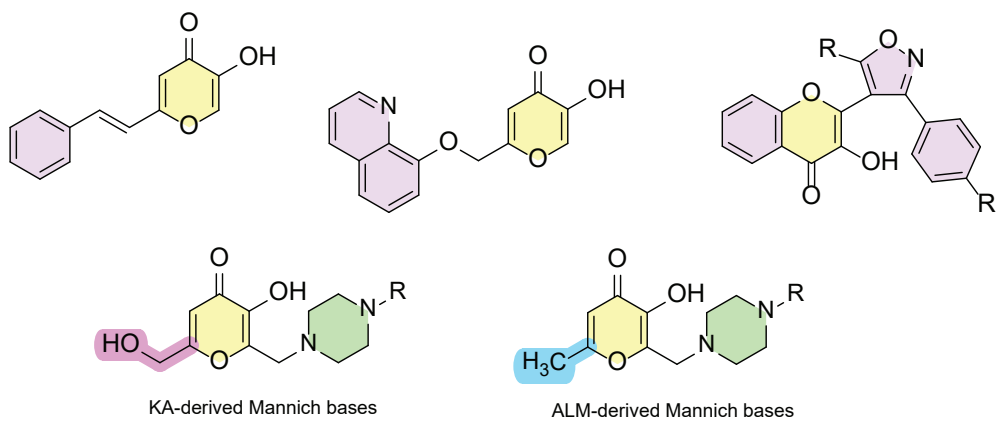


Figure 1. Chemical structures of KA derivatives with anticancer potential.

Piperidine rings are key structures in many important therapeutic compounds, showing various biological and pharmacological effects, including anticancer properties (El-Sayed Ebead et al., 2023) A series of arylidenes derivatives was synthesized under ultrasonic methodology via Knoevenagel condensation reaction of cyanoacetohydrazide derivative with the appropriate aldehydes and/or ketone. The anticancer properties of the newly synthesized compounds were tested against four different human cancer cell lines (HEPG-2, MCF-7, HCT-116, and PC-3. They are effective against many cancers, including drug-resistant prostate and ovarian cancers, by inducing mitotic arrest, terminal differentiation, and apoptosis (Mutahir et al., 2023).

These considerations led us to evaluate the cytotoxic effects of KA-based Mannich bases, specifically those incorporating a piperidine ring, across various cancer cell lines. Our chemical design was guided by previous structure–activity observations, which suggested that substitution at the Mannich position critically affects both potency and selectivity. The resulting compounds were assessed for their ability to induce apoptosis, with particular attention paid to their chemical structure–activity relationships. In this study, Compound 1 was synthesized and characterized for the first time using ^{1}H-NMR, ^{13}C-NMR, and LC-MS analysis techniques. The other derivatives (Compounds 2–9) were re-synthesized according to our previously reported procedures (Aytemir & Çalış, 2010; Karakaya et al., 2022) to evaluate their comparative anticancer activities. Their cytotoxic potential was tested on a panel of human cancer cell lines (SK-MEL, MCF-7, MDA-MB-231, A549, HeLa, Hep3B, HT-29, and Neuro2A) and the normal Vero cell line. For the most potent compound, it was investigated how it induces apoptosis, a type of programmed cell death, using the Annexin V method, and performed cell cycle analysis to understand its mechanism of action.

MATERIALS AND METHODS

Chemistry

All chemical reagents used in the synthesis were purchased from Merck and Aldrich Chemical Co. Melting points were determined using a Büchi M-560 Melting Point Apparatus and are reported without correction. NMR spectra were recorded on a Varian Mercury 500 MHz spectrometer in DMSO-d_6, with tetramethylsilane (TMS) serving as an internal standard. Mass spectra were obtained on a Micromass ZQ LC-MS instrument using the electrospray ionization (ESI) technique. HPLC analyses were performed on a Waters Alliance system equipped with a C18 column. Elemental analyses were conducted in the Central Laboratory of the Faculty of Pharmacy, Ankara University, using a Leco CHNS-932 analyzer. The purity of the compounds was verified by thin-layer chromatography (TLC) on Kieselgel 60 F_{254} plates (Merck®, Germany).

Synthesis of Compound 1

4-Phenyl-3,6-dihydropyridine (3 mmol) was mixed with formaldehyde under continuous stirring. KA (3 mmol) was then added to the reaction mixture, followed by 20 mL of methanol, and the solution was stirred at room temperature for 15–25 minutes. The resulting precipitate was collected by vacuum filtration, washed with cold methanol, and recrystallized from suitable solvents. The purity of the compound was confirmed by thin-layer chromatography (TLC) using a chloroform:methanol (9:1, v/v) solvent system.

3-Hydroxy-6-(hydroxymethyl)-2-((4-phenyl-3,6-dihydropyridin-1(2*H*)-yl)methyl)-4H-pyran-4-one (Compound 1)

$C_{18}H_{19}NO_4$ (M.W.: 313.35 g/mol); white solids, yield: 60%, M.p. 90-192°C; ^{1}H-NMR δ (DMSO$_{d6}$, 500 MHz) 2.50 (2H; t; *J*= 1.75; pyridine-*H*^{5}); 2.74 (2H; t; *J*= 5.65; pyridine-*H*^{6}); 3.17 (2H; d; *J*= 2.75; pyridine-*H*^{2}); 3.65 (2H; s; pyrane-C*H*$_2$-pyridine); 4.32 (2H; s; -C*H*$_2$-OH); 6.13 (1H; s; pyridine-*H*^{3}); 6.35 (1H; s; pyrane-*H*^{5}); 7.24 (1H; m; Ar-*H*^{4}); 7.32 (2H; m; Ar-*H*^{3,5}); 7.40 (2H; d; *J*= 8.4; Ar-*H*^{2,6}); ^{13}C-NMRδ (DMSO$_{d6}$, 125 MHz) 27.73, 50.06, 52.63, 53.54, 60.08, 109.41, 122.22, 125.01, 127.46, 128.80, 134.35, 140.54, 144.17, 147.12, 168.14, 174.11. ESI-MS (m/z): 160, 314 (%100, M^{+}+H).

Biological activity studies

The following instruments were used in bioactivity sudies: CO_2 incubator (Thermo Fisher Scientific, USA), Class II laminar flow cabinet (ESCO, Singapore), orbital shaker incubator (Biosan, Türkiye), flow cytometer (BD Accuri C5, BD Biosciences, USA), inverted phase-contrast microscope (Zeiss, Germany), light microscope (Olympus, Germany), spectrophotometer (Thermo Fisher Scientific, USA), bench centrifuge (Nüve, Türkiye), refrigerated microcentrifuge

(Hettich, Germany), water bath (LKTC-L Controller, Türkiye), vortex mixer (Thermo Fisher Scientific, USA), liquid nitrogen storage tank (50 L, Cryogenic), ultra-low freezer (−86 °C, Eppendorf, Germany), and refrigerator (+4°C, Uğur, Türkiye).

Cell culture and maintenance under two-dimensional conditions

Seven human cancer cell lines — SK-MEL (melanoma), MCF-7 and MDA-MB-231 (breast carcinoma), A549 (lung carcinoma), HeLa (cervical adenocarcinoma), Hep3B (hepatocellular carcinoma), HT-29 (colon adenocarcinoma), and one murine line, Neuro-2A (neuroblastoma) — together with one normal cell line, Vero (African green monkey kidney epithelial cells), were used in this study. Cells were cultured in RPMI-1640 or DMEM/Ham's F-12 media (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100 IU/mL penicillin, and 1 µg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

When cell confluency reached approximately 80%, the cells were sub-cultured using 0.25% trypsin-EDTA at 37°C for 3 minutes. Detached cells were collected by centrifugation at 1000 rpm for 5 minutes and re-suspended in fresh medium.

For long-term storage, cell stocks were frozen either in culture medium containing 5% dimethyl sulfoxide (DMSO) or in freezing medium composed of 10% DMSO in fetal bovine serum, and preserved at −86°C or in liquid nitrogen (−196°C) until further use (Sevimli-Gur et al., 2013; Üçkan et al., 2023).

Determination of cytotoxicity of synthesized compounds in 2D cell culture

Cell viability assay

The cytotoxic potential of the synthesized compounds was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, a colorimetric method based on the reduction of tetrazolium salts by mitochondrial dehydrogenase enzymes of viable cells. The conversion of MTT (Sigma Chemicals, USA) into an insoluble blue formazan product was measured spectrophotometrically at 570 nm using a microplate reader (VersaMax™ Tunable Microplate Reader, Molecular Devices, USA). The percentage of cell viability was calculated relative to untreated controls as previously described (Sevimli-Gur et al., 2010, 2011, 2013).

Evaluation of apoptosis-inducing potential in 2D cell culture by Annexin V–FITC/PI assay

Apoptosis induction was assessed using the Annexin V–FITC/PI double-staining method, which detects the externalization of phosphatidylserine from the inner to the outer leaflet of the plasma membrane—a hallmark of early apoptosis. Annexin V, a calcium-dependent phospholipid-binding protein, binds to exposed phosphatidylserine with high affinity, allowing the detection of apoptotic cells when conjugated with a fluorochrome.

MCF-7 cells in the logarithmic growth phase were seeded in 6-well plates at a density of 2.5 × 10^5 cells/mL and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Following incubation, cells were treated with compound 4 concentrations of 3.125, 1.562, and 0.781 µg/mL for 24 and 48 hours. After treatment, supernatants were discarded, and cells were washed with PBS and detached using trypsin-EDTA. The cell suspensions were collected and washed three times with PBS (2000 rpm, 5 min, 4°C). Cells were resuspended in 100 µL binding buffer (1×), stained with 1 µL FITC-conjugated Annexin V (BD Biosciences, USA), and incubated in the dark for 15 minutes. After washing twice with PBS, samples were resuspended in 200 µL PBS and analyzed by flow cytometry (BD Accuri C5, BD Biosciences, USA) using the FL1 (525 nm) channel (Bogurcu et al., 2011).

Cell cycle distribution was determined using the same Annexin V–FITC staining protocol to evaluate the effects of Compound 3 on MCF-7 cell proliferation. The proportion of cells in each phase (G_0/G_1, S, and G_2/M) was quantified by flow cytometry. Data were analyzed using BD Accuri C6 software to assess possible G_1-phase arrest in correlation with apoptotic induction.

Statistical analysis

All experiments were performed independently in triplicate. Results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using Microsoft Excel. Differences between groups were evaluated using one-way ANOVA followed by Dunnett's multiple comparison test. Significance between paired data was determined using the Student's t-test. Differences were considered statistically significant at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (Sevimli-Gur et al., 2013).

RESULTS AND DISCUSSION

In medicinal chemistry, heterocyclic amines like piperidine and piperazine are key pharmacophores. They are vital for receptor binding and maintaining a favorable pharmacokinetic balance. Piperidine, a saturated six-membered heterocycle, offers a conformationally flexible, electron-rich scaffold. This feature is critical for influencing metabolic stability and molecular recognition. The Mannich reaction is powerful for structural diversification of KA because the resulting Mannich base introduces an b-amino methyl functionality at the C-6 or C-7 position of the pyrone ring. This modification significantly alters physicochemical properties such as lipophilicity, hydrogen-bonding capacity, and electronic distribution. Such modifications can improve cellular permeability and interaction with biological macromolecules, thereby enhancing biological activity (Roman, 2015).

Compounds 2-9 have been reported in our previous studies (Aytemir & Çalış, 2010; Karakaya et al., 2022). For this study, they were resynthesized, and their structures were confirmed (Figure 2.). Compound 1, however, is an original compound characterized for the first time in this study and included in the study to strengthen the understanding of the structure-activity relationships. Its chemical structure has been confirmed by appropriate spectral analysis methods.

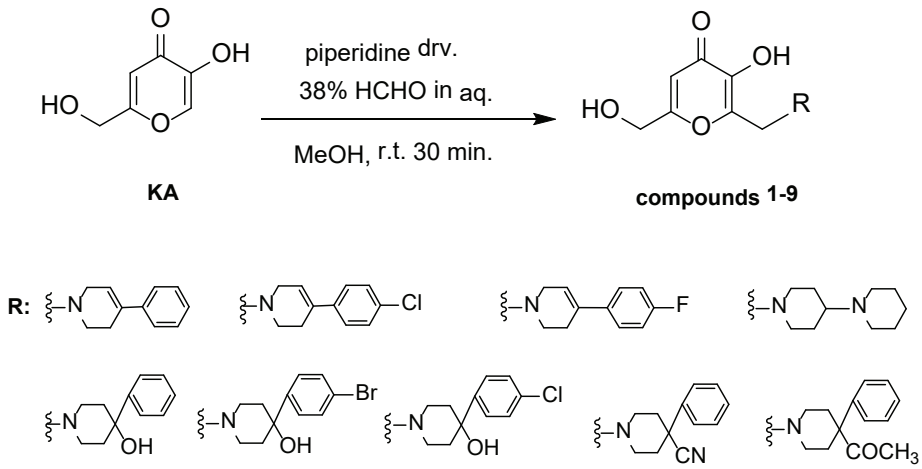


Figure 2. General synthetic route of KA–based Mannich base derivatives.

All target compounds were synthesized rapidly and in high yields under mild, room-temperature conditions. The spectral and physicochemical data supported the proposed molecular structures.

The cytotoxic effects of the Mannich bases synthesized within the scope of this study were evaluated on seven cancer cell lines — SK-MEL (human melanoma), MCF-7 and MDA-MB-231 (human breast carcinoma), A549 (human lung carcinoma), HeLa (human cervical adenocarcinoma), Hep3B (human hepatocellular carcinoma), HT-29 (human colon adenocarcinoma), and Neuro2A (mouse neuroblastoma) — and one normal cell line, Vero (African green monkey kidney epithelial cells). Cytotoxicity was determined using the MTT assay after 72 hours of incubation.

Doxorubicin (10 µg/mL), a clinically used chemotherapeutic agent, served as the positive control, while cells treated with 0.1% DMSO were used as the negative control. All compounds were tested at four concentrations — 12.5, 6.25, 3.125, and 1.56 µg/mL — to assess dose-dependent responses.

When the bioactivity results were evaluated on a cell line-specific basis, the Hep3B human hepatocellular carcinoma cell line proved to be the least responsive to the tested compounds, followed by the HeLa human cervical adenocarcinoma cell line. Intriguingly, the cytotoxic profiles of the compounds showed a similar trend against SK-MEL and Neuro2A cells, a parallelism that was not observed for the reference drug, doxorubicin.

In anticancer drug discovery, an ideal candidate should exert potent cytotoxic effects against tumor cells while exhibiting minimal toxicity toward healthy cells. Therefore, assessing the effects of the synthesized compounds on the Vero normal cell line is of particular importance. From this perspective, none of the synthesized compounds caused greater cytotoxicity to normal cells than doxorubicin at the lowest tested dose. These findings suggest that the newly synthesized compounds may represent promising and relatively safe anticancer candidates with selective activity toward cancer cells.

Considering all results together, Compounds 9 and 4 emerged as the most promising lead candidates among the synthesized KA-based Mannich derivatives. Although Compound 9 showed lower cell viability values against the MCF-7, SK-MEL, and Neuro2A cell lines at the lowest tested concentration, this effect was dose-independent. Rather than exhibiting the anticipated decrease in cell viability with increasing concentration, Compound 9 displayed an atypical, concentration-independent cytotoxic behavior.

In contrast, Compound 4 demonstrated consistent dose-dependent activity across a broader range of concentrations and achieved lower IC_{50} values (Figure 3 and Table 1.). Given its predictable and favorable dose-activity profile, Compound 4 was selected for further mechanistic evaluation to better elucidate its apoptosis-inducing potential. These investigations include the Annexin V–PI apoptosis assay and cell cycle analysis.

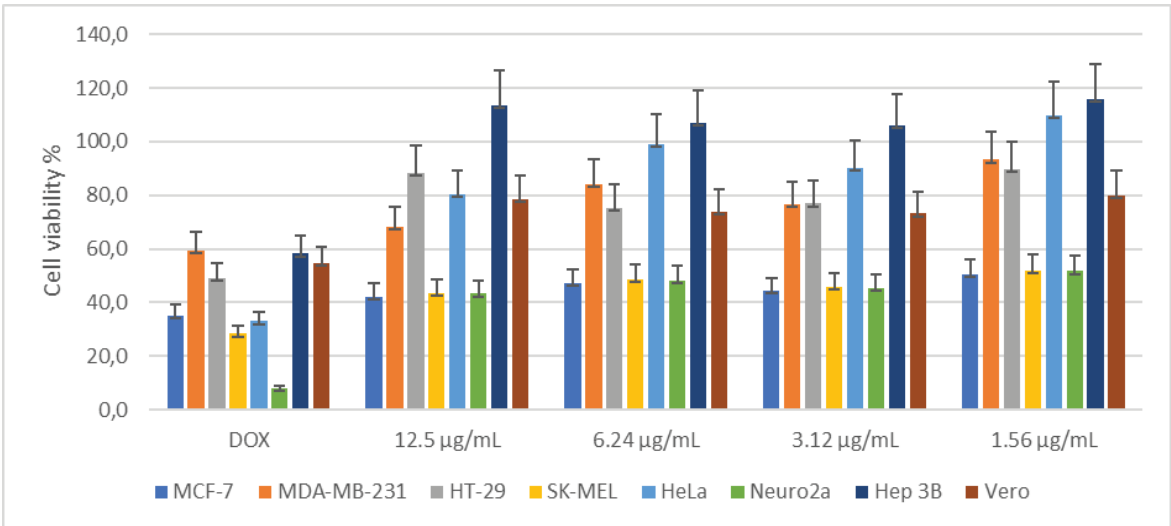
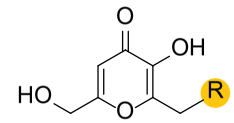


Figure 3. Cell viability results for Compound 4 at all tested doses

Table 1. The IC_{50} (μM) values of the compounds' cytotoxicity against the cell lines tested*



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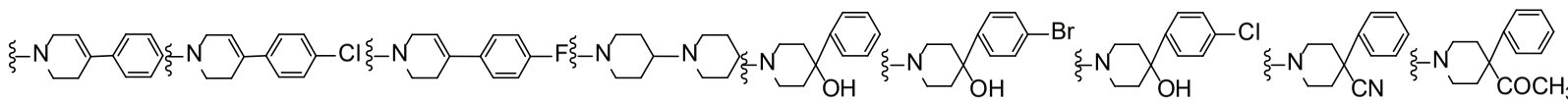

Cell line	Comp. 1	Comp. 2[a]	Comp. 3[b]	Comp. 4[b]	Comp. 5[a]	Comp. 6[a]	Comp. 7[a]	Comp. 8[a]	Comp. 9[a]	DOX
MCF7	140.93 ± 13.05	132.58 ± 12.31	90.12 ± 8.03	**5.02 ± 0.09**	124.06 ± 11.41	86.41 ± 7.85	100.60 ± 9.16	114.17 ± 10.46	35.06 ± 0.39	4.05 ± 0.18
MDA-MB-231	147.60 ± 11.27	129.50 ± 9.86	135.00 ± 10.26	132.66 ± 10.05	129.86 ± 9.84	112.76 ± 8.60	119.79 ± 9.08	98.69 ± 7.26	88.16 ± 6.44	3.68 ± 1.66
HT-29	147.50 ± 15.35	74.18 ± 7.30	121.63 ± 12.52	115.97 ± 11.88	133.48 ± 13.85	98.21 ± 10.12	125.99 ± 13.09	44.07 ± 3.94	126.61 ± 13.15	3.86 ± 0.74
SK-MEL	140.93 ± 8.36	132.58 ± 7.91	90.12 ± 5.01	**5.92 ± 0.12**	124.06 ± 7.27	86.41 ± 4.97	100.60 ± 5.82	114.17 ± 6.64	71.91 ± 3.89	4.78 ± 0.18
HeLa	147.60 ± 9.51	129.50 ± 8.31	135.00 ± 8.66	132.66 ± 8.47	129.86 ± 8.30	112.76 ± 7.26	119.79 ± 7.65	98.69 ± 6.08	88.16 ± 5.37	-
Neuro2a	140.93 ± 7.24	132.58 ± 6.87	90.12 ± 4.32	**5.80 ± 0.06**	124.06 ± 6.31	86.41 ± 4.29	100.60 ± 5.03	114.17 ± 5.76	71.91 ± 3.30	-
Hep 3B	137.20 ± 6.57	89.48 ± 4.02	138.68 ± 6.73	69.82 ± 2.85	126.48 ± 6.04	115.37 ± 5.61	86.85 ± 3.94	77.15 ± 3.32	91.13 ± 4.14	-
Vero	137.20 ± 14.20	89.48 ± 9.00	138.68 ± 14.43	69.85 ± 6.73	126.48 ± 13.07	115.37 ± 12.02	86.82 ± 8.75	77.15 ± 7.61	91.13 ± 9.21	3.81 ± 1.29

*Concentration range studied = 12.5–1.5 μg/mL (1/2 serial dilution, 4 concentrations for each compound) a: (Aytemir & Çalış, 2010). b: (Karakaya et al., 2022)

When all synthesized compounds were compared for their cytotoxic effects against tumor cell lines, compound 4 exhibited the highest potency and the broadest spectrum of activity. Its antitumor efficacy was approximately 7.7–28.8 times greater than that of the other compounds in the series. The calculated therapeutic index (TI) values of Compound 4 were 13.90 μg/mL for MCF-7 (human breast adenocarcinoma), 11.79 μg/mL for SK-MEL (human malignant melanoma), and 12.04 μg/mL for Neuro2A (mouse neuroblastoma) cells, indicating a favorable selectivity profile.

The IC_{50} values obtained for compound 5 ranged between 124.06 and 133.48 μM, while those for compound 3 were in the range of 90.12-138.68 μM. Compound 7 exhibited IC_{50} values between 86.82 and 125.99 μM across the same cell lines. Compound 9 displayed IC_{50} values ranging from 35.06 to 126.61 μM, with the lowest value recorded for the MCF-7 cell line, suggesting that this cell line was the most sensitive to its effects.

For compound 4, IC_{50} values varied between 5.02 and 132.66 μM, confirming it as the most potent cytotoxic agent among all synthesized derivatives. The most responsive cell lines to this compound were MCF-7, Neuro2A, and SK-MEL, with IC_{50} values of 5.02, 5.80, and 5.92 μM, respectively, whereas the corresponding IC_{50} value for the normal Vero cell line was 69.85 μM.

When all compounds and cell lines were collectively evaluated, the MCF-7 cell line emerged as the most sensitive, followed by Neuro2A and SK-MEL, while Hep3B was the least affected. In contrast, all compounds showed relatively low cytotoxicity toward the normal Vero cells, supporting their potential safety as anticancer agents.

Evaluation of the IC_{50} values reveals significant insights into the structure-activity relationships (SAR) of the synthesized Mannich bases. The most striking finding is the superior potency of Compound 4, which contains a bis-piperidine skeleton, compared to the phenyl-substituted derivatives (Compounds 1–3 and 5–9). While the phenyl-bearing compounds generally exhibited IC_{50} values above 35 μM against MCF-7 cells, the replacement of the aromatic phenyl ring with a saturated, pharmacologically flexible piperidine moiety in Compound 4 drastically improved the cytotoxicity (IC_{50}: 5.02 μM). This suggests that the absence of the rigid aromatic ring and the presence of a secondary piperidine group may enhance cellular uptake or binding affinity to the target protein.

A key observation lies in the electronic nature of the substituents at the 4-position of the piperidine ring. Compound 5, which bears a hydroxyl group (typically considered an electron-donating and polar group), exhibited relatively low cytotoxicity (IC_{50} > 120 μM). Interestingly, replacing this group with electron-withdrawing moieties altered the biological profile. The introduction of an acetyl group (-$COCH_3$) in Compound 9 significantly improved the cytotoxic potency (IC_{50}: 35.06 μM against MCF-7), suggesting that electron-withdrawing substituents at this position contribute positively to the anticancer activity compared to electron-donating ones. However, the strongest activity was achieved not by these substitutions, but by the unique bis-piperidine skeleton of Compound 4. This implies that while electronic properties are important, the conformational flexibility and specific steric features provided by the piperidinopiperidine moiety may be the primary drivers of the superior potency observed in this series.

Determination of the apoptotic effects of compound 4

Since Compound 4 exhibited the most potent cytotoxic activity among the synthesized derivatives, we aimed to investigate its mode of cell death to determine if the observed cytotoxicity was mediated through apoptosis, a preferred mechanism for anticancer agents, rather than non-specific necrosis. To investigate the potential cell death mechanism of Compound 4, MCF-7 cells were stained with Annexin V-FITC/PI following 24 and 48 hours of treatment. As shown in

Figures 4 and 5, treatment with Compound 4 resulted in a slight increase in the apoptotic cell population compared to the negative control. Specifically, at the 48th hour, a moderate elevation in early apoptotic cells was observed. However, unlike the positive control doxorubicin, Compound 4 did not exhibit a sharp, dose-dependent increase in the apoptotic population. The overlapping error bars and the modest increase in Annexin V-positive cells indicate that the apoptotic effect is limited under the tested conditions. These findings suggest that while Compound 4 possesses strong cytotoxic potential, its primary mechanism of cell death may involve pathways other than, or in addition to, classical apoptosis. Therefore, these flow cytometry results should be interpreted as preliminary evidence of a potential apoptotic process, which warrants further validation through extensive molecular studies, such as caspase activation assays or gene expression analysis.

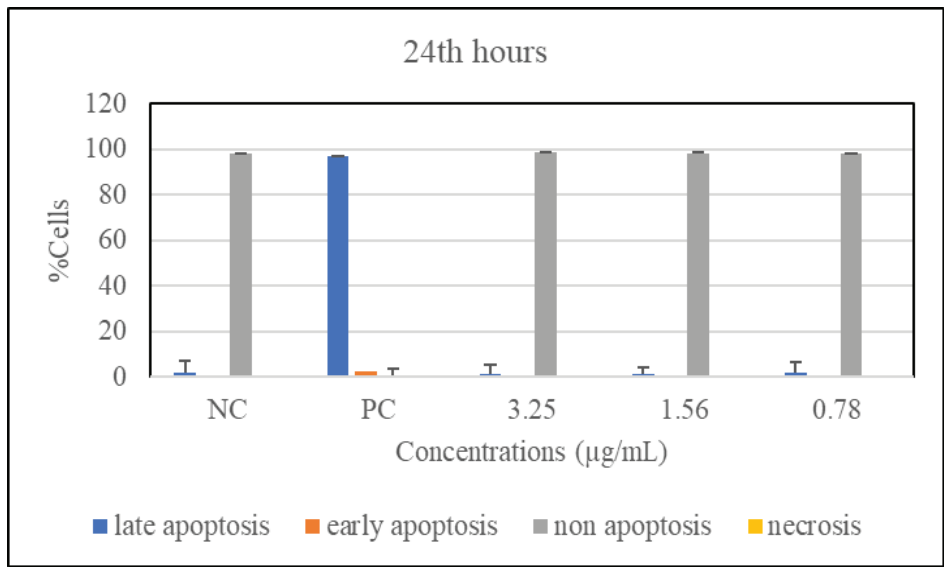

Figure 4. Determination of Apoptotic Effects of Compound 4. The results of 24 hours of treatment with 3.125, 1.56, and 0.78 µg/ml compound 4, the negative control (NC), and the positive control (PC) doxorubicin.

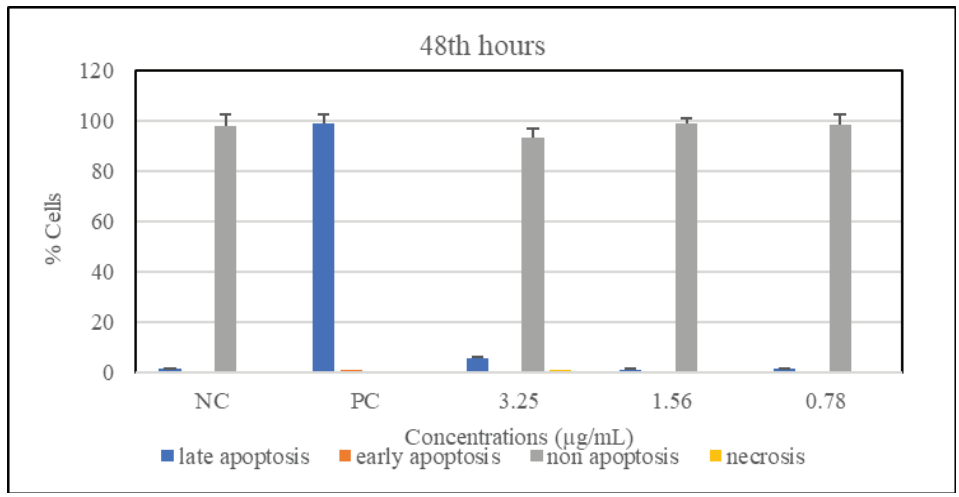

Figure 5. Determination of Apoptotic Effects of Compound 4. The results of 48 hours of treatment with 3.125, 1.56, and 0.78 µg/ml compound 4, the negative control (NC), and the positive control (PC) doxorubicin.

CONCLUSION

A series of KA-Mannich base derivatives was evaluated for their cytotoxic effects on various cancer cell lines using doxorubicin as a reference drug. Compound 4, which contains a piperidinopiperidine moiety, exhibited the strongest and broadest cytotoxic activity, particularly against MCF-7, Neuro2A, and SK-MEL cell lines, with IC_{50} values of 5.02, 5.80, and 5.92 μM, respectively. The compound showed low toxicity toward healthy Vero cells, indicating favorable selectivity. SAR analysis revealed that electron-withdrawing substituents, such as the cyano and acetyl groups, significantly enhance cytotoxic potency. Crucially, the absence of an aromatic ring, a feature unique to Compound 4, further optimized this activity. Subsequent flow cytometric analyses indicated that Compound 4 triggers a moderate and time-dependent apoptotic response in MCF-7 cells, rather than a strong dose-dependent effect.

Collectively, these results underscore Compound 4 as a promising lead molecule. Future studies will focus on clarifying its complete molecular mechanism and verifying the mode of cell death using more sensitive quantitative techniques such as RT-PCR and Western blot analyses.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept – G.K., C.S.G., M.D.A.; Design – G.K., C.S.G., M.D.A.; Supervision – M.D.A.; Resources – G.K., C.S.G., M.D.A.; Materials G.K., C.S.G., M.D.A.; Data Collection and/or Processing – G.K., C.S.G.; Analysis and/or Interpretation – G.K., C.S.G., M.D.A.; Literature Search – G.K.; Writing –G.K., C.S.G., M.D.A.; Critical Reviews – G.K., C.S.G., M.D.A.

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