Original Article

Synthesis of 4-Phenyl-4,5-dihydropyranopyrazolone Derivatives with Activated Potassium Carbonate: Evaluation of Anticancer Activity on Cancer Cell Lines and Apoptosis Mechanism

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Abstract

A green and efficient one-pot, four-component synthesis of 4-phenyl-4,5-dihydropyranopyrazolone derivatives **5a–5l** is described in ethanol-water with activated potassium carbonate and evaluation of anticancer activity on cancer cell lines and apoptosis mechanism is also investigated. This method provides several advantages such as environmental friendliness, shorter reaction time, excellent yields, and simple workup procedure. The *in vitro* cytotoxic activity of the synthesized compounds was investigated against cancer cell lines (PC-3, MCF-7, and A-2780) in comparison with doxorubicin, a well-known anticancer drug, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide colorimetric assay. Also apoptosis studies were investigated by caspase-3 and caspase-9 enzymes and mitochondrial membrane potential. Our compounds showed acceptable and moderate cytotoxicity compared with doxorubicin in the studied cell lines. The compounds **5g** in PC3 cell line (half maximal inhibitory concentration (IC_{50} = 104 µM)), **5g** and **5i** in MCF7 cell line (IC_{50} = 23 and 87 µM, respectively), and **5g–5i** in A2780 cell line (IC_{50} = 60, 50, and 31 µM, respectively) showed the best results close to the control drug doxorubicin. The compound **5h** showed significant result in the activation of caspase-3 and caspase-9 enzymes in comparison with the control. Only the **5g** in MCF7 cells and the **5g–5i** derivatives in A2780 cells caused increased mitochondrial membrane potential compared to the control group.

Keywords: *Caspase, cytotoxic activity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, synthesis, pyranopyrazolone*

Introduction

Cancer is a disease of worldwide importance and its incidence is rising. According to information from the World Health Organization, more than 11 million people are diagnosed with cancer, and also more than 13% of overall deaths are directly caused by cancer every year worldwide.[1] Cancer cells are characterized by unlimited replicative potential, self-sufficiency ingrowth signals and insensitivity to antigrowth signals, sustained angiogenesis, metastasis, and evasion of apoptosis.[2] Apoptosis or programmed cell death is an important phenomenon for remission of damaged cells. Apoptosis can be activated by two major pathways: the extrinsic pathway, in which specific cell death receptors located on the cell surface membrane are activated by specific ligands, and the intrinsic pathway, where primarily mitochondria are involved.[3] Apoptosis plays a vital role in normal embryonic development as well as in adult life, such as elimination of dispensable or excess cells. It has been known that defects in the apoptosis pathways and the ability to evade cell death is one of the hallmarks of cancers, which results in uncontrollable tumor cell growth, as well as tumor resistance to chemotherapeutic agents.[4] Therefore, finding of new therapeutic agents for neoplastic diseases with focus on the apoptosis pathways is one of the top subjects in this area of research. It has been well documented that many of the clinically useful cytotoxic agents induce apoptosis in cancer cells. The proapoptotic chemotherapeutic agents that target tubulin polymerization such as Taxol and vinca alkaloids, including vincristine, vinblastine, and vinorelbine, are among the most potent and commonly prescribed antineoplastic agents. The development of chemoresistance as well as dose-limiting neurologic and bone marrow toxicity, however, has limited the use of tubulin-targeting agents. This clearly highlights the urgent need for novel chemotherapeutic agents for more effective treatment of cancer.[5]

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Pyranopyrazoles are the fused heterocyclic compounds that show bactericidal,[6] molluscicidal,[7,8] and analgesic activity,[9] and act as hypoglycemic and anticancer agents.[10,11] They are also potential inhibitors of human Chk1 kinase.[12] Celecoxib (**1**), sulfaphenazole (**2**), 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDPPB or mGluR5 Ligand) (**3**), linazolac (**4**), mepiprazole (**5**), and rimonabant (**6**) are some of the pyrazole-based drugs available today in the market [Figure 1].[13] Owing to their biological significance, there has been considerable interest in developing synthetic methods for the preparation of pyranopyrazole derivatives.[13-24]

Green chemistry techniques continue to grow in importance. Alternative processes help to conserve resources and to reduce costs. The replacement of conventional solvents with water, which is harmless to health and is available in large quantities, is an interesting basic approach along these lines.[25-27] One of the tools used to combine economic aspects with the environmental ones is the multicomponent reaction strategy, the process consists of two or more synthetic steps, which are carried out without isolation of any intermediate, thus reducing time, saving money, energy, and raw materials.[28] As a part of our program aimed at developing new selective and environmentally friendly methodologies for the preparation of fine chemicals and developing anticancer agents,[29] we performed the synthesis of 4-phenyl-4,5-dihydropyranopyrazolone derivatives **5a–5l** through a four-component reaction with activated potassium carbonate as catalyst [Scheme 1]. Then, the *in vitro* cytotoxic activity of the synthesized compounds **5a–5l** was investigated against PC3, MCF7, and A2780 cancer cell lines in comparison with doxorubicin, a well-known anticancer drug, using MTT colorimetric assay. Considering the importance of apoptotic cell death as a key factor of a potential antitumor drug, in the next set of experiments, the apoptotic potentials of the most potent compounds were investigated.

Materials and Methods

Chemistry

All starting materials, reagents, and solvents were purchased from Merck and Sigma-Aldrich Chemical Companies, Kermanshah University of Medical Sciences, Kermanshah, Iran. All yields referred to isolated yield. The structure of compounds was characterized by Infrared (IR), Nuclear Magnetic Resonance (^{1}H NMR) spectra, and mass spectrometry (MS). Merck silica gel 60 F254 plates were used for Thin layer chromatography (TLC). ^{1}H-NMR spectra were recorded using a Bruker 500 spectrometer, and chemical shifts were expressed as (ppm) with tetramethylsilane as internal standard using Deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO)-d_6 as solvent. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks). The melting point was taken on a Kofler hot stage apparatus and was uncorrected. The mass spectra

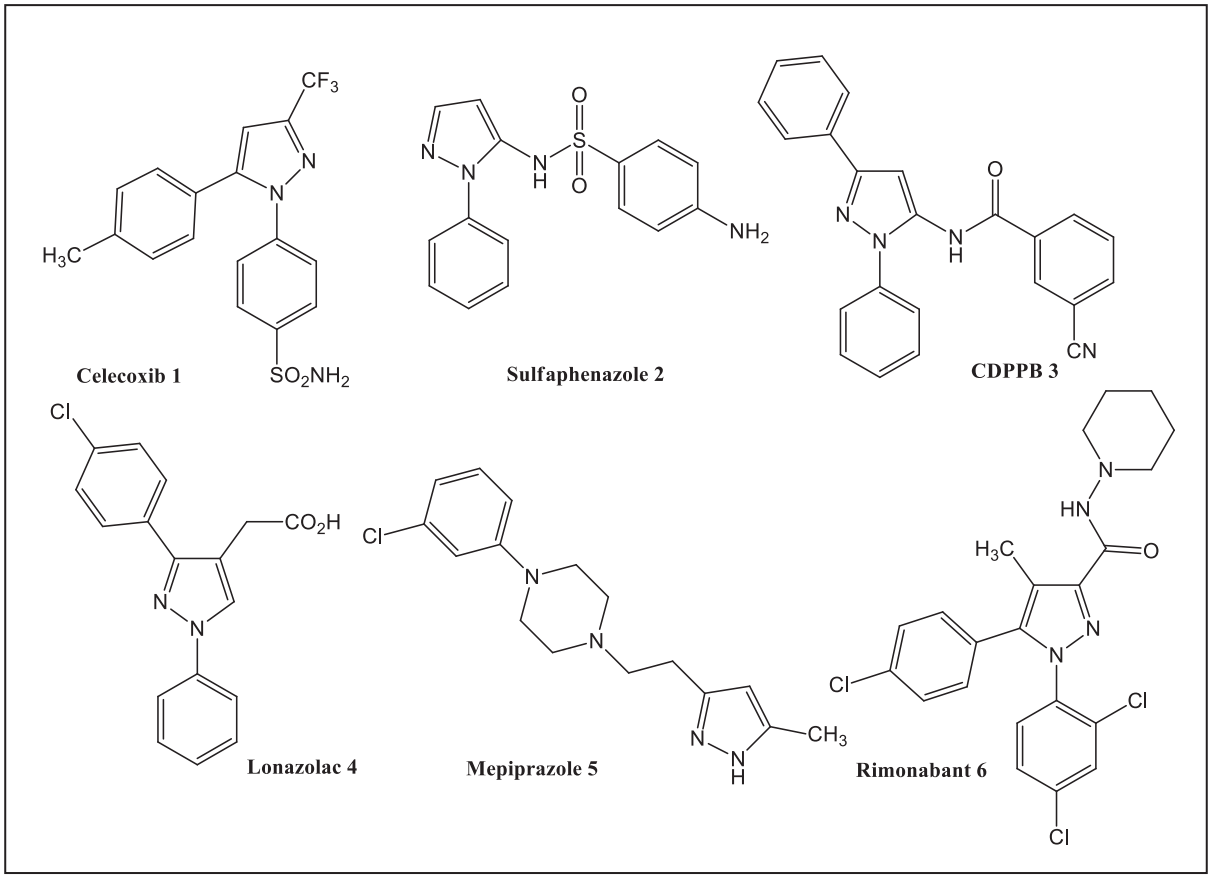


Figure 1: Structures of pyrazole derivatives used as biologically active compounds

were run on a Finigan TSQ-70 spectrometer at 70 eV, Kermanshah University of Medical Sciences, Kermanshah, Iran.

General procedure for the synthesis of pyranopyrazolones 5a–5l with activated potassium carbonate as the catalyst in water-ethanol

Pyranopyrazole derivatives **5a–5l** were synthesized by the one-pot four-component condensation of an aromatic aldehyde (1.0 mmol, **2a–2l**), methyl acetoacetate (1.0 mmol, **4**), hydrazine hydrate (1.0 mmol, **3**), Meldrum's acid (1.0 mmol, **1**), and activated potassium carbonate (10 mol%) as the catalyst, in water-ethanol (5 mL, 1:1) for a designated time listed in Table 1 (120–240 min). After completion of the reaction followed by TLC analysis, the reaction mixture was cooled and the precipitated solid was filtered and washed several times with cool water. The obtained products (**5a–5l**) were crystallized in appropriate solvent (ethanol) and the corresponding pyranopyrazolones **5a–5l** were obtained in 59%–85% yields [Scheme 1, Table 1].[29] The spectral data of the compounds **5a–5j** are as following:

*4-(3-Methoxyphenyl)-3-methyl-4,5-dihydropyrano[2,3-c] pyrazol-6(2H)-one (**5a**)*

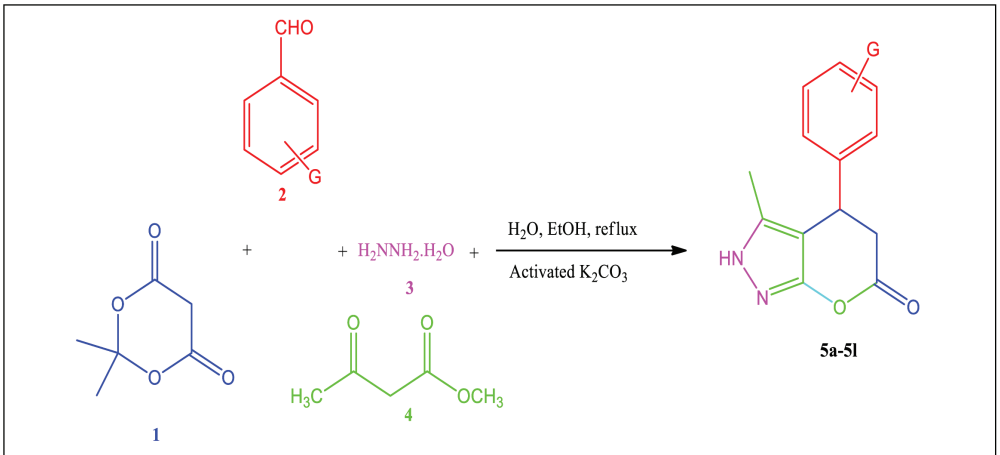
Chemical formula: $C_{14}H_{14}N_2O_3$; molecular weight: 258.27 (g/mol); yield: 66%; melting point: 105°C; Fourier-transform infrared spectroscopy (FT-IR) (KBr, cm^{-1}): υ 3448 (stretch NH), 3012 (stretch CH, aromatic), 2935 (stretch CH, aliphatic), 1604 (stretch C=O ring), 1573 (stretch C=C), 1477 (stretch C=C), 1435 (binding CH); 1265 (stretch C–O), 1045 (stretch C–O), 786 (binding CH, OOP), 690 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 11.5 (*s*, 1H, NH), 7.50 (*t*, 2H, J = 8.5 Hz, $H_{5aromatic}$), 7.05 (*s*, 1H, $H_{2aromatic}$), 6.8 (*m*, 2H, $H_{4,6aromatic}$), 4.70 (*t*, 1H, J = 7.5 Hz, CH), 3.86 (*s*, 3H, OCH_3), 2.91 (*m*, 2H, CH_2), 2.05 (*s*, 3H, CH_3); MS (*m*/*z*, %): 258 (M), 217 (M-41, 100), 161 (M-97, 71.7), 135 (M-123, 76.9), 91 (M-167, 27.3), 71 (M-181,48,7).

*4-(4-Methoxyphenyl)-3-methyl-4,5-dihydropyrano[2,3-c] pyrazol-6(2H)-one (**5b**)*

Chemical formula: $C_{14}H_{14}N_2O_3$; molecular weight: 258.27 (g/mol); yield: 65%; melting point: 145°C; FT-IR (KBr, cm^{-1}): υ 3367 (stretch NH), 2931 (stretch CH, aliphatic), 1600 (stretch C=O ring), 1508 (stretch C=C), 1462 (stretch C=C), 1369 (binding CH); 1249 (stretch C–O), 1026 (stretch C–O), 833 (binding CH, OOP), 675 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 11 (*s*, 1H, NH), 7.80 (*d*, 2H, J = 10 Hz, $H_{2,6aromatic}$), 7.05 (*d*, 2H, J = 10 Hz, $H_{3,5aromatic}$), 4.10 (*t*, 1H, J = 7.5 Hz, CH), 3.68 (*s*, 3H, OCH_3); 2.90 (*s*, 3H, CH_3), 2.03 (*m*, 2H, CH_2); MS(*m*/*z*, %): 258 (M), 217 (M-41, 100), 185 (M-73, 31.3), 161 (M-97, 41.5), 91 (M-167, 16.9), 77 (M-181, 17.7).

*4-(2,3-Dimethoxyphenyl)-3-methyl-4,5-dihydropyrano [2,3-c]pyrazol-6(2H)-one (**5c**)*

Chemical formula: $C_{15}H_{16}N_2O_4$; molecular weight: 288.30 (g/mol); yield: 60%; melting point: 150°C; FT-IR (KBr,



Compound	G	Compound	G
5a	3-OCH_3	**5g**	2,5-$(OCH_3)_2$
5b	4-OCH_3	**5h**	3,4-$(OCH_3)_2$
5c	2,3-$(OCH_3)_2$	**5i**	3,5-$(OCH_3)_2$
5d	2-OH-4-OCH_3	**5j**	2,3,4-$(OH)_3$
5e	2,4-$(OH)_2$	**5k**	2,3,4-$(OCH_3)_3$
5f	2,4-$(OCH_3)_2$	**5l**	3,4,5-$(OCH_3)_3$

Scheme 1: Reagents and conditions for the synthesis of pyranopyrazolones 5a–5l

Table 1: Synthesis of pyranopyrazolones using activated potassium carbonate in water-ethanol and the corresponding IC50 (μM) values against different carcinoma cell lines

Entry	Compound	Yield (%)[a]	Time (min)	IC_{50} (μM)		
				A2780	MCF7	PC3
1	**5a**	66	120	150	165	90
2	**5b**	65	180	150	NA[b]	165
3	**5c**	60	150	150	120	100
4	**5d**	80	180	NA	NA	NA
5	**5e**	85	210	150	NA	75
6	**5f**	68	240	NA	101	NA
7	**5g**	74	150	104	87	60
8	**5h**	75	180	150	NA	50
9	**5i**	70	120	NA	23	31
10	**5j**	80	180	NA	NA	NA
11	**5k**	60	240	NA	NA	NA
12	**5l**	59	210	NA	NA	NA
13	Dox	-	-	3.7	4.76	5.25

[a]Yield related to isolated product after purification
[b]NA = not active

cm^{-1}): $\bar{\upsilon}$ 3448 (stretch NH), 3047 (stretch CH, aromatic), 2943 (stretch CH, aliphatic), 1624 (stretch C=O ring), 1593 (stretch C=C), 1489 (stretch C=C), 1438 (binding CH); 1265 (stretch C–O), 1087 (stretch C–O), 821 (binding CH, OOP), 678 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 8.67 (*s*, 1H, NH), 7.85 (*d*, 1H, J = 7.9 Hz, $H_{4aromatic}$), 7.55 (*d*, 1H, J = 8.2 Hz, $H_{6aromatic}$), 4.65 (*t*, 1H, J = 7.5 Hz, CH), 3.54 (*s*, 3H, OCH_3); 3.30 (*s*, 3H, OCH_3), 3.28 (*s*, 3H, CH_3), 2.14 (*m*, 2H, CH_2); MS (*m*/*z*, %): 288 (M), 222 (M-66, 100), 204 (M-84, 100), 194 (M-94, 50.8), 94 (M-194, 17.7), 77 (M-211, 11.8).

*4-(2-Hydroxy-4-methoxyphenyl)-3-methyl-4,5-dihydropyrano [2,3-c]pyrazol-6(2H)-one (**5d**)*

Chemical formula: $C_{14}H_{14}N_2O_4$; molecular weight: 274.27 (g/mol); yield: 80%; melting point: 164°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3417 (stretch NH), 3066 (stretch OH), 2939 (stretch CH, aliphatic), 1612 (stretch C=O ring), 1566 (stretch C=C), 1462 (stretch C=C), 1431 (binding CH); 1257 (stretch C–O), 1018 (stretch C–O), 802 (binding CH, OOP), 640 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 11.5 (*s*, 1H, NH), 7.77 (*d*, 1H, J = 8.5 Hz, $H_{6aromatic}$), 6.95 (*d*, 1H, J = 8.5 Hz, $H_{5aromatic}$), 6.77 (*s*, 1H, $H_{3aromatic}$), 6.47 (*s*, 1H, OH), 4.70 (*t*, 1H, J = 7.5 Hz, CH), 3.84 (*s*, 3H, OCH_3), 2.68 (*m*, 2H, CH_2), 1.95 (*s*, 3H, CH_3); MS (*m*/*z*, %): 274 (M), 271 (M-3, 27), 171 (M-103, 38.9), 135 (M-139, 96), 127 (M-147, 51.5), 92 (M-182, 70.3), 77 (M-197, 100).

*4-(2,4-Dihydroxyphenyl)-3-methyl-4,5-dihydropyrano[2,3-c] pyrazol-6(2H)-one (**5e**)*

Chemical formula: $C_{13}H_{12}N_2O_4$; molecular weight: 260.25 (g/mol); yield: 85%; melting point: 270°C (decomposition); FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3514 (stretch NH), 3468 (stretch OH), 3217 (stretch OH), 1616 (stretch C=O ring), 1558 (stretch C=C), 1504 (stretch C=C), 1454 (binding CH); 1257 (stretch C–O), 848 (binding CH, OOP), 648 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 11.36 (*s*, 1H, NH), 8.71 (*s*, 1H, $H_{3aromatic}$), 7.69 (*d*, 1H, J = 10 Hz, $H_{6aromatic}$), 7.36 (*d*, 1H, J = 10 Hz, $H_{5aromatic}$), 6.70 (*s*, 1H, OH), 6.29 (*s*, 1H, OH), 4.55 (*t*, 1H, J = 7.5 Hz, CH), 2.73 (*m*, 2H, CH_2), 2.05 (*s*, 3H, CH_3); MS: (*m*/*z*, %): 260 (M), 255 (M-5, 51.6), 206 (M-54, 76.2), 162 (M-98, 100), 94 (M-166, 15.2), 76 (M-184, 12.7).

*4-(2,4-Dimethoxyphenyl)-3-methyl-4,5-dihydropyrano [2,3-c]pyrazol-6(2H)-one (**5f**)*

Chemical formula: $C_{15}H_{16}N_2O_4$; molecular weight: 288.30 (g/mol); yield: 68%; melting point: 201°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3443 (stretch NH), 3008 (stretch CH, aromatic), 2943 (stretch CH, aliphatic), 1604 (stretch C=O ring), 1577 (stretch C=C), 1500 (stretch C=C), 1419 (binding CH); 1269 (stretch C–O), 1026 (stretch C–O), 829 (binding CH, OOP), 678 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 8.77 (*s*, 1H, NH), 7.85 (*s*, 1H, $H_{3aromatic}$), 6.60 (*m*, 2H, $H_{5.6Aromatic}$), 4.60 (*t*, 1H, J = 7.0 Hz, CH), 3.82 (*s*, 3H, OCH_3), 3.79 (*s*, 3H, OCH_3), 2.77 (*m*, 2H, CH_2), 2.11 (*s*, 3H, CH_3); MS (*m*/*z*, %): 288 (M), 222 (M-66, 100), 194 (M-94, 50), 94 (M-194, 17), 77 (M-211, 11).

*4-(2,5-Dimethoxyphenyl)-3-methyl-4,5-dihydropyrano [2,3-c]pyrazol-6(2H)-one (**5g**)*

Chemical formula: $C_{15}H_{16}N_2O_4$; molecular weight: 288.30 (g/mol); yield: 74%; melting point: 145°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3444 (stretch NH), 3001 (stretch CH, aromatic), 2943 (stretch CH, aliphatic), 1620 (stretch C=O ring), 1581 (stretch C=C), 1492 (stretch C=C), 1423 (binding CH), 1261 (stretch C–O), 1045 (stretch C–O), 813 (binding CH, OOP), 667 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 10.77 (*s*, 1H, NH), 8.86 (*s*, 1H, $H_{6aromatic}$), 7.44 (*d*, 1H, J = 7.2 Hz, $H_{4aromatic}$), 7.05 (*s*, 1H, J = 7.2 Hz, $H_{3Aromatic}$), 3.79 (*s*, 3H, OCH_3), 3.72 (*s*, 3H, OCH_3), 2.28 (*m*, 2H, CH_2), 1.95 (*s*, 3H, CH_3); MS (*m*/*z*, %): 288 (M), 222 (M-66, 100), 204 (M-84, 80), 194 (M-94, 51), 94 (M-194, 18), 77 (M-211, 12).

*4-(3,4-Dimethoxyphenyl)-3-methyl-4,5-dihydropyrano[2,3-c]pyrazol-6(2H)-one (**5h**)*

Chemical formula: $C_{15}H_{16}N_2O_4$; molecular weight: 288.30 (g/mol); yield: 75%; melting point: 175°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3429 (stretch NH), 2958 (stretch CH, aliphatic), 1624 (stretch C=O ring), 1581 (stretch C=C), 1462 (binding CH); 1261 (stretch C–O), 1141 (stretch C–O), 813 (binding CH, OOP), 678 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 8.57 (*s*, 1H, NH), 8.23 (*s*, 1H, $H_{2aromatic}$), 7.30 (*d*, 1H, J = 6.5 Hz, $H_{6aromatic}$), 7.01 (*d*, 1H, J = 7.00 Hz, $H_{5aromatic}$), 4.50 (*t*, 1H, J = 7.1 Hz, CH), 3.78 (*s*, 6H, 2(OCH_3)), 2.93 (*m*, 2H, CH_2), 2.12 (*s*, 3H, CH_3); MS (*m*/*z*, %): 288 (M), 286 (M-2, 57.6), 247 (M-41, 29.6), 191 (M-97, 100), 164 (M-124, 34.7), 92 (M-196, 33), 77 (M-211, 43.2).

*4-(3,5-Dimethoxyphenyl)-3-methyl-4,5-dihydropyrano[2,3-c]pyrazol-6(2H)-one (**5i**)*

Chemical formula: $C_{15}H_{16}N_2O_4$; molecular weight: 288.30 (g/mol); yield: 70%; melting point: 140°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3448 (stretch NH), 3005 (stretch CH, aromatic), 2947 (stretch CH, aliphatic), 1681 (stretch C=O ring), 1597 (stretch C=C), 1454 (binding CH); 1292 (stretch C–O), 1064 (stretch C–O), 844 (binding CH, OOP), 678 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 8.59 (*s*, 1H, NH), 7.00 (*s*, 2H, $H_{2.6aromatic}$), 6.61 (*s*, 1H, $H_{4aromatic}$), 4.70 (*t*, 1H, J = 7 Hz, CH), 3.75 (*s*, 6H, 2(OCH_3)), 2.99 (*m*, 2H, CH_2), 2.1 (*s*, 3H, CH_3); MS:(*m*/*z*, %): 288 (M), 269 (M-19, 25.4), 285 (M-3, 16.94), 205 (M-83, 70.3), 191 (M-97, 100), 164 (M-124, 72.8), 92 (M-196, 26.2), 77 (M-211, 47.4).

*3-Methyl-4-(2,3,4-trihydroxyphenyl)-4,5-dihydropyrano[2,3-c]pyrazol-6(2H)-one (**5j**)*

Chemical formula: $C_{13}H_{12}N_2O_5$; molecular weight: 276.24 (g/mol); yield: 80%; melting point: 280°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3414 (stretch NH), 3271 (stretch OH), 3078 (stretch CH, aromatic), 2924 (stretch CH, aliphatic), 1612 (stretch C=O ring), 1585 (stretch C=C), 1496 (stretch C=C), 1411 (binding CH); 1296 (stretch C–O), 1056 (stretch C–O), 821 (binding CH, OOP), 663 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 11.01 (*s*, 1H, NH), 8.60 (*s*, 3H, OH), 7.21 (*d*, 1H, J = 8.5 Hz, $H_{5aromatic}$), 6.81 (*d*, 1H, J = 8.5 Hz, $H_{6aromatic}$), 4.55 (*t*, 1H, J = 7.1 Hz, CH), 3.32 (*m*, 2H, CH_2), 2.46 (*s*, 3H, CH_3); MS (*m*/*z*, %): 276 (M), 248 (M-28, 100), 231 (M-45, 42.3), 105 (M-171, 98.3), 77 (M-199, 57.6).

*3-Methyl-4-(2,3,4-trimethoxyphenyl)-4,5-dihydropyrano[2,3-c]pyrazol-6(2H)-one (**5k**)*

Chemical formula: $C_{16}H_{18}N_2O_5$; molecular weight: 318.32 (g/mol); yield: 60%; melting point: 186°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3448 (stretch NH), 3016 (stretch CH, aromatic), 2939 (stretch CH, aliphatic), 1612 (stretch C=O ring), 1589 (stretch C=C), 1492 (stretch C=C), 1411 (binding CH); 1288 (stretch C–O), 1091 (stretch C–O), 810 (binding CH, OOP), 671 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 8.71 (*s*, 1H, NH), 7.70 (*d*, 1H, J = 7 Hz, $H_{6aromatic}$), 6.89 (*d*, 1H, J = 10 Hz, $H_{5aromatic}$), 3.84 (*s*, 6H, 2(OCH_3)), 4.68 (*t*, 1H, J = 7.2 Hz, CH), 3.74 (*s*, 3H, OCH_3), 2.92 (*m*, 2H, CH_2), 2.16 (*s*, 3H, CH_3); MS (*m*/*z*, %): 318 (M), 311 (M-7, 11.8), 210 (M-108, 31.3), 179 (M-139, 100), 151 (M-167, 91.5), 94 (M-224, 22.8), 77 (M-241, 44.9).

*3-Methyl-4-(3,4,5-trimethoxyphenyl)-4,5-dihydropyrano[2,3-c]pyrazol-6(2H)-one (**5l**)*

Chemical formula: $C_{16}H_{18}N_2O_5$; molecular weight: 318.32 (g/mol); yield: 59%; melting point: 184°C; FT-IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3437 (stretch NH), 2935 (stretch CH, aliphatic), 1620 (stretch C=O ring), 1577 (stretch C=C), 1504 (stretch C=C), 1415 (binding CH); 1234 (stretch C–O), 813 (binding CH, OOP), 621 (binding NH, OOP); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ (ppm) 8.61 (*s*, 1H, NH), 7.17 (*s*, 2H, $H_{2.6aromatic}$), 4.58 (*t*, 1H, J = 7.5 Hz, CH), 3.80 (*s*, 6H, 2(OCH_3)), 3.69 (*s*, 3H, OCH_3), 2.82 (*m*, 2H, CH_2), 2.30 (*s*, 3H, CH_3); MS:(*m*/*z*, %): 318 (M), 315 (M-3, 23.7), 299 (M-19, 36.4), 221 (M-97, 100), 194 (M-124, 72.8), 91 (M-227, 54.2).

Cell culture conditions

PC3, MCF7, and A2780 cell lines were purchased from Pasteur Institute of Iran, Tehran, Iran. Cell lines were grown and maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. Cells were cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium) supplemented with 10% (V/V) heat inactivated fetal bovine serum, and antibiotics (100 IU/mL penicillin and 100 µL/mL streptomycin) at 37°C in 95% CO_2 humidified incubator. Doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), penicillin, and streptomycin were purchased from Sigma-Aldrich. The test compounds were dissolved in DMSO, diluted with media, and stored as the stock solutions with a concentration of 1.0 mg/mL at –20°C (the concentration of DMSO was less than 1%).[30-32]

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide cytotoxicity assay

Cytotoxic effects of the compounds **5a–5l** were studied in MCF7, PC3, and A2780 cell lines evaluated by MTT assay. Exponentially growing mammalian cells (on 96-well plates) were exposed for 24 h to different concentrations of compounds. Control cells were kept in medium containing 0.1% DMSO. After 24 h of incubation, the medium was removed and 0.1 mg/well of MTT was added to the cells, and the plates were further incubated for 3 h at 37°C. The formazan crystals were solubilized in 0.1 mL of DMSO and the optical density (OD570) was measured using a microplate reader (BioTek Instruments, Kermanshah University of Medical Sciences, Kermanshah, Iran). Half maximal inhibitory concentration (IC_{50}) values were calculated by plotting the log10 of percent cell viability versus drug concentrations. After 24 h, the cells were washed, trypsinized, stained by trypan blue (0.4%), and the number of viable and dead cells was scored.[30-32]

Measurement of mitochondrial membrane potential

In this study, mitochondrial membrane potential (MMP) was evaluated by rhodamine 123 fluorescent dye. Depolarization

of MMP during cell apoptosis results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence intensity. After treatment, cells were incubated with rhodamine 123 for 30 min at 37°C. The florescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a florescence microplate reader (BioTek, H1M).[30-32]

Determination of caspase-3 and 9 activities

The caspase-3 and 9 activities were measured by commercial caspase assay kit (Sigma-Aldrich), according to the manufacturer's protocol. The kit is based on the hydrolysis of the peptide substrate Ac-DEVD-pNA by caspase-3, AcDEVD-pNA (for caspase-3), and Ac-LEHD-pNA (for caspase-9), resulting in the release of the *p*-nitroaniline (pNA) moiety. Briefly, 1×10^6 cells were collected and lysed with 50 µL of chilled lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at maximum speed for 5 min at 4°C. Then, 10 µL of cell lysate was combined with an equal amount of substrate reaction buffer containing caspase-3 and caspase-9 colorimetric substrates. After 2-h incubation at 37°C, the pNA light emission was quantified using a microplate reader at 405 nm (BioTek, H1M). Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allowed determination of the fold increase in caspase-3 and caspase-9 activities. The protein content was determined by the Bradford method using the bovine serum albumin as a standard.[30-32]

Results and Discussion

Chemistry

Although the reported methods for the synthesis of pyranopyrazolone derivatives are effective, they are confronted with certain drawbacks of environment compatibility by the use of toxic and expensive catalysts. In this approach, we used water-ethanol instead of large amounts of organic solvents, workup was simplified, and the reaction times were considerably decreased. In this reaction, the one-pot synthesis was optimized by a mixture of methyl acetoacetate (**4**) (1.0 mmol), hydrazine hydrate (**3**) (1.0 mmol), Meldrum's acid (**1**) (1.0 mmol), and aromatic aldehydes (**2a–2l**) (1.0 mmol) in the presence of activated potassium carbonate (10 mol%) in water-ethanol (5 mL, 1:1) and was stirred at reflux for the time listed in Table 1. The experimental procedure is very simple and convenient, and under the reaction conditions used, it can tolerate a variety of functional groups. Simply by adding catalyst to a mixture of reactants, rapid and convenient condensation is achieved at reflux in a mixture of water-ethanol. After the completion of the reaction (monitored by TLC), the residue was filtered and washed with cooled water, then it was purified by recrystallization from ethanol to produce the desired solid and it was confirmed by physical and spectral data.

Cytotoxic and apoptosis mechanism

The *in vitro* cytotoxic activity of pyranopyrazolone derivatives **5a–5l** was evaluated by MTT assay on PC3, MCF7, and A2780 cell lines [Table 1]. The dose–effect curves were generated and the sensitivity to pyranopyrazolone derivatives was expressed as a drug concentration that caused 50% growth inhibition. Pyranopyrazolone derivatives efficiently inhibited all cell growth tested in a dose-dependent manner. The data for doxorubicin as a positive control were included for comparison. The most potent of the compounds was **5g** against PC3 cell line (IC_{50} = 104 µM), **5f**, **5g**, and **5i** against MCF7 cell line (IC_{50} = 101, 87, and 23 µM, respectively), **5e**, **5g**, **5h**, and **5i** against A2780 cell line (IC_{50} = 75, 60, 50, and 31 µM, respectively), which seems to be the best ones relative to the control drug. It seems electron-donating substituents (methoxy and hydroxy) are effective on the potency of the test compounds so that the best results were obtained for these groups. All of the compounds **5a–5l** have less cytotoxic activity compared to the control drug. The compounds **5g–5i** proved to be outstandingly potent cytotoxic agents, especially against A2780 and MCF7 cells. Therefore, in subsequent experiments, **5g–5i** were adopted for the identification of mechanisms of action on A2780 and MCF7 cell lines.

The apoptosis induction capacity instead of necrotic induction is considered to be the important factor of a potential anticancer drug. Therefore, the mechanism involved in cytotoxic effects of selected compounds was evaluated using well-characterized apoptosis markers. Activation of caspase cascade is critical in the initiation of apoptosis in various biological systems. A member of this family, caspase-3 and caspase-9 enzymes, has been identified as being a key mediator of apoptosis. Our results showed that 24-h treatment with IC_{50} concentration of **5h** increased caspase-3 and caspase-9 activation in A2780 cell line [Figure 2]. In addition, the compound **5g** increased caspase-9 activity significantly in MCF7 cell line [Figure 3].

Mitochondria are double-membrane-bound organelles that play significant roles in activating apoptosis through intrinsic pathway in mammalian cell. During apoptosis, the MMP decreases. A decrease in MMP leads to matrix condensation and the release of cytochrome *c* from the mitochondrial intermembrane space. The release of cytochrome *c* triggers the activation of caspase-9, the apical proteases in intrinsic pathway of apoptosis, which goes on to activate the effector

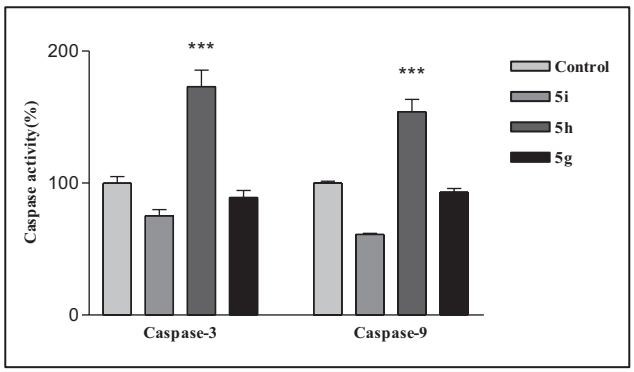


Figure 2: The effect of the compounds 5g–5i on mitochondrial membrane potential in A2780 cell line. Data are expressed as the mean ± standard error of mean (SEM) of three separate experiments (n = 3). *** = p < 0.001

caspases. The result indicated that **5g** was able to significantly decrease MMP and also increase caspase-9 activity in MCF7 cell line, thus implying that mitochondrial pathway of apoptosis is involved in apoptotic induction of **5g** [Figure 4]. In the A2780 cells, the compound **5h** could decrease MMP significantly [Figure 5]. In addition, this compound was able to increase caspase-9 activity significantly in A2780 cells, indicating that **5h** induces apoptosis via mitochondrial pathway.

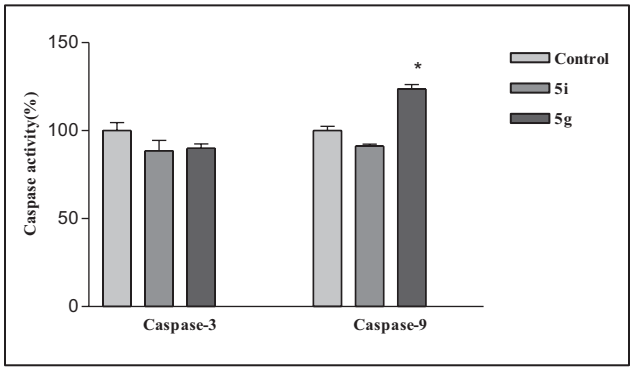


Figure 3: The effect of the compounds 5g–5i on caspase-3 and caspase-9 activity in A2780 cell line. Data are expressed as the mean ± standard error of mean (SEM) of three separate experiments (*n* = 3). * = *p* < 0.05

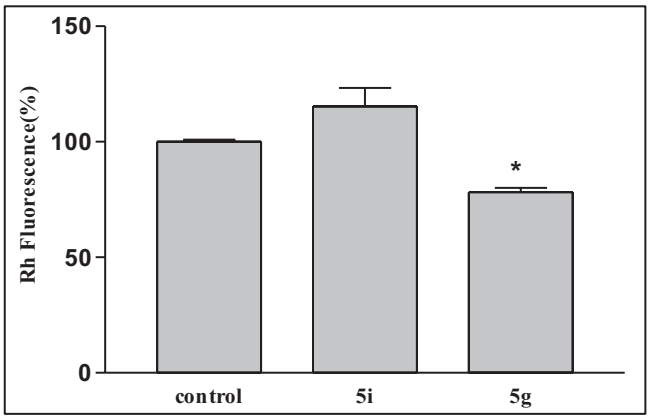


Figure 4: The effect of the compounds 5g and 5i on caspase-3 and caspase-9 activity in MCF7 cell line. Data are expressed as the mean ± standard error of mean (SEM) of three separate experiments (*n* = 3). * = *p* < 0.05

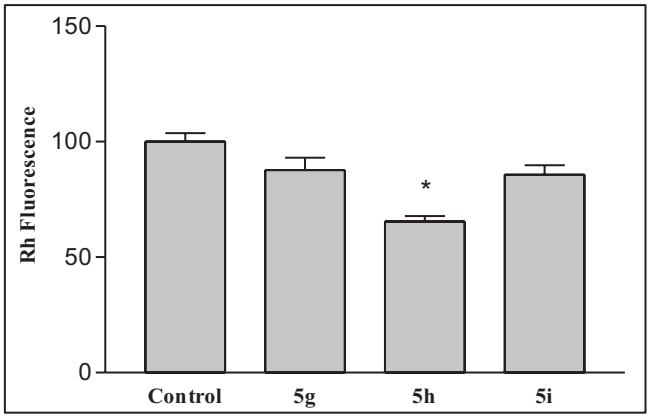


Figure 5: The effect of the compounds 5g and 5i on mitochondrial membrane potential in MCF7 cell line. Data are expressed as the mean ± standard error of mean (SEM) of three separate experiments (*n* = 3). * = *p* < 0.05

Conclusion

In conclusion, we have been able to introduce an efficient and environmentally friendly approach for the synthesis of pyranopyrazolones via a four-component condensation, using activated potassium carbonate as a solid base catalyst in heterogeneous conditions. The reactions are characterized by noncorrosiveness, safety, low cost and waste, ease of separation, high yields, and short reaction times. Furthermore, cytotoxic activity of the test compounds was evaluated against a panel of cancer cell lines *in vitro* comparable to doxorubicin. Some of the pyranopyrazolones showed good tumor-specific cytotoxicity, indicating a new drug candidate for cancer chemotherapy.

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Conflicts of interest

There are no conflicts of interest.

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