Synthesis and Cytotoxic Activity of Novel 9-[Hydroxy(Substitutedphenyl) Methyl]-2,2-Dimethyl-2,3,8,9-Tetrahydro-4*H*,10*H*-Pyrano [2,3-*f*]Chromene-4,10-Diones

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ABSTRACT: *Chromanone derivatives demonstrate remarkable cytotoxity against a varieties of cancer cell lines. Novel 9-[hydroxy(substitutedphenyl)methyl]-2,2-dimethyl-2,3,8,9- tetrahydro-4H,10H-pyrano[2,3-f]chromene-4,10-diones as Glyasperin analogues were synthesized in four steps from known 4-chromone 1. The key step was the preparation of chromane dione 5a by regioselective intramolecular cyclization reaction in 85% yield. Condensation of 5a with substituted aromatic aldehydes afforded corresponding alpha hydroxybenzyl analogues 6a-6e. The cytotoxic study of the synthesized compounds against breast cancer human cell line (T47D) showed moderate cytotoxic activities (IC_{50}=16-40 μM) compared to the positive control drug vincristin (IC_{50}=2.5 μM).*

KEY WORDS: *Chromane, Aldol reaction, Cytotoxicity, Chromanone, Glyasperin analogues.*

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INTRODUCTION

The discovery and development of new anticancer drugs is needed due to problems with currently available medicines, like toxicities and drug resistance [1].

Apoptosis or programmed cell death is a normal process that organisms use to eliminate dispensable or excess cells and is important in animal development, as well as in tissue homeostasis. It has been known that inadequate or abnormal inhibition of apoptosis, which leads to unchecked cell proliferation and results in cell accumulation, is a hallmark of cancer [2]. It has been well documented that many of the clinically useful cytotoxic agents induce apoptosis in cancer cells. The pro-apoptotic 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 chemo-resistance, as well as dose-limiting neurologic and bone marrow toxicity, however, has limited the use of tubulin targeting agents. This clearly highlights the need for novel chemotherapeutic agents for effective treatment of cancer [3].

Chromanone analogues demonstrate impressive cytotoxicity as well as a remarkable ability to inhibit tubilin polymerization. These compounds and related derivatives have diverse biological activities, including antitumor, leishmanicidal, and bacteriostatic that makes these compounds attractive for further investigations and screening as a novel therapeutic agent [4,5]. Based on previous work [6-9], the design and synthesis of cytotoxic compounds similar to a Glyasperin was undertaken (Fig. 1). The chromone molecular framework was modified by the introduction of substituted benzyl rings into the alpha position of carbonyl group of compound 5a.

CHEMISTRY

The desired compounds 6a-6e were prepared according to Scheme 1. Reaction of 7-hydroxy-4-chromone 1 with 3-bromopropanol 2 in refluxing acetone afforded primary alcohol 3. Oxidation of compound 3 with PDC failed [10,11]. However, Jones oxidation gave the corresponding carboxylic acid 4 in almost quantitative yield. Treatment of carboxylic acid 4 with Bronsted and Lewis acids like CF_3COOH, $AlCl_3$ and $POCl_3/ZnCl_2$ to perform intramolecular cyclization failed and the starting material was decomposed [11,12]. However,

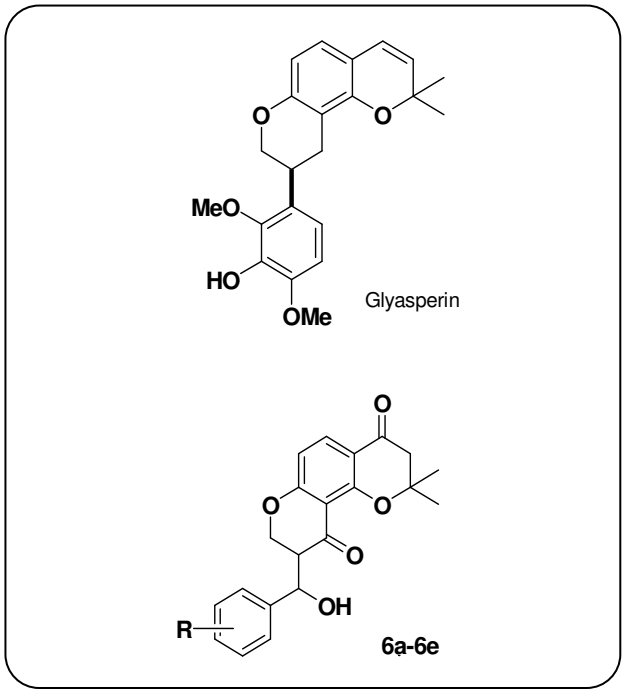


Fig. 1: Structures of glyasperin and 6a-6e compounds.

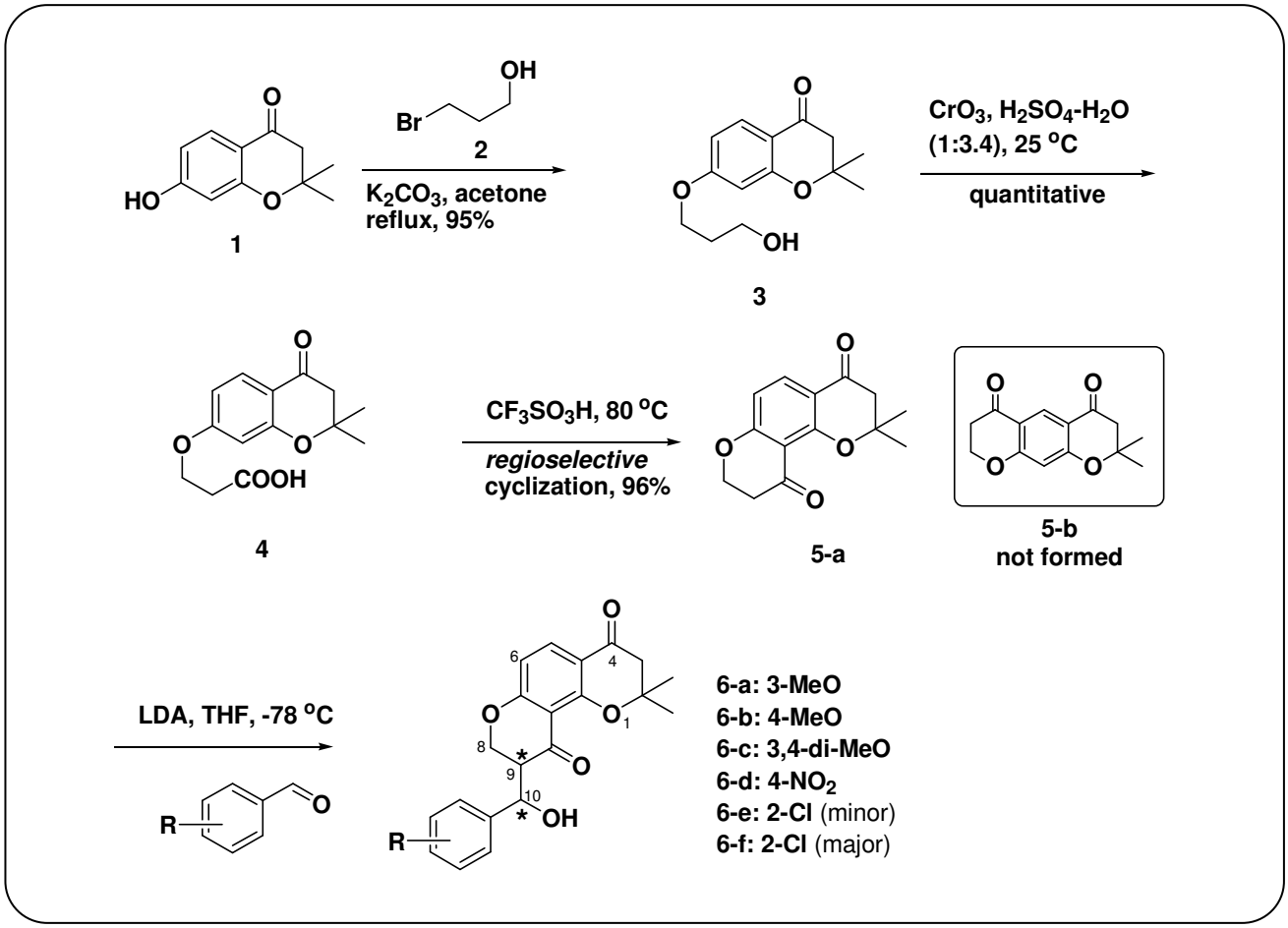
trifluoromethanesulfonic acid, gave pyranochromane 5 regioselectively [6,14]. Using two equivalent of this acid at 80 °C afforded 5-a in high yield (96%), and the other regioisomer 5-b was not formed. Treatment of diketone 5-a with LDA at -78 °C in THF in the presence of HMPA gave selective deprotonation of the less hindered alpha carbonyl proton in 9-pyrano ring position[15], and subsequent treatment of this anion with substituted benzaldehyde gave corresponding alpha hydroxyl benzyl analogues 6a-6e (Scheme 1).

The aldol products 6a-f were obtained as mixtures of two diasteromers, the assighment of diastereomers will be reported in the forthcoming paper. Compounds 6e and 6f were separated using preparative TLC from the crude diastromeric mixture.

CYTOTOXIC ASSAY [16]

Cell line and culture

Breast cancer human cell line, T47D (human breast duct carcinoma; ATCC HTB-133 estrogen receptor-positive, ER+), was obtained from Pasture institute, Tehran (Iran). T47D cells were maintained in RPMI 1640 with added 10% FBS, 1% L-Glutamine, and Penicillin-Streptomycin, and then incubated at 37°C under a 5% concentration of CO_2.



Scheme 1: Synthesis of compounds 6a-6f.

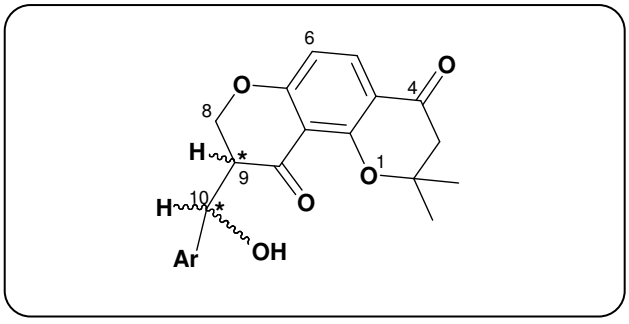


Fig. 2: (R^*R^*/S^*S^*) and (R^*S^*/S^*R^*) Diastereomers of 6a-6f.

Cells were harvested by Trypsin-EDTA and resuspended in fresh medium and seeded in 96-well culture plates at 4000 cells/well. After 2 days incubation, in order to cells attaching, different concentrations of test compounds were prepared by serial dilution (1:2) and each concentration added to wells as triplicate from 1 to10 µg/mL. In each plate, there were control wells (cells without test compounds) and blank wells (the medium only or 0.1%DMSO). On day 3, the medium was removed and phenol red-free medium with FBS was added to cells.

Viability Assay

Cell survival was determined by MTT colorimetric assay. Exponentially growing cells (4×10^4 cells/well) were seeded in 96-well plates in RPMI with 10% FBS and incubated for 24 h. The cells were treated by different concentrations of test compounds for 24 h at 37°C. After treatment, the medium was removed and phenol red-free medium with FBS was added to cells. Then MTT solution was added to each well (2 mg/mL), followed by 4 h incubation. The viable cell number is directly proportional to the production of formazan, which, following solubilization with isopropanol, can be measured spectrophotometrically at 492 nm by an ELISA plate reader.

Morphological study of the apoptotic cells

Acridine Orange/Ethidium Bromide (AO/EB) staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis [17].

Cells were washed in cold PBS and adjusted to a cell density of 1×10^7 cell/ml of PBS. Acridine orange/ ethidium bromide solution (1:1 v/v) was added to the cell suspension in a final concentration of 100 µg/mL. The cellular morphology was evaluated by Axoscope 2 plus fluorescence Spmicroscopy from ZEISS (Germany).

Results

The percentage of T47D viability versus controls was assessed by the formula [1 - (absorbance of treated cells/absorbance of control cells)] × 100. The IC_{50} was defined as the concentration of drug required to decrease 50% of cell viability and were determined by linear regression analysis.

Significance of results was assayed by using ANOVA, followed by Dennett's Comparison of Treatment against Control with $p < 0.001$ evaluated as statistically significant.

As shown in Table 1, compounds 6b-6f showed moderate activities against T47D cell line (range of IC_{50}=16 to 40 µM) relative to positive drug control vincristin with IC_{50}=2.5 µM.

The inhibition percentage of treated cells were plotted against compounds concentrations (Fig. 3). Acridine orange/ethidium bromide double staining enables distinguishing between apoptotic and necrotic cells. The treated cells by the studied compounds showed obvious nuclear condensation after 4 h treatment. Control cells showed bright green nucleus with uniform intensity without taking up ethidium bromide, where the apoptotic cells appeared orange in color (Fig. 4). This study indicates that the synthesized compounds showed moderate cytotoxic activities against the breast cancer human cell line (T47D) compared to vincristin positive control drug.

EXPERIMENTAL SECTION

Chemicals and reagents were obtained from Merck or Sigma-Aldrich Chemical. ^{1}H-NMR spectra were reported using a Bruker Avance 500 MHz spectrometer. Chemical shifts are expressed in (ppm) with tetramethylsilane as internal standard. The IR spectra were taken using Nicolet FT-IR Magna 550 spectrophotometer (KBr disks). MS spectra were obtained by a Finnigan MAT TSQ-70 spectrometer. The results of elemental analyses (C, H, N) were within ±0.4% of theoretical values for C, H, and N.

Table 1: IC_{50} (µM) of the synthesized compounds against T47D (breast cancer human cell line).

Compound	R	IC_{50} (µM)
6-a [a]	4-MeO	ND [b]
6-b	3-MeO	40
6-c	3,4-di-MeO	20
6-d	2-NO_2	20
6-e (minor)	2-Cl	16
6-f (major)	2-Cl	20
Vincristin		2.5

a) Mixture of diasteromers. b) Not determined.

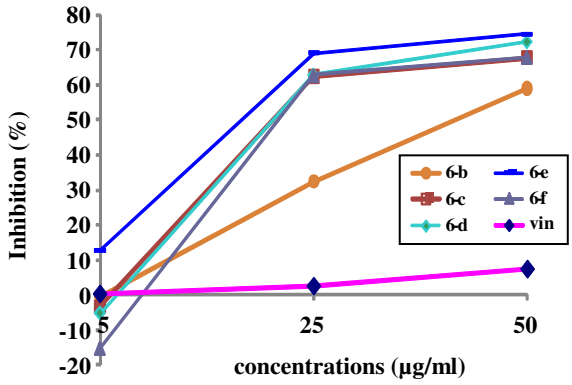


Fig. 3: The Inhibition versus concentration of five compounds.

7-(3-Hydroxypropoxy) -2,2- dimethyl -2,3- dihydro -4H-chromen-4-one (3)

To a stirred solution of compound 2 (1.06 g, 5.52 mmol), in dry acetone (30 mL) and K_2CO_3 (0.92 g, 6.62 mmol), was added 3-bromo-1-propanol (1.6 mL, 6.07 mmol). The resulting mixture was stirred for 24 h at room temperature. The mixture was filtered off and evaporated. To the crude mixture water was added and aqueous phase was extracted with EtOAc. The organic phase was washed with brine, dried (Na_2SO_4) and filtered. Solvent was evaporated under reduced pressure, the residue was purified by flash chromatography (silica gel = 30 g,

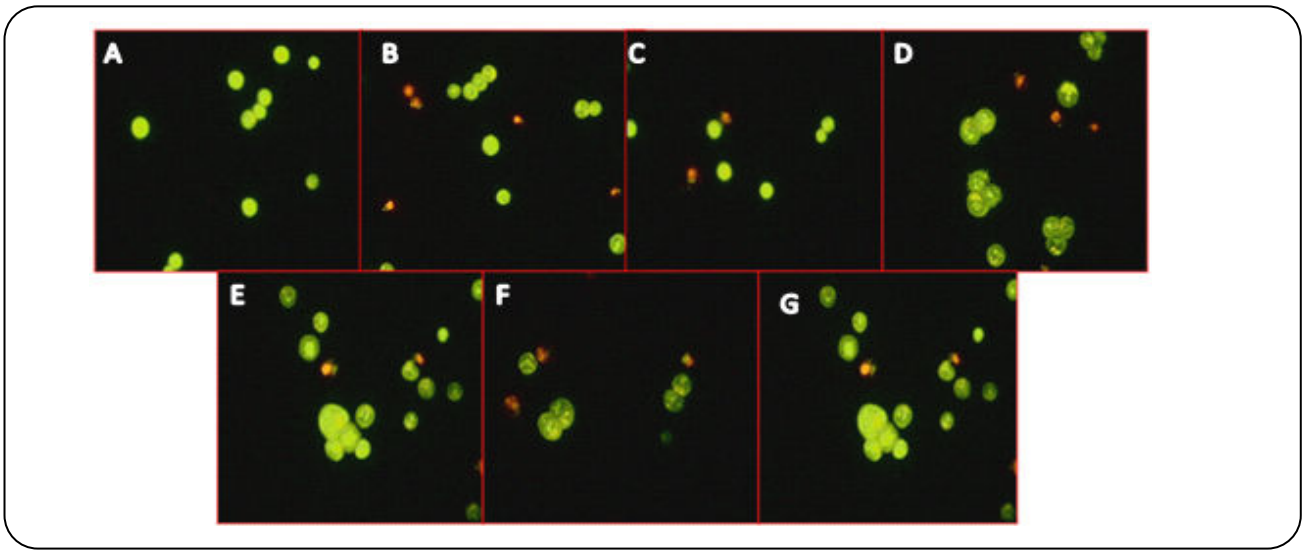


Fig. 4: Fluorescence microscopic analysis of treated T47D cells by the synthetic compounds. T47D cells were incubated by IC_{50} concentrations of compounds for 4h. A mixture of acridine orange and ethidium bromide was used. The differential uptake of these two dyes allows the identification of viable and non-viable cells. Acridine orange penetrates into living and dead cells, emitting green fluorescence. Ethidium bromide stains the nuclei of dead cells (orange color). Control T47D (A), cells treated with vincristin (B), compounds 6-b (C), 6-c (D), 6-d (E), 6-e (F) and 6-f (G).

hexane / EtOAc = 5:1) to give (1.25 g, 90 %) of compound 3 as red crystals (mp= 118-120 °C). ^{1}H-NMR (80MHz, $CDCl_3$) δ1.41 (s, 6H), 2.01 (doft, 2H, *J*=6.0Hz), 2.63 (s, 2H), , 3.81 (quintet, 2H, *J*=5.9Hz), 4.1 (t, 2H, *J*=6.0Hz), 6.43 (s, 1H), 6.47 (d, 1H, *J*=8.8 Hz) 7.75 (d, 1H, *J*=8.7 Hz), FT-IR (KBr) ν (cm^{-1}) 3406, 1655. Anal. Calcd for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25. Found: C, 67.24; H, 7.18.

3- [(2,2-Dimethyl -4- oxo -3,4- dihydro -2H- chromen-7-yl) oxy]propanoic acid (4)

To a solution of Jones reagent (3.6 mL, 27.21 mmol) in acetone (34 mL) compound **3** (2.27 g, 9.07 mmol) in acetone (30 mL) was slowly added over 5 min at 0 °C. The stirring was continued for 30 min at 0 °C. The resulting mixture was gradually warmed to room temperature over 1 h and the stirring was continued for an additional 5 h at room temperature. The reaction mixture was filtered and evaporated and water was added. The aqueous phase was extracted by EtOAc. The combined organic phase was concentrated and the residue was purified by column chromatography (silica gel, hexane/EtOAc = 6:1 then 2:1) to give 2.53 g (quantitative yield) of 4 (mp= 136-138 °C). ^{1}H-NMR (80MHz, $CDCl_3$) δ 1.45 (s, 6H), 2.67 (s, 2H), 2.86 (t, 2H, *J*=6.1Hz), 4.27 (t, 2H, *J*=6.1Hz), 6.42 (s, 1H), 6.52 (d, 1H, *J*=8.7 Hz), 7.80 (d, 1H, *J*=8.7 Hz), FT-IR (KBr) ν (cm^{-1}) 3406, 1692. Anal. Calcd for $C_{14}H_{16}O_5$: C, 63.63; H, 6.10. Found: C, 63.56; H, 6.24.

2,2- Dimethyl -2,3,8,9- tetrahydro -4H,10H- pyrano[2,3-f]chromene-4,10-dione (5a)

To the acid 4 (7.5 g, 28.3 mmol) was added trifluoromethanesulfonic acid (5 ml, 56.7 mmol) in one portion. The solution was warmed to 80 °C for 30 min, cooled to room temperature over 15 min, and poured into chloroform (100 mL). The solution was slowly added into water (30 mL), and the organic layer was separated. The aqueous layer was extracted with 2 x 20 ml of $CHCl_3$. The combined organic layers were washed with brine, dried (Na_2SO_4) and filtered. After concentration, the residue was purified by flash chromatography (silica gel, hexane/EtOAc = 8:1 then 3:1) and the desired compound was crystallized from EtOAc-hexane to give 6.6 g, 96% of 5a (mp= 104-105 °C). ^{1}H-NMR (80MHz, $CDCl_3$) δ 1.53 (s, 6H), 2.71 (s, 2H), 2.79 (t, 2H, *J*=6.4 Hz), 4.56 (t, 2H, *J*=6.4Hz), 6.56 (d, 1H, *J*=8.8 Hz), 8.48 (d, 1H, *J*=8.8 Hz), FT-IR (KBr) ν (cm^{-1}) 1682; MS *m/z* (%): 246 (79), 231 (61), 191 (100), 162 (61), 121 (14), 94 (25), 69 (18). Anal. Calcd for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.35; H, 5.89.

General Procedure for Synthesise of 6a-6e.

A solution of compound **5** (100 mg, 0.4 mmol) in dry THF (0.5 mL) was added to a solution of LDA (4 ml, 0.8 mmol) in THF (3 ml) at -78 °C and stirred for 30 min. To this solution was added the corresponding aladehyde (0.48 mmol) and HMPA (14 mL, 0.8 mmol). The reaction mixture was stirred for 2 h, then quenched with satd. aq. NH_4Cl (5 mL). The mixture was extracted with EtOAc (2 × 20 mL). The extract was washed successively with water (3 mL), 10% HCl (3 mL), water, satd. aq. $NaHCO_3$ (2 mL) and sat. NaCl. Organic phase was dried (Na_2SO_4), filtered and evaporated in *vacuo* to give a diasteremeric mixture of alcohols as a brown viscous oil. After concentration, the residue was purified by flash column chromatography (silica gel, hexane/EtOAc, 7 : 4) to give 6a-6e.

9-[Hydroxy (4-methoxyphenyl)methyl] -2,2- dimethyl-2,3,8,9- tetrahydro -4H,10H- pyrano[2,3- f]chromene-4,10-dione (6a)

6a (53%). ^{1}H-NMR (500MHz, $CDCl_3$) δ: as a mixture of diasteromers (major: minor, 66:34): 1.49 and 1.52 (2s, 3H), 1.56 and 1.58 (2s, 3H), 2.71 (d, 1H, *J*= 17 Hz), 2.77 (d, 1H, *J*= 17 Hz), 3.00 (doft, *J*= 7.5 Hz, *J*= 9 Hz), 3.08 (octet, *J*= 2.3 Hz, *J*= 5.5 Hz, *J*= 11.5 Hz), 3.81 (s, 3H), 4.04 (d, *J*= 7.5 Hz), 4.38 (dd, *J*= 5.5 Hz, *J*= 11.5 Hz), 4.57 (t, *J*= 11.5 Hz), 4.92 (d, *J*= 9 Hz), 5.62 (d, *J*= 2.3 Hz), 6.52 (d, 1H, *J*= 8.8 Hz), 6.91 (d, 2H, *J*= 8.4), 7.30 (d, 2H, *J*= 8.4), 7.95 (d, 1H, *J*= 8.8 Hz), 8.00 (d, 1H, *J*= 8.8 Hz). IR (KBr): ν (cm^{-1}) 3426, 1682. MS *m/z* (%):382 (M^+, 11), 364 (5), 246 (100), 231 (63), 203 (28), 191 (89), 162 (50), 135 (77), 107 (20), 94 (17), 77 (32). Anal. Calcd for $C_{22}H_{22}O_6$: C, 69.10; H, 5.80. Found: C, 69.20; H, 5.79.

9- [Hydroxy(3- methoxyphenyl)methyl] -2,2- dimethyl-2,3,8,9- tetrahydro-4H,10H -pyrano[2,3-f]chromene-4,10-dione (6b), obtained exclusively as one diastereomer.

Yield (65%), ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.52 (s, 3H, CH_3), 1.57 (s, 3H), 2.71 (d, 1H, *J*= 16.6 Hz), 2.77 (d, 1H, *J*= 16.6 Hz), 3.01 (doft, 1H, *J*= 7.5 Hz, *J*= 9 Hz), 3.82 (s, 3H), 4.06 (d, 2H, *J*= 7.5 Hz), 4.94 (d, 1H, *J*= 9 Hz), 6.53 (d, 1H, *J*= 8.8 Hz), 6.88-7.29 (m, 4H), 8.00 (d, 1H, *J*= 8.8 Hz). IR (KBr): ν (cm^{-1}) 3452, 1685. MS *m/z* (%): 383 (M^++1, 17), 382 (M^+, 37), 364 (32), 309 (9), 246 (97), 231 (63), 217 (9), 203 (33), 191 (100), 163 (52), 147 (10), 136 (83), 107 (25), 94 (19), 77 (32). Anal. Calcd for $C_{22}H_{22}O_6$: C, 69.10; H, 5.80. Found: C, 69.32; H, 5.73.

9-[Hydroxy(3,4-dimethoxyphenyl)methyl] -2,2-dimethyl-2,3,8,9- tetrahydro -4H,10H- pyrano [2,3-f]chromene-4,10-dione (6c), obtained exclusively as one diastereomer.

Yield (50%), ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.53 (s, 3H), 1.60 (s, 3H), 2.72 (d, 1H, *J*= 16.8 Hz), 2.79 (d, 1H, *J*= 16.8 Hz), 3.04 (doft, 1H, *J*= 7.0 Hz, *J*= 9.2 Hz), 3.90 (s, 3H), 3.92 (s, 3H), 4.06 (d, 2H, *J*= 7.0 Hz), 4.92 (d, 1H, *J*= 9.2 Hz), 6.55 (d, 1H, *J*= 8.8 Hz), 6.86 (m, 2H), 6.97 (s, 1H), 8.03 (d, 1H, *J*= 8.8 Hz). IR (KBr): ν (cm^{-1}) 3452, 1685. MS *m/z* (%): 412 (M^+, 16), 394 (33), 360 (11), 310 (10), 246 (100), 231 (55), 219 (11), 203 (27), 191 (71), 177 (14), 166 (76), 151 (12), 135 (19), 95 (31), 77 (29). Anal. Calcd for $C_{23}H_{24}O_7$: C, 66.98; H, 5.87. Found: C, 66.85; H, 5.73.

9-[Hydroxy(4-nitrophenyl)methyl]-2,2-dimethyl-2,3,8,9-tetrahydro-4H,10H-pyrano[2,3-f]chromene-4,10-dione (6d), obtained exclusively as one diastereomer.

Yield 45%; mp= 179-181 °C (colorless crystals); ^{1}H-NMR (500MHz, $CDCl_3$), δ: 1.52 and 162 (2s, 6H), 2.71 (d, 1H, *J*= 16.5 Hz), 2.80 (d, 1H, *J*= 16.5 Hz), 3.16 (octet, 1H, *J*= 2.3 Hz, *J*= 5.2 Hz, *J*= 11.5 Hz), 4.27 (dd, 1H, *J*= 5.2 Hz, *J*= 11.5 Hz), 4.59 (t, 1H, *J*= 11.5 Hz), 5.84 (d, 1H, *J*= 2.3 Hz), 6.55 (d, 1H, *J*= 8.8 Hz), 7.56 (d, 2H, *J*= 8.8 Hz), 8.03 (d, 1H, *J*= 8.8 Hz), 8.28 (d, 2H, *J*= 8.6). IR (KBr): ν (cm^{-1}) 3460, 1679. MS *m/z* (%): 398 (M^++1, 12), 397 (M^+, 45), 379 (13), 341 (20), 324 (20), 246 (100), 231 (73), 217 (13), 203 (47), 191 (98), 177 (12), 162 (61), 150 (50), 134 (17), 120 (11), 105 (19), 94 (20), 77 (49). Anal. Calcd for $C_{21}H_{19}NO_7$: C, 63.47; H, 4.82; N, 3.52. Found: C, 63.26; H, 4.79; N, 3.68.

9-[(2- Chlorophenyl)(hydroxy)methyl] -2,2- dimethyl-2,3,8,9- tetrahydro -4H,10H- pyrano [2,3-f]chromene-4,10-dione (6e/6f)

The mixture of diasteromers were separated by TLC plate (Silica gel) 6e: minor diasteromer (oil, 18%), ^{1}H-NMR (500MHz, $CDCl_3$) δ: 1.52 (s, 3H), 1.61 (s, 3H), 2.67 (d, 1H, *J*= 16.4 Hz,), 2.76 (d, 1H, *J*= 16.4 Hz),

2.91 (brs,1H), 3.33 (octet, 1H, *J*= 2.2, *J*= 5.4 Hz, *J*= 11.5 Hz), 4.29 (dd, 1H, *J*= 5.4 Hz, *J*= 11.5 Hz), 4.62 (t, 1H, *J*= 11.5 Hz), 6.07 (brs, 1H), 6.54 (d, 1H, *J*= 8.8 Hz), 7.25-7.37 (m, 3H), 7.61 (m, 1H), 8.01 (d, 1H, *J*= 8.8 Hz). IR (KBr): νν (cm^{-1}) 3452, 1687. MS *m/z* (%): 388 (M^++2, 16), 387 (M^++1, 11), 386 (M^+, 45), 351 (100), 333 (10), 295 (17), 246 (20), 231 (39), 203 (42), 191 (91), 175 (10), 163 (34), 141 (22), 113 (12), 105 (13), 86 (18),77 (42). Anal. Calcd for $C_{21}H_{19}ClO_5$: C, 65.20; H, 4.95. Found: C, 64.95; H, 5.19.

6f: (oil, 40%), major diasteromer, ^{1}H-NMR (500MHz, CDCl3) δ: 1.53 (s, 3H), 1.61 (s, 3H), 3.13 (d, 1H, *J*= 16.6 Hz), 3.15 (d, 1H, *J*= 16.6 Hz), 3.14 (octet, 1H, *J*= 5.3 Hz, *J*= 11.6 Hz, *J*= 9.0 Hz), 4.08 (dd, 2H, J= 5.3 Hz, *J*= 11.6 Hz), 4.34 (t, 1H, *J*= 11.6 Hz), 5.54 (d, 1H, *J*= 9.0 Hz), 6.54 (d, 1H, *J*= 8.8 Hz), 7.25-7.37 (m, 3H), 7.61 (d, 1H), 8.01 (d, 1H, *J*= 8.8 Hz). IR (KBr): ν (cm^{-1}) 3452, 1687. MS *m/z* (%): 388 (M^++2, 18), 386 (M^+, 52), 369 (32), 351 (100), 333 (22), 295 (15), 277 (15), 247 (60), 231 (45), 203 (32), 191 (89), 175 (10), 163 (31), 147 (20), 115 (15), 105 (55), 77 (57). Anal. Calcd for $C_{21}H_{19}ClO_5$: C, 65.20; H, 4.95. Found: C, 64.95; H, 4.73.

CONCLUSIONS

In conclusion, we have synthesized a novel series of 9- [hydroxy(substitutedphenyl)methyl] -2,2- dimethyl-2,3,8,9- tetrahydro-4*H*,10*H*-pyrano[2,3-*f*]chromene-4,10-diones as Glyasperin analogues using a 4 steps methodology starting from 7-hydroxy-2,2-dimethyl-2,3-dihydro -4*H*-chromen-4-one. The cytotoxic activity of the synthesized compounds against breast cancer human cell line (T47D) showed moderate cytotoxic activities compared to the positive reference drug vincristin.

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