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SYNTHESIS, SPECTROSCOPIC CHARACTERIZATIONS AND CYTOTOXIC ACTIVITIES OF SOME NOVEL 1,2-BIS-(TETRASUBSTITUTED-BENZYLIDENE) HYDRAZINE ANALOGUES

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ABSTRACT. The present work describes an efficient and convenient synthesis of a library of novel 1, 2-bis-(tetrasubstituted-benzylidene) hydrazine an analogue (3-7) 1,2-bis (3-methoxy-2-hydroxybenzlidene) hydrazine (**3**) and 1,2-bis (6-bromo-3-methoxy 2-hydroxybenzylidene) hydrazine (**4**), were obtained via opening of ester coumarin derivatives (**1** and **2**) with hydrazine hydrate under reflux. Diazotization of compound **4** with aryldiazonium chloride led to the formation of 1,2-bis (6-bromo-5-arylazo-3-methoxy-2-hydroxybenzylidene) hydrazine (**6a,b**). Acetylation of compounds **4** and **6**a with acetic anhydride afforded the corresponding 1,2-bis (6-bromo-5-substituted-3-methoxy-2-acetoxy benzylidene) hydrazines (**5** and **7**). The cytotoxicity screening of some synthesized 1,2-bis (tetra substituted benzylidene) hydrazines (**4**-**7**) against breast cancer cell lines (MCF-7), and it was found several active compounds. Meanwhile compound **5** exhibited cytotoxic activity compared to reference drug. The DNA flow cytometry on MCF-7 cells of compound **5** was determined, it was found to cause G2/M phase arrest and induce apoptosis in all G1/M phases. In addition, compound **5** has been tested in other trials against aromatase inhibitors and tyrosinase inhibitors.

KEY WORDS: Synthesis, Hydrazine derivatives, ^{1}H NMR, Mass spectra, MCF-7 cell lines, Cytotoxicity

INTRODUCTION

Cancer is one the deadliest fatal diseases which has been conquering lives worldwide [1] and an important barrier to increasing life expectancy in every country of the world [2]. Breast cancer exceeds lung cancer in terms of mortality rate, with more than two million patients every year (more than 6-9% mortality rate (3:4). According to global cancer statistics, approximately 19.3 million deaths by cancer were reported in 2020 its global burden is expected to be 28.4 million cases in 2040 [3]. There is no doubt that there are many organic compounds with good biological and pharmacological activities [4, 5]. Nitrogen containing compounds are one of the most important compounds found in organic chemistry, as well as in the pharmaceutical industry [6]. Anticancer properties of compounds containing nitrogen and/or sulfur supreme dominating stuffs which can be employed in medicinal and pharmaceutical field [7]. Hydrazine and their derivatives are a series of highly active organic molecules [8]. They have important biological activities and this functional groups are found as a core structure in many chemotherapeutic agents having antimicrobial, antimalarial, antileishmanial, antiviral and anticancer effects [9].

They are widely used as lead compounds for the research and development of new anticancer drugs as they exhibit antiproliferative activities with the ability to prevent cell progression in cancerous cells through different mechanisms [10]. A variety of hydrazines have been utilized as anticancer drugs. Representative members of this class are zorubicin and bisantrene which are widely used for treatment of different cancer types as shown in Chart 1 [11]. Hydrazone derivatives displayed a strong ability to inhibit tubulin polymerization [11] and capable of inducing apoptosis through capase-3 activation [12]. Based on the above facts, and as a continuation of our search for new anticancer agents [13], new 1,2-bis (substituted benzylidene)

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hydrazine derivatives (Chart 2) were designed and synthesized. All the synthesized molecules were evaluated for their *in vitro* cytotoxic activity against the breast MCF-7 cancer cell line. Additionally, the most active molecule **5** was further evaluated for aromatase, tyrosinase inhibitor and cell cycle analysis.

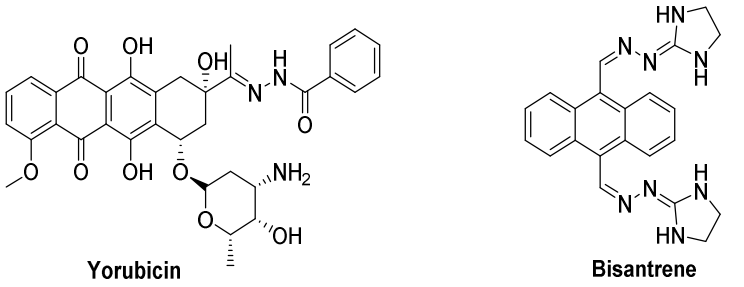


Chart 1. Chemical structures of know hydrazine linkage based anticancer drugs.

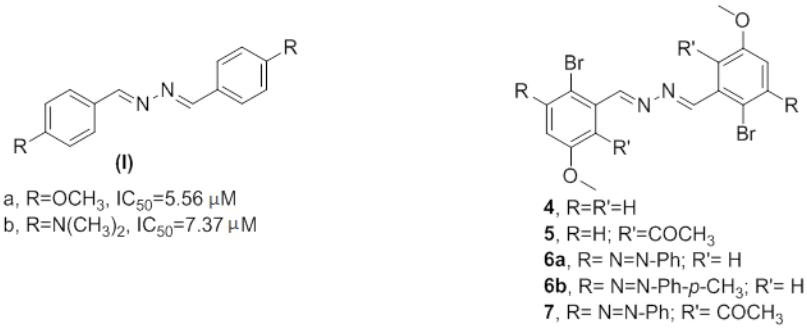
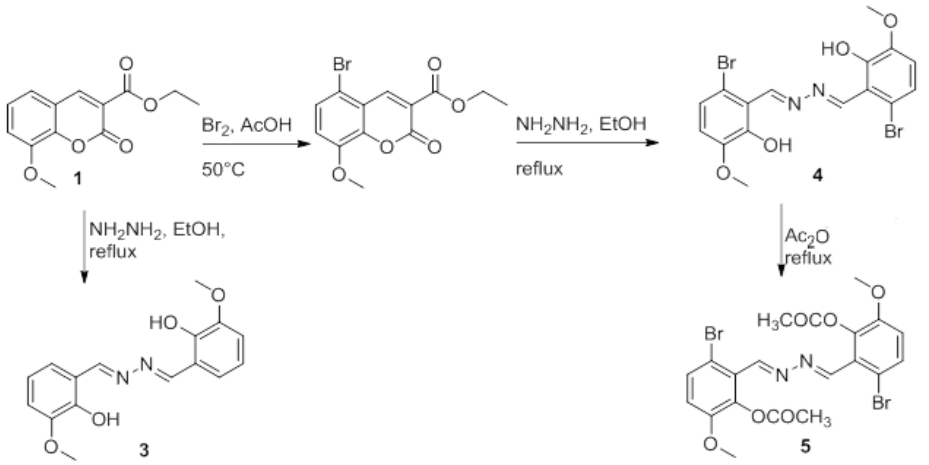


Chart 2. Structure of 1,2-bis (substituted benzylidene) hydrazine derivatives.

RESULTS AND DISCUSSION

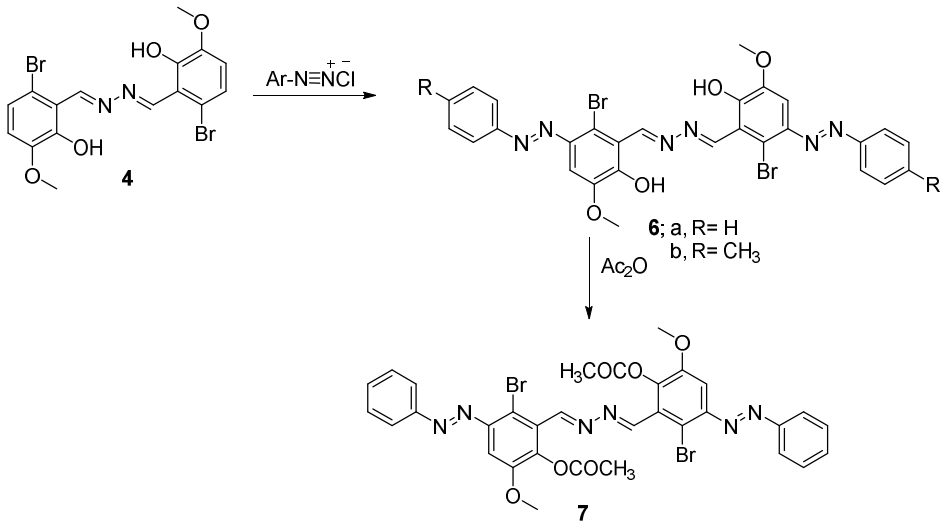
Chemistry

We have synthesized title compounds of 1,2-bis (5,6-disubstituted-3-methoxy-2-hydroxy benzylidene) hydrazine analogues (Scheme 1 and 2). Ethyl 8-methoxy coumarin-3-carboxylate (**1**) was obtained by means of 3-methoxy-2-hydroxybenzaldehyde reaction with diethyl malonate under fusion in the presence of piperidine as base catalyst according to the literature method, as a key staring material. Halogenation of ester coumarin (**1**) with bromine in glacial acetic acid and with stirring at 50 °C, lead to the formation of ethyl 5-bromo-8-methoxy coumarin-3-carboxylats (**2**). 1,2-bis (2-hydroxy-3-methoxy benzylidene) hydrazine (**3**) and 1,2-bis (6-bromo-3-methoxy-2-hydroxybenzylidene) hydrazine (**4**) were synthesized via the ring opening of ester coumarin derivatives (**1** and **2**) by boiling with hydrazine hydrate in ethanol. Acetylation of compound **4** with acetic anhydride under reflux to yield 1,2-bis (6- bromo-3-methoxy-2-acetoxybenzylidene) hydrazine (**5**, Scheme 1).



Scheme 1. Synthesis of 1,2-bis (6-substituted-3-methoxy-2-hydroxy (or acetoxy) benzylidene) hydrazines (3-5).

Treatment of compound **4** with aryldiazonium chloride (namely, phenyl diazonium chloride and 4-methylphenyl diazonium chloride) at 0–5 °C with stirring to yield the corresponding 1,2-bis-(6-bromo-5-anyldiazinyl-3-methoxy-2-hydroxy benzylidene) hydrazines (**6a, b**). Boiling of compound **6** with acetic anhydride under reflux led to the formation of 1,2-bis (2-acetoxy-3-methoxy-5-phenyldiazenyl-6-bromobenzylidene) hydrazines (**7**) (Scheme 2). The chemical structures of all the synthesized 1,2-bis (substituted benzylidene) hydrazines. (**3-7**) were characterized by ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry.



Scheme 2. Synthesis of 1,2-bis (tetrasubstituted benzylidene) hydrazines (**6, 7**).

*NMR spectra study of compounds **3**, **4** and **6***

From the current study the ^{1}H and ^{13}C NMR spectra of compounds 1,2-bis (tetrasubstituted benzylidene) hydrazine (**3, 4** and **6**), showed the structure of these compounds in *E* and *Z* stereoisomers as shown in Chart 3.

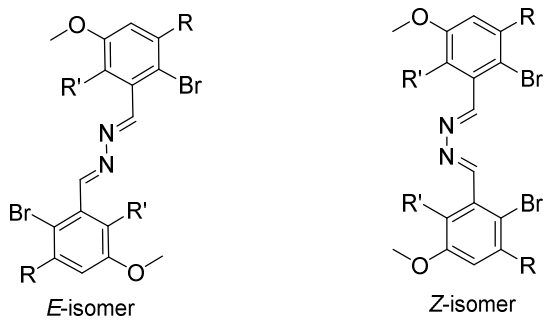
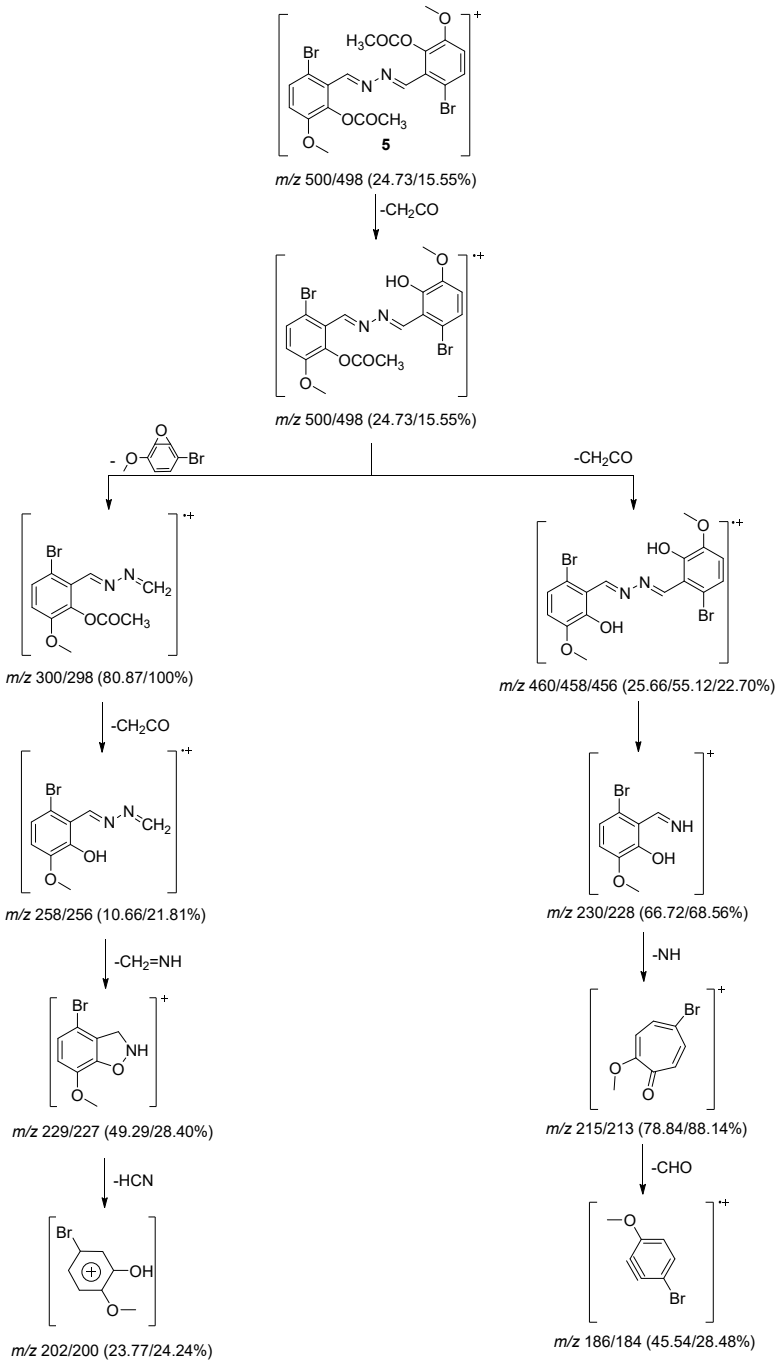


Chart 3. E and Z isomers of 1,2 - bis (tetrasubstituted benzylidene) hydrazine derivatives (**3**, **4** and **6**).

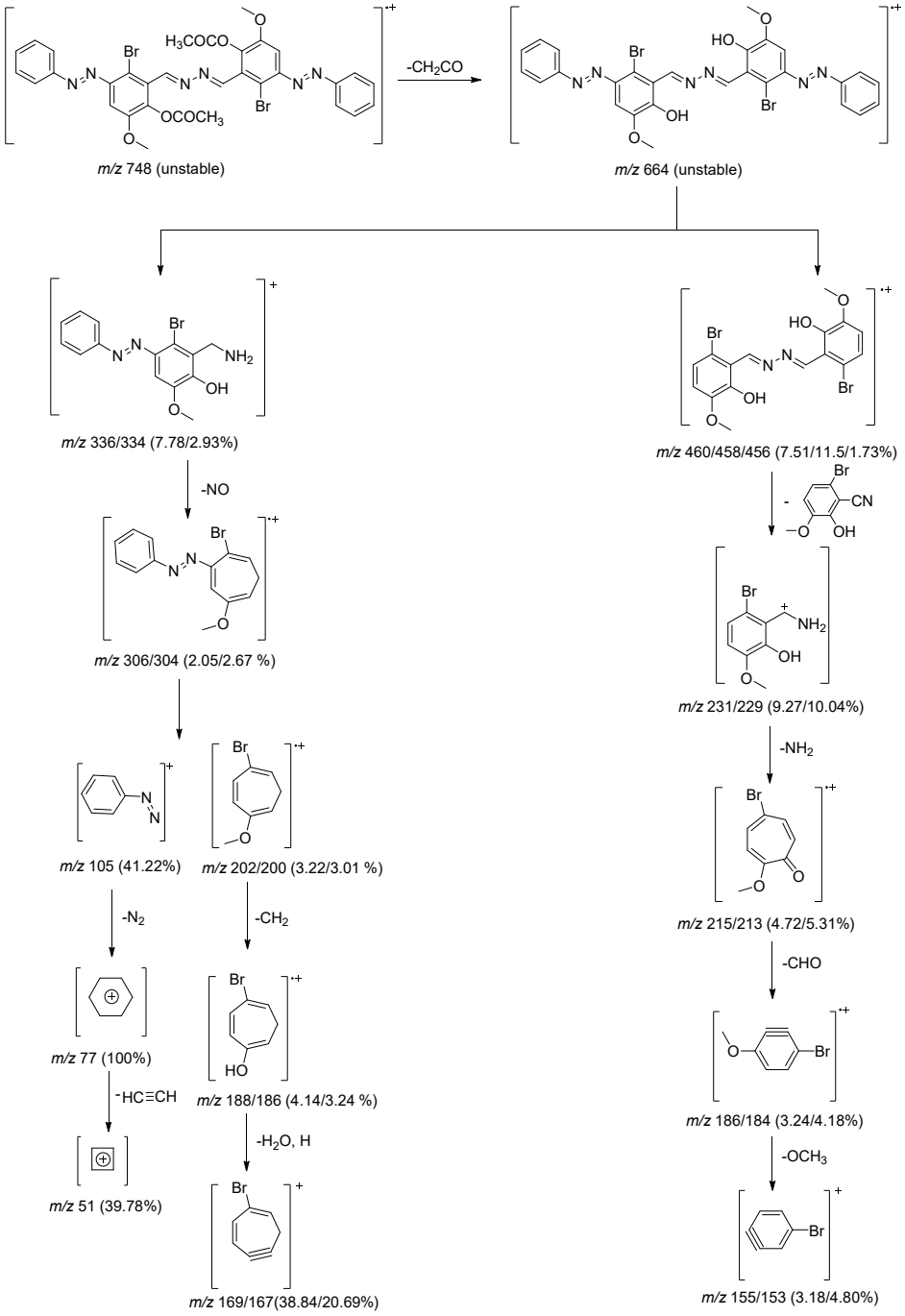
From the study of the ^{1}H-NMR spectrum of compounds **3** showed three singlet signals, at δ 10.90, 8.99 and 3.84 ppm due to the protons of hydroxyl (OH, azomethine (CH=N) and methoxy (OCH_3) groups of the *E*-isomer, while the protons of these groups of *Z*-isomer appeared at δ 12.47, 9.12 and 3.96 ppm. From the calculation of proton integration of the aromatic protons was confirmed the presented that of compound **3** in the *E* and *Z* isomers. Also, the ^{1}H NMR spectrum of compound (**6a**) exhibited three singlet signals at δ 10.46, 9.12 and 3.84 ppm refer to the hydroxyl (OH), azomethine CH=N and methoxy groups of *E*-isomers. While the protons of these groups in Z-isomer appeared at δ 12.09, 9.12 and 3. 92 ppm**.** The ^{1}H-NMR spectrum of compound **6b** displayed five singlet signals at δ 12.17 (OH), 9.22, 9.06 (2 × CH=N), 3.84 (OCH_3) and 3.19 (CH_3) of the *E*-isomer of this compound, while the *Z*- isomer of compound **6b**, the ^{1}H NMR spectrum showed five signals at δ 12. 10 (OH), 8.91, 8.90 (2 × CH=N), 3.84 (OCH_3) and 2.30 (CH_3) ppm. The ^{13}C-NMR spectrum of compound **3** gave eight carbon signals of two isomers at δ 163.41 (2 × C=N), 149.00, 148.46 (4 × C-O), 122.56, 119.78, 118.83, 115.83 (8 aromatic carbons) and 56.42 (2 × OCH_3) ppm. The ^{1}H-NMR spectrum of compound **5** showed the two singlet signals at δ 2.97 and 2.31 ppm refer to the acetoxy (O $COCH_3$) group of *E* and *Z*-isomers. Also, in the ^{1}H NMR spectrum of compound **5** exhibited four singlet signals at δ 9.22, 9.06, 8.90 and 8.69 ppm due to the proton of azomethine (CH=N) of *E* and *Z*-isomers. In the case of compound **7**, the ^{1}HNMR spectrum showed the singlet signal of azomethine at δ 10.28 and singlet signal at δ 1.86 ppm of acetoxy group of *E*-isomer.

*Mass spectra of compounds **5**, **6** and **7***

The mass spectral decomposition modes of the prepared, 1,2-bis (tetra substituted benzylidene) hydrazines have been investigated. The mass spectrum of compound **5** showed unstable molecular ion peak at m/z 540, corresponding to the molecular formula $C_{20}H_{18}Br_2N_2O_6$. The molecular ion peak of compound **5** (Scheme 3) underwent fragmentation with rearrangement to produce peak at m/z 498 by losing ketene (CH_2CO) molecule. The loss of ketene molecule from the ion at m/z 498 resulted in an ion at m/z 456 referring to the molecule ion peak of compound **4**, corresponding to the molecular formula $C_{16}H_{14}Br_2N_2O_4$ for compound **4**. The ion at m/z 456 was broken to give



Scheme 3. Main fragmentation pathway of compound **5**.



Scheme 4. Main fragmentation pathway of compounds **6** and **7**.

ion peak at m/z 228. The ion at m/z 228 underwent loss of NH and formyl (CHO) groups to give peaks at m/z 213 and m/z 184, respectively. Also, the ion at m/z 498 underwent fragmentation with rearrangement to produce stable ion peak at m/z 298. The base ion peak at m/z 298 underwent loss of ketene (CH_3CO) molecule, CH_2=NH and hydrogen cyanide (HCN) to give peaks at m/z 256, 227 and 200, respectively.

From the mass spectra of compounds **6a** and **7**, it was concluded that the molecular ions at m/z 664 and 748 are unstable. The molecular peak at m/z 748 (unstable) of compound **7** was broken by loss two molecule of ketene (CH_2CO) to give molecular ion peak m/z 664 (unstable) of compound **6a**. The ion m/z 664 underwent fragmentation to produce peak at m/z 334. The ion of m/z 334 broke to give an ion at m/z 304 which lost NO. Ion of m/z 304 fragmented to produce an ion of m/z loss. The ion at m/z 105 underwent loss of nitrogen molecule (N_2) to give stable ion peak at m/z 77. Also, the ion of m/z 304, which further broke to give an ion at m/z 200. Ion of m/z 200 fragmented to give peaks at m/z 186 and 167, which lost a methylene (CH_2) and hydronium ion (H_3O^+). The ion at m/z 664 underwent fragmentation to produce ion peak at m/z 456, corresponding to the molecular ion peak of compound **4**. The ion of m/z 456 underwent fragmentation to produce the peak at m/z 229 by losing 6-methoxy-3-bromo-2-cyanophenol. It further underwent loss of amino (NH_2), formyl (CHO) and methoxy (OCH_3) groups to give peaks at m/z 213, 184 and m/z 153, respectively (Scheme 4).

Cytotoxicity screening

In *vitro* cytotoxicity of the synthesized compounds **4**–**7** was identified by MTT assay utilizing human breast (MCF-7) cancer cell lines. Doxorubicin (Doxo) was utilized as a standard drug; the results are shown in Table 1. In agreement with the MTT results, compound **5** was discovered to be the most effective compound with IC_{50} value 30.64 µg/mL, in MCF-7 cell line. Inspected against normal breast cell (MCF-10A) with IC_{50} value 22.09, was the most effective compound **5** and the result showing that the compound was safe for normal cell.

Table 1. IC_{50} values of tested 1,2-bis (tetrasubstituted benzylidene) hydrazines (**4**-**7**) against MCF-7 cancer cell lines.

Compound No.	IC_{50} values (µg/mL) MCF-7 cell lines
4	80.29 ±0.73
5	30.64 ±1.81
6a	43.19 ±1.71
6b	80.14 ±0.37
7	86.49 ±0.84
Doxorubicin	3.83 ±0.42

*Aromatase activity assay of compound **5**.*

Aromatase (CYP19A, Ec 1.14.14.15) is a member of the cytochrome P450 monoxides (CYP) family of microsomal xenobiotic metabolism enzymes. Aromatase plays a critical role in steroidogenesis, catalyzing the conversion of androgenic hormones, into estrogens. Inhibitors of aromatase are used to treat estrogen dependent breast cancer, as estrogens promote the expression of peptide growth factors responsible for tumorigenesis. The inhibitory activity of the compound **5** was at different concentrations (100, 10, 1, 0.1 and 0.01 µg/mL) against aromatase assay ketoconazole is used as standard drug for comparison purposes. The results are listed in Table 2. It was found that the compound **5** showed good activity with IC_{50} value 0.276 µg/mL, compared with IC_{50} value of standard (0.062. µg/mL) (Figure 1).

Table 2. Aromatase assay of compound **5** against MCF.7 cell lines.

Compound No.	K-Activity					IC_{50} value (µg/ mL)
	100	10	1	0.1	0.01	
5	8.55	21.01	38.98	70.33	97.54	0.276±0.012
Ketoconazole	7.33	14.49	21.58	57.67	82.24	0.06±0.003

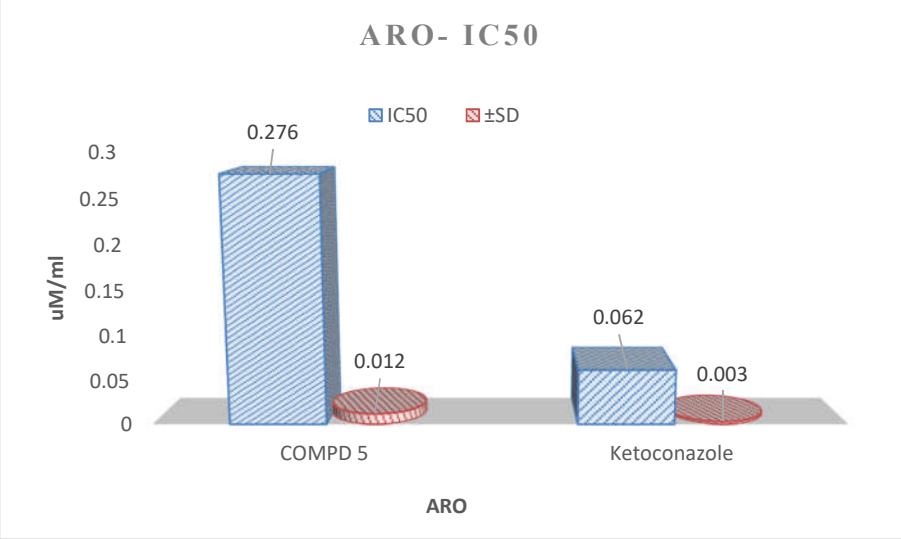


Figure 1. In vitro inhibition percentage of aromatase for compound **5** at its IC_{50} compared to the control inhibition ketoconazole.

*Tyrosinase inhibitor screening of compound **5***

Tyrosinase of polyphenol oxidase (EC 1.14.18.1), is an oxidoreductase that participate in the biosynthesis of melanin, as ubiquitous biological pigment found in hair, eyes, skin, etc. The inhibition of tyrosinase has been a long-time target in skin health research, cosmetics, and agricultural industries because of its role in browning reactions in skin pigmentation and during fruit harvesting and handing. Skin whitening and bleaching products utilize natural synthetic tyrosinase inhibitors to lighten the skin color. Polyphenols, benzaldehyde derivatives, long-chain lipids, steroids, and natural compounds have been used as tyrosinase inhibitors. Tyrosinase catalyzes the oxidation of tyrosine producing a chromophore that can be detected at 310 nm. The tyrosinase inhibitor screening of compound **5** was measured at different concentrations (100, 10, 1, 0.1 and 0.01 µg/mL). Kojic acid is used as a stand and reference for comparison purposes. The results are reported in Table 3. The tyrosinase inhibitor activity of compound **5** was found good activity with IC_{50} value 28.33 µg/mL of these compound compared with IC_{50} value 8.76 (µg/mL) of Kojic acid standard reference (Figure 2).

Table 3. Tyrosinase inhibitor activity of compound **5**.

Compound No.	K-Activity					IC_{50} value (µg/ mL)
	100	10	1	0.1	0.01	
5	19.64	41.89	79.74	98.44	115.82	28.3±1.61
Kojic acid	11.94	23.30	52.24	93.74	111.05	8.76±0.45

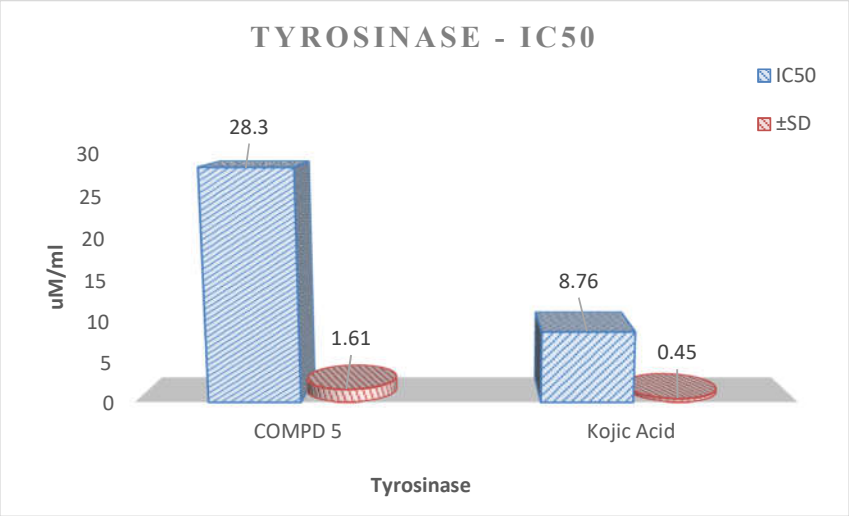


Figure 2. In vitro inhibition percentage of Tyrosinase for compound **5** at its IC50 compared to the control inhibition kojic acid.

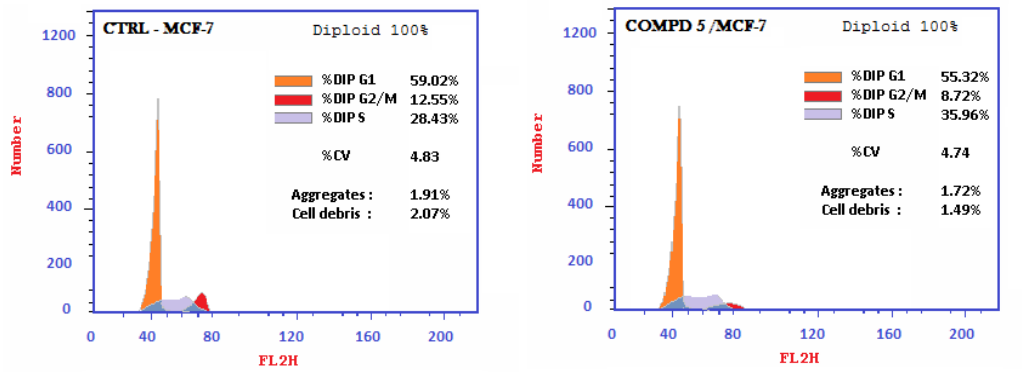
Cell cycle analysis

The cell cycle distribution was determined by DNA flow cytometric analysis. MCF-7 cells were treated with compound **5**, increased the content in GO/G1 from 59.02 to 55.32%. And S-phase from 28.43 to 35.96%. Meanwhile, the DNA content in G2/M phase decreased from 8.72 to 12.55% in the control cells. Additionally, exposure of MCF-7 cells to the investigated compounds significantly increased the cells percentage at the pre-G1 phase. These outcomes confirmed that compound **5** caused a distinct G1/S phase arrest pattern, which could be attributed to the DNA fragmentation (Figure 3).

Annexin V-FITC/PI screening

The synthesized compound **5** had a probable cytotoxicity effect on MCF-7 cell propagation. The capability of the target compound to persuade apoptosis in MCF-7 cells was detected using an exact assay, Annexin - V-FITC/PI apoptosis/necrosis method was utilized to in-depth explanate the killing and/or inhibition effect of this compound on the cancer cells growth. As the results in Figure 4, revealed that the increased the apoptosis percentages at the total stage from 1.88 to 33.19% and the early stage from 0.43 to 21.16 compared to the control one. Furthermore, the apoptosis percentage at the late phase amended from 0.18 to 9.41% compared to the control. Therefore, the examined compound **5** could be considered as a significant inducer of apoptosis in the MCF-7 Cell line.

A



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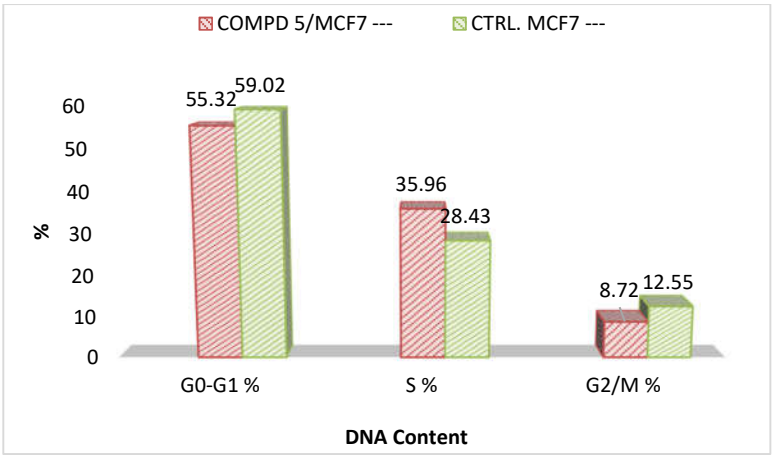

Figure 3. **A**: Cell cycle distribution after compound **5** treatment against MCF-7 cell lines at its IC50 (μM). **B**: Graphical representation of cell cycle analysis profile of compound **5** against MCF-7 cell lines.

Molecular docking study

Molecular docking is a vital toll in drug design [14, 15] to get a better understanding of the above-described activity relationships, we performed docking simulations of the complexes active compound **5** against breast cancer protein (PDB: 3HB5). The docking results showed a potential structure activity relationship between compound **5** against 3HB5 protein as shown in Table 4 and Figure 5. Compound **5** showed the highest binding interaction against the k amino acids of the 3HB5 with docking scores -0.3, -1.8 kcal mol^{-1} and distance 3.58, 3.01. Compound **5** exhibited a potential interaction toward 3HB5 receptor via the H- donor and H-acceptor, which compatible with cytotoxicity and biological results.

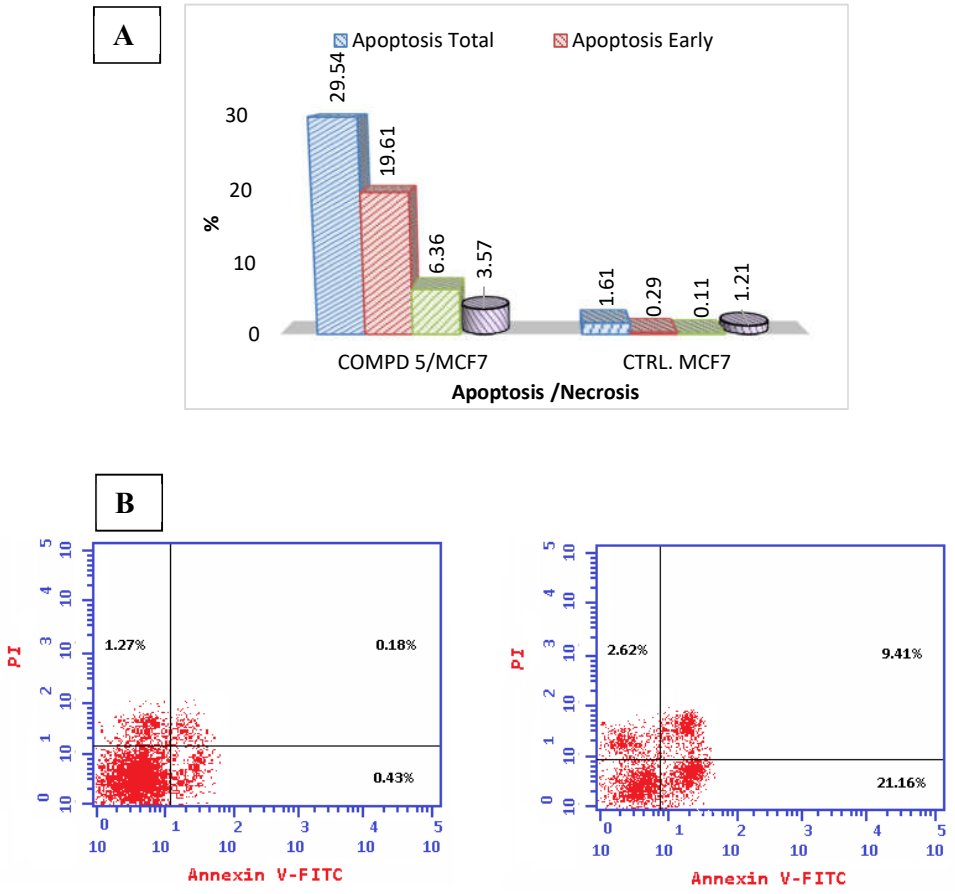

Figure 4. **A**: The percentage of apoptosis and necrosis caused by compound **5** using MCF-7 cell lines. **B**: Representative dot plots of MCF-7 cells treated with compounds **5** and at its IC50 for 24 h analyzed using flow cytometer after double staining of the cells by annexin V-FITC and PI.

Table 4. Docking interactions of compound **5** into human breast cancer (3HB5).

Compound	S (kcal $mol^{-1)}$	Ligand			Amino acid	Type of interaction	Distance
	-0.3	Br-9			Ala-1	H-donor	3.85
		-1.8	N-15	Thr-3	H-acceptor		3.01

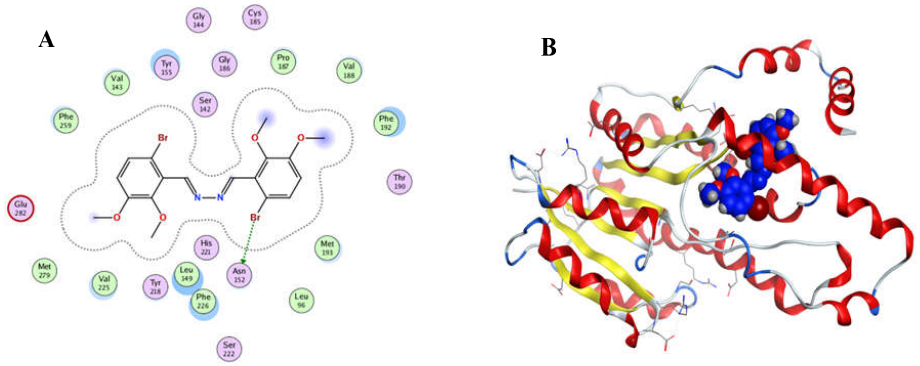

Figure 5. Interactive conformations (2D) and binding mode (3D) of compound **5** in the active binding breast cancer protein (3HB5).

EXPERIMENTAL

^{1}H NMR spectra and ^{13}C NMR spectra were recorded on Bruker spectrometers AVANCE 400 MH_2 and 100 MH_2. The Chemical shifts are referenced to solvent signals (CD Cl_3: δ ^{1}H = 7.26, δ ^{13}C = 77.0 and DMSO: δ ^{1}H = 2.50, δ ^{13}C = 39.7). Mass spectra were recorded on a Finnigan MAT 95 XP (Thermo Election Corporations). Ionization was performed by electron impact (70 eV). Infrared spectra were obtained on an Avatar Series FTIR spectrophotometer as KBr disks (Thermo Nicolet, Waltham, MA, USA). Melting points were measured using a Mel-Temp melting point apparatus and are uncorrected. Satisfactory elemental analysis (0.30 of the calculated values) was obtained for the synthesized compounds using a Perkin-Elmer PE 2400 CHN analyzer. Commercially available reagents were purchased from Fluka, Aldrich and Alfa Aesar and purified it necessary.

Synthesis

*Synthesis of ethyl 8-methoxy coumarin-3-carboxylate (**1**).* Ethyl 8-methoxy coumarin-3-carboxylate (**1**) was obtained via condensation of 3-methoxy-2-hydroxybenzaldehyde with diethyl malonate in the presence of base catalyst, as colorless crystals, yield 85% m.p. 98 °C. IR (KBr) ν_{max} = 1739, 1725 (C=O), 1605, 1583 (C = C), 1083, 1032 (C-O) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.32 (t, 3H J = 7.2 Hz, CH_3), 3.92 (s, 3H, OCH_3), 4.32 (q, 2H, J = 8.8 Hz, OCH_2), 7.30-7.45 (M, 3H), 8.71 (s, 1H, H-4 of coumarin ring) ppm. ^{13}C-NMR (DMSO-d_6) δ: 164.45, 163.04 (2 × C=0), 157.15, 154.41 (2 × C-O), 149.14 (C-4 of coumarin ring), 134.95, 130.75, 125.30, 118.76, 118.24, 116.60 (C-aromatic, 4 carbon and C-3 of coumarin ring), 61.72 (OCH_2), 56.60 (OCH_3), 14.55 (CH_3) ppm.

Synthesis of ethyl 5-bromo-8-methoxy coumarin-3-carboxylate. A solution of compound (0.01 mol) in 20 mL glacial acetic acid, then 10 mL of bromine (0.01 mol) glacial acetic acid was added dropwise to compound **1** with stirring at 50-60 °C. After 5-10 min, the bromine color was discharged, and yellow solution remained. At this point, add 0.5-1 mL of bromine-AcOH solution while stirring at room temperature for 1.5-2 h. The reaction mixture was poured into water with stirring, the resulting solid was collected via filtration, washed with water, dried and crystallized from ethanol to give compound **2** as pale-yellow crystals, yield 76%, m.p. 155 °C. IR (KBr) ν_{max} = 1745, 1721 (C=O), 1610, 1588 (C = C), 1091, 1036 (C-O) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.33

(t, 3H, J = 7.20 Hz, CH_3), 3.93 (s, 3H, OCH_3), 4.32 (q, 2H, J= 8.8 Hz, OCH_2) 7.365-7.387 (d, 1H, J = 8.8 Hz, Ar-H), 7.637-7.89 (d, 1H, J = 8.8 Hz, Ar-H), 8.58 (s, 1H, H-4 of coumarin ring) ppm. MS/m/z (%) = 328 (M^{+2}, 67.03), 326 (M, 100), 300 (6.81), 299 (6.31), 283 (21.40), 281(8.52), 255 (11.30), 254 (18.87), 248 (8.14), 247 (1.11), 219 (7.18), 181 (7.60), 156 (6.39), 119 (9.14), 105 (9.30), 104 (25.72). Anal. calcd. for $C_{13}H_{11}BrO_3$ (Mwt: 326); C, 47.85; H, 3.77. Found: C, 47.61, H, 3.22.

*Synthesis of 1,2 -bis (6-substituted -3-methoxy- hydroxy benzylidene) hydrazines (**3** and **4**)*

To a solution of ester coumarin derivatives (1 and 2, 0.01 mol) in ethanol (30 mL) was added hydrazine hydrate (0.02 mol). The reaction mixture was heated under reflux for 4 h, then cooled, poured into water, and neutralized with diluted hydrochloric acid (2%). The resulting precipitate was separated by filtration, washed with water, dehydrated any recrystallized from ethanol to give compound **3** and **4**.

*1,2-Bis-(3-methoxy-2-hydroxy benzylidene) hydrazines (**3**)* as yellow crystals, yield 68%, m.p. 225 C. IR (KBr) ν_{max} = 3351 (br-OH), 1635 (C=N), 1605, 1591 (C = C), 1121, 1063 (C-O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.84 (s, 6H, 2 × OCH_3), 6.903-6.94 (t, 2H, J = 8.0 Hz, 2 × Ar-H), 7.127-7.144 (d, 2H, J = 6.8 Hz, 2 × Ar-H), 7.287-7.304 (d, 2H, J = 6.8 Hz, 2 × Ar-H) 8.99 (s, 2H, 2 × CH=N) (*E*-isomer, 62.15%) ppm. ^1H NMR (DMSO-d_6) δ: 3.95 (s, 6H, 2 × OCH_3), 7.062-7.084 (d, 2H, J = 8.8 Hz, 2 × Ar-H), 7.196-7.218 (d, 2H, J = 8.8 Hz, 2 × Ar-H), 7.361-7.407 (t, 2H, J = 8.2 Hz, 2 × Ar-H), 9.06 (s, 1H, CH=N), 9.126 (s, 1H, CH=N) (*Z*-isomer, 37.85%) ppm. ^{13}C NMR (DMSO-d_6) δ: 163.23 (2 × C=N), 149.00, 148.46 (4 × C-O), 132.56, 119.78, 118.83, 115.83 (2 × 4 carbon aromatic), 56.40 (2 × OCH_3) (*E*- isomer) ppm. ^{13}C NMR (DMSO-d_6) δ: 164.42, 163.41 (2 × C=N), 151.49 (2 × C-O), 130.41 (2 × C-O), 123.83, 123.65, 122.82, 121.73, 119.88, 115.63, 115,33 (C- aromatic), 56.410 (2 × OCH_3), (*Z*-isomer) ppm. Anal. calcd. for $C_{14}H_{16}N_2O_4$ (Mwt = 276): C, 60.87; H, 5.79; N, 10.14. Found: C, 60.60; H, 5.51; N, 10.01.

*1,2-Bis-(6-bromo-3-methoxy-2-hydroxybenzylidene) hydrazine (**4**)* as pale-yellow crystals, yield 72% m.p. 26 C. IR (KBr) ν_{max} = 3392 (br.OH), 1633 (C=N), 1608, 1592 (C=C), 1117, 1063, 1021 (C-O) cm^{-1}. NMR (DMSO-d_6) insoluble. Anal. calcd. for $C_{16}H_{14}Br_2N_2O_4$ (Nwt = 456): C, 42.11, H; 3.07; N, 6.14. Found: C, 42.01, H, 2.46; N, 6.04.

*3.1.4 Synthesis of 1,2-bis (6-bromo-5-substituted-3-methoxy-2-hydroxybenzylidene) hydrazines (**6a, b**)*

A solution of compound **4** (0.01 mol) in aqueous sodium hydroxide (15 mL, 10%) was chilled in ice 0-5 °C. A cold aqueous solution of aryl diazonium salt at 0-5 °C (namely, phenyl diazonium chloride and 4-methylphenyl diazonium chloride, 0.02 mol) was added dropwise with stirring during 15 min. After addition, the reaction mixture was stirred for further 30 min, and then left for 2 h in a refrigerator. The precipitate product was collected via filtration, washed with water, dried and purified by crystallization with ethanol to give **6.**

*1,2-Bis (6-bromo-5-phenyl diazinyl-3-methoxy-2-hydroxy-benzylidene) hydrazine (**6a**)* as red powder, yield 83%, m.p. 268 °C. IR (KBr) ν_{max} = 3360-3320 (br-OH), 1633 (C=N), 1605, 1593 (C=C), 1063, 1033 (C-O) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.84 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 7.37-7.96 (m, 12H, Ar-H), 9.12 (s, 2H, 2 × CH=N), 10.46 (s, 1H, OH), 12.09 (s, IH, OH) ppm. MS: m/z (%) = 664 (M^+, unstable), 337 (1.74), 336 (8.45), 335 (7.22), 334 (11.96), 333 (2.66), 332 (2.42), 300 (1.28), 299 (1.18), 298 (1.05), 259 (3.24), 258 (2.00), 257 (3.50), 256 (1.33), 245 (2.35), 244 (1.44), 242 (1.08), 232 (1.04) 232 (4.83), 231 (7.10), 230 (2.22), 229 (13.13), 228 (3.42), 227 (2.78), 226 (2.27), 225 (1.32), 214 (4.82), 213 (2.03), 212 (1.34), 202 (1.47), 200

(1.59), 188 (2.72), 187 (1.64), 186 (2.49), 185 (1.56), 184 (2.80), 183 (1.45), 169 (4.51), 168 (5.95), 167 (4.13), 166 (2.25), 158 (1.70) 157 (1.18), 156 (1.63), 155 (2.76) 154 (1.66), 153 (4.84) 152 (5.28), 151(2.31), 150 (2.11), 141 (1.66), 139 (3.43), 138 (1.21), 129 (1.48), 128 (3.91), 127(3.99), 126 (2.52), 119 (1.31), 118 (1.02), 107 (4.87), 106 (2.69), 105 (14.44), 96 (4.83), 95(2.27), 94 (7.35), 93 (5.66), 92 (3.42), 91 (2.00), 79 (15.55), 78 (12.14), 77 (100), 76 (10.29), 75 (5.48), 65 (4.55), 64 (3.63), 63 (6.10), 51(30.91), 50 (10.68). Anal. calcd. for $C_{28}H_{22}Br_2N_6O_4$ (Mwt = 664): C, 50.60, H, 3.31 N, 12.65. Found: C, 50.38; H, 3.23; N, 12.42.

*1,2-Bis-(6-bromo-5-(4-methylphenyl) diazinyl-3-methoxy-2-hydroxy benzylidene) hydrazine (**6b**)* as orange powder, yield 76%, m.p. 258 °C. IR (KBr) ν_{max} = 3395-3342 (br. OH), 1636 (C=N), 1605, 1582 (C=C), 1068, 1023 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ = 2.30 (s, 3H CH_3), 2.319 (s, 3H CH_3), 3.84 (s, 6H, 2 × OCH_3), 7.109-7.73 (m, 10H, Ar-H) 8.69 (s, 1H, CH=N) 8.91(s, 1H, CH=N), 9.06 (s, 1H, CH=N), 9.22 (s, 1H, CH=N), 12.10 (s, 1H, OH), 12.178 (s, 1H, OH), ^{13}C NMR (DMSO- d_6) δ: 165.83, 162.86 (C=N), 152.31, 150.46, 148: 48, (C-O and C-N), 131.61, 124.21, 123.50, 119.63, 118.50 (C-aromatic), 56.58 (2 × OCH_3), 20.92 (2 × CH_3) ppm. MS: m/z (%) = 692 (M^2, unstable), 490 (10.07), 487 (1.47), 482 (3.93), 480 (4.23) 476 (1.26), 477 (3.82) 451 (3.32) 450 (1.75), 439 (2.48), 427 (3.34) 423 (4.09) 421 (5.95), 400 (2.43), 399 (4.05) 398 (3.32), 385 (3.45), 380 (3.89), 368 (3.48), 366 (2.51), 349 (2.74), 346 (7.57), 339 (2.34) 337 (3.70), 335 (4.71), 334 (2.31), 329 (2.79), 326 (7.82), 322 (4.12), 320 (2.18), 315 (2.19), 309 (4.17), 301 (5.42), 299 (17.76), 294 (4.56), 283 (5.16), 276 (3.04), 275 (2.92), 274 (3.03), 272 (5.93) 269 (4.70), 267 (3.36), 266 (3.05) 265 (4.92), 256 (8.18), 255 (6.21) 254 (4.18) 252 (3.27), 245 (3.64), 242 (2.78), 740 (2.91), 233 (2.41), 232 (3.57), 198 (3.34) 197 (3.06), 190 (10.12), 187 (3.4), 174 (2.46), 172 (3.49), 169 (4.45), 168 (2.77), 167 (10.56), 160 (4.8), 159 (4.90), 158 (4,07), 156 (4.20), 154 (5.20), 152 (11.36) 150 (14.02), 146 (4.87), 144 (15.34), 135 (9.16), 134 (10.12), 133 (19.01) 131 (5.67), 124 (10.58), 122 (34.84), 121 (7.46), 120 (5.73), 119 (10.74) 117 (5.24), 115 (5.67), 113 (12.28) 110 (8.97), 109 (20.39), 108 (84.57) 107 (66.57) 106 (100%) 105 (29.71) 104 (3.28) 95 (24.12), 94 (10.21), 93 (28.33), 92 (23.49) 91 (23.49), 90 (5.87), 87 (21.87), 86 (16.93), 85 (14.93), 83 (46.91), 82 (22.44), 81 (25.09), 80 (61.15), 79 (75.97), 78 (66.71), 77 (60.88), 69 (27.62) 67 (23.24), 66 (32.83) 65 (52.46), 63 (61.53), 62 (31.55), 55 (43.67), 52 (74.58), 51(29.45). Anal. calcd. for $C_{30}H_{26}Br_2N_6O_4$ (Mwt = 692): C, 52.02; H, 3.76; N, 12.14. Found: C, 51.87; H, 3.53; N, 12.01.

*General procedure for acetylation reactions. Formation of acetoxy derivatives (**5** and **7**)*

A solution of compound **4** and 6 (0.01 mol in 20 mL acetic anhydride) was refluxed for 2 h, then cooled and poured into iced water with stirring. The reaction mixture was left 24 h, and the solid formed was isolated by filtration, cleaned with water, and dehydrated. Finally, the crude product crystallized from a proper solvent to provide **5** and **7**.

*1,2-Bis (2-acetoxy -3-methoxy-6-bromobenzylidene) hydrazine (**5**)* as pale-yellow crystals, yield 69% m.p. 168 °C. IR (KBr) ν max = 1758 (C=O), 1635 (C=N), 1605, 1583 (C=C), 1126, 1058 (C-O) cm^{-1}. ^{1}H NMR (DMSO- d_6) δ: 2.29 (s, 3H, $COCH_3$), 2.31 (s, 3H, $COCH_3$), 3.844 (s, 3H, OCH_3), 3.849 (s, 3H, OCH_3), 7.101 7.09 (dd, 8H, Ar-H of two isomers), 8.69 (s, 1H, CH=N), 8.90 (s, 1H, CH=N), 9.065 (s, 1H, CH=N), 9.22 (s, 1H, CH=N) (of two isomers) ppm. ^{13}C NMR (DMSO-d_6) δ: 168.58 (CO), 161.63, 159.32 (2 × C=N), 153.20, 148.30, 147.19 (C-O), 131.61, 131.48, 123.20, 122.51, 118.30 (C- aromatic), 56.92 (OCH_3), 56.62 (OCH_3), 20.90 (2 × $COCH_3$) ppm. MS: m/z (%) = 540 (M^+, unstable), 450 (24.73), 499 (3.28), 498 (15.58), 485 (1.95), 483 (2.51), 461 (12.58), 460 (25.65), 459 (10.57), 4.58 (55.12), 457 (4.33), 456 (22.70), 449 (1.29), 448 (3.47), 443 (5.92), 442 (1.36), 441 (68.39), 440 (3.23), 439 (3.81), 422 (12.02), 421 (19.19), 420 (12.69) 419 (18.45), 418(5.34), 411(2.09), 410 (2.96), 409 (1·11), 380 (21.95), 379 (59.98), 378 (27.00), 377 (68.57), 364 (25.76), 363 (6.87), 362 (5.31), 361 (8.57), 349 (3.56), 348 (3.33), 347(3.47), 345 (4.03), 342 (2.87), 341 (9.43) 340 (2.99), 337 (6.42), 336 (23.87), 335 (6.97), 334

(22.70) 326 (4.33), 318 (3.20), 317 (4.02), 301 (15.05), 300 (80.87), 299 (77.75), 298 (100), 297 (43.03), 284 (24.19), 283 (45.64) 282 (14.01), 281 (27.07), 280 (63.09), 279 (11.97), 270 (9.58), 269 (10.96), 268 (10.37), 267 (19.23), 266 (9.12), 265 (11.95), 264 (7.45), 258 (10.66), 257 (16.91), 256 (21.81), 255 (31.91), 254 (16.43), 253 (13.05), 251 (10.75), 242 (8.99), 241 (10.80), 240 (13.59), 239 (15.02), 238 (10.16), 237 (13.06), 736 (7.56), 232 (14.65), 231 (29.88), 230 (66.72), 229 (49.24), 228 (68.56), 227 (28.40), 226 (19.03), 225 (13.31), 217 (12.55), 216 (14.68), 215 (78.84), 214 (33.51), 213 (88.14), 212 (28.63), 211 (16.89), 203 (11.87), 202 (23.77), 201 (19.36), 200 (24.24), 199 (18.24) 198 (10.56), 197 (14.19), 188 (33.56), 187 (14.48), 186 (45.45), 185 (21.28), 184 (28.48), 183 (11.37), 177 (16.94), 175 (11.69), 171 (10.66) 170 (12.02), 169 (12.49), 168 (16.76), 167 (10.71) 160 (12.89), 159 (18.40), 158 (19.14), 157 (15.21), 156 (12.05), 153 (20.63), 152 (25.44), 151 (13.15), 150 (63.07), 149 (34.57), 148 (13.22), 145 (12.04), 143 (14.89), 137 (19.25), 136 (16.03). 135 (57.60), 134 (20.22), 133 (16.99), 129 (19.56), 127 (14.35), 123 (20.02), 122 (26.02), 121 (30.88) 120 (27.24), 119 (17.01), 111 (15.86), 110 (8.63), 109 (15.73), 108 (27.49), 107 (36.48), 106 (47.45), 105 (41.15), 104 (13.53), 98 (13.60), 97 (21.70), 96 (17.42), 95 (25.98), 94 (12.24), 93 (26.01), 92 (17.60), 91 (30.79), 85 (16.58), 84 (18.84), 83 (28.97), 82 (14.28), 81 (30.19), 79 (54.14) 78 (27.33), 77 (76.92), 76 (12.71), 71 (20.43), 69 (50.15), 67 (24.15), 65 (21.73), 57 (31.62), 51 (19.22). Anal Calced for C_{20} H_{18} Br_2 N_2 O_6 (Mwt = 540): C 44.44; H, 3.33; N; 5.16. Found: C, 44.11; H, 3.11; N, 5.02.

1,2-Bis (2-acetoxy-3-methoxy-5-(phenyl) diazinyl-6-bromo benzylidene) hydrazine (7) as pale red powder, yield 76%, m.p. 198 °C. IR (KBr) ν_{max} = 1753 (C=O), 16.31 (C=N), 1610, 1591(C=C), 1086, 1031 (C-O). ^{1}H NMR (DMSO-d_6) δ: 1.86 (s, 6H, 2 × $COCH_3$), 3.82 (s, 6H, 2 × OCH_3), 7.268-7.999 (m, 12H, Ar-H), 10.28 (s, 2H, 2 × CH=N), ppm. ^{13}C NMR (DMSO-d6) δ: 173.42 (C=O), 166.31, 165.93 (2 × C=N), 153.86, 153.21 (2 × C-O), 143.87 (2 × C=N), 136.21, 129.99, 129.63, 129.50, 123.06, 122.26, 122.09, 121.03, 101.32 (C-aromatic), 55.41 (2 × OCH3), 22.33 (2 × COCH3) ppm. MS: m/z (%) = 748 (M^+, unstable), 460 (2.51), 459 (1.36), 458 (1.65), 457 (1.35), 476 (1.73), 410 (1.39) 409 (1.08), 396 (1.18), 395 (1.22), 394 (1.29), 379 (1.18), 378 (2.35), 377 (3.67), 360 (1.61), 358 (1.10), 337 (1.94), 336 (7.79), 335 (3.31), 334 (12.93), 333 (25.4), 321 (2.66) 306 (2.05), 305 (1.25), 304 (2.60), 303 (1.83), 287 (1.87), 286 (2.58), 259 (2.22), 258 (1.14), 257 (3.41), 256 (1.72), 255 (3.60), 246 (3.00), 245 (17.50), 244 (8.24), 243 (3.30), 231 (9.27), 230 (6.81), 229 (10.04), 228 (6.95), 216 (3.42), 215 (4.72), 214 (5.47), 213 (5.31), 212 (3.38), 203 (2.82), 202 (3.22), 201 (3.28), 200 (3.01), 199 (1.90), 188 (4.14), 187 (2.32) 186 (3.24), 184 (4.18), 183 (2.26), 170 (5.54), 169 (38.84), 168 25.49), 167 (20.96), 166 (4.17), 157 (2.56), 156 (2.01), 155 (3,18), 154 (3.58), 153 (4.80) 152 (7.63), 151 (3.78), 149 (5.13), 141 (3.11), 140 (3.84), 139 (10.35), 129 (2.02), 128 (5.27), 127 (4.09), 122 (3.19), 119 (2.48), 118 (2.95), 117 (2.47), 116 (2.56), 107 (5.21), 106 (7.67), 105 (41.22), 104 (3.82), 103 (4.10), 94 (8.86) 93 (14.82), 92 (63.6), 91 (7.62), 89 (3.04), 79 (19.07), 78 (17.95), 77 (100), 76 (14.67), 75 (7.03), 66 (9.16), 65 (8.66) 63 (8.98), 62 (4.21), 60 (10.12), 51(39.78), 50 (6.93). Anal. calcd. for $C_{32}H_{26}Br_2N_6O_6$ (Mwt = 748): C, 51.33; H, 3.47; N, 11.23. Found: C, 51.11; H, 3.23; N, 11.02.

Biology assay

In vitro cytotoxicity screening

By using MTT assay, the cytotoxicity of the 1,2-bis (tetrasubstituted benzylidene) hydrazines (**4-7**) was screened against human breast cancer cell lines (MCF-7). All cell lines were supplemented with 10% fetal bovine serum and maintained in culture flasks in an atmosphere of 5% CO_2. Cell lines were incubated in 96, well plates (1×10^4 cells/well) and allowed to attach for 24 h before treating. Different concentrations of each compound (0.01, 0.1, 1, 10 and 100 µg/mL) were added to wells containing cell lines. Cells were incubated with compounds at 37 ºC for 48 h. After 48 h,

cells were fixed, washed, and stained with MTT stain. The color intensity was measured at 570 min in an ELISA reader [16].

*Aromatase inhibitor assay of compound **5***

In vitro aromatase inhibition assay was performed for the investigated compound **5**. Aromatase inhibition was measured by quantifying the fluorescent intensity of fluorescein standard using the Aromatase (CYP19A). Activity assay kit (Biovision) according to the manufacturer protocol. In brief, the tested compound **5** was pre-incubated with reaction mixture of the enzyme and substrate for 6 min at 37 °C, then fluorescence was measured by TECAN fluorescence spectrophotometer at 488 nm (excitation) and 527 nm emission). IC_{50} values were calculated using graph padprism 8 analysis software (Graph-Pad, San Dego, CA, USA) from the dose-response curves of 5 concentrations of each test compound. Serial dilutions from exemestane, as reference standard [17].

*Tyrosinase inhibitor screening of compound **5***

Using tyrosinase inhibitor screening kit (K 575 = 100 assays) dissolve the compound **5** in ethanol, dilute to 5× the desired test concentration with tyrosinase assay buffer use, add 20 µL diluted test inhibitor, inhibitor control working solution, of tyrosinase assay buffer into wells assigned as a test inhibitor (sample, S), inhibitor control (IC) of tyrosinase enzyme control (EC) wells, respectively. Additional wells with serial dilutions of the test inhibitors may be prepared at this time if desired, containing 20 µL in each candidate well. For each well, prepare 50 µL tyrosinase enzyme solution (48 µL tyrosine as assay buffer, 2 µL tyrosinase, mixed well and add 50 µL/well into wells containing test inhibitor, inhibitor control and enzyme control. Mix incubated for 10 min at 25 °C. For each well, prepare 30 µL of tyrosinase substrate solution (23 µL tyrosinase assay buffer, 2 µL tyrosinase substrate and 5 µL tyrosinase enhancer). Mix and add 30 µL of tyrosine substrate solution into each well. Mix well measure the absorbance in kinetic mode for 30-60 min at 510 nm. Choose two time points (T_1 and T_2) in the linear range of the plot and obtain the corresponding values for the absorbance (Abs 1 and Abs 2). Calculate the slope for all samples, including enzyme activity control (EC) by dividing the net as Δ Abs (Abs2 - Abs1) values by the time T (T2 - T1). Calculate (%) relative inhibition as follows:

$$\% \text{ Relative Inhibition} = \frac{(\text{Slope EC} - \text{Slope S})}{\text{Slope of EC}} \text{ x } 100$$

Cell cycle analysis

The cell cycle phases were determined by using flow cytometry with propidium iodide (PI). Staining using BioVision EZ cell™ cell cycle analyses kit (Cat NO. K920 -100). MCF-7 cells (2×10^5 well) treated with compound **5** in an incubator with 5% CO_2 at 37 C for 48h, and cells incubated in medium without the compound as control. After Incubation, cells were separated with 0.5% trypsin. The separated and suspended cells were harvested in a complete medium and centrifuged at 300 g for 5 min. Pellets were washed with PBS twice and fixed with ice cold 70% ethanol for 1h at 20 C, treated with nuclear dye and stained with propidium iodide. Fluorescence intensity was detected and recorded on BD flow cytometer in FL-2 channel [18].

Annexin V-FITC/PI assay

Apoptosis was detected by flow cytometric analysis with annexin V staining. The Bio Vision Annexin V-FITC apoptosis detection Kits (Cat No: K101-25) was used MCF-7 cells (2×10^5 well) were exposed to compound **5** at the IC_{50} concentration. Cells were harvested by trypsin, washed with PBS, and resuspended in 500 µL of binding buffer. Cells were then incubated with

5 mL of Annexin V-FITC and 5 µL of propidium iodide (PI) for 45 min at room temperature in the dark place. The cells were immediately analyzed by a flow cytometer using the FITC signal detector [19].

CONCLUSION

In conclusion, novel series of 1,2-bis(tetrasubstituted benzylidene) hydrazines (**3**-**7**) were synthesized via ring opening of ethyl 5-substituted-8-methoxy coumrin-3-carboxylate with hydrazine hydrate under reflux to yields compounds **3** and **4**. Compounds **5** and 6 were obtained from the compound **4** via the treatment with acetic anhydride and diazonium chlorides. All the produced compounds (**4**-**7**) were assayed for the cytotoxicity performance against breast cancer cell lines (MCF-7) and show good inhibitory efficiency. In vitro assay of compound **5** exhibited cell cycle arrest at G2/M phase and, induced at pre-G1 phase as indicated by the annexin V test. Compound **5** have expressed a good inhibitory activity against aromatase and tyrosinase screening.

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