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Synthesis, Characterization, Theoretical Study, Antioxidant Activity and in Vitro Cytotoxicity Study of Novel Formazan Derivatives Toward MCF-7 Cells

Hasanain Gomhor J. Alqaraghuli,[a,*] Zainab Y. Kadhim,[b] Ameena N. Seewan[a]

[a]*Department of Sciences, College of Basic Education, Al-Muthanna University, Al-Muthanna, Iraq.*

[b]*College of Veterinary Medicine, Al-Muthanna University, Al-Muthanna, Iraq.*

Abstract

New formazan derivatives **F-1** and **F-2** were synthesized and tested as antioxidant and anticancer agents. Chemical structures of compounds were proved by spectroscopic methods (FT-IR, 1H-NMR and GC-Mass) and elemental analysis (CHN). The cytotoxicity activity of formazan derivatives was estimated against human breast cancer (MCF-7) cells. The synthesized compounds exhibited significant cytotoxic activity toward MCF-7 cell lines. Compound **F-1** showed the highest toxicity toward MCF-7 cells. MTT assay demonstrated that 16 µg/ml of compound **F-1** reduced cell growth by 88.33%, after 48 hours. Hemolysis study demonstrated that the hemolysis percentage of compounds **F-1** and **F-2** at (10 mg/ml) concentration was (4.05% to 4.18%), this result indicates the safety of their use inside the body. The antioxidant activity of the formazan compounds against DPPH radicals was tested in vitro. The results displayed that compound **F-1** has stronger antioxidant activity than compound **F-2**. The new compounds were investigated in the gas phase using HyperChem software, applying semi-empirical methods and molecular mechanics. The heat of formation, binding energy, HOMO-LUMO, energy gab and dipole moment were calculated, and the results showed that compound **F-1** was more stable and more polar than **F-2**.

Keywords: Formazanderivatives; breast cancer; anticancer activity; antioxidant activity; theoretical study; MCF-7.

1. Introduction

Breast cancer is the common class of cancer that affects females and results in the highest number of death cases around the world [1, 2]. Cancer is strongly opponent to modern healthcare, even when cancer is diagnosed at early stages, modern treatments sometimes fail to treat the patient completely [3]. Chemotherapy is one of the most effective treatments to extend a patient's life. Approximately 60% of anti-cancer drugs are natural origin, such as plants (*i.e.* irinotecan and vincristine) and microorganisms (*i.e.* bleomycin, doxorubicin and dactinomycin) [4]. However, many chemotherapeutic drugs are in a dilemma due to the problem of drug resistance [5]. Chemotherapeutic agents also exert toxicity to healthy cells, which in turn causes undesirable side effects to the patients. For these reasons, the development for new classes of anticancer agents that demonstrate effective and selective toxicity on cancer cells is attracting increased attention [6].

Formazan compounds are an important class of organic colored compounds that contain the characteristic chain of atoms -N-C=N-NH- [7, 8]. Formazan derivatives were studied widely due to their pharmaceutical and biological activities

*Corresponding author e-mail: hgomhor@gmail.com.
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such as antibacterial [9], anti-parkinsonian, antimicrobial [10], anti-fertility [11], antifungal [12], antihyperglycemic, anticonvulsant [13], anti-tubercular, anticancer [14] and anti-HIV [15]. Recently, formazans displayed promising anti-cancer activity in photodynamic therapy activity toward cancer cells [16].

Turkoglu G. and Akkoc S. reported the synthesis of para -H, -F, -Cl, -Br substituted formazan compounds. Turkoglu examined the anticancer activity for the prepared compounds against colon cancer cells, breast cancer cells (MDA-MB-231) and liver cancer cell lines using MTT assay. However, the results demonstrated that all the prepared compounds exhibited more cytotoxic activity in the colon cell line in comparison with the other two cell lines [14].

In this article, we present the synthesis and structural characterization with various spectroscopic methods of new formazan derivatives. Furthermore, anti-proliferative activity studies of the formazancompounds were tested toward breast cancer cell (MCF-7) lines using MTT assay. According to the resulting data, formazan compounds exhibited good cytotoxic activity against MCF-7 cell line. The antioxidant activities of the synthesized formazan compounds were evaluated, the results confirmed that the **F-1** and **F-2** have potent DPPH radical antioxidant effects.

2. Experimental

2.1. Materials and instruments

All chemicals were purchased from Merck (Germany) and Sigma-Aldrich (USA). FT-IR spectra were obtained using KBr discs (4000–400) cm^{-1} on FTIR-8000, single beam path laser, Shimadzu Fourier transform infrared spectrophotometer.^{1}HNMR spectra were recorded on Bruker (500 MHz) NMR spectrophotometer, using DMSO-d_6 as a solvent. GC-mass spectra were recorded on a Fisons Trio 1000 spectrometer. Elemental analyses CHN was performed by EM-017 analyzer.

2.2. Synthesis of Schiff bases

The synthesis of Schiff bases H-1 and H-2 was carried out via a modified procedure described by *Azabet al*. [17]Compounds H-1 and H-2 were prepared by the reaction of 0.01 mole of aldehyde dissolved in absolute ethanol (40 ml) with 0.01 mole of phenylhydrazine with three drops of glacial acetic acid, then the mixture was refluxed for 4 h. The solid product that separated after cooling was filtered, dried and recrystallized using methanol.

1-((2-phenylhydrazineylidene)methyl)naphthalen-2-ol (H-1)

It was prepared by reaction the mixture of (1.72 gm, 0.01 mole) of 2-Hydroxy-1-naphthaldehyde with (1.08 gm, 0.01 mole) of phenyl hydrazine. Yellow solid material, yield 75%, m.p. 191-192 ^{0}C, R_f = 0.81.Elemental analysis **Calc**. C, 77.84; H, 5.38; N, 10.68. **Found** C, 77.01; H, 4.55; N, 9.71. **FT-IR** [cm^{-1}]: *υ* (O-H str.) 3485.49 b *υ* (N-H str.) 3321.53 *m;υ* (aromatic C-H str.) 3051.49-3022.55*m*; *υ* (C=N) 1600.97s; *υ* (N-H bend) 1535.39*s*; *υ* (aromatic C=C str.) 1494.88-1469.81*s*; *υ* (C-N str.) 1255.10 s.

3-bromo-4-((2-phenylhydrazineylidene)methyl)phenol (H-2)

It was synthesized by reacting (2.01 gm, 0.01 mol) of 2-bromo-4-hydroxybenzaldehyde with (1.08 gm, 0.01 mole) of phenyl hydrazine. Brown solid material, yield 79%, m.p. 137-139 ^{0}C, R_f = 0.93. Elemental analysis **Calc**.C, 53.63; H,5.38 ; N,9.62.**Found** 54.37; H, 4.77; N, 9.96. **FT-IR** [cm^{-1}]: *υ* (O-H str.) 3298.36*;* *υ* (N-H str.) 3122.96*m*; *υ* (aromatic C-H str.) 3051.49*m*; *υ* (C=N) 1600.97*s*; *υ* (N-H bend) 1533.49*s*; *υ* (aromatic C=C str.)1494.88-1448.59; *υ* (C-N str.) 1273.06*m*.

2.3. Synthesis of formazan compounds

The formazan derivatives **F-1** and **F-2** were synthesized via the modified method described by *Mariappan et al.*[18]. (1.98 gm, 0.01 mol) of 4,4′diaminodiphenylmethane was dissolved in (3 ml) of concentrated HCl and (10 ml) of distilled

water chilled in an ice bath with constant stirring at 0-5 ^{0}C temperature. Subsequently, (10 ml) (1.69 gm, 0.02 mol) of sodium nitrite solution was added dropwise, and the reaction mixture was cooled in an ice bath below 5°C for 15 min. The product solution was added dropwise with stirring to (0.02 mol) of compounds (H-1 and H-2) dissolved in 20 ml pyridine, then the mixture was stirred for 20 min. The colored solid which was separated is filtered and washed with distilled water followed by diethyl ether.

5,5'-(Methylenebis(4,1-phenylene))bis(3-(2-hydroxynaphtha ene-1-yl)-1-phenylformazan) (F-1)

It was synthesized by reacting (5.24 gm, 0.02 mole) of compound H-1 with (1.98 gm, 0.01 mole) of 4,4'-diaminodiphenylmethane diazonium salt solution. Dark brown solid material.Yield 77 %, m.p. 193-195 ^{0}C. **R_f**= 0.69Elemental analysis. **Calc.** C, 75.01; H, 3.91; N, 14.11. **Found** C, 75.79; H, 4.87; N, 15.04. **FT-IR** [cm^{-1}]: *υ* (O-H str.) 3483.96*b*; *υ* (N-H str.) 3323.46*m*; *υ* (aromatic C-H str.) 3059.20-3009.05*w*; *υ* (aliphatic C-H str.) 2965.91; *υ* (C=N) 1622.19*s*; *υ* (N-H bend) 1600.97*s*; *υ* (aromatic C=C str.) 1562.39*s* -1533.49; *υ* (N=N) 1490.52*s*; *υ* (C-N str.) 157.63*m*.**^{1}H-NMR** (500 MHz, DMSO-d_6, δ/ppm): δ (3.79-3.84) ppm (*d, J*=25, 1H, Ph-**CH$_2$**-Ph), δ (3.91-3.96) ppm (*d, J*=25, 1H, Ph-**CH$_2$**-Ph), δ (6.71-8.94) ppm (*m*, 30H, Ar-H), δ 10.54 ppm (*s*, 2H, NH), δ 11.88 ppm (*s*, 2H, OH).***m/z*:** 744 (M$^+$, R%20), 774 (R%10), 149 (R%31), 104 (R%15), 76 (R%10), 57 (R%77), 41 (R%100).

5,5'-(Methylenebis(4,1-phenylene))bis(3-(2-bromo-4-hydroxyphenyl)-1-phenylformazan) (F-2)

It was synthesized by reacting (5.82 gm, 0.02 mole) of compound H-2 with (1.98 gm, 0.01 mole) of 4,4'-Diaminodiphenylmethane diazonium salt solution. Dark brown solid material.Yield 86 %, m.p. 202-204 ^{0}C. **R_f**= 0.84.Elemental analysis. **Calc.** C, 58.01; H, 18.78; N, 12.01. **Found** C, 58.37; H, 19.91; N, 13.96. **FT-IR** [cm^{-1}]: *υ* (O-H str.) 3443.05b*;* *υ* (N-H str.) 3319.50*m*; *υ* (aromatic C-H str.) 3028.34*w*; *υ* (aliphatic C-H str.) 2914.54; *υ* (C=N) 1614.47*s*; *υ* (N-H bend) 1521.89*s*; *υ* (aromatic C=C str.)1477.52*s;* *υ* (N=N) 1425.66.49; *υ* (C-N str.) 1334.91*m***. ^{1}H-NMR** (500 MHz, DMSO-d_6, δ/ppm): δ (3.76) ppm (*s*,2H, Ph-CH_2-Ph), δ (6.82-8.48) ppm (*m*, 24H, Ar-H), δ 10.47 ppm (*s*, 2H, NH), δ 10.53 ppm (*s*, 2H, OH).***m/z*:** 802 (M$^+$, R%38), 796 (R%55), 775 (R%38), 696 (R%65), 630 (R%70), 600 (R%16), 665 (R%80), 556 (R%50), 546 (R%50).

2.4. Hemolysis assay of formazans

Healthy human blood specimens were centrifuged at 1350 rpm for 10 min. The red blood cells (RBCs) were washed three times and suspended in normal saline (0.9%) to obtain the concentration of 2% w/v RBCs. The suspension of 2% RBCs (1.25 m) was dispersed in an equivalent volume of distilled water (positive control) and in the same volume of 0.9% normal saline (negative control). Compounds **F-1** and **F-2** at (0.1, 1 and 10 mg/ml) concentration (0.15 ml) were incubated at 37 ºC with 2% RBCs (1.25 ml) and normal saline (1.1 ml) for three hours. The sample was centrifuged at (900 rpm) for 12 min, and the supernatant was utilized for the hemolytic ratio (HR) evaluation. The released hemoglobin concentration was estimated by measuring the absorption of the supernatant solution at 538 nm [19, 20]. HR was calculated using the following equation:

$$\textbf{Hemolysis (\%)} = (D_s - D_n)/(D_w - D_n) \times 100$$

where D_s, D_n, and D_w are the absorbance of the sample, saline, and distilled water, respectively.

2.5. Antioxidant activity

The synthesized formazan derivatives **F-1**, **F-2** and ascorbic acid (as a standard) were tested for the scavenging effect on 1,1-Diphenyl-2-Picry hydroxyl (DPPH) radical methods, as reported in Muthuvel*et al*[21]. The test sample solution was prepared in different concentrations (15, 30, 60, 125, 250 and 500) µg/ml and added to an equivalent volume of 0.1 mMmethanolic solution of DPPH. The reaction mixture was incubated for 1 hr at room temperature. The absorbance was measured for the mixture at 517 nm, which gives the antioxidant activity. The percentage of inhibition was calculated utilizing the following equation:

$$\textbf{\% DPPH scavenging activity} = (A_{\text{control}} - A_{\text{test sample}} / A_{\text{control}}) \times 100$$

2.6. Anticancer activity

2.6.1 Cell Culture

MCF-7 cells were used, which are adherent epithelial adenocarcinomas obtained from the mammary gland. Breast cells (non-triple negative) are derived from the site of metastatic pleural effusion. The cell line was seeded in 75-cm^2 tissue culture flasks and maintained in Dulbecco's MEM supplemented with (10% heat) in activated fetal bovine serum, 100 U mL-1 penicillin and 100 µg mL-1 streptomycin. Every two days the medium was renewed and the cell cultures were incubated at (37 °C) in a humid atmosphere (95% air) and (5% CO_2) [22][23] .

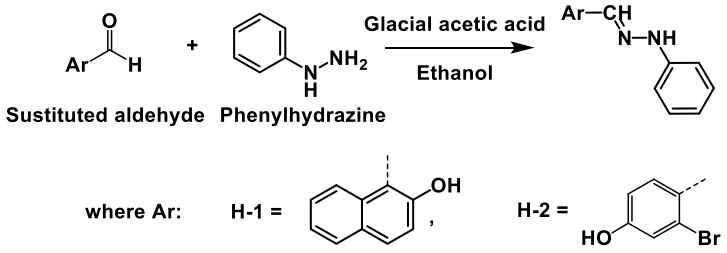
2.6.2. Cell proliferation inhibition assay

Proliferation inhibition effects on cell line by synthesized formazan derivatives **F-1**, **F-2** and doxorubicin (as a standard drug) were evaluated at various concentrations (1, 2, 4, 8 and 16) µg/ml utilizing MTT method [24].

3. Results and Discussion

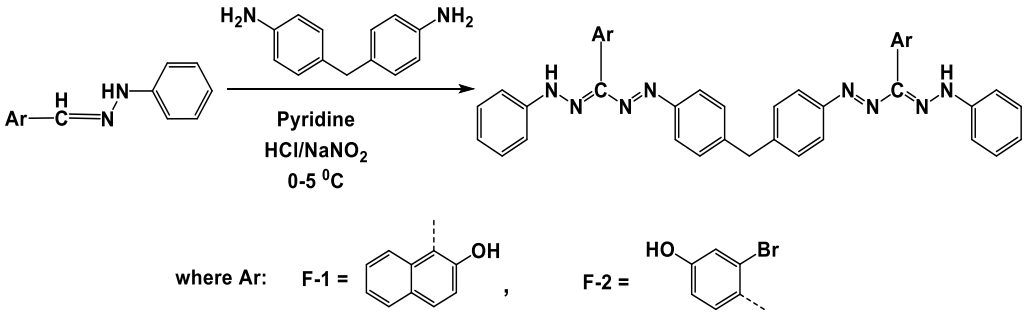
3.1. Chemistry

Formazan compounds were synthesized by two steps. The first step consisted of the formation of Schiff bases H-1 and H-2 through reaction of phenylhydrazine with substituted aldehyde (2-hydroxynaphthalaldehyde, 4-(dimethylamino)benzaldehyde and 2-bromo-4-hydroxybenzaldehyde), respectively as summarized in Scheme 1.



Scheme 1: Synthesis of Schiff base compounds

The second step involved a coupling reaction between 4,4'-diaminodiphenylmethane diazonium saltand the imine group in a basic medium (sodium hydroxide) [25] to produce formazan compounds **F-1** and **F-2** (Scheme 2).



Scheme 2: Synthesis of formazan compounds

3.2. Structure determination

The chemical structures of the synthesized compounds were determined basis on the FT-IR, ^{1}H-NMR, GC-Mass, spectroscopic techniques and CHNanalysis, which are provided in the experimental section.

FT-IR spectra of compounds H-1 and H-2 explained by the presence of stretching vibrations corresponding to the (HC=N) band at 1600.97 cm^{-1} which is the functional group in the Schiff base compounds [26]. The spectra of H-1 and H-2 are also characterized by appearing a broad medium band due to the stretching vibration of (N-H) at (3122.96 - 3490.71) cm^{-1}. The spectra of compounds H-1 and H-2 displayed a broadband at (3485.49 and 3298.36)

cm^{-1}, respectively due to the stretching vibration of (O-H) group. FT-IR spectra of formazan compounds **F-1** and **F-2** revealed the presence of new absorption bands for stretching vibrations corresponding to the (N=N) bonds at (1490.52 - 1425.66) cm^{-1}[27]. The spectrum of compound **F-1** showed a broadband for hydroxyl group at 3483.96 cm^{-1}.

The formation of compounds **F-1** and **F-2** has been proved by the ^{1}H-NMR spectra. The protons of (N-H) group were recorded in (10.54 and 10.44) ppm for **F-1** and **F-2** respectively. The proton of methylene group (Ph-CH_2-Ph) of compound **F-1** appeared at δ (3.82 and 3.93) ppm (*d, J*=25), while in the compound **F-2** appeared at δ (3.90 and 3.75) ppm as a singlet. The proton of (O-H) group appeared at (11.88 and 10.52) ppm as a singlet for compounds **F-1** and **F-2** respectively. ^{1}H-NMR spectra displayed a sharp peak at 2.50 and 3.33 ppm due to DMSO-d_6 solvent [28].

The chemical structure of synthesized formazan derivatives was further confirmed by the GC-mass spectra. The GC-mass spectra of formazan compounds showed the correct molecular ion peaks. The molecular ion peaks appeared at m/z: 744 (M+, R%20) and m/z: 802 (M+, R%38) was for compounds **F-1**and **F-2** respectively.

3.3. Hemolysis Study

The hemolysis ratio of synthesized formazan derivatives was estimated by measuring hemoglobin released from the RBCs. The hemolysis ratio percentages of compounds **F-1** and **F-2** at (5 mg/ml) concentration were (4.05% and 4.18%), respectively. The hemolysis ratio percentages of all formazans were less than 10%, this result indicates the safety of their use inside the human body [19].

Table 1. The results of hemolysis study for compounds **F-1** and **F-2** at different concentration.

Compound code	HR (%)		
	0.1 mg/ml	1 mg/ml	10 mg/ml
F-1	0.53%	2.17%	4.05%
F-2	0.71%	2.49%	4.18%

3.4. Theoretical studies

HyperChem is important molecular modeling software utilized to draw molecules by choosing the internal coordinates of the molecules and then predicting their spectral properties. This semi-empirical software can provide precise solutions for the experimental difficulties during the study of some highly sensitive, hazardous materials and very active materials [29, 30].

In the present work the heat of formation ($\Delta H°_f$), binding energy (ΔE_b) dipole moment (μ), IR and UV spectra in addition to molecular orbital energy (E_{HOMO}-E_{LUMO}) were calculated using HyperChem8.03software for the semi-empirical and molecular mechanic calculation.

All computationalchemistry techniques explain that the molecule with the lowest energy is the most stable. Thus, the shape of molecule corresponds to the shape with the lowest energy[31, 32]. The heat of formation ($\Delta H°_f$), binding energy (ΔE_b), HOMO, LUMO and dipole moment(μ) for formazan derivatives were calculated by PM3 method (Table 1).

Table 2. Conformation energetic (in K.J.mol^{-1}) and dipole moment (in Debye) for synthesized compounds.

Comp.	$\Delta H°f$	ΔE_b	μ	HOMO	LUMO	ΔE_{gap}
H-1	-32288.98	-48690.75	1.94	-5.45	-.120	5.33
H-2	-31266.70	-47681.77	2.20	-5.30	-0.11	5.19
F-1	-90760.48	-13649.19	11.87	-8.67	-1.94	6.73
F-2	-87166.10	-38930.30	7.78	-7.275	-1.1	6.18

The heat of formation of compound **F-1** is smaller than other compounds. Thus, compound **F-1** is expected to be more thermodynamically stable than compound **F-2**. The molecular orbital energy (E_{HOMO} and E_{LUMO}) and the energy gap were calculated. The energy gap is representing the energy between the HOMO and LUMO orbitals, when ΔE_{gap} is large, the molecule is more stable [33]. According to the results, the energy gap for all compounds was arranged as follow:

$$\Delta E_{gab}\mathbf{F\text{-}1} > \Delta E_{gab}\mathbf{F\text{-}2} > \Delta E_{gab}\mathbf{H\text{-}1} > E_{gab}\mathbf{H\text{-}2}$$

3.5. Antioxidant activity

DPPH is a standard and stable free radical that is commonly utilized to measure the ability of synthesized compounds to act as free radical scavengers and to estimate antioxidant activity. The DPPH intensity is reduced by the acceptance of electron or hydrogen [34]. The *in vitro* radical scavenging ability of the **F-1** and **F-2**is evaluated by DPPH radical scavenging assays. Reduced activity of the samples was measured by changing the color of DPPH from the initial deep purple solution to yellow. The results of antioxidant activity of samples are determined and compared with the standard antioxidant ascorbic acid as shown in Table 3. At concentration 30–500 µg/ml, **F-1** show 29% to 86%, **F-2** show 25 to 83% and standard ascorbic acid show 33–91%. The above observed activity is lower than that of the **F-1** and **F-2** compared to the standard vitamin C. The results for DPPH free radical scavenging activity of **F-1**, **F-2**and standard, are presented in Table 3.

Table 3. The results of antioxidant activity of F-1, F-2 and standard vitamin C.

Concentrations (µg/ml)	**F-1**	**F-2**	Standard
15	17	15	20
30	29	25	33
60	42	38	49
125	55	49	66
250	74	69	79
500	86	83	91

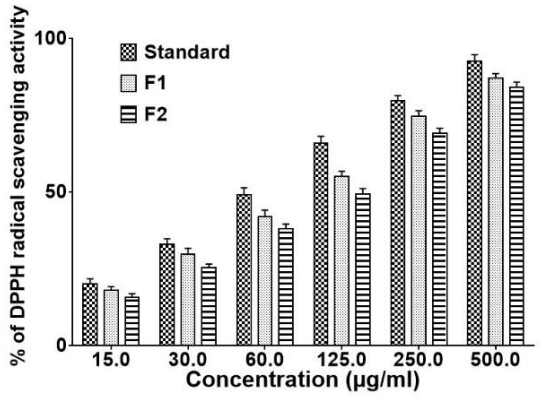


Figure 1. The results of antioxidant activity of **F-1**, **F-2** and standard ascorbic acid

3.6. Anticancer activity

The cytotoxic activity of synthesized formazan compounds **F-1** and **F-2** was assessed against the MCF-7 cells at various concentrations (1, 2, 4, 8 and 16) µg/ml utilizing the MTT assay. After 48 hours, the IC_{50} values of doxorubicin and **F-1** and **F-2** were estimated at 3.35, 4.68 and 7.16 µg/ml, respectively. The results suggested that the activity of **F-1** against the MCF-7 cell line was more significant compared to other compounds. Figure 1 shows the toxicity of doxorubicin, **F-1** and **F-2** in the MCF7 cell line. MTT assay demonstrated that 16 µg/ml of doxorubicin, **F-1** and **F-2** reduced cell growth by 96%, 88.33% and 76.33% respectively after 48 hrs. The prepared compounds gave a high response to inhibiting and killing cancer cells due to the presence of the formazan group (-N=N-C(R)=N-NH-), which has been proven in previous studies to have the greatest role in the toxicity of compounds towards cancer cells [35]. Our results exhibited that **F-1** and **F-2** have the ability to inhibit the proliferation of MCF-7 cells and the potential to act as an anti-breast agent.

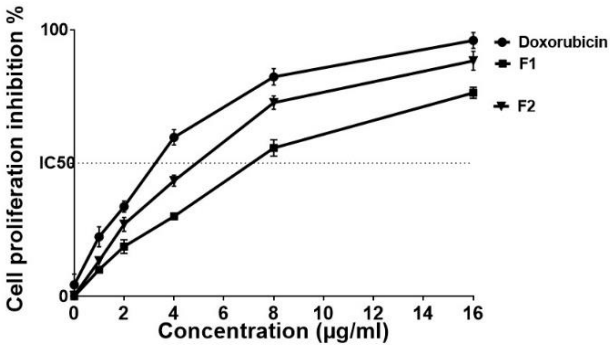


Figure 2. Proliferation inhibition effect of doxorubicin, **F-1** and **F-2** in MCF-7 cell line

Conclusions

The new formazan compounds **F-1** and **F-2** were successfully synthesized in good yields (77-82%), and exhibiting antioxidant and anti-proliferative activity toward breast cancer (MCF-7) cells. The chemical structure of the synthesizedcompounds was proved using CHN, FT-IR, ^{1}HNMR, and GC-Mass.Hemolysis study demonstrated the safety of formazans useinside the body. The MTT results showed good cytotoxicity of **F-1** and **F-2** in the MCF7 cell line comparing with standard drug doxorubicin. Compound **F-1** showed the highest toxicity against MCF-7 cells.The antioxidant studies on synthesized compounds confirmed that the **F-1** has a strong DPPH radical antioxidant effect.The

calculation of energies in the gas phase provided information about the most stable structure of the formazans, and our result demonstrated that the stability of formazan structures is arranged as follows (**F-1> F-2**). The HOMO-LUMO, energy gab and dipole moment results indicated that compound **F-1** less reactive and more polar from the other compounds.

Conflicts of Interest

There are no conflicts to declare.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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