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Synthesis, Molecular Docking Study, Anti-Oxidant and Cytotoxicity Evaluation of New Spiro Six Membered Ring Derivatives of 5-Nitro Isatin

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Abstract

Spiro 5-nitro isatin six-membered ring compounds (quinazoline-4-one, thiazine-4-one and oxazine-4-one) were produced by a cycloaddition of 5-nitro isatin Schiff bases (1–5) with anthranilic acid, o-mercaptobenzoic acid and salicylic acid. ^{1}HNMR and ^{13}CNMR as well as Fourier-transform infrared spectroscopy, were used to identify the structures of the obtained compounds. Spiro-5-nitro isatins are of interest to researchers due to their potential antioxidant and anticancer properties. The MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)) assay was used to examine in vitro bioactivity testing against breast cancer (MCF-7) cell lines which showed compound 14 (IC_{50}=79.85 mM) after 24 hours have good cytotoxicity compared with reference tamoxifen (IC_{50}=91.16 mM). The synthesized compounds (6-20) were evaluated for their antioxidant activity showed no significant activity. Molecular docking, compound 14 showed good PLP's fitness with 81% which agree with MTT assay data so make it a promising compound in the treatment of breast cancer.
Keywords: 5-nitro isatin , Spiro quinozolin-4-one, Spiro thiazine-4-one, Spiro oxazine-4-one, MCF-7 assay, DPPH assay.

تحضير،دراسة الالتحام الجزيئي، تقييم مضادات الأكسدة والسمية الخلوية لمشتقات جديدة من الحلقات العقدية السداسية من ٥-نيترو إيزاتين

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الخلاصة

تم تحضير مركبات ذات حلقات عقدية سداسية تحتوي على ٥-نيترو ايزاتين من (كوينازولين-٤-اون، ثايازين-٤-اون واوكازين-٤-اون) على التوالي بواسطة الغلق الحلقي لقواعد شيف (١-٥) مع انثرانليك اسد، اورثو مركبتو بنزويك اسيد وسالسيلك اسيد. تم استخدام طيف الرنين المغناطيسي البروتوني والكربوني بالإضافة الى طيف الأشعة تحت الحمراء لتحديد الصيغة التركيبية للمركبات التي تم الحصول عليها. كما تم تقييم الفعالية المضادة للأكسدة والسرطان بسبب خصائصها المضادة. تم استخدام اختبار MTT لفحص اختبار النشاط الحيوي ضد سرطان الثدي حيث اظهر المركب ١٤ قيمة (IC_{50}=79.85 µg/mL) بعد ٢٤ ساعة سمية خلوية جيدة مقارنة مع عقار تاموكسفين كمرجع (IC_{50}=91.16 µg/mL). تم تقييم المركبات المحضرة (٦-٢٠) من حيث نشاطها المضاد للأكسدة ولم تظهر أي نشاط معنوي. في الالتحام الجزيئي، اظهر المركب ١٤ نتيجة جيدة في الالتحام الجزيئي بنسبة ٨١٪ والتي تتفق مع اختبارMTT مما يجعله مركبا واعدا في علاج سرطان الثدي.
الكلمات المفتاحية: ٥-نيترو إيزاتين , حلقة عقدية كوينازولين-٤-اون , حلقة عقدية ثايازين-٤-اون , حلقة عقدية اكازين-٤-اون , فحص MCF-7 , فحص DPPH.

Introduction

The second biggest cause of mortality is breast cancer, which is the most prevalent malignancy in women. Over a million new cases of breast cancer are recorded each year, and the incidence of the disease has been rising for many years. Compared to other malignancies, it affects more women. [(1)] Based on estrogen or progesterone receptor expression and human epidermal growth factor 2 ERBB2 gene amplification, breast cancer is divided into three major tumor subtypes. [(2)] There are a substantial number of modifiable and non-modifiable risk factors for breast cancer [(3)].

Due to their biological activity and widespread occurrence in the nuclei of natural products, isatins are receiving a lot of interest among the several hetero- cycliccompounds used to treat cancer. Researchers have extensively studied the anti-cancer properties of isatin and its derivatives [(4,5)].Isatin (1H-indol-2,3-dione) is a known natural alkaloid that is obtained from plants of the Isatis genus and other plants as a yellow - orange powder. It can also be discovered in the secretions of the Bufo frog and the Australian mollusk Dicathais orbita, as well as in the brains of mammals all over the world.

As a metabolite of tryptophan or epinephrine and an endogenous substance in humans, [6,7] it is also widely distributed throughout the central nervous system (CNS) [8]. These plants have indole chemicals, which are already utilized as anti-inflammatory and anti-cancer agents and have demonstrated therapeutic qualities, making them appropriate for use in the synthesis and design of drugs [9,10].To create effective and selective pharmaceutical agents, many isatin derivatives have been created using various substituents at various locations across the isatin's basic structure. Compounds' properties were modulated by a variety of substituents, such as electrophilic aromatic substitution at positions C-5 and C-7 of the ring, N substitutions, nucleophilic additions onto the more reactive C-3 carbonyl group, chemoselective reductions, oxidations, ring expansions, and spiro-annulations, and others, as shown in Figure 1 [11-13].

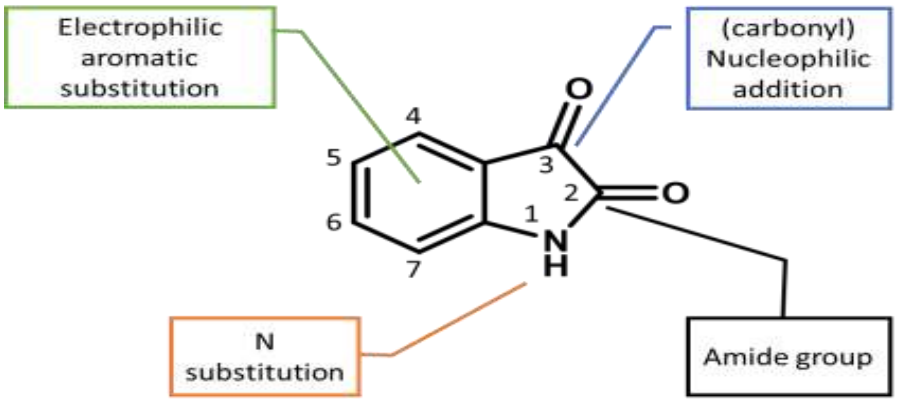


Figure 1. Isatin structure-reactivity relationship

The name "spiro" refers to polycyclic compounds in which one carbon is a common member of two dissimilar rings [14] carrying one common carbon atom to two rings are structurally significant; these substances are appreciated for their broad range of biological functions [15]. The reactive carbonyl group's play role in the synthesis of spiro-heterocycles so it is great interest to organic scientists and reported variety of synthesis processes [16]. Although spirocyclic structures are unusual scaffolds for biologically active compounds, recent advances in the isolation and identification of novel compounds from natural products and new synthesized routes to spiro basic building block have made it easier for these molecules to be incorporated into more molecules with medicinal applications, such as anti-diabetic, anti-cancer, and anti-drugs. Alzheimer's in some cases, these molecules have even been successfully implemented as commercial drugs [17] such as their anti-cancer, diuretic, anti-inflammatory, anti-convulsant, and anti-hypertensive effects, the classes of fused heterocycles quinazoline and thiazine are of great interest [18]. The reactions of intermediate compounds with anthranilic acid and o-mercapto benzoic acid led to the synthesis of spiro quinazoline and thiazine, respectively [19-21]. In this article, we have to get into detail on how to create the spiro six-membered ring compounds (quinazoline-4-one, thiazine-4-one and oxazine-4-one) of 5-nitro isatin, which has significant bioactivities against cancer cells in vitro and antioxidants using the DPPH method. In order to investigate the potential mechanism underlying the anticancer effect, molecular docking experiments were also conducted.

Materials and Methods

Chemicals and materials

Materials: All 4-substituted aniline (BDH, England), 5- nitro isatin (Sigma Aldrich, German), all solvents (Alpha Chemika, India), materials were used without further purification.

General procedure: melting point equipment was used in the experiment by capillary method on Gallenkamp's electro-thermal (UK). Using the (ATR) tensor 27 Bruker (USA), in the spectral region of (4000-600) cm^{-1}, infrared spectra were obtained and was performed in College of Pharmacy / Mustansiriyah University. Using near magnetic resonance technology from Bruker (USA), Ultra-shield (300 MHz) for ^{1}HNMR and Ultra-shield (76 MHz) for ^{13}CNMR spectra were collected and was performed in Iran. Chemical shifts are recorded in parts per million (δ) deshield. Hydrogen signals splitting forms are indicated for the multiplicities: s= singlet, d= doublet and m= multiplet. Shimadzu Spectrophotometer, a Japanese company, recorded UV-VIS spectra to measure the antioxidant activity and was performed in Department of Chemistry/ College of Science/ University of Baghdad. MTT assay was performed in college of biotechnology, Al-Nahrain university. GraphPad Prism was used to

create the graphics. The molecular docking studies were achieved applying the CCDC GOLD Suite.

General procedure for Synthesis of 3-(4-substituted phenylimino)-5-nitro-1H-indolin-2-one (1-5]): -

5-nitro isatin (1.0 g, 5.2 mmol) was dissolved in (7 mL) absolute ethanol and (3 mL) dimethylformamide (DMF) in a 50 mL round-bottom flask, adding 3-4 drops of glacial acetic acid and stirring the mixture for 15 minutes. Several paras substituted aniline compounds (p-toluidine, p-nitro aniline, p-chloro aniline, p-aminodiphenyl amine, aniline) (5.2 mmol) in 3 mL of absolute ethanol were added. About 12- 16 hours were spent refluxing and stirring the mixture checked by TLC (hexane:ethylacetate 6:4). Then the solution was poured into crushed ice, filtered, and re-crystallization with ethanol-water [(22)].

5-nitro-3-(4-tolylimino)-indolin-2-one (1)

[$C_{15}H_{11}N_3O_3$]: bright orange powder, 75% yield; m.p. 292-294 °C; Rf=0.62; FTIR (ATR) (cm^{-1}): 3228 (NH), 3093,3047 (Ar-H), 2996,2922 & 2857 (CH aliphatic), 1743 (C=O), 1658 (C=N), 1528 & 1337 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 2.41 (s,3H, CH_3), 6.98-8.38 (m,7H, Ar-H), 11.70 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 164.29, 159.17, 153.50, 152.70, 150.99, 147.42, 145.77, 145.64, 143.06, 142.91, 141.96, 135.67, 130.57, 130.53, 130.41, 129.29, 122.95, 121.02, 120.79, 118.22, 117.91, 116.07, 112.19, 111.4, 21.18, 21.10.

5-nitro-3- [(4-nitro phenyl) imino]-indolin-2-one (2)

[$C_{14}H_8N_4O_5$]: orange powder, 76% yield; m.p. 141-143 °C; Rf=0.69; FTIR (ATR) (cm^{-1}): 3339 (NH), 3072 (Ar-H), 1725 (C=O), 1653 (C=N), 1530 & 1300 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.73-8.46 (m,7H, Ar-H), 11.69 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 162.69, 156.15, 155.64, 143.04, 136.05, 133.52, 126.84, 120.04, 118.55, 112.96, 112.81.

5-nitro-3- [(4-chloro phenyl) imino]-indolin-2-one (3)

[$C_{14}H_8ClN_3O_3$]: orange powder, 85% yield; m.p. 190-191 °C; Rf=0.69; FTIR (ATR) (cm^{-1}): 3314 (NH), 3090 (Ar-H), 1729 (C=O), 1659 (C=N), 1523 & 1336 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.62-8.48 (m,7H, Ar-H), 11.73 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 164.05, 159.11, 154.27, 152.87, 151.37, 148.76, 147.39, 141.93, 133.51, 130.76, 130.46, 130.34, 130.17, 130.03, 129.02, 128.76, 122.21, 121.95, 120.85, 120.01, 118.22, 115.88, 112.29, 111.59.

5-nitro-3-[(4-(phenylamino) phenyl) imino] indolin-2-one (4)

[$C_{20}H_{14}N_4O_3$]: deep purple powder, 90% yield; m.p. 230-232 °C; Rf=0.66; FTIR (ATR) (cm^{-1}): 3389,3338 & 3243 (NH), 3093 (Ar-H), 1733 (C=O), 1655 (C=N), 1513 & 1322 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 5.26 (s,1H, -NH-), 6.67-8.81 (m,12H, Ar-H), 11.65 (s,1H, NH isatin). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 164.69, 160.35, 160.02, 152.27, 151.25, 149.37, 145.37, 143.31, 143.26, 143.03, 142.85, 141.95, 141.11, 138.53, 133.52, 129.99, 129.78, 129.75, 127.91, 121.75, 120.84, 120.04, 119.00, 118.55, 117.80, 117.40, 116.84, 116.26, 115.02, 112.97, 112.01, 110.92.

5-nitro-3-(phenylimino) indolin-2-one (5)

[$C_{14}H_9N_3O_3$]: pale orange powder, 92% yield; m.p. 238-240 °C; Rf=0.73; FTIR (ATR) (cm^{-1}): 3281 (NH), 3064 (Ar-H), 1746 (C=O), 1648 (C=N), 1554 & 1312 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.96-8.48 (m,8H, Ar-H), 11.71 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 164.21, 157.01, 153.86, 152.75, 150.16, 141.93, 135.68, 130.89, 130.59, 130.22, 128.81, 126.16, 125.93, 120.90, 120.14, 118.38, 118.06, 117.79, 115.95, 112.20, 111.50.

General procedure for Synthesis 3'-(4-substituted phenyl)-1'H-5-nitro spiro (3,2'-indolin-quinazoline]-2,4'-dione (6-10), 3'-(4-substituted phenyl)-5-nitro spiro (3,2'-indolin-benzo[e]-1',3'-thiazine)-2,4'-dione (11-15), 3'-(4-substituted phenyl)-5-nitro spiro (3,2'-indolin-benzo[e]-1',3'-oxazine)-2,4'-dione (16-20): -

Schiff base derivatives [1–5] (0.9 mmol) were dissolved in (8 mL) THF in a round bottom flask (50 mL), and then (0.12 g, 0.0009 mol) anthranilic acid, (0.14 g, 0.0009 mol) o-mercapto benzoic acid and (0.13 g, 0.0009 mol) salicylic acid were added. For around (12-18) hours, the mixture was refluxed mixture checked by TLC (hexane:ethylacetate 1:2) and then it was neutralized with sodium bicarbonate (5%) ($NaHCO_3$). The finished product was filtered, dried, and re-crystallization using ethanol [(23)]

3'-(4-tolyl)-1'H-5-nitro spiro-3,2'-indolin-quinazoline-2,4'-dione (6)

[$C_{22}H_{16}N_4O_4$]: deep orange powder, 80% yield; m.p. 270-272 °C; Rf=0.6; FTIR (ATR) (cm^{-1}): 3227 (NH), 3092 (Ar-H), 2919 & 2850 (CH aliphatic), 1728 (C=O amide), 1698 (C=O quinazoline ring), 1528 & 1337 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 2.41 (s,3H, CH_3), 3.62 (s,1H, NH quinazoline ring), 6.98-8.38 (m,11H, Ar-H), 11.36 (s,1H, NH indoline). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 164.51, 163.66, 153.63, 153.07, 151.27, 147.45, 141.88, 135.64, 130.57, 129.29, 121.01, 120.79, 118.22, 116.11, 112.24, 100.32, 21.10.

3'-(4- nitro phenyl)-1'H-5-nitro spiro-3,2'-indolin-quinazoline-2,4'-dione (7)

[$C_{21}H_{13}N_5O_6$]: deep brown powder, 65% yield; m.p. 211°C (decompose); Rf=0.65; FTIR (ATR) (cm^{-1}): 3313 (NH), 3077 (Ar-H), 1725 (C=O

amide), 1690 (C=O quinazoline ring), 1546 &1313 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 3.92 (s,1H, NH quinazoline ring), 6.37-8.66 (m,11H, Ar-H), 11.66 (s,1H, NH indoline). 13C NMR (76 MHz, DMSO) δ/ppm: ^{13}CNMR (76 MHz, DMSO) δ 13C NMR (76 MHz, DMSO) 169.73, 163.66, 134.79, 129.31, 126.47, 119.90, 114.74, 112.19, 99.98.

3'-(4-chloro phenyl)-1'H-5-nitro spiro-3,2'-indolin-quinazoline-2,4'-dione (8)

[$C_{21}H_{13}ClN_4O_4$]: pale brown powder, 60% yield; m.p. 210-212 °C; Rf=0.55; FTIR (ATR) (cm^{-1}): 3185 (NH), 3094 (Ar-H), 1733 (C=O amide), 1675 (C=O quinazoline ring),1526 & 1337 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 3.37 (s,1H, NH quinazoline ring), 7.00-8.47 (m,11H, Ar-H), 11.69 (s,1H, NH indoline). 13C NMR (76 MHz, DMSO) δ/ppm: ^{13}CNMR (76 MHz, DMSO) δ 13C NMR (76 MHz, DMSO) δ 164.14, 161.37, 154.38, 152.96, 148.84, 143.01, 141.99, 133.33, 130.83, 130.56, 130.29, 130.20, 128.80, 121.95, 120.90, 120.02, 118.28, 116.02, 112.34, 99.52.

3'-[4-(phenylamino) phenyl]-1'H-5-nitro spiro-3,2'-indolin-quinazoline-2,4'-dione (9)

[$C_{27}H_{19}N_5O_4$]: purple powder, 70% yield; m.p. 213-215 °C; Rf=0.65; FTIR (ATR) (cm^{-1}): 3415,3390 & 3243 (NH), 3094 & 3055 (Ar-H), 1736 (C=O amide), 1663 (C=O quinazoline ring), 1514 & 1335 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 3.99 (s,1H, NH quinazoline ring), 5.38 (s,1H, -NH-), 6.83-8.72 (m,16H, Ar-H), 11.58 (s,1H, NH indoline). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 163.48, 160.93, 154.41, 142.45, 138.94, 129.77, 129.72, 129.53, 121.22, 120.96, 120.16, 118.22, 117.91, 117.40, 115.10, 107.73.

3'-phenyl-1'H-5-nitro spiro-3,2'-indolin-quinazoline-2,4'-dione (10)

[$C_{21}H_{14}N_4O_4$]: orange powder, 75% yield; m.p. 268-270 °C; Rf=0.53; FTIR (ATR) (cm^{-1}): 3250 (NH), 3061 (Ar-H), 1727 (C=O amide), 1691 (C=O quinazoline ring), 1526 & 1337 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 4.34 (s,1H, NH quinazoline ring), 6.39-8.36 (m,12H, Ar-H), 10.79 (s,1H, NH indoline). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 166.12, 162.85, 155.09, 150.87, 150.44, 141.19, 132.18, 131.02, 130.67, 130.18, 125.95, 120.86, 120.06, 117.85, 116.22, 115.83, 114.33, 112.83, 103.38.

3'-(4-tolyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-thiazine-2,4'-dione (11)

[$C_{22}H_{15}N_3O_4S$]: brown powder, 80% yield; m.p. 232-234 °C; Rf=0.42, FTIR (ATR) (cm^{-1}): 3259 (NH), 3017 (Ar-H), 2922 & 2857 (CH aliphatic), 1728 (C=O amide), 1699 (C=O thiazine ring), 1529 & 1337 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 2.33 (s,3H, CH_3), 6.94-8.51 (m,11H, Ar-H), 10.42 (s,1H, NH).

3'-(4-nitro phenyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-thiazine-2,4'-dione (12)

[$C_{21}H_{12}N_4O_6S$]: pale brown, 55% yield; m.p. 98-100 °C; Rf=0.55; FTIR (ATR) (cm^{-1}): 3216 (NH), 3081 (Ar-H), 1729 (C=O amide), 1689 (C=O thiazine ring), 1502 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.59-8.51 (m,11H, Ar-H), 11.00 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 166.01, 162.07, 146.82, 140.55, 136.05, 128.98, 126.88, 116.52, 112.83, 99.10.

3'-(4- chloro phenyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-thiazine-2,4'-dione (13)

[$C_{21}H_{12}ClN_3O_4S$]: pale brown, 60% yield; m.p. 116-118 °C; Rf=0.61; FTIR (ATR) (cm^{-1}): 3277 (NH), 3163 (Ar-H), 1733 (C=O amide), 1683 (C=O thiazine ring), 1508 & 1338 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.56-8.65 (m,11H, Ar-H), 11.45 (s,1H, NH).

3'-[4-(phenylamino) phenyl]-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-thiazine-2,4'-dione (14)

[$C_{27}H_{18}N_4O_4S$]: purple, 70% yield; m.p. 213-215 °C; Rf=0.44; FTIR (ATR) (cm^{-1}): 3342 (NH), 3057 (Ar-H), 1727 (C=O amide), 1679 (C=O thiazine ring), 1512 & 1334 (NO_2); ^{1}HNMR (DMSO-*d*6, 300 MHz,) δ/ppm: 5.73 (s,1H, -NH-), 6.75-8.63 (m,16H, Ar-H), 11.68 (s,1H, NH indoline). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 168.16, 162.77, 152.30, 151.41, 151.31, 143.59, 139.41, 133.66, 132.05, 129.75, 128.74, 126.41, 125.45, 121.74, 121.73, 120.96, 117.81, 117.43, 115.10, 115.07, 97.23.

3'-(4-phenyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-thiazine-2,4'-dione (15)

[$C_{21}H_{13}N_3O_4S$]: orange, 75% yield; m.p. 246°C (decompose); Rf=0.72; FTIR (ATR) (cm^{-1}): 3326 (NH), 3083 (Ar-H), 1735 (C=O amide), 1709 (C=O thiazine ring), 1523 & 1337 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.83-8.74 (m,12H, Ar-H), 11.72 (s,1H, NH).

3'-(4- tolyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-oxazine-2,4'-dione (16)

[$C_{22}H_{15}N_3O_4$]: deep orange powder, 65% yield; m.p. 232-234 °C; Rf=0.41; FTIR (ATR) (cm^{-1}): 3221 (NH), 3091 (Ar-H), 2917 & 2850 (CH aliphatic), 1728 (C=O amide), 1701 (C=O oxazine ring), 1528 & 1339 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 2.13 (s,3H, CH_3), 6.60-8.27 (m,11H, Ar-H), 11.46 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 170.71, 163.70, 157.25, 148.11, 139.40, 134.83, 130.75, 130.39, 129.18, 120.74, 120.66, 118.30, 117.06, 113.35, 21.08.

3'-(4- nitro phenyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-oxazine-2,4'-dione (17)

[$C_{21}H_{12}N_4O_7$]: deep brown powder, 50% yield; m.p. 308-310 °C; Rf=0.58; FTIR (ATR) (cm^{-1}): 3294 (NH), 3071 (Ar-H), 1732 (C=O amide), 1695 (C=O oxazine ring), 1588 & 1320 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.60-8.53

(m,11H, Ar-H), 11.64 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 169.09, 160.90, 148.30, 135.24, 131.88, 130.47, 128.87, 126.86, 120.77, 117.20, 116.55, 116.24, 112.84, 112.67.

3'-(4- chloro phenyl)-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-oxazine-2,4'-dione (18)

[$C_{21}H_{12}ClN_3O_5$]: pale brown powder, 55% yield; m.p. 268-270 °C; Rf=0.63; FTIR (ATR) (cm^{-1}): 3320 (NH), 3066 (Ar-H), 1729 (C=O amide + C=O oxazine ring overlap), 1527 & 1338 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.56-8.31 (aromatic ring), 11.13 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm:167.77, 162.82, 149.18, 147.88, 141.98, 140.61, 135.27, 132.14, 130.96, 130.62, 130.07, 129.96, 128.94, 128.74, 121.85, 120.80, 120.49, 120.16, 116.85, 116.47, 116.27, 113.18.

3'-[4-(phenylamino) phenyl]-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-oxazine-2,4'-dione (19)

[$C_{27}H_{18}N_4O_5$]: purple powder, 75% yield; m.p. 223-225 °C; Rf=0.53; FTIR (ATR) (cm^{-1}): 3414,3386 & 3237 (NH), 3045 & 3022 (Ar-H), 1735 (C=O amide), 1695 (C=O oxazine ring), 1513 & 1333 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 5.84 (s,1H, -NH-), 6.68-8.44 (m,16H, Ar-H), 11.27 (s,1H, NH indoline). ^{13}CNMR (76 MHz, DMSO) δ/ppm: 168.37, 163.75, 145.82, 143.78, 142.29, 138.81, 135.56, 130.34, 129.72, 129.52, 121.15, 120.96, 120.41, 118.61, 118.41, 118.22, 117.97, 117.34, 115.35, 115.10.

3'-phenyl-5-nitro spiro-3,2'-indolin-benzo[e]-1',3'-oxazine-2,4'-dione (20)

[$C_{21}H_{13}N_3O_5$]: orange powder, 75% yield; m.p. 288-290 °C; Rf=0.47; FTIR (ATR) (cm^{-1}): 3246 (NH), 3068 (Ar-H), 1728 (C=O amide), 1680 (C=O oxazine ring), 1528 & 1313 (NO_2); ^{1}HNMR (DMSO-d_6, 300 MHz,) δ/ppm: 6.59-8.58 (m,12H, Ar-H), 11.76 (s,1H, NH). ^{13}CNMR (76 MHz, DMSO) δ/ppm:169.29, 162.91, 150.82, 139.53, 135.26, 132.07, 131.91, 130.89, 130.57, 130.04, 128.94, 125.59, 120.80, 120.57, 118.04, 117.26, 117.03, 116.75, 116.26, 113.52.

MTT anticancer assay

In vitro method was performed to investigate the effect of compounds (9, 14, 19) on MCF-7 cell line. After the cells in the vessel made confluent monolayer, the following protocol was performed: PBS was used to wash the cell sheet after the growth medium was aspirated then two to three ml Trypsin/ versine solution were added to the cell was added. The vessel was turned over to cover the monolayer completely with gentle rocking. The vessel allowed incubating at 37 °C for 1 to 2 minutes. Until the cells
had detached from the vessel. Fresh complete RPMI medium (15-20 ml) was added and cells were dispersed from the wedding surface into growth medium by pipetting. Cells were reordered at required concentration into culture vessels, flasks or plates whatever needed and incubated at 37 °C in 5 % CO2 incubator. Cell concentration was accomplished by counting the cells using the haemocytometer and applying the formula:

Total Cell Count/ml: cell count × dilution factor (or sample volume) ×104

The cytotoxic effect of different concentrations (25, 50, 100, 200, and 400 µg/mL) from (9, 14, 19) was performed using MTT ready to use kit contain MTT solution 1ml ×10 vials and solubilization solution 50 ml× 2 bottle. Tumor cells (1x104–1x106 cells/ml) were grown in 96 flat well micro-titer plates, in a last volume of 200 µL complete culture medium per each well. The microplate was surrounded by sterilized parafilm and shacked gently. Plates were incubated at 37 °C, 5 % CO_2 for 24 hrs. Medium was removed and two-fold serial dilutions of the desired concentrations of (9, 14, 19) (25, 50, 100, 200, and 400 µg/mL) were added to the wells. Triplicates were used for each concentration as well as the controls (cells treated with serum free medium). Plates were incubated at 37 °C, 5 % CO_2 for selected exposure time (24 hours). 10 µl of the MTT solution was added to each well. Then Plates were further incubated at 37 °C, 5 % CO_2 for 4 hours. After incubation, the media were cautiously removed and 100 µL of solubilization solution was added per each well for 5 min. By using an ELISA reader for measurement at 575 nm wave length. The data of optical density was subjected to statistical analysis in order to evaluate the concentration of compounds required to cause 50 % reduction in cell viability for each cell line [24-25].

Anti-oxidant activity test: DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) method used to determined antioxidant activity of final compounds by the ability of compounds to scavenge free radicals. Using this technique, antioxidants removed the purplish color from the PPH solution which purple-colored solution made by dissolving 24 mg of DPPH in 100 mL of methanol then combined with 1.6 mL of synthesized compounds in methanol at various concentrations (50, 100, 150, and 200 g/mL). After 30 minutes of darkness and room temperature, the solution absorbance at 517 nm was measured. The reference employed was ascorbic acid. As a negative control, 2.4 mL of DPPH solution and 1.6 mL of methanol were combined. The following equation was used to get the antioxidant percentage: -

$$\%\boldsymbol{DPPH\ radical\ scavenging\ activity} = \left(\frac{\boldsymbol{A_0 - A_1}}{\boldsymbol{A_0}}\right) \times \mathbf{100}$$

Where A0 was absorbance of the control, A1 was absorbance of the sample [26].

Molecular docking experiments

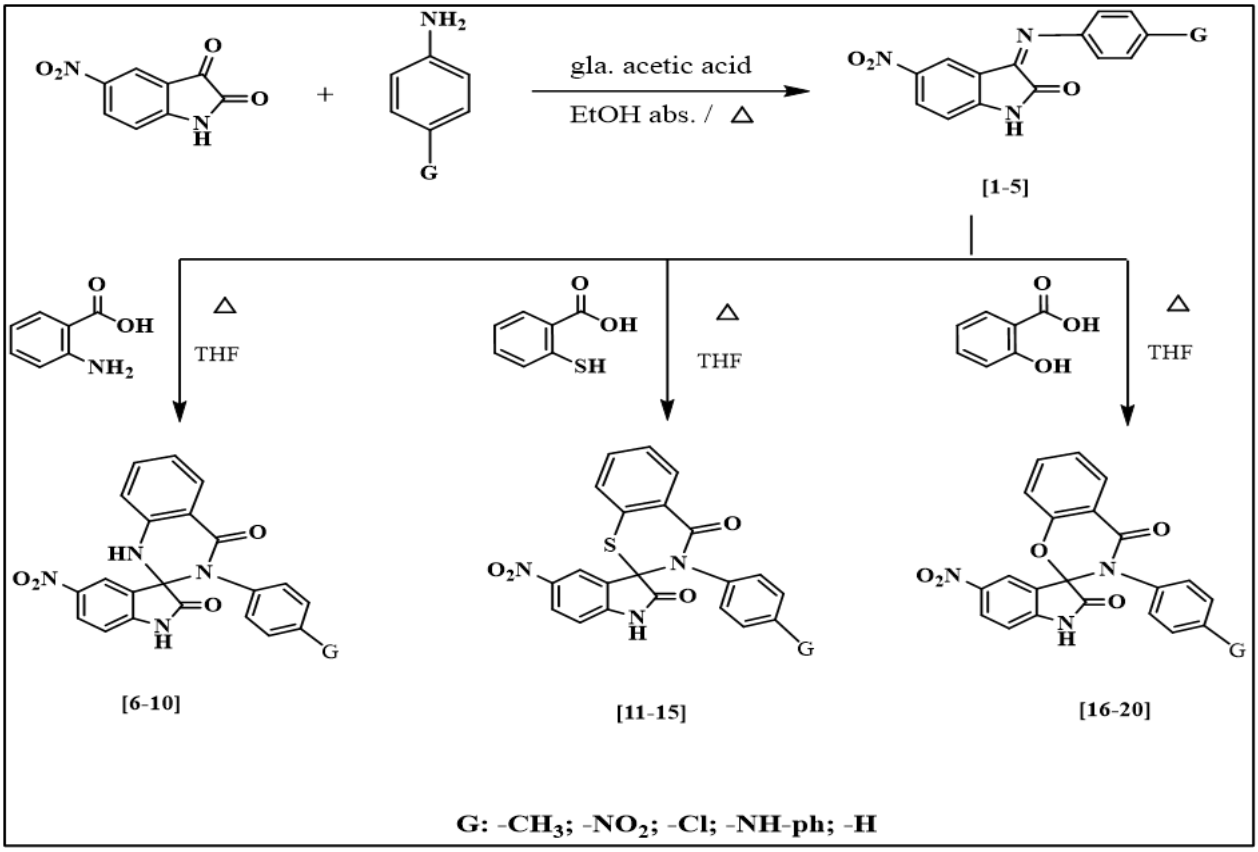
The CCDC GOLD Suite was used for the compounds' molecular docking investigations (v. 5.6.2). The CCDC Hermes visualizer program (v. 1.9.2) was employed to visualize the protein, ligands, hydrogen bonding interactions, brief contacts, and bond length measurement. The chemical structures of our ligands were shown using Chem Bio Office (v. 19.1) software [27-29].

Statistical analysis

The statistical were performed in triplicate and data were expresses as mean ± standard deviation (SD). Significant variances (ns: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$) between each compound with Tamoxifen were analysed by two-way ANOVA (Tukey) and student's t-test (one-tailed unpaired) using GraphPad Prism version 9.0 (GraphPad Prism software Inc., La Jolla, CA, USA). [24].

Results and Discussion

The synthesis processes were done to obtain spiro quinozolin-4-one 6–10; spiro thiazine-4-one 11–15; spiro oxazine-4-one 16–20 started by a nucleophilic addition reaction to the carbonyl group to have Schiff's bases 1–5 were created when the primary aromatic amine reacts with 5-nitro isatin as a ketone using acid that is catalyzed under reflex and ethanol and DMF as solvents, followed by cyclization of Schiff base derivatives with anthranilic acid, 2-mercapto benzoic acid, and salicylic acid, respectively under reflex (Scheme 1). Nuclear magnetic resonance (^{1}H and ^{13}C) and Fourier-transform infrared were used to characterize the structure of the compounds that had been prepared.



Scheme 1.

According to FTIR data, the signal at 1773 cm-1 for 5-nitroisatin vanished, and a peak at 1659–1648 cm-1, which is consistent with the compounds' imine groups' (C = N) vibrations, emerged (1–5). After the synthesis of spiro quinozolin-4-one (6–10), spiro thiazine-4–one (11-15) and spiro oxazine–4-one (16-20) absorption band formed at 1663–1698 cm-1, 1679–1709 cm-1, and 1680–1729 cm-1 respectively while peaks of (C = N) bands disappeared, and seen peaks at 1725–1736 cm-1 for the 5-nitro isatin's (C=O amide).

^{1}HNMR and 13CNMR are used to identify 5-nitro isatin and prepared compounds. All of the compounds were identified by 1HNMR, it was resulted in a new signal at 2.13-2.41 ppm that binds to the CH3 group of compounds 1, 6, 11, and 16, as

well as new values for the new benzene rings, which overlap with the original benzene ring values for 5-nitro isatin also new signal at 3.62-4.34 ppm for NH of quinozolin-4-one ring. The most accurate measurements for interpretation are the 13CNMR readings. In 13CNMR, we noticed that Schiff bases derivatives appeared signal of C=N at 156-160 and disappeared signal at 182 for C=O keto group in 5-nitro isatin. When synthesis spiro compounds the imine group disappeared and new signal at ppm for 160.93-163.66 ppm, 162.07-162.77 ppm and 160.90-163.75 for C=O of quinozolin-4-one, thiazine-4-one and oxazine-4-one rings respectively while signal at 163.48-170.71 ppm for C=O of amide group of 5-nitro isatin.

Biological evaluation

Using the MTT method, compounds (9, 14 and 19) were tested for their ability to inhibit MCF-7 breast cancer cells in vitro. For those molecules that in this experiment indicated considerable growth proliferation inhibiting effects.

Antiproliferative activity

The test of 3-(dimethylthiazl-2-yl)-2,5-diphenyltetrazolium bromide (MT) was accomplished to conclude the cytotoxic effect of compounds (9, 14, 19) on breast cancer cell line (MCF-7). MTT Assay was made to calculate the cell viability and inhibition rate on the tumor cell line by using different concentrations of compounds (9, 14, 19). The percentage viability of treated cells was calculated in a comparison with normal cell line HdFn. The cytotoxic effect of compounds (9, 14, 19) in concentration ranged from (25-400 µg/ml) on MCF-7 cells after 24 hours, 48 hours and 72 hours (Table 1-3) presented a decrease in cell viability in a dose-dependent pattern. The cell viability is reduced by increasing the concentration of compounds (9, 14, 19) that showed significant with reference drug ($p \leq 0.01$). So, after 24 hours, compound 14 showed lower (IC50=79.85) than reference drug Tamoxifen with (IC50=91.16) as shown in Figure 2, while after 48 hours still compound 14 have lower IC50 compared with other compounds as shown in Figure 3 but after 72 hours the IC50 value of compounds 9,14 and 19 have not good cytotoxicity as shown in Figure 4. According to data the spiro thiazine-4-one of 5-nitro isatin moiety with substituted 4-(phenylamino) in ring give good cytotoxicity against breast cancer.

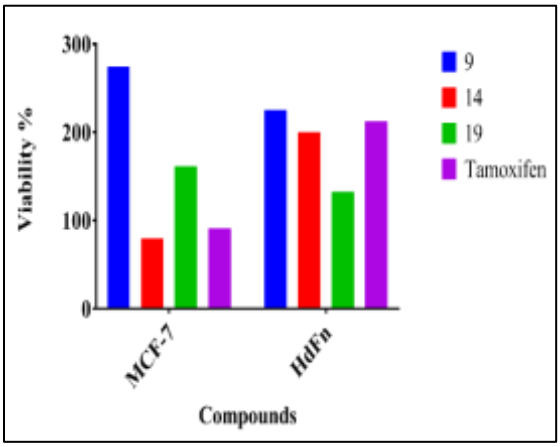


Figure 1. Cytotoxic activities of 9, 14, 19 and Tamoxifen toward MCF-7 and HdFn after 24h

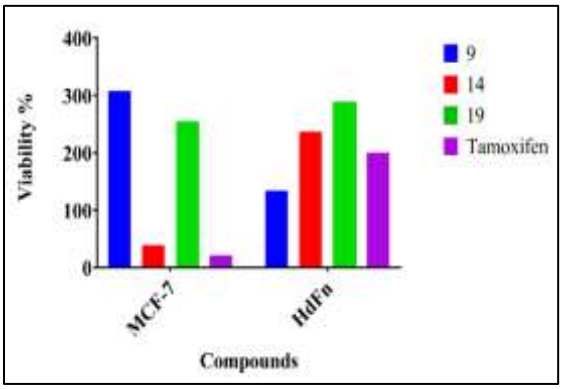


Figure 2. Cytotoxic activities of 9, 14, 19 and Tamoxifen toward MCF-7 and HdFn after 48h

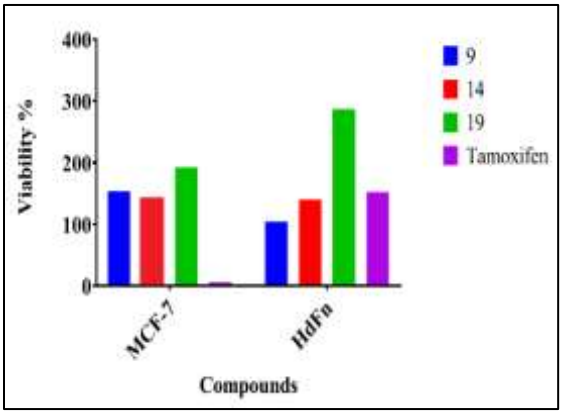


Figure 3. Cytotoxic activities of 9, 14, 19 and Tamoxifen toward MCF-7 and HdFn after 72h

Table 1. Cytotoxicity effect of compounds 9, 14, 19 and reference drug tamoxifen on MCF-7 and HdFn cells after 24 hours incubation at 37ºC

Comp. No.	Con. (µg/mL)	Viable cell count of MCF-7 cells line Mean± S.D.	Viable cell count of HdFn cell line Mean± S.D.	IC_{50} of MCF-7 cells line	Sig.	IC_{50} of HdFn cells line	Sig.
9	400	71.91 ± 1.30	77.96 ± 4.79	274.2	**	225.6	**
	200	82.02 ± 0.64	85.94 ± 4.07		**		ns

	100	90.97 ± 2.91	92.21 ± 0.47		**		ns
	50	94.83 ± 2.43	95.72 ± 0.64		**		ns
	25	97.96 ± 1.39	95.41 ± 1.14		**		ns
14	400	65.93 ± 2.79	72.30 ± 3.55	79.85	**	200.5	ns
	200	72.44 ± 5.91	83.72 ± 1.39		**		ns
	100	77.62 ± 2.70	94.52 ± 0.71		**		ns
	50	88.27 ± 4.31	95.45 ± 0.64		**		ns
	25	94.17 ± 3.44	95.06 ± 1.24		**		ns
19	400	68.67 ± 5.27	81.60 ± 2.42	161.4	**	132.6	**
	200	77.12 ± 3.68	83.26 ± 1.86		**		ns
	100	88.85 ± 0.35	92.82 ± 1.01		**		ns
	50	94.60 ± 1.95	94.95 ± 1.12		**		ns
	25	94.41 ± 3.06	94.83 ± 0.97		**		ns
Tamoxifen	400	15.05 ± 2.17	68.56 ± 2.76	91.16	212.7		
	200	21.37 ± 7.42	83.26 ± 3.85				
	100	37.23 ± 4.77	93.98 ± 2.12				
	50	48.19 ± 3.28	94.64 ± 0.57				
	25	59.38 ± 2.55	94.56 ± 0.50				

Significant difference between each compound with Tamoxifen. ns: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$

Table 2. Cytotoxicity effect of compounds 9, 14, 19 and reference drug tamoxifen on MCF-7 and HdFn cells after 48 hours incubation at 37ºC

Comp. No.	Con. (µg/mL)	Viable cell count of MCF-7 cells line Mean± S.D.	Viable cell count of HdFn cell line Mean± S.D.	IC_{50} of MCF-7 cells line	Sig.	IC_{50} of HdFn cells line	Sig.
9	400	61.342±1.97	67.32±3.82	307.6	**	134.3	*
	200	75.57±2.5	72.801±2.40		**		ns
	100	85.687±2.43	85.60±2.5		**		**
	50	93.904±0.406	93.4±0.64		**		ns
	25	95.332±0.74	94.52±0.24		**		ns
14	400	49.537±1.116	65.316±3.79	39.09	**	236.6	ns
	200	55.247±0.37	77.314±1.72		**		ns
	100	59.68±1.54	86.304±2.022		**		**
	50	72.801±0.41	94.136±0.7		**		ns
	25	85.571±1.35	94.63±0.52		**		ns
19	400	74.0±1.04	72.8±3.51	251.1	**	286.5	**
	200	84.72±2.00	86.034±0.85		**		**
	100	88.7±1.70	93.287±1.10		**		ns
	50	93.71±0.707	95.023±0.87		**		ns
	25	95.216±0.24	94.599±1.6		**		ns
Tamoxifen	400	12.34±0.52	60.378±0.813	21.51	200		
	200	15.47±2.087	77.08±5.52				
	100	26.852±1.3	93.59±2.1				
	50	34.87±2.66	94.174±1.5				
	25	48.109±3.26	94.05±0.37				

Significant difference between each compound with Tamoxifen. ns: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$

Table 3. Cytotoxicity effect of compounds 9, 14, 19 and reference drug tamoxifen on MCF-7 and HdFn cells after 72 hours incubation at 37ºC

Comp. No.	Con. (µg/mL)	Viable cell count of MCF-7 cells line Mean± S.D.	Viable cell count of HdFn cell line Mean± S.D.	IC_{50} of MCF-7 cells line	Sig.	IC_{50} of HdFn cells line	Sig.
9	400	52.08±1.63	57.523±5.8	153.5	**	104.2	ns
	200	63.31±1.5	62.92±2.33		**		ns

	100	76.15±1.7	77.662±1.40		**		ns
	50	86.227±3.3	87.4±1.42		**		ns
	25	94.44±0.50	94.71±0.67		**		ns
14	400	48.7±5.6	52.73±2.85	138.6	**	140	ns
	200	65.27±2.82	65.12±1.38		**		ns
	100	74.88±2.19	73.8±1.21		**		ns
	50	84.83±1.50	88.19±2.69		**		ns
	25	93.24±0.83	93.9±0.64		**		ns
19	400	64.81±1.86	72.83±3.51	191.1	**	286.5	**
	200	74.614±1.96	86.034±0.85		**		**
	100	85.26±0.63	93.287±1.10		**		**
	50	92.36±1.4	95.023±0.87		**		ns
	25	94.753±0.75	94.599±1.6		**		ns
Tamoxifen	400	8.02±1.90	53.858±2.74	5.68	151.8		
	200	12.53±1.7	65.625±0.75				
	100	19.17±1.3	78.588±5.73				
	50	27.662±2.32	90.741±3.47				
	25	40.278±2.80	93.82±0.43				

Significant difference between each compound with Tamoxifen. ns: non-significant, **: $p \leq 0.01$, *: $p \leq 0.05$

Anti-oxidant

In this section, the use of DPPH to identify the antioxidant capabilities of compounds (6–20) is discussed. The maximum absorption at free-radical DPPH interaction with an odd electron occurs at 517 nm (purple color). The reaction between DPPH and a free-radical scavenger antioxidant produces DPPHH, which has a lower absorbance than DPPH due to the lower hydrogen content. This radical form exhibits decolorization (a yellow color) in compared to the DPPH-H as the quantity of electrons gathered rises. Table 4 and Figure 5 shows that compounds 6-20 had not good antioxidant activity, except compounds (11,13,14,15) with very low antioxidant percentages.

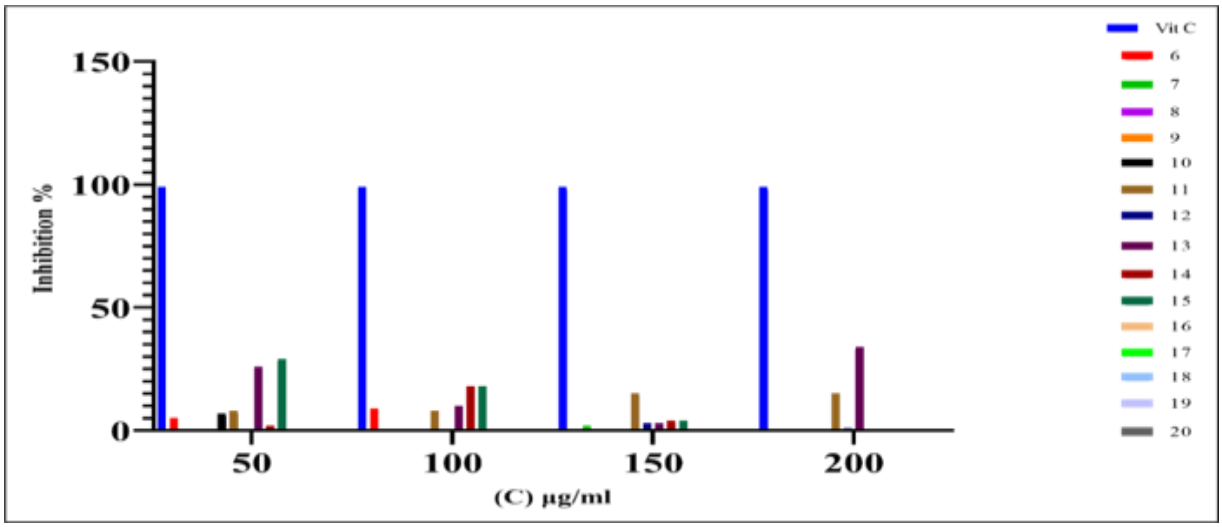


Figure 4. Effect of the synthesized compounds toward DPPH.

Table 4. DPPH Scavenging Assay

Compounds	% Inhibition activity of different concentration			
	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL
6	5	9	0	0
7	0	0	2	0
8	0	0	0	0
9	0	0	0	0
10	7	0	0	0
11	8	8	15	15

12	0	0	3	1
13	26	10	3	34
14	2	18	4	0
15	29	18	4	0
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
Ascorbic acid (Vit C)	99	99	99	99

Molecular docking studies

A genetic Computational technique called GOLD (Genetic Optimization for Ligand Docking) is used to dock flexible ligands into protein binding sites. Perfect performance for posture prediction and outstanding outcomes for virtual screening have been demonstrated by the GOLD Suite [27-29]. The experimental section contains a thorough description of the steps taken to carry out these theoretical experiments. By analyzing the contact interactions between the active binding sites of the protein and the target molecules, the docking results forecast the selectivity and binding energies of the ligands for the human estrogen alpha receptor. Based on their PLP fitness, the final compounds (6-20) and tamoxifen as a reference medication were rated. According to (table 5), the docked compounds' PLP fitness ranged from 56.20 to 92.3 on the human estrogen alpha receptor. Docking analysis indicted those compounds 9, 14, 19 interact through hydrogen bonding and short contacts with our final ligand's library, and show promising anti-breast cancer activity. Compound 14 is shown bound to the human estrogen alpha receptor in Figure 6 (14) by hydrogen bonds of GLU 353, which are bonds length was ≤3 Aº. ILE 424 (3), PHE 404 (5), MET 388 (4), LEU 384, LEU 387 are examples of amino acid residues with short contact interactions that support the more prevalent hydrogen-bonding interactions.

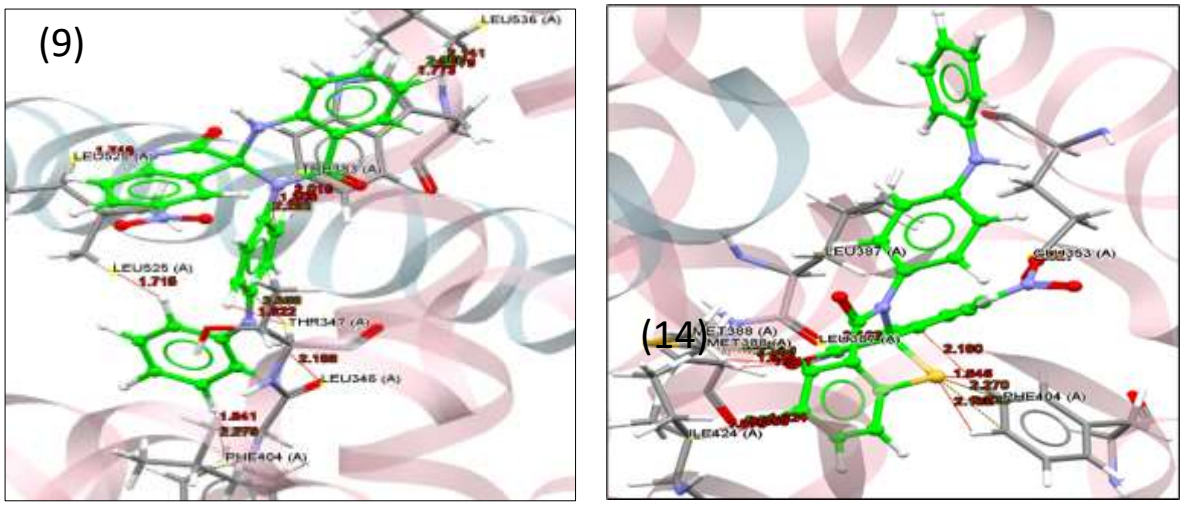
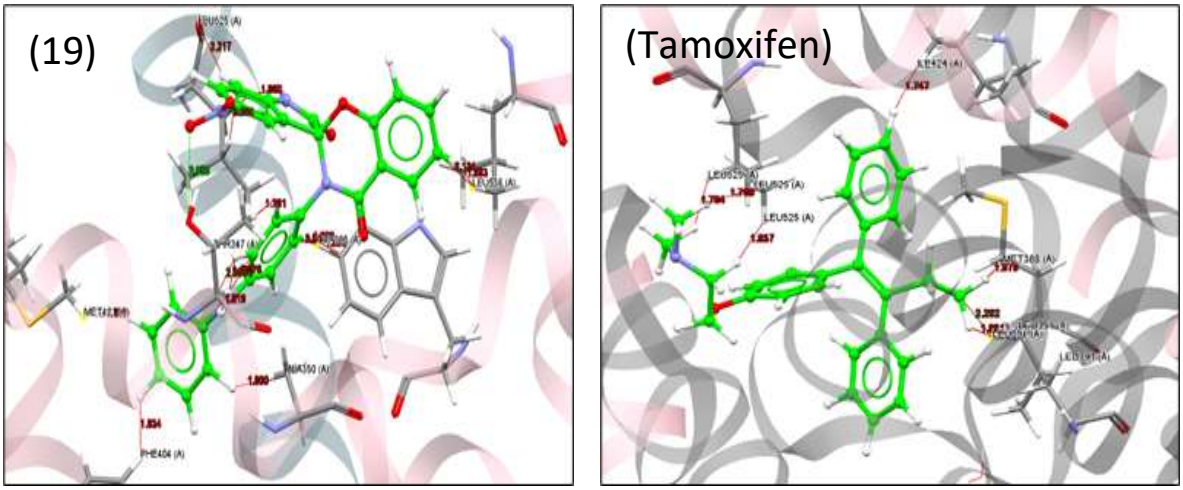


Figure 5. Molecular docking results for interactions of compounds 9,14,19 and tamoxifen with human estrogen alpha receptor (1ERR) breast cancer



Continued Figure (5)

Table 5. Molecular docking results for the interaction between 6-20, Tamoxifen and human estrogen alpha receptor

Compounds	Binding energy (PLP fitness)	H-bond interaction Amino acids residues	Short contact interaction Amino acids residues
6	56.7	-	TRP 383 (3), LEU 525 (2), GLY 521, ILE 424 (3), GLU 353 (2), LEU 346
7	66.22	GLU 353, ALA 350	LEU 387 (2), LEU 384, PHE 404, MET 421, MET 388 (5), ILE 424 (2), LEU 428 (6)
8	56.81	THR 347	PRO 535, LEU 354 (2), TRP 383 (3), LEU 525, THR 347(3)
9	74.66	LEU 536	LEU 525 (2), TRP 383 (3), LEU 536 (3), THR 347 (2), LEU 346, PHE 404
10	58	-	TRP 383 (3), LEU 387, LEU 346 (7), ILE 424 (2), MET 388 (3)
11	66.78	ALA 350, GLU 353	PHE 404 (3), LEU 384 (2), ILE 424 (6), MET 388 (5)
12	63.6	ALA 350, GLU 353	THR 347, LEU 387 (2), PHE 404, MET 388 (5), ILE 424 (3), LEU 428 (2)
13	61.76	-	LEU 539, LEU 354, THR 347, LEU 525 (4), TRP 383
14	81.99	GLU 353	ILE 424 (3), PHE 404 (5), MET 388 (4), LEU 384, LEU 387
15	66.46	ALA 350, GLU 353	LEU 349, LEU 387 (2), PHE 404, TRP 383, LEU 525, LEU 384 (2), MET 388 (5), ILE 424 (2)
16	59.61	-	ILE 424 (3), GLU 353 (2), LEU 384, PHE 404, MET 421
17	63.64	-	PHE 404 (2), MET 388 (5), ILE 424 (3), MET 421, PHE 425
18	56.20	ALA 350	LEU 346, PHE 404 (2), MET 388 (3), ALA 350 (2), LEU 387 (5), LEU 384, LEU 428 (3), ILE 424 (3), MET 421
19	72.43	THR 347	ALA 350, THR 347 (5), TRP 383, MET 421, LEU 525 (3), PHE 404, LEU 536
20	61.44	-	ILE 424 (2), MET 388, LEU 525, MET 421 (2), ALA 350 (2), LEU 387 (2), LEU 346
Tamoxifen	92.3	-	ILE 424, LEU 525 (3), LEU 391 (2), MET 388

Conclusion

Spiro six membered ring of 5-nitro isatin were synthesized via [2+2] cycloaddition reaction between different Schiff bases of 5-nitro isatin and anthranilic acid, o-mercapto benzoic acid and salicylic acid. Purification, structural characterization, and in vitro cytotoxicity testing against cancer cell line (MCF-7) were performed on every produced molecule. compounds (9,14,19) showed significant against tamoxifen ($p \leq 0.01$) but according to IC50, after 24hours and 48hours, compound 14 showed good anti-cell proliferation behavior against the cancer cell line compared to Tamoxefin. DPPH assay anti-oxidant activity shows not good antioxidant.

Conflict of Interest

There is no conflict of interest in the manuscript.

Funding

The work has no funding

Author Contribution

The authors confirm contribution to the paper. all authors reviewed the results and approved the final version of the manuscript

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