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# Comparison of *in vitro* Anticancer efficacy of o-diaminocyclohexane Schiff-base ($H_2L$) and Pd (II) complex (PdL) on MCF-7 Breast Cancer Cell Line

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## ABSTRACT

**Background:** Breast cancer is prevalent in women, with high mortality rates worldwide. Males can also develop it, accounting for less than 1% of all breast cancer cases. Chemotherapy often leads to multidrug resistance, highlighting the need for more effective treatments. Schiff bases have attracted attention due to their strong coordination ability with metal ions, enhancing biological activities, including anticancer effects. Palladium (Pd(II)) chelate complexes are widely explored for their distinct anticancer properties. **Aim of the study:** This study investigates the in vitro anticancer properties of o-diaminocyclohexane Schiff-base ($H_2L$) and its Pd(II) complex (PdL) against the MCF-7 cell line, focusing on cytotoxicity and selectivity. **Materials and Methods:** The anticancer efficacy of $H_2L$ and PdL was assessed on MCF-7 cells utilizing the MTT assay, with dose-dependent cytotoxicity measured after 48 hours. Selectivity was assessed by comparing $IC_{50}$ values against normal human skin fibroblast (HSF) cells. **Results:** The results confirmed that these two newly synthesized compounds reduced cell viability in a way that was dependent on concentration. In particular, PdL exhibited greater cytotoxicity than $H_2L$ against MCF-7 cells, having an $IC_{50}$ of 118.48 ± 6.03 µg/mL. However, its potency was approximately threefold lower than doxorubicin's ($IC_{50}$ = 38.998 ± 0.008 µg/mL), highlighting the need for further structural optimization to enhance its therapeutic potential. **Conclusions:** These findings highlight the potential of PdL and $H_2L$ as lead compounds for novel anticancer drug development. Further structural modifications and mechanistic studies are warranted to enhance selectivity and potency.

## 1. Introduction

Cancer is a major worldwide health issue [1-4], typified by uncontrolled cell proliferation and spread [5, 6]. It progresses through three stages: initiation (DNA damage by carcinogens), promotion (faulty cell proliferation), and progression (tumor metastasis). Breast, colorectal, lung, skin, prostate, and stomach cancers are among the common types of cancer [7, 8]. Cancer causes more deaths than AIDS, malaria, and tuberculosis combined, with rising incidence rates [9, 10]. By 2030, cases are expected to reach 21.4 million, with 13.2 million deaths annually [7]. In women, breast cancer is the most prevalent type of cancer and a major contributor to cancer-related death [11-14]. In Egypt, it is the most frequently diagnosed cancer among women, with approximately 46,000 new cases projected by 2050 [15]. It also remains the primary cause of cancer-related deaths among women globally, accounting for an estimated 680,000 fatalities annually [16].

The breast cancer survival rate in Egypt is relatively low, ranging from 28% to 64%, a trend largely attributed to limited public awareness, delayed healthcare-seeking behavior, and inadequate screening programs [16]. A meta-analysis conducted by Azim et al. [15], which included over 15,067 patients across 12 studies, reported a mean age at diagnosis of 50.4 years in the Egyptian population. Notably, 57% of cases occurred in premenopausal or perimenopausal women, suggesting a younger age of onset compared to Western populations. Breast cancer can metastasize to vital organs, including bones, heart, lungs, and brain [11]. Early detection and treatment improve outcomes [9].

The challenge of cancer treatment lies in selectively eliminating cancer cells while preserving healthy tissue [17]. Currently, available therapies include immunotherapy, hormone therapy, targeted therapy, chemotherapy, and surgery [18, 19]; but these face challenges such as toxicity, recurrence, and multidrug resistance (MDR) [12, 18, 20, 21]. A key MDR mechanism is the overreaction of expression of ATP-binding cassette (ABC) transporters, including ABCB1 and ABCC3, which actively expel therapeutic agents from cancer cells,

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reducing intracellular drug accumulation [19, 22, 23]. This is particularly relevant for Doxorubicin, a widely used topoisomerase II inhibitor in breast cancer treatment, which induces DNA strand breaks and oxidative stress but is often rendered ineffective due to MDR mechanisms [24-26]. Furthermore, Doxorubicin's clinical application is constrained by dose-dependent cardiotoxicity [27, 28], necessitating the search for alternative therapies with improved efficacy and safety [12, 29].

Despite significant advances in cancer therapy over the past few decades [30, 31], breast cancer remains globally one of the top causes of mortality [32, 33]. To overcome these limitations, researchers are exploring alternative strategies, including metal-based therapeutics. Among them, Schiff-base complexes of transition metals have drawn attention because of their enhanced selectivity and cytotoxicity against cancer cells [34]. Schiff bases are organic compounds with significant biological and pharmacological properties, making them a focus of active research [35]. Schiff bases coordinate with metal ions through both nitrogen and oxygen forming stable complexes [36]. These complexes have shown anticancer potential, targeting various cancer cell lines. They exhibit promising anticancer activity by interfering with DNA replication, inducing apoptosis, and inhibiting key enzymatic pathways [18]. Transition metal complexes, particularly palladium (Pd(II)) chelates, show strong anticancer activity [3, 37]. Due to their complexes' structural resemblance to those containing Pt(II), Pd(II) is an excellent choice [34]. It was previously discovered that several Pd(II) complexes exhibit comparable or even superior activity to Pt(II)-containing medications. Pd(II) complexes show reduced Cisplatin cross-resistance, high specificity, and decreased toxicity [3]. Hence scientists' attention has been drawn to Pd(II) coordination compounds for anticancer investigations [34, 38]. Schiff bases have been employed as anticancer agents and are known to enhance the possibilities of complex design when combined with palladium metal [37, 39]. Schiff base-Pd(II) coordination enhances drug design potential by improving selectivity and cytotoxicity [34]. The current study aimed to synthesize a novel anticancer agent, consisting of o-diaminocyclohexane Schiff base ($H_2L$) and its Pd(II) complex (PdL), and assess their in vitro anticancer efficacy on the MCF-7 breast cancer cell line, focusing on selectivity in targeting cancer cells over normal human skin fibroblast (HSF) cells, in addition to cell viability and cytotoxicity in comparison to Doxorubicin using $IC_{50}$ values. MCF-7 cells are widely used in breast cancer research due to their well-characterized hormone receptor status and responsiveness to chemotherapy. They serve as an ideal model for evaluating novel anticancer agents [40].

## 2. Materials and methods

### 2.1. Materials

All analytical grade chemical substances and reagents used in this work were provided by the following suppliers and utilized without additional purification: paraformaldehyde ($(CH_2O)_n$) sourced from Roth, o-vanillin (o-Val), 1,2-diaminocyclohexane (DACH), 4-methoxypyridine (4-MeOPy) obtained from Sigma-Aldrich, and anhydrous $ZnCl_2$ procured from GRUSSING GmbH.

### 2.2. Instrumentation

A BUCHI melting point B-540 device was utilized to determine melting points, which were recorded in open glass capillaries without correction. A BRUKER Tensor-37 FT-IR spectrophotometer was used to gather FTIR spectra, scanning from 400 to 4000 $cm^{-1}$ on KBr discs with a resolution of 2 $cm^{-1}$ in the 4000–550 $cm^{-1}$ range. Broad (br), sharp (sh), weak (w), medium (m), strong (s), and very strong (vs.) were the symbols used to indicate the intensities of the signals. $^1$H-NMR and $^{13}$C-NMR spectra were measured using Bruker Avance DRX200 (200 MHz) and DRX500 (125 MHz) spectrometers, respectively. Signal multiplicities were symbolized by the following acronyms: s for singlet, d for doublet, t for triplet, q for quartet, and m for multiplet. Mass spectra of the synthesized compounds were obtained using two instruments: a BRUKER Ultraflex MALDI-TOF system and a UHR-QTOF maXis 4 G (Bruker Daltonics). The MALDI-TOF equipment utilized a nitrogen laser operating at 337 nm with a frequency of 10 Hz. Both devices were employed to analyze positive ions in linear mode.

### 2.3. Synthesis of 5-chloromethyl-vanline (CMV, 1)

In a 250 mL two-necked round-bottom flask (RBF), a combination of paraformaldehyde (4.5 g, 150.0 mmol), anhydrous $ZnCl_2$ (3 g, 22.0 mmol), and concentrated hydrochloric acid (50 mL) was vigorously agitated at ambient temperature under an HCl atmosphere for 30 minutes until the solids completely dissolved. Subsequently, a solution of o-vanillin (15 g, 98.6 mmol) in benzene (75 mL) was introduced dropwise with constant stirring under an HCl atmosphere. The reaction mixture was subjected to a continuous flow of HCl gas for an additional 4 hours and kept at a temperature < 20°C. Subsequently, a mixture of water (50 mL) and crushed ice (50) was added to the content. This led to the formation of two distinct phases. The benzene layer was then carefully extracted through aspiration to eliminate resinous byproducts. It was then thoroughly rinsed with deionized water until the pH of the rinse water reached a neutral value of 7. Following drying over anhydrous sodium sulfate, distillation was used to remove the benzene. The thick, dark brown-black oil that was produced underwent two rounds of crystallization using petroleum ether with a boiling range of 100-120 degrees, yielding CMV as orange needles (11.08 g, 56%) with a melting point of 40-41°C. FT-IR (KBr, $cm^{-1}$): 3441 (s, br, phenolic O-H), 3091 (m, sh, aromatic CH), 3039 (m, sh, aromatic CH), 2969 (m, sh, aliphatic $CH_2$), 2871 (m, sh, aldehyde CH), 1647 (s, sh, aldehydic carbonyl), 1272 (s, sh, aryl-O), 688 (s, sh, C-Cl). $^1$H-NMR ($CDCl_3$, 200 MHz) δ (ppm): 11.19 (s, 1H, phenolic O-H), 9.92 (s, 1H, aldehyde CH), 7.41-7.17 (m, 2 H, 2 aromatic C-H), 4.59 (s, 2 H, benzylic $CH_2$), 3.97 (s, 3 H, $OCH_3$). $^{13}$C-NMR ($CDCl_3$, 126 MHz) δ (ppm): 196.49, 151.98, 148.79, 124.51, 121.01, 119.02, 118.42, 56.79, and 46.19.

### 2.4. Preparation of 1-((vanillyl)methyl)4-methoxypyridinium chloride (VMMPC, 2)

A solution of CMV (1.97 g, 9.83 mmol) in anhydrous toluene (25 mL) was added in a dropwise manner over 30 minutes to a vigorously agitated mixture of 4-MeO-Py (1.07 g, 9.75 mmol) in anhydrous toluene (15 mL) at ambient temperature under nitrogen atmosphere. After that, the mixture was heated to 60 °C and stirred under nitrogen for 24 hours. After cooling, the obtained products were thoroughly washed with anhydrous toluene (5 x 5 mL) and ether (5 x 10 mL) to eliminate unreacted materials. The desired product (2) was dried under vacuum and used in subsequent preparations without further purification. The pale-yellow solid compound 2 was obtained with a yield of 91% (5.50 g) and had a melting point of 69-71 $^{o}$C. FTIR (KBr, cm$^{-1}$): 3371 (m, br, phenolic O-H), 3142 (m, sh, aromatic C-H), 2939 (m, sh, aliphatic $CH_2$), 2873 (m, sh, aldehyde CH), 1643 (vs, sh, aldehyde C=O), 1571 (s, sh, bending C=N of pyridine), 1269 (s, sh, aryl-O), 1139 (s, sh, bending H-C=N and H-C=C of pyridinium ring). $^{1}$H-NMR (DMSO-$d_6$, 200 MHz) δ (ppm): 10.51 (s, 1H, phenolic O-H), 10.29 (s, 1H, aldehyde CH), 8.99 (d, *J* = 7.4 Hz, 2H, 2 pyridine-H), 7.67 (d, *J* = 7.4 Hz, 2H, 2 pyridine-H), 7.61 (d, *J* = 2.1 Hz, 1H, aromatic-H), 7.39 (d, *J* = 2.1 Hz, 1H, aromatic-H), 5.63 (s, 2H, benzylic $CH_2$), 4.05 (s, 3H, $OCH_3$), 3.89 (s, 3H, $OCH_3$). $^{13}$C-NMR (DMSO-$d_6$, 126 MHz) δ (ppm): 191.09, 170.99, 151.87, 148.96, 146.41, 126.01, 123.21, 120.29, 118.18, 114.21, 61.29, 58.60, and 56.91. A positive mode ESI-MS: peak at m/z = 274.2 a.m.u. ($[C_{15}H_{16}NO_4]^+$, M - Cl).

### 2.5. Synthesis of N,N′-Bis[5-((4-methoxypyridinium)methylene)-3-methoxy-salicylidene)-cis-1,2-cyclohexanediamine dichloride ($H_2L$, 3)

In a nitrogen-rich environment, a methanolic solution (10 mL) containing DACH (2.0 mmol) was introduced dropwise from a Schlenk tube into a 100 mL Schlenk flask comprising a solution (25 mL) of ionic liquid **2** (1.25 g, 4.05 mmol) in methanol. Under a nitrogen atmosphere, the solution was refluxed and agitated for 7 hours. Following this, the solvent was partially removed using vacuum distillation, and the addition of ethyl acetate led to the precipitation of yellow products of 3. The mixture was refrigerated overnight. After decanting the solvent, the resulting crude product underwent sonication in $Et_2O$ (3 X 25 mL) for 15 min. The $Et_2O$ was evaporated, and the resultant solid was extensively rinsed with a mixture of methanol and $Et_2O$ (1:3 ratio) to remove any unreacted components. Subsequently, the solid was dissolved again in methanol. Ethyl acetate was slowly added over 15 minutes, causing the desired product (**3**, $H_2L$) to precipitate as a yellow-orange solid. Filtration was employed to isolate the solid, which was then subjected to vacuum drying. The yield was 1.25 g (77.5%), with a melting point of 179-181 $^{o}$C. FTIR (KBr, cm$^{-1}$): 3398 (m, br, phenolic O-H), 3153 (w, sh, aromatic C-H), 2934 (m, sh, aliphatic $CH_2$), 1631 (vs, sh, azomethine H-C=N), 1551 (s, sh, bending C=N of pyridine), 1267 (s, sh, aryl-O), 1144 (s, sh, bending H-C=C+H-C=N of pyridine). $^{1}$H-NMR (DMSO-$d_6$, 200 MHz) δ (ppm): 13.49 (s, 2H, 2 phenolic O-H), 9.18 (d, *J* = 7.2 Hz, 4H, 4 pyridine-H), 8.58 (s, 2H, 2× azomethine H-C=N), 7.67–7.37 (m, 5H, 5 pyridine-H and aromatic-H), 7.29-7.05 (m, 3H, aromatic-H), 5.09 (s, 4H, 2 benzylic $CH_2$), 4.18 (s, 6H, 2 $OCH_3$), 3.91 (s, 6H, 2 $OCH_3$), 3.88-3.78 (m, 2H, 2× Cyclohexyl-H), 1.81–1.45 (m, 8H, 8× Cyclohexyl-H). $^{13}$C-NMR (DMSO-$d_6$, 126 MHz) δ (ppm): 170.82, 165.59, 151.31, 149.00, 146.31, 137.43, 132.23, 129.97, 126.15, 124.63, 114.15, 110.51, 65.23, 58.59, 56.62, 51.91, 26.17, 21.24, 19.03. A positive mode ESI-MS: peaks at m/z = 662.2 a.m.u for $C_{36}H_{42}ClN_4O_6^+$ [M – $Cl^-$] and 313.3 for $C_{36}H_{42}N_4O_6^{2+}$ [M – 2 $Cl^-$]. Anal. calcd for $C_{36}H_{42}Cl_2N_4O_6$ (M = 697.65 g/mol): C, 61.98; H, 6.07; N, 8.03%. Found: C, 61.93; H, 6.11; N, 7.98%.

### 2.6. Synthesis of cis-[[2,2′-][(1,2-cyclohexanediyl)bis(nitrilomethylidyne)]bis[4-(methoxy-pyridinium)methylene-3-methoxy-phenolat]-[N,N′,O,O′] palladium (II) dichloride monohydrate (PdL)

An ethanolic solution (10 mL) containing the free ligand ($H_2L$) (1 mmol) and 1 mL of concentrated HCl was prepared. To this, a solution of $PdCl_2$ (0.126 g, 1 mmol/5 mL EtOH) was added gradually. The resulting mixture was then heated under reflux conditions with continuous stirring for 6 hours. Subsequently, the solvent was removed under vacuum, leaving behind an oily residue. This residue was solidified by introducing petroleum ether (40–60) and storing it in a refrigerator overnight. The resulting solid products were separated by filtration and washed three times with 3 mL of a cold mixture of MeOH/$Et_2O$ (1:2) to obtain the $PdLCl_2$ complex. Obtained as a faint brown powder (69%). FTIR (KBr, cm$^{-1}$): 3430 (m, br, water O-H), 3142 (w, sh, aromatic C-H), 2932 (m, sh, aliphatic $CH_2$), 1623 (vs, sh, azomethine H-C=N), 1549 (s, sh, bending C=N of pyridine), 1259 (s, sh, aryl-O), 1145 (s, sh, bending H-C=C+H-C=N of pyridine) 1259, 542 (m, sh, Pd-O), 465 (w, sh, Pd-O). ESI-MS: peaks at m/z = 766.6 a.m.u. for $C_{36}H_{42}ClN_4O_6^+$ [M – $Cl^-$] and 365.5 for $C_{36}H_{42}N_4O_6^{2+}$ [M – 2 $Cl^-$]. Anal. calcd for $C_{36}H_{40}Cl_2N_4O_6Pd$ (M = 802.06 g/mol): C, 53.91; H, 5.03; N, 6.99%. Found: C, 53.87; H, 5.09; N, 6.95%.

### 2.7. In vitro anticancer activity

#### *2.7.1.* Cell Lines and Compounds

The human skin fibroblast (HSF) normal cell line and MCF-7 breast cancer cell line were obtained from the National Research Centre, Egypt. Dulbecco's modified Eagles medium (DMEM) with 5% fetal bovine serum, 1% L-glutamine, and an antibiotic-antimycotic mixture containing (10,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B, and 10,000 U/mL potassium penicillin) was used to cultivate the cells. With 5% $CO_2$, the cells were maintained at 37 $^{o}$C. Toxicity has been measured according to cell viability and cell morphology.

#### 2.7.2. Cytotoxicity assay

Cell viability was determined by measuring the mitochondrial-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

from yellow to purple formazan [41, 42]. Samples with varying concentrations (6.25-100 µg/mL) were applied to the cells and incubated for 48 h. Additionally, the MCF-7 breast cancer cell line was utilized to compare the effect to that of the standard drug, Doxorubicin. The safety of the synthetic compounds was examined using the HSF normal cell line as a noncancerous cell line model.

#### 2.7.3. Selectivity index (SI)

The investigated chemicals' selectivity index values on cancer cells were calculated using the methodology outlined by Mahdy et al. [43] and Taghour et al. [44]. The selectivity index (SI) is the ratio between a compound's toxic and effective concentrations (SI = $IC_{50}$ on non-cancer cells/$IC_{50}$ on cancer cells). Ideally, the compound's SI value should be at least 10. A low SI (<1) indicates that the studied substance is poisonous and unfit for use as a safe medication. If the SI value is within the range of 1 and 10, additional examination utilizing different biological systems is advised to corroborate the findings [44].

#### 2.7.4. Cell cultures and cytotoxic effect on human cell lines

The study's treatment groups were as follows: Cells alone provide negative control. Positive control: cells + Doxorubicin. Test groups: cells + PdL and cells + $H_2L$. The same manner of handling was pursued for HSF normal cell lines. Using a Laminar flow biosafety cabinet Class II A2 (made by Labconco), each of the subsequent procedures was performed in a sterile environment. Using a $CO_2$ incubator (Sartorius stedium, Biotech), cells were suspended in DMEM media with 5% fetal bovine serum, 1% L-glutamine, and 1% antibiotic-antimycotic mixture containing 10,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B, and 10,000 U/mL potassium penicillin at 37 ºC under 5% $CO_2$. After being batch cultured for 10 days, the cells were seeded in 96-well plastic plates with fresh complete growth medium at a concentration of $10x10^3$ cells/well. They were then kept at 37 ºC under 5% $CO_2$ for 24 hours, either by themselves (as a negative control) or in combination with various drug concentrations to provide a final concentration of (100, 50, 25, 12.5, 6.25 µg/mL). The medium was aspirated after 48 hours of incubation, and each well received 20 µL of MTT salt (2.5 µg/mL). The wells were then incubated for an additional four hours at 37 ºC under 5% $CO_2$. 200 µL of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl was added to each well to stop the reaction and dissolve the formed crystals. The wells were then incubated at 37 ºC overnight. As a known cytotoxic agent, utilized doxorubicin as a positive control [44, 45]. A microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) was then utilized at 595 nm to measure the absorbance using a wavelength of 620 nm as a reference. Every experiment was conducted in triplicate, and the mean values were determined. The cytotoxic impact of each experienced chemical on cells was calculated. Viability is drug absorbance divided by control absorbance times 100. Cytotoxicity = 100 - viability. The term "$IC_{50}$ value" refers to the medication concentration at which a 50% reduction in viable cells was observed [46]. Then the $IC_{50}$ concentration of these compounds was estimated by plotting a linear regression curve (y = mx + n), and the value of x with y set to 50 was calculated [5].

### 2.8. Statistical analysis

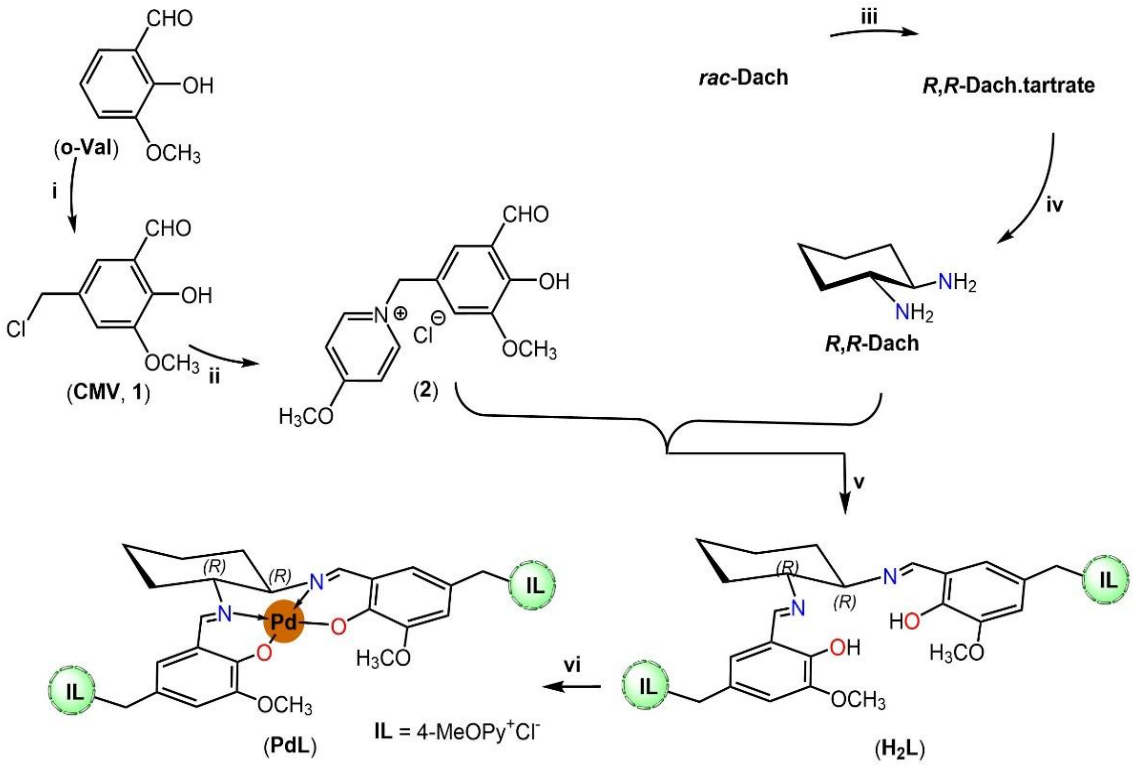
Each of the three replications' results was expressed as mean ± SD. Variance analysis ANOVA (one-way) followed by Tukey's test was applied to statistically analyze the data. Version 20.0 of the IBM SPSS software program for Windows (Armonk, NY: IBM Corp.) was utilized to estimate the level of statistical significance across groups. If a difference's p-value was less than 0.05, it was considered statistically significant.

## 3. Results and discussion

### 3.1. Chemistry of the synthesis protocol

**Scheme 1** illustrates the detailed procedure employed for producing a new ionic liquid (IL), vanillyl-pyridinium chloride (Val(4-MeOPy$^+Cl^-$, 2), and its corresponding o-diaminocyclohexane Schiff base ($H_2L$). This method integrates chloromethylation, quaternization, and Schiff base condensation techniques to yield the desired ligand. The synthesis of Val(4-MeOPy$^+Cl^-$) (2) begins with o-Val and involves two sequential reactions. The first step entails chloromethylation of o-Val using a mixture of paraformaldehyde and aqueous HCl, along with a catalytic amount of $ZnCl_2$, under a flow of HCl gas. This process results in the formation of 5-chloromethylvaniline (5-CMV, **1**) with an acceptable yield and high purity. Subsequently, the quaternization of 4-methoxylpyridine was conducted using 5-CMV as a quaternizing agent in the $N_2$ environment, producing o-Val-(4-MeOPy$^+Cl^-$) (2) with excellent yield and high purity. The final stage involves the Schiff-base condensation of compound 2 with o-diaminocyclohexane, generating the $H_2L$ ligand. This ligand functions as a chelating agent for Pd(II) ions in the preparation of the Pd(II) saldach complex ($PdLCl_2$).

High yields were achieved in the synthesis of both the unbound ligand and its palladium(II) complex. Their structures were confirmed through a combination of microanalytical and spectroscopic techniques, including Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) spectroscopy ($^1H$ and $^{13}C$), and electrospray ionization mass spectrometry (ESI-MS). The experimental section contains the details of these analyses.




(**i**) $CH_2O$, $ZnCl_2$, $HCl_{aq}$, $HCl_g$, stirr, r.t; (**ii**) 4-MeO-Py, toluene, stir, 80 °C, $N_2$; (**iii**) L-(+)-tartaric acid, milli-Q $H_2O$, 100% AcOH, stir, 80 °C, 2h (**iv**) NaOH, dist. H2O, CH2Cl2, stir, 30 min, (**v**) EtOH, reflux, stir, $N_2$; (**vi**) $PdCl_2$, EtOH, stir, 50 °C, 8h.

**Scheme 1**: Synthesis protocol for vanillyl-methoxypyridinium chloride ionic liquid (o-Val-(4-MeOPy$^+$Cl), 2), o-diaminocyclohexane Schiff-base ($H_2L$) and Pd(II) complex (PdL).

### 3.2. In vitro cytotoxicity

As of the twenty-first century, cancer is globally the second most prevalent cause of death [43]. Breast cancer is the primary cause of death among Egyptian women. The 5-year survival rate in Egypt stayed low, ranging from 28 to 68%, as a result of delayed diagnosis and an inadequate prognosis, despite great progress in many developing countries [28]. Numerous anticancer medicines are available; however, their widespread use has been limited due to toxicity, adverse effects, limited effectiveness, no specificity, and poor water solubility [43, 47]. To terminate the cancer apparition, there is thus a persistent need for the development of innovative and promising cancer chemotherapeutic medications [43]. An in vitro assessment of the recently synthesized compounds' cytotoxicity activity was conducted on human breast adenocarcinoma (MCF-7) cancerous cell lines as well as human skin fibroblast (HSF) normal cell lines utilizing the MTT assay. MCF-7 and HSF were exposed to varying concentrations (6.25, 12.5, 25, 50, 100 µg/mL) of PdL and $H_2L$ for 48 h to evaluate compounds with the potential to be toxic that alter the morphology and fundamental activities of cells. The effect induced was also compared to the standard drug Doxorubicin ($C_{27}H_{29}NO_{11}$, 543.52 g/mol). The results revealed that the investigated compounds showed a cytotoxic impact on MCF-7 cancerous cells as they decreased the viability of the MCF-7 cell line in a manner reliant on dosage. However, these cytotoxic effects of the tested compounds were inferior to the effect induced by Doxorubicin, a typical chemotherapy medication, that is frequently used in treating breast cancer (Figures 1, 2 & 3), respectively. Specifically, when treating the MCF-7 cell lines with 100 µg/mL of PdL or $H_2L$, the MCF-7 cell viability was just 54.5% and 55.5% respectively. However, Doxorubicin exhibited the highest cytotoxic impact towards MCF-7 breast cell lines with a viability of only 2%. On the other hand, in treating the HSF cell lines with 100 µg/mL of PdL or $H_2L$ for 48 hours, the HSF cell viability was 94.2% and 89.47%, respectively. In comparison, Doxorubicin exhibited the highest cytotoxic activity towards HSF cell lines with a viability of 66.5%. The results of the MTT assay for PdL and $H_2L$ revealed their acceptable effective cell growth inhibition activity as they decreased the percent viability towards MCF-7 breast cell lines; also, they were highly safe towards HSF cell lines. However, for both MCF-7 and HSF cell lines, Doxorubicin was more cytotoxic than the other compounds that were evaluated. Similarly, in the study undertaken by Faghih et al. [48], the cytotoxicity of four Pd(II) complexes based on Schiff base ligands made from ortho-vanillin was evaluated on three cancer cell lines: A549 (lung carcinoma), SKOV3 (ovarian carcinoma), and MCF-7 (breast carcinoma), and juxtaposed with those of cisplatin. From the tested compounds, $Pd(L1)_2$ displayed the highest anti-proliferative impact on these studied malignant cell lines and was more efficient than cisplatin. Comparable to our findings, Hayder et al. [37] revealed that synthesized novel nano Schiff base ligand (LH) by the

reaction of 1, 2-diphenyl-2-(thiazol-2-ylimino) ethan-1-one with 1-(4-((2-hydroxybenzylidene) amino) phenyl) ethenone and 4,4'-methylene dianiline and its Pd(II) complex possess remarkable anti-cancer properties and can be used as a medication to prevent cancer in the field of medicine.

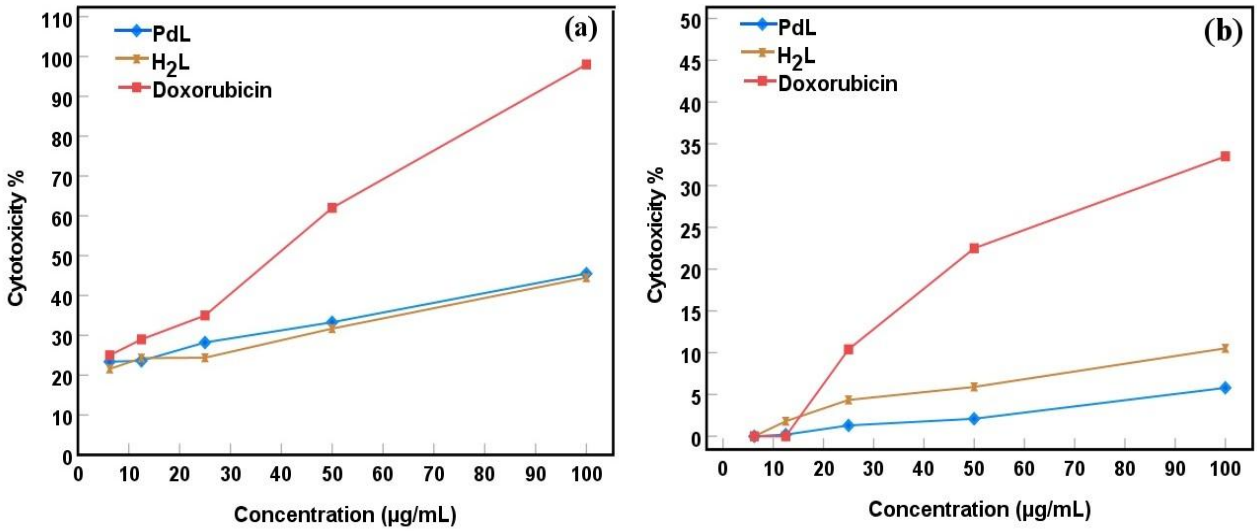



**Figure (1):** Concentration & cytotoxicity effects for the tested compounds and Doxorubicin against **(a)** the human MCF-7 cancerous cells and **(b)** the human HSF normal cells.

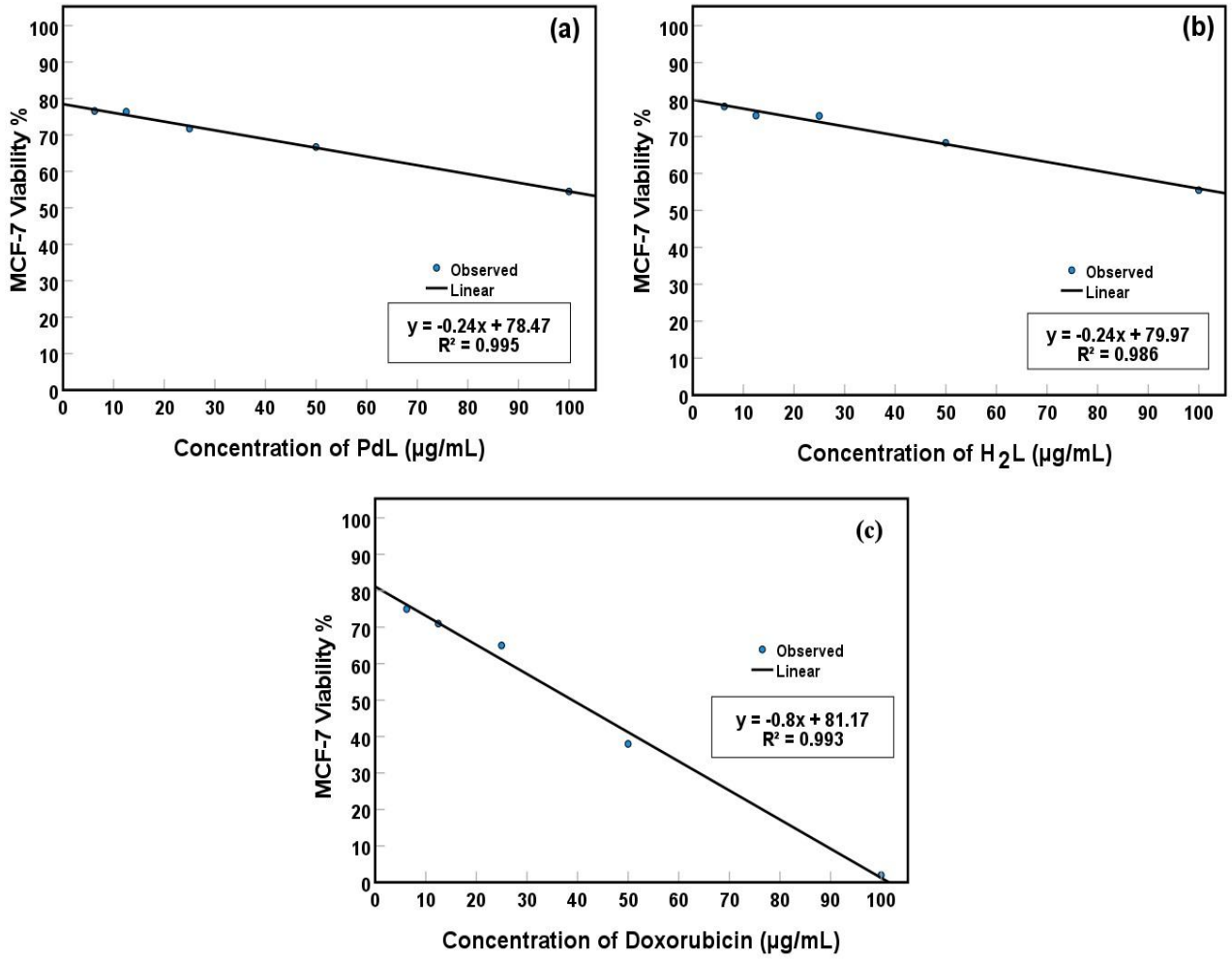



**Figure (2):** Linear regression curve of MCF-7 cell line viability % of different concentrations of **(a)** PdL, **(b)** $H_2L$, and **(c)** Doxorubicin.

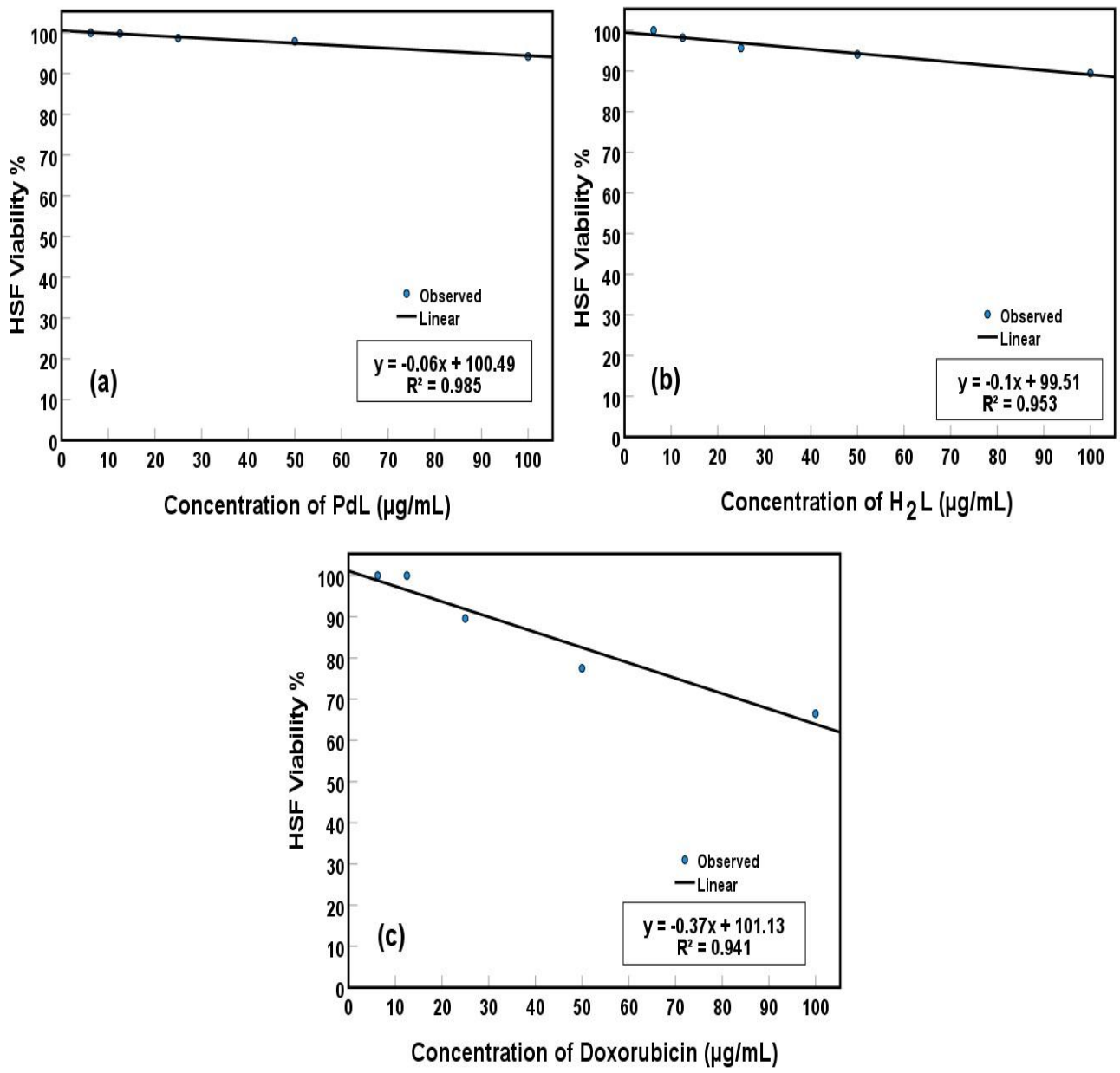



**Figure (3):** Linear regression curve of HSF cell line viability % of different concentrations of **(a)** PdL, **(b)** $H_2L$, and **(c)** Doxorubicin.

The $IC_{50}$ values for PdL and $H_2L$ were investigated against MCF-7 breast cancer cells and contrasted with the common medication Doxorubicin (Table 1, Figure 4). The results showed that every compound can suppress the growth of MCF-7 breast cancer cells with a different degree depending on their structure and concentration. Both PdL and $H_2L$ showed a capacity to prevent the MCF-7 breast cancer cell lines from growing. The $IC_{50}$ value against MCF-7 has been slightly reduced from 123.37 ± 5.85 for $H_2L$ to 118.48 ± 6.03 µg/mL for PdL. Meanwhile, the $IC_{50}$ value for the standard drug Doxorubicin against MCF-7 cells was 38.998 ± 0.008 µg/mL. So, Doxorubicin still showed the greatest amount of cytotoxic action towards MCF-7 breast cell lines, as its $IC_{50}$ is about three-fold lower than that of PdL and $H_2L$. Our findings suggest that PdL and $H_2L$ have strong cytotoxic impacts on the human breast cancer cell line MCF-7 and may provide useful anti-cancer treatment options, but they are not superior to Doxorubicin. However, more research is needed utilizing different biological systems to corroborate the findings and to investigate their potential mode of anticancer action. It is important to note that the planarity of the complex can influence its cytotoxicity by influencing its capacity to engage non-covalently (by groove binding, phosphate clamping, stacking, or intercalation) with the bioactive substrates, especially DNA. Cell death is the result of these interactions between the complex and the cancer cell's DNA, which cause significant alterations in the DNA's structural properties and replication capacity [43]. The results of the MTT assay of the previous study of Hussain

et al. [34] disclosed that all palladium complexes exhibited substantial activity against MDA-MB-231 and MCF-7 cells. The complex, [Pd(L1) ($CH_3CN$)], showed the greatest activity against the two breast cancer cell lines, with corresponding $IC_{50}$ values of 25.50 ± 0.30 µM and ± 20.76 ± 0.30 µM. Another study done by Fahmy et al. [3] in which they synthesized new Schiff base ligands derived from 2-hydroxybenzohydrazide and (E)-1-(2-(p-tolyl) hydrazono) propan-2-one, and their Pd(II) and Zn(II) complexes. The anticancer activity was tested against the MCF-7 cell line, revealing that the Zn(II) complex outperformed Cisplatin and had $IC_{50}$ of 1.40 µg/ml, which is extremely low, whereas the Pd(II) complex showed roughly the same Cisplatin $IC_{50}$.

**Table (1):** The $IC_{50}$ for the tested compounds and Doxorubicin on MCF-7 and HSF cell lines

| Compounds | $IC_{50}$ (µg/mL) | |
|---|---|---|
| | MCF-7 cell line | HSF cell line |
| PdL | $118.48^{c}$ ± 6.03 | $813.07^{bc}$ ± 4.21 |
| $H_2L$ | $123.37^{c}$ ± 5.85 | $455.82^{ac}$ ± 3.62 |
| Doxorubicin | $38.998^{ab}$ ± 0.008 | $131.62^{ab}$ ± 2.76 |
| **F** | **286.043*** ** | **27198.102*** ** |
| **P** | **< 0.001** | **< 0.001** |

$IC_{50}$: Inhibitory concentration of the sample which causes the death of 50% of cells in 48 hours.
Data were expressed as mean ± SD.
F: F for the ANOVA test, and the post hoc test (Tukey) was used to compare each of the two compounds pairwise.
p: p-value for comparing the study's tested compounds.
a: Significant with PdL compound.
b: Significant with $H_2L$ compound.
c: Significant with Doxorubicin standard drug.
***: Statistically highly significant at p ≤ 0.001.

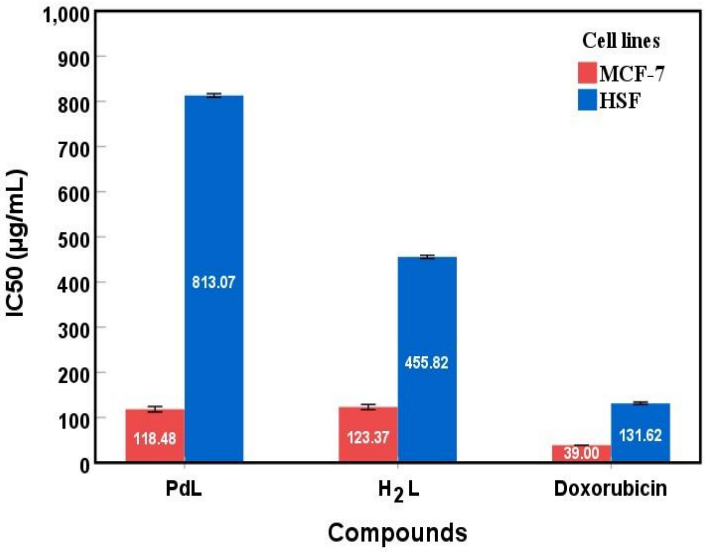



**Figure (4):** The $IC_{50}$ (µg/mL) for the tested compounds and Doxorubicin against the human MCF-7 cancerous cells and the human HSF normal cells.

The selectivity of the PdL and $H_2L$ compounds was investigated by measuring the $IC_{50}$ values of the examined compounds against HSF normal human cells over MCF-7 breast cancer cells, as shown in Table 2. All tested samples exhibited higher $IC_{50}$ values toward HSF normal cells (813.07 ± 4.21 and 455.82 ± 3.62 µg/mL, respectively) as compared to their values against MCF-7 cancer cells (118.48 ± 6.03 and 123.37 ± 5.85 µg/mL, respectively), indicating the high safety of these compounds toward HSF normal human cells. Meanwhile, Doxorubicin was more cytotoxic than the tested compounds on both the MCF-7 cell line and HSF cell line with $IC_{50}$ values of 38.998 ± 0.008 and 131.62 ± 2.76, respectively. Moreover, PdL is the least cytotoxic compound for HSF cells followed by $H_2L$, while Doxorubicin is the most toxic one for HSF cells. As shown in Figure 5, the SI values demonstrated that PdL (SI = 6.86) showed the highest selectivity for MCF-7 cancer cells as compared to the other compound $H_2L$ (SI = 3.69) or with Doxorubicin (SI = 3.38). Thus, the PdL and $H_2L$ could act as promising selective anti-breast cancer candidates. However, additional examination utilizing different biological systems is advised to corroborate the findings.

**Table (2):** The Selectivity index (SI) for the tested compounds and Doxorubicin on MCF-7 and HSF cell lines

| Compounds | Selectivity index (SI) (SI = $IC_{50}$ on non-cancer cells/$IC_{50}$ on cancer cells) |
|---|---|
| PdL | 6.86 |
| $H_2L$ | 3.69 |
| Doxorubicin | 3.38 |

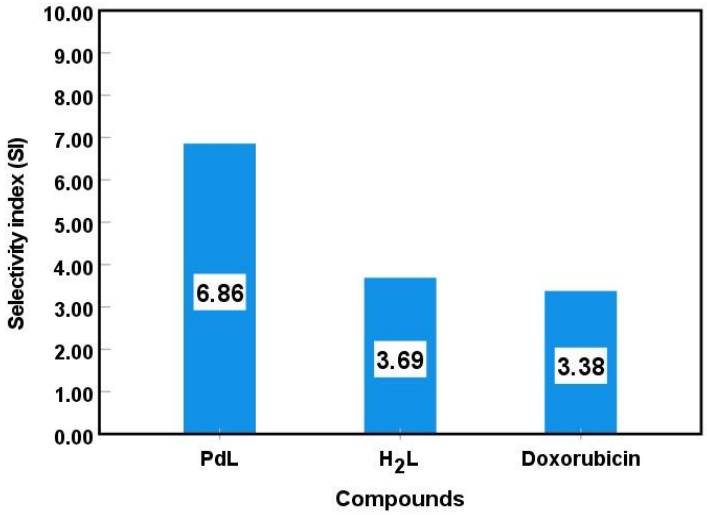



**Figure (5):** Selectivity index (SI) for doxorubicin and the chemicals under test.

The cells were microscopically inspected after 48 hours to visually assess the cytotoxicity impact of the tested compounds, particularly at the concentration of 100 µg/mL. Correspondingly, morphological changes were

observed by the tested compounds and the standard drug compared with those of the negative control. The MCF-7 cells showed shrinkage and membrane blebbing; the cells contracted became deformed, and detached, as shown in Figure 6. In terms of inducing MCF-7 cell death, both PdL and $H_2L$ were effective. For instance, the PdL ($IC_{50}$ = 118.48 µg/mL) has a slightly stronger growth-inhibitory effect than the $H_2L$ ($IC_{50}$ = 123.37 µg/mL). The present study emphasizes that Schiff bases possess significant potential to inhibit carcinoma cells, which is slightly enhanced upon complexation with palladium metal. PdL demonstrates enhanced interaction with cancer cells due to the presence of palladium, which increases DNA-binding efficiency and oxidative stress. There is hope for novel advancements in the treatment of cancer due to the intricate relationship between robust anti-cancer potential and ligand design for palladium complex synthesis [34]. Cell morphological changes in MCF-7 breast cancer cells proved that the tested compounds can either cause the MCF-7 cells to die or stop growing. These tested compounds were discovered to be somewhat similar to the morphological alterations induced by Doxorubicin, a common chemotherapy medication, on MCF-7 breast cancer cells. However, in the normal HSF cell line, PdL and $H_2L$ compounds showed nearly slight or no observed morphological changes when compared with the HSF negative control. On the other hand, Doxorubicin showed observed morphological changes such as shrinkage and membrane blebbing according to Figure 7. These findings showed the high safety of the tested compounds toward HSF normal human cells while Doxorubicin was more cytotoxic than the tested compounds. So, additional detailed studies would be required to evaluate the molecular mechanism leading to the cytotoxic effects of the tested compounds against the MCF-7 cells. In the future, we will examine the role of these compounds in cellular apoptosis, proliferation defects, and cell cycle arrest utilizing different biological systems.

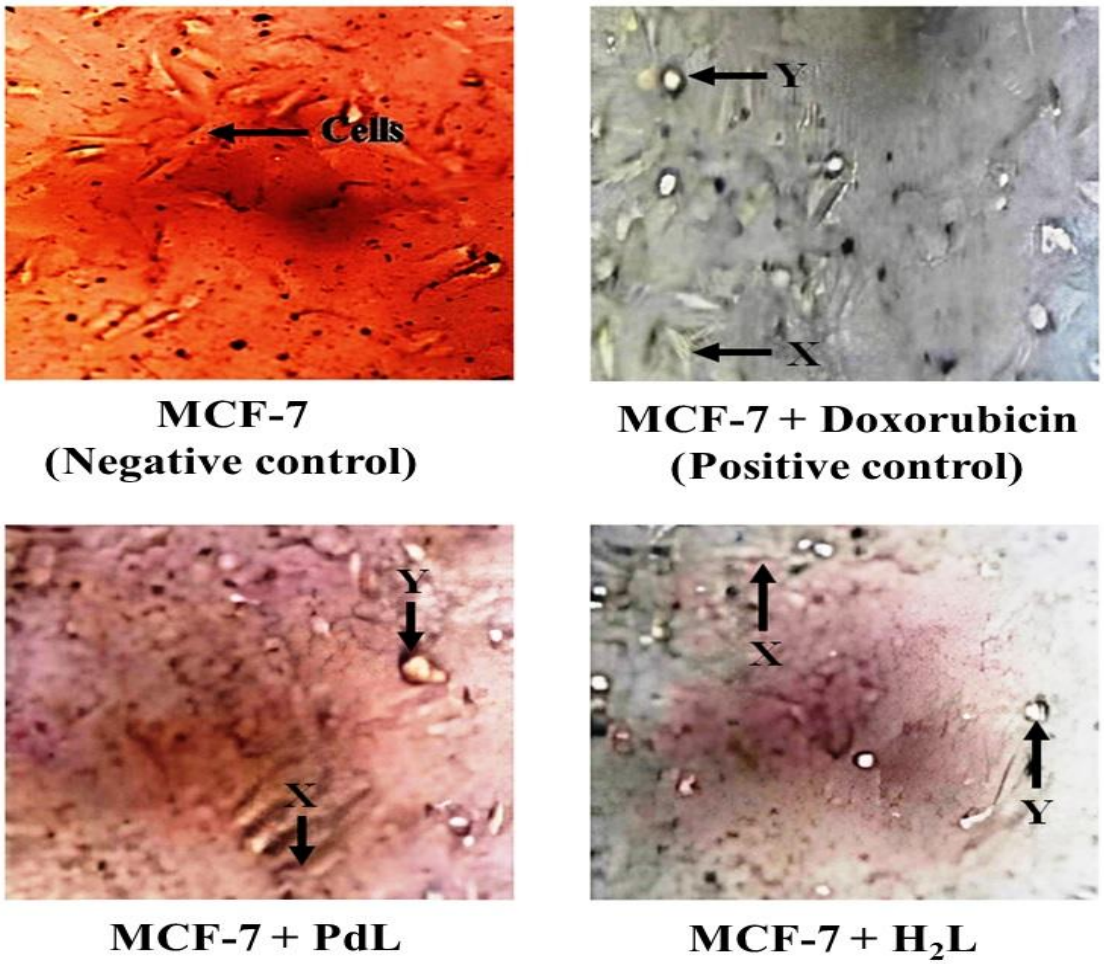



**Figure (6):** Morphological changes of MCF-7 breast cancer cell lines after 48 h. X: Cellular shrinkage; Y: Membrane blebbing (magnification for MCF-7 cell lines was 20X).

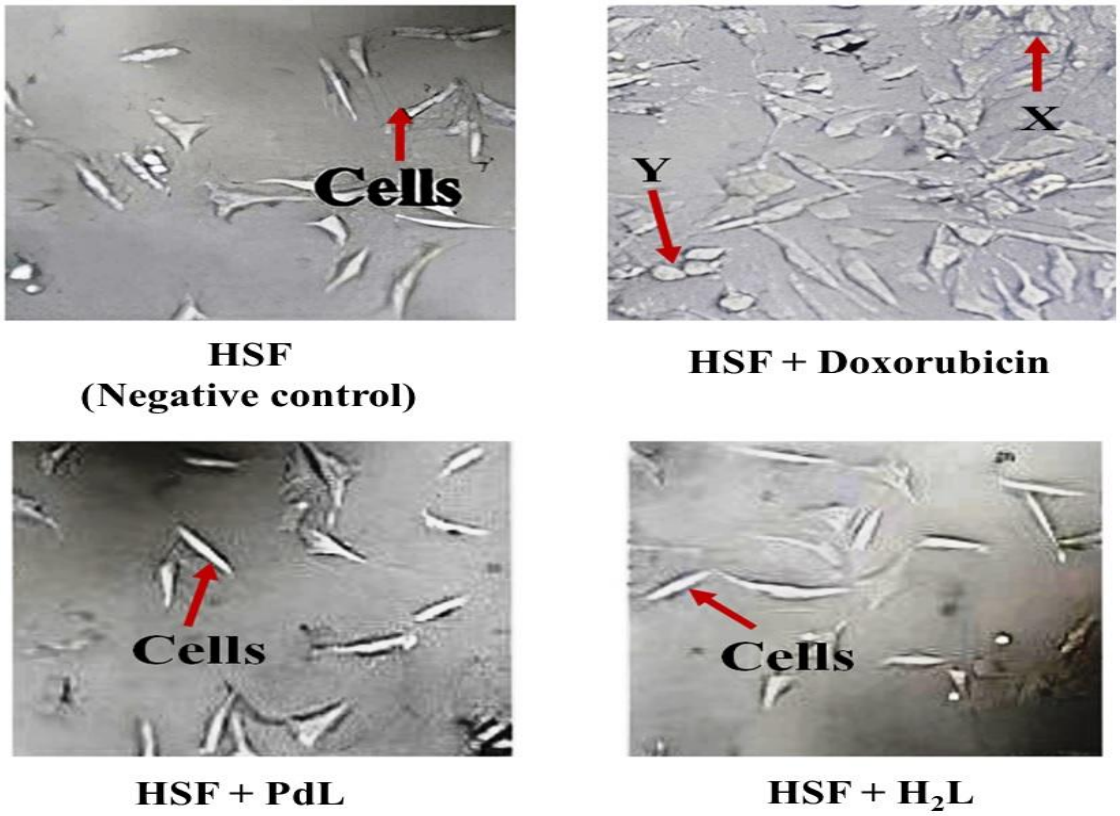



**Figure (7):** Morphological changes of HSF normal cell lines after 48 h. X: Cellular shrinkage; Y: Membrane blebbing (magnification for HSF cell lines was 20X).

## 4. Conclusion

In conclusion, the current study added a few new cell viability-inhibiting compounds to chemotherapy: o-diaminocyclohexane Schiff-base ($H_2L$) and Pd(II) complex (PdL) for the MCF-7 breast cancer cell line. These chemicals' synthesis was thoroughly verified using a range of analytical methods, including Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) spectroscopy ($^1H$ and $^{13}C$), and electrospray ionization mass spectrometry (ESI-MS). The study's findings demonstrated the anticancer properties of PdL and $H_2L$ compounds. These two newly synthesized compounds reduced cell viability in a way that was dependent on concentration. In particular, PdL showed higher cytotoxicity activities than $H_2L$ for the MCF-7 breast cancer cell line with an $IC_{50}$ of 118.48 µg/mL. However, PdL was not superior to Doxorubicin (38.998 µg/mL). Acceptably, PdL and $H_2L$ do not harm the HSF healthy cell line when compared to the current medication, Doxorubicin. By suppressing the MCF-7 breast cancer cell line, the tested treatments' selectivity demonstrated a promising anticancer potential. As a result, this study not only demonstrates the fascinating anticancer potential of PdL and $H_2L$ but also their utility in selective anti-tumor medications that reduce harm to healthy tissues. These findings encouraged us to conduct the following investigations on PdL and $H_2L$ utilizing different biological systems. In order to verify therapeutic efficacy, future research should concentrate on in vivo assessments and clinical trials.

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**Declaration of conflict of interest**
The authors declare no conflict of interest.

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