Synthesis, Cytotoxicity Evaluation and Molecular Docking Study of *N*-Phenylpyrazoline Derivatives

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Abstract: *The synthesis of N-phenylpyrazolines* ***1-5*** *was performed by the cyclocondensation of phenylhydrazine and appropriate chalcones that have been synthesized from our previous work. All of the compounds were elucidated for their structure using GC-MS, FTIR, ^{1}H, and ^{13}C-NMR spectrometers. Their anticancer activity was evaluated against breast cancer cell line (T47D) and colorectal cancer cell line (WiDr). Compound* ***4*** *(4-(3-(4-chlorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)-2-methoxyphenol) was found to be the most potent compound with IC_{50} value of 13.11 µg/mL in T47D cell line and 3.29 µg/mL in WiDr cell line. Docking study was conducted to evaluate the interaction between all compounds and EGFR receptor on cancer cells. Among the tested compounds, compound* ***4*** *is the only compound that has interaction with MET769 residue through hydrogen bonding due to the presence of hydroxyl group on its structure. Our findings suggest that the synthesized N-phenylpyrazolines in this study have a promising anticancer activity.*

Keywords: *N-phenylpyrazoline; anticancer; cytotoxic activity; molecular docking*

■ INTRODUCTION

Cancer is one of the leading diseases that cause death worldwide, according to the World Health Organization (WHO). Cancer incidence and mortality are rapidly growing throughout the world due to the growth and aging of the population, particularly in less developed countries [1]. In 2018, GLOBOCAN estimated 18.1 million new cancer cases and 9.6 million cancer deaths worldwide [2]. Chemotherapy is one of the effective approaches in cancer treatment. However, in most cases, the toxicity properties of chemotherapeutic agents and the occurrence of drug-resistant hinder the successful outcomes of cancer treatment [3]. Therefore, the search for novel anticancer agents is urgently needed.

Pyrazoline is 5-membered heterocyclic compound with two nitrogen atoms at 1–2 positions and one endocyclic double bond. Pyrazoline derivatives possess numerous biological and pharmacological activities, thus making it an important class of heterocycles for carrying it out in further drug research. It was reported that different pyrazolines possess different activities such as anticancer [4], anti-inflammatory [5], antidepressant [6], antimalarial [7], antifungal [8], antibacterial [9], antioxidant [10], antitubercular [11], analgesic [12], and insecticidal [13] activities. The cyclocondensation reaction of chalcones with hydrazines is one of the most popular methods to synthesize pyrazoline derivatives [14].

Cytotoxicity evaluation and molecular docking study of pyrazoline derivatives show that these compounds inhibit the epidermal growth factor receptor (EGFR) [15-16]. Protein EGFR overexpression activates the signal transduction pathway that responsible for the occurrence of many types of cancer such as breast, colorectal, ovarian, lung, glioblastomas, head, and neck [17]. The molecular docking study informs that *N-N* bond and various functional groups on pyrazolines hold a significant role in affecting their anticancer activity [16].

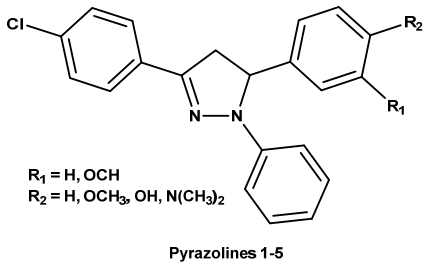

Fig 1. Chemical structure of pyrazolines **1**-**5**

Pyrazoline derivatives containing phenyl group on nitrogen atom that is called as *N*-phenylpyrazolines are reported to exhibit good cytotoxicity toward various cancer cell lines [16,18-20].

There are many studies reporting the synthesis of pyrazolines derivatives as anticancer agents; however, the synthesis of our targeted *N*-phenylpyrazolines (Fig. 1) and the effect of chloro, methoxy, hydroxyl, and dimethylamino group against breast and colorectal cancer cell line (T47D and WiDr) has not been reported yet. Given the above facts and in continuation to our previous work [21], herein, we reported the synthesis of *N*-phenylpyrazolines **1-5** via cyclocondensation reaction between reported chalcones **1**-**5** [21] and phenylhydrazine. Their cytotoxicity was evaluated against T47D and WiDr. Furthermore, we performed molecular docking study with EGFR receptor to understand their molecular interaction and the substituent effect on the compounds.

■ EXPERIMENTAL SECTION

Materials

The reagents and solvents used were obtained from Merck with pro analysis grade without further purification, i.e., phenylhydrazine, glacial acetic acid, ethanol absolute, methanol, *n*-hexane, dichloromethane, ethyl acetate, and dimethyl sulfoxide. Chalcones **1**-**5** were obtained from our earlier work [21]. Thin-layer chromatography was performed using aluminum plates (20 × 20 cm) coated with silica gel 60 F_{254} (Merck), while column chromatography was carried out using silica gel 60 (0.063–0.200 mm) from Merck. Materials for cytotoxicity assay were microwell plate 96, breast cancer cell line (T47D), colorectal cancer cell line (WiDr), normal cell line (Vero), Roswell Park Memorial Institute Medium (RPMI 1640), Medium 199 (M199), Fetal Bovine Serum (FBS), phosphate buffer solution (PBS), penicillin-streptomycin (Pen-Strep), HEPES, sodium hydrogen carbonate, amphotericin B, trypsin-EDTA solution, sodium dodecyl sulfate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Instrumentation

Structure elucidation of pyrazolines **1-5** was carried out using GC-MS (Shimadzu QP2010S, EI), FT-IR (Shimadzu Prestige-21, KBr discs), ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) (JEOL JNMECA, standard internal TMS) spectrometers. Cytotoxicity evaluation of pyrazolines **1-5** was performed using laminar air flow (Labconco, Purifier Delta Series Class II), 5% CO_2 incubator (Heraeus), inverted microscope (Axiovert 25), centrifuge (Janetzki T5), ELISA reader (BIO-RAD Benchmark), hemocytometer (Neubauer) and micropipette 2-20 µL, 20–200 µL (VWR brand), 100–1000 µL (AccuBioTech).

Procedure

Synthesis of pyrazolines 1-5

3-(4-chlorophenyl)-1,5-diphenyl-4,5-dihydro-1*H*-pyrazole (Pyrazoline 1). A solution of 0.18 g (0.76 mmol) chalcone **1** ((*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one) in 5 mL of absolute ethanol was put into the round-bottom flask. Next, 5 mL of glacial acetic acid and 0.075 mL of phenylhydrazine (0.76 mmol) were added respectively. The mixture was refluxed for 6.5 h and monitored by TLC. After completion, the mixture was poured into ice-cold water and was left in the refrigerator for 24 h. The resulting precipitate was filtered off using Buchner vacuum filtration, washed with cold water and dried in a vacuum desiccator. The purification was performed using column chromatography with *n*-hexane:dichloromethane as the eluent to give pyrazoline **1** as a light green solid, yield: 47.54%, purity: 100%. FTIR (KBr, cm^{-1}): 3062 (C_{sp2}-H str.), 1597 (C=N str.), 1512 and 1496 (Ar C=C str.), 1388 (Ar C-N str.), 1126 (C-N str.), 1087 (Ar C-Cl str.). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 3.14 (1H, *dd*, *J* = 7.25, 16.75 Hz, CH_2), 3.84 (1H, *dd*, *J* = 12.5, 17 Hz, CH_2), 5.32 (1H, *dd*, *J* = 7.25, 12.5 Hz, CH), 6.84 (1H, *m*, ArH), 7.12, 7.22 (4H, *m*, ArH), 7.34

(7H, *m*, ArH), 7.68 (2H, *m*, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ 43.6 (CH_2), 64.8 (CH), 113.6 (2 CHAr), 119.5 (CHAr), 126.0 (2CHAr), 127.1 (2CHAr), 127.9 (CHAr), 128.9 (2CHAr), 129.1 (2CHAr), 129.4 (2CHAr), 131.5, 134.4, 132.5, 144.8 (4C Ar), 145.7 (C). Mass spectrum (EI): m/z 334 (M+2, ^{37}Cl, 25%), 332 (M, ^{35}Cl, 75), 255 (^{35}Cl, 40), 228 (^{35}Cl, 5), 104 (20), 91 (100), 77 (45).

3-(4-chlorophenyl)-5-(4-methoxyphenyl)-1-phenyl-4, 5-dihydro-1*H*-pyrazole (Pyrazoline 2). It was prepared by following the procedure as described for pyrazoline **1** from a mixture of chalcone **2** ((*E*)-1-(4-chlorophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one) (0.51 g, 1.875 mmol), 5 mL of absolute ethanol, 5 mL of glacial acetic acid and 0.185 mL of phenylhydrazine (1.875 mmol). The mixture was refluxed for 7 h. The product of pyrazoline **2** was obtained as light green solid, yield: 44.19%, purity: 100%, m.p. 90–91 °C. FTIR (KBr, cm^{-1}): 3055 (C_{sp2}-H str.), 2939 (C_{sp3}-H str.), 1597 (C=N str.), 1512 and 1496 (Ar C=C str.), 1381 (Ar C-N str.), 1126 (C-N str.), 1249 and 1033 (C-O asymm. and sym. str.), 1087 (Ar C-Cl str.). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 3.05 (1H, *dd*, *J* = 7, 17 Hz, CH_2), 3.75 (3H, *s*, OCH_3), 3.76 (1H, *dd*, *J* = 12.5, 17 Hz, CH_2), 5.22 (1H, *dd*, *J* = 7, 12.5 Hz, CH), 6.78 (1H, *m*, ArH), 6.84 (2H, *d*, *J* = 8.0 Hz, ArH), 7.05 (2H, *d*, *J* = 7.0 Hz, ArH), 7.16, 7.22 (4H, *m*, ArH), 7.33 (2H, *d*, *J* = 8.0 Hz, ArH), 7.62 (2H, *d*, *J* = 7.0 Hz, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ 43.6 (CH_2), 55.5 (CH_3-O), 64.3 (CH), 113.6 (2CHAr), 114.7 (2CHAr), 119.5 (CHAr), 127.1 (2CHAr), 127.2 (2CHAr), 128.9 (2CHAr), 129.1 (2CHAr), 131.6, 134.4, 134.6, 144.85 (4 CAr), 145.7 (C), 159.2 (CAr). Mass spectrum (EI): m/z 365 (M+2, ^{37}Cl, 15%), 362 (M, ^{35}Cl, 35), 255 (^{35}Cl, 10), 193 (5), 91 (100), 77 (45).

3-(4-chlorophenyl)-5-(3,4-dimethoxyphenyl)-1-phenyl -4,5-dihydro-1*H*-pyrazole (Pyrazoline 3). It was prepared by following the procedure as described for pyrazoline **1** from a mixture of chalcone **3** ((*E*)-1-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one) (0.23 g, 0.76 mmol), 5 mL of absolute ethanol, 5 mL of glacial acetic acid and 0.075 mL of phenylhydrazine (0.76 mmol). The mixture was refluxed for 4 h. The product of pyrazoline **3** was obtained as yellowish white solid, yield: 50.24%, purity: 100%, m.p. 143–144 °C. FTIR (KBr, cm^{-1}): 3055 (C_{sp2}-H str.), 2931 (C_{sp3}-H str.), 1597 (C=N str.), 1512 and 1496 (Ar C=C str.), 1381 (Ar C-N str.), 1126 (C-N str.), 1257 and 1026 (C-O asymm. and sym. str.), 1087 (Ar C-Cl str.). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 3.07 (1H, *dd*, *J* = 7, 17.5 Hz, CH_2), 3.75 (1H, *dd*, *J* = 12.5, 17.5 Hz, CH_2), 3.83 (3H, *s*, OCH_3), 3.78 (3H, *s*, OCH_3), 5.18 (1H, *dd*, *J* = 7, 12.5 Hz, CH), 6.81 (4H, *m*, ArH), 7.06 (2H, *d*, *J* = 8 Hz, ArH), 7.16 (1H, *m*, ArH), 7.32 (2H, *d*, *J* = 8 Hz, ArH), 7.62 (2H, *d*, *J* = 7.5 Hz, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ 43.7 (CH_2), 56.1, 56.1 ($2CH_3$-O), 64.9 (CH), 108.7 (CHAr), 111.7 (CHAr), 113.7 (2CHAr), 118.2 (CHAr), 119.6 (CHAr), 127.1 (2CHAr), 128.9 (2CHAr), 129.1 (2CHAr), 131.5, 134.5, 135.1, 145.1, 145.9, 148.6 (6 CAr), 149.8 (C). Mass spectrum (EI): m/z 394 (M+2, ^{37}Cl, 20%), 392 (M, ^{35}Cl, 50), 361 (^{35}Cl, 5), 257 (^{37}Cl, 5), 255 (^{35}Cl, 20), 193 (5), 178 (5), 164 (10), 91 (100), 77 (40).

4-(3-(4-chlorophenyl)-1-phenyl-4,5-dihydro-1*H*-pyrazol-5-yl)-2-methoxyphenol (Pyrazoline 4). It was prepared by following the procedure as described for pyrazoline **1** from a mixture of chalcone **4** ((*E*)-1-(4-chlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one) (0.22 g, 0.76 mmol), 5 mL ethanol absolute, 5 mL glacial acetic acid and 0.075 mL phenylhydrazine (0.76 mmol). The mixture was refluxed for 4 h. The product of pyrazoline **4** was obtained as yellow solid, yield: 11.03%, purity: 100%, m.p. 144–145 °C. FTIR (KBr, cm^{-1}): 3502 (O-H str.), 3055 (C_{sp2}-H str.), 2931 (C_{sp3}-H str.), 1651 (C=N str.), 1512 and 1496 (Ar C=C str.), 1381 (Ar C-N str.), 1126 (C-N str.), 1265 and 1033 (C-O asymm. and sym. str.), 1087 (Ar C-Cl str.). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 3.10 (1H, *dd*, *J* = 7.8, 16.85 Hz, CH_2), 3.78 (1H, *dd*, *J* = 12.3, 16.55 Hz, CH_2), 3.81 (3H, *s*, OCH_3), 5.19 (1H, *dd*, *J* = 7.8, 12.3 Hz, CH), 5.57 (1H, *s*, OH), 6.77 (1H, *d*, *J* = 1.3 Hz, ArH), 6.81 (1H, *t*, ArH), 6.85 (1H, *m*, ArH), 6.89 (1H, *d*, *J* = 7.8 Hz, ArH), 7.08 (2H, *d*, *J* = 7.75 Hz, ArH), 7.19, 7.35, 7.64 (6H, 3*m*, ArH). ^{13}C-NMR (*100* MHz, $CDCl_3$, ppm): δ 43.8 (CH_2), 56.2 (CH_3-O), 65.051 (CH), 108.0 (CHAr), 113.7 (2CHAr), 114.9 (CHAr), 119.0 (CHAr), 119.6 (CHAr), 127.1 (2CHAr), 128.9 (2CHAr), 129.1 (2CHAr), 131.5, 134.5, 134.7, 145.1, 145.3, 145.9 (6 CAr), 147.4 (C). Mass spectrum (EI): m/z 380 (M+2, ^{37}Cl, 20%), 378 (M, ^{35}Cl, 40), 255 (^{35}Cl, 20), 241 (5), 91 (100), 77 (40).

4-(3-(4-chlorophenyl)-1-phenyl-4,5-dihydro-1*H*-pyrazol-5-yl)-*N*,*N*-dimethylaniline (Pyrazoline 5). It was prepared by following the procedure as described for pyrazoline **1** from a mixture of chalcone **5** ((*E*)-1-(4-chlorophenyl)-3-(4-(dimethylamino)phenyl)prop-2-en-1-one) (0.28 g, 1 mmol), 10 mL of absolute ethanol, 5 mL of glacial acetic acid and 0.1 mL of phenylhydrazine (1 mmol). The mixture was refluxed for 7 h. The product of pyrazoline **5** was obtained as yellow solid, yield: 75.53%, purity: 96.07%, m.p. 135-136 °C. FTIR (KBr, cm^{-1}): 3070 (C_{sp2}-H str.), 2939 (C_{sp3}-H str.), 1597 (C=N str.), 1519 and 1496 (Ar C=C str.), 1350 (Ar C-N str.), 1126 (C-N str.), 1087 (Ar C-Cl str.). ^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ 2.92 (6H, *s*, 2xCH_3), 3.09 (1H, *dd*, *J* = 7.1, 17.2 Hz, CH_2), 3.75 (1H, *dd*, *J* = 12.3, 17.2 Hz, CH_2), 5.21 (1H, *dd*, *J* = 7.1, 12.3 Hz, CH), 6.68 (2H, *d*, *J* = 8.4 Hz, ArH), 6.77 (1H, *m*, ArH), 7.09 (2H, d, *J* = 8.45 Hz, ArH), 7.16 (2H, *d*, *J* = 8.4 Hz, ArH), 7.18 (2H, *m*, ArH), 7.34 (2H, *d*, *J* = 8.45 Hz, ArH), 7.64 (2H, *d*, *J* = 7.8 Hz, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ 40.7 (2CH_3), 43.7 (CH_2), 64.5 (CH), 113.1 (2CHAr), 113.7 (2CHAr), 119.3 (CHAr), 126.9 (2CHAr), 127.0 (2CHAr), 128.9 (2CHAr), 129.1 (2CHAr), 130.1, 131.8, 134.2, 145.0, 145.8 (5 CAr), 150.2 (C). Mass spectrum (EI): m/z 377 (M+2, ^{37}Cl, 20%), 375 (M, ^{35}Cl, 60), 255 (^{35}Cl, 15), 147 (100), 121 (15), 91 (80), 77 (35).

Cytotoxicity evaluation of pyrazolines 1-5 against breast (T47D) and colorectal (WiDr) cancer cell lines

Cancer cell lines (T47D dan WiDr) and normal cell line (Vero) were cultured in medium of RPMI/10% FBS and M199/10% FBS at 37 °C under 5% CO_2 water-saturated atmosphere. The cell suspensions (10^6/mL) were prepared. A total of 100 µL/well were inserted into 96-well plate giving 10^4 cells/well. Incubation process was carried out for 24 h to allow cells to reattach. All five pyrazolines were prepared in DMSO at a concentration of 10^5 µg/mL. Each pyrazoline samples were diluted into culture medium in 6 serial concentration: 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/mL. A total of 100 µL of respective test concentration was then filled into the wells. The incubation was performed for the next 24 h, and the MTT assay was carried out to assess the cell viability. The PBS was then used to remove and to wash the remaining culture medium on the plates. The MTT solution in PBS was prepared (50 mg/10 mL) and 1 mL of this aliquot was diluted by the addition of 9.5 mL of culture medium. An aliquot (100 µL) of diluted MTT was inserted into each well. The incubation was conducted for another 4 h. Finally, 100 µL of SDS stopper 10% in 0.1 N HCl was filled into each well. The plates were kept at room temperature overnight. The ELISA reader was used to record absorbance readings at 595 nm.

Molecular docking study of pyrazolines 1-5

The 3D structure of pyrazolines **1**-**5** was modeled using GaussView 5.0.8 [22], and the optimization was done using density functional theory (DFT) with B3LYP method and 6-31G basis set using Gaussian 09 software [23]. The crystal structure of complexes EGFR (PDB ID: 1M17) with Erlotinib was obtained from RCSB Protein Data Bank. The docking simulations were carried out with Autodock Tools [24] and Autodock 4 using a Lamarckian Genetic Algorithm (LGA). The cubic grid box of 45 Å size (x, y, z) with a spacing of 0.375 Å were created. Docking analysis gives the best result with RMSD value < 2 Å [25]. A total of 100 molecular docking poses for individual ligand were listed according to the docking score. We use a scoring function in AutoDock to estimate the binding affinity of the tested pyrazolines to the EGFR receptor. The most suitable conformation was selected from the lowest binding energy. The visualization of the docking result was performed using Autodock Tools (ADT) and Discovery Studio Visualizer (DSV).

■ RESULTS AND DISCUSSION

Synthesis of Pyrazolines 1-5

The cyclocondensation reaction of chalcones **1**-**5** [21] and phenylhydrazine with glacial acetic acid as a catalyst has been done following the procedure from Suma et al. [26] with slight modification. The synthetic scheme of pyrazolines **1**-**5** was shown in Fig. 2.

The ^{1}H-NMR spectra of all pyrazolines proved the formation of pyrazolines had been successfully completed by the presence of three characteristic peaks with a splitting pattern as doublet of doublet at 3, 4 and 5 ppm that occurred due to diastereotopic nature of methylene

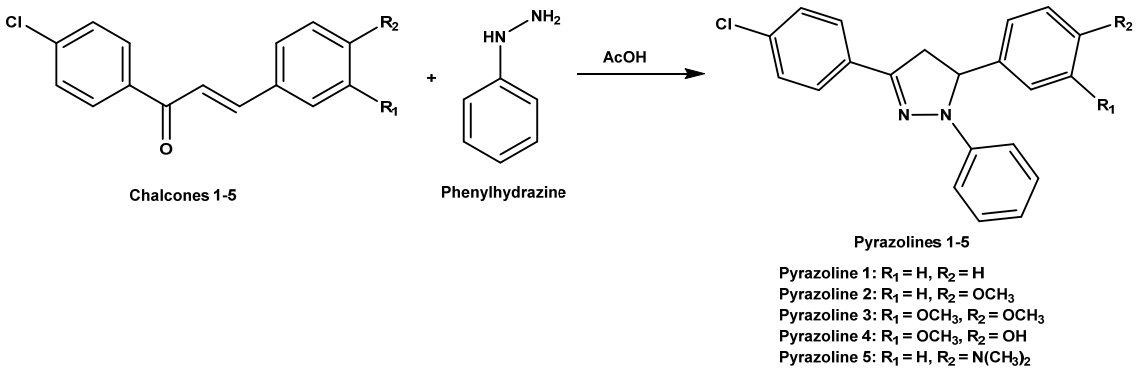

Fig 2. Synthesis of pyrazolines **1**-**5**

protons on pyrazoline ring [8]. The IR spectra of all pyrazolines also confirmed the formation of pyrazolines by the appearance of absorption bands at 1597 cm^{-1} for C=N stretching and at 1388 and 1126 cm^{-1} for C-N aromatic and aliphatic stretching. The synthetic products also showed the disappearance of an absorption band at 978 cm^{-1} for *trans*-disubstituted alkene bending from chalcones as a reactant. The molecular weight of the desired pyrazolines with the presence of chlorine isotopes was proved by the mass spectra. The characteristic molecular ion M^+ for ^{35}Cl and M^{+2} for ^{37}Cl were displayed with the height ration of 3:1. In addition, the presence of carbon atoms in the pyrazoline ring and the absence of *trans*-alkene carbon from chalcone were clarified by ^{13}C-NMR spectra.

Cytotoxicity Evaluation

All of the products were obtained in excellent purity hence satisfy the condition for the cytotoxicity evaluation. Cytotoxicity of pyrazolines **1**-**5** and two commercial drugs were evaluated by MTT method against breast cancer cell line (T47D), colorectal cancer cell line (WiDr) and normal cell line (Vero). Two commercial drugs as positive control that were used in this evaluation were doxorubicin for breast cancer [27] and 5-fluorouracil for colorectal cancer [28].

The IC_{50} values of all pyrazolines are displayed in Table 1. Evaluation of pyrazoline **1** and **2** against both cancer cell lines (T47D and WiDr) showed that the addition of one methoxy group did not make any significant changes to their activity. However, the addition of two methoxy groups on pyrazoline **3** greatly increased its anticancer activity that was showed by the decrease of IC_{50} value. This result is in accordance with Ciupa et al. [18], which reported that the addition of several methoxy groups on pyrazoline derivatives might affect their activity on inhibition of cancer cells. Evaluation of pyrazoline **4** against both tested cancer cell lines gave the lowest IC_{50} value compared to the other pyrazolines. It means that the addition of one hydroxyl and one methoxy group has a better effect on increasing the anticancer activity. On the other hand, the addition of the dimethylamino group on pyrazoline **5** increased the IC_{50} values against both cancer cell lines; therefore, has poor inhibition activity.

Classification of compounds activity on their inhibition of cell growth was categorized into three types: (i) active if IC_{50} value is less than 20 μg/mL, (ii) moderate if IC_{50} value is between 20–100 μg/mL, (iii) inactive if IC_{50} value is greater than 100 μg/mL [29]. According to the reference, it can be seen that pyrazolines **1**-**5** have different selectivity against both cancer cell lines (T47D and WiDr). The results of cytotoxicity evaluation against T47D showed that pyrazolines **1**-**3** have moderate activity, pyrazoline **4** has a good activity, and pyrazoline **5** is inactive. While the result against WiDr showed that pyrazolines **1**-**4** have good activity and pyrazoline **5** has moderate activity. It can be seen that all of the synthesized pyrazolines in this study have better inhibition toward colorectal cancer cell WiDr and the most active compounds against both cancer cell lines is pyrazoline **4**.

However, the compound selectivity toward normal cell line (Vero) is also needed to take into consideration in order to conclude the best compound as the candidate of the anticancer agent in this study. It was reported that that compound with SI (selectivity index) value higher than 6 has high selectivity, SI value between 3–6 has moderate selectivity, and SI value lower than 3 is nonselective [30]. From Table 1, it can be said that pyrazolines **1**-**5** have poor potential in breast cancer treatment caused by T47D. However, they have good potential in colorectal cancer treatment caused by WiDr, especially pyrazolines **1**-**4**. Pyrazolines **2** and **3** have good activity against WiDr and high selectivity to normal cell line, while pyrazolines **1** and **4** have high activity against WiDr and moderate selectivity to normal cell line. From the IC_{50} and SI values in this study, it can be concluded that pyrazolines **3** and **4** are the most promising compound as an anticancer agent.

Molecular Docking Study

Molecular docking study of pyrazolines **1**-**5** was performed against EGFR as the receptor. This receptor

Table 1. IC_{50} values and selectivity index of pyrazolines **1**-**5**

Compound	IC_{50} (μg/mL)			SI	
	T47D	WiDr	Vero	T47D	WiDr
1	62.35	7.90	46.59	0.75	5.90
2	66.26	9.92	79.74	1.20	9.04
3	26.82	3.93	27.52	1.03	7.01
4	13.11	3.29	16.55	1.26	5.03
5	>100	20.84	>100	0.85	5.70
Doxorubicin[1]	10.58	-	-	-	-
5-Fluorouracil[2]	-	25.57	-	-	-

Note: [1]Positive control for breast cancer. [2]Positive control for colorectal cancer

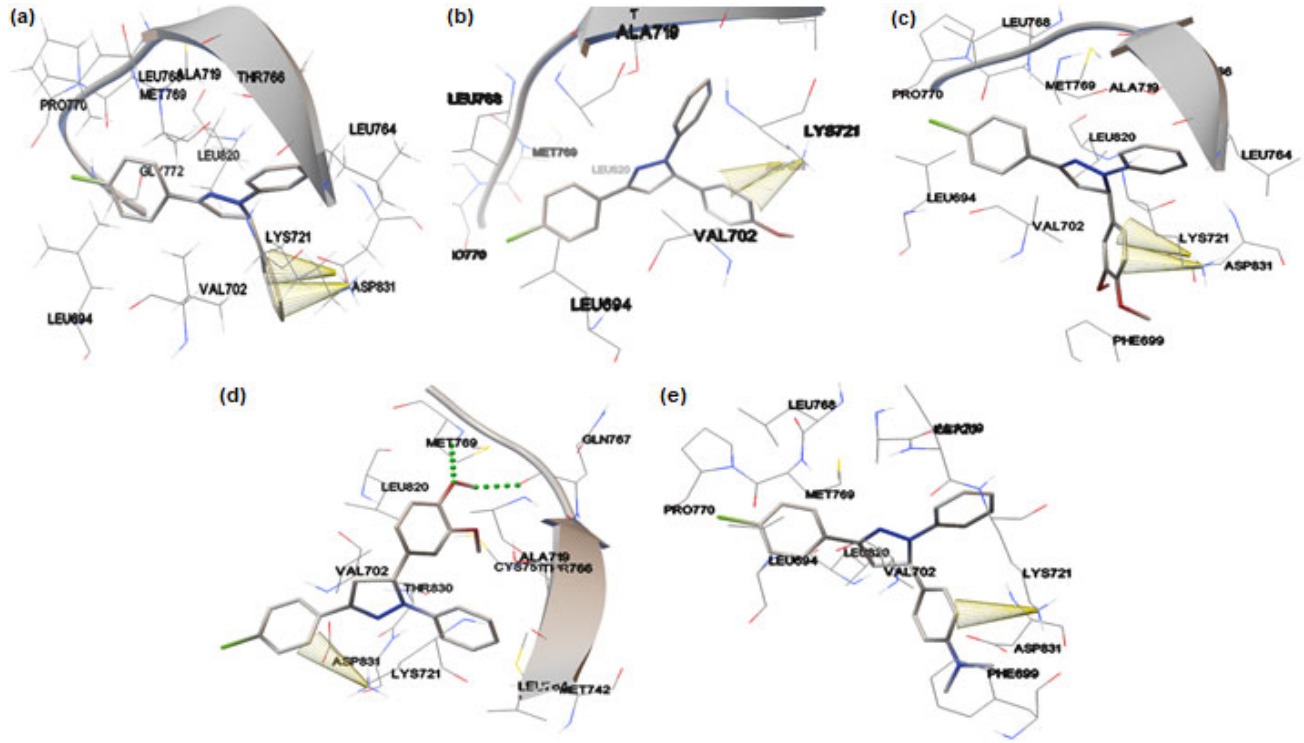

Fig 3. The molecular docking model of EGFR receptor with (a) pyrazoline **1**, (b) pyrazoline **2**, (c) pyrazoline **3**, (d) pyrazoline **4**, and (e) pyrazoline **5**

Table 2. Molecular docking result of pyrazolines **1**-**5**

Compound	Energy (kcal/mol)	Interaction
1	-7.85	π-cation: Lys721(*) Alkyl/π-alkyl: Ala719, Lys721, Leu768, Leu820 π-anion: Asp831 π-σ: Leu694, Val702 van der Waals: Phe699, Ile720, Met742, Leu764, Thr766, Met769, Pro770, Gly772, Thr830
2	-7.94	π -cation: LYS721(*) H-Bond (C): Asp831 Alkyl/π-alkyl: Ala719, Lys721, Leu768, Leu820 π-anion: Asp831 π-Sulfur: Met742 π-σ: Leu694, Val702 Halogen: Pro770 van der Waals: Phe699, Ile720, Leu764, Ile765, Thr766, Met769, Gly772, Thr830
3	-8.03	π-cation: LYS721(*) H-Bond (C): Arg817, Asn818, Asp831 Alkyl/π-alkyl: Ala719, Lys721, Leu768, Leu820 π-anion: Asp831 π-Sulfur: Met742 π-σ: Leu694, Val702 Halogen: Pro770 van der Waals: Phe699, Ile720, Leu764, Ile765, Thr766, Met769, Gly772
4	-8.04	π -cation: LYS721(*) H-Bond: GLN767 (1.728 Å), MET769 (1.789 Å) (*) H-Bond: Lys721, Gln767, Met769 Alkyl/π-alkyl: Val702, Ala719, Leu742, Leu764 π-anion: Asp831 π-σ: Leu820 π-π: Phe699 van der Waals: Glu738, Cys751, Leu753, Thr766, Leu768, Thr830
5	-7.97	π -cation: LYS721(*) Alkyl/π-alkyl: Val702, Ala719, Lys721, Leu768 π-anion: Asp831 π-Sulfur: Met742 π-σ: Leu694, Phe699 Halogen: Pro770 van der Waals: Ile720, Leu764, Thr766, Met769, Gly772
Erlotinib	-7.45	π-cation: LYS721(*) H-Bond: MET769 (1.946 Å) (*) CHB: PRO770 Halogen: GLU738 π-anion: ASP831 π-σ: LEU694 Alkyl/π-alkyl: LYS721, LEU820, VAL702

(*) Visualized by Autodock Tools

was used in this study since EGFR is often overexpressed in various cancer types, including breast and colorectal cancer [17]. From the result of redocking analysis between EGFR and Erlotinib as the original ligand, the

binding site on MET769 residue was then used to conduct the docking study of all the synthesized pyrazolines **1**-**5**. The visualization of their interaction is presented in Fig. 3 and summarized in Table 2.

Erlotinib is nicely bound to EGFR via two hydrogen bonds with MET769 and CYS773 residues and via one π-cation interaction with LYS721 residue. Compare to these interactions, all of the tested pyrazolines also have π-cation interaction between LYS721 residue and ring B on pyrazoline. However, only pyrazoline **4** that has hydrogen bond interaction that occurred between MET769 residue and oxygen atom on hydroxyl group (distance: 1.789 Å). In addition to that, pyrazoline **4** also has another hydrogen bond interaction that occurred between GLN767 and hydrogen atom on hydroxyl group (distance: 1.728 Å). From this data, it can be seen that molecular docking results of pyrazolines with EGFR are consistent with their cytotoxicity data. This analysis showed that hydroxyl group on pyrazoline **4** could enhance the anticancer activity due to its capability to form hydrogen bonding with the receptor; hence pyrazoline **4** has the lowest IC_{50} value.

■ CONCLUSION

The series of high purity *N*-phenylpyrazolines **1**-**5** were successfully synthesized via cyclocondensation reaction between chalcones and phenylhydrazine. Cytotoxicity evaluation showed that pyrazoline **4** has the most potent activity as an anticancer agent against colorectal cancer cell WiDr. The molecular docking study revealed the addition of hydroxyl group on pyrazoline **4** greatly enhanced its activity due to its interaction to the EGFR receptor via two hydrogen bonds on MET769 and GLN767 residues.

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