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Synthesis, biological evaluation and molecular docking of methoxy *n*-phenylpyrazoline derivatives as anticancer agents

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Abstract: This research aims to synthesize some *N*-phenylpyrazoline derivatives with methoxy substituents and study their activity as potent anticancer agents as well as their interaction with the EGFR receptor on cancer cells. The synthesis of *N*-phenylpyrazolines was carried out via cyclocondensation reaction of chalcones and phenylhydrazine. All products were elucidated using GC-MS, FT-IR, ^{1}H- and ^{13}C-NMR spectrometers. The cytotoxicity evaluation was performed against cancer cell lines (HeLa, MCF-7, T47D, WiDr) and normal cell lines (Vero) using MTT assays. A molecular docking study of pyrazolines was conducted toward EGFR protein as a receptor. Cyclocondensation reactions yielded *N*-phenylpyrazolines in 63-91%. The presence of methoxy substituents on synthesized pyrazolines enhanced their potency as an anticancer agent. The best pyrazolines with high cytotoxicity and selectivity are compound 2e against HeLa cell line, 2d against WiDr cell line and 2f against MCF-7 cell line. Compound 2g is the most promising anticancer agent with a broad spectrum activity toward several cancer cell lines. A molecular docking study showed that the binding energy value of compound 2g with EGFR receptor is -8.4 kcal/mol and has interaction with Met769 residue. This study presented a convenient method for preparing *N*-phenylpyrazoline derivatives as promising anticancer candidates.

Keywords: *N*-phenylpyrazoline, anticancer, molecular docking, EGFR protein.

INTRODUCTION

Cancer is one of the most formidable diseases in the world due to its rapid pathological proliferation of abnormal cells. The main obstacles to cancer treatment are cancer cells' resistance, the lack of selectivity and the severe adverse effects of the commercial anticancer agents (Falzone *et al*., 2018; Schirrmacher, 2019). Hence, there is an urgent need to develop potent and cost-effective anticancer drugs.

N-phenylpyrazoline is one of the derivatives of 4,5-dihydro-1H-pyrazoles. *N*-phenylpyrazoline derivatives have been found to have various biological activities such as antibacterial (Aksoz *et al*., 2020), antioxidant (Rana *et al*., 2021), antidiabetic (Ibraheem *et al*., 2020), DNA probe (Lin *et al*., 2020), anti-inflammatory (Mantzanidou *et al*., 2021) and anticancer (Matiadis and Sagnou, 2020). Large attention was given to *N*-phenylpyrazoline as a potential anticancer agent. *N*-phenylpyrazoline derivatives have potent activity against EGFR, comparable with the positive control Erlotinib (Yang *et al*., 2013). The ability of compounds to inhibit the kinase activity of EGFR offers a promising appeal as new anticancer agents (Lichtner *et al*., 2001; Al-Obeidi and Lam, 2000).

A series of pyrazolines as potential inhibitors of cancer cells has been reported. The best inhibition activity was achieved from compounds whose B-ring contains a minimum of two methoxy groups to have optimal inhibition if it is compared with one or without any methoxy group (Yang *et al*., 2016). The hydrogen group at A-ring could inhibit HeLa cells (Neethu and Yusuf, 2014). The presence of a hydrophilic group on A-rings, such as hydroxyl at 4-position, gave the best inhibition activity toward lung cancer cell NCI-H460 (Congiu *et al*., 2010).

Due to the potential of *N*-phenylpyrazoline as an anticancer, researchers are interested in the synthesis of novel benzaldehyde analogs that contain *N*-phenylpyrazoline moiety. Synthesis of *N*-phenylpyrazoline derivatives was carried out via cycloaddition of chalcone with phenylhydrazine. Numerous phenylation methods have been published for the preparation of *N*-phenylated pyrazoline derivatives. More than 83% yield of some series of new 1,3,5-triaryl-2-pyrazoline have been synthesized by Li *et al*. (2007). Xu *et al*. (2017) have successfully synthesized pyrazoline by refluxing chalcone and phenylhydrazine for 4 h with a TBAB catalyst. Gupta *et al*. (2010) have prepared an 80-90% yield of *N*-phenylpyrazoline derivatives by sonicating chalcone, phenylhydrazine and glacial acetic acid for 30-100 min.

In the present research, we aimed to synthesize some *N*-phenylpyrazoline derivatives with variations of methoxy groups and study their cytotoxicity against various cancer cell lines and their selectivity against normal cell lines. Further, a molecular docking evaluation was performed to study their interaction against a receptor on cancer cells.

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MATERIALS AND METHODS

The chemicals were purchased from Merck with analytical grade. The monitoring of reactions was done by Thin Layer Chromatography on Merck pre-coated aluminum TLC plates $60F_{254}$ under UV light exposure. Evaluation of the cytotoxic activity on HeLa, MCF-7, T47D and WiDr cancer cell lines and also normal cell line (Vero) was performed at the Faculty of Medicine, Universitas Gadjah Mada, Indonesia.

IR analysis of KBr disks was recorded on a Shimadzu Prestige 21 FT-IR spectrophotometer. Melting points of all products were determined on Electrothermal-9100 and are uncorrected. The GC-MS analysis was operated using a Shimadzu QP 2010S. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) analyses were performed on a JEOL JNM ECA 500 spectrometer in $CDCl_3$ with an internal standard of tetramethylsilane. ELISA reader (Microplate Reader, Benchmark) was used to measure the absorbance.

Synthesis of 5-(3,4-dimethoxyphenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (2a)

Chalcone (*E*)-3-(3,4-dimethoxyphenyl)-1-phenylprop-2-en-1-one (1a) (0.268 g, 1 mmol) was prepared in 5 mL of ethanol and poured into a round bottom flask. An amount of 0.3 mL phenylhydrazine (3 mmol) and 2 mL glacial acetic acid was added to the solution. Then, the mixture was refluxed for 4 hours. After that, the solution was poured onto ice-cold distilled water and was stirred quickly. The formed solid was filtered off, washed with cold distilled water and cold ethanol consecutively and dried in a vacuum desiccator to give the 2a.

Synthesis of 2-(5-(3,4-dimethoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2b)

Pyrazoline 2b was prepared with the same procedure for pyrazoline 2a. However, the preparation used chalcone (*E*)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (1b) (0,284 g, 1 mmol) that was dissolved in 5 mL of ethanol with the addition of 0.3 mL of phenylhydrazine (3 mmol) and 2 mL of glacial acetic acid.

Synthesis of 4-(5-(3,4-dimethoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2c)

Pyrazoline 2c was prepared with the same procedure for pyrazoline 2a. However, the preparation used chalcone (*E*)-3-(3,4-dimethoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (1c) (0.284 g, 1 mmol) that was dissolved in 5 mL of ethanol with the addition 0.3 mL of phenylhydrazine (3 mmol) and 2 mL of glacial acetic acid.

Synthesis of 4-(1,5-diphenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2d)

Chalcone (*E*)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one (1d) (0.22 g, 1 mmol) was dissolved in 0.1 mL of glacial acetic acid/ethanol (10 mL). The mixture was stirred and 0.1 mL of phenylhydrazine (1 mmol) was added, then it was refluxed for 6 hours. The mixture was cooled at room temperature for 24 hours, filtered and recrystallized with ethanol to give the pyrazoline 2d.

Synthesis of 4-(5-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2e)

Chalcone (*E*)-1-(4-hydroxyphenyl)-3-(4-methoxyphenyl) prop-2-en-1-one (1e) (0.51 g, 2 mmol) was dissolved in 10 mL of ethanol with 0.2 mL of phenylhydrazine (2 mmol) and 2 mL of NaOH 20% (w/v) solution and poured into a round bottom flask. Then, it was refluxed for 5 hours. The solution was poured into distilled water ice and stirred quickly. The formed solid was filtered, washed using cold distilled water and dried by placing it inside a vacuum desiccator. The residue was dissolved with ethanol to give the 2e.

Synthesis of 3-(4-methoxyphenyl)-1,5-diphenyl-4,5-dihydro-1H-pyrazole (2f)

Pyrazoline 2f was prepared with the same procedure for pyrazoline 2d. However, the preparation used chalcone (*E*)-1-(4-methoxyphenyl)-3-phenylprop-2-en-1-one (2f) (0.23 g, 1 mmol) with 0.1 mL of phenylhydrazine (1 mmol) and 0.1 mL of glacial acetic acid.

Synthesis of 3,5-bis(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazole (2g)

Pyrazoline 2g was prepared with the same procedure for pyrazoline 2e. However, the preparation used chalcone (*E*)-1,3-bis(4-methoxyphenyl)prop-2-en-1-one (1g) (0.54 g, 2 mmol) with 0.2 mL of phenylhydrazine (2 mmol) and 2 mL of glacial acetic acid.

Synthesis of 3-(3,4-dimethoxyphenyl)-5-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazole (2h)

Pyrazoline 2h was prepared with the same procedure for pyrazoline 2e. However, the preparation used chalcone (*E*)-1-(3,4-dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (1h) (0.60 g, 2 mmol) with 0.2 mL of phenylhydrazine (2 mmol) and 2 mL of glacial acetic acid.

Cytotoxicity evaluation

The newly synthesized pyrazolines 2a-2h were evaluated on their *in vitro* inhibition of four human cancer cell lines, namely, cervical (HeLa), breast (MCF-7 and T47D) and colon (WiDr), using MTT assay. The cells were seeded in a 96-well plate for 24 h. This is to ensure the attachment of cells to the wall of each well. A series of concentrations of each synthesized compound (400, 200, 100, 50, 25, 12.5 and 6.25 μg/mL) was added to the cell. The cells were further incubated for 24 h at 37 °C with an atmosphere of 5% CO_2. After 24 h, all wells of a 96-well plate were washed with Phosphate Buffer Saline (PBS) and added with MTT solutions. The cells were further incubated for 4 h and stopped with Sodium Dodecyl Sulphate (SDS). The intensity of the formed formazan crystal color was recorded in an ELISA reader.

Molecular docking study

A molecular docking study of pyrazolines 2a-2h was performed with a similar procedure from the previous work (Suma *et al*., 2019). Briefly, all synthesized compounds were docked in EGFR (PDB ID: 1M17) with AutoDock Vina (Trott and Olson, 2010) and AutoDockTools 1.5.6 (Morris *et al*., 2009). A total of 128 conformations resulted in each docking step being listed according to the docking score. The lowest binding energy conformation was selected as the most proper conformation of the interaction. The docking results were displayed on Discovery Studio Visualizer (DSV).

STATISTICAL ANALYSIS

The correlation between the drug concentration and surviving cells was calculated to get the IC_{50} value of each cancer cell line by probit analysis using IBM SPSS Statistics version 23.0.

RESULTS

Synthesis

The synthesis of *N*-phenylpyrazolines 2a-2h from chalcones 1a-1h according to the pathway illustrated in fig. 1 was summarized in table 1.

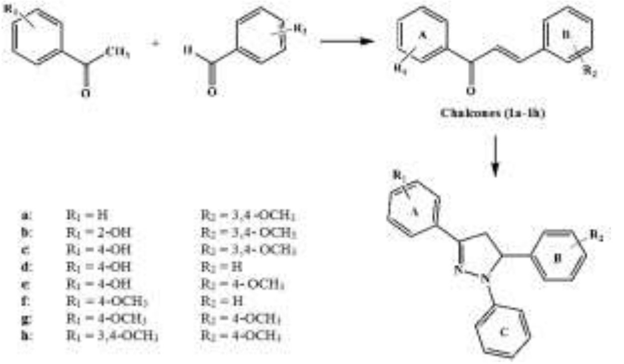

Fig. 1: General synthesis of *N*-phenylpyrazoline derivatives (2a-2h)

Spectral characterization of the synthesized compounds

5-(3,4-dimethoxyphenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazol (2a), brownish white solid, yield: 75%, M.p. 130-131 °C. Mass Spectrum (EI): m/z 358 [M^+], 327, 221, 151, 91 (base peak), 51. IR (KBr, cm^{-1}): 3078 (Csp^2-H), 2931 (Csp^3-H), 1597 (C=N), 1141 (C-N). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 3.12 (1H, dd *J* 8.0 and 16.8 Hz-CH_2 (H_a)), 3.80 (1H, dd, *J* 12.0 and 14.8 Hz, -CH_2 (H_b)), 3.81 (3H, s, O-CH_3), 3.84 (3H, s, O-CH_3), 5.18 (1H, dd, *J* 8.0 and 12.3 Hz, -CH_2 (H_x)), 6.77 (1H, d, *J* 6.5 Hz, -Ar_c-H), 6.79 (1H, d, *J* 7.5 Hz, -Ar_b-H), 6.81 (1H, d, *J* 2.5 Hz, -Ar_b-H), 6.87 (1H, dd, *J* 8.5 and 2.5 Hz, -Ar_b-H), 7.08 (2H, d, *J* 7.5 Hz, -Ar_c-H), 7.16 (2H, t, -Ar_c-H), 7.31 (1H, d, *J* 7.5 Hz, -Ar_a-H), 7.36 (2H, t, -Ar_a-H), 7.71 (2H, d, *J* 9.0 Hz, -Ar_a-H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 43.91 (-CH_2), 56.11 (-OCH_3), 64.86 (-CH-N), 108.79, 111.65, 113.71, 118.24, 119.42, 125.93, 128.76, 128.82, 129.07, 132.93, 135.43, 145.36, 147.09, 148.55, 149.79.

2-(5-(3,4-dimethoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2b), white solid, yield: 63%, M.p. 162-164 °C. Mass Spectrum (EI): 374 [M^+], 357, 237, 151, 91 (base peak), 51. IR (KBr, cm^{-1}): 3425 (O-H), 3062 (Csp^2-H), 2970 (Csp^3-H), 1597 (C=N), 1141 (C-N). ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 3.03 (1H, dd, *J* 8.0 and 17.0 Hz, -CH_2 (H_a)), 3.70 (3H, s, O-CH_3), 3.72 (3H, s, O-CH_3), 3.81 (1H, dd, *J* 12.0 and 17.5 Hz, -CH_2 (H_b)), 5.27 (1H, dd, *J* 8.0 and 12.5 Hz, -CH_2 (H_x)), 6.82 (1H, d, *J* 7.0 Hz, -Ar_c-H), 6.84 (1H, s, -Ar_b-H), 6.86 ((1H, d, *J* 8.0 Hz, -Ar_b-H), 6.88 (2H, dd, *J* 2.5 and 7.8 Hz, -Ar_c-H), 6.96 (2H, d, *J* 8.0 Hz, -Ar_c-H), 7.04 (1H, d, *J* 7.5 Hz, -Ar_b-H), 7.11 (1H, dd, *J* 1.5 and 7.8 Hz, -Ar_a-H), 7.18 (1H, d, *J* 7.5 Hz, -Ar_a-H), 7.20 (1H, d, *J* 7.5 Hz, -Ar_a-H), 7.26 (1H, d, *J* 7.5 Hz, -Ar_a-H), 10.80 (1H, s, OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 44.31 (-CH_2), 56.12 (-OCH_3), 63.75 (-CH-N), 108.75, 111.74, 113.72, 116.55, 116.82, 118.36, 119.61, 120.28, 127.37, 129.21, 129.29, 130.67, 144.55, 148.79, 149.91, 150.01, 157.41.

4-(5-(3,4-dimethoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2c), brownish white solid, yield: 74%, M.p. 157-159 °C. Mass Spectrum (EI): m/z 374 [M^+], 357, 237, 151, 91 (base peak), 51. IR (KBr, cm^{-1}): 3425 (O-H), 3078 (Csp^2-H), 2970 (Csp^3-H), 1597 (C=N), 1134 (C-N). ^1H NMR (500 MHz, DMSO) δ (ppm): 3.03 (1H, dd, *J* 7.0 and 17.3 Hz, -CH_2 (H_a)), 3.70 (6H, s, O-CH_3), 3.81 (1H, dd, *J* 12.0 and 17.3 Hz, -CH_2 (H_b)), 5.27 (1H, dd, *J* 7.0 and 11.8 Hz, -CH_2 (H_x)), 6.68 (1H, t, -Ar_c-H), 6.75 (1H, dd, *J* 2.0 and 8.0 Hz, -Ar_b-H), 6.81 (2H, *J* 8.5 Hz, -Ar_c-H), 6.88 (1H, d, *J* 8.5 Hz, -Ar_b-H), 6.92 (1H, d, *J* 2.0 Hz, -Ar_b-H), 6.97 (2H, d, *J* 8.0 Hz, -Ar_c-H), 7.13 (2H, t, -Ar_a-H), 7.57 (2H, d, *J* 9.0 Hz, -Ar_a-H), 9.75 (1H, s, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 43.42 (-CH_2), 55.45 (-OCH_3), 63.11 (-CH-N), 109.61, 112.07, 112.87, 115.50, 117.72, 118.13, 123.41, 127.42, 128.78, 135.12, 145.02, 147.74, 147.94, 149.04, 158.28.

4-(1,5-diphenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2d), brown solid, yield: 83.87%, M.p. 145-147 °C. Mass spectrum (EI) : m/z 314 (M^+), 237, 210, 104, 91 (base peak), 77, 51. IR (KBr, cm^{-1}): 3417 (-OH), 3032 (C_{sp2}-H), 1597 (-C=N), 1496 (-C=C), 1249 (-C-N). ^1H-NMR (500 MHz, $CDCl_3$) δ (ppm): 3.11 (1H, dd, *J* 7.5 and 17.0 Hz, -CH_2 (H_a)), 3.80 (1H, dd, *J* 12.0 and 15.0 Hz, -CH_2 (H_b)), 5.21 (1H, dd, *J* 7.5 and 12.0 Hz, -CH_2 (H_x)), 5.27 (1H, t, -Ar-H), 6.83 (3H, m, -Ar-H and OH), 7.16 (2H, s, -Ar-H), 7.28 (2H, t, -Ar-H), 7.32 (4H, m, -Ar-H), 7.60 (2H, d, m, -Ar-H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ (ppm): 43.78 (-

CH$_2$), 64.46 (-CH-N), 115.57, 125.88, 127.38, 127.50, 128.71, 128.93, 129.11, 142.70, 145.13, 146.77, 156.20.

4-(5-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (2e), yellow solid, yield: 81.41%, M.p. 145-147 °C. Mass spectrum (EI) : m/z 344 (M$^+$), 237, 121, 91 (base peak), 77, 51. IR (KBr, cm^{-1}): 3186 (C$_{sp2}$-H), 2924(C$_{sp3}$-H), 1597 (-C=N), 1496 (-C=C), 1249 (-C-O-C), 1172 (-C-N). ^{1}H-NMR (500 MHz, CDCl$_3$) δ (ppm): 3.05 (1H, dd, *J* 7.5 and 17.0 Hz, -CH$_2$ (H$_a$)), 3.75 (3H, s, -OCH$_3$), 3.77 (1H, dd, *J* 12.0 and 14.75 Hz, -CH$_2$ (H$_b$)), 5.16 (1H, dd, *J* 7.5 and 12.0 Hz, -CH$_2$ (H$_x$)), 6.74 (1H, t, -Ar$_c$-H), 6.82 (2H, d, *J* 9.5 Hz, -Ar$_c$-H), 6.84 (2H, d, *J* 8.5, -Ar$_a$-H), 7.04 (2H, d, *J* 7.5 Hz, -Ar$_b$-H), 7.15 (2H, t, -Ar$_b$-H), 7.22 (2H, t, -Ar$_c$-H), 7.59 (2H, d, *J* 8.5 Hz, -Ar$_a$-H). ^{13}C-NMR (125 MHz, CDCl$_3$) δ (ppm): 44.06 (-CH$_2$), 55.49 (-OCH$_3$), 64.21 (-CH-N), 113.50, 114.65, 115.71, 119.00, 125.91, 127.28, 127.62, 129.06, 135.01, 145.42, 147.00, 156.40, 159.10.

3-(4-methoxyphenyl)-1,5-diphenyl-4,5-dihydro-1H-pyrazole (2f), brown solid, yield: 78.12%, M.p. 144-147 °C. Mass spectrum (EI) : m/z 328 (M$^+$), 251, 91 (base peak), 77, 51. IR (KBr, cm^{-1}): 3032 (C$_{sp2}$-H), 2924 (C$_{sp3}$-H), 1597 (-C=N), 1496 (-C=C), 1257 and 1018 (-C-O-C), 1172 (-C-N). ^{1}H-NMR (500 MHz, CDCl$_3$) δ (ppm): 3.11 (1H, dd, *J* 7.5 and 16.8 Hz, -CH$_2$ (H$_a$)), 3.82 (1H, dd, *J* 5.5 and 16.8 Hz, -CH$_2$ (H$_b$)), 3.83 (3H, s, -OCH$_3$), 5.21 (1H, dd, *J* 7.5 and 12.5 Hz, -CH$_2$ (H$_x$)), 6.75 (1H, t, -Ar-H), 6.91 (2H, d, *J* 7.0 Hz, -Ar$_a$-H), 7.04 (2H, d, *J* 8.3 Hz, -Ar-H), 7.16 (2H, t, -Ar-H), 7.32 (4H, m, -Ar-H), 7.37 (1H, t, -Ar-H), 7.66 (2H, d, *J* 7.0 Hz, -Ar$_a$-H). ^{13}C-NMR (125 MHz, CDCl$_3$) δ (ppm): 43.80 (-CH$_2$), 55.33 (-OCH$_3$), 64.47 (-CH-N), 104.74, 113.22, 113.98, 118.79, 125.89, 127.21, 127.49, 128.86, 129.10, 142,75, 145.17, 146.77, 160.08.

3,5-bis(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazole (2g), yellow solid, yield: 67.67%, M.p. 139-141°C. Mass spectrum (EI) : m/z 358 (M$^+$), 251, 121, 91 (base peak), 77, 51. IR (KBr, cm^{-1}): 3070 (C$_{sp2}$-H), 2931 (C$_{sp2}$-H), 1597 (-C=N), 1504 (-C=C), 1249 (-C-O-C), 1172 (-C-N). ^{1}H-NMR (500 MHz, CDCl$_3$) δ (ppm): 3.09 (1H, dd, *J* 7.5 and 17.0 Hz, -CH$_2$ (H$_a$)), 3.76 (1H, dd, *J* 12.0 and 17.0 Hz, -CH$_2$ (H$_b$)), 3.82 (6H, s, -OCH$_3$), 5.18 (1H, dd, *J* 7.0 and 12.0 Hz, -CH$_2$ (H$_x$)), 6.82 (1H, t, -Ar$_c$-H), 6.89 (2H, d, *J* 8.5 Hz, -Ar$_c$-H), 6.95 (2H, d, *J* 8.5, -Ar$_b$-H), 7.12 (2H, d, *J* 8.0 Hz, -Ar$_a$-H), 7.22 (2H, d, *J* 7.5 Hz, -Ar$_b$-H), 7.27 (2H, d, 8.5 Hz, -Ar$_c$-H), 7.70 (2H, d, *J* 9.0 Hz, -Ar$_a$-H). ^{13}C-NMR (125 MHz, CDCl$_3$) δ (ppm): 43.96 (-CH$_2$), 55.37 (-OCH$_3$), 55.44 (-OCH$_3$), 64.10 (-CH-N), 113.44, 114.14, 114.58, 118.89, 125.73, 127.22, 127.35, 129.00, 134.96, 145.38, 146.95, 159.05, 160.22.

3-(3,4-dimethoxyphenyl)-5-(4-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazole (2h), yellow solid, yield: 90.82%, M.p. 87-89 °C. Mass spectrum (EI) : m/z 388 (M$^+$), 373, 281, 121, 91 (base peak), 77, 51. IR (KBr, cm^{-1}): 3001 (C$_{sp2}$-H), 2931 (C$_{sp2}$-H), 1597 (-C=N), 1504 (-C=C), 1249 (-C-O-C), 1172 (-C-N). ^{1}H-NMR (500 MHz, CDCl$_3$) δ (ppm): 3.07 (1H, dd, *J* 7.5 and 17.0 Hz, -CH$_2$ (H$_a$)), 3.75 (3H, s, -OCH$_3$), 3.78 (1H, dd, *J* 12.0 and 17.0 Hz, -CH$_2$ (H$_b$)), 3.92 (3H, s, -OCH$_3$), 5.17 (1H, dd, *J* 12.5 and 7.5 Hz, -CH$_2$ (H$_x$)), 6.75 (1H, t, -Ar$_c$-H), 6.81 (2H, d, *J* 8.5 Hz, -Ar$_c$-H), 6.84 (1H, d, *J* 9.5, -Ar$_a$-H), 7.01 (2H, dd, *J* 2.5 and 8.5 Hz, -Ar$_b$-H), 7.05 (2H, d, *J* 8.0 Hz, -Ar$_a$-H), 7.16 (2H, t, -Ar$_b$-H), 7.23 (2H, t, -Ar$_c$-H), 7.48 (1H, d, *J* 1.5 Hz, -Ar$_a$-H). ^{13}C-NMR (125 MHz, CDCl$_3$) δ (ppm): 44.01 (-CH$_2$), 55.46 (-OCH$_3$), 56.13 (-OCH$_3$), 64.27 (-CH-N), 108.31, 110.85, 113.52, 114.65, 119.06, 119.22, 126.08, 127.25, 129.05, 134.97, 145.32, 147.08, 149.33, 150.05, 159.15.

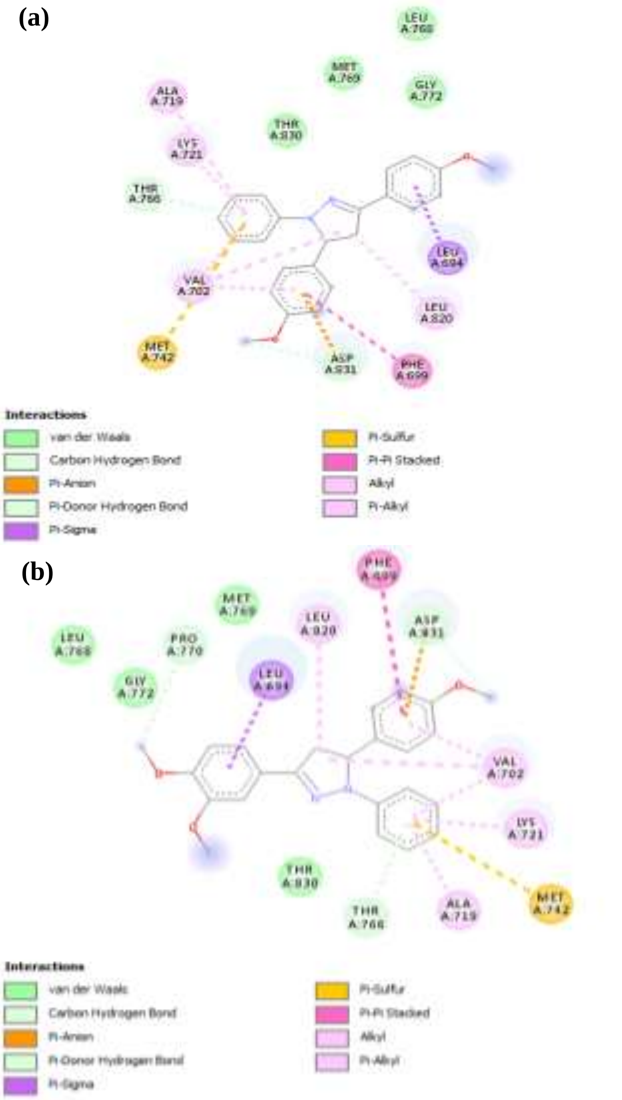

Fig. 2: The molecular docking model of EGFR receptor with (a) pyrazoline 2g and (b) pyrazoline 2h.

Cytotoxic activity

The *in vitro* cytotoxicity evaluation of the synthesized *N*-phenylpyrazolines 2a-2h on several cancer cell lines was summarized in table 2.

Molecular docking of compounds 2g and 2h

The molecular docking of compounds 2g and 2h on the EGFR receptor active site was conducted. The result was presented in table 3 and the visualization of their interaction was shown in fig. 2.

DISCUSSION

Chemistry

Synthesis of the target compounds 2a-2h was achieved through the straight pathway. The cyclization of chalcone was performed with phenylhydrazine and acid or base as a catalyst to obtain pyrazolines 2a-2h. Pyrazolines were purified by washing with ethanol. The mixtures of chalcones 1a-1h, phenylhydrazine and catalyst were refluxed for 4-6 h to produce pyrazolines 2a-2h in 63-91% yields.

All of the synthesized pyrazolines were elucidated with GC-MS, FTIR, ^{1}H- and ^{13}C-NMR spectrometers. The formation of pyrazolines 2a-2h was characterized by the presence of C=N bonds at 1597 and C-N bonds at 1134-1141 cm^{-1} in the IR spectra. According to GC-MS analysis, all synthesized pyrazoline produced molecular ions (M^+), which were suitable for the molecular weight of target compounds. In ^{1}H-NMR spectra, aromatic protons were observed at δ 6.70-7.70 ppm. The characteristic of pyrazoline is the appearance of signals from the geminal and vicinal proton of the pyrazoline ring with a splitting pattern doublet of a doublet in the upfield area. Two signals of CH_2 (Ha and Hm) appeared at 3.03-3.12 and 3.80-3.81 ppm, while the proton signal of CH (Hx) in pyrazoline rings gave chemical shifts at 5.18-5.27 ppm. According to ^{13}C-NMR analysis, the characteristic of pyrazolines was confirmed by the presence of carbon from CH_2, CH-N and C=N at 43-44, 63-64, 149-150 ppm, respectively.

Cytotoxicity Evaluation of Pyrazolines 2a-2h

All cytotoxic activities of pyrazolines 2a-2h were tested against some cancer cell lines (HeLa, WiDr, T47D, MCF-7) and Vero (normal cell line). Doxorubicin, cisplatin and 5-fluorouracil (5-Fu) are commercial anticancer drugs used as positive controls for each cancer cell (Ghosh, 2019; Sritharan and Sivalingam, 2021; Ghafouri-Fard *et al.*, 2021).

Compound 2a has high activity against WiDr cell lines. Compound 2b has high and moderate activity and selectivity against WiDr and T47D cell lines. Compound 2c has high cytotoxicity toward all four cancer cell lines but is nonselective toward the normal cell. Therefore, compound 2c is not a good anticancer agent candidate. Similar to 2c, compound 2d has high cytotoxicity toward all four cancer cell lines, but its high selectivity on normal cells is only against WiDr cell lines. Compound 2e-2h are inactive against T47D cell lines. Compounds 2e and 2g have high activity and selectivity against HeLa, WiDr and MCF-7 cell lines. Compound 2f has high activity against HeLa and MCF-7 cell lines and moderate activity against WiDr cell lines. Compound 2h has high activity against HeLa and WiDr cell lines and moderate cytotoxicity toward MCF-7 cell lines.

Overall, functional groups on ring A of the synthesized pyrazolines increase their cytotoxic activity. The presence of 4-OH substituent on ring A (compound 2c-2e) could increase their cytotoxic activity but decrease its selectivity toward normal cells. The presence of OCH_3 substituent on ring A (compound 2f-2h) could bring a more positive impact on their cytotoxic activity as well as their selectivity toward normal cells. However, two substituents of OCH_3 on ring A (compound 2h) could slightly decrease its activity, possibly due to the steric effect. In this study, the functional groups on B-rings of the synthesized pyrazolines have inconsistent effects on their cytotoxic activity against different types of the tested cancer cell line. However, the addition of 4-OCH_3 substituent (compound 2d vs. 2e, 2f vs. 2g) on ring B could decrease its cytotoxicity against normal cells; thus, its selectivity is slightly increased.

According to the results, it can be concluded that all synthesized pyrazolines (except for pyrazoline 2c) have great potential in the treatment of WiDr cancer cell lines. On the other hand, all synthesized pyrazolines are not preferable to treat T47D cancer cell lines. The best compounds to inhibit the growth of different cancer cell lines with high selectivity toward normal cells are 2e against HeLa, 2d against WiDr and 2f against MCF-7. The synthesized pyrazolines that possess a broad spectrum of cytotoxicity on several cancer cell lines with good selectivity toward normal cell lines are compounds 2f, 2g and 2h.

Molecular Docking Study

The molecular docking evaluation was conducted to learn the interaction of the synthesized pyrazolines with a receptor protein on cancer cell lines. Compounds 2g and 2h, which have great potential as anticancer candidates on several cancer cell lines, were selected as the ligand, while EGFR protein is overexpressed in various cancer cells (Sunayana *et al.*, 2017) was selected as the receptor.

The redocking result between the ligand Erlotinib and the EGFR receptor revealed that it has a binding site on Met769 residue (Suma *et al.*, 2019). Compared to this interaction, pyrazolines 2g and 2h also interact with Met769 via Van der Waals interaction. These compounds interacted with EGFR receptors with a -8.4 and -8.1 kcal/mol binding energy values. The lower binding energy value of compound 2g showed that EGFR receptors interact with 2g better than with 2h. This result is consistent with their cytotoxicity data. Compound 2g possesses better activity compared with compound 2h.

Table 1: Results of the synthesized pyrazolines 2a-2h.

Compd.	R_1	R_2	Reaction time (h)	Yield (%)	Melting point (°C)
2a	H	3,4-OCH_3	4	75	130-131
2b	2-OH	3,4-OCH_3	5	63	162-164
2c	4-OH	3,4-OCH_3	4	74	157-159
2d	4-OH	H	6	84	145-147
2e	4-OH	4-OCH_3	5	81	145-147
2f	4-OCH_3	H	6	78	144-147
2g	4-OCH_3	4-OCH_3	5	68	139-141
2h	3,4-OCH_3	4-OCH_3	5	91	87-89

Table 2: IC_{50} values and selectivity index of pyrazolines 2a-2h.

Compound	IC_{50} (µg/mL)*					SI**			
	HeLa	WiDr	T47D	MCF7	Vero	HeLa	WiDr	T47D	MCF7
2a	94.41	7.04	>100	>100	>100	1.34	17.97	0.44	0.37
2b	>100	14.09	35.05	>100	>100	0.27	8.59	3.45	0.32
2c	18.77	6.31	9.87	10.45	18.25	0.97	2.89	1.85	1.75
2d	4.09	1.72	8.06	2.42	12.14	2.97	7.06	1.51	5.02
2e	2.55	15.98	>100	16.44	>100	54.31	8.67	0.32	8.42
2f	4.83	21.47	>100	12.35	>100	>100	74.32	2.73	>100
2g	3.29	15.42	>100	15.42	>100	>100	>100	3.91	>100
2h	6.53	17.41	>100	28.09	>100	18.32	6.87	0.26	4.26
Doxorubicin	-	-	10.55	0.13	-	-	-	-	-
Cisplatin	8.15	-	-	-	-	-	-	-	-
5-Fu	-	24.65	-	-	-	-	-	-	-

*Active: IC_{50} <20 µg/mL, moderate: IC_{50} 20-100 µg/mL, inactive: IC_{50} >100 µg/mL (Tanamatayarat *et al*., 2003)
**High selectivity: SI >6, moderate selectivity: SI 3-6, nonselective: SI <3 (Amin *et al*., 2013)

Table 3: Molecular docking results of pyrazolines 2g and 2h.

Compound	Energy (kcal/mol)	Interaction
2g	-8.4	van der Waals: Met769, Thr830, Leu768, Gly772 CHB/π-HB: Thr766, Asp831 π-anion: Asp831 π-σ : Leu694 π-sulfur: Met742 π-π stacked: Phe699 alkyl/π-alkyl: Ala719, Lys721, Val702. Leu820
2h	-8.1	van der Waals: Met769, Leu768, Gly772, Thr830 CHB/π-HB: Pro770, Asp831, Thr766 π-anion: Asp831 π-σ : Leu694 π-sulfur: Met742 π-π stacked: Phe699 alkyl/π-alkyl: Ala719, Lys721, Val702. Leu820

CONCLUSION

In summary, a series of *N*-phenylpyrazoline with methoxy groups were synthesized, characterized and evaluated as anticancer agents. The result showed that the cyclocondensation reaction of chalcones 1a-1h with phenylhydrazine produced pyrazolines 2a-2h in 63-91% yields. The addition of OCH_3 substituent on pyrazolines enhanced their potency as an anticancer agent. Compound 2g has the most potent anticancer activity and is nicely bound to the EGFR receptor via interaction with Met769 residue.

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