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Synthesis, anticancer activity, and docking study of *N*-acetyl pyrazolines from veratraldehyde

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ABSTRACT

N-acetyl pyrazoline derivatives **A–C** containing methoxy and chloro/hydroxyl substituents were synthesized and tested for their cytotoxic activities. The precursor chalcones **A–C** which were obtained from the condensation reaction between veratraldehyde and acetophenone derivatives were reacted with hydrazine hydrate in the presence of glacial acetic acid to give pyrazolines **A–C** with excellent yield and purity. Characterization of all products was done using Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometers. (GC-MS). Cytotoxicity evaluation of pyrazolines revealed that pyrazoline **A** has moderate activity against breast cancer cell line MCF7 (IC_{50} 40.47 µg/ml), breast cancer cell line T47D (IC_{50} 26.51 µg/ml), and cervical cancer cell line HeLa (IC_{50} 31.19 µg/ml). Pyrazoline **B** is inactive against all tested cancer lines (IC_{50} > 100 µg/ml). Pyrazoline **C** has moderate activity against MCF7 (IC_{50} 94.02 µg/ml), but inactive against T47D and HeLa. Docking study showed the interaction between pyrazolines and EGFR receptor via hydrogen bonds and π-cation interactions.

INTRODUCTION

Cancer is a group of diseases defined by the rapid, uncontrolled, and pathological proliferation of the abnormal cells (Karabacak *et al*., 2015). Globally, following cardiovascular disorders, cancer is the second-leading disease that causes death (Patel *et al*., 2011). In 2013, the two types of cancer with the most cases which occurred in Indonesia were cervical and breast cancer with 98,692 cases and 61,682 cases, respectively (Kementerian Kesehatan, 2015). Resistance, lack of selectivity, and the occurrence of side effect to chemotherapeutic agents remain challenging problems in the medical field. Therefore, novel anticancer agents must be developed to suppress this issue.

Pyrazolines, a class of electron-rich nitrogen heterocyclic compounds, have versatile uses in the medicinal chemistry. They possess a wide range of pharmacological functions such as anticancer, antitubercular, antimalarial, antibacterial, antifungal, anti-inflammatory, analgesic, anticonvulsant, and antioxidant activities (Ahmad *et al*., 2016; Beyhan *et al*., 2017; Karad *et al*., 2016; Kumar *et al*., 2013; Lv *et al*., 2010; Rani *et al*., 2012; Viveka *et al*., 2015).

Molecular docking studies of pyrazoline derivatives against two types of epidermal growth factor receptor kinase, which are epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER-2) were reported (Mubeen *et al*., 2015; Yang *et al*., 2013). In many cases, those proteins are often overexpressed in breast and cervical cancer (Masuda *et al*., 2012; Tian *et al*., 2016). The molecular docking studies revealed the important factors influencing their activity as anticancer agents that were in the N–N bond of the pyrazoline ring and the various functional groups attached to the benzene rings (Yang *et al*., 2013). Pyrazoline derivatives containing an acetyl group attached to the nitrogen atom, called *N*-acetyl pyrazolines, are reported to produce excellent cytotoxic activity. Moreover, the presence of a methoxy substituent on the benzene B-ring enhanced its activities together with the presence of chloro and hydroxyl substituent on the *para* position of the benzene A-ring (Sharma *et al*., 2014).

A well-known method to synthesize pyrazolines is through a cyclocondensation reaction between hydrazines with α,β-unsaturated aldehydes or ketones. In this method, intermediate

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hydrazones are formed that undergo cyclization in the presence of a cyclizing reagent such as acetic acid to yield pyrazolines (Shaaban *et al*., 2012).

In the light of the above findings, we aimed to synthesize *N*-acetyl pyrazoline derivatives bearing methoxy, chloro, and hydroxyl substituents (Fig. 1). Their cytotoxicity was evaluated against breast cancer cell lines (MCF7 and T47D) and the cervical cancer cell line (HeLa) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents used were pro analysis grade originated from Merck with no further purification, i.e., 4-chloroacetophenone, 4-hydroxyacetophenone, veratraldehyde, hydrazine hydrate, sodium hydroxide, hydrochloric acid, glacial acetic acid, ethanol, methanol, ethyl acetate, dichloromethane, hexane, and dimethyl sulfoxide (DMSO). Thin layer chromatography was carried out using aluminum plates 20 × 20 cm (Merck) coated with silica gel 60 F_{254}. The materials used for the cytotoxicity evaluation were breast cancer cell line (MCF7 and T47D), cervical cancer cell line (HeLa), normal cell line (Vero), Dulbecco's Modified Eagle Medium (DMEM), Medium 199 (M199), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium hydrogen carbonate, Fetal Bovine Serum (FBS), penicillin-streptomycin (Pen-Strep), amphotericin B, trypsin-EDTA solution, phosphate buffered saline (PBS) pH 7.4, MTT, and sodium dodecyl sulfate (SDS).

Instrumentations

All melting points were uncorrected and determined in open capillary tubes by digital melting point apparatus (Electrothermal 9100). Mass spectra and purity of all synthesized compounds were taken from GC-MS spectrometer on Shimadzu QP2010S (EI). IR spectra were recorded with Shimadzu Prestige-21 using KBr discs. ^{1}H and ^{13}C-NMR spectra were recorded with JEOL JNMECA [500 MHz (^{1}H) and 125 MHz (^{13}C)] using tetramethylsilane (TMS) as a standard internal. The cytotoxicity evaluation were performed using microwell plate 96 (Biologix), micropipette 2–20 µl (VWR brand), micropipette 20–200 µl (VWR brand), micropipette 100–1,000 µl (AccuBioTech), laminar air flow (Labconco, Purifier Delta Series Class II), a 5% CO_2 incubator (Heraeus), a hemocytometer (Neubauer), an inverted microscope (Axiovert 25), a centrifuge (Janetzki T5, and an enzyme-linked immunosorbent assay (ELISA) reader (BIO-RAD Benchmark).

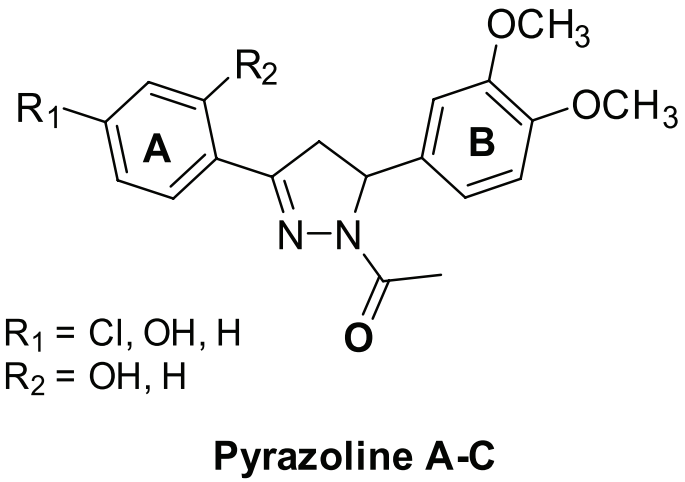

Figure 1. Chemical structure of *N*-acetyl pyrazoline **A**–**C**.

Synthesis of (E)-1-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl) prop-2-en-1-one (chalcone A)

To a solution of veratraldehyde (1.54 g, 10 mmol) in 30-ml absolute ethanol, 4-chloroacetophenone (1.3 ml, 10 mmol) was added with constant stirring. Droplets of 5-ml aqueous sodium hydroxide 30% (w/v) were also added into it. The reaction was stirred for 4 hours at the room temperature. Then, the stirring was stopped and the mixture was kept overnight in refrigerator at 15°C. Vacuum filtration of the mixture was performed to obtain solid precipitate. It was washed with cold water and dried in vacuum desiccator to obtain chalcone **A**. Yellowish green solid, m.p. 99.2°C–99.6°C, yield: 93.19%, purity: 97.72%. IR (KBr) ν_{max} (cm^{-1}): 2,954 (C-H str.), 1,658 (C=O str.), 1,581 and 1,512 (aromatic C=C str.), 1,280 (C-O methoxy str.), 1,157 and 1,026 (C-Cl str.), and 987 (trans C=C bend.). ^{1}H-NMR (500 MHz) δ (ppm): 3.93 (3H, *s*, OCH_3), 3.95 (3H, *s*, OCH_3), 6.89 (1H, *d*, J = 8.4 Hz, ArH), 7.14 (1H, *d*, J = 1.95 Hz, ArH), 7.23 (1H, *dd*, J = 1.95, 8.45 Hz, ArH), 7.33 (1H, *d*, J = 15.55 Hz, C=CH trans), 7.46 (2H, *d*, J = 8.4 Hz, ArH), 7.76 (1H, *d*, J = 15.55 Hz, C=CH trans), and 7.95 (2H, *d*, J = 8.45 Hz, ArH). ^{13}C-NMR (125 MHz) δ (ppm): 56.1 (OCH_3), 56.2 (OCH_3), 110.3 (CH), 111.3 (CH), 119.6 (CH), 123.5 (C=C), 127.8 (C), 128.6 (CH), 130.0 (CH), 136.9 (C), 139.1 (C), 145.7 (C=C), 149.4 (C), 151.8 (C), and 189.4 (C=O). MS (EI) m/z: 304 (^{37}Cl, 30), 302 (^{35}Cl, 80), 289 (^{37}Cl, 10), 287 (^{35}Cl, 40), 273 (^{37}Cl, 10), 271 (^{35}Cl, 50), 261 (^{37}Cl, 5), 259 (^{35}Cl, 15), 191 (30), 163 (20), 141 (^{37}Cl, 15), 139 (^{35}Cl, 50), 113 (^{37}Cl, 30), and 111 (^{35}Cl, 100).

Synthesis of (E)-3-(3,4-dimethoxyphenyl)-1-(4-hydroxyphenyl) prop-2-en-1-one (chalcone B)

A mixture of 4-hydroxyacetophenone (0.68 g, 5 mmol) in 10-ml ethanol and 2-ml aqueous NaOH 40% (w/v) was prepared. To this mixture, a solution of veratraldehyde (0.83 g, 5 mmol) in 10-ml ethanol was added dropwise with constant stirring. The reaction mixture was stirred for 48 hours, then it was poured into iced-cold water and acidified with HCl 10% (v/v). Vacuum filtration of the mixture was performed to obtain solid precipitate. It was washed with cold water and dried in vacuum desiccator. Recrystallization with ethanol was done to obtain chalcone **B**. Yellow solid, m.p. 199°C–201°C, yield: 84.51%, purity: 100%. IR (KBr) ν_{max} (cm^{-1}): 3,240 (OH str.), 1,643 (C=O str.), 1,597 and 1,512 (aromatic C=C str.), 1,265 (C-O methoxy str.), and 987 (trans C=C bend.). ^{1}H-NMR (500 MHz) δ (ppm): 3.93 (3H, *s*, OCH_3), 3.95 (3H, *s*, OCH_3), 5.70 (1H, *s*, OH), 6.90 (1H, *d*, J = 8.4

Hz, ArH), 6.93 (1H, *d*, *J* = 9.05 Hz, ArH), 7.15 (1H, *d*, *J* = 1.95 Hz, ArH), 7.23 (1H, *dd*, *J* = 1.95 and 8.45 Hz, ArH), 7.39 (1H, *d*, *J* = 15.55 Hz, C=CH trans), 7.76 (1H, *d*, *J* = 15.55 Hz, C=CH trans), and 8.00 (1H, *d*, *J* = 8.45 Hz, ArH). ^{13}C-NMR (125 MHz) δ (ppm): 55.9 (OCH_3), 55.9 (OCH_3), 110.2 (CH), 111.2 (CH), 115.5 (CH), 119.9 (C=C), 123.1 (CH), 128.1 (C), 130.1 (C), 131.1 (CH), 144.4 (C=C), 149.2 (C), 151.3 (C), 161.9 (C), and 189.7 (C=O). MS (EI) m/z: 284 (100), 269 (50), 253 (40), 241 (30), 191 (20), 121 (90), 93 (50), and 65 (90).

Synthesis of (E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one (chalcone C)

A mixture of 2-hydroxyacetophenone (0.68 g, 5 mmol) in 5-ml methanol and 5-ml aqueous NaOH 40% (w/v) was prepared. To this mixture, a solution of veratraldehyde (0.83 g, 5 mmol) in 5-ml methanol was added dropwise with constant stirring. The reaction mixture was heated under reflux for 4 hours, then it was poured into iced-cold water. The mixture was acidified with HCl 10% (v/v) and kept overnight in refrigerator at 15°C. Vacuum filtration of the mixture was performed to obtain the solid precipitate. It was washed with cold water and dried in vacuum desiccator. Recrystallization with methanol-water (4:1) was done to obtain chalcone **C**. Yellow solid, m.p. 103.2°C–106.0°C, yield: 45.07%, purity: 100%. IR (KBr) ν_{max} (cm^{-1}): 3,448 (OH str.), 1,635 (C=O str.), 1,573 and 1,512 (aromatic C=C str.), 1,265 (C-O methoxy str.), and 979 (trans C=C bend.). ^{1}H-NMR (500 MHz) δ (ppm): 3.94 (3H, *s*, OCH_3), 3.96 (3H, *s*, OCH_3−), 6.90 (1H, *d*, *J* = 8.45 Hz, ArH), 6.92 (1H, *d*, *J* = 7.15 Hz, ArH), 7.02 (1H, *d*, *J* = 8.40 Hz, ArH), 7.18 (1H, *s*, ArH), 7.26 (1H, *d*, *J* = 8.45 Hz), 7.47 (1H, *d*, *J* = 8.40 Hz, ArH), 7.50 (1H, *d*, *J* = 14.90 Hz, C=CH trans), 7.88 (1H, *d*, *J* = 15.55 Hz, C=CH trans), 7.92 (1H, *d*, *J* = 7.75 Hz, ArH), and 12.90 (1H, *s*, OH). ^{13}C-NMR (125 MHz) δ (ppm): 56.1 (OCH_3), 56.1 (OCH_3), 110.4 (CH), 111.3 (CH), 117.9 (C=C), 118.7 (CH), 118.9 (CH), 123.8 (CH), 129.7 (CH), 136.3 (CH), 120.2 (C), 127.7 (C), 145.8 (C=C), 19.4 (C), 151.9 (C), 163.6 (C), and 193.7 (C=O). MS (EI) m/z: 284 (35), 269 (5), 253 (10), 241 (5), 121 (65), 93 (40), and 65 (100).

Synthesis of 1-(3-(4-chlorophenyl)-5-(3,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)ethan-1-one (pyrazoline A)

Hydrazine hydrate (0.2 ml, 4 mmol) with glacial acetic acid (15 ml) was heated under reflux for 4 hours to give acetyl hydrazine. Chalcone **A** (3.02 g, 1 mmol) was added into it and it was heated under the reflux for another 5 hours. Then, the mixture was poured into iced-cold water and left overnight. Vacuum filtration of the mixture was performed to obtain solid precipitate. Recrystalization with ethanol was done to obtain pyrazoline **A**. White solid, m.p. 128°C–129.3°C, yield: 97.28%, purity: 91.90%. IR (KBr) ν_{max} (cm^{-1}): 1,660 (C=O str.), 1,597 (C=N str.), 1,257 (C-O methoxy str.), 1,142 (C-N str.), and 1,026 (C-Cl str.). ^{1}H-NMR (500 MHz) δ (ppm): 2.42 (3H, *s*, $COCH_3$), 3.12 (1H, *dd*, *J* = 4.50 and 17.50 Hz, CH_2), 3.70 (1H, *dd*, *J* = 12.50 and 17.50 Hz, CH_2), 3.82 (3H, *s*, OCH_3), 3.84 (3H, *s*, OCH_3), 5.54 (1H, *dd*, *J* = 4.50 and 12.5 Hz, CH), 6.75 (3H, *m*, ArH), 7.39 (2H, *d*, *J* = 8.40 Hz, ArH), and 7.76 (2H, *d*, *J* = 8.40 Hz, ArH). ^{13}C-NMR (125 MHz) δ (ppm): 22.1 (CH_3), 42.5 (CH_2), 56.1 (CH), 60.1 (OCH_3), 60.1 (OCH_3), 109.2 (CH), 111.6 (CH), 117.6 (CH), 126.5 (CH), 129.1 (CH), 128.0 (C), 134.7 (C), 136.5 (C), 148.7 (C), 149.4 (C), 153.0 (C), and 169.2 (C=O). MS (EI) m/z: 358 (^{35}Cl, 5), 315 (^{35}Cl, 15), 221 (^{35}Cl, 25), 206 (^{35}Cl, 15), 179 (^{35}Cl, 10), 138 (^{35}Cl, 10), and 43 (100).

Synthesis of 1-(5-(3,4-dimethoxyphenyl)-3-(4-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)ethan-1-one (pyrazoline B)

A mixture of chalcone **B** (0.54 g, 2 mmol), glacial acetic acid (10 ml), hydrazine hydrate (0.4 ml, 10 mmol), and 10-ml methanol was heated under the reflux for 6 hours. Then, it was poured into iced-cold water and kept in refrigerator overnight at 15°C. Vacuum filtration of the mixture was performed to obtain solid precipitate. Column chromatography was performed using dichloromethane-ethyl acetate as an eluent to give pyrazoline **B**. White solid, m.p. 219°C–221°C, yield: 88.28%, purity: 83.66%. IR (KBr) ν_{max} (cm^{-1}): 3,425 (OH str.), 1,651 (C=O str.), 1,604 (C=N str.), 1,234 (C-O methoxy str.), and 1,141 (C-N str.). ^{1}H-NMR (500 MHz) δ (ppm): 2.38 (3H, *s*, $COCH_3$), 2.43 (1H, *s*, OH), 3.08 (1H, *dd*, *J* = 4.55 and 17.50 Hz, CH_2), 3.64 (1H, *dd*, *J* = 11.70 and 17.50 Hz, CH_2), 3.79 (3H, *s*, OCH_3), 3.80 (3H, *s*, OCH_3), 5.45 (1H, *dd*, *J* = 4.55 and 11.7 Hz, CH), 6.72 (2H, *d*, *J* = 6.45 Hz, ArH), 6.76 (1H, *d*, *J* = 9.05 Hz, ArH), 6.81 (2H, *d*, *J* = 9.05 Hz, ArH), and 7.57 (2H, *d*, *J* = 6.45 Hz, ArH). ^{13}C-NMR (125 MHz) δ (ppm): 22.0 (CH_3), 42.7 (CH_2), 55.9 (OCH_3), 56.0 (OCH_3), 59.8 (CH), 109.3 (CH), 111.6 (CH), 115.8 (CH), 115.9 (CH), 117.8 (CH), 128.5 (CH), 128.6 (CH), 123.0 (C), 134.7 (C), 148.6 (C), 149.3 (C), 159.3 (C), 155.0 (C), and 169.3 (C=O). MS (EI) m/z: 340 (15), 297 (30), 221 (20), 190 (5), 161 (15), 135 (10), 120 (20), 93 (5), and 43 (100).

Synthesis of 1-(5-(3,4-dimethoxyphenyl)-3-(2-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)ethan-1-one (pyrazoline C)

A mixture of chalcone **C** (0.142 g, 0.5 mmol), glacial acetic acid (10 ml) and hydrazine hydrate (0.2 ml, 4 mmol) was heated under the reflux for 24 hours. Then, it was poured into iced-cold water and kept in refrigerator overnight at 15°C. Vacuum filtration of the mixture was performed to obtain solid precipitate. Column chromatography was performed using hexane-ethyl acetate as eluent to give pyrazoline **C**. White solid, m.p. 137.2°C–139.8°C, yield: 93.14, purity: 98.96%. IR (KBr) ν_{max} (cm^{-1}): 3,448 (OH str.), 1,658 (C=O str.), 1,597 (C=N str.), 1,265 (C-O methoxy str.), and 1,142 (C-N str.). ^{1}H-NMR (500 MHz) δ (ppm): 2.39 (3H, *s*, $COCH_3$), 3.29 (1H, *dd*, *J* = 4.55 and 17.50 Hz, CH_2), 3.84 (3H, *s*, OCH_3), 3.86 (3H, *s*, OCH_3), 3.94 (1H, *dd*, *J* = 12.5 and 17.50 Hz, CH_2), 5.50 (1H, *dd*, *J* = 4.55 and 12.5 Hz, CH), 6.76 (2H, *d*, *J* = 6.60 Hz, ArH), 6.80 (1H, *d*, *J* = 6.50 Hz, ArH), 6.92 (1H, *d*, *J* = 7.80 Hz, ArH), 7.06 (1H, *d*, *J* = 8.45 Hz, ArH), 7.22 (1H, *d*, *J* = 8.45 Hz, ArH), 7.35 (1H, *t*, ArH), and 10.28 (1H, *s*, OH). ^{13}C-NMR (125 MHz) δ (ppm): 22.9 (CH_3), 42.9 (CH_2), 56.1 (OCH_3), 56.1 (OCH_3), 58.4 (CH), 109.3 (CH), 111.6 (CH), 117.2 (CH), 117.8 (CH), 120.0 (CH), 128.6 (CH), 132.5 (CH), 115.3 (C), 133.9 (C), 148.9 (C), 149.4 (C), 157.8 (C), 156.6 (C), and 1,698.1 (C=O). MS (EI) m/z: 340 (50), 325 (5), 298 (30), 297 (65), 281 (35), 267 (10), 161 (55), and 43 (100).

The cytotoxic activity assay

All cell lines were cultured in 5% CO_2 water-saturated atmosphere at 37°C with medium DMEM/10% FBS for cancer

cell line (MCF7, T47D, and HeLa) and M199/10% FBS for normal cell line (Vero). Cell suspensions (10^6/ml) were prepared, and 100 µl/well were dispensed into a 96-well plate giving 10^4 cells/well. The plates were incubated for 24 hours to allow the cells to reattach. Pyrazolines were initially formulated at 10^5 µg/ml in DMSO. These samples were diluted with a culture medium to give six serial concentration: 200, 100, 50, 25, 12.5, and 6.25 µg/ml. Aliquots (100 µl) of each concentration were added to the wells. After further incubation for 24 hours, the cell viability was evaluated using the MTT assay. The culture medium on plates was removed and washed with PBS. A solution of MTT in PBS was prepared at 50 mg/10 ml. Aliquots (1 ml) of MTT solution were diluted with 9.5-ml culture medium. Aliquots (100 µl) of diluted MTT were added to the wells and incubated for 4 hours. A total of 100 µl SDS stopper 10% in 0.1 N HCl was added into each well and left overnight. Absorbance readings were performed by ELISA reader at 595 nm. Cell viability and IC_{50} value were calculated.

Docking study

The three-dimensional (3D) structure of pyrazolines **A–C** was drawn using GaussView 5.0 and optimized using Gaussian 09 with DFT/B3LYP method and 6-31G basis set. The 3D crystal structure of the EGFR domain bound to Erlotinib was retrieved from the protein data bank (PDB ID: 1M17). The preparation of ligand and protein was conducted using Chimera. Redocking and docking were performed with Autodock Tools (ADT) and Autodock 4 in a 45 × 45 × 45 Å grid box with a spacing of 0.375 Å using Lamarckian Genetic Algorithm. The redocking analysis was successfully performed when the RMSD value was <2 Å (Huey *et al*., 2007). A hundred molecular docking poses for each ligand were ranked based on their docking score. The scoring function in AutoDock was used to predict the binding affinity of the ligand to the receptor. The conformation with the lowest binding energy was chosen as the most suitable conformation. The docking result was visualized using ADT and Discovery Studio Visualizer.

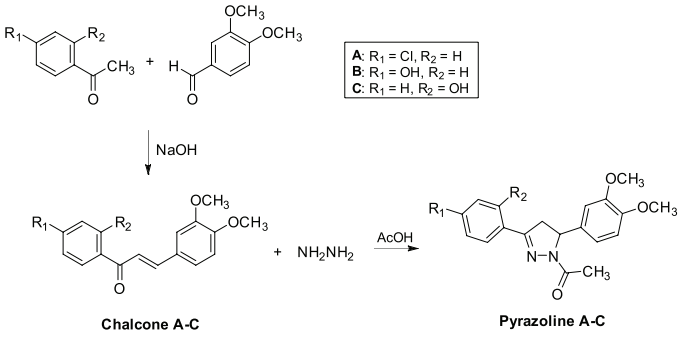

Figure 2. A synthetic scheme of pyrazoline **A–C**

RESULTS AND DISCUSSION

The synthesis of chalcone **A–C** (Fig. 2) was accomplished via the aldol condensation reaction with the addition of sodium hydroxide as a basic catalyst to afford products in excellent yield and purity. Those syntheses were carried out according to the literature (Susanti *et al*., 2012) with slight modification. Table 1 presents the physical data of the chalcones. The characterization of the synthesized chalcones was conducted by GC-MS, FTIR, and NMR spectrometers. The MS spectra confirmed the molecular weight of the desired chalcones. Characteristic molecular ion peaks (M^+ and M^{+2}) with the height ratio of 3:1 were observed in chalcone **A**. This confirmed the presence of two chlorine isotopes, ^{35}Cl and ^{37}Cl. The IR spectra displayed some important peaks. The presence of C_{sp2}-H bending band at 979–987 cm^{-1} for trans-disubstituted alkene and the absence of C-H aldehyde stretching bands confirmed that chalcone **A–C** were correctly achieved. The ^{1}H-NMR spectra of chalcone displayed two doublet signals with *J* (coupling constant) value of 15.55 Hz proving the presence of alkene protons with a trans coupling (Raza *et al*., 2016). Signals that corresponded with the trans-alkene carbons were also observed in ^{13}C-NMR spectra.

The cyclocondensation reaction of the chalcone **A–C** and hydrazine hydrate with the addition of glacial acetic acid produced *N*-acetyl pyrazoline **A–C** (Fig. 2). The obtained pyrazolines have high yield and purity. Table 1 reports the physical data of these products. The structure of pyrazoline **A–C** was confirmed by several important spectral changes. The mass spectra confirmed the molecular weight of the synthesized pyrazolines. The IR spectra showed the absence of trans-disubstituted alkene at 978 cm^{-1} as well as the presence of new absorption peaks for C=N (~1,597 cm^{-1}) and C-N (~1,142 cm^{-1}) confirming the formation of a pyrazoline ring. The ^{1}H-NMR spectra displayed the existence of characteristic signals with AMX spin system due to the diastereotopic nature of methylene protons on the pyrazoline ring (Suma *et al*., 2017). Thus, their splitting patterns came as a doublet of doublets at the region of δ 3 and 4 ppm. Furthermore, the CH proton on the pyrazoline ring arose as a doublet of doublet at the region of δ 5 ppm owing to vicinal coupling with methylene protons. The ^{13}C-NMR spectra confirmed the absence signals of trans-alkene carbons and the appearing signals of carbons on the pyrazoline ring.

Table 1. Physical data of all synthesized compounds.

Compound	R_1	R_2	Molecular formula	Molecular weight	Yield (%)	Purity (%)	Melting Point (°C)
Chalcone A	Cl	H	$C_{17}H_{15}ClO_3$	302.75	93.19	97.72	99.2–99.6
Chalcone B	OH	H	$C_{17}H_{16}O_4$	284.31	84.51	100.00	199.0–201.0
Chalcone C	H	OH	$C_{17}H_{16}O_4$	284.31	45.07	100.00	103.2–106.0
Pyrazoline A	Cl	H	$C_{19}H_{19}ClN_2O_3$	358.82	97.28	91.90	128.0–129.3
Pyrazoline B	OH	H	$C_{19}H_{20}N_2O_4$	340.38	88.128	83.66	219.0–221.0
Pyrazoline C	H	OH	$C_{19}H_{20}N_2O_4$	340.38	93.14	98.96	137.2–139.8

The synthesized pyrazolines **A–C** were evaluated as anticancer agents using MTT against breast cancer lines (MCF7 and T47D), the cervical cancer line (HeLa), and the normal cell line (Vero). Table 2 summarizes the percentage of cell growth inhibition of pyrazolines **A** and **B**. This result shows a correlation between sample concentrations with the percentage of cell growth inhibition: higher concentration resulted in less cell growth. Thus, the inhibition percentage is increasing; this is understandable because typically the more concentrated the compound is, the more toxic it becomes.

The percentage of cell growth inhibition was converted into the IC_{50} value. The activity of compounds on their cell growth inhibition is classified into three categories, i.e., active (IC_{50} < 20 µg/ml), moderate (IC_{50} 20–100 µg/ml), and inactive (IC_{50} > 100 µg/ml) (Tanamatayarat *et al*., 2003). Table 3 shows the IC_{50} values of the synthesized pyrazoline **A–C** and commercial drugs. According to the reference stated above, pyrazoline **A** has moderate activity in the inhibition of all tested cancer cell line MCF7 (IC_{50} 40.47 µg/ml), T47D (IC_{50} 26.51 µg/ml), and HeLa (IC_{50} 31.19 µg/ml). The most potent activity of pyrazoline **A** is against the breast cancer cell line T47D. Pyrazoline **B** is inactive in the inhibition of all tested cancer cell lines (IC_{50} > 100 µg/ml). Despite its inactivity, pyrazoline **B** is deemed selective as compared with its activity against normal cell Vero (IC_{50} 44 × 10^3 µg/ml). This compound has a selectivity index of 64.6, 16.3, and 5.8 against MCF7, T47D, and HeLa, respectively. On the other hand, pyrazoline **C** has moderate activity in the inhibition of MCF7 (IC_{50} 94.02 µg/ml); however, it is inactive against T47D and HeLa. Pyrazoline **C** is also deemed selective as compared with normal cell Vero (IC_{50} 9 × 10^3 µg/ml) with a selectivity index of 98.1, 12.1, and 34.2 against MCF7, T47D, and HeLa, respectively. Based on this result, the IC_{50} values of pyrazoline **A** against all tested cancer cell lines are shown to be lower than the IC_{50} values of pyrazolines **B** and **C**. Thus, pyrazoline **A** is a better anticancer candidate than the others. The difference in their activities is due to the presence of functional groups attached to the molecule. Pyrazoline **A** with the presence of the halogen *p*-Cl substituent on the benzene A-ring displayed more potent activity. Meanwhile, the presence of a hydroxyl substituent on the benzene A-ring of pyrazoline **B** and **C** decreased their activity.

Table 2. Percentage of cell growth inhibition.

Pyrazoline	Concentration (µg/ml)	Cell growth inhibition (%)		
		MCF7	T47D	HeLa
A	200	91.81	99.04	99.22
	100	77.92	97.12	81.99
	50	56.40	75.08	64.29
	25	28.92	44.83	47.28
	12.5	18.85	15.02	16.89
	6.25	4.96	4.79	5.36
B	200	31.96	-	13.52
	100	31.61	10.81	10.42
	50	27.46	10.91	9.04
	25	15.18	4.14	6.02
	12.5	11.25	1.68	3.86
	6.25	8.51	1.82	-

Table 3. IC_{50} values of the tested compounds.

Entry	IC_{50} value (µg/mL)		
	MCF7	T47D	HeLa
Pyrazoline A	40.47	26.51	31.19
Pyrazoline B	680.00	2702.00	7642.00
Pyrazoline C	94.02	764.89	269.47
Doxorubicin	3.04	10.58	-
Cisplatin	-	-	18.39

A molecular docking study was performed to understand how pyrazolines interact with the receptor. An EGFR receptor was used as the protein is often overexpressed in breast and cervical cancer (Masuda *et al*., 2012; Mubeen *et al*., 2015). Other studies also reported the use of the EGFR receptor on the docking molecule of pyrazoline derivatives (Lv *et al*., 2010; Yang *et al*., 2013). The redocking analysis of EGFR with the original ligand (Erlotinib) showed its binding site on the MET769 residue. This binding site was then used to perform a docking simulation of the pyrazoline compounds. The binding models of pyrazolines with EGFR are shown in Fig 3 and the result was summarized in Table 4.

Pyrazoline **A** and **C** is nicely bound to the EGFR receptor through hydrogen bond with the side chain of MET769 residue. The binding energy of pyrazoline **A** is −7.24 kcal/mol. The oxygen atom on *m*-OCH_3 of ring B contributes to the hydrogen bonding interaction with the hydrogen atom on the side chain of MET769 (distance: 1.946 Å). The π-cation interaction occurred between ring A and the LYS721 residue indicates that the presence of ring A on pyrazoline has enhanced the interaction of ligand and receptor. Compared with pyrazoline **A**, the interaction between pyrazoline **C** and EGFR receptor is less preferable which is

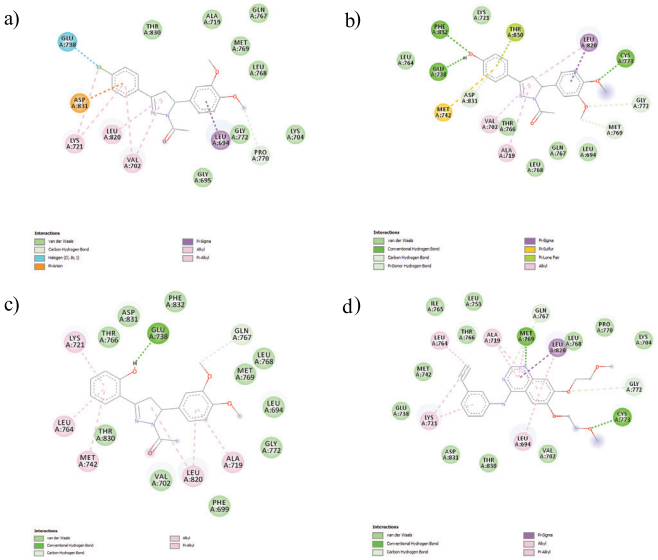

Figure 3. 2D molecular docking model of the EGFR receptor with (a) pyrazoline **A**, (b) pyrazoline **B**, (c) pyrazoline **C**, and (d) Erlotinib.

Table 4. Docking molecular result.

Compound	Energy (FEB) (kcal/mol)	Interaction
A	−7.24	H-Bond: MET769 (1.946 Å)*
		π-cation: LYS721*
		CHB: PRO770
		Halogen: GLU738
		π-anion: ASP831
		π-σ: LEU694
		Alkyl/π-alkyl: LYS721, LEU820, VAL702
B	−7.49	H-Bond: GLU738 (1.608 Å)*, CYS773 (2.074 Å)*
		π-cation: LYS721*
		H-Bond: GLU738, CYS773, PHE832
		CHB/π-HB: ASP831, MET769, GLY772
		π-σ: LEU820
		π-Sulfur: MET742
		π-Lone Pair: THR830
		Alkyl: VAL702, ALA719, LEU820
C	−7.15	H-Bond: GLU738 (1.916 Å)*, MET769 (1.986 Å)*
		π-cation: LYS721*
		H-Bond: GLU738
		CHB: GLN767
		Alkyl/π-alkyl: ALA719, LYS721, LEU764, MET742, LEU820
Erlotinib	−7.45	H-Bond: MET769 (1.946 Å)*
		π-cation: LYS721*
		CHB: PRO770
		Halogen: GLU738
		π-anion: ASP831
		π-σ: LEU694
		Alkyl/π-alkyl: LYS721, LEU820, VAL702

*Visualized by Autodock Tools.

showed from its binding energy of −7.15 kcal/mol. The hydrogen bonding interaction occurred between oxygen atom on *m*-OCH_3 of ring B with the hydrogen atom on the side chain of MET769 residue (distance: 1.986 Å). The π-cation interaction is observed between ring A and the LYS721 residue. Meanwhile, there is no interaction between pyrazoline **B** and MET769 residue which led to the conclusion that pyrazoline **B** is the less active compound among the others. The molecular docking result together with the cytotoxicity evaluation data infers that pyrazoline **A** is a better anticancer agent.

CONCLUSION

The novel *N*-acetyl pyrazoline derivatives containing methoxy groups obtained from veratraldehyde were successfully synthesized in excellent yield and purity by the cyclocondensation of chalcones and hydrazine hydrate in glacial acetic acid. Cytotoxicity evaluation revealed that the presence of a halogen substituent such as chloro on a pyrazoline derivative (pyrazoline **A**) increased its cytotoxic activity against some cancer cell lines, while the presence of hydroxyl substituent (pyrazoline **B** and **C**) decreased the anticancer activity. The molecular docking study showed that pyrazoline **A** could nicely bind to the active site of the EGFR receptor via hydrogen bonding with MET769 and π-cation interaction with LYS721.

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