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RESEARCH ARTICLE

Synthesis, Anticancer activity and molecular modelling of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

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ABSTRACT:
Bis(arylidene) cyclohexanone-based compound has been known for possessing various biological activity, especially as potential anticancer agent. The scaffold mimicking the structure of curcumin without the diketo group, thus eliminating the tautomeric group which could affect its potency. This study aimed to synthesize one of its derivate, 2,6-bis-(4-nitrobenzylidene) cyclohexanone and determine its anticancer activity against A549 pulmonary cancer cell line as well as its cytotoxicity against normal Vero cell. In addition, molecular docking study was performed to predict its binding mechanism in EGFR receptor. Synthesis was performed using aldol condensation with cyclohexanone and 4-nitrobenzaldehyde as starting material. This reaction was carried out in basic condition under microwave irradiation. Afterwards, the compound was tested its cytotoxic activity using MTT assay against A549 and Vero cell line. Ultimately, molecular docking was done using Vina 1.2.3. against EGFR receptor (PDB ID: 1M17). The results showed that 2,6-bis-(4-nitrobenzylidene) cyclohexanone has been successfully synthesized using this approach with acceptable yield. The compound also possesses anticancer activity against pulmonary cancer cell IC_{50} = 0.48±0.05mM) with negligible cytotoxicity against normal cell. Molecular docking result suggested that this compound targets EGFR receptor as it yielded low binding energy better than erlotinib, the natural ligand of EGFR. Further developments are needed to optimize its potency.

KEYWORDS: 2,6-bis-(4-nitrobenzylidene) cyclohexanone, EGFR, molecular docking, pulmonary cancer.

INTRODUCTION:
Lung cancer is one of the most common cancers in the world, where it has caused approximately 1.8 million deaths across the globe[1]. This type of cancer can be classified into two categories according to its histology, namely small cell lung cancer and non-small cell lung cancer. The latter is the more dominant type with prevalence of 85% of lung cancer[2]. Overall survival rates of this cancer remain low, despite the current development of therapy[3]. Bis(arylidene) cycloalkanone based compound has been commonly found in various fields, namely polymer sciences[4], agrochemicals, perfume industry, and pharmaceuticals[5].

This compound could also potentially be utilized as starting point for developing novel drug, since this scaffold possess various biological activity[6,7]. This class of compound possess almost identical scaffold to curcumin, one of the well-known constituents in Curcuma genus plant[8]. Several studies of structure modifications of curcumin have suggested the replacement of diketo moiety with cycloalkanone ring, namely[9-12]. One of the reasons for this modification is to eliminate tautomeric functional group, which could affect bioactivity[13] and stability[14] of curcumin-based compound (figure 1).

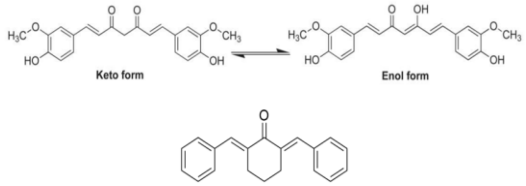

Figure 1: (above) tatutomeric keto-enol form of curcumin. (below) bis(benzylidene) cyclohexanone class of compound

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Various research has been performed to confirm the bioactivity of this class of compound. It has been shown that cyclohexanone-curcumin analogs with methoxy group yielded anti-cancer activity against various cancer cell line such as carcinoma in gastric, esophagus[10], prostate[11], and colon[12]. Nakhjiri et al. have designed various asymmetric bis(nitro-benzylidene) cyclohexanone and tested their bioactivity against various cancer cell-line. The result showed that most of the analogs possessed better anti-cancer activity[9]. This class of compound can be synthesized in a facile manner using aldol condensation from cyclohexanone and benzaldehyde derivates. Normally this reaction is performed under strong acid or strong base condition (eg. HCl and NaOH)[7,15]. Many other catalysts have also been utilized to further improve the outcome of this condensation process, such as p-toluenesulfonic acid[16], sulfamic acid[17], magnesium bisulfate[18], boron trifluoride-diethyl etherate complex[19], ionic liquid[20], and NMSDSA[5]. Interestingly, some of the reactions explained previously were performed under microwave irradiation. This method has significant advantage over conventional reaction, especially increasing product yield and minimizing use of chemical reagents[21].

In this study, we aimed to synthesize 2,6-bis-(4-nitrobenzylidene) cyclohexanone and evaluated its anticancer property. This compound belongs to symmetric bis(nitrobenzylidene) class and has been previously evaluated its bioactivity as antioxidant and anti-inflammatory agent[7]. Synthesis process was performed using microwave-assisted aldol condensation in basic condition by using 10% NaOH. Afterwards, the product was tested against lung cancer cell A549. In addition, molecular docking study was performed to verify the possible mechanism of interaction between designed compound and its molecular target[22].

MATERIALS AND METHODS:

Instruments and Chemicals:

Synthesis procedure was performed in the Laboratory of Organic Chemistry, Faculty of Pharmacy, University of Surabaya. All chemicals and reagents used in the synthesis process was purchased from Merck Millipore (Singapore). Commercial microwave (LG MH6548FR) was used for synthesis process. Thin Layer Chromatography was used to monitor the completion of reaction (Type 60 F254, Merck). Detection of the spots was aided by UV lamp in 254nm. Melting point determination of the product was performed using Sybron-Thermolyne-MP12615. Infrared and Ultraviolet spectra measurement was done using Agilent and Shimadzu, respectively. These data were obtained from Research Laboratory, Faculty of Pharmacy, University of Surabaya. NMR spectra measurements were conducted using JEOL ECS-400 spectrophotometer (400 MHz) in Institute of Tropical Disease, Airlangga University. HRMS-TOF spectra was recorded in Padjadjaran University. Anticancer activity against A549 and Vero cell line was evaluated in Gadjah Mada University. Ultimately, molecular docking was performed in standard personal computer specification (Intel Core i7-9700F 3.00 GHz, RAM 16 GB) using Vina 1.2.3[23].

Synthesis and structure identification:

4.82mmol cyclohexanone (0.5mL) was put to Erlenmeyer flask over an ice bath, then added by 8mL of 10% NaOH solution. Afterwards, 9.66mmol 4-nitrobenzaldehyde (1.46g) was weighed and dissolved in ethanol 96%, separately. The flask containing cyclohexanone was then set on an ice bath while slowly 4-nitrobenzaldehyde solution was added dropwise. The mixture was then irradiated with 200W microwave for twenty seconds. This irradiation process was replicated five times, to ensure the completion of reaction (figure 2). The reaction was monitored using TLC with n-hexane/chloroform system of 2:5. The product was then acidified in HCl 2M to yield solid product. After washing the crystal with water and ethanol, it was recrystallized using chloroform-ethanol solvent and dried in oven. The final product was then characterized using various instrument mentioned previously (melting point apparatus, UV spectrophotometer, infrared spectrophotometer, NMR, and HRMS-TOF).

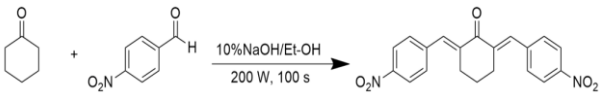

Figure 2: Synthesis of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

Anticancer bioassay:

Anticancer activity was evaluated in vitro against A549 and Vero cell lines. Stock solution of 50.000ppm was made by dissolving the test compound, positive control (erlotinib), and negative control with DMSO. This solution was then diluted in several concentration using RPMI media to make working solution for normal cell. A549 and Vero cell lines were seeded into 96-well plates and then incubated overnight in 5% CO_2 incubators. Various concentration of test compound, positive, and negative compound were added into the well plates in triplicate. At the end of incubation, each well was added with 100µL of 0.5mg/mL 3-(4,5-*dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT), followed by incubation for 3 h. The reaction was quenched by adding 100µL of 10% sodium lauryl sulfate solution in HCl 0.1M into each well. Afterwards, the plates were reincubated at 37°C overnight. Enzyme-linked immunosorbent assay (ELISA) reader was used to quantify the formation of formazan blue color from MTT at 595nm. The IC_{50} values were obtained using probit analysis and compared to positive control[24].

Molecular docking study:

Prediction of molecular interaction between test compound and protein target was done using molecular docking approach. EGFR was selected as macromolecule target (PDB ID: 1M17)[25]. It is argued that EGFR can be found in non-small cell lung cancer, such as A549 cell line[26]. This protein was prepared by stripping of its native ligand (erlotinib) followed by adding hydrogen and Kollman charges. Meanwhile, the native ligand and designed compound was also prepared by ensuring the correct three-dimensional structure with addition of Gasteiger charge. The first step performed was to validate the method by redocking the native ligand back to the original binding site in the protein. Grid box size of 16x16x16 $Å^3$ was built centered on the binding site of erlotinib. Vina use Monte-Carlo stochastic search algorithm in combination with BFGS optimization to produce the most optimal docking pose [23]. RMSD value was utilized to evaluate the process, where less than 2Å was considered acceptable. Afterwards the test compound was docked to the designated binding site and then evaluated its binding energy with Vina-based scoring function. In addition, ligand interaction with amino acid residues within the binding site using Discovery Studio Visualizer.

RESULT AND DISCUSSION:

Synthesis and structure identification:

To the best of our knowledge, 2,6-bis-(4-nitrobenzylidene) cyclohexanone has been previously synthesized[5,7] and utilized as a starting material for synthesis potential anticancer agent[27]. In this study, the target compound was synthesized via Claisen-Schmidt aldol condensation with the assistance of microwave irradiation, in contrast to the previous studies which utilized reflux conditions. This method has helped in increasing the reaction rate significantly, where the product can be obtained in just less than 2 minutes. The compound (2,6-bis-(4-nitrobenzylidene) cyclohexanone) obtained from this reaction has a physical characteristic of yellow crystal without any observable by-product, as indicated by TLC and NMR results.

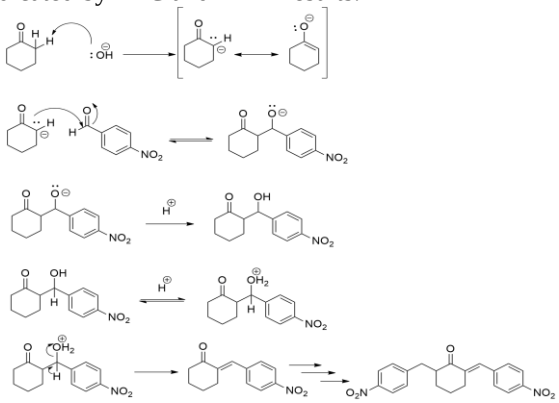

Figure 3: Reaction mechanism of synthesis of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

2,6-bis-(4-nitrobenzylidene) cyclohexanone:

Yield: 85%, yellow crystal (Rf = 0.40); m.p. = 204°C; UV (λ max) = 333nm; IR(ATR) (cm^{-1}-ν: 3060 (C-H aromatic), 2924(C-H cycloalkane), 1690(C=O), 1662 (C=C alkene), 1584 (C=C aromatic), 1511(-NO_2), 1446 (C=C aromatic), 1336(-NO_2), 829(p-disubstituted benzene); ^{1}H-NMR(400 MHz,$CDCl_3$): δ (in Hz) 1.85 (q,*J* = 2.4 Hz, 2H), 2.93 (t,*J* = 10.3 Hz, 4H), 7.59 (d,*J* = 8.7 Hz, 4H), 7.79 (s, 2H), 8.26 (d,*J* = 8.7 Hz, 4H); ^{13}C-NMR (100MHz,CDCl3): δ (in Hz) 22.92, 28.46, 123.78, 130.91, 134.99, 138.74, 142.20, 147.44, 189.32; HRMS m/z ESI+ ($M+H^+$): 365.1135 (figure 4-8)

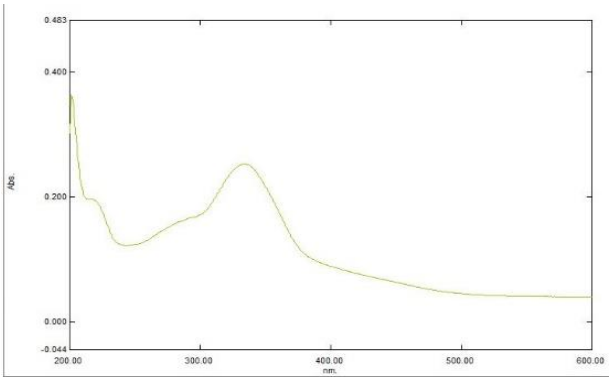

Figure 4: UV spectrum of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

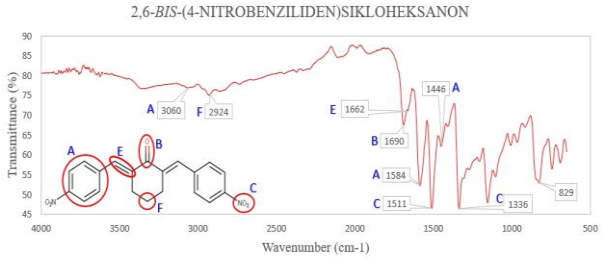

Figure 5: IR spectrum of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

Anticancer bioassay:

Several analogs of benzylidene-cyclohexanone have exhibited promising anti-cancer properties[9-12]. However, this compound possesses anticancer activity only on millimolar scale against A549 cell line. Similar result was observed for erlotinib as well which also yielded millimolar inhibitory activity. The findings indicated that both the tested compound and erlotinib are considered not adequate to inhibit A549 cell line. Although erlotinib is known to possess potent inhibitory activity against EGFR receptor and has been approved for usage against non-small-cell lung cancer by FDA[28], its potency against A549 cell line was found to be minimal in other study[29]. On the other hand, the results from Vero cell line bioassay showed that both compounds indicating the non-toxic characteristic against normal cell line.

Table 1: IC_{50} of *2,6-bis-(4-nitrobenzylidene) cyclohexanone* and erlotinib

Compound Name	A549 cell line (mM)	Vero cell line (mM)
2,6-bis-(4-nitrobenzylidene) cyclohexanone	0.48 ± 0.05	> 1
Erlotinib	0.28 ± 0.03	> 0.2

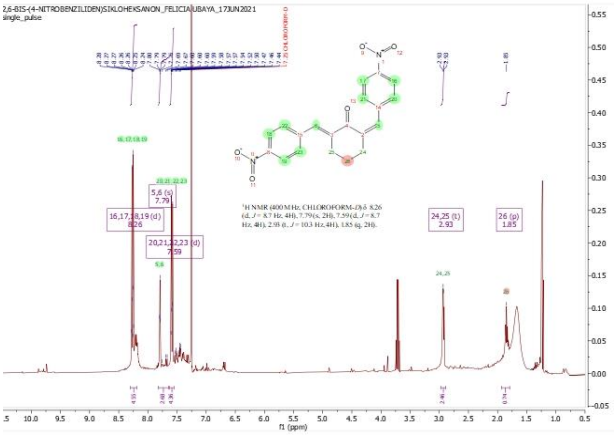

Figure 6: 1 H-NMR spectrum of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

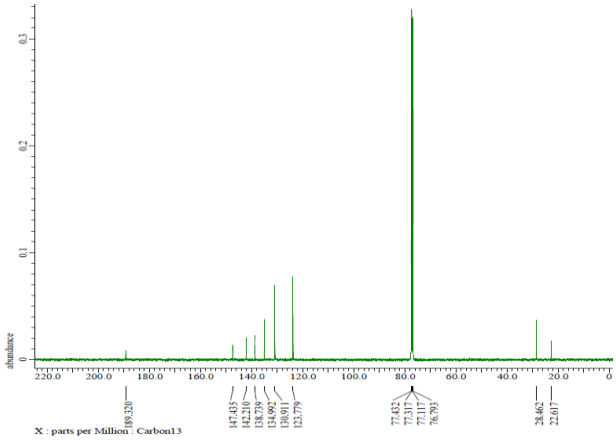

Figure 7: 13C-NMR spectrum of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

Figure 8: Mass spectrum of 2,6-bis-(4-nitrobenzylidene) cyclohexanone

Molecular docking:

This study aims to predict the binding interaction between designed compounds and evaluate its binding energy compared to erlotinib. Prior to the docking process, method validation was performed by redocking its native ligand (erlotinib). This process has yielded 10 best docking poses ranked according to their binding energy. However, RMSD value obtained from all of the best scoring poses was found to be higher than the commonly acceptable value of 2Å. It is possible that this result was due to the flexibility of erlotinib which has 10 rotatable bonds, mainly on the methoxyethoxy group, which caused difficulty for docking algorithm in reproducing original docking pose[30]. In this case, we decided to select docking pose which is closer to the original conformation rather than choosing the first ranked pose[31]. The selected pose has an RMSD value of 2.21 Å with binding energy value of -6.80. Meanwhile, the docking result of designed compound also yielded 10 docking poses. After evaluating every conformation based on its interaction with amino acid residues, it was decided to choose the best scoring pose with binding energy value of -8.25. It is also found that although 2,6-bis-(4-nitrobenzylidene) cyclohexanone cannot form hydrogen bond with any amino acid, the ligand possesses π-alkyl interactions with VAL702, ALA719, LYS721, and CYS773 (Table 2). Despite the similar result with previous study of another curcumin-cyclohexanone analogue[22] it did not correlate well with in vitro cell based assay against A549 cell.

Table 2: Molecular docking result of 2,6-bis-(4-nitrobenzylidene) cyclohexanone and erlotinib against EGFR receptor (PDBID:1M17)

Ligand	Docking Score	Hydrogen Bond Interaction	π-Alkyl Interaction
2,6-bis-(4-nitrobenzylidene) cyclohexanone	-8.25	-	VAL702 ALA719 LYS721 CYS773
Erlotinib	-6.80	MET769 CYS773	VAL702 LYS721

CONCLUSION:

Compound 2,6-bis-(4-nitrobenzylidene) cyclohexanone can be synthesized using microwave assisted aldol condensation in a time-efficient manner. Anticancer activity of this compound was found to be nominal. Further structure modifications are needed in order to improve its bioactivity as anticancer agent.

CONFLICT OF INTEREST:

All authors declare no conflict of interest.

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REFERENCES:

1. Sung H. Ferlay J. Siegel RL. Laversanne M. Soerjomataram I. Jemal A. et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021; 71(3): 209–49, https://doi.org/https://doi.org/10.3322/caac.21660.
2. Molina JR. Yang P. Cassivi SD. Schild SE. Adjei AA. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc. 2008; 83(5): 584–94, https://doi.org/10.4065/83.5.584.
3. Hogea P. Tudorache E. Fira-Mladinescu O. Pescaru C. Manolescu D. Bratosin F. et al. Bronchial Microbiota and the Stress Associated with Invasive Diagnostic Tests in Lung Cancer vs. Benign Pulmonary Diseases: A Cross-Sectional Study. Diagnostics. 2023. https://doi.org/10.3390/diagnostics13142419.
4. Homocianu M. Serbezeanu D. Macsim AM. Vlad-Bubulac T. From cyclohexanone to photosensitive polyesters: Synthetic pathway, basic characterization, and photo-/halochromic properties. J Mol Liq. 2020; 316: 113888, https://doi.org/https://doi.org/10.1016/j.molliq.2020.113888.
5. Nakhaei A. Morsali A. Davoodnia A. An efficient green approach to aldol and cross-aldol condensations of ketones with aromatic aldehydes catalyzed by nanometasilica disulfuric acid in water. Russ J Gen Chem. 2017; 87(5): 1073–8, https://doi.org/10.1134/S1070363217050292.
6. Cui J. Crich D. Wink D. Lam M. Rheingold AL. Case DA. et al. Design and synthesis of highly constrained factor Xa inhibitors: amidine-substituted bis(benzoyl)--diazepan-2-ones and bis(benzylidene)-bis(gem-dimethyl)cycloketones. Bioorg Med Chem. 2003; 11(16): 3379–92, https://doi.org/10.1016/s0968-0896(03)00332-8.
7. Lam KW. Tham CL. Liew CY. Syahida A. Abdul Rahman MB. Israf DA. et al. Synthesis and evaluation of DPPH and anti-inflammatory activities of 2,6-bisbenzylidenecyclohexanone and pyrazoline derivatives. Med Chem Res. 2012; 21(3): 333–44, https://doi.org/10.1007/s00044-010-9521-0.
8. Sun W. Wang S. Zhao W. Wu C. Guo S. Gao H. et al. Chemical constituents and biological research on plants in the genus Curcuma. Crit Rev Food Sci Nutr. 2017; 57(7): 1451–523, https://doi.org/10.1080/10408398.2016.1176554.
9. Nakhjiri M. Safavi M. Alipour E. Emami S. Atash AF. Jafari-Zavareh M. et al. Asymmetrical 2,6-bis(benzylidene)cyclohexanones: Synthesis, cytotoxic activity and QSAR study. Eur J Med Chem. 2012; 50: 113–23, https://doi.org/10.1016/j.ejmech.2012.01.045.
10. Alibeiki F. Jafari N. Karimi M. Peeri Dogaheh H. Potent anti-cancer effects of less polar Curcumin analogues on gastric adenocarcinoma and esophageal squamous cell carcinoma cells. Sci Rep. 2017; 7(1): 2559, https://doi.org/10.1038/s41598-017-02666-4.
11. Mapoung S. Suzuki S. Fuji S. Naiki-Ito A. Kato H. Yodkeeree S. et al. Cyclohexanone curcumin analogs inhibit the progression of castration-resistant prostate cancer in vitro and in vivo. Cancer Sci. 2019; 110(2): 596–607. https://doi.org/10.1111/cas.13897.
12. Rahim NFC. Hussin Y. Aziz MNM. Mohamad NE. Yeap SK. Masarudin MJ. et al. Cytotoxicity and Apoptosis Effects of Curcumin Analogue (2E,6E)-2,6-Bis(2,3-Dimethoxybenzylidine) Cyclohexanone (DMCH) on Human Colon Cancer Cells HT29 and SW620 In Vitro. Molecules. 2021; 26(5). https://doi.org/10.3390/molecules26051261.
13. Priyadarsini KI. Chemical and structural features influencing the biological activity of curcumin. Curr Pharm Des. 2013; 19(11): 2093–100. https://doi.org/10.2174/138161213805289228.
14. Shen L. Ji H-F. The pharmacology of curcumin: is it the degradation products? Trends Mol Med. 2012; 18(3): 138–44, https://doi.org/10.1016/j.molmed.2012.01.004.
15. Handayani S. Budimarwanti C. Haryadi W. Microwave-assisted organic reactions: Eco-friendly synthesis of dibenzylidenecyclohexanone derivatives via crossed aldol condensation. Indones J Chem. 2017; 17(2): 336–41. https://doi.org/10.22146/ijc.25460.
16. Gall E Le. Texier-Boullet F. Hamelin J. Simple Access to α, β Unsaturated Ketones by Acid-Catalyzed Solvent-Free Reactions. Synth Commun. 1999; 29(20): 3651–7. https://doi.org/10.1080/00397919908086000.
17. Rostami A. Ahmad-Jangi F. Sulfamic acid: An efficient, cost-effective and green catalyst for crossed-aldol condensation of ketones with aromatic aldehydes under solvent-free. Chinese Chem Lett. 2011; 22(9): 1029–32. https://doi.org/https://doi.org/10.1016/j.cclet.2011.03.015.
18. Salehi P. Ali Zolfigol M. Shirini F. Baghbanzadeh M. Silica Sulfuric Acid and Silica Chloride as Efficient Reagents for Organic Reactions. Curr Org Chem. 2006: 2171–89. https://doi.org/http://dx.doi.org/10.2174/138527206778742650.
19. Narender T. Papi Reddy K. A simple and highly efficient method for the synthesis of chalcones by using borontrifluoride-etherate. Tetrahedron Lett. 2007; 48(18): 3177–80. https://doi.org/https://doi.org/10.1016/j.tetlet.2007.03.054.
20. Wan Y. Chen X-M. Pang L-L. Ma R. Yue C-H. Yuan R. et al. Synthesis and Fluorescence Properties of α,α′-Bis(substituted-benzylidene)cycloalkanones Catalyzed by 1-Methyl-3(2-(sulfooxy)ethyl)-1H-imidazol-3-ium Chloride. Synth Commun. 2010; 40(15): 2320–8. https://doi.org/10.1080/00397910903243781.
21. Santosa H. Yuniarta TA. Kesuma D. Putra GS. 2-Hydroxy-N′-(4-fluorobenzoyl)benzohydrazide. Molbank. 2020; 2020(1). https://doi.org/10.3390/M1103.
22. Xu Y-Y. Cao Y. Ma H. Li H-Q. Ao G-Z. Design, synthesis and molecular docking of α,β-unsaturated cyclohexanone analogous of curcumin as potent EGFR inhibitors with antiproliferative activity. Bioorg Med Chem. 2013; 21(2): 388–94. https://doi.org/10.1016/j.bmc.2012.11.031.
23. Eberhardt J. Santos-Martins D. Tillack AF. Forli S. AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. J Chem Inf Model. 2021; 61(8): 3891–8. https://doi.org/10.1021/acs.jcim.1c00203.
24. Kesuma D. Medika A. Yuniarta TA. Synthesis and cytotoxic activity of N-(2,4-dichloro)benzoyl-N'-phenylthiourea against human breast cancer cell line. Thai J Pharm Sci. 2022; 46(2): 173–6.
25. Stamos J. Sliwkowski MX. Eigenbrot C. Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J Biol Chem. 2002; 277(48): 46265–72. https://doi.org/10.1074/jbc.M207135200.
26. Sari S. Andayani TM. Endarti D. Widayati K. Health-related quality of life in non-small cell lung cancer (Nsclc) patients with mutation of epidermal growth factor receptor (egfr) in indonesia. Res. J. Pharm. Technol. 2020; 13(1): 443–7. https://doi.org/10.5958/0974-360X.2020.00086.4.
27. Insuasty B. Becerra D. Quiroga J. Abonia R. Nogueras M. Cobo J. Synthesis of Novel Pyrimido[4,5-b]quinolin-4-ones with Potential Antitumor Activity. J Heterocycl Chem. 2013; 50(3): 506–12. https://doi.org/https://doi.org/10.1002/jhet.1510.
28. Dowell J. Minna JD. Kirkpatrick P. Erlotinib hydrochloride. Nat Rev Drug Discov. 2005; 4(1): 13–4. https://doi.org/10.1038/nrd1612.
29. Greve G. Schiffmann I. Pfeifer D. Pantic M. Schüler J. Lübbert M. The pan-HDAC inhibitor panobinostat acts as a sensitizer for erlotinib activity in EGFR-mutated and -wildtype non-small cell lung cancer cells. BMC Cancer. 2015; 15(1): 947. https://doi.org/10.1186/s12885-015-1967-5.
30. Wang Z. Sun H. Yao X. Li D. Xu L. Li Y. et al. Comprehensive evaluation of ten docking programs on a diverse set of protein–ligand complexes: the prediction accuracy of sampling power and scoring power. Phys Chem Chem Phys. 2016; 18(18): 12964–75. https://doi.org/10.1039/C6CP01555G.
31. Ramírez D. Caballero J. Is It Reliable to Take the Molecular Docking Top Scoring Position as the Best Solution without Considering Available Structural Data? Molecules. 2018; 23(5). https://doi.org/10.3390/molecules23051038.