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***In-silico*, synthesis, structure elucidation and anticancer activity study of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one**

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Abstract: The research aims to synthesize 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one and evaluate its anticancer activity against MCF-7. This compound was selected based on *in-silico* study conducted against several dihalophenylbenzoxazinone analogues using molecular docking towards Methionyl-tRNA synthetase. Synthesis of target compound was carried out using anthranilic acid and 3,4-dichlorobenzoyl chloride. The resulting compound was characterized using various spectroscopic analysis. 1D and 2D NMR, infrared and MS. *In-silico* studies was performed by MVD. Several designed compounds were docked into the active site on Methionyl-tRNA Synthetase (1PG2). Anticancer activity was evaluated by MTT Assay against MCF-7. 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one has been successfully synthesized with decent amount of yield 88 %. Its spectroscopic analysis 1D and 2D NMR, IR, MS, FTIR has proven the chemical structure of compound. *In-silico* studies toward the enzyme showed docking score of -76.04 Kcal/mol, higher than its native ligand (-93.50 Kcal/mol). Meanwhile, MTT assay result against MCF-7 showed IC₅₀ value of 68.59ppm. Based on preliminary *in-silico* studies inhibited Methionyl-tRNA Synthetase, 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one was synthesized and tested *in-vitro* against MCF-7. Albeit the compound does not possess better docking score than native ligand, it is still argued that benzoxazine ring can be considered as a potential anticancer agent, as showed by MTT assay result which indicated moderate cytotoxicity.

Keywords: Anticancer, benzoxazinone, elucidation, molecular docking, MTT assay.

INTRODUCTION

Cancer is one of the most fatal disease worldwide. It is caused by abnormal and uncontrolled growth of cells (American Cancer Society, 2019). Based on WHO data, breast cancer is one of the most prevalent types which occurs in 2.1 million women annually (Ferley *et al.*, 2019; Kesuma *et al.*, 2018). There are numerous mechanisms behind this disease, one of which is over expression of human methionyl-tRNA synthetase (MRS). This enzyme belongs to aminoacyl-tRNA synthetase group which acts as a coupling agent between tRNA with the appropriate amino acid. The amino acid-tRNA cognate is then utilized to build peptide. Over expression of methionyl-tRNA synthetase has been found in several different type of cancer (Kim *et al.*, 2011; Bharathkumar *et al.*, 2015) Therefore, MRS can become one of therapeutic target to develop novel selective anticancer agents. Studies showed that benzoxazine-a heterocyclic compound-possesses selective activity in inhibiting human MRS. It has also been found that this class of compound to be cytotoxic in breast cancer cell MCF-7. Recently, 2-phenyl-4H-benzo[d][1,3]oxazin-4-one derivatives possess moderate cytotoxicity against several cancer cell line (El-Azab *et al.*, 2010; Kesuma *et al.*, 2020). In this study,

several analogues of dihalo-2-phenyl-4H-benzo[d][1,3]oxazin-4-one were predicted *in silico* using molecular docking against human MRS. The best predicted compound was then synthesized and underwent MTT assay against breast cancer cell MCF-7 to verify its activity *in vitro*. Comprehensive chemical structure characterization was also performed to verify the correctness of synthesized compound.

MATERIALS AND METHODS

Molecular docking study

Molecular docking was performed using MVD (Molegro® Virtual Docker version 5.5) (Thomsen & Christensen, 2006). The compounds were built initially in 2D and then optimized geometrically into 3D using MFF94 (Halgren, 1996). These compounds were then docked into the active site of human methionyl-tRNA synthetase (MRS) (PDB ID: 1PG2)(Bharathkumar *et al.*, 2015; Crepin *et al.*, 2003). Redocking of native ligand (2-(6-amino-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol) was performed in order to ensure the validity of molecular docking method. The method was considered valid when RMSD value obtained less than 2.0 Å. After redocking process, all benzo[d][1,3]oxazin-4-

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one derivatives were docked into active site of MRS enzyme. MolDock score was utilized as ligand-protein scoring function (Thomsen & Christensen, 2006). In addition, re-ranking was performed based on Lennard-Jones steric energy (Thomsen & Christensen, 2006). The compound which yielded the lowest docking score was chosen to be synthesized and evaluated of its anticancer activity through bioassay against MCF-7 cell.

Synthesis and structure elucidation

Chemicals used for synthesis including: Anthranilic acid (Merck); 3,4-dichlorobenzoyl chloride (Sigma Aldrich); Pyridine (Merck); Distilled water; Sodium Bicarbonate (Merck); Ethanol (Merck); n-Hexane (Merck); Ethyl acetate (Merck); Chloroform (Merck); Acetone (Merck).

Characterization of target compound was performed using various spectroscopic method. NMR spectra measurements were conducted using JEOL ECS-400 spectrometer, MS spectra were measured in QSTAR XL NanoSpray™ using Electrospray ionization (ESI) mode. FT-IR spectra was obtained in Jasco FT-IR 5300, Ultraviolet spectra was recorded using Shimadzu UV-Vis Spectrophotometer 1800. In addition, melting point of the compound was determined using Fisher-John Electrothermal Mel-Temp.

Anthranilic acid 1.37g (10mmol) was dissolved in pyridine, then added slowly with 3,4-dichlorobenzoyl chloride (12mmol). The reaction was performed in 0°C for 15 minutes. Afterwards, the mixture was put in the room temperature up to one hour. The whole process was taken place under constant stirring. In order to evaluate the completion of reaction, TLC was employed using silica gel 60 GF254 (Merck) and equimolar mixture of n-hexane-ethyl acetate as eluent. Afterwards, the mixture was added by 10% solution of sodium bicarbonate followed by distilled water. The solid phase was obtained after vacuum-filtration, then recrystallized in ethanol-acetone (5:1) solution.

Anticancer activity test

The anticancer activity study was performed using MTT assay approach. The MCF-7 cells were seeded into 96-well plates and incubated for 24 hours in 5% CO₂. There were three groups prepared consisted of positive, negative and control group. Positive group contains the mixture of cancer cell with the synthesized compound in several different concentration (7.00 until 250.00µg/mL, each concentration was replicated five times). Meanwhile, negative and control group only contain cancer cell and medium, respectively. In the end of incubation period, each well was added with 100µL of 0.5mg/mL MTT, followed by another 3 hours of incubation time. MTT reagent will be converted by live cells into formazan which yields dark blue color. This reaction was quenched by adding 100µL of 10% SDS in 0.01 N HCl into each

well. The micro plates were then wrapped in paper and incubated at 37°C for 24 hours. Elisa reader was utilized to identify the absorption at λ =595nm. Ultimately, percentage of cell viability was calculated to determine IC₅₀ value of the compound (Riss *et al.*, 2016).

$$\% \text{ cell viability} = \frac{\text{Abs.Positive controls} - \text{Abs Media controls}}{\text{Abs.Negative controls} - \text{Abs Media controls}} \times 100$$

RESULTS

Molecular docking study

Synthesis and structure elucidation

This study has successfully synthesized 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one with good yield. Furthermore, spectroscopic characterizations (fig. 3) have concluded the validity of the synthesized compound.

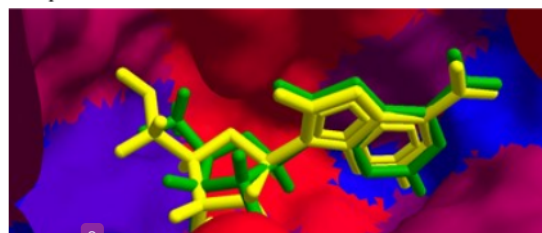


Fig. 1: Comparison of its native ligand (green) with the docking result simulation (yellow) by Molegro Virtual Docker (MVD) software Ver.5.5. The RMSD is 0.84Å

Anticancer activity study

The result of MTT assay shown in table 3. The calculation IC₅₀ value of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one is 70.74±3.95µg/mL equivalent to 2.35 mM.

DISCUSSION

In-silico study

Molecular docking study has been long employed as a tool to predict ligand binding affinity towards receptor. Here, this method was applied as a preliminary study to predict the most potent compound among several 2-dihalophenylbenzoxazinone analogues against MRS. MolDock score was implemented as a scoring function to assess the predicted activity of compounds. This scoring function is based on guided differential evolutionary algorithm, where it employs PLP function with an improvement in terms of hydrogen bond and charge schemes (Thomsen & Christensen, 2006; de Azevedo, 2010). Furthermore, post-process re-ranking was introduced based on weighted approximation of Lennard-Jones 12-6 potential to better characterize steric interaction. The result showed that native ligand possess docking score of -93.48±0.07 kcal/mol with hydrogen bond interaction predicted with Glu27; His28; Gly294;

Table 1: Molecular Docking result of compounds and its native ligand on active site human methionyl-tRNA synthetase

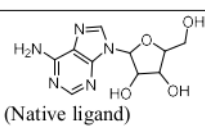
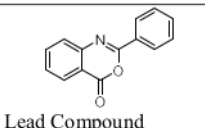
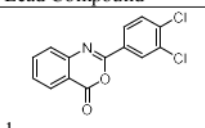
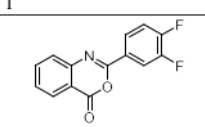
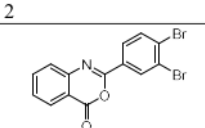
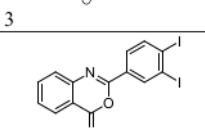
Compound	Rerank Score (Kcal/mol)	Doked Pose	Hydrogen Bond	Amino acids residues	Steric Interaction	Amino acids residues
 (Native ligand)	-93.48 ±0.07	√	5	Glu 27 His 28 Gly 294 Asp 296 Val 326	2	His 21 Glu 27
 Lead Compound	-74.50 ±0.03	√	-	-	3	His 24 Gly 27 His 323
 1	-74.74 ±0.05	√	-	-	4	His 24 Gly 27 His 323 Val 326
 2	-68.39 ±0.02	√	-	-	4	Ala 12 His 24 His 323 Val 326
 3	-69.05 ±0.06	√	-	-	4	Ala 12 Leu 13 His 323 Val 326
 4	-67.69 ±0.05	√	-	-	4	Ala 12 Leu 13 His 24 Val 326

Table 2: Summary spectra data 1D NMR (¹H and ¹³C-NMR) and 2D NMR (HMQC, HMBC, COSY) and MS, FTIR and UV of 2-(3,4-dichlorophenyl)-4H-benzo[1,3]oxazin-4-one

Kinds of Spectra	Characteristics
¹ H-NMR Spectrum (400 MHz, DMSO- <i>d</i> ₆)	δ 8.27 (d, <i>J</i> =2.0 Hz, 1H), 8.14 (dd, <i>J</i> =7.9, 1.2 Hz, 1H), 8.10 (dd, <i>J</i> =8.5, 1.8 Hz, 1H), 7.97-7.91 (m, 1H), 7.85 (d, <i>J</i> =8.5 Hz, 1H), 7.72 (d, <i>J</i> =8.0 Hz, 1H), 7.63 (t, <i>J</i> =7.6 Hz, 1H). There are 7 atoms of Hydrogen
¹³ C-NMR Spectrum (100 MHz, DMSO- <i>d</i> ₆)	δ 159.0, 155.0, 146.4, 137.6, 136.0, 132.5, 132.0, 131.3, 129.8, 129.6, 128.7, 128.3, 127.6, 117.6. There are 14 atoms of Carbon.
COSY Spectrum (DMSO- <i>d</i> ₆)	(8.14; 7.63); (8.10; 7.85); (7.95; 7.72) and (7.95; 7.63); (7.85; 8.10); (7.72; 7.75); (7.63; 8.14) and (7.63; 7.95)
HMQC Spectrum (DMSO- <i>d</i> ₆)	(8.27;129.8); (8.14;128.3); (8.10;128.7); (7.95;137.6); (7.85;132.0); (7.72;127.6); (7.63;129.6)
HMBC Spectrum (DMSO- <i>d</i> ₆)	(8.27; 136.0; 132.5); (8.14; 159.0; 146.4; 117.6); (8.10; 131.3);(7.95;146.4); (7.85; 155.0; 132.5; 131.3); (7.72; 129.6; 117.6); (7.63; 137.6; 117.6)
Mass Spectrum ESI/MS <i>m/z</i> values (Rel. abundance)	C ₁₄ H ₈ O ₂ NC ₂ . [M] ⁺ = 292 (100%); [M] ⁺ =295 (65%); [M] ⁺ = 296 (10%).fragments <i>m/z</i> :217; 173; 175; 177,
FT-IR Spectrum (KBr, ν max, cm ⁻¹)	1760 (C=O lactone); 1621 and 1474 (C=C aromatic); 3090 (=C-H aromatic); 1620 (C=N); 1324 (C-N); C-Cl (770) and C-O-C (1255)
Ultraviolet Spectrum	λ max (nm) 288; 300, in ethanol 70 % solution with 50.0 ppm concentration

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Table 3: Anticancer activity of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one **27** against Human Breast Cancer Cell Line MCF-7.

Concentrations ($\mu\text{g/mL}$)	Absorbance	Cell Viabilities (%)	IC ₅₀ (ppm)
7.81	0.504 \pm 0.013	68.54 \pm 1.87	70.74 \pm 3.95
15.25	0.469 \pm 0.005	62.66 \pm 0.77	
31.25	0.465 \pm 0.008	61.97 \pm 1.44	
62.50	0.463 \pm 0.021	61.52 \pm 3.66	
125.00	0.346 \pm 0.005	41.62 \pm 0.79	
250.00	0.289 \pm 0.001	31.76 \pm 1.68	
Negative Control	0.688 \pm 0.006	100	

Absorbance media control= 0.103 \pm 0.001

Table 4: Cross-peaks in heteronuclear multiple-bond correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one **19**

No	HMQC		Cosy	HMBC
	H-NMR	C-NMR		
1	8.14 (dd, $J=7.9, 1.2$ Hz, 1H)	128.3	H2	C5;C6;C7
2	7.63 (t, $J=7.6$ Hz, 1H)	129.6	H1;H3	C4;C6
3	7.97 - 7.91 (m, 1H)	137.6	H2;H4	C5;C6;C7
4	7.72 (d, $J=8.0$ Hz, 1H)	127.6	H3	C2;C6
5	-	146.4	-	-
6	-	117.6	-	-
7	-	159.0	-	-
8	-	155.0	-	-
9	-	131.3	-	-
10	8.10 (dd, $J=8.5$ Hz, 1H)	132.0	H11	C8;C9;C13
11	8.10 (dd, $J=8.5, 1.8$ Hz, 1H)	128.7	H10	C9
12	-	136.0	-	-
13	-	132.5	-	-
14	8.27 (d, $J=2.0$ Hz, 1H)	129.8	-	C10;C12

Asp296; Val 326 and steric interaction with His21 and Glu27. On the other hand, all of designed compound does not show any interaction but steric interaction with several residues (His24; Gly27; His323; Val326; Ala12; Leu13) in the active site.

This indicates the addition of halo substituents only create steric interaction with receptor, which manifested in higher docking scores than native ligand. Among several designed analogues, 3,4-dichloro compound possess the best docking score of -74.74 \pm 0.05kcal/mol. Therefore, it is chosen to be evaluated further *in-vitro* to verify its potency as anticancer agent.

Synthesis and structure elucidation **15**
Compound 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one was obtained by reacting anthranilic acid (1)

(10mmol) with 3,4-dichlorobenzoyl chloride (2) (1.2 mmol) in pyridine (fig. 4). The mixture was constantly stirred at 0°C for 1 hour to yield 88% \pm 2% (n=6).

The reaction was taken place in two steps. Initially, amine group of (1) as nucleophile attacks carbonyl center of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one via SN-acyl. Pyridine aided in the deprotonation process of the hydrogen atom of amine group. This yielded to a tetrahedral intermediate product which continue to form 3' (fig. 5). Subsequently, pyridine acted as deprotonating agent for carboxylic moiety of 3' to yield carboxylate anion which then proceeded to attack the resulting amide group intramolecularly. This cyclization resulted to a benzoxazine ring (3) (Putra *et al.*, 2017; Noolvi *et al.*, 2011; Noolvi *et al.*, 2013) (fig. 6).

Determination of compound 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one was confirmed by ¹H-NMR, ¹³C-NMR, various 2D-NMR analysis (COSY, HMQC, HMBC), MS and IR spectral data. In addition, UV spectral data was also provided to determine the maximum wavelengths of the compound (288 and 300 nm) where it is possible to measure the linearity of concentration and absorbance according to Lambert-Beer equation.

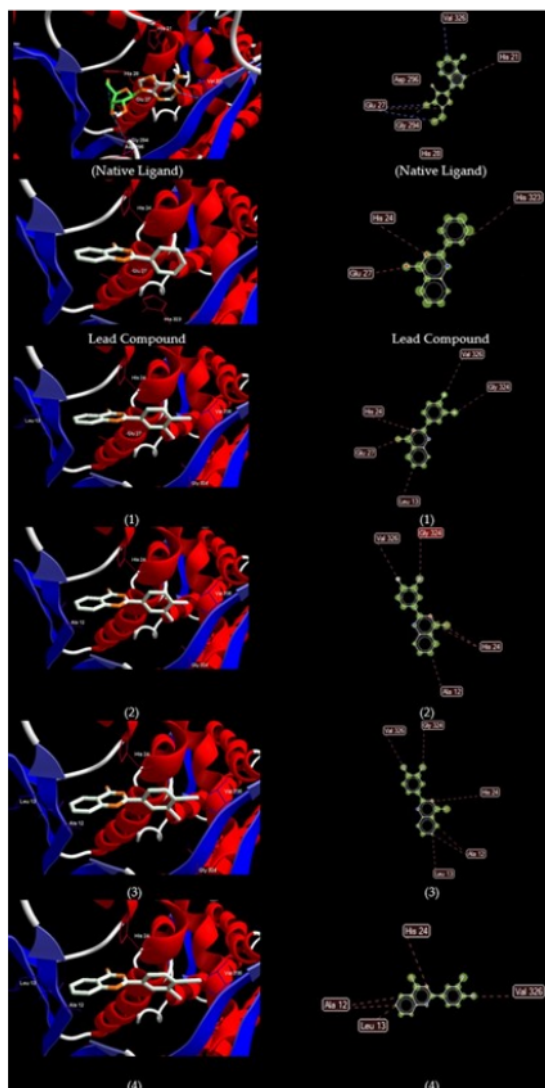


Fig. 2: The Interaction between native ligand, lead compound and compounds 1-4 into active site human methionyl-tRNA synthetase

The ¹H-NMR spectrum of compound 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one showed

peaks around δ 6.5-8 ppm, indicating the existence of 7 protons of aromatic ring (fig. 3).

Meanwhile, ¹³C-NMR similarly showed the presence of two aromatic rings (fig. 3). Furthermore, the presence of carbonyl fragment at δ 159 ppm and imine fragment at δ 155 ppm pointed the formation of benzoxazinone ring.

2D NMR analysis further confirmed the correlation of proton and carbon atom of the structure. Based on HMQC analysis, 7 protons of compound 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one have been assigned to their counterpart carbon atoms (Benzoxazinone ring: 8.14 ppm-128.3 ppm; 7.63 ppm-129.6 ppm; 7.95 ppm-137.6 ppm; 7.71 ppm-127.6 ppm, Dichlorophenyl ring: 8.27 ppm-129.8 ppm; 7.85 ppm-132.0 ppm; 8.10 ppm-128.7 ppm) shown in fig. 3.

Furthermore, HMBC spectral data showed long range interaction between H atom at position C-10 with C-8 (N=C-O) indicating the creation of C-C bond between dichlorophenyl and benzoxazinone ring (fig. 3). Correlation spectroscopy (COSY) shown in fig. 3, the result pointed that all of the hydrogen position is in accordance with the proposed structure (table 2).

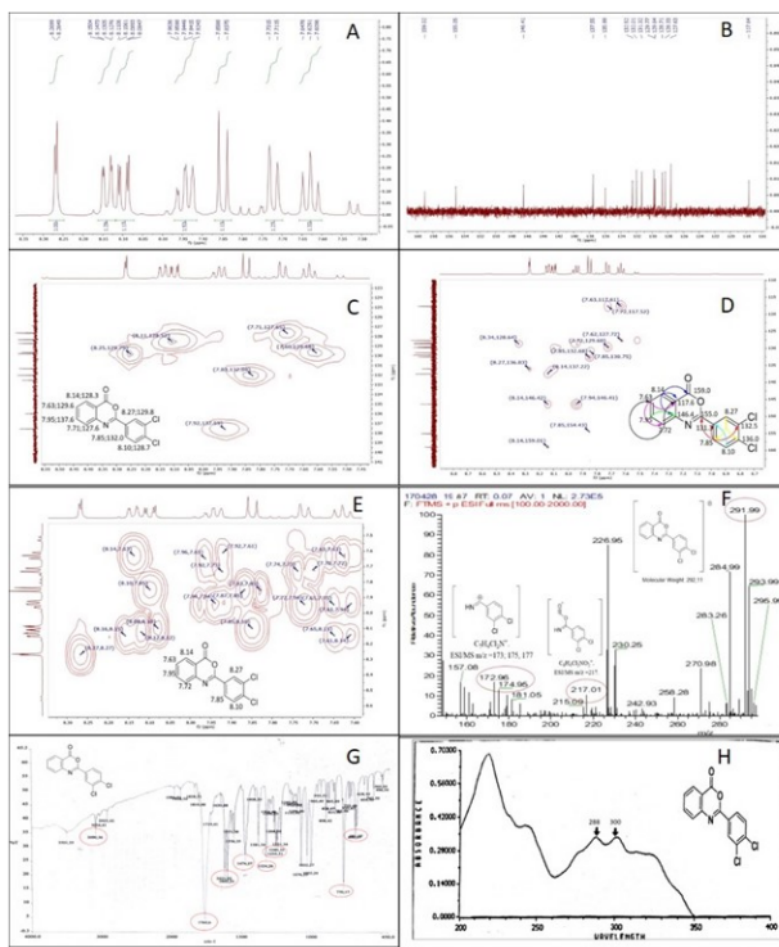
Mass spectroscopy confirmed the molecular weight of 291.99 ([M+H]⁺), with mass deviation of 0.13 (<5mmu) from theoretical molecular weight. The fragmentation pattern confirmed the molecular formula of C₁₄H₈O₂NCl₂. The presence of chlorine atom was confirmed by the molecular peaks with m/z = 292 (M; 100%); m/z = 294 (M+2; 65%); m/z = 296 (M+4; 10%) shown in fig. 3.

The relative abundance pattern of 10:6:1 indicates that the compound possesses two chlorine atoms (Pavia *et al.*, 2009). Furthermore, the formation of N=C-C₅H₃Cl₂ fragment with m/z value of 173 and 175 confirmed the formation of target compound (fig. 3). Ultimately, infrared spectral data showed the formation of benzoxazinone ring manifested in absorption band at 1621 cm⁻¹ (-C=N bond) and 1760 cm⁻¹ (C=O lactone bond) (Putra *et al.*, 2017; Pavia *et al.*, 2009) shown in fig. 3.

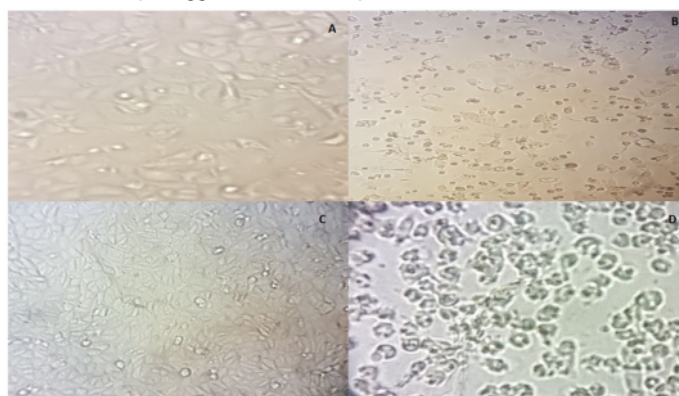
Anticancer activity study

Based on the anti-cancer activity result, 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one possess anticancer activity againsts Human Breast Cancer cell line MCF-7, with IC₅₀ value of 70.74±3.95µg/mL. This result is classified as low cytotoxicity activity based on MTT assay criteria (50-100µg/mL) (Batista *et al.*, 2009; Cos *et al.*, 2006; Weerapreeyakul., 2012; Indrayanto *et al.*, 2020) Based on previous research (Kesuma *et al.*, 2020), it is known that benzoxazinone scaffold without any substituent has IC₅₀ value of 65.43±2.7µg/mL against A549 cell line (fig. 7).

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7 **Fig. 3:** A. $^1\text{H-NMR}$ (400 MHz) spectrum in DMSO- d_6 . B. $^{13}\text{C-NMR}$ (400 MHz) spectrum in DMSO- d_6 . C. HMQC spectrum. D. HMBC Spectrum. E. COSY spectrum. F. MS spectrum (ESI Method). G. FT-IR spectrum in KBr pellet. H. Ultraviolet Spectrum in ethanol 70% (50.0 ppm concentration)



6 **Fig. 7:** MCF-7 cells before administration of a test compound: living cells condition (A and C) and MCF-7 cells after administration of a test compound with a dose of 250 $\mu\text{g/mL}$ (B) and of 125 $\mu\text{g/mL}$ (D): the presence of dead cells after administration of a test compound (B and D).

This finding indicates that addition of halo- substituent yields no significant impact in improving anticancer activity of benzoxazinone compound. This is also in accordance with molecular docking result, in which there is no difference in docking score between 2-phenylbenzoxazinone with its substituted counterpart. Generally, it is evident that 1,3-benzoxazine scaffold.

Possess anticancer activity and study has shown several analogues can be considered as potent as anticancer agent with good cytotoxicity (El-Azab *et al.*, 2010). Therefore, it is necessary to re-examine which substitution pattern is the most important in order to improve the activity of this scaffold.

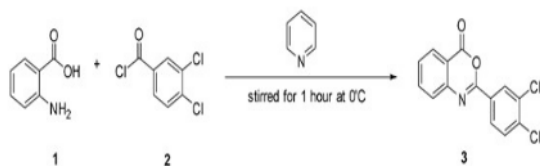


Fig. 4: Synthesis of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one from anthranilic acid (1) and 3,4-dichlorobenzoylchloride (2)

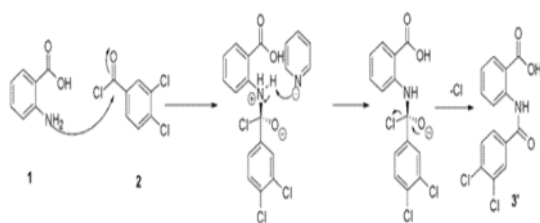


Fig. 5: Reaction mechanism between anthranilic acid (1) and 3,4-dichlorobenzoyl chloride to form 2-(3,4-dichlorobenzamido)benzoic acid (3')

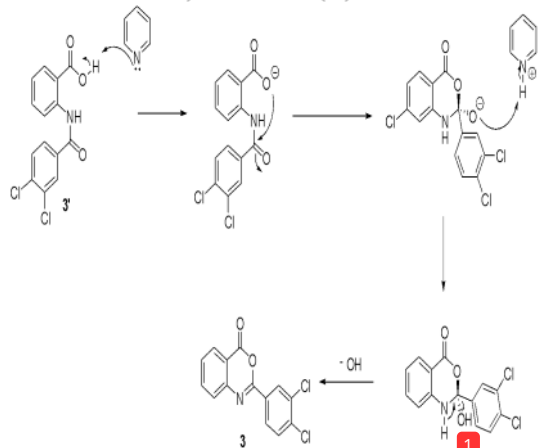


Fig. 6 : Reaction mechanism of the formation of 2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one (3)

CONCLUSION

2-(3,4-dichlorophenyl)-4H-benzo[d][1,3]oxazin-4-one has been synthesized with a very good yield (88%±2%) and has been characterized with various spectroscopic analysis (1D NMR (H and C-NMR); 2D NMR (COSY, HMQC, HMBC), MS, FTIR). Based on in vitro assay, this compound is classified as low cytotoxic against MCF-7 cell line with IC₅₀ value of 70.74±3.95µg/mL. The result was in accordance with molecular docking outcome, which indicated no significant difference between the compound and unsubstituted 2-phenylbenzoxazinone.

ACKNOWLEDGEMENT

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