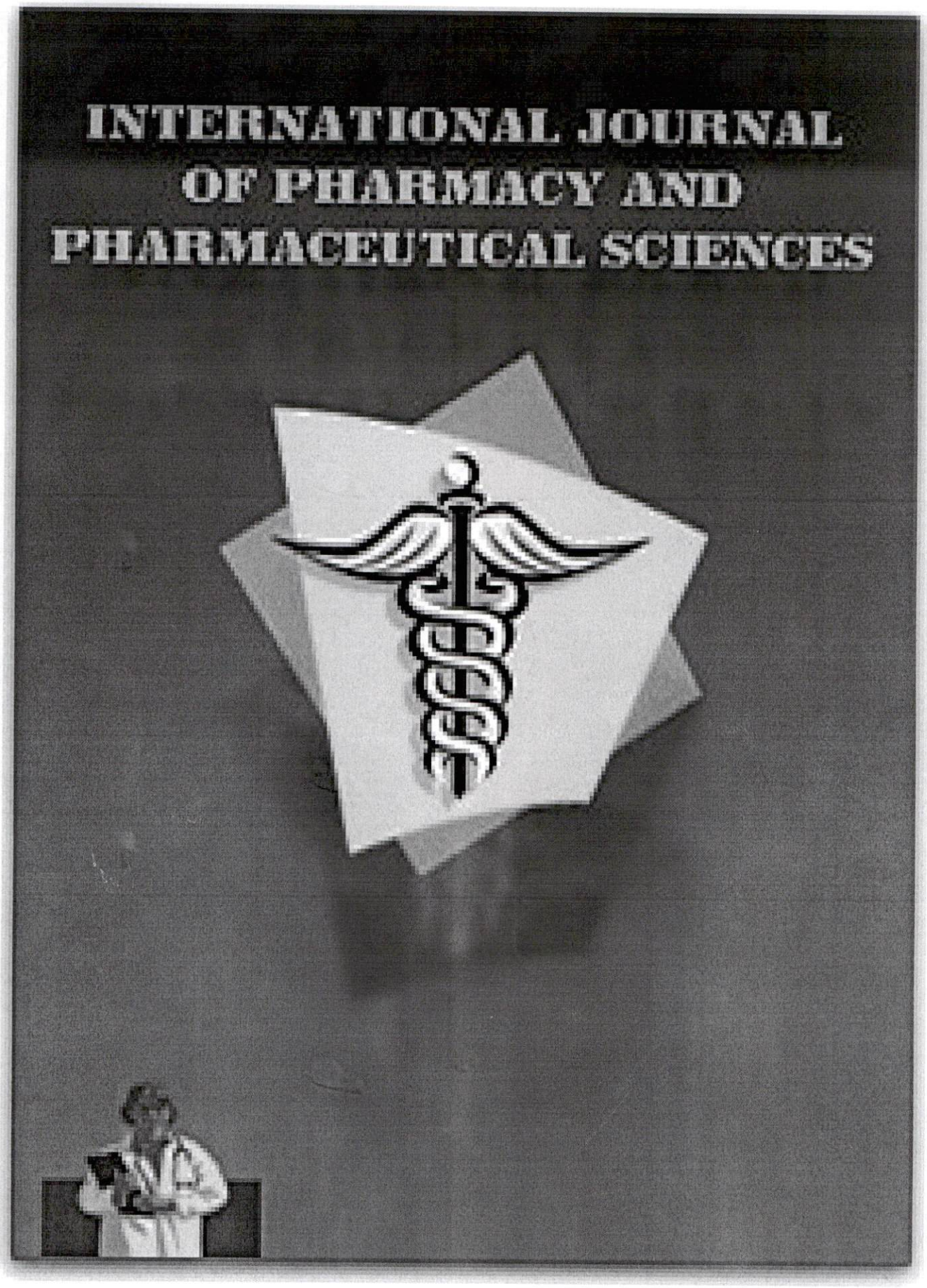
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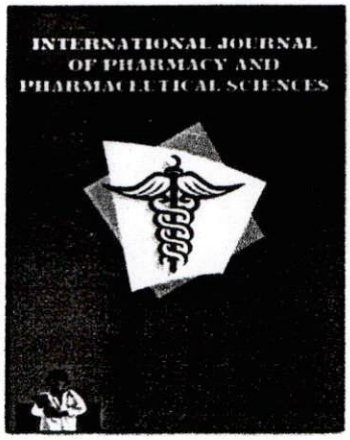
# INTERNATIONAL JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES

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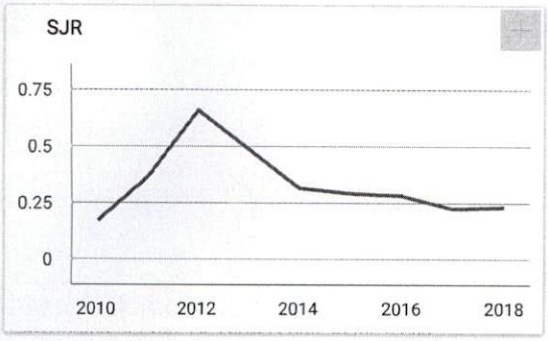


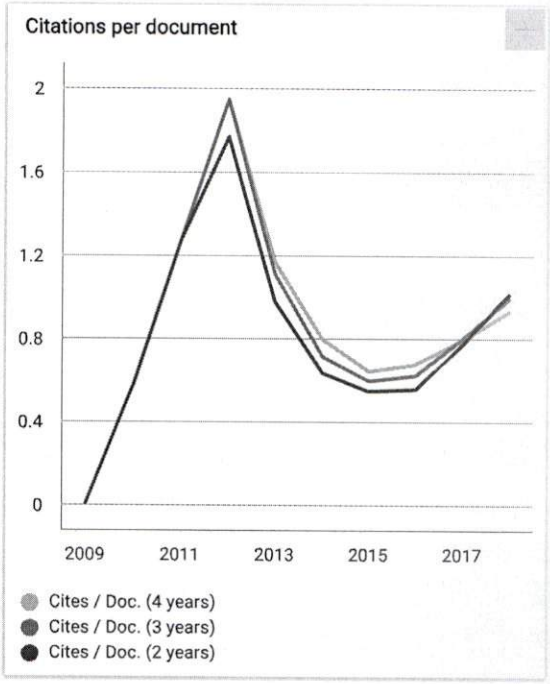


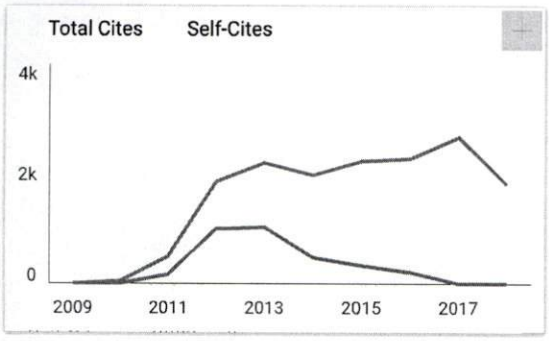


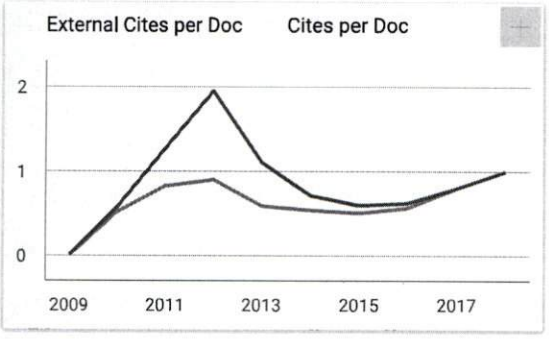


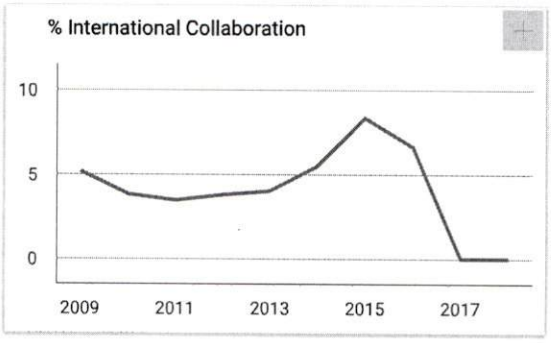


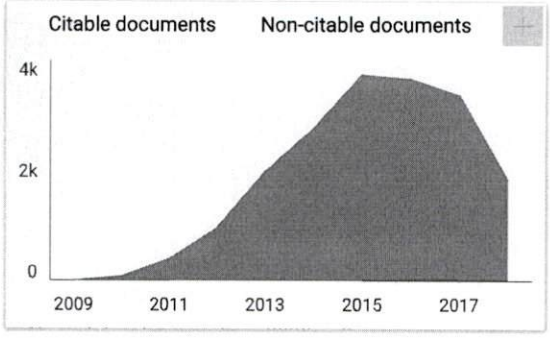


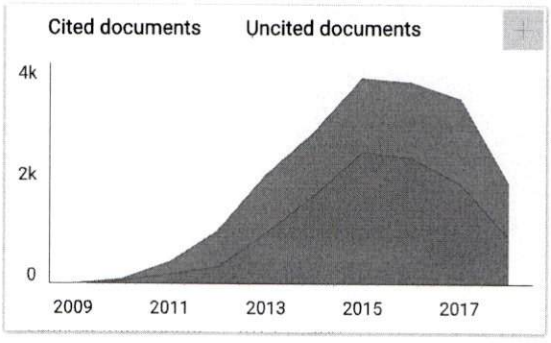


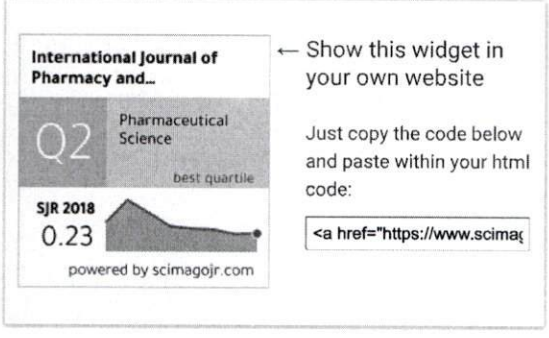


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Research Article

# SYNTHESIS AND ANTITUMOR ACTIVITY EVALUATION OF *N,N'*-DIBENZOYL-*N,N'*-DIETHYLUREA AGAINST HUMAN BREAST CANCER CELL LINE (MCF-7)

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**ABSTRACT**

Objective: To find new antitumor agents a serie of ring-substituted *N,N'*-Dibenzoyl-*N,N'*-Diethylureas (3a-d) were designed and synthesized. The study was conducted to synthesize *N,N'*-Dibenzoyl-*N,N'*-Diethylureas and examine their antitumor activity against human breast carcinoma cell line (MCF-7).

Methods: The compounds were synthesized from reaction of N,N'-diethylurea with ring-substitued-benzoylureas. The molecular structures were confirmed by FTIR, $^1$H-NMR, $^{13}$C-NMR and MS spectroscopy. Their antitumor activities were tested *in vitro* against human breast carcinoma cell line (MCF-7) using MTT assay. *In silico* molecular modeling was carried out through docking the compounds into binding site of ERK2 (PDB. 1TVO).

Results: The tested compounds exhibited antitumor activity higher than reference drug hydroxyurea. One of them (3c) displayed the highest activity among the tested compounds with $IC_{50}$ 0.56 μM, twenty-fold more active than hydroxyurea ($IC_{50}$= 11.58 μM). This compound more fitting to the enzyme's binding site than hydroxyurea could explain its better inhibitory activity.

Conclusion: Four ring-substituted *N,N'*-Dibenzoyl-*N,N'*-Diethylurea derivatives had been synthesized and one of them is highly potential as antitumor agents against human breast carcinoma cell line (MCF-7).

**Keywords**: *N, N'*-Dibenzoyl-*N,N'*-Diethylurea, Antitumor, MCF-7 cell line, Docking, ERK2.

## INTRODUCTION

Cancer is one of the major causes of death in developing countries and also in the worldwide. In Indonesia, the cancer became the third largest contributor to death after heart disease. Since 2007 breast cancer reached first ranks for in-patients in hospitals, followed by cervical cancer [1]. It is still ongoing efforts to find new anticancer drugs because long used drugs have gradually become less effective and there is a tendency of resistance [2]. Cancer chemotherapy has entered a new era of molecularly targeted therapeutic agents, which targeting mutant oncogenic Growth Factor Receptor and non-receptor tyrosine kinases involved in mitogenic or proliferative signal transduction pathways in cancer cells [3]. The mitogen activated protein (MAP) kinase family regulates biological processes including cell growth, proliferation, differentiation, and programmed cell death. The extracellular signal-regulated kinase (ERK 1/2) is one of the three main members of MAP kinases which are important mediators of cell proliferation [4]. Constitutive activation of the ERK proteins plays an important role in the proliferation of some human cancers including breast, ovarian, colon, and prostat [5]. MAP kinases may be promising targets of new cancer chemotherapy.

Urea derivatives have been used as anticancer drugs, such as carmustine/BCNU (bis-chloroethylnitrosourea), ENU (*N*-ethyl-*N*-nitrosourea), and hydroxyurea. The presence of alkyl groups in could increase the hydrophobic character of the nitrosourea derivatives so that the compound can penetrate the blood-brain barrier [2]. Hydroxyurea could acted as antimetabolite that inhibit the ribonucleotide reductase and also targeted histone deacetylase and matrix metalloproteinase [6,7,8]. New urea derivatives have also been reported as a tyrosine kinase inhibitor and has become an important class of anticancer drugs [9]. Therefore it is still interesting to synthesis of urea derivatives as anticancer compounds. A serie of ring-substituted benzoylurea derivatives had synthesized and showed CNS depressant activity in mice [10]. Further reseach reported that those benzoylurea derivatives were more active than hydroxyurea according to Brine Shrimp Lethality Test so they were potential to develop as anticancer agent. Quantitative Structure Activity Relationship and *in silico* study of the benzoylureas using ribonucleotide reductase (3HNC) revealed that binding affinity of benzoylurea derivatives to the enzyme were better than hydroxyurea. Their cytotoxic activity was influenced by steric (MR) and hydrophobic (log P) factors and the presence of aromatic ring in the structure of urea had increased its bioactivity [11]. In order to find more potent urea antitumor agents, we modified the urea structure by attached two ethyl groups and substituted aromatic ring on the two N atom generated some *N,N'*-dibenzoyl-*N,N'*-diethylurea derivatives. The antitumor activity of the new compounds were evaluated against human breast cancer cell line (MCF-7). The molecular interaction model for their cytotoxicities were performed by docking the molecules into the X-ray crystallographic structure of ERK2 in complex with an inhibitor [12] using Molegro Virtual Docker.

## MATERIALS AND METHODS

### Materials

The *N,N'*-Dibenzoyl-*N,N'*-Diethylureas **(3a-d)** were synthesized from the reaction of starting material *N,N'*-diethylurea **(2)** with several ring-substituted benzoyl chlorides **(1)** (Fig.1). The structure of synthesized compound were identified by IR, $^1$H-NMR, $^{13}$C-NMR, and MS spectroscopy; whereas their purity were determined by melting point and TLC tests.

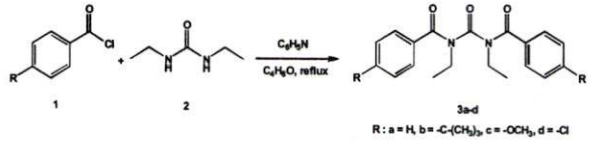



**Fig. 1: Scheme of synthesis reaction of *N,N'*-dibenzoyl-*N,N'*-diethylurea**

### General Experimental

All reagents and solvents were purchased from standard commercial suppliers. Melting points were measured with an Electrothermal melting point apparatus without correction. IR spectra were recorded in KBr on Jasco FT-IR 5300 and major absorption was listed in cm$^{-1}$. $^1$H-NMR and $^{13}$C-NMR spectra were taken at JEOL ECS-400 spectrometer (400MHz), and chemical shift were reported in ppm on the δ-scale from internal standard $Me_4Si$. MS spectra were measured with Agilent 6890N spectrometer using EI methods. TLC was carried out on aluminium plates coated with silica gel $F_{254}$ (E Merck) using UV lamp 254 nm to spot detection.

**Synthesis of *N,N'*-Dibenzoyl-*N,N'*-Diethylureas**

To *N,N'*-diethylurea in tetrahydrofuran (THF) and a few drops of pyridine, an equimolar of benzoyl chloride were added slowly at ice bath while stirring. Then the temperature was raised to 60 °C and the mixture were refluxed for 3 hours. Reflux was continued for 20 hours at room temperature. The mixture was concentrated by evaporating THF and the product was washed with water and saturated sodium bicarbonate respectively. The resulting solid was recrystalised from hot ethanol-water (1:1) to give *N,N'*-Dibenzoyl-*N,N'*-Diethylurea compounds **3a-d** (Fig.1).

**Antitumor Activity**

*In vitro* antitumor activity against MCF- 7 cancer cell lines was assayed by MTT method and expressed in $IC_{50}$, concentration of the compounds inducing a 50% inhibition of cell growth of treated cells compared to the growth of control cells. Hydroxyurea (HU) was used as the reference drug. Cancer cell lines were seeded at a density of $5x10^3$ cells/well in 96-well microplates. After 24 hours, exponentially growing cells were exposed to the test compounds in DMSO at final concentration ranging from 100 to 600 µg /mL. After 48 hours incubated in a 5 % $CO_2$ incubator at 37 °C, cell survival was determined by the addition of MTT solution (100 µL of 0.5 mg/ml MTT in PBS). Once formazan was formed, 100µL 10 % SDS in 0.1 N HCl was added and plates were incubated in the dark at 37 °C overnight. The absorbance was observed at 595 nm on ELISA-reader and survival ratio of living cells were exppressed in percentages with respect to untreated cells. Each experiment was performed at least three times. The $IC_{50}$ value of test compounds were shown in Table 1.

**Molecular Docking**

**Software and program**

CS ChemBioDraw Ultra ver 11.0 (Cambridge Soft) was employed to built 3D structure of compounds and Molegro Virtual Docker (MVD) ver 5.0 (CLC Bio) was the docking program used in this research.

**Docking Study**

The structure of ERK2 (PDB ID = 1TVO, at 2,5 A° resolution)was obtained from the Protein Data Bank (www.pdb.org). Compound structures were built with ChemBioDraw Ultra 11.0 and their geometry optimization were performed using the MMFF94 method in the program and saved as Sybyl Mol2 format. In the molecular docking, the test compound were placed into binding site of 1TVO (cavity-1) by align method to the reference ligand (FR180204). FR180204 is a selective inhibitor against ERK2 which complexed with this enzyme (13). The binding affinity between ligand and enzyme (docking score) was predicted using MolDock Score and post analysed as Rerank Score (RS). The highest-scoring pose (lowest energy) should represent the best-found binding mode. Evaluation of the interaction was based on the their RS which is the sum of ligand-protein interaction energy and internal ligand energy, including hydrogen bonds between ligands and protein. The validation of docking was carried out by redocking the FR180204 into cavity-1 of 1TVO.

A more negative binding affinity indicates stronger binding. The best docked structures have to follow these criteria : i) they have the lowest binding energy (highest-scoring pose); ii) geometrically, they must occupy the same cavity in the enzyme similar to FR180204. This can be observed visually by comparing the structure of docked molecule with crystal structure of FR180204 inside the binding site. The ligand-protein complexes of the top-scoring poses were used for further visual inspection (Fig. 2)

**RESULTS AND DISCUSSION**

**Synthesis of *N,N'*-dibenzoyl-*N,N'*-diethylurea derivatives**

Four *N,N'*-dibenzoyl-*N,N'*-diethylurea derivatives **(3a-d)** were synthesized from *N,N'*-diethylurea in one steps. All compounds are white solids and water insoluble substances. The structure of the synthesized compounds were identified by IR, 1H-NMR,13C-NMR,and MS spectroscopy as follows :

*N,N'*-carbonylbis(*N*-ethylbenzamide)=*N,N'*-dibenzoyl-*N,N'*-diethylurea **(3a)**. Yield 30% as white crystal, m.p. 147 °C. $^1$H-NMR ($CDCl_3$, 400 MHz) : 1,1 (6H, t, *J*=7.2 Hz, $CH_3$); 3,2 (4H, m, $CH_2$); 7,3 (10H, m). NMR-$^{13}$C ($CDCl_3$, 400 MHz) : 13,47 ($CH_3$); 42,99 ($CH_2$); 127,65 (=C aril); 128,67 (=C-C); 132,21 (=C-C); 136,03 (=C-C); 159,03 (C=O); 170,96 (C=O aril). IR (KBr),ν maks ($cm^{-1}$) : 3087 ($Csp_2$–H), 2977 (C–H), 1717 (C=O ar), 1676 (-C=O(-N-)), 1580 (C=C), 858 (C–H aril). MS m/z EI : 324($M^+$), Calculated Mass $C_{19}H_{20}N_2O_3$ 324.15.

*N,N'*-carbonylbis(4-*tert*-butyl-*N*-ethylbenzamide)=*N,N'*-di(4-*tert*-butylbenzoyl)-*N,N'*-diethylurea **(3b)**. Yield 20% as white crystal, m.p. 151 °C. $^1$H-NMR ($CDCl_3$, 400 MHz) : 1,2 (6H, t, *J* = 6,8, $CH_3$); 1,3 (18H, s, -$CH_3$ t-butyl); 3,4 (4H, m, $CH_2$); 7,3 (4H, m, =CH aril); 7,5 (4H, m, =CH aril). NMR-$^{13}$C ($CDCl_3$, 400 MHz) : 13,47 ($CH_3$); 31,25 (-$CH_3$ t-butyl); 35,26 ($CH_2$); 43,04 (–C– t-butyl); 125,55 (=C aril); 127,76 (=C aril); 130,16 (=C aril); 155,76 (=C aril); 159,03 (C=O); 171,33 (C=O aril). IR (KBr),ν maks ($cm^{-1}$) : 3066 ($Csp_2$–H), 2968 (C–H), 1701 (C=O ar), 1683 (-C=O(-N-)), 1605 (C=C), 856 (C–H aril). MS m/z EI : 436 ($M^+$), Calculated Mass $C_{27}H_{36}N_2O_3$ 436.27

*N,N'*-carbonylbis(*N*-ethyl-4-methoxybenzamide)=*N,N'*-di(4-methoxybenzoyl)-*N,N'*-diethylurea **(3c)**. Yield 22% as white waxy solid, m.p. 79 °C. $^1$H-NMR ($CDCl_3$, 400 MHz) : 1,1 (6H, t, *J*= 6,8, $CH_3$); 3,8 (4H, m, -$CH_2$); 4,3 (6H, s, -O-$CH_3$); 6,9 (4H, m, =CH aril); 8,04 (4H, m, =CH aril). NMR-$^{13}$C ($CDCl_3$, 400 MHz) : 15,22 ($CH_3$); 35,43 (-$CH_3$ on ar-O-$CH_3$); 42,37 ($CH_2$); 114,22 (=C aril); 121,36 (=C aril); 128,32 (=C aril); 132,92 (=C aril); 162,38 (C=O); 164,65 (C=O aril). IR (KBr),ν maks ($cm^{-1}$) : 3060 ($Csp_2$–H), 2979 (C–H), 1774 (C=O ar), 1711 (-C=O(-N-)), 1609 (C=C), 838 (C–H aril). MS m/z EI : 352 ($M^+$), Calculated Mass $C_{21}H_{24}N_2O_3$ 352.18.

*N,N'*-carbonylbis(4-chloro-*N*-ethylbenzamide)=*N,N'*-di(4-chlorobenzoyl)-*N,N'*-diethylurea **(3d)**. Yield 28% as white crystal, m.p. 141 °C. $^1$H-NMR ($CDCl_3$, 400 MHz) : 1,1 (6H, t, *J*= 6,8, $CH_3$); 3,4 (4H, m, -$CH_2$); 7,3 (8H, m, =CH aril). NMR-$^{13}$C ($CDCl_3$, 400 MHz) : 13,55 ($CH_3$); 43,25 ($CH_2$); 129,06 (=C aril); 134,06 (=C aril); 138,76 (=C aril); 158,37 (C=O); 170,18 (C=O aril). IR (KBr),ν maks ($cm^{-1}$) : 3056 ($Csp_2$–H), 2974 (C–H), 1786, 1681 (-C=O amida), 1588 (C=C), 860 (C–H aril). MS m/z EI : 352 ($M^+$), Calculated Mass $C_{21}H_{24}N_2O_3$ 352.18.

The *N,N'*-dibenzoyl-*N,N'*-diethylureas were produced by nucleophilic acylation between benzoyl chlorides and *N,N'*-diethylurea, where the nucleophile was *N,N'*-diethylurea. The structural change of **(2)** to *N,N'*-dibenzoyl-*N,N'*-diethylureas was characterized by the conversion of –NH– moeity of diethylurea to –N–C=O(Ar). As there are two symmetric -NH- moeity, the new compounds bear two symmetric substituted-benzoyl moeities, each attach on their N atom. It could be observed in $^1$H-NMR spectra which didn't showed proton peak at –NH region, supported by IR spectra without N-H bands.

**Antitumor Activity**

Intending to find antitumor agent for breast cancer, we examined antitumor activity of the compounds *in vitro* on human breast carcinoma cell line (MCF-7). The results were listed in Table 1.

**Table 1: $IC_{50}$ *in vitro* cytotoxicity of the tested compounds against human breast cancer cell (MCF-7) and their docking scores for ERK2 binding site**

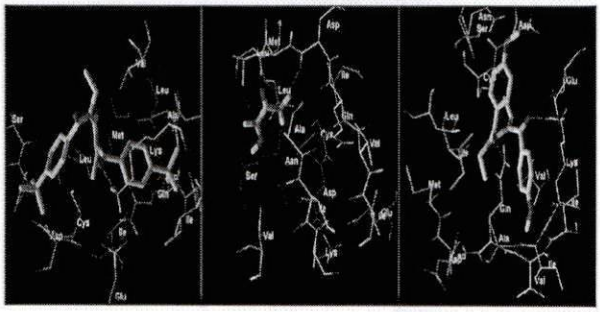
| Compound | $IC_{50}$ cytotoxicity (µM) | Rerank Score (RS) |
|---|---|---|
| 3a | 2,12 | -69,157 |
| 3b | 1,29 | -90,857 |
| 3c | 0,56 | -81,665 |
| 3d | 1,70 | -77,189 |
| HU | 11,58 | -38,297 |

Table 1 show that all tested compounds have $IC_{50}$ lower than anticancer drug hydroxyurea. Those indicated that all tested compounds had antitumor activity against MCF-7 cells higher than HU. It seems that benzoyl and ethyl group on the structure of urea may increase antitumor activity. HU that does not contain benzoyl group has low activity. Substituents on the benzene ring can also enhance the anticancer activity, depend on character of the

substituents. Compound without substituent on benzene ring **(3a)** show the lowest activity among the synthesized compounds. Compound with methyl substituents on the para-position **(3c)** exhibit the highest activity with $IC_{50}$ of 0.56 μM, about twenty-fold more active than HU with $IC_{50}$ of 11.58 μM. These results indicated that the *N,N'*-dibenzoyl-*N,N'*-diethylurea derivates were potential as antitumor agents against breast carcinoma.

**Docking Study**

The capability of tested compounds to interact with target ERK2 was investigated by docking study, and the results were compared to HU (Table 2). Human breast cancer were known to overexpress in MAP kinase which is represented by ERK2 in this study, and HU could interacted with this enzyme [13]. Table 2 display better score (RS) for all *N,N'*-dibenzoyl-*N,N'*-diethylurea derivatives compared to the HU.



A B C

**Fig. 2: Docked 3D conformation of best docked compound 3b (Fig. 2A), reference drug HU (Fig. 2B), and most bioactive compound 3c (Fig. 2C) showing hydrogen bonds with amino acid residues in ERK2 binding site (cavity-1). Pictures were created by Molegro version 5.5**

The results indicate high predicted affinity of the new compounds to ERK2, with best values obtained for compound **3b**. The docking studies have shown more favorable interactions for the new dibenzoylurea derivatives with the target ERK2. Compound **3a** without substituent on benzene ring has the lowest affinity among the synthesized compound. It may explain that substituent on benzene ring contribute to their inhibitory activity. Interaction of molecules **3b, 3c**, and HU with amino acid residues in binding site of ERK2 are shown in Figure 2.

Fig. 2A illustrate hydrogen bonding interaction between compound **3b** (best-docked molecule) and amino acid residue Lys 54, Fig. 2B show hydrogen bonding interactions of HU (reference drug) with Asp 106 and Met 108, and Fig. 2B display hydrogen bonds performed between compound **3c** (highest bioactivity) with Asn 154, Asp 167, Gln 105, and Lys 54. Hydrogen bonds performed by all synthesized compounds with amino acids in binding site of ERK2 were summarized in the Table 3.

**Table 3: Hydrogen bonds performed by interaction between tested compounds and amino acids in binding site of ERK2**

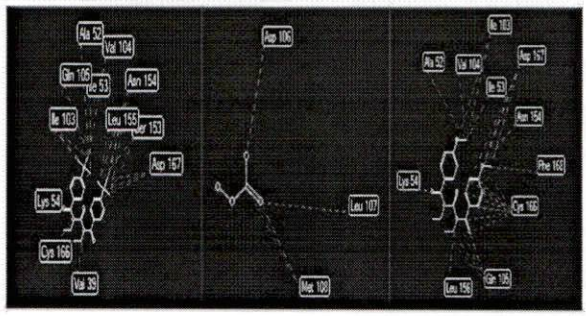
| Amino acid | Compound | | | | |
|---|---|---|---|---|---|
| | HU | 3a | 3b | 3c | 3d |
| Asn 154 | - | - | - | + | - |
| Asp 106 | + | - | - | - | - |
| Asp 167 | - | - | - | + | - |
| Cys 166 | - | + | - | - | + |
| Gln 105 | - | ++ | - | + | + |
| Lys 54 | - | + | + | + | - |
| Met 108 | + | - | - | - | - |

+ = hydrogen bond; ++ = two hydrogen bonds

– = no hydrogen bond

In Table 3 there can be seen variation of amino acids bound to each compound, but all of these amino acids perform hydrogen bonds with N–CO–N moeity of urea in the same binding site (cavity-1). It appears from the presence of substituted-benzoyl group in synthezised compounds which doesn't exist in HU. These interaction denoted the importance of nitrogen atom for binding and the subsequent inhibitory capacity. Besides hydrogen bonding, there are steric interactions (van der Waals) performed by compounds with amino acid residues as shown in Fig. 3.

The *in silico* study indicated that different substituents on the aromatic ring could result in variations of hydrogen bonds and steric interactions. Such steric interactions supported the better score (RS) for compound **3b** compared to the HU (Table 2) although **3b** performed only one hydrogen bond. The methoxy substituent in compound **3c** performing additional hydrogen bonds with Asn 154 and Asp 167 (Fig. 3C) may contribute to its high bioactivity. The comparative docking study of the *N,N'*-dibenzoyl-*N,N'*-diethylurea derivatives with anticancer drug hydroxyurea supports the molecular design of this serie of compounds.



A B C

**Fig. 3: 2D pictures showing hydrogen bonds (blue dashed-line) and steric interaction (red dashed-line) of best-docked compound 3b (Fig. 3A), reference drug HU (Fig. 3B), and most potent compound 3c (Fig. 3C) with amino acid residues in ERK2 binding site. Images were obtained from Molegro version 5.5.**

**CONCLUSION**

The research concluded that four derivatives of *N,N'*-dibenzoyl-*N,N'*-diethylurea showed *in vitro* antitumor activity against human breast cancer cell line (MCF-7) higher than anticancer drug hydroxyurea. One of them (compound **3b**) displayed the highest activity among the tested compounds with $IC_{50}$ 0.22 μM, twenty-fold higher than hydroxyurea ($IC_{50}$= 11.58 μM). *In silico* study could explained that their better inhibitory activity related to their higher affinity with ERK2 binding site. The presence of substituents on the aromatic ring increased the binding affinity and their bioactivity.

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