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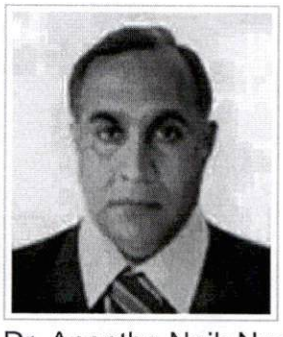

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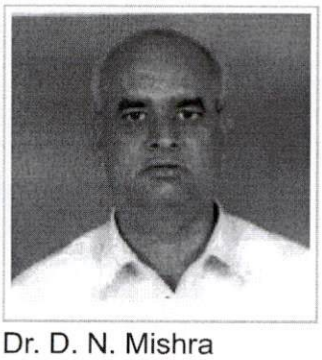

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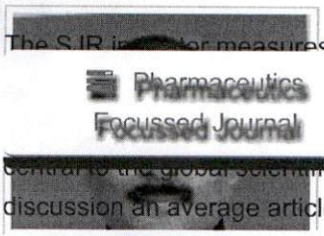

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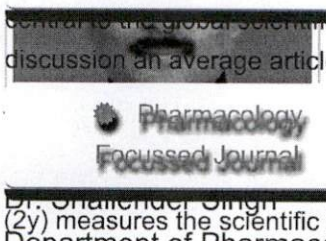

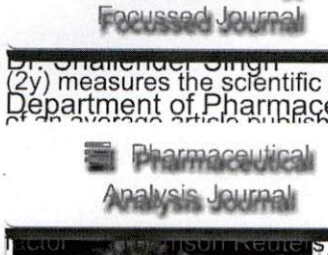

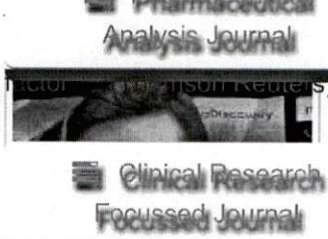

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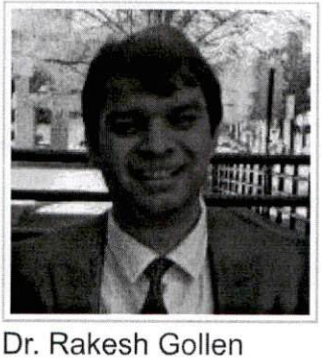

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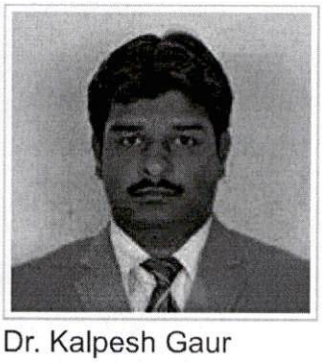

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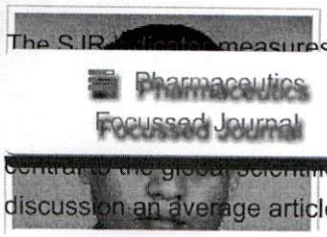

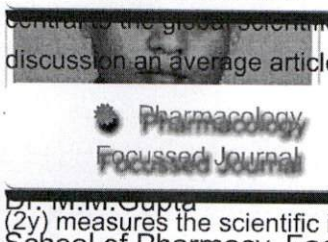

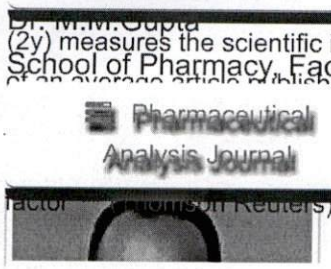

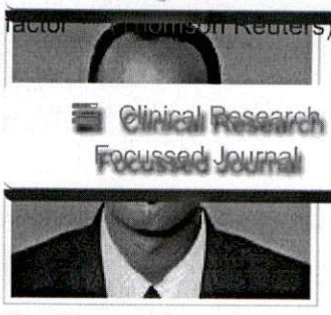

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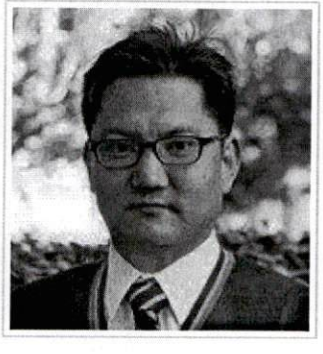

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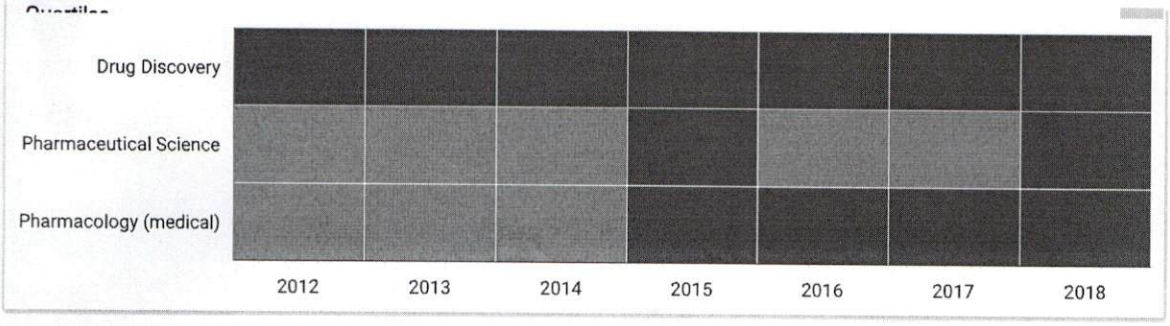
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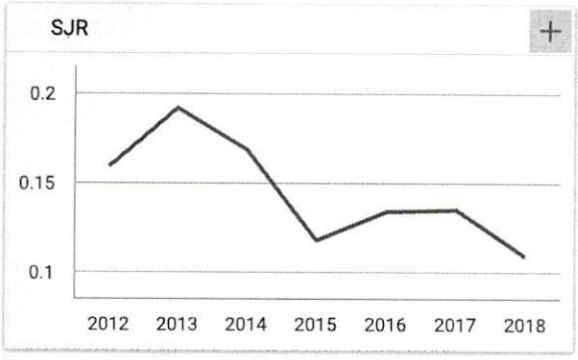
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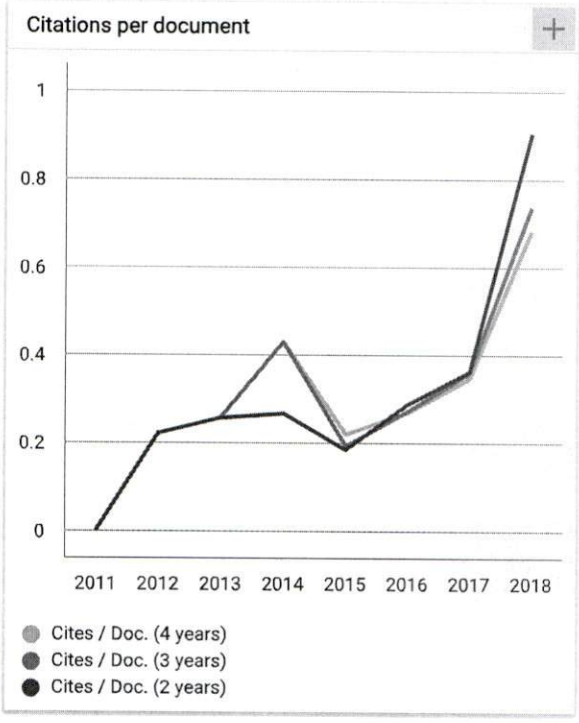
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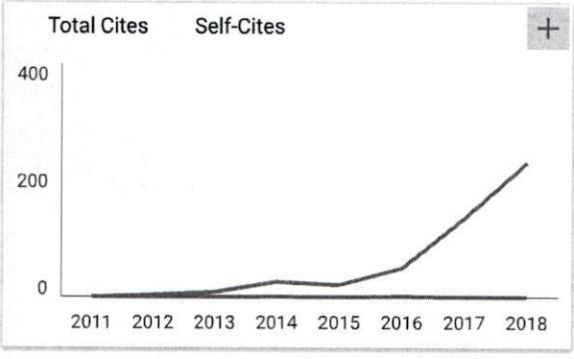
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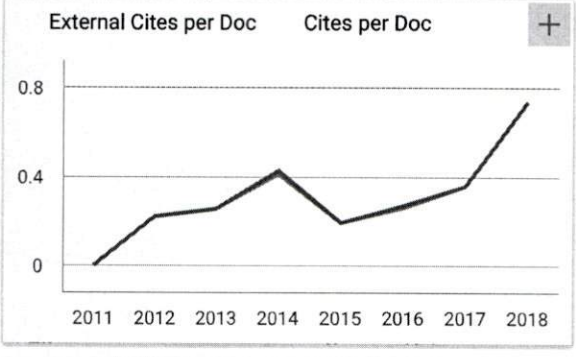
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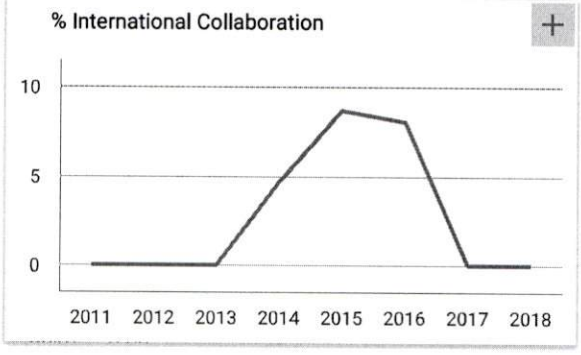
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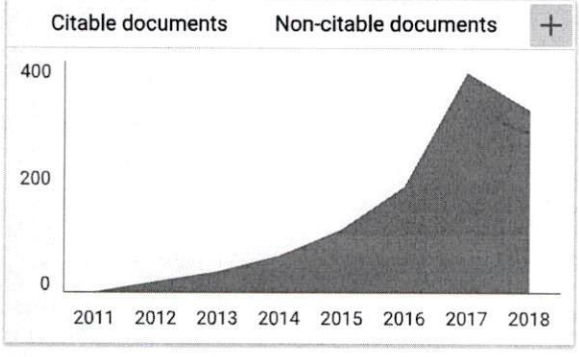
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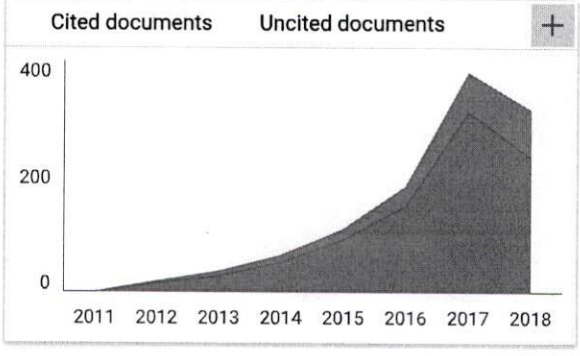
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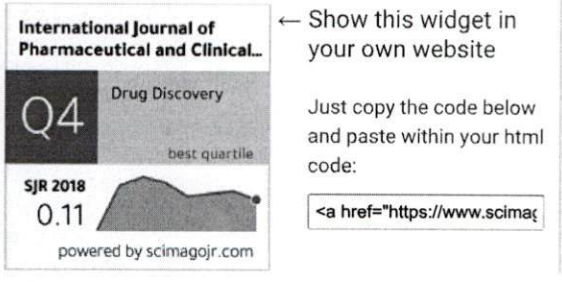
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Research Article

Syntheses, Molecular Docking Study and Anticancer Activity Examination of *p*-Methoxycinnamoyl Hydrazides

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Available Online: 8th June, 2016

ABSTRACT

In this study, we attempted to develop a potential anticancer drug by synthesizing some of *p*-methoxycinnamoyl hidrazides. The compounds were synthesized from the ethyl *p*-methoxycinnamate (EPMC), isolated from rhizome of Kaemferia galanga Linn. The structures of the compounds were confirmed by UV-vis spectrophotometry, 1H-NMR, 13C-NMR, FT-IR, and MS spectroscopic methods. The study was followed by anticancer activity evaluation of the compounds by in silico study using Molegro® ver. 5.5 and by in vitro assay against human breast cancer cells (T47D) by 3-(4,5-Dimethylthiazol-2-yl)-2-5-Diphenyltetrazolium Bromide (MTT) method. The yield of derivatives of p-methoxycinnamoylhidrazide was around 25 to 90%. The result showed that 3-(4-methoxyphenyl)-N'-(3-(4-methoxyphenyl) acryloyl) acrylohydrazide has the highest value of rerank score (-124.81). In addition, from the in vitro assay, it was revealed that 2-hydroxybenzohydrazide has the lowest IC50 (0.2 x 106 nM) against T47D as the most effective compound than the others. *p*-Methoxycinnamoyl hidrazides have been synthesized as low as 25% yields. Among the tested compounds, 2-hydroxybenzohydrazide is the most effective compound against T47D (human breast cancer) cell line in vitro. While in silico study result showed that 3-(4-methoxyphenyl)-N'-(3-(4-methoxyphenyl) acryloyl)acrylo- hydrazide has better activity than the lead compound, EPMC.

Keywords: ethyl *p*-methoxycinnamate, hydrazides, anticancer, molecular docking, MTT assay

INTRODUCTION

Cancer is the biggest threat to human health both in the developed and developing countries. According to the WHO (2014), breast cancer became the most common cancer in women and the second biggest cause of death after heart disease in the world[1,2]. This fact makes many researchers in the world try to design a new effective anticancer drug. Based on IMS (2002), drugs for cancer therapy are a class of drugs most widely used by patients in the world[3]. Breast cancer is a multi-factorial disease caused by epigenetic changes and genetic mutations that occur in genes directly involved in the process of cell division and programmed cell death[4]. in the last decade, it has been reported that epmc isolated from indonesian plant, kaempferia galanga linn., showed significant anticancer activity by inhibiting cyclooxygenase-2 (cox-2)[5-7]. therefore, in this study, we used an approach based on the structure to design the compounds as anticancer drugs through inhibition of cox-2, which is known as docking[8].based on the docking results between the ligand and protein from cox-2, it was identified that there were 4 points of pharmacophore groups of cox-2, such as hydrogen bond donors and acceptors, a hydrophobic group and two aromatic groups[9]. cox-2 inhibitor increased apoptosis and inhibit angiogenesis of cancer cell[10,11]. epmc is a compound that potentially can be used as a lead compound for synthesizing new anticancer drugs, due to its bioavailability in indonesia's plants that are relatively large, its effortless isolation process, and its nature and chemical structure. based on its chemical structure, epmc

is a propenoate that consists of alkyl aromatic ring, olefin groups, and ester groups, which are possible tc be experienced into various functional group transformations. after obtaining epmc from rhizome of k. galanga by soxhletation, the next stage of this study was synthesizing *p*-methoxycinnamoyl hydrazide derivatives. the following step was performing molecular docking simulations, and then the derivatives of *p*-methoxycinnamoyl hydrazides would be examined in terms of its in vitro anticancer activity against human breast cancer cells (t47d) using mtt method. the data obtained from the docking study and the in vitro assays were then compared to reach conclusions.

MATERIALS AND METHODS

All reagents and solvents were purchased from standard commercial suppliers. The structure of the synthesized compounds were confirmed by 1H-NMR, 13C-NMR, IR and LRMS spectral data, the purity was ascertained by melting point and TLC tests. Melting points were

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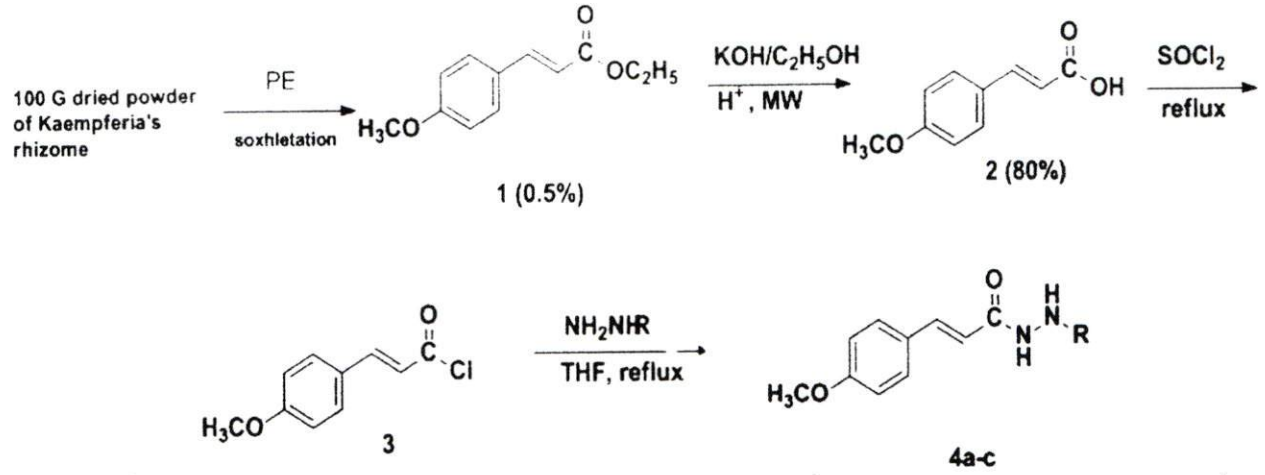


Figure 1: Synthesis of *p*-methoxycinnamoyl hidrazides.

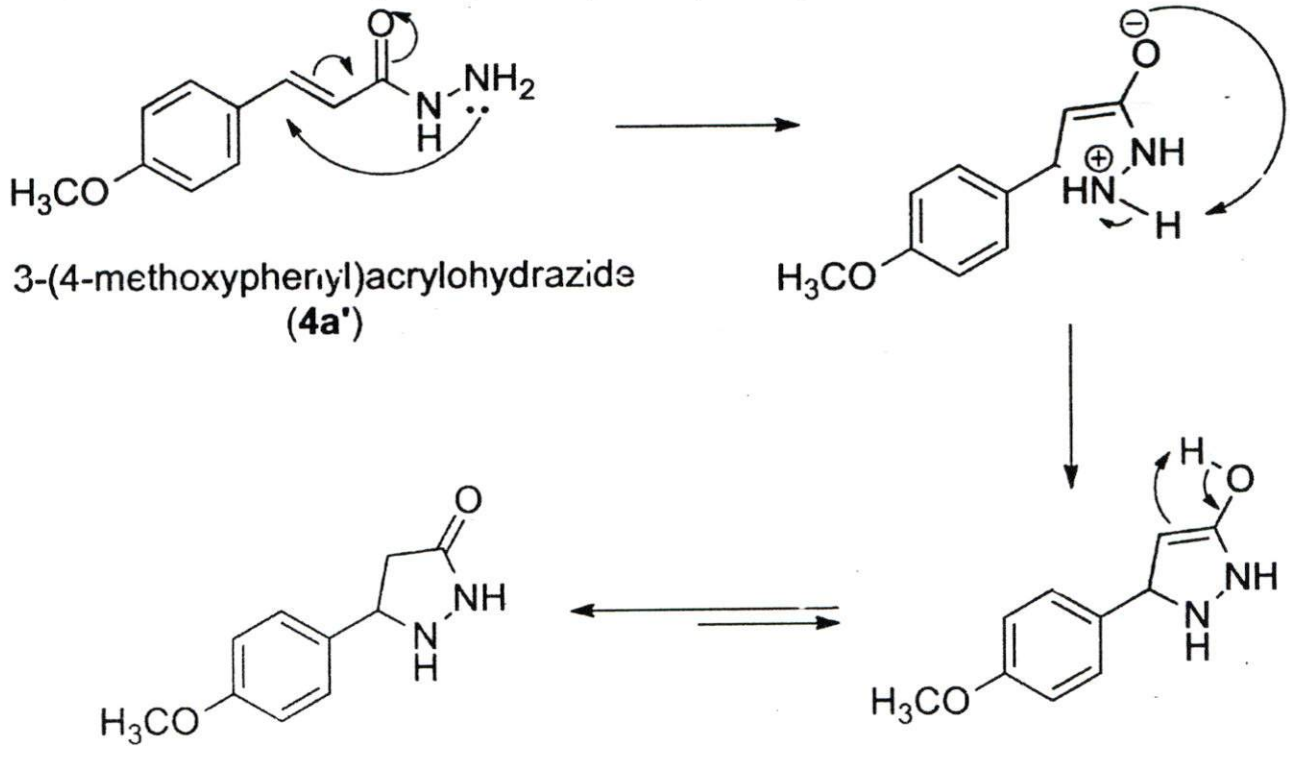


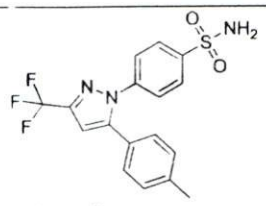
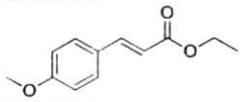
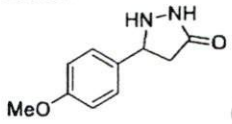
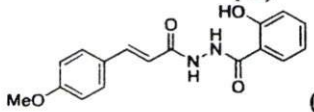
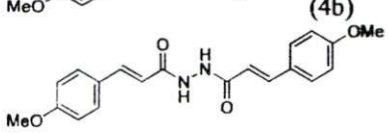
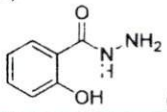
Figure 2: Cyclization of 3-(4-methoxyphenyl)acrylohydrazide.

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. 1H-NMR and 13C-NMR spectra were obtained on a BRUKER instrument, and chemical shifts were reported in ppm on the δ-scale from internal Me4Si. MS spectra were measured with a JEOL JMS 600 spectrometer by using the ESI methods. TLC was carried out on glass plates coated with silica gel F254 (Merck). Spot detection was performed with UV 254 nm.

Syntheses of p-methoxycinnamoyl hidrazides

0.5 grams of Ethyl *p*-methoxycinnamate (EPMC) (1) was isolated from 100 grams dried powder of K. galanga by soxhletation with PE, bp.40-60°C (0.5% yield). EPMC in 5% KOH/ethanol was being irradiated by microwave then acidified to produce *p*-methoxycinnammic acid (2). To *p*-methoxycinnamic acid in THF and one drop of pyridine, 5 eq. of thionyl chloride was added. The mixture was refluxed to produce *p*-methoxycinnamoyl chloride (3). Without further purification, 3 was diluted in THF and added to the solution of 5 eq. hidrazines. The mixture was stirred several times to obtain solid precipitates. The

Table 1: Molecular Docking Value of EPMC and the tested compounds.

Compounds	Rerank Score (kcal/mol)
 Celecoxib	-125.09
 EPMC	-95.63
 (4a)	-71.19
 (4b)	-90.41
 (4c)	-124.81
 (4d)	-60.56

obtained compounds were recrystalized with appropriate solvents to give *p*-methoxycynnamoyl hidrazide derivatives (4) (see figure 1). In addition, 2-hydroxybenzohydrazide (4d) was synthesized by reacting an ester, methyl salicylate with 4 eq. hydrazine hydrate using domestic microwave (400 watt, 10 minutes), with 80% yield. The obtained compound, 4d, had similar spectra data as noted in the reference[12].

RESULTS AND DISCUSSION

In this study, the desired products were 3-(4-methoxyphenyl)acrylohydrazide (4a`), 2-hydroxy-N'-(3-(4-methoxyphenyl)acryloyl)benzohydrazide (4b), and 3-(4-methoxyphenyl)- N'-(3-(4-methoxyphenyl)acryloyl)acrylo-hydrazide (4c). Compound 4b was obtained by reacting p-methoxycinnamoyl chloride (3) with 2-hydroxybenzohydrazide (4d) through acyl nucleophilic substitution with 35% yield. Reaction between p-methoxycinnamoyl chloride (3) and hydrazine hydrate, which always produced its disubstituted derivative and independent of the molecular equivalency of the reacting substrate, yielded 4c (25 %). Unfortunately, 4a' could not be generated since it cyclized to became 5-(4-methoxyphenyl)pyrazolidin-3-one (4a) via nucleophilic addition of unsaturated α, β-carbonyl or known as Michael addition[15] The compound 4a was produced with 90% yield (see figure 2). The detailed physicochemical and spectral data of the obtained compounds are as follows:

5-(4-metoksifenil)pirazolidin-3-on) (4a)

(yield 90%) as pink crystal (m.p. 138-140°C). ESI-MS m/z, [M+H]+= 193, Calc. Mass (C10H12N2O2)= 192. 1H NMR (CDCl3, ppm) 2.37 (1H, dd, J = 15.6, 7.7 Hz), 2.67 (1H, dd, J = 15.0, 7.3 Hz), 3.64 (3H, s), 4.20 (1H, s), 4.50 (1H, t, J = 7.5 Hz), 6.83 (2H, d, J = 8.3 Hz), 7.24 (2H, d, J = 8.3 Hz), 9.30 (1H, s). 13C NMR (CDCl3, ppm) 176.79, 159.00, 128.39 (2C), 114. 84, 114.28 (2C), 60.07, dan 55.53 (2C). IR (KBr) 3224, 3025, 1765, 1614, 1474, 1308, 1259 cm-1. All these spectral data are in agreement with the structure of compound 4a.

2-hydroxy-N'-(3-(4-methoxyphenyl)acryloyl)benzohydrazide (4b)

(yield 35 %) as white crystal (m.p. 260-262 °C). ESI-MS m/z, [M+Na]+ = 335. Calc. Mass C17H16N2O4 = 312.

Molecular Docking Study

To estimate the anticancer activity of derivatives of p-methoxycinnamoyl hidrazide, molecular docking study was performed using Molegro Virtual Docker (MVD) Ver.5.5. We used receptor Celecoxib-2 or COX-2 (PDB code: 1CX2) as the target protein. 1CX2 is a receptor model of inhibitor celecoxib, with the ligand code: SC58. The most stable chemical conformation of the target compounds was determined. The active conformation of ICX2 and SC58 was selected for the preparation step. Cavity was detected to select the pocket binding site (active site of the enzyme). EPMC and the p-methoxycinnamoyl hidrazides (4a-c) were then docked on to the protein, on the same cavity. The results, concluded which conformation produced the lowest energy state when bound to the target protein, were shown as Rerank Score. In this study, we also examined compound 4d, since this compound was also potential to be developed as an anticancer agent.

In vitro Anticancer Activity Assay

The in vitro anticancer activity examination of compound EPMC, 4a, 4b, 4c and 4d were determined by MTT assay method. T47D cells were seeded in 96-well plates (5000 cells/well) and divided into control and treatment group. A series of concentration of each tested compound was prepared with the range of 35 – 5260 nM. The compounds were dissolved in DMSO (1 mg/mL) as stock solution and diluted in culture medium. After 24 h incubation, it was removed and cells were washed using PBS. A 5 mg/mL of MTT was diluted by culture medium (1:10) and 100 µl of it was added into wells. After being incubated for 4 h, formazan was generated, and it was stopped by adding 10% SDS. The plate was covered and incubated in a dark place for overnight. After that, the plate was shaken for 10 minutes and the absorbance of the mixture was measured by ELISA reader at λ 595 nm to determine the IC50 value of each compound. The cytotoxic activity of doxorubicin on T47D cell line was determined using MTT assay in the previous study with the exactly same method and gave IC50 value of 16 nM[13,14].

1H NMR (DMSO, ppm) δ 11.86 (s, 1H), 10.77 (s, 1H), 10.52 (s, 1H), 7.89 (d, J = 7.9 Hz, 1H), 7.64 (d, J = 8.7 Hz, 1H), 7.51 (dd, J = 27.2, 12.2 Hz, 3H), 7.41 (t, J = 7.7 Hz, 1H), 7.04 – 6.85 (m, 5H), 6.61 (d, J = 15.8 Hz, 1H), 4.20 – 4.06 (m, 1H), 3.76 (d, J = 3.4 Hz, 3H), 3.67 – 3.56 (m, 1H), 1.75 (ddd, J = 28.0, 12.9, 5.3 Hz, 1H).13C NMR (DMSO, ppm) δ 166.24, 164.14, 161.19, 159.12, 140.66, 134.51, 130.70, 129.92, 129.22, 119.69, 117.76, 117.01, 114.99, 114.89, 55.82. IR (KBr) 3445, 3061, 1660, 1602, 1519, 1280, 1255 cm-1. All these spectral data are in agreement with the structure of compound 4b.

3-(4-methoxyphenyl)-N'-(3-(4-methoxyphenyl)acryloyl)-acrylohydrazide(4c)

(yield 25%) as white crystal. (m.p. 290-293°C). ESI-MS m/z, 375 [M+Na]+. Calc. Mass C20H20N2O4 = 352. 1H NMR (DMSO, ppm) δ 3.75 (3H, s), 6.59 (1H, d, J = 15.8 Hz), 6.95 (2H, d, J = 8.5 Hz), 7.41-7.50 (2H, m), 7.52 (1H, s), 10.30 (1H, s). 13C NMR (DMSO, ppm) δ 163.98, 161.10, 140.29, 129.84 (2 C), 127.74, 117.36 (2C), 114.97, 55.80. IR (KBr) 3449, 3319, 3060, 1649, 1604, 1541, 1451, 1438, 1280, 1250 cm-1. All these spectral data are in agreement with the structure of compound 4c.

Table 2: Anticancer Activity Assay of the Tested Compounds.

Compunds	IC_{50} Value (nM)
4a	13.3×10^6
4b	2.0×10^6
4c	0.9×10^6
4d	0.2×10^6
EPMC	0.74×10^6
Doxorubicin*	16

Molecular Docking Study Result

The molecular docking study of tested compounds with receptor 1CX2 is shown in table 1. Rerank score is a parameter which strictly determines potential activity of drug-receptor. It is also a logarithmic cumulative energy between drug-receptor interaction by hydrogen, electronic, and steric bond interaction. The smaller rerank score shows the smaller amount of energy required in forming drug-receptor interaction that give an assumption the drug is more suitable to occupying active site of the receptor. Therefore, compound which has the lower rerank score than the ligand, to be estimated has activity stronger than the ligand's. Table 1 showed the rerank scores of the tested compounds, including the ligand, Celecoxib. The affinity on COX-2's binding site of compound EPMC, 4a, 4b, and 4d were lower than celecoxib. Those compounds still had capability to inhibit COX-2 although their interactions to the enzyme were not selective than celecoxib. Compound 4c has rerank score (-124.81 kcal/mol) similar to celecoxib's (-125.09 kcal/mol). Compound 4c possessed the greatest potential for anticancer activity compared to other compounds included the lead compound, EPMC. On the other hand, compound 4d had the lowest potential for anticancer activity among the examined compounds, since it had rerank score: -60.56 kcal/mol. Based on the rerank score, compound 4c was predicted has potential to inhibit COX-2 as effective as celecoxib.

In vitro anticancer activity

The tested compounds were being evaluated in terms of its anticancer activity against T47D cell line by MTT method. The result of anticancer activity could be seen in Table 2 below. Based on data (shown in table 1 and 2), the rerank scores of *p*-methoxycinnamoyl hidrazides (especially 4a, 4b and 4c) are in accordance with their IC_{50} values. Both predicted activity and in vitro anticancer assay *p*-methoxycinnamoyl hidrazides are 4c >4b>4a. The data above exhibited that compound 4d had the highest anticancer activity among the tested compounds, with IC_{50} 0.2 x 106 nM, despite the fact that it had the highest rerank score binding with COX-2, which means that 4d was predicted to have the lowest anticancer activity. Nevertheless, compound 4d still had a lower anticancer activity than the standard anticancer agent, Doxorubicin. The possible explanation for these results is that compound 4d has a different mechanism in inhibiting cancer cell growth compared to the other compounds (4a-4c). Compound 4d has similar chemical structure with phenylurea that was reported to inhibit Check Point kinase 1[16-8]. Further study is needed to clarify the mechanism of the anticancer activity of compound 4d.

CONCLUSION

In this study, we had synthesized derivatives of p-methoxycinnamoyl hidrazides, with the range of 25% to 90% yields. From in silico study data, 3-(4-methoxyphenyl)-N'-(3-(4-methoxyphenyl) acryloyl) acrylohydrazide (4c) with the lowest Rerank score -124.81 has the highest potential as anticancer agent amongst the studied compounds. Meanwhile in vitro activity data revealed that 2-hydroxybenzohydrazide (4d) was the most effective compound against T47D cell line with an IC50 value of 0.2 x 106 nM.

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