

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

by Juni Ekowati

Submission date: 21-Dec-2019 03:35PM (UTC+0800)

Submission ID: 1237723266

File name: cancer_Activity_Examination_of_p-Methoxycinnamoyl_Hydrazides.pdf (363.47K)

Word count: 3041

Character count: 15890

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

Melanny Ika Sulistyowaty^{1*}, Nugroho A E², Putra G S¹, Ekowati J¹, Budiati T¹

¹Faculty of Pharmacy, Universitas Airlangga, Dharmawangsa Dalam Street Surabaya, 60286, Indonesia

²Hoshi University, Ebara 4-2-1 Tokyo, 1428501, Japan

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 *Kaempferia 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*-methoxycinnamoylhydrazide 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 world³. 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 death⁴. 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 docking⁸. 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 groups⁹. COX-2 inhibitor increased apoptosis and inhibited 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 propanoate that consists of alkyl aromatic ring, olefin groups, and ester groups, which are possible to be experienced into various functional group transformations. After obtaining EPMC from rhizome of *K. galanga* by Soxhlet extraction, 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 was confirmed by ¹H-NMR, ¹³C-NMR, IR and LRMS spectral data, the purity was ascertained by melting point and TLC tests. Melting points were

*Author for Correspondence

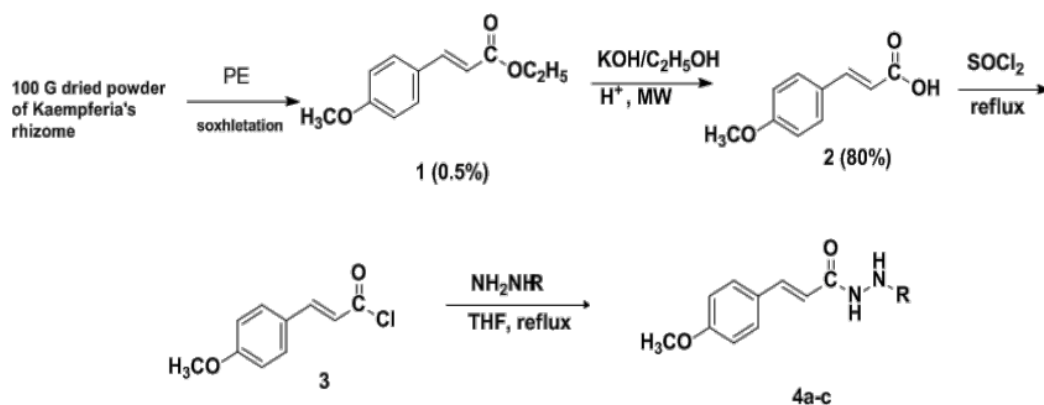
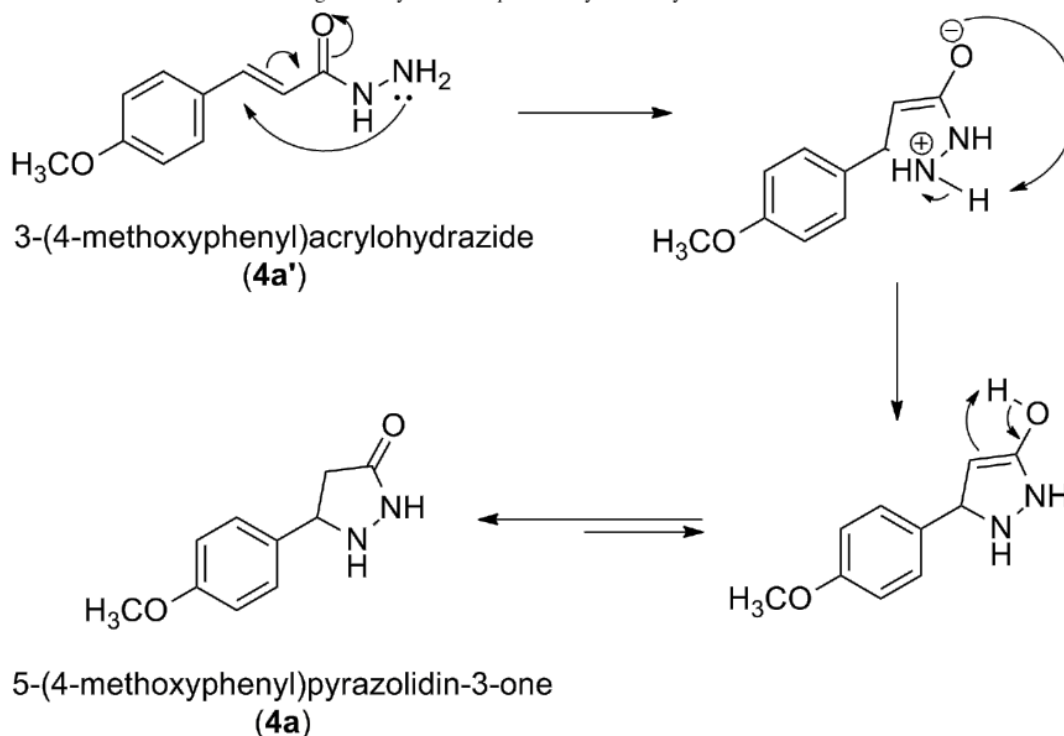
Figure 1: Synthesis of *p*-methoxycinnamoyl hydrazides.

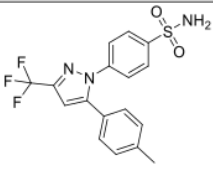
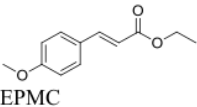
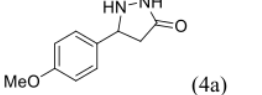
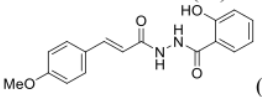
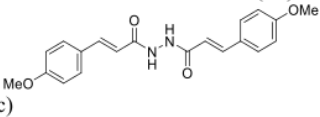
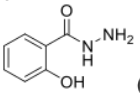
Figure 2: Cyclization of 3-(4-methoxyphenyl)acrylohydrazide.

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} . $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra were obtained on a BRUKER instrument, and chemical shifts were reported in ppm on the δ -scale from internal Me_4Si . 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 hydrazides

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*-methoxycinnamic 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. hydrazines. 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)
	-125.09
Celecoxib	
	-95.63
EPMC	
	-71.19
(4a)	
	-90.41
(4b)	
	-124.81
(4c)	
	-60.56
(4d)	

obtained compounds were recrystallized with appropriate solvents to give *p*-methoxycinnamoyl hydrazide 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 reference¹².

RESULTS AND DISCUSSION

In this study, the desired products were 3-(4-methoxyphenyl)acryloylhydrazide (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 become 5-(4-methoxyphenyl)pyrazolidin-3-one (4a) via nucleophilic addition of unsaturated α , β -carbonyl or known as Michael

Molecular Docking Study

To estimate the anticancer activity of derivatives of *p*-methoxycinnamoyl hydrazide, 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 hydrazides (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 μ l 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}.

addition¹⁵ 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.

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.

Table 2: Anticancer Activity Assay of the Tested Compounds.

Compounds	IC ₅₀ Value (nM)
4a	13.3 x 10 ⁶
4b	2.0 x 10 ⁶
4c	0.9 x 10 ⁶
4d	0.2 x 10 ⁶
EPMC	0.74 x 10 ⁶
Doxorubicin*	16

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

ACKNOWLEDGMENTS

The authors are grateful to Pharmacy Faculty of Universitas Airlangga Surabaya Indonesia for its financial support in year 2014.

REFERENCES

- Santos CA, Silva BGS, Noronha SAAC, Noronha SMR, Shimuta SI, Nakaie CR, et al. Angiotensin-(1-7) Changes Apoptosis-Related Genes Expression in Human Breast Cancer Cell Line T47D. *J Cancer Ther* 2014; 5:1412-22.

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 (2C), 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.

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 IC₅₀ 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 IC₅₀ 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 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.

- American Cancer Society. *Cancer Facts & Figures* 2015. Atlanta: American Cancer Society; 2015.
- Michael Kleinrock. *The Global Use of Medicines: Outlook Through 2016*. USA: The IMS Institute for Healthcare Informatics; 2012.
- Daidone MG, Paradiso A, Gion M, Harbeck SF, and Schmitt M. Biomolecular Features of Clinical Relevance in Breast Cancer. *Eur J Nucl Med Mol Imaging* 2004; 31: 3-14.
- Sae-wong C, Ridditid W, Rean-mongkol W, Wongnawa M. Anti-nociceptive Activity of The Methanolic Extract of *Kaempferia galanga* and Its

- Possible Mechanisms in Experimental Animals. Thai J Pharmacol 2008; 30: 26-34.
6. Aroonkerk N, Kamkaen N. Anti-inflammatory Activity of *Quercus Infectoria*, *Glycyrrhiza Uralensis*, *Kaempferia Galanga* and *Coptis Chinensis*, The main Components of Thai Herbal Remedies for Aphthous Ulcer. J Health Res 2009; 23 Suppl 1: 17-22.
 7. Ekowati J, Sukardiman, Sasaki S, Siswandono, Budiati T. Structure Modification of Ethyl p-methoxycinnamate and Their Bioassay as Chemopreventive Agent Against Mice's Fibrosarcoma. Int J Pharm Pharm Sci 2012; 4 (suppl 3):528-32.
 8. Rajakrisnan V, Manoj VR, Subba RG. Computer-aided, Rational Design of A Potent and Selective Small Peptide Inhibitor of Cyclooxygenase 2 (COX2). J Biomol Struct Dyn 2008; 25: 535-42.
 9. Michaux C, de Leval X, Fabien J, Dogne JM, Pirotte B, Francois D. Structure-based Pharmacophore of COX-2 Selective Inhibitors and Identification of Original Lead Compounds from 3D Database Searching Method. Eur J of Med Chem 2006; 41: 1446-55.
 10. Sobolewski C, Cerella C, Dicato M, Ghibelli L, Diederich M. The Role of Cyclooxygenase-2 in Cell Proliferation and Cell Death in Human Malignancies. Intern. J. of Cell Biology 2010; Article ID 215158.
 11. Goodsell DS. The molecular Perspective: Cyclooxygenase-2. Oncologist 2000; 5: 169-71.
 12. Budiati T, Stephanie DA, Widjajakusuma EC. Rapid Solvent Free Microwave Assisted Synthesis of Some N-benzylidene Salicylic Acid Hydrazides. Indo J Chem 2012; 12: 163-6.
 13. Armandari I, Palupi KD, Farida S, Hermawan A, Susidarti RA, Meiyanto E. Synergistic Combination of Ciplukan (*Physalis angulata*) Herbs Ethanolic Extract and Doxorubicin on T47D Breast Cancer Cells. Indonesian Journal of Cancer Chemoprevention 2010; 1: 26-31.
 14. Fitriasari A, Junedi S, Hermawan A, Susidarti RA, Meiyanto E. The Improvement of Doxorubicin Activity on Breast Cancer cell Lines by Tangeretin through Cell Cycle Modulation. Orient Pharm Exp Med 2011; 11: 183-90.
 15. Mc Murry J. Organic Chemistry. 7th ed. USA: Thomson Learning Inc. 2008.
 16. Mónica C, Sánchez-Archidona AR, David S, Lucía D, Ruiz MA, Sara C, Francesco S, et al. Different role of COX-2 and Angiogenesis in Canine Inflammatory and Non-inflammatory Mammary Cancer. The Veterinary Journal 2013; 197 : 427-32.
 17. Basu G, Winnie S, Dietrich A, Lee T, Christopher R, Barbara A, et al. A Novel Role for Cyclooxygenase-2 in Regulating Vascular Channel Formation by Human Breast Cancer Cells. Breast Cancer Res 2006; 8: R69.
 18. Song D, Na-Na D, Yue-Ming W, Wei-Ying H, En-Zhu J, Shi-Xiang C, et al. Synthesis and Activity Evaluation of Phenylurea Derivatives as Potent Antitumor Agents. Bioorganic and Medical Chemistry 2009; 17: 3873-8.

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

ORIGINALITY REPORT

14%

SIMILARITY INDEX

11%

INTERNET SOURCES

11%

PUBLICATIONS

0%

STUDENT PAPERS

PRIMARY SOURCES

1	ijcc.iregway.com Internet Source	2%
2	file.scirp.org Internet Source	1%
3	www.patentjp.com Internet Source	1%
4	repository.unair.ac.id Internet Source	1%
5	www.freepatentsonline.com Internet Source	1%
6	Edy Meiyanto, Aditya Fitriasaki, Adam Hermawan, Sendy Junedi, Ratna Asmah Susidarti. "The improvement of doxorubicin activity on breast cancer cell lines by tangeretin through cell cycle modulation", Oriental Pharmacy and Experimental Medicine, 2011 Publication	1%
7	www.leighday.co.uk	

Internet Source

1%

8

patents.google.com

Internet Source

1%

9

doaj.org

Internet Source

1%

10

www.dovepress.com

Internet Source

1%

11

www.google.com

Internet Source

<1%

12

physicalchemistry.ir

Internet Source

<1%

13

repository.uhamka.ac.id

Internet Source

<1%

14

repositorio.ufjf.br

Internet Source

<1%

15

www.patentsencyclopedia.com

Internet Source

<1%

16

www.faqs.org

Internet Source

<1%

17

Alan R. Katritzky, Daniela C. Oniciu, Ion Ghiviriga. " Benzotriazole-Assisted Synthesis of $-(\alpha\text{-Cyanoalkyl})\text{sulfonamides}$ ", Synthetic Communications, 1997

<1%

18

Hideo Kigoshi, Kiyotake Suenaga, Masaki Takagi, Atsushi Akao, Kengo Kanematsu, Noriyuki Kamei, Youko Okugawa, Kiyoyuki Yamada. "Cytotoxicity and actin-depolymerizing activity of aplyronine A, a potent antitumor macrolide of marine origin, and its analogs", *Tetrahedron*, 2002

Publication

<1%

19

Yingfei Wang, Xiaojing Zhang, Changjiang Xu, Gaoxiao Zhang, Zaijun Zhang, Pei Yu, Luchen Shan, Yewei Sun, Yuqiang Wang. "Synthesis and Biological Evaluation of Danshensu and Tetramethylpyrazine Conjugates as Cardioprotective Agents", *CHEMICAL & PHARMACEUTICAL BULLETIN*, 2017

Publication

<1%

20

Mourad Ounissi, Abdelkrim Kameli, Chafia Tigrine, Fatma Zohra Rachedi. "Computer-aided identification of natural lead compounds as cyclooxygenase-2 inhibitors using virtual screening and molecular dynamic simulation", *Computational Biology and Chemistry*, 2018

Publication

<1%

21

Juni Ekowati, Suko Hardjono, Iwan Sahrial Hamid. "Ethyl p-methoxycinnamate from *Kaempferia galanga* inhibits angiogenesis

<1%

through tyrosine kinase", *Universa Medicina*,
2016

Publication

22

J Vasse. "Chiral biomimetic NADH models in the benzo[b]-1,6-naphthyridine series. A novel class of stable, reactive and highly enantioselective NADH mimics", *Tetrahedron*, 2003

<1%

Publication

Exclude quotes Off

Exclude matches Off

Exclude bibliography On

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

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

Instructor

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5
