International Journal of Pharma Research & Review, April 2016;5(4):1-11 ISSN: 2278-6074

Research Article

Synthesis, Molecular Docking Study and Brine Shrimp Lethality Test of Benzoxazine and Aminomethyl Derivatives from Eugenol

Galih Satrio Putra[1]*, Tegar Achsendo Yuniarta[2], Achmad Syahrani[1], Marcellino Rudyanto[1,2]

1. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia.
2. Institute of Tropical Diseases, Airlangga University, Surabaya, Indonesia.

ABSTRACT

The specific objective of this research is to study the reaction of eugenol with formaldehyde and aniline and to perform in silico and biological activity studies on the obtained products. All the obtained structure compounds was confirmed by ^{1}H-NMR, ^{13}C-NMR, IR, MS and UV-Vis spectroscopic methods and then were tested for biological activity screening using in silico study by Molegro virtual Docker v 5.5 as a software. Docking process was used by Check point kinase 1 receptor (PDB ID: 2YWP) for screening biological anticancer activity. We have been using brine shrimp lethality test (BST) as in vitro study for anticancer activity screening The result of synthesis, compound (2):4-Allyl-2-methoxy-6-((phenylamino)-methyl)phenol, compound (3):6-Allyl-8-methoxy-3-phenyl-3,4-dihydro-2*H*-benzo-[e][1,3]oxazine and compound (4):6,6'-(Phenylazanediyl)bis(methylene)bis(4-allyl-2-methoxyphenol) were obtained by mannich reaction on eugenol using formaldehyde and aniline was stirred at room temperature for 0,5 h and reflux at 65 ^{o}C for 4 h with the yield product were 26 %, 52 % and 18 %. The results of docking process were benzoxazine and aminomethyl derivatives from eugenol have potential as anticancer activity based on their rerank score. The result of BST, It was found that all compounds have potential anticancer activity based on Meyer's criteria, therefore benzoxazine and aminomethyl derivatives have potential to be further studied for their bioactivity.

Keywords: Aminomethyl, benzoxazines, BST, eugenol, molecular docking, synthesis

Received 14 Feb 2016 Received in revised form 30 March 2016 Accepted 8 April 2016

***Address for correspondence:**
Galih Satrio Putra,
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia.
E-mail: Galih_satrio_putra@yahoo.co.id

1. INTRODUCTION

Cancer is a group of more than 100 different diseases that are characterized by uncontrolled cellular growth, local tissue invasion,and distant metastases. The four most common cancers are prostate, breast, lung, and colorectal cancer. Lung cancer is the most common cause of cancer death worldwide, with around 1,590,000 deaths from lung cancer in 2012 (19% of the total) [1,2]. Smoking is the main risk factor cause bronchus and lung cancer [2]. Indonesia is the world's largest producer and user of clove (*Eugenia caryophyllata* Thumb. or *Syzygium aromaticum* L. Merr.) [3,4]. Clove is used mostly in kretek-cigarette industry. Our research aims to develop new chemicals by synthesis method to find compounds that have the potential for selective receptor cancer cells with minimum side effects. The final goal to provide solutions for industrial kretek-clove cigarettes to explore not only produce kretek/ cigarettes but also the development of new APIs (Active Pharmaceutical Ingredients) which is the source of cloves as anticancer agents to prevent or treat lung cancer due to smoking habit.

Clove consist of eugenol, It is a colorless to pale yellow oil liquid extracted from certain essential oils especially from clove. It is present in concentrations of 80–90% in clove bud oil and at 82–88% in clove leaf oil [5,6]. Eugenol is a member of the phenylpropanoids class of chemical compounds that has potential local anesthesia has been medically used by the

dentists. There is a phenol functional group that have the potential antioxidant, anti-inflammatory, antiallergy, antithrombotic, antimicrobial and antineoplastic activity [7, 8].

Phenol functional group of eugenol can be reacted by Schiff base to produce Mannich base derivatives using Mannich reaction [9]. Mannich reaction is a reaction between compound that could form an enol derivatives with iminium salt (schiff base) to produce β-amino alkyl carbonyl / alkylaminomethyl / Mannich base derivatives [9,10]. Iminium salt (Schiff base) is a reaction between primary or secondary amines with formaldehyde [9-11].

Japanese group has previously reported the synthesis of benzoxazine and aminomethyl derivatives of phenols. Benzoxazine derivatives were studied for antihypertensive [12], antimicrobial [13], antifungal [14, 15], antimycobacterial [16, 17, 18] analgesic, and antiinflammatory activities [19, 20]. Aminomethyl derivatives were reported had potential activity as antimalarial [21], analgesic and antiinflammatory activities [22, 23], antimicrobial [24], anticonvulsant and also anticancer [25, 26]. Indonesian group has previously reported the synthesis some of aminomethyl sourced of eugenol from the following process reaction also obtained benzoxazine derivatives. Aminomethyl and benzoxazine derivatives from eugenol have been reported potential anticancer activity to be further studied for their bioactivity [27].

One of them is aminomethyl and benzoxazine derivatives from mannich reaction on eugenol with aniline and formaldehyde. The process of stirring and reflux for 48 hours can be resulted of benzoxazine derivatives. Benzoxazine derivatives could be hydrolisis in acidic condition to produce aminomethyl derivatives shown on (**Figure 1**). All these compounds have given good result for screening anticancer agent by brine shrimp lethality test (BST) and they have a potential to be further studied for their bioactivity [27].

Bujnowski et al had found simple and efficient synthetic method about mechanistic investigation of a Mannich reaction of phenol that produce aminomethyl derivatives without hydrolysis process [28]. Benzoxazine and aminomethyl derivatives can be obtained by one-pot reaction with variety of result products that depend on time to reaction [28] shown (**Figure 2**). Several of the methods for the synthesis of aminomethyl derivatives , we choose one-pot reaction without hydrolysis. The synthesis method on this research, Mannich reaction on eugenol using formaldehyde and aniline was stirred at room temperature for 0.5 hours and reflux at 65 ^{o}C for 4 hours. Based on the graphic, reaction time for 4 hours can be obtained benzoxazine and aminomethyl derivatives to studied for their anticancer activity.

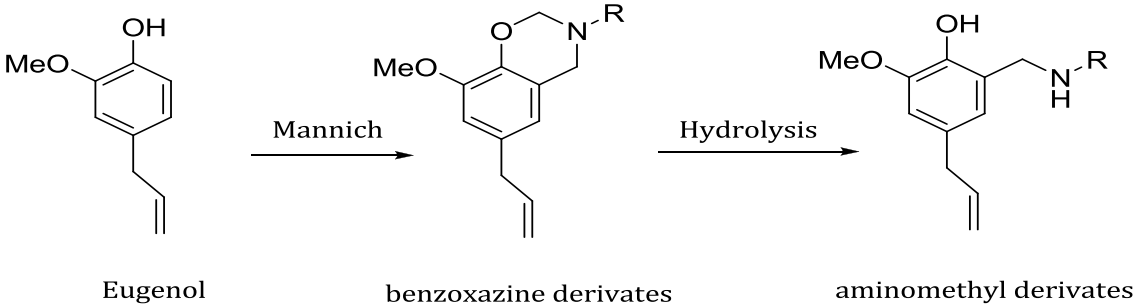


Figure 1: Transformation of eugenol to 1,3-benzoxazine and aminomethyl derivatives [27]

2. MATERIALS AND METHODS

General Commercially available materials were used as received. Eugenol, aniline and were purchased from Merck. Reactions were monitored with TLC. Purification of products was carried out by column chromatography on silica gel using hexane-ethyl acetate (5:2). IR spectra were obtained using a Perkin Elmer Spectrum One spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on JEOL JNM-ECS 400 (^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100

MHz) instrument for solutions in $CDCl_3$. Mass spectra were measured with a JEOL JMS 600 spectrometer.

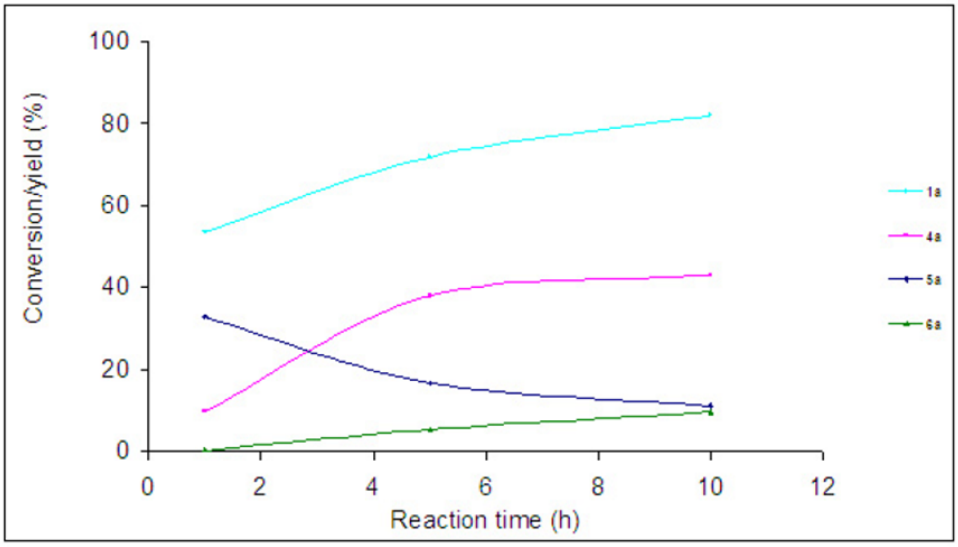
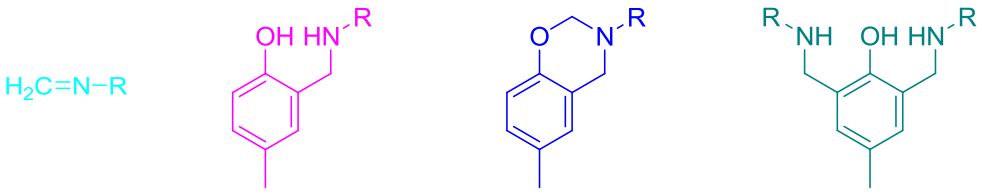


Figure 2 : Corelation between resulf of Mannich product and time to reaction [28]

Note:



(1a) (4a) (5a) (6a)

2.1 Synthesis

Eugenol (2.0 g, 12.2 mmol) was dissolved in methanol (24 mL). The mixture was cooled in ice bath. To the mixture were added 37% formaldehyde solution (3.0 mL, 36.5 mmol) and aniline (1.84 mL, 24.3 mmol). The resulting mixture was stirred at room temperature for 0,5 h and at 65 ^{o}C for 4 h. The mixture was concentrated by evaporation of the solvent under reduced pressure. The crude product was purified using column chromatography with eluen hexane: ethyl acetate (5:2) [27]

2.2 Docking Study

All the obtained compounds from reaction of eugenol with formaldehyde and aniline by Mannich reaction were drawn using ChemOffice Ultra 2010 v12 and saved as mol file. Before the molecular docking, preparation steps must be done as follow converting the 2D structure of ligands to their 3D form, addition and removing of polar hydrogen atoms, energy minimized using the MMFF94x force field. MMFF94x was reported as the efficient force field for minimizing ligand-protein complexes [29]. All Molecular docking analysis and docking calculation were performed in Molegro Virtual Docker (MVD) Ver.5.5. The program operated on an AMD A6 Vision CPU @ 1.4 GHz , 4 GB of RAM.

Docking was performed against to the active site of Check point kinase 1 Receptor. Check point kinase 1 downloaded from protein data bank website (www.pdb.org). This receptor code is 2YWP. Base on reference that reseptor can use for screening anticancer activity because Check point kinase 1 is an enzyme that plays a role in regulating the growth and division of cells undergoing DNA damage.

The protein crystal structure was prepared for docking via removing of water molecules, addition and removal of polar hydrogen atoms then isolation of the active site. The active site was considered to be the site where co-crystalline ligand A42. The active site had been defined as the area

within 157.18 Å^3 around the co-crystallized ligand (**Figure 3**).

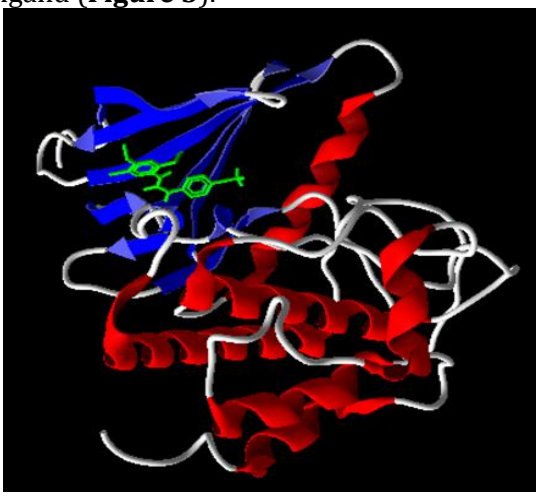


Figure 3: The ribbon diagram showing of the overall structure of Check point kinase 1 with co-crystalline ligand

The co-crystalline ligand was re-docked in the active site to insure the docking method was efficient and the active site was saved as mol. file to be used for docking simulation of the selected compounds (**Figure 4**). Results indicated that the X-ray crystallography conformer was nearly identical to the docked conformer, as deduced from superimposition of the two structures that displayed an RMSD Root Mean Square Deviation < 2,0 Å [30, 31].

The docking result are moldock and rerank score as prediction and simulation between drug-receptor both of them. The moldock and rerank scores were expressed in negative energy terms the lower the binding free energy, the better the binding affinity and the ligand interactions (hydrogen bonding, electrostatic interaction hydrophobic interaction) with Check point kinase 1 [32].

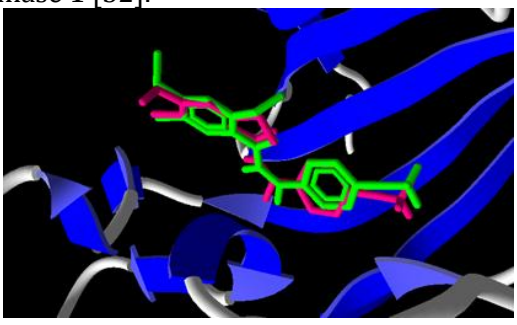


Figure 4: The co-crystalline ligand in the active site of Check point kinase 1 from X-ray crystal structure (green) and from MVD (purple) with RMSD value 0.97 Å

2.3 Brine Shrimp Lethality (BST) Test

The BST assay was carried out according to Meyer [33] with minor modifications. Eggs of Artemia sp. (about 30 mg) were placed into hatching chamber and kept under constant aerator for 24 h. After hatching, active nauplii were collected with Pasteur pipette to be used for assay. Test samples were prepared as follows. 50.0 mg of each synthesized compounds were accurately weighed and dissolved in 50.0 ml methanol to give stock solution with concentration of 1000 ppm. From the stock solution, a variety of solution concentrations were prepared as, 0.50 ppm, 1.0 ppm, 2.0 ppm, 5.0 ppm, 10.0 ppm, 50.0 ppm each in triplicate. 5 ml each of these dosages were transferred into small vials and prepared in triplicate. The vials used for control experiment was stained with 1 ml methanol. All vials containing the dosages and the control were left overnight for the methanol to vaporize, leaving only the sample as residue. To each of the vials containing the tested compounds, 5 drops of DMSO 1% were added to redissolved the dosage followed by distilled sea water up to 5 ml. For the control, control test of each sample was added DMSO 1% and sea water up to 5 ml. Then, 10 nauplii were introduced into each test vials, sea water was added to make volume of 5 mL. After 24 h incubation, the vials were observed using a magnifying glass, and followed by counting the numbers of survivors and calculating percentages of deaths. Larvae were considered dead if they did not show any movement during several seconds of observation The resulting data were converted to probit analysis method for determination of the lethal dose 50% (LC_{50}) values for the tested compounds.

3. RESULTS AND DISCUSSION

3.1 Synthesis result

Mannich reaction on eugenol using formaldehyde and aniline was stirred at room temperature for 0,5 h and heated at 65 ^oC for 4 h, obtained benzoxazine and aminomethyl derivatives. The Benzoxazine derivatives from that reaction is compound (2),6-allyl-8-methoxy-3-phenyl-3,4-dihydro-2*H*-benzo[e]-[1,3]oxazine and the aminomethyl derivatives are compound

(3), 4-allyl-2-methoxy-6-((phenylamino)-methyl)phenol and compound (4), 6,6'-(Phenylazanediyl)bis(methylene)-bis(4-allyl-2-methoxyphenol). Investigation of the mechanistic theory of Mannich reaction of phenol by Bujnowski et al was proven at this research [28]. Reaction time reasonably influences the yields of products. If it is sufficiently short, benzoxazine derivatives (3) is the dominant product that can possibly be isolated. Only after appropriately long reaction time the maximum yield of benzylamine (2) is observed. In this research, the reaction excess of aniline (24.3 mmol) and formaldehyde (36.5 mmol) make yield of major product was obtained compound (2) 52 % and compound (3) 26 % because the reaction time for 4 hours. this condition reaction was also obtained compound (4) as minor product. To explain the process reaction can show (**Figure 5-8**).

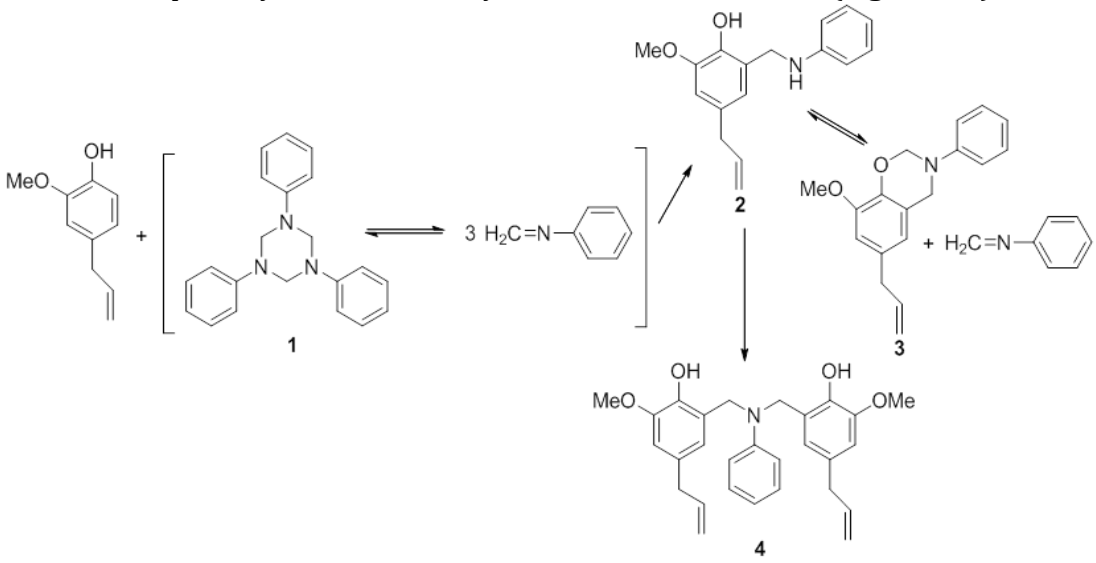


Figure 5: Mannich reaction on eugenol using formaldehyde and aniline

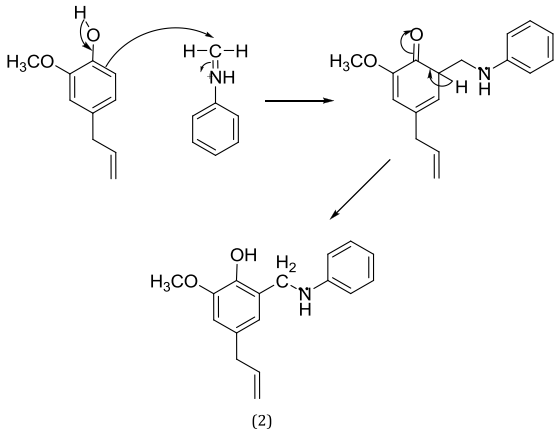


Figure 6: The mechanism reaction forming compound (2)

Detailed physicochemical and spectral data of the obtained compounds are as follows:

1,3,5-Triphenyl-1,3,5-triazinane

Obtained as intermediate product a crystalline and stable. IR (KBr) cm^{-1}: 1519 (Ar C=C), 1083 (C-N) ^{1}H-NMR ($CDCl_3$, δ, ppm): 7.27-7.21 (6H, m), 6.94-6.89 (6H, m), 6.79-6.73 (3H, m), 5.41 (6H, s). ^{13}C-NMR ($CDCl_3$, δ, ppm): 148.6(3C), 129.6 (6 C), 121.9 (3C), 114.3 (6), 84.1 (3C) HRMS calculated for $C_{21}H_{21}N_3$ 315.4126; found 315.4353. All the spectral data are in agreement with the structure of compound 1.

4-Allyl-2-methoxy-6-((phenylamino)-methyl)phenol

Obtained as yellow oil in 52% yield. IR (KBr) cm^{-1} : 3500 (O-H), 3017 (C-H sp^2), 3003(N-H), 2938 (C-H sp^3), 1638 (aliph C=C), 1519 (Ar C=C). 1267 (C-O), 1083 (C-N). ^{1}H-NMR ($CDCl_3$, δ, ppm): δ 7.15-7.19(2H, m), 6.70-6.75 (4H, m), δ 6.62 (1H, s), δ 5.87-5.97 (1H, m), δ 5.02-5.11 (2H, m), δ 4.33 (2H, s), δ 4.33 (2H, s), δ 3.86 (3H, s), δ 3.27 (2H, d, J=6,8 Hz). ^{13}C-NMR ($CDCl_3$, δ, ppm): 148.0, 146.6, 142.3, 137.7, 131.2, 129.1 (2C), 124.2, 120.9, 118.1, 115.5, 113.7 (2C), 110.3, 55.9, 44.3, 39.8. HRMS calculated for $C_{17}H_{19}NO_2$ 269.1416, found 269.1422. All these spectral data are in agreement with the structure of compound 2.

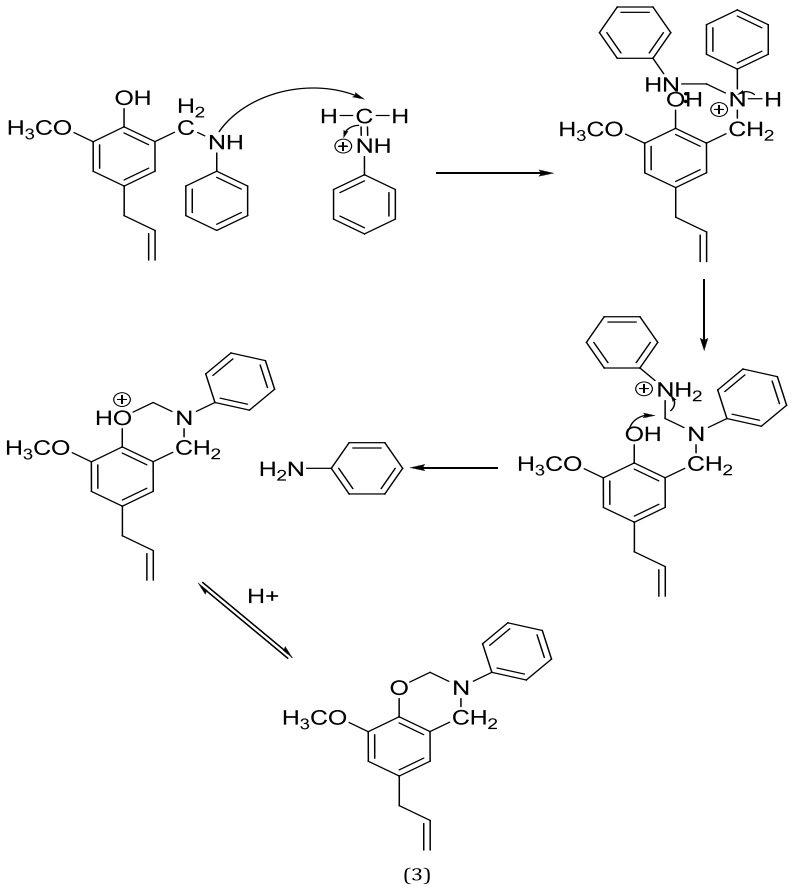


Figure 7: The mechanism reaction forming compound (3)

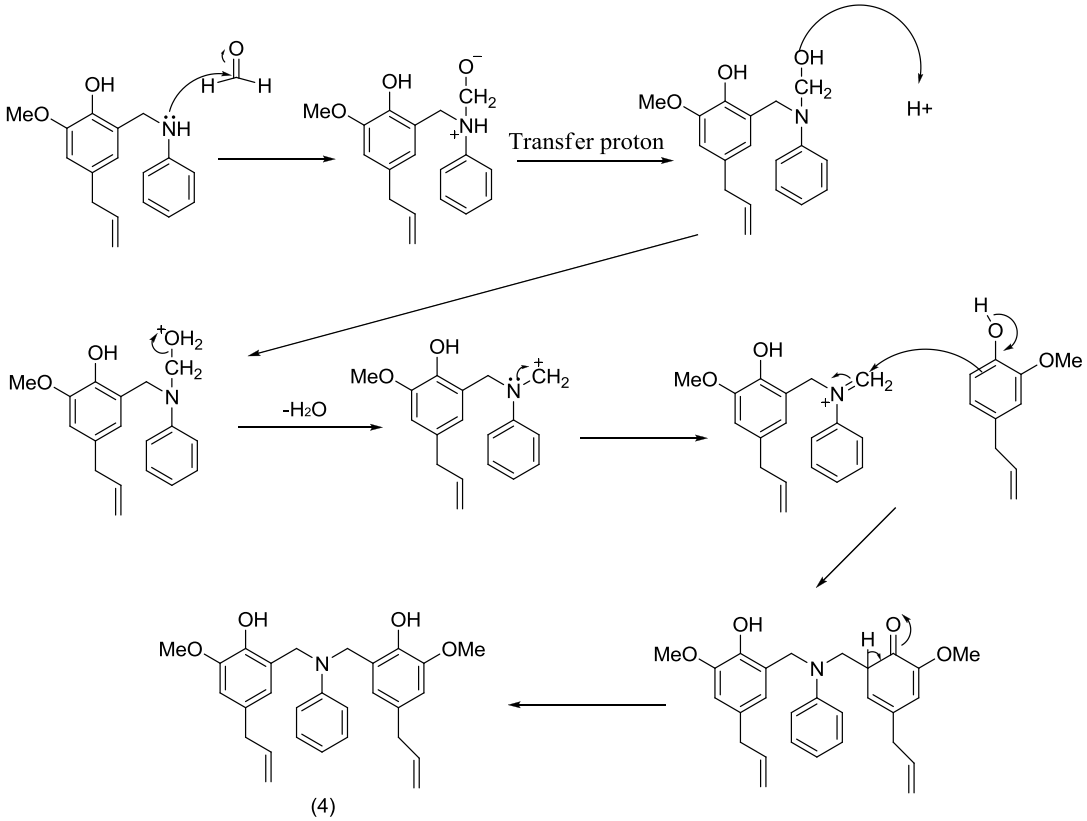


Figure 8: The mechanism reaction forming compound (4)

6-Allyl-8-methoxy-3-phenyl-3,4-dihydro-2*H*-benzo-[e][1,3]oxazine

Obtained in 26% yield as a orange oil. IR (KBr) cm^{-1} : 3414 (O-H), 3060 (C-H sp^2), 2902 (C-H sp^3), 1639 (aliph C=C), 1455 (Ar C=C), 1265 (C-O), 1083 (C-N) ^{1}H-NMR ($CDCl_3$, δ, ppm): δ 7.10-7.12 (2H, m), δ 7.22-7.26 (2H, m), δ 6.89-6.93(1H, m), δ 5.87-5.96 (1H, m), δ 5.41 (2H, s), δ 5.04-5.10 (2H, m), δ 4.60 (2H, s), δ 3.83 (3H, s), δ 3.28 (2H, d, J=6,8 Hz). ^{13}C-NMR ($CDCl_3$, δ, ppm): 148.3, 147.9, 141.9, 137.4, 131.9, 129.1 (2C), 121.3, 121.1, 118.2, 118.0 (2C), 115.7, 109.9, 79.6, 55.7, 50.2, 39.8. HRMS calculated for $C_{18}H_{20}NO_2$ 282.1494; found 282.1513. All the spectral data are in agreement with the structure of compound 3.

6,6'-(Phenylazanediyl)bis(methylene)-bis(4-allyl-2-methoxyphenol)

Obtained in 18% yield as a orange oil. IR (KBr) cm^{-1} : 3498 (O-H), 3070 (C-H sp^2), 3007 (N-H), 2942 (C-H sp^3), 1636 (aliph C=C), 1520 (Ar C=C). 1265 (C-O), 1083 (C-N).^{1}H-NMR ($CDCl_3$, δ, ppm): 7,15-7,19 (2H, m),δ 6,95-6,97 ppm (2H, m), δ 6,79-6,83 ppm (1H, m), δ 5,83-5,93 ppm (2H, m), δ 4,98-5,05 ppm (4H, m), δ 4,47ppm (4H, s), δ 3,84 ppm (6H, s), δ 3,23 (4H, d, J=6,8 Hz). ^{13}C-NMR ($CDCl_3$, δ, ppm): 149.8 (2C), 149.6, 141.3 (2C), 136.5 (2C), 134.8 (2C), 123.5(2C),129,6 (2C), 121,9, 124.6 (2C), 114,3 (2C), 115.9 (2C), 112.8 (2C), 60.5 (2C), 56.1 (2C), 40.1 (2C). HRMS calculated for $C_{28}H_{31}NO_4$ 445,5413; found 445.5518. All the spectral data are in agreement with the structure of compound 4.

3.2 Molecular Docking Result

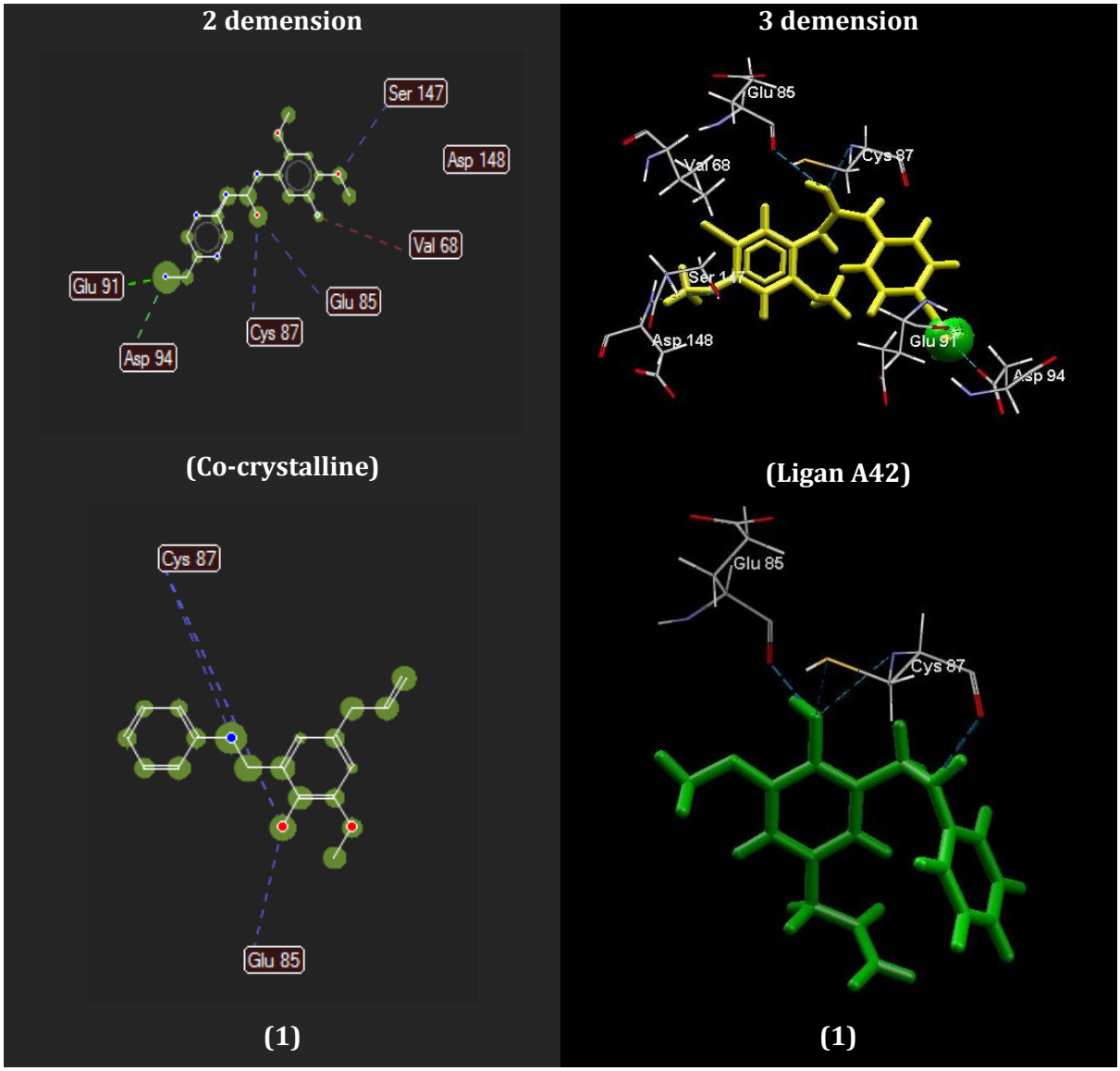
Table 1: Molecular Docking Value of co-crystalline ligand and the tested compounds

Docking process	MolDock Score (kcal/mol)	Rerank Score (kcal/mol)
Co-crystalline ligand	-139.91	-108.84
Compound (2)	-120.76	-82.98
Compound (3)	-123.33	-80.27
Compound (4)	-169.52	-108.51

The result of molecular docking value which performed on compound 2, 3 and 4 then compared with ligand molecular docking value of the receptor check point kinase 1 as a positive control resulted to have a potential anticancer activity. Rerank score is a parameter which strictly determines potential activity of drug-receptor. Rerank score is also a logarithmic cumulative energy between drug-receptor interaction by hydrogen, electronic, and steric bond interaction. The smaller the rerank score, the smaller the activity potential. The smaller the rerank score also shows the smaller amount of energy required to form drug-receptor interaction, therefore it is assumptive that it is more compatible between the drug-receptor interaction. In silico test result of compound 4 shows that it possesses a greater anticancer activity compared to the compound 2 and 3 (**Table 1**). The interaction co-crystalline ligand on binding site of 2YWP occured as the hydrogen bonds on residu Glu 85 and Cys 87, electrostatic bonds on residu Lys 38, Glu 55, Asp 148 and hydrophobic bonds on residu Lys 38, Glu 85, Cys 87 and Leu 137 with value of rerank score -108.84 kcal/mol. The interaction compoud 2 on binding site of 2YWP occured as the hydrogen bonds on residu Glu 85 and Cys 87 with value of rerank score -82.98 kcal/mol. The interaction compoud 3 on binding site of 2YWP occured as the hydrogen bond on residu Cys 87 and hydrophobic bonds on residu Leu 84 and Cys 87 with value of rerank score -80.27 kcal/mol. The interaction compoud 4 on binding site of 2YWP occured as the hydrogen bonds on residu Glu 85, Cys, 87 Asn 135, and Ser 147 and hydrophobic bonds on residu Leu 15, Tyr 20, Val 23, Leu 84, Glu 85, Cys 87, Glu 134, Asn 135, Ser 147 and Asp 148 with value of rerank score -108.51 kcal/mole (**Table 2 and Figure 9**).

Table 2: Docking analysis on Check point kinase 1

Compounds	Rerank Score kcal/mol	Hydrogen bond	Residual involved	Electronic interaction	Residual involved	Steric interaction	Residual involved
Co-crystalline Ligand	-108.84	3	Glu 85 Cys 87 Ser 147	3	Lys 38 Glu 55 Asp 148	4	Lys 38 Glu 85 Cys 87 Leu 137
(2)	-82.98	2	Glu 85 Cys 87	0	-	0	-
(3)	-80.27	1	Cys 87	0	-	2	Leu 84 Cys 87
(4)	-108.51	4	Glu 85 Cys 87 Asn 135 Ser 147	0	-	7	Leu 15 Tyr 20 Val 23 Leu 84 Glu 85 Cys 87 Asp 148



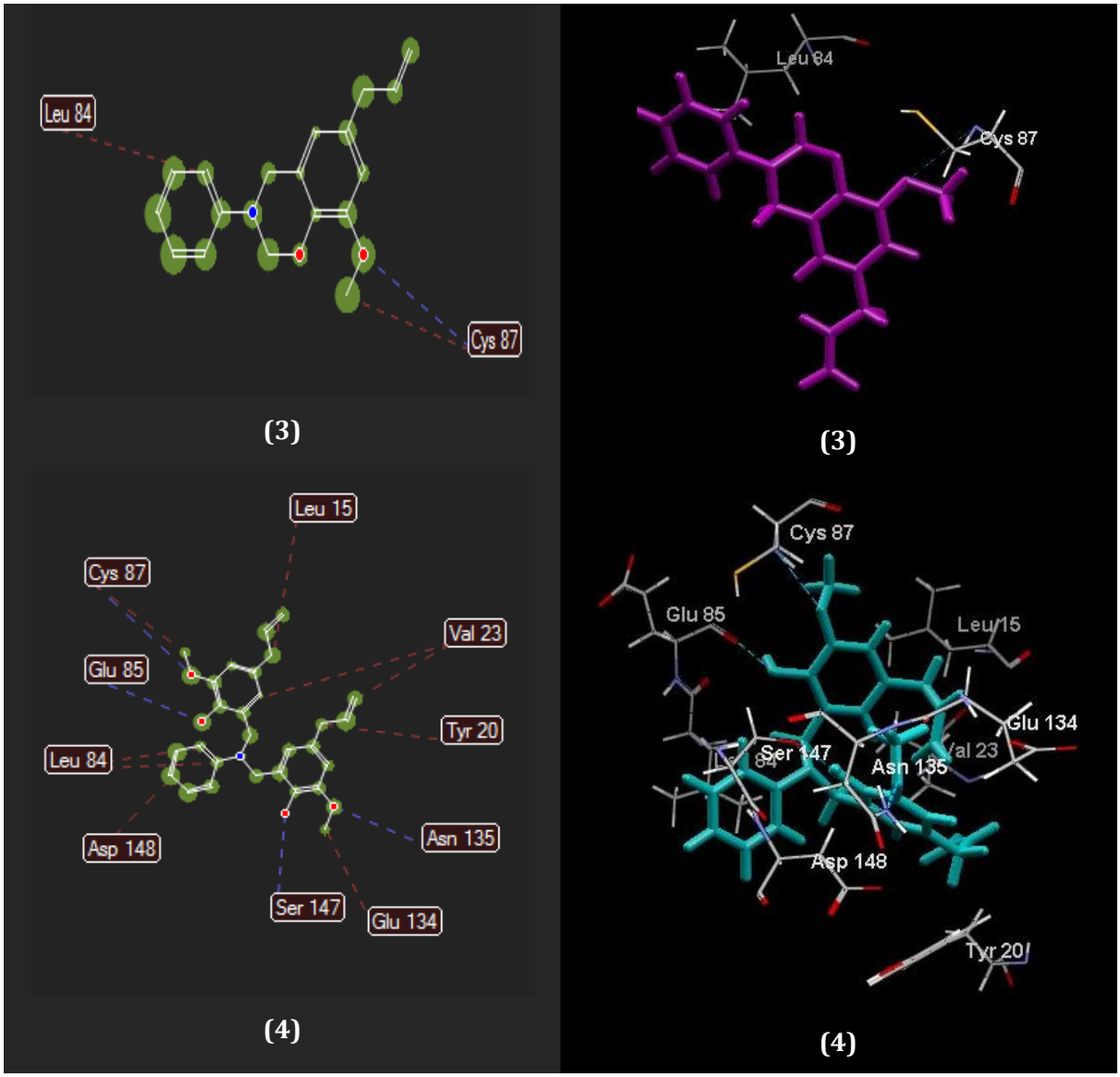


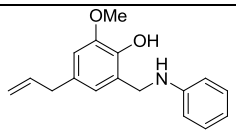
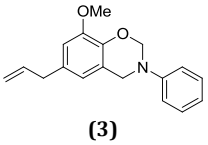
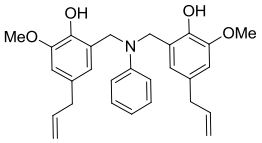
Figure 9: The interaction co-crystalline ligand and the tested compounds on binding site of 2YWP

3.2 Brine Shrimp Lethality Test Result

The results of toxicity test of all the obtained compounds 2, 3, and 4 derivatives of eugenol against *Artemia sp*. are shown in (**Table 3**).

The docking study result of test compounds on binding site of Check point kinase 1 was in line with the in vitro Brine shrimp lethality test result. Based on Meyer's criteria [33] that a pure substance considered toxic if the LC_{50} value is less than 30 ppm. Compounds 2, 3, and 4 are toxic and potential to be studied further (**Table 3**). Compound 4 has the highest toxicity than others.

Table 3. Results of Brine Shrimp Lethality Test

Compounds	Rerank Score (kcal/mol)	LC_{50} (ppm)
 **(2)**	-82.98	5.4
 **(3)**	-80.27	13.6
 **(4)**	-108.51	1.6

4. CONCLUSION

1. Compound (2), (3) and (4) were obtained by mannich reaction on eugenol using formaldehyde and aniline was stirred at room temperature for 0,5 h and reflux at 65 ^{o}C for 4 h with the yield product were 26 %, 52 % and 18 %.
2. Based on molecular docking study the value of rerank score, all compounds are identic with value of rerank score co-crystalline ligand, that assume are all compounds have potential anticancer activity.
3. Based on Meyer's criteria from result of Brine Shrimp Lethality Test, all compound have potential anticancer activity just for screening step.

ACKNOWLEDGMENT

This work was financially supported by Faculty of Pharmacy, Airlangga University. The authors kindly thank Prof. Dr. Siswandono MS., Apt. for valuable discussions, and Ganes Aji Laksono for the assistance in the preparation of this paper.

REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 (link is external) [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Accessed August 2015.
2. Dipiro, Joseph., Robert L., Gary C., Gary R., Barbara G., L. Michael P. Pharmacotherapy: a pathophysiologic approach. New York, McGraw-Hill Medical. 2008 ;pp 2085-2096.
3. Baietto, Manuela. Bud Fall Induction in Clove (Syzygium Aromaticum). Academic Research International. 2014; 5: 23-29.
4. Razafimamonjison, Gaylo., Michel J, Thierry D, Panja R, Fanja F, Pascal D. Bud, leaf and stem essential oil composition of Syzygium aromaticum from Madagascar, Indonesia and Zanzibar. International Journal of Basic and Applied Sciences. 2014; Volume 3 : 224-233.
5. Gulcin, Ilhami., Mahfuz E., Hassan Y. A. Antioxidant activity of clove oil – A powerful antioxidant source. Arabian Journal of Chemistry. 2012; Volume 5 :489–499.
6. Hidalgo, Maria., Carlos D., Hector C., Wilson C., Claudio G., Antioxidant capacity of eugenol derivatives. Quim. Nova, 2009; 32 :1467-1470.
7. Siswandono. & Soekardjo. The Stucture relationship cancer activity, Medical Chemistry Vol 2, Surabaya:Airlangga University Press. 2000 :163-181.
8. Kurti, L. And Czako, B. Stategic Applications of Named Reactions in Organic Synthesis: Background and Detailed Mechanism. USA: Elsevier Inc, 2005:pp. 274-275.
9. Clayden, J., Greeves, N., Warren, S. Organic Chemistry. New York: Oxford University Press. 2001:pp. 135-136, 712-715.
10. Solomon, G.T.W., Fryhke C.B. Organic Chemistry. New York: John Wiley and Sons, Inc. 2008.
11. Dewick,P.M.,Essentials of Organic Chemistry. England : John Wiley and Sons Ltd, 2006:pp.369-370.
12. Kumar, G. Naveen, N. Siva , M. Ramadevi, CH. Maheswara R, B.Vivekananda, Moghul Z. Comparative evaluation of calcium channel blocking activity of novel amlodipine containing benzoxazine nucleus against amlodipine. Indo American Journal of Pharmaceutical Research. 2013; 3 : 8146-8153.
13. Dilesh, Indorkar., Chourasia O, Limaye S. PC-Model Studies of 7 methoxy- 2H-3-Aryl-3, 4-dihydro-1,3 benzoxazine-Aryl-3, 4-dihydro-4-methyl 7 methoxy -1, 3 benzoxazine Biological activity. Research Journal of Pharmaceutical Sciences. 2013 ; 2(3), 17-25.
14. Hayta, S. Alper., E. Akı, B. Tekiner, I. Yıldız, O. Temiz, I. Yalcın, N. Altanlar. Synthesis, antimicrobial activity and QSARs of new benzoxazine-3-ones. European Journal of Medicinal Chemistry. 2006; 41 : 1398-1404.
15. Habib, Osman., Hussein M., Ahmed E. Studies on Some Benzoxazine-4-one Derivatives with Potential Biological Activity. American Journal of Organic Chemistry 2012; 2 : 45-51.
16. Petrlíkova, Eva., Karel W, Rafael D, Pavel H, Jiří G, Jiří K, Jarmila K. Antimycobacterial 3-phenyl-4-thioxo-2H-1,3-benzoxazine-2(3H)-ones and 3-phenyl-2H-1,3-benzoxazine-2,4(3H)-dithiones substituted on phenyl and benzoxazine moiety in position 6. Chemical Papers 2011; 65 (3) : 352–366.
17. Gallegos, Ana., Ramon C, Robert P, Karel W. Similarity approach to QSAR Application to antimycobacterial benzoxazines. International Journal of Pharmaceutics 2004; 269 : 51–60.
18. Nemeček, Peter., Ján M, Jozef L, Karel W. Prediction of anti-tuberculosis activity of 3-phenyl-2H-1,3-benzoxazine-2,4(3H)-dione derivatives. Chemical Papers 2013; 67 (3): 305–312.
19. Khalaj, Ali., Mohammad A, Abbas K, Neda A, Zahra P, Sara R. The antinociceptive and antiinflammatory activities and lack of

ulcerogenicity of a benzodioxin-4-one and its analog benzoxazine as cyclic acetal-like derivatives of salicylic acid and salicylamide in mice and rats. Indian Journal of Pharmacology 2002; 34: 184-188.
20. Tomimoto M, Ikeda H, Oka Y, Yurugi S, Miyazaki N, Funado M,Matsumoto N, Chiba S, Kawai K. Synthesis of 1,3-benzoxazine derivatives and their physiological activities. Takeda Kenkyushoho 1975; 34 (4): 455-466.
21. Bajwa GS, Hartman KE, Joullie MM. Antimalarials. Heterocyclic analogs of N-substituted naphthalenebisoxazines. J Med Chem 1973; 16 (2): 134-138.
22. Chien, M.H., An, R.L., Pei, H.K., and Wen, H.H. Synthesis of Mannich Base Derivatives of Oroxylin A Endowed with Anti-inflammatory Activity. J Med. Sci. 2010; 30: 041-046.
23. Gokce, E., Bakir, G., Sahin, M.F., Kupeli, E., Yesilada, E.. Synthesis of new mannich bases of arylpyridazinones as analgesic and anti-inflammatory agents. Arzneim. Forsch. 2005; 55 :318-325.
24. Idhayadhulla, A., Kumar, R.S., Nasser, A.J.A, Manilal, A.. Synthesis and Antimicrobial Activity of Some New Mannich Base Derivatives. J. Chem. Pharm. 2010; 3 : 904-911.
25. Choi, B.G., Seo, H.K., Chung, B.H., Choi, S.U., and Lee, C.O. Synthesis of Mannich Bases of Antineoplaston A10 and their Antitumor Activity. Arch. Pharm. Res.1994. 17; 467-469.
26. Holla, B.S., Veerendra, B., Shivananda, M.K., Poojary, B. Synthesis characterization and anticancer activity studies on some mannich bases derived from 1,2,4-triazoles.Eur. J. Med.Chem. 2003; 38: 759-767.
27. Rudyanto, M., Ekowati, J., Widiandani, T., Honda, T. Synthesis and brine shrimp lethality test of some benzoxazine and aminomethyl derivatives of eugenol Internasional Jornal of Pharmacy and Phamaceutical Sciences. 2014; 6 (2): 465-467
28. Bujnowski,K., A,m Synoradski, L. O-Aminomethylderivatives., of phenols with N-methylenealkylaminies. ARKIVOC. 2008; XIII :106-114.
29. Sawy, Eslam., Heba M., Khalied M., Eman S., Amira M., Adel H. Synthesis, anticancer activity and molecular docking study of novel 1, 3-diheterocycles indole derivatives. International Journal of Pharmacy and Pharmaceutical Sciences. 2015. 7 : 379-385.
30. Dai, Yujie., Qiang W., Xiuli Z., Shiru J., Heng Z., Dacheng F., & Peng Yu. Molecular docking and QSAR study on steroidal compounds as aromatase inhibitors. European Journal of Medicinal Chemistry. 2010; XXX : 1-9.
31. Suvannang, Naravut., Chanin N., Chartchalerm I., Virapong P. Molecular Docking of Aromatase Inhibitors. Molecules. 2011; 16 : 3597-3617.
32. Lensink MF, Méndez R, Wodak SJ. Docking and scoring protein complexes. CAPRI 3rd Edition. Proteins 2007; 69 :704-18.
33. Meyer BN, Ferrigni NR, McLaughlin JL. Brine Shrimp: A convenient general bioassay for active plant constituents.Planta Med 1982; 45: 31-34.