CHAPTER IV

RESULTS AND DISCUSSION

In this research, syntheses of eugenol derivatives have been reported in several methods such as *O*-alkylation at hydroxy group of eugenol, demethylation at methoxy group of eugenol, electrophilic aromatic substitution at aromatic ring of eugenol, smiles rearrangement and allylation*via* Grignard reaction. The eugenol derivatives in solution form will be preliminarily tested the anesthetic activity in aquatic animals such as *L. vannamei*,*L. calcarifer* and *C. macrocephalus*. The general structures of eugenol derivatives were as shown in Figure 28. The commercially available eugenol derivatives **3**, **16** and **20**accepted from Fluka, Merck and Acros Organics or Aldrich Chemical Co., Ltd.

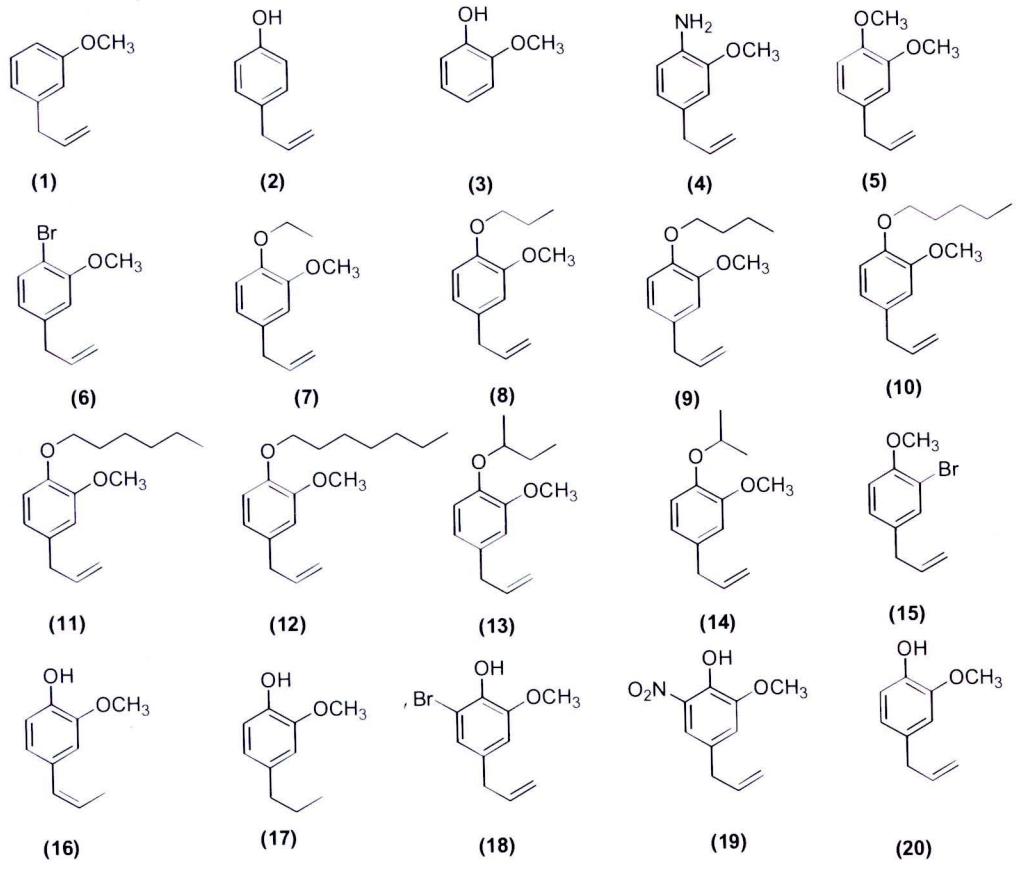


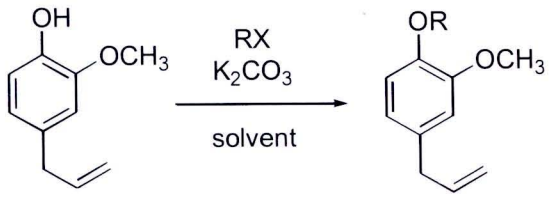
Figure 28 Chemical structure of eugenol (20) and derivative (1-19)

Synthesis of eugenol derivatives

1. Synthesis of eugenol derivatives *via O*-alkylation reaction (5, 7-14)

Synthesis at hydroxyl group of derivatives (**5**, **7-14**) was previously reported by Allen and Gates.[42] The results obtained by alkylation reaction at hydroxyl group of eugenol with halogenated hydrocarbons under potassium carbonate catalysis are summarized in Table 11.

With potassium carbonate as a base catalyst and methyl iodide, methyleugenol (**5**) was formed in 90% yield from eugenol (entry 1). The others derivatives using bromopropane, bromobutane, bromopentane, bromohexane and bromoheptane as reagent provided the desired products 7-14 in a good yield (entries 3-7). This reaction occurred under general nucleophilic substitution mechanism. In case of bromoethane, it gave only acceptable moderate yield. For 2-bromobutane and 2-bromopropane, moderate yield was obtained as expected because of steric hindrance of secondary butyl and isopropyl structure. (entries 8 and 9).



(20) **(5, 7-14)**

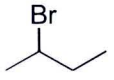
R = Hydrocarbon chain (alkyl groups)

Figure 29 Synthesis of eugenol derivatives *via O*-alkylation reaction (5, 7-14)

Table 11 Synthesis of eugenol derivatives *via O*-alkylation reaction

Entry	RX	%Yield (Compounds)
1	CH_3I	90% (**5**)
2		55% (**7**)
3		86% (**8**)

Table 11 (Cont.)

Entry	RX	%Yield (Compounds)
4		91% (**9**)
5		81% (**10**)
6		98% (**11**)
7		72% (**12**)
8		57% (**13**)
9		68% (**14**)

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Note: Reaction conditions: K_2CO_3, Acetone, reflux, 10 h.

2. Synthesis of 3-allylanisole (1)

In this research, the 3-allylanisole (**1**) was prepared by allylation reaction *via* Grignard reagent in Figure 30. Grignard reaction was prepared from 3-bromoanisole, which was changed to aryl magnesium bromide by using magnesium powder is aprotic solvent (anhydrous tetrahydrofuran) yielded the product in 96% yield [43, 44, 45, 46].

In Table 12, the Grignard reaction was screened with variety of conditions solvents, reagents and catalysts. At preliminary studies, it was revealed that typical coupling conditions gave no product (Table 12, entry 1-5) at any reagent and catalysts, implying that the coupling reaction was too sensitive to humid and difficult to occur at corresponding condition. For the use of tributyltin chloride (Bu_3SnCl) and allylmagnesium chloride (AllylMgCl) as reagent, it was found that the product mixture was unable to purify due to the similarity in dipole moment of the mixture. (entry 6). However, at the last try, the condition using Mg and allylbromide in THF afforded the

desired product in 96.1% yield (entry 7). And finally, 3-allylanisloe was successfully prepared and further used for the model reaction to attach the allyl group to the aromatic ring in this study

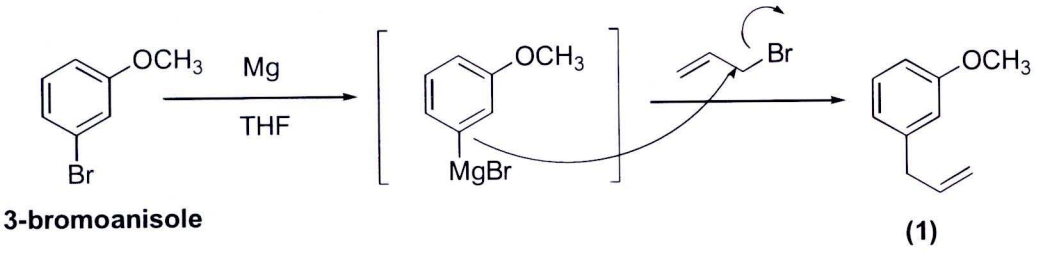


Figure 30 Mechanism of 3-allylanisole (1) *via* allylation Grignard reaction

Table 12 Study of optimal condition in synthesis of 3-allylanisole (1)

Entry	Catalyst	Alkyl halides/ Solvent	Temp (°C)	Time (h)	Yield (%)
1	$FeCl_3$	allylMgCl/ THF	0	24	NR
2	Mg, $AgNO_3$	Allylbromide/ Ether	0	24	NR
3	Mg, $CoCl_2$	allylMgCl/ THF	35	24	NR
4	Mg, $CoCl_2$	Allylbromide/ THF	35	24	NR
5	Mg	Allylbromide/ Ether	0	24	NR
6	Mg, Bu_3SnCl	allylMgCl/ THF	35	24	Mixture
7	Mg	Allylbromide/ THF	35	24	96

Note: NR = No reaction

3. Synthesis of 4-allylphenol (2)

The most easily synthetic plan of 4-allylphenol formation was removal of methyl group from commercially available 4-allylanisole. Thus, the demethylation reaction of methoxy group was substantial reviewed. The results of the demethylation of 4-allylphenol (**2**) (Figure 31) were summarized in Table 13. The reaction was usually operated by Lewis acids or base such as aluminum chloride ($AlCl_3$), sodium ethanethiolate (NaSEt) and others catalysts. The $AlCl_3$ reagent promoted the demethylation reaction in various solvent (entry 1-3) as shown in Table 13 which had not found the product or cannot be easily purified.[47] However, these conditions are not compatible with compounds having acid-sensitive functional groups like allyl moiety. Thus, it is necessary to develop a new method for the selective cleavage of methyl group as shown in entry 4. Lithium chloride is an ideal choice to catalyze the selective cleavage of methoxy group.[48] The microwave radiation condition was attempted in this reaction to cleavage aryl methyl ethers in the presence of lithium chloride/N,N,-dimethylformamide (LiCl-DMF) system.[37] However, the result showed that it was not found the product in our effort.

Afterwards, Trimethylsilyl chloride ($(CH_3)_3SiCl$) in pyridine and *tetra*-n-butyl ammonium bromide (TBAB) in the mixture of 48%HBr/acetic acid was used (entry 5 and 6)[49], unfortunately, it still was not found the desired product. No reaction occurred with sodium metal (entry 7), sodium/1,4,7,10,13,16-hexaoxacyclooctadecane (18-Crown-6) mixture (entry 8)[51] and iodocyclohexane (entry 10). Therefore, the condition has been developed with using of methyl magnesium iodide (MeMgI) in THF which gave compound **2** in a good yield (70%) (entry 9) [50]. However, in the use of sodium ethanethiolate (NaSEt), the desired product was occurred but it was not unable to purify the mixture products (entry 11). Consequently, the above studies prompted us to select the use of MeMgI in THF under reflux temperature to the demethylation reaction of 4-allylpheol.

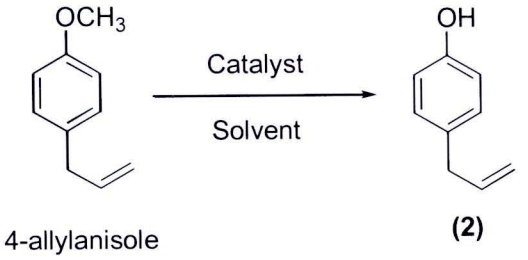


Figure 31 Demethylation reaction of 4-allylphenol (2)

Table 13 Optimization of reaction conditions for demethylation of 4-allylphenol (2)

Entry	Catalyst	Solvent	T (°C)	t (h)	Yield (%)
1	$AlCl_3$	CH_3CN	0	24	NR
2	$AlCl_3$	CH_2Cl_2	0-35	24	NR
3	$AlCl_3$	EtOAc	50	24	NR
4	LiCl	DMF	MW	72	NR
5	$(CH_3)_3SiCl$	Pyridine	35	24	NR
6	TBAB	HBr/AcOH	reflux	24	NR
7	Na	THF/NH_3	35	48	NR
8	Na/18-Crown-6	THE	35	24	NR
9	MeMgI	THF	reflux	24	70
10	Iodocyclohexane	DMF	reflux	48	NR
11	NaSEt	DMF	reflux	3	mixture

Note: NR = No reaction, MW = Microwave (100 W, 160 °C)

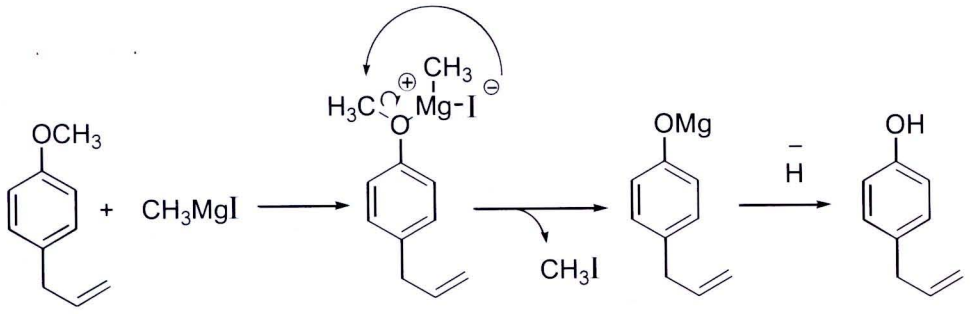


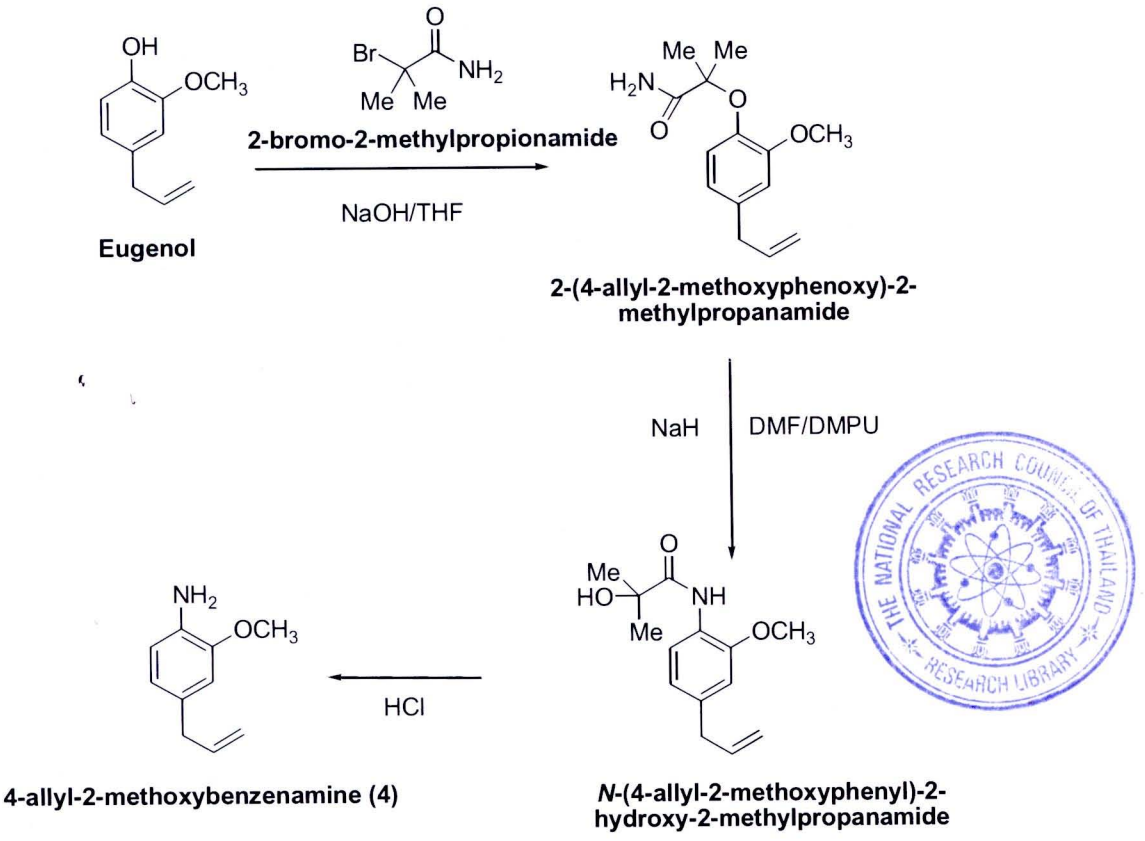
Figure 32 Proposed mechanism of catalytic demethylation of 4-allylphenol (2)

The proposed possible mechanism of demethylation reaction by using CH_3MgI in THF was demonstrated in Figure 32. The process probably occurs by coordination of oxygen atom with magnesium and then nucleophilic attack of CH_3MgI on the methyl group followed by the release of CH_3I. Therefore, the occurrence of this cleavage may be attributed to a nucleophilic attack resulting from the strongly electron-withdrawing effect [35].

4. Synthesis of 4-allyl-2-methoxybenzenamine (4)

The one-pot reaction of 4-allyl-2-methoxybenzenamine (**4**) was synthesized starting from commercially available eugenol. The mechanism of this one step reaction occurred *via* Smiles rearrangement which is an electron withdrawing group rearrangement on aromatic ring. The eugenol was etherified to 2-(4-allyl-2-methoxy phenoxy)-2-methylpropanamide with 2-bromo-2-methylpropionamide in sodium hydroxide in dry tetrahydrofuran (THF) for 4 h (Figure 33). The derived 2-(4-allyl-2-methoxyphenoxy)-2-methylpropanamide was subsequently submitted to the Smiles rearrangement by treatment with sodium hydride in a refluxing 10:1 mixture of *N,N*-dimethylformamide (DMF) and *N,N'*-dimethyl-*N,N'*-propylen-urea [DMPU; 1,3-dimethyl-2-oxo-hexahydropyrimidine]. The resultant *N*-(4-allyl-2-methoxyphenyl)-2-hydroxy-2-methylpropanamide was eventually converted into 4-allyl-2-methoxy benzenamine (**4**) in 46% yield upon treatment with 6M HCl for 2 h at 100 °C.[41,52] The mechanism of this reaction was divided into two steps. The first step was a protonate proton of hydroxyl group of eugenol with base. It indicates that the hydroxyl group is a good nucleoplilic attack at carbon-bromide group of 2-bromo-2-methyl

propionamide to give the intermediate A. In the next step, the intermediate A was abstract proton with HCl and *via* Smiles rearrangement reaction (Figure 34).



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Reagents and Conditions; 1) NaOH, THF, N_2, 3 h, 30 °C, 2) 2-bromo-2-methylpropionamide, 4 h, 100 °C, 3) DMPU:DMF (1:10), NaH, 2 h, 100 °C, 4) 6 M HCl, 2 h, 100 °C.

Figure 33 Synthesis of 4-allyl-2-methoxybenzenamine (4)

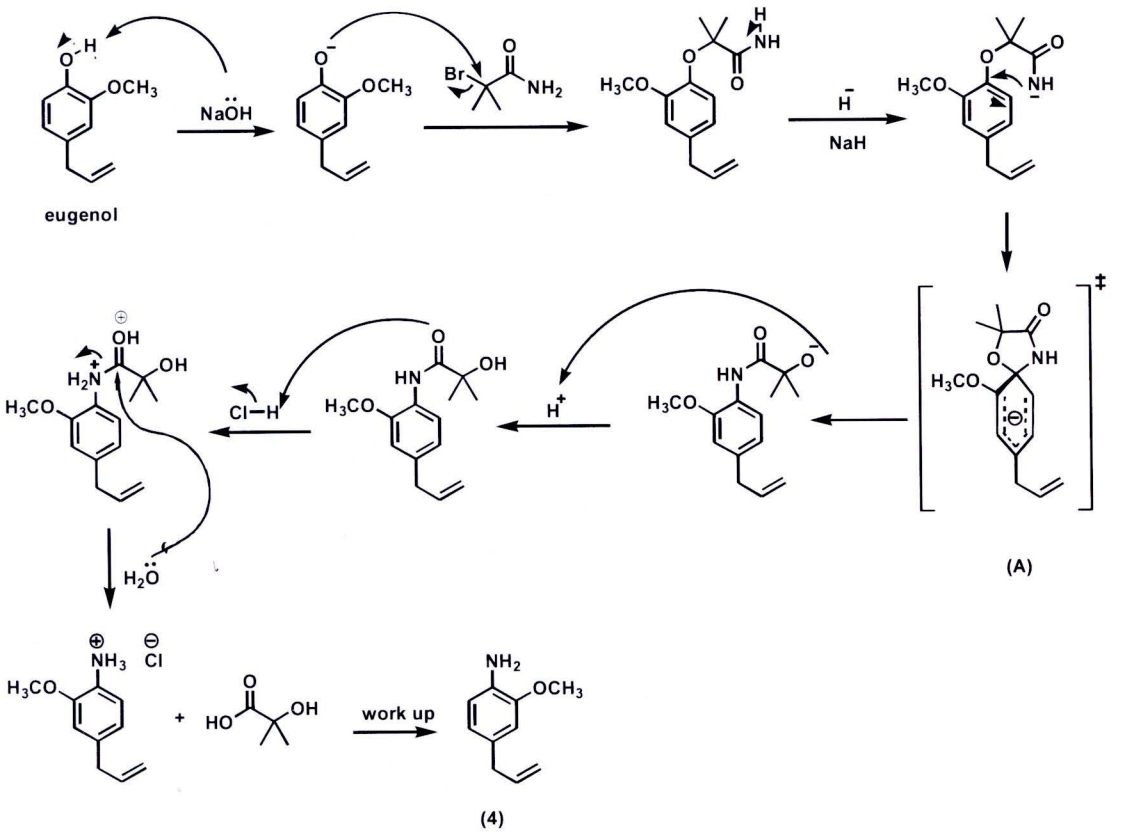
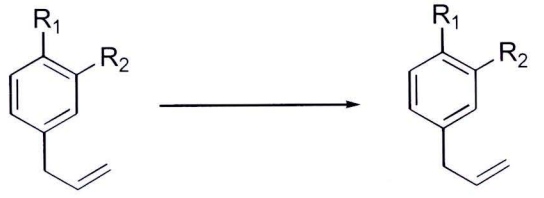


Figure 34 Mechanism of Smiles rearrangements to give 4-allyl-2-methoxybenzenamine (4)

5. Synthesis of 4-allyl-1-bromoanisole (6)and4-allyl-2-bromoanisole (15)

The synthesis of 4-allyl-1-bromoanisole (**6**) and 4-allyl-2-bromoanisole (**15**) was successfully obtained by bromination reaction according to the literature modification. In the Table 14, it was shown the optimized conditions for the synthesis of compound **6** and **15**. The use of *N*-bromosuccinimide (NBS) in variety solvents (entry1-3) such as dimethylfomamide, acetonitrile, and tetrahydrofuran was not provided the desired product as expected. Then, the selective catalyst such as *iso*-propylamine was used for induced the electrophilic substitution at *ortho*-phenol derivatives from the literature review of Fujisaki S. and co-worker [53] was undertaken as shown the Figure 36. The mixture of *N*-bromosuccinimide and diisopropylamine generate an intermediate; *N*-bromo-*N*-isopropylpropane-2-amine, this intermediate can be achieved hydrogen bonding interaction between nitrogen and hydrogen atom of hydroxyl group. Then seemly the substitution can occurred

at*ortho*position of phenol derivatives. However, the experimental in our hands with allylanisole (entry 4,5) cannot formed the product because methoxy group (OCH_3) did not form favorable hydrogen bonding interaction with catalysts.



R_1 = H, R_2 = OCH_3 ; 3-allylanisole **R_1 = Br, R_2 = OCH_3 ; (6)**

R_1 = OCH_3, R_2 = H ; 4-allylanisole **R_1 = OCH_3, R_2 = Br ; (15)**

Figure 35 Synthesis of 4-allyl-1- bromoanisole (6) and 4-allyl-2-bromoanisole (15)

Table 14 Optimization of conversion of 4-allyl-1- bromoanisole (6) and 4-allyl-2-bromoanisole (15)

Entry	Reagent	Catalyst	Solvent	T (°C)	t (h)	%yield
1	NBS	-	DMF	0 -35	24	NR
2	NBS	-	MeCN	35	24	NR
3	NBS	-	THF	0-35	24	NR
4	NBS	$(i\text{-Pr})_2NH$	CH_2Cl_2	0-35	24	NR
5	NBS	*p*-TsOH	MeCN	35	24	NR
6	HBr	-	DMSO	35	24	NR
7	HBr	CH_3COOH	DMSO	35	24	NR
8	Br_2	$FeCl_3$	MeCN	0-35	12	NR
9	$Py{\cdot}HBr_3$	Zn	-	35-60	48	55 (**6**), 48 (**15**)

Note: NR= No reaction

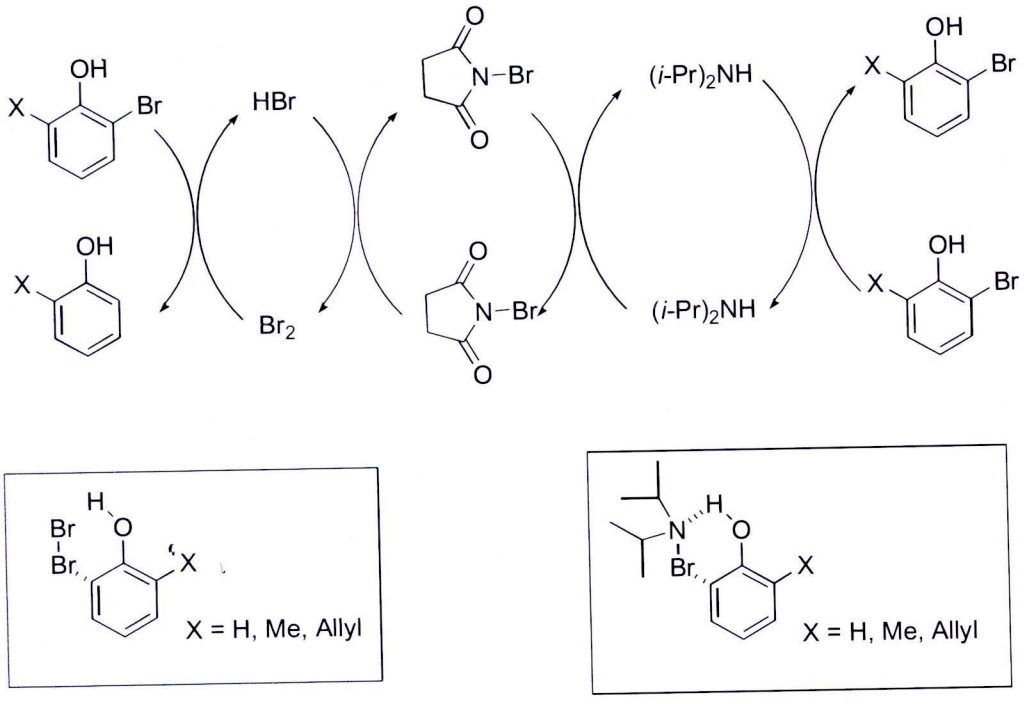


Figure 36 Mechanism of brominationby using diisopropylamine is catalyst

Bovonsombat, P. and co-worker [54] reported the use of regioselectivity catalyst such as *p*-toluenesulfonic acid (*p*-TsOH) for their effectiveness in promoting *para*-substitution selectivity. The hydrogen bond is believed to exist between the acidic hydrogen of *p*-TsOH and the phenol OH group (Figure 37). Since the nearby of the sulfonic acid to the phenol ring, the *ortho* position is effectively blocked to any approaching reagent such as NBS. The halonium-donating species are themselves large groups that could impose additional steric blocked to the *ortho* position of phenol. However, the experimental in entry 5, it gave not the desired product. It might be that the basic and acidic condition could not work in allyl species.

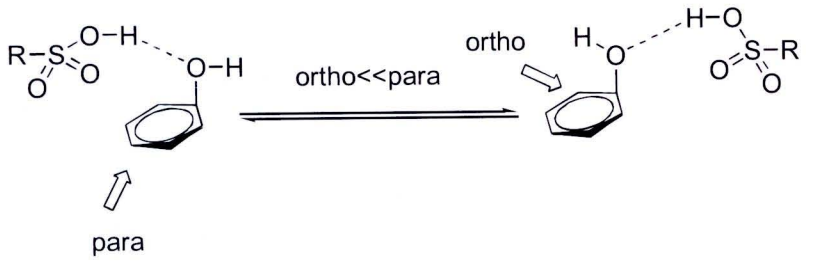


Figure 37 Regioselectivities of bromine by using *p*-toluenesulfonic acid

After that, aqueoushydrobomic acid (HBr) in dimethyl sulfoxide (DMSO) was used instead in entry 6.[55] The following mechanism shows the formation of bromosulfonium bromide from DMSO and HBr *via* dimethylsulfonium bromide (Figure 38). The bromination of **6** and **15** with aqueous hydrobromic acid and dimethyl sulfoxide was not gave the desired product as well. However, the use of acetic acid as a co-solvent for enhances the reaction rate (entry 7) and basic catalyzed condition by using iron trichloride ($FeCl_3$) in acetonitrile (entry 8) still was not provided any the product.

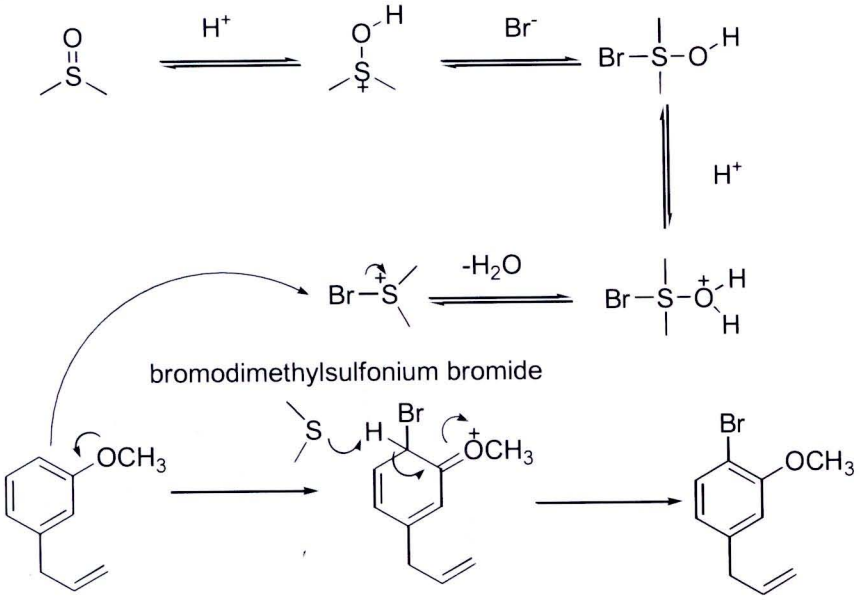


Figure 38 Mechanism of bromination *via* dimethylsulfonium bromide

The experimental by using 2.8 equiv of pyridinium hydrobromide perbromide instead of 2.4 equiv as previously reported, 4-allyl-1-bromoanisole (**6**) and 4-allyl-2-bromoanisole (**15**) could be obtained with 55 and 48% yield respectively after further debromination by zinc (entry 9).[39] The mechanism is dividing in to two steps; first, alkene group was attacked by bromine to give dibromo intermediate together with electrophilic aromatic substitution by bromine at the *ortho*-position. Next step is the debromination of dibromo intermediate with zinc catalyst as shown in the Figure 39.

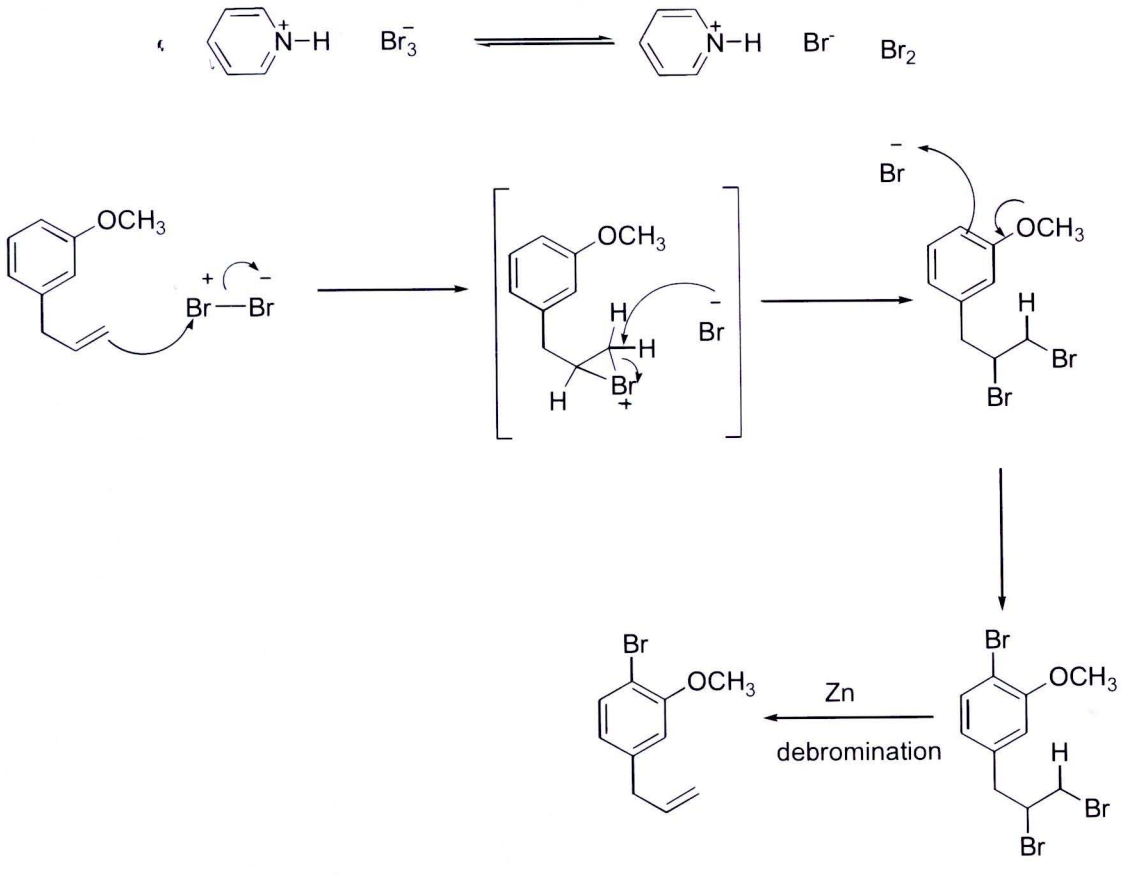
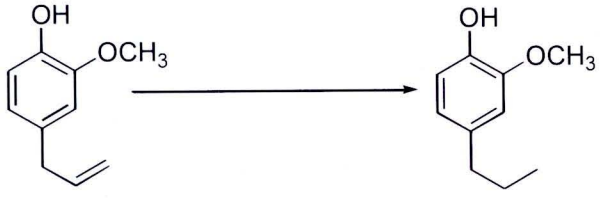


Figure 39 Mechanism of bromination *via* pyridinium hydrobromide perbromide

6. Synthesis of 2-methoxy-4-propylphenol (PMP) (17)

The synthesis of 2-methoxy-4-propylphenol (PMP) (**17**) was shown in Figure 40. Simple hydrogenation reaction of eugenol (**20**) by palladium in trifluoro acetic acid (TFA) under H_2 atmosphere[31] was performed resulting of reduction of allyl group on eugenol **20** to give PMP in 82% yield.



(20) **(17)**

Reagents and Conditions; Pd, TFA, H_2, rt, 24 hr.

Figure 40 The synthetic scheme of-methoxy-4-propylphenol (PMP) (17)

The mechanism of hydrogenation reaction of alkene was divided into three steps (Figure 41). The first step, hydrogen molecules react with the metal atoms at the catalyst surface. The relatively strong H-H sigma bond is broken and replaced with two weak metal-H bonds. Next step, the pi bond of the alkene interacts with the metal catalyst weakening the bond. A hydrogen atom is transferred from the catalyst surface to one of the carbons of the double bond. In the last step, the pi bond of the alkene interacts with the metal catalyst weakening the bond. A second hydrogen atom is transferred from the catalyst surface forming the alkane.[56]

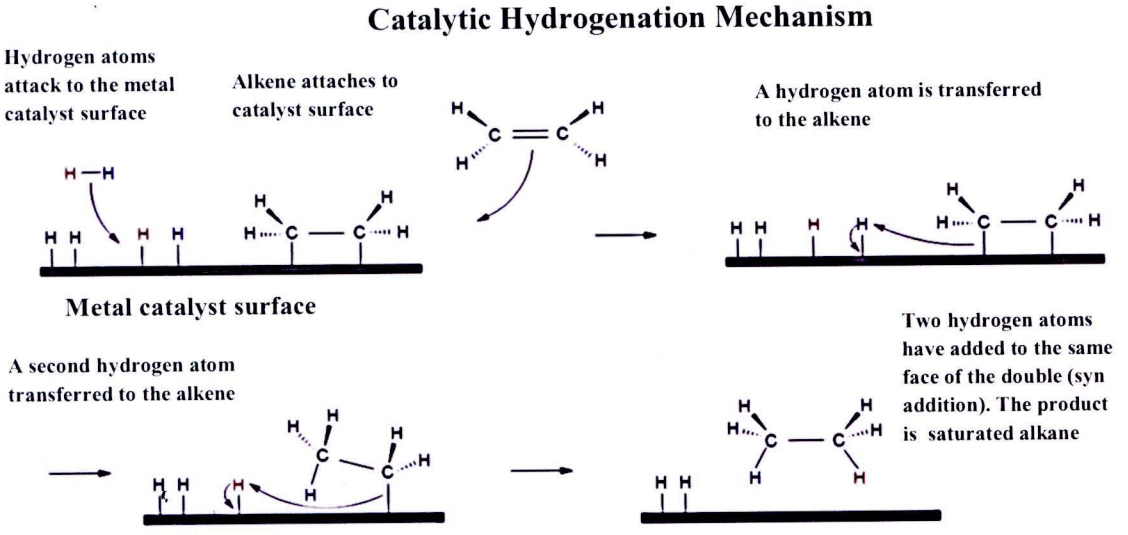
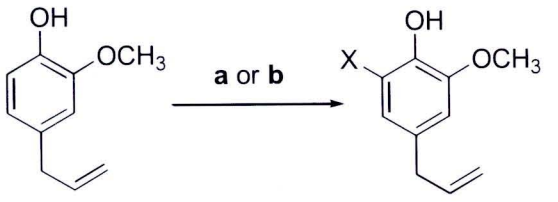


Figure 41 Mechanism of catalytic hydrogenation mechanism [57]

7. Synthesis of 4-allyl-2-bromo-6-methoxyphenol (bromoeugenol) (18) and 4-allyl-2-methoxy-6-nitrophenol (nitroeugenol) (19)

Electrophilic aromatic substitutions reaction at the *ortho*-position of eugenol such as nitration and bromination can be provided the bromoeugenol and nitroeugenol in moderate yield. The bromination of eugenol (Figure 42) was initially carried out using excess *N*-bromosuccinimide (NBS) in dimethylformamide (DMF) at 80 °C [58-60] afforded the desired compound **18** in 48 % yield. Synthesis of 4-allyl-2-methoxy-6-nitrophenol (**19**) *via* nitration reaction [61] of eugenol using urea nitrate gave the desired product in 48 %yield.



(20) **(18,19)**

X= Br (**18**), NO_2 (**19**)

Reagents and Conditions; a) NBS, DMF, 0 °C, 0.5 h (**18**) b) urea nitrate, acetic acid, 60 °C, 24 h (**19**)

Figure 42 The synthetic scheme of 4-allyl-2-bromo-6-methoxyphenol (bromoeugenol) (18) and 4-allyl-2-methoxy-6-nitrophenol (nitroeugenol) (19)

Interestingly, bromoeugenol **18** was successfully synthesized by the using NBS, whereas the synthesis of **6** and **15** was not succeeding with the same condition. It was due to the more electron-releasing group from hydroxyl moiety of compound **20** could lead to appropriate electronic effect in the bromination reaction with NBS.

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Preliminary anesthetic activity test in aquatic animals

This study was performed to demonstrate that the synthesized eugenol derivatives (**1-20**) comparing with the standard (Tween 80/Span 80; **CT**; control) can be positively affected to anesthesia and supported the live transportation for the surrogate aquatic animals. The anesthetic test was divided into 3 parts as operated in *L. vannamei, L. calcarifer* and *C. macrocephalus* respectively.

(A) (B) (C)

Figure 43 (A) *L. calcarifer*, (B) *L. vannamei* and (C) *C. macrocephalus*

1. Anesthetic test in Post larvae and adult *L. vannamei*

The procedure of both experiment in post larvae (PL) and adult was done in the same manner as explained in chapter III. And the results indicated that the eugenol derivatives number **1**, **6**, **8**, **9**, **10**, **13**, **15** and **17** (Figure 44) were the good representative groups. Their activities were effective than those of the other derivatives and control in terms of shorter period of anesthesia, longer period of recover and high Survival rates after recovery (Figures 45, 46 and 47). However, the derivative **8** was the most considerably effective in both ages of *L. vannamei*.

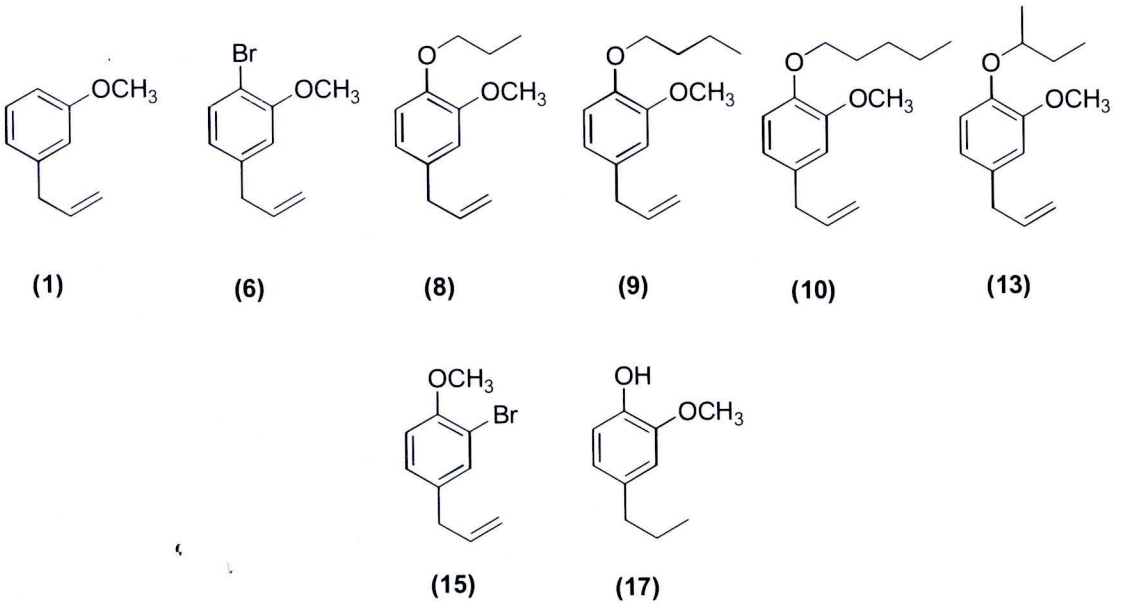

Figure 44 Chemical structures of the most effective eugenol derivatives for post larvae and adult *L.vannamei*

It was found that the most derivatives had the efficiency to anesthesia in this post larvae and adult *L.vannamei*. It was implied that the important active size should be on the allyl group and aromatic of eugenol when considering from overall structure of effective derivatives. The modification of the hydroxyl group in eugenol by displacement of hydrogen (**1**), H-acceptor group (**6**) and alkyation of hydroxyl group (**8**, **9**, **10** and **13**) has a tendency to be still anesthesia in *L.vannamei*.

The derivative **8** was the most effective on post larvae and adult *L. vannamei* compared to eugenol**20**. The post larvae *L. vannamei* showed the introduce time within 7.5 and 4.3 min (Figure 45), time of recovery 4.3 and 3.3 min (Figure 46), and 100% of Survival rates (Figure 47) at 25 and 50 ppm, respectively. And the adult *L. vannamei* showed the introduce time within 4.3 and 4.4 min (Figure 48), time of recovery 39.0 and 40.2 min (Figure 49), and 100% of Survival rates (Figure 50) at 25 and 50 ppm, respectively.

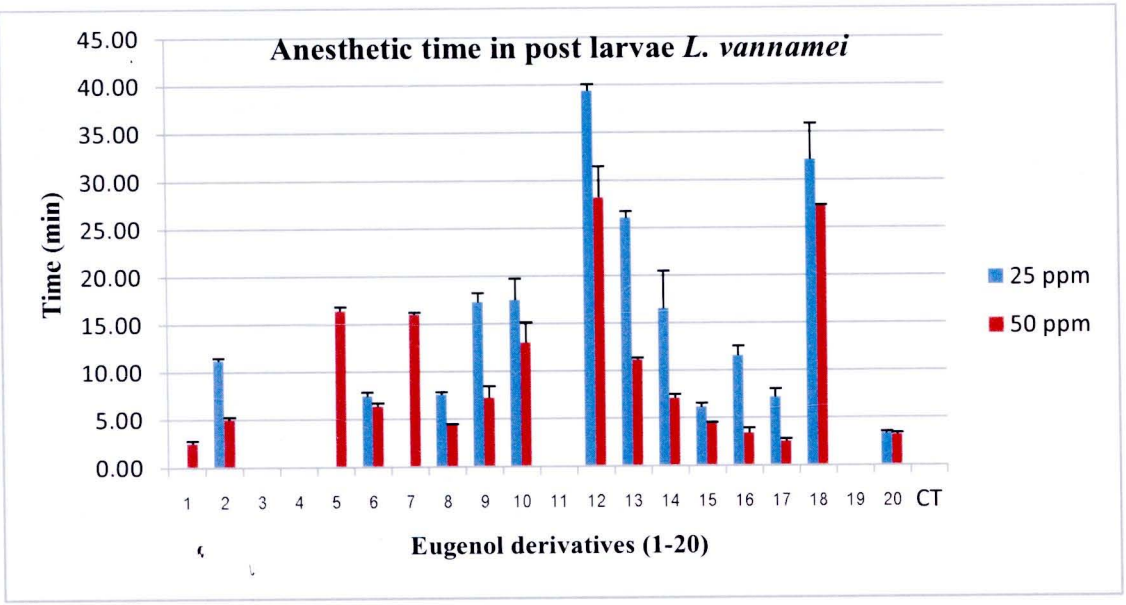

Figure 45 Anesthetic period of Post larvae *L. vannamei* using eugenol derivatives at 25 and 50 ppm

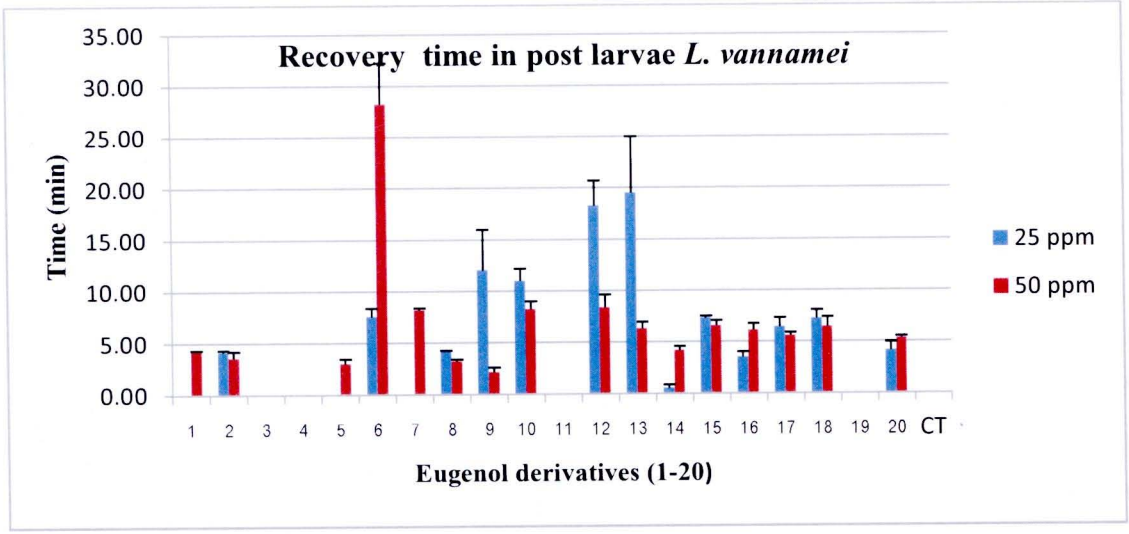

Figure 46 Recovery period of Post larvae *L. vannamei* after anesthetic to stage 2 using eugenol derivatives at 25 and 50 ppm

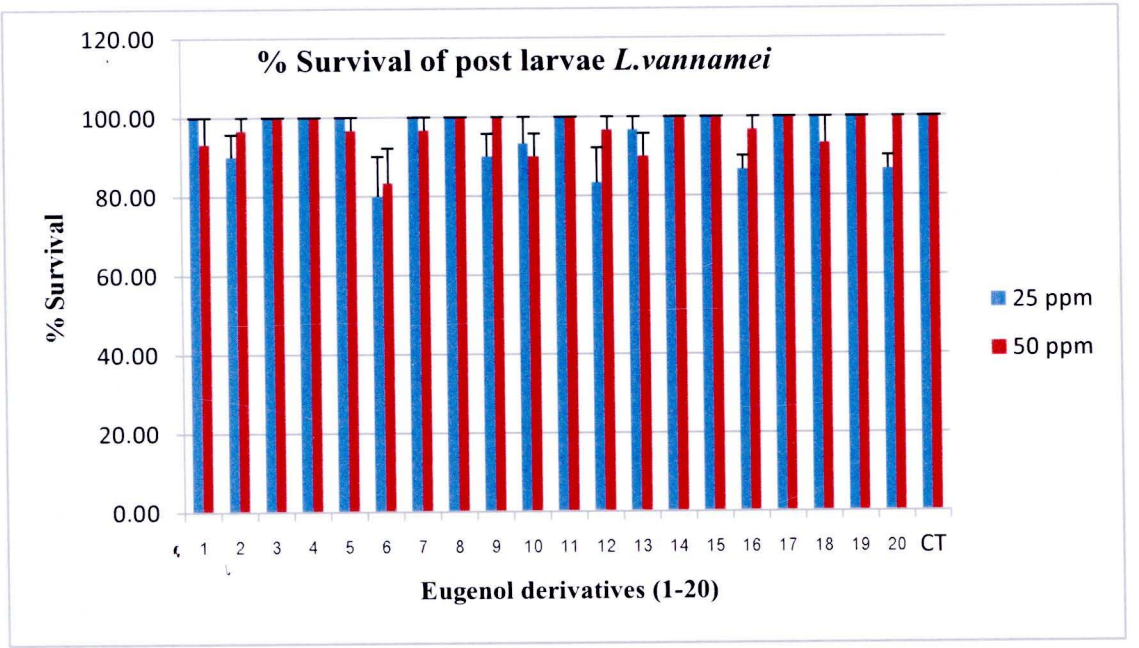

Figure 47 Survival rates of recoverable Post larvae *L. vannamei* after using eugenol derivatives at 25 and 50 ppm

The Survival rates of post larvae after transportation were not different ($p>0.05$) among using concentrations 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 ppm of derivative **8** and negative control (Table 15).

Table 15 Survival rates of PL *L. vannamei* after transportation for 24 hours using different concentrations of derivative 8

Entry	Concentration of derivative 8 (ppm)	% survival
1	2.0	53.3
2	2.5	40
3	3.0	60
4	3.5	73.3
5	4.0	73.3
6	4.5	66.6
7	5.0	60

Table 15 (Cont.)

Entry	Concentration of derivative 8 (ppm)	% survival
8	5.5	60
9	6.0	20
10	6.5	53.3
11	Negative control	100
12	Positive control	20

Note: Negative control = without derivative **8**, Positive control = at 25 ppm of derivative **8**

At the concentration 3.5 ppm of derivative **8**in the transportation test was the most appropriate concentration for PL *L. vannamei* because of the highest percent Survival rates at 73.3%, this range of percent survival is acceptably compared to negative and positive control (at 2.0-3.0 ppm). Due to the PL *L. vannamei* have anesthesia in stage 1, partial loss of reaction to external stimuli and the long time experiment (24 h) may lead to cannibalism between PL *L. vannamei* themselves, and low percent Survival rates (range 4.0-6.5 ppm). The total loss of reactivity or loss of all reflexes (in stage 4) makes the PL *L. vannamei* gradually die. It is due to the range of this concentration may be too high for post larvae. So the concentration at 3.5 ppm was chosen for used as anesthesia in PL *L. vanname* transportation.

In this thesis study, the transportation of post larvae in plastic bag for 24 h by using the derivative **8** at 3.5 ppm compared with negative control was found that the derivative **8** gave low die rate or high percent Survival rates (98.3%). Therefore, the derivative **8** can be the anesthesia agent in PL *L. vannamei* for 24 h at concentration 3.5 ppm and gave high percent Survival rates (100%).

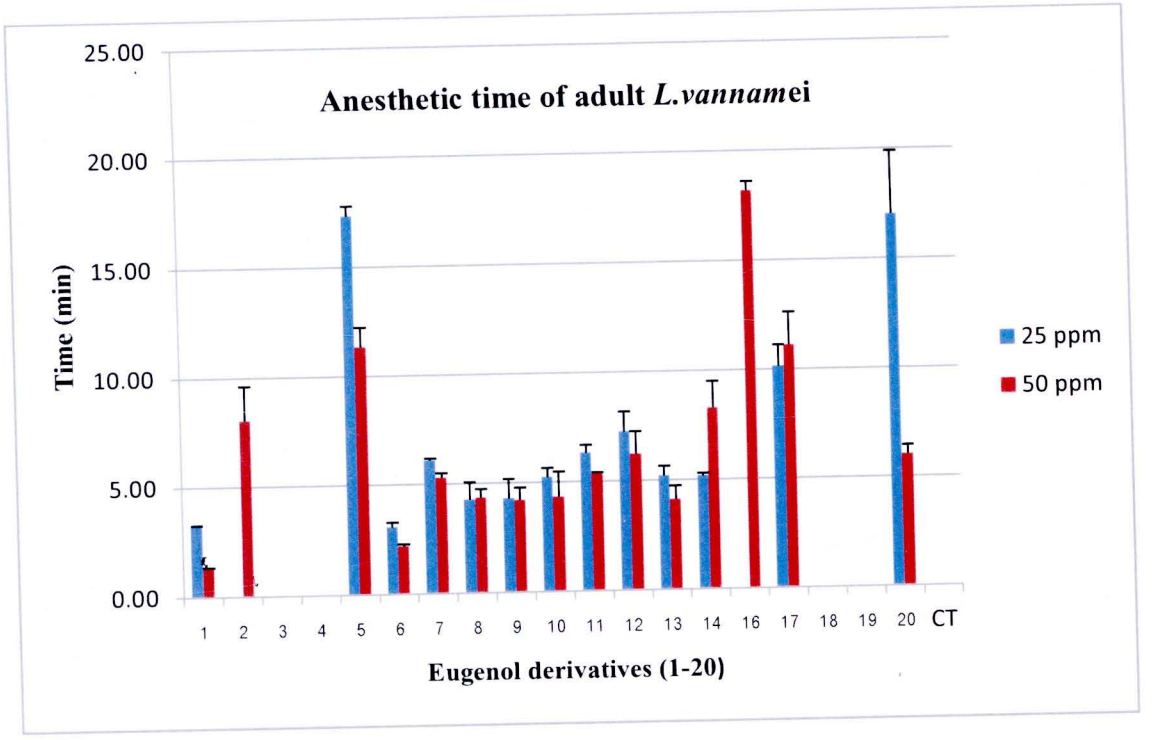

Figure 48 Anesthetic period of Adult *L. vannamei* using eugenol derivatives at 25 and 50 ppm

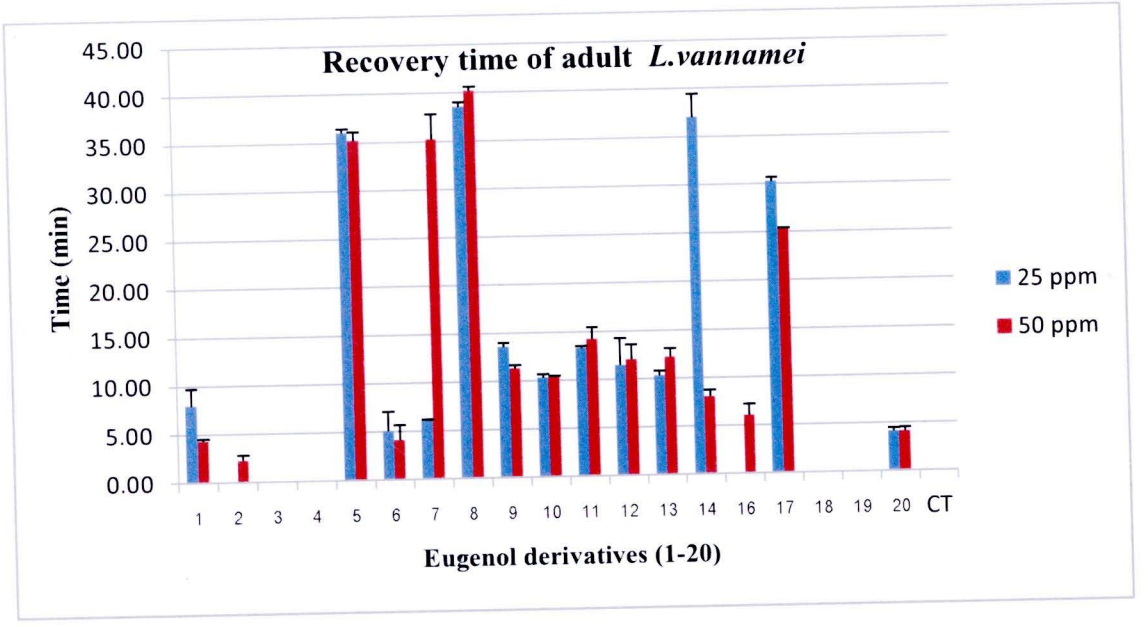

Figure 49 Recovery period of Adult *L. vannamei* after anesthetic to stage 2 using eugenol derivatives at 25 and 50 ppm

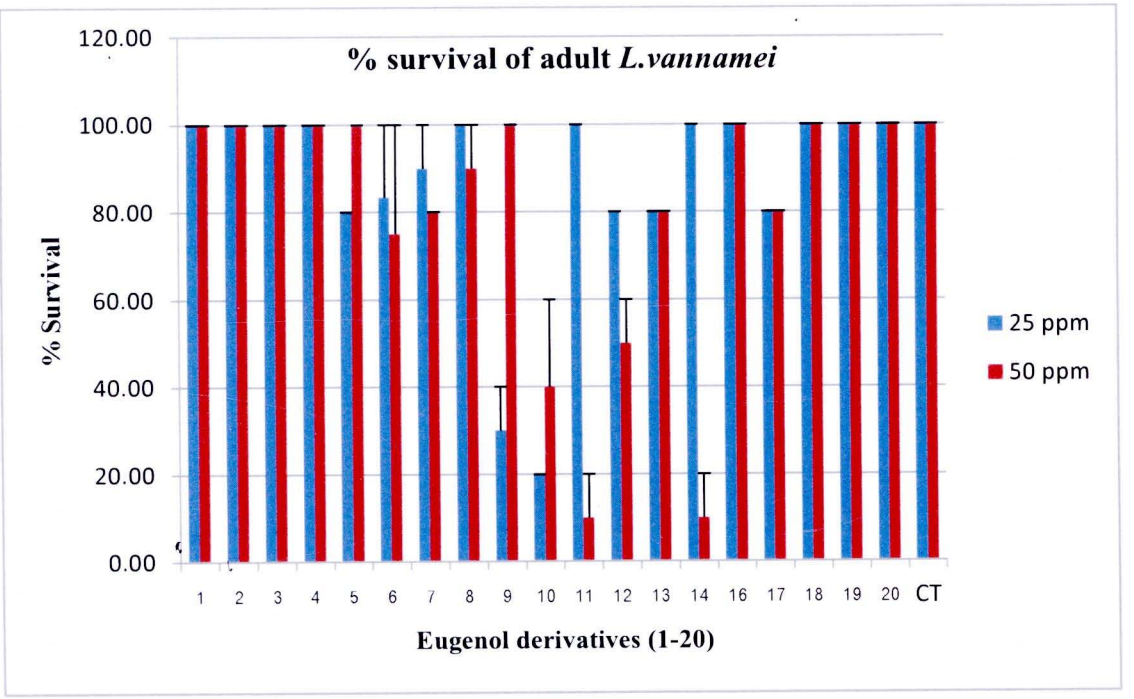

Figure 50 Survival rate of recoverable Adult *L. vannamei* after using eugenol derivatives at 25 and 50 ppm

The survival rate of adult after transportation were not different ($p>0.05$) among using concentrations 2.5, 5 and 10 ppm of derivative **8** and negative control (Table 16).

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Table 16 Survival rate of adult *L. vannamei* after transportation for 24 hours using different concentrations of derivative 8

Entry	Concentration of derivative 8 (ppm)	%survival
1	2.5	83.3
2	5	0
3	10	0
4	Negative control	76.3
5	Positive control	72.3

Note: Negative control = without derivative **8**, Positive control = at 25 ppm of derivative **8**

In Table 16, 2.5 ppm of concentration was the most appropriate concentration because at 5.0 and 10 ppm all of adult *L. vannamei* die, so this concentration was not safe for Adult *L. vannamei*. Finally, concentration at 2.5 ppm was chosen to real test by transportation in plastic bags within 24 h.

The result of transportation in plastic bag within 24 h revealed that adult *L. vannamei* can became anesthesia with derivative **8** at 2.5 ppm of concentration when compared with negative control. Percent Survival rates for 2.5 ppm concentration, positive control and negative control were 85.3± 3.4% 72.7± 2.6% and 73.3± 4.4 respectively ($p<0.05$)(Table 17).

Therefore, derivative **8** can cause anesthesia in Adult *L. vannamei* within 24 h by using the low concentration (2.5 ppm) with high percent Survival rates (85.5%).

Table 17 Survival rate of Adult *L. vannamei* after transportation for 24 hours using different concentrations of derivative 8in plastic bag

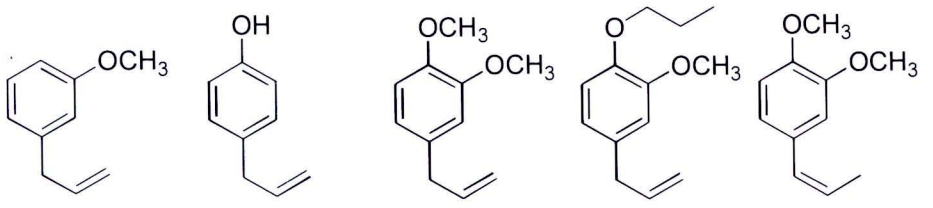
Concentration of derivative 8 (ppm)	%survival
2.5	85.3
Positive control	72.7
Negative control	73.3

Note: Negative control = without derivative **8**, Positive control = at 25 ppm of derivative **8**

2. Anesthetic test in post larvae *L. calcarifer*

The procedure of experiment in post larvae (PL) *L. calcarifer* was done as explained in chapter III. The results shown that the eugenol derivatives **1**, **2**, **5**, **8** and **16** (Figure 51) were the good representative groups. Their activities were effective than those of the other derivatives and control in terms of shorter period of anesthesia, longer period of recover and 100% Survival rates after recovery (Figures 52, 53 and 54). However, the derivative **8** was the most favorable effective candidate as same as in *L. vannamei*.

The comparisons of eugenol **20** with high potential derivative **8** in PL *L. calcarifer*, it was found that the derivative **8** can make PL *L. calcarifer* to anesthesia within 3.1 and 3.0 min (Figure 50), time of recovery at 14.1 and 6.3 min (Figure 51), and Survival rates at 96.6 and 50% (at concentration 25 and 50 ppm), respectively. The derivatives **1**, **2**, **5** and **16** were similar effect to derivative **8**, they had introduce time to anesthesia at 25 ppm at 2.4, 3.4, 1.2 and 0.5 min, respectively, but had short recovery time at 0.2, 0.4, 5.2 and 2.5 min, respectively compared to eugenol **20** and it has low Survival rates with acceptable.



(1) (2) (5) (8) (16)

Figure 51 Chemical structures of the most effective eugenol derivatives for post larvae *L. calcarifer*

The anesthetic result of post larvae *L. calcarifer* was the same trend with post larvae and adult *L. vannamei*. The derivative **1** which replace by hydrogen atom on hydroxyl and derivative **2** which replace by hydrogen atom on methoxy group still can introduce to anesthesia. This demonstrated to the important of allylic aromatic part of these derivatives. For the alkylated derivative with various hydrocarbon chains (**5** and **8**), derivative **8** had a high potential to be anesthesia agent considering from introduce time (Figure 52), recovery time (Figure 53) and survival rate (Figure 54). However, the derivative **16** which modified at allyl group of eugenol can cause anesthetic as well.

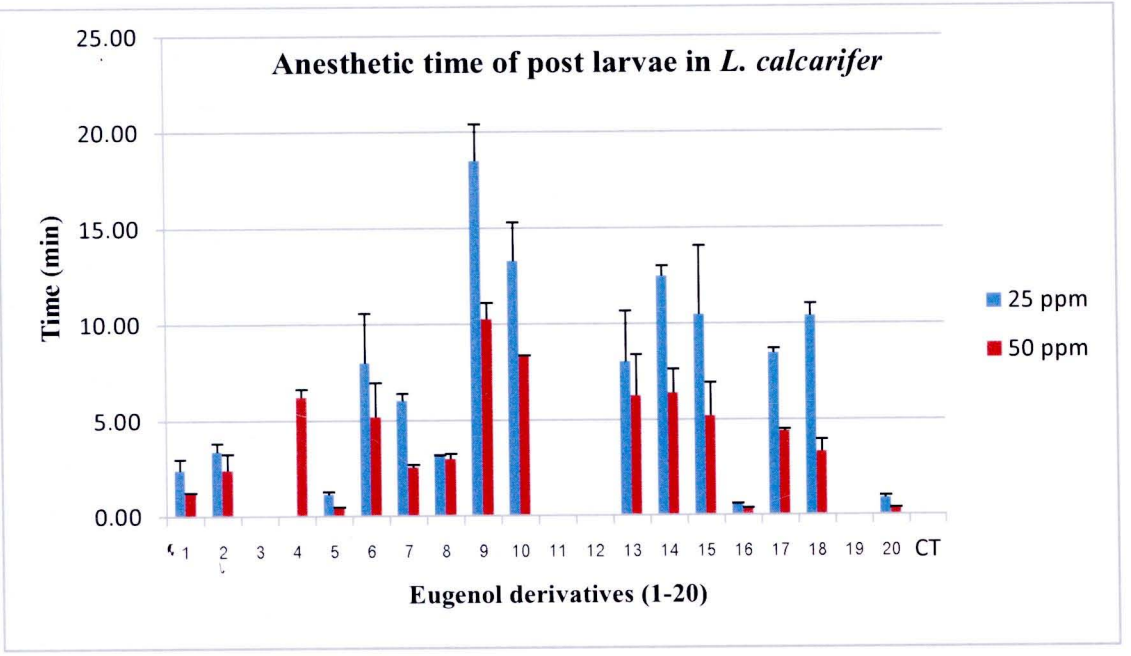


Figure 52 Anesthetic period of post larvae *L. calcarifer* using eugenol derivatives at 25 and 50 ppm

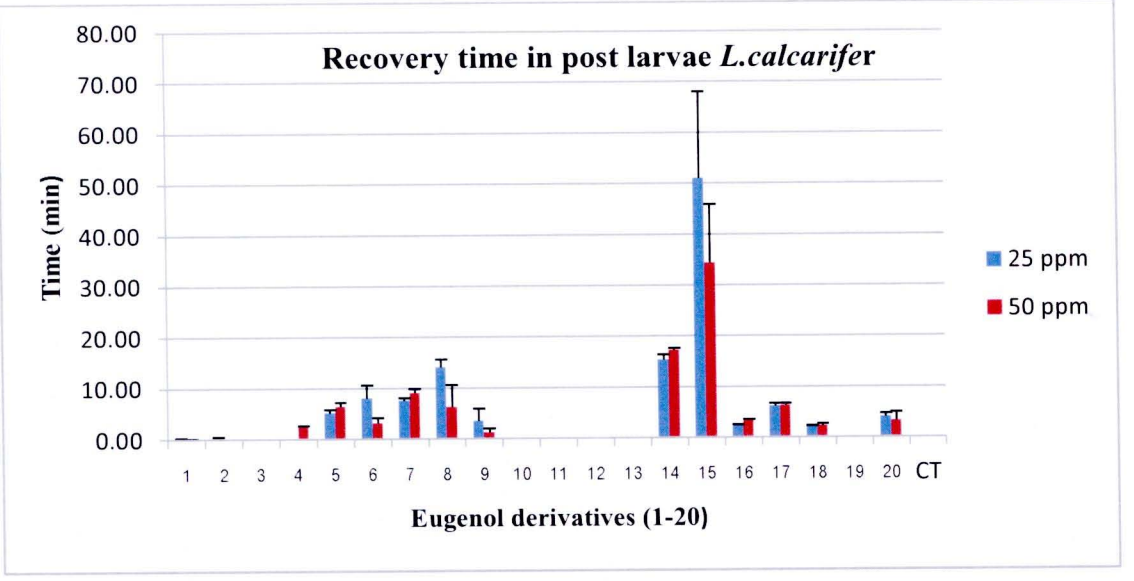


Figure 53 Recovery period of post larvae*L. calcarifer*after anesthetic to stage 2 using eugenol derivatives at 25 and 50 ppm

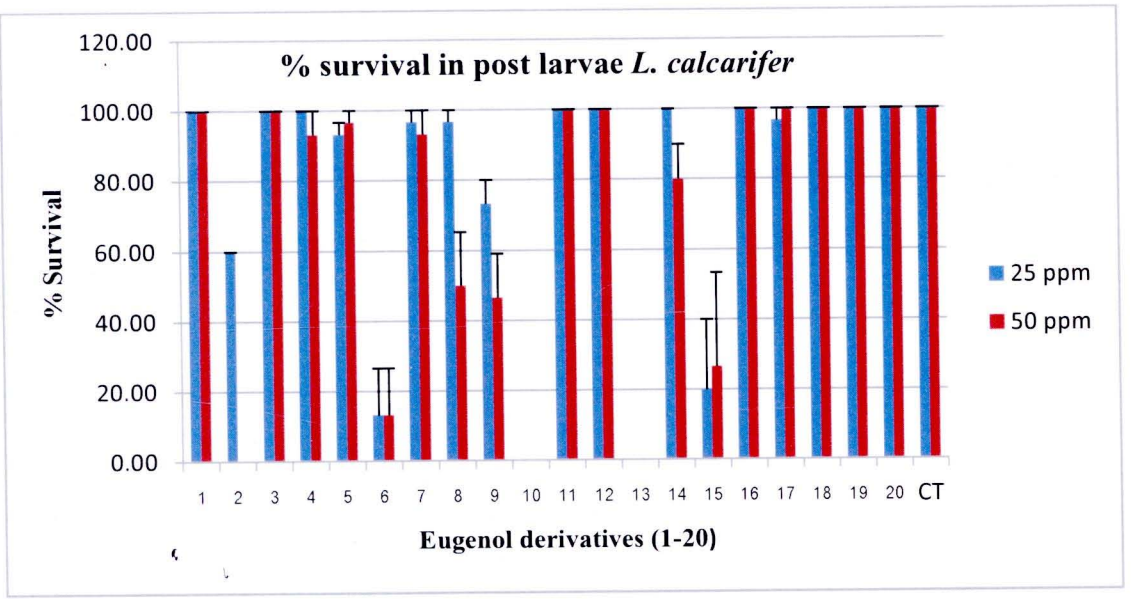


Figure 54 Survival rate of recoverable post larvae *L. calcarifer* after using eugenol derivatives at 25 and 50 ppm

From this experiment, it can be concluded that derivative **8** is an effective candidate for post larvae *L. calcarifer* anesthetic. Derivative **8** was chosen to study the concentration level that compared with negative control within 24 h.

The effect of concentration of derivative **8** on PL *L. calcarifer* can be concluded that at concentrations 4.5, 5.5 and 6.5 ppm is not different to negative control ($p>0.05$) and at 2.0, 3.0, 3.5, 4.0, 5.0 and 6.0 ppm ($p>0.05$) similar to positive control. (Table 18)

Table 18 Survival rate of post larvae *L. calcarifer* after transportation for 24 hours using different concentrations of derivative 8

Entry	Concentration of derivative 8 (ppm)	%survival
1	2.0	93.3
2	2.5	100
3	3 .0	86.6
4	3.5	80
5	4.0	86.6
6	4.5	46.6
7	5.0	86.6
8	5.5	40
9	6.0	73.3
10	6.5	66.6
11	Negative control	70
12	Positive control	100

Note: Negative control = without derivative **8**, Positive control = at 25 ppm of derivative **8**

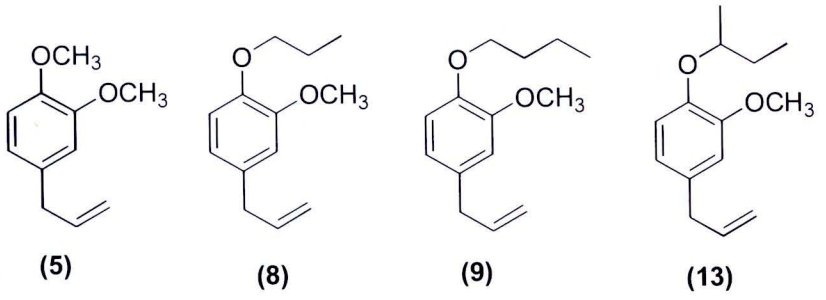
At concentration 2.5 ppm of derivative **8** was the appropriate concentration for PL *L. calcarifer* because the percent Survival rates was 100%. Due to PL *L. calcarifer* have anesthesia in stage 1 in the long time (24 h), it may cause cannibalism between PL *L. calcarifer*, which effect on percent Survival rates (at 4.5, 5.5 and 6.5 ppm). The total loss of reactivity or loss of all reflexes (in stage 4) makes the PL *L. calcarifer* gradually die. The range of this concentration may too high for post larvae. So the concentration at 2.5 ppm was chosen for used as anesthesia in transportation.

In this study, the transportation of PL *L. calcarifer* in plastic bag for 24 h by using the derivative **8** at 2.5 ppm compared with the negative control (76.6%). It was found that the derivative **8** had low die rate or high percent Survival rates (83.3%). Therefore, the derivative **8** can be anesthetic agent in PL *L. calcarifer* for 24 h at concentration 2.5 ppm and make PL *L. calcarifer* 83.3 percent Survival rates.

The experimental of PL *L. vannamei* and adult and PL *L. calcarifer* revealed that derivative **8** which were *O*-alkylated at –OH group had a tendency to be an anesthetic agent better than other group.

3. Anesthetic test in post larvae and adult *C. macrocephalus*

The procedure of both experiment in post larvae (PL) and adult *C. macrocephalus* was done in the same manner as explained in chapter III. The anesthetic experimental in post larvae and adult *C. macrocephalus* was tested in 25 and 50 ppm of **5, 7-14, 17** and **20** and control (**CT**). It was found that the high potential derivatives of eugenol were compose of compounds **5**, **8**, **9** and **13** (Figure 55) which had a good efficiency for used as anesthesia test in PL and adult *C. macrocephalus*.



(5) (8) (9) (13)

Figure 55 Chemical structures of the most effective eugenol derivatives for post larvae and adult *C. macrocephalus*

The comparisons of eugenol **20** with high potential derivative **8** in larvae and adult of *C. macrocephalus*, it was found that the derivative **8** can make post larvae to anesthesia within 3.0 and 2.1 min (Figure 56), period recovery time 37.1 and 43.5 min (Figure 57), and the period of Survival rates is 100% (Figure 58) (at concentration 25 and 50 ppm), respectively. The adult, it was found that anesthesia within 4.3 and 3.2min (Figure 59), period recovery time 31.0 and 40.5 min (Figure 60), and the period of Survival rates is 100% (Figure 61) (at concentration 25 and 50 ppm), respectively.

The anesthetic result of post larvae and adult *C. macrocephalus* was the same trend with *L. vannamei* and *L.calcarifer*. The alkylated derivative with various

hydrocarbon chains (**5, 8, 9 and 13**) showed the good anesthetic properties especially derivative **8** which had a high potential to be anesthesia agent considering from introduce time (Figure 56), recovery time (Figure 57) and survival rate (Figure 58).

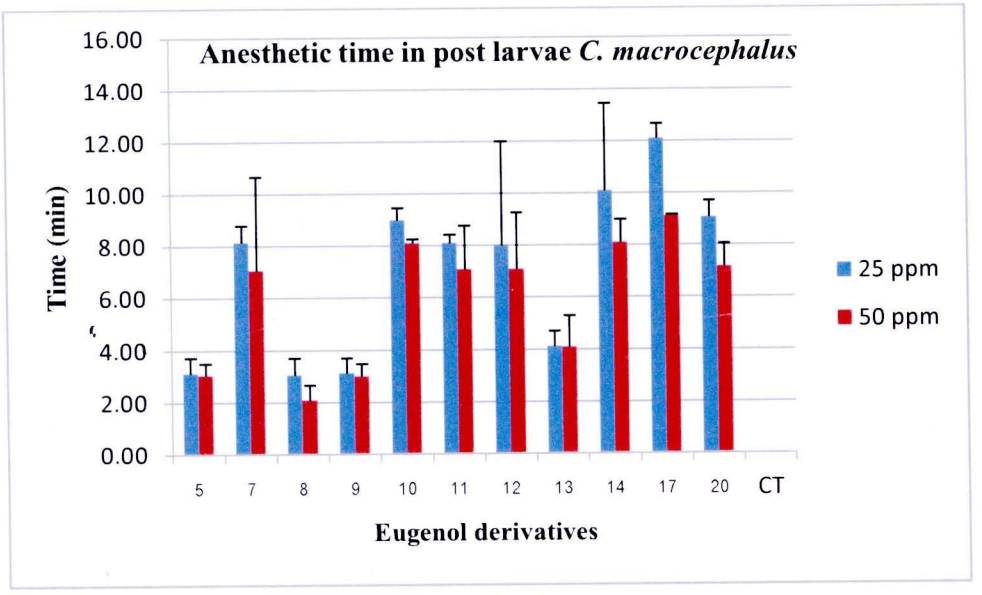


Figure 56 Anesthetic period of post larvae *C. macrocephalus* using eugenol derivatives at 25 and 50 ppm

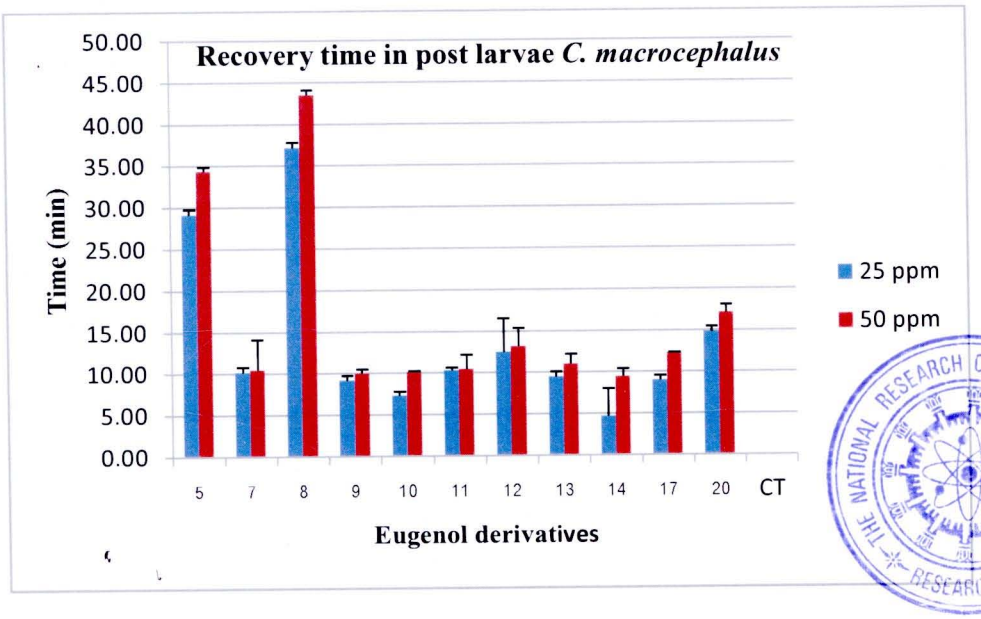


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Figure 57 Recovery period of post larvae *C. macrocephalus* after anesthetic to stage 2 using eugenol derivatives at 25 and 50 ppm

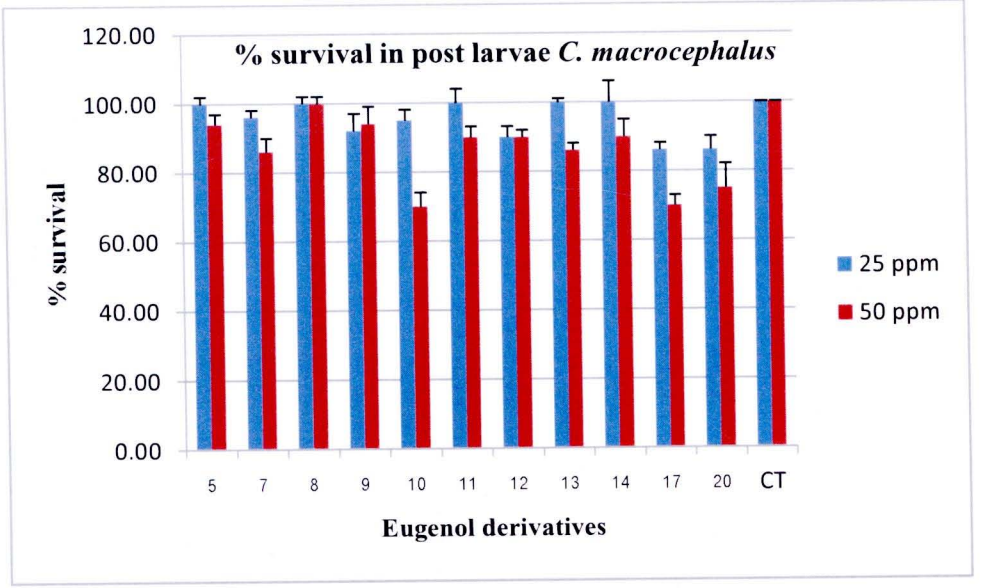

Figure 58 Survival rate of recoverable post larvae *C. macrocephalus* after using eugenol derivatives at 25 and 50 ppm

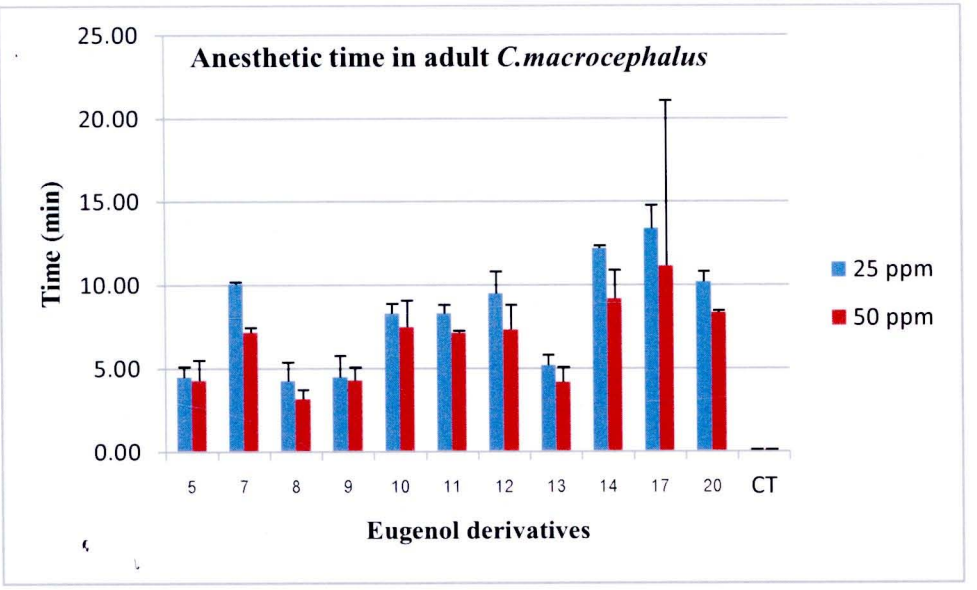

Figure 59 Anesthetic period of adult *C. macrocephalus* using eugenol derivatives at 25 and 50 ppm

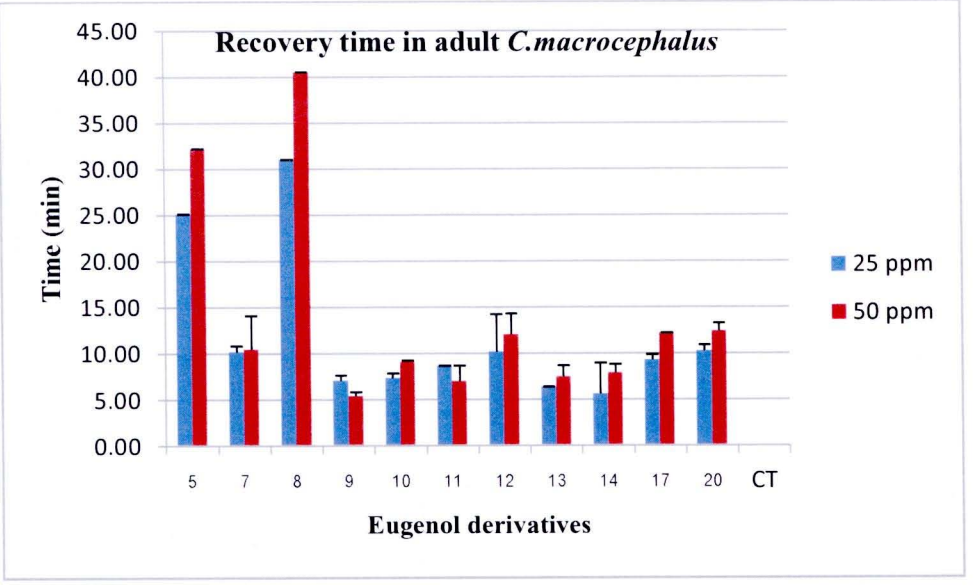

Figure 60 Recovery period of adult *C. macrocephalus* after anesthetic to stage 2 using eugenol derivatives at 25 and 50 ppm

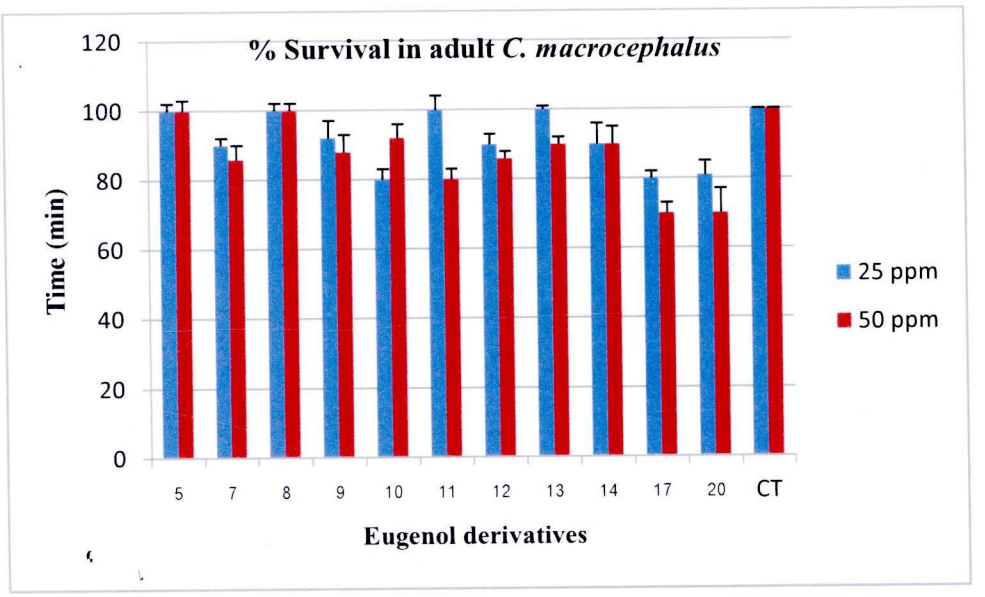

Figure 61 Survival rate of recoverable adult *C. macrocephalus* after using eugenol derivatives at 25 and 50 ppm

In the result of experimental found that derivative **8** which was *O*-alkylated at the –OH group of eugenol **20** (derivatives **5**, **7-14**), was the best anesthesia when compared to eugenol**20** and the same group of other derivative. Both larvae and adult of *C. macrocephalus* are same result that means derivative **8** can faster introduce time to anesthesia, longer recover time and higher percent survival rates than eugenol**20**.

From the experiment, it can be concluded that derivative **8** is an effective candidate for anesthetic. Derivative **8** was chosen to study the concentration level that compared with negative control (without derivative **8**) within 10 h.

The effect of concentration of derivative **8** on the post larvae and adult *C. macrocephalus* can be concluded that at concentrations 10 ppm is not different to negative control ($p>0.05$) (Table 19)

At concentration 10 ppm of derivative **8** was the appropriate concentration for post larvae and adult *C. macrocephalus* because the percent survival rate was 80.6%. Due to post larvae and adult *C. macrocephalus* have anesthesia in stage 2 in the long time (10 h), it may cause cannibalism between the post larvae and adult *C. macrocephalus*, which effect on percent survival rates (at 5 and 20 ppm). The total

loss of reactivity or loss of all reflexes (in stage 4) makes the larvae and adult gradually die. The range of this concentration may too high for the post larvae and adult *C. macrocephalus*. So the concentration at 10 ppm was chosen for used as anesthesia in transportation.

Table 19 Survival rate of post larvae and adult *C. Macrocephalus* after transportation for 24 hours using different concentrations of derivative 8in plastic bag

Try	Concentration of derivative 8 (ppm)	%survival
1	5	63.3
2	10	80.6
3	20	0
4	Negative control	100

Note: Negative control = without derivative **8**

In this study, the transportation of the post larvae and adult *C. macrocephalus* in plastic bag for 24 h by using the derivative **8** at 10 ppm was performed comparing with the negative control. It was found that the derivative **8** had low die rate or high percent Survival rates (100%). Therefore, the derivative **8** can be anesthetic agent for the post larvae and adult of *C. macrocephalus* for 24 h at concentration 10 ppm and make the post larvae and adult of *C. macrocephalus* 100 percent survival rates.

When considering the suitable chemical structure of eugenol derivatives for the correspondance aquatic animals (post larva and adult *L. vannamei*, post larvae *L. calcarifer* and post larva and adult *C. macrocephalus*), it can be concluded that the majority of derivatives except for **3**, **4**, **11**, **18** and **19** were efficient to anesthesia (Figure 28). By considering an important active size in the chemical structure of the anesthetic substances, it was found that the absence of hydroxyl (-OH) and methoxy group ($-OCH_3$) group respectively in the derivatives **1** and **2** which was less affect to anesthesia for aquatic animals. It was implied that the important active size should be

on the allyl part and aromatic ring of derivatives. The disappearances of allyl group in derivative **3** showed less efficient. Therefore, to maintain the efficiency of anesthesia properties, it should be remain the allyl group within eugenol derivatives.

To modified the hydroxyl group in eugenol, the displacement of H-donor group ($-NH_2$) on hydroxy group was investigated, it was found that the derivative **4** was not effect to anesthesia which was possible that H-bonding of amino group cannot appropriately react in size and electronic factor with the neuro-receptor of aquatic animal. On the other hand, at high concentration (50 ppm), the displacement by H-bond acceptor as methoxy group and bromo group (derivative **5** and **6**) could introduce to anesthesia. The methoxy-substitution derivative **5** showed the same tendency as the alkylated derivatives with various hydrocarbon chains (derivatives **7-14**). Derivative **11** which is modification of –OH group by hexyl chain give the different anesthesia properties compared to the other derivatives. Other derivatives can be achieved in anesthesia but they were not in the trend line. It was indicated that the appropriate size chain (between 2-5 carbon chains) on hydroxyl group in derivatives fit with the active size for anesthesia purpose. Derivative **8** is the most effective agent on aquatic animal when considering from anesthetic and recovery periods and percent survival rates. While the derivatives **15**are displacements of methoxy group of eugenol with H-acceptor and the derivatives **16** and **17**are modification of allyl group of eugenol respectively, it found that, they had efficiency for anesthesia due to the similar structural size to eugenol. The derivatives **18** and **19** were adding of eugenol with bromo- and nitro- groups, the derivative **18** have more efficiency than derivative **19** (50 ppm). Therefore, the addition of bromo group into aromatic ring of eugenol had more effective than nitro group but resulting in more toxicity.

As a result above, the derivatives **8** were the most effective on the fast introduce time for anesthesia, long time of recovery and 100% survival rates compared to the other derivatives and eugenol. Moreover it is easy to synthesis, low cost and high percent yield. In this thesis study, the derivative **8** was chosen as a best of choice anesthesia agent for transportation of aquatic animal.