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Synthesis of Copolymer Eugenol-Trithiol-Divinylbenzene via Photoinitiated Cross-linking Reaction as Antibacterial Compound

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Abstract. The Copolymer networks derived from eugenol and divinylbenzene was carried out through the photoactivated thiol-ene reaction. Divinylbenzene with two vinyl groups indicated can be a good cross-linker to form copolymer compound. Meanwhile, eugenol the main material from the copolymer is being known to have an excellent potential of antibacterial activity. The photoactivated thiol-ene reaction is a fast and straightforward method to prepare new material with advanced physical and chemical properties as an antibacterial agent. The mixtures were irradiated with UV light and produce a transparent resin film. The thiol-ene reaction of copolymer network E-3T-DVB was successfully carried out as seen from FTIR spectra where the intensity of the -SH group in 2577 cm^{-1} and C=C group in 1657 cm^{-1} decreased after being irradiated by UV light. The presence of copolymer product was characterized using FTIR, TGA, and tensile strength to obtain the physical and chemical properties of the copolymer. The antibacterial activity of the copolymer network E-3T-DVB was investigated against *Escherichia coli* and *Staphylococcus aureus* by turbidimetric method. The copolymer was more effective in inhibiting *E. Coli* than *S. Aureus*, with the perform inhibition of bacterial growth from *E. Coli* and *S. Aureus* of 72.9% and 0.6%, respectively for 12 h incubation time, while for 24 h incubation time, the copolymer ability to inhibit the bacterial growth of *Escherichia coli* and *Staphylococcus aureus* are 9.3% and 47.1%, respectively.

INTRODUCTION

The copolymer materials with environmentally friendly properties, long-term sustainability, and wide bioactivity are still being improved and have gained widespread attention [1]. One of these copolymers is a copolymer made from eugenol. Eugenol is well-known to have versatile bioactivity [1]. One method of forming copolymers from eugenol is to use a thiol-ene polymerization reaction [2]. Thiol-ene polymerization is the polymerization reaction between double bond carbon from “ene” compound that reacted radically with thiol monomers, result in cross-linked bond thiol-ether between the monomers [2]. This reaction is regioselective [3], high reaction rate allows the development of a more homogeneous network structure [4] and not inhibited by the presence of oxygen which can slow down the polymerization process, need a simple condition of reaction, even can be carried out with or without solvent reaction [5].

Thiol-ene reactions can be catalyzed using metals, oxidants, bases, or light [3]. In the presence of light, thiol-ene photoinitiated polymerization is known to take place through the mechanism of free radical formation followed by propagation through chain transfer to thiol [6]. Termination of the thiol-ene reaction occurs by bimolecular

recombination between the two radicals. This reaction allows the use of various types of thiols and ene monomers to produce three-dimensional cross-linked chains [6].

Eugenol (4-allyl-2-methoxyphenol) is one of the phenolic compounds that mostly found in clove oil extracted from the leaves and flower petals of clove plant [7]. This compound has many biological activities, one of its well-known is antibacterial activity against wide range of gram-negative and gram-positive bacteria [8]. Eugenol has an allyl group which allows it to be further modified by thiol-ene reaction to form copolymer [9]. Copolymer of eugenol itself is expected to have better physical and chemical stability but still maintain its bioactivity.

Some sources of thiol that can be used in the thiol-ene reaction are thiol propionate esters, thiol ethers, thiol glycolate esters, alkyl thiols and mercaptant [10]. Trithiol is an alkyl thiol which is versatile in terms of synthetic reactions. Having three -SH groups, trithiol can play a role as an effective source of thiol in copolymerization reactions forming cross-linking with three-dimensional space with good thermal stability and mechanical properties [10].

In the synthesis of copolymers as new materials, both chemical and physical properties and applications can be improved. Divinylbenzene have two vinyl group that potentially act as cross-link. Divinylbenzene in polymer synthesis increases resistance of stress cracking, chemicals, heat distortion, hardness, and strength. This compound also increases the thermal stability of the copolymer formed [11].

In this research, E-3T-DVB copolymer synthesized from mixture of eugenol as antibacterial agent, divinylbenzene as cross-linker, trithiol as monomer and darocur 1173 as photoinitiator. Synthesis of copolymer E-3T-DVB involved thiol-ene reaction where initiation step UV light changes the photoinitiator as reactive radical then reacts with trithiol result thiyl radical [10]. Propagation step, thiyl radical reacts with C=C double bond where the chain transfer occurs and the unpaired electron from the carbon-center radical reacts with another thiol group on the trithiol so that another radical is formed [10], this propagation step became like a cycle. Until it reaches the termination stage where a radical combination occurs [5].

MATERIALS AND METHODS

Materials and Instrument

All materials in the experiments were used without further purification. Trimethylolpropane tris (3-mercaptopropionate) (Trithiol) $\geq 95\%$, eugenol for synthesis, divinyl benzene for synthesis, 2-hydroxy-2-methylpropiophenone 97% (Darocur 1173), aquadest, chloroform, ethanol, ethyl acetate, *n*-hexane, benzene, dimethyl sulfoxide (DMSO) for analysis, alcohol 70%, bacterial stock of *Escherichia coli* and *Staphylococcus aureus*, yeast, pepton and nutrient agar. While the instruments used in this research were hotplate, magnetic stirrer, oven, laboratory glasswares, ose needle, autoclave, shaker, incubator, Laminar Air Flow (LAF), UV Lamp 366 nm 2×8 W, Perkin Elmer 10.4.00 Frontier Fourier Transform Infrared (FTIR), Thermo Scientific™ GENESYS 10S UV-Vis spectrophotometer, Thermogravimetric Analyzer (TG7300) at a temperature range of 50–600°C with a 5 °C heating rate per minute and platinum as the reference material.

Synthesis of Eugenol-Trithiol-Divinylbenzene copolymer (E-3T-DVB)

Synthesis E-3T-DVB copolymer was prepared following the method reported in the previous study [8, 9]. Eugenol (1.5 g; 1.5 mL; 9×10^{-3} mol), DVB (3.5 g; 3.8 mL; 2.7×10^{-2} mol) and trithiol (5.3811 g; 4.5 mL; 1.35×10^{-2} mol) were mixed into a beaker glass. Darocur 1173 (3% wt) was then added as photoinitiator. The mixture was stirred vigorously using magnetic stirrer for 10 minutes at room temperature. The mixture was then poured into a petri dish, irradiated with UV light on 366 nm for an hour at room temperatures then heated for 30 minutes at 60°C. Solubility test of the copolymer was carried out with several solvents. The copolymer formed was characterized by FTIR, tensile strength, Thermogravimetric Analysis-Differential Scanning Calorimetry (TGA-DSC) and antibacterial activity.

Antibacterial Activity of *E. coli* and *S. aureus*

Bacteria Propagation of E. coli and S. aureus

Yeast (0.125 g), pepton (0.025 g) and nutrient agar (1 g) were dissolved in 50 mL aquadest. The mixture was stirred and sterilized for 45 minutes using autoclave with ose needle and reaction tube. Bacteria propagation was done in Laminar Air Flow (LAF). Agar media was poured into reaction tube and placed in an inclined position until

condense. Bacteria colony was planted into media using ose needle and incubated for 24 hours at 37°C. Bacteria stock was then stored in refrigerator.

Preparation of Bacteria Inoculum

Yeast (0.125 g) and pepton (0.025 g) were dissolved in 50 mL aquadest. The mixtures were stirred and sterilized for 45 minutes using autoclave with ose needle. Preparation of bacteria inoculum was done in LAF. Bacteria stock after incubation for 24 hours was then suspended in nutrient medium using ose needle. The suspension bacteria were then incubated for 3 hours in shaker incubator with 125 rpm. Absorbance was measured using UV-Vis Spectrophotometer ($\lambda = 600$ nm) to obtain absorbance of 0.5 McFarland standard solution.

Antibacterial Test using Turbidimetric Method

Bacteria suspension (100 μ L) which has equivalent turbidity with 0.5 McFarland standard solution was taken into each liquid media (0.125 g yeast and 0.025 g pepton in 50 mL aquadest). There were 4 liquid media with two of them as control (negative and positive). Afterwards, the absorbance was measured using UV-Vis spectrophotometer ($\lambda = 600$ nm) as the initial absorbance. The copolymer sample was placed into each nutrient media then incubated in shaker incubator with 125 rpm. The absorbance was measured after 12 hours and 24 hours of incubation. The absorbance data obtained were used to determine the percentage inhibition (% inhibition) of the copolymer. The formula of % inhibition is:

$$\% \text{ inhibition} = \frac{(Ac - As)}{Ac} \times 100$$

Where Ac is the absorbance of negative control and as is the mean absorbance of copolymer sample.

RESULTS AND DISCUSSION

E-3T-DVB copolymer networks was synthesized by thiol-ene reaction with trithiol as a source of thiol, eugenol, divinyl benzene as a cross linking agent and Darocur 1173 as an alpha-hydroxy ketone-based photoinitiator. This procedure is an easy synthetic methodology for the preparation of networks with variable properties as it was previously described [10]. Thiol-ene reaction is a reaction through anti-Markovnikov addition [12].

The E-3T-DVB copolymer formation reaction showed in Fig. 1. The yield of E-3T-DVB copolymer was 76.82%. The formed copolymer has the shape as a colorless thin sheet. The solubility test proof that the E-3T-DVB only partially dissolved in ethyl acetate, chloroform and DMSO, while in other solvents it is insoluble copolymer. The solubility test result showed in the Table 1.

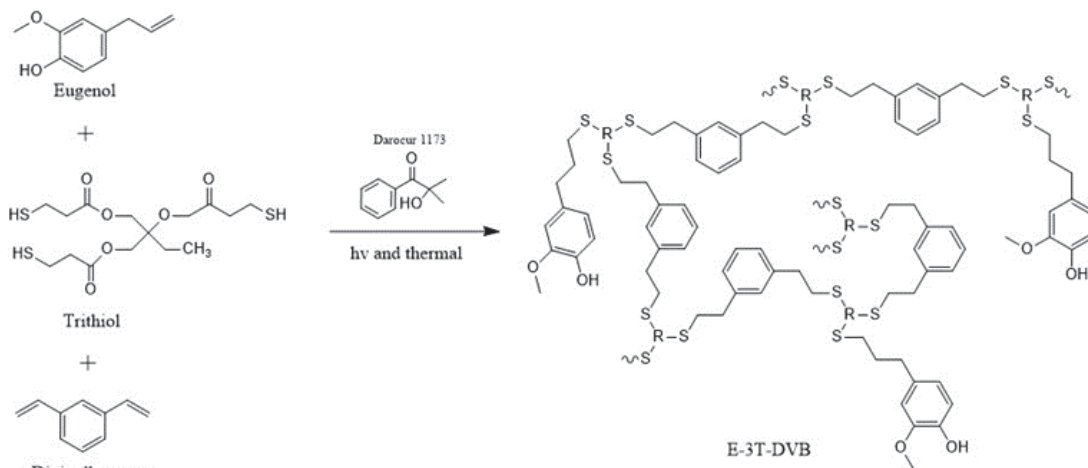
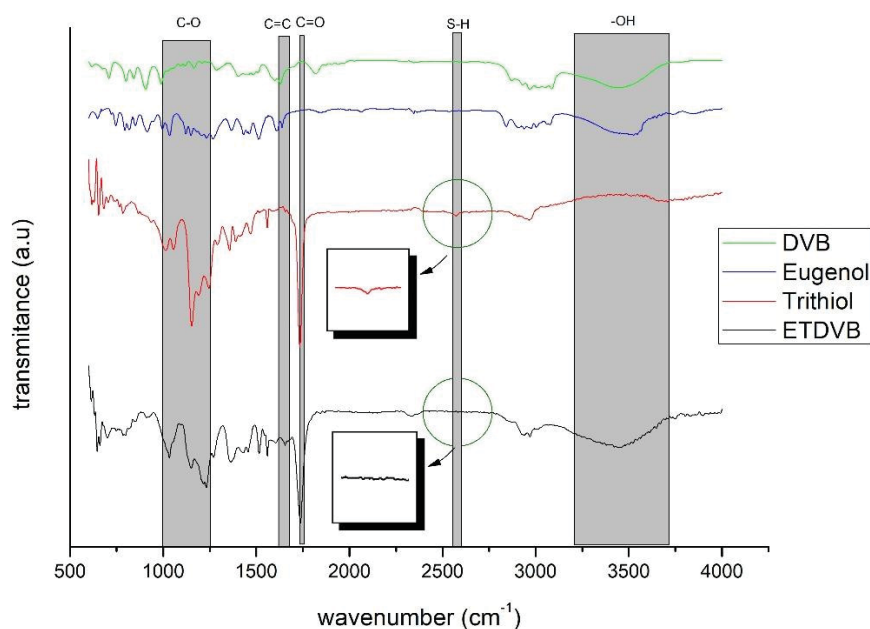


FIGURE 1. The thiol-ene reaction to form E-3T-DVB Copolymer

TABLE 1. The solubility of E-3T-DVB copolymer

Solvent	E-3T-DVB
Aquadest	Insoluble
Ethanol	Insoluble
Ethyl acetate	Partially soluble
Chloroform	Partially soluble
Benzene	Insoluble
<i>n</i> -hexane	Insoluble
DMSO	Partially soluble

Fig. 2 shows the comparison of FTIR spectrum between eugenol, divinylbenzene, trithiol and E-3T-DVB copolymer. Eugenol and divinyl benzene show absorptions in 1631 cm^{-1} and 1640 cm^{-1} as C=C group stretching, respectively. Trithiol shows typical weak absorption in 2577 cm^{-1} as -SH group stretching and strong absorption in 1739 cm^{-1} as C=O ester stretching. The formation of the E-3T-DVB copolymer seen from the absorption intensity of -SH and C=C groups. The formed of the copolymer was confirmed by the decrease of -SH and C=C intensity. after trithiol reacts with eugenol and divinylbenzene to form the copolymer, then the -S-H group on the trithiol will be disappeared due to reaction of S-H group with the functional groups from eugenol and divinylbenzene to form S-C bonding. As seen in FTIR spectrum, the intensity of -SH group of E-3T-DVB in the in range of $2550\text{-}2600\text{ cm}^{-1}$ was disappear. On the other hand, the intensity of C=C group still existed. This is probably happened due to some divinylbenzene that might be attached at the end of polymer chain. The copolymer has λ_{max} of 285 nm as shown in Fig. 3.

**FIGURE 2.** FTIR Spectrum of Divinylbenzene, Eugenol, Trithiol and E-3T-DVB Copolymer

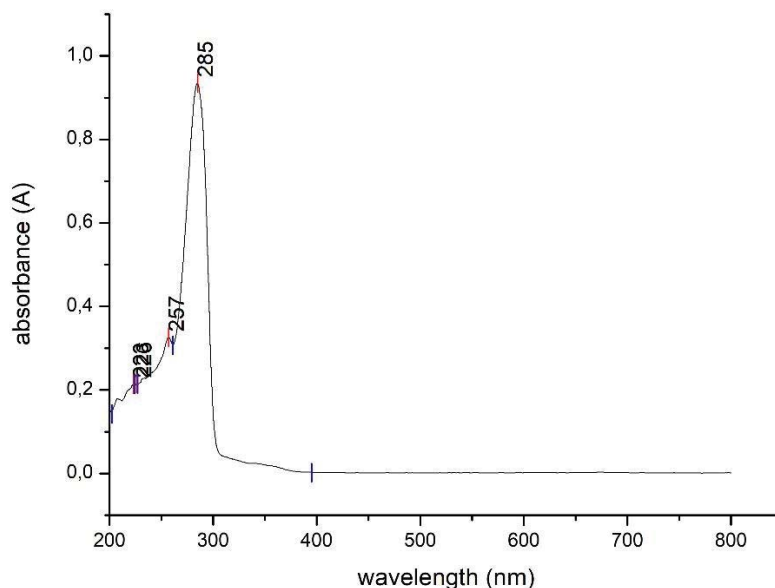


FIGURE 3. UV-Vis Spectra of E-3T-DVB Copolymer

The thermal properties of E-3T-DVB copolymer shown in Fig. 4. It is found that E-3T-DVB the copolymer exhibits four stage decomposition under nitrogen atmosphere. The first stage on the temperature 100-300°C, the degradation on this stage is responsible for weight loss of 3.4%. The weight loss of 3.4% is possible from the degradation of water adhering to the copolymer. The second and the third stages were the main stage in thermal copolymer degradation. In the second degradation stage that occurs at 300-400°C. It is probably caused by the degradation of crosslinked polymer structure. Therefore, there was a very significant weight loss at this stage. Then in the third stage, further degradation happened which produce a shorter carbon chain. The total weight loss in the second and third stages was measured 87.7% of the total weight loss. The third stage (above 500°C) corresponds to the gradual degradation of the char. The DSC curve of the copolymer shows that the copolymer exhibits T_g value at 31°C. The endothermic decomposition process was seen at a peak at 329.5°C with ΔH 36.8 mJ/mg that corresponded to the mass loss from TGA experiment.

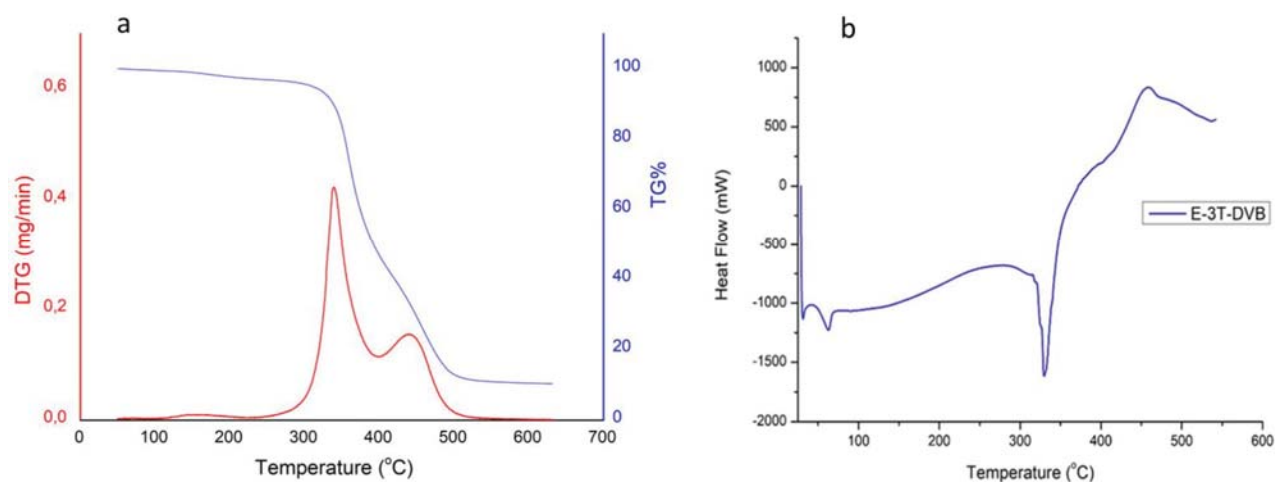


FIGURE 4 (a) TGA and DTG curves of E-3T-DVB Copolymer (b) DSC curve of E-3T-DVB Copolymer

The mechanical properties of E-3T-DVB copolymer was obtained from tensile strength test to show a tensile strength value of 0.164 MPa. The elongation value is rather low (6%) due to the formation of an interconnected network of branching polymer chains [13]. From previous study, the increase in the content of divinylbenzene as a cross-linking agent, will also increase the tensile strength of the copolymer. Cross-linking agent will increase the strength of the polymer network and reduces chain mobility so that the copolymer density increases. Increasing the density of copolymer requires more energy to start the tensile strength process, so the presence of cross-linking agent will increase the main tensile strength of the copolymer [14].

The bacteria used in the antibacterial test are pathogenic bacteria of *S. aureus* (gram positive) and *E. coli* (gram negative). The turbidimetric method was used with the number of colonies for the two test bacteria was 2.5×10^8 CFU/mL. The results obtained is showed in Table 2. The turbidimetric method showed the percent inhibition of bacterial growth with observations at 12 and 24 hours [15].

TABLE 2. The Antibacterial Activity of E-3T-DVB

Bacteria	% Inhibition			
	<i>E. coli</i>		<i>S. aureus</i>	
Incubation Time	12 h	24 h	12 h	24 h
E-3T-DVB (0.15 gr)	72.9	9.3	0.6	47.1
Control + (Ciprofloxacin 4000 ppm)	96.6	99.8	99.3	99.0

In the observation of *E. coli* bacteria at 12 hours inhibited growth by 72.9% while for 24 hours had a percentage of inhibition of 9.3%. At 24 hours of observation there was a decrease in the inhibition of the compound against *E. coli* meaning that after 12 hours the bacteria would grow back marked by the high turbidity produced. Based on these results, it can be said that the copolymer is effective in inhibiting *E. Coli* for 12 hours of incubation. Based on the copolymer structure, the phenol group is able to interact with the lipid bilayer of *E. Coli* bacteria causing damage to the *E. Coli* bacteria wall, but after 12 hours the bacteria can grow back [16]. While against *S. aureus*, the copolymer performs the highest percentage of inhibition of 47.1% at 24 hours. It indicates the ability of the copolymer to inhibit *S. aureus* bacteria of up to 24 hours of incubation. *S. aureus* is a gram-positive bacterium which on the outside of the membrane is teichoic acid and lipoteichoic acid [17].

In gram-negative bacteria, the peptidoglycan layer on the cell wall is thin and surrounded by lipoproteins, lipopolysaccharides (LPS), phospholipids and some proteins [18]. However, when viewed from the results of the percent inhibition of the two test bacteria, the copolymer was more effective in inhibiting *E. Coli* for 12 hours than *S. Aureus*. Gram-negative *E. Coli* bacteria have an outer lipid membrane [18], making them more able to interact with hydrophobic copolymer, so it is predicted that the inhibition mechanism is to damage the cell wall which will cause cell leakage and loss of cell integrity [19]. The bacterial cell wall determines the characteristic shape and functions to protect the inside of the cell against changes in osmotic pressure and other environmental conditions [20]. The presence of the Thiol-ene group of the E-3-T-DVB copolymer derived from the reaction between thiol and DVB also greatly affects the effectiveness as an antibacterial [21]. The presence of a thiol-ene group and an aromatic group increased the hydrophobicity of copolymer which made it effective in inhibiting gram-negative at 12 hours but had the ability to inhibit gram-positive at 24 hours, although not as much as gram-negative. Based on these results, the thiol-ene and aromatic groups interact more quickly with gram-negative and provide greater inhibition than gram-positive.

CONCLUSION

The photoactivated thiol-ene reaction was fast and straightforward with UV light irradiation to help the reaction happened rapidly and no solvent needed. The transparent film copolymer of E-3T-DVB was successfully synthesized and confirmed by FTIR spectra. The copolymer has λ_{max} 285 nm and the yield of E-3T-DVB copolymer was 76.82%. The copolymer has a glass transition temperature at 31°C and resistant to thermal treatment of up to 300 °C. Tensile strength and elongation at break of the copolymer are 0.164 MPa and 6%, respectively. The copolymer was more effective in inhibiting *E. Coli* than *S. Aureus*. The best incubation time for antibacterial activity of E-3T-DVB copolymer was investigated and the result show that the copolymer has antibacterial activity with % inhibition value of 79.2% after 12 hours incubation time against *E. Coli* and 47.1% after 24 h against *S. Aureus*.

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