Synthesis of para-menthane 3,8 - diol from *Eucalyptus citriodora* essential oil for application in mosquito repellent products

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Abstract. To repel mosquitoes, there have been many highly effective chemical repellent products, but they carry the potential risk of being unsafe for humans and harmful to the environment. Insect repellent products derived from natural sources that are safe for humans and environmentally friendly are becoming increasingly popular. PMD is a broad-spectrum and effective insect repellent but is present in small amounts in the *Eucalyptus citriodora* essential oil (EO). In this study, we investigated and evaluated the conditions for synthesizing PMD from EO raw materials in an acidic environment to achieve the highest synthesis efficiency of 76.3%, and the PMD product was 96.4% pure after being converted for 5 hours at 50°C in a 0.25% H_2SO_4 solution. Additionally, the study also separated the two cis and trans isomers of PMD by column chromatography and determined their structures using infrared spectroscopy (IR) and nuclear magnetic resonance (NMR).

1 Introduction

Due to the impact of climate change, the Earth is warming up, leading to increased air and water temperatures, more rainfall and floods, and creating conditions for the development of many disease-carrying insects. Insects (mosquitoes, ticks, fleas, etc.) can become disease vectors when they carry infectious agents such as viruses (causing Dengue hemorrhagic fever, Zika, Chikungunya, etc.), bacteria (causing Lyme disease, bubonic plague, etc.), and parasites (causing Malaria, Leishmaniasis disease, etc.). Each year, there are over 700,000 deaths due to vector-borne diseases, which account for more than 17% of all infectious disease cases [1]. According to the comprehensive analysis by Fan (2014), for every 1°C increase in temperature, cases of dengue fever will increase by 35% [2]. According to the WHO, the actual number of cases of this disease has increased 30-fold in the past 50 years [3].

Mosquitoes are attracted to carbon dioxide and lactic acid present in sweat, as well as the scent produced by the human body, which is detected by chemosensory organs located in their antennae [4]. When an odorant binds to an odorant receptors (ORx), Orco ion channel is opened, ultimately activating sensory nerve cells that detect the scent [5].

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Therefore, ligands and allosteric antagonists targeting ORx and Orco can act as potential repellents by disrupting the insect's olfactory perception. This forms the basis for developing insect repellent products based on olfactory perception - products that have great potential for repelling and fighting insects.

Since World War II, synthetic insect repellent products such as DEET, picaridin, and IR3535 have been proven to be highly effective and widely used. However, they have raised some concerns about environmental and human health risks, especially in children [6], [7]. PMD is a component in EO (*Eucalyptus citriodora* essential oil), has been officially recognized by the Centers for Disease Control (CDC) as an insect repellent compound similar to DEET, and is the only natural product allowed to be used in the US and Europe as a mosquito repellent with no side effects [8]. PMD has a lower vapor pressure and slower evaporation rate than other components in essential oils, therefore providing high efficacy in repelling insects over a broad range, with longer protection time. PMD is effective in repelling various mosquito species such as *Aedes aegypti*, *Aedes albopictus*, *Culex nigripalpus*, *Culex pipiens*, *Anopheles gambiae*, *Ochlerotatus triseriatus*, *Ochlerotatus taeniorhynchus*, as well as other blood-sucking species such as *Haemadipsa sylvestris*, *Haemadipsa picta*, *Stomoxys calcitrans*, *Ixodes ricinus* [7]. PMD exists in 4 stereoisomeric forms in nature: (±)-cis and (±)-trans. Commercial products are formulated as a combination of (±)-cis/(±)-trans isomers in a 2:1 ratio. Barasa (2002) showed that all 4 stereoisomers of PMD were equally effective against *Anopheles gambiae* mosquitoes at any concentration, and the insect repellent activity of each stereoisomer and the mixture of all four stereoisomers were all equivalent [9]. However, the results differ from those reported by Borrego (2021) which demonstrated that among the stereoisomers, (1R)-(+)-cis-PMD had higher repellent index against *Aedes albopictus* mosquitoes compared to other stereoisomers [7].

PMD is a broad-spectrum insect repellent with significant potential as a personal protection measure against insect bites, but is present in small amounts (about 1%) in EO. To synthesize PMD, Yuasa (2000) converted citronellal in 0.25% sulfuric acid at 50 °C for 11 h, giving a conversion of 97.9%, a selectivity of 92.3%. The crude products were crystallized in n-heptane at 50 °C for 20 h to yield PMD with 80% efficiency and high purity [10]. However, citronellal materials are often more expensive and not as readily available as essential oils because of the steps involved in extracting citronellal from essential oils. To limit that, Drapeau (2011) synthesized PMD from EO material by treating it with citric acid in a two-phase medium (water/essential oil). As a result, 82% (+) - citronellal was converted to PMD with a selectivity of 80% [11]. This method is environmentally friendly, but the conversion and synthesis efficiency of PMD is not as high as that of Yuasa's method using sulfuric acid (2000). Therefore, in this study, we used the available EO source in Vietnam containing high citronellal content (72.87%) and treated with sulfuric acid to synthesize PMD.

2 Materials and Methods

2.1. Chemicals

- *Eucalyptus citriodora* essential oil of Vietnam was supplied by Hanoi natural essential oil joint stock company with the main ingredients including citronellal 72.87%, isopulegol 12.02%, citronellol 4.01%.
- Chemicals: para-menthane 3,8-diol 95% (Usolf, China); silica gel 60 (Merck). Citronellal (95%) and other chemicals are in pure form from Sigma-Aldrich.

2.2 Methods

2.2.1. Investigation of the reaction conditions for PMD synthesis

- Based on references [10], [11], experiments were designed to investigate the reaction conditions, including the ratio of acid/EO (ratios of 3:1 and 5:1 by weight), H_2SO_4 concentration (0.1%, 0.25%, and 0.5%), and conversion temperature (30°C, 50°C, and 70°C). The reaction time for synthesis was then studied at 0, 2; 3,5; 5; 8 and 11 hours to determine the appropriate duration.
- PMD synthesis process : Add 25 g of EO into a 250 ml 2-necked flask containing the investigated amount of sulfuric acid and stir well at the investigation temperature for 11 hours. After the reaction is complete, neutralize the mixture with a 10% $NaHCO_3$ solution. Then transfer the entire mixture into a 250 ml pear-shaped flask. Add 42.5 ml of n-hexane, shake well, and let it settle for 10 minutes. Collect the upper layer of the solution. Continue to add 25 ml of distilled water to the obtained extract, shake well and allow to settle for 10 min, collecting the supernatant. Transfer the extract to a 100 ml conical flask. Add 2.5 g of anhydrous Na_2SO_4 and let it stand for 30 minutes to remove any residual water from the organic phase. Then filter the upper layer of the solution into a new 100 ml flask and store it at -25°C for 48 hours to crystallize the product. After 48 hours, discard the upper layer of the solution, collect the precipitate, wash it with 5 ml of n-hexane, and then dry it at 50°C for 7 hours. The crystallized product is weighed and its purity is preliminarily checked using TLC method to select the reaction conditions for high PMD synthesis efficiency.

2.2.2. Purify the cis and trans isomers of PMD

- Use silicagel chromatography column (column size 40 cm x 2 cm) to separate the two isomers of PMD.
- Chromatographic conditions: sample volume 0.50 g, use 50.00 g silica gel 60, the mobile phase solvent system: 4 n-hexane: 1 ethyl acetate (v/v).

2.2.3. Thin Layer Chromatography (TLC) method

Samples were diluted in n-hexane. Using Merck's Silica gel 60 F254 plate, thickness 0.25mm as the stationary phase, and the mobile phase is a solvent system of 4 n-hexane: 1 acetone (v:v). Chromatography was conducted for about 10 min, followed by drying. The spots on TLC plate are detected using a vanillin sulfate reagent, and can be dried at 100°C for about 5 minutes if necessary.

2.2.4. GC-MS method (Gas Chromatography/Mass Spectroscopy)

Analysis of PMD was performed on the Thermo Scientific™ ISQ™ 7000 Single Quadrupole GC-MS system. The GC separation was performed on a TraceGOLD TG-5SilMS column (30 m x 0.25 mm, 0.25 µm, containing 5% phenylmethylpolysiloxane). The helium flow rate was 5 ml/min. The sample injection volume was 1.0 µl. The injection temperature was maintained at 230ºC in splitless mode (0.5 min) and split flow 100 ml/min. The column temperature was set as follows: initial temperature of 40 ºC for 1.5 min, followed by a stepwise increase to 180 ºC at 25 ºC/min, then to 280 ºC at 5 ºC/min, and finally to 300 ºC at 10 ºC/min, with a hold time of 5 min.The MS ionization process was carried out in electron ionization mode. The mass spectrum was obtained at 70 eV. The transfer line and ion source temperatures were set at 280 and 200ºC, respectively. Each

sample was analyzed in triplicate. The Chromelon 7 software was used for data processing and analysis. The cis-PMD and trans-PMD contents in the analysis solution were determined based on the PMD standard curve constructed in the range of concentrations from 40-200 mg/l

2.2.5. Spectral methods.

Infrared spectroscopy (IR) was recorded on an Impac-410 FTIR machine using a KBr crystal disk. NMR spectra, including 1H-NMR, 13C-NMR, HSQC, and HMBC, were measured on a BRUKER AVANCE-500M machine at a frequency of 500MHz, using TMS as an internal standard and DMSO-d6 as the solvent. Chemical shift values were calculated in ppm.

The melting point was determined using a Stuart melting point apparatus.

3 Results and Discussion

3.1. Investigate conditions for PMD synthesis from EO

Citronellal accounts for a large amount of 60-85% in EO, so the study also used EO materials available in Vietnam to synthesize PMD. The survey results of acid/EO ratio, H_2SO_4 concentration and temperature give the results in Table 1.

Table 1. Survey results of PMD synthesis reaction conditions

Experiment	Ratio of acid/EO	Concentration H_2SO_4 (%)	Temperature (°C)	Amount of crystallization (g)	Efficiency (%)
1	3:1	0.1	70	13.25	65.13
2	5:1	0.1	70	11.32	55.64
3	**3:1**	**0.25**	**50**	**15.48**	**76.09**
4	5:1	0.25	50	15.42	75.79
5	3:1	0.5	30	9.23	45.37
6	5:1	0.5	30	8.98	44.14

The results in Table 1 show that in Experiment 3: the acid/EO ratio of 3:1; H_2SO_4 concentration of 0.25%; and temperature of 50°C resulted in the highest synthesis efficiency of 76.09% with 15.48 g of crystallized PMD. The results were similar to the publication by Yuasa (2000) who found that the optimal reaction temperature was 50°C and the optimal H_2SO_4 concentration was 0.25% [10]. According to Yuasa (2000): when synthesizing PMD at low temperatures (25-35°C) and high acid concentrations (0.5-10%), the formation of by-products such as acetals resulted in a low yield of crystallized PMD; whereas low acid concentrations (below 0.15%) resulted in a slower PMD synthesis rate, lower PMD selectivity even at higher temperatures and longer reaction times [10].

The TLC image in Figure 1 shows that the converted solutions after the reaction almost does not show the horizontal spot of citronellal standard, while the corresponding spot in the EO lane is very clear, indicating that citronellal has been almost completely converted. In all converted solutions and crystallized products, two horizontal spots of PMD standard appear (the spot that runs faster at the top is the cis isomer, the trans isomer at the bottom is lower), and no other bands are visible in the crystallized product lane, indicating that PMD has been successfully synthesized in all six experiments and the resulting PMD samples are pure.

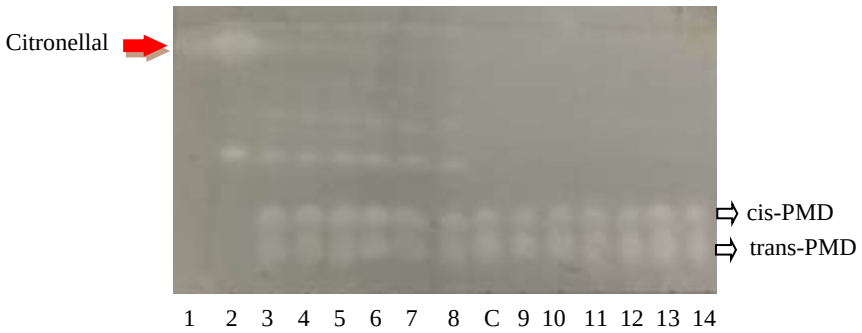

Figure 1. The TLC image after staining with vanillin sulfate of the converted and crystallized product in experiments: 1. citronellal standard; 2. EO; 3-8: corresponding converted samples in experiments 1-6; C. PMD standard (95%); 9-14: PMD product after crystallization in experiments 1-6.

3.2 Selecting the reaction time for PMD synthesis

To select an appropriate reaction time for PMD synthesis, experiments were conducted to collect samples after 2; 5; 8; 11; 14 hours of reaction. The results of testing the converted solutions after synthesis over time on Figure 2 showed that in the initial sample, there was a clear citronellal spot (lane 2). After 2 hours of reaction (lane 3), the citronellal spot decreased significantly while two clear bands of PMD appeared. After 5, 8, 11, and 14 hours, no citronellal spot was observed as it had been converted into PMD. The products obtained after crystallization at different time intervals all appeared two spots of PMD, and no impurity spot was observed on the TLC plate, indicating that the obtained crystallized products were pure PMD.

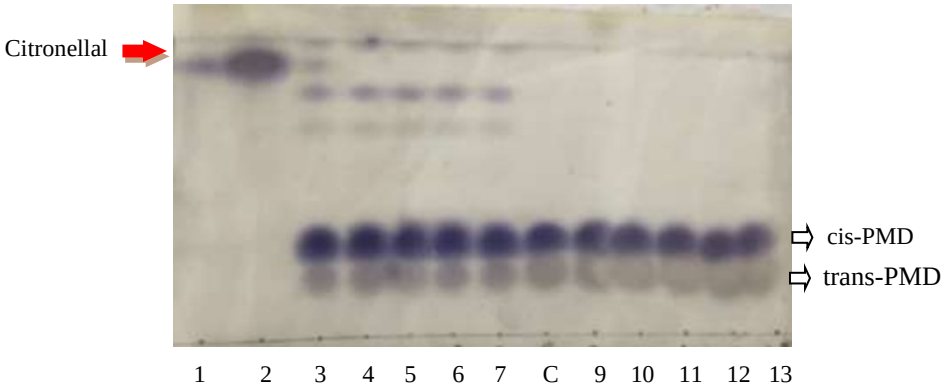

Figure 2. TLC of PMD synthesis products over time: 1: citronellal standard; 2-7: the corresponding converted samples at 0, 2, 3.5, 5, 8, and 11 hours; C: 95% PMD standard; 8-12: crystallized PMD products from samples after 2, 3.5, 5, 8, and 11 hours of conversion.

The crystallized products from samples converted over time were also weighed to determine the amount of PMD obtained after drying. From the graph of the results obtained in Figure 3, it can be observed that the conversion rate was fast in the first 3.5 hours, especially after 2 hours of conversion, the amount of crystals produced reached 11.35 g (synthesis efficiency 55.79%). After 3.5 hours, the reaction rate slowed down. The highest amount of crystals obtained was approximately 15.51 g (PMD synthesis efficiency of approximately 76.23%) after 5 hours of conversion and was maintained in the following hours (with negligible differences), indicating that the reaction could be stopped after 5 hours. These results are consistent with the TLC results in Figure 2.

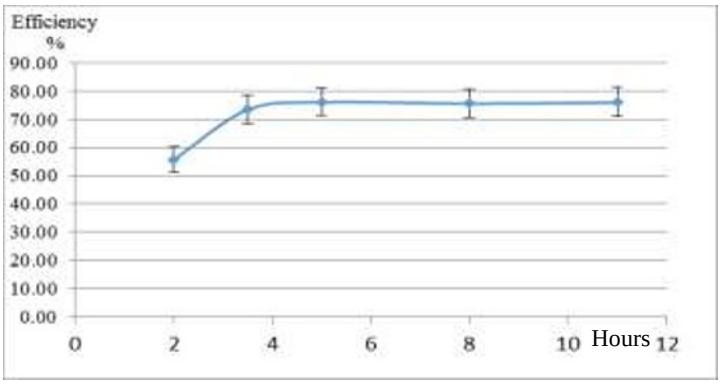

Figure 3. Graph showing the amount of PMD crystallized over time.

Thus, through the survey, the reaction conditions were selected: the ratio of H_2SO_4 0,25%/EO is 3/1 (w/w), converted at 50°C for 5 hours for the best PMD synthesis efficiency.

3.3. Quantification of PMD

The synthesized PMDs were used for GC-MS analysis to determine their components and concentrations based on the PMD calibration curve constructed in the concentration range from 40-200 mg/l. The results of GC-MS analysis of synthesized PMD samples at a concentration of 50 ppm are presented in Table 2 and Figure 4.

Table 2. GC-MS analysis results of synthesized PMD at a concentration of 50 ppm.

Run	Area (counts*min)			
	cis-PMD	trans-PMD	Total	Rate cis/trans
1	32797023.70	15044506.28	47841529.98	2.18
2	33069035.50	14762962.28	47831997.78	2.24
3	33785676.20	15287636.29	49073312.49	2.21
Average	33217245.13	15031701.62	48248946.75	2.21

The purity of the synthesized PMD product can be calculated from the total PMD standard curve and the data obtained from analyzing the synthesized PMD (Table 2) using the following formula:

$$\omega = \frac{48248946.75 + 7001742.9}{1089183.9 \times 50} \times 95\% = 96,38\%$$

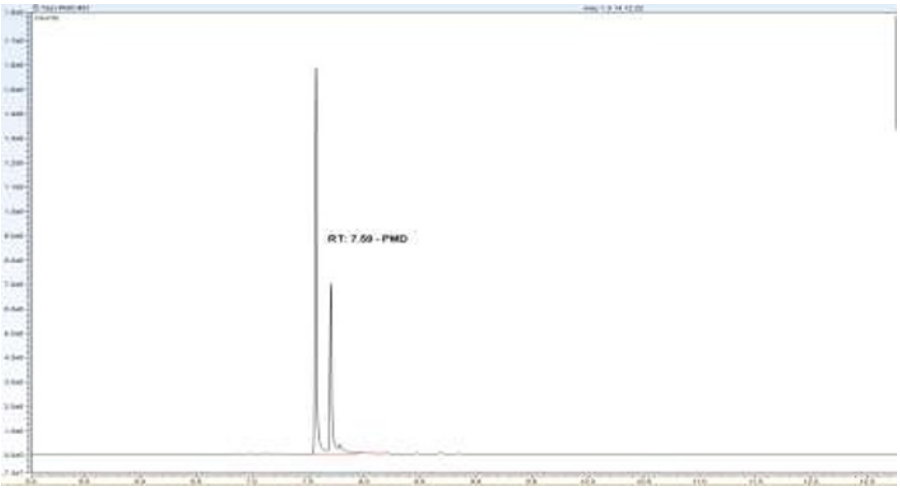

Figure 4. GC-MS chromatogram of the purified PMD sample at a concentration of 50 ppm

The GC-MS results in Figure 4 show that the purified PMD sample only appears 2 peaks (cis and trans PMD isomers respectively), showing that the purified PMD is pure.

3.4 Purification of PMD isomers

The synthesized PMD product was purified through silicagel chromatography column to separate the cis and trans PMD isomers (results shown in Figure 5).

Figure 5. TLC image of purified cis and trans PMD isomers through silicagel column. LC : synthesized PMD; 15, 16, 23, 24, 32, 33, 39, 41: the respective numbered purity fractions.

The result of purification on Figure 5 shows that from the sample applied onto the column containing 2 cis and trans spots. After purification, purified cis-PMD fractions were obtained from fractions 15-24; purified trans-PMD fractions were obtained from fractions 32-39. The purified cis-PMD fractions (15-24) will be combined and rotary evaporated to dryness. The same procedure will be carried out with purified trans-PMD fractions (32-39). The purified cis-PMD and trans-PMD samples will be analyzed by spectral analysis to determine their structures.

3.5 Determination of melting point and structure of PMD

Spectral data of cis-p-menthane-3,8-diol: mp 82°C, IR(KBr) ν) 3234 cm^{-1}. **^{1}H NMR** 0.8-0.87 (d, 3H, CH3), 0.95 (t, 1H, H5), 1.08 (t, 3H, CH3), 1.17 (s, 3H, CH3), 1.5 (m, 2H, H6), 1.68 (m, 2H, H6), 1.7 (m, 1H, H2), 4.16 (s, 1H, H1), 4.37 (s, OH), 4.54 (d, 1H, OH). **^{13}C**

NMR 20.27 (CH2), 22.26 (CH3), 25.22 (CH, C2), 28.29 (CH3), 28.52 (CH3), 34.74 (CH2), 66.15 (C1), 48.8 (C5), 71.52 (C).

Spectral data of trans-p-menthane-3,8-diol: mp 74 °C, IR(KBr) ν) 3270 cm^{-1}. **^{1}H NMR** 0.8 (m, 3H, CH3), 1.06 (s, 3H,CH3) 1,09(s, 3H, CH3), 1.2 (t, 1H, H5), 1.35 (m, 1H, H1), 1.6 (t, 2H, H4), 3.5 (m, 1H, H2), 5.28 (s, 1H, C-OH), 5.5 (s, 1H, CH-OH). **^{13}C NMR** 21.99 (CH3), 24.24 (CH3), 26.3 (CH), 29.4 (CH3), 30.74 (C1), 44.6 (C2), 52.86 (C5), 71.58 (C1), 73.2 (C).

The research group combined 1H, 13C NMR, and IR spectral analysis methods to determine the structures of two compounds, cis-p-menthane-3,8-diol and trans-p-menthane-3,8-diol, which were synthesized. The obtained results are in agreement with the previous publications [7], [10]. It is proved that PMD has been successfully synthesized and 2 isomers cis and trans PMD isolated.

4 Conclusions

The study investigated the conditions of PMD synthesis and selected it at a temperature of 50^{o}C, synthesized in 5 hours and the ratio of 0.25% H_2SO_4/EO was 3/1 for high PMD synthesis efficiency.

Synthesized PMD has a purity of 96.38%. The study also isolated and determined the structure of 2 isomers: cisPMD and transPMD.

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