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Quantitative Analysis of Eugenol Content in Clove Oil (*Eugenia caryophyllus*) Extracted from Flower, Stem, and Leaf using GC-MS Instrument

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ABSTRACT

The quantification of eugenol in clove oil (*Eugenia caryophyllus* Linn.) is crucial due to its extensive application in the agricultural industries as a bio-based pesticide, given its efficacy in controlling insect pests, nematodes, pathogenic fungi, and bacteria. The antimicrobial mechanisms of eugenol include disrupting cell membrane function, inactivating enzymes, inhibiting chitin synthesis, nucleic acid and protein synthesis, and blocking ATP (adenosine triphosphate) production. Despite its importance, there is a lack of comprehensive studies comparing eugenol content across different parts of the clove plant. This study aims to fill this gap by conducting a quantitative analysis of eugenol content in clove oil extracted from the flower, stem, and leaf of the plant. The objective is to determine the variation in eugenol concentration among these plant parts using Gas Chromatography-Mass Spectrometry (GC-MS). The methodology involved the extraction of clove oil from the flower, stem, and leaf, followed by analysis with the Agilent 7890B GC-MS instrument. The results revealed average eugenol concentrations of 97.24% in the flower, 93.42% in the stem, and 79.74% in the leaf. These findings suggest that the flower contains the highest concentration of eugenol, which has significant implications for optimizing the extraction process in commercial applications. The study contributes to the understanding of the distribution of eugenol within clove plants and provides a basis for selecting the most potent plant parts for industrial extraction.

Keywords: Bio-pesticide, caryophyllic acid, essential oil, phenylpropene, plant eugenol

Introduction

Eugenol, a phenolic compound predominantly found in clove oil (*Eugenia caryophyllus* Linn.), has garnered significant attention due to its multifaceted applications in various industries, including pharmaceuticals, cosmetics, and agriculture (Abdul Aziz et al., 2023; Tavvabi-Kashani et al., 2024; Wang et al., 2021). Its broad spectrum of biological activities, such as antimicrobial, antioxidant, and anti-inflammatory properties, makes eugenol a valuable natural product (Nisar et al., 2021). In recent years, there has been an increasing interest in exploring its potential as a bio-based pesticide (Zuo et al., 2024), driven by the need for sustainable and environmentally friendly pest management strategies in agriculture (Fernandes et al., 2020). The ability of eugenol to control a wide range of pathogens and pests, including nematodes (Boyko & Brygadyrenko, 2023; Nasiou & Giannakou, 2020), fungi (Ben et al., 2023; Cui et al., 2021; Maximino et al., 2020), bacteria (Oluoch et al., 2022), and insects (Abenaim et al., 2022; Yan et al., 2021), positions it as a promising candidate for integrated pest management (IPM) programs (Sousa et al., 2022). The application of bio-based pesticides like eugenol in agriculture is not only aligned with the global push towards sustainable farming practices but also addresses the growing concerns over the negative impacts of synthetic pesticides on human health and the environment (Mujoko et al.,

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2022; Sagala & Kusuma, 2023; Souto et al., 2021). Synthetic pesticides, while effective, have been associated with a plethora of issues, including the development of pesticide resistance, contamination of soil and water resources, and harm to non-target organisms, including beneficial insects (Sharma et al., 2020). As such, the shift towards using natural compounds such as eugenol offers a viable alternative that can enhance agricultural productivity while minimizing ecological footprints (Milićević et al., 2022).

Eugenol's mode of action as a biopesticide is diverse, encompassing mechanisms such as disruption of cell membrane integrity, inhibition of enzymatic activity, and interference with the synthesis of vital cellular components like chitin, nucleic acids, and proteins (Silva-Beltran et al., 2023). These mechanisms are not only effective against a wide array of pathogens but also reduce the likelihood of resistance development, a major drawback associated with conventional pesticides (Ulanowska & Olas, 2021). Moreover, eugenol has demonstrated significant efficacy in controlling various pests and diseases in major crops, including *Phytophthora palmivora* in pepper (Noveriza & Manohara, 2023), *Fusarium oxysporum* in banana (Nurmansyah et al., 2024), and *Sitophilus zeamais* in stored grains (Ertürk, 2021).

Given the promising pest control properties of eugenol, there is a need to understand its distribution and concentration in different parts of the clove plant, as this could influence its effectiveness as a biopesticide (dos Santos et al., 20220. Previous studies have indicated that the Agro-morphologies and phytochemical properties of eugenol varies significantly among the flower bud, stem, and leaf of the clove plant, which could impact the efficiency and cost-effectiveness of its extraction for agricultural applications (Hariyadi et al., 2020). Therefore, a detailed quantitative analysis of eugenol content in these different plant parts is essential to optimize its utilization in pest management. The current study aims to fill this knowledge gap by performing a quantitative analysis of eugenol content in clove oil extracted from the flower, stem, and leaf using Gas Chromatography-Mass Spectrometry (GC-MS). This approach not only provides precise measurements of eugenol concentrations but is also expected to offer valuable insights into the optimal extraction processes and the most suitable plant parts for biopesticide production.

Material and Methods

Plant and chemical materials

Clove plant samples (*E. caryophyllus*) were obtained from a clove plantation located in Karang Asem, Sidomulyo, Pengasih Subdistrict, Kulon Progo Regency, Special Region of Yogyakarta, Indonesia (55652). The parts of the clove plant utilized for extraction in this study included the flower buds, stems, and leaves. For the chemical analysis, a eugenol standard 99.8 % (Sigma) and Ethyl alcohol (CH_3CH_2OH) (Sigma) used as a chemical materials in the extraction and analysis processes. Additionally, inert helium gas (Agilent, USA) was employed as the carrier gas during the GC-MS analysis, ensuring optimal separation and detection of the eugenol content in the samples.

Essential oil extraction from E. Caryophyllus

In this study, the extraction of essential oil from clove plant materials, specifically the buds, stems, and leaves, was carried out using the hydrodistillation method followed Tambe and Gotmare, (2020). Two types of distillation apparatus were utilized: conventional distillation apparatus and a Clevenger-type apparatus. The selection of hydrodistillation as the extraction technique was based on its efficiency in isolating volatile compounds, particularly eugenol, from plant materials. To begin the extraction process using the conventional distillation apparatus, 125 grams of each dried clove sample (bud, stem, and leaf) were accurately weighed. These samples were then transferred into a distillation flask, to which 500 milliliters of distilled water were added. The mixture was initially heated to 80 °C using a heating mantle, then gradually increased and maintained at 100 °C to facilitate the complete extraction of essential oil. To remove any

residual water, the oil was dried over anhydrous sodium sulfate, then filtered through a 0.22-µm filter paper to eliminate any particulate matter or impurities that might have been present. The purified essential oil was stored in sealed vials at 4 °C in a dark environment to prevent oxidation and degradation of the sensitive compounds.

Preparation of sample and standard eugenol solution

Initially, a 25 µL aliquot of clove oil extract was carefully measured and subsequently diluted to a final volume of 5 mL using methanol of mass spectrometry (MS) grade quality. The solution was then subjected to vortex mixing for 1 minute to achieve complete homogeneity of the sample. Following this, the prepared samples were transferred into GC-MS vials were then placed on tray 1 of the CTC PAL auto-sampler system in preparation for injection into the GC-MS instrument. To construct a calibration curve for the quantification of eugenol in the clove oil samples, standard solutions of eugenol with a purity of 99.8 %, was used to prepare solutions at three different concentrations: 0.495 µL.L−1, 0.99 µL.L−1, and 1.98 µL.L−1. The prepared standard solutions were also subjected to the same GC-MS analytical procedure as the samples, ensuring consistency and comparability in the results.

GC/MS instrumentation

GC-MS Instrumentation The quantitative analysis of eugenol content in the extracted clove oils was performed using Gas Chromatography-Mass Spectrometry. The analysis was conducted on an Agilent 7890B GC-MS system (Agilent Technologies, USA), which was equipped with a split-splitless injector and a CTC-PAL auto-sampler. The system was coupled with an apolar HP-5 capillary column (5 % phenyl polymethyl siloxane, Agilent 19091J-413:1) featuring dimensions of 30 meters in length, 320 micrometers in internal diameter, and a film thickness of 0.25 micrometers. The column was interfaced with a mass detector, allowing for the precise identification and quantification of the eugenol compound present in the samples. The carrier gas used in the GC-MS system was high-purity helium, maintained at a constant flow rate of 1 mL per minute. The split ratio was set to 1:100, which provided optimal separation of the sample components. The injector temperature was maintained at 275 °C, with a pressure setting of 7.1 psi. To ensure sample integrity, the septum purge flow was set at 3 mL per minute. The temperature of the mass detector was held at 300 °C, while the column temperature was initially maintained at 120 °C for 2 minutes. This was followed by a linear temperature ramp from 120 °C to 240 °C at a rate of 10 °C per minute, after which the column was kept isothermal at 240 °C for an additional 2 minutes.

The transfer line between the GC and MS components was heated to 280 °C to prevent condensation of volatile compounds. Mass spectra were acquired in Electron Impact (EI) mode with an ionization energy of 70 eV. The mass spectrometer was operated in scan mode, covering a mass range of 30 to 600 m/z, which allowed for the detection of both small and large molecular weight compounds. For each analysis, 1 microliter of the sample, dissolved in ethanol, was injected into the system. Eugenol within the clove oil was achieved by comparing the obtained mass spectra with those available in the National Institute of Standards and Technology (NIST) library.

Results and Discussion

Extraction results of essential oil from E. Caryophyllus

The extraction of essential oil from the clove plant involved obtaining oil from three different parts of the plant: leaves, stems, and flowers. This process yielded essential oils with varying colors, which may indicate differences in chemical composition and the concentration of active compounds in the oils from each plant part (Figure 1). The essential oil extracted from clove leaves exhibited the darkest color among the three plant parts tested. This darker hue could be attributed to the high concentration of dissolved compounds, such as eugenol and other phenolic components, which are typically more abundant in the leaves (Hariyadi et al., 2020; El Ghallab et

al., 2020). Additionally, since the leaves are the primary site of photosynthesis, they may contain higher amounts of volatile compounds that contribute to the deeper color of the oil (Letchamo et al., 1995). In contrast, the essential oil obtained from the clove stems showed a lighter color compared to the leaves, yet still darker than the oil from the flowers. This suggests that while the stems also contain eugenol and other components, their concentration may be higher than in the leaves (Saran et al., 2023). The lighter color could indicate a higher content of non-volatile compounds, as well as structural differences in the stem tissues that affect the composition of the essential oil. The essential oil from clove flowers exhibited the lightest color among the oils from leaves and stems. This lighter hue is likely due to the predominance of lighter, more volatile compounds in the flower oil, which have a different chemical structure than the components in leaves and stems (Mahulette et al., 2020). The clove flowers, being reproductive organs, may contain more specific compounds intended to attract pollinators, which also influence the color of the essential oil produced (Robustelli della Cuna et al., 2021).

The extraction of clove essential oil was carried out using Clevenger's apparatus, a widely adopted technique for hydrodistillation. This apparatus is preferred due to its efficiency in isolating essential oils by distillation with water or steam (Alfikri et al., 2020). The process involves the condensation of volatile compounds, including eugenol, which are then collected in a side arm of the apparatus. The use of the Clevenger apparatus ensures that the volatile components are separated from the non-volatile residue, resulting in a concentrated essential oil extract. This method is particularly effective for clove oil extraction as it preserves the integrity of eugenol and other volatile compounds, ensuring a high yield of pure essential oil (Ben Hassine et al., 2021).

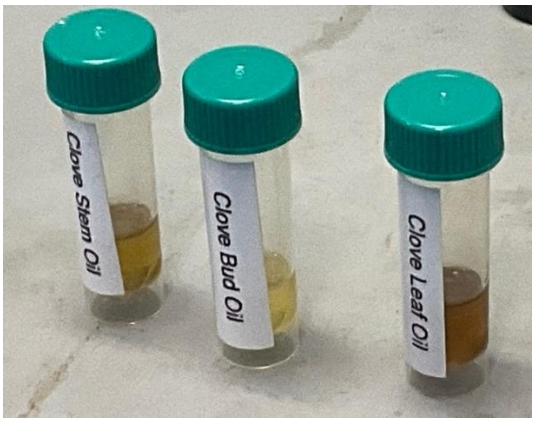

Figure 1. Extraction results of essential oil from different parts of the clove plant (Leaves, stems, and flowers)

Standard curve analysis

In this study, the quantification of eugenol in the clove oil samples was conducted through a standard curve method. The standard curve was constructed using three different concentrations of the eugenol standard: 0.495 %, 0.99 %, and 1.98 % (Figure 2). Each concentration corresponded to a specific peak area obtained from the GC-MS analysis, which was used to create a linear relationship between the concentration of eugenol and the area under the curve. The retention time for the eugenol standard was consistently recorded at 6.303 minutes. The peak areas corresponding to the concentrations of 0.495 %, 0.99 %, and 1.98 % were 15,362.30, 31,855.10, and 65,828.30, respectively. The correlation coefficient (R^2) of the standard curve was found to be 0.99978, indicating a very high degree of linearity and reliability in the quantification method (Dini et al., 2020). The residual standard deviation was calculated to be 729.11939, demonstrating the precision of the measurements. The standard curve generated from this data yielded the equation $y = m\,x + b$, where m = 33333.79442 and b = − 633.81836. In this equation, y represents

the peak area, and x denotes the concentration of eugenol in percentage. This linear equation allows for the accurate determination of eugenol concentration in unknown samples based on their GC-MS peak areas. The strong linear correlation and low residual standard deviation highlight the robustness of the standard curve method for the quantitative analysis of eugenol in clove oil, ensuring that the results are both precise and reliable (Pati et al., 2021).

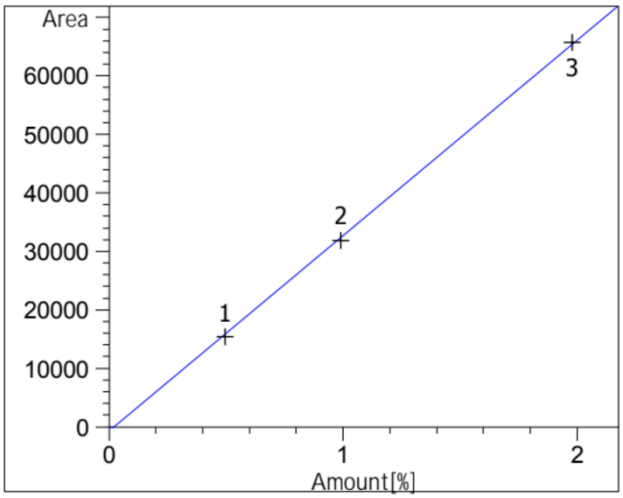

Figure 2. Calibration Curve of Eugenol Standard at Different Concentrations. Eugenol at (1) 0.495 %, (2) 0.99 %, (3) 1.985 % with Retention Time: 6.303

GC/MS Detection and Quantification of Eugenol in E. Caryophyllus

The detection and quantification of eugenol in the hydro-distillated oil extracted from the leaves, stems, and flower buds of *E. caryophyllus* were performed using GC-MS. The relative percentage of eugenol in the essential oil was determined by calculating the total ion chromatogram (TIC) response and comparing it with the standard curve obtained from known concentrations of eugenol. Identification of eugenol in the clove oil samples was confirmed by comparing the mass spectra with the National Institute of Standards and Technology (NIST) library spectra, which provided a reliable match. Furthermore, the fragmentation pattern observed in the mass spectral data was consistent with those reported in the literature, further validating the presence of eugenol as the major component in the clove oil extracts (Teles et al., 2021). The results showed distinct peaks in the chromatograms, indicating the presence of eugenol, a major bioactive compound in clove oil. The retention times and areas under the peaks were used to quantify the eugenol content in each plant part based on a previously established standard curve. For the hydro-distillated oil extracted from the leaves, as shown in Figure 3, the GC-MS chromatogram displayed a peak at a retention time (RetTime) of 6.282 minutes, with a corresponding area of 52,527.8. The concentration (amount) read by GC-MS was 1.59483, and after applying the standard curve, the average eugenol content was calculated to be 79.74 %.

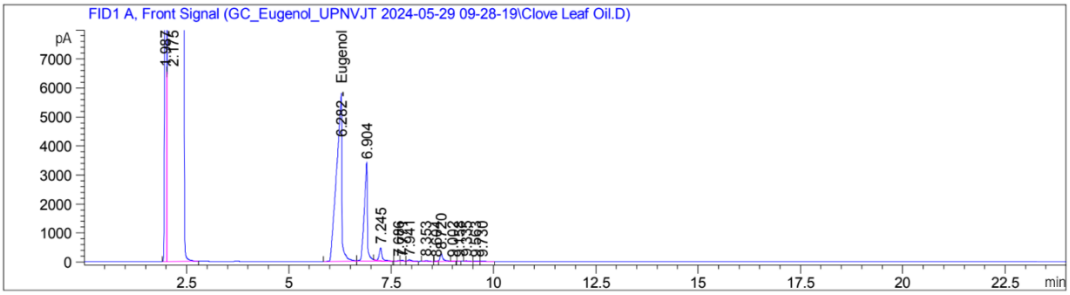

Figure 3. Total Ion Chromatogram (TIC) of Clove Oil Extract from Leaves Showing Eugenol as the Major Peak (RT 6.282)

This indicates that the leaf extract contains a moderate amount of eugenol, making it a potential, though not the most concentrated, source of the compound. In the stem extract, a peak was observed at a retention time of 6.293 minutes, with an area of 61,644.0 (Figure 4). The GC-MS analysis recorded a concentration (amount) of 1.86831, which, after calculation using the standard curve, resulted in an average eugenol content of 93.42 %. This demonstrates a significantly higher concentration of eugenol in the stem compared to the leaves, highlighting the stem as a richer source of eugenol.

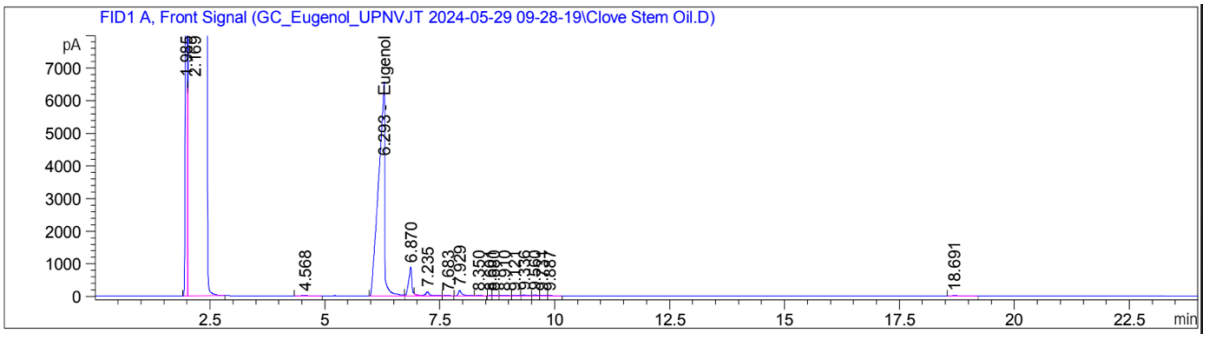

Figure 4. Total Ion Chromatogram (TIC) of Clove Oil Extract from Stems Showing Eugenol as the Major Peak (RT 6.293)

The essential oil extracted from the flower buds exhibited the highest concentration of eugenol, with a peak at a retention time of 6.303 minutes and an area of 64,195.7 (Figure 5.). The concentration (amount) detected by GC-MS was 1.94486, and the calculated eugenol content, based on the standard curve, was 97.24 %.

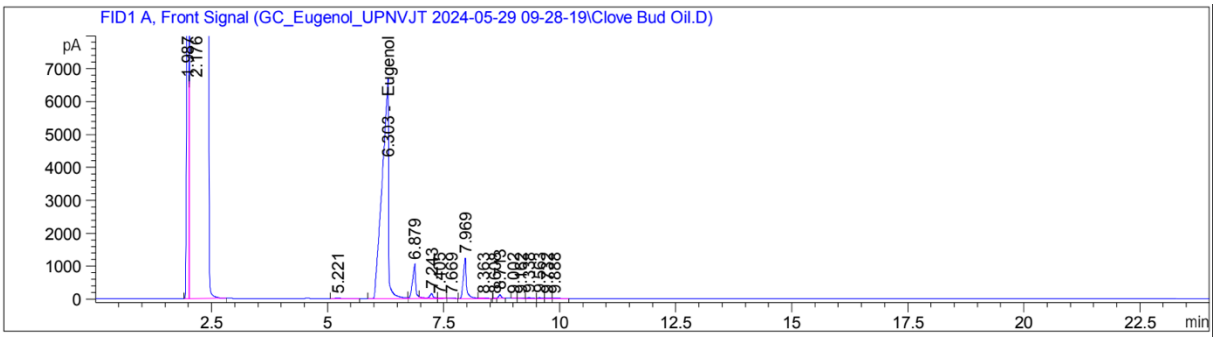

Figure 5. Total Ion Chromatogram (TIC) of Clove Oil Extract from Flower Bud Showing Eugenol as the Major Peak (RT 6.303)

This confirms that the flower buds of *E. caryophyllus* are the most potent source of eugenol among the plant parts studied, as previously reported in the literature (Uddin et al., 2017). The high eugenol content in the flower buds makes them an ideal source for industrial applications, especially in the production of biopesticides. This aligns with the growing interest in using natural, eco-friendly compounds for pest management in sustainable agriculture (Matos et al., 2020).

The detection and quantification of eugenol in essential oils extracted from *E. caryophyllus* is a critical step in ensuring the purity and effectiveness of the oil. The complexity of essential oils lies in the diverse compounds they contain, making it essential to accurately identify and quantify these compounds to ensure the oil's purity and therapeutic efficacy (Do et al., 2015). The findings of this study not only confirm the high yield of eugenol from clove oil but also underscore its potential utility in developing bio-based solutions for pest and disease control in crop management.

Conclusion

This study successfully quantified the eugenol content in essential oils extracted from the leaves, stems, and flower buds of clove (*E. caryophyllus*) using hydrodistillation and GC-MS analysis. The results revealed that clove oil extracted from the flower buds had the highest concentration of eugenol at 97.24 %, followed by the stems at 93.42 %, and the leaves at 79.74 %. These findings confirm that clove flower buds are the most potent source of eugenol, making them the most valuable part of the plant for commercial essential oil production. The optimized GC-MS method, which was employed in an Agilent 7890B GC-MS system and HP-5 capillary column and a gradient oven temperature, provided accurate and efficient detection of eugenol, confirming the suitability of this analytical technique for essential oil analysis. The use of a standard curve allowed for precise quantification of eugenol across different sample concentrations, demonstrating the reliability of this method for routine analysis of eugenol in essential oils. This research is significant for both the agricultural and commercial sectors. As eugenol is known for its potent biological properties, including its role as a natural biopesticide, these findings highlight the potential for using clove oil, particularly from the flower buds, in pest and disease management strategies. By utilizing a natural compound like eugenol, farmers and agricultural industries can reduce dependence on synthetic chemicals, promoting more sustainable and eco-friendly farming practices. Moreover, the high eugenol content in clove oil offers opportunities for its application in various industries, including pharmaceuticals, cosmetics, and food preservation, where eugenol's antimicrobial and antioxidant properties are highly valued.

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