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Research Article

Preliminary study on the rapid detection of eugenol in selected plants using thin layer chromatography

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Abstract
Eugenol, a naturally occurring phenolic compound with significant pharmacological properties, is found in various aromatic plants. This study aims to develop and optimise a thin-layer chromatography (TLC) method for qualitative detection of eugenol in selected plant species. Samples were collected from *Piper betle*, *Syzygium aromaticum*, *Cinnamomum zeylanicum*, *Zingiber officinale*, *Mitragyna speciosa,* and *Cymbopogon citratus*. The plant extracts were prepared using a Soxhlet apparatus with 95% methanol as extraction solvent and subjected to TLC analysis. Silica gel plates were employed as the stationary phase, and a solvent system of dichloromethane: methanol (99:1) was optimised for the mobile phase. The detection of eugenol was carried out using ultraviolet (UV) light and fast blue BB spray for visualisation. The Rf values obtained were consistent with the standard eugenol reference, confirming the presence of eugenol in the selected plants. This TLC method offers a simple, cost-effective, and efficient approach for the rapid screening of eugenol in various plant materials.

Keywords: eugenol, plants, thin-layer chromatography, rapid detection

Introduction

Eugenol (IUPAC: 2-methoxy-4-prop-2-enylphenol) is a naturally occurring simple phenolic compound whose chemical formula is $C_{10}H_{12}O_2$ (**Figure 1**). Eugenol is classified as an aromatic compound due to the presence of an aromatic ring in its structure, and it is characterized by its pleasant smell [1, 2]. Eugenol has attracted significant attention in recent years due to its diverse and valuable properties, which include antioxidant, antimicrobial, and anticancer activities, making it a useful component to study.

Antioxidant properties constitute some of eugenol's studied properties. Antioxidants protect the body from oxidative stress, which results from the effects of reactive oxygen species (ROS) and free radicals, which are generated as byproduct of adenosine triphosphate production by the mitochondria when cells use oxygen to generate energy. Oxidative stress is characterized by an imbalance of antioxidant defense against excessive ROS production. Phenolic groups found in various compounds, including eugenol, facilitate antioxidant activity, thereby increasing the overall effectiveness of the antioxidant defense mechanism [2]. However, while eugenol minimizes ROS-mediated oxidative stress, it has also been observed to act as a prooxidant at higher concentrations, thus further enhancing ROS production [3].

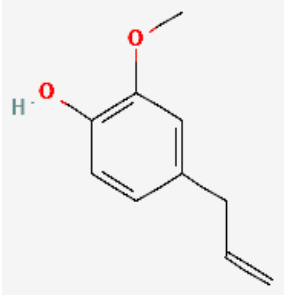

Figure 1. 2-methoxy-4-prop-2-enylphenol, courtesy of PubChem

A study of toxin-producing fungi in bread dough growth found that higher concentrations of eugenol

inhibited fungi, while at lower eugenol concentrations, fungi growth was stimulated [4]. These findings highlight the need for more detailed studies on eugenol dose–response relationships, as our understanding of its complex interactions in different systems remains incomplete.

Eugenol has also been found to enhance the shelf life of food products [2]. A study by Kerosenwala et al. [5] explored the potential use of eugenol in this area by integrating it into polymers, thereby creating products with better shelf lives.

Eugenol exhibits excellent antimicrobial properties against a wide range of bacteria, including *Staphylococcus aureus* and *Escherichia coli* [6]. The compound's primary mode of action appears to be the disruption of the cytoplasmic membrane, which leads to increased nonspecific membrane permeability and altered transport of ions and ATP [7]. A study by Devi et al. [8] investigated the antibacterial properties of eugenol against *Salmonella typhi* along with its mechanism of action, finding that eugenol anti salmonella activity is primarily attributed to its effects on the bacterial cell membrane. Eugenol initially alters membrane permeability, leading to hyperpermeability. This is followed by ion leakage and a substantial loss of other cellular contents, ultimately resulting in cell death.

Eugenol was also found to exhibit anticancer properties. A study by Begum et al. [9] proved that simplest phenolic compounds are effective in inhibiting cancer cell growth due to their ability to modulate ROS levels and enhance tumor expression protein expression.

Zinc oxide-eugenol (ZOE) was widely used as dental paste for its anti-inflammatory properties. This effect is attributed to its ability to inhibit the production of inflammatory mediators through interference with arachidonic acid metabolism [10]. However, its use has been associated with potential cytotoxic effects [11].

Eugenol has been identified and documented in several plant species, including cloves, cinnamon, allspice and basil essential oils [12]; however, data on its presence in other plants, particularly plants that are abundantly available around households in tropical countries such as Malaysia, remain limited. Understanding the distribution of eugenol across differing plant materials can provide a deeper understanding of the plant processes themselves—the function of chemicals such as phenolic compounds in a plant and their contribution to growth and development, which includes their importance in protection against pests or diseases.

Several studies concerning eugenol have been carried out with thin layer chromatography (TLC). The wavelength of the visualized TLC plate in this study differs from that of a study by Balkrishna et al. [13] using 280 nm for eugenol detection. Another study found that eugenol absorbs UV light at wavelengths greater than 290 nm; thus, it visualized the TLC plate at 366 nm [14]. TLC is a cost-effective and simple method for detecting target compounds in plant extracts. This technique allows for simultaneous analysis of multiple samples, which makes it a valuable analytical tool for eugenol detection in various plant species [15].

This study aims to confirm and assess eugenol presence in *Syzygium aromaticum* (clove buds), *Cymbopogon citratus* (lemongrass bulbs), *Piper betle* (betel leaves), *Mitragyna speciosa* (kratom leaves), *Cinnamomum zeylanicum* (cinnamon barks), and *Zingiber officinale* (ginger rhizome) using a TLC method as well as to optimize a TLC method for eugenol detection.

Materials and Methods

Chemicals and reagents

Methanol (CH_3OH) was of HPLC grade from Elite Advanced Material Sdn. Bhd. (Rawang, Selangor, Malaysia). Dichloromethane (CH_2Cl_2) and 0.1 M NaOH were from MERCK (Darmstadt, Germany). Eugenol standard ($C_{10}H_{12}O_2$) and Fast Blue BB salt were purchased from Sigma-Aldrich Co. LLC (Germany). The TLC plate used was 20x20 cm TLC Silica gel 60 F_{254} was also by Sigma-Aldrich (Germany). Water used was distilled using the ELGA PURELAB® Chorus 1 Complete (Model PC110COBPM1) and ELGA 30 Litre Reservoir (Model LA758) (United Kingdom).

Plant materials

The plant materials shown were acquired from markets in areas around Kota Bharu, Kelantan, Malaysia. All six plant materials in **Table 1** were dried and ground in a blender except for *Syzygium aromaticum* and *Cinnamomum zeylanicum*. Then, 25 g of the materials were measured and subjected to Soxhlet extraction with an extraction solvent of 95% methanol.

Each plant's materials were prepared according to their types. For leaf materials such as *Piper betle* and *Mitragyna speciosa,* the leaves were washed thoroughly with running tap water to remove any impurities before being subjected to the drying process for 48 hours. *Mitragyna speciosa* leaves were dried by lyophilization, whereas other fresh materials were dried in a hot air oven at 40°C. Rhizome of *Zingiber officinale* and *Cymbopogon citratus* buds were sliced into smaller portions to encourage even

Table 1. Six plant materials used in the study

Common Name	Scientific Name	Parts Used
Betel (Sireh)	*Piper betle*	leaves
Cinnamon (Kulit kayu manis)	*Cinnamomum zeylanicum*	barks
Clove (Bunga cengkih)	*Syzygium aromaticum*	buds
Ginger (Halia)	*Zingiber officinale*	rhizome
Kratom (Ketum)	*Mitragyna speciosa*	leaves
Lemongrass (Serai)	*Cymbopogon citratus*	bulbs

dryness in the hot air oven. Additionally, for materials that were already acquired in a dried form, such as *Syzygium aromaticum* buds and *Cinnamomum zeylanicum* barks, the materials were ground and stored at room temperature until further analysis. The plants subsequently underwent extractions with the Soxhlet extraction method after drying.

Plant extraction

The preparation of methanol extracts of the plant materials was conducted in the Extraction Room, Pharmacology Laboratory, Department of Pharmacology, School of Medical Sciences, USM, Health Campus, Kelantan.

The solvent for Soxhlet extraction was prepared by using 500 ml of 95% methanol from the mixture of pure methanol and distilled water. First, 475ml of absolute methanol is measured in a measuring cylinder and 25 ml of distilled water was then added in the measuring cylinder. The solution was then poured into a 500 ml round-bottom flask. The solution is ready to be put on a heating mantle for Soxhlet extraction. Afterwards, the plant extracts were dried in a rotary evaporator at 60°C for 1 hour and a hot air oven at 50°C for 48 hours. The colour of plant extracts after rotary evaporation was recorded.

The plant extracts were initially prepared at a 1:1 ratio with methanol for spotting, but this resulted in improper separation due to the high concentration. The plant extracts were then adjusted and diluted to a ratio of 1:3 (plant extract: methanol).

Preparation of eugenol standard for TLC spotting

The solution of the eugenol standard was prepared according to a modified procedure from Murugananthan et al. [14]. Initially, the pure eugenol (density 1.067 g/ml) in liquid form was mixed with pure methanol at a 1:1 ratio (eugenol: methanol). However, the ratio has proven to be very unbalanced, and the concentration of eugenol was too high, causing it to separate more vigorously on the TLC plate to the point that it interferes and pushes other extracts away. To address this, a new serial dilution of 10x and 100x eugenol was done and used as the positive control. 100x dilution of eugenol gives excellent separation in the mobile phase used.

Fast Blue BB salt (FBBBS) reagent reaction

Fast Blue BB Salt hemi(zinc chloride) salt (FBBBS) was used as a colorimetric assay in addition to the TLC technique. It is a diazonium salt that is commercially available as the zinc chloride double salt [16].

It contains a diazonium group (-N≡N+), which possesses electrophilic reactivity, and this reacts with the nucleophilic phenol, phenoxide ion, or amine group [17]. In this study, FBBBS allows determination of eugenol at Rf 0.80 with characteristic yellow ochre colour.

Thin layer chromatography analysis

The extracts and eugenol standard were spotted using a semi-automatic CAMAG® TLC Spotter and observed under the CAMAG® UV light at $\lambda = 254$ nm and 366 nm after developing in the mobile phase. The plate was then sprayed with Fast Blue BB salt (FBBBS) reagent.

The solvent system employed was modified from Bisergaeva et al. in which dichloromethane was used [1]. Initially, a ratio of 95:5 (dichloromethane: methanol) was tested, but it did not provide meaningful separation for eugenol. The mobile phase was optimised to a ratio of 99:1 (dichloromethane: methanol) with 9.9 ml of dichloromethane added to 0.1 ml of methanol. The mixture was equilibrated for 15 minutes in a 10×10 cm CAMAG® twin trough chamber. After the development in the mobile phase, the TLC visualiser provided by CAMAG® allows detection under UV light with wavelengths of 254 nm and 366 nm.

Fast Blue BB salt reagent was sprayed onto the developed TLC plate to allow visual confirmation of eugenol when a colour reaction was observed near the Rf value of the target compound and can range from orange to purple colour [18]. The detection process using Fast Blue BB Salt reagent does not require any heating steps, either before or after application. The

developed TLC plate was simply sprayed with the reagent in a fume hood and then left to air dry at room temperature. Additionally, the colour reaction with Fast Blue BB Salt reagent was recorded. The Rf value was calculated by the WinCATS software integrated with the CAMAG® TLC Visualizer. WinCATS is a planar chromatography manager that provides computer-aided TLC analysis [19]. This software-assisted analysis allowed for the qualitative determination of eugenol presence in the plant extracts. The Rf values of the plant extracts were compared to the eugenol standard to confirm the presence or absence of eugenol.

Results and Discussion

Colour observations after rotary evaporation

Rotary evaporation of leaves such as *Mitragyna speciosa* and *Piper betle* gave rise to a solution colored dark green. The *Mitragyna speciosa* leaves did not form sludge, unlike those the *Piper betle* extract, as shown in **Table 2**. Other extracts formed a relatively viscous solution that was then further dried in a hot air oven. A review article by Khalil et al. [20] indicated that the batch extraction method may provide higher extraction efficiency than the Soxhlet extraction method.

TLC analysis

In the present study, the initial mobile phase of dichloromethane: methanol (95:5 v/v) resulted in broad and slightly tailing bands for eugenol, particularly in samples with high pigment content, which interfered with visual detection. Increasing the non-polar component to 99:1 improved band sharpness, resolution, and separation from interfering pigments. Thus, the mobile phase with a ratio of 99:1 v/v (dichloromethane: methanol) was used throughout the study. **Table 3** presents a comparison of TLC-based eugenol detection methods from the present study and selected literature.

The resulting developed TLC plate was visualized under UV light at wavelengths of 254 nm and 366 nm, as indicated by **Figures 2** and **3**, respectively. The plate was then sprayed with Fast Blue BB salt reagent (FBBBS), which was prepared beforehand by mixing 100 mg of FBBBS into 20 ml of 0.1 M NaOH (cold). This was then vortexed and filtered at low temperature. **Figure 4** illustrates the visualization of the plate after derivation of FBBBS.

A band with an Rf value of 0.80, which represents the eugenol standard diluted at 100x factor, was observed (rightmost) when visualized under UV λ = 254 nm. Bands with the same Rf of 0.80 were also visualized for the observed *Piper betle, Cinnamomum zeylanicum* and *Syzygium aromaticum* extracts. This suggests that the three extracts may have eugenol presence. Visualization under UV λ = 366 nm did not produce any banding for the eugenol standard. Therefore, it is not used to determine eugenol. An analysis of the eugenol standard after derivation with Fast Blue BB produced a yellow, ocher color that was observed at an Rf of 0.80.

The FBBBS spray method allows for higher selectivity for eugenol, a phenolic compound. However, as seen in the results for the betel extract, the reagent also reacts with another compound, which heavily disrupts the proper observation of eugenol. This is further compounded by the separation of other components due to the mobile phase. Thus, the concoction of a revised mobile phase composition is proposed in order to achieve better separation and prevent this error in future studies.

The observation of eugenol under UV at a wavelength of 366 nm did not provide any substantial banding. This contrasts a previous study finding that eugenol absorbs UV light at wavelengths greater than 290 nm [14]. This may be due to differences in preparation, procedures, and materials.

Table 2. Colour observations after rotary evaporation (60 °C, 1 hour)

	Betel	Cinnamon	Clove	Ginger	Kratom	Lemongrass
Extract						
Appearance	Dark green sludge	Dark amber	Dark brown with precipitate	Light brown	Dark green	Pale yellow

Table 3. Comparison of TLC-based eugenol detection methods from the present study and selected literature

Study	Plant Source	Mobile Phase Composition	Detection Wavelength	Detection Reagent	Rf Value for Eugenol
Present study	*Syzygium aromaticum* (clove buds), *Cymbopogon citratus* (lemongrass bulbs), *Piper betle* (betel leaves), *Mitragyna speciosa* (kratom leaves), *Cinnamomum zeylanicum* (cinnamon barks) and *Zingiber officinale* (ginger rhizome)	dichloromethane: methanol (99:1, v/v)	254 nm and 366 nm	Fast Blue BB salt	0.8
Murugananthan et al. [14]	Cinnamon leaves (*Cinnamomum verum*) and Clove (*Eugenia caryophyllat*a)	petroleum ether: toluene: ethyl acetate (7:2:1, v/v)	UV light with 366 nm	Not reported (UV visualization only)	Not reported
Zhou et al. [21]	Syzygium aromaticum (L.) (flower buds)	cyclohexane/ethyl acetate (8:2, v/v)	UV light at 254 nm	10 % sulfuric acid/ethyl alcohol solution	Not reported

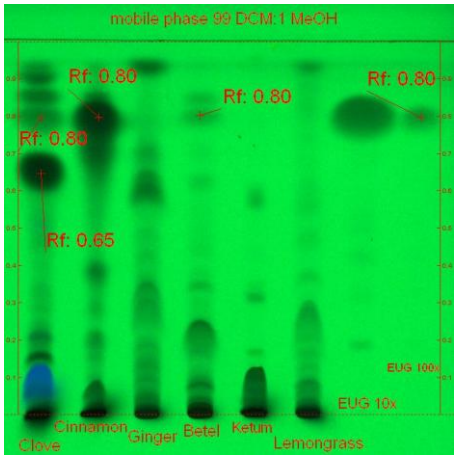

Figure 2. Visualisation of plant extracts under the wavelength of 254 nm

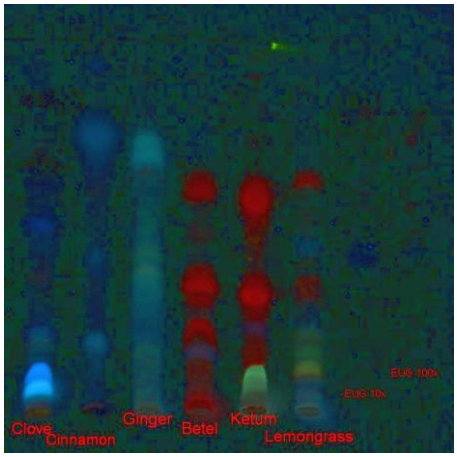

Figure 3. Visualisation of plant extracts under the wavelength of 366 nm

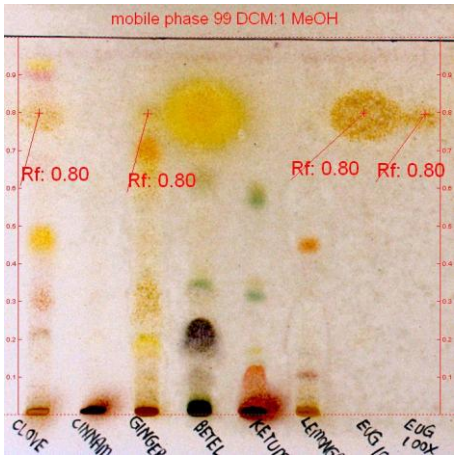

Figure 4. Visualisation of derivatized TLC plate with Fast Blue BB salt

Initial analysis under λ = 254 nm resulted in a banding observed for *Piper betle, Cinnamomum zeylanicum*, and *Syzygium aromaticum* extract for an Rf value of 0.80 (the eugenol standard). Only Syzygium aromaticum and *Zingiber officinale* had a reaction spot close to Rf 0.80 after derivation with FBBBS. The Piper betle extract separation prevented the objective determination of eugenol due to another pigment; therefore, it was unconfirmed. The results suggest that eugenol is present in *Syzygium aromaticum* and *Zingiber officinale*, which exhibit yellow ocher spots after being sprayed with FBBBS reagent. The FBBBS reagent was used to specifically detect alkaloids. When used in combination with techniques such as TLC, the reagent allows for the visualization and identification of various bioactive compounds, including cannabinoids and other phenolic substances that possess aromatic rings. As eugenol is a phenolic compound, it is likely to react with the reagent, thus providing a specific detection.

The amount of target eugenol seemed higher in *Syzygium aromaticum* compared to the *Zingiber officinale* extract, as evidenced by the difference in their band sizes. This aligns with findings in a previous study in which eugenol was determined to be a main compound in cloves [22]. Based on this study, the presence of eugenol was determined in *Syzygium aromaticum* (clove) and *Zingiber officinale* (ginger).

The results indicating that *Syzygium aromaticum* is eugenol positive is consistent with previous studies [12,22,23]. The presence of eugenol in ginger also conforms with the findings of previous studies, which confirm that eugenol is present in ginger, albeit in low concentrations [20]. The study was limited to six plant materials: *Syzygium aromaticum* (clove buds), *Syzygium aromaticum* (lemongrass bulbs), *Piper betle* (betel leaves), *Mitragyna speciosa* (kratom leaves), *Cinnamomum zeylanicum* (cinnamon barks), and *Zingiber officinale* (ginger rhizome).

The *Piper betle* leaf is the most widely used and studied part of betel vines. The practice of chewing betel leaves is part of the cultural traditions of various countries. This habit is widely believed to be beneficial for oral health benefits. Chewing betel leaves was assumed to fortify gums and aid in dental preservation [24]. In a previous study, the presence of eugenol in *Piper betle* leaf was determined to be 17.85 mg/g [25,26].

Ceylon (scientific name: *Cinnamomum zeylanicum*), or "true" cinnamon from a cinnamon tree, was determined to possess a eugenol content of around 5%–10% in the bark [27]. Eugenol was largely found in methanolic extracts obtained from *Syzygium aromaticum* processing [6]. It is a major component of cloves, with past studies confirming that 3.03 g of eugenol can be successfully extracted from 100 g of ground clove [22]. Eugenol is also a major component of clove, cinnamon, allspice, and basil essential oils [12].

Zingiber officinale, or ginger, is a medicinal plant that has been documented to contain eugenol [20]. It was consumed as a traditional remedy to relieve common health problems, such as pain and vomiting, and it is also popular as a flavoring or fragrance in dishes [28]. *Mitragyna speciosa*, commonly known as kratom, has been a subject of regulatory concern in Southeast Asia. In Malaysia, for instance, possessing kratom leaves or kratom drinks is an offense, as it raises addiction and dependencies, leading to its abuse [29]. To date, no data on the presence of eugenol in *Mitragyna speciosa* has been published, and to our knowledge, this is the first study investigating presence in *Mitragyna speciosa*.

Cymbopogon citratus is a plant from the genus Cymbopogon, which covers around 55 species. Commonly known as lemongrass, the bulbs, stalks, and leaves are widely used in a variety of dishes to improve aromas and flavors. Previous studies have reported that the plant contains phytocompounds such as flavonoids and phenolic compounds. However, the presence of eugenol has not been determined [30].

The optimized TLC method provides a reliable, robust, highly reproducible, and accurate detection technique. This study's findings also identify a reliable source of eugenol, as reported previously. This may enhance further research into eugenol's possible uses and applications, particularly its antioxidant, antimicrobial, and anticancer activities. Furthermore, this study provides a foundation for future research in the areas of phytochemical identification, such as eugenol detection, in selected plants.

This study aimed to establish and optimize a simple, rapid method for the qualitative detection of eugenol, rather than to perform quantitative or semi-quantitative estimation at this stage. Future studies will incorporate densitometric measurements to complement the qualitative findings and provide a more comprehensive analysis of eugenol content.

Confirmatory techniques such as HPLC, FTIR, or co-TLC/spiking could further strengthen the specificity of eugenol identification, and these approaches are recommended for future studies to validate and complement the present qualitative findings.

Conclusion

This study successfully developed a TLC method for the qualitative detection of eugenol in selected plant species. The method utilized a 99:1 dichloromethane: methanol mobile phase and a silica gel 60 matrix plate stationary phase. This method for screening eugenol in selected plant materials was deemed simple, cost-effective, and efficient. The study determined the presence of eugenol in *Syzygium aromaticum* (clove) and *Zingiber officinale* (ginger) extracts.

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