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EXTRACTION, IDENTIFICATION OF PHYTOCHEMICALS, AND MICROBIAL ANALYSIS OF *EUCALYPTUS GLOBULUS* ESSENTIAL OIL

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Abstract

As medicinal prices continue to surge and bacterial resistance to antibiotics remains a prevalent issue, exploring alternative methods of treatment in medicine has become imperative. Often, natural remedies are more readily available and affordable than synthesized medications. This study aims to identify and isolate the major substituents in Eucalyptus globulus essential oil (EGO) and determine its antimicrobial properties. Various techniques are utilized in this study including: Soxhlet extraction, rotary evaporation, thin layer chromatography (TLC), column chromatography, and Kirby-Bauer antimicrobial testing of Escherichia coli and Staphylococcus epidermidis. Ultimately, it was concluded that 1,8-cineole is the major substituent of EGO, and the various oil components work collectively to produce the antimicrobial effects observed during the Kirby-Bauer test. Although 1,8-Cineole was not the only chemical within EGO that leads to its anti-microbial properties. As reflected by the antimicrobial properties of EGO, natural remedies such as eucalyptus oil should be considered when developing treatment regimens for optimal patient care.

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Introduction

Investigating potent antimicrobial compounds capable of countering bacterial infections has garnered immense interest as bacteria continue to mutate and develop antibiotic resistance.[1] As the efficacy of natural remedies is met with growing recognition, research into herbal antimicrobial and other pharmacological properties is paramount. However, major concerns regarding herbal medicine quality control, adverse side effects, and supervision over prescriptions continue to persist.[2-4] Despite apprehensions, many patients turn towards herbal medications as a more affordable alternative to synthetic drugs, though exceptions exist.[5-8] Moreover, the escalating cost of synthetic medications severely limits access to life-saving drugs for a growing number of people.[9] Another dilemma encompasses prescription medicine supply and demand. Numerous drugs exhibit shortages each year, and this drug shortage list continues to expand as supply decreases yet demand increases.[10-12] For example, demand for Ozempic exceeds supply, thus many individuals with diabetes are unable to obtain this medication to manage their condition as others use the drug for its off-label weight loss side effects.[13-15] Thus, exploring alternative methods of treating medical conditions can prove worthwhile.

Research has shown EGO possesses many numerous pharmacological effects including: antimicrobial, antidiabetic, anthelmintic, antiviral, antihistaminic, anti-inflammatory, anti-malarial, antioxidant, cytotoxic effects, anti-allodynic, and treating respiratory diseases.[16-27] The major substituents of EGO that have been extracted and used for medicinal purposes include, but are not limited to α-pinene, β-pinene α-terpineol, limonene, β-myrcene, and 1,8-cineole, the most abundant component.[16,25] Eucalyptol, also known as 1,8-cineole, has been shown to benefit patients suffering from acute respiratory infections and chronic respiratory diseases.[16,27-32] Additionally, 1,8-cineole has been distinguished for its hallmark anti-inflammatory, antinociceptive, and antioxidant properties.[32-34] By analyzing treatment methods used in historical societies, the benefits of EGO highlighted through research are further reaffirmed.

For centuries, a myriad of societies including Chinese, Ayurvedic, Greek, European, and Aboriginals from Australia have utilized the pharmacological properties of EGO to aid in various healing processes. EGO has been harnessed as a multi-faceted antidote to promote wound healing, diminish headaches, provide neuralgia relief and fever reduction. Additionally, EGO has been used to treat influenza and other infections such as bronchitis or ones of fungal lineage, as well as dermatomycosis, diabetes, and more.[35-38] Further, literature reflects the use of EGO as an antiseptic as noted in England during the 1800s to clean urinary catheters.[39] Consequently, EGO has historically proven to contain tremendous potential in treating sick individuals and combatting infectious pathogens. It is hypothesized that the most abundant component of Eucalyptus globulus volatile oil will be 1,8-cineole and EGO will have anti-microbial resistance to growth of E. coli and S. epidermis as shown in the Kirby-Bauer test for zone of inhibition.

In this study, we explore the antimicrobial effects of Eucalyptus globulus oil and its components on the inhibition of growth of Escherichia coli and Staphylococcus epidermidis. We separate through chromatography and then identify through mass spectrometry, the presence of 1,8-Cineole as one of the chemicals found in EGO. We find that 1,8-Cineole is partly responsible for EGO's antimicrobial properties but show that it is not the lone contributor. We find that EGO is an alternative home remedy to deal with bacterial infections.

Materials and Methods

Soxhlet extraction of Eucalyptus globulus essential oil

The extraction solvent consisted of approximately 500 mL of methanol (Fischer Scientific, ≥ 99.8%) along with boiling chips

were added to the round-bottom flask (RBF) of the Soxhlet extraction apparatus (Cole-Parmer). Next, 22.0 g of dried Eucalyptus globulus leaves (Junhengtianji Trade Company) were weighed and placed in between a top and bottom layer of cotton in the Soxhlet thimble. The device was run for 12 hours under reflux until a runny, dark green mixture was present.

The round bottom flask of the rotary evaporator (Cole-Parmer) was weighed, and the extracted EGO and methanol mixture was transferred to the flask. The rotary evaporator was run at a high-speed setting and a water bath temperature of 89 ^{O}C until the mixture became flaky and visibly dry, indicating total methanol evaporation. The flask was re-weighed, and the percent recovery was calculated.

Thin Layer Chromatography (TLC)

The EGO flakes were placed in small vials and diluted with methanol. Extracted EGO was run against the standard 1,8-cineole (Toyko Chemical Industry America, ≥ 98.0%) which was diluted in a ratio of 1 drop of 1,8-cineole standard per 1 mL of methanol. The TLC plates (Sigma-Aldrich) were coated in a silica gel 60 matrix and a fluorescent indicator. A 93:7 toluene-ethyl acetate solvent (EM Science, ≥ 99.9% and Fisher Scientific, ≥ 99.5%, respectively) along with an ethanolic (Fisher Scientific, ≥ 99.5%) vanillin-H_2SO_4 (Acros Organics, ≥ 99.0% and Fisher Scientific, ≥ 95.0% - 98.0%, respectively) reagent stain was utilized. The vanillin stain comprised of 15.0 g vanillin, 250 mL ethanol, and 2.5 mL H_2SO_4.[40] After the TLC plate was ran and dipped into the vanillin stain, the plate was dried with a heat gun and the Rf values were calculated. The presence of the major substituent, 1,8-cineole, was confirmed. The target, 1,8-cineole, was selected in this study as the literature on EGO denotes this ether as the major substituent in the oil.

Column Chromatography

Wool was placed at the bottom of the 2.5 cm diameter column and the 93:7 toluene-ethyl acetate solvent was mixed with white sand and silica gel (Select Scientific, 100-200 particle size) through the “slurry” packing method. Once gas bubbles were eliminated, solvent was allowed to pass through the column. Exactly 1.00 mL of the extracted EGO was transferred onto the slurry mixture and the solvent was continuously pipetted onto the oil, and ultimately the slurry mixture once the oil was pulled down. It was ensured that the solvent line did not depress below the slurry mixture line. Each fraction was collected in 5 mL test tubes and 28 fractions were collected from the column. The fractions containing isolated 1,8-cineole were green in color, whereas yellow fractions indicated the presence of an unknown compound that did not show up on the TLC plate with the stain. Consequently, fractions 22-26 were most notable as they produced the most consistent results on the TLC plate when run against the EGO. Fractions 20 and 21 were yellow, thus contained a compound other than the target, 1,8-cineole.

Gas chromatography / Mass Spectrometry

Samples were run with a Hewlett Packard Series II 5890 gas chromatograph with a running rate of run at 0.5 ml/min, or 5 psi using Helium as the carrier gas. Methanol was the solvent of choice, and 1.5 µL of the EGO solution was loaded into the column and allowed to run for 1 hour. A delay of 5 minutes was allotted as the first compound to boil off, methanol, was already known. The final oven temperature was set to 250 °C and the initial temperature was 40 °C. The temperature was increased by 10 degrees per minute until reaching 250 °C. Results of gas chromatography were analyzed using the local data file, eucal. Each major peak was enhanced and the 1,8-cineole peak, peak #2 which is the largest peak (shown in Figure 3), was converted into a mass spectrum on the Hewlett Packard Mass Selective Detector 5972 Series. The peaks within the gas chromatograph were identified using the National Institute of Standards and Technology (NIST) Database via a search method. If a compound displayed a high probability match to a compound in the NIST database, the compound was listed.

Mueller Hinton Agar Preparation

To forge the Mueller Hinton agar (Carolina Biological), 750 mL of distilled water was poured into an Erlenmeyer flask and 28.5 g of Muller Hinton agar was weighed out. Three, 250 mL beakers were used to distribute the volume of mixture, and each was heated to a boil and allowed to stay at this state for 1 min. Each beaker was then autoclaved at 125 ^{O}C for 47 min and allowed to cool for 10 min before being poured into the petri dishes. Petri dishes were placed on a shelf upside-down for storage.

Kirby-Bauer Antimicrobial Testing

Four variables were tested in their efficacy of reducing microbial growth: Eucalyptus globulus essential oil (1), isolated 1,8-cineole, or fractions 22-26 (2), 1,8-cineole standard (3), and the leaf of the Eucalyptus globulus plant (4). Small, round filter paper hole punches were dipped into each variable vial, except for the leaf. The filter papers were placed in their respective corners of two petri dishes with a plain filter paper hole punch located in the center of each dish as the control. One petri dish contained a bacterial lawn of Escherichia coli (Fisher Scientific) and the other, Staphylococcus epidermidis (Fisher Scientific). The two petri dishes were then incubated at 37 °C for 24-48 hours upside down and results were analyzed.

Results and Discussion

Purification and Identification of EGO components

EGO was extracted from Eucalyptus globulus leaf matter and

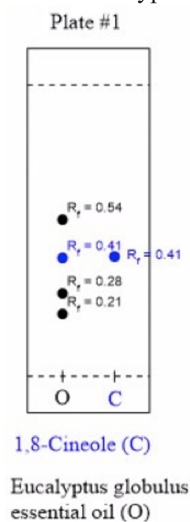

Figure 1: Chemdraw representation of EGO run against dilute 1,8-cineole standard.

had a percent recovery of 35.5%. A TLC plate of the EGO and a 1,8-Cineole standard were run and compared as schematically depicted in Figure 1. The EGO had components with a retention factor (R_f) of 0.21, 0.28, 0.41, and 0.54. The R_f value of 0.41 matched the R_f of 1,8-Cineole, confirming the presence of 1,8-Cineole in the EGO.

Column chromatography of the EGO was completed and then confirmed via TLC. Figure 2 represents the TLC collected from the 26 fractions from column chromatography. Fractions 20-26 were determined to have 1,8-Cineole present. However, fractions 20 and 21 were found to contain other components from EGO, so only fractions 22 through 26 will be used for further analysis. A summary of R_f values from the TLC plates is shown in Table 1.

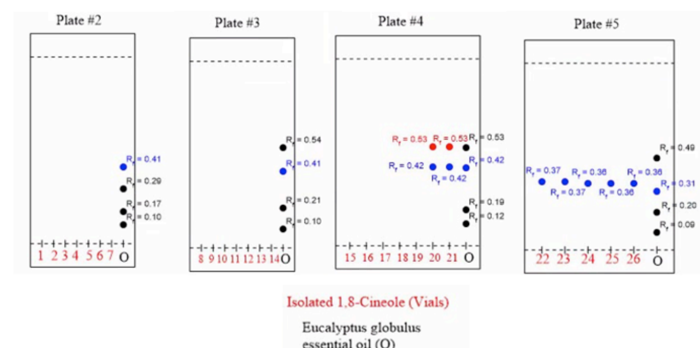

Figure 2: Chemdraw representation of the TLC plates comparing 26 fractions of EGO from column chromatography compared to EGO not ran through column chromatography to properly isolate 1,8-Cineole.

Table 1: Summarization of TLC Plates of EGO Run Against 1,8-Cineole Standard and Isolated 1,8-Cineole

Plate Number (1-5)	EGO Components Rf Value	1,8-Cineole Standard Rf Value	1,8-Cineole Isolate Rf Value	Column Chromatography Fraction Number (20-26)
1	0.21 0.28 0.41 0.54	0.41	N/A	N/A
2	0.10 0.17 0.29 0.41	N/A	N/A	N/A
3	0.10 0.21 0.41 0.54	N/A	N/A	N/A
4	0.12 0.19 0.42 0.53	N/A	0.42 0.53	20 21
5	0.09 0.2 0.31 0.49	N/A	0.37 0.37 0.36 0.36 0.36	**22** **23** **24** **25** **26**

Gas Chromatography-Mass Spectrometry (GCMS) was also used to help positively identify the presence of 1,8-Cineole (aka Eucalyptol) in EGO extracted from Eucalyptus globulus leaves. Many peaks in the gas chromatogram were identified as shown in Figure 3. The 1,8-Cineole (labeled as peak 2) had a retention time on the column of approximately 7 minutes. The mass spectrum shown in Figure 4 was analyzed and confirmed to be 1,8-Cineole through comparison to the NIST database.

Kirby-Bauer antimicrobial testing

Kirby-Bauer antimicrobial testing was conducted on the EGO, 1,8-Cineole isolated from vials 22 through 26 from column chromatography, standard 1,8-Cineole, and the Eucalyptus leaf. The zones of inhibition from E. coli and S. epidermis are shown in Figure 5 and summarized in Table 2. The zone of inhibition for EGO was 2.4(+/- 0.5) and 3.2 (+/-0.3) cm for E. coli. and S. epidermis, respectively. The EGO was more effective at inhibiting S. epidermis growth than it was E. coli. The isolated and standard 1,8-Cineole showed very similar response for E. coli with zones of inhibition of 1.3 (+/- 0.3) and 1.8 (+/- 0.9) cm, respectively. As well as a similar response with S. epidermis with a zone of inhibition of 1.4 (+/- 0.4) and 1.3 (+/- 0.4) cm for the isolated 1,8-Cineole and standard 1,8-Cineole, respectively. The Eucalyptus leaf

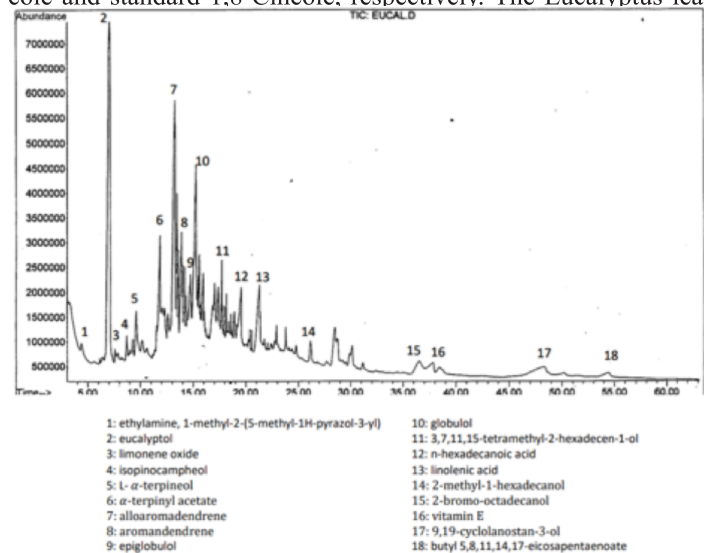

Figure 3: Gas Chromatogram of Eucalyptus globulus Essential Oil. Eighteen peaks were identified and listed below the chromatogram

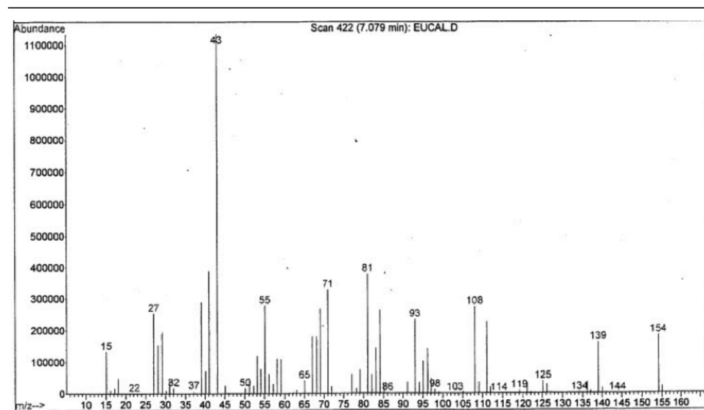

Figure 4: Mass Spectra of 1,8-Cineole extracted from Eucalyptus globulus leaf matter.

showed very similar results to the EGO with values of 1.7 (+/- 0.1) and 2.8 (+/- 0.6) cm for E. coli and S. epidermis, respectively.

The 1,8-Cineole, in either the experimentally isolated or standard reference form, showed a lower zone of inhibition than the EGO or eucalyptus leaf. The 1,8-Cineole showed at minimum half of the zone of inhibition that the EGO or eucalyptus leaf which shows 1,8-Cineole plays a major role into the antimicrobial properties of the eucalyptus oil and leaf. However, the increase in the zone of inhibition for the leaf and the EGO suggests that other components of the oil also have antimicrobial properties as well. Other studies have shown that the EGO has a reduced the zone of inhibition than that of 1,8-cineole.[41,42]

Interestingly, the EGO and eucalyptus leaf both showed a higher zone of inhibition for S. epidermis than for E. coli. The zone of inhibition was higher for E. coli in the EGO or leaf than it was for 1,8-Cineole however the S. epidermis showed a much greater increase in the zone of inhibition than E. coli. A chemical within the EGO and the eucalyptus leaf has antimicrobial properties that are more selective to S. epidermis than E. coli which is shown by this discrepancy between the two bacterial strands. This is in agreement with previous reports that has shown 1,8-cineole has a wide range of efficacy for different strands of bacteria.[43] The EGO and eucalyptus leaf is more effective at inhibiting S. epidermis growth than E. coli. The identity of the chemical within EGO

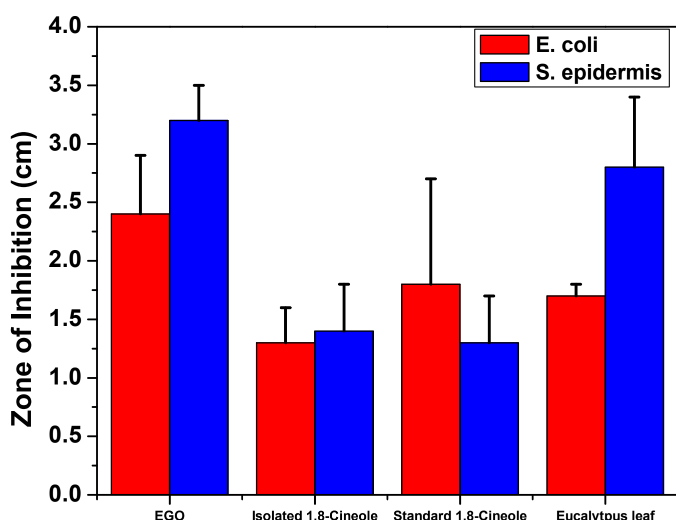

Figure 5: The zone of inhibition caused by EGO (essential oil), 1,8-Cineole isolated from EGO, 1,8-Cineole reference, and a eucalyptus leaf on Escherichia coli and Staphylococcus epidermidis.

Table 2: Results of the Zones of Inhibition from Collection of Petri Dishes

	Zones of Inhibition (cm)	
	E.Coli Avg (+/- SD)	S. Epidermis Avg +/- SD
EGO	2.4 (+/- 0.5)	3.2 (+/- 0.3)
Isolated 1,8-Cineole	1.3 (+/- 0.3)	1.4 (+/- 0.4)
Standard 1,8-Cineole	1.8 (+/-0.9)	1.3 (+/- 0.4)
Eucalyptus Leaf	1.7 (+/- 0.1)	2.8 (+/- 0.6)

and the eucalyptus leaf that is having this increased resistance to S. epidermis is outside the scope of this study but is of interest for further understanding of natural remedies.

One may also notice that the EGO showed a greater zone of inhibition than the eucalyptus leaf. This is most likely due to the local concentration of EGO which is lowered in the eucalyptus leaf due to other components of the leaf that are not EGO. Another explanation may be that the EGO is not as available in the case of the eucalyptus leaf due to its confinement within specialized structures within the leaf. Although the leaf itself has antimicrobial properties, to enhance the medicinal ability of EGO, it should be extracted from the plant however 1,8-Cineole should not be completely isolated from the EGO due to other antimicrobial chemicals within the EGO.

Conclusion

The presence of 1,8-cineole as a major substituent within Eucalyptus globulus essential oil was confirmed. Furthermore, the oil does possess antimicrobial properties due to 1,8-Cineole and other chemicals within the EGO. The other chemicals within EGO have an enhanced antimicrobial properties against S. epidermis that is not shown in E. coli. This shows that EGO is more effective at inhibiting certain bacterial strains over others. This inhibition shows that the use of Eucalyptus globulus in medicine is further affirmed and should be considered as an alternative route to antibiotics.

Future studies could investigate the other chemicals identified within EGO to assign the contribution to the antimicrobial properties of EGO. Furthermore, studies may be completed to further understand the mechanism behind inhibition of microbial growth in the presence of EGO.

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