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EXTRACCIÓN DE ACEITE ESENCIAL DE HOJAS DE EUCALIPTO (*EUCALYPTUS GLOBULUS*) POR DESTILACIÓN CON VAPOR DE AGUA Y SU CARACTERIZACIÓN FISICOQUÍMICA Y MICROBIOLÓGICA.

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RESUMEN

El propósito de esta investigación fue estudiar el efecto de la masa y la humedad de las hojas de eucalipto en el rendimiento de extracción de aceite de *Eucalyptus Globulus* por destilación con vapor. Se utilizó un diseño unifactorial, utilizando diferentes masas de hojas de eucalipto alimentadas a la cámara de extracción mientras se mantenían las condiciones de operación. Se ha obtenido un aceite con una densidad de 0,906 g/ml, índice de refracción de 1,462 y viscosidad absoluta de 2,28 cp. Se obtuvo un rendimiento máximo de 1,28% m/m con una humedad de 60,95%. Se determinó que a medida que disminuye la humedad de las hojas para la extracción de aceite, se obtiene un mayor rendimiento. Las condiciones óptimas para la extracción fueron las siguientes: flujo de agua de enfriamiento: 102,5 L/h; caudal de vapor para extracción: 2,67 L/h; volumen de la cámara de extracción: 0,016 m^3; lo que dio como resultado un volumen de aceite de 56,32 mL para una masa de 4 kg de hojas. El volumen de aceite extraído depende directamente de la masa de las hojas de eucalipto e inversamente proporcional a la humedad de las hojas. El aceite de eucalipto mostró actividad antimicrobiana contra Staphylococcus aureus y eficacia antifúngica contra los hongos Rhizopus stolonifer y Aspergillus niger.

Palabras clave: Eucalyptus Globulus; aceite esencial; destilación al vapor; actividad antimicrobiana; eficacia antifúngica.

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EXTRACTION OF ESSENTIAL OIL FROM EUCALYPTUS LEAVES (*EUCALYPTUS GLOBULUS*) BY STEAM DISTILLATION AND ITS PHYSICOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION

ABSTRACT

The purpose of this research was to study the effect of mass and humidity of eucalyptus leaves on the oil extraction performance of Eucalyptus Globulus by steam distillation. A unifactorial design was used, using different masses of eucalyptus leaves fed to the extraction chamber while maintaining operating conditions. An oil with a density of 0,906 g/ml has been obtained, refractive index of 1,462 and absolute viscosity of 2,28 cp. A maximum yield of 1,28% m/m was obtained with a humidity of 60,95%. It was determined that as the humidity of the leaves for oil extraction decreases, a higher yield is obtained. The optimal conditions for extraction were the following: cooling water flow: 102,5 L/h; steam flow rate for extraction: 2,67 L/h; extraction chamber volume: 0,016 m^3; which resulted in an oil volume of 56,32 mL for a mass of 4 kg of leaves. The volume of oil extracted depends directly on the mass of eucalyptus leaves and inversely proportional to the humidity of the leaves. Eucalyptus oil showed antimicrobial activity against *Staphylococcus aureus* and antifungal efficacy against the fungi *Rhizopus stolonifer* and *Aspergillus niger*.

Keywords: *Eucalyptus Globulus*; essential oil; steam distillation; antimicrobial activity; antifungal efficacy.

INTRODUCTION

Eucalyptus essential oil (EEO) has been used for centuries for its medicinal and aromatic properties. It was extracted from the leaves of the eucalyptus tree, native to Australia, and is used in a wide variety of fields such as traditional medicine, aromatherapy, and the cosmetic industry. Eucalyptus is rich in components such as eucalyptol, geraniol, and alpha-terpineol, possesses antiseptic, anti-rheumatic, balsamic, and decongestant properties, making it a valuable active component with remarkable medicinal properties[1]. In Peru, there are several varieties of eucalyptus trees such as *Eucalyptus globulus*, *Eucalyptus camaldulensis*, *Eucalyptus citriodora*, and *Eucalyptus deglupta*.

The process of extracting EEO employs different methods, such as steam distillation, microwave-assisted hydrodistillation, solvent-free microwave extraction, and ultrasound-assisted extraction[2], solvent extraction, cold pressing, and supercritical fluid extraction. Steam distillation and microwave-assisted hydrodistillation usually provide the highest yields[3]. Moreno et al (2010) reported that fresh leaves tend to produce a lower oil yield, while dried leaves can contain up to 3% essential oil[3]. Young leaves (3-6 months) were found to have a higher essential oil content. yield between 1,25-1,5% than mature leaves (12-18 months) between 1,1 and 1,4%, and that the chemical composition of the essential oil varies significantly with the age of the leaves, with a greater the α-pinene content in young leaves and a higher eucalyptol content in mature leaves, with greater antimicrobial activity against pathogenic bacteria[4-7].

Therefore, this study reports the effect of the feed mass and moisture content of eucalyptus leaves on the yield and volume of oil extracted by steam distillation, as well as its structural, physicochemical, and microbiological properties, with the aim of using it in the formulation for obtaining bioplastics.

EXPERIMENTAL PART

Material

The eucalyptus (Eucalyptus globulus) leaves came from Cerro la Meza surrounding the city of Huancayo; these were fresh leaves, harvested between August and September 2023. KOH c.p. from Merk Peruana S.A. was used. Distilled water was used in the chemical analyses.

Steam distillation process.

It begins with the selection of young leaves, then the removal of leaves from the eucalyptus branches, followed by weighing the fresh *Eucalyptus globulus* leaves with an average moisture content of 60,95 ± 1,37%; which are then placed inside the extraction chamber. The boiler tank was filled with ten liters of potable water and connected to the extraction chamber, and the latter was connected to the heat exchanger, which has an inlet and outlet for cooling water. Then, the boiler tank is turned on to generate steam, which will pass through the extraction chamber, where the vapors solubilize the essential oil by internal heating of the water and destroy the plant cells, causing the rupture of the glands and the oil receptacles. This mixture of steam and essential oil passes through the heat exchanger for condensation (See Figure 1). The distillate was received in a Florentine flask, until the change in oil volume is negligible, which occurs after 80 minutes. The obtained essential oil was separated using decantation equipment. Finally, it was stored in an amber bottle and refrigerated for further chemical and microbiological analysis.

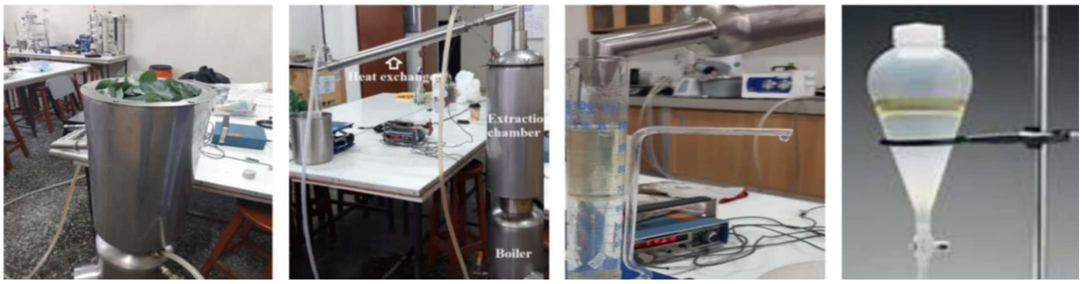

Figure 1. Eucalyptus essential oil extraction process.

The main method used is steam distillation, however, there are other methodologies. The steam distillation method does not employ organic solvents, as is the case with the Soxhlet method, in which organic solvents are used, which can extract a variety of compounds in addition to the desired substance[8]. The oil extraction yield depends on the extraction method and the quality of the leaves (fresh, dry, origin, and species).

Experimental design

This research had a quantitative approach, with an explanatory level. A single-factor design was used to study the effect of the feed mass of eucalyptus leaves on the extraction system on the yield and volume of essential oil obtained. On the other hand, the effect of the moisture content of the eucalyptus leaves on the yield was studied. The operating conditions were kept constant.

Characterization

The taxonomic characterization was carried out according to the APG IV Classification System (2016) and performed at the Natural History Museum (San Marcos Herbarium - USM).
For the structural characterization, a Nicolet protege 8700 equipment with ATR accessory and ZnSe crystal was used, performing thirty scans from 4000 to 650 cm^{-1}, and a resolution of 4 cm^{-1}. An analysis of the chemical composition of the eucalyptus oil was performed by gas chromatography. The equipment used was a SHIMADZU GC-2010 Plus gas chromatograph, equipped with a SHIMADZU AOC-6000 autosampler, and a SHIMADZU GCMS-QP210 Ultra mass spectrometry detector.
The viscosity and density of the eucalyptus oil were determined using the Ostwald viscometer and the pycnometer, respectively.
The method described in the Peruvian Technical Standard (NTP) 319.075 was used to determine the refractive index of essential oils, using an Abbe refractometer.
The acidity was determined following the procedure described in NTP 319.088 for essential oils, using the acid index (expressed in milligrams of potassium hydroxide required to neutralize the free acids present in one gram of essential oil).
To evaluate antimicrobial activity, Kirby-Bauer tests were performed under standardized conditions. Two nutrient agar plates were prepared. Escherichia coli was inoculated onto one plate and Staphylococcus aureus onto the other, both at a concentration of 1x10^8 CFU/mL. Two wells with a diameter of 6 mm were made on each plate. One well was used to place the gentamicin antibiotic disk as a positive control. The other well received 40 µL of eucalyptus oil. The plates were incubated at a temperature between 35 and 37 °C for 19 hours. Subsequently, the diameter of the inhibition zones was measured.
For the antifungal activity test, a sensitivity test of fungi such as *Rhizopus stolonifer* and *Aspergillus niger* was performed on a sample of eucalyptus oil and an antibiotic such as nystatin, as a positive control, using the Kirby Bauer method.

RESULTS AND DISCUSSION

Taxonomic analysis

According to the study carried out by the San Marcos Herbarium of the sample consisting of branches, leaves and flowers, which were collected during the flowering of the plant. The sample was studied and classified as: *Eucalyptus globulus* Labill. and has the following taxonomic position, according to the APG IV Classification System (2016):
Order: Myrtales Juss. Ex Bercht & J. Presl

Family: Myrtaceae Juss.
Genus: Eucalyptus L'Hér.
Species: *Eucalyptus globulus Labill*.

Structural analysis

Figure 2 provides information on the chemical composition and molecular structure of eucalyptus oil. There is an absorption band at a wavenumber of 3448 cm^{-1} due to the hydroxyl groups present in alcohols, phenols, and carboxylic acids. It presents prominent bands at 2923 cm^{-1} and 2958 cm^{-1}, due to the stretching vibrations of the C-H bonds in the methyl ($-CH_3$) and methylene ($-CH_2$) groups present in the components of eucalyptus oil, such as terpenes. The band at 1635 cm^{-1} corresponds to the stretching vibrations of the C=C bond present in the unsaturated compounds of the oil, such as monoterpenes and sesquiterpenes. The absorption bands at 1460 cm^{-1} and 1383 cm^{-1} are associated with the deformation vibrations of the C-H bonds in the methyl and methylene groups, respectively. The bands at 1103 cm^{-1} and 1035 cm^{-1} are attributed to the stretching vibrations of the C-O bonds present in oxygenated compounds, such as alcohols, ethers, or esters, which are common components in eucalyptus oil. Finally, the band at 668 cm^{-1} is attributed to the out-of-plane deformation vibrations of the aromatic C-H bonds, suggesting the presence of aromatic compounds in eucalyptus oil.

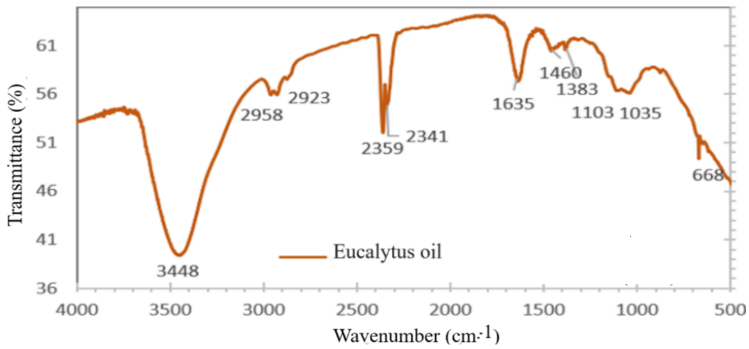

Figure 2. Infrared spectrum of eucalyptus oil (*Eucalyptus globulus*)

Analysis by Gas Chromatography - Mass Spectrometry.

Eucalyptus oil is a complex mixture of more than one hundred different compounds, and the exact composition may vary depending on the eucalyptus plant species, the part of the plant used, and the extraction method. In the present study, the chemical composition of eucalyptus oil was determined by gas chromatography-mass spectrometry (GC-MS). Table 1 reports the twenty-eight components identified in eucalyptus oil. Eucalyptus oil is composed of several compounds, monoterpenes and their oxygenated derivatives. Among the four most abundant are: 1,8-Cineole or Eucalyptol (67,39%), considered the main component of eucalyptus oil and an oxygenated monoterpene that gives it its characteristic fresh, medicinal, and camphor-like aroma; α-Pinene (20,73%), a bicyclic monoterpene very common in the essential oils of conifers and some aromatic plants; α-Terpinyl acetate (1,73%), an ester of α-terpineol with a fruity, slightly floral and herbaceous aroma; α-Terpineol (1,22%), an oxygenated monoterpene with a floral, slightly pungent and balsamic aroma, and L-β-Pinene (0,86%), an isomer of α-pinene, also a bicyclic monoterpene with similar aromatic properties (See Table 1). It is important to note that the extraction method influences the chemical composition of these oils, although it should be emphasized that climatic and genetic conditions also impact the species. Various studies have evaluated the presence of eucalyptol in the essential oil of eucalyptus from different species and origins as the major component. In *eucalyptus globulus* leaves, percentages of eucalyptol of 72,3%[9], 68,2%[10], a majority value of 75%[11], and 63,1%[12] of eucalyptol were found. In conclusion, through the present study, the presence of eucalyptol as one of the main components of eucalyptus essential oil is verified, with percentages ranging between 63% and 75% in the *Eucalyptus globulus* species.

The presence of α-pinene and L-β-pinene also exhibits activity against various bacteria, including *Escherichia coli*. On the other hand, the chemical composition of the essential oil varies significantly with the age of the leaves, and they are found with a higher content of eucalyptol in mature leaves[6]; and with a higher content of α-pinene in young leaves[8].

Table 1. Chromatographic data of the components of eucalyptus essential oil

Peak	Retention Time	Area %	Name
1	8,00	20,73	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
2	10,68	0,86	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
3	12,38	0,69	Beta-Myrcene
4	16,93	67,39	Eucalyptol
5	20,59	0,43	Gamma-Terpinene
6	39,42	0,28	Terpinen-4-ol
7	40,81	1,22	Alpha-Terpineol
8	45,97	0,36	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
9	50,67	0,05	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-, acetate
10	51,05	0,73	Alpha-Terpinyl acetate
11	51,99	0,05	Isocedrene
12	52,94	0,25	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-
13	53,73	0,28	1H-Cyclopropa[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-
14	54,70	0,03	1H-Cyclopropa[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-
15	55,05	1,62	Aromadendrene
16	56,03	0,44	(1R,9R,E)-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene
17	57,28	0,05	Alpha-Farnesene
18	57,59	0,34	Gamma-Murolene
19	58,57	0,04	Aromadendrene, dehydro-
20	60,22	0,38	(1aR
21	60,51	0,14	2,5-Cyclodiol
22	60,96	0,10	1H-Cyclopropa[e]azulene-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1S-
23	61,23	1,66	1H-Cyclopropa[e]azulene-4-ol, decahydro-1,1,4,7a-tetramethyl-, [1aR-(1a.alpha.,4.alpha.,7.beta.)]-
24	61,51	0,56	2,6-Cyclodiol
25	61,61	0,09	2-(3R,3aR,3bS,4R,7R,7aS)-3,7-Dimethyloctahydro-1H-cyclopenta[a][3]k
26	61,94	0,16	2-(4aS,8R,8aR)-4a,8-Dimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalene-2
27	62,49	0,05	5,8,11,14-Eicosatetraenoic acid, ethyl ester, (all-Z)-
28	63,21	0,21	3,5,11,23-Trimethyl-2,3,4,5,6,8,8-heptacyclo[3.3.1.12.9.03,7.09,13]heptalen-2-yl)propan-2-ol

Physicochemical Properties

In this research, Eucalyptus oil was a transparent, light-yellow liquid and was obtained with an absolute density of 0,906 g/mL (ISO 770:2002, which establishes a range of 0,905 g/cm^3 to 0,925 g/cm^3 at 20°C); absolute viscosity of 2,28 cp; refractive index of 1,4622 (20°C) and an acidity index of 4,38 mg KOH/g of Essential Oil, a value slightly lower than the values reported by other authors[13].
Finally, the dynamic viscosity was 2,28 cp at 20°C, which was determined by Ostwald Viscometry.
Eucalyptus globulus essential oil has an acidity index of approximately 5,2 mg KOH/g of oil, which was attributed to the presence of acetic acid and butyric acid[14]. An acidity index value of approximately 4,8 mg KOH/g of oil, which was related to the presence of acetic acid and propionic acid[15]; 5,5 mg KOH/g of oil associated with the presence of acetic acid and butyric acid.

Extraction Yield

To determine the extraction yield, equation 1 was used.

$$Extraction\ yield\ (\%) = \frac{Essential\ oil\ mass\ (g)}{leaf\ mass\ (g)} * 100\% \qquad (1)$$

Figure 3 shows the graph of the volume of oil extracted vs. extraction time. It can be observed that as the employed mass increases, the greater the volume of oil extracted and at an extraction time of 80 min, the maximum extraction volume is reached.

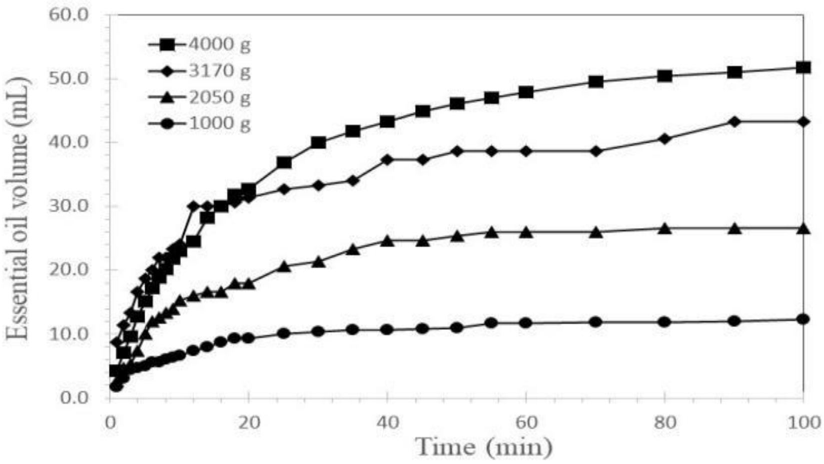

Figure 3. Graph of the volume of eucalyptus essential oil extracted vs. extraction time.

Likewise, the yield of eucalyptus essential oil is influenced by the age of the tree and the environmental conditions of the site where it grows[16].
Table 2 shows the results of the apparent density, percentage yield, extracted volume of oil for all experimental runs.

Table 2. Results of apparent density, yield and extracted oil volume.

Masa (g)	Apparent density	Yield (%)	Extracted Volumen	Oil mass. (g)
4000	0.2690	1.28	56.32	51.03
3950	0.2739	1.09	47.33	42.88
3170	0.2692	1.24	43.33	39.26
2050	0.1352	1.18	26.67	24.16
1000	0.1229	1.15	12.67	11.48
1000	0.1545	1.09	12.00	10.87

Analysis of Variance for Yield

Table 3 shows the analysis of variance for yield. It was found that the amount of mass has no significant effect on yield, with a $p > 0,05$. However, it is important to note that the mass of 4000g of leaves was necessary to completely fill the extraction chamber and with this mass of leaves a higher volume of eucalyptus oil was obtained with a value of 56,32 mL.

Table 3. Analysis of variance for oil extraction yield.

Source	df	Adj SS	MC Adj.	F-value	p-value
mass	4	0,028483	0,007121	3,96	0,358
Error	1	0,001800	0,001800		
Total	5	0,030283			

Significance level (p<0,05)

Analysis of variance for oil extraction volume

Table 4 shows the analysis of variance for volume. It was found that the amount of mass has a significant effect on the volume of oil extracted. However, it is important to note that the mass of 4000g of leaves is necessary to completely fill the extraction chamber and thus obtain a higher volume of eucalyptus oil with a value of 56,32 mL.

Table 4. Analysis of variance for volume.

Source	df	Adj SS	MC Adj.	F-value	p-value
mass	4	1750,02	437,504	1949,23	0,017*
Error	1	0,22	0,224		
Total	5	1750,24			

*Significance level (p<0,05)

Figure 4 shows that the volume of eucalyptus oil is directly dependent on the amount of mass of eucalyptus leaves.

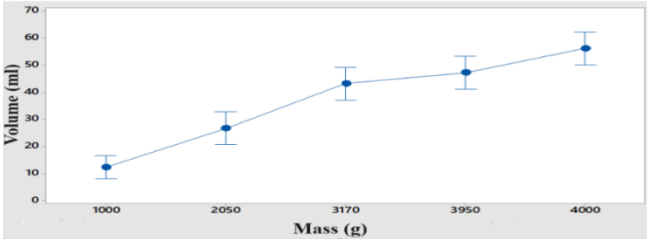

Figure 4. Graph of oil volume vs. mass of eucalyptus leaves.

Effect of leaf moisture on yield.

To study the effect of eucalyptus leaf moisture on yield, fresh leaves containing 60,95% moisture were dried in a convection oven at 40°C for 4 hours and 8 hours, to achieve moisture contents of 41,88% and 12,10%, respectively (See Table 5).
Table 5 shows the results of the extraction yield when using young eucalyptus leaves with different moisture values. It was found that as the moisture of the eucalyptus leaves decreases, the % extraction yield of the oil increases, and it presents a linear relationship. Therefore, by extrapolating the line, there would be an extraction volume of 23,34 ml of essential oil for eucalyptus leaves with a moisture content of 0%, in which a maximum yield of 2,34% would be obtained. It has been found that by decreasing the moisture content of Eucalyptus globulus leaves from 65% to 35%, the yield of essential oil extraction by steam distillation increases from 1,3% to 2,2%[17]. Therefore, it is proven that there is an increase in yield when the leaves are previously dried to decrease the moisture content. However, there may be a change in the chemical composition of the essential oil. The drying temperature of Eucalyptus globulus leaves has a significant impact on the chemical composition of the essential oil[18]. When the leaves were dried at higher temperatures (60°C), a decrease in the content of 1,8-cineole and an increase in the concentration of spathulenol and globulol were observed compared to drying at lower temperatures (25°C). Likewise, it is reported in the literature that the extraction yield of essential oils using dried leaves with a moisture content of 0% is higher than that reported for fresh sample[19].
Eucalyptus oils with similar physicochemical properties, such as density, viscosity, refractive index, and acidity, were obtained. This is attributed to the low drying temperature to which the leaves were subjected before oil extraction.

Table 5. Results of extraction yield for the extraction of eucalyptus oil from eucalyptus leaves with different moisture contents.

Mass (g)	Moisture (%)	Extraction volumen (ml)	Extraction yield (%)
1000	12,10	22,77	2,06
1000	41,88	17,77	1,61
1000	60,95	12,00	1,09

Microbiological analysis.

The antimicrobial efficacy of eucalyptus oil against *Escherichia coli* and *Staphylococcus aureus* microorganisms was evaluated by the Kirby Bauer method (agar diffusion method). Through the antimicrobial efficacy analysis shown in Table 6, the eucalyptus

oil sample presented an inhibition halo against *Staphylococcus aureus* of 28,96 mm. However, it did not show antimicrobial efficacy against Escherichia coli.
The antimicrobial properties are related to the phenols and monoterpenes in its composition, which allows it to interact with the pathogen's cytoplasm, potentially incorporating into the lipids of the cell membrane, destabilizing the bacteria and its ions[20].
The bacterium *Escherichia coli* is a gram-negative bacterium and has an outer membrane composed of phospholipids and lipopolysaccharides. This outer membrane acts as a protective barrier that makes it difficult for antimicrobial substances, including eucalyptus oil, to penetrate.
On the other hand, the bacterium *Staphylococcus aureus* is a gram-positive bacterium and has a different cell membrane structure that facilitates interaction with eucalyptus oil. The absence of an outer membrane in gram-positive bacteria allows for greater exposure of cellular components to eucalyptus oil, increasing the effectiveness of its antimicrobial properties.
The differences in the efficacy of eucalyptus oil against *E. coli* and *S. aureus* can be attributed to the different cell wall structures of these bacteria. *E. coli* has an additional outer membrane that makes it difficult for the antimicrobial components of the oil to penetrate[21]. In contrast, *S. aureus* does not have this outer membrane, making it more susceptible to the action of the oil[22].
Several studies have confirmed that different components of the oil, such as limonene, eucalyptol, alpha-pinene, beta-pinene, and cymene, contribute to this antimicrobial activity. The synergy between these components enhances their individual effect, making eucalyptus oil a promising natural alternative to combat infections caused by *Escherichia coli*.
Through the microbiological study, it has been found that eucalyptus oil has greater antimicrobial efficacy against *R. stolonifer* with an inhibition halo of 21 mm than against *A. niger* (inhibition halo of 16 mm) (Ver Table 6). Its inhibition is of interest for its application in food conservation, since the antifungal property of eucalyptus oil is proven, which would allow the handling and conservation of post-harvest fruits. The antimicrobial effect and strong antifungal activity are mainly due to the high content of 1,8-cineole. Therefore, this finding suggests its potential application as a natural preservative in the food industry.

Table 6. Evaluation of antimicrobial efficacy of eucalyptus oil against microorganisms.

Microorganisms	Diameter of the inhibition halo (mm)	
	Eucalyptus oil	Gentamicina
Escherichia Coli	6,00	19,59
Staphylococcus aureus	28,96	29,03
Microorganisms	Diameter of the inhibition halo (mm)	
	Eucalyptus oil	nystatin
Rhizopus stolonifer.	21,00	26,00
Aspergillus niger.	16,00	24,00

CONCLUSIONS

A light yellow and transparent essential eucalyptus oil was obtained, with an absolute density of 0,906 g/mL, an absolute viscosity of 2,28 cp, a refractive index of 1,4622 and an acidity index of 1,566 mg of KOH per gram of eucalyptus essential oil (AEE).
Eucalyptus essential oil was extracted from leaves of the *Eucalyptus globulus* species using the steam distillation method, with a yield of 1,28%. This yield is not related to the mass of eucalyptus leaves used in the extraction process.
The volume of essential oil obtained increases proportionally to the mass of eucalyptus leaves used in the extraction. 56,32 mL of oil were extracted using 4 kg of leaves in a process that lasted approximately 80 minutes.
The obtained eucalyptus essential oil contains 67,29% 1,8-cineole or eucalyptol, along with other monoterpenes, oxygenated derivatives, and phenols, which are responsible for its antimicrobial properties against *Staphylococcus aureus*. However, it did not show antimicrobial activity against *Escherichia coli* due to the lack of limonene and cymene in its chemical composition.
Eucalyptus oil showed an antifungal effect against the fungi *Rhizopus stolonifer* and *Aspergillus niger*, which cause the deterioration of different fruits and therefore.

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