



L-L EXTRACTION MIXTURE BETWEEN EUCALYPTUS-PELARGONIUM ESSENTIAL OILS AND ITS EFFECTIVENESS ON *Klebsiella pneumoniae*

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ABSTRACT

Objective: The synergistic or adjuvant effect on the antimicrobial effectiveness of pelargonium essential oil (PEO) in combination with eucalyptus essential oil (EEO) has been the objective of this work.

Theoretical Framework: The small amounts of oil in pelargonium (difficultly extractable by vapor drag) have motivated a liquid PEO-liquid EEO (L-L) extraction. EEO has been used as an extractant for PEO, giving a final proportion of 5:2.

Method: Both essential oils were obtained by steam stripping with water. Analysis by GC-FID chromatography gave 80.5% w/w of 1,8-cineole (eucalyptol), 3.05% w/w of limonene, 5.4% w/w of α -pinene, and antioxidant capacity of 76.0%. For the ME-E5P2 mixture, 56.4% of 1,8-cineole and 9.30% of citronellol were obtained from the essential oil of *Pelargonium spp.* with an antioxidant capacity of 78.6%. Due to the small amount of PEO (0.033% in the wet base of the plant) compared to EEO (5%), a direct soxhlet extraction of PEO with EEO as extractant was developed, until obtaining a proportion equivalent to EEO (5): PEO (2), so that volatility losses (typical of hydro distillation) were avoided. This mixture has been called the L-L EXTRACTION MIXTURE (ME-E5P2).

Results and Discussion: In vitro, the effectiveness of ME-E5P2 against *Klebsiella pneumoniae* had better effectiveness against *K. pneumoniae* ATCC 13883 with a minimum bacterial concentration (MBC) of 60.5 mg/mL, a minimum inhibitory concentration (MIC) of 130 mg /mL and an excellent antimicrobial sensitivity (AMS) between 15.0 to 29.0 mm compared to Imipenem and oxacillin. It was found that the combination of the essential oils of *Eucalyptus globulus Labill* and *Pelargonium spp.* in a ratio of 5:2 has better antimicrobial effectiveness against *Klebsiella pneumoniae*.

Research Implications: The practical and theoretical implications of this research are discussed, providing information on how the results can be applied or influence practices in the field of medicine and biology.

Originality/Value: The relevance and value of this research is evident in providing an efficient and natural alternative to the indiscriminate use of chemical antibiotics, avoiding microbial resistance.

Keywords: Effectiveness, Essential Oil, Eucalyptus, Pelargonium, *Klebsiella pneumoniae*.

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MISTURA DE EXTRAÇÃO L-L ENTRE EUCALIPTO-PELARGÔNIO ÓLEOS ESSENCIAIS E SUA EFICÁCIA SOBRE *Klebsiella pneumoniae*

RESUMO

Objetivo: O efeito sinérgico ou adjuvante na eficácia antimicrobiana do óleo essencial de pelargônio (PEO) em combinação com óleo essencial de eucalipto (AEE) foi o objetivo deste trabalho.

Referencial Teórico: As pequenas quantidades de óleo de Pelargonium (difícil de extrair por extração a vapor) motivaram uma extração de PEO líquido-EEO líquido (L-L). O EEO tem sido utilizado como extrator de PEO, dando uma proporção final de 5:2

Método: Ambos os óleos essenciais foram obtidos por decapagem a vapor. A análise por cromatografia GC-FID deu 80,5% p/p de 1,8-cineol (eucaliptol), 3,05% p/p de limoneno, 5,4% p/p de α -pineno e uma capacidade antioxidante de 76,0%. Para a mistura ME-E5P2 foram obtidos 56,4% de 1,8-cineol e 9,30% de citrônol a partir do óleo essencial de Pelargonium spp. com capacidade antioxidante de 78,6%. Devido à pequena quantidade de PEO (0,033% na base úmida da planta) em comparação com EEO (5%), foi desenvolvida uma extração direta de PEO em soxhlet com EEO como extratante, até obter uma proporção equivalente a EEO (5):PEO (2), para que fossem evitadas perdas de volatilidade (típicas da hidrodestilação). Esta mistura foi chamada de MISTURA DE EXTRAÇÃO L-L (ME-E5P2).

Resultados e Discussão: In vitro, a eficácia do ME-E5P2 contra *Klebsiella pneumoniae* teve melhor eficácia contra *K. pneumoniae* ATCC 13883 com concentração bacteriana mínima (MBC) de 60,5 mg/mL, concentração inibitória mínima (CIM) de 130 mg/mL e excelente sensibilidade antimicrobiana (AMS) entre 15,0 a 29,0 mm em comparação com Imipenem e Oxacilina. Verificou-se que a combinação dos óleos essenciais de *Eucalyptus globulus* Labill e *Pelargonium* spp. na proporção de 5:2 apresenta melhor eficácia antimicrobiana contra *Klebsiella pneumoniae*.

Implicações da Pesquisa: São discutidas as implicações práticas e teóricas desta pesquisa, fornecendo informações sobre como os resultados podem ser aplicados ou influenciar práticas no campo da medicina e da biologia.

Originalidade/Valor: A relevância e o valor desta pesquisa são evidentes ao fornecer uma alternativa eficiente e natural ao uso indiscriminado de antibióticos químicos, evitando a resistência microbiana.

Palavras-chave: Eficácia, Óleo Essencial, Eucalipto, Pelargonium, *Klebsiella pneumoniae*.

MEZCLA DE EXTRACCIÓN L-L ENTRE ACEITES ESENCIALES DE EUCALIPTO- PELARGONIUM Y SU EFICACIA SOBRE *Klebsiella pneumoniae*

RESUMEN

Objetivo: El efecto sinérgico o coadyuvante sobre la eficacia antimicrobiana del aceite esencial de pelargonio (PEO) en combinación con el aceite esencial de eucalipto (AEE) fue el objetivo de este trabajo.

Marco Teórico: Las pequeñas cantidades de aceite del Pelargonio (difícilmente extraíble mediante arrastre de vapor) han motivado una extracción de PEO líquido-EEO líquido (L-L). Se ha utilizado EEO como extractante de PEO, dando una proporción final de 5:2

Método: Ambos aceites esenciales se obtuvieron mediante decapado con vapor de agua. El análisis mediante cromatografía GC-FID dio un 80,5 % p/p de 1,8-cineol (eucaliptol), un 3,05 % p/p de limoneno, un 5,4 % p/p de α -pineno y una capacidad antioxidante del 76,0 %. Para la mezcla ME-E5P2 se obtuvo 56,4% de 1,8-cineol y 9,30% de citrônol a partir del aceite esencial de Pelargonium spp. con una capacidad antioxidante del 78,6%. Debido a la pequeña cantidad de PEO (0.033% en la base húmeda de la planta) en comparación con EEO (5%), se desarrolló una extracción soxhlet directa de PEO con EEO como extractante, hasta obtener una proporción equivalente a EEO (5):PEO (2), de modo que se evitaron pérdidas de volatilidad (típicas de la hidrodestilación). Esta mezcla se ha denominado MEZCLA DE EXTRACCIÓN L-L (ME-E5P2).



Resultados y Discusión: In vitro, la efectividad de ME-E5P2 contra *Klebsiella pneumoniae* tuvo mejor efectividad contra *K. pneumoniae* ATCC 13883 con una concentración bacteriana mínima (MBC) de 60,5 mg/mL, una concentración mínima inhibitoria (CIM) de 130 mg/mL y una excelente sensibilidad antimicrobiana (AMS) entre 15,0 a 29,0 mm en comparación con Imipenem y Oxacilina. Se encontró que la combinación de los aceites esenciales de *Eucalyptus globulus Labill* y *Pelargonium spp.* en una proporción de 5:2 tiene mejor eficacia antimicrobiana contra *Klebsiella pneumoniae*.

Implicaciones de la Investigación: Se discuten las implicaciones prácticas y teóricas de esta investigación, proporcionando información sobre cómo los resultados pueden aplicarse o influir en las prácticas en el campo de la medicina y la biología.

Originalidad/Valor: La relevancia y valor de esta investigación se evidencian en dar una alternativa eficiente y natural frente al uso indiscriminado de antibióticos químicos, evitando la resistencia microbiana.

Palabras clave: Efectividad, Aceite Esencial, Eucalipto, Pelargonium, *Klebsiella pneumoniae*.

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1 INTRODUCTION

In the context of biodiversity in Peru and phytomedicines, some plant species' essential oils (EO) present antimicrobial compounds for treating infectious diseases of the respiratory tract. The EOs of species such as Eucalyptus (*Eucalyptus globulus Labill*) and Pelargonium (*Pelargonium spp.*) from the Cusco region present medicinal properties against acute respiratory infections with anti-cold, expectorant, antiseptic effects, pleasant aroma and probably with antibacterial and antiviral. It is currently used in industrial preparations to combat colds; analgesic and anti-inflammatory properties are attributed to pelargonium leaves (Bussmann & Sharon, 2015).

The interest of the present investigation was determining the in vitro antimicrobial effectiveness of *Klebsiella pneumoniae* of the mixture of the two essential oils by determining the MBC, MIC, and AMS. Due to the small amount of PEO (0.033% on the wet basis of the plant) compared to EEO (5%), a direct liquid-liquid (L-L) extraction in soxhlet of PEO with EEO as extractant was developed until an equivalent proportion was obtained to EEO (5): PEO (2) so that volatility losses (typical of hydro distillation) are avoided. This mixture has been called the L-L EXTRACTION MIXTURE (ME-E5P2)



2 THEORETICAL FRAMEWORKS

2.1 EUCALYPTUS GLOBULUS LABILL

The eucalyptus is a tall tree with more than 700 species; 300 contain essential oils in their leaves. Eucalyptus trees, sometimes exceeding 40 meters in height, have a straight and poorly branched trunk, usually letting off thin layers of bark, aromatic, simple, alternate, entire leaves. Flowers and fruits are relatively small and unremarkable. They belong to the botanical family *Myrtaceae*, a family of trees rich in essential oils; they are native to Australia and Tasmania. Many introductions of different species have been made in America, such as *Eucalyptus globulus Labill*. The various species of eucalyptus are used interchangeably as popula medicine or for uses as wood.

These trees have evergreen, grayish-green leaves, and evident foliar dimorphism. Those located on young branches or in grafts of old branches are opposite, sessile, with an oval and thin horizontal blade covered by a waxy layer. Those situated on old branches are alternate, with a twisted and wrinkled petiole (2-3 cm), entire, leathery, glabrous, with an elliptical or slightly falciform-shaped blade, relatively thick (up to 25 cm in length and 5 cm in width) , which hangs vertically. The central nerve is yellowish-green, and the lateral ones are thinner and anastomose at the edge, forming a continuous line. Both on the upper side and on the underside, there are small, irregularly distributed dark brown spots and glands secreting the essential oil. It has solitary, short-pedunculated flowers, with absent sepals and welded petals forming a hemispherical structure that, when detached, reveals numerous long stamens curved inwards. As fruits, it has globose capsules with four ribs, more acute at the base.

Figure 1

Eucalyptus globulus Labill plant



Source: The Vargas Herbarium (CUZ) of the National University of San Antonio Abad of Cusco



Eucalyptus essential oil contains 1,8-cineole (45.4%), limonene (17.8%), p-cymene (9.5%), γ -terpinene (8.8%), α -pinene (4.2%) and α -terpineol (3.4%), while, in the vapor, 1,8-cineole (34.6%), limonene (29.9%), p-cymene (10, 5%), γ -terpinene (7.4%), α -pinene (4.0%) and α -phellandrene (2.4%) were identified (Tyagi & Malik, 2011). They are also finding two monoterpene glycosides, conjugated with gallic acid [globulusin A, B], along with four known compounds, cypellocarpin A, eucaglobulin, cuniloside and (1 S, 2 S, 4 R) - trans - 2-hydroxy-1,8-cineole β - d-glucopyranoside, determined by spectroscopic analysis (Hasegawa et al., 2008).

The effectiveness of 1,8-cineole for acute bronchitis is also related to its ability to increase mucociliary clearance and its anti-inflammatory activity. The molecular mechanisms associated with plant extracts' antiviral effects may vary between viruses. However, the potential of the plant extract to boost the human body's inherent antiviral defense involving an intricate immune system could utilize common pathways. Thus, the immunostimulatory properties of plant extracts with antiviral properties have been explored. Mukhtar et al., 2008).

2.2 PELARGONIUM SPP.

An herbaceous, perennial plant, 30 - 50cm high. Erect stem with alternate, simple, aromatic leaves, lobular margin. Its veins indicate it is palmate, and its leaf base is cordate. The flowers are in an umbel; they can be simple or double; they are showy and aromatic; the calyx has five free sepals; the corolla has five free petals, 10-15 stamens (not all fertile), and a super ovary.

Pelargonium leaves are used in throat inflammation, diarrhea, bleeding, and toothache cases. Other uses: ornamental and as an insecticide to eliminate fleas. Its form of preparation is infusion; gargling is recommended for throat inflammations. In case of pain, apply the ground leaves to the affected area.



Figure 2

Pelargonium spp. plant



Source: The Vargas Herbarium (CUZ) of the National University of San Antonio Abad of Cusco

The essential oils of *Pelargonium* contain thirty-two compounds that constitute 99.23% of the essential oil identified; the main ones were citronellol (29.9%), trans-geraniol (18.0%), 10-epi- γ - eudesmol (8.27%), isomanthone (5.44%), linalool (5.13%), geranyl acetate (4.52%), γ -Cadinene (2.89%), geranyl butyrate (2.53%), geranyl tiglate (2.50%) and gemacrene D (2.05%). Geranium essential oil is rich in oxygenated components and commercial rhodinol (linalool + citronellol + geraniol). C galloyl glycosidic flavones, non-galloyl flavones, phenolics (flavonoids and tannins), benzoic and cinnamic acid derivatives were reported in the aerial parts, and the diterpene (reniformin), which has a new diterpene skeleton linked to a unique poxyphenetanesulfonic acid in the roots of *Pelargonium reniform*—the new ellagitannins with a 1- C₄. Glucopyranose core (pelargoniins) and 4-allyl-2,5-dimethoxyphenol-1-b-glucoside were also found. Tartaric acid is a characteristic component of the genus *Pelargonium*.

The occurrence of coumarin sulfates (5,6-dimethoxycoumarin 7-sulfate and a mixture of 6- and 8-monosulfate 6,8-dihydroxy-5,7-dimethoxycoumarin), coumarin glycosides and proanthocyanidins was limited to *P. Sidoides*. The presence of structurally related umckalin (coumarins) as an active ingredient in Umckaloabo (herbal extract derived from the plant *Pelargonion Sidoides*) decreases upper respiratory tract infection and flu, alleviates the severity of symptoms by improving the immune system. Antimicrobial activity of *Pelargonium* extracts and its constituents was reported on (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*), fungi (*Microsporum canis*, *Microsporum gypseum*, *Aspergillus*



fumigatus, *Mucor racemosus*, *Rhizopus nigricans*) and pathogens, as well as opportunistic yeasts such as *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Cryptococcus neoformans* (J et al., 2011). *Pelargonium odoratissimum* (scented geranium), from the leaves and flowers of the species, presents flavonoids such as quercetin, kaempferol, and myricetin. The essential oil of its leaves is rich in methyl-eugenol, limonene, and fencone (Stashenko et al., 2007).

2.3 ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

In various studies carried out, Correa et al. (2009) show that the antimicrobial activity presented by essential oils is due to terpenoids. Following in order of activity the terpenoids that contain alcohol groups, then those that have aldehydes, and finally those that have ketonic groups. In the same way, authors propose that oils with a high percentage of terpenoid compounds of the phenolic type have notable antimicrobial properties. The antimicrobial effects of essential oils are related to their composition of phenols and monoterpenes that they possess due to their direct interaction with the cytoplasm of the pathogen or due to their hydrophobicity that can be incorporated into the lipids of the bacterial cell membrane and cytotoxic effects, which cause damage to the cell membrane (Montero-Recalde et al., 2019b).

Essential oils are not pure compounds but mixtures of a multitude of substances (it can be more than 100 different chemical substances) that are found in different proportions and that together provide the essential oil with its characteristics. On the other hand, essential oil is the product obtained from the plant after physicochemical treatment. Among the components are terpenes, up to 75-90% of the total weight; they contribute little to the overall aroma. Those responsible for the aroma are usually substances that are found in a lower proportion, such as organic compounds with functional groups such as ketone, ester, alcohol, aldehyde, and ether, which are often found in small quantities that make up the aromatic fingerprint of each essential oil (Sanchez, 2006).

Respiratory infections are high-frequency events in the population, defined as a condition that compromises one or more parts of the respiratory system for no more than 15 days. The leading causes are viral and bacterial origin; they are the first cause of antibiotic indication worldwide. Acute respiratory infections (ARI) are classified according to the anatomical site affected as high and low, with the epiglottis being the separation point. Among the high ones, we have rhinopharyngitis, pharyngotonsillitis, sinusitis, and acute otitis media, and the low ones include epiglottitis, laryngotracheobronchitis, bronchiolitis, and pneumonia.



The incidence of mortality from ARI in developing countries is 14-24% in children under five years of age (Valero et al., 2009).

2.4 MINIMUM INHIBITORY CONCENTRATION (MIC) OR BACTERIOSTATIC EFFECT

The minimum concentration of antimicrobial in µg/ml inhibits the visible growth of a microorganism after 24 hours of incubation at 37° C. The MIC has been established as a “Gold Standard” compared to other methods that evaluate antimicrobial susceptibility, in addition to confirming unusual resistance, definitive responses when the result obtained by other methods is indeterminate (Horna et al., 2005).

2.5 MINIMUM BACTERICIDAL CONCENTRATION (MBC) OR BACTERICIDAL EFFECT

The minimum antimicrobial concentration eliminates more than 99.9% of viable microorganisms after a specific incubation time (generally 24 hours) (Horna et al., 2005).

2.6 ANTIMICROBIAL SENSITIVITY (AMS)

Sensitivity tests or antibiograms determine the susceptibility of a microorganism to antimicrobial drugs based on the exposure of a standardized concentration of the germ to these drugs. Sensitivity tests can be done for bacteria, fungi, or viruses (Herrera, 1999).

2.7 LIQUID-LIQUID (L-L) EXTRACTION OF ESSENTIAL OILS

The steam stripping method is frequently used for essential oil contents in green plants greater than 0.1%, as in the case of EEO. For plants with lower contents, solvent extraction is the most appropriate even though there are risks of incorporating contaminants.

The L-L extraction is appropriately adapted to Soxhlet extraction equipment, as shown in Figure 3. The chopped plant material packed in filter paper is introduced into the body, and the weight and volume of the solvent (EEO) is loaded into the balloon. After several cycles of evaporation-condensation-siphoning, the extraction is exhausted in the plant material and with new recharges until an increase in the volume of the solvent is obtained in the required proportion.



3 METHODOLOGY

3.1 MICROBIOLOGICAL MATERIAL:

- *Klebsiella pneumoniae* ATCC 13883 KWIK STIK Microbiology

Essential oils:

- *Eucalyptus globulus* Labill essential oil (50ml) - EEO

The fresh leaves were from the district of San Salvador, province of Calca, Department of Cusco, Coordinates 13°29'29" S / 71°46'30" W 3,118 m altitude. The sample selected for this investigation was taken by simple random steam extraction sampling.

- The leaves of Pelargonium (*Pelargonium spp.*) – PEO

Coming from the district of Wanchaq, province of Cusco, Department of Cusco, Adolfo Guevara Velasco National Hospital coordinates 13°31'33" S / 71°57'31" W 3,340 m altitude. The sample selected for this research was taken by simple random sampling.

- L-L extraction mixture of PEO with EEO: obtained in Soxhlet extraction equipment, according to section 2 criteria, proportion EEO (5): PEO (2). Table 1 shows the laboratory instruments used for extraction, equipment, and culture media.

Table 1

Lab's materials, equipment, and culture media of the study

Lab's materials	Equipment	Culture media
- Analytical balance - Decanting bulb, 500 ml. 2 units - Universal support, two units - Tweezers, four units - Spatula, 1 unit - 10 ml volumetric pipette, four units - Test tubes of 25ml, 50 ml, 100 ml, 250 ml - Beaker of 1000ml, 500ml, 250 ml - 250ml glass funnels - 250 ml Erlenmeyer - 10 ml amber bottles, 30 units with airtight lid - 10 Kg gas cylinder. - 12x15mm test tubes - 10x12mm test tubes - 100x20mm Petri dishes	- Modular essential oil extraction equipment - Steam generator - Soxhlet micro-extraction equipment (25ml) - Analytical balance: Capacity 120 g, precision 0.0001 g, level bubble, and adjustable feet, 41 glass cases with sliding doors and weighed in grams - Stopwatch, 1 unit - Incubator - Autoclave - Bunsen lighter - 10-100ul micropipettes - 100-1000ul micropipettes	- Muller Hintong Agar - Mannitol Salt Agar - Mac Conkey Agar - TSA Broth - TSB agar - Mc Farland scale 0.5%

Source: Own elaboration



3.2 EXPERIMENTAL PLAN

The research used a completely randomized experimental design, with a 2-factor factorial arrangement (AxB) (Table 2), with two levels for factor A and four levels for factor B (Table 3), with three repetitions, making a total of 8 treatments, with 24 experimental units; a Tukey test of means at a significance level of 0.05, according to the following table.

Table 2

Study factors evaluated the effectiveness of eucalyptus and pelargonium essential oil

Study factors	
D _i : Essential oils	C _i : Concentration
d ₁ : Aceite de <i>Eucalyptus globulus</i>	c ₁ : 12.5%
d ₂ : Aceite de <i>Eucalyptus-Pelargonium</i> 5:2	c ₂ : 25%
	c ₃ : 50%
	c ₄ : 100%

Source: Own elaboration

Table 3

Factorial combination of the experiment

Concentration (C _i)	C ₁	C ₂	C ₃	C ₄
Essential oils (D _i)				
d ₁	d ₁ C ₁	d ₁ C ₂	d ₁ C ₃	d ₁ C ₄
d ₂	d ₂ C ₁	d ₂ C ₂	d ₂ C ₃	d ₂ C ₄

Research treatments

T1: d₁C₁ (Eucalyptus oil 12.5 %)

T2: d₁C₂ (Eucalyptus oil 25.0 %)

T3: d₁C₃ (Eucalyptus oil 50.0 %)

T4: d₁C₄ (Eucalyptus oil 100 %)

T5: d₂C₁ (Eucalyptus oil plus pelargonium oil 5:2 al 12.5 %)

T6: d₂C₂ (Eucalyptus oil plus pelargonium oil 5:2 al 25.0 %)

T7: d₂C₃ (Eucalyptus oil plus pelargonium oil 5:2 al 50.0 %)

T8: d₂C₄ (Eucalyptus oil plus pelargonium oil 5:2 al 100 %)



3.2.1 Physical-chemical determinations and in vitro effectiveness of the essential oils of *Eucalyptus globulus* Labill and Pelargonium sp. envelope *Klebsiella pneumoniae* ATCC 13883

The physical-chemical analysis and the M.I.C., C.M.B., and S.A.M. tests of the Essential Oils of *Eucalyptus globulus* Labill and *Pelargonium* spp. were carried out in the "FRACTAL QUIMICOS E.I.R.L." Laboratory.

3.2.2 Determination of the in vitro effectiveness of the essential oils of *Eucalyptus globulus* Labill and Pelargonium spp. Envelope *Klebsiella pneumoniae* ATCC 13883

Activation of A.T.C.C. strains. The strain of *Klebsiella pneumoniae* ATCC 13883 KWIK STIK Microbiology was activated according to the manufacturer's recommendations, for which the following steps were followed:

- The swab that comes with the ATCC strain was saturated in the hydrated material and transferred into selective culture media for each microorganism.
- For *Klebsiella pneumoniae* ATCC 13883 KWIK STIK Microbiologist on Mac Conkey agar.
- It was incubated at 35-37 °C for 24-48 hours.

3.2.3 Determination of the Minimum Inhibitory Concentration (M.I.C.) modified in this research (Gil et al., 2012)

After activation, the ATCC strains were pre-hydrated in B.H.I. broth and incubated for 24 hours at 37°C until reaching the standard turbidity of 0.5% of the Mac Farland scale equivalent to 1.5×10^8 cfu/ml.

For the dilutions, the macro dilution technique in tubes was used with some adaptations because it is a natural product different from common antibiotics, carried out by Gil et al. (2008) and proceeded as follows:

- For each ATCC bacteria used, a battery of 10 tubes of 2ml capacity was prepared with 200µl of B.H.I. broth to make 10-point dilutions, a negative control, and a positive control.
- Dilutions of the essential oil of *Eucalyptus globulus* Labill. They were prepared in proportions of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$, $\frac{1}{256}$.



- In the same way, the essential oil of *Eucalyptus globulus* L. and *Pelargonium spp.*, in a 5:2 proportion, proportions of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$, $\frac{1}{256}$.
- Then, 10 μ l of the A.T.C.C. strain suspension at 0.5% of the Mac Farland Escla was added to each tube and incubated at 37°C for 24-48 hours.
- Turbidity observation was carried out in each tube compared to the controls.
- Subsequently, 10 μ l of each tube was inoculated onto plates with the selective medium of Mac Conkey Agar for *Klebsiella pneumoniae* ATCC 13883
- It was incubated at 37°C for 24-48 hours.
- Colony counting was then performed to determine the Bacteriostatic effect (M.I.C.) using the following criteria:

-

+++ > 100,000 cfu/ml

++ 50,000 – 100,000 cfu/ml

+ <50,000 cfu/ml

- 0 cfu/ml

(1)

3.2.4 Determination of the Minimum Bactericidal Concentration (M.B.C.), according to Gil (2012) modified in this research

- For each A.T.C.C. bacteria used, a battery of 10 tubes of 2ml capacity was prepared with 200 μ l of B.H.I. broth to make 10-point dilutions, a negative control, and a positive control.
- Dilutions of the essential oil of *Eucalyptus globulus* Labill. They were prepared in proportions of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$, $\frac{1}{256}$.
- In the same way, the essential oil of *Eucalyptus globulus* L. and *Pelargonium spp.*, in a 5:2 proportion, proportions of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$, $\frac{1}{256}$.
- Then 10 μ l of the A.T.C.C. strain suspension at 0.5% of the Mac Farland Scale was added to each tube and incubated at 37°C for 24-48 hours.
- Turbidity observation was carried out in each tube compared to the controls.
- Subsequently, 10 μ l of each tube was re-inoculated with 2ml of B.H.I. broth previously prepared in 10ml capacity tubes.
- It was incubated at 37°C for 24-48 hours.



- Then, the turbidity reading was carried out to determine the bactericidal effect (C.M.B.) using the following criteria: + Apparent turbidity, - No turbidity

3.2.5 Antimicrobial Sensitivity (SAM), according to the dug-well method (Castresan et al., 2013) modified in the present research

- Preparation of concentrations of *Eucalyptus globulus Labill* essential oil. Concentrations of 100%, 75%, 50%, 25%, and 12.5% diluted in absolute ethanol were prepared from the essential oil.
 - Preparation of concentrations of *Eucalyptus globulus Labill* and *Pelargonium spp.* essential oil in a 5:2 proportion. Concentrations of 100%, 75%, 62.5%, 50%, 37.5%, 25%, and 12.5% diluted in absolute ethanol were prepared from the mixture of essential oils in a 5:2 proportion.
 - After activating the ATCC strains in selective agar for each one.
 - A suspension was prepared with 3-5 colonies in 5ml of TSB broth incubated at 37°C for 2-5 hours compared to the Mac Farland scale of 0.5%.
 - Three 5.5mm diameter perforations were made in previously prepared Muller Hinton agar at 22mm from each well.
 - Each ATCC strain was shown homogeneously by spreading it on the surface with the help of a sterile swab.
 - 50µl of each prepared concentration of the essential oils of *Eucalyptus globulus* and *Eucalyptus globulus L.* and *Pelargonium sp.* were applied. In a 5:2 ratio, in triplicate on plates with Muller Hinton agar.
- They were incubated at 37°C for 24-72 hours.

4 RESULTS AND DISCUSSIONS

4.1 IDENTIFICATION OF BOTANICAL SPECIES

The Vargas Herbarium (CUZ) of the National University of San Antonio Abad of Cusco identified the botanical species under study in this research as:

- *Eucalyptus globulus Labill.*
- *Pelargonium spp.*



4.2 EXTRACTION OF ESSENTIAL OILS

The optimal results found in the essential oil extraction process by the steam drag method are presented below:

Table 4

Optimal parameters found for the extraction of eucalyptus essential oil by Steam Drag

Parameter	Values
In the boiler-mass flow of propane gas fuel	2.8Kg/h
Volume of water in the extraction chamber =	6 liters
Optimal temperature in distillation head	98-105°C
Condensate temperature	19°C
First condensation start time	20 min
Volume of flowered water (hydrolate)	1,400 ml
Total extraction time per run	1 hour
Weight of chopped raw material of eucalyptus leaves	3Kg
Yield of eucalyptus oil from 3 Kg. of eucalyptus leaves	25ml/3Kg

Source: Own elaboration

Table 5

Optimal parameters found for the extraction of essential oil by pelargonium by Steam Drag.

Essential oil	Efficiency
<i>Eucalyptus globulus</i> Labill	0.833%
<i>Pelargonium</i> spp.	0.005 %

Source: Own elaboration

4.3 HPLC-DAD AND GC-FID CHEMICAL ANALYSIS OF AEE AND ME-E5P2 ESSENTIAL OILS

Table 6

Analysis results

ESSENTIAL OILS	Total Flavonoids ^a (%p/p)	Total Phenols ^b (%p/p)	Antioxidant capacity ^c DPPH	1,8 cineol ^d (%p/p)	Citronelol ^e (%p/p)
Eucalyptus (EEO) (100:0)	ND	ND	76,0 ± 2,0	80,5 ± 1,0	-
Eucalyptus: Pelarg (E5P2) (70:30) = (5:2)	ND	ND	78,6 ± 2,0	56,4 ± 0,7	9,30 ± 0,06

ND: LD = 000010 %.

^{a, b} Analysis by HPLC-DAD chromatography

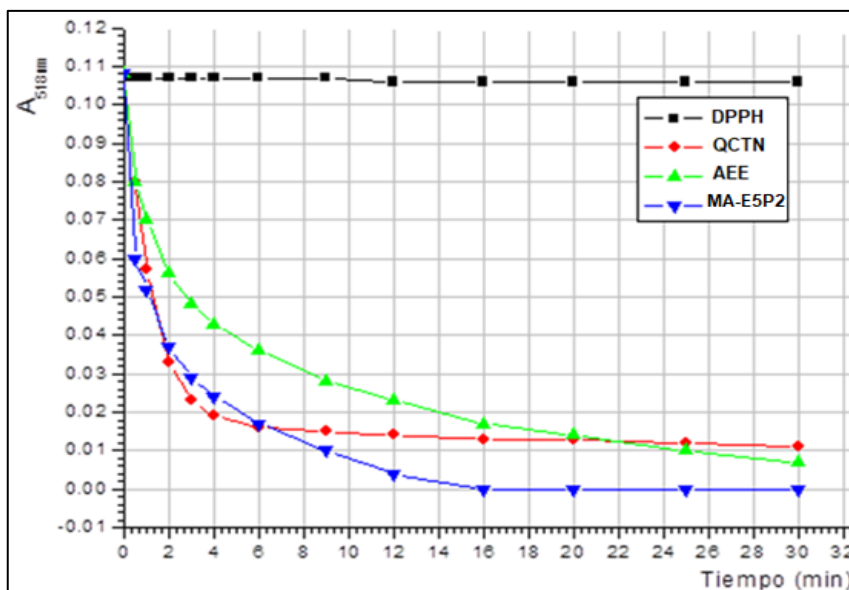
^c Antioxidant capacity determined after 30 minutes and compared to Quercetin.

^{d, e} Analysis by gas chromatography GC-FID.



Figure 3

Kinetic determination in an ethanolic medium of the antioxidant capacity



Kinetic determination in an ethanolic medium of the antioxidant capacity of AEE and MA-E5P2 in comparison with QCTN at 30 minutes. Initial concentrations in the reaction volume: DPPH = 20ppm; QCTN = 2.00 ppm; AEE = 2.50ppm and ME-E5P2 = 2.50ppm. Measurements in VISIBLE spectrophotometer at 518nm

4.4 EFFECTIVENESS OF THE MIXTURE OF ESSENTIAL OILS OF *EUCALYPTUS GLOBULUS LABILL* AND *PELARGONIUM SPP.* IN PROPORTION 5:2 (MA-E5P2) ON *KLEBSIELLA PNEUMONIEAE*

4.4.1 Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration of the essential oil Eucalyptus-Pelargonium 5:2 on *Klebsiella pneumoniae* ATCC 13883 was found to be 1/16 (60.5mg/ml) at 24 hours, as shown in Table 7 and Figure 5.

Table 7

Minimum Bactericidal Concentration for Klebsiella pneumoniae ATCC 13883 with Eucalyptus-Pelargonium essential oil 5:2

Time	Treatments									
	Pure	1/2	1/4	1/8	1/16	1/32	1/64	1/128	C+	C-
24h	-	-	-	-	+	+	+	+	+	-
48h	-	-	-	-	+	+	+	+	+	-

(+) Apparent turbidity (-) No turbidity



Figure 5

MBC of Eucalyptus-Pelargonium essential oil



MBC of Eucalyptus-Pelargonium essential oil 5:2 against *Klebsiella pneumoniae*. Turbidity is observed from concentration 1/16

4.5 MINIMUM INHIBITORY CONCENTRATION (MIC)

The Minimum Inhibitory Concentration of the essential oil of Eucalyptus-Pelargonium 5:2 on *Klebsiella pneumoniae* ATCC 13883 reached 1/8 (129.6 mg/ml) at 24 hours, as shown in Table 8 and Figure 6.

Table 8

Minimum Inhibitory Concentration for Klebsiella pneumoniae ATCC 13883 with Eucalyptus-Pelargonium essential oil 5:2

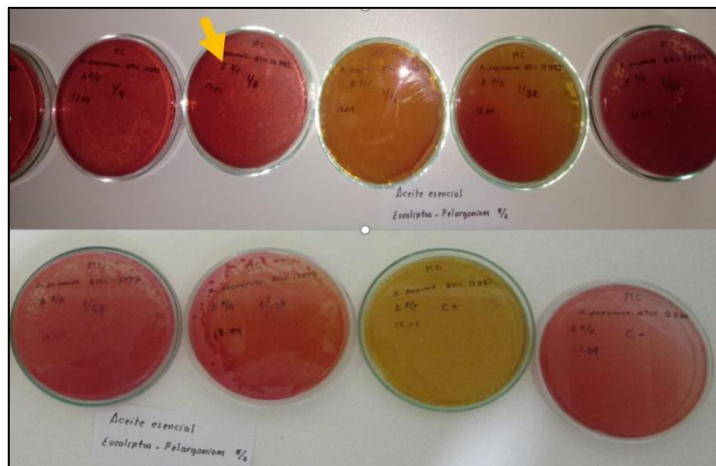
Time	Treatments									
	Pure	1/2	1/4	1/8	1/16	1/32	1/64	1/128	C+	C-
24h	-	-	-	-	++	++	+++	+++	+++	-
48h	-	-	-	-	++	++	+++	+++	+++	-

(-) 0 ufc/ml, (+) <50,000 ufc/ml, (++) 50,000-100,000 ufc/ml, (+++) >100,000 ufc/ml



Figure 6

MIC of Eucalyptus-Pelargonium essential oil



MIC of Eucalyptus-Pelargonium essential oil 5:2 against *Klebsiella pneumoniae*

4.6 ANTIMICROBIAL SENSITIVITY

The results of the in vitro antimicrobial sensitivity carried out using inhibition zones of the essential oils of Eucalyptus-Pelargonium 5:2 is presented below: The in vitro antimicrobial activity of Eucalyptus-Pelargonium Essential Oil 5:2 against *Klebsiella pneumoniae* ATCC 13883 showed a more excellent significant inhibition zone of 15.01mm – 29.04mm at 25% - 50% concentration at 50%, which is close to the control of Imipenem of 43.23mm and at a concentration of 62.5% or more it surpasses the control, the distilled water blank maintains halos of 5.5mm. In comparison, control Ethanol (Control Et-OH) used as diluent found a slight halo of 7.39mm, as shown in Table 9 and Figure 7.



Table 9

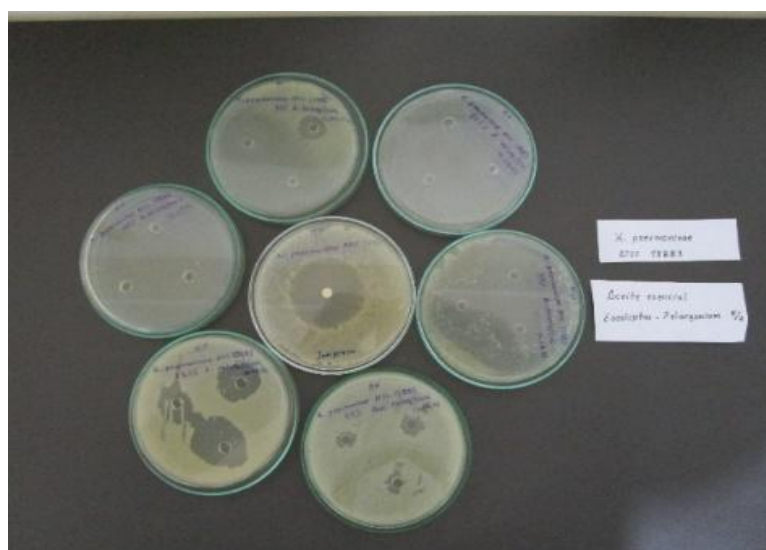
Inhibition zones of the in vitro antimicrobial activity of the essential oil Eucalyptus-Pelargonium

% essential oil	Inhibition diameter (mm) against <i>Klebsiella pneumoniae</i> ATCC 13883			Average
	1	2	3	
100	47	47	47	47.00
75	46.24	46.39	50.56	47.73
62.5	46.28	47.68	48.95	47.64
50	34.35	36.15	41.29	37.26
37.5	34.88	27.23	25.02	29.04
25	15.29	15.47	14.28	15.01
12.5	12.79	16.23	18.27	15.76
Water blank	5.5	5.5	5.5	5.50
Control Et-OH	6.82	7.6	7.75	7.39
Imipenem	43.84	40.76	45.1	43.23

Inhibition zones of the in vitro antimicrobial activity of the essential oil Eucalyptus-Pelargonium 5:2 against *Klebsiella pneumoniae* ATCC 13883

Figure 7

Diameter measurement of inhibition zones of Eucalyptus-Pelargonium essential oil



Diameter measurement of inhibition zones of Eucalyptus-Pelargonium essential oil 5:2 against *Klebsiella pneumoniae*. In the center, the imipenem reference, and on the right side with three wells, the MA-E5P2 mixture.

5 CONCLUSIONS

The present works studied the synergistic or adjuvant effect on the antimicrobial effectiveness of pelargonium essential oil (PEO) in combination with eucalyptus essential oil (EEO). The small amounts of oil in pelargonium (difficultly extractable by vapor drag) have motivated a liquid PEO-liquidAEE (L-L) extraction. EEO has been used as an extractant for PEO, giving a final proportion of 5:2.



Analysis by CG-FID chromatography gave 80.5% w/w of 1,8-cineole (eucalyptol), 3.05% w/w of limonene, 5.4% w/w of α -pinene, and antioxidant capacity of 76.0%. For the ME-E5P2 mixture, 56.4% of 1,8-cineole and 9.30% of citronellol were obtained from the essential oil of *Pelargonium spp.* with an antioxidant capacity of 78.6%.

The in vitro effectiveness of *Eucalyptus globulus Labill* essential oil against *Klebsiella pneumoniae* ATCC 13883 demonstrated a Minimum Bactericidal Concentration (MBC) of 1/16 (60.50g/ml), Minimum Inhibitory Concentration (MIC) of 1/8 (125.58 mg /mL) and an antimicrobial sensitivity lower than the Imipenem standard (41.04mm), with an average inhibition zone of 13.47mm. Eucalyptus oil had better effectiveness against *Staphylococcus aureus* ATCC 25923 (Gram-positive) than *Klebsiella pneumoniae* ATCC 13883 (Gram-negative).

The essential oil of *Eucalyptus globulus Labill* and *Pelargonium sp.* 5:2 had better in vitro effectiveness against *Klebsiella pneumoniae* ATCC 13883 with an MBC of 1/16 (60.47mg/mL), a MIC of 1/18 (129.58mg/mL) and an excellent antimicrobial sensitivity between 25-37.5 % (15.01-29.04mm) compared to the Imipenem standard (43.23mm). Finally, it was shown that combining both oils in a 5:2 ratio was more effective than pure *Eucalyptus* essential oil.

ACKNOWLEDGEMENTS

The authors sincerely acknowledge to Andean University of Cusco through the vice-rector for Research.

REFERENCES

- Ács, K., Balázs, V. L., Kocsis, B., Bencsik, T., Böszörményi, A., & Horváth, G. (2018). Antibacterial activity evaluation of selected essential oils in liquid and vapor phase on respiratory tract pathogens. *BMC Complementary and Alternative Medicine*, 18. <https://doi.org/10.1186/s12906-018-2291-9>
- Alarcón, M. E. T., Conde, C. G., & Mendez, G. L. (2019). Extracción, caracterización y actividad antioxidante del aceite esencial de *Eucalyptus globulus Labill*. *Revista Cubana de Farmacia*, 52(1), Article 1. <http://www.revfarmacia.sld.cu/index.php/far/article/view/266>
- Barry, B., & Bernard, S. (2018). Infecciones de las vías respiratorias superiores. *EMC - Tratado de Medicina*, 22(2), 1-8. [https://doi.org/10.1016/S1636-5410\(18\)89310-2](https://doi.org/10.1016/S1636-5410(18)89310-2)



- Bouteleux, B., Henrot, P., Ernst, R., Grassion, L., Raherison-Semjen, C., Beaufils, F., Zysman, M., & Delorme, M. (2021). Respiratory rehabilitation for Covid-19 related persistent dyspnoea: A one-year experience. *Respiratory Medicine*, 189, 106648. <https://doi.org/10.1016/j.rmed.2021.106648>
- Bussmann, R. W., & Sharon, D. (2015). Plantas medicinales de los Andes y la Amazonia. La Flora mágica y medicinal del Norte del Perú. 292-292. <http://dx.doi.org/10.32859/era.15.1.001-293>
- Calle, R., & Sánchez, D. B. B. y A. G. (2019). Actualización de las infecciones respiratorias en Urgencias. *Medicine - Programa de Formación Médica Continuada Acreditado*, 12(88), 5170-5179. <https://doi.org/10.1016/j.med.2019.10.013>
- Castresan, J. E., Rosenbaum, J., & González, L. A. (2013). Estudio de la efectividad de tres aceites esenciales para el control de áfidos en pimiento, *Capsicum annuum* L. *Idesia (Arica)*, 31(3), 49-58. <https://doi.org/10.4067/S0718-34292013000300007>
- Cedeño, A., Moreira, C., Muñoz, J., Muñoz, A., Pillasaguay, S., & Riera, M. A. (2019). Comparación de métodos de destilación para la obtención de aceite esencial de Eucalipto. *Colón Ciencias, Tecnología y Negocios*, 6(1), 1-13. <http://dx.doi.org/10.48204/j.colonciencias.v7n1a1>
- Díez, Ó., Batista, N., Bordes, A., Lecuona, M., & Lara, M. (2007). Diagnóstico microbiológico de las infecciones del tracto respiratorio superior. *Enfermedades Infecciosas y Microbiología Clínica*, 25(6), 387-393. <https://doi.org/10.1157/13106964>
- García, S. del P. (2015). *Efecto antibacteriano In vitro del aceite esencial de pelargonium hortorum sobre la cepas de pseudomonas aeruginosa atcc 27853 y staphylococcus aureus atcc25923* [tesis de grado]. Universidad Privada Antenor Orrego - UPAO. <https://repositorio.upao.edu.pe/handle/20.500.12759/1309>
- Gil, M., Perelli, A., Alvarado, R., Arias, Y., & Blumenthal, E. (2012). Actividad bacteriostática y bactericida de la tintura de propóleos sobre bacterias enteropatógenas. *Salus*, 16(3), 021-025.
- Hasegawa, T., Takano, F., Takata, T., Niiyama, M., & Ohta, T. (2008). Bioactive monoterpene glycosides conjugated with gallic acid from the leaves of *Eucalyptus globulus*. *Phytochemistry*, 69(3), 747-753. <https://doi.org/10.1016/j.phytochem.2007.08.030>
- Hemmer, C. J., Löbermann, M., & Reisinger, E. C. (2021). [COVID-19: Epidemiology and mutations: An update]. *Der Radiologe*, 61(10), 880-887. <https://doi.org/10.1007/s00117-021-00909-0>
- Herrera, M. L. (1999). Pruebas de sensibilidad antimicrobiana: Metodología de laboratorio. *Revista Médica del Hospital Nacional de Niños Dr. Carlos Sáenz Herrera*, 34, 33-41. <http://dx.doi.org/10.51481/amc.v56i3.841>
- Horna, G., Silva Díaz, M., Vicente Taboada, W., & Tamariz Ortiz, J. (2005). Concentración mínima inhibitoria y concentración mínima bactericida de ciprofloxacina en bacterias uropatógenas aisladas en el Instituto Nacional de Enfermedades Neoplásicas. *Revista Medica Herediana*, 16(1), 39-45. <http://dx.doi.org/10.20453/rmh.v16i1.862>



- J, S., K, V., Baburao, N., Hilal, M. H., & Rani, A. R. (2011). Phytopharmacological importance of Pelargonium species. *Journal of Medicinal Plants Research*, 5(13), 2587-2598.
- López, M. T. (2004). Los aceites esenciales. *Offarm*, 23(7), 88-91.
- Mieres-Castro, D., Ahmar, S., Shabbir, R., & Mora-Poblete, F. (2021). Antiviral Activities of Eucalyptus Essential Oils: Their Effectiveness as Therapeutic Targets against Human Viruses. *Pharmaceuticals*, 14(12), 1210-1210. <https://doi.org/10.3390/ph14121210>
- MINSA. (2022a). Boletines epidemiológicos. CDC MINSA. <https://www.dge.gob.pe/portalnuevo/publicaciones/boletines-epidemiologicos/>
- MINSA. (2022b). Covid 19 en el Perú—Ministerio del Salud. https://covid19.minsa.gob.pe/sala_situacional.asp
- Montani, D., Savale, L., Noel, N., Meyrignac, O., Colle, R., Gasnier, M., Corruble, E., Beurnier, A., Jutant, E.-M., Pham, T., Lecoq, A.-L., Papon, J.-F., Figueiredo, S., Harrois, A., Humbert, M., Monnet, X., & COMEBAC Study Group. (2022). Post-acute COVID-19 syndrome. *European Respiratory Review: An Official. Journal of the European Respiratory Society*, 31(163), 210185. <https://doi.org/10.1183/16000617.0185-2021>
- Montero-Recalde, M., Morocho-Núñez, M. J., Avilés-Esquivel, D., Carrasco-Cando, Á., & Erazo-Gutierrez, R. (2019a). Eficacia antimicrobiana del aceite esencial de eucalipto (*Eucalyptus* spp) sobre cepas de *Escherichia coli* y *Staphylococcus aureus* subsp. *Aureus*. *Revista de Investigaciones Veterinarias del Perú*, 30(2), 932-938. <https://doi.org/10.15381/rivep.v30i2.16099>
- Montero-Recalde, M., Morocho-Núñez, M. J., Avilés-Esquivel, D., Carrasco-Cando, Á., & Erazo-Gutierrez, R. (2019b). Eficacia antimicrobiana del aceite esencial de eucalipto (*Eucalyptus* spp) sobre cepas de *Escherichia coli* y *Staphylococcus aureus* subsp. *Aureus*. Antimicrobial efficacy of eucalyptus essential oil (*Eucalyptus* spp) on *Escherichia coli* and *Staphylococcus aureus* subsp. *aureus* strains., 30(2), 932-938. <https://doi.org/10.15381/rivep.v30i2.16099>
- Moo, C.-L., Osman, M. A., Yang, S.-K., Yap, W.-S., Ismail, S., Lim, S.-H.-E., Chong, C.-M., & Lai, K.-S. (2021). Antimicrobial activity and mode of action of 1,8-cineol against carbapenemase-producing *Klebsiella pneumoniae*. *Scientific Reports*, 11(1), 1-13. <https://doi.org/10.1038/s41598-021-00249-y>
- Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R. J., Wigdahl, B., & Parveen, Z. (2008). Antiviral potentials of medicinal plants. *Virus Research*, 131(2), 111-120. <https://doi.org/10.1016/j.virusres.2007.09.008>
- Navas Ortega, E. P. (2017). *Estudio invitro del efecto anti fúngico del aceite esencial del pelargonium graveolens (geranio) al 25%, 50%, 75% y 100% sobre cepas de candida albicans atcc ® 10231TM*. <http://www.dspace.uce.edu.ec/handle/25000/9731>
- Ospina, L. M. P., Peláez, J. A. M., Borrego-Muñoz, P., Cardona, W. F. C., & Villamizar, L. B. (2016). Composición química y actividad antimicrobiana del aceite esencial de *Pelargonium odoratissimum* (l) l'her (geraniaceae). *Revista Facultad de Ciencias Básicas*, 12(1), 74-83. <https://doi.org/10.18359/rfcb.1856>



- Sánchez, M. F. O. (2006). *Manual práctico de aceites esenciales, aromas y perfumes*. Aiyana ediciones.
- Schürmann, M., Oppel, F., Gottschalk, M., Büker, B., Jantos, C. A., Knabbe, C., Hütten, A., Kaltschmidt, B., Kaltschmidt, C., & Sudhoff, H. (2019). The Therapeutic Effect of 1,8-Cineol on Pathogenic Bacteria Species Present in Chronic Rhinosinusitis. *Frontiers in Microbiology*, 10. <https://www.frontiersin.org/article/10.3389/fmicb.2019.02325>
- Stashenko, E. E., Martínez, J. R., Pérez, D. L. M., & Santoyo, M. C. O. (2007). Composición química de los metabolitos secundarios volátiles de pelargonium graveolens, en función del método de extracción y época de recolección del material vegetal. *Scientia Et Technica*, XIII(33), 183-184. <http://dx.doi.org/10.4322/sc.2017.003>
- Tyagi, A. K., & Malik, A. (2011). Antimicrobial potential and chemical composition of Eucalyptus globulus oil in liquid and vapour phase against food spoilage microorganisms. *Food Chemistry*, 126(1), 228-235. <https://doi.org/10.1016/j.foodchem.2010.11.002>
- Valero, N., Larreal, Y., Arocha, F., Gotera, J., Mavarez, A., Bermudez, J., Moran, M., Maldonado, M., & Espina, L. M. (2009). Etiología viral de las infecciones respiratorias agudas. *Investigación Clínica*, 50(3), 359-368.
- Vargas, J. A. L., & Toro, L. M. E. (2010). *K. pneumoniae*: ¿la nueva “superbacteria”? *Patogenicidad, epidemiología y mecanismos de resistencia*. 23(2), 9. <http://dx.doi.org/10.17533/udea.iatreia.11129>