Chemical composition of brazilian pennyroyal essential oil (*Mentha pulegium* L.) and evaluation of the antimicrobial effect on *Staphylococcus aureus* and *Escherichia coli*

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ABSTRACT

The extraction of the EOs was first obtained by hydrodistillation, then their chemical composition was determined by GC/MS and their antimicrobial activity was evaluated. In the analysis and identification of the hydrodistilled essential oil of Mentha pulegium L. by gas chromatography and mass spectroscopy, 12 compounds were separated, representing 99.31% of the total mass of the essential oil. The majority component was pulegone (80.553%), other components present in appreciable levels were; Piperitone (5,430%); Cyclohexanone,5-methyl-2-(1-methyl)-,(2R-cis) (3,304%); trans-Isopulegone (2.945%); α-Humulena (1,950%). In addition, the screening of the essential oil reveals that there were appreciable results regarding its antibacterial activity against S. aureus 20 µg/mL and E. coli 2.5 µg/mL . This result was obtained by the undiluted essential oil. However, more research on the factors that influence the biosynthesis and bioactivity of essential oils is needed as essential oils gain

important applications in the food and pharmaceutical industry.

Keywords: Antioxidant, Lamiaceae, Antibacterial properties, CBA, CG/MS.

1 INTRODUCTION

A variety of different chemical and synthetic compounds have been used as antimicrobial agents. However, the indiscriminate and widespread use of antimicrobials has led to a number of ecological and medical problems. Thus, one of the most important therapeutic challenges involves the treatment of infectious diseases caused by bacteria. After the discovery of penicillin and the subsequent expansion of its application in treatment, new antibiotics are continuously developed to fight infections. This has resulted in the expansion of the use of natural products such as synthetic antibiotics in the therapy of infections. However, the overuse of these antimicrobials is associated with increased resistance to different antibiotics in most bacteria (SALES *et al*., 2015; SALES *et al*., 2017). Several authors have tested essential oils from aromatic plants to extend the shelf life of foods (KOSTAKI et al., 2009), while others have focused on antioxidant and antimicrobial action, and the properties of plant extracts and essential oils (CAO *et al*., 2009).

The Lamiaceae family is known for harboring several species of menthol plants, mainly in temperate countries, with a global distribution, containing 7,886 species and are appreciated for their antibacterial, antifungal, antioxidant, antiviral, and biopesticide properties (SANTOS, 2022; SCHELLENBERGER *et al*., 2023). In Brazil, this group is represented by 71 genera and 589 species, mainly found in open plant formations (OLIVEIRA *et al*., 2023). This botanical family contains many species that possess distinct organoleptic properties due to the presence of secondary metabolites, especially essential oils found in the leaves, flowers, and fruits. This gives this family a great economic appeal, especially in sectors such as the food industry. In addition, in the perfume industry, species such as lavender (*Lavandula angustifolia* Mull.) and mint (*Mentha spp.*) are highly valued for their aromatic essential oils (OLIVEIRA, 2022; ANTAR *et al*., 2023).

The genus *Mentha* has 30 species that grow in temperate regions of Eurasia, Australia, South America, and South Africa. Mint species have great relevance both from a medicinal and commercial point of view. The leaves, flowers, and stems of *Mentha spp*. are often used in herbal teas or as additives in commercial spice blends for various foods, providing aroma and flavor. In addition, *Mentha* spp. has been employed as a folk remedy for the treatment of conditions such as nausea, bronchitis, flatulence, anorexia, ulcerative colitis and liver problems, due to its anti-inflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue and anticatarrhal properties (DESCHAMPS *et al*., 2006; HADJLAOUI, 2009; VILAR *et al*., 2023).

Pennyroyal (*Mentha pulegium* L.) is a herbaceous, perennial plant that has evergreen leaves. The flowers, with a typically bilabiate gamopetala corolla, are grouped in small clusters. The fruit is a small hard tetra-achene, the secretory structures are located on the leaves and stems, can reach up to half a meter in height, is naturalized in America and thrives in Western, Southern and Central Europe, Asia, Iran, Arab countries and Ethiopia (ALAPETITE, 1981; GRUENWALD *et al*., 2000). It is a kind of great popularity and has already had a wide use in the food industry as a preservative ingredient. The antimicrobial properties of this plant are due to the potent compounds pulegone and 1-8 cineole (MAHMODI *et al*., 2011). In addition, it is known for its perfume and flavor in traditional medicine (RODRIGUES *et al*., 2013). The essential oil and dry parts in the form of infusions, teas or powder, are used in medicine to treat digestive disorders, liver and gallbladder problems, amenorrhea, gout, colds, increased urination, skin diseases and also as abortifacients (AHMED *et al*., 2018). They are also used in gastronomy, aromatherapy and the cosmetics industry, highlighting their versatility and applicability in different areas (GRUENWALD *et al*., 2000; AGNIHOTRI *et al*., 2005).

The essential oil of Mentha pulegium *L. has demonstrated several pharmacological effects, including abortifacient activity in the myometrium of rats, cytotoxicity against different human cell lines, and antioxidant properties, the antibacterial effect of* M. pulegium in Gram-positive bacteria is greater than in Gram-negative bacteria (MAHBOUBI; HAGHI, 2008; JAVANMARD *et al*., 2018).

Detailed studies have revealed that the qualitative composition of *Mentha pulegium L. oils* depends on the geographical origin and the specific ecological sites from where the plant material is collected for distillation. Additionally, the quantitative composition of the oil can vary greatly depending on the growing region and the country of origin. For example, *Mentha pulegium* L. oil from Bulgaria was found to have a high percentage of pulegone (42.9-45.4%), while oil from Uruguay contained mostly pulegone (73.4%) and isolontone (12.9%). In Egypt, the oil contained pulegone (43.5%) and pipestone (12.2%), while in Tunisia, the main constituents were pulegone (41.8%) and isolontune (11.3%). In Iran the main constituents of *M. pulegium* were piperiteone (38.0--%), piperitenone (33.0%). These studies identified three distinct chemotypes of *Mentha pulegium L.,* characterized by the following main components of the oil: (1) pulegone, (2) piperitenone and/or piperiteone and (3) isomentone/neoisomenthol, in Algeria the marjoritarian compounds were pulegone (38.81 %) and menthol (19.24 %) (BIGO DE GROSSO; MOYNA, 1985; STOYANOVA *et al*., 2005; EL-GHORAB, 2006; MKADDEM *et al*., 2007; MAHBOUBI; HAGHI, 2008; TOPALOV; DIMITROV 1969, COOK *et al*., 2007; BOUKHEBTI *et al*., 2011).

TABLE 1 – MAIN COMPONENTS OF THE ESSENTIAL OIL OF *M. pulegium* IN DIFFERENT COUNTRIES OF CULTIVATION

Compound	Country of origin	Author
the moon (73.4%)	Uruguay	Lorenzo *et al*. (2002)
The moon (43.5%)	Egypt	El-Ghorab (2006)
power (42,9–45.4%)	Bulgaria	Stoyanova *et al*. (2005)
The moon (41.8%)	Tunisia	Mkaddem *et al*. (2007)
the moon (38.81%)	Algeria	BoukhebtI, *et al*. (2011)
piper (38.0%)	Iran	Mahboubi; Haghi (2008)

Source: Author's production (2023).

Biologically active compounds (BAC) found in plants have aroused increasing interest as highly desirable natural agents in various applications (LUGO, 2010). CBAs found in plants are characterized by their significant amounts of organoleptic and biochemical properties and play a key role in sensory quality in their health benefits (CODEVILLA, 2015). In addition, the secondary metabolites of plants are widely used in traditional medicine due to their well-established potential in terms of pharmaceutical and biological properties, including anti-inflammatory, neuroprotective, antioxidant, and antidiabetic activities. (PACHECO; ALVES, 2020).

The use of essential oils (EOs) by humans dates back more than 60,000 years, with its main focus on medicinal practices and religious rituals. Essential oils are aromatic and volatile liquids, which means that they evaporate quickly when exposed to rising temperatures. They consist of a complex mixture of organic compounds, containing only carbon, hydrogen, and oxygen atoms. These components can belong to a variety of classes of compounds, but the classes commonly found are terpenes and phenylpropenes. They are often extracted from plant materials such as leaves, flowers, roots, bark, and seeds. These oils are known for their distinctive and generally pleasant scents, and they have a wide range of applications in aromatherapy, the fragrance industry, health care, and more (SILVA *et al*., 2009; SILVEIRA *et al*., 2012),

The antibacterial properties of essential oils and their components are explored in several commercial products, such as dental root canal sealants, antiseptics, and feed supplements for lactating sows and weaned piglets. Some food preservatives containing EO are already commercially available (BURT, 2004).

The antimicrobial activity of EOs depends on their chemical composition. They are usually characterized by two or three main components in fairly high concentrations (up to 80%) compared to other components present only in trace amounts (FERNANDES, 2017). However, there is evidence that EOs are more strongly antimicrobial than is explained by the additive effect of their main antimicrobial components (LIMA, 2022). The smaller components seem to play a significant role. Thus, it is not clear which constituents or mixtures of them are mainly responsible for their antimicrobial activity (SANTOS, 2020). Thus, this study investigated the chemical composition of the essential oil of the leaves and stems of *Brazilian M. pulegium* and the antibacterial effects on *Staphylococcus aureus* and *Escherichia coli*.

2 MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL

The pennyroyal plants were collected at the Jaqueira Agroecology Site (20°45′S - 41°31′W), about 287m above sea level, located in the municipality of Alegre/ES. On August 26, 2023, at 7:30 a.m., with cloudy skies and overcast by rain, on the new moon. The material was collected and stored in a black plastic bag and taken to the Chemistry Laboratory 4 of the Federal University of Espírito Santo – Alegre Campus.

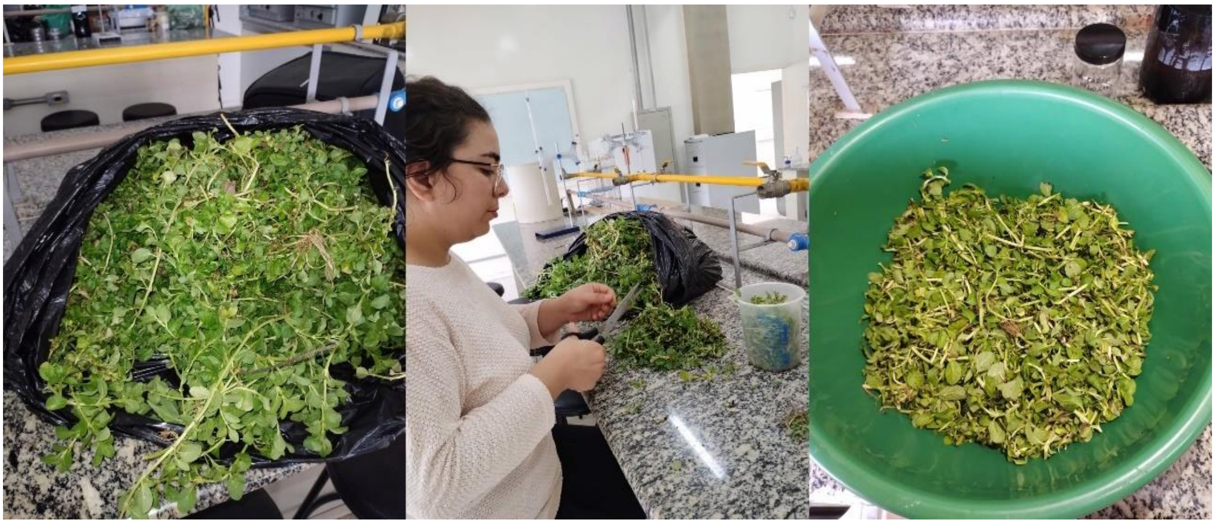
FIGURE 1 – COLLECTION SITE OF THE PLANT SPECIES *M. pulegium*

Source: Authors' collection (2023).

2.2 EXTRACTION OF *MENTHA PULEGIUM ESSENTIAL OIL*

The plant parts were then washed under running water to eliminate soil and other surface contaminants. After removing the excess water with paper towels, the plant material was cut into small pieces with the help of scissors. 910g of leaves and stems of *M. pulegium were used*.

FIGURE 2 – PREPARATION OF THE RAW MATERIAL FOR THE EXTRACTION OF THE EO



Source: Authors' collection (2023).

The methodology used in this study for EO extraction was adapted to the technique employed by Behbahani *et al*., (2013), in which the hydrodistillation technique was used for 16 hours with 1,500 ml of distilled water in a modified Clevenger device. The chopped parts were placed in natura in the 1 L round bottom flask and covered by distilled water, as a heat source an electric blanket was used and the distillation process was carried out by steam drag to extract the essential oil of *M. pulegium.* The oil obtained was collected and stored in an amber glass flask with screw cap in a 5°C refrigerator before the analyses.

FIGURE 3 – STEAM DRAG DISTILLATION PROCESS

Source: Authors' collection (2023).

2.3 CHEMICAL COMPOSITION OF PENNYROYAL ESSENTIAL OIL BY GAS CHROMATOGRAPHY (GC/MS)

The essential oil of *M. pulegium* was analyzed by gas chromatography-gas coupled to mass spectrometry (GC/MS) in the Shimadzu QP2010-Plus device, located in the Analytical Center of the Food and Nutrition Engineering Building. The following chromatographic conditions were used in both analyses: fused silica capillary column (30 m x 0.25 mm) with Rtx-5MS® stationary phase (0.25 µm film thickness); N2 (in the GC/FID analysis) and He (in the GC/MS analysis) as carrier gas with a flow rate of 3.0 mL/min; the oven temperature followed a schedule in which it remained for 3 minutes at the initial temperature of 40 °C and then gradually increased by 3 °C/minute until it reached 240 °C, remaining at this temperature for 5 minutes; injector temperature of 250 °C; detector temperature 280°C; Split ratio of 1:30.

GC/MS analyses were performed on equipment operating with electronic impact with impact energy of 70 eV, scan speed of 1000, scan interval of 0.50 fragments/second and detected fragments from 29 to 400 (m/z). The identification of the chemical components of the essential oil was performed by comparing its mass spectra with those available in the Willey7, NIST05 and NIST05s spectrolibraries databases with the co-injection of standards and by the RI (Linear Temperature Programmed Retention Indexes) retention indexes. A mixture of linear n-alkanes (C7 to C40) was used to calculate Id. The RI calculated for each compound was compared with values in the literature (ADAMS, 2007). The relative percentage of each compound was calculated for all constituents of the sample. Gas chromatography analyses obtained data with a flame ionization detector (GC/FID). Compounds with a relative area greater than 1% were considered for the essential oils under study.

FIGURE 4 – SHIMADZU QP2010-PLUS DEVICE

Source: Authors' collection (2023).

2.4 BACTERIAL STRAINS

The bacterial strains used in this study were *Escherichia coli (ATCC 25922) and* Staphylococcus aureus *(ATCC 25923).*

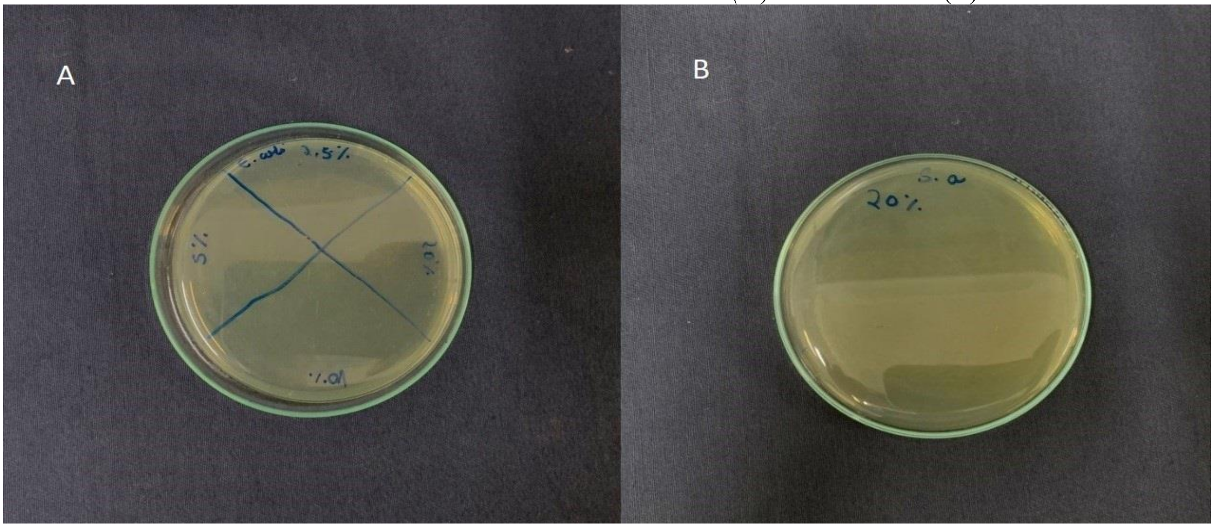
2.5 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The determination of the MIC was performed by the modified broth microdilution method (CLSI, 2015). The assays were performed in sterile polystyrene microplates with 96 round-bottomed wells, in triplicate. The final volume of the final reaction mixture was 200 µL, the culture medium used was Mueller Hinton Broth (CMH) and the final concentration of the inoculum in the test was standardized to 5x 105 CFU/ml. The tested EO concentrations ranged from 0.01% to 20% (v/v) [(the final concentration was 0.005% to 10% (v/v)]. The EO solutions were added with 2.5% DMSO to facilitate the solubilization of EO in aqueous solution. After assembly, the microplates were incubated at 35±2ºC for 20-24 h. The MIC was considered to be the lowest EO concentration capable of completely inhibiting bacterial growth (absence of precipitate or turbidity in the culture medium or precipitate ≤ 2mm) after the 24-hour incubation period. The microplate had a sterility control (no inoculum), growth control (inoculum + culture medium), solvent control (inoculum + culture medium with DMSO) and a positive control (inoculum + culture medium with DMSO + gentamicin), all subjected to the same growing conditions.

2.6 EVALUATION OF THE ACTION OF ESSENTIAL OIL IN THE INHIBITION OF BACTERIAL GROWTH: BACTERICIDAL OR BACTERIOSTATIC ACTION

After the microdilution test in liquid medium, the MIC wells were plated, 2 concentrations higher than the MIC of the essential oil and the positive control, in Petri dishes containing 25 ml of nutrient agar. The plates were incubated at 35±2oC for 24 hours. The result was defined by the presence or absence of colony formation, and the action was classified as bacteriostatic or bactericidal, respectively.

FIGURE 5 – NUTRIENT AGAR SUBCULTURE TO DETERMINE THE MINIMUM BACTERICIDAL CONCENTRATION OF PENNYROYAL ESSENTIAL OIL ON *E. coli* (A) AND *S. aureus* (B).



Source: Authors' collection (2023).

3 RESULTS AND DISCUSSION

3.1 CHEMICAL COMPOSITION OF *MENTHA PULEGIUM ESSENTIAL OIL*

12 compounds were separated by GC/MS, representing 99.31% of the total mass of essential oil of *M. pulegium.* The general chemical profile of the essential oil, retention time, retention index, and oil area are summarized in (Table 2).

TABLE 2 – CHEMICAL COMPOSITION OF *M. pulegium essential oil*

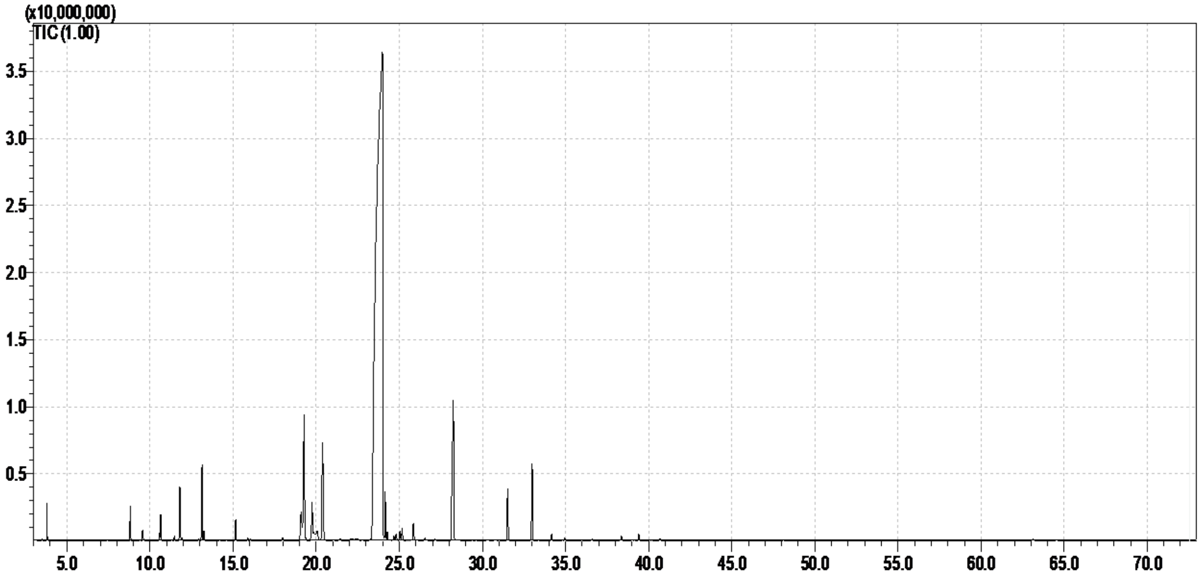
COMPOUND	TR*	GO**	%***
1. Pulegona	1265	1233	80,553
2. Piperitenona	1363	1340	5,430
3. Ciclohexanona,5-methyl-2-(1-methylethyl)-,(2R-cis)	1172	0	3,304
4. trans-Isopulegona	1185	1179	2,945
5. α-Humulena	1478	1452	1,950
6. D-Limonene	1028	1024	1,501
7. β-cariofileno	1442	1417	1,269
8. 3-octanol	1000	0	1,036
9. α-Pinene	929	932	0,588
10. Cyclohexanone	1186	0	0,582
11. β-Pinene	973	974	0,418
12. β-methylpropyl	796	721	0,415

*Retention time (default)
**Retention rate
Area %
Source: Authors' production (2023).

The antimicrobial activity of pennyroyal essential oil has been attributed to its main compounds, such as the high content of pulegon, iso-mentone, menthol, and piperite as reported by Hadjlaoui *et al*., 2009, or to the high concentration of piperite and the synergistic effects of other constituents (MAHBOUBI; HAGHI, 2008). Even the content of α-Humulena has been presented as an explanation for the antimicrobial activity of this EO (INOUYE *et al*., 2001). Whatever the case, in general, oxygenated monoterperenes are significantly more active than monoterpene hydrocarbons (CARSON; RILEY, 1995), have generally been found in significant concentrations in M. *pulegium EOs*.

In the analysis by gas chromatography coupled to mass spectrometry (GC/MS) (Figure 6), it was evidenced that the essential oil extracted from *M. pulegium* used in this study contains the following major components: pulegone (80.553%); Piperitone (5,430%); Cyclohexanone,5-methyl-2-(1-methyl)-,(2R-cis) (3,304%); trans-Isopulegone (2.945%); α-Humulena (1,950%).

FIGURE 6 – GC/FID PROFILES OF *M. pulegium* L. essential oil.



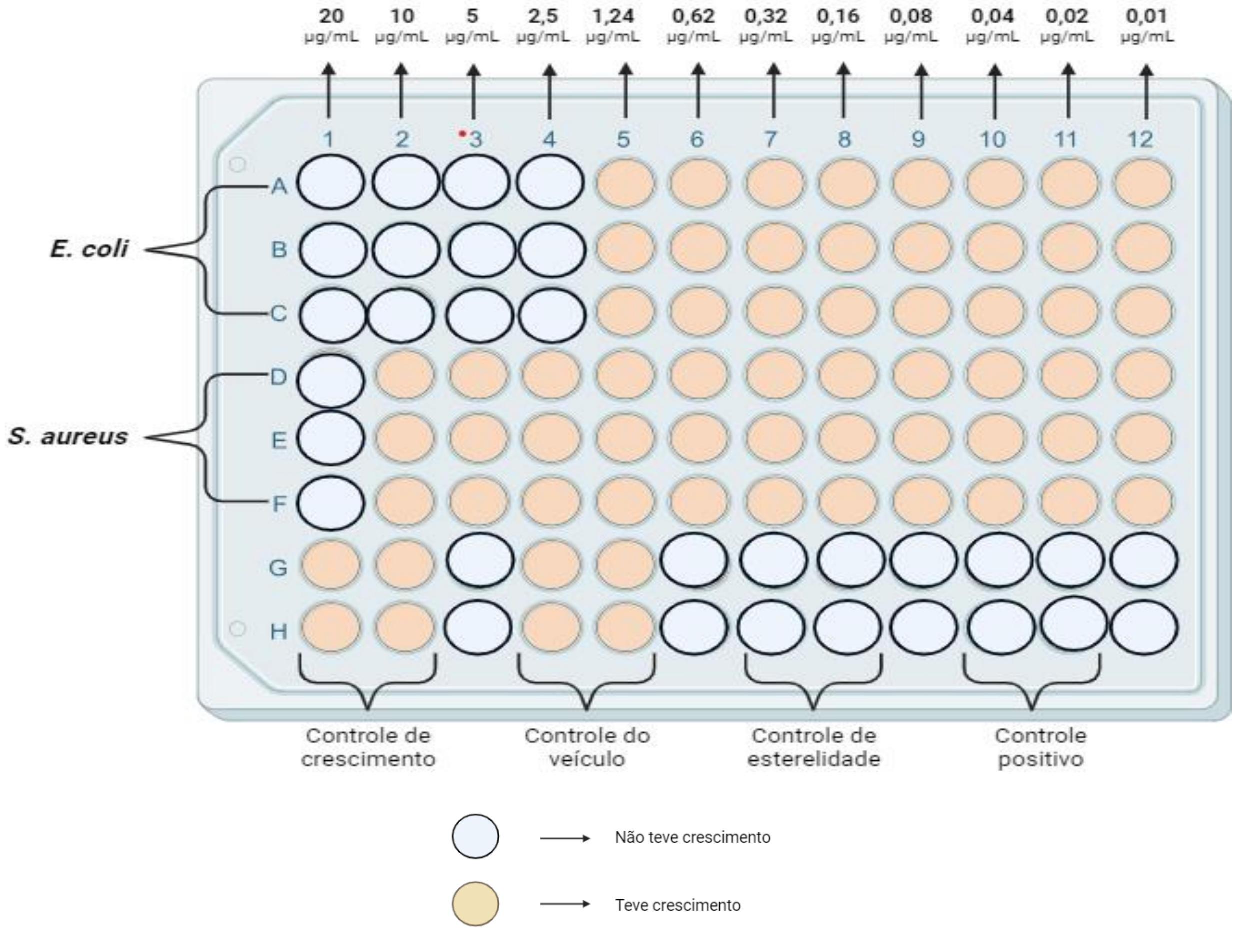
Source: Authors' production (2023).

Regarding the compounds found, a series of studies already published on *M. pulegium* reveal a great variability in its chemical profile. The results of the study identified which chemotype studied is familiar to those observed, with pulegone as the main marjoritarian component. In Uruguay (Lorenzo et al., 2002*), Tunisia (Hajlaoui* et al., 2009) and Morocco (Benayad, 2008) with pulegone content 73.4%, 61.11% and 73.33% respectively. In addition, results published by Vian *et al.* (2008) reported that pulegone (83.70%) was the major component of *M. pulegium*'s essential oil, while Mahboubi; Haghi (2008) reported that pipestone (38.0%) is the main constituent. Compared to oils tested with published data on the oil composition of other M. *pulegium* L. samples, there are some quantitative and qualitative differences. These chemical differences can probably be explained by the existence of different chemotypes, as well as the geographical distribution of this plant significantly influences the chemical composition of its essential oils.

3.2 ANTIMICROBIAL ASSESSMENT

The antibacterial activities of *M. pulegium* essential oil against Gram-negative E. coli *bacteria and against Gram-positive bacteria* S. aureus *are described in (Figure 7).*

FIGURE 7 – SCHEMATIC REPRESENTATION OF THE MICROPLATE AFTER 24 HOURS OF INCUBATION



Source: Authors' production (2023).

The results of the MIC obtained in this assay were 2.5 µg/mL for *E. coli* and 20 µg/mL for *S. aureus*. MBC was coincident with MIC, which highlights the bactericidal activity of pennyroyal essential oil.

TABLE 3 – MINIMUM INHIBITORY CONCENTRATION AND MECHANISMS OF ACTION OF PENNYROYAL ESSENTIAL OIL AGAINST *E. coli* AND *S. aureus bacteria*

Micro-organism	MIC (µg/mL)	CBM (µg/mL)
E. coli	2,5	2,5
S. aureus	20	20

Source: Authors' production (2023).

According to CLSI standards, microbial sensitivity is observed when, in conventional therapies, a drug is able to stop bacterial growth. On the other hand, a resistant microorganism becomes more challenging to control, even with the use of normally effective concentrations (WAYNE, 2005). The data from this study indicated that *E. coli* was the most sensitive strain tested in the EO *of Brazilian M. pulegium and also indicate that the EO* of M. pulegium *may be an effective inhibitor agent of the strains studied. Mentha pulegium* is considered a medicinal plant due to its pharmacological and biological properties (GHAZGHAZI *et al*., 2013).

The results showed that the essential oil of *Mentha pulegium* L. brasileiro has higher antibacterial activity against *E. coli 2.5 (µg/mL) than against* S. aureus *20 (µg/mL*). Boukhebti *et al.* (2011), reported that Gram-positive bacteria are more susceptible to essential oils than Gram-negative bacteria, but the results shown in this study showed that *E. coli,* which is a Gram-negative, was more susceptible to the essential oil of *Brazilian M. pulegium* L., using a microdose of lower inhibition concentration than for *S. aureus.*

Pennyroyal could be a center of interest in the food industry with flavoring, antioxidant and antimicrobial properties (GHAZGHAZI *et al*., 2013). It is notable that the effect of *M. pulegium* L. essential oil against some bacterial strains is close to or superior to that of standard antibiotics.

4 CONCLUSIONS

The search for new products to control pathogens is a promising area of research. *Mentha pulegium* L. may be a good candidate for the development of new antimicrobials and can be used in important applications in the food and pharmaceutical industry. Despite increasing research in this area, more studies on the antimicrobial activity or chemical composition of essential oils are still needed.

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