# Antioxidant and Antimicrobial Activities of Essential Oil of *Satureja calamintha* ssp. *nepeta* (L.) Briq. from the Northwest of Algeria

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**Summary**

*Satureja calamintha* ssp. *nepeta* (L.) Briq. is a medicinal and aromatic plant used for long time in Algerian traditional medicine. This study was carried out to determine physical characteristics (density and refractive index), total phenolic content (Folin-Ciocalteau assay), antioxidant (DPPH free radical scavenging method) and antimicrobial (disc diffusion assay against *Staphylococcus aureus* (ATCC 29273), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231)) activities of essential oil of *Satureja calamintha* spp. *nepeta* (Lamiaceae). The essential oil of aerial parts of *S. calamintha* was isolated by the hydrodistillation method. Concerning the physical characteristics, essential oil gave a value of density of 0.910 and revealed an refractive index of 1.480. The total phenolic content was in the order of 219±0.06 mg Gallic Acid Equivalents per g of sample. This oil had antioxidant power with a percentage of inhibition of the free radical DPPH (91.81 %) at 500 μg $mL^{-1}$. Furthermore, the antimicrobial results revealed that this oil exhibit antimicrobial activity with minimum inhibitory concentration that ranged from 0.09 to 1.56 μL $mL^{-1}$. Overall, the results obtained are promising and open up new perspectives in the field of natural applications which can be used in the food, pharmaceutical and cosmetic industries.

**Key words**

antimicrobial activity, antioxidant activity, medicinal plant, polyphenol

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## Introduction

The chemical additives-associated harmful effects on human health, besides the microbial resistance and the increasing demand for healthy food with less synthetic ingredients led the scientific community to search for natural agents able to be used as "safe" additives (Yap et al., 2014; Sharma et al., 2018). Recently, natural substances such as essential oils have attracted the attention of researchers due to their great beneficial effects (Maccelli et al., 2020). Essential oils have become more significant in human life nowadays since their discovery thousands of years back (Yi Peng et al., 2012). They have been largely employed due to their biological activities, such as antibacterial, antiviral, antifungal, antioxidant, insecticidal and anti-herbivorous (Yi Peng et al., 2012). Essential oils such as biopesticide have some advantages, where pathogenic microorganisms are not likely to develop resistance against them, little or no mammalian toxicity and are not accumulated in soils (Gormez et al., 2015). Essential oils, which are the secondary metabolites produced within the various tissues of medicinal plants/herbs, are complex mixtures of volatile compounds such as terpenes (mostly monoterpenes and sesquiterpenes), phenolics and alcohols (Rezvanpanah et al., 2011). In nature, essential oils play an important role in the protection of the plants as antibacterials, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants (Bakkali et al., 2008). They also may attract some insects to favor the dispersion of pollens and seeds, or repel undesirable others (Bakkali et al., 2008). They can be synthesized in all plant organs: buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al., 2008).

The Lamiaceae family has about 233 genera and 6900 species (Gormez et al., 2015). *Satureja* L. (Lamiaceae) includes about 200 species of herbs and shrubs, often aromatic, and widely diffused in the Mediterranean area, Asia and boreal America (Kaya et al., 2009). *Satureja* species have been traditionally used in the treatment of various diseases such as nausea, indigestion, cramps, diarrhea, infectious diseases and muscle pains (Bojovic et al., 2018). *Satureja calamintha* ssp. *nepeta* (L.) Briq. is used as a remedy for coughs, indigestion and mild respiratory infections (Padrini and Lucheron, 1996). It has been reported that *Satureja* species extracts and fractions possess various therapeutic effects. They have antioxidant, anti-inflammatory, antidiarrheal, antifungal, antibacterial and antispasmodic activities (Babajafari et al., 2015). *Satureja* essential oil provides the basis for a wide range of biological and industrial applications thanks to the content of biologically active phytochemicals (Maccelli et al., 2020). Furthermore, it is often used in Mediterranean cooking preparations and even as natural antibacterial agent in food packaging (Maccelli et al., 2020). In Algeria the genus *Satureja* is present with four subgenera, among them the subgenus *calamintha* which comprises five species and three subspecies (Kerboucheab et al., 2013). To the best of our knowledge there are no studies on *Satureja calamintha* ssp. *nepeta* oil antioxidant and antimicrobial activities of this species growing in the northwest (Mascara region). However, some studies on *Satureja* oils compositions and biological activities in the central and eastern regions of Algeria have been conducted (Kerboucheab et al., 2013 ; Labiod, 2016). In the Northwest of Algeria (Mascara region), *Satureja calamintha* ssp. *nepeta* is used as a remedy for many illnesses such as coughs and respiratory infections. Therefore, the present study was conducted to study the physical characteristics, the antimicrobial and antioxidant activities of the essential oil of *Satureja calamintha* ssp. *nepeta* from the Northwest of Algeria.

## Materials and Methods

### Plant Material

Fresh samples of aerial parts (leaves and flowers) of *Satureja calamintha* spp. *nepeta* were collected at the flowering stage from Bouhanifia Zmaacha station (Mascara province) in the Northwest Algeria (35° 18' 58'' north, 0° 02' 54'' west).

### Extraction Process

Essential oil was extracted by hydrodistillation (100 g of aerial parts of *Satureja calamintha* spp. *nepeta*) for 4 hours using a Clevenger according to the method described in the European pharmacopoeia. The oil was dried over anhydrous sodium sulfate and kept in dark at 4°C until use (Ismaili et al., 2015).

### Extraction Process Kinetics

To study the extraction kinetics of the essential oil of *Satureja calamintha* ssp. *nepeta*, corresponding quantities of essential oil were recovered at intervals of 60 min. ranging from 0 to 240 min (4 hours). The quantities of oil obtained were exploited in order to calculate the yield at each time interval (AFNOR, 1986). All measurements were performed in triplicate.

### Physical Characteristics

Physical parameters of the essential oil extracted were determined using the methods described by AFNOR (1986).

### Density

Density measurement was taken using a numerical balance of very high degree of accuracy by double weighing at 20 °C.

### Refractive Index at 20 °C

Refractive index is the measurement of refraction of light rays as these pass through the material. This was measured with the help of a refractometer. 2-3 drops of essential oil under test were put between the 2 prisms of the instrument at a known temperature (20 °C) and the index was measured.

### Total Phenolic Content

The total phenolic content of the essential oil was determined using spectrophotometric analysis with Folin-Ciocalteu reagent (Georgé et al., 2005; Tsai et al., 2008 in Bourkhiss et al., 2010). Folin-Ciocalteu reagent (1 ml previously diluted with distilled water) was added to the essential oil (0.2 ml) and held for 3 min. Then, 3 ml of 7.5 % (w/v) sodium carbonate solution were added and allowed to stand at room temperature for 30 min. The absorbance at 765 nm was measured. The total phenolic content was calculated by a standard curve prepared with gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per gram of essential oil.

### Antioxidant Activity

The antioxidant activity of essential oil of *S. calamintha* was determined by the DPPH essay (Bouhdid et al., 2008; Arulpriya et al., 2010; Amarti et al., 2011; Mansouri et al., 2011).

Briefly, the sample was prepared by dissolution in absolute methanol (31.25, 62.5, 125, 250 and 500µg $mL^{-1}$). The reaction was carried out in a total volume of 2 mL containing 0.4 mL of DPPH (0.5 mM) solubilized in ethanol. The mixture was incubated in the dark at room temperature for 30 min. The absorbance of the mixture was then measured at 517 nm. Ascorbic acid was used as a positive control. The ability of the sample to scavenge DPPH radical was determined from:

$$AA\ \% = ([Abs\ control - Abs\ test]/Abs\ control) \times 100,$$

where AA %: percentage of antioxidant activity,
Abs: absorbance measured at 517 nm.

### Antimicrobial Activity

The antimicrobial activity of essential oil against *Staphylococcus aureus* (ATCC 29273), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231) was evaluated by diffusion disk method in Mueller Hinton Agar (MHA) plates by recording the inhibition zones and minimum inhibitory concentration (MIC) through the microdilution method.

### Diffusion Disk Method

The diffusion disk method was performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility (LaPierre et al., 2020). The test organisms were inoculated in nutrient broth and incubated overnight at 37 °C to adjust the turbidity to 0.5 McFarland standards. The microbial suspension was spread on the MH agar medium. The essential oil was dissolved in DMSO (dimethyl sulfoxide). The disks (6 mm diameter) were individually impregnated with essential oil at all the prepared concentrations (undiluted essential oil (100 %), 50 % and 25 %) and placed on the culture medium surface inoculated with different microbial strains. The treated Petri dishes were placed at 4°C for 1-2h. After that, the Petri dishes were incubated at 37°C for 18-24h. DMSO without the essential oil was used as a negative control. Cefazolin (30 µg $disk^{-1}$), aztreonam (30 µg $disk^{-1}$) and spiramycin (10 µg $disk^{-1}$) for bacteria and spiramycin (10 µg $disk^{-1}$) for yeast were used as standard antibiotics for positive control in the same conditions as the tested oil. After incubation, the zones of inhibition were measured and the results reported in millimeters (mm). Each test assays were repeated in triplicate (Amhis et al., 2001; Bereksi et al., 2018).

### Determination of MIC

The minimum minimum inibitory concentration was evaluated by the microdilution method with a 96 microwell plate. The 96-well plates were prepared by dispensing into each well 50 µl of nutrient broth. 50µl of the essential oil solution was added to the wells of the 2nd column. Finally, 50 µl of microbial inoculum were added. The wells of the 1st column containing the culture medium and the microbial suspension served as a control. The plates were shaken and incubated for 24 h at 37 °C. Microbial growth was determined by absorbance at 620 nm using an ELISA reader. The lowest concentration required to completely inhibit the growth of the tested strain was designated as the MIC and expressed in µl $mL^{-1}$ (Kahlmeter and Turnidge, 2012).

## Results and Discussion

### Extraction Process Kinetics and Yield

Although several studies have been undertaken to investigate the yield, chemical composition and the biological activities of essential oils extracted from an important number of medicinal species, the kinetics of essential oils extraction has not been well studied. Kinetic models along with essential oil yield and composition are important from both technological and economical points of view (Milojević et al., 2013; Kusuma and Mahfud, 2016; Nascimento et al., 2020)

The purpose of this study was to determine the time needed to extract the maximum amount of oil, avoiding the waste of time and energy. Considering this, aerial part of *S. calamintha* was submitted to extraction for 4 h by continuous hydrodistillation and aliquots were collected at different times (60 min, 120 min, 180 min, and 240 min).

According to Fig. 1, the rate of extraction was increased as the extraction time increased until it reached a plateau from 150 min (yield = 1.2 %), arriving at 1.3 % after the 240 min (4 hours).

The previous work has shown that the extraction rate was increased as the extraction time increased until it reached a plateau or constant. It was fast at the beginning and slow until the end of the extraction process (Kusuma and Mahfud, 2016; Nascimento et al., 2020). The oil recovery trend of *S. calamintha* relative to total yield (100 %) as function of time is represented as follows: after 60 min (69.23 %), 120 min (23.07 %) and 180 min (07.69 %).

The maximum essential oil yield and the duration of extraction to attain it varied from one plant material to another and under the applied operational conditions (Milojević et al., 2013).

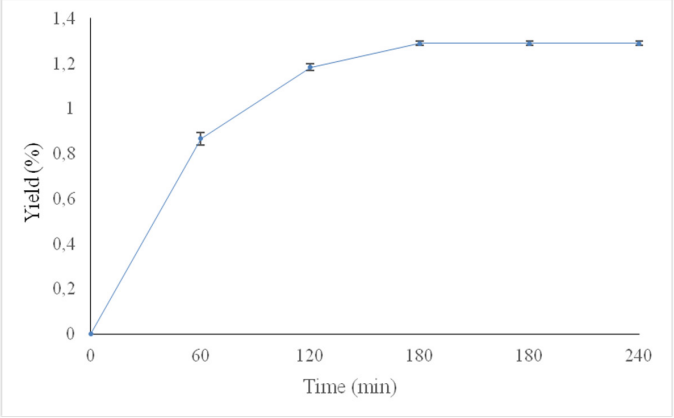



**Figure 1.** Yield (%) of *S. calamintha nepeta* (L.) Briq. essential oil as function of time (min)

The oil obtained was highly volatile, with a strong, pleasant and mentholated, aromatic odour, a liquid appearance and a light yellow colour.

The variations in yields were obtained in samples of *S. calamintha* extracted by hydrodistillation using a Clevenger from different regions in Algeria (from 1.48 % to 2.54 %) (Labiod, 2016). The yield obtained in this study represents an average value compared to other plants that are industrially exploited as a source of essential oil. It is higher than that of rose (0.1 – 0.35 %) and lower than that of thyme (2.25 %) (Benchaqroun et al., 2012). These differences can be attributed to several factors such as the nature of the soil, the geographical area, the harvest period, the quality of the plant material used and the method of extraction (Barra, 2009).

### Physical Characteristics

The measurements of the physical characteristics is a valuable analytical tool in determining composition and purity of an essential oil (Clarke, 2008). The AFNOR standard advocates a density of 0.906 for a low quality oil and 0.990 for oils of very high quality (Belkhodja el al., 2016). With a density of 0.910 for the essential oil analyzed, it is suggested that this oil has acceptable quality.

As for the refractive index, the results obtained showed the value of 1.480. Indeed, AFNOR provides for the essential oil refractive index between 1.495 for high-quality oils and 1.513 to lesser quality oils (Belkhodja et al., 2016).

The refractive index of the essential oil is a weighted average of the reftactive index of each of its components, which takes into account the molar fractions of the components in the oil (Ospina et al., 2016). In general, the refractive index changes essentially with the content of monoterpenes and oxygenated derivatives. A high content of monoterpenes will give a high index. According to the results obtained and the GC/MS, the essential oil tested was rich in monoterpenes (polegone 73.54 %) (Bouzidi et al., 2018).

Considering that published scientific works on the physicochemical characteristics of this oil are non existent, it is difficult to make any further discussion concerning the results obtained. But according to the AFNOR standard, it appears that the essential oil from this study is of good quality.

### Total Phenolic Content

Phenolics which exist naturally in an approximated number of 8000, share the identical prevalent structure composed of one or more aromatic hydroxyl nuclei (Hatami et al., 2014). So far, plant phenolics form one of the main groups of compounds working as primary antioxidants or free radical scavengers (Miguel, 2010; Viuda-Martos et al., 2011 Hatami et al., 2014). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. They may also have a metal chelating potential (Javanmardi et al., 2003).

Total phenolic contents were determined using a spectrophotometric technique, based on the Folin-Ciocalteau reagent, and calculated as gallic acid equivalents per gram of essential oil. The Folin–Ciocalteu assay has been proposed as a standardized method for use in the routine quality control and measurement of antioxidant capacity of food products and dietary supplements (Ainsworth and Gillespie, 2007). This assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm (Ainsworth and Gillespie, 2007).

The calibration curve was plotted using data combining the measured optical densities of each solution with already known concentration of gallic acid. The content of phenolics was calculated from the regression equation of the calibration curve ($R^2$ =0.943, y = 0.6502x + 0.0367), expressed in GAE as milligrams per gram of the extract. The total phenolic content of essenial oil of *S. calamintha* was 219±0.06 mg gallic acid equivalents per g of essential oil.

A high phenol content was found in *S. calamintha* from the Annaba area (482,666 ±2.516 µg GAE $mg^{-1}$ extract), while that of the Jijel area is considered a less rich source of total phenols (264,666 ± 0.577µg GAE $mg^{-1}$ extract extract) (Labiod, 2016).

In general, polyphenol content varies qualitatively and quantitatively from plant to plant, which can be attributed to plant and environmental factors (Dorman and Deans, 2000; Holley and Patel, 2005).

### Antioxidant Activity

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Javanmardi et al., 2003). The DPPH radical scavenging method was used to evaluate the antioxidant activity of essantial oil of *S. calamintha* ssp. *nepeta* in comparison to that of the standard antioxidant ascorbic acid. In this test, the DPPH· of purple color is reduced in a yellow compound, the diphenylpicrylhydrazine, whose intensity of the color is inversely proportional to the reducing capacity of antioxidants present in the medium (Arulpriya et al., 2010).

The oil tested showed a significant effect in inhibiting free radicals produced by DPPH, reaching up to 91.81 % at 500 µg $mL^{-1}$ (Fig. 2) with an $IC_{50}$ value of 31.25 µg $mL^{-1}$. This capability was decreased with the decrease of oil concentration. Ascorbic acid showed a higher percentage of inhibition (97,92 %) at the same concentration (500 µg $mL^{-1}$).

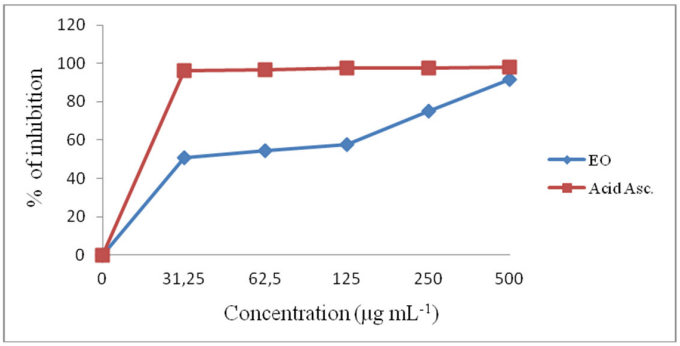



**Figure 2.** The antioxidant activity of the essential oil of *S. calamintha* ssp. *nepeta* (L.) Briq.

A previous work carried out on the essential oil of *S. calamintha* from different regions has shown an antiradical activity with a percentage of inhibition between 20 and 50 % for a concentration of 1000 µg $mL^{-1}$ (Labiod, 2016).

Babajafari et al. (2015) established that different *Satureja* species exerted anti-oxidant, lipid peroxidation inhibitory, and anti-inflammatory activities.

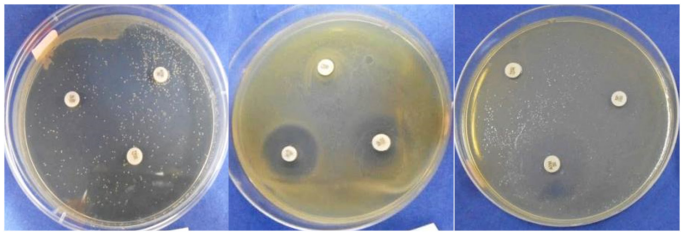
Antioxidant activity is expressed as the ability to inhibit the oxidation as well as eliminate free radicals (El-Ghorab, 2006). However, the molecular mechanism of radical scavenging activity could be attributed to the presence of polyphenolic compounds (Kaur and Kapoor, 2002; Javanmardi et al., 2003; Salehi et al., 2005; El-Ghorab, 2006; Kizil et al., 2010; Hatami et al., 2014). It has already been shown that polyphenolic compounds were responsible for radical scavenging activity in Lamiaceae family due to ease of their hydrogen atom donation to active free radicals (Salehi et al., 2005). It has earlier been reported that plant phenols can behave as ROS (Reactive Oxygen Species) scavengers, metal chelators and enzyme modulators and prevent lipid peroxidation (Kizil et al., 2010).

Ouakouak et al. (2015) report that the antioxidant activity of plant essential oils containing terpenes is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. The most powerful scavenging compounds were reported to be monoterpene ketones (Kizil et al., 2010; Viuda-Martos et al., 2011). It is well known that pulegone (73.54 %), predominant in the oil tested, has considerable antioxidant power (Labiod, 2016).

### Antimicrobial Activity

Recently, the emergence of microbial resistance mechanisms against conventional food preservatives and increasing awareness of their residual toxicity or possible side effects have highlighted the importance of the essential oils as effective alternative to overcome this problem (Quinto et al., 2019). Several studies have reported that essential oils, derived from different species of *Satureja* genus, possess considerable antibacterial and antifungal activities against different microorganisms (Demirci et al., 2011; Ghorbanpour et al., 2016; Bojovic et al., 2018 ; Radi et al., 2019 ; Maccelli et al., 2020).

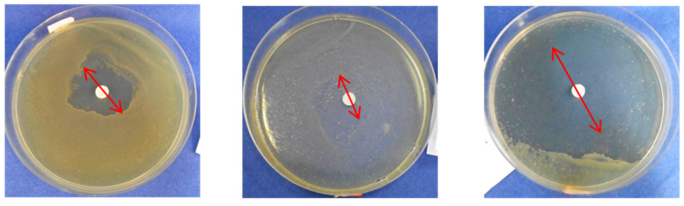
The inhibition zone above 8 mm in diameter was regarded as a positive result (Tekwu, 2012). As shown in Table 1, the essential oil exhibited the antimicrobial activity against *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* with 85 mm, 57 ±1,73 mm and 24,67 ± 1,53mm of inhibition zones diameters, respectively (Fig. 3 and 4).



*Candida albicans* *Staphylococcus aureus* *Pseudomonas aeruginosa*

**Figure 3.** Effect of the antibiotics on *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa*

The results obtained from the disc diffusion method, followed by measurements of MIC, indicated that *Candida albicans* was the most sensitive microorganism tested, with the lowest MIC value (0.09 µL $mL^{-1}$). Strong inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was observed with MIC values (0.78 and 1.56 µL $mL^{-1}$). However, *Pseudomonas aeruginosa* was less sensitive to this oil compared to the other strains tested.



*S. aureus* *P. aeruginosa* *C. Albicans*

**Figure 4.** Effect of 50 % of essential oil of *S. calamintha* ssp. *nepeta* (L.) Briq. against the tested bacterial and fungal strains

**Table 1.** Results of antibiogram and antimicrobial activity of essential oil of *Satureja calamintha* ssp. *nepeta* (mm)

| | *Candida albicans* | *Staphylococcus aureus* | *Pseudomonas aeruginosa* |
|---|---|---|---|
| Antibiotics | | | |
| Cefazolin (30 µg $disk^{-1}$) | | 8 ± 0.10 | 7 ± 0.30 |
| Aztreonam (30 µg $disk^{-1}$) | 0 | 28 ± 0.15 | 23 ± 0.20 |
| Spiramycin (10 µg $disk^{-1}$) | | 15 ± 0.21 | 19 ± 0.25 |
| Essential oil | | | |
| 25 % | 25.0 ± 20.00 | 17.33 ± 1.15 | 9.67 ± 1.15 |
| 50 % | 47.33 ± 0.58 | 24.67 ± 1.53 | 19.67 ± 0.58 |
| 100 % (undiluted essential oil) | 85.00 ± 0.00 | 57.00 ± 1.73 | 24.67 ± 1.53 |
| MIC (µL $mL^{-1}$) | 0.09 | 0.78 | 1.56 |

These differences in the sensitivity of the microorganisms against the essential oil analyzed can be explained by the quantity and quality of the bioactive molecules or the nature and composition of the cell wall as well as the power of the enzymatic system of the cell which controls its metabolism (Matasyoha et al., 2007).

Many of the previous studies have demonstrated that essential oils show a considerable antimicrobial activity due to the presence of chemical compounds containing mainly aromatic oxygenated monoterpenes and high phenolic contents (Gormez et al., 2015; Maccelli et al., 2020). So, the high antimicrobial activity of *S. calamintha* ssp. *nepeta* essential oil could be explained through the high level of pulegone, well known for hav¬ing antibacterial activity (Flamini et al., 1999; Oumzil et al., 2002; Radi et al., 2019).

Essential oil of *S. calamintha* ssp. *nepeta* tested contains pulegone (73.5 %) and isomenthone (7.9 %), cis piperitone oxide (2.3 %), limonene (1.8 %), trans isopulegone (1.1 %), spathulenol (0.8 %) as major components (Bouzidi et al., 2018).

Oxygenated monoterpenes have a broad spectrum of antimicrobial activity (Salamci et al., 2007). The presence of an oxygenated function increases the antimicrobial properties of terpenoids (ketones) (Dorman and Deans, 2000). As a result of their lipophilic character, cyclic monoterpenes influence the permeability of cell membranes (Cox et al., 2000; Zouari et al., 2010). Pulegone has a similar structure to carvone which has been shown to affect the cell membrane by dissipation of pH gradient and membrane potential of cells (Salehi et al., 2005; Kumar et al., 2016).

Although the mechanism of antimicrobial activity of terpenes is not entirely known, it seems that these lipophilic compounds may alter the structural and functional integrity of the cell membrane in Gram-negative and Gram-positive bacteria as well as in Fungi (Maccelli et al., 2020). However, other components and the possible interaction between these substances could also affect the antimicrobial activities. In fact, the antimicrobial activity of essential oils may be the result of synergy, antagonism, or additive effects of their components which possess various potencies of activity (Ardalan Alizadeh, 2015).

## Conclusion

Plants are an immense source of complex natural molecules exploited by humankind. This study was carried out to determine physical characteristics, the total phenolic content, antioxidant and antimicrobial activities of essential oil of *Satureja calamintha* spp. *nepeta* (Lamiaceae).

The essential oil of *Satureja calamintha* spp. *nepeta* presented the yield of 1.3 %, it had density of 0.910 and refractive index of 1.480. According to these physical characteristics, it is suggested that this oil has a good quality according to AFNOR standards.

In addition, for the evaluation of the antioxidant activity, essential oil showed a high scavenging power of free DPPH radical (91.81 %) at 500µL $mL^{-1}$. This oil also presented a good antimicrobial activity against the tested fungal and bacterial strains.

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