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Clinopodium nubigenum (Kunth) Kuntze essential oil: Chemical composition, antioxidant activity, and antimicrobial test against respiratory pathogens

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The essential oil of leaves and flowers of *Clinopodium nubigenum* (Kunth.) Kuntze (Lamiaceae) collected in the province of Pichincha-Ecuador was steam distilled and analyzed by gas chromatography mass spectrometry (GC/MS) to determine its chemical composition. The majority of the compounds identified were carvacrol acetate (42.1%), carvacrol (20.6%), pulegone (6.3%) and thymol (5.5%). Antioxidant activity was assessed by the assays of diphenylpicrylhydrazyl (DPPH) (IC_{50}: 1.8 µl / ml), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (IC_{50}: 0.3 µl / ml) and β-carotene (IC_{50}: 0.031 µl / ml) compared to *Thymus vulgaris* and butylated hydroxyanisole (BHA) as referents. The specie also shows a promising medicinal potential exhibiting significant antibacterial activity at different concentrations against *Staphylococcus aureus* (2.5% v/v), *Streptococcus pyogenes* (0.6% v/v), *Streptococcus pneumoniae* (0.6% v/v) and *Streptococcus mutans* (0.6% v/v), suggesting an interesting natural alternative in the fight against bacteria that generate resistance to other antibiotics.

Key words: *Clinopodium nubigenum*, gas chromatography mass spectrometry (GC/MS), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), diphenylpicrylhydrazyl (DPPH), antimicrobial test.

INTRODUCTION

A minimal fraction of the known biodiversity has been sufficiently studied to know its properties and potentialities for the multiple benefits for humans (Estrella, 2005). The high biological and cultural diversity of the Ecuador have let it become one of the countries with great potential in terms of traditional therapeutics. Therefore, it is important to establish different aspects of importance, such as the methods of use of each plant and the curative benefits of traditional medicine to the different communities in the country (Zambrano et al., 2015). Moreover, if we consider Andean cultures to possess a wealth of knowledge about the use of plants and the quantity of species used, traditional medicinal practices could be more diverse than hitherto documented and published; Therefore, it is fundamental to continue with ethnobotanical studies that allow systematizing and disseminating this valuable knowledge (Ansaloni et al., 2010) in order to achieve greater use of both technical and economic resources, considering the traditional management them and the state of

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conservation (Ocampo, 1994).

According to Myers et al. (2000), the Tropical Andes is believed to contain at least 20,000 known plant endemics, and many more species, probably thousands, remain to be discovered there. However, it is important to mention that due to the increase in global climate change the native species that inhabit sensitive ecosystems such as those of the paramo are threatened because they will react by means of displacement, adaptation (either in terms of evolutionary changes or physiological adaptations) or local extinction of the species that form it, and locally these mechanisms could interact and lead to alterations of their compositions (Aguirre et al., 2014).

The *Clinopodium nubigenum* (Kunt) Kuntze may be found in this bio diverse zone. The genus *Clinopodium* belongs to the *Lamiaceae* family and comprises of 271 described species, with 142 being accepted as such. Within this group, *C. nubigenum* traditionally known as "sunfo" or "tipo de llano", is an aromatic medicinal plant native to Ecuador, which has been reported in the provinces of Carchi (Nudo del Boliche), Pichincha (Paso de Guamaní), Tungurahua (Páramo de Minza-Chica), Cañar (Páramo de Biblián), Azuay (Páramo de Tinajillas), El Oro (Páramo de Corredores) and Loja (Saraguro) (Epling and Jativa, 1964; Pulgar et al., 2010; Ansaloni et al., 2010; Missouri Botanical Garden, 2017).

It is an herbaceous plant that can reach approximately 15 cm in height and it is possible to identify it by its characteristic of being covered with small white hairs on its leaves, the stem is quadrangular and reddish brown color (Aguilar et al., 2009). This aromatic plant is also known with the synonyms of *Thymus nubigenus* Kunth, *Micromeria nubigena* (Kunth) Benth, and *Satureja nubigena* (Kunth) Briq (Gilardoni et al., 2011). According to Cantino and Wagstaff (1998), after making a generae reassessment, based on molecular data and some herbarium studies, they recommend that the genera *Satureja* and *Micromeria* should be considered in a narrow sense and restricted way for the Old World, while most of the specimens of the New World form a clade including the genera *Clinopodium* and *Calamintha*, all in order to group and facilitate further studies given their similarities.

Aiming at its medicinal usefulness, there are several reports of use as a hot infusion of flowers and leaves with anti-inflammatory, stomachal, anti-influenza and anti-infective activity against dysentery and attenuating menstrual syndromes (Gilardoni et al., 2011). They offer relief of general malaise and to counteract the cold (Ansaloni et al., 2010). Their use is also cited to prevent urinary incontinence in children (de la Torre et al., 2008). The study of Lituma and Molina (2008) is of the opinión that the “sunfo” has analgesic activity. Another study by Jerves-Andrade et al. (2014) details the ethnopharmaceutical uses for stomach conditions and gastritis. The species in question denotes a promising medicinal potential, and the aim of this study was to evaluate and elucidate the chemical composition, and its antioxidant and antimicrobial activities.

MATERIALS AND METHODS

Plant material and essential oil distillation

The plant material studied was collected from the paramos of the parish of Píntag (27.5 Km S.E. de Quito) in the province of Pichincha, Ecuador. The plant was identified as *C. nubigenum* (Kunth.) Kuntze by the National Herbarium of Ecuador. In order to obtain the essential oil, the vegetable sample of approximately 6 Kg was distilled by steam trapping in a distiller with a capacity of 40 L, the process took 5 h.

Essential oil characteristics

For characterization of the essential oil obtained, the percentage of yield and the different organoleptic (odour, colour, taste) and physicochemical parameters were gotten (density, refractive index, pH) at 20°C.

Gas chromatography mass spectrometry (GC/MS) analysis

The sample analyzed by GC/MS was prepared by dissolving 10 µl of essential oil in 1 ml of dichloromethane, the volume of injection was 2 µl. The analysis was carried out on a Varian 3900 chromatograph, a Factor Four® column (5% phenyl-95% dimethylpolysiloxane 30 mx 0.25 µm) and helium carrier gas was used at a flow rate of 1 ml/min with a Split at 1:50, oven programming is shown in Table 1. In the Varian Saturn 2100 mass spectrometer, the conditions were set to a current emission of 10 µAmp, ionization voltage 70 eV, mass range 35 to 400 Da, scanning speed 1 scan/min, trap temperature 220°C and temperature transfer line 260°C. The total GC-MS analysis time was 90 min. The chemical identification of the essential oil was done by comparing the mass spectra, using the commercial database of chemical compounds of the National Institute Standard and Technology NIST. In addition, experimental lineal retention indexes were determined in relation to the retention times of a series of C_8 to C_{20} alkanes, later compared to the theoretical retention indexes of Adams (2007).

DPPH and ABTS assays

The methods of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) developed by Brand-Williams et al. (1995) modified by Noriega et al. (2015) as well as the method with 2,2'-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) used by Kuskoski et al. (2004) were used. For both evaluations, the essential oil of *T. vulgaris* was a natural reference and as a positive control for Butylhydroxyanisole (BHA).

The DPPH reagent was prepared by dissolving 19.6 mg DPPH in 500 ml of 96% ethanol. For the positive control, BHA dilutions were performed to which 2.9 ml of DPPH reagent were added, the samples were prepared by taking 20 µl of the oils dissolved in 180 µl of dimethyl-sulfoxide (DMSO), then to prepare a range of concentrations to which 2.9 ml of reagent were added DPPH. Both the control dilutions and the oil samples were stirred for 30 min. Subsequently, the absorbances were measured at ʎ 517 nm, 96% ethanol was used as a blank.

For the preparation of the ABTS reagent, solution A (27 mg ABTS in 25 ml distillated H_2O) was prepared, to which 250 µl of

Table 1. GC-MS oven program.

Initial temperature (°C)	Increase (°C/min)	Time (min)	Final temperature (°C)
50	1	50	100
100	5	30	250
250	-	10	250

solution B (188.2 mg $K_2S_2O_8$ in 10 ml distillated H_2O) was added, subsequently after 24 h the reagent was adjusted with 96% ethanol to obtain an absorbance of 0.7±0.02 at ʎ 754 nm. A series of BHA dilutions were prepared to which 0.9 ml of ABTS reagent was added. Samples were prepared by taking 4 µl of the essential oils dissolved in 196 µl of DMSO. To prepare a series of concentrations, 0.9 ml of ABTS reagent was added. Absorbances were measured at ʎ 754 nm, 96% ethanol was used as a blank. For the calculation of the percentage of inhibition of the free radical DPPH and ABTS, the following formula was used:

$$Inhibition\ \% = \frac{A - B}{A} \times 100$$

Where:

A is equal to the absorbance of the blank, and B is the absorbance of the sample.

β-carotene assay

The β-carotene test was performed based on the method developed by Miller (1971) with certain modifications in the concentrations. The essential oil of *T. vulgaris* was used as a natural reference and BHA as a positive control. An emulsion of β-carotene was prepared as follows:

β-carotene 4 ml of 1000 ppm solution in chloroform to which 400 µl of a solution of linoleic acid was added in 8 ml Tween 20®.

The chloroform was evaporated at 40°C for 15 min and flushed at 1 L. For the positive control, a series of 5 ml BHA dilutions were prepared, samples were made by dissolving 50 µl of essential oils in 1 ml Tween 20® to subsequently perform a series of concentrations by adding 5 ml of the β-carotene emulsion. A blank solution (20 µl Ac. 400 µl linoleic + Tween 20 + 50 ml H2O + 0.1 M Tris-HCl pH 7.4) was used. An absorbance reading was performed at ʎ 470 nm followed by a new reading after 60 min at 50°C. For the calculation of the antioxidant activity, the following formula was used:

$$Antioxidant\ activity\ \% = \frac{DRC - DRS}{A} \times 100$$

Where:

DRC is the percent degradation of the control and DRS corresponds to the percent degradation of the sample.

To determine the respective percentages of degradation, the following formula was applied:

$$DR = \frac{\ln(\frac{a}{b})}{60} \times 100$$

Where:

a is the initial absorbance and b the absorbance after 60 min at 50°C.

Antimicrobial susceptibility test

For the test of antimicrobial resistance, four certified strains ATCC (American Type Culture Collection) of Gram-positive bacteria were acquired: *Staphylococcus aureus* ATCC®: 25923™, *Streptococcus pyogenes* ATCC®: 19615™, *Streptococcus pneumoniae* ATCC®: 49619™ and *Streptococcus mutans* ATCC®:25175™. Their subsequent inoculation was carried out on tryptic soy agar medium (TSA) except for *S. pneumoniae* requiring lamb's blood agar, the necessary environmental conditions were followed for each microorganism (24 H, 37 °C); *S. aureus* in aerobiosis and *Streptococcus* strains in anaerobiosis. The inoculum was obtained in tryptic soybean broth (TSB) (18 H, 35°C), followed by the measurement of the absorbance at ʎ 625 nm of 0.08 to 0.11 for standardization of the initial inoculum. To test for microbial susceptibility, the Well Diffusion assay was used for which 1 ml of inoculum in plaque (TSA) was dispersed for the case of *S. aureus* and for the *Streptococcus* strains brain heart infusion plaques (BHI). In each plate, 4 wells of 6 mm φ were made, in which 0.8 µl of the different experimental materials were placed: the dilutions of the essential oil (2,5-1,25-0,6-0,3-0,15 % v/v) in DMSO as well as the positive control Penicillin (106 IU), and the negative control (DMSO). The plates were incubated at 37°C 24 h under the above-described conditions to finally carry out measurement of the inhibition halos.

RESULTS AND DISCUSSION

Essential oil characteristics

The oil characteristics obtained by distillation (Table 2) allow to establish differences between other oils. Those properties are subject of change, attributed to the environmental conditions, soil factors, life cycle of the specie and the extraction method employed. This could be affirmed considering for example the research lead by Ruiz et al. (2010), plants collected in a province from Ecuadorian south sierra region showed major yield (1.42%) although the physical properties were similar. Other characteristic is the pungent odor present in the oil according to Jyoti (2016), due to the presence of carvacrol a monoterpenoid phenol and its derivatives as it occurs in the *Origanum sp.*

Chemical composition of the oil

In the essential oil of *C. nubigenum*, 25 compounds were found, representing 98.09% of the essential oil

Table 2. Characteristics of *Clinopodium nubigenum* essential oil.

Parameter	Characteristic / Value	
Yield	0.27 % (p/p)	
Odor	Citrus pungent	
Color	Amber	
Taste	Bitter	
Density	0.91 g/cm^3	
Refraction index	1.479	20°C
pH	5.6	

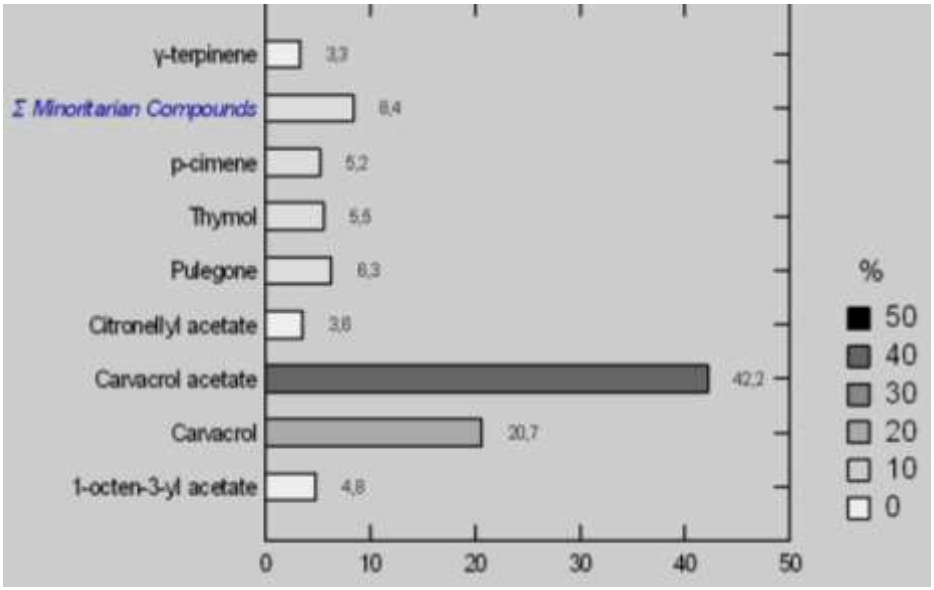


Figure 1. Amount of *C. nubigenum* (Kunth.) Kuntze essential oil main constituents.

composition (Figure 1). The preliminary GC/MS study (Table 3) reveals the presence of carvacrol acetate (40,95%), carvacrol (21,21%), pulegone (6,09%) and thymol (5,67%) as the main components. The chemical identification agrees with the research done by Ruiz et al. (2010), defining carvacrol acetate as the major component. Other studies have determined a significant difference in chemical composition, showing thymol and carvacrol as major components (El-Seedi et al., 2008), these changes in chemical composition could be attributed to climatic differences, soil composition, vegetative cycles, plant age and cultivation conditions (Gilardoni et al., 2011).

DPPH, ABTS and β-carotene assays

The capacity of the DPPH radical for the essential oil of *C. nubigenum* increased with the major concentration of essential oil in the prepared dilutions. The DPPH was scavenged by the antioxidant molecules forming the reduced form DPPH-H because of this reason, the color changes from purple to yellow in the reduction process quantified by the spectrophotometric method by decreasing the absorbance at 517 nm. The IC_{50} value for the DPPH radical assay (Figure 3) was 1,812±3,0 E-002 µl/ml, in contrast to the natural reference of *T. vulgaris* essential oil IC_{50} DPPH 0,759±1,0 E-002 µl/ml and BHA IC_{50} DPPH 5,2 E-003±1,3 E-005 µl/ml. However, the ABTS is radicalized in the presence of $K_2S_2O_8$ forming a blue-green compound which is decolorized by its reduction in the presence of the antioxidant molecules, a process quantified by spectrophotometry. In this way, the IC_{50} for ABTS was 0,3375±9.5 E-004 µl/ml in contrast to *T. vulgaris* IC_{50} ABTS 0,2107±3.3 E-004 µl/ml and BHA IC_{50} ABTS 1,22 E-003±4,0 E-005 µl/ml (Figure 3). In the third antioxidant test, the technique is based on oxidative discoloration of β-carotene in the presence of linoleic acid. Discoloration occurs when β-carotene reacts with the free radicals generated by linoleic acid. The presence

Table 3. Preliminary chemical composition of *Clinopodium nubigenum* essential oil analyzed by GC/MS and reported bioactivity of its phytochemical compounds.

S/N	Compound name	LRI[a]	LRI[b]	Preliminary identification	Relative composition (%)	Reported bioactivity	References
1	Tricyclene	916	926	RT, MS 70 eV, Adams and NIST databases	0.35	-	-
2	α-thujene	922	930	RT, MS 70 eV, Adams and NIST databases	0.59	-	-
3	α-terpinene	1014	1017	RT, MS 70 eV, Adams and NIST databases	0.33	-	-
4	p-cimene	1022	1024	RT, MS 70 eV, Adams and NIST Databases	3.80	Antifungal	Ksouri et al. (2017)
5	Limonene	1025	1029	RT, MS 70 eV, Adams and NIST databases	0.67	-	-
6	B-ocimene trans	1052	1050	RT, MS 70 eV, Adams and NIST databases	1.31	-	
7	γ-terpinene	1054	1059	RT, MS 70 eV, Adams and NIST databases	2.43	Anti-tumor and prooxidative, anti-inflammatory	Özkan and Erdoğan (2017); Leelarungrayub et al. (2017)
8	1-octen-3-yl acetate	1110	1112	RT, MS 70 eV, Adams and NIST databases	4,28	Repellent, insecticide, fungistatic, acaricide	Kihara et al. (2014); Re et al. (1999); Yang and Lee (2012)
9	Pulegone	1236	1237	RT, MS 70 eV, Adams and NIST databases	6.09	Antifungal	Ebadollahi et al. (2017)
10	Thymol	1298	1290	RT, MS 70 eV, Adams and NIST databases	5.67	*Antimicrobial*, anti-dermatophyte, anti-cancer, *antioxidant*	Ruiz-Rico et al. (2017); Mahboubi et al. (2017); Li et al. (2017); Llana-Ruiz-Cabello et al. (2015)
11	Carvacrol	1308	1299	RT, MS 70 eV, Adams and NIST databases	21.21	*Antibacterial*, neuroprotector, antifungal, herbicide	Engel et al. (2017); Dati et al. (2017); Hosseini et al. (2016); Hazrati et al. (2017)
12	δ-elemene	1333	1338	RT, MS 70 eV, Adams and NIST databases	0.96	-	-
13	Thymol acetate	1359	1352	RT, MS 70 eV, Adams and NIST databases	0.60	-	-
14	Citronellyl acetate	1364	1352	RT, MS 70 eV, Adams and NIST databases	3.54	-	-
15	Carvacrol acetate	1377	1372	RT, MS 70 eV, Adams and NIST databases	40.95	*Antibacterial*, anthelmintic, *antioxidant*, anti-inflammatory	Andre et al. (2016); Pires et al. (2014); Damasceno et al. (2014)
16	β-cubebene	1387	1388	RT, MS 70 eV, Adams and NIST databases	0.15	-	-
17	β-elemene	1388	1390	RT, MS 70 eV, Adams and NIST databases	0.42	-	-
18	β-caryophyllene	1411	1419	RT, MS 70 eV, Adams and NIST databases	0.35	-	-
19	β-Gurjunene	1423	1433	RT, MS 70 eV, Adams and NIST databases	0.21	-	-
20	α-humulene	1451	1454	RT, MS 70 eV, Adams and NIST databases	0,14	-	-
21	γ-muurolene	1477	1479	RT, MS 70 eV, Adams and NIST databases	0,31	-	-
22	Bicyclogermacrene	1491	1500	RT, MS 70 eV, Adams and NIST databases	1,85	Anti-inflammatory, anticancer	Morshedloo et al. (2017)
23	δ-amorphene	1504	1512	RT, MS 70 eV, Adams and NIST databases	0.13		
24	δ-cadinene	1513	1516	RT, MS 70 eV, Adams and NIST databases	1,32	Acaricide	Guo et al. (2017)
25	Spathulenol	1579	1578	RT, MS 70 eV, Adams and NIST databases	0.44	-	-
	Total				98.09	-	-

LRI[a] Lineal experimental retention index calculated in comparison to alkane series (C_8 to C_{20}) retention time. LRI[b] Theoretical Retention Index (Adams, 2007)..

of antioxidant substances prevents oxidative discoloration of the emulsion by the neutralization of free radicals. The antioxidant activity evaluated with the β-carotene bleaching assay obtained an IC_{50} de 0,031±3,0 E-003 µl/ml in contrast to the IC_{50} 0,022±1,0 E-003 µl/ml of *T. vulgaris* and IC_{50} 7,479 E-006±4,8 E-006 µl/ml of BHA. The results of this study show that the antioxidant activity is comparable to the natural reference *T. vulgaris*. If we review the data in Table 3, it can be deduced that within the preliminary chemical compounds of the essential oil we found limonene, δ-elemene, γ-murolene, carvacrol acetate y thymol as the possible antioxidant agents about which there is a reference to their activity with both thymol and

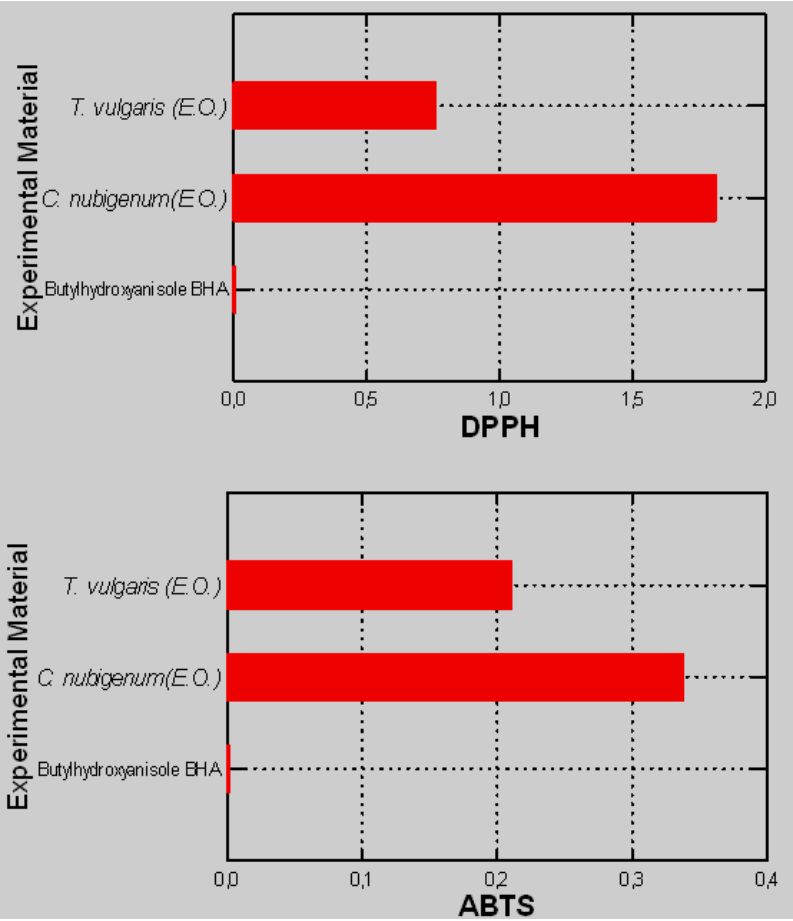


Figure 2. Free radical DPPH and ABTS scavenging activity. Comparison of IC_{50} mean between *Thymus vulgaris*, *Clinopodium nubigenum* and BHA control using both DPPH and ABTS methods.

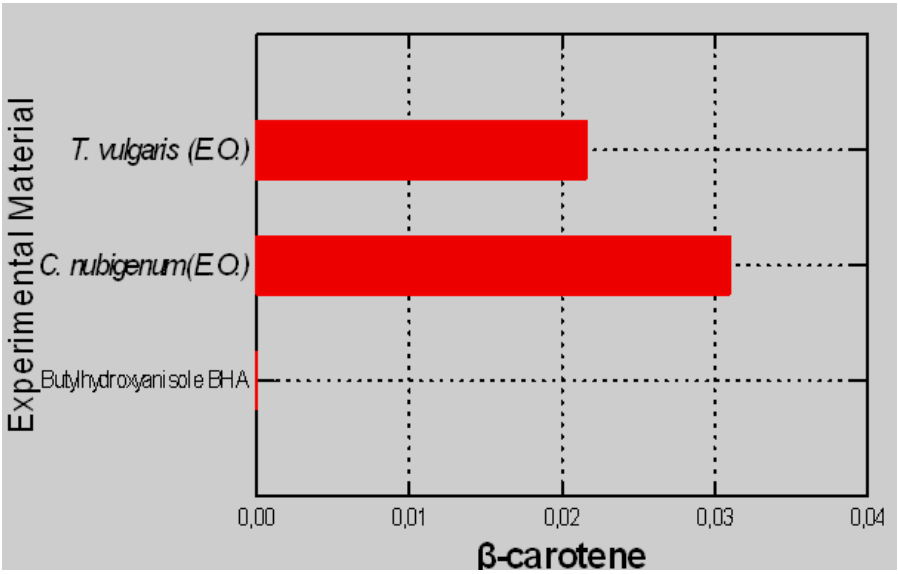
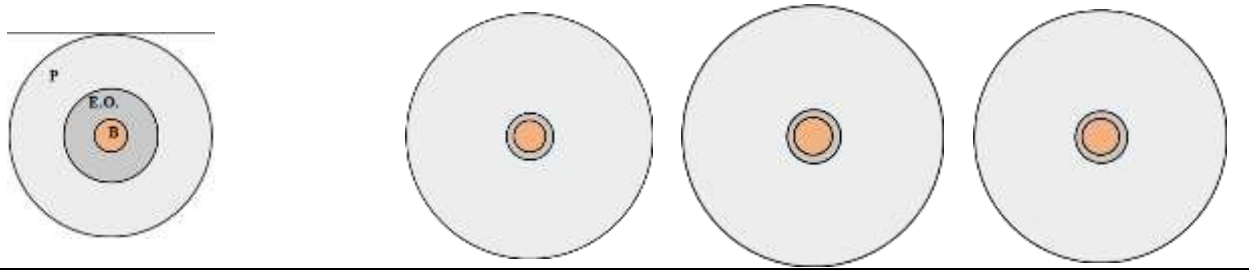


Figure 3. Inhibitory concentration (IC_{50}) results applying β-carotene antioxidant activity test.

Table 4. Comparative data between inhibition halos developed by Penicillin and *Clinopodium nubigenum* essential oil.

Variable	Microorganism			
	S. aureus	***S. mutans***	***S. pyogenes***	***S. pneumoniae***
Essential oil concentration to show m.o. inhibition	2.5%	0.3%	0.3%	0.3%
Essential oil halo diameter	12.68 mm	8.80 mm	8.46 mm	8.46 mm
Penicillium halo diameter	40.00 mm	43.58 mm	41.53 mm	40.51 mm
DMSO halo diameter			6.00 mm	
Well diameter			6.00 mm	
				

P: Penicillin halo (Positive control: 10^6 I.U.); E.O.: *Clinopodium nubigenum* essential oil halo; B: Initial well diameter equal to DMSO (Negative Control).

carvacrol acetate being two major compounds. However, all components can act synergistically, since from a chemical point of view essential oils are very complex matrixes and it is difficult to attribute their antioxidant activity to one or a few compounds (Shakeri et al., 2017), although generally the majority of those being responsible for the biological effects (Ksouri et al., 2017).

Antimicrobial susceptibility test

Statistical analysis showed that the antimicrobial results presented activity at different concentrations of essential oil (Table 4) 2.5% for *S. aureus* and 0.3% for *S. mutans, S. pyogenes and S. pneumoniae*. These results are in line with El-Seedi et al. (2008), presuming that this activity is mainly due to the presence of carvacrol. According to the study of Magi et al. (2015), this compound exerts a direct bactericidal activity causing damage to the bacterial cell membrane. In addition, it was shown that carvacrol is not prone to generate bacterial resistance in some species of *Streptococcus* and could exhibit a synergistic behavior in combination with other antibiotics such as erythromycin. However, the chemical composition also highlights thymol, carvacrol acetate and limonene that can accentuate this activity.

Conclusion

It was evidenced that the essential oil of *C. nubigenum* (Kunth.) Kuntze showed a significant antioxidant activity possibly attributed to the presence of one of its major components, carvacrol acetate, in addition to other compounds such as thymol, limonene, δ-elemene and γ-murolene, for this reason, it is interesting to know the relationship between compounds that potentiate this effect. Regarding microbial activity, the effect for all pathogens tested was observed, being more effective against *S. mutans*, *S. pyogenes*, *S. pneumoniae* and less so for *S. aureus* strains. The compounds carvacrol and carvacrol acetate are those of greater presence in the essential oil and bibliographically can be indicated as the main antimicrobial agents. These data suggest an interesting natural alternative in the fight against bacteria that generate resistance to other antibiotics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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