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Composition And Antimicrobial Activity Of The Essential Oil Obtained From Satureja Brevicalyx E. Leaves

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Abstract

The genus Satureja is characterized for being aromatic shrubs of worldwide distribution, of ancestral use by diverse cultures due to its medicinal properties which are attributed to the hydrocarbon compounds as secondary metabolites that can be extracted in the essential oil of its aerial parts. In this study, the essential oil was extracted from the leaves of S. brevicalyx E. by steam distillation and the relative amount of the metabolites was identified by GC-MS, and their antibacterial activity against Bacillus cereus, Listeria monocytogenes, and Escherichia coli was determined by agar diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. Thirty-one compounds were identified in the oil, of which 90,3 % were found in proportions of less than 6 %, with the highest number of oxygenated monoterpenes menthone at 32,7 %, followed by pulegone (20,89 %) and isomenthone (10,71 %). The oil showed higher activity against gram-positive bacteria with MIC equal to MBC at 781 and 938 µg/ml for L. monocytogenes and B. cereus respectively, while E. coli was less sensitive, with MIC of 1 875 µg/ml and MBC of 3 750 µg/ml. This type of shrub can be a source of isolation of monoterpenes, of great utility in the cosmetic, pharmaceutical, and food industry and its conservation, as an alternative for the inhibition of the growth of pathogens with the potential to form biofilms and cause food-borne diseases.

Keywords: antimicrobial activity,essential oil, oxygenated monoterpenes, satureja brevicalyx, ,

Introduction

The genus Satureja belongs to the Lamiaceae family and is characterized for being annual herbs and aromatic shrubs or perennial of arid, sunny, rocky habitats, which there are 200 species with adaptative features for the temperate and tropical regions worldwide (Flores et al., 2013; Soltanzadeh et al., 2018; Čopra-Janićijević et al., 2020). It is known for its medicinal properties, the aerial parts are used to make infusions and produce antiflatulent digestive tonics, antiseptics, analgesic or expectorants for cold and others (Soltanzadeh et al., 2018).

Several studies denominate different species of this genus as Satureja bachtiarica, S. boliviana, S. laxiflora, S. hortensis, S. Khuzestanica, S. m2QW ontana, S. ovobata, S. parvifolia, among others. In addition, denominate them as antiviral, antibacterial, and/or fungal, antioxidant, antiparasitic, antidiarrheal, vasodilator activity, among others (de Rojas et al., 1996; Yamasaki et al., 1998; Hajhashemi et al., 2000; Hajhashemi et al., 2002; Sonboli et al., 2004; Abad et al., 2008; Soltanzadeh et al., 2018; Vitanza et al., 2018; Mohammed et al., 2019; Bagher & Khodaei, 2020). Satureja brevicalyxEpling is an endemic aromatic plant located in sandy, clay, and rocky soils hillsides from 3 300 to 3 800 m.a.s.l in Peruvian Andes. It is a 15 m high evergreen shrub and is characterized by small, spatulate, sessile, whorled, opposite, margin entire leaves, its flowers are white, axillary, tetramerous, bilabiate, with didynamous stamens, superior ovary, apical style, and simple stigma that flower in spring and summer (Flores et al., 2013). In Peru, S. brevicalyx is known commonly as wayra muña, muña - muña, inca muña, muña del inca, among others names. In addition, it is known by its old use in the culinary area as a flavoring and in medicine as an analgesic, anti-inflammatory, antimicrobial, antispasmodic and to treat gastrointestinal diseases, (Flores et al., 2013; Vásquez & Alvarado-García, 2016).

In this sense, it was established that some secondary metabolites produced by the plants are responsible for their old effective use in medicine. The essential oils are composed of vegetable metabolism and volatile organic that could contain between 50 to 300 hydrocarbons, which belong to terpenic, alcohols, aldehydes, ketones, ethers and esters, phenols, phenylpropanoids groups, etc. These are responsible for the plants' aroma and carry out an ecological role as pollinator attractants and seed dispersers, and in chemical defense, by repelling pests. Likewise, according to the benefit of human consumption, they have become more important over time in the food industry as flavorings; in the cosmetic area, as fragrances and other properties for skincare products; and in the pharmacological area, as antiseptics, anti-inflammatory, antispasmodic, antitussive, expectorant, antirheumatic, cicatrizant, and others (López, 2004; Ruiz et al., 2015).

The diversity of medicinal and agricultural properties of plants have motivated diverse studies, both to identify responsible chemical compounds of its activity and to define the sensibility reaction in different pathogenic microorganisms, expanding the possibilities of their isolation and mass production, either for their application as pest control in culture or at the pharmacological level, and thus, in order to prevent nosocomial infections, or foodborne diseases (FBD), among others.

In this regard, there are few studies in the literature about S. brevicalyx, neither the specificity and sensibility of its antibiotic action nor the compounds that are part of its essential oil are known, that's why, it was proposed as a preliminary aim of this study to obtain the essential oil from the S. brevicalyx E leaves, to identify its compounds, and to determine its antibacterial activity against Bacillus cereus, Listeria monocytogenes and Escherichia coli that are bacterias known to be part of FBD etiologic agents and can also form biofilms in different environments, such as ready-to-eat food production areas or hospital care areas by contaminating stainless steel metal surfaces (Cáceres et al., 2019; Park et al., 2019; Ramires et al., 2021).

Materials and methods

Essential oil extraction

In the mountainous Sierra area of the Cotabambas district of the Apurimac department of Peru, located at 3 400 m.a.s.l approximately. 3 200 k of S. brevicalyx E. leaves were collected that were moved to the School of Engineering of Universidad Nacional Micaela Bastidas de Apurímac (Micaela Bastidas National University of Apurímac), where were washed with abundant clean water, separating into small bunches to eliminate impurities. Then, they were put in an aluminum tray and the withered, infected, and perforated leave were

thrown away. The selected leaves were left in trays and outdoors under sunlight and then, indoors at room temperature between 20 and 25 °C, for about three days, until dry.

The dried leaves were crushed until become a greenish dry powder that was saved in an amber glass bottle and was moved to the Organic chemistry laboratory of the School of Pharmacy and Biochemistry of Universidad Peruana Cayetano Heredia, Lima (Peruvian University Cayetano Heredia), where it was proceeded with the essential oil extraction using the steam entrainment distillation technique following the methodology described by Stashenko (2009) in an airtight stainless-steel distiller. In the separatory funnel, it was dehydrated with Na_2SO_4 anhydrous.

Physicochemical characterization

The color, smell, and taste of the extracted essential oil were noted. The yield obtained in dry weight and room temperature of 21 °C were determined following the recommendations of the Peruvian technical standards for oils and fats, then the relative density (NTP 319.081:1974) and refractive index were determined (NTP 319.075:1974). Subsequently, it proceeded to identify the chemical compounds through gaschromatography with an ionization detector (GC-FID) and the volatile composition with a gas chromatograph coupled to mass spectrometry (GC-MS).

GC-FID analysis was made in a Hewlett-Packard 6890 chromatograph computerized with a 0,1 ml sample of the essential oil, diluted at 1 % in ethanol and with Supelcowax TM 10 columns, and methylsilicone SE-30 (30m x 0,25 mm, 0,25 m film thickness), using helium as the carrier gas. The injection temperature was 250 °C and the oven temperature started with 60 °C to 220 °C (for 10 minutes). The compounds were identified by comparing the database NIST 98 with literature reports.

Antibacterial activity using agar diffusion method

The antibacterial activity of essential oil extracted from S. brevicalyx was determined using the agar diffusion method with Listeria monocytogenes, Bacillus cereus, and Escherichia coli from ATCC 19118 freeze-dried strains, 11778 and 25922 respectively, which were reactivated in tryptic soy agar (TSA) (L. monocytogenes and E. coli) and nutrient agar (B. cereus) at 37 °C for 24 hours. From isolated colonies, the concentration for an inoculum of 1,5 x 10^8 inoculum colony forming units per ml (CFU/ml) was adjusted with the swab and physiological solution (NaCl) at 0,9 % by serial dilution and swabbed in Petri dishes with Mueller-Hinton agar in three directions to ensure homogeneous distribution.

Then, sterile Whatman filter paper discs No. 3 of 6 mm were put in the agar pressing gently and added 12 µl essential oil samples were diluted in dimethyl sulfoxide (DMSO) at 25, 50, 75, and 100 % v/v in the filter papers, and they were incubated in upside down for 24 hours at 37 °C. The positive control was a tetracycline solution, and the negative control was DMSO.

The inhibition degree of the bacterial growth is equal to diameters in millimeters of the growth inhibition halos that formed around the discs, which are composed in ranges: no antimicrobial activity (diameters less than 6 mm), low activity (halos between 6 to 8 mm), medium activity (over 8 to 10 mm), and high activity (above 10 mm).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The strains reactivated in TSA medium and nutrient agar adjusted in 0,9 % NaCl to1,5 x 10^8 CFU/mL, were further diluted in serial dilution to 1,5 x 10^5 CFU/mL in Tryptic Soy Broth (TSB). The same way, the serial dilution of the essential pure oil in 50 µl TSB + 0,15 % agar + 0,02 % Tween 80 was made in microtiter plates for final concentration/well from 50 µg/ml to 0,029 µg/ml. Then, 50 µl of bacterial suspension previously adjusted was sowed and incubated at 37 °C for 24 hours.

As time passed, 50 µl iodonitrotetrazolium chloride (INT) to 4mg/ml were added, incubating for 1 hour at 37 °C. The wells where no violet or pink color was formed determine the inhibition limit of the bacterial growth, defining MIC as a minimum concentration of oil, where the bacterial growth is not enough to change the color. Subsequently, these wells of bacterial growth not visible were feathered by streaking with Kolle handle loop in TSA and blood agar media and incubated for 24 hours at 37 °C to determine the MBC; that is, the minimum concentration of essential oil where no viable bacteria are found, the oil eliminates 99 % of the bacteria.

The assays were performed in triplicate and a control culture was maintained for each strain to verify its viability.

Results and Discussion

The essential oil got from the S. brevicalyx leaves was characterized by having a strong and lasting smell, being translucent and slightly yellowish, tasting bitter with a dry weight yield of 0,968 %, relative density (RD) of 0,913 gr/ml, and a refractive index (RI) of 1,470. Similar results to those reported by Enciso et al. (2018) in fresh leaves, RD= 0,897 g/ml and RI= 1,471 with a yield of 0,95 %. However, several authors have demonstrated that the extraction yields depend on factors like the sample type, if were leaves, steems, or flowers, dry or fresh, growth stage of the plant, extraction method, and chemotypes (Agostini et al., 2009; Mossi et al., 2012; Linde et al., 2016).

Despite this, the yield obtained is in the averages reported by different authors. Therefore, Agostini et al. (2009) reported between 0,1 and 2 % of yield from dried leaves of seven different Lamaiaceae species using the same extraction procedure of this study. Mossi et al. (2012) obtained yield between 0,2 and 2 % using CO_2 pressure extraction process with dried leaves of Cunila galliodes Benthe (Lamiaceae) coming from different zones. In addition, as an example of a species from another family, Linde et al. (2016) obtained yields of around 0,6 % in steam distillation for fresh leaves and between 0,4-2,8 % in dry weight for dry leaves.

According to the composition of S. brevicalyx essential oil, 31 compounds were identified, of which 90,32 % were in proportions of less than 6 %, containing terpene especially, the highest number of oxygenated monoterpenes were found, such as menthone at 32,7 %, followed by pulegone (20,89 %) and isomenthone (10,71 %) (Table 1). It is noteworthy that diethyl phthalate was found in 0,34 %, it is a synthetic compound derived from plastic, which probably represents an industrial pollutant indicator that arises in the water of the zone.

However, similar results to those observed in this study, it is reported by Flores et al., (2013) in their qualitative evaluation of the essential oil of S. brevicalyx leaf samples collected in the Ayacucho department, Peru, who observed mentone, pulegone, and linalool as the main compounds and to that reported by Matailo et al., (2019) in hydrodistilled oil from leaves of Clinopodium browneisynonym of Satureja brownei (Sw.). Briq. collected in Ecuador, mainly composed of oxygenated monoterpenes: pulegone (48 %), menthone (34 %), and acorenol (3,4 %).

Table 1. Composition of S. brevicalyx essential oil

Compounds	**%**	**Retention time (minutes)**
Isomenthone	10,71	26,78
Menthone	32,74	27,15
Pulegone	20,89	29,59
Isopulegone	3,15	27,43
Carvacol	6,27	30,92
β-linanool	5,80	24,55
ρ- cimol	2,79	21,87
1,8- cineol	1,71	32.15
Thymol	0,35	31,22
α- terpinene	1,33	23,13
β – pinene	0,25	18,23
α - pinene	0,24	20,09
Limonene	0,40	23,62
Sabinene	0,11	23,62
β – myrcene	0,20	20,08
3 – octanol	0,11	20,61
Biciclogermacrene	3,17	37,51
β – caryophyllene	3,54	27,15
Piperiterone	0,48	30,08
Piperitone	0,27	32,77
Geranill acetate	0,27	39,70
Caryophyllene oxide	0,15	34,27
Spathulenol	0,26	39,75
α - humulene	0,30	37,43
Trans – isopulegone	0,11	20,61
ϒ – terpinene	1,33	23,13
Aromadendrene	0,17	36,56
Germacrene β	0,24	41,38
Diethyl phthalate	0,42	39,07
α – phellandrene	0,30	17,87

Epiglobulol	0,22	41,69

However, the proportion of the identified compounds in these studies is contradictory compared to what has been reported for different species of the genus Satureja in other latitudes. Bagher and Khodaei (2020) report carvacrol as the main component with 87 %, and S. bachtiarica with thymol at 34 %, followed by carvacrol (14 %), borneol (13 %), and linanool (12 %) in samples collected in Iran of S. khuzestanica. On the other hand, Capriopli et al. (2018) obtained in the subspecies 22 % carvacrol, 17 % ρ-cimene, and 17 % thymol evaluating twosubspecies of S. montana from Italy, while for the subspecies montana, the formula is altered to 62 % carvacrol, 10% ρ-cimene and 8 % ϒ-terpinene. However, Vitanza et al. (2019) observed 44 % of carvacrol, followed by 7 % of thymol, 3 % of borneol, and 3 % of caryophyllene in commercial essential oil samples of S. montana, showing representativeness of monoterpenes in 24 %. However, with aerial parts of S. montana, but collected in Bosnia and Herzegovina, Čopra-Janićijević et al. (2020) reported a different formula and the oxygenated monoterpenes with 24 % of linalool, followed by 15 % of α-terpineol, 22 % of cis-sabinene hydrate, and 18 % of p-cymene.

The specific chemical characteristics of the essential oil vary according to culture area, determined by the environmental conditions, including soil. That's why the comparison and reproducibility of the results can be difficult when adding the related factors with the collection, drying, and processing to obtain the oil and evaluation of extracts. Nevertheless, it is confirmatory that the presence of terpenes is responsible for the medicinal properties to which the old cultures of so many different populations of the world, have attributed to their endemic plant species.

According to the antimicrobial activity of the extracted S. brevicalyx oil, it was observed that Bacillus cereus was the most sensitive species with the largest inhibition halo diameters, corresponding to the high antibacterial activity category with inhibition halos greater than 10 mm, in all dilutions of the oil evaluated (Table 2).

Table 2. Growth inhibition halos of S. brevicalyx essential oil.

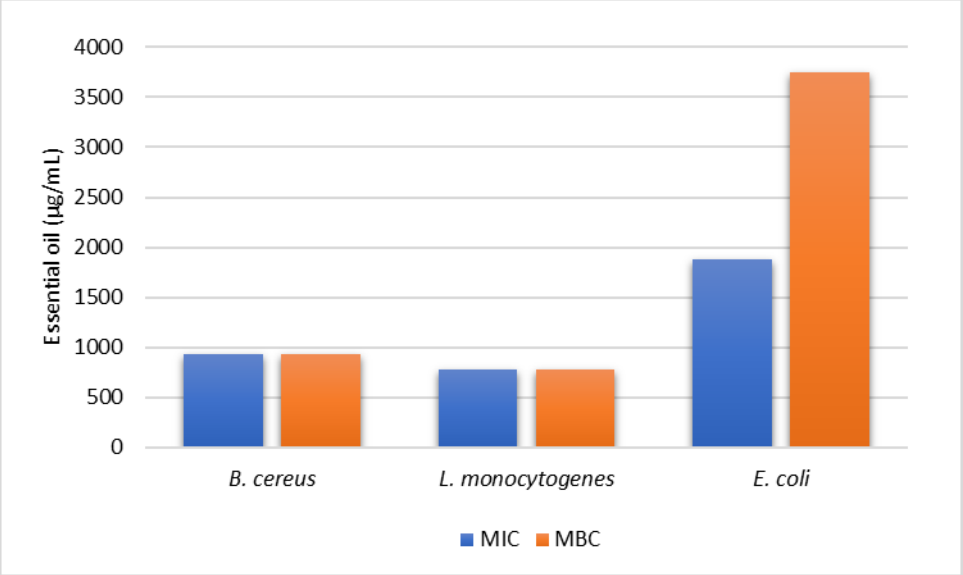
The Essential oil	**Reference bacteria ***		
in DMSO	**L. monocytogenes**	**B. cereus**	**E. coli**
25 %	8,33 ± 0,33	11,67 ± 0,67	5,67 ± 0,57
50 %	10,33 ± 0,33	13,67 ± 0,67	7,33 ± 0,57
75 %	11,67 ± 0,33	17,67 ± 0,67	9,67 ± 0,57
100 %	17,67 ± 0,33	21,67 ± 0,67	11,67 ± 0,57

mm*: average ± standard deviation in millimeters

On the other hand, Escherichia coli proved to be the most resistant species, with low or no activity observed at concentrations of 25 % and 50 % of oil and high activity with pure oil. Subsequently, when valuing MIC and MBC coincidences for B. cereus and L. monocytogenes was observed in the concentrations of 938 and 781 µg/ml of the oil respectively, while for E. coli the, MBC was higher than MIC with 3750 µg/ml and 1875 µg/ml (Figure 1). Reports about the antibacterial activity of brevicalyx were not found in the literature to establish a direct comparison. When comparing, the S. brevicalyx oil was as effective as the results reported by Vitanza et al., (2019) with S. montana oil about the reference strain L. monocytogenes,

where it is observed MIC of 780 µg/ml and MBC of 1560 µg/ml and in the uropathogenic strain of E. coli, MIC of 1560 µg/ml and MBC of 3125 µg/ml, considering that the oil was composed by 44 % of carvacrol.

Figure 1. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the essential oil of S. brevicalyx



Otherwise, results reported by Bagher and Khodaei (2020) with S. khuzestanica and S. bachtiarica oil essential, in which their inhibitory and bactericidal concentrations were lower in the order of 12,5 and 25 µg/ml and 25 and 100 µg/ml, respectively against E. coli. It should be noted that, in this case, the composition of the oil corresponded to 87 % carvacrol.

The proportion of the aromatic compounds differs according to the extraction type to which the plant material is subjected if they are oil extraction processes by steam distillation or hydrodistillation against the extraction with alcohol-based on the solubility and polarity of the compounds. Taking into account, the study of Soltanzadeh et al., (2018) observed antimicrobial activity by agar diffusion in the extraction of processes with methanol and ethanol of S. khuzestanica leaves, in the range of 0,025 a 0,050 µg/ml with average inhibition halos of 14 mm, being more sensitive, once again; the gram-positive bacteria B. Subtilis and Staphylococcusaureus, compared to E. coli, indicate that the aqueous extract did not show activity and the methanolic extract was the most effective.

Mohammed et al., (2019) on extracts from S. hortensis with ethanol, methanol, and dichloromethane, reported MIC between 25 and 800 µg/ml for Staphylococcus aureus, Enterococcus faecalis, E. coli, Pseudomonas aeruginosa, andAcinetobacter baumannii, classifying oil as weakly effective in the concentrations greater than 625 µg/ml and a different specificity of inhibition, according to the extract type, which depends on the composition type of the compound's types extracted in each one.

According to the S. brevicalyx oil composition obtained in this study, its main compounds such as monoterpenes, menthone, pulegone, and isomenthone have been associated mainly with the mint smell and bitter taste in many varieties of plants, as well as its antihistamine properties, that is, as anti-inflammatory immunomodulator, working well in skin conditions such as atopic dermatitis, antispasmodic (muscle relaxant); also, it was reported that can inhibit the development of Alzheimer's disease in mice (Choi et al., 2018; Matailo et al., 2019).

Therefore, with the oil obtained, strong antibacterial activity was observed against gram-positive bacteria like L. monocytogenes and B. cereus, and moderate antibacterial activity against E. coli, which can be sources of isolation, being useful as natural food preservatives. Therefore, it can be used not only in the food industry but also in cosmetics and pharmaceuticals as an alternative for the inhibition of the growth of pathogens with the potential to form biofilms and be a cause of FBD.

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