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EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF *SALVIA PURPUREA* CAV.

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Abstract

Salvia purpurea Liebm. (Labiateae) is popularly used against gastrointestinal and skin infections. The antibacterial activity of *Salvia purpurea* Liebm. was evaluated using the resazurin method. The plant was collected in the State of Mexico, left to dry, and the flowers and a mixture of the flowers–leaves and of the flowers–leaves–stems were ground and macerated consecutively in hexane, dichloromethane, methanol, and water for 48 h. A solution of 10 mg/mL was prepared and dilutions were performed and added to crops with 4 x 10^6 Colony-Forming Units [CFU]/mL, resazurin sodium (0.675%), and Mueller–Hinton medium. The bacteria used were: *Bacillus subtilis*; *Staphylococcus aureus*; *Escherichia coli*; *Salmonella typhimurium*; *Shigella flexneri*; *Salmonella typhi*, and *Proteus mirabilis*. As negative and positive control, 0.8% DMSO and Penicillin-Streptomycin solution were used, respectively. The cultures were incubated for 20 h at 37°C.

The hexanic extracts of flower and flower–leaf inhibited the growth of *S. aureus* and *B. subtilis*, the latter presenting the lowest Minimal Inhibitory Concentration (MIC) 0.041 mg/mL. The leaf–flower methanolic extract showed the greatest activity by inhibiting *S. aureus*, *B. subtilis*, *P. mirabilis*, and *S. typhi* with an MIC of 0.33-2.66 mg/mL. The aqueous extract of either material inhibited *P. mirabilis* and *S. typhi* at concentrations ranging from 0.33-1.33 mg/mL. The dichloromethane extract of flower or leaf–flower inhibited only *B. subtilis*. The growth of *S. typhimurium*, *S. flexneri*, and *E. coli* was not inhibited by any extract. The results support the use of the plant against gastrointestinal infections.

Keywords: Sage, Antibacterial activity, Enterobacteria

Introduction

According to the Instituto Nacional de Geografía y Estadística (INEGI), National Institute of Statistics and Geography, in the year 2019 in Mexico, of the 589 deaths due to acute diarrheic diseases in children younger than 5 years of age, the main cause of death was classified as diarrhea and gastroenteritis of presumed infectious origin, in that it represented 89.3% of cases. As second cause were intestinal infections due to other specified organisms with 8.2% of cases (1). In this population there also were present acute respiratory infections, particularly pneumonia, as the most important cause, with 92% of registered cases, followed by bronchitis and acute bronchiolitis. Treatment is based on the administration of antibiotics, many of which have lost their effectiveness due to that some bacteria have developed resistance to the antibiotics in clinical use (2). The latter has motivated the search for novel antimicrobials derived from natural sources, such as medicinal plants.

One of the genera that has been studied to detect antimicrobial activity is the genus *Salvia*, which belongs to the Labiataceae family. The term Salvia derives from the Latin word *salvare*, signifying to heal, this in reference to the curative properties of various species. The leaves and stems are the most frequently employed parts in the form of infusions, decoctions, poultices, or principally in baths. Plants of the genus *Salvia* have been utilized to treat headache, epilepsy, flu, bronchitis, hemorrhage, tuberculosis, menstrual disorders, inflammation, infections, and cancer. The pharmacological actions of the plants of this genus are associated with essential oils that the plants contain the following: monoterpenes; carbohydrates; oxygenated monoterpenes; sesquiterpenes, and diterpenes, Additionally, other non-terpenoid compounds have been described. Many species of this family secrete volatile aromatic compounds (3).

The essential oil of *Salvia chloroleuca* moderately inhibits the development of *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* in minimal concentrations of 3.75, 3.75, and 7.5 mg/mL, respectively, and activity associated with the major components of the following essential oil: 1,3-cineol, α-pinene, β-pinene, β-caryophyllene, and carvacrol (4). In addition, it has been reported that the essential oil of *Salvia fruticosa* inhibits the growth of phytopathogenic fungi, including *Fusarium oxyporum*, *Fusarium proliferatum*, *Fusarium solani*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (5). Li and collaborators, on working with the *Salvia* species most frequently employed in China, including *Salvia plebeia*, *Salvia prionitis*, and *Salvia chinensis*, reported that the diterpenes of the oils inhibit the development of *Staphylococcus aureus* and *Micrococcus luteus* (6). Also, Rota et al. reported that *S. fruticosa*, *Salvia tormentosa*, *Salvia lavandulafolia*, and *Salvia officinalis* inhibit bacterial growth (7).

In traditional Mexican medicine, *Salvia coccinea* (Wild myrtle), *Salvia polystachya* (Hierba chica), *Salvia microphylla* (Myrtle), *Salvia hispanica* (Chia), *Salvia reptans* (Hierba de pozuña), and *Salvia leucantha* (Royal Salvia) have been utilized for the treatment of gastrointestinal alterations such as dysentery, diarrhea, stomachache, inflammation, pain, cutaneous infections, vomiting, cephalea, vertigo, cancer, and insomnia. Despite the plant's widespread distribution in Mexico, in that it is localized in 24 of the 32 states of the country, there are few pharmacological studies on these plants (8). In Mexico, the antibacterial and antifungal activity of *Salvia apiana*, whose ethanolic extracts inhibited to *S. aureus*, *Streptococcus pyogenes*,

and *Enterococcus faecalis* and the yeast *Candida albicans* (9).

One scarcely studied plant, but one utilized in traditional Mexican medicine, is *Salvia purpurea* Cav., whose name in popular usage is field myrtle (*Santomexochitl*). Its leaves, stem, or flowers are habitually employed in infusions, postpartum baths, and nasal hemorrhage. Detected in these plant parts have been ursolic acid, betulinic acid, (triterpenes), and β-sitosterol (phytosterol). *Salvia purpurea* Cav. is a plant with ovate, green-yellowing leaves; Its inflorescences arise during mid-autumn, flower in winter, and regularly grow in mountainous zones and temperate forest. In Mexico, the plant is widely distributed and is recommended for the treatment of gastrointestinal infections (10). Therefore, in the present study, we proposed to know the effect possessed by the extracts of the aerial structures of *Salvia purpurea* Cav. in terms of the proliferation of gastrointestinal infections and cutaneous infections in Mexico.

Methods

Plant material

The plant was collected in the Bosque Esmeralda of Amecameca, State of Mexico, in November 2015. Sample identification was carried out by Profs. Jorge Santana and Reyna Cerón of the "Ramón Riba y Nava Esparza" Herbarium of the Metropolitan Autonomous University in Mexico City, where the samples were deposited.

Preparation of the extracts

The aerial structures of the plants were left to dry at room temperature and were then ground; there was 500 g of pulverized material that was separated into three batches: a) Flower; b) Flower–Leaf, and c) Flower–Leaf–Stem. These were macerated consecutively at room temperature during 48 h in three liters of hexane, dichloromethane, methanol (J.T. Baker, USA), and water. The extracts were filtered, the solvents were eliminated at reduced pressure in a rotavapor (Buchi RII, Switzerland), and the water was eliminated by evaporation in a double boiler. With the solid extract, we prepared 5.0 mg/mL solutions in dimethylsulfoxide 10% (DMSO)/H2O (J.T. Baker, USA). The recovery percent of the extracts was assessed, we performed a preliminary phytochemical study by means of colorimetric and precipitation reactions, and we determined total protein content by the method of Lowry (11).

Bacterial strains

The bacteria employed were *Salmonella typhimurium* ATCC 13311, *Shigella flexneri* ATCC 29003, *Salmonella typhi* ATCC 6539, *Escherichia coli* SOS, *Proteus mirabilis, Bacillus subtilis*, and *Staphylococcus aureus* ATCC 6538. To determine the Minimal Inhibitory Concentration (MIC), we followed the protocol of Drummond and Waigh, modified by Satyajit in 96-multiwell plates and with resazurin as viability indicator. This method is based on the capacity of reduction of the resazurin into resorufin by the oxido-reductase enzymes of the surviving bacteria. When the extract inhibits bacterial growth, there is no oxido-reductase activity; thus, resazurin remains blue in color, while when it survives, the reduction of resorufin stains the culture medium pink by means of a color-changing indicator (12).

Antibacterial activity of the extracts

The bacteria were seeded by means of the crossstreak method on plates with Muller–Hinton Agar medium (Bioxón, Mex) and incubated for 24 h at 370C. One colony was seeded in duplicate in 50 ml of Muller–Hinton medium (Bioxón, Mex) and was incubated for 24 h at 370C. After this, a 2.5-ml aliquot as taken, which was centrifuged (SOL-BAT, Mex) at 2,500 g during 5 min, the supernatant was eliminated, and the cellular pellet was resuspended in the same volume of sterile physiological saline (SPS) solution. The bacterial concentration was adjusted to 4 x 106 Colony Forming Units (CFU)/mL, with the 0.5 turbidity pattern of the McFarland Nephelometer and incubated for 24 h at 37°C. With the solid extracts, we separately prepared an initial solution of 532 mg/mL in dimethyl sulfoxide (DMSO; J.T. Baker, USA) at 0.8% and performed double dilutions. We deposited 50 µL/well on 96-well plates (Nunclon), 10 µL of the bacterial suspension (4 x 106 CFU/mL), 10 µL of resazurin sodium (0.675% p/v-distilled water) and 30 µL Muller–Hinton Broth 3X

medium (320 milliosmoles [mOsm]). As negative control, we utilized DMSO 0.8% -H2O, and as positive control, a Penicillin-Streptomycin solution 1 x 104 IU/mL-1 x 104 mg/mL (Sigma Chemical Co., USA). The culture plates were incubated at 370C during 22 h in an atmosphere of 5% of CO2 and 90% relative humidity (LabLine, USA). Each extract was tested in triplicate on at least three occasions (13).

Results

The yield, the total protein concentration, and the preliminary phytochemical study are presented in Table 1.

Antibacterial Activity

We evaluated three types of extract for each solvent against seven bacterial strains, that is, a total of 21 assays per solvent. The MIC of the study was 0.04 mg/ml (40 µg/mL), presented by the hexanic extract of flower–leaf on *P. mirabilis*. The hexanic extracts of flower, and flower–leaf–stem also inhibited this bacterium with an MIC of 0.08 mg/mL. The three hexanic extracts also inhibited *S. aureus* in concentrations ranging from 0.66-2.66 mg/mL. This bacterium was also inhibited by the methanolic extract of flower–leaf with the 2.66-mg/mL concentration (Table 2).

P. mirabilis was the most susceptible bacterium, in that it was inhibited by the hexanic, dichloromethane, and methanolic extracts with MIC of 0.04, 0.16, and 0.33 mg/mL, respectively. The dichloromethane extract presented the least activity, because it only inhibited one of the seven bacteria utilized. The methanolic extract exhibited the greatest activity, inhibiting four of the seven bacteria and presenting 10 inhibitory extracts for a total of 21 assayed. The aqueous and methanolic extracts inhibited *P. mirabilis* and *B. subtilis* with MIC of 1.33 and 0.33 mg/mL, respectively. The methanolic extract demonstrated 10 inhibitory extracts, followed by the hexanic and aqueous extracts, which presented six inhibitory extracts, and the dichloromethane extract, with three inhibitory extracts. The enterobacteria *E. coli*, *S. flexneri*, and *S. typhimurium* were not inhibited by any of the extracts evaluated (Table 2).

Discussion

Among the outstanding components of the genus *Salvia* are found the essential oils, alkaloids, phenols, tannins, and saponins, compounds that possess antibacterial activity. In our study, we were able to observe that the antibacterial activity in the extracts obtained is found in low- or null-chemical-polarity solvents, such as in hexane and dichloromethane extracts and in extracts obtained with chemically polar solvents such as methanol and water. In the former group, the presence can be assumed of terpenic compounds such as ursolic acid and betulinic acid (triterpenes). Additionally, β-sitosterol (phytosterol) have been reported in *S. purpurea* (10).

The hexanic and dichloromethane extracts were more active against Gram-positive bacteria such as *S. aureus* and *B. subtilis*. The antibacterial and antifungal action of the terpenes and the β-sitosterol is associated with the capacity to alter the functions of the cellular membrane, affecting cellular viability. In the reports on the antibacterial activity of the essential oils, the latter are obtained through hydrodistillation. In our study, the hexanic and dichloromethane extracts, in which the essential oils are found, were obtained by maceration at room temperature and during 42 h; it is noteworthy that both methods for obtaining these are very different. The activity shown in the hexanic and dichloromethane extracts could be related with that of the various species of Salvia that contain chemically non-polar compounds, such as terpenoids, which are responsible for the antibacterial activity of the essential oils, containing compounds such as camphor, cineol, α-pinene, linalool, terpinene, among others that, on being isolated from *Salvia verticillata*, inhibited the development of *B. subtilis*, *S. aureus*, and *Enterococcus faecalis*.

The usefulness of the essential oil of plants of the genus *Salvia* can be exemplified by the antibacterial effect of *Salvia macrochlamys*, which is capable of inhibiting human pathogenic bacteria resistant to antibiotics such as the Mycobacteria (14). The antibacterial activity of the essential oils of *Salvia sclarea* was demonstrated, among others, by Haiying and collaborators, who reported that the

essential oil of the plant alters the integrity of the cellular membrane, in turn the permeability of same, thus causing the release of the cellular content and the lysis of *S. aureus* and *E. coli*. The release of Adenosine triphosphate (ATP) and of fragments of DNA is manifested as a marked diminution of these molecules intracellularly, which renders them unviable. These authors report that the susceptibility of the bacteria to the essential oil is considerably greater in Gram-negative than in Gram-positive bacteria (15).

In contrast, in our study, the hexanic and dichloromethane extracts more effectively inhibited the Gram-positive bacteria, that is, *S. aureus* and *B. subtilis*, than the Gram-negative bacteria. The greater susceptibility of Gram-positive bacteria to extracts from Salvia is also reported by Pierozan et al., when these authors employed the essential oil of *S. officinalis*, *S. sclarea*, and *Salvia lavandulifolia* (16). In Table 2, it can be observed that the methanolic and aqueous extracts demonstrated greater activity than the activity obtained with hexane and dichloromethane.

Our results with the former can be compared with those obtained by Firuzi et al., who also procured the extracts of seven species of *Salvia* by means of maceration with methanol and methanol 80% during 48 h, but with the addition of a change of solvent at 24 h. In that study, the authors obtained an MIC of 0.66. In our case, the MIC was 2.66 mg/mL; this difference can be explained as due to that, in our study, the material was previously treated with hexane and dichloromethane, considered a material with fewer components than that employed by Firuzi and collaborators (17). In these extracts, the activity could be attributed to the presence of phenolic acid-type phenolic compounds (caffeic, ferulic, or chlorogenic) that, in addition to their being cytotoxic for transformer cells, have exhibited antibacterial activity (18). Another group of compounds with antimicrobial activity present in plants of the genus *Salvia* are tannins, which are soluble in water and in other polar solvents, because the tannins of the plant can form hydrogen bridges with nucleic acids and proteins. They precipitate these, modifying their activity as structural or enzymatic proteins, even giving rise to that the bacteria cannot utilize the nutrients of the culture and to inhibiting their proliferation (19). For the majority of the phenolic compounds, the hydroxyl group
(-OH) allows the introduction of the aryl and alkyl groups into the proteins, modifying the three-dimensional (3D) structure of the proteins. The OH-group can also affect DNA stability when the group interacts with the purine- and pyrimidine-based amino and carbonyl groups, forming new hydrogen bonds, which inhibits the functionality of the microorganisms (20).
Our results show that there is a relation between the popular use by indigenous populations of Mexico of this plant and its antibacterial activity *in vitro*. The inhibitory activity of the growth that the plant presents has also been observed in normal bone-marrow and mouse gall-bladder cells (results not included), which suggests an unreported cytotoxic effect for the plant. The differential effect of the genus *Salvia* on bacterial growth can be attributed principally to the particular properties of the species, to the geographic localization of its growth, and to the method of obtaining the test materials. The extract of the flower–leaf mixture presented the best antibacterial activity. The antibacterial activity of the flower is reinforced with the presence of the leaf.

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Table 1. Recovery percent, total protein content, and phytochemical analysis of the extracts of *Salvia pupurea* Cav.

Structures/ Metabolites	Recovery (%)	Proteins µg/mL	Tannins	Flavonoids	Reducing sugars	Saponins
Flower Hexane	1.40	0.40	-	++	+	-
Flower–Leaf Hexane	0.82	7.12	-	++	+	-
Flower–Leaf–Stem Hexane	0.22	12.13	-	+	+	-
Flower Dichloromethane	2.82	7.55	±	++	+	±
Flower–Leaf Dichloromethane	2.49	18.12	±	++	+	±
Flower–Leaf–Stem Dichloromethane	0.43	22.25	±	+	++	±
Flower Methanol	3.85	10.80	++	+	+	+++
Flower–Leaf Methanol	3.52	16.20	++	++	+++	+++
Flower–Leaf–Stem Methanol	2.03	23.5	++	++	++	++
Flower Water	10.49	12.70	++	++	++	+++
Flower–Leaf Water	6.66	16.20	++	++	++	+++
Flower–Leaf–Stem Water	4.25	28.30	++	+	++	+++

Table 2. Antibacterial activity of the extracts of the aerial structures of *Salvia purpurea* Cav.

BACTERIA/EXTRACTS	A	B	C	D	E	F	G
HEXANE							
Flower	2.66	-	0.08	-	-	-	-
Leaf–Flower	0.665	-	0.04	-	-	-	-
Leaf–Flower–Stem	1.33	-	0.08	-	-	-	-
DICHLOROMETHANE							
Flower	-	-	0.66	-	-	-	-
Leaf–Flower	-	-	0.16	-	-	-	-
Leaf–Flower–Stem	-	-	0.16	-	-	-	-
METHANOL							
Flower	-	1.33	2.66	0.33	-	-	-
Leaf–Flower	2.66	1.33	0.33	0.66	-	-	-
Leaf–Flower–Stem	-	1.33	0.66	0.66	-	-	-
AQUEOUS							
Flower	-	1.33	-	0.332	-	-	-
Leaf–Flower	-	1.33	-	0.665	-	-	-
Leaf–Flower–Stem	-	1.33	-	0.33	-	-	-

A = *Staphylococcus aureus*, B = *Proteus mirabilis*, C= *Bacillus subtilis*, D = *Salmonella typhi*, E= *Escherichia coli*, F = *Salmonella typhimurium*, G = *Shigella flexneri.*