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Effect of methanol extracts from *Salvia purpurea* CAV. on the proliferation of bacteria and hematopoietic cells

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Abstract
The effect of the methanolic extract of *Salvia purpurea* Cav. (Labiatae) was evaluated. Consecutive macerations were prepared with the hexane, dichloromethane, methanol, and water of three mixtures of the aerial structures: flower, flower–leaf, and flower–leaf–stem. The antibacterial activity was assessed by the method of resazurin. The bacteria employed were: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*; *Shigella flexneri*, *Salmonella typhi*, and *Proteus mirabilis*. Hematopoietic activity was evaluated in bone-marrow and spleen mouse cell cultures utilizing 1, 10, and 100 µg/mL. The extracts of flower and flower–leaf presented a minimal inhibitory concentration (MIC) of 0.33 µg/mL vs. *S. typhi* and *B. subtilis*, respectively. The flower–leaf extract stimulated better cell proliferation, increasing by 166, 178, and 171% in the cellularity with 1, 10, and 100 µg/mL, respectively, vs. the control (p <0.025). In the spleen cell cultures, all extracts stimulated the cell proliferation.

Keywords: Hematopoietic activity, antibacterial activity, *salvia* genus, labiatae, cell proliferation.

Introduction
The Lamiaceae family is made up of 236 genera and 7,173 known species. Due to its wealth, it occupies sixth place worldwide and it one of the greatest riches in Mexico. At the worldwide level, it is distributed in six great regions: the Mediterranean and in the South West (SW) of Central Asia; Africa and Madagascar; China; Australia, and South America and North America including Mexico. At present, 10 sub-families are recognized [1]. This family is one of those that contains the greatest number of botanical species in Mexico. The presence has been reported of 598 native or naturalized species belonging to three sub-genera and six sub-families. One of these is the Nepetoideae family, to which the following genera belong: *Scutellaria*, *Stachys*, *Hyptis*, *Hedeoma*, and *Salvia.*
The *Salvia* genus is the major and most diverse genus with 306 species, with the Mexican state of Oaxaca that with the greatest number of genera and of the plant species of this family, but the state of Jalisco is that which contains the highest number of endemic species. In Mexico, the *Salvia* species have adapted, developing into diverse types of vegetation such as the following: arid zones; desert zones; tropical forest zones, and deciduous, sub-deciduous, and temperate-climate forests, which develop at altitudes above 100 meters above sea level (MASL). The majority of the species are found between 1,500 and 2,500 MASL. Among the growth forms, the following are included: annual and perennial plants; shrubs, and occasionally, climbing shrubs [2].
The leaves and the branches are the parts most frequently employed in the form of an infusion, decoction, cataplasm, or in baths, among other uses. Plants of the *Salvia* genus have been used to treat headache, epilepsy, influenza bronchitis, hemorrhage, tuberculosis, menstrual disorders, inflammation, infections, and cancer. The pharmacological actions of the *Salvia* genus have been linked with the essential oils that contain the following: monoterpenes; hydrocarbons; oxygenated monoterpenes; sesquiterpenes, and diterpenes, highlighting 1,8 cineol, borneol α-pinene, β-pinene, linanool, and geranyl acetate. Additionally, other non-terpenoid compounds have been described [3]. Many of the *Salvia* species secrete volatile aromatic compounds; thus, their use is appreciated, as culinary, medicinal, and in the perfume industry. Some Mexican species merit attention due to their use, such as chia (*Salvia hispanica* L.) because of its nutritious value, and ska pastora (leaves of the shepherdess) (*Salvia divinorum*), for its psychoactive effect [4].

There are few pharmacological studies of *Salvias* that are employed empirically in Mexico in the treatment of gastrointestinal alterations such as dysentery, diarrhea, stomach ache, inflammation, pain, cutaneous infections, cancer, and insomnia. There are activities that have been associated with the presence of monoterpenes, sesquiterpenes, diterpenes, triterpenes, phenolic compounds, and flavonoids among the essential oils of the plants of the genus *Salvia*. At the worldwide level, there are multiple studies on the antibacterial and antifungal activity of various species of this genus. However, one of these that has been little studied is *Salvia purpurea* Cav., whose popular name is field myrtle, Santomexochitl in the Nahuatl language, which is utilized in Traditional Mexican Medicine, employing the leaves, stems, or flowers in infusions, postpartum baths, and nasal hemorrhage. Ursolic acid, betulinic acid (triterpenes), and β-sitosterol (a phytosterol) have been detected. *Salvia purpurea* Cav. is a plant whose leaves are ovulated, green-yellowish in color; its inflorescences arise at mid-autumn, it flowers in winter, and regularly grows in mountainous zones and in temperate forests. It is widely distributed in Mexico [5].

As mentioned previously, some species are used as antitumor for its cytotoxic effects on transformed cells; therefore, they are considered potentially useful against cancer. In the present work, we have proposed the effect of methanolic extracts of the aerial parts of the plant on the proliferation of bacteria and hematopoietic cells as a model of continuous reproduction in which the cytotoxic effect of the plant can be better appreciated [6].

Materials and Methods

Plant Material

The plant was collected at Bosque Esmeralda in Amecameca, Mexico, in November 2017, and was authenticated at the Herbario Metropolitano "Ramón Riba y Nava Esparza" of the Universidad Autónoma Metropolitana Iztapalapa, where a voucher specimen of the plant is stored.

Experimental animals

Male CD_1, mice, 8-12-weeks in age, from the UAM-Iztapalapa Animal Facilities, were used. Four mice per box were housed at a constant temperature of 24ºC with a 12-h light/12-h dark photoperiod with free access to food and sterilized water. The handling of laboratory animals and experimental procedures were performed according to national and international regulations (U.S. National Institutes of Health [NIH] Guidelines for the Handling and Care of Animals), including the Official Mexican Regulation 2001. In addition, the study was approved by Institution's Ethical Committee.[7]

Preparation of the extracts

The aerial parts were dried at room temperature, protected from dust and sunlight. Three portions of a) flowers, b) flowers–leaves, and c) flowers–leaves–stems were ground; 100 g of these materials was separately macerated with 600 ml of hexane for 72 h at room temperature. After that, the extract was filtered with the recovered plant material, and the process was repeated using dichloromethane and methanol (J.T. Baker, USA) and water. The organic solvents were evaporated to dryness under reduced pressure at 35ºC in a rotary evaporator (Buchi RII, Switzerland). The water was evaporated by heating it at 56ºC in a water bath. The methanolic extract was diluted to a concentration of 20 mg/mL with Dimethyl sulfoxide (100% DMSO); each of these solutions was diluted 1:10 initially with bidistilled water, and another two decimal dilutions were performed with DMSO 10%, thus containing the test concentrations of 2,000, 200, and 20 µg/mL in 10% DMSO was determined. Prior to each assay, a dilution was performed of each decimal using RPMI-1640 medium supplemented with 10% newborn calf serum (NCS), therefore obtaining summary test concentrations of 200, 20, and 2 µg/mL of the extract/DMSO 1%.

Phytochemical screening

A phytochemical study of the extracts was performed by coloration and precipitation assays as reported[8]. Proteins were determined by the Lowry method. In addition, the total polyphenols were quantified of the methanolic extracts using a colorimetric oxide-reduction reaction. The oxidizing agent utilized was the Folin–Ciocalteau reagent. A standard solution of gallic acid (0.1 mg/mL) was employed. Absorbance was measured at 760 nm. Results are expressed in mg as the equivalent of gallic acid per g of the extract (mg/GA7g extract) [9].

Antibacterial activity

The bacteria used 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 antibacterial activity and minimal inhibitory concentration (MIC), we followed the protocol of Drummond and Waigh, modified by Satyajit, which employed 96-multiwell plates and Resazurin as an indicator of viability. This method is based on the reduction of the Resazurin-to-Resorufin capability by the oxide-reductase enzymes of the surviving bacteria: when the extracts inhibit bacterial growth, there is rust-reductase bacterial activity; thus, they are blue in color, while when they survive, a reduction-to-resorufin yields a pink color to the culture medium by the shift in the indicator [10].

Assay of the antibacterial activity of extracts

The bacteria were grown in Mueller–Hinton broth for 24 h and the concentration was adjusted to 4 x 10^6 colony-forming units (CFU)/mL, with a turbidity of 0.5 (McFarland Nephelometer), and this was incubated for 24 h at 37°C. From the solid methanolic extracts, an initial dissolution of 5 mg/mL was performed in Dimethyl sulfoxide (DMSO; J.T. Baker, USA) to 0.8% and comprised two-fold dilutions. We deposited, in 50-µl well plates, the 10 µl bacterial suspension; 10 µl of sodium Resazurin was added (0.675% w/v distilled water), as well as 30 µL of the Müller–Hinton médium. As negative control physiological saline solution was used and, as positive control, a 1 x 10^4 Penicillin–Streptomycin solution IU/mL-1 x 10^4 mg/mL (Sigma Chemical Co., USA). The culture plates were incubated at 37ºC for 22 h.

Hematopoietic activity (bone-marrow cultures)

To know the effect of the extracts on the proliferation of normal hematopoietic cell cultures of bone marrow and spleen were performed. Mice were sacrificed in a CO_2 chamber, the femur was isolated under sterile conditions, the epiphysis and diaphysis were cut, 1 mL of physiological saline solution was injected through the bone-marrow channel, and the cells were collected in a 4.8-mL cryotube (Nunc, USA). A cell-suspension aliquot was diluted with the Turk solution (1:20) in white-cell pipettes to count the total nucleated cells with the aid of a hemocytometer under a clear field microscope.

Cell viability was determined using 0.2% of Trypan Blue. The cell concentration was adjusted to 4.5 x 10^5 in RPMI-1640 medium-FCS-1%. One hundred µl was added to the 96-plate wells containing 100, 10, and 1 µg/mL of each extract in DMSO 0.5%, RPMI-1640 medium-10% fetal calf serum (FCS), and incubated at 37ºC during 72 h into an atmosphere of CO_2 5% and 90% humidity.[11]

Spleen-cell cultures

A needle of a 3-ml syringe with 3 ml of RPMI 1640 medium with 10% FCS was introduced into the spleen: the cells were collected in plastic tubes and centrifuged at 1,500 rpm at room temperature for 5 min. The supernatant was removed by decantation, and the cell button was resuspended with 1 ml of the same medium. Each cell suspension obtained was quantified for nucleated cells, and cell viability was determined with Turk and Trypan Blue solutions 0.2%, respectively. The final concentration was adjusted to 4.5 x 10^5cells/mL in RPMI 1640 medium with 10% of FCS. Additionally, DMSO 10% was employed as the control solution. Ready-made plates were maintained under incubation in anticipation of cell suspension for the crop (bone-marrow cells) or spleen cells. Each experiment included extract-free cultures. To evaluate cell proliferation, the Sulphorhodamine B (SRB) method was used.[12] Results are expressed as mean ± standard deviation. The extract-treated cultures were compared with control cultures utilizing ANOVA analysis.

Results

The recovered material, total proteins, and phytochemical analysis results are shown in Table 1.

Table 1: Recovery of and phytochemical studies of methanolic extracts of the aerial structures of –No alkaloids were detected.

Structure/ Metabolites	Recovery (%)	Protein content µg/mL	Total polyphenols %	Tannins	Flavonoids	Reducing sugars	Saponins
Flower	3.85	8.5	1.3	+	+	+	+
Flower–leaf	3.52	16	2.5	+	++	+++	++
Flower–leaf–stem	2.03	23.5	2.9	+	++	++	++

Antibacterial Activity

The minimal concentration (MIC) of the extracts that inhibited bacterial growth was 0.33 µg/mL, exhibited by extracts a (flower) and b (flower–leaf) against *Salmonella typhi* and *Bacillus subtilis*, respectively. The flower–leaf extract presented the greatest activity, in that it inhibited the development of four of the seven bacteria utilized. The flower–leaf extract was the only one that inhibited the growth of *Staphylococcus aureus* with an MIC of 2.66 µg/mL. The enterobacteria *Escherichia coli*, *Salmonella typhimurium*, and *Shigella flexneri* were not inhibited by any of the extracts evaluated (Table 2).

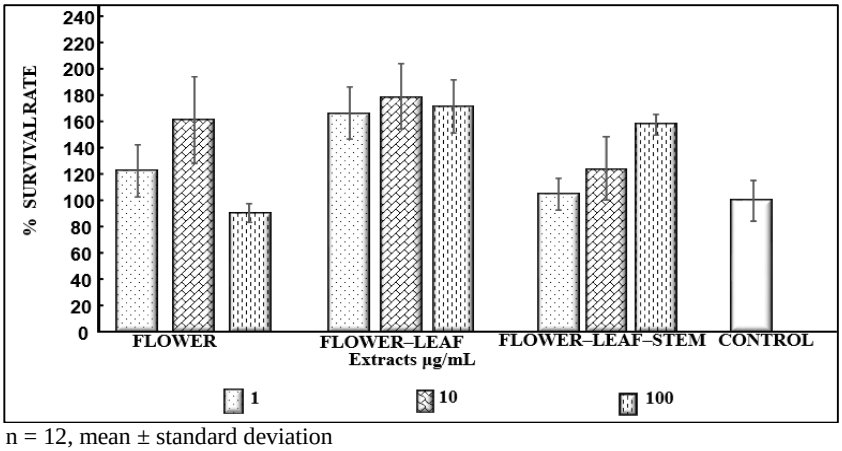
Table 2: Antibacterial activity of the methanolic extracts of *Salvia purpurea* Cav.

Bacteria	A	B	C	D	E	F	G
Flower	-	2.66	1.33	0.33	-	-	-
Flower–leaf	2.66	0.33	1.33	0.66	-	-	-
Flower–leaf–stem	-	0.66	1.33	0.66	-	-	-

A= *Staphylococcus aureus*, B= *Bacillus subtilis*, C= *Proteus mirabilis*
D= *Salmonella typhi*, E= *Escherichia coli*, F= *Salmonella typhimurium*, G= *Shigella flexneri*. -= No inhibitory activity.

Hematopoietic Activity

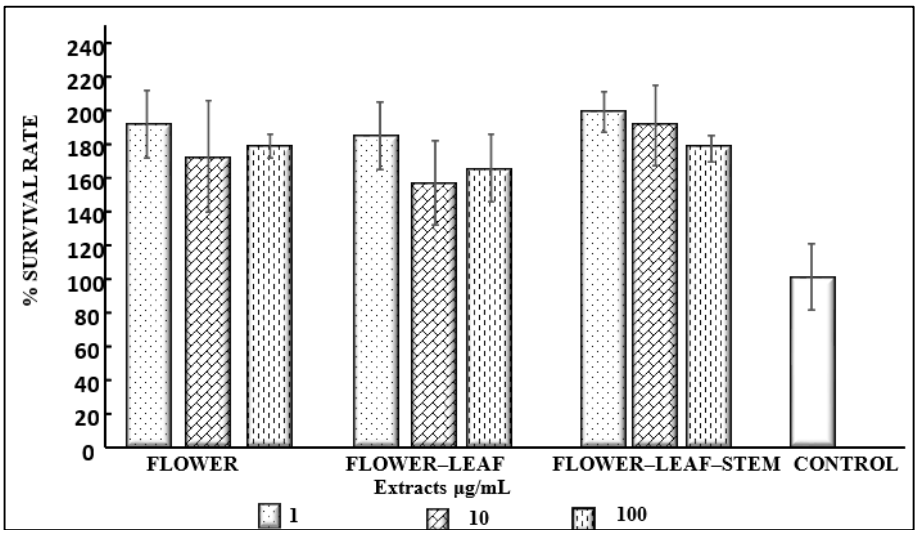
In the bone-marrow cultures, the flower–leaf extract was that which best stimulated cell proliferation, giving rise to increases of 166, 178, and 171% in the cellularity of the concentrations of 1, 10, and 100 µg/mL, respectively. We did not observe statistically significant diferences among the concentrations of the extract, but these did exist with respect to the control on considering the cell proliferation as 100% OJO 100% (p<0.025). The flower–leaf–stem extract stimulated cell proliferation in dose-dependent fashion, while the flower extract at the concentration of 10 µg/mL stimulated cell proliferation but did not present a behavioral pattern. However, the 1 and 100% concentrations of same did not present statistically significant differences with respect to the control (Figure 1).



n = 12, mean ± standard deviation

Fig 1: Effect of the methanolic extracts of *Salvia purpurea* Cav. on the proliferation of mouse bone-marrow cells.

In the gall-bladder culture cells, all of the extracts stimulated cell proliferation, causing increases ranging from 160-196% in the number of cells cultivated with respect to the control culture. Increases that were statistically significant (p <%0.001). However, diferences were not found among the activities of the extracts nor among the concentrations employed. That is, all of the extracts of the concentrations used presented stimulant activity that is similar among them (p <0.1) (Figure 2).



n = 12, mean ± standard deviation

Fig 2: Effect of the methanolic extracts of *Salvia purpurea* Cav. on the proliferation of mouse spleen cells.

Discussion

According to the Mexican National Institute of Geography and Statistics (INEGI), in the year 2015 in Mexico, diarrheic infections and those of gastrointestinal origin caused 5.1% deaths in children aged 1-4 years of age [13] Treatment was based on the administration of antibiotics, many of which have lost their effectiveness due to that some bacteria have developed resistance to antibiotics of clinical use, which has motivated the search for antimicrobials from natural sources, such as medicinal plant However, only some of these have been studied chemically and biologically with the object of knowing their pharmacological action and the constituents [14]. One of the genera that studied to detect antimicrobial activity is the genus *Salvia*, this activity is associated with the essential oils of the plant. It was demonstrated that the oil of *Salvia chloroleuca* moderately inhibits the development of *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* at minimal concentrations of 3.75, 3.75, and 7.5 mg/mL, respectively. This latter activity is associated with the major compounds of the essential oils: 1,3 -cineol; α-pinene; β-pinene; β-caryophyllene, and carvacrol [15]. In addition, it was reported that the essential oil of *Salvia fruticosa* inhibits the growth of the phytopathogenic fungi: *Fusarium oxyporum*; *F. proliferatum*; *F. solani*; *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* [16]. Li and collaborators worked with the most frequently utilized *Salvia* species in China as follows: *Salvia plebeia*; *Salvia prionitis*, and *Salvia chinensis*, concluding that the diterpenes of the oils inhibit the development of *S. aureus* and *Micrococcus luteus* [17]. Rota *et al*. reported that *S. fruticosa*, *Salvia tormentosa*, *Salvia lavandulafolia*, and *Salvia officinalis* inhibit bacterial growth [18, 19].

In Mexico, the antibacterial and antifungal activity of *Salvia apiana* have been studied, whose ethanolic extracts inhibited *S. aureus*, *Streptococcus pyogenes*, and *Enterococcus fecalis*, and the yeast *Candida albicans.*[5] In Traditional Mexican Medicine, *Salvia purpurea* is recommended for the treatment of gastrointestinal infections. In the present study, the susceptibility is demonstrated of bacteria associated with gastrointestinal infections such as *Proteus vulgaris* and *Salmonella typhi*, which supports the use of the plant against different infections. In the majority of reports on antibacterial and/or antifungal activity, the is associated with the essential oils of the plant. This is in contrast with our extract, which was obtained by masceration in methanol, a chemically polar solvent, which implies that the compounds responsible for the antibacterial activity of our extracts cannot be associated with the presence of terpenoids (non-polar substances), but instead with phenolic-type compounds that are soluble in water and in other polar solvents. The antibacterial activity could be related with the tannins of the plant, in that they can form hydrogen bridges with nucleic acids and proteins. These are precipitated, modifying their activities as structural or enzymatic proteins, even the bacteria to utilize the nutrients of the culture and inhibit proliferation of the bacteria.

It the other hand, has been reported that specific species of *Salvia* are cytotoxic for transformed cells; therefore, they are considered potentially useful against cancer. *S. officinalis* inhibits the development of renal adenocarcinoma cells. However, it does not inhibit the development of MCF-7 cells (human mammary carcinoma) and LNCaP cells (hormone-dependent prostatic carcinoma cells) [20]. and, in the same manner, for the UMSCCI cells of the oral cavity [21]. It is reported that the combination of three bioactive compounds of the essential oil of *Salvia libanetica*, (including linalyl acetate, terpeniol, and camphor) suppresses the growth of HCT-16 cells up to 64%.[22] *Salvia leriifolia* inhibits the development of ACHN renal adenocarcimona cells, amelanotic melanoma, and malignant melanoma A375 cells with IC_{50} values of 6.3 and 6.5 µg/mL, while *Salvia acetabulosa* and *Salvia leiifolia* possess no antiproliferative activity on the 142BR fibroblasts of the skin [23]. It was also reported that the extract of the root of *Salvia tebesana* inhibits the growth of the transformed cells of the A2780 (ovarian) and MCF-7 (breast) cells, and that DU145 (prostate) lines inhibit the development of cells with an IC_{50} of less than 50 µg/mL, while the dichloromethanic extract demonstrated an IC_{50} of less than 6.25 on DU145 cells [24].

Notwithstanding that it was demonstrated that some species of *Salvia* are cytotoxic, in our study it was shown that *Salvia purpurea* is not cytotoxic for bone-marrow cells, (the precursors of blood cells and platelets). In Figure 1, it can be observed that the flower–leaf extract in the concentrations employed and in the concentrations of 10 and 100 µg/mL of flower extracts and of flower–leaf–stem extracts, respectively, stimulated cell proliferation. However, no extract achieved significant increases in the number of cells, the latter referring that there were cell divisions with regard to the extracts. The remaining extracts did not present a significant difference with respect to the control group, the latter presenting cytostatic activity.

In Figure 2, we may observe that all of the extracts stimulated cell proliferation. However, only concentrations of 100 and 10 µg/mL doubled the cell count with respect to the extract-free control culture, increasing the cell count as a result of the product of the cellular multiplication. The extracts possess greater stimulation on the lymphopoietic activity (spleen) than myelopoietic activity (bone-marrow cells), suggesting an immunostimulant role for the extracts evaluated. Results not included show that the dichloromethanic and aqueous extracts of the flower–leaf mixture are frankly stimulants of the proliferation of spleen cell, giving rise to four and three increases in the cell population, respectively. Our results confirm that plants of the *Salvia* genus are selectively cytotoxic for normal or transformed cells. In this first approximation of the study of *S. purpurea* Cav., the latter did not reveal cytotoxic activity, which is important because the plant could be ingested by patients with a cancer after treatment with radio- or chemotherapy, then restoring the hematopoiesis in that condition created by a state of immunodeficiency.

Conclusions

The extract of the flower–leaf mixture presented the best antibacterial activity.

The methanolic extracts of *S. purpurea* are not only cytotoxic for the hematopoietic cells, but also the extracts of the flower–leaf mixture are stimulants of hematopoiesis, particularly of the gall bladder.

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Conflict Of Interest

The authors declare no conflicts of interest.

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