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Research Article

Effect of *Salvia purpurea* cav. on the Proliferation of Hematopoietic Cells *In Vitro*

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Abstract

In Mexico, some plants of the *Salvia* genus are employed against digestive problems, circulatory problems, infections, and some hematological alterations. We evaluated the effect of *Salvia purpurea* Cav. (Labiatae) on the proliferation of mouse hematopoietic cells. The extracts were prepared by consecutive maceration with hexane, dichloromethane, methanol, and water from three mixtures of the aerial structures of the plant: flower; flower-leaf, and flower-leaf-stem. Their hematopoietic capacity was determined in cultures of mouse bone marrow and spleen utilizing concentrations of 1, 10, and 100 µg/mL of each extract. The 100-µg/mL concentration of the flower extracts obtained with hexane, dichloromethane, and water established the proliferation of bone-marrow cells. The 10-µg/mL concentration of the hexanic and dichloromethanic extracts reduced cellularity by 34 and 33%, respectively ($p < 0.005$). Likewise, the 10-µg/mL concentration of the hexanic extract reduced the cell count by 30% ($p < 0.025$). The 100-µg/mL concentration of the four flower-leaf extracts increased the concentration from 135–174%. Of the 36 extracts tested in bone marrow, 14 were stimulants, 19 were cytostatic, and three, cytotoxic. The remaining extracts increased the cellular concentration from 127 to 199.6%. In spleen cultures, the 100-µg/mL concentration of the flower-leaf-stem, and the hexanic and methanolic extracts increased the cellular concentration. The 100-µg/mL concentration of the flower-leaf extracts obtained with dichloromethane and water gave rise to 4.0 and 3.4 increments in the cell count, respectively. The phytochemical study revealed the presence of phenolic and saponin compounds.

Keywords: Hematopoietic activity, Genus *Salvia*, *Salvia purpurea* Cav, Bone marrow, Spleen.

INTRODUCTION:

The plants of the genus *Salvia* have been employed empirically in Mexico to treat dysentery, diarrhea, stomachache, inflammation, skin infections, blood alterations, and cancer. These effects have been associated with the presence of the monoterpenes, sesquiterpenes, diterpenes, triterpenes, phenolic compounds, and flavonoids present in the essential oils of the plants of this genus¹. The antitumor capacity of the *Salvia* genus has been reported, particularly that of *Salvia officinalis*, whose cytotoxicity on various cancerous cell lines and on animal models of cancer has been demonstrated.²

Additionally, it has been reported that *Salvia* tea impedes the initiation of carcinogenesis of the colon. The extracts of *S. officinalis* have exhibited proapoptotic and inhibitory effects on the growth of cancer cell lines (MCF-7) breast, (HeLa) cervical adenocarcinoma, (HCT-116, HCT15, C0115, and HT29) colorectal, (RINm5F) insulinoma, (Hep-2) lung, (A549) melanoma (A375, M14, A2058, and B16) melanoma, and squamous cells of the oral cavity, in addition to carrying out the release of α-TNF and nitric oxide from macrophages,

increasing with this its cytotoxic effect. Activities that can be related with cytotoxic and anticancerigenous compounds are those of the terpenes and terpenoids, caryophyllene and alpha-humulene, isolated from *S. officinalis*.³ Itani and collaborators reported that the combination of linaline acetate, terpinol, and camphor from the essential oil of *Salvia libanotica*, suppress the growth of HCT-116 cells up to 64% in 48 h.⁴ *Salvia leriifolia* has an inhibitory effect on renal adenocarcinoma ACHN cells and malignant melanoma A375. However, *S. leriifolia* and *Salvia acetabulosa* do not have antiproliferative activity on the 142BR cells of skin fibroblasts.^{5,6}

One of the poorly studied species that is found in Mexico is *Salvia purpurea* Cav., whose popular name is *Mirto del campo* (field myrtle), *Santomexochitl*, which is frequently utilized in Traditional Mexican Medicine. The most used parts are the leaves, stems or flowers, in infusions, decoctions, baths, or plasters. In *Salvia purpurea* Cav., the following have been detected: ursolic and betulinic acids (triterpenes) and β-sitosterol (phytosterol). The plant presents ovulated green-

yellowish leaves, it flowers in winter, and it regularly grows in mountainous zones and template forests. Its distribution extends from the State of Mexico to Central America.⁷ It has been reported that the methanolic extract of the aerial parts has antibacterial activity *in vitro*.⁸ Considering that various plants of the *Salvia* genus inhibit the growth of cancerous, then we studied such activity of *Salvia purpurea* Cav on normal mouse cells. The goal of the present study was to know the effect of the extracts of the plant's aerial structures on the proliferation of normal hematopoietic cells as a cellular model of continuous reproduction, in which we can better appreciate the cytotoxic effect of the plant and determine its impact on these cells that are responsible for the specific and nonspecific mechanisms of defense of the individual.

MATERIAL 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 CD₁ mice, aged 8-12-weeks, from the UAM-Iztapalapa Animal Facilities, were used in the study. Four mice per box were housed at a constant temperature of 24°C with a 12-h light/12-h dark photoperiod and free access was allowed to food and sterilized water by filtration through 0.22-µm Millipore membranes (USA). The handling of laboratory animals and experimental procedures was 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 2001⁹ In addition, the study was approved by the Institution's Ethical Committee.

Preparation of extracts:

The aerial parts were dried at room temperature and were protected from dust and sunlight. Three portions of a) flowers, b) flowers-leaves, and c) flowers-leaves-stem were ground; 100 g of these materials was macerated separately with 600 ml of hexane during 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 35°C in a rotavapor (Buchi RII, Switzerland). The water was evaporated by heating at 56°C in a water bath. The extracts were diluted to a concentration of 20 mg/mL with Dimethylsulfoxide (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 obtaining test concentrations of 2,000, 200, and 20 µg/mL, which had been determined in 10% DMSO. Prior to each assay, a dilution was performed of each decimal part using RPMI-1640 medium supplemented with 10% newborn calf serum (NCS), in this manner obtaining summary test concentrations of 200, 20, and 2 µg/mL of the DMSO extract 1%.

Phytochemical screening:

A preliminary phytochemical study of the extracts was performed by coloring and precipitation assays, as reported. Also, proteins were determined by the Lowry method¹⁰

Hematopoietic activity:

Bone-marrow cell cultures

In order to know the effect of the extracts on the proliferation of normal cells, cultures of hematopoietic cells from bone marrow and spleen were performed. Mice were sacrificed in a CO₂ 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 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% Trypan Blue. The cell concentration was adjusted to 4.5×10^5 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 37°C during 72 h in an atmosphere of CO₂ 5% and 90% humidity.

Spleen-cell cultures:

The 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 decanting, and the pellet was resuspended in 1 ml of the same medium. Each cell suspension obtained was quantified with total nucleated cells, and cell viability was determined with Turk and Trypan Blue solutions 0.2%, respectively. Finally, the concentration was adjusted to 4.5×10^5 /mL in RPMI 1640 medium with 10% FCS. Additionally, DMSO 10% was employed as the control solution (Note: Wells framing the plate were filled with 200 µl of sterile distilled water). 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. Each extract was tested five times in triplicate in independent experiments. To evaluate cell proliferation, the Sulphorhodamine B (SRB) method was employed^{11,12} Results are expressed as mean $\pm$ standard error. The extract-treated cultures were compared with control cultures utilizing ANOVA analysis.

RESULTS:

The percentage of recovery, the concentration of proteins, and the presence of reducing sugars, tanins, flavonoids, and saponins are presented in Table 1.

Hematopoietic Activity on Bone-Marrow Cells:

Flower:

The 100-µg/mL concentration of the hexanic, dichloromethanic, and aqueous extracts of the flower stimulated cellular proliferation, causing 144, 156 and 136% increments in the cellular concentration, respectively, in comparison with the control cultures, considered as 100% of the cellular proliferation, differences that are statistically significant ($p < 0.025$). The greatest stimulating activity was found with the 10-µg/mL concentration of the methanolic extract, increasing the cellular concentration by 165%. Due to the dispersion of the data, statistically significant differences are not demonstrated among the previously mentioned extracts. In counterpoint, the 10-µg/mL concentration of the hexanic and dichloromethanic extracts reduced cellularity by 34 and 33%, that is, the extracts exhibited cytotoxic behavior ($p < 0.005$). The remaining extracts and concentrations assayed did not present differences with respect to the control; their behavior was cytostatic. Table 2.

Flower-Leaf:

The 100 µg/mL concentration of the four extracts increased the cellular concentration from 135% to 174%. A statistically

significant difference was not observed among these, but there was a difference with respect to the control ($p < 0.005$). Additionally, the three concentrations of the methanolic extracts stimulated cellular proliferation from 173,174 to 182%, the latter presenting greater stimulating activity in the bone marrow. On the other hand, the 10- $\mu\text{g}/\text{m}$ extract of the hexanic extract reduced the cell count by 30%, that is, it acted cytotoxically ($p < 0.0025$). The remaining extracts and concentrations evaluated presented differences with the control. Table 2.

Flower-Leaf-Stem:

The 100- $\mu\text{g}/\text{mL}$ concentrations of the hexanic and methanolic extracts increased the cellular concentration by 156 and

162%, respectively. In the same manner, the 1- $\mu\text{g}/\text{mL}$ concentration of the dichloromethanic and aqueous extracts also stimulated cellular proliferation in the same manner as the previously mentioned extracts, showing, in all cases, statistically significant differences with regard to the control culture. The remaining employed extracts and concentrations showed no difference with respect to the control, these acting cytostatically. None of the extracts presented cytotoxic activity. It should also be pointed out that no extract duplicated the cellular concentration. Of the 36 extracts evaluated in bone-marrow cultures, 14 were stimulating, 19 were cytostatic, and three were cytotoxic. Table 2.

Table 1: Recovery, a phytochemical analysis of the extracts on *Salvia purpurea* Cav.

STRUCTURES/ METABOLITES	RECOVERY (%)	PROTEINS $\mu\text{g}/\text{mL}$	TANINS	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	$\pm$	++	+	$\pm$
Flower-Leaf /Dichloromethane	2.49	18.12	$\pm$	++	+	$\pm$
Flower-Leaf-Stem/Dichloromethane	0.43	22.25	$\pm$	+	++	$\pm$
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: Effect of the extracts of *Salvia purpurea* Cav. on the proliferation of bone-marrow cells

FLOWER				
EXTRACT $\mu\text{g}/\text{mL}$	HEXANE	DICHLOROMETHANE	METHANOL	WATER
100	144.5 $\pm$ 15.02	156.03 $\pm$ 15.00	93.02 $\pm$ 8.16	136.85 $\pm$ 20.00
10	66.5 $\pm$ 12.50	65.77 $\pm$ 7.50	165 $\pm$ 30.25	74.18 $\pm$ 12.50
1	92.72 $\pm$ 12.00	117.26 $\pm$ 20.00	118 $\pm$ 19.50	113.66 $\pm$ 17.50
FLOWER-LEAF				
100	168.82 $\pm$ 17.52	135.57 $\pm$ 17.51	174.00 $\pm$ 21.02	160.43 $\pm$ 29.01
10	70.50 $\pm$ 2.55	81.24 $\pm$ 15.00	182.20 $\pm$ 25.50	128.53 $\pm$ 20.00
1	106.47 $\pm$ 20.00	128.26 $\pm$ 35.05	173.35 $\pm$ 24.03	158.83 $\pm$ 27.50
FLOWER-LEAF-STEM				
100	156.03 $\pm$ 20.01	135.57 $\pm$ 21.61	162.35 $\pm$ 11.20	82.01 $\pm$ 13.30
10	91.44 $\pm$ 11.60	112.64 $\pm$ 21.00	127.55 $\pm$ 28.30	117.98 $\pm$ 28.31
1	87.60 $\pm$ 16.61	161.79 $\pm$ 21.60	107.53 $\pm$ 14.20	145.80 $\pm$ 21.60
	CONTROL 100 + 15.00			

$n = 9$. Mean $\pm$ Standard deviation

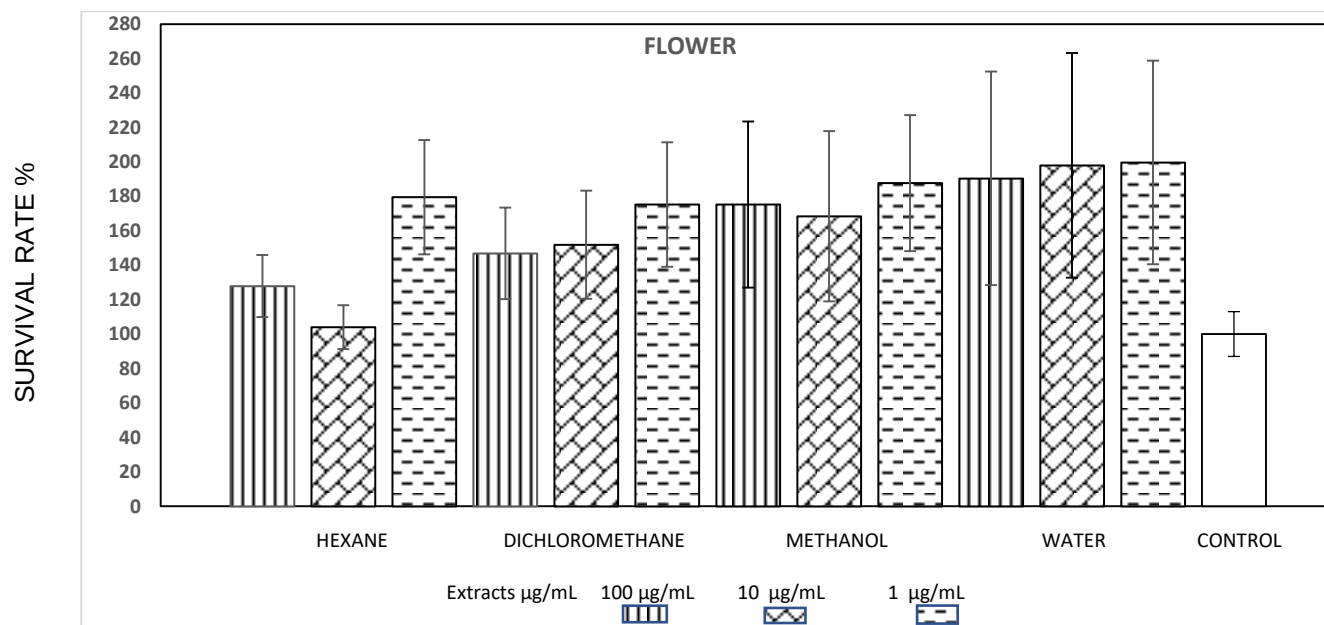
Hematopoietic Activity on Spleen Cells:

Flower:

Except for the 10- $\mu\text{g/mL}$ concentration of the hexanic extract that exhibited cytostatic behavior, the remaining extracts stimulated cellular proliferation, increasing the cellular concentration from 127% to 199.6%. No statistically significant difference was observed between the extracts and

the concentrations employed. It is noteworthy that aqueous extract presented the greatest stimulating activity, giving rise to increases of 190, 198, and 199% in the cell count with concentrations of 100, 10, and 1 $\mu\text{g/mL}$, respectively. This is in contrast with that observed in bone-marrow cultures, in which the flower extracts did not inhibit the proliferation of the spleen cells. Figure 1.

Figure 1: Effect of the extracts of flower on the proliferation of mouse spleen cells.



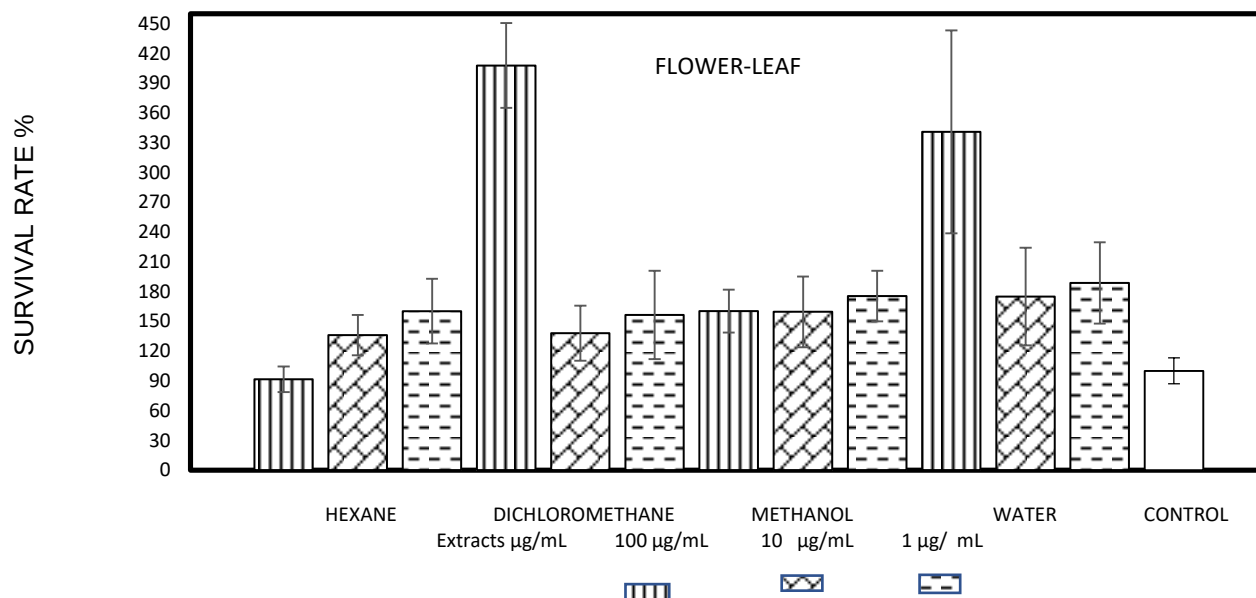
$n = 9$, Mean $\pm$ Standard deviation.

Flower-Leaf:

The 100- $\mu\text{g/mL}$ concentration of the dichloromethanic and aqueous extracts presented frank stimulating activity of cellular proliferation, generating 4.0 and 3.4 increases in the cell count, respectively. No statistically significant difference

was observed between the activity of both of these ($p < 0.05$). Nonetheless, a difference was observed with respect to the other extracts, which slightly stimulated cellular proliferation. The hexanic extract in the 10- $\mu\text{g/mL}$ concentration presented similar activity to that of the control, behaving cytostatically ($p < 0.5$). The activity of the leaf-flower extracts is greater than that corresponding to the flower extract. Figure 2.

Figure 2: Effect of the flower-leaf extracts on the proliferation of mouse spleen cells.



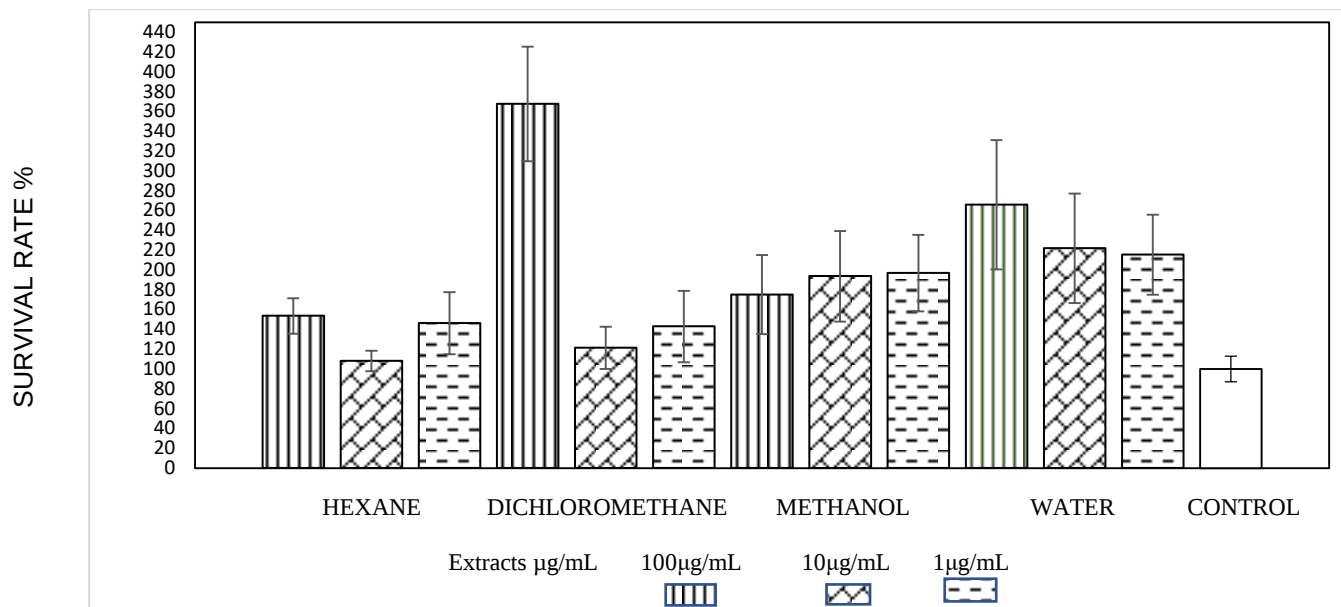
$n = 9$. Mean $\pm$ Standard deviation.

Flower-Leaf-Stem:

The dichloromethanic and aqueous extracts at the 100- $\mu\text{g/mL}$ concentration caused one, 3.7 and two, 2.5 increments in the cell count. Both stimulated cellular proliferation. However, the latter did not present a difference with respect to methanolic and aqueous extracts in the 10- and 1- $\mu\text{g/mL}$ concentrations.

The hexanic and dichloromethanic extracts at the 10- and 1- $\mu\text{g/mL}$ concentrations behaved cytostatically. The flower-leaf-stem mixture presented less activity than its equivalents, that is, flower, and flower-leaf. Therefore, we can deduce from this that the stem does not contribute to the activity of the extracts. Figure 3.

Figure 3: Effect of the extracts of flower-leaf-stem on the proliferation of mouse cells.



$n = 9$. Mean $\pm$ Standard deviation.

DISCUSSION:

Based on cytotoxicity bioassays, more than 400 compounds have been identified, among these vinblastine, vincristine, etoposide, and taxol, derived from plants with proven anticancerigenous action. The majority of these compounds possess limited activity in solid cancers, in addition to having secondary effects. Therefore, the development is necessary of novel and more effective antineoplastics¹³. In our study, we evaluated the effect of different extracts of *Salvia purpurea* on the proliferation of bone-marrow and spleen hematopoietic cells, as models of proliferation and constant renovation that allow for maintaining the functionality and effectiveness of the specific (immunological) and nonspecific mechanisms of defense of an individual in the face of an offending agent.

In preliminary studies, we reported that the flower-leaf methanolic extract and the 10- and 100- $\mu\text{g/mL}$ concentrations of flower and flower-leaf-stem, respectively, stimulated the proliferation of bone-marrow cells. However, no extract caused significant increases in the number of cells that support there being cell divisions in response to the extracts⁸. In the present study, among the extracts of the flower of *S. purpurea* in bone-marrow cultures, three were cytotoxic, as depicted in Table 2.

Regarding the effect of the extracts on spleen-cell proliferation, none of these extracts exhibited cytotoxicity. The behavior of the methanolic extract was similar to that of the previous study. However, the dichloromethanic and aqueous extracts of the flower-leaf mixture presented the greatest stimulating activity, giving rise to four and three increases in the cellular population, respectively, demonstrating that there were various cellular divisions stimulated by these extracts (Figures 1-3).

Alimpié et al. utilized aqueous extracts of aerial parts obtained through maceration at room temperature, as in our study. On studying six species of the *Salvia* genus, these authors demonstrated that *Salvia amplexicaulis* and *Salvia ringens* are

cytotoxic *in vitro* for K562 cells (chronic myelocytic leukemia) at concentrations of 151- and 173 $\mu\text{g/mL}$, respectively¹⁴ and, in our case, except for the 10- $\mu\text{g/mL}$ concentrations of the flower aqueous extract and of the 100- $\mu\text{g/mL}$ concentration of flower-leaf-stem, which exhibited cytostatic behavior in bone-marrow cultures. The remaining aqueous extracts stimulated bone-marrow and spleen cellular proliferation (in their majority lymphocytes). The difference in cytotoxicity between our work and that of Alimpié and collaborators can be attributed to, among other causes, that those authors obtained the extracts by direct maceration in water, while in our study, the aqueous extract resulted from previous maceration with hexane, dichloromethane, and methanol, which could be considered a residual extract and one possibly less chemical components than the aqueous extract obtained by those investigators¹⁴. Something similar presents between our methanolic extracts and those obtained by Amirghofran et al., who reported the cytotoxic activity of four species of *Salvia* on different lines of cancerous cells, reporting that greatest cytotoxic activity was presented by *Salvia reuterana*, with a cytotoxicity index higher than 50% at a 21- $\mu\text{g/mL}$ concentration on Raji cells (Burkitt's lymphoma), followed by the activity of *Salvia macrosiphon*¹⁵.

Based on the cytotoxicity reports of various species of *Salvia*^{16,17}. We expected higher cytotoxicity, because the presence has been reported of cytotoxic compounds such as salvicin, salvinal, and tanshinone in various species of the genus. On the other hand, in our work, the stimulation of proliferation was greater in spleen cultures (lymphopoietic activity) than in the bone-marrow cultures (myelopoietic activity), suggesting an immunostimulating role of *S. purpurea*. The latter activity was previously reported by Amirghofran and collaborators for *Salvia mirzayanii*, which at low concentrations stimulates the proliferation of lymphocytes activated by the peripheral blood and the plant possesses an inhibitory effect at higher concentrations, causing DNA fragmentation in such cells.¹⁸

In Figure 2, it can be observed that the flower-leaf extracts, obtained with dichloromethane and water, present frank stimulating activity, in a similar manner to that of the dichloromethanic flower-leaf-stem extract. In this case, such activity could be attributed to the terpenoid compounds present in the essential oils of the *Salvia* genus. From among the activities described that can support these results, we find the use of *Salvia* for the formation of scar tissue, while the activity of the aqueous extract could be attributed to hydrosoluble bioflavonoids that act as immunostimulants.^{19,20} The cytotoxic activity observed at the 10 µg/mL concentration of the hexanic and dichloromethanic extracts of flower and the 10-µg/mL concentration of the flower-leaf extract in bone-marrow cultures could be related to the presence of phenolic acids (phenylpropanoids), such as ferulic, caffeic, chlorogenic, and neochlorogenic acids, with proven cytotoxic action on transformed cells.²¹

Our results confirm that plants of the *Salvia* genus are selectively cytotoxic for normal or transformed cells. In this first approximation, *S. purpurea* Cav. did not show cytotoxic activity. This is important, because the plant could be ingested by patients with cancer after treatment with radio- or chemotherapy, in order to restore hematopoiesis in that condition, which creates an immunodeficient state.

CONCLUSIONS:

The extracts of the flower-leaf mixture stimulate the proliferation of spleen cells; cytotoxic activity was observed solely in the bone marrow. Stimulation of the flower extracts is observed to be reinforced by the presence of the leaf; the flower-leaf extracts presented the greatest stimulating activity.

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CONFLICT OF INTEREST:

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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