

**HEMATOPOIETIC ACTIVITY OF *Smilax aristolochiaefolia*  
*in vitro* AND *in vivo***

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**Summary**

In Mexican traditional medicine, *Smilax aristolochiaefolia* (Liliaceae), is empirically used against tumors, leprosy, anemia and as a tonic. Although this plant is used as antianemic, there is not scientific information about its hematopoietic properties. Then, we prepared water, methanol, chloroform and hexane extracts from pulverized roots by heating for 3 hours at reflux temperature. 0.1 ml of dilutions of 0.4, 0.2, 0.1 and 0.05 g/mL were added to cultures of bone marrow and spleen cells (only 0.4, 0.2 g/mL) of CD1 strain male mice aged 8 to 12 weeks. Cell cultures were incubated at 37°C for 72 hours, and cells were counted using a hemocytometer. Aqueous and methanolic extracts were administered orally at a single daily dosage of 0.8, 0.4 0.2 and 0.1 g/mL to groups of 10 mice. A control group received saline solution only. Forty eight hours after the last dose was administered, erythrocytes, leukocytes, platelets and nucleated bone marrow cells (femoral) were counted. Doses of 0.4 y 0.2 g/mL of aqueous extract increased 2 y 1.4 times the cell concentration on bone marrow cultures, respectively, while methanol extract stimulated the cell proliferation at the four doses tested as compared with control cultures ( $p < 0.01$ ). On the other hand, chloroform and hexane extract did not stimulate the cell proliferation. Water and methanol extracts at doses of 0.4 and 0.2 g/mL increased 3 and 1.9 times and 5.7 y 5 times, respectively cell concentration in spleen cells cultures. Respect the *in vivo* assays, the aqueous extract at dose of 0.8 increased the concentration of erythrocytes and significant increases in hemoglobin and hematocrit values ( $p < 0.01$ ) at doses tested. Methanol extract increased leucocytes at all doses ( $p < 0.001$ ). No effect was observed on erythrocytes and platelets concentration.

Key words: Hematopoiesis, Anemia, *Smilax aristolochiaefolia*

### Introduction

*Smilax aristolochiaefolia* (Liliaceae) is a perennial woody climber growing to 5 m. It has broadly ovate leaves, tendrils, and a small greenish flower (1). This plant is commonly known as zarzaparilla, alambrilla, cocolmeca, etc. It is widely distributed in Mexico (2). The form more commonly employed is the decoction of the roots that is drunk to fight; gastric ulcer, fever, leprosy, eczema, anemia, and also to purify and reinforce the blood (3,4,5). In scientific literature and in the database of the NAPRALERT of the University of Illinois in Chicago (6), do not exist reports regarding the capacity of this plant to stimulate the production of blood cells and their in the bone marrow. Then, the purpose of the present work is to evaluate the ability of extracts from *Smilax aristolochiaefolia* to stimulate the proliferation of hematopoietic cells *in vitro* and *in vivo*.

### Methods

The plant was acquired in the “Sonora” herbal medicine market in Mexico City. The roots were dried at room temperature, protected from dust and sunlight and grounded in a hand mill (Victoria, Colombia). 500 g of the ground material were heated using 10 L of water, methanol, chloroform or hexane (J. T. Baker, USA), under reflux for 3 hours. The extracts were filtered and the last three were evaporated to dryness under reduced pressure, at 35°C in a Savant Speed Vac Plus SC210A concentrator (Farmingdale, USA). The aqueous extract was lyophilized. Afterwards, 0.40, 0.20 0.10 and 0.05 g/mL dilutions were prepared using saline solution (85% NaCl) for the aqueous and methanol extracts, and corn oil (Mazola, Mex) for the chloroform and hexane extracts. All extracts were placed in vials and frozen at -20°C until its injection into the mice or use in cultures.

### Experimental Animals

Male CD<sub>1</sub> mice from 8-12 weeks-old from Universidad Autónoma Metropolitana, Campus Iztapalapa were used and housed under constant temperature of 24°C with a 12 h alternating periods of light and darkness and allowed free access to food and water according to the statutes of the CICUAL (Comité Institucional para el Uso y Cuidado de los Animales de Laboratorio) of the official Mexican norms for the production and maintenance of laboratory animals NOM-062-200-1999.

***In vitro* Assays**

**Bone Marrow Cultures.** Mice were sacrificed by cervical dislocation, the femur was isolated under sterile conditions, the epiphysis were cut, 1 ml of physiologic 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's solution (1:20) in white cell pipettes to count the total number of nucleated cells with the aid of a hemocytometer under a clear field microscope. Cell viability was determined using 0.2% trypan blue. Cell concentration was adjusted to  $2.0 \times 10^6$  cells/mL using Leibovitz medium (L-15). Afterwards, 0.1 ml of this suspension was added to 0.60 ml of L-15, pH 7.2 (Gibco-BRL), 0.20 ml of heat inactivated horse serum (Sigma, Saint Louis, MO) and 0.1 ml of the extract dilution to be tested. Then, 0.50 ml of this mixture were placed in 24-well plates (Nunc, USA) and incubated at 37°C for 72 hours in a humid atmosphere with 5% CO<sub>2</sub> and 95% air. Control cultures without extracts were prepared simultaneously. The cultures were harvested and centrifuged at 3000 rpm for 10 minutes at room temperature; cells were washed twice with phosphate buffer saline solution (PBS) at pH 7.2. The cell pellet was resuspended in 0.5 ml PBS, and cells were counted with the aid of a hemocytometer by clear field microscopy (7). Each extract was assayed five times in triplicate.

**Spleen Cultures.** Mice were sacrificed by cervical dislocation; the spleen was isolated and mechanically dispersed using tweezers and scissors.  $\alpha$ -MOPS medium (Gibco-BRL) 30 ml were added with 10% heat inactivated horse serum (In vitro-Mex), and 1% antibiotic solution (100 IU/mL Penicillin-100  $\mu$ l/mL Streptomycin; Sigma, Saint Louis, MO). Cells were dispersed with the aid of a pipette, washed with  $\alpha$ -MOPS medium, and centrifuged at 3500 rpm for 15 minutes at room temperature. The cell pellet was resuspended in  $\alpha$ -MEM medium (Sigma, Saint Louis, MO) (pH 7.0), supplemented with 10% horse serum. Cells were counted in a hemocytometer, and cell viability was determined using 0.2% trypan blue. Cell concentration was adjusted to  $2.0 \times 10^6$  cells/mL in  $\alpha$ -MEM medium. One volume of this suspension was added with 6 volumes  $\alpha$ -MEM medium, 2 volumes of heat inactivated horse serum (Sigma, Saint Louis, MO), and 1 volume of aqueous or methanol extract (0.4 and 0.2 g/mL). 0.50 ml of this mixture placed (duplicate) in 24-well plates (Nunc, USA), then incubated at 37°C for 48 hours in humid atmosphere with 5% CO<sub>2</sub> and 95% air.

Cells were then harvested, washed with PBS (pH 7.2), centrifuged twice at 3000 rpm, and finally resuspended in 0.5 ml PBS (pH 7.2). Cells were counted with a hemocytometer (8). Each extract was assayed five times in triplicate. All experiments included extract-free cultures as control.

### ***In vivo Assays***

Doses of 0.4, 0.2, 0.1 and 0.05 g/mL/saline solution of the aqueous and methanolic extracts were orally administered (0.1 ml) to groups of 10 animals during three consecutive days. Control groups received saline solution. Two days after the last dose, all animals were anesthetized with ether and bled through a cardiac puncture to determine the erythrocyte, leucocyte and platelet concentration in blood. The whole blood was diluted with Dacie's (1:200) fluid in a Thomas pipette to count the erythrocytes. The blood was diluted (1:20) with Turk's solution in order to count the leucocytes. The cells were counted with a hemocytometer using a light microscope. The blood was also diluted (1:200) with 1% ammonia oxalate to count platelets with a phase contrast microscope (9). The hemoglobin concentration was quantified by the cyanmethemoglobin method and the hematocrit by centrifugation of the blood in a 1200 x g capillary tube (National Labnet, Co, USA) for ten minutes at room temperature (10). All results are expressed as mean  $\pm$  standard error. The comparison among groups was made using variance analysis (ANOVA) and the LSD Fischer test. A  $p \leq 0.05$  value was considered statistically significant.

## **Results**

### **Hematopoietic activity *in vitro***

The doses of 0.4 and 0.2 g/mL of the aqueous extract increased 2 and 1.4 times, respectively in bone marrow cultures. ( $p < 0.01$ ). With the methanolic extract, the increases were 2.5, 2.4, 1.8 and 1.5 times with doses of 0.4, 0.2, 0.1 and 0.05 g/mL of extract, respectively. Such increases were statistically significant as compared control. Figure 1.

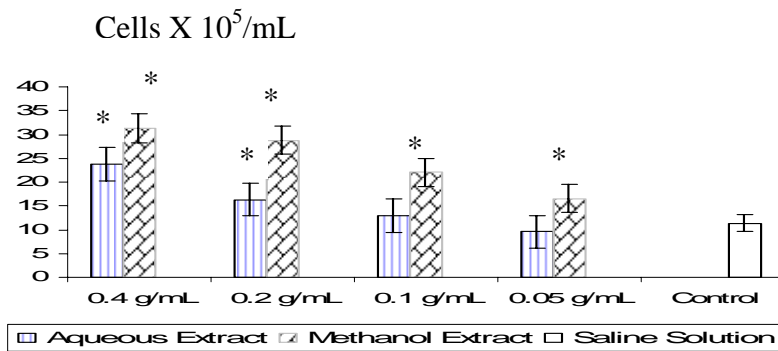


Figure 1. Hematopoietic activity of aqueous and methanol extracts of *Smilax aristolochiaefolia* on mice bone marrow cultures. Mean ± E.E. \*p<0.01.

In bone marrow cultures exposed to chloroform and hexane extracts no increase in the cell number were observed. Figure 2.

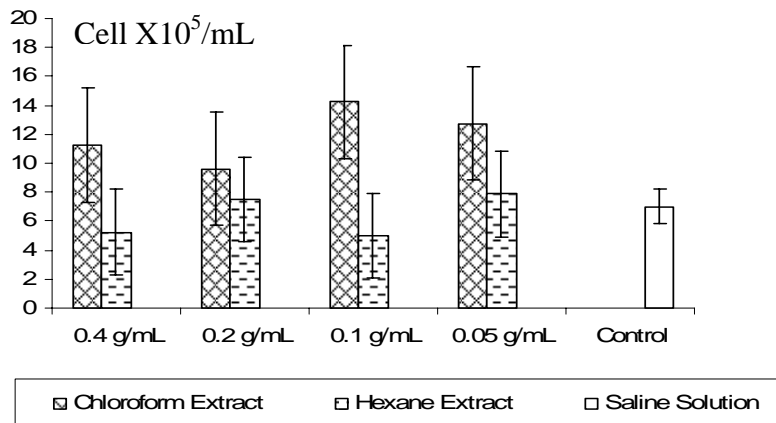


Figure 2. Hematopoietic activity of chloroform and hexane extracts of *Smilax aristolochiaefolia* on mice bone marrow cultures. Mean ± E.E. p> 0.05 Non statistical significance as compared with control.

In spleen cultures doses of 0.4 and 0.2 g/mL of the aqueous extract increased 3.1 and 1.9 times, respectively, the number of cells in culture. Meanwhile with the same doses of methanolic extract the increases were 5.7 and 5 times, respectively. All increases were statistically significant as compared with control. Figure 3.

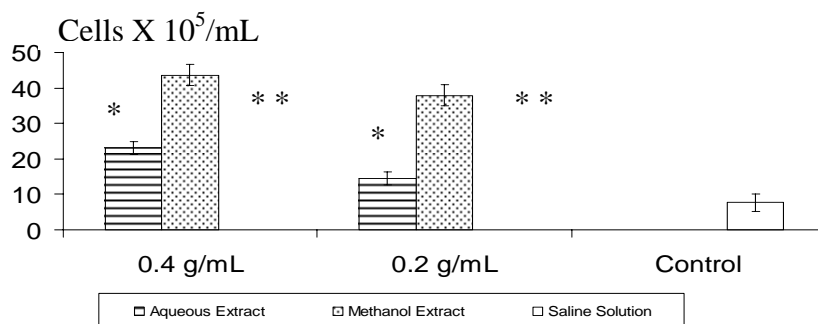


Figure 3. Effect of the aqueous and methanolic extracts of *S. aristolochiaefolia* on the proliferation of mouse spleen cells in culture. Mean  $\pm$  E.E. \* $p < 0.01$ , \*\* $p < 0.001$  as compared with controls.

### Discussion

Considering that we worked with raw extracts (complex), it is not possible to attribute to any compound or group of compounds the hematopoietic capacity of the aqueous and methanolic extracts *in vitro*. However, from the phytochemical studies of *S. aristolochiaefolia*, it is noticed that the plant contains saponins, steroids and alkaloids as constant constituents. This specie is rich in the triterpenic and steroidal saponins; sarsaparrilloside, parrillin(11).

It is well-known that triterpenoid or steroidal saponins being compounds highly polar, they are soluble in water, ethanol, methanol and other polar solvents, for what is possible have been present in the aqueous and methanolic extracts evaluated in our study. Then, the hematopoietic effect of our extracts it could be attributed to compounds belonging this chemical family which could act at least to two mechanisms.

Table 1. Effect of the aqueous and methanolic extracts of *Smilax aristolochiaefolia* on the erythrocyte concentration and other erythroid parameters in mice. Mean  $\pm$  E.E., (n=10). \*p<0.01 as compared with control

Dose g/mL	Aqueous Extract			Methanolic Extract		
	Erythrocyte Hematocrit X10 <sup>12</sup> /l	Hemoglobin g/dl	%	Erythrocyte Hematocrit X10 <sup>12</sup> /l	Hemoglobin g/dl	%
<b>0.8</b>	10.15 $\pm$ 0.63	12.29 $\pm$ 2.02*	7.87 $\pm$ 6.20*	11.60 $\pm$ 1.43	23.70 $\pm$ 2.20	54.59 $\pm$ 5.30
<b>0.4</b>	8.72 $\pm$ 0.63	13.65 $\pm$ 0.89*	44.12 $\pm$ 0.73*	11.70 $\pm$ 1.15	22.81 $\pm$ 1.11	42.85 $\pm$ 4.27
<b>0.2</b>	9.21 $\pm$ 0.97	13.90 $\pm$ 2.28*	41.82 $\pm$ 2.55*	12.50 $\pm$ 1.80	22.82 $\pm$ 0.92	43.12 $\pm$ 3.85
<b>0.1</b>	10.14 $\pm$ 1.14	15.07 $\pm$ 1.00*	43.85 $\pm$ 4.58*	12.60 $\pm$ 0.47	27.49 $\pm$ 2.30	46.11 $\pm$ 3.11
<b>Control</b>	10.20 $\pm$ 0.91	9.59 $\pm$ 1.10	30.31 $\pm$ 0.41	13.45 $\pm$ 0.36	26.72 $\pm$ 1.15	45.02 $\pm$ 5.10

Table 2. Effect of the aqueous and methanolic extracts of *Smilax aristolochiaefolia* on the leucocytes and platelets concentration in mice. Mean  $\pm$  E.E., (n=10). \*p<0.01, \*\*p< 0.001 as compared with control

Dose g/mL	Aqueous Extract		Methanolic Extract	
	Leucocytes X 10 <sup>12</sup> /l X 10 <sup>9</sup> /l	Platelets	Leucocytes X 10 <sup>9</sup> /l X10 <sup>12</sup> /l	Platelets
<b>0.8</b>	21.34 $\pm$ 2.10	1.85 $\pm$ 0.68	8.28 $\pm$ 0.92*	1.86 $\pm$ 0.30
<b>0.4</b>	12.96 $\pm$ 2.35	2.45 $\pm$ 0.25	9.50 $\pm$ 1.55*	2.54 $\pm$ 0.20
<b>0.2</b>	7.63 $\pm$ 1.25	1.43 $\pm$ 0.53	9.01 $\pm$ 1.36*	1.76 $\pm$ 0.30
<b>0.1</b>	8.70 $\pm$ 1.70	2.66 $\pm$ 0.23	11.86 $\pm$ 2.08**	1.71 $\pm$ 0.20
<b>Control</b>	15.24 $\pm$ 2.65	2.47 $\pm$ 0.44	5.02 $\pm$ 0.69	1.88 $\pm$ 0.30

One of them, is by the interaction between saponins and cellular membranes that cause transitory changes in the structure of the membrane (segregation, activation/inhibition of ionic channels). These effects depend on the used concentration and of the structure of the saponins. However, the specific interaction between saponins and the components of the cellular membrane is still ignored. Melzing and collaborators (12) added different commercial saponins to cultures of endothelial cells, and four days later they demonstrated the cellular acidification, expressed as hydrolysis of ATP, liberation of products of the glycolysis including lactic acid and concluded that the residual carbohydrate of the saponin seems to play a paper in interaction saponin-cellular membrane. This author considers that, the saponins interacting with cholesterol and other components of the membrane must have to a specific structure that allows form pores in the membrane making it permeable to the ions, especially to the effluxes of potassium and influxes of sodium. Meanwhile, other authors (13) mention that the effect of the saponins on the cellular proliferation is creating channels that permit the transit of different compounds which stimulate or inhibit the cellular proliferation. The ability of the saponins inhibit the growth of transformed cells (cytotoxicity) or to stimulate the proliferation of those no transformed, do not depend alone of the class of aglicone, but rather of the residual carbohydrate (14).

In support to the paper of the saponinas to recognize to the receiving membranales and to act as possible mitogens, is the fact that an extract of *Phytolacca americana* containing triterpenic saponins is used to stimulate the proliferation of lymphocytes for the production of interferon, hematopoietic growth factors and cytokines (15).

On the other hand, the *iv vivo* assays with the aqueous extract only increased the hemoglobin content and the hematocrit, meanwhile the methanolic extract increased the number of leucocytes. It could be because the extract was administered orally, then exposed to the attack of the enzymes of the saliva, digestive tract or sanguine plasma, causing the loss of hematopoietic activity. The ability of *S. aristolochiaefolia* to stimulate lymphocyte (spleen cells) proliferation as we report here, suggests an immunologic activity of this plant, although the role in the immune function remains to be elucidated. Our results *in vivo* might explain the ethnomedicinal use of *S. aristolochiaefolia* in the treatment of anemia.



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