

Achyrocline satureioides (LAM.) DC (Marcela): Antimicrobial activity on *Staphylococcus* spp. and immunomodulating effects on human lymphocytes

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ABSTRACT. *Achyrocline satureioides* (LAM.) DC (Compositae) is a sub-bush original from America and distributed in Europe and Africa. It is mainly used in infusions, as digestive, sedative among others and has antimicrobial and antiviral properties. A research was made into the anti-microbial activity of the *A. satureioides* decoction on the *Staphylococcus* spp strains. They were isolated from 18 patients with acne lesions and from 7 patients infected with *Staphylococcus* spp. (5 strains were taken from catheters and 2 from wounds). The strains were classified through biochemical tests and then were seeded in triptein-soy agar with or without decoction to observe the antibacterial activity. On the other hand, cultures of lymphocytes were made from those patients who displayed infections caused by *Staphylococcus* spp. and from 12 control non-infected individuals. The lymphocytes were stimulated with decoction or PHA-M. Among the expanded, CD8+ T cells, with anti-human CD8 monoclonal antibody were the outstanding ones by indirect IF. The *A. satureioides* decoction inhibited 95% of the isolated *Staphylococcus* spp. strains and stimulated the lymphocyte expansion, of which 40% were CD8+ T cells. The *A. satureioides* decoction showed anti-microbial activity and resulted to be an immunostimulating agent on CD8+ T cells, with lesser mitogenic effects than PHA-M.

Key words: *Achyrocline satureioides* (LAM) D.C., *Staphylococcus* spp., anti-microbial activity, immunomodulator, mitogen.

RESUMEN. *Achyrocline satureioides* (LAM.) DC (Compositae) es un sub-arbusto originario de América y distribuido en Europa y África. Este vegetal es comúnmente utilizado en infusiones como digestivo, sedativo, entre otras propiedades y tiene actividad antimicrobiana y antiviral. En este trabajo investigamos la actividad antimicrobiana de la decocción de *A. satureioides* sobre cepas de *Staphylococcus* spp. Las cepas se aislaron de 18 pacientes con lesiones de acné y de 7 pacientes con infecciones causadas por *Staphylococcus* spp. (5 cepas fueron tomadas de catéteres y 2 de heridas). Las mismas se clasificaron a través de pruebas bioquímicas y luego se sembraron en agar tripteína-soya con o sin el agregado de la decocción para observar la actividad antibacteriana. Por otro lado, se realizaron cultivos de linfocitos de aquellos pacientes que presentaron infecciones causadas por *Staphylococcus* spp. y de 12 individuos controles, no infectados. Los linfocitos fueron estimulados con decocción o PHA-M. Luego se realizó el recuento de LT CD8+ por IF indirecta, utilizando un anticuerpo monoclonal anti-CD8 humano. La decocción de *A. satureioides* inhibió el 95% de las cepas de *Staphylococcus* spp. aisladas y estimuló la proliferación de linfocitos, de los cuales el 40% perteneció a la subpoblación de LT CD8+. La decocción de *A. satureioides* mostró actividad antimicrobiana y resultó un agente inmunoestimulador sobre los LT CD8+ con menor efecto mitogénico que PHA-M.

Palabras clave: *Achyrocline satureioides* (LAM) D.C., *Staphylococcus* spp., actividad antimicrobiana, inmunomodulador, mitógeno.

INTRODUCTION

In Argentina, medicine plants are numerous; we can mention the Compositae, Labiatae, Plantaginaceae, Valerianaceae, Verbenaceae and Usneaceae-families. One of the most numerous vascular plants is the Compositae, herbs, bushes and less frequently trees and lianas with 19,000 species. This group is divided into two subgroups, Asteraceae with 12 tribes and Cichorioidea with only one tribe. In the first sub-group we can found the *Achyrocline*

satureioides (LAM.) DC specie (Fig. 1). *A. satureioides* is a 30-50 cm height sub-bush, quite branched, very aromatic, usually known as “Marcela hembra”, “Marcela” or “Marcelita”. It has downy stems, 3-5 cm long-lineal lanceolate leaves with downs on both sides of grey whitish appearance. Flowers are gathered in chapters of 4 to 8 each. Chapters are numerous, small, cylindrical, yellowish or reddish, arranged in dense terminal glomeruli. Original from America and distributed in Europe and Africa, in Argentina this specie is most frequently found in sandy and wet soils of the centre of our country. It blooms and blossoms in the spring – summer season.²⁶

It is mainly used in infusions, as digestive, sedative, anti-inflammatory, anti-spasmodic, analgesic, diuretic and bronchus dilatation medicine.³⁵ This last property explains that it has been used as infusion in the treatment of the asthma crisis.¹⁰ Anti-microbial,^{5,24} anti-viral,⁴⁰ inhibitor of high glucose levels in blood,⁶ liver protecting¹⁸ and

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antioxidant^{2,10,29} properties have been discovered. Other researchers have found immunomodulating and cytotoxic properties against tumour cells in the vegetal fractions.^{33,34}

Diverse species of the Compositae group were effective on *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis*. Extracts, decoctions and oils evidenced capacity to inhibit Gram (+) and Gram (-) bacteria, and also anti-fungic activity.^{5,15}

Most of antibiotics they have antimicrobial effects but they depress the immunological system by different routes. As much for the medicinal product laboratory like for the medical therapist, it would be a manage to count on a product that, on the one hand, alters the growth of the microorganism and in addition enhanced the immunological system to the infected individual. It will be of election the drug that better exerts its effects on these two fundamental points: antibacterial and immunomodulator. According to the mentioned thing previously, this work was made to prove the anti-microbial activity of the *Achyrocline satureioides* decoction on *Staphylococcus* spp. isolated from human infections. Furthermore, it was aimed at proving the immunomodulating properties of *Achyrocline satureioides* on the human lymphocyte proliferation and determining the cell sub-population sensitive to the antigenic or mitogenic stimulation of the plant decoction.



Figure 1. Specimen of *Achyrocline satureioides* (LAM) DC, "Marcela", in herbarium from Systematic Botanic Area, Department of Natural Sciences, at the National University of Río Cuarto, Argentina.

MATERIAL AND METHODS

Obtaining of samples

Samples were taken from 25 patients (14-26 years old) in the Allergy Service of the Municipal Health Facility and Río Cuarto Medical Center and from adult's students volunteers of the Universidad Nacional de Río Cuarto. Two groups were formed, one made up of 18 patients with youthful acne skin lesions and the other of 7 patients with different infections by *Staphylococcus* spp. (5 by catheters and 2 by wounds). In each case, the sample was taken in sterility and introduced in tubes with triptein-soy agar broth. Samples were incubated for 18 hours at 37°C.³⁷

To carry out the immunological study, 13 of the 25 patients were evaluated. In addition, 12 individuals without infections were evaluated as controls. Ten milliliters of venous peripheral blood were obtained from each one and collected in sterile tubes containing heparin. According to Ethics, they were properly informed about the study and signed conformity to make the test.

Strain isolation

The bacteria isolation was made from its growth in triptein-soy agar broth and seeded in stria by consumption in triptein-soy agar and salted manitol agar (Chapman). Incubation was made for 24 hours at 37°C. Characteristic colonies were Gram stained. Those colonies belonging to Gram-positive coccus were selected.

In order to preserve the strains, the Gram-positive coccus were smeared in jagged tubes containing triptein-soy agar and kept at 4° C until their subsequent classification.¹⁶ The control strain *Staphylococcus aureus* ATCC 29213 was used.

Strain identification

Dichotomic codes were designed according to Bergey's. Groups were divided into according to their opposing characteristics and those tests with 90-95% of positive or negative results were selected. The staphylococcus was divided into 3 groups: I. Coagulase positive, novobiocin sensitive, II. Coagulase negative, novobiocin resistant and III. Coagulase negative, novobiocin sensitive.^{16,32}

In order to classify the *Staphylococcus* species of the coagulase positive – novobiocin sensitive group, the following tests were performed: production of acetoin grown at 45°C, and maltose and production of pigments as confirming tests of Collins, C. & P. Lyne. 1989.⁷

To classify the *Staphylococcus*, the following metabolic tests were carried out:

The catalase test was carried out according to Branson, D. 1974;⁶ b) Test of resistance to bacitracin was carried out according to Falk, D. & S. Guering. 1983;¹³ c) The use of glucose was realized as it indicates by Smibert, M. & N. Krieg. 1981.³⁶

In order to group the *Staphylococcus* strains, the following additional tests were made:

a) Novobiocin sensibility test was realized according to Kloos, W. & P. Schleifer 1975.²⁰

b) Evaluation of the production of free coagulase or staphylocoagulase was realized as it indicates by Devriese, L. et. al., 1985.¹¹ c) Culture in Blood Agar was carried out according to Holt J. et al., 1994.¹⁶

Vegetal material processing

Leaves of *Achyrocline satureioides*, collected in Alpha Corral, Province of Cordoba, in February 2004 were used. Professor Dr. Margarita Grosso from the Systematic Botanic area, Department of Natural Sciences, at the Universidad de Río Cuarto, classified the plants.

The material was washed with distilled water, placed on graphite paper and dried at room temperature. Then, it was grinded in electric processing machine and kept in flasks at 4°C.

To obtain a plant decoction, 10 gr. of dry and grinded plant were suspended in 200 ml. of distilled water (5% final concentration). The mixture was heated at boiling temperature for 20 minutes. First, it was filtered with filter paper and then in clarifying filter (0.4 µm). The concentration of the decoction was of 11 mg/ml.

The filtering was fractioned in caramel-coloured flasks and sterilized by autoclave for 20 minutes at 115°C, keeping it at - 20°C until being used.²⁴

Anti-microbial activity of *Achyrocline satureioides* on the isolated strains

Trial in plates per radial stria for the analysis of the plant decoction: 4 ml from the filtering of sterilized plant decoction was mixed with 16 ml of sterilized cast triptein-soy agar (final concentration: 2, 2 mg/ml). Once homogenized, it was put on Petri plates. The antimicrobial activity was determined by seeding the so prepared plates with the different strains in radial stria. Likewise, controls on triptein-soy agar were made without plant decoction. All plates were incubated for 24 hours at 37°C and the development or inhibition of the bacteria growth was observed.¹²

Lymphocyte cultures

Blood samples were diluted 1/3 with Hanks' balanced saline solution (HBSS) (Sigma, St. Louis, US), placed over Hystopaque® – 1077 (Sigma, St. Louis, US) and centrifuged at 2000 rpm for 20 min at room temperature. For obtaining lymphocytes, the interface was collected and washed 3 times using RPMI – 1640 (Sigma, St. Louis, US).

The assays of cellular proliferation took place following the colorimetric method according to Mosmann²⁵ using the Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes Invitrogen Detection Technologies, Eugene, Oregon, USA). Cells (2 x 10⁵/ml), in a final volume of 200 µl, were cultured in the 96-wells sterile microplates (NUNCLO®, Delta Nunc Inter Med, made in Denmark) containing RPMI-1640 amended with 25 mM of Hepes (Gibco Laboratories, Life Technologies Inc. Grand Island, NY, USA), 2 mM of L-glutamine (Parafarm, Industria Argentina) 5% de calf foetal serum (CFS) (Gibco BDRL), 50 mM of 2-mercaptoethanol (2-ME) and 1% of antibiotics (100 µg/ml streptomycin and 100 µg/ml ampicilin). Cultures were stimulated with Phytohemagglutinin-M (PHA) (10 µg/ml), and different concentrations of the *A. satureioides* decoction (2.8; 1.4; 0.7 y 0.35 mg/ml). Control lymphocyte cultures were performed using RPMI 1640 alone. Cells were incubated during 72 hs to 37°C with 5% CO₂ in atmosphere humidified. After incubation, the plate was centrifugated and supernatants were placed in ependof tubes to -80°C until use in the IFNγ measurement assay. Then they added 100 µl of freshly RPMI-1640 and 10 µl of MTT solution (1 mg/ml of MTT in PBS 0.01 M pH 7.2) per well and the plate was incubated to 37°C with 5% CO₂ during 4 hs. Then they added 50 µl of dimetil-sulfoxide (DMSO, Sintorgan^o Industria Argentina) per well, in order to dissolve the crystals of formazan that result from the conversion of the salt of tetrametil-tetrazolium (MTT, or 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide). The result by reading in spectrophotometer (Labsystems Multiskan MS) was interpreted to 570/690 nm. The cellular expansion reached about the classic mitogens was compared with the produced one by the different concentrations from EO of *M. verticillata* calculating the Proliferation Index (PI) according to the following equation:

$$PI = \frac{\text{stimulated cells}}{\text{none stimulated cells}} \geq 1.20$$

According to Tuchscherer et al 2002 an IP ≥ 1,20 he is indicative of cellular proliferation.³⁸

CD8 (+) T cells score by indirect IF

CD8 (+) T cells present in the cultures stimulated with *A. satureioides* decoction were scored by indirect IF technique. A 1×10^6 viable mononuclear cell/ml suspension was used. Five μl of anti-human CD8 monoclonal antibody (Sigma St. Louis, USA) were added to the cell suspension. Cells were incubated at 18°C - 22°C for 30 minutes. Then, they were washed twice with 2 ml of Azide-PBS, suspended in 100 μl FITC-conjugated anti- γ -globulin (Sigma St. Louis, USA) and diluted 1/15000 with Evans Blue stain. The suspension was incubated at 18°C - 22°C for 30 minutes, in the dark. After this, cells were washed twice using 2 ml of Azide-PBS and the supernatant was thrown away. Cells were then suspended in 15 μl of glycine buffer and a drop (5-10 μl) of the cellular sediment was placed in a microscope slide. Cells were scored in an UV light epifluorescence microscope (1000X magnification). The percentage of fluorescent cells was determined over 100 lymphocytes. CD8(+) T cells were distinguished by a bright fluorescence in their cellular membrane. CD8(+) T cells levels that were between 20 and 25 % are considered as normal in peripheral blood.²³

RESULTS

Strain identification

From the bacteria isolation study it resulted that among the extracted samples 13 strains (52%) were isolated from patients with acne and 7 strains (28%) were isolated from clinical material, which showed the following characteristics: Growth in salted manitol agar (CINa 7.5%), Gram positive coccus, immovable, positive catalasa, resistant to bacitracin and glucose fermentation; therefore these strains were considered as belonging to the *Staphylococcus* type. 100% of the strains isolated from patients with acne, and 71% of the strains isolated from clinical materials were novobiocin sensitive.

46% of the strains isolated from patients with acne, and 29% of strains isolated from clinical materials were coagulase positive. 38% of the strains isolated from patients with acne, and 71% of the strains isolated from clinical materials evidenced alpha hemolytic effect.

Anti-microbial activity of *A. satureioides* decoction

A. satureioides decoction (2.2 mg/ml) showing inhibiting effect on 95% of the isolated strains of the staphylococcal type. The 5% of coagulase negative -non-haemolytic strains isolated from clinical material was not inhibited (Figs. 2 and 3).

Immunomodulating properties

The blastogenic response without stimuli or mitogen adding was quite positive in groups of patients with acne and less positive in the control group, although with statistical difference.

Cells from patients with acne shown lymphocyte proliferation indexes higher than those of the control group cells, regardless of the culture considered: without stimuli, with PHA-M or with *A. satureioides* decoction. Differences were statistically significant. The proliferation indexes of cells stimulated with PHA-M were within the normal values in all cases. Higher indexes were observed in the group of patients with acne. The cell proliferating response before the *A. satureioides* decoction was significantly higher than the spontaneous one ($p < 0.001$), but lower than that before PHA-M ($p < 0.02$) in both groups (Table 1).

The tried concentrations of decoction that showed mitogenic effects were similar (2.8; 1.4; 0.7; 0.35 mg/ml) than the concentration that had antimicrobial activity (2.2 mg/ml).

The proliferated cells displayed altered morphologic characteristics: to optical microscope were seen with the unrolled chromatin and the different stage from mitosis, but some of them did not show increase in their size at highest concentration (Fig 4).

It is possible that the observed cytotoxic effects could have to the high used concentrations. Concentrations in the order of the micrograms did not show mitogenic effects but the cells did not show toxicity signs (data non show).

A high positive correlation was observed in the proliferation indexes resulting from stimulation with PHA-M and *A. satureioides* decoction, both in the acne group ($r = 0.78$) and the control group ($r = 0.81$). The index of cells identified as lymphocytes T CD8(+) was 42%.

DISCUSSION AND CONCLUSIONS

The above mentioned tests for the *Staphylococcus* classification were enough to classify the isolated strains. The test of bacitracin resistance using 0.04 U disks was highly sensitive and specific.³ The test of lysostaphin sensitivity was not used because of a variation of the susceptibility among the *Staphylococcus* species.¹⁴ The test of furazolidone resistance was not used because, although the *Staphylococcus* species are resistant, several authors have noted that some strains have shown low levels of resistance, which could lead to wrong classifications.⁹

The tests of coagulase and novobiocin allow dividing the staphylococcal strains into three groups: I. Coagulase positive, Novobiocin sensitive; II Coagulase positive, No-

vobiocin resistant, and III Coagulase negative, Novobiocin sensitive.⁹ Within the first group there exists a pathogen important in human and animal pathologies, *S. aureus*.

It was considered that the staphylococcal-type microorganisms are the most commonly isolated skin infection pathogens. In this study 54% of the strains isolated from patients with youthful acne and 43% of the strains isolated from clinical materials were coagulase negative and novobiocin sensitive staphylococcal, result that shows the importance of

these microorganisms in the worsening of this pathology. Within this group, diverse species are classified such as *S. epidermidis*. This species is normal flora of the human skin and agent cause of clinical conditions especially in the urinary tract.³⁷ It should be noted that two of the strains are alpha hemolytic, virulence factor prominent in some coagulase negative species such as *S. haemolyticus*, among others.²⁸

Coagulase negative and novobiocin sensitive staphylococcal microorganisms are the main agents causing in-

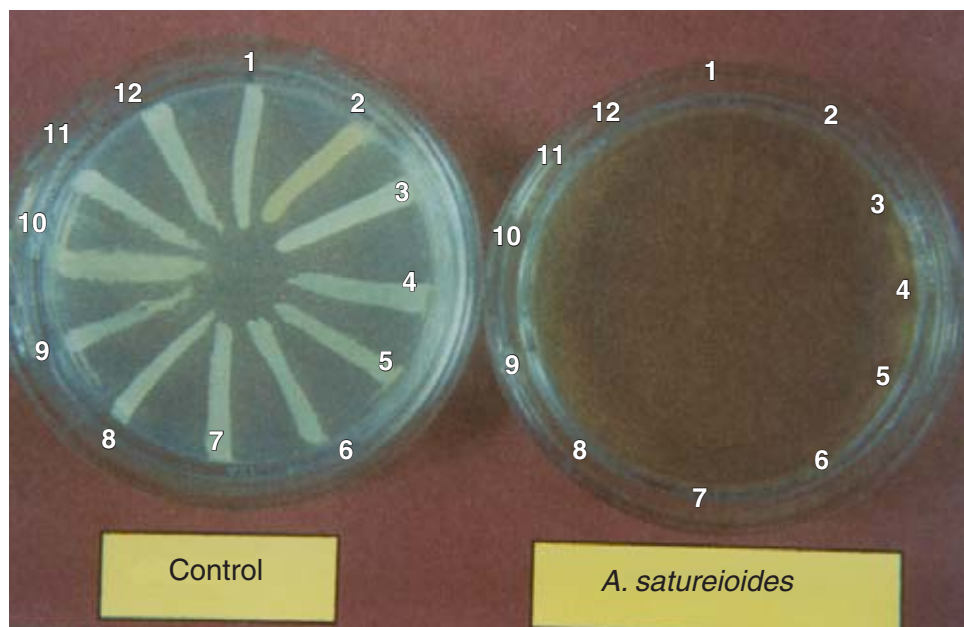


Figure 2. Antimicrobial effect of Achyrocline satureioides (LAM) DC decoction on staphylococci strains isolated from patients with acne.

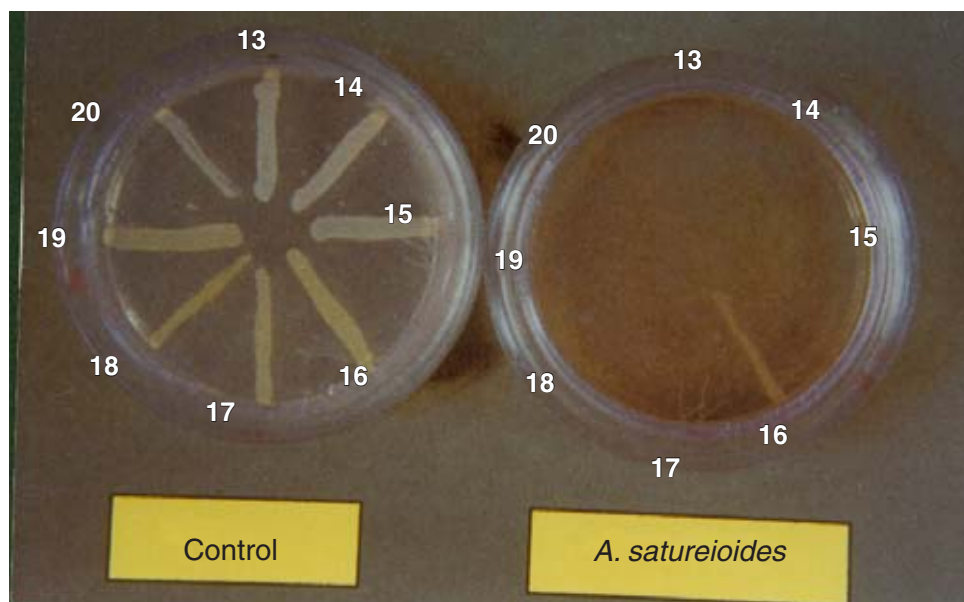


Figure 3. Antimicrobial effect of Achyrocline satureioides (LAM) DC decoction on staphylococci strains isolated from clinical materials.

vesting, as well as the concentrations that were used, factors that influence their biological effects and could explain these results.³⁰

When analyzing cells that proliferated in lymphocyte cultures stimulated by *A. satureioides* decoction, IFI technique revealed that the vegetal derivate might be modulating the Th1 response, expanding the CD8 (+) T cells. These results do not differ from those found by other authors who have tested similar products derived from different medicinal species.^{19,21}

Although *A. satureioides* decoction in this study did not show lymphocytes proliferation effect like polyclonal mitogen, its action was more effective than that of a superantigen.

The vegetal derivative when stimulating the expansion of T cells starts up immunomodulators mechanisms

and activation of phagocytosis and the half-full cytotoxicity by T and NK cells and activation of the differentiation of LTh cells. This population cooperates by Th1 cells derived those that participate in order to cooperate with the cytotoxic effects of T CD8 cells and by the Th2 deviation to cooperate with the cells B those that become in antibodies producers plasmatic cells. *A. satureioides* decoction, in the concentrations tried in this study, could be used *in vitro* like alternative mitogen to stimulate the lymphocytes proliferation, in tests diagnoses of lymphocyte functionality.

Our findings justify futures studies on cytotoxicity *in vitro* and *in vivo* of the *A. satureioides* decoction for their use like phytopharmacological product in individuals with infections by staphylococci.

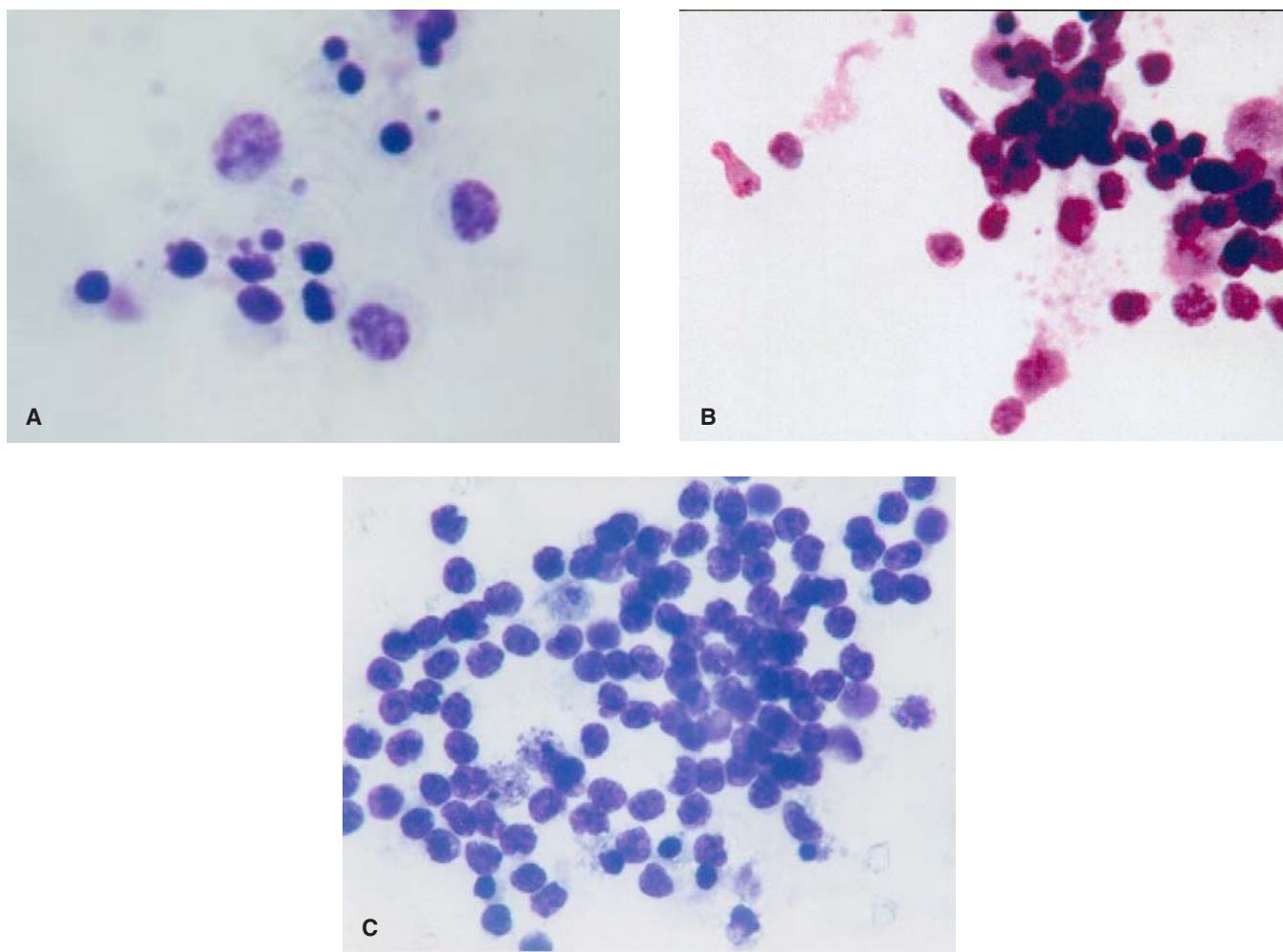


Figure 4. Microphotography of cultured human lymphocytes stimulated with *A. satureioides* decoction A) 0.7 mg/ml; B) 1.4 mg/ml y C) 2.8 mg/ml. The proliferated cells were seen with the unrolled chromatin and the different stage from mitosis, but some of they did not show increase in his size (1000X).

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