



# Leukocyte death incited by propolis toxicity

## *Muerte leucocitaria por toxicidad del propóleo*

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### ABSTRACT

**Objective:** The aim of the present study was to assess the extent of propolis' *in vitro* cytotoxicity on polymorphonuclear leukocytes. **Materials and methods:** The present study was an *in vitro*, controlled, experimental endeavor. Statistical procedure entailed variance analysis of repeated measures as well as Scheffe's *post-hoc*. A  $p < 0.05$  statistical significance was established. To obtain leukocytes, 10 mL of peripheral venous blood was harvested from six randomly selected, healthy, 20-30 year old subjects, of both genders. **Results:** Scheffe's analysis with 95% reliability for comparison between control and experimental groups. Significant with  $p < 0.0001$  for propolis 1 control 1 and propolis 2 control 2. For propolis 1 propolis 2  $p < 0.5002$ /control 1 control 2  $p < 0.9621$ . **Conclusions:** In the present experiment propolis at a 1:4 dilution applied for 1-2 hours to polymorphonuclear leukocytes caused 70% cellular death. This resulted in statistical significance.

**Key words:** Cytotoxicity, propolis, neutrophil polymorphonuclear leukocytes.

**Palabras clave:** Citotoxicidad, propóleo, leucocitos polimorfonucleares neutrófilos.

### RESUMEN

**Objetivo:** El propósito de este estudio fue evaluar el grado de citotoxicidad *in vitro* que presenta el propóleo sobre leucocitos polimorfonucleares. **Material y métodos:** Estudio experimental, controlado, *in vitro*. Procedimiento estadístico de: análisis de varianza de repetidas medidas y *post-hoc* de Scheffe, estableciendo una significancia estadística de  $p < 0.05$ . Se tomaron 10 mL de sangre venosa periférica de seis individuos sanos, ambos sexos entre 20 y 30 años de edad escogidos al azar para obtener los leucocitos. **Resultados:** Análisis de Scheffé con 95% de confiabilidad para comparación entre grupo control y grupo experimental: significativo con  $p < 0.0001$  para propóleo 1, control 1 y propóleo 2, control 2, para propóleo 1, propóleo 2,  $p < 0.5002$ /control 1, control 2,  $p < 0.9621$ . **Conclusiones:** El propóleo en dilución 1:4 aplicado a leucocitos polimorfonucleares durante 1-2 horas en el experimento provocó muerte celular en un 70%; dando significancia estadística.

### INTRODUCTION

Recently, in the area of natural products research, a material called propolis stands out; scientific literature reports this materials is analgesic, fungicide, anti-inflammatory, healing as well as anti-cariogenic.<sup>1</sup>

Bees produce this resin through a process of mixing substances gathered from budding plants, flower buds and resinous exudates. They thus produce a material fit to close gaps, embalm dead insects within the beehive, as well as protect them from micro-organism and insect invasion.<sup>2</sup> Inca tribes used it to cure febrile infections. In XIII and XIV century Europe, it was used to treat sores. Its therapeutic action was attributed to the several phenolic compounds which conform it, among which the main are flavonoids, as well as some phenolic acids, esters, aldehydes, alcohols and ketones.<sup>3,4,5</sup>

Propolis physical and chemical composition is as follows:

- Resins and aromatic balsams 50-80%.
- Essential oils and other volatile substances (4.5 to 15%).
- Waxes (12-15%).
- Pollen (5-11%).
- Flavones, flavonoids, flavanones, dihydroflavones.
- Benzyl alcohol, benzalcohol, benzoic acid.
- Cinnamic alcohol derivatives, coumarins, phenolic triglycerides.
- Other aromatic elements, monoterpenes, hexaterpenes, triterpenes.

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- Polyunsaturated fatty acids and linoleic acid.
- Vitamins A, B1, B2, B6, C, E, Nicotinic acid, pantothenic acid.
- Copper, manganese, magnesium, nickel, silver, silica, vanadium zinc.

When a subject is born, it is exposed to numberless microorganisms heretofore unknown to him. They can become part of his normal flora population, or they can cause illness which will elicit as a consequence a certain type of immune response, which can be non-specific, where neutrophil polymorphonuclear leukocytes intervene (NPML), or a specific response where there is interacting of T and B lymphocytes.<sup>6,7</sup>

NPML are the main phagocytic cells found in the peripheral blood, they conform 50-70% of total white blood cells<sup>7</sup>. They are considered the second defense line of the human body, the first line of defense being skin and mucosa.<sup>6</sup> Average life is 8-20 hrs in circulation, which increases when entering infected or inflamed tissues.<sup>8</sup>

NPML transit from the capillaries until reaching the lesion site experiences several phases: 1 margination NPML contact with endothelial walls. 2. Endothelial adherence through selectins and integrins. 3. Diapedesis. trans-endothelial migration. For this to happen, chemotaxis is necessary, that is to say NPML must be attracted towards the infection focus through different molecules such as IL-8 (interleukin 8), complement C5a factor of the complement, LTB4 (leukotriene B4) among others. Phagocytosis and cellular death. Destruction of the micro-organism within the neutrophil takes place through two mechanisms; one oxygen dependent and one oxygen-independent.<sup>9</sup>

The neutrophil dies, once it has fulfilled its function, following an apoptosis procedure (programmed cellular death). This entails certain characteristic alterations such as the increase in phosphatidylserine surface markers' expression. This helps in the elimination undertaken by macrophages, avoiding thus possible cytotoxic content release into the extra-cellular medium. This represents a benefit of the apoptotic neutrophil inasmuch as it reduces inappropriate inflammatory response. The model is based upon the strategy of providing benefits to excessive responses in a microbial infection.<sup>10</sup> In our NPML research we have developed a model to isolate and purify NPML from peripheral blood so as to later confront them to different substances, propolis in this case, to measure its ability to exert cytotoxic effect.

## MATERIALS AND METHODS

This study was an *in vitro*, controlled, experimental study with the following statistical procedure: repeated measurement variance analysis and Scheffe's *post-hoc*  $p < 0.05$  statistical significance. Harvested blood was mixed with heparin (Inhepar) in the following proportion: 5,000 U per blood ml, to avoid coagulation and leucocyte entrapment in 16 x 150 test tubes with 3 mL of 3% Dextran (Sigma co). From the previous tube, blood was passed onto another tube where 3% Dextran was previously incorporated. This procedure must be carefully performed with sterile Pasteur pipette, attempting to achieve blood sliding on the tube's walls so that red blood cells do not lyse. Incubation was conducted at 37°C for one hour, in an attempt to replicate human body conditions. Plasma rich in leukocytes was gathered in 13 x 100 tubes. Upon withdrawing plasma from the incubator, two phases could be observed: a leukocyte-rich upper or supernatant phase and a lower or sediment phase mainly composed of red blood cells and platelets. Blood was centrifuged at 180g (1,200 rpm) during three minutes (Ficher Scientific centrifuge). Cell free supernatant material was discarded, and leukocytes remained agglutinated at the bottom of the tube. In order to eliminate Dextran excess, three rinses were performed with RPMI 1640 commercial culture medium. Cells were then gently separated to then undertake counting. Obtained cell population was adjusted to a  $12 \times 10^8$  cell concentration per mL. 5% acetic acid was incorporated to achieve better leukocyte counting; the following were used as adjuvants: a Neubauer camera, Leica and Van Guard light microscopes as well as a manual counter

Experimental design.

Our *in vitro* system counted with the following reactive agents:

1. **Problem sample tube** containing propolis, cells RPMI-1640 medium tested at one and two hours. Propolis was at 1:4 solution rate (minimum inhibitory concentration obtained in former experiments performed by Lara, Tinoco et al).<sup>11</sup>
2. **Viability control** conducted with cells and RPMI-1640 medium as control group. A sample of each group at one and two hours was left in the incubator at 37°C. Results were observed by counting dead and live cells using 5% Trypan blue dye. Blue-dyed cells were considered dead or non-viable, due to the entrance of the dye through membrane and cellular walls. Live, refringent cells showed no coloring and no cellular damage. The tubes then

received 0.5 mL propolis at 1.4 concentration, then 0.5 mL cells, then medium. After incubating for one and two hours images were observed. Images exhibited provoked agglutination as well as blue-tinted polymorphonuclear leukocytes which were evidence of cellular death.

**3. Purity in NPML obtention.** The separation of neutrophil polymorphonuclear leukocytes was undertaken with 95 to 100% purity as demonstrated by Wright Stain procedure, under microscope at 40x objective.

**RESULTS**

In *figure 1* we can observe a leukocyte obtained through Dextran purification technique, dyed with Wright stain. Viability range with this method was fairly high: it reached 95-100% purity.

In *figure 2*, here we can observe live leukocytes not affected by propolis since they appeared hyaline and did not incorporate Trypan blue stain. Dead NPML would result dyed in blue.

In *figure 3*, strong agglutination elicited by propolis can be observed, in the *figure 4*. Dead leukocytes can be observed when incorporating 5% Trypan blue stain.

Quantified results previously shown statistically express the following based on six performed repetitions:

In *table 1* we find results of the six experiments, where the number of live and dead cells is counted after one hour and two hour periods. Control was basal count of 12,000,000 Neutrophil polymorphonuclear leukocytes. (NPML). Subsequently, in all experiments, the number of dead and live NPML were counted after one and two hour periods. Results of the present experiment showed, when viability was assessed in the control group, a population approximately three times greater than in the group where propolis was present.

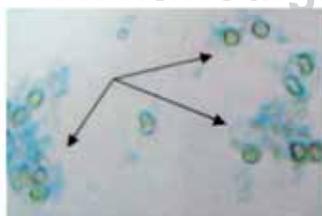
**Table I.** Live and dead cell calculation after one and two hour periods.

<p>Experiment 1:</p> <p>Tube one: propolis + cells= basal measurement 12,000,000.                  After one hour: live cells: 3,600,000, dead cells 8,400,000                  After two hours: live cells 3,000,000, dead cells 9,000,000.                  Tube two: cells + medium = basal measurement 12,000,000                  After one hour: live cells 11,400,000; dead cells 600,000.                  After two hours: live cells 11,400,000; dead cells 600,000</p> <p>Experiment 2.</p> <p>Tube one: propolis + cells = basal measurement 12,000,000.                  After one hour: live cells: 3,600,000, dead cells 8,400,000.                  After two hours: live cells : 3,000,000; dead cells 9,000,000.                  Tube two: cells + medium = basal measurement 12,000,000.                  After one hour: live cells: 11,600,000, dead cells 840,000.                  After two hours: live cells 10,00,000, dead cells 1,200,000.</p> <p>Experiment 3.</p> <p>Tube one: propolis + cells = basal measurement 11,000,000.                  After one hour: live cells 4,620,000, dead cells 6,380,000.                  After two hours: live cells 4,400,000, dead cells 6,600,000.                  Tube two: cells + medium = basal measurement 12,000,000.                  After one hour: live cells 10,890,000, dead cells 110,000.                  After two hours: Live cells 9,900,000; dead cells: 1,100,000.</p> <p>Experiment 4</p> <p>Tube one: propolis + cells = basal measurement 12,000,000.                  After one hour: live cells, 3,600,000, dead cells 8,400,000.                  After two hours: live cells: 3,000,000. Dead cells: 9,000,000.                  Tube two: cells + medium = basal measurement 12,000,000.                  After one hour: live cells 11,640,000. Dead cells 360,000.                  After two hours: live cells 11,520,000, dead cells 480,000.</p> <p>Experiment 5</p> <p>Tube one: propolis + cells = basal measurement 12,000,000.                  After one hour: Live cells 1,560,000, dead cells 10, 440,000.                  After two hours: live cells 1,200,000, dead cells 10,800,000.                  Tube two: cells + medium = basal measurement 12,000,000.                  After one hour: live cells: 11,400,000, dead cells 600,000.                  After two hours: live cells 11,400,000, dead cells 600,000.</p> <p>Experiment 6</p> <p>Tube one: propolis + cells = basal measurement 12,000,000.                  After one hour, live cells 3,240,000, dead cells 9,960,000.                  After two hours: live cells 600,000, dead cells 11,400,000.                  Tube two: cells + medium = basal measurement 12,000,000.                  After one hour: live cells 11,400,000, dead cells 600,000.                  After two hours: live cells 11,400,000, dead cells 600,000.</p>
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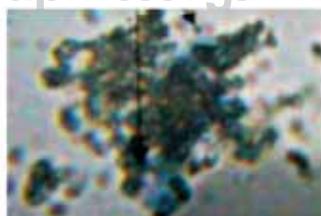
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**Figure 1.** Wright stain leukocyte.



**Figure 2.** Live Leukocytes.



**Figure 3.** Agglutinated dead leukocytes.



**Figure 4.** Blue stained leukocytes, cellular death.

**Table II.** Scheffe's analysis with 95% reliability performed between control and experimental groups.

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Propolis 1 propolis 2 = mean diff. 836666.667 p-value 0.5002
Propolis 1 control 1 = mean diff -8018333.333 p-value < 0.0001
Propolis2 control 2 = mean diff -8570000.000 p-value < 0.0001
Control 1 control 2 = mean diff 285000.000 p-value 0.9621

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Initial basal measurement, in all experiments, was adjusted to 12,000,000 NMPL. Population was thus standardized to avoid fluctuations which might have affected results.

Statistical analysis showed significant statistical difference between propolis 1 (measured at 1 hour of incubation time) and control 1 with  $p < 0.0001$  value. The same could be said for propolis 2 (measured at two hours of incubation time) when compared to control two with a  $p < 0.0001$  value.

Analysis showed the following (*Table II*):

### DISCUSSION

The antimicrobial ability of propolis has been widely researched and demonstrated by Bretz et al, Koo et al, Hegazi AG, El Hardy FK as well as Drago M et al.<sup>2,4,11-13</sup> Propolis's toxicity has not been extensively researched and few studies on live cells have been recorded. This is one of the reasons behind the use of polymorphonuclear leukocytes in the present experiment since they are easily recoverable cells of peripheral blood as well as second line of defense of the human body; they are equally involved in anti-bacterial and anti-infectious processes, which are essential characteristics mentioned here for propolis as well.

Leukocyte separation was based on the method used by Arce Mendoza, Tinoco Carbiales (1984) when they measured leukocyte chemotaxis on tetracycline effect.<sup>14,15</sup>

Results reflect the fact that cell harvesting purity was adequate, since cells dyed following Wright's method appeared with a purplish hue with well-circumscribed nuclei (*Figure 1*). Our viability control was high (95-100%). In it, leukocytes appeared refractile, therefore alive.

In the test tube where propolis was incorporated into the cells a patent agglutination could be observed. The present study thus proposes this might be one of the anti-bacterial effect mechanisms subject of study by several authors such as Koo, Parl et al.<sup>4,16</sup>

Cell populations used in the present study (basal measurement) were constant (standardized), that is, 12,000,000 leukocytes as initial cell population. Upon counting, control group exhibited three times the

viability than that observed when propolis was used. This result was repetitive in the different quantified samples. There was no apparent significant difference with results obtained at one or two hours. This would substantiate the theory that propolis is indeed acting with constant toxicity on the cells. This situation coincides with reports of Scheller et al: working with mice they applied ethanol and propolis extract and found pathological changes in the liver which were transitory and reversible in a period of two to four weeks after intra-venous administration.

Some authors, like Ramirez et al<sup>17</sup> support the theory that high doses of propolis orally administered to animals (10 to 15 mg per kg of bodyweight) do not elicit toxic effects or pathological disorders, even in the long term. This might show certain relationship to a dosage-effect response.

The results obtained in the present study were not significant when considering the time variable. The differences in the control group after one hour showed  $p < 0.0001$  statistically significant differences. This was also the case for the two hour group and its control  $p < 0.0001$ . In the light of results reported by Magro and Philo and Carballo<sup>18</sup> this would result paradoxical since they reported the fact that propolis elicited accelerated epithelial repair after rat extraction.

Seheller et al, in a study conducted on dogs, found stimulation in the regenerative process. Stojko and Seheller, in a study conducted on dogs reported osteogenesis acceleration as well as bone tissue regeneration with pronounced anti-bacterial effect. It should be noted that the aforementioned authors were not looking for measurements of cellular death. We could surmise they were using different ways for propolis preparation or extraction, which could alter results. Propolis, as an alternative product does not have precise standardization.

Finally, it must be pointed out that experiments conducted by us were *in vitro*. When interacting *in vivo*, elements implicated in the present experiment could sustain a buffer effect elicited by the organism, since, when propolis is diluted in our human system its toxic effect on NPML could be reduced to a minimum. Nevertheless, this would not be the case for its bactericidal or anti-inflammatory effect.

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