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CLINICAL EVALUATION OF THE ANTI-INFLAMMATORY EFFECT OF *BACCHARIS DRACUNCULIFOLIA* PROPOLIS GEL ON CERVICITIS

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Abstract

Propolis is a natural product produced by bees and used widely to treat inflammatory and infectious diseases. Cervicitis is an inflammation of the uterine cervix, normally caused by sexually transmitted infections or by non-infectious causes, such as contraceptive diaphragms, allergy to latex condoms or intrauterine devices. We have developed a propolis formulation patented in Brazil PI0904121-4A2 on 01/09/2006. The objective of this work is to evaluate the anti-inflammatory effect of standardized Brazilian propolis (G1) gel on cervicitis. Twenty eligible women with recurrent cervicitis were randomly assigned to the treatment of our study. This study was performed on the group with cervicitis as evaluated by neutrophil analysis of cervico-vaginal material before and after treatment with G1 during 7 or 14 days. These women received G1 and were instructed to use it for the next seven days. We demonstrated that treatment for seven days resulted in a significant decrease in the number of neutrophils in the collected cervico-vaginal material. After seven and fourteen days, respectively, the level of inhibition in the vulva was 55±5% for both, 38±4% and 60±5% in the vagina, and 39±4% and 37±5% in the cul-de-sac. In the colon (data after seven days), the inhibition was 55±6%, and, in contrast, inhibition in the endocervix was 49±5%. The results follow the same pattern as our recent results showing that the anti-inflammatory effect of propolis may be due to the inhibition of iNOS gene expression, through interference in NF-κB sites in the iNOS promoter in association with decrease of prostaglandin E2 production. These results suggest that G1 may be an important new bioproduct to use during chronic cervicitis.

Keywords: Brazilian propolis. Anti-inflammatory. Cervicitis.

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INTRODUCTION

Propolis is a natural product produced by bees and is widely used to treat several diseases. Bees collect plant resins and balsamic substances, which are transported to beehives and used to close the apertures and to protect the beehives against bacterial and fungal contamination. On beehives, this resinous substance is called propolis.[1] The propolis is re-collected, cleaned, triturated, and percolated under standard conditions to create an extract. The propolis extract is used to prepare various pharmaceutical formulations, such as capsules, creams, oral spray, and shampoos, among others.[2]

In Central and Eastern Europe, the main botanical source of propolis is *Populos nigra*. This propolis is brown, and its chemical composition is based mainly in caffeic acid derivatives such as caffeic acid phenyl ethyl esther (CAPE) and aryl derivatives (Allergenic components in European *Populus* propolis).[3] In Brazil, there are many regions containing many types of forests. The main regions (and forests) are: North Brazil (Amazonian Forest, where propolis still unknown), Central Brazil (Pantanal), Southern Brazil (containing two forests: Araucarian Forest in the mountains and Atlantic Forest at the coast, where brown propolis has been extensively studied[4]), Northeast Brazil (also containing two forests: mangue, where red propolis have being studied because of preliminary trials showing cancer cell toxicity, and cerrado, where the propolis remains mostly unstudied), and Southeast Brazil (Minas Gerais State, containing the most studied propolis, as the mountains and forests contain much *Baccharis dracunculifolia,* the main botanical source of green propolis[5]).

Brazilian propolis resin or crude extract productions are organized in the South and Southeast, and the best Brazilian propolis is exported to Japan and Europe. The Brazilian pharmaceutical industry has recently tried to improve the production process to increase the value of Brazilian propolis. The majority of Brazilian propolis is brown and comes from two botanical sources: *Araucaria angustifolia* and *Eucalipto citriodora*. Green propolis is produced from *Baccharis dracunculifolia*. Brazilian green propolis is produced by an interesting

mechanism: *Baccharis dracuncuclifolia,* during a specific time of the year, a pathological process similar to cancer. During this time, honeybees collect the resin from buds of *Baccharis dracuncuclifolia* in order to repair the hive and to protect it from bacteria and various diseases.[6]

The chemical composition of propolis varies by region and also depends on which honeybees collect the resin. The general chemical composition is: 55% resins and balsams, 30% wax, 10% pollen, and secondary metabolites including phenolic acids and its esters, flavonoids, terpenes, beta-steroids, aromatic acids and alcohols, sesquiterpenes, and derivatives from estilbene, among others.[7]

Our results with propolis show that it causes reduction in inflammation that, in combination with a decrease in angiogenesis, may have an inhibitory effect on tumor growth or metastasis.[8] Tumor cells induce an inflammatory response during the first stage of their growth. This effect is important because it leads to an increase in the local blood flow and other factors such as vasorelaxation, increase of local temperature and the induction of vascular endothelial growth factor (VEGF) or matrix metalloproteinase (MMP), which induce neoangiogenesis and additional nutritional support to cancer cells.[8] An important approach to decreasing the size of tumors and inhibiting metastasis is to decrease the inflammatory events associated with the onset of cancer.[9,10] Inflammation is produced through several pathways such as Lipopolysaccharide (LPS) or other inflammatory mediators that interact with a specific receptor, such as Toll-like receptor (TLR), in the cell membrane. This receptor starts a phosphorylative intracellular cascade.[11] This effect activates the kinase of inhibitory kappa B (IKK) and the phosphorylation of inhibitory NF-κB protein (IκB). After this, NF-κB is activated and initiates the nuclear translocation and/or activation of specific inflammatory enzymes and proteins, including the cytokines IL1, IL12, and TNF-a, and cyclooxygenase 2, with the consequent increased production of PGE2.[12] Together, these cascades produce inflammation, an increase of vascular permeability, vasorelaxation and cell migration, and they establish the perfect conditions for tumor growth and metastasis.[11]

Several groups have studied the effects of propolis on angiogenesis. Mirzoeva and Calder (1996)[13] studied the effect of propolis and its components on eicosanoid production during the inflammatory response and showed that propolis reduces the prostaglandin and leukotriene production during the experimental inflammatory response. Liao et al. (2003)[14] described the inhibitory effect of caffeic acid phenylethyl ester on angiogenesis, tumor invasion, and metastasis. The authors suggest that CAPE decreases expression of MMP-2, MMP-9, and VEGF in CT26 cells. Abdel Latif et al. (2005)[15] showed that the anti-inflammatory effects of CAPE on *Helicobacter pylori*-induced NF-κB and AP-1 in the gastric epithelial cell line AGS were related to the inhibition of degradation of IκB-α protein. They also showed that these were linked to the suppression of *H. pylori*-induced cell proliferation and the production of the cytokines TNF-α, IL-8 and COX-2 expression. The author proposes that the inhibition of such transcription by CAPE could result in the suppression of many genes during *H. pylori*-induced inflammation and also provides new insights into the anti-cancer and anti-inflammatory properties of CAPE. Ansorge et al. (2003)[16] reported that propolis and some of its constituents down-regulate DNA synthesis and inflammatory cytokine production but induce TGFb1 production in human immune cells.

The most important results have been shown by our research group. In a recent publication, we evaluated the analgesic and anti-inflammatory effects of *Baccharis dracunculifolia* Brazilian propolis. Propolis reduced the edema induced by carrageenan, cell migration, and release of inflammatory cell mediators such as nitric oxide, PGE2, and cytokines (e.g., IL1 and TNFα). We also showed that Brazilian green propolis and Artepillin C are potent NF-κB inhibitors in HEK cells and can modulate CO_2 and nitric oxide production in RAW 264.7 cells.[17]

Chronic cervicitis is the most frequently encountered of all pathologic conditions, both in the clinic and in the laboratory. The last medical consensus review from the World Health Organization (WHO) restricted the terms cervicitis, exocervicitis, endocervicitis and cervicitis with or without ectropium and erosion

to cervical inflammation (CID-10, 1996) and frequently increase the risk factor to cervical cancer.[18]

The objective of this work is the clinical evaluation of the anti-inflammatory effect of standardized Brazilian propolis gel (green propolis gel) on cervicitis to prevent the development of precancerous lesions and cervical cancer.

MATERIALS AND METHODS

Preparation and botanical and chemical characterization of ethanol extract of propolis

The propolis sample was collected in March of 2002 in Minas Gerais, Brazil (Nectar Farmacêutica Ltda; sample number SBN-54), and it was subsequently frozen and stored in our laboratory. The material was prepared for examination according to the laboratory protocol of Nectar Farmacêutica. The resulting sediments were identified as the insoluble parts of propolis, which were used to prepare the slides. The elements present in the green propolis sediment showed that the botanical origin was *Bacharis dracunculifolia*. The propolis was crushed and macerated with an extractive solution containing 96 GL alcohol for 10 days, being stirred for 10 min each day. Afterwards, the solvent was evaporated, and a stock solution was prepared in 96 GL alcohol and labeled G1. Before use, the extract was prepared as a gel formulation containing 5% crude extract.

The chemical composition of G1 was determined by high performance liquid chromatography using a Merck-Hitachi apparatus (Germany) equipped with a pump (model L-7100, Merck-Hitachi) and a diode array detector (model L-7455, Merck-Hitachi). Separation was performed in a Lichrochart 125-4 column (Merck, Darmstadt, Germany) as previously described.[19] Detection of the components was monitored at 280 nm, and standard compounds were co-chromatographed with the extract. Data analysis was performed in a Merck-Hitachi D-7000 (Chromatography Data Station - DAD Manager).

Experimental procedure

This study was performed at the Universidade do Sul de Santa Catarina (Southern University of Santa Catarina), and all volunteers were informed of the risks of the use of the propolis gel. The target population were women 28-40 years old) after exams and anatomo-histopathological diagnosis. Women were excluded from the project if they presented any of the following: diabetes, pregnancy, contact dermatitis, allergies, or super sensitivity to propolis or apiderivatives.

Confirmation of inflammatory disease in the cervicovaginal material in various regions such as the vulva, vagina, cul-de-sac, exocervix, or endocervix was made by means of neutrophil analysis, as defined by the Di-Prever System. After clinical evaluation and biochemical analysis, the women received the propolis gel (5%) (1 tube each containing 100 g) and were instructed to use it for the next seven days. All the women evaluated were submitted to a test in which a sample of cervicovaginal material was collected three times for quantification of neutrophil content. The sample was collected three times, before treatment, 7 days after initiating treatment, and 14 days after initiciating treatment.

Ethical guide for the use of the protocol

The technical procedures were developed in accordance with Resolution 251 (August 7th, 1997) of the National Health Council (MS-Brazil), which authorizes the research and development of new pharmaceuticals. This study also followed the ethical guidelines of the National Health Council and was approved by the UNISUL Commission on Ethics in Research.

Drugs and reagents

In this experiment, we used standardized green propolis extract from Brazil (Nectar Farmacêutica Ltda, Belo Horizonte, Brazil), carbopol, methylparaben, and propylparaben (Galena, São Paulo, Brazil). For other reagents, the suppliers are as indicated.

Statistical analysis

For this study, the results are presented as the mean ± S.E.M. Differences between the experimental groups were evaluated using analysis of variance followed by Dunnett's multiple comparison test or Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

The chemical composition of G1 was evaluated by HPLC analysis, which showed high levels of the phenolic compounds 3,5-diprenyl-4-hydroxycinnamic acid (DHCA) and the derivatives 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran (DPB), 3-prenyl-4-hydroxycinnamic acid (PHCA), *p*-coumaric acid (PCUM), caffeic acid (CAF), and caffeoylquinic acids, in addition to cinnamic acids and the flavonoids pinobanksin and kaempferol (Table 1). The flavonoid content corresponded to 22.37 mg/g of dried extract.

Seven days of treatment with Brazilian green propolis gel resulted in a significant decrease in the number of neutrophils in the cervico-vaginal material. This response was seen in the vulva, with 55±5% inhibition after 7 days, but it increased again after 14 days (Figure 1). When we evaluated the number of neutrophils in the vagina, we observed a decrease of 38±4%; this effect was sustained for 7 more days after treatment, with a similar inhibitory effect (60±5%, Figure 2). The anti-inflammatory effect of Brazilian green propolis on the cul-de-sac was similar. After 7 days, the application of propolis gel (5%) produced a 39±4% reduction in the number of neutrophils, and, after 14 days, the inhibitory effect was 37±5% (Figure 3). When we evaluated the number of neutrophils in the cervico-vaginal material, from the exocervix, we observed a significant inhibitory effect after 7 days (55±6%); in contrast, after 14 days, there was no significant effect (Figure 4). On the other hand, when we evaluated the number of neutrophils in the cervico-vaginal material, from the endocervix, the application of propolis gel did not reduce the neutrophil after 7 days, but reduced 49±5% after 14 days (Figure 5).

DISCUSSION

Brazilian propolis is sub-divided into four types based on multivariate statistical analysis of the composition of a series of standardized ethanol extracts determined by HPLC.[19] The propolis sample employed in the present work is classified as a subtype of BRPX, or G1, since it contains a high percentage of the bioactive compounds from phenolic acids. This mixture is characteristic of propolis samples collected in Southeastern Brazil, especially in the state of Minas Gerais. The botanical source of this propolis is *Baccharis dracunculifolia*.[20,21] G1 and another propolis sample from Brazil, labeled P1, from Southern Brazil, showed significant analgesic[22] and anti-inflammatory effects in mouse models.[23]

In each of the models of inflammation, the propolis extract was administered both intraperitoneally and orally. Our results indicate a satisfactory oral absorption of the bioactive components of G1, leading to effective anti-inflammatory activity. It has been reported that several vegetal extracts with important effects lose their activity when administered orally rather than intraperitoneally.[24]

Mediators of inflammatory processes are involved in cervicitis and in other inflammatory diseases.[22,25] The anti-inflammatory effect of G1 is possibly secondary to its anti-inflammatory activity; i.e., the process of cell migration, involving interference by active principals present in the extract of the synthesis of such mediators. Supporting this hypothesis, Mirzoeva and Calder reported that the ethanol extract of propolis suppressed prostaglandin and leukotriene generation by peritoneal macrophages *in vitro* as well as during zymosan-induced acute peritoneal inflammation in mice.[13]

We have recently demonstrated that Brazilian propolis produced a significant anti-inflammatory effect mediated, at least in part, by PGE_2 and the inhibition of the NF-κB pathway and nitric oxide production.[17] This inflammatory factor is involved in the control of inflammatory responses, interfering with the production of iNOS and of the inducible isoform of COX-2.[24] Phenolic compounds

found in high content in G1 present, among their broad range of properties, the ability to act as antioxidant agents, scavenging free radicals, including nitric oxide radicals, and also interfering with inflammatory processes by inhibiting iNOS and COX-2 activities.[26,27] Based on these results, we propose that green propolis gel might inhibit neutrophil migration during chronic cervicitis, thus making it useful in controlling the chronic inflammatory process in cervico-vaginal pathologies.

Despite the existence of several pharmacological studies with propolis extracts, the present work demonstrates that a typical Brazilian propolis extract, at low concentrations, induces anti-inflammatory effects in clinical assays in human cervicitis. These observations, together with the *in vitro* results, support the hypothesis of Song and coworkers[28] that the anti-inflammatory effect of propolis may be due to inhibition of iNOS gene expression, through interference at NF-κB sites in the iNOS promoter. The propolis components responsible for the pharmacological activities are currently not known, but the phenolic acids are strong candidates. Further experiments are now in progress aiming to discover the contributions of these compounds of Southeastern Brazilian green propolis towards its pharmacological properties.

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Table 1: Amount in mg/g of each compound identified by HPLC analysis of crude propolis (referring to the sample described previously).

Compound identified in propolis	**mg/g of dried extract**
2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopiran	3.17
2,2-Dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid	9.77
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 1)	1.73
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 2)	0.72
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 3)	1.04
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 4)	1.70
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 5)	3.70
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 6)	2.46
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 7)	1.85
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 8)	1.66
3,5-Diprenyl-4-hydroxycinnamic acid (derivative 9)	2.89
3,5-Diprenyl-4-hydroxycinnamic acid (Artepillin C ®)	27.77
3-Prenyl-4-hydroxycinnamic acid	5.71
Caffeic acid	2.43
Caffeic acid (derivative 1)	11.35
Cinnamic acid derivative	65.98
Ferulic acid	7.21
Kaempferol (derivative 1)	20.45
p-Coumaric acid	18.02
Pinobanksin	31.48
Total amount (mg/g of crude resin)	**221.10**

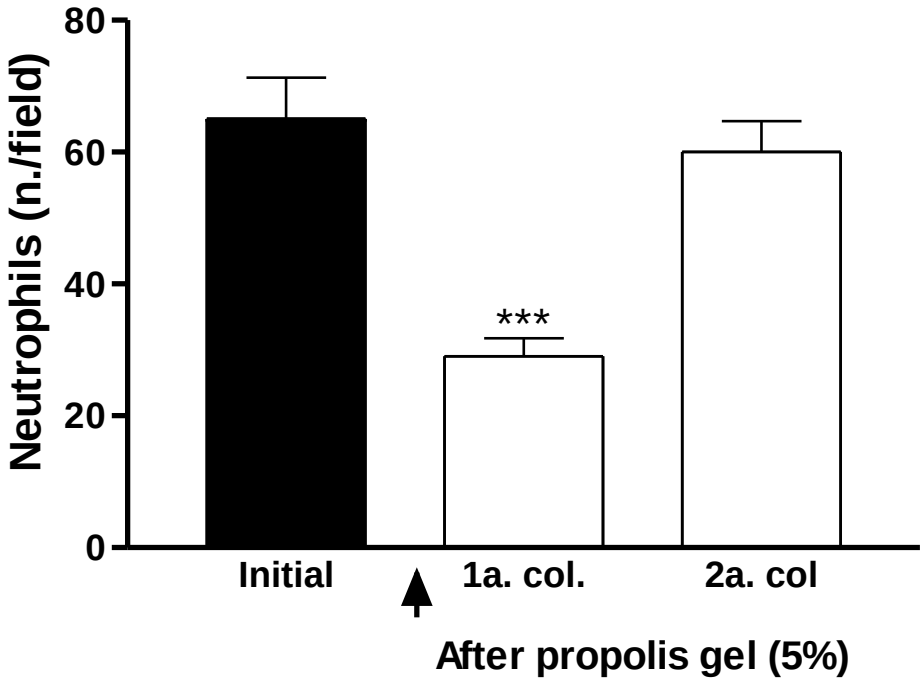

Fig. 1: Effect of Brazilian green propolis gel (5%) in the number of neutrophils in the cervico-vaginal material, from vulva, collected from women diagnosed with chronic cervicitis in absence of treatment (full bars) or 7 or 14 days (open bars) of treatment. Each value represents the mean ± S.E.M. of 6 women, and asterisks indicate significant inhibition in the absolute number of neutrophils in relation to the untreated group, $P < 0.05$.

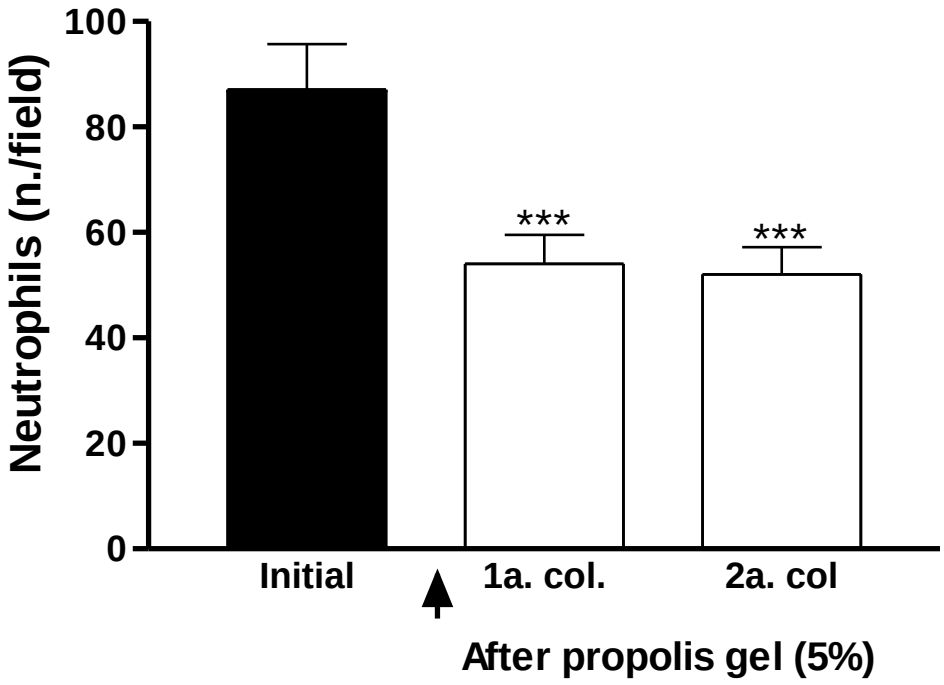

Fig. 2: Effect of Brazilian green propolis gel (5%) on the number of neutrophils in the cervico-vaginal material, from the vagina, collected from women diagnosed with chronic cervicitis in absence of treatment (full bars) or 7 or 14 days (open bars) of treatment. Each value represents the mean ± S.E.M. of 6 women, and asterisks indicate significant inhibition in the absolute number of neutrophils in relation to the untreated group, $P < 0.05$.

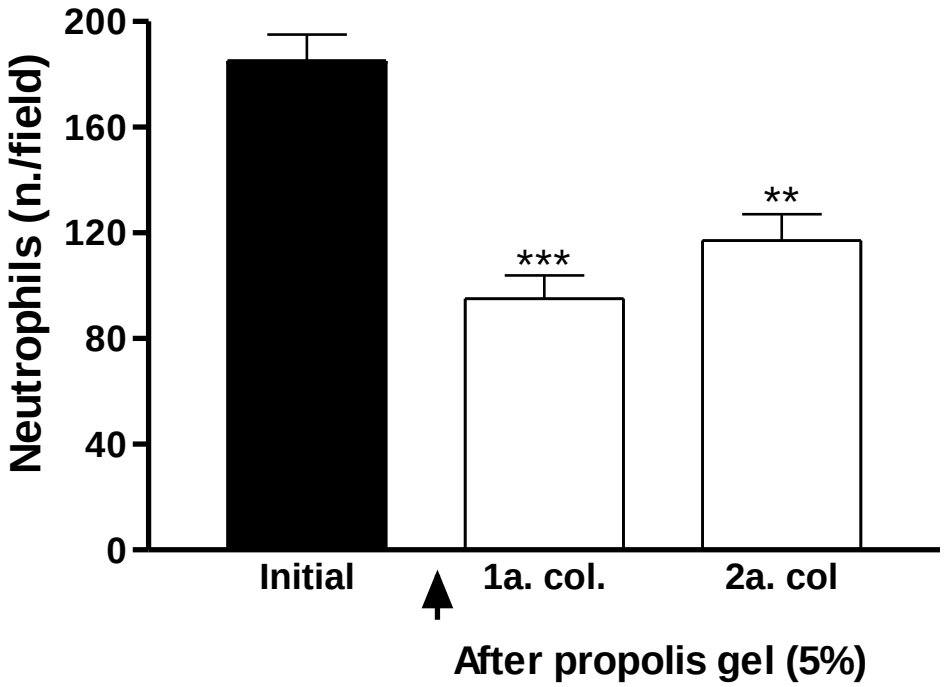

Fig. 3: Effect of Brazilian green propolis gel (5%) on the number of neutrophils in the cervico-vaginal material, from the cul-de-sac, collected from women diagnosed with chronic cervicitis in absence of treatment (full bars) or 7 or 14 days (open bars) of treatment. Each value represents the mean ± S.E.M. of 6 women, and asterisks indicate significant inhibition in the absolute number of neutrophils in relation to the untreated group, $P < 0.05$.

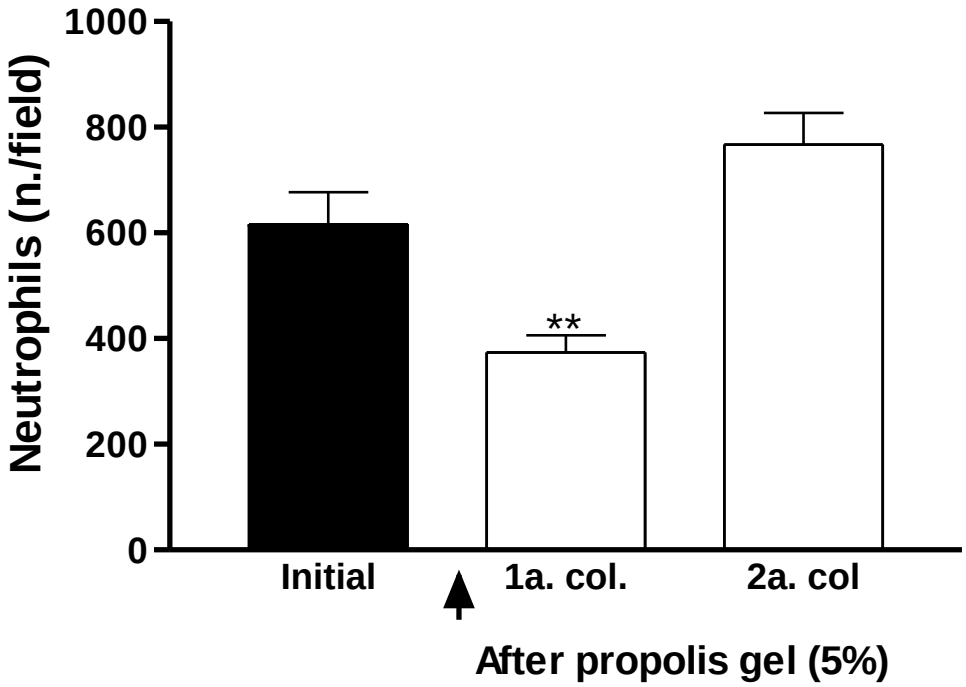

Fig. 4: Effect of Brazilian green propolis gel (5%) on the number of neutrophils in the cervico-vaginal material, from the exocervix, collected from women diagnosed with chronic cervicitis in absence of treatment (full bars) or 7 or 14 days (open bars) of treatment. Each value represents the mean ± S.E.M. of 6 women, and asterisks indicate significant inhibition in the absolute number of neutrophils in relation to the untreated group, $P < 0.05$.

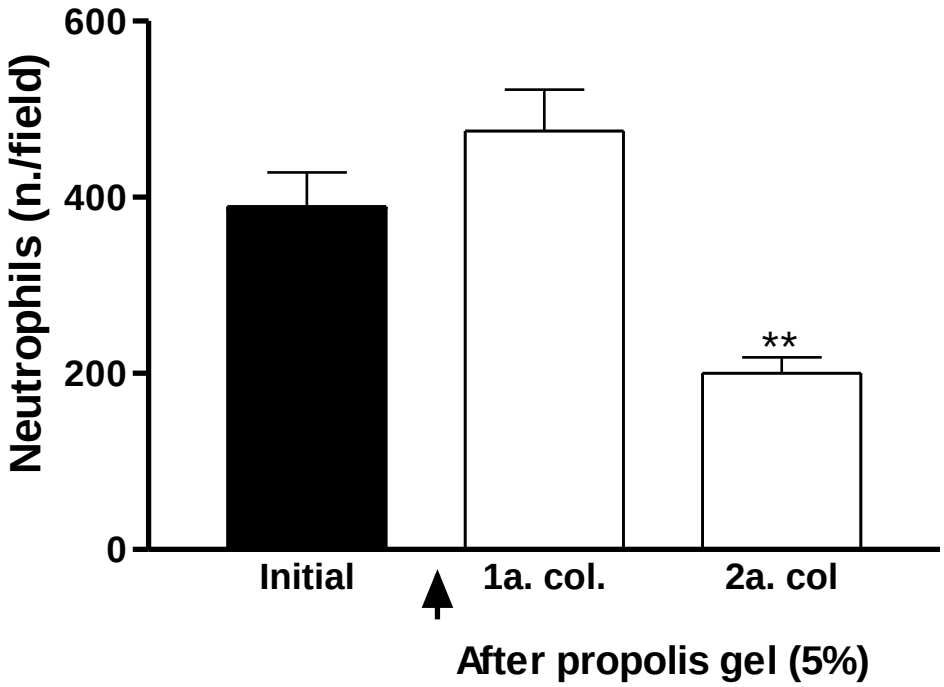

Fig. 5: Effect of Brazilian green propolis gel (5%) in the number of neutrophils in the cervico-vaginal material, from the Endocervix, collected from women diagnosed with chronic cervicitis in absence of treatment (full bars) or 7 or 14 days (open bars) of treatment. Each value represents the mean ± S.E.M. of 6 women, and asterisks indicate significant inhibition in the absolute number of neutrophils in relation to the untreated group, $P < 0.05$.