

Original Research Article

Study of the effect of a propolis solution on the macrophage cultures: A cellular analysis

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A B S T R A C T

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Propolis is a natural product collected by honey bees from various plant sources. We aimed to determine the possible effects of propolis on macrophage cells. Methods: Macrophages cells were obtained by *Wistar* rats and treated with propolis solution during 30 and 60 minutes. Morphology microscope analysis has been done. Results: Due to the analysis of the results it was observed that in the quantitative analysis was not observed the apoptotic effect of the propolis solution although in the qualitative analysis this effect was observed in the cells culture treated during 30 and 60 minutes. Conclusion: it may be suggested that prenylflavanones, propolin A and propolin B, Artepillin C together with other components, depend on the concentration level, might have the capable of inducing apoptosis in cell.

Introduction

The function of the normal cell applies for a balance between the physiologic demands and the limitations of the cellular structure and of the metabolic capacity; the result is a stable state or homeostasis. The cells can alter his functional state in reply to a moderate stressor maintain his stable state. More excessive physiologic stresses or pathological averse stimuli (injury) turn in adaptations, reversible injury or irreversible injury and cellular death. The adaptations take place when physiologic or pathological stresses induce a new state that alters the cell, which

preserves his viability in reply to extern stimuli as hyperplasia, hypertrophy, atrophy and metaplasia. The cellular reversible injury shows cellular pathological alterations that can be restored to the normality if the stimulus will be withdrawn or if the cause of the injury was not serious. The irreversible injury takes place when the stimulus exceeds the capacity of the cell to be adapted and shows pathological constant alterations that cause the cellular death. There are two morphological and mechanical standards of cellular death:

necrosis and apoptosis (Hortelano et al, 2001; Amarante-Mendes, 2003; Tabas, 2005; Mitchellet al, 2006).

The necrosis is the type of the commonest cellular death, it wraps great cellular edema, denaturizing and coagulation of proteins, degradation of organelles cellular and break of cells, loss of the entirety of the plasmatic membranes, cytoplasmatic disorganization and nuclear debauchery. In general, a great number of cells in the adjacent cloth is affected. The apoptosis takes place when the cell dies due to the activation of a program of "suicide" controlled internally, what wraps an orchestrated disturbance of cellular components; there takes place a least break of the adjacent cloth.

Morphologically, it takes place to condensation and the fragmentation of the chromatin with degradation of the DNA genomic in fragments oligonucleisomas, loss of the volume and increase of the cellular granulocyte, maintenance of the structure of the organelles, formation of "pleats" in the plasmatic membrane and consequent cellular fragmentation in apoptotic bodies. In different cellular populations, a great similarity is observed in the phenotype of the apoptosis, same in quite different physiologic situations. This phenotype is resultant, principally, of the action in members' waterfall of a special family of proteases cistein-aspartate. In different cellular populations, a great similarity is observed in the phenotype of the apoptosis, same in quite different physiologic situations. This phenotype is resultant, principally, of the action in members' waterfall of a special family of proteases cistein-aspartate, called Caspases. These are divided in two groups, being the executioners responsible for the execution of the process in you.

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If the sign of stress is very violent, which takes place in general in pathological situations, a given cell has no choice, not being to suffer a necrotic death, impossible of genetics to be controlled or pharmacology, and quickly to lose the entirety of his cellular membrane, releasing his cytoplasmatic content and inducing an inflammatory answer in the busy cloth. On the other side, when the stress is more pleasant, the produced sign will be going to be analyzed by the set of mitochondria, which act like a sensor and have the responsibility of deciding if this cell will be going to keep on living or will have to be removed by mechanisms apoptotic. In this case, the mitochondria suffer a dysfunction of the respiratory chain that will cause the liberation especially of the citocrom c for the cytosol, which will activate the waterfall of caspases what will be going to execute the program of cellular package known how apoptosis.

In certain situations where the cells apoptotic were not removed for phagocytes, it can take place cellular necrosis called secondary necrosis or powders - apoptosis. In this case, apoptotic they break the membranes of the cells with

liberation of intra-cellular components, as proteases, what induce inflammatory answer and tissue harm. In macrophages, the apoptotic phenotype shows a sequence of alterations in several parameters, like loss of cellular adhesion and asymmetry of the plasmatic membrane, disorganization of the cytoskeleton and internucleisoma fragmentation of the DNA, together with activation of the caspases. Properties chemical-physical general of the cell are modified during the apoptosis (Tabas, 2005; Mitchellet al, 2006).

Propolis is a natural product collected by honey bees from various plant sources. It exhibits pronounced antimicrobial and anti-inflammatory effects. Kismet et al (2008) described that propolis administration reduced hepatocyte apoptosis as well as it showed a significant hepatoprotective effect in this experimental obstructive jaundice model. Draganova et al (2008) related that The present study demonstrates that high concentrations of propolis and CAPE cause apoptosis-induced cell death in McCoy-Plovdiv cells.

Propolis is one of the major hive products of bees and is rich in flavonoids, which are known for antioxidant activities. It is well known that the chemical properties of phenolic acids or flavonoids, in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers, predict their antioxidant properties. Alyane et al (2008) demonstrate that antioxidants from natural sources may be useful in the protection of cardiotoxicity in patients who receive doxorubicin and as reported for its claimed beneficial effect on human health by biomedical literature. A flavonoids-free Brazilian propolis (type 6) showed biological effects against mutans streptococci and inhibited the activity of

glucosyl transferases. Duarte et al (2006) suggested that the cariostatic properties of propolis type 6 are related to its effect on acid production and acid tolerance of cariogenic streptococci; the biological activities may be attributed to its high content of fatty acids. Hayacibara et al (2005) suggested that the putative cariostatic compounds of propolis type-3 and -12 are mostly non-polar; and H-fr should be the fraction of choice for identifying further potentially novel anti-carries agents. Gebaraa et al (2003) observed that sub gingival irrigation with propolis extract as an adjuvant to periodontal treatment was more effective than conventional treatment both by clinical and microbiological parameters. *Streptococcus mutans* triggers dental caries establishment by two major factors: synthesis of organic acids, which demineralize dental enamel, and synthesis of glucans, which mediate the attachment of bacteria to the tooth surface. *Baccharis dracunculifolia* DC (Asteraceae), a native plant from Brazil, is the most important botanical origin for the production of green propolis (Brazilian propolis) by honeybees.

However, whether *B. dracunculifolia* (Bd) has an anticariogenic effect, like green propolis, remains unknown. In a research it was observed that Bd leaf rinse and green propolis extracts have similar inhibitory effects on the *S. mutans* cariogenic factors evaluated herein, and allowed us to suggest that Bd leaves may be a potential source for pharmaceutical products employed for this purpose (Leitão et al, 2004).

In recent years, propolis has been the object of extensive research for its antibacterial, antioxidant, anti-inflammatory, and antitumoral activities.

Doğanyığıt et al. (2013) demonstrated that the administration of propolis could have a protective effect against changes of both genomic stability values and methylation profiles, and it minimized the increase in MDA and tissue damage caused by LPS, the application of propolis prior to LPS-induced endotoxemia has shown to reduce hepatic damage. The aim of this work was to evaluate the effect of the water of propolis solution at cultures of macrophages obtained of Wistar rats.

Materials and Methods

In the present work macrophages were obtained of Wistar rats (*Rattus norvegicus albinus*), n=5. The animal used in the work was obtained of the enterprise Biocampo 2000 Biological Product Ltda (enterprise supplier of biological products, including animals for inquiries, done not notlinked to the specific institution and what has an own vivarium in appropriate conditions of functioning). In the sequence for attainment of macrophages, first there was carried out the preparation of the way of culture where the cells would be put. A liter of distilled water was used. There happened, then, the preparation of the least essential way of Eagle whose pH is 7.0. A bottle of the powder of the way was poured in 1 liter of distilled sterile water.

There was carried out positive filtration of the way Eagle through sterile membrane of 0.22 μm . 100 mg of penicillin were weighed G benzatine and 100 mg of sulphate of streptomycin. 121 were obtained in the end mg/l of penicillin and 230 mg/L of streptomycin (both the commercial mark Sigma-Aldrich, St Louis, USES). The antibiotics were put in the middle of culture, since East because of being a rich, even sterile way can become contaminated. Concentration of 1

was used mL of the antibiotic for each 50 mL of the way (concentration of 50 times). After getting the way of culture, the animals were put in a bottle containing cotton and chloroform that was closed. After the death of the animals, there was carried out the injection of the way of culture in the peritoneum of the same thing.

The edema obtained through this maneuver attracts macrophages. The macrophages were inhaled by syringe and transferred to tubes with way of culture. These tubes were stipulated in ice. Brackets of the content were transferred to a blade to observation of the cells. For the microscopic analysis, there was used the blade of Neubauer, which allows the placing of the material collected in a compartment of the blade to make easy the subsequent counting of the cells that in our case are the macrophages. The counting was carried out through a hemocitometry. To the end 25 was obtained ml of solution with macrophages. It was considered that 0.8×10^5 cells/mL for 1 mL, soon 200×10^5 is for 25 mL in proportion. It was obtained 2×10^7 cells of the rats in 5 minutes. These are migratory cells. In 100 μL have 1/10 of this value. Serum was added fetal bovine to 20 %, which contains adesines what make easy the adhesion of the macrophages in the plate.

There were put brackets of the way of culture containing the macrophages in the wells of the plates for cultivation. The plates were put in a drier with environment of CO₂ (distilled water, bicarbonate of sodium (Na₂HCO₃) and sulphuric acid) creating an environment of anaerobiose. The drier was left in stove to 37°C. It was used 2 micro plates of test for cellular culture, with 6 orifices each one (Zellkultur Test platte).

In a second stage, it broke for the exhibition of the macrophages to the preservative ways made used in the dental avulsion. The preservative ways being used were:

- .preservative was the propolis spray
- .positive control was the way with serum fetal bovine
- .negative control was the way containing Antimicina A (used like lethal substance)

The preservative was the propolis spray (Makrovit, Rio De Janeiro, Brazil). For the negative control, there was used the Antimicin A (Sigma-Aldrich, St Louis, USES) what acts inhibiting the cellular oxidation, it inhibits the citocrom (complex I, NADH desidrogenase) and so there is no production of ATP in the breathing mitochondrial of the macrophages, causing the cellular death. The toxic dose of the Antimicin A (C₂₀H₄₀N₂O₄) is of 2 µg/ml and the lethal dose is 200DL50.

The ways were gathered of the plates. The environments contained in 6 spaces of two other plates were put in test tubes. The two plates with 6 wells had his environments collected and put in test tubes and the preservatives were put in the numbered wells from 1 to 6 in the plate itself respectively with his numbers established-daily pay. These plates are numbered from 1 to 3 in the superior and part from 4 to 6 in the inferior part. One of these plates was put for 30 minutes and other one for 60 minutes in the drier and when was left in the stove 37° C. When the respective times of 30 and 60 were spent, the preservatives were gathered and the plates washed with PBS quickly. The Blue thing of Tripan to 0.5 % was added in all the wells and left for 1 minute. The wells were washed by water and subsequently methanol was added and then Panotic to fix the coloring.

After colored and fixed, the plates were observed to the microscope. Not much ruddy or clearer cells are those that show up cheer and the ruddiest or when they fled well, these one are the dead one.

Results and Discussion

The macrophages were collect from *Wistar* rats and they were treated with different types of solutions. After a period of time the survive of these cells was analyzed and expressed by values in percentage.

Due to the analysis of the results it was observed that there was not difference between the group treated with propolis solution (30 min and 60 min) to the control group (p=0,047; Dunn's Multiple Comparison Test and Kruskal-Wallis test, by the Prism 5, statistical program analysis).

Table.1 Effect of water of propolis in the survive of macrophages cells

Preservative ways	30 min	60 min
Positive control	100%	100%
Propolis	71%	37%

In the figures of 1 to 7 (400X), could be observed that the extract of propolis was capable of inducing to apoptosis with passing of the time (t=60min) exactly not being identified such difference in statistical level.

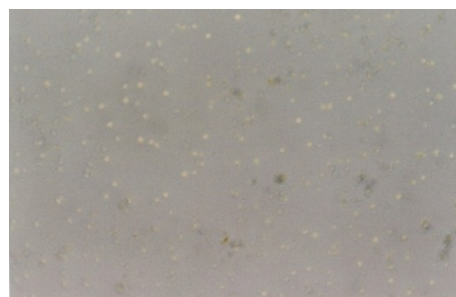


Figure.1 Positive control; Practically all the alive cells. (100X)



Figure.2 Negative control. All the dead cells. (100X)

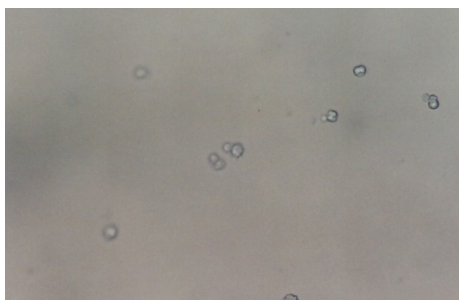


Figure.3 Propolis in 30 minutes. Alive cells. (100X)

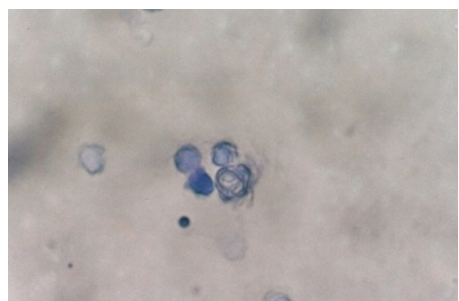


Figure.4 Propolis in 30 minutes. Alive cells together with deformed cells. The propolis causes increase of the cell for probable increase of the permeability of the cellular membrane. (400X)

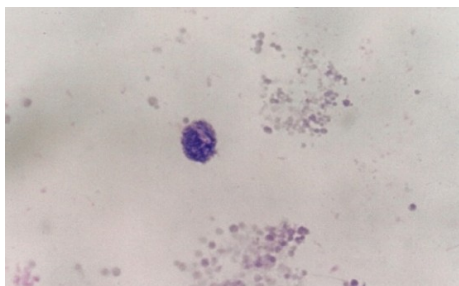


Figure.5 Propolis after 60 minutes. It is observed deformed necrotic cells, also observed apoptosis. Nuclear spalling. (400X)

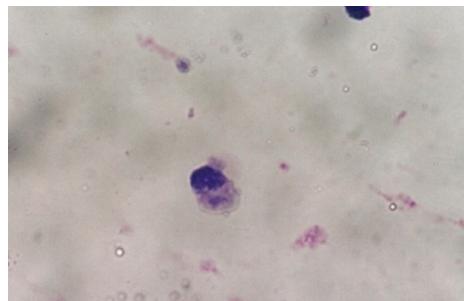


Figure.6 Propolis after 60 minutes of exposition. Cytoplasmic alteration. Nucleus starting to break up. Apoptose. (400X)

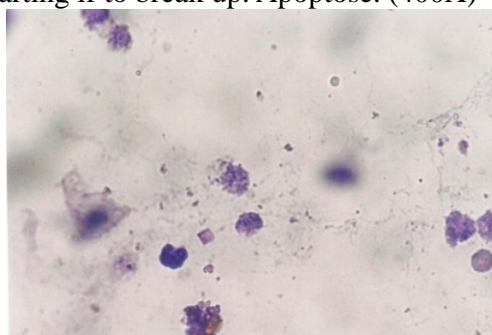


Figure.7 Propolis after 60 minutes of exposition. Necrosis of the cell can be perceived. Scars in the cell occur, what cause necrosis. The necrosis stimulates the inflammatory process. (400X)

Huang et al (2007) described that the isolation and characterization of propolin G from Taiwanese propolis (TP) have demonstrated for the first time that this compound is a potent inducer of apoptosis in brain cancer cells and that this compound and TP extract exhibit a protective effect against oxidative stress in rat cortical neurons. Complementary Inokuchi et al (2006) described that Brazilian green propolis has neuroprotective effects against retinal damage both in vitro and in vivo, and that a propolis-induced inhibition of oxidative stress may be partly responsible for these neuroprotective effects.

Propolis is a resinous substance which is used by bees to repair and maintain their hives. It has more than 180 compounds including flavonoids, phenolic acids and

its esters which have anti-inflammatory, antibacterial, antiviral, immunomodulatory, antioxidant and anti-proliferative effects.

Propolis is shown to inhibit cell division and protein synthesis. However the exact mechanism underlying antitumor effect is not clearly described. On the other hand progressive telomere shortening to a critical level results with senescence of normal cells by inducing apoptosis and telomerase prevents erosion of telomeres. Propolis may show antitumor and apoptotic effect via inhibiting telomerase expression besides the mechanisms which have been described previously (Gunduz et al, 2005). In this work we observed that propolis solution was not capable to induce apoptosis in a quantitative analysis in the different times evaluated. Hernandez et al (2007) described that constituents of *Sonoran propolis* samples possess a strong antiproliferative activity on cancer cell lines. Chen et al (2004) related that *Taiwanese propolis* is a rich source of propolins A, B, C, D, E and F and that TP induces apoptosis in human melanoma cells due to its high level of propolins.

Vegetables and fruit help the prevention and the therapy of several kinds of cancer because they contain micronutrients, a class of substances that have been shown to exhibit chemopreventive and chemotherapeutic activities. Scifo et al (2004) pointed out the anticancer activity of resveratrol and propolis extract in human prostate cancer, exerting their cytotoxicity through two different types of cell death: necrosis and apoptosis, respectively. They suggested the possible use of these micronutrients both in alternative to classic chemotherapy, and in combination with very low dosage of vinorelbine.

Chen et al (2004) had demonstrated that two prenylflavanones, propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced apoptosis in human melanoma cells and significantly inhibited xanthine oxidase activity. The isolation and characterization of propolin C from bee propolis are described for the first time, and this compound is a powerful inducer of apoptosis in human melanoma cells.

Oncag et al (2006) revealed that propolis had good *in vitro* antibacterial activity against *E. faecalis* in the root canals, suggesting that it could be used as an alternative intracanal medicament.

Sabir et al (2005) suggest that direct pulp capping with propolis flavonoids in rats may delay dental pulp inflammation and stimulate reparative dentin.

According to Hayacibara et al (2005) the putative cariostatic compounds of propolis type-3 and -12 are mostly non-polar; and H-fr should be the fraction of choice for identifying further potentially novel anticaries agents.

Botushanov et al (2001) described that Propolis is bee-produced substance with pronounced anti-inflammatory effect. It is an ingredient of many drugs; it is added to toothpastes as a prophylactic component for periodontal diseases and that the toothpaste showed very good plaque-cleaning, plaque-inhibiting and anti-inflammatory effect. Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) has a molecular weight of 300.40 and possesses antibacterial activity. When artepillin C was applied to human and murine malignant tumor cells *in vitro* and *in vivo*, artepillin C exhibited a cytotoxic effect and the growth of tumor cells was clearly

inhibited. The cytotoxic effects of artepillin C were most noticeable in carcinoma and malignant melanoma. Apoptosis, abortive mitosis, and massive necrosis combined were identified by histological observation after intratumor injection of artepillin C. It was indicated that artepillin C activated the immune system, and possesses direct antitumor activity (Kimoto et al, 1998). Due to Kimoto et al (1998) Artepillin Cis an active ingredient of Brazilian propolis that possesses anti-tumor activity. When Artepillin C was applied to human leukemia cell lines of different phenotypes, namely, lymphocytic leukemia (7 cell lines of T-cell, 5 cell lines of B-cell), myeloid and monocytic leukemia and non-lymphoid non-myeloid leukemia cell lines *in vitro*, Artepillin C exhibited potent cytotoxic effects and induced marked levels of apoptosis in all the cell lines.

The most potent effects were observed in the T-cell lines. Apoptotic bodies and DNA fragmentation were induced in the cell lines after exposure to Artepillin C. DNA synthesis in the leukemia cells was clearly inhibited and disintegration of the cells was confirmed microscopically. Apoptosis of the leukemia cells may be partially associated with enhanced Fas antigen expression and loss of mitochondrial membrane potential. In contrast, although Artepillin C inhibited the growth of pokeweed mitogen (PWM)-stimulated normal blood lymphocytes, it was not cytotoxic to normal unstimulated lymphocytes. These results suggested that Artepillin C, an active ingredient of Brazilian propolis, has anti-leukemic effects with limited inhibitory effects on normal lymphocytes. Pinocembrin is the most abundant flavonoids in propolis, and has been proven to have antioxidant,

antibacterial and anti-inflammatory property. According to Gao et al (2008) Pinocembrin could also down-regulate the expression of p53 protein, and inhibit the release of cytochrome c from mitochondria to cytosol. Thus they concluded that pinocembrin exerts its protective effects in a glutamate injury model partly by inhibiting p53 expression, thus Bax-Bcl-2 ratio, and the release of cytochrome c. Related to the apoptosis induced by the propolis solution, observed in the qualitative analysis, it may be due to the high concentrations of propolis and CAPE which may cause apoptosis-induced cell death (Draganova et al, 2008). It is known that caffeic acid phenethyl ester (CAPE) is one of the most effective components of propolis which is collected by honey bees. The cytotoxic and apoptotic effects of CAPE were verified by Avci et al (2007). Chen et al (2007) demonstrated that two new prenylflavanones, propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced cytotoxicity effect in human melanoma A2058 cells and shows a strong capability to scavenge free radicals.

In fact they suggested that propolin A and propolin B may activate a mitochondria-mediated apoptosis pathway. On the other hand, our data show that propolin B inhibited xanthine oxidase activity more efficiently than propolin A or CAPE. However, CAPE suppressed ROS-induced DNA strand breakage more efficiently than propolin A or propolin B. All these results indicated that propolin A and propolin B may trigger apoptosis of A2058 cells through mitochondria-dependent pathways and also shown that propolin A and propolin B were strong antioxidants. In a study Mishima et al (2005) have previously reported that Brazilian propolis

extracts inhibited growth of HL-60 human myeloid leukemia cells, which is partly attributed to the induction of apoptosis associated with granulocytic differentiation. They have suggested that the number of the caffeoyl groups bound to quinic acid plays an important role in the potency of the caffeoylquinic acid derivatives to induce granulocytic differentiation.

These findings would support that although in a quantitative analysis it was not identified apoptosis significantly different of it we observed in a morphological qualitative analysis the capable of propolis inducing apoptosis in the macrophages cultures. Our results can be related with a study developed by Markiewicz-Ukowska et al. (2013), whom demonstrated that the ethanolic extract of propolis presents cytotoxic properties and may cooperate with temozolomide synergistically enhancing its growth inhibiting activity against glioblastoma U87MG cell line. This phenomenon may be at least partially mediated by a reduced activity of NF-kappaB.

According to the results obtained we may suggested that apoptosis cells may be partially associated with enhanced Fas antigen expression and loss of mitochondrial membrane potential, although in contrast, as described by other author, is known that Artepillin C inhibited the growth of pokeweed mitogen stimulated normal blood cells and that these effects would support the apoptotic effect of propolis which may be related to the Artepillin C, an active ingredient of Brazilian propolis, which has apoptotic effects. In the light of results we may suggest that apoptotic effect observed in the qualitative analysis would be related to the concentration level of Artepillin C

together with other flavonoids as pinocembrin as well as other components as CAPE besides of the time of exposing.

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