Medical and Applied Malacology
Crossing Boundaries

Medical and Applied Malacology
Crossing Boundaries:
Integrative Approaches to Malacology

Edited by

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CAMBRIDGE SCHOLARS

P U B L I S H I N G

To Dr *Toshie Kawano*, one of the most important Brazilian researchers in Malacology who always encouraged Malacological studies and left, as a legacy of her career, a huge literature in the area and numerous graduates.

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FOREWORD

The current organizing committee gratefully received and accepted the invitation from Cambridge Scholars Publishing to produce this volume, which contains papers presented at XI International Congress on Medical and Applied Malacology: "Crossing boundaries: Integrative Approaches to Malacology". The congress was held at the State University of Rio de Janeiro between the 25th and 29th of September 2012. This meeting brought together people from different countries - Argentina, Brazil, Chile, Colombia, Hungary, Jamaica, Japan, Peru, Philippines, Poland, Russia, Spain, Sultanate of Oman, Taiwan, Thailand, Uruguay and the USA.

This book provides an overview of the 11th ICMAM, presenting papers on bioprospecting methods, medical malacology, mollusk culture, biomonitoring, alien species, reproductive biology, trends in malacological research and teaching and education.

There were many lecturers at the congress, not all of whom we could invite to join us on this journey. Select papers were chosen to be published, along with those that received student awards.

To honor the memory of Toshie Kawano, the Brazilian Society of Malacology decided to create the Toshie Kawano Award. This prize was to be given to a renowned female researcher, well-known for relevant scientific work and successful efforts in teaching. We were overjoyed to grant the prize to Maria Cristina Dreher Mansur. The best oral and poster presentations on medical malacology were both awarded in association with Dr. Wladimir Lobato Paraense Awards and best presentations in all other malacological fields were granted by Dr. William H. Heard Awards.

We would like to thank all the authors who submitted papers for review, those who provided manuscripts for inclusion in this publication, and all members of the congress committee (scientific, organizing and executive) for the achievement of making XI ICMAM an unforgettable event. We are also grateful to Instituto Oswaldo Cruz, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Rio de Janeiro (FAPERJ) and Ministério da Saúde and Universidade do Estado do Rio de Janeiro (UERJ).

—The Editors

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CHAPTER ONE

CELL DEATH MECHANISMS INDUCED
BY *PHYLLOCAULIS BORACEIENSIS* MUCUS

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Introduction

In order to improve the study of bioactive molecules, many groups around the world are nowadays performing scientific research on snails and slugs. These sorts of animals are repositories for a large amount of molecules that have the potential to be used in medicine as a source of compounds to treat diseases such as cancers (Dwek *et al.* 2001), scars (Badiu *et al.* 2008; Ledo *et al.* 1999), burns (Tsoutsos *et al.* 2009), hepatitis (Liu *et al.* 2013) and certain sorts of bacteraemia (Díaz-Marrero *et al.* 2012) and fungimia (Iijima *et al.* 1994).

Phyllocaulis boraceiensis (Thomé, 1972) is a Brazilian slug that has been studied as a potential source of natural compounds. Mucus released by this mollusc is capable of inducing cell proliferation and the remodelling of tissue (Toledo-Piza *et al.* 2013), thus enhancing the wound healing process in mouse skin (Toledo-Piza and Maria *in press*). In addition, it enhances angiogenesis in cultures of endothelial cells and fibroblasts (Toledo-Piza and Maria 2013).

The process of apoptosis, or programmed cell death is common during both pathological situations contributing to inflammation and organ dysfunction, such as under physiological conditions, and during embryogenesis and other transformation processes. These processes can be divided into two main categories: the intrinsic mitochondrial pathway, and

the extrinsic or death receptor pathway (Qiao and Wong 2009). Induction of apoptosis can be caused by several factors including nutrient depletion, temperature, concentration of dissolved oxygen and toxic by-products (Meneses-Acosta *et al.* 2001). Caspases belong to a family of cysteine proteases that are active in both pathways, with crucial functions, and their activation also results in the implementation of apoptosis (Salvesen and Riedl 2008).

The Bcl-2 family consists of two proteins; pro- and anti-apoptotic. It is known that an excessive increase in the expression of the anti-apoptotic Bcl-2 protein neutralizes apoptotic signals, while the pro-apoptotic protein, BAX, transfers a signal that begins the process of apoptosis. This event is also essential for cell death in uncontrolled cell elimination. Various events, for example DNA damage or abnormality, can lead to chromosomally controlled apoptosis in normal cells. Tumour cells acquire mutations in their genomes that allow them to survive these apoptotic signals (Soussi and Wiman 2007).

Necrosis occurs when there is a breakdown of cellular homeostasis, at which point the cell undergoes extreme variations in its physiological conditions, such as hypothermia or hypoxia, and lyses the cell membrane, subsequently releasing its intracellular components. As a result of this catastrophic process, necrosis often causes inflammation. In contrast, apoptosis is an active cellular death process that occurs physiologically in response to a variety of stimuli and physiological or pathological conditions; the cell participates in its own destruction. Normally, because there is no loss of membrane integrity, apoptosis is not accompanied by a drastic inflammatory reaction (Solá *et al.* 2001).

The goal of this work was to study the pathways of apoptosis and necrosis induced by *Phyllocaulis boraceiensis* mucus in order to evaluate how this natural compound affects cell death. The expression of receptors Annexin-V/PI, Bcl-2 and Caspase 3 on the surface of fibroblasts and endothelial cells treated with the mucus was analysed.

Methods

Collection and preparation of mucus

The specimens of *Phyllocaulis boraceiensis* were taken from a captive breeding programme at the Butantan Institute. Animals were kept in plastic boxes in a pollution-free, temperature-controlled laboratory environment (Toledo-Piza *et al.* 2013). They were fed daily.

Mucus was obtained by stimulating the pedal tissues (Toledo-Piza *et al.* 2012). Experiments were performed using lyophilized samples hydrated in an organic solvent.

Cultures of human fibroblasts and endothelial cells

Normal human fibroblasts were isolated from an eyelid blepharoplasty performed at the Clinical Hospital of the University of São Paulo (research project number 921/06).

Skin fragments were transferred to a 35mm Petri dish containing RPMI-1640 with antibiotics, and washed to remove excess blood and red blood cells. Fat tissue was removed with the aid of forceps and scissors. The fragments were cut and distributed into three Petri dishes containing culture medium.

Cultures of human umbilical vein endothelial cells were obtained from a commercial strain (ATCC CRL-1730). Both types of cell were cultured in bottles ($75cm^2$) with RPMI-1640 supplemented with 10% inactivated fetal bovine serum, 2mM L-glutamine and antibiotics. They were incubated at 37 °C in an atmosphere of 5% CO^2, and examined under an inverted microscope three times a week. The culture medium was changed three times a week. When cells reached confluence, they were trypsinized (0.005% trypsin) to allow detachment from the culture bottles. They were centrifuged at 324,000g twice and rehydrated in culture medium, and the cell concentration was adjusted to 5 x 10^5 cells/ml.

After the cells had been transferred to 96-well plates, they were allowed 24 hours to adhere to the plate and reach confluence, and were then treated with *P. boraceiensis* mucus at concentrations of 0.012µg/µl and 0.18µg/µl (Toledo-Piza *et al.* 2013).

Expression analysis of cell markers involved in pathways of necrosis and apoptosis

Expression analysis of cell markers involved in apoptosis and necrosis pathways was performed by studying the expression profile of the markers annexin V/PI, Bcl-2 and Caspase-3. This analysis aimed to quantitatively determine the percentage of cells undergoing apoptosis by virtue of the ability to bind annexin V and exclude propidium iodide (PI).

After centrifugation and detachment, the cells were treated with *Phyllocaulis boraceiensis* mucus. They were solubilized in 100µl of binding buffer and incubated for 30 minutes with 1µl of annexin V-FITC

(Santa Cruz Biotechnology, Santa Cruz, California, USA) and 20µg/ml of propidium iodide.

Aliquots of 100µl of cell suspensions were incubated for one hour at 4 °C with 1µl of the antibody specific for the cell markers Bcl-2 and Caspase-3 (Santa Cruz Biotechnology, Santa Cruz, California, USA). Then, 10µl of 0.1% Triton X-100 was added to permeabilize the cell membranes. Cells were centrifuged for 10 minutes at 1,500rpm and washed with iced PBS. The supernatants were discarded and the "pellet" solubilized in PBS containing paraformaldehyde (0.1%). Assessment was performed on a FACSCalibur ™ flow cytometer in fluorescence intensity FL-1 or FL-2 according to the antibody. The results obtained were analysed using WinMDI 2.8 software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, USA), and the statistical significance of differences between groups was determined using unpaired one-way ANOVA. The means were compared by using Tukey's multiple comparison tests. Regression analysis was used to examine the data obtained and infer relationships between dependent and independent variables. Comparisons were considered to be either non-significant (NS) or significant at $*\rho < 0.05$, $**\rho < 0.005$, or $***\rho < 0.0001$.

Results

Expression analysis of cell markers involved in pathways of necrosis and apoptosis

Human fibroblasts

The percentage of cells expressing the cellular markers involved in cell death by apoptosis and necrosis in human fibroblasts after treatment with concentrations of 0.18 µg/µl and 0.012 µg/µl of *Phyllocaulis boraceiensis* mucus are shown as mean values for chart analysis (Fig. 1.1). In addition, dot plots (Fig. 1.2) were provided in order to analyze and compare the control and treated groups. Both treatments induced necrotic cell death, the cytotoxic effects presenting at various times. However, after 72 hours of treatment, no further significant increases in cell necrosis were observed. There were no significant differences in the numbers of cells that became apoptotic early and late.

Human fibroblasts were monitored after treatment with mucus for the activation of Caspase-3 and for activation of the intrinsic mitochondrial pathway using Bcl2 as a marker (Fig. 1.3 and 1.4). In this cell lineage, activation of Caspase-3 (Fig. 1.5 and 1.6) occurred only in the group of cells treated with 0.18µg/µl *Phyllocaulis boraceiensis* mucus after 72 hours of exposure, and was accompanied by a significant decrease in Bcl2 expression.

Endothelial cells

The proportion of endothelial cells killed was determined by the expression of phosphatidylserine (Annexin V) and propidium iodide (PI) as detected by flow cytometry of the cells. There were significant differences in the number of necrotic and apoptotic cells following treatment with *Phyllocaulis boraceiensis* mucus.

The proportion of cells killed by apoptosis was significantly higher after 48 hours at concentrations of 0.18µg/µl and 0.012µg/µl than that of the control group, which also showed apoptotic cells (Figs. 1.7 and 1.8). This increase occurred after 48 hours of treatment and continued up to 72 hours at both concentrations of mucus. In this cell lineage, the expression of the anti-apoptotic protein Bcl-2 was significantly reduced compared to the control group (Fig. 1.9 and 1.10). However, Caspase-3 activation did not significantly differ according to examination time or protein concentration used (Fig. 1.11 and 1.12).
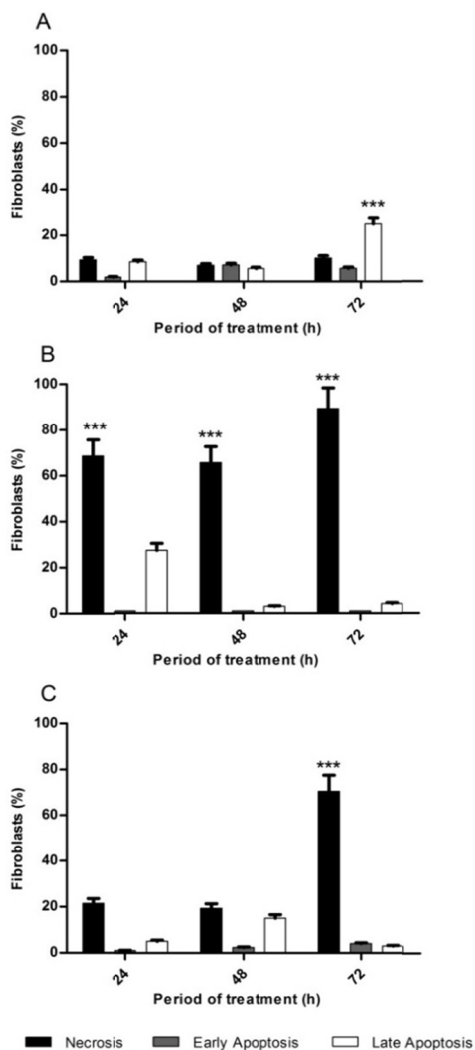
Figure 1.1. Detection of Annexin V/PI by flow cytometry in fibroblasts treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus. Bar graphs representing the means (%) and standard deviations (±) of marker expression: (A) control experiment, (B) treated with 0.18μg/μl *P. boraceiensis* mucus, and (C) treated with 0.012μg/μl *P. boraceiensis* mucus. Data are presented as NS (not significant) or significant at *ρ < 0.05, **ρ < 0.005, or ***ρ < 0.0001. FSC, forward scatter
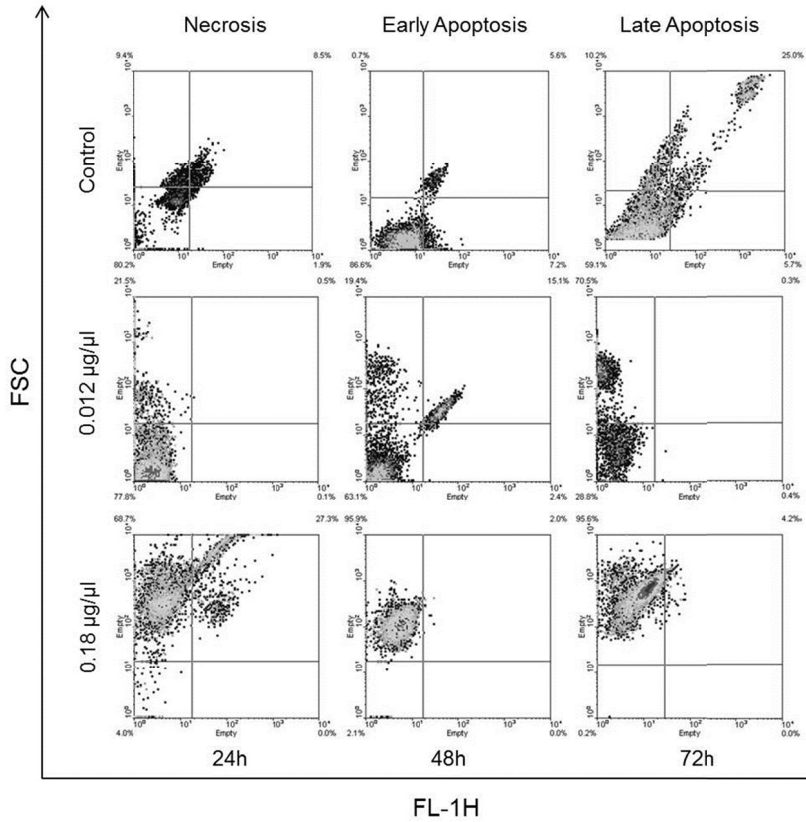
Figure 1.2. Representative histograms of control fibroblasts and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus and stained with Annexin V/PI
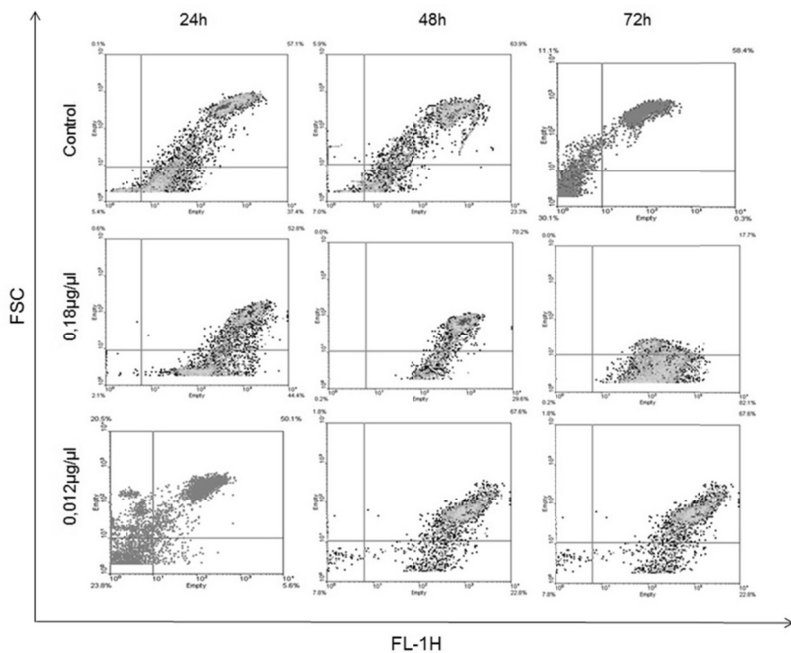
Figure 1.3. Representative histograms of detection by flow cytometry of control fibroblasts and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus and stained with Bcl2
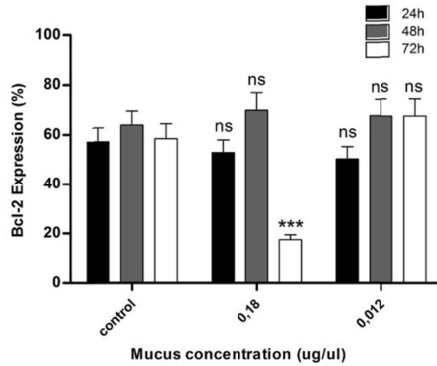
Figure 1.4. Detection of Bcl2 by flow cytometry of fibroblasts treated with 0.18 and 0.012μg/μl *P. boraceiensis* mucus. Bar graphs represent the means (%) and standard deviations (±) of marker expression. The data are presented as NS (not significant) or significant at *$\rho < 0.05$, **$\rho < 0.005$, or ***$\rho < 0.0001$. FSC, forward scatter

Figure 1.5. Detection of Caspase-3 by flow cytometry of fibroblasts treated with 0.18 and 0.012μg/μl *P. boraceiensis* mucus. Bar graphs represent the means (%) and standard deviations (±) of marker expression. The data are presented as NS (not significant) or significant at *$\rho < 0.05$, **$\rho < 0.005$, or ***$\rho < 0.0001$. FSC, forward scatter
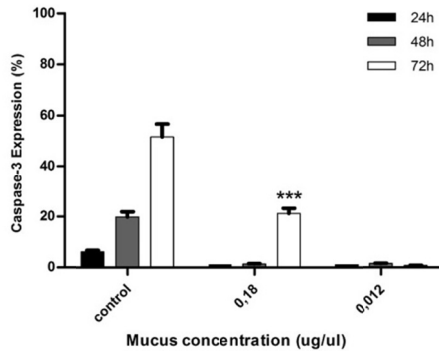
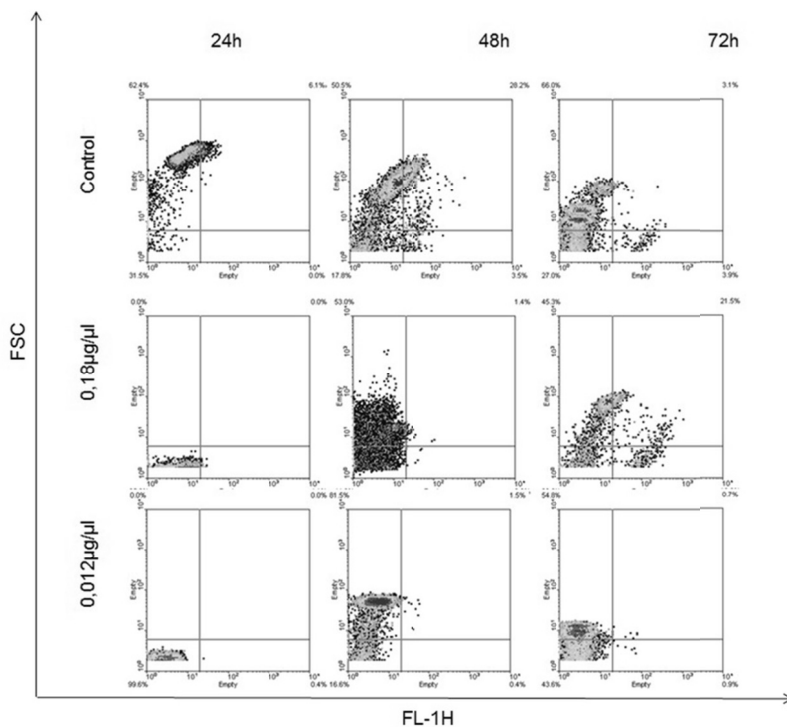**Figure 1.6.** Representative histograms of detection by flow cytometry of control fibroblasts and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus and stained with Caspase-3

Figure 1.7. Representative histograms of control endothelial cells and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus and stained with Annexin V/PI

Figure 1.8. Detection of Annexin V/PI by flow cytometry of endothelial cells treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus. Bar graphs represent the means (%) and standard deviations (±) of marker expression: (A) control experiment, (B) treated with 0.18μg/μl *P. boraceiensis* mucus, and (C) treated with 0.012μg/μl *P. boraceiensis* mucus. The data are presented as NS (not significant) or significant at *$\rho < 0.05$, **$\rho < 0.005$, or ***$\rho < 0.0001$. FSC, forward scatter

Figure 1.9. Representative histograms of detection by flow cytometry of control endothelial cells and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus and stained with Bcl-2
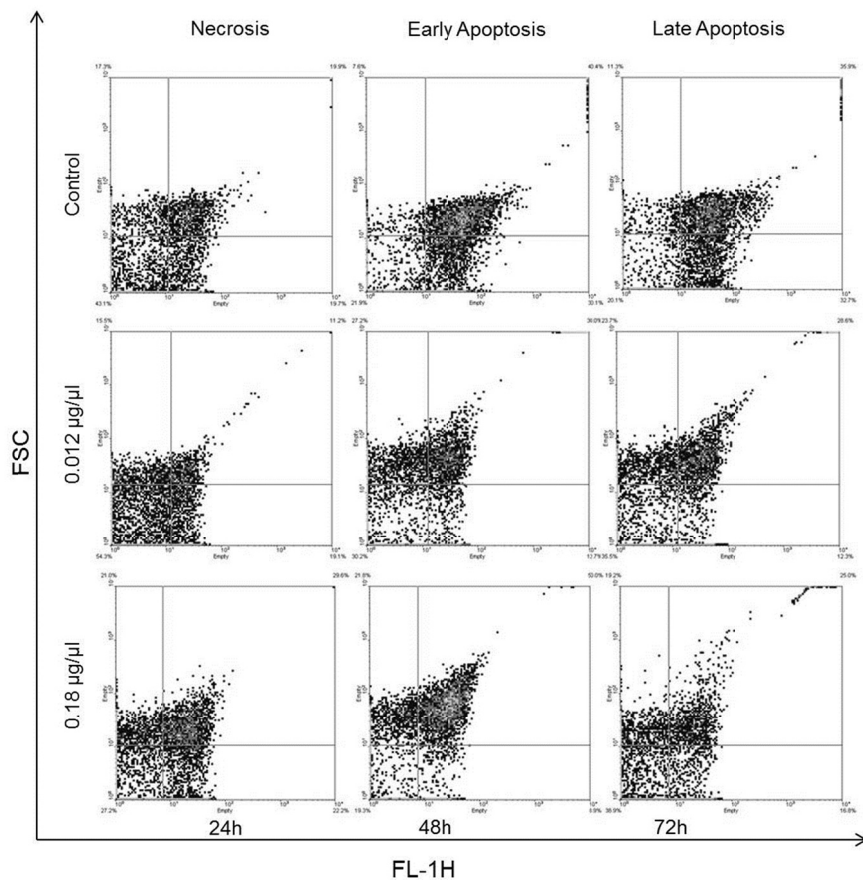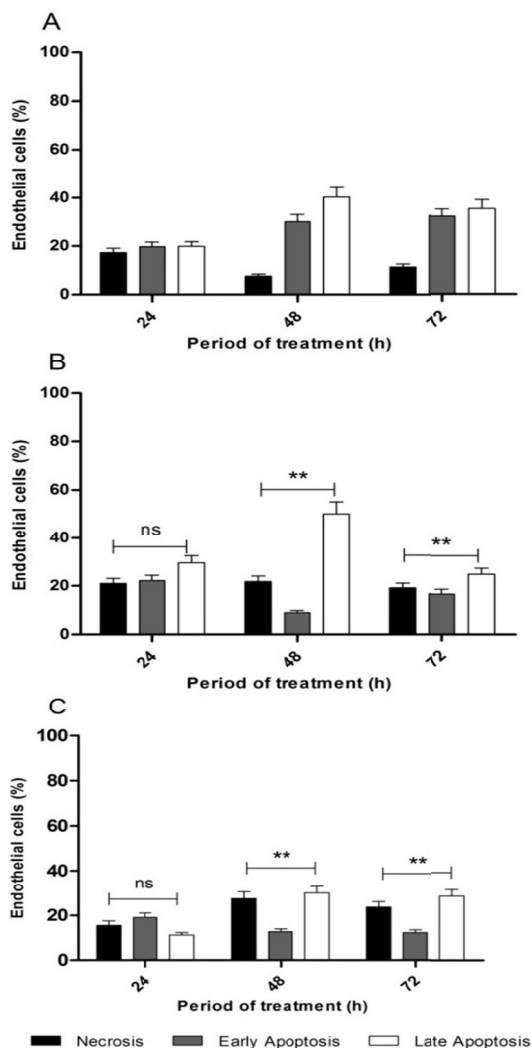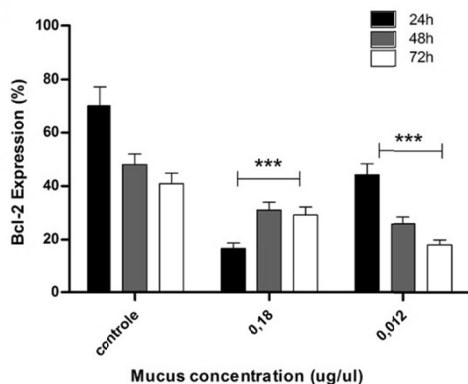
Figure 1.10. Detection of Bcl-2 by flow cytometry of control endothelial cells and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus. Bar graphs represent the means (%) and standard deviations (±) of marker expression. The data are presented as NS (not significant) or significant at *$\rho <$ 0.05, **$\rho < 0.005$, or ***$\rho < 0.0001$. FSC, forward scatter
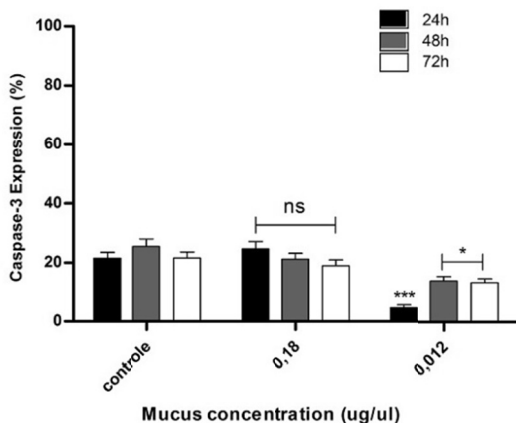
Figure 1.11. Detection of Caspase-3 by flow cytometry of control endothelial cells and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus. Bar graphs represent the means (%) and standard deviations (±) of marker expression. The data are presented as NS (not significant) or significant at *$\rho < 0.05$, **$\rho < 0.005$, or ***$\rho < 0.0001$. FSC, forward scatter
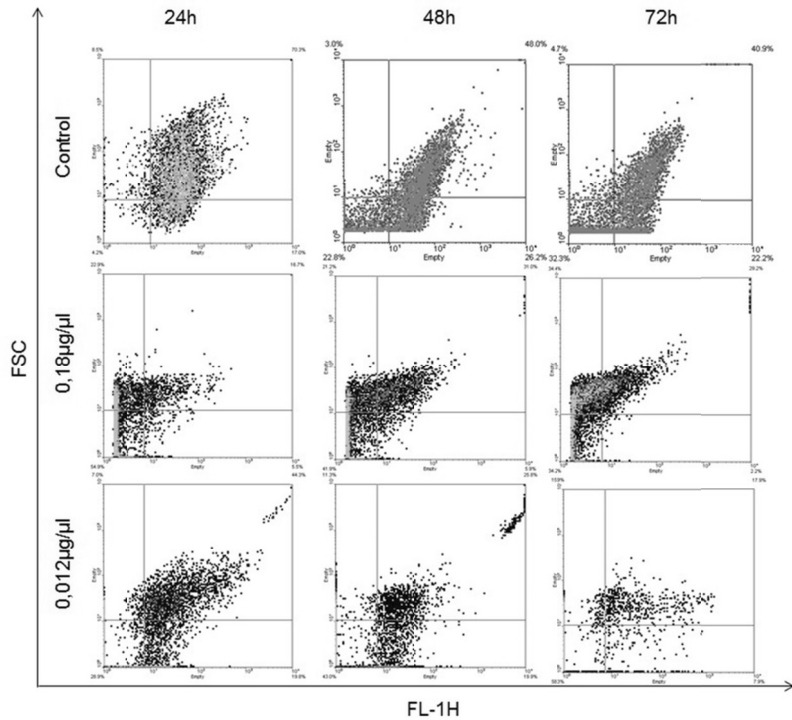
Figure 1.12. Representative histograms of detection by flow cytometry of control endothelial cells and those treated with 0.18 and 0.012μg/μl *Phyllocaulis boraceiensis* (Thomé, 1972) mucus and stained with Caspase-3
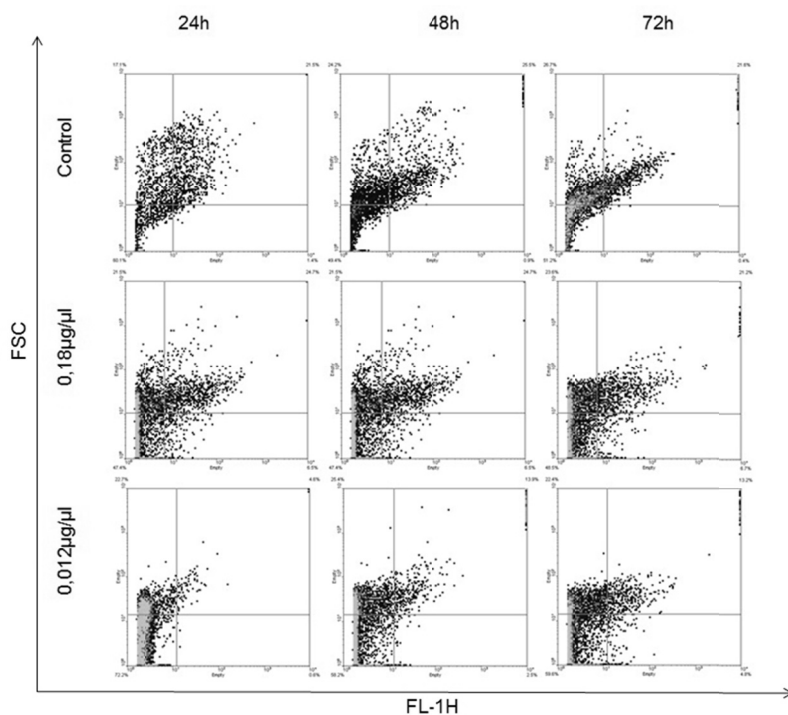
Discussion

Apoptosis is an active mechanism of cell death which occurs under genetic control and involves a highly regulated program (Mendonça *et al.* 2002). This process is also used to determine the percentage of cell death after exposure to external conditions such as the presence of a drug in the culture medium.

Studies of the actions of pro- and anti-apoptotic compounds are used in public health to compile accurate figures about their effects and possible damage to humans. This study aimed to demonstrate the apoptotic and/or necrotic effects of *P. boraceiensis* mucus, a natural product widely studied for its potential application as an ointment for facilitating wound healing (Toledo-Piza and Maria *in press*). This same compound was used in angiogenesis experiments performed on *in vitro* endothelial cells (Toledo-Piza and Maria 2013).

Formulations containing the mucus of *H. aspersa* accelerate the apoptosis process in dermis tissues damaged by surface burns and also promote epithelialization of partial deep burns (Tsoutsos *et al.* 2009).

The long-term oxidative stress caused by the overproduction of free radicals can induce necrosis. For example, the high reactivity of the hydroxyl radical is considered one of the most powerful drivers of necrotic cell death. Free radicals play a major role in determining redox status in the apoptosis process, and also in many forms of apoptosis induction that involve reactive oxygen species and nitrogen. Some antioxidants, such as tocopherol and carotenes, glutathione peroxidase, superoxide dismutase, and DNA repair mechanisms, have been used as defences, minimizing the deleterious effects of free radicals and reducing apoptosis (Harman 2001; Pollack and Leeuwenburgh 2001). Under this assumption, we calculated the production of free radicals by *P. boraceiensis* mucus, and our results showed there is a significant decrease in the rate of free radicals produced by fibroblasts and endothelial cells when treated with this compound (Toledo-Piza *et al.* 2013).

The cells that express phosphatidylserine on the outer surface of their membranes bind Annexin V, and cells with compromised membranes allow propidium iodide to bind to the cellular DNA. The resulting cells, when immediately analysed by flow cytometry, belonged to one of four groups: living cells unlabelled by a fluorochrome, necrotic cells labelled only with PI, cells in early apoptosis that are labelled only with FITC-labelled Annexin V and double-labelled cells (propidium iodide and Annexin V) in late stage apoptosis (Faiao-Flores *et al.* 2013). Our results showed that most fibroblasts were in the late stage of apoptosis.

Mitochondria play a key role in the activation of apoptosis in mammalian cells. Members of the Bcl-2 family regulate the release of proteins from the space between the inner and outer mitochondrial membranes. Once in the cytosol, these proteins activate caspase proteases, which initiate cellular destruction and signal cells for phagocytosis (Wang and Youle 2009).

This process involves mitochondrial pro-apoptotic members of the Bcl-2 family, which regulate the suppression of apoptosis in various cellular systems: cell death is regulated by controlling the permeability of the mitochondrial membrane and functions in a feedback cycle with Caspase-3. The Caspase-3 marker is one of the proteins involved in the cascade of sequential activation of caspases, and plays a central role in the implementation phase of apoptosis. The caspases reside in the cell in inactive pro-forms and, in most cases, are activated by proteolytic cleavage. This activation involves the removal of the pro-domain and the amino-terminal cleavage of the remaining polypeptide, resulting in a large subunit and a small subunit which together form the functional enzyme. The majority of activation via "executioner caspases" takes place following the action of other proteases designated "initiator caspases", requiring the help of adapter proteins, which bind to the protein interaction motifs located on the pro-domains. The connection of the adapter therefore promotes the activation of initiator caspases, which can then cleave and activate the executioner caspases (Tsujimoto 1998).

The results of the experiments, performed to determine the expression of cell death markers, showed a significant increase in death by necrosis of human fibroblasts treated with *P. boraceiensis* mucus at a concentration of 0.18μg/μl. Those treated with 0.012μg/μl *P. boraceiensis* mucus showed a significant increase only after 72 hours of treatment. Endothelial cells treated with the same concentrations also showed an increase in the number of cells undergoing apoptosis.

It was suggested that we examine the ultrastructure of cells to evaluate morphological changes (Mendonça *et al.* 2009), so in a supplemental study (Toledo-Piza *et al.* 2013) we evaluated the cell cycle phases of these same cell lines when subjected to various concentrations of *Phyllocaulis boraceiensis* mucus. At cytotoxic concentrations, cell death was observed and the cells' morphological changes were monitored by scanning electron microscopy. However, it is not possible to infer whether the changes were due to apoptosis or necrosis.

In the future, these experiments will be reproduced using tumour cells in order to evaluate their potential for apoptotis.

Conclusion

Phyllocaulis boraceiensis mucus is a natural compound that, in cytotoxic concentrations, can induce significant apoptosis (Toledo-Piza *et al.* 2013) in fibroblasts. These results support the premise that, at non-cytotoxic concentrations, the compound can induce cell proliferation accompanied by a physiological cell death without any injury to the cell or to the organism as a whole.

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