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*Original Article*

# APOPTOTIC ACTIVITY OF BOSWELLIA CARTERII EXTRACT ON HUMAN NEUTROPHILS.

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## Abstract

**Background:** Neutrophil activation is required for the initiation of the defence mechanisms which include phagocytosis. Paradoxically, neutrophils also represent one of the main mediators of tissue injury in various human diseases. The resolution of inflammation requires getting rid of excess inflammatory cells through natural cell death and phagocyte clearance. Frankincense, an oleogum resin of different species of the genus *Boswellia* (*Burseraceae*), has long been used in eastern countries' traditional medicine to alleviate pain and inflammation. Although it was demonstrated that boswellic acids are potent activators of polymorphonuclear cells, little is known about the effects of the total extract on the human phagocytes' apoptosis.
**Aims:** To undertake a characterization of the *Boswellia carteri* resin extract (BCE) effects on human neutrophils activity and viability in vitro.
**Methods:** Oxidative burst after stimulation with BCE was evaluated by reduction of nitroblue tetrazolium (NBT) colorimetric method for superoxide anion radical in the the presence of different compounds (N-ethyl maleimide, diltiazem, chelerythrine and wortmannin). Neutrophils viability was assessed monitoring the uptake of the vital mitochondrial dye 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT). Flow analyses were performed on neutrophils stained with propidium iodide (PI) and annexin V-FITC.
**Results:** our results show that BCE induces the release reactive species of oxygen in human neutrophils in a dose dependent manner. The superoxide anion radical is principally produced via NADPH oxidase since inhibitors of the enzyme may prevent it. $Ca^{+2}$ depletion reduce the magnitude of activation and PKC and PI3-K are also apparently involved in the process. The BCE has also cytotoxic activity revealed by the MTT assay. This effect seems to be produced by an apoptotic process as shown by the exclusion of the dye propidium iodide and the annexin V-FITC binding in neutrphils.
**Conclusion:** The capacity of the *Boswellia carterii* extract to accelerate the cellular death by an apoptotic process suggest that either the extract or its active compounds could have applications for the resolution of some inflammatory conditions.

## INTRODUCTION

Neutrophils are the most abundant circulating leukocytes and play a critical role in the inflammatory process. These cells are rapidly recruited to sites of infection or lesion at the onset of the process, where they act as the first line of defence against invading pathogens. Neutrophil activation is required for the initiation of the defence mechanisms which include phagocytosis. Invading agents are destroyed by a combination of reactive oxygen species (ROS) generated by the NADPH oxidase system and release of various microbicidal molecules by degranulation and production of pro-inflammatory cytokines (Nathan, 2006). In addition, ROS may induce apoptosis (Simon et al, 2000).

Paradoxically, neutrophils also represent one of the main mediators of tissue injury in various human diseases (Abraham, 2003; Tsukamoto et al, 2010). Under normal conditions, the lifespan of these cells is very short in the circulating blood because they are obliged to undergo programmed cell death or apoptosis that is regulated by intrinsic and extrinsic mechanisms. (Akgul et al, 2001; Taylor et al, 2007). However, during acute inflammation neutrophil life span becomes significantly extended due to the action of proinflammatory mediators and bacterial membrane components. This neutrophil survival is associated with the accumulation of activated neutrophils contributing to ongoing inflammation and consecutive host tissue damage.

The resolution of inflammation requires getting rid of excess inflammatory cells through natural cell death and phagocyte clearance. This process can be influenced by different factors that affect how neutrophils, a type of inflammatory cell, die and are removed. Some of these factors are lipids, proteins, and inhibitors that come from essential fatty acids, autacoids, and cyclins. They can enhance the resolution of inflammation by triggering neutrophil apoptosis and facilitating their efferocytosis (Dade et al, 2016; El Kebir & Filep, 2013; Filep & El Kebir, 2009; Hallett et al, 2008; Savill, 2000).

Frankincense, an oleogum resin of different species of the genus *Boswellia* (*Burseraceae*), has long been used in eastern countries' traditional medicine to alleviate pain and inflammation (Moussaieff & Mechoulam, 2009). The resins from both *B. serrata* and *B carterii* trees and its derivatives, particularly the boswellic acids, have shown various biological activities, including inhibitory activity of leukotriene biosynthesis and antitumor activity (Singh et al, 2008). They also exhibited an immunomodulatory activity of human lymphocytes (Poeckel & Werz, 2006) and inhibited the growth of different leukemic cells lines (Glaser et al, 1999)

The mechanism of this action is due to some boswellic acids and is different to other NSAIDs in that they do not target cycloxygenases but inhibit 5-lipoxygenase (Safayhi et al, 1992). However, other factors such as cytokines (interleukins and TNF-alpha), the complement system, leukocyte elastase and oxygen radicals are also biological targets (Ammon, 2006). Although it was demonstrated that boswellic acids are potent activators of polymorphonuclear cells (Altmann et al, 2004), little is known about the effects of the total extract on the human phagocytes' apoptosis. The ethnomedicinal importance of *Boswellia carteri* resin (Moussaieff & Mechoulam, 2009) and the pivotal role of neutrophils clearance in the resolution of inflammation prompted us to undertake a characterization of the BCE in vitro effects on the human activity and viability of neutrophils.

## MATERIAL AND METHODS

### *Boswellia carterii* extracts

*Boswellia serrata* (Burseraceaea) resin was obtained from H15© tablets (Gufic Chemicals, India). They consist of a standardised extract rich in the 5-LOX inhibitor acetyl-11-keto-β-boswellic acid (Glaser et al, 1999). The tablets were powdered using a mortar and pestle and extracted with absolute ethanol. Excipients were removed by centrifugation and the clear supernatant taken for experiments.

### Isolation of human neutrophils

Neutrophils were isolated from fresh anti-coagulated peripheral blood obtained from healthy volunteers, then purified using a standard protocol. Briefly, erythrocytes were sedimented by adding an equal volume of dextran/saline solution (3% dextran T-500 in 0.9% NaCl) at room temperature for 30 min. The leukocyte-rich upper layer of the suspension was then collected and centrifuged on a density gradient with

Histopaque®-1077 (Sigma-Aldrich) according to manufacturer's instructions. Residual erythrocytes were removed by hypotonic lysis (cold water). Neutrophils were washed twice in phosphate-buffered saline (PBS) pH 7.4, then resuspended in the same buffer containing 1 mg/mL glucose, 0.4 mM Mg2+, and 1.20 mM Ca2+. Cell viability was determined by the trypan blue dye exclusion method and always exceeded 98% (Dade et al, 2016).

### Superoxide production

Oxidative burst was evaluated by reduction of nitroblue tetrazolium (NBT) colorimetric method for superoxide anion radical ($O^{2-}$) (Montesinos et al, 1995). Cells, ($2.5\times10^6$) were incubated for 30 minutes in 0,05% NBT in PBS-glucose pH 7.4 in the presence of BCE (10-200 µg/ml). After centrifugation, the formazan crystals were dissolved in DMSO: HCl (95:5), left overnight, and the absorbance was determined at 560 nm in a plate reader. TPA was used as a reference compound. For mechanistic studies on $O^{2-}$ release the neutrophils were pre-incubated for 30 minutes in the presence of different compounds: N-ethyl maleimide, (NEM) (50 µM), diltiazem (0.5 mM), chelerythrine (20 µM) and wortmannin (150 nM) prior to addition of NBT. The results were expressed as ΔAbsorbance / $2.5\times10^6$ cells.

### Mitochondrial viability assay

Neutrophils viability was assessed monitoring the uptake of the vital mitochondrial dye 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT). The assay is based on the reduction of the dye by mitochondrial dehydrogenase to a purple formazan product (Mosmann, 1983). For the assay, $2,5\times10^6$ cells/tube were treated with different concentrations of BC extract at different times. DMSO was used as control. MTT dye solution was added to each tube (final concentration 0, 5 mg/ml in PBS). The tubes were incubated at $37^0$C in the dark for 4 h. Formazan produced by PMN cells in each tube was dissolved in solubilization solution (10% SDS in 0,01 M HCl) and the tubes were incubated overnight. The solubilized formazan product was quantified by absorbance at 570 nm, with 630 nm-reference filter.

### Flow cytometry analyses

Propidium iodide (PI) staining was assessed according to Riccardi & Nicoletti (2006) with slight modifications. Aliquots of $2.5\times10^6$ cells/tube were suspended in ice-cold 70% ethanol and stored at –20°C for at least 30 min, then the cells were washed twice in PBS at 4°C and resuspended in 500 µl of DNA staining solution (20 µg/ml PI plus 0.2 mg/ml RNAse A in PBS). The cells were incubated in the dark at room temperature for 30 min and the fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson). A minimum of 20,000 events were counted per sample. The percentage of apoptotic cell nuclei (hypodiploid DNA peak) was calculated.

Phosphatidylserine (PS) exposure was measured by the binding of annexin V-FITC. After the appropriate incubations with BCE, cells ($2.5\times10^6$) were washed twice with cold PBS and then resuspended in binding Buffer (10 mM Hepes/NaOH pH 7.4,140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of $1\times10^6$ cells/ml. Then, $1\times10^5$ cells were transferred to a 5 ml culture tube, 5 µl of Annexin V-FITC and 10 µl of PI solution (50 µg/ml in PBS) were added to the cells and incubated for 15 min at room temperature in the dark. After incubation, 400 µl of binding Buffer were added to each tube and analyzed by flow cytometry (Schinella et al, 2008).

### Statistical analysis

Results are expressed as means ± SD for the indicated number of observations or illustrated by an observation representative of results from several different experiments. Statistical analysis used ANOVA followed by Dunnett's t-test for multiple comparisons using the Prism analysis program (GraphPad). Differences were considered significant when $P<0.05$.

## RESULTS AND DISCUSSION

Phagocytosis is considered one of the most important processes during the innate immune response against bacterial and fungal infections. Phagocytosis of such microbes at the infection site triggers the activation of several neutrophil functions, such as the release of proteases, bactericidal peptides and reactive oxygen species (ROS). ROS production, i.e., oxidative burst, is initiated by the generation of $O_2^{\cdot-}$ by the phagocyte NADPH oxidase (Belambri et al, 2018; Nguyen et al, 2017).

The effect of *Boswellia carterii* extract on neutrophils activation (release of ROS) and cytotoxicity was assayed in vitro using different concentrations of extract (10-200 µg/ml). In our experiments BCE activated the PMNs dose-dependently in the 50 -200 µg/ml range as measured by NBT reduction (Figure 1).

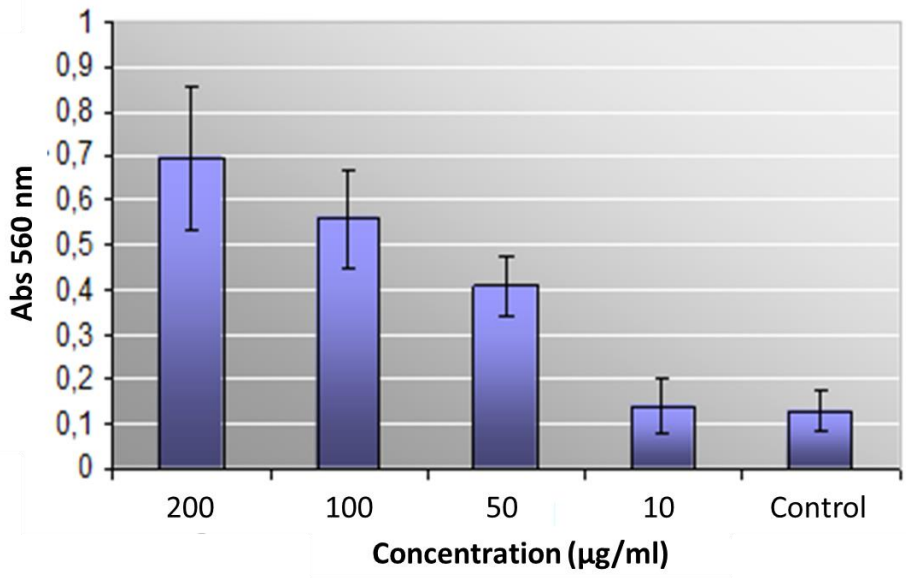


**Figure 1. Effect of *B. carterii* ethanolic extract on the production of ROS in PMNs as measured by NBT reduction.**

In order to clarify the mechanisms of action of the stimulatory effect of BCE at the fixed concentration of 100 µg/ml we pre/co-incubated the cells with several drugs (Figure 2).

Preincubation of cells with NEM inhibited de oxidative burst in a very high percentage confirming that NADPH oxidase -the major source of ROS in human neutrophils- is indeed involved in the stimulatory effect of BCE.

Calcium Channel Blockers such nifedipine and diltiazem are known to regulate some plasma membrane $Ca^{2+}$ channels in excitable cells, can also regulate $Ca^{2+}$ release from intracellular stores in PMN and that this regulation may have significant effects on PMN function (Rosales & Brown, 1992).

As is known, PMN activation is a $Ca^{2+}$-dependent process and boswellic acids were previously shown to stimulate signaling pathways that control functional responses of leukocytes, including the formation of ROS, among others (Altmann et al, 2004). Our results show that BCE induce a oxidative burst in human neutrophils and that this effect is inhibited by diltiazem, a compound that is able to regulate the release of calcium from intracellular stores.

Neutrophil activation is accompanied by the activation of many other protein kinases such as PKC, and PI3K. The activated kinases phosphorylate several proteins with important cellular functions in neutrophils, including the NADPH oxidase components (Belambri et al, 2018). We also investigated the role of PKC and PI3-K in the BCE-induced neutrophils activation by pre-incubation of cells in the presence of cheleritryne, a PKC inhibitor, or wortmannin, a PI3-K inhibitor. They both reduced ROS production by 50% and 66% respectively, suggesting that PKC and PI3-K are involved in the activation of NADPH oxidase by BCE.

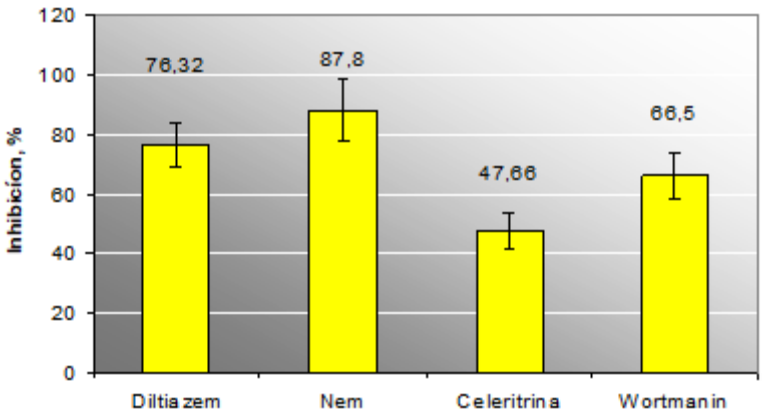


**Figure 2. Effect of *B. carterii* ethanolic extract (100 µg/ml) on the production of ROS in PMNs in the presence of selected inhibitors.**

The MTT assay revealed that BCE decreased the mitochondrial viability of PMNs in a time and dose-dependent manner (Figure 3). A concentration of 100 µg/ml produced the death of approximately 80% of the cells after 3 hours of incubation. Using flow cytometry, we determined the PMN cell membrane integrity by exclusion of propidium iodide in presence and absence of BCE.

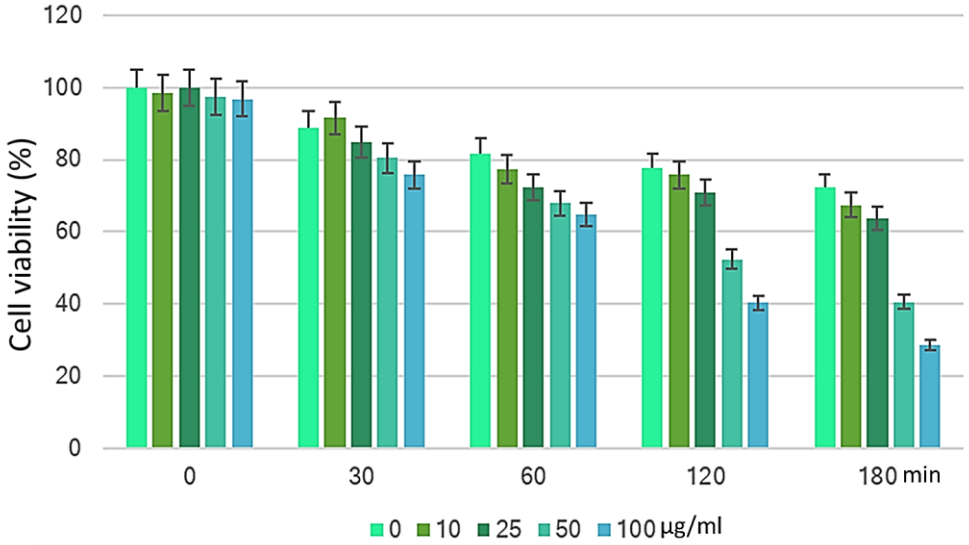


**Figure 3. Effect of *B. carterii* ethanolic extract on the mitochondrial viability of PMNs.**

The nature of the cytotoxicity was evaluated with different flow cytometry methods, including the development of hypodiploid nuclei by propidium iodide and the exposure of membrane phosphatidylserine through Annexin V-FITC binding.

Apoptotic nuclei were analyzed by PI staining. In our work, PMNs were incubated with increasing concentrations of BCE (10-100 µg/ml) at different times (30-180 minutes). The cell population in the sub G1 region represents cells with hypodiploidal DNA, an indicator of apoptosis. When cells were incubated with 100 µg/ml of BCE, the cell population in the sub-G1 region increased, suggesting a time-dependent increase in the population of apoptotic cells (Figure 4A).

The results in Figure 4B show the magnitude of the BCE concentration-dependent changes in the percentage of cell population with hypodiploid DNA. These results show the decrease in cytoplasmic volume and chromatin condensation that are early events of the apoptotic process (Elmore, 2007).

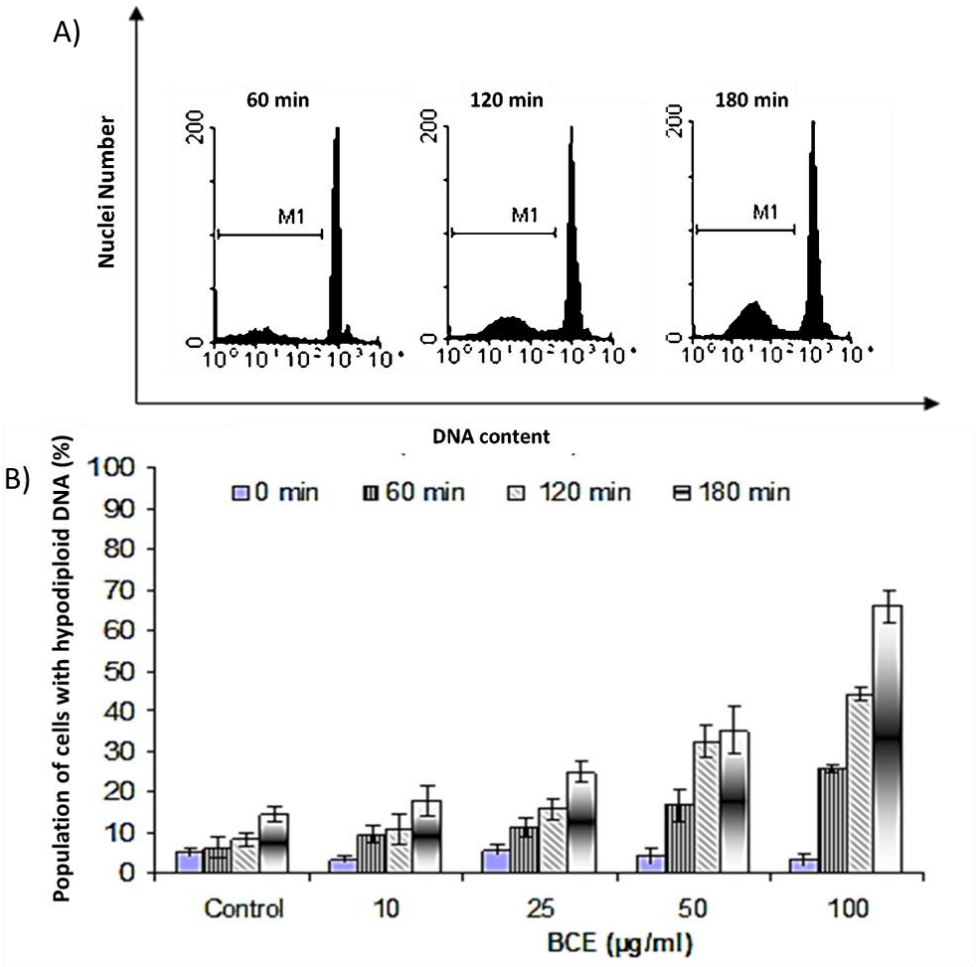


**Fig. 4: Promotion of neutrophils apoptosis by BCE. A) Typical histograms showing cells in sub-G1 phase indicating the range of cells with hypodiploid DNA (M1). B) Population of cells with hypodiploid DNA (%) treated with *B. carterii* ethanol extract (10-100 µg/ml) during 60-, 120- and 180-min. Bars represent Mean±SD, n=3.**

Early events during the process of apoptosis are characterized by the loss of membrane phospholipids asymmetry. The translocation of phosphatidylserine to the outer plasma membrane takes place long before the classical appearance of DNA fragmentation. In our experiments, the ability of BCE to induce apoptosis was also analyzed by flow cytometry using a double labeling technique with Annexin-V and PI to distinguish between apoptotic and necrotic cells. When the cells were incubated in the presence of BCE at a concentration of 100 µg/ml during 60 min, a significant number underwent apoptosis when compared to untreated cells (An+/ PI-: 92 ± 4% vs. 3 ± 1% of control, $P<0.05$) (Figure 5).

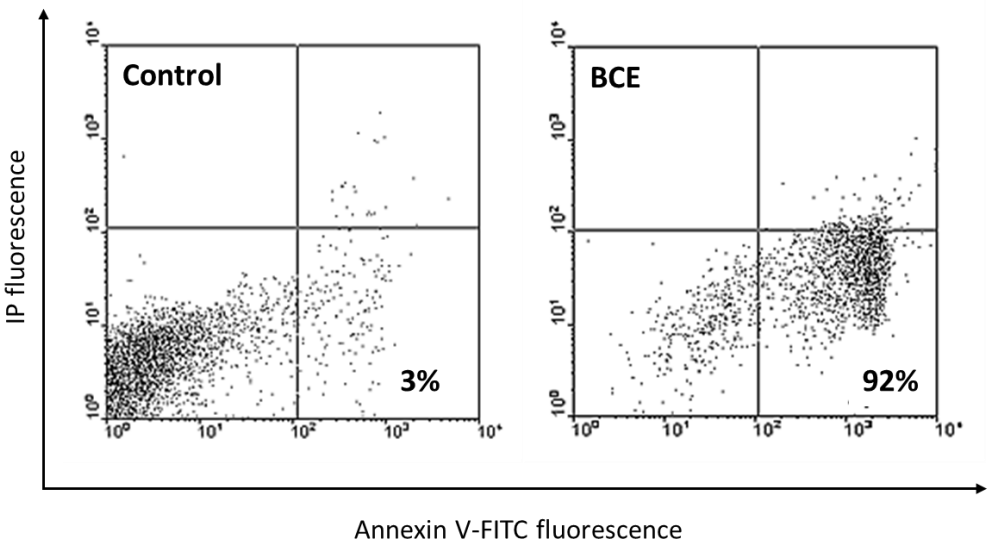


**Fig. 5. Representative dot plots of annexin V/propidium iodide (PI) staining are shown. The lower left quadrant contains the vital (double negative) population. The lower right quadrant contains the apoptotic (annexin V+/PI–) population. Finally, cells in the top right quadrant (annexin V+/PI+) are in later stages of apoptosis and necrosis. Graph shows a representative effect of the *Boswellia carterii* extract (BCE) at 100 µg/ml incubated during 180 min, n=3.**

Neutrophil apoptosis is essential for inflammation resolution and clearance of effector cells. Various agents modulate this process through diverse receptor-signalling pathways. NADPH oxidase activation and ROS generation potentiate neutrophil apoptosis. Depending on ROS level and location, NADPH oxidase can also induce necrosis or survival of neutrophils upon different stimuli. (El Kebir & Filep, 2013; Filep, 2022; Filep & El Kebir, 2009; Geering & Simon, 2011; Zeng et al, 2019)

This study demonstrates that BCE activated PMN inducing an increase in the generation of $O_2^{\cdot-}$ by NADPH oxidase and is inhibited by diltiazem, a compound that is capable of regulating the release of calcium from intracellular stores. Furthermore, our data show that the activation of PKC and PI3-K dependent pathways are involved. The ability of *Boswellia carterii* extract to accelerate cell death through an apoptotic process, this process could be carried out through multiple and complex receptor signaling pathways. Although the results of this research are promising, more studies are necessary to demonstrate its efficacy and safety “in vivo.”

## CONCLUSION

Taken together our results permit to asseverate that the extract of *Boswellia carterii* is able to induce the release of reactive species of oxygen in human neutrophils in a dose-dependent manner. The superoxide anion radical is principally produced by NADPH oxidase since inhibitors of the enzyme may prevent it. $Ca^{+2}$ depletion reduce the magnitude of activation and PKC and PI3-K are also apparently involved in the process.

The BCE has also cytotoxic activity revealed by the MTT assay, and this effect seems to be produced by an apoptotic process. The apoptotic effect was demonstrated by the exclusion of the dye propidium iodide and the annexin V-FITC binding.

The capacity of the *Boswellia carterii* extract to induce an oxidative burst that may accelerate the cellular death by an apoptotic process suggests that either the extract or its active compounds may have applications for the resolution of inflammation in some clinical conditions.

### Conflicts of Interest

The authors declare no personal or financial conflict of interest related to this work.

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This article is dedicated to Horacio Tournier on the occasion of his retirement. His dedication to pharmacology and his kindness inspired us.

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**Authors contribution**

Conceptualization GS, JMP; MD, GS; Validation MD, JMP, FF, GS; Methodology, Investigation, Formal analysis, Visualization MD, GS; Resources JMP, FF, GS; Writing - Original Draft: JMP, GS; Writing - Review & Editing JMP, FF, GS; Supervision JMP, GS; Project administration: GS; Funding acquisition: FF, GS.

**Supplementary materials**

Authors did not provide Supplementary Materials.

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