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# Antimicrobial Activity and Nutraceutical Potential of Tuscan Bee-Pollens on Oxidative and Endoplasmic Reticulum Stress in Different Cell-Based Models †

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**Abstract:** Bee-pollen is an apiary product of great interest owing to its high nutritional and therapeutic properties. This study aimed to assess the cellular antioxidant activity and the anti-hemolytic effects of *Castanea, Rubus,* and *Cistus* bee-pollens on oxidized human erythrocytes. Besides, the antimicrobial potential of each sample was tested on three Gram-negative and two Gram-positive bacteria. Finally, the effect of *Castanea* bee-pollen, showing better phytochemical content, was analyzed on human microvascular endothelial cells (HMEC-1) exposed to thapsigargin, used to induce endoplasmic reticulum stress (ER-stress). Our results showed good biological activities of all bee-pollen samples that, under oxidative conditions, significantly improved the erythrocytes antioxidant activity and limited cell lyses. Moreover, all samples exerted antimicrobial activity with different selectivity among tested microorganisms with minimal inhibitory concentration values ranging from 5 to 10 mg/mL. Finally, thapsigargin treatment increased the intracellular ROS production and up-regulated the expression of factors involved in the ER-stress and inflammatory pathway. Conversely, *Castanea* bee-pollen was effective in reducing gene over-expression as well as the oxidation process arising from thapsigargin treatment, with maximum protective effect at 10 μg/mL. In conclusion, bee-pollens, mainly *Castanea* species, represent good natural antibacterial and potential nutraceutical products useful in the prevention of free radical and ER-stress associated diseases.

**Keywords:** nutraceutical; bee-pollen; antioxidant and anti-hemolytic effect; CAA-RBC; antimicrobial activity; MIC; HMEC-1; ER-stress

## 1. Introduction

Apicultural products have been used for centuries in alternative medicine, in diets, or as dietary supplementation for their health and positive implications. Among others, bee-pollen is an apiary product that is receiving great attention as a functional food for its high nutritional value and therapeutic properties including antioxidant, anti-inflammatory, antimicrobial, antifungal, anti-mutagenic, and antitumor effects; besides, bee-pollen is an important source of energy, bioactive compounds and proteins for human nutrition [1–5]. To the best of our knowledge, no data on bee-pollen effects on endoplasmic reticulum stress are available in the literature.

This study aimed to assess, on oxidized human erythrocytes, the antioxidant activity and the anti-hemolytic effects of *Castanea, Rubus*, and *Cistus* bee-pollens by CAA-RBC (Cellular Antioxidant Activity in Red Blood Cells) and hemolysis assays. Besides, we tested the antimicrobial activity, expressed as the minimum inhibitory concentration (MIC), of each pollen sample on three Gram-

negative (*Enterobacter aerogenes, Escherichia coli*, and *Salmonella enterica* ser. *Typhimurium*) and two Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) strains. Finally, we analyzed the effects of *Castanea* bee-pollen, having the highest phytochemicals content, on the functional properties of human microvascular endothelial cells (HMEC-1) exposed to thapsigargin, a plant-derived sesquiterpene lactone used to induce endoplasmic reticulum (ER) stress.

## 2. Materials and Methods

### *2.1. Cellular Antioxidant Activity (CAA) and Erythrocytes Oxidative Hemolysis*

Bee-pollen samples (50 mg/mL) were extracted in 95% ethanol according to Barbieri et al. [6]. The cellular antioxidant activity of 100 μg/mL bee-pollen extracts and the anti-hemolytic properties of increasing concentrations (20, 50, 100, and 200 μg/mL) of bee-pollen extracts were detected ex vivo on oxidized human erythrocytes as previously described by Frassinetti et al. [7].

### *2.2. Minimum Inhibitory Concentration*

The minimum inhibitory concentration (MIC) of increasing concentration of ethanolic bee-pollen samples (range 0.01–1 mg/mL) was determined according to Frassinetti et al. [8] on selected pathogenic bacteria, mainly three Gram-negative (*Enterobacter aerogenes, Escherichia coli*, and *Salmonella enterica* ser. *Typhimurium*) and two Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) strains. The lowest concentration of bee-pollen extracts able to inhibit the microorganisms' growth was defined as the MIC value.

### *2.3. Human Microvascular Endothelial Cells (HMEC-1) Treatment*

Cells were grown according to Gabriele et al. [9]. The *Castanea* ethanolic extract was lyophilized under vacuum, resuspended in DMSO 0.1% in water, and used on HMEC-1 cell culture. Following 1 h pre-treatment with increasing concentration of *Castanea* bee-pollen (1, 10, 100, and 200 μg/mL), HMEC-1 were stimulated for 2 h with or without 0.3 μM thapsigargin. The cell viability was carried out by MTT assay as previously described [10].

### *2.4. Gene Expression and ROS Production*

Quantitative Real-Time PCR was performed using the SsoFastTM EvaGreen® Supermix (Bio-Rad, CA) in a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA). Samples were assayed in triplicate and the gene expression was calculated by the $2 - \Delta\Delta CT$ relative quantification method. The β-actin was used as the housekeeping gene.

Cellular reactive oxygen species (ROS) were detected using the 2′-7′dichlorodihydrofluorescein diacetate (DCFH-DA) as previously described [11].

### *2.5. Statistical Analysis*

Results were expressed as mean ± standard deviation (SD) of at least three replicates. Differences between bee-pollen samples were examined by one-way analysis of variance (ANOVA) with Tukey post hoc test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). A $p < 0.05$ was considered as statistically significant.

## 3. Results and Discussion

In a previous study, we investigated the botanical origin, the chemical and antioxidant compounds profile, as well as the free-radical scavenging activity of polyfloral Tuscan bee-pollen composed by three botanical species, specifically *Castanea* sp., *Rubus* sp., and *Cistus* sp. A strong in vitro antioxidant activity has been highlighted and, among them, *Castanea* bee-pollen showed the highest phytochemicals content [12].

In the present study, the antioxidant activities of *Castanea, Rubus*, and *Cistus* ethanolic extracts were screened on human erythrocytes under oxidative condition using the CAA-RBC and the

hemolysis test. Both CAA-RBC and hemolysis tests are based on the use of AAPH, an oxidizing agent whose thermal decomposition in peroxyl radicals causes damage to the erythrocytes membrane through lipids and proteins peroxidation and, at high doses, erythrocytes lysis.

As shown in Figure 1A, our findings revealed that all bee-pollen pre-treatments improved of about 50% the erythrocytes antioxidant activity compared to the control (AAPH-treated cells, CAA = 0; *** $p < 0.001$), with CAA values lower than quercetin (8 μM, ~92%) used a standard. Moreover, no significant differences in the CAA values among all the analyzed pollen types were found.

As shown in Figure 1B, all bee-pollen pre-treatments exerted a dose-dependent hemolysis inhibition compared to AAPH-treated erythrocytes. Besides, our results demonstrated comparable anti-hemolytic activities following *Castanea* and *Cistus* pre-treatment, with higher percentages of hemolysis inhibition than *Rubus* bee-pollen at similar doses. *Castanea* and *Cistus* extract from 50 to 200 μg/mL showed greater anti-hemolytic effects than the trolox used as a standard. These results are probably related to increased levels of polyphenols, flavonoids, and flavonols detected in *Castanea* and *Cistus* bee-pollen than the *Rubus* ones [12].

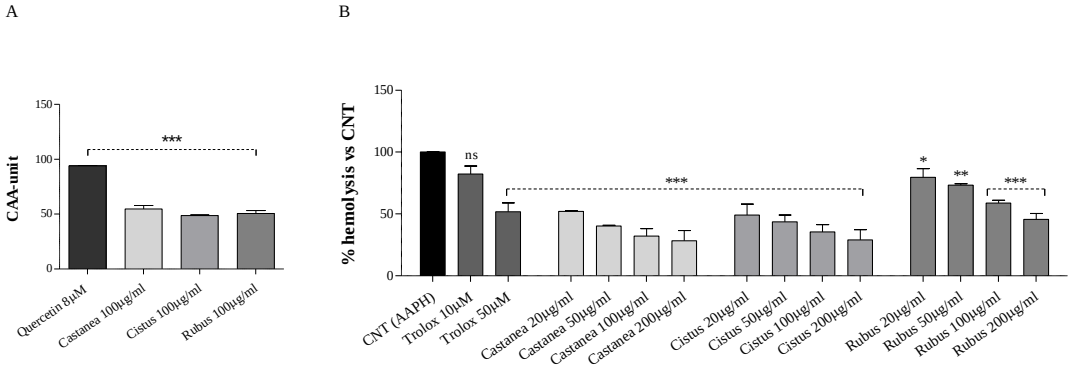


**Figure 1.** (**A**) Effects of *Castanea*, *Cistus,* and *Rubus* bee-pollen extracts (100 μg/mL) on the cellular antioxidant activity (CAA) of oxidized human erythrocytes. Quercetin (8 μM) was used as the reference standard. (**B**) Effects of increasing concentrations (20, 50, 100, and 200 μg/mL) of *Castanea*, *Cistus* and *Rubus* bee-pollen extracts on erythrocytes AAPH-induced oxidative hemolysis. Trolox (10 and 50 μM) was used as a standard. Results were expressed as mean ± SD. One-way ANOVA with Tukey's multiple comparison test: * significantly different from CNT (AAPH-treated cells), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The antibacterial potential of increasing doses of *Castanea, Cistus*, and *Rubus* bee-pollen extracts was tested on selected pathogenic bacterial strains and MIC was used as a parameter of bacterial growth inhibition. All bee-pollen extracts exerted antimicrobial activity with different selectivity among tested microorganisms and MIC values ranging from 5 to 10 mg/mL (Table 1). Our findings revealed that the most sensitive Gram-positive strain *S. aureus* being inhibited at 5 and 10 mg/mL by the *Cistus* and *Castanea*/*Rubus* extract, respectively. While *Cistus* bee-pollen exhibited antibacterial action against all tested bacteria, *Castanea* inhibited selectively *E. coli, S. typhimurium*, and *S. aureus* growth. On the contrary, *Rubus* bee-pollen was effective only on the Gram-positive strains (*S. aureus* and *E. faecalis*) herein tested. Moreover, the Gram-negative strain *E. areogenes* was selectively inhibited only by the *Cistus* bee-pollen.

**Table 1.** Minimum inhibitory concentration (MIC) values of *Castanea, Cistus*, and *Rubus* bee-pollen extracts on selected pathogen strains growth (O.D. 660 nm).

| | ***Minimum Inhibitory Concentration (MIC) Values*** | | |
|---|---|---|---|
| ***Strains*** | ***Castanea*** | ***Cistus*** | ***Rubus*** |
| *Escherichia coli* | 10 mg/mL | 10 mg/mL | - |
| *Salmonella Typhimurium* | 10 mg/mL | 10 mg/mL | - |

| | | | |
|---|---|---|---|
| *Enterobacter erogene* | - | 10 mg/mL | - |
| *Enterococcus faecalis* | - | 5 mg/mL | 10 mg/mL |
| *Staphylococcus aureus* | 10 mg/mL | 5 mg/mL | 10 mg/mL |

Finally, to the best of our knowledge, this study aimed to investigate, for the first time, the protective effect of *Castanea* bee-pollen, showing the highest phytochemical content, on human microvascular endothelial cells (HMEC-1) under ER-stress condition by evaluating cell viability, intracellular ROS production, as well as the expression of factors involved in ER-stress, inflammation, endothelial dysfunction and activation. Specifically, HMEC-1 cells were stimulated for 2 h with or without 0.3 μM thapsigargin, following 1 h pretreatment with increasing concentrations of *Castanea* bee-pollen extract. Overall our results demonstrated that thapsigargin exposure induced ER-stress, ROS overproduction, and up-regulated IL-6, COX-2, and ICAM-1 expression. Besides, our findings demonstrated that lower concentrations of *Castanea* bee-pollen were effective in reducing CHOP, IL-6, COX-2, and ICAM-1 expression, as well the oxidation process arising from thapsigargin exposure, with the maximum protective effect at 10 μg/mL, while higher doses of *Castanea* bee-pollen (100 and 200 μg/mL) showed pro-oxidant effects (data not shown).

## 4. Conclusions

Our results showed a significantly higher cellular antioxidant activity following all bee-pollen pre-treatments and better erythrocytes hemolysis protection by *Castanea* and *Cistus* bee-pollens, suggesting good ex vivo biological activity as free radical scavengers and natural antioxidants. Moreover, all bee-pollen extracts exerted antimicrobial activity with different selectivity among tested microorganisms with MIC values ranging from 5 to 10 mg/mL. Finally, thapsigargin treatment did not affect the HMEC-1 viability, while increased the intracellular ROS production and up-regulated the expression of factors involved in the ER-stress and inflammatory pathway. Conversely, *Castanea* bee-pollen was effective in reducing gene overexpression as well as the oxidation process arising from thapsigargin treatment, with maximum protective effect at 10 μg/mL. In conclusion, bee-pollens, mainly *Castanea* species, represent a good natural antibacterial and a potential nutraceutical product useful in the prevention of free radical and ER-stress-associated diseases.

**Author Contributions:** Conceptualization, M.G. and L.P.; formal analysis, M.G.; investigation, M.G. and S.F.; data curation, M.G. and S.F.; writing—original draft preparation, M.G.; writing—review and editing, M.G., S.F. and L.P.; visualization, M.G.; project administration, M.G. and L.P.; funding acquisition, M.G. and L.P. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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