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 antihemolytic effects of Castanea, Rubus, and Cistus bee-pollens on oxidized human erythrocytes. In addition, 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, which, under oxidative conditions, significantly improved the erythrocytes’ antioxidant activity and limited cell lyses. Moreover, all samples exerted antimicrobial activity with different selectivity among the tested microorganisms, with minimal inhibitory concentration values ranging from 5 to 10 mg/mL. Finally, thapsigargin treatment increased intracellular ROS (reactive oxygen species) production and up-regulated the expression of factors involved in the ER-stress and inflammatory pathways. Conversely, Castanea bee-pollen was effective in reducing gene overexpression, as well as the oxidation process arising from thapsigargin treatment, with a maximum protective effect at 10 µg/mL. In conclusion, Castanea bee-pollen, 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 antihemolytic 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, antimutagenic, 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’s effects on endoplasmic reticulum stress are available in the literature.

This study aimed to assess, on oxidized human erythrocytes, the antioxidant activity and the antimicrobial effects of Castanea, Rubus, and Cistus bee-pollens by CAA-RBC (cellular antioxidant activity in red blood cells) and hemolysis assays. In addition, 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]. Erythrocytes were collected from healthy blood donors upon informed consent for the use of residual blood for research purposes, according to the Italian regulations and, in particular, the regulations of “Fondazione G. Monasterio CNR-Regione Toscana”. The cellular antioxidant activity of 100 µg/mL bee-pollen extracts and the antihemolytic 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 Frassineti et al. [7].

2.2. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of increasing concentrations 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 an HMEC-1 cell culture. Following 1 h pre-treatment with increasing concentration of Castanea bee-pollen (1, 10, 100, and 200 µg/mL), the HMEC-1 culture was stimulated for 2 h with or without 0.3 µM thapsigargin. The cell viability was carried out by an 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−ΔΔCT relative quantification method. 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, USA). p < 0.05 was considered as statistically significant.

3. Results and Discussion

In a previous study, we investigated the botanical origin, chemical and antioxidant compounds profile, and 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 was 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 conditions using the CAA-RBC and hemolysis tests. Both the 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 lipid and protein peroxidation and, at high doses, erythrocytes’ lysis.

As shown in Figure 1A, our findings revealed that all bee-pollen pre-treatments improved about 50% of the erythrocytes antioxidant activity compared to the control (AAPH-treated cells, CAA = 0; *** \( p < 0.001 \)), with CAA values lower than the quercetin (8 µM, ~92%) used as 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 antihemolytic activities following *Castanea* and *Cistus* pre-treatment, with higher percentages of hemolysis inhibition than *Rubus* bee-pollen at similar doses. *Castanea* and *Cistus* extracts from 50 to 200 µg/mL showed greater antihemolytic effects than the trolox used as a standard. These results are probably related to increased levels of polyphenols, flavonoids, and flavonols detected in the *Castanea* and *Cistus* bee-pollen than in the *Rubus* ones [12].

![Figure 1](image-url)

**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), ** \( p < 0.01 \), *** \( p < 0.001 \).

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*, was inhibited at 5 and 10 mg/mL by the *Cistus* and *Castanea* bee-pollen extracts, respectively. While *Cistus* bee-pollen exhibited antibacterial action against all tested bacteria, *Castanea* selectively inhibited *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).

| Strains                        | Minimum Inhibitory Concentration (MIC) Values |
|-------------------------------|---------------------------------------------|
|                               | Castanea | Cistus | Rubus |
| Escherichia coli              | 10 mg/mL | 10 mg/mL | -     |
| Salmonella Typhimurium        | 10 mg/mL | 10 mg/mL | -     |
| Enterobacter aerogenes        | -        | 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 and intracellular ROS production, as well as the expression of factors involved in ER-stress, inflammation, and 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 and ROS overproduction, and up-regulated IL-6, COX-2, and ICAM-1 expression. In addition, 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 it increased the intracellular ROS production and up-regulated the expression of factors involved in the ER-stress and inflammatory pathways. Conversely, Castanea bee-pollen was effective in reducing gene overexpression as well as the oxidation process arising from thapsigargin treatment, with a maximum protective effect at 10 µg/mL. In conclusion, bee-pollens, mainly Castanea species, represent a good natural antibacterial and potential nutraceutical product useful in the prevention of free radical and ER-stress-associated diseases.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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