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Estudio de la actividad antioxidante y antitumoral del propóleo

Study on the antioxidant and antitumoral activity of propolis

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Conflicto de interés
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RESUMEN

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Introducción: El propóleo es la sustancia que protege a la colmena, es una resina de composición compleja y viscosa que las abejas utilizan en la reparación y protección de la colmena. El material del que procede el propóleo son las resinas, brotes y pecíolos de las hojas de diferentes vegetales, por ello presenta una composición química muy compleja que varía en función de la flora de recolección de las abejas. Posee capacidad antimicrobiana, antiinflamatoria que está relacionada con su poder antioxidante, inmunomoduladora, entre otras.

Objetivos: En este trabajo se estudian las actividades antioxidantes y antitumorales de propóleos de distintas zonas de la provincia de Málaga comparándolos con uno de la región de Bohemia al sur de la República Checa.

Material y métodos: La actividad antioxidante se evaluó según el método $ABTS^{+}/S_2O_8K_2$. Además se estimó la cantidad de proteínas totales a partir del contenido de nitrógeno y posteriormente se determinó la citotoxicidad y actividad antitumoral del propóleo del Puerto de la Torre, al norte de Málaga, según el método del bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio.

Resultados: Se observó que el propóleo presenta una elevada actividad antioxidante, aunque tiene una menor cantidad de proteínas. El propóleo presenta elevada toxicidad y mayor actividad antitumoral frente al cáncer de colon que al de leucemia.

Conclusión: Con todos estos datos obtenidos se puede concluir que el propóleo presenta diferentes actividades de interés para la industria alimentaria o cosmética, entre las que destaca su elevado poder antioxidante y su capacidad como antitumoral.

Palabras clave: antioxidante, antitumoral, abeja, miel, propóleo, toxicidad.

ABSTRACT

Introduction: Propolis is the substance that protects the hive, a resin of complex and viscous composition bees use in the repair and protection of the hive. The material from which propolis arises are the resins, shoots and petioles of the leaves of different plants, so it has a very complex chemical composition that varies depending on the flora of the bees collection. It offers an antimicrobial, anti-inflammatory capacity related to its antioxidant, immunomodulatory power, among others.

Aims: In this work, antioxidant and antitumoral activities of different propolis collected from different areas of the province of Malaga, comparing them with one from the Bohemian region to the south of the Czech Republic are studied.

Material and methods: Antioxidant activity was determined according to the ABTS+/$S_2O_8K_2$ method. In addition, the quantity of total proteins from the nitrogen content and subsequently the cytotoxicity and antitumoral activity of the propolis of Puerto de la Torre, north of Malaga, are measured according to the 3-(4,5-dimetiltiazol-2-il)-2,5-diphenyl tetrazolium bromide method.

Results: It was observed that propolis has high antioxidant activity, although it has a lower amount of proteins. Propolis has high toxicity and higher antitumoral activity against colon cancer than leukemia.

Discussion: With all these data, it can be concluded that propolis offers different activities of interest, for the food and cosmetic industry, among which the high antioxidant and antitumoral capacity.

Keywords: antioxidant, antitumoral, bee, honey, propolis, toxicity.

INTRODUCTION

The term propolis is derived from the Greek "pro" meaning "entrance" and "polis", meaning community or city, and is the substance that defends the hive. It is a waxy resin, of complex composition and viscous consistency, produced by the bees (*Apis mellifera*) and it is used in the construction, repair, isolation and protection of the hive[1]. The hive, due to their temperature and humidity conditions, is a prolific environment for the development of viruses and bacteria; therefore, propolis acts as a microbicidal and disinfectant agent, responsible for guaranteeing the asepsis of the hive[2-3]. Its chemical composition varies depending on the flora present in the collection area. Alcohols, aldehydes, amino acids, aliphatic acids, aromatic acids, aromatic esters, fatty acids, flavonoids, p-prenylated coumaries, caffeine-linoic acids, lignans, diterpene, triterpene, steroids and sugar acids[4-5] have been identified as main components.

Propolis has been known from the antiquity and it has been widely used by different cultures for various purposes. The Egyptians benefited from their properties, like their anti-putrefaction capacity, to embalm their dead. Greek and Roman doctors used it as an antiseptic and healing agent. In Inca culture, it was used as an antipyretic and in the pharmacopoeias of London in the seventeenth century it appears as an official medicine[6].

Currently, it has been demonstrated to possess an antimicrobial, anesthetic, healing and anti-inflammatory capacity directly related to its antioxidant power and its free radical retention[7], immunostimulating, immunomodulatory[8], antiulcerous and hepatoprotective[9] capacity.

Due to the wide range of biological activities, propolis has begun being used as an ingredient and additive in the food industry; although the most of the propolis is consumed associated with other bee products, such as honey. The presence of propolis provides greater bacteriostatic activity, thus improving the properties of the processed product.

A large number of medicinal and aromatic herbs performs the biosynthesis of phytochemical compounds with antioxidant activity, which can be used as a natural source of compounds that eliminate free radicals. The majority of these plants are used by bees to collect the resins used in propolis, through which these bioactive components of plant origin can be transmitted to propolis. Consequently, the properties of propolis from different locations may be expected to be different due to vegetation. Thus, the composition and antioxidant capacity of propolis depends on the source of the flower used, seasonal factors and the environment[10].

Both genetic and environmental factors play an important role in the appearance of cancer[11]. In recent decades standard cancer treatments have involved surgery, radiation therapy and chemotherapy. However, the disease often arises again even after surgery, and even systemic treatment leads to side effects. Chemotherapeutic drugs have a great problematic burden because they are not selective in killing cancer cells but induce the cell death of normal cells. Although the mode of action of these drugs is to affect the synthesis of nucleic acids and/or proteins, types of drug-resistant cancers have been found[12].

Propolis is one of the most promising candidates for use as an antitumoral agent. The phenols and flavonoids present in propolis have been described as inducing cell death by apoptosis in several cancer cell lines; however, it can affect non-cancerous cells during the DNA replication, transcription and recombination process[13]. The antitumoral activity of propolis has been studied in several cell lines, such as breast cancer (CAM), human epithelial carcinoma (HeLa) and human leukemia (HL-60, CI41, U937)[14-15]. Propolis and its phenolic compounds exert considerable cytotoxicity both in tumor cells that exhibit chemo resistance and in non-tumoral cells; antimetastatic and antitumoral effects have been reported in mice and rats[16].

The purpose of this study is determination of antioxidant activity of different propolis and cytotoxicity and antitumoral activity of propolis from Puerto de la Torre.

MATERIAL AND METHODS

Different types of propolis from the province of Malaga (Andalusia, Spain), supplied by Bee Garden Malaga and one from Třeboň, in the Bohemia region in the south of the Czech Republic. The used material is summarized in Table 1.

Preparation for propolis extraction

The crude propolis samples are refrigerated at -20°C, where they are solidified for better handling. A 100 mL of pure ethanol is added for each 30g of propolis. The solution was stirred on a magnetic stirrer for 10 days in the absence of light and room temperature. After 10 days it was cen-

trifuged in order to take the supernatant and filter it. The samples were then rotated all night to completely remove the ethanol in the sample. The extracted propolis was lyophilized for 48 hours and stored at -20°C for different assays.

Evaluation of the total antioxidant capacity in a water-soluble medium according to the ABTS^{+}/$S_2O_8K_2$ method

Antioxidant activity was measured by using ABTS+/$S_2O_8K_2$ method according to Re *et al*., 1999[17]. For this, an aqueous solution containing 7 mM of ABTS^{+} and 2.45 mM of potassium persulfate was prepared and kept in the dark at room temperature for 12-16 hours prior to performing the assay, thus ensuring the complete formation and stability of the ABTS^{+} radical. Absorbance measurements were taken at 413 nm before the addition of the possible antioxidant substance (propolis extract) and once added, it was well shaken and readings were taken the minute of reaction, for duration of 8 minutes to observe the drop in absorbance. The calculation of the antioxidant activity was determined from the ratio, expressed as a percentage (% AA), between the natural absorbance drop of ABTS^{+} at 8 minutes of reaction and recorded for each volume of the substance with possible antioxidant capacity. Trolox was used as a reference antioxidant at concentrations between 5 µM and 20 µM, expressing the final results in µmol Trolox g^{-1} of dry weight (DW).

Table 1. List of propolis studied

Name / Zone	Geographical coordinates	Date	Type extraction	Vegetation
Puerto de la Torre	36.73N, 4.50W	29 / 03 / 2016	Scrape	Pine trees, Oaks, poplar
Yunquera	36.72,N 4.96 W	03 / 08 / 2012	Mesh	Pine trees, Oaks, poplar
La Mosca	36.74 N 4.37 W	05 / 12 / 2015	Scrape	Pine trees, Oaks, poplar
Třeboň	49.01N 14.77E	05 / 2015	Scrape	Oak trees, Beech trees, Apple trees
Třeboň	49.01N 14.77E	05 / 2016	Scrape	Oak trees, Beech trees, Apple trees

Total protein content

The total nitrogen content of the samples was determined on a C:N:H autoanalyzer (Leco CHNS-932 Elemental Analyzer with O VTF-900 Analyzer, Corporation, Michigan, USA) from the Central Support Services to the Research (SCAI) of the University of Malaga. The nitrogen content was determined and expressed as a percentage. The protein content was obtained from the nitrogen content, expressed as a percentage, multiplied by a factor of 6.25 according to Kieldahl (1883)[18].

Evaluation of cytotoxicity and antitumoral activity according to the 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium MTT or bromide method

The MTT method was performed according to Denizot and Lang (1986)[19]. This assay is based on the metabolic reduction of MTT through the mitochondrial enzyme succinate dehydrogenase in a blue-colored compound (formazan), allowing the mitochondrial function of the treated cells to be determined. This method has been highly used to measure cellular survival and proliferation. The amount of live cells is proportional to the amount of formazan produced. Cytotoxicity and antitumoral activity was conducted only in the propolis of Puerto de la Torre.

For the preparation of the treatments, 2 mg of the Puerto de la Torre propolis was weighed and initially dissolved in 10 µl of propylene glycol, since the propolis does not dissolve well directly in the culture medium, starting thus with an initial concentration of 2 mg mL^{-1}. Different cellular lines were used for RAW 264.7 (ATCC® TIB-71) cytotoxicity and HCT-116 (ATCC® CCL-247) and U-937 (ATCC® CRL-1593.2) for antitumoral activity.

Depending on each assay a number of cells were placed, whereby the cell count was fitted to each assay. In the evaluation of cytotoxicity with RAW 264.7, 2500 cells mL^{-1} were used; whereas in the case of anti-tumoral activity 2000 cells ml^{-1} of HTC-116 and 3500 cells mL^{-1} of U-937 were used.

The optical density was determined at 550 nm in a microplate spectrophotometer (Eon Fluorescence Plate Read-

er, BioTek). The results were expressed as a percentage (%) of survival with respect to the untreated control and as a concentration inhibitor of 50% (IC_{50}); it is the concentration of the compound permitting the survival of 50% of the cellular population.

Statistic analysis

An ANOVA statistical study was performed with SigmaPlot software version 11.0 (Systat Software Inc. San José, USA). Assays were performed with eight replicates (n = 8) and the results are expressed as the mean ± SD (SD) of the eight independent samples.

RESULTS

Performance of the propolis extraction

The extractions made from the different types of propolis have obtained a yield, detailed in Table 2. The yield extraction ranged from 53.91% in La Mosca to 1.51% in Třeboň (2015).

Table 2. Yield from the extraction

Name	Gross weight (waxes with propolis) (g)	Lyophilized weight (g)	Relative yield
Puerto de la Torre	15.9062	1.7662	11.10 %
Yunquera	12.9559	4.1748	32.22%
La Mosca	20.8103	11.2224	53.91%
Třeboň (2015)	5.5964	0.0847	1.51%
Třeboň (2016)	44.2381	17.2524	39%

Antioxidant capacity according to the ABTS+/S8O8K2 method

Table 3 shows the antioxidant activity of the different samples, expressed in the Trolox equivalent, which is the control antioxidant. The antioxidant activity differs depending on samples. The highest antioxidant activity was observed in La Mosca propolis followed by Propolis from Yunquera and Puerto de la Torre. The lowest levels were reached in Třeboň samples.

Table 3. Antioxidant activity

Name	Antioxidant activity (µmol TE/g) ± standard deviation
Puerto de la Torre	49.73 ± 2.06
Yunquera	47.27 ± 1.40
La Mosca	63.45 ± 0.24
Třeboň (2015)	16.66 ± 2.63
Třeboň (2016)	29.75 ± 2.15

Total protein content

The amount of total proteins in each sample studied is presented in Figure 1. The highest levels of proteins are found in Puerto de la Torre propolis followed by La Mosca and Yunquera whereas the lowest levels were found in Třeboň samples.

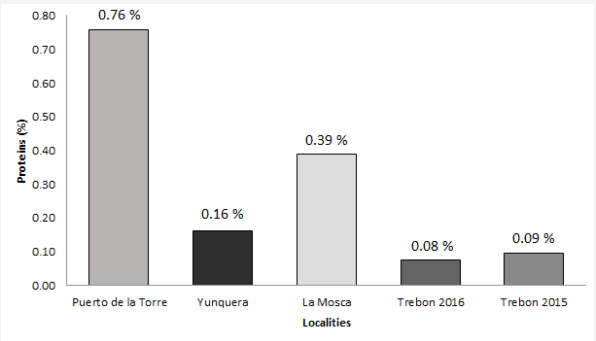

Figure 1. Percentage of total proteins in the propolis from different localities

Cytotoxicity according to the MTT method

Propolis demonstrates cytotoxicity, being the IC_{50} 22.92 µg mL^{-1}. The cellular death is proven in the first concentrations followed by a survival as the concentration of administered propolis decreases (Fig. 2).

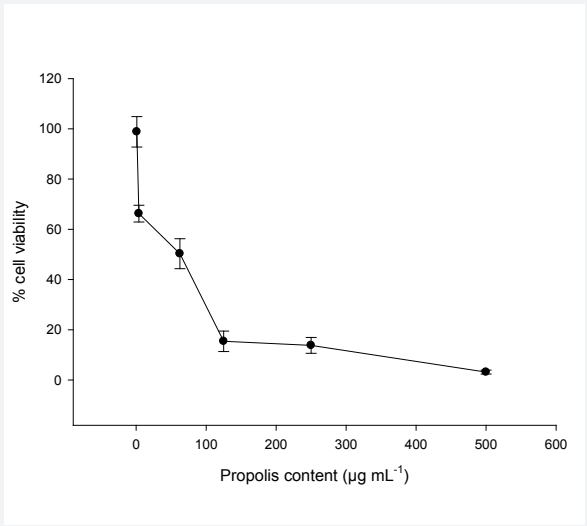

Figure 2. Citotoxicity expressed as cell viability (%) of propolis from Puerto de la Torre.

Antitumoral activity according to the MTT method

Colon cancer: HTC-116 cell line

In the case of propolis, activity against colon cancer cells is demonstrated, with an IC_{50} of 89.97 µg mL^{-1}. The death of tumor cells in the highest concentrations of propolis is observed (Fig. 3).

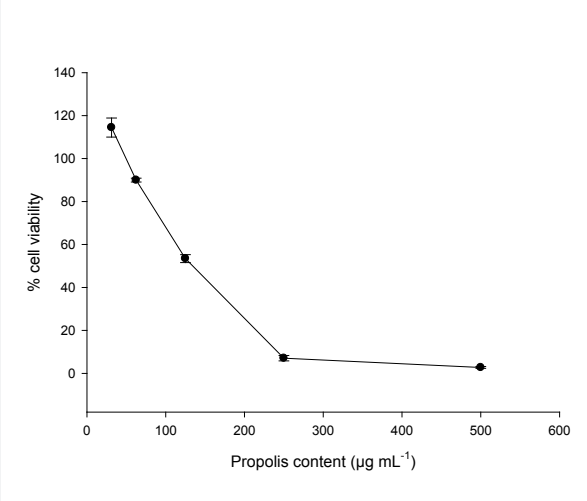

Figure 3. Antitumoral activity against colon cancer of the propolis from Puerto de la Torre

Leukemia Cancer: U-937 cell line

The propolis of Puerto de la Torre used as a study treatment has an IC_{50} of 132.33 µg mL^{-1} and therefore presents activity against human leukemia cells. It is possible to observe how the cells die at the highest concentrations of administered propolis and survive as the concentration decreases (Fig. 4).

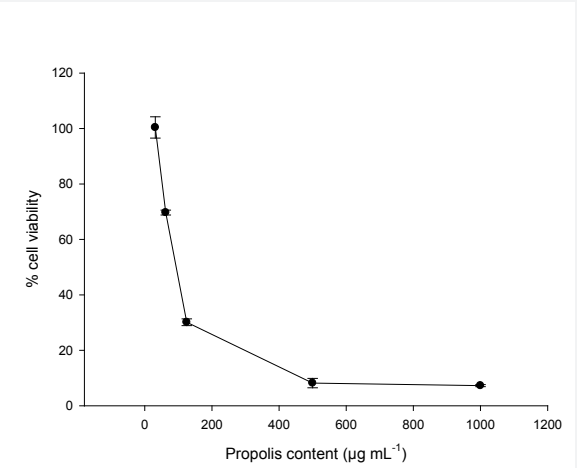

Figure 4. Antitumoral activity against leukemia cáncer of the propolis from Puerto de la Torre

DISCUSSION

The main objective of this study was to evaluate the antioxidant activity of different types of propolis and study the antitumoral activity of propolis from one of them (Puerto de la Torre), in order to determine properties of interest for its possible use in the cosmetic and pharmaceutical industry. A small bioprospection of different types of propolis from the province of Malaga was performed in order to be able to select the one with the most optimal properties and thus be used for biotechnological applications in the area of food, nutraceutic and biomedicine.

The first study performed was the antioxidant activity via the ABTS method. Different antioxidant activity was observed. Presenting the highest values the propolis from Malaga compared to that from the south of the Czech Republic. The content of antioxidant substances such as carotenoids or phenols is expected to be higher in Malaga vegetation than in the south of the Czech Republic because of a higher radiation dose in Malaga than in Třeboň[20]. This is supported by different studies where different types of Andalusian propolis have reflected percentage inhibition data ranging from 23 to 71.2%[21].

Bees do not collect only the resins from the trees found in the environment of the hive, but have ample capacity for

flight, which allows them to make the selection of resins they specifically need[22]. Probably, this selection is related to the phytogeographic characteristics[13] and to the antimicrobial activity of the resin, since the bees use propolis as an antiseptic and antimicrobial element. So it has been demonstrated that it will be very difficult to find two hives that produce identical propolis, even though located in the same geographical area; what is more significant is that the propolis from different parts of the hive does not have exactly the same chemical composition[23]. These variations are not given by the diversity of the elements present in their composition stable in the greater proportion, but in the quantities present in the sample for each of them. Protein content showed also differences between the different localities, probably due to the vegetal origin from which each case arises.

Cytotoxicity is expressed as the percentage of survival relative to the control, which are the cells without treatment. In the case of propolis this concentration is 22.92 μg mL^{-1}, so it can be concluded that our studied propolis is toxic to the cells. Propolis is mostly used as a dietary supplement, since they boast multiple properties such as antibacterial, antifungal, antimicrobial, antitumoral, anesthetic, anti-inflammatory, antioxidant, among others[13]. According to the EFSA (European Food Safety Authority) the maximum amount of propolis ingested must be between 0.7-1.3 g kg^{-1} day^{-1}. However, propolis is considered as food supplement, where it is difficult to have a standard figure since, as described above according to the vegetation from which it arises, one type or another of propolis with different characteristics is obtained. Therefore, food supplements vary in the amount of the propolis that they present, although most are usually diluted. The studied propolis demonstrates toxicity at low concentration, thus its alimentary use should be marketed at low concentration and its dilution should be recommended when ingesting it, to reduce the concentration in which it is consumed.

The antitumoral activity was studied in two cell lines, colon cancer and leukemia. The propolis of Puerto de la Torre presented activity against the two tumoral lines. This antitumoral activity may be due to the different properties. The antioxidant capacity of the Puerto de la Torre propolis presents a percentage of inhibition of 64.31% at a concentration of 5000 μg mL^{-1}, so that the ability to decrease the pro-oxidant potential of some molecules present in the propolis is not expected to be very high. However, it must be considered that under certain conditions antioxidant substances can be converted into pro-oxidants. The antitumoral activity of the propolis could be due to the presence of a variety of compounds considered as promising antitumoral agents, among which are found caffeic acid (CA) and its derivative phenethyl ester of caffeic acid (CAPE), artepillin C and others. CAPE has several therapeutic effects, including anti-inflammatory and cytotoxic antimicrobial, antioxidants properties[24]. Artepillin C was also isolated from the Brazilian propolis, demonstrating cytotoxic activity against in vitro cultured tumor cells[25]. This opens new lines of study for this propolis, in order to learn what properties are the one that bring about this antitumoral activity.

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