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Are by-products from beeswax recycling process a new promising source of bioactive compounds with biomedical properties?

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ABSTRACT

During the process of beeswax recycling, many industrial derivatives are obtained. These matrices may have an interesting healthy and commercial potential but to date they have not been properly studied. The aim of the present work was to evaluate the proximal and phytochemical composition, the antioxidant capacity and cytotoxic effects of two by-products from beeswax recycling process named MUD 1 and MUD 2 on liver hepatocellular carcinoma. Our results showed that MUD 1 presented the highest ($P < .05$) fiber, protein, carbohydrate, polyphenol and flavonoid concentration, as well as the highest ($P < .05$) total antioxidant capacity than the MUD 2 samples. MUD1 exerted also anticancer activity on HepG2 cells, by reducing cellular viability, increasing intracellular ROS levels and affecting mitochondrial functionality in a dose-dependent manner.

We showed for the first time that by-products from beeswax recycling process can represent a rich source of phytochemicals with high total antioxidant capacity and anticancer activity; however, further researches are necessary to evaluate their potentiality for human health by *in vivo* studies.

1. Introduction

Food production is one of the most important economic sectors in the world, which in turn is responsible for a great amount of industrial waste. Food waste, defined as the leftover material from animal or vegetable origin used in food and beverage production, has become a worldwide burden (Oreopoulou and Russ, 2007). Some decades ago, food wastes were considered neither a cost nor a benefit since they were sent for composting, brought to landfills and used as animal feed (Baiano, 2014). However, nowadays, this attitude is becoming to change because of several reasons, such as the substantial disposal costs, the need to reduce the waste impact on human health, the awareness of the potential benefit of biocomponents present in food waste and the environmental concerns related to this matter (Laufenberg et al., 2003). In particular, increasing ecological awareness and waste accumulation, which are economically and environmentally costly to dispose (e.g. landfill, incineration), have prompted the wider scientific community to find other uses such as biofuels or carbon sources for cattle feed. However, growing public concerns about hunger, conserving the environment, and the effect of socioeconomic factors have accelerated research into food waste (Stuart, 2009). At the present, the food industry is focused on the reduction of the energy and water consumption, and, in a residual way, on the recovery of energy from waste, so that there is an urgent need to invest in research, new recovery technologies and/or new production lines for the reuse of biocomponents present in food waste (Baiano, 2014); currently, there is

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still a great wastage of food by-products prompting us to seek alternative uses of this waste.

The recovery of added value biologically active compounds from leftover materials used in food and beverage production and its valorization has many advantages: it provides a rich source of phytochemicals and biologically active compounds which could be incorporated back in the food chain or used for medicinal purposes. For example, collagen derived from fish skin, bones and fins can be used for the delivery of anticancer drugs or genes that promote bone and cartilage formation; animal brains and nervous systems are a rich source of cholesterol while heparin can be extracted from the liver. At the same time, nutraceuticals obtained from seaweed or plants are usually used as antioxidants or modulators or appetite (Baiano, 2014).

Apiculture (from Latin: apis "bee") is the maintenance of honey bee colonies, commonly in hives, by humans. Bee products include honey and other products that the hive produces like beeswax, propolis, pollen, venom, royal jelly, and others. It is of great interest to study the various products that are obtained from the hive in order to add value to the beekeeping sector while contributing, at the same time, to the study of potential molecules of biomedical interest. The recycling of the beeswax results in substances, hitherto considered as industrial waste, which, however, could have an important value in biomedicine, although they have not been properly studied to date. The aim of this study was to evaluate the proximal, nutritional and phytochemical composition, as well as the antioxidant capacity of different beeswax recycling by-products; the cytotoxic effects on HepG2 were also assessed, by evaluating the cellular viability, intracellular ROS production and the mitochondrial functionality.

2. Materials and methods

2.1. Sample collection and preparation

In the factory wax, during the recycling process of the wax honeycombs, the combs were collected and subjected to a heating process by steam. A sediment with inorganic and organic waste was separated from wax. This waste included pollen, molting debris of baby bees, etc. This sediment, called MUD 1, represented about 50% of the initial weight of the product that arrived at the factory. The remaining wax was then passed to a continuous decanter, where a fine sediment was generated: this new sediment was called MUD 2.

Five samples of MUD 1 and MUD 2 were randomly collected from total MUDs and used for the hydrophilic extraction, performed as previously reported for honey (Alvarez-Suarez et al., 2010) by diluting 1 g of each sample of MUD 1 or MUD 2 in 10 mL of distilled water and filtered through Minisart filter of 45 μm (PBI International).

2.2. Characterization of MUD samples

2.2.1. Determination of proximal composition

The proximal analysis consisted in the determination of humidity in the samples by drying them and further calculation by gravimetry. Ashes were calculated by sample incineration in a Mufla oven at 550 °C. Protein analysis was performed by using the Kjeldahl method, with previous acid digestion, and calculation were done by using a conversion factor of 6.25. The analysis of fat was performed after the method of Soxhlet before acidic hydrolysis. Fiber analysis was done by a gravimetric enzymatic method, while calculation of carbohydrates was done by difference. Humidity, ashes, protein, fat and fiber were calculated following official methods from the AOAC (A.O.A.C, 2000). Free amino acid of each extract from both types of MUD were quantified by the Cd-ninhydrin method published by Doi et al. (1981).

2.2.2. ICP-MS analysis of chemical elements

Samples for quantitative determination of metals were first lyophilized in a vacuum pump (Telstar, Madrid, Spain) and then prepared by attack with nitric acid and hydrogen peroxide of supra-pure quality, in a microwave digester (Milestone, Sorisole, Italy). Determination of Mg, Al, Si, P, K, Ca, Sc, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Y, Mo, Cd, Au, Hg and Pb total content in muds was performed by an ICP-MS instrument (Agilent 7500, Agilent Technologies, Tokyo, Japan) fitted with a Meinhard type nebuliser (Glass Expansion, Romainmotier, Switzerland) and equipped with a He collision cell. A Milli-Q system (Millipore, Bedford, MA, USA) was used to obtain deionized water (18 MΩ). All reagents used were of the highest available purity. Hydrogen peroxide and nitric acid were supra-pure quality from Merck (Darmstadt, Germany). A standard solution of 100 μg/l of Li, Mg, Sc, Co, Y, In, Ce, Ba, Pb, Bi, and U in 1% (v/v) HNO_3 was prepared from a 1.000 mg/1 multi-element stock standard solution (Merck) and used for daily optimizing of the ICP parameters. Single-element standard solutions for ICP-MS containing 1.000 μg/ml of calcium or vanadium were also purchased from Merck. The samples were prepared with nitric acid and hydrogen peroxide in a microwave digestion system. The extracts were collected and made up to a final volume for subsequent analysis. Calibration curves were prepared using Ga as an internal standard and by the dilution of stock solutions of 1.000 mg/l in 1% HNO_3. The accuracy of this method was evaluated by recovery studies after complete digestion of spiked muds with multi-element standards. The calculated recoveries for each element were between 95% and 105% in all cases.

2.2.3. Measurement of total phenolic and flavonoid content

The Folin–Ciocalteu method was used to determine the total phenol content (TPC) of the extracts from both types of MUD, as reported by Singleton et al. (1999), while flavonoid content (Flavo) of the extracts from both types of MUD was determined using a colorimetric method described previously by Chang et al. (2002).

2.2.4. UPLC-DAD/ESI-MS characterization of MUD samples

The UPLC instrument consisted of a Waters Acquity H class UPLC system (Waters Co., Milford, MA, USA) coupled to a SYNAPT G2-Si mass spectrometer with a quadrupole time-of-flight (TOF) configuration (Waters Co., Milford, MA, USA) in negative electrospray ionisation (ESI) mode. Instrument operation was carried out using MassLynx 4.1 software. The chromatographic separation was achieved on a Waters ACQUITY UPLC HSS C18 column from Waters (3 × 30 mm, 1.8 μm). The column temperature was set at 40 °C. The mobile phases were 0.5% acetic acid in water (A) and acetonitrile (B). A flow rate of 0.4 mL min-1 was used and an injection volume of 10 μL of the phenolic extracts. The elution gradient established was the following: 0 min 5% B; 0–15 min, 5–95% B. Finally, the B content was decreased to the initial conditions (5%) in 10s and the column re-equilibrated for 3 min. ESI source operated with a capillary voltage of 0.5 kV, source temperature 120 °C, cone gas flow 50 L h − 1, desolvation gas at 450 °C, 1000 L h − 1. Resolution of first quadrupole was LM 4.9, HM 15. TOF scan range was 50–1200 u. Analyzer operated in resolution mode (FWHM ≈ 18,000) at 1 scans/s, calibrated with sodium formate solution (mixture Of 100 L NaOH 0.1 M, 200 L formic acid 10%, and 20 mL acetonitrile/water 80/20, v/v). MS detector was programmed to perform scans at 50–1800 m/z range. The identification of the phenolic compounds present in the analyzed samples was based on retention time data, extracted ion chromatograms and comparing the MS spectra with previously published results.

2.2.5. Evaluation of the total antioxidant capacity (TAC)

TAC of the extracts from both types of MUD was assessed using the Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The TEAC assay was carried out according to the modified method of Re et al. (1999). Each sample was analyzed in three replicates and TEAC results are expressed as mmol of Trolox equivalents/kg (mmol TEq/kg). Data are reported as a mean value ± standard error of the mean (SEM) for four measurements. The FRAP