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Chemical composition and evaluation of antioxidant, antimicrobial and antiproliferative activities of *Tuber* and *Terfezia* truffles

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ABSTRACT

Ten truffle species of *Tuber* and *Terfezia* genera were chemical characterized, assessing their proximate composition, individual nutrient compounds and some bioactive molecules. The bioactive properties of these species were also evaluated, namely their antioxidant, antimicrobial and cytotoxic potential. Carbohydrates were the main macronutrients present in truffles, followed by proteins. Furthermore, the levels of polyunsaturated fatty acids (PUFA), subsequently presented as a percentage, were higher in truffles (38.2–79.3%) except in *Tuber magnatum* and *Terfezia arenaria*, which have a more saturated fatty acids (SFA) profile (70.7% and 53.7%, respectively). Comparing the species, *T. magnatum* revealed the highest levels of total phenolic compounds (TPC) (290 mg GAE/100 g truffle), as also the best results in the four methods used to evaluate the antioxidant activity. On the other hand, only five extracts obtained from some studied truffle species (*Terfezia magnusii*, *Tuber aestivum*, *Tuber gennadii*, and *Tuber melanosporum*) showed a slight inhibition of microbial growth, tested against different bacteria. *Terfezia* and *T. gennadii* extracts, showed potential to inhibit the cellular growth of NCI-H460, HeLa, HepG2, and MCF-7 cell lines (GI_{50} concentrations range: 19–78, 33–301, 83–321 and 102–321 μg/mL, respectively), indicating anti-proliferative activity. Nevertheless, *T. arenaria* revealed some potential hepatotoxicity, inhibiting the growth of PLP2 cells (GI_{50} concentration of 220 μg/mL), a primary cell culture obtained from porcine liver.

1. Introduction

Edible fungi can be classified in two groups, epigeous carpophores commonly known as mushrooms, and hypogeous species that grow underground, known as truffles (Lee et al., 2020). The most economically important genus of truffles is the genus *Tuber*, which includes the species *T. magnatum* Pico (white truffle), *Tuber melanosporum* Vittad. (black truffle), or *Tuber aestivum* Vittad. (summer truffle), highly appreciated for their distinctive aroma (Costa et al., 2015; Culleré, Ferreira, Venturini, Marco, & Blanco, 2013). These species are traditionally harvested in Italy, France, and Spain. Outside Europe, whereas *Tuber indicum* Cooke & Massee is exclusively harvested in Asia (Reyna & Garcia-Barreda, 2014). Other genera, such as *Terfezia* or *Tirmania*, have culinary interest, but their organoleptic qualities are less appreciated worldwide. These genera, also called desert truffles, are mostly endemic to the arid and semi-arid areas of the Mediterranean basin (Zambonelli et al., 2014).

The investigation of their nutritional profile showed that fresh

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mushrooms, including truffles, are a rich source of carbohydrates and proteins (Kalač, 2013). Some minor compounds such as minerals, amino acids, and fatty acids were generally determined in truffles (Lee et al., 2020). However, other minor chemical constituents, specifically phenolic compounds or tocopherols, only have been studied in *T. aestivum* and *T. magnatum* truffles (Beara et al., 2014; Shah, Usvalampi, Chaudhary, Seppänen, & Sandesh, 2020). Some of these compounds, even in synergy with others, have been reported to have biological activity, namely free radical scavenging activity, metal chelation, or inhibition of lipid oxidation against reactive oxygen species (ROS) (Sánchez, 2017). Furthermore, some reports on bioactivities (antiviral, antimicrobial, antimutagenic, antioxidant and anti-inflammatory) of desert truffles belonging to *Terfezia* and *Tirmania* spp. have been investigated (Dahham, Al-Rawi, Ibrahim, Abdul Majid, & Abdul Majid, 2018; Stojković et al., 2013; Vahdani, Rastegar, Rahimzadeh, Ahmadi, & Karmostaji, 2017).

As far as we know, the biological activity of *Tuber* genus was scarcely explored in three species. There are studies reporting that the aqueous and ethanolic extracts from *T. aestivum* exhibit an antimutagenic effect (Fratianni, Luccia, Coppola, & Nazzaro, 2007), while its methanolic extract inhibited ABAP (2′-azobis-(2-amidinopropane)-dihydrochloride)-induced lipid peroxidation (Villares, García-Lafuente, Guillamón, & Ramos, 2012). Also, *T. magnatum* showed antioxidant, anti-inflammatory and cytotoxic activities (Beara et al., 2014) and *T. indicum* showed antioxidant activity (Guo, Wei, Sun, Hou, & Fan, 2011). Recently, we reported anti-immunomodulatory properties in *T. melanosporum* (Tejedor-Calvo, Morales, García-Barreda, et al., 2020). Besides, several studies have demonstrated the antimicrobial activity of different extracts of several epigeous carpophores (Venturini, Rivera, Gonzalez, & Blanco, 2008). Regarding hypogeous fungi, the methanolic and ethanolic extracts of *Terfezia* truffles showed antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Janakat, Al-Fakhiri, & Sallal, 2004). Within this genus, the species *T. arenaria* (Moris) Trappe showed antimicrobial activity against *Enterococcus faecalis* and *Escherichia coli,* among other pathogenic bacteria, yeast and filamentous fungi (Harir et al., 2019). Nevertheless, to the best of our knowledge, there are no reports on the biological activities of *Tuber brumale* Vittad., *T. gennadii* (Chatin) Pat., *Tuber oligospermum* (Tul. & C. Tul.) Trappe*, Terfezia leptoderma* Tul. & C. Tul., and *Terfezia magnusii* Mattir.

Consequently, this study aimed to perform for first time a comprehensive investigation of the proximate analysis, chemical composition (*i.e.* fatty acids, organic and phenolic acids, proteins, tocopherols, and soluble sugars), as well as antioxidant, antimicrobial and anti-proliferative bioactivities of ten truffles species belonging to *Tuber* and *Terfezia* genera.

2. Materials and methods

2.1. Truffle samples

Fresh hypogeous edible carpophores belonging to ten species were studied, seven of the genus *Tuber* (*T. aestivum*, *T. brumale*, *T. indicum*, *T. gennadii*, *T. magnatum*, *T. melanosporum*, and *T. oligospermum*), and three of the genus *Terfezia* (*T. arenaria*, *T. leptoderma*, *T. magnusii*). *T. aestivum*, *T. brumale*, and *T. melanosporum* were directly harvested from the orchard. The rest was obtained from natural truffle grounds, except *T. indicum* and *T. magnatum* that were supplied by Espora Gourmet Company. The studied species origin, harvest season, scientific and common names are listed in Table 1.

Carpophores were identified on the field based on the location and host plant, and their macroscopic features. Then, truffles were transported to the laboratory in insulated boxes with ice packs and were refrigerated at 4 °C until their processing (between 3 and 12 h). This first classification was confirmed at the laboratory by microscopic identification according to the morphology of ascus and spores. Subsequently,

Table 1
Species of analysed truffles: scientific name, common name, harvest season and country of origin.

Scientific name	Common name	Harvest season	Country
Tuber aestivum	Summer truffle	June-October	Spain
Tuber brumale	Winter truffle	November-March	Spain
Tuber indicum	Chinese truffle	November-March	China
Tuber gennadii	Cheese truffle	January-May	Spain
Tuber magnatum	White truffle	August-January	Italy
Tuber melanosporum	Black truffle	November-March	Spain
Tuber oligospermum	Desert truffle	October-May	Spain
Terfezia arenaria	Desert truffle	March-April	Spain
Terfezia leptoderma	Desert truffle	March-April	Spain
Terfezia magnussi	Sheep truffle	August-April	Spain

the samples were brushed with a wet soft brush, rinsed with tap water and forced air-dried in a laminar cabinet. Qualitative selection of the carpophores was made by discarding those truffles with soft texture or those parasitized or damaged during harvest under a stereomicroscope, as described by Rivera, Venturini, Marco, Oria, and Blanco (2011). Maturity of each fruiting body was determined calculating the ratio between the number of ascii containing melanized spores and the total number of ascii by microscopic observation. The degree of maturation of the ascocarps was defined according to the percentage of asci-containing mature spores in the following categorized stages: stage 0 = 0–5%, stage 1 = 6–30%, stage 2 = 31–70%, and stage 3 = 71–90% (Zeppa et al., 2002). After the classification, five ascocarps (≈10 – 20 g) were selected for each species. The ascocarps were then cut into thin slices using a sharp knife and blended in order to obtain a homogeneous sample that can be considered as representative of these truffles' species (Culleré et al., 2013). All truffle samples were previously frozen (-80 °C, 24 h), and disposed in a freeze dryer (Cryodos-50 Telstar, Barcelona, Spain) to lyophilize them at 50 °C and vacuum of < 10 mbar for 48 h. Then samples were ground sieved to obtained particle size lower than 0.5 mm, vacuum sealed in polyethylene bags (Oriented Polyamide/Polypropylene, 15/65, 80 μm (Orved, Musile di Piave, Italy) with a VM-12 vacuum sealer (Tecnotrip), and kept at −80 °C until further use. For total phenolic compounds (TPC) analysis and antioxidant activity determination, fresh samples were used.

2.2. Reagents

The eluents *n*-hexane 95% and ethyl acetate 99.98% were of HPLC grade from Lab-Scan (Lisbon, Portugal). Methanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). Tocopherol standards (α-, β-, γ- and δ-), sugars standards (L(+)-arabinose, D(−)-fructose, D(+)-galactose, D(+)-glucose anhydrous, lactose 1-hydrate, maltose 1-hydrate, D(+)-mannitol, D(+)-mannose, D(+)-melezitose, D(+)-melibiose monohydrate, L(+)-rhamnose monohydrate, D(+)-sucrose, D(+)-trehalose and D(+)-xylose), phenolic compounds standards (apigenin-6-C-glucoside, apigenin-7-O-glucoside, chlorogenic acid, ellagic acid, hesperetin, naringenin, p-coumaric acid, protocatechuic acid and quercetin-3-O-glucoside) and the standards used in the antioxidant activity assays: BHT (3,5-(Dimethylethyl)-4-hydroxytoluene) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Chemical composition

2.3.1. Proximate analysis

The proximate analysis of truffle samples was assessed (protein, fat, carbohydrates and ash) using the AOAC procedures (AOAC 2016). The crude protein content ($N \times 4.38$) of the samples was estimated by the macro Kjeldahl method (AOAC 978.04). The crude fat was determined by extracting a known weight of powdered mushroom sample with petroleum ether, using a Soxhlet apparatus (AOAC 920.85). The ash

content was determined by incineration at 600 ± 15 °C (AOAC 923.03). Total carbohydrates were calculated by difference. Total energy was calculated according to Regulation (EC) number 1169/2011 of the European Parliament and of the Council, of 25 October 2011, on the Provision of Food Information to Consumers, as: Energy [(kcal/100 g; dry basis) = 4 × ($g_{protein}$ + $g_{carbohydrates}$) + 9 × (g_{fat})]. (The European Parliament and Council, 2011)

2.3.2. Determination of nutrient compounds: Fatty acids, tocopherols and soluble sugars

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC–FID)/capillary column based on the ISO 5509:2000 trans-esterification method. The fatty acid profile was analysed with DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey-Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm d_f). Extraction procedures and analysis conditions were performed following Barros et al. (2013) method. The results were expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. A Sigma (St. Louis, MO, USA) mixture of 37 FAMEs (standard 47885-U) was used. Some fatty acid isomers were identified with specific standards, also purchased from Sigma.

Tocopherols were determined using a HPLC-Fluorescence (FP-2020, Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. Extraction procedures and analysis conditions were performed according to the protocol previously described by Pereira, Barros, and Ferreira (2015). The quantification was performed by comparison to the fluorescence signal obtained from the commercial standards (α-, β-, γ-, and δ-isoforms) of each compound; a racemic tocol was used as internal standard. The results were expressed in μg per 100 g of dw.

Soluble sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC–RI). Extraction procedures and analysis conditions were performed according to Barros, Baptista, Correia, Sá Morais, and Ferrerira (2007). The results were expressed in g/100 g of dry weight, calculated by internal normalization of the chromatographic peak area. Sugar identification was made by comparing the relative retention times of sample peaks with standards.

2.3.3. Determination of bioactive compounds: Organic acids and phenolic compounds

2.3.3.1. Organic acids. Organic acids were determined based on a protocol described by Dias et al. (2015). The organic acids were analysed using the Ultra-Fast Liquid Chromatography (UFLC, Shimadzu 20A series, Kyoto, Japan) coupled with a diode array detector (DAD), using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The separation was achieved on a Sphere Clone reverse phase C18 column thermostated at 35 °C. The organic acids found were quantified using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g (dry basis).

2.3.3.2. Total phenolic compounds (TPC). Firstly, the methanolic extract (ME) of the different truffle species was prepared. For that, 1 g of fresh truffle species were extracted with 30 mL of methanol and homogenized with an ultraturrax (IKA model DI 25, Staufer, Germany) during 30 s. The extract was centrifuged at 2000 rpm for 15 min at 4 °C in a centrifuge (Heraeus Megafuge 1.0, Buckinghamshire, England). The supernatant was evaporated with a rotavapor (Heildolph Instruments GmbH & Co model Laborota 4000, Schwabach, Germany), re-suspended with 10 mL of methanol: water 80:20, filtered through a 45 μm nylon membrane and stored at −18 °C prior to further use, forming the ME. The TPC was determined by the *Folin-Ciocalteu* method (Guo et al., 2011) with some modifications. Briefly, an aliquot (1 mL) of the ME or of a standard solution of quercetin (0–250 mg/L) was added to a 10 mL volumetric flask and mixed with 1 mL of *Folin-Ciocalteu* reagent. After 5 min, 1 mL of 10% Na_2CO_3 water solution was added and the solution was diluted to 10 mL with deionized water and incubated 1 h at room temperature (~20 °C) in darkness, the absorbance was determined at 760 nm with a spectrophotometer (Unicam UV 500, England). TPC was expressed as mg gallic acid equivalents (GAE) per 100 g of fresh weight.

2.3.3.3. Phenolic compounds profile. For the phenolic acids (and related compounds) identification, 1 g of each dried powdered sample was extracted with 30 mL methanol under magnetic stirring for 1 h at room temperature (~20 °C). Then, the residue was re-extracted maintaining the same operational conditions. The combined extracts were evaporated at 40 °C in a rotary evaporator (Büchi R-210, Flawil, Switzerland) to remove alcohol and be lyophilized. The lyophilized extracts were redissolved at 30 mg/mL in methanol:water (20:80, v/v) and filtered through a 0.2 μm nylon filter.

Phenolic acids analysis was carried out using the system previously described for organic acids determination (UFLC-DAD) and following a procedure already described (Reis et al., 2016). Detection was carried out in the photodiode array detector (DAD), using 280 nm as the preferred wavelength and in a mass spectrometer (MS) connected to HPLC system *via* the DAD cell outlet. Separation was achieved using a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm) column thermostatted at 35 °C. The quantification of the phenolic compounds identified was made by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from the commercial phenolic standards. The results were expressed as mg per 100 g of dw.

2.4. Testing of the bioactivities

2.4.1. Antioxidant activity

Four methods were used to assess the antioxidant activity of the truffles: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, the ferric reducing antioxidant power (FRAP) assay, the oxygen radical antioxidant capacity (ORAC) and the Reducing power. The same extract conditions used to obtain TPC methanol extracts (ME) were followed.

2.4.1.1. DPPH assay. The DPPH assay is based on the method used by Llorach, Martínez-Sánchez, Tomás-Barberán, Gil, and Ferreres (2008) with modifications. Briefly, 900 μL of ME or their dilutions were mixed with 900 μL of DPPH (133 μM in methanol). The free radical scavenging activity was evaluated by measuring the variation in absorbance at 515 nm after 2 h and 30 min of reaction. The calibration curve was made with Trolox (0–60 μM) as a standard, and the results were expressed as mmol Trolox equivalents (TE)/100 g dry weight and as a percentage of reduction with extract concentration of 25 mg/mL.

2.4.1.2. FRAP assay. The FRAP assay was done according to the procedure described by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Hawkins Byrne (2006). Absorbance at 595 nm was measured after 30 min in a microplate reader (Tecan Trading AG, Switzerland). The standard solution was made with Trolox (0–1000 μM) and the results were expressed as mmol TE/100 g dry weight.

2.4.1.3. ORAC assay. The ORAC method was described by Zulueta, Esteve, and Frígola (2009). The assay was carried out on a microplate spectrofluorometer (Tecan Trading AG, Switzerland). APPH (2,2-azobis (2-amidinopropane) dihydrochloride; 221 mM) was added and the fluorescence decay was measured every 150 s during 120 min.

2.4.1.4. Reducing power assay. It was measured according to the

method of Yen and Chen (1995). The absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. Butylhydroxytoluene (BHT) was used as positive control. The reducing capacity was obtained directly from the absorbances, and the results were presented using the equivalent to the extract concentration corresponding to 0.5 of absorbance as reference value (Ferreira, Baptista, Vilas-Boas, & Barros, 2007; Guo et al., 2011). This was obtained by interpolating each species to a linear or quadratic model, depending on which one conforms more closely to the assumptions of regression models (homogeneity of variances, linearity and normality). It has been performed by using RStudio 1.2.1335 (RStudio Team, 2019) using R version 3.6.1.

2.4.2. Antimicrobial activity

Sixteen microbial species were tested in this study. These comprised nine gram-negative bacteria: *Chronobacter sakazakii* (ATCC 29544), *Escherichia coli* (ATCC 8739), *Escherichia coli* 0157:H7 (ATCC 35150), *Salmonella enterica* subs. *enterica* (ATCC 49214), *Salmonella typhimurium* (ATCC 13311), *Shigella flexneri* (ATCC 12022), *Shigella sonnei* (ATCC 11060), *Yersinia enterocolitica* (ATCC 27729) and *Pseudomonas aeruginosa* (ATCC 14053); four gram-positive bacteria: *Bacillus cereus* (ATCC 10876), *Listeria monocytogenes* (ATCC 13932), *Staphylococcus aureus* (ATCC 25923) and *Micrococcus luteus* (ATCC 9341a); one mold: *Byssochlamys nivea* (ATCC 22260) and two yeasts: *Candida albicans* (ATCC 14053) and *Cryptococcus neoformans* (CBS 54). The microbial isolates were supplied by the Spanish Type Culture Collection (Burjassot, Valencia, Spain). The test strains were inoculated into Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) supplemented with 0.6% yeast extract (YE) (Merck) and incubated at 37 °C for 24 h for the bacteria and at 25 °C for 48–72 h for the molds and yeasts. The fresh samples (5 g) were extracted by Venturini et al. (2008) method. All extracts used in this study were subject to microbial analysis for quality control. No colonies were isolated from any of the truffle extracts.

Antimicrobial activity was determined with the agar diffusion method using Mueller-Hinton agar (Oxoid, Hampshire, England). Each plate was inoculated with 0.1 mL of the appropriate microbial suspension: bacteria (10^8 cfu/mL), yeasts or molds (10^6 cfu/mL). Then, 15 μl of each extract (deionized sterilized distilled water, methanol, hexane and ethyl acetate) was added to a 6 mm filter paper disk (Filter-Lab, Spain) and placed on the surface of the inoculated plates. The plates were incubated for 24–72 h at the optimal conditions for each microorganism, and the inhibition zones were then measured in millimeters with an automatic device (Flash and Go, IUL instruments, Barcelona, Spain). Results obtained by the agar diffusion method are qualitative, and the microorganisms were classified as: not sensitive (−) for a diameter equal to 8 mm or below; sensitive (+) for a diameter between 8 and 14 mm; very sensitive (++) for a diameter between 14 and 20 mm and extremely sensitive (+++) for a diameter equal to or larger than 20 mm (Elaissi et al., 2011). Control plates were prepared by placing sterile water, ethanol, hexane and ethyl acetate on disks for negative controls. Each inhibition zone diameter was measured three times, and the average was taken. The standard deviations were less than 5%.

2.4.3. Anti-proliferative activity

The anti-proliferative activity of the truffle samples was performed on four human tumor cell lines: NCI-H460 (non-small cell lung cancer), HeLa (cervical adenocarcinoma), HepG2 (hepatocellular carcinoma) and MCF-7 (breast adenocarcinoma). The cytotoxic activity was evaluated in one non-tumor primary cell culture obtained from porcine liver (PLP2). For both, 6 concentrations of the extracts were used (400, 200, 100, 50, 25, 12.5 μg/mL), following the same extraction conditions as for phenolic compounds, but re-dissolving the lyophilized extracts at 8 mg/mL. The analyses were performed for each of the dilutions of the extract following the Sulforhodamine B (SRB) assay, as previously described by Corrêa et al. (2015). The results were expressed as GI_{50} value (μg/mL), which corresponds to the concentration of extract that inhibited 50% of cell proliferation.

Solvents used for extraction and detection methods for all analysis performed are referenced in Supplementary Table 1.

2.5. Statistical analysis

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 25 (IBM Corporation, New York, USA). Three samples were used for each preparation and all the assays were carried out in triplicate. The results were expressed as mean values ± standard deviation (SD). An analysis of variance (ANOVA) was applied, and the typical requirements, homoscedasticity by the Levene test and normal distribution by the Shapiro Wilk's test, were preliminarily performed. The Welch test was applied to verify the existence of statistically significant differences. The ANOVA results were classified using the Tukey HSD test or Tamhane's T2, when homoscedasticity was verified or not, respectively.

3. Results and discussion

3.1. Chemical composition of truffles

3.1.1. Proximate analysis

The results of the proximate analysis and estimated energetic value obtained for the ten truffle species are shown in Table 2. Protein was found in high levels and varied between 14.04 and 24.15 g/100 g dw, the highest levels registered by *T. magnatum* and *T. arenaria*. This late species also revealed the highest amounts of crude fat (5.10 g/100 g dw). However, the fat content was relatively low for all species, ranging between 0.99 and 5.10 g/100 g dw. Ash content varied between approximately 0.01 and 4.3 g/100 g dw. Carbohydrates, calculated by difference, were the main macronutrient and ranged from 69.0 and 80.7 g/100 g dw. Carbohydrates are usually the principal component of the fruiting bodies, also including fiber such as the structural

Table 2
Proximate chemical composition and energetic value of ten truffle species (mean ± SD; $n = 9$).

Species	Protein (g/100g dw)	Fat (g/100g dw)	Ash (g/100g dw)	Carbohydrates (g/100 g dw)	Energy (Kcal/100 g dw)
T. aestivum	19.9 ± 0.4^{e}	0.99 ± 0.04^{g}	0.0074 ± 0.0001^{d}	79.1 ± 0.2^{c}	404.9 ± 0.1^{f}
T. brumale	22.12 ± 0.04^{d}	2.28 ± 0.02^{e}	0.010 ± 0.001^{d}	75.60 ± 0.05^{f}	411.34 ± 0.07^{d}
T. gennadii	16.93 ± 0.04^{g}	2.67 ± 0.01^{d}	0.006 ± 0.001^{d}	80.39 ± 0.02^{b}	413.31 ± 0.03^{c}
T. indicum	16.7 ± 0.1^{h}	2.6 ± 0.2^{d}	0.0075 ± 0.0001^{d}	80.7 ± 0.2^{a}	412.7 ± 0.6^{c}
T. magnatum	24.15 ± 0.06^{a}	1.52 ± 0.06^{f}	0.0112 ± 0.0005^{d}	74.32 ± 0.09^{g}	407.5 ± 0.2^{e}
T. melanosporum	22.43 ± 0.06^{c}	2.3 ± 0.3^{e}	0.00793 ± 0.00003^{d}	75.3 ± 0.2^{f}	411.3 ± 0.9^{d}
T. oligospermum	19.38 ± 0.03^{f}	3.76 ± 0.01^{b}	0.010 ± 0.001^{d}	76.85 ± 0.01^{e}	418.76 ± 0.05^{a}
T. arenaria	23.2 ± 0.2^{b}	5.10 ± 0.07^{a}	4.2 ± 0.2^{b}	76.6 ± 0.2^{e}	394.4 ± 0.3^{h}
T. leptoderma	14.04 ± 0.09^{i}	3.4 ± 0.7^{c}	4.3 ± 0.2^{a}	78.3 ± 0.7^{d}	400 ± 2^{g}
T. magnusii	16.94 ± 0.09^{g}	2.2 ± 0.2^{e}	2.66 ± 0.02^{c}	69.0 ± 0.2^{h}	414.9 ± 0.2^{b}

Different letters in each column indicate significant differences between the samples ($p < 0.05$). dw- dry weight

polysaccharides β-glucans, chitin, hemicelluloses and pectic substances (Kalač, 2013). However, it is known that the compounds contents of truffles are affected by several factors, namely the species, within individual carpophores, the stage of development, the part sampled and the location (Harki, Bouya, & Dargent, 2006; Tejedor-Calvo, Morales, García-Barreda, et al., 2020). Truffles, as many mushroom studies report (Kalač, 2013), are a good source of carbohydrates and proteins and have low amounts of fat, making them low-energy foods.

3.1.2. Nutrient composition of truffles – Fatty acids, tocopherols and soluble sugars

The fatty acids profile, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied truffles are shown in Table 3. The major fatty acids found in the studied samples were linoleic acid (C18:2$n-6$) and oleic acid (C18:1$n-9$), followed by stearic acid (C18:0) and palmitic acid (C16:0), and the concentrations varying between species. Palmitic acid levels stand out in *T. magnatum* and *T. arenaria* (42.4 and 25.3% respectively).

Table 3
Fatty acids composition (mean ± SD; % of total FA; $n = 9$).

	T. aestivum	*T. brumale*	*T. gennadii*	*T. indicum*	*T. magnatum*	*T. melanosporum*	*T. oligospermum*	*T. arenaria*	*T. leptoderma*	*T. magnusii*
C6:0	0.94 ± 0.09^{c}	0.047 ± 0.002^{g}	0.13 ± 0.01^{f}	1.6 ± 0.1^{b}	2.6 ± 0.3^{a}	0.05 ± 0.04^{g}	0.27 ± 0.01^{e}	0.82 ± 0.02^{d}	nd	0.241 ± 0.001^{e}
C8:0	0.28 ± 0.05^{c}	0.182 ± 0.001^{e}	0.14 ± 0.01^{f}	0.78 ± 0.01^{b}	1.24 ± 0.05^{a}	0.124 ± 0.008^{f}	0.14 ± 0.01^{f}	0.24 ± 0.05^{d}	nd	0.17 ± 0.01^{e}
C10:0	0.06 ± 0.01^{c}	0.06 ± 0.01^{c}	0.06 ± 0.01^{c}	0.094 ± 0.001^{b}	0.17 ± 0.02^{a}	0.034 ± 0.001^{d}	0.039 ± 0.001^{d}	0.012 ± 0.001^{f}	0.036 ± 0.009^{d}	0.026 ± 0.001^{e}
C12:0	0.15 ± 0.02^{c}	0.12 ± 0.01^{d}	0.14 ± 0.01^{c}	0.19 ± 0.02^{b}	0.66 ± 0.06^{a}	0.086 ± 0.001^{e}	0.11 ± 0.01^{d}	0.073 ± 0.001^{ef}	0.056 ± 0.007^{f}	0.065 ± 0.004^{f}
C14:0	0.56 ± 0.05^{c}	0.34 ± 0.03^{f}	0.32 ± 0.02^{f}	0.66 ± 0.02^{b}	1.903 ± 0.01^{a}	0.211 ± 0.001^{g}	0.38 ± 0.02^{e}	0.40 ± 0.02^{d}	0.16 ± 0.01^{h}	0.22 ± 0.01^{g}
C14:1	0.049 ± 0.004^{fg}	0.035 ± 0.001^{gh}	0.030 ± 0.001^{h}	0.787 ± 0.004^{b}	3.30 ± 0.06^{a}	0.025 ± 0.001^{h}	0.235 ± 0.006^{c}	0.079 ± 0.004^{e}	0.182 ± 0.005^{d}	0.059 ± 0.001^{f}
C15:0	0.32 ± 0.02^{b}	0.19 ± 0.01^{e}	0.118 ± 0.001^{g}	0.20 ± 0.01^{d}	0.99 ± 0.02^{a}	0.135 ± 0.002^{f}	0.287 ± 0.004^{c}	0.183 ± 0.001^{e}	0.075 ± 0.007^{h}	0.071 ± 0.004^{h}
C16:0	20.6 ± 0.5^{c}	10.0 ± 0.3^{i}	16.0 ± 0.2^{e}	13.69 ± 0.06^{g}	42.4 ± 0.2^{a}	11.17 ± 0.07^{h}	15.58 ± 0.03^{f}	25.3 ± 0.5^{b}	10.0 ± 0.1^{i}	17.3 ± 0.1^{d}
C16:1	0.64 ± 0.02^{c}	0.39 ± 0.03^{f}	0.52 ± 0.01^{d}	0.11 ± 0.01^{h}	0.82 ± 0.02^{b}	0.388 ± 0.004^{f}	0.290 ± 0.001^{g}	0.43 ± 0.03^{e}	0.109 ± 0.004^{h}	1.10 ± 0.02^{a}
C17:0	0.600 ± 0.001^{b}	0.39 ± 0.03^{c}	0.234 ± 0.004^{h}	0.28 ± 0.01^{g}	1.02 ± 0.01^{a}	0.33 ± 0.01^{e}	0.355 ± 0.008^{d}	0.295 ± 0.004^{f}	0.17 ± 0.01^{i}	0.145 ± 0.001^{j}
C18:0	5.59 ± 0.08^{f}	3.35 ± 0.03^{i}	5.143 ± 0.004^{g}	20.96 ± 0.09^{a}	15.4 ± 0.2^{d}	2.46 ± 0.02^{j}	10.8 ± 0.2^{e}	19.3 ± 0.2^{b}	18.7 ± 0.1^{c}	4.96 ± 0.01^{h}
C18:1n9	28.6 ± 0.3^{b}	4.023 ± 0.004^{j}	19.5 ± 0.5^{d}	49.2 ± 0.2^{a}	13.1 ± 0.1^{h}	18.19 ± 0.03^{e}	14.7 ± 0.4^{g}	12.9 ± 0.6^{i}	15.4 ± 0.5^{f}	22.26 ± 0.03^{c}
C18:2n6	37.1 ± 0.2^{g}	78.3 ± 0.2^{a}	55.4 ± 0.7^{c}	6.91 ± 0.03^{j}	10.1 ± 0.2^{i}	61.12 ± 0.03^{b}	52.3 ± 0.3^{d}	30.9 ± 0.6^{h}	51.3 ± 0.2^{e}	49.7 ± 0.1^{f}
C18:3n3	0.18 ± 0.01^{e}	0.622 ± 0.001^{c}	0.161 ± 0.001^{f}	0.052 ± 0.001^{i}	0.13 ± 0.01^{g}	3.648 ± 0.004^{a}	0.594 ± 0.006^{d}	1.39 ± 0.06^{b}	0.195 ± 0.001^{e}	0.107 ± 0.008^{h}
C20:0	0.34 ± 0.03^{d}	0.31 ± 0.01^{f}	0.25 ± 0.01^{i}	0.33 ± 0.01^{e}	0.49 ± 0.01^{c}	0.296 ± 0.001^{g}	0.498 ± 0.006^{c}	1.34 ± 0.02^{a}	0.544 ± 0.001^{b}	0.262 ± 0.004^{h}
C20:1	0.28 ± 0.01^{e}	0.226 ± 0.003^{g}	0.28 ± 0.01^{e}	0.83 ± 0.01^{a}	0.21 ± 0.02^{h}	0.25 ± 0.01^{f}	0.502 ± 0.006^{b}	0.05 ± 0.02^{i}	0.403 ± 0.004^{c}	0.392 ± 0.006^{d}
C20:2	0.29 ± 0.02^{e}	0.258 ± 0.001^{f}	0.398 ± 0.004^{d}	0.135 ± 0.001^{h}	0.094 ± 0.01^{i}	0.43 ± 0.02^{c}	0.66 ± 0.04^{b}	0.23 ± 0.06^{g}	0.85 ± 0.01^{a}	0.27 ± 0.02^{ef}
C20:3n3+C21:0	0.41 ± 0.01^{c}	0.13 ± 0.01^{h}	0.20 ± 0.01^{f}	0.44 ± 0.02^{b}	0.72 ± 0.03^{a}	0.067 ± 0.001^{j}	0.245 ± 0.002^{e}	0.33 ± 0.03^{d}	0.10 ± 0.02^{i}	0.170 ± 0.008^{g}
C20:5n3	0.06 ± 0.02^{c}	0.052 ± 0.001^{d}	0.084 ± 0.003^{b}	nd	nd	0.062 ± 0.001^{c}	0.187 ± 0.004^{a}	nd	nd	nd
C22:0	2.1 ± 0.1^{d}	0.88 ± 0.01^{g}	0.56 ± 0.01^{h}	2.34 ± 0.07^{c}	3.65 ± 0.02^{b}	0.58 ± 0.03^{h}	1.51 ± 0.07^{e}	5.60 ± 0.02^{a}	1.4 ± 0.2^{f}	0.912 ± 0.007^{g}
C22:1n9	0.11 ± 0.03^{c}	nd	0.040 ± 0.002^{e}	0.11 ± 0.02^{c}	0.79 ± 0.05^{a}	0.074 ± 0.004^{d}	0.132 ± 0.001^{b}	0.05 ± 0.01^{e}	nd	0.128 ± 0.003^{b}
C23:0	0.054 ± 0.001^{d}	0.075 ± 0.002^{c}	nd	0.035 ± 0.001^{f}	nd	0.041 ± 0.004^{e}	0.075 ± 0.004^{c}	0.08 ± 0.01^{b}	0.094 ± 0.006^{a}	0.053 ± 0.002^{d}
C22:6n3	0.12 ± 0.02^{a}	nd	0.037 ± 0.001^{c}	nd	nd	0.056 ± 0.006^{b}	nd	nd	nd	nd
C24:0	0.23 ± 0.01^{a}	0.10 ± 0.01^{f}	0.075 ± 0.004^{g}	0.119 ± 0.001^{e}	0.21 ± 0.01^{c}	0.064 ± 0.004^{h}	0.219 ± 0.004^{b}	0.14 ± 0.01^{d}	0.219 ± 0.002^{b}	0.23 ± 0.02^{a}
C24:1	0.32 ± 0.01^{b}	nd	0.17 ± 0.01^{c}	0.16 ± 0.02^{c}	nd	0.115 ± 0.002^{d}	nd	nd	0.09 ± 0.02^{e}	1.14 ± 0.01^{a}
SFA (relative percentage)	31.8 ± 0.5^{d}	16.0 ± 0.3^{i}	23.2 ± 0.3^{h}	41.3 ± 0.2^{c}	70.7 ± 0.3^{a}	15.58 ± 0.07^{j}	30.23 ± 0.04^{f}	53.7 ± 0.2^{b}	31.4 ± 0.2^{e}	24.7 ± 0.1^{g}
MUFA (relative percentage)	30.0 ± 0.2^{b}	4.66 ± 0.03^{j}	20.5 ± 0.5^{d}	51.2 ± 0.2^{a}	18.2 ± 0.1^{f}	19.04 ± 0.02^{e}	15.8 ± 0.4^{h}	13.5 ± 0.7^{i}	16.2 ± 0.4^{g}	25.07 ± 0.01^{c}
PUFA (relative percentage)	38.2 ± 0.2^{g}	79.3 ± 0.3^{a}	56.3 ± 0.8^{c}	7.54 ± 0.01^{j}	11.1 ± 0.2^{i}	65.38 ± 0.05^{b}	54.0 ± 0.4^{d}	32.8 ± 0.5^{h}	52.4 ± 0.3^{e}	50.3 ± 0.1^{f}

Different letters in each row indicate significant differences between the samples ($p < 0.05$). SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids. nd- not detected. Caproic acid (C6:0); caprylic acid (C8:0); capric acid (C10:0); lauric acid (C12:0); myristic acid (C14:0); myristoleic acid (C14:1); pentadecanoic acid (C15:0); palmitic acid (C16:0); palmitoleic acid (C16:1); heptadecanoic acid (C17:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6); α-linolenic acid (C18:3n3); arachidic acid (C20:0); eicosenoic acid (C20:1); *cis*-11,14-eicosadienoic acid (C20:2); *cis*-11,14,17-eicosatrienoic acid and heneicosanoic acid (C20:3n3 + C21:0); *cis*-5,8,11,14,17-eicosapentaenoic acid (C20:5n3); behenic acid (C22:0); erucic acid (C22:1n9); tricosanoic acid (C23:0); Docosahexaenoic acid (C22:6n3); lignoceric acid (C24:0); nervonic acid (C24:1).

Stearic acid reached levels of about 20% in *T. indicum* and two *Terfezia* truffles, *T. arenaria* and *T. leptoderma.* PUFA were predominant in the studied species, except for *T. indicum,* where MUFA content were higher, and *T. magnatum* and *T. arenaria*, where SFA predominated. For these two last species, SFA ranged from 53.7% to 70.7%. This fact is consistent with data found in the literature, which reports that, in mushrooms and truffles, unsaturated fatty acids predominate over saturated fatty acids (Kalač, 2013; Reis, Barros, Martins, & Ferreira, 2012).

The tocopherols content of truffles is shown in Table 4. *T. brumale* and *T. melanosporum* had the highest content of α-tocopherol (160.5 and 54.2 μg/100 g dw, respectively). β-Tocopherol was the most abundant vitamer in the analysed species, and the highest contents were obtained for the same species (401 and 272 μg/100 g dw, respectively). γ-Tocopherol was only found in two species, *T. brumale* and *T. oligospermum*. Moreover, δ-tocopherol was preferentially identified in *T. magnatum* (96 μg/100 g dw). Up to now, tocopherols had only been found in *Tirmania* truffles (Stojković et al., 2013). However, there is still no information about the four vitamins (α-, β-, γ- and δ-tocopherols) in *Tuber* species. Tocopherols are one of the most important natural antioxidants because of their ability to scavenge free radicals, involving a tocopherol–tocopheryl semiquinone redox system. Furthermore, tocopherols were shown to exhibit various beneficial effects on degenerative diseases, among them, Alzheimer's disease or certain types of cancer (Saldeen & Saldeen, 2005).

The soluble sugars profile is presented in Table 5. All the studied truffles presented mannitol and trehalose as the main sugars, reaching up to 2.6 and 1.4 g/100 g dw, respectively. However, mannitol was not detected in *T. arenaria,* and trehalose was only reported in five species (*T. aestivum, T. brumale, T. gennadii, T. arenaria* and *T. leptoderma*). In addition, fructose was only found in three species, *T. indicum*, *T. melanosporum* and *T. magnusii*. The presence of these sugars has been already reported in *Tirmania* truffles (Stojković et al., 2013). It should be highlighted that trehalose is a storage carbohydrate and transport sugar with many other biological functions such as protection against nutrition deficiency, hyperosmotic, saline stress and glucose homeostasis modulator (Yaribeygi, Yaribeygi, Sathyapalan, & Sahebkar, 2019). Given the relatively high content in mannitol and trehalose, *T. leptoderma*, *T. aestivum*, and *T. brumale* species were the ones that presented higher values of total sugars (Table 5).

3.1.3. Truffle bioactive compounds

The results obtained for the organic acids profile are also presented in Table 6. The most abundant organic acid was oxalic acid in *Terfezia* truffles (*T. arenaria* and *T. magnussi* with 141.9 and 37 mg/100 g dw, respectively). Fumaric acid was only detected in *T. melanosporum* (≈ 0.03 mg/100 g dw). In the remaining species, organic acids were detected in trace amounts, in contrast to other studies that reported citric acid as the most abundant organic acid in *Tirmania* genus (1.26 g/100 g dw) (Stojković et al., 2013).

Total phenolic acid (TPC) levels (Table 7) varied between different species, from 22 to 290 mg GAE/100 g. *T. magnatum* was the species with the highest TPC values following by *T. melanosporum* and *T. leptoderma* (138 and 134 mg GAE/100 g). In contrast, *T. indicum* species obtained the minor levels. The phenolic acids profile of the studied truffles is also presented in Table 7. The highest levels of gallic, *p*-hydroxybenzoic and *p*-coumaric acids were reported for *T. melanosporum*. This species also revealed high levels of TPC. The related compound cinnamic acid was only detected in *T. arenaria* species. In a previous study, only *p*-hydroxybenzoic acid, baicalein, kaempferol, and secoisolariciresinol were reported in *T. aestivum* extracts, while gallic acid, quercetin, quercetin-3-*O*-glucoside, hyperoside and rutin were mainly found in *T. magnatum* (Beara et al., 2014). Also, gallic, *p*-hydroxybenzoic, *o*-hydroxybenzoic and a dihydroxybenzoic acid isomer were detected in *T. melanosporum* ethanolic extracts (Savini et al., 2017). In general, the results obtained for the phenolic acids

Table 5
Soluble sugars composition (mean ± SD; g/100 g dw; $n = 9$).

Species	Fructose	Mannitol	Trehalose	Total sugars
T. aestivum	nd	1.44 ± 0.05^{d}	1.4 ± 0.1^{a}	2.81 ± 0.08^{b}
T. brumale	nd	1.73 ± 0.04^{c}	1.09 ± 0.04^{c}	2.8 ± 0.1^{b}
T. gennadii	nd	1.32 ± 0.01^{e}	1.05 ± 0.04^{c}	2.37 ± 0.02^{c}
T. indicum	0.310 ± 0.001^{a}	1.8 ± 0.1^{b}	nd	2.1 ± 0.1^{d}
T. magnatum	nd	0.23 ± 0.01^{h}	nd	0.23 ± 0.01^{i}
T. melanosporum	0.200 ± 0.001^{b}	1.24 ± 0.05^{f}	nd	1.44 ± 0.05^{f}
T. oligospermum	nd	0.83 ± 0.04^{g}	nd	0.83 ± 0.04^{g}
T. arenaria	nd	nd	0.63 ± 0.01^{d}	0.63 ± 0.01^{h}
T. leptoderma	nd	2.6 ± 0.1^{a}	1.18 ± 0.06^{b}	3.8 ± 0.1^{a}
T. magnusii	0.18 ± 0.01^{c}	1.48 ± 0.04^{d}	nd	1.66 ± 0.04^{e}

Different letters in each column indicate significant differences between the samples ($p < 0.05$). nd- not detected.

Table 6
Organic acids composition (mean ± SD; mg/100 g dw; $n = 9$).

Species	Oxalic acid	Fumaric acid	Total organic acids
T. aestivum	87.1 ± 0.9^{b}	tr	87.1 ± 0.9^{b}
T. brumale	tr	tr	tr
T. gennadii	tr	tr	tr
T. indicum	tr	tr	tr
T. magnatum	tr	tr	tr
T. melanosporum	tr	0.030 ± 0.001	tr
T. oligospermum	tr	tr	tr
T. arenaria	141.9 ± 0.8^{a}	tr	141.9 ± 0.8^{a}
T. leptoderma	tr	tr	tr
T. magnusii	37 ± 2^{c}	tr	37 ± 2^{c}

Different letters in each column indicate significant differences between the samples ($p < 0.05$). tr- trace amounts.

Table 4
Tocopherols composition (mean ± SD; μg/100 g dw; $n = 9$).

Species	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
T. aestivum	0.71 ± 0.01^{g}	nd	nd	34.4 ± 0.4^{c}	35.1 ± 0.4^{f}
T. brumale	160.5 ± 0.1^{a}	401 ± 13^{a}	17 ± 1^{b}	18 ± 1^{f}	597 ± 13^{a}
T. gennadii	nd	nd	nd	83.0 ± 0.5^{b}	83.0 ± 0.5^{e}
T. indicum	0.20 ± 0.01^{h}	nd	nd	22.0 ± 0.7^{e}	22.2 ± 0.7^{g}
T. magnatum	15.9 ± 0.5^{c}	266.1 ± 0.2^{c}	nd	96 ± 7^{a}	378 ± 8^{b}
T. melanosporum	54.2 ± 0.4^{b}	272 ± 11^{b}	nd	nd	326 ± 11^{c}
T. oligospermum	14 ± 1^{d}	104 ± 3^{d}	131 ± 7^{a}	28.6 ± 0.8^{d}	278 ± 12^{d}
T. arenaria	2.7 ± 0.1^{e}	nd	nd	10.8 ± 0.1^{h}	13.5 ± 0.3^{h}
T. leptoderma	0.88 ± 0.03^{g}	nd	nd	12.8 ± 0.4^{g}	13.7 ± 0.5^{h}
T. magnusii	1.5 ± 0.1^{f}	nd	nd	22.5 ± 0.4^{e}	24.0 ± 0.3^{g}

Different letters in each column indicate significant differences between the samples ($p < 0.05$). nd- not detected.

Table 7
Total phenolic compounds (TPC; mean ± SD; mg GAE/100 g fw) and phenolic acids and related compounds composition (mean ± SD; g/100 g dw; $n = 9$).

Species	TPC	Gallic acid	Protocatechuic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid	Cinnamic acid
T. aestivum	67 ± 1^{c}	2.92 ± 0.06^{c}	2.27 ± 0.04^{b}	0.86 ± 0.01^{d}	0.53 ± 0.02^{e}	nd
T. brumale	55 ± 13^{c}	nd	1.99 ± 0.02^{bc}	0.70 ± 0.01^{d}	nd	nd
T. gennadii	54 ± 13^{c}	2.08 ± 0.09^{d}	1.60 ± 0.05^{cd}	nd	nd	nd
T. indicum	22 ± 10^{d}	1.99 ± 0.09^{d}	1.99 ± 0.06^{bc}	0.60 ± 0.03^{de}	nd	nd
T. magnatum	290 ± 5^{a}	5.1 ± 0.2^{b}	1.20 ± 0.05^{de}	2.34 ± 0.04^{b}	2.03 ± 0.06^{b}	nd
T. melanosporum	138 ± 5^{b}	8.3 ± 0.3^{a}	2.2 ± 0.1^{b}	4.5 ± 0.1^{a}	4.1 ± 0.2^{a}	nd
T. oligospermum	50 ± 3^{c}	2.96 ± 0.05^{c}	3.7 ± 0.1^{a}	4.2 ± 0.1^{a}	1.05 ± 0.01^{d}	nd
T. arenaria	74 ± 9^{c}	0.46 ± 0.02^{g}	0.84 ± 0.01^{e}	1.51 ± 0.01^{c}	0.33 ± 0.01^{e}	0.35 ± 0.01
T. leptoderma	135 ± 3^{b}	1.28 ± 0.06^{e}	2.22 ± 0.06^{b}	0.63 ± 0.01^{de}	1.84 ± 0.04^{c}	nd
T. magnusii	61 ± 15^{c}	0.66 ± 0.01^{f}	0.76 ± 0.01^{e}	0.210 ± 0.001^{ef}	nd	nd

Different letters in each column indicate significant differences between the samples ($p < 0.05$). GAE: gallic acid equivalents. nd- not detected.

profile, as well as total phenolic content of the truffle species studied, were in reasonable accordance with other research studies (Tejedor-Calvo, Morales, Marco, et al., 2020; Tejedor-Calvo et al., 2019; Villares et al., 2012). Phenolic acids are generally considered as good antioxidants due to their antioxidant activity as chelators and free radical scavengers with special impact over hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites (Carocho & Ferreira, 2013).

3.2. Testing of the bioactivities

Truffles are an important source of natural bioactive compounds that could be used as potential therapeutic agents (Wang & Marcone, 2011). Specifically, these bioactive compounds, such as fatty acids, organic acids, phenolics, polysaccharides, sterols and terpenoids, have been identified as antidiabetic, anti-inflammatory, antimicrobial, antioxidant, antitumor, and hepatoprotective agents (Lee et al., 2020; Patel, Rauf, Khan, Khalid, & Mubarak, 2017).

The correlation between different bioactivities (antioxidant, antimicrobial and anti-proliferative activities) directly with one or a group of specific bioactive molecules its difficult. So that, in this study, we performed a thorough characterization of the biological activity of potential bioactive compounds in the truffle species analysed.

3.2.1. Evaluation of truffle antioxidant activity

The antioxidant activity was studied using four different methods because no single method is able to provide a complete overall evaluation (Thaipong et al., 2006). When the antioxidant activity was measured using the DPPH method, *T. magnatum* showed the highest content followed by *T. indicum* (4.3 and 3.9 mmol TE/100 g fw respectively), being *T. gennadii* the species with the lowest value (0.18 mmol TE/100 g fw) (Table 8). To make comparisons with the results of a large number of authors, the percentage reduction of DPPH activity must be determined. In our case, *T. magnatum* (83.2%) had the highest value, while *T. gennadii* obtained the lowest (8%), both for a concentration of 25 mg/mL. However, Guo et al. (2011) reported values of around 98% reduction for different *T. indicum* extracts with a concentration of 30 mg/mL, indicating the powerful antioxidant effect of this truffle species.

Turning to the FRAP method of measuring antioxidant activity, the highest values were given by *T. magnatum* followed by *T. indicum* and *T. melanosporum,* ranging from 5.6 to 8.8 mmol TE/100 g fw. *T. gennadii* again had the lowest values (1.54 mmol TE/100 g fw). For *Terfezia* truffles, values range obtained were between 2.6 and 5.3 mmol TE/100 g fw, similar to those obtained in our study for species of the same genus (Al-Laith, 2010). Antioxidant behaviour quantified using the ORAC method gave very similar results to those given by the FRAP test. The highest values once again corresponded to *T. magnatum* and *T. indicum* (3.3 and 3.2 μmol TE/g fw), and the lowest to *T. gennadii* (0.06 μmol TE/g fw) which had a value considerably lower than the other species.

Regarding to the reducing power, once again *T. magnatum* (37 mg/mL) presented the lowest values which reflects the highest capacity. The highest values, indicating lower reducing capacity, were given by *T. aestivum* (146 mg/mL). Guo et al. (2011) obtained a value of 30 mg/mL for *T. indicum,* lower than the value shown in our study (57 mg/mL) for the same species. The reducing power could be due to the ability to donate hydrogen ions. Consistent with this, *T. magnatum,* which has greater reducing power, would be capable of reacting with free radicals, stabilising, and blocking radical chain reactions. However, the BHT reducing power value is noticeably lower (<5 mg/mL) than those of the fungi extracts analysed, suggesting that artificial antioxidants have higher reducing capacity than the antioxidant extracts taken from the truffles.

In truffles, the antioxidant-conferring agents were catechin, ferulic acid, p-coumaric acid, and cinnamic acid, among others (Beara et al., 2014; Villares et al., 2012). *T. magnatum* was the most promissory species presenting the highest TPC levels and the best antioxidant activity

Table 8
Antioxidant activity (DPPH, FRAP, ORAC, and Reducing power) of ten species of truffles.

Species	DPPH		FRAP (mmol TE/100 g fw)	ORAC (μmol TE/g fw)	Reducing power (mg truffle/mL)[b]
	(mmol TE/100 g fw)	% reduction[a]			
T. aestivum	0.9 ± 0.1^{f}	21 ± 2^{e}	3.1 ± 0.2^{d}	1.2 ± 0.3^{cd}	146.0
T. brumale	0.84 ± 0.02^{fg}	19 ± 1^{ef}	5.3 ± 0.3^{c}	0.7 ± 0.3^{de}	94.5
T. indicum	3.9 ± 0.1^{b}	53 ± 1^{b}	8.0 ± 0.3^{b}	3.2 ± 0.7^{a}	56.7
T. gennadii	0.18 ± 0.01^{h}	8 ± 2^{g}	1.54 ± 0.05^{g}	0.06 ± 0.01^{e}	127.3
T. magnatum	4.3 ± 0.2^{a}	83 ± 2^{a}	8.8 ± 0.3^{a}	3.3 ± 0.5^{a}	37.0
T. melanosporum	2.2 ± 0.2^{c}	34.2 ± 0.6^{c}	5.6 ± 0.1^{c}	2.2 ± 0.1^{b}	60.4
T. oligospermum	1.2 ± 0.2^{e}	19.4 ± 0.8^{e}	2.7 ± 0.4^{ef}	1.79 ± 0.08^{bc}	117.2
T. arenaria	1.6 ± 0.1^{d}	24.5 ± 0.6^{d}	2.6 ± 0.4^{f}	1.75 ± 0.06^{bc}	118.7
T. leptoderma	2.14 ± 0.02^{c}	29 ± 2^{c}	5.3 ± 0.4^{c}	1.9 ± 0.3^{bc}	84.2
T. magnusii	0.69 ± 0.06^{g}	15 ± 3^{ef}	2.76 ± 0.07^{e}	1 ± 1^{cd}	44.2
BHT	–	–	–	–	< 5

Data expressed as mean ± standard deviation (SD) of 3 samples. The different superscript letters within the same column indicate statistically significant differences at $p < 0.05$.
a: extract concentration of 25 mg/mL.
b: Reducing power was calculated as the concentration at which the absorbance is 0.5. It was calculated with an interpolation on a quadratic regression model for all species, except for *T. aestivum*, *T. gennadi* and *T. oligospermum* that were interpolated on a lineal regression model. All selected regression models accomplish $R^2 > 0.99$.
TE: Trolox equivalents

(highest mmol TE/100 g and lowest reducing power values). Also, it has high tocopherol content. In contrast, *T. indicum* was one truffle with lower TPC and tocopherols values; however, showed positive antioxidant properties in all test carried out probably due to other compounds. However, as Savini et al. (2017) indicated, antioxidant activity in truffle extracts could decreased during storage regardless of the packaging used.

The studied samples are sources of powerful antioxidants such as phenols and tocopherols, which could be enriched with emerging technologies as stated in recent studies (Tejedor-Calvo, Morales, García-Barreda, et al., 2020), and use them against diseases related to oxidative stress, dermatological applications, cosmetics, as well as supplements in the food industry.

3.2.2. Evaluation of truffle antimicrobial activity

The antimicrobial activity study was carried out on ten truffle species with four extracts (deionized sterilized distilled water, methanol, hexane and ethyl acetate) over sixteen micro-organisms, giving a total of 640 tests. Table 9 shows details of just the five extracts, belonging to four species, in which activity was detected, representing only 0.8% of the total. An inhibitory effect was only found for the aqueous extract of *T. magnusii* with an inhibition zone around the paper disc of 19.7 mm for *S. aureus* and of 8.1 mm for *M. luteus*; for methanol extract of *T. gennadii* against *S. flexneri* (8.1 mm); for aqueous extract of *T. melanosporum* against *S. aureus* (8.7 mm), and for ethyl acetate extract of *T. aestivum* against *L. monocytogenes* (8.5 mm). According to Elaissi et al. (2011) method, *S. aureus* would be highly sensitive to the aqueous extract of *T. magnusii*, while the other cases would be moderately sensitive.

The potential antimicrobial activity on truffles has been scarcely studied. There are only reports in *Tirmania nivea* and *Terfezia claveryi* against different bacteria (Janakat et al., 2004). However, several studies reported positive antimicrobial activity of edible hypogeous fungi compared to edible epigeous species. For example, Venturini et al. (2008) reported 50.2% positive extracts in a study of 49 wild and cultivated mushroom species against various food pathogens. Several authors have attributed this noticeable antimicrobial activity to phenol compounds (Beara et al., 2014; Palacios et al., 2011). Two reasons could be responsible for the low antimicrobial activity of truffles. One is that their mycorrhizal or symbiotic association with the host tree provides them with high resistance or defences so that the development of antimicrobial systems is unnecessary (Culleré, Ferreira, Marco, Venturini, & Blanco, 2017; Molinier et al., 2013). Another is that the abundant microbiota on the surface or peridium of the truffles (Rivera et al., 2011) are mainly made up of gram-negative bacteria that contribute to their development and growth and should not, consequently, be inhibited.

A recent study revealed that *T. arenaria* extracts obtained by the Soxhlet method with dichloromethane presented a significant antibacterial and antifungal activity (Harir et al., 2019). These results agree with those reported by Neggaz et al. (2015) who found that the Soxhlet extract of *T. claveryi* showed greater antimicrobial activities against Gram-positive, Gram-negative, and yeast than that of the maceration extract. According to these studies, extraction method is a key for antimicrobial activity. For that, other extraction methods or solvents could be more effective for antimicrobial activity determination in our samples.

3.2.3. Evaluation of anti-proliferative activity

Cancer is the second leading cause of death worldwide. Phytochemicals, among others, have been reported as crucial agents against the development of tumor cells (Li, Wang, Luo, Zhao, & Chen, 2017). The potential of truffle species extracts for inhibiting tumor cell growth was evaluated in four different tumor cell lines and the results obtained are presented in Table 10, and graphically in the Supplementary Fig. 1. The *Terfezia* and *T. gennadii* extracts revealed anti-proliferate activity in all tumor cells assessed in concentrations ranging from 19 ± 1 to 78 ± 4 μg/mL (in NCI-H460); 33 ± 1 to 206 ± 11 μg/mL (in HeLa); 83 ± 5 to 321 ± 4 μg/ml (in HepG2), and 102 ± 6 to 321 ± 4 μg/mL (in MCF-7). In addition, *T. brumale*, *T. indicum* and *T. oligospermum* extracts showed anti-proliferate activity only in HeLa tumor cells with higher GI_{50} values (194 ± 11 to 301 ± 15 μg/mL). GI_{50} values were above 400 μg/mL in all tumor cells tested with *T. aestivum*, *T. magnatum*, and *T. melanosporum* extracts. On the other hand, only *T. arenaria* reveal toxicity for PLP2, a non-tumor liver cell primary culture (220 ± 2 μg/mL). Given the results obtained, the species under study may be tested for different applications, namely in the food industry, except *T. arenaria*, which presented some toxicity. Overall, the results obtained reveal that *Terfezia* extracts were more active (lower GI_{50} values) against the tumor cells tested than *Tuber* extracts. According to that, *T. claveryi* showed cytotoxic activity against (MCF-7, HT-29, U-87 MG and PC3) cell lines demonstrated with an MTT assay (Dahham et al., 2018). Beara et al. (2014) also tested the aqueous and methanol extracts of *T. magnatum* and *T. aestivum* against tumor cell lines (HeLa, MCF7, and HT-29). The results demonstrated a prominent activity of aqueous extracts towards breast adenocarcinoma (MCF-7), with IC_{50} values ranging from 2.3 to 34.5 μg/mL (Beara et al., 2014). In addition, *T. claveryi* extracts were tested on 4 types of cancer cell lines (U-87 MG, HT 29, MCF-7 and PC3) showing moderate anticancer activities (Dahham et al., 2018). This study speculated that the anticancer activity of *T. claveryi* is due to the presence of these main chemical constituents and the synergistic effect between them, as per only stigmasterol and beta-Sitosterol forms about 47% of the extract. Zhao et al. (2014) studied 52 *Tuber* polysaccharides from a *Tuber*

Table 9
Inhibition of microbial growth by extracts obtained from truffle species.[a]

Species	Extract	Microorganism	Inhibition zone diameter (mm)[b]
T. magnusii	Water	*S. aureus* (ATCC 25923)	19.7 ± 0.8 (++)
T. magnusii	Water	*M. luteus* (ATCC 9341a)	8.1 ± 0.1 (+)
T. aestivum	Ethyl acetate	*L. monocytogenes* (ATCC 13932)	8.5 ± 0.2 (+)
T. gennadii	Methanol	*S. flexneri* (ATCC 12022)	8.1 ± 0.5 (+)
T. melanosporum	Water	*S. aureus* (ATCC 25923)	8.7 ± 0.5 (+)

Positive inhibition of the growth of different micro-organisms in several truffle extracts at a concentration of 0.1 g/mL. Microorganisms were classified as: not sensitive (–) for diameter equal to 8 mm or below; sensitive (+) for diameter between 8 and 14 mm; very sensitive (++) for diameter between 14 and 20 mm and extremely sensitive (+++) for diameter equal to or larger than 20 mm.

[a] Only extracts with antimicrobial activity are included.

[b] Values for zone of growth inhibition measured as the diameter (mm) of the clear zone around the paper disc are averages of three replicates. The diameter of the paper disc (6 mm) is included.

Table 10
Cytotoxic activity of the methanol extracts of truffles against human tumor cell lines and non-tumor cells (mean ± SD; GI_{50} values; n = 9).

Species	Tumor cell lines				Non-tumor cells
	NCI-H460	HeLa	HepG2	MCF-7	PLP2
T. brumale	>400	300 ± 8[a]	>400	>400	>400
T. gennadii	63 ± 2[c]	148 ± 6[f]	231 ± 3[c]	172 ± 6[c]	>400
T. indicum	>400	192 ± 5[d]	>400	>400	>400
T. oligospermum	>400	228 ± 5[b]	>400	>400	>400
T. arenaria	18.9 ± 0.7[d]	33.2 ± 0.4[g]	82 ± 3[d]	103 ± 3[d]	220 ± 2
T. leptoderma	78 ± 2[a]	177 ± 7[e]	283 ± 6[b]	308 ± 5[b]	>400
T. magnusii	74 ± 3[b]	206 ± 6[c]	320 ± 2[a]	321 ± 2[a]	>400

Different letters in each column indicate significant differences between the truffle species ($p < 0.05$). GI_{50} - concentration corresponding to 50% of growth inhibitory activity.

fermentation system, and 12 among them exhibited relatively higher *in vitro* antitumor activity against HepG2, A549, HCT-116, SK-BR-3, and HL-60 cells than those from *Tuber* fruiting body. This study concluded that polysaccharide fractions could promote antitumor activity. According to our results, truffles samples contained high carbohydrates levels (>60 g/100 g) and could be related with anti-proliferative activity. As indicated in recent studies, truffles contained sterols and polysaccharides (Tejedor-Calvo, Morales, García-Barreda, et al., 2020), however further studies are necessary to correlate the bioactivity with these compounds. To the best of our knowledge, there are no studies of the anti-proliferative activity of the remaining truffle species.

4. Conclusions

Tuber and *Terfezia* truffles highlighted by their PUFA, β-tocopherols and phenolic acids profile and contents especially *T. brumale* and *T. melanosporum* for the first two compounds, and *T. magnatum* for the phenol content. However, in respect to the anti/proliferative effects against human tumor cell all *Terfezia* species revealed better effects than *Tuber* ones. Thus, it was not possible to select a particular compound or group of compounds as responsible for the species' bioactivity that could be probably marked by synergistic effects between the different compounds. Therefore, this study presents some preliminary results that may be the basis of future investigations about the potential application of these species as dietary supplements or other applications in the food industry such as antioxidants.

CRediT authorship contribution statement

Eva Tejedor-Calvo: Investigation, Writing - original draft. **Khira Amara:** Investigation, Writing - original draft. **Filipa S. Reis:** Investigation, Methodology, Writing - original draft. **Lillian Barros:** Conceptualization, Methodology, Data curation, Writing - review & editing. **Anabela Martins:** Supervision, Writing - review & editing. **Ricardo C. Calhelha:** Investigation, Methodology. **Maria Eugenia Venturini:** Supervision, Project administration. **Domingo Blanco:** Funding acquisition, Supervision. **Diego Redondo:** Investigation, Methodology. **Pedro Marco:** Conceptualization, Investigation, Writing - review & editing. **Isabel C.F.R. Ferreira:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declarations of interest.

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.110071.

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