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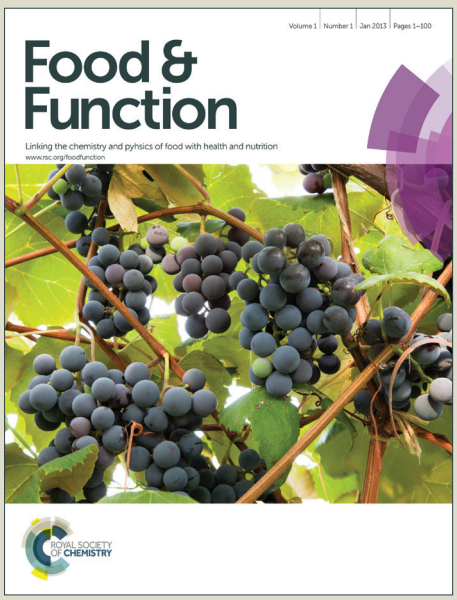

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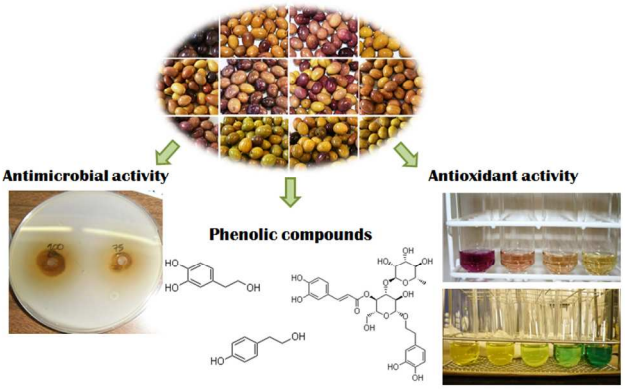

Natural fermented table olives as source of phytochemicals with bioactive properties

Bioactivity and phenolic composition from natural fermented table olives

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Abstract

In the present work, phenolic composition, antioxidant and antimicrobial properties of twenty four samples of naturally fermented table olives from Northeast of Portugal were evaluated.

The analysis of phenolics composition was performed by HPLC/DAD, and ten compounds were identified, being hydroxytyrosol, verbascoside derivate and hydroxytyrosol glycol the most abundant. Total phenolics content varied between 2.37 and 64.17 μg/mg of extract. The IC_{50} values from the antioxidant activity methods tested varied between 0.30 and 1.66 mg/mL for reducing power, and between 0.13 and 0.83mg/mL for DPPH. The results obtained in the antioxidant activity were extremely significantly correlated with the main phenolic compounds as well as with total phenolics content.

A principal component analysis allowed grouping samples according to their phenolic composition and antioxidant potential. Table olives extracts were able to inhibit some pathogenic microorganisms, mainly Gram-positive bacteria. Higher antimicrobial inhibition was recorded in the extracts rich in phenolic compounds and higher antioxidant potential.

Keywords: antimicrobial activity / antioxidant activity / natural fermented table olives / phenolic composition

Introduction

Table olives are among the most popular fermented food products worldwide, and are an important ingredient of the Mediterranean diet. In the last two decades, according to the data released by the International Olive Council (IOC), the worldwide consumption of table olives increased continuously being predicted a consumption of 2 668 000 tonnes during 2013/2014 season.[1] Such popularity is related with their sensorial characteristics and health promoting properties. The beneficial and healthy aspects attributed to table olives are mainly related with their fatty acids composition, and minor compounds content, namely tocopherols, sterols and phenolic compounds.[2-4]

The olive fruits phenolic composition is well studied and documented. According to Amiot et al.[5] phenolic compounds in olive fruits account for approximately for 1 to 2% of the fresh drupe. Oleuropein is the main phenolic compound found in green unprocessed olives, it is a 3,4-dihydroxy-phenylethanol ester with a β-glucosylatedelenolic acid, being responsible for the natural bitterness of the fruit. During the physiological development of the fruit oleuropein content decreases drastically, being observed a prevalence of hydroxytyrosol and its derivates in mature olives. This compounds are associated with diversified bioactive properties, acting as antioxidants,[6] and as antimicrobial agents.[7] The phenolic composition of olive fruits and table olives is affected by several factors that change the bioactivity of table olives as well. Olive cultivar,[8,9] olive maturation,[10] and the technological process applied to turn olives edible[11,12] are among the issues that most affect olives and table olives phenolic composition. In order to achieve edibility olives need to overcome a technological process. In the international market there are three representative kinds of table olives: Spanish-style green olives in brine, Greek-style naturally black olives in brine, and Californian black ripe olives. Among these three technological processes, table olives

from the Californian-style are those who present lower phenolic content, while Spanish- and Greek-style methods provide higher and appreciable amounts of phenolic compounds.[13]

In Portugal, Trás-os-Montes (North-eastern region of Portugal) is the second most important olives producing region. In this region table olives are mainly produced by natural fermentation, being an important socio-economic aspect for producers. The characterization of these table olives is being carried out, and phenolic composition and bioactivity of the natural fermented table olives from Trás-os-Montes has never been assessed before.

In the present work the main objective is to characterize the phenolic composition of natural fermented table olives from Trás-os-Montes region (Northeast of Portugal) as well to study their antioxidant activity and antimicrobial properties. Phenolics from table olives were obtained by aqueous extraction and their profile was determined by HPLC-DAD (high-performance liquid chromatography with a diode array detector). The antioxidant activity was evaluated by reducing power and scavenging effects on DPPH radical assays and the obtained data were correlated with the amount of phenolics found in each sample. The antimicrobial activity was screened using Gram-positive (*Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus epidermis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*).

Results and discussion

Phenolic profile of natural fermented table olives

The phenolic composition of 24 samples of natural fermented table olives from Trás-os-Montes region (detailed information about samples in Table 1) was studied and the obtained profile is reported in Table 2. Among the 24 samples, ten individual phenolic compounds were found (Figures 1 and 2), namely, three phenolic alcohols (hydroxytyrosol glycol, hydroxytyrosol, and tyrosol), three flavones (luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, and apigenin), two hydroxycinnamic acids (verbascoside derivate and verbascoside), one phenolic acid (5-*O*-caffeoylquinic acid), and one flavonol (rutin). The results revealed differences among samples, both in compounds identified as well as in their amounts. Hydroxytyrosol was the most abundant phenolic compound identified in the table olives (average value of 10.76 μg/mg of extract), followed by verbascoside derivate (average of 3.26 μg/mg), and hydroxytyrosol glycol (average of 3.13 μg/mg). Hydroxytyrosol was present in all the 24 samples studied and its content ranged between 0.63 μg/mg (sample 24) and 34.17 μg/mg (sample 17). This compound is reported as the main phenolic compound in processed table olives.[11,12] Hydroxytyrosol could be formed, in part, during olives maturation due to the action of β-glucosidases, esterases and polyphenol oxidase, but also during fermentation of olives. Its formation is due to the hydrolytic cleavage of the ester bond on oleuropein,[14] explaining the absence of oleuropein in the final table olives. During fermentation, oleuropein, the main phenolic compound present in unprocessed olive fruits is converted in several oleuropeinderivates, including hydroxytyrosol. This high content in hydroxytyrosol may confer important properties to table olives since to this compound are ascribed several bioactive properties: antioxidant activity,[15,16] reduction in atherosclerosis development,[17] reduction in the risk of

thrombosis,[18] reduction in oxidative stress,[19] reduce the risk of heart disease,[20] antimicrobial properties,[7] and anti-cancer properties.[21]

Hydroxytyrosol glycol (3,4dihydroxyphenylglycol) was also present in the 24 table olives samples. Its content varied between 0.29 µg/mg (sample 15) and 16.56 µg/mg (sample 22). This C_6-C_2 phenolic compound is not only present in table olives but also in unprocessed olives, olive oil and olive mill waste waters.[22-24] This compound exerts an even higher antioxidant activity than hydroxytyrosol.[25]

Verbascoside derivate was absent in only one sample (sample 16), and varied between 0.05 µg/mg (sample 16) and 19.12 µg/mg (sample 22). Verbascoside and tyrosol were present in considerable amounts, with median values of 2.72 and 1.88 µg/mg, respectively. Apigenin 7-*O*-glucoside, 5-*O*-caffeoylquinic acid, and rutin were present in small amounts, being absent or not quantifiable in many samples (Table 2). Luteolin 7-*O*-glucoside and apigenin were identified in some table olives samples but their amount was below the limit of quantification, being impossible to quantify them.

Concerning total phenols content, they varied between 2.37 µg of total phenols/mg of extract (sample 24), and 64.17 µg/mg (sample 23). Such differences in total phenols content are related mainly with three aspects: i) olive cultivar; ii) the maturation stage of the olive fruits at the harvest time; and iii) the state of the fruits at fermentation. Concerning this last aspect, sometimes prior to fermentation, olive fruits are split lengthwise by cutting into the skin and part of the flesh in order to facilitate brine introduction. This leads to a higher lixiviation of phenolic compounds to the brine, which explains the low content of total phenols observed in samples 6, 7, 11, 15 and 24 (in the remaining samples fruits were fermented as natural as possible).

Antioxidant activity of fermented table olives

The antioxidant activity of the aqueous extracts of natural fermented table olives was assessed by two different chemical assays: reducing power and scavenging effect on DPPH free radicals. In the first method, the presence of reducers and their capacity to reduce the Fe^{3+}/ferricyanide complex to the ferrous form is evaluated in table olives. The more greenish or bluish is the test solution, higher the reducing power displayed by the tested extract. In the second method the antiradical potential of the extracts is evaluated. The loss of absorbance at 517 nm is indicative of scavenging capacity, showing the test solution a yellow-transparent coloration instead of the violet color of the blank solution. Higher the loss in the absorbance, higher the presence of natural antioxidants able to scavenge the free radicals of DPPH, indicative of high antiradical activity.

The results obtained are expressed as IC_{50} values (mg/mL) and as quantity of olive pulp (mg) and are reported in Table 3. In both methods tested a concentration-dependent activity was observed (Figure 3). In the table olives extracts high reducing power was observed at low concentrations. The IC_{50} values varied between 0.30 mg/mL and 1.66 mg/mL in samples 22 and 18 respectively. Among all samples, sample 24 revealed lower reducing power. For this sample, it was only possible to calculate the IC_{25} value (2.67 mg/mL). When IC_{50} values were converted in mass of olive pulp, sample 22 reported 5.05 mg while sample 18 reported 27.94 mg. Concerning sample 24, the IC_{25} value correspond to 44.86 mg of olive pulp (Table 3).

The results observed in the DPPH method are in accordance to those obtained in the reducing power. Sample 22 reported lower IC_{50} value, 0.13 mg/mL, consequently displaying higher antiradical activity, while sample 18 reported lower activity, reporting though higher IC_{50} value, 0.83 mg/mL. As observed in the reducing power method, for sample 24 it was only possible to calculate the necessary extract concentration to

scavenge 25% of the free radicals of DPPH (1.85 mg/mL). When IC_{50} values for DPPH method were converted in mass of olive pulp, sample 22 reported 2.12 mg, sample 18 reported 13.90 mg, and sample 24 reported 31.15 mg which correspond to the IC_{25} value for the same aqueous extract.

Comparatively to other table olives from the Northeast of Portugal, natural fermented table olives extracts revealed similar antioxidant activity than commercial "alcaparras" table olives,[26] but higher activity than monocultivar "alcaparras" table olives.[8] Our results are also comparable to the antioxidant activity of Portuguese table olives from different olive cultivars and processed by different technological treatments.[11] In fact these authors observed that natural fermented table olives were those who reported higher total phenols content and higher antioxidant activity. Comparatively to Greek commercial table olives,[27] a lower quantity of olive pulp is needed to reach the IC_{50} values (DPPH method) in the Portuguese table olives, revealing higher antiradical potential

When pure phenolic compounds were tested (hydroxytyrosol, tyrosol and verbascoside; Figure 3 and Table 3), it was observed that the aqueous extracts of table olives were more active than tyrosol in both methods assayed. When we tested tyrosol in both antioxidant methods even at the highest concentration tested the IC_{50} value was not reached. Hydroxytyrosol revealed extremely high antioxidant activity, with IC_{50} values of 0.034 and 0.014 mg/mL respectively for reducing power and DPPH methods. Among the phenolic compounds tested, verbascoside reported intermediate antioxidant activity with IC_{50} values of 0.121 and 0.030 mg/mL respectively for reducing power and DPPH methods.

The antioxidant activity of the table olives is partially related with the phenolic composition of the extracts. In fact, when a regression analysis was established between

the phenolic profile and total phenols content with the IC_{50} values of both antioxidant assays tested, correlations were established (Table 4). 5-*O*-caffeoylquinic acid wasn't correlated with the antioxidant activity displayed in both methods, as well as apigenin 7-*O*-glucoside in DPPH method. The remaining phenolic compounds as well as total phenols content reported very significant or extremely significant correlations. The equations obtained from the regression analysis revealed negative slope values. In this case, a negative slope indicates that as higher is the content of a determined phenolic compound lower are the IC_{50} values, which means higher antioxidant activity.

In order to summarize the data obtained in the phenolic profile and antioxidant activity of the 24 aqueous extracts of natural fermented table olives a principal component analysis (PCA) was performed. 64.42% of the total variance of the data can be explained by using two principal factors (Figure 4). Samples were gathered in three main groups: one group represented in the negative region of the first principal factor (samples 17, 21, 22 and 23); a second group represented in the central region of the figure; and a third group composed only by sample 24. Sample 24 is represented in both positive regions of the principal factors due its high values obtained in the IC_{25}, and is represented in the extreme opposite region of the first group because sample 24 was the one with lower content on total phenols content (Table 2). Samples 17, 21, 22 and 23 are represented in the negative region of the first principal factor because are the samples with higher total phenols content and those who reported lower IC_{50} values which correspond higher antioxidant activity. Even inside this group, samples 17 and 21 are represented in the positive region of the second principal factor due to being richer in hydroxytyrosol and tyrosol. By other hand samples 22 and 23 are separated from samples 17 and 21 because they are characterized by high content in hydroxytyrosol glycol, verbascoside and its derivate (Figure 4). Therefore, the phenolic composition of

the aqueous extracts is a critical aspect for the contribution of the antioxidant potential of the table olives.

Antimicrobial activity of natural fermented table olives

The antimicrobial activity was tested in the aqueous extracts of six table olives samples (samples 7, 8, 12, 13, 21 and 22). The choice of the samples to be tested was based on their antioxidant potential and extracts availability (Table 3). Samples 7 and 13 revealed, among the samples studied, the lowest antioxidant potential in both antioxidant chemicals assays (sample 24 was not chosen due to extremely low performance). Samples 8 and 12 reported an intermediate antioxidant potential, while samples 21 and 22 were among those samples that exhibited extraordinary high antioxidant capacity.

The antimicrobial assays were tested against four Gram-positive bacteria (*B. cereus*, *B. subtilis*, *S. aureus*, and *S. epidermis*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*). The minimal inhibitory concentration (MIC) values for the tested bacteria were determined to evaluate the antimicrobial potential of the aqueous extracts of table olives samples and are reported in Table 5. The extracts revealed antimicrobial activity against all the microorganisms tested (except some extracts in *E. coli*), in a dose-dependent manner for each microorganism and according to the extract assayed. The results obtained revealed that Gram-positive bacteria were more susceptible to the table olives extracts. For *Bacillus* genus MIC varied among 12.5 and 25 mg/mL for *B. subtilis* and 12.5 and 50 mg/mL for *B. cereus*. Higher growth inhibition for both bacteria were observed in samples 8, 21 and 22 (MIC of 12.5 mg/mL). By other hand, samples 7 and 13 reported lower inhibition growth at higher concentrations (25 and 50 mg/mL, respectively for *B. subtilis* and *B. cereus*) comparatively with the remaining extracts.

Concerning *Staphylococcus* genus, generally, the bacteria tested were more resistant than *Bacillus*. Among *Staphylococcus*, *S. aureus* was more inhibited than *S. epidermis*. For *S. aureus*, sample 21 reported good inhibition growth at 12.5 mg/mL, followed by samples 22 and 8 (25 mg/mL), and finally sample 7 reported lower inhibition growth at 50 mg/mL, the same pattern observed for the *Bacillus* genus bacteria. Meanwhile, the results obtained in *S. epidermis* revealed high MIC values, 50 mg/mL, reporting sample 8 higher inhibition at this concentration. In this bacteria, sample 13 reported lower inhibition, with MIC value of 75 mg/mL.

When we studied Gram-negative bacteria, the differences among table olives extracts were even more notorious (Table 5). *Pseudomonas aeruginosa* was more inhibited by samples 21 and 22, with MIC values of 25 mg/mL, reporting sample 22 higher inhibition growth. Surprisingly, samples 7 and 13 inhibited more *P. aeruginosa* growth than samples 8 and 12, but with the same MIC value (50 mg/mL). *Escherichia coli* were the most resistant bacteria among all tested. Only samples 8, 21 and 22 were capable to inhibit these bacteria at MIC values of 50 mg/mL. Samples 7, 12 and 13 even at 100 mg/mL (maximum concentration tested) were unable to inhibit the bacterial growth.

The results obtained are mainly related to two crucial aspects: i) the microorganisms tested; and ii) extracts composition. Gram-positive bacteria were more susceptible than Gram-negative bacteria, a result in agreement with several works that studied the antimicrobial potential of different plant extracts;[28-31] and table olives.[11,32] Such fact is related with the bacterial cell wall structure. Gram-negative bacteria possess an outer-membrane composed by lipopolysaccharides[33] that protect the microorganisms acting as a permeability barrier, enabling the antimicrobial agents to enter in the bacterial cell,[34] a fact not shared by Gram-positive microorganisms. By other hand, the extracts that exhibited higher antimicrobial potential reported medium-high antioxidant activity, with

total phenols content above 50 μg/mg of extract (samples 21 and 22). As observed in the results of antioxidant activity, the antimicrobial activity of the aqueous extracts is related with the phenolic composition. In fact, the antimicrobial capacity of phenolic compounds is well known.[7,35,36] Thus, in order to assess the possible role of the major phenolic compounds in aqueous extracts, hydroxytyrosol, tyrosol and verbascoside were also tested for their antimicrobial activity. Little antimicrobial capacity was observed for these individual and isolated compounds (data not shown). Such results could be related with their individuality, since according to Borchers et al.[37] extracts may possess higher bioactivity than isolated compounds, because a bioactive individual component can change its properties in the presence of other compounds in the extract, increasing the overall bioactivity displayed.

Comparatively to other table olives, the antimicrobial potential of the aqueous extracts of natural fermented table olives is similar to that presented by "alcaparras" table olives,[32] a particular kind of stoned table olives produced in Trás-os-Montes (Northeast of Portugal). However our results revealed higher bioactivity than table olives from Trás-os-Montes produced by several technological processes.[11]

Experimental

Standards and reagents

Methanol, 2,2-diphenyl-1-picrylhydrazyl, iron (III) chloride, and agar-agar were obtained from Sigma-Aldrich (St. Louis, USA). Methanol (HPLC grade), sodium dihydrogen phosphate dihydrate, potassium hexacyanoferrate (III), formic acid 98-100%, and glucose were purchased from Merck (Darmstadt, Germany). Hydrochloric acid, sodium chloride, and di-sodium hydrogen phosphate 2-hydrate were obtained from Panreac (Barcelona, Spain). Standards used for phenolic profile identification were obtained from Sigma (St. Louis, USA) and Extrasynthèse (Genay, France). Yeast extract, peptone and tryptone were obtained from Himedia (Mumbai, India).The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Table olives sampling

Twenty four samples of natural fermented table olives were collected directly from producers of Trás-os-Montes region (Table 1). Per producer, a sample of 2 kg of olives was collected being transported to laboratory and frozen at -20 ºC until extraction and analysis.

Preparation of the aqueous extracts

For each sample the table olives were freeze-dried at -110 ºC (CoolSafe 110-4 Scanvac, LaboGene, Lynge, Denmark). After freeze-drying samples were mashed and two sub-samples were constituted, being submitted to an aqueous extraction as described by Sousa et al.[32] and Malheiro et al.[8]. Briefly, ≈5 g of table olives (20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman No. 4 paper. The obtained aqueous solutions were frozen and freeze-dried in order to

obtain the aqueous extracts. The extracts were then dissolved in water in concentrations ranging from 0.01 and 5 mg/mL for antioxidant activity assays, 50 mg/mL for phenolic profile evaluation, and between 12.5 and 100 mg/mL for antimicrobial activity.

Phenolic compounds analysis

For identification and quantification purposes of the phenolic compounds of the natural fermented table olives each lyophilized aqueous extract was redissolved in water, filtered (0.2 μm Nylon membrane (Whatman)) and 20 μL were injected in an analytical HPLC Knauer Smartline separation module equipped with a Knauer Smartline autosampler 3800, a cooling system set to 4 ºC and a Knauer Diode Array Detector (DAD). A reversed-phase Spherisorb ODS2 column was used (250 x 4 mm id, 5 μm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel) maintained at 30 ºC. Chromatographic separation was carried out as reported previously [9] using a gradient that consisted on a solvent A (water/formic acid (19:1)) and solvent B (methanol), applied at a flow rate of 0.9 mL/min, as follows: 5% B at 0 min, 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 40% B at 39 min, 45% B at 42 min, 45% B at 45 min, 47% B at 50 min, 48% B at 60 min, 50% B at 64 min and 100% B at 66 min. Detection was achieved with a DAD. Spectral data from all peaks were accumulated in the range 200-600 nm, and chromatograms were recorded at 280, 320, 330 nm and 350 nm. Data acquisition and remote control of the HPLC system was done by ClarityChrom® software (Knauer, Berlin, Germany). The compounds in each extract were identified by comparing their retention times and UV-Vis spectra in the 200-600 nm range with authentic standards analyzed under the same conditions and with the library of spectra previously compiled by the authors. Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Hydroxytyrosol glycol, hydroxytyrosol and tyrosol were determined at 280 nm; 5-*O*-caffeoylquinic acid at 320 nm, verbascoside derivative and verbascoside at 330 nm and all the other compounds at 350 nm. Hydroxytyrosol glycol was quantified as hydroxytyrosol. 5-*O*-caffeoylquinic acidwas quantified as chlorogenic acid. Verbascoside derivative was quantified as verbascoside. The remaining compounds were quantified as themselves.

Antioxidant activity

Reducing power assay

Reducing power was determined according to a previously described procedure.[38] Various concentrations (from 0.01 to 5 mg/mL) of sample extracts (1 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was shaken vigorously and then incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10 % trichloroacetic acid (w/v) was added and then the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge (Centorion K24OR-2003, 4 °C), for 8 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured spectrophotometrically at 700 nm. The extract concentration providing 0.5 of absorbance (IC_{50}) was calculated from the graph of absorbance registered at 700 nm against the correspondent extract concentration. The experiments were performed in triplicate per extract.

DPPH radicals scavenging effect

The ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to the method reported by Malheiro et al..[8] Various concentrations (from 0.01 to 5 mg/mL) of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L).The mixture was shaken vigorously and left to stand in the dark at room temperature until stable absorption values at 517 nm were obtained (60 min). DPPH radical scavenging effect was calculated as the percentage of DPPH discoloration using the following equation:

% DPPH radical scavenging capacity = $[(A_{DPPH} - A_S)/A_{DPPH}]\times100$, where A_S was the absorbance of the solution when the sample extract was added and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of scavenging effect percentage against the extract concentration.The experiments were performed in triplicate per extract.

Antimicrobial activity

For the antimicrobial activity assays 6 table olives samples (7, 8, 12, 13, 21 and 22) were selected according to their availability and the results obtained in the preliminary antioxidant chemical assays.

Microorganisms and culture conditions

Four Gram-positive (*Bacillus cereus, Bacillus subtilis*, *Staphyloccocus aureus* and *Staphyloccocus epidermis*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains were used. All the microorganisms were obtained from the Biology Department of University of Minho (Braga, Portugal). Bacterial stocks cultures were maintained at 4 ºC on LB agar [tryptone 1% (w/v), NaCl 1% (w/v) and agar 2% (w/v)], being sub-cultured periodically at 37 ºC.

380

381 **Preliminary assays for antimicrobial activity**

382 The screening for natural fermented olives activities against Gram-positive and
383 Gram-negative bacteria as well as the determination of the minimal inhibitory
384 concentration (MIC) values was achieved by an adaptation of the agar streak dilution
385 method based on radial diffusion.[39] Suspensions of the microorganisms were prepared
386 and mixed with molten agar (0.8%, w/v) in order to contain approximately 10^6 cfu/mL.
387 A volume of 8 mL of this mixture was seeded as a lawn onto the surface of plates
388 containing the LB assay medium for bacteria. Samples to be tested for antimicrobial
389 potential were placed (85 μL) in a hole made in the center of the solid medium (3 mm
390 depth, 5 mm diameter). The MIC was considered to be the lowest concentration of the
391 tested sample (12.5, 25, 50, 75 and 100 mg/mL) able to inhibit the growth of bacteria
392 (after 24 h at 37 ºC). The diameters of the inhibition zones were measured using a ruler,
393 with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times
394 (in three different plates) and the results were expressed as an average of the radius of
395 the inhibition zone in mm. Plates inoculated with each sensitive indicator
396 microorganism were used as controls.

397

398 **Statistical analysis**

399 **Linear regression analysis**

400 A regression analysis, using Excel from Microsoft Corporation, was established
401 between the individual phenolics identified as well as for total phenols content of the
402 twenty four samples of natural fermented table olives with the IC_{50} values obtained in
403 both antioxidant chemical assays tested (DPPH and reducing power).

404

405 **Principal component analysis**

406 Principal components analysis (PCA) was applied for reducing the number of variables
407 (hydroxytyrosol glycol, hydroxytyrosol, tyrosol, 5-*O*-caffeoylquinic acid, verbascoside
408 derivate, verbascoside, rutin, apigenin 7-*O*-glucoside, total phenols content, and IC_{50}
409 values obtained in both antioxidant assays tested) to a smaller number of new derived
410 variables (principal component or factors) that adequately summarize the original
411 information, i.e., the phenolic composition and antioxidant potential of 24 samples of
412 natural fermented table olives. Moreover, it allowed recognizing patterns in the data by
413 plotting them in a multidimensional space, using the new derived variables as
414 dimensions (factor scores).

415 The aim of the PCA is to produce components suitable to be used as predictors or
416 response variables in subsequent analysis. The number of factors to keep in data
417 treatment was evaluated by the Scree plot, taking into account the eigenvalues, which
418 should have: values greater than one for retaining the factor in the analysis, high values
419 of total percentage of variance explained by the number of components selected internal
420 consistency by means of α-Cronbach's value which should be positive.[40]

421

Conclusions

Natural fermented table olives from Trás-os-Montes revealed to possess phenolic compounds with bioactive properties. The antioxidant activity of the table olives aqueous extracts was directly related with their phenolic composition. The same was verified for the antimicrobial potential. Table olives with high phenolic content and high antioxidant activity displayed higher microbial growth inhibition. Such results highlight the importance of the consumption of natural fermented table olives, being this product capable to prevent diseases in which free radicals are involved as well as to inhibit the growth of most common microorganisms.

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511 **Figures Legends**

512 **Figure 1.** HPLC-DAD of phenolic compounds in natural fermented olives.Detection at
513 280, 320, 330 and 350 nm. Peaks: (**1**) hydroxytyrosol glycol; (**2**) hydroxytyrosol; (**3**)
514 tyrosol; (**4**) 5-*O*-caffeoylquinic acid; (**5**) verbascoside derivative; (**6**) verbascoside; (**7**)
515 luteolin-7-*O*-glucoside; (**8**) rutin; (**9**) apigenin-7-*O*-glucoside; (**10**) apigenin.

516

517 **Figure 2.** Chemical structures of the phenolic compounds identified. (**1**) hydroxytyrosol
518 glycol; (**2**) hydroxytyrosol; (**3**) tyrosol; (**4**) 5-*O*-caffeoylquinic acid; (**6**) verbascoside;
519 (**7**) luteolin-7-*O*-glucoside; (**8**) rutin; (**9**) apigenin-7-*O*-glucoside; (**10**) apigenin.

520

521 **Figure 3.** Reducing power and DPPH scavenging effect of aqueous extracts of samples
522 of natural fermented table olives and hydroxytyrosol, tyrosol and verbascoside (mean ±
523 SE; n = 2).

524

525 **Figure 4.** Principal component analysis obtained from the phenolic composition and
526 antioxidant activity recorded in the samples of natural fermented table olives. PCA
527 factors explain 64.42% of the total variance. RP – reducing power; Hyd. glycol –
528 hydroxytyrosol glycol; 5OCqA – 5-*O*-caffeoylquinic acid; A7Ogl – apigenin 7-*O*-
529 glucoside.

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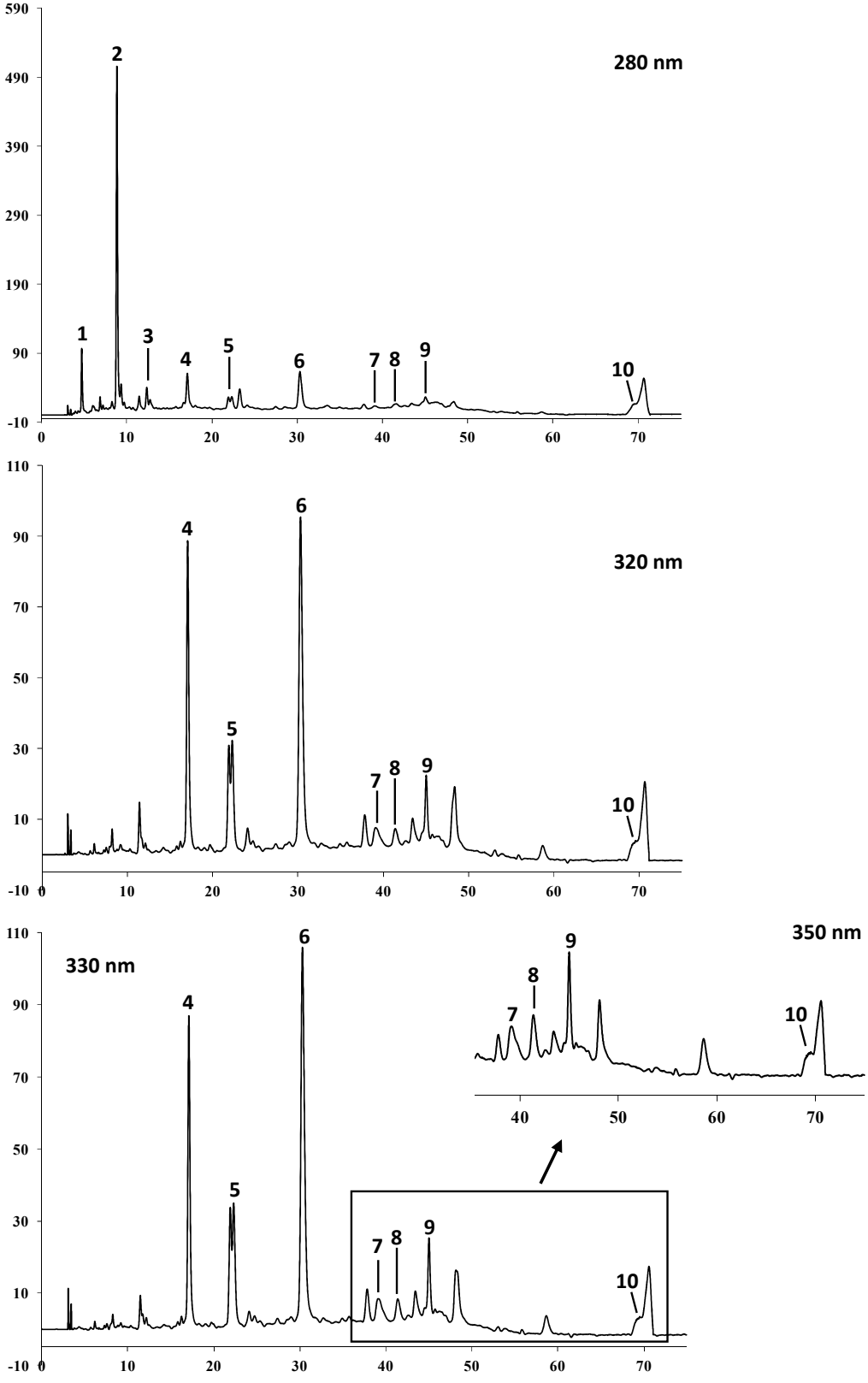
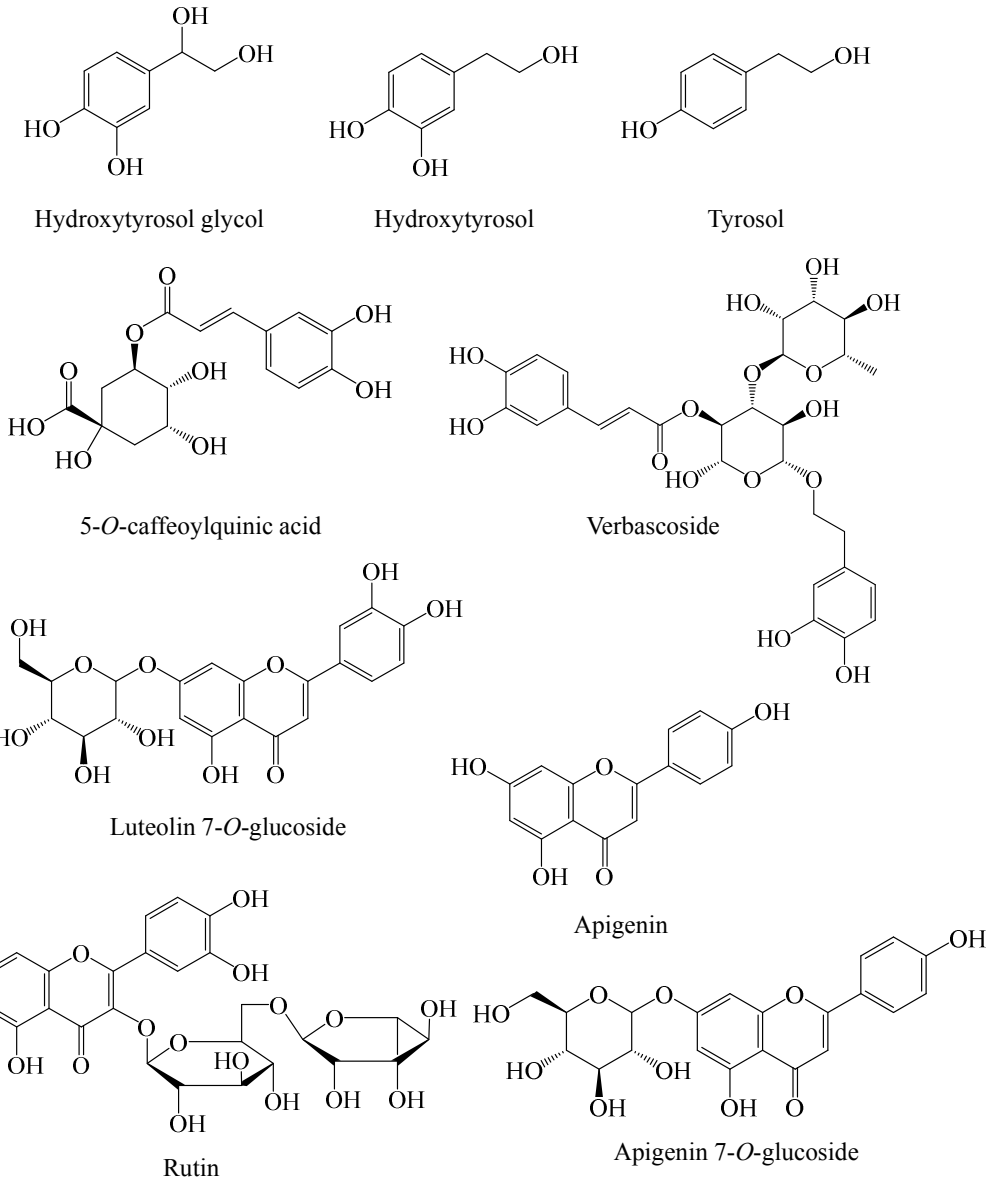


Figure 1.



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534 **Figure 2.**

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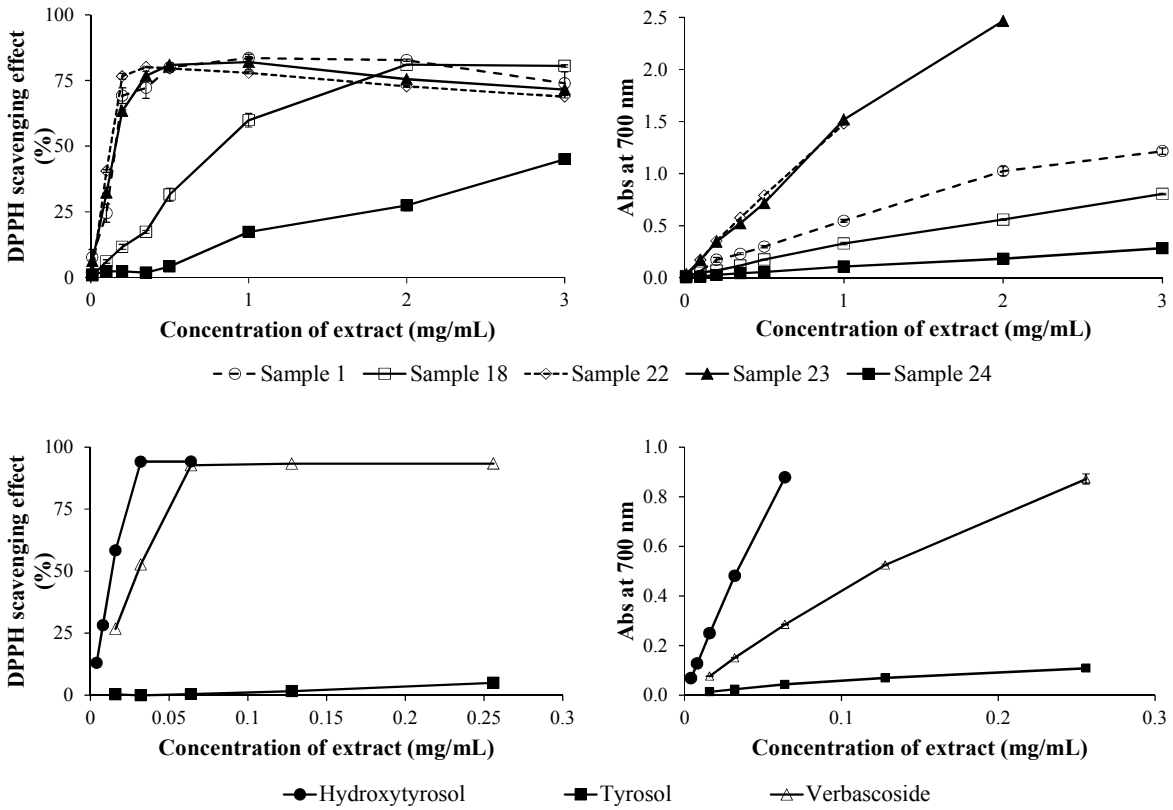

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538 **Figure 3.**

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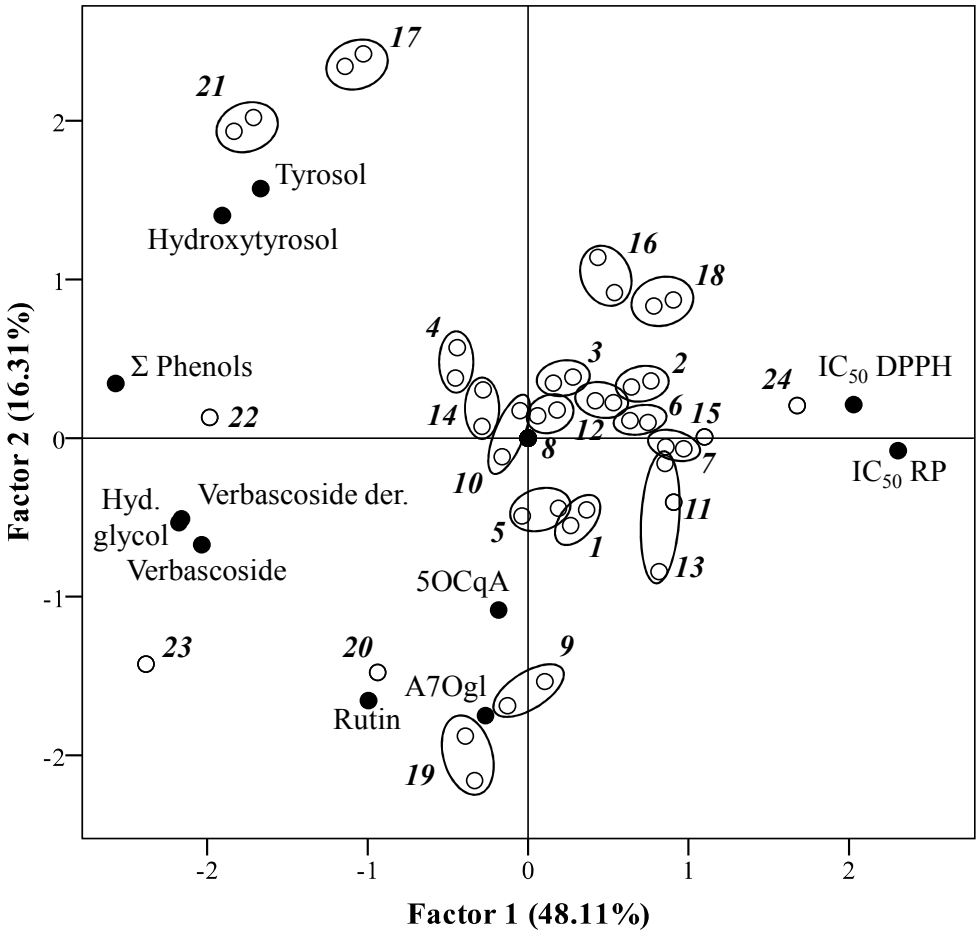

Figure 4.

546 **Table 1.** Basic characteristics of samples.

Sample	Region	Cultivar	Type of olives*	Pulp/stone ratio
1	Mirandela	Cobrançosa	Black olives	5.60 ± 0.78
2	Mirandela	Cobrançosa	Green olives	3.87 ± 0.45
3	Mirandela	Cobrançosa	Olives turning color	5.39 ± 0.52
4	Mirandela	Cobrançosa	Green olives	4.74 ± 0.73
5	Mirandela	Cobrançosa	Olives turning color	5.40 ± 0.51
6	Mirandela	Cobrançosa	Olives turning color	4.77 ± 0.79
7	Valpaços	Cobrançosa	Green olives	3.30 ± 0.67
8	Mirandela	Cobrançosa	Green olives	4.63 ± 0.57
9	Valpaços	Cobrançosa	Olives turning color	5.41 ± 0.60
10	Valpaços	Cobrançosa	Green olives	4.76 ± 0.64
11	Valpaços	Cobrançosa	Olives turning color	5.27 ± 0.77
12	Valpaços	Cobrançosa	Green olives	4.96 ± 0.57
13	Valpaços	Cobrançosa	Olives turning color	5.31 ± 0.51
14	Mirandela	Cobrançosa	Olives turning color	4.87 ± 0.66
15	Valpaços	Negrinha de Freixo	Olives turning color	5.38 ± 1.11
16	Mirandela	Cobrançosa	Olives turning color	5.62 ± 0.68
17	Moncorvo	Negrinha de Freixo	Green olives	5.80 ± 0.76
18	Moncorvo	Negrinha de Freixo	Green olives	4.80 ± 0.58
19	Valpaços	Cobrançosa	Olives turning color	5.20 ± 0.86
20	Mirandela	Cobrançosa	Black olives	5.40 ± 0.78
21	Mirandela	Cobrançosa	Green olives	6.72 ± 1.31
22	Mirandela	Cobrançosa	Olives turning color	5.32 ± 0.73
23	Mirandela	Cobrançosa	Olives turning color	4.65 ± 0.69
24	Mirandela	Cobrançosa	Olives turning color	5.44 ± 0.85

547 *Classification according to Trade Standard Applying to Table Olives (International Olive Council) COI/OT/NC no.
548 1 December 2004.

549

Table 2. Phenolic compounds in natural fermented olives aqueous extracts (μg compound/mg aqueous extract)[a]

Sample	Hydroxytyrosol glycol	Hydroxytyrosol	Tyrosol	5-*O*-caffeoylquinic	Verbascoside derivate	Verbascoside	Luteolin 7-*O*-glucoside	Rutin	Apigenin 7-*O*-glucoside	Apigenin	Total Phenols
1	0.48 ± 0.03	7.70 ± 0.90	0.83 ± 0.08	0.26 ± 0.03	1.40 ± 0.23	1.99 ± 0.35	n.q.	n.q.	0.05 ± 0.01	n.q.	12.70 ± 1.55
2	0.61 ± 0.03	9.40 ± 0.40	1.16 ± 0.01	0.18 ± 0.01	0.29 ± 0.05	0.20 ± 0.02	-	-	n.q.	n.q.	11.84 ± 0.43
3	1.67 ± 0.03	10.38 ± 0.26	1.34 ± 0.08	0.31 ± 0.01	2.25 ± 0.10	1.32 ± 0.13	n.q.	-	n.q.	n.q.	17.26 ± 0.55
4	0.37 ± 0.01	14.84 ± 0.26	1.69 ± 0.03	0.23 ± 0.01	0.89 ± 0.04	9.36 ± 0.09	-	n.q.	n.q.	n.q.	27.37 ± 0.09
5	0.36 ± 0.01	9.11 ± 0.36	0.91 ± 0.06	0.20 ± 0.00	1.09 ± 0.07	4.81 ± 0.61	-	n.q.	0.13 ± 0.01	n.q.	16.60 ± 1.07
6	0.61 ± 0.02	5.59 ± 0.11	0.81 ± 0.06	0.17 ± 0.01	0.65 ± 0.07	0.71 ± 0.07	-	n.q.	-	n.q.	8.55 ± 0.10
7	1.34 ± 0.05	7.34 ± 0.60	1.06 ± 0.07	0.29 ± 0.01	0.20 ± 0.02	0.23 ± 0.01	-	n.q.	n.q.	n.q.	10.46 ± 0.68
8	0.79 ± 0.05	12.51 ± 0.11	1.57 ± 0.20	n.q.	1.11 ± 0.09	2.69 ± 0.12	-	n.q.	0.15 ± 0.01	n.q.	18.83 ± 0.27
9	1.74 ± 0.47	13.18 ± 1.39	1.55 ± 0.12	0.88 ± 0.35	1.21 ± 0.18	2.67 ± 0.44	n.q.	0.59 ± 0.06	0.22 ± 0.02	n.q.	22.03 ± 2.95
10	1.63 ± 0.05	9.63 ± 0.03	0.83 ± 0.04	0.24 ± 0.01	3.09 ± 0.70	3.30 ± 0.55	-	n.q.	n.q.	n.q.	18.73 ± 1.15
11	1.52 ± 0.01	3.59 ± 0.16	0.69 ± 0.10	0.14 ± 0.01	0.85 ± 0.02	0.57 ± 0.03	n.q.	0.18 ± 0.02	-	n.q.	7.54 ± 0.25
12	2.00 ± 0.06	5.32 ± 0.42	0.65 ± 0.07	n.q.	0.34 ± 0.26	0.30 ± 0.13	n.q.	n.q.	n.q.	n.q.	8.63 ± 0.89
13	0.62 ± 0.18	4.91 ± 0.36	0.57 ± 0.06	0.33 ± 0.12	0.75 ± 0.19	1.08 ± 0.35	n.q.	n.q.	0.10 ± 0.02	n.q.	8.35 ± 1.28
14	1.77 ± 0.07	13.48 ± 0.47	1.33 ± 0.39	0.48 ± 0.03	1.76 ± 0.13	2.20 ± 0.32	n.q.	n.q.	n.q.	n.q.	21.02 ± 0.93
15	0.29 ± 0.02	3.57 ± 0.21	0.82 ± 0.06	-	0.05 ± 0.00	0.09 ± 0.01	n.q.	n.q.	n.q.	n.q.	4.83 ± 0.25
16	0.59 ± 0.01	12.29 ± 0.92	2.17 ± 0.20	-	-	-	-	-	-	n.q.	15.05 ± 1.01
17	4.33 ± 0.31	34.17 ± 0.58	10.48 ± 0.39	-	0.20 ± 0.03	0.22 ± 0.06	-	-	n.q.	n.q.	49.40 ± 0.36
18	0.42 ± 0.03	9.21 ± 0.73	1.61 ± 0.14	n.q.	0.11 ± 0.03	0.14 ± 0.03	-	-	-	n.q.	11.49 ± 0.76
19	2.62 ± 0.00	7.42 ± 0.08	0.97 ± 0.06	n.q.	4.37 ± 0.09	3.85 ± 0.08	-	1.34 ± 0.06	0.18 ± 0.00	n.q.	20.76 ± 0.37
20	11.81 ± 0.69	5.32 ± 0.56	-	n.q.	9.44 ± 0.06	2.64 ± 0.04	n.q.	n.q.	0.49 ± 0.05	n.q.	29.71 ± 1.18
21	6.47 ± 0.62	27.29 ± 2.23	7.29 ± 0.34	-	8.21 ± 1.61	4.74 ± 0.36	n.q.	n.q.	n.q.	n.q.	54.01 ± 4.95
22	16.56 ± 0.03	14.81 ± 0.27	1.98 ± 0.09	-	19.12 ± 4.96	8.24 ± 1.81	n.q.	n.q.	-	n.q.	60.72 ± 6.98
23	15.24 ± 0.57	16.50 ± 0.04	2.56 ± 0.06	0.46 ± 0.06	17.51 ± 1.25	8.60 ± 0.88	n.q.	3.31 ± 0.32	-	n.q.	64.17 ± 2.83
24	1.28 ± 0.07	0.63 ± 0.03	0.37 ± 0.01	n.q.	0.10 ± 0.01	-	n.q.	-	n.q.	n.q.	2.37 ± 0.10

[a] Results are expressed as mean ± standard deviation (SD) of two independent determinations; n.q. – not quantifiable

Table 3. Extraction yield and IC_{50} values (mg/mL) of aqueous extracts of natural fermented table olives.

Sample	Extraction yield (%)	Reducing power (IC_{50}[a])	Olive pulp (mg)[b]	DPPH (IC_{50}[c])	Olive pulp (mg)[d]
1	17.19 ± 0.14	0.91 ± 0.07	15.32 ± 1.10	0.16 ± 0.00	2.65 ± 0.06
2	24.74 ± 8.96	1.33 ± 0.13	22.45 ± 2.18	0.53 ± 0.06	8.88 ± 1.08
3	30.95 ± 0.07	1.26 ± 0.04	21.17 ± 0.64	0.38 ± 0.03	6.44 ± 0.44
4	22.66 ± 0.00	0.63 ± 0.01	10.67 ± 0.19	0.44 ± 0.00	7.38 ± 0.07
5	18.47 ± 18.63	0.79 ± 0.05	13.29 ± 0.90	0.42 ± 0.01	7.05 ± 0.23
6	23.85 ± 0.34	1.26 ± 0.12	21.13 ± 2.04	0.47 ± 0.07	7.97 ± 1.14
7	42.49 ± 26.60	1.46 ± 0.08	24.56 ± 1.39	0.68 ± 0.03	11.42 ± 0.54
8	25.95 ± 0.27	0.88 ± 0.03	14.74 ± 0.55	0.37 ± 0.03	5.94 ± 0.85
9	26.51 ± 14.05	1.32 ± 0.04	22.30 ± 0.58	0.50 ± 0.02	7.13 ± 1.35
10	20.59 ± 0.01	0.71 ± 0.05	11.94 ± 0.81	0.28 ± 0.01	4.69 ± 0.22
11	23.90 ± 0.24	1.30 ± 0.04	21.83 ± 0.74	0.75 ± 0.03	12.56 ± 0.44
12	17.50 ± 2.55	0.80 ± 0.09	13.46 ± 1.56	0.42 ± 0.04	7.07 ± 0.65
13	20.44 ± 3.27	1.46 ± 0.10	24.57 ± 1.73	0.72 ± 0.06	12.07 ± 0.98
14	10.61± 6.51	0.49 ± 0.08	8.21 ± 1.39	0.22 ± 0.06	3.66 ± 1.03
15	33.32 ± 4.01	1.59 ± 0.03	26.81 ± 0.44	0.72 ± 0.06	9.25 ± 0.69
16	22.06 ± 0.24	0.96 ± 0.08	16.15 ± 1.31	0.22 ± 0.06	8.98 ± 0.60
17	18.77 ± 3.31	0.36 ± 0.01	6.10 ± 0.24	0.19 ± 0.00	3.20 ± 0.05
18	28.61 ± 1.59	1.66 ± 0.24	27.94 ± 3.97	0.83 ± 0.10	13.90 ± 1.74
19	28.44 ± 5.88	0.63 ± 0.09	10.54 ± 1.56	0.38 ± 0.02	6.44 ± 0.37
20	22.05 ± 5.20	0.38 ± 0.00	6.35 ± 0.05	0.19 ± 0.02	3.23 ± 0.37
21	17.20 ± 0.38	0.41 ± 0.02	6.87 ± 0.31	0.18± 0.01	2.98 ± 0.19
22	26.10 ± 7.82	0.30 ± 0.01	5.05 ± 0.16	0.13± 0.00	2.12 ± 0.05
23	16.33 ± 0.39	0.34 ± 0.03	5.75 ± 0.41	0.16± 0.00	2.63 ± 0.09
24	21.73 ± 0.84	2.67 ± 0.11*	44.86 ± 1.88**	1.85± 0.29*	31.15 ± 4.88***
Standards					
Hydroxytyrosol	-	0.034 ± 0.000	-	0.014 ± 0.000	-
Verbascoside	-	0.121 ± 0.001	-	0.030 ± 0.000	-

[a] IC_{50} (mg/mL): effective concentration at which the absorbance is 0.5;
[b] Quantity of fresh olive pulp necessary to reach the absorbance of 0.5;
[c] IC_{50} (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged;
[d] Quantity of fresh olive pulp necessary to scavenge 50% of the free radicals of DPPH;
* IC_{25} value (mg/mL);
**Quantity of fresh olive pulp necessary to reach the absorbance of 0.25;
***Quantity of fresh olive pulp necessary to scavenge 25% of the free radicals of DPPH.

Table 4. Correlation between phenolic composition of natural fermented table olives and respective antioxidant activity.

	IC_{50} DPPH			IC_{50} Reducing Power		
Phenolic compound	**Equation**	**R^2**	***P***	**Equation**	**R^2**	***P***
Hydroxytyrosol glycol	$y = -0.031x + 0.562$	0.160	***	$y = -0.067x + 1.220$	0.314	***
Hydroxytyrosol	$y = -0.025x + 0.727$	0.264	***	$y = -0.044x + 1.477$	0.345	***
Tyrosol	$y = -0.053x + 0.577$	0.119	**	$y = -0.109x + 1.238$	0.208	***
5-*O*-caffeoylquinic acid	$y = -0.196x + 0.493$	0.045	n.s.	$y = -0.123x + 1.074$	0.005	n.s.
Verbascoside derivate	$y = -0.028x + 0.556$	0.178	***	$y = -0.059x + 1.202$	0.312	***
Verbascoside	$y = -0.042x + 0.508$	0.291	***	$y = -0.108x + 1.211$	0.406	***
Rutin	$y = -0.151x + 0.626$	0.757	***	$y = -0.315x + 1.321$	0.820	***
Apigenin 7-*O*-glucoside	$y = -0.430x + 0.455$	0.109	n.s.	$y = -1.463x + 1.183$	0.324	**
Total phenols	$y = -0.011x + 0.714$	0.325	***	$y = -0.023x + 1.496$	0.513	***

n. s. – not significant;
* $P \leq 0.05$ – significant correlation;
** $P \leq 0.01$ – very significant correlation;
*** $P \leq 0.001$ – extremely significant correlation.

Table 5. Antimicrobial activity of the samples tested of natural fermented table olives. Results expressed as MIC (minimum inhibitory concentration; mg/mL).

Samples	*B. subtilis*	*B. cereus*	*E. coli*	*S. epidermis*	*S. aureus*	*P. aeruginosa*
7	25 (+)	50 (+)	(-)	50 (+)	50 (+)	50 (++)
8	12.5 (++)	12.5 (++)	50 (+)	50 (++)	25 (++)	50 (+)
12	25 (+)	25 (+)	(-)	50 (+)	25 (+++)	50 (+)
13	25 (++)	50 (+)	(-)	75 (++)	50 (+++)	50 (++)
21	12.5 (++)	12.5 (++)	50 (+)	50 (+)	12.5 (++)	25 (+)
22	12.5 (++)	12.5 (++)	50 (+)	50 (+)	25 (++)	25(++)

(-) inhibition zone < 1 mm; Slight antimicrobial activity (+) inhibition zone 2-3 mm; Moderate antimicrobial activity (++) inhibition zone 4-5 mm; High antimicrobial activity (+++) inhibition zone 6-9 mm; Standard deviation ± 0.5 mm.