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Synthesis and recovery of high bioactive phenolics from table-olive brine process wastewater

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ABSTRACT

The generated wastewater of table-olive brine processing contains a high amount of polyphenols which are endowed with interesting biological activities. The ethyl acetate extract (EAE) of such wastewater shows high hydroxytyrosol (HT) and tyrosol concentrations of 690 and 98 mg g^{-1} dry weight extract, respectively. Phenolic compounds analysis was performed by gas chromatography–mass spectrometry (GC–MS). The antioxidant activity was evaluated by the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonate] radicals. Total polyphenol content was estimated with the Folin–Ciocalteu assay. Hydroxytyrosol has DPPH and ABTS radicals scavenging activities higher than 2,6-di-*tert*-butyl-hydroxytoluene (BHT), while triacetylated hydroxytyrosol (triAcHT) was devoid of any antioxidant activity. The assessment of these antioxidant compounds in biological systems was carried out by the determination of their in vitro cytotoxicity against two different human cancer cell lines (HeLa and DG75) and normal peripheral blood lymphocytes (PBL) using the MTT assay. The 50% cytotoxic concentrations were ranged between 27 and 210 μg mL^{-1} for Hela and DG75. At the same concentration range, the EAE and the pure HT and triAcHT exhibited an insignificant cytotoxicity against PBL. Incubation of HeLa and DG75 cells with non-cytotoxic concentrations of EAE, HT or triAcHT resulted in a remarkable protection from the oxidative stress induced by Fe^{2+}. The antimicrobial activity evaluated by the broth dilution NCCL method using Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli, Salmonella enterica*) bacteria and (*Candida albicans*, *Aspergillus niger*) fungi showed a broad spectrum bactericidal and fungicidal effect of table-olives-EAE, HT and triAcHT. The MICs vary from 125 to 500 μg mL^{-1} for bacteria and from 500 to 2000 μg mL^{-1} for fungi.

1. Introduction

Table olives are a traditional product and one of the most important components of the Mediterranean diet. They are well-known sources of phenolic compounds with important biological properties.[1] In addition to the monounsaturated fats; the benefits of table olives in nutrition are associated with minor constituents, such as phenolic compounds.[2]

The world production of table olives is estimated to surpass a million tons per year,[3] with the Mediterranean countries being the main producers. In Tunisia, the table-olives production is highly important for the country's economy, constituting one of the major agro-industrial activities. According to statistical data,[4] the Tunisian table-olive annual production is about 23,000 tons. Meski olive cultivar is characterised by a higher fruit weight (6.4 g) and a good pulp quality, especially at green stage. These physical parameters showed that Meski cultivar have the best criteria of table olives.[4] At the same time, both table-olives and oil-olives processes resulted in notoriously polluting wastewaters which are difficult to treat mainly due to their high polyphenols[5,6] and organic contents.[7,8] There are three principal types of table olives: green, black and black through oxidation. Half of the table-olives production corresponds to Spanish-style green table olives.[9] The

Abbreviations: EAE, ethyl acetate extract of table-olive wastewater; HT, hydroxytyrosol; triAcHT, triacetylated hydroxytyrosol; BHT, butylhydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonate]; BSTFA, bis(trimethylsilyl)-acetamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; OMW, olive mill wastewater; FRAP assay, ferric reducing antioxidant power assay; TBARS assay, thiobarbituric acid-reactive substances assay; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; GC–MS,gas chromatography– mass spectrometry; FPLC, fast performance liquid chromatography.

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procedure consists in treating the fruits with dilute NaOH solution to hydrolyse their natural bitterness (oleuropein), followed by one or two water washes to remove the excess alkali and, finally, a spontaneous lactic acid fermentation in brine for several months.[10] The washing solutions contain a high content of sugar and phenolic compounds, particularly hydroxytyrosol (3,4-dihydroxyphenyl ethanol).[11] These solutions are not accepted in municipal sewers because of their very high organic load and high concentrations of phenolic compounds, which are known to cause toxic effects to living organisms. In all stages, large quantities of clean water are consumed and wastewater is produced at about 0.9–1.9 $m^3\,t^{-1}$ for black olives and 3.9–7.5 $m^3\,t^{-1}$ for green olives, depending on the olive variety, its maturity and the treatment process.[12]

It would be of interest to obtain high-value products from these washing waters, such as organic acids (lactic acid) and polyphenols, particularly hydroxytyrosol and *ortho*-diphenols with a high antioxidant activity[13] and nutritional properties.[14] Because hydroxytyrosol is unstable, particularly in solution, it has to be preserved dried, in darkness and in the absence of air. A recent study demonstrated that triacetyl derivative [4-(acetoxyethyl)-1,2-diacetoxy benzene] was the most suitable compound which had the biological properties like the parent hydroxytyrosol in protecting human cells from oxidative stress-induced toxicity, after the metabolization by esterases, although it is devoid of any chemical antioxidant activity.[15,16]

The phenolic content of olive oil has been under investigation for many years. However, table olives have not been studied to an equal extent. There are several studies about the quantitative and qualitative compositions.[17] Nevertheless, there are only a few studies that present the total antioxidant capacity and cytotoxicity of the phenolic fraction of table olives.[18] Polyphenols belong to the category of natural antioxidants and are the most abundant antioxidants in our diet.[1] They play an important role in human nutrition as preventative agents against several diseases through their protection of the body tissues against oxidative stress. Many studies indicate an antioxidant capacity of these polyphenols with respect to the oxidation of low-density lipoproteins[19] and oxidative alterations due to free radical and other reactive species.[20] Polyphenols intake is beneficial for human health because their antioxidant activity has been associated with a lower risk of coronary heart disease,[21,22] some types of cancer,[2,23] inflammation[22] and inhibition of platelet-activating factor activities.[24]

The aim of this study is to examine the green Meski table-olive processing wastewater extract for its high added value polyphenols that may contribute to the benefits of the human diet. First, the antioxidant capacities of EAE, HT and triAcHT were evaluated by DPPH and ABTS radical-scavenging assays. Then, the chemical antioxidant properties of the tested compounds were compared with their protective effects against the oxidative stress in HeLa and DG75 cell lines. The non-cytotoxic doses used were determined beforehand by the MTT cytotoxic test. Finally, the antimicrobial properties of the extract as well as the two pure compounds were tested against a spectrum of bacterial and fungal strains.

2. Results and discussion

2.1. Physicochemical parameters of wastewaters from table-olives processing

After the debittering and washing steps, the olive fruit loses the major part of its phenolic content which is transferred into wastewaters. Table 1 shows the results of common physicochemical parameters and the chemical analysis of the typical wastewater from the Meski table-olives processing. The dry matter (1.80% (w/v)) of the table-olive processing wastewater is high albeit, its colour is fade and its total phenols concentration is slightly low. The mean values of total polyphenols and flavonoids contents (Table 1) were expressed as milligram of gallic-acid equivalent and quercetin equivalent by milligram of dry weight of extract, respectively. The electrical conductivity (10.17 mS cm^{-1}) was high due to the presence of salts.

Table 1
Common physicochemical parameters and chemical analysis of wastewaters from table-olives processing

Physicochemical parameters	Contents
pH	9.25 ± 0.18
Electrical conductivity (mS cm^{-1})	8.51 ± 0.30
Colour	1.20 ± 0.08
Dry matter (%)	1.80 ± 0.10
Mineral matter (%)	1.36 ± 0.09
Volatile matter (%)	0.44 ± 0.05
Total sugars (mg L^{-1})	1200 ± 56.00
Hydroxytyrosol mg g^{-1} (dry weight extract)	690 ± 4.32
Total phenols GAE mg g^{-1} (dry weight extract)	920 ± 9.00
Total flavonoids CE mg g^{-1} (dry weight extract)	43.41 ± 2.27

The data are displayed with mean standard deviation of three replications.

2.2. Identification and quantification of phenolic compounds in the EAE

A reversed phase high performance liquid chromatographic technique was used to identify and quantify the major phenolic compounds of the crude EAE.

For this purpose, the standards mixture solutions of phenolic compounds were analyzed. Sample concentrations were calculated on the basis of peak areas compared to those of each of the external standards, as described in the Section 4. A representative chromatogram of table-olive wastewater extract (EAE) obtained after the HPLC analysis is given in Figure 1. This chromatogram shows that the EAE is mainly made up of hydroxytyrosol, tyrosol and *p*-coumaric acid, which were confirmed by the GC–MS analysis (Table 2). In addition, two other phenolic compounds, caffeic acid and ferulic acid, were identified by GC–MS analysis. The obtained mass fragments are in accordance with those described previously.[25]

The quantification and identification of phenolics by HPLC revealed that hydroxytyrosol which occurs during the debittering stage and is transferred to wastewaters during the washing stages, is the major compound with the highest amount (690 mg g^{-1} dry weight extract). In fact, this is in agreement with the literature where hydroxytyrosol was found to be the most abundant identified phenolic compound in table-olives wastewater.[26] This compound results from the hydrolysis of oleuropein, which is the major phenolic in fresh olive fruits.[27] Oleuropein is responsible for the olive fruits bitter taste and, to become edible, the fruits need to lose, at least partially, their natural bitterness which is immediately hydrolysed by the high alkalinity of wastewater. Consequently, oleuropein cannot be found in the wastewater of processed fruits. Tyrosol is also detected but its concentration is very low in comparison with other phenolics.

For the hydroxytyrosol recovery, a liquid–liquid extraction procedure using the ethyl acetate solvent was applied on olive brine. It was reported that ethyl acetate is the most convenient solvent for the phenolic monomers extraction.[28,29] The obtained extract was subjected to a preparative fast performance liquid chromatography (FPLC) system which gives a pure HT (Fig. 2). In this context, sev-

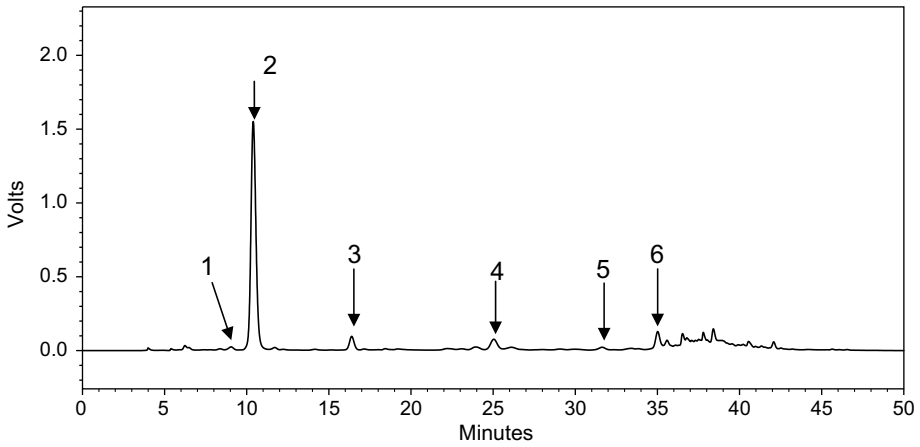

Figure 1. Ethyl acetate extract of table-olive wastewater **1**: protocatechuic acid; **2**: hydroxytyrosol; **3**: tyrosol **4**: *p*-coumaric acid; **5**: caffeic acid; **6**: ferulic acid.

Table 2
Abbreviated mass spectra of major phenolics identified in table-olive wastewater extract (EAE)

TMS derivatives	Mass spectra (*m*/*z* and % of the base peak)
Tyrosol	282(M^+, 18); 267(13); 193(15); 179(100); 73(42)
Hydroxytyrosol	370(M^+, 39); 267(90); 193(25); 179(12); 73(100)
Protocatechuic acid	370(M^+, 50); 311(15); 281(10); 223 (16); 193(100); 147(20); 73(75)
caffeic acid	396(M^+, 100); 381(25); 307(12); 239(11); 219(92); 191(13); 73(55)
para-Coumaric acid	308(M^+, 81); 293(100); 249(44); 219(82); 179(13); 73(51)
Ferulic acid	338(M^+, 90); 323(50); 308(41); 293(31); 267(35); 249(45); 147(20); 73(100)

eral methods have been developed to produce hydroxytyrosol. The method developed by Allouche et al.,[28] consisted in a continuous procedure for the extraction of hydroxytyrosol from Olive mill wastewater (OMW). It was also reported that hydroxytyrosl was produced by the enzymatic and acid hydrolysis of olive leaf extracts.[30,31] Hydroxytyrosol in its native form has a major problem because it is chemically unstable, unless preserved dried in the absence of air and in the darkness. On the basis of these considerations, triacetylhydroxytyrosol was produced in order to obtain a chemically more stable derivative able to be biochemically converted in vivo into its original active form. To prepare the acetylated derivatives of hydroxytyrosol, acetyl chloride was used. Several conditions were tested including different quantities of acetyl chloride, pyridine, temperatures and incubation times. Three hydroxyl groups exist in the hydroxytyrosol structure, and therefore different acetyl derivatives were expected. Under our experimental conditions, the triacetylated derivative was obtained resulting in 96.8% initial hydroxytyrosol conversion. The acetylated raw material was further purified by a preparative fast performance liquid chromatography (FPLC) system. A typical HPLC profile of triacetylated hydroxytyrosol derivative is shown in Figure 2. Its identification was confirmed using a GC–MS apparatus. The spectrum exhibited a molecular ion at *m*/*z* 280 (M^+) with fragments at *m*/*z* 220, 196, 178, 136, 123, 107 and 77, which was consistent with the known fragmentation scheme for triacetylhydroxytyrosol.[32] It has been reported that the hydroxytyrosol acetyl derivatives are chemically stable and effective, like the native compound in preventing the ROS-mediated molecular oxidative alterations and cytotoxicity in human cells, despite the fact that they are completely devoid of chemical antioxidant activity.[15,16]

2.3. Antioxidant potential

The EAE, HT and triAcHT were screened for their antioxidant capacity by DPPH and $ABTS^{\bullet+}$ radicals scavenging assays (Table 3). Hydroxytyrosol exerted a strong antioxidant activity (IC_{50} = 3.71 μg mL^{-1}), which was higher than BHT (IC_{50} = 7.94 μg mL^{-1}). However, EAE was also effective and showed an antioxidant activity comparable to that of BHT. In contrast, triAcHT derivative was completely inefficient as a hydrogen donor, indicating that it was devoid of any chemical antioxidant activity. The extract and the two pure compounds showed similar activity trends in both ABTS and DPPH radical-scavenging tests (Table 3). This finding confirms that the *ortho*-diphenolic structure of hydroxytyrosol is particularly crucial for its antioxidant activity. Indeed, the triAcHT which lacks the *ortho*-diphenol moiety is totally inactive as a hydrogen donor and therefore does not exert any antioxidant effect. Our results are in agreement with those previously reported by Capasso et al.[15] and Manna et al.[16] These authors demonstrated that HT is endowed with a high antioxidant capacity, whereas the acetylated compound does not show any ferric reducing activity, as measured using the FRAP assay.

2.4. Cytotoxicity assay

To investigate their cytotoxic effects on HeLa and DG75 human cell lines as well as on normal peripheral blood lymphocytes, cells were treated with various concentrations of EAE, HT and triAcHT for 48 h, and then submitted to the MTT test. As far as we know, no data are available in the literature concerning the study of cytotoxic properties of polyphenols in olive brine wastewater using the cell culture model system.

As shown in Figure 3, EAE, HT and triAcHT have growth inhibition effects on human cells in a dose-dependent manner. HeLa and DG75 cell lines showed higher sensitivity to the cytotoxic actions of EAE, HT and triAcHT than are normal peripheral blood lymphocytes, suggesting a protective activity of these samples against cancer. In fact, antioxidants are considered as promising cancer chemopreventive compounds which are able to lower the rate of malignant transformation.[33,34] Our results are in agreement with that reported by Fabiani et al.,[35] who have demonstrated that HT extracted from virgin olive oil inhibited the cancer cells proliferation by inducing apoptosis as evidenced by flow cytometry, fluorescence microscopy and internucleosomal DNA fragmentation. Besides, these authors reported that no effect on apoptosis was observed after similar treatment of freshly isolated human lymphocytes and polymorphonuclear cells.[35] In contrast, Della Ragione

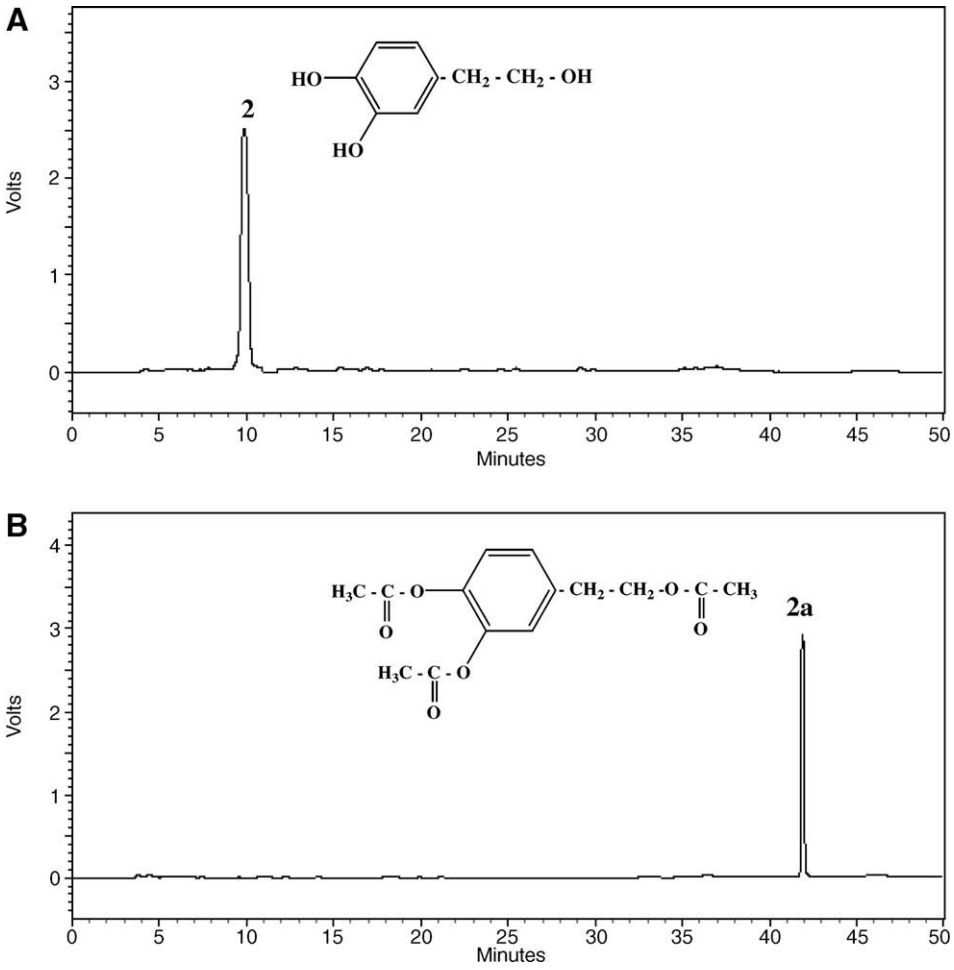

Figure 2. Chromatograms (A) of pure hydroxytyrosol (**2**) and (B) of pure triacetylated hydroxytyrosol (**2a**).

Table 3
DPPH and ABTS scavenging radicals of table-olive wastewater extract (EAE), hydroxytyrosol (HT), triacetylated hydroxytyrosol (TriAcHT) and butylhydroxytoluene

Compound	IC_{50} (μg mL^{-1})	TEAC (mmol)
EAE	8.91 ± 1.12	2.06 ± 0.11
HT	3.71 ± 1.20	3.40 ± 0.09
TriAcHT	NA	NA
BHT	7.94 ± 1.07	2.37 ± 0.15

NA: not active.

et al.[36] found that HT actively induces apoptosis in white blood cells (both transformed and normal cells) as a consequence of a rapid cytochrome c release from mitochondrial intermembrane space, explaining the presumably anti-inflammatory effect of olive oil. This discrepancy could be due to the different assays used to assess apopotosis. Della Regioni et al.[36] have particularly measured apoptosis by annexin staining of phosphatidylserine translocation on the cell surface, which is an early event in the apoptosis processs.[37]

In another hand, TriAcHT have exhibited a higher cytotoxicity than HT, toward peripheral blood lymphocytes and DG 75 cell line (Fig. 3, Table 4). This could be due to the liberation of acetyl group (CH_3CO) in the cells, after conversion of TriAcHT to HT by enzymatic hydrolysis. Such phenomenon was not observed with HeLa, probably due to the higher resistance of this adherent cell line. Further studies will be necessary to clarify this important point.

The 50% cytotoxic concentration of EAE, HT and triAcHT are represented in Table 4. Hence, doses under these concentrations were used for antioxidant activity investigation.

2.5. Biological antioxidant activity in human cell cultures

The investigation of the biological antioxidant activity of EAE, HT and triAcHT was carried in two different human cell lines: HeLa, an adherent epithelial cell line and DG75, a lymphoblastic non adherent cell line. Human cells were cultured with or without addition of EAE, HT and triAcHT. After 4 hours, oxidative stress was induced by adding 100 μM Fe^{2+} solution (as Fe_2SO_4) in PBS for 1 h. Malondialdehyde production, a lipid peroxidation marker, was evaluated. The oxidative treatment resulted in at least a four-fold increase in TBARS concentration compared with control cells. As shown in Figure 4, a significant protection against ROS inducing damage was obtained with the two selected compounds and the extract treatments.

EAE has an efficient antioxidant effect at both 50 and 100 μg/mL toward both cell lines, which was in correlation with its high content in hydroxytyrosol and other phenolic compounds such as tyrosol, caffeic acid, ferulic acid and *p*-coumaric acid. It has been reported that polyphenols such as caffeic acid and ferulic acid significantly inhibit lipid peroxidation, exhibit DNA protective effects in normal PBMCs exposed to H_2O_2-induced oxidative stress and protect cells from oxidative stress-induced apoptosis.[38] On the other hand, isolated pure HT at both concentration 10 and 20 μg/mL exhibited an antioxidant effect by decreasing

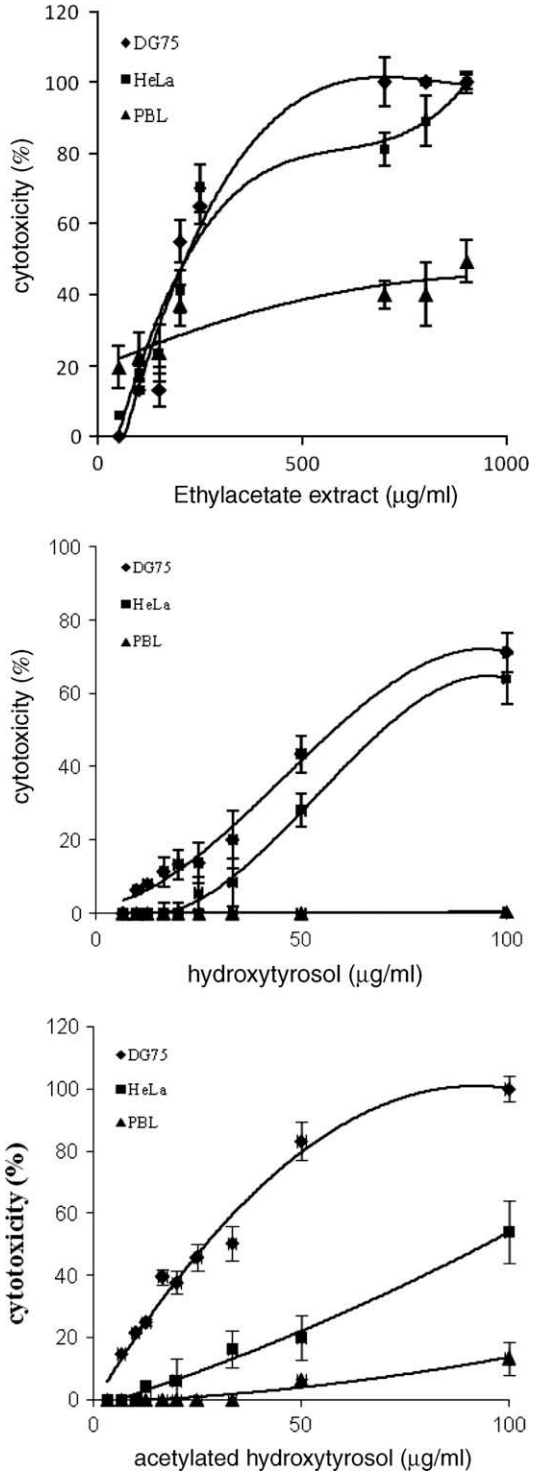

Figure 3. Cytotoxic effect of HT, TriAcHT and ethyl acetate extract on Hela, DG75 cell lines and human PBL. The inhibitory effects of different doses on cell growth were determined by MTT essay. Cells were treated with ethyl acetate extract at concentrations ranging from 50 to 1000 μg mL^{-1}, as well as with hydroxytyrosol and acetylated hydroxytyrosol at concentration ranging from 5 to 100 μg mL^{-1}. The percent growth reduction was calculated from the extinction difference between treated cell culture and the control. Results are the means of three repetitions.

Table 4
The 50% cell-inhibitory concentration of table-olive wastewater extract (EAE), hydroxytyrosol (HT) and triacetylated hydroxytyrosol (TriAcHT)

	EAE (μg mL^{-1})	HT (μg mL^{-1})	TriAcHT (μg mL^{-1})
HeLa	210	70	92
DG 75	210	58	27
PBL	—	—	—

(—): not reached.

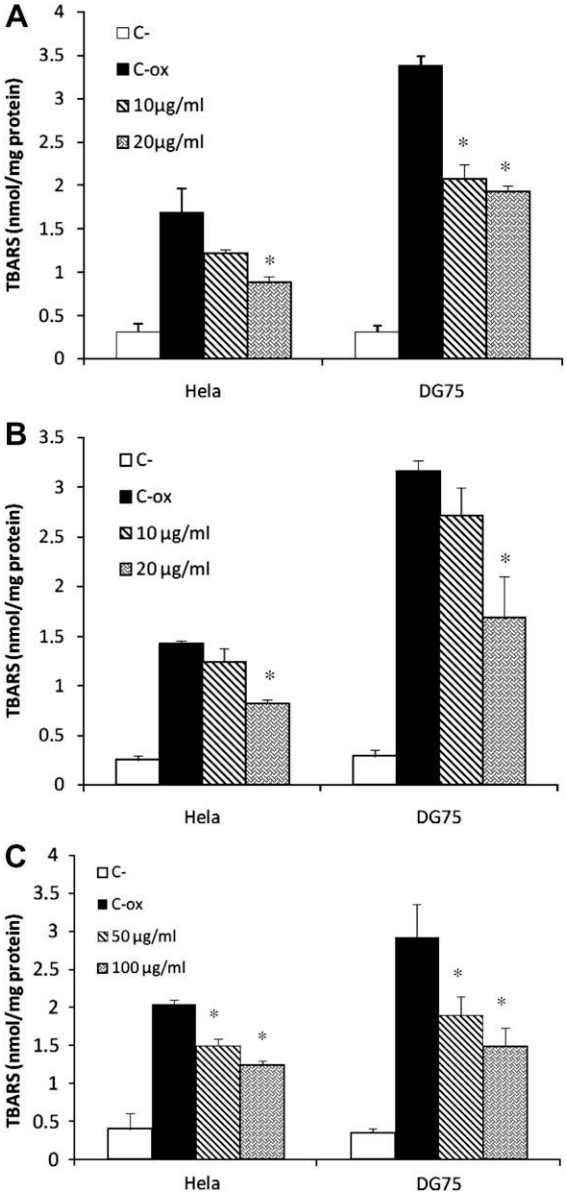

Figure 4. MDA levels in HT (A), TriAcHT (B) and ethyl acetate extract (EAE) (C) supplemented Hela and DG75 cell lines. Cells were cultured in 25 cm^2 flasks with HT (10 μg mL^{-1}and 20 μg mL^{-1}), TriAcHT (10 μg mL^{-1} and 20 μg mL^{-1}) and EAE (50 μg mL^{-1} and 100 μg mL^{-1}) for 4 h. Oxidative stress was induced by addition of Fe^{2+} to the cells, for 1 h at a final concentration of 100 μM. Results were compared to untreated cell (c) and cell treated with Fe^{2+} alone (c-ox). $^{*}p < 0.05$.

TBARS formation in both cell lines, with a preferential response with DG75. In fact, adherent cells have always showed more resistance to drug treatment than non adherent cells due to the strong expression of adhesion molecules on the cell surface.[39,40] In this study adherent cell resistance was observed not only with antioxidant assay, but also during Fe^{2+} stress induction and cytotoxic tests particularly those of HT and TriAcHT (Figs. 3 and 4).

At the same concentrations as HT, the TriAcHT compound exhibited a decrease in TBARs levels, which was significant only with 20 μg/mL for both cell lines.

The antioxidant capacity of HT in the biological system was previously described by Schaffer et al.,[41] who demonstrated that hydroxytyrosol-rich olive mill wastewater extracts prevent oxidative stress in murine brain cells in vitro and ex vivo. Manna et al.,[42,43] have also shown that olive oil hydroxytyrosol protects human erythrocytes, as well as Caco-2 cell line against oxidative damages. Triacetylated hydroxytyrosol was devoid of chemical antioxidant activity. It was found to be metabolised by cellular esterase leading to the improvement of the bioavailability of hydroxytyrosol which is the effective antioxidant compound as has been reported by Manna et al.[16] In addition to its contribution to the hydroxytyrosol bioavailability, the acetylation is thought to take part in preserving the stability of such antioxidant.

Our results are in agreement with previous reports[15,16] because an important protection against oxidative stress was also observed with triAcHT in HeLa and DG75 cell lines, suggesting its possible utilisation, as well as the EAE at non-toxic doses, in a wide range of biological matrices such as cosmetic and pharmaceutical preparations or in foods.

2.6. Antimicrobial assay

The antimicrobial activities of the EAE, HT and triAcHT were evaluated against a large range of microorganisms represented by five bacterial strains and two fungal strains of different genus. The minimum inhibitory concentration values are reported in Table 5. The results show that, all the tested bacteria and fungi are inhibited by EAE, HT and triAcHT. According to the NCCLS method, MICs values (Table 5) are also minimum bactericidal concentrations MBCs after sub-culturing, all tubes showing no visible growth. The extract and the two pure compounds exhibited similar bactericidal and fungicidal effects with a slight advantage for HT. Depending on microorganisms sensitivity to HT, triAcHT and the phenolic compounds present in the EAE, the obtained MICs values vary from 125 to 250 μg mL^{-1} and from 500 to 1000 μg mL^{-1} for bacteria and fungi, respectively.

Contrary to what has been reported in previous studies the Gram-positive bacteria are more susceptible to plants extracts than the Gram-negative one. A similar action on the two kinds of bacteria has been detected. The resistance of the Gram-negative bacteria and fungi to antibacterial compounds is normally, due to the high density of lipopolysaccharides in their cell wall.[44,45] The Gram-negative antibacterial and antifungal, efficiency of EAE, HT and triAcHT may be explained by their double properties, that is, lipophilic and hydrophilic.[46] As expected, the MIC values are higher for fungi than for bacteria. This is unexceptional since the antifungal activity requires considerable lipophilicity while EAE, HT and triAcHT are essentially hydrophilic. Furthermore, fungi are known for their high resistance to phenolic compounds.[47,48]

Table 5
Minimum inhibitory concentration of table-olive wastewater extract (EAE), hydroxytyrosol (HT) and triacetylated hydroxytyrosol (TriAcHT) against standard microbial strains

Microbial species	MIC (μg mL^{-1})		
	EAE	HT	TriAcHT
Pseudomonas aeruginosa ATCC 15442	250–500	125–250	250–500
Escherichia coli ATCC 10536	250–500	125–250	250–500
Salmonella enterica CIP 80.39	250–500	125–250	250–500
Bacillus subtilis ATCC 6633	125–250	62.5–125	125–250
Staphyloccocus aureus ATCC 9144	125–250	62.5–125	125–250
Candida albicans ATCC 10231	500–1000	125–250	500–1000
Aspergillus niger ATCC 16404	1000–2000	250–500	500–1000

Data are reported as the means of two tests each carried out in duplicate after 24–72 h of incubation at microbial appropriate temperature (28 °C for fungi, 30 °C for *Bacillus* and *Pseudomonas* and 37 °C for *Staphylococus*, *Escherichia* and *Salmonella*).

The antimicrobial action of phenolics is well known and related to their ability to denaturize proteins.[49] They act by causing the leakage of cytoplasmic constituents such as proteins or minerals and testifying their ability to cross the cells wall.[50] It was reported that the iron depletion (because of the iron-chelation properties of phenolic compounds), affect microbial growth, inhibiting the metallo-enzyme activities.[51] Polyphenols are also known to bind to the peptidoglycan leading to the breaking of the bacterial cell-wall integrity.[52] Ciafardini and Zullo[53] reported that the phenolic compounds react through the disruption of the bacterial cells wall proteins. Sun et al.[54] observed the effects of the phenolic compounds on the ultra-structure of *Candida albicans* under scanning electron microscopy and transmission electron microscopy. They found that the cell-wall changes profoundly and showed some wrinkles, excoriation and fracture of the cell surface. These ultrastructural changes demonstrated the antifungal effects and provided the hint of the most potential mechanism of antifungal activities. The cellular action of polyphenolic compounds may involve the modulation of transcriptional factors. The results of Maggi-Capeyron et al.[55] suggest that phenolic acids may act directly on cell signalling via inhibition of transcriptional activity (protein-1 activator). Some plants extracts showed the efficiency of their action against fungal species as phenolic compounds of *Olea europaea* L. against *Phytophthora megasperma* and *Cylindrocarpon destructans*.[56]

The MICs results are particularly important, because they show the much extended action oprotf EAE on a wide range of microorganisms. Genus like *Aspergillus*, *Candida*, *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Salmonella*, and *Bacillus* are responsible for numerous diseases. These microorganisms are all sensitive and may be destroyed by EAE, HT and triAcHT. Additional MIC s data show that EAE may be a good candidate for its employment as an antimicrobial agent and phytoncide against numerous pathogenic bacteria and fungi.

3. Conclusion

The results obtained in the present work denote that EAE may constitute a good source of healthy compounds or phenols intake in the diet, suggesting that it could be useful in the prevention of diseases induced by free radicals. Moreover, and as far as we know, this is the first report concerning the antioxidant potential of 'Meski' table-olives wastewater phenolics. This study has demonstrated that triAcHT was devoid of any DPPH and ABTS radical-scavenging activity. In the cell culture experiments, triAcHT has shown a similar antioxidant capacity to HT and with the latter being more effective. Further studies are needed to clarify the ripening stage used to make Meski table-olives cultivar in its high phenolic composition and antioxidant potential. The acquired data suggest that olive table wastewaters could be exploited to obtain HT-rich extracts with promising biological effects and could be proposed as a therapeutic agent in pharmacological preparations.

4. Experimental

4.1. Wastewater used and extraction

Fresh debittering, washing and brine wastewaters were obtained from the Agro-industrial Cooperation of Chaouat (Mater, North of Tunisia). Samples taken in the 2006–2007 harvest seasons (November 2006) were generated from Meski olive cultivar processing. They were stored immediately at −20 °C to avoid the auto-oxidation and subsequent polymerisation of the phenolic

compounds. Wastewaters were taken from tank 1, from the same batch of olives which were debittered for about 6 h and washed once with water for about 12 h. The extraction of phenolics was achieved by using ethyl acetate.

4.2. Analytical methods and chemicals used

Electrical conductivity and pH, was determined according to standard protocols.[57] The colour of the wastewater was determined by the difference of absorbances at 440 and 700 nm in 1 cm pathlength cells.[9] Hydrxytyrosol was prepared as described previously by Bouaziz and Sayadi.[31] The phenolic compounds were dissolved in a mixture of methanol/water (8:2 v/v). Pure HPLC solvents were used in all cases. 2,2-Diphenyl-1-picrylhydrozyl (DPPH), 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate] (ABTS) were purchased from Sigma–Aldrich, USA.

4.3. Chromatographic purification of hydroxytyrosol

Ethyl acetate extract of brine wastewaters (2 g) was chromatographed on a C-18 silica gel (LiChroprep RP-18; 25–40 μm) column (2.5 × 30 cm, Merk) under medium pressure. Phenolic compounds elution was carried out with the same gradient solvent as used in the HPLC. The flow rate was adjusted to 0.3 mL/min and 5 mL fractions were collected. These fractions were measured by optical density at 280 nm and the chromatogram (optical density versus fraction number) was represented (data not shown).

4.4. Hydroxytyrosol acetylation

Hydroxytyrosol (0.65 mmol, 100 mg) was dissolved in diethyl ether (20 mL) and mixed with pyridine (165 μL) in glass vial equipped with magnetic stirrer. Then acetyl chloride 164 μL (2.3 mmol) in 10 mL of diethyl ether was added dropwise. The mixture was stirred at 0 °C for 10 h and a white precipitation appears (pyridine chloridrate). The formed precipitate was filtered and the obtained solution was dried at 35 °C under vacuum to give triacetylated hydroxytyrosol as a pale brown residue.

4.5. Chromatographic purification of triacetylated hydroxytyrosol

Triacetylated hydroxytyrosol-rich extract was chromatographed on a C-18 silica gel (LiChroprep RP-18; 25–40 μm, Merk) column (2.5 × 15 cm) under high pressure using a preparative fast performance liquid chromatography (FPLC) system (Amersham Biosciences) equipped with a UV detector. Phenolic compound elution was carried out with the same solvent gradient as used in HPLC. The flow rate was adjusted to 0.7 mL/min.

4.6. High performance liquid chromatography

The identification and quantification of phenolic monomers were carried out by HPLC analysis as described by Bouaziz et al.[58] The assays were performed on a Shimadzu apparatus composed of an LC-10ATvp pump and an SPD-10Avp detector. Eluates were detected at 280 nm. The column was 4.6 × 250 mm (Shimpack VP-ODS) and its temperature was maintained at 40 °C. The flow rate was 0.6 mL min^{-1}. The mobile phase was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 50 min. The elution conditions applied for monomeric phenols were: 0–25 min, 10–25% B; 25–35 min, 25–80% B; 35–37 min, 80–100% B; 37–40 min, 100% B. Finally, the column was subjected to washing and reconditioning steps for (40–50 min) with a linear gradient of 100–10% B.

4.7. GC-MS analysis

GC–MS analysis was performed with an HP model 5975B inert MSD, equipped with a capillary DB-5MS column (30 m in length; 0.25 mm i.d.; 0.25 μm film thickness (Agilent Technologies, J&W Scientific Products, USA). The carrier gas was He, used with a 1 mL min^{-1} flow rate. The oven temperature program was as follows: 1 min at 100 °C, ramped from 100 to 260 °C at 4 °C min^{-1}, and 10 min at 260 °C. The chromatograph was equipped with a split/splitless injector used in the split mode. The split ratio was 100:1. One hundred microlitres of bis(trimethylsilyl)acetamide (BSTFA) was added to 100 μL of the ethyl acetate extract of table-olive brine process wastewater sample. The obtained solution was incubated for 60 min at 80 °C.

4.8. Determination of total phenolics

The concentration of total phenolics was measured by the method described by Singleton and Rossi[59] with some modifications. Briefly, an aliquot (1 mL) of appropriately diluted extracts or standard solutions of gallic acid (20, 40, 60, 80 and 100 mg L^{-1}) was added to a 25 mL volumetric flask containing 9 mL of ddH_2O. A reagent blank using ddH_2O was prepared. One millilitre of Folin & Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 7% Na_2CO_3 solution was added with mixing. The solution was then immediately diluted to volume (25 mL) with ddH_2O and mixed thoroughly. After incubation for 90 min at room temperature, the absorbance versus prepared blank was read at 750 nm. Total phenolic contents of extracts were expressed as mg gallic-acid equivalent (GAE) per gram of EAE. All samples were analyzed in five replications.

4.9. Determination of total flavonoids

Total flavonoids were measured by a colorimetric assay developed by Zhishen et al.[60] One millilitre aliquot of an appropriately diluted sample or standard solutions of catechin (20, 40, 60, 80 and 100 mg L^{-1}) was added to a 10 mL volumetric flask containing 4 mL ddH_2O. At zero time, 0.3 mL 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At 6 min, 2 mL (1 M NaOH) was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 mL of ddH_2O and thoroughly mixed. Absorbance of the mixture, pink in colour, was determined at 510 nm versus prepared water blank. Total flavonoids of extract were expressed on a fresh weight basis as mg catechin equivalents (CE) per gram of extract. Samples were analyzed in five replications.

4.10. DPPH radical-scavenging assay

The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple coloured methanol solution of DPPH. This spectrophotometer assay (UV-1650PC Shimadzu, Japan) uses stable radical diphenylpicrylhydrazyl (DPPH) (Sigma, Aldrich) as a reagent.[61] Aliquots (50 μL) of various concentrations of the test compound in methanol were added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent ($I\%$) was calculated in following way: $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test com-

pound. Test compound concentration providing 50% inhibition (IC_{50}, expressed in μg mL^{-1}) was calculated from the graph plotted inhibition percentage against extract concentration. Synthetic antioxidant reagent butylated hydroxytoluene (BHT) was used as positive control and all tests were carried out in triplicate.

4.11. Measurement of the trolox equivalent antioxidant capacity (TEAC)

The ABTS radical-scavenging activity was determined according to Re et al.[62] The ABTS radical cation was prepared by reacting an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45 mM, final concentration), which was kept in the dark at 25 °C for 12–16 h. The solution was diluted in ethanol to an absorbance of 0.70 (±0.020) at 734 nm before use. Aliquots of trolox or sample in water (20 μL) were added into 2.0 mL of this diluted solution, and the absorbance at 734 nm was determined at 30 °C, exactly 6 min after initial mixing. Appropriate solvent blanks were run in each assay. The antioxidant solution reduces the radical cation to ABTS, which reduces the colour. The extent of decolourization is calculated as percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the equivalent trolox concentration. The activity of each antioxidant was determined at three concentrations, within the range of the dose–response curve of trolox, and the radical-scavenging activity was expressed as the trolox equivalent antioxidant capacity (TEAC), defined as mM of trolox per gram of sample.

4.12. MTT cytotoxicity assay

4.12.1. Cell lines and culture condition

Two continuous human cell lines were investigated for cytotoxicity and antioxidant effect of olive waste extract (EAE): HeLa (cervical cancer line, adherent) and DG75 (Burkitt like lymphoma cell line, nonadherent).[63] These cell lines were grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamin in tissue culture flasks (Nunc). They were passaged twice a week and kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

4.12.2. Lymphocyte preparation for cytotoxicity assay

Three normal volunteer donors were recruited into the study after obtaining their informed consent. Peripheral blood mononuclear cells (PBL) were isolated from heparinised venous blood by sedimentation in Ficoll-hypaque (Sigma, Germany). Cells were washed three times in PBS (phosphate buffered saline) and immediately used for MTT test.

4.12.3. MTT test

The proliferation rates of PBL, HeLa and DG75 cells after treatment with EAE, HT and triAcHT were determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[64] The yellow compound MTT is reduced by mitochondrial dehydrogenases to the water-insoluble blue compound formazan, depending on the viability of cells.

PBL (10^6 mL^{-1}), HeLa (5×10^4 mL^{-1}) and DG75 (5×10^4 mL^{-1}) cells were incubated in 96-well plates (200 μL of cell suspension/well) for 48 h in the presence and absence of EAE, HT and triAcHT with serial dilutions (5–100 μg mL^{-1} for HT and triAcHT and 50–1000 μg mL^{-1} for EAE). Twenty microlitres MTT solution (Sigma) (5 mg mL^{-1} in PBS) were added to each well. The plate was incubated for 4 h at 37 °C in a CO_2-incubator. One hundred and eighty microlitres of medium was removed from every well without disturbing the cell clusters. A 180 μL methanol/DMSO (50:50) was added to each well, and the preparations were mixed thoroughly on a plate shaker with the cell containing formazan crystals. After all of the crystals were dissolved, the A_{570} values were determined with a microplate reader (EL_X 800).

4.13. Biological tests for the antioxidant activity in HeLa and DG75 cell lines

4.13.1. Induction of oxidative stress

Cells were adjusted to 5×10^5 cells mL^{-1} in 25 cm^2 flasks, and incubated at 37 °C. Oxidative stress was induced, after 48 h, by addition of Fe^{2+} (as Fe_2SO_4) to the cells at a final concentration of 100 μM, for 1 h. The oxidation was performed in phosphate buffered saline (PBS). Cells without any treatment were used as a control.

4.13.2. Determination of lipid peroxidation

Malondialdehyde (MDA) was used as a marker of lipid peroxidation. For evaluation of MDA rate production, thiobarbituric acid-reactive species (TBARs) assay was used. Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 500 μL of deionized water and lysed by five cycles of sonication during 20 s at 35% (Sonisc, vibracell). One millilitre of TBA solution (15% trichloroacetic acid, 0.8% thiobarbituric acid, 0.25 N HCl) was added. The mixture was heated at 95 °C for 15 min to form MDA–TBA adduct. Optical density (OD) was measured by a spectrophotometer (Biochrom, Libra S32) at 532 nm. Values were reported to a calibration curve of 1,1,3,3-tetraethoxypropane (1.1.3.3 TEP).

4.13.3. Antioxidant effect

To assay the capacity of EAE and the two pure compounds HT and triAcHT to protect HeLa and DG75 cells from ROS-mediated oxidative injury, cells were preincubated for 4 h in the presence of EAE (50 μg mL^{-1}, 100 μg mL^{-1}), HT (10 μg mL^{-1}, 20 μg mL^{-1}) and triAcHT (10 μg mL^{-1}, 20 μg mL^{-1}).

At the end of the preincubation time, the medium was changed before the addition of the oxidative stress-inducing agent. Finally, the above mentioned marker was evaluated.

4.13.4. Proteins quantification

Protein levels were determined using the Protein Assay Kit from Bio-Rad (France) and bovine serum albumin served as the standard.

4.14. Antimicrobial assay

The antimicrobial activity of EAE, HT and triAcHT was evaluated against Gram-positive and Gram-negative bacteria, fungi and yeast. The minimum inhibitory concentrations (MICs) were determined by the broth dilution[65] modified method. The EAE, HT and triAcHT were serially diluted twofold in Nutrient Broth (Difco Laboratories, USA) for bacteria or in Malt Extract Broth (Difco Laboratories, USA) for fungi. Duplicate tubes of each dilution (8000, 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.813, and 3.906 μg mL^{-1}) were inoculated with about 10^6 CFU mL^{-1} of the bacterial and yeast cultures in the exponential phase of growth and of the fungal spores solution. The tubes were incubated at the appropriate temperature of each strain for 18 h. A tube containing only broth inoculated with the organism was kept at +4 °C in a refrigerator overnight and used as standard for the determination of complete inhibition. MIC was taken as the highest dilution (least concentration) of EAE, HT and triAcHT showing no detectable growth.

The MIC determination by broth dilution method can be converted to determine the minimum bactericidal concentrations (MBC) by sub-culturing, all tubes not showing visible growth.

4.15. Statistical analysis

All data presented are means ± SE. Statistical differences were calculated using a one-way analysis of variance (ANOVA), followed by the Student's test.

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