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Eco-Extraction of Phenolic Compounds from Moroccan Olive Fruits and Leaves and their Potential use as Antimicrobial Agents

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Abstract

In Morocco, the olive harvest generates regenerates a lot of waste such as leaves and olive fruits. Valuation by the extraction of polyphenols from this waste could be a promising source. In our work, we have prepared an olive tree extract from this waste, our extract contains 148 g/l of polyphenols, 8.4 g/l of flavonoids and 39.11 g/l of o-diphenols. Polyphenols, major natural antioxidants play a key role in hundreds biological reactions. The antioxidant activity test revealed great antioxidant potential of our extract with high ORAC value 3 848 100 μmol Te/kg. The present work has as objective to evaluate the antimicrobial activity. The olive tree extract showed broad-spectrum antibacterial activity against Escherichia coli, Escherichia coli TG1, Escherichia coli DH5α, Staphylococcus aureus, Bacillus cereus, Bacillus cereus MED5 and Streptococcus agalactiae. While pure phenolic compounds (caffeic acid, ascorbic acid and quercetin) showed more limited activity. The antifungal effect of the olive tree extract exceed the antibiotics at a concentration of 3 mg/disc (p<0.05). Industrial technology can therefore exploit this extract, rich in polyphenols, in order to use instead of a synthetic antioxidants and antibiotics that could be dangerous. This would lead Morocco to enhance the olive harvest waste as an important economic source.

Keywords: Olive leaves; Olive fruit; Polyphenols; Antioxidants; Antibacterial; Antifungal.

Introduction

The olive oil industry is very important in the Mediterranean area, both in terms of wealth and tradition, where Spain, Italy, Greece, Tunisia and Morocco are the main producing countries. In Morocco, the olive harvest generates large amounts of waste such as leaves and olives fruits. This waste has the ability to become a low-cost starting materiel rich in polyphenols. Which can be extracted and used as natural antibiotics and antioxidants. Its valorization of by production of natural active compounds can be an important source economic and a good way to capitalize on this problematic waste.

Several studies have shown that olive polyphenols have various biological activities, such as inflammatory (Pacheco Y.M., 2007; Brunelleschi S., 2007 ; Rahman I., 2006 ; Martinı´nez-Domı´nguez E., 2001), anti-diabetic (Hamden K., 2009), antitumor, anti-proliferative (Bouallagui Z., 2011; Fabiani R., 2002) and anti-atherogenic activities (Covas M.I., 2006). Howover, act as natural antioxidants to prevent human diseases.

In addition to these activities, phenolic compounds also have antiviral, antibacterial and antifungal properties (Karaosmanoglu H, 2010; Yamada K, 2009; Zhao G, 2009; Battinelli L., 2006; Medina E., 2006). Moreover, several studies have shown the capacity of hydroxytyrosol to delay and/or inhibit the growth of a range spectrum of bacteria and fungi, including pathogenic bacteria (human pathogens). It was reported that the water fruit resulting from olive oil extraction was toxic to phytopathogenic bacteria such as *Pseudomonas syringae* (Gram-negative) and *Corynebacterium michiganense* (Gram-positive) (Capasso et al., 1995). Bisignano et al. (1999) studied the in vitro susceptibility of several pathogens from human's respiratory and intestinal tract to hydroxytyrosol and oleuropein. The minimum inhibitory concentration (MIC) reported in this study showed a wide antimicrobial activity of hydroxytyrosol against these bacterial strains (MIC between 0.24 and 7.85 mg/ml for standard strains and between 0.97 and 31.25 mg/ml for clinically isolated strains). These results suggest that hydroxytyrosol may be useful in the antimicrobial treatment of intestinal and respiratory tracts in human infections.

Thus, the aim of the present study was to describe the olive tree extraction and identification. In addition, it deals with the evaluation of antioxidant potential and the determination of antibacterial and antifungal activities of this extract, polyphenols standards as well as antibiotics.

Materials and Methods

Material and Chemicals

Folin–Ciocalteu reagent, Anhydrous sodium carbonate, Methanolic aluminum trichloride, Sodium molybdate dehydrate, 2,2 -diphenyl-1-picrylhydrazyl Mueller Hinton, Potato dextrose broth, Dextrose and Methylene blue were purchased from Sigma-Aldrich Chemie (Paris, France). Trolox, gallic acid, quercetin, ascorbic acid, hydroxytyrosol and caffeic acid were supplied by Sigma-Aldrich Chemie (Paris, France).

Bacterias: Escherichia coli, Escherichia coli TG1, Escherichia coli DH5α, Staphylococcus aureus, Bacillus cereus, Bacillus cereus MED5 and Streptococcus agalactiae and fungis: Candida albicans and Aspergillus niger were originally obtained from Biotechnology Laboratory collection (Faculty of Science Dhar El Mahraz - Fez – Morocco).

All antimicrobial disks: Amoxicillin/Ac. Clavulanic (20/10 μg); Chloramphenicol (30 μg); Flucloxacillin (5 μg); Kanamycin (30μg); Gentamicin (10 μg), Amphotericin B (10 μg) and ketoconozole (15 μg) were obtained from Sigma-Aldrich Chemie (Paris, France).

Phenolic Extract Preparation - Fresh leaves and olives (RI:1,88) were obtained during the harvest from the company ATLAS OLIVE OILS (Atlas Olive Oils Ltd., 110 Bd. Yacoub El Mansour, 20370 Casablanca, Morocco). They were transported to the laboratory and intensively washed with distilled water at 20ºC. Leaves and olives are oven-dried (BENDER oven) at 40°C (away from light) until loss of 70% of the total weight. Then the leaves and fruits, dried, were ground using a propeller mill, type "electric coffee grinder" in order to obtain an olive powder (flour).

The solid-liquid extraction of the phenolic compounds was carried out three times by water at 45°C. The mixture of olive powder/water (w/v, 10, 20 and 30%) was stirred at room temperature, protected from light, for 24 hours. Total polyphenols were determined in the supernatant after centrifugation (6000 tr/min, 20 min) and oil removing (n-hexane, 2v/v) each 2h during extraction. All the collected supernatants (aqueous phase) were concentrated in a rotary evaporator at 40°C and freeze-dried to remove water. An extract of olive powder as obtained.

Physicochemical Characterization of Extract - Total solids, mineral matter and fat content were determined according to the experimental protocol described in Rodier (2009). All the experiments were carried out in triplicate.

Determination of Total Phenol Content - Total phenols were determined according to the modified Folin–Ciocalteu (FC) method (De Marco E., 2007).A 100μl aliquot of the olive tree extract was added to a 9,9ml of water. FC reagent (0.5 ml) was added and the contents were mixed thoroughly. After 5 min, 2 ml of a 20%, anhydrous sodium carbonate solution (w/v) was added, and then the mixture was allowed to stand for 30 min. The absorbance of the blue-coloured samples was measured at 750 nm. The total phenolic content was determined as gallic acid equivalents (GAE) and values are expressed as g of gallic acid /l.

Determination of Flavonoids Content

The amount of total flavonoids in the extracts was measured following a previously reported method (Goulas, V., 2010) 1 ml of 2% methanolic aluminum trichloride ($AlCl_3$) solution was mixed with the same volume of the olive tree extract. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430. The concentrations of flavonoid compounds expressed as mg quercetin equivalent per g (mg QE/g) of extract were calculated according to the standard quercetin graph.

Determination of o-Diphenols Content

The determination of o-diphenols was performed according to the method used by Mateos (Mateos, 2001). A mixture of 4 mL of the olive tree extract with 1 mL of a 5% solution of sodium molybdate dihydrate in ethanol/water (1:1) was shaken vigorously. After15 min, the absorbance at 370 nm was measured. A blank was obtained by measuring a mixture of 4 mL of phenolic solution with 1 mL of ethanol/water (1:1).

The measurements were repeated on triplicate samples.

HPLC Analysis of the Olive Tree Extract

Phenolic compounds of olive tree extract were analyzed by reverse HPLC using a binary gradient elution. The HPLC (Shimadzu prominence) device consisted of two pumps LC-10ADVP, a photodiode array detector SPD-20A/20AV (UV-VIS Detectors) operating in the range of 190–900 nm, and an automatic injector SIL-10ADVP. The modified method of Hrncirik and Fritsche (2004) was used to separate the mixture on revers phase C18 Shimadzu column CLC-ODS (M) (250 mm L. × 4.6 mm I.D., 5 μm) at 27°C with a flow rate of 1 ml/min and a mixture of 0.5% orthophosphoric acid (v/v) in water (solution A) and methanol/acetonitril (50:50, v/v) (solution B). The following gradient program (in %) with a total analysis time of 75 min was used: A/B = 95/5; t = 15 min, A/B = 70/30; t = 15 min,

A/B = 65/35; t = 7 min, A/B = 60/40; t = 13 min, A/B = 30/70; t = 5 min, A/B = 0/100, held for 5 min, starting ratio (for 7 min) and the column equilibration (8 min). Components were detected at 280 nm.

Determination of Antioxidant Activity

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH• (2,2-diphenyl-1-picrylhydrazyl) (Berrin Bozan**,** 2008). Briefly, 3.9 ml from DPPH (63.4 mM) solution added to a series of different concentrations of the olive tree extract (375– 2.5 mg/l). The mixtures were incubated at 37°C for 30 min in dark, and then analyzed in the spectrophotometer at 517 nm. Ascorbic acid was used as positive control and hydroxytyrosol was tested as a pure phenolic compound for comparison to the olive extract. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula:

$$\text{Scavenging activity } (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

Where $A_{control}$ is the absorbance of the control at t= 0 min, and A_{sample} the absorbance of the antioxidant at t= 30 min. The results were compared to the DPPH radical-scavenging activity of standard concentrations of Trolox, assuming as unit of ORAC the DPPH radical-scavenging activity of 1 mmol of Trolox (Zullo and Ciafardini, 2008).

Determination of Antimicrobial Activity

Antimicrobial activity was tested against a panel of microorganisms, including seven bacteria strains: Escherichia coli, Escherichia coli TG1, Escherichia coli DH5α, Staphylococcus aureus, Bacillus cereus, Bacillus cereus MED5 and Streptococcus agalactiae and two fungis Candida albicans and Aspergillus niger.

The disc diffusion method was used to determine the antimicrobial activities of the olive tree extract and polyphenol standards (caffeic acid, ascorbic acid and quercetin). On Mueller Hinton agar for Bacterias and on Mueller Hinton supplemented with 2% dextrose and 0.5 mg/L methylene blue for fungis. The bacterial test organisms were grown in nutrient broth for 24h and used for further study. The fungal organisms are grown on potato dextrose broth (PDB) for 72h and used for further experiment. Mueller Hinton agar plates are prepared, sterilized and solidified. After solidification, 100μl overnight culture of each organism was spreaded on the petriplates using a sterile glass rod to prepare bacterial lawns. Microbial cultures were diluted in sterile distilled water in order to give a population of approximately 10^6 CFU/plate. Four sterile paper disks (6 mm in diameter) were placed on the surface of each agar plate and 10 μl of each standard, extract or controls was added to the paper discs. Disks embedded as the final concentration for the olive tree extract and polyphenols standards were 0.5 mg/disk, 1 mg/disk, 2 mg/disk, 3 mg/disk, and 4 mg/disk, respectively.

Each experiment was carried out in triplicate. Petri dishes were incubated at 37°C for 16–18h for bacteria, at 35°C for 24h for fungi. The diameter of the inhibition zone was measured in mm (including disc) with callipers, three replicates were performed (three different plates) and the average was taken. A disk impregnated with sterile distilled water served as negative controls and disks with an antibiotics served as a positive control. Five antimicrobial disks were included for antibacterial activity: Amoxicillin/Ac. Clavulanic (20/10 μg); Chloramphenicol (30 μg); Flucloxacillin (5 μg); Kanamycin (30μg); Gentamicin (10 μg). And amphotericin B (10 μg) and ketoconozole (15 μg) were used for antifungal activity.

Statistical Analysis

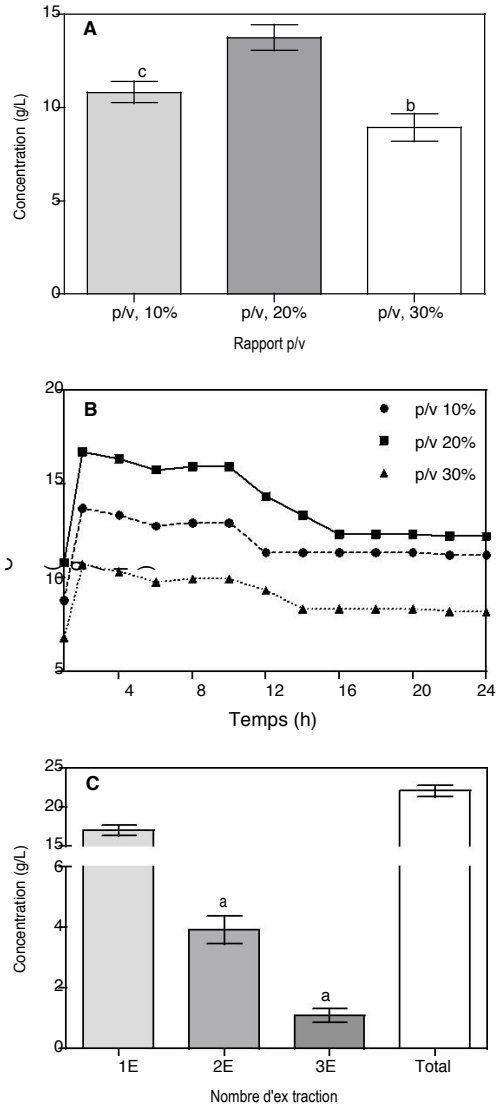
Statistical analyzes were performed using GraphPad Prism software version 6.00 (GraphPad Inc., San Diego, California). Data were analyzed by analysis of variance (ANOVA Analysis of Variance) followed by post-hoc Dunnet test if the sample distribution follows normal distribution or by the Kruskal-Wallis if the sample distribution does not follow the normal law. Values between groups were considered statistically significant for at $P<0.05$.

Results and Discussions

Olive Phenolic Extract

Results plotted on the first graph of the figure 1 show that the extract obtained with 20% ratio (w/v) contains a significantly high amount of polyphenols compared to other used ratios (P <0.05 and <0.01 compared to the 10 and 30% ratios).

Figure 1: Optimization of extraction conditions of polyphenols from olive powder, (a) p/v, (b) time of extraction and (c) number of extraction cycles. Mean ± standard deviation. The letters indicate the significance of the difference compared to controls, [a] P <0.001, [b] P <0.01 and [c] P <0.05.



The evolution of total polyphenol concentration of the three used ratios was studied through 24 hours. The curves of the figure 1b show that the temporal evolution of the polyphenol content of these three extracts has followed similar pace. Thus, the system (olive powder/water) tends towards equilibrium after two hours for the three used ratios (w/v), where the polyphenols content of the olive extract is higher.

Retaining the optimal extraction conditions (w/v ratio of 20% and 2 hours of extraction) we chose to perform extractions from a larger quantity of olive powder in order to exhaust the plant material. For that reason, 100 g of olive powder was extracted three times with tepid water (45°C) for 2 hours in the dark and at room temperature. To avoid the phenomena of possible degradation of bioactive compounds, the resulting extract was collected (and stored carefully) after each cycle and

only the plant matrix will undergo a new extraction cycle. The figure (1c) shows the results recorded for the three extraction cycles. As can we deduced, over 77% of olive extract polyphenols was obtained during the first extraction cycle compared to the last two cycles (P <0.001). The three fractions were, therefor, combined and subsequently concentrated using rotary evaporation at 40 °C (Buchi Rotavapor). Table below presents the average values of the main constituents of the olive extract.

The gross characterization of the extract is summarized in Table 1. The prepared extract was a Dark brown powder with a characteristic odor of processed olives.

Table 1: Organoleptic characteristics of the olive extract

Description	Characteristic
Appearance	Dark brown powder
Odor	Processed olives
Solubility	98%
pH	5,64

The eco-extraction of bioactive compounds from olive powder was carried out by the water at 45 °C (laundering) for 2 hours using a w/v ratio of 20%. The physical and chemical characterization (table 2) revealed that the olive extract is rich in polyphenols (over 15%), especially hydroxytyrosol (2%) without having to resort to any purification treatment.

Table 2: Total phenols and main constituents of olive phenolic extract. Mean ± standard deviation

Biophenol content	Concentration
Dry matter (g/100g of powder)	97.96 ±7.83
Mineral matter (g/100g of powder)	12.9 ±0.7
Fat (g/100g of powder)	< 1
Total phenolic content (g GAE/l)	159.8 ± 1.9
Flavonoids (g QE/l)	8.4 ± 0.6
o-Diphenols (g CAE/l)	39.11 ± 0.9
Hydroxytyrosol (g GAE/l)	2.09 ±1.4

Actually, the olive fruit has been known for several years as a source of phenolic compounds with high biological activities. However, combining the olive fruits and leaves to extract bioactive molecules (ecologically without use of chemical solvents) not only contributes to the improvement (quantitative and qualitative) of extraction yield, but also is a way to valuate of olive leaves generated during the olive harvest (10% of the total weight of olives produced) and trees pruning. Leaves and olive fruits were dried moderately, at a temperature of 40 ± 2 °C until loss of 70% of their weight. In fact, desiccation allows the reduction of the water content, the inhibition of the cellular enzymes activity (hydrolases and oxidases) and therefore increasing the extraction yield (Ahmad et al-Qasem, 2013. Groubert, 1984). Simple drying at 40 °C, although slow, allows the preservation of the original content bioactive molecules from the plant material compared to other recently used techniques (drying under hot air flow or infrared and lyophilization) (Ahmad- Qasem et al., 2013). Furthermore, use of tepid water (at 45°C) as extraction solvents induces, also, enzymes inactivation (denaturation) and increases the permeability of the cell membranes, the solubility of the extracted material and the diffusion coefficient. Finally, the temperature decreases the viscosity of the obtained phases (water and oil), which not only facilitates the passage of water through the mass of the solid substrate, but also the subsequent operations of separation (Leybros and Frémeaux 1990 Binbenet, et al., 2007). On the other hand, the rotary evaporation by vacuum concentration at 40°C will increase the final concentration of the polyphenols but also the protection of hydroxytyrosol and other phenolic compounds, who the stability is proportional with their concentration in a aqueous medium (Zafra-Gómez et al., 2011)

Biophenols Content

The table 3 shows the phenolic retention time of the olive tree extract.

Table 3: the polyphenol compound retention time

Polyphenol	Retention time (min)
Acide gallique	0,698
Rutine	0,769
Myricetine	0,976
Quercetine	1,493
Lutéoline	1,643
Kaempferole	2,252
Acide Chlorogenique	2,459
Acide Caféique	2,524
Apigénine	2,642
Isorhamnetin	2,642
Acide p-Comairique	3,508
Hydroxytyrosol	3,893
Acide Férulique	4,310
Tyrosol	5,190
Oleuropéine	12,400

These results analysis revealed a complex mixture of phenolic compounds, among which the components identified as oleuropein, hydroxytyrosol (3′, 4′-DHPEA), tyrosol (*p*-DHPEA) were the most intense. Flavonoids such as rutin, quercetin, luteolin and apigenin were also founded. It is worth noting that oleuropein and these secoiridoid derivatives are the predominant phenol commonly found in *Olea europaea* fruits, leaves and virgin olive oil (Mateos et al., 2001)

In fact, the most important source of these compounds is the olive fruit (Montedoro et al., 1993). However, they are also present in the leaves (Japón-Luján et al., 2007). The most abundant polyphenols identified in extracts of leaves and olive fruits were oleuropein, hydroxytyrosol and its secoiridoids derivative, and flavonoids (Benavente-García et al., 2000). This explains these results, where hydroxytyrosol, tyrosol, apigenin, luteolin and caffeic acid was the major phenolic compounds in the olive extract. Furthermore, these molecules provide a strong antioxidant activity to olive extract, resulting in high ORAC value (table). Moreover, these compounds impart several biological properties for olive extracts, such as antioxidant (Benavente-García et al., 2000), antiproliferative (Taamalli et al., 2012) and antimicrobial (Micol et al., 2005).

The olive tree extract also showed strong antioxidant resulting high ORAC value, which 3848100 ± 38481μmol Te/kg (tested using DPPH radical assay according to the protocol described by Zullo and Ciafardini (2008)). Actually, in the past decade, polyphenol-rich foods and herbs have become a topic of increasing interest not only to food and health science researchers or medical experts, but also to the general public. It has received particular attention due to their various biological effects including antioxidant activity. The phenolic compounds found in olives, olive oil, and Olive Mill Waste Water have been reported to possess strong antioxidant activity (Obied HK, 2007; Lafka T-A, 2011).

Antimicrobial Activity

The results of the study of the antimicrobial effect of the olive extract, obtained after 24 and 72h of incubation for bacteria and fungi, are summarized in Table 3.

The results of Table 3 show that the antimicrobial effect of the olive tree extract is dose-dependent, where the maximum activity was recorded for the dose of 4 mg/disc. At this dose, the antimicrobial activity of the olive extract was significantly higher (P <0.05) compared to that of antibiotics used as positive controls. However, surprisingly, none of the standard molecules used in the current study had a significant effect on microorganism growth (data not show). Moreover, none of the strains studied (bacteria and fungi) showed resistance to olive extract, even at low concentrations, 0.5 (data not shown) and 1 mg/disc. This behavior could be due to the fact that the aqueous extract of the

olive tree (subject of study) is rich in bioactive molecules with antimicrobial effect; i.e. phenolic compounds whose antimicrobial and antifungal activity has been elucidated by several authors (Medina et al., 2009; Keceli et al., 2002; Capasso et al., 1995).

Generally, the antimicrobial activity of an extract (or essential oils) is closely related to its chemical composition, particularly the minor compounds that act synergistically. Thus, the results obtained in this study show that the olive tree extract has a significant antimicrobial activity against the tested bacteria and fungi. This activity is probably due to its chemical composition rich in phenolic compounds, especially hydroxytyrosol, oleuropein and secoiridoids derivatives (dialdehydic form of decarboxymethyl oleuropein/ligstrosid aglycon). Actually, bactericidal, bacteriostatic and antifungal effect of these molecules has been reported in several studies (Karaosmanoglu et al., 2010; Medina et al., 2006; Battinelli et al., 2006). However, Romero et al (2007) have reported that only the dialdehydic forms of oleuropein and ligstroside (aglycons) showed inhibitory activity towards *Helicobacter pylori*. In addition, these authors have correlated the antimicrobial activity of the olive oils and extracts to their phenolic profiles and, more particularly, to the synergistic effect of these molecules.

Furthermore, several studies have shown that the phenolic compounds of the olive have antimicrobial properties by denaturing proteins and microbial enzymes (Cushnie and Lamb, 2005). This activity is strongly related to their structure (aromatic ring attached to the hydroxyl groups in different positions). Ultee et al. (2002) showed that this structure allows these compounds to form hydrogen bonds with the SH-groups in the active sites of target enzymes, which leads to the deactivation of these enzymes in *Bacillus cereus*.

Moreover, Medina et al (2009) compared the antibacterial activity of the dialdehydic form of oleuropein and ligstroside (3, 4-DHPEA-EDA and *p*-HPEA-EDA) to the glutaraldehyde and ortho-phthalaldehyde (OPA and GTA, known biocides), which had a similar dialdehydic structure. OPA and GTA interact, strongly, with the amino acids, proteins and membrane molecules (lipids), which increases the permeability of the membrane and causes cell lysis (Simoes et al., 2006). The results showed that the antibacterial activity (on a range of bacteria, including *Staphylococcus aureus*) of phenolic compounds with dialdehydic residues in their structure was higher than that of other polyphenols and, surprisingly, similar to that of two commercial biocides (OPA and GTA). Although dialdehydic structure is essential for their antimicrobial activity, the rest of the molecule also influences this activity since the OPA has a stronger activity than GTA.

Table: Antibacterial and antifungal activities of olive tree extract. Results expressed as diameter of the inhibition zone (mm). Mean values (standard deviation)

	Olive extract concentrations (mg/disc)				Antibiotics (μg/disc)						
	1	**2**	**3**	**4**	**Am/Cl (20/10)**	**Ch (30)**	**Fl (5)**	**Ka (30)**	**Ge (10)**	**Am (10)**	**Ke (15)**
Bacteria											
E. coli	15.00 (1.00)	20.33 (1.52)	25.33 (1.52)	29.67 (1.52)	^{a}11.66 (0.52)	^{a}11.66 (0.53)	^{a}18.00 (0.00)	^{a}20.00 (0.00)	^{a}11.69 (0.53)	-	-
E. coli TG1	14.00 (1.00)	19.67 (0.53)	25.00 (0.00)	30.33 (0.53)	^{a}19.64 (0.57)	^{a}14.67 (0.53)	^{a}24.66 (0.57)	^{a}23.33 (0.53)	^{a}17.00 (0.00)	-	-
E. coli DH5α	15.33 (0.57)	20.66 (0.57)	26.66 (0.57)	30.33 (0.53)	^{a}23.00 (0.00)	^{a}18.00 (0.00)	^{a}25.67 (0.57)	^{a}25.67 (0.77)	^{a}17.33 (0.53)	-	-
S. aureus	10.33 (0.57)	13.67 (0.57)	17.33 (0.57)	20.66 (0.57)	^{a}17.60 (0.57)	^{a}17.33 (0.53)	24.00 (0.00)	^{ns}21.67 (0.55)	^{a}17.00 (1.00)	-	-
B. cereus	14.67 (0.55)	16.67 (0.58)	22.00 (0.00)	30.33 (0.57)	^{a}13.63 (0.51)	^{a}10.00 (0.00)	^{a}22.33 (0.53)	^{a}21.67 (0.53)	^{a}13.66 (0.57)	-	-
B cereus MED5	16.67 (0.53)	20.63 (0.77)	25.67 (0.53)	31.00 (0.00)	^{a}13.00 (0.00)	^{a}9.33 (1.15)	^{a}24.33 (0.50)	^{a}20.00 (0.00)	^{a}15.00 (0.00)	-	-
S. agalactiae	18.00 (0.43)	20.33 (0.57)	21.33 (0.58)	22.33 (0.57)	^{a}18.00 (0.00)	^{a}12.00 (0.00)	24.00 (0.00)	23.66 (0.57)	^{a}20.33 (0.55)	-	-
Fungi											
A. niger	10.67 (0.53)	11.77 (0.55)	13.67 (1.15)	16.68 (1.51)	-	-	-	-	-	18.00 (0.00)	^{ns}16.66 (0.53)
C. albicans	10.33 (0.57)	12.00 (0.21)	16.67 (1.52)	19.71 (1.52)	-	-	-	-	-	^{a}14.67 (0.57)	^{ns}19.67 (0.57)

Am/Cl, Amoxicillin/Ac. Clavulanic ; **Ch**, Chloramphenicol ; **Fl**, Flucloxacillin ; **Ka**, Kanamycin ; **Ge**, Gentamicin ; **Am**, Amphotericin ; **Ke**, Ketoconazole.

Letters indicate significant difference at $P< 0.05$; ns, not significant. (-) no effect

Conclusion

Thus, the olive extract, obtained by a simple and environmentally technic, which closes an extraordinary amount of antimicrobial phenolic compounds inhibited the growth of a range of bacteria and fungi (*in vitro*). This inhibition included two ubiquitous opportunistic bacteria frequently responsible for food intoxications, *Staphylococcus aureus* and *Bacillus cereus*. These experimental data, although preliminary, lead us to think about future use of olive bio-extract as a food additive to protect food against these opportunistic germs but also with high nutritional value (through the biological effects of olive polyphenols). However, further work on the determination of minimum inhibitory and bactericidal concentrations (MCI and MCB) as well as the action of this extract mode are needed for this kind of use.

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