# CLINICAL STUDIES

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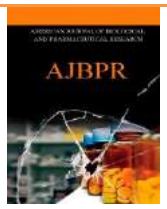


# EFFECTS OF RICH POLYPHENOLS OLIVE TREE EXTRACT ON INFLAMMATION AND PAIN IN PATIENTS WITH RHEUMATOID ARTHRITIS: A 8-WEEKS RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL

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Rheumatoid arthritis; Olive tree extract; Inflammatory biomarkers; Pain intensity; Randomized clinical trial.

**ABSTRACT**

Olive tree polyphenols have been known as natural anti-inflammatory agents. The aim of the current clinical trial was to assess the therapeutic effect of an olive tree extract with high polyphenols content on inflammatory process and pain intensity in rheumatoid arthritis (RA) patients. This is a randomized, double-blind, placebo-controlled clinical trial. Ninety RA patients were randomized into two groups; treated group received a daily dose of 3 g of olive extract (6 capsules, 500 mg each) during 8 weeks, while patients in placebo group received capsules with maltodextrin. Laboratory analysis, questionnaires administration, pain intensity, and inflammatory biomarkers were determined at the baseline and the end of the trial. Doctors assessed potential adverse effects of olive tree extract through the period of study. Significant decrease in disease activity score has shown at the end of intervention in the treated group and between groups ($P<0.0001$). Compared to the placebo group, inflammatory biomarkers decreased significantly in treated participants ($P<0.0001$). The changes from baseline in treated group were -1.37 mg/L (CI, -2.71 to -1.57 mg/L), -2.14 pg/mL (CI, -2.71 to -1.57), -1.046 pg/mL (CI, -1.50 to -0.59) and -1795 pg/mL (CI, -2283 to -1308) for hs-CRP, IL-6, TNF-α and PGE2 respectively. Pain relief and global participants satisfaction increased significantly ($P<0.0001$) after 8 weeks of olive tree extract supplementation. Results obtained after 2 months of supplementation demonstrate for the first time the potential therapeutic effect of olive tree extract with high polyphenols content against inflammation and associated pain in RA.

## INTRODUCTION

Rheumatoid arthritis (RA) is chronic autoimmune inflammatory disease responsible for joint destruction that contributes to functional impairment. RA remain the most common joint illness, occurring in 0.5-1% of worldwide population [1-2]. Several factors are involved to triggering the disease: tobacco [3-4], microbiome [5], hormonal factors [6], genetic background and environmental factors [7]. Schematically, the physiopathology of RA can be divided into three distinct phases: (1) initiation phase, (2) inflammation of the synovial membrane (synovitis), and (3)

joint destruction due to the pseudotumoral proliferation of synovial cells under cytokine actions. In fact, chronic synovial inflammation is the hallmark of RA that involves complex interactions between T and B lymphocytes, macrophages, and fibroblast-like synoviocytes, including a network of cytokines, chemokines and others molecules [8-9]. In RA, there is an imbalance between pro and anti-inflammatory cytokines. For instance, Nuclear Factor kappa β (NF-κβ) is activated in the synovium inflammatory cells and induced cytokines expression, including tumor necrosis factor-alpha (TNF-α), interleukine-1 (IL-1β), IL-6, IL-15, IL-18, but also metalloproteinase (MMP-1) and small osteochondral destruction molecules like prostaglandin $E_2$ ($PGE_2$) and nitric oxide [10]. These inflammatory biomarkers are present with high concentrations in the synovial fluid and serum of patients with RA, which clinically manifests in swelling, pain and tissue destruction. Through the last few years, blockage of cytokines network has taken a substantial proportion in clinical management of RA, more interestedly inhibition of produced TNF-α, IL-6, and IL-1 [8-11]. Useful molecules blocking these cytokines are mainly represented by the monoclonal antibodies or recombinant proteins (e.g. infliximab, Etanercept) [11-12]. Although their simple clinical use, these substances had some unexpected effects (including, efficacy, toxicity and even pharmacodynamics), e.g. catastrophic effects of the first-into-human administration of TGN1412 [12].

Beside the existing armamentarium therapeutic for RA disease, natural products represent a source of innovative treatments that could revolutionized the management of inflammatory diseases. Salminen et al. (2002) [13] reported that 33% to 75% of RA patients believed in alternative and complementary therapies, as dietary food that can delay the disease symptoms [14]. In this sense, several clinical trials have elucidated the effectiveness of olive polyphenols, as principal components of Mediterranean diet, on some inflammatory chronic diseases, including RA [15-16], and stable coronary heart disease [17-18]. Thus, adherence to the Mediterranean diet decreased inflammatory activity, increased in physical function, and improved vitality in RA patients. Hydroxytyrosol (3,4-DHPEA) is one of the most extensively studied olive polyphenols for its anti-inflammatory properties and various pharmacological activities, suggesting their potential use for the development of functional food [19]. In fact, hydroxytyrosol, tyrosol (*p*-HPEA) and oleuropein (3,4-DHPEA-EA) exert *in vitro* inhibitor effects on $PGE_2$, $LTB_4$, TNF-α, IL-6, IL-1 and high-sensitivity C-reactive protein (hs-CRP) [20, 21, 22]. Beauchamp et al. (2005) [23] have been signaled that the anti-inflammatory effect of oleocanthal was similar to the NSAID ibuprofen.

Although *in vitro* findings may be the first stitch in the chain of shift from natural product to synthetic molecule based drug, more results from the clinical trials are needed. Thus, we presented here results from randomized clinical trial regarding the effects of an olive tree extract supplementation on the inflammatory biomarkers, pain intensity, and disease activity of Moroccan patients having RA.

## MATERIALS AND METHODS

### Subjects

Men and women were recruited from October 2012 to April 2013 among of those referred to rheumatology service of a clinic in Fez, Morocco. To be enrolled in the current study, subjects had to have rheumatoid arthritis for more than one year diagnosed based on the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) [24]. Study was thoroughly explained to the voluntary participants. Patients were not eligible if they were under the age of 20 years or over the age of 80 years, pregnant, lactating, receiving contraceptive, smoking, being diagnosed with metabolic syndrome as defined by the Adult Treatment Panel III [25], having inflammatory disorders, receiving NSAIDs and/or cytokine inhibitors, had a white blood cell count $\leq 3.5\times10^9$/L, hemoglobin (Hb) level ≤8.5g/dl, platelet count $\leq 100\times10^9$/L, creatinine level ≥2.0 mg/dl, and aspartate aminotransferase (AST) levels ≥2.5 times the upper limit of normal. Exclusion criteria involved also the consumption of olive antioxidants or other antioxidant supplements ≤3 weeks before the intervention, history of allergy or intolerance to olive products. Before to be enrolled to this study, written informed consent was obtained from all voluntary participants.

### Study design and intervention

The current study was planned as a double-blind, randomized, placebo-controlled trial (fig. 1). Eligible participants were randomly assigned to Olive Tree Extract (OTE) supplement group or placebo group using a computer-generated random-number sequence. Researchers, participants and clinical staff were blinded to the treatment codes of each group. Candidates were invited by telephone to the clinic after an overnight fasting at last 12h to attend a screening visit including tender and swollen joints examination. The baseline examination included the assessment of adherence to the Mediterranean Diet according to the modified questionnaire of Estruch et al. (2006) (appendix table 1), evaluation of physical exercise by the International Physical Activity Questionnaire (Physical exercise was categorized as high, moderate, or low). Participants were asked to maintain their habitual diet during the period of study, and avoid the consumption of olive products (including olive oil, olive table) and nutrients with high n-3 PUFA contents (i.e. fish), the use of all herbs or products known to affect inflammation and immune function (including antioxidant and probiotic

supplements). Dietary changes was monitored trough a 3-day dietary records at baseline, 4 and 8 weeks after treatment and placebo intervention. Necessary explanations were provided about how to estimate food intake and record the estimations. Anthropometric and blood pressure measurements were performed and a sample of 8 ml fasting blood was obtained from each participant's antecubital vein. We repeated all examinations and measurements after 8 weeks.

During the study, all participants and investigators had free and continuous access to clinic for advice and consultation.

Participants who fulfilled all the inclusion criteria were received 500-mg study capsules (identical capsules for supplement and placebo group). Participants received also instructions concerning capsules taking and storage. Patients were asked to administrate 6 capsules per day before each meal and they were contacted every week to monitor supplement intake. Aqueous olive tree extract (OTE) and maltodextrin excipient were enclosed in soluble vegetal capsules. The placebo capsules contained only maltodextrin. OTE was obtained from different olive tree parts, fruits, olive tree young branches, and leaves using a purely natural and physical extraction. Olive trees are planted in the middle a rocky desert of Morocco, free of pollution, free of industrial activity, and under drought-stress (temperatures up to 52°C). OTE is OLIVIE FORCE marketed in Belgium as OLIVIE RICHE (see more in www.olivie.ma). Table 1 illustrate the main components of OTE extract.

**Laboratory measurements**

Anthropometric measures was performed using calibrated scales and wall-mounted stadiometer with a precision of 0.1 cm; systolic and diastolic blood pressure were measured using a semi-automatic oscillometer (Boso Medicus smart Semi automatic Blood Pressure Monitor, Germany). Blood samples were collected in EDTA and SST tubes. The obtained erythrocytes, plasma, serum and urine samples were aliquoted into 1 mL microtubes and stored at -80°C until further analysis. Energy, nutrient intake and participants' diets assessment was carried out by Nutritionist 4.3 software (First Databank, Hearst Corp, San Bruno, CA).

High-sensitivity enzyme - linked imminosorbent assay kits (DiaSource, Belgium) were used to quantify $PGE_2$, leukotriene $B_4$ ($LTB_4$), TNF-α and cytokines IL-1 and IL-6 in serum according to the manufacturer's guidelines. Serum's hs-CRP level was determined by Turbidometric assay (Modular™, Roche Diagnostics, France) using a commercial kits at a wavelength of 500 nm. Clinical indication of disease activity and laboratory parameters of study participants were measured at the baseline and at the end of study according the internal methods of the clinic laboratories.

Urinary hydroxytyrosol was quantified by High Performance Liquid Chromatography (HPLC) as markers of OTE intake. Briefly, hydroxytyrosol was extracted from acidified urine (hydrochloric acid, 0.6 N of final concentration) as described previously [26] and analyzed in a Shimadzu chromatograph device equipped with a reverse phase C18 column (250 mm L. × 4.6 mm I.D., 5 µm).

Pain intensity was evaluated at baseline, 4 and 8 weeks (end of study), using visual analog scale (VAS) according to the protocol defined by DeLoach et al. (1998) [27]. Participants are instructed to indicate pain intensity by marking a 100-mm line, 0= no pain and 100=most severe pain. Pain relief was assessed using a 5-point verbal rating scale (VRS) where 0=no relief, 1=a little (perceptible) relief, 2=some (meaningful) relief, 3=a lot of relief, and 4 = complete relief. Disease Activity Score (DAS28) was determined according to the EULAR [28], based on number of tender and swollen joint (TJC and SJC), serum hs-CRP concentration, and the result of Global Health (GH) assessed by the patient on a 10-cm VAS. DAS28 was calculated as follows:

$$DAS28\ (CRP) = [0.56\ \sqrt{TJC}] + [0.28\ \sqrt{SJC}] + [0.36\ Ln\ (CRP + 1)] + [0{,}014\ (GH)]$$

Doctors assessed potential adverse effects of OTE administration over the period of study including mouth symptoms, digestive disorders, fullness, allergic skin response, and other intervention-related symptoms. Finally, global satisfaction assessment in response to treatment (GAST) (including anxiety) was evaluated using a 5-point categorical scale (0 = poor, 1 = fair, 2 = good, 3 = very good, and 4 = excellent). The current study was directed according to the guidelines approved by Helsinki Declaration.

**Statistical analysis**

Data were statistically analyzed using GraphPad Prism version 5.00 (GraphPad Prism Inc, San Diego, California). For the baseline characteristics, continuous variables are expressed as mean values ± standard deviation (SD), and categorical variables are expressed as frequencies (percent). For inflammatory biomarkers, pain intensity, and pain relief mean values are expressed with 95% confidence intervals (CIs). Normal distribution of data was checked using the Kolmogorov-Smirnov test.

The difference between baseline groups characteristic was performed by, the independent t test, the Mann-Whitney U test, and the $\chi 2$ test for normally continuous data, not normally continuous data, and categorical data, respectively. The independent t test was also used to compare the mean changes from baseline to the end of the study (8 weeks) between OTE and placebo group. Results with two-sided P values of $<0.05$ were considered statistically significant.

## RESULTS

One hundred one eligible patients were enrolled, and 11 were excluded from the study for several reasons (Figure 1). Five participants were dropped out of analysis (2 in OTE-group and 3 in placebo-group) because they were unable to follow study protocol. Good compliance was showed in OTE-group (95.55%) and placebo-group (93.33%), without any study-intervention observed adverse. Urinary hydroxytyrosol determined as biomarker of compliance was quantified by HPLC. Results plotted in the graph of figure 2 illustrate the changes from pre-intervention periods for placebo and OTE (at 4 and at the end of study) group. The concentration of hydroxytyrosol determined in urine of OTE participant's group was significantly different ($P$<0.0001) compared to that of placebo group. However, it is worth noting that literature data on olive phenols absorption, metabolism, and excretion are not in agreement [29-30].

Table 2 shows the baseline characteristics of the 90 participants who randomized into the OTE and placebo group. Statistical analysis reveal no significant differences between the two study groups with regard to any of the baseline characteristics, including the degree of adherence to Mediterranean diet ($P$=0.296).

Results of dietary questionnaires represented in table 3 show that there was no significant difference in diet intake at the baseline and after eight weeks of OTE and placebo supplement. Result of table 3 show also that the PUFAs intake was maintained constant ($P$ value of 0.611 and 0.741 for OTE and placebo group), since the presence of n-3 PUFAs quantity may be useful for the treatment of inflammatory in RA disease [31]. We also reported in table 3 change in participant's weight, no significant differences has been observed over the period of study for OTE group ($P$=0.976) and placebo group ($P$= 0.759). This is appropriate to this study as the adipose tissue is an active endocrine organ that secretes inflammatory cytokines [32]. Generally, the level of macronutrient intakes was held constant during the study course, which could not affect inflammatory response and biomarkers of inflammation level in the patient's sera. However, a significant difference ($P$=0.045) was reported for MUFAs intake, due to an excessive consumption of olive oil (high content of MUFAs, e.g. oleic acid) at the last week of study intervention by tow participants of placebo group that was considered not affect the current study results. Indeed, all participants met the daily diet recommended by the researchers for this study by avoiding the consumption of olive products and any other products known to have anti-inflammatory effects.

Rheumatoid arthritis is a chronic autoimmune inflammatory disease characterized by joint swelling, joint tenderness and destruction of synovial joints. The clinical outcome is pain, warmth, redness, and loss of function. Inflammation of synovial membrane is believed to be the main cause involved in RA outcomes. High concentration of circulating markers of inflammation, such as cytokines (IL-6, IL-1, TNF-α) and hs-CRP correlate with propensity to join destruction in RA.

Graphs of figure 3 show the changes from baseline values in inflammatory biomarkers IL-6, IL-1, TNF-α, and hs-CRP concentrations in the two study groups. The CRP concentration decreased significantly in participants who were received OTE after 4 ($P$=0.014) and 8 weeks ($P$<0.0001) compared with participants in the placebo group. The average change of hs-CRP levels were -0.55 (CI, -0.92 to -0.18) and -1.37 mg/L (CI, -2.71 to -1.57 mg/L) after 4 and 8 weeks, respectively.

Significant decrease was also observed in plasma levels of IL-6 and TNF-α ($P$<0.0001). The adjusted between-group changes was -2.14 pg/mL (CI, -2.71 to -1.57) and -1.046 pg/mL (CI, -1.50 to -0.59) for IL-6 and TNF-α at the end of the study. Nevertheless, no significant difference from baseline ($P$ value of 0.929 and 0.206 at 4 and 8 weeks) was observed for IL-1 concentrations. The significant decrease in plasma IL-6 may leads to the stabilization of circulating IL-1, which can explain the results of figure 3d. Otherwise, RA has often been accompanied by high-intensity chronic pain. Graphs of figure 4 summarized the changes in pain intensity and pain relief from baseline in the OTE and placebo groups.

**Figure 1. Study flow diagram.**

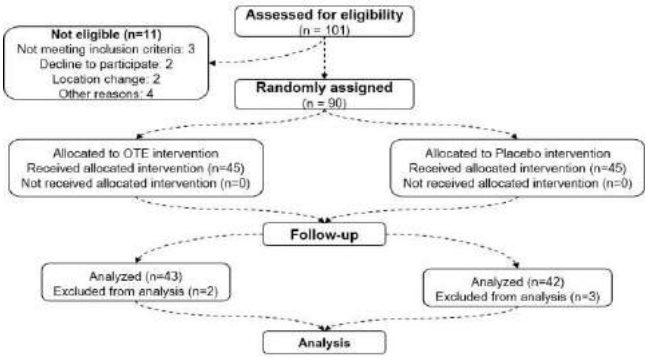



**Figure 2. Degree of compliance checked by the change from baseline in urinary hydroxytyrosol excretion. Mean with 95% CI. [a]$P$<0.0001, between OTE-group (at 4 or 8 weeks); [b]$P$= 0.003, between OTE-group at 4 and 8 weeks.**

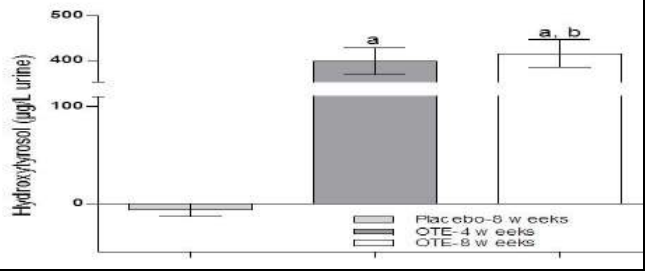
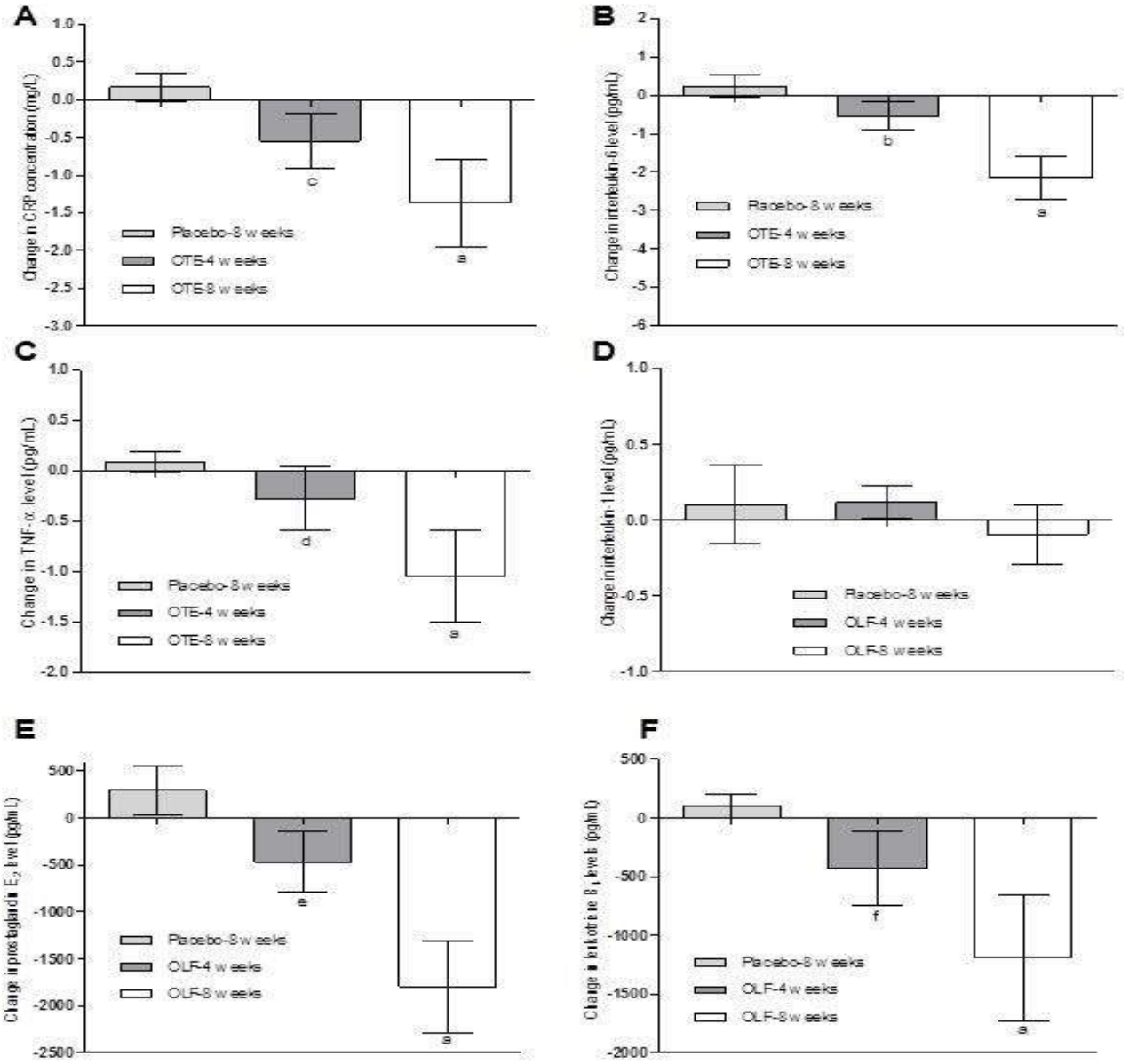


**Figure 3. Change from baseline in circulating inflammatory biomarkers level in the tow study groups, (a) hs-CRP, (b) IL-6, (c) TNF-α, (d) IL-1, (e) $PGE_2$, and (f) $LTB_4$ (a). Error bars are 95% CIs. [a]*P*<0.0001, [b]*P*=0.014, [c]*P*=0.009, [d]*P*=0.0247, [e]*P*= 0.0004, and [f]*P*=0.0017 vs. placebo group.**




Significant decrease (*P*<0.0001) in pain intensity score has been recorded in OTE-group compared to the placebo group. The evaluation of the post-intervention pain intensity shows a decrease of -12.94 ± 4.970 (CI, -15.50 to -10.39) after 4 weeks, and -26.71 ± 9.29 (CI, -31.49 to -21.93) at the end of the trial. Thus, the pain intensity (100-mm pain scale) decrease from 75.51 ± 9.81 to 48.80 ± 4.16 after 8 weeks of OTE intake. A similar trend to pain intensity response was observed for pain relief score. Participants of OTE-group had significantly recorded high pain relief scores compared to those in the placebo group (*P*<0.0001), even after 4 weeks of intervention. Pain relief score increased by 2.61 ± 0.48 (CI, 2.37 to 2.37), which correspond to an average value of 3.26 ± 0.66 (CI, 2.92 to 3.60) in the 5-point VRS after 8 weeks of OTE administration. We should underline that 30% of the OTE-group participants were declared a lot of pain relief (pain relief score ≥3), whereas others OTE-group members perceived a meaningful pain relief (pain relief score ≥2) at the end of the study.Similarly, significant differences between OTE and placebo groups (*P*<0.0001) have been reported for DAS28 scores. Patients in OTE group with baseline active RA (DAS28 score ≥ 3.2) showed good therapeutic response (decrease in DAS28 by 1.23) (figure 4). DAS28 score recorded at the end of the trial for OTE group was 2.23 ± 0.40, signaling a RA remission (≤ 2.6). Figure 4 summarized also global satisfaction assessment in response to treatment, including patient's anxiety. Participants who were allocated to OTE-group had a satisfaction score of 3.206 ± 0.53 (corresponding to a very good in the 5-point categorical scale), compared (*P*<0.0001) with those of placebo group. Such degree of satisfaction correlate with a significant decrease in circulating inflammatory biomarkers level and increase in pain relief score and DAS.

**Figure 4**. Change from baseline in pain intensity (a), pain relief (b), DAS28 (c, d), and GART (e) scores. Mean with 95% CIs. [a]*P*<0.0001, significant difference between OTE and placebo groups.

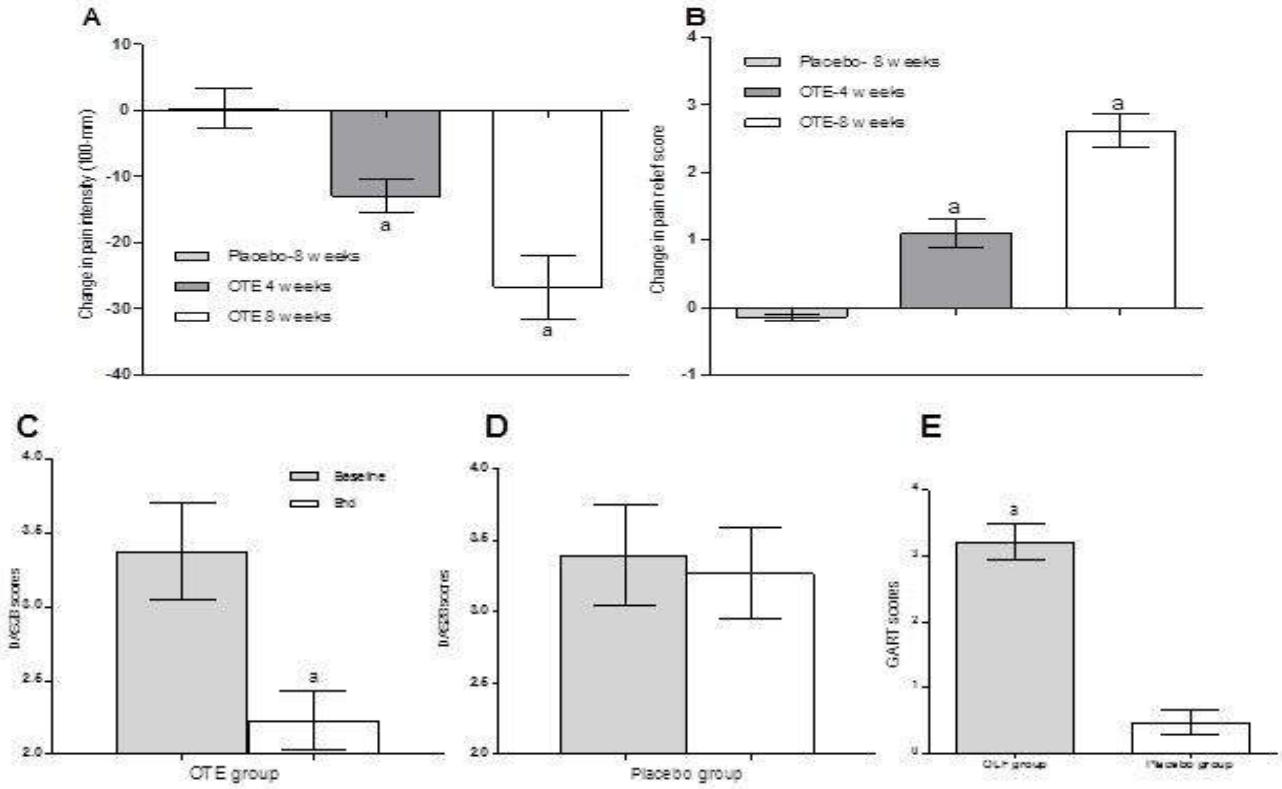



**Table 1. Main constituents of olive extract (OTE) expressed in percentage (g/100g). Mean ± standard deviation.**

| Parameter | Average value |
|---|---|
| Total solids | 97.96 ± 7.83 |
| Total volatiles (mineral) | 12.9 ± 0.7 |
| Total lipids | < 1 |
| Total polyphenols | 15.98 ± 1.9 |
| Hydroxytyrosol | 2.09 0.14 |

**Table 2. Baseline characteristics of participants.**

| Parameter | OTE group (n=45) | Placebo group (n=45) | *P* value [a] |
|---|---|---|---|
| Age (years) | 53.27 ± 1.61 | 55.73 ± 1.97 | 0.346 |
| Female, n (%) | 42 (93.33) | 41 (91.11) | 0.915 |
| Weight (kg) | 67.15 ± 3.86 | 67.65 ± 3.99 | 0.944 |
| BMI (kg/m$^2$) | 28.17 ± 1.662 | 27.83 ± 1.815 | 0.851 |
| Disease duration (years) | 6.67 ± 0.421 | 7.50 ± 0.563 | 0.366 |
| Medical history of disease, n (%) | 19 (40.00) | 15 (33.33) | 0.106 |
| Family history of disease, n (%) | 9 (20.00) | 10 (22.22) | 0.698 |
| Exercise activity habits, n (%) | 14 (31.11) | 13 (28.88) | 0.788 |
| Alcohol drinking habits, n (%) | 4 (8.88) | 2 (4.44) | 0.293 |
| 15-item Mediterranean diet score | 2.05 ± 0.15 | 2.40 ± 0.20 | 0.296 |
| DAS28 | 3.374 ± 0.6625 | 3.392 ± 0.7132 | 0.940 |
| Pain VAS (0–100 mm) | 75,51 ± 9,814 | 76.65 ± 10.12 | 0.741 |

Value are expressed as mean ± standard deviation or in percentage.
[a] *P* value (<0.05) by independent t-test or Mann-Whitney test, as appropriate.

**Table 3. Change in energy and macronutrients intake at baseline and end of the study for tow study groups. Data are expressed as mean ± standard deviation**

| Parameter | OTE group (n=45) | Placebo group (n=45) |
|---|---|---|
| **Energy (cal)** | | |
| Baseline | 1695.00 ± 219,80 | 1729.00 ± 100.7 |
| 8 weeks | 1702.00 ± 225,30 | 1685.00 ± 318.6 |
| *P* value [a] | 0.576 | 0.745 |
| **Fat (g)** | | |
| Baseline | 65.90 ± 11.04 | 68.23 ± 14.37 |
| 8 weeks | 69.07 ± 12.35 | 70.40 ± 14.35 |
| *P* value [a] | 0.547 | 0.780 |
| **PUFAs (g)** | | |
| Baseline | 13.23 ± 1.41 | 12.90 ± 1.33 |
| 8 weeks | 13.73 ± 2.89 | 13.07 ± 1.91 |
| *P* value [a] | 0.611 | 0.741 |
| **MUFAs (g)** | | |
| Baseline | 20.57 ± 1.85 | 20.07 ± 1.77 |
| 8 weeks | 21.57 ± 2.09 | 21.57 ± 1.62 |
| *P* value [a] | 0.110 | 0.045 |
| **SFAs (g)** | | |
| Baseline | 13.73 ± 1.49 | 13.73 ± 2.07 |
| 8 weeks | 14.23 ± 1.63 | 13.57 ± 2.09 |
| *P* value [a] | 0.415 | 0.849 |
| **Weight (kg)** | | |
| Baseline | 67.15 ± 3.86 | 67.65 ± 3.99 |
| 8 weeks | 67.31 ± 3.87 | 69.31 ± 3.46 |
| *P* value [a] | 0.976 | 0.759 |

PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids. [a] Paired Student *t* test ($P < 0.05$).

## DISCUSSION AND CONCLUSION

Patient with chronic RA (according to the ACR/ELUAR) were allocated to a treatment by an aqueous olive tree extract during 8 weeks, by receiving a daily dose of 3 g of olive tree extract (6 capsules, 500 mg each). No adverse sign and laboratory parameters fluctuation have been observed during the study and 3 weeks of the post-intervention (data not shown). Results obtained after 2 months of clinical trial demonstrate for the first time the potential therapeutic effect of high polyphenols content extract (OTE) against inflammation in RA disease. Thus, 8 weeks of OTE administration reduced pro-inflammatory cytokines level (TNF-α, IL-6, but not significantly IL-1), hs-CRP concentration and pain intensity.

Through the RA progression, an excessive influx of inflammatory cells has occurred into the synovial membrane (transformed to an autonomous tissue, pannus) where chronic inflammation take place leading to cartilage damage and bone destruction (mediated by osteoclasts). Taken together, the inflammatory process and osteoclasts differentiation were the consequence of cytokines activation, particularly TNF-α, IL-6, IL-1 and other inflammation mediators such as eicosanoids (especially $PGE_2$ and $LTB_4$) (Smolen et Redlich, 2014; Boissier et al., 2012). Thus, inhibition of the overproduction of pro-inflammatory cytokines is the principal target of anti-inflammatory agents, including glucocorticoids and NSAIDs. Smolen et Redlich (2014) reported that inhibition of TNF-α and IL-6 production seems to be more important to predict inflammation, while IL-1 inhibition appears to be minor. Results herein obtained show that the supplementation by a rich polyphenols extract (15% of total polyphenols and 2% of hydroxytyrosol) contribute to the reduction of TNF-α, IL-6, hs-CRP, $PGE_2$, and $LTB_4$ (figure 3) in patients with RA. This result may be a direct consequence of OTE polyphenols (particularly hydroxytyrosol), who act directly on DNA to reduce expression of inflammatory mediators or inhibit their biosynthesis pathways through a similar mechanism of glucocorticoids and/or NAIDs.In this sense, hydroxytyrosol inhibit the expression of inducible cyclooxygenase (COX-2) (key enzyme that catalyzed biosynthesis of $PGE_2$ from arachidonic acid during inflammation process) and, therefore, $PGE_2$ level in isolated human monocytes [22, 33, 34] and murine macrophages [21]. Similar effect on COX-2 and $PGE_2$ was observed *in vivo* when mice with DSS-induced colitis are treated by olive oil with high hydroxytyrosol content [35] or by oleuropein [36]. Beside

this, pure hydroxytyrosol or contained in its natural matrix (olive products such as, aqueous olive tree extracts and olive oil) exerts an inhibitor effect on $LTB_4$, TNF-α, IL-6, IL-1 and hs-CRP [20, 21, 22, 37]. The effect of olive polyphenols on inflammatory markers has been emphasized in patients with stable coronary heart disease who received olive oil with different polyphenols concentrations [17-18]. Results of the current study were in agreement with the *in vitro* and *in vivo* investigations (literature cited above), suggesting the therapeutic effect of hydroxytyrosol and other OTE polyphenols against inflammation in RA. Additionally, the decrease in both $PGE_2$, $LTB_4$, TNF-α, IL-6, IL-1 and hs-CRP concentration may be the direct consequence of COX-2 inhibition. However, the repression of COX-2 gene leads to a decrease in IL-6 production and a relationship between increased macrophage $PGE_2$ and IL-6 level is reported *in vitro* [38-39]. In turn, one of the known biological roles of IL-6 is the activation of produced inflammatory proteins, which can explain the decrease in hs-CRP level. Similar mechanism has already described for NSAI drugs. Nevertheless, NSAIDs (celecoxib, rofecoxib, diclofenac) increased TNF-α production in rheumatoid synovial membrane cultures [33-40], while our results indicate a significant decrease in plasma TNF-α. This could be due to another signaling pathway induced by hydroxytyrosol and/or other OTE polyphenols. By assuming that, the potential effect of hydroxytyrosol (and other olive polyphenols) on NF-κβ has been previously elucidated by several authors. NF-κβ occupied a central upstream position in the inflammatory process, since it triggers the expression of more than 150 genes [41]. Among of them, those encoding cytokines, TNF-α, IL-1, and IL-6 herein studied. Hydroxytyrosol from aqueous olive extract inhibit the expression of NF-κβp65, and the authors suggest that this inhibitor effect may be the cause of cytokines reduction in murine macrophages [21]. Furthermore, hydroxytyrosol suppressed NF-κβ expression in human monocyte (TPH-1) and altered its translocation into the nucleus [42]. Beside this, hydroxytyrosol decreases NF-κβ activity in endothelial [43] and neural cells [44]. Thus, aqueous olive extract (OTE) most likely exert its anti-inflammatory effect in patients with RA by reducing the expression of NF-κβ and/or COX-2 enzyme.

On the other hand, an excessive angiogenesis ensues from progression of inflammatory process in synovium, and leads to pannus proliferation and RA symptoms complication. Neovascularization (angiogenesis) represent a major contributor in the development and maintain of inflammation in RA [45], and a correlation between RA progression and VEGF level (Vascular Endothelial Growth Factor, most important pro-angiogenic factor) has been observed in patients with RA [46]. The activation of VEGF and Angiopoietins-1 (Ang-1), another pro-angiogenic factor, is a shared and multi-targeted mechanism, including cytokines dependent NF-κβ (IL-1β and TNF-α) and COX-2 expression [43-47]. Our unpublished data elucidate that Hydroxytyrosol from olive fruit inhibit, *in vitro*, angiogenic response of endothelial cells, by repressing VEGF (isoforms A, B, and C), Ang-1 and Ang-2 gene expression. This was in agreement with results of previous studies [43-48]. Otherwise, pain intensity has been long since known as main clinical manifests of inflammatory process in RA. Pain intensity in RA was associated to an increase of $PGE_2$ level [49-50], which explain the effectiveness of NSAIDs as pain relief agents. Beauchamp et al (2005) [23] have reported similar findings for oleocanthal (phenolic compound from olive oil). As a result, the decrease in circulating inflammatory markers, particularly in $PGE_2$ level, is likely the major cause of pain intensity reduction observed in OTE-group.

In conclusion, results from clinical trial suggest the effectiveness of olive tree extract with high polyphenols content as anti-inflammatory agents in patients with RA. The resolution of inflammatory process in RA is exerted through plausible mechanisms, including cytokines (IL-6 and TNF-α) dependent NF-κβ inhibition, COX-2, VEGF and Ang-1 repression. The net outcomes are decrease in pain intensity, disease activity score and joint protection.

This provides evidence the pleiotropic effects of hydroxytyrosol on inflammation, particularly when it was transported in its natural environment (the olive tree as a whole). Despite their various targets, more information are needed regarding anti-angiogenic activity of hydroxytyrosol in synovial membrane, which could represent a future target of new anti-inflammatory drugs based on hydroxytyrosol structure. In addition, the potential effect of olive polyphenols on T-cell co-stimulation and B-cell depletion must be clarified. The current findings are in agreement with those obtained *in vitro* or *in vivo* in several clinical studies about anti-inflammatory effects of olive polyphenols, suggesting the potential role of these natural compounds for "functional foods" conception.

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OLIVIE

THE BENEFITS OF THE OLIVE TREE

# CLINICAL STUDY N°2

# Anticancer effect of an olive tree extract through his cytotoxic, antioxidant and antiangiogenic activities

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**Abstract**

The aim of the current study was to evaluate the anticancerous potential of a phenolic olive tree extract through his cytotoxic, antioxidant and antiangiogenic activities. The *in vitro* cytotoxic activity of a crude olive tree extract and his major constitutive components has been evaluated against human KB, HL60 cancer cell lines using MTS and flow cytometry. ROS production was estimated with the DCFH-DA assay. Antiangiogenic activity was evaluated *in vitro* on endothelial cells tube formation and the pro-angiogenic factor expression was quantified using qRT-PCR. *In vitro* cellular assays have demonstrated the cytotoxic effect of the crude olive tree extract. This extract reduces significantly ($p$<0.05) ROS produced in cells exposed to oxidative stress. Beside this, olive tree extract has demonstrated a strong anti-angiogenic activity, which was correlated with a significant decrease ($p$<0.05) in VEGF, angiopoietin and HIF1α expression. Basically, the evaluation of anti-proliferative, antioxidant and anti-angiogenic activities could be the first step to formulate an efficient pharmaceutical product with preventive and/or curative properties against cancer.

**Key words**: Olive tree extract; Anticancerous; Cytotoxic; Antioxidant; Antiangiogenic.

## Introduction

Olive is a generic name for about 35 species of evergreen shrubs and trees of *Olea* genus and *Oleaceae* family, native to tropical and warm temperate regions. The name is particularly used to describe *Olea europaea* L. space cultivated for its edible fruits (olives). The use of olive fruits and leaves for alimentary, therapeutic and cosmetic purposes was a well-known practice in the Mediterranean basin for more than 7 000 years (Evagelia et al., 2004). Although polyphenols are present in olive leaves, olive fruits has been known for several years as a major source of phenolic compounds with high biological activities (Japón-Luján et al., 2007; Mario-Casas et al., 2003; Visioli et al., 2000). Actually, combined of olive fruits and leaves (10% of the total weight of the olives produced generated during olive harvest and olive tree size) to biophenols extracting enhance both qualitative and quantitative extraction yield.

The phenolic fraction of olive tree (fruits, leaves and very young leaves) is a complex mixture of chemical compounds with different structures and molecular weight obtained using mainly liquid-liquid extraction (methanol-water). Olive tree polyphenols belong generally to the following classes: (a) tyrosol, hydroxytyrosol and their secoiridoide derivatives; (b) 4-hydroxybenzoic acid, 4-hydroxy-phenylacetic acid and 4-hydroxycinnamic acid derivatives; (c) lignans and (d) flavonoids. These various compounds have antioxidant (Nardia et al., 2014; Benavente-García et al., 2000), antimicrobial (Micol et al., 2005) and antiproliferative (Taamalli et al., 2012) properties. Today, data collected from *in vitro*, *in vivo* and clinical studies reveal the benefits of these bioavailable compounds for human health. These antioxidant compounds are useful agents for preventing cellular aging and age-related diseases by improving mitochondrial function (Raederstorff et al., 2010). The benefits of olive phenolic compounds on the cardiovascular system have been well documented (Covas, 2007, 2006; Esturch et al., 2006). Beside, these compounds also modulate the inflammatory response associated to several diseases (Killeen et al., 2014; Scoditti et al., 2012; Richard at al., 2011).

Published data have reported that apoptosis and/or necrosis are implicated in the cytotoxicity of olive tree polyphenols. Thus, it has been demonstrated that oleuropein and hydroxytyrosol induce apoptosis in HT-29 human colon adenocarcinoma, HL-60 promyelocytic leukemia) and MCF-7 breast cancer cell lines (Fabiani et al. 2002,

2006, Han et al., 2009). More recently, LeGendre et al. (2015) stated that oleocanthal rapidly and selectively induces apoptotic or necrotic processes via lysosomal membrane permeabilization. Beside this, through an *in vivo* study, Hamdi and Castellon (2005) showed that oleuropein inhibits tumor growth in tumor-bearing mice. Additionally, many epidemiological studies suggest the possible correlation between the consumption of olive products and the incidence of breast cancer (Giacosa et al., 2013).

In the present study, we investigated the cytotoxic effect of an olive tree extract against several cancer cell lines through commonly used cellular assays. Additionally, the current study includes the determination of antiangiogenic and antioxidant effects of olive phenolic compounds contained in their stable and natural matrix.

## Materials and methods

### Extract preparation

Olive leaves and fruits were mixed to obtain the aqueous olive tree extract (OTE) by solid-liquid extraction 100% natural, 100% mechanical using water (no use of alcohol, no use of ethanol, no use of chemicals). Actually, OTE is marketed in the world (France, Switzerland, Belgium, …) under the brandname OLIVIE FORCE / OLIVIE RICHE (see more in www.olivie.ma). The extract consists in a dark brown solution whose major constituents have been determined (table 1). It comes from organic olive trees that are planted in the middlle of a rocky desert where trees suffer and trigger a self-defence mechanism where they produce abnormally high quantities of anti-oxydants to survive.

**Table 1**. Total phenols and major constituents of olive tree extract. Mean ± standard deviation *(corresponds to the initial first version of OLIVIE FORCE / OLIVIE RICHE)*.

| Parameter | Average value (g per100g) |
|---|---|
| Density | 1.3 |
| Dry matter | 98.0 ± 7.8 |
| Mineral matter | 12.9 ± 0.7 |
| Fat | < 1 |
| Total polyphenols | 7.90 ± 1.9 |
| Hydroxytyrosol | 2.09 ± 0.14 |
| ORAC (µmol Te kg)* | 3848100 ± 38481 |

*Antioxidant activity determined by ORAC assay (Oxygen Radical Absorbance Capacity).

**Cell culture**

The HSkMEC (human skin immortalized endothelial cells) was kindly provided by C Kieda (UPR4301 CNRS, Orleans, France) (Kieda 2002), the KB cell line (human nasopharyngeal epidermis carcinoma) was obtained from NCI and HL60 (promyelocytic leukemia) and HT-29 cells (human colon adenocarcinoma) were purchased from ATCC. KB cells in D-MEM, HL60 and HT29 in RPMI and HSkMEC in OPTI-MEM were cultured in complete medium containing 10% (except HSkMEC 2%) fetal bovine serum, penicillin, streptomycin and fungizone in a humidified incubator under 5% $CO_2$ atmosphere at 37°C. Phenolic compounds (used as reference molecules in this study): oleuropein, hydroxytyrosol, tyrosol, cafeic acid, apigenin, and luteolin were purchased from Sigma Alderich (Paris, France).

**MTS assay**

HSkMEC, KB and HL60 cell lines were used to determine the cytotoxicity of OTE and reference molecules (phenolic compounds used as standards). Cells were plated in 96-well tissue culture microplates ($10^3$ to $2.10^3$ cells/well in 200 µl of culture medium). After 24 hours of incubation at 37°C under 5% $CO_2$ atmosphere, cells were treated with increasing concentrations of OTE (diluted in $H_2O$) and phenolic compounds dissolved in DMSO (1, 10, and 100 µM). Control cells were treated with either water or DMSO (1% of final volume). These operations were carried out using an automated workstation (Biomek 3000 Laboratory Automation Workstation, Beckman-Coulter). After 72h of exposure to olive tree extract or chemicals, MTS reagent (CellTiter 96® $AQ_{ueous}$ One, Promega) was added and incubated for 3 h at 37 °C. Experiments were performed in triplicate: the absorbance was monitored at 490 nm and results were expressed as the inhibition of cell proliferation calculated as the ratio [(1-(OD490 treated/OD490 control)) x 100].

**FACS analysis**

Apoptotic and necrotic cells were analyzed by Fluorescence-Activated Cell Sorting (FACS). KB cells ($10^4$ cells in 100 µL medium/well in 96-well microplate) were exposed for 24 and 48 hours at 37°C under 5% $CO_2$ to olive tree extract at different concentrations. 50nM doxorubicin was used as positive control. Microplates were centrifuged to collect floating cells. Adherent cells were trypsinazed, washed with PBS, mixed with floating cells and spun down. Cells were resuspended into 100µl

medium prior the addition of 50µl of freshly prepared reaction mixture consisting in the fluorescent agent 7-aminoactinomycin D (7-AAD) (6.5 µL of 1 mg/ml ethanol solution) and recombinant human protein annexin-V-PE (6.5 µL, Bender). After 20 min of incubation in the dark at room temperature, cells were analyzed with a Guava EasyCyte flow cytometer (Millipore). Cells were classified according to their fluorescence and the results were expressed as percentage of cells in each group.

**LDH release assay.**

Necrosis was estimated through the release of LDH in the culture medium. 20,000 KB cells were incubated for 24 and 48 h in the presence of OTE in 96-well microplates containing 100 µL medium. After centrifugation at 300g for 1 min, 25 µL of culture medium was added with 25 µL Cytotox-ONE reagent (Promega) and kept in the dark at room temperature for 20 min. Fluorescence was recorded (exc 560 nm, em 590 nm): results are expressed as the residual activity in the presence of OTE compared to activity in the presence of vehicle alone.

**Caspase activity assay**

Caspase activity was determined in KB cells after 24 and 48h of treatment by olive tree extract. Cells ($20.10^3$ cells in 180 µl of complete medium per well) were plated in a 96-well microplate under 5% $CO_2$ atmosphere at 37°C. Lysis buffer (20 µL of a 10x stock solution consisting of 250 mM HEPES buffer, pH 7.5, 5 mM EDTA, 0.5% NP40, 0.1% SDS and 50 mM dithiothreitol) was added before adding caspase-3 substrate (DEVD-AMC at final concentration of 50 µM). The plates were incubated at 37°C and fluorescence was recorded ($\lambda_{ex}$ 360 nm, $\lambda_{em}$ 465 nm) after 0, 30, 60, 120, and 180 min. The reaction rate was calculated from the slope of the linear regression (fluorescence *vs* time) and expressed as the ratio of activation compared to the control (cells treated with DMSO). Doxorubicin (1µM) was used as positive control.

**ROS production**

HT29 cells were growth in 200 µl RPMI complete medium ($20.10^3$ cells/well in black 96-well microplates). After 24 hours, cells were incubated with increasing concentrations of olive tree extract (1 to 100% diluted in $H_2O$) and 100 µM of reference phenolic compounds (hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin, caffeic acid dissolved in DMSO). Control cells received DMSO (1% of final

volume). The culture medium was removed and cells were washed with PBS buffer before addition of 100 µl of RPMI containing 50 mM of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were subsequently incubated for 30 minutes at 37°C before washing with PBS. The fluorescence was monitored after the addition of 100 µl of PBS ($\lambda_{ex}$ 485 nm, $\lambda_{em}$ 530 nm). The positive control was untreated HT29 cells incubated in PBS containing 1 mM of $H_2O_2$.

**In vitro endothelial cell tube formation**

In this study, the *in vitro* test of tube formation on Matrigel was used to assess the anti-angiogenic activity of olive tree extract. The collagen matrix (Matrigel) was diluted in ice-cold Opti-MEM without fetal calf serum and 30µl of this mix were transferred into a 96-well microplate. The plate was incubated at 37°C under 5% $CO_2$ atmosphere for 60 min to form the gel. HSkMEC cells ($50.10^3$ cells/well) were seeded in 200µl medium containing increasing concentrations of olive tree extract and phenolic compounds (100µM). Endothelial cells cultured on Matrigel formed a three-dimensional micro-network of tubes within 16 h of culture. Cell organization was documented photographically with an inverted microscope at $\times$10 magnification.

**Quantification of pro-angiogenic factors by qRT-PCR**

The quantitative RT-PCR was used to measure expression levels of genes coding for the pro-angiogenic factors, vascular endothelial growth factor (VEGF: A, B and C isoforms), hypoxia-inducible factor (HIF-1α), angiopoietin (ANGPT 1 and 2 isoforms) and VEGF receptor (VEGFR-2) as previously reported (Kieda et al, 2006, Kieda et al, 2013). The cells were lysed and total RNA was extracted from each well of the 96-well microplate using RNA extraction kit according to manufacturer's instructions (Promega, Madison, WI). Two micrograms of RNA were used to prepare cDNA with oligo (dT) and primers of Myeloblastoma Avian Virus (AMV) and reverse transcriptase (Promega). Quantitative real-time PCR was performed using the Roche Light Cycler system and kit FastStart DNA Master SYBR Green-I. Primers were supplied by Qiagen. The data were analyzed with LightCycler 480 Software and values were normalized to the relative amounts of the cDNA of GAPDH gene.

**Statistical analysis**

All cellular tests were performed in triplicate. Statistical analyzes were performed using GraphPad Prism version 5.00 (GraphPad Inc., San Diego, California). Data were analyzed by analysis of variance (ANOVA Analysis of Variance) followed by post-hoc Dunnet test for normal distributed data or by the Kruskal-Wallis if the sample distribution does not follow the normal law. Differences between groups were considered as statistically significant at $p<0.05$.

## Result

### OTE cytotoxicity

A cytotoxic extract is defined as a substance (or mixture of several phytochemical compounds) that is toxic for the cell, acting at several levels from simple metabolic disorders to apoptosis and/or necrosis induction. Actually, studying of plant extract's cytotoxicity is essential to determine appropriate dose prior to its biological uses. Nevertheless, the cytotoxic character is often sought in pharmaceutical field during the research for new drugs with various biological properties. The results presented in this section are those obtained with the commercial MTS test, e.g. the formazan formation followed at 490 nm. The measured absorbance values are directly proportional to the number of living cells and the results were expressed as the inhibition of cell proliferation and viability (Figure 1). Due to its intrinsic color which can quench or conceal measurements, serial dilutions of OTE were applied to cells: the highest concentration 100 indicates that the pure OTE was used at a final concentration of 1µl OTE into 100µl of medium, whereas the lowest concentration 1 indicates that the pure OTE was diluted 1 to 100 and used at a final concentration of 1µl diluted OTE into 100µl of medium.

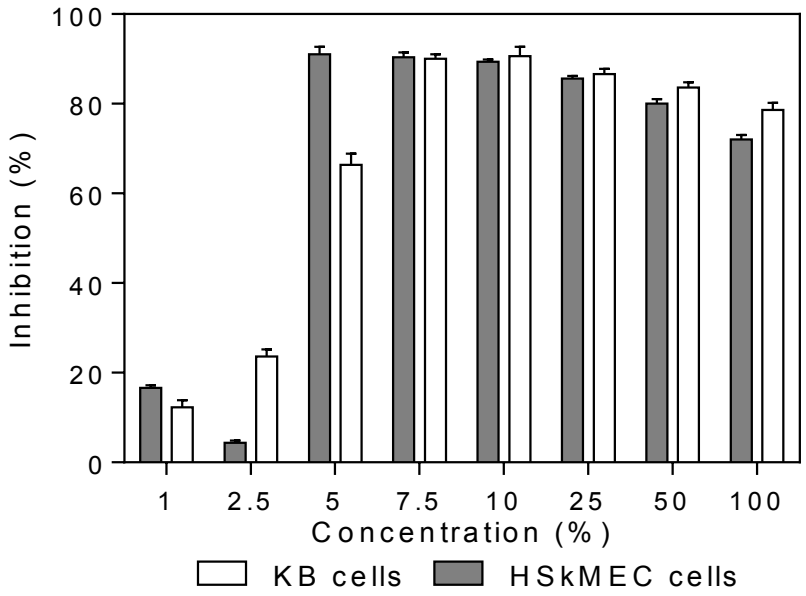


**Figure 1**. Inhibition of cell viability (%), illustrating the cytotoxicity of olive tree extract in KB cell line.

After 72 hours of treatment, the olive tree extract showed a strong cytotoxic effect on both KB and HSkMEC cell lines. Thus, the olive tree extract was toxic up to 5% dilution with a percentage cell viability inhibition over than 80%. The $EC_{50}$, concentration of the olive tree extract corresponding to 50% of cell viability inhibition, was subsequently evaluated. KB cells were more sensitive to the olive tree extract ($EC_{50}$ = 39%) than endothelial cells ($EC_{50}$ = 43%), although the observed difference was not significant. Such toxic effect was also observed against other human cancer cells (HL60, MCF-7, HCT116) and non-cancerous cell lines (MRC5 and EPC data not shown). This cytotoxicity activity could be attributed to the phenolic fraction which is present in olive tree extracts (fruits and leaves). Actually, the profile of the olive tree extract revealed a complex mixture of phenolic compounds. Among of them, the components identified as oleuropein, hydroxytyrosol (3′,4′-DHPEA) and tyrosol (*p*-DHPEA) were the most represented and flavonoids such as rutin, quercetin, luteolin and apigenin were present in lower concentrations (Laaboudi et al., 2015). For this reason, oleuropein, hydroxytyrosol, tyrosol, apigenin, luteolin, and caffeic acid were used as pure individual molecules for comparison in the current study (Table 2).

**Table 2**. Inhibition of cell proliferation induced by pure molecules contained in OTE on KB cells. Results are the mean ± SD of experiments performed in triplicate and expressed as a percentage of inhibition compared to cells treated with DMSO only.

| Compound | Concentration | | |
|---|---|---|---|
| | 100 µM | 10 µM | 1 µM |
| Caffeic acid | 0 ± 10 | 3 ± 5 | 6 ± 6 |
| Apigenin | 83 ± 1 | 30 ± 5 | 5 ± 1 |
| Luteolin | 81 ± 1 | 22 ± 6 | 4 ± 2 |
| Oleuropein | 0 ± 9 | 0 ± 1 | 0 ± 10 |
| Tyrosol | 0 ± 6 | 5 ± 3 | 0 ± 8 |
| Hydroxytyrosol | 11 ± 11 | 1 ± 10 | 0 ± 6 |

Results of table 2 show, surprisingly, that only flavonoids (apigenin and luteolin) were potent inhibitors of KB cell proliferation, while other molecules (tyrosol, hydroxytyrosol, oleuropein and caffeic acid) had no or negligible effect. However flavonoids have no more antiproliferative activity at 1µM. Similar results were obtained with other studied cell lines, including HSkMEK (data not shown). This does not exclude the effect on cell proliferation of non-identified compounds (at this stage) present in OTE, including secoiridoids derivatives of hydroxytyrosol and tyrosol especially oleacein (3',4'-DHPEA-EDA) and oleocanthal (*p*-DHPEA-EDA).

**FACS analysis of cell death**

Apoptosis and necrosis are two distinct and redundant forms of cell death occurring in response to chemical aggression. These two cellular processes featured different and complementary modes of action: apoptosis (or programmed cell death) is a tightly regulated cell death program that plays a pivotal role in a variety of biological processes, generally executed in animal cells via the activation of cellular proteases leading to the cleavage of chromatin into nucleosomal fragments [43], while necrosis involves the destruction of the plasma membrane leading to the release of cytosolic enzymes and cofactors into the external medium. Flow cytometry was used at first to investigate the cell death process: it enabled to discriminate between intact "healthy" cells, apoptotic cells and necrotic cells in an advanced state of degradation.

In line with cytotoxicity experiments, KB cells were treated by diluted OTE (50-1%) for 24 and 48h and analyzed by FACS. According to the fluorescence monitored in FACS, cells can be classified as intact, in early apoptosis, in late apoptosis or necrotic (dead cells). The percentage of cells into each group is shown in Table 3. As it can be inferred from results of table 3, significant changes were observed in cell population distribution after 24 h of exposure to OTE. Intact cell population progressively decreased when the OTE concentration increased, associated with a transient raise in early apoptotic cells at low OTE concentration and a massive outburst of necrotic cells at higher OTE concentrations. After 48h of exposure, the necrotic cell population predominated even with low OTE concentrations to the detriment of intact and early apoptotic cell populations. Therefore it can be concluded that the effects of olive tree extract are dose- and time-dependent.

**Table 3**. Quantification by FACS of cell death induced in KB cell lines by olive tree extract.

| **Cell population (%)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Concentration (%)** | **Intact** | | **Early apoptotic** | | **Late apoptotic** | | **Necrosis** | |
| | 24h | 48h | 24h | 48h | 24h | 48h | 24h | 48h |
| DMSO | 90 | 90 | 3.1 | 2.1 | 0.5 | 1.2 | 6.5 | 7.2 |
| Doxo 100 mM | 53 | 32 | 36 | 45 | 0.2 | 1.0 | 11 | 21 |
| OTE 50 | 3.4 | 12 | 4.1 | 1.8 | 0.1 | 0.3 | 92 | 86 |
| OTE 25 | 3.6 | 3.9 | 6.6 | 2.2 | 0.8 | 0.3 | 89 | 94 |
| OTE 10 | 20 | 2.3 | 34 | 8.0 | 0.4 | 0.4 | 46 | 89 |
| OTE 7.5 | 37 | 3.4 | 57 | 12 | 0.2 | 0.4 | 6.0 | 84 |
| OTE 5 | 54 | 19 | 42 | 33 | 0.2 | 0.4 | 3.4 | 47 |
| OTE 2.5 | 86 | 84 | 7.6 | 8.1 | 0.7 | 2.0 | 5.4 | 57 |
| OTE 1 | 83 | 82 | 11 | 9.5 | 0.3 | 0.9 | 4.7 | 7.2 |

Caspases (cysteine-aspartic acid proteases) are regarded as the most common apoptotic effector activated during apoptosis in many cell types. It was reported that caspase-3 is the terminal effector in the apoptotic cascade and is the major contributor to cellular DNA fragmentation.[33] To go further in the elucidation of the cell death pathway induced by OTE, the catalytic activity of caspase-3 was explored in KB cells treated for 24 and 48 h with OTE at the same concentrations as in FACS analysis. Caspase-3 activity can be determined by the cleavage of the pro fluorescent DEVD peptide which is catalyzed by both caspase-3 and 7, albeit more efficiently by the former. As expected, 1µM doxorubicin elicited a potent activation of caspase-3 activity after a 48 h treatment (figure 3A). Although FACS analysis showed the apoptotic effect of olive tree extract at low concentrations (7.5 and 5%), results of figure 3A show that caspase-3 activity was moderately increased after a 48h exposure of KB cells to OTE. This suggest that apoptotic activity of olive tree extract was marginal compared to its necrotic effect and led us to evaluate the release of cytosolic release of lactic dehydrogenase used as a hallmark for necrosis.

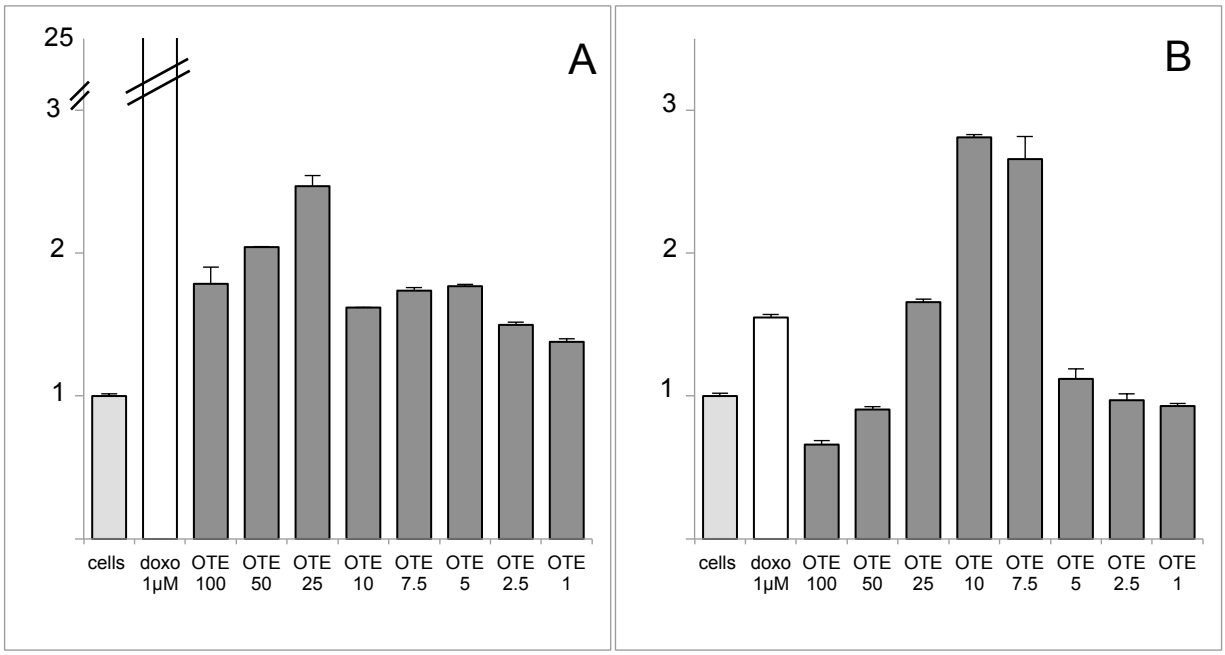


**Figure 2**. Activation of caspase-3 (A) and release of LDH (B) from KB cells after 48h of treatment with olive tree extract, vehicle only (cells) and doxorubicin (1µM).

Results are expressed as the relative activity in duplicate experiments ± SD, compared to activity in untreated KB cells.

As shown in figure 2B, the release of LDH was enhanced after 48h of exposure to increasing OTE concentrations, before to decline at high concentrations, indicative of a complete cell destruction. This confirms that the olive tree extract induces a transient and moderate activation of apoptosis preceding a massive necrotic cell death.

**ROS production**

The production of reactive oxygen species can be estimated with the DCFH-DA assay using the fluorogenic probe 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA). Briefly, the non-fluorescent DCFH-DA diffuses spontaneously into cells and is deacetylated by cellular esterase to 2',7'-Dichlorodihydrofluorescin (DCFH), which is rapidly oxidized by ROS to the highly fluorescent 2',7'-dichlorodihydrofluoresceine (DCF). The emitted fluorescence intensity ($I_{rel}$) is proportional to the levels of ROS produced into cell cytosol.

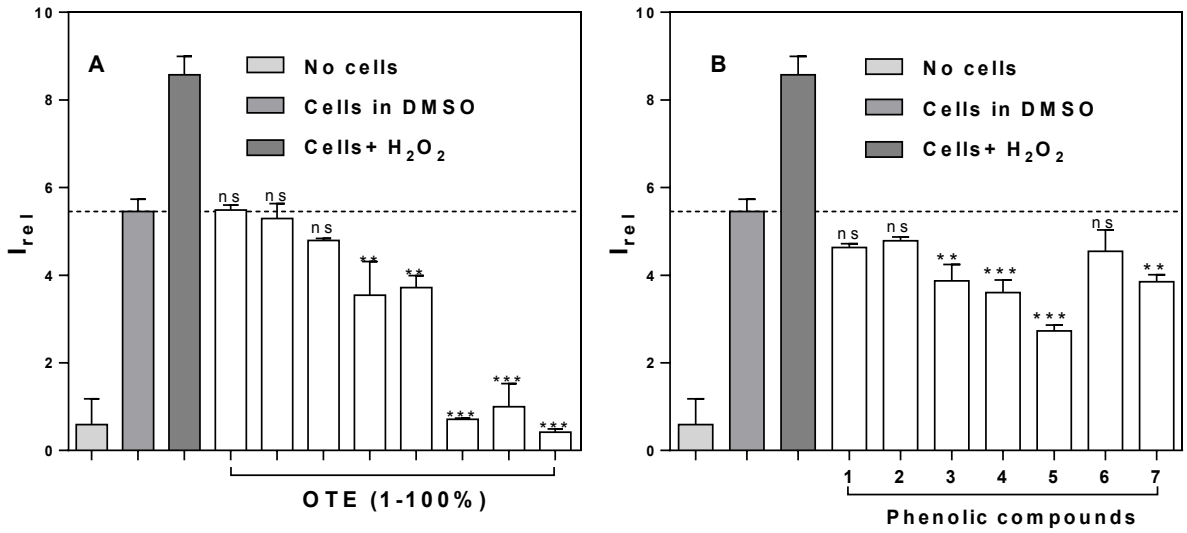


**Figure 3**. Intracellular ROS production after incubation of HT29 cells for 24h with (A) increasing concentrations of olive tree extract and (B) pure phenol compounds (100 µM). Results are the mean ± SD of 3 individual assays. *** ($p$ <0.001), ** ($p$ <0.01) * ($p$ <0.05); ns, not significant from fluorescence produced in cells treated with vehicle only. (1), oleuropein; (2), tyrosol; (3), hydroxytyrosol; (4), apigenin; (5), luteolin; (6), protocatechuic acid; (7) cafeic acid.

In the presence of 1mM $H_2O_2$, the emitted fluorescence intensity was significantly increased (Figure 3). Conversely, a progressive decrease in the quantity of produced ROS was observed in cells treated with increasing concentrations of OTE to reach an almost complete protection against ROS at highest concentrations. On the other hand, no significant effect on ROS production were noticed with 100µM tyrosol, oleuropein and protocatechuic acid, while hydroxytyrosol, apigenin and luteolin significantly reduced the amount of ROS produced during cell incubation. Thus, the anti-radical effect of olive tree extract could be the result of the antioxidant activity (synergistic action) of these phenolic compounds, although the determination of the role of other compounds (not identified at this stage) is required to confirm these observations.

**In vitro anti-angiogenic effect on tube formation.**

Endothelial cells grown on an extracellular matrix spontaneously form a capillary network in the presence of growth factors present in the fetal calf serum. This *in vitro* test allow to determine the capacity of a molecule or substance to inhibit the formation of capillary structures. It is considered to be close to *in vivo* tests and to require the proliferation, migration and degradation of the basement membrane of endothelial cells. The photographs taken after a 16h of endothelial cells incubation on Matrigel at 37 °C and 5% $CO_2$ in the presence of increasing concentrations of olive tree extract and phenolic compounds (100 µM) are presented in figure 4.

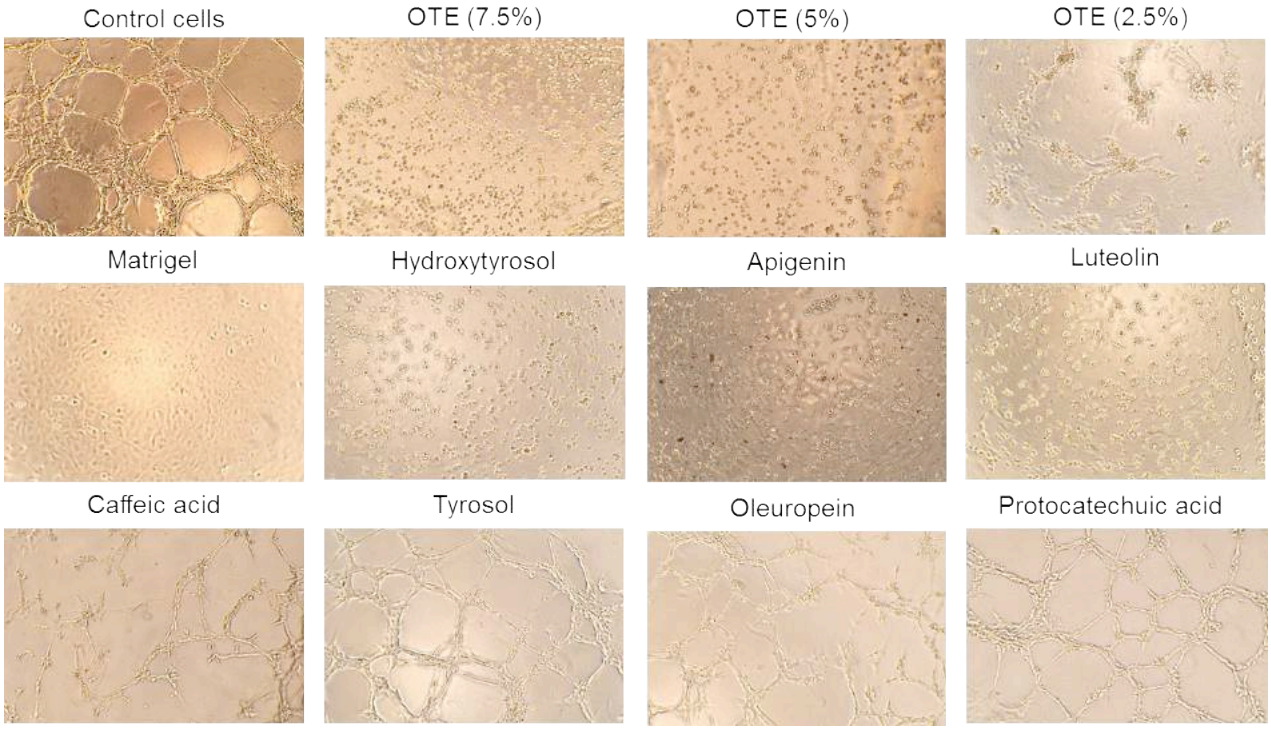


**Figure 4**. Representative photographs of the network structure formed by endothelial cells (HSkMEC) on Matrigel in the presence of increasing concentrations of olive tree extract and pure phenolic compounds used at 100 µM. Control cells are incubated without Matrigel.

Photographs of figure 4 show that endothelial cells grew in complete medium containing 2% FCS as a continuous monolayer (figure 4 control cells) whereas they form capillary structures when laid down on Matrigel. This capacity to form capillary structure was severely impaired in the presence of olive tree extract, suggesting its dose-dependent anti-angiogenic effect. The OTE effect has been observed at concentration 2.5% to be complete at concentration 7.5%. Indeed, referring to the antiproliferative capacity of OTE, the anti-angiogenic activity could be due to a direct cytotoxic effect of OTE on HSkMEC cells. However, the results of the cytotoxicity indicate that the olive tree extract diluted at concentration 2.5 does not affect the proliferation and viability of HSkMEC cells, whereas the anti-angiogenic effect was evident at this concentration. Therefore the anti-angiogenic effect of pure molecules identified as major phenolic compounds present in olive tree extract was investigated in the same conditions (figure 4). Among these pure compounds hydroxytyrosol, luteolin, apigenin and to a lesser extent caffeic acid at a 100µM concentration elicited a marked inhibitory effect on capillary network formation of HSkMEC cells. On the

other hand, tyrosol, oleuropein and protocatechuic acid moderately affect the organization of endothelial cells. Thus, we may conclude that the synergistic action of these molecules on endothelial cells might account for the OTE anti-angiogenic effect.

Indeed, the ability of endothelial cells to organize themselves into three-dimensional space to form new capillaries proceeded through cell migration, proliferation and vessel stabilization governed by the secretion of chimiotactiles factors (called pro-angiogenic factors). Several proteins are involved to support angiogenesis phenomena, such as VEGF factors (A, B and C isoforms), angiopoietin (1 and 2), HIF and the VEGF receptor-2. The expression rate of genes coding for these pro-angiogenic factors was, therefore, measured using quantitative real time RT-PCR (Figure 5) and summarized in table 4.

Analytical data plotted on figure 5 were consistent with observation of micro-capillary formation on Matrigel. Basically, the expression of pro-angiogenic genes was significantly affected in the presence of hydroxytyrosol which was the molecule having the highest inhibitory effect on microtube formation, and to a lesser extent by caffeic acid which is a weaker microtube disruptor. The two modest inhibitors of microtube formation tyrosol and oleuropein did not affect the expression of VEGF A and B. It is noteworthy that none of these compounds modify the expression of the VEGF receptor-2 which binds VEGF-A. VHL is a tumor suppressor which binds to HIF-1α to allow its degradation by the proteasome. Its expression is regulated by HIF-1α through an HRE element present in its promoter: thus HIF could promote its own degradation by the induction of VHL gene expression (Luczak et al 2011, Karhausen et al 2005). This is consistent with the co-regulation observed in HSkMEC cells exposed to phenolic compounds and presented in figure 5 and table 4.

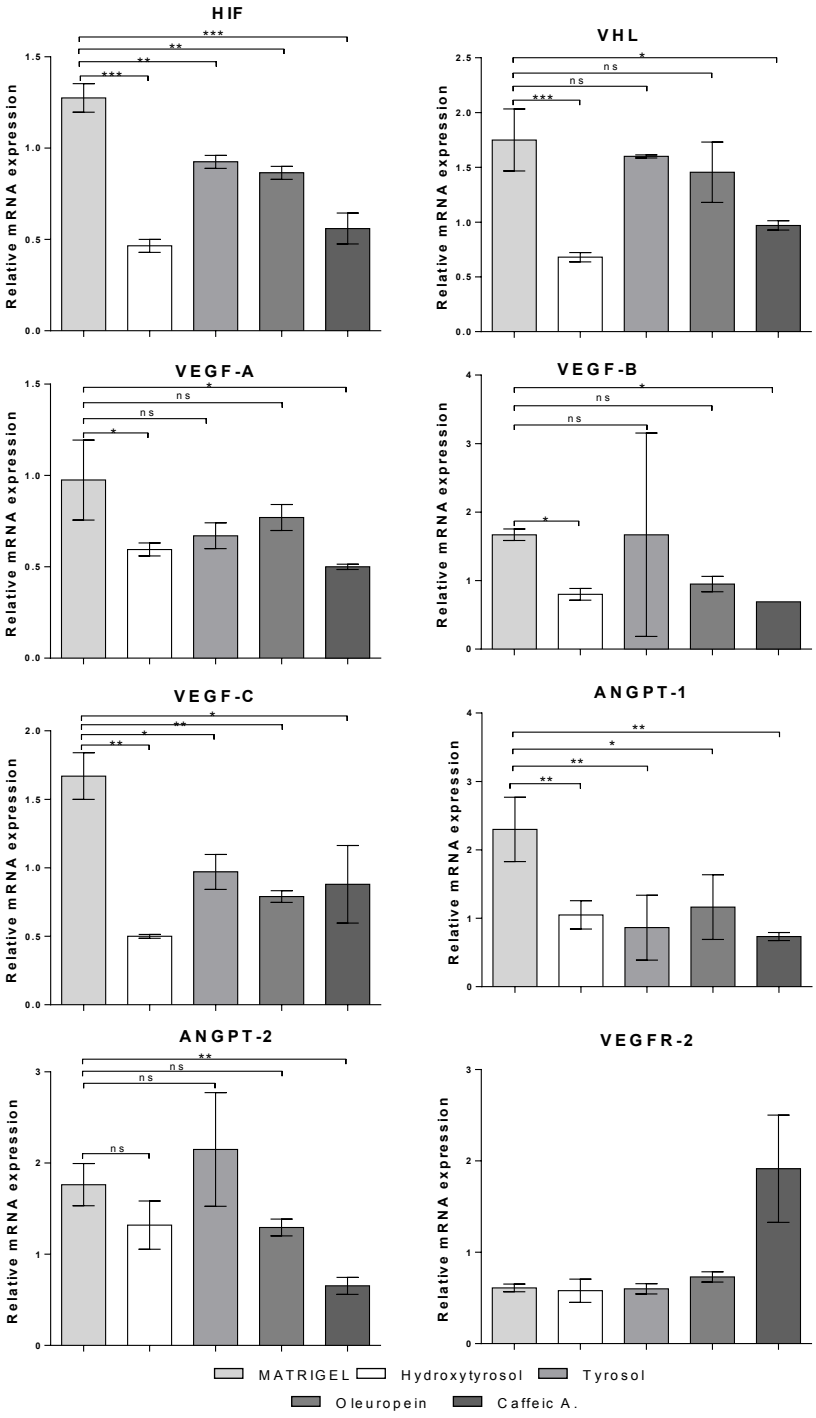


**Figure 5**. Effect of phenolic compounds in olive tree extract on the expression of pro-angiogenic agents. HIF Hypoxia Inducible Factor-1α; VHL protein of Von Hippel-Lindau; VEGF (A, B and C), Vascular Endothelial Growth Factor; ANGPT (1 and 2), Angiopoietin; VEGFR-2, cell receptor for VEGF proteins. Values are expressed as mean ± SD (n = 3). *** (P <0.001), ** (P <0.01) * (P <0.05); ns, not significant difference.

**Table 4**. Summay of anti-angiogenic effect of the major polyphenols present in olive tree extract. (+), Inhibitory effect of angiogenesis; (-), No effect.

| Molecule (100 µM) | HIF | VHL | VEGF-A | VEGF-B | VEGF-C | ANGPT-1 | ANGPT-2 | VEGFR-2 |
|---|---|---|---|---|---|---|---|---|
| Hydroxytyrosol | +++ | +++ | + | + | ++ | ++ | - | - |
| Tyrosol | ++ | - | - | - | + | ++ | - | - |
| Oleuropein | ++ | - | - | - | ++ | + | - | - |
| Caffeic acid | +++ | + | + | + | + | ++ | ++ | - |

All together the lower expression of VEGFs, angiopoietin and HIF-1α can explain the anti-angiogenic of active phenolic compounds present in the olive tree extract, individually and/or synergistically.

**Discussion**

The olive tree extract tested in this study, displayed potent cytotoxic activity against human cancer cells through the activation of necrotic and/or apoptotic pathways. This is consistent with several previous studies have reported that crude extracts from olive leaves and phenolic compounds demonstrate high cytotoxic activity against various cancer cell lines derived from blood, colon and breast cancers (Fu et al, 2010;. Hashim et al., 2008; Abaza et al, 2007). This cytotoxic activity was, particularly, remarkable in HER-2 positive breast cancer cells. Olive tree extract reduces the overexpression of HER-2 and decreases resistance to trastuzumab, an anti-HER-2 monoclonal antibody used clinically for breast cancer treatment (Menendez et al., 2007). Independently, Taamalli et al. (2012) have shown that the inhibition of breast cancer cells viability was depended on the polyphenol content in an olive leaves extract.

As expected our olive tree extract is rich in hydroxytyrosol and its secoiridoid derivatives (oleacein and oleocanthal), and in flavones such as luteolin and apigenin. Therefore, the individual effect of these compounds to explain the cytotoxicity toward cancer cells of this crude extract deserves more attention in future studies. Interestingly the whole OTE effect might not result from the abundance of a single compound, but rather from the synergistic interaction between different compounds

including those that are not already identified. In this respect, authors have recently pointed out that food polyphenols who exert their pharmacological effects through synergistic interactions involve the implication of multiple targets (Efferth and Koch, 2011; Wagner, 2011).

Thus, the effects of molecules abundant in OTE like hydroxytyrosol (50 $\mu g.ml^{-1}$) and oleuropein (200 $\mu g.ml^{-1}$) were demonstrated in MCF-7 cells (Han et al., 2009), and confirmed *in vitro* on a variety of human cancer cell lines together with apigenin (Kim et al, 2011; Bulotta et al, 2011; Fabiani et al 2006, 2002), and *in vivo* in tumor-bearing mice (Hamdi and Castellon, 2005). Moreover, many epidemiological studies suggest a potential correlation between the consumption of olive products (olive fruits and olive oil) and a low incidence of breast cancer in Mediterranean area, suggesting a protective effect of olive compounds against cell proliferation e.g. cancer (Corona et al., 2007). Thus, we can conclude that the OTE's cytotoxic effect is mainly due to these phenolic compounds.

ROS play a crucial role in tumorigenesis as suggested by Sabharwal and Schumacker (2014) who reported that signaling pathway(s) triggered by ROS contributes to proliferation and tumor survival of many cancers. These authors propose that improving mitochondria potential redox is a promising target for future cancer therapies. In accordance with Nardi et al. (2014), we reported herein that olive tree extract and some of its phenolic components showed elevated antioxidant activity in HT-29 cells, giving them a central role in the fight against carcinogenic risks. Likewise, Deiana et al. (1999) reported that hydroxytyrosol reduced biochemical effects of peroxynitrite, such as the deamination of adenine and guanine in certain cell lines. ROS generated during oxidative stress may be directly involved in DNA damage and/or in the intrinsic apoptotic pathway induction (mitochondria-dependent). Thus, the intracellular accumulation of ROS may be involved in the partial disruption of the mitochondrial membrane causing the release of mitochondrial material (cytochrome-c, pro-apoptotic factors) in the cytosol, finally leading to the activation of the caspase cascade (Eloy et al., 2012). Moreover, cellular ROS production by different metabolic pathways has been associated with cell aging phenomenon call the "radical" hypothesis of aging (Barouki, 2006). Thus, we can postulate the antioxidant activity of OTE and these phenolic compounds has a double effect, i) the elimination of carcinogenic ROS and ii) the prevention of cell aging.

Angiogenesis is a critical process implicated in the proliferation and dissemination of cancer cells by metastasis. The fast and limitless multiplication of cancer cell requires an important and incremental supply in oxygen and nutrients through the blood circulation. The spreading of circulating cancer cells is highly facilitated by the density of blood vessels and the cell aggressiveness enabling the metastasis formation.

Angiogenesis is under the control of multiple regulation pathways, among them are the vascular endothelial growth factors which stimulate vasculogenesis and angiogenesis (Jiang et al., 2000) and facilitate the formation of new capillary vessels in association with angiopoietins reviewed by Rak et al., 2000. The transcription of VEGF is stimulated by Hypoxia Inducing Factor 1-α present in the cytosol as a heterodimer with VHL allowing its degradation by the proteasome. The key role of VEGFs was also highlighted by a clinical report which positively correlated polymorphism in the VEGF-A gene (-1154 A/G, -2578 C/A and -460 T/C) and the lower susceptibility to develop breast cancer in Moroccan individuals (Rahoui et al., 2014).

Data reported herein clearly demonstrate the inhibitory effect of olive tree extract and of some of its phenolic components (hydroxytyrosol, apigenin, luteolin and caffeic acid) on the formation of new capillaries from human endothelial cells. This inhibition was consistent with previous observations made on various human cells, animal tumors and *in silico* (Ambasta et al., 2015; Simon Silvan et al., 2013; Fortes et al., 2012; Gacche et al., 2011; Engelmann et al. 2002). Two mechanisms could explain the anti-angiogenic effect of olive tree extract and phenolic compounds: a severe restriction of cell proliferation reducing the number of endothelial cells required for building the capillary structure and a lack of pro-angiogenic factors production thus restricting cell migration and assembly. Clearly, hydroxytyrosol oleuropein and caffeic acid had no effect on cell proliferation, but are potent down-regulator of HIF, VEGF and angiopoietin suggesting their direct anti-angiogenic effect. Additionally, Kim et al (2011) had shown that apigenin has an anti-angiogenesis effect through a down-regulation of VEGF and MMP-8 release in hepatocellular carcinoma cells. Therefore, OTE’s effect is the result of the cumulative effect of individual phenolic compounds reducing the cell supply and thus restricting the tumor growth.

On the other hand, an excessive angiogenesis ensues from progression of inflammatory process in synovium, and leads to pannus proliferation and rheumatoid

arthritis symptoms complication. Angiogenesis is a major contributor in the development and maintain of inflammation in rheumatoid arthritis (Semerano et al., 2011), and a correlation between rheumatoid arthritis progression and VEGF level has been observed in patient with RA (Sone et al., 2001). Thus, the inhibition of angiogenesis could be a potential therapeutic target for the treatment of rheumatoid arthritis (Ghanam et al., 2015). However, the VEGF-dependent antiangiogenic effect of olive tree polyphenols, in both cancer and rheumatoid arthritis diseases, need to be confirmed in future *in vivo* studies.

## Conclusion

The cellular assays have demonstrated the effectiveness of phenolic olive tree extract as an antioxidant in cells exposed to oxidative stress. This extract has also demonstrated a strong VEGF-dependent antiangiogenic activity. Indeed, ROS elimination, apoptosis and/or necrosis activation and angiogenesis inhibition make this extract an excellent agent to prevent cancer diseases. The characterization of bioactive molecules in this extract, as well as the contribution of each to the global biological activity, could provide the chemical basis for the development of new anticancer drugs. Further studies are needed to extent the antiangiogenic activity of phenolic compounds derived from studied olive tree extract (hydroxytyrosol, oleacein and oleocanthal) in case of angiogenesis-dependent diseases.

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THE BENEFITS OF THE OLIVE TREE

# CLINICAL STUDY N°3

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Original Article

# HYPOGLYCEMIC AND HYPOLIPIDEMIC EFFECTS OF PHENOLIC OLIVE TREE EXTRACT IN STREPTOZOTOCIN DIABETIC RATS

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**ABSTRACT**

**Objective:** The aim of the present study was to determine the effects of an olive tree extract with high polyphenols content on blood glucose level and other related parameters in streptozotocin-induced diabetic rats.

**Methods:** Diabetes was induced in rats by intraperitoneal injection of streptozotocin (55 mg/kg bw). 72h after injection, rats with fasting blood glucose higher than 2 g/l were used for the experiments. Olive tree extract was administered for 28 d and blood glucose level was measured every 4 d. Total cholesterol, triglycerides, HDL-cholesterol, creatinine, urea, total protein, uric acid, aspartate aminotransferase and alanine aminotransferase levels, were determined at the end of the experiment.

**Results:** The oral administration of olive tree extract contributes to blood glucose level decreasing in diabetic rats group, which was significantly lower at 4th week compared to the diabetic control rats. Moreover, supplementation by olive tree extract decreased significantly ($p<0.05$) the values of total cholesterol, triglycerides, HDL-cholesterol, creatinine, urea, total protein, uric acid, aspartate aminotransferase and alanine aminotransferase resulting from damage caused by streptozotocin treatment. Beside this, significant reduce ($p<0.05$) in heart disease risk ratio was observed for treated group (4.1±0.14) compared to untreated group (7.64±0.36), which was quite similar to normal rats (4.50±0.36). Studied olive tree extract effects were similar to those of glibenclamide, a well-known antidiabetic drug.

**Conclusion:** Results herein obtained reveal the hypoglycemic effect of this olive tree extract, suggesting his potential use as a natural antidiabetic agent.

**Keywords:** Olive leaves, Olive fruit, Polyphenols, Antidiabetic, Streptozotocin, *In vivo*


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## INTRODUCTION

There has been a tragic increase in diabetes mellitus across the world. Diabetes mellitus is caused by complete or partial deficiencies in insulin production and/or insulin action coupled with chronic hyperglycemia and metabolism disruption [1]. It is considered as one of the most important clinical risk factors involved in some disorders like nephropathy, retinopathy, neuropathy, and cardiovascular diseases, which its prevalence is predicted to be increased daily [2-6]. Therefore, it is great urgency to find better treatments and novel prevention strategies regarding this worldwide health problem. The most common treatment is insulin and drugs with hypoglycemic effects [7]. However, there is an increasing demand by patients to use natural products, due to the side effects associated with insulin and oral hypoglycemic agents [8, 9]. The study of such products may offer a natural alternative to diabetes management in the future.

Among the plants used for their antidiabetic effect, an olive tree (*Olea europeae*) is of paramount importance. Currently, the implication of the olive tree byproduct extracts in pharmacology and food industries is due to the presence of some important phenolic components. The olive tree has been recognized for a long time as a source of bioactive polyphenols, such as oleuropein, hydroxytyrosol, oleuropein aglycone, and tyrosol [10-12]. Furthermore, olive wastes are considered as a cheap raw material for extracting of high-added value products [13]. Several studies have shown that olive tree possessed a wide range of pharmacological and health-promoting properties including the reduction of coronary heart disease risk [14, 15], anti-inflammatory [16-20], antitumor, anti-proliferative [21, 22], antidiabetic [23-26], antibacterial and antifungal properties [27-30]. Many of these properties have been described as resulting from the antioxidant character of polyphenols [31]. Gonzalez *et al.* [1] have previously reported that olive polyphenols had an antihyperglycemic effect on diabetic rats, although the mechanism by which they attenuate hyperglycemia is still not well known. However, particular attention has been paid to hydroxytyrosol [32], which occurs naturally in olive byproducts. This *o*-diphenol, like the majority of the olive polyphenols such as tyrosol, has been proven to have significant antitumor, anti-proliferative [21, 22] and antiviral activities [33].

Since the waste of olive has been recommended in the literature [23-26], as a remedy for the treatment of diabetes, it was considered worthwhile to investigate the influence of an olive tree extract, administered orally during 4 w, in normal and streptozotocin-induced diabetic rats. For this purpose, serum glucose level, lipids, renal and hepatic profiles were measured during the current study.

## MATERIALS AND METHODS

### Plant material and chemicals

Streptozotocin; glucose; glibenclamide (glyburide) were purchased from Sigma-Aldrich (Paris, France). Glucometer (BIONIME blood glucose monitoring system right GM300) and strips (BIONIME blood glucose test strip rightet GS300) were purchased from Bionime distributor (Casablanca, Morocco).

The olive tree extract (OTE) was obtained from Moroccan olive fruits and leaves using an eco-extraction, free of chemical solvents or toxic additives, according to the previously described protocol [19]. OTE is marketed in the world as under the brand name OLIVIE FORCE/OLIVIE RICHE (see more in www.olivie.ma).

### Animals

Male adult 'Wistar' rats (200-250 g) obtained from the animal breeding unit (located in the Faculty of Science Dhar El Mahraz-Fez–Morocco) were used in this study. The animals were housed in clean

plastic cages and maintained under environmentally controlled breeding room (temperature, 22±2 °C; humidity, 40±5%; 12 h dark/light cycle) and had free access to food and water. Housing conditions and *in vivo* experiments were approved according to the guidelines established by the European Union on Animal care (CEE Council 86/609). The animals were used after an acclimatization period of two weeks to the laboratory environment and fasted overnight before experiments.

**Experimental design**

Diabetes was induced in rats by single intraperitoneal injection of a freshly prepared solution of streptozotocin (55 mg/kg body weight) in citrate buffer 0.1M (pH 4.5). For three days, rats received 5% of glucose in the drinking water. 72h after injection, rats with fasting blood glucose higher than 2 g/l were used for the experiments and received daily the adequate treatment for a period of 4 w.

Rats were divided into four different groups (10 rats in each group n= 10). "Control rats": negative control rats were received orally 10 ml/kg of 0.9% NaCl solution; "Diabetic rats": positive control rats were administered orally 10 ml/kg of 0.9% NaCl solution; "Diabetic rats+glibenclamide": rats were treated by 0.3 mg/kg of glibenclamide and "Diabetic rats+OTE": rats received daily oral dose of 1 g/kg of OTE.

**Biochemical analysis**

During this study, blood glucose level was measured every 4 d, at the same time in the morning. Glycemia was measured in blood from the tail vein using the commercial glucometer.

After 28 d of treatments, bold samples were collected into heparinized tubes and centrifuged at 3000 tr/min for 10 min. Plasma samples were separated and transferred in Eppendorf tubes for analysis. Total cholesterol (Total CT), triglycerides (TG), high-density lipoproteins cholesterol (HDL-CT), creatinine, urea, total protein, uric acid, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, were determined using commercial kits (Sigma-Aldrich; Paris, France) according to the manufacturer's guidelines. Low lipoproteins cholesterol (LDL-CT) level was calculated by the following Friedewald equation [34]:

$$\mathrm{LDL \cdot CT} = Total \cdot CT - HDL \cdot CT - \frac{TG}{5}$$

**Statistical analysis**

Statistical analyses 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). Values between groups were considered statistically significant for at $P<0.05$.

**RESULTS**

**Blood glucose level**

Results plotted in the graph of fig. 1 illustrates an OTE's effect on blood glucose through the period of treatment. The blood glucose levels of normal and diabetic (untreated) rats were between 0.9±0.11 and 4.5±0.62 g/l, and 1.1±0.10 and 5.23±0.66 g/l throughout the period of treatment. However, a significant decrease in blood glucose levels was observed in supplemented diabetic rats from the first intake of OTE (after 4 d). Diabetic rats in the treated (by OTE) group reach the normal blood glucose after 20 d of OTE administration (1 g/kg), while this level decreases to the same value of normal rats (~1 g/l) at the end of the study. Thus, the OTE was found to have a similar hypoglycemic effect to glibenclamide effect at the end of the study.

**Lipid profile**

Total cholesterol, triglycerides and HDL-cholesterol levels were measured at the end of the experiment; obtained results are showed in the graph of fig. 2.

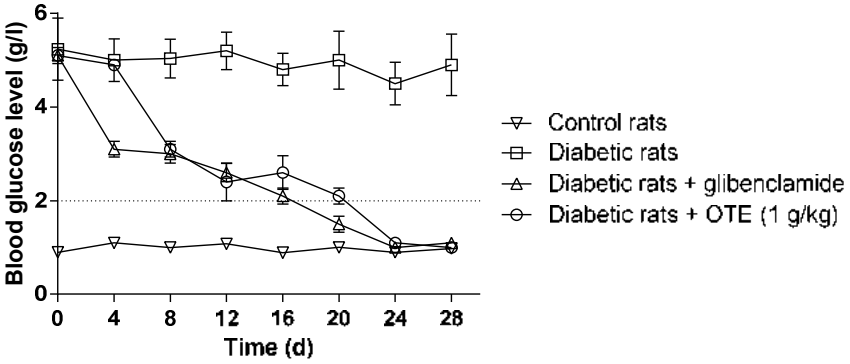


**Fig. 1: Effect of OTE on blood glucose levels in diabetic and non-diabetic rats after 4 w of daily admission (n= 10)**

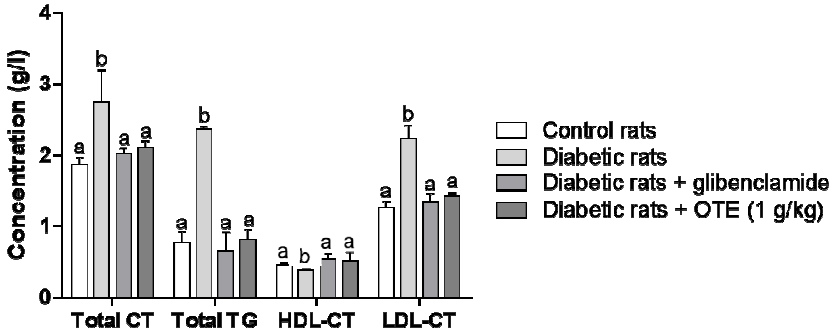


**Fig. 2: Effect of OTE on total cholesterol (CT), triglycerides (TG), high-density lipoproteins cholesterol (HDL-CT) and low lipoproteins cholesterol (LD-CT) in diabetic and normal rats after 4 w of daily admission (n= 10). Different letters [a–b] indicate significant differences ($p<0.05$)**

The analytical values of TC (2.75±0.44 g/l), TG (2.36±0.03 g/l) and LDL-CT (2.23±0.18 g/l) recorded in untreated diabetic rats showed a significant ($p<0.05$) increase at the end of the study compared to those of rats in control or treated groups (fig. 2). However, the values of TC, TG and LDL-CT drop significantly ($p<0.05$) to 2.1±0.01, 0.81±0.14 and 1.43±0.04 g/l, respectively, in the blood of rats supplemented by 1 g/kg of studied olive tree extract. As a net result, OTE administration was able to restore the lipid profile and correct the hypercholesterolemia associated with hyperglycemia. Nevertheless, OTE administration had no significant effect on HDL-CT of treated diabetic rats (0.51±0.12 g/l) compared to control rats (0.45±0.04 g/l) or diabetic rats treated by glibenclamide (0.54±0.07 g/l).

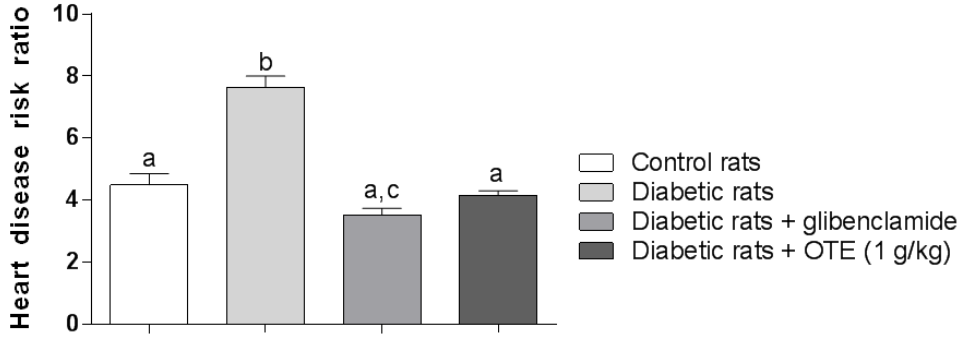


**Fig. 3: Heart disease risk ratio in diabetic and normal rats after 4 w of OTE daily admission (n=10). Different letters [a–b] indicate significant differences ($p<0.05$)**

On the other hand, the overall improvements in the blood lipid profile of rats treated by OTE had positively influenced heart disease risk ratio parameter (Total CT/HDL-CT) [35]. In this sense, results of fig. 3 show a significant difference ($p<0.05$) in favor of rats treated by OTE (4.1±0.14) and glibenclamide (3.53±0.21) compared to untreated diabetic rats (7.64±0.36). We should also underline that the heart disease risk parameter of rats supplemented for 28 d by the studied olive tree extract was quite similar to that of control animals (4.50±0.36).

**Renal function tests**

Table 1 shows the effect of OTE on creatinine, urea, total protein and uric acid in rats allocated to the study groups. The results showed that creatinine, urea, and uric acid increased in streptozotocin-induced diabetic rats compared with control rats ($p<0.05$).

However, OTE and glibenclamide administration have significantly ($p<0.05$) stabilized these parameters at the normal values compared to rats in control group. These results suggest, in fact, that supplementation by OTE has an appropriate regulating effect directed to each of the biochemical parameters associated with hyperglycemia. Moreover, serum levels of AST and ALT (and AST/ALT ratio) are commonly measured clinically as biomarkers for liver health. Results showed in table 2 (combined with those of table 1) indicate that OTE administration had no adverse effect on renal function of treated rats.

**Table 1: Effect of OTE on Creatinine, urea, total protein and uric acid in diabetic rats after 4 w of daily administration**

| | Control rats | Diabetic rats | Diabetic rats+glibenclamide | Diabetic rats+OTE (1 g/kg) |
|---|---|---|---|---|
| Creatinine (mg/l) | 6±1 [a] | 11±1.73 [b] | 6±1[a] | 6±1[a] |
| Urea (g/l) | 0.28±0.03[a] | 0.45±0.06[b] | 0.22±0.05[c] | 0.29±0.03[a] |
| Total proteins (g/l) | 74±4.36[a] | 59±3.46[b] | 77±3.60[a] | 76±2[a] |
| Uric acid (mg/l) | 40±2[a] | 72±1[b] | 44±3.60[a] | 45±4[a] |

[a–c]Values in the same row for each rats group with different superscripts are significantly different ($p<0.05$) [mean±SD, n= 10].

**Table 2: Effect of OTE on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in diabetic rats after 4 w of daily administration**

| | Control rats | Diabetic rats | Diabetic rats+glibenclamide | Diabetic rats+OTE (1 g. kg$^{-1}$) |
|---|---|---|---|---|
| AST (UI/l) | 22±2.64[a] | 86±1.73[b] | 31±4[c] | 36±2.64[c] |
| ALT (UI/l) | 28±2.28[a] | 90±5.56[b] | 35±4.58[a] | 40±3.60[a] |

[a–c]Values in the same row for each rats group with different superscripts are significantly different ($p<0.05$) [mean±SD, n= 10].

## DISCUSSION

Streptozotocin is the most prominent diabetogenic chemical agent in diabetes research. In 1963, Rakieten *et al.* reported that streptozotocin is diabetogenic [36]. Since then, it has been chosen for diabetes mellitus induction in animal models [37]. Actually, streptozotocin inhibits insulin secretion and causes a state of insulin-dependent diabetes mellitus, due to its specific chemical properties, namely its alkylating potency [37]. Streptozotocin has been known to cause specific necrosis of the pancreatic beta cells, which is similar to the feature of the later stage of type 2 diabetes [38, 39] Results of this study showed that blood glucose of diabetic rats increases significantly three days after the intraperitoneal streptozotocin injection. This was similar with the researches that have been done throughout the world for diabetes induction [37, 40, 41].

A significant decrease in blood glucose of diabetic rats treated by OTE –compared with that of the diabetic rats– was observed from the first day of the study to the 28 d (first OTE intake). The OTE was found to have a similar hypoglycemic effect to glibenclamide at the end of the study. The OTE's hypoglycemic activity may result from two mechanisms: (i) potentiation of glucose-induced insulin release, and (ii) increasing peripheral uptake of glucose [1]. Beside this, OTE –like olive leaf extract– might produce its hypoglycemic effect is

through the inhibition of pancreatin amylase activity [24]. In this sense, olive leaf extract was found to inhibit the activities of α-amylases from human saliva and pancreas [24]. In Animal models studies, the hypoglycemic effect of OTE could be facilitated by the reduction of starch digestion and absorption. Moreover, hydroxytyrosol, oleuropein and their Seco iridoids derivatives –major phenolic compounds of OTE [42] – had a hypoglycemic and antioxidant *in vitro* and in rats [23]. These compounds may (i) protect pancreatic cells from progressive damage caused by streptozotocin, (ii) enhance insulin secretion by several mechanisms [43], (iii) active some enzymes – hexokinase and pyruvate kinase– implicated in glucose metabolism [23], and (iv) protect pancreatic cells from oxidative damage –through their strong antioxidant activity– caused by the increase of insulin secretion [44].

The lipid profile levels are usually raised in diabetics, which represents a risk factor for coronary heart disease [45]. It has been showed that high levels of total cholesterol and LDL-cholesterol are cardiovascular risk factors. However, increased level of HDL-cholesterol assured anti-inflammatory properties [46]. The present results showed that OTE exhibited a significant decrease in the level of lipid parameters in diabetic rats. OTE improves lipid profile because of high concentration of phenolic compounds having a lipid lowering action and prevented LDL-cholesterol oxidation. Epidemiological studies also suggested that the Mediterranean diet, rich in polyphenol, decreases the cardiovascular disease risk factors [47-49].

The total protein level was decreased in diabetic rats. This may lead to muscle wasting and an increased release of purine, the main source of uric acid as well as in the activity of xanthine oxidase. Moreover, the increase of uric acid level may be due to a metabolic disturbance in diabetes reflected in the high activities of xanthine oxidase, lipid peroxidation, and triglycerides and cholesterol increasing [50]. However, results herein presented show that the OTE decreases the creatinine, serum urea, and uric acid levels and increase total protein level in diabetic rats. In fact, elevation of the serum's urea and creatinine, as significant markers, are related to renal dysfunction in diabetic hyperglycemia [51].

Serum enzymes, including AST and ALT, are studied to evaluate the hepatic profile. An increase in these enzyme activities reflected liver damage. High transaminases levels are caused by hepatocellular inflammation [52]. Streptozotocin treatment has a significant role in the alteration of liver functions since the activity of AST and ALT were significantly higher than those of normal values. In the present study, a reduction in AST and ALT levels were found in diabetic rats treated by OTE. It may be the result of the olive polyphenols anti-inflammatory activity, which allowed OTE to regulate transaminases levels [19, 20].

By the end of the study period, all of the evaluated parameters in rats treated by OTE exhibited a significant restoration, which was similar to the normal control rats. In our previous studies, we found that OTE was endowed with important antioxidant and anti-inflammatory activities [19, 42]. These properties allow OTE to be efficient in the protection against some metabolic diseases related to oxidative stress such as diabetes. In fact, it has been demonstrated that antioxidant-based therapy is promising to minimize the complications associated with oxidative stress in diabetes mellitus [42, 53-56].

## CONCLUSION

Based on the findings of this study, we demonstrate that olive tree extract with high polyphenols content has the same effects –in comparison with glibenclamide– regarding blood glucose and other related parameters regulation in diabetes illnesses. The current findings are in agreement with those obtained *in vitro* or *in vivo* in several pre-clinical and clinical studies about anti-diabetic effects of olive polyphenols and/or olive tree extracts, suggesting the potential role of these natural compounds for "functional foods" conception to help in diabetes management.

## CONFLICTS OF INTERESTS

All authors have none to declare

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OLIVIE

THE BENEFITS OF THE OLIVE TREE

# CLINICAL STUDY N°4

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Original Article

# ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF OLIVE TREE EXTRACT

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**ABSTRACT**

**Objective:** The aim of this study was the *in vivo* evaluation of analgesic and anti-inflammatory effect of an olive tree extract with high polyphenols content.

**Methods:** This olive tree extract was obtained from Moroccan olive fruits and leaves using an eco-extraction free of chemical solvents and toxic additives. Anti-inflammatory activity was evaluated using carrageenan and histamine-induced paw edema methods. Analgesic activity of the olive tree extract was estimated against a hot plate, acetic acid induced writhing and formalin tests.

**Results:** The extracts showed significant anti-inflammatory and analgesic activities with a dose-dependent manner. Anti-inflammatory activity of olive tree extract at 250 and 500 mg/kg doses was more important compared to the used standard drugs ($p<0.05$), in both carrageenan and histamine-induced paw edema tests. In analgesic assays, results showed that 500 mg/kg dose of olive tree extract has a significant analgesic effect through both peripheral and central mechanisms.

**Conclusion:** Our findings suggest that olive tree extract is safe and has potential anti-inflammatory and analgesic activities, which promote this use as a food supplement against pain and inflammation related to inflammatory diseases.

**Keywords:** Olive leaves, Olive fruit, Polyphenols, Anti-inflammatory, and Analgesic, *In vivo*



## INTRODUCTION

The correlation between inflammatory response and many chronic diseases like rheumatoid arthritis, cardiovascular diseases and some types of cancer has been reported [1, 2]. Excessive production and accumulation of oxygen and reactive nitrogen species (ROS and RONS), and arachidonic acid metabolites play a key role in the pathogenesis of cardiovascular diseases and cancer [3, 4]. In the process of inflammation, arachidonic acid is converted by cyclooxygenase (COX) and lipooxygenase (LOX) to prostaglandins, thromboxanes, prostacyclin, and leukotrienes [5]. These inflammatory mediators are responsible for pain and other inflammatory symptoms. C-reactive protein concentration rises during inflammation [6]. Plasma thromboxane B2 (TXB2) increases platelet aggregation and leukotrienes B4 (LTB4) promotes migration of neutrophils to inflamed tissue [7]. Oxidative stress enhances inflammation by activating nuclear factor kappa B (NF-κβ) and activator protein-1 (AP-1) transcription factors and affecting cellular signaling processes [8]. NF-kB activation is associated with cancer progression. Thereby, ROS, COX-1/2 and NF-κβ inhibitors, may have a potential therapeutic effect on inflammation-depending diseases [9, 10].

In this sense, olive phenolic compounds are well known for their potential health benefits including the reduction of coronary heart disease risk, the prevention of some cancers and for their anti-inflammatory properties [11-13]. In fact, olive phenolic compounds are considered to possess anti-inflammatory properties, and therefore, were proposed as a alternative natural approach to prevent and/or treat chronic inflammatory diseases [14, 15]. Anti-inflammatory mechanisms of olive polyphenols are suggested to include: inhibition of pro-inflammatory enzymes, such as COX-2, LOX [16]. Its also inducible nitric oxide synthase (iNOS); inhibition of phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinases, NF-kB, and AP-1; and down-regulation of various pro-inflammatory cytokines such as chemokines, tumor necrosis factor alpha (TNF-a), interleukins including (IL-1 b, IL-6, IL-8), and monocyte chemotactic protein-1 (MCP-1) [14, 17-21]. It is reported that olive polyphenols relieved pain and have an analgesic activity in the case of some inflammatory diseases [22, 23].

In the present study, we have examined the possible antinociceptive and anti-inflammatory effects of a phenolic olive tree extract using *in vivo* experimental models. Namely, the carrageenan and histamine-induced hind paw oedema model tests for the anti-inflammatory activity and hot plate, acetic acid-induced abdominal writhing and formalin tests to assess the analgesic activity. Indomethacin and diclofenac sodium (with known anti-inflammatory effects), and tramadol hydrochloride and acetylsalicylic (with known analgesic properties) were used as positive controls.

## MATERIALS AND METHODS

### Plant materials

The olive tree extract (OTE) was ecologically obtained from Moroccan olive fruits and olive leaves/young sprouts, according to the previously described protocol [24]. They have been taken from stressed olive trees planted in a rocky desert harsh environment of Morocco. These suffering olive trees belong to a farm of the renowned Company Atlas Olive Oils. Actually, OTE is marketed in the world (France, Switzerland, Belgium, etc.) under the brand name OLIVIE FORCE/OLIVIE RICHE (see more at www.olivie.ma).

### Chemicals

Carrageenan; histamine; indomethacin; diclofenac sodium (DFS); acetylsalicylic acid (ASA); tramadol hydrochloride (Tramadol Hcl); formalin; acetic acid were purchased from Sigma-Aldrich (Paris, France).

### Animals

For studying the acute toxicity and the *in vivo* activities, male adult Wistar rats (120-180 g) and Swiss albino mice (20-25 g) of both sexes were obtained from the animal breeding unit of the Faculty of Science Dhar El Mahraz-Fez–Morocco. They were housed in polypropylene cages with free access to food and water. The animals were maintained under controlled conditions of temperature (22±2 °C) with a 12 h light-dark cycle. The animals were used after an acclimatization period of 7 d in the laboratory environment. Housing conditions and *in vivo* experiments were approved according to the

guidelines established by the European Union on Animal care (CEE Council 86/609). Animals fasted overnight before any experiments.

**Acute toxicity study**

Swiss albino mice (male) weighing 20-25 g were divided into two groups (five per group). Mice in the experimental group were received increasing OTE's dose (100, 250, 500 and 1000 mg/kg) while those in the control group received only 0.9% NaCl solution (10 ml/kg). The mortality rate was determined and the LD50 was estimated within the 24h following orally administration, according to the method described by Creton *et al.* [25].

***In vivo* anti-inflammatory activity**

**Carrageenan-induced rat paw oedema**

OTE's anti-inflammatory activity was evaluated using carrageenan-induced paw oedema in rats. Male Wistar rats were divided into 8 groups of five animals each. (1) Control group (10 ml/kg of 0.9% NaCl solution); (2) and (3) groups received reference drugs (10 mg/kg of indomethacin and diclofenac sodium); (4), (5), (6), (7) and groups (8) were orally administered OTE in 50, 100, 250, 500 and 1000 mg/kg doses, respectively.

Animals were pre-treated with drug and OTE 60 min before injection of carrageenan. Inflammation of the hind paw was induced by injecting 0.1 ml of 0.5% carrageenan suspension into the sub-plantar surface of the right hind paw. Measures of the paw circumference were determined at 3, 4, 5 and 6h (after edematogenic agent injection) intervals later (St) using the method of Bamgbose and Noamesi [26]. The difference between St (3, 4, 5 and 6h) and S0 was taken as the oedema size. The inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls and calculated by the following equation:

$$\%\ \text{inhibition} = \frac{(\text{St} - \text{S0})\text{control} - (\text{St} - \text{S0})\text{treated}}{(\text{St} - \text{S0})\text{control}} \times 100$$

**Histamine-induced rat paw oedema**

Eight groups of rats (five rats each) were used for this test. Group (1) served as a control group (10 ml/kg of 0.9% NaCl solution), animals in groups (2) and (3) were orally treated with indomethacin and diclofenac sodium (10 mg/kg), while rats in groups (4), (5), (6), (7) and (8) received the OTE at doses of 50, 100, 250, 500 and 1000 mg/kg, respectively.

Animals were treated by drug controls and OTE 1h before histamine injection (0.1 ml of a 1% solution in 0.9% NaCl solution) into the plantar region of the right-hand paw. Paw size was measured before injection of histamine and at 3, 4, 5 and 6h, after injection. The paw oedema was measured using the cotton thread method according to Farshid *et al.* [27]. The average increase in paw size of each group was determined. The percentage inhibition was obtained using this formula:

$$\%\ \text{inhibition} = \frac{(\text{St} - \text{S0})\text{control} - (\text{St} - \text{S0})\text{treated}}{(\text{St} - \text{S0})\text{control}} \times 100$$

With St = the paw size for each group after histamine treatment. And S0 = paw size for each group before histamine injection.

***In vivo* analgesic activity**

**Hot plate test**

Twenty-five Swiss albino mice (20-25 g) were divided into 5 groups of five mice per group. Group (1) received control solution (0.9% NaCl solution), group (2) received tramadol hydrochloride (10 mg/kg), while groups (3), (4) and (5) received 100, 250 and 500 mg/kg of OTE, respectively. 1h after the orally administration, mice were placed onto a hot plate (55±2 °C), and the reaction time for licking of paw or jumping for the control and treated mice was recorded (in seconds) [28]. A cutoff time of 15 s was used to avoid damage to the paw [29]. The percentage increase in reaction time was determined thus:

$$\%\ \text{increase in reaction time} = \frac{\text{Tt} - \text{T0}}{\text{T0}} \times 100$$

**Aceta writhing test**

Overnight fasted mice were divided into five groups of five each. Groups (1) and (2) received control solution (0.9% NaCl solution) and acetylsalicylic acid (10 mg/kg), while groups (3), (4) and (5) received 100, 250 and 500 mg/kg of OTE, respectively. 1h after, the animals were intraperitoneally injected with acetic acid (0.6%, v/v in 0.9% NaCl solution) [30], the analgesic activity was quantified by counting the total number of writhes over a period of 25 min after a latency period of 5 min [31]. The percentage of analgesic activity was calculated as follows:

$$\%\ \text{inhibition} = \frac{\text{number of writhes (control)} - \text{number of writhes (test)}}{\text{number of writhes (control)}} \times 100$$

**Formalin licking test**

Formalin licking test was carried out using male mice under same experimental conditions of acetate writhing test. 1h after orally OTE administration, 20 µl of 1 % formalin solution (in 0.9% NaCl solution) was injected subcutaneously into the plantar surface of the right hind paw of each mouse. Licking the injected paw time was measured over 30 min divided into two phases. The early phase was observed during the first 5 min and the late phase was recorded in 15-30 min [32]. These phases represented neurogenic and inflammatory pain responses, respectively [33]. The percentage of inhibition was obtained by the following formula:

$$\%\ \text{inhibition} = \frac{\text{reaction time (control)} - \text{reaction time (treated)}}{\text{reaction time (control)}} \times 100$$

**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 posthoc Dunnet test if the sample distribution follows a 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**

**Toxicity test**

Results (data not shown) showed no signs of toxicity which could be attributed to the administrated material (OTE), even at high doses. On the other hand, OTE, containing more than 15% of polyphenols (w/w), had no evidence of toxicity in mice and rats (mortality, tremor, convulsions, loss of reflex, sedation, and diarrhea). This, in fact, was in concordance with Soni *et al.* (2006) findings, that reported NOAEL (No Observed Adverse Effect Level) in rats after aqueous olive fruit extract administration (with high polyphenols content), even though at high doses as 2 g/kg/day. As a result, we suppose that OTE's orally administration (to mice) at the doses of 100, 250 and 500 mg/kg will be safe.

**Anti-inflammatory activity**

Results plotted in tables 1 and 2 illustrate the dose-dependent OTE's effect on paw oedema formation after induction by carrageenan (table 1) and histamine (table 2).

The subplantar injection of carrageenan-induced a progressive local oedema reaching its peak at the 3rd hour (table 1). The orally administration of the OTE showed a dose-dependent reduction in carrageenan-induced paw oedema from the 3rd to the 6th hour. The highest OTE's inhibition activity (80 %) was recorded after 4h at 500 mg/kg dose, compared to reference drugs indomethacin (53.24%) and diclofenac (69.34%) ($p<0.05$). Besides, no significant difference ($p<0.05$) was observed when the treatment dose, rise to 1 g/kg (compared to 500 mg/kg dose).

Similarly, the paw oedema induced by histamine was reduced after OTE administration (table 2). High inhibition activity was observed for the 500 and 1000 mg/kg doses (76.00 and 78.22%), but with no significant difference ($p<0.05$). Thus, OTE's administration has a significant anti-inflammatory effect compared to the used reference drugs, even at low doses like 250 mg/kg.

**Table 1: Effect of olive tree extract on carrageenan-induced rat paw oedema in rats**

| | Inhibition (%) | | | |
|---|---|---|---|---|
| **Dose (mg/kg)** | **3h** | **4h** | **5h** | **6h** |
| 50 | [a]0.27±0.13 | [a]10.00±2.20 | [a]20.21±2.21 | [a]0.45±0.05 |
| 100 | [b]44.45±3.45 | [b]60.00±2.33 | [b]64.24±2.22 | [b]16.67±1.00 |
| 250 | [b]50.27±3.61 | [b]75.00±2.76 | [c]73.40±1.56 | [c]37.50±1.11 |
| 500 | [c]66.76±3.69 | [c]80.00±2.11 | [c]78.73±1.72 | [d]50.00±4.27 |
| 1000 | [c]67.81±5.06 | [c]81.97±2.63 | [c]78.79±1.18 | [d]50.00±3.00 |
| Indomethacin* | [d]19.05±2.20 | [b]53.24±2.54 | [d]60.99±2.33 | [e]8.34±1.22 |
| Diclofenac sodium* | [b]47.11±2.22 | [b]69.34±3.25 | [d]63.12±1.65 | [e]3.34±0.18 |

[a–e] Values in the same column with different superscripts are significantly different ($p$<0.05) [mean±SD, n= 6]. * Reference drugs (indomethacin 10 mg/kg and diclofenac sodium 10 mg/kg).

**Table 2: Effect of olive tree extract on histamine-induced rat paw oedema in rats**

| | Inhibition (%) | | | |
|---|---|---|---|---|
| **Dose (mg/kg)** | **3h** | **4h** | **5h** | **6h** |
| 50 | [a]0.13±0.03 | [a]8.90±0.55 | [a]18.96±0.59 | [a]0.26±0.04 |
| 100 | [b]25.00±2.43 | [b]52.00±1.54 | [b]60.50±1.35 | [b]12.00±1.67 |
| 250 | [c]51.00±2.39 | [c]70.90±1.67 | [c]69.00±2.22 | [c]34.00±1.50 |
| 500 | [d]62.70±0.84 | [d]76.00±2.03 | [d]76.30±0.70 | [d]45.50±1.34 |
| 1000 | [d]63.98±0.57 | [d]78.22±1.18 | [d]77.10±0.86 | [d]47.00±2.37 |
| Indomethacin | [e]15.50±0.50 | [b]51.00±0.59 | [b]56.80±1.04 | [a]3.21±0.79 |
| Diclofenac sodium | [f]43.90±1.01 | [e]64.96±1.66 | [b]58.60±1.09 | [a]2.60±0.10 |

[a–f] Values in the same column with different superscripts are significantly different ($p$<0.05) [mean±SD, n= 6]. * Reference drugs (indomethacin 10 mg/kg and diclofenac sodium 10 mg/kg).

**Analgesic activity**

Graphs of fig. 1, 2 and 3 show results related to the OTE's analgesic activity assessed by means of the hot plate, acetic acid-induced abdominal writhing and formalin *in vivo* tests. Considering the results of anti-inflammatory tests, analgesic tests were performed at three OTE's doses (100, 250 and 500 mg/kg).

OTE administration has increased the insensibility to pain without loss of consciousness (analgesic activity) in mice placed onto the hot plate (55±2 °C). The observed OTE's analgesic effect was dose-dependent, reaching its maximum (36.77%) at 500 mg/kg dose (fig. 1). This effect was, however, compared to that observed (35.84%) for tramadol hydrochloride (no significant difference at $p$<0.05).

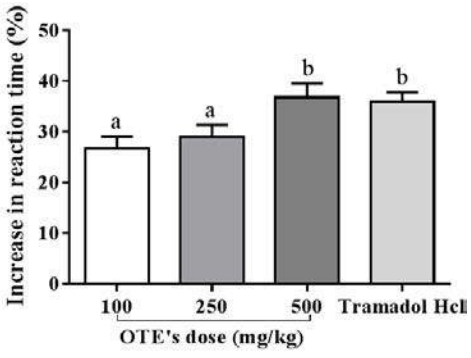


**Fig. 1: Effect of olive tree extracted on hot plate-induced pain in mice. Data is expressed as means±SD [n= 5]. Different letters indicate significant differences ($p$<0.05)**

On the writhing response in mice, OTE induced a potent dose-dependent antinociceptive activity at all used doses (fig. 2). This activity was up to 68% for 500 mg/kg dose, which was similar to that shown by the reference drug significantly (no differences at $p$<0.05).

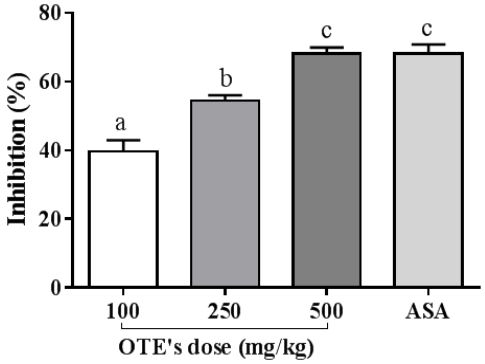


**Fig. 2: Olive tree extract's analgesic activity in mice treated with acetic acid. Data are expressed as means±SD [n= 5]. Different letters (a-c) indicate significant differences ($p$<0.05)**

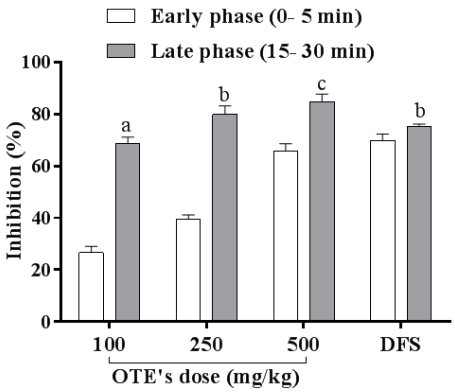


**Fig. 3: Effects of olive tree extract on the formalin-induced licking response in mice. Data is expressed as means±SD [n= 5]. Different letters (a-c) indicate significant differences ($p$<0.05)**

Two phases showed in fig. 3 represented neurogenic and inflammatory pain responses, respectively [31]. The subcutaneous

injection of formalin solution into the plantar surface of the right hind paw of mice produced an analgesic response of licking of the treated paw. OTE showed dose-dependent effect in both early and late phases. The OTE's analgesic activity was incomparable to that of reference drug (diclofenac sodium) during the early phase (corresponding to the neurologic pain) (fig. 3). However, this effect was significantly higher in mice treated with 500 mg/kg dose (84.70 %) compared to diclofenac sodium (75.20 %) ($p$<0.05) in the late phase (corresponding to the inflammatory pain).

## DISCUSSION

The carrageenan-induced paw oedema is frequently used as an experimental model for acute inflammation studying [34]. The inflammatory reaction carrageenan-induced (in rats) is a biphasic response, (i) oedema formation involving the production of inflammatory mediators such as histamine, serotonin, and kinins; (ii) the biosynthesis of prostaglandin and other autacoids release and attributed to the induction of cyclooxygenase (COX)-2 in the tissue [35-37]. Actually, the results of this study suggest that the OTE could antagonize the action and/or inhibit the production of the circulating inflammation mediators. On injection, histamine acts as an inflammatory mediator, which increases vascular permeability [38-40]. Results herein presented show the anti-oedomatogenic effect of orally administered olive tree extract in rats injected by Histamine. This could be, in fact, attributed to the anti-inflammatory activity of studying extract through an antihistamine mechanism. The rich phenolic olive tree extracted may act by inhibiting the release and/or histamine action, which can explain its inhibitory activity on oedema development.

Thus, observed OTE's Anti-inflammatory effects could be related to its phenolic composition. Actually, OTE is a rich phenolic extract (15%, w/w), particularly hydroxytyrosol (2%, w/w). In general, olive phenolic compounds have been known to inhibit both COX-1/2 inflammatory enzymes (in a dose-dependent manner) more efficiently than ibuprofen drug [16]. In a clinical trial, Ghanam *et al.* [14] recently reported that olive tree extract relieved pain in rheumatoid arthritis patients and decreased the circulating inflammatory biomarkers such as hs-CRP, IL-6, TNF-α, and PGE2 significantly.

Moreover, hydroxytyrosol impedes PGE2 synthesis by indirectly blocking of inducible nitric oxide synthase and COX-(2) enzymes. This effect was raised by the prevention of transcription factors activation as NF-κβ, interferon regulatory factor-1 and transducer and activator of transcription 1a, which prevents the activation of mouse macrophages J774 [41]. It is also known that hydroxytyrosol is capable of bringing about arylating/alquilant adducts in the cysteine residues of NF-κβ. The action of hydroxytyrosol on this factor blocks COX-(2) and 5-lipooxygenase transcription, reducing the PGE2 synthesis and, thus, the chronic inflammation associated with inflammatory diseases such as cancer [42]. Beside this, hydroxytyrosol possesses significant anti-inflammatory actions in inflammation animal models through the inhibition of pro-inflammatory cytokines expression (TNF-α and IL-1β) [20, 23].

Results from the current study reveal the effectiveness of natural OTE as oedema inhibitor compared to reference drugs (indomethacin and diclofenac sodium). These classical non-steroidal anti-inflammatory drugs (NSAIDs) mainly inhibit COXs. However, they have side effects such as irritation of the gastric mucosa, caused by the inhibition of prostaglandin biosynthesis, which has a protective role in the gastrointestinal tract. Many NSAIDs are also acids that may cause additional harm in the gastrointestinal tract [43]. Thus, the use of natural anti-inflammatory agents is one of the proposed solutions to overcome problems caused by side effects of NSAIDs.

Hot plate test was selected to investigate the central analgesic effect of OTE, which is known to elevate the pain threshold of mice towards heat [44]. It also indicates narcotic involvement with opioid receptor and measures the complex response to a non-inflammatory acute nociceptive input [45, 46]. The high percentage inhibition (36.77%) shown by OTE in this test suggests that OTE is a centrally acting analgesic. Acetic acid-induced writhing response is useful for the evaluation of peripherally acting analgesics. Acetic acid stimulates the tissue to produce several inflammatory mediators such as histamine, serotonin, cytokines, and eicosanoids with an increase in peritoneal fluid levels of these mediators [47]. In this sense, OTE inhibited mice abdominal writhes, suggesting that OTE's antinociceptive activity could be related to the reduction of inflammatory mediator's liberation and/or to direct blockage of receptors resulting in peripheral analgesic response.

The formalin test is a valid model of pain and analgesic research for clinical pain compared to tests using phasic thermal or mechanical stimulation. It has two distinctive phases, reflecting different types of pain [45]. The early phase (0–5 min) reflects centrally mediated pain, which was a result of direct stimulation of nociceptors and believed to be a non-inflammatory pain.

The late phase (15–30 min) persistent period caused by local tissue inflammation. Experimental results demonstrated that substance P and bradykinin participate in the early phase, while histamine, serotonin, prostaglandins, nitric oxide, and bradykinin are believed to be involved in the late phase of the formalin test response. Our results show that OTE has an inhibitory effect on the analgesic response of both early and late phases of the formalin test. Moreover, significant pain relief activity observed in the late phase (compared to the early phase) indicates the peripherally acting protective effect of OTE, which was correlated with anti-inflammatory tests results. In that way, OTE attenuate pain response better than diclofenac sodium (NSAID), commonly used as a reference due to its anti-inflammatory and analgesic effects. This drug has the ability to reduce inflammation, swelling and pain by inhibiting either the release of arachidonic acid or the prostaglandin synthesis [48, 49]. This fact corroborates with published data about, *in vivo*, phenolic compounds antinociceptive effects, mainly attributed to flavonoids [50, 51] and hydroxytyrosol [52]. It has also reported in a rodent model of opiate tolerance, that antiradical activity of olive phenolic compounds (hydroxytyrosol and oleuropein, amongst others) reinstates the analgesic action of morphine [22].

## CONCLUSION

Results of pharmacological tests performed in the present study suggest that olive tree extract with high polyphenols content is safe and presented potential anti-inflammatory and analgesic activities, which are comparable with the reference drugs. This might be correlated with the phenolic compound composition of this extract, particularly hydroxytyrosol. Considering high consumer demand due to the beneficial health effects, olive tree extract can be beneficially used as a natural food supplement to contend inflammation and pain in the case of inflammatory diseases.

## CONFLICTS OF INTERESTS

All authors have none to declare

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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 $\mu$mol 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 $\mu$g); Chloramphenicol (30 $\mu$g); Flucloxacillin (5 $\mu$g); Kanamycin (30$\mu$g); Gentamicin (10 $\mu$g), Amphotericin B (10 $\mu$g) and ketoconozole (15 $\mu$g) 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 100$\mu$l 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 $\mu$m) 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, 100$\mu$l 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 $\mu$l 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 $\mu$g); Chloramphenicol (30 $\mu$g); Flucloxacillin (5 $\mu$g); Kanamycin (30$\mu$g); Gentamicin (10 $\mu$g). And amphotericin B (10 $\mu$g) and ketoconozole (15 $\mu$g) were used for antifungal activity.

**Statistical Analysis**

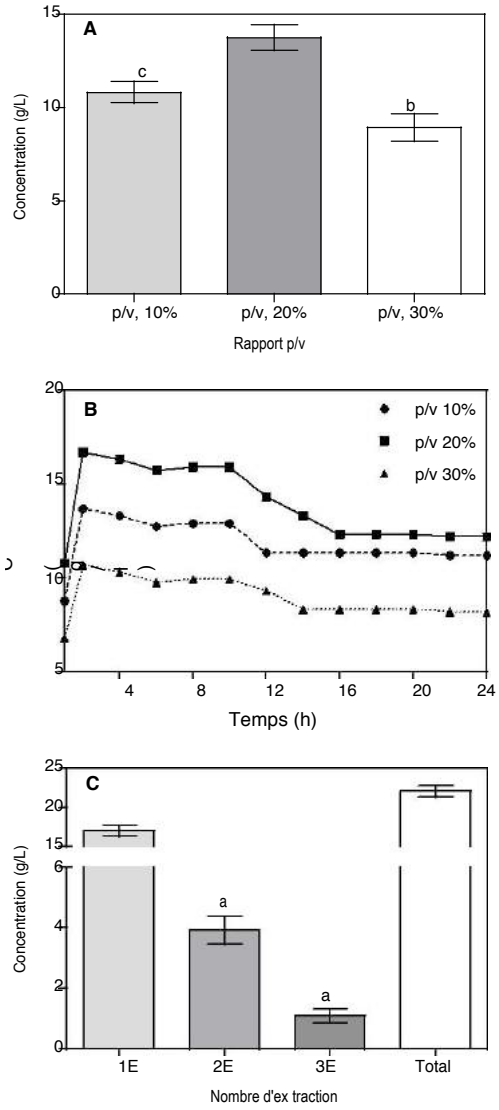
Statistical analyzes were performed using GraphPad Prism software version 6.00 (GraphPad Inc., San Diego, California). Data were analyzed by analysis of variance (ANOVA Analysis of Variance) followed by post-hoc Dunnet test if the sample distribution follows normal distribution or by the Kruskal-Wallis 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 ± 38481$\mu$mol 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 ($\mu$g/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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# Supplementation with Rich-Polyphenols Olive Tree Powder Improves Fasting Blood Glucose and Insulin Resistance in Patients with Type 2 Diabetes Mellitus: A 14-Weeks Randomized, Double-Blind, Placebo-Controlled Clinical Trial

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**Abstract:** ***Despite the fact that olive tree extracts have been used for long time as antidiabetics in Mediterranean folk medicine, there are few studies providing support to this view. To assess the effect of rich-polyphenols olive extract on glucose metabolism and insulin resistance, a randomized, double-blinded, placebo-controlled trial was conducted on human subjects with type 2 diabetes. 80 T2DM patients were randomized to receive 3 g of olive tree powder or placebo during 14 weeks. changes from baseline in glucose metabolism, insulin resistance, and lipid profile were determined and compared between groups. The lipid profile levels of treated group have been decreased significantly (p< 0.0001 vs. placebo group), while the value of HDL-cholesterol raises to 51.5 ± 9.4 mg/dL (p= 0.007 vs. placebo). The administration of rich-polyphenols olive tree powder resulted in a significant reduction (vs. placebo) in HbA1c (p< 0.0001), fasting glucose (p< 0.0001), and insulin resistance (p= 0.0002). The average value of fasting glucose of the treated group was under the normal level defined by the American Diabetic Association (114.2 ± 15.2 mg/dL) by the end of the 14 weeks. Supplementation with olive tree extract was associated to a net improvement in fasting plasma glucose, insulin resistance, and lipid profile in subjects with T2DM, suggesting its potential therapeutic effect as an antidiabetic.***

**Keywords**: Olive tree extract-Type 2 diabetes mellitus-Insulin resistance -Glucose control- Randomized clinical trial.

## 1. Introduction

The most recent data published by the World Health Organization suggest that 422 million people already had diabetes by 2014 [1], while the projections predict a continuous increase in the global incidence of diabetes to reach 552 million patients by 2030 [2]. This makes the pandemic of type 2 diabetes (T2DM) one of the enormous public health problems. T2DM is a chronic degenerative disease of metabolic disorders (most notably glucose metabolism), that progressively affects the optimal function of cardiovascular system, eyes, kidneys, nervous system and other organs such as the skin, liver and gut[2].

Regarding T2DM, one third of patients use alternative medicine to delay the disease outcomes, even without any scientific evidence supporting these uses [3]. Data from comprehensive meta-analyses reported, in fact, inverse correlations between adherence to Mediterranean diet and risk of type 2 diabetes, as well as significant improvements in glycemic control [4, 5]. The main features of this kind of diets is the predominance of plant foods and -notably- the high consumption of olive products. Olive polyphenols are reportedly responsible for the health benefits associated with the Mediterranean diet [6, 7], as the analysis of the results from the PREDIMED trial showed an inverse correlation between polyphenol excretion and fasting glucose [8]. The most well studied phenolic compounds present in olive tree products are the catecholic derivatives, oleuropein and hydroxytyrosol, which show -according to *in vitro* and animal studies- antioxidant, anti-inflammatory, hypoglycemic, antihypertensive, antimicrobial, and anti-atherosclerotic properties [9]. For this reason, -in 2012- the European Union recognize that a daily intake of 20 g of virgin olive oil containing, at least, 5 mg of hydroxytyrosol and its derivatives (notably, oleacein), contributes to improve human health and well-being [10]. Additionally, the European Food Safety Authority has already endorsed the health claim that ‘‘the consumption of olive oil polyphenols contributes to the protection of blood lipids to oxidative damage’’ in 2006 [11]. This make exploring of the potential health benefits of olive products (rich in polyphenols) an expanding nutraceutical market. However, more studies on cultured cells, animals and -notably- humans are needed to provide compelling evidence that olive polyphenols are possible candidates for prevention and therapy of metabolic syndrome, particularly T2DM.

For this purpose, we conducted a randomized, double-blinded, placebo-controlled trial to assess the effect of olive tree powder on glucose metabolism in human subjects with T2DM. The main monitored outcomes were glycemic control and plasma biomarkers involved in the development of cardiovascular disease.

## 2. Materials and methods

### 2.1 Subjects

Men and women were recruited from October 2016 to February 2017 among of those referred to an outpatient clinic in Fez, Morocco. To be enrolled in the current study, subjects had to have been diagnosed with T2DM since at least one year based on the American Diabetes Association

(ADA) [12] criteria for the diagnosis of diabetes (A hemoglobin A1c (HbA1c) level of 6.5% or higher; A fasting plasma glucose (FPG) level of 126 mg/dL or higher; A 2-hour plasma glucose level of 200 mg/dL or higher during a 75-g oral glucose tolerance test (OGTT); A random plasma glucose of 200 mg/dL or higher in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis). Study was thoroughly explained to the voluntary participants. Patients were not eligible if they were under the age of 20 years or over the age of 80 years;practicing >1 hour of physical activity per week with participation in weight-reduction programs;on insulin therapy; they had hepatic or renal dysfunction; they had history of malignancy; they had a clinically important hematological disorder or severe autoimmune disease;they were pregnant (or planned to be), or breastfeeding during the trial period;receiving contraceptive; smoking; drug or alcohol abuse.Exclusion criteria involved also the consumption of olive antioxidants or other antioxidant supplements ≤3 weeks before the intervention, history of allergy or intolerance to olive products. Before to be enrolled to this study, written informed consent was obtained from all voluntary participants.

### 2.2 Study design and intervention

The current study was planned as a double-blind, randomized, placebo-controlled trial (Figure 1). It was directed according to the guidelines approved by Helsinki Declarationand the protocol wasapproved by the local ethics committee of theUniversity Sidi Mohammed Ben Abdellah. Eligible participants were randomly assigned to Olive Tree Extract (OTE) supplement group or placebo group using a computer-generated random-number sequence. Researchers, participants and clinical staff were blinded to the treatment codes of each group. The enrolled participants were invited by telephone to the clinic after an overnight fasting (between 8 and 14 h)to attend a screening visit (baseline analyses) including the assessment of adherence to the Mediterranean Diet (according to the modified questionnaire of Estruch et al. [6] and the evaluation of physical exercise by the International Physical Activity Questionnaire (Physical exercise was categorized as high, moderate, or low).

Participants were asked to maintain their habitual diet during the period of study, avoid the consumption of olive products (including olive oil, olive table), and the use of all herbs or products known to affect glucose metabolism (synthetic or natural antioxidants). Dietary changes were monitored trough a 3-day dietary records at baseline and 14 weeks after intervention. Necessary explanations were provided about how to estimate food intake and record the estimations. We repeated all examinations and measurements after 14 weeks. During the study, all participants and investigators had free and continuous access to clinic for advice and consultation. Participants who fulfilled all the inclusion criteria were received 500-mg study capsules (identical capsules for supplement and placebo group). Participants received also instructions concerning capsules taking and storage. Patients were asked to administrate 6 capsules per day before each meal and they were contacted every week to monitor supplement intake. Olive tree extract powder (OTE) was enclosed in soluble vegetal capsules. The placebo capsules contained only maltodextrin. OTE was obtained from different olive tree parts, including fruits, olive tree young branches, and leaves using a purely bio-extraction [13]. Plant material used for extraction derived from specific olive trees planted in the middle of a rocky desert in Morocco. This environment is free of pollution, free of industrial activity, and under drought-stress (with temperatures up to 52°C). OTE is encapsulated in slight variations through the brand OLIVIE such as for example OLIVIE RICH/FORCE and marketed in Belgium as OLIVIE RICHE (see more in www.olivie.ma).

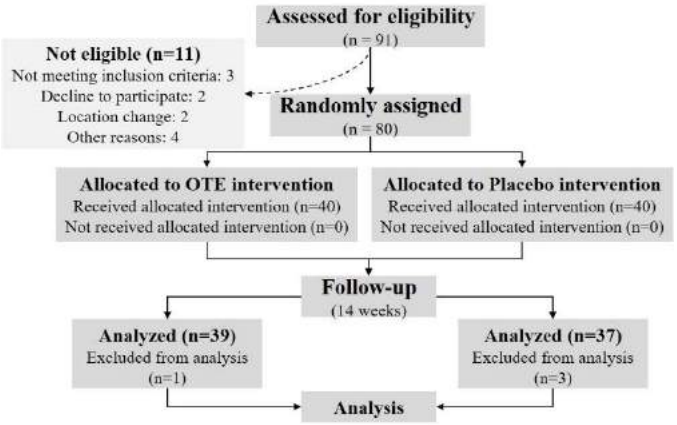


**Figure 1**: Study flow diagram

### 2.3 Laboratory measurements

Anthropometric measures were performed using calibrated scales and wall-mounted stadiometer with a precision of 0.1 cm; systolic and diastolic blood pressure were measured using a semi-automatic oscillometer (BosoMedicus smart Semi automatic Blood Pressure Monitor, Germany). Energy, nutrient intake and participants' diets assessment was carried out by Nutritionist 4.3 software (First Databank, Hearst Corp, San Bruno, CA). Blood samples were collected in EDTA and SST tubes. The obtained erythrocytes, plasma, serum and urine samples were aliquoted into 1 mL microtubes and stored at -80°C until further analysis. The fasting plasmaglucose(mg/dl) was assayed by the glucose oxidase method (Beckman Glucose Analyzer).The following parameters were measured: $HbA_{1C}$ (%), TC (mg/dl), HDLcholesterol (HDL-c) (mg/dl), LDL cholesterol (LDL-c) (mg/dl), TGs (mg/dl), hemoglobin (g/dl), hematocrit (%) and erythrocytes (mil./mm3). TC, VLDL and TG weremeasured using enzymatic testsin a contract clinical laboratory. LDL-c levels were calculated by the Friedewald equation, HDL-c was measured by using theheparin-manganese precipitation method. High-sensitivity enzyme-linked imminosorbent assay kits (DiaSource, Belgium) were used to quantify serum levels of insulinaccording to the manufacturer's guidelines. Fasting insulin resistance was assessed with homeostasis model assessment and calculated with the following formula, according to Matthews et al. (1985) [14]: *fasting plasma glucose (mg/dL)*fasting serum insulin (µU/mL)/405*. High scores indicate high insulin resistance.Urinary hydroxytyrosol was quantified by High Performance Liquid Chromatography (HPLC) as markers of OTE intake. Briefly, hydroxytyrosol was extracted from acidified urine (hydrochloric acid, 0.6 N of final concentration) as described previously [15] and analyzed in

a Shimadzu chromatograph device equipped with a reverse phase C18 column (250 mm L. × 4.6 mm I.D., 5 µm).

Doctors assessed potential adverse effects of OTE administration over the period of study including mouth symptoms, digestive disorders, fullness, allergic skin response, and other intervention-related symptoms. Finally, global satisfaction assessment in response to treatment (GAST) (including anxiety) was evaluated using a 5-point categorical scale (0 = poor, 1 = fair, 2 = good, 3 = very good, and 4 = excellent).

### 2.4 Statistical analysis

Data were statistically analyzed using GraphPad Prism version 6.00 (GraphPad Prism Inc, San Diego, California). For the baseline characteristics, continuous variables are expressed as mean values ± standard deviation (SD), and categorical variables are expressed as frequencies (percent). Normal distribution of data was checked using the Kolmogorov-Smirnov test. The difference between baseline groups characteristic was performed by, the independent t test, the Mann-Whitney U test, and the $\chi 2$ test for normally continuous data, not normally continuous data, and categorical data, respectively. the independent t test was also used to compare the mean changes from baseline to the end of the study between treated and placebo groups. Results with two-sided P values of <0.05 were considered statistically significant.

## 3. Results

### 3.1 Baseline characteristics

Ninety-one eligible patients were enrolled, and 11 were excluded from the study for several reasons (Figure 1). Four participants were dropped out of analysis because they were unable to follow study protocol (Figure 1), due to higher fasting plasma glucose, total cholesterol and LDL-C levels than participants who completed the study.Good compliance was showed in treated-group (94.6%) and placebo-group (92.3%), without any observed study-intervention adverse. Urinary hydroxytyrosol determined as biomarker of compliance was quantified by HPLC. Results of the Figure 2graph illustrate the changes from pre-intervention periods for placebo and treated (at 4 and at the end of study) group. The concentration of hydroxytyrosol founded in urine of treated participants was significantly higher ($P$<0.0001) compared to that of placebo group. However, it is worth noting that literature data on olive phenols absorption, metabolism, and excretion are not in agreement [16, 17].
Table 1 shows the baseline characteristics of the 80 participants who randomized into the treated and placebo group. Statistical analysis reveals no significant differences in demographic and clinical measurements among the two study groups, including the degree of adherence to Mediterranean Diet ($P$=0.326).

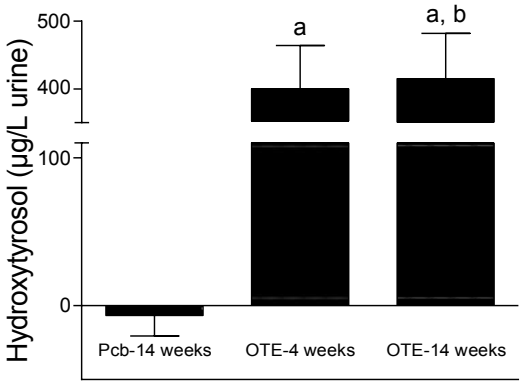


**Figure 2:** Change from baseline in urinary hydroxytyrosol excretion. Mean with SD. [a]$P$<0.0001, between OTE-group (at 4 or 14 weeks) and placebo-group (Pcb); [b]$P$=0.003, between OTE-group at 4 and 14 weeks.

**Table 1:** Baseline characteristics of participants

| *Parameter* | *Intervention group (n=40)* | *Placebo group (n=40)* | *P value* [a] |
|---|---|---|---|
| Age (years) | 53.27 ± 1.61 | 55.73 ± 1.97 | 0.346 |
| Female, n (%) | 17 (42.5) | 15 (37.5) | 0.915 |
| Weight (Kg) | 88.81 ± 3.55 | 86.15 ± 4.06 | 0.631 |
| BMI (Kg/m$^2$) | 30.5 ± 5.1 | 29.8 ± 4.7 | 0.175 |
| BMI >30 (Kg/m$^2$), n (%) | 28 (70) | 24 (60) | 0.632 |
| BMI <25 (Kg/m$^2$), n (%) | 7 (17.5) | 9 (22.5) | 0.539 |
| Disease duration (y) | 4.67 ± 1.4 | 3.50 ± 0.7 | 0.366 |
| Family history of disease, n (%) | 9 (22.5) | 10 (25.0) | 0.699 |
| Diet, n (%) | 7 (17.5) | 9 (22.5) | 0.813 |
| OAH + Diet, n (%) | 33 (82.5) | 31 (77.5) | 0.784 |
| $HbA_{1C}$ (%) | 7.79 (0.8) | 7.46 (1.1) | 0.663 |
| $HbA_{1C}$ level > 7%, n (%) | 27 (67.5) | 23 (57.5) | 0.558 |
| Glucose (mg/dL) | 166.9 ± 10.8 | 162.4 ± 9.8 | 0.764 |
| Insulin (µU/mL) | 13.1 (5.6) | 14.1 (6.4) | 0.432 |
| HOMA-IR | 5.4 (2.8) | 5.7 (3.1) | 0.698 |
| Total cholesterol | 201.7 ± 14.6 | 199.3 ± 18.3 | 0.923 |
| LDL-C | 127.7 ± 13.8 | 133.9 ± 14.9 | 0.765 |
| HDL-C | 45.9 ± 6.2 | 43.6 ± 5.2 | 0.784 |
| TGs | 131.5 ± 11.7 | 127.1 ± 11.5 | 0.799 |
| Systolic BP (mm Hg) | 130.9 ± 11.4 | 129.3 ± 12.4 | 0.589 |
| Diastolic BP (mm Hg) | 81.3 ± 7.2 | 80.7 ± 7.7 | 0.643 |
| 15-item Mediterranean diet score | 2.05 ± 0.15 | 2.40 ± 0.20 | 0.326 |

Value are expressed as mean ± SD or in percentage.[a] P value (<0.05) by independent t-test or Mann-Whitney test. **BP**: Blood Pressure; **BMI**: Body Mass Index; **$Hb_{A1c}$**: Hemoglobin A1c; **(L)HDL**:(Low)High-Density Lipoprotein;**TGs**:Triglycerides; **HOMA-IR**: Homeostasis Model Assessment of Insulin Resistance; **OAH**: Oral Antihyperglycemic agents.

Results of dietary questionnaires represented in Table 2 show that there was no significant difference in diet intake at the baseline and after 14 weeks of OTE and placebo supplement. The MUFAs and PUFAs –main components of the Mediterranean Diet–intake was maintained constant, which was good for the study since these nutrients affect (positively) plasma lipids and glucose metabolism ofT2DM patients [5, 18]. We also reported in Table 2 change in participant's weight, with a slight decrease observed at the

end of the intervention period in participant of treated group (but still not significant, *P*=0.176). The level of macronutrient intakes was held constant during the study course and all participants met the daily diet recommended by the researchers by avoiding consumption of olive products and any other products known to affect glucose metabolism.

**Table 2:** Change in energy and macronutrients intake at baseline and end of the study for tow study groups. Data are expressed as mean ± standard deviation.

| *Parameter* | *OTE group (n=45)* | *Placebo group (n=45)* |
|---|---|---|
| **Energy (cal)** | | |
| Baseline | 1755 ± 209.8 | 1809 ± 200.7 |
| 14 weeks | 1832 ± 202.3 | 1445 ± 318.6 |
| *P* value [a] | 0.506 | 0.695 |
| **Fat (g)** | | |
| Baseline | 75.90 ± 9.9 | 69.2 ± 12.4 |
| 14 weeks | 69.7 ± 12.4 | 71.4 ± 15.4 |
| *P* value [a] | 0.507 | 0.680 |
| **PUFAs (g)** | | |
| Baseline | 9.2 ± 1.4 | 8.9 ± 1.3 |
| 14 weeks | 9.7 ± 2.9 | 10.7 ± 1.9 |
| *P* value [a] | 0.711 | 0.651 |
| **MUFAs (g)** | | |
| Baseline | 22.7 ± 1.5 | 20.1 ± 1.7 |
| 14 weeks | 21.7 ± 2.9 | 21.6 ± 1.2 |
| *P* value [a] | 0.510 | 0.450 |
| **SFAs (g)** | | |
| Baseline | 15.3 ± 1.9 | 13.6 ± 2.7 |
| 14 weeks | 15.2 ± 1.6 | 14.1 ± 2.9 |
| *P* value [a] | 0.655 | 0.844 |
| **Weight (kg)** | | |
| Baseline | 88.81 ± 3.55 | 86.15 ± 4.06 |
| 14 weeks | 86.31 ± 3.87 | 87.31 ± 3.46 |
| *P* value [a] | 0.176 | 0.359 |

PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.[a] Paired Student *t* test (p<0.05).

### 3.2 Clinical measurement

At the end of the 12-week study period, weight and BMI were reduced in the intervention group, but with no significant difference compared to the control group (Table 3).

However, the lipid profile levels of treated group decrease significantly (*vs.* placebo group) for Total cholesterol (p< 0.0001), LDL-C (p< 0.0001), and TGs (p< 0.0001), while the value of HDL-C raises to 51.5 ± 9.4 mg/dL (p= 0.007). The daily supplementation with the rich-polyphenols olive tree extract was associated to a significant reduction (vs. placebo) in HbA1c(p< 0.0001), fasting glucose (p< 0.0001), HOMA (p= 0.0002). The average value of fasting glucose has dropped to 114.2 ± 15.2 mg/dL at the end the intervention, which is significantly below normal level defined by the ADA (13).

The fasting insulin levels increased over time for the treated group (even the difference still no significant compared to the placebo group p= 0.251), suggesting an improve in insulin secretion as well (taking together with the decrease in fasting glucose).

**Table 3:** Results from generalized linear model analysis describing changes in clinical and laboratory measurements between baseline and 14-monthfollow-up examinations. See legend of Table 1 for the abbreviations.

| *Variable* | *Intervention group (n=39)* | | *Placebo group (n=37)* | | *P value* [c] |
|---|---|---|---|---|---|
| | *14-weeks* | *Δ study end* | *14-weeks* | *Δ study end* | |
| Weight (Kg) | 86.3 ± 3.8 | ↓2.5 | 87.3 ± 3.4 | ↑1.2 | 0.593 |
| BMI (Kg/m$^2$) | 28.1 ± 4.6 | ↓2.4 | 30.8 ± 3.9 | ↑1 | 0.332 |
| $HbA_{1C}$ (%) | 6.08 ± 1.2 | ↓1.3 | 8.6 ± 1.3 | ↑ 1.04 | <0.0001 |
| Glucose(mg/dL) | 111.2±15.2 | ↓55.7 | 172.7±17.1 | ↑ 10.3 | <0.0001 |
| Insulin (μU/mL) | 14.5 ± 2.5 | ↑1.4 | 13.6 ± 3 | ↓0.5 | 0.251 |
| HOMA-IR | 3.9 ± 1.2 | ↓1.4 | 5.8 ± 2.0 | ↑0.1 | 0.0002 |
| Total cholesterol (mg/dL) | 150.9 ± 26.4 | ↓50.8 | 234.8±37.3 | ↑ 35.5 | <0.0001 |
| LDL-C (mg/dL) | 106.9± 20.1 | ↓20.8 | 150.9±26.4 | ↑ 17 | <0.0001 |
| HDL-C (mg/dL) | 51.5 ± 9.4 | ↑5.6 | 41.7± 11 | ↓1.9 | 0.007 |
| TGs (mg/dL) | 87.1 ± 11.2 | ↓44.4 | 148.8±19.4 | ↑ 21.7 | <0.0001 |
| GAST | 3.4 ± 0.6 | | 2.2 ± 0.4 | | 0.04 |

The fasting insulin levels increased over time for the treated group (even the difference still no significant compared to the placebo group p= 0.251), suggesting an improve in insulin secretion as well (taking together with the decrease in fasting glucose).

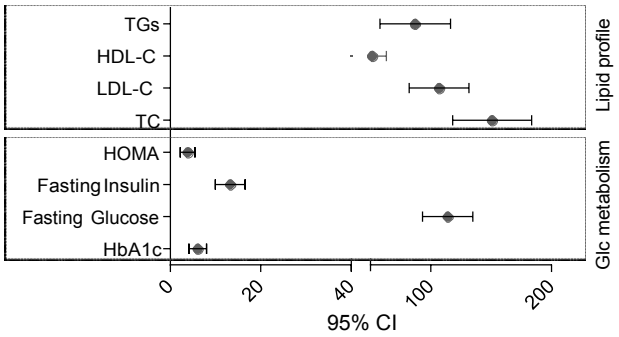


**Figure 3:** The horizontal line joins the lower and upper limits of the 95% CIof each corresponding parameter measured in the intervention group.

Additionally, almost all participants in the treated group have reported a very good satisfaction of the treatment, by answering to the GASTquestionnaire (Table 3).

## 4. Discussion

In this placebo-controlled trial, patient with T2DM were allocated to a treatment by an aqueous olive tree extract during 14 weeks, by receiving a daily dose of 3 g (6 capsules, 500 mg each). No adverse signs and laboratory parameters fluctuation have been observed during the study period and within the three post-intervention weeks (data not shown). We found that the supplementation with rich- polyphenols OTE

modulates carbohydrate and lipid metabolism, attenuate hyperglycemia, dyslipidemia, and insulin resistance.

T2DMis group of metabolic disorders characterized byinsulin resistanceand impaired carbohydrate metabolism. Several authors reported that Mediterranean Diet (rich in olive polyphenols) and polyphenol-rich foods (olive oil, tea, cocoa, cinnamon, grapes, and berries) modulatecarbohydrate metabolism, and attenuate hyperglycemia, dyslipidemia, and insulin resistance [5, 19, 20,21]. We have already shown -as well as many others authors- that a daily supplementation with olive polyphenols exert a hypoglycemic response inanimal models [22, 23,24,25]. Furthermore, diabetic rats consuming0.5 mg/kg olive leaves extract for 30 days showed improved blood glucose, and insulin secretion [26]. More interesting, the anti-hyperglycemic effects of olive polyphenols were also demonstrated in prediabetics and diabetics human volunteers through several research groups [27,28,29, 30].

The first goal of T2DM treatment is to target glycemic control by maintaining HbA1c as close as possible to normal value (≤ 7%). Here, OTE supplementation for 14 weeks was associated with a reduction of HbA1c by ≈22% (more than 62% of participants have had a normal value by the end of the intervention). Similar effect was also observed in diabetic patients consuming a daily dose of olive leaves extract of 500 mgfor 14 weeks, but the HbA1c values decreased only by 10% [29]. It is to be noted that the OTE represent the full spectrum of the tree, not only a single part of the olive tree such as isolated leaves. Thus, there is a positive synergetic effect produced by the mix of all the polyphenols present inside the olive tree in their natural proportions. How polyphenols influence the level of circulating glycatedplasma proteins is still not so clear. However, it was suggested that the antioxidant properties might diminish the production of advanced glycosylated end products such as HbA1c [31]. T2DM is also associated with deregulation of lipid metabolism, which can be positively targeted by olive polyphenols. In the well-known large multi-centre crossover trial (200 healthy men), Estruch et al. [7] demonstrated the dose dependent improvements in plasma HDL status after administration of olive oilswith increasing polyphenol concentrations.Supplementary, modulation of glucose metabolism would reduce the accumulation of lipids in the liver (as observed in a cholesterol fed rat model) and potentially offset de-novo lipogenic pathways [32]. This might explain reduced dyslipidaemia (reduction in total cholesterol, LDL-C, TGs, and improvement in HDL-C) of the participants allocated to OTE. The supplementation with rich-polyphenols olive leaves extract improves fasting glucose in T2DM diabetic subjects [29], and both insulin sensitivity and secretion in overweight middle-aged-men [28]. Similar effects were observed at the end of this intervention with an improvement in fasting glucose, insulin resistance, and insulin secretion by over 33, 27 and 11%, respectively. We should underline, in fact, that we have used an olive tree powder (not an olive leaves extract) at high daily dose in comparison to de Bock' [28]and Wainstein'[29] studies. Additionally, the treatment by OTE might have an exaggerated response in patients who had already T2DM compared to prediabetic subjects [28], which can explain the results herein obtained. However, we all assume that polyphenols contained in our extract powder are responsible of the observed hypoglycemic effects. In this sense, it has been reported that a daily supplementation with rutin (500 mg) reduces fasting glucose levels by over 10% in diabetic patients after 4 and 8 weeks [33].

Because T2DM is a multifactorial disease, olive polyphenols might have multifaceted anti-hyperglycemic effects. Firstly, hydroxytyrosol and oleuropein have been shown, *in vitro*, a strong inhibition of amylase andα-glucosidase [34, 35]. Actually, our unpublished data show the same effect of the studied olive tree extract (rich in hydroxytyrosol) on α-glucosidase and α-mannosidase. On the other hand, polyphenols can act as direct suppressors of the proteins involved inthe intestinal transport of dietary carbohydrate [36].This wouldresult in the suppressed digestion of starch and therefore a lower glycemic response to foods.Furthermore, Polyphenols might affect glucose metabolism via a reduction of glucose release from the liver or a stimulation of cellular glucose uptake, which lead to reduced plasma glucose [36, 37].Oleuropein and hydroxytyrosol (two phenols abundant in the studied extract) enhance glucoseinduced insulin secretion following oral glucose challenge in human subjects [28], and protect insulin-secreting β-cells against toxic $H_2O_2$ by maintaining normal redox homeostasis during an oxidative stress [37].

## 5. Conclusion

Overall, results herein obtained demonstrate that the administration of rich-polyphenols extract from olive tree was associated to significant hypoglycemic effects in patients with type 2 diabetes. We suggest that olive polyphenols -as natural components of olive tree extract powder- exert an hypoglycemiceffect, mainlyby i) improving glucose-induced insulin secretion, and ii) increasing peripheral glucose uptake. Further researchesshould compare hypoglycemic effect of pure polyphenols (from this olive tree powder) to conventional T2DM therapy (e.g. metformin) to better understanding the mechanism(s) by which these molecules contribute to glucose metabolism control.

## 6. Acknowledgements

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