



# CLINICAL STUDIES

Realized on our OLIVIE products

DESERT EMPOWERED  
HYDROXYTRYROSOL



BEFORE



AFTER



We made 8 clinical studies on the OLIVIE products. More than 1'000'000 US\$ spent on science for the benefits of the consumers. Just the Joints anti-inflammation study costed us 140'000 US\$. In the next pages you will find all the clinical studies about the OLIVIE products.

As matter of fact, OLIVIE products are backed by deep sciences as we have realized 8 clinical studies (2 more are underway) on:

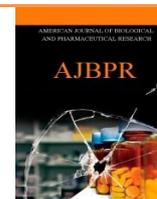
- lowering **diabetes** (2 studies realized)
- lowering **arthritis and arthrosis** pain and inflammation for patient suffering from **joints inflammation** (2 studies realized in this).
- lowering **cancer** tumor growth-anti-microbial effect.
- lowering **Alzheimer** incidence and improving **brain cognition**.

Last but not least, the story behind this powder is unique:

We produce the OLIVIE powder from suffering olive trees in a rocky desert. Because of high temperatures (up to 52°C in summer), a lot of rocks in the soil making the trees not able to grow their roots to look for nutrients, nearly no rain throughout the year, our olive trees stress and think they will die. So they trigger a self-defense mechanism through which they product abnormally high quantity of antioxidants. We think these antioxidants, born because of this harsh environment, are more active, more potent and this is the main reason why we obtained excellent results (illustrated by the clinical studies we made) for anti-inflammation, anti-pain, anti- diabetic effect, anti-microbial and anti-alzheimer effect.



## **CLINICAL STUDY N°1**



## **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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### **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- $\alpha$  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)

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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  $\beta$  (NF- $\kappa$  $\beta$ ) is activated in the synovium inflammatory cells and induced cytokines expression, including tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukine-1 (IL-1 $\beta$ ), IL-6, IL-15, IL-18, but also metalloproteinase (MMP-1) and small osteochondral destruction molecules like prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) 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- $\alpha$ , 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<sub>2</sub>, LTB<sub>4</sub>, TNF- $\alpha$ , 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  $\leq 3.5 \times 10^9/L$ , hemoglobin (Hb) level  $\leq 8.5$ g/dl, platelet count  $\leq 100 \times 10^9/L$ , creatinine level  $\geq 2.0$  mg/dl, and aspartate aminotransferase (AST) levels  $\geq 2.5$  times the upper limit of normal. Exclusion criteria involved also the consumption of olive antioxidants or other antioxidant supplements  $\leq 3$  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 through 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 administer 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](http://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 immunoassay kits (DiaSource, Belgium) were used to quantify PGE<sub>2</sub>, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), TNF- $\alpha$  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.  $\times$  4.6 mm I.D., 5  $\mu$ m).

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:

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

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  $\pm$  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 ( $P = 0.296$ ).

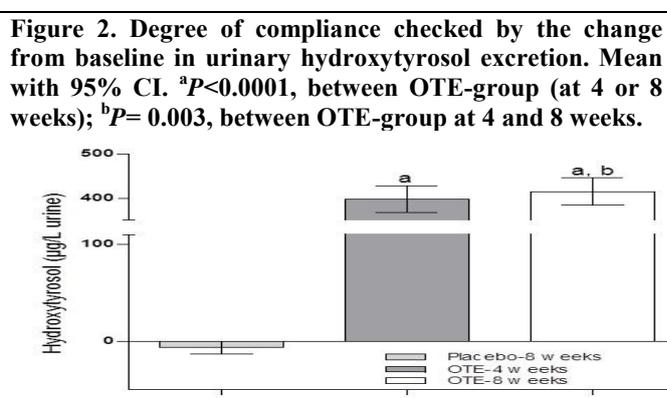
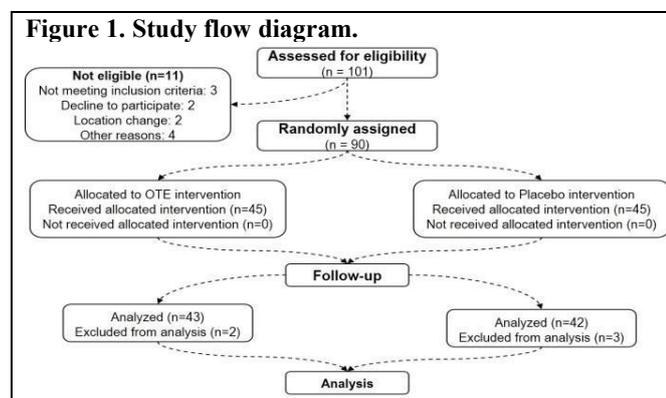
Results of dietary questionnaires represented in table 3 show that there was no significant difference in diet intake at the baseline and after eight weeks of OTE and placebo supplement. Result of table 3 show also that the PUFAs intake was maintained constant ( $P$  value of 0.611 and 0.741 for OTE 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.

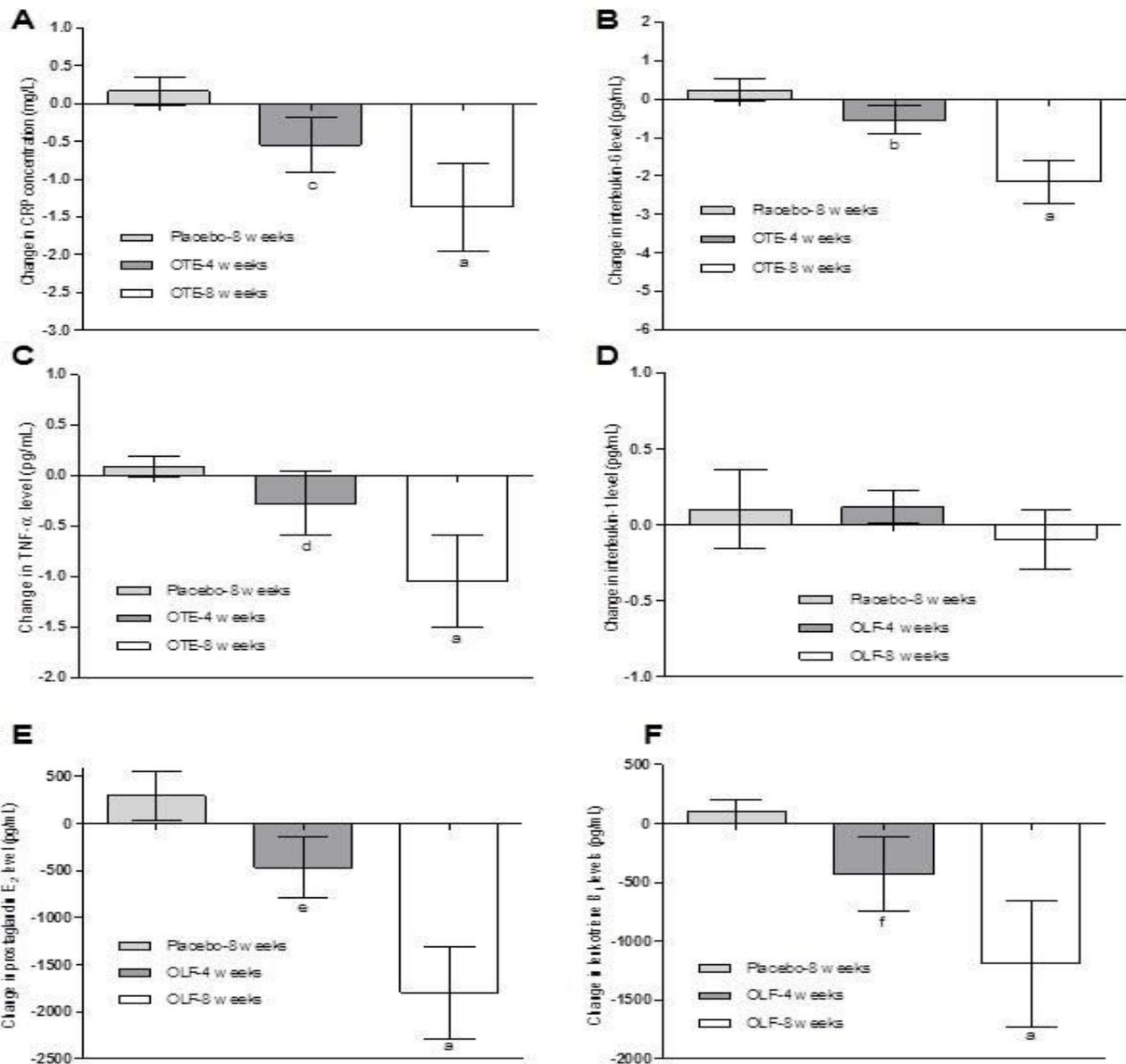
Rheumatoid arthritis is a chronic autoimmune inflammatory disease characterized by joint swelling, joint tenderness and destruction of synovial joints. The clinical outcome is pain, warmth, redness, and loss of function. Inflammation of synovial membrane is believed to be the main cause involved in RA outcomes. High concentration of circulating markers of inflammation, such as cytokines (IL-6, IL-1, TNF- $\alpha$ ) 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- $\alpha$ , 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- $\alpha$  ( $P < 0.0001$ ). 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- $\alpha$  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.



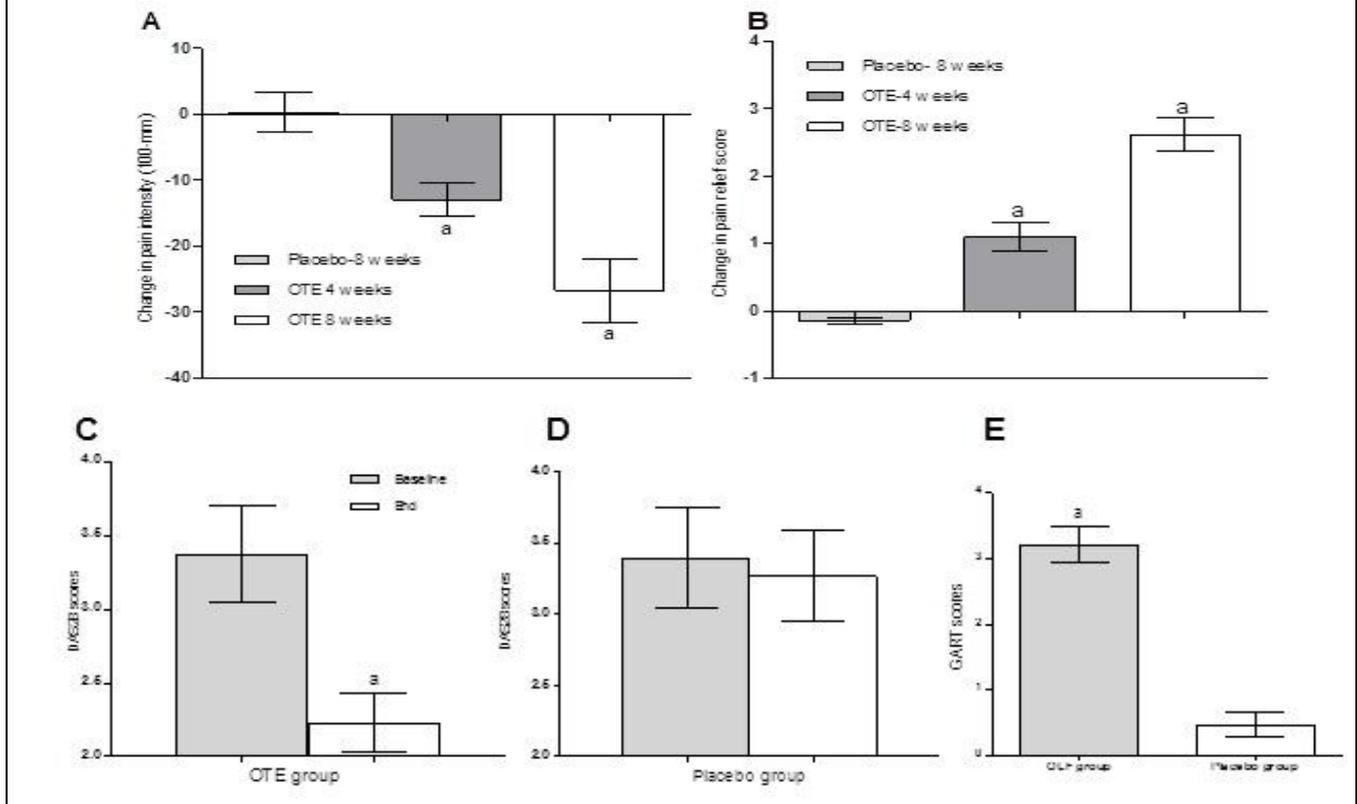
**Figure 3. Change from baseline in circulating inflammatory biomarkers level in the tow study groups, (a) hs-CRP, (b) IL-6, (c) TNF- $\alpha$ , (d) IL-1, (e) PGE<sub>2</sub>, and (f) LTB<sub>4</sub> (a). Error bars are 95% CIs. <sup>a</sup> $P<0.0001$ , <sup>b</sup> $P=0.014$ , <sup>c</sup> $P=0.009$ , <sup>d</sup> $P=0.0247$ , <sup>e</sup> $P=0.0004$ , and <sup>f</sup> $P=0.0017$  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  $-12.94 \pm 4.970$  (CI,  $-15.50$  to  $-10.39$ ) after 4 weeks, and  $-26.71 \pm 9.29$  (CI,  $-31.49$  to  $-21.93$ ) at the end of the trial. Thus, the pain intensity (100-mm pain scale) decrease from  $75.51 \pm 9.81$  to  $48.80 \pm 4.16$  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  $2.61 \pm 0.48$  (CI,  $2.37$  to  $2.37$ ), which correspond to an average value of  $3.26 \pm 0.66$  (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  $\geq 3$ ), whereas others OTE-group members perceived a meaningful pain relief (pain relief score  $\geq 2$ ) 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  $\geq 3.2$ ) 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  $2.23 \pm 0.40$ , signaling a RA remission ( $\leq 2.6$ ). 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  $3.206 \pm 0.53$  (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. <sup>a</sup>*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)	<i>P</i> value <sup>a</sup>
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 <sup>2</sup> )	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.

<sup>a</sup> *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  $\pm$  standard deviation**

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

PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids. <sup>a</sup> 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- $\alpha$ , 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- $\alpha$ , IL-6, IL-1 and other inflammation mediators such as eicosanoids (especially PGE<sub>2</sub> and LTB<sub>4</sub>) (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- $\alpha$  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- $\alpha$ , IL-6, hs-CRP, PGE<sub>2</sub>, and LTB<sub>4</sub> (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<sub>2</sub> from arachidonic acid during inflammation process) and, therefore, PGE<sub>2</sub> level in isolated human monocytes [22, 33, 34] and murine macrophages [21]. Similar effect on COX-2 and PGE<sub>2</sub> 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<sub>4</sub>, TNF- $\alpha$ , 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<sub>2</sub>, LTB<sub>4</sub>, TNF- $\alpha$ , 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<sub>2</sub> 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 NSAID drugs. Nevertheless, NSAIDs (celecoxib, rofecoxib, diclofenac) increased TNF- $\alpha$  production in rheumatoid synovial membrane cultures [33-40], while our results indicate a significant decrease in plasma TNF- $\alpha$ . 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- $\kappa$ B has been previously elucidated by several authors. NF- $\kappa$ B 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- $\alpha$ , IL-1, and IL-6 herein studied. Hydroxytyrosol from aqueous olive extract inhibit the expression of NF- $\kappa$ Bp65, and the authors suggest that this inhibitor effect may be the cause of cytokines reduction in murine macrophages [21]. Furthermore, hydroxytyrosol suppressed NF- $\kappa$ B expression in human monocyte (THP-1) and altered its translocation into the nucleus [42]. Beside this, hydroxytyrosol decreases NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B (IL-1 $\beta$  and TNF- $\alpha$ ) 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<sub>2</sub> 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<sub>2</sub> 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- $\alpha$ ) dependent NF- $\kappa$ B 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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## **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 HIF1 $\alpha$  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](http://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 middle 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  $\pm$  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 $\pm$ 7.8
Mineral matter	12.9 $\pm$ 0.7
Fat	< 1
Total polyphenols	7.90 $\pm$ 1.9
Hydroxytyrosol	2.09 $\pm$ 0.14
ORAC ( $\mu$ mol Te kg)*	3848100 $\pm$ 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<sub>2</sub> atmosphere at 37°C. Phenolic compounds (used as reference molecules in this study): oleuropein, hydroxytyrosol, tyrosol, caffeic 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<sub>2</sub> atmosphere, cells were treated with increasing concentrations of OTE (diluted in H<sub>2</sub>O) 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<sup>®</sup> AQueous 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  $[(1-(OD490 \text{ treated}/OD490 \text{ control})) \times 100]$ .

## **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<sub>2</sub> to olive tree extract at different concentrations. 50nM doxorubicin was used as positive control. Microplates were centrifuged to collect floating cells. Adherent cells were trypsinized, 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 ( $20 \cdot 10^3$  cells in 180 µl of complete medium per well) were plated in a 96-well microplate under 5% CO<sub>2</sub> 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 ( $20 \cdot 10^3$  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<sub>2</sub>O) 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  $\mu$ l 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  $\mu$ l of PBS ( $\lambda_{\text{ex}}$  485 nm,  $\lambda_{\text{em}}$  530 nm). The positive control was untreated HT29 cells incubated in PBS containing 1 mM of H<sub>2</sub>O<sub>2</sub>.

### **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 30 $\mu$ l of this mix were transferred into a 96-well microplate. The plate was incubated at 37°C under 5% CO<sub>2</sub> atmosphere for 60 min to form the gel. HSkMEC cells (50.10<sup>3</sup> cells/well) were seeded in 200 $\mu$ l medium containing increasing concentrations of olive tree extract and phenolic compounds (100 $\mu$ M). 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-1 $\alpha$ ), 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.

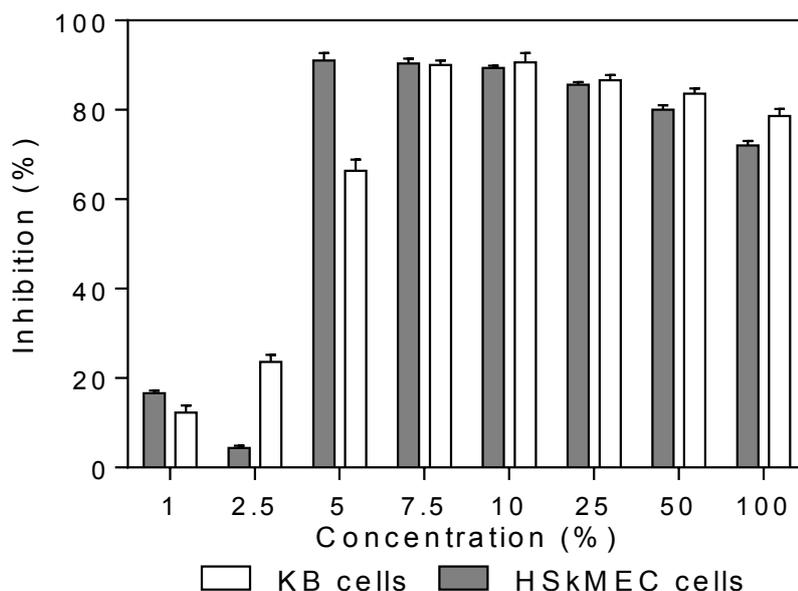
### **Statistical analysis**

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 1 $\mu$ l OTE into 100 $\mu$ l of medium, whereas the lowest concentration 1 indicates that the pure OTE was diluted 1 to 100 and used at a final concentration of 1 $\mu$ l diluted OTE into 100 $\mu$ l 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  $\pm$  SD of experiments performed in triplicate and expressed as a percentage of inhibition compared to cells treated with DMSO only.

Compound	Concentration		
	100 $\mu$ M	10 $\mu$ M	1 $\mu$ M
Caffeic acid	0 $\pm$ 10	3 $\pm$ 5	6 $\pm$ 6
Apigenin	83 $\pm$ 1	30 $\pm$ 5	5 $\pm$ 1
Luteolin	81 $\pm$ 1	22 $\pm$ 6	4 $\pm$ 2
Oleuropein	0 $\pm$ 9	0 $\pm$ 1	0 $\pm$ 10
Tyrosol	0 $\pm$ 6	5 $\pm$ 3	0 $\pm$ 8
Hydroxytyrosol	11 $\pm$ 11	1 $\pm$ 10	0 $\pm$ 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 1 $\mu$ M. 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

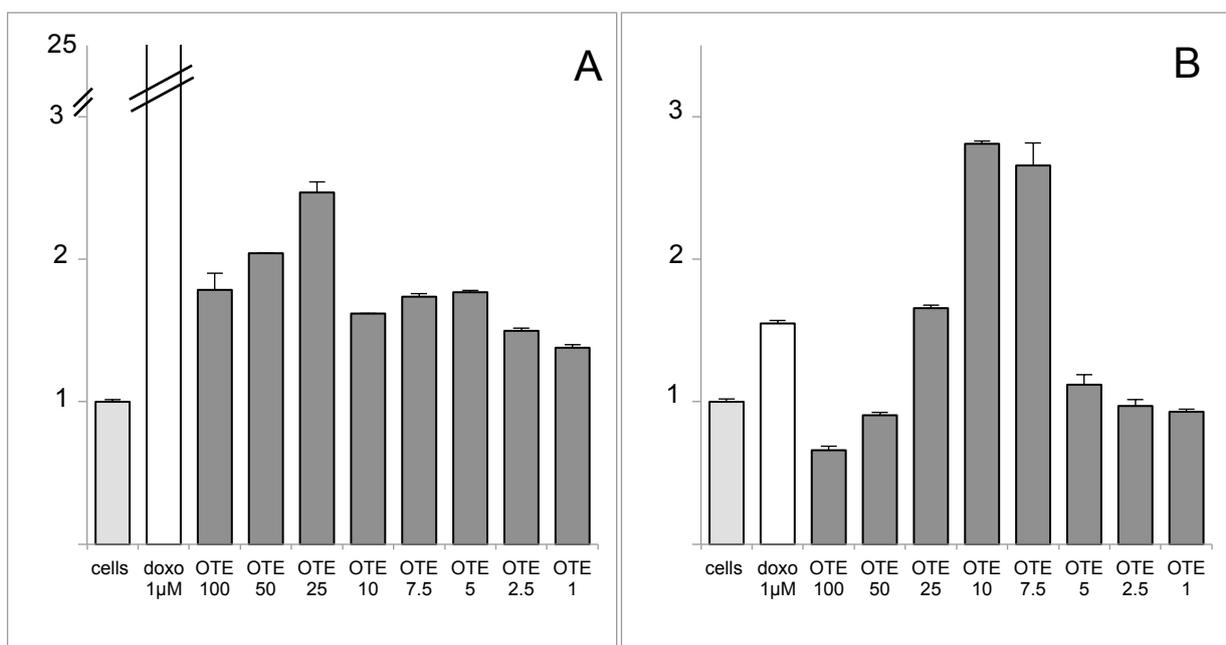
Apoptosis and necrosis are two distinct and redundant forms of cell death occurring in response to chemical aggression. These two cellular processes featured different and complementary modes of action: apoptosis (or programmed cell death) is a tightly regulated cell death program that plays a pivotal 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.<sup>[33]</sup> 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, 1 $\mu$ M 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.



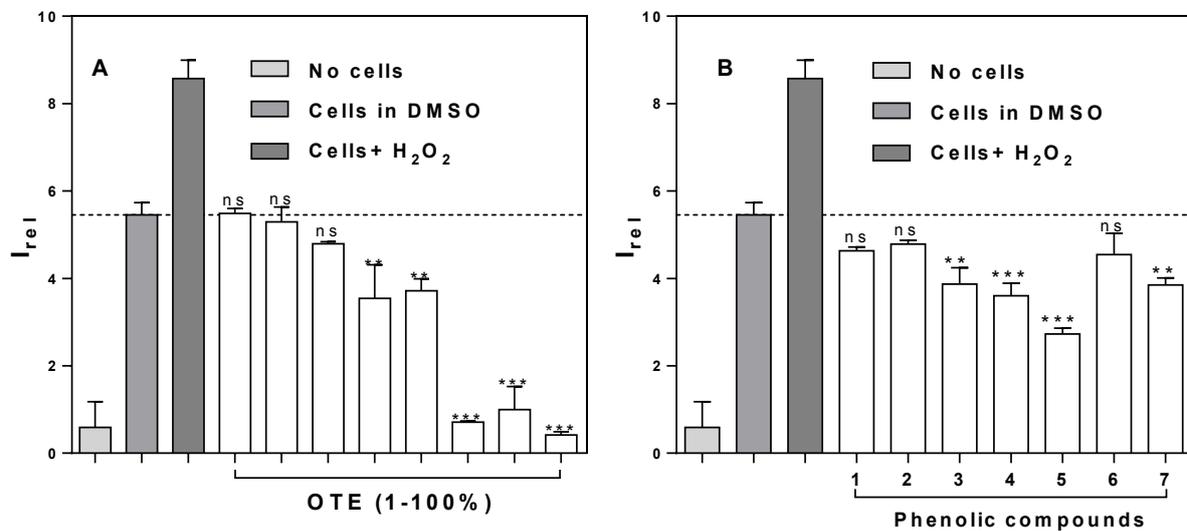
**Figure 2.** Activation of caspase-3 (A) and release of LDH (B) from KB cells after 48h of treatment with olive tree extract, vehicle only (cells) and doxorubicin (1 $\mu$ M).

Results are expressed as the relative activity in duplicate experiments  $\pm$  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'-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, the non-fluorescent DCFH-DA diffuses spontaneously into cells and is deacetylated by cellular esterase to 2',7'-Dichlorodihydrofluorescein (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.

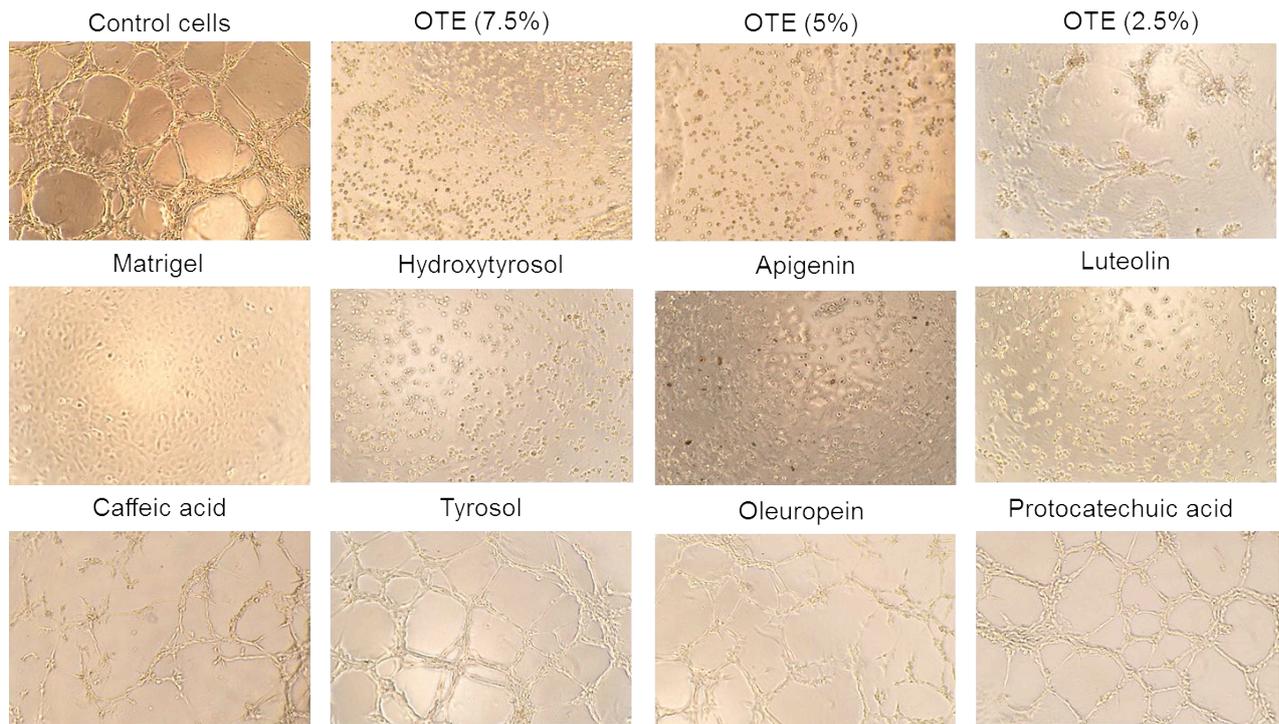


**Figure 3.** Intracellular ROS production after incubation of HT29 cells for 24h with (A) increasing concentrations of olive tree extract and (B) pure phenol compounds (100  $\mu$ M). Results are the mean  $\pm$  SD of 3 individual assays. \*\*\* ( $p < 0.001$ ), \*\* ( $p < 0.01$ ) \* ( $p < 0.05$ ); 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) caffeic acid.

In the presence of 1mM H<sub>2</sub>O<sub>2</sub>, 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<sub>2</sub> in the presence of increasing concentrations of olive tree extract and phenolic compounds (100 µM) are presented in figure 4.



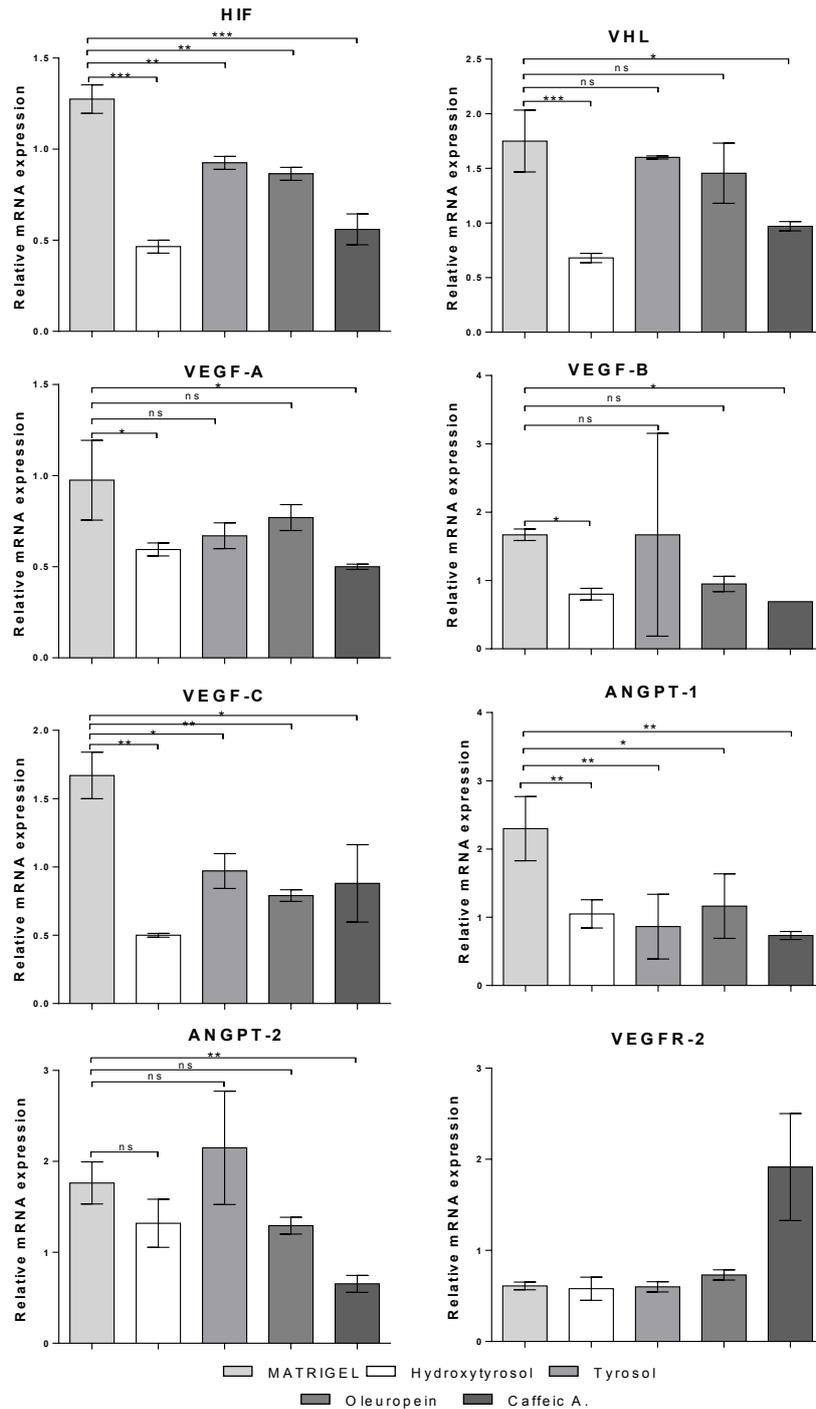
**Figure 4.** Representative photographs of the network structure formed by endothelial cells (HSkMEC) on Matrigel in the presence of increasing concentrations of olive tree extract and pure phenolic compounds used at 100  $\mu$ M. Control cells are incubated without Matrigel.

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 100 $\mu$ M 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 chemotactic 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-1 $\alpha$  to allow its degradation by the proteasome. Its expression is regulated by HIF-1 $\alpha$  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-1 $\alpha$ ; VHL protein of Von Hippel-Lindau; VEGF (A, B and C), Vascular Endothelial Growth Factor; ANGPT (1 and 2), Angiopietin; VEGFR-2, cell receptor for VEGF proteins. Values are expressed as mean  $\pm$  SD (n = 3). \*\*\* (P <0.001), \*\* (P <0.01) \* (P <0.05); ns, not significant difference.

**Table 4.** Summary 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-1 $\alpha$  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 ( $50 \mu\text{g}\cdot\text{ml}^{-1}$ ) and oleuropein ( $200 \mu\text{g}\cdot\text{ml}^{-1}$ ) 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- $\alpha$  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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## **CLINICAL STUDY N°3**

**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 ( $4.1 \pm 0.14$ ) compared to untreated group ( $7.64 \pm 0.36$ ), which was quite similar to normal rats ( $4.50 \pm 0.36$ ). 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 europaea*) 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](http://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,  $22 \pm 2$  °C; humidity,  $40 \pm 5\%$ ; 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]:

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

### 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  $P < 0.05$ .

## 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  $0.9 \pm 0.11$  and  $4.5 \pm 0.62$  g/l, and  $1.1 \pm 0.10$  and  $5.23 \pm 0.66$  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 ( $\sim 1$  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.

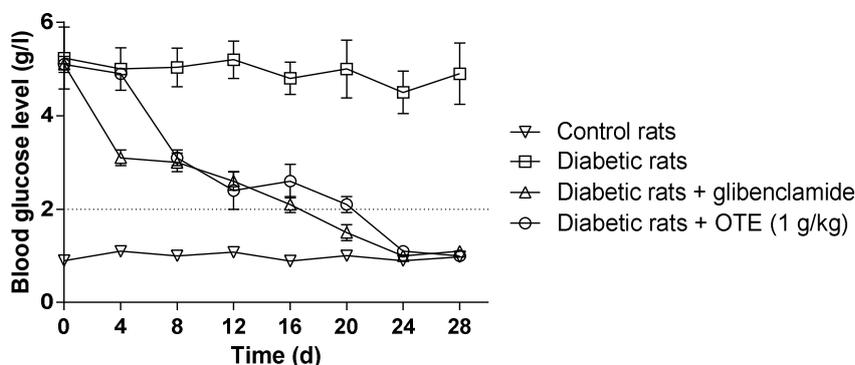


Fig. 1: Effect of OTE on blood glucose levels in diabetic and non-diabetic rats after 4 w of daily admission ( $n=10$ )

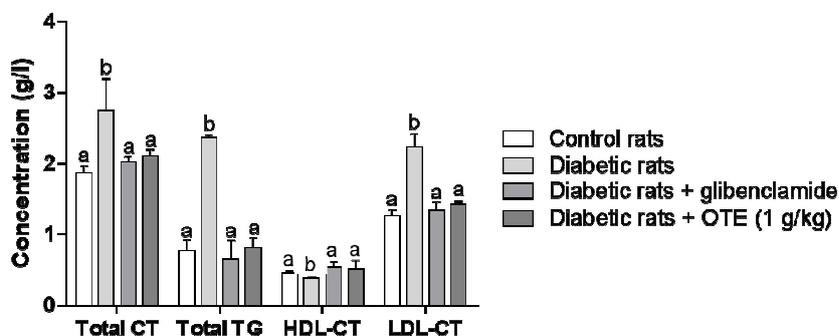


Fig. 2: Effect of OTE on total cholesterol (CT), triglycerides (TG), high-density lipoproteins cholesterol (HDL-CT) and low lipoproteins cholesterol (LD-CT) in diabetic and normal rats after 4 w of daily admission ( $n=10$ ). Different letters  $a-b$  indicate significant differences ( $p < 0.05$ )

The analytical values of TC ( $2.75 \pm 0.44$  g/l), TG ( $2.36 \pm 0.03$  g/l) and LDL-CT ( $2.23 \pm 0.18$  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  $2.1 \pm 0.01$ ,  $0.81 \pm 0.14$  and  $1.43 \pm 0.04$  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 ( $0.51 \pm 0.12$  g/l) compared to control rats ( $0.45 \pm 0.04$  g/l) or diabetic rats treated by glibenclamide ( $0.54 \pm 0.07$  g/l).

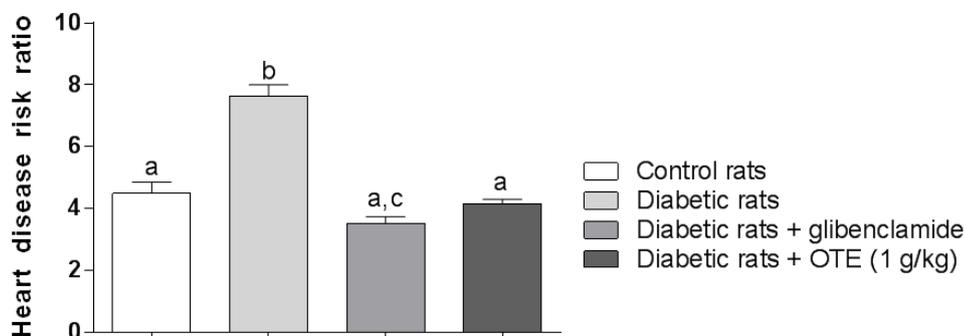


Fig. 3: Heart disease risk ratio in diabetic and normal rats after 4 w of OTE daily admission (n=10). Different letters <sup>a-b</sup> indicate significant differences ( $p < 0.05$ )

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 ( $4.1 \pm 0.14$ ) and glibenclamide ( $3.53 \pm 0.21$ ) compared to untreated diabetic rats ( $7.64 \pm 0.36$ ). 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 ( $4.50 \pm 0.36$ ).

#### 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)	$6 \pm 1^a$	$11 \pm 1.73^b$	$6 \pm 1^a$	$6 \pm 1^a$
Urea (g/l)	$0.28 \pm 0.03^a$	$0.45 \pm 0.06^b$	$0.22 \pm 0.05^c$	$0.29 \pm 0.03^a$
Total proteins (g/l)	$74 \pm 4.36^a$	$59 \pm 3.46^b$	$77 \pm 3.60^a$	$76 \pm 2^a$
Uric acid (mg/l)	$40 \pm 2^a$	$72 \pm 1^b$	$44 \pm 3.60^a$	$45 \pm 4^a$

<sup>a-c</sup>Values in the same row for each rats group with different superscripts are significantly different ( $p < 0.05$ ) [mean $\pm$ 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 <sup>-1</sup> )
AST (UI/l)	$22 \pm 2.64^a$	$86 \pm 1.73^b$	$31 \pm 4^c$	$36 \pm 2.64^c$
ALT (UI/l)	$28 \pm 2.28^a$	$90 \pm 5.56^b$	$35 \pm 4.58^a$	$40 \pm 3.60^a$

<sup>a-c</sup>Values in the same row for each rats group with different superscripts are significantly different ( $p < 0.05$ ) [mean $\pm$ 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  $\alpha$ -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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## **CLINICAL STUDY N°4**

**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*

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**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 lipoxygenase (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- $\kappa$ B) and activator protein-1 (AP-1) transcription factors and affecting cellular signaling processes [8]. NF- $\kappa$ B activation is associated with cancer progression. Thereby, ROS, COX-1/2 and NF- $\kappa$ B 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 an 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- $\kappa$ B, and AP-1; and down-regulation of various pro-inflammatory cytokines such as chemokines, tumor necrosis factor alpha (TNF- $\alpha$ ), interleukins including (IL-1 b, IL-6, IL-8), and monocyte chemoattractant 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](http://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 $\pm$ 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:

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

#### 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:

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

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:

$$\% \text{ increase in reaction time} = \frac{T_t - T_0}{T_0} \times 100$$

### 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 3<sup>rd</sup> hour (table 1). The orally administration of the OTE showed a dose-dependent reduction in carrageenan-induced paw oedema from the 3<sup>rd</sup> to the 6<sup>th</sup> 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**

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

<sup>a-e</sup> 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**

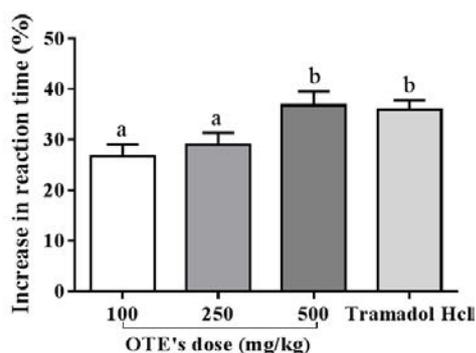
Dose (mg/kg)	Inhibition (%)			
	3h	4h	5h	6h
50	<sup>a</sup> 0.13±0.03	<sup>a</sup> 8.90±0.55	<sup>a</sup> 18.96±0.59	<sup>a</sup> 0.26±0.04
100	<sup>b</sup> 25.00±2.43	<sup>b</sup> 52.00±1.54	<sup>b</sup> 60.50±1.35	<sup>b</sup> 12.00±1.67
250	<sup>c</sup> 51.00±2.39	<sup>c</sup> 70.90±1.67	<sup>c</sup>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

<sup>a-f</sup> 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

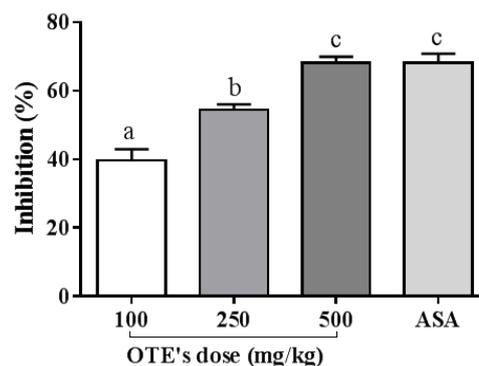
Graphs of fig. 1, 2 and 3 show results related to the OTE's analgesic activity assessed by means of the hot plate, acetic acid-induced abdominal writhing and formalin *in vivo* tests. Considering the results of anti-inflammatory tests, analgesic tests were performed at three OTE's doses (100, 250 and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

OTE administration has increased the insensibility to pain without loss of consciousness (analgesic activity) in mice placed onto the hot plate ( $55 \pm 2$  °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  $p < 0.05$ ).

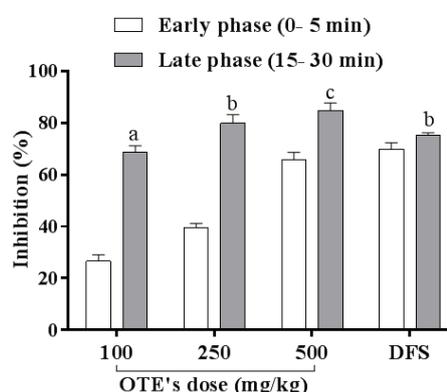


**Fig. 1: Effect of olive tree extracted on hot plate-induced pain in mice. Data is expressed as means±SD [n= 5]. Different letters indicate significant differences ( $p < 0.05$ )**

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-oedematogenic 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- $\alpha$ , 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- $\kappa$ B, 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- $\kappa$ B. The action of hydroxytyrosol on this factor blocks COX-(2) and 5-lipoxygenase 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- $\alpha$  and IL-1 $\beta$ ) [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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## **CLINICAL STUDY N°5**

## **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  $\mu\text{mol Te/kg}$ . 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* DH5 $\alpha$ , *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 material 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 ; Martínez-Domínguez E., 2001), anti-diabetic (Hamden K., 2009), antitumor, anti-proliferative (Bouallagui Z., 2011; Fabiani R., 2002) and anti-atherogenic activities (Covas M.I., 2006). However, 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* DH5 $\alpha$ , *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus cereus* MED5 and *Streptococcus agalactiae* and fungus: *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 ketoconazole (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 20°C. 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<sub>3</sub>) 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. After 15 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 Hrnčirik and Fritsche (2004) was used to separate the mixture on reverse 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:

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

Where  $A_{\text{control}}$  is the absorbance of the control at  $t = 0$  min, and  $A_{\text{sample}}$  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* DH5 $\alpha$ , *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus cereus* MED5 and *Streptococcus agalactiae* and two fungus *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 fungus. 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<sup>6</sup> 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 ketoconazole (15  $\mu$ g) were used for antifungal activity.

### **Statistical Analysis**

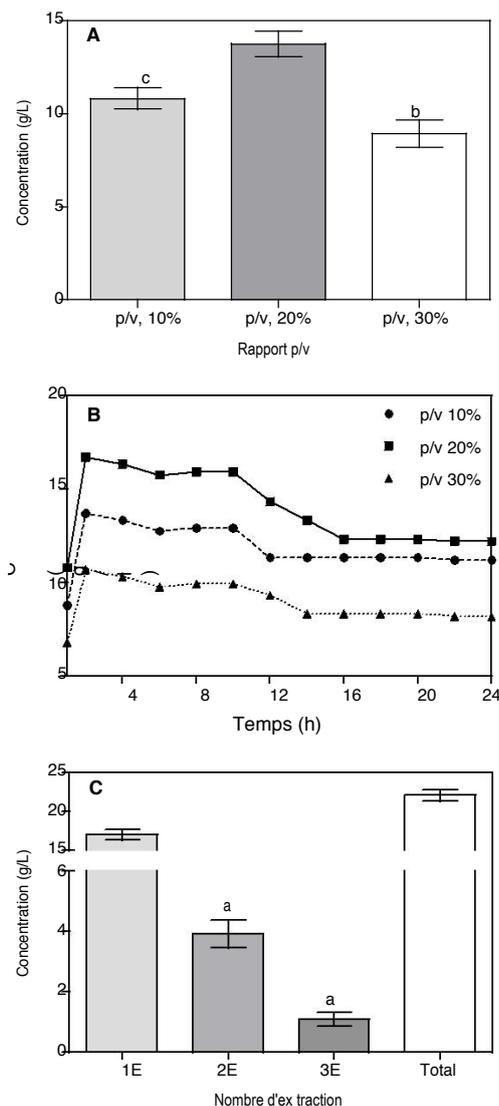
Statistical analyzes were performed using GraphPad Prism software version 6.00 (GraphPad Inc., San Diego, California). Data were analyzed by analysis of variance (ANOVA Analysis of Variance) followed by post-hoc Dunnet test if the sample distribution follows normal distribution or by the Kruskal-Wallis 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  $\pm$  standard deviation. The letters indicate the significance of the difference compared to controls, <sup>a</sup>  $P < 0.001$ , <sup>b</sup>  $P < 0.01$  and <sup>c</sup>  $P < 0.05$ .



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, therefore, 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  $\pm$  standard deviation

Biophenol content	Concentration
Dry matter (g/100g of powder)	97.96 $\pm$ 7.83
Mineral matter (g/100g of powder)	12.9 $\pm$ 0.7
Fat (g/100g of powder)	< 1
Total phenolic content (g GAE/l)	159.8 $\pm$ 1.9
Flavonoids (g QE/l)	8.4 $\pm$ 0.6
o-Diphenols (g CAE/l)	39.11 $\pm$ 0.9
Hydroxytyrosol (g GAE/l)	2.09 $\pm$ 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  $\pm$  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 Féulique	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  $3848100 \pm 38481 \mu\text{mol Te/kg}$  (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 (µg/disc)						
	1	2	3	4	Am/Cl (20/10)	Ch (30)	Fl (5)	Ka (30)	Ge (10)	Am (10)	Ke (15)
<b>Bacteria</b>											
<i>E. coli</i>	15.00 (1.00)	20.33 (1.52)	25.33 (1.52)	29.67 (1.52)	<sup>a</sup> 11.66 (0.52)	<sup>a</sup> 11.66 (0.53)	<sup>a</sup> 18.00 (0.00)	<sup>a</sup> 20.00 (0.00)	<sup>a</sup> 11.69 (0.53)	-	-
<i>E. coli</i> TGI	14.00 (1.00)	19.67 (0.53)	25.00 (0.00)	30.33 (0.53)	<sup>a</sup> 19.64 (0.57)	<sup>a</sup> 14.67 (0.53)	<sup>a</sup> 24.66 (0.57)	<sup>a</sup> 23.33 (0.53)	<sup>a</sup> 17.00 (0.00)	-	-
<i>E. coli</i> DHSa	15.33 (0.57)	20.66 (0.57)	26.66 (0.57)	30.33 (0.53)	<sup>a</sup> 23.00 (0.00)	<sup>a</sup> 18.00 (0.00)	<sup>a</sup> 25.67 (0.57)	<sup>a</sup> 25.67 (0.77)	<sup>a</sup> 17.33 (0.53)	-	-
<i>S. aureus</i>	10.33 (0.57)	13.67 (0.57)	17.33 (0.57)	20.66 (0.57)	<sup>a</sup> 17.60 (0.57)	<sup>a</sup> 17.33 (0.53)	24.00 (0.00)	<sup>ns</sup> 21.67 (0.55)	<sup>a</sup> 17.00 (1.00)	-	-
<i>B. cereus</i>	14.67 (0.55)	16.67 (0.58)	22.00 (0.00)	30.33 (0.57)	<sup>a</sup> 13.63 (0.51)	<sup>a</sup> 10.00 (0.00)	<sup>a</sup> 22.33 (0.53)	<sup>a</sup> 21.67 (0.53)	<sup>a</sup> 13.66 (0.57)	-	-
<i>B. cereus</i> MED5	16.67 (0.53)	20.63 (0.77)	25.67 (0.53)	31.00 (0.00)	<sup>a</sup> 13.00 (0.00)	<sup>a</sup> 9.33 (1.15)	<sup>a</sup> 24.33 (0.50)	<sup>a</sup> 20.00 (0.00)	<sup>a</sup> 15.00 (0.00)	-	-
<i>S. agalactiae</i>	18.00 (0.43)	20.33 (0.57)	21.33 (0.58)	22.33 (0.57)	<sup>a</sup> 18.00 (0.00)	<sup>a</sup> 12.00 (0.00)	24.00 (0.00)	23.66 (0.57)	20.33 (0.55)	-	-
<b>Fungi</b>											
<i>A. niger</i>	10.67 (0.53)	11.77 (0.55)	13.67 (1.15)	16.68 (1.51)	-	-	-	-	-	18.00 (0.00)	<sup>ns</sup> 16.66 (0.53)
<i>C. albicans</i>	10.33 (0.57)	12.00 (0.21)	16.67 (1.52)	19.71 (1.52)	-	-	-	-	-	<sup>a</sup> 14.67 (0.57)	<sup>ns</sup> 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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## **CLINICAL STUDY N°6**

# 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  $51.5 \pm 9.4$  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 ( $114.2 \pm 15.2$  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  $\leq 3$  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 Declaration and the protocol was approved by the local ethics committee of the University 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 through 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](http://www.olivie.ma)).

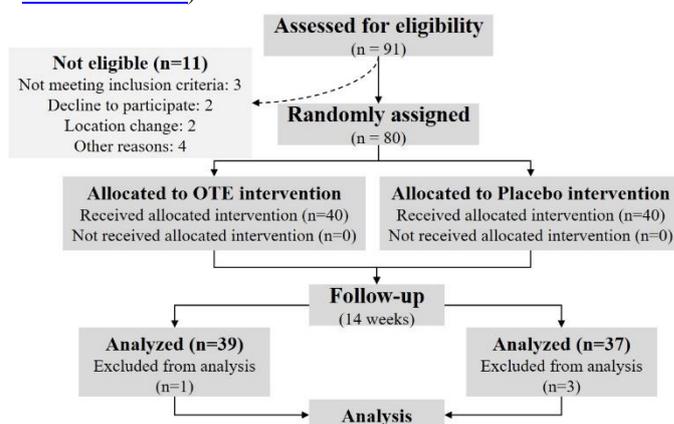


Figure 1: Study flow diagram

## 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 plasma glucose (mg/dl) was assayed by the glucose oxidase method (Beckman Glucose Analyzer). The following parameters were measured: HbA<sub>1c</sub> (%), TC (mg/dl), HDL cholesterol (HDL-c) (mg/dl), LDL cholesterol (LDL-c) (mg/dl), TGs (mg/dl), hemoglobin (g/dl), hematocrit (%) and erythrocytes (mil./mm<sup>3</sup>). TC, VLDL and TG were measured using enzymatic tests in a contract clinical laboratory. LDL-c levels were calculated by the Friedewald equation, HDL-c was measured by using the heparin-manganese precipitation method. High-sensitivity enzyme-linked immunosorbent assay kits (DiaSource, Belgium) were used to quantify serum levels of insulin according 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]:  $\text{fasting plasma glucose (mg/dL)} \times \text{fasting serum insulin } (\mu\text{U/mL}) / 405$ . 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).

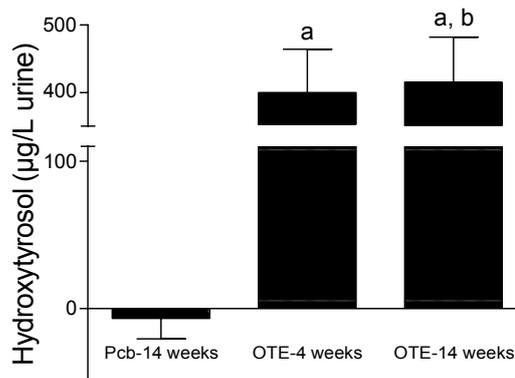
### 2.4 Statistical analysis

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 2 graph 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. <sup>a</sup> $P < 0.0001$ , between OTE-group (at 4 or 14 weeks) and placebo-group (Pcb); <sup>b</sup> $P = 0.003$ , 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 <sup>a</sup>
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 <sup>2</sup> )	30.5 ± 5.1	29.8 ± 4.7	0.175
BMI >30 (Kg/m <sup>2</sup> ), n (%)	28 (70)	24 (60)	0.632
BMI <25 (Kg/m <sup>2</sup> ), 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 <sub>1c</sub> (%)	7.79 (0.8)	7.46 (1.1)	0.663
HbA <sub>1c</sub> 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. <sup>a</sup> P value (<0.05) by independent t-test or Mann-Whitney test. **BP:** Blood Pressure; **BMI:** Body Mass Index; **HbA<sub>1c</sub>:** 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 of T2DM 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  $\pm$  standard deviation.

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

PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.<sup>a</sup> 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  $51.5 \pm 9.4$  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  $114.2 \pm 15.2$  mg/dL at the end the intervention, which is significantly below normal level defined by the ADA (13).

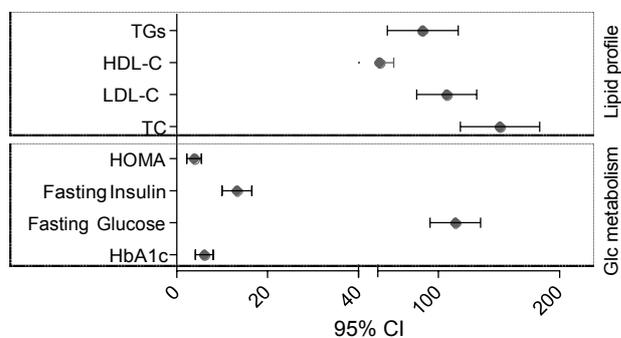
The fasting insulin levels increased over time for the treated group (even the difference still no significant compared to the placebo group  $p = 0.251$ ), suggesting an improve in

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-month follow-up examinations. See legend of Table 1 for the abbreviations.

Variable	Intervention group (n=39)		Placebo group (n=37)		P value
	14-weeks	$\Delta$ study end	14-weeks	$\Delta$ study end	
Weight (Kg)	86.3 $\pm$ 3.8	$\downarrow$ 2.5	87.3 $\pm$ 3.4	$\uparrow$ 1.2	0.593
BMI (Kg/m <sup>2</sup> )	28.1 $\pm$ 4.6	$\downarrow$ 2.4	30.8 $\pm$ 3.9	$\uparrow$ 1	0.332
HbA1c (%)	6.08 $\pm$ 1.2	$\downarrow$ 1.3	8.6 $\pm$ 1.3	$\uparrow$ 1.04	<0.0001
Glucose(mg/dL)	111.2 $\pm$ 15.2	$\downarrow$ 55.7	172.7 $\pm$ 17.1	$\uparrow$ 10.3	<0.0001
Insulin ( $\mu$ U/mL)	14.5 $\pm$ 2.5	$\uparrow$ 1.4	13.6 $\pm$ 3	$\downarrow$ 0.5	0.251
HOMA-IR	3.9 $\pm$ 1.2	$\downarrow$ 1.4	5.8 $\pm$ 2.0	$\uparrow$ 0.1	0.0002
Total cholesterol (mg/dL)	150.9 $\pm$ 26.4	$\downarrow$ 50.8	234.8 $\pm$ 37.3	$\uparrow$ 35.5	<0.0001
LDL-C (mg/dL)	106.9 $\pm$ 20.1	$\downarrow$ 20.8	150.9 $\pm$ 26.4	$\uparrow$ 17	<0.0001
HDL-C (mg/dL)	51.5 $\pm$ 9.4	$\uparrow$ 5.6	41.7 $\pm$ 11	$\downarrow$ 1.9	0.007
TGs (mg/dL)	87.1 $\pm$ 11.2	$\downarrow$ 44.4	148.8 $\pm$ 19.4	$\uparrow$ 21.7	<0.0001
GAST	3.4 $\pm$ 0.6		2.2 $\pm$ 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% CI of 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 GAST questionnaire (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.

T2DM is a group of metabolic disorders characterized by insulin resistance and 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) modulate carbohydrate 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 in animal models [22, 23, 24, 25]. Furthermore, diabetic rats consuming 0.5 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 ( $\leq 7\%$ ). Here, OTE supplementation for 14 weeks was associated with a reduction of HbA1c by  $\approx 22\%$  (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 mg for 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 glycosylated plasma 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 oil with 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 and  $\alpha$ -glucosidase [34, 35]. Actually, our unpublished data show the same effect of the studied olive tree extract (rich in hydroxytyrosol) on  $\alpha$ -glucosidase and  $\alpha$ -mannosidase. On the other hand, polyphenols can act as direct suppressors of the proteins involved in the intestinal transport of dietary carbohydrate [36]. This would result 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 glucose-induced insulin secretion following oral glucose challenge in human subjects [28], and protect insulin-secreting  $\beta$ -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 hypoglycemic effect, mainly by i) improving glucose-induced insulin secretion, and ii) increasing peripheral glucose uptake. Further research should 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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## **CLINICAL STUDY N°7**

**Supplementation with a rich-polyphenols olive tree powder reduces circulating inflammatory markers, disease activity, and pain intensity in patients with rheumatoid arthritis: a 9-weeks randomized, double-blind, placebo-controlled clinical trial**

**Ghanam Jamal and Benlemlih Mohammed**

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**Abstract**

*Background-* Notwithstanding the way that olive fruits polyphenols and olive leaf polyphenols have been known for their natural anti-inflammatory effect in the Mediterranean countries, there is little deep scientific study to confirm these benefits.

*Objective-* To assess the effect of rich-polyphenols olive tree powder (made of leaves, baby leaves, olive fruit and olive oil) on inflammatory process and pain intensity, a randomized, double-blinded, placebo-controlled trial was conducted on human subjects with rheumatoid arthritis (RA).

*Methods-* Seventy-nine RA patients were randomized to get either 1 g per day of olive tree powder or 1 g of placebo powder during 9 weeks. Laboratory analysis, questionnaires administration, pain intensity, disease activity score, and inflammatory biomarkers were determined at the baseline and at the end of the trial. Specialists have monitored eventual side effects and antagonistic impacts of taking the olive tree powder through the period of the study.

*Results-* Good compliance (over 95%) with the treatment was observed, without any side effect or study-intervention adverse. Significant decrease in disease activity score has shown at the end of intervention within the treated group, and between groups ( $P < 0.0001$ ). Compared with the placebo group, inflammatory biomarkers decreased significantly in treated participants ( $P < 0.0001$ ). Here are the changes noticed from baseline in treated group were -1.25 mg/L (CI, -1.75 to -0.75), -2.09 pg/mL (CI, -2.63 to -1.54), -0.82 pg/mL (CI, -1.14 to -0.49) and -1769 pg/mL (CI, -2254 to -1283) for hs-CRP, IL-6, TNF- $\alpha$  and PGE2 respectively. Additionally, it is important to note that pain

relief and global participants satisfaction increased significantly ( $P<0.0001$ ) after 9 weeks of olive tree powder supplementation.

**Conclusion-** A net improvement in circulating inflammatory markers, disease activity, and pain intensity was observed in RA patients allocated to rich-polyphenols olive tree powder food supplement.

**Keywords:** Rheumatoid arthritis; olive tree powder; Inflammatory biomarkers; Pain intensity; Disease activity

## 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 0.7-1% of worldwide population (Prado et al., 2018; Hresko et al., 2018). Its etiology is still not completely understood. However, several risk factors have been previously identified to be associated to RA development. Genetic factors can contribute by 50% to RA development beside other non-modifiable risk factors (presence of RA-related auto-antibodies, rheumatoid factor, and cyclic citrullinated peptide), while modifiable risk factors related to lifestyle (smoking, obesity, low fish intake, and poor dental health) participate by 41% (Nielen et al., 2004; Rantapaa-Dahlqvist et al., 2003).

Nowadays, Blockage of cytokines network –mediators of chronic inflammation– has taken a substantial proportion in the clinical management of inflammatory diseases like RA, which explain the progress of treatments from traditional nonsteroidal anti-inflammatory drugs to disease modifying anti-rheumatic drugs and biological drugs, since 1990s (Hresko et al., 2018). However, these drugs still had some unexpected adverse effects related to immune system impairment (Westra et al., 2014). Meanwhile, researchers have been also focusing on natural products as a source of alternative treatment that can stop the inflammatory flux associated to RA. In this sense, several *in vitro*, *in vivo*, and clinical studies have elucidated the effect of olive tree polyphenols –as principal components of Mediterranean Diet– on the inflammation process that characterizes RA (McKellar et al., 2007; Sköldstam et al., 2003). Thus, adherence to the Mediterranean diet decreased inflammatory activity, increased in physical function, and improved vitality in RA patients. Moreover, several *in vitro* studies have demonstrated the anti-inflammatory effect of hydroxytyrosol, tyrosol and

oleuropein (polyphenols coming olive leafs, baby leafs, olive fruits and olive oil) by acting directly on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, IL-1 and high-sensitivity C-reactive protein (hs-CRP) (Camargo et al., 2014; Richard et al., 2011; Zhang et al., 2009). Here, we should also underline the similar anti-inflammatory effect of oleocanthal and the ibuprofen (drug from NSAIDs family) discovered by Beauchamp et al. (2005).

Although these promising results from *in vitro* and preclinical studies, more data from clinical trials are needed to prove the real anti-inflammatory effect of olive tree polyphenols in pathological cases like RA. Thus, the purpose of the present study was to verify whether supplementation with an olive tree powder rich in polyphenols could improve clinical and laboratory parameters of disease activity in Moroccan patients who have RA.

## **Methods**

### **Study population**

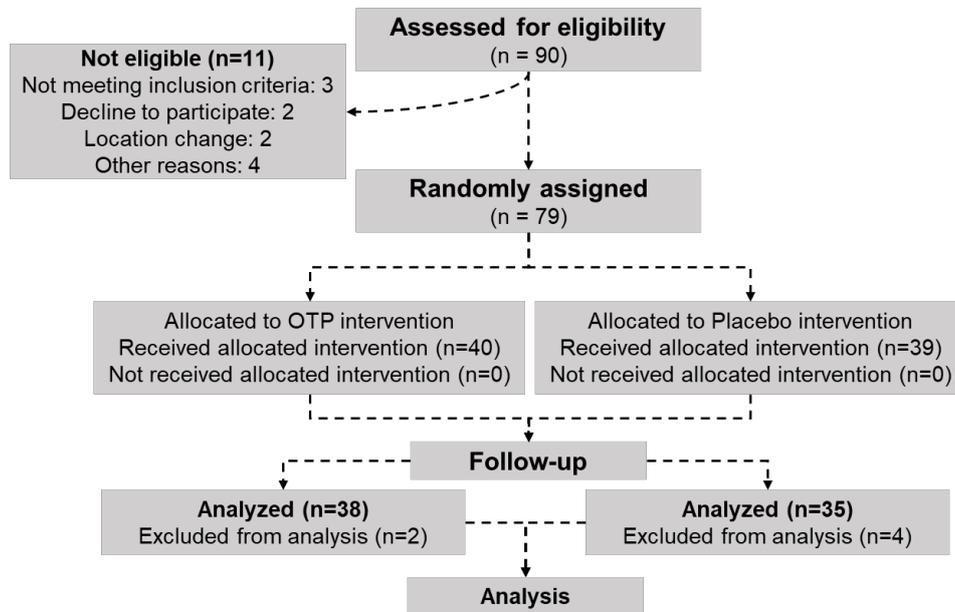
90 patients were recruited during July 2018 among those referred to rheumatology service of clinic ESSEHA in Casablanca, Morocco. In order to be enrolled to this study, patients have to be diagnosed with rheumatoid arthritis for more than one year according to the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) (Aletaha et al., 2010). The content of this study was explained in details to the voluntary participants. Patients that were not eligible were falling under the following categories: if they were pregnant, lactating, receiving contraceptive, smoking, if they were under the age of 20 years or over the age of 80 years, being diagnosed with metabolic syndrome as defined by the Adult Treatment Panel III, having inflammatory disorders, receiving NSAIDs and/or cytokine inhibitors, and having a white blood cell count  $\leq 3.5 \times 10^9/L$ , hemoglobin (Hb) level  $\leq 8.5g/dl$ , platelet count  $\leq 100 \times 10^9/L$ , creatinine level  $\geq 2.0$  mg/dl and aspartate aminotransferase (AST) levels  $\geq 2.5$  times the upper normal limit. Subjects also agreed to avoid consumption of olive antioxidants and any other antioxidant supplements  $\leq 3$  weeks before and during the intervention. The history of allergy or intolerance to olive products was also considered during participants selection. Finally, a written consent was obtained from all voluntary participants before to be officially enrolled to this study.

### **Study design and intervention**

The current study is a double-blind, randomized trial controlled by a placebo (Figure 1). Eligible participants were randomly assigned to be supplemented by the Olive Tree Powder (OTP) or the placebo using a computer-generated random-number sequence. Investigators, participants, and study personal were blinded to the type of supplement used by each group. After an overnight fasting (at least 12h), participants were invited by telephone to the clinic to undergo a screening visit including tender and swollen joints examination. The pre-examination has also included the adherence to the Mediterranean Diet by the modified questionnaire of Estruch et al (2006), the assessment of physical exercise by the International Physical Activity Questionnaire (Physical exercise was categorized as high, moderate, or low). Normal habitual diet of participants has been maintained during the study period whilst avoiding olive tree products intake (olive oil, olive table fruits) as well as nutrients with high n-3 PUFA contents (e.g. fish). The use of all herbs and products known to affect inflammation and immune function had also to be avoid throughout the study period. Dietary changes were monitored trough a 3-day dietary records at baseline, 4 and 9 weeks after treatment and placebo interventions. Study personnel provided necessary explanations about food intake estimations. Anthropometric and blood pressure data were measured and a sample of 8 ml fasting blood was obtained from each participant's antecubital vein. All examinations and measurements have been repeated at the end of the study. Participants and all study personnel (including investigators) had free and continuous access to clinic services for advices and consultations during this study.

Illegible participants have received 500 mg HPMC study capsules (the capsules for supplement and the capsules for the placebo were both the same, HPMC vegetal capsules of 500 mg). Participants received instructions about capsules taking (and storage) and were asked to administrate two capsules per day (equivalent to a dose of  $1 \text{ g}\cdot\text{day}^{-1}$ ) of either OTP or placebo (maltodextrin excipient) before each meal. Supplement and placebo intake was weekly controlled. Both OTP and placebo components were enclosed in a soft soluble HPMC vegetal capsules. OTP was obtained from olive leafs, olive baby leafs, olive fruits and olive oil using a purely physical extraction without the use of: solvents, purification process, or any chemicals. The temperatures implied in the process were cold (freeze drying) to preserve all the polyphenols potency. It is worth to note that this olive tree powder comes from olive trees planted in a specific rocky desert environment where temperatures attain  $127 \text{ }^{\circ}\text{F}$

in the summer, where it nearly never rains and where only little water in the dwells allow the tree survival. Also, the massive quantity of rocks do not allow olive tree roots to look for nutrients. These specific olive trees are under stress and produce abnormally high quantity of antioxidants (mainly hydroxytyrosol) to defend themselves and survive. For more details, please see [www.olivie.ma](http://www.olivie.ma).



**Figure 1.** Study flow diagram.

### Laboratory measurements and outcomes

Clinical indication of disease activity and laboratory parameters of study participants were measured at the baseline and at the end of study using internal methods of clinic ESSEHA and its associated laboratories. A calibrated scales and wall-mounted stadiometer with a precision of 0.1 cm, and a semi-automatic oscillometer (Boso Medicus smart Semi-automatic Blood Pressure Monitor, Germany) were used to measure anthropometric parameters and blood pressure. Blood samples were collected in EDTA and SST tubes. All erythrocytes, plasma, serum and urine samples were stored as 1 mL aliquots at  $-80^{\circ}\text{C}$  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). Serum quantification of  $\text{PGE}_2$ ,  $\text{LTB}_4$ ,  $\text{TNF-}\alpha$  and cytokines IL-1 and IL-6 was performed using High-sensitivity enzyme-linked immunosorbent assay kits (DIA, Belgium), while the serum's hs-CRP level was determined by a turbidometric assay using a commercial kit at a wavelength of 500 nm.

Urinary hydroxytyrosol content, considered as marker of OTP intake, was measured by High Performance Liquid Chromatography (HPLC). First, hydroxytyrosol was extracted from acidified urine (hydrochloric acid, 0.6 N of final concentration) and then quantified by a Shimadzu chromatograph device equipped with a reverse phase C18 column (250 mm L. × 4.6 mm I.D., 5 µm) according to the protocol described by Visioli et al (2000).

A visual analog scale (VAS) has been considered to evaluate the pain intensity in this study, according to the protocol defined by DeLoach et al (1998). Participants have been instructed to draw a 100-mm line to describe their pain, 0 mm= no pain and 100 mm=most severe pain. Pain relief was assessed using a 5-point verbal rating scale (VRS) as, 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) established by the EULAR (Wells et al., 2009), 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 was calculated as follows:

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

Possible adverse effects of OTP administration through the period of study (mouth symptoms, digestive disorders, fullness, allergic skin response, and other intervention-related symptoms) were assessed by doctors and study personnel. Finally, a 5-point categorical scale (0 = poor, 1 = fair, 2 = good, 3 = very good, and 4 = excellent) was used to assess the global satisfaction assessment in response to treatment (GAST) (including anxiety). This study was conducted according to the guidelines approved by Helsinki Declaration.

### **Statistical analysis**

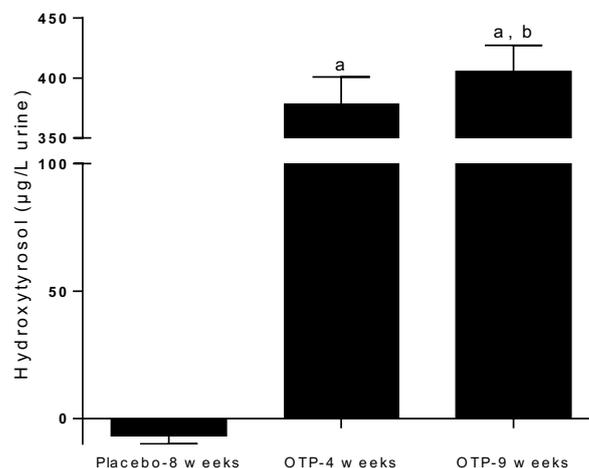
Data were statistically analyzed using GraphPad Prism version 6.00 (GraphPad Prism Inc, San Diego, California). For the baseline characteristics, continuous variables are expressed as mean values ± standard deviation (SD), and categorical variables are expressed as frequencies (percent). For inflammatory 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 χ<sup>2</sup> 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 (9 weeks) between OTP and placebo group. Results with two-sided P values of <0.05 were considered statistically significant.

## Results

### Study compliance and adverse effects

Out of the ninety eligible patients enrolled to this study, 11 were excluded before the intervention for several reasons (Figure 1). Moreover, six participants were dropped out of analysis (2 in OTP-group and 4 in placebo-group) because they were unable to follow the study protocol. No adverse reactions or side effects related to the intervention were observed and 95% compliance to the supplementation was reported. Results of Figure 2 illustrate the changes in urinary Hydroxytyrosol concentrations (used as biomarker of compliance) from initial values for both placebo and OTP groups. The content of hydroxytyrosol found in urine of OTP participant's group was significantly higher ( $P < 0.0001$ ) compared to that of placebo group. However, it is worth noting that literature data regarding olive phenols metabolism, and excretion are not in agreement (Covas et al., 2006; Visioli et al., 2003).



**Figure 2.** Change from baseline in urinary hydroxytyrosol excretion. Mean with 95% CI. <sup>a</sup> $P < 0.0001$ , between OTP-group (at 4 or 8 weeks); <sup>b</sup> $P = 0.003$ , between OTP-group at 4 and 8 weeks.

### Baseline characteristics

The baseline characteristics of the 79<sup>th</sup> participants who were randomized into OTP and placebo groups are shown in table 2. No significant differences have been

revealed between the two study groups for all the baseline parameters. This include the adherence to the Mediterranean diet ( $P=0.296$ ).

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

Parameter	OTP group (n=40)	Placebo group (n=39)	P value <sup>a</sup>
Age (years)	56.73 ± 1.61	54.31 ± 1.97	0.306
Female, n (%)	32 (80)	27 (69.23)	0.935
Weight (kg)	65.43 ± 3.06	63.54 ± 4.34	0.957
BMI (kg/m <sup>2</sup> )	28.17 ± 1.662	27.83 ± 1.815	0.901
Disease duration (years)	7.77 ± 0.42	6.33 ± 0.56	0.356
Medical history of disease, n (%)	15 (37.50)	12 (30.76)	0.142
Family history of disease, n (%)	8 (20)	10 (25.64)	0.708
Exercise activity habits, n (%)	16 (40)	18 (45)	0.822
Alcohol drinking habits, n (%)	2 (5)	3 (7.69)	0.233
15-item Mediterranean diet score	2.04 ± 0.20	1.91 ± 0.15	0.307
DAS28	4.07 ± 0.67	4.40 ± 0.71	0.911
Pain VAS (0–100 mm)	77.11 ± 9.84	75.65 ± 9.12	0.710

Value are expressed as mean ± standard deviation or in percentage.

<sup>a</sup> P value (<0.05) by independent t-test or Mann-Whitney test.

### Food, Energy, and Nutrient Intake

Results of table 3 show that there was no significant difference in diet intake at the baseline and after nine weeks of OTP and placebo supplement. Importantly, PUFAs intake was maintained constant ( $P$  value of 0.711 and 0.802 for OTP and placebo group). Actually, n-3 PUFAs intake may play a substantial role in the resolution of inflammation in RA (Park et al., 2013). Results of Table 3 indicate also a slight increase in participant's weight, which was no significant for both OTP ( $P=0.794$ ) and placebo ( $P=0.906$ ) groups. However, this was suitable for the current study, since the adipose tissue is also an active source of inflammatory cytokines (Lu et al., 2014). In general, all participants have met the daily recommended diet for this study, and the consumption of olive products and any other products known to have anti-inflammatory effects was avoided.

### Inflammatory markers

Inflammation of synovial membrane is believed to be the main cause involved in RA outcomes. High concentration of circulating inflammatory markers, such as cytokines (IL-6, IL-1, TNF- $\alpha$ ) and hs-CRP correlate with propensity to pain, warmth, redness associated to the joint destruction. Changes from baseline in these markers are illustrated if the graphs of Figure 2. Significant decrease in the average hs-CRP of

participants allocated to OTP treatment compared to those in the placebo group ( $P$  value of 0.014 and  $<0.0001$  after 4 and 9 weeks) has been observed. The mean changes from baseline in the hs-CRP levels were  $-0.56$  (CI,  $-0.91$  to  $-0.18$ ) and  $-1.25$  mg/L (CI,  $-1.75$  to  $-0.74$ mg/L) after 4 and 9 weeks, respectively. Circulating interleukin-6, and TNF- $\alpha$  concentrations decreased only in the treated group,  $P<0.0001$  for both parameters. The adjusted within treated group changes in IL-6 and TNF- $\alpha$  were  $-2.08$  pg/mL (CI,  $-2.63$  to  $-1.53$ ) and  $-0.81$  pg/mL (CI,  $-1.14$  to  $-0.49$ ). Compared to other parameters, changes in IL-1 were not so significant ( $P$  value of 0.413 and 0.084 at 4 and 9 weeks). However, this can be due to the significant decrease of plasma IL-6, leading to the stabilization of circulating IL-1 (Figure 2).

**Table 3.** Mean energy and nutrient intake at baseline and end of the study for two study groups. Data are expressed as mean  $\pm$  standard deviation.

Parameter	OTP group (n=45)	Placebo group (n=45)
<b>Energy (cal)</b>		
Baseline	1355.00 $\pm$ 209,54	1809.00 $\pm$ 130.3
9 weeks	1402.00 $\pm$ 205,11	1695.00 $\pm$ 318.4
$P$ value <sup>a</sup>	0.603	0.445
<b>Fat (g)</b>		
Baseline	63.90 $\pm$ 11.40	78.32 $\pm$ 13.47
9 weeks	64.17 $\pm$ 11.95	75.06 $\pm$ 12.95
$P$ value <sup>a</sup>	0.747	0.833
<b>PUFAs (g)</b>		
Baseline	11.33 $\pm$ 2.01	11.90 $\pm$ 1.22
9 weeks	12.77 $\pm$ 2.91	10.67 $\pm$ 1.43
$P$ value <sup>a</sup>	0.711	0.802
<b>MUFAs (g)</b>		
Baseline	22.15 $\pm$ 1.87	23.22 $\pm$ 1.34
9 weeks	23.52 $\pm$ 2.23	22.54 $\pm$ 1.57
$P$ value <sup>a</sup>	0.193	0.114
<b>SFAs (g)</b>		
Baseline	13.11 $\pm$ 1.52	12.93 $\pm$ 1.97
9 weeks	15.86 $\pm$ 1.62	15.57 $\pm$ 2.11
$P$ value <sup>a</sup>	0.515	0.749
<b>Weight (kg)</b>		
Baseline	65.43 $\pm$ 3.06	63.54 $\pm$ 4.34
9 weeks	66.01 $\pm$ 4.77	63.97 $\pm$ 4.66
$P$ value <sup>a</sup>	0.794	0.906

PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids. <sup>a</sup> Paired Student  $t$  test ( $p<0.05$ ).

### Pain intensity and disease activity

RA has often been accompanied by high-intensity chronic pain. Table 4 summarizes the changes from baseline in pain intensity, pain relief as well as DAS28 in both OTP

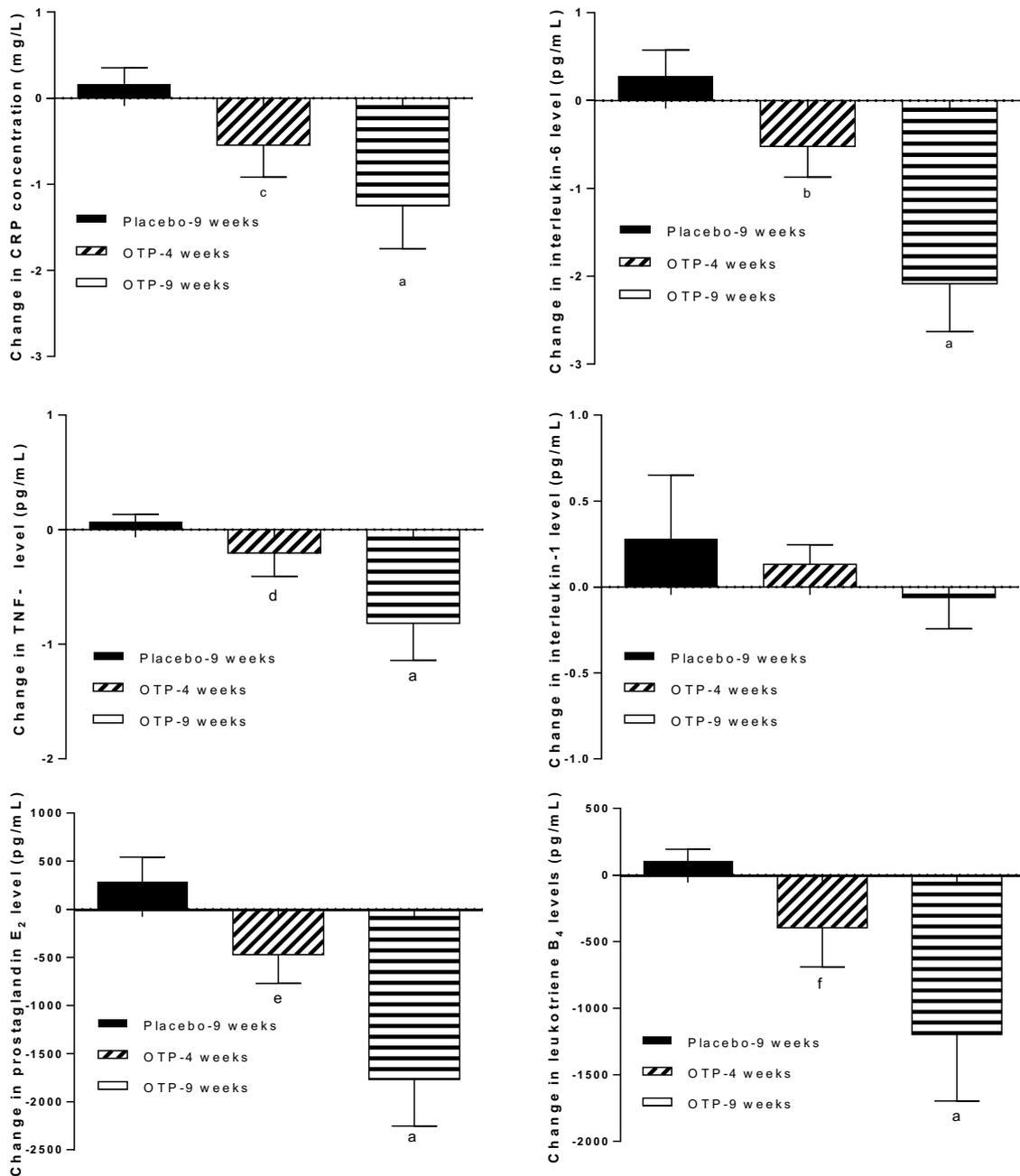
and placebo groups. VAS (100-mm pain scale) values for the OPT-group ( $50.41 \pm 8.86$  mm) were significantly lower than those of the placebo group ( $76.03 \pm 10.46$  mm) at 9 weeks ( $P < 0.0001$ ). Similarly, pain relief score was significantly higher in the treated group compared to the placebo ( $P < 0.0001$ ), even after 4 weeks of intervention (Table 4). Here, we should underline that more than 30% of the OPT-group members have declared a high pain relief score ( $\geq 3$ ), while the rest of the participants reported a meaningful pain relief (score  $\geq 2$ ) by the end of the study.

**Table 4.** Change from baseline in pain intensity, pain relief, DAS28. Data are expressed as mean and (95% CIs).

Parameter	OTP group (n=38)	Placebo group (n=35)	P value
Pain intensity	-26.70 (-30.39 to -20.90)	0.38 (-2.26 to 3.02)	<0.0001
Pain relief	2.14 (2.13 to 2.71)	-0.11 (-0,21 to -0,11)	<0.0001
DAS28	-1.84 (-2.43 to -2.03)	-0,13 (-0,21 to 0,09)	<0.0001
GART*	3.21 (2.93 to 3,48)	0.47 (0.28 to 0.65)	<0.0001

\* Global satisfaction assessment in response to treatment. Only the average value measured at the end of the study.

Data for DAS28 show a significant decrease in disease activity reported by the treated participants compared to those allocated to the placebo ( $P < 0.0001$ ). Patients in OTP group with baseline active RA (DAS28 score  $\geq 3.2$ ) showed good therapeutic response by the end of the study (DAS28 score of  $2.23 \pm 0.54$ ), which signal a RA remission (DAS28 score  $\leq 2.6$ ). Table 4 shows also the global satisfaction assessment in response to treatment, included the assessment of patient's anxiety. Very good satisfaction regarding the intervention (score of 3.21 of the 5-point categorical scale) was reported by participants received OTP, compared ( $P < 0.0001$ ) to those of placebo group. High degree of participant's satisfaction was correlated to a significant decrease in circulating inflammatory biomarkers level, pain intensity, and disease activity score, indicating the efficacy of the treatment with OTP.



**Figure 3.** Change from baseline in circulating inflammatory biomarkers level in the two study groups, hs-CRP, IL-6, TNF- $\alpha$ , IL-1, prostaglandin E<sub>2</sub>, and leukotriene B<sub>4</sub>. Error bars are 95% CIs. <sup>a</sup>(P<0.0001), <sup>b</sup>(P=0.014), <sup>c</sup>(P=0.009), and <sup>d</sup>(P=0.0247), <sup>e</sup>(P=0.0017), <sup>f</sup>(P= 0.0004).

## Discussion

In the present study, rheumatoid arthritis patient (according to the ACR/ELUAR) were treated by a phenolic olive tree powder for 9 weeks, by receiving a daily dose of 1 g. No adverse reactions signs have been observed during the study as well as 3 weeks after (data not shown). We found that the olive tree polyphenols -carried in natural olive

tree powder- display a strong therapeutic effect against inflammation, disease activity, and joint pain associated to RA.

RA is characterized by a continuous and excessive influx of inflammatory cells into the synovial membrane. Chronic inflammation leads to cartilage damage and bone destruction (mediated by osteoclasts), which cause -finally- the loss of function. However, inflammatory reaction and osteoclasts differentiation are both mediated by a complex network of cytokines, mainly TNF- $\alpha$ , IL-6, IL-1, as well as other simple molecules such as eicosanoids (PGE<sub>2</sub> and LTB<sub>4</sub>) (Smolen et Redluch, 2014; Boissier et al., 2012). RA treatment was evolved to the use advent of biologic treatments targeting specific immunologic pathways. Thus, it has been reported that the inhibition of TNF- $\alpha$  and IL-6 seems to be more efficient to predict inflammation compared to IL-1 (Smolen et Redlich, 2014). Current results demonstrate that supplementation by OTP (14% of total polyphenols and 10% of hydroxytyrosol) reduces the circulating TNF- $\alpha$ , IL-6, hs-CRP, PGE<sub>2</sub>, and LTB<sub>4</sub> in patients with RA (Figure 2).

However, this result can be a direct consequence of OTP's polyphenols (particularly hydroxytyrosol), who may (i) act directly on DNA to reduce expression of inflammatory mediators or (ii) inhibit their biosynthesis pathways through a similar mechanism of glucocorticoids and/or NAIDs. In this sense, several studies have demonstrated the inhibitory effect that can be played by hydroxytyrosol on PGE<sub>2</sub> levels through the repression of inducible cyclooxygenase (COX-2, key enzyme of PGE<sub>2</sub> biosynthesis pathway from arachidonic acid) in isolated human monocytes (Rosignoli et al., 2013; Zhang et al., 2009a; Lu et al 2005) and murine macrophages (Richard et al., 2011). Moreover, hydroxytyrosol and oleuropein display a strong *in vivo* inhibitory effect against COX-2 and PGE<sub>2</sub> in mice with DSS-induced colitis (Sánchez-Fidalgo et al., 2011; Giner et al., 2011). Additionally, results from other cell culture models show that pure hydroxytyrosol -or carried in its natural matrix, like OTP and olive oil- can inhibit the synthesis of LTB<sub>4</sub>, TNF- $\alpha$ , IL-6, IL-1 and hs-CRP (Camargo et al., 2014; Richard et al., 2011; Zhang et al., 2009a; Bitler et al., 2005; Maiuri et al., 2005). More importantly, results from clinical studies have highlighted such inhibitory effect against inflammatory markers in patients with stable coronary disease (Fitó et al., 2007; Estruch et al., 2006). Actually, results of the current study were consistent with previous *in vitro* and *in vivo* investigations (literature cited above), which explain the observed positive effect of rich-polyphenols powder from olive tree in patients with RA.

Besides, *in vitro* COX-2 targeting reduces PGE<sub>2</sub> level, which leads, in turn, to a decrease in circulating IL-6 in human macrophages and synovial fibroblasts (Inoue et al., 2002; Hinson et al., 1996). However, it has been known that IL-6 play an important role in the activation of inflammatory proteins like hs-CRP, which may explain the decrease in hs-CRP levels in treated participants. This was similar to the anti-inflammatory mechanism of NSAIDs drugs. Nevertheless, treatment with NSAIDs (celecoxib, rofecoxib, diclofenac) causes an increase in TNF- $\alpha$  contents in rheumatoid synovial membrane cultures as well as in blood (Rosignoli et al., 2013; Page et al., 2010), unlike our findings indicating a significant decrease of TNF- $\alpha$  levels. This mean that hydroxytyrosol and/or other OTP's polyphenols target other immunological pathway(s) leading to the decrease of IL-6 and TNF- $\alpha$ . Actually, Nuclear factor kappa  $\beta$  (NF- $\kappa$  $\beta$ ) signaling and chronic inflammatory diseases has been in depth reviewed by Killeen et al (2014). These authors underlined the potential of hydroxytyrosol to drive new therapeutic opportunities by reducing NF- $\kappa$  $\beta$  activation and its nuclear translocation. NF- $\kappa$  $\beta$  triggers the expression of more than 150 genes including those encoding cytokines, TNF- $\alpha$ , IL-1, and IL-6 (Makarov, 2001). Richard et al (2011) found that the decrease of cytokines levels in murine macrophages after treatment with Hydroxytyrosol form aqueous olive extract was correlated to low expression of NF- $\kappa$  $\beta$ p65. Hydroxytyrosol affects also NF- $\kappa$  $\beta$  activity in endothelial (Scoditti et al., 2012) and neural cells (St-Laurent-Thibault et al. 2011). Thus, one would think that OTP's polyphenols anti-inflammatory effect is, mainly, exerted by i) inhibiting COX-2 enzyme, and/or ii) reducing the expression of NF- $\kappa$  $\beta$ .

Otherwise, in RA, chronic inflammation induces proliferation of the synovium leading to formation of pannus and joint destruction, where neovascularization (angiogenesis) is major contributor (Semerano et al., 2011; Lee et al., 2001). A high correlation between RA progression and VEGF level (Vascular Endothelial Growth Factor, most important pro-angiogenic factor) has been observed in RA patients (Lee et al., 2001; Sone et al., 2001). Pro-angiogenic factors (VEGF and Angiopoietins-1) activation in the synovial membrane is a multi-targeted mechanism involving both cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) and COX-2 (Scoditti et al., 2012; Pettit et al., 2001). Our data (under publication process) elucidate that Hydroxytyrosol from the studied OTP modulates the angiogenic response of endothelial cells by repressing VEGF (isoforms A, B, and C), Ang-1 and Ang-2 gene expression, as it has been already stated (Scoditti et al., 2012; Fortes et al., 2012).

On the other hand, pain is a cardinal symptom in any illness state and especially the rheumatic diseases. In RA, pain intensity is strongly correlated to high PGE<sub>2</sub> level (Procházková et al., 2009; Scher et al., 2007; Kamei et al., 2004), explaining the effectiveness of NSAIDs as pain relief agents. Similar pain relief effect of Ibuprofen (most known NSAIDs) has been reported for oleocanthal (phenolic compound from olive oil) by Beauchamp et al (2005). The same applies to pain relief reported by participants received OTP. The decrease in circulating inflammatory markers, particularly in PGE<sub>2</sub> level, is likely the major responsible of pain intensity reduction observed in that group.

### **Conclusion**

In summary, chronic inflammation and pain are the hallmark of RA. Our current findings demonstrate that the administration of rich-polyphenols extract from olive tree was associated to significant decrease of circulating inflammatory markers, pain intensity, and disease activity in RA patients. We suggest that the anti-inflammatory effect of olive polyphenols -as natural components of OTP- is linked to i) NF- $\kappa$ B-dependent inhibition of cytokines (IL-6 and TNF- $\alpha$ ), ii) like-NSAIDs inhibition of COX-2, and iii) VEGF and Ang-1 repression. Further research should focus on the anti-angiogenic activity of hydroxytyrosol in synovial membrane, as future target of new anti-inflammatory drugs based on hydroxytyrosol structure.

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## **CLINICAL STUDY N°8**

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# Dietary consumption of desert olive tree pearls reduces brain A $\beta$ content and improves learning and memory ability in aged mice

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## ABSTRACT

Recently, the number of patients with neurodegenerative diseases, such as Alzheimer's disease (AD), has increased with aging of the population. Desert Olive Tree Pearls (DOTP), one of the olive oil products rich in functional compounds such as polyphenols is considered to have beneficial effect on neuronal activities. In this study, our study showed that pre-treatment of SH-SY5Y cells with DOTP ameliorated A $\beta$ -induced cytotoxicity. Moreover, the Morris water maze test indicated that DOTP improves learning and memory in aged mice. Also, DOTP induced a decrease in A $\beta$ <sub>42</sub> and inflammatory cytokine levels and increase in neurotransmitter levels in limbic system and serum of aged mice. These results suggest that DOTP may have anti-oxidative effects and decreased oxidative stress associated with overproduction of pro-inflammatory cytokines in A $\beta$  accumulations by aging, thus protecting neurons. Generally, the results of the present study suggest that DOTP is a promising dietary ingredient for improvement of cognitive function.

## 1. Introduction

Currently, approximately 50 million individuals worldwide have dementia-related problems. Due to aging populations, this number is expected to reach 66 million by 2030 and 115 million by 2050 (Peprah and McCormack, 2019). Much research has been conducted on the pathology of Alzheimer's disease (AD), but treatment for the most common forms of dementia is still unclear. Current treatments target symptoms and do not affect the disease progression. AD is characterized by accumulation of amyloid  $\beta$  (A $\beta$ ) plaques in the brain parenchyma and cerebral blood vessels (Selkoe, 2001). Accumulation of A $\beta$  is associated with pathological changes associated with AD (Hardy, 2009). A $\beta$  is a product of amyloid precursor protein (APP) that is processed to produce A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub>. Currently, acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) and glutamate receptor antagonists (memantine) are available as treatments for symptoms of AD (Casey et al., 2010). However, these treatments result in several improvements in symptoms but are limited, and none of them have therapeutic or disease-modifying effects (Casey et al., 2010). Therefore, much research is needed to develop therapeutic approaches that have AD-modifying

effects to prevent the pathology of AD and enhance cognitive function.

Diet therapy is one of the oldest and traditional methods of prevention and cure of all known diseases that makes up the majority of some Greek, Arab, and Islamic health systems based on herbs and diets (Saad, 2015). Particularly, several epidemiological studies have reported various health benefits of the Mediterranean diet. Interestingly, the prevalence of AD is lower in the Mediterranean region than in other countries (Solfrizzi et al., 2003; Panza et al., 2004; Scarmeas et al., 2009). Several systematic reviews and meta-analyses have also shown that the Mediterranean diet reduces the incidence of AD (Singh et al., 2014; Aridi et al., 2017; Hill et al., 2019). One of the most representative components of the Mediterranean diet is olive oil products such as olive oil and Desert Olive Tree Pearls (DOTP), which have many bioactive compounds, especially hydroxytyrosol, with many health benefits. Recently, several reports of Mediterranean diet have suggested that its consumption ameliorates and decrease the risk for many chronic diseases, such as cardiovascular disease (Estruch et al., 2013), diabetes (Filippatos et al., 2016), and age-related memory decline (Feart et al., 2013). Moreover, hydroxytyrosol, main component of DOTP, have the potential to exert the neuroprotective effect due to its potent anti-

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oxidant and anti-inflammatory activities (Omar et al., 2017). In our previous study, we reported for the first time the neuroprotective effect against A $\beta$ -induced neuronal cell death in olive oil (Villareal et al., 2016). However, although DOTP, which also contains many bioactive compounds such as hydroxytyrosol related to antioxidants and anti-inflammatory compounds, is expected to have the potential to exert physiological activities equal to or higher than that of olive oils, there are no reports about its physiological activities.

Thus, in the present study we studied the effects of DOTP on cell viability and neuroprotection against A $\beta$ <sub>42</sub>-induce neuronal cell death in SH-SY5Y human neuro-blastoma cells. Moreover, to investigate the possibility of using DOTP as a medical food in AD, in the present study, we hypothesized that oral administration of DOTP enhances spatial learning and memory ability by reducing A $\beta$ <sub>42</sub> content and attenuating A $\beta$ <sub>42</sub>-associated biomarker changes. To the best of our knowledge, this is the first study to investigate the effect of using DOTP as a possible medical food with current AD medication.

## 2. Materials and methods

### 2.1. Desert Olive Tree Pearls (DOTP) samples

The Desert Olive Tree Pearls (DOTP) used in this study was obtained from ATLAS OLIVE OILS company in Morocco. DOTP, one of the olive oil products, is made from leaves, baby leaves, and fruits of olive trees by cold agitation and high-pressure mechanical manufacturing methods and contains a lot of polyphenols, such as hydroxytyrosol, tyrosol, oleocanin, and oleacein (analyzed using HPLC by Sidi Mohamed Ben Abdellah University, Morocco) (Table 1). Particularly, DOTP contains high amount of hydroxytyrosol (16.12 mg/g) compared with other polyphenols (Table 1). In the present study, for in vitro experiments, DOTP was dissolved in serum-free Eagle's minimum essential medium (OPTI-MEM; Gibco, Japan) with homogenization and sonication and for in vivo experiment, DOTP was dissolved in milliQ water with homogenization and sonication.

### 2.2. SH-SY5Y cell culture

The human neuroblastoma SH-SY5Y cell line was purchased from the American Type Culture Collection (ATCC). SH-SY5Y cells were cultured in a 1:1 (v/v) mixture of Dulbecco's modified Eagle Medium and Ham's F-12 medium (Gibco, Japan) supplemented with 15% heat-inactivated fetal bovine serum (Bio West, U.S.A) and 1% penicillin (5000  $\mu$ g/ml)-streptomycin (5000 IU/ml) (PS) (Lonza, Japan) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. SH-SY5Y cells were cultured in 100-mm petri dishes or 96-well plates. Serum-free Eagle's minimum essential medium (OPTI-MEM; Gibco, Japan) was used to culture the cells for the cell viability assay.

### 2.3. MTT assay

Cell viability and mitochondrial activity were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to check for effects of DOTP (10, 25, 50, 75, and 100  $\mu$ g/mL) and A $\beta$ <sub>42</sub> (3  $\mu$ M) on cytotoxicity. SH-SY5Y cells were seeded at  $2 \times 10^5$  cells/mL in 96-well plates (BD BioCoat, U.S.A.) and incubated for 24 hr. After

24 hr incubation, SH-SY5Y cells were treated DOTP for 72 hr. To evaluate the neuroprotective effects of DOTP against A $\beta$ -induced cytotoxicity, SH-SY5Y cells were pre-treated with DOTP for 24 hr before 3  $\mu$ M A $\beta$ <sub>42</sub> monomer treatment. After sample treatment, a solution of 5 mg/ml MTT dissolved in PBS was added (10  $\mu$ l/well) and incubated for another 24 hr. The resulting MTT formazan was dissolved in 100  $\mu$ l of 10% SDS (w/v) and the absorbance was measured using a microtiter plate reader (Dainippon Sumitomo Pharma Co., Ltd., Japan).

### 2.4. RNA isolation from SH-SY5Y cells

SH-SY5Y cells were seeded at  $3.7 \times 10^5$  cells/mL in a 10 cm<sup>2</sup> dish (BD BioCoat) and incubated at 37 °C for 24 h. The SH-SY5Y cells were then pre-treated with DOTP (100  $\mu$ g/mL) at 37 °C for 24 hr. After DOTP treatment, A $\beta$ <sub>42</sub> solution (final concentration: 3  $\mu$ M) was added and incubated for a further 24 hr at 37 °C. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions as per a previous study (Villareal et al., 2016).

### 2.5. Measurement of MAPK-related genes using real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed to evaluate the effect of DOTP on MAPK-related genes expression in SH-SY5Y cells. In this study, 100  $\mu$ g/mL DOTP showed the highest effect on neuroprotective effect against A $\beta$ <sub>42</sub> toxicity. Thus, we used 100  $\mu$ g/mL of DOTP for the effect of DOTP on MAPK-related genes expression. The TaqMan probe (ThermoFisher Scientific, USA) was used for the quantification of gene expression. Using a superscript III reverse transcriptase kit (ThermoFisher Scientific) a complementary DNA (cDNA) solution was synthesized following the manufacturer's instructions. For quantification of transcript amounts, TaqMan real-time RT-PCR amplification reactions were performed using the Applied Biosystems 7500 Fast Real-Time System (ThermoFisher Scientific). All primer sets and the TaqMan Universal PCR Master Mix were obtained from ThermoFisher Scientific. Specific primers for actin beta (Hs1060665\_m1), Poly (ADP-ribose) polymerase 1 (PARP1) (Hs00242302\_m1), Mitogen-Activated Protein Kinase Kinase 4 (MAP2K4) (Hs00387426\_m1), Mitogen-activated protein kinase 14 (MAPK14) (Hs01051152\_m1), Mitogen-Activated Protein Kinase 8 (MAPK8) (Hs01548508\_m1), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) (Hs00907957\_m1), AKT Serine/Threonine Kinase 1 (AKT1) (Hs00178289\_m1), and caspase-3 (CASP3) (Hs00234387\_m1) were used.

### 2.6. Animals and sample treatment

Male C57BL/6J mice, aged 10 weeks and 18 months, were obtained from Japan SLC, Inc. (Shizuoka, Japan) and housed under conditions of controlled temperature and humidity and unrestricted access to food and water with a 12-hr light/dark cycle. The protocol for this animal experiment was approved by the Animal Care and Use Committee of the University of Tsukuba (19–363). Because estrogens are well known for their robust enhancement on cognition about learning and memory in female rodents (Korol & Pisani, 2015), to avoid the effect of estrogens, we used male mice in the present study. After 1 week of acclimatization to the laboratory conditions, the mice were divided into four groups: 10-week-old water-administered group (n = 8) (Young control), 10-week-old DOTP-administered group (n = 8) (Young test), 18-month-old water-administered group (n = 8) (Aged control), and 18-month-old DOTP-administered group (n = 8) (Aged test). In this study, based on the previous study about animal experiment of olive oil study (Hernández-Rodas et al., 2017) and morris water maze (MWM) experiment of aging study (Sasaki et al., 2019; Sasaki et al., 2021), the concentration and the period of oral administration of DOTP were decided. The DOTP-administered group was administered 50 mg/kg DOTP mixed with MilliQ water for 30 days by oral gavage using a tube and syringe.

**Table 1**  
Polyphenols contents in Desert Olive Tree Pearls (DOTP).

	Content (mg/g)
Hydroxytyrosol	16.12
Tyrosol	3.2
Oleocantal	4.46
Oleacein	1.68
Total polyphenols	55.44

Water-administered groups were administered an equivalent volume of MilliQ water.

### 2.7. Morris water maze (MWM) test

To evaluate the effects on spatial learning and memory of DOTP administration, the MWM test was performed as previously described (Sasaki et al., 2019; Sasaki et al., 2021). The apparatus consisted of a circular water tank (120 cm in diameter and 45 cm in height) that contained water ( $23 \pm 2$  °C) to a depth of 30 cm and divided into four quadrants: north, east, west, and south. The escape platform (10 cm in diameter) was submerged 1 cm below the water surface and placed at the midpoint of any quadrant so that it was invisible at the water level. All mice received training for 5 consecutive days using a single hidden platform in one quadrant, with the start point rotating around the other three quadrants. The latency to escape from the water maze (find the hidden escape platform) was measured for each trial. Then, the probe test was conducted on day six after the MWM test training session, the platform was removed, and each mouse was allowed to swim freely for 60 s. The respective time spent by each mouse in the target quadrant and the number of crossings over the platform location (where the platform was located during the training) were calculated. The time spent by each mouse in the target quadrant and the number of times crossing the virtual platform were considered to represent the degree of memory consolidation.

### 2.8. Protein extraction from the limbic systems of mouse brain

Furthermore, to evaluate the molecular markers of spatial learning and memory improvement effects of DOTP, animals were sacrificed, and the brain was carefully removed after the behavioral test. The limbic system of the mouse brain was carefully dissected, frozen in liquid nitrogen, and stored at  $-80$  °C until use. Moreover, 100 mg of the limbic system was homogenized in 1 mL of RIPA buffer with protease inhibitor (Santa Cruz Biotechnology, Japan) using a glass-Teflon homogenizer and centrifuged (10000g, 30 min, 4 °C). After centrifugation, the supernatant was collected for protein samples. Protein estimation was conducted using 2-D quant kit (GE Healthcare Inc., Japan) according to procedure of the kit, and the data were expressed as pg/ $\mu$ g protein.

### 2.9. $A\beta_{42}$ analysis in the limbic systems of mouse brain

$A\beta_{42}$  levels in the limbic system of the mouse brain were measured using commercial ELISA kits (Wako, Japan) in accordance with the manufacturer's instructions. Briefly, protein samples or standards were used to determine  $A\beta_{42}$  levels. After treatment with the  $A\beta_{42}$  antibody, a second incubation was performed with streptavidin-horseradish peroxidase conjugate solution. After addition of substrate and stop solution,  $A\beta_{42}$  levels were determined by measuring the absorbance at 450 nm.  $A\beta_{42}$  levels were expressed as nmol/ $\mu$ g protein.

### 2.10. TNF- $\alpha$ and IL-6 analysis in the serum and limbic systems of mouse

Commercial ELISA kits (R&D Systems, USA) were used to measure TNF- $\alpha$  and IL-6 levels in the serum and limbic systems of the mouse brain. Briefly, serum, protein samples, or standards were used according to the manufacturer's instructions for TNF- $\alpha$  or IL-6 ELISA kit. TNF- $\alpha$  and IL-6 levels were computed as pg/mL (serum) or pg/ $\mu$ g protein (protein).

### 2.11. Dopamine, noradrenaline, serotonin, gamma-aminobutyric acid (GABA), and glutamate analysis in the limbic systems of mouse brain

Dopamine, noradrenaline, serotonin, GABA, and glutamate levels in the limbic systems of the mouse brain were measured using commercial ELISA kits (ImmuSmol, Inc., France). Briefly, protein samples or

standards were used for catecholamine extraction, acylation, and determination according to the manufacturer's instructions. After optimal color development, the reaction was stopped, and the absorbance at 450 nm was recorded. Dopamine, noradrenaline, serotonin, GABA, and glutamate levels were computed by correcting for the protein concentration, and the data were expressed as ng/ $\mu$ g protein.

### 2.12. Pro-BDNF and BDNF analysis in serum and the limbic systems of mouse brain

Pro-BDNF and BDNF levels in the serum and limbic systems of the mouse brain were measured using commercial ELISA kits (R&D Systems, Inc., Minneapolis, USA) in accordance with the manufacturer's instructions. Briefly, serum, protein samples, or standards were used to determine the Pro-BDNF and BDNF levels in the brain. After treatment with the antibody, a second incubation was performed with streptavidin-horseradish peroxidase conjugate solution for 60 min. After addition of substrate and stop solution, Pro-BDNF and BDNF levels were determined by absorbance at 450 nm. The pro-BDNF and BDNF levels were normalized to the determined protein concentration. The data for pro-BDNF and BDNF were expressed as pg/mL (serum sample) and pg/ $\mu$ g protein (protein samples).

### 2.13. Statistical analysis

Statistical analysis of the results obtained from the MWM was conducted using two-way ANOVA followed by the Tukey's post-hoc test. In another experiment, comparisons between treatment groups of animal models were performed using one-way ANOVA followed by Tukey's post hoc test for normally distributed data. was also conducted. A P-value < 0.05 was considered statistically significant.

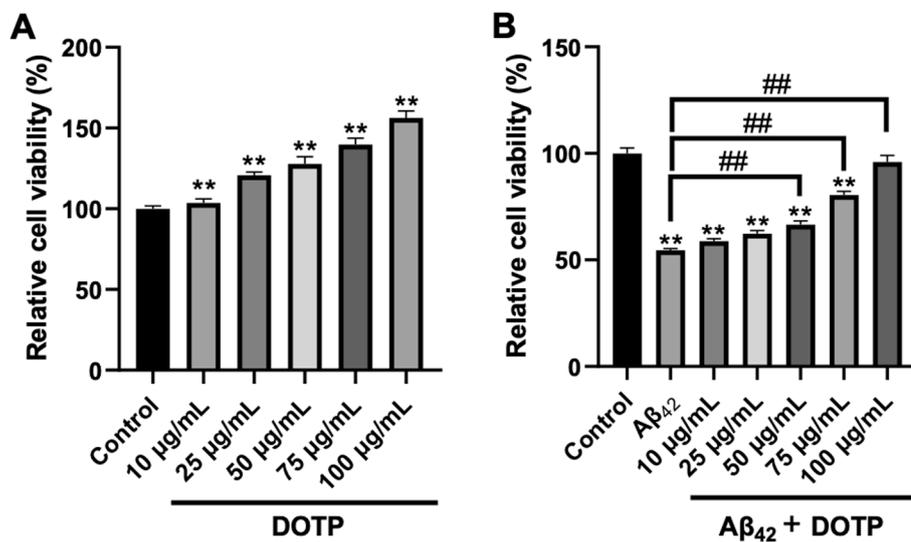
## 3. Results

### 3.1. Desert Olive Tree Pearls (DOTP) induced cell viability and inhibited $A\beta_{42}$ -induced cell death on human neuroblastoma SH-SY5Y cells

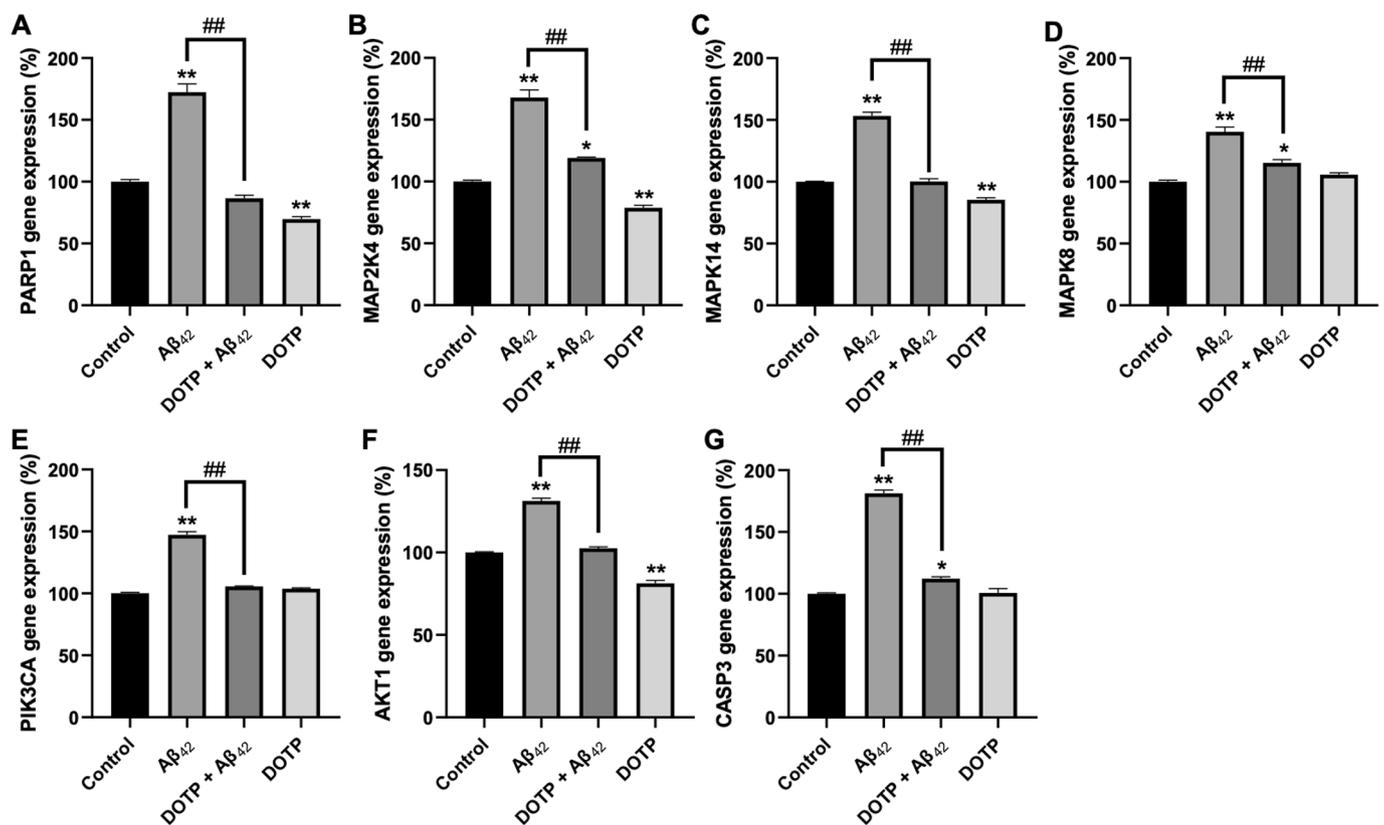
SH-SY5Y cells were treated with DOTP (10, 25, 50, 75, and 100  $\mu$ g/mL) for 72 hr and cell viability measured with the MTT assay. Our MTT results showed that only DOTP (25, 50, 75, and 100  $\mu$ g/mL) treated cells induce a significant increase in cell proliferation ( $120.8 \pm 4.3\%$ ,  $127.8 \pm 7.8\%$ ,  $139.7 \pm 7.1\%$ , and  $156.3 \pm 8.8\%$ , respectively) ( $P < 0.01$ ) compared with non-treated cells (Fig. 1A). Moreover, to evaluate the neuroprotective effect of DOTP SH-SY5Y cells were pre-treated with DOTP (10, 25, 50, 75, and 100  $\mu$ g/mL) for 24 hr. And then,  $A\beta_{42}$  was added (final concentration: 3  $\mu$ M) and co-treated with DOTP for 48 hr; and cell viability was measured with the MTT assay. In our present study, when we assessed the optimal concentration of  $A\beta_{42}$ , 3  $\mu$ M-treatment showed a reduction in cell viability of approximately 50% (Data was not shown). Therefore, we decided that 3  $\mu$ M  $A\beta_{42}$  treatment is optimal concentration for evaluating neuroprotective effect. As shown in Fig. 1B, the  $A\beta_{42}$ -treated group showed a significantly reduction in cell viability compared to the non-treated group ( $54.6 \pm 1.7\%$ ). In contrast, pre-treatment with DOTP (25, 50, 75, and 100  $\mu$ g/mL) ameliorated  $A\beta_{42}$ -induced cytotoxicity ( $114.2 \pm 5.9\%$ ,  $121.9 \pm 7.4\%$ ,  $147.3 \pm 7.9\%$ , and  $175.8 \pm 11.2\%$  compared to 100% in  $A\beta_{42}$ -treated cells, respectively,  $P < 0.01$ ) (Fig. 1B).

### 3.2. Effect of Desert Olive Tree Pearls (DOTP) on gene expression related MAPK signaling in $A\beta_{42}$ -treated SH-SY5Y cells

To evaluate the effects of DOTP on MAPK-related genes expression was measured by real-time RT-PCR in  $A\beta_{42}$ -treated SH-SY5Y cells. SH-SY5Y cells were treated with 100  $\mu$ g/mL of DOTP for 24 hr. After DOTP treatment, SH-SY5Y cells were stimulated with  $A\beta_{42}$  for further 24 hr. As shown in Fig. 2, the  $A\beta_{42}$ -treated cells showed significantly



**Fig. 1.** Effect of Desert Olive Tree Pearls (DOTP) on (A) the cell viability and (B) Amyloid- $\beta$  ( $A\beta$ )-induced changes in human neuroblastoma SH-SY5Y cells viability. For the evaluation of cell viability, SH-SY5Y cells were treated with DOTP for 72 hr. And for the evaluation of neuroprotection, SH-SY5Y cells were pre-treated with DOTP for 24 hr, and then, the cells were treated with 3  $\mu$ M  $A\beta$  for 48 hr. Each bar represents the mean  $\pm$  SEM ( $n = 5$  independent experiments). \*\*  $P < 0.01$  vs control cells, ##  $P < 0.01$  vs  $A\beta_{42}$ -treated cells.



**Fig. 2.** Effect of Desert Olive Tree Pearls (DOTP) on gene expression of (A) PARP1, (B) MAP2K4, (C) MAPK14, (D) MAPK8, (E) PIK3CA, (F) AKT1, and (G) CASP3 in human neuroblastoma SH-SY5Y cells. SH-SY5Y cells were pre-treated with 100  $\mu$ g/mL DOTP for 24 h. Following that, cells were treated with  $A\beta_{42}$  (final concentration: 3  $\mu$ M) for further 24 h. After treatment, the gene expression of PARP1, MAP2K4, MAPK14, MAPK8, PIK3CA, AKT1, and CASP3 were evaluated using real-time RT-PCR. Values are expressed as the mean  $\pm$  SD of triplicate experiments and are expressed relative to the percentage of control cells. \*\*  $P < 0.01$  vs control cells, ##  $P < 0.01$  vs  $A\beta_{42}$ -treated cells.

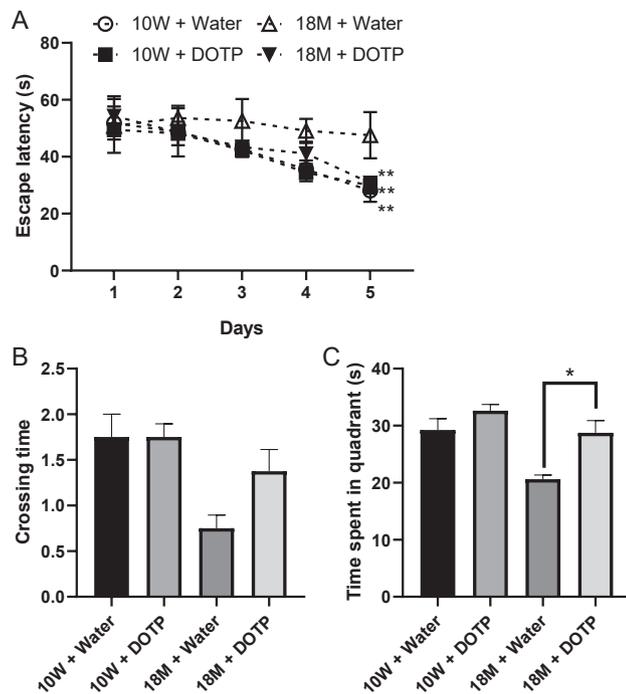
increase ( $P < 0.01$ ) PARP1 gene expression by  $172.4 \pm 11.7\%$  (Fig. 2A), MAP2K4 (Fig. 2B) gene expression by  $167.9 \pm 10.6\%$ , MAPK14 (Fig. 2C) gene expression by  $153.3 \pm 5.2\%$ , MAPK8 (Fig. 2D) gene expression by  $140.5 \pm 6.5\%$ , PIK3CA (Fig. 2E) gene expression by  $147.3 \pm 4.2\%$ , AKT1 (Fig. 2F) gene expression by  $131.3 \pm 2.8\%$ , and CASP3 (Fig. 2G) gene expression by  $181.2 \pm 4.8\%$  compared to that in non-treated cells. However, the 100  $\mu$ g/mL DOTP-treated cells showed a significantly lower gene expression of PARP1 by 50.2% (Fig. 1A), MAP2K4 (Fig. 2B)

by 71.0%, MAPK14 (Fig. 2C) by approximately 65.4%, MAPK8 (Fig. 2D) by 82.0%, PIK3CA (Fig. 2E) by 71.7%, AKT1 (Fig. 2F) by 78.0%, and CASP3 (Fig. 2G) by approximately 62.0% compared to that in  $A\beta_{42}$ -treated SH-SY5Y cells.

### 3.3. Administration of Desert Olive Tree Pearls (DOTP) improved spatial learning and memory in aged mice

To evaluate the potential effect of DOTP on spatial learning and memory impairment, 50 mg/kg of DOTP was orally administered to 10-week-old mice (young mice) and 18-month-old mice (aged mice) for 30 days. Spatial learning and memory were evaluated using the Morris water maze (MWM) test, a widely used tool to assess spatial learning and memory in rodents. As shown in Fig. 3A, the escape latency time of the Aged control group did not show any decrease from the 1st to 5th day training (Day 1, 50.11 ± 6.31 s; Day 2, 50.87 ± 7.27 s; Day 3, 46.83 ± 6.83 s; Day 4, 49.08 ± 4.23 s; Day 5, 46.13 ± 5.75 s). However, on day 5, both the Young control group (31.75 ± 3.57 s) and Aged test group (33.0 ± 5.27 s) showed a statistically significant decrease in escape latency compared with the Aged control group (46.13 ± 5.75 s) (Fig. 3A). In contrast, in the Young test group (Young mice fed with DOTP group) also showed a decrease (31.33 ± 4.89 s) in escape latency; however, this decrease was not different from that in the Young control group (31.75 ± 3.57 s).

Moreover, to verify whether the effect of spatial learning and memory improvement by administration of DOTP is sustainable and whether learning and memory ability of mice was dependent on spatial information, a probe test was conducted. In the probe test, the observation of the behavior of the mouse is performed under the same condition except removing the platform. As a result of the probe test, the Aged test group showed a significantly increased swimming time in the quadrant (28.75 ± 4.25 s) compared with the Aged control group (20.50 ± 3.51 s). Furthermore, the Aged test group showed an increase in the number of crossings of the platform, but the difference was not significant. Moreover, Young control group and Young test group showed a significant increase both in the number of crossing times across the virtual platform (Young control group, 1.75 ± 0.50; Young test group,



**Fig. 3.** Effect of Desert Olive Tree Pearls (DOTP) administration on spatial learning and memory as evaluated by escape latency of young (10 weeks) mice, aged (18 months) mice, and mice 50 mg/kg DOTP - and water-treated group by Morris water maze test (A). Effect of DOTP on numbers of crossings of platform (B). Effect of DOTP on the time spent in the target quadrant by DOTP-treated or water-treated mice (C). \*P < 0.05, \*\*P < 0.01 Compared with Aged control group by one-way ANOVA.

1.67 ± 0.26) and the swimming time in the quadrant (Young control group, 29.25 ± 3.92; Young test group, 30.50 ± 3.96) where the platform was installed compared with Aged control group (number of crossing of the platform, 0.75 ± 0.29; time spent in quadrant, 20.50 ± 3.51 s) (Fig. 3B and 3C). Conversely, there was no difference between the Young control and Young test groups in terms of both the number of crossings of the platform and time spent in quadrant.

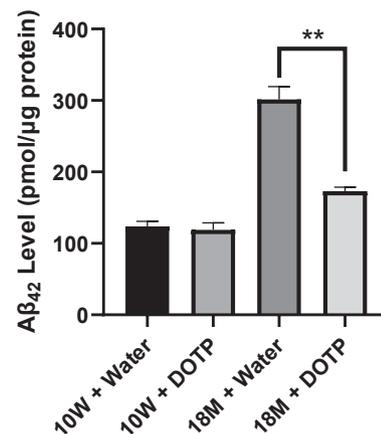
### 3.4. Administration of Desert Olive Tree Pearls (DOTP) reduced Aβ<sub>42</sub> content in limbic systems of mouse brain

Then, we assessed whether oral administration of DOTP reduced the levels of Aβ<sub>42</sub> in the limbic system of the mouse brain. The ELISA results showed that the Aβ<sub>42</sub> levels in the limbic systems of mouse brains were significantly (P < 0.05) increased in the Aged control group (301.41 ± 36.06 pmol/μg protein) compared to that in the Young control group (123.69 ± 12.44 pmol/μg protein) (Fig. 4). However, administration of DOTP reversed this increase of Aβ<sub>42</sub> levels (173.31 ± 9.54 pmol/μg protein) in aged mice (Aged test group). Furthermore, in young mice (10-weeks-old mice), Aβ<sub>42</sub> levels (Young control group, 123.69 ± 12.44 pmol/μg protein; Young test group, 119.13 ± 17.05 pmol/μg protein) compared with the Aged control group, but between the water-treated group and DOTP-treated group, there was no significant difference (Fig. 4).

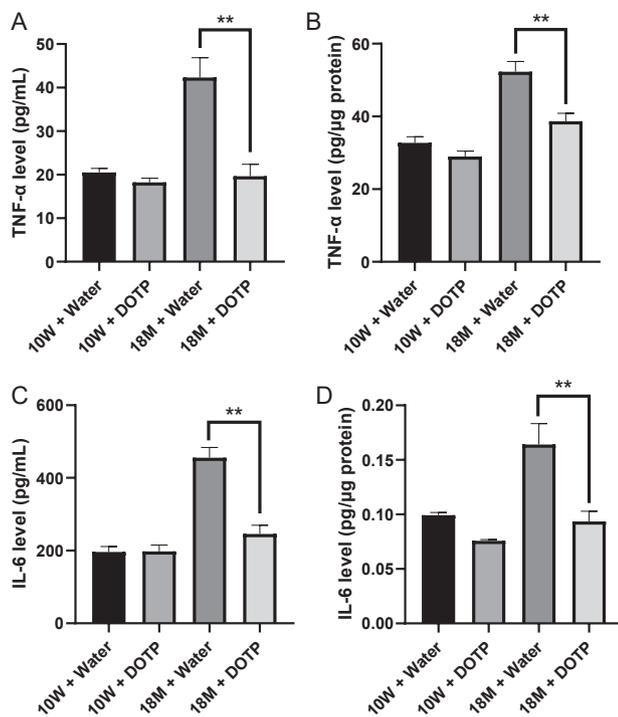
### 3.5. Desert Olive Tree Pearls (DOTP) suppressed the levels of neuroinflammatory factors in serum and limbic systems of mouse brain

To investigate if altered neuroinflammation by the increase in Aβ<sub>42</sub> may have contributed to spatial learning and memory improvement of DOTP, quantification of inflammatory cytokines in serum and limbic systems of mice brain was performed. Quantification using the commercial ELISA kits showed that TNF-α and IL-6 levels, which are major inflammatory cytokines, were higher in the serum of the Aged control group (TNF-α, 42.30 ± 7.86 pg/mL; IL-6, 455.92 ± 48.10 pg/mL) than in the Young control groups (TNF-α, 20.52 ± 1.57 pg/mL; IL-6, 197.83 ± 30.38 pg/mL) (Fig. 5A and 5B). However, in the Aged test group, a decrease in the serum TNF-α levels (19.67 ± 5.44 pg/mL) was observed (Fig. 3A). Moreover, administration of DOTP significantly decreased serum IL-6 levels (57.56% compared to 100% in the SAMP8 water-administered group) (Fig. 5C).

Next, we evaluated whether administration of DOTP could reduce the TNF-α and IL-6 levels in the limbic systems of aged mice brains. As



**Fig. 4.** Effect of Desert Olive Tree Pearls (DOTP) on Aβ<sub>42</sub> level in the limbic systems of young (10 weeks) and aged (18 months) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. The Aβ<sub>42</sub> level was determined by ELISA kit. Each value is presented as mean ± SD. \*\*P < 0.01 compared to Aged control group by one-way ANOVA.

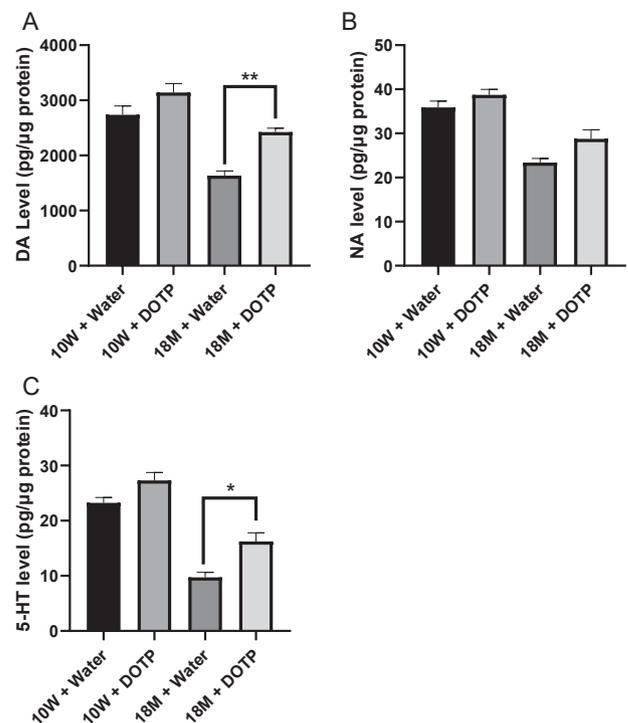


**Fig. 5.** Effect of Desert Olive Tree Pearls (DOTP) on levels of (A, B) tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and (C, D) interleukin-6 (IL-6) in serum and the limbic systems of young (10 weeks old) and aged (18 months old) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. The TNF- $\alpha$  and IL-6 levels were determined by ELISA kit. Each value is presented as mean  $\pm$  SD. \*\* $P < 0.01$  compared to Aged control group by one-way ANOVA. ##  $P < 0.01$  compared to Young control group by one-way ANOVA.

shown in Fig. 5B and 5D, the TNF- $\alpha$  and IL-6 levels in the limbic systems from the Aged control group (TNF- $\alpha$ ,  $52.32 \pm 6.31$  pg/ $\mu$ g protein; IL-6,  $0.164 \pm 0.033$  pg/ $\mu$ g protein) were significantly higher than those in the Young control group (TNF- $\alpha$ ,  $32.74 \pm 3.34$  pg/ $\mu$ g protein; IL-6,  $0.099 \pm 0.005$  pg/ $\mu$ g protein). In contrast, the TNF- $\alpha$  and IL-6 levels in the Aged test group (TNF- $\alpha$ ,  $38.69 \pm 3.83$  pg/ $\mu$ g protein; IL-6,  $0.094 \pm 0.016$  pg/ $\mu$ g protein) were significantly reduced compared with the Aged control group ( $p < 0.01$ ).

### 3.6. Desert Olive Tree Pearls (DOTP) attenuated the decline in neurotransmitter level in the limbic systems of mouse brain by aging

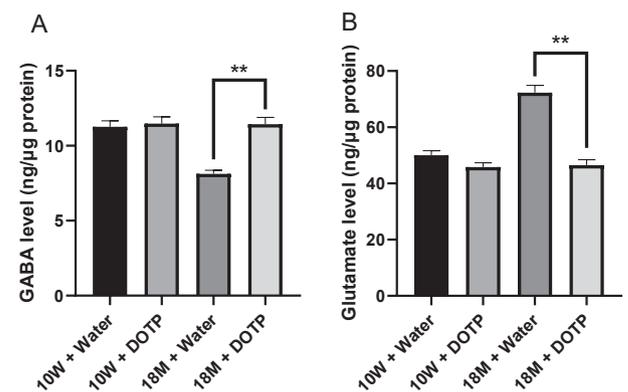
To further probe the potential mechanisms contributing to the spatial learning and memory improvement of DOTP, we quantified neurotransmitters (dopamine, noradrenaline, serotonin) in the limbic systems of mice brain. Using commercial ELISA kits, we found that the levels of dopamine ( $1635.41 \pm 165.15$  ng/ $\mu$ g protein), noradrenaline ( $23.40 \pm 2.03$  ng/ $\mu$ g protein), and serotonin ( $9.75 \pm 1.57$  ng/ $\mu$ g protein) in the limbic systems were significantly lower in the aged mice (Aged control group) compared with those in the young mice (Young control group) ( $2739.23 \pm 275.37$  ng/ $\mu$ g protein,  $35.88 \pm 2.78$  ng/ $\mu$ g protein, and  $23.25 \pm 1.69$  ng/ $\mu$ g protein, respectively) (Fig. 6). However, in the limbic systems of the Aged test group, dopamine ( $2426.14 \pm 120.80$  ng/ $\mu$ g protein) and serotonin ( $16.26 \pm 2.67$  ng/ $\mu$ g protein) levels were significantly higher compared with those in the Aged control group (Fig. 6). The noradrenaline levels ( $28.75 \pm 3.57$  ng/ $\mu$ g protein) in the Aged test group were also higher compared with those in the Aged control group, but this was not significant. In contrast, we did not find any significant change in these neurotransmitters between the Young control and Young test groups.



**Fig. 6.** Effect of Desert Olive Tree Pearls (DOTP) on (A) dopamine (DA), (B) noradrenaline (NA), and (C) serotonin (5-HT) levels in the limbic systems of young (10 weeks) and aged (18 months) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. These neurotransmitters levels were determined by ELISA kit. Each value is presented as mean  $\pm$  SD. \*\* $P < 0.01$  compared with Aged control group by one-way ANOVA.

### 3.7. Desert Olive Tree Pearls (DOTP) enhanced the GABA production in the limbic systems of mouse brain

In parallel with the increased dopamine and serotonin levels, we also evaluated the effect on glutamate and GABA levels in the limbic systems of the mouse brain. In the Aged control group, the GABA level (Fig. 7A) was significantly decreased ( $8.13 \pm 0.47$  ng/ $\mu$ g protein), and the glutamate level (Fig. 7B) was significantly increased ( $72.32 \pm 5.78$  ng/ $\mu$ g protein) compared to those in the Young control group ( $11.27 \pm 0.91$  ng/ $\mu$ g protein;  $50.01 \pm 3.24$  ng/ $\mu$ g protein). DOTP treatment induced an increase in GABA levels ( $11.45 \pm 0.89$  ng/ $\mu$ g protein) in the limbic



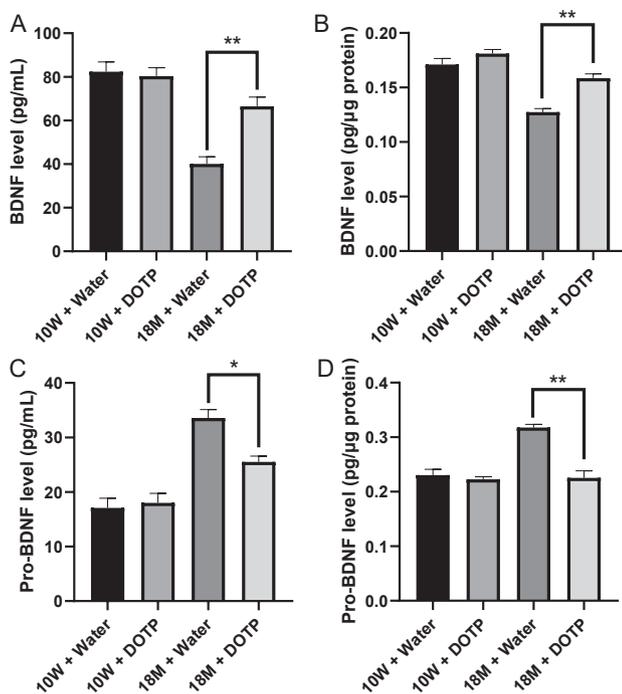
**Fig. 7.** Effect of Desert Olive Tree Pearls (DOTP) on (A) GABA and (B) glutamate levels in the limbic systems of young (10 weeks) and aged (18 months) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. These neurotransmitter levels were determined by ELISA kit. Each value is presented as mean  $\pm$  SD. \*\* $P < 0.01$  compared with Aged control group by one-way ANOVA.

systems of 18-month-old mice compared with the Aged control group. Furthermore, administration of DOTP induced the decrease in glutamate level ( $46.51 \pm 4.79$  ng/ $\mu$ g protein) in limbic systems of 18-month-old mice compared with Aged control group.

### 3.8. Desert Olive Tree Pearls (DOTP) enhanced the brain-derived neurotrophic factor (BDNF) production in serum and limbic systems of mouse brain

Commercial ELISA assays were run to measure the proBDNF and BDNF levels in the serum of young (10 weeks) and aged (18 months) mice with or without DOTP. As shown in Fig. 8A and 8B, there was a significant increase in the serum proBDNF level and a significant reduction in the serum BDNF level in the Aged control group (proBDNF,  $33.59 \pm 3.07$  pg/mL; BDNF,  $40.14 \pm 6.49$  pg/mL) compared with the Young control group (proBDNF,  $17.07 \pm 3.56$  pg/mL; BDNF,  $82.40 \pm 8.97$  pg/mL). Further analyses of the DOTP-treated group revealed that the proBDNF levels in serum in the Aged test group ( $25.52 \pm 2.12$  pg/mL) were significantly lower than those in the Aged control group. Moreover, the serum BDNF levels in the Aged test group ( $66.58 \pm 8.19$  pg/mL) were significantly higher than that in the Aged control group.

We further analyzed the protein levels of proBDNF and BDNF in the limbic systems of young and aged mice with or without DOTP (Fig. 8C and 8D). The proBDNF levels in the limbic systems of the Aged control group ( $0.318 \pm 0.013$  pg/ $\mu$ g protein) was significantly higher than that in the Young control group ( $0.231 \pm 0.023$  pg/ $\mu$ g protein). The BDNF levels in the limbic systems of the Aged control group ( $0.127 \pm 0.007$  pg/ $\mu$ g protein) were significantly lower than those in the Young control group ( $0.171 \pm 0.012$  pg/ $\mu$ g protein). However, the Aged test group showed significantly decreased proBDNF levels ( $0.225 \pm 0.029$  pg/ $\mu$ g protein) and increased BDNF levels ( $0.159 \pm 0.009$  pg/ $\mu$ g protein) in the limbic systems of the brain compared to the Aged control group.



**Fig. 8.** Effect of Desert Olive Tree Pearls (DOTP) on levels of (A, B) brain-derived neurotrophic factor (BDNF) and (C, D) BDNF precursor (proBDNF) in the serum and limbic systems of young (10 weeks) and aged (18 months) mice. Mice were orally administered with 50 mg/kg DOTP for 30 days. The BDNF and proBDNF levels were determined by ELISA kit. Each value is presented as mean  $\pm$  SD. \*\*\*  $p < 0.01$  compared to Aged control group by one-way ANOVA.

## 4. Discussion

Dementia triggers behavioral disorders and memory impairment and is generally caused by neurodegeneration and age-related neuronal cell death. Among the dementias, AD is the major neurodegenerative disease associated with aging. Aging is the most important risk factor for the onset of neurodegenerative diseases, and most neuro-degenerative disorders develop in the elderly population (Kritsilis et al., 2018). Thus, there is an urgent need to develop neuroprotective agents against A $\beta$ -induced toxicity from natural products to prevent damage or death of neural cells, both neurons and glia. Several previous studies have reported that natural products, especially from plant sources, show biological activities, such as neuroprotective effects (Daulatzai, 2013; Pak et al., 2016). Natural resource-derived products have multiple bioactive properties and could be useful for the prevention of neurodegeneration and improvement of cognitive function (Sowndhararajan and Songmun, 2017). Moreover, olive oil products have been established as a dietary ingredient that promotes health. Evidence reports the beneficial properties of olive oil products in the prevention of cardiovascular disorders and improvement of cognitive function (Scarmeas et al., 2011). In the present study, we focused on Desert Olive Tree Pearls (DOTP), which is one of the olive oil products, that are rich in natural bioactive substances, such as hydroxytyrosol (233 mg/kg). It has been reported that hydroxytyrosol treatment showed neuroprotective effect due to the reduction of aggregates-membrane interaction on SH-SY5Y cells by stabilizing A $\beta_{42}$  oligomers and fibrils (Leri et al., 2019). We evaluated the effect of DOTP on learning and memory ability by MWM and A $\beta_{42}$  levels in aged mice. We also focused on major pro-inflammatory cytokines and neurotransmitters, which are affected by A $\beta_{42}$  toxicity. Our MWM test results showed that 30 days administration of DOTP improves spatial learning memory in aged mice. Moreover, the probe test results showed that spatial learning and memory ability were improved by DOTP administration. Therefore, our present result of MWM revealed that DOTP has the potential to prevent or improve the loss of learning and memory ability by aging.

As aging progresses, the production of A $\beta$ , which leads to a large number of amyloid plaques, was increased. This overproduction can reduce the number and plasticity of synapses, and the resulting synapses can also be abnormal in shape and composition. It is well known that the most important A $\beta$  peptides in AD are the proteolytic products of APP metabolism, A $\beta_{40}$  and A $\beta_{42}$ . Particularly, A $\beta_{42}$  is more hydrophobic and viscous than A $\beta_{40}$ , and because it has high amyloid-forming properties, it easily forms oligomers (Jeong, 2017; Takahashi et al., 2017). In the present study, treatment of DOTP showed the neuroprotection against A $\beta_{42}$ -induce neurotoxicity in human neuroblastoma SH-SY5Y cells. Moreover, DOTP also induced the regulation of up-regulation of genes-related MAPK signaling, which activate A $\beta_{42}$ -treatment. Therefore, we focused on the effect of DOTP on A $\beta_{42}$  levels in limbic systems of the mouse brain. Ahlemeyer and colleagues reported that A $\beta$  was present at high levels in the 15-month-old mice and they also suggest the aged mouse as a model to study A $\beta$  plaque formation (Ahlemeyer et al., 2018). Actually, our results showed that A $\beta_{42}$  level was significantly increased in the limbic systems of 18-month-old mice (aged mice) brain compared with 10-week-old mice (young mice). Therefore, we considered that because A $\beta_{42}$  is a common feature found in both our in vitro neuroprotection model and in vivo aged mice model, the results from in vitro and in vivo are correlated. In the present study, oral administration of DOTP induced a decrease in A $\beta_{42}$  levels in the limbic systems of mice brain compared with aged mice treated with water. Many natural food-derived compounds have been reported may be useful for AD therapeutics because its effect on A $\beta$ . For example, food derived polyphenols such as rosmarinic acid, ferulic acid and curcumin showed the significantly inhibition of A $\beta$  aggregation (Ono et al., 2004) and (-)-epicatechin, abundant in various fruits, showed the reduction of A $\beta$  in a transgenic mouse model of AD (Cox et al., 2015). Thus, our results suggest that the decrease in the amount of A $\beta_{42}$  in the brain due to

administration of DOTP contributed to the improvement of spatial learning memory in aged mice. However, further mechanistic analysis of the reduction in A $\beta$ <sub>42</sub> levels in the brain following DOTP administration is needed for future investigation.

Aged mice develop cognitive impairment due to the damage of oxidative stress and neuroinflammation due to an increase in inflammatory cytokines (Perkins et al., 2021), which are relatively similar to the physiological symptoms found in AD. Neuroinflammation and overactivation of microglia are closely interrelated, and the overactivation of microglia is widely accepted as a major hallmark of ROS generation in the central nervous system (Block et al., 2007; Sorce and Krause, 2009). Intercellular ROS generation leads to the release of inflammatory mediators via MAPKs and NF- $\kappa$ B signaling molecules in the microglia (Pan et al., 2008). Microglia-derived increase in free radical generation damages neuronal cells and is implicated in neurodegeneration (Parvathenani et al., 2003). It has also been reported that excessive production of inflammatory cytokines, which are downstream molecules of MAPK and NF- $\kappa$ B signaling, induces mitochondrial dysfunction and damage to dopaminergic neurons (Hunter et al., 2007). In the present study, we confirmed that aged mice treated with water had significantly increased TNF- $\alpha$  and IL-6 levels in the serum and limbic systems of mouse brain. However, DOTP administration decreases the levels of these pro-inflammatory cytokines in aged mice. Actually, DOTP treatment induced the decrease of the increase MAPK-related genes expressions on A $\beta$ <sub>42</sub>-treated SH-SY5Y cells in this study. And we also found that DOTP administration regulated the decreases in dopamine, serotonin, and GABA levels in aged mice. Moreover, oral administration of DOTP induced a decrease in glutamate levels in the limbic systems of mouse brain. In the brain, dopamine is synthesized by specific dopamine neurons and plays several important roles in the brain through four distinct pathways: mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways. These pathways are related to the regulation of mood and aid in cognitive and motor function. Impairment of this dopaminergic system potentially causes depression (Daily et al., 2004), memory loss (Sawaguchi et al., 1988), and impaired motor control in patients with AD. Serotonin is involved in a wide range of physiological, emotional, and cognitive functions (Lucki, 1998). Serotonin dysfunction has been implicated in several psychiatric and neurological diseases across the life span (Lucki, 1998), including age-related diseases, such as AD (Blin et al., 1993). Glutamate is a key molecule in cellular metabolism, the most abundant excitatory neurotransmitter in the brain, and the principal neurotransmitter of cortical efferents. GABA is the main inhibitory neurotransmitter of the central nervous system, which allows them to communicate with both the immune (Lee et al., 2011) and nervous systems (Heja et al., 2012). Generally, defective functioning of brain glutamate and GABA is also considered responsible for AD pathology (Zhang et al., 2016). Thus, our results of the neuro-transmitter quantification indicated that oral administration of DOTP may have the potential to improve the impairment of neurotransmitters in aged mice. Taken together, our results suggest that DOTP may have anti-inflammatory effect associated with the regulating the overproduction of inflammatory cytokines during aging, thus protecting neurons through suppression of neuroinflammation.

BDNF, a member of the neurotrophin family of proteins, is an important component of brain function, including neuronal survival, learning and memory, and synaptic plasticity (Arancio & Chao, 2007). BDNF is involved in the pathophysiology of neuropsychiatric disorders, such as depression (Castrén & Kojima, 2017), anxiety (Wang et al., 2015), schizophrenia (Zhang et al., 2012), and various neurodegenerative disorders, such as Parkinson's disease (Wang et al., 2016) and AD (Hock et al., 2000). The BDNF gene product is composed of three different forms, including mature BDNF, BDNF precursor (proBDNF), and BDNF prodomain, and performs various biological functions (Hempstead, 2015). Mature BDNF is well known for its neurotrophic and neuroprotective functions, but proBDNF and pro-domains may have opposite functions to BDNF (Hempstead, 2014). Thus, the results of

many studies suggest that an imbalance or insufficient proBDNF transformation into mature BDNF plays a key role in the pathogenesis of neurodegenerative disorders by impairing neuronal plasticity (Wang et al., 2021). In fact, we found that the BDNF levels decreased and proBDNF levels increased in both the serum and limbic systems of aged mice. Moreover, treatment with DOTP ameliorated this change in BDNF and proBDNF levels. Therefore, our findings suggest that administration of DOTP improves the imbalance between BDNF and proBDNF due to aging.

## 5. Conclusion

All these findings suggest that the Desert Olive Tree Pearls (DOTP), one of the olive oil products, probably has an effect on anti-neuroinflammation and neuroprotection associated with A $\beta$  reduction and accumulation by aging and increases learning and memory and brain neurotransmitters. To best of our knowledge our present study may be the first report on the anti-neuroinflammatory effect of olive oil products (DOTP) associated with a decline of brain A $\beta$  in aged mice. It could be used as a new therapeutic agent for the treatment and prevention of neurodegenerative diseases related to the aging process. And it has also been reported that the phenolic fraction of DOTP contains antioxidants and anti-inflammatory compounds, such as hydroxytyrosol, which are associated with the reduction of A $\beta$  pathology (Leri et al., 2019; Nardiello et al., 2018). Thus, it is crucial to continue the study and discovery of the effects of DOTP and its active compounds on other aspects of neural health and pathology.

## 6. Ethics statement

Animal experiment was approved by the Animal Care and Use Committee of the University of Tsukuba (19–363).

## CRedit authorship contribution statement

**Kazunori Sasaki:** Investigation, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Hiroko Isoda:** Conceptualization, Funding acquisition, Writing – review & editing.

## Declaration of Competing Interest

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

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