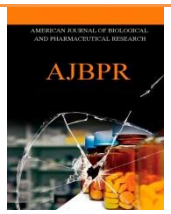
e-ISSN - 2348-2184
Print ISSN - 2348-2176

AMERICAN JOURNAL OF BIOLOGICAL AND PHARMACEUTICAL RESEARCH

Journal homepage: www.mcmed.us/journal/ajbpr



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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Article Info

Received 09/01/2015
Revised 16/02/2015
Accepted 01/03/2015

Key words: -
Rheumatoid arthritis; Olive tree extract; Inflammatory biomarkers; Pain intensity; Randomized clinical trial.

ABSTRACT

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Subjects

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

Study design and intervention

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

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

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

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

Laboratory measurements

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

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

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

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

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

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

Statistical analysis

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

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

RESULTS

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

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

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

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

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

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

Figure 1. Study flow diagram.

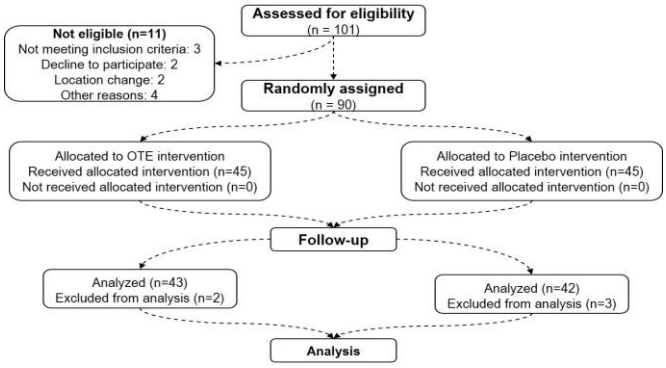


Figure 2. Degree of compliance checked by the change from baseline in urinary hydroxytyrosol excretion. Mean with 95% CI. [a]P<0.0001, between OTE-group (at 4 or 8 weeks); [b]P= 0.003, between OTE-group at 4 and 8 weeks.

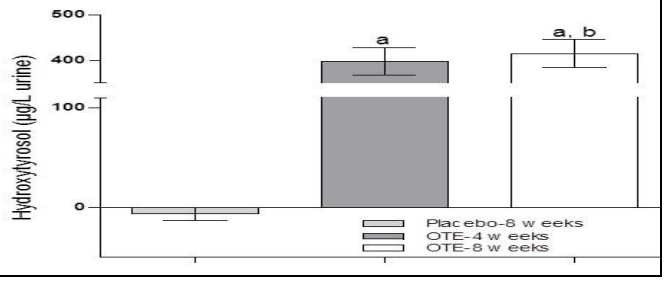
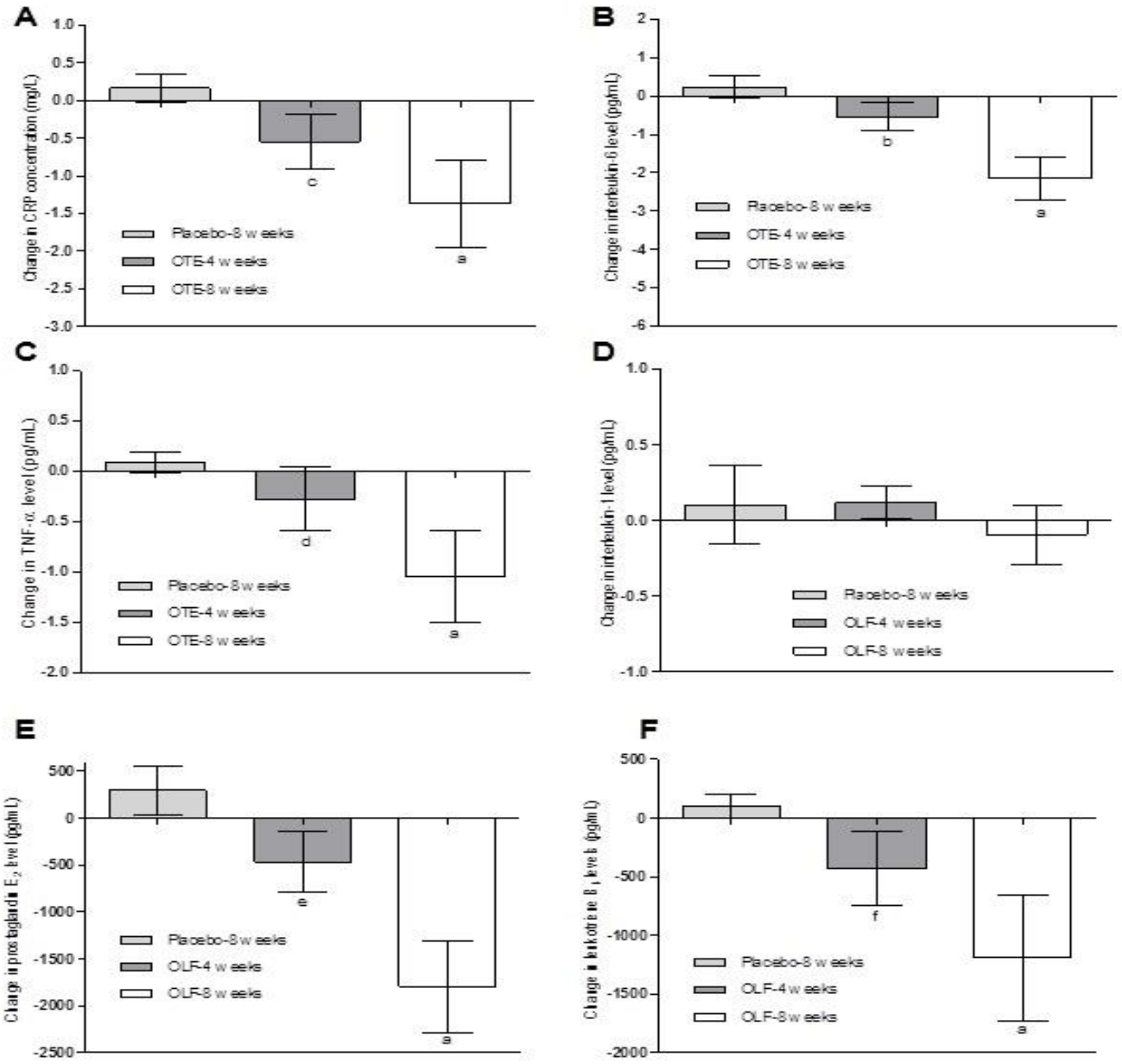


Figure 3. Change from baseline in circulating inflammatory biomarkers level in the tow study groups, (a) hs-CRP, (b) IL-6, (c) TNF-α, (d) IL-1, (e) PGE_2, and (f) LTB_4 (a). Error bars are 95% CIs. [a]*P*<0.0001, [b]*P*=0.014, [c]*P*=0.009, [d]*P*=0.0247, [e]*P*= 0.0004, and [f]*P*=0.0017 vs. placebo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



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

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

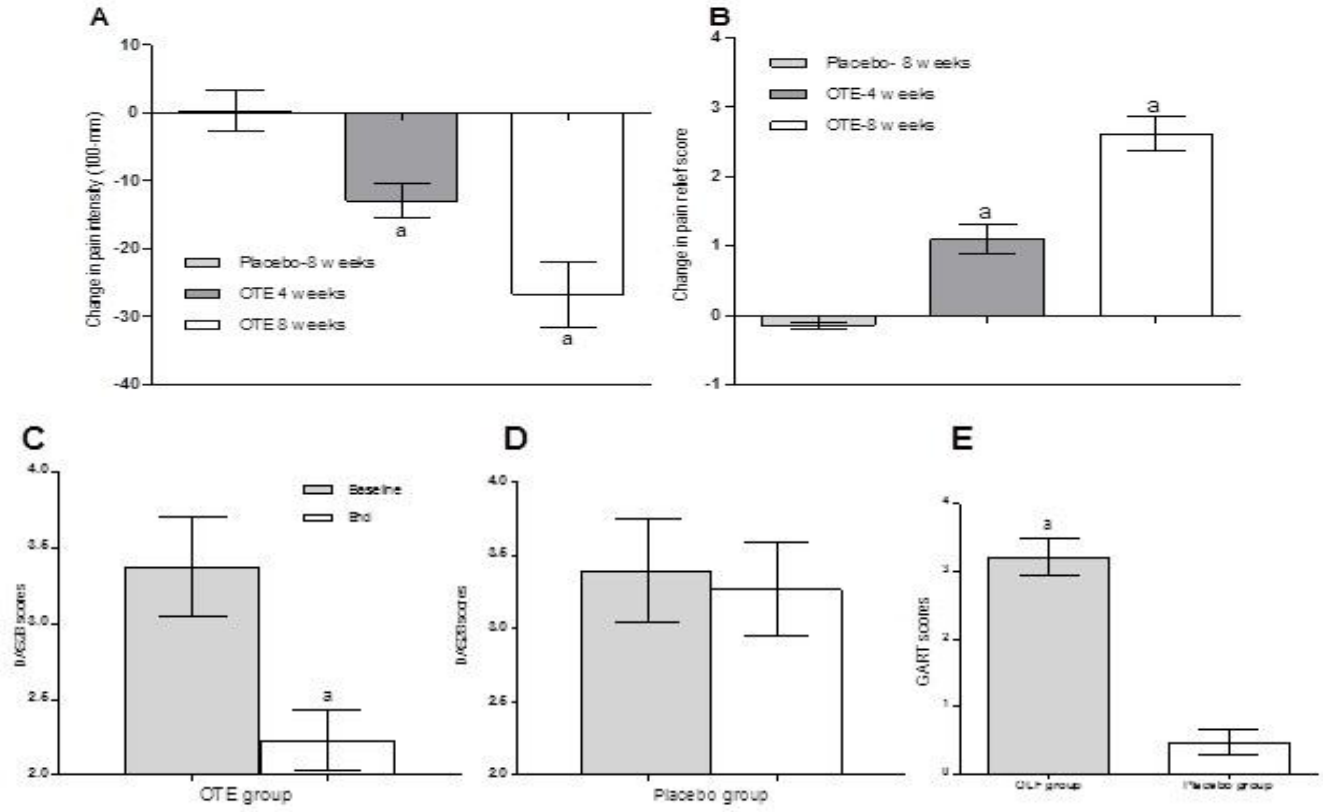


Table 1. Main constituents of olive extract (OTE) expressed in percentage (g/100g). Mean ± standard deviation.

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

Table 2. Baseline characteristics of participants.

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

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

[a] *P* value (<0.05) by independent t-test or Mann-Whitney test, as appropriate.

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

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

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

DISCUSSION AND CONCLUSION

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

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

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

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

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

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

ACKNOWLEDGEMENTS

Special tanks are due to the clinical staff, including doctors, nurses, and laboratory technicians. The authors specially thank Atlas Olive Oils Company (110 Bd. Yacoub El Mansour, 20 370-Casablanca-Morocco) for its efforts to providing olive tree extract.

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