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The effects of polyphenols in olive leaves on platelet function

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Abstract *Introduction:* The phenolic compounds of olive leaves and olive oils in the Mediterranean diet have been associated with a reduced incidence of heart disease. Accordingly, antioxidant-rich diets may prevent the deleterious effects of oxidative metabolism by scavenging free radicals, thus inhibiting oxidation and delaying atherosclerosis. The process involves phospholipase C activation and arachidonic acid metabolism, and is thought to reduce hydrogen peroxide (H_2O_2). In our study, an extract of *Olea europaea* L. leaves was used. The active phenolic compounds in this extract are part of the secoiridoid family, known for their capacity to scavenge H_2O_2. The results from this study will help to improve our understanding of effects of polyphenol antioxidants in olive leaf extract on platelet function.
Methods: Full blood examination (FBE), platelet aggregation, and ATP release were performed on samples from fasting, normal, healthy male subjects. Platelet function at increasing concentrations of oleuropein was investigated through measures of platelet aggregation and ATP release from activated platelets.
Results: Blood analysis ($n = 11$) revealed a significant dose-dependant reduction in platelet activity with olive extract concentrations of 1.0% v/v ($P < 0.001$). ATP Release showed a similar pattern ($P = 0.02$).
Conclusions: Olive leaf polyphenols derived from *O. europaea* L. leaves inhibited *in vitro* platelet activation in healthy, non-smoking males. Further bioavailability studies need to be undertaken to determine the *in vivo* effect of extract on platelet function and to validate the present results.

Background

Olive leaves and olive oils in the Mediterranean diet have been the focus of many epidemiological studies and have been shown to reduce the

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incidence of heart disease [1–3]. Adherence to the traditional Mediterranean diet is associated with a significant reduction in total mortality [4]. For example, Estruch et al. [5] found that compared with a low-fat diet, Mediterranean diets supplemented with olive oil or nuts have beneficial effects on cardiovascular risk factors. The Lyon Diet Heart Study tested a Mediterranean-type diet over long term and suggested that a comprehensive strategy to decrease cardiovascular morbidity and mortality should include primarily a cardioprotective diet [6].

The flavonoid polyphenols in olive are natural antioxidants that have a host of health beneficial effects [7]. Hydroxytyrosol and tyrosol are some of the many phenol compounds in olives that contribute to bitter taste, astringency, and resistance to oxidation [8–10]. The active phenolic compounds in the olive leaf extract are part of the secoiridoid family, known for their capacity to scavenge H_2O_2 [11]. Pignatelli et al. demonstrated that following stimulation by collagen, there is a burst of hydrogen peroxide (H_2O_2) in the process of platelet activation [12]. H_2O_2 activates the enzyme phospholipase C, which brings about arachidonic acid metabolism and platelet aggregation.

There is a growing interest in the use of natural antioxidants as bioactive components in food, and such foods have been termed ''functional foods'' [13]. Due to their ability to scavenge reactive oxygen species (ROS), antioxidants are capable of inhibiting the process of low-density lipoprotein (LDL) cholesterol oxidation subsequently decreasing the risk of cardiovascular diseases [14]. Although oxidation of LDL can be prevented by the addition of synthetic antioxidants, greater attention is now focused on natural antioxidants because of their better safety compared to that of synthetic compounds [15]. The protective effects of these diets, which are rich in fruits and vegetables, against CHD and certain cancers have been attributed partly to the antioxidants found within them, particularly to polyphenols [13].

In the current study, we used extract of *Olea europaea* L. leaves to determine the effect of olive leaf extract on platelet function in healthy human subjects.

Materials and methods

Participants

Following approval by the RMIT University Human Research Ethics Committee, 11 healthy male volunteers between the ages of 18 and 54 years were recruited for this study. All participants provided written informed consent to undertake the study.

Criteria for subject recruitment for the study included male, non-smoking, and healthy, with no history of cardiovascular disease or diabetes. Subjects were screened by means of a questionnaire, which requested information regarding the level of physical activity, medical history, diet and use of aspirin-type products, non-steroidal anti-inflammatory drugs, blood pressure and other drugs. During the testing phase, subjects were screened for aspirin intake via platelet aggregation response to the agonist, arachidonic acid. Subjects were requested not to vary their habitual diet during the study period. Subjects were required to complete a food record (diary) by food frequency questionnaire over a 7-day period. Diaries were analysed to monitor their intake of polyphenol rich food such as omega-3 polyunsaturated fatty acid rich foods, alcohol and cocoa products and to confirm that study participants were not consuming antioxidant supplements. Subjects on medication, antioxidant supplements, and who had high dietary intakes of alcohol, seafood and cocoa products were also excluded from the study.

This study did not involve any additional dietary supplement intake.

Study design

A randomised single blinded study involving 11 healthy male volunteers was conducted in the Division of Laboratory Medicine, School of Medical Sciences, RMIT University. Subjects were overnight fasted before blood collection.

Whole blood, collected on two occasions at weekly intervals, was first screened for baseline platelet count (PLT) and mean platelet volume (MPV) before analysis with and without the *in vitro* addition of commercially available olive leaf extract (Olive Leaf Australia Pty. Ltd., QLD). The extract was diluted to produce various concentrations, and the amount of platelet aggregation induced at each concentration was investigated. The optimal dose was determined from the dose response curves constructed.

Olive leaf extract

The product *Olive Leaf Extract* was provided by Olive Leaf Australia Pty. Ltd., QLD. Previous composition investigation studies by the company have shown it to be 98% pure leaf extract of *O. europaea* L. leaves. The product was stated to contain 5.40 mg/mL of oleuropein as its active ingredient. The actual product used was dark brown fluid with no solvents. It contained 50% water-extracted olive leaf extract (from fresh *O. europaea* leaves) and

50% vegetable glycerine. The product was aqueous and could therefore be added directly to the blood.

Blood collection

A total of 22 mL of venous blood per volunteer was collected by a registered nurse at RMIT Health Services (Bundoora campus) using a tourniquet and 21-gauge Vacuettes® (Greiner bio-one GmbH, Kremsmünster, Austria). Blood was collected into 2 mL tri-potassium ethylene diamine tetra-acetic acid (EDTA) (1.8 mg/mL), and 4 mL tri-sodium citrate (3.8%) tubes (Greiner bio-one GmbH, Kremsmünster, Austria). The EDTA tubes were collected before the tri-sodium citrate tubes to avoid the risk of collecting platelets activated by venipuncture. Care was taken to ensure minimal specimen handling and agitation.

Full blood examination

EDTA anti-coagulated whole blood was analysed for PLT and MPV parameters using Beckman Coulter A^c.T™ 5diff analyser (Coulter Corporation, Miami, FL, USA) within 15—20 min of venipuncture. Prior to full blood examination (FBE) each day, the performance of the analyser was validated using Coulter A^c.T™ 5diff Control Plus, low, normal and high controls (Beckman Coulter™, Miami, FL, USA). The analyser was also pre-calibrated with the A^c.T™ 5diff Cal Calibrator (Beckman Coulter™, Miami, FL, USA). The PLT and MPV parameters on all subjects were checked to ensure that they fell within a reference range for healthy adults ($150—400 \times 10^9$/L and 6—10 fL, respectively).

Sample preparation

Five volume-per-volume (v/v) concentrations of olive leaf extract were added to the blood, i.e. 0.1%, 0.3%, 0.5%, 0.7%, and 1.0% (Table 1). The concentrations of oleuropein from the extract were calculated to be 5.4 μg/mL, 16.2 μg/mL, 27.0 μg/mL, 37.8 μg/mL, and 54.0 μg/mL, respectively. Whole blood without additives (containing 0% v/v olive extract or 0 μg/mL oleuropein) was used as a control and baseline measurement.

Platelet aggregation

The appropriate volume of olive leaf extract was added to the blood in cuvettes (Chrono-Log Corporation, Philadelphia, PA, USA) and incubated at 37 °C ± 1.0 for 30 min. Siliconised stir bars (Chrono-Log Corporation, Philadelphia, PA, USA) were then added, and the samples were diluted 1:1 with pre-warmed 0.9% (w/v) saline (NaCl, BDH AnalaR®, MERCK, Pty. Ltd, VIC). The samples were then placed inside the heating block testing wells of the aggregometer and the respective electrodes were immersed into samples for 2 min at 37 °C ± 1.0 to ensure stability before analysis. Platelet aggregation was recorded for 6 min. These investigations were carried out within 2 h post-venipuncture.

Table 1 Volumes of olive leaf extract added to whole blood to achieve v/v concentrations of 0.1%, 0.3%, 0.5%, 0.7% and 1.0% olive leaf extract concentrations *in vitro*

Volume of olive leaf extract (μL)	Volume of whole blood (μL)	%Concentration of extract (v/v)	Concentration of oleuropein (μg/mL)
0	1000	0 (Baseline)	0
1	999	0.1	5.4
3	997	0.3	16.2
5	995	0.5	27.0
7	993	0.7	37.8
10	990	1.0	54.0

Statistics

Statistical analyses were performed using Microsoft® Excel 2000 (Microsoft Corporation, Redmond, WA, USA) and SPSS® version 12.0 (SPSS Inc., Chicago, IL, USA); a significance level of $P < 0.05$ was applied. Analysis of variance (ANOVA) adjusted with Sidak simultaneous tests using SPSS® were performed to find significant differences between the concentrations of olive leaf extract tested. One-way ANOVA using Microsoft® Excel 2000 was applied for results from ATP release curve.

Results

Subjects

Baseline FBE results on 11 subjects showed PLT count of $214 \pm 34 \times 10^9$/L and MPV of 8.0 ± 0.8 fL. All parameters fell within normal reference ranges. All subjects had avoided aspirin as normal platelet aggregation curves were obtained using arachidonic acid as an agonist.

Whole blood platelet aggregation

Platelet aggregation fell in a dose-dependent fashion with a steep fall between 0.7% v/v and

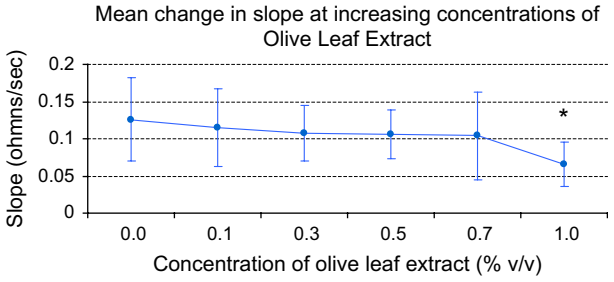

Figure 1 The effect of increasing concentrations of olive leaf extract on platelet aggregation (Ω/s). ANOVA (Sidak simultaneous tests), significantly inhibited platelet aggregation observed at 1.0% v/v olive leaf extract (adjusted $P = 0.0001$).

1.0% v/v concentrations of olive leaf extract. Sidak 95.0% simultaneous confidence intervals found 1.0% v/v (54.0 μg/mL) olive leaf extract to be significantly different from baseline (Fig. 1).

ATP release

ATP release from platelets was similarly antagonised at the optimal dose of 0.7% v/v and 1.0% v/v concentrations of olive leaf extract, determined from the earlier dose response curves (Fig. 2).

Discussion

The findings from this study demonstrate that polyphenols found in olive leaf extract are capable of inhibiting *in vitro* platelet activation in healthy, non-smoking male individuals. This finding is in agreement with previous work by Petroni et al. [16] and Karantonis et al. [17] and provides additional information as to how platelets react to olive leaf polyphenols *in vitro*. Petroni et al. found that 2-(3,4-dihydroxyphenyl)-ethanol (DHPE) components of the phenolic fraction of olive oil can inhibit platelet function and eicosanoid formation

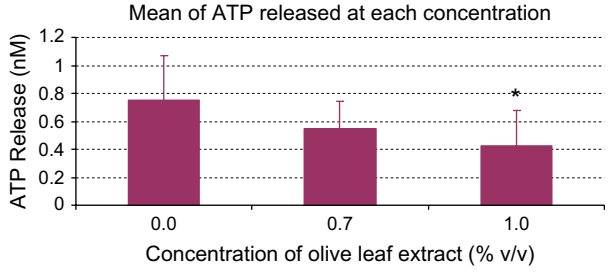

Figure 2 Mean concentration of ATP released (nM) at each concentration of olive leaf extract (% v/v). One-way ANOVA, significantly inhibited ATP release observed at 1.0% v/v olive leaf extract ($P < 0.02$).

in vitro [16]. Karantonis et al. reported that platelet-activating factor (PAF) antagonists in olive oil exert significant antiatherosclerotic activity in rabbits [17]. Both these studies (Petroni et al. and Karantonis et al.) used platelet rich plasma, whereas the current investigation used whole blood. As previously mentioned platelet analysis by the whole blood impedance method decreases the processing and handling time of the specimen, thereby preserving its integrity, and is ultimately a better indication of platelet activity *in situ*.

Previous studies have demonstrated that oleuropein and hydroxytyrosol, due to their capacity to scavenge H_2O_2, inhibited the respiratory burst of human neutrophils elicited by phorbol 12-myristate 13-acetate (PMA) in a dose-dependant fashion [18]. Hence, while the mechanism of action whereby these polyphenols are capable of inhibiting platelet function is not fully understood. It is hypothesized that this is due to their ability to scavenge H_2O_2, which is produced during the arachidonic acid metabolism cascade, which leads to platelet aggregation [12].

This *in vitro* study does not take into account several variables such as absorption and metabolism of the supplement. There are insufficient data in the literature to fully understand the bioavailability of polyphenols such as oleuropein, hydroxytyrosol and tyrosol, which are found in olive leaf extract. It is known that oleuropein is poorly absorbed due to its large size and planar configuration [19]. It has, however, been hypothesized that since oleuropein is a glycoside, it could probably access a glucose transporter like a sodium-dependent glucose transporter (SGLT1) found on the epithelial cells of the small intestine, permitting its entry into the cells. Conversely, Hollman et al. postulated that the absorption of the quercetin glycoside (a similar polyphenolic), involved active sugar transporters [20]. Gee and co-workers also showed that polyphenols are capable of interacting with SGLT1, adding further support to this theory [21].

Although oleuropein is the only active ingredient found in the olive leaf extract used in this study, reports in the literature indicate that oleuropein is not the only phenolic found in *O. europaea* L. leaves. Benavente-Garcia et al. [22] quantified various polyphenols found in *O. europaea* L. leaves and reported that oleuropein was found to be the largest fraction. Other polyphenols like hydroxytyrosol, caffeic acid, luteolin and rutin were also isolated from the leaves [22]. The flavanol catechin was also found in olive leaf extract. Data from our laboratory demonstrated that catechin is capable of inhibiting platelet function because of its antioxidant activity [23]. Hydroxytyrosol, caffeic

acid, luteolin and rutin are also established to have antioxidant activity and H_2O_2 scavenging properties [24]. Polyphenols also display a synergic behaviour in mixed form, as occurs in olive leaf extract with a high content of oleuropein and other active polyphenols [22,24—26]. Therefore, the observed platelet inhibition could be attributed to a synergistic effect of various polyphenols, as opposed to oleuropein alone. For this reason, oleuropein concentrations were not reported in the dose response curves. The investigations involving this particular olive leaf extract can therefore only be considered a primary but valuable study. Further work is required for a profile on all the polyphenols found in this olive leaf extract. The results of present study will need to be validated with *in vivo* evaluation of platelet activation such as urinary thromboxane B2 excretion and evaluation of oxidative stress markers such as isoprostanes (derived from the non enzymatic peroxidation of arachidonic acid) to provide insight into the mechanisms responsible for the inhibition of platelet function by polyphenols.

In conclusion, we demonstrate that polyphenols from olive leaf extract significantly inhibited platelet aggregation *in vitro*, possibly via their H_2O_2 scavenging properties. This has important benefits for the food industry as the antiplatelet effects in olive leaves may offer a degree of protection from thrombosis and other cardiovascular diseases. Follow-up *in vivo* studies will be undertaken to validate the results of current study and to establish the bioavailability of these polyphenols. These polyphenols could also be purified and concentrated followed by further studies on platelet aggregation to see their pure effect *in vitro* and *in vivo*.

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