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In vitro antioxidant activity of the total polyphenolic fraction of extra virgin olive oil (*Olea europaea* L.), blend type and its protective effect against rat erythrocyte haemolysis

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Summary

The intake of extra virgin olive oil (EVOO) has been associated with a lower incidence of degenerative diseases, due at least in part to its polyphenol content. The objective of this study was to examine the functional properties of the total polyphenolic fraction (TPF) of four EVOOs from Argentina. To our knowledge, there are no studies available that examine the antioxidant properties of EVOO TPF in biological membranes. The TPF was evaluated by the following assays: total polyphenol content (*TPC*), antioxidant activity (*AA*) against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the protective effect of TPF on oxidative damage induced by both hypotonic solution and the radical initiator 2,2'-azo-bis(2-amidinopropane) dichlorohydrate (AAPH) in rat erythrocytes. The *TPC* ranged from 125.7 ± 1.8 mg·kg^{-1} to 213.2 ± 2.9 mg·kg^{-1}. *AA* values were 340.0 ± 8.7 mmol·kg^{-1} to 533.2 ± 8.7 mmol·kg^{-1}. TPF moderately inhibited haemolysis induced by AAPH and hypotonic solution. These findings suggest that EVOO TPF is an important source of natural antioxidants; it has effective *AA* against diverse reactive species and protects rat erythrocytes against oxidative damage. The data obtained provide new experimental evidence of the protective role of *TPC* in EVOO derived from a blend of olive varieties against oxidative stress in biological systems.

Keywords

Argentina; extra virgin olive oil; *Olea europaea*; rat erythrocyte; polyphenol; membrane stabilization

The olive tree (*Olea europaea* L. Oleaceae) has historical and commercial importance in several countries of the world, including Argentina. Olive production is mainly located in the provinces bordering the Andes Mountains in the centre-west and north-west of the country, including Catamarca, La Rioja, San Juan and Mendoza [1]. The characteristics of the Argentine soil and climate allow the cultivation of numerous varieties of olives; among them, the local Arauco variety is the main exported variety and Arbequina is the main oil variety. The Argentine olive industry uses 75 % of its olives for the production of virgin and extra virgin olive oil (EVOO) [2]. Olive oil is composed of two fractions called saponifiable (majority) and unsaponifiable (minority). Phenolic compounds constitute the unsaponifiable fraction of olive oil and have been extensively studied to geographically characterise the origin and authenticity of oils, as well as for their health benefits [3, 4]. Around 30 phenolic compounds have been identified in EVOO, which belong to the phenylpropanoid and secoiridoid classes [5]. Among them, the most notable are simple phenols such as hydroxytyrosol and tyrosol; polyphenols such as flavonoids, apigenin, and luteolin; lignans; and secoiridoids, including oleuropein, elenolic acid, ligstroside and oleocanthal can be found in EVOO [6].

These compounds are characterised by a wide range of health benefits, including antioxidant, anti-inflammatory, anti-allergic, anti-atherogenic,

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anti-thrombotic and anti-mutagenic effects [4, 6], among many others. It should be noted that the unique properties of olive oil polyphenols have been evaluated mainly using in vitro models [7–14]. Thus, for example, previous studies examined the functional properties of a total polyphenolic fraction (TPF) of EVOO and pure hydroxytyrosol to determine their antioxidant and anti-inflammatory effects at the cellular level in endothelial cells and myoblasts [10]. Their protective effect on oxidative alterations induced by hydrogen peroxide or peroxyl radicals in human erythrocytes was also investigated [8, 12, 13]. However, to date, the functional activity of polyphenols in EVOO produced in Argentina has not been evaluated using rat erythrocytes.

Therefore, the aim of this study was to determine the antioxidant and anti-inflammatory activity of the TPF of four extra virgin olive oils, of the blend type, produced in Argentina, using rat erythrocytes. Given the polyphenolic composition of the EVOO extracts, effective protection against oxidation-inducing agents would be expected. 2,2'-azo-bis(2-amidinopropane) dichlorohydrate (AAPH) was used as a free radical initiator to induce oxidative damage in erythrocytes. The total polyphenol content (*TPC*) in the unsaponifiable fraction was also determined.

MATERIALS AND METHODS

Reagents

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, 95%), 2,2'-azo-bis(2-amidinopropane) dichlorohydrate (AAPH, ≥98%), Folin-Ciocalteu reagent, ethanol (96%), methanol (≥99.9%), sodium carbonate and other chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Indomethacin was sourced from Montpellier laboratory (Química Montpellier, Buenos Aires, Argentina). Re-distilled water was used for the preparation of the experimental solutions.

Commercial extra virgin olive oils

In this study, total polyphenols were determined in four commercial oils and their bioactivity was subsequently evaluated under in vitro conditions [15]. They were selected taking into account the availability of different EVOOs in shops in Argentina. The proportion of each varietal in the blend is unknown (Tab. 1).

Total polyphenolic fraction

The sample was prepared in a 10 ml screw-capped test tube by weighing 2.0 g of olive oil, adding 5 ml of the methanol/water extraction solution and shaking for 1 min, then centrifuging at 2 500 ×*g* for 25 min [16]. Once the hydroalcoholic fraction was separated, it was used for the determination of *TPC* by the Folin-Ciocalteu method and antioxidant activity (*AA*) by the DPPH method, as well as for bioactivity assays in cellular systems.

A vortex shaker (Velp Scientifica, Usmate, Italy), a refrigerated centrifuge Sorvall ST8R (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a microplate reader Multiskan GO (Thermo Fisher Scientific,) were used for the experimental assays.

Total polyphenol content

In this test, 50 μl of Folin-Ciocalteu reagent (1 mol·l^{-1}) was mixed with 10 μl of sample, blank or standard and 600 μl of distilled water; then 150 μl of sodium carbonate solution (200 g·l^{-1}) was added [17]. This was brought to a final volume of 1 ml with distilled water. After 2 h incubation at room temperature (cca 21 °C) in the dark, absorbance was measured at 750 nm using spectrophotometer 5100B (Shanghai Metash Instruments, Shanghai, China), in triplicate. Gallic acid was used as the standard (35–65 μg·ml^{-1}); the calibration curve equation used was:

$$y = 0.8029x + 0.0637; R^2 = 0.99874 \quad (1)$$

where y is absorbance at 750 nm, x is the concentration of phenolic compounds expressed as micrograms of gallic acid per millilitre of solution, and R is the correlation coefficient.

Tab. 1. List of commercial extra virgin olive oil blends analysed.

Trade name	Producer	Varietal composition	Province of origin
Nucete	José Nucete e Hijos, Guaymallén, Mendoza, Argentina	Arbequina, Arauco	San Juan
Cortijo	Las Perdices, Agrelo, Mendoza, Argentina	Frantoio, Nevadillo, Arauco	Mendoza
Toscana	Finca La Toscana, Maipu, Mendoza, Argentina	Arbequina, Barnea, Coratina	La Rioja
Zuelo	Finca Maipú, Maipú, Mendoza, Argentina	Arbequina, Genovesa, Picual	Mendoza

TPC was expressed as milligrams of gallic acid equivalent (GAE) per kilogram of sample (Tab. 2).

Tab. 2. Total polyphenol content of the extra virgin olive oil samples.

Trade name	*TPC* [mg·kg^{-1}]
Nucete	125.7 ± 1.8^{a}
Cortijo	143.6 ± 3.1^{a}
Toscana	144.6 ± 1.2^{a}
Zuelo	213.2 ± 2.9^{b}

Values represent mean ± standard deviation ($n = 3$). Means followed by different lowercase superscript letters in a column are significantly different (Tukey's test, $P < 0.05$).
TPC – total polyphenol content (expressed as milligrams of gallic acid equivalents per kilogram of sample).

Tab. 3. Antioxidant activity of extra virgin olive oil samples.

Trade name	*AA* [mmol·kg^{-1}]
Nucete	340.0 ± 8.7^{a}
Cortijo	384.5 ± 7.9^{a}
Toscana	393.8 ± 6.9^{a}
Zuelo	533.2 ± 8.7^{b}

Values represent mean ± standard deviation ($n = 5$). Significant differences according to Tukey's test in the same column are indicated by different letters ($P < 0.05$).
AA – antioxidant activity (expressed in millimoles of Trolox equivalents per kilogram of sample).

Antioxidant activity

A 40 μl aliquot of the working solution was mixed with 960 μl of DPPH solution (0.6 mmol·l^{-1} methanol). It was incubated for 30 min in the dark at room temperature (cca 21 °C) [18, 19]. The absorbance was read at 517 nm using spectrophotometer 5100B (Shanghai Metash Instruments) and the determination was made in quintuplicate. With the measurements taken for the samples and the blank, the percentage of inhibition (I) of the radical was calculated using the equation:

$$I = \frac{A_0 - A_S}{A_0} \times 100 \quad (2)$$

where A_S is the absorbance of the sample after 30 min and A_0 is the absorbance of the blank. Trolox was used as a positive control standard (100–800 μm).

The calibration curve equation used was:

$$y = 0.0828x + 3.4342; R^2 = 0.9955 \quad (3)$$

where y is inhibition (in percent), x is concentration of Trolox (in millimoles), and R is the correlation coefficient.

AA was expressed as millimoles of Trolox equivalent (TE) per kilogram of sample (Tab. 3).

Animals

The research protocol was approved (Res. No. 0019) by the Animal Research Ethics Committee of the Faculty of Medicine of Universidad Nacional del Nordeste (Corrientes, Argentina, 2024). Six adult male Wistar rats weighing 175–200 g (sixty days old) from the laboratory animal facility at Universidad Nacional del Litoral (Santa Fe, Argentina), were used. The rats were housed under controlled environmental conditions (23 ± 2 °C temperature, 60 ± 10 % humidity, 12/12 hours light/dark cycle). Animals received food (Ganave, Mar del Plata, Argentina) and water ad libitum. At the time of euthanasia, blood was collected from all anaesthetised animals (ketamine and xylazine, 75–100 mg·kg^{-1}). The samples were centrifuged to obtain the erythrocytes as described in the following section.

Tests on cellular systems

Hypotonic solution-induced haemolysis

To assess the stabilising capacity of the TPF, the technique previously described by other researchers was used, with some modifications [20, 21]. Blood was collected from healthy rats, centrifuged at 800 ×g and the red blood cells were washed with phosphate buffer saline, pH 7.4. The final cell suspension (40 %) of red blood cells was then prepared. For the assay, aliquots of the erythrocyte suspension were mixed with 5 ml (final volume) of a hypotonic solution (9 g·l^{-1} NaCl) containing the TPF of the EVOOs at concentrations of 100 μg·ml^{-1}, 200 μg·ml^{-1}, and 400 μg·ml^{-1}. Control samples were mixed with drug-free hypotonic solution. For the haemolysis control (100 %), the TPF was replaced with an equivalent volume of dimethyl sulfoxide (DMSO). The mixtures were incubated for 20 min at room temperature (cca 21 °C), centrifuged for 3 min at 800 ×g and then the absorbance of the supernatant was measured at 540 nm using spectrophotometer 5100B (Shanghai Metash Instruments). The commercial anti-inflammatory drug indomethacin and gallic acid were used as controls (100 μg·ml^{-1}, 200 μg·ml^{-1}, 300 μg·ml^{-1}). Five independent experiments were used for these calculations. The haemolysis (H) percentage was calculated using the formula:

$$H = \frac{A}{B} \times 100 \quad (4)$$

where A is the absorbance of the test sample and B is the absorbance of the haemolysis control.

Oxidative haemolysis inhibition assay

For this assay, AAPH reagent was used as a stable radical generator. Different concentrations of the TPF of the EVOOs (100 μg·ml^{-1}, 200 μg·ml^{-1} and 400 μg·ml^{-1}) were mixed with the red blood cell suspension and the 50 mmol·l^{-1} AAPH solution previously activated at 40 °C for 10 min [21]. The reaction mixture was incubated at 37 °C for 75 min, then centrifuged at 800 ×g for 3 min. The absorbance of the supernatant was evaluated at 545 nm using spectrophotometer 5100B (Shanghai Metash Instruments). Gallic acid was used as a reference antioxidant (50–300 μg·ml^{-1}). The percentage inhibition (I_H) was determined using the formula:

$$I_H = 100 - \left(\frac{A_0 - A_S}{A_0} \times 100\right) \quad (5)$$

where A_0 is the absorbance of the control (without EVOO extract) and A_S is the absorbance in the presence of the EVOO TPF.

Statistical analysis

All assays were performed in triplicate or quintuplicate and the results represent the means ± standard deviation from up to six independent extractions. The data were evaluated by analysis of variance (ANOVA) and Tukey's test for comparison of means, with a significance level of $\alpha = 0.05$. Statistical analyses of data were carried out using the InfoStat software (InfoStat Group, National University of Córdoba, Córdoba, Argentina).

RESULTS AND DISCUSSION

In this study, the phenolic compounds of four different commercial EVOO blends were quantified. Factors influencing the polyphenol content of EVOO include the variety and ripeness of the olives and soil conditions, among others [2]. The extraction and storage conditions of the oil also have a substantial effect on the concentration of phenolics in the final product [3].

Tab. 2 shows the *TPC* of the TPF of the 4 EVOOs analysed (expressed as GAE). The values range from 125.7 ± 1.8 mg·kg^{-1} to 213.2 ± 2.9 mg·kg^{-1}). These results are within the range of values reported by other authors for EVOOs made from Coratina, Barnea or Arbequina and their blends (Barnea and Coratina) [2, 22]. The amount of phenolic compounds was higher in the extract of the EVOO of the Zuelo brand, made from the blend of Arbequina, Genovesa and Picual, while the EVOO of the Nucete brand, made from the Arauco and Arbequina varietals, presented the lowest *TPC* value. Studies conducted on varieties grown in other countries reported *TPC* levels similar to those obtained in this research, ranging from 197 mg·kg^{-1} to 318 mg·kg^{-1} in other varieties such as Picual, Frantoio and Nevadillo [3, 6]. These small differences can be attributed to the environmental characteristics of each growing area, genotype and the conditions in which the oils are obtained [23].

As shown in Tab. 3, EVOO TPFs showed radical scavenging activity (expressed as Trolox equivalent). The TPF obtained from Zuelo had the highest activity against the DPPH radical, followed by Toscana, Cortijo and Nucete ($P < 0.05$). The values found for the TPFs in this test ranged from 340.0 ± 8.7 mmol·kg^{-1} to 533.2 ± 8.7 mmol·kg^{-1} (Tab. 3). It is important to highlight the relationship between *TPC* and *AA* of the EVOO TPFs studied. However, these TPF of EVOO consist of a heterogeneous mixture of compounds, with hydroxytyrosol and hydroxytyrosol acetate (catechol phenolic compounds) exhibiting the highest DPPH radical scavenging activity [2, 23–25].

To verify the biological relevance of the antioxidant activity of the TPF of EVOOs, the rat erythrocyte was used as a cellular model system. Red blood cells (RBC) haemolysis is considered an appropriate model for determining oxidative damage caused by free radicals to the cell membrane and for evaluating anti-inflammatory action [19]. Thus, exposure of RBCs to harmful substances, such as hypotonic medium or APPH, induces alterations of their membrane and causes both haemolysis and haemoglobin oxidation. The haemolytic effect of the hypotonic solution is linked to excessive fluid accumulation within the RBCs, which causes their membrane to rupture and subsequent damage due to free radical-induced lipid peroxidation [20].

Haemolytic assays showed that the *TPC* present in TPFs does not induce haemolysis, i.e. the phenolic compounds do not cause a lytic effect on RBCs in the concentration range evaluated. Fig. 1 and Fig. 2 show that, in the presence of a hypotonic solution or AAPH, the percentage of haemolysis decreased in a concentration-dependent manner when TPF was applied (between 100 μg·ml^{-1}, 200 μg·ml^{-1} and 400 μg·ml^{-1}). Nucete TPF showed the highest percentage of inhibition of haemolysis induced by hypotonic solution (68.2 ± 1.4 %).

Meanwhile, TPF Toscana showed higher activity than the other samples in the APPH assay (71.7 ± 1.0 %). As in previous studies, the other TPFs also showed suppression of haemolysis induced by hypotonicity or AAPH [8–13]. However,

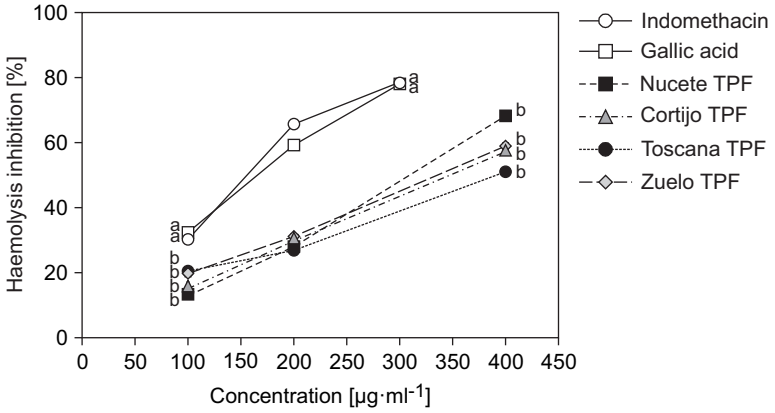


Fig. 1. Effect of the total polyphenolic fraction concentration of extra virgin olive oil samples on hypotonicity-induced haemolysis.

Data are represented as means ± standard deviation (n = 5). Means with different letters within the same concentration were significantly different ($P < 0.05$).
TPF – total polyphenolic fraction.

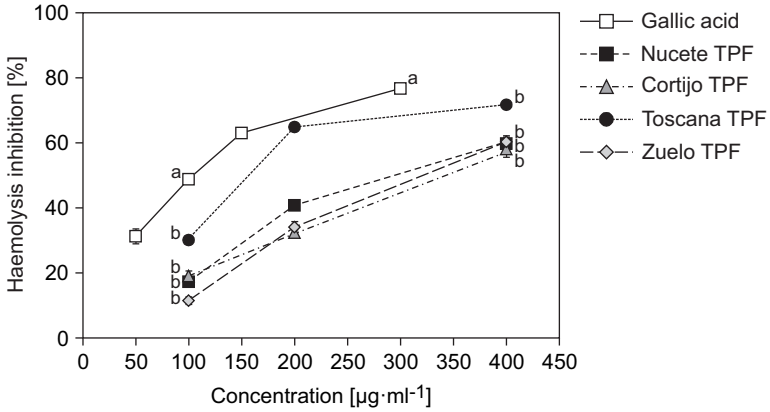


Fig. 2. Effect of the total polyphenolic fraction concentration of extra virgin olive oil samples on AAPH-induced haemolysis in erythrocytes.

Data are represented as means ± standard deviation (n = 5). Means with different letters within the same concentration were significantly different ($P < 0.05$).
TPF – total polyphenolic fraction.

in both methods applied, the bioactivity of TPFs was lower than that of the controls, indomethacin and gallic acid, respectively. This suppression is probably due to the fact that the peroxyl radicals generated by AAPH were eliminated by the phenolic compounds in the aqueous phase until the antioxidant agent was depleted [8–14]. However, the results obtained indicate that there is no relationship between the inhibition of AAPH-induced oxidative haemolysis and the percentage content of phenolic compounds.

This is probably because the *TPC* in TPF can interact with each other, increasing or decreasing their antioxidant capacity [11, 14, 18]. According to the scientific literature, this capacity depends on factors such as the reactivity of hydrogen or electron donor agents, the ability to stabilise and delocalise unpaired electrons originating from subsequent reactions, reactivity towards other antioxidant compounds, and the chelating potential of transition metals [24–26].

Hydroxytyrosol is among the main phenolic constituents of the *TPC*s studied. This compound has been reported to be a scavenger of aqueous peroxyl radicals near the surface of the RBC membrane [26]. Oleuropein has also been reported to act as a scavenger of lipid peroxyl radicals that propagate the chain within biological membranes [26]. Toscana is a blend of Coratina, Arbequina and Barnea. A previous study reported that these

varieties contain 3.06 $mg \cdot kg^{-1}$, 3.0 $mg \cdot kg^{-1}$ and 1.8 $mg \cdot kg^{-1}$ of hydroxytyrosol, respectively [22]. However, another study analysed the hydroxytyrosol content of different varieties produced in the same region, including those studied here, and detected a lower hydroxytyrosol content, which would be associated with less haemolysis suppression [2].

Therefore, it seems likely that the protective activity of *TPC* is mainly related to the total hydroxytyrosol content. This is because hydroxytyrosol has a different antioxidant potential related to its chemical structure and probably a different ability to bind or interact with the RBC membrane [8, 10, 12, 13]. However, in addition to hydroxytyrosol, TPFs contain other minor compounds that may affect their bioactivity in various ways. Therefore, it is likely that both the antioxidant capacity and the ability to protect RBCs depend not only on the overall phenolic composition of TPFs, but also on the type and amount of individual phenolic compounds, mainly hydroxytyrosol [11, 14]. Flavonoids may also be involved in this effect, which would partly explain the differences observed in the various erythrocyte assays [13]. The most common flavonoids detected in EVOO are apigenin and luteolin, both of which have antioxidant and anti-inflammatory properties [26–28].

CONCLUSIONS

The results showed the antioxidant and free radical scavenging capacity of the TPF of various EVOOs (blended type), both in chemical assays and in cellular models, in vitro. This effect can be attributed in part to the higher levels of phenolic compounds, and to the chemical profile of the TPF of each particular EVOO. The latter factor depends on both the varietals present in the blend and the method of oil extraction, which favours the preservation of phytocompounds with specific attributes in each of the EVOOs. To the best of our knowledge, this is the first work to report on the anti-haemolytic properties of the TPF of EVOO in rat biological membranes.

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