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Determination of pentacyclic triterpenes and polyphenols from table olives in colon and plasma and their chemopreventive effects on 1,2-dimethylhydrazine-induced preneoplastic lesions in rat colon

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Table olives are a rich dietary source of pentacyclic triterpenes (PT) and polyphenols (P), many of which have demonstrated significant antiproliferative and proapoptotic activities. This study aimed to evaluate the effect of this food on the early stages of colon carcinogenesis induced by 1,2-dimethylhydrazine (DMH) at 20 mg kg^{-1}. Male Sprague-Dawley rats were administered either water or a suspension of Arbequina table olives (OA; 3.85 g kg^{-1}) by gavage at 10 mL kg^{-1} for 49 days. Each group was then divided into two subgroups that received subcutaneous injections of the carcinogen (DMH+/Olives- and DMH+/Olives+) or the solvent (DMH–/Olives– and DMH–/Olives+) on days 8, 15, and 22. Analysis by LC-MS of AO enabled us to calculate the administered doses of PT (12.38 mg kg^{-1}) and P (4.02 g kg^{-1}) as well as the colon content of these compounds. At the end of the intervention, we found 5.1% of PT and 0.2% of P of the administered dose in the colonic content of the DMH+/Olives+ group. The highest concentrations were for maslinic and oleanolic acids (321 ± 67 and 84.8 ± 14.3 nmol g^{-1}, respectively) followed by hydroxytyrosol (3.31 ± 0.24 nmol g^{-1}). The supplementation with AO reduced aberrant crypt foci by 54.1%, and mucin depleted foci by 35.7% compared to the control group. The daily consumption of table olives exerts chemopreventive activities by reducing preneoplastic intestinal lesions, which might be explained, at least in part, by the significant concentrations of PT and P remaining in the colon.

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1. Introduction

Foods derived from *Olea europaea* L. have attracted considerable scientific interest due to their distinctive profile of bioactive compounds, which have been found to confer significant health benefits.[1] Olive oil has been extensively investigated and has been shown to have cardioprotective, anti-inflammatory and antitumour properties, among others.[1] In contrast, table olives have received relatively little attention, despite sharing the same origin as olive oil and even having a higher phytochemical content.[2] Consequently, our recent research has focused on how dietary supplementation with table olives can elicit health benefits by providing an adequate supply of bioactive compounds.[3–5] Arbequina table olives (AO) are rich in pentacyclic triterpenes (PT, approximately 3 g kg^{-1}) and polyphenols (P, around 1 g kg^{-1}).[6] Among them, maslinic acid and hydroxytyrosol stand out for their high concentrations and significant antitumoral activities.[7,8] These chemopreventive effects have been described in human colon cancer cell lines for PT,[9,10] as well as for P.[11,12] The antitumor effects against colorectal cancer have also been observed *in vivo* for PT[13–15] as well as for P.[16,17] Furthermore, it is widely established that adherence to a "healthy" dietary pattern is associated with a lower risk of developing colorectal cancer.[18] Notably, table olives are one of the common foods in the Mediterranean diet that are characterised by a high content of PT and P. Therefore, we set out to study whether regular intake of the right amount of AO could achieve protective effects on colon cancer.

Consequently, an *in vivo* model of colon cancer was established using Sprague-Dawley rats in which preneoplastic lesions are induced by 1,2-dimethylhydrazine (DMH). This carcinogen leads to the formation of aberrant crypt foci (ACF), and mucin-depleted foci (MDF) that are dysplastic lesions exhibiting characteristics comparable to those observed in the most common form of intestinal neoplasia, sporadic non-

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familial colorectal cancer.[19,20] Using this model, we investigated the effect of AO at a dose of 3.85 g of destoned olives per kg body weight which is the equivalent to human consumption of 30 AO and double the serving size recommended by the Mediterranean diet pyramid.[21] To this aim, the concentrations of PT and P were determined in AO as well as in plasma and colon content. Finally, we studied the effect of the regular intake of AO on the number of preneoplastic lesions in the colon of rats. The reductions of these carcinogenic markers could provide a scientific basis for recommending table olive supplementation as a dietary source rich in bioactive compounds with chemopreventive properties.

2. Materials and methods

2.1. Chemicals and reagents

Hydroxytyrosol and hydroxytyrosol acetate were supplied by Seprox Biotech S.L. (Madrid, Spain). Caffeic acid, catechol, *o*-coumaric acid, *p*-coumaric acid, 2-(3-hydroxyphenyl) ethanol (Internal Standard (IS) of P), erythrodiol, maslinic acid, oleuropein, (+)-pinoresinol, quercetin, rutin, salidroside, vanillic acid, and verbascoside were supplied from Sigma-Aldrich (Tres Cantos, Spain). Apigenin, betulinic acid (IS of PT), luteolin, luteolin-7-*O*-glucoside, oleanolic acid, tyrosol, ursolic acid, and uvaol were purchased from Extrasynthèse (Genay, France). L-Ascorbic acid, 10% buffered formalin pH 7.4, phosphate-buffered solution pH 7.4 (PBS) were provided by Sigma-Aldrich. Acetone, acetonitrile, isopropanol, methanol, and tetrahydrofuran were from Panreac Química S.L.U. (Castellar del Vallès, Spain). Ethyl acetate and ethanol were acquired from J. T. Baker (Deventer, Holland) and glacial acetic acid was from Merck (Darmstadt, Germany). All chemicals were of analytical grade, and the solvents were of liquid chromatography-mass spectrometry (LC-MS) grade. Ultrapure water was employed in all experiments (Millipore, Madrid, Spain).

2.2. Animals and diets

Adult male rats of the Sprague-Dawley strain (7 weeks old) were supplied from the Animal House Facility of the Facultat de Farmàcia i Ciències de l'Alimentació (Universitat de Barcelona, UB). Animals were housed in cages (n = 2 per cage) and kept under controlled conditions of temperature (22 ± 2 °C), relative humidity (50 ± 10%), and a dark-light cycle of 12 h. Rats were maintained on a 2014 Teklad Global 14% Protein Rodent Maintenance diet (Envigo RMS Spain S.L., Barcelona, Spain) and water *ad libitum*. The commercial diet contained (g kg^{-1}): 150.0 protein, 40.0 fiber, 480.0 carbohydrate and 40.0 lipid, with a total metabolizable energy content of 290 kcal per 100 g of feed. All rat manipulations were performed in the morning to avoid the effects of any circadian rhythm. All animal experiments complied with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. Handling and killing were in full accordance with the ethical requirements established by the Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Ethics Committee of Animal Experimentation of the UB (ref. 106/17) and the Generalitat de Catalunya (ref. 9468).

2.3. Arbequina table olives and dose preparation

The variety of table olives used was the Arbequina from the 2016/2017 harvest (Cooperativa del Camp, Foment Maialenc SCCL, Maials, Spain). The trees of *Olea europaea* L. were cultivated in Ribera d'Ebre (Tarragona, Spain) in orchards submitted to drip irrigation. Olives were picked in the green-yellow stage of maturation and were processed as natural olives in brine following the Greek style. The composition of AO consisted of (g kg^{-1} of olives): 210 lipids, 16 proteins, 72 fiber, and 41.3 salt. The total metabolizable energy content per 100 g of olives was of 211 kcal.

AO were administered orally as a homogenous suspension at a dose of 3.85 g of destoned olives per kg of rat body weight every day for 49 days. The animal dose is equivalent to the human intake of 30 small-size Arbequina olives, calculated following the body surface area normalization method described by Reagan-Shaw *et al.*[22] Hence, Milli-Q water was added to the edible part of the olive before being carefully ground with 6 short pulses of 30 s using a Polytron homogenizer (PT 20 TS rotor, setting 5; Kinematica AG, Lucerne, Switzerland). The olive suspension was prepared every two days and kept at 4 °C protected from light.

2.4. Determination of pentacyclic triterpenes and polyphenols in Arbequina table olives

2.4.1. Simultaneous extraction of pentacyclic triterpenes and polyphenols. The content of PT and P in AO was determined in 6 independent aliquots of the homogenous suspension, as previously described.[6] Hence, 1 g of the homogeneous olive suspension was mixed with 6 mL of methanol–ethanol (1 : 1; v/v) that contained betulinic acid (IS of PT) and 2-(3-hydroxyphenyl) ethanol (IS of P). The mixture was vigorously vortex-mixed for 5 min and centrifuged at 3345*g* at 4 °C for 30 min (Megafuge 1.0R, Heraeus Instruments GmbH, Hanau, Germany). After removing the supernatant, the pellet was submitted to two additional extractions with 3 mL of methanol–ethanol (1 : 1; v/v). The pooled supernatants were centrifuged at 25 000*g* for 30 min at 2 °C (Centrifuge 5417R, Eppendorf Ibérica S.L., San Sebastián de los Reyes, Spain) and filtered with a 0.45 μm PTFE syringe filter. Prior to LC-MS analysis, the filtrate was submitted to a 1 : 50 dilution with methanol 80% to quantify maslinic acid, oleanolic acid, hydroxytyrosol, verbascoside, and luteolin. The other polyphenols were measured in a 1 : 4 dilution. Finally, erythrodiol, ursolic acid, and uvaol were assessed in a non-diluted sample.

2.4.2. Instrumentation. Analyses were performed in an Acquity ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to an Applied Biosystems API 3000 triple quadrupole MS (AB Sciex, Toronto, ON, Canada). The UPLC autosampler was refrigerated and kept at 10 °C, with the automatic injector set at 10.0 μL. The binary pump provided a flow rate of 0.80 mL min^{-1}. The equipment was controlled by the Analyst software 1.4.2 (AB Sciex)

which also carried out data acquisition and processing. The instrumentation was located at the Scientific and Technological Centers of the UB (CCiTUB).

2.4.3. LC-APCI-MS determination of pentacyclic triterpenes. The separation of PT was performed on a Zorbax Eclipse PAH column (150 mm × 4.60 mm, 3.50 μm) kept at 40 °C, and protected with a guard column of the same material (12.5 mm × 4.60 mm, 5.00 μm) (Agilent Technologies, Santa Clara, CA, USA). The mobile phase was formed by a phase A consisting of Milli-Q water with acetic acid at 0.05%, and a phase B of methanol with acetic acid at 0.05%. The elution program was: 0 min, 17% A and 83% B; 22.5 min, 17% A and 83% B; 23.0 min, 0% A; and 100% B. The column was washed for 4 min before setting the gradient back to the initial conditions. A 6-minute delay was implemented between injections to ensure equilibration of the column. Ionization of acids was achieved using an atmospheric pressure chemical ionization (APCI) source that operated in negative ion mode at 500 °C from 0 to 17 min, while alcohols were ionized at 450 °C in positive ion mode from 17 to 23 min. PT were determined in the selected ion monitoring (SIM) mode, monitoring the $[M - H]^-$ at *m*/*z* 471.3 for maslinic acid and 455.3 for oleanolic acid, ursolic acid, and betulinic acid (IS), with a dwell time of 1000 ms for all them. Triterpenic alcohols were measured at $[M + H–H_2O]^+$ at *m*/*z* 425.3 with a dwell time of 4000 ms.

2.4.4. LC-ESI-MS/MS determination of phenolic compounds. Analysis was carried out in a Zorbax Eclipse XDB-C18 analytical column (150 mm × 4.60 mm, 5.00 μm) coupled to a guard column (12.5 mm × 4.60 mm, 5.00 μm) of the same material, both from Agilent Technologies. The temperature of the column was kept at 30 °C. Mobile phase A comprised Milli-Q water with 0.025% acetic acid, while phase B was acetonitrile with 5% acetone. The elution gradient was: 0 min, 97.5% A and 2.5% B; 2 min, 97.5% A and 2.5% B; 3 min, 90% A; and 10% B; 8 min, 35% A and 65% B; 8.5 min, 0% A and 100% B. Carryover was prevented by programming three column wash cycles of 2 min with 100% organic mobile phase interleaved with re-equilibration to initial conditions. Before the next injection, the system was re-equilibrated at initial conditions for 6 min. The MS analysis was carried out using an electrospray ionization (ESI) source, with temperature set at 350 °C, and multiple reactions monitoring (MRM). P were detected in negative mode with 60.0 ms as dwell time for the MRM transitions indicated in Table 1.

2.4.5. Identification and quantification of pentacyclic triterpenes and polyphenols in table olives. The identification was performed by comparing the retention times and the corresponding *m*/*z* to those of the analytical standards. Individual stock solutions of each PT and P were first prepared in 80% methanol at 250 μM and stored at −20 °C. These stocks were used to make two different working solutions, one containing 5 PT and another with 17 P, prepared at the concentrations of 1.00; 10.0; and 50.0 μM.

Quantification was performed using the standard addition method. Therefore, the working solutions were spiked into the filtered olive supernatant diluted either at 1 : 50 or 1 : 4 to match the samples. Then, calibration standards were at concentrations of 0; 0.5; 1.0; 1.5; 2.0; and 3.0 μM. Both working solutions and calibration standards were freshly prepared before each analysis. In every analytical run, a complete set of standards, including a reagent blank, was injected. Results were expressed in milligrams per kilogram of destoned olives (mg kg^{-1}).

Calibration standards were used to validate the process following the recommendations of the FDA[23] for accuracy, precision, linearity, and carry-over. Matrix effect was determined as described by Matuszewski[24] in terms of relative standard line slope.

2.5. Experimental design

Rats were stratified by body weight and distributed into 4 groups to avoid differences in the mean initial weight. The following experimental groups were established: DMH−/Olives− (negative control: no carcinogen/no test agent; n = 3), DMH−/Olives+ (no carcinogen/Arbequina table olives; n = 3), DMH+/Olives− (positive control: DMH/no test agent; n = 6) and DMH+/Olives+ (DMH/Arbequina table olives; n = 6). Animals in the Olives+ groups were orally administered 3.85 g of destoned olives per kg rat body weight through a stainless-steel animal feeding tube (18 gauge × 76 mm, ref. FTSS-18S-76, Instech Laboratories, Inc., PA, USA) at 10 mL kg^{-1} every day for 49 days, whereas the ones assigned to the Olives− groups only received water. The DMH+ groups were given a subcutaneous injection of the carcinogen (20 mg DMH per kg dissolved in EDTA 1 mmol L^{-1}, pH 6.5; 1 mL kg^{-1}) on days 8, 15, and 22 of the experimental period. The rats in the DMH− groups were injected subcutaneously with the solvent (EDTA 1 mmol L^{-1}, pH 6.5; 1 mL kg^{-1}). The carcinogen was freshly prepared immediately before each administration.

Throughout the study, the body weight was registered daily, whereas food and water consumption were checked once per week. Food consumption was monitored by weighing the pellets before being placed on the cage grill, and any remaining food was weighed again after one week. Additionally, the cages were inspected daily, and any residual food that had fallen to the bottom was collected and weighed. This approach allowed us to accurately account for any potential food spillage and ensure reliable calculations of total food intake. Food conversion efficiency (FCE) was determined as a percentage by dividing the weekly body weight gain by the weekly food consumption.

2.6. Sample collection

After 49 days of supplementation with AO, samples were collected between 17–19 h following the last oral administration. Hence, overnight fasted rats were anesthetized with 90 mg kg^{-1} of ketamine (Imalgene 100 mg mL^{-1}, Merial Laboratorios S.A., Barcelona, Spain) and 10 mg kg^{-1} of xylacine (Rompun 20 mg mL^{-1}, Bayer Hispania S.L., Barcelona, Spain). Blood samples were obtained by cardiac puncture and collected into 4 mL EDTA-K_3 tubes (Sarstedt, Nümbrecht, Germany). The samples were then centrifuged at 3345*g* for 15 min at 4 °C

Table 1 Concentrations of pentacyclic triterpenes and polyphenols in Arbequina table olives as well as plasma and colon content obtained between 17 to 19 h after the last oral administration of 3.85 g of destoned olives per kg of body weight for 49 days

			Plasma (nM)		Colon content (nmol g^{-1})	
Analyte	*m/z*	Arbequina table olives (mg kg^{-1})	DMH−/Olives+	DMH+/Olives+	DMH−/Olives+	DMH+/Olives+
Pentacyclic triterpenes						
Maslinic acid	471.3	2342 ± 82.2	3.64 ± 0.27	10.1 ± 2.74*	55.5 ± 5.22	321 ± 67.0*
Oleanolic acid	455.3	862 ± 44.0	2.25 ± 0.17	3.19 ± 0.58	22.8 ± 1.30	84.8 ± 14.3*
Ursolic acid	455.3	<LOD	φ	φ	φ	φ
Erythrodiol	425.3	10.4 ± 0.11	φ	φ	0.13 ± 0.01	1.19 ± 0.27*
Uvaol	425.3	<LOD	φ	φ	φ	φ
Total		**3214 ± 123**	**5.89 ± 0.10**	**13.2 ± 3.32**	**78.4 ± 6.52**	**407 ± 81.2**
Phenolic compounds						
Apigenin	269.0/117.1	4.52 ± 0.17	φ	φ	0.47 ± 0.07	0.37 ± 0.02
Caffeic acid	179.1/135.1	4.64 ± 0.14	φ	φ	0.76 ± 0.23	0.73 ± 0.19
Catechol	108.8/91.0	φ	φ	φ	φ	φ
o-Coumaric acid	163.2/119.2	<LOD	φ	φ	φ	φ
p-Coumaric acid	163.2/119.2	5.65 ± 0.10	φ	φ	0.92 ± 0.20	1.83 ± 0.67
Hydroxytyrosol	153.2/122.8	475 ± 11.8	2.11 ± 0.02	2.18 ± 0.54	1.36 ± 0.30	3.31 ± 0.24*
Hydroxytyrosol acetate	195.0/59.0	26.9 ± 0.71	φ	φ	<LOQ	<LOQ
Luteolin	285.2/133.2	89.6 ± 2.97	φ	φ	0.49 ± 0.19	0.43 ± 0.26
Luteolin-7-*O*-glucoside	447.3/285.2	11.1 ± 1.74	φ	φ	<LOQ	<LOQ
Oleuropein	539.5/275.0	12.6 ± 0.24	φ	φ	φ	φ
(+)-Pinoresinol	357.2/151.1	3.08 ± 0.22	φ	φ	<LOQ	<LOQ
Quercetin	301.1/151.1	6.49 ± 0.16	φ	φ	0.23 ± 0.03	0.23 ± 0.07
Rutin	609.5/301.0	26.0 ± 3.14	φ	φ	<LOQ	<LOQ
Salidroside	299.2/119.2	17.4 ± 0.98	φ	φ	φ	φ
Tyrosol	137.1/106.2	23.1 ± 0.58	φ	φ	1.17 ± 0.10	1.46 ± 0.19
Vanillic acid	167.0/152.0	3.56 ± 0.06	φ	φ	1.00 ± 0.13	1.12 ± 0.11
Verbascoside	623.5/161.3	334 ± 30.8	φ	φ	0.16 ± 0.002	0.20 ± 0.01
Total		**1043 ± 46.9**	**2.11 ± 0.02**	**2.18 ± 0.54**	**6.56 ± 0.08**	**9.68 ± 0.77**

Results are expressed as means ± SEM in the analysis of table olives (n = 6) as well as the samples of plasma and colon content from DMH−/Olives + (n = 3) and DMH+/Olives + (n = 6) groups. Pentacyclic triterpenes and polyphenols in plasma and colon content were compared by Student's unpaired t test. Different from DMH− rats: *$p < 0.05$; φ, not detected; LOD, limit of detection (signal-to-noise ratio of 3 : 1); LOQ, limit of quantification (signal-to-noise ratio of 5 : 1).

(Megafuge 1.0R) to separate the plasma, which was kept at −80 °C until analysis by LC-MS.

Immediately following blood collection, a gross necropsy was performed. The liver, brain, lungs, kidneys, testicles, spleen, heart, and thymus were removed, separated from any adherent mesenteric tissue, and their wet weights were immediately recorded. Results are expressed as organ weight relative to 100 g of body weight (%).

Then, the colon was excised, and the intestinal lumen was flushed with 1 mL of cold PBS (pH 7.4) to obtain the intestinal content that was stored at −80 °C until analysis by LC-MS. Subsequently, the colonic tissue was washed with PBS, trimmed from mesenteric tissue, and divided into three segments of similar length: proximal (close to the cecum), medial, and distal (close to the rectum).

2.7. Determination of pentacyclic triterpenes and polyphenols in plasma and colon content

PT and P were quantified in both DMH−/Olives+ and DMH+/Olives+ groups using the validated methods established for plasma by Kundisová *et al.*[25] and colon content by Lozano-Mena *et al.*[26]

2.7.1. Simultaneous extraction of pentacyclic triterpenes and polyphenols in plasma. Briefly, 200 μL of plasma was mixed with 10 μL of ascorbic acid (10%, v/v), 10 μL of acetic acid (1%, v/v), and 10 μL of betulinic acid (IS of PT) and 10 μL of 2-(3-hydroxyphenyl) ethanol (IS of P). Ethyl acetate (2 mL) was added, and the mixture was strongly mixed in a vortex (5 min), placed into an ultrasonic bath (10 min), and centrifuged (3345*g*, 10 min, 2 °C; Megafuge 1.0R). After centrifugation, a second extraction was carried out. Subsequently, ethyl acetate extracts were pooled, added with 10 μL of ascorbic acid, and evaporated to dryness (Concentrator 5301). Then, the residue was dissolved in 100 μL of methanol (80%, v/v), shaken in a vortex (5 min), sonicated (2 min), and centrifuged (25000*g*, 30 min, 4 °C; Centrifuge 5417R), and the clear supernatant was placed in an amber vial.

2.7.2. Simultaneous extraction of pentacyclic triterpenes and polyphenols in colon content. Colon content (1 g) was mixed with 4 mL of methanol–water (80 : 20; v/v) with betulinic acid (IS of PT) and 2-(3-hydroxyphenyl) ethanol (IS of P). Samples were homogenized using 2 pulses of 30 seconds each in a Polytron homogenizer (PT 10 TS rotor, setting 6). The tip was rinsed four times with 1 mL of 80% methanol to collect the remaining solid particles and to obtain a final pooled volume of 8 mL. Then, samples were shaken in a vortex (5 min), sonicated (10 min), and centrifuged (16400*g*, 30 min, 2 °C; Centrifuge 5417R). Before LC-MS analysis, the clear

supernatant was filtered with a 0.45 μm PTFE syringe. For the analysis of maslinic acid and oleanolic acid, the filtered supernatant was diluted 1 : 25. The non-diluted filtered supernatant was used for the determination of the other compounds.

2.7.3. Instrumentation. Analyses were carried out in an Agilent 1260 LC (Agilent Technologies) coupled to an AB Sciex QTRAP 4000 MS (AB Sciex) located in the CCiTUB. The LC was equipped with an autosampler at 10 °C, and a quaternary pump that delivered a flow rate of 0.80 mL min^{-1}. The Analyst software version 1.6.2 (AB Sciex) controlled the system and analysed the data.

2.7.4. LC-APCI-MS determination of pentacyclic triterpenes. The LC-APCI-MS conditions were based on the ones described in the subheading 2.4.3 except for some adjustments. The gradient elution was modified to allow the separation of the metabolites in plasma and colon content as follows: 0 min, 95% A and 5% B; 2 min, 95% A and 5% B; 4 min, 40% A and 60% B; 19 min, 17% A and 83% B; 40 min, 17% A and 83% B; 42.5 min, 0% A and 100% B. Then, the column was washed for 4 min with 100% B and the system was re-equilibrated for 6 min. The APCI source was programmed in two periods: from 0 to 35.5 min operated at 500 °C in negative mode to determine the acids, and from 35.5 to 42.5 min, the source was kept at 450 °C and positive mode for the analysis of the alcohols.

2.7.5. LC-ESI-MS/MS determination of phenolic compounds. P in plasma and colon content were analysed as indicated in the subheading 2.4.4 but the LC-ESI-MS/MS conditions were slightly modified. The injection volume was set at 2 μL, and the mobile phases eluted with the following gradient: 0 min, 95% A and 5% B; 1 min, 90% A and 10% B; 10 min, 35% A and 65% B; 10.5 min, 0% A and 100% B. Solvent B was maintained at 100% for 5 min to prevent carry-over. A 6 min equilibration period preceded each new injection. The ESI source operated in negative mode, and the temperature was set at 600 °C.

2.7.6. Identification and quantification of pentacyclic triterpenes and polyphenols in plasma and colon content. Concentrations in plasma and colon content were calculated by interpolating the peak area ratio of the analytes to IS on a calibration curve. Calibration standards were prepared using blank plasma or blank colon content from overnight fasted rats never exposed to AO or bioactive compounds. Then, blank plasma was spiked with working solutions to obtain the final concentrations of 0; 1; 2.5; 5; 7.5; 10; and 25 nM, which were processed as indicated in 2.7.1. Results were expressed in nmol per liter of plasma (nM). Finally, blank colon content was processed as described in 2.7.2. Increasing concentrations of PT and P were added to the clear blank supernatants yielding the final concentrations of 0; 0.05; 0.25; 0.5; 1; 1.5; and 2 μM. Results were expressed in nmol per g of colon content (nmol g^{-1}). Metabolites were identified with the *m*/*z* indicated in Table 2 and were assumed to possess a similar LC-MS response as the parent compound. Therefore, the concentrations of metabolites were quantified using the standard curve of the parent compound. Calibration standards made in blank plasma and colon content were used to validate the process as described in the subheading 2.4.5.

2.8. Aberrant crypt foci in colon

Aberrant crypt foci (ACF) were assessed following the method described by Bird.[27] Proximal, medial, and distal colon segments were opened along the longitudinal median, pinned flat onto a polystyrene board and fixed in 10% buffered formalin (pH 7.4) for at least 24 h. Fixed segments were washed out with PBS, then stained with methylene blue 0.2% for 8 min (proximal) or 10 min (medial and distal). Excess dye was removed by rinsing the tissues with PBS. For analysis, segments were placed mucosa-side up on slides and observed under a light microscope at 10× magnification (BX41, Olympus Corporation, Tokyo, Japan). ACF were identified based on the criteria described by Bird.[27] Lesions are characterized by a thickened epithelial layer that displays a more intense stain, distorted, slit-like luminal openings, and larger size (2–3 times that of normal surrounding crypts). Aberrant crypts (AC) were determined as a single altered crypt or as a cluster of altered crypts that form a focus termed ACF. The number of ACF was counted in the proximal, medial, and distal segments of each rat. Crypt multiplicity was assessed as the number of AC per focus. The scores were checked by two independent observers who were blinded to the treatments. Images of the mucosal surface were taken with an XC 50-UTV1X-2 camera (Olympus Corporation, Tokyo, Japan).

2.9. Mucin depleted foci in colon

After counting ACF, colon segments were kept in PBS at 4 °C until being dyed with the high-iron diamine/alcian blue/neutral red staining (HID-AB) to assess mucin production.[28] The segments were first washed with PBS and stained with high-iron diamine for 18–24 h at room temperature, protected from the light. Then, tissues were rinsed in PBS and stained with alcian blue 1% in acetic acid 3% for 5 min. Subsequently, tissues were washed again, and finally stained for 2 min in neutral red 0.1% with acetic acid 0.002%. Then, the stained segments were placed in slides with the mucosal side up and examined under a light microscope at 20× magnification. Mucin-depleted foci (MDF) were identified by their lack of mucins, distorted lumen openings, and elevated lesions above the colon surface.[28] The number of MDF and the multiplicity expressed as mucin-depleted aberrant crypts (MDAC) per aberrant focus were determined. Scores were evaluated by two independent, blinded observers.

2.10. Statistical analysis

Results are presented as means ± standard error of the means (SEMs). Outliers were rejected using Chauvenet's criterion. Data evaluation, statistical analysis, and elaboration of graphs were done using Prism version 6 (GraphPad Software Inc., San Diego, CA, USA). Normality was assessed with the Kolmogorov–Smirnov test and based on the results, a parametric or non-parametric analysis was performed. Statistical analysis was performed by two-way ANOVA followed by Tukey's

Table 2 Concentrations of the metabolites of maslinic acid, oleanolic acid, hydroxytyrosol, and luteolin in plasma and colon content obtained between 17 to 19 h after the last oral administration of 3.85 g of destoned olives per kg of body weight for 49 days

		Plasma (nM)			Colon content (nmol g^{-1})		
Analyte	*m/z*	RT (min)	DMH−/Olives+	DMH+/Olives+	RT (min)	DMH−/Olives+	DMH+/Olives+
Metabolites of maslinic acid							
Monohydroxylation							
MA-M1a	487.3	13.8	2.01 ± 0.65	3.71 ± 1.33	14.0	17.2 ± 1.48	35.1 ± 2.38*
MA-M1b		—	φ	φ	15.0	12.1 ± 0.31	18.3 ± 1.05*
Monohydroxylation and dehydrogenation							
MA-M2	485.3	—	φ	φ	16.7	14.0 ± 2.05	15.6 ± 0.63
Dihydroxylation							
MA-M3	503.3	—	φ	φ	10.8	10.6 ± 0.22	11.1 ± 0.08
Dihydroxylation and dehydrogenation							
MA-M4	501.3	—	φ	φ	13.8	10.6 ± 0.32	11.1 ± 0.16
Metabolites of oleanolic acid							
Monohydroxylation							
OA-M1	471.5	17.0	φ	3.56 ± 0.78	—	φ	φ
Monohydroxylation and dehydrogenation							
OA-M2	469.5	13.8	φ	0.37 ± 0.05	14.0	5.54 ± 0.32	7.32 ± 0.09
Sulfation							
OA-M3a	535.5	—	φ	φ	13.6	4.40 ± 0.01	4.55 ± 0.04
OA-M3b		—	φ	φ	14.3	4.43 ± 0.04	4.53 ± 0.04
Metabolites of hydroxytyrosol							
Sulfation							
Hty-M1a	233.0/153.2	5.50	1.55 ± 0.1	2.15 ± 0.66	5.40	0.22 ± 0.05	0.33 ± 0.04
Hty-M1b		5.65	3.17 ± 0.2	5.84 ± 2.46	—	φ	φ
Glucuronidation							
Hty-M2	329.0/153.2	—	φ	φ	6.64	0.06 ± 0.001	0.06 ± 0.005
Metabolites of luteolin							
Sulfation							
Lu-M1	365.0/285.2	—	φ	φ	10.8	0.04 ± 0.02	0.05 ± 0.01
Glucuronidation							
Lu-M2	461.2/285.2	—	φ	φ	—	0.007 ± 0.001	0.009 ± 0.001

Results are expressed as means ± SEM in the analysis of the samples of plasma and colon content from DMH−/Olives+ (n = 3) and DMH+/olives+ (n = 6) groups. Data were analysed by Student's unpaired t test. Different from DMH− rats: *$p < 0.05$; φ, not detected.

multiple comparison test. When, body weight, water intake, food consumption, and FCE were the dependent variables, the two-way ANOVA analysis used experimental model (DMH− or DMH+) and treatment (water or AO) as the independent variables. For ACF and MDF counts as dependent variables, the analysis incorporated the intestinal segments (proximal, medial or distal) and treatment (water or AO) as independent variables. For multiplicity of ACF and MDF, the analysed considered the number of crypts (1, 2, 3 or ≥4) and treatment (water or AO) as independent variables. Significant differences of ACF and MDF in total colon, as well as AC and MDAC were compared using Student's unpaired t test. Differences in the concentration of PT and P and their metabolites in plasma and colon content were established by Student's unpaired t test. For all tests three levels were considered as significant: *$p < 0.05$; ** $p < 0.01$; and ***$p < 0.001$.

3. Results

3.1. Determination of pentacyclic triterpenes and polyphenols in Arbequina table olives

3.1.1. Validation. No matrix effect was observed, as evidenced by the slope ratios of calibration curves prepared in post-extracted olives compared to those obtained in 80% methanol, which ranged from 0.8 to 1.2. The limit of quantification (LOQ) was below 15 nM for PT and P, except for erythrodiol (35 nM), pinoresinol (30 nM), uvaol (65 nM) and catechol (100 nM). Linearity was assessed using calibration standards from 0.05 to 3.00 μM, obtaining correlation coefficients (R^2) above 0.998 for all compounds. Precision, as the percent relative standard deviation (%RSD) of 6 replicates and accuracy, as the percentage of relative error of the measured concentration compared to the nominal values, both met the established 15% limit. No carry-over was detected, as blank reagents injected after the highest calibration standards showed no peaks at the retention times of PT, P, or IS.

3.1.2. Identification and quantification. The Arbequina variety is characterized by its small size, with an average weight per olive of 1.56 ± 0.03 g per fruit (n = 77). Once pitted, the mean weight per fruit is 1.11 ± 0.03 g, corresponding to 70.7 ± 0.35% of the total olive weight. The dry weight was 0.36 ± 0.01 g (n = 77).

The total PT in AO were 3214 ± 123 mg kg^{-1}, exceeding the total P content of 1043 ± 46.9 mg kg^{-1}. Analysis of the PT revealed three compounds: maslinic acid accounting for 72.9%, oleanolic acid which represented a 26.8% and erythrodiol constituting 0.3% of the total PT content (Table 1).

Regarding the total P content, 15 compounds were identified. The most abundant was hydroxytyrosol accounting for 45.5% of the total of P, followed by verbascoside comprising 32%, and luteolin representing an 8.6%. Twelve additional phenolic compounds were identified, collectively accounting for 13.9% of total P (Table 1).

3.2. Body weight, food and water consumption, and food conversion efficiency

Animals in the DMH−/Olives− group showed an initial body weight of 244 ± 12 g which increased to 335 ± 22 g after 7 weeks, representing a weight gain of 91.0 ± 0.5 g (Fig. 1A). In contrast, the DMH−/Olives+ group had an initial body weight of 246 ± 2 g that rose to 315 ± 12 g by week 7. The DMH-/Olives+ rats gained 69.0 ± 9.5 g, which was 24% lower ($p < 0.05$) than the weight gain observed in the DMH−/Olives− group.

A similar trend was observed in the groups treated with DMH (Fig. 1A). The DMH+/Olives− rats had an initial mean body weight of 253 ± 9 g that increased to 337 ± 9 g by day 49. Over the same period, the body weight of the DMH+/Olives+ group rose from 250 ± 7 g to 311 ± 7 g. Hence, the DMH+/Olives− rats gained 84.2 ± 2.6 g while the DMH+/Olives+ rats increased 61.3 ± 6.9 g, which was 27% lower ($p < 0.05$) than the positive control group.

Concerning the food consumption or water intake, no significant differences were found between the control and the treated groups. Daily energy consumption was monitored throughout the study period. In the olive-treated groups, the total energy intake comprised both dietary food consumption and energy provided by olive gavage. Daily energy intake was consistent across all experimental groups, with mean values of 54.2 ± 0.9 kcal in DMH−/Olives− rats, 50.6 ± 0.8 kcal in DMH−/Olives + rats, 53.0 ± 0.9 kcal in DMH+/Olives− rats, and 49.7 ± 1.2 kcal in DMH+/Olives+ rats.

FCE was highest during the first week, decreased during the second and third weeks, and remained constant until the end of the experiment (Fig. 1B). No significant differences were observed for FCE in both DMH− and DMH+ groups.

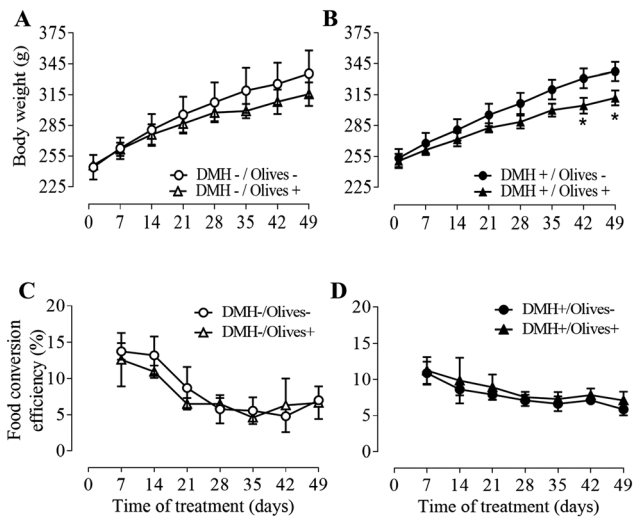

Fig. 1 Body weight and food conversion efficiency of male Sprague-Dawley rats. (A and C) depicts the animals that did not receive the carcinogen (DMH−) and were orally administered with solvent (Olives−) or table olives at 3.85 g kg^{-1} (Olives+). (B and D) displays the groups that received 1,2-dimethylhydrazine (DMH+) once a week for three weeks along with solvent (Olives−) or table olives at 3.85 g kg^{-1} (Olives+). Results are expressed as means ± SEMs (n = 3–6) and were analysed by two-way ANOVA, followed by Tukey's multiple comparisons test. Asterisks indicate differences from the control group: *$p < 0.05$.

3.3. General observation and gross necropsy

All rats were routinely monitored throughout the study, and no adverse effects or mortality were found. Stool consistency was pelleted and firm, with no visible differences between groups. At the end of the experiment, animals were submitted to a thorough post-mortem examination of the internal organs. No macroscopic changes in size, colour, or texture were observed in any of the groups, upon examination of the liver, brain, lungs, kidneys, testicles, spleen, heart, and thymus. The supplementation with AO at a dose of 3.85 g kg^{-1} did not significantly affect the final relative weight (g per 100 g body weight) of the examined organs ($p > 0.05$).

3.4. Determination of pentacyclic triterpenes, polyphenols, and their metabolites in rat plasma

3.4.1. Validation. The results obtained in the validation indicated the absence of a matrix effect. The method showed adequate sensitivity for the analysis of PT since the LOQ were below 0.2 nM for maslinic acid, oleanolic acid, and ursolic acid, 2 nM for erythrodiol and 4.60 nM for uvaol. Regarding P, the LOQ was lower than 1 nM, except for hydroxytyrosol acetate and catechol which were 4.66 nM and 9.71 nM, respectively. Calibration curves were linear with R^2 above 0.997 for PT and P. Precision and accuracy were below 15% and no carry-over was observed.

3.4.2. Identification and quantification. Maslinic acid was found in the plasma of the Olives+ groups at 3.64 ± 0.27 nM in the DMH− animals and 10.1 ± 2.74 nM in the DMH+ rats (Table 1). In a targeted analysis, one peak was identified as a monohydroxylated derivative (MA-M1) representing in the DMH− group a 35.5% of the total, whereas in the DMH+ group accounted for a 26.9% (Table 2).

Oleanolic acid was detected at 2.25 ± 0.17 nM in DMH+/Olives− rats and no metabolites were found (Table 1). In contrast, in DMH+/Olives+ rats, the parent compound (3.19 ± 0.58 nM) was accompanied by two Phase I metabolites: a monohydroxylated derivative (50.0%) and a monohydroxylated and dehydrogenated derivative (5.24%) (Table 2).

The plasma concentrations of hydroxytyrosol were around 2 nM in both groups (Table 1). This compound underwent extensive Phase II metabolism, mainly forming two sulfate (Hty-M1) isomers (Table 2). A similar pattern was observed in DMH−/Olives+ and DMH+/Olives− groups, with the parent compound representing around 25%, with the two sulfate isomers representing approximately 22% and 52% respectively.

3.5. Determination of pentacyclic triterpenes, polyphenols, and their metabolites in colon content

3.5.1. Validation. No matrix effect was observed. The LOQ for PT ranged from 0.33 nM for maslinic acid to 3.09 nM for uvaol. LOQs for P were within 0.16 (luteolin-7-*O*-glucoside) to 2.85 nM (*p*-coumaric acid), except for hydroxytyrosol acetate, tyrosol, and catechol that presented values of 4.28, 6.73 and 16.2 nM, respectively. The linearity was observed over the concentrations assayed (0.05–2.00 μM), with R^2 above 0.996 for all compounds. Precision and accuracy were within the ±15% established in the guidelines and no carryover was observed.

3.5.2. Identification and quantification. Chromatogram analysis identified 3 PT (Fig. 2) and 13 P (Fig. 3). However, only 9 of the 13 phenolic compounds were quantified because hydroxytyrosol acetate, luteolin-7-*O*-glucoside, (+)-pinoresinol, and rutin were below the LOQ.

Maslinic acid was the predominant bioactive compound in AO and in the colon content, found at 55.5 ± 5.22 nmol g^{-1} in the DMH−/Olives+ group and 321 ± 67.0 nmol g^{-1} in the DMH+/Olives+ rats (Table 1). Metabolite screening identified 5 compounds, all from Phase I reactions (Table 2). Despite searching for Phase II derivatives, none of the most common conjugates were found. The identified metabolites were categorized into four groups: monohydroxylated (MA-M1, including two isomers), monohydroxylated and dehydrogenated (MA-M2), dihydroxylated (MA-M3), and dihydroxylated and dehydrogenated (MA-M4). In the DMH−/Olives+ group, maslinic acid constituted 46.2% of the total, with metabolites making up 53.8%. In contrast, in the DMH+/Olives+ group, maslinic acid represented 78.0%, and metabolites accounted for only 22.0% (Table 2).

Oleanolic acid, the second most prevalent bioactive compound in AO, was also the second most abundant in the colon content, at concentrations of 22.8 ± 1.30 nmol g^{-1} in the DMH−/Olives+ rats, and 84.8 ± 14.3 nmol g^{-1} in the DMH+/Olives+ animals. Three metabolites were identified: one Phase I (monohydroxylated and dehydrogenated, OA-M2) and two Phase II sulfate conjugates (OA-M3). The parent compound was predominant in both groups, with higher relative abundance in DMH+/Olives+ rats (61.3%) compared to DMH−/Olives+ rats (83.8%).

Hydroxytyrosol was the third most abundant compound in colon content with concentrations of 1.36 ± 0.30 nmol g^{-1} in the DMH−/Olives+ rats and 3.31 ± 0.24 nmol g^{-1} in the DMH+/Olives+ animals, respectively (Table 1). This phenolic alcohol underwent Phase II biotransformation, resulting in two sulfate conjugates (Hty-M1) and one glucuronide conjugate (Hty-M2). In both groups, the sulfate derivative comprised 12%, the glucuronide 3%, and hydroxytyrosol 85% of the total.

Interestingly, although *p*-coumaric acid is present in olives in minor quantities, it was the fourth most abundant compound in the colon, with concentrations of 0.92 ± 0.20 nmol g^{-1} in DMH−/Olives+ rats and 1.83 ± 0.67 nmol g^{-1} in DMH+/Olives+ rats. Other bioactive compounds were present at similar concentrations across both groups, ranging from 1.46 ± 0.19 nmol g^{-1} for tyrosol to 0.16 ± 0.002 nmol g^{-1} for verbascoside (Table 1).

3.6. Aberrant crypt foci in colon

No preneoplastic ACF were observed in the DMH−/Olives− and DMH−/Olives+ groups, so the results focus on the DMH-treated groups only (Fig. 4).

ACF exhibited a regional distribution pattern along the colon in DMH-treated rats, with fewer lesions in the proximal colon that progressively increased towards the distal colon

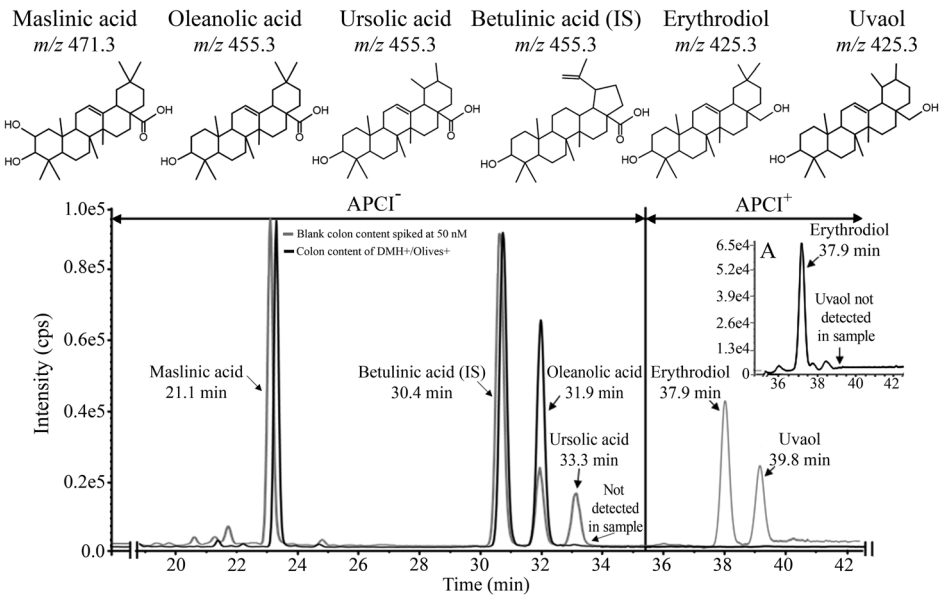

Fig. 2 Total ion chromatograms (TICs) of pentacyclic triterpenes in rat colon content obtained by LC-APCI-QTRAP-MS. The representative chromatograms display rat blank colon content spiked with a mixture of standards (50 nM) and betulinic acid (IS, 200 nM) (grey trace) and colon content from a rat in the DMH+/olives+ group (black trace). The insert (A) depicts a representative extracted ion chromatogram (XIC) at *m/z* 425.3 of an undiluted sample from the DMH+/olives+ group.

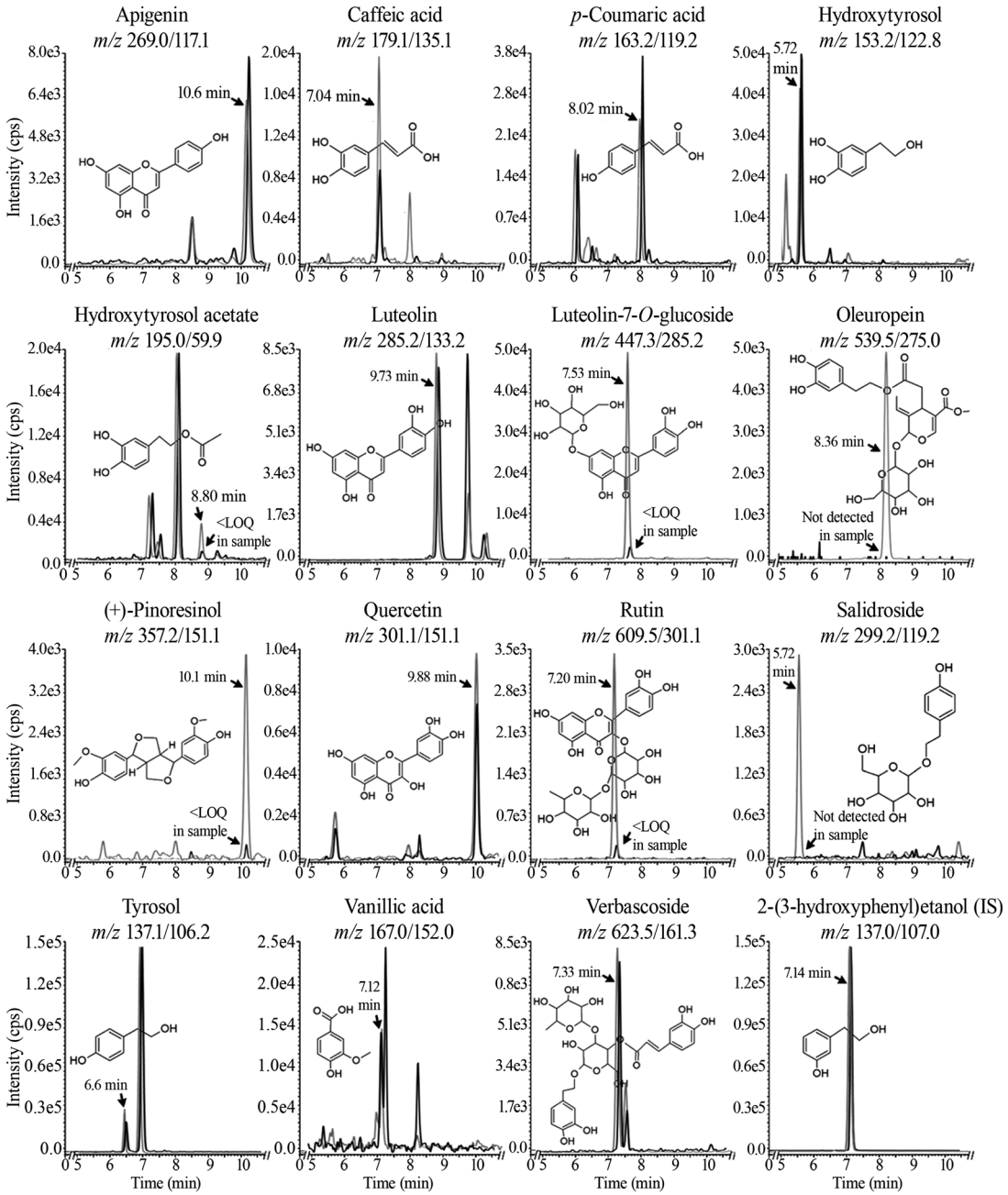

Fig. 3 Extracted ion chromatograms (XICs) of polyphenols in rat colon content obtained by LC-ESI-QTRAP-MS. The chromatograms show rat blank colon content spiked with a mixture of standards (50 nM) and 2-(3-hydroxyphenyl)-ethanol (IS, 5000 nM) (grey trace) and of the colon content of a representative rat from the DMH+/olives+ group (black trace).

(Fig. 4A). This distribution pattern was consistent across all groups. Oral administration of table olives at 3.85 g kg^{-1} for 49 days reduced ACF numbers by 43.5% ($p < 0.05$), 45.9% ($p < 0.05$), and 60.0% ($p < 0.01$) in the proximal, medial, and distal colon segments, respectively (Fig. 4A). Overall, ACF numbers in the total colon decreased by 54.1% ($p < 0.01$), from 114 ± 14 crypts in the DMH+/Olives− group to 52 ± 5 crypts in the DMH+/Olives+ group (Fig. 4B).

Crypt multiplicity analysis revealed that most ACF in both DMH-treated groups, consisted of single crypts, with fewer foci containing 2, 3, or ≥4 crypts (Fig. 4C). AO supplementation reduced ACF with 1, 2, 3, and 4 crypts in the total colon by 52.0% ($p < 0.001$), 56.1% ($p < 0.01$), 63.0% ($p < 0.01$), and 38.5% ($p > 0.05$), respectively. Total aberrant crypts in the colon dropped by 55.3% from 174 ± 23.2 to 78 ± 5.8 crypts ($p < 0.01$) with AO supplementation.

3.7. Mucin depleted foci in colon

MDF showed a distribution pattern along the colon similar to ACF but in lower numbers (Fig. 5). The consumption of AOs

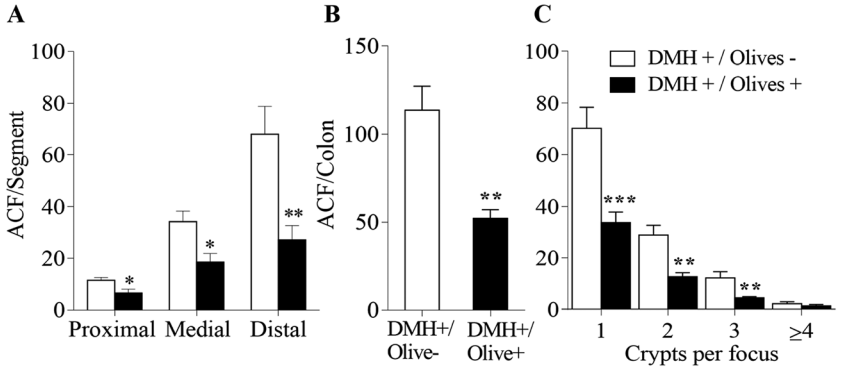

Fig. 4 Effects of the daily intake of Arbequina table olives for 49 days on the development of aberrant crypt foci (ACF) in colonic segments (A), in total colon (B), and number of crypts per focus in total colon (C). Results are expressed as means + SEMs (n = 6). ACF in colonic segments and crypt multiplicity were analysed by two-way ANOVA, followed by Tukey's multiple comparisons test, whereas ACF in total colon were compared using Student's unpaired t test. Asterisks indicate differences from the control group: *p < 0.05, **p < 0.01, and ***p < 0.001.

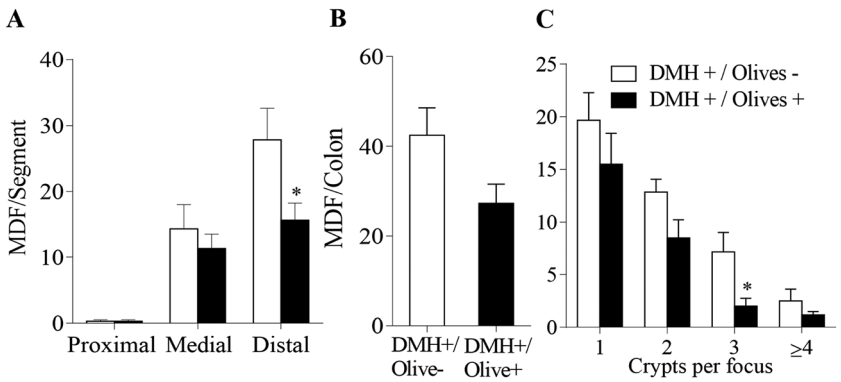

Fig. 5 Effects of the daily intake of Arbequina table olives for 49 days on the development of mucin depleted foci (MDF) in colonic segments (A), in total colon (B), and number of crypts per focus in total colon (C). Results are expressed as means + SEMs (n = 6). MDF in colonic segments and crypt multiplicity were analysed by two-way ANOVA, followed by Tukey's multiple comparisons test, whereas MDF in total colon were compared using Student's unpaired t test. Asterisks indicate differences from the control group: *p < 0.05.

reduced MDF by 20.9% (p > 0.05) and 43.7% (p < 0.05) in the medial and distal colon segments, respectively (Fig. 5A), with a total MDF decrease of 35.7% (p > 0.05) (Fig. 5B).

Crypt multiplicity was also analysed for MDAC (Fig. 5C). AO reduced MDAC with 1 crypt by 21.2% (p > 0.05); of 2 crypts by 33.8% (p > 0.05), of 3 crypts by 72.1% (p < 0.05), and ≥4 crypts by 53.3% (p > 0.05). Furthermore, the total number of MDAC was reduced in a 43.9% (p < 0.05) after the supplementation with AO.

4. Discussion

Colorectal cancer is one of the most prevalent cancers worldwide. While genetics play a role in individual risk, the incidence is largely influenced by modifiable environmental factors, particularly lifestyle and dietary habits.[18] Key preventive measures include adopting a diet rich in fruits and vegetables, which provide essential micronutrients, dietary fiber, and phytochemicals.[18] In this context, table olives could be incorporated into a dietary pattern aimed at decreasing the risk of developing colorectal cancer, due to their unique profile of beneficial compounds, including PT and P.[2] Therefore, this study aims to evaluate the effect of AO on the development of early markers of colon carcinogenesis in DMH-treated rats. Moreover, to better understand the potential role of bioactive compounds from AO in colorectal cancer prevention, we analysed PT and P by LC-MS. First, the determination was performed in the food itself to quantify their concentration in the administered dose. Subsequently, we measured them in plasma and colon content, to assess how much of PT and P reached the target organ.

The analysis of AO revealed distinct differences in PT and P content and align with previous studies on Greek-style processed table olives across various cultivars. Our observed PT values (around 3 g kg^{-1}) fall within the previously reported range (2.10 to 3.28 g kg^{-1}) for cultivars such as Empeltre, Manzanilla, Cellina di Nardò, Hojiblanca, Conservolea, and Kalamata.[6,29,30] Similarly, our P content (around 1 g kg^{-1}) is consistent with the range (0.35 to 1.20 g kg^{-1}) reported for cul-

tivars including Marfil, Empeltre, Cellina de Nardò, Bella di Cerignola, Termite di Bitetto, and Manzanilla.[6,31–33] The supplementation was equivalent to the human consumption of 30 AO, which doubles the recommended serving size according to the Mediterranean diet pyramid.[21] Consequently, rats received a dose of 3.85 g AO per kg, supplying 12.38 mg of PT per kg body weight and 4.02 mg of P kg^{-1}. Among them, the most abundant was maslinic acid (9.02 mg kg^{-1}), followed by oleanolic acid (3.32 mg kg^{-1}), hydroxytyrosol (1.83 mg kg^{-1}), verbascoside (1.29 mg kg^{-1}) and luteolin (0.34 mg kg^{-1}). Hence, AO provided substantial amounts of PT and P with demonstrated colon cancer chemopreventive activities *in vivo*.[13–17]

After administration, the analysis of PT and P in plasma yielded low concentrations of only three parent compounds (Table 1): maslinic acid, oleanolic acid and hydroxytyrosol at the end of the 7-week intervention and 17–19 hours after the final dose of AO. In our targeted metabolomics approach, we searched for both Phase I and Phase II metabolites of the main pentacyclic triterpenes and polyphenols. Phase I metabolism primarily involves oxidation, reduction, and hydrolysis reactions, whereas Phase II metabolism includes conjugation reactions.[34] These metabolic processes occur predominantly in the liver, although other organs can also contribute to metabolize different compounds from the diet. Analysis of plasma samples revealed that 73% of maslinic acid remained unaltered, while 27% was present as a monohydroxylated metabolite, consistent with previous studies showing limited Phase I biotransformation of this compound.[35] Conversely, oleanolic acid and hydroxytyrosol underwent significant biotransformation upon absorption and only approximately 45% of oleanolic acid and a mere 21% of hydroxytyrosol were detected in their unaltered forms in plasma. Oleanolic acid yielded two Phase I metabolites whereas hydroxytyrosol underwent Phase II transformation, resulting in two sulfate conjugates. These findings agree with those obtained in rats after the administration of pure compounds.[5,36,37] Our results in plasma confirm that the bioactive compounds from Arbequina table olives are effectively absorbed, reaching the blood.

Although previous studies have reported on the plasma concentrations of PT and P following table olive consumption,[5,25,38] there is a lack of data regarding the disposition of these compounds in colon content after oral administration of this food. To address this, we employed an analytical method previously developed for the analysis of maslinic acid in intestinal content.[26] The validation of the procedure indicated a lack of matrix effect, as well as adequate sensitivity, linearity, precision and accuracy thus demonstrating its suitability for the measurement of PT and P in colon content. The application of the analytical method showed that three PT and nine P from those determined in AO, were detected in the colon content at higher amounts than in plasma. Previous research by Cameron *et al.*[39] reported that rats injected with DMH exhibited significantly prolonged transit times of 36.4 ± 3.8 hours compared to 25.4 ± 4.2 hours in negative control animals not exposed to DMH. This delayed transit in the DMH-treated group could allow for an augmented exposure duration of bioactive compounds in the intestinal lumen before excretion occurs. To get insight into the bioavailability and potential efficacy of these compounds at the target site for colon cancer prevention, we calculated the percentage of PT and P reaching the colon relative to the amount supplemented to the rats. The most abundant compound in the colonic content in DMH+/olives+ was maslinic acid that was found at 321 nmol g^{-1}, which represented a 5.6% of the administered dose. In the colonic content, 78% of maslinic acid remained unaltered, while five distinct Phase I metabolites represented 22% of the total maslinic acid detected. The second compound in abundance was oleanolic acid (84.8 nmol g^{-1}) that was found at 3.9% from the amounts ingested in the olives. This PT was detected intact in an 84%, whereas a 16% underwent transformation, yielding one Phase I and two Phase II metabolites. Concerning PT, erythrodiol that was found in Arbequina table olives at very low amounts was detected in the colonic content at 1.19 nmol g^{-1} which constitutes a 4.3% of the dose administered. Unlike the PT, where all three compounds present in table olives were detected in the colon content, P did not exhibit the same pattern, and only a 0.2% of the administered dose were found in the colon. The most abundant P in Arbequina table olives were not the ones with the higher concentrations in the colon. The compounds detected in the colon included hydroxytyrosol, *p*-coumaric acid, tyrosol, vanillic acid, caffeic acid, luteolin, apigenin, quercetin, and verbascoside. Notably, the results for *p*-coumaric acid, vanillic acid, caffeic acid, and apigenin in the colon content are particularly significant, given that these compounds were present in AO at very low concentrations. Additionally, we did not detect hydroxytyrosol acetate, rutin, salidroside, oleuropein or luteolin-7-*O*-glucoside. Our results agree with studies on the effects of the gastrointestinal digestion on table olives through a simulated *in vitro* gastrointestinal digestion, which describe a progressive decrease in the concentrations of P along the gut, reaching the lowest amounts in the large intestine.[40,41] Furthermore, our findings are in accordance with D'Antuono *et al.*[40] who reported the breakdown of oleuropein into hydroxytyrosol, verbascoside into hydroxytyrosol and caffeic acid, salidroside into tyrosol or comselogoside into *p*-coumaric acid, among others. These transformations, may explain, in part, our results where various P appeared in colonic content that were originally present in AO at very low concentrations, and the absence of other P initially present in the food.

The cancer chemopreventive activities of AO was investigated using an experimental design previously established in our laboratory consisting in three subcutaneous injections of DMH, followed by an observation period of 4 weeks.[42] This protocol led to the formation of preneoplastic lesions, ACF and MDF, in a number that was consistent with previous studies in our group[15,41] as well as for other authors.[13,14,16,17] No signs of toxicity were observed during the experimental period, and neither food nor water intake was affected by either AO or the DMH treatment. Body weight remained unaffected by DMH administration. However, supplementation with AO induced a

small decrease in total body weight of 24% (DMH−/olives+) and 27% (DMH+/olives+) compared to their respective controls. These findings agree with the weight reducing effects of different olive oils enriched in pentacyclic triterpenes and polyphenols elicited in various experimental models.[43–45]

The administration of AO over a 49-day period exerted a chemopreventive effect, as evidenced by a 54.1% reduction in the total number of ACF and a 35.7% decrease in MDF in the colon. Furthermore, AO influenced the multiplicity (number of crypts per focus) of ACF and MDF, with a decrease in the number of foci containing 1, 2, 3, and ≥4 aberrant crypts. We assessed the development of ACF in the DMH−/olives+ group to confirm that table olive supplementation alone did not promote the formation of these preneoplastic lesions. Previous research has demonstrated that caloric restriction, which leads to reduced body weight, significantly inhibits the formation of new ACF and delays the progression of existing ACF in the AOM-induced colorectal cancer model in rats.[46] Consistent with these findings, the reduced body weight observed in rats supplemented with AO may have contributed, in part, to the decreased ACF number and multiplicity found in our study. Moreover, the significant reduction in ACF coincides with the presence of bioactive compounds in the colon content, suggesting a potential relationship between their concentration and chemopreventive effects. Maslinic acid was the bioactive compound that we have found in higher concentration in the colon (321 nmol g^{-1}) after the supplemented by gavage of AO that contained a dose of 9 mg kg^{-1} of this compound and resulted in a reduction of ACF of 54%. A previous study from our group, addressing the effect of maslinic acid in the same animal model, revealed that when administered by gavage at 10 mg kg^{-1}, the concentration achieved in the colon was lower (90 nmol g^{-1}) and the reduction of ACF was only of a 18%.[15] The higher reduction achieved with AO compared to the pure compound may be attributed to the greater concentration of bioactive compounds in the colon, correlating with *in vitro* findings in HT29 and Caco-2 colon cancer cell lines.[9,10] These studies showed a dose-dependent relationship, with increasingly pronounced antiproliferative and pro-apoptotic effects at higher concentrations of maslinic acid. However, in evaluating the results, we cannot rule out the effect of the other compounds from this food, since in addition to PT and P, AO are rich in unsaturated fatty acids, essential amino acids, minerals, vitamins and fiber.[2]

There is no previous data on the effects of regular table olive intake on colon cancer *in vivo*, although this murine model has been used to evaluate the chemopreventive properties of olive oil.[47–49] While Femia *et al.*[47] did not find any effect of extra virgin olive oil (EVOO) on colon carcinogenesis, regardless of its P content, França *et al.*[48] observed a reduction in the number of ACF. This finding was corroborated by Nanda *et al.*,[49] who reported that EVOO lowered tumour incidence and inhibited tumour development. Although the reduction of colonic tumorigenesis has been associated with the high content of monounsaturated fatty acids, particularly oleic acid, the presence of other minor compounds cannot be disregarded.[48,49] In this regard, the effects on DMH-induced colon carcinogenesis in rats have been evaluated for various PT, including maslinic acid[15] and oleanolic acid[13,14] as well as P like *p*-coumaric acid[17] and luteolin.[16] The research on the chemopreventive properties of AO reveals a complex interplay of multiple bioactive compounds that act synergistically, producing a more potent effect than individual components alone, thus highlighting the importance of studying whole foods.

The chemopreventive activity of AO could be associated not only with the anti-tumour properties but also with their positive influence on gut microbiota. Studies have shown that supplementation with table olives can increase beneficial bacterial genera and probiotic strains,[4] contributing to their chemopreventive effects by reshaping the gut microbiota, which is crucial since gut dysbiosis is associated with colorectal cancer development.[50] Moreover, the anti-inflammatory properties described for PT and P could also play a role.[8,51] These bioactive compounds have also been reported to suppress the pro-inflammatory NF-κB pathway implicated in colon cancer progression, and by downregulating iNOS and COX-2 expression, they can target the local inflammation detected in early mucin-depleted foci stages.[8,51] Moreover, the inflammation and Wnt/β-catenin pathway activation in the colonic mucosa leads to increased transcription of proliferative genes like *c-Myc*, *c-Jun*, *cyclin-D1* as well as anti-apoptotic *Bcl-2*, *Bcl-xl*, *p53* that could be modulated by PT and P[8,51] contributing to the antitumoral activity induced by OA.

5. Conclusion

Our findings demonstrate that bioactive compounds from AO exhibit differential bioavailability and colonic exposure. Table olives appear to be a particularly effective vehicle for delivering bioactive compounds to the colon, thus 5.1% of the PT and 0.2% of P from the administered dose arrived unmetabolized to this region. This localized exposure holds significance as these phytochemicals may contribute to the cancer chemopreventive activities exerted by AO that diminished early preneoplastic lesions, both ACF and MDF in rat colon. Thus, these results suggest that the daily consumption of table olives may confer health benefits, particularly concerning colon cancer prevention.

Author contributions

Rocío Moreno-González: investigation, formal analysis, data curation, writing – original draft, writing – review and editing, visualization. M. Emília Juan: investigation, methodology, formal analysis, data curation, validation, writing – original draft, writing – review and editing, visualization, supervision. Joana M. Planas: conceptualization, methodology, formal analysis, data curation, validation, writing – original draft, writing – review and editing, visualization, supervision,

resources, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data availability

Data for this paper, including body weight, food and water consumption, organ weight, ACF, MDF and LC-MS data of pentacyclic triterpenes and polyphenols in Arbequina table olives, plasma and colon content are available at the Science Data Bank at **https://doi.org/10.57760/sciencedb.12841**.

Conflicts of interest

The authors declared no conflict of interest.

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