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Olive juice dry extract containing hydroxytyrosol, as a nontoxic and safe substance: Results from pre-clinical studies and review of toxicological studies

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ABSTRACT

Products derived from olives, such as the raw fruit and oils, are widely consumed due to their taste, and purported nutritional/health benefits. Phenolic compounds, especially hydroxytyrosol (HT), have been proposed as one of the key substances involved in these effects. An olive juice extract, standardized to contain 20% HT ("OE20HT"), was produced to investigate its health benefits. The aim of this study was to demonstrate the genotoxic safety of this ingredient based on *in vitro* Ames assay and *in vitro* micronucleus assay. Results indicated that OE20HT was not mutagenic at concentrations of up to 5000 μg/plate, with or without metabolic activation, and was neither aneugenic nor clastogenic after 3-hour exposure at concentrations of up to 60 μg/mL with or without metabolic activation, or after 24-hour exposure at concentrations of up to 40 μg/mL. To further substantiate the safety of OE20HT following ingestion without conducting additional animal studies, a comprehensive literature review was conducted. No safety concerns were identified based on acute or sub-chronic studies in animals, including reproductive and developmental studies. These results were supported by clinical studies demonstrating the absence of adverse effects after oral supplementation with olive extracts or HT. Based on *in vitro* data and the literature review, the OE20HT extract is therefore considered as safe for human consumption at doses up to 2.5 mg/kg body weight/day.

1. Introduction

Olive fruit from *Olea Europea* L. has been widely consumed for centuries, mostly in European countries [1]. The olive sector is a growing market and olive consumption continues to progress. The Centre for the Promotion of Imports from developing countries (CBI), states that the European consumption of table olives has slightly increased over the past 5 years, progressing at an annual rate of 1% [2]. According to the CBI, European consumption of table olives in 2020/2021 was estimated at 595 thousand tons, representing approximately 20% of the total worldwide consumption. In Europe, the main olive consumers of table olives are Spain, with a 31% share in 2020, followed by Italy (17%), France (12%), and Germany (8%) [2]. Regarding the consumption *per capita*, the CBI states that the largest *per capita* consumers of table olives in Europe are in Cyprus, with 4.4 kg per inhabitant per year, followed by Spain (4.1 kg). According to the European Food Safety Authority (EFSA) comprehensive European Food Consumption Database, the range of means among European union surveys for olive products consumption was 0.02–0.50 g/kg body weight/day for adults and the range means among European Union surveys for table olive consumption was 0.03–0.28 g/kg body weight /day for adults [3]. Olive is also widely consumed as olive oil. According to the International Olive Council, the

Abbreviations: 2AA, 2-aminoanthracene; 9AA, 9-aminoacridine; CBI, Centre for the Promotion of Imports from developing countries; CP, cyclophosphamide monohydrate; EFSA, European Food Safety Authority; HT, hydroxytyrosol; MF, mutation factor; MMC, mitomycin C; MMS, methyl-methanesulfonate; NDP, 4-nitro-1,2-phenylene-diamine; NOAEL, no observed adverse effect level; OE20HT, olive juice dry extract titrated 20% hydroxytyrosol; OECD, Organization for Economic Co-operation and Development; PD, population doubling; RICC, relative increase in cell count; RPD, relative population doubling; SAZ, sodium azide.

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olive oil production and consumption has risen by 1 million tonnes in the last 25 years, growing from 1.8 million tonnes at the beginning of the 1990 s to 3 million tonnes in the 2016 crop year [1].

The nutritional and non-nutritional composition of olives is mainly influenced by agronomical factors, particularly the cultivar, the ripening stage, and the processing method employed [4–6]. In the past few years, the beneficial effects of minor components of olives and olive oil have raised a growing interest, in particular hydrophilic phenolics of the unsaponifiable fraction [7,8]. Many health benefits, including a decrease in cardiovascular risk factors [9–12], hypoglycemic effects [13], reduction in the development of neurodegenerative diseases [14] and cancers [15,16], an improvement in women-specific health issues [17], and thyroid-modulating activity [18] have been proposed to be attributed to this fraction. Among these phenolic compounds, secoiridoids are the most abundant in extra virgin olive oil [6]. Among secoiridoids, oleuropein and ligastroside, which are glucosides containing an ester bond between either *p*-hydroxy-phenylethyl alcohol (tyrosol) or 3,4-dihydroxy-phenylethyl alcohol, (hydroxytyrosol (HT)), and elenolic acid or its demethylated form, are the main secoiridoids found in olive leaves and olive fruit [6]. However, flavonoids (*e.g.*, apigenin) and lignans (*e.g.*, pinoresinol) are also present [6].

HT (Fig. 1) has been reported to be the main simple phenolic compound in olive fruit and olive oil [19]. According to Neveu et al., the mean content of free HT in olive oils is 3.5 mg/kg for virgin olive oils and 7.7 mg/kg for extra-virgin olive oils, while it is increased by a factor 100 in table olives, reaching 659.3 mg/kg for black olives and 555.7 mg/kg for green olives [20]. Based on these data, the European Food Safety Authority (EFSA) has estimated the mean daily intake of free HT in adults in Europe to be approximately 0.0105–0.28 mg/day from olive oil consumption and 12.95–51.33 mg/day from table olive consumption, for a 70-kg adult [3]. The safety of HT has been evaluated by EFSA in 2017 and, on the basis of the toxicological data presented to EFSA, the Panel adopted a no observed adverse effect level (NOAEL) of 50 mg/kg body weight/day for HT [3]. In the United States (US), olive extracts and/or HT itself are permitted as additives for use in various foodstuffs that result in estimated daily intakes of up to approximately 55 mg/day (Generally recognized as safe notifications: GRN876 [US FDA, 2020]; GRN 600 [US FDA, 2015]).

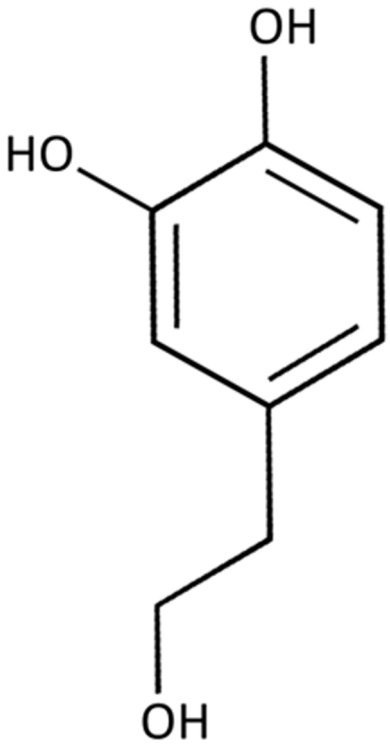

Fig. 1. Hydroxytyrosol structure.

In human trials, the oral supplementation of HT, alone or combined with others compounds, has been reported to be associated with various beneficial health outcomes, such as improvement of cardiovascular parameters [21–30], insulin sensitivity [31,32], gut microbiota [23,33], regulation of various inflammatory diseases [21,22,29,34], decrease in body weight and improvement in body composition [21,23,33,35–38].

As HT is found principally in olive fruits, as compared to olive oil, during the production of virgin olive oil, olive waste products, rich in HT are generated and these by-products are not always utilized. In the current context of attempting to reduce agri-food waste and putting to use these components, companies in the agri-food industry are developing solutions to extract value for some of these by-products, such as HT. Nevertheless, the safety of these new foods must be demonstrated. As such, this investigation was conducted to assess the safety of an olive extract produced from olive juice, a by-product of olive oil production, standardized to 20% HT, called "OE20HT". Two tests have been performed with OE20HT to demonstrate the absence of mutagenic and genotoxic potential: (i) an Ames assay according to the Organization for Economic Co-operation and Development (OECD) 471 guideline and (ii) an *in vitro* micronucleus assay in mammalian cells, according to OECD 487. In addition, to further substantiate the safety of OE20HT, under the philosophy of reducing the need for animal testing, the safety of olive extracts containing HT and the safety of HT were substantiated based on a comprehensive literature review.

2. Material and methods

2.1. Pre-clinical studies

2.1.1. Products

The test item, olive juice dry extract titrated to 20% HT (OE20HT), was provided by NATAC Biotech S.L. (Spain).

Briefly, the product is obtained from olive juice, as a by-product of olive oil production. The microfiltered olive juice is loaded into a column filled with an adsorbent resin. HT is weakly adsorbed on the resin (physical interaction) and then recovered by elution with water. After discarding a first fraction of water containing other soluble components of olive fruit, a second fraction of water containing HT and other polyphenols is collected. This fraction is concentrated under vacuum and spray dried. Due to stability and technological constraints, fractions containing HT (35 or 70% HT) is then combined with maltodextrin and silica in order to obtain extracts standardized to 10 or 20% HT.

Bacterial strains used for the bacterial reverse mutation test (*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, *Escherichia coli* WP2 *uvrA* 07) were all purchased from MOLTOX- Molecular Toxicology Inc. (NC, USA). Chemicals used as positive controls in the Ames assay, 4-nitro-1,2-phenylene-diamine (NPD), sodium azide (SAZ), methylmethanesulfonate (MMS) and 2-aminoanthracene (2AA) were provided by SIGMA-ALDRICH, whereas 9-aminoacridine (9AA) were purchased from MERCK. Distilled water was purchased from MAGILAB Kft. (Hungary) and dimethylsulfoxide (DMSO) 100% from VWR. Minimal glucose agar plates were purchased from MERCK. Phenolic compounds, including hydroxytyrosol, suffer the phase I of metabolism in enterocytes and subsequently go through phase II of metabolism, where they are transformed into glucuronide, methylated and sulphate byproducts [39]. To consider, in the Ames assay and micronucleus assay, the effects of the product and its metabolites, the assay have been performed in presence and absence of metabolic activation that mimic this important metabolism. The post-mitochondrial fraction (S9 fraction) was prepared by the Microbiological Laboratory of Charles River Laboratories Hungary Kft. according to Ames et al. [40] and Maron and Ames [41]. Chemicals used as positive controls for the *in vitro* micronucleus assay, mitomycin C (MMC), colchicine and cyclophosphamide monohydrate (CP) were purchased from Sigma-Aldrich Co.

2.1.2. Rat liver homogenate S9 fraction preparation

Phenobarbital and β-naphthoflavone-induced rat liver S9 was used as the metabolic activation system for both the Ames assay and *in vitro* micronucleus assay. The S9 was prepared from 17 to 20-week-old male Wistar rats (weight approx. 444–672 g) induced by administration of phenobarbital and β-naphthoflavone at a dose of 80 mg/kg body weight/day by oral gavage for three consecutive days. Rats were euthanized and liver was extracted 24 h after the last treatment. Livers were homogenized with 0.15 M KCl solution (MERCK) and homogenates were centrifugated for 10 min at 9000 g. The supernatant was aliquoted, frozen quickly, and stored at -80 ± 10 °C. The protein concentration of the S9 fraction was 21.15 g/L. The biological activity in the *Salmonella* assay of S9 was characterized in each case using the two mutagens 2-aminoanthracene and benzo(*a*)pyrene, which requires metabolic activation by microsomal enzymes.

2.1.3. S9-mix preparation

Complete S9-mix was freshly prepared for the treatment on the day of use. S9-mix was prepared with 40% (v/v) of S9 fraction, 20% (v/v) D-glucose-6-phosphate Na (Sigma-Aldrich Co.), 20% (v/v) β-nicotinamide-dinucleotide-phosphate (Sigma-Aldrich Co.), and 20% (v/v) potassium chloride (MERCK). S9-mix was added to cell culture to provide a S9 fraction final concentration of 2%.

2.1.4. Bacterial reverse mutation test (Ames test)

This study was performed according to OECD Guideline for Testing of Chemicals No. 471, Bacterial Reverse Mutation Test, adopted 21st July 1997, consistent with Commission Regulation (EC) No. 440/2008, B13/14, and EPA Health Effects Test Guidelines, OPPTS 870.5100 (EPA 712-C-98–247, 1998), as well as the principles of good laboratory practice, according to Hungarian GLP Regulations (42/2014. (VIII. 19.)) EMMI decree of the ministry of human capacities which corresponds to the OECD good laboratory practices (ENV/MC/CHEM [98] 17).

Distilled water was used as solvent to prepare the stock solution (100 mg/mL) of the test material. Test solutions were freshly prepared at the beginning of the experiments in the testing laboratory by diluting the stock solution using the selected solvent. The stock solution was ultrasonicated for 2 min. *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 uvrA were incubated with OE20HT at 6 concentrations spaced by factors of approximately $\sqrt{10}$ (C1: 5000 μg/plate; C2: 1581 μg/plate; C3: 500 μg/plate; C4: 158.1 μg/plate; C5: 50 μg/plate, and C6: 15.81 μg/plate), with or without S9 fraction, according to both the direct plate incorporation and pre-incubation procedures. Doses have previously been validated in TA98 and TA100 strains (Supplementary Table 1). Plates were incubated for 48 h (± 1 h) at 37 °C and colonies were counted. The assay was performed in triplicate along with vehicle and positive controls. Each bacterial strain culture was mixed with the test item either with metabolic activation system mix (S9) or without metabolic activation system mix (phosphate buffer was used instead). In the direct incorporation procedure, the mixture was immediately poured over a minimal agar medium plate and incubated at 37 °C for 48 h, whereas in the pre-incubation procedure, the mixture was incubated for 20 min at 37 °C prior to be poured over the minimal agar medium plate. Cytotoxicity evaluation of HT was based on the decrease in the number of revertant colonies, or a clearing or diminution of the background lawn. Results were expressed as mean revertant for triplicate and as mutation factor (MF) calculated as follow:

$$MF = \frac{\text{mean revertants(test item)}}{\text{mean revertants(solvent control)}}$$

2.1.5. In vitro micronucleus assay

This study was performed according to OECD Guidelines for Testing of Chemicals No. 487, *In vitro* Mammalian Cell Micronucleus Test. adopted 29th July 2016 and the principles of good laboratory practice according to Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the ministry of human capacities which corresponds to the OECD good laboratory practices (ENV/MC/CHEM [98] 17.

L5178Y TK$^{+/-}$ 3.7.2 C mouse lymphoma cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and frozen stocks were maintained in liquid nitrogen. Thawed stock cultures were propagated in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 5 or 10% heat inactivated horse serum (Life Technologies (Gibco)), and 100 UI/mL penicillin, 0.1 mg/mL streptomycin, 0.25 μg/mL amphotericin B (Sigma-Aldrich Co.), 2 mM L-glutamine (Sigma-Aldrich Co), 0.5 mg/mL Pluronic-F68 (Sigma-Aldrich Co), 0.2 mg/mL pyruvic acid (Life Technologies (Gibco)). Three treatment conditions were tested. A 3-hour treatment with or without metabolic activation, followed by 21 h of recovery, and a 24-hour treatment without metabolic activation. Treatments were performed in duplicate cultures. Cells were treated with 3 different concentrations of OE20HT (C1: 60 μg/mL, C2: 30 μg/mL, and C3: 15 μg/mL for the 3-hour test in the presence of metabolic activation, C1: 50 μg/mL, C2: 20 μg/mL and C3: 7.5 μg/mL for the 3-hour test in the absence of metabolic activation, and C1: 40 μg/mL, C2: 15 μg/mL and C3: 7.5 μg/mL for the 24-hour test without activation). Theses doses were established based on a preliminary test performed to determine the cytotoxic dose (Supplementary Tables 2 and 3). Treatment concentrations for the main test were selected on the basis of results of the performed preliminary test (data not shown) and according to the OECD No. 487 guideline instructions (up to the cytotoxic limit). Higher concentrations were expected to elicit a survival rate lower than the threshold 40% Relative Increase in Cell Count (RICC). For the experiments, 1×10^6 cells (starting cell count (N0)) were placed in 25 cm^2 sterile flasks in RPMI-1640 medium supplemented with 5% serum at 37 °C ± 1 °C in a humidified atmosphere (approximately 5% CO_2 in air) and exposed to OE20HT, negative and positive controls items, with or without metabolic activation. Cells were incubated for either 3 or 24 h in presence of OE20TH. After incubation, the cell cultures were centrifuged at 2000 rpm (approximately 836 g) for 5 min, washed with tissue culture medium, and suspended in 10 mL RPMI-1640 10% serum. Cells were transferred into flasks for growth through the recovery period (21 h) at 37 °C ± 1 °C in a humidified atmosphere (approximately 5% CO2 in air) for the three-hour treatments or immediately counted using haemocytometer to estimate number of surviving cells (N). Cytotoxicity was assessed as follows: for each culture, the relative population doubling (RPD) was calculated according to the equation shown below and used relative to that of the vehicle control.

$$RPD = \frac{(N°\,of\,population\,doublings\,in\,treated\,cultures)}{(N°\,of\,population\,doublings\,in\,control\,cultures)} \times 100$$

$$Population\,doublings(PD) = [\log(\frac{N}{N0})]\log 2$$

$$Mean\,PD\,as\%\,of\,the\,control = \frac{mean\,PD\,treated}{mean\,PD\,vehicle\,control} \times 100$$

Any cytotoxicity induced by a treatment was evaluated based on the decrease in the PD, when compared to the vehicle control (mean % PD of the vehicle control set to 100%).

$$Decrease\,in\,PD(\%) = 100 - Mean\,PD\,as\%\,of\,the\,control$$

Additionally, the Relative Increase in Cell Count (RICC; expressed as %) was also calculated for each culture according to the equation shown below.

$$RICC = \frac{(increase\,in\,number\,of\,cells\,in\,treated\,cultures(final - starting))}{(increase\,in\,number\,of\,cells\,in\,control(final - starting))} \times 100$$

Cells were then treated for 3 min with hypotonic KCl solution at room temperature and fixed in a methanol/acetic-acid 3:1 (v/v) mixture, before being kept at 2–8 °C overnight. Cells suspension was placed onto microscope slides and air-dried before staining with 5% (w/v) Giemsa solution (Sigma-Aldrich Co.) for 5 min. Slides were washed

with distilled water and air-dried at room temperature for at least 12 h. Two thousand cells were counted per replicate to assess the ratio of micronucleated cells and expressed as a percentage. The slide analysis was conducted under the control of the Principal Investigator in compliance with Good Laboratory Practice as required by the United Kingdom GLP Compliance Regulations 1999 (SI 1999 No. 3106, as amended 2004, SI No. 0994) and which follow the OECD Principles of Good Laboratory Practice (as revised in 1997).

2.1.6. Statistical analysis

Micronucleated cells in treated cultures were compared to that of the negative (vehicle) control cultures. Statistical analysis (Fisher's exact test) was performed. Statistical significance cut-off was set at 5%.

2.2. Literature search

A comprehensive literature review of olive products, olive extracts, and hydroxytyrosol safety was performed using different databases (MEDLINE and Google Scholar) on March 10, 2022. The keywords used for the search included (olive OR hydroxytyrosol), filtering publications written in English with no limitation regarding the publication date. After elimination of duplicates, a total of 1618 publications were screened for their relevance based on their title and abstract. Studies that were omitted included those that did not evaluate any aspect of HT safety, or in which the HT-content of the test article was not quantified and could not be estimated. Ninety-one publications related to the safety evaluation of either olive extracts or hydroxytyrosol were included in the analysis.

3. Results

3.1. Ames assay

Cytotoxicity was evaluated in the *S. typhimurium* TA98 an TA100 strains by the direct incorporation procedure, with or without metabolic activation system (± S9). In total, seven concentrations were prepared by successive dilutions of the stock solution. Concentrations were spaced by factors of 2, 2.5, and 4 times (approximately $\sqrt{10}$), starting at 10 μg/plate up to 5000 μg/plate. No inhibitory or cytotoxic effects were detected in the two bacterial strains at a concentration of up to 5000 μg/plate (Supplementary Table 1). Positive controls elicited a requisite increase in revertants and MF in both direct incorporation and preincubation assays (Tables 1 and 2), which confirm the validity of the test system. None of the concentrations of "OE20HT" evaluated elicited an increase in the number of revertants in any of the strains, as compared to the solvent control samples, with or without S9-mix.

All results remained within the historical control range for the laboratory, and no dose-response relationship or relevant increase of revertant compared to the solvent controls, were noted.

3.2. In vitro micronucleus assay

3.2.1. Cytotoxicity evaluation

The concentrations evaluated of the test article in the cytotoxicity assay were 80, 70, 60, 30, 15, 7.5, and 3.75 μg/mL (3-hour treatment with metabolic activation), 50, 40, 30, 20, 15, 7.5, and 3.75 μg/mL (3-hour treatment without metabolic activation), and 50, 40, 30, 15, 7.5, and 3.75 μg/mL (24-hour treatment without metabolic activation). There were no substantial changes in the pH or osmolality and no insolubility was observed in the final treatment medium in any of the experiments. Marked cytotoxicity was observed in the 3-hour treatment with metabolic activation at concentrations of 80, 70, 60, and 30 μg/mL (RICC values were 1%, 22%, 38% and 58%, respectively) (Supplementary Table 2). Marked cytotoxicity was also observed in the 3-hour treatment without metabolic activation at concentrations of 50 and 40 μg/mL (RICC values were 49% and 64%, respectively) (Supplementary Table 2). The same effect was observed in the 24-hour treatment without metabolic activation at concentrations of 50, 40, and 30 μg/mL (RICC values were 37%, 43% and 56%, respectively) (Supplementary Table 3). Therefore, concentrations of 60, 30 and 15 μg/mL were selected for the 3-hour treatment with metabolic activation, 50, 20 and 7.5 μg/mL were selected for the 3-hour treatment without metabolic activation, and 40, 15 and 7.5 μg/mL were selected for the 24-hour treatment without metabolic activation.

3.2.2. Micronucleus test results

The three positive control substances, cyclophosphamide (a mutagenic and clastogenic agent that requires metabolic transformation by microsomal enzymes), mitomycin C (mutagenic and clastogenic agent), and colchicine (aneugenic agent), all induced a significant increase of mean number of micronucleated cells, supporting the validity of the test system (all $p < 0.001$ compared to vehicle control) (Tables 3 and 4). None of the OE20HT concentrations elicited a biologically or statistically significant increase in the number of micronucleated cells as compared to the negative (vehicle) control value in the experiments with and without metabolic activation (Tables 3 and 4).

4. Discussion and safety review

4.1. Olive composition

The olive fruit is divided into three parts: the epicarp or skin, the mesocarp or pulp, and the endocarp or stone. The pulp represents 84–90% of the total fruit mass. The olive fruit is composed primarily of water (~50%), and oil (~22%), followed by carbohydrate (19.1%), cellulose (5.8%), protein (1.6%), inorganic substances (1.5%), and phenolic compounds (1.3%) [42]. Other important compounds present in olive fruit are pectin, organic acids, and pigments. It is also a potential source of antioxidant enzymes, such as catalase and superoxide dismutase [43]. The composition of olive fruit is highly dependent on the harvesting period and the fruit maturity [44], but, interestingly, moisture and total phenolic content were not substantially affected by the maturation of olive fruit.

The growing interest in olive fruit is mainly related to the presence of phenolic compounds, which may play a role in various beneficial health effects. The most common phenolic compounds are HT and its derivatives, such as tyrosol, as well as oleuropein, rutin, verbascoside, apigenin, luteolin, luteolin-7-glucoside, quercetin-3- glucoside, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, vanillic acid, caffeic acid, and vanillin [42,45–50]. Depending on the specific variety, the fruit an contain 1000–2700 mg of phenolic compounds per kg fresh fruit, while the oil has a dramatically lower concentration (35–170 mg/kg oil) [50].

4.2. Genotoxicity and mutagenicity

4.2.1. Mutagenicity

The mutagenic potential of OE20HT have been evaluated in *S. typhimurium* and *E. coli* strains according to OECD Guidelines for Testing of Chemicals n°471. There were no biologically relevant increases in the number of revertants at concentrations of up to 5000 μg/plate using the direct incorporation and preincubation methods, with or without metabolic activation. This upper limit concentrations was equivalent to 1000 μg/plate of HT. These results are in accordance with findings from previous studies showing no mutagenic potential in Ames assays performed with either olive aqueous extracts containing HT or pure HT [51–53]. Only one study was identified in the literature in which a mutagenic effect was reported for an olive extract called HIDROX®, containing 2.4% HT and 6% phenolic compounds. However, the results were considered equivocal by investigators due to the presence of toxicity and precipitates at doses demonstrating a potential mutagenic response [54,55].

In a somatic mutation and recombination test (SMART) in the wings

Table 1
Direct incorporation Ames test results performed on Salmonella typhimurium TA98, TA100, TA1535, TA1537 and Escherichia coli WP2 UvrA, with (+) or without (-) metabolic activation (S9) after exposure to OE20HT. Results are presented as mean values of three replicates ± standard deviation (SD). Mutation factor (MF) was calculated as the ratio of mean revertants in the test item conditions on the mean revertants of the corresponding solvent control. 2AA: 2-aminoanthracene; 9AA: 9-aminoacridine; MMS: methyl-methanesulfonate; NDP: 4-nitro-1,2-phenylene-diamine; OE20HT: Olive fruit dry extract 20% HT; SAZ: Sodium azide.

S9	Strain	Compound	Concentrations (μg/plate)	Mean values of revertants/plate (± *SD*)	MF
S9 (−)	*Salmonella typhimurium* TA98	Untreated (control)	-	16.7 (*0.58*)	0.96
		DMSO (control)	-	18.3 (*1.53*)	1.06
		Distilled water (control)	-	17.3 (*1.53*)	1.00
		OE20HT	5000	18.0 (*1.00*)	1.04
		OE20HT	1581	19.0 (*1.00*)	1.10
		OE20HT	500	17.0 (*1.00*)	0.98
		OE20HT	158.1	16.0 (*0.00*)	0.92
		OE20HT	50	17.0 (*1.00*)	0.98
		OE20HT	15.81	15.3 (*1.53*)	0.88
		NDP	4	417.3 (*12.22*)	22.76
	Salmonella typhimurium TA100	Untreated (control)	-	91.7 (*8.50*)	0.94
		DMSO (control)	-	92.0 (*5.29*)	0.94
		Distilled water (control)	-	98.0 (*5.57*)	1.00
		OE20HT	5000	100.3 (*3.51*)	1.02
		OE20HT	1581	99.3 (*8.14*)	1.01
		OE20HT	500	100.0 (*4.00*)	1.02
		OE20HT	158.1	99.0 (*2.65*)	1.01
		OE20HT	50	98.0 (*6.08*)	1.00
		OE20HT	15.81	93.0 (*6.08*)	0.95
		SAZ	2	1093.3 (*33.31*)	123.80
	Salmonella typhimurium TA1535	Untreated (control)	-	11.3 (*1.15*)	0.94
		DMSO (control)	-	11.3 (*0.58*)	0.94
		Distilled water (control)	-	12.0 (*0.00*)	1.00
		OE20HT	5000	13.7 (*0.58*)	1.14
		OE20HT	1581	13.3 (*1.15*)	1.11
		OE20HT	500	12.7 (*2.31*)	1.06
		OE20HT	158.1	12.7 (*1.53*)	1.06
		OE20HT	50	12.0 (*0.00*)	1.00
		OE20HT	15.81	12.7 (*0.58*)	1.06
		SAZ	2	1228.0 (*22.27*)	102.33
	Salmonella typhimurium TA1537	Untreated (control)	-	7.3 (*0.58*)	0.96
		DMSO (control)	-	8.0 (*2.00*)	1.04
		Distilled water (control)	-	7.7 (*0.58*)	1.00
		OE20HT	5000	8.0 (*1.73*)	1.04
		OE20HT	1581	8.7 (*1.53*)	1.13
		OE20HT	500	7.3 (*0.58*)	0.96
		OE20HT	158.1	8.0 (*1.73*)	1.04
		OE20HT	50	8.3 (*1.53*)	1.09
		OE20HT	15.81	8.0 (*0.00*)	1.04
		9AA	50	410.7 (*16.65*)	51.33
	Escherichia coli WP2 uvrA	Untreated (control)	-	43.7 (*1.53*)	0.96
		DMSO (control)	-	46.0 (*2.00*)	1.01
		Distilled water (control)	-	45.3 (*1.53*)	1.00
		OE20HT	5000	47.7 (*2.52*)	1.05
		OE20HT	1581	47.0 (*1.73*)	1.04
		OE20HT	500	42.7 (*1.53*)	0.94
		OE20HT	158.1	43.3 (*0.58*)	0.96
		OE20HT	50	42.7 (*1.15*)	0.94
		OE20HT	15.81	43.7 (*4.93*)	0.96
		MMS	2 μL	1062.7 (*66.01*)	23.44
S9 (+)	*Salmonella typhimurium* TA98	Untreated (control)	-	18.3 (*1.53*)	0.98
		DMSO (control)	-	19.7 (*0.58*)	1.05
		Distilled water (control)	-	18.7 (*0.58*)	1.00
		OE20HT	5000	18.3 (*0.58*)	0.98
		OE20HT	1581	17.7 (*2.08*)	0.95
		OE20HT	500	19.3 (*1.15*)	1.04
		OE20HT	158.1	19.3 (*1.15*)	1.04
		OE20HT	50	20.3 (*0.58*)	1.09
		OE20HT	15.81	18.7 (*1.15*)	1.00
		2AA	2	2434.7 (*12.22*)	123.80
	Salmonella typhimurium TA100	Untreated (control)	-	103.0 (*6.24*)	0.97
		DMSO (control)	-	104.0 (*6.93*)	0.98
		Distilled water (control)	-	105.7 (*2.08*)	1.00
		OE20HT	5000	97.7 (*2.52*)	0.92
		OE20HT	1581	97.3 (*6.11*)	0.92
		OE20HT	500	105.0 (*3.00*)	0.99
		OE20HT	158.1	93.3 (*3.51*)	0.88
		OE20HT	50	103.7 (*3.06*)	0.98
		OE20HT	15.81	101.0 (*1.00*)	0.96
		2AA	2	2440.0 (*12.00*)	23.50
	Salmonella typhimurium TA1535				

(*continued on next page*)

Table 1 (*continued*)

S9	Strain	Compound	Concentrations (μg/plate)	Mean values of revertants/plate (± *SD*)	MF
		Untreated (control)	-	11.3 (*0.58*)	0.97
		DMSO (control)	-	12.0 (*0.00*)	1.03
		Distilled water (control)	-	11.7 (*1.53*)	1.00
		OE20HT	5000	14.7 (*0.58*)	1.26
		OE20HT	1581	13.7 (*1.15*)	1.17
		OE20HT	500	13.7 (*0.58*)	1.17
		OE20HT	158.1	13.7 (*0.58*)	1.17
		OE20HT	50	12.3 (*1.53*)	1.06
		OE20HT	15.81	13.0 (*0.00*)	1.11
		2AA	2	213.0 (*6.00*)	17.75
	Salmonella typhimurium TA1537	Untreated (control)	-	8.7 (*0.58*)	1.08
		DMSO (control)	-	7.0 (*1.00*)	0.88
		Distilled water (control)	-	8.0 (*1.00*)	1.00
		OE20HT	5000	8.3 (*2.08*)	1.04
		OE20HT	1581	7.7 (*0.58*)	0.96
		OE20HT	500	7.0 (*1.00*)	0.88
		OE20HT	158.1	8.0 (*0.00*)	1.00
		OE20HT	50	9.0 (*1.00*)	1.13
		OE20HT	15.81	8.3 (*2.31*)	1.04
		2AA	2	205.0 (*7.21*)	29.29
	Escherichia coli WP2 uvrA	Untreated (control)	-	45.7 (*1.15*)	0.99
		DMSO (control)	-	46.0 (*1.00*)	0.99
		Distilled water (control)	-	46.3 (*0.58*)	1.00
		OE20HT	5000	50.3 (*1.53*)	1.09
		OE20HT	1581	50.7 (*1.53*)	1.09
		OE20HT	500	49.3 (*2.08*)	1.06
		OE20HT	158.1	42.3 (*4.51*)	0.91
		OE20HT	50	47.0 (*1.00*)	1.01
		OE20HT	15.81	42.7 (*1.53*)	0.92
		2AA	50	279.7 (*9.45*)	6.08

of *Drosophila melanogaster,* no genotoxic, nor mutagenic activity of extra virgin olive oil or of the three distinctive compounds (triolein, tyrosol and squalene) from different origins, were observed [56].

4.2.2. Genotoxicity In vitro

Genotoxicity of OE20HT has been evaluated in this study according to OECD Guidelines for Testing of Chemicals n°487. Accordingly to OECD 487 guidelines, the doses were selected based in the cytotoxic potential of the product, the highest dose tested inducing a slight cytotoxic effects, as reported by a decrease of the RICC. According to results obtained in the *in vitro* micronucleus assay, no aneugenic nor clastogenic potential was observed in mouse lymphoma cells (L5178Y $TK^{+/-}$ 3.7.2 C) after exposure to OE20HT at doses up to 60 μg/mL after 3 h of exposure in presence of metabolic activation, up to 50 μg/mL after 3 h of exposure in absence of metabolic activation, and up to 40 μg/mL after 24 h of exposure without metabolic activation. These concentrations consisted of 12 μg/mL, 10 μg/mL, and 8 μg/mL of HT respectively.

Based on the literature review, these results are in accordance with findings from an *in vitro* micronucleus assay performed in Chinese hamster ovary cells wherein, HT of different sources (pure HT, diluted HT, and olive extracts) at concentrations of 12.5 μg/mL, 15.6 μg/mL or 18 μg/mL, respectively, was not associated with any significant increases in the numbers of binucleated cells with micronucleus compared to control, either after 4-hour exposure with metabolic activation, or 24-hour exposure without metabolic activation [51]. In the same study, higher concentrations of HT (from pure HT or extracts containing HT) were associated with an increase of micronucleated cells; however, in these cases, concomitant increases in cytotoxicity and cytostasis were also observed at this concentrations with a dose-dependent increase in hydrogen peroxide production. This was postulated by the researchers to be formed after interaction between HT and components of the growth medium. In this context, the authors stated that the genotoxic effect observed at high doses was more likely related to the effects of hydrogen peroxide than HT itself [51].

Chromosomal aberrations induction potential of different olive extracts and HT have also been evaluated in different cellular models. An *in vitro* study performed in *Allium cepa* root meristematic cells showed variable results after cells exposure to extracts from olive mill wastewater, olive wet husk, and olive brine [53]. Despite a significant decrease in the mitotic index values in the highest concentration conditions, no significant increases in anaphase aberrations (*i.e.*, bridges, laggard chromosomes, and fragments) were measured, except for the 500 μg/L gallic acid equivalent concentration of olive wet husk compared to negative control (2.35 ± 0.42 *vs.* 0.35 ± 0.13, $p < 0.05$). This study highlighted a potential toxic effect of olive extracts on mitosis that is not systematically associated with a clastogenic potential in *Allium cepa* root meristematic cells [53]. This effect at very high concentrations of certain extracts was also noted in a mammalian cellular model [54]. Christian et al., demonstrated that in the presence of metabolic activation, HIDROX®, a hydrolyzed aqueous olive pulp extract containing 24 μg/mL of HT, induced a significant increase in the mean percentage of aberrant cells compared to the negative control (29% *vs.* 5%, $p < 0.05$) in Chinese hamster ovary cells (CHO) after exposure to 1000 μg/mL. Nevertheless, according to the authors, these positive results were considered equivocal, due to the high cytotoxicity and the presence of precipitates and were not confirmed or replicated in an *in vivo* micronucleus assay [54,55]. This clastogenic or aneugenic potential could partly be associated with the presence of HT. Indeed, in an *in vitro* chromosomal aberration test performed in primary human lymphocytes, it was demonstrated that high concentrations of HT (287.7 μg/mL and 503.5 μg/mL) induced a significant increase in aberrant cells percentage (excluding gaps) compared to negative control, both in the presence and absence of metabolic activation [52]. The doses showing clastogenic or aneugenic potential far exceeding those attainable after intake. Consequently, the authors concluded that there were not genotoxic potential of HT at doses achievable after intake [51, 52].

Overall, the *in vitro* genotoxic potential of HT-containing olive extracts and HT itself has been evaluated in various studies. Overall, the results were negative with the only positive findings observed at very high concentrations and/or in the presence of marked cytotoxicity/precipitates that confounded establishing a clear positive response. These results are in accordance with results presented in this study, demonstrating the absence of OE20HT genotoxic or mutagenic

Table 2
pre-incubation Ames test results performed on Salmonella typhimurium TA98, TA100, TA1535, TA1537 and Escherichia coli WP2 UvrA, with (+) or without (-) metabolic activation (S9) after exposure to OE20HT. Results are presented as mean values of three replicates ± standard deviation (SD). Mutation factor (MF) was calculated as the ratio of mean revertants in the test item conditions on the mean revertants of the corresponding solvent control. 2AA: 2-aminoanthracene; 9AA: 9-aminoacridine; MMS: methyl-methanesulfonate; NDP: 4-nitro-1,2-phenylene-diamine; OE20HT: Olive fruit dry extract 20% HT, SAZ: Sodium azide.

S9	Strain	Compound	Concentrations (μg/plate)	Mean values of revertants/plate	MF
S9 (−)	*Salmonella typhimurium* TA98	Untreated (control)	-	17.7 (*2.08*)	0.95
		DMSO (control)	-	17.0 (*1.73*)	0.91
		Distilled water (control)	-	18.7 (*1.15*)	1.00
		OE20HT	5000	16.7 (*1.53*)	0.89
		OE20HT	1581	20.3 (*1.53*)	1.09
		OE20HT	500	18.0 (*1.00*)	0.96
		OE20HT	158.1	16.7 (*0.58*)	0.89
		OE20HT	50	16.3 (*0.58*)	0.88
		OE20HT	15.81	17.3 (*1.15*)	0.93
		NDP	4	406.7 (*19.73*)	23.92
	Salmonella typhimurium TA100	Untreated (control)	-	85.7 (*1.53*)	1.03
		DMSO (control)	-	85.7 (*3.21*)	1.03
		Distilled water (control)	-	83.0 (*3.61*)	1.00
		OE20HT	5000	44.7 (*20.21*)	0.54
		OE20HT	1581	84.7 (*5.03*)	1.02
		OE20HT	500	86.0 (*4.36*)	1.02
		OE20HT	158.1	88.0 (*4.93*)	1.06
		OE20HT	50	84.3 (*4.93*)	1.02
		OE20HT	15.81	86.0 (*2.65*)	1.04
		SAZ	2	120.3 (*52.05*)	14.52
	Salmonella typhimurium TA1535	Untreated (control)	-	12.0 (*2.00*)	0.90
		DMSO (control)	-	12.7 (*1.15*)	0.95
		Distilled water (control)	-	13.3 (*1.15*)	1.00
		OE20HT	5000	13.0 (*1.00*)	0.98
		OE20HT	1581	14.0 (*0.00*)	1.05
		OE20HT	500	10.3 (*2.52*)	0.78
		OE20HT	158.1	11.3 (*1.53*)	0.85
		OE20HT	50	11.0 (*1.73*)	0.83
		OE20HT	15.81	11.0 (*1.73*)	0.83
		SAZ	2	1172.0 (*30.20*)	87.90
	Salmonella typhimurium TA1537	Untreated (control)	-	11.7 (*0.58*)	0.95
		DMSO (control)	-	12.3 (*0.58*)	1.00
		Distilled water (control)	-	12.3 (*0.58*)	1.00
		OE20HT	5000	11.7 (*0.58*)	0.95
		OE20HT	1581	11.0 (*1.73*)	0.89
		OE20HT	500	12.0 (*1.00*)	0.97
		OE20HT	158.1	12.3 (*0.58*)	1.00
		OE20HT	50	10.7 (*0.58*)	0.86
		OE20HT	15.81	10.7 (*1.15*)	0.86
		9AA	50	408.0 (*16.00*)	33.08
	Escherichia coli WP2 uvrA	Untreated (control)	-	45.7 (*3.21*)	0.99
		DMSO (control)	-	46.0 (*2.00*)	1.00
		Distilled water (control)	-	46.0 (*2.00*)	1.00
		OE20HT	5000	44.7 (*3.06*)	0.97
		OE20HT	1581	46.3 (*2.08*)	1.01
		OE20HT	500	48.7 (*1.15*)	1.06
		OE20HT	158.1	47.3 (*1.15*)	1.03
		OE20HT	50	48.3 (*2.08*)	1.05
		OE20HT	15.81	46.3 (*3.79*)	1.01
		MMS	2 μL	1093.3 (*36.07*)	23.77
S9 (+)	*Salmonella typhimurium* TA98	Untreated (control)	-	18.7 (*1.15*)	0.97
		DMSO (control)	-	17.0 (*1.00*)	0.88
		Distilled water (control)	-	19.3 (*1.15*)	1.00
		OE20HT	5000	15.0 (*1.00*)	0.78
		OE20HT	1581	17.0 (*1.00*)	0.88
		OE20HT	500	18.7 (*0.58*)	0.97
		OE20HT	158.1	17.3 (*2.08*)	0.90
		OE20HT	50	18.3 (*0.58*)	0.95
		OE20HT	15.81	18.7 (*0.58*)	0.97
		2AA	2	2414.7 (*31.07*)	142.04
	Salmonella typhimurium TA100	Untreated (control)	-	92.7 (*1.15*)	1.00
		DMSO (control)	-	93.7 (*4.51*)	1.01
		Distilled water (control)	-	92.7 (*4.16*)	1.00
		OE20HT	5000	99.3 (*1.53*)	1.07
		OE20HT	1581	97.0 (*2.65*)	1.05
		OE20HT	500	94.0 (*6.08*)	1.01
		OE20HT	158.1	97.7 (*4.51*)	1.05
		OE20HT	50	100.3 (*2.52*)	1.08
		OE20HT	15.81	97.7 (*2.52*)	1.05
		2AA	2	2397.3 (*62.14*)	22.59
	Salmonella typhimurium TA1535				

(*continued on next page*)

Table 2 (*continued*)

S9	Strain	Compound	Concentrations (µg/plate)	Mean values of revertants/plate	MF
		Untreated (control)	-	14.3 (*0.58*)	0.98
		DMSO (control)	-	15.0 (*1.00*)	1.02
		Distilled water (control)	-	14.7 (*0.58*)	1.00
		OE20HT	5000	12.7 (*0.58*)	0.86
		OE20HT	1581	14.0 (*0.00*)	0.95
		OE20HT	500	13.0 (*1.00*)	0.89
		OE20HT	158.1	12.3 (*0.58*)	0.84
		OE20HT	50	12.0 (*0.00*)	0.82
		OE20HT	15.81	13.0 (*1.00*)	0.89
		2AA	2	209.3 (*4.16*)	13.96
	Salmonella typhimurium TA1537	Untreated (control)	-	13.7 (*0.58*)	1.00
		DMSO (control)	-	13.7 (*0.58*)	1.00
		Distilled water (control)	-	13.7 (*0.58*)	1.00
		OE20HT	5000	12.0 (*2.00*)	0.88
		OE20HT	1581	13.3 (*0.58*)	0.98
		OE20HT	500	13.3 (*0.58*)	0.98
		OE20HT	158.1	13.0 (*1.00*)	0.95
		OE20HT	50	12.7 (*0.58*)	0.93
		OE20HT	15.81	13.3 (*1.15*)	0.98
		2AA	2	215.3 (*7.02*)	15.76
	Escherichia coli WP2 uvrA	Untreated (control)	-	48.7 (*1.53*)	0.97
		DMSO (control)	-	49.3 (*2.52*)	0.98
		Distilled water (control)	-	50.3 (*1.53*)	1.00
		OE20HT	5000	49.3 (*3.06*)	0.98
		OE20HT	1581	49.0 (*2.65*)	0.97
		OE20HT	500	48.7 (*1.15*)	0.97
		OE20HT	158.1	48.3 (*3.21*)	0.96
		OE20HT	50	47.7 (*0.58*)	0.95
		OE20HT	15.81	49.3 (*2.31*)	0.98
		2AA	50	244.0 (*9.17*)	4.95

Table 3
micronucleated count results for 3 h treatment and 21 h recovery, performed on mouse lymphoma L5178Y TK$^{+/-}$ 3.7.2 C cells, with (+) or without (-) metabolic activation (S9), after exposure to OE20HT. Results are presented as mean number of micronucleated cells per 1000 cells counted of two replicates. *** : statistically significant at $p < 0.001$ level when compared to negative (vehicle) control, Fisher's test. Not applicable: cell counts at harvest were lower than at the start of the treatment. CP: Cyclophosphamide; MMC: Mitomycin C; OE20HT: Olive fruit dry extract 20% HT, RICC: relative increase in cell count; VC: vehicle control.

S9	Compound	Concentrations	RICC (%) (vs. distilled water)	Mean number of micronucleated cells
S9 (+)	Distilled water (VC)	2%	100	1.5
	OE20HT	60 µg/mL	38	2.5
	OE20HT	30 µg/mL	58	2.5
	OE20HT	15 µg/mL	75	1.0
	CP	6 µg/mL	15	80.5 ***
S9 (-)	Distilled water (VC)	2%	100	1.5
	OE20HT	50 µg/mL	49	4.5
	OE20HT	20 µg/mL	101	1.0
	OE20HT	7.5 µg/mL	105	2.5
	MMC	0.5 µg/mL	44	102.0 ***
	Colchicine	0.5 µg/mL	Not applicable	77.5 ***

Table 4
micronucleated count results for 24 h treatment, performed on mouse lymphoma L5178Y TK$^{+/-}$ 3.7.2 C cells, without (-) metabolic activation (S9), after exposure to OE20HT. Results are presented as mean number of micronucleated cells per 1000 cells counted of two replicates. *** : statistically significant at $p < 0.001$ level when compared to negative (vehicle) control, Fisher's test. Not applicable: cell counts at harvest were lower than at the start of the treatment. MMC: Mitomycin C; OE20HT: Olive fruit dry extract 20% HT, RICC: relative increase in cell count; VC: vehicle control.

S9	Compound	Concentrations	RICC (%) (*vs.* distilled water)	Mean number of micronucleated cells
S9 (-)	Distilled water (VC)	2%	100	2.0
	OE20HT	40 µg/mL	43	3.0
	OE20HT	15 µg/mL	73	2.0
	OE20HT	7.5 µg/mL	89	1.5
	MMC	0.5 µg/mL	7	80.0 ***
	Colchicine	0.5 µg/mL	Not applicable	97.5 ***

potential.

4.2.3. Genotoxicity in vivo

Although some equivocal findings have been noted for HT and HT-containing olive extracts in some *in vitro* genotoxicity assays, no positive effects were elicited in several *in vivo* micronucleus assays [51,54], nor in an *in vivo* bone marrow chromosome aberration test [57]. To confirm the genotoxic potential of HIDROX®, following equivocal *in vitro* results, Christian et al. conducted an *in vivo* micronucleus assay in rats following single and repeated gavage dosing with up to 2000 mg /kg body weight (single dose) or 5000 mg/kg body weight/day (28 days of dosing), providing, respectively, 48 mg/kg body weight and

120 mg/kg body weight/day of HT [54]. No significant increases in polychromatic erythrocytes counts were reported in any group dosed with olive aqueous extract (HIDROX®) after single or repeated dosing, as compared to the negative control [54]. Similar negative results were observed in rats in a chromosome aberration test after a single oral dose of 2000 mg HT/kg body weight [57] or after repeat oral dosing for 4 weeks at a daily dose of 561 mg HT/kg body weight [49]. After acute oral supplementation with H40, an extract nominally containing 40% HT, negative results were also reported at doses providing up to 2000 mg/kg body weight of HT [51]. None of the assays showed statistically significant or biologically relevant increases in the micronuclei frequency in polychromatic erythrocytes [51,57], despite the marked increase of plasmatic HT concentrations as measured in one of the studies [51]. However, in this same study, exposure to 250 and 500 mg HT/kg body weight/day, corresponding to the middle and highest dose respectively, from an extract nominally containing 35% HT for 90 days, was associated with a significant increase of micronuclei in males rats bone marrow for high dose and in females rats bone marrow for middle dose [51]. Due to certain shortcomings in the conduct of the study, and not described, the authors questioned the results obtained in this 90-days *in vivo* micronucleus [51]. Overall, *in vivo* micronucleus and bone marrow tests support the absence of genotoxic potential of HT and olive extracts following ingestion and rule out the equivocal findings from *in vitro* assays.

4.2.4. Acute toxicity

From the literature review, several acute toxicity studies were identified, being conducted in different *in vivo* models. In mice, no mortality, nor morbidity were noted after a single dose of HIDROX® (2000 mg/kg body weight, providing 48 mg/kg body weight of HT) [54]. In rats, no mortality occurred after oral administration of 5000 mg/kg body weight of HIDROX® (providing 120 mg/kg body weight of HT). However, at 1500 and 2000 mg/kg body weight (36 and 48 mg/kg body weight/day of HT), weight gain was significantly reduced in females but without significant differences in food consumption compared to controls. No similar effects were observed in male rats. The acute median lethal dose levels (LD_{50}) were set to $>$ 2000 mg /kg body weight in mice and $>$ 5000 mg/kg body weight in rats, the highest doses tested [54]. Similar results on the body weight, were noted in acute *in vivo* micronucleus test conducted in rats wherein 2000 mg HT/kg body weight (from H40 extract) administrated by gavage, was well- tolerated and only associated with mild clinical signs (reduced activity, abdominal position, ruffled fur) [51]. In an acute toxicity study conducted in rats in accordance with OECD Guidelines for Testing of Chemicals n°420, a single dose of virgin olive oil extract (300 mg/kg body weight, providing 45 mg HT/kg body weight) had no adverse effects on body weight gain, clinical evaluations (incidence of tremors, convulsions, numbness, salivation, diarrhea, alteration of the skin, hairs or eyes, or the urine color), or macroscopic or microscopic appearance of organs and tissues, compared to controls [58]. However, some effects were reported in relation to hematological and biochemical parameters, including an increase in monocytes counts (1.9 ± 0.1% *vs.* 3.5 ± 0.3%, $p = 0.017$) and creatinin kinase isoenzyme MB (374.6 ± 36.3 U/L *vs.* 549.8 ± 22.14 U/L, $p = 0.041$), and a decrease in hemoglobin content (14.0 ± 0.4 g/dL *vs.* 10.1 ± 2.7 g/dL, $p = 0.029$) and percentage of packed red blood cell volume (38.5 ± 1.2% *vs.* 27.7 ± 7.8%, $p = 0.033$) in the dosed group compared to controls. These variations remained within the historical range of values for these parameters in rats and therefore were not considered by the researchers to be biologically relevant [58]. Similar results were observed in male and female rats following acute administration of phosphatidyl-HT, a phospholipid carrier form of HT, at gavage dose of 2000 mg/kg body weight. No mortality, abnormal clinical signs, behavioral changes, body weight changes, macroscopic findings, organ weight differences, hematological or biochemical markers, or histopathological changes were reported [59]. In the same study, the gavage dosing of 2000 mg/kg body weight/day in rats for 14 days, did not elicit any adverse effects in relation to any of the same parameters evaluated as in the acute study.

4.3. Repeated dose oral toxicity

Sub-chronic toxicity of olive extracts and pure HT have been evaluated in different *in vivo* studies [54,58,60,61]. Overall, the data obtained from the repeated dose studies showed that oral supplementations with olive extracts or HT did not induce toxic effects *in vivo*. Since the variations observed were small, transient, not always dose-dependent and the values remaining within the normal values for each parameter tested, these variations were not considered to be relevant from a toxicological point of view.

4.3.1. Sub-Chronic

Martínez et al. (2018) conducted a repeated dose 28-day oral toxicity study in male and female Wistar rats to evaluate the safety of phosphatidyl-HT, a more bioavailable HT form. The 28-day administration of 2000 mg phosphatidyl-HT/kg body weight/day, was not associated with any mortality, changes in body weights, body weight gains or food consumption. No significant hematological or clinical pathologic alterations were noted compared to controls. Both gross and histopathological examinations did not reveal any treatment-related changes. The authors concluded that the NOAEL in rats was 2000 mg/kg body weight/day, the highest dose tested [59].

4.3.1.1. Growth, water, and food consumption. The daily gavage administration of 2000 mg HIDROX®/kg body weight/day (corresponding to 48 mg HT/kg body weight/day) in Sprague Dawley rats for 90 days did not affect final body weights, or body weight gains, nor feed intake of male or female rats, as compared to controls [54]. Regarding these parameters, similar results were obtained in other sub-chronic studies after oral supplementation of Wistar rats with doses of virgin olive oil extracts up to 2000 mg/kg body weight/day (providing 300 mg/kg body weight/day HT) [58].

However, at study termination, a significant reduction in body weights, of approximatively 9%, was noted in male rats after supplementation with olive extract H35 at doses providing 500 mg/kg body weight/day of HT [61]. Transient but statistically significant differences in body weight gain were also noted in this same group at different timepoints during the study. Nevertheless results from recovery study indicated that these effects were reversible following cessation of dosing (reduction in body gain in male rats at 13 weeks = 17% and at the end of the recovery period at week 17 = 6%) [61]. These results are in accordance with results obtained after supplementation with pure HT at doses up to 500 mg/kg body weight/day, where a similar decrease of absolute body weight and body weight gains was observed following week 13 in male Wistar rats with a decrease of 14% in body weight gain [60]. However, these observations were not associated with other indications of toxicity and were concluded to be non-adverse by the authors.

4.3.1.2. Clinical signs. Regarding clinical signs, small alopecia, scabs, and desquamation were observed with lower doses of either virgin olive oil extract (100 and 300 mg/kg body weight/day, providing 15 and 45 mg/kg body weight/day of HT) or HT (5 mg/kg body weight/day) in a few numbers of animals. In both studies, authors considered that these effects were not related to the test items [58,60]. Mild to moderate salivation was observed in different studies in tested groups after dosing with HIDROX® (dose-dependent increase), H35 providing 250 or 500 mg HT/kg body weight/day, and after administration of pure HT at a daily dose of 500 mg/kg body weight/day [54,60,61]. Nevertheless, due the absence of histological or neurobehavioral effects that would be indicative of an autonomic imbalance, these findings were considered by the authors as non-adverse treatment-related effect [61] and attributed to either the increased viscosity of tested solutions [54] or the bitter

taste of HT [60].

Overall, none of the olive extracts, or HT test articles elicited any serious adverse clinical effects and no findings were noted following necropsy observations [54,58,60].

4.3.1.3. Hematological biomarkers. Christian et al. concluded that no toxicologically relevant effects on hematological were observed following the administration of HIDROX®. Nevertheless, they noted that a potential slight stimulation of the bone marrow was suspected due to a significant dose-dependent increase in red blood cell count was measured in female rats, which reached statistical significance at the highest dose of 2000 mg/kg body weight/day [54]. In the study conducted by Rodriguez et al., the highest tested dose of virgin olive oil extract (*i.e.* 1000 mg/kg body weight/day corresponding to 150 mg HT/kg body weight/day) was associated with a significant decrease of mean corpuscular hemoglobin (18.85 ± 0.22 *vs.* 16.17 ± 0.96, $p = 0.001$), mean corpuscular hemoglobin concentration (34.50 ± 0.305 g hemoglobin/dL *vs.* 28.85 ± 1.86 g hemoglobin/dL, $p = 0.001$), neutrophil percentage ($16.0 \pm 1.2\%$ *vs.* $8.26 \pm 1.28\%$, $p = 0.001$), and an increase of the mean platelet volume (6.13 ± 0.06 fL *vs.* 7.19 ± 0.30 fL, $p = 0.001$) as compared to the control group. A significant decrease of platelet count (684.5 ± 70.9 10^3/μL *vs.* 424.8 ± 72.2 10^3/μL, $p = 0.016$), but an increase of mean platelet volume (6.13 ± 0.06 fL *vs.* 7.23 ± 0.38 fL, $p = 0.001$) was also observed in the lowest dose group (100 mg/kg body weight/day corresponding to 15 mg/kg body weight/day of HT) when compared to control group [58]. Despite these significant variations in hematological biomarkers, values remained within normal ranges. Similar variations in hematological parameters were noted after administration of H35, which provided up to 500 mg HT/kg body weight/day [61]. These variations were inconsistent and not dose-dependent, except for minor differences in males at the highest dose, which returned to control levels after a recovery period. The variations observed were overall not considered to be toxicologically significant [61]. Regarding the specific effect of HT on these parameters, Auñon-Calles et al., measured statistically significant differences within hematological biomarkers in groups of rats administered pure HT as compared to the control group. In males, only a significant decrease of red blood cell distribution width in the highest dose group was observed compared to controls (2.64 ± 0.24 g/dL *vs.* 2.42 ± 0.12 g/dL, $p < 0.05$). Nevertheless, values remained within the normal historical control range. In females, the opposite results to the findings of Christian et al. were noted regarding the mean corpuscular hemoglobin, which was significantly increased at the 50 and 100 mg/kg body weight/day doses (17.9 ± 0.3 pg *vs.* 18.7 ± 0.6 pg and 18.6 ± 0.5 pg respectively, all $p < 0.05$) and was associated with an increase of the mean corpuscular volume (55.3 ± 0.8 fL *vs.* 57.0 ± 1.4 fL and 57.5 ± 1.2 fL, $p < 0.05$ and $p < 0.01$ respectively). In parallel, the highest dose induced a statistically significant increase in reticulocytes with high fluorescence compared to the controls (0.079 ± 0.025 *vs.* 0.128 ± 0.042, $p < 0.05$), and a statistically significant increase in white blood cells (3.0 ± 0.8 *vs.* 3.9 ± 1.1, $p < 0.05$) and eosinophils counts ($0.0 \pm 0.0 \times 10^6$ cells /L *vs.* $0.1 \pm 0.0 \times 10^6$ cells /L, $p < 0.05$). These results remained within the normal range values for these parameters for rats and were therefore concluded to be not toxicologically relevant. Moreover, these results were in accordance with results from Christian et al. and could suggest a slight stimulation of hematopoiesis.

4.3.1.4. Biochemical biomarkers. Christian et al., demonstrated that administration of HIDROX® was associated with a statistically significant decreases in the levels of alanine aminotransferase, aspartate aminotransferase, sorbitol dehydrogenase, and cholesterol levels [54]. These decreases were noted in females at 1500 and 2000 mg/kg body weight/day for sorbitol dehydrogenase and in males and females at all doses for alanine aminotransferase [54]. These results are not indicative of toxic effects, but may in fact reflect health benefits, notably on liver function. Similarly, statistically significant variations in some biochemical parameters were noted after dosing with virgin olive oil extract at a dose of 300 mg/kg body weight/day (providing 45 mg HT/kg body weight/day) compared to controls, including decreases in plasmatic glucose levels (151.23 ± 10.52 mg/dL *vs.* 180.99 ± 9.58 mg/dL), decreases in plasmatic protein concentration (5.88 ± 0.17 g/dL *vs.* 6.36 ± 0.06 g/dL, $p = 0.024$), and increases in bilirubin concentration (0.77 ± 0.004 mg/dL *vs.* 0.69 ± 0.005 mg/dL, $p < 0.001$). Moreover in this study, at all three doses (100, 300, and 1000 mg/kg body weight/day, providing 15, 45, and 150 mg HT/kg body weight/day, respectively), an increase in sodium concentration was noted (100 mg: 237.14 ± 8.64 mg/kg body weight/d, 300 mg: 214.06 ± 7.75 mg/kg/dL and 1000 mg: 226.28 ± 8.16 mg/kg/dL, $p = 0.001$, *vs.* 183.60 ± 4.52 mg/kg/dL) [58]. After supplementation with H35, a statistically significant increase in total protein was noted in males at the doses of 125 and 500 mg HT/kg body weight/day (125 mg: 6.63 ± 0.28 g/dL and 500 mg: 6.74 ± 0.24 g/dL, 6.26 ± 0.30 g/dL; all $p < 0.05$). A significant increase in albumin was noted for all doses tested in males group (125 mg: 4.53 ± 0.16 g/dL, 250 mg: 4.51 ± 0.09 g/dL and 500 mg: 4.74 ± 0.10 g/dL, $p < 0.01$, $p < 0.05$ and $p < 0.01$ respectively *vs.* 4.36 ± 0.13 g/dL), as well as a significant increase in the phosphorus levels at the two highest doses (250 mg: 6.25 ± 0.39 mg/dL and 500 mg: 6.39 ± 0.92 mg/dL, all $p < 0.05$ *vs.* 5.42 ± 0.80 mg/dL). In females, a significant increase in alkaline phosphatase was noted in the highest dose group (29.39 ± 6.20 IU/L *vs.* 42.99 ± 13.17 IU/L, $p < 0.05$), and significant decreases of both sodium and chloride in the lowest and highest doses groups without significant change in the intermediate dose group were measured. Nevertheless, similar to the hematological parameter findings, all variations in the biochemical markers were either not dose- dependent, transient, resolved during the recovery period, were of a spontaneous nature and/or their values remained within the normal ranges for these parameters. Thus, these variations were not considered of toxicological relevance.

These results are in accordance with findings with pure HT at doses of up to 150 mg/kg body weight/day that were not associated with any toxicologically relevant variations of biochemical parameters. Thus, in male rats, the highest dose of pure HT (500 mg/kg body weight/day) was associated with significant lower glucose (7.66 ± 0.65 mM/L *vs.* 6.82 ± 0.52 mM/L, $p < 0.05$) and creatinine (28.0 ± 5.1 μM/L *vs.* 21.3 ± 3.4 μM/L, $p < 0.01$) and higher albumin values (42.2 ± 1.5 g/L *vs.* 45.3 ± 1.9 g/L, $p < 0.01$) with respect to the control group. Elevated calcium values were observed in males in the 50 and 500 mg/kg body weight/day groups (2.50 ± 0.26 *vs.* 2.67 ± 0.08 and 2.67 ± 0.08, all $p < 0.05$) and higher aspartate aminotransferase values were observed in the lowest and intermediate dosed groups (67.4 ± 36.1 U/L for the control *vs.* 98.3 ± 18.3 U/L for the lowest dose or 99.4 ± 26.6 U/L for the intermediate dose, $p < 0.05$ compared with the control group). Statistically significant differences were recorded in potassium in males from the low dose group. No relevant differences were observed in females in any of the same biochemical parameters. Both potassium and aspartate aminotransferase increase cannot be considered of toxicological relevance in the absence of a dose–responsive relationship and absence of effects in females. [60]. The authors concluded that based on the absence of observable adverse effects at the highest dose the NOAEL was considered to be 500 mg HT/kg body weight/day, the highest dose tested [60].

4.3.1.5. Histopathological examination. HIDROX® supplementation was not associated with any gross nor histological changes. Only, focal, minimal or mild hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach was present in the stomach of some male and female rats at the dose of 2000 mg/kg body weight/day and was considered to be due to local irritation by the large intubated volume of the viscous, granular formulation [54]. The absence of any

dose-dependent macro- and micro-scopic histological effects was also reported in relation to the cerebellum, heart, esophagus, salivary glands, gonads, bone, bone marrow, nerve, pancreas, skin, windpipe and thymus in rats dosed with virgin olive oil extract, providing up to 150 mg HT/kg body weight/day, as compared to control group animals [58]. Similar conclusions were made for H35 extract, providing 500 mg HT/kg body weight/day and pure HT at doses of up to 500 mg/kg body weight/day, which did not induce any modification or alteration of the organs evaluated at the histological level [60,61].

4.3.1.6. Organs weights. The terminal and relative (to body weight) weights of the brain, liver, kidneys, adrenals, spleen, thymus, thyroid, heart, testes, epididymides, uterus and ovaries were comparable among the four groups receiving HIDROX® and did not significantly differ [54].

Regarding the effects of pure HT on these parameters, supplementation with the daily dose of 500 mg/kg body weight/day was associated in males and females with a significant higher relative (*vs.* body weight) kidney and mandibular salivary gland weights, in males with a significant higher relative brain and epididymis weights, and in females with a significant higher relative heart and liver weights. Significant and dose-dependent increases in relative weights (*vs.* body weight) of the heart and testes was also noted in males as compared to controls. After a recovery period, higher relative and absolute weight of testes in males and liver and kidney in females were observed compared to the controls. The absence of macroscopic or microscopic alterations of these organs supported that conclusion that that these observations were not toxicologically relevant.

4.3.1.7. Other information

4.3.1.7.1. Neurological observations. In three studies, the neurological effects of olive extracts/HT administration in rats was specifically examined. Heilman et al. conducted weekly neurobehavioral observations including home cage observations (posture and convulsions); observations during removal and handling (ease of removal from the cage, handling reactivity, lacrimation, palpebral closure, eye abnormalities, skin abnormalities, piloerection, salivation); and open field observations (gait, mobility, rearing, respiration, arousal level, urination, defecation, vocalizations, stereotypy, bizarre behavior, and clonic or tonic movements) as well as functional observational battery including sensory reactivity measurements (approach response, touch response, click response, tail-pinch response, pupil response, air righting reflex), landing hind limb foot splay, grip strength and motor activity. Results from these observations showed that none of the doses of H35, providing up to 500 mg HT/kg body weight/day, were associated with neurobehavioral abnormalities [61]. Similar results were noted after supplementation with virgin olive oil extract at doses of up to 1000 mg/kg body weight/day, providing up to 150 mg HT/kg body weight/day [58]. In this study, no altered clinical sign of central nervous system stimulation or depression, or the way of walking (with the abdomen moving backwards) were observed in any groups, during the intervention period. In addition, no trembling, convulsions, or numbness of limbs were observed [58]. Regarding the effects of pure HT, despite a slightly significant effect at the lowest dose (5 mg/kg body weight/day) on forelimb grip strength (lower strength), no effect was noted at higher doses or after a recovery period. In the absence of a dose effect, this result was not considered as toxicologically relevant [60]. In the same study, a minimal decrease in locomotor activity was recorded in males at the highest dose at 30, 40, 50, and 60 min and at the two lower doses at 20 min, but these differences were considered not toxicologically significant and could not be attributed to the treatment in absence of a dose-related effect [60].

4.3.1.8. Ophthalmological observations. Some studies were interested in the ophthalmological effects of olive extract or HT supplementation. Overall, no ocular alterations were recorded in any of the ophthalmoscopic examinations [60,61].

4.3.1.9. No-observed adverse effect levels (NOAEL). A summary of the NOAEL derived from each study is presented in Table 8. In rats, the NOAEL for HIDROX® was 2000 mg/kg body weight/day, corresponding to 48 mg HT/kg body weight/day [54]. For pure HT, the NOAEL was concluded to be 500 mg/kg body weight/day in rats [60]. This NOAEL was decreased by EFSA in its evaluation of HT safety [3] to 50 mg/kg body weight/day due to the persistence of significant liver and kidney weight reduction in females rats following a recovery period. Moreover, the 500 mg/kg body weight/day NOAEL was higher than the NOAEL proposed in rats for H35 (691 mg/kg body weight/day), which provides 250 mg HT/kg body weight/day. In this study, the highest dose providing 500 mg/kg body weight/day of HT was considered as the lowest-observed adverse effect level due to the decrease of body weights and body weight gains [61]. Finally, the NOAEL for virgin olive oil containing 15% HT was estimated to be 1000 mg/kg body weight/day, providing 150 mg HT/kg body weight/day [58].

4.4. Reproductive toxicity and toxicity during pregnancy and lactating period

Some studies have evaluated the effects of supplementation with either olive extracts or HT on the reproductive performance, offspring development, and pregnancy outcomes in rodents [62,63].

Christian et al. conducted a reproductive and developmental toxicity study in rats after oral administration to HIDROX® [54]. In the reproductive toxicity study, performed with doses up to 2000 mg/kg body weight/day (providing 48 mg/kg body weight/day of HT), in males, mating and fertility parameters, as well as terminal body weights and paired epididymal and testicular weights, were comparable among the dosed groups, whereas in females, HIDROX® did not affect the number of estrous stages nor any mating, fertility, gestation, parturition, lactation or necropsy parameters [54]. Moreover, no delivery or litter parameters were affected by the olive pulp extract. In the developmental toxicity study, in pups exposed at day 21 after birth to HIDROX® during 7 days, no death, no clinical signs, no body weight alteration nor gross necropsy were observed at doses up to 2000 mg/kg body weight/day (providing 48 mg/kg body weight/day of HT) [54]. Pharmacokinetic study performed in these conditions showed that HT was not detected in the maternal milk nor in plasma obtained from pups 9 days after the beginning of lactation [54]. Regarding the developmental toxicity study, female rats were exposed, from day 6 through day 21 of gestation to 1000, 1500, or 2000 mg/kg body weight/day of HIDROX® providing respectively 24, 36 or 48 mg/kg body weight/day of HT. No adverse clinical or necropsy observations or differences in maternal body weights, body weight gains, gravid uterine weights, corrected maternal

Table 8
No-observed adverse effects levels (NOAEL) defined in sub-chronic studies.

Species	Product	NOAEL	Equivalent HT	Comments	References
rats	HIDROX®	2000 mg/kg body weight/d	48 mg/kg body weight/d	-	[54]
rats	Pure HT	500 mg/kg body weight/d	500 mg/kg body weight/d	EFSA considers the NOAEL 50 mg/kg body weight/d	[60]
rats	H35	691 mg/kg body weight/d	250 mg/kg body weight/d	-	[61]
rats	Virgin olive oil	1000 mg/kg body weight/d	150 mg/kg body weight/d	-	[58]

body weights or body weight gains or absolute (g/day) or relative (g/kg body weight/day) feed consumption values were attributable to administration of HIDROX® as high as 2000 mg/kg body weight/day. All values were comparable among the four dose groups and did not statistically differ [54]. These results are in accordance with results from Heilman et al. that showed no difference between all groups on fertility parameters. Regarding male fertility parameters, sperm motility or percent of abnormal sperm were not affected by the supplementation with H35 providing doses of HT up to 500 mg/kg body weight/day. Moreover, no significant changes in homogenization resistant spermatid count were observed in testicular and cauda epididymis of H35 treated male rats compared with controls [61]. Regarding female fertility parameters, estrus cycle length and pattern of all treated female rats were comparable with female rats of the control group, and no differences were noted between groups on the ovary and uterine (horns and cervix) absolute or relative weights [61].

The absence of effect of olive extract on reproductive parameters, as well as development *in utero* and pregnancy outcomes, was also partly supported by two studies, performed in pigs, which evaluated the effects of maternal HT supplementation on placental gene expression and other parameters related to intrauterine growth restriction [62,64]. In these studies the effect of 1.5 mg HT/kg body weight/day during pregnancy and lactation period was evaluated. On day 35 of pregnancy, animals were fed a diet fulfilling 50% of their daily requirements in order to affect fetal development, including lower birthweight in the newborns. Half of the sows received HT. The results indicated that maternal supplementation with HT during pregnancy had no deleterious effects on the reproductive traits of the sows (*i.e.,* prolificacy, homogeneity of the litter, percentage of stillborn and low-birthweight piglets) and the postnatal features of the piglets (*i.e.*, growth patterns, adiposity, and metabolic traits). Despite the conflicting results between the two studies regarding the piglets birth-weight; one study showing that HT was associated with higher mean birth weight and lower incidence of piglets with low birth weight [64], while the second study showed that piglets in the control group had a higher mean birth-weight and a larger abdominal circumference than piglets from the HT-supplemented group (1.3 ± 0.03 *vs.* 1.2 ± 0.03 kg and 18.7 ± 0.25 *vs.* 17.8 ± 0.25 cm, respectively; $p < 0.05$ for both) [62]; differences between groups remained weak and results from the second study have to be interpreted with caution due to the employment of omega-3 fatty acids in addition to HT. Moreover, the results observed in the second study were non-persistent and piglets from the supplemented sows showed higher average daily weight gain and fractional growth rate [62]. No other toxicologically relevant variations between test and control groups were noted.

When evaluating the pharmacokinetics of HT in pregnant and lactating rats, Christian et al. observed that HT plasma levels for pregnant and lactating rats were comparable to non-pregnant rats. Minimal levels of HT crossed the placenta. Analysis made on maternal milk and plasma from nursing pups were below the detection limit [54].

Overall, neither pure HT, nor olive extracts containing HT at different concentrations adversely affected any of the mating, fertility, delivery, or litter parameters investigated in oral supplementation reproduction studies in rats. Adverse effects were also absent in a rat developmental toxicity study in which pregnant dams were dosed with 1000, 1500 or 2000 mg/kg body weight/day of HIDROX® on day 6 through 20 of gestation. Moreover, despite a slight decrease of body weight gain in piglets, no major adverse effects related to piglet development was noted following maternal supplementation with a dose of 1.5 mg HT/kg body weight/day. These data support the safety of HT and HT-containing extracts during pregnancy and lactation periods.

4.5. Other toxic effect

4.5.1. Hormone-modulating properties

Some evidence suggests that phenolic compounds present in olive products possess hormone-modulating properties [18,65–69]. Indeed, in rats subjected to restraint stress or a high-casein diet, the oral supplementation with olive oil or oleuropein was associated with a significant decrease of corticosterone levels [65,69]. In the study conducted by Nassef et al., oleuropein which is composed with hydroxytyrosol, was associated with an increase of urinary noradrenaline and testicular testosterone levels in rats fed a high-casein diet [65,69], whereas oleuropein aglycone was shown to dose-dependently increase the plasma luteinizing hormone level in male Sprague-Dawley rats [65].

In another study, the combination of olive leaf extract and oleuropein was orally administered to healthy mice, resulted in a statistically significant increase in the serum follicle-stimulating hormone concentration [67]. After oral supplementation with 100 mg/kg body weight/day of olive leaf extract and oleuropein, in male mice exposed to cyclophosphamide and cisplatin, two chemotherapeutic agents responsible of testicular toxicity, damage caused by chemotherapeutic agents were mitigated [67]. In the same study, the group supplemented with both oleuropein and olive leaf extract showed a significant increase in serum follicle-stimulating hormone concentration compared to control group (control: 4.68 ± 0.31 *vs.* 100 mg/kg body weight/day of oleuropein + 100 mg/kg body weight/day of olive leaf extract: 7.56 ± 0.37, $p < 0.001$) [67]. In these studies, supplementation was associated with a normalization of serum testosterone, luteinizing hormone, and/or follicle-stimulating hormone levels, compared to mice not supplemented but exposed to chemotherapeutic agents [67,68].

These effects on serum testosterone and luteinizing hormone levels were replicated in a clinical study performed in 60 young and healthy male volunteers aged between 23 and 40 years old [65]. Results showed that the young men consuming extra virgin olive oil (doses not reported, but stated as containing sterols) for 3 weeks, had a statistically significant increase in serum testosterone and luteinizing hormone levels [66]. These effects are considered as a positive action on the androgen hormonal profile of men [75] and cannot be only attributed to phenolics compounds due to the additional presence sterols in the product used in this study [70].

Thyroid-stimulating properties of olive oil, olive leaf extract, and solid olive residue have been substantiated in different studies performed *in vivo*, in euthyroid animals as well as in animals exposed to heat stress or with chemical-induced hypothyroidism [18]. According to Pang et al., there is a concurrent improvement of thyroid function and oxidative status in animals with hypothyroidism upon supplementation with olive derivatives; however, the causal relationship has not been determined. Olive oil and leaf extract are also postulated to induce a higher rate of conversion of inactive T4 to biologically active T3 in euthyroid animals [18].

4.5.2. Allergenicity

The olive tree belongs to the *Oleaceae* family, native to the coastal areas of the eastern Mediterranean. Olive is a major component of the agriculture and gastronomy throughout Mediterranean countries. That is the reason olive pollen is a very important cause of respiratory allergic reactions in this area [71], involving Ole e 1 as the most clinically relevant sensitizing allergen [72]. Olive fruit is also used as the raw material to obtain olive oil, which has been implicated in allergic contact dermatitis, contact urticaria, and allergic airway disease due to inhalation of olive particles [71,73]. Thirteen olive allergens have been identified, among them, Ole e 13 is the only allergen described in the olive fruit. It belongs to the family of the thaumatin-like proteins, which are involved in host defense processes [74].

However, food allergy due to olive fruit is a rare pathology described in the literature, despite widespread consumption [75]. Nevertheless, sporadic case reports of olive food allergy have been identified[71].

One case report focused on a 28-year-old male who presented with palatal itching and generalized urticaria following ingestion of olive 3 years after being diagnosed with olive pollinosis [75]. The results of prick tests and prick-to-prick tests for olive fruit were positive, as were

those of specific immunoglobulin E tests to olive pollen and fruit. Nasal provocation with olive pollen also gave a positive response; however, an open label oral provocation test with olive oil did not cause any symptoms [76]. In another case report, a 19-year-old woman, had a four-year-long history of episodes of facial, neck, and hands angioedema, and intense palm itch [76]. In one of the episodes, the angioedema affected the tongue as well, and the itch was generalized. The symptoms abated in 24 h. On each occasion, the patient had ingested olives 15–30 min beforehand. She tolerated olive oil and had no rhinitis/asthma during the pollen season. Prick-by-prick tests with three different olive brands, gave a positive result. A prick test with *Olea europaea* was negative. The immunoglobulin E antibody levels specific to olive was 1.7 kU/L, and 0.73 kU/L to *Olea europaea* [77]. Finally, the case of a 21-year-old woman with a history of house dust mite allergic rhinitis and asthma treated with sublingual immunotherapy was reported after the patient experienced three episodes of oropharynx and palm itching, cough and dyspnea, a few minutes after eating a snack with onion, gherkin, red pepper, and olive [74]. These symptoms subsided with β2-agonists and antihistamines some hours later. She did not have a history of any food allergy. Skin prick tests were positive to different mites and negative to pollens (including olive pollen). Prick-by-prick with olive fruit gave a positive result with a wheal of 25 mm × 20 mm, and the patient suffered a general skin itching that improved with antihistamines. Five control subjects showed a negative test with olive fruit. Total immunoglobulin E was 2534 kU/L and specific immunoglobulin E was negative to pollens and foods [75].

Nevertheless, these cases represent a very minor occurrence of allergic responses int eh context of the widespread consumption of olive products around the world. Moreover, regarding allergic reactions, olive extracts, and particularly HT, have been reported to elicit protective effects, by inhibiting the mast cell degranulation induced by immune and non-immune pathways in rats [78] or by increasing suppressive immune response towards an allergen, by increasing interleukin 10 expression in human primary peripheral blood mononuclear cells [79].

4.6. Safety evaluation in humans

In humans, the consumption of olive products, such as olives or olive oil is common and widespread. Olive oil is the main source of lipids in Mediterranean countries and is associated with the health benefits of the Mediterranean diet [35]. Many studies have evaluated the effects of oral supplementation with olive extracts or HT in humans.

In a randomized double-blinded, placebo-controlled study, 105 individuals (55–75 years old) with osteoarthritis and rheumatoid arthritis were randomly allocated to receive either 400 mg/day of freeze-dried polyphenolic-rich olive extract (providing 10 mg/day of phenolic compounds) or placebo for 8 weeks [79]. Safety was assessed as additional outcomes by clinical and biochemical tests, physical examinations, and any adverse events reported by patients. No adverse effects related to the test item were reported. No adverse changes in the biochemical markers or kidney (serum blood urea nitrogen and creatinine) and liver (serum blood aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin) function was reported. An improvement of basal abnormal levels of kidney and liver functions markers was noted. Considering the fact that subjects, due to their condition, were under different medications, the safety of the extract combined with patient's other medications was substantiated by the kidney and liver function markers [80]. Comparable results have been noted in a similar population in a more recent randomized, double-blinded and placebo-controlled study after 12 weeks supplementation with 500 mg/day of olive fruit extract, providing 50 mg/day of maslinic acid (HT level not known), where no adverse effect related to the test item was reported, and no biologically and toxicologically relevant changes in blood parameters were reported [81]. In the same year, Pais et al., have also conducted a randomized, double-blinded, and placebo-controlled study evaluating the impact of 11 days supplementation with either 250 or 500 mg/day of olive fruit extract (Proliva®), providing respectively 50 or 100 mg/day of HT, on 36 subjects (45–66 years) at risk for arterial stiffness [27]. No difference in adverse events between test item groups and placebo group was noted. No change in markers of liver and kidney functions, and other biochemical analysis parameters was noted (*i.e.*, glucose, uric acid, cholesterol, triglycerides, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol, urea, creatinine, blood urea nitrogen, total bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, gamma-glutamyl transferase, total protein, albumin, globulin, albumin/globulin ratio, iron, calcium, phosphorus, sodium, potassium and chlorine) [28]. The addition of 3.75 mg/day of polyphenols from olives, equivalent to 20 g/day of olive oil, added to a meat product, was not associated with any adverse effects in 35 healthy subjects after 4 weeks supplementation. Kidney (blood urea and creatinine levels) and liver (blood aspartate aminotransferase and alanine aminotransferase levels) function markers were not altered. Beneficial modulation of some biochemical markers were noted in a sub-group of patients with at least two biochemical or anthropometric elements of cardio-metabolic risk (reduced glucose ($p < 0.002$), insulin levels ($p = 0.03$), total cholesterol ($p < 0.009$), triglycerides ($p < 0.005$), low density lipoprotein-cholesterol ($p < 0.01$) and oxidized-low density lipoprotein ($p < 0.01$) compared to baseline values) [82]. Moreover, no modification of the major lymphocyte population was noted after supplementation [82].

The absence of adverse effects or the absence of change in hematological or biochemical markers, after intake of HT from olive extracts or from synthetic origin, in healthy populations were also supported by other clinical studies [83–86].

A randomized, double-blinded, placebo-controlled, crossover, comparative study, performed in 30 healthy subjects (35–65 years old) compared the effects of 30 mg/day of HT provided either as synthetic HT or as/in olive fruit extract after 4 weeks of supplementation. The tolerability of the products was very good and no change in blood pressure was noted. Some adverse effects (headache and common cold followed by musculoskeletal problems such as back pain) were reported in the olive extract group (13 subjects reported 20 adverse events), in the pure HT group (10 subjects reported 13 adverse events), and in the placebo group (15 subjects reported 28 adverse events) without relation to the test items [86]. Similarly to the study of Peroulis et al. [82], the supplementation with HT, from olives water extract, was not associated with changes in blood lipids levels in healthy individuals, despite a significant decrease of low-density-lipoprotein cholesterol levels observed only in the synthetic HT group [86]. A decrease of LDL-cholesterol, being considered a physiological benefit, HT up to 30 mg/day as olive extract or as a pure the ingredient appears safe for healthy humans. Similar results have already been observed in a randomized, double-blinded, placebo-controlled and crossover study, after supplementation with an olive mill waste water extract providing lower doses of HT (5 mg/day or 25 mg/day) [83]. In a mild hyperlipidemic population ($n = 14$), 8 weeks supplementation with 45 mg/day of pure HT was associated with slight changes in hematological and biochemical parameters. A slight decrease in lactate dehydrogenase and a slight increase in creatine phosphokinase enzyme levels were observed *vs.* baseline, but the values remained within the normal ranges and the changes were considered to have limited clinical relevance. Regarding hematological parameters, only a slight increase in mean corpuscular volume was noted. Vitamin and mineral assessment showed an increase in serum vitamin C concentration, whereas ferritin, serum and red blood cell folate were reduced. According to these results, authors concluded that HT at 45 mg/day was safe and did not influence markers of cardiovascular disease, blood lipids, inflammatory markers, liver or kidney functions or electrolyte balance [85]. Finally, in a very recent prospective, randomized, double-blinded and placebo-controlled study, evaluating the effects of long term (6 months) of oral supplementation with 15 and 5 mg/day of HT among 29 overweight/obese women, no adverse

events were reported by the subjects. The product was considered safe and well-tolerated by the authors [36]. Note that in this study, statistically significant weight and visceral fat mass loss (%weight loss: $p = 0.012$, %visceral fat loss: $p = 0.006$) were observed in the group receiving the maximum HT dose as compared to the placebo group after 4 weeks of the intervention, with attenuation of these findings at 12 and 24 weeks [36].

Overall, the identified human data substantiates the safety of oral supplementation, with olive extracts or pure HT at daily doses of up to 45 mg HT/day.

5. Conclusion

The present study aims to substantiate the safety of OE20HT after oral exposure in humans, based on genotoxicity and mutagenicity data obtained from *in vitro* assays conducted with the ingredient, as well a comprehensive review of published safety/toxicology data.

OE20HT was not mutagenic in the Ames assay, performed in accordance with OECD guidelines, at concentrations of up to 5000 μg/plate, corresponding to 1000 μg/plate of HT. OE20HT did not induce gene mutations by base pair changes or frameshifts in the genome of the *S. typhimurium* or *E. coli* strains used. The cytotoxicity observed in our study, highlighted by a reduced background lawn development, was possibly attributed to the antibacterial properties that has been reported for olive phenolics [87]. This result is in accordance with previous studies assessing the mutagenicity of HT and olive extracts.

The absence of genotoxicity of OE20HT has been substantiated based on an *in vitro* micronucleus assay performed according to OECD guidelines, at concentrations of up to 60 μg/mL, equivalent to 12 μg/mL of HT. In this study, no increase in the frequency of micronucleated cells was measured following exposure to OE20HT, substantiating the absence of clastogenic or aneugenic potential of the ingredient. These results are in accordance with previous data obtained in *in vitro* micronucleus assays after exposure to pure HT at equivalent doses or other olive extracts providing similar amount of HT. Some observed clastogenic or aneugenic results of these test substances noted in previously published studies was considered to be mainly due to a high cytotoxic effects induced by olive extracts, or HT, in these studies, which can interfere with the proper genotoxic potential evaluation. Moreover, no genotoxic effects were observed from *in vivo* micronucleus assays performed with HT or olive extracts, despite a significant increase in HT in animal blood, highlighting a sufficient exposure of bone marrow to the product.

OE20HT did not induce clastogenic, aneugenic nor mutagenic effects *in vitro* [51]. This combination of tests (*i.e.* Ames assay and *in vitro* micronucleus assay) fulfils the basic requirements to cover the three genetic endpoints relevant in toxicology; the bacterial reverse mutation assay covering gene mutations and the *in vitro* micronucleus test covering both structural and numerical chromosome aberrations. These two tests are reliable for detection of most potential genotoxic substances [88].

In addition to these assessments, the safety of OE20HT in human after oral intake is supported based on data identified in a comprehensive review of the peer-reviewed literature.

Repeated dose oral toxicity studies have highlighted a potential effect of olive extract and pure HT in relation to decreases in body weights and/or body weight gain in rodents [60,61], without alteration on food consumption or any other toxicologically relevant findings. Reduction in body weight gain is one of the benefits of olive oil in the context of Mediterranean diet and has been reported in a clinical trial [36]. Several mechanisms have been proposed to explain the weight loss after polyphenols supplementation, such as increased lipolysis and activated β-oxidation through downregulation of the expression of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ and by activation of 5'AMP-activated protein kinase and lipase; a prebiotic effect for gut microbiota [89–91]; inducing satiety; an inhibition of adipocyte differentiation; and a promotion of adipocyte apoptosis [92]. Overall, the body weight gain reduction noted in different sub-chronic rodent studies appears as a slight and non-toxicologically relevant effect due to the absence of other signs of toxicity in these studies. Moreover, this effect is considered as one of the main beneficial effects of Mediterranean diet and was only transiently observed in a clinical study.

The NOAEL determined based on repeated oral dosing in rodents, were reported to be between 48 and 250 mg HT/kg body weight/day. As presented previously, some effects were noted in *in vivo* studies after oral supplementation with more than 50 mg/kg body weight/day of HT. Considering the 50 mg/kg body weight/day as point of departure in animals and applying uncertainties factors allowing to consider the interspecies variations and the interindividual variations within human, an acceptable daily intake of HT for human is proposed to be 0.5 mg/kg body weight/d. Considering that the product OE20HT provides 20% HT, a dose of 2.5 mg/kg body weight/day of OE20HT can be considered as safe.

Olive extracts or HT were not reproductive or developmental toxins. Despite a slight decrease in body weight gain noted in piglets after sows were supplemented with HT during pregnancy, no toxicologically relevant adverse effects were noted on pregnancy outcomes or in offspring [62,64].

Some hormone-modulating properties were associated with olive phenolic compounds, such as oleuropein, in animals or humans [65,66,69]. However, these effects were not considered as toxic and none were specifically associated with HT [65,66,69]. The involvement of sterols contained in olive extracts cannot be excluded as responsible for the hormone-modulating effects, the hormone-modulating properties being more likely due to sterols than phenolic compounds.

The absence of mutagenic and genotoxic potential of the OE20HT product was substantiated in this study by an Ames assay and *in vitro* micronucleus assay, both performed in accordance with OECD guidelines. Neither olive extracts containing HT nor pure HT were associated with any significant toxicologically relevant effects in acute, subchronic, reproductive, and developmental toxicity studies conducted in rodents and pigs. The OE20HT extract is therefore considered as safe for human consumption at doses up to 250 mg/kg body weight/day.

CRediT authorship contribution statement

ML: Conceptualization, Investigation, Writing – original draft. **MPL**: Writing – review & editing. **OM**: Writing – review & editing. **MR**: Writing – review & editing. **JCQ**: Writing – review & editing. **JLB**: Writing – review & editing, Supervision.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. MPL and JCQ are employed by NATAC Biotech S.L. which produces the product OE20HT presented in these studies. ML, OM, MR and JLB are employee of Nutraveris, a Food Chain ID company, a consulting firm involved in the request for authorization of the OE20HT as novel food in European Union under the regulation n°2015/2283.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.toxrep.2023.02.003.

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