Investigation of the Cytotoxic, Genotoxic, and Apoptosis-Inducing Effects of Estragole Isolated from Fennel (Foeniculum vulgare)

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · MARCH 2014

Impact Factor: 3.8 · DOI: 10.1021/np400653p · Source: PubMed

7 AUTHORS, INCLUDING:

Milena Villarini
Università degli Studi di Perugia
78 PUBLICATIONS **1,051** CITATIONS
SEE PROFILE

Luca Dominici
Università degli Studi di Perugia
34 PUBLICATIONS **178** CITATIONS
SEE PROFILE

Cristina Fatigoni
Università degli Studi di Perugia
46 PUBLICATIONS **591** CITATIONS
SEE PROFILE

Massimo Moretti
Università degli Studi di Perugia
85 PUBLICATIONS **1,249** CITATIONS
SEE PROFILE

JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Investigation of the Cytotoxic, Genotoxic, and Apoptosis-Inducing Effects of Estragole Isolated from Fennel (*Foeniculum vulgare*)

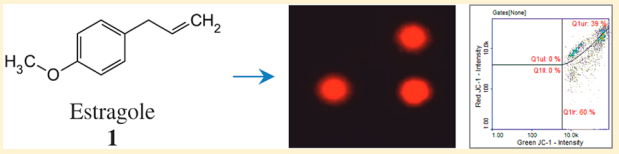
Milena Villarini,[†] Rita Pagiotti,[‡] Luca Dominici,[†] Cristina Fatigoni,[†] Samuele Vannini,[†] Sara Levorato,[†,§] and Massimo Moretti*[,†]

[†]Department of Pharmaceutical Sciences (Unit of Public Health), University of Perugia, Via del Giochetto, 06122 Perugia, Italy

[‡]Department of Pharmaceutical Sciences (Unit of Plant Biology), University of Perugia, Borgo XX Giugno, 06121 Perugia, Italy

[§]School of Advanced Studies (Area: Life Sciences and Public Health; Ageing and Nutrition), University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy

ABSTRACT: The present study was undertaken to evaluate, in the HepG2 human hepatoma cell line, the in vitro cytotoxic, genotoxic, and apoptotic activities of estragole (**1**), contained in the essential oil of *Foeniculum vulgare* (fennel) and suspected to induce hepatic tumors in susceptible strains of mice. Toward this end, an MTT cytotoxicity assay, a trypan blue dye exclusion test, a double-staining (acridine orange and DAPI) fluorescence viability assay, a single-cell microgel-electrophoresis (comet) assay, a mitochondrial membrane potential ($\Delta\psi$m) assay, and a DNA fragmentation analysis were conducted. In terms of potential genotoxic effects, the comet assay indicated that estragole (**1**) was not able to induce DNA damage nor apoptosis under the experimental conditions used.



Foeniculum vulgare Mill. (fennel), a perennial herb with a characteristic aniseed flavor, belongs to the Apiaceae family, being native to the Mediterranean area, yet naturalized in northern Europe, Australia, and North America, and cultivated worldwide. For centuries, fennel fruits (commonly referred to as "seeds") have been used as a traditional herbal medicine in Europe and mainland China.[1] A number of beneficial properties have been attributed to fennel fruits. Among the best characterized are anti-inflammatory, analgesic, antibacterial, and antioxidant properties.[2,3] Fennel infusion, with a mild flavor and good tolerance, is currently regarded as a first-choice treatment in infants with dyspeptic disorders.[4]

The pharmacological activity of sweet fennel fruits is traceable to an essential oil, mostly consisting of *trans*-anethole (50−75%), putatively endowed with anticancer and antioxidant properties.[2] Furthermore, the essential oil contains estragole (**1**) in amounts of 3.5−12% and no higher than 7.5% of fenchone.[5] Other minor constituents (such as limonene, β-pinene, β-myrcene, and *p*-cymene),[6] as well as nonvolatile constituents including flavonoids and coumarins,[7] are found in fennel fruit essential oil. The relative amounts of the different constituents may vary considerably, depending on phenological factors and fruit origin.[8]

In the face of those beneficial constituents, estragole (**1**) and its metabolites, also present in *F. vulgare* fruits, have been reported to produce hepatic tumors in susceptible strains of mice. Estragole (**1**) is, indeed, a naturally occurring genotoxic carcinogen in experimental animals.[9−11] As a result, a Scientific Committee on Food of the European Commission recommendation restricts the use of estragole (**1**)-containing remedies, in that a maximum safe-exposure threshold cannot be determined.[12] On the other hand, the Joint FAO/WHO Expert Committee on Food Additives has recently conducted a comprehensive review of the literature and reported that although evidence of carcinogenicity to rodents given high doses of estragole (**1**) exists, further research is needed to assess the potential risk to human health from low-level dietary exposure to this compound present in foods and essential oils and used as flavoring agents.[13]

The Scientific Committee of the European Food Safety Authority (EFSA) draft guidance document[14] states that, whenever herbal ingredients include substances that are both genotoxic and carcinogenic, the "margin of exposure" (MoE) approach[15] should be applied covering the botanical(s) under examination and any other dietary sources of exposure. The MoE approach uses a "reference point" (usually derived from data obtained in experimental animals) that represents the dose causing a low but measurable carcinogenic response. More specifically, the MoE is the ratio between a reference point of carcinogenic potency descriptors [i.e., the $BMDL_{10}$, which refers to the corresponding lower limit of a one-sided 95% confidence interval on a benchmark dose (BMD) for a 10% increase in tumor incidence] and the estimated human exposure. When the MoE is higher than 10 000, the compound is considered to be of low priority for risk management actions.[16]

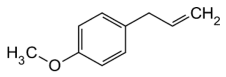
Rietjens et al.[17] have reported a BMD analysis, performed with the BMDS software version 1.4.1c, on the data by Miller et al.[11] regarding the incidence of hepatomas in female mice fed an estragole (**1**)-containing diet for 12 months. The authors concluded that the $BMDL_{10}$ value for estragole (**1**) varies in

Received: August 9, 2013

dx.doi.org/10.1021/np400653p | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

mice between 9200 and 32 700 μg/kg per day. In order to make an accurate estimate of dietary exposure to estragole, Raffo et al.[18] have quantified the content of **1** in some commercial fennel herbal teas. Considering a daily consumption of three portions of herbal tea in adults and 100 mL (one baby-bottle) in infants, the authors calculated a maximum exposure level to estragole (**1**) of 10 μg/kg/day for adults and up to 51 μg/kg/day for infants when using teabags and of up to 23 μg/kg/day on using instant teas. The resulting MoE values for infants ranged from 400 to 1470, which calls for some concern relative to safety in humans. Meanwhile, criticisms have been raised on the extrapolation of carcinogenicity data from animal models to humans. The major issue, in this specific regard, relates to the transfer of data obtained in animal models to human pathophysiology.

The present study was undertaken to evaluate the in vitro cytotoxic, genotoxic, and apoptotic activities of purified estragole (**1**) in the HepG2 human hepatoma cell line. For this purpose, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay, a trypan blue dye exclusion test, a double-staining fluorescence (acridine orange and 6,4′-diamidino-2-phenylindole) viability assay, a single-cell microgel-electrophoresis (comet) assay, a mitochondrial membrane potential (Δψm) assay, and a DNA fragmentation analysis were applied.



1

RESULTS AND DISCUSSION

Cell Viability: MTT Assay, Trypan Blue Dye Exclusion Assay, and AO/DAPI Double-Staining Test. Estragole (**1**) was dissolved in MEM (84.55 μg/mL), according to solubility data reported in the literature,[19] and, to determine its eventual cytotoxic effects, HepG2 cells were treated with eight increasing doses (84.55−0.66 μg/mL) of this compound for 4 h. Cell viability was determined using the conventional MTT assay. HepG2 cell viability was not affected after 4 h of exposure by any of the concentrations tested (Table 1). To prevent false-negative results, besides damage in mitochondrial dehydrogenases (as assessed by the MTT assay), the loss of membrane integrity was quantified, another parameter related to cell death. Thus, the three highest noncytotoxic concentrations in the MTT assay were investigated further by the trypan blue dye exclusion assay and double staining of cells with acridine orange (AO) and 6,4′-diamidino-2-phenylindole (DAPI) (Table 1). No significant differences were observed in the proportions of trypan blue unstained (viable) cells in cultures treated for 4 h with **1** compared to negative controls. Double staining of cells with AO and DAPI confirmed the results obtained with the trypan blue dye exclusion assay, and, after treatment with estragole, the viability of HepG2 cells was always higher than 90%.

Genotoxicity Testing: Comet Assay. Guidelines for in vitro genotoxicity testing suggest a minimum of 70% viable cells for the comet assay.[20] For estragole (**1**), the three highest tested concentrations (i.e., 84.55, 42.28, and 21.14 μg/mL) for cytotoxicity were tested also for genotoxicity, and the results obtained in HepG2 cells are shown in Figure 1. The positive control (1 μM 4-nitroquinoline *N*-oxide, 4NQO) demonstrated the sensitivity and validity of the test system by inducing statistically significant increases in the extent of primary DNA damage in exposed cells as compared with negative controls. None of the three tested concentrations of estragole (**1**) produced any statistically significant increase in the extent of DNA strand-breakage as measured by the standard alkaline comet assay when compared with negative controls.

Table 1. Cell Viability Assessed by the MTT Assay (% of Negative Control), Trypan Blue Dye Exclusion Assay (% of Total Cells), and AO/DAPI Double Staining (% of Total Cells) in HepG2 Cells Treated with Different Concentrations of Estragole (1)[a]

	viability		
estragole (**1**) (μg/mL)	MTT	trypan blue	AO/DAPI
84.55	102.8 ± 14.9	85.0 ± 3.8	90.2 ± 0.9
42.28 (1:2)[b]	108.2 ± 13.1	93.3 ± 1.2	91.7 ± 1.7
21.14 (1:4)	109.6 ± 15.4	92.7 ± 0.7	90.9 ± 0.9
10.57 (1:8)	116.8 ± 9.5		
5.28 (1:16)	104.0 ± 10.2		
2.64 (1:32)	100.9 ± 15.1		
1.32 (1:64)	94.8 ± 12.3		
0.67 (1:128)	106.3 ± 10.7		
negative control[c]		91.7 ± 1.5	92.9 ± 1.6

[a]Each value represents the mean ± SEM of three independent experiments. [b]Dilution factor. [c]Negative control: MEM, complete medium.

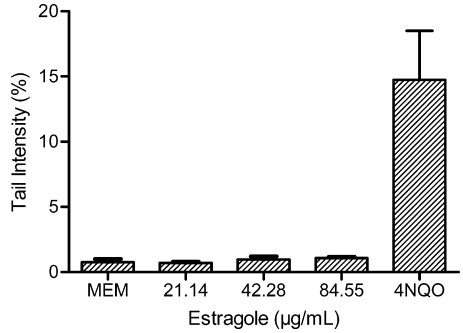


Figure 1. DNA strand-breakage (i.e., tail intensity) as evaluated in the comet assay for genotoxicity assessment of estragole (**1**) in HepG2 cells. Results are expressed as the means ± SEM of three independent experiments. Negative control: MEM, complete medium; positive control: 1 μM 4NQO.

Early Apoptosis: Mitochondrial Membrane Potential (Δψm). For detecting estragole (**1**) capabilities in decreasing mitochondrial membrane potential (Δψm), treated HepG2 cells were subjected to JC-1 staining followed by analysis with a NucleoCounter NC-3000 analysis system. In early apoptotic cells, where the mitochondrial membrane potential collapses, the monomeric JC-1 remains cytosolic and stains the cytosol with a green color. On the other hand, in nonapoptotic cells, JC-1 impulsively forms complexes with intense red fluorescence. As shown in Table 2, it was found that, when compared with the control group, estragole treatment did not produce any significant collapse of the mitochondrial membrane potential, indicating unaltered mitochondrial function after treatment with this compound.

Late Apoptosis: DNA Fragmentation. The late events of apoptosis were evaluated by DNA content analysis. The results

dx.doi.org/10.1021/np400653p | J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 2. Percentage of Apoptotic HepG2 Cells after 4 h Treatment with Estragole (1): Early (Mitochondrial Membrane Potential; Δψm) and Late (DNA Fragmentation) Apoptosis[a]

estragole (1) (μg/mL)	depolarized Δψm (% of cells)	fragmented DNA (% of sub-G_1 cells)
84.55	13.2 ± 1.4	4.9 ± 3.3
42.28	12.3 ± 1.0	3.7 ± 2.5
21.14	15.9 ± 2.1	3.4 ± 1.9
negative control	11.7 ± 1.2[b]	2.9 ± 1.7[b]
positive control	40.3 ± 1.6[c]	21.1 ± 4.6[d]

[a]Each value represents the mean ± SEM of three independent experiments. [b]Negative control: MEM, complete medium. [c]Positive control: 0.5 μM valinomycin. [d]Positive control: 1 μM staurosporine.

(Table 2) indicated that no sub-G_1 cells were observed in control cells and in HepG2 cells treated with estragole (1).

Final Remarks. The evaluation of toxic, cytotoxic, and genotoxic damage caused by plant compounds is of pivotal importance in minimizing the possible risks arising from exposure to these agents, especially when they are part of long-term treatment or exposure with the diet.[21] Fennel fruit decoctions are a very common remedy used by Italian mothers, and, should these infusions contain hepatocarcinogenic substances, pediatric liver cancer incidence might occur.[21] On the contrary, in Italy (and worldwide as well) hepatic tumors are extremely rare in children, with only 34 new cases of hepatomas in the time frame 2003−2008 in children (age 0−14 years), corresponding to 1.1% of incident pediatric neoplasms in Italy.[22]

Although estragole (1) has been reported as being a potent hepatocarcinogen in rodents,[11] genotoxicity tests have given equivocal results,[23,24] and the mechanism of estragole-induced hepatocarcinogenicity remains unclear. However, it has been proposed that estragole carcinogenicity is linked to its metabolic conversion mediated by sulfotransferases (SULT).[10,25] In fact, estragole itself is not reactive, but the reason for its hepatocarcinogetic properties is a specific metabolism leading to 1′-hydroxylation of the side chain. In in vitro studies, Jeurissen et al.[26] identified cytochrome P450 1A2 (CYP1A2) and CYP2A6 as the most important enzymes involved in the 1′-hydroxylation of estragole. A further biotransformation step with sulfuric acid is mediated by SULT enzymes, giving rise to an electrophilic metabolite of estragole (1) (1′-sulfooxyestragole). 1′-Sulfooxyestragole is unstable in an aqueous environment and is subjected to degradation, leading to loss of the sulfate group to form a carbocation.[25] This carbocation reacts easily with DNA and proteins and may cause DNA adducts and liver tumors.[26]

However, the metabolism of estragole (1) can follow other pathways. It can be detoxified via *O*-demethylation to 4-allylphenol or via epoxidation to estragole 2,3-oxide.[27] The main metabolite, 1′-hydroxyestragole, can be detoxified via oxidation to 1′-oxoestragole or undergo glucuronide conjugation, with the corresponding metabolite being excreted with urine.[28] In human liver cells, by 24 h, about 12.5% of estragole (1) is converted by specific uridine diphosphate glucuronosyltransferases to 1′-hydroxyestragole glucuronide.[28]

Metabolic pathways are regulated by dose following a nonlinear relationship between the dose itself and profiles of metabolic activation.[17] In mice and rats, as the dose is increased, the extent of *O*-demethylation decreases, while 1′-hydroxylation increases;[29] in humans, at higher estragole (1) doses, no relative increase in the formation of 1′-sulfooxyestragole was observed to occur, mainly due to saturation of the 1′-hydroxylation pathway.[30]

To provide further data for the interpretation of the pattern of toxicity in vitro, this study explored, in human HepG2 cells, cytotoxic, genotoxic, and apoptotic effects of estragole (1).

In HepG2 cells, transcript levels of CYP1A2 (phase I enzyme mainly implicated in bioactivation of estragole) have been reported to have a good correlation with activity observed in primary human hepatocytes. Moreover, genes coding for SULT1A1 enzymes are expressed significantly in HepG2 cells.[31]

In the in vitro model (i.e., human hepatoma HepG2 cell line) used in this study, the estragole (1) metabolic pathway leading to formation of the hepatocarcinogenic metabolite 1′-sulfooxyestragole (i.e., phase I 1′-hydroxylation by CYP1A2 and phase II sulfonation by SULT1A1) is potentially active. However, treatment of HepG2 cells with estragole did not induce either cytotoxic effects or DNA damage at all concentrations tested.

In a study examining in vitro metabolic pathways of estragole (1) in humans, a significant prevalence has been reported of the detoxification (oxidation) of 1′-hydroxyestragole compared to the reaction rate of its formation in the human population,[32] and the probability of formation of metabolites of **1** that directly damage DNA has been considered as being low. Consequently, in humans, the risk of estragole acting as a carcinogen might be considered extremely small.[17,33]

The interspecies differences in estragole (1) metabolism highlight the difficulties in using data obtained from conventional rodent species to predict human potential drug-induced toxicity. Cytotoxicity was evaluated by the MTT assay, the trypan blue dye exclusion assay, and the double staining of cells with AO and DAPI, all widely accepted methods for cell injury evaluation. Taken together, the results of these assays indicate that estragole (1) is noncytotoxic. As regards genotoxic effects, the comet assay indicated that estragole was not able to induce DNA damage in HepG2 cells under all conditions used in the experiments conducted. Moreover, in this study, it was shown that the concentrations tested of estragole did not induce apoptosis.

In a recently published article, Martins et al.[34] have reported similar results obtained by testing estragole (1) in vitro in a dose range similar to that used in the present study; similar to the outcomes described herein, they have found estragole did not induce either significant cytotoxicity in the MTT assay or apoptosis. On the contrary, Martins et al.[34] have found estragole-induced DNA damage in the comet assay in a dose-dependent manner; however, the in vitro model they have adopted comprised rodent cells (i.e., V79 and CHO hamster cells) and in which abnormal metabolic pathways, as discussed above, might be involved in the rodent in the metabolic activation of estragole to its final genotoxic metabolites.

In conclusion, the present results demonstrate that estragole (1) is not cytotoxic, genotoxic, nor apoptotic in human cells (i.e., the hepatic HepG2 cell line). On the basis of these findings obtained data support the opinion that fennel fruit decoction [and, consequently, the exposure to estragole (1) resulting from consumption of food] does not raise significantly the risk of primary liver cancer. In addition, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has critically analyzed the results retrieved in the literature for

dx.doi.org/10.1021/np400653p | J. Nat. Prod. XXXX, XXX, XXX−XXX

estragole and concluded that exposure to this alkenylbenzene from food, mainly as spices or added as such, does not pose a significant cancer risk to humans.[27]

EXPERIMENTAL SECTION

Test Compound. Estragole (**1**) (IUPAC name: 1-methoxy-4-prop-2-enylbenzene; CAS No: 140-67-0) was purchased from Sigma-Aldrich Italia (Milan, Italy), with its purity certified as ≥98.5%.

Chemicals and Reagents. All reagents used were of analytical grade. Hydrochloric acid (HCl), dimethyl sulfoxide (DMSO), ethanol, ethylenediaminetetracetic acid disodium (Na_2EDTA) and tetrasodium (Na_4EDTA) salt, sodium chloride (NaCl), and sodium hydroxide (NaOH) were purchased from Carlo Erba Reagenti Srl (Milan, Italy). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide (EtBr), low- and normal-melting-point agarose (LMPA and NMPA, respectively), 4-nitroquinoline *N*-oxide (4NQO), tris(hydroxymethyl)aminomethane (Tris base), Triton X100, staurosporine, and valinomycin were obtained from Sigma-Aldrich Srl (Milan, Italy). Gibco Eagle's minimum essential medium (MEM), fetal calf serum (FCS), L-glutamine, antibiotics (penicillin and streptomycin), sodium pyruvate, Dulbecco's phosphate buffered saline, pH 7.4 (PBS), trypsin-EDTA, and trypan blue dye were purchased from Invitrogen Srl (Milan, Italy). Acridine orange (AO), 6,4′-diamidino-2-phenylindole (DAPI), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolecarbocyanine iodide (JC-1) were purchased from ChemoMetec A/S (Allerød, Denmark). Conventional microscope slides and coverslips were supplied by Knittel-Glaser GmbH (Braunschweig, Germany). Distilled water was used throughout the experiments.

Cell Cultures. Human Caucasian hepatocyte carcinoma HepG2 cells were obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (Brescia, Italy). HepG2 cells retain many characteristics of hepatocytes such as the activities of phase I and phase II drug-metabolizing enzymes and reflect the metabolism of xenobiotics in the human body better than other metabolically incompetent cells.[35,36] In particular, HepG2 cells have been reported to express cytochrome P450 (CYP) 1A2-dependent enzymes (active in phase I reactions) and sulfotransferase (SULT) 1A1 (active in phase II reactions) involved in estragole (**1**) metabolism.[37] Cells were grown as monolayer cultures in 25 cm^2 tissue flasks (Orange Scientific, Braine-l'Alleud, Belgium), in MEM added with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. HepG2 subcultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell stocks were routinely frozen and stored in liquid nitrogen. Under these conditions, the doubling time for HepG2 cells was about 24 h. For this study, cells were used at 101−122 passages.

Cell Treatment. For viability/cytotoxicity testing, subconfluent HepG2 cultures were collected by trypsin treatment and suspended in MEM without phenol red. In the MTT assay, cells (1×10^5 per well) were dispensed within 96-well culture plates (VWR International PBI Srl, Milan, Italy) in 100 μL volumes; for the trypan blue dye exclusion test and double-staining fluorescence (AO−DAPI) viability assay, cells (5×10^5 per well) were dispensed within six-well culture plates (Becton Dickinson Italia SpA, Milan, Italy) in 5 mL volumes. Cells were maintained in culture for 24 h to form a semiconfluent monolayer and then treated with 2% Triton X100 (positive control) or estragole (**1**) over a range of eight concentrations. The cells were exposed for 4 h.

For DNA damage and apoptosis testing, 48 h prior to test compound treatment, HepG2 cells in early log-phase growth were trypsinized and seeded (approximately 5×10^5 cells/well) in six-well plates. Cells were then treated for 4 h with the three highest noncytotoxic concentrations of estragole; cell subcultures were also treated with 1 μM 4NQO (positive controls).

Cytotoxicity Testing. To examine the cytotoxicity induced by estragole (**1**), changes in viability of HepG2 cells after a 4 h exposure were screened in a preliminary manner by assessing mitochondrial activity and membrane integrity (MTT reduction) in the MTT viability assay. The three highest noncytotoxic doses out of the eight tested in the MTT assay were investigated further using the trypan blue dye exclusion test and the double-staining fluorescence (AO−DAPI) viability assay. The MTT viability assay is based on the reduction of MTT by the mitochondrial dehydrogenases of intact cells to a purple formazan product. The MTT assay was conducted according to the ISO 10993-5 guidelines.[38] Briefly, after HepG2 cell incubation with test compounds, 50 μL of 1 mg/mL MTT (in fresh MEM without supplements and without phenol red) was added to each well, and the plates were further incubated at 37 °C in a 5% CO_2 atmosphere for 2 h. Then, the MTT solutions were aspirated from all the wells, and the formazan crystals formed were dissolved by adding 100 μL of 2-propanol into each well. A spectrophotometric analysis was run at 570 nm using a Sunrise (Tecan Italia Srl, Milan, Italy) microplate reader with background subtraction at 650 nm. Cell viability was calculated as the percentage (%) of the viable cells compared with untreated controls. The trypan blue dye exclusion assay is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged; therefore, all the cells that exclude the dye are considered to be viable.[39] In this assay, viable cells will have a clear cytoplasm, whereas nonviable cells will have a blue cytoplasm. Cytotoxicity using the trypan blue dye exclusion assay was measured using a Countess (Invitrogen Srl, Milan, Italy) automated cell counter. Briefly, aliquots of cell suspensions (~1×10^6 cells/mL) were mixed with equal volumes of 0.4% trypan blue, with 10 μL loaded onto a Countess cell counting chamber slide. The instrument is equipped with a camera that acquires images from cell samples on the chamber slide, and the image analysis software automatically analyzes acquired cell images and measures cell count and viability. Cytotoxicity was analyzed further by double staining of cells with AO and DAPI. A NucleoCounter NC-3000 (ChemoMetec A/S, Allerød, Denmark) automated system using fluorescence microscopy and image analysis was used to determine cell number and viability.[40] Double staining with two fluorescent dyes allows a cell stain (AO) for cell detection and a nucleic acid stain (DAPI) for detecting nonviable cells; DAPI cannot penetrate the cell membrane; hence it only stains cells with a permeable cell membrane (i.e., nonviable cells). Briefly, Via1-Cassette (ChemoMetec A/S, Allerød, Denmark) devices were loaded with approximately 60 μL of a cell suspension. The inside of the Via1-Cassette devices is coated with the two fluorescent dyes, AO and DAPI, which were dissolved and stained respectively the entire cell population (represented by green cells) and the nonviable cells (represented by blue cells). After placement of Via1-Cassette devices in the NucleoCounter NC-3000, the stained cell samples were automatically measured, the fluorescent images were recorded, and a report showing the results for cell counts and their viability was generated.

Genotoxicity Testing. The single-cell microgel-electrophoresis (comet) assay is a simple and fast technique allowing the detection of DNA strand-breakage.[20] For this test, a suspension of isolated cells is embedded into an agarose microgel onto a microscope slide and subsequently lysed by detergents at high salt concentration. The liberated DNA is then exposed to alkali to unwind it from the strand-breakage sites and electrophoresed under alkaline conditions. In the presence of DNA strand breaks, electrophoresis at high pH and staining with EtBr results in structures resembling comets with the tail length or tail fluorescence content reflecting the frequency of DNA strand breaks and hence DNA damage.[41] The standard alkaline procedure (lysis at pH 10, unwinding, and electrophoresis at pH >13) allows the detection of both single- and double-strand DNA breaks as well as apurinic/apirimidinic sites (i.e., alkali labile lesions) that are expressed as frank strand breaks in the DNA under the alkaline conditions of the assay. Immediately after exposure, HepG2 cells were processed in the comet assay under alkaline conditions, basically following the original procedure,[20,42] with minor modifications.[43] Briefly, at the end of treatment with estragole (**1**) the cells were

dx.doi.org/10.1021/np400653p | J. Nat. Prod. XXXX, XXX, XXX−XXX

washed twice with 5 mL of ice-cold PBS (pH 7.4) and detached with 300 μL of 0.05% trypsin solution. After about 3 min, trypsinization was terminated by adding complete culture medium (700 μL). Aliquots of 500 μL from each treatment well were taken and collected by centrifugation at 1000 rpm for 8 min. Pellets were gently resuspended in 300 μL of 0.7% LMPA maintained at 37 °C, and 65 μL was immediately spread onto microscope slides precoated with 1.0% NMPA. Agarose microgels were allowed to set for 10 min at 4 °C before addition of a final layer of LMPA (75 μL). After a further 10 min at 4 °C the slides were immersed in ice-cold lysis solution (10 mM Tris-HCl, 2.5 M NaCl, 100 mM Na_2EDTA, and 1% Triton X100; pH 10) and left to stand for 18 h at 4 °C, protected from light. After lysis, the slides were placed in an electrophoresis tank and left for 20 min at 4 °C in the high-pH (>13) electrophoresis buffer (300 mM NaOH, 1 mM Na_4EDTA, pH > 13) to allow the DNA to unwind. Electrophoresis was then performed, on ice, in the same buffer for 20 min at 1.0 V/cm and 300 mA. Afterward, the slides were washed twice with the neutralization buffer (0.4 M Tris-HCl, pH 7.5) and fixed in 70% ethanol for 5 min. The slides were dried and stored at least overnight before microscopic observation. Staining of slides was performed immediately before analysis using 65 μL of EtBr (10 μg/mL). For each experimental point duplicate slides were prepared, and 100 cells were screened per each sample (50 cells from each slide) at 200× magnification with an epifluorescent microscope (Olympus BX41, Tokyo, Japan) under a 100 W high-pressure mercury lamp (HSH-1030-L, Ushio, Japan) using appropriate optical filters (excitation filter 510−550 nm and emission filter 590 nm). The extent of induced DNA damage was measured as the percent of fluorescence migrated in the comet tail (i.e., tail intensity)[44] by a computer-based image analysis system (Comet Assay III, Perceptive Instruments, Haverhill, UK).

Early Apoptosis: Mitochondrial Membrane Potential (Δψm). Loss of the mitochondrial membrane potential is known to precede apoptosis and chemical-hypoxia-induced necrosis. The lipophilic cationic dye JC-1 displays potential-dependent accumulation in the mitochondria and provides a fluorescence-based method for distinguishing between healthy and apoptotic cells. In healthy cells, in fact, the negative charge established by the intact mitochondrial membrane potential facilitates the accumulation of JC-1 in the mitochondrial matrix. At high concentrations, JC-1 forms aggregates and becomes red fluorescent. In apoptotic cells the mitochondrial potential collapses and JC-1 localizes to the cytosol in its monomeric green fluorescent form.[45] Δψm was estimated by using a NucleoCounter NC-3000 analysis system after staining cells with JC-1 and DAPI fluorescent dyes according to the manufacturer's instructions. Mitochondrial depolarization was revealed as a decrease in the red/green fluorescence intensity ratio. In the test necrotic cells were detected as blue fluorescent (DAPI) ones. The green and red fluorescence of the viable cells was displayed in a scatterplot. Gates in the scatterplot were used to demarcate depolarized/apoptotic cells. Results are expressed as percentage of cells excluded by the gate. Valinomycin (0.5 μM) for 4 h was used as a positive control to depolarize the Δψm.

Late Apoptosis: DNA Fragmentation. During apoptosis calcium- and magnesium-dependent nucleases are activated to degrade DNA. The consequence of nicks and double-strand breaks formed along the DNA molecule is DNA fragmentation.[46,47] This late event of apoptosis was detected by DNA content analysis to discriminate cells having less than 2 C DNA (DNA content of a diploid somatic nucleus), so-called sub-G_1 cells. The sub-G_1 method relies on the fact that after DNA fragmentation small DNA molecules are able to diffuse out of the cells following washing (e.g., with PBS). Thus, after staining with a quantitative DNA-binding dye, such as DAPI, cells having lost DNA will take up less stain and will appear left of the G_1 peak in a DNA content histogram.[48] For the test, the cells were first permeabilized with ethanol, and during this procedure the low-molecular-weight DNA inside the apoptotic cells leaks out and is removed from the sample during a subsequent washing step. The high-molecular-weight DNA retained in the cells was stained with DAPI; the fluorochrome binds only to double-stranded DNA, and the intensity of DAPI will correspond to the amount of DNA (thus the G_1/G_0 peak will have half the amount of fluorescent intensity of the G_2 peak). Cellular fluorescence was quantified by a NucleoCounter NC-3000 automated image analysis system. The G_1/G_0 peak was defined manually by the user; the percentage of sub-G_1 cells (i.e., the cells below the marker defining the G_1/G_0 peak) was quantified by NucleoView software. Staurosporine (1 μM) was used as a positive control.

Statistical Analysis. All the assays were carried out in triplicate. The results were expressed as mean values and standard error of means (SEM). After testing the normal distribution of data with the Kolmogorov−Smirnov test, statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnet's post hoc analysis to examine where the differences actually occurred; Pearson's correlation coefficients (r) were calculated to investigate concentration−response relationships. The level of significance was set at $p < 0.05$ for all statistical analyses. The statistical package SPSS (SPSS Inc., Chicago, IL, USA) was used for the analyses.

AUTHOR INFORMATION

Corresponding Author

*Tel: +39 075 5857324. Fax: +39 075 5857342. E-mail: massimo.moretti@unipg.it.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Prof. P. Puccetti, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, for reading the manuscript and offering constructive comments.

REFERENCES

(1) Garland, S. *The Complete Book of Herbs & Spices*; Frances Lincoln, Ltd.: London, 1979.

(2) Shahat, A. A.; Ibrahim, A. Y.; Hendawy, S. F.; Omer, E. A.; Hammouda, F. M.; Abdel-Rahman, F. H.; Saleh, M. A. *Molecules* **2011**, *16*, 1366−1377.

(3) Aprotosoaie, A. C.; Hancianu, M.; Poiata, A.; Tuchilus, C.; Spac, A.; Cioana, O.; Gille, E.; Stanescu, U. *Rev. Med. Chir. Soc. Med. Nat. Iasi* **2008**, *112*, 832−836.

(4) Bub, S.; Brinckmann, J.; Cicconetti, G.; Valentine, B. *J. Am. Med. Dir. Assoc.* **2006**, *7*, 556−561.

(5) Speijers, G.; Bottex, B.; Dusemund, B.; Lugasi, A.; Toth, J.; Amberg-Muller, J.; Galli, C. L.; Silano, V.; Rietjens, I. M. *Mol. Nutr. Food Res.* **2010**, *54*, 175−185.

(6) Brand, N. Foeniculum. In *Hagers Handbuch der Pharmazeutischen Praxis*, 5th ed.; Hänsel, R.; Keller, K.; Rimpler, H.; Schneider, G., Eds.; Springer-Verlag: Berlin, 1993; Vol. *5*, pp 156−181.

(7) Murray, R. D. H.; Mendez, J.; Brown, S. A. *The Natural Coumarins: Occurence, Chemistry and Biochemistry*; John Wiley & Sons: New York, 1982.

(8) Piccaglia, R.; Marotti, M. *J. Agric. Food Chem.* **2001**, *49*, 239−244.

(9) Muller, L.; Kasper, P.; Muller-Tegethoff, K.; Petr, T. *Mutat. Res.* **1994**, *325*, 129−136.

(10) Phillips, D. H.; Reddy, M. V.; Randerath, K. *Carcinogenesis* **1984**, *5*, 1623−1628.

(11) Miller, E. C.; Swanson, A. B.; Phillips, D. H.; Fletcher, T. L.; Liem, A.; Miller, J. A. *Cancer Res.* **1983**, *43*, 1124−1134.

(12) EC (European Commission). *Opinion of the Scientific Committee on Food on Estragole (1-Allyl-4-Methoxybenzene)*; Brusseles, 2001.

(13) FAO/WHO. *Joint FAO/WHO Expert Committee on Food Additives - Sixty-ninth Meeting - Summary and Conclusions*; Rome, June 17−26, 2008, 2008.

(14) EFSA. *EFSA J.* **2009**, *7*, 1249.

(15) EFSA.. *EFSA J.* **2005**, *282*, 1−31.

(16) Dybing, E.; O'Brien, J.; Renwick, A. G.; Sanner, T. *Toxicol. Lett.* **2008**, *180*, 110−117.
(17) Rietjens, I. M.; Punt, A.; Schilter, B.; Scholz, G.; Delatour, T.; van Bladeren, P. J. *Mol. Nutr. Food Res.* **2010**, *54*, 195−207.
(18) Raffo, A.; Nicoli, S.; Leclercq, C. *Food Chem. Toxicol.* **2011**, *49*, 370−375.
(19) FFHPVC (The Flavor and Fragrance High Production Vol. Consortia). *Revised Robust Summary & Test Plan: Estragole*; United States Environmental Protection Agency: Washington, DC, 2005.
(20) Tice, R. R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J. C.; Sasaki, Y. F. *Environ. Mol. Mutagen.* **2000**, *35*, 206−221.
(21) Gori, L.; Gallo, E.; Mascherini, V.; Mugelli, A.; Vannacci, A.; Firenzuoli, F. *Evid.-Based Complementary Altern. Med.* **2012**, *2012*, 860542.
(22) Group, A. W.; Group, A. W. *Epidemiol. Prev.* **2013**, *37*, 2−24.
(23) Nesslany, F.; Parent-Massin, D.; Marzin, D. *Mutat. Res.* **2010**, *696*, 1−9.
(24) Zeiger, E.; Anderson, B.; Haworth, S.; Lawlor, T.; Mortelmans, K.; Speck, W. *Environ. Mutagen.* **1987**, *9* (S9), 1−109.
(25) Suzuki, Y.; Umemura, T.; Ishii, Y.; Hibi, D.; Inoue, T.; Jin, M.; Sakai, H.; Kodama, Y.; Nohmi, T.; Yanai, T.; Nishikawa, A.; Ogawa, K. *Mutat. Res.* **2012**, *749*, 23−28.
(26) Jeurissen, S. M.; Punt, A.; Boersma, M. G.; Bogaards, J. J.; Fiamegos, Y. C.; Schilter, B.; van Bladeren, P. J.; Cnubben, N. H.; Rietjens, I. M. *Chem. Res. Toxicol.* **2007**, *20*, 798−806.
(27) Smith, R. L.; Adams, T. B.; Doull, J.; Feron, V. J.; Goodman, J. I.; Marnett, L. J.; Portoghese, P. S.; Waddell, W. J.; Wagner, B. M.; Rogers, A. E.; Caldwell, J.; Sipes, I. G. *Food Chem. Toxicol.* **2002**, *40*, 851−870.
(28) Iyer, L. V.; Ho, M. N.; Shinn, W. M.; Bradford, W. W.; Tanga, M. J.; Nath, S. S.; Green, C. E. *Toxicol. Sci.* **2003**, *73*, 36−43.
(29) Zangouras, A.; Caldwell, J.; Hutt, A. J.; Smith, R. L. *Biochem. Pharmacol.* **1981**, *30*, 1383−1386.
(30) Punt, A.; Jeurissen, S. M.; Boersma, M. G.; Delatour, T.; Scholz, G.; Schilter, B.; van Bladeren, P. J.; Rietjens, I. M. *Toxicol. Sci.* **2010**, *113*, 337−348.
(31) Wilkening, S.; Stahl, F.; Bader, A. *Drug Metab. Dispos.* **2003**, *31*, 1035−1042.
(32) Punt, A.; Paini, A.; Boersma, M. G.; Freidig, A. P.; Delatour, T.; Scholz, G.; Schilter, B.; van Bladeren, P. J.; Rietjens, I. M. *Toxicol. Sci.* **2009**, *110*, 255−269.
(33) Zeller, A.; Horst, K.; Rychlik, M. *Chem. Res. Toxicol.* **2009**, *22*, 1929−1937.
(34) Martins, C.; Cacao, R.; Cole, K. J.; Phillips, D. H.; Laires, A.; Rueff, J.; Rodrigues, A. S. *Mutat. Res.* **2012**, *747*, 86−92.
(35) Sassa, S.; Sugita, O.; Galbraith, R. A.; Kappas, A. *Biochem. Biophys. Res. Commun.* **1987**, *143*, 52−57.
(36) Knasmuller, S.; Parzefall, W.; Sanyal, R.; Ecker, S.; Schwab, C.; Uhl, M.; Mersch-Sundermann, V.; Williamson, G.; Hietsch, G.; Langer, T.; Darroudi, F.; Natarajan, A. T. *Mutat. Res.* **1998**, *402*, 185−202.
(37) Donato, M. T.; Lahoz, A.; Castell, J. V.; Gomez-Lechon, M. J. *Curr. Drug Metab.* **2008**, *9*, 1−11.
(38) ISO (International Organization for Standardization). *Biological Evaluation of Medical Devices. Part 5: Tests for In Vitro Cytotoxicity*; Geneva, Switzerland, 2009.
(39) Freshney, R. *Culture of Animal Cells: A Manual of Basic Technique*; Alan R. Liss, Inc.: New York, 1987.
(40) Shah, D.; Naciri, M.; Clee, P.; Al-Rubeai, M. *Cytotechnology* **2006**, *51*, 39−44.
(41) Dominici, L.; Moretti, M.; Villarini, M.; Vannini, S.; Cenci, G.; Zampino, C.; Traina, G. *Int. J. Probiotics Prebiotics* **2011**, *6*, 179−196.
(42) Singh, N. P.; McCoy, M. T.; Tice, R. R.; Schneider, E. L. *Exp. Cell Res.* **1988**, *175*, 184−191.
(43) Moretti, M.; Cossignani, L.; Messina, F.; Dominici, L.; Villarini, M.; Curini, M.; Marcotullio, M. C. *Food Chem.* **2013**, *140*, 660−665.
(44) Collins, A. R. *Mol. Biotechnol.* **2004**, *26*, 249−261.
(45) Salvioli, S.; Ardizzoni, A.; Franceschi, C.; Cossarizza, A. *FEBS Lett.* **1997**, *411*, 77−82.
(46) Nagata, S. *Exp. Cell Res.* **2000**, *256*, 12−18.
(47) Nagata, S.; Nagase, H.; Kawane, K.; Mukae, N.; Fukuyama, H. *Cell. Death Differ.* **2003**, *10*, 108−116.
(48) Kajstura, M.; Halicka, H. D.; Pryjma, J.; Darzynkiewicz, Z. *Cytometry, Part A* **2007**, *71*, 125−131.