ORIGINAL ARTICLE

Thymol and *Thymus vulgaris* extract protects human keratinocyte cell line (HaCaT) from UVA and UVB damage

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ABSTRACT

Objective: The aims of our study were to characterize ultraviolet (UV)A- and UVB-induced damages in a keratinocytes cell line (HaCaT), and to evaluate the protective capacities two plant-derivative compounds, namely *Thymus vulgaris* L leaf extract and thymol, its major component. A polyphenol rich diet has gained wide attention and it is now considered to be a protective agent for human skin, which can be over-exposed to environmental factors and in particular UV light.

Methods: Cells were pretreated for 1 h, in serum-free medium, with thymol (1 µg/ml) or *Thymus vulgaris* L (1.82 µg/ml) then exposed to different UVA (8-24 J/cm²) or UVB doses (0.016-0.72 J/cm²). Immediately after the UV exposure the intracellular redox status was evaluated by reactive oxygen species quantification and apoptotic events. Genotoxic aspects were evaluated 24 h after the end of irradiations using the alkaline comet assay and the immunostaining of phosphorylated H2AX histone protein (detected 1 h after the end of UV exposure).

Results: The pre-treatment of our experimental model with the two substances confirmed an antioxidant action and anti-apoptotic effect by reducing the cells percentage (sub-G_1 phase). Furthermore, thymol and extract of *Thymus vulgaris* L were able to reduce genotoxic damage. The alkaline comet assay showed that the two substances were capable to decrease DNA damage. Also in this case, *Thymus vulgaris* L extract is more effective than thymol in decreasing genotoxicity markers.

Conclusions: Our results confirmed the more oxidant UVA and more genotoxic UVB effects. Regarding the protective effect of thymol and *Thymus vulgaris* L extract, data obtained proved their antioxidant and free-radical scavenging ability as known for phenolic (which our compounds belong to) and polyphenolic compounds. Thymol and mainly *Thymus vulgaris* L extract were also able to reduce the direct genotoxic damage.

INTRODUCTION

Polyphenols are the most abundant dietary antioxidants and are constituents of many plant food sources, including fruits, vegetables and cereals. These natural compounds are secondary metabolites of plants and may be classified into different groups according to their chemical structures (phenolic acids, flavonoids, stilbenes and lignans) [1-3].

Several studies suggest that polyphenols play an important role in the prevention of degenerative diseases; involved in the defense against the effects of various pollutants and chemicals, hostility from pathogens and radiation, thanks to their antioxidant, anti-inflammatory, immunomodulatory and DNA repairing properties [4-7]. Therefore, a long-term consumption of a polyphenol rich diet has gained large attention and it is now considered to be a protective agent for human skin, which can be over-exposed to environmental factors and, particularly, ultraviolet (UV) light. The latter can cause some disorders, such as sunburn, photoaging, immunosuppression, inflammation, oxidative damage and cancer [8-10].

The UV radiation is a component of the solar spectrum and it is divided into three categories, depending on the wavelength: UVA (400-320 nm), UVB (320-290 nm) and UVC (290-100 nm) [11]. UVA rays comprise the largest spectrum of the solar UV radiation (90-95%) and penetrate deeply into epidermis and dermis [12]. They cause significant damage to cellular components (proteins, DNA and biological membranes) through an oxidative process, by releasing reactive oxygen species (ROS) [13]; uncontrolled producing of ROS is involved in the pathogenesis of a sheer number of human skin disorders including photoaging, pigmentation and cutaneous neoplasia by inducing oxidative DNA injuries (8-oxo-dG or thymine-glycol) and single-strand breaks [14-17].

The UVB radiation constitutes approximately 5% of the total solar UV light; it is less penetrating than UVA rays, it acts mainly in the epidermal layer of the skin and is mainly responsible for a variety of cutaneous cancers [18]. It induces, particularly, direct damage to DNA, leading to the formation of mutagenic lesions: cyclobutane-pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts ((6-4)-PP) [19]. The first are the most frequent injuries and they could give rise to UV-signature C to T and CC to TT transition mutations [20]. UVB can also cause an indirect damage, provoking reactive oxygen and nitrogen species (ROS and RNS) production and creating inflammation, skin photoaging, sunburn and erythema [21-23]. Finally, the UVC radiation is largely absorbed by the atmospheric ozone layer and does not reach the surface of the earth and, thus, its involvement in cutaneous cancerogenesis is irrelevant [24, 25]

In order to protect themselves from UV-damages, human cells are provided with defense and repair endogenous mechanisms, such as antioxidant systems [26] and

DNA repair systems (NER: Nucleotide Excision Repair system, which removes UVB-induced lesions; BER: Base Excision Repair system, for oxidative DNA damage) [27-29]. When these systems are not capable to counteract ultraviolet-induced damages, polyphenols can be an efficient support, exercising their health-promoting ability [3]. In our study, we focused the attention on two plant-derivative compounds: *Thymus vulgaris* L leaf extract and thymol, its major component; the latter confers to the thyme extract with many biological activities, such as anti-inflammatory, antibacterial, antispasmodic, expectorant and antitussive properties [30-31].

In vitro and *in vivo* studies have confirmed the ability of thymol to prevent oxidative stress damages, exhibiting antioxidant and scavenging activity [32-34], but also to reduce apoptotic cells percentage in systems subjected to γ-radiations treatment [35]. Furthermore, this phenolic compound has proved to be able to reduce the production of inflammation mediators (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8) and to induce the anti-inflammatory cytokines gene expression, such as IL-10 [36]. Regarding genotoxicity, previous literature has shown that high concentrations (mM) of thymol and a prolonged time of exposure to it (6-48 h) can cause chromosomal aberrations [37-40]. Although, recent experimentations [33, 41] have demonstrated the anti-genotoxic effect of low concentrations of thymol (μM), that decrease DNA damages.

Therefore, the two aims of our study were to *(i)* characterize UVA- and UVB-induced damages in a keratinocytes cell line (HaCaT), and *(ii)* to evaluate the protective capacities at low concentrations of thymol and thyme extract in respect of this injury, by analyzing the cell cycle progression, oxidative stress and genotoxicity.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin solution, non-animal L-glutamine minimum essential medium (MEM) vitamin solution 100x, non-essential amino acid solution 100x, 3-(4-5-dimethylthiazol-2-11)-2,5-diphenyl tetrazolium bromide (MTT), tert-butylhydroperoxide solution (TBHP), dimethylsulfoxide (DMSO), 2',7'-dichlorofluorescin diacetate (DCFDA), trypsin-EDTA solution 1x (trypsin), low-melting-point agarose (LMA), agarose for routine use, propidium iodide (PI), sodium chloride (NaCl), tris(hydroxymethyl) aminomethane, sodium hydroxide (NaOH), potassium chloride (KCl), Hepes, Triton X-100, trichloroacetic acid (TCA), thiobarbituric acid (TBA), hydrochloric acid (HCl), sodium-citrate, ethidium bromide, citric acid and sucrose were obtained from Sigma-Aldrich (Milan, Italy). Fetal bovine serum (FBS) was purchased from Biochrom (Milan, Italy).

Thymol and *Thymus vulgaris* L leaf extract

Thymol (5-methyl-2-(1-methylethyl)-phenol) was purchased from Sigma-Aldrich (purity ≥ 99.5%). Powder was dissolved in DMSO (120 mg/ml) and then diluted in serum-free culture medium to obtain the 1μg/ml final concentration. DMSO concentration did not exceed 0.1%.

Thymus vulgaris L leaf extract was kindly donated by 'EPO S.r.l. Istituto Farmochimico Fitoterapico, Milan'. It is a dry water-soluble extract obtained by *Thymus vulgaris* L leafs' hydrodistillation (purity 65-85%, 30% maltodextrin). Extract powder was dissolved in DMSO (150 mg/ml) and then diluted in serum-free culture medium to obtain the 1.82 μg/ml final concentration. DMSO concentration did not exceed 0.1%. The concentration of *Thymus vulgaris* L extract used contains a comparable dosage of Thymol (1μg/ml).

Cell cultures

HaCaT keratinocytes, a spontaneously immortalized human epithelial cell line [42], were purchased from Istituto Zooprofilattico di Brescia (Brescia, Italy). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 4 mM non-animal L-glutamine, 1% penicillin-streptomycin solution, 1% MEM vitamin solution 100x and 1% non-essential amino acid solution 100x, in a humidified incubator, aerated with 5% CO_2, at 37°C. Culture medium was changed twice a week.

Ultraviolet irradiation system

The illuminator system consisted of four UVA lamps (each lamp 6 Watt, peak 365 nm) closed two-by-two, and two closed UVB lamps (each 6 Watt, peak 312 nm) (TRIWOOD 31/36, Helios Italquartz, Milan, Italy). Each UVA and UVB group lamps are equipped with a switch for the separate (UVA or UVB) or coupled (two UVA groups) functioning. The irradiances of the UVA (at the distance of ∼ 7 cm) and UVB lamps (at the distance of ∼12 cm) were respectively 2.25 mW/cm² and 0.8 mW/cm². The emitted intensity was measured using the HD 2302.0 radiometer (Delta OHM; Padova, Italy) with a specific UVA or UVB detector.

Keratinocytes treatment

To evaluate the protective effect of the two natural compounds against ultraviolet radiations, HaCaT cells were pre-treated (1 h, 37°C) with thymol (6.6 μM corresponding to 1μg/ml) or *Thymus vulgaris* L extract (1.82 μg/ml) in serum-free medium. Then, the medium was removed, cells were rinsed once with phosphate-buffered saline (PBS 1x), covered with a thin PBS layer and irradiated with different UVA (16 and 24 J/cm²) or UVB (0.016-0.72 J/cm²) doses. To prevent the PBS overheating during irradiation, plates were kept on ice. Control cells were treated the same way as the experimental ones, but they were not exposed to UV rays. UV doses were chosen applying to real exposition doses, to which the human skin could be undergone daily (10

$J/cm^2 \approx 30$ min of sun exposition, at 12 a.m. in Central Europe; 0.032 $J/cm^2 \approx 1$-2 min at the same conditions). Cytotoxicity of ultraviolet radiation and polyphenols compound in exam was assessed immediately at the end of the treatment and 24 h after.

To examine genotoxic effect of UV rays, samples were irradiated with 16-24 J/cm^2 UVA or 0.016-0.032 J/cm^2 UVB; later, cells were incubated for 24 h in culture medium, necessary time to reveal genotoxic damage and repair of the latter. Instead, to carry out oxidative stress tests, samples were exposed to 16-24 J/cm^2 UVA or to 0.064 (the last dose which was not able to induce oxidative damage)-0.72 (the first dose that induced a significant ROS production) J/cm^2 UVB; at least, immediately after irradiation, cells were collected to execute the analysis.

Cell cycle analysis

Cell cycle analysis was carried out by flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA) to determine cells distribution in different phases of the proliferative cycle and to highlight possible modifications in the correct progression of cell cycle. HaCaT cells were plated in 28 cm^2 culture dishes (Corning, NY, USA) and, after the pretreatment with thymol or *Thymus vulgaris* L extract, were exposed to UVA (16-24 J/cm^2) or UVB (0.016-0.032 J/cm^2).

After 24 h in culture medium, samples supernatant was collected; cells were washed with PBS, detached with trypsin, collected with their supernatant, and finally centrifuged for 5 min at 2000 rpm. Cells were re-suspended in 1 ml of PBS + 2% FBS, counted by the trypan blue method (1 x 10^6 cells/ml) and centrifuged again. Samples supernatant was discarded and cell cycle solution (80% PI 50 µg/ml in PBS, 10% RNAsi 0.5 mg/ml in PBS 1x, 10% Nonidet® P 40 substitute 0.1% in PBS) was added and kept for 30 min at room temperature (RT) in the dark. At least, samples were analyzed by flow cytometry at an excitation wavelength of 535 nm and an emission wavelength of 617 nm [43].

Apoptosis

Apoptosis, the 'programmed cells death', occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. This process is also considered a defense mechanism in immune reactions or when cells are damaged by disease or noxious agents [44].

Annexin V-fluorescein isothiocyanate (FITC) detection of apoptosis

In apoptotic cells, phosphatidylserine (PS) is translocated from the inner to the outer layer of the plasma membrane. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. We studied the protective effect of thymol and *Thymus vulgaris* L extract against UVA- and UVB-induced apoptosis using the Alexa Fluor 488 Annexin

V/Dead cell apoptosis kit (Invitrogen), according to the manufacturer's instructions, with some modifications. The kit contains Annexin V conjugated with the bright fluorophore Alexa Fluor 488 and the red-fluorescent PI nucleic acid binding dye, which is impermeable to live and apoptotic cells; so, it only stains dead cells. Cells were seeded in 28 cm^2 culture dishes, treated with thymol or *Thymus vulgaris* L extract and exposed to UVA (16 and 24 J/cm^2) or UVB (0.016-0.032 J/cm^2).

After 24 h, supernatant samples were picked. Cells were washed with PBS, detached with trypsin and, finally, collected with their supernatant. Cells were centrifuged for 5 min at 2000 rpm and re-suspended in 1 ml of PBS + 5% FBS, counted by the trypan blue method (1 x 10^6 cells/ml) and centrifuged again. Supernatant was discarded and 100 µl of Annexin Binding Buffer (1:10 in sodium citrate 0.1%) were added to each sample (except for control cells and only PI stained cells). HaCaT cells were double stained with 5 µl of Alexa Fluor 488 Annexin V and 1 µl of PI (100 µg/ml) and kept for 15 min at RT, in the dark. After the incubation, 400 µl of Annexin Binding Buffer 5x (1:10 in sodium citrate 0.1%) were added to each sample and the samples were analyzed by flow cytometry at an excitation wavelength of 496 nm and an emission wavelength of 516 nm.

Oxidative stress

Overproduction of ROS or reduction in the ability of endogenous antioxidants to neutralize them results in oxidative stress, which can lead to damage of lipids, proteins and DNA. This condition is traceable by measuring ROS, lipid peroxidation, protein oxidation and DNA fragmentation, or cell death.

Reactive oxygen species generation

ROS formation was quantified according to Wang and Joseph [45] with some modifications, using the cell-permeable, non-fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFDA), which is de-esterified intracellularly and turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. Cells were seeded in black 96-well plates, pre-treated with thymol or *Thymus vulgaris* L extract for 1 h, then exposed to UVA (16-24 J/cm^2) or UVB (0.064-0.72 J/cm^2). The cells were, at least, incubated with 25 µM DCFDA at 37°C for 30 min. After incubation medium culture containing probe was discarded, cells were washed with PBS and fluorescence was measured using a microplate reader (Multilabel counter Victor Wallac 1420; Perkin-Elmer, Monza, Italy) at 485 nm_{ex}/530 nm_{em}. The results were calculated as fluorescence units (FU)/mg of cell protein, according to Lowry *et al* [46].

Genotoxicity

Alkaline single-cell gel electrophoresis (SCGE, comet assay)

The comet assay is a simple and sensitive method for quantifying and analyzing DNA damage in single cells.

Cells, embedded in agarose on a microscope slide, are lysed with a solution containing detergent and high salt to form nucleoids with supercoiled loops of DNA. The DNA is allowed to unwind under alkaline conditions in the electrophoresis buffer. Following the unwinding, the DNA undergoes electrophoresis at high pH ($\geq$ 13), allowing fragmented DNA to migrate away from the nucleus. Once the slides were dry, they were stained with a DNA-specific fluorescent dye such as PI and observed by fluorescence microscopy. Undamaged cells appear as intact nucleoids, damaged cells as comets. The extent of DNA migrated from the head of the comet is directly proportional to the amount of DNA damage. Brighter and longer is the tail, higher is the level of DNA damage.

Experiments were carried as described in our previous studies [47-48] with minor modifications. Cells were plated in 28 cm^2 culture dishes and after the pretreatment with thymol or *Thymus vulgaris* L extract were exposed to UVA (16-24 J/cm^2) or UVB (0.016-0.032 J/cm^2). After 24 h in culture medium, cells were collected by trypsin. Trypsinization was stopped with 1 ml of culture medium and the cell suspension was centrifuged for 5 min at 2000 rpm. Supernatant was removed and the pellet re-suspended in 1 ml of culture medium and kept on ice. A total of 2 x 10^4 cells/ml (counted by the trypan blue method) were re-suspended in 200 μl of 0.5% low-melting-point agarose (LMA) in PBS; then, they were transferred onto pre-coated microscope slides with 1% agarose for routine use in PBS, and covered with a coverglass. Slides were prepared with a first layer of cell suspension in 0.5% LMA and a second layer only of 0.5% LMA, and stored at 4°C for 10 min to allow solidification. The coverglasses were gently removed, and slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, pH 10) at 4°C for 1 h. Slides were then rinsed with neutralization solution (0.4 M Tris, pH 7.5) and placed in a horizontal gel electrophoresis tank filled with ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM Na-EDTA, pH 13) for 20 min, on ice and in the dark, to allow DNA to unwind.

Electrophoresis was done at 25 V and 300 mA for 30 min, followed by 5 min neutralization with 2 ml of neutralization solution, then fixation with ethanol (Carlo Erba; Val de Reuil, France) at -20°C for 5 min. When the slides were dry, they were stained with 500 μl PI (20 μg/ml) and analyzed using a fluorescence microscope (Axioplan 2, Zeiss; Milan, Italy) at 25-fold magnification. For each sample, 50-60 randomly selected nucleoids were acquired. Images of the fluorescently stained cell nuclei were analyzed using TriTek Comet Score Imaging Software 1.5 (Wilmington, DE, USA). Nucleoids were first classified in five categories (A, B, C, D, E) on the basis of the area and intensity of the tail staining, then DNA damage was quantified as the percentage of DNA in the tail, tail length and tail moment (calculated as the product of the two previous parameters).

Alkaline SCGE (comet assay) with endonuclease III (Endo III)/formamidopyrimidines DNA glycosylase (FPG)

Making the alkaline comet assay more sensitive and specific is possible by introducing the extra step of digesting the nucleoids with an enzyme that recognizes a particular kind of damage. Endo III and FPG are used to detect oxidative DNA injuries; they remove oxidized DNA bases leaving an apurinic/apyrimidinic site, which is then converted to a break.

Experiment was carried out following the above described protocol of SCGE comet assay, with a modification: after lysis phase, nucleoids were incubated for 30 min at 37°C with 25 µl of enzyme dissolved in a buffer (0.1 M KCl, 0.1 mM Na-EDTA, 40 mM Hepes, 0.2 ml FBS; ENDO III 1 µg/ml in buffer, FPG 1 µg/ml in buffer) or with 25 µl of buffer only. The oxidative damage presence is highlighted by the increase of tail intensity in samples incubated with enzyme [49].

Gamma-H2AX staining

Phosphorylation on the Ser-139 residue of the histone variant H2AX (γ-H2AX) is an early cellular response to the induction of DNA double-strand breaks (DSB); detection of this event has emerged as a highly specific and sensitive marker for monitoring DNA damage initiation and resolution [50-52]. The presence of phosphorylated H2AX can be measured, immunocytochemically or by immunofluorescence, in the form of distinct nuclear foci where each focus is assumed to correspond to a single DSB [53]. Test was performed according to the protocol described by Wischermann *et al* [54], with some modifications.

Cells were seeded on cover glasses slides. After pretreatment with thymol or *Thymus vulgaris* L extract, HaCaT cells were irradiated with UVA (24 J/cm^2) or UVB (0.016-0.032 J/cm^2) and incubated in culture medium for 1 h at 37°C. After 1 h, cells were fixed with ice-cold methanol for 10 min at RT, washed three times in PBS 1x and permeabilized with Triton X-100 (0.5% in PBS) for 20 min at RT. Samples were washed three times with PBS 1x, and then aspecific sites were blocked by 3% BSA (3% BSA in PBS 1x) for 30 min at RT. The cells were incubated with the γ-H2AX primary antibody (mouse anti-phospho-Histone-H2AX (Ser 139), clone JBW301, Millipore, 1 mg/ml) 1:150 in 1% BSA for 2 h at RT. After three washings in PBS 1x, cells were incubated with the secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG 2 mg/ml; Life Technologies™, Carlsbad, CA, USA) 1:500 in BSA 1% for 1 h at RT. At this point, cells were washed three times with PBS 1x and once with distilled water. Finally, samples were mounted with VECTASHIELD® mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA, USA). Cells were analyzed at 64x amplification by confocal microscopy (Zeiss LSM 510-Meta). Images of randomly selected cells were analyzed using ImageJ software and counting the number of γ-H2AX foci/nucleus in order to distinguish between cells with a basal number of foci (0-5 foci/nucleus), low number of foci (6-10 foci/nucleus) and with high number of foci (>10 foci/nucleus).

Statistical analysis

Results of each test are expressed as mean ± standard deviation of at least three experiments. In particular, for the comet assay, results are expressed as mean of median

± standard deviation. Statistical analysis was performed with Graphpad Prism 5.0 (Graphpad, La Jolla, CA, USA). For all assays, One-Way Anova plus Dunnett's post hoc test or Two-Way Anova followed by Bonferroni post hoc test were used.

RESULTS

Thymol and *Thymus vulgaris* L extract cytotoxicity

Thymol and *Thymus vulgaris* L extract cytotoxicity was quantified by MTT test (data not shown) immediately at the end of treatment and 24 h after. Results showed that cell viability of samples exposed to thymol (1-8 µg/ml) and *Thymus vulgaris* L leaf extract (1.82-14.69 µg/ml) never decreased under 80%, both immediately or 24 h after the treatment.

UVA and UVB doses

Ultraviolet radiation cytotoxic effect was assessed by MTT test (data not shown), both immediately after irradiation and 24 h later. Immediately after UV exposure, UVA (16-24 J/cm²) and UVB (0.016- 0.72 J/cm²) did not cause any significant decrease in cell viability; instead, 24 h after the irradiation there was a 20% reduction in cell viability with 24 J/cm² UVA, and a 50% reduction with UVB (0.064-0.72 J/cm²), in a dose-dependent manner.

Cell cycle analysis

The cell cycle progression of samples was altered by UV exposure. UVA irradiation (16-24 J/cm²) caused an increase of cells in S phase and a simultaneously decrease of cells percentage in G_0/G_1 phase. Pretreatment with thymol and *Thymus vulgaris* L extract of samples exposed to irradiation induced a new increment of cells in G_0/G_1 phase, a decrease of cells percentage in Sub-G_1 and S phases and, consequently, a boost of cells in G_2/M phase. UVB rays (0.016-0.032 J/cm²) provoked a marked reduction of cells in G_0/G_1 phase and an increase of cells percentage in Sub-G1 phase, in a dose-dependent manner. Treatment with natural compounds was effective in decreasing cells in Sub-G1 phase, in favor of an increment of cells in G_2/M phase. (Table 1 shows data only related to 24 J/cm² and 0.032 J/cm²).
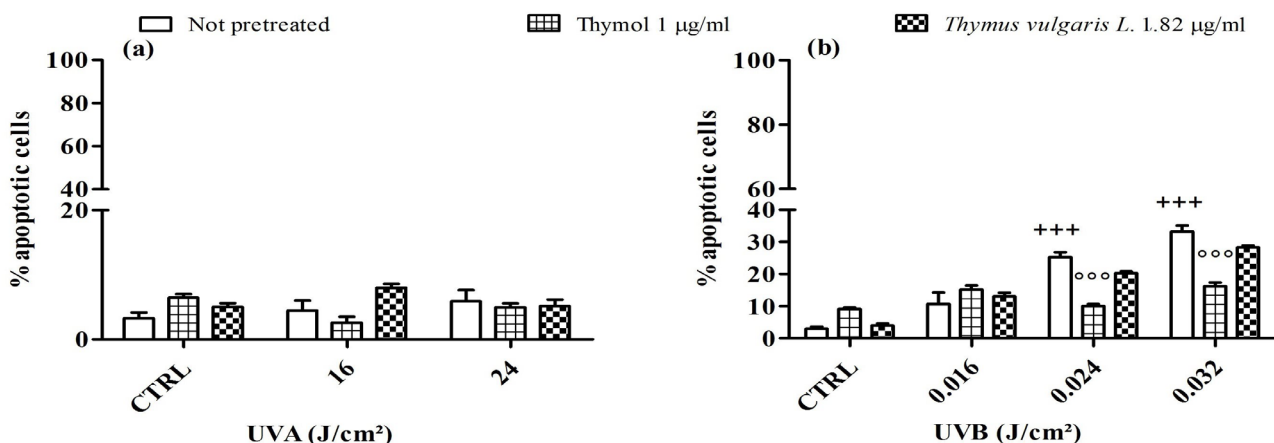
Apoptosis (Annexin V assay)

The increase of apoptotic cells (Figure 1) was significant only in samples irradiated with UVB at 0.024-0.032 J/cm² (P < 0.001 *vs* control). *Thymus vulgaris* L extract pretreatment induced a small and not significant decrease of apoptotic cells; instead, thymol pretreatment was able to reduce significantly the apoptosis (P < 0.001 at 0.024 and 0.032 J/cm²).

Table 1. Assessment of irradiated cells distribution in the different phases of the cell cycle and effect evaluation of *Thymus vulgaris* L extract (1.82 µg/ml) and thymol (1 µg/ml) pretreatment (1 h) immediately after the end of UVA (24 J/cm²) or UVB (0.032 J/cm²) irradiation

	% Sub-G_1	% G_0/G_1	% S	% G_2/M
CTRL	2.05	46.81	32.68	18.47
CTRL + Thymol 1 µg/ml	1.73	41.2	27.71	29.36
CTRL + *Thymus vulgaris* L 1.82 µg/ml	1.73	42.8	28.06	27.41
UVA 24 J/cm²	13.21*		48.17	18.3
UVA 24 J/cm² + Thymol 1 µg/ml	4.28	24.36***	40.46	30.9
UVA 24 J/cm² + *Thymus vulgaris* L 1.82 µg/ml	3.46	31.58***	33.32	31.64
UVB 0.032 J/cm²	18.5**	38.13*	26.71	16.67
UVB 0.032 J/cm² + Thymol 1 µg/ml	9.2	36.26*	31.54	23
UVB 0.032 J/cm² + *Thymus vulgaris* L 1.82 µg/ml	12.11	38.05*	28.22	21.62

Results are shown as cell percentage in the various phases of the cell cycle (P < *0.05, **0.01, and ***0.0001)

Figure 1. Effect of thymol (1 µg/ml) and *Thymus vulgaris* L extract (1.82 µg/ml) pretreatment (1 h) on (a) UVA (16-24 J/cm²), or (b) UVB (0.016-0.032 J/cm²)-induced apoptosis. Annexin V test was assessed 24 h after end of the irradiation. Results are shown as percentage of apoptotic cells compared to negative control cells (CTRL) and are means ± SD of three independent experiments. +++P < 0.001 vs CTRL (One-Way ANOVA test, Dunnett's Multiple Comparison Test); °°°P < 0.001 vs the same treatment w/o thymol or Thymus vulgaris L extract (Two-Way ANOVA test, Bonferroni post-test).

Oxidative stress (ROS generation)

HaCaT cells exposure to UVA and UVB radiation produced a significant dose-related increase in ROS generation, more evident after UVA irradiation (P < 0.001 at 16 and 24 J/cm^2) and after UVB irradiation only at 0.72 J/cm^2 (P < 0.001) (Figure 2).

Thymus vulgaris L (P < 0.01 at 16 J/cm^2; P < 0.001 at 24 J/cm^2; P < 0.001 at 0.72 J/cm^2) and thymol (P < 0.05 at 16 J/cm^2; P < 0.001 at 0.72 J/cm^2) pretreatment showed their protective effect towards both UVA- and UVB-induced ROS production.

Genotoxicity

Comet assay

DNA damage was more evident in samples exposed to UVB radiation, which showed a significant increase of tail length (P < 0.01 at 0.024 J/cm^2; P < 0.001 at 0.032 J/cm^2) (Figure 3). These doses not determine ROS formation. Thymol (P < 0.01 at 0.024 and 0.032 J/cm^2) and thyme extract (P < 0.01 at 0.024 J/cm^2; P < 0.001 at 0.032 J/cm^2) pretreatment was efficient to reduce the tail length significantly (Figure 3).

Comet assay with endonuclease III

The comet assay with Endo III, assessed to detect oxidized DNA bases, showed a significant difference between damage in samples exposed to UVA radiation and treated with Endo III, and in samples treated with dilution buffer only (P < 0.001 at 24 J/cm^2). The significant increase of tail length in cells incubated with the enzyme suggests that UVA rays are able to induce genotoxic damage of oxidative nature (Figure 4). On the contrary, this injury was not observed in samples treated with buffer (and in samples only irradiated), probably for the repair mechanisms activation (which are able to remove DNA oxidative damage in 24 h). Thymol and thyme extract pretreatment exercised a protective effect against oxidative DNA damage, but differences between cells incubated with Endo III or with buffer only were not observed.

H2AX staining

Immunostaining of phosphorylated H2AX histone protein was performed as a marker of DSBs presence. Irradiation of samples with UVA (24 J/cm^2) and UVB (0.016-0.032 J/cm^2) caused the formation of DSBs, as showed by the significant increase of cells with more than 10 *foci* (P < 0.05 at 24 J/cm^2; P < 0.01 at 0.016 and 0.024 J/cm^2; P < 0.001 at 0.032 J/cm^2). Pretreatment with thymol and *Thymus vulgaris* L extract did not show any significant protection. This is probably because, after 1 h only, the DNA repair mechanisms have not been stimulated and activated yet by the action of the natural compounds (Figure 5).

DISCUSSION

Ultraviolet radiation exposure is considered one of the major risk factors for skin disease outbreak. Over recent years, the decrease in stratospheric ozone layer and the increase of outdoor human activities and of sun lamps use have caused a considerable increment of cutaneous cancer incidence [15, 55, 56]; consequently, ultraviolet rays (UVA and UVB) has been classified as a Class I carcinogen by the International Agency for Research on Cancer (IARC) [14]. Our data confirmed the different mechanism of UVA and UVB damage also in this cellular model. The HaCaT cells are spontaneously immortalized and are characterized by high differentiation degree, elevated mitotic activity and great keratinization of the plasmatic membrane; they are considered as an optimal system for studying the molecular events of neoplastic degeneration of skin cells and for analyzing effects of the ultraviolet radiation exposure [42, 57, 58]. The results obtained through the oxidative stress tests showed that UV increase of ROS production, depending on their wavelength [59]. In particular, UVB significantly increased ROS only at higher irradiation doses than that used for evaluating genotoxic damage Therefore we confirm the different mechanisms of genotoxic damage in these cells. Regarding genotoxicity, our data demonstrate that UVA are capable to induce a weakly DNA damage at the highest irradiation dose (24 J/cm^2) only. This result could be attributed to DNA repair systems that,
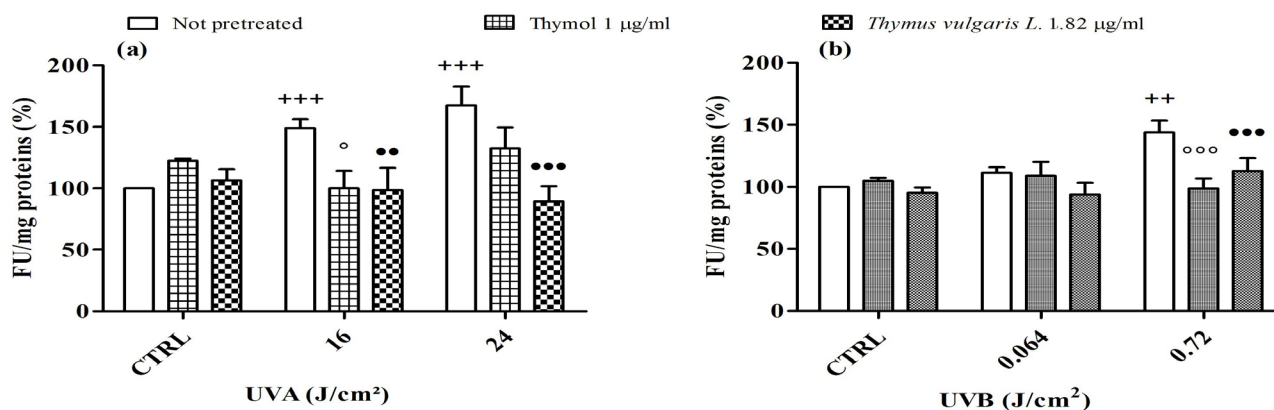
Figure 2. Protective effect of thymol (1 µg/ml) and *Thymus vulgaris* L extract (1.82 µg/ml) pretreatment (1 h) on ROS formation immediately after the end of (a) UVA (16-24 J/cm²), or (b) UVB (0.064-0.72 J/cm²) irradiation. Results are shown as fluorescence units (FU)/mg proteins (%) and are mean values of three independently produced experiments ± SD. $^{++}$P < 0.05, $^{+++}$P < 0.001 vs CTRL (One-Way ANOVA test, Dunnett's Multiple Comparison Test); $^{\circ}$P < 0.05, $^{\circ\circ\circ}$P < 0.001, $^{\cdot\cdot}$P < 0.01, $^{\cdot\cdot\cdot}$P < 0.001 vs the same treatment w/o thymol or Thymus vulgaris L extract (Two-Way ANOVA test, Bonferroni post-test).

24 h after the UVA exposure (necessary time to reveal genotoxic damage), could be able to remove efficiently DNA oxidative injuries.

Our hypothesis can be validated by the data obtained in different assays. Unlike what we had seen in another line of NCTC keratinocytes, which were shown much more sensitive to oxidative damage by UVA highlighting in addition to an effect of lipid peroxidation also important in DNA damage [60]. Gamma-H2AX histone test showed the presence of DSBs, at the highest dose of irradiation (24 J/cm^2). Carrying out the experiment 1 h after the end of irradiation, was possible to still detect DNA lesions

and to assume that, in this time, repair mechanisms did not reach full efficiency. Simultaneously, the cell cycle analysis (assessed 24 h after the end of irradiation) demonstrated the presence of a block in S phase of UVA irradiated cells. From this result, it is possible to suppose that the cells, damaged by ultraviolet rays, accumulate in this phase because of the DNA damage checkpoints and repair systems activation, which attempt to eliminate injuries and to advance cells in their proliferative cycle.

In addition, the alkaline comet assay with Endo III allowed conferring again to reparative mechanisms a fundamental role in the elimination of UVA genotoxic damage. In alkaline comet assay, the UVA rays genotoxicity was detected only in small percentage; instead, the treatment of irradiated samples with Endo III (which has the ability to identify DNA oxidized bases, to cut DNA strand at the lesion and to prevent, as a consequence, the repair), showed a significant increase of damage in genetic material. The comparison between the two variants of comet assay gives further confirmation about the UVA oxidizing power and allows highlighting the efficiency of HaCaT repair enzymes [61]. In fact, they are able, during 24 h, to restore the stability of DNA.

On the other hand, UVB rays demonstrated a major genotoxic potential than UVA in all genotoxicity assays. Probably, this is due to the capability of UVB to damage directly the DNA double helix, thanks to its wavelength. When DNA injuries are too extensive to be repaired, the cells put to use the programmed cell death process, in order to prevent the fixation of DNA mutations, which may lead to the carcinogenesis. The event of apoptosis was evaluated through the Annexin V test. UVA irradiated samples did not show positivity to the assay; also in this case, we could suppose that UVA-induced DNA damage is efficiently repaired by endogenous protective systems. Furthermore, a study conducted by He *et al* [58] reports that a chronical and repeated exposition of cutaneous cells to this UV component can cause resistance from apoptosis. UVB rays, instead, revealed a high apoptotic activity, leading to a significant increase in the apoptotic
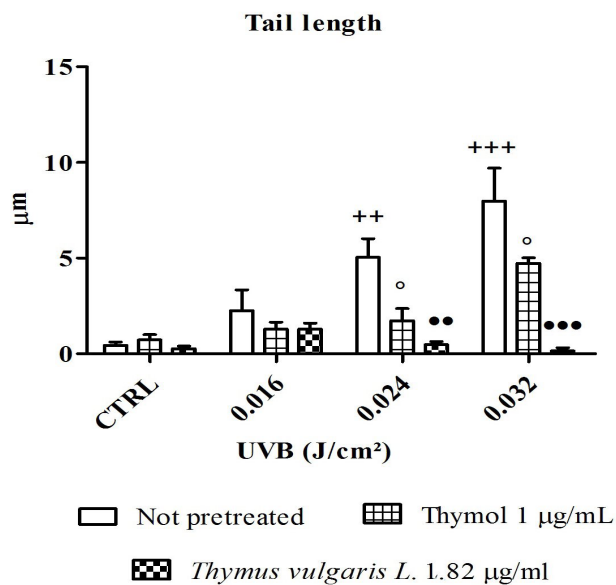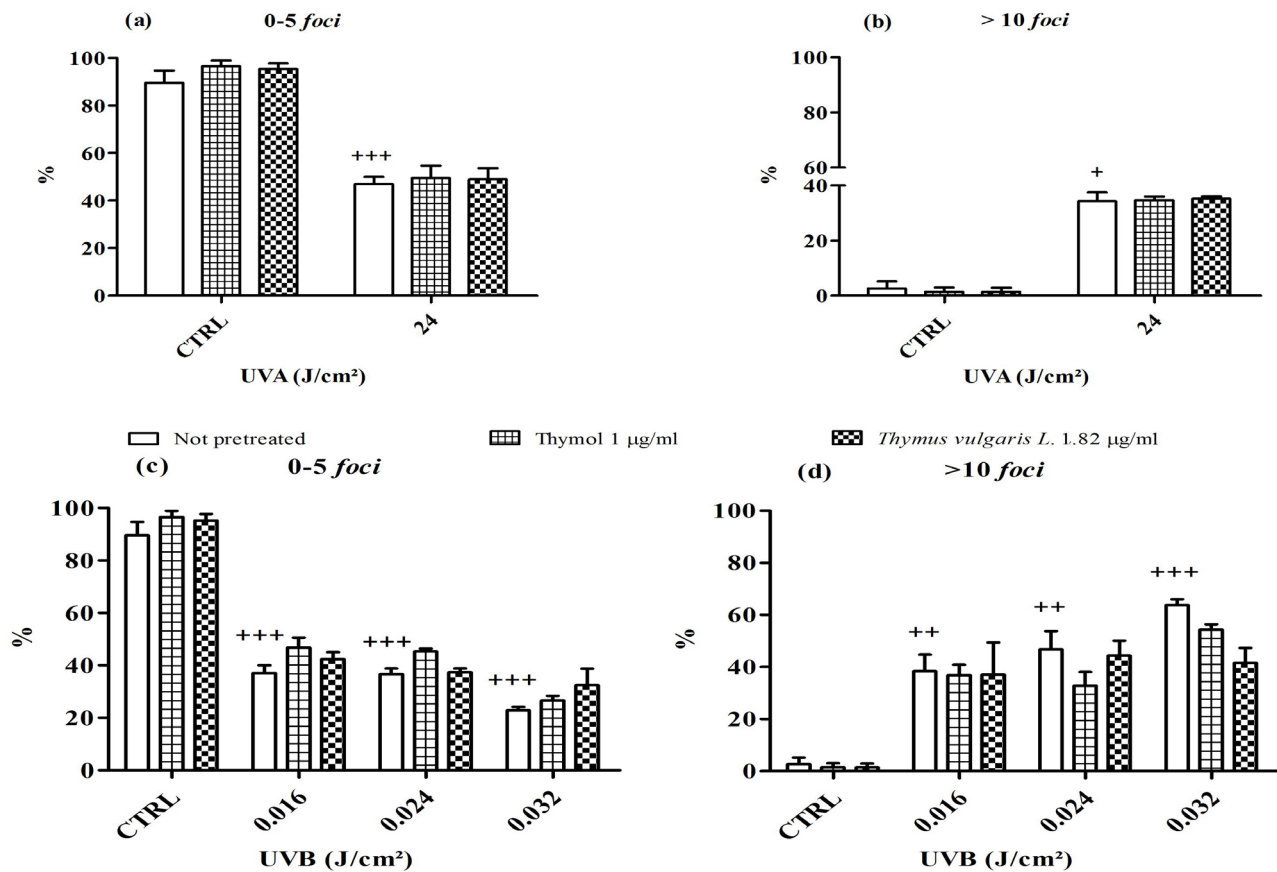

Figure 3. Protective effect of thymol (1 µg/ml) and *Thymus vulgaris* L extract (1.82 µg/ml) pretreatment (1 h) on UVB (0.016-0.032 J/cm²)-induced DNA damage. Genotoxicity was assessed by alkaline comet assay (pH ≥ 13) 24 h after the end of irradiation. DNA damage is reported as tail length of the cells, and results are mean of medians ± SD of three independent experiments. ++P < 0.01, +++P < 0.001 vs CTRL (One-Way ANOVA test, Dunnett's Multiple Comparison Test); °P < 0.05, °°P < 0.01, °°°P < 0.001 vs the same treatment w/o thymol or *Thymus vulgaris* L extract (Two-Way ANOVA test, Bonferroni post-test).
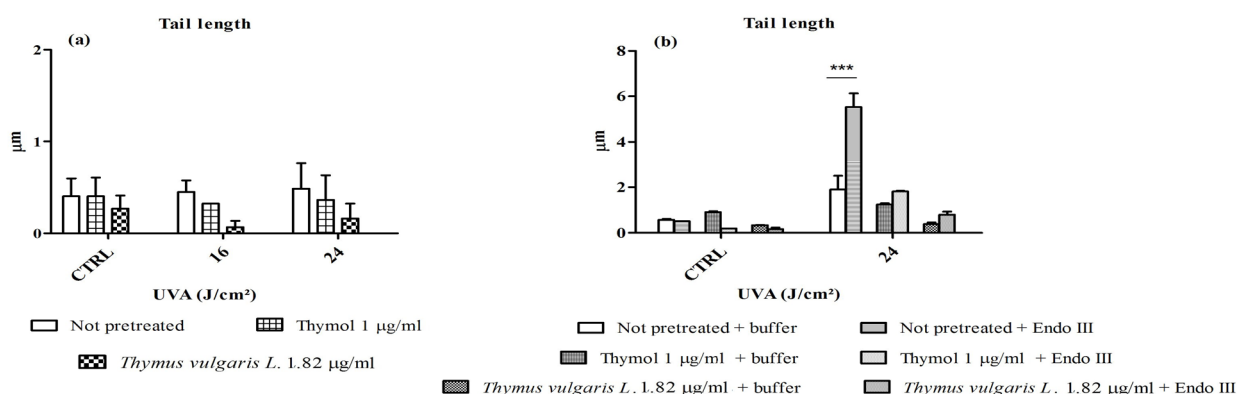
Figure 4. (a) Protective effect of thymol (1 µg/ml) and *Thymus vulgaris* L extract (1.82 µg/ml) pretreatment (1 h) on UVA (16-24 J/cm²)-induced DNA damage. Genotoxicity was assessed by alkaline single-cell gel electrophoresis (pH ≥ 13) 24 h after the end of irradiation. DNA damage is reported as tail length of the cells, and results are mean of medians ± SD of three independent experiments. (b) Evaluation of genotoxic damage and protective effect of thymol (1 µg/ml) and *Thymus vulgaris* L extract (1.82 µg/ml) pretreatment (1 h) in cells exposed to UVA (24 J/cm²) and incubated w/o Endonuclease III (1 µg/ml, 30 min). Genotoxicity was assessed by alkaline single-cell gel electrophoresis (pH ≥ 13) with Endo III 24 h after the end of irradiation. Results are reported as the tail length of the cells and are expressed as a comparison between the tail length of the cells treated or no with Endo III. Data are elaborated with Two-Way ANOVA test, Bonferroni post-test (***P < 0.001)

Figure 5. Protective effect of thymol (1 μg/ml) and *Thymus vulgaris* L extract (1.82 μg/ml) pretreatment (1 h) on UVA (24 J/cm²) or UVB (0.016-0.032 J/cm²)-induced double strand breaks formation. Presence of DSBs was evaluated with immunostaining of γ-H2AX histone protein 1 h after the end of irradiation. Results are expressed as percentage of cells with 0-5 and more than 10 foci (%), and are means ± SD of three independent experiments. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs CTRL (One-Way ANOVA test, Dunnett's Multiple Comparison Test).

cells percentage. This UVB property was also supported by the results obtained with the cell cycle analysis, which shown a dose-dependent enhancement of cells in Sub-G_1 phase.

Another important aspect of our study, in addition to the characterization of UV damage, was the evaluation of protective effects of two natural compounds. To date, the healthy properties of these substances (such as antioxidant, anti-inflammatory and chemopreventive activities) are well known [6]; these features, combined with the ability of natural molecules to exert the same beneficial effects of synthetic compounds (causing minor toxicity in a chronic treatment), motivate the increasing interest from many authors [35].

Among the natural substances, polyphenols are one of the most studied classes. They originate from the secondary metabolism of plants and are found in fruit, vegetables and cereals. Their protective effect against UV is given by the capability to directly absorb the rays, act as a 'scavenger' of free radicals, inhibit inflammation, induce endogenous antioxidant enzymes activity, and enhance DNA repair systems [62].

The natural compounds tested in our project research were a dry extract of *Thymus vulgaris* L and its main components, thymol. The pretreatment of our experimental model with the two substances allowed to confirm, in the evaluation test of intracellular oxidation

state, the antioxidant action conferred to them. They were both able to reduce the amount of oxygen free radicals in the irradiated cells, but the most protective effect is attributed to the *Thymus vulgaris* L extract, probably for a possible synergy between thymol and the various components of the extract (*e.g.* carvacrol). Furthermore, thymol and extract of *Thymus vulgaris* L were able to reduce genotoxic damage. This property could be derived from the capability of polyphenolic compounds to stimulate DNA repair mechanisms (and, especially, NER system), responsible for the elimination of specific UV-induced lesions (CPDS and 6-4 PPs) [5]. At least, regarding the apoptotic process, both of the compounds act by reducing the cells percentage that are direct to the programmed death. This behavior was observed also in the cell cycle analysis, in which thymol and thyme extract reduce the cells in Sub-G_1 phase.

In conclusion, seeing the good protective capacity of polyphenolic compounds used in our study, it is possible to affirm that the results obtained could be the basis for future evaluations. It would be interesting to analyze the mechanism underlying the protective effect of thymol (as single compound) and *Thymus vulgaris* L extract (such as complex mixture) against the genotoxic damage induced by UV radiation and, in addition, to examine in depth their bioavailability, for using them as photo-protective compounds in food supplements or sunscreen.

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