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Sublethal UVB Induces DNA Lesions and Pro-Apoptotic Gene Transcription in Human Keratinocytes: Attenuation by a Mixture of Plant Extracts

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Abstract Ultraviolet B (UVB) radiation is a potent environmental factor in skin pathogenesis as it modulates oxidative burst, gene expression and proliferation/apoptosis of human normal keratinocytes. In the absence of cell apoptosis, sublethal UVB is shown to induce significant increase of mitochondrial and nuclear DNA lesions as quantified by increased apurinic/apyrimidinic sites together with transcriptomic modulation of pro-apoptosis/survival genes. These effects may be attenuated by cell pretreatment with extracts from three various plants: *Papaver rhoeas, Passiflora incarnata and Anchusa arvensis* or their mixture. Extracts variably decreased UVB-induced mitochondrial and nuclear DNA lesions in normal human keratincoytes. When mixed together, higher reduction of DNA lesions has been observed at lower extracts' concentrations, compared to each extract alone which suggested their synergy. Furthermore, sublethal UVB-induced transcriptomic modulation was significantly prevented by cell preincubation with extract mixture through coordinated reduction of pro-apoptotic gene transcription while increasing pro-survival related transcripts. Finally, extract mixture protected keratinocytes against lethal UVB dose. This work reveals sublethal UVB-mediated lesions at both DNA and RNA levels in human keratinocytes and the ability of appropriate plant-derived sunscreen to reduce cell injury.

Keywords: *UVB, plant extract, keratinocyte, photoprotection, apoptosis*

1. Introduction

Steadily increasing incidence of skin cancers, the most common malignancies occurring in Caucasian populations, has brought much attention to the process by which these tumors develop and how they can be prevented [1]. Skin is constantly exposed to ultraviolet (UV) radiation, which lead to the production of reactive oxygen species (ROS), transcriptional modifications, and the acceleration of cutaneous photoaging [2,3]. Solar UV radiation comprises approximately 1-10% UVB and 90-98% UVA. UVB is largely absorbed in the protective ozone layer but it is experimentally more genotoxic and about 1000 times more capable of causing sunburn than UVA [1]. Indeed, increases in the more hazardous band 280 to 315nm (UVB) of the solar UVR spectrum at the Earth's surface result from the decrease in stratospheric ozone levels. UVB modulates multiple intracellular targets that acting together to determine whether the UVB damaged cell will survive, proliferate or die [2]. UVB directly activates pro-apoptotic cell surface receptors such as CD95/Fas by inducing receptor clustering, without a need for specific ligands [4]. Correlation of DNA damage severity and reduction of apoptosis by enhancement of DNA repair enzymes [5] substantiates the idea that UVB-induced DNA damage activates the apoptotic pathway including mitochondrial compartment [6].

To counter UVB effects, keratinocytes have multiple protective strategies such as inflammation, antioxidants enzymes, increased expression of pro-survival genes and DNA repair [2]. Potential deleterious effects on health of UVB lead for searching effective strategies that increase skin's own protective mechanisms. Application of natural substances has been extensively investigated as therapeutic/prevention approach [7]. Preliminary analysis of multiple plant extracts led us to investigate, in this study, the protective potential of extracts from *Papaver rhoeas* petals (PR) [8] and *Passiflora incarnata* (PI) petals [9] and *Anchusa arvensis* (AA) [10] on sublethal UVB-irradiated human normal keratinocytes. PR and PI petals have already been analysed for their chemical content [8], [9] and shown to display similar bioactivities including anti-oxydant properties [11,12]. PR L. (Papaveraceae) is an annual herb indigenous to Iran and many other regions with wide range use as anti-inflammatory, anti-diarretic, mild sedative effect [13] and the reduction of opioid addiction [14].

In present work, we first attested the innocuity of various extracts on human keratinocytes before adding them to sublethal UVB-irradiated human keratinocytes at

different doses, separately or together. Cells were then analysed for the levels of mitochondrial/nuclear DNA lesions and the transcription of various apoptosis/survival genes.

2. Materials and Methodes

2.1. Extracts

Papaver rhoeas (PR, poppy) extract was obtained from petals, rinsed, soaked into cold water (pH 4.6/6.6), before being dried, pulverized, and put in maceration in water during 48h at a rate of 100g per liter. The aqueous part is settled, the filter cake is expressed, the product obtained is faded with coal, and adequate conservative is added and adjusted diluted in water. AA et PI extracts were prepared from flowering tops, dried, pulverized, and percolated at a rate of 100g of plant for 500ml water; the extract accounts for approximately 5% of the plant. Filter cake is expressed, liquors put together and diluted at 100g per liter of water. The concentrations were then adjusted to 10g per litre (10mg/mL) final dilution and, when added to cell cultures, concentrations indicated the weight of dry extract per mL.

2.2. Cells and Treatments

Peripheral blood was obtained from blood transfusion leukocyte filters from healthy volunteers with their informed consent. The study was approved and strictly followed the ethics guidelines of Medical Ethical Committees at the Bordeaux Segalen University, Bordeaux, France, and conducted under full compliance with government policies and the Helsinki Declaration. Peripheral blood-derived mononuclear leukocytes (PBL) were isolated on Ficoll gradient and lymphocytes were induced by 10^{-6} M phytohemagglutinin-P (PHA) (5µg/mL; Murex Biotech Ltd, Dartford, UK) exactly as mentioned elsewhere [15]. Keratinocytes were isolated from normal human skin in patients undergoing plastic surgery, grown in MCDB153 medium, and irradiated at a dose of 60 or 100mJ/cm^{-2} doses using a Biotronic device (Vilber Lourmat, Marne la Vallée, France), equipped with a dosimeter, in which the UVB lamp emitted a continuous spectrum between 280 and 380nm (major peak at 312nm) [16]. In parallel, non-irradiated cells were treated similarly and kept in the dark in a cell incubator. Cultures were also incubated with various extracts during 3 hours, washed and subsequently irradiated.

2.3. Cell viability and DNA Analysis

Cell viability and proliferation were analyzed using a methyl thiazol tetrazolium (MTT) assay. The colorimetric assay is based on the ability of live cells to reduce the yellow MTT reagent (Sigma, St Louis, MO, USA) to a purple formazan product. Nuclear and mitochondrial (mtcDNA) DNA lesions were analysed by Apurinic/apyrimidinic (AP) site quantification. AP sites are one of the major DNA lesions formed during the course of base excision and repair of oxidized or alkylated bases. The level of AP sites is a good indicator of DNA damage and the initiation of apoptosis/transformation [17]. Nuclear (Wizard® Genomic DNA Purification Kit, Promega, Charbonnières les Bains, France) or Mitochondrial DNA (mtDNA, Mitochondrial DNA isolation kit; Biovision, Mountain view, CA) were isolated and quantified (Molecular Probes, Life technologies, St Aubin, France). For each sample, 0.5 µg DNA was used in triplicates to determine the density of AP sites. To detect cell apoptosis/necrosis, externalization of inner membrane phosphatidylserine and plasma membrane permeability were investigated by flow cytometry using a fluorescein-conjugated annexin V and propidium iodide kit (Immunotech, Marseille, France).

2.4. Transcriptomic Analysis

For transcriptomic quantification, total RNA was extracted from cultures using RNeasy kit (Qiagen, Hilden, Germany) and analysis were performed using real-time reverse transcriptase-polymerase chain reaction (PCR) to detect mRNA species encoding apoptosis/necrosis factors (PAHS-141 array, SuperArray, Frederick, USA). Synthesis, purification and hybridization of biotin-labeled cDNA (6 µg) addition to array plates were performed according to manufacturer's recommendations. After local background subtraction, average signal intensity from duplicate wells was compared to values obtained for housekeeping genes. For each gene, modulation was defined as the relative expression value for treated compared to control sample.

2.5. Statistical Analysis

Comparisons were assessed using Fischer's exact test for proportions and Mann-Whitney-U test for quantitative values. Results were analyzed and compared using the Student *t*-test for paired data. $P < 0.05$ was considered to be significant.

3. Results

3.1. Extracts Did Not Affect Normal Human Cell Growth and Survival

Prior to evaluate activity, *Papaver rhoeas*, *Passiflora incarnate*, *Anchusa arvensis* or their mixture have been tested for their effects on the survival and the proliferation of human lymphocytes [15] and keratinocytes. We did not observe any variation of cell numbers (direct counting) or viability (MTT) of inactivated (< 4.5%, data not shown) or mitogen-induced normal human PBLs (non significant variations in two distinct donors, $p > 0.09$, Figure 1 Upper panel) cultured with various extracts' dilutions. Furthermore, no significant decrease in HNKC viability (Figure 1, central panel) was observed following their incubation with extracts corroborating the absence of apoptosis (Figure 1, Lower panel). These results confirmed the absence of direct toxic or proliferative effects of above extracts on normal human cell growth *in vitro*.

3.2. Various Plant Extracts Decreased UVB-Induced DNA Lesions in Human Keratinocytes

Keratinocytes were irradiated with sublethal doses of UVB 60 (mJ/cm^{-2}). This dose did not induce cell apoptosis

(see below) but caused DNA lesions as quantified by the number of apurinic/apyrimidinic (AP) sites. UVB increased the number of AP sites at both mitochondrial (from 0.65±0.09 to 1.34±0.22/10^5 base pairs) and nuclear (from 0.45±0.10 to 1.12±0.14/10^5 base pairs) levels. Addition of various extracts causes variable but significant reduction of ($p < 0.05$) of DNA lesions as optimal reduction was observed with cells treated with AA at 80 µg/ml (-20%), PI at 300 µg/ml (-32%) or PR at 170 µg/ml (-30%) (Figure 2). Of Interest, the mixture of these extracts showed significantly higher inhibition of UVB-induced DNA damage (-83±9 for nuclear and -56±10% for mtcDNA) compared to the optimal values found with each extract dilution alone ($p < 0.002$). Optimal effect was observed with cells incubated with 26 µg AA, 100 µg PI and 170 µg PR (Figure 2). Addition of only 1/10 of above extracts showed significantly higher reduction of DNA lesions compared to optimal reduction observed with each extracts alone. Accordingly, addition of the effects of 3, 10 and 6 µg/ml of AA, PI and PR respectively did not bypass 7% reduction of DNA lesions. This clearly indicate that these extracts synergizes in inhibition of DNA lesions when mixed at low dilutions ($p < 0.0004$).

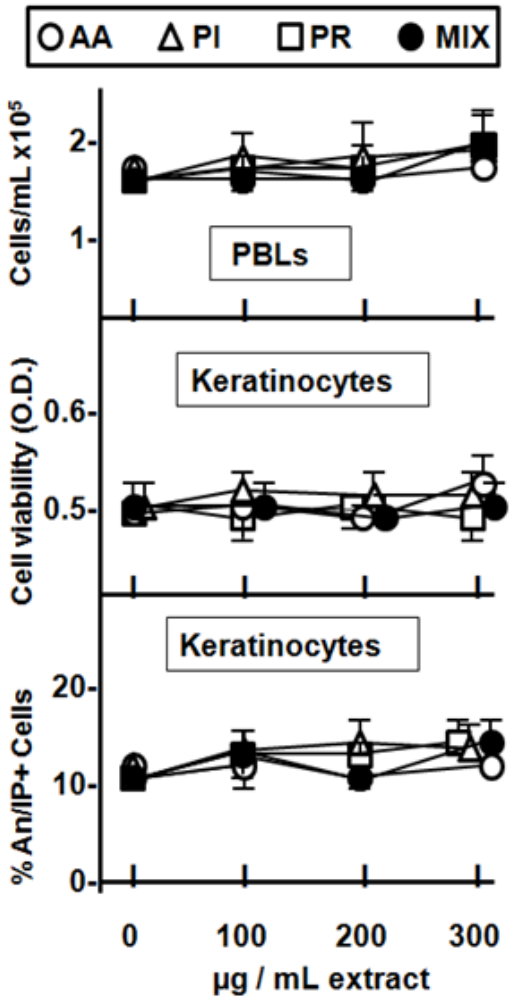

Figure 1. Absence of direct toxicity by various plant extracts or their mixture. Extracts added to PHA-treated peripheral blood leukocytes (upper panel) or keratinocyte cultures. Following 3 day incubation, the number of leukocytes (upper panel), cell viability (MTT test, central panel) and apoptosis/necrosis (lower panel) of keratinocytes were quantified. Mean±SD from two distinct donors, each done in triplicate

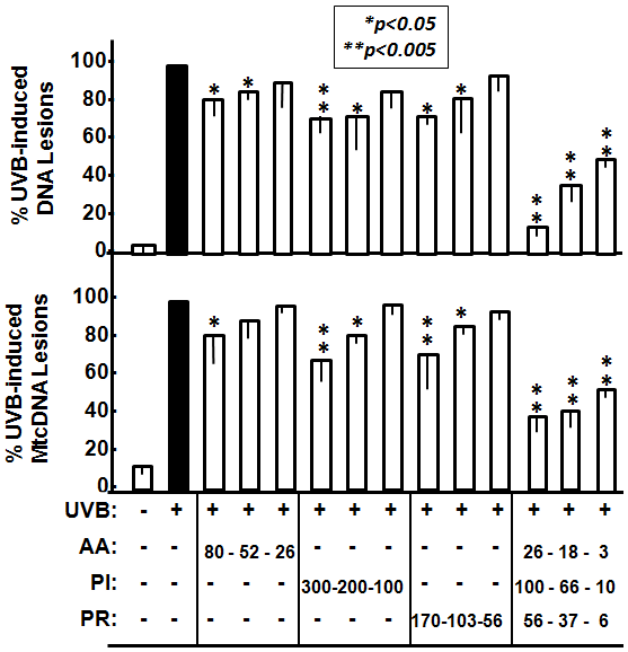

Figure 2. Plant extracts decreased nuclear and mitochondrial DNA lesions in UVB-irradiated keratinocytes. HNKC were first pre-treated during 3h with AA, PI, PR or MIX (values shown as µg/mL) before irradiation with UVB (60mJ cm^{-2}). AP sites lesions of nuclear (upper panel) and mtDNA (lower panel) were quantified 24h later and results are shown as the percentage of lesions, compared to untreated UVB-irradiated cells (black column). *p* values compared data from treated to untreated irradiated HNKC. Data show mean from 3 ± SD from distinct HNKC preparations

3.3. Attenuation of UVB-Mediated Modulation of Gene Transcription by Various Plant Extracts

To enforce the protective effect of various extracts and their mixture on UVB-induced modulation of keratinocytes, we have selected another approach to analyse cell modifications by measuring transcriptomic modulation of 84 pro-apoptosis/survival genes (RT-PCR array SuperArray, Frederick, USA) in sublethal UVB-irradiated cells pretreated with/without various extracts or MIX. As expected, UVB clearly modulated gene transcription in HNKC as 14 distinct mRNAs were significantly increased or inhibited (> 2 folds) when compared to non irradiated keratinocytes ($p < 0.0001$)(Table 1, first column). UVB increased the expression of genes encoding transformation/pro-apoptotic proteins including Bcl-2-associated-X (BAX) protein [18], FAS [4], tumor necrosis factor (TNF), TNFRSF16, CASP8 [19], RAB25 [20], cystein proteases CPAN2 and CPAN5 [21], and receptor-interacting serine-threonine kinases RIPK2 [22] (Table 1). In contrast, significant inhibition of the levels of pro-survival/anti-apoptotic NFκB1 and CYLD [23] mRNAs was observed. Finally, UVB highly increased the expression of PPIA [24], MAG [25] and ATP6V1G2 [26] genes with unknown role in keratinocytes (Table 1). Together, these data confirmed deleterious effect of sublethal UVB on keratinocytes at transcription levels. The effect of preincubation with plant extracts on UVB-induced transcriptional modulations was then tested. Prior to these experiments, extracts or their mixture were added (100-300 µg/ml) to non-irradiated HNKC and low or no

transcriptional modulation was observed (<35% changes) except to 2 folds NFκB1 increase with >200µg/ml of PR. When used alone, extracts variably affected UVB-mediated gene modulation (Table 1) while addition of extract mixture significantly reduced UVB-induced transcriptional variations ($p < 0.0005$). As lowest concentration of mixture (Table 1) was used in this experiment, these data further support synergistic activity of extracts ($p < 0.001$). However, MIX did not modify UVB-mediated TNF or FAS mRNAs increase which further pointed to the specificity of extract activity (Table 1). MIX effects on other genes will be detailed under discussion.

3.4. Extract Mixture Protects Keratinocytes Against UVB-Induced Apoptosis

HNKC were irradiated with pro-apoptotic UVB dose (100mJ cm^{-2}) with/without pretreatment with extract mixture. As shown in Figure 3, in contrast to UVB60 (Figure 3, upper panel), UVB100 induced cell apoptosis and decreased cell viability as early as 24h incubation (Figure 3, lower panel). Cells treated with extract mixture significantly reduced the percentage of apoptotic, annexin+ cells ($p < 0.009$). These data clearly indicate that extracts inhibited the apoptosis/necrosis of human keratinocytes following high dose UVB irradiation.

Table 1. The ability of plant extracts or their mixture to reverse UVB-mediated modulation of mRNA levels in human keratinocytes

		Folds mRNA /Control[1]					
Unigene (Hs)	GENE	UVB	+ PR 170 µg	+AA 80 µg	+PI 300 µg	+ Mix*	Mix/UVB[2]
Pro-apoptotic							
624291	BAX: Bcl-2–associated X protein	2	1,4	1,3	1,05	1.27	-62%
244139	FAS : Fas , TNF-R superfamily	2	-	-	-	2	--
241570	TNF : Tumor necrosis factor	2	-	-	-	2	--
448588	TNFRSF16: TNF-R superfamily	3	2,5	3,1	2,2	1,5**	- 65%
558218	CASP8 : Caspase 8 associated protein 2	2	1,5*	1,8	1,8	1,1**	- 90%
632469	RAB25 : RAS oncogene family	7	10,6	6,9	4,6	4,5**	-230%
248153	CAPN5 : cysteine proteases	2	1,6*	1,7*	1,7*	1,3**	- 79%
350899	CAPN2 : cysteine proteases	2	1,5	2	1,4	-1,1***	- 276%
103755	RIPK2 : Receptor-interacting serine-threonine kinase 2	2	1,62	1,76	1,73	1,14**	- 57%
Survival/Anti-apoptotic							
654408	NFKB1 : Nuclear factor of kappa B-cells 1	-2,5	1,2*	-1,6	-1,1	1,6***	+ 436 %
578973	CYLD : Cylindromatosis	-6,3	-4,2*	-2,2**	-1,4**	1,2***	+ 643%
Unknown function in HNKC apoptosis							
249227	ATP6V1G2 : ATPase	6,4	9	7,5	8,6	4,3**	- 210%
356331	PPIA : Peptidylprolyl isomerase A, cyclophilin A	5	5	5	1,5***	2,2**	- 333%
643440	MAG : Myelin associated glycoprotein	5,8	5,8	5,8	2**	1,2***	- 450%

[1]Values compared to gene expression, as quantified by RT-PCR, in non-irradiated normal keratinocytes. Compared to UVB alone, best reversion values were sub-lined. *Mixture contained 6, 3 and 10 µg/ml of PR, AA and PI respectively.
[2]Last column shows 1% MIX-mediated modifications compared to irradiated cells (data first column).
*$p < 0.03$, **$p<0.002$, ***$p<0.0003$ compared to UVB column.

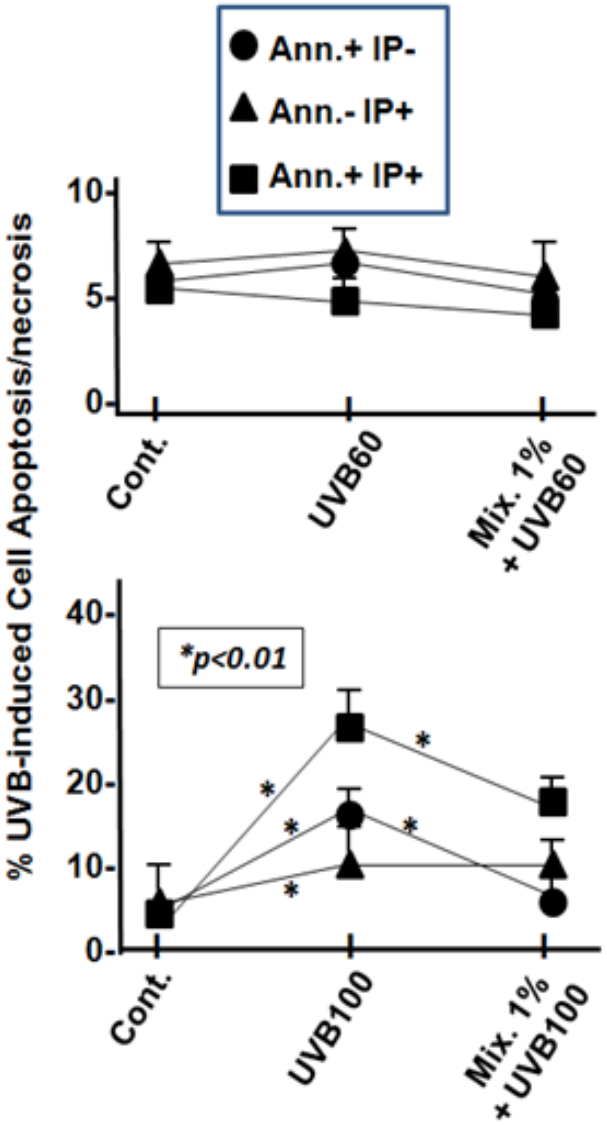

Figure 3. Plant mixture decreased HNKC apoptosis following exposure to apoptotic UVB dose. HNKC were first pre-treated during 3h with MIX before irradiation with UVB (60 or 100mJ cm^{-2}). HNKC were analysed 24h later for the percentage of pro-apoptotic (Annexine+, IP-), apoptotic (Annexine+, IP+), and necrotic (Annexine-, IP+). In contrast to UVB60, higher dose (UVB100) increased the percentage of apoptotic cells and cell death. Pretreatment with MIX significantly reversed cell apoptosis/necrosis at lower extract dilutions (3, 10 and 6 µg/ml of AA, PI and PR respectively). Data show mean±SD from 3 distinct HNKC preparations

4. Discussion

DNA may directly absorb UVB photons that cause their damage such as breakage/mutation and initiation of apoptosis [1,17]. Most of the DNA lesions caused by sublethal solar UV radiation are corrected by the nucleotide excision repair system [27] and, in the absence of appropriate DNA repair, permanent mutations may lead to cancer initiation [1]. UVB protection by application of natural substances has been extensively investigated as preventive approach [7]. Early reports detailed the modulation of ROS by various sunscreens because these radicals are directly increased by UVB, and known to initiate other intracellular modifications [1,2]. The present study clearly demonstrates that sublethal UVB induced DNA lesions at both nuclear and mitochondrial levels and modulated the expression of various apoptosis/cell repair genes in normal human keratinocytes. As sublethal doses do not eliminate irradiated cells, lesions may be deleterious and their accumulation initiates cell transformation [2]. By the use of a mixture of extracts from three distinct natural plants, we have shown their ability to significantly reduce UVB-mediated DNA lesions.

High repression of CYLD expression by UVB irradiation may inhibit pathways leading to the activation of NFκB [23], critical transcription factor for normal cell cycle progress and cytokinesis [19]. This corroborates NFκB1 gene inhibition in UVB-irradiated cells (Table 1). CYLD inhibition may also favors long term cell transformation as it has been shown to inhibit tumorigenesis/metastasis by blocking JNK/AP1 signaling [23]. Plant extracts reduced CYLD and NFκB1 gene repression with x6 and x4 increase respectively, compared to non-treated cells. MIX also inhibited most UVB-induced pro-apoptotic gene expression including BAX, TNFRSF16, CASP8, CAPN2 and CAPN5. MIX decreased the expression RIPK2, potent inducer of apoptosis through attenuation of NFκB pathway [22]. Furthermore, we found that other genes, namely PPIA [24], MAG [25] and ATP6V1G2 [26], were significantly increased following UVB irradiation and subsequently decreased by MIX, while the role of these genes in HNKC apoptosis or physiology is still to be elucidated. Meanwhile, MIX was unable to modulate UVB-induced TNFα or FAS gene expression which supported the specificity of MIX effects. Thus, significant down-regulation of UVB-mediated pro-apoptotic/pro-survival gene modulation may account for the reduction of DNA lesions.

The precise intracellular mechanisms at protein level of the protective activity of plant mixture, ROS modulation in particular, are under investigation. ROS production in UVB-irradiated skin and its contribution to cellular lesions have been well documented and supported by the protective effect of some anti-oxidants [3]. PR and PI have very similar traditional use for their anti-inflammatory and anti-addiction properties. The major compounds of PR extract are rhoeadine, rhoeadic acid, papaveric acid, rhoeagenine and anthocyanins [14,28]. PI flower extracts have been used to reduce anxiety and insomnia, confirmed in experimental models [9]. It was also helpful in the treatment of substance addictions as amphetamine, nicotine, marihuana and alcohol [9]. Phytochemical research carried out on PI petals had lead to the isolation of several bioactive metabolites such as luteolin, quercetin, chrysin, apigenin, homoorientin, vitexin, kaempferol, isovitexin, orientin, isoorientin, schaftoside, isoschaftoside, harman, harmol, harmine, harmalol, and harmaline [9]. A. arvensis, is a part of Boraginaceae family bears small blue tubular flowers which contain rosmarinic acid, known for its photoprotective property on human keratinocytes [11]. It also contains high levels of the ω-6 series EFAs that are particularly important in skin structure and function and authors reported its ability to inhibit atopic dermatitis [10]. Finally, Boraginaceae-derived alkannin was recently reported to protect human keratinocyte cell line against UVB-induced apoptosis, through the induction of protective HSP70 expression [29].

PI and PR contain various antioxidant anthocyanins and/or flavonoids such as luteolin and quercetin, known to inhibit cell accumulation of ROS and NO [30,31,32]. AA, may contribute to UVB-protection through rosmaric acid and akennin content, together with its various lipids, all reported to be photoprotective and/or anti-inflammatory for keratinocytes [10,11]. Interestingly, bioactivities found in above extracts were obtained with comparatively higher concentrations than those used in the present study within their mixture.

Finally, addition of plant extract mixture significantly decreased apoptotic cell number following irradiation with apoptotic UVB dose. Elimination of damaged cells allows prevention of transformed cell accumulation and accelerates tissue renewal while excessive apoptosis is harmful for human skin [1]. It is thus important to define the degree of lesions in irradiated cells to confirm the utility of sunscreen application in later case. Altogether, this study clearly showed that UVB-mediated DNA lesions and transcriptomic modulation may be limited by synergistic activity of a mixture of non-toxic extracts from three distinct plants.

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Conflict of Interest

The authors declare no conflict of interest.

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