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Protective Effects of *Pinus halepensis* Bark Extract and Nicotine on Cigarette Smoke-induced Oxidative Stress in Keratinocytes

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Abstract. *Background/Aim:* Cigarette smoke (CS) is a major environmental health threat. The oxidative stress induced by CS on keratinocytes and the possible protective effect of nicotine, its receptor inhibitors, and *Pinus halepensis* bark extract in relation to known antioxidants were investigated. *Materials and Methods:* Primary mouse keratinocytes were exposed to cigarette smoke in the presence and absence of *Pinus halepensis* bark extract (1 µg/ml), rutin (50 µM) and ascorbic acid (250 µM), nicotine (1 µM) with or without mecamlamine (5 µM) and α-bungarotoxin (0.1 µM). Keratinocyte viability and oxidative stress were evaluated by MTT and fluorescence assays. *Results:* *Pinus halepensis* bark extract decreased the oxidative stress and increased the viability of keratinocytes, and moreover, these effects were more pronounced compared to the mixture of rutin and L-ascorbic acid. Nicotine significantly enhanced the viability potentiation of the beneficial effect induced by *Pinus halepensis* bark extract. Mecamlamine and α-bungarotoxin showed no specific effect. *Conclusion:* *Pinus halepensis* bark extract in combination with nicotine may successfully reverse skin damage induced by cigarette smoke.

Cigarette smoke (CS) is a global problem of our society and according to the World Health Organization “the tobacco epidemic is one of the biggest public health threats the world has ever faced, killing more than 8 million people a year” (1). The Lancet Commission on pollution and health characterized CS as more dangerous than AIDS, tuberculosis, and malaria (2). CS is associated with cardiovascular diseases, higher risk of skin diseases, such as squamous cell carcinoma and atopic dermatitis, and is connected with decreased wound healing rate (3-7). The skin is the most important target for the harmful effect of CS pollution. Both solid and gas products of CS contain aniline, *N*-nitrosodimethylamine, benzopyrenes, acrolein, formaldehyde, *N*-nitrosopyrrolidine that are considered to be toxic, causing serious oxidative damage (8). CS produces reactive oxygen and nitrogen species oxidizing important biomolecules such as DNA, proteins and lipids leading to tissue injury which is translated to enhanced inflammatory phenomena (9-14).

One of the main constituents of CS is nicotine; it is an agonist of acetylcholine and acts on the epidermis *via* the keratinocyte nicotinic acetylcholine receptors (nAChRs) (15). nAChRs are large (290 kDa) pentameric transmembrane complexes, which are ligand-gated ion channels (LGICs) permeable to Na, K and Ca ions (16, 17). Nicotine activity is doubtful as it has been shown to exert both toxic and beneficial effects; it has been reported as neurotoxic and neuroprotectant (18), toxic to muscles and lung epithelium (19, 20), beneficial in colitis (21), while its potential activity on mitochondria apparently requires further investigation (22). Concerning inflammatory and wound healing processes, the effect of nicotine is considered rather negative, while topical nAChR antagonists have been shown to improve these negative effects (18, 23, 24). Topically applied antioxidants like L-ascorbic acid and α-tocopherol, as well as rutin, correctly formulated, seem to protect skin against environmentally induced oxidative damage (25, 26).

This article is freely accessible online.

†This manuscript is dedicated to our beloved Professor Georgios Deliconstantinos, who passed away recently.

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Key Words: Oxidative stress, cigarette smoke, keratinocytes, *Pinus halepensis* bark extract, nicotine.

On the other hand, there are many published studies, where the researchers evaluate the antioxidant activity of extracts of various plant extracts. Among them, *Pinus halepensis*, also known as Aleppo pine, is a Mediterranean pine with a bark extract rich in phenolic antioxidants, such as procyanidins and phenolic acids, which appears to effectively protect against skin inflammation induced by X-rays and UV radiation, *in vivo* (27, 28). Moreover, *Pinus maritima* bark extract has been shown to protect skin against tobacco smoke and UV radiation (29-31).

The present study aimed to evaluate the antioxidant effect of *Pinus halepensis* bark extract in comparison to the known synthesized antioxidants, such as rutin and ascorbic acid, on the CS-induced oxidative damage on keratinocytes. In addition, the role of nicotine and its receptor antagonists, α -bungarotoxin and mecamylamine, in the CS-induced oxidative stress was also investigated. Since epidermal cells come into direct exposure with CS, it would be of great interest to examine possible mechanisms to prevent the induced damage.

Materials and Methods

Chemicals. The chemicals nicotine, hydrochloride mecamylamine, α -bungarotoxin (toxin, snake from *Bungarus multicinctus*), rutin hydrate and L-ascorbic acid (sodium salt), Dulbecco's phosphate buffered saline (DPBS) were purchased from Sigma-Aldrich (Darmstadt, Germany). All compounds were of analytical grade. Cetavlon (Cetrimide 20% w/v) was obtained from Cana Laboratories S.A. (Athens, Greece), accutase containing 0.5 mM EDTA from Chemicon International (Temecula, CA, USA), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (St Louis, MO, USA). The dispase (1.12 units/mg), the keratinocyte serum-free medium (SFM), bovine pituitary extract, recombinant epidermal growth factor (rEGF) and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) were purchased from Gibco-Life Technologies (ThermoFisher Scientific, USA).

CS environment. The cigarette smoking environment was simulated by using the smoking apparatus from CH8152, type MCF (Ismatec SA, Glattbrugg, Switzerland). The oxidative stress was measured by using a Fluostar Galaxy spectrometer, fluorescence and chemiluminescence meter, BMG Labtech, Germany.

***Pinus halepensis* bark extract preparation.** *Pinus halepensis* bark was collected from Kaisariani forest (Athens, Greece) and extracted as previously described (27). In brief the bark was powdered, extracted with distilled water, freeze dried and dissolved in keratinocyte SFM (1 μ g/ml).

Cell isolation and culture. Primary keratinocytes were obtained from neonatal (≤ 24 h old) SKH-HR1 and SKH-HR2 mice. Briefly, the excised skin was immersed in isopropanol 70% solution, rinsed with PBS, and dipped into PBS solution containing gentamycin (7 μ g/ml; Schering-Plough Ltd, New Jersey, USA). Subcutaneous adipose tissue and skin main vessels were carefully removed and

skin was immersed in a Petri dish containing dispase-PBS (0.2 U/ml). Skin was cut in small pieces and incubated under stirring (250 rpm) for at least 1 h; then, the mixture was centrifuged at 1000 rpm, the dispase solution was discarded and the remaining cells were grown in keratinocyte SFM with low calcium chloride concentration (0.045 mM), containing 20-30 μ g/ml bovine pituitary extract, and 0.1-0.2 ng/ml rEGF. Cells were maintained in a humidified atmosphere of 5% CO₂, at 37°C. Tissue culture treated flasks (75 cm²), centrifuge tubes (50 ml) and cell culture dishes (60 mm \times 15 mm style) were obtained from Corning Incorporated (Corning, NY, USA).

Keratinocytes were sub-cultured using accutase (70% v/v in DPBS). At 70-80% confluence, cells were seeded into 96-well culture treated plates at a density of 35 \times 10³ cells/well, for further treatment and exposure to CS.

Exposure to CS. The keratinocyte SFM was exposed to CS in the smoking apparatus connected to a custom-made chamber (Figure 1). The device was adjusted to produce a combination of sidestream smoke (89%) and mainstream smoke (11%) as previously described (30). Briefly, CS was generated from ASSOS Papastratos brand cigarettes (ASSOS Papastratos, Xanthi, Greece; 10 mg tar, 0.7 mg nicotine, and 6 mg CO per cigarette). The above experiments were performed by following the standard Federal Trade Commission conditions (puff volume: 35 ml, puff duration: 3 s, puff frequency: 1/min). The keratinocyte SFM was exposed to the smoke of 8 cigarettes for 45 min; the resulting medium was then diluted in a ratio 1:5 in order to be used on cells as CS-conditioned medium. Undiluted CS-conditioned medium was highly toxic, leading mouse keratinocytes immediately to necrosis; thus, the dilution 1:5 was selected as the lowest effective dose was used (based on data about keratinocyte viability and induced oxidative stress in relation to exposure time; data not shown).

Cell treatment. In order to assess the effects of CS on keratinocyte viability and oxidative stress, keratinocyte cells were incubated in CS-conditioned media for 0.5 h, 1 h, 2 h and 3 h. Furthermore, aiming to examine the potential protective effects of several compounds on keratinocytes, cells were pre-incubated for 24 h with keratinocyte SFM containing one of the following: i) nAChR inhibitors (α -bungarotoxin 0.1 μ M and hydrochloride mecamylamine 5 μ M), ii) ascorbic acid (250 μ M) and rutin hydrate (50 μ M), iii) *Pinus halepensis* bark extract (1 μ g/ml); this concentration was selected as the lowest effective dosage (data not shown) iv) nicotine 1 μ M, v) mixture of ascorbic acid (250 μ M) with rutin hydrate (50 μ M) and nAChR inhibitors (α -bungarotoxin 0.1 μ M and hydrochloride mecamylamine 5 μ M), vi) *Pinus halepensis* bark extract (1 μ g/ml) and nAChR inhibitors, vii) nicotine 1 μ M and nAChR inhibitors (α -bungarotoxin 0.1 μ M and hydrochloride mecamylamine 5 μ M), viii) nicotine 1 μ M with ascorbic acid (250 μ M) and rutin hydrate (50 μ M), ix) nicotine 1 μ M and *Pinus halepensis* bark extract (1 μ g/ml), x) nicotine 1 μ M with ascorbic acid (250 μ M), rutin hydrate (50 μ M) and nAChR inhibitors (α -bungarotoxin 0.1 μ M and hydrochloride mecamylamine 5 μ M), xi) nicotine 1 μ M with *Pinus halepensis* bark extract (1 μ g/ml) and nAChR inhibitors (α -bungarotoxin 0.1 μ M and hydrochloride mecamylamine 5 μ M). Then, each group was incubated with CS-conditioned media containing the respective compound, for an extra period of 24 h. After 48 h of total incubation of keratinocytes the viability was estimated for each experimental condition by performing the MTT assay. Cells incubated with keratinocyte SFM served as control.

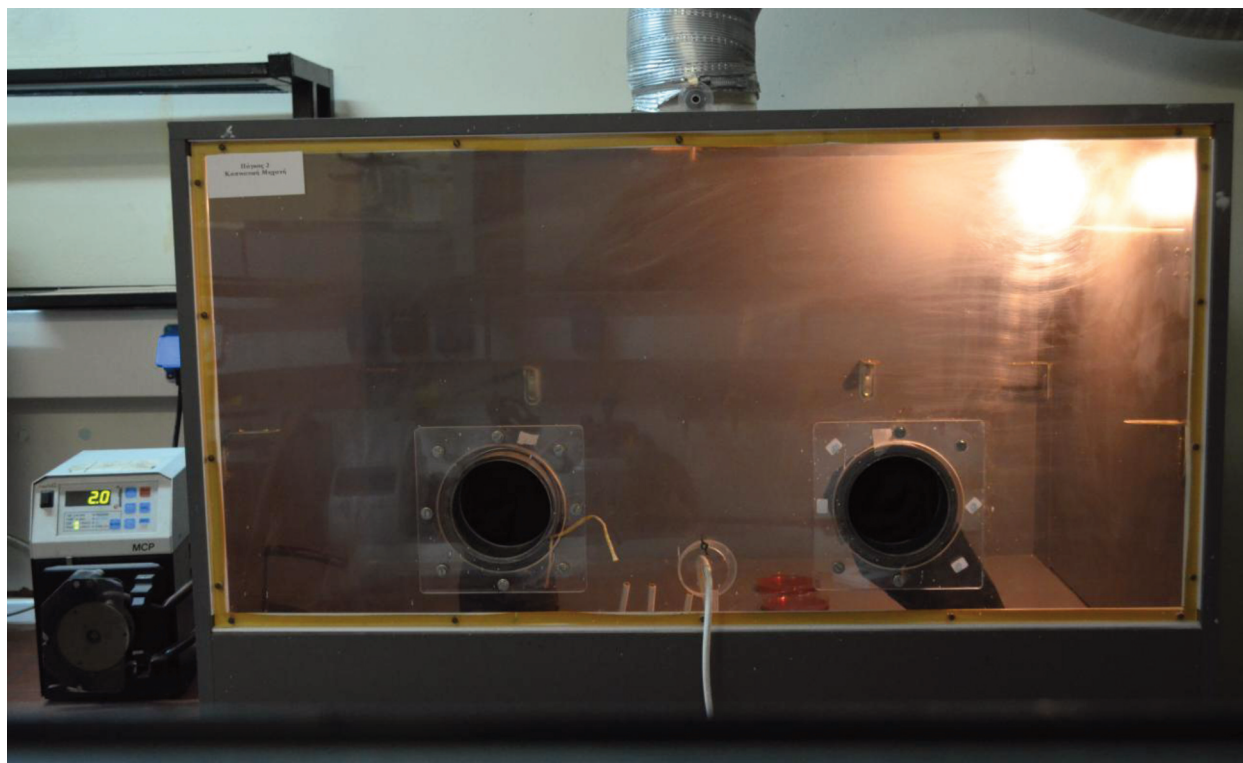


Figure 1. Plexiglass chamber connected to smoking device, which was used for the exposure of keratinocyte media to cigarette smoke.

Furthermore, keratinocyte cultures were incubated with CS-conditioned media with or without *Pinus halepensis* bark extract (1 $\mu\text{g/ml}$), for 1h, 2 h and 3 h in order to examine its effects on keratinocyte viability and oxidative stress.

Detection of reactive oxygen species (ROS) formation. Oxidative stress was evaluated on keratinocytes using chloro-methyl derivative of dichlorodihydrofluorescein (CM-H₂DCFDA). For this purpose, keratinocytes were cultured under the appropriate experimental condition (as mentioned above), in 96-well plates, for 4 and 24 h. After the treatment, the cells were washed and 200 μl of DPBS buffer was added in each well where 15 μl of a fluorescent solution containing 0.083 g/l CM-H₂DCFDA was added. The fluorescence of keratinocyte cells was monitored with the microplate reader using 485 nm excitation and 520 nm emission wavelengths. Fluorescence images obtained using a Canon PC1049, 16X (Tokyo, Japan) camera with Boligor (Tokyo, Japan) and Carl Zeiss 426126 and MC 80DX-1.0x (Oberkochen, Germany) for Axiovert 25 microscope Carl Zeiss (Oberkochen, Germany) adapters, with fluorescent light MBQ 52ac LEJ (Jena, Germany) emitter.

Cell viability assay. Cell viability was evaluated in keratinocytes after 48 h of CS-conditioned treatment using the MTT colorimetric assay. MTT diluted in DPBS was added to 100 μl of keratinocyte SFM at a final concentration of 0.5 mg/ml in each well of 96-well plates. After 3 h of incubation 150 μl of acidified isopropanol is added. The resulting absorbance of the formazan in each well was measured at 540 nm with the microplate reader.

Statistical analysis. The results were expressed as mean values \pm SD. All conditions (control and treated cells) were tested in five replications. Statistical differences were estimated using one-way analysis of variance (ANOVA with Bonferroni *post-hoc* test and were considered significant when $p < 0.05$).

Results

Our experiments showed that exposure of keratinocytes to CS-conditioned medium for 2 h significantly decreased the viability ($p < 0.001$) and increased the oxidative stress ($p < 0.05$), reaching maximum values after 2 h of exposure. Keratinocyte viability was significantly decreased ($p < 0.001$) after 3 h of exposure to CS-conditioned medium (Figure 2). Exposure of keratinocytes in CS-conditioned medium for 4 h significantly increased the oxidative stress ($p < 0.05$), while further exposure, up to 24 h, had no significant influence, most likely due to decrease of cell viability (Figure 3).

The protective effects of the antioxidants and/or nicotine and/or nAChR inhibitors on the viability of keratinocytes exposed to CS-conditioned medium are shown in Figure 4. According to the results, the presence of the antioxidants L-ascorbic acid and rutin, as well as nAChR inhibitors did not show any protective effect against the impact CS-conditioned medium on cell viability. On the contrary, *Pinus*

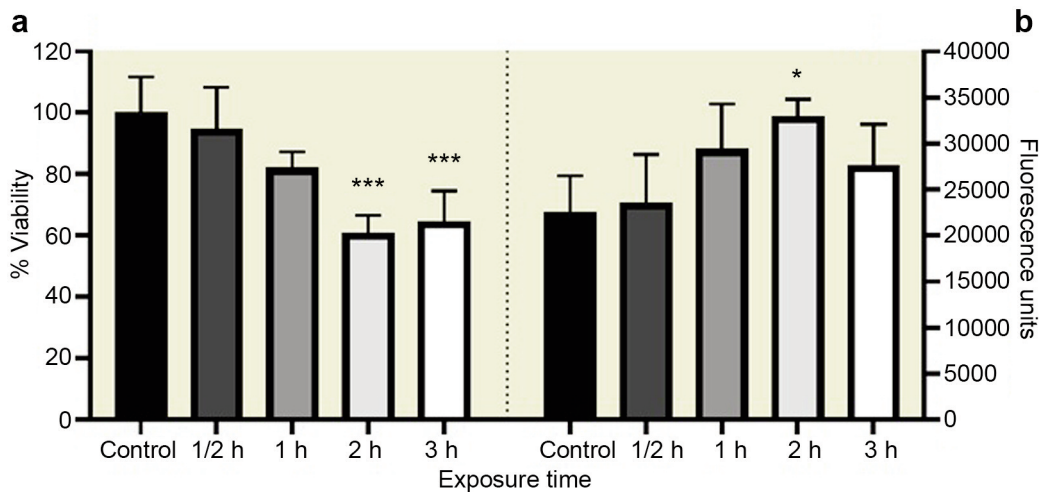


Figure 2. Kinetics of keratinocyte viability (a) and oxidative stress (b) after exposure to cigarette smoke-conditioned keratinocyte medium diluted 1:5. * $p < 0.05$; *** $p < 0.001$, compared to the control cells incubated with keratinocyte serum-free medium.

halepensis bark extract showed a significant protection of keratinocytes from CS-conditioned medium-induced damage, since it significantly increased their viability ($p < 0.05$, compared to cells treated only with CS-conditioned medium). Nicotine combined with *Pinus halepensis* bark extract in the presence or absence of nAChR inhibitors significantly protected keratinocytes from the CS-conditioned medium-induced damage ($p < 0.001$, compared to cells treated only with CS-conditioned medium). It is worth noting that this mixture of nicotine with *Pinus halepensis* bark extract enhanced keratinocyte viability even compared to non-exposed to CS-conditioned medium control cells ($p < 0.01$). It seems that nicotine could act synergistically even in the case of the non efficient by itself mixture of ascorbic acid, rutin and nAChR inhibitors ($p < 0.05$; Figure 4).

The protective effect of *Pinus halepensis* bark extract on keratinocyte viability and oxidative stress after 1, 2, and 3 h of exposure to CS-conditioned medium is presented in Figure 5. *Pinus halepensis* bark extract significantly protected the viability of keratinocytes; however, the data reached statistical significance only at 1 h of exposure ($p < 0.001$; Figure 5a). It is clearly shown that incubation of keratinocytes with CS-conditioned media with *Pinus halepensis* extract for 1, 2, and 3 h significantly reduced oxidative stress ($p < 0.001$), even the endogenous one ($p < 0.05$), as this is expressed by the non treated control keratinocytes (Figure 5b). Fluorescence images confirmed the ability of *Pinus halepensis* bark extract to significantly reduce oxidative stress produced by CS-conditioned medium in different time points, included the endogenous one (Figure 6).

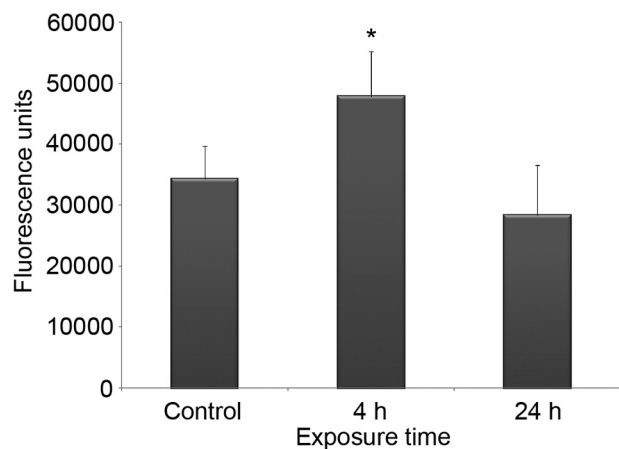


Figure 3. Oxidative stress in keratinocytes exposed to cigarette smoke-conditioned keratinocyte medium, diluted 1:5, for 4 h and 24 h. * $p < 0.05$, compared to the control cells incubated with keratinocyte serum-free medium.

Discussion

CS is well recognized as a strong oxidizing agent, producing substantial oxidative stress and inflammation mediators (8, 32). It exerts a strong carcinogenic effect (3), which is potentiated by UV radiation (7, 30, 33). In the present study, epidermal cells (keratinocytes) exposed even in a diluted medium concentration of CS (1:5), had increased oxidative stress and decreased viability. Relevant results for the effect of CS have been previously observed by using similar assays on HaCat keratinocytes and oral keratinocytes co-cultured

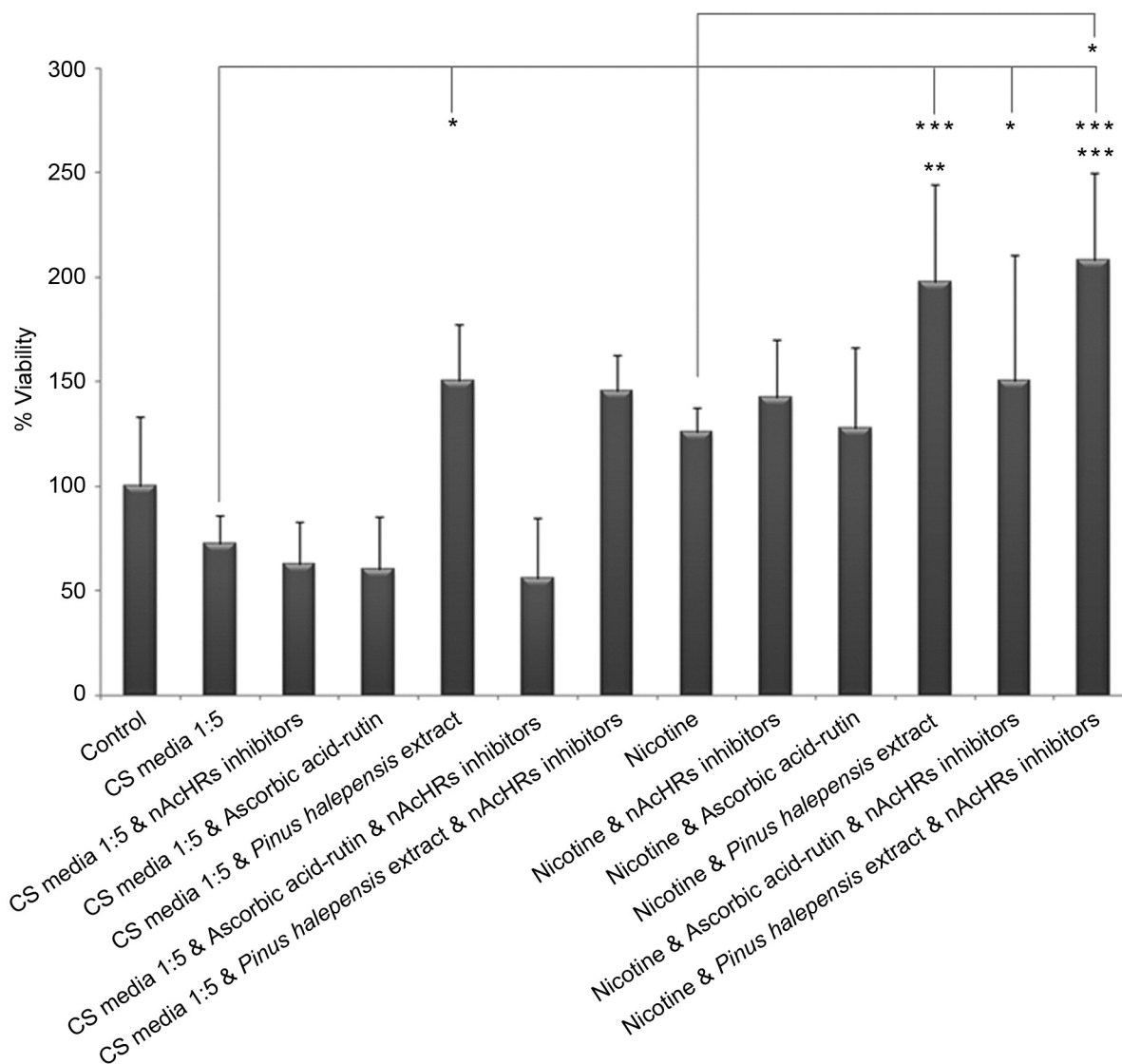


Figure 4. Keratinocyte viability (%) in the presence of cigarette smoke conditioned keratinocyte medium diluted 1:5, nicotine, mixture of mecamylamine and α -bungarotoxin (nAChRs inhibitors), mixture of ascorbic acid and rutin, *Pinus halepensis* bark extract and their mixtures either with nicotine or mecamylamine and α -bungarotoxin (nAChRs Inhibitors). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (compared to the CS-treated cells and the untreated control cells).

with fibroblast tissues (EpiOral or EpiGingival) (34-36). Particular efforts have been made to address the negative effects of CS either on reducing or even eliminating the oxidative stress. In this context, *Pinus halepensis* bark extract has been used, which showed significant antioxidant and anti-inflammatory properties upon tobacco smoke, UV or X-rays irradiation of mouse skin, *in vivo* (27-29, 37). Herein, our observations indicated that *Pinus halepensis* bark extract was able to protect primary mice keratinocytes from the effects of CS on both viability and oxidative stress, compared to other antioxidants or to the control (non treated)

group. These results are in accordance with previous *in vivo* studies, which demonstrated that topical administration of *Pinus halepensis* protected the skin of hairless mice from the damage induced by CS and UV radiation (27, 37). It is noteworthy that the antioxidant mixture of ascorbic acid (250 μ M) with rutin (50 μ M), which was used as reference, did not show any protection on keratinocytes (Figure 4), although the same concentrations as those described in literature were used (38-40). Moreover, known antioxidants such as resveratrol, and a furaldehyde isolated from the extract of the Japanese apricot which decrease lipid

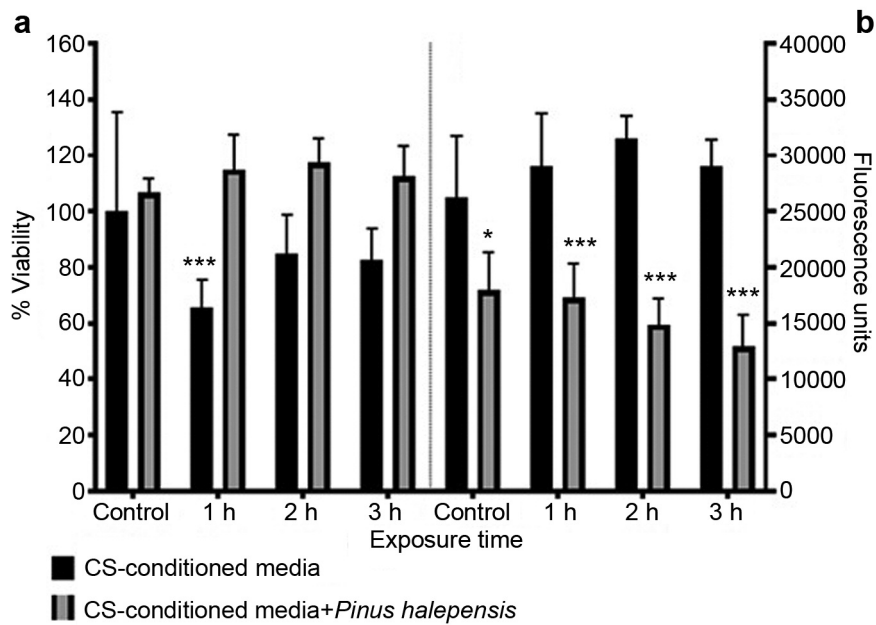


Figure 5. Protective effect of *Pinus halepensis* bark extract on the viability (a) and oxidative stress (b) of keratinocytes, exposed to cigarette smoke-conditioned media for 1, 2, or 3 h. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (compared to the cells treated only with CS-conditioned media, at each time point).

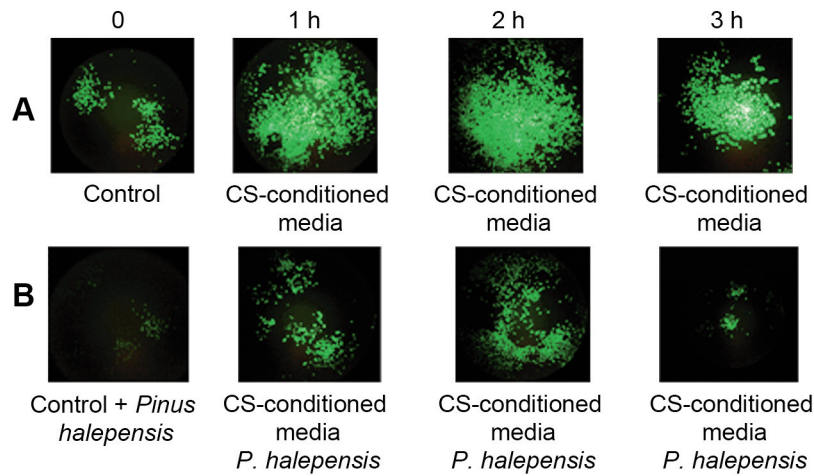


Figure 6. Fluorescence images of keratinocyte cultures before (0) and after exposure of 1, 2 and 3 h to CS-conditioned medium alone (A) or CS-conditioned medium plus *Pinus halepensis* bark extract (B). The green areas illustrate the oxidative stress induced by cigarette smoke.

peroxidation derived aldehydes and *N*-acetyl cysteine which attenuated the damage provided by CS have been shown to partially protect skin cells from the oxidative effects of CS (36, 41, 42). However, the antioxidant-protective effect of *Pinus halepensis* bark extract seems to be even superior, since it is an agent able to absolutely inhibit oxidative stress or enhance viability even after the serious oxidative stress induced by CS-conditioned media (Figures 2-6).

Concerning the decisive answer on the *in vitro* effects of nicotine on skin, our data support its protective role on skin cells, especially when combined with antioxidants (Figure 4). This effect was enhanced when nicotine was combined with *Pinus halepensis* bark extract. It has been suggested that nicotine may act through the nAChRs by reducing the secretion of pro-inflammatory cytokines as $TNF\alpha$ and IL6 (43, 44). It has also been reported that nicotine has no

antioxidant activity and in many cases has been used as an oxidizing agent creating reactive oxygen species in human endometrial cells or enhancing lipid peroxidation in brain and testis *in vivo* in rats (45, 46). However, in our experiment, the nAChR inhibitors did not show significant effects on keratinocytes (Figure 4). This could be due to either lower concentration or longer exposure (24 h) of keratinocytes to nAChR inhibitors, which were used in this study compared to the literature (47, 48).

In consistence with our results, other investigators have reported that nicotine inhibited the inflammation, thus minimizing the risk of atopic dermatitis, *in vitro* as well as *in vivo*, in keratinocytes and BALB/c mice, respectively (43). Regarding the role of nicotine, the results are controversial, since there is data showing toxic effects of nicotine on oral keratinocytes or fibroblasts. One possible explanation for this inconsistency is that higher doses of nicotine were used in literature (44) compared to those in the present study.

The kinetic study on the effect of CS on control keratinocytes, showed an increase of oxidative stress and decrease of cell viability already in the first hours of exposure (Figures 2, 3). The lowest effective dose of *Pinus halepensis* bark extract showed a significant protection against CS-induced oxidative stress, while it also decreased the endogenous ROS formation (Figure 5b). The results suggest that co-administration of *Pinus halepensis* bark extract with nicotine could be useful for *in vivo* preclinical studies, since this mixture is shown to exert enhanced protective effect against CS damage.

Conclusion

CS is highly harmful to keratinocytes, affecting both viability and oxidative stress. The negative effects of CS on viability and oxidative stress of keratinocytes were successfully inhibited by *Pinus halepensis* bark extract, while nicotine significantly enhanced their viability. The protective activity on keratinocytes was further enhanced when these two agents were used in combination, at well-established doses. The results seem promising for the use of *Pinus halepensis* bark extract to minimize the damaging effects of CS on skin.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

Pavlou P contributed to the realization of the study and writing of the manuscript. Antoniadou I, Peraki A, Vitsos A, Dallas P, and Papaioannou G contributed to the realization of this work. Mostratos D. worked on results, performed the statistical tests, and contributed

to the figures. Deliconstantinos G, Grando SA, and Rallis M contributed to the conception writing and realization of the study.

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