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Mechanism of Aloe Vera extract protection against UVA: shelter of lysosomal membrane avoids photodamage

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Keywords

Protective effect, photodamage, *Aloe barbadensis mil*, antioxidant, oxidative stress, lysosomal instability, liposomes, giant unilamellar vesicles.

Abbreviations

AO, Acridine orange; AV, Aloe Vera; CF, carboxyfluorescein; DCF, dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; DMMB, 1,9-dimethyl methylene blue; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; FBS, fetal bovine serum; GUVS, giant unilamellar vesicles; H_2DCFDA, dichlorodihydrofluorescein diacetate; HDCF, dichlorodihydrofluorescein; LMP, lysosomal membrane permeabilization; LTG, LysoTracker® Green DND-26; LTR, LysoTracker® Red DND-99; MB, Methylene blue; NR, Neutral red.

ABSTRACT

The premature aging (photoaging) of skin characterized by wrinkles, leathery texture and mottled pigmentation is a well-documented consequence of exposure to sunlight. UVA is an important risk factor for human cancer also associated with induction of inflammation, immunosuppression, photoaging and melanogenesis. Although herbal compounds are commonly used as photoprotectants against the harmful effects of UVA, the mechanisms involved in the photodamage are not precisely known. In this study, we investigated the effects of Aloe Vera (*Aloe barbadensis mil*) on the protection against UVA-modulated cell killing of HaCaT keratinocytes. Aloe Vera exhibited remarkable ability of reducing both *in vitro* and *in vivo* photodamage, even though it does not have anti-radical properties. Interestingly, the protection conferred by Aloe Vera was associated with the maintenance of membrane integrity in both mimetic membranes and intracellular organelles. Increased lysosomal stability led to a decrease in lipofuscinogenesis and cell death. This study explained why Aloe Vera extract offered protection against photodamage at cellular level in both UV and visible spectrum, leading to its beneficial use as a supplement in protective dermatological formulations.

INTRODUCTION

The pathogenic effects of ultraviolet (UV) radiation in skin includes sunburn, immune suppression, cancer, and premature photoaging[1]. Most (>95%) of the solar UV energy incident on human skin is in the UVA range (315–400 nm), and cutaneous exposure to chronic UVA-radiation is an established causal factor in photocarcinogenesis and photoaging[2–4].

UVA effects on human skin involve induction of photooxidative stress mediated by the direct generation of reactive oxygen species (ROS)[5], and indirect effects resulting from the release of free iron[6,7]. More recently, it was shown that cells activate autophagy in attempt to maintain cellular homeostasis following UVA photodamage[8,9]. Autophagy has an important role in the intracellular degradation of damaged proteins and entire organelles with a pro-survival role[10]. Target proteins and organelles are surrounded by a double membrane structure called as autophagosome. The contents of the autophagosome are degraded upon fusion of these LC3-II positive vesicles with lysosomes[10]. Therefore, targeting autophagy UV-irradiated cells counteract the damage promoted by excessive lipid oxidation[8]. Recent studies demonstrated that due to UVA-promoted lysosomal dysfunction, there is a conversion of protective autophagy into destructive one[11,12]. Damage in lysosomes also causes the release of iron pools increasing the redox misbalance after UVA exposure[13]. Therefore, a possible route to protect cells from UVA photodamage would be to protect lysosomes. In fact, recent work has shown that the flavonoids epicatechin and its derivative methylated epicatechin protected lysosomes and avoided liberation of intracellular iron[14]. As we will discuss later, we found that Aloe Vera extract also offers lysosome protection, however by a different mechanism.

In recent years, natural compounds have emerged with considerable interest as protective agents for reducing UVA-induced skin damage[15–19]. Usually, these herbs and herbal preparations have a high protective role predominantly due to their antioxidants, such as vitamins (vitamin C, vitamin E), flavonoids, and phenolic acids[20–22]. Although isolated plant compounds have a high potential in skin protection, whole herbs extracts showed better potential due to their complex composition[21]. Among several natural extracts Aloe Vera (AV extract) from *Aloe barbadensis mil* appears as the most commercialized herb in the cosmetic and toiletry industry[23], whose biological activities include promotion of wound healing, anti-oxidant effects, antifungal activity, antiinflammatory, anticancer, immunomodulatory and gastroprotective properties[21,24,25].

The natural chemical constituents found in the AV extract are mainly mono- and polysaccharides (Table 1), showing a low concentration of the phototoxic component aloin[26]. It is important to highlight the absence of flavonoids, allowing investigation of mechanisms of cell protection that differ from those already reported[4,6,14,27].

Many of the benefits of AV may be attributed to the polysaccharides contained in the gel of the leaves[28]. However, several other natural chemical constituents of AV extract have been associated to its health benefits: amino acids, anthraquinones, enzymes, chromones, organic and inorganic compounds, lipids, carbohydrates, lignin, salicylic acid, saponins, sterols, and vitamins (B1, B2, B6, C, β-carotene, choline, folic acid, α-tocopherol)[23]. The polysaccharides, mannose-6-phosphate, and complex anthraquinones are known to contribute synergistically to the benefits of AV extract[23,29,30]. The aqueous extract from AV extract exhibits inhibitory capacity against Fe^{3+}/ascorbic acid induced phosphatidylcholine liposome oxidation, scavenged stable $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and superoxide anion radicals,

and acted as reductant[31]. The components ascribed to the antioxidant activity of AV extract comprise total phenols, flavonoids, ascorbic acid, β-carotene and α-tocopherol[31].

Despite the emerging use of AV extract in the prevention of radiation dermatitis[32], the specific molecular protective effects on skin cells remain largely undefined. In fact, there is a little scientific proof of the effectiveness of AV extract for any cosmetic or medicinal purposes, and the positive evidences are frequently challenged by other studies[33]. Here, we aim to provide an important contribution on the mechanism of action of Aloe Vera. For this, AV extract was used to analyze its protection role against UVA irradiation.

MATERIALS AND METHODS

Cell lines and cell culture. The human immortalized skin keratinocyte (HaCaT)[34] was gently supplied by Butantan Institute, Brazil. The cells were cultured in Dulbecco Modified Eagle Medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a 37°C incubator at a moist atmosphere of 5% carbon dioxide.

Bioextract Aloe Vera. Powdered AV whole-leaf extract was supplied by FarmaService BioExtract (São Paulo, Brazil). The characterization of the chemical components was performed by using HPLC and colorimetric assays. As described in several literature reports the chemical constituents found in AV extract are mainly mono- and polysaccharides (Table 1).

Equipment. For irradiation, we employed a homemade UVA simulator with spectral range (280-400 nm). The UVA light irradiator (Novatecnica, Brazil) included temperature and humidity sensors, as previously described by our group[35]. Both variables were maintained stable during the experiments. The UVA irradiance (Wm^{-2}) was measured at eight different areas in this irradiator using a dosimeter (VLX-3W, France). To provide 2, 6 and 18 J/cm^2,

HaCaT keratinocytes were irradiated for 10, 30 and 90 min, respectively, at irradiance of 33.3 Wm^{-2}.

Determination of the total phenol contents. The total concentration of phenols was estimated by the Folin-Ciocalteu method with slight modifications. Following dilution (30-fold) an aliquot of an aqueous solution of AV extract (20 mg/mL) was mixed with 0.1 M Folin & Ciocalteu′s phenol reagent (SigmaAldrich) and 3% (w/v) sodium carbonate solution. After 2 hours of incubation at room temperature, the absorbance at 780 nm was measured using an Infinite M-200 Tecan microplate reader (Männedorf, Switzerland). The results were expressed as Gallic Acid equivalents (mg) per mL of the AV extract (Table 1).

DPPH radical scavenging capacity. The free radical scavenging capacity of the AV extract, based on the stable DPPH radical, was carried out according to the procedure described previously[36] with modifications. An aliquot of ethanol solution prepared from AV extract at different concentrations (0.1 to 5.0 mg/mL) was added to freshly prepared 0.1 M DPPH ethanol solution. After 4 hours of incubation at room temperature, the absorbance at 515 nm was measured using an Infinite M-200 Tecan microplate reader (Männedorf, Switzerland). The percentage of $DPPH^{\bullet}$ remaining at the steady state was calculated by the formula: [1-(Abs control – Abs sample)/Abs control] × 100. The radical scavenging ability was defined as the amount of antioxidant necessary to decrease the initial $DPPH^{\bullet}$ concentration by 50% (Efficient Concentration = EC_{50}), which was estimated using a nonlinear regression algorithm. All test analyses were run in triplicate. Trolox (6-Hydoxy-2,5,7,8-tetra- methyl-chromasn-2-carboxilic acid) was used as a positive control and for calibration of a standard curve (0.0125 – 0.075 mg/mL).

Cell viability assays. Twenty-four hours before treatment, exponentially growing human HaCaT cell lines were seeded in 96-well microtiter culture dish (Corning®) at a density of 6 x 10^4 cells/cm^2. Prior to UVA irradiation, HaCaT keratinocytes were washed with phosphate

saline buffer (PBS) twice. AV extract 5% (w/v) was dissolved in PBS and diluted to the required concentration in PBS, following UVA irradiation at indicative dose (J/cm^2). Dishes were irradiated in presence or absence of PBS with AV extract in a dose-dependent manner. In parallel, non-irradiated cells were treated similarly. 48 hours after photodamage in presence or absence of AV extract the survival rates were estimated by MTT and CVS assays that were carried out independently. The survival rates were normalized to the absorbance values of dark condition. Briefly, 48 hours after photodamage we added MTT (SigmaAldrich) at 50 µg/mL to cells containing medium and incubated for 2 hours at 37°C. Next, the MTT reduced-product formazan was solubilized in DMSO (SigmaAldrich), and absorbance values were read at 550 nm, using an Infinite M-200 Tecan microplate reader (Männedorf, Switzerland). For the CVS assay, fixed cells from NRU-assay were stained with Crystal Violet (CV, SigmaAldrich) at 0.02% (w/v) for 5 minutes at room temperature. After washing, CV was eluted with 0.1 M sodium citrate in 50% (v/v) ethanol, and the absorbance values were recorded at 585 nm.

Quantification of autophagy arbitrary units. Next, to quantify cell death closely associated with autophagy we applied a recent strategy based on a numeric variable AAU (autophagy arbitrary units)[37]. Briefly, its conceptual framework is based on the fact that lysosomes accumulated in cells with impaired autophagy[37,38]. The uptake of the acidophilic lysosomotropic Neutral Red, NR (SigmaAldrich) allowed us to measure lysosomal content. 48 hours after photodamage, HaCaT keratinocytes were stained with NR (30 µg/mL) and incubated at 37°C for 2 hours. After washing, NR was eluted with an alcoholic-based 1% (v/v) acetic acid fixing solution and measured at 540 nm using an Infinite M-200 Tecan microplate reader (Männedorf, Switzerland). These fixed cells after washing with water were used for CVS (Crystal Violet Staining) assay. To calculate AAU, the NR-uptake was

weighted by the average of cell survival measured by MTT and CVS assays, as previously described[37].

Evaluation of oxidation of H_2DCF UVA-generated. The radical scavenging effect of AV extract in UVA irradiated cells was monitored by dichlorodihydrofluorescein assay. The polar, pre-fluorescent dichlorodihydrofluorescein diacetate (H_2DCFDA) undergoes deacetylation by cytosolic esterases to form dichlorodihydrofluorescein (H_2DCF), which under intracellular oxidation gives rise to dichlorofluorescein (DCF). The green fluorescence was monitored at specific excitation/emission wavelengths 488/517 nm using an Infinite M-200 Tecan microplate reader (Männedorf, Switzerland). Before irradiation in presence or absence of AV extract (0.5 mg/mL), HaCaT keratinocytes were incubated with H_2DCFDA (10 μM) for 15 min at 37°C. Following UVA irradiation (18 J/cm^2), HaCaT keratinocytes were washed with PBS twice and maintained on ice for DCF fluorescence measurement. After detection of DCF fluorescence, cells were fixed and submitted to CVS assay to determine cellular density. Next, the DCF fluorescence was normalized by CVS results.

Staining and flow cytometry analysis of lysosomes. Acridine orange (AO) is a lysosomotropic weak base (pKa= 10.3) that is retained (protonated form AOH^+) due to proton trapping in lysosomes (pH 4.5–5.5). On excitation with blue light (λ = 488 nm), AO dimers (high AO concentration) exhibit red and monomers (low AO concentration) green fluorescence[39]. The end stage of lysosomal damage may be evaluated by the AO uptake method, as previously described[40]. Immediately after photodamage by UVA- (18 J/cm^2), HaCaT keratinocytes were exposed for 15 min to AO (10 μg/mL) in a standard culture medium at 37°C, such that AO accumulated in stable lysosomes. Cells were then rinsed with culture medium in the dark and incubated under standard culture conditions for another 10 min to reduce cytosolic AO. Next, after washing twice in PBS, we collected at least 20,000

events for flow cytofluorometric analysis of AO (FL1 an FL3) using BD FACS Verse™ and FlowJo software. Since AO will only accumulated in intact lysosomes, the extent of lysosomal rupture was assessed by comparing the amount of red fluorescence in irradiated-cells compared to control in presence or absence of AV extract (0.5 mg/mL).

Membrane mimic systems: Photoinduced CF release from liposomes. To evaluate the ability of AV extract to protect membranes from photodamage we measured the leakage of fluorescent carboxyfluorescein (CF, SigmaAldrich) entrapped in unilamellar soy lecithin liposomes[41] as a function of irradiation time. This protocol was based on DMMB (1,9-dimethyl methylene blue) triggered membrane-damage, as assessed by leakage of CF entrapped in unilamellar liposomes[42]. Briefly, to prepare unilamellar liposomes we dried phospholipid mixtures (30 mg, lecithin soy) under argon chloroform. Then, the dried film was suspended in 10 mM Tris-NaCl buffer at pH 8.0 containing CF (50 mM) and the dispersion was sonicated and vortexed intermittently for at least 20 minutes at room temperature. The resultant phospholipid concentration was about 30 mM. Free carboxyfluorescein was removed by passage of the dispersion through a column of Sephadex G-50 where the vesicles eluted with the void volume. Aliquots of the liposomal stock preparations were incubated with DMMB (15 μM) and submitted to irradiation in the presence of AV extract or Trehalose (0 – 10 mg/mL). Membrane damage quantification was carried out in a 96-well fluorescence microplate (Greiner Bio One—Frickenhausen, Germany). The whole microplate was irradiated with a red light emitting diode (LED), with maximum emission wavelength at 633 nm and 35 Wm^{-2} irradiance. The CF fluorescence at 517 nm was monitored as function of irradiation time at a controlled temperature with an Infinite M-200 Tecan microplate reader (Männedorf, Switzerland), operating with excitation at 480 nm. Prior LED irradiation, the CF fluorescence was detected for both DMM and control condition, and considered as time zero[42].

Giant unilamellar vesicles (***GUVs) preparation.*** GUVs were prepared by the electroformation procedure. Briefly, ~20 µL of 2 mg/mL lipid (DOPC) stock chloroform solution were spread on the surfaces of two conductive glass slides coated with indium tin oxide. The glass slides were placed with their conductive sides facing each other and separated by a 2 mm thick Teflon frame. The chamber was filled with 0.2 M sucrose solution. The glass plates were connected to a function generator and an alternating voltage of 2 V with a 10 Hz frequency was applied for 2 h at room temperature (22-24°C)[43]. The vesicles were then diluted in 0.2 M glucose containing the desired amount of AV extract or 20 µM Methylene Blue (MB)-containing AV extract solution and immediately observed by phase contrast optical microscopy. The osmolarity of the sucrose and glucose solutions were measured with a cryoscopic osmometer Osmomat 030 (Gonotec, Germany) and matched to avoid differences in osmotic pressure. All measurements were done at room temperature. An inverted microscope Axiovert 200 (Carl Zeiss, Jena, Germany) equipped with a Plan Neo-Fluar 63X Ph2 objective (NA 0.75) and A-plan 10X Ph1 (NA 0.25) was used and the images were recorded with an AxioCam HSm digital camera (Carl Zeiss, Jena, Germany). Irradiation of the samples was performed with the 103 W Hg lamp (HXP 120, Kubler) of the microscope using an appropriate filter for photo activation of MB (λ_{ex} = 665 nm; λ_{em} = 725 nm). Visualization of the effect elicited by MB and AV on GUVs was accompanied by optical contrast fading and quantified by taking into account the changes in the brightness intensity through the Image J software (NIH, USA).

MB Photosensitization in HaCaT keratinocytes. MB photosentization protocol[44] was used to generate known quantities of intracellular singlet oxygen and to test the effect of protection specifically against this kind of harm. Basically, HaCaT keratinocytes were exposed to 633 nm LED at 11 J/cm^2 after incubation with different MB concentrations (0 – 3.0 µM) for 1 hour in a 37°C incubator at a moist atmosphere of 5% carbon dioxide. Prior to LED

irradiation, MB treated and untreated cells were washed with phosphate saline buffer (PBS) twice. Next, dishes were irradiated for 40 minutes in presence or absence of AV extract (0.5 mg/mL) diluted in PBS. Sample irradiation was carried out with a LED with maximum emission wavelength at 633 nm and 46 Wm^{-2} irradiance. In parallel, non-irradiated cells were treated similarly. 48 hours after photodamage in presence or absence of AV extract the survival rates were estimated by MTT assay as described before.

LC3-II and P62 immunoassaying. After photodamage slides were washed twice in PBS and fixed in formaldehyde at 4.0 % (w/v) for 15 minutes at 4°C. They were then rinsed three times in PBS for 5 minutes at room temperature and blocked with PBS containing 5.0 % (w/v) of bovine serum albumin (BSA) and Triton X-100 at 0.3% (v/v) for at least 60 minutes at room temperature. After blocking, we incubated slides with primary rabbit monoclonal antibodies against microtubule-associated proteins 1B (LC3B, Cell Signaling Technology) and ubiquitin binding protein sequestosome 1 (SQSTM1/p62, Cell Signaling Technology) both diluted 1:200 in PBS containing 1.0 % (w/v) of BSA and 0.3% (v/v) of Triton X-100 overnight at 4°C in a humid light-tight box. The stained cells were washed and incubated for 2 h at room temperature with secondary antibody (Alexa 488-goat fluorochrome-conjugated anti-rabbit antibody (Molecular Probes) diluted 1:500 in PBS containing 1.0 % (w/v) of BSA and 0.3% (v/v) of Triton X-100). The slides were then washed three times with PBS, counterstained in Prolong Gold antifade reagent with DAPI, visualized using a confocal microscope (Zeiss™ Axiovert 200 LSM 510 Laser and Confocor Modules, Germany) and imaged using Image J Software (National Institutes of Health, Bethesda).

Labeling of lysosomes in live cells. After the photodamage, the acidic compartments were evaluated after indicative times using acidophilic lysosomal probes LysoTracker® Green (LTG, Molecular Probes) and LysoTracker® Red (LTR, Molecular probes) [45,46]. In brief, after the photodamage we incubated HaCaT with 200 ηM LTG or 200 ηM LTR in DMEM 1%

(v/v) FBS at 37°C for 1 hour. After washing, we immediately analyzed live-cells under confocal microscope as described above. Since LTR remained in lysosomes even after aldehyde fixation, we performed confocal microscopy following P62 immunostaining. Alternatively, we collected at least 20,000 events to flow cytofluorometric analysis of LTG (BD FACS Verse™) using FlowJo software.

Lipofuscin-related autofluorescence. Autophagic degradation of endogenous aggregates such as lipofuscin can be monitored in HaCaT keratinocytes utilizing the autofluorescence of lipofuscin particles[46]. At 48 hours after UVA photodamage induction upon AV extract treatment (0.5 mg/mL), autofluorescence of lipofuscin particles (FL1) was determined by cytofluorometric analysis (FACSVerse, BD Biosciences) using FlowJo software.

Statistics. Comparative statistical analysis of independent samples was performed using IBM® SPSS Statistics version 20. Data obtained from at least three independent experiments (n=3) were expressed as mean values ± standard error. To perform comparative statistical analysis, we first analyzed the variance between groups. Then, multiple comparisons were performed using one-way analysis of variance (ANOVA) with Dunnett's T3 or Bonferroni post-hoc test, depending on homogeneity of variance. The analysis of correlation was done using Pearson's coefficient (r). P-values lower than 0.05 were considered significant.

RESULTS

The protective effect of AV extract against UVA irradiation in human keratinocytes

We first characterized the UVA phototoxicity (2 to 18 J/cm^2) on HaCaT keratinocytes at 24 hours after irradiation (Figure 1a). Note that there was a significant reduction of cell survival as function of UVA dose. Interestingly, with the decrease in cell survival there was an

increase in the lysosomal content, which was calculated in terms of the arbitrary autophagy units (AAU)[37], (Figure 1b). This finding supported the concept of autophagy-associated cell death induced by UVA[8,9,12].

Cell viability 48 hours after UVA measured by MTT reduction significantly increased from 57.8% in control to 82.5% when cells were irradiated in presence of AV extract (Figure 1c). Consequently, AV extract, which did not affect cell viability in the dark, decreased the phototoxic effects of UVA on HaCaT keratinocytes, leading to a significant cellular rescue (Figure 1c). Accordingly, at 48 hours after UVA the level of PI (+) cells decreased from 22% to 14%, indicating maintenance of cytoplasm membrane integrity and cellular protection against UVA photodamage (Figure 1d).

Since AV extract seems to minimize the loss of membrane integrity (Figure 1d) induced by UVA parallel to a pro-survival role on HaCaT keratinocytes in a long-term response (Figure 1c), we asked if the protective effects would avoid morphological changes (reduction of cytoplasmic volume and pyknosis) immediately induced by UVA exposure (data not shown). For this purpose, we performed flow cytofluorometric analysis of cell size (FSC-Height) and granularity (SSC-Height) immediately after UVA irradiation. As shown in scatter-plots, there was a considerable retraction of keratinocytes (low cell size) parallel to low granularity in 62.1% of irradiated cells compared to control (34.8%) (Figure 1e). Noteworthy, AV extract prevented such morphological changes leading to a significant recovery of keratinocyte´s morphology (high cell size and high granularity), as revealed in Figure 1e-ii.

In an attempt to ascribe the protective role of AV extract to its ability of filtering photophysical membrane damage, we analyzed AV extract´s light-spectrum (Supplementary Information). By measuring the absorbance of the light in a range of 300 to 800 nm, we saw that AV extract did not absorb any light even at large concentrations. Consequently, AV

extract cellular protection (Figure 1c-e) was not due to a filter effect. Other possible explanation for the protection effect of AV is some sort of anti-oxidant action.

The protective role of AV extract against UVA irradiation at cellular level

UVA triggers cell death mainly by generating oxidative stress and that causes translocation of cathepsins related to lysosomal membrane permeabilization (LMP)[47,48]. To link the protective effects of AV extract against UVA photodamage to the antioxidant capacity of AV extract[30], we monitored the generation of oxygen reactive species in terms of H_2DCF oxidation (Figure 2a) using HaCaT keratinocytes. Even though DCF fluorescence of irradiated cells significantly increased (164%), in presence of AV extract it diminished in 50% compared to UVA (Figure 2a). Noteworthy, even upon basal condition AV extract significantly suppressed H_2DCF oxidation at 73%.

The most important intermediate of the photodamage induced by UVA is singlet oxygen, which can also be generated by irradiation of photosensitizers, such as MB, by using visible light. Note that after irradiation the oxidation of H_2DCF significantly increased as a function of MB concentration (Figure 2b). However, in the presence of AV extract the DCF fluorescence significantly decreased reaching a maximum scavenging level of 45% (Figure 2b). Interestingly, this reduction of redox misbalance significantly associated with increase in cellular rescue (Figure 2c). Therefore, AV extract significantly decreased the redox misbalance and cell death induced by photosensitization both in the UVA and in the visible (Figures 1c and 2a-c).

The suppression of H_2DCF oxidation due to UVA or visible light (in the presence of MB) does not seem to result from a direct anti-oxidant activity of AV extract[30]. The free radical 1,1-Diphenyl-2-picrylhydrazyl $DPPH^{\bullet}$ is suppressed by known antioxidant agents such as Gallic Acid and Trolox[49], and this suppression is commonly used to characterize natural antioxidants[36,49]. We measured the percentage of $DPPH^{\bullet}$ radicals remaining at the

steady state as a function of concentration (Figure 2d). Both Gallic Acid and Trolox showed significant radical scavenging ability with EC_{50} of 1.0 μg/mL and 4.0 μg/mL, respectively (Figure 2d), while neither AV extract nor Trehalose showed any action in terms of suppressing $DPPH^{\bullet}$. The fact that Trehalose did not suppress $DPPH^{\bullet}$ was expected since Trehalose is a non-reducing disaccharide, and therefore was not expected to have anti-radical action[50]. Some preparations of AV may have antioxidant properties, since the chemical composition may depend on the extraction method[30]. However, the AV extract used here show no effect of suppressing radical species. The lack of antioxidant effect was compatible with the low amount of total polyphenols detected in AV extract (Table 1).

To check if AV extract prevents damages in membrane organelles, we evaluated the loss of lysosomal membrane integrity following UVA exposure[51]. Right after UVA irradiation we evaluated the stability of lysosome membranes based on the retention of the lysosomotropic weak base Acridine orange (AO)[40]. In fact, there was a significant decrease of almost 45% in the red AO fluorescence compared to control (dark) condition (Figure 3a). Note that in the presence of AV extract, AO red fluorescence increased by 82% compared to the photodamage without AV (Figure 3a). Therefore, in the presence of AV lysosomes maintained their intrinsic ability of proton trapping even after photodamage by UVA.

To further evaluate the effects of photodamage on lysosomes we investigated LMP after photoactivation of MB using HaCaT cells (Figure 3). LMP was measured in terms of the fluorescence reduction of the lysosomotropic dye LysoTracker® Red DND-99 (LTR) parallel to release of Cathepsin B (CTSB) to cytosol[51]. Note that the loss of lysosomal membrane integrity was measured in terms of decrease in LTR-loading in lysosomes[51,52] (Figure 3b-i). Parallel to lysosomal membrane instability we observed cytosolic increase in CTSB 30 minutes after MB photosensitization (Figure 3b-ii), which decreased under AV extract treatment (Figure 3b-ii, line scans). Within 3 hours after MB-photosensitization, we found a

subset of cells AV-treated (lined area) with relevant increase in the lysosomal LTR-loading (Figure 3c). Cytofluorometric analysis using the lysosomotropic dye LysoTracker® Green DND-26 (LTG) to stain stable lysosomes also supported this finding (Figure 3d). AV extract significantly suppressed the photodamage triggered by MB on lysosomes in both times of evaluation. Therefore, AV extract maintained the intrinsic ability of proton trapping by lysosomes even after photodamage by MB (Figures 3b-d).

AV extract suppresses the negative effects of UVA on autophagy

Autophagy is induced by UVA and promotes removal of oxidized phospholipids and protein aggregates in epidermal keratinocytes[8]. It has been shown that lysosomal impairment causes perceived accumulation of autolysosome cargo in human keratinocytes and that lysosomes are one of the main biological targets of UVA[11,12,53]. Therefore, we decided to investigate whether AV extract had any impact on inhibition of autophagy flux UVA-induced. Note that UVA caused relevant accumulation of acidic vacuoles parallel to increase in the immunostaining of autophagic cargos (LC3-II and P62), which were significantly reduced by AV extract (Figures 4a and 4b).

As UVA promoted loss of lysosomal function[11,12], we asked if this reduction at long-term response would lead to the formation of endogenous aggregates commonly associated with cell aging, such as lipofuscin[54]. This accumulation had been observed in other cell models exposed to UVA such as fibroblasts and retinal pigment epithelial cells[12,55,56]. Indeed, 48 hours after UVA irradiation, lipofuscin significantly accumulated in 61% of irradiated HaCaT keratinocytes compared to non-irradiated cells (39%) (Figure 4c-i). AV extract significantly reduced the promotion of lipofuscinogenesis triggered by UVA, resulting in a significant increase of 3-fold in the frequency of living cells [PI (-)] without lipofuscin-loading [Lipofuscin (-)] compared to irradiated-cells (Figure 4c-ii).

Cells irradiated with UVA activate autophagy in an attempt to maintain cellular homeostasis or to orchestrate cell death through a programmed mechanism[8,9]. However, since UVA jeopardizes lysosomal function (Figure 4), autophagic clearance fails on its pro-survival role leading to activation of autophagy-associated cell death[11,12,55]. In this scenario, AV extract acted at cellular level with pro-survival effect (Figures 1c-d) most likely by protecting lysosome membranes (Figure 3a), since it did not present any antioxidant activities (Figure 2d). The decrease in generation of oxygen reactive species by AV extract (Figure 2a) seems to result more from effective autophagic clearance of oxidized proteins and organelles promoted by UVA rather than from an actual antioxidant activity of AV extract components (Figure 2d). Thus, we proposed that the occurrence of functional autophagy under UVA exposition (Figure 4) is only possible because of the protective role of AV extract components on lysosome function at molecular level.

In order to test if the mechanism underlying the AV extract protective effect on lysosomal function integrity was due to photo-protection of the membranes, we investigated the interaction of AV extract with more simplistic membrane mimetic systems, i.e., liposomes and giant unilamellar vesicles (GUVs). These mimetic models allowed us to investigate how *in situ* photo-generated oxidative species affect the phospholipid bilayer.

AV extract reduced the loss of membrane integrity after photodamage

After photoactivation of DMMB single oxygen is generated, causing lipid oxidation and increasing the permeability of carboxyfluorescein[42]. In fact, this was observed in our experimental setting. Note that CF fluorescence significantly increased in liposomes as a function of time, showing elevated Pearson´s linear coefficient in comparison with control vesicles that had no leakage (Figure 5a-i). Note also that AV extract remarkably decreased the CF release in a concentration dependent manner (Figure 5a-ii). At 10 mg/mL CF release was reduced by 75%, which is a strong evidence that AV really protects membranes from

photodamage. In another set of experiments, we compared the efficiency of AV to protect membranes with that of Gallic acid, a known and standard anti-oxidant[49]. Note that both agents efficiently protect the membranes against photodamage (Figure 5b). However, the Gallic acid solution showed a high level of polyphenols (data not shown), which is not the case of the AV extract, what is in agreement with the lack of $DPPH^{\bullet}$ suppression showed in figure 2d. Therefore, it seems that the protective role of AV extract was not associated with presence of polyphenols (Table 1).

Since the alpha-linked non-reducing disaccharide Trehalose has a strong stimulatory effect on autophagy[57–59] and exhibits strongly ability of cell protection related to its exclusive interaction with membranes[60–62], we decided to compare the protective role of AV extract to this natural disaccharide by evaluating damage of liposomes after DMMB-photosensitization (Figure 5c). Note that Trehalose was also able to protect the membrane even though it did not have any anti-oxidant activity as showed before in Figure 2d (Figure 5c),by binding together the headgroups of two lipids[60,62]. Therefore, it seems that this physical effect could explain the effect of AV in terms of UVA and visible light protection.

We then asked if AV could also directly protect giant unilamellar vesicles membranes made of dioleoyl-sn-glycero-3-phosphocholine (DOPC) from photosensitization with visible light[63] (Figure 5d). We thus pursued an analysis of AV extract-mediated photoprotection by evaluating the response of the membrane to oxidative stress. Initially we noted that GUVs dispersed in AV-containing solution in the absence of a photosensitizer (upper panel) were not altered by light irradiation in contrast with those dispersed in MB solutions (middle panels). In the latter case, the lipid photo-oxidation significantly affected the GUVs shape/size ending up to membrane disruption in a short period of irradiation (less than 3 minutes). On the other hand, by adding AV extract in MB solution (lower panel) the lipid photo-oxidation was less pronounced andGUVs remained intact after up to 30 minutes of continuous

irradiation. There was just an increase in membrane permeability evidenced by fading in the phase contrast (see Fig 5d-*l*), after 30 minutes of MB-photoactivation. Therefore, these observations supported that AV extract was able to protect lipid membranes from oxygen reactive species generated by irradiation in the surrounding environment.

DISCUSSION

UVA caused cell death, lysosome damage and autophagy inhibition in HaCaT keratinocytes, all of which were lessened by AV extract. AV also decreased the redox misbalance induced both by UVA and by MB photoactivation at λ = 633 nm. These redox effects could not be explained by a molecular anti-oxidant role of AV, since it has very little amounts of phenolic compounds and no measurable activity of radical suppression. AV binds and brings together the head-groups of two phospholipids avoiding that membrane leaks and decreasing exposition of reactive sites of the membrane[62]. In fact, the cell protection could be correlated with membrane protection, which was partially mimicked by a simple solution of Trehalose, a non-reducing sugar that is known to protect membranes[50,60,62,64]. By comparing the responses of the AV extract with that of a threalose solution, we do not want to suggest that there actually are trehalose molecules inside the AV extract. We just infer that the mechanism of membrane protection induced by the AV extract is similar to that known to be induced by threalose.

Previous reports have showed that herbal compounds suppressed the UVA-induced ROS production, which results in a decrease in intracellular lipid peroxidation, augmentation of endogenous antioxidant capacity and increase in cellular viability[16,17,49,65]. Most of them showed direct antioxidant properties[16,17], though plant sterols showed protective effects towards UVA-induced upregulation of ICAM-1, by reducing the ceramide formation from sphingolipids⁄cholesterol-rich microdomains (rafts)[66].

The reduction of the UVA photodamage on lysosome membranes mediated by natural compounds was first reported by Basu-Modak and colleagues[14]. They showed that flavonoid constituents of many plant-derived foods remarkably protect against lysosomal destruction by UVA. Here we show that the cellular protective effects of AV extract against photodamage are intrinsically related to the prevention of photo-oxidation of lipid membranes and protection of lysosome membrane. Even in visible light AV extract significantly protected human keratinocytes against remarkable MB-driven photodamage.

AV extract promoted restriction of photo-oxidation of lipid membranes, showing a key role on autophagic cellular rescue upon lethal oxidative stress. Indeed, the impact of AV extract on lysosomes led to a significant reduction of lipofuscinogenesis UVA-triggered, maintaining cellular homeostasis and increasing cell survival. Our data suggest that the lysosome membrane shelter triggered by AV extract would allow a more robust protection against solar radiation, and may represent a new paradigm in terms of sun protection. We hope this paper contributes to future work on this direction.

CONCLUSION

In this study, we characterized the protective role of natural compounds extracted from leaves of Aloe Vera (*Aloe barbadensis mil*) and originally raised the concept that AV extract protects against UVA at cellular level by protecting membranes.

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CONTRIBUTIONS

D.R. and A.C.V. contributed equally to this work; W.K.M. conceived and designed the experiments; W.K.M, D.R., A.C.V., R. C. and A.G. performed research; W.K.M., R.I., A.G. and M.S.B analyzed data; D.S. contributed in DPPH and Polyphenol assays; and W.K.M. wrote the main manuscript text, and all authors reviewed the manuscript.

Table 1: Constituents of Aloe Vera extract.

Chemical Component	Concentration (mg/mL)
Glycose free	6.3 ± 0.04
Mannose free	1.1 ± 0.01
Mannose polysaccharide-conjugated	0.6 ± 0.1
Total carbohydrates	7.5 ± 0.1
Gallic acid	0.02 ± 0.002
Malic acid	1.3 ± 0.03

Source: FarmaService Bioextract LTDA, Brazil

Figures

Figure 1. Photodamage and cellular protection mediated by AV extract in human keratinocytes (HaCaT). 24 hours after UVA exposure with 33.3 Wm^{-2} irradiance: (a) cell viability was determined by MTT reduction and CVS assays as a function of UVA dose (J/cm^2); (b) Lysosomal content measured in terms of autophagy arbitrary units (AAU) correlated to cell survival determined by MTT assay; (c) Cell viability (MTT reduction) carried out in the absence and presence of AV extract (0.5 mg/mL); (d) cell size as a function of Propidium Iodide (PI) incorporation. Gating according to FSC-Height (cell size) and PI

(FL3). In (i) representative 5% contour scatter-plots showing cell size (FSC) and PI labeling. Representation of the mean frequency of cell subpopulations by bars representation (ii). (e) Evaluation of the morphology of HaCaT cells by flow cytometry immediately after UVA exposure (18 J/cm^2) carried out in the presence and absence of AV extract (0.5 mg/mL). In (i) representative 5% contour scatter-plots showing cell size (FSC) and granularity (SSC). The mean frequency of HaCaT cells with high cell size and high granularity (upper right quadrant) or low cell size and low granularity (lower left quadrant) was represented by graph bars (ii). Mean ± standard error of three independent experiments are shown, *P≤0.01; **P<0.001, ***P<0.0001.

Figure 2. Photodamage and protection against generation of reactive oxygen species mediated by AV extract in human keratinocytes (HaCaT). (a) Total DCF fluorescence (λexc = 488nm and λemi = 515nm) was determined immediately after UVA exposure (18J/cm^2) carried out in the presence and absence of AV extract (0.5 mg/mL). (b) $DCFH_2$ oxidation promoted by photodynamic system was determined as a function of MB concentration (2.0 and 3.0 μM) in the presence or absence of AV extract (0.5 mg/mL). (c) 48 hours after photodamagein presence or absence of AV extract (0.5 mg/mL), the cell survival was determined by MTT assay as function of MB concentration (0 - 3.0 μM). (d) The percentage of $DPHH^{\bullet}$ remaining at the steady state (4 hours of reaction) was determined in the presence or not of Gallic acid (0 – 2.1 μg/mL), Trolox (0 – 6.3 μg/mL), AV extract (0 – 1.0 mg/mL) and the disaccharide Trehalose (0 – 1.0 mg/mL). The concentration that suppresses 50% of the $DPHH^{\bullet}$ concentration (EC_{50}) was calculated. (Mean ± standard error of three independent experiments. *P≤0.01; **P<0.001, ***P<0.0001.

Figure 3. Mechanism of AV extract protection: shelter of lysosomal membrane avoids photodamage in human keratinocytes (HaCaT).

(a) Uptake of the lysosomotropic dye Acridine Orange (AO) immediately after UVA exposure (18J/cm^2) carried out in the presence and absence of AV extract (0.5 mg/mL). Lysosome membrane stability was monitored in terms of the lysosomal red fluorescence of AO. (b) After 30 minutes of MB-photoactivation (2 μM) in absence or presence of AV extract (0.5 mg/mL), HaCaT cells were stained with LysoTracker® Red DND-99 (LTR, in red) and immunostained for total CTSB (green). Micrographs of photodamaged cells (traced line) showing elevated CTSB in cytosol associated with decrease in LTR-loaded lysosomes (i). Fluorescence plot profiles represented as line scans of LTR-loaded lysosomes and CTSB (ii). (c) 3 hours after photosensitization of MB (2 μM) in absence or presence of AV extract (0.5 mg/mL) cells were stained with LTR and evaluated under confocal microscope. (d) Alternatively, 2 and 3 hours after photosensitization of HaCaT cells with MB (2 μM) in absence or presence of AV extract (0.5 mg/mL) the lysosomes stained with LysoTracker® Green DND-26 (LTG) were quantified by FACS. Bars show the percentage of LTG-loading lysosomes after photodamage in comparison to control (100%). Mean ± standard error of three independent experiments. *P≤0.01; **P<0.001, ***P<0.0001.

Figure 4. Cellular protection mediated by AV extract in human keratinocytes (HaCaT). 48 h after photodamage (UVA 18 J/cm^2) in the presence or absence of AV extract (0.5 mg/mL): (a) Accumulation of autophagy cargo by immunostaining of LC3-II. (i) Upper images show confocal micrographs and bottom images show surface plots. (ii) Mean intensity of LC3-II. (b) Keratinocytes were exposed to UVA (18 J/cm^2) in the presence or absence of AV extract (0.5 mg/mL). Following staining of lysosome with LTR (red), cells were immunostained for P62 (green) 48 hours after the photodamage (i). Bars show the average fluorescence intensity from multiple cells (ii). (c) Cytometry analysis to determine the frequency of lipofuscin-loading (λexc = 488 nm and λemi = 527/32 nm). In (i) representative 5% contour scatter-plots showing lipofuscin (FL1) and Propidium Iodide (FL3). In (ii) the

mean frequency of cell subpopulations showing (+) or not (-) lipofuscin autofluorescent and PI incorporation. Mean ± standard error of three independent experiments, *P≤0.01; **P<0.001, ***P<0.0001. Scale bars in (a-i) are 10 μm.

Figure 5. Photodamage and protection of membrane mimetic unilamellar liposomes. (a) Loss of membrane integrity evaluated by CF. In (i) Liposomes were damaged by reactive oxidative species after photoactivation with DMMB (15 μM) under irradiation (λexc = 639 nm) for the periods indicated in the x axis. In (ii) DMMB-driven membrane photodamage in the presence of increasing concentrations of AV extract (0.5, 4 and 10 mg/mL). (b) Membrane protection against photodamage DMMB-triggered after 120 minutes (expressed as percentage of protection) in the presence or absence of AV extract (20 mg/mL) and of the anti-oxidant agent Gallic Acid (5 μg/mL). The ponderation of membrane protection according to total polyphenol quantified by Folin-Ciocalteu method is represented by gray bars. (c) Comparative analysis of protective effects of AV extract (0.5 and 10 mg/mL) and of the non-reducing disaccharide Trehalose (0.5 and 10 mg/mL) on liposomes after photodynamic activation of DMMB for 120 minutes. (d) In (i) DOPC GUVs snapshots obtained in phase contrast mode at increasing incubation times (indicated in the right corner of each figure) with a solution containing AV (0.5 mg/mL) in the absence (upper panel) and presence of MB (20 μM) (bottom panel) under continuous irradiation at λ = 633 nm. MB-containing GUVs solutions under irradiation were displayed in the middle panel for comparison. Mean ± standard error of three independent experiments, *P≤0.01; **P<0.001, ***P<0.0001. Scale bars in (d-i) are 10 μm.

Supplementary information: Spectrum analysis of AV extract as a function of concentration (mg/mL). Mean ± standard error of three independent experiments.

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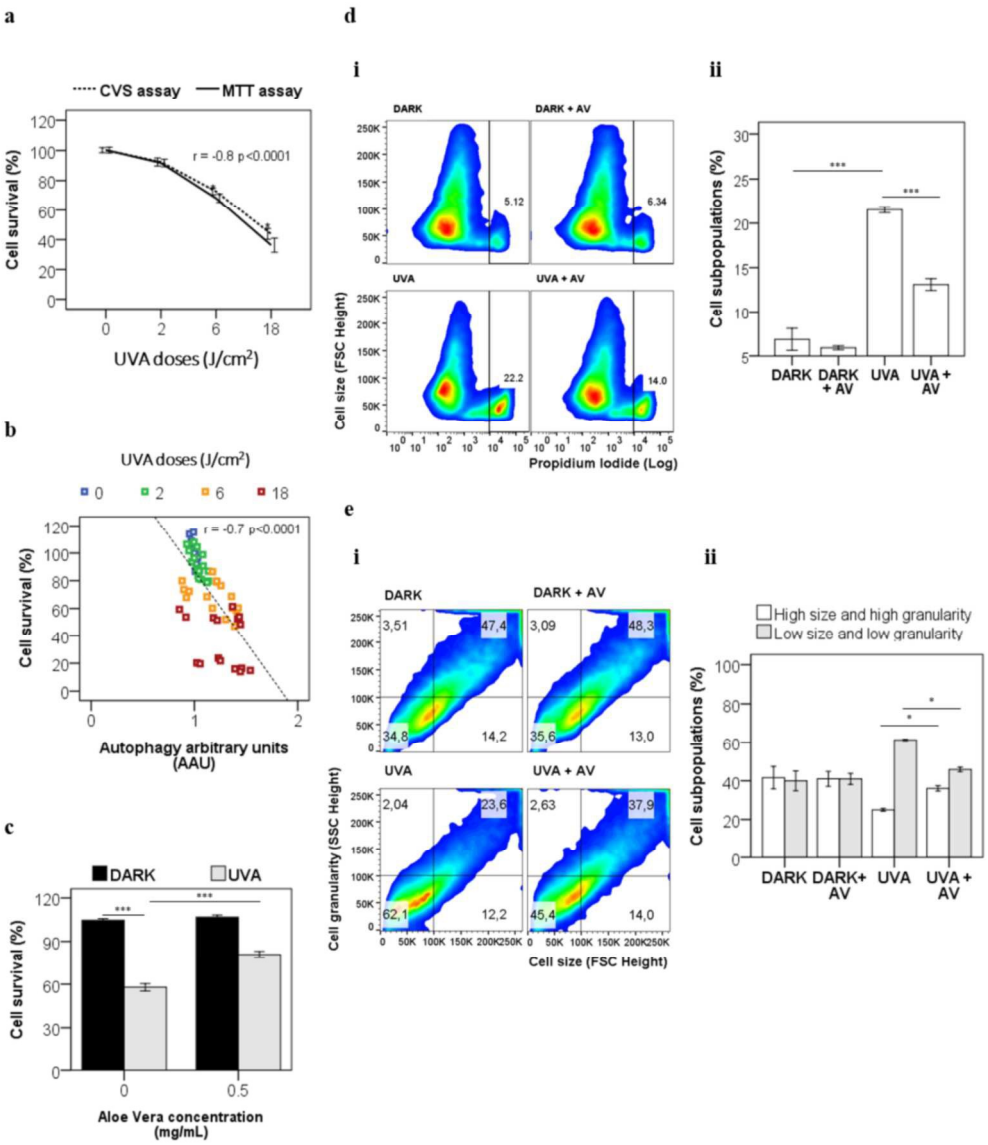

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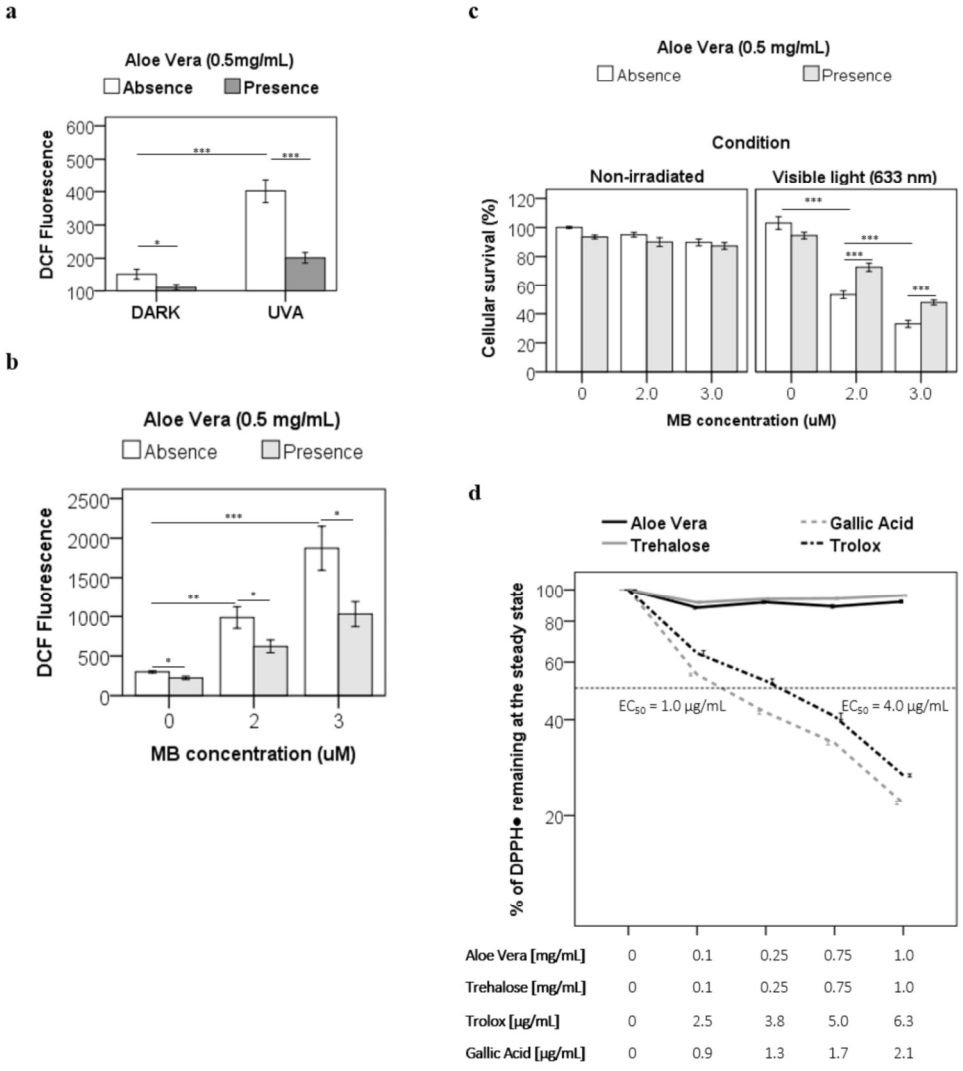

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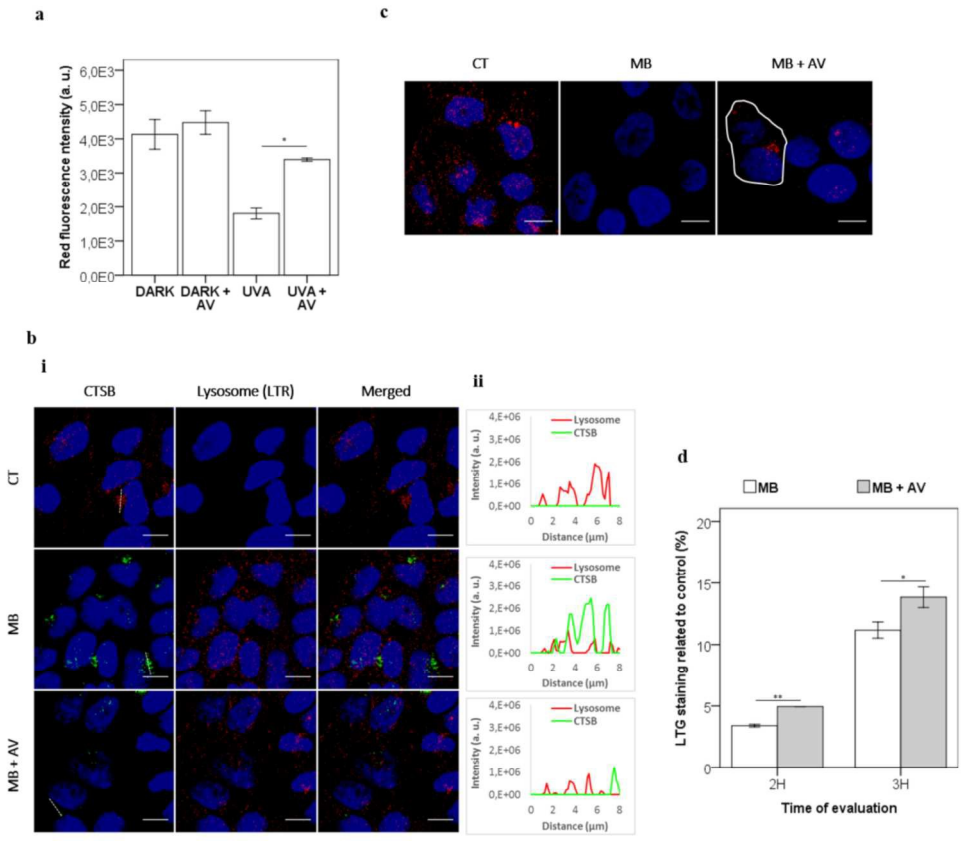

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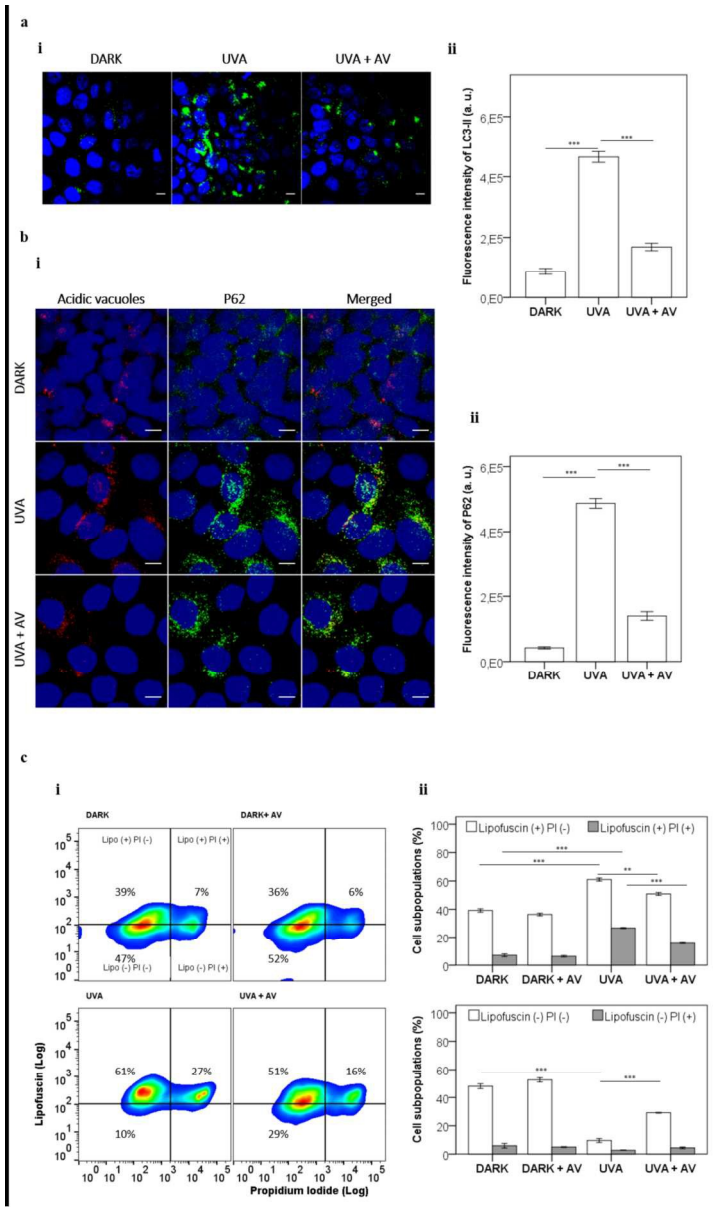

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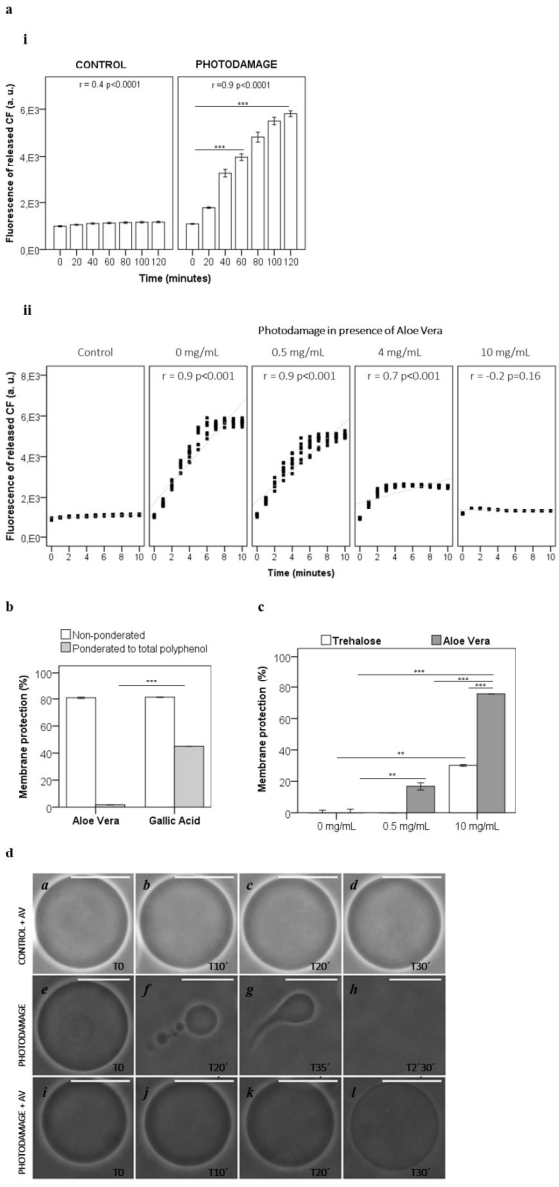

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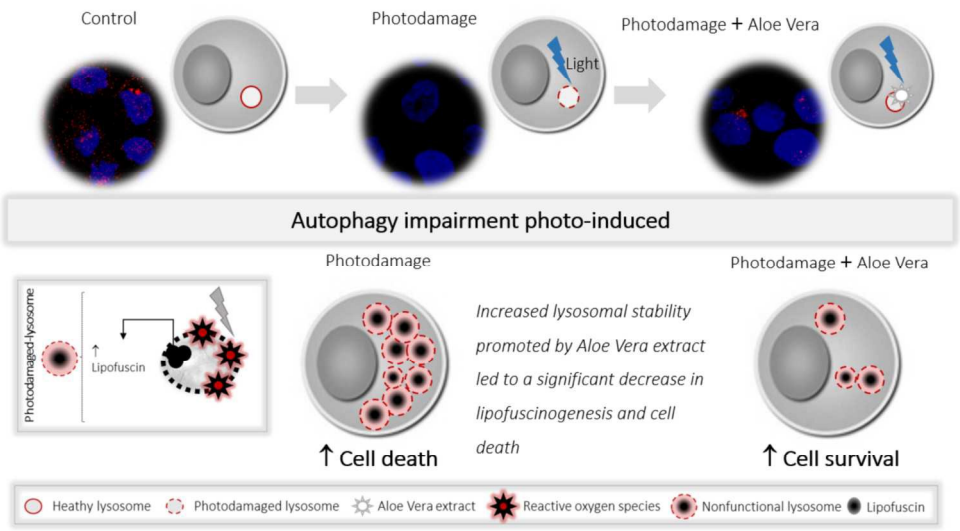

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