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Preliminary ethnobotanic screening in HaCaT cells and skin explants of medicinal plants from the Wadi Araba region in Jordan

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Abbreviations:
UV – Ultraviolet;
DCFDA – 2',7' – Dichlorofluorescin diacetate;
ROS – Reactive oxygen species;
PBS – Phosphate buffered saline;
DMEM – Dulbecco's modification of Eagle's medium

A B S T R A C T

Medicinal plants have been traditionally used to address and treat a variety of pathologies including skin diseases. Six medicinal herbs, grown in Jordan, were evaluated for their ability to reduce UVB-induced skin damage, a major contributor to erythema, skin aging and cancer formation. Ethanolic, acetonic and aqueous extracts were prepared from the stems and leaves of the plants. Human keratinocyte HaCaT cells were incubated without or with the different extracts for 24 hr, exposed to UVB irradiation and taken for viability and apoptosis assessment by resazurin and caspase-3 determinations, respectively. Selected extracts were further evaluated by the DCFDA method (ROS production measurement) and on pig and human skin explants. From the 18 extracts screened, only *Citrillus colocynthis* extracts showed significant protective properties against UVB-induced apoptosis. UV protective activity was found in the water extracts of both stem and leaf preparations of the plant. UV protection was independent of ROS production. Importantly, *Citrillus colocynthis* extracts were also able to attenuate the photodamage in both *ex vivo* skin models used in this study. Therefore, the current preliminary screening indicates that Jordanian medicinal plants might be valuable sources for novel skin care compounds. This study also suggests that *Citrillus colocynthis* extracts exhibit significant photoprotection properties and may attenuate the deleterious effects of UVB radiation.

1. Introduction

Herbs and plants have been used routinely for medicinal purposes for millennia (Verpoorte, 2009). Natural plant products still play a highly significant role in drug discovery and development processes. However, the majority of traditional herbal medicines lack scientific evidence based examination (Ramawat et al., 2009). The Hashemite kingdom of Jordan is composed of several distinct ecosystems ranging from dense forest to arid desert. There are more than 2,500 wild plant species from 700 genera - of those 100 are endemic and 375 are rare or extremely rare species (Aburjai et al., 2007; Afifi-Yazar et al., 2011; Al-Qura'n, 2005). The Dead Sea region of Jordan, and the Wadi Araba region specifically, are home to numerous tribes and villages with ethnobotanic knowledge and practices that are yet to be fully explored (Afifi-Yazar et al., 2011; Sinha and Hader, 2002).

In order to adapt to extreme environmental conditions, desert plants have developed unique properties and metabolism. Specifically, plants in the Araba region adapted to the arid desert were exposed to UV radiation (UVR), high temperatures and

shortage of water. Therefore, others and we have hypothesized that these plants might synthesize unique compounds to protect themselves from UV damage (Rewald, 2012). Indeed, several studies have demonstrated that plants in such extreme environments increase the synthesis of phenolic compounds that absorb the radiation and low molecular weight ROS scavengers that increase their antioxidant defense (Rewald, 2012).

The current preliminary screening of the medicinal properties of the extracts was focused on their ability to protect human cells and skin explants against ultraviolet B (UVB)-induced damage. Although low levels of UVR have several beneficial effects, such as the formation of melanin and vitamin D3 (Brenner and Hearing, 2008; Reichrath and Rass, 2014), high dosage of radiation may hamper normal skin function. High or chronic exposure to UVR results in the induction of inflammatory response in the form of erythema, or sunburn and may even lead to carcinogenesis and apoptosis in a reactive oxygen species (ROS)-dependent manner (Clydesdale et al., 2001). Therefore, the present study aimed to investigate the protective capacity of the extracts against UVB-induced damage. The specific goal was to determine whether the extracts reduce apoptosis induction and attenuate ROS production. The current data suggests that *Citrillus colocynthis* extracts have a unique capacity to protect against the harmful effects of UVB in a ROS independent manner.

2. Materials and Methods

2.1. Materials

Culture media and sera were purchased from Biological Industries (Beit-Haemek, Israel). Unless specified, reagents and analytical grade substrates were from Sigma-Aldrich (Rehovot, Israel). Caspase-3 substrate was purchased from Calbiochem (Darmstadt, Germany). DCFDA was obtained from Invitrogen-Molecular Probes (Eugene, OR, USA).

2.2. Plant extracts preparation

Acetonic, ethanolic and aqueous extracts of the plants listed in Table 1 were prepared in Jordan as follow: The leaves and stems of all plants were thoroughly washed with tap water to reduce dust and non-plant materials and dried under shade for six days. The materials were grounded by mortal and pastel and 50 g of each sample were added to 50 ml of double distilled water, ethanol or acetone. The solutions were gently mixed for 16 hr at constant shaking in reduced light. Non soluble solids were removed by centrifugation (2,000 G, 4ºC, 30 min). The supernatants of the aqueous samples were sterilized by filtration (0.22 μM filter; Pall Corporation, Ann Harbor, MI, U.S.A.). The acetone extracts were evaporated and re-suspended in equal volume of ethanol to reduce vehicle cytotoxicity. All samples were aliquoted and stored at -80°C until used.

2.3. Human and pig skin preparation and HaCaT cell culture

Human skins were obtained with permission from 20-60 year-old healthy women undergoing aesthetic abdomen surgery or breast reduction, after signing an informed consent. All experiments were conducted with approval of the IRB (Helsinki Committee) of Soroka Medical Center, Be'er Sheva, Israel (number 0228-13SOR). Pig ears were purchased from the Lahav Clinical Research Organization CRO (Kibbutz Lahav, Israel). Both skin explants preparations were used no longer than 24 hr after obtained. The skins were thoroughly washed with phosphate buffered saline (PBS) and cleaned from residual hypodermis tissue by scalpel. A mechanical skin press was used to section the skin to 0.8x0.8 cm^2 pieces, as previously described (Cohen et al., 2013; Wineman et al., 2012; Portugal-Cohen et al., 2011; Portugal-Cohen et al., 2009). The skin pieces were laid in 6-well culture plates containing skin culture medium (Dulbecco's modification of Eagle's medium [DMEM] supplemented with 100U/ml penicillin and 100 μg/ml streptomycin), dermal side down in the medium and epidermis up. All samples were used after an overnight recovery.

HaCaT cells (human immortalized keratinocyte cell line) were purchased from CLS Cell Lines Service (Eppelheim, Germany). The cells were grown and maintained in DMEM supplemented with 10% FBS, 100U/ml penicillin and 100 μg/ml streptomycin, as described by others (Boukamp et al., 1988).

2.4. UVB challenge

HaCaT cells were seeded at 96 well ($3*10^5$ cell/well) in complete growth medium. After 48 hr, the medium was replaced and the cells were incubated for 24 hr without or with 0.01% (v/v) of the herbal extracts. Then, the cells were washed three times with PBS and exposed to 25 mJoule UVB (λ~290 nm). The cells were allowed to recover in complete growth medium for 5 hours before subjected to the functional tests. Similarly, human and pig skin explants were treated topically with 2.5 μl (1 g/ ml) of the extracts for 24 hr. The samples were then washed with PBS and exposed to 400 or 200 mJoule UVB, respectively, as previously described (Wineman et al., 2012). The explants were allowed to recover overnight at standard conditions.

2.5. Apoptosis measurement

Caspase-3 activity assay was performed in order to monitor the apoptosis levels in both HaCaT cells and skin explants, as

described before (Kleszczynski et al., 2013). Briefly, 5 hours after UVB exposure the HaCaT cells medium was aspirated and 100 µl/well caspase-3 substrate solution (1:50 500mM DTT, 1:200 10% Triton x, 1:1000 caspase-3 substrate in PBS) was added. The degree of apoptosis was measured repeatedly and read 20 times at 2 minute intervals (ex. 355 nm, em. 460 nm), according to the suppliers instructions. The kinetic slope of each sample was calculated. Measurements of tissue apoptosis levels of human and pig epidermis samples were performed as described previously (Cohen et al., 2013). In order to separate the epidermis, the skin pieces were incubated at pre-wared PBS (56°C) for 1 min. Then, the epidermis layer was carefully peeled using a scalpel. After 1 hr recovery in PBS, the epidermis was transferred to a black 96 fluorescent plate that contained the caspase-3 mixture, as described above. Experiments were performed in triplicates in three independent experiments.

2.6. Resazurin viability measurement

Following the recovery period, the medium was replaced and both cells and epidermis samples were incubated with 3% resazurin prepared in serum free medium at 37°C for 1 hr. The fluorescence intensity of resazurin's reduced form, resorfin, was then measured (ex. 544 nm, em. 590 nm) according to the manufacturer's instructions. Experiments were performed in triplicates in three independent experiments.

2.7. DCFDA reactive oxygen species assay

HaCaT cells at 80% confluence were incubated with 25 µM of the green fluorescence dye 5, (and 6) -carboxy-2' 7'-dichlorodihydrofluoresceine diacetate (DCFDA) for 20 minutes, according to the manufacturer's protocol. Following the mounting period, epidermis was peeled as described above and fluorescence was determined immediately (ex. 485 nm, em. 538 nm). HaCaT cells without treatment were used as negative control. HaCaT cells treated with hydrogen peroxide served as positive control.

2.8. Statistical Analysis

Results are given as Mean ± SEM of three independent experiments. Statistical analyses were performed using Student's t test. Results with $p<0.05$ were considered significant.

3. Results

3.1. UVB protection of desert plants extracts on HaCaT cells

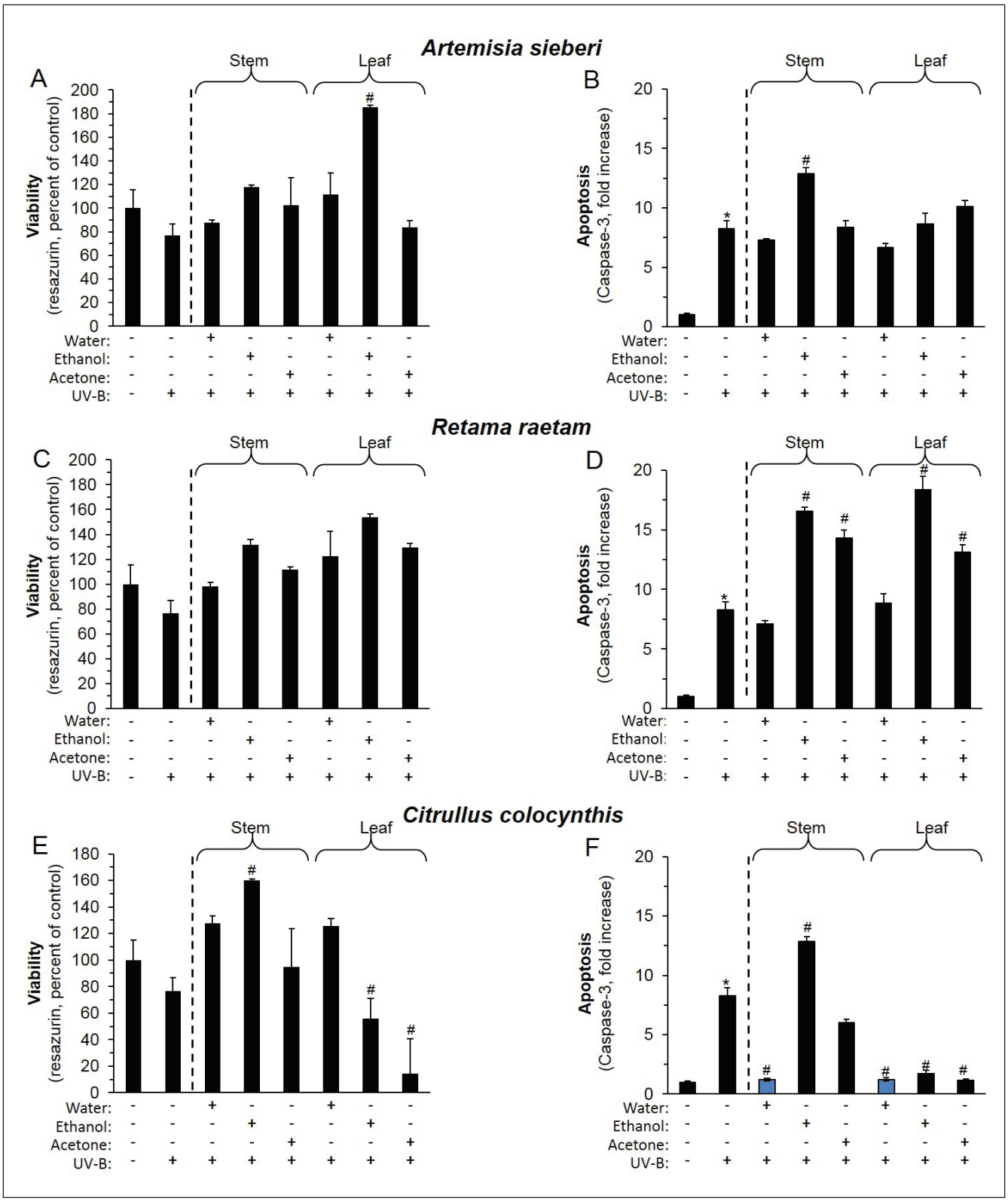
In the search for natural products with medicinal properties, six plants traditionally used in the Wadi Arabah region were selected following oral survey of local inhabitants (Table 1). To exclude bias, the questions asked were either general, regarding their knowledge of local medicinal plants, or alternatively, samples of local plants were presented to locals who were asked about the plants' medicinal properties. The acetonic, ethanolic and aqueous extracts of the herbs were prepared from both stems and leaves.

In order to investigate the extracts' ability to protect against UV radiation, the human immortalized keratinocyte cell line, HaCaT, was incubated without or with the different extracts for 24 hr and then exposed to UVB radiation. Following recovery, cellular viability and apoptosis levels were measured using resazurin and caspase-3 assays, respectively. As expected, the short UVB challenge alone slightly reduced cell viability and induced a dramatic eight-fold increase in caspase-3 activity (Figure 1). Of note, the vehicle solvents used, DDW and ethanol (0.1% in the culture medium), had no noticeable effect on both tests (95.78 ± 7.34 and 104.1 ± 7.97 for DDW and ethanol in the resazurin assay, and 1.06 ± 0.51 and 0.98 ± 0.47 in the caspase-3 assay, respectively).

The data in figure 1 shows the impact of the different extracts on the viability and apoptosis levels of the cells; the aqueous and acetonic extracts prepared from *Artemisia sieberi* had no noticeable effect on both parameters. However, treatment with the ethanolic extract from its leaf resulted in two-fold increase in viability (Figure 1 A&B). Interestingly, ethanolic and acetonic extracts of *Retama raetam* showed an increase in apoptosis rates (Figure 1 C&D). This suggests pro-apoptotic compound(s) are eluted from *R. raetem.*

Importantly, as clearly demonstrated in figure 1 E&F, treatment with the aqueous extracts from both stem and leaf of *Citrullus colocynthis* significantly attenuated Caspase-3 induction, and counteract the reduction in the cell viability thus blocking UVB-induced damage to the cells. Although the ethanolic and acetonic leaf extracts of the plant also show reduced Caspase-3 activity, cell viability was simultaneously reduced, indicating a toxic response of these extracts and not a protective effect.

All extracts derived from *Pergularia tomentosa* and *Atripex halimus* showed no significant effect on the HaCaT cell viability and apoptosis rate (Figure 1 G&J). In addition, several *Peganum harmala* extracts significantly increased cell viability, without any effect on apoptosis (Figure 1 K&L).



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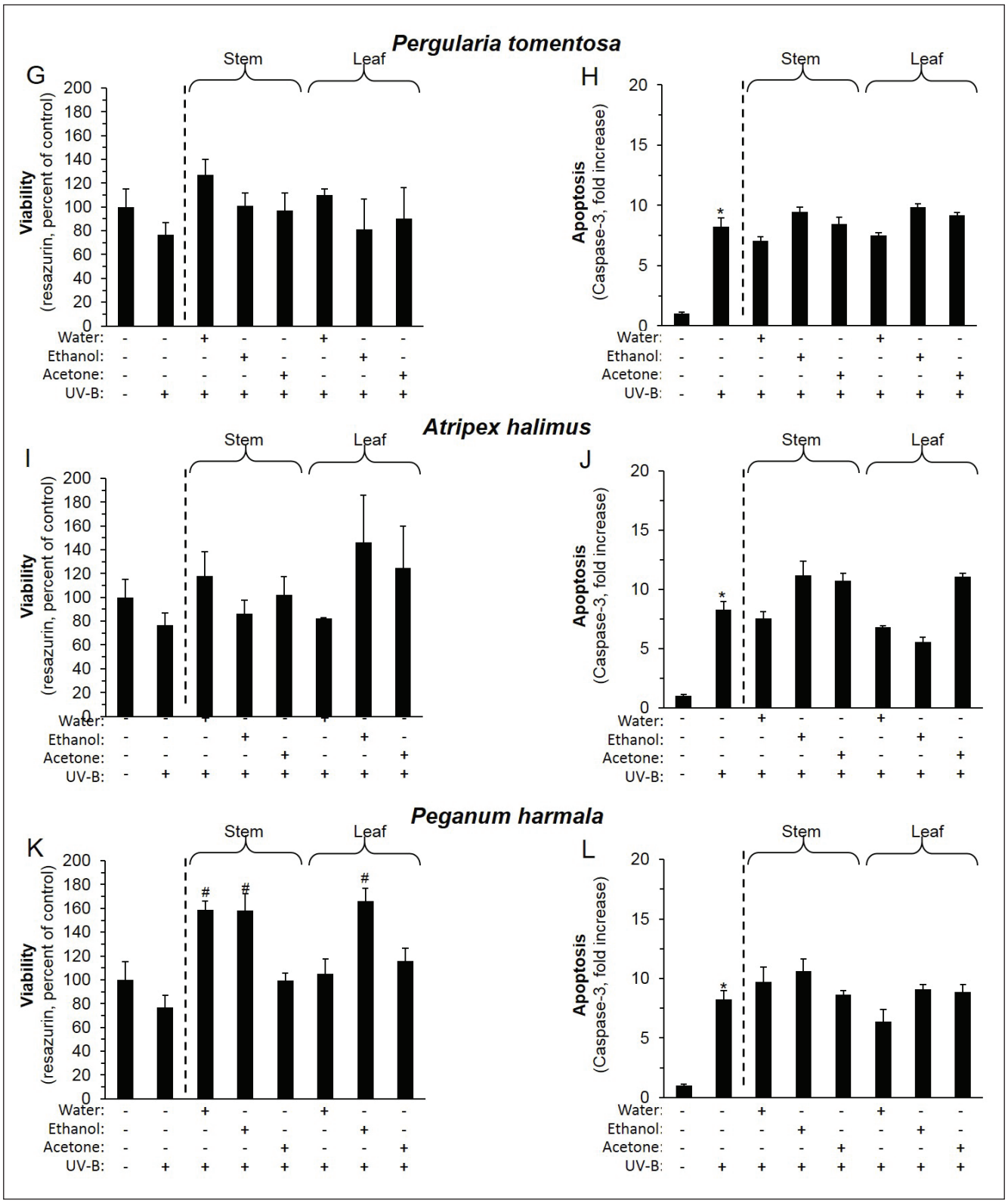

Figure 1: Extract screening in HaCaT cells. The cells were incubated without or with 0.01% (vol/vol) of the indicated extracts for 24 hr. Then, the cells were washed in PBS and exposed to 25 mJoule UVB (λ~290 nm). After recovery, cell viability and apoptosis were measured, as described in the "Methods" section. ***A&B** Artemisia sieberi extracts; **C&D** Retama raetam extracts; **E&F** Citrullus colocynthis extracts; **G&H** Pergularia tomentosa extracts; **I&J** Atripex halimus extracts; **K&L** Peganum harmala extracts;* *p<0.05 for difference from untreated cells; #p<0.05 for difference from the respective UBV-treated control group. Result are shown as Means ± SEM; n=3.

3.2. The photoprotective effect of Citrullus colocynthis is ROS independent

One of the major pathways underling UV radiation damage is the production of ROS (Heck et al., 2003). The hypothesis that *C. colocynthis* extracts increase the cell's antioxidant capacity was therefore tested. HaCaT cells were treated with the different *C. colocynthis* extracts and ROS generation was measured by the DCFDA method. Cells treated with exogenous hydrogen peroxide (1 mM) served as positive control. As clearly shown in figure 2, ROS production was unaltered by the different *C. colocynthis* extracts.

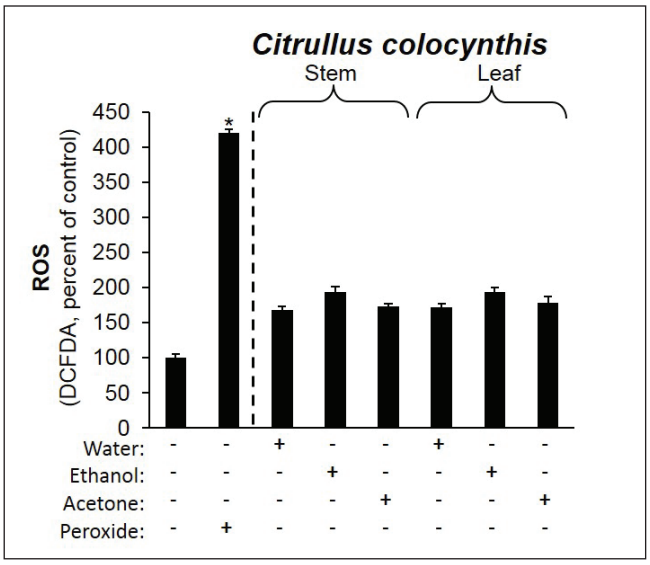

Figure 2: *Citrullus colocynthis* photoprotection is ROS independent. HaCaT cells were incubated without or with 0.01% (vol/vol) of ***Citrullus colocynthis*** extracts for 24 hr. Cells were then washed in PBS and exposed to 25 mJoule UVB (λ~290 nm). After recovery, ROS production was measured with the DCFDA compound, as written in the "Methods" section; *p<0.05 for difference from untreated cells. Result are shown as Means ± SEM; n=3.

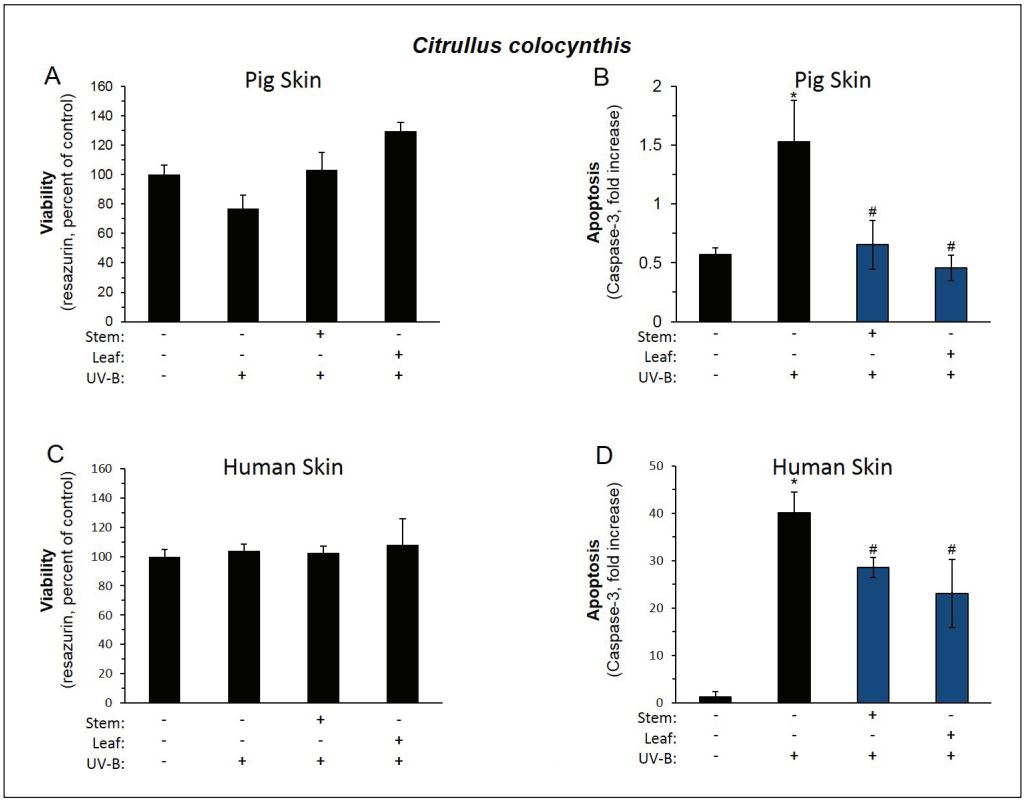

Figure 3: *Citrullus colocynthis* attenuates UVB-induced apoptosis. Pig skin (A&B) or Human skin explants (C&D) were incubated without or with 2.5µl of Citrullus colocynthis extracts topically for 24 hr. The samples were then washed with PBS and exposed to 200 mJoule UVB. After recovery, epidermis viability and apoptosis were measured, as described in the "Methods" section; *p<0.05 for difference from untreated cells; #p<0.05 for difference from the respective UBV-treated control group. Result are shown as Means ± SEM; n=3.

3.3. Citrullus colocynthis reduce UVB-induced damage in human and pig skin tissues

To further investigate the photoprotective effects of *C. colocynthis*, its ability to protect normal human and pig skin explants against UVB challenge was investigated. Figure 3 (A&B) shows that both stem and leaf extracts markedly attenuated UVB-induced apoptosis. Importantly, the aqueous extracts also attenuate apoptosis in human skin explants (Figure 3 C&D). Collectively, these results indicate that *Citrullus colocynthis* extracts effectively protect skin from UVB irradiation.

4. Discussion

This work has demonstrated for the first time the skin photoprotective effects of medicinal plants of Jordan in *in vitro* and *ex vivo* skin models. Of the six plants tested, only *C. colocynthis* showed significant photoprotective properties.

Many tribes and villages around the world still rely on locally grown and cultivated plants to help remedy and cure a wide variety of diseases and conditions (Verpoorte, 2009). In the Wadi Araba region in Jordan, copious tribes were consulted and provided information regarding traditional plants that are used to this day by the local elders. Yet, the scientific evaluation of those plants on skin models and on other medicinal properties are scarce.

Several studies have reported on medicinal properties of *Citrullus colocynthis*. Heydari *et al*. and Shafaei *et al.* have recently found anti-diabetic properties of the plant extracts *in vitro* and *in vivo* (Heydari et al., 2015; Shafaei et al., 2014). However, to our knowledge, this is the first study that demonstrates skin photoprotective properties of the plant. Interestingly, petroleum ether extracts of *C. colocynthis* have been reported to block androgen-induced alopecia (Dhanotia et al., 2011). Therefore, the plant may exhibit several beneficial skin care effects.

UV radiation is considered a major etiological factor in erythema formation, skin aging and skin cancer (Ichihashi et al., 2000; Larroque-Cardoso et al., 2015). High dosage or cumulative exposure to radiation may hamper normal skin function and induce cellular damage by several distinct mechanism. First, UV may directly cause covalent modification of macromolecules. If absorbed by the DNA, cyclobutane–pyrimidine dimers (CPDs) or 6–4 photoproducts pyrimidine dimers may be formed that subsequently results in genotoxic damage (Ichihashi et al., 2000). UVB-induced oxidative stress and ROS generation is another major contributor to cellular damage. Augmented ROS levels and an imbalance between prooxidant and antioxidant levels after UV stimuli may cause non-enzymatic modified proteins and phospholipids, augmented generation of lipid peroxidation products that contributes to the induction of apoptosis and cell death (Aldini et al., 2007). Our data suggest that *C. colocynthis* extracts protect against UVB radiation in a ROS independent mechanism. Therefore, it is possible that the herbal extracts reduce the genotoxic damage, either by direct absorbance of the radiation or by the induction of the cellular repair mechanism. Further work is required in order to investigate this hypothesis.

The three solvents that were tested had very different polarities and as such, different compounds were extracted from each plant. The results showed unique apoptotic profiles, with ethanol samples yielding the highest apoptosis values, as suggested by Reichardt (Reichardt, 2004). The *C. colocynthis* leaf samples behaved in a similar fashion in all three solvents and reduced the apoptosis levels. The plant stem samples had contradictory effects on HaCaT cells. While the aqueous and acetonic extracts were shown to have anti-apoptotic effects, the ethanolic extract had a pro-apoptotic effect. Viability was not affected in the *C. colocynthis* samples extracted in water as opposed to those extracted in less polar solvents. This indicates that non-aqueous extracts may contain a toxin(s) present in the *C. colocynthis* plant. Interestingly, previous reports indicate that *C. colocynthis* is moderately toxic (Aburjai et al., 2007), therefore, solvent preparation may separate these deleterious effects from its beneficial ones.

The current study also found that the ethanolic and acetonic extracts of *Retama raetam* induce a marked increase in apoptosis in HaCaT cell, but not its aqueous extracts. Pro-apoptotic compounds are needed in medicine for various conditions that include auto-immune diseases and cancer. Moreover, essential oils of *R. raetam* have been proposed as antimicrobial compounds that attenuated the growth of six different bacteria species (Awen et al., 2011). Collectively, this data suggest that *R. raetem* may be used as a source for new botanic based drugs. Further studies are needed to ascertain its therapeutic value.

In summary, this work reports for the first time the effects of important ethnobotanic species from Jordan on skin care. In the various screens carried out on HaCaT cells as well as on human and pig skin explants, different samples were found to potentially modulate the skin response to UVB exposure. Future work is needed to isolate the specific bioactive element(s) from these plants that could lead to the development of new therapeutics.

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