Natural dietary antioxidants containing flavonoids modulate keratinocytes physiology: In vitro tri-culture models

Natural dietary antioxidants containing flavonoids modulate keratinocytes physiology: *in vitro* tri-culture models.

Running title: role of natural compounds on keratinocyte metabolism.

Eleonora Bassino[1*], Franco Gasparri[2], Luca Munaron[1]

[1]Department of Life Sciences and Systems Biology, University of Turin, Italy. [2]Department of Pharmacy, University of Salerno, Italy.

Abstract:

ETHNOPHARMACOLOGICAL RELEVANCE: Several traditional medicinal herbs are widely used for dermatologic and cosmetic preparations. The beneficial skin repair activity is detected in various phases of wound-healing process, such as cell-cell, cell-matrix interactions or collagen synthesis.

AIM OF THE STUDY: The study assessed the effects of *Opuntia ficus-indica* (L.) Mill. (*Opuntia*) and Milk Thistle (MT) (*Silybum marianum* (L.) Gaerth) on adult keratinocytes (HaCaT) functioning under basal condition or in the presence of mechanical damage (wounded cells).

MATERIALS AND METHODS: The role of the natural compounds was tested on HaCaT grown in mono-culture and tri-culture configurations. In tri-cultures models, HaCaT were treated with the conditioned media (CM) obtained by Human Normal Dermal Fibroblast (NHDF) and Human Dermal Microvascular Endothelial cells (HMVEC) co-cultures. Specifically, were tested cell viability, oxidative stress mechanisms (cytokines release and lipid peroxidation) and cellular remodelling (growth factors release or metalloproteinase modulation). Moreover, the migratory potential of HaCaT was analysed by the use of wound healing *in vitro* assay.

RESULTS: *Opuntia* and MT differently modified the metabolism (EGF, MMP-9), and the migratory properties of HaCaT both under physiological conditions or upon mechanical damage (wounded cells). Moreover, both compounds modulated HaCaT response to oxidative stress. The response to the natural compounds were modified, and in some cases potentiated, in tri-culture configuration systems.

CONCLUSIONS: The data demonstrated that *in vitro* tri-culture approach is suitable to characterize the role of natural compounds on the complex communication between dermal-epidermal cellular components and microvascular endothelium. Specifically, *Opuntia* and MT are good alternatives to synthetic compounds in skin repair promotion.

KEYWORDS:

endothelial cells, keratinocytes, *Opuntia ficus-indica* (L.) Mill., milk thistle, wound-healing.

***Corresponding author**:

Correspondence to:
Eleonora Bassino, Ph.D.
Dept. Life Sciences & Systems Biology
University of Torino

Via Accademia Albertina 13

10123 Torino, ITALY

eleonora.bassino@unito.it

1. INTRODUCTION

Human skin acts as an environmental barrier to physical, chemical and biological agents. It is directly exposed to environmental pollutants, solar radiation, and other mechanical and chemical insults, which trigger free radical generation (Poljšak et al., 2012; Waris et al., 2006). Several traditional herbs and plant parts are reported to be effective in oxidative stress conditions and on dermal wounds. These include extracts from *Aloe vera* (L.) Burm f. leaves, that promote healing by enhancing collagen turnover in the wounded tissue (Chithra et al., 1998), *Sedum telephium* L. leaves (Raimondi et al., 2000), *Cinnamomum zeylanicum* bark (Kamath et al., 2003), and *Calendula officinalis* L. (Leach 2008). The beneficial skin repair activity is detected in various phases of wound-healing such as cell-cell, cell-matrix interactions and collagen synthesis (Gescher et al., 2011; Parente et al., 2012; Pirbalouti et al., 2010). Of particular interest is *Opuntia ficus-indica* (L.) Mill. (*Opuntia*), a species of cactus plant that grows in arid and semi-arid regions of the world. Due to its biological and pharmacological properties, this plant has been employed for different medicinal purposes. In Sicily folk medicine, *Opuntia* cladodes are used for the treatment of gastric ulcer (Barbera et al., 1993). It has been shown that *Opuntia* contain poly-phenolic compounds such as flavonoids and phenolic acids which provide antioxidant activity (Corral-Aguayo et al., 2008; Sànchez et al., 2014). Most of the studies focus on the protective activity of fruits and stems, but all plant parts display attractive biological activities and may be used for the production of promising bioactive compounds. *Opuntia* extracts are reported to protect against neuronal damage produced under oxidant conditions (Kim et al., 2006), or against renal and hepatic alterations caused by mycotoxins (Zourgui et al., 2008). An ethanol extract of cladodes decreased the oxidation of linoleic acid and showed a dose-dependent free-radical scavenging activity (Lee et al., 2002). *Opuntia* has anti-diabetic and anti-cancer properties (Frati-Munari et al., 1990), and its cladodes have been used in the treatment of several skin disorders, including ulceration (Galati et al., 2006) and skin wound healing (Park et al., 2001).

The topically application of *Opuntia* polysaccharides ((MW)>10(4) Da) induced beneficial effects on cutaneous repair accelerating reepithelization phase in rats wound models (Trombetta et al., 2006). Mucilaginous and methanol extracts of *Opuntia* accelerated wound contraction and remodeling phases in rats wound models (Ammar et al., 2015). In wound healing experiments similar results were exerted by the low molecular weight of *Opuntia* polysaccharides (Di Lorenzo et al., 2017). In Human keratinocytes *Opuntia* exerted anti-oxidant activities suggesting its beneficial role in treating barrier-disrupted skin disorders (Nakahara et al., 2015). Moreover, similar anti-oxidant beneficial effects were observed in UVA stressed keratinocytes (Petruk et al., 2017).

Milk thistle (MT) (*Silybum marianum* (L.) Gaerth.) is a medicinal plant, originating from the Mediterranean area and used in the treatment of liver diseases (Ligeret et al., 2008). Milk thistle root, young shoots and leaves being consumed as food (Post-White et al., 2007). It has been reported that polyphenols (i.e. flavonolignans) in MT seeds exerted antioxidant, anti-inflammatory, hypolipidemic, and anti-carcinogenic effects (Katiyar 2005). Silibinin, one of the major active constituents of silymarin extracted from MT, is bioactive (Lee et al., 2003). In the work of Fehér et al., (2016) was evidenced that topical formulations containing silymarin inhibit UVB irradiation induced oxidative stress in guinea pig and HaCaT keratinocytes (Fehér et al., 2016). Skin cream containing MT seed oil, palmitoyl peptides, vitamin E improved facial wrinkles, elasticity, dermal density and skin tone (Hahn et al., 2016). Recently, it has been reported that MT was effective in limiting the direct DNA damage, reducing lipid membrane peroxidation mechanism and increasing cellular antioxidants (Di Caprio et al., 2017). While both plants (*Opuntia* and MT) are associated with a beneficial effect on human skin, little is known about the regulation of dermal-epidermal cross-talk and its interaction with dermal microvascular endothelium. In the present study we tested the effects of both the natural compounds on cellular physiology of adult keratinocytes using *in vitro* tri-cultures (Bassino et al., 2017) to better characterize the paracrine communication between

different cells types that populate the dermal/epidermal layers and sub-cutaneous vasculature upon injury.

2. Materials and Methods

2.1. Cell cultures

Human Adult Keratinocyte cell line (HaCaT) was obtained from Cell Line Services (CLS Cell Lines Service, Germany). HaCaT were grown in Dulbecco's Modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic (Invitrogen, Grand Island, NY).

Human microvascular endothelial cell line (HMVEC) was purchased from Lonza and grown in Endothelial Cell Grown Medium (EGM 2-MV medium, Lonza, Basel, Switzerland).

Normal human dermal fibroblast (NHDF) was purchased from Lonza and grown in FGMTM-2 Fibroblast Growth Medium-2 (Lonza) that contains 2% serum and 1% antibiotic/antimycotic (Invitrogen, Grand Island, NY).

All cell types were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Compounds

Opuntia (*Opuntia ficus-indica* (L.) Mill.: highly-concentrated dry extract of *Opuntia ficus-indica* cladodes) (BIONAP, Catania, Italy) Composition (*Opuntia* % W/W): Polysaccarides from *Opuntia ficus indica* cladodes) = 40-60. (For more chemical details see Di Lorenzo et al., 2017).

Milk Thistle (MT) (*Sylibum Marianum* (L.) Gaerth. fruit, Dray Extract). Silymarin calculated as silibinin min. 80% (adapted spectrophotometric method). Silymarin calculated as silibinin min. 65% (HPLC DAB method) (EUROMED S.A., Barcelona, Spain).

2.3. Co-cultures

HMVEC were thawed from frozen stock and seeded in 24 well plates at a density of $3x10^3$ cells/well. For cell viability in co-culture, NHDF were thawed, centrifuged, recounted, and seeded at a density of $3x10^3$ cells/well for 2 days in DMEM 10% FCS until 100% confluence on transwell

clear polyester membrane inserts (pore size 0.4 μm, area 0.3 cm^2; Corning). Natural compounds were added to HMVEC grown in presence of NHDF for 24 hrs (Table 1 and scheme in Figure 2).

2.4. Tri-cultures

To establish the tri-cultures, HaCaT were seeded in 24 or 96 well plates for 24 hrs in DMEM 10%; then DMEM 10% was substituted with DMEM 0, 10%, or with CM obtained from co-cultures (NHDF+HMVEC grown with or without natural compounds). CM1: NHDF+HMVEC; CM2: NHDF+HMVEC (*Opuntia* at three concentrations); CM3: NHDF+HMVEC (MT at three concentrations) (see Table 1 and scheme in Figure 2).

2.5. Cell viability.

For viability assays in tri-cultures, HaCaT were plated in 24 well plates at the density of $3x10^3$ cells/well. Cell viability was analysed after 24 hrs of incubation of HaCaT with DMEM 0-10%, *Opuntia*, MT or CM obtained from the co-cultures (CM1, CM2 and CM3) (See table 1). Cell viability was evaluated by the CellTiter 961 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS). The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. Formazan product was measured with a FilterMax F5 microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm as absorbance is directly proportional to the number of viable cells.

2.6. Enzyme-linked immunosorbent assays (ELISA)

MMP9, EGF and IL-1β levels were assessed by enzyme-linked immunosorbent assay (ELISA) in medium and cell lysates using commercially available kits (Sigma-Aldrich ELISA kit, Invitrogen ELISA Kit). Briefly, 100 μl of medium or cell lysates were incubated into an antibody-coated 96-

well plate at room temperature for 2.5 hrs. The wells were washed four times with wash buffer solution. Then 100 µl anti-human MMP9, EGF and IL-1β antibody was added and the samples were again incubated for 1 hr at room temperature. The plate was washed four times, 100 µl of streptavidin-peroxidase conjugated was applied for 1 hr at room temperature. After a final washing, 100 µl tetramethylbenzidine substrate was added and allowed to develop for 30 minutes in the dark at room temperature. After stopping the reaction with 50 µl stop solution containing citric acid 2.0 mML, absorbance was read at 450 nm with a F5 FilterMax microplate reader 550 (Molecular Devices, Sunnyvale, CA). Sample concentration was calculated from the standard curve.

2.7. Lipid peroxidation

The Click-iT® Lipid Peroxidation Imaging Kit-Alexa Fluor® 488 (ThermoFisher) leverages copper-catalyzed click chemistry and the linoleamide alkyne (LAA) reagent (alkyne-modified linoleic acid) for detection of lipid peroxidation-derived protein modifications in fixed cells. Briefly, Click-iT® LAA stock solution was added to the cells at a final concentration of 50 µM, thereafter we treated HaCaT with H_2O_2 used alone or in association to *Opuntia*, MT or CM for 24 hrs. Afterwards, cells were washed 3 times with PBS to remove free Click-iT® LAA from cells, immediately fixed (3.7% formaldehyde in PBS for 20 min) and permeabilized (0.5% Triton for 10 min). The permeabilized cells were then blocked by adding 1% BSA in PBS solution for 30 min Finally, 125 µl of Click-iT® reaction cocktail is added to each well for 30 min at room temperature, protected from light. Fluorescence (excitation/emission maxima: Alexa Fluor® 488 azide: 495/519 nm) was acquired using Nikon T-E microscope (10-40x objective). Cell fluorescence was measured with ImageJ software.

2.8. Scratch wound healing

HaCaT were seeded in 24 multi-well plates and culture until confluent. A scratch was made in the confluent monolayer with a plastic disposable pipette tip (10 µl) (Bassino et al., 2017). Debris was removed from the culture by gently washing with sterile PBS. Hereafter, HaCaT were cultured in DMEM 10%, DMEM 0%, *Opuntia*, MT, or CM for 24 hrs. Experiments were performed using a Nikon T-E microscope (4x objective). Cells were kept at 37°C and 5% CO_2 for all experiments. Photos were taken every 4 hrs using Metamorph software. Cell migration was measured with ImageJ software. At least three fields for each condition were analyzed in each independent experiment.

2.9. Statistical analysis

Statistical significance of all experiments was evaluated by GraphPad (GraphPad Software Inc., San Diego, CA, USA) using nonparametric test for all experiments. The Dunn's multi-comparison test was chosen because data were not normally distributed. All values were presented as the mean ± standard deviation (SD). For each experimental condition 5 technical and 3 biological replicates were performed. Results with p-values<0.05 are considered statistically significant: (* or § $p<0.05$).

3. RESULTS

3.1. Effects of Opuntia, MT and CM on HaCaT viability: mono-culture and tri-culture configurations.

The high dose of *Opuntia* significantly prevented cell death induced by serum deprivation (DMEM 0%) (24 hrs) (Fig. 1a), while only lower doses (12.5 μg/ml, 1.25 μg/ml) of MT resulted effective (Fig. 1b). In tri-culture configurations systems (Fig. 2), the incubation of HaCaT with CM1 significantly enhanced cell survival (Fig. 2a, b). Treatment with CM2 (CM2-m) increased cell number compared to CM1 (Fig. 2a) suggesting a pro-mitogenic effect, while no further variations were exerted by all concentrations of CM3 (CM3-h, CM3-m and CM3-l) (Fig. 2b).

3.2. Antioxidant activities of natural compounds

The antioxidant activity was determined by different assays based on the damaging effects of H_2O_2 on HaCaT. MT (lower doses) treatment significantly prevented IL-1β release induced by H_2O_2 (500 μM, 24 hrs), while *Opuntia* was ineffective (Fig. 3 a-b).

In tri-cultures, CM1 reduced of IL-1β release and a similar effect was observed for all the concentrations of CM2 (CM2-h, CM2-m and CM2-l) (Fig. 3c). Conversely, CM3-h was more effective (Fig. 3d). CM1 significantly reduced lipid peroxidation in HaCaT exposed to oxidative stress (Fig. 4a), and a similar activity was exerted by low concentrations of *Opuntia* and MT (12.5 μg/ml and 1.25 μg/ml), while the highest dose (5 mg/ml) enhanced lipid peroxidation (Fig. 4c). Treatment with lower doses of CM2 or CM3 (CM2-m, CM2-l, CM3-m and CM3-l) strongly reduced lipid peroxidation, while the highest concentration (CM2-h and CM3-h) resulted ineffective (Fig. 4d, e).

3.3. Regulation of cellular remodelling by Opuntia and MT in monoculture and tri-culture configurations.

Opuntia did not significantly modify the release of MMP-9 from HaCaT in intact monolayer (Fig. 5a). On the other hand, all concentrations of *Opuntia* significantly increased MMP-9 release from damaged cells (wounded monolayer) maintained in DMEM 0% (Fig. 5b). CM1 increased MMP-9 production (Fig. 5c) while all concentrations of CM2 exerted the opposite effect (Fig. 5c). Mechanical damage (24 hrs wound) inhibited MMP-9 release (Fig. 5d) with a further reduction induced by CM2 (CM2-h, CM2-m, CM2-l) (Fig. 5d). MT (5 mg/ml and 12.5 μg/ml) slightly reduced MMP-9 from HaCaT after 24 hrs of treatment (Fig. 6a). Conversely, MT drastically reduced MMP-9 in wounded cells (Fig. 6b). In tri-culture configuration, CM3-h, CM3-m and CM3-l drastically reduced MMP-9 secretion compared to the cells treated with CM1, suggesting a potential role of MT in the modulation of matrix metalloprotease when added to a more complex system (Fig. 6c). In wounded monolayer, CM3-h and CM3-m strongly reduced the MMP-9 secretion compared to CM1 (Fig. 6d).

3.4. Role of natural compounds in the regulation of wound closure

To evaluate the role of compounds and CM in the wound healing rate of HaCaT, we employed an established *in vitro* scratch wound healing assay. Wound closure was evaluated by observing the re-populated area between the wound margins at different time intervals (0-24 hrs) after the lesion. Control sample (DMEM 10%) followed the physiological healing process, reaching approximately 55% of closure at 24 hrs after injury (Fig. 7a-c). All doses of MT and *Opuntia* increased HaCaT migration when added to poor medium (DMEM 0%) (Fig. 7a-c). Lower concentrations of *Opuntia* (12.5 μg/ml and 1.25 μg/ml) strongly promoted wound healing (Fig. 7c).

In the tri-cultures, CM1 enhanced healing, reaching 90% of closure at 24 hrs after injury (Fig. 8a-c). CM3-m significantly increased HaCaT migration respect to DMEM10%, while the other two doses reduced cell migration compared to CM1; the wound-healing effect was similar to DMEM 10% (Fig. 8a, b). CM2-h completely inhibited cell migration, whereas the other doses did not further modify CM1 response (Fig. 8c).

3.5. Role of Opuntia and MT on growth factors release

Opuntia did not modify EGF production by intact monolayer respect to the CTRL condition (DMEM 10%) (Fig. 9a). In the wounded monolayer, the highest dose of *Opuntia* significantly increased EGF production after 24 hrs of treatment (Fig. 9b). MT (12.5 μg/ml and 1.25 μg/ml) slightly increased EGF production in intact monolayer, and exerted a positive effect at the highest concentration (5 mg/ml) on wounded monolayer (Fig. 9c, d). In tri-cultures, CM1 enhanced the production of EGF by intact HaCaT monolayer upon treatment for 24 hrs (Fig. 10a). CM2-m and CM2-l further increased the effect (Fig. 10a). Interestingly, EGF production was also induced upon mechanical damage (wounded HaCaT monolayer) (Fig. 10b). In these experimental conditions, CM2-m and CM2-l promoted a release of EGF significantly higher than CM1 (Fig. 10b). Similar results were obtained for CM3-h and CM3-m both on intact and wounded monolayer (Fig. 10c, d).

4. DISCUSSION

Dermatologically active herbal ingredients are directly incorporated into topical formulations or added to oral dietary supplements (Allemann et al., 2009). Recent studies evidenced that plant-derived secondary metabolites improve wound healing. In particular, *in vitro* approaches allow to investigate the influence of plant extracts on the metabolic activity of normal and damaged keratinocytes. Polyphenols act against wound infection and stimulated keratinocyte proliferation (Werdin et al., 2009; Deters et al., 2001). Moreover, polysaccharides promote keratinocyte growth, stimulated extracellular matrix formation (Deters et al., 2005) and enhance dermal tissue remodelling by induction of fibroblast differentiation (Zippel et al., 2009). On human umbilical vein endothelial cells (HUVECs), non-cytotoxic micromolar concentrations of betalain (a pigment of *Opuntia*) dampen the expression of cell adhesion molecules such as ICAM-1 (Gentile et al., 2004).

In the present work we focalised our attention on the complex crosstalk between the several skin cellular components. The effects of *Opuntia* and MT were characterized on the paracrine communication between cells that populate the dermal and epidermal layers and microvascular endothelium. For this study *in vitro* co-culture and tri-culture setups were validated in our laboratory (Bassino et al., 2015; Bassino et al., 2017).

Recently, we showed that several natural compounds, including flavonoids, are active in vasculature re-growth during HF disruption, but their effects have not been yet evaluated directly on microvascular endothelial cells (Bassino et al., 2016). The results obtained in the present work reveal the ability of both *Opuntia* and MT to modify keratinocyte metabolism both on intact monolayers and in the presence of mechanical or chemical injuries (respectively the application of a wound and the treatment with H_2O_2). Both compounds improve wound healing of HaCaT in tri-culture models. *Opuntia* promotes EGF release and HaCaT migration. These results are in nice agreement with literature: indeed, topical application of *Opuntia* extracts on skin lesions accelerates the reepithelization affecting cell-matrix interactions and modulating laminin deposition (Trombetta

et al., 2006). A recent study reports that *Opuntia* is able to reduce oxidative stress in keratinocytes and to increase the expression of epidermal barrier proteins, potentially preventing the skin barrier disruption found in atopic dermatitis or eczematous disorders (Nakahara et al., 2015). Moreover, we observed that MT strongly prevents MMP9 release, not only in physiological conditions but particularly in wounded monolayers. Accordingly, recent studies report the role of Silibinin in the inhibition of MMP9 expression by cancer cells (Kim et al., 2011; Lee et al., 2007). In addition, Silymarin attenuates mast cell recruitment thereby decreasing the MMP-2 and -9 expression in rat liver carcinogenesis (Ramakrishnan et al., 2009). Moreover, Roy et al., (2012) showed that the topical administration of silibinin protects against UVB-induced photodamage: this evidence provides a strong rationale to investigate the possibility that this bioactive compound may reduce the risk of non-melanoma skin cancer (Roy et al., 2012).

Finally, we unveiled the protective role of both compounds against oxidative stress. Specifically, MT and *Opuntia* decrease cytokine production when added in tri-cultures and abrogate lipid peroxidation, a biochemical event usually leading to a functional damage of cell membranes.

5. CONCLUSIONS

In summary, the results of this study suggest a role for both *Opuntia* and MT in the regulation of keratinocytes physiology. In particular, they differentially modify the paracrine communication between cells located in the dermal-epidermal layers and the sub-cutaneous vasculature, not only in physiological conditions but also upon mechanical or chemical damages.

Conflicts of interest

The authors declare not conflict of interest

Authors' Contributions

E.B. designed the research study, performed the research, analysed the data, and wrote the paper. F.G. contributed essential reagents or tools and designed the research study. L.M. designed the research study, analysed the data, wrote the paper, and contributed essential reagents or tools.

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Figure legends

Figure 1. **Role of *Opuntia,* MT on HaCaT viability**. Schematic configurations of *in vitro* mono-culture systems. a) *Opuntia* (5 mg/ml) prevented cell death; b) MT (12.5 μg/ml and 1.25 μg/ml) slightly but not significantly prevented the reduction of viability.

Figure 2. **Role of CM on HaCaT viability.** Schematic configuration of in vitro tri-culture systems. a) CM1 and CM2 (all tested doses) significantly prevented cell death; b) CM1 and CM3 prevented cell viability.

Figure 3. Modulation of cytokines production. a) *Opuntia* failed to prevent IL-1β production after the treatment with H_2O_2; b) MT (12.5 μg/ml and 1.25 μg/ml) significantly reduced IL-1β; c) CM1 and all CM2 concentrations significantly reduced IL-1β release; d) CM3-h and CM3-l reduced IL-1β production.

Figure 4. Regulation of lipid peroxidation mechanism in H_2O_2 stressed HaCaT. a) CM1 prevented lipid peroxidation after 24 hrs of incubation; b, c) *Opuntia* and MT (5 mg/ml) further increased oxidative stress induced by H_2O_2. The lower doses of both compounds completely blocked lipid peroxidation mechanism. d, e) CM2-h and CM3-h increased lipid peroxidation; the lower doses drastically reduced oxidative stress.

Figure 5. **Regulation of metalloproteinase production by *Opuntia* (24 hrs).** a) *Opuntia* did not modify MMP-9 production under basal conditions; b) in wounded cells all concentrations of *Opuntia* prevented MMP-9 reduction observed in DMEM 0%; c) CM1 significantly increased MMP9 production respect to DMEM 0%. No further variations were observed in presence of CM2; d) In wounded HaCaT, CM2 (all concentrations) drastically reduced MMP-9 production.

Figure 6. **Regulation of metalloproteinase production by MT (24 hrs).** a) All doses of MT did not significantly modify MMP-9 production under basal condition; b) All doses of MT drastically reduced MMP-9 production upon wound application; c) CM3 (all doses) did not modify MMP-9 production respect to the cells maintained in DMEM 0%; d) CM3-h and CM3-m drastically reduced MMP-9 production upon wound application.

Figure 7. Effects of *opuntia and* MT on HaCaT migration. a) Representative microphotographs of wound healing: untreated or treated (*Opuntia* and MT) monolayers at 0 hrs and 24 hrs after injury (4×magnification); b, c) Wound healing quantification. The closure percentage values are referred to 24 hrs of treatment.
a-c) *Opuntia* and MT increased cell migration when added to DMEM 0% (24 hrs). The percentage of wound closure was lower compared to that observed in cells maintained in DMEM 10%.

Figure 8. Effects of CM on HaCaT migration. a) Representative microphotographs of wound healing: untreated and CM-treated HaCaT monolayers at 0 hrs and 24 hrs after injury (4×magnification); b, c) Wound healing quantification. The closure percentage values are referred to 24 hrs of treatment.
a-b) CM1 and CM3 (-m, -l) increased cell migration when added to DMEM 0% (24 hrs). The percentage of wound closure was lower compared to that observed in cells maintained in DMEM 10%. a-c) CM1 and CM2 (-m, -l) increased cell migration when added to DMEM 0% (24 hrs). The percentage of wound closure was lower compared to that observed in cells maintained in DMEM 10%.

Figure 9. Regulation of EGF release by *Opuntia and MT*. a) O*puntia* did not modify EGF production under basal conditions; b) In wounded cells *Opuntia* (5 mg/ml) increased EGF production; c) MT (all doses) did not significantly modified EGF production under basal conditions; d) all doses of MT did not modify EGF production upon wound application.

Figure 10. Regulation of EGF release by CM. a) CM1 significantly increased EGF production, a further increment was induced by CM2-m; b) In wounded HaCaT, CM2-h, CM2-m, and CM2-l increased EGF production similar to CM1; c) CM3-h, CM3-m and CM3-l increased EGF production, the values were higher respect CM1; d) CM3-h strongly increased EGF production upon wound application.