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Honeydew honey: biological effects on skin cells

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Abstract Honey is a natural product well known by humankind and now reconsidered for its use as topical agent for wound and burn treatments. Floral honey is made by honeybees from the nectar of blossoms, while honeydew honey is prepared from secretions of plants or excretions of plant-sucking insects. Chemical composition is different between blossom and honeydew honeys and there is very few information about the biological properties of honeydew honey. So, this study was specifically designed to explore the potential wound healing effects of the honeydew honey. We used in vitro scratch wound healing model consisting of fibroblasts and keratinocytes. Data showed that honeydew honeys is able to increase wound closure by acting both on fibroblasts and keratinocytes. Based on our findings, honeydew honey has the potential to be useful for clinical settings.

Keywords Chemotaxis · Honeydew honey · Scratch wound assay · Wound repair

Introduction

There is a long and intriguingly history between men and honey; in particular, honey has been utilized from the beginnings as a traditional medicine by different cultures to treat a plethora of disorders [1].

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Nowadays, the scientific interest towards honey is increasing and it is currently accepted as effective agent for wounds and burns [1].

Floral honey is made by honeybees from the nectar of blossoms, while honeydew honey is prepared from secretions of living parts of plants or excretions of plant-sucking insects on the living part of plants.

Differentiation between floral and honeydew honey is a response to consumer demands; in many countries, nectar honey is valued more highly than honeydew honey but, in other countries, honeydew honey is preferred [2].

In fact, chemical composition is considerably different from blossom honey. Differences are mostly created because of passage of juice through insect's intestine. So, honeydew honey contains enzymes of saliva glands and intestine that induce differences in the spectre of carbohydrates in respect to blossom honey. Honeydew honey has been found to contain higher di- and trisaccharide contents, as well as lower mean contents of glucose and fructose that nectar honey. Moreover, honeydew honey also presents a high polyphenol content, antioxidant and antibacterial activity [3].

In respect to blossom honey [4], there are very few and anectodical information about the biological effects of honeydew honeys. Slovak fir honeydew honey has recently showed excellent antibacterial activity against multi-drug-resistant clinical isolates of *Stenotrophomonas maltophilia* and wound pathogens [5]. Therefore, given the wide traditional use and the dermatological properties of honeys, this study was specifically designed to explore the potential wound healing effects of the honeydew honey.

To this aim, we used in vitro scratch wound healing model consisting of fibroblasts and keratinocytes [6, 7].

Over the last decades, ethno-pharmacological studies have included in vitro assays as a replacement for

experiments using tissues or whole animals [8]. These in vitro tests are very useful in research because of ethical reasons and of their usefulness in bioactive-guided fractionation and determination of active compounds [9].

The wound healing mechanism is a well-orchestrated process composed of several overlapping phases, during which different cell types are regulated and involved. For this reason, we included in our experiments two cell types playing main roles in skin restoration, i.e. keratinocytes and fibroblasts.

Our data showed that honeydew honeys is able to increase wound closure by acting both on fibroblasts and keratinocytes. Results pointed out a Ca^{2+}-dependent process and to require the involvement of several cell signalling pathways. Based on our findings, honeydew honey has the potential to be useful for clinical settings.

Materials and methods

Honeydew honey samples

Samples of Japanese and Bulgarian honeydew honeys were obtained from Yamada Apiculture Center, Inc. (Tomata-Gun, Okayama, Japan).

Cell culture and reagents

All reagents of analytical grade were from Sigma-Aldrich, unless otherwise indicated. HaCaT cells, a non-tumorigenic human keratinocyte cell line [6], and fibroblasts NIH 3T3 (American Type Culture Collection, ATCC CRL-1658) [10] were maintained at 37 °C, 5% CO_2, in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic mixture (1% penicillin–streptomycin).

Calcein-AM assay

The lipophilic, nonfluorescent calcein-acetoxymethylester (calcein-AM) penetrates cell membranes and is then cleaved by intracellular esterases, yielding the hydrophilic fluorescent dye. Cells were settled overnight in 96-well plates (8000 cells/well), incubated with honey for 24 h, washed with PBS, and then incubated for 30 min at 37 °C with a solution of 2.5 μM calcein-AM in PBS. Plates were read in a fluorescence plate reader (Infinite 200 Pro, Tecan, Wien, Austria), by using 485-nm exc and 535-nm em filters.

Scratch wound assay

Scratch wounds were created in confluent cell monolayers by using a sterile 0.1–10 μL pipette tip. After washing away suspended cells, cultures were refed with medium in the presence of different concentrations of honey for 24 h. Cells were fixed in 3.7% formaldehyde in PBS for 30 min, and then stained with 0.1% toluidine blue at room temperature for 30 min. The width of the wound space was measured at wounding and at the end of treatments, using an inverted microscope equipped with a digital camera (Leica Microsystems). Digitized pictures of wounds were analysed using the NIH ImageJ software. Wound closure rates were determined as the difference between wound width at 0 and 24 h.

Cell migration assay

A cell migration assay was performed in transwell plates (8 μm pore size, Costar, Cambridge, MA). A total of 1×10^5 cells per well were seeded in the upper compartment of filters. After 24 h, filters were removed and stained for 10 min with 0.5% crystal violet (145 mM NaCl, 0.5% formal saline, 50% ethanol), and then washed twice with water. The upper side of filters was scraped using a cotton swab to remove cells that had attached but not migrated. Following PBS washing of filters, the dye was eluted from cells with 33% acetic acid, and measured at 540 nm in a plate reader.

Statistical analysis

Data were analysed by ANOVA and the post hoc Tukey's test, using the Instat software package (GraphPad Software, Inc, San Diego, CA). Median (EC_{50}) and minimum (EC_{05}) effective concentrations and their 95% confidence intervals were determined by using a downhill logistic dose–response curve.

Table 1 Values of EC_{05} and EC_{50} (% v/v) determined in HaCaT cells for different honeydew honeys by the calcein-AM endpoint at 24 h

	EC_{05}	EC_{50}
Bulgarian	**2.30** (1.58–3.36)	**7.80** (6.6–11.23)
Japanese	**3.50** (2.25–5.46)	**10.67** (9.34–12.19)

Experiments were carried out in triplicate with a minimum of eight replicates each. 95% CI are given in parentheses

Table 2 Values of EC_{05} and EC_{50} (% v/v) determined in NIH 3T3 cells for different honeydew honeys by the calcein-AM endpoint at 24 h

	EC_{05}	EC_{50}
Bulgarian	**4.5** (2.8–7.10)	**17.74** (15.05–20.03)
Japanese	**2.55** (1.59–4.1)	**22.1** (18.9–15.73)

Experiments were carried out in triplicate with a minimum of eight replicates each. 95% CI are given in parentheses

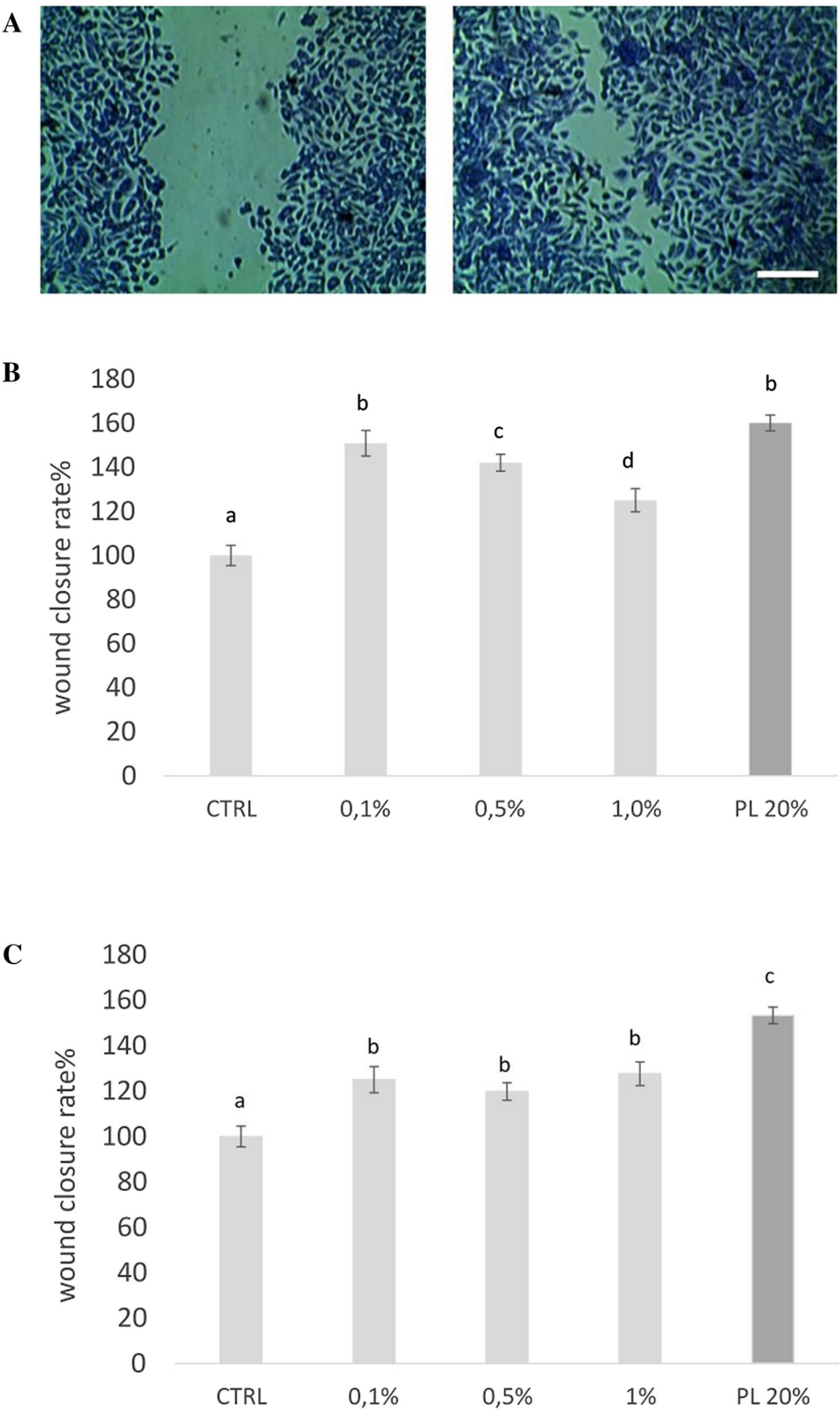

Fig. 1 Scratch wound healing of HaCaT confluent monolayers. Cells were cultured in 24-well plates and mechanically scratched with a sterile 0.1–10 µL pipet tip, and then allowed to re-epithelialize for 24 h at 37 °C in the presence of different honeydew honey concentrations (v/v). One sample was exposed to 20% platelet lysate (PL) as positive control. **a** micrographs of scratch wounded HaCaT monolayers incubated under control conditions (*left*) or in the presence of 0.5% Japanese honeydew honey (*right*) and then stained with blue toluidine and observed 24 h after wounding. *Scale bar* 200 µm. Measurements of wound closure after Japanese (**b**) or Bulgarian (**c**) honeydew honey exposure expressed as the difference between wound width at 0 and 24 h. *Bars* represent mean ± SD of two independent experiments, each with $n = 25$. The mean of control has been set to 100. *Different letters* on *bars* indicate significant differences according to the Tukey's test ($p < 0.01$)

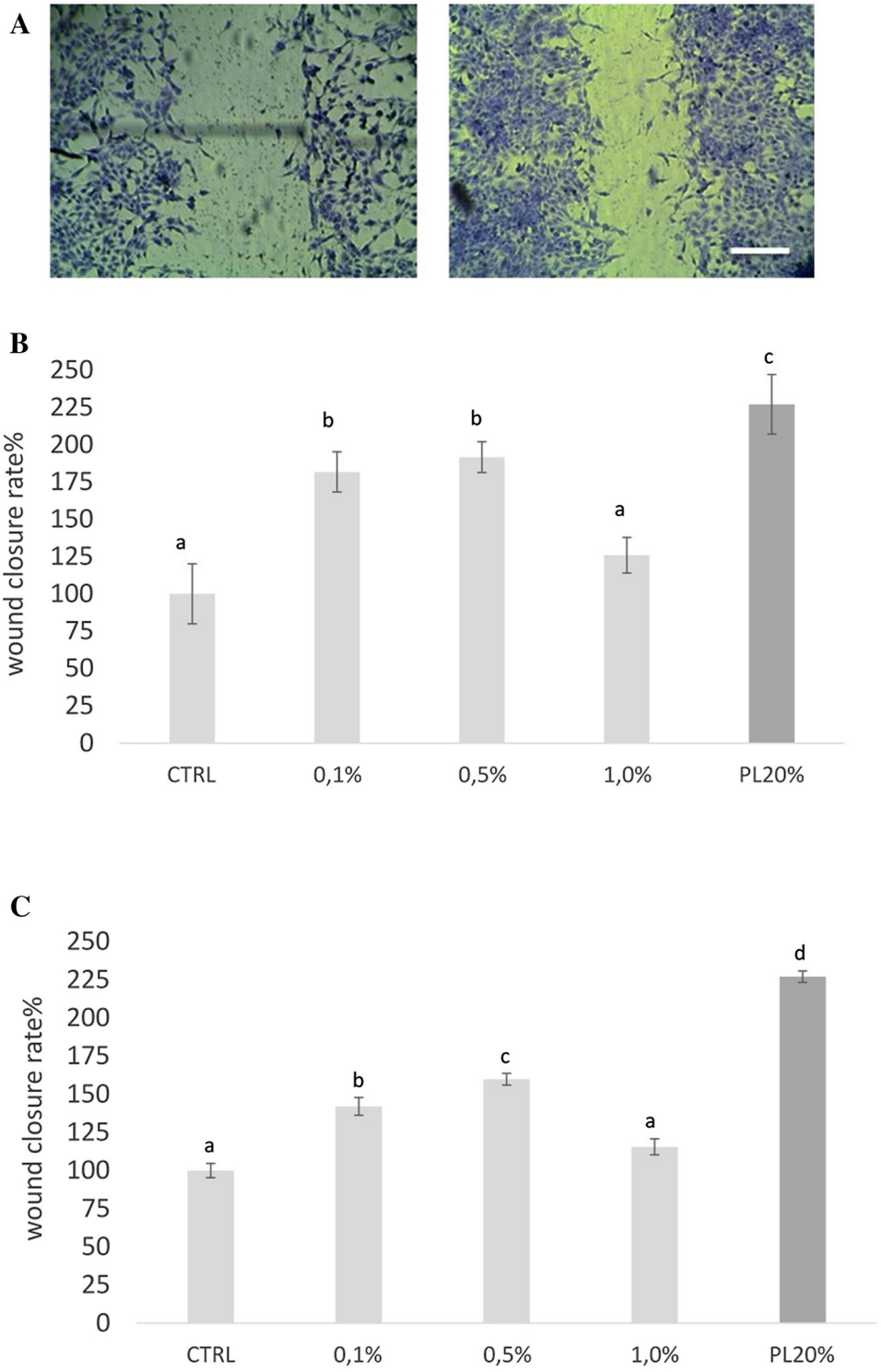

Fig. 2 Scratch wound healing of NIH 3T3 confluent monolayers. Cells were cultured in 24-well plates and mechanically scratched with a sterile 0.1–10 µL pipet tip, and then allowed to re-epithelialize for 24 h at 37 °C in the presence of different honeydew honey concentrations (v/v). One sample was exposed to 20% platelet lysate (PL) as positive control. **a** micrographs of scratch wounded NIH 3T3 monolayers incubated under control conditions (*left*) or in the presence of 0.5% Japanese honeydew honey (*right*) and then stained with blue toluidine and observed 24 h after wounding. *Scale bar* 200 µm. Measurements of wound closure after Japanese (**b**) or Bulgarian (**c**) honeydew honey exposure expressed as the difference between wound width at 0 and 24 h. *Bars* represent mean ± SD of two independent experiments, each with $n = 25$. The mean of control has been set to 100. *Different letters* on *bars* indicate significant differences according to the Tukey's test ($p < 0.01$)

Results

Cell proliferation

Two honeydew honey samples were utilized from different origins (Japan and Bulgaria), in order to assess the possible difference in inducing biological effects.

Cytotoxicity tests were carried out on HaCaT and 3T3 cell lines in order to optimize the dosages for in vitro scratch wound analyses.

Calcein-AM assay, as reported in Table 1 and Table 2, showed low toxicity levels for the two honeydew honey samples for HaCaT and NIH 3T3 cells. Based on these data, in subsequent experiments, honey concentrations

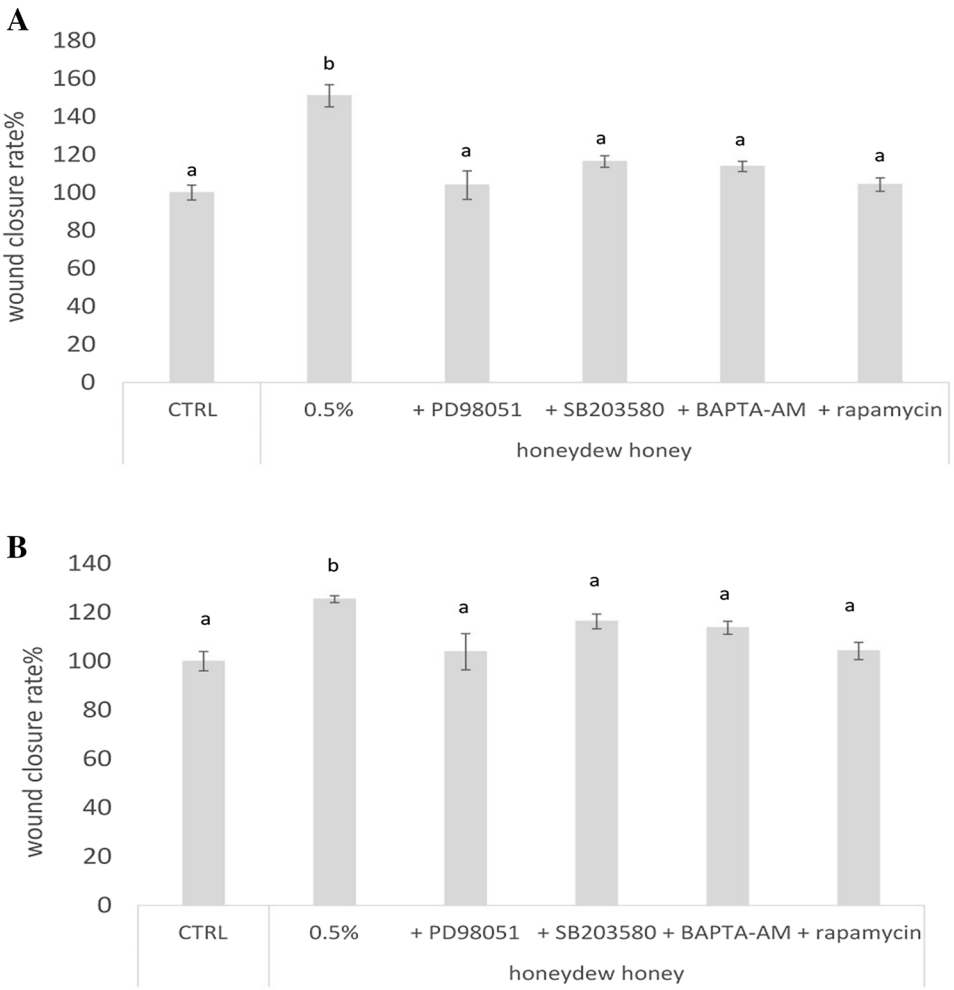

Fig. 3 Effect of different inhibitors on honeydew honey-induced scratch wound repair of HaCaT monolayers. Data were recorded 24 h after scratch wound healing of cells exposed to 0.5% honeydew honey (**a** Japanese, **b** Bulgarian), in the presence or absence of various inhibitors. *Bars* represent mean ± SD of percent wound closure inhibitions recorded in two independent experiments, each with $n = 20$. *Different letters* on *bars* indicate significant differences among groups according to the Tukey's test ($p < 0.01$)

below EC_{05} were used, and these concentrations are similar to that of an analogous study with honeys on keratinocytes and fibroblasts [4].

Scratch wound repair of HaCaT and NIH 3T3 in the presence of honeydew honey

Confluent monolayers of keratinocytes and fibroblasts were scratched and then incubated or not with honeydew honey samples. As a positive control, we used 20% (v/v) of a platelet lysate (PL), which had been previously shown to promote wound healing in these cells [6, 11]. The PL was obtained from blood samples as described in Ranzato et al. [12].

For keratinocytes, cells exposed to Japanese honeydew showed significantly higher wound closure rates at 24 h with respect to controls, while Bulgarian honeydew induced a significant but lower effect (Fig. 1).

For NIH 3T3 fibroblasts, cell exposed to Japanese honeydew honey showed a strong increase in wound closure. This increase is also present for at lower concentrations of Bulgarian honeydew honey (Fig. 2).

Effects of inhibitors on scratch wound assay

To investigate honey mechanism of action on wound closure, we performed a scratch wound battery experiments at 24 h in the presence of specific signal transduction pathway inhibitors, such as PD98059 (ERK kinase, 10 μM), SB203580 (p38 kinase, 20 μM), rapamycin (mTOR inhibitor, 100 nM), and BAPTA-AM (cell-permeant calcium chelator, 30 μM).

These inhibitors did not alter the basal wound closure rate in the absence of honeydew honey. Moreover, the vehicle alone (0.1% DMSO) produced no influence on wound closure, either in the presence or absence of honeydew honeys ($p > 0.05$) (data not shown).

For keratinocytes, we observed a similar pattern (Fig. 3), with the maximum inhibition exerted by the PD98059 and the BAPTA-AM, while the other inhibitors

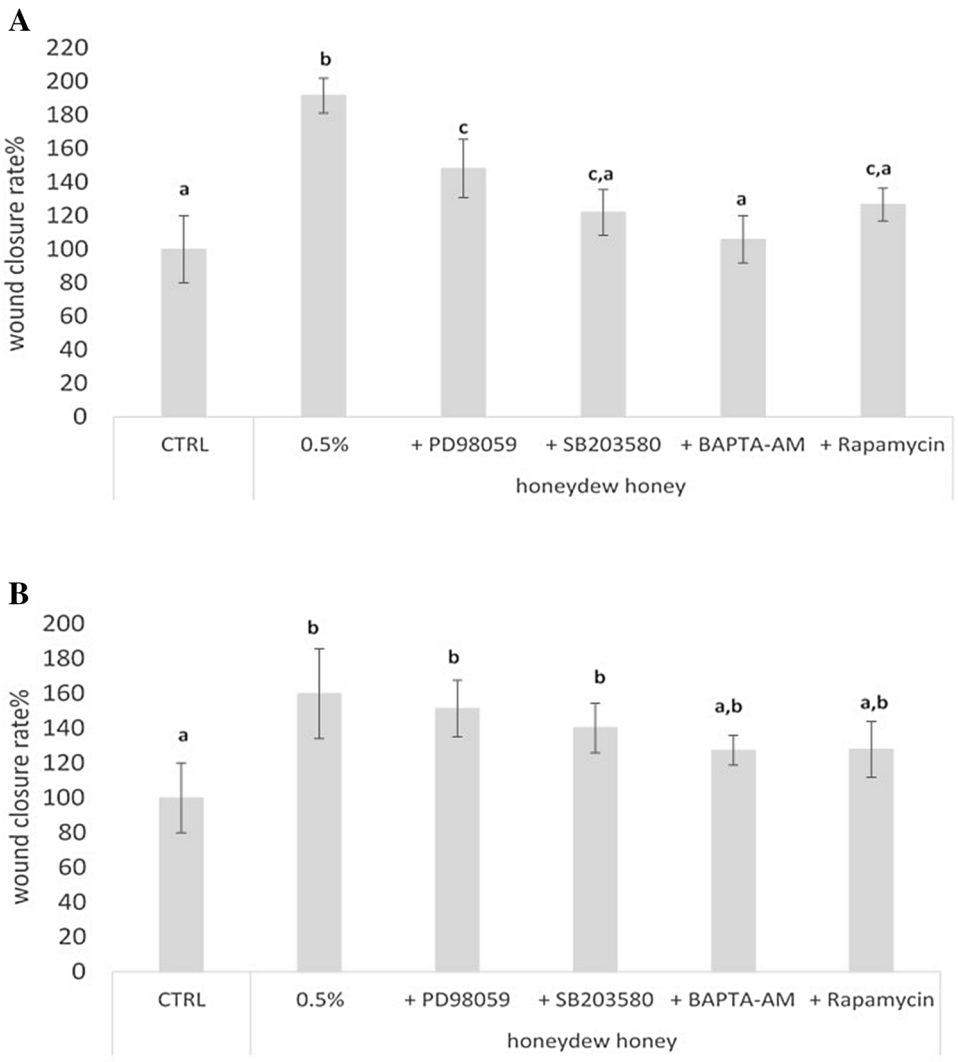

Fig. 4 Effect of different inhibitors on honeydew honey-induced scratch wound repair of NIH 3T3 monolayers. Data were recorded 24 h after scratch wound healing of cells exposed to 0.5% honeydew honey (**a** Japanese, **b** Bulgarian), in the presence or absence of various inhibitors. *Bars* represent mean ± SD of percent wound closure inhibitions recorded in two independent experiments, each with $n = 20$. *Different letters* on *bars* indicate significant differences among groups according to the Tukey's test ($p < 0.01$)

are still effective. And the behaviour is very similar for both samples of honeydew honeys.

Using NIH 3T3 fibroblasts, with both honeydew honeys, BAPTA-AM almost completely abolished the healing effect, while the other drugs induced different inhibitory effects (Fig. 4), resulting minimally sensitive to PD98059.

Chemoattractant effect of honeydew honey

To assess whether honeydew honeys influence cell migration rates, we used a chemotaxis assay. The results for both keratinocytes and fibroblasts showed that in the presence of 0.5% v/v honeydew honeys (of Bulgarian and Japanese origin), the number of migrating cells was significantly increased in the presence of honeydew honeys with respect to control ($p < 0.01$) (Fig. 5).

Moreover, we repeated wound healing assay in the presence of 10 μg/mL mitomycin C (MMC) for 2 h prior to the scratch assay. MMC inhibits mitosis of the cells and allowed us to distinguish between migration and proliferation. For 24-h exposure time, the main driving factor of wound closure is migration than proliferation, as demonstrated for both keratinocytes and fibroblasts (Fig. 6).

Discussion

Our data have showed that both honeydew honeys induce low cytotoxicity on HaCaT keratinocytes and NIH 3T3 fibroblasts, in accordance with previous results on keratinocytes and fibroblasts with blossom honey [13], suggesting that honeydew honey can be considered as non-toxic compound, and used safely not only for external applications on healthy skin, but also for wound healing procedures.

In fact, our scratch wound experiments showed that honeydew honey induces an increase of the wound repair capabilities of both keratinocytes and fibroblasts. We also

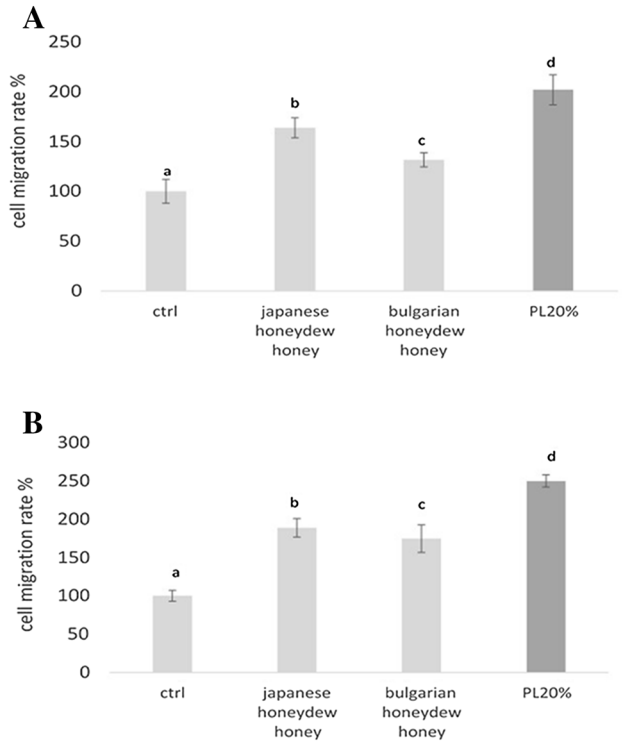

Fig. 5 Effect of 0.5% honey and of 20% platelet lysate (PL) as positive control on HaCaT cells (**a**) and NIH 3T3 cells (**b**) migration evaluated by transwell assay (see "Materials and methods" section). Data are mean ± SD ($n = 5$) of cell migration rate (see text) expressed as percentage variation with respect to control. *Different letters* on *bars* indicate significant differences among groups according to the Tukey's test ($p < 0.01$)

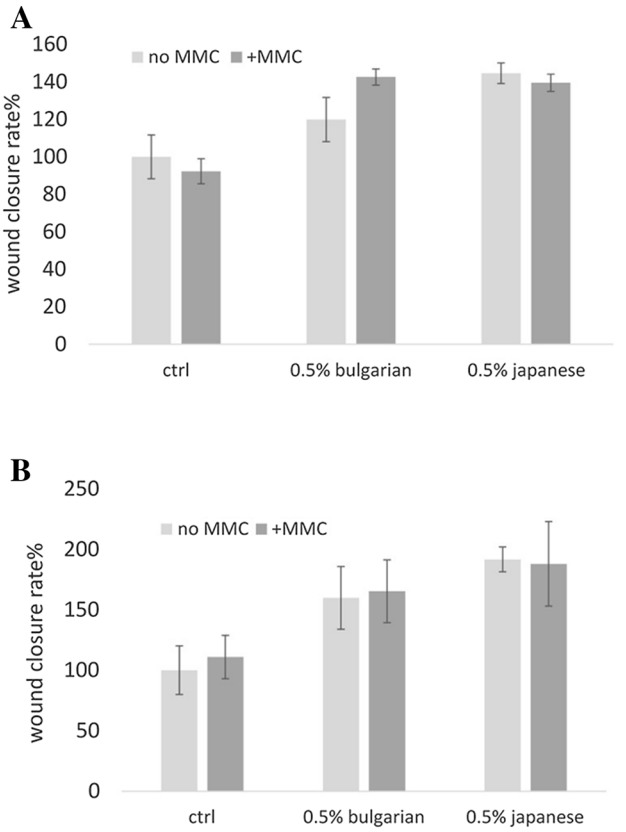

Fig. 6 Effect of mitomycin C (MMC) on wound repair of HaCaT **a** and fibroblasts **b** monolayers. Data were recorded 24 h after scratch wound healing of cells exposed to 0.5% honeydew honeys, in the presence or absence of MMC (10 μg/mL, 2 h prior to the scratch assay). *Bars* represent mean ± SD of percent wound closure inhibitions recorded in two independent experiments, each with $n = 20$

compared this effect to the platelet lysate (PL) stimulation that is already used as a dressing to improve wound repair [6, 14].

Japanese and Bulgarian honeydew honeys showed a dose-dependent effect in inducing the wound closure of the scratch, with the maximum of the effects at 0.5% (v/v), and a clear decrease after 1%. However, the induction of wound closure was lower than the effects induced by PL, whereas honeys (such as manuka), as already demonstrated [4], achieved similar positive effects [15].

Hence, in order to have better knowledge into the honeydew-stimulated wound healing, we performed scratch wound assay using a battery of well-known cell signal translation inhibitors.

The results of these analyses suggest that different pathways are involved in the mechanism of action of honeydew honeys. Furthermore, the pattern of inhibition was very similar (with slight differences for Japanese honeydew honey) among keratinocytes and fibroblasts, suggesting that honeydew honeys acts through the same mechanism in both cell types.

Furthermore, by means of BAPTA-AM inhibition results, we observed that cell calcium plays a basic role, as observed also in similar studies [6, 12]. The use of SB203580 and PD98059 provided evidence for the involvement of p38 and ERK1/2, two well-known pathways regulating cell migration and wound healing (see e.g. [16]).

An intriguing observation is that rapamycin, a mTOR inhibitor, is able to block the wound closure induced by honeydew exposure. The mTOR pathway represents an integral component of the normal wound healing process [9, 17], while its pharmacological inhibition with rapamycin delays wound closure [18].

mTOR is known to regulate cellular growth by integrating nutrient and hormonal signals, and we suggest that this rapamycin-sensitive effect of honeydew honeys on wound healing come from the role of some honeydew components as agent able to induce effects similar to nutrient stimulation.

Moreover, our cell migration assay showed that honeydew honeys improve both fibroblast and

keratinocytes wound repair capabilities. We know that cellular motility plays a pivotal role in tissue restoration phenomena, but both cell migration and proliferation are involved in wound healing, but their role depend on timing. To try to discriminate between these two phenomena in our timing (i.e. 24 h), we incubated cells with MMC prior to the scratch in order to prevent cell proliferation (mitosis). Therefore, we have observed that wound closure under honeydew honeys exposure was not significantly affected.

Wound healing is a complex process, and there are many mechanisms involved, but for 24 h exposure time, the main driving factor of wound closure is migration than proliferation and therefore, its induction could explain the ability of honeydew honeys to promote the scratch wound healing, similarly to what reported for blossom honeys [4].

Taken together our findings provide, for the first time, a scientific characterization of honeydew honeys properties on skin cells, suggesting that they could be successfully used in clinical settings.

However, other studies on the active components of honeydew honeys are strongly required to identify not only single effective molecules but also synergistic interactions between the huge number of active compounds interactions mediating biological effects.

In summary, our present data on honeydew honeys and previous findings on blossom honeys [13] indicate that honeys are very active in inducing and supporting wound healing processes, thus confirming a bulk of anecdotal and scientific evidence [19].

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