CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649044

Experimental Investigation of Honey as Wound Repair Enhancer by *In Vitro* Time-Lapse Microscopy

Flora Ascione[a], Antonio Zuorro*[b], Marco Fidaleo[b], Roberto Lavecchia[b], Stefano Guido[a,c,d], Sergio Caserta[a,c,d]

[a] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Università Federico II, P.le Tecchio 80, 80125 Napoli, Italy.
[b] Dipartimento di Ingegneria Chimica, Materiali e Ambiente, Università "La Sapienza", Via Eudossiana 18, 00184 Roma, Italy
[c] CEINGE Biotecnologie Avanzate, Via Sergio Pansini, 5, 80131 Napoli, Italy.
[d] Consorzio Interuniversitario Nazionale per la Scienza e Tecnologia dei Materiali (INSTM), UdR INSTM Napoli Federico II, P.le Tecchio, 80, 80125 Napoli, Italy.

Honey is widely used for treating wounds, burns and ulcers, due to its antimicrobial and anti-inflammatory properties. Recently, it has been shown that it can also promote tissue repair. However, the mechanisms of action in the wound healing process are still far from full comprehension. In this work we propose an experimental methodology to investigate the potential role of honey as wound repair enhancer, by analyzing *in vitro* cell motility and proliferation, that are the main mechanisms involved in the wound repair. Our methodological approach is based on the well-assessed *in vitro* Wound Healing (WH) assay, coupled with Time-Lapse Microscopy (TLM) and image analysis techniques. Our methodology was applied to test the potential healing activities of several kinds of honeys and mixtures of selected honeys, on monolayers of HT1080 human fibrosarcoma cells. The honeys were also characterized for their antimicrobial activity. Overall, this work is focused on raising new therapeutic interest in the valuable natural product honey.

1. Introduction

Honey is a viscous, supersaturated sugar solution produced by bees from nectar of plants as well as from honey dew. It contains water, sugars (30% glucose, 40% fructose, 8% maltose, 2% sucrose) and other active components, including aminoacids, vitamins, minerals, enzymes, proteins, carbohydrates, flavonoids, phenolic acids, and other phytochemicals (Sato and Miyata, 2000). The specific composition of honey depends on the flowers available to the bees and varies according to its geographical origin as well.
Honey has been shown to have antimicrobial and anti-inflammatory activity. The ability of honey to inhibit bacterial growth is related to several factors, the most important being an acidic pH (3–4), the osmotic action resulting from the high sugar content, the production of hydrogen peroxide by an enzyme added to the nectar by the bees, the presence of flavonoids and antioxidants (Bogdanov, 1997). Some Italian honeys of different floral origins, including eucalyptus, thyme, forest and honeydew honeys, have been found to have antimicrobial and anti-quorum sensing activities against pathogens commonly associated with burn and wound infections (Fidaleo et al., 2015). In addition, the anti-inflammatory action of honey has been assessed (Subrahmanyam, 1998). Due to its antimicrobial and anti-inflammatory potential, honey is very effective in treating burns and a range of wounds, including surgical incisions, pressure ulcers, catheter exit sites. Furthermore, many studies describing the role of honey in promoting wound healing have been recently reported (Sell et al., 2012). Honey was found to accelerate wound healing in several rat models when applied topically (Ker-Woon et al., 2014). The hypertonicity of honey and its low pH are considered the main factors in promoting wound healing (Iftikhar et al., 2010). However, some factors that can contribute to the healing property of honey are still largely unexplored. For example, it has not been clarified whether honey plays a role in increasing cell motility and growth during the wound closure process.
In this work, we propose a novel experimental methodology to quantify *in vitro* the potential effect of honey on wound healing kinetics. In particular, the methodological approach here proposed is based on the classical

Please cite this article as: Ascione F., Zuorro A., Fidaleo M., Lavecchia R., Guido S., Caserta S., 2016, Experimental investigation of honey as wound repair enhancer by in-vitro time-lapse microscopy, Chemical Engineering Transactions, 49, 259-264 DOI: 10.3303/CET1649044

Wound Healing (WH) assay coupled with live cell imaging by Time-Lapse Microscopy (TLM), and image analysis techniques. The WH assay, also known as scratch test, is a method extensively used for the quantitative characterization of cell dynamics *in vitro*. In this assay, an artificial scratch is created by mechanically scraping off an area of cells on a confluent cell monolayer. Stimulated by the empty space, the cells on the edges of the wound move and proliferate to cover the gap (Liang et al., 2007). The spreading of the two cell sheets dynamically evolving one toward the other, can be observed by using TLM image acquisition (Ascione et al., 2014; Caserta et al., 2013), that allows direct visualization of active bio-soft matter dynamics *in vitro*. This technique is based on iterative image acquisition of selected regions within the sample by means of a motorized video-microscope, equipped with an incubating system that allows keeping the sample in a controlled environment to ensure cell viability during the experiment.
Our methodological approach was applied to the evaluation of the potential influence of several kinds of honeys and mixtures of selected honeys on cell motility and proliferation, that are both relevant in the wound healing process. Honeys characterized for their antimicrobial activity were added to cell culture medium in different concentrations. HT1080 fibrosarcoma cells were used as a model system.
The experimental data were interpreted according to mathematical models based on transport phenomena concepts. Some preliminary results are reported here.

2. Materials and methods

2.1 Honeys

Two honeydew (fir HD and Metcalfa HD) and two nectar (eucalyptus and thyme) honeys were obtained from Rigoni di Asiago (Asiago, VI, Italy). They were collected from beehives in different locations in Italy and cold processed at temperatures below 30 °C. Honey samples were placed, as received, in glass containers and stored in the dark at room temperature until use.
An artificial honey (AH), mimicking the sugar content of honey, was prepared according to the following composition (wt%): 40 % fructose, 30 % glucose, 8 % maltose, 2 % sucrose, 20 % water.

2.2 Cell cultures and bacterial strains

HT1080 human fibrosarcoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) and antibiotics (50 units/mL penicillin, 50 µg/mL streptomycin) and maintained in a humidified incubator at 37 °C under an atmosphere of 5 % CO_2 in air.
Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 29887) and *Pseudomonas aeruginosa* (ATCC 10145) were obtained from KairoSafe (Duino Aurisina, Italy). Methicillin-resistant *Staphylococcus epidermidis* (MRSE), isolated from an infected surgical wound, was obtained from Tor Vergata University Hospital (Rome, Italy).

2.3 Determination of total phenolics and flavonoids

Total phenolics were determined by the Folin Ciocalteu's method with some modifications. Five mL of 0.1 M HCl, 150 µL of Folin-Ciocalteu's reagent and 200 µL of the sample to be tested were poured into a graduated glass vial and an aqueous sodium carbonate solution (10% w/v) was added to a final volume of 10 mL. The vial was shaken and kept at room temperature in the dark for 1 h. Then, the absorbance at 750 nm was measured. The results were expressed as gallic acid equivalents (GAE) using a calibration curve obtained with gallic acid standards.
Total flavonoids were determined according to the procedure described by Zuorro and Lavecchia (2014). Briefly, 300 µL of the sample, 900 µL of methanol, 60 µL of an aluminum chloride aqueous solution (10 % w/v), 60 µL of sodium acetate (1 M) and 1.7 mL distilled water were poured into an optical glass cuvette. The cuvette was shaken and kept in the dark at room temperature for 30 min. Then, the absorbance at 415 and 700 nm was measured. Measurements were made by a double-beam UV-VIS spectrophotometer (Lambda 25, Perkin Elmer, USA) against a blank of distilled water. The results were expressed as quercetin equivalents (QE) using a calibration curve obtained with quercetin standards.

2.4 Antibacterial activity assay

Antibacterial activity of honeys was determined by the agar-well diffusion method (Zuorro et al., 2010) following NCCLS (National Committee for Clinical and Laboratory Standards) guidelines. Briefly, bacterial cells from an exponential-phase culture grown in Mueller–Hinton (MH) broth were spread on the surface of agar (Mueller–Hinton Agar 2) plates using a sterile swab soaked in the bacterial suspension. Test plates were then prepared by cutting wells with a diameter of 9 mm in the agar layer. The wells were filled with 150 µL of honey. After 18-h incubation at 37 °C, the plates were examined and the diameter of the inhibition zone measured. Chloramphenicol (30 µg) and methicillin (5 µg) were used as positive controls.

2.5 *In vitro* WH assay

HT1080 fibrosarcoma cells were plated on uncoated 24-well culture dishes at a density of 1.5×10^5 cells/well. The cells were incubated at 37 °C for 24 h in order to allow them to attach and spread, creating continuous

monolayers. The samples were then scratched manually with a sterile p200 pipette tip. The medium containing cell debris was removed and the cells were washed twice with phosphate-buffered saline (PBS). Before starting the experiments, the wounded cell monolayers were covered with fresh culture medium supplemented or not with different honeys or honey mixtures at 0.1 % or 1 % (v/v).

2.6 Time-Lapse Microscopy

The wound closure process was dynamically captured by using a TLM workstation consisting of an inverted microscope (Zeiss Axiovert 200; Carl Zeiss, Jena, Germany) with a long working distance 5× objective (Zeiss, Ph1). The microscope is caged in a homemade incubating system that allows to keep the sample at constant temperature (37 °C) and under 5 % CO_2, 100% humidified atmosphere to ensure cell viability. The microscope is also equipped with motorized stage and focus, that allow the automatic and iterative image acquisition at different locations (fields of view) within the samples using an high-resolution high-sensitivity monochromatic CCD video camera (Orca AG; Hamamatsu, Japan) and a homemade control software in Labview. In particular, two fields of view corresponding to different regions along the wound were selected for each cell sample. The delay time between two consecutive images of the same field of view was 15 min and the overall experiment length was about 24 h.

2.7 Image analysis

A home-made automated image analysis algorithm was used to measure the size of the wound (A) through image segmentation for each time step. The wound width (l) was quantified considering the area of an equivalent rectangle. Specifically, l was calculated as the ratio between A and the height of the image, for each time step. The position of the invading cell front was then calculated as $x = \frac{l_0 - l}{2}$, where l_0 is the wound width at time t=0.

In Figure 1, two images of a WH experiment, corresponding to t=0 h and t>0 h, are reported on the left. The dotted lines indicate the wound edge on the right and left side of the scratch. The wound area is shown in black. The wound width (l_0 and l for t=0 and t>0, respectively) is also indicated. x_0 is the position of the advancing cell front at time t=0; x(t) is the position of the advancing cell front on the left edge, which changes in time along the wound closure direction x.

The position of the invading cell front (x) was reported over time for all cell samples. The curves describing the progression of x in time showed a linear trend, with the slope of the line corresponding to the velocity of cell front propagation (v) (Figure 1). This suggests that the wound clusure process occurs at constant speed.

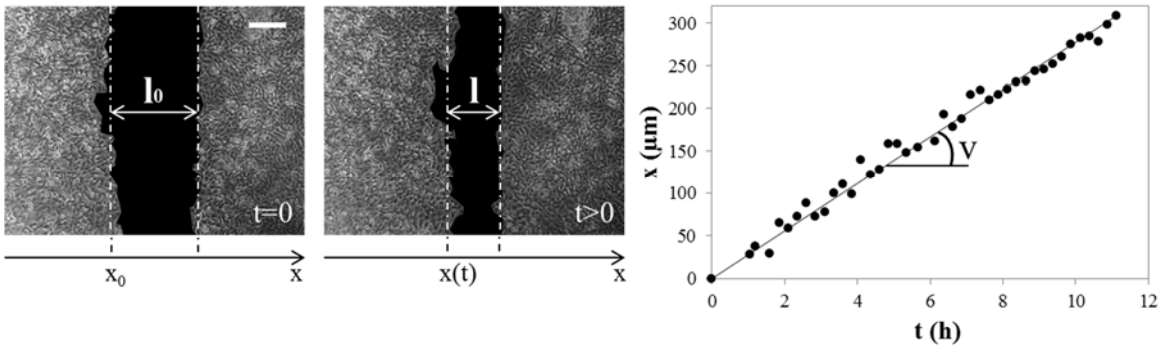

Figure 1: Schematic representation of WH assay quantitative analysis. On the left, images acquired during a WH experiment at the time t=0 and t>0 are reported (scale bar = 200 µm). On the right, the position of the invading cell front (x) is reported as a function of time for one of the cell samples. A linear fit of the experimental data is also reported

3. Results and discussion

This work was mainly addressed to test the potential healing activity of honey. Four pure honeys (eucalyptus, thyme, fir HD and Metcalfa HD) and two ternary mixtures were used. These mixtures were made, respectively, of eucalyptus and the two HD honeys, or thyme and the two HD honeys. Each mixture contained the three components in equal proportions by weight.

The phenolic and flavonoid contents of pure honeys and honey mixtures are reported in Table 1. The amount of phenolic compounds in honey samples ranged from 43.8 to 80.4 mg GAE/100 g and that of flavonoids from 18.6 to 31.8 mg QE/100 g. Compared to nectar honeys, honeydew honeys were richer in both classes of compounds, with Metcalfa HD showing the highest content of total phenolics and flavonoids.

Table 1: Phenolic and flavonoid contents of pure honeys and honey mixtures

Honey	Phenolic content (mg GAE/100 g)	Flavonoid content (mg QE/100 g)
Eucalyptus	46.5 ± 1.2	21.3 ± 0.8
Thyme	43.8 ± 1.1	18.6 ± 0.7
Fir HD	60.1 ± 1.6	27.2 ± 0.9
Metcalfa HD	80.4 ± 2.4	31.8 ± 1.1
Fir HD–Metcalfa HD–Eucalyptus	63.2 ± 1.8	27.4 ± 1.3
Fir HD–Metcalfa HD–Thyme	61.5 ± 1.6	25.2 ± 1.2

The antibacterial activity of honeys and their mixtures was evaluated against Gram-positive (*S. aureus*, *S. epidermidis*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria, including an antibiotic-resistant strain (MRSE) isolated from a clinical sample. The results are shown in Figure 2. As can be seen, the honeys tested were all active, but to different degrees, against the five bacterial strains. On average, Metcalfa HD was the most effective, followed by thyme, eucalyptus and fir HD honeys. We also note that Gram-positive bacteria were more susceptible to honeys than Gram-negatives. Interestingly, MRSE, one of the most important causative agent of nosocomial and device-related infections, was the most sensitive to honeys. The lower sensitivity of Gram-negative bacteria to the honeys examined is in agreement with previously reported results (Fidaleo et al., 2011; Zainol et al., 2013) and could be attributed to the presence, in these microorganisms, of an outer lipopolysaccharide membrane acting as a physical barrier to lipophilic compounds and thus hindering the penetration of bioactive honey components into the cell (Pagès et al., 2008).

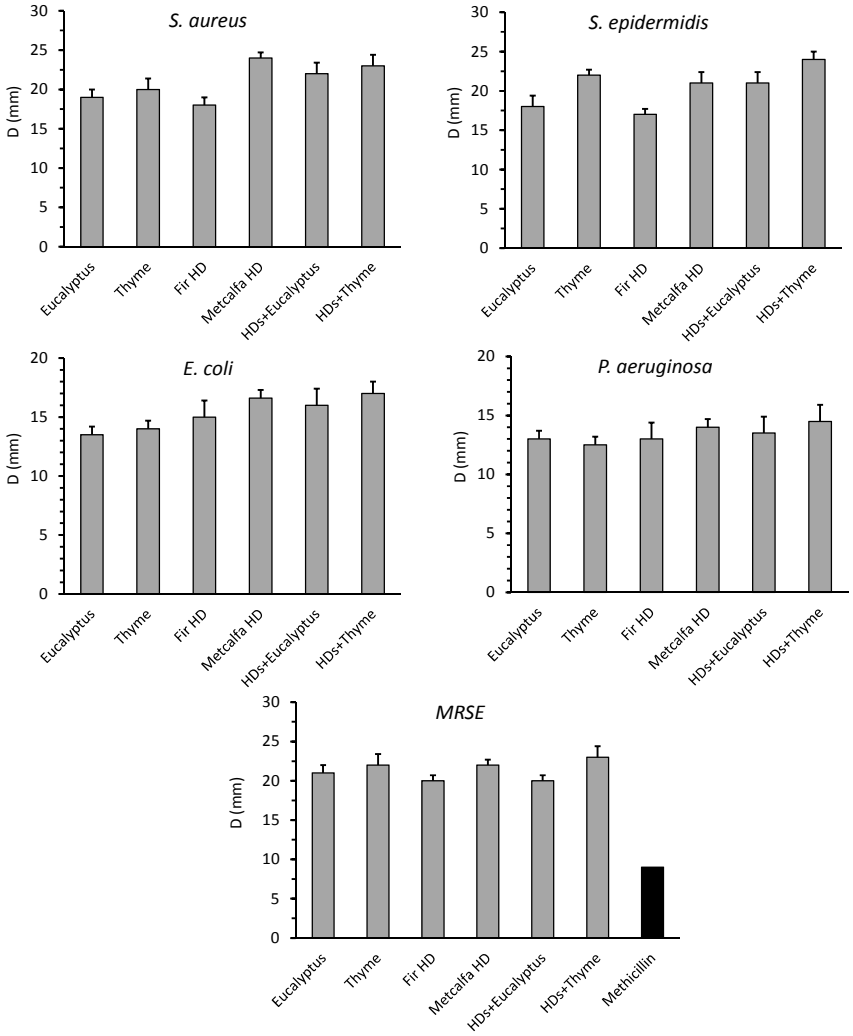

Figure 2: Antibacterial activity of honeys against the pathogens tested. D is the diameter of inhibition zone

The potential correlation between the antibacterial properties of honey, as well as the phenolic and flavonoid content, and its healing activity were analyzed.

In order to investigate the effect of the honeys and honey mixtures on wound repair, we proposed an experimental methodology based on *in vitro* WH assays performed on HT1080 human fibrosarcoma cells, coupled with Time-Lapse Microscopy and image analysis techniques.

In order to determine the optimal honey concentration to use for WH experiments, honey cytotoxicity was tested plating the HT1080 cells in presence of different concentrations on the honeys under investigation (0.1 %, 1 %, 2 %, 5 %, 10 % v/v). Cell dynamic behavior was observed for 72 h by using TLM image acquisition. Pure honeys and honey mixtures at 5 % and 10 % (v/v) resulted to be toxic for the cells; the same results were obtained testing the artificial honey (AH) on the cells.

An *in vitro* WH assay was performed to investigate the effect of artificial honey at two concentrations (0.1 % and 1 %) on wound repair; TLM visualization was used to monitor the wound closure process. HT1080 fibrosarcoma cells treated with the AH at both 0.1 % and 1 % showed a reduced wound closure velocity compared to the control cells, thus suggesting that the wound healing property of honey is not related to its sugar content.

A TLM WH experiment was carried out on HT1080 cells in the absence (control sample) and in the presence of the four honeys and the two mixtures at two concentrations, i.e. 0.1 % and 1 %. In Figure 3 the velocity of cell front propagation (v) during the wound closure process, obtained as described in the Materials and methods section, is reported for each type of honey and mixture under investigation. The bars represent the average of two independent measurements obtained for two fields of view relative to the same cell sample, the standard deviations are reported as error bars. The velocity of cell front propagation was lower than the control experiment (bar shown in black in Figure 3) for each type of honey.

In light of these results, the antibacterial properties of pure honeys and their mixtures, as well as the phenolic and flavonoid contents, do not seem to be correlated with the healing activity of honey. Our results are not consistent with previous works, which highlight the role of honey as enhancer of the wound healing process *in vitro*. In particular, Sell et al. (2012) reports that Manuka honey accelerates the closure of a wound generated in a monolayer of human fibroblasts. Moreover, Ranzato et al. (2013) showed that acacia, Manuka and buckwheat honey activates dermal fibroblast wound closure process. However, previous works were not based on detailed quantitative analysis of the WH assay, and could be affected by reproducibility and analysis errors.

Further extensive experimental investigations are needed, in order to assess the effect of honey on cell motility and proliferation, that are the main mechanisms involved in tissue dynamics. Future works will include the use of other types of honey, that have not been tested in this work. In addition, WH assays could be carried out on different types of cells, such as dermal fibroblasts and keratinocytes.

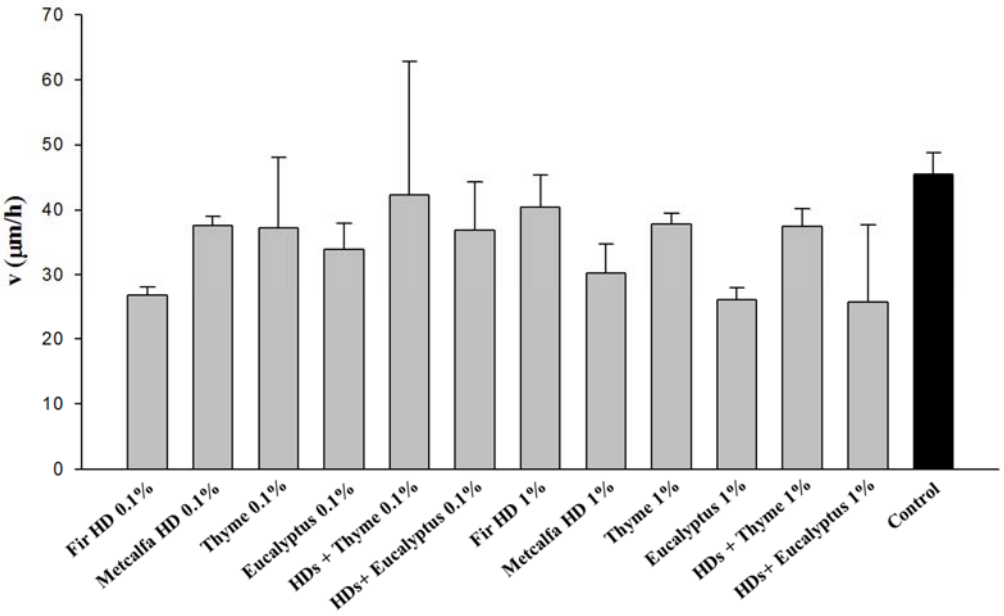

Figure 3: Velocity of cell front propagation (v) in the WH experiment for each honey under investigation

5. Conclusions

In this work, we proposed an experimental methodology to investigate the potential effect of honey on wound repair *in vitro*. Our methodological approach is based on Wound Healing (WH) assay, combined with Time-Lapse Microscopy (TLM) and image analysis techniques. The WH assay is a well-assessed and straightforward method widely used for the quantitative characterization of cell motility and proliferation *in vitro*. TLM image acquisition is a powerful analytical tool that allows direct visualization of active bio-soft matter dynamic evolution. This experimental technique allows to regularly observe over time exactly the same wound region, thus enabling stable and consistent measurements of cell front progression over time. The application of image analysis techniques allows the quantification of the wound closure process. The experimental data are then interpreted according to mathematical models, based on transport phenomena concepts.
Our methodological approach was used to test the potential healing activities of four pure honeys and two ternary mixtures, whose antimicrobial effect was assessed. The antibacterial activity of pure honeys and their mixtures here investigated, as well as the phenolic and flavonoid contents, do not seem to be correlated with the healing property of honey, contrary to what is reported in previous works. Further extensive experimental investigations will include the use of other types of honey and different cell types.

Acknowledgements

This work was financially supported by the Italian Ministry of Research (PRIN 2008, prot.2008W3WBMH). This study is related to the activity of the European network action COST MP1106 “Smart and green interfaces - from single bubbles and drops to industrial, environmental and biomedical applications".

References

Ascione F., Caserta S., Perris R., Guido S., 2014, Investigation of cell dynamics in vitro by time lapse microscopy and image analysis, Chemical Engineering Transactions, 38, 517–522, DOI: 10.3303/CET1438087.
Bogdanov S., 1997, Nature and origin of the antibacterial substances in honey, LWT-Food Sci. Technol., 30, 748–753.
Caserta S., Campello S., Tomaiuolo G., Sabetta L., Guido S., 2013, A methodology to study chemotaxis in 3-D collagen gels, AIChE J., 59, 4025–4035.
Fidaleo M., Zuorro A., Lavecchia R., 2011, Antimicrobial activity of some Italian honeys against pathogenic bacteria, Chemical Engineering Transactions, 24, 1015–1020, DOI: 10.3303/CET1124170.
Fidaleo M., Lavecchia R., Zuorro A., 2015, Antibacterial and anti-quorum sensing activities of selected Italian honeys against antibiotic-resistant pathogens, Online J. Biol. Sci., 15, 236–243.
Iftikhar F., Arshad M., Rasheed F., Amraiz D., Anwar P., Gulfraz M., 2010, Effects of acacia honey on wound healing in various rat models, Phytother. Res., 24, 583–586.
Ker-Woon C., Ghafar N.A., Hui C.K., Yusof Y.A.M., Luan N.S., 2014, Proliferative capacity of in vitro corneal epithelium: role of Acacia honey in the initial step of wound healing, J. Med. Bioeng., 3, 107–112.
Liang C-C, Park A.Y., Guan J-L, 2007, In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro, Nat. Protoc., 2, 329–333.
Pagès J.M., James C.J., Winterhalter M., 2008, The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria, Nat. Rev. Microbiol., 6, 893–903.
Ranzato E., Martinotti S., Burlando B., 2013, Honey exposure stimulates wound repair of human dermal fibroblasts, Burns Trauma, 1, 32–38.
Sato T., Miyata G., 2000, The nutraceutical benefit, Part III: Honey, Nutrition, 16, 468–469.
Sell S.A., Wolfe P.S., Spence A.J., Rodriguez I.A., McCool J.M., Petrella R.L., Garg K., Ericksen J.J., Bowlin G.L., 2012, A preliminary study on the potential of manuka honey and platelet-rich plasma in wound healing, Int. J. Biomater., Article ID 313781.
Subrahmanyam M., 1998, A prospective randomised clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine, Burns, 24, 157–161.
Zainol M.I., Mohd Yusoff K., Mohd Yusof M.Y., 2013, Antibacterial activity of selected Malaysian honey, BMC Complement. Altern. Med., 13, Article ID 129.
Zuorro A., Fidaleo M., Lavecchia R., 2010, Solubility enhancement and antibacterial activity of chloramphenicol included in modified β-cyclodextrins, Bull. Korean Chem. Soc., 31, 3460–3462.
Zuorro A., Lavecchia R., 2014, Recovery of phenolic antioxidants from the peel fraction of bilberry (*Vaccinium myrtillus* L.) processing waste, Biotechnology: An Indian Journal, 9, 273–279.