An *in vitro* examination of the antioxidant and anti-inflammatory properties of buckwheat honey

• **Objective:** Hydroxyl radical and hypochlorite anion formed at the wound site from superoxide anion produced by activated polymorphonuclear neutrophils (PMNs) are considered important factors in impaired wound healing. Superoxide anion may also react with nitric oxide produced by macrophages to form peroxynitrite, a third strong oxidant that damages surrounding tissue. In order to select honey for use in wound-healing products, different samples were compared for their capacity to reduce levels of reactive oxygen species (ROS) *in vitro*.

• **Method:** Honey samples were tested in assays for inhibition of ROS production by activated human PMNs, antioxidant activity (scavenging of superoxide anion in a cell-free system) and inhibition of human complement (reducing levels of ROS by limiting formation of complement factors that attract and stimulate PMNs). For buckwheat honey (New York, US), moisture and free acid content were determined by refractive index measurement and potentiometric titration respectively. Honey constituents other than sugars were investigated by thin layer chromatography, using natural product reagent to detect phenolic compounds. Constituents with antioxidant properties were detected by spraying the chromatogram with DPPH.

• **Results:** Although most honey samples were shown to be active, significant differences were observed, with the highly active honey exceeding the activities of samples with minor effects by factors of 4 to 30. Most pronounced activities were found for American buckwheat honey from the state of New York. Phenolic constituents of buckwheat honey were shown to have antioxidant activity.

• **Conclusion:** As buckwheat honey was most effective in reducing ROS levels, it was selected for use in wound-healing products. The major antioxidant properties in buckwheat honey derive from its phenolic constituents, which are present in relatively large amounts. Its phenolic compounds may also exert antibacterial activity, whereas its low pH and high free acid content may assist wound healing.

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reactive oxygen species; complement; moisture content; acidity; phenolic components

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The antibacterial potency has been reported to vary by as much as 100-fold between honeys of different floral sources.[1,2] Unifloral honeys are obtained from a single flowering plant species. Unlike multifloral honey, different batches of an unifloral honey contain identical constituents in more or less similar concentrations, and exhibit the same range of biological activities. The ensuing reliability in terms of clinical efficacy means that unifloral honeys are preferred for medicinal use.

Many publications on clinical wound healing have stated that honey rapidly eradicates infection with no adverse effects,[3-6] and may stimulate the healing process.[1] Research to support these clinical observations has mainly focused on honey's antibacterial properties, and has shown that certain types, such as Manuka honey (obtained from *Leptospermum scoparium*), stop bacterial growth, even methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[7]

Reduced inflammation observed in the clinic following the application of honey is supported by histological evidence of reduced numbers of inflammatory cells present in wound tissue.[1] Furthermore, pasture honey (multifloral) and unifloral Manuka honey at concentrations of 1% have been reported to stimulate monocytes *in vitro* to release tumour necrosis factor-alpha, a regulatory cytokine that may induce fibroblast collagen synthesis, thereby initiating healing.[8,9]

The non-adaptive division of the immune system, also known as the innate immune system, is immediately activated in response to foreign substances and invading microorganisms, and so plays a role in the wound-healing process.[10] The cellular part of this first line of defence is constituted by polymorphonuclear neutrophils (PMNs), macrophages and monocytes.

In activated PMNs, NADPH oxidase generates superoxide anion by transfer of electrons to oxygen,[11] which is converted into hydrogen peroxide in the wound site.[11-13] Myeloperoxidase, an enzyme

released by activated PMNs, catalyses the conversion of hydrogen peroxide into hypochlorite anion, a potent bactericidal agent that kills invading microorganisms.[14] When ferrous ion is present, hydrogen peroxide may also be converted into hydroxyl radical, a very strong but short-lived oxidant.[15] The free radicals (superoxide anion and hydroxyl radical) and the non-radicals (hydrogen peroxide and hypochlorite anion) are collectively known as reactive oxygen species (ROS).

Activation of PMNs results in both intracellular and extracellular generation of ROS. Intracellularly, the combined action of ROS and proteolytic enzymes kills ingested bacteria, preventing wound infection.[12,13] Extracellularly, however, excessive generation of ROS has detrimental effects on surrounding tissue. Superoxide anion easily reacts with nitric oxide, a radical produced, for instance, by macrophages at the wound site. This results in the formation of peroxynitrite, another major oxidant that causes tissue damage.[16]

Reactive oxygen species play an important role in impaired wound healing.[17-19] Although in some cases ROS are considered to possess certain beneficial antimicrobial properties and second messenger abilities,[20] prolonged exposure to elevated levels of ROS causes cell damage and may eventually inhibit healing of both acute and chronic wounds. Typically, burn injuries show excessive activity of free radicals.[21,22]

It has been suggested that the antioxidant activity of Jambolan honey (from *Syzygium cumini*) initiates healing by controlling free radicals.[21] Free radicals have been implicated in hypertrophic scar formation following burn injuries.[22] Therefore, it is likely that reducing levels of free radicals and other oxidants in the wound bed will aid wound management.

We therefore assessed the antioxidant and anti-inflammatory properties of several honeys of different floral sources using *in vitro* assays to test their ability to inhibit ROS production by activated human PMNs and to scavenge superoxide anion in a cell-free system.

Complement is part of the humoral response of the innate immune system to foreign matters.[23] Complement activation proceeds through a series of proteolytic steps in either the classical, alternative or lectin pathway. The terminal stage of each pathway results in the formation of a macromolecular membrane attack complex that kills invading microorganisms through lysis.[24]

Complement factor C3b is an opsonin, a molecule that enhances phagocytosis by binding to foreign cells. Since PMNs have receptors for C3b, these phagocytic cells can easily detect, ingest and destroy opsonised microorganisms.[25]

In addition, the complement cascade results in the generation of small split products that mediate many immunoregulatory effects, such as complement factor C5a. This activates PMNs and attracts them to the wound site.[26]

Inhibition of complement may thus lower ROS levels in the wound by limiting the factors involved in PMN recruitment and activation.

Honeys of different floral sources were tested *in vitro* for their ability to reduce oxidant levels and inhibit activated human complement, with a view to selecting the most active variety for use in the development of wound-management products.

Materials and method

Honey samples tested

Testing included two varieties used in commercial wound-healing products:

- Unifloral Manuka (*Leptospermum scoparium*) honey, which is highly valued for its medicinal properties and has been the subject of several studies[1,4,7] Supplied by Comvita (Bay of Plenty, New Zealand)
- Special multifloral honey (Miel de Abeja organica) from the Maule Region of Chile, which is recommended for use on wounds due to its high glucose oxidase content (W. Verkruisen, personal communication). This was supplied by Sociedad Apicola Verkruisen, an organic honey producing and exporting company (San Javier, Chile).

Other varieties tested were:

- Two samples of dark-brown American buckwheat honey (*Fagopyrum esculentum*); these were supplied by bee keepers in the US states of New York and North Dakota respectively, and were obtained from Dutch Gold Honey (Lancaster, Pennsylvania, US)
- Two exotic varieties from Hawaii: a brown macadamia honey (*Macadamia integrifolia)* and an almost white, rare kiawe (*Prosopis pallida*) honey. These were supplied by Hawaii Island Honey Company and Volcano Island Honey Company respectively
- A Canadian mixture of clover (*Trifolium* species) and alfalfa (*Medicago sativa*) honey, which was selected as an alternative light-coloured honey to Hawaiian kiawe honey. This was sourced from Golden Acres, Three Hills, Alberta, Canada.

Inhibition of factors increasing oxidant levels

All of the above varieties of honey were tested in the following bioassays, which are commonly used to screen anti-inflammatory activity. The following bioassays were performed, and have been described in detail elsewhere.[27]

- **Inhibition of ROS** This *in vitro* test assesses the potential anti-inflammatory effects of a substance, based on inhibition of ROS production by inflammatory cells. Honey samples were tested for their ability to inhibit production of ROS by zymosan-activated human neutrophils (PMNs), using luminol as the chemiluminescent probe. When luminol reacts with ROS, in particular the hypochlorite anion, an excited oxidation product is

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formed, which emits light (chemiluminescence) when it returns to its ground state.

In summary, in a 96-well plate freshly isolated human PMNs[28] were stimulated by the addition of opsonised zymosan. The resulting production of ROS was detected as luminol-dependent chemiluminescence using a Titertek Luminoskan luminometer. Chemiluminescence was monitored for 0.5 seconds every two minutes over a 30-minute period at 37°C. Peak levels of chemiluminescence measured for honey samples and controls (identical incubations without honey present) were used to calculate the inhibition of ROS production.[29]

- **Antioxidant activity** Although superoxide anion does not cause tissue damage as such, its conversion into hydroxyl radical, hypochlorite anion and/or peroxynitrite may eventually have detrimental effects. Thus, the ability of honey to scavenge superoxide anion may contribute to its wound-healing properties.

The capacity of honey samples to scavenge superoxide anion was determined in a cell-free system as inhibition of chemiluminescence; lucigenin, which detects superoxide anion, was used as the chemiluminescent marker.

In summary, in a 96-well plate superoxide anion production was initiated by adding xanthine oxidase to a mixture of hypoxanthine, lucigenin and honey samples. The chemiluminescence signal generated was monitored for 0.5 seconds every three minutes over 30 minutes using a Fluoroskan Ascent Fluorescence and Luminescence reader. Activity of the honey samples was calculated, based on the extent of the chemiluminescence signal that was inhibited by superoxide dismutase.[27]

- **Inhibition of human complement** The ability of the honey samples to inhibit human complement, activated via the classical pathway, was determined using a microtiter plate method.[30]

Serial dilutions of honey samples were prepared in Veronal buffer supplemented with calcium and magnesium ions, and incubated with human pooled serum as the source of complement for 30 minutes. Sheep erythrocytes were then added; these had been sensitised by incubation with amboceptor as classical pathway complement activity can only be triggered by particles or cells coated with immunoglobulins. Activation of complement eventually results in formation of membrane attack complex, causing lysis of the erythrocytes. The amount of haemoglobin released, spectrophotometrically determined at 405nm using an automatic ELISA reader, served as a measure for classical pathway complement activity.

Presentation of activities determined in bioassays — RIC50

In general, inhibitory activity is expressed as IC50, which is the amount of sample per ml (mg/ml) in the test system (bioassay) that achieves 50% inhibition. Therefore, low IC50 values represent strong activities. However, diagrams showing test results as IC50 often cause confusion by having low bars that represent high activity and high bars that represent minor effects. Therefore, activities are presented here as RIC50 (reciprocal IC50), 1/IC50 in ml/g, which is the volume (ml) to be added per gram of sample giving 50% inhibition. In this way, samples with stronger activities requiring more dilution (increased volume) show higher RIC50 values. High bars that correspond with strong activities greatly improve interpretation of test results when presented in bar charts. RIC50 values presented are the mean ± standard deviation (SD).

Results

All honey samples tested showed inhibition in the bioassay for ROS produced by activated human PMNs, with activities ranging from 160 to 130ml/g (buckwheat honey from New York [NY] and North Dakota [ND] respectively) to 40ml/g (Hawaiian kiawe honey) (Fig 1). No differences were found between Chilean and Manuka honey, each showing a RIC50 value of approximately 110ml/g.

Differences in superoxide anion scavenging capacity were more pronounced (Fig 2). Again, the highest activity was shown by NY buckwheat honey (RIC50: 290ml/g), which exceeded the RIC50 value of the least active Hawaiian kiawe honey (10ml/g) by a factor of almost 30. Although it showed half the activity of the New York sample, when compared with the remaining samples, the superoxide anion scavenging capacity of North Dakota buckwheat honey (RIC50: 150ml/g) can still be considered strong. Again, no significant difference was found between activities for Chilean (59ml/g) and Manuka honey (48ml/g).

Results for inhibition of human classical pathway complement activity are given in Fig 3. Although NY buckwheat honey showed the strongest inhibition (RIC50: 120ml/g), this was not significantly different to ND buckwheat (102ml/g), Hawaiian macadamia (115ml/g) and Manuka honey (111ml/g). Here, the Chilean sample (RIC: 33ml/g) scored worse than Manuka honey, and the same as Hawaiian kiawe honey (RIC50: 34ml/ml).

New York buckwheat honey showed the most pronounced activities (Figs 1–3), both in terms of its ability to inhibit human complement, which results in reduced formation of factors that attract and stimulate PMNs, and to scavenge oxidants produced by PMNs upon activation.

Based on its superior *in vitro* activities, and after it had been established that heavy metals, pesticides and antibiotics were all below detection limits or present in acceptable amounts, NY buckwheat honey was considered for use in wound-healing prod-

Fig 1. Inhibition of ROS produced by human PMNs

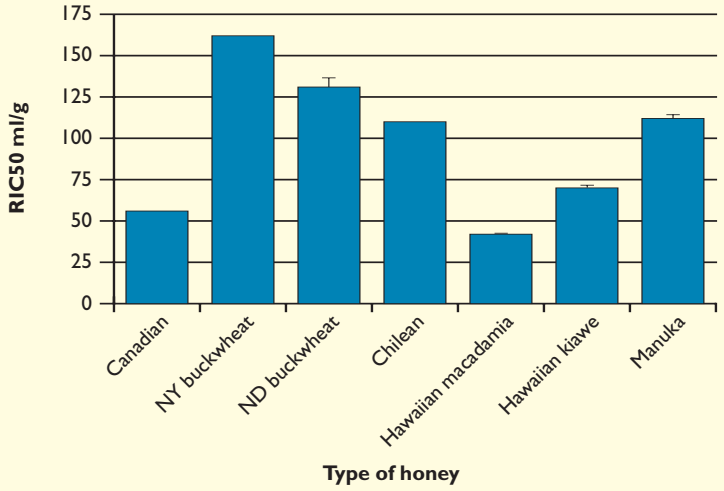
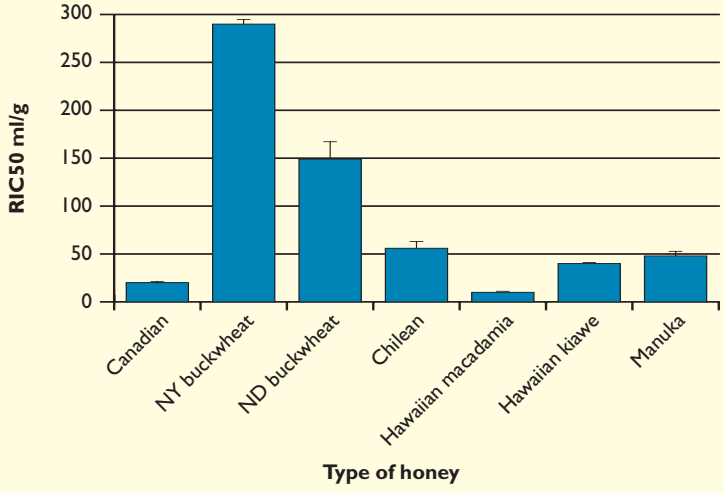


Fig 2. Superoxide anion scavenging



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ucts, MelMax and MelDra (Dermagenics Europe BV, Kaatsheuvel, the Netherlands). It was therefore subjected to further research, which is outlined below.

Method

Physical characteristics of buckwheat honey

● **Moisture content** To prevent growth of high osmolarity-resistant yeast species, the water content of honey should not be more than 20%.[31] Although honey products may be gamma-ray sterilised, growth of yeasts before sterilisation may affect their medicinal properties.

The amount of water present was determined according to the European Pharmacopoeia[31] by measuring the refractive index (RI), which increases with increasing sugar concentration. Refractive index is commonly denoted as n_D with D referring to the wavelength of 589nm used (the so-called D-line emitted by a sodium vapour lamp). Moisture content was derived from RI corrected for temperature, using a table of n_D values (at 20°C) for which the corresponding water content is listed.

● **Acidity** Since acidity may contribute to wound healing, the pH and free acid content were also determined.[31]

A solution of 25g buckwheat honey in 75ml carbon dioxide-free demineralised water was used to determine the pH. The latter was measured as the potential difference (voltage) between a glass electrode and silver chloride reference electrode.[31]

Free acid content is the volume (in ml) of 1M potassium hydroxide solution required to neutralise the acid components present in 1kg of honey, and is expressed in meq/kg (milliequivalents/kg). Free acids were quantified by potentiometric acid-base titration with a known concentration of potassium hydroxide solution.[32] This endpoint is generally determined using a visual indicator such as phenolphthalein, which is colourless in acid solution but turns pink above pH 8.2. However, a visual indicator could not be used for the dark-brown buckwheat honey. Therefore, potentiometric measurement, as described above, was used to determine the point of equivalence at pH 8.3.

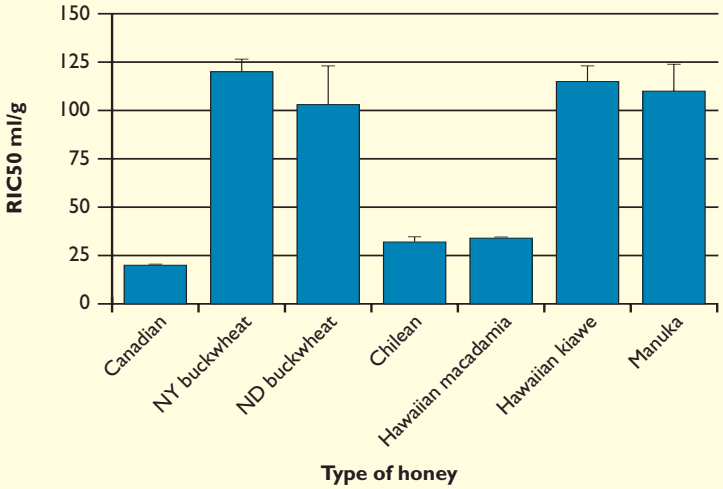
Investigation of phenolic constituents

Thin layer chromatography (TLC) is a rapid analytical method for detecting separate compounds present in complex mixtures. This process is illustrated in Fig 4. A multicomponent sample is applied to a TLC plate at the start position, and a solvent is then used as a mobile phase to separate the components of interest, which migrate through the thin sorbent layer (stationary phase). The retention factor is the distance (a) travelled by a compound from the start point (Fig 4) divided by the entire distance (b) allowed for the solvent migration (ie, from start point to solvent front; Fig 4). Constituents are detected as coloured spots under ultraviolet light and/or after spraying the chromatogram with specific reagents (Fig 4). Thus, TLC enables characterisation of compounds by retention-factor value and specific colour reactions.

● **Extraction and analysis of the phenolic components** As the phenolic components in honey are limited, they can only be detected after separation from the dominant sugars. Therefore, phenolic constituents were extracted from honey before TLC analysis as follows: 25g honey was dissolved in 75ml of water, and the solution was extracted with ethyl acetate (3 x 50ml). The combined ethyl acetate extracts were dried over anhydrous magnesium sulphate. After filtration and evaporation of the solvent under reduced pressure, the residue was taken up in 10ml of ethanol (96% and subjected to TLC analysis.

Ethanol solutions containing phenolic constituents extracted from NY buckwheat honey and Chilean honey, which is particularly high in glucose oxidase content, were applied in 30µl amounts to TLC plates pre-coated with silica gel. The solvent system used was acetonitrile/water (87:13, saturated chamber). After the development of the chromatogram, the plate was sprayed with natural product reagent (2-aminoethyldiphenyl borinate), and examined under ultraviolet light (366nm). Natural product reagent detects phenolic constituents, including flavonoids, which become visible as blue and green fluorescent spots. Spraying (another) chromatogram with DPPH (2,2-diphenyl-1-picryl-hydrazyl radical; 0.2mg/ml dissolved in absolute ethanol) showed the presence of constituents with antioxidant activity as yellow spots on a purple background (examination in daylight). DPPH is a relatively stable purple radical that turns into a yellow reduction product (DPPH-H) by accepting hydrogen atoms donated by antioxidants.

Fig 3. Inhibition of human CP complement activity



Results

Physical characteristics of NY buckwheat honey

- **Moisture content** Refractive index of NY buckwheat honey measured at 22.1°C was n_D 1.4939. Correction for temperature resulted in n_D 1.4944, corresponding with a moisture content of 16.8% (w/w), which is below the upper limit of 20% required by European legislation[33] and European Pharmacopoeia.[31]
- **Acidity** New York buckwheat honey was found to have relatively strong acid properties, represented by a low pH and high free-acid content.

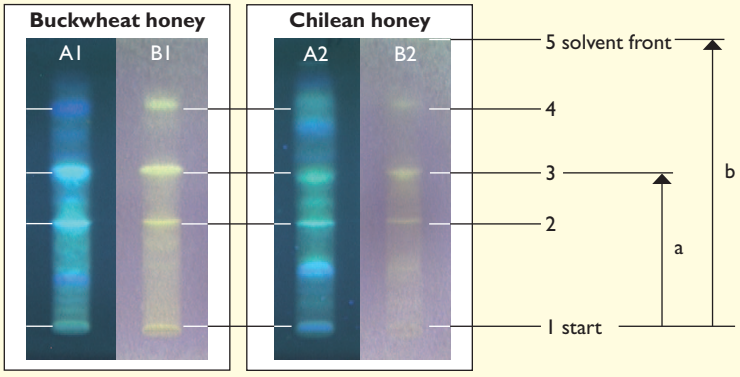
Measurement of pH using a solution of NY buckwheat honey in water (25% w/v) showed a pH 3.3, which is lower than the average pH 3.9 for non-tropical honeys, where the typical range is 3.4–6.1.[34]

Determination of free acids in NY buckwheat honey showed a content of 50meq/kg, which is the maximum allowed by European legislation for honey intended for human consumption.[33]

- **Investigation of phenolic constituents** Thin layer chromatography analysis with natural product reagent detection showed blue and green-fluorescent spots, indicating phenolic compounds, including flavonoids, extracted from NY buckwheat honey (Fig 4, TLC-A1). Detection of antioxidants by DPPH showed the presence of some strong antioxidant constituents that immediately turned purple DPPH yellow (Fig 4, TLC-B1).

In Fig 4, A1 and A2, and B1 and B2 refer to parts of two separate chromatograms A and B, which were developed under similar experimental conditions. Consequently, identical constituents of NY buckwheat honey on TLC-A1 and TLC-B1 have the same retention factor value, as indicated by 1–4 on the figure. Since fluorescent constituents corresponded with those detected by DPPH, phenolic compounds present in NY buckwheat honey were concluded to be major antioxidant principles. In addition, the phenolic fraction may also have antibacterial activity.[35]

Fig 4. Thin layer chromatography of phenolic constituents extracted from equal amounts of NY buckwheat (A1 and B1) and Chilean honey (A2 and B2)



A = Detection with natural product reagent, showing flavonoids and other phenolic components as green and blue fluorescent spots under ultraviolet light of 366nm
B = Detection with DPPH reagent, showing constituents with antioxidant activity as yellow spots on a purple background in daylight; 3 = major antioxidant constituent of Chilean, as well as NY buckwheat honey with R_f 0.53; retention facor (R_f) = (distance a)/(distance b)

We also found that amounts of antioxidants in NY buckwheat honey (Fig 4, TLC-B1) greatly outmeasured those in Chilean honey (Fig 4, TLC-B2). The component indicated by 3 with a retention factor of 0.53 was a major antioxidant constituent of both NY buckwheat and Chilean honey, although the latter contained a relatively minor amount, only slightly reducing DPPH (Fig 4, TLC-B2). The Chilean honey is also used in wound-care products, such as HoneySoft (Mediprof BV, Rijswijk, the Netherlands), but was selected on basis of its high glucose oxidase content.

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Discussion

Since elevated levels of ROS may cause cell damage and inhibit wound healing,[21,22] honeys of different floral sources were tested *in vitro* for their ability to lower oxidant levels, including radicals. As NY buckwheat honey showed superior effects, it was selected for medicinal application. Compared with Manuka and Chilean honey, which have both been used in commercial wound-healing products, NY buckwheat honey was found to be the strongest scavenger of superoxide anion and the most effective inhibitor of ROS produced by stimulated human PMNs. It inhibited classical pathway complement activity as well, but ND buckwheat, Hawaiian macadamia and Manuka honey showed similar activities with no significant differences.

With 16.8% (w/w) water present, NY buckwheat honey amply met the legislatory requirements for moisture content. Due to its generally high osmotic value (high sugar content), honey draws wound exudate to the wound surface, creating a moist environment that results in a non-adherent interface between the dressing and wound bed.[4] Reports on the significance of high osmolarity for antibacterial activity of honey are controversial (see below). However, in this study the moisture content was not determined to serve as an antibacterial indicator for NY buckwheat honey, but rather to exclude the possibility that yeasts resistant to high osmolarity would have affected its properties.

The value of honey as an antibacterial has long been recognised. Hydrogen peroxide, whether residual and/or generated by glucose oxidase activity on dilution of honey, and the phenolic constituents of honey are considered to be its major antibacterial factors.[1,35] We could detect neither glucose oxidase nor residual hydrogen peroxide in NY buckwheat honey selected for application to wounds (unpublished results). With sugar contents above 80% (w/w), the high osmolarity of undiluted honey is sufficient to stop all microbial growth.[1] In practice, however, this may be of minor importance. Depending on the type of wound, dilution by exudate may eventually result in loss of osmotic antibacterial activity.[1,4] Somewhat contradictorily, it has been claimed that high osmolarity is responsible for the antimicrobial activity of honey if it has a low pH.[36,37] However, the free acid content, rather than the pH, of non-peroxide honey has been found to significantly correlate with antibacterial activity against *Staphylococcus aureus* and *Micrococcus luteus*.[38] The low pH and, particularly, the high free acid content[38] of NY buckwheat honey may contribute to its antibacterial activity.

Lysed platelet concentrates, pre-incubated close to pH 5.0 (as opposed to pH 7.0) have been found to contain increased amounts of available platelet-derived growth factor (PDGF), and show increased capacity to stimulate fibroblast proliferation *in vitro*.[39] Although this was not studied, showing relatively strong acidic properties, NY buckwheat honey may promote healing of chronic wounds by effecting a low pH at the wound site, resulting in fibroblast proliferation.

Compared with other types, buckwheat honey is a rich source of phenolic antioxidants.[40] Since phenolic compounds have antibacterial properties,[35] lack of glucose oxidase activity or hydrogen peroxide in NY buckwheat honey may well be compensated by its relatively abundant phenolic constituents.

Pilot clinical studies

After selection based on *in vitro* biological activity and the quality control described above, NY buckwheat honey was used in wound-healing products, which were tested in pilot clinical experiments.

In a study involving 21 burn patients with difficult-to-treat wounds, application of MelMax (a wound dressing impregnated with NY buckwheat honey and an ointment containing a synthetic blend of metal ions and citric acid)[27] resulted in an overall full wound closure (spontaneous) of 94%. The mean healing time was 25.6 days (range 6–63). Reduced exudate and slough, and recruitment of a controlled amount of granulation tissue, with progressive epithelialisation from the wound edges was observed, leading to stable wound closure. In addition, control of contamination was noted, with variable, often reduced, microbiological quantities and species.[41]

In another study involving 60 patients, treatment of venous leg ulcers with MelMax showed similar and sometimes better results to silver dressings.[42]

Conclusion

Compared with honeys from other floral sources, NY buckwheat honey showed pronounced *in vitro* activities leading to decreased levels of oxidants, including radicals. Antioxidant properties of NY buckwheat honey mostly derive from its phenolic constituents. Its relatively low pH and high free acid content may also contribute to its ability to heal chronic wounds.

Initial clinical pilot experiments have shown that MelMax containing NY buckwheat honey was successfully applied to burn wounds and venous leg ulcers. Although often mentioned as major wound-healing factors, residual hydrogen peroxide or glucose oxidase could not be detected. Instead, buckwheat honey was found to contain large amounts of antioxidant phenolic constituents, which may also have antibacterial activity, compensating absence of hydrogen peroxide or its generation by glucose oxidase.

In wound management, bacteria resistant to antibiotics are becoming an increasing problem. Although results obtained so far are most promising, and indicate NY buckwheat honey to be an effective antimicrobial wound-healing product, final proof will only be provided by full clinical trials. ■

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