

The effect of Malaysian honey and its major components on the proliferation of cultured fibroblasts

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Background/aim: To examine, for the first time, the effect of a selected Malaysian honey and its major components on the proliferation of cultured fibroblasts.

Materials and methods: Honey and some of its components, which include the sugars, the proteins, the hydrogen peroxide produced, and the phenolics, were exposed to cultured fibroblasts. The MTT colorimetric assay was used to assess cell viability and proliferation.

Results: The stimulatory effect of honey on fibroblast proliferation was observed to be time- and dose-dependent. The continuous production of hydrogen peroxide by the honey-glucose oxidase system also acts to stimulate cell proliferation in a time- and dose-dependent manner. The presence of phenolics with antioxidant properties, on the other hand, renders protection to the cells against the toxic effect of hydrogen peroxide. However, the presence of a growth factor-like substance in honey could not be ascertained.

Conclusion: For the first time, honey and its major components were shown to exert stimulatory effects on cultured fibroblasts. Honey is therefore potentially useful in medicinal practices.

Key words: Honey, protein, hydrogen peroxide, phenolics, fibroblasts

1. Introduction

Honey has been reported to be effective in the treatment of wounds and ulcers

(1–3). It has been found to promote wound healing by stimulating processes such as granulation tissue formation (4) and epithelialization (5,6). A number of animal studies have demonstrated that honey stimulates tissue growth and cellular components involved in the healing processes (7). Honey has been shown to stimulate angioblastic activity (8,9), and reduce inflammation (8,10). Microscopic examinations (8,9) and biochemical evaluations (11) have shown that honey activates fibroblast proliferation and increases collagen deposition and other extracellular matrix contents.

Most of the beneficial effects of honey on wound healing were attributed to its antibacterial activity; however, the above findings indicated that honey also has a stimulatory effect on tissue growth. Sugars showed a lesser stimulatory effect than honey, indicating that the chemical constituents of honey are responsible for its action (2). Growth factors, as well as hydrogen peroxide, were presumed to play a role in the stimulatory effect of honey (2,3).

Although the antibacterial activity of honey has been widely studied, there are no reports on the mechanism of how honey stimulates tissue growth. Therefore, the aim of this study was to establish the effect of honey and its components on the proliferation of cultured fibroblasts, which play a very important role in the tissue repair processes (12). Attempts were also made to examine the presence of growth factors in honey.

2. Materials and methods

2.1. Honey and its components

A Malaysian gelam (*Melaleuca* sp.) honey, which was sterilized by gamma-irradiation (25 kGy), was used. A sugar solution containing equivalent concentrations of the major sugars measured in natural honey was prepared using commercial sugars in sterile distilled water. The honey's proteins were extracted using a dialysis membrane with a molecular weight cut-off of 3.5 kDa. Hydrogen peroxide in honey was measured according to the method described by Kerkvliet (13). Honey's phenolics were extracted using the method described by Aljadi and Kamaruddin (14).

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2.2. Cell line

The 3Y1 rat fibroblast cell line was a generous gift from Professor Rohana Yusof of the Molecular Biology Lab, Department of Molecular Medicine, Faculty of Medicine, University of Malaya. The cell line was originally obtained from the Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, UK.

2.3. Chemicals

All reagents used were of analytical grade.

2.4. Cell culture and treatment

Cell culturing and treatment were carried out according to the established methods performed in tissue culture laboratories (15).

2.5. Cell cultures

The 3Y1 fibroblasts were grown in an RPMI 1640 media (Flowlab, Australia) supplemented with 10% fetal bovine serum (Flowlab). Cells were cultured in 25- and 75-mm² flasks and kept in a humidified incubator with 5% CO₂ at 37 °C. The pH of the media was monitored at 7.40. Cell growth was monitored periodically by viewing the culture flask under an inverted microscope (Olympus, Japan). Cells that were ready for harvesting (confluent flasks) were washed with 5 mL of phosphate buffered saline (PBS) (Amresco, USA), followed by the addition of 1 mL of 25% trypsin (Flowlab) and centrifugation (250 × g, 10 min).

2.6. Treatment

The harvested cells were resuspended in the growth media, counted, and plated in 96-well microtiter plate at a density of 1×10^4 cells/100 μL^{-1} well⁻¹, using a multichannel pipette. After an overnight incubation under the culturing conditions (to recover from handling), cells were treated with the following:

- Honey as a whole;
- Sugar solution (fructose 37%, glucose 31%, and sucrose 2%);
- Protein solution;
- Hydrogen peroxide (Merck, Germany);
- Mixture of sugar, protein extract, and phenolic

extract. Throughout this paper, the abbreviation EM is used to represent this extract mixture.

The cells were exposed for 2, 6, 12, and 24 h at the doses indicated below (Table), with a final volume of 200 μL per well. At the end of the exposure periods the media were removed, and cells were washed and reincubated in a fresh growth media for a total period of 48 h. For each variable, control cells (untreated cells) were run in parallel and were tested on 5 replicate wells. Each set of the experiments was repeated 3 times.

Cell viability and proliferation were then assessed by colorimetric MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide) (16,17).

2.7. MTT assay

The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) to purple formazan in living cells (16,18). This assay is commonly used to evaluate cell viability and proliferation.

MTT (Sigma, USA) was prepared in PBS at 5 mg/mL. At the end of the incubation period, 20 μL of MTT solution was added to each well; after 4 h of incubation at 37 °C, the formation of the formazan product was viewed under an inverted microscope, and the media were gently removed from each well and 150 μL of pure spectral grade dimethyl sulphoxide (Amresco) was added to solubilize the MTT-formazan product (17). After thorough mixing with an automated plate mixer, the absorbance measured at 550 nm with a microtiter plate reader (Bio-Tek Instruments, Inc., USA). Reagent blanks that contained the treatment agents (honey, H₂O₂, etc.) prepared in the growth media without cells were also treated with MTT and run in parallel, to minimize any interference by those components.

2.8. Statistical analysis

The collected data were expressed as mean \pm SD. Statistical analysis was performed by Student's t-test to express the difference between the groups of interest using SPSS.

Table. Detail of different doses used for cells treatment.

Honey (mg/mL)	195	19.5	1.95	0.195
Sugar solution* (mg/mL)	132	13.2	1.32	0.132
Protein extract (mg/mL)	6.33	0.633	0.063	0.0063
Hydrogen peroxide (μM)	57	5.7	0.57	0.057
Extract mixture (EM)	15%	1.5%	0.15%	0.015%
Sugar and protein extract (same as above)				
Phenolic extract (mg/mL)	1.95	0.195	0.0195	0.00195

*: Sugar solution was a mixture of fructose (37%), glucose (31%), and sucrose (2%), the major sugars left in the honey sample after the protein extraction procedure was done.

3. Results

3.1. Effect of honey on the growth of fibroblasts

The effect of honey on the proliferation rate of cultured fibroblasts is shown in Figure 1. It was observed that honey was most effective on rat fibroblasts at a concentration of 1.95 mg/mL and the effect was strongest at 6 h after treatment. At this concentration, honey treatment resulted in 35% increase in cell viability over the control ($P < 0.0001$). Treatment with the same concentration resulted in 19% ($P < 0.01$), 25% ($P < 0.001$), and 9% ($P > 0.05$) increase in cell proliferation at 2, 12, and 24 h, respectively. Cells treated with 19.5 mg/mL honey showed 22.5% ($P < 0.01$) and 25% ($P < 0.01$) increase in their proliferation over the control at 2 and 6 h, respectively; however, their proliferation decreased to 12% ($P < 0.05$) and 6% ($P > 0.05$) over the control at 12 and 24 h, respectively. Treatment with 0.195 mg/mL honey resulted in nonsignificant ($P > 0.05$) increase in cell proliferation at all of the treatment periods. In addition, treatment with 195 mg/mL honey resulted in nonsignificant ($P > 0.05$) increase in cell proliferation at 2 h after treatment; however, at 6, 12, and 24 h after treatment, the proliferation of the cells showed no obvious difference from that of the control.

3.2. Effect of sugar on the growth of fibroblasts

Figure 2 shows the effect of sugars in honey on the proliferation of cultured fibroblasts. While treatment of cells with 132 mg/mL of sugar solution resulted in a significant 12% ($P < 0.05$) increase in cell proliferation at 6 h, treatment with other dilutions resulted in nonsignificant ($P > 0.05$) increases in cell proliferation at 2 and 6 h after treatment, as compared to the controls. Again, treatment of

cells with 1.32 and 0.32 mg/mL of sugar solutions resulted in nonsignificant ($P > 0.05$) increases in their proliferation at 12 and 24 h after treatment. However, treatment with doses of 132 and 13.2 mg/mL resulted in significant ($P < 0.05$) and highly significant ($P < 0.01$) increases in cell growth at 12 and 24 h after treatment, respectively. This effect was greatest for 132 mg/mL at 24 h, where there was 16.6% increase in cell proliferation over the control. The highest effect of sugar solution was significantly ($P < 0.01$) lower than the highest stimulation effect caused by honey.

3.3. Effect of protein extract on the growth of fibroblasts

The effect of honey protein extract on the growth of cultured fibroblasts is shown in Figure 3. Results showed that the protein fractions of the tested honey had no significant effect on the cell growth when they were added for periods of up to 24 h, suggesting that the protein fraction of honey has no direct effect on the growth of cultured fibroblasts.

3.4. Effect of preformed hydrogen peroxide on the growth of fibroblast

The effects of bolus addition of hydrogen peroxide on the growth of fibroblasts are illustrated in Figure 4. It was observed that the addition of preformed hydrogen peroxide had both stimulatory and inhibitory effects on the growth of cultured fibroblasts. Treatment of cells with 5.7 μM H_2O_2 for 2 h resulted in a nonsignificant ($P > 0.05$) increase in cell proliferation, whereas treatment with 0.57 μM H_2O_2 for the same period of time resulted in a significant ($P < 0.05$) increase in cell proliferation. At these particular conditions, there was 11.5% increase in cell growth over the control. Although these 2 doses (5.7 and 0.57 μM) of H_2O_2 had stimulatory effects at 2 h, their effects

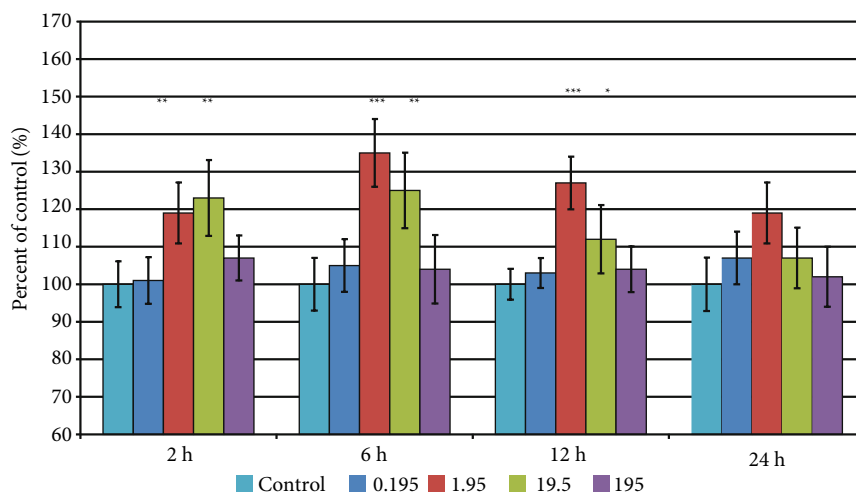


Figure 1. Cell proliferation of 3Y1 fibroblasts under the influence of various doses (mg/mL) of gelam honey at the time points indicated. The ability of the treated cells to reduce MTT to formazan was estimated and expressed as a percentage of unexposed control cells. Each value represents the mean \pm SD of 5 independent measurements. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

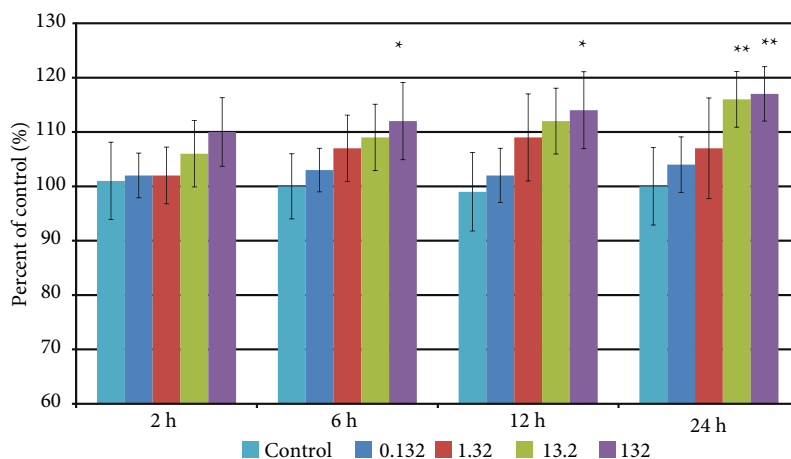


Figure 2. Cell proliferation of 3Y1 fibroblasts under the influence of various doses (mg/mL) of sugar solution at the time points indicated. The ability of the treated cells to reduce MTT to formazan was estimated and expressed as a percentage of unexposed control cells. Each value represents the mean \pm SD of 5 independent measurements. *: $P < 0.05$, **: $P < 0.01$.

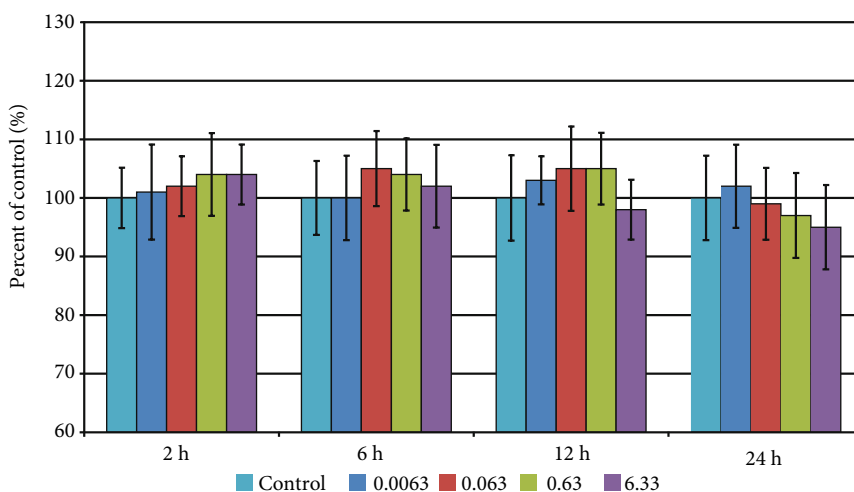


Figure 3. Cell proliferation of 3Y1 fibroblasts under the influence of various doses (mg/mL) of protein extract at the time points indicated. The ability of the treated cells to reduce MTT to formazan was estimated and expressed as a percentage of unexposed control cells. Each value represents the mean \pm SD of 5 independent measurements.

became lower at 6 h and they had nonsignificant ($P > 0.05$) inhibitory effects at 12 and 24 h after treatment. Treatment of cells with $0.057 \mu\text{M}$ H_2O_2 had no significant effects on growth at any of the incubation periods. However, a dose of $57 \mu\text{M}$ H_2O_2 exerted a toxic effect on the cell growth, as indicated by the reduction in cell viability at all of the treatment periods. The greatest toxic effects caused by this dose ($57 \mu\text{M}$) were at 12 and 24 h, where there was a 14% ($P < 0.01$) and 18.6% ($P < 0.01$) reduction in the viability of the treated cells, respectively, as compared to the controls. These results indicated that increasing exposure time to hydrogen peroxide increased its toxic effect. However,

hydrogen peroxide, when present in lower doses for a specific time, has the ability to stimulate cell proliferation.

3.5. Effect of continuous generation of hydrogen peroxide on the growth of fibroblasts

The effect of a honey extract mixture on the growth of cultured fibroblasts is shown in Figure 5. Similar to the bolus addition of H_2O_2 , continuous H_2O_2 generated by the honey extract mixture had both stimulatory and inhibitory effects on the growth of fibroblasts in vitro. While a 0.015 EM dilution mixture showed no effect on cell growth, the 0.15 dilution showed the highest stimulatory effect on the proliferation of fibroblasts. Cells treated with a 0.15 EM

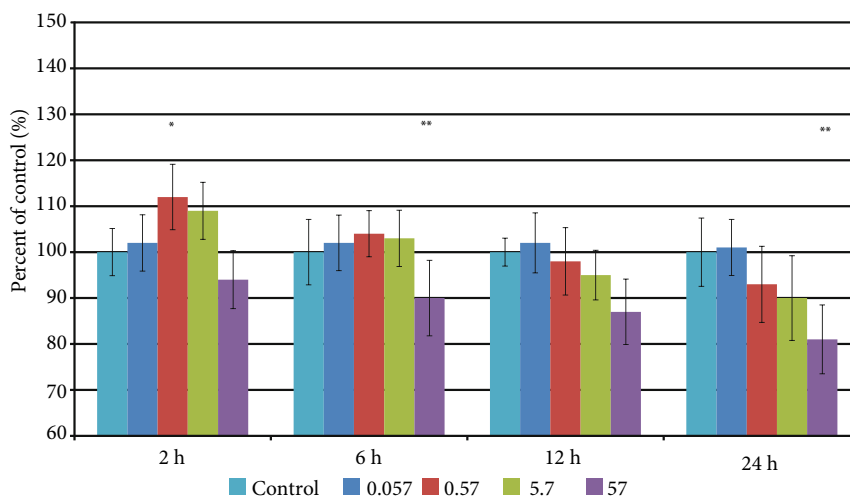


Figure 4. Cell proliferation of 3Y1 fibroblasts under the influence of various doses (μM) of preformed H_2O_2 at the time points indicated. The ability of the treated cells to reduce MTT to formazan was estimated and expressed as a percentage of unexposed control cells. Each value represents the mean \pm SD of 5 independent measurements. *: $P < 0.05$, **: $P < 0.01$.

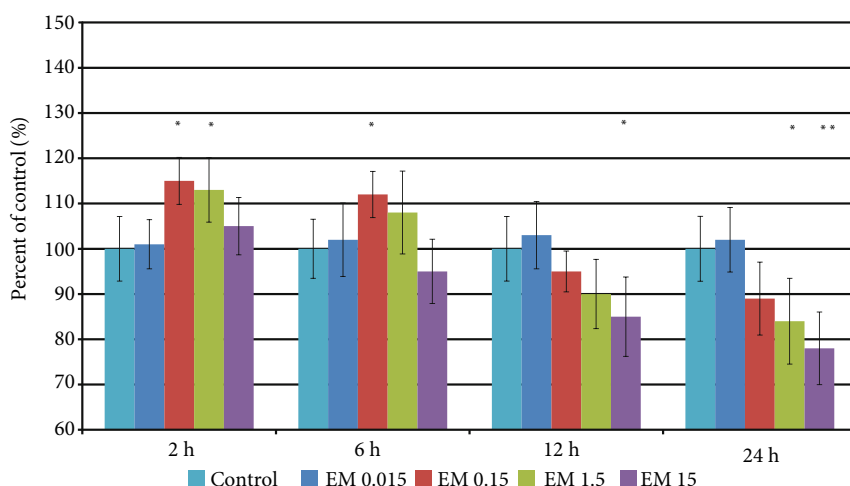


Figure 5. Cell proliferation of 3Y1 fibroblasts under the influence of various doses (mg/mL) of extract mixture (EM) at the time points indicated. The ability of the treated cells to reduce MTT to formazan was estimated and expressed as a percentage of unexposed control cells. Each value represents the mean \pm SD of 5 independent measurements. *: $P < 0.05$, **: $P < 0.01$.

dilution mixture showed a significant ($P < 0.05$) increase in their proliferation at 2 and 6 h after treatment. The highest rate obtained was at 2 h, where a 14.12% increase in cell growth was observed over the control. The same dilution showed nonsignificant ($P > 0.05$) inhibitory effects at 12 and 24 h after treatment. Cells treated with a 1.5 EM dilution of honey mixture showed significant 11.76% ($P < 0.05$) and nonsignificant 8.16% ($P > 0.05$) increases in their proliferation at 2 and 6 h, respectively. On the other hand, the same dilution showed nonsignificant ($P > 0.05$)

and significant ($P < 0.05$) inhibition effects on cell growth at 12 and 24 h, respectively.

Cells treated with a 15 EM dilution mixture for 2 h showed viability close to that of the control cells. However, the same dose caused nonsignificant inhibitory effects on cell viability at 6 h after treatment, and, moreover, the highest inhibitory effects were shown at 12 and 24 h, where there was, respectively, a 16% ($P < 0.05$) and a 22% ($P < 0.01$) reduction in the viability of the treated cells compared to those of the control.

4. Discussion

A number of *in vitro* studies based on fibroblast proliferation have been produced that elucidate the effects of different agents as wound healing promoters (19,20). Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The MTT assay offers a fast and accurate quantitative method for the evaluation of cell response to different agents, whether it is an increase in proliferation, no effect, or a decrease in viability (16,17,21).

The present study is the first *in vitro* study describing the effect of honey and some of its components on cultured fibroblasts. A dilution of 15% (v/v) honey, at 195 mg/mL, was chosen as a maximum test dose because honey showed bactericidal action at this dilution (unpublished observations). Results showed that the highest stimulation could be achieved by using from 19.5 to 1.95 mg/mL honey dilutions for 2–6 h under the assay conditions. Additionally, the maximum stimulation was obtained by the addition of 1.95 mg/mL for 6 h. The use of lower concentrations resulted in a low response, whereas the use of a high dose (195 mg/mL) seems to have had a nonsignificant negative effect. Therefore, the stimulatory effect of honey is dose- and time-dependent, which is not surprising, since most of the growth factors have their maximum stimulatory effect at a specific dose and must be secreted at the right moment.

The maximum stimulatory effect of sugar solution was significantly lower than that of honey, indicating that sugars enhance cell proliferation and, as a source of energy, play a role in the stimulatory action of honey. However, the stimulatory action of honey seems not to be due to sugar content alone, but is also due to other chemical constituents of honey. The present results are in agreement with that reported *in vivo* by Postmes (22), who found that the wound-healing ability of honey was superior to that of sugar.

Growth factors were suspected to be present in honey. Most of the growth factors have molecular weights of 6 kDa and above (23), and therefore they should be retained (if present) by the dialysis membrane with molecular weight cut-off of 3.5 kDa that was used in this study. Cells treated with protein fractions showed no significant response, suggesting the absence of growth factor-like activity in the protein fraction of the tested honey. However, some of the growth factors have no direct effect but rather act through secondary messengers, and thus the presence of such factors with growth factor-like activity remains to be speculated upon.

Hydrogen peroxide was presumed to be one of the honey's factors that stimulate fibroblast proliferation (2). Addition of preformed hydrogen peroxide to cultured fibroblasts resulted in a combination of stimulation,

inhibition, and no response effects. Addition of doses of 0.57 μM and 5.7 μM H_2O_2 to the cells for 2 h exerted significant and nonsignificant stimulation effects, respectively, but not thereafter. These levels were assumed to be the average level of H_2O_2 that was generated in 0.15% and 1.5% (v/v) of the tested honey. As noted, the same doses exerted nonsignificant inhibitory effects at 12 and 24 h after addition. Bolus addition of 57 μM H_2O_2 (average level that was generated in 15% v/v of the tested honey) showed nonsignificant inhibitory effects at 2 and 6 h but caused highly significant inhibition at 12 and 24 h after addition.

Previous reports have indicated that low concentrations (from 10 nM to 1.0 μM) of hydrogen peroxide can stimulate growth or growth responses in a variety of mammalian cell types when added exogenously to the cultured medium (24,25). Davies (26) reported that 3–15 μM hydrogen peroxide causes a significant stimulatory response, with 25%–45% growth stimulation of mammalian cells. However, higher concentrations of 250–400 μM cause permanent growth arrest, which has often been confused with cell death. The present results are in agreement with the previous findings and lead to the suggestion that hydrogen peroxide may act as a growth stimulus through biochemical processes similar to natural growth factors.

Continuous generation of hydrogen peroxide, created by the honey's protein, sugar, and phenolic mixture, also has a biphasic effect on fibroblasts under the present assay conditions. It has higher stimulatory effects and less toxicity than preformed doses at preparations equivalent to 0.15% and 1.5% (v/v) honey added for 2 and 6 h. However, it also showed higher inhibitory effects at 12 and 24 h after addition. The flux of hydrogen peroxide generated enzymatically was found to be less toxic to the host tissue than the injection of bolus hydrogen peroxide (27). Moreover, addition of bolus hydrogen peroxide to Jurkat cells was able to induce apoptosis, while the continuous presence of hydrogen peroxide inhibited the execution of the apoptotic process regardless of the initiation agent being hydrogen peroxide or other inducers (28).

Most of the H_2O_2 toxicity is mediated by transition metal ions, mainly iron and/or copper, which are able to catalyze the formation of the highly reactive hydroxyl radicals (HO^*) by Fenton-type reactions (29). Therefore, the phenolic compounds that are present in the mixture act to minimize the toxicity of hydrogen peroxide via antioxidant mechanisms.

The significant inhibitory effects of the EM mixture at 12 and 24 h is thought to be due to long-term exposure to hydrogen peroxide and imbalance of the antioxidant system. On the other hand, the stimulatory effects of honey were higher than that of its EM-mixtures. This could be attributed to the fact that honey as a whole contains a wide range of nutrients including 11 to 21 amino acids and

several essential vitamins and minerals, and the addition of these nutrients was shown to accelerate tissue growth (30).

In conclusion, the present study established the effect of honey and its major components on the growth of cultured fibroblasts. The stimulatory effect of honey on fibroblast proliferation was not directly proportionally to the dose and time of exposure, but it was time- and dose-dependent. Major sugars (fructose and glucose) as well as other nutritional elements play important roles in the stimulatory mechanism of honey. Continuously generated hydrogen peroxide in honey also acts to stimulate cell

proliferation in a time- and dose-dependent manner. The presence of antioxidant substances protects the cells from hydrogen peroxide toxicity. The bactericidal level tested in this study was not toxic to the cultured fibroblasts under the assay conditions, indicating that honey can be applied to wounds at a bactericidal level that is not toxic to the host tissue. The presence of growth factor-like substances in honey could not be ascertained in this study.

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