Acacia honey and chrysin reduce proliferation of melanoma cells through alterations in cell cycle progression

ELENA PICHICHERO¹, ROSELLA CICCONI², MAURIZIO MATTEI², MARCO GALLINELLA MUZI³ and ANTONELLA CANINI¹

¹Department of Biology, Honey Research Center, University of Rome ‘Tor Vergata’ Via della Ricerca Scientifica 1; ²Centro di Servizi Interdipartimentale Stazione per la Tecnologia Animale - STA University of Rome ‘Tor Vergata’, Via Montpellier 1; ³Department of Surgery, University Hospital Tor Vergata, Viale Oxford 81, I-00133 Rome, Italy

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Abstract. Honey has long been used in medicine for different purposes. Only recently, however, its antioxidant property and preventive effects against different diseases, such as cancer, have been highlighted. Chrysin (5,7-dihydroxyflavone) is a natural flavone commonly found in acacia honey. It has previously been shown to be an anti-tumor agent. In this study, we investigated the antiproliferative role of honey or chrysin on human (A375) and murine (B16-F1) melanoma cell lines. The results of the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the trypan blue exclusion test showed that both the tested compounds were able to induce an antiproliferative effect on melanoma cells in a dose- and time-dependent manner. Flow cytometry analysis indicated that cytotoxicity induced by melanoma cells in a dose- and time-dependent manner. Further investigation is needed to validate the contribution of chrysin in tumor therapy in vivo.

Introduzione

Honey is the natural sweet substance produced by honeybees from nectar or from the secretion of living part of plants. It is composed of at least 300 compounds and is basically a solution supersaturated in sugars, fructose and glucose being the most important. In addition, there is a great variety of minor components, including phenolic acids and flavonoids, different enzymes, carotenoids, organic acids and proteins (1-3). Honey may be considered a ‘functional’ food, also coming under the category of ‘nutraceuticals’ or ‘therapeutic’ food. In fact it satisfies both the classical concept of ‘nutritionally adequate food’, which is a food that provides nutrients in sufficient quantities to satisfy particular organic needs, and the concept of ‘optimal nutritional food’, which includes, besides the above, potential of food to promote health, improve general well-being, and reduce the risk of developing certain illnesses (4,5). Honey has a long tradition of use in folk medicine for various purposes and has been referred to extensively in the medical literature of Egypt, and Greece (6,7). Recent times have seen a revival of interest in the medical properties of honey-bee products, because they are thought to exhibit a broad spectrum of biological activities, including antibacterial, antifungal, cytostatic, wound healing, antitumor and anti-inflammatory effects (8-10). An important property of honey is its antioxidant capacity; this is mainly due to the presence of flavonoids and phenolic acids, although the exact action mechanism is unknown. Among the mechanisms proposed are free radical sequestration, hydrogen donation, or a combination of these acting as substrates for radicals such as superoxide and hydroxyl (11-13). The antioxidant activity of honey is linked to the observed anticancer and antiatherosclerosis effects of honey. Until 1990, the chemopreventive action of honey was attributed to its hydrogen peroxide releasing properties, through induction of cell apoptosis, although recent finding point to a complementary role of the phytochemical antioxidant which can act either with or independently from the release of H₂O₂ (14,15). Chrysin (5,7-dihydroxyflavone) is a secondary metabolite of the class of flavones, this natural compound is a marker of acacia honey because it represents the largest fraction of flavonoids present in this unifloral honey (16,17). Flavones are a subclass of flavonoids, these compounds being widely present in fruits, vegetables, beverages, cereals and herbs. Many experiments have strongly demonstrated that flavones possess many useful properties for human health care, these including enzymes inhibition as well as anti-inflammatory, antioxidant, and cytotoxic antitumor activity. Antioxidant scavenging activity is linked to the prevention of many chronic and age-dependent pathological conditions like cancer, diabetes, atherosclerosis, cataract and chronic neurological conditions (18-21). Like other flavones, such as apigenin and luteolin, chrysin also shows beneficial...
effect on health. Chrysin has been shown to be a potent inhibitor of the enzyme aromatase (22), and to modulate GABA_A and GABA_C receptors (23). Chrysin also possesses anti-inflammatory activity related to the pro-inflammatory activities of COX-2 (24). It has been found to possess cancer chemopreventive properties through inhibiting malignant cell growth, as well as inducing cell death and perturbing cell cycle progression (25-27).

Cancer cells that grow uncontrollably, such as melanoma, are largely resistant to chemotherapy. One of the strategies of cancer management is to inhibit cell proliferation. Since research in melanoma cancer therapy is focused on the discovery of novel drugs able to reduce melanomas proliferative capacity, in this study we have investigated the antiproliferative activity of honey and chrysin in murine B16 and human A375 melanoma cell lines.

Materials and methods

Materials. An acacia honey sample was obtained directly from beekeepers during the 2006-2007 harvest. The floral origin was specified by pollen analysis carried out in our laboratory. Chrysin was purchased from Sigma (St. Louis, MO). The purity of Chrysin was about 96.0% (HPLC).

Cell cultures and treatments. A B16-F1 mouse melanoma cell line and a A375 human melanoma cell line were purchased from American Type Culture Collection. The cells were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 100 U penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. The cells were passaged twice weekly. Acacia honey or chrysin were added to cells in complete medium after 24 h of growth. Honey was diluted at twice weekly. Acacia honey or chrysin were added to cells in complete medium after 24 h of growth. Honey was diluted at a final concentration of 0.01, 0.02, 0.025, 0.03, 0.05, 0.1 and 0.2 g/ml, while chrysin was diluted in methanol at the final concentration of 10, 25, and 50 μM. Cells were also treated with a solution of sugar or with methanol as solvent control.

MTT assay. The effect on cell proliferation, of honey or chrysin, was measured using MTT colorimetric assay as described by Mosman (28). In brief, 2×10^4 cells/well, both human and murine melanoma cells, were grown in 96-well plates and treated as previously described. After 24-, 48- and 72-h treatment 20 μl MTT reagent (5 mg/ml PBS) was added to each well. The cells were further incubated at 37°C for 4 h. After careful removal of the medium, 100 μl of buffered Dimethyl sulfoxide (DMSO) was added to each well, and the plates were shaken. The cellular metabolism was determined by recording absorbance of samples at 570 nm in a micro ELISA reader. The effect of each compound on cell growth was assessed as a percentage of cell viability where vehicle-treated cells were taken as 100% viable. IC_{50} (concentration needed to inhibit growth of 50%) value were estimated following 72-h treatment.

Trypan blue exclusion test. A375 cells and B16 cells were seeded at a density of 1×10^4 cells/well in 24-well plates. After 24 h cells were treated with honey or chrysin and the plates were incubated at 37°C and 5% CO_2 for 24, 48 and 72 h. After treatment time, floating cells in the medium of each well were transferred to centrifuge tubes; adherent cells were washed with PBS, collected by trypsinization and mixed with the corresponding floating cells before centrifugation. Cells were stained with 0.4% of trypan blue and counted in triplicate by optic microscope to estimate the number of live and dead cells.

Cell cycle analysis by FACS (fluorescence-activated cell sorting) analysis. B16 and A375 cell were seeded at density of 2×10^4 cells/well in 24-well plates. After 24 h cells were treated with honey or chrysin as previously described. After 24-, 48- and 72-h treatment and incubation at 37°C and 5% CO_2, cells were washed with PBS harvested with trypsin and centrifuged. Cells were stained with propidium iodide (PI) solution (50 μg/ml PI, 0.5% RNase A and 0.1% Triton X-100) and incubated for 30 min at 4°C in the dark. After this period of incubation cells were fixed with paraformaldehyde (PFA) 1% in PBS for 15 min. Cell cycle distribution was analyzed by flow cytometry on a FACSCalibur (Becton-Dickinson, Mountain View, CA). PI was excited at 488 nm, and fluorescence analyzed at 620 nm. A total of 10,000 events in each sample was acquired. Using CellQuestPro software, the percentages of cells at different phases of the cell cycle were determined.

Results

Honey and chrysin reduces metabolic activity of melanoma cells. In the first set of experiments, the anti-neoplastic activity of honey or chrysin in melanoma cell lines was measured using MTT assay in basic culture conditions. All the doses of honey used in this assay were able to induce a significant reduction of metabolic activity of both melanoma cell lines. The inhibitory effect was observed in a time- and dose-dependent manner (Fig. 1A and B). The estimated IC_{50} value was equal to about 0.02 g/ml of acacia honey for both human and murine cell lines. Honey significantly reduced the metabolic activity of tested cells after only 24-h exposure time; in fact the intermediate doses inhibited cellular growth of 50 and 40% in B16 and A375 cells, respectively. In B16-F1 cells the highest doses tested (0.2 and 0.1 g/ml) showed a cytotoxic effect on cells. Indeed, a reduction of metabolic activity of >90% was observed after only 24-h treatment. In A375 line this cytotoxic effect was observed after 48- and 72-h exposure of cells to 0.2 and 0.1 g/ml doses of acacia honey. We performed the same experimental plan used for acacia honey with different concentrations of chrysin (10, 25 and 50 μM). The antiproliferative activity of chrysin in both A375 and B16-F1 melanoma cells was measured by MTT assay to evaluate any level of cell growth. The result showed that all concentrations of chrysin were able to induce a moderate but significant reduction of metabolic activity in both melanoma cell lines in a time-dependent manner (Fig. 1C and D). The estimated IC_{50} (50% inhibition of cell growth after 72-h treatment) value was equal to about 50 μM chrysin for both human and murine cell lines. After 24-h treatment was observed a significant reduction of metabolic activity only for 25 and 50 μM chrysin, the percentages of reduction respect to control being equal to about 10 and 20%
in murine cells, and 15 and 25% in human cells, respectively. The compound significantly reduced metabolic activity, in a dose-dependent manner up to 50% after 72-h treatment with respect to the control in both human and murine cells.

Acacia honey and chrysin inhibit cell proliferation of melanoma cells. Since MTT assay is an indirect assay of cell viability a direct one such as the trypan blue exclusion test was also carried out. Acacia honey inhibited the growth of B16 cells significantly at all doses tested. In fact, as compared with the control, after 24, 48 and 72 h an inhibition of 60, 70 and 80%, 42, 50 and 60% and 14, 30 and 40% at the doses of 0.025, 0.02 and 0.01 g/ml was observed (Fig. 2A). In the same way, honey significantly reduced the proliferation of A375 cells (Fig. 2B), accounting for 42, 57 and 68%, 35, 43 and 53% and 20, 32 and 46% of inhibition at doses of 0.025, 0.02 and 0.01 g/ml after 24-48- and 72-h exposure, respectively. IC50 values at 72 h were also obtained through the trypan blue test and were equal to about 0.015-0.01 g/ml in both the B16 and the A375 cell lines. The inhibitory effect was time- and dose-dependent. 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acacia honey induced a time-dependent arrest of cell cycle progression in the G0/G1 phase in both melanoma cell lines, when compared with the control sample, with reduction of S and G2/M phase population and also an increment of Sub-G0 cell population (Fig. 3). Analysis of the cell cycle showed a further interesting effect caused by prolonged exposure of melanoma cells to intermediate concentrations of honey.
Following treatment with 0.025, 0.03 and 0.05 g/ml doses of acacia honey for 48 and 72 h, flow cytometric analysis showed an increase of cells in G2/M phase, followed by a decline of this population with the appearance of hyperploid cells in both A375 (Fig. 4) and B16-F1 cell lines (Fig. 5). Hyperploid cell accumulation did not occur in cells that were similarly stored but not incubated with acacia honey, indicating that this is not an artifact of cell sorting (Figs. 4A and 5A). After 24-h treatment with chrysin, we observed by FACS analysis a strong and dose-dependent arrest of cell cycle in the G0/G1 phase in B16 and A375 cell lines, with reduction of the S and G2/M phase populations (Fig. 6A). In regard to the amount of B16 cells, in G0/G1 phase it was equal to 75% in 50 μM chrysin-treated cells compared with about 50% in control cells (Fig. 6B). Flow cytometry analysis also showed an increase in the number of cells undergoing sub-G0 phase, this effect being dose- and time-dependent. After 72-h treatment the amount of cells in sub-G0 phase was approximately equal to 10, 25 and 35% compared to control, for 10, 25 and 50 μM chrysin, respectively (Fig. 6C). Similarly, in A375 cells, a G0/G1 arrest was observed at the 50 μM dose after 24 h (Fig. 7A), the amount of human melanoma cells exposed to chrysin in G0/G1 phase was equal to about 70% in respect to the untreated control (Fig. 7B). As in the case of murine melanoma cells an increase of cell population in sub-G0 was also observed in the A375. After 72-h treatment the amount of cells in this phase is approximately equal to 8, 20 and 30% compared to control, for 10, 25 and 50 μM chrysin, respectively (Fig. 7C). An accumulation of melanoma cells in the G0/G1 phase was also detected after 48-h treatment in both the cell lines for all concentration tested and also after 72 h only for 10 μM chrysin, although the differences with respect to the control were less significant than after 24 h of incubation (data not shown). Following 72-h treatment with chrysin, flow cyto-

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Figure 5. DNA content flow cytometric histograms of B16-F1 cells (brackets, 1, G0/G1 phase; 2, S phase; 3, G2/M phase; 4, hyperploid cells). (A) Untreated A375 cells growth for 72 h. (B-D) Induction of hyperploid progression in B16-F1 melanoma cells after 72-h exposure to acacia honey treatment (B) 0.025 g/ml, (C) 0.03 g/ml and (D) 0.05 g/ml. After treatment, cells were stained with propidium iodide and flow cytometric analysis was performed as described in Materials and methods. The data shown are representative of three independent analysis.

Figure 6. Effect of chrysin (10-25-50 μM) on cell cycle progression of B16-F1 cells. (A) DNA content flow cytometric histograms of melanoma cells after 24-h treatment with chrysin. Ctr, untreated control. (B) The percentage of cell cycle distribution of melanoma cells after 24-h treatment with chrysin. (C) The percentage of cell population in sub-G0 phase after 24-, 48- and 72-h treatment. After treatment, cells were stained with propidium iodide and flow cytometric analysis was performed as described in Materials and methods. Results are the mean ± SD from three independent experiments. *P<0.05 versus vehicle control-treated cells. The data shown are representative of three independent analysis.
metric analysis showed that there is an increase of cells in G2/M phase, followed by a decline of this population with the appearance of hyperploid cells in both A375 and B16-F1 cell lines (Fig. 8). Hyperploid cell accumulation did not occur in cells that were similarly stored but incubated without chrysin, indicating that this is not an artifact of cell sorting.

**Discussion**

In a previous work we demonstrated that chrysin is the main secondary metabolites present in acacia honey, we demonstrated also that the presence of secondary metabolites is closely related to the antioxidant power of honey (16). Considering these assumptions we decided to test the antitumor efficacy of both acacia honey and chrysin on two different melanoma cell lines *in vitro*. Initially we analyzed the metabolic activity of honey- or chrysin-treated cells by using the MTT assay. The MTT test is widely used to determine cytotoxicity of potential medicinal agents and other toxic materials, since those agents can cause cell toxicity and therefore metabolic dysfunction. This assay is based upon the selective ability of live cells to reduce the yellow soluble salt to purple-blue insoluble formazan precipitate in active mitochondria (29). These reductions take place only when reductase enzymes are active, and therefore conversion is often used as a measure of viable cells. Our results indicated that both acacia honey and chrysin inhibited the metabolic activity of melanoma cells, resulting in inhibition of cell proliferation. This effect was time-and dose-dependent, but was not line-specific. The data obtained showed that high doses of acacia honey (0.2 and 0.1 g/ml) had a cytotoxic effect on melanoma cells, while, in contrast the highest concentration of chrysin used (50 μM) showed no significant cytotoxic effects. The high toxicity observed after treatment with high doses of acacia honey was probably due to the high concentration of sugars present in the tested compounds.
Indeed the same effect was found in the control treatment where cells were exposed to the same concentration of sugar provided in 0.2 and 0.1 g/ml doses of acacia honey (data not showed). Since MITT is an indirect colourimetric assay, the trypan blue test was then used to directly assess the cell survival rate in melanoma lines following treatment. Antineoplastic activity of acacia honey and chrysin was confirmed, although IC₅₀ values obtained after a 72-h treatment were considerably lower than those from the MITT test. This discrepancy in test sensitivity could be explained by the fact that MITT assay is based on intracellular dehydrogenases whose activity could be altered by the tested compound. Furthermore it is known that flavonoids might directly reduce the MITT without living cells (30,31).

By direct cell counting it is possible to distinguish between the cytostatic and the cytotoxic effect; in the latter case the results of trypan blue test confirmed the cytotoxic activity exerted by higher doses of acacia honey after 24-h treatment due to a higher percentage of dead cells than the number of viable cells respect to control. Moreover, a cytostatic effect exerted by the other doses are recorded in both the cell lines. The results obtained also confirmed the cytostatic activity exerted by chrysin, at least in the experimental conditions used. However, we cannot rule out that higher concentration of this molecule and longer incubation times could exert cytotoxic activity, since our experimental observations were extended for up to 72 h of culture and at a maximum dosage of 50 μM.

In order to assess whether acacia honey- or chrysin-induced cell growth inhibition is mediated via alterations in cell cycle progression, cell cycle analysis in response to treatments was performed. In this study, we observed that acacia honey and chrysin induced a time-and dose-dependent G₀/G₁ phase arrest in melanoma cells. The accumulation of cell population in G₀/G₁ phase was also observed in other cancer cell lines after honey or chrysin treatments. Honey-induced cell growth inhibition through accumulation of cell population in G₁ phase was also observed in T24 bladder cancer cell lines (32). Chrysin-induced G₁ arrest in C6 glioma cells has been demonstrated to be caused by increased p21Waf/Cip₁ protein levels and suppression of CDK4 and CDK2 kinase activities, which mediate the phosphorylation of Rb (27). Other secondary metabolites of class of flavones, such as apigenin, luteolin and tangertin, with anticancer properties blocked the cell cycle either in the G₀/G₁ phase (33,34) or in the G₂/M phase (35,37). Since most of the antineoplastic drugs in clinical use block the cell cycle in the S or G₂/M phases whereas chrysin blocks the cell cycle in the G₁ phase, a combination of chrysin with currently used drugs might possibly improve melanoma therapies. Flow cytometric analysis of acacia honey and chrysin-treated cells also showed that there is an increase of cell in G₂/M phase, followed by a decline of this population with the appearance of hyperploid cells in both A375 and B16 cells after 72-h treatment. Most cancer therapies target cell cycle checkpoints by activating checkpoint-mediated cell death or by enhancing chemical sensitivity due to loss of checkpoint function (38). Since malignant tumor cells generally have an impaired checkpoint function, therapeutic agents induce premature transition of checkpoint border, resulting in massive cell death (39). However, it is well known that treatment with cancer therapeutic agents also causes drastic phenotypic changes in surviving cancer cells. The mitotic spindle checkpoint monitors spindle microtubule structure, chromosome alignment on the spindle, and chromosome attachment to kinetochores during mitosis. The spindle checkpoint delays the onset of chromosome segregation during anaphase until any defects in the mitotic spindle are corrected (40,41). The molecular mechanism of the spindle checkpoint has implicated the activity of mitotic checkpoint protein encoded by the BUB and MAD genes, including BubR1, Bub1, Bub3, Mad1, and Mad2. A number of drugs important in clinical and biomedical research disrupt the assembly (e.g., nocodazole and vincristine) or disassembly (e.g., paclitaxel and docetaxel) of microtubules and in doing so, invoke the spindle checkpoints (42,43). Hyperploidy can be induced in mammalian cells by many anti-cancer drugs, such as nocodazole, vincristine and taxol (44), including inhibitors of the microtubule organization and various serine/threonine kinases (45). These anti-microtubule drugs (AMDs) generally induce mitotic arrest followed by massive cell death in many cancer cell types, and thus they are frequently used to treat multiple human cancers. However, it is possible that a fraction of cancer cells are able to survive the prolonged mitotic stress and resume cell cycle progression. It has been reported that prolonged exposure to AMDs induces cells to exit mitosis and enter the G₁ phase without undergoing complete chromosome segregation and cell division; this process is known as ‘mitotic slippage’ or ‘adaptation’ (46). While normal cells are arrested at the G₁ phase after prolonged mitotic spindle damage, many cancer cells are not arrested at the G₁ phase and enter the subsequent S phase with a 4N DNA content. Those cells undergoing premature DNA replication consequently form hyperploid cells; this process is known as endoreduplication (47). Previous reports showed that molecules involved in the G₁ checkpoint, such as p53, p21 and Rb, play an important role in prevention of hyperploidy formation after prolonged mitotic spindle damage (46,48,50). However, the mechanism that generates these hyperploid cells remains unknown. Even more uncertain is whether hyperploidy formation is the effect or the consequence of the malignant progression. Therefore, knowledge of the cellular mechanism behind hyperploidy is pivotal to our understanding of cancer progression and cancer therapy (44). Hong et al (45) demonstrated that the S-phase re-entry without cell division leading to hyperploidy could be inhibited by the inhibitor of G₁-specific Cdks. These results provide a unique opportunity to develop a therapy that is specific against tumors lacking the G₁ checkpoint.

In conclusion, the present study demonstrates that acacia honey has an antiproliferative effect on human and murine melanoma cells. In our opinion, this effect is mainly due to the presence of high concentrations of chrysin. Our results suggest that chrysin may be considered a potential candidate for both cancer prevention and treatment, although further investigation is needed to validate the contribution of chrysin in tumor therapy in vivo. Honey exerts a wide range of beneficial effects on human health, honey's secondary metabolites content and antioxidant potential being closely...
related. As such, honey should be considered non only for its nutritional properties but also as a functional food to prevent tumors.

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