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INFLUENCE OF VITAMIN C AND RIBWORT PLANTAIN EXTRACT ADDITION TO THE PROPOLIS EXTRACT ON THE VIABILITY OF FIBROBLASTS IN CELL CULTURE *IN VITRO*

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Propolis is a honey bee product rich in biologically active substances that have been proved to have beneficial effects on human health. It is widely used in traditional medicine for the treatment of various not only respiratory but also skin disorders. Propolis can be used alone as a pure extract or with the addition of multiple plant extracts and antioxidants to achieve synergistic effects. The aim of this study was to examine the effects of different propolis extracts, commercially available on the market, on the viability of fibroblasts in cell culture *in vitro*. We examined the effect of three different propolis extracts: pure propolis extract (25%), propolis extract (10%) with added vitamin C and propolis extract (10%) with Ribwort Plantain extract and added vitamin C, on the viability of L929 fibroblasts, using the MTT test and microscopically. Concentration-dependent effect of all examined propolis extracts on the viability of fibroblasts was observed. Also, differences in the effect of examined extracts on cell viability were noticed related to the additions to the propolis extract, and the pattern was different in lower compared to the higher examined concentrations. The addition of vitamin C and Ribwort Plantain extract influences the effects of pure propolis. Using propolis in combination with plant extracts and bioactive substances may have beneficial effects, but it should be considered based on the indications for which these products are intended and the effects to be achieved.

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Key words: *propolis, ribwort plantain, vitamin C, fibroblasts, in vitro*

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Introduction

Propolis is a natural, resinous substance produced by bees from substances they collect from different parts of plants and exudates. The chemical composition of propolis, biological and pharmacological activity vary greatly depending on the plant species from which it originates and the geographical origin (1). Propolis is mainly composed of resins and vegetable balsam (50%), wax (30%), essential oils (10%), pollen (5%), sugars, amino acids, vitamins, and minerals (5%) (2–9). Except for resins and waxes, the main groups of chemical compounds found in propolis are phenols (e.g., flavonoids, polyphenols, phenolic acids and other phenolic compounds) and their esters, terpenes and terpenoids, steroids, aromatic acids, aromatic esters, aldehydes, alcohols, sugars and their alcohols and acids, amino acids, vitamins, fatty acids, hydrocarbons, mineral elements and alcohols (7, 9–12). Flavonoids are a major group of phenolic compounds in propolis which greatly contribute to the biological and pharmacological activities of propolis. Propolis has been known in the traditional medicine of many countries for its therapeutic properties for a long time, so in some countries, it is used to treat a wide range of diseases and is also used for cosmetic purposes (13). It is known that propolis has an antibacterial, antiviral and antifungal activity (8, 9, 13), so it is used to treat infections of the upper respiratory tract as well as superficial wounds in the form of topical formulations (14). Propolis acts as an antioxidant and anti-inflammatory agent, so it is also used in the treatment of inflammatory processes and disorders (8, 9, 13). Since propolis has been shown to have a beneficial effect on wound

healing (13, 15), it is used in the form of topical formulations for healing wounds and burns on the skin and in dental preparations for the treatment of gingivitis (16). Nowadays, propolis is used in the form of drops and sprays for oral and *per os* use, in the form of creams, gels, lotions, capsules, lozenges, toothpastes and others.

Vitamin C is a well-known antioxidant that is added to various products in order to achieve a synergistic effect and to potentiate their antioxidant capacity. In addition to its antioxidant effect, vitamin C stimulates fibroblast proliferation and collagen synthesis (17), so it is often added to preparations for skin care and wound healing. In cosmetology and dermatology, vitamin C is commonly used as a component of various creams and serums because it has a protective effect on skin damage caused by various harmful effects and slows down the ageing process (18). The addition of vitamin C to various formulations can contribute to and stimulate their beneficial properties.

Ribwort Plantain (*Plantago lanceolata* L.) has been known for its beneficial effects on human health since ancient times. *Plantago lanceolata* L. is a species from the genus *Plantago*, family Plantaginaceae, that is the most commonly used for medicinal purposes. Ribwort Plantain is registered as a natural medicine (herbal medicinal product) by the European Medicines Agency (EMA). EMA defines the oral, oromucosal and cutaneous use of the leaf of Ribwort Plantain (lat. *Plantaginis lanceolatae folium*) for the following indications: as a demulcent for the symptomatic treatment of oral or pharyngeal irritations and associated dry cough, for the relief of cough associated with cold and for the treatment of minor inflammation of the skin (19). In many countries, it is used in combination with other plant species to treat various disorders. It is used in the form of extracts, syrups, teas, lozenges and tablets by oral and topical route for the treatment of inflammation of the upper and lower parts of the respiratory tract, inflammation of the skin and for the healing of skin and mucous membranes wounds (20). Ribwort Plantain has been shown to possess anti-inflammatory (21), antioxidant (22), antibacterial (23), and antiviral activity (24), to stimulate epithelization and to act as a spasmolytic agent (25). It is one of the main plants used in cough remedies and has anti-inflammatory effects, protects the liver, and is used in the treatment of cancer (26). The main biologically active compounds of *Plantago lanceolata* L. are: iridoid glycosides (aucubin and catalpol), phenylethanoid glycosides, polysaccharides, flavonoids (apigenin and luteolin), polyphenols, alkaloids, terpenoids, fatty acids, phytosterols, phenylethanoids (acteoside, plantamajoside) and tannins, organic acids, mucilaginous substances, mineral salts, and pectins (26–29). Ribwort Plantain extract can successfully be used as an effective ingredient in cosmetic products (30).

In biomedical research, where biological activities of various substances and pharmaceutical products are examined, studies on cell cultures *in vitro* are of fundamental importance and represent the first step in examining the biological activities. In order to investigate the potential effects, such as wound healing, on the skin and mucous membranes, the most commonly used model for this type of investigation is fibroblast cultures. The cell line that is most suitable for this purpose is the L929 cell line, a permanent cell line of fibroblasts obtained from the mouse skin.

The aim of this study was to examine the influence of the addition of vitamin C and Ribwort Plantain extract to the propolis extract on the viability of fibroblasts in cell culture *in vitro*.

Materials and Methods

Propolis extracts

Three different propolis ethanol extracts available on the market were tested: highly purified propolis extract (25%) in ethanol solution (Propolis drops—extra 25%), highly purified propolis extract (10%) in ethanol solution with added vitamin C (Propolis drops with vitamin C), highly purified propolis extract (10%) and *Plantaginis lanceolatae folium* extract in an ethanolic solution with added vitamin C (Propolis drops with Ribwort Plantain and vitamin C), all from producer Sinefarm d.o.o. Serbia. Extracts were diluted in complete DMEM, and final dilutions 1/100, 1/200, 1/500, 1/1,000 and 1/2,000 (v/v) were examined.

Cell culture and viability assay

The effect on cell viability was examined on the L929 cell line (obtained from the American Type Culture Collection—ATCC), which was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, antibiotic-antimycotic solution and stable glutamine (which makes complete DMEM medium), all purchased from Capricorn Scientific GmbH, Germany. Cells were seeded in 96-well culture plates (Greiner Bio-One, Germany) in complete DMEM so that 20,000 cells were seeded per well per 100 µl of medium, and left for 24 hours in an incubator with 5% CO_2, in humidified atmosphere, at 37 ^{o}C, to adhere and to adapt to the environment. After that, 100 µl of prepared dilutions of examined propolis extracts (twice as high as those finally tested) were added to the cells. Cells were incubated with different concentrations of the examined propolis extracts for the next 24 hours, and after that MTT test was performed to assess the effect of the extracts on cell viability. MTT test is commonly used for viability testing and is based on the

ability of viable cells to reduce the yellow tetrazolium salt MTT by mitochondrial dehydrogenases to violet formazan crystals, which are further dissolved in 2-propanol. The intensity of the colored solutions obtained from the dissolved formazan crystals was in direct correlation with the number of viable cells. Absorbance was measured on a multichannel spectrophotometer, Multiscan Ascent (ThermoLab Systems, Finland), at the wavelength of 540 nm with the correction at 650 nm. Each concentration was tested in tetraplicates and the experiment was repeated twice. As a control, we used cells that were incubated under the same conditions in complete medium but without the examined extracts (untreated cells).

Microscopical analysis

Cells were analyzed microscopically using an inverted light microscope (Observer Z1, Carl Zeiss, Germany), under phase contrast. The images of cells were acquired before incubation with extracts and 24 hours after incubation, before the MTT test, using the camera AxioCam HR in the software ZEN 2 blue edition (Carl Zeiss, Germany).

Statistical analysis of the results

The results of the MTT test are shown as a percentage of cell viability in relation to the control (untreated cells), for which the viability was considered to be 100%, with relative standard deviations. The percentage of cell viability was obtained from the mean values of the measured absorbances for each examined concentration and control. Statistically significant differences were analyzed by one-way ANOVA (analysis of variance) test, and values for which $p < 0.05$ were considered significant.

Results

The effects of examined propolis extracts on the viability of L929 fibroblasts were assessed by MTT test and microscopically. The results of the MTT test are shown in Figure 1.

Statistically significant difference in the effect of examined propolis extracts on L929 cell viability was observed at all examined dilutions. Significant decrease in cell viability, i.e., cytotoxicity, was noticed for all examined dilutions of P-25 and PC extracts, with more pronounced cytotoxic effect of dilutions from 1/100 to 1/1,000. PCP extract was shown to be the least cytotoxic among the examined extracts. Dilutions 1/1,000 and 1/2,000 of PCP extract were not cytotoxic for L929 cells. The trend of action of examined extracts in dilutions 1/100 and 1/200 was the same; the PCP extract was the most cytotoxic, while the P-25 extract was the least toxic at these concentrations. With a decrease in the concentration of all three extracts, i.e., increase in dilution, the trend of action changed, so the most cytotoxic effect was noticed for the P-25 extract, while the least cytotoxic was the PCP extract.

The effect of the examined extracts on cell morphology and number was examined microscopically, and the results are shown in Figure 2. In higher examined concentrations (dilutions from 1/500 to 1/100), cells were round in shape, which indicates toxic effects, with some cells becoming elongated in the case of 1/500 dilution of PCP extract. At dilution 1/1,000 differences among examined extracts were noticed, with the most pronounced cytotoxic effect reflected on the cell morphology in the case of P-25 extract, followed by PC extract where there were some cells elongated in shape and PCP extract with most of the cells elongated in shape, typical of fibroblasts. These observations were in accordance with the results of the MTT test. At dilution 1/2,000, differences in cell density besides differences in cell morphology could be noticed, which were in accordance with the results of MTT test in terms that PCP extract did not exert toxic effects, which was visible as the highest cell density on microscopical images and morphology of cells typical for fibroblasts.

Solvent (ethanol at dilution 1/100) neither influenced the cell morphology and number of cells, nor acted cytotoxic (% of cell viability measured by the MTT test was 112% which was slightly, but significantly stimulated parameter of cell viability compared to the control). This means that solvent did not cause cytotoxic effects on cells.

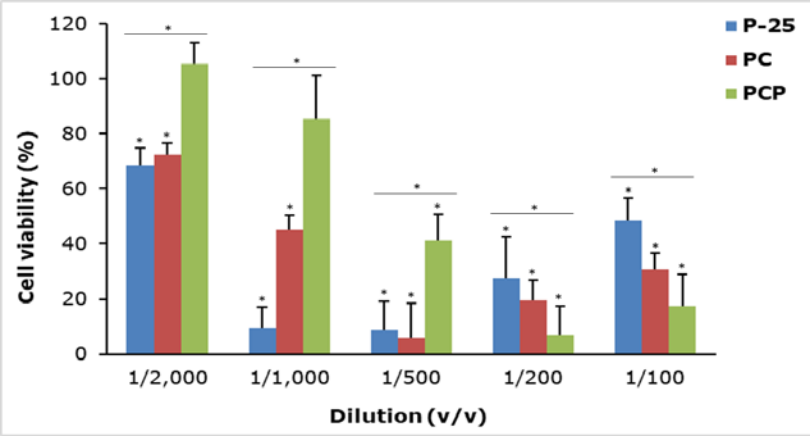

Figure 1. The effect of different propolis extracts: pure propolis extract 25% (P-25), propolis extract with added vitamin C (PC) and propolis extracts with Ribwort Plantain and added vitamin C (PCP), on the viability of L929 cells expressed as a percentage of cell viability compared to the control (untreated cells); (*) $p < 0.001$

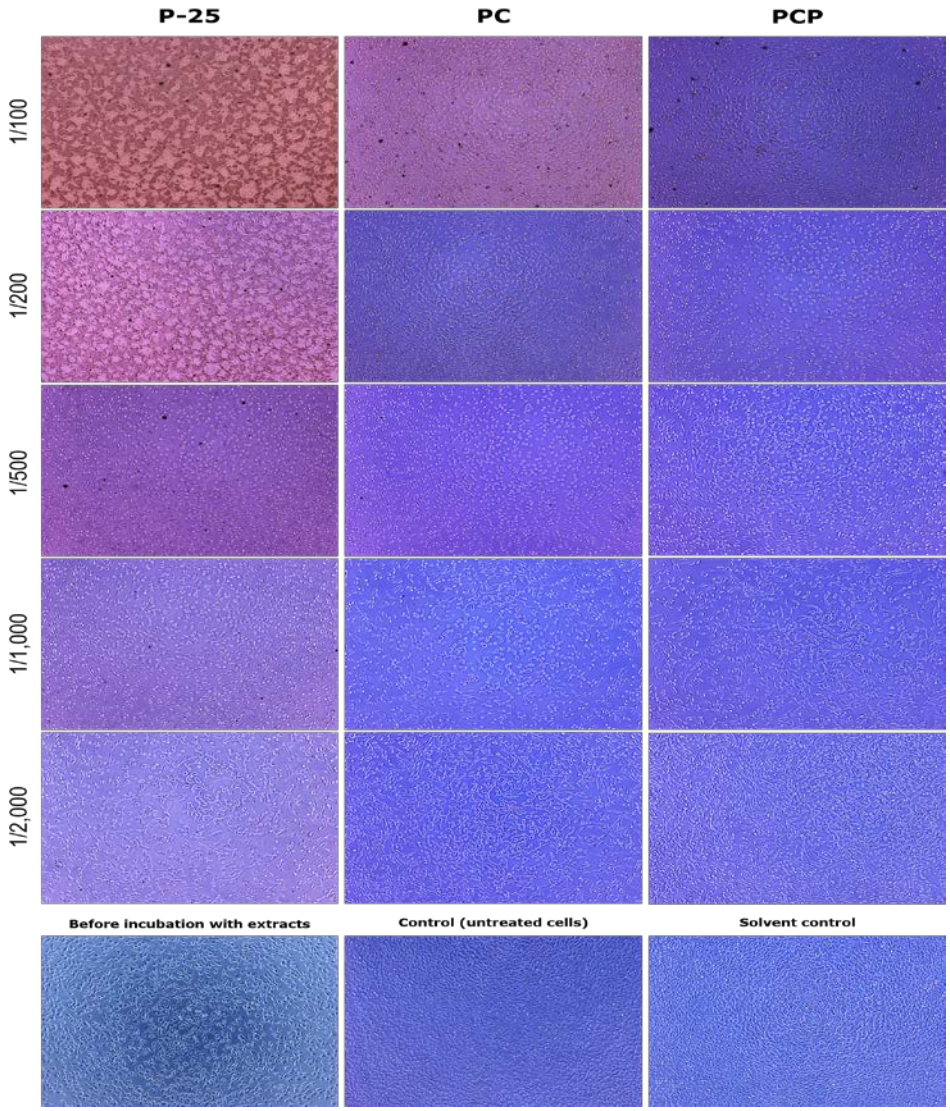

Figure 2. Morphological appearance of L929 fibroblasts before incubation with extracts, 24 hours after incubation with different dilutions of pure propolis extract 25% (P-25), propolis extract with added vitamin C (PC), propolis extract with Ribwort Plantain and added vitamin C (PCP), control (untreated cells) and solvent control.

Discussion

The obtained results show that all examined propolis extracts exhibited a concentration-dependent effect. A different effect on cell viability was obtained in the treatment of cells with different propolis extracts. At higher dilutions of the extracts, e.g., lower concentrations of the extracts, the protective effect of vitamin C and Ribwort Plantain extract on cell viability was pronounced, compared to the effect of pure propolis extract. At lower dilutions, i.e., higher concentrations of extracts, the cytotoxic effect was present regardless of the type of extract used. In order to rule out the potential effect of the solvent used for extract preparation, the cells were incubated with the same dilutions of ethanol, which was used as a solvent in all examined extracts, where even the highest concentration of ethanol, a dilution of 1/100, was not cytotoxic for L929 fibroblasts, moreover, it slightly increased the reduction of MTT by fibroblasts. The concentration of ethanol in all examined dilutions of propolis extracts was less than 1%.

The cytotoxic and antiproliferative effect of propolis and extracts of *Plantago* spp. was demonstrated on cancer cell lines, which gives these extracts a potential application in chemotherapy (31, 32). This effect was mainly demonstrated on epithelial cancer cells.

The ethanolic extracts of Malaysian and Brazilian red propolis show potential to assist in wound healing, depending on their concentration, in an *in vitro* cell model using normal human fibroblast cell line CRL-7522 (33). In the study where Portuguese 30% propolis ethanolic extract was examined on human dermal fibroblasts and keratinocytes, it was found that concentrations of propolis extract below 1 mg/mL were well-tolerated by fibroblasts and moderately tolerated by keratinocytes, which, together with good antimicrobial and antibiofilm effect, suggests that propolis extract could have a good applicability in the form of topical formulations for antibacterial treatment of infected skin disorders (14).

Vitamin C is important for collagen synthesis by fibroblasts and is a very popular addition to creams and other topical formulations for preventing skin damage and ageing, as well as different products for preventing gum retraction. In a study where human gingival fibroblasts were rinsed with 0, 10, 20 and 50 μg/mL of L-ascorbic acid for 7 min, three times per day in the experiment that lasted for two days, it was shown that rinsing the fibroblasts with 50 μg/mL of L-ascorbic acid significantly reduced the cell viability, evaluated by MTT test, while concentrations bellow 50 μg/mL did not influence the cell viability (34). In our study, propolis extract with added vitamin C (PC) led to decreased cell viability at dilutions from 1/100 to 1/500, compared to the pure propolis extract (P-25) without vitamin C. The concentration of vitamin C in extracts PC and PCP examined in our study, was 3 g/100 mL according to the data available on the website of the manufacturer, which means that 1/500 dilution of PC extract contains 60 μg/mL of vitamin C which is close to the concentration of vitamin C used in the mentioned study. In lower concentrations (dilutions 1/1,000 and 1/2,000) propolis extract with added vitamin C was shown to be less cytotoxic compared to the pure propolis extract. The important difference was also that in the mentioned study, fibroblasts were only rinsed with vitamin C, while in our study, fibroblasts were incubated for 24 hours with propolis extracts containing vitamin C. Also, we used a permanent cell line, while in the mentioned study, a primary culture of gingival fibroblasts was used.

Extracts from *Plantago ovata* have been shown to have a beneficial effect and to stimulate the proliferation of fibroblasts *in vitro* (35). Extracts of *Plantago lanceolata* L. were shown to be a valuable source of bioactive substances that have beneficial effects on fibroblasts due to high antioxidant properties, UV protecting activity and stimulation of skin regeneration, which makes those extracts a good additive to the pharmaceutical formulations that are used as natural cosmetics (26). Wound healing activity of aqueous extract of Ribwort Plantain was demonstrated in an open wound rat model (36). It was also shown that different solvents influence different effects of the Ribwort Plantain extracts on fibroblasts. For example, extracts using glycol and glycerin allowed the isolation of some bioactive compounds that were not present in ethanolic and aqueous extracts, while the ethanolic extract of Ribwort Plantain at concentrations of 50 mg/mL reduced the viability of fibroblasts by 45% (26). In our study, propolis ethanolic extract with Ribwort Plantain and added vitamin C was examined and was shown to be the least cytotoxic among examined propolis extracts, with absence of cytotoxic activity at lower examined concentrations (1/1,000 and 1/2,000), while pure propolis extract and propolis extract with added vitamin C were cytotoxic at those dilutions.

There are numerous propolis extracts on the market. Differences in the action of propolis extracts that can be found in the literature can be explained by the different composition of propolis from different countries, which is inevitable, because the composition of propolis depends primarily on the type of plants and their parts from which bees collect it. Combining propolis extract with plant extracts may have beneficial effects on human health if the combination is properly designed. It is also very important to consider the potential application of propolis extracts prior to mixing with other biologically active compounds. Further studies with propolis extracts that we examined are necessary, in order to analyze the effects of those extracts on

wound healing both *in vitro* and *in vivo*, before making any conclusions regarding the potential use of those extracts in the treatment of skin disorders.

Conclusion

Based on the obtained results, it can be concluded that there is a concentration-dependent effect of all examined propolis extracts and that there is a difference in the effect of the extracts concerning the composition. The addition of vitamin C to the propolis extract, as well as the combination of propolis extract with Ribwort Plantain, changes the effect of propolis on the viability of fibroblasts in cell culture. When choosing the propolis extracts with additions of other extracts or vitamin C, the potential indication and application of that extract should be considered. Further *in vitro* and *in vivo* studies are needed before recommendations on the use of these combinations in the treatment of skin disorders.

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UTICAJ DODATKA VITAMINA C I EKSTRAKTA BOKVICE EKSTRAKTU PROPOLISA NA VIJABILNOST FIBROBLASTA U ĆELIJSKOJ KULTURI *IN VITRO*

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Propolis je pčelinji proizvod izuzetno bogat biološki aktivnim supstancama za koje je dokazano da blagotvorno utiču na zdravlje ljudi. Propolis ima široku primenu u tradicionalnoj medicini u lečenju raznih respiratornih, kao i kožnih oboljenja. Propolis se može koristiti samostalno kao prečišćeni ekstrakt ili sa dodatkom raznih biljnih ekstrakata i antioksidanasa kako bi se ostvarili sinergistički efekti. Cilj ovog istraživanja bio je da se ispitaju efekti koje različiti ekstrakti propolisa dostupni na tržištu imaju na održivost fibroblasta u ćelijskoj kulturi *in vitro*. Pomoću MTT testa ispitan je efekat triju različitih ekstrakata propolisa – čistog ekstrakta propolisa (25%), ekstrakta propolisa (10%) sa dodatkom vitamina C i ekstrakta propolisa (10%) sa bokvicom i dodatkom vitamina C – na održivost L929 fibroblasta. Uočeno je da je efekat svih ispitivanih ekstrakata propolisa na održivost fibroblasta zavisio od koncentracije. Takođe, uočene su razlike u uticaju ispitivanih ekstrakata na održivost ćelija koje su bile povezane sa dodatkom ekstraktu propolisa, a obrazac u nižim koncentracijama razlikovao se od onog u višim koncentracijama. Dodatak vitamina C i ekstrakta bokvice ekstraktu propolisa utiče na delovanje čistog propolisa. Premda upotreba propolisa u kombinaciji sa biljnim ekstraktima i bioaktivnim supstancama može imati blagotvorne efekte, te kombinacije najpre treba razmotriti na osnovu indikacija za koje su ovi proizvodi namenjeni i željenih efekata koje je potrebno postići.

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***Ključne reči**: propolis, bokvica, vitamin C, fibroblasti, in vitro*