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In vitro analysis of the biological activity of royal jelly on different cell lines

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Abstract

Royal jelly is a bee product that is traditionally used as a dietary supplement but also as a potential remedy. Although it is widely used, experimental data that supports its therapeutic potential is lacking. Also, many studies have examined individual fractions and isolated substances from royal jelly, but there are very few studies that examine the native form of royal jelly, the one used in the daily diet. Our aim was to examine the effects of royal jelly, in the form of lyophilized powder product commercially available on the market, on the viability and proliferation of different cell lines in vitro. Our results showed that examined royal jelly product did not influence the cell viability of examined cell lines in examined concentrations while acted anti-proliferative in concentration-dependent manner on HeLa, cancer cell line but not on MDCK, non-cancer cell line. We can conclude that royal jelly contains some compounds that could exert certain activity towards cancer cells indicating its potential to which further studies should be directed.

Key words: royal jelly; in vitro; HeLa cells; MDCK cells; proliferation; viability.

INTRODUCTION

Royal jelly is natural product of the secretion of the hypopharyngeal and mandibular glands of the honey bees (*Apis mellifera*) and serves as a food for larvae and adult queens. It is collected and used by humans as a dietary supplement or mixed with honey in everyday nutrition, because it contains numerous components that may have a beneficial effect on health. It is also used in the cosmetics industry to make skin care products as well as natural cosmetics. In holistic treatment and alternative medicine, royal jelly is considered to slow down aging and this effect is attributed to its components, primarily amino acids and a wide range of vitamins and minerals.

The main components of royal jelly are water (60-70%), carbohydrates (7-18%), proteins (9-18%) and lipids (3-8%) but royal jelly also contains free amino acids, peptides, vitamins, minerals, flavonoids, enzymes, hormones and other biologically active substances

[1,2]. About 80% of total proteins belong to the group of major royal jelly proteins (MRJPs) responsible for many biological activities of the royal jelly [3,4]. Royal jelly contains free amino acids and the most abundant are lysine and proline [4-6]. About 80 to 90% of the lipid fraction are free fatty acids, and the rest being neutral lipids and sterols [4]. 10-hydroxide-2-decenoic acid (10H2DA), an unsaturated fatty acid, and 10-hydroxydecanoic acid (10-HDA), the saturated counterpart of 10H2DA, are major and unique lipid components of royal jelly largely responsible for many of the biological activities of royal jelly [7]. Carbohydrates take up 15% and among them fructose, glucose and sucrose are mainly present, but maltose, trehalose, melibiose, ribose and erlose can also be found in traces [2,4,8,9]. Royal jelly also contains minerals in various amounts. Potassium followed by magnesium, sodium and calcium, occurs in the highest concentrations in royal jelly while the most abundant trace elements are Zn, Cu and Mn while Al, Fe, Ni are present only in traces

[2,10]. The most abundant vitamins in royal jelly are vitamin B5 (pantothenic acid), followed by B3 (niacin) and B6 (pyridoxine) while small amounts of vitamins A, C, E, B1 (thiamine), B2 (riboflavin), B8 (biotin), B9 (folic acid), and B12 were found in royal jelly [2,7].

The effects of royal jelly, as well as the mechanism of its action have not been sufficiently studied, but in recent years there is more and more data on its action and potential mechanisms, both *in vivo* and *in vitro*. Many biological activities are attributed to major royal jelly proteins (MRJPs) and free fatty acids [4]. Reported biological activities of royal jelly are antioxidant, antitumor, anti-aging, neurotrophic, anti-inflammatory and antimicrobial effects [11-14]. Some studies have shown that royal jelly and its components have estrogenic activity [2,15]. It has been reported that royal jelly has beneficial effect on female reproductive health due to estrogen-like activity, has anti-aging and wound healing activity and may be used as protective agent in neurodegenerative and aging diseases [14]. Some activities such as antitumor, immunomodulatory, estrogenic and neurogenic are attributed to royal jelly fatty acids [16]. Animal studies showed that royal jelly may be used for prevention of osteoporosis and for the improvement of bone strength due to the effects of royal jelly on bone metabolism [2,17,18]. Some studies have shown that royal jelly stimulates the growth of glial cells [19] and neurogenesis of stem cells in the brain [20]. There is also preliminary evidence that royal jelly lowers blood cholesterol levels [21,22] and has anti-hypertensive effect [2]. Immunomodulatory activity of royal jelly is attributed to the fatty acids [23,24] that are the main lipid components of royal jelly. Studies have shown that 10H2DA, inhibits VEGF-induced angiogenesis *in vitro* [25] and has a protective effect on oxidative stress caused by cisplatin in rats [26] while 3,10-dihydroxy-decanoic acid (3,10-DDA) was proved to stimulate maturation and Th1 polarizing capability of human monocyte-derived dendritic cells *in vitro*, which could be beneficial for anti-tumour activity [27]. In addition to having a potentially beneficial effect, on both healthy people and people suffering from certain diseases, royal jelly may exert some side effects, because it has many allergens [28], so it can cause allergic reactions, asthma, and even anaphylaxis [29].

In our recent study, we made a review of the characteristics and biological activities of propolis, bee product rich in biologically active substances and widely used in traditional medicine [30], which is often combined with other bee products such as royal jelly. Chemical composition and subsequent biological activity of propolis are reported to vary to great extent based on geographical origin and plant sources from which bees collect the material to produce propolis [30]. Unlike propolis, composition of royal jelly is reported to be independent of these environmental factors [16], and its composition is more or less standard-

ized in terms of present compounds, however, some authors reported the differences in the quantity of some compounds [31] which may or may not influence the biological activity.

A large number of studies of the biological effects of royal jelly *in vitro* have been performed with its fractions or isolated compounds. Since royal jelly is used as a dietary supplement in a mixture with different substances, in its fresh state mixed with honey or in the form of different products like lyophilized powder, there is a need for examining royal jelly in the form it is used. Fresh royal jelly contains 60-70% of water which requires specific storage conditions. The best way to overcome this issue is to perform lyophilization which will enable easier storage with preservation of the chemical composition and activity. It has been shown that lyophilization of royal jelly maintains its chemical characteristics and activities [31].

Our aim was to examine the effects of commercially available product of royal jelly in the form of lyophilized powder. The field of research that requires special attention is the effect of royal jelly on epithelial tissues, because bee products are mainly applied to epithelium through the oral or topical route. Therefore, in this study we used two epithelial cell lines commonly used as models for studying the effects of biologically active substances on epithelial cells. One of the used cell lines is cancer (HeLa) and the other is non-cancer cell line (MDCK). With this choice of cell lines, we also wanted to examine any potential selective effect towards cancer cells since it was suggested in some literature data that royal jelly contains compounds that may have anti-cancer activity.

MATERIAL AND METHODS

Cell lines

For *in vitro* testing on cell cultures, two epithelial-like cell lines were used: MDCK (Madin-Darby canine kidney cells) and HeLa (human cervical cancer cells). These cell lines are the most commonly used as models for epithelial cells. Both cell lines were obtained from ATCC (American Type Culture Collection). Cells of both cell lines were cultured in DMEM (Dulbecco's Modified Eagle's Minimal Essential Medium) supplemented with 10% fetal bovine serum (FBS), 2 mM stable glutamine and 1% antibiotic-antimycotic solution (complete DMEM) at 37 °C in a humidified atmosphere containing 5% CO₂. All media components were purchased from PAA Laboratories GmbH, Austria.

Preparation of royal jelly product for *in vitro* testing on cell cultures

Commercially available lyophilized royal jelly product (International Trade System (ITS), Belgrade, Serbia) was

examined in this study. It was dissolved directly in cell culture medium (complete DMEM) and examined in the concentration range from 32 to 2,000 µg/mL.

Viability and proliferation assay

HeLa and MDCK cells were detached using trypsin-EDTA solution and appropriate cell density was adjusted using Trypan Blue Dye Exclusion method. HeLa cells were seeded at density 100,000 cells per well (in viability assay) and 20,000 cells per well (in proliferation assay) while MDCK cells were seeded at density 60,000 cells per well (in viability assay) and 15,000 cells per well (in proliferation assay), in 96 well plates (Greiner Bio-One, Germany). Twenty-four hours after cultivation of cells under the standard cell culture conditions, royal jelly was added to the cells in examined concentrations. Cells cultured in standard cell culture medium (complete DMEM) without royal jelly (untreated cells) under the same conditions, were used as control cells. Each concentration of royal jelly, as well as control medium, was tested in four to eight replicates, and experiment was repeated three times. Cells were incubated with royal jelly and complete medium for the next 24 hours (in viability assay) or 72 hours (in proliferation assay), under the standard cell culture conditions, respectively. The cells were observed and photographed before and after the incubation with royal jelly, on inverted light microscope (Observer Z1, Carl Zeiss, Germany).

MTT test

After incubation of cells ended in both assays, cell viability and proliferation were assessed by MTT test. The cells were first washed with phosphate buffer saline followed by addition of 100 µL of MTT solution (concentration 1 mg/mL). The cells were incubated with MTT for the next three hours at 37 °C followed by removal of MTT solution and dissolution of formed formazan crystals with 100 µL of 2-propanol per well. The amount of formed formazan crystals is in direct correlation with the percentage of viable cells. The absorbance of dissolved formazan was measured on Multiskan Ascent Plate Reader (ThermoLab Systems, Finland) at a wavelength of 540 nm. The mean absorbance values were calculated for each examined concentration, as well as for the control cells. The results are expressed as a percentage of cell viability that was calculated according to the formula: % of viable cells / cell proliferation = (absorbance of treated cells/absorbance of control cells)*100.

Statistical analysis

The results of MTT test were analyzed using one-way analysis of variance (ANOVA) and expressed as a percentage of cell viability/proliferation with relative

standard deviation, calculated according to the control culture of cells for which cell viability and proliferation rate were considered to be 100%. As statistically significant differences we considered those for which $p < 0.05$.

RESULTS

The results of viability assay are presented in **Figure 1** while the results of proliferation assay are presented in **Figure 2**.

Royal jelly did not affect significantly the cell viability of the used cell lines at examined concentrations and conditions.

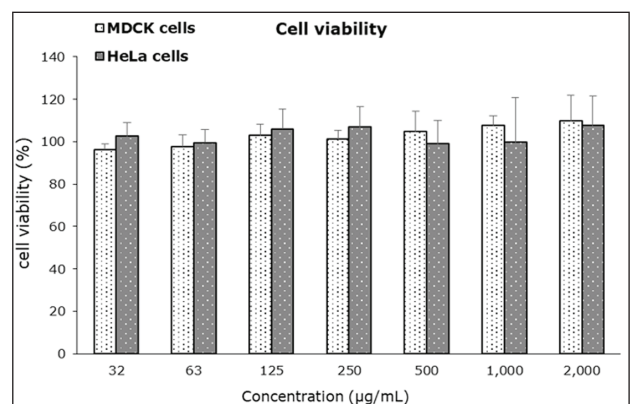


Figure 1. The effect of royal jelly on cell viability.

Royal jelly showed concentration-dependent effect on cell proliferation and acted anti-proliferative on HeLa cells at higher examined concentrations (1,000 and 2,000 µg/mL) but not on MDCK cells. This decrease in HeLa cells' proliferation was significant ($p < 0.01$) when compared to % of MDCK cells' proliferation at concentrations 1,000 and 2,000 µg/mL as well as at a concentration 32 µg/mL, although the difference in the response of cells of both cell lines was noticed at all examined concentrations.

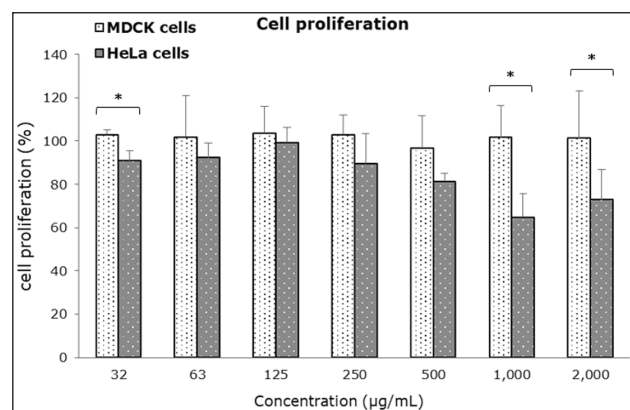


Figure 2. The effect of royal jelly on cell proliferation; (*) $p < 0.01$.

DISCUSSION

In recent years, many researches have focused on biological properties of bee products in general and their beneficial effects on human health. Royal jelly is used as a dietary supplement and is usually combined with honey which preserves the fresh royal jelly. Although it has great history of being used as functional food and wide traditional use, research on the potential benefits on human health has been intensifying in recent years.

There are very few studies on the effects of royal jelly in its original natural form, as a mixture of biologically active substances, on cell growth *in vitro*. Most of the literature data reported the action of individual fractions and isolated components of royal jelly on cells in culture. Our results are unique since they show how royal jelly in its complete composition affects the growth of different epithelial cells. This is especially important to know if you keep in mind that preparations of bee products, including royal jelly, are applied mainly to the epithelium. Also, we analyzed commercially available product, in the form of lyophilized powder, and we show that it is biologically active. There is an emerging requirement for searching the potential compounds, favorable from natural sources that would have potential to inhibit the growth of cancer cells that can be used as addition to the standard chemotherapy. That was the reason why we choose two cell lines, one cancer and the other non-cancer cell line, to examine possible selective effect on the cancer cells. Although the results are preliminary, and for any concrete conclusion we need to perform more studies on other cancer cell lines and non-cancer cells but also to examine the royal jelly in different *in vitro* cell culture systems as well as *in vivo* on experimental animals, obtained results may be indicative and towards the course of further research in that direction.

In one of the rare studies of the effects of royal jelly on cell proliferation, an inhibitory effect of royal jelly on bisphenol-A-induced breast cancer cell proliferation was found, without an anti-proliferative effect on non-induced cell proliferation [32]. Izuta *et al.* found that bee products, including royal jelly, act by suppressing the proliferation of HUVEC cells induced by VEGF, as well as the process of angiogenesis [33]. The above findings on the negative effect of royal jelly fractions on the development of blood vessels, including our findings on the anti-proliferative action of royal jelly on cancer cells, suggest that royal jelly should be further examined for potential use as addition to chemotherapy. Moubarak *et al.* showed that royal jelly alone is relatively non-toxic to normal cells, but decreased the viability of human breast cancer cell line MDA-MB-231 in higher concentrations (200 µg/mL) as estimated by Trypan blue exclusion assay, while this decrease in cancer cells' viability was enhanced when royal jelly was combined with thymoquinone [34].

When it comes to the testing of the activity of certain fractions obtained from royal jelly, data have been published on the cytotoxicity of some protein fractions on HeLa cells [35]. On the other hand, the 57-kDa protein fraction has been shown to act as a mitogen, causing increased DNA synthesis in rat hepatocytes, with increased albumin synthesis in these cells. The authors of this study saw the potential role of royal jelly in liver regeneration and hepatocyte cytoprotection in this finding [36].

Having in mind the results of our study and reported literature data, it can be concluded that the effect of royal jelly depends on the cell type on which it is examined, the dose/concentration, the way of application of royal jelly and system and method used for assessing the effects.

CONCLUSION

Based on obtained results we can conclude that royal jelly exerts concentration-dependent effect on cell cultures *in vitro*. In examined concentrations, royal jelly did not affect cell viability, however, acted anti-proliferative on HeLa cells in higher examined concentrations that may suggest its selective effect toward cancer cells. Since we used only one cancer and one non-cancer cell line, we could not make strong conclusions about specific activity. Further analysis on greater number of different cell lines but also on more complex systems such as 3D cell cultures and animal models *in vivo*, together with detailed chemical profiling is needed.

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***In vitro* analiza biološke aktivnosti matičnog mleča na različitim ćelijskim linijama**

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HeLa, kancerske ćelije, ali ne i na MDCK, ne-kancerske ćelije. Možemo zaključiti da matični mleč sadrži neka jedinjenja koja bi mogla da ispolje određenu aktivnost prema kancerskim ćelijama što ukazuje na potencijal matičnog mleča na koji bi trebalo usmeriti dalja istraživanja.

Ključne reči: matični mleč; *in vitro*; HeLa ćelije; MDCK ćelije; proliferacija; vijabilnost.

Kratak sadržaj

*Matični mleč je pčelinji proizvod koji se tradicionalno koristi kao dodatak ishrani, ali i kao lek. Iako je u širokoj upotrebi, nedostaju eksperimentalni podaci koji podržavaju njegov terapeutski potencijal. Takođe, mnoge studije su se bavile ispitivanjem pojedinih frakcija i izolovanih supstanci iz matičnog mleča, ali retke su studije u kojima se ispituje nativna forma matičnog mleča, ona koja se koristi u svakodnevnoj ishrani. Naš cilj je bio da ispitamo efekat matičnog mleča, u obliku proizvoda koji sadrži liofilizovani matični mleč i koji je komercijalno dostupan na tržištu, na vijabilnost i proliferaciju ćelija dve različite ćelijske linije *in vitro*. Naši rezultati su pokazali da ispitivani proizvod matičnog mleča nije uticao na vijabilnost ćelija ispitivanih ćelijskih linija u ispitivanim koncentracijama, dok je delovao antiproliferativno na*