Chapter

Royal Jelly and Human Interferon-Alpha (HuIFN-αN3) Affect Proliferation, Glutathione Level, and Lipid Peroxidation in Human Colorectal Adenocarcinoma Cells In Vitro

Bratko Filipič, Lidija Gradišnik, Klemen Rihar, Adriana Pereyra, Damir Đermić and Hrvoje Mazija

Abstract

The purpose was to investigate the influence of RJ-F(M), 10-hydroxy-2-decenoic acid and HuIFN-αN3 on the proliferation of CaCo-2 cells and ascertain their effects on intracellular glutathione level and lipid peroxidation. The antiproliferative (AP) activity of RJ-F (M) (0.1 g/10 mL PBS), HuIFN-αN3 (1000 IU mL^{-1}), 10-HDA (100.0 μmol L^{-1}) and their combinations, in the ratios 1:1, 1:2, and 2:1 on CaCo-2 cells were measured. Single RJ-F (M) had a low AP activity: 2.0 (0.5 mg mL^{-1}). HuIFN-αN3 had an AP activity of 2.5 (208.33 IU mL^{-1}), while 10-HDA had an AP activity of 1.5 (37.5 μmol mL^{-1}). AP activity of 3.8 was obtained when RJ-F(M) and HuIFN-αN3 were in the ratio 2:1. In it, the level of GSH was 24.9 ± 2.4 nmol g^{-3} of proteins (vs. 70.2 ± 3.2 nmol g^{-3} in the control), and level of MDA was 72.3 ± 3.1 nmol g^{-3} (vs. 23.6 ± 9.1 nmol g^{-3} in the control). 10-HDA, together with HuIFN-αN3, is responsible for the inhibition of CaCo-2 cell proliferation *in vitro*. RJ-F (M) and HuIFN-αN3 applied at 2:1 decreased level of GSH and increased lipid peroxidation *via* MDA in CaCo-2 cells. Future studies are needed whether these GSH- and MDA-related activities of RJ-F (M), HuIFN-αN3, 10-HDA, and their combinations may decrease the tumorigenicity index and tumorigenic potential of tumor cells *in vitro*

Keywords: antiproliferative activity, antitumor activity, CaCo-2 cells, 10-hydorxy-2-decenoic acid, malondialdehyde, HuIFN-αN3, RJ-F (M)

1. Introduction

Royal jelly (RJ) is a milky acid colloid of a pH between 3.6 and 4.2, produced by hypo pharyngeal and mandible glands of young worker bees between sixth and twelfth days of their life. Fed as a sole nutrient, it is the exclusive food for the bee colonies' queen honey bee (*Apis mellifera*) larva, developing into a sexual mature

queen bee. RJ has a crucial role in the life span of bees: worker bee lives around 45 days, while a queen bee can be alive up to 5 years. During this time, she is able to spawn daily an equivalent of her body weight in eggs (approximately 2000–3000 eggs/day for up to 5 years) [1, 2]. Chemically, RJ is composed by water (50–60%), different proteins (18.0%), carbohydrates (15.0%), lipids (3–6%), mineral salts (1.5%), and vitamins, together with bioactive substances, such as 10-hydroxy-2-decenoic (10-HDA) acid [3]. 10-HDA exerts an inhibitory effect on vascular endothelial growth factor-induced angiogenesis in cellular models of tumor growth, partly by inhibiting cell proliferation and migration [4]. RJ contains different immunomodulative and antiproliferative substances as well as substances with possible antitumor activities. It also contains proteins, like 350-kDa protein, that stimulate proliferation of human monocytes [5–7]. In addition, RJ protein$_{30}$ can be found; it has shown an prominent cytotoxic effect on HeLa cells in vitro [8]. A protein component of RJ belong to the family of major royal jelly proteins (MRJPs) or an albumin. In this families, eight proteins (MRJP 1–8) were identified and found that they have molecular masses between 49 and 87 kDs. MRJPs have an important role in the diet of the queen bee [9]. Other proteins in RJ that are in much lower amount are royalisin, jelleines, aspimin, and royalactin. Researchers showed antibacterial properties of royalisins against Gram-positive bacteria and proposed their use as potential antimicrobial [10]. Royalisins are composed of 51 residues with a net charge of +2; even their origin is unknown. It was supposed that they could originate directly from honeybee. It was also shown that the RJ's protein royalactin is needed for queen bee differentiation. It activates the epidermal growth factor receptor (EGFR) signaling pathway in honeybee larvae, which leads to queen development. Corona et al. [11] found that royalactin induces queen development through shortening her development time and increases longevity of the queen bee by the EGFR signaling pathway.

In RJ, glucose oxidase enzyme (GOx) was also detected. GOx catalyzing the oxidation of glucose to hydrogen peroxide was showing a relatively high antibacterial activity [12].

Interferons (IFNs) are multifunctional glycoproteins/proteins produced and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites, or tumor cells. There are three classes of IFNs, designated as Types I, II, and III. HuIFN-αN3, a Type I interferon, is a protein composed from 13 subtypes [13, 14] having antiviral, antiproliferative, and antitumor activities [15]. Type I IFN isoforms were found since its discovery. These, coded by a single exon, include IFN-α (composed from 13 subtypes: IFN-α1, IFN-α2, IFN-α4, IFN-α5, IFN-α6, IFN-α7, IFN-α8, IFN-α10, IFN-α13, IFN-α14, IFN-α16, IFN-α17, and IFN-α21), IFN-β, IFN-ε, IFN-κ, and IFN-ω [16]. The latter was used from the beginning for the treatment of different forms of cancers, despite its molecular mechanism of cytoreductive action being far from clear. HuIFN-αN3's antiproliferative activity plays a central role in its chemotherapeutic effect. The research explained its action in apoptosis as a way of antitumor action [17]. In addition, HuIFN-αN3 shows a direct cytotoxic effect on malignant cells and different tumor cell lines *in vitro* [18]. It was also shown that endogenous type I IFN prevents the growth of primary carcinogen-induced and transplantable tumors [19]. One of the earliest described immunoregulatory functions of HuIFN-αN3 is its ability to regulate NK cell functions that is one of the major mechanisms of regulation of tumor growth by the endogenous type I IFN. The role of HuIFN-αN3 in regulating tumor cell proliferation, apoptosis, and autophagy was only recently begun to be investigated in depth [20]. In addition, the ability of oncogenes such as Ras and HPV16 E6E7 for downregulating the IFN-inducing innate receptors, RIG-I and TLR9, is a suggestion for a role of HuIFN-αN3 in modulating the infectiveness of cell-transforming ability of oncogenic viruses [21].

The low-molecular-weight thiol, glutathione (GSH) with a role in the control of the thiol/disulfide redox state in the cells, is important for cellular redox signaling and also for CaCo-2 cells. Intracellular concentration of GSH, cell proliferation, and apoptosis are mutually connected. GSH in a relatively high level enhances the cell proliferation. In contrary, its depletion results in the inhibition of CaCo-2 cell growth and proliferation, because of the increase of apoptosis [22].

Oxidative degradation of lipids or lipid peroxidation (LP) is a process where free radicals "rob" electrons from cellular membrane lipids and induce cell damage by proceeding with the free radical chain reaction mechanism. The LP's end products are reactive aldehydes with a relatively strong carcinogenic potential.

Malondialdehyde (MDA) is one of the important reactive aldehydes. It is a bioactive marker of the LP with a different biological activity similar to reactive oxygen species [23, 24].

The aim of the present study was to explore the effect of RJ-F (M) and 10-HDA on the HuIFN-αN3-induced inhibition of CaCo-2 cell proliferation in vitro. Also important is to ascertain their influence on the level of the GSH in the cells and also on the lipid peroxidation via the MDA activity. Such an experimental approach might serve to explain some of the antiproliferative/antitumor mechanisms in CaCo-2 cells. All this could be also important for the developing of future antiproliferative treatments based on the use of the mentioned bioactive compounds.

2. Materials and methods

2.1 Materials

During the experiments, the following materials were used: (1) HuIFN-αN3 (Institute of Immunology, Zagreb, Croatia) was applied at 1000 IU mL^{-1}. This concentration was found in our previous experiments [25]. (2) Fresh royal jelly (Mižigoj) (RJ-F (M)) (Medex D.o.o., Ljubljana, Slovenia) was applied in a concentration of 0.1 g/10 mL [26]. (3) 10-hydroxy-2-decenoic acid (10-HDA) (Sigma-Aldrich, Missouri, USA) was applied in a concentration of 100 μmol L^{-1} [27–29]. All reagents were dissolved in phosphate buffer saline (PBS) having a pH of 7.2, centrifuged at 2500 g (Centric, Tehtnica D.o.o., Železniki, Slovenia) for 15 minutes, and the supernatants were sterilized by filtration through 0.2 μm syringe filter (Millipore, USA). Sterilized reagents were stored on −20°C until their use in the experiments.

2.2 Cell culture

CaCo-2 cells were cultivated in a complete Eagle's medium supplemented with 10% of fetal calf serum (FCS) (Sigma-Aldrich, Missouri, USA). The cells were multiplied before the experiments, so that their viability and capability for proliferation can be assayed with MTT Cell Proliferation Assay Kit (K299-100) (BioVision, Milpitas, California, USA). In performed tests, positive cells were further cultivated in a 96-well flat microtiter plates (Sterilin, Sigma-Aldrich, Missouri, USA) or in 25 cm^2 flasks (Sterilin, Sigma-Aldrich, Missouri, USA) in 5% CO_2 at 37°C until the monolayer appeared before they can be used for the experiments.

2.3 The studies' design

The experiments were designed as follows: (1) RJ-F (M), HuIFN-αN3, and 10-HDA in the previously stated concentrations were added alone in a volume of 100 μL/well. (2) In various combinations between them (RJ-F (M), HuIFN-αN3,

and 10-HDA) for ratio 1:1, 100 + 100 μL/well were added. For ratio 1:2, 66.8 + 133.2 μL/well were added, and for ratio 2:1, 133.2 + 66.8 μL/well were added.

2.4 Antiproliferative (AP) activity

In the AP assay that was performed in a way according to Sugarman et al. [30], single substances RJ-F (M), HuIFN-αN3, and 10-HDA and their combinations in ratios 1:1, 1:2, and 2:1 were added. In the first well in a row of a 96-well microtiter plate, 200 μL of sample volume was added. This was serially transferred per 100 μL from 1:2 to 1:4096. To these, CaCo-2 cells in a concentration of 10^4 cells/well/100 μL in a complete Eagle's medium with 10% FCS were added. Separately, with CaCo-2 cells without being added alone or in their combinations of 1:1, 1:2, and 2:1, substances served as the negative control. The single tested substances RJ-F (M), HuIFN-αN3, and 10-HDA were used as a positive control. Microtiter plates with substances, as single or in combinations 1:1, 1:2, and 2:1, and CaCo-2 cells were incubated for 72 hours at 37°C in a 5% CO_2 atmosphere. Afterward, the supernatants were discarded, and cells were fixed with 100 μL/well of 10% formalin (Sigma-Aldrich, Missouri, USA) in PBS. After 2 hours, the fixative was removed and cells washed twice with the PBS. Then, 2% Rhodamine (Sigma-Aldrich, Missouri, USA) in a volume of 100 μL/well was added for 15 minutes. This was then removed, and the cells were washed twice with the PBS and air-dried. Optical density (OD) at 550 nm was measured on dried microtiter plates on the Synergy HTX Multi-Mode Reader with Gen 5 software (Biotek, Winooski, USA). The AP activities was determined with the well in rows where 50% cell growth inhibition were found. The AP_{50} was calculated for each separate substance (RJ-F (M), HuIFNαN3, and 10-HDA) or their combinations (1:1, 1:2, and 2:1).

2.5 Glutathione (GSH) determination

According to Watson et al. [24], the GSH assay was performed. The CaCo-2 cells were cultivated in flasks having 25 cm^2 (Sterilin, Sigma-Aldrich, Missouri, USA) in a complete Eagles' medium with 10% FCS. To the monolayer of CaCo-2 cells, the substances of RJ-F (M), HuIFN-αN3, and 10-HAD alone in volumes of 1.0 mL, or their combinations of 1:1, 1:2, and 2:1 in volumes of 2.0 mL, were added. The treatment took place for 24 hours at 37°C and 5% CO_2. Then, the cells were removed by Trypsin (Sigma-Aldrich, Missouri, USA) and treated with 1 mL of mmol mL^{-1} Tris–HCl solution (pH = 6.0) containing 0.5 mol mL^{-1} diethylene triamine pentacetic acid (DTPA) (Sigma-Aldrich, Missouri, USA). After such treatment, the cells were lysed by the syringing through the insulin syringe. The quantities of total cellular proteins were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA) having a standard bovine serum albumin (BSA) (Sigma-Aldrich, Missouri, USA).

To determine the amount of GSH, 50 μL of the lysed CaCo-2 cells, 100 μL of 25 μmol L^{-1} DL-Dithiothreitol (DTT), and 150 μL of 0.1 mol mL^{-1} Tris-HCl (pH 8.5) were added. This mixture was incubated for 30 minutes on ice. Then, 750 μL of 2.5% (wt. /vol) 5-sulfosalicylic acid was added to precipitate proteins. The precipitate was centrifuged at 13000 g (Centric, Tehtnica D.o.o., Železniki, Slovenia) for 5 minutes at 4°C, and the supernatants were used in GSH Assay Kit (Sigma-Aldrich, Missouri, USA) to measure the GSH level at OD at 412 nm on Synergy HTX Multi-Mode Reader with Gen 5 software (Biotek, Winooski, USA) and express it as nmol of GSH g^{-3}/proteins.

2.6 Lipid peroxidation assay

CaCo-2 cells were cultivated in 25 cm^2 flasks (Sterilin, Sigma-Aldrich, Missouri, USA) in a complete Eagles' medium with 10% FCS in 5% CO_2 and 37°C. When monolayer was formed, the cells were treated with substances either alone or their combinations as described previously, in a quantity of 1.0 mL when single substances were added, and a total of 2.0 mL when their combinations (1:1, 1:2, and 2:1) were added. The cells were incubated for 24 hours at 37°C and 5% CO_2. The medium was removed, and cells were detached with trypsin, washed, and resuspended in 5 mL of PBS. The total cell protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA) with a BSA as a standard. A measure of 1 mL of thiobarbituric acid (TBA) reagent (0.38% 2-TBA, 15.0% TBA, 0.3 mol mL^{-1}HCl) was added to the cell suspension, and these were heated at 95°C for 20 minutes, chilled to room temperature, and centrifuged at 1500 g for 10 minutes (Centric, Tehtnica d.o.o., Železniki, Slovenia). The quantity of TBA reactive substances (RS) developed by lipid peroxidation was measured at OD at 535 nm in Synergy HTX Multi-Mode Reader with Gen 5 software, (Biotek, Winooski, USA). The obtained results were expressed as MDA nmol g^{-3} of protein.

2.7 Statistical evaluation

For statistical significance determination ($^{*}p < 0.1$, $^{**}p < 0.05$), the t-test was used. All the data are shown as a mean value ± standard deviation. The tests were performed in triplicate, and each was repeated three to four times.

3. Results and discussion

3.1 Antiproliferative (AP) activity

The results of AP activity and their concentrations at AP_{50} in case of single substances were found as follows: RJ-F (M), 0.157 (0.5 mg mL^{-1}); HuIFN-αN3, 0.741 (208.33 IU mL^{-1}; and 10-HDA, 0.487 (37.5 μmol L^{-1}). The AP activities of used single substances (RJ, HuIFN-αN3, and 10-HDA) were relatively low. Such AP activity expressed as growth rate index (GRI) of their combinations (1:1, 1:2, 2:1) is shown in **Figure 1**.

When a combination between RJ-F (M) and HuIFN-αN3 in ratio 2:1 was used, the highest AP activity was found. Their AP activity expressed as GRI was 1.006. The combinations of 10-HDA and HuIFN-αN3 2:1 show the AP activity expressed as GRI 0.952, which was lower than this in combination of RJ-F (M) and HuIFN-αN3 (2:1). These results suggest that RJ-F (M) should contain some other components responsible for the relatively strong AP activity of the combination of the RJ-F (M) and HuIFN-αN3 (2:1). The RJ $protein_{30}$, a water soluble fraction showing the cytotoxic effect on the HeLa cells by decreasing the cell population by 50%, should be taken into account.

The AP activity of RJ-F (M), HuIFN-αN3, and 10-HDA on the CaCo-2 cells is connected to the induction of apoptosis and cytotoxicity. They also influence the glutathione level and lipid peroxidation in CaCo-2 cells [31].

3.2 GSH determination and measurement of lipid peroxidation via MDA

The results obtained in our study show that RJ-F (M), HuIFN-αN3, 10-HDA, and their combinations (1:1, 1:2, and 2:1) decreased the intracellular level of the GSH

and increased the lipid peroxidation via the MDA. The data in extent are shown in **Figure 2** and **Table 1.**

The GHS has an important role in various intracellular processes like cellular differentiation, proliferation, apoptosis, and cancer. The decrease of the GSH/glutathione disulfide (GSSG) ratios consequently lead to the enhanced susceptibility to oxidative stress and the progression of cancer. In addition, the increased GSH levels enhance the antioxidant capacity and resistance to oxidative stress that can be found in transformed cells. Some of the possible antitumor mechanisms of RJ-F (M) are linked to the modulation of the oxidative stress and induction of apoptosis [32]. Similar effects were found during the treatment of the PaCa-44 cells with 10-HDA, where induction of the apoptosis was also confirmed [33]. Furthermore, the antitumor activity of the HuIFN-αN3 is connected with the induction of apoptosis and the modulation of the oxidative stress in rats having breast cancer [34].

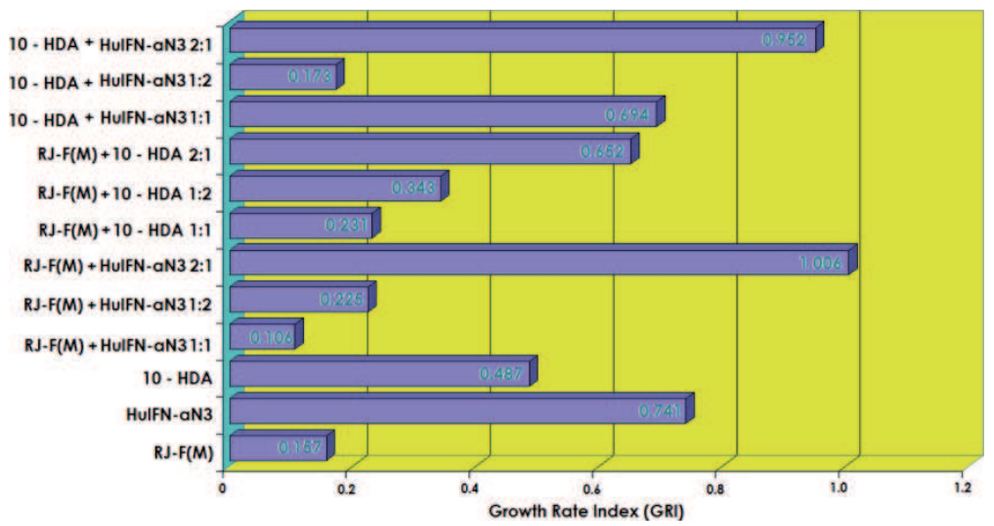

Figure 1.
The antiproliferative (AP) effect expressed as growth rate index (GRI = $GR_{in.}$ – GR_{8d}/GR_{8d}) of RJ-F (M), HuIFN-αN3, 10-HDA, and their combinations: 1:1, 1:2, and 2:1 on the CaCo-2 cells. The inhibition of proliferation of CaCo-2 cells treated with RJ-F (M) (0.1 g/10 mL PBS), HuIFN-αN3 (1000 IU mL^{-1}), 10-HDA (100 μmol mL^{-1}), and different combinations between them (1:1, 1:2, and 2:1) were analyzed. The AP activity was determined by Rhodamine B staining method after 72 hours of incubation at 37°C and 5% CO_2.

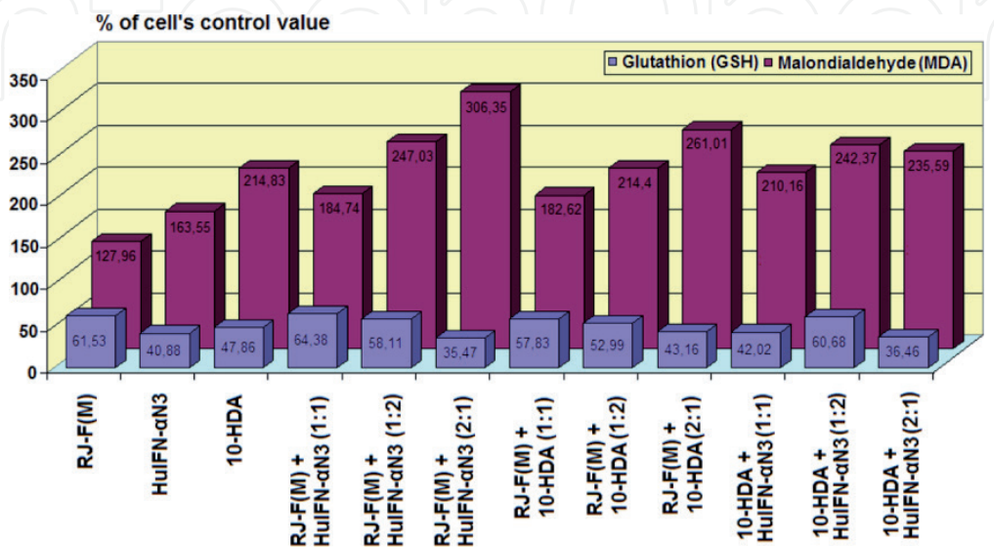

Figure 2.
The percent of CaCo-2 cell control value of glutathione (GSH) or malondialdehyde (MDA) after treatment with RJ-F (M), HuIFN-αN3, 10-HDA, and different combinations between them (1:1, 1:2, and 2:1).

Values for	Cell Control	RJ-F (M) b)	Hu IFN-αN3 d)	10-HDA d)	RJ-F (M) + HuIFN-αN3 (1:1)	RJ-F (M) + HuIFN-αN3 (1:2)	RJ-F (M) + HuIFN-αN3 (2:1)	RJ-F (M) + 10-HDA (1:1)	RJ-F (M) + 10-HDA (1:2)	RJ-F (M) + 10-HDA (2:1)	10-HDA + HuIFN-αN3 (1:1)	10-HDA + HuIFN-αN3 (1:2)	10-HDA + HuIFN-αN3 (2:1)
Glutathion (GSH) (Mean±SE) a)	70.2 ± 3.2	43.8 ± 2.8	28.7 ± 6.4	33.6 ± 5.8	45.2 ± 4.7	40.8 ± 3.1	24.9 ± 2.4	40.6 ± 4.5	37.2 ± 2.1	30.3 ± 3.7	29.5 ± 1.7	42.6 ± 5.3	25.6 ± 3.1
T-test: T		37.33	58.68	51.76	35.35	41.57	64.06	41.86	46.66	56.42	56.14	37.19	64.88
T-test: p		0.00 035	0.00 014	0.00 018	0.00 04	0.00 028	0.00 012	0.00 028	0.00 022	0.00 031	0.00 031	0.00 072	0.00 023
T-test: r		p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05
Malondi-aldehyde (MDA) (Mean±SE) a)	23.6 ± 9.1	30.2 ± 4.3	38.6 ± 4.2	50.7 ± 4.6	43.6 ± 4.1	58.3 ± 5.2	72.3 ± 3.1	43.1 ± 2.6	50.6 ± 4.5	61.6 ± 5.2	49.6 ± 4.2	57.2 ± 2.6	55.6 ± 6.2
T-test: T		-7.88	-19.70	-36.72	-31.42	-54.52	-76.52	-26.03	-42.42	-52.06	-50.11	-45.87	-50.28
T-test: p		0.01 572	0.00 256	0.0007 4	0.00 1011	0.00 0336	0.00 0171	0.00 1472	0.00 0535	0.00 0369	0.00 0398	0.00 0475	0.00 0395
T-test: r		p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05	p<.05

Table 1.
Glutathione (GSH) determination and the measurement of lipid peroxidation (MDA) after the CaCo-2 cell treatment with RJ-F(M), HuIFN-αN3, 10-HDA, and their combinations of 1:1, 1:2, and 2:1.

The 10-HDA, the most important constituent of the RJ-F (M) and HuIFN-αN3, had a complementary active point due to its antitumor activity, which can be enhanced when they were combined in a proper ratio. The most effective was the ratio of 2:1 of the RJ-F (M) and HuIFN-αN3. Using this, the level of GSH was 24.9 ± 2.4 nmol g^{-3}, and the level of MDA was 72.3 ± 3.1 nmol g^{-3}. The following were the control values: for GSH it was 70.2 ± 3.2 nmol g^{-3} and for MDA it was 23.6 ± 9.1 nmol g^{-3}. The RJ-F (M) and HuIFN-*α*N3 applied at 2:1 ratio and HuIFN-*α*N3 applied in combination with 10-HDA in 2:1 ratio caused a decrease in the level of GSH and increase in lipid peroxidation indicator level (MDA) in CaCo-2 cells in

comparison with the control group. On the other hand, it was observed that these combinations had the highest antiproliferative effect. In addition, it is suggested that antiproliferative effects of RJ-F (M), HuIFN-αN3, and 10-HDA on the CaCo-2 cells can be connected not only with the induction of apoptosis and cytotoxicity but also with their influence on the pro- and anti-oxidative balance [35].

4. Conclusion

Future experiments will show whether these GSH- and MDA-related activities of the RJ-F (M), HuIFN-αN3, 10-HDA, and their combinations may cause the decrease of the tumorigenicity index of different tumor cells in vitro as a result of their tumorigenic potential, as it has already been reported in the literature [36–39]. This is important for practical use of the RJ-F (M), 10-HDA, and HuIFN-αN3 in a proper combination that could be of value for future development of possible tumor therapy based on the use of these bioactive compounds.

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Conflict of interests

No conflicts of interest were declared by all authors.

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