The analysis of the effects of propolis products as food supplements on the viability of baby hamster kidney (BHK-21) and murine macrophage (RAW 264.7) cells by spectrophotometric MTT assay

Research Article

Serol Korkmaz[1a]
Ayşe Parmaksız[2b]
Ahmet Sait[2c]
İrem Omurtag Korkmaz[3d]

[1]Institute of Heath Sciences, Marmara University, Istanbul Turkey
[2]Pendik Veterinary Control Institute, Istanbul, Turkey
[3]Institute of Heath Sciences, Marmara University, Istanbul Turkey

ORCİD-
[a]0000-0001-8970-6883
[b]0000-0003-1242-7987
[c]0000-0001-7658-8793
[d]0000-0001-7918-6212

orrespondence
Serol Korkmaz
serolkorkmaz@yahoo.com

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ABSTRACT

Propolis is beeswax with rich bioactive compound content. On the market, there are many propolis products as food supplements for the consumers. This study aimed to investigate the effect of food supplement products based on propolis at several concentrations on the viability of baby hamster kidney cells and murine macrophage cell lines (BHK-21 and RAW 264.7). For this purpose, both cell lines were treated with the two-fold serial dilutions (from 20 to 2-10) of each six propolis products (P1, P2, P3, P4, P5 and P6) after reaching monolayer cell in 96-well microplates. The viability and inhibition of cells were spectrophotometrically determined by MTT assay after 24 h. For BHK-21, the CC50s of P1, P2, P3, P4, P5 and P6 were calculated as 0.003, 0.178, 0,082, 0.451, 0.278 and 0.384 %, respectively. For RAW 264.7, the CC50 of P1, P2, P3, P4, P5 and P6 were calculated as 0.260, 0.218, 0.115, 0.257, 0.207 and 0.265%, respectively. The CC50 value was higher for RAW 264.7 cells than for BHK-21 cells. So, the low cytotoxic effect was determined in RAW 264.7 cells. Propolis products containing some additives (aroma, flavoring) had lower the CC50 and the lower viability of BHK-21 cells. So, Additives in the propolis food supplement might be an effective factor on cell viability as much as dilution factor and propolis content.

Keywords: BHK-21, Cytotoxicity, Food Supplement, Propolis, RAW 264.7

INTRODUCTION

Propolis is a beeswax containing natural ingredients, which bees produce from poplar and coniferous, clusia of flowers or tree and the hive cells (Gojmerac 1980; Valcic et al, 1999). Its bioactive compounds are generally flavonoids, ferulic acids, and terpenoids which come from resin, vegetable balsam, wax, essential aromatic oils, salivary secretions and pollen (Burdock, 1998; Yaghoubi et al., 2007; Bogdanov, 2014). But its composition varies related to the plant species, climate, season, harvest time, and geographic area (Markham et al. 1996; Sforcin et al. 2000). According to its chemical composition, propolis has many biological effects on anti-inflammatory and cellular immunity, wound healing and antioxidant metabolisms. It was shown that it has antimicrobial effects and also suggested that it might be used against COVID-19 (Burdock, 1998; Al-Shaher et al., 2004; Bedier, 2016; Martini and Mahendra, 2019; Berretta et al., 2020)

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Recently, there are many propolis product as food supplement for the consumers. In addition to propolis, these products contain various ingredients such as honey, royal jelly and plant flavoring. The bioactive compounds and concentrations in them are varied related to the solvents and their form (Moussa and Temirek, 2018; Berretta et al., 2020).

There are several test methods to determine the medical using safety of food or feed supplements before commercial production. Cytotoxicity assay is one of the most applied methods for establishing their safety in preclinical studies. Especially for the cytotoxicity methods, cell lines originated from mammalians such as kidney (baby hamster kidney, BHK-21) and blood (murine macrophage, RAW 264.7) were mostly preferred to understand the etiology of the diseases and to detect the levels of cellular toxicity in ethnobotanical and apitherapy studies. (Moussa and Temirek, 2018; Berretta et al., 2020). In this study, the aim was to investigate the effect of food supplement products based on propolis at several concentrations on the viability of two different cell lines (BHK-21 and RAW 264.7) by the spectrophotometric assay for assessing cell metabolic activity (tetrazolium-based colorimetric assay, MTT assay).

MATERIAL and METHOD

Materials

Trypan blue solution (0.4%, sterile-filtered, Sigma-Aldrich), DMEM (500ml, Dulbecco's Modified Eagle Medium (w/o L-glutamine, w/o sodium pyruvate)), L-alanyl-L-glutamine (200 mM), penicillin (10,000 units ml^{-1})-streptomycin (10 mg ml^{-1})-amphotericin B (0.025 mg ml^{-1}) solution, fetal bovine serum (FBS, European grade), trypsin-EDTA solution (w/o phenol red) and Dulbecco's Phosphate Buffered Saline (PBS w/o calcium magnesium) were purchased (Biological Industries, USA). For cell culture, the 75 cm2-flasks, (EasYFlask, Thermo Scientific) and 96-well microplates (CellStar, Greiner Bio-One, Germany), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolium bromide (MTT, research-grade, Serva, Germany) was commercially supplied. The syringe filters (sterile, 0.22 µm, Merck Millipore, Germany), Sterile centrifuge tubes (ISOLAB, Germany) were also purchased.

Propolis samples

Six different propolis products were purchased from retail-markets and pharmacies in Istanbul, Turkey. All products were solved in water by their manufacturers and contained propolis at several concentrations. The ingredients of propolis products were shown in Table 1 and they were coded as P1, P2, P3, P4, P5 and P6. They were kept at 4 °C or room temperature in terms of manufacturers' recommendations for further analysis.

Cell culture

Baby hamster kidney cells (BHK-21) and murine macrophage cells (RAW 264.7) were from American Type Culture Collection (ATCC), Manassas, VA, USA). Fetal bovine serum was inactivated by heating up in a water bath at 56 ◦C for 30 minutes before use. All medium and solutions were heated to 37 °C before the process of cell cultivation. Both cells were cultured in DMEM supplemented with fetal bovine serum (10%), L-alanyl-L-glutamine and 1% penicillin-streptomycin at 37 °C. They were maintained in 75 cm^2 cell culture flasks in the incubator with the condition of 37 °C and 5% CO2 for 24 h to confluence. After the incubation of 24 h, all waste medium were discarded and the monolayer cells were disaggregated with trypsin-EDTA in the incubator for 3 min. The suspension was centrifuged in a refrigerated centrifuge at 1200 rpm/15 min. The supernatant was discarded and the pellet was suspended in a fresh medium. Then, viable and dead cells were counted by the method of trypan blue (0.4%) staining with a haemocytometer. For the preparation of 96-well microplates, 100 µl of the

stock viable cell suspension (3 x 10^5 cell ml^{-1}) was seeded in each well (3 x 10^4 cell) and kept in the incubator for 24 h to confluence at least 90%.

Propolis treatment on cell culture

All propolis solutions (P1, P2, P3, P4, P5 and P6) were 2-fold serially diluted with DMEM supplemented FBS + antibiotics from 2^0 to 2^{-10} (from 1 to 1/2048). Then, 100 µl of each dilution was added to six-replicated wells of the 96-well microplate seeded with the cell culture. DMEM solution was only added to cell control wells (medium + cell) and blank wells (only medium). The microplates were incubated at 37 °C and 5% CO2 for 24 h. The inert microscopy (Olympus ix71, Tokyo, Japan) was used to observe the morphological changes of the cells before the MTT assay.

Determination of cell viability

The MTT assay was used to determine the cell viability and inhibition by spectrophotometry. MTT solution was prepared at 5mg/ml concentration in PBS, filtered by a syringe filter (sterile, 0.22 µm) and stored at -20 °C.

After the incubation of 24 h, the sample solution in wells was discarded all and 50µl of MTT solution was added to all wells. The microplates were gently shaken and incubated for 4 hours at 37 °C in 5% CO2. The solvent was discarded after incubation. 50 µl of DMSO was added and the microplates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader (Absorbance 96, Byonoy, Germany) at a wavelength of 570 nm.

Data analysis

The percentage of cell viability and inhibition was calculated in Table 2, Figure 1 and 2 using the formula as following,

Cell viability (%) = $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100$

Cell inhibition (%) = $100 - [(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100]$

A_{sample} = absorbance value of test compound, $A_{control}$ = absorbance value of control (cell), A_{blank} = absorbance value of blank (medium)

The means of data and the standard deviations (SD) were calculated for each group using SPSS 21 software (SPSS Inc., IL, USA). The effects on cell viability were analysed by one-way analysis of variance (ANOVA). Differences among absorbance data means of cell control and propolis experiments were compared using the Tukey post hoc test at a $P < 0.05$ level of significance. The linearity plots were demonstrated by Excel 2016 (Microsoft, USA). The half maximal cytotoxic concentration (CC50) of all products was calculated using the slope-intercept equations as "y = mx + b" in each figure (Fig. 1 and 2).

RESULTS

The OD absorbance data means and cell viability results of each products were calculated and compared with the results of their own cell controls and blanks in each microplates.

All propolis supplements had shown significant inhibition effects on the viability of both cell lines at different concentrations. When compared with the cell controls, P1, P2, P3, P4, P5 and P6 affected significantly the viability of BHK-21 cells up to the dilutions of 1/128, 1/16, 1/32, 1/16, 1/1 and 1/1, respectively ($p<0.05$ and $p<0.01$, Table 1). Both P2 and P4 products affected the BHK-21 cells approximately at the dilution ratio of 1/16. But, the highest inhibition was determined in P1 (93%) and P3 (91%) ($p<0.01$) and the lowest was observed in P6 ($p<0.05$) on BHK-21 cells (Figure 1). Figure 1 showed that there was a strong linear relation between the % cell inhibition and concentrations of P5 and P6 (R^2=0.980 and R^2=0.898

respectively), however, weak linearity for P1, P2, P3 and P4. The CC50s of P1, P2, P3, P4, P5 and P6 were calculated as 0.003, 0.178, 0,082, 0.451, 0.278 and 0.384 % for BHK-21, respectively.

Table 1. The description of propolis products used in the study

Products	Solvent	Form	Propolis Content (%)	Other Contents	Origin
P1	Water	Liquid	6 %	Honey, menthol, glycol	Turkey
P2	Water	Liquid	15 %	Glycol	Turkey
P3	-No data-	Liquid	-No data-	-No data-	Turkey
P4	Water	Liquid	15 %	Glycol	Turkey
P5	Water	Liquid	23 %	Honey, glycerol, licorice, eucalyptus aroma	Brasil
P6	Water	Liquid	5 %	Organic propolis	Turkey

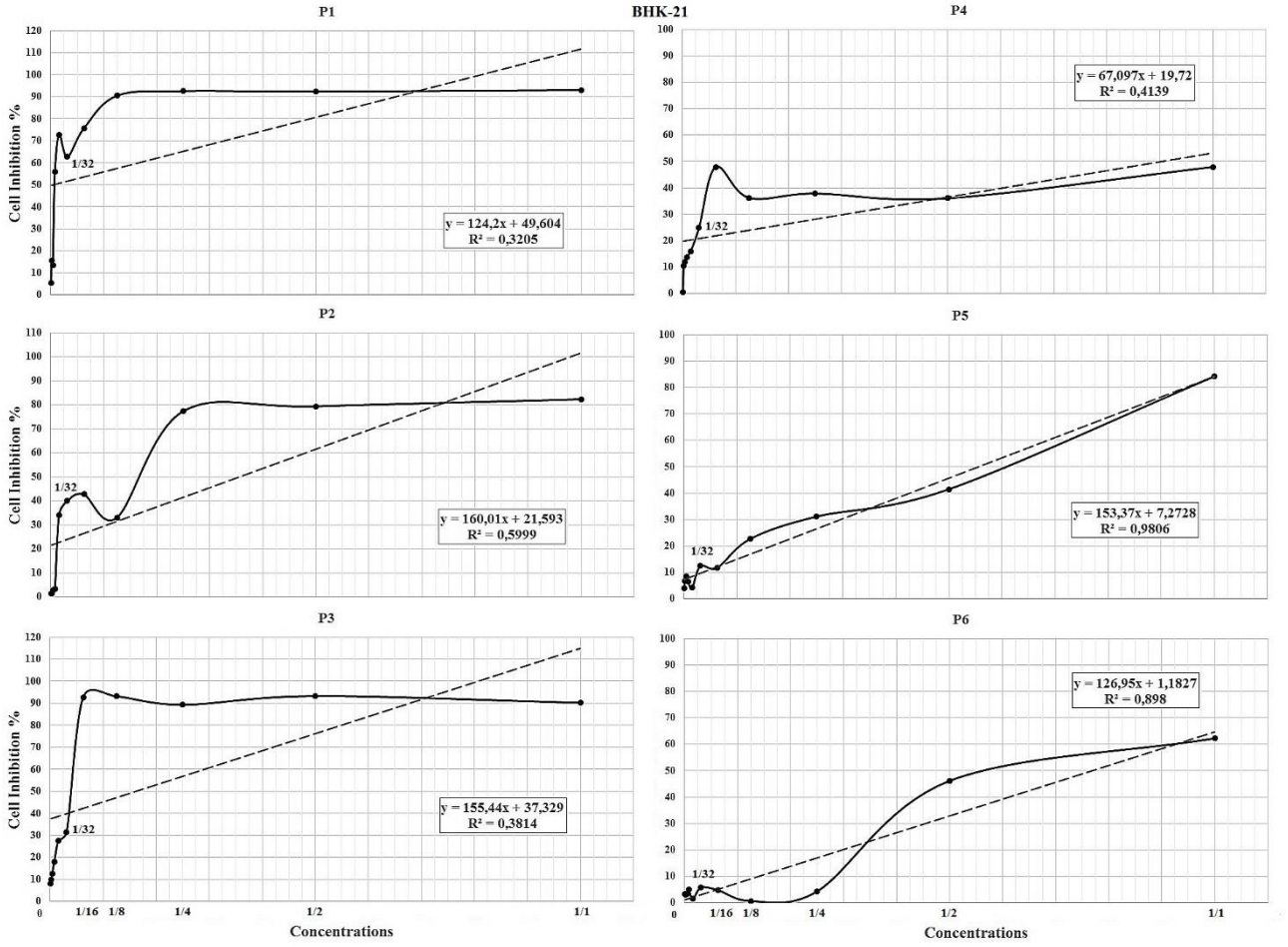

Figure 1. The inhibition effect of six propolis food supplements on BHK-21 cell line

On the viability murine macrophage cells (RAW 264.7), all propolis supplements had shown significant inhibition effects at the different dilution factors. When compared with the cell controls, P1, P2, P3, P4, P5 and P6 affected significantly the viability of macrophage cell line up to the dilutions of 1/2, 1/8, 1/64, 1/8, 1/2 and 1/2, respectively ($p<0.05$ and $p<0.01$, Table 2). Both P2 and P4 products adversely affected the RAW 264.7 cells at the dilution ratio of 1/8 ($p<0.05$). But, the % cell inhibition increased in P3 despite of decreasing its concentration up to 1/64 ($p<0.01$). The highest % cell viability was significantly determined in P6 (49% on 1/2 dilution) ($p<0.05$, Table 2). Figure 2 showed that there was a strong linear relation between the %

cell inhibition and concentration of P1, P5 and P6 (R^2 = 0.809, R^2=0.816 and R^2=0.944 respectively), however, weak linearity for P2, P3 and P4. The CC50 of P1, P2, P3, P4, P5 and P6 were calculated as 0.260, 0.218, 0.115, 0.257, 0.207 and 0.265% for RAW 264.7, respectively.

Table 2. The effects of six propolis products on viability of BHK-21 cell line

Dilutions	P1		P2		P3		P4		P5		P6	
	Abs. ± SD (nm)	**Cell Viability (%)**	**Abs. ± SD (nm)**	**Cell Viability (%)**	**Abs. ± SD (nm)**	**Cell Viability (%)**	**Abs. ± SD (nm)**	**Cell Viability (%)**	**Abs. ± SD (nm)**	**Cell Viability (%)**	**Abs. ± SD (nm)**	**Cell Viability (%)**
1/2	**0.137 ± 0.057**	7.0	**0.190 ± 0.033**	17.8	**0.151 ± 0.175**	9.8	**0.610 ± 0.090**	52.1	**0.171 ± 0.020**	15.9	**0.335 ± 0.060**	37.8
1/4	**0.147 ± 0.064**	7.7	**0.214 ± 0.154**	20.7	**0.113 ± 0.180**	6.7	**0.742 ± 0.282**	63.9	0.551 ± 0.088	58.5	0.468 ± 0.016	53.9
1/8	**0.144 ± 0.043**	7.5	**0.231 ± 0.171**	22.7	**0.162 ± 0.307**	10.7	**0.722 ± 0.136**	62.1	0.643 ± 0.256	68.9	0.815 ± 0.352	95.7
1/16	**0.176 ± 0.084**	9.5	0.603 ± 0.177	67.0	**0.115 ± 0.097**	6.9	**0.740 ± 0.151**	63.8	0.719 ± 0.101	77.3	0.846 ± 0.514	99.3
1/32	**0.409 ± 0.422**	24.3	0.522 ± 0.195	57.4	**0.119 ± 0.085**	7.2	**0.610 ± 0.058**	52.1	0.816 ± 0.394	88.3	0.811 ± 0.150	95.2
1/64	**0.613 ± 0.719**	37.3	0.545 ± 0.256	60.1	**0.875 ± 0.086**	68.6	0.865 ± 0.125	75.0	0.810 ± 0.344	87.6	0.803 ± 0.308	94.3
1/128	**0.456 ± 0.413**	27.3	0.595 ± 0.226	66.1	0.923 ± 0.135	72.5	0.965 ± 0.113	83.9	0.883 ± 0.368	95.8	0.839 ± 0.327	98.5
1/256	**0.719 ± 0.305**	44.0	0.852 ± 0.137	96.7	1.041 ± 0.156	82.1	0.991 ± 0.039	86.3	0.863 ± 0.239	93.6	0.810 ± 0.510	95.0
1/512	1.389 ± 0.261	86.6	0.856 ± 0.217	97.2	1.108 ± 0.213	87.5	1.010 ± 0.217	88.0	0.845 ± 0.123	91.5	0.823 ± 0.428	96.7
1/1024	1.354 ± 0.425	84.4	0.867 ± 0.127	98.5	1.142 ± 0.096	90.2	1.027 ± 0.239	89.5	0.861 ± 0.143	93.3	0.825 ± 0.139	96.9
1/2048	1.514 ± 0.594	94.6	0.868 ± 0.497	98.6	1.162 ± 0.066	91.9	1.137 ± 0.095	99.4	0.885 ± 0.217	96.0	0.825 ± 0.201	96.8
Cell Control	1.599 ± 0.063	100.0	0.880 ± 0.201	100.0	1.262 ± 0.095	100.0	1.144 ± 0.054	100.0	0.920 ± 0.112	100.0	0.851 ± 0.114	100.0
CC50 % in dilution)	0.003		0.178		0.082		0.451		0.278		0.384	

Table 3. The effects of six propolis products on viability of BHK-21 cell line

Dilutions	P1		P2		P3		P4		P5		P6	
	Abs. ± SD (nm)	Cell Viability (%)	Abs. ± SD (nm)	Cell Viability (%)	Abs. ± SD (nm)	Cell Viability (%)	Abs. ± SD (nm)	Cell Viability (%)	Abs. ± SD (nm)	Cell Viability (%)	Abs. ± SD (nm)	Cell Viability (%)
1/1	0,710 ± 0,057	29,6	0,178 ± 0,030	8,7	0,647 ± 0,175	25,1	0,517 ± 0,134	33,8	0,459 ± 0,034	14,7	0,295 ± 0,047	11,724
1/2	0,540 ± 0,064	22,5	0,918 ± 0,318	48,5	0,570 ± 0,180	22,1	0,668 ± 0,265	44,3	0,505 ± 0,044	16,3	1,182 ± 0,135	49,632
1/4	1,501 ± 0,043	62,5	0,943 ± 0,181	49,9	0,490 ± 0,307	19,0	0,770 ± 0,405	51,4	1,559 ± 0,380	52,5	1,456 ± 0,154	61,343
1/8	1,828 ± 0,084	76,1	0,604 ± 0,089	31,6	0,248 ± 0,097	9,6	0,572 ± 0,053	37,6	2,078 ± 0,858	70,3	2,333 ± 0,584	98,823
1/16	1,964 ± 0,422	81,8	1,589 ± 0,124	84,6	0,554 ± 0,085	21,5	1,139 ± 0,577	77,0	2,115 ± 0,729	71,6	2,219 ± 0,429	93,923
1/32	2,187 ± 0,719	91,1	1,530 ± 0,772	81,5	1,351 ± 0,086	52,4	1,217 ± 0,120	82,4	2,624 ± 0,745	89,1	2,203 ± 0,709	93,253
1/64	2,293 ± 0,413	95,5	1,738 ± 0,327	92,7	1,423 ± 0,135	55,2	1,222 ± 0,282	82,7	2,405 ± 0,438	81,5	2,227 ± 0,723	94,265
1/128	2,241 ± 0,305	93,3	1,823 ± 0,399	97,2	2,245 ± 0,156	87,1	1,185 ± 0,290	80,1	2,509 ± 0,749	85,1	2,200 ± 0,453	93,128
1/256	2,276 ± 0,261	94,8	1,867 ± 0,407	99,6	2,478 ± 0,213	96,1	1,105 ± 0,303	74,6	2,916 ± 0,234	99,1	2,140 ± 0,345	90,561
1/512	2,332 ± 0,425	97,1	1,827 ± 0,669	97,5	2,521 ± 0,096	97,8	1,319 ± 0,272	89,4	2,915 ± 0,628	99,0	2,357 ± 0,586	99,821
1/1024	2,321 ± 0,594	96,7	1,847 ± 0,424	98,5	2,508 ± 0,066	97,3	1,404 ± 0,397	95,4	2,937 ± 0,068	99,8	2,360 ± 0,287	99,963
Cell Control	2,401 ± 0,032	100,0	1,874 ± 0,180	100,0	2,578 ± 0,547	100,0	1,471 ± 0,291	100,0	2,943 ± 0,054	100,0	2,361 ± 0,180	100,000
CC50 % in dilution)	0,260		0,218		0,115		0,257		0,207		0,265	

DISCUSSION

Many studies suggested that propolis could have biological effects such as antibacterial, anti-inflammatory, tumorocidal and immunomodulator (Bogdanov, 2014; Bedier et al. 2016). The studies have focused on the effects of propolis on fibroblast cell lines such as BHK-21, gingival, skin and retinal because these cell lines exhibit fibroblast morphology for many studies (Wardati et al., 2014; Kartika et al., 2015, Bedier et al. 2016; Kurniati et al., 2018; Widjiastuti et al., 2020).

Ethanolic extract of propolis (100µg/ml, approx. 0.01%) with mineral trioxide aggregate increased the viability of BHK-21 cells for 24 h, however, the cell line was not affected by the propolis supplementation at 72 h and 7 days (Bedier et al. 2016). The propolis extracts and products such as oral gel had shown more biocompatibility and protective effects on BHK-21, odontoblastic and foreskin fibroblast cells against the cytotoxic effects of H2O2 (Aliyazicioglu et. al., 2011; Wardati et al., 2014; Kurniati et al., 2018). Human periodontal ligament (PDL) fibroblast cells were preserved by propolis at up to 50 ug/ml (approx. %0,005) concentrations for 24 h. (Al-Haj Ali, 2016). Murase et al. (2013) determined that the water extract of Brazilian green propolis increased the viability of mouse retinal or human skin fibroblast cells against the UVA-induced cell

damage. Likewise, Turkish propolis ethanolic extract showed dose-dependly an antioxidant activity on human fibroblast cells (Misir et al., 2018).

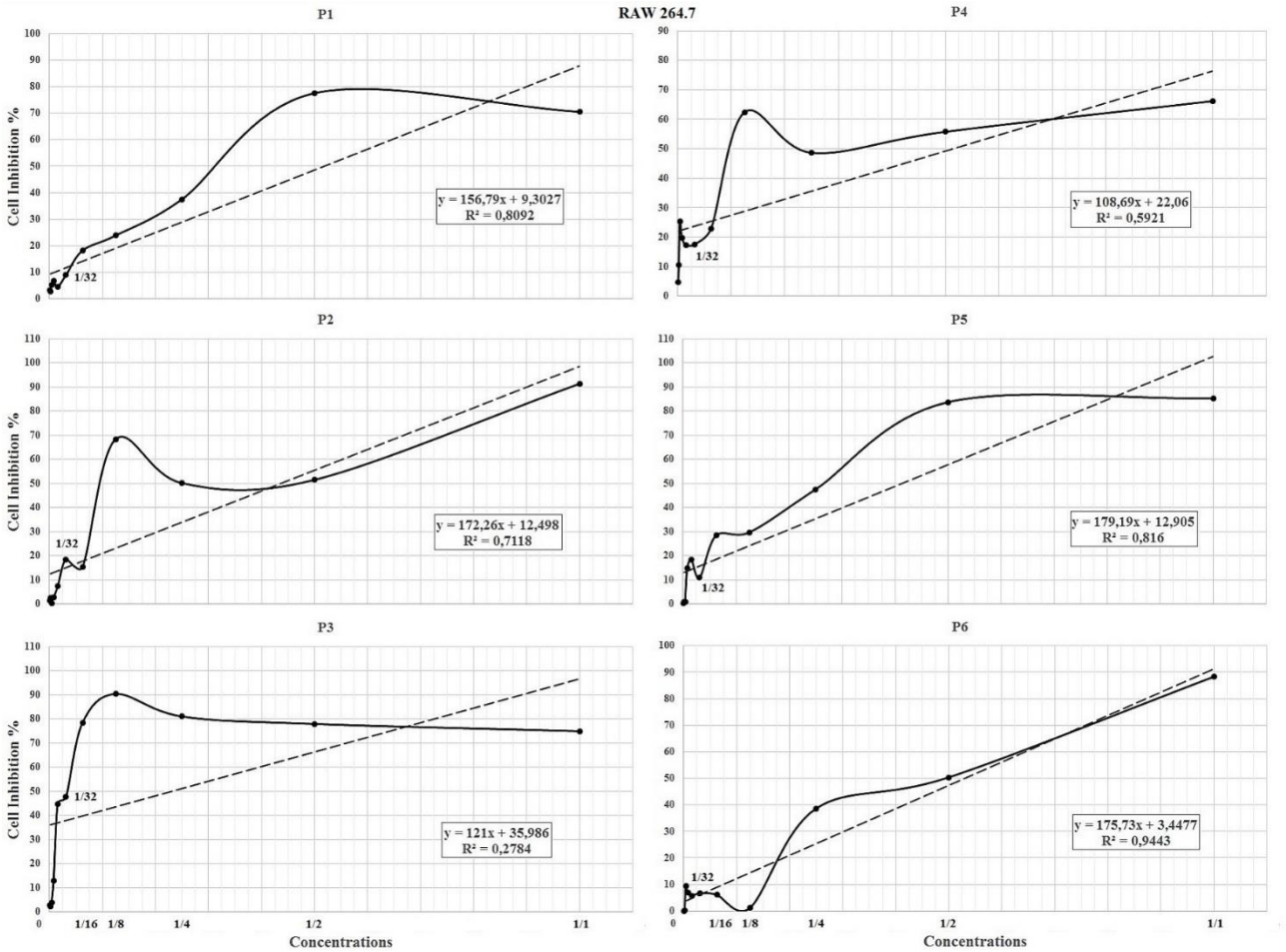

Figure 2. The inhibition effect of six propolis food supplements on RAW 264.7 cell line

Al-Haj Ali et al. (2016), Mooduto et al. (2018) and Ugur Aydın et al. (2018) suggested propolis were cytotoxic respectively at the concentration of 50 ug/ml (approx. 0.005%), 92.70 µg/ml (approx. 0,00927%) or greater and 15% (in ethanol) for gingival fibroblast cells. Likewise, Kartika et al. (2015) observed that 20ug/ml (approx. 0,002%) propolis decreased the % viability of dental pulp fibroblast from 80% to 32% (Widjiastuti et al., 2020). However, most of the studies have generally introduced that propolis and its extracts have low genotoxic, cytotoxic effects and are biocompatible on fibroblast cell lines (Wardati et al., 2014; Kartika et al., 2015; Al-Haj Ali et al., 2016; Kurniati et al., 2018; Mooduto et al., 2018). In this study, some propolis products contained flavouring, sweetener and aroma (such as menthol, glycol, liconice, eucalyptus) while some did not contain any additive. So, organic propolis and propolis products with only glycol had higher the CC50 and high viability of BHK-21 cells.

Propolis at 150ug/mL (approx. 0.015%) and 200ug/mL (0.02) reduced the viability of RAW 264.7 but propolis at 6.25–50ug/mL did not affect and was not toxic on RAW 264.7 cells when compared negative cell control (Sahlan et al., 2021).

Han et al. (2002) suggested that the water extract of Korean Propolis from 2.5 pg/ml to 25 pg/ml had no toxic effect on RAW 264.7. However, Kim et al. (2019) observed that ethanolic extract of South Korean propolis decreased dose-dependently (10 to 40 ug/ml) the

viability of RAW 264.7 independently of region. Asgharpour et al. (2019) determined the CC50 of ethanol extract of propolis as 15±3.2 µg/ml (approx. 0.0015%) for RAW 264.7. when compared water and methanolic extracts of Brazilian propolis, the water extract was lower toxic and had higher CC50 levels on murine macrophage-like J774 cells (Myint 2003). In this study, the CC50s of the products were higher for RAW 264.7 cells than for BHK-21 cells. So, low cytotoxic effect was determined in RAW 264.7 cells even at high concentrations of the propolis products.

CONCLUSION

Despite their rich bioactive compound content, the propolis products affected both baby hamster kidney cells and murine macrophage cells at different concentrations. The results of many studies suggested that alcoholic extracts of propolis or containing other solvents and additives adversely affected the cell viability. Similarly, the results of this study introduced that the dilution factors and additives might be cytotoxicity-determining factor of the propolis products.

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