Anti-proliferation and Insulin Resistance Alleviation of Hepatocellular Carcinoma Cells HepG2 *in Vitro* by Chinese Propolis

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Abstract   Previous studies suggest affirmative effects of propolis on cancers and diabetes, due to its abundant flavonids. But information is rarely available with regard to its effects on hepatocellular carcinoma cells (HCC), particularly on cell proliferation and insulin resistance (IR). We measured the flavonids of ethanol extract from Chinese propolis (EEP) by HPLC. IR model of HepG2 cells were established, which were treated with different kinds and doses of propolis. The anti-proliferation and IR alleviation of HepG2 cells were studied. As result, EEP contained many flavonoids. Compared with other two solvents (PEG and DMSO), olive oil (dosage: 200 μg/mL) could significantly inhibit cell proliferation and alleviate IR of HepG2 cells. EEP dissolved in DMSO (mass ratio: 2:1; dosage: 300 μg/mL) exhibited anti-proliferative effect on HepG2 cells. When EEP was dissolved in olive oil (mass ratio: 2:1; dosage: 300 μg/mL), IR of HepG2 cells was greatly alleviated. As a conclusion, olive oil may be more suitable as auxiliary solvent of propolis as to improve its biological activities. High-dose propolis could effectively inhibit the proliferation and alleviate the IR of HepG2 cells in vitro.

Keywords: cell proliferation, flavonoids, HepG2 cells, insulin resistance, propolis

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common therioma, ranking third only to gastric cancer and esophageal cancer. In addition, it is very hard to diagnose in the early phase of symptom development due to lack of awareness, leading to the seriously affected life quality of patients [1]. HCC is mainly characterized by abnormal cycles and limitless proliferation of tumor cells. Therefore, its most widely accepted therapy is the interference with cell cycles, including the inhibiting the proliferation and growth of tumor cells, or inducing the apoptosis of tumor cells [2]. Bortezomib has been found to inhibit the cell proliferation in two different HCC cell lines, making it attractive for future experimentation in animal models [3]. Vasoative intestinal peptide can also inhibit the proliferation of HepG2 cells *in vitro*, and its anti-proliferative effects may offer a novel and promising approach of suppressing HCC [4]. Insulin resistance (IR) is a state in which a given concentration of insulin produces a less-than-expected biological effect. The liver and its peripheral tissues are target organizations and main lesion tissues of IR [5]. Moreover, IR is mainly characterized by reduced glucose consumption of target cells with a normal insulin level [6]. The situation of IR can be successfully mitigated by improving the insulin

digestion. Eriodictyol and 7-O-methylaromadendrin, which are both flavonoids, have been found to increase glucose uptake and alleviate IR, suggesting anti-diabetic properties of flavonoids [7,8]. Flavonoids are the main components in Chinese propolis, and some researchers have reported the anti-diabetic effects of propolis from different geographic regions [9,10]. Based on these reports, studies on anti-proliferation and IR alleviation of human HCC cells have important theoretical and practical significance.

Propolis is a mixture of various amounts of beeswax and resins collected by honeybees from secretions of plants, particularly from branches, buds or callus. These secretions are interfused with other secretions from maxillary and wax glands of honeybee. Finally, an aromatic solid gelatinous substance is successfully processed, which is called as propolis [11,12]. According to historical records, propolis has been always applied in drugs since centuries ago. Egyptians pioneered the use of propolis to retard carcass decay [13]. In Greece and Ancient Roma, doctors used propolis as antiseptic, disinfectant and cicatrizant for wound treatment. Ancient Europeans paid more attentions to the anti-bacterial properties of propolis [14,15]. Propolis possesses many biological activities due to its abundant flavonoids, such as broad-spectrum bactericidal, antioxidant, immune regulation, anti-inflammation and anti-cancer [14]. Especially, propolis has some affirmative effects on

different cancers, such as laryngeal carcinoma, renal adenocarcinoma and prostate cancer [16,17]. Despite the broad anti-cancer potential of propolis, information is rarely available with regard to its effects on HCC cells, particularly on cell proliferation and IR. In the current investigation, we established an IR model of HepG2 cells using supra-physiological concentrations of insulin and glucose solutions. Furthermore, the capacity of anti-proliferation and IR alleviation by propolis was also assessed in liver cancer cells *in vitro*.

2. Materials and Methods

2.1. Instruments and Reagents

Thermostatic oscillator (THZ-C) was purchased from Aiwa Optoelectronic Technology Co., Ltd. (Wuxi, China). Ultraviolet visible spectrophotometer (752N) was provided by Hanker International Group Co., Ltd. (China). Centrifuge (5804R) was obtained from Eppendorf Co., Ltd. (China). SANYO carbon dioxide incubator (MIR-253) was supplied by Biotechnology Co., Ltd. (Shanghai, China).

The standards used in HPLC analysis, tryosin and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, USA). Thiazolyl blue tetrazolium bromide (MTT), glucose and insulin were obtained from Beijing Chemical Reagent Company. Human hepatoma HepG2 cell lines were kept in our laboratory. Dulbecco's modified eagle medium (DMEM) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Glucose reagent kit was produced by Randox Laboratories Ltd. (the United Kingdom).

2.2 Preparation of Ethanol Extract from Propolis (EEP)

Raw propolis was collected from honeybee hive (*Apis mellifera* L.) in Linyi (35.24°N, 118.53°E) locating in North China in April, 2012, and it mainly origins from poplar (*Populus* sp.). Raw propolis was ground into fine powder, mixed with 95% (v/v) ethanol (solid-liquid ratio was 1:10) in a beaker flask and incubated at a constant temperature (40°C) in a water bath for 24 h. During the period of incubation, the beaker flask was gently wobbled every 2 h. Subsequently, the supernatant was obtained through the filtration on Whatman No. 4 filter paper. The collected supernatant was evaporated in a rotary evaporator under a reduced pressure at 55°C. The extract was dried in an oven until it reached an invariable weight. The final extract was stored at -20°C prior to further analysis.

2.3. Measurement of Flavonoid Content with HPLC

Chromatographic analyses of flavonoid content were carried out using the method of Chinese Standard (GB/T 19427-2003) and operated on an Agilent HPLC system, which was equipped with a vacuum degasser G1322A, a quaternary pump G1311A, an autosampler G1329A, a programmable variable wavelength detector G1314B and a thermostatted column compartment G1316A. Flavonoid separation was performed on Sepax GP-C18 column (4.6 mm × 150 mm, 3 μm) at a detection wavelength of 270 nm at 28°C. The mobile phases consisted of methanol and water at a ratio of 58/42 (v/v), and the pH of the mobile phase was adjusted to 3 with phosphoric acid at a flow rate of 0.7 mL/min. All sample solutions were filtered through 0.22-μm membrane filters, and the injection volume was 10 μL.

2.4. Setting of Drug Treatment Groups

The drug treatment groups were divided into propolis groups (sample groups) and non-propolis groups (solvent groups, including polyethylene glycol, PEG; dimethyl sulfoxide, DMSO; olive oil). Table 1 lists detailed information.

Table 1. The setting of the drug treated groups

Drug treated groups	Drug treated sub-groups	Material (mass ratio)	Dosage (μg/mL)
PL	PLp	PEG	20
	PLs	PEG + propolis (2:1)	30
PM	PMp	PEG	100
	PMs	PEG + propolis (2:1)	150
PH	PHp	PEG	200
	PHs	PEG + propolis (2:1)	300
DL	DLp	DMSO	20
	DLs	DMSO + propolis (2:1)	30
DM	DMp	DMSO	100
	DMs	DMSO + propolis (2:1)	150
DH	DHp	DMSO	200
	DHs	DMSO + propolis (2:1)	300
OL	OLp	Olive oil	20
	OLs	Olive oil + propolis (2:1)	30
OM	OMp	Olive oil	100
	OMs	Olive oil + propolis (2:1)	150
OH	OHp	Olive oil	200
	OHs	Olive oil + propolis (2:1)	300

P, D, O separately represented PEG, DMSO, and Olive oil.
L, M, H separately represented low, mid, high concentration.
p, s separately represented solvent groups, sample groups.

2.5. Cytological Experiments

2.5.1. Cell Recovery

F*rozen HepG2 cells were* thawed in a 37°C water bath for 2 min and resuspended at 40°C on shaker at 60 rpm. Subsequently, the cell suspension was centrifuged at 800 rpm for 5 min.

2.5.2. Cell Culture

The supernatant was removed, and cell pellet was resuspended in 10 mL DMEM containing 10% FBS and 1% penicillin-strptomycin. Cells were then cultured at a saturated humidity of 5% CO_2 and 37°C for 24 h.

2.5.3. Cell Digestion

Culture medium was removed, HepG2 cells were trypsinized with 0.25% trypsin, and then the reaction was terminated by adding 10 mL DMEM containing 10% FBS.

2.5.4. Cell Vaccination

Briefly, cells were seeded into 96-well culture plates at a density of 3×10^4 cells/mL, and 200 μL of cell suspension was added into each well. The plates were incubated at a saturated humidity of 5% CO_2 and 37°C for 24 h. Six wells without the drug treatment were used as CK (control check) groups.

2.5.5. IR Model

After 24 h, culture medium was replaced with 100 μL of DMEM containing 10^{-6} M insulin, 1% FBS and 25 mM glucose. Cells were cultured at *37*°C with a *5% CO_2 saturation humidity for* another 36 h, and the culture medium was refreshed every 18 h. Six wells without the drug treatment were separated as MG (model group) groups.

2.5.6. Drug Treatment

Drug treatment was carried out according to Table 1. Each drug treatment group included six wells, and treated cells were cultured at *37*°C with a *5% CO_2 saturation humidity* for 24 h.

2.5.7. Measurement of Relative Cell Proliferation

Briefly, 20 μL of MTT solution (5 mg/mL, dissolved in phosphate-buffered saline, PBS) was added into each well, and the culture plates were incubated at *37*°C with a *5% CO_2 saturation humidity* for 4 h until purple precipitate was clearly visible. The supernatant was then discarded, 150 μL DMSO was added into each well, and the plates were shaken at medium speed for 5 min. The cell proliferation rate was determined according to the absorbance at a wavelength of 570 nm using the microplate reader. The relative cell proliferation rate of MG groups was defined as 100%, whereas it of other groups was determined accordingly.

2.5.8. Measurement of Relative Glucose Consumption Capability

The supernatant was removed, and the wells were washed twice with PBS. Subsequently, 10 mL DMEM containing 10^{-6} M insulin and 25 mM glucose was added into each well. The plates were incubated at *37*°C with a *5% CO_2 saturation humidity* for 24 h. The change of glucose content in the supernatant, which corresponded to the glucose consumption capacity, was determined by the glucose kit. The relative glucose consumption capacity of MG groups was defined as 1, whereas it of other groups was determined accordingly.

2.6. Statistical Analysis

Data were processed by SPSS statistical software version 18.0 (SPSS, Inc., Chicago, IL, USA). Analysis of variance was performed using one-way ANOVA method, and differences within treated groups were assessed using paired *t*-tests. A *P* value of < 0.05 was considered statistically significant, and a *P* value of < 0.01 was considered statistically extremely significant. Bivariate correlation method was used to conduct correlation analysis. All of the figures were constructed using Microsoft Excel.

3. Results

3.1. Flavonoid Content of EEP

Figure 1 shows that the chromatographic profiles of EEP and flavonoid standards were recorded at 270 nm, and their levels were shown in Table 2. The total flavonoid content of EEP was 167.00 ± 7.00 mg/g. Eight most representative flavonoids of EEP were also analyzed, including rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin. The HPLC revealed that seven flavonoids (except for myricetin) were detected in EEP.

Table 2. Concentrations of flavonoids presented in EEP

No.	Components	Acquisition time (min)	mg/g of EEP[a]
1	Rutin	26.993	0.32±0.01
2	Myricetin	28.795	0.00
3	Quercetin	36.357	12.09±0.51
4	Kaempferol	44.012	13.88±0.58
5	Apigenin	46.234	14.02±0.59
6	Galangin	46.247	44.01±1.84
7	Pinocembrine	48.031	34.94±1.46
8	Chrysin	48.584	47.73±2.00

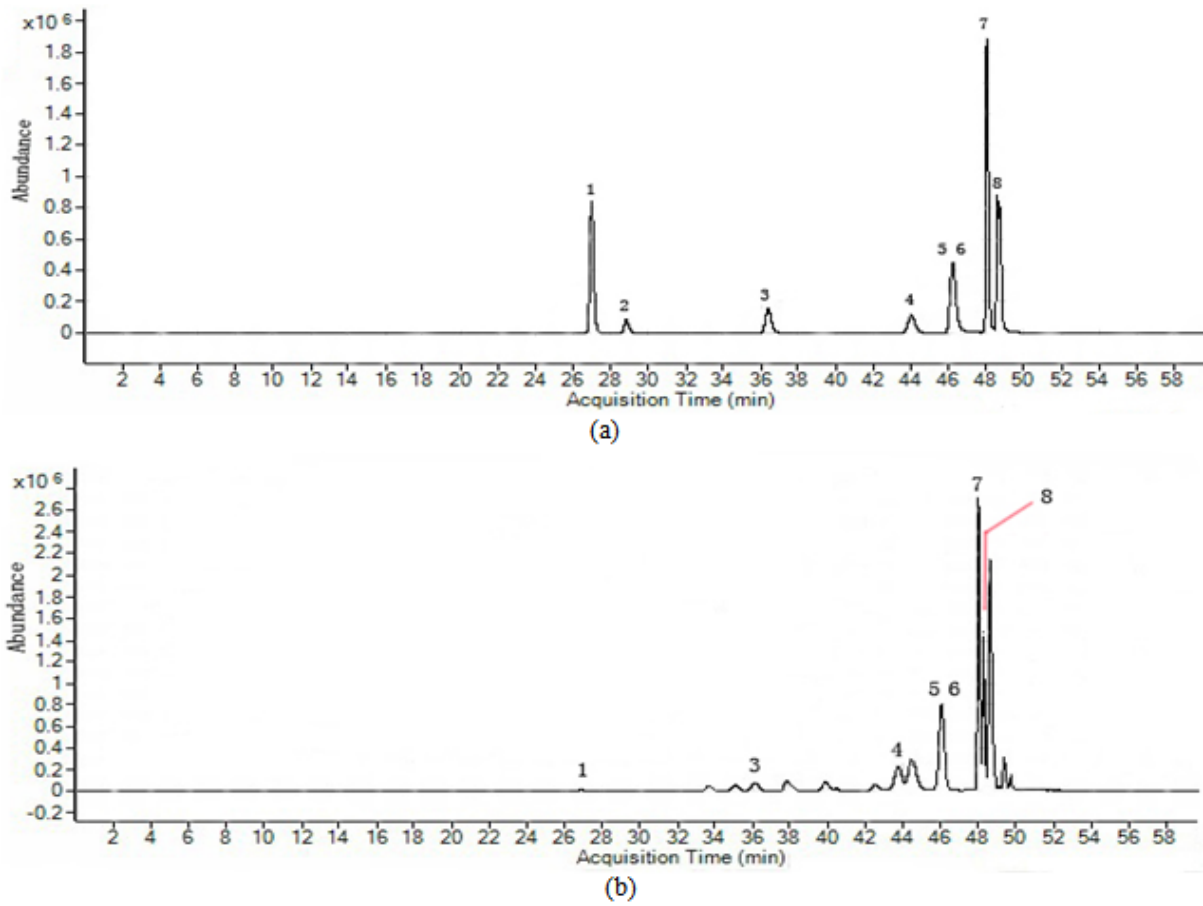[a]Reported values are the means ± SD (n=3).

Figure 1. HPLC chromatogram of flavonoids standards (a) and EEP (b). Peaks: 1 rutin; 2 myricetin; 4 quercetin; 5 apigenin; 6 pinocembrin; 7 chrysin; 8 galangin

3.2. Influence of Propolis on Proliferation of HepG2 Cells

Figure 2 shows that the relative cell proliferation rate of OMp and OHp groups was 84.67% ± 10.50% and 73.60% ± 10.38% respectively, reflecting a greater anti-proliferative effect compared with other solvent groups. In addition, the proliferation of HepG2 cells was significantly inhibited in all sample groups, and the anti-proliferative effect was in a concentration-dependent manner. The minimum relative cell proliferation rate was 79.29% ± 5.03%, 49.30% ± 6.45% and 19.14% ± 3.39 when the propolis concentration was 10 μg/mL, 50 μg/mL and 100 μg/mL, respectively. Table 3 shows the P values between different groups. In solvent groups, the relative cell proliferation rate of only OHs group was significantly different from that of CK or MG groups, with a P value of 0.031 or 0.010, respectively. Except for the OLs group, extremely significant differences in terms of the relative cell proliferation rates of all other sample groups were observed compared with CK or MG groups. Moreover, the relative cell proliferation rate of sample groups at the middle (150 μg/mL) and high (300 μg/mL) concentrations was also compared with all solvent groups, and the differences all reached an extremely significant level ($P <$ 0.01). The pairwise comparisons showed that the relative cell proliferation rate was in a concentration-dependent manner. Namely, a greater anti-proliferative effect was observed once the propolis concentration was increased.
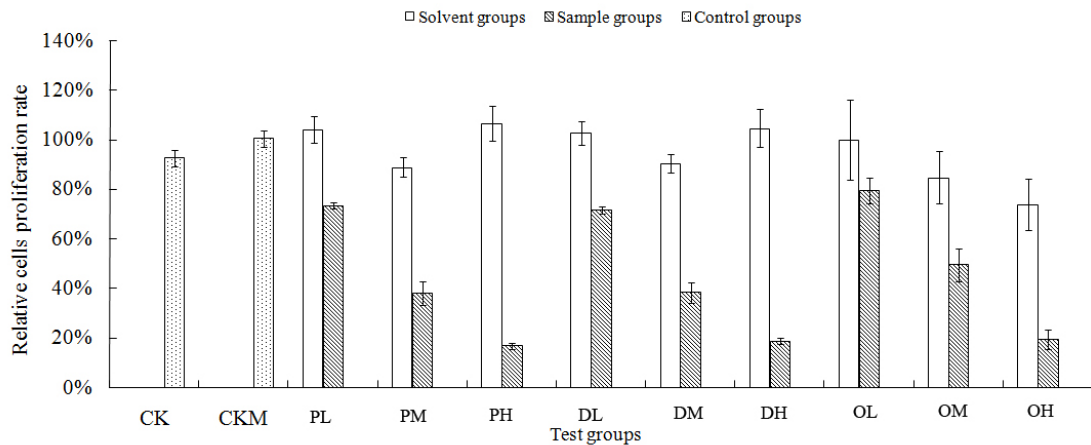

Figure 2. Relative cells proliferation rate of HepG2 cells of treated groups

3.3. Influence of Propolis on IR of HepG2 Cells

Figure 3 shows the relative glucose consumption capacity of all groups. In all solvents groups, OMp and OHp groups exhibited some relieving effects on IR of HepG2 cells, with a relative glucose consumption capacity of 1.31 ± 0.12 and 1.47 ± 0.18, respectively. In all sample groups, the IR of HepG2 cells was significantly reduced when the propolis concentration was increased. Particularly, the relative glucose consumption capacity of OMs and OHs groups was 1.73 ± 0.14 and 4.11 ± 0.58, respectively.
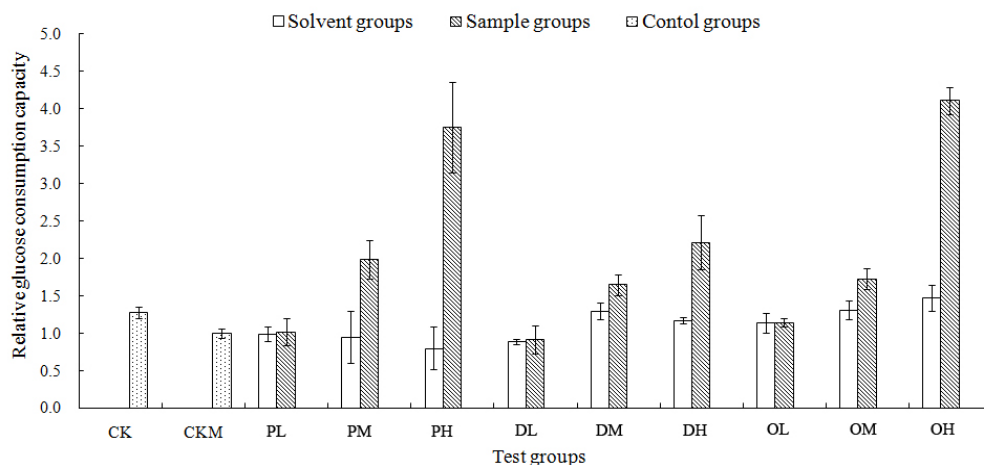

Figure 3. Relative glucose consumption capacity of HepG2 cells of treated groups

Table 3. Comparison of relative cells proliferation rate of HepG2 cells between treated groups

	CK	MG	PLp	PMp	PHp	DLp	DMp	DHp	OLp	OMp
CK	—	0.042*	0.032*	0.280	0.035*	0.036*	0.483	0.063	0.482	0.290
MG		—	0.377	0.017*	0.256	0.539	0.024*	0.430	0.953	0.069
PLp			—	0.016*	0.666	0.757	0.021*	0.911	0.693	0.047*
PMp				—	0.019*	0.017*	0.665	0.033*	0.315	0.561
PHp					—	0.489	0.024*	0.788	0.557	0.041*
DLp						—	0.023*	0.721	0.786	0.054
DMp							—	0.042*	0.375	0.438
DHp								—	0.666	0.056
OLp									—	0.247
OMp										—
OHp										
PLs										
PMs										
PHs										
DLs										
DMs										
DHs										
OLs										
OMs										
OHs										

	OHp	PLs	PMs	PHs	DLs	DMs	DHs	OLs	OMs	OHs
CK	0.031*	0.001**	0.000**	0.000**	0.001**	0.000**	0.000**	0.019*	0.000**	0.000**
MG	0.010**	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.003**	0.000**	0.000**
PLp	0.008**	0.001**	0.000**	0.000**	0.001**	0.000**	0.000**	0.004**	0.000**	0.000**
PMp	0.608	0.003**	0.000**	0.000**	0.002**	0.000**	0.000**	0.061	0.001**	0.000**
PHp	0.008**	0.001**	0.000**	0.000**	0.001**	0.000**	0.000**	0.005**	0.000**	0.000**
DLp	0.009**	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.004**	0.000**	0.000**
DMp	0.046*	0.002**	0.000**	0.000**	0.002**	0.000**	0.000**	0.040*	0.001**	0.000**
DHp	0.011*	0.002**	0.000**	0.000**	0.002**	0.000**	0.000**	0.009**	0.001**	0.000**
OLp	0.072	0.047*	0.003**	0.001**	0.044*	0.003**	0.001**	0.104	0.007**	0.001**
OMp	0.243	0.134	0.002**	0.000**	0.119	0.002**	0.000**	0.469	0.008**	0.001**
OHp	—	0.961	0.004**	0.000**	0.868	0.004**	0.001**	0.403	0.021*	0.001**
PLs		—	0.000**	0.000**	0.562	0.000**	0.000**	0.112	0.003**	0.000**
PMs			—	0.002**	0.000**	0.913	0.002**	0.000**	0.067	0.006**
PHs				—	0.000**	0.001**	0.115	0.000**	0.001**	0.341
DLs					—	0.000**	0.000**	0.090	0.003**	0.000**
DMs						—	0.001**	0.000**	0.067	0.004**
DHs							—	0.000**	0.001**	0.806
OLs								—	0.003**	0.000**
OMs									—	0.002**
OHs										—

* represented significant difference ($P<0.05$)
** represented extremely significant difference ($P<0.01$)

Table 3 shows the *P* values between different groups. After the insulin treatment at high concentrations, an extremely significant difference ($P < 0.01$) in terms of the relative glucose consumption capacity of the CK group was observed compared with the MG group. It indicated that IR condition emerged and MG group was a successful model. Moreover, no extremely significant difference ($P < 0.01$) was observed when the relative glucose consumption capacity of all solvent groups was compared with that of the MG group. However, when the relative glucose consumption capacity of DMSO groups (DLp, DMp, DHp) and olive oil groups (DMp, DHp) was compared with that of the MG group, the differences all reached a significant level ($P < 0.05$). In addition, when the relative glucose consumption capacity of all sample groups at the middle (150 µg/mL) and high (300 µg/mL) concentrations was compared with that of the MG group, the differences reached an extremely significant level ($P < 0.01$). Besides, the highest difference in terms of the relative glucose consumption capacity was detected between OHs and MG groups. Furthermore, the pairwise comparisons between sample groups revealed that the relative glucose consumption capacity was in a concentration-dependent manner. Extremely significant differences ($P < 0.01$) in terms of the relative glucose consumption capacity were detected between all sample groups at the high concentration (300 µg/mL) and solvent groups.

3.4. Correlation Analysis

Figure 4 shows that the cell proliferation was negatively correlated with the IR level (Kendall's tau rank: r=-0.851, n=27, p=0.000). A negative correlation was found between (olive oil + propolis) groups and the cell proliferation (Kendall's tau rank: r=-0.998, n=9, p=0.04). Moreover, a positive correlation was found between (DMSO+propolis) groups and the IR level (Kendall's tau rank: r=1.000, n=9, p=0.004). However, all other groups did not have any correlation with the cell proliferation or IR level. These results indicated that propolis exerted distinct effects on the cell proliferation and IR level, and the selection of a proper solvent played a crucial role.
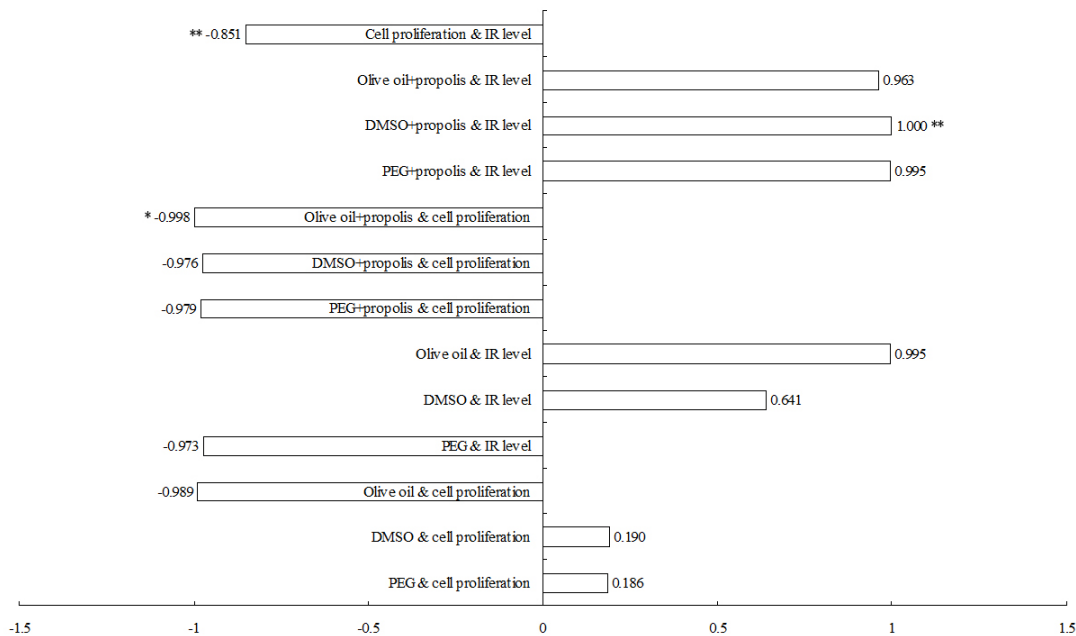

Figure 4. Correlation analysis of treated groups and the 2 indictors (* represented significant difference (*P*<0.05), ** represented extremely significant difference (*P*<0.01).)

4. Discussion

As a nutritious food, propolis consists of abundant bioactive components, such as flavonoids, terpenes and phenolic acids. It plays a beneficial role in protecting liver from various pathological changes. However, its underlying internal mechanism still remains unclear. Recently, food safety and quality issues have become globally prominent. Therefore, a great deal of attention has been attracted on the safety of propolis consumption. The raw propolis used in this study was collected from North China, of which the main botanical source is well known as poplar (*Populus* sp.) [18]. The EEP of our samples contained many flavonoids, such as rutin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin. Although different in chemical constituents or plant sources, the biological activities of many propolis used in other studies are always similar [19,20,21]. In the present study, we showed that almost all sample groups exhibited an inhibited proliferation of HepG2 cells and a reduced IR level *in vitro*, and these effects were in a propolis concentration-dependent manner, which was consistent with other studies. Artepillin C is one of the principal phenolic acids specifically found in Brazilian propolis, and it is highly cytotoxic to a variety of malignant human and murine tumor cell lines *in vitro* [22]. Furthermore, same results have been identified in tumor-transplanted athymic and thymic mice [23]. Brazilian propolis was used to treat 10-week-old Otsuka Long-Evans Tokushima Fatty (OLETF) rats, and the results suggested that propolis is an effective and functional food to prevent the IR development [24]. The hypolipidemic

effect of Brazilian propolis has also been examined in a *mouse model* of diet-induced *obesity*, and data showed that propolis can significantly reduce levels of serum glucose and tends to mitigate the IR level [25]. The propolis samples used in previous studies were mostly collected from Brazil, and their effects on inhibiting cell proliferation and relieving IR have been systemically and exhaustively investigated. Until now, only few studies about Chinese propolis have been reported in these aspects. In our previous study, we have shown that different from Brazilian propolis, Chinese propolis consists of flavonoids, trepenes, esters, alcohols and so on [26]. Therefore, there must be some biological differences between Chinese propolis and Brazilian propolis. Although propolis from different geographic origins may possess different chemical compositions, they should have similar biological activities [27]. Our study provided the theoretical basis for the future medical research on propolis. Nevertheless, since we used only *in vitro* cultured cells as our experimental materials, it is necessary to confirm these findings through further verifications on animal experiments and clinical applications.

As solvents for propolis, their dissolution with propolis and oral safety should be concerned. Búfalo et al. reported that 70% ethyl alcohol has no effect either on cell morphology or on cell viability [28]. Sonmez et al. showed that 10% raw Turkish propolis and Sigma propolis (dissolved in 70% ethyl alcohol and propylene glycol) can inhibit the periodontopathogen microorganisms to some extent. Although not as effective as Australian and USA propolis, these propolis samples are safe for gingival fibroblasts. Moreover, synergism between propolis and anti-bacterial agents has been also observed [29]. In this regard, Noori et al. found that there is synergism between propolis and anti-microbial drugs against *S. aureus*, and its underlying mechanism is the interference with bacterial protein synthesis by those agents [30]. Therefore, we assumed that right solvent could improve the function of propolis. In the present study, only olive oil was found to have a significant effect on cell proliferation and IR of HepG2 cells among three types of solvents, and propolis could be easily dissolved in oils. Based on these findings, olive oil was a suitable auxiliary solvent of propolis. Moreover, tocopherols and phenolic compounds of virgin olive oil have a strong antioxidant activity and free radical-scavenging properties, resulting in an improved biological function of propolis. However, the main vegetal sources of propolis are *Salix babylonica* L., *Quercus palustris* Muncch. and *Alnus japonica* (Thunb.) Steud. in China [31]. More work is needed in future studies in order to clarify the influence of plant source on the biological function of propolis.

5. Conclusion

The raw propolis used in this study was collected from North China, its ethanol extract contained many flavonoids, such as rutin, quercetin, kaempferol, apigenin, pinocembrin, chrysin and galangin. Almost all EEP samples applied on HepG2 cells exhibited an inhibited proliferation of HepG2 cells and a reduced IR level in vitro, and these effects were in a propolis concentration-dependent manner. Moreover, only olive oil was found to

have a significant effect on cell proliferation and IR of HepG2 cells among three types of solvents, and propolis could be easily dissolved in oils. So olive oil was a suitable auxiliary solvent of propolis. Nevertheless, since we used only in vitro cultured cells as our experimental materials, it is necessary to confirm these findings through further verifications on animal experiments and clinical applications.

Acknowledgements

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