

Water Soluble Propolis and Royal Jelly Enhance the Antimicrobial Activity of Honeys and Promote the Growth of Human Macrophage Cell Line

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Abstract: Due to the overuse and misuse of antibiotic, an increase in antibiotic resistance of pathogenic bacteria is evolving. Attention should be focused on natural alternatives to antibiotics, like propolis, royal jelly (RJ) and honeys. They all have strong antibacterial properties due to the active substances they contain. This study investigated the effect of combination of water soluble propolis (WSP) Greit120 or fresh royal jelly (F-RJ) (Mižigoj) and Forest honeys as antibacterial against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Candida albicans*. These substances are also cell growth promoters for human macrophage (TLT) cell line. WSP Greit120, F-RJ (M) and different Forest honeys were prepared in saline as 10% solutions. The antimicrobial activity was expressed as the minimal inhibitory concentration (MIC) in mg/mL. The growth promotion activity was measured at optical density (OD) 595 nm. The combination of WSP Greit120 with different Forest honeys is better than F-RJ (M) in same combination with different Forest honeys. The best antibacterial/antifungal activity was found with the combination of 10% WSP Greit120 in the Forest honey (1:10) from Italy or Spain. When measuring the growth promoting activity of TLT cell line, the best activity was detected at the combination of 10% WSP Greit120 in the Forest honey from Italy ($GI_3 = 0.796 \pm 0.014$ and $GI_5 = 1.133 \pm 0.022$). Antimicrobial and growth promoting activities are correlated and WSP-dependent.

Key words: Propolis, royal jelly, Forest honey, antimicrobial activity, human macrophage cell line, cell growth promoting activity.

1. Introduction

Antibiotic resistance of different bacteria was attributed to the overuse and misuse, as well as the inappropriate use of antibiotics that led to many forms of bacterial resistance, thereby limiting the use of these agents in strains of microorganisms resistant to antibiotics [1]. Research on natural alternative antibacterial products, such as, propolis, royal jelly (RJ) and honey, as a part of complementary medicine, is of great importance, as they could be successfully

used against certain acquired resistant pathogenic microorganisms [2, 3]. Among them, propolis is a mixture of balsamic resinous substances produced by honeybees and used by humans as a remedy because of its health properties, such as, in wound treatment, burns and sore throat [4]. It is also used as a substance applied in medicine and cosmetics due to its antiviral, antibacterial and antifungal activities containing various chemical components [5]. In general, propolis contains 50% resins and plant balsam, 30% of wax, 10% of essential and aromatic oils, 5% pollen and 5% of other various substances, including organic debris. The composition of propolis depends on the place and

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time of collection [6]. Phenolic substances, flavonoids and cinnamonic acid derivatives are the major bioactive components of propolis, while its antimicrobial properties are related to the synergistic effect of its components. Among bioactive constituents, flavonoids have the highest role in antimicrobial activity of propolis. They impede the multiplication of the bacteria by damaging bacterial cytoplasm causing bacterial cell lyses [7-9].

The hypopharyngeal and the mandible glands in the head of nurse bees produce RJ. It has a thick, milky appearance, with a slightly acid, pungent odour and a somewhat bitter taste. Its water content is usually uniform, greater than 60%, and it has a water activity (a_w) above 0.92. The dry matter of RJ is composed of protein (27%-41%, including free amino acids), carbohydrates (approximately 70%), lipids (8%-19%), trace elements and some vitamins [10]. Its antibacterial activity against both Gram-positive and Gram-negative bacteria is attributed to the antibacterial activity mainly to present fatty acids, such as trans-10-hydroxydec-2-enoic acid, 3-hydroxydodecanoic acid, 11-oxododecanoic acid and 11-S-hydroxydodecanoic acid [11]. Short extension of the peptides, their poor cytolytic and mast cell degranulating activities, together with their broad spectrum of antibiosis, make them attractive models for the development of new antibiotic agents [12]. RJ also contains specific antibacterial peptide royalisin that display certain antibacterial activities against Gram-positive bacteria [13-15].

Honey is a complementary remedy for the treatment of infected wounds, particularly where conventional modern therapeutic agents fail. Among honey types, certainly the most effective Manuka honey was reported to exhibit antimicrobial activity against pathogenic bacteria, such as, *Staphylococcus aureus* and *Helicobacter pylori*, making it a promising agent for the treatment of wounds or stomach ulcers [16]. The beneficial role of honeys is attributed to the antibacterial property that is comprised of high

osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) as well as non-peroxide components, i.e., the presence of phytochemical components, like methylglyoxal (MGO). H_2O_2 is the most important antimicrobial agent in honey. Its concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower pollen [17, 18]. Most types of honeys generate H_2O_2 when diluted, because of the activation of the enzyme glucose oxidase that oxidizes glucose to gluconic acid and H_2O_2 , which thus attributes the antimicrobial activity [19]. The healing properties of honey can be ascribed to the fact that it offers antibacterial activity, maintains a moist wound environment to promote healing and has a high viscosity that helps to provide a protective barrier to prevent infection [20, 21]. On the other hand, glycosylated proteins can induce tumor necrosis factor-alpha (TNF- α) secretion by macrophages, and this cytokine is known to induce the mechanism of wound healing. Furthermore, the ability of honey to reduce "reactive" intermediates release may limit the tissue damage caused by activated macrophages during wound healing. Thus, the immunomodulatory property of honey is relevant to wound repair [22].

The present experiments were aimed, at very first time, to compare the enhancement of different Forest honey's antimicrobial activity in combination with water soluble propolis (WPS) Greit120 or fresh royal jelly (F-RJ) (Mižigoj). In addition, these combinations were analysed for growth enhancing activity of human macrophage (TLT) cell line *in vitro* as a possible model of wound healing.

2. Materials and Methods

2.1 Samples

2.1.1 Species of Bacteria and Candida albicans Used in the Experiments

Different clinical isolates of Gram-negative bacteria: *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*,

Gram-positive bacteria: *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, *Streptococcus agalctiae* and fungus (yeast) *Candida albicans* were obtained from microbe collection of the Institute for Microbiology and Immunology, Medical Faculty in Ljubljana. All microbes were first cultivated on Mueller Hinton agar at 37 °C for 48 h. Afterward, they were transferred to Mueller Hinton broth, until the concentration of 0.5 McFarland was obtained.

2.1.2 TLT Cells

TLT cell line was obtained from Lidija Gradišnik (Institute for Biomedical Sciences, Medical Faculty in Maribor, University of Maribor). Cells were cultivated serially in the Eagle's minimal essential medium (EMEM) + 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin and gentamycin) in 75 cm² plastic flasks (Bio-One, BioScience, Greiner, Werneberg, Austria) for 3 d at 37 °C and 5% CO₂. In the cell growth experiments, TLT cells were put into 96 wells, flat bottom, plates (Bio-One, BioScience, Greiner, Werneberg, Austria).

2.1.3 WSP Greit120

The WSP Greit120, (BNatural, Corbetta, Italy) was used in dilution 1:10 w/v in saline.

2.1.4 RJ

F-RJ (M) (MEDEX d.o.o., Slovenia) was used in dilution 1:10 w/v in saline.

2.1.5 Honeys

Three different sorts of honeys were used, i.e., Bio-Forest (Plattner, Binenhof, der GRAMM, Gramm, Italy), Forest Italy (AlceNero SPA, Monterenzino, Bolzano) and Forest Spain (Delimet Foods SLU, Corbera, Barcelona) were used in dilution 1:10 w/v in saline.

2.1.6 Control Antibiotics/Antimycotics

In the experiments, the following antibiotics were used: 10% solution of penicillin in EMEM w/v, 10% solution of streptomycin in EMEM w/v and 10% solution of gentamycin in EMEM w/v. All antibiotics were resuspended in EMEM without FCS and

antibiotics. As control antimycotic, 10% solution of nystatin in EMEM w/v without FCS was used.

2.2 Methods

2.2.1 Scheme of the Experiments

The following combinations between F-RJ (M), WSP Greit120, Bio-Forest honey from Italy, Forest honey from Italy and Forest honey from Spain were used in the experiments: 10% solution of F-RJ (M) in saline (1:10 w/v), 10% solution of WSP Greit120 in saline (1:10 w/v), 10% solution of Bio-Forest honey from Italy in saline (1:10 w/v), 10% solution of Forest honey from Italy in saline (1:10 w/v), 10% solution of Forest honey from Spain in saline (1:10 w/v), 90% of Bio-Forest honey from Italy (1:10 w/v) + 10% of F-RJ (M) (1:10 w/v), 90% of Forest honey from Italy (1:10 w/v) + 10% F-RJ (M) (1:10), 90% of Forest honey from Spain (1:10 w/v) + 10% of F-RJ (M) (1:10 w/v), 90% of Bio-Forest honey from Italy (1:10 w/v) + 10% of WSP Greit120 (1:10 w/v), 90% of Forest honey from Italy (1:10 w/v) + 10% of WSP Greit120 (1:10 w/v), 90% of Forest honey from Spain (1:10 w/v) + 10% of WSP Greit120 (1:10 w/v).

2.2.2 Determination of the Minimal Inhibitory Concentration (MIC) in mg/mL

The MIC values determinations were performed according to Resazurin method [23-25] as follows. Here, 96 well "U" profile microtitre plates (8 × 12 wells) were used. Into the 2nd column (eight wells) until the 12th column, the 50 µL of saline was put. Into the 1st column (eight wells), 100 µL of samples were put as follows: in the first plate samples, in wells 1-4 and into wells 5-8 in the 1st column; in the 2nd plate samples, in wells 9-11 + untreated control in the 1st column; into the 3rd plate, penicillin, streptomycin, gentamycin and nystatin were put into wells in duplicate in the 1st column. Then, 50 µL was transferred from the 1st column into the 2nd, 3rd and until 11th, where 50 µL was discharged. Into each plate on each well, 50 µL of tested microorganism in the concentration of 0.5 McFarland was added, and the

plate was wrapped into aluminium foil and incubated for 48 h at 37 °C. After incubation finished, 20 µL of Resazurin (Sigma-Aldrich) (0.0028 g of Resazurin dissolved in 10 mL of distilled water, to which 90 mol of EMEM were added) and plates were wrapped into aluminium foil and incubated at room temperature for 4 h. The following is the growth inhibition of bacteria after the staining with Resazurin. The resazurin has blue colour. The absence of microbe's growth inhibition results in the change of the colour of Resazurin from blue to pink. MIC was determined as the highest dilution, resulting in no or the minimal change in colour. For example, in the 7th well, there is a change from pink to blue. So, colour change in the 7th well with dilution 1:64, MIC = $1 \times 10/64 = 0.156$ mg/mL (Table 1).

2.2.3 Percent of Bacteria/Fungi Growth Inhibition

The percent of bacterial/fungal growth inhibition was measured by the two-fold dilution of the substance, from the 1st to 11th well in the row, and then bacteria/fungi were added (0.5 McFarland). After 24 h of incubation at 37 °C, 20 µL of Resazurin was added. The plates were incubated and wrapped in aluminium foil for 4 h at room temperature. After the incubation, it was detected, in which well the growth inhibition occurred. The percent of bacterial/fungal growth inhibition was calculated as follows: if the growth inhibition was found in the 4th well, the percent of bacterial/fungal growth inhibition = $4/12 \times 100 = 33.33$ (Table 1).

2.2.4 Determination of TLT Cells Growth Index (GI)

The determination of TLT cells growth index (GI) and % of growth inhibition index (%GI-INH) measuring the influence of WSP or RJ in combination with different honeys on it, were performed by the method described by Miller et al. [26] and Khellaf and Zerdaoui [27]. At first, the TLT cells were cultivated in plastic flasks of 75 cm² (Bio-One, BioScience, Greiner, Werneberg, Austria) in EMEM + 10% FCS + antibiotics (penicillin, streptomycin, gentamycin) at

37 °C. When reached the confluence, the cells were split and resuspended into 90 mL of EMEM + 10% FCS + antibiotics (penicillin, streptomycin, gentamycin). Then, they were distributed into 96 flat well microtiter plates. Into each well, 100 µL of cell suspension was put. Plates were incubated for 24 h at 37 °C and 5% CO₂. On next day, the experimental samples (1.6 mL) in EMEM + 2% FCS + antibiotics (penicillin, streptomycin, gentamycin) were added into two columns ($8 \times 2 = 16$ wells \times 100 µL = 1.6 mL). On the separate plate, there were two columns (8×2 wells) with cells, which were immediately fixed with 2.5% glutaraldehyde (initial number of cells). Plates with experimental samples were incubated for 3 d and 5 d at 37 °C and 5% CO₂, and then fixed with 2.5% glutaraldehyde for 15 min at room temperature, washed with phosphate buffered saline (PBS) pH = 7.2, and stained with crystal violet. After 15 min, the stain was removed, and cells were washed with PBS and air-dried. Dried cells were measured in the spectrometer for microtiter plates at 595 nm. Then the GI values at 3 d and 5 d were calculated as Eqs. (1) and (2):

$$GI_3 = \text{No. of cells after 3 d} / \text{initial number of cells} \quad (1)$$

$$GI_5 = \text{No. of cells after 5 d} / \text{initial number of cells} \quad (2)$$

2.3 Statistical Analysis

The statistical significance of differences in susceptibilities was calculated using the two-tailed Student's *t*-test. In order to compare the difference in susceptibility between honey alone and honey with the addition of RJ or propolis, MIC for different honey samples for individual bacteria were pooled.

3. Results and Discussion

3.1 Antimicrobial Activity of F-RJ (M) or WSP Greit120 and Forest Honeys

3.1.1 The Antimicrobial Activity of F-RJ (M)

In the performed experiments, the antimicrobial activity of 10% solution of F-RJ (M) in saline was

Table 1 Antimicrobial and percent of growth index inhibition activity of F-RJ (M), WSP Greit120 and Forest honeys from Italy and Spain.

Samples	Gram-positive bacteria								Gram-negative bacteria								Yeast	
	MRSA		<i>S. aureus</i>		<i>S. pyogenes</i>		<i>S. agalactiae</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>P. mirabilis</i>		<i>A. baumannii</i>		<i>C. albicans</i>	
	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)
F-RJ (M) (1:10)	2.500 ± 0.260	25.00	2.500 ± 0.260	25.00	1.250 ± 0.070	33.33	1.250 ± 0.070	33.33	2.500 ± 0.260	25.00	2.500 ± 0.260	25.00	1.250 ± 0.070	33.33	0.625 ± 0.016	41.66	2.500 ± 0.260	25.00
WSP Greit120 (1:10)	1.250 ± 0.070	33.33	2.500 ± 0.260	25.00	1.250 ± 0.070	33.33	1.250 ± 0.070	33.33	0.625 ± 0.016	41.66	0.625 ± 0.016	41.66	0.312 ± 0.04	50.00	0.312 ± 0.040	50.00	2.500 ± 0.260	25.00
Bio-Forest Italy (1:10)	0.312 ± 0.040	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66
Forest-Italy (1:10)	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00	0.078 ± 0.080	66.66	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.625 ± 0.016	41.66	0.078 ± 0.008	66.66	0.312 ± 0.040	50.00
Forest-Spain (1:10)	0.312 ± 0.070	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00
Penicillin (1:10)	5.000 ± 0.016	16.66	2.500 ± 0.260	25.00	0.312 ± 0.040	50.00	2.500 ± 0.260	25.00	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00		
Streptomycin (1:10)	0.019 ± 0.004	83.33	0.009 ± 0.004	91.66	0.019 ± 0.004	83.33	0.009 ± 0.004	91.66	0.039 ± 0.008	75.00	0.009 ± 0.004	91.66	0.009 ± 0.004	91.66	0.009 ± 0.004	91.66		
Gentamycin (1:10)	0.019 ± 0.004	83.33	0.009 ± 0.004	91.66	0.039 ± 0.008	75.00	0.009 ± 0.004	91.66	0.078 ± 0.008	66.66	0.009 ± 0.004	91.66	0.009 ± 0.004	91.66	0.019 ± 0.004	83.33		
Nystatin (1:10)																	0.019 ± 0.004	83.33

GI-INH (%): % of growth index inhibition; MIC: minimal inhibitory concentration, expressed in mean ± standard deviation.

analysed and presented in Table 1. It shows relatively weak antimicrobial activity: MIC for Gram-positive bacteria lies between 1.25 mg/mL and 2.5 mg/mL. The bests are *S. pyogenes* and *S. agalactiae* with MIC = 1.25 ± 0.07 mg/mL and 33.33% of bacterial growth inhibition. For *C. albicans*, the MIC was 2.5 mg/mL, and percentage of growth inhibition was 25.00%. For Gram-negative bacteria, the MIC value is between 0.625 mg/mL and 2.5 mg/mL. The best was *A. baumannii* with value for MIC 0.625 ± 0.016 mg/mL and percentage of growth inhibition 41.66% (Table 1).

3.1.2 The Antimicrobial Activity of WSP Greit120

Recently, WSP became important because of its antitumor activity. Besides, WSP shows also an antimicrobial activity, which is presented in Table 1. For the Gram-positive bacteria, the range of MIC is between 1.25 mg/mL and 2.5 mg/mL. For *C. albicans*, the MIC is 2.5 mg/mL. Against Gram-negative bacteria, MIC of WSP lies between 0.312 mg/mL and 0.625 mg/mL. The best are *P. mirabilis* and *A. baumannii* with the MIC 0.312 ± 0.04 mg/mL and percentage of growth inhibition of 50.00%. It is quite unusual that the antimicrobial activity of 10% WSP Greit120 is stronger against Gram-negative than against Gram-positive bacteria and *C. albicans*, because the antibacterial activity of propolis is usually stronger against Gram-positive bacteria and *C. albicans*.

3.1.3 Antimicrobial Activity of Honeydew (Forest) Honeys

The two Italian honeys (Bio-Forest honey and Forest honey) and one Spain Forest honey were tested as shown in Table 1. In concentration of 10% in saline, the antibacterial activity of Italian Forest honey was stronger for Gram-positive bacteria, MIC = 0.078-0.312 mg/mL, the best was *S. pyogenes* with MIC = 0.078 ± 0.08 mg/mL and percentage of growth inhibition was 66.66%; while for Gram-negative bacteria, MIC = 0.078-0.625 mg/mL, the best was *A. baumannii* with the MIC = 0.078 ± 0.08 mg/mL and percentage of growth inhibition 66.66%. The MIC for

yeast *C. albicans* was 0.312 mg/mL. The Bio-Forest honey was surprisingly weaker. The MIC for Gram-positive bacteria was in a range of 0.156-0.312 mg/mL, and for *C. albicans* was 0.625 mg/mL. For Gram-negative bacteria, the range was between 0.156 mg/mL and 0.625 mg/mL. It is interesting that the data obtained from the tests of Forest honey from Spain show the range of MIC = 0.156-0.312 mg/mL for Gram-positive bacteria and MIC = 0.312 mg/mL for *C. albicans*. The MIC range for Gram-negative bacteria was between 0.156 mg/mL and 0.625 mg/mL. Both Forest honeys were better than Bio-Forest honey from Italy. It can be provisionally concluded that the composition of the honey depend on plant composition due to geographical differences.

3.1.4 Enhancing Effect of F-RJ (M) on Antimicrobial Activity of Honeydew (Forest) Honeys

When the F-RJ (M) was combined with the Bio-Forest honey (Italy), Forest Honey (Italy) or Forest honey from Spain, the best antibacterial activity was found in case of Forest honey from Spain, where the MIC range for Gram-positive bacteria was between 0.039 mg/mL and 0.156 mg/mL with percent of growth inhibition of 75%. For *C. albicans*, the MIC was 0.156 ± 0.04 mg/mL and 58.33% of growth inhibition. In case of Gram-negative bacteria, the range of MIC was between 0.078 mg/mL and 0.156 mg/mL with percentage of growth inhibition of 66.66%. The statistical evaluation of the effect of F-RJ (M) does not confirm the fold decrease from median of the enhancement of the antibacterial activity of Bio-Forest honey (Italy), Forest Honey from Italy or Forest from Spain (Table 2).

3.1.5 Enhancing Effect of WSP Greit120 on Antimicrobial Activity of Honeydew (Forest) Honeys

When the combination of the WSP Greit120 with Bio-Forest honey, Forest honey from Italy and Forest honey from Spain, the best antibacterial activity was found in Forest honey from Spain, where the range of MIC for Gram-positive bacteria was between 0.009 mg/mL and 0.156 mg/mL. The maximal percentage of

Table 2 Statistical evaluation of the influence of F-RJ (M) on the antimicrobial activity of Forest honey from Italy and Spain.

Samples	Gram-positive bacteria								Gram-negative bacteria								Yeast	
	MRSA		<i>S. aureus</i>		<i>S. pyogenes</i>		<i>S. agalactiae</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>P. mirabilis</i>		<i>A. baumannii</i>		<i>C. albicans</i>	
	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)
Bio-Forest Italy (1:10)	0.312 ± 0.040	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66
Forest-Italy (1:10)	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00	0.078 ± 0.080	66.66	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.625 ± 0.016	41.66	0.078 ± 0.008	66.66	0.312 ± 0.040	50.00
Forest-Spain (1:10)	0.312 ± 0.070	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66	0.312 ± 0.07	50.00	0.625 ± 0.016	41.66	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00
Median	0.312		0.312		0.156		0.156		0.312		0.625		0.625		0.156		0.312	
90% Bio-Forest Italy (1:10) + 10% F-RJ (M) (1:10)	0.625 ± 0.016	41.66	0.312 ± 0.040	50.00	0.625 ± 0.016	41.66	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.156 ± 0.040	58.33	0.078 ± 0.008	66.66	0.625 ± 0.016	41.66
90% Forest-Italy (1:10) + 10% F-RJ (M) (1:10)	0.312 ± 0.070	50.00	0.039 ± 0.008	75.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66	0.312 ± 0.070	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00
90% Forest-Spain (1:10) + 10% F-RJ (M) (1:10)	0.039 ± 0.008	75.00	0.039 ± 0.008	75.00	0.078 ± 0.008	66.66	0.156 ± 0.040	58.33	0.078 ± 0.008	66.66	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33
Median	0.312		0.039		0.312		0.156		0.156		0.312		0.156		0.156		0.312	
Fold decrease (from median)	1		8		1		1		2		2		4		1		1	
<i>t</i> test	0.731		0.116		0.264		0.371		0.551		0.089		0.055		1.00		1.00	

GI-INH (%): % of growth index inhibition; MIC: minimal inhibitory concentration, expressed in mean ± standard deviation.

Table 3 Statistical evaluation of the influence of WSP Greit120 on the antimicrobial activity of Forest honeys from Italy and Spain.

Samples	Gram-positive bacteria								Gram-negative bacteria								Yeast	
	MRSA		<i>S. aureus</i>		<i>S. pyogenes</i>		<i>S. agalactiae</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>P. mirabilis</i>		<i>A. baumannii</i>		<i>C. albicans</i>	
	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)	MIC (mg/mL)	GI-INH (%)
Bio-Forest Italy (1:10)	0.312 ± 0.040	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66
Forest-Italy (1:10) (4)	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00	0.078 ± 0.080	66.66	0.156 ± 0.040	58.33	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.625 ± 0.016	41.66	0.078 ± 0.008	66.66	0.312 ± 0.040	50.00
Forest-Spain (1:10) (5)	0.312 ± 0.070	50.00	0.312 ± 0.040	50.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66	0.312 ± 0.070	50.00	0.625 ± 0.016	41.66	0.156 ± 0.040	58.33	0.312 ± 0.040	50.00
Median	0.312		0.312		0.156		0.156		0.312		0.625		0.625		0.156		0.312	
90% Bio-Forest, Italy (1:10) + 10% WSP Greit120 (1:10)	0.039 ± 0.004	75.00	0.156 ± 0.040	58.33	0.039 ± 0.004	75.00	0.078 ± 0.008	66.66	0.039 ± 0.008	75.00	0.156 ± 0.040	58.33	0.156 ± 0.040	58.33	0.078 ± 0.008	66.66	0.039 ± 0.008	75.00
90% Forest-Italy (1:10) + 10% WSP Greit120 (1:10)	0.019 ± 0.004	83.33	0.039 ± 0.008	75.00	0.019 ± 0.003	83.33	0.009 ± 0.004	91.66	0.039 ± 0.008	75.00	0.019 ± 0.004	83.33	0.078 ± 0.008	66.66	0.019 ± 0.004	83.33	0.019 ± 0.004	83.33
90% Forest-Spain (1:10) + 10% WSP Greit120 (1:10)	0.039 ± 0.008	75.00	0.156 ± 0.040	58.33	0.009 ± 0.004	91.66	0.039 ± 0.008	75.00	0.019 ± 0.004	83.33	0.039 ± 0.008	75.00	0.078 ± 0.008	66.66	0.078 ± 0.008	66.66	0.009 ± 0.004	91.66
Median	0.039*		0.156*		0.019*		0.039*		0.039*		0.039*		0.078*		0.078		0.019*	
Fold decrease (from median)	8		2		8		4		8		16		8		2		16	
<i>t</i> test	0.012		0.007		0.017		0.005		0.021		0.016		0.018		0.093		0.020	

GI-INH (%): % of growth index inhibition; MIC: minimal inhibitory concentration, expressed in mean ± standard deviation.

* Statistical significant difference at $P < 0.05$ compared to pooled Forest honey sample.

bacterial growth inhibition was 91.66%. The same MIC (0.009 ± 0.004 mg/mL) was found at *C. albicans*. When the Gram-negative bacteria were studied, the range of MIC was between 0.019 mg/mL and 0.078 mg/mL and the percentage of bacterial growth inhibition was 83.33%. The statistical evaluation of the effect of the WSP Greit120 on the fold decrease from median and *t*-test of the enhancement of the antibacterial/antifungal activity of Bio-Forest honey and Forest honey from Italy or Forest honey from Spain was performed (Table 3). There were more significant differences in MIC especially in case of *P. aeruginosa* and *C. albicans* with fold decrease of value for MIC 16 \times , and the least in case of *S. aureus* and *A. baumannii* with fold decrease of value for MIC 2 \times (Fig. 1).

3.2 The Growth Promoting Activity of TLT Cell Line by the Combination of F-RJ (M) or WSP Greit120 with Honeydew (Forest) Honeys

To measure the growth promoting activity of TLT

cell line, the combinations of RJ or WSP with different Honeydew (Forest) honeys were used. As the measure, the level of GI was used. The highest level of growth promoting activity ($GI_3 = 0.796$ after 3 d and $GI_5 = 1.133$ after 5 d) was found, when WSP added to the Honeydew (Forest) honey from Italy. The Honeydew (Forest) honey from Italy alone or in combination with F-RJ (M), both had lower growth promoting activity ($GI_3 = 0.506$, $GI_5 = 0.996$ and $GI_3 = 0.279$, $GI_5 = 0.501$, respectively) than in combination with propolis (Fig. 2).

RJ was demonstrated to pose different physiological activities, like growth of human monocytes [28, 29]. The proteins and peptides from RJ can participate in the direct antimicrobial action or indirectly by the induction of different cytokines. The most abundant is peptide albumin 1 that showed the total inhibition of *S. aureus*, *Listeria monocytogenes* and *Salmonella typhimurium* in concentration about 200 μ g/mL [30]. Weak antimicrobial activity of RJ is probably consequence of its low concentration.

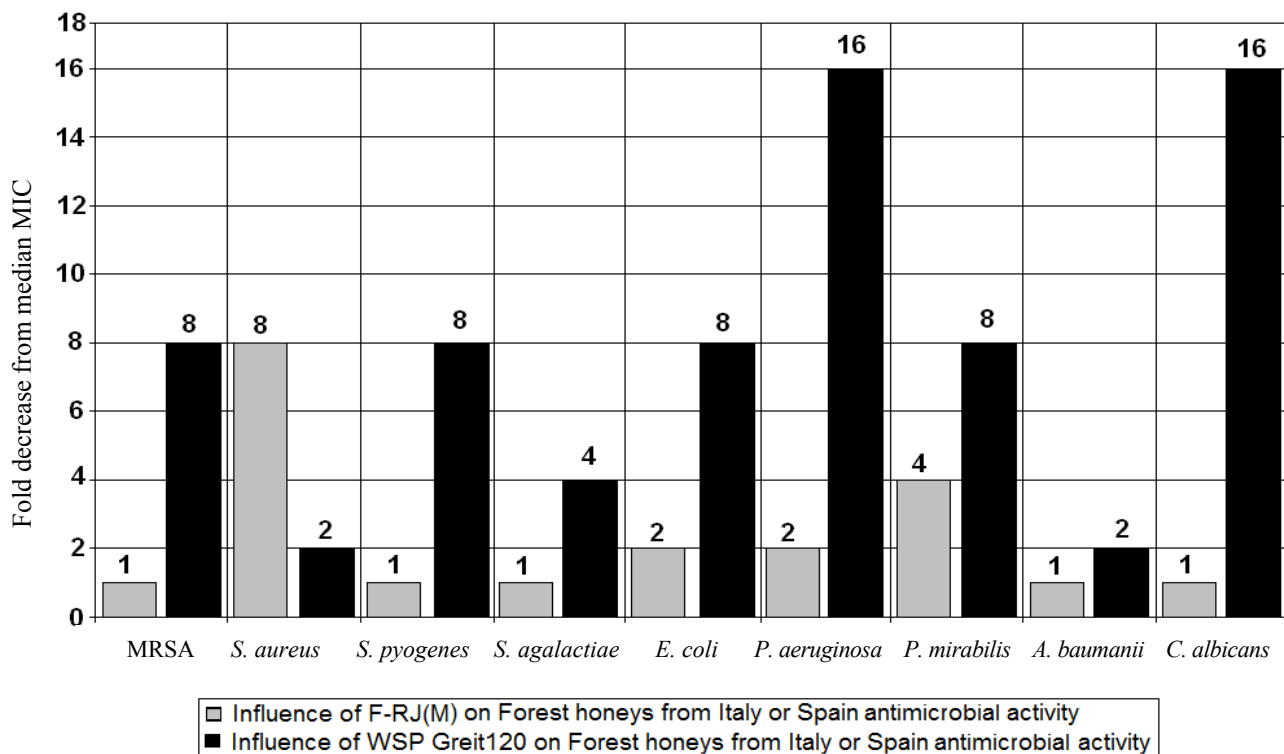


Fig. 1 Influence of F-RJ (M) or WSP Greit120 on the antimicrobial activity of Forest honeys from Italy or Spain.

**Water Soluble Propolis and Royal Jelly Enhance the Antimicrobial Activity of honeys
and Promote the Growth of Human Macrophage Cell Line**

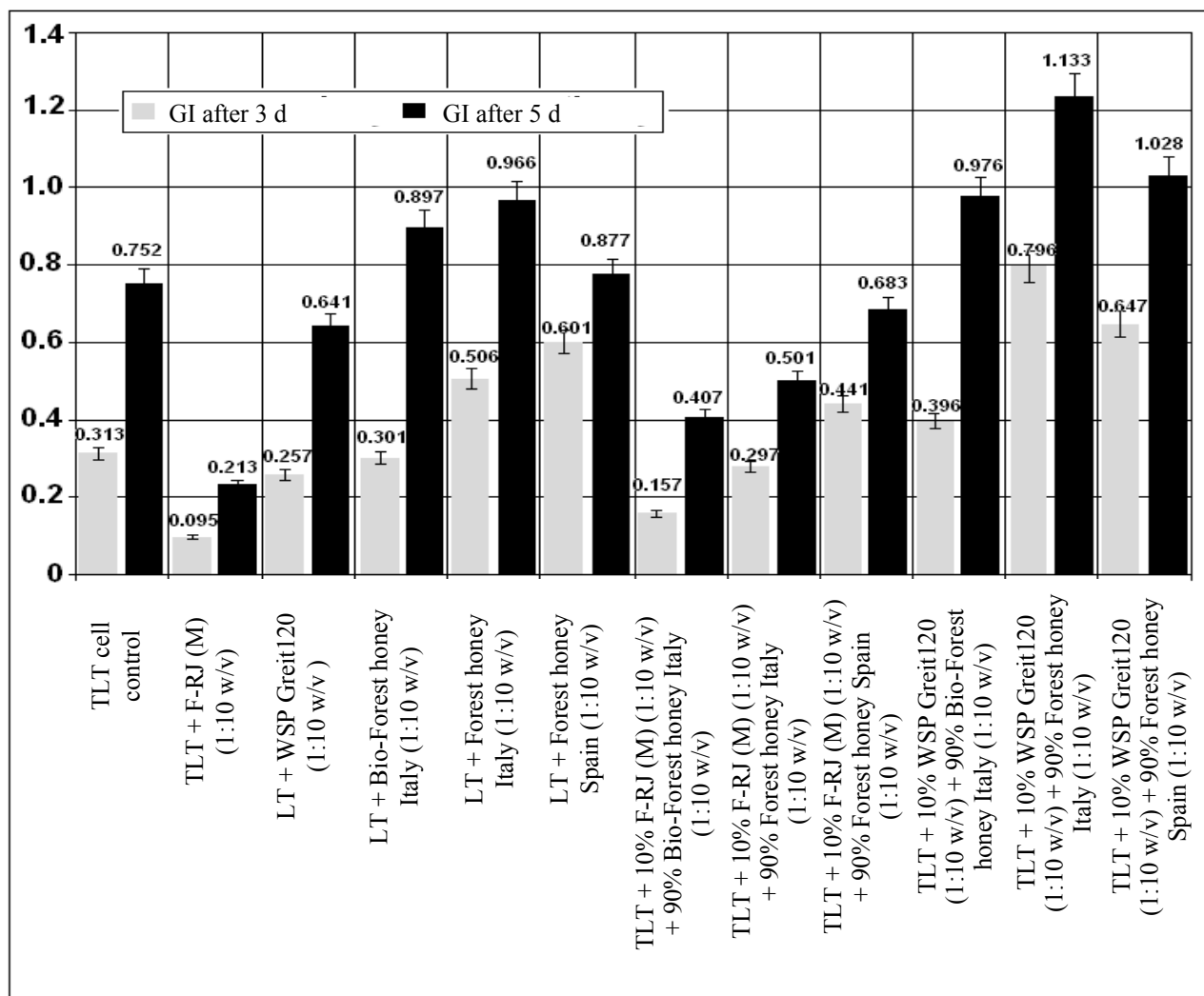


Fig. 2 Effect of the combinations of Forest honeys with F-RJ (M) and WSP Greit120 on the growth of TLT cell line.

There are two types of propolis extracts—ethanol and water. Recently, the water extract became important, especially because it can kill the tumour cells and originate the growth of remaining normal (no transformed) cells, despite it allows having quite low antimicrobial growth [31, 32]. Predominantly identified compounds in WSP Greit120 were phenolic acids, which contribute about 40% of total radical scavenging activity. This extract inhibits the growth and reproduction of all tested microorganisms. Antimicrobial activity of some extracts was equal or exceeded the antimicrobial effect of ethanol extract. Extracts made in pure water or oil only at room temperature, contained more than 5-10 fold lower amount of phenolic compounds, and demonstrated

weak or no antimicrobial activity [33]. The honey is a natural remedy for the treatment among other of infected wounds. The results indicate that Honeydew (Forest) honey has high *in vitro* activity against Gram-positive and Gram-negative bacteria. In general, Gram-positive bacteria were more susceptible to Forest honeys than Gram-negative bacteria [34]. Some Italian honeys of different floral origin and one Honeydew (Forest) were investigated for their potential as natural antimicrobials against different pathogens commonly associated with wound or burn infections. *S. aureus*, *Staphylococcus epidermidis*, *E. coli*, *P. aeruginosa* and *P. mirabilis* were used as test microorganisms [35]. Similarly, it was found for Spanish Forest honeys [36]. For both types of

honeys—Italian and Spanish Forest honeys, it was found to rapidly clear the infection in the wound, and promote and clean healthy granulation tissue. The antimicrobial activity of the combination of RJ and honey was studied [37, 38], and the additive activity of RJ and honey against *P. aeruginosa* was found, as comparison with the experiments in this study, where the water soluble F-RJ (M) did not affect the Forest honey in term of statistically stronger antimicrobial activity. Al-Waili et al. [39] also analysed the antimicrobial activity of the combination of ethanol extract of propolis and honey from Saudi Arabia, and found the synergism between the ethanol extract of Saudi propolis or Egyptian propolis with honey from Saudi Arabia in term of killing of MRSA, *E. coli* and *C. albicans*. These data are comparable with this study, despite the WSP Greit120 was used in combination with Forest honey. Therefore, it seems that the geographical origin of propolis and honeys have an important role.

The TLT cell line represents a useful model system for granulation tissue in wounds. The proliferation increase of TLT cells can mimic the operation of the immune system, more precisely the proliferation of phagocytes and B- and T-lymphocytes. WSP Greit120 enhanced the antimicrobial activity of Forest honey [40, 41]; while on other side, can enhance proliferation of phagocytes, B- and T-lymphocytes in a concentration up to 10%. In this way, the WSP Greit120 enhancement of antimicrobial activity of Forest honey could be connected with the proliferation of phagocytes and B- and T-lymphocytes. The WSP Greit120 in combination with Forest honey represents a tool of value for further research connected with wounds, because of the inhibition of *P. aeruginosa*, *A. baumannii*, *S. aureus* and MRSA, the important pathogens of the wound.

4. Conclusions

The F-RJ (M) combined with the Bio-Forest honey (Italy), Forest Honey (Italy) or Forest honey from

Spain enhances antibacterial activity against Gram-positive bacteria with fold decrease from median MIC for MRSA (1×), *S. aureus* (8×), *S. pyogenes* (1×) and *S. agalactiae* (1×). For Gram-negative bacteria, the fold decrease from median MIC were found for *E. coli* (2×), *P. aeruginosa* (2×), *P. mirabilis* (4×), *A. baumannii* (1×) and yeast *C. albicans* (1×). Unfortunately decrease from median MIC was not statistical significant (*t* test).

The WSP Greit120 somehow enhances the Forest honey's antimicrobial activity against Gram-positive bacteria with fold decrease from median MIC of Forest honeys for MRSA (8×), *S. aureus* (2×), *S. pyogenes* (8×), and *S. agalactiae* (4×). When Gram-negative bacteria were analysed, the fold decrease from median MIC of Forest honeys were found for *E. coli* (8×), *P. aeruginosa* (16×), *P. mirabilis* (8×), *A. baumannii* (2×) and yeast *C. albicans* (16×). The WSP Greit120 in combination with Forest honey on other site stimulates the monocytes/macrophages, according to the data of TLT cells, to release cytokines important for wound healing, together with the activation of the macrophage and neutrophils and protecting the healing tissue from pathogenic infection.

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