Arch. Biol. Sci., Belgrade, 64 (3), 927-934, 2012 DOI:10.2298/ABS1203927K

THE ANTIMICROBIAL ACTIVITY OF HONEY, BEE POLLEN LOADS AND BEESWAX FROM SLOVAKIA

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Abstract - The aim of this study was to test the antimicrobial activity of propolis, bee pollen loads and beeswax samples collected in the year 2009 from two locations in Slovakia to pathogenic bacteria, microscopic fungi and yeasts. The antimicrobial effect of the bee product samples were tested using the agar well diffusion method. For extraction, 99.9% and 70% methanol (aqueous, v/v) and 96% and 70% ethanol (aqueous, v/v) were used. Five different strains of bacteria, i.e. *Listeria monocytogenes* CCM 4699, *Pseudomonas aeruginosa* CCM 1960; *Staphylococcus aureus* CCM 3953; *Salmonella enterica* CCM 4420, *Escherichia coli* CCM 3988, three different strains of microscopic fungi, *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger,* and seven different strains of yeasts *Candida krusei*, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Geotrichum candidum*, *Rhodotorula mucilaginosa*, were tested. After 48 hours *S. aureus* was the bacterium most sensitive to the 70% ethanol extract of pollen, *A. fumigatus* was the most sensitive microscopic fungus (70% ethanol) and *C. glabrata* the most sensitive yeast (70% methanol). Microorganisms most sensitive to propolis extracts were *L. monocytogenes*, *A. fumigatus* (70% ethanol) and *G. candidum* (70% methanol). Most sensitive to beeswax extracts were *E. coli*, *A. niger* and *C. tropicalis*.

Key words: Antimicrobial activity, bee products, propolis, bee pollen, beeswax, pathogenic bacteria, microscopic fungi, yeasts

INTRODUCTION

Beekeeping is an important component of agriculture, rural employment, human nutrition and economic development (Molan, 1999). Propolis (bee glue) is a resinous or sometimes wax-like beehive product that has been used by man since ancient times for its pharmaceutical properties (Walker, 1987). It is still used as a remedy in folk medicine (Kujungjev, 1999) as a constituent of 'bio-cosmetics', 'health foods' and for numerous other purposes (Wollenweber, 1997). Bees use this material to seal hive walls and entrances, to strengthen the border

of the combs, and embalm dead invaders. The antibacterial and antifungal activities are the most popular and among the most extensively investigated biological actions of propolis (Marcucci, 1995). The chemical composition of propolis is extremely complex, containing more than 150 components such as flavonoids, phenolic acids and their esters, alcohols, ketones, amino acids, and inorganic compounds (Hagazi, 2000; Banskota, 2001b; Marcucci, 2001; Bankova, 2005).

Pollen is a fine, powder-like material produced by flowering plants and gathered by bees. A pollen grain contains the male gametophyte. Pollen is the bee's primary food source, being rich in nutrients and phytochemicals such as carotenoids, flavonoids and phytosterols (Broadhurst, 1999). Bee-collected pollen and pollen products have been successfully used for the treatment of benign prostatitis and for oral desensitization of children who have an allergy (Campos, 1997; Mizrahi, 1997). In addition, bee pollen has antimicrobial effects (Haas, 1992).

The honeybee's wax has an extremely wide spectrum of useful applications and occupies a very special position among waxes of plant and animal origin. The major part of beeswax produced is used for technical purposes, candles, modeling, polishes, etc.), but is also utilized in cosmetics, food processing (food packaging, processing and preservation – natural food additive E 901) and medicine (coating pills, antibiotic properties) (Krell, 1996).

The aim of this study was to investigate the antimicrobial activities of propolis, bee pollen and beeswax samples from different locations in Slovakia against five strains of pathogenic bacteria, three strains of microscopic fungi and seven strains of yeasts.

MATERIALS AND METHODS

For each bee product, one sample was prepared by mixing several partial samples. Bee product samples were obtained from 20 cultivated honeybee hives placed in 2 localities in the central part of Slovakia (Detva region). Partial samples were collected several times during the period of April to August in the year 2009. Bee pollen (P) samples containing mainly monofloral pollen loads from sunflower (*Helianthus annuus*), poppy (*Papaver somniferum*) and rape (*Brassica napus)* were stored frozen. Propolis (Pr) samples were frozen and subsequently milled to powder. Beeswax samples from combs 1-2 years old were cut into small pieces and used for extraction. Bee products (10g) were extracted in 80 mL of solvent. Four different solvents were used: 99.9 and 70% (v/v) aqueous methanol, designated MEh and MEl, respectively, and 96 and 70% (v/v) aqueous ethanol, designated Eh and El, respectively. MEl und El were acidified with hydrochloric acid to pH 1.5 and 2, respectively. Samples were extracted at 80°C under reflux for 1 h. The following extracts were used:

1. PMEh – pollen 99.9 % methanolic extract,
2. PMEl – pollen 70.0% methanolic extract,
3. PEEh – pollen 96.0% ethanolic extract,
4. PEEl – pollen 70.0% ethanolic extract
5. PrMEh – propolis 99.9 % methanolic extract,
6. PrMEl – propolis 70.0% methanolic extract,
7. PrEEh – propolis 96.0% ethanolic extract,
8. PrEEl – propolis 70.0% ethanolic extract,
9. WMEh – beeswax 99.9 % methanolic extract,
10. WMEl – beeswax 70.0% methanolic extract,
11. WEEh – beeswax 96.0% ethanolic extract,
12. WEEl – beeswax 70.0% ethanolic extract.

After chilling, the mixture was centrifuged and the solvent of the supernatant evaporated under re-

duced pressure at 40-45°C. The residue was dissolved in 160 mL of solvent mixture ethyl acetate (1:1, v/v) and shaken for 30 min. The organic (ethyl acetate) phase was separated, the solvent evaporated, and the residue dissolved in 10 mL 99.9% methanol.

The bacterial strains were purchased from the Czech Collection of Microorganisms (CCM). Microscopic fungi and yeasts, all clinical isolates, were provided by the University Hospital in Martin (Slovak Republic).

The antimicrobial effects of the extracts were tested using the agar well diffusion method in Mueller-Hinton agar (MHA) for bacteria and Sabouraud agar (SA) for microscopic fungi and yeasts. After 30 min of initial drying, agar plates were inoculated with 200 μL of microorganism suspension at a density of 10^7 CFU mL^{-1} in saline solution and spread on the surface. Subsequently, four equidistant wells, 9 mm in diameter each, were punched into the inoculated medium with sterile glass. Bacteria were incubated at 37°C and fungi at 25°C. Inhibition zones in mm around the disks were measured after 48 h of cultivation. As positive controls for bacteria, chloramphenicol was used, for fungi 40% phenol solvent and 99.9% methanol was evaluated for comparison. Five different strains of bacteria; two Gram-positive strains (*Listeria monocytogenes* CCM 4699; *Staphylococcus aureus* CCM 3953) and three Gram-negative strains (*Pseudomonas aeruginosa* CCM 1960; *Salmonella enterica* CCM 4420; *Escherichia coli* CCM 3988), three different strains of microscopic fungi (*Aspergillus fumigatus*; *Aspergillus flavus*; *Aspergillus niger*), and seven different strains of yeasts (*Candida krusei*; *Candida albicans*; *Candida glabrata*; *Candida parapsilosis*; *Candida tropicalis*; *Geotrichum candidum*; *Rhodotorula mucilaginosa*) were tested in sets of plates, which were simultaneously processed for each strain. All the experiments were repeated twice, including a control with chloramphenicol. After incubation, the zones of growth inhibition of the bacteria, microscopic fungi and yeasts around the disks were measured. The mean values of three trials and standard deviations were calculated.

RESULTS AND DISCUSSION

Bee pollen loads

The antibacterial activities of the pollen extract *in vitro* test against different Gram-positive and negative pathogenic bacteria, microscopic fungi and yeasts are shown in Table 1. According to analysis among the tested bacteria, *Escherichia coli* CCM 3988 was the most sensitive during 48 h of PMEh, and the sensitivity of the bacteria decreased as follows: *Staphylococcus aureus* CCM 3953 > *Salmonella enterica* CCM 4420 > *Pseudomonas aeruginosa* CCM 1960 > *Listeria monocytogenes* CCM 4699. *Escherichia coli* CCM 3988 was the most sensitive also in PMEl. *Staphylococcus aureus* CCM 3953 was the most sensitive in PEEh (96.0 %). *Escherichia coli* CCM 3988 was the most sensitive using PEEl.

Aspergillus fumigatus (2.00±2.65) mm was the most sensitive during 48 h with PMEh and the sensitivity of the microscopic fungi decreased as follows: *Aspergillus niger* > *Aspergillus flavus*. *Aspergillus niger* was the most sensitive in PMEl, *Aspergillus fumigatus* (4.17±1.44) mm was the most sensitive in PEEl. According to analysis among the tested yeasts, *Candida albicans* (2.17±0.29) mm was the most sensitive in PMEh, *Candida glabrata* in PMEl, *Candida krusei* in PEEh, *Rhodotorula mucilaginosa* in PEEl. The control with chloramphenicol proved that the methanol and phenol used in the extractions did not have inhibiting action.

Different patterns of sensitivity in pollen loads are due to different phenolic compounds in pollen, as shown in the studies of Almeida-Muradian et al. (2005) and Carpes et al. (2007). In these studies, the antioxidant activity, phenolic content and antibacterial activity of pollen extracts obtained with different concentrations of ethanol were compared among pollen samples. The best extraction conditions, relating to biological properties, were PEE at 70% ethanol solution. As different extracts exhibited different antioxidant and antibacterial activities, there may be different kinds of phenolic content in different pollen extracts. According to the results obtained in these

Table 1 Inhibitory effects of pollen extracts against the pathogenic bacteria, molds and yeasts (inhibition zone diameter in mm). **PMEh** – pollen 99.9% methanolic extract; **PMEl** – pollen 70.0% methanolic extract; **PEEh** – pollen 96.0% ethanolic extract; **PEEl** – pollen 70.0% ethanolic extract; **Cm** – Chloramphenicol

Microorganisms	Controls		Extracts			
	Methanol 99.9%	Cm or Phenol 40.0%	PMEh	PMEl	PEEh	PEEl
Listeria monocytogenes CCM 4699	<1.00	10.66±1.15	1.67±1.04	2.67±1.15	2.00±0.87	2.33±0.58
Pseudomonas aeruginosa CCM 1960	<1.00	7.00±0.00	1.67±0.58	1.00±1.00	1.00±1.00	1.17±1.26
Staphylococcus aureus CCM 3953	<1.00	10.00±0.00	1.67±0.58	2.33±0.58	2.17±0.29	1.67±1.53
Salmonella enterica CCM 4420	<1.00	10.67±1.15	2.00±0.00	2.67±1.53	2.17±0.76	1.67±0.29
Escherichia coli CCM 3988	<1.00	12.33±2.08	2.33±0.00	2.67±1.53	1.83±0.29	3.00±0.00
Aspergillus fumigatus	<1.00	10.33±9.29	2.00±2.65	1.00±1.00	2.67±0.58	4.17±1.44
Aspergillus flavus	<1.00	15.33±4.51	1.00±1.00	1.67±2.08	0.33±0.58	1.00±1.00
Aspergillus niger	<1.00	17.67±4.04	1.67±1.53	2.00±1.73	1.67±1.53	2.67±1.15
Candida krusei	<1.00	8.33±4.73	1.00±1.00	2.67±1.15	2.67±1.15	1.00±1.00
Candida albicans	<1.00	12.33±0.58	2.17±0.29	3.00±1.73	1.67±0.58	2.50±0.50
Candida glabrata	<1.00	11.33±1.15	2.00±1.00	3.50±1.32	1.33±0.58	2.50±0.50
Candida parapsilosis	<1.00	14.33±0.58	1.83±0.29	2.50±1.32	1.67±1.15	2.00±1.00
Candida tropicalis	<1.00	9.67±2.52	2.00±0.00	2.67±0.58	1.67±1.15	2.00±1.00
Geotrichum candidum	<1.00	12.67±4.04	0.67±1.15	1.67±0.58	1.00±1.00	2.67±2.89
Rhodotorula mucilaginosa	<1.00	17.33±3.06	1.33±0.58	2.83±0.76	2.00±1.00	3.00±1.00

studies as well as in our study, pollen seems to have interesting biological properties, and can be considered as a functional food. Due to the great biodiversity of pollen-producing plants, more studies are necessary for a better understanding of the functional properties of pollen.

Propolis

The antibacterial activities of the propolis extracts *in vitro* test against different Gram-positive and negative pathogenic bacteria, microscopic fungi and yeasts are shown in Table 2. The inhibition zones varied among the propolis methanolic extracts (PrME) and propolis ethanolic extract (PrEE). *Listeria monocytogenes* CCM 4699 was the most sensitive in PrMEl, *Staphylococcus aureus* CCM 3953 in PrEEl and *Listeria monocytogenes* CCM 4699 in PrEEl. According to analysis among the tested microscopic fungi, *Aspergillus fumigatus* (3.33±0.58) mm was the most sensitive during 48 of PrMEh, and the sensitivity of the microscopic fungi decreased: *Aspergillus niger* > *Aspergillus flavus*. *Aspergillus fumigatus* has the same results in the same extracts during 48 h. According to analysis among the tested yeasts, *Candida albicans* (4.67±2.52)mm was the most sensitive during 48 h of PrMEh, *Geotrichum candidum* in PrMEl, *Candida albicans* in PrEEh, and *Candida krusei* in PrEEl. The controls (methanol, phenol) showed an inhibitory effect on none of the tested bacteria.

A number of studies have presented evidence that propolis has strong antimicrobial properties (Ozcan, 2000; Banskota, 2001; Sforcin, 2007; Viuda-Martos, 2008). Bankova et al. (1995) examined the activity of different fractions of Brazilian prop-

Table 2 Inhibitory effects of propolis extracts against the pathogenic bacteria, molds and yeasts (inhibition zone diameter in mm)
PrMEh – propolis 99.9% methanolic extract; **PrMEl** – propolis 70.0% methanolic extract; **PrEEh** – propolis 96.0% ethanolic extract; **PrEEl** – propolis 70% ethanolic extract; **Cm** - Chloramphenicol

Microorganisms	Controls		Extracts			
	Methanol 99.9%	Cm or Phenol 40.0%	PrMEh	PrMEl	PrEEh	PrEEl
Listeria monocytogenes CCM 4699	<1.00	10.66±1.15	4.00±1.00	6.00±1.00	4.33±0.58	6.33±2.08
Pseudomonas aeruginosa CCM 1960	<1.00	7.00±0.00	1.33±1.15	2.66±0.58	1.67±2.89	3.67±3.52
Staphylococcus aureus CCM 3953	<1.00	10.00±0.00	3.33±1.53	5.67±1.53	5.67±2.08	6.00±2.65
Salmonella enterica CCM 4420	<1.00	10.67±1.15	2.33±2.52	3.83±1.26	2.50±2.50	5.00±1.00
Escherichia coli CCM 3988	<1.00	12.33±2.08	2.33±2.52	3.00±2.65	3.67±2.08	4.33±2.08
Aspergillus fumigatus	<1.00	10.33±9.29	3.33±0.58	5.67±1.15	4.00±1.00	6.00±1.73
Aspergillus flavus	<1.00	15.33±4.51	2.00±2.00	3.33±1.15	2.33±0.58	4.00±2.00
Aspergillus niger	<1.00	17.67±4.04	3.00±1.73	3.67±3.51	3.00±3.61	3.00±2.00
Candida krusei	<1.00	8.33 ±4.73	2.67 ±0.58	3.00 ±2.65	2.50 ±0.50	5.33 ±2.31
Candida albicans	<1.00	12.33±0.58	4.67±2.52	4.50±0.87	3.00±1.00	4.17±1.44
Candida glabrata	<1.00	11.33±1.15	3.00±1.00	4.00±1.73	2.83±1.26	4.00±1.00
Candida parapsilosis	<1.00	14.33±0.58	2.00±0.00	3.83±1.04	2.67±2.08	3.83±1.26
Candida tropicalis	<1.00	9.67 ±2.52	3.33 ±1.15	4.67 ±1.15	2.83 ±0.29	3.33 ±1.15
Geotrichum candidum	<1.00	12.67±4.04	3.00±1.00	6.67±2.08	2.50±0.50	2.67±2.52
Rhodotorula mucilaginosa	<1.00	17.33±3.06	3.67±1.53	4.00±1.73	2.50±0.50	4.50±3.04

olis towards *Staphylococcus aureus*, and observed that the antibacterial activity is mainly due to polar phenolic compounds. Kujumgiev et al. (1999) reported that all the propolis samples used in their experiments were active against the Gram-positive bacteria. Castaldo and Capasso (2002) reported that propolis samples showed *in vitro* antimicrobial activity mainly against Gram-positive (*Staphylococcus* spp. and *Streptococcus* spp.) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*). The antibacterial activity of propolis extract was found to be higher than that of pollen extract (Castaldo and Capasso, 2002). The variation in the antibacterial activities of tested extracts may be due to the different substance compounds and their phenolic constituents (Castaldo and Capasso, 2002; Nagai, 2003; Kuzawa et al., 2004; Basim et al., 2006). *S. aureus*, *S. epidermidis*, *B. cereus* and *L. monocytogenes* strains were sensitive against all the PrEE tested in study of Kalogeropoulos (2009). Melliou et al. (2007) reported that the volatiles of Greek propolis inhibited four different species of Gram-negative bacteria (*E. coli, E. cloacae, K. pneumoniae, P. aeruginosa*). The ethanolic extract of Bulgarian propolis inhibited 90.9% of Gram-negative bacteria tested (Boyanova et al., 2006). Bankova et al. (1996) found no inhibitory activity of Brazilian and Bulgarian propolis extracts against a strain of the Gram-negative bacterium *E. coli*. In addition, Brazilian and Korean propolis extracts inhibited the Gram-negative bacterium *S. typhimurium* ATCC 13311, but failed to inhibit the Gram-negative *Pseudomonas aeruginosa* ATCC 15523 (Choia et al., 2006).

Table 3 Inhibitory effects of beeswax extracts against the pathogenic bacteria, molds and yeasts (inhibition zone diameter in mm)
WMEh – beeswax 99.9% methanolic extract; **WMEl** – beeswax 70.0% methanolic extract; **WEEh** – beeswax 96.0% ethanolic extract; **WEEl** – beeswax 70% ethanolic extract; **Cm** - Chloramphenicol

Microorganisms	Controls		Extracts			
	Me 99.9%	Cm or Phe 40.0%	WMEh	WMEl	WEEh	WEEl
Listeria monocytogenes CCM 4699	<1.00	10.66±1.15	0.33±0.58	2.67±2.31	0.33±057	4.43±3.79
Pseudomonas aeruginosa CCM 1960	<1.00	7.00 ±0.00	1.67 ±1.53	2.33 ±2.08	1.67 ±1.53	2.67 ±2.31
Staphylococcus aureus CCM 3953	<1.00	10.00±0.00	2.00±0.00	1.67±1.53	1.67±2.08	1.00±1.00
Salmonella enterica CCM 4420	<1.00	10.67±1.15	2.67±0.58	2.67±0.58	2.17±1.89	3.67±0.58
Escherichia coli CCM 3988	<1.00	12.33±2.08	1.50±1.32	4.67±2.52	1.67±1.53	4.67±0.58
Aspergillus fumigatus	<1.00	10.33±9.29	2.33±0.58	2.67±1.15	2.00±2.00	2.50±1.32
Aspergillus flavus	<1.00	15.33±4.51	0.67±1.15	1.67±0.58	1.67±0.58	2.00±1.00
Aspergillus niger	<1.00	17.67±4.04	2.33±0.58	3.00±0.00	3.00±1.73	4.00±1.73
Candida krusei	<1.00	8.33±4.73	2.00±1.00	1.83±1.76	2.50±2.18	4.00±3.46
Candida albicans	<1.00	12.33±0.58	2.33±2.08	3.67±1.15	2.67±1.15	3.33±0.58
Candida glabrata	<1.00	11.33±1.15	2.00±1.73	2.67±2.08	2.67±1.53	4.83±1.26
Candida parapsilosis	<1.00	14.33±0.58	2.00±1.00	2.67±1.15	1.33±1.15	3.00±3.61
Candida tropicalis	<1.00	9.67±2.52	3.00±1.00	4.67±0.58	2.00±0.00	3.67±0.58
Geotrichum candidum	<1.00	12.67±4.04	2.33±0.58	2.67±2.31	2.33±1.53	4.17±2.47
Rhodotorula mucilaginosa	<1.00	17.33±3.06	2.33±0.58	2.50±1.32	1.67±0.58	2.33±0.58

The inhibition activity of ethanol extract of propolis against mycotoxigenic fungi was studied by Grigoryan et al. (2010), who proved that ethanol propolis extract and mix with hydrogen peroxide showed a stable antifungal effect. The PrEE inhibition percentage of *A. flavus* mycelial growth was 40%-60%. The recent review of Simone-Finstrom and Spivak (2010) also summarizes the importance of the antimicrobial action of propolis in regards of bee health.

The antibacterial activities of pollen and propolis extracts at different concentrations *in vitro* against different plant pathogenic bacteria were tested in the work of Basim et al. (2006). The inhibition zones were varied, related to the different concentrations of pollen and propolis extracts. The inhibitory effect of propolis was found to be higher than that of pollen.

Beeswax

The antibacterial activities of the beeswax extract *in vitro* test against different Gram-positive and negative pathogenic bacteria, microscopic fungi and yeasts are shown in Table 3. The inhibition zones varied amongst the beeswax methanolic extracts (WME) and propolis ethanolic extracts (WEE). According to analyses among the tested bacteria, *Salmonella enterica* CCM 4420 (2.67±0.58) mm was the most sensitive during 48 h of WMEh, and the sensitivity of the bacteria decreased as follows: *Staphylococcus aureus* CCM 3953 > *Pseudomonas aeruginosa* CCM 1960 > *Escherichia coli* CCM 3988 > *Listeria monocytogenes* CCM 4699. *Escherichia coli* CCM 3988 was the most sensitive during 48 h in WMEl, *Salmonella enterica* CCM 4420 in WEEh and *Escherichia coli* CCM 3988 in WEEl. Accord-

ing to analyses among the tested microscopic fungi, *Aspergillus niger* (2.33±0.58) mm was the most sensitive during 48 h of WMEh, *Aspergillus niger* in WMEl and WEEh. According to analyses among the tested yeasts, *Candida tropicalis* was the most sensitive during 48 h of WMEh, *Candida glabrata* in WEEh as well as WEEl. The controls (absolute methanol and phenol) did not show an inhibitory effect on any of the test microorganisms.

The antimicrobial character of beeswax has been documented in European and Asian holistic remedies for centuries. Bogdanov (2004) summarizes the use of bee's wax today, including cosmetics, and cites earlier works on its antibacterial properties. It was found to be particularly active against *B. alvei, Proteus vulgaris, Salmonella gallinarum* and *B. subtilis*. Its effectiveness dropped by half with *Salmonella pullorum*, *S. dublin*, *E. coli* and *Bacillus larvae*. Puleo and Keunen (1991) in their study correlated some of the compounds of plant origin from beeswax that have antimicrobial activity. Al-Waili (2005) studied the effect of a honey mixture prepared by mixing natural honey, beeswax and olive oil on the growth of *C. albicans* and *S. aureus*; no growth of *S. aureus* or *C. albicans* was obtained on media containing honey whereas mild to moderate growth was obtained on media containing beeswax. In any case, this study is one of the first where an antimicrobial property of beeswax against various pathogenic species of microorganisms is quantified in more detail.

CONCLUSION

The present research has shown that a combination of methanolic and ethanolic extracts of bee products studied here possess antibacterial and antifungal effect on bacteria, fungi and yeasts. The inhibition effect of three bee product extracts was found to be solvent-dependent. From the tested extracts, the best inhibiting effects were shown by the propolis ethanolic extract in 70% concentration. The most sensitive microorganism was *Geotrichum candidum* in the propolis ethanolic extract. Due to the great biodiversity of propolis and pollen sources, which also influence the secretion of beeswax, more studies are necessary for a better understanding of the functional properties of bee products. This study is one of the first where an antimicrobial property of beeswax against various pathogenic species of microorganisms is quantified.

Acknowledgments - This study was supported by Cultural and Educational Grant Agency no. 053 SPU-4/2011 and 013 SPU-4/2012.

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