Supplementary information to the paper:

Novel micellar form of poplar propolis with high cytotoxic activity

by

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Measurements
DLS measurements were conducted with a Zetamaster analyzer (Malvern Instruments, UK). All samples were measured at 25 ^{o}C with a scattering angle of 90^{o}. Three measurements of each sample were conducted. TEM analysis were conducted with a JEOL 2100 electron microscope at an accelerating voltage of 200 kV, equipped with a digital camera. A drop of sample solution was deposited on a TEM copper grid coated with a Carbon film, and the solvent was allowed to evaporate. UV–vis absorption spectra of propolis were recorded in the 240–500 nm range using a DU 800 spectrophotometer (Beckman Coulter).

Total flavone and flavonol content
The analysis was performed as described in (Popova et al., 2004). Briefly, 2 mL of the propolis solution, 20 mL methanol and 1 mL 5 % $AlCl_3$ in methanol (w/v) were mixed in a volumetric flask and the volume was made up to 50 mL with methanol. The mixture was left to rest for 30 min and the absorbance at 425 nm was measured. Calibration was performed using galangin as reference compound.

Total flavanone and dihydroflavonol content
The analysis was performed as described in (Popova et al., 2004). Briefly, 1 mL of the propolis solution and 2 mL of 2,4- dinitrophenylhydrazine (DNP) solution (1 g DNP in 2 mL 96 % sulfuric acid, diluted to 100 mL with methanol in a volumetric flask) were heated at 50 ◦C in 2 mL 96 % sulfuric acid, diluted to 100 mL with methanol in a volumetric flask) were heated at 50 ^{o}C for 50 min (water bath). After cooling to room temperature the mixture was diluted to 10 mL with 10 % KOH in methanol (w/v). One mL of the resulting solution was added to 10 mL methanol and was diluted to 50 mL with methanol (volumetric flasks). Absorbance was measured at 486 nm. Calibration was performed using pinocembrin as reference compound.

Total phenolic content
The analysis was performed as described in (Popova et al., 2004). Briefly, 1 mL of the propolis solution was transferred to a 50 mL volumetric flask containing 15 mL distilled water, and 4 mL of Folin-Ciocalteu reagent and 6 mL of a 20 % sodium carbonate solution (w/v) were added. The volume was made up with distilled water to 50 mL. The sample was left for 2 h and the absorbance at 760 nm was measured. Calibration was performed using a reference mixture of pinocembrin and galangin, in 2:1 (w/w) for calibration.

GC–MS analysis:
Analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness DB-17HT capillary column. The temperature was programmed from 80 to 320 °C at a rate of 5 °C min^{-1}, and a 10 min hold at 320 °C. Helium was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. The split ratio was 1:10, the injector temperature 280 °C, the interface temperature 300 °C, and the ionisation voltage 70 eV. The

identification of the compounds was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds. Semiquantification was carried out based on Total Ion Currant by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100 % area. The ion current generated depends on the characteristics of the compound concerned and is not a true quantification.

In vitro release studies

In vitro release of propolis from the micelles was examined in a phosphate buffer (pH 7.4). The freshly prepared micellar propolis was introduced into a dialysis membrane bag (MWCO = 12 000) that further was placed into 100 mL of phosphate buffer. The medium was stirred (50 rpm) and the temperature was maintained constantly during the study (37 ^{o}C). At predetermined time intervals the medium outside the dialysis bag was replaced by fresh one and the concentration of the released propolis was determined by UV–vis spectrophotometry.

In vitro cytotoxicity

The cytotoxicity of free propolis, propolis loaded in micelles and blank micelles was evaluated by MTT test on HL-60, REH and HEP-G2 cell lines. The cells were cultivated in RPMI-1640 medium supplemented with L-glutamine and 10 % fetal bovine serum. Logarithmically growing cells were seeded in 96 flat bottomed tissue culture plates at a density of 3 x 10^5 cells per mL. The cultivation was performed at 37 ^{o}C under a humidified atmosphere with 5 % CO_2 for 24 hours. After this period, the investigated samples were added to the cells in different concentrations. After incubation for 72 h, 10 µL of the MTT-solution was added to each well and the plates were further incubated for 4 h at 37 ^{o}C. Then, formazan crystals were dissolved by the addition of 5 % formic acid in 2-propanol and absorption was measured at 540 nm using a Labexim LMR-1 microplate reader. Each experiment was repeated six times.

References to ESI:

M. Popova, V. Bankova D. Butovska, V. Petkov, B. Nikolova-Damyanova, A.G. Sabatini, G.L. Marcazzan, S. Bogdanov. Validated methods for the quantification of biologically active constituents of poplar-type propolis. *Phytochem Anal*., 2004, **15**: 235-40.

Table S1. Chemical composition of ethanol extract of propolis sample.

Compound	**% of TIC**
Aromatic acids	**6.6**
Benzoic zcid	0.6
Cinnamic acid	0.1
p-Hydroxybenzoic acid	0.1
p-Methoxycinnamic acid	0.2
p-Coumaric acid	0.6
Dimethoxycinnamic acid	1.1
Ferulic acid (Z)	0.8
Ferulic acid (E)	0.8
Caffeic acid	0.2
Caffeic acid	2.1
Phenolic acid esters	**13.0**
Pentenyl coumarate	0.1
Pentenyl coumarate (isomer)	0.2
3-Methyl-3-butenyl ferulate	0.1
3-Methyl-3-butenyl caffeate	1.5
2-Methyl-2-butenyl ferulate	0.1
3-Methyl-2-butenyl ferulate	0.9
2-Methyl-2-butenyl caffeate	0.6
3-Methyl-2-butenyl caffeate	1.9
Phenylethyl p-coumarate	0.1
Benzyl caffeate	2.3
Benzyl ferulate	0.2
Cinnamyl caffeate	1.3
Phenylethyl caffeate	3.7
Chalcones	**1.6**
Pinocembrin chalcone	1.0
Trihydroxymonomethoxy chalcone m/z=502	0.2
Pinobanksinacetate chalcone	0.4
Flavanones and dihydroflavonols	**37.3**
Pinobanksin	5.2
Pinocembrin	10.4
Sakuranetin	0.2
Dihydroxymethoxyflavanone	2.1
3-Acetylpinobanksin	14.4
Pinobanksin butanoate	0.7
Pinobanksin propanoate	1.6
Pinobanksin pentanoate	1.8
Alpinone	0.2
Pinobanksin pentenoate	0.7
Flavones and flavonols	**22.6**
Galangin	8.2
Chrysin	7.6
Dihydroxymonomethoxy flavone	0.8
Kaempferol	0.9
Quercetin	1.1
Kaempferol methyl ether	1.3
Quercetin-methyl ether	1.2
Quercetin-methyl ether (isomere)	0.7
Quercetin-methyl ether (isomere)	0.6
Dihydroxymonomethoxy flavone	0.2
Others	**0.2**
Phenylethyl alcohol	0.1
Hexadecanoic acid	0.1
Sugars	**3.8**

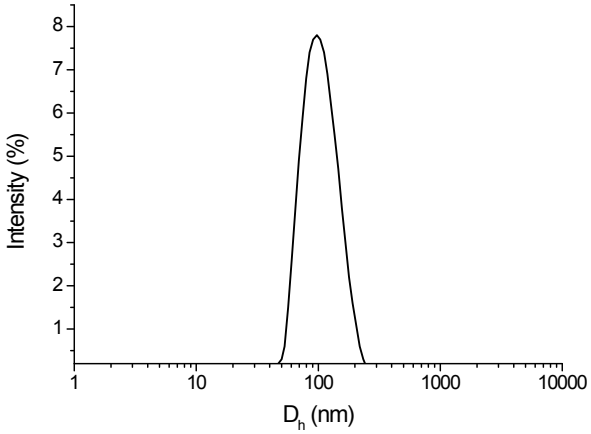

Figure S1. Particle size distribution of micellar propolis in water prepared at $PEO_{26}PPO_{40}PEO_{26}$ /propolis wt. ratio 2 and concentration of propolis 1 mg mL^{-1}.

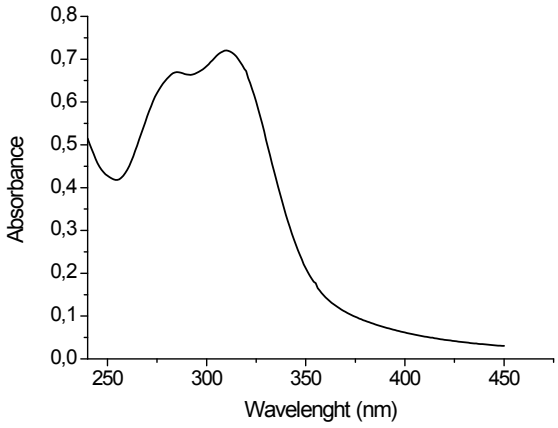

Figure S2. UV-vis absorption spectra of propolis in phosphate buffer (pH = 7.4). Concentration of propolis 0.4 mg mL^{-1}

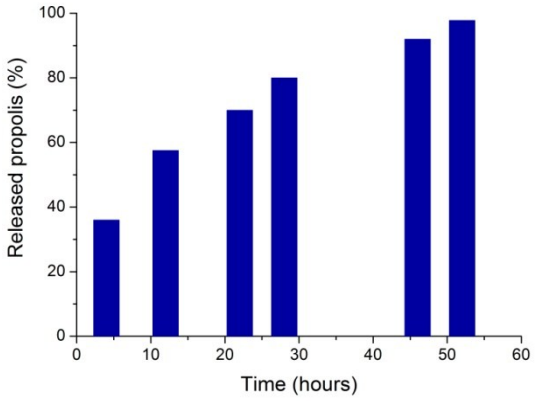

Figure S3. *In vitro* release of propolis from $PEO_{26}PPO_{40}PEO_{26}$ micelles in phosphate buffer (pH 7.4). The sample was prepared at $PEO_{26}PPO_{40}PEO_{26}$/propolis wt. ratio 5 and concentration of propolis 1 mg mL^{-1}.