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Antiviral activity of propolis special extract GH 2002 against *Varicella zoster* virus *in vitro*

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Received July 26, 2018, accepted October 3, 2018

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Pharmazie 73: 733–736 (2018) *doi: 10.1691/ph.2018.8672*

Propolis is a generic name for a biological substance produced by bees used for multiple purposes in folk medicine. Propolis special extract GH 2002 is crude propolis highly purified by a special procedure and freed from the accompanying substances like pollen, wax, resins. The cytotoxic and antiherpetic effect of propolis extracts against *Varicella zoster* virus (VZV) was analysed in cell culture, and revealed a moderate cytotoxicity on lung fibroblasts with a CC_{50} of 380 µg/ml. The 50 % inhibitory concentration (IC_{50}) of GH 2002 propolis extract for VZV plaque formation was determined at 64 µg/ml. The propolis extract exhibited high levels of antiviral activity against VZV in viral suspension tests, infectivity was significantly reduced by 93.9 % and a direct concentration-dependent antiviral activity could be demonstrated. In order to determine the mode of virus suppression by propolis, the extract was added at different times during the viral infection cycle. Addition of propolis to uninfected cells (pretreatment cells) prior to infection or to infected cells (replication) during intracellular replication had no or only minor effect on virus multiplication. However, propolis exhibited high anti-VZV activity when viruses were pretreated with propolis prior to infection thus indicating an unspecific interaction between the virus and propolis. The antiviral activity is comparable to acyclovir.

1. Introduction

Propolis is manufactured by bees while adding their saliva to the resinous plant exudate, subsequently the partially digested material is additionally mixed with beewax (Banskota et al. 2001). Bees use propolis (bee glue) as a building material and also to keep pathogenic bacteria and fungi out of the hive. Thus, action against microorganisms is an essential characteristic of propolis. Raw propolis undergoes secondary processing to remove resins and other impurities before being used in a variety of pharmaceutical and natural health care products. Propolis mainly consists of resins, beeswaxes and fatty acids, essential oils, pollen and some other components such as minerals, vitamins, and sugar. The chemical components of propolis are qualitatively and quantitatively variable, depending on the geographic origin and regional plant ecology.

Propolis is a well-known substance in folk medicine and is used for multiple purposes. The scientific literature describes the natural substance as biologically active with antibacterial, antiphlogistic and antiviral effects (Amoros et al. 1992; Astani et al. 2013; Borrelli et al. 2002; Boyanova et al. 2005; Burdock et al. 1998; Holcova and Hladikova, 2015, Kujumgiev et al. 1999; Papova et al. 2005; Reichling et al. 2009). A pharmacological activity against several viral infections has been demonstrated, e.g. influenza (Serkedjieva et al. 1992), HIV (Ito et al. 2001), and *Herpes simplex* virus (Schnitzler et al. 2010; Nolkemper et al. 2010). Also other natural products, e.g, essential oils or plant extracts have demonstrated high activity against herpetic infections (Schnitzler et al. 2007; Astani et al. 2010; Astani et al. 2011; Schnitzler and Reichling 2011a; Astani et al. 2012).

The propolis special extract GH 2002 is obtained from the natural product propolis, which comes from only one bee pasture in Central Europe. It is highly purified in a special procedure and freed from the accompanying substances like wax, resins and pollen. In this way, a fraction of the propolis, a concentrated pure native extract enriched with substances like flavonoids, polyphenols and phenyl carboxylic acids, is formed that is involved in the antiviral efficacy (Nolkemper et al. 2010; Schnitzler et al. 2010).

Varicella zoster virus (VZV) is a DNA virus related to the *Herpes simplex* virus. Primary VZV infection results in varicella also known as chickenpox. The virus then remains latent in dorsal root ganglia. When cellular immunity decreases (age over 50 or under immunosupression), VZV can reactivate and cause a vesicular rash typically distributed in a single sensory dermatom known as shingles. Vesicular rash during both chickenpox and shingles period is caused by local replication of VZV in the skin (Zerboni et al. 2014). The most common complications of shingles are secondary bacterial infections and postherpetic neuralgia especially in patients over 60 years of age (Dworkin et al. 2003; Gnann et al. 2002; Kost et al. 1996; Oaklander 2008).

In the current study we have investigated the antiviral activity of the special propolis extract GH 2002 against VZV *in vitro* and determined the mode of antiviral action of propolis against VZV.

2. Investigations, results and discussion

2.1. *Cytotoxicity*

Propolis extract was serially diluted and added to cell culture medium to examine the effect on growth and viability of LEP cells. Cell monolayers were grown in medium containing different concentrations of propolis. After 9 days of incubation, cell viability of LEP cells was determined with the neutral red assay. The maximum noncytotoxic concentration of propolis was determined at 100 µg/ml, the CC_{50} value was 380 µg/ml (Fig. 1). Experiments to assess the cytotoxicity of propolis extracts for cultured eucaryotic cells indicate a moderately toxic behaviour of propolis in cell culture.

2.2. *Antiviral action*

VZV replication is characterized by a complex sequence of different steps at which antiviral agents might interfere. In order to investigate the inhibitory effects on VZV in detail, propolis extract was added at different stages during viral infection. The

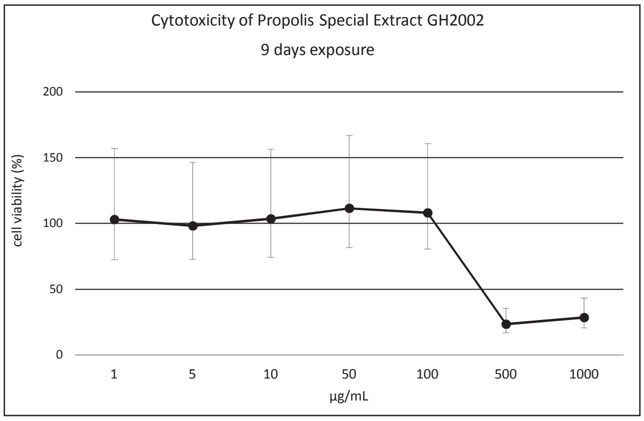

Fig. 1: Cytotoxicity of propolis extract, results are depicted as rate of mean values of negative control. The propolis special extract GH2002 revealed a moderate cytotoxicity on lung fibroblasts with a CC_{50} of 380 µg/ml. Experiments were repeated independently, data are presented as mean±SD of three independent experiments, each time in duplicate.

propolis extract did not show any effect on VZV when host cells were pretreated (pretreatment cells) prior to infection. Direct incubation of VZV together with the propolis extract (pretreatment virus) caused a significant suppression of VZV multiplication, the IC_{50} is 64 µg/ml. At the maximum noncytotoxic concentration of propolis (100 µg/ml), infectivity was reduced by 93.9 % with 95 % confidence interval 89.3-97 % (Fig. 2). Complete inhibition of virus replication was not observed and a minor cytopathic effect was observed. In contrast, when the extract was added to the overlay medium after penetration of the viruses into the host cells, plaque formation was not significantly reduced. Acyclovir showed the highest antiviral activity when added during the replication period with a complete inhibition of viral replication, no plaques or cytopathic effect were observed. This drug inhibits specifically the viral DNA polymerase during the replication cycle when new viral DNA is synthesized.

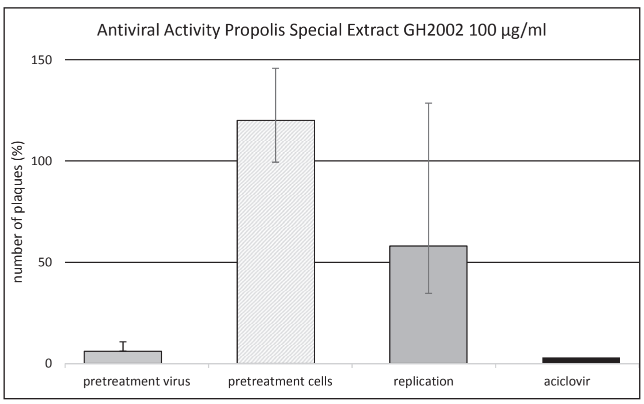

Fig. 2: Antiviral activity of propolis against *Varicella zoster* virus (VZV). Bars represent rate of mean values for plaque forming units (pfu) of the propolis or acyclovir treated assays. A statistical significance of $p < 0.05$ was shown for propolis in the pretreatment virus assay. The IC_{50} with propolis special extract GH2002 against VZV plaque forming units was reached at 64 µg/ml. Experiments were repeated independently, data are presented as mean±SD of three independent experiments, each time in duplicate.

3. Discussion

Propolis reveals a broad spectrum of biological activities, is used in food and folk medicine, thus there is a renewed interest in its antiviral potential. It is well understood that bees collect propolis to seal their hive and to prevent the decomposition of creatures which have been killed by bees after an invasion of the hive. Thus, propolis is considered to possess antimicrobial activity. Only little information on the effects of propolis against infections with VZV is available. Several drugs are currently available for the management of VZV infections such as acyclovir. Acyclovir and related synthetic nucleosides analogues interfere with viral DNA replication through activation by viral thymidine kinase (De Clercq 2004). VZV causes chicken pox and remains as chronic, persistent infection that might reactivate and presents as *Herpes zoster* (shingles). Holcova and Hladikova (2015) performed a clinical study and treated shingle patients topically with propolis extract GH2002 quite efficiently.

Besides antimicrobial and antiviral activities, there is broad spectrum of biological activities that were reported for propolis extract. Propolis has many targets and may attack also other diseases. Bueno-Silva et al. (2016) have demonstrated, that Brazilian red propolis is a promising anti-inflammatory natural product which mechanism seems to be by reducing leukocyte rolling and adhesion. Bee products are considered as source of natural antioxidants such as flavonoids, phenolic acids and terpenoids. Oxidative stress is the underlying pathogenesis of several diseases, like neurodegenerative disorders, cancer, diabetes and atherosclerosis which might be counteracted by propolis (Kocot et al. 2018). Cao et al. (2017) have reported the induction of the expression of antioxidant-related genes by propolis, which has great implications for the potential of propolis to alleviate oxidative stress in wound tissues. Dietary propolis improves insulin sensitivity in insulin resistant rats and blood pressure in the early stage in development of insulin resistance, which might be mediated by suppression of metabolic acidosis (Aoi et al. 2013). Even the progression of type 2 Diabetes mellitus may be delayed by propolis through anti-inflammation effects and anti-oxidant effects (Chen et al. 2018). When metal implants were removed from Russian propolis extracts, toxicity to mammalian cells was drastically reduced, suggesting a potential for medical applications (Ambi et al. 2017). So far, no side effects of the large multi-specific activity of propolis is known, thus propolis might be applied for the treatment and prevention of a large array of different diseases and ailments.

Our *in vitro* results suggest that propolis extract interferes with virion envelope structures or is masking viral compounds which are necessary for adsorption or entry into host cells. Polyphenols and tannins of the propolis GH 2002 extract demonstrate unspecific antiviral effects by precipitating proteins of the top tissue layers of skin or mucous membrane. This leads to a coherent, firmly attached membranous protein layer and reduces the penetration of viruses and microorganisms (Ueda et al., 2013; Zhao et al., 2015). Apparently, VZV is very sensitive to propolis extract and the inhibition of VZV appears to occur before entering the cell but not after penetration of the virus into the cell. Several experiments have been performed earlier to elucidate the antiviral activity and mode of antiviral action against herpes simplex virus. Our results are consistent with findings of Schnitzler et al. (2010) and Nolkemper et al. (2010), where propolis was directly effective against the *Herpes simplex* virus (HSV), another member of the family Herpesviridae, which is closely related to VZV. It remains to be determined whether the inhibitory effect is due to binding of some constituents of the extract to viral proteins involved in host cell adsorption and penetration or is due to damage of the virions, possibly their envelopes, thereby impairing their ability to infect host cells. The positive antiviral control acyclovir showed the highest antiviral activity when added during the intracellular replication period. This drug inhibits specifically the viral DNA polymerase during the intracellular replication cycle when new viral DNA is synthesized. In contrast to propolis extracts, no effect on viral replication was detected when cells or viruses were pretreated with acyclovir. Amoros et al. (1992) investigated the *in vitro* antiviral activity of resin balsam against HSV and could detect a virucidal effect when herpesvirus was pretreated with propolis, but pretreatment of cells with propolis did not inhibit viral replication. These data are in accordance with our results. Administration of propolis before infection yielded the most significant inhibitory effect. Thus, the antiviral activity is probably due to prevention of virus adsorption to host cells. Debiaggi et al. (1990) investigated propolis-derived flavonoids and reported concentration-dependent reduction of HSV replication for quercetin, when high drug concentrations were used. A synergy could be demonstrated when binary flavone-flavonol combinations of propolis compounds were tested against HSV, thus explaining why propolis is more active than its individual compounds (Scheller et al. 1999). Consequently the extract containing many different components are more effective against HSV than single isolated components and exhibited significant higher antiherpetic effects as well as higher selectivity indices than single isolated constituents (Schnitzler et al. 2010). Propolis extracts might be suitable for topical application against herpes infection.

Our in vitro results are absolutely in accordance with the quite promising clinical study, where propolis extract was very effective in *Herpes zoster* patients. Furthermore, the exact mode of antiviral action was elucidated with cell culture experiments. The low cytotoxicity of propolis in cell culture was also demonstrated in these patients who tolerated this propolis extract well.

4. Experimental

4.1. Propolis special extract GH2002

Propolis, the bee glue of *Apis mellifera*, was collected at Moravia, Czech Republic and has a defined composition, quality and provenance and contains flavonoids and phenylcarboxylic acids. The ethanolic extract GH 2002 was prepared with a special procedure to remove the wax and resin components. Subsequently, propolis was extracted with 90% ethanol resulting in a native viscous extract with a drug/extract ratio 2:1; the extract corresponds to about 200% of the primary raw propolis material. Five grams of propolis special extract GH 2002 were diluted in 50 ml of 96% ethanol overnight in room temperature covered to prevent light exposure. All tests were performed with this stock solution, which was further diluted in cell culture medium.

4.2. Cultivation of varicella zoster virus (VZV)

A cell line of human embryonic lung fibroblasts (LEP) was used in all the tests. Passages of the LEP cell culture used were always low to maintain the best conditions for virus susceptibility. Cells were cultivated at 37 °C and 5 % CO_2 atmosphere. VZV strain Právník was obtained from a patient suffering from chickenpox in 2015. Wild strains and OKA (strain used in vaccine) strains differ in specific single nucleotide polymorphisms, which are target sites for restriction endonucleases. Wildtype genotype of strain Právník was shown with previously published methods (La Russa et al. 1992; Loparev et al. 2000). PCR amplification of three different loci of VZV genome (2 sites from gene ORF 62 and 1 from ORF 38 genes) was followed by restriction analysis. To preserve the attributes of wild type virus low passaged virus was used.

Fresh viral stock was prepared for all the tests. Briefly, a confluent LEP cells layer was infected with the VZV virus and cultivated at 37 °C until the presence of a cytopathic effect. Tissue culture was washed two times with PBS, trypsinized and resuspended in tissue culture medium. The cell suspension was then used as an inoculum for the tests. All the tests were performed with three different virus dilutions (1:50, 1:100, 1:500). RPMI 1640 (Lonza, Verviers, Belgium) with 10% Fetal Bovine Serum (Biochrom, Berlin, Germany), 50000 U/mL penicillin (Biotika, Slovenska Lupca, Slovakia), 50 mg/ml streptomycin (Sigma Aldrich) and 5mg/ml gentamicin (Sandoz, Ljubljana, Slovenia).

4.3. Cytotoxicity

Cells (LEP) were seeded into 96-well cultivation plates in the concentration of 3 x 10^5 cells per well in 200 µl of the cultivation medium. When more than 80% confluent (after 24 h), cells were washed with PBS and covered with medium including serial dilution of the ethanolic extract of propolis (8 wells per 1 dilution). After 9 days cells were stained with neutral red and after fixation absorbance at 530 nm was measured. The result was expressed as percentage of mean value of untreated negative control optical density (OD). Experiments were repeated independently, data are presented as mean ±SD of three independent experiments, each time in duplicate.

4.4. Mode of antiviral action

In order to determine the mode of antiviral action for propolis extract, cells were pre-treated with propolis extract before viral infection (pretreatment cells), viruses were incubated with propolis before infection (pretreatment virus) or infected cells were incubated together with propolis extract after penetration of the virus into the host cells (replication). Propolis was always used at the maximum non-cytotoxic concentration. Cell monolayers containing 5 x 10^5 cells were pretreated with the drugs prior to inoculation with virus by adding propolis at a concentration of 100 µg/ml to the culture medium and subsequent incubation for 1 h at 37 °C (pretreatment cells). Serial dilutions of the fresh virus stock (1:50; 1:100; 1:500) were seeded into pretreated cultivation plates, 0.5 ml of virus stock dilution per well. After 1 h at 37 °C and 5% CO_2, the fluid was removed and cell culture was overlaid with cultivation medium. For pretreatment of virus, VZV was incubated in medium containing 100 µg/ml propolis extract for 3 h at room temperature prior to infection of LEP cells. After 1 h of adsorption at 37 °C, the inoculum was removed and medium without propolis was added. The effect of propolis extract against VZV was also tested during the replication period by adding propolis after viral penetration to the overlay medium, as typical performed in antiviral susceptibility studies. Serial dilutions of the fresh virus stock (1:50; 1:100;1:500) were seeded into 6-well cultivation plates with confluent LEP tissue culture, 0,5 mL of virus stock dilution per well. After 1 h at 37 °C and 5 % CO_2, the fluid was removed and cell culture was overlaid with cultivation medium. After 4 h the cultivation medium was replaced with cultivation medium including propolis at 100 µg/ml. The cultivation plates were stained at day 9 after inoculation with neutral red and plaques were counted. Each assay was run in triplicate, the number of plaques of drug-treated cells or viruses were compared to untreated controls to calculate the extent of plaque reduction (in % of control = pfu %). All untreated controls were incubated with culture medium instead of the drug. Acyclovir (Mediproduct, Lipany, Slovakia) in a concentration 50 µg/ml was used as positive control. Experiments were repeated independently, data are presented as mean±SD of three independent experiments, each time in duplicate.

4.5. Statistical analysis

SPSS 23.0. (SPSS Inc., Chicago, IL) and R (R Core Team (2015), softwares were used for data analysis. Poisson regression served as statistical model. Significance level α was established on 0.05. For multiple analysis Šidák correction of level α (α = 0.0253) was used. For determination of IC_{50} logistic regression curve was counted by non-linear minimal square method.

Acknowledgement: We thank Dr. Paul Schnitzler for critically reading the manuscript.

Conflicts of interest: None declared.

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