Effects of Propolis on Hypoxanthine–Xanthine Oxidase-Induced Toxicity in Cultivated Human Cells and on Neutrophil Elastase Activity

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The inhibition of the proliferation rate of the immortalized human cell line ECV 304 after oxidant damage by oxygen radicals generated in a hypoxanthine–xanthine oxidase system and the protection provided by various propolis extracts was determined. Best inhibition was demonstrated by 60—80% ethanolic extracts (IC50 of approximately 2 μg dry weight/ml) and by the ethyl acetate extract (IC50 of 6.9 μg dry weight/ml). The beneficial effect of polar extracts was quite weak. Human neutrophil elastase activity was inhibited distinctly by ethanolic (60 to 96 %) and ethyl acetate extracts (IC50 of approximately 2 μg dry weight/ml). Both activities seem to be responsible for the anti-inflammatory effect of the extract.

Key words propolis; radical scavenger activity; ECV 304 cell; neutrophil elastase; anti-inflammatory activity

Propolis is used by bees—Apis mellifera—as a glue to seal and to protect the beehives against outside invaders and enemies. However, bees also take advantage of its biological action. The antibacterial and antifungal properties of propolis are responsible for the lower incidence of bacteria and moulds within the hive. Propolis is a resinous substance collected from different plant sources—the main source in Europe is popular besides birch, oak, alder, willow and hazel—and contains, depending on the region of collection different polyphenolic compounds such as flavonoids, phenolic acids and their esters, fatty acids, diterpenic acids and other compounds.1,2) But just this seems to be the main problem in the application of propolis in modern medical therapy. Due to the wide variability of its chemical composition depending on its origin the chemical standardisation is extremely difficult. On the other hand, propolis is reported to possess the incidence of contact sensitivity in persons using it in external preparations, cosmetics and self treatment of various diseases.3,4) Among the substances isolated from propolis especially the caffeic acid derivatives, flavonoids and cinnamic acid caused positive allergic reactions.5,6) Propolis as a traditional remedy in folk medicine is appreciated for its antibacterial, antifungal, antiviral, anti-inflammatory and immunostimulating activities.3,7) These effects may be induced by the potent free radical scavenger activity of flavonoids and caffeic acid derivatives, which are the typical constituents of propolis of the temperate zone of Europe, Asia and North America.7) Reactive oxygen species (ROS) involved in energy production, regulation of cell growth and the synthesis of biological important compounds play on the other side a damaging role and can induce peroxidative degradation of biomembranes, protein modification and DNA damage. The oxidative damage by ROS has been related to different diseases like inflammatory diseases, myocardial infarction, atherosclerosis, as well as aging and cell death.8) Endothelial cells as an element of the vascular system seem to be a suitable target of the cytotoxicity by oxidant stress induced by hypoxanthine–xanthine oxidase.9) We tested the decline of human tumour cells expressing endothelial properties (ECV 304-cells) after oxidant damage by hypoxanthine–xanthine oxidase and determined the protection provided by different propolis extracts to find out, if propolis is capable of reducing toxic effects. In addition we tested the inhibitory potency of different propolis extracts against the neutrophile elastase. The serine endopeptidase elastase is of particular importance in the degradation of connective tissue and high elastase activity levels are considered to be one indicator for inflammation. The interaction of both neutrophil elastase and reactive oxygen species play a role in inflammation and cartilage degradation as well as in ischemic injuries of lung and liver.10—12)

MATERIALS AND METHODS

Chemicals Hypoxanthine, xanthine oxidase, soybean trypsin inhibitor and the enzyme substrate MeO-Suc-Ala-Ala-Pro-Val-pNA were purchased from Sigma (Germany). Neutrophil elastase from human leucocytes (E.C.3.4.21.37) was supplied by Fluka (Germany). Propolis was a gift from Länderinstitut für Bienenkunde, Hohen Neuendorf e.V. (Brandenburg, Germany). It was collected in spring 2001 in the north-eastern part of Brandenburg. A specimen (P1/01) is deposited in the herbal drug collection of the Institute of Pharmacy at Free University in Berlin. The cell culture media were supplied by Biochrom (Berlin, Germany).

Preparation of the Extracts One gram propolis was extracted with 10 ml of the respective extracting agent (1 : 10, w/v) for 20 h at room temperature and then for 20 min by sonification. The mixture was centrifuged and 6 ml of the supernatant were filtered by a sterile filter (0.2 μm). Five milliliters of the filtrate were stored at 4 °C until use. The determination of the dry weight of the extracts starting from 1 g propolis results in app. 150 mg for ethanol (96% v/v), 101 mg for ethanol (80% v/v), 76 mg for ethanol (70% v/v), 62 mg for ethanol (60% v/v), 12 mg for ethanol (40% v/v), 10 mg for ethanol (20% v/v), 200 mg for water, 154 mg for acetone, 151 mg for dichlormethane, and 83 mg for ethyl acetate.

Cell Culture ECV 304-cells (ATCC No. ACC 310) were cultivated in Eagle Medium 199 supplemented with 10% fetal calf serum. Subcultivation was performed once per week in 70 cm² flasks (Greiner, Germany). For the experi-
ments the cells were cultivated in 96-well plates (Greiner, Germany) in an atmosphere of 5% CO₂ at 37 °C in a humidified environment.

**Cytotoxicity Assay**  Cells were detached with trypsin in order to release them from the culture flasks, counted by means of the cell analyser CASY (Schärfe Systeny, Germany) and plated into 96-well plates at a density of approximately 1000 cells per cavity. After 24 h, medium was removed and cells were exposed to 100 μM hypoxanthine, 2 mU/ml xanthine oxidase and to the test substance (propolis extract) in PBS for 1 h. As the solubility of the most propolis extracts in PBS was very low, cloudy suspensions were used. A control without test substance as well as a control containing only 100 μl PBS was carried out in the same manner. Then, the supernatant was removed and the cells were cultivated in Eagle Medium 199 supplemented with 10% fetal calf serum for 4 d. The viability was determined by reduction of thiazolyl-blue (MTT) using DMSO solving the purple formazan.[13]

We tested various concentrations of the propolis extracts and determined their influence on the viability of the ECV 304-cells in relation to the test with hypoxanthine–xanthine oxidase on its own and to the test with PBS. Quercetin was used as positive control. EC₅₀ values, i.e., the concentration which inhibits 50% of the toxicity of hypoxanthine–xanthine oxidase under assay conditions, were estimated after transformation of dose-effect-curves by linear regression analysis performed using Microsoft Excel software.

**Elastase Assay**  The determination of neutrophil elastase activity was performed with human leucocyte elastase and a peptide substrate.[14] The substrate stock solution was 10 mM MeO-Suc-Ala-Ala-Pro-Val-pNA in 60 mM Tris–HCl, pH 7.5, containing 5% DMSO. Elastase was dissolved in 1000 μl sterile water. Then 125 μl substrate solution was mixed with 395 μl buffer, 60 μl test solution and 20 μl elastase solution. The samples were vortexed and the reaction was allowed to proceed for 60 min at 37 °C. The reaction was stopped by addition of 500 ml soybean trypsin inhibitor solution (0.2 mg/ml 60 mM Tris–HCl-buffer, pH 7.5) and placed in an ice bath. After vortexing the absorbance was measured at 405 nm.[15] The inhibition rates were calculated in comparison to the inhibitor-free control.

**Statistics**  Data are presented as means from at least four independent experiments with 8 parallel samples in the case of the cytotoxicity assay and with 3 parallel samples in the case of the elastase assay, respectively. The results are expressed as mean value ± SD. The U-test was used to test significance (p < 0.05).

RESULTS AND DISCUSSION

**Inhibition of Hypoxanthine–Xanthine Oxidase-Induced Toxicity in Cultivated Human Cells**  Our studies demonstrated that the ECV 304-cells have been very sensitive against the toxicity induced by hypoxanthine–xanthine oxidase. An optimal damaging effect (cytotoxicity of approximately 80%) was received by exposure to 100 μM hypoxanthine and 2 mU/ml xanthine oxidase for 1 h. Hypoxanthine and xanthine oxidase on its own were not toxic in this concentrations (non-significant decrease of viability of 3% and non-significant increase of 1%, respectively; data not shown).

In order to investigate the influence of a variety of propolis extracts on the hypoxanthine–xanthine oxidase-induced toxicity, different concentrations of the extracts were tested. In these experiments, the cells were incubated with both hypoxanthine–xanthine oxidase and test substance for 1 h. After the removal of the supernatant the cells were cultivated for additional 4 d. The extracts on their own were non-toxic in the tested concentrations and under the experimental conditions as described (data not shown).

Figure 1 indicates that the extent of the inhibition of the oxidative damage by various propolis extracts was quite different. Best inhibition was demonstrated by 60—80% ethanolic extracts. A restitution of ECV cells of 50% was achieved by concentrations of approximately 25 nl extract/ml, this corresponds to approximately to 2 μg dry weight per ml (Table 1). The ethyl acetate extract of propolis also showed a remarkable effect of protection (IC₅₀ 82.7 nl/ml, this corresponds to 6.9 μg dry weight per ml). The beneficial effect of 20% ethanolic and the water extract was quite weak.
(inhibition of cytotoxicity of 12 and 7%, respectively at a concentration of 0.1 µl/ml).

The data give evidence that the scavenging activity against the hypoxanthine–xanthine oxidase-induced cytotoxicity on human cells depends on the polarity of the extracting agent and as a result on the polarity of the isolated compounds. Our former investigations indicate that in addition to the *in vitro* evaluated structure–activity relationship of flavonoids and phenolic compounds the lipophilicity of the tested substances seems to be of particular importance for the radical scavenging properties in this assay using cultivated human cells.¹⁰) The radical scavenging effect of lipophilic flavonoids was also determined in an endothelial cell model by attenuation the daunomycin-induced toxicity via radical scavenging.¹⁶)

In general, the antioxidant activity of flavonoids depends on the structure and substitution pattern of the hydroxyl groups. The essential requirement for effective radical scavenging is the 3',4'-orthodihydroxy configuration in the B ring and the 4-carbonyl group in the C ring. The presence of the 3-OH group or 3- and 5-OH groups, giving a catechol-like structure in ring C, is also beneficial for the antioxidant activity of flavonoids. The presence of the C2–C3 double bond configured with a 4-keto is known to be responsible for electron delocalization from the B ring, and it increases the radical scavenging activity. In the absence of the o-dihydroxy structure in the B ring, a catechol structure on the A ring can compensate for the flavonoid antioxidant activity.¹⁷)

Taking into consideration that propolis contains mainly apolar flavonoids the marked antioxidative and protecting potential of the ethanolic extracts confirms our observations. Additionally caffeic acid phenethyl ester (CAPE) seems to account for a part of the anti-oxidative principle. Our results correspond to former investigations on the antioxidant capacity of propolis extracts in cell-free assays, taking regard to the variability of the chemical composition of these propolis samples. The determination of the antioxidant activity of ethanolic and aqueous extracts of propolis from Minas Gerais, Brazil, by measuring the coupled oxidation of β-Caroten and linoleic acid demonstrated the greatest antioxidant activity for 70 and 80% ethanolic extracts, whereas the 20% ethanolic and the aqueous extract showed the lowest antioxidant activity.¹⁸) The chemiluminescence produced by superoxide generated from the xanthine–xanthine oxidase reaction was 50% inhibited by 5 µg/ml and 9.5 µg/ml, respectively, of an ethanolic extract of two types of Cuban propolis.¹⁹) Contrary, Basnet et al.²⁰) demonstrated a pronounced anti-oxidative potential of an aqueous extract of Brazilian propolis. The isolated phenolic compound propol was more potent than vitamin C and E in the DPPH free radical assay system. In addition to radical inactivation by scavenging also a direct inhibition of the xanthine oxidase activity by flavonoids²¹) was reported and has to be discussed. These results support our opinion that flavonoids are an important part of the antioxidative activity of propolis.

**Inhibition of Neutrophil Elastase Activity** Figure 2 and Table 2 demonstrate the inhibition of neutrophil elastase activity by different propolis extracts. Elastase was distinc-
vitively inhibited by both ethanolic (60 to 96%) and nonpolar extracts (dichloromethane, acetone and ethyl acetate). Best effects were shown by the ethyl acetate and the 70% ethano-
lic extract (IC₅₀ of approximately 2 µg/ml). The inhibition by an aqueous extract and 20% and 40% ethanolic extracts was weaker (IC₅₀ of the water extract 4.5 µl/ml, this corresponds to 22 µg dry weight per ml). The effect of the non-polar propolis extracts on elastase activity is remarkable taking into consideration that an extract of *Drosera madagascariensis*, which is mainly used in the therapy of infections of the respiratory tract, inhibited human neutrophil elastase with an IC₅₀ of 9.4 µg/ml.²²)

In former investigations we found that flavonoids and cinnamic acid derivatives (cafeic acid esters) inhibit neutrophil elastase activity²³,²⁴) by non-competitive interaction with the enzyme. Especially compounds with catechol structure elements showed a strong inhibitory activity against neutrophil elastase.

The compounds of propolis extracts can interfere with platelet aggregation and eicosanoid synthesis²⁵) by suppression of the lipoxygenase pathway of the arachidonic acid metabolism and inhibition of the prostaglandin and leukotrien generation.²⁶) One additional mechanism of the
anti-inflammatory effect of propolis seems to be the attenuation of proteolytic damage of tissue by the inhibition of neutrophil elastase. The serine proteinase neutrophil elastase released in inflammatory processes effects on the destruction of connective tissue and cleaves various collagen types, elastin and other proteins. It is also important for the enzymatic degradation of the bacterial membrane proteins. However, in a chronic inflammatory process neutrophil elastase promotes inflammation and inhibits the process of healing.\(^{23}\)

In the airway epithelium neutrophil elastase induces the expression of a major respiratory mucin gene (MUC5AC) by an oxidant dependent mechanism.\(^{27}\) With simultaneous consideration of the good radical scavenger activity of propolis the inflammation process seems to be influenced by both systems.

Non-polar and medium polar extracts of propolis show remarkable effects on the inhibition of neutrophil elastase and on the protection against oxidant damage by oxygen radicals in cultivated human cells. The benefit of propolis seems to be the result of effects at a variety of pharmacological targets including inhibition of different enzymes and radical scavenging. These activities synergistically influence the anti-inflammatory effect of the extract. This is of considerable interest with respect to therapeutic application of propolis extracts and offers a pharmacological explanation for the documented therapeutic activity of propolis preparations.

REFERENCES


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